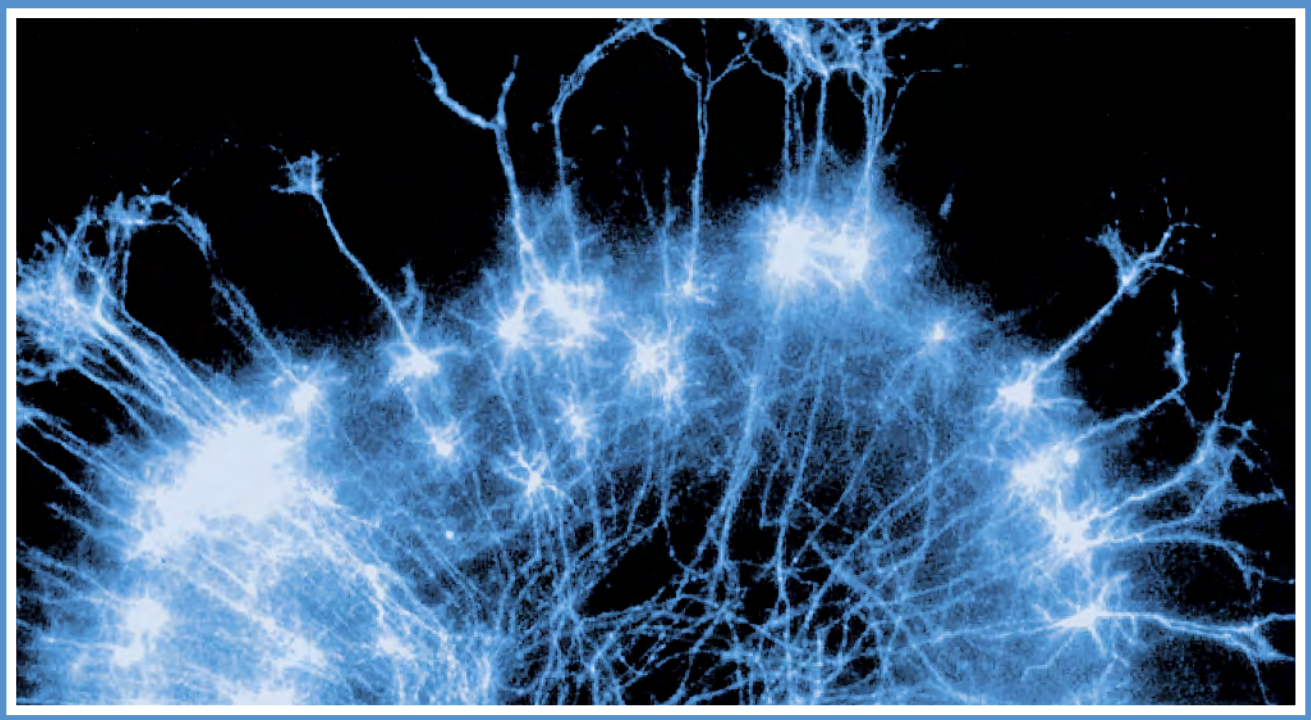
Protocols for Neural Cell Culture

THIRD EDITION



Edited by

Sergey Fedoroff *and* Arleen Richardson

Humana Press

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Preface

The first edition of *Protocols for Neural Cell Culture* was published in 1992 and the second edition in 1997. Originally, the publication grew out of protocols used in the Tissue Culture Course given at the University of Saskatchewan. The course was patterned on those given by the Tissue Culture Association, first in Toronto, Canada, in 1948, then in Cooperstown, NY, then Denver, CO, and finally in Madison, WI, where the course ended in 1964. The course in Saskatchewan began in 1963 as a month-long international course that included both animal and plant tissue cultures. Over the years the course underwent specialization, first being limited to animal tissue culture, then to an intensive one-week general course. This led to one-week courses especially designed for tissue culture for the study of cancer or of the cardiovascular or the nervous system. In 1989, the Saskatchewan course became part of the Tissue Culture Training Facility of the Neuroscience Network of the Canadian Network of Centres of Excellence. The course and the Training Facility ceased to exist in 1997. The faculty for the Saskatchewan course was drawn from the best laboratories in the world and laboratory protocols from those centers were thoroughly tested in a student laboratory setting for many years.

The first and second editions of *Protocols for Neural Cell Culture* were composed entirely of protocols used in the Saskatchewan course. The third edition includes new protocols that, although not tested in the Saskatchewan course, all come from well-established tissue culture laboratories with proven records. Four chapters in the second edition have been omitted and 11 new chapters introduced. The protocols in the four deleted chapters that deal with the preparation of cultures for EM, immunocytochemistry, subculturing cell lines, and the care of dissecting instruments are incorporated into the remaining chapters. Currently, immunostaining is a routine procedure in most tissue culture laboratories and most chapters in the manual deal with aspects of it. The 16 chapters carried over from the second edition have been extensively revised and updated.

The new chapters deal with preparation of substrata, use of serum-free media, maintaining hybridomas, and the production and purification of monoclonal antibodies. Additions include the listing of available neuron and glia cell lines and their characteristics and discussion of setting up multiple-well plate biological assays and cell migration assays. The chapter on myelination is considerably revised and a new chapter on olfactory ensheathing cells, which can remyelinate and promote the long distance growth of regenerating axons in adult mammalian CNS *in situ*, is included. A major recent discovery is that the brain maintains throughout life a population of stem cells that can form replacements for neurons and glia in specific regions of the CNS. Therefore, an extensive new

chapter deals with the isolation, growth, and characterization of neural stem cells and several chapters discuss the glia progenitor cells and how they can be manipulated.

In the second edition we added a chapter on organotypic cultures in which the *in situ* cellular organization is preserved and the differentiation of cells continues in the cultures. When slice cultures are combined with explant cultures from regions of the brain that are normally interconnected with the cells in the slice culture, it is possible to reconstruct specific afferent and efferent projections under in vitro conditions. The third edition adds two new chapters in this area. They deal with hippocampal slice cultures and slice cultures for the study of microglia. The chapter on procedures for transfection of neurons in culture with nontoxic mutant herpes simplex virus has been expanded. Procedures for use of adenovirus have been added because the adenovirus can also be used as a vector for the transfer of genes to nondividing neurons.

The first edition of *Protocols for Neural Cell Culture* dealt entirely with embryonic and neonatal rodent cells. In the third edition the contributors deal with embryonic as well as adult cells of rodent and human origin. A few authors discuss cryopreservation of neural cells. For scientists not trained in neuroanatomy, dissection of the brain and spinal cord presents considerable difficulties; thus in the third edition most chapters describe detailed dissection procedures, some with illustrations, which should be of help.

To assist users of this volume, we present protocols as they are applied in the laboratories of origin, even though this has resulted in some duplication and some differences in the details of similar procedures, from chapter to chapter. Throughout the manual, procedures and important discussions are embedded and these might be missed unless the whole chapter is read. Therefore we advise the user to consult the index. As with the first and second editions, the manual is intended to be a practical companion in the neural cell culture laboratory, and in its updated and expanded version should be an even more valuable resource.

Dr. Richard P. Bunge, one of the pioneers in neural cell in vitro biology, was a faculty member of the Saskatchewan Tissue Culture Course and a contributor to the first and second editions of *Protocols for Neural Cell Culture*. He died in 1997. His colleagues, Dr. Mary I. Johnson and Dr. Patrick M. Wood, have here extensively revised and updated the chapter on myelination that was co-authored by Dr. Bunge and dedicate it to his memory. The editors of this volume join them in paying tribute to Richard P. Bunge.

In preparation of the third edition of *Protocols for Neural Cell Culture* we received considerable help with proofreading from M. E. Fedoroff, Irene Partridge, and Gregg Parchomchuk. We are very grateful for their contribution.

Sergey Fedoroff
Arleen Richardson

Preface to the First Edition

In recent years tissue culture has become one of the major methodologies of biomedical research. It has applications in clinical diagnosis, toxicology, and industrial biotechnology. In the future, its usefulness will undoubtedly expand into gene and cell therapies.

Every worker in tissue culture has favorite methods for doing things. The procedures in this book by no means represent the only ways of achieving desired results. However, certain principles prevail. Only proven protocols that are used routinely in well-established laboratories have been selected for the present volume. Most of the protocols have been used successfully for many years in teaching tissue culture at the University of Saskatchewan. Four chapters deal with culturing neurons from the central and peripheral nervous systems and include a protocol for the study of myelination by oligodendrocytes and Schwann cells. Three chapters cover procedures for isolating and growing glia cells, including astrocytes, oligodendrocytes, and microglia from mice, rats, and humans. Protocols for quantification of cells in culture and growth assays, as well as protocols for biological assays for neuroactive agents, are included. Procedures are described for the application of immunocytochemistry to the study of neural cells, the identification of antigens, the preparation of cultures for electron microscopic analysis, and the care and use of dissecting instruments.

To assist users of this volume, we have presented protocols as they are used in the laboratories of origin, even though this has resulted in some duplication and differences in details of similar procedures from chapter to chapter. We believe that good sterile techniques are much more important as preventive measures against contamination than the routine use of antibiotics; therefore, we have avoided inclusion of antibiotics as part of the culture media. We have included general discussion, where appropriate, based on experience gained in our tissue culture laboratory during more than forty years of research and teaching.

This volume is designed to be kept close at hand as a ready reference and a guide to laboratory procedures. It is based on tissue culture manuals used for a number of years at international courses on tissue culture at the University of Saskatchewan, made possible by the generous support of the Canadian Council of Animal Care and the Medical Research Council of Canada.

Sergey Fedoroff
Arleen Richardson

Introduction

The neural organ—brain, spinal cord, eye, and peripheral ganglia—comprises a wide variety of different cell types. There are neuronal, glial, endothelial, epithelial, connective, stem, and other cell elements. There are thousands of different subsets of neurons, each with a cell body in one location and neuritic processes in another, some extending over a meter in length. Glial cells can be astroglia, oligodendroglia, or microglia in the central nervous system or Schwann cells in the peripheral nervous system. Each neuronal and glial cell type can be further subdivided into subtypes based on anatomical location, developmental age, and the repertoire of genes expressed. Neural cell properties can be regulated by molecules that physically interact with the cells. Such environmental signals often change in response to normal function or insult, and thus, the expressed cellular characteristics can vary temporally.

This astonishing complexity of nervous tissue presents unique problems to the neuroscientist attempting to identify individual neural cells or cooperative cell communities and to understand how both contribute to the proper functioning of the nervous system. Nevertheless, a driving force in neuroscience research is the belief that a detailed understanding of neural cells will lead to the development of rationales for treating the disturbingly wide range of developmental abnormalities, pathologies, and traumatic injuries that are imposed on the human nervous system.

When neural tissue or tissue derivatives are transferred to a culture vessel, the tissue loses, to various extents, the humoral environment, physical connection with other cells, and three-dimensional structure that originally existed in vivo. In vitro, the cell culture medium and gas phase provide a new humoral environment and artificial growth surfaces provide re-anchorage. The culture medium and substratum can be defined before they are used. However, medium and substratum will become less defined after the cellular elements are introduced, because neural cells will remove certain components and contribute others. A common way to delay and minimize cellular modifications of the culture environment is to provide the medium and substratum in large excess relative to the number of neural cells. Although there can be no perfect succedaneum for the loss of specific intercellular interactions, cultures can be set up to retain or re-establish connections that mimic the original tissue.

The two major experimental advantages of using in vitro methods to characterize the nervous system are reduced cellular complexity and the ability to manipulate the cellular environment. The major disadvantage, promulgated for all in vitro studies, is the impossibility of re-establishing in vitro most of the properties of the neural cell in its original in vivo environment. Nevertheless,

there is overwhelming evidence that neural cells can express many of the same properties in vivo and in vitro. Examples include:

1. Astroglial cell production of vimentin, glial fibrillary acidic protein, and neurotrophic factors,
2. Oligodendroglial and Schwann cell elaboration of myelin, and
3. The postmitotic condition of neurons as well as neuronal expression of action potentials, neurofilaments, and growth cones.

The advantages of reducing neural tissue complexity and manipulation of the cellular environment in vitro have permitted scientists to predict and regulate the behavior of neural cells in vivo. For example, the purification, characterization, and cloning of neurotrophic factors (NTFs) depended almost entirely on the use of quantitative in vitro methods to measure factor biological activity. The biological activities of almost all the NTFs, such as promotion of cell survival and the enhancement of growth and maturation, were first observed using in vitro methods, and then later observed in vivo.

In practical terms, the following considerations are important in using neural cultures. The source of neural tissue must be accurately defined, such as animal species, animal age, and anatomic site of tissue. The type of culture must be chosen, such as organ, slice, explant (fragment), disaggregate, reaggregate, or cell line. Finally, the extrinsic environment must be defined, such as basal medium, medium supplements, substratum, and gas phase, and the temperature must be controlled.

Most studies have used neural tissues from invertebrates, chickens, and rodents. Such organisms are popular because of the convenience of maintaining colonies, the ability to predict the developmental age of their nervous systems, and the specific similarities of their nervous systems with the less accessible human one. Just about every neural organ and tissue from these animals at almost every age has been used for in vitro studies. Particularly favored tissues include the chick embryo sympathetic, parasympathetic, and sensory ganglia; rodent sensory and sympathetic ganglia; and specific chick and rodent spinal cord and brain components. Detailed characterizations of these neural tissues and their component cells make the above systems ideal for continued study.

Organ, slice, and explant (fragment) cultures have been preferred preparations for electrophysiological investigations because they retain the greatest degree of original histiotypic organization and can be initiated from the same tissue from both developing and adult animals. Disaggregated or reaggregated cells and cell lines have been preferred by investigators who require replicate cultures, cell accessibility, and cell subset purification.

Most neural cultures are maintained at body temperature and in a 100% air or 5% carbon dioxide/95% air gas phase. However, the basal medium, supplements, and substratum may vary considerably depending on what aspects of the cells are being studied. Usually, except for some invertebrate cells that can be grown in simple salt media, neural cells require specific ions, amino acids, vitamins, cofactors, hormones, mitogens (for proliferating cells), and other growth- and maintenance-promoting substances. Generally, these requirements are met by supplying the cells with a basal nutrient medium containing fetal bovine, horse, or human serum and/or crude tissue extracts. Chemically defined media have been developed that allow a more rigorous definition of the chemical requirements of neural cells and permit neuroscientists to manipulate the chemical environment to

favor the survival and/or growth of selected cell subsets. The overall aim of this manual is to give the reader an introductory understanding of the most important aspects of neural cell cultures and to provide enough basic information to allow the reader to set up and use neural cell cultures competently.

Marston Manthorpe

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Abbreviations and Definitions

Ad	adenovirus
anhyd	anhydrous
Ara-C	cytosine arabinoside
A/2	half the normal area
A2B5	monoclonal antibody that identifies common sialogangliosides and sulfatides in cell surface membranes of neurons, neuroendocrine, and glial cells
B18	serum-free medium supplement
BAC	bacterial artificial chromosome
5-BUdR	5-bromodeoxyuridine
bis	bisacrylamide
BSA	bovine serum albumin
BSS	balanced salt solution
CD14	cluster of differentiation 14; anti-CD14, monoclonal antibody that identifies cell surface membrane glycoprotein in human microglia and monocytes
CD45	cluster of differentiation 45; anti-CD45, monoclonal antibody that identifies leukocyte common antigen
$CD45^{hi}$	leukocyte cell population having a high expression of common leukocyte antigen
cDNA	complementary DNA
C.I.	color index
CNS	central nervous system
CPE	cytopathic effect
cryl	acrylamide
CSF-1	colony stimulating factor-1, also known as macrophage colony stimulating factor (MCSF)
CUSA	Cavitron™ ultrasonic aspiration
DAB	3,3'-diaminobenzidine
DDSA	dodecenyl succinic anhydride
DEAE	diethylaminoethyl
DIC	differential-interference-contrast optics
DlL-ac-LDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanate perchlorate, labels low-density lipoproteins in lysosomes

DM5	chemically defined serum free medium
DME(M)	Dulbecco's modified Eagle's medium
DME(M)/F12	Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12
DMP	2,4,6-tri(dimethylaminomethyl)phenol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPBS	Dulbecco's phosphate-buffered saline
DRG	dorsal root ganglion
DRGN	dorsal root ganglion neuron
E	embryonic age (e.g., E18 = 18-d-old embryo)
EBM	Eagle's basal medium
EBSS	Earle's balanced salt solution
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid (versene)
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EM	electron microscope
EMEM	Eagle's minimum essential medium
F4/80	cell surface membrane antigen of unknown function, restricted to cells of mononuclear phagocyte system
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
fM	femtomoles
FUdR	5'-fluoro-2'-deoxyuridine
G-5	defined medium supplement for glial cells
GalC, GalCer	galactocerebroside expressed on cell surface membrane in oligodendroglia
GBSS	Gey's balanced salt solution
GD3	a ganglioside, major glycolipid component of immature neuroectodermal cells and immature oligodendroglia
GFAP	glial fibrillary acidic protein, protein of intermediate filaments in astroglia
GHAP	glial hyaluronic acid protein
GQ1c	a ganglioside expressed on cell surface membrane, used as marker for oligodendroglia precursor cells
GS	glutamine synthetase
Ham's F12	Ham's nutrient mixture F-12
HBS	HEPES-buffered saline
H-Buffer	homogenization buffer
HEBM	Eagle's basal medium with supplements
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, used as buffer in media
HS	horse serum

HSV	herpes simplex virus
IgG	immunoglobulin G
IgM	immunoglobulin M
L1	neural cell–cell adhesion molecule
IL	interleukin, a cytokine, usually identified by number (e.g., IL-1)
L-15	Leibovitz's L-15 medium
Leu-M5	human monocyte, macrophage, and microglia antigen, a subunit of CD11c heterodimer
LPS	lipopolysaccharide (endotoxin)
Mac-1	monoclonal antibody that identifies complement 3 receptor (CR3), used as marker for microglia and macrophages
Mac-3	glycoprotein on mouse mononuclear phagocytes outside bone marrow
MAP2	microtubule-associated protein 2, present in neurons
MAP5	microtubule-associated protein 5, present in neurons
MBP	myelin basic protein
MEM	minimum essential medium
MEMg	minimum essential medium with glucose
MEMgi	minimum essential medium with glucose and insulin
MHC	major histocompatibility complex
μCi	microcurie
mMEM	modified Eagle's minimum essential medium
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N1	defined culture medium supplement
N2	defined culture medium supplement
N-CAM	neural cell adhesion molecule
NF H	neurofilament (180–200 kDa)
NF L	neurofilament (60–70 kDa)
NF M	neurofilament (130–170 kDa)
ng	nanogram
NGF	nerve growth factor
nm	nanometer
n*M*	nanomolar
NMA	nadic methyl anhydride
NS-1	Ag4-1–mouse non-secreting myeloma cell line
NTF	neurotrophic factor
O-2A	oligodendrocyte progenitor cell
O4	monoclonal antibody, recognizes sulfide, used as an oligodendroglia marker
OD	optical density
OPM	oligodendroglial precursor cell medium
OPM-G	oligodendroglial presursor cell medium with glucose

p75	tumor necrosis factor receptor
PCR	polymerase chain reaction
PDL	poly-D-lysine
PFU	plaque-forming unit
PVP	polyvinylprolidone
P/G	penicillin/glutamine
P0	postnatal d 0 (day of birth)
RD	nutrient medium, 1:1 mixture of RPMI 1640 and Dulbecco's modified Eagle's medium.
RPMI-1640	Rosewell Park Memorial Institute, cell culture medium, originally formulated for culture of blood cells. Used now for lymphocytes and hybridoma cultures.
SDS	sodium dodecyl sulfate-polyacrylamide-gel electrophoresis
S-100	calcium-binding protein found in glia
X-gal	a histochemical substrate for galactosidase
XTT	2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide

Chapter One

In Vitro Assays for Axonal Growth and Targeting

Valérie Castellani and Jürgen Bolz

1. INTRODUCTION

In vitro assays are important tools for investigating how the intricate network of neuronal connections is established during brain development. We have been using different in vitro systems to study the cellular strategies involved in the formation of neuronal projections between the cerebral cortex and subcortical areas, and to examine mechanisms that control the assembly of local cortical circuits (for review, *see* Bolz et al., 1993; Bolz, 1996; Bolz and Castellani, 1997). Results from these studies indicated that diffusible and membrane-associated molecules provide crucial information for the establishment of layer-specific extrinsic and intrinsic cortical connections.

Until recently, very little was known about the molecular nature of these signals. During the past few years, however, several new gene families (netrins, semaphorins, and ephrins) have been discovered, which code for proteins that regulate the growth, targeting, and synapse formation of axons in different regions of the brain (for review, *see* Tessier-Lavigne and Goodman, 1996). Based on these findings, in vitro assays have now been used to determine the possible contribution of defined molecules on the specification of neuronal connections in the developing cortex.

First results indicate that these molecules exert cell type-specific effects, and have a wide and complex range of action. Some molecules selectively affect elongation or branching or guidance of axons, others affect any combination thereof. For example, ephrin-A5, a ligand for Eph receptor tyrosine kinases, acts as a repulsive axonal guidance signal for neurons in the upper cortical layers, and as a branch-promoting signal for axons of neurons in the deep cortical layers (Castellani et al., 1998).

Other molecules have opposing effects of different sets of cortical neurons: They either induce or inhibit axonal branching, or they attract or repel growing axons (Castellani and Bolz, 1999). Members of the semaphorin gene family can function as diffusible and as substrate-bound guidance signal, and in some cases the response of a given population of cortical axons depends critically on the spatial distribution of semaphorins (Bagnard et al., 1999, 2000). Thus, molecules that control the formation of cortical connections function in many alternative ways. Because it is not

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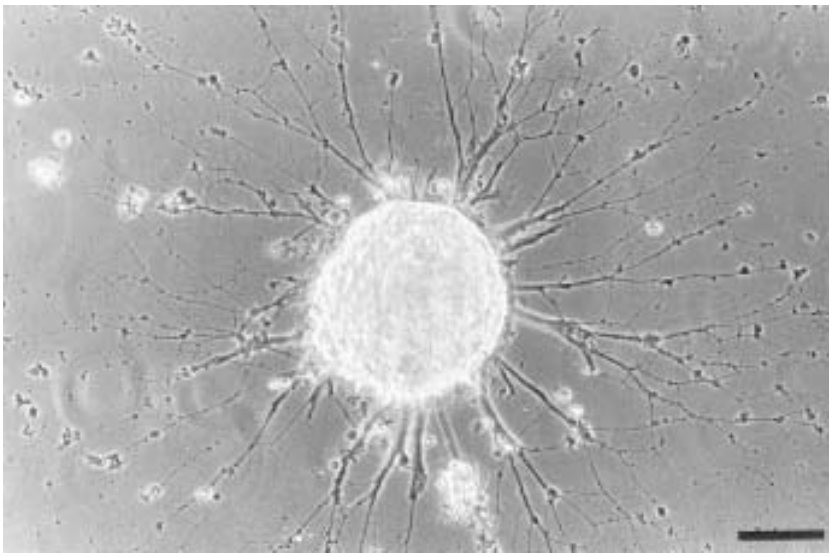

Fig. 1. Phase- contrast micrograph of fiber outgrowth in 2-D cultures. Cortical explants were prepared at embryonic d 16 and placed on coverslips coated with postnatal cortical membranes. Cultures were fixed after 3 d in vitro. Scale bar: 100 μm.

possible to classify these signals as “attractive” vs “repulsive” signals, or as “guidance factors,” “branching factors,” and so on, the authors have proposed to call such, molecules, collectively “wiring molecules” (Bolz and Castellani, 1997).

Here the focus is on two in vitro systems that were applied to characterize the function of known and unknown wiring molecules: a two-dimensional (2-D) outgrowth assay for explant cultures (Fig. 1), and a three-dimensional system for cortical slice cultures (Fig. 2). In general, both culture systems are well suited for study of axonal outgrowth from embryonic tissue, axonal growth from postnatal tissue is very low in 2-D cultures. As described below, axonal outgrowth from postnatal cortical explants or slices is markedly increased when the tissue is embedded in a plasma clot and cultured in a roller tube (Castellani and Bolz, 1999).

2. OUTGROWTH ASSAY

In the 2-D outgrowth assay, small explants are placed on substrate-coated coverslips in Petriperm dishes with culture medium. This permits the observation and quantification of axonal growth patterns with phase-contrast optics, continuously over time, without staining of the axons. Either defined substrates, for example, cell adhesion molecules, or complex substrates, such as membrane preparations, can be used. In several studies, membranes from target and nontarget regions of specific populations of axons have been examined, and wiring molecules that regulate target innervation were characterized by enzymatic treatment or antibody incubations of the membranes (Walter et al., 1987; Henke-Fahle et al., 1996; Mann et al., 1998). It has also been possible to prepare membranes from isolated cortical layers, and to identify wiring molecules that regulate the formation of layer-specific cortical circuits (Castellani and Bolz, 1997, 1999). Finally, substrates, consisting of membranes from cell lines transfected with expression vectors, were used to study the effects of defined molecules on axonal growth and targeting (Drescher et al., 1995; Bagnard et al., 1998; Castellani et al., 1998; Mann et al., 1998).

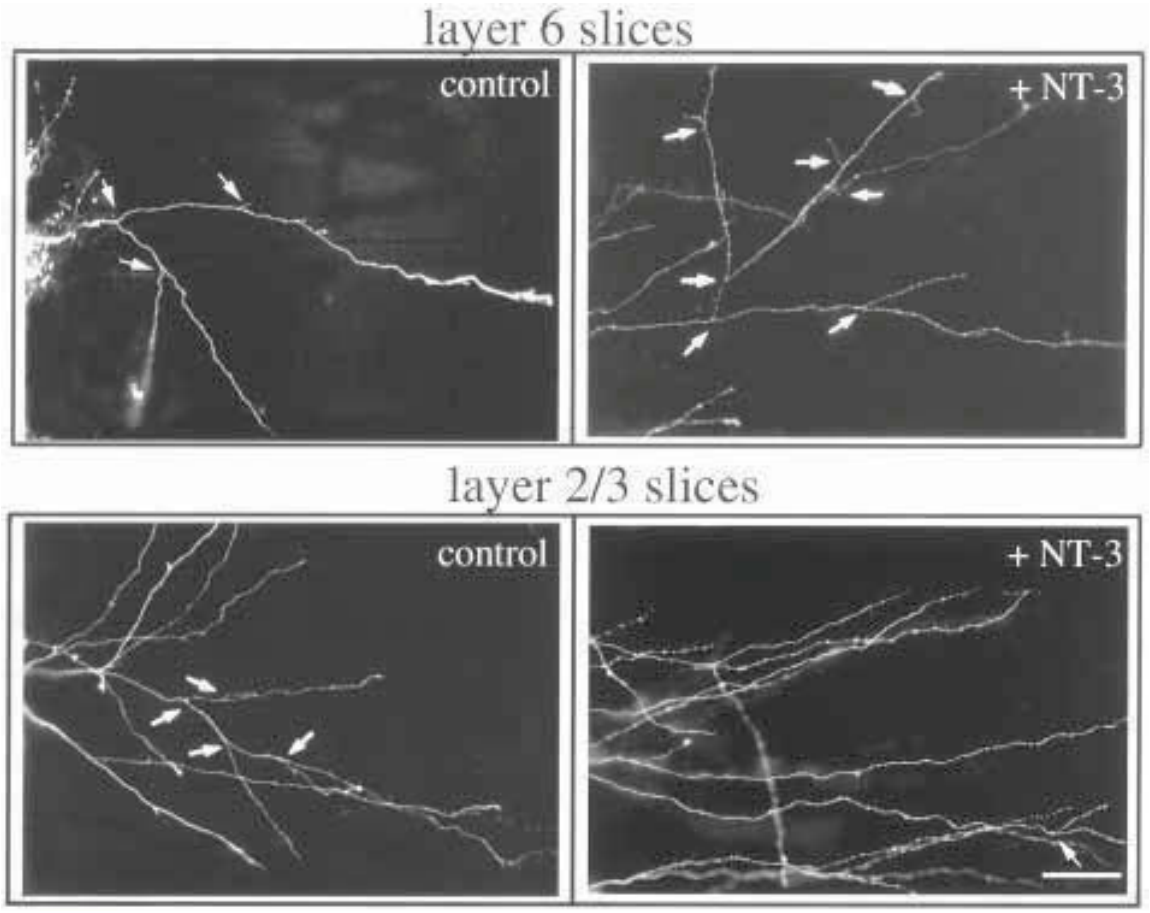

Fig. 2. Fluorescent micrograph of axonal outgrowth in three-dimensional cultures. Layer 6 and layers 2–3 explants were dissected from cortical slices prepared at postnatal d 7, and embedded in a plasma clot. After 2 d in the roller tube, the cultures were fixed and axons were labeled with the fluorescent dye, DiI. This experiment illustrates the dual effect of neurotrophin-3 (NT-3) on axonal branching: Addition of 200 ng/mL NT-3 to the medium increased branching of layer 6 axons and decreased branching of layers 2/3 axons. Some branches are indicated by the arrows. Scale bar: 20 μm.

2.1. Preparation of Substrata

1. Materials:
   Forceps (1), fine.
   Culture medium (3 mL).
   Phosphate buffer (25 mL), calcium- and magnesium-free (CMF).
   Membrane solution (100 μL).
   Laminin-polylysine solution (300 μL).
   Petri dishes (2), 100-mm.
   Petri dish (1), sterile, glass, 100-mm.
   Petriperm dishes (2).
   Coverslips (4), 11 × 22 mm.
   Sterile pipet tips.

2. Preparation of a laminin–polylysine substrate.
   The extracellular matrix protein laminin has proven to be a good substrate for axonal growth; however, different types of axon populations respond differently to the growth-promoting properties of laminin. Polylysine is sticky; it enhances the attachment of the explants, which is necessary for fiber extension, but it also might reduce axonal growth. Therefore, different combinations of laminin-polylysine, as well as laminin alone, should be tested.
   a. Sterilize forceps using the hot bead sterilizer. When the temperature reaches 250°C, insert dry and clean instruments for at least 5–10 s, depending on their size. When sterile, put the forceps into a sterile glass Petri dish, and keep the ends covered with the lid.
   b. With fine forceps, place two cleaned and sterile coverslips on the bottom of a 100-mm Petri dish. Pipet 100 µL laminin–polylysine solution onto each coverslip. Prepare two sandwiches by covering each coverslip with a second one.
   c. Cover the Petri dish, and incubate the sandwiches for at least 30 min at 37°C in a humid atmosphere of 5% CO_2, 95% air.
3. Preparation of membrane carpets.
   Different components of cortical cell membrane preparations promote or inhibit the growth of cortical and thalamic axons, and some of these molecules act differentially on these two axon populations. Moreover, cortical cell membranes, prepared at different developmental stages, revealed that their expression can be developmentally regulated. The preparation of cell membranes (in which molecules from the extracellular matrix co-purify) is described in the appendix.
   a. Open one of the two sandwiches with a forceps, and rinse each coverslip with phosphate-buffered saline (PBS), twice with 1 mL on the side coated with laminin–polylysine solution, and once with 1 mL on the other side.
   b. Place one coverslip on the bottom of another 100-mm Petri dish, coated side up. Pipet 100 µL membrane solution on this coverslip. Again make a sandwich by placing the second coverslip, coated side down, on the first one.
   c. Place the two Petri dishes (one with laminin–polylysine sandwich, and one sandwich with membrane solution) for 2 h in the incubator.
   d. After incubation, open the laminin–polylysine sandwich and rinse the two coverslips with PBS, as in step 3a above. Place both coverslips, coated side up, in a Petriperm dish, and add 750 µL culture medium.
   e. Open the sandwich with membrane preparation. Let the membrane solution drip briefly, but do not rinse. Place both coverslips, coated side up, in a Petriperm dish, and add 750 µL culture medium. Store the two Petriperm dishes in the incubator until the explantation of the cortical tissue.

2.2. Cortical Explants

Dissection of the fetuses involves removing the brain, trimming it down to isolate the cerebral cortex, and cutting the block of cortex twice at orthogonal orientations with the McIlvain tissue chopper (Mickle Laboratory Engineering, Mill Works, UK). This produces many tissue cubes of roughly the same size.

1. Dissecting out fetuses.

Note: The following procedure is performed outside of the laminar flow hood; extra caution should be exercised to prevent contamination.

a. Materials:
   Pregnant rat with E16–E18 fetuses.
   Scissors (1), large.
   Scissors (1), medium.
   Forceps (1), large.
   Forceps (1), small.
   Petri dish (1), glass, 100-mm.
   Gauze squares (4), 2 × 2 in.
   70% ethanol.
   Cork board and eight pins.
b. Euthanize the pregnant rat by CO_2 inhalation, followed by cervical dislocation.
c. Pin the rat down on a cork board, ventral side up. Prepare the ventral surface of the rat by soaking with 70% ethanol.
d. Using the large sterile scissors and large forceps, cut laterally across the lower abdomen just above the vaginal orifice. Cut through the skin, but not the underlying muscle. Then cut up the left side and laterally across the chest. Move the skin to the side and pin. Again soak the exposed area with 70% ethanol.
e. Using the medium sterile scissors and small forceps, make an incision, as before, through the muscle layer, taking care not to pierce the intestines. Grasp one horn of the uterus, dissect it out of the abdominal cavity, and transfer the intact uterus to the 100-mm glass Petri dish. Quickly transfer this glass Petri dish to the laminar flow hood.

Note: All subsequent procedures are performed in the laminar flow hood.

2. Removal of fetuses from uterus.
   a. Materials:
      Scissors (1), small.
      Forceps (1), small.
      Petri dish (1), 35-mm.
      Petri dish (1), sterile, glass, 100-mm.
   b. Sterilize instruments, using the hot bead sterilizer. When the temperature reaches 250°C, insert dry and clean instruments for at least 5–10 s, depending on their size. When sterile, put the instruments into a sterile glass Petri dish, and keep the ends covered with the lid.
   c. Using the small scissors and forceps, cut through the length of the uterus and separate the fetuses from their placentas and accompanying amniotic membrane. Transfer the fetuses to a 35-mm Petri dish.
3. Preparation of cortical explants.
   a. Materials.
      Scissors (1), small.
      Forceps (2), Dumont.
      Scalpel blade (1).
      Spatula (1), large.
      Spatula (1), small.
      Culture medium (15 mL).
      Gey's balanced salt solution/glucose (GBSS/glucose) (30 mL).
      Petri dish (1), 100-mm.
      Petri dish (1).
      Ice bucket and ice.

b. Sterilize instruments, using the hot-bead sterilizer. When the temperature reaches 250°C, insert dry and clean instruments for at least 5–10 s, depending on their size. When sterile, put the instruments into a sterile glass Petri dish, and keep the ends covered with the lid.
c. Place the GBSS/glucose in an ice bath. Pipet six drops (1 mL each) of this solution, about equally spaced in a 100-mm Petri dish.
d. Decapitate three embryos with small scissors, and put each head in one drop of GBSS/glucose.
e. Under a dissecting microscope, remove the skin and skull together, with two fine forceps. Take out the whole brain with a small spatula, and put it in a second drop of GBSS/glucose. Cut between the two hemispheres. Place one hemisphere pia side down, and hold the tissue with forceps placed in the striatum. With a scalpel, make a small cut at the anterior and posterior pole of the hemisphere, then unfold the hemisphere with a second forceps. Make two lateral cuts to obtain a rectangular block of (neo)cortex. Transfer each block to a third drop of GBSS/glucose.
f. Transfer all blocks of cortex to the disk on the tissue chopper, cut at 200 µm, rotate the disk by 90°, and cut again at 200 µm.
g. Collect the cortex cubes with a large spatula, and place them in a 35-mm dish half-filled with culture medium. Incubate the tissue for 1–2 h at 37°C in a humidified atmosphere of 5% CO_2/95% air, which allows the diffusion of proteolytic enzymes released by damaged cells.
h. Under the dissecting microscope, explant the cortex cubes with a pipet in 15 µL medium on the coated coverslips in the Petriperm dishes. Repeat this until there are enough explants on the four coverslips (two coated with laminin–polylysine, two coated with membrane solution), but allow a reasonable distance between the explants. To keep the volume of the medium (750 µL) constant, remove 15 µL from the medium each time you explants are transferred on one of the coverslips (the explants do not adhere to the substrate, if there is too much fluid).
i. Wait 10 min, then carefully transfer the Petriperm dishes into the incubator.

Note: Most explants have not yet adhered to the substrate, so do not shake or tilt the dishes.

Incubate for 30 min at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Most explants should then have adhered to their substrate.

j. Remove the explants from the incubator, and slowly add 1250 µL culture medium to each dish. Return to the incubator.

Note: Examine fiber outgrowth after 2–4 d in vitro. To confirm the neuronal origin of the fibers, explants can be stained with antibodies directed against neuronal markers, e.g., microtubular-associated protein 5 (Sigma, St. Louis, MO), SMI31 (Sternberger Monoclonals, Baltimore, MD), and glial markers, e.g., glial fibrillary acidic protein (Bioscience Products AG, Emmenbrecke, Switzerland) and vimentin (Sigma).

3. CORTICAL SLICE CULTURES

In slice cultures prepared from the cerebral cortex, the intrinsic organization is organotypically preserved, and the differentiation and maturation of cortical neurons continues in this culture system (Fig. 3). By culturing cortical slices next to explants from regions of the brain that are interconnected with the cortex in vivo, it is possible to reconstruct specific afferent and efferent projections under in vitro condition (for review, *see* Bolz et al., 1993).

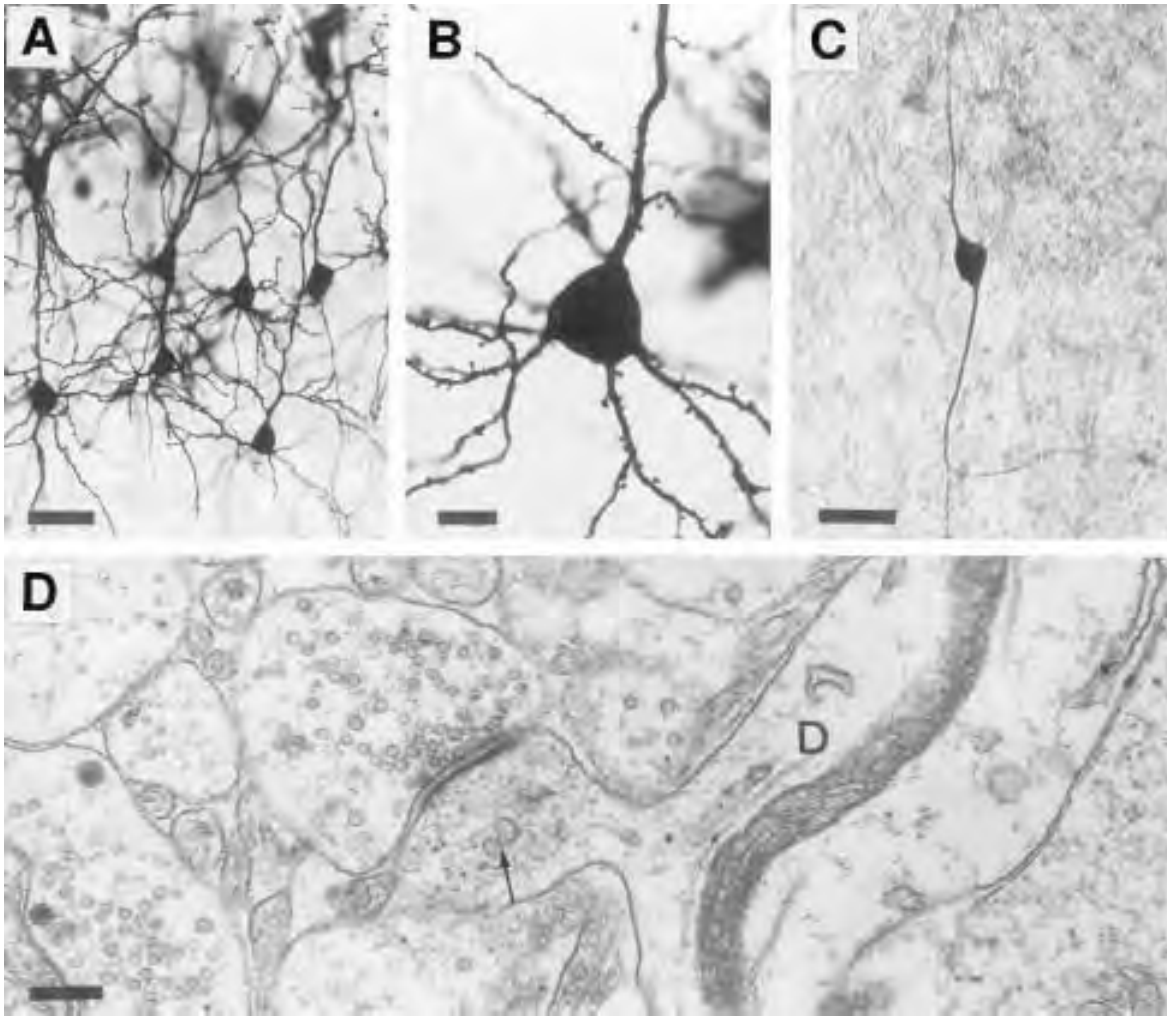

Fig. 3. Organotypic organization of cortical slice cultures. Slices were prepared from 6-d-old rats and kept for 2 wk IV. **(A, B)** Golgi-stained pyramidal cells. **(C)** GABA immunoreactive nonpyramidal cell. **(D)** Electron micrograph showing a synapse on a dendritic spine. Scale bars: 200 μm in (A), 50 μm in (B,C), and 0.5 μm in (D).

Different techniques have been applied to sustain slices in vitro long enough for neurons to establish connections with co-cultured targets. According to one protocol, slices are placed on collagen-coated membranes and kept stationary in an incubator (Stoppini et al., 1991). On these membranes, however, there is only little spontaneous fiber outgrowth from postnatal cortical slices. Axons invade cocultured explants only when the slices are in direct contact with each other, or when migrating cells form bridges between the two cultures. In a procedure initially described by Gähwiler (1981), slices are embedded in a plasma clot on glass coverslips and cultured in a roller tube, to alternate exposure of the tissue to air and medium. The plasma clot provides a good substrate for fiber outgrowth, and, in addition, it also stabilizes diffusible molecules. Co-cultures of cortical explants, with aggregates of cells transfected with expression vectors for secreted semaphorins, demonstrated that these molecules can act as chemoattractant or as chemorepellent guidance signals for cortical axons (Bagnard et al., 1998). In these short-term experiments, co-

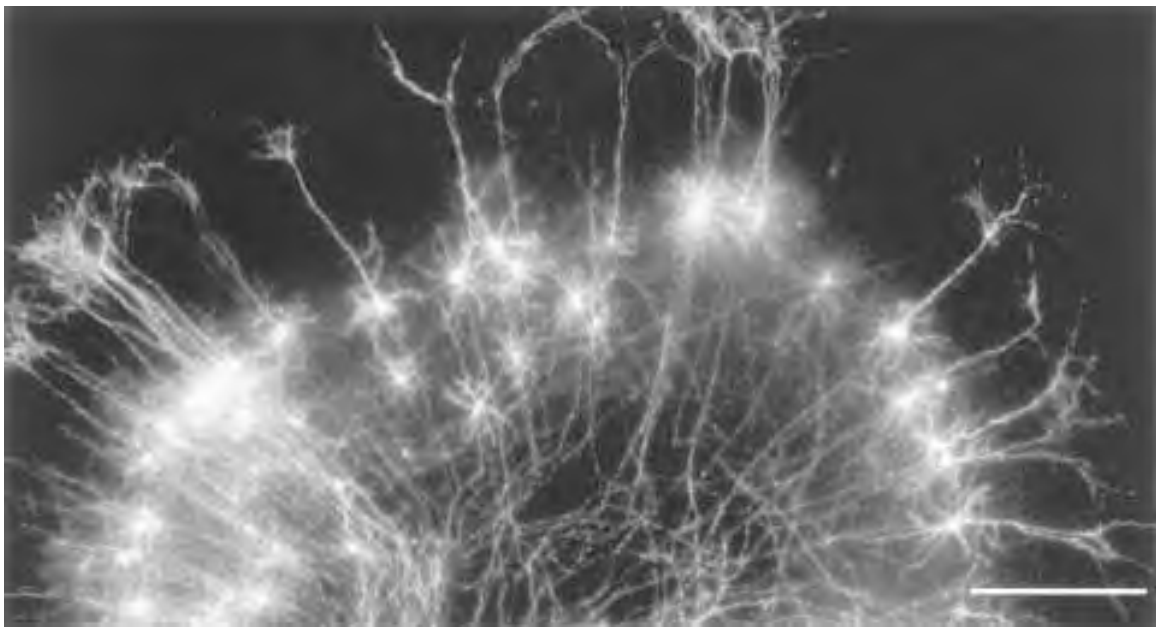

Fig. 4. Cortical projection neurons in a cortical slice co-cultured with an explant from the superior colliculus. The co-culture was kept for 12 d in the incubator under continuous rotation. The fluorescent dye, DiI, was then placed in the tectal explant, to label cortical neurons that established connections with their target in vitro. The laminar position and the cell morphology of cortical projection neurons in vitro is similar to their counterparts in vivo. Scale bar: 500 μm.

cultures were kept stationary in the incubator for 48 h, and axonal outgrowth was then analyzed after fixation with 4% paraformaldehyde and 3% sucrose dissolved in PBS (Fig. 4).

3.1. Materials

Scissors (1), large.
Scissors (1), small.
Forceps (2), fine.
Scalpel blade.
Spatula (1), large.
Spatulas (2), small.
Medium (3.0 mL).
GBSS/glucose (30 mL).
GBSS (2 mL).
Chicken plasma (20 μL).
Thrombin solution (20 μL).
Petri dishes (2), 100-mm.
Petri dish (1), sterile, glass, 100-mm.
Coverslips (4), 11 × 22 mm.
Plastic tubes, Nunc, flat, 110 x 16-mm.
Ice bucket and ice.
Roller drum.
Tissue chopper (McIlvain).

3.2. Preparation of Cortical Slices

1. Preparation for dissection:
   a. Sterilize instruments, using the hot bead sterilizer. When the temperature reaches 250°C, insert dry and clean instruments for at least 5–10 s, depending on their size. When sterile, put the instruments into a sterile glass Petri dish, and keep the ends covered with the lid.
   b. Place the GBSS/glucose in an ice bath. Pipet four, drops (1 mL each) of this solution about equally spaced in a 100-mm Petri dish.
2. Dissection:
   a. Anesthetize a young postnatal rat by Metofane inhalation, then decapitate the animal with large scissors. Using forceps, hold the head on the bottom of the Petri dish. With small scissors, cut the skin along the midline and unfold the skin with two forceps. Then cut the skull along the midline and remove the whole brain with a large spatula, and place it in a drop of GBSS/glucose in the Petri dish.
   b. Cut the two hemispheres apart with a scalpel blade, and place them pia side down in a drop of GBSS/glucose. Under a dissecting microscope, hold the tissue with forceps placed in the striatum. With a scalpel, make a small cut at the anterior and posterior poles of the hemisphere, then unfold the hemisphere with a second forceps. Make two lateral cuts to obtain a rectangular block of (neo)cortex. Transfer each block to a third drop of GBSS/glucose. Remove the pia carefully with two forceps.
3. Cortical slices:
   a. Add 5 mL cold GBSS/glucose to a 100-mm Petri dish.
   b. Place a block of cortex, with pia side down, on the tissue chopper, remove fluid with a pipet, and cut 300-µm-thick slices.
   c. Transfer the slices with a large spatula into a new 100-mm Petri dish containing cold GBSS and glucose. Under a dissecting microscope, carefully separate the slices with a small spatula.
   d. Store the slices at 4°C for 30–45 min. This allows the diffusion of enzymatic factors released by damaged cells.

3.3. Preparation of Plasma Clot

1. Place four glass coverslips in a 100-mm Petri dish; pipet 20 µL chicken plasma solution into the center of each coverslip. Transfer a slice into the plasma drop (*see* Section 4.6.).
2. Pipet 20 µL thrombin solution next to the plasma clot, and gently hold the coverslip with forceps on the bottom of the Petri dish (Section 4.7.). Then carefully mix the two drops with a small spatula, and spread the mixture over the entire surface of the coverslip. Keep the slices in the Petri dish 30–45 min. to allow coagulation of the plasma.
3. Place the coverslip in a Nunc plastic tube. and add 750 µL culture medium (*see* Section 4.1., item 2.)
4. Transfer the tubes in a roller-drum incubator (10 revolutions/h) at 37°C in dry air.

3.4. Maintenance of the Cultures

1. Change the medium every 2–5 d, depending on the size and metabolic activity of the slices, as indicated by a change in color of the medium from orange to yellow.
2. After 2–3 d in vitro, to prevent excessive growth of glia and other neuronal cells, 7.5 µL mitotic inhibitor solution is added to each culture tube for 24 h.

4. APPENDIX

4.1. Medium

1. Culture medium for outgrowth assay.
   100 mL Eagle's basal medium (Gibco-BRL, cat no. 41100025), 50 mL Hank's balanced salt solution, 50 mL horse serum.
   a. Mix Eagle's basal medium and Hanks' balanced salt solution.
   b. Add 0.8 g (4 mg/mL) methylcellulose (Sigma, cat no. M-7027). Agitate at 4°C for 2 h, or until dissolved.
   c. Inactivate the horse serum at 56°C for 30 min.
   d. Add 0.1 m*M* glutamine and 6.5 mg/mL glucose.
   e. Add antibiotics (streptomycin 10 mg/mL; penicillin, 10,000 U/mL); antifungic (amphotericin B 2.5 mg/mL); and the horse serum.
   f. Sterilize by filtration through a 0.2-μm filter.
2. Medium for cortical slices.
   Same medium as for outgrowth assay, but without the methylcellulose. Annis et al. (1990) described a serum-free medium for organotypic slice cultures.
3. GBSS/glucose.
   a. GBSS (Gibco-BRL, cat. no. 14260-020).
   b. Add 6.5 mg/mL glucose.
   c. Sterilize by filtration through a 0.2-μm filter.

4.2. Laminin and Polylysine

1. 1 mg/mL Laminin (Sigma, cat. no. L-2020; Gibco-BRL, cat. no. 23017-015).
   a. Sterilize with a 0.2-μm filter (nonpyrogenic, hydrophilic).
   b. Aliquot, and store at –20 °C.
2. Poly-L-lysine hydrobromide (Sigma, cat. no. P1399).
   a. Dilute to 1 mg/mL with high-quality distilled water (dH_2O).
   b. Sterilize by filtration through a 0.2-μm filter.
   c. Aliquot into 10-μL amounts, and store at –20°C.
3. Laminin-polylysine solution.
   Add 3 μL laminin and 3 μL polylysine stock solution to 300 μL GBSS.

4.3. Homogenization Buffer (H-Buffer)

Tris-HCl, pH 7.4	10.0	m*M*
$CaCl_2$	1.5	m*M*
Spermidine (Sigma, cat. no. S-4139)	1.0	m*M*
Aprotinine (Sigma, cat. no. A-1153)	25.0	μg/mL
Leupeptine (Sigma, cat. no. L-2884)	25.0	μg/mL
Pepstatine (Sigma, cat. no. P-4265)	5.0	μg/mL
2,3-Dehydro-2-deoxy-*N*-acetyl-neuraminic acid (Sigma, cat. no. D-4019)	15.0	μg/mL

4.4. Membrane Preparation

1. Dissect the postnatal cortex (P0–P7) in GBSS supplemented with glucose (6.5 mg/mL), and remove the pia.
2. Pool blocks of cortex in an ice-cold H-buffer.

3. Homogenize the cortex first with a 1-mL pipet tip, then with a 27-gage injection canula.
4. Centrifuge for 10 min at 50,000*g* with a Beckman ultracentrifuge (rotor TLS 55) in a sucrose step gradient (upper phase, 150 µL 5% glucose; lower phase, 350 µL 50% sucrose).
5. Wash the interband containing the membrane fraction twice in CMF PBS, which also contains the inhibitors, leupeptine, pepstatine, aprotinine, and 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (same concentration as in the H-buffer), at approx 12,000*g* in an Eppendorf Biofuge.
6. After resuspension in CMF PBS without the inhibitors, the concentration of the purified membranes is determined by its optical density (OD) at 220 nm with a spectrophotometer. Adjust the concentration of the membrane suspension, so that an aliquot diluted 1:15 in 2% sodium dodecyl sulfate has an OD of 0.1.
7. The membranes can be frozen in a solution of 50% CMF PBS and 50% glycerol solution. In this case, adjust the OD to about 0.2 and make aliquots of 200 µL. Before use, add 1 mL CMF PBS, recentrifuge, and resuspend in 300 µL PBS. Adjust the OD to 0.1.
8. To prepare membranes from transfected cell lines, Petri dishes (9 cm diameter) with confluent cells are used. The culture medium is replaced by 1 mL H-buffer, and the Petri dish is placed for 1 min on dry ice. Cells are then detached from the dish with a cell scrapper, and the cell suspension is collected in a small plastic tube. We usually combine cells from four Petri dishes. Cells are homogenized with a 27-gage injection needle, and membranes are prepared according to the protocol described above for cortical tissue.

4.5. Mitotic Inhibitor Solution

1. Dissolve 2.46 mg 5-fluoro-2-deoxyuridine (Sigma, cat. no. F-0503) in 10 mL dH_2O.
2. Dissolve 2.8 mg cytosine-D-arabinofuranoside (Sigma, cat. no. C-6645) in 10 mL dH_2O.
3. Dissolve 2.4 mg uridine (Sigma, cat. no. U-3750) in 10 mL dH_2O.
4. Mix 5-fluoro-2-deoxyuridine, uridine, and cytosine-D-arabinofuranoside 1:1:1. Sterilize by filtration, using a 0.2-µm filter.
5. Store at –20°C in 200-µL aliquots.

4.6. Plasma

Chicken plasma (Sigma, cat. no. P-3266) is reconstituted with 1 mL sterile dH_2O. Sterilize by filtration, using a 0.2 µm filter. Five-hundred microliter aliquots are stored frozen at –20°C.

4.7. Thrombin

1. Reconstitute thrombin (10,000 U; Sigma, cat. no. T-4648) with 8 mL dH_2O.
2. Centrifuge for 30 min at 600*g*.
3. Sterilize by filtration, using a 0.2-µm filter.
4. Store 30-µL aliquots frozen at –20°C.
5. Before use, add one 30 µL aliquot to 1 mL GBSS.

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Chapter Two

Interface Organotypic Hippocampal Slice Cultures

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1. INTRODUCTION

In complex tissues such as the central nervous system, differentiation and functional activity often require temporally and spatially dynamic epigenetic cues that cannot be reproduced in dissociated cell cultures. Organotypic cultures and, among them, hippocampal slice cultures represent in vitro models that keep the different cell types and retain the complex three-dimensional organization of the nervous tissue. In addition, much of the appropriate synaptic circuitry, physiology, and neurotransmitter receptor distribution of the intact hippocampus is preserved in this type of culture (Bahr, 1995; Gahwiler et al., 1997). Functional activities of neurons in slice culture were found to be similar to their *in situ* counterparts. These cultures can be grown for many weeks without any passages. More mature neurons (until 21–23 d postnatal) can be grown with slice culture, but only embryonic or neonatal neurons can be grown with dissociated cell culture techniques. Finally, if needed, even more complexity can be achieved by co-culturing different brain target areas.

Two classical techniques have been previously described for preparing organotypic cultures, the Maximov chamber procedure (Toran-Allerand, 1990) and the roller tube method. Gahwiler (1981) has characterized the latter in detail. In both of these techniques, the basic procedure involves the attachment of a tissue explant onto a glass coverslip.

The principle of the interface type of culture we will described below is to maintain nervous tissue on a porous and transparent membrane at the interface between the culture medium and the atmosphere (Stoppini et al., 1991). No plasma clots or roller drums are used. The important factor to ensure good tissue survival is to add the culture medium only below the membrane of the culture insert. By capillarity, the culture medium crosses the membrane and covers slices by a thin film of medium. In this condition, explants do not dry out, and remain well-oxygenated (Fig. 2A).

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2. SLICE CULTURES ON MEMBRANES

2.1. Animals

Brain tissue from both rats and mice (wild-type or transgenic mice) is routinely used for the preparation of slice cultures. The best results, in terms of reproducibility, survival, and morphological organization of hippocampal slice cultures, have been obtained with animals between 0 and 15 d old for rats, and between 0 and 5 d for mice.

2.2. Materials

Scissors (1), large, 16 cm.
Scissors (1), fine, 9 cm.
Forceps (1), curved, fine (Dumont-type, no. 7).
Forceps (1), straight, fine (Dumont-type, no. 5).
Holder (1), for razorblade knives, curved (Aesculap, cat. no. BA 290).
Razorblades.
Spatulas (4), metal with polished edges, straight or angled.
Minimum essential medium (MEM) (Gibco-BRL, Eur. cat. no. 11012-010).
Horse serum, mycoplasma-screened (Gibco-BRL, Eur. cat. no. 26050-047).
Note: Serum is stored at –20°C for up to 18 mo and thawed slowly at room temperature. Horse serum is heat-inactivated in a water bath at 56°C for 30 min, before using to inactivate complement.
Penicillin—streptomycin solution (10,000 U penicillin—10,000 µg streptomycin; Gibco-BRL, Eur. cat. no. 15140-114).
Hanks' balanced salt solution (HBSS) (Gibco-BRL, Eur. cat. no. 042-0460M).
Insert, culture with 3-mm membrane (Millipore, cat. no. PICMOR G50) or Insert, 25-mm, 4.15 cm^2 (Nunc, cat. no. 161395).
Multiwell plates, 6-well (Falcon, cat. no. 3046).
Petri dishes, 35-mm, plastic, sterile (Falcon, cat. no. 1008).
Petri dishes, 100-mm, plastic, sterile (Falcon, cat. no. 1029).
Membrane punch (8-mm).
Syringes (2, 5, and 10 mL).
Needles (20-gage).
Pasteur pipet (1), fire-polished, cut.
Note: Cut pipets should have openings of 2–5 mm; the size of the opening depends on the age of the animal.
Pasteur pipet (1), curved, fire-polished (to unroll hippocampus).
Stereo microscope.
CO_2 incubators (2), one set at 36°C, the other at 33°C.
Tissue chopper (McIlwain, Brinkmann, Westbury, NY).

2.3. Preparation for Slice Cultures on Membranes

1. Media:
   a. Serum-based medium (100 mL).
      25 mL 2X MEM, with 25 m*M* HEPES. 2X MEM may be stored at 4°C for up to 6 mo.
      25 mL Horse serum.
      25 mL 10X HBSS. HBSS may be stored at room temperature for up to 6 mo.

1 mL 100X penicillin—streptomycin (if needed).
60 mg Tris (Fluka, cat. no. 93349) (5 m*M* final concentration).
7.5% $NaHCO_3$ (Fluka, cat. no. 71627 (460 µL).
Sterile, double-distilled water (DW) of high quality to 100 mL.

b. Dissection medium.
For the dissection of brain tissue and the preparation of the cultures, the authors' experience has been that the pH of the regular culture medium is not sufficiently stable. Therefore, a dissection medium of the following composition is used: MEM 100% + Tris (10 m*M*), pH 7.2.

c. Filtering.
Dissecting and culture media are filtered into 500-mL sterile bottles, using bottle-top filters (Millipore Steritop 0.22-µm SCGPT05RE). Sterile dissecting medium is made fresh for each experiment; culture medium can be kept refrigerated at 4°C for 2 or 3 wk, or stored at –20°C for several months.

2. Preparation of the culture inserts.
One millileter culture medium is added to each well in a multiwell plate. A culture insert is placed into each well. Both the Millipore and Nunc culture membranes should become completely transparent when wetted. The multiwell plate is placed in the CO_2 incubator 1 h prior to explantation, to warm up and to adjust the pH of the culture medium.

2.4. Dissection

Decapitation of the rat and dissection of brain tissue are carried out under sterile conditions.

1. Using small scissors, make an incision along the midline of the head, to simultaneously cut the skin and the skull to expose the brain. Move away the sectioned skull with forceps. Remove the brain with a spatula, while cutting the cranial nerves (Fig. 1B,C).
2. Drop the brain into precooled dissecting medium. Make a saggital cut through the interhemispheric sulcus, and separate the two hemispheres (Fig. 1D).
3. Hold one hemisphere with forceps placed in the frontal part of the brain. Cut off the neighboring thalamus and basal ganglia situated on top of the hippocampus. Remove the pia carefully with two forceps (Fig. 1E).
4. Unroll the hippocampal structure by placing a curved, fire-polished glass pipet underneath the hippocampus and the underlying cortex, and sever the septo-hippocampal connections with a scalpel (Fig. 1F and G).

2.5. Hippocampal Slices Using the Tissue Chopper

Although the vibratome approach can be used to slice brain tissue, we found that, for the hippocampal structure, the chopper device is more appropriate.

1. Place one or two hippocampi on a Teflon stage of a McIlwain-type tissue chopper. Aspirate the excess buffer medium. The hippocampi will adhere to the Teflon plate.
2. Align the hippocampus perpendicular to the chopper blade. Slices (250–400 µm) thick are rapidly cut (2/s) by the tissue chopper.

Note: The thickness of the slices will depend on the age of the animal. The older the animal, the thinner should be the slice, with a compromise around 300 µm.

3. Using a cut, fire-polished Pasteur pipet, transfer the slices to a Petri dish containing dissection medium. Place the Petri dish under a dissecting microscope, so that slices can be gently separated under visual control.

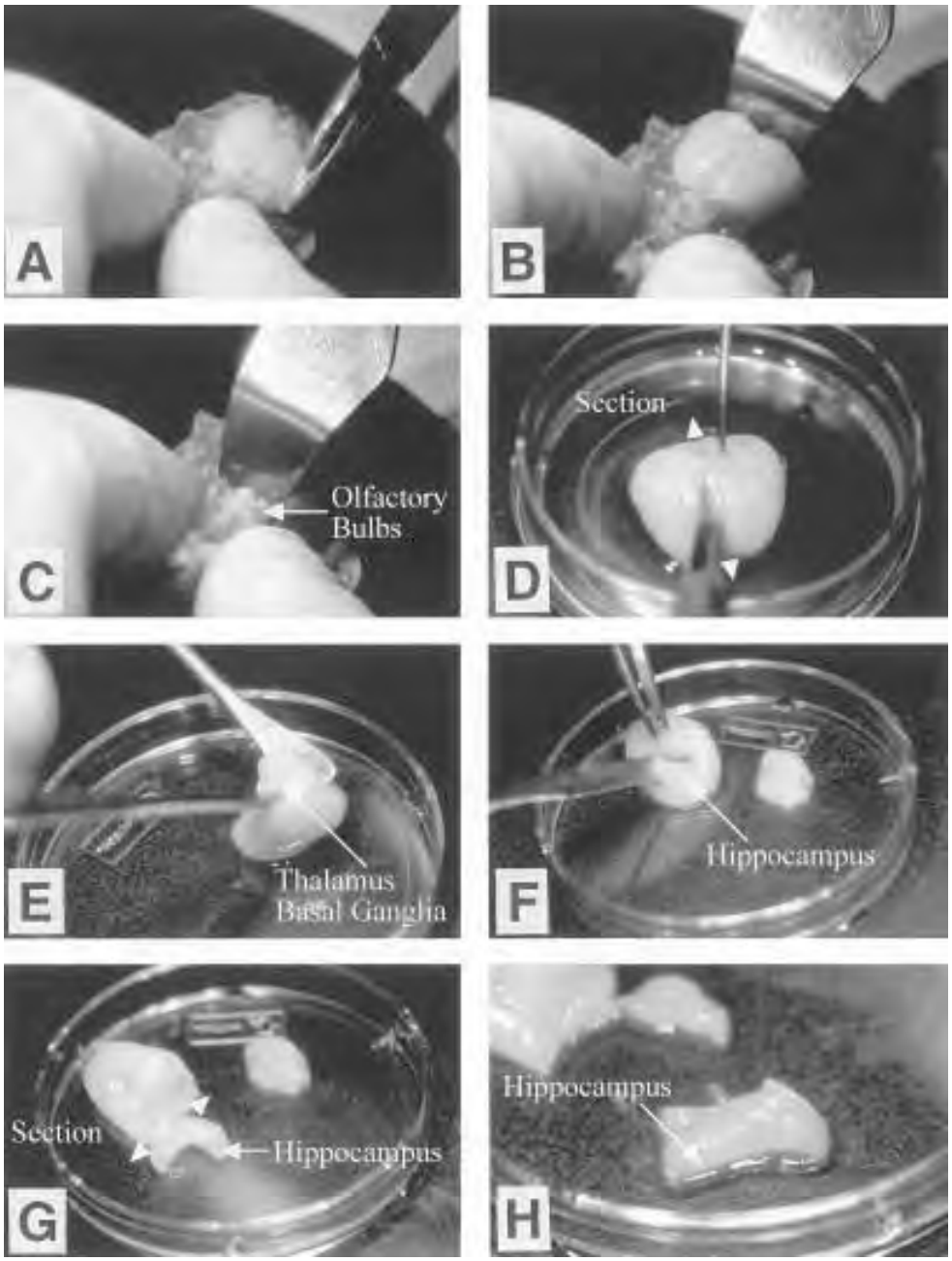

Fig. 1. Dissection protocol. The sectioned skull is moved away with forceps **(A)**. The cerebellum **(B)** and the olfactory bulbs **(C)** are cut with a curved spatula. The brain is removed with a spatula and dropped into precooled dissecting medium. A saggital cut is made through the interhemispheric sulcus to separate the two hemispheres **(D)**. One hemisphere is held with forceps placed in the frontal part of the brain, while neighboring thalamus and basal ganglia, situated on top of the hippocampus are cut off **(E)**. The hippocampal structure is unrolled by placing a flat spatula underneath the hippocampus **(F)**, and the underlying cortex and the septo—hippocampal connections are severed with a scalpel **(G)** to detach the hippocampus **(H)**.

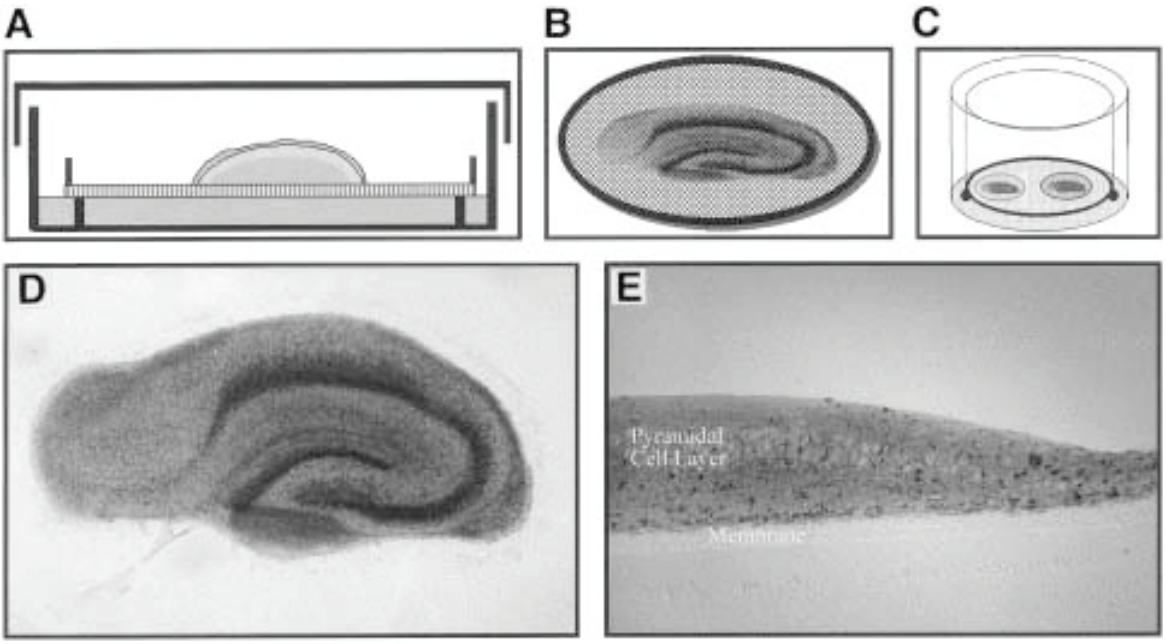

Fig. 2. **(A)** Representation of a transversal scheme a slice culture grown onto the surface of a culture insert placed in a Petri dish. An alternative procedure is to grow slices onto precut patches of membrane **(B)**. These slices on disks are then placed onto the membrane of classical culture inserts **(C)** or onto the surface of solid agarose medium (no show). The general organization of the HC from a 10-d-old rat is preserved in a slice after 2 wk in culture **(D)**. The pyramidal cell layer has spread into a horizontal layer in the middle of the slice **(E)**.

4. Transfer selected individual slices (using a cut, fire-polished Pasteur pipet), with a drop of dissection medium, onto a Millipore insert, or on a 25-mm Nunc filter, in one of the wells of the prepared multiwell plate (Fig. 2A).

Note: Four hippocampal slices can be plated on a single insert.

Remove the excess dissection medium on the insert, using a regular Pasteur pipet (Fig. 2A).

5. Immediately transfer the multiwell plate into an incubator equilibrated with 5% CO_2 in air at a temperature of 37°C.

2.6. Maintenance of Cultures

During the first 4 d, slice cultures are maintained in a CO_2 incubator at a temperature of 37°C. The medium is changed the day after preparation of the cultures, then twice a week. For long-term cultures, we found that maintaining the cultures after the first week in a CO_2 incubator set at 33–34°C yields better results. No irradiation or antimitotic treatments are necessary.

Horse serum is required during the first few days, to allow the slice cultures to recover from the explantation trauma. Serum-free media can then be used to maintain slice cultures for several days. The recovery processes (microglial cell phagocytic activities) are speeded up at 37°C, although, when growing slice cultures from older animals (>15-d-old), tissue cultures should be transferred directly into an incubator, with the temperature set at 33°C, to ensure a better survival.

From a thickness of 400 µm, slice cultures will flatten to 150 µm after 1 wk in culture. Pyramidal neurons will spread out, and will remain organized into a 3–4-cell layer surrounded by a dense network of apical or basal dendrites and glial processes (Fig. 2E).

3. PRECUT PATCHES OF MEMBRANE

3.1. Precut Patches of Membrane in Culture Insert

For experiments in which slice cultures will later be transferred to specific chambers, such as for electrophysiological recordings or for histological studies, we use small patches of polytetrafluoroethylene (PTFE) membranes (6, 8, or 10 mm in diameter; Millipore, cat. no. FHLC0477). Other types of membrane can also be used, e.g., Whatman Cyclopore Polyethylene terephthalate (PET) (1.0 μm, Whatman, cat. no. 70690310) and Whatman Anodisc membranes (13 mm, 0.2 μm; Whatman, cat. no. 68097023).

Note: Precut patches of PTFE and PET membranes, 6–10 mm in diameter, can only be purchased from BioCell-Interface (www.BioCell-Interface.com).

1. At the time of preparation of the cultures, the patches of membranes are humidified with culture medium and placed inside a Petri dish.
2. The slice of brain tissue (normally, one slice per disk) is then transferred, with a drop of dissection medium, onto the disk of membrane, and the excess of medium is removed while keeping the slice on the membrane (Fig. 2B). Do not let slices dry out.
3. The patch of membrane bearing the hippocampal slice is then transferred onto the membrane of the culture insert (Fig. 2C).
4. Comment: Within a day, the slice cultures will adhere to the membrane patches, then can easily be transferred to any place by simply picking up the precut patches with fine tweezers. The patches, as well as the membrane culture inserts, can be treated with poly-L-ornithine or poly-L-lysine to increase adherence of the slice culture to the membrane. We find, however, that this is not routinely necessary.

3.2. Interface Slice Cultures on Disks of Membrane onto Solid Agarose Medium

Instead of using culture inserts, the precut patches of membrane bearing tissue slices can also be transferred onto solid medium, using, e.g., agarose to gel the culture medium. The procedure to transfer the hippocampal slice onto the patch of membrane is similar to that described above. The disk of membrane with the slice of hippocampus is laid down onto the surface of the solid agarose medium. Transfer slice cultures on patches onto a new agarose medium every 2 wk (*see* Appendix 7.5).

4. ELECTROPHYSIOLOGY

The viability and functional connections in hippocampal slice cultures on membranes can be analyzed either in submerged or in interface types of chambers. While the regions of the slice cultures enriched in glia cells are getting thinner, neuronal layers remain thick, and thus allow recordings of large field potentials. See Section 7 for preparation of artificial cerebrospinal fluid.

4.1. Electrophysiological Chambers

1. Slice cultures are placed in an interface type of chamber (Dunwiddie and Lynch, 1978), gassed with a humid atmosphere composed of 95% O_2/5% CO_2, and continuously perfused with the artificial cerebrospinal fluid.
2. The temperature is maintained at 34°C by warming a bath filled with dH_2O. The cultures can be kept for several hours in this chamber.
3. The stimulation electrodes, made of twisted nichrome wires, are usually placed on a group of CA3 neurons of the hippocampus and synaptic responses are recorded extracellularly, using pipets filled with saline (about 5 Mohm resistance) in the CA2–CA1 region.

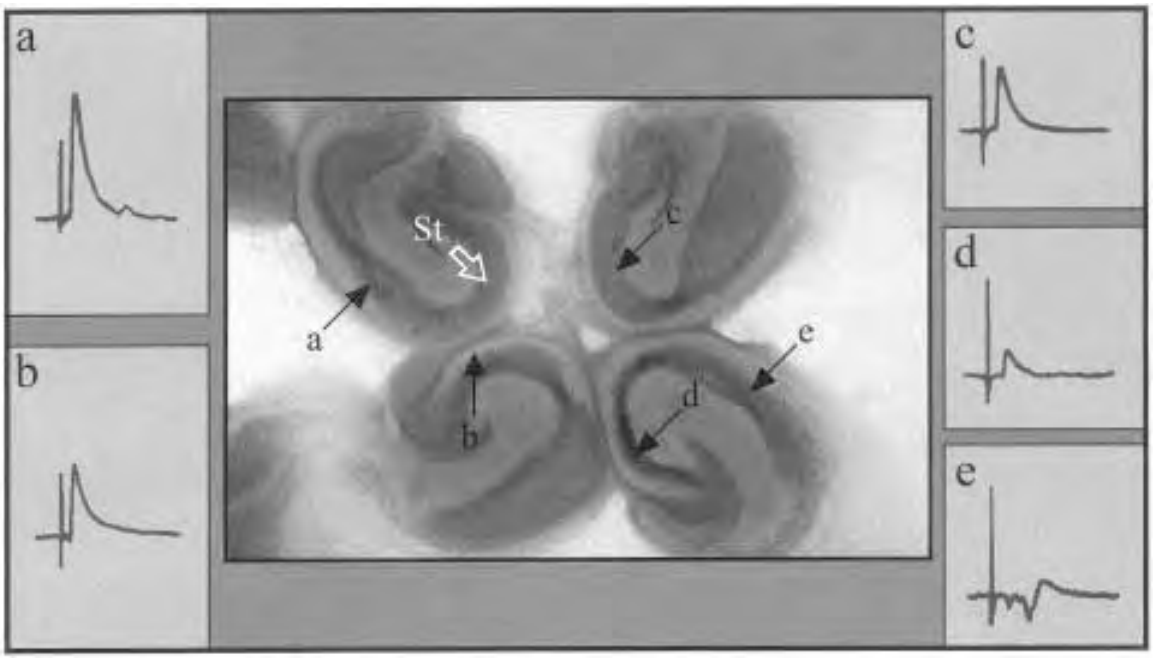

Fig. 3. After 1 wk in co-culture, electrophysiological studies were performed on explants. Electrodes of stimulation were placed in the CA3 area of the upper left slice; the recording electrode was placed either in the CA1 of the same culture (a) or in the three other slice cultures, in different areas (b–e). We observed that, in each case, evoked responses were elicited, indicating functional connections.

4. The maximal size of evoked synaptic responses recorded in the cell body or dendritic layer are used as an index of the number of functional synaptic contacts that were activated by stimulation of a group of neurons.

Note: Figure 3 shows an example of evoked synaptic activities recorded in a co-culture of four hippocampal slice cultures.

4.2. Comment

After 2–3 wk in culture, some slice cultures can present spontaneous epileptical activities. These rhythmic activities may induce neuronal loss, a phenomenon that can be precluded by adding glutamate receptor antagonists (Pozzo-Miller et al., 1994).

5. IMMUNOSTAINING

5.1. Solutions

1. Paraformaldehyde 4%.
2. Methanol (biochemistry grade, Fluka, cat. no. 65535).
3. Phosphate-buffered saline (PBS) 0.1 *M*.
4. PBS 0.1 *M* with 0.2% Triton X-100 (Merck, cat no. 12298).

5.2. Fixation of Tissue

1. Paraformaldehyde 4%.
   a. Dip the tissue for 30 min into a 4% paraformaldehyde solution.

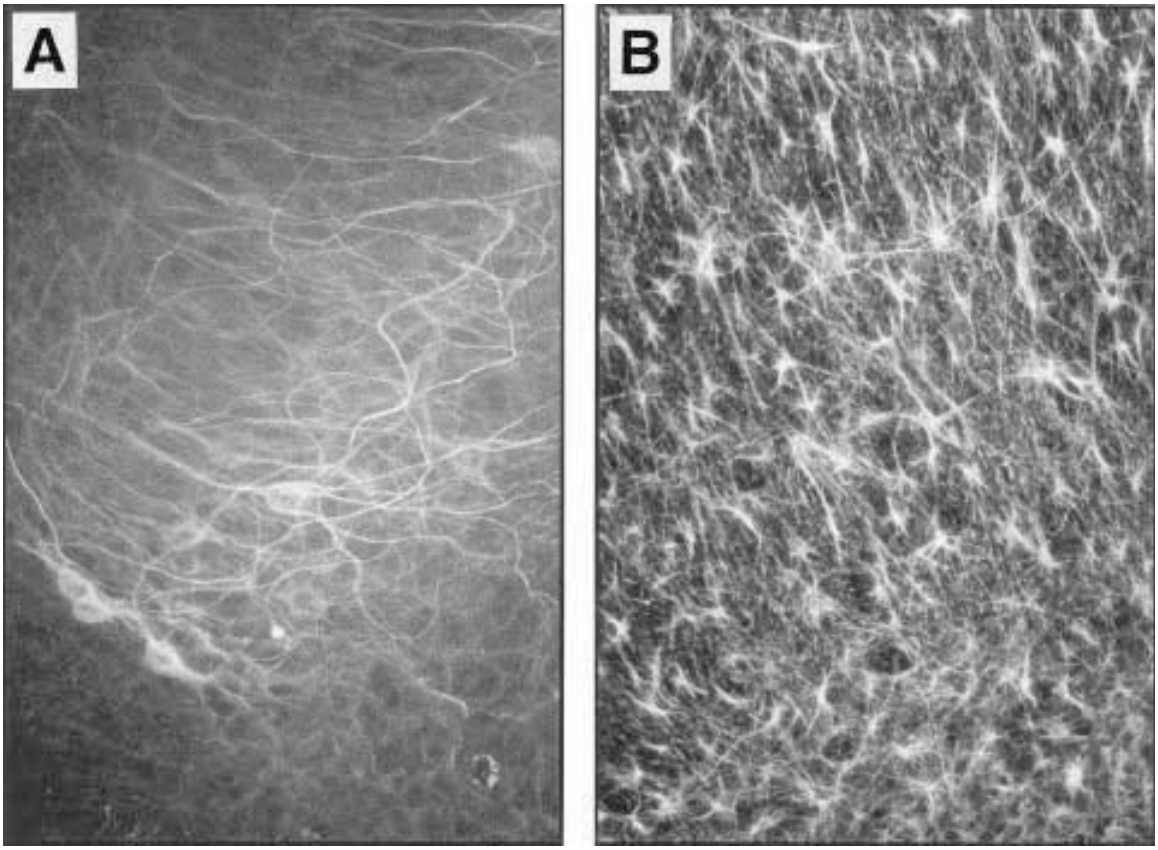

Fig. 4. Immunostainings of a pyramidal neuron using neurofilament antibodies **(A)**, and astrocytes using glial fibrillary acidic protein antibodies **(B)**. Astrocyte are principally localized on top and below the pyramidal cell layer.

   b. Rinse 2× with PBS (0.1 *M*) for 10 min at room temperature.
   c. Rinse 1× with PBS 0.1 *M* containing Triton X-100 detergent for 10 min, to make the membrane permeable to antibodies (Abs).
2. Methanol (–20°C).
   Dip the tissue for 5 min into pure methanol precooled at –20°C.

Note: This type of fixation works well for cytoskeleton staining.

3. Paraformaldehyde 4% and methanol (–20°C).
   This is a combination of the two above-described methods that allows some double staining.
   a. Dip the tissue for 30 min into a 4% paraformaldehyde solution.
   b. Rinse the slice culture with PBS 0.1 *M* for 30 min.
   c. Dip the tissue into methanol –20°C for 5 min.

5.3. Antibody Incubation

Incubation can last from a few hours to several days, depending on the type of antibodies and the thickness of the slice.

1. Primary antibodies.
   a. As examples, slice cultures were incubated overnight at 4°C with neurofilament antibodies (Sigma, 68 kDa monoclonal antibody, dilution 1:50 or neurofilament antibody,

Boehringer, cat. no. 814326) (Fig. 4A) or with glial fibrillary acidic protein antibodies (Dako, cat. no. Z0334; dilution 1:50) (Fig. 4B).

b. Slices were left on the membrane during the whole immunostaining procedure.
c. A 50 μL drop of antibodies was placed on both sides of the membrane and trapped with parafilm.

2. Secondary antibodies.
   After three rinses with PBS, reactive sites were visualized by an antimouse rhodamine secondary antibody (Boehringer, cat. no. 1214608; dilution 1:10, incubation time 2 h, protected from light).
3 After the last wash in PBS, the slice culture grown on a membrane patch is placed on a glass slide and mounted with SlowFade antifade kit (Molecular Probes, cat. no. S-2828). The whole mount is then glued with nail polish.

6. TRANSMISSION ELECTRON MICROSCOPE PROCESSING OF HIPPOCAMPAL ORGANOTYPIC CULTURES

A simple method is described here to allow observation of hippocampal organotypic cultures at the level of electron microscopy. Tissue slices cultured on hydrophilic PTFE membrane disks can be easily manipulated individually for classical fixation, and a step of flat embedding allows precise transverse, as well as longitudinal sectioning.

6.1. Materials

Tweezers (1), small (Regine S.A.).
Scissors (1).
Razor blades.
Potassium hydroxide (KOH), granules.
Bottles (2), glass, 25 mL, with waterproof caps.
Glass slides.
Petri dish, glass, paraffin-filled, with a black cover.
Petri dish, glass, paraffin-filled.
Pasteur pipets.
Copper grids (200-mesh).
Empty gelatin capsules.
Plastic foil (3), approx 10 × 10 cm, transparent, heat resistant.
Note: One 10 × 10 cm sheet will have a hole of about 2 cm diameter in the middle. The plastic foils must be tested before the first use. To test, put 1 mL resin epoxy between two plastic foils, and let polymerize at 60°C. The plastic foils should not undulate.
Magnetic mixing bar.
Drying oven.
Ultramicrotome (we use Reichert-Jung) with glass and a Diatome diamond knife.

6.2. Preparation of Solutions (Quantities for One Culture)

1. Fixation solution (10 mL): glutaraldehyde,1.5%, and paraformaldehyde, 1.0%, in PBS 0.1 *M*, pH 7.4, 4°C.
2. Wash solution (50 mL): PBS 0.1 *M*, pH 7.4.
3. Postfixation solution (5 mL): Osmium tetroxyde 1% in PBS 0.1 *M*, pH 7.4.
4. Dehydration alcohols.

   a. Ethanol solutions (15 mL each) at 25, 50, 75, and 95%.
   b. 100% Ethanol, 50 mL.
5. Pre-embedding solution:
   a. 20 mL Propylene oxide (Fluka, cat. no. 82320) 100% to 26 mL EPON.
   b. To prepare 26 mL EPON, add 16 g epoxy embedding medium (Fluka, cat. no. 45345), 5.7 g dodecenyl succinic anhydride (Fluka, cat. no. 45346), and 9 g MNA (Fluka, cat. no. 45347) into a glass bottle. Mix gently with magnetic stir bar, add 0.45 g 2,4,6-tri(dimethylaminomethyl) phenol-30 (EMS, cat. no. 13600), and mix again. EPON can be conserved for 1 mo at –20°C.
6. Sectioning solution: EPON colorant solution (400 mL): Mix 2 g methylene blue, 2 g Azur II, and 2 g Borax in 400 mL dH_2O. Filter and conserve at room temperature.
7. Staining solutions:
   a. Uranyl acetate, 5%.
      i. 1 g uranyl acetate in 20 mL water.
      ii. Store at 55°C in a sealed metal box. The solution is light-sensitive and radioactive.
   b. Lead citrate.
      i. 1.33 g $Pb(NO_3)_2$ (lead nitrate), 1.76 g $Na_3(C_6H_5O_7)\cdot 2H_2O$ (sodium citrate), 30 mL dH_2O.
      ii. Shake vigorously for 30 min, then add 8 mL NaOH 1 *N* and dilute the suspension to 50 mL with dH_2O.
      iii. Centrifuge if any faint turbidity is present.
      iv. Store at 5°C.

6.3. Protocol

1. Fixation.
   Take a slice culture on its membrane (if needed, cut the surrounding membrane), and put in a glass bottle containing 1.5% glutaraldehyde and 1% paraformaldehyde in PBS 0.1 *M*, pH 7.4, at 4°C for 1 h 30 min.
2. Wash 3× for 10 min each, with PBS 0.1 *M*, pH7.4, at 4°C.
3. Postfixation.
   Postfix in the dark for 1 h at 20°C with 1% osmium tetroxide in PBS 0.1 *M*, pH 7.4.
4. Wash 3× for 15 min each, with PBS 0.1 *M*, pH 7.4 at 20°C.
5. Dehydration (all steps at 20°C):
   25% Ethanol: 5 min.
   50% Ethanol: 5 min.
   75% Ethanol: 10 min.
   95% Ethanol: 10 min.
   100% Ethanol 3× for 20 min each.
6. Pre-embedding:
   a. 50% Ethanol in propylene oxide: 5 min.
      100% Propylene oxide: 3× for 5 min each.
   Note: These steps must be completed as quickly as possible to avoid drying.
   b. Then pre-embed into:
      i. A mixture of 50% propylene oxide in EPON for 2 h.
      ii. Then in a mixture of 30% propylene oxide in EPON for another 2 h.
      iii. Then overnight in a new bottle containing pure EPON.

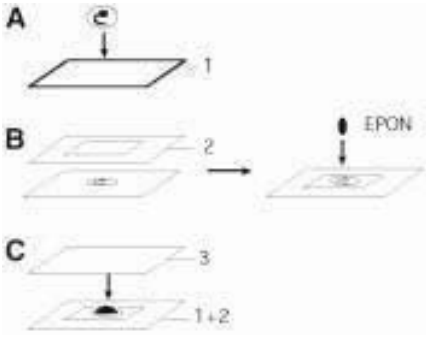

Fig. 5. Flat-embedding.

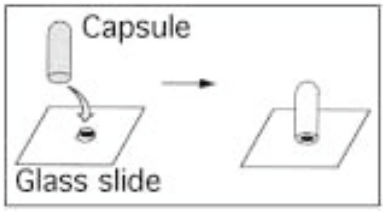

Fig. 6. Embedding a longitudinal section.

7. Flat-embedding (Fig. 5):
   a. Place the culture on the first plastic foil (Fig. 5A).
   b. Place the second, perforated foil on top of the culture, and add a drop of EPON on the culture (Fig. 5B).
   c. Finally, place the third plastic foil on top, press gently to flatten the culture (Fig. 5C), and let polymerize at 60°C overnight.
8. Embedding:
   a. Remove the plastic foil numbers 1 and 3. The culture will be surrounded by polymerized EPON and stay in the hole of the second plastic foil.
   b. Cut the surrounding membrane of the culture, and dip the culture for approx 5 min in EPON.

Note: For longitudinal sections, place the culture on a glass slide, membrane down, and cover with a gelatin capsule filled with EPON (Fig. 6). For transverse sections, traditional embedding in a silicone matrix can be used. Polymerize at 60°C for 48 h. The gelatin capsule and the tissue can be removed from the glass slide by heating at about 70°C for 5 min.

9. Sectioning:
   a. Trim the block in the gelatin capsule, using a clean razorblade.
   b. Sections 2 µm-thin can be stained with the EPON colorant solution for 1–5 min at 50°C for light microscopic observations of the tissue and final trimming in the region of interest.
   c. Ultrathin sections of approx 60 nm are realized on an ultramicrotome and mounted on uncoated copper grids (200-mesh).
10. Staining:
    a. Place drops of uranyl acetate on the surface of a paraffin-filled Petri dish. Stain the grids on top of a drop for 25 min under light protection of a black cover, then wash with jets of dH_2O and allow to dry for about 30 min.
    b. Place drops of lead citrate on the surface of a paraffin-filled Petri dish in the presence of KOH granules, to avoid CO_2 dissolution in lead citrate and carbonate precipitation. Stain the grids for 35 s and wash them with 0.02 *N* NaOH in dH_2O, then with dH_2O alone, and allow to dry.

Note: Figure 7 shows an example of an electron microscopic picture of a hippocampal slice culture neuropil taken in the stratum radiatum region.

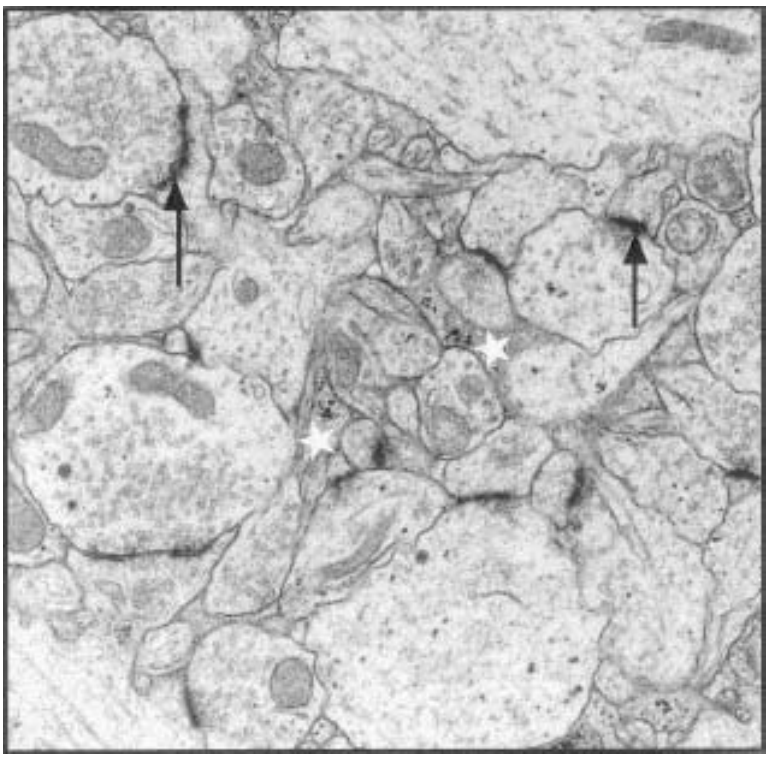

Fig. 7. Electron microscopy picture showing the parenchyma of a hippocampal slice culture in the stratum radiatum region. The neuropil is dense and the different types of synapses can be found (arrows), as well as glial cell processes (stars). Bar = 2 μm.

7. APPENDIX

7.1. MEM (Gibco-BRL, Eur. cat. no. 11012)

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	140.00
KCl	400.00
KH_2PO_4	60.00
$MgSO_4$ (anhydrous)	97.67
NaCl	7500.00
Na_2HPO_4 (anhydrous)	47.88
Other components:	
D-Glucose	1000.00
HEPES	5958.00
Phenol red	10.00
Amino acids:	
L-Arginine·HCl	126.00
L-Cystine 2HCl	31.29
L-Glutamate	292.00

L-Histidine HCl H_2O	42.00
L-Isoleucine	52.00
L-Leucine	52.00
L-Lysine·HCl	72.50
L-Methionine	15.00
L-Phenylalanine	32.00
L-Threonine	48.00
L-Tryptophan	10.00
L-Tyrosine, disodium salt	52.09
L-Valine	46.00
Vitamins:	
D-Calcium pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal·HCl	1.00
Riboflavin	0.10
Thiamine·HCl	1.00

7.2. HBSS (10X) (Gibco-BRL, Eur. cat. no. 14060)

Components	g/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	1.40
KCl	4.00
KH_2PO_4	0.60
$MgCl_2$ $6H_2O$	1.00
$MgSO_4$ $7H_2O$	1.00
NaCl	80.00
Na_2HPO_4 $7H_2O$	0.90
Other components:	
D-Glucose	10.00
Phenol Red	0.10

7.3. PBS (Gibco-BRL, Eur. cat no. 20012)

Components	g/L
KH_2PO_4	0.21
NaCl	9.00
Na_2HPO_4 $7H_2O$	0.726

7.4. Artificial Cerebrospinal Fluid (aCSF)

1. Prepare 10X (10-fold concentrated) stock solution.
   a. The composition for 1 L is as follows:

Component	Final concentration	
NaCl (mol wt 58.44)	124.0 m*M*	72.5 g
KCl (mol wt 74.56)	1.6 m*M*	1.2 g
$NaHCO_3$ (mol wt 84.01)	24.0 m*M*	20.16 g

KH_2PO_4 (mol wt 136.09)	1.2 m*M*	1.7 g
Ascorbic acid (mol wt 176.13)	2 m*M*	3.52 g
Glucose		1.0 g

b. QSP: 1 L with mono-DW.
c. Keep at 4°C.

2. Prepare 10 mL 1 *M* solution of $CaCl_2$.

Component	Final concentration	
$CaCl_2$ (mol wt 219.08)	2.5 m*M*	2.19 g

3. Prepare 10 mL 1 *M* solution $MgCl_2$.

Component	Final concentration	
$MgCl_2$ (mol wt 203.31)	1.5 m*M*	2.033 g

4. Working solution (100 mL).
Add 250 µL 1 *M* $CaCl_2$ and 150 µL 1 *M* $MgCl_2$ to 10 mL stock solution, and complete to 100 mL with mono-dH_2O. The final pH is 7.4 after its saturation with a 95% O_2 / 5% CO_2 gas mixture.

7.5. Solid Agarose Medium (100 mL)

1. Autoclave 25 mL regular agarose (Argarose type II; Sigma, cat. no. A-6877) 3.9% in dH_2O, keep warmed at 56°C.
2. Mix the following compounds, and warm the solution to 56°C:

2X MEM with 25 m*M* HEPES 2X (Sigma, cat. no. H-3375)	25 mL
Tris (Fluka, cat. no. 03349), 5 m*M*	60 mg
Penicillin—streptomycin (100X)	1 mL
$NaHCO_3$ (7.5%)	460 mL
Horse serum (Gibco-BRL, cat. no. 26050-047)	25 mL
Hanks' balanced salt solution	25 mL

3. Add 25 mL warm agarose, while stirring at 56°C (final concentration of agarose: 0.9%).
4. Pour the mixture into Petri dishes to a depth between 8 and 10 mm of culture medium.
5. Let the solution solidify at room temperature, then put the Petri dishes into the CO_2 incubator (prepare the solid medium 1 d before explantation).

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Chapter Three

Slice Cultures for Study of Microglia

Carol A. Colton, Meggan Czapiga, and Toby N. Behar

1. INTRODUCTION

Culture of isolated microglia from dissociated cortical tissue has promoted the in vitro study of microglial function and morphological characteristics (Giulian and Baker, 1986; Streit and Kincaid-Colton, 1995). However, cultures prepared in this manner demonstrate characteristic "ameboid" morphology, and are generally considered to be more activated than the normal, resting microglia found *in situ* (Sedgwick et al., 1991). To reduce this problem, as well as to recreate a cellular architecture more typical of in vivo conditions, we have utilized an organotypic tissue slice method for culture of microglia (Czapiga and Colton, 1999; Gahwiler et al., 1997). This technique allows visualization of microglial morphology and the determination of certain microglial functional parameters, in a culture environment more reminiscent of the in vivo brain.

2. TISSUE SLICES

Tissue slices can be prepared from rat, hamster, or mouse brain. We have used fetal d 16 and postnatal d 2 (PN2) pups. The upper age limit of the pups required for the successful production of slice cultures, using this specific technique, has not yet been determined.

2.1. Materials

Dissecting pins (8–10).
Scissors (2), fine-point, 3-in.
Spatula (1), weighing (small, used to scoop brain out of skull).
Razorblade, single-edge, sterilized by wiping with 80% ethanol.
Forceps (2), Dumont no. 5.
Forceps (2), curved, medium.
Dissecting medium.
Slicing medium (ice-cold).
Note: Slicing medium must be assembled on the day of dissection.
Culture medium.
Petri dishes, 35-mm, sterile plastic (Falcon, cat. no. 1008).
Note: 5–6 are needed, depending on the number of pups used in the preparation. We ordinarily use 2–3 pups for each experiment.

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Multiwell culture plates, 6-well (Costar, cat. no. 3506).
Transparent membranes, 30-mm diameter, 0.4 μm (Millicell-CM, Millipore, MA, cat. no. PICM 03050).
Note: The number will depend on the number of slices required for the experiment. We generally place three slices/filter.
Ethanol, 80%, in squeeze bottle.
Gloves, sterile.
Transfer pipet (1), 3-mL, sterile, disposable (Falcon, cat. no. 7575) with end cut off.
Note: A perfect cross section is cut, using sterile scissors. The end of the pipet must be cut off to create an opening large enough to accommodate the slice without damaging it. Thus, the size of the opening will depend on the size of the brain. An embryonic d-17 mouse brain slice will fit through a smaller opening, a PN4 mouse brain slice will require a larger opening. Therefore, for a larger brain, one may need to make the cut above the tapered portion of the pipet.
Quick set epoxy (one tube for adhering brain to vibratome block).
Styrofoam mounting board (1).
Absorbent bench paper cut to fit mounting board.
Dissecting microscope, low-power.
Vibratome, ethanol-sterilized.

2.2. Preparation for Dissection

1. Sterilize dissection instruments and pins.
2. Cool dissecting medium and slicing medium to 4°C.
3. Add 2 mL dissecting medium to each 35-mm Petri dish.
4. Set up mounting board.
5. Set up dissecting microscope for visualization of cortices in 35-mm dishes.
6. Set up vibratome.

Note: The authors have used several different vibratomes. The brand of vibratome is not critical. It is important, however, to have a metal block on which the brain is set, which can be submerged in cold slicing media (Fig. 1A).

a. It is important to maintain cleanliness of the chamber and the vibratome, to reduce contamination of the slice preparation. This should include alcohol sterilization (80% ethanol for 30 min) of the metal block and plastic chamber, each time prior to use. Rinse with sterile dH_2O or air-dry under the sterile hood after ethanol treatment.
b. Cutting settings for the vibratome will be empirically determined. We use 300-μm slices, although, under some circumstances, sections as thin as 100 μm have been cut.

2.3. Dissection

Note: This section of the protocol is done outside of the sterile tissue culture hood. To prevent contamination of the slice preparations with fungus (most likely), bacteria, or yeast, use sterile solutions and sterile instruments, and take precautions to maintain sterility, including the wearing of sterile gloves. Speed is also important, and the likelihood of contamination increases with increased preparation time.

1. When using 2-d-old neonates, quickly decapitate by cutting across the neck with the scissors. Discard the body. For dissection of fetal central nervous system, *see* Chapter 22.
2. Pin the head at the tip of the nose to the Styrofoam mounting board, dorsal surface (cortices) upward.

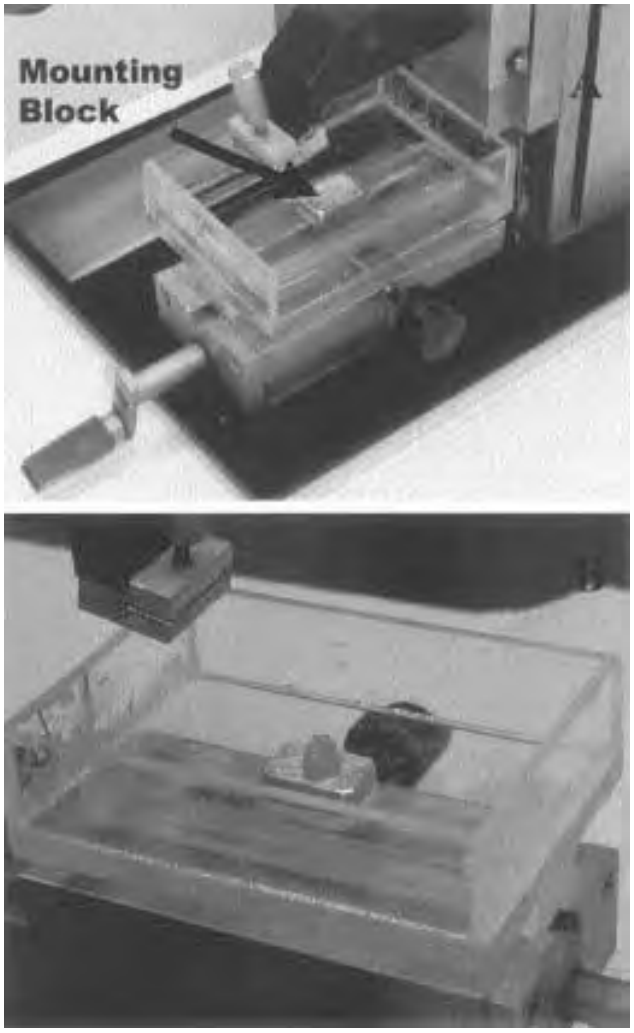

Fig. 1. Vibratome for preparation of slice cultures. **(A)** View of mounting block and vibratome chamber. **(B)** View of neonatal brain placement on mounting block.

3. Using sterile scissors, insert tips at the cut base of the skull, and cut up the midline of the skull to the nose. Make a superficial cut and be careful not to cut the underlying brain tissue. The bone is fairly soft, and can be either clipped away with the scissors or simply peeled back from the brain, using the curved forceps.
4. Place the spatula under the brain, and gently scoop the brain out of the skull. Immediately place it into the 35-mm sterile Petri dish containing the dissection medium.
5. Repeat for each pup.
6. Under the dissecting microscope, and using the no. 5 Dumont forceps, remove the meninges from the brains. The meninges will appear as a delicate membrane containing blood vessels.
7. Remove the cerebellum by cutting at a 90° angle with the sterile razorblade. Place the remaining cleaned brain in a new 35-mm dish containing fresh, cold dissection medium.

2.4. Cutting the Slices

Note: The success of the slice culture technique depends in large part on the vibratome cut of the sections. This technique will need some degree of practice to achieve consistent results.

1. Place the 30-mm diameter transparent membranes (0.4 μm porosity) into the 6-well culture plates. Add 1.5 mL/well ice-cold slicing medium. Keep the plate ice-cold.
2. Place a drop of epoxy on the cutting block.
3. Using the spatula and the curved forceps, gently scoop up a cleaned brain and place it on the block, with the caudal end (i.e., the side from which the cerebellum has been removed) placed into the epoxy. The rostral side (the olfactory region) will be oriented upward at a 90° angle from the surface of the block (Fig. 1B). Be careful to orient the cortices so that an even cut can be made with the vibratome.
4. Place the cutting block, with the affixed brain on the vibratome, into the vibratome chamber. Fill the vibratome chamber with ice-cold slicing medium, covering the brain completely. Take care not to displace brain.
5. Using the manual or automatic advance setting for the vibratome, cut 300-μm sections, starting from the rostral-most region of the brain to the caudal-most region. We chose to use slices taken from the caudal-most appearance of the lateral ventricle to the rostral-most appearance of the lateral ventricle. This specific area of the cortex was used primarily because of ease of visualization. However, microglia are known to be distributed in all regions of the brain (Perry and Gordon, 1991; Murabe and Sano, 1983; Rio-Hortega, 1932), and other regions could be chosen for study.
6. After each cut, using the sterile transfer pipet, transfer the slice to the membrane in the multiwell plate, as prepared in step 1. Generally, three slices are placed on each 30-mm diameter membrane.
7. Once the slices are transferred, carefully remove the excess fluid that was transferred with the slice. This is essential to ensure adequate adherence of the slice to the membrane, and to prevent it from floating.
8. Place the multiwell plate in a 4°C environment for 2 h to allow the cells to recover from the initial injury.
9. Place the 6-well plate containing the slices in the laminar flow hood. Add 1.5 mL culture medium to the new sterile, 6-well plate. Then, using sterile curved forceps, transfer the inserts containing the slices to the multiwell plate containing the culture medium. The air–medium interface should provide enough surface tension to hold the slice in place without floating.

Note: Complete submersion of the slice in medium will promote displacement of the slice from the membrane, and will impair oxygenation at the air–medium interface.

10. Place in a humidified atmosphere of 5% CO_2/95% air at 37°C. Change medium every 2 d. Feed cultures by adding 1.2–1.5 mL fresh culture medium to each well of a new 6-well plate. Using sterile forceps, transfer the membranes to the new plate.

2.5. Cultures

Immediately after slicing, and up to 4 d in culture, the microglia typically assume a rounded morphology and demonstrate characteristics of activated microglia (Czapiga and Colton, 1999). In many instances, however, the microglia appear ramified within the interior of the slice (Fig. 2A). Under normal conditions, longer culture times (10 d) are associated with ramified microglia throughout the slice. The use of serum-free culture conditions is particularly helpful in limiting the microglial reactions that are commonly seen in other slice techniques (Hailer et al., 1996).

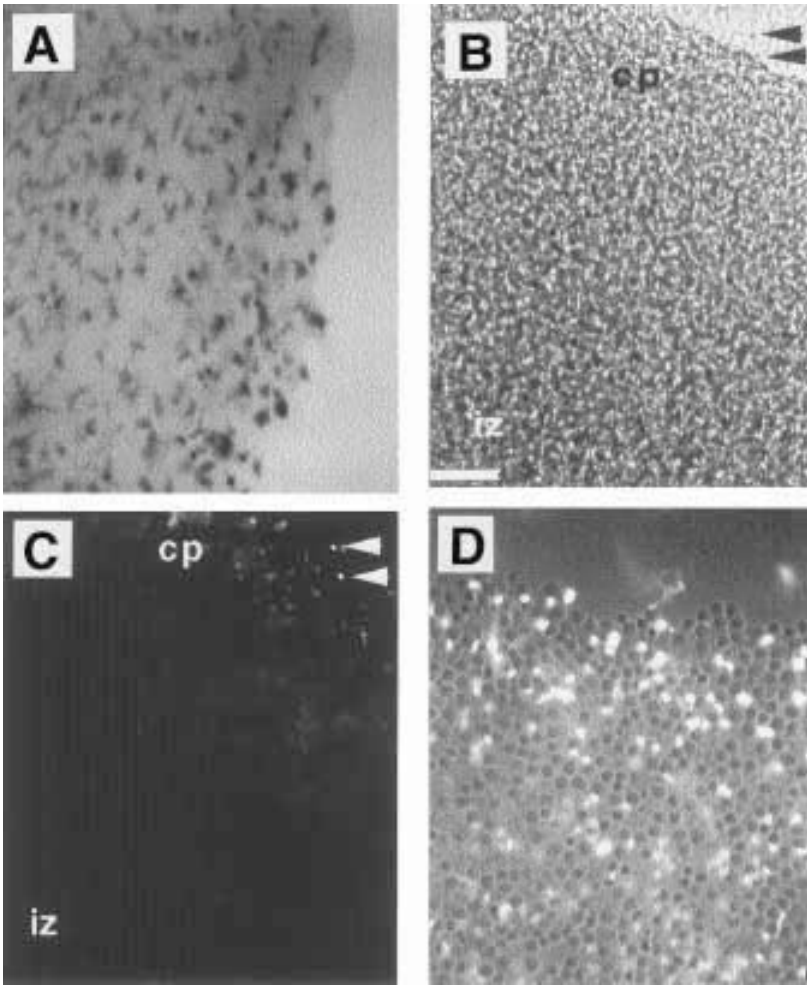

Fig. 2. Micrographs of slices stained for microglia and for cell viability. **(A)** Images of a hamster neonatal slice cultured for 4 d and stained with GS-1 for microglia. **(B,C)** Images of an unfixed fetal mouse slice cultured for 3 d and viewed under bright field (**B**) and fluorescent (**C**) optics. A few propidium iodide (PI)-labeled cells (**B**, arrows) are arrayed along the periphery of the slice. The two PI+ cells indicated in **(B)** (white arrows) are identified in **(C)** by black arrows. **(D)** Phase contrast image of a slice intentionally maintained under suboptimal conditions for several hours has PI-labeled cells (white cells) throughout the cerebral wall. cp = cortical plate; iz = intermediate zone. Bar = 80 μm.

3. IDENTIFICATION OF MICROGLIA

Microglia can be identified in the slice culture by immunocytochemistry, or by their reaction with specific lectins (Czapiga and Colton, 1999). Because of the ease of staining, the authors most commonly use the lectin, *Griffonia simplicfolia* (GS-1) (Sigma, cat. no. L-3019), also known as *Bandeiraea simplicfolia* (BS-1), to identify rodent microglia (Colton et al., 1992). However, staining cells within a slice differs from staining isolated cells in culture. Most important, longer equilibration times must be used to allow the fixative or other material to fully enter the tissue. This time must be determined empirically, and depends on the thickness of the slice.

3.1. Materials

Forceps (1), curved medium.
Scalpel (1), small.
Buffered formalin (Sigma, cat. no. F-5391).
BS-1, peroxidase labeled (Sigma, cat. no. L-3383).
Lectin buffer.
Phosphate buffered saline (PBS), 1X (Gibco-BRL, cat. no. 20012-027).
Chromagen.
Glycerol/Tris mounting media.
Microscope slides.
Coverslips, round, 25-mm (Fisher, cat. no. 12 545-102).
Clear finger nail polish for sealing coverslips.

3.2. Staining Procedure

1. Remove the porous membrane insert, containing the slices, from the 6-well plate, using curved forceps. Carefully replace the medium in the plate with 1.5 mL buffered formalin. Replace the insert in the plate, and equilibrate at room temperature for 60 min. Replace formalin with PBS for 10 min, repeating 2×.
2. Dilute lectin into lectin buffer at an initial value of 1:50 (20 μg/mL). The exact dilution will need to be determined empirically for the slice.

Note: Take care to observe safety rules for use of a lectin, as instructed by the manufacturer.

3. Replace the PBS in the 6-well plate with the lectin solution prepared above. Equilibrate the slices with the lectin solution for 8 h at room temperature.
4. After 8 h, replace the lectin solution with fresh PBS, and equilibrate at room temperature for 10 min. Repeat PBS wash 2×.
5. Prepare Sigma Fast DAB (3,3′-diammobenzidine tetrahydrochloride) according to manufacturer's instructions. Replace PBS with DAB, and equilibrate at room temperature until color is developed (usually 20 min for a 300-μm slice). Microglia will appear as blue-black staining regions in the slice (Fig. 2A).
6. Wash with PBS for 10 min.
7. Using forceps, transfer the inserts from the 6-well plate to a solid surfaces (i.e., a piece of Parafilm placed on the lab bench). Carefully cut out the bottom of the membrane insert, using a scalpel. Using the forceps, pick up the cut membrane containing the slice, and place on the microscope slide, slice up.
8. Add a drop of glycerol/Tris to the slice, and place the coverslip over the slice. Seal around the circumference of the coverslip with the clear fingernail polish.
9. Visualize the microglia, using a standard light microscope.

4. EVALUATION OF CELL DEATH IN SLICE CULTURES

The viability of the cells in the slice culture can be routinely assessed using propidium iodide (PI), a fluorescent dye that intercalates into the DNA of cells (Jones and Senft, 1985; Behar et al., 1999). Nonviable cells (dead cells) are unable to exclude PI, and contain bright orange-red nuclei, when observed with a fluorescent microscope, using standard fluorescein or rhodamine filters.

4.1. Materials

Scalpel, small (1).
Forceps (1), curved, medium.
PI solution (Molecular Probes no. P3566) diluted to 50 μg/mL in PBS.
PBS.
Glycerol/Tris mounting media.
Microscope slides.
Fluorescent microscope equipped with fluorescein or rhodamine filters.

4.2. Treatment with PI

1. Using forceps, transfer the inserts from the 6-well plate to a solid surface (i.e., a piece of Parafilm placed on the lab bench). Carefully cut out the bottom of the membrane insert, using a scalpel. Using the forceps, pick up the cut membrane containing the slice, and place it on the microscope slide, slice up.
2. Prepare a positive control by incubating one set of slices at room temperature for 4 h prior to transferring to a microscope slide.
3. Once the slices have been transferred to the microscope slide, add enough PI solution to cover each slice. Equilibrate for 5 min at room temperature.
4. Add a drop of glycerol/Tris mounting media to the surface of each slice, and place coverslip on the slice.
5. View immediately on low power using the fluorescein or rhodamine filters.
6. Dead cells appear as bright orange-red.

5. APPENDIX

5.1. Media

Note: All media must be sterile. Prepare under a sterile, tissue culture hood and pass through a 0.2-μm filter, if in doubt.

1. Slicing media:
   a. Krebs-Ringer buffer stock (10X stock).
      NaCl (120 m*M*) 35.3 g
      KCl (5 m*M*) 1.2 g
      KH_2PO_4 (1.2 m*M*) 0.8 g
      Glucose (14 m*M*) 12.7 g
      $NaHCO_3$ (26 m*M*) 10.8 g
      Phenol red 50 mg
      i. Bring up to 500 mL with dH_2O.
      ii. Store at 4°C until used.
   b. Magnesium stock:
      i. $MgSO_4 \cdot 7\ H_2O$ (31 m*M*): 3.8 g/100 mL dH_2O.
      ii. Store at 4°C.
   c. Slicing media.
      i. *On day of tissue slice preparation*, make slicing media by adding:
         15.0 mL 10X Krebs-Ringer buffer stock
         1.2 mL Magnesium stock
         450.0 mg Bovine serum albumin (BSA) (Sigma, cat. no. A-4503)
      ii. Bring up to 150 mL with dH_2O.

iii. Sterilize the solution by passing it through a 0.2-μ filter (Nalgene, cat. no. 125-0200).
iv. Store at 4°C or on ice until used in the tissue slice preparation.

Note: Calcium is excluded from the slicing media, in order to reduce neuronal death from entry of calcium into the cells. Magnesium is included to reduce *N*-methly-D-aspartate channel activation, and the BSA is used to make the solution slightly hypertonic, thereby reducing swelling of the cells during the preparation process. These changes help to reduce neuronal loss in the culture, and are essential to successful slice preparation.

2. Serum-free culture media:
   200 mL Neurobasal™ medium (Gibco-BRL, cat. no 21103-049).
   2 mL Glutamine (100X), 200 m*M* (Gibco-BRL, cat. no. 25030-081).
   4 mL B-27 (50X) supplement (Gibco-BRL, cat. no. 17504-010).
   0.2 mL Gentamicin (50 mg/mL) (Gibco-BRL. cat. no. 15750-060).
3. Dissecting media:
   100 mL Leibovitz's medium (L-15) (Gibco-BRL, cat. no. 11415-064).
   1 mL Penicillin–streptomycin (100X) (Gibco-BRL, cat. no. 15070-063).
   1 mL Fungizone antimycotic (Gibco-BRL, cat. no. 15290-018).

5.2. Solutions

1. Lectin buffer:

NaCl (150 m*M*)	0.87 g
HEPES (10 m*M*) (Gibco-BRL, cat. no. 15630-080)	0.26 g
$CaCl_2$ (10 m*M*)	0.15 g
$MgCl_2$ (1 m*M*)	0.02 g
$MnCl_2$ (1 m*M*)	0.198 g
Triton X-100 (Fisher, cat. no. CS282-100)	0.1%

   Add to 100 mL of dH_2O and adjust pH to 7.4.
2. Chromagen: Sigma Fast DAB with metal enhancer (Sigma, cat. no. D0426). Follow manufacturer's instructions for preparation of chromagen.
3. 50 m*M* Tris-HCl solution, pH 7.5: Dilute 0.5 mL 1 *M* Tris-HCl solution (Gibco-BRL, cat. no. 15567-027) in 10 mL dH_2O.
4. PBS without calcium and magnesium (Biofluids, cat. no P313-500): If 10X, then dilute 1:10 with dH_2O and filter-sterilize.

5.3. Glycerol/Tris Mounting Media

8.5 mL Glycerol.
1.5 mL 50 m*M* Tris-HCl.

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Chapter Four

Microexplant Cultures of the Cerebellum

Bernard Rogister and Gustave Moonen

1. INTRODUCTION

This chapter is devoted to a simple method for culturing developing rat cerebellum. The method can also be used for rat hippocampus embryonic age 18 d (E18), cerebral cortex (E18), and even spinal cord, but at an earlier stage of development (E14). This method was initially designed to obtain long-term survival of cerebellar macroneurons, Purkinje cells, and deep nuclear macroneurons (Moonen et al., 1982; Neale et al., 1982). Indeed, in cultures in which a single cell suspension is seeded, virtually all the neurons die between 5 and 10 d, but significant survival is obtained if small aggregates of cells (microexplants), rather than single cells, are seeded. The term "microexplant" was used to stress the difference from the classic cerebellar explants, which consist of a thin cerebellar slice, which are much more organized tissue samples.

Microexplant cultures have also been adapted to serum-free conditions for short-term experiments of <1 wk duration (Section 3.1.). These preparations have been used for the study of survival factors for cerebellar neurons (Grau-Wagemans et al., 1984), protease release by neural cells, proliferation of astroglia cells, neuronal (Selak et al., 1985; Magyar-Lehmann et al., 1995a) or oligodendroglial (Schmidt et al., 1997) migration on different substrates, modulation of neuritic behavior (Magyar-Lehmann et al., 1995b; Fischer et al., 1986; Schmidt et al., 1996), and electrophysiology recordings of cerebellar granule and Purkinje neurons (Zempel and Steinbach, 1995).

2. PREPARATION OF CEREBELLUM

2.1. Materials

Newborn rats, 0-2 d old.
Forceps (1), large.
Forceps (1), medium, curved.
Scissors (1), large.
Scissors (1), small.
Forceps (1), fine, curved.
Forceps (1), 10-cm, fine.

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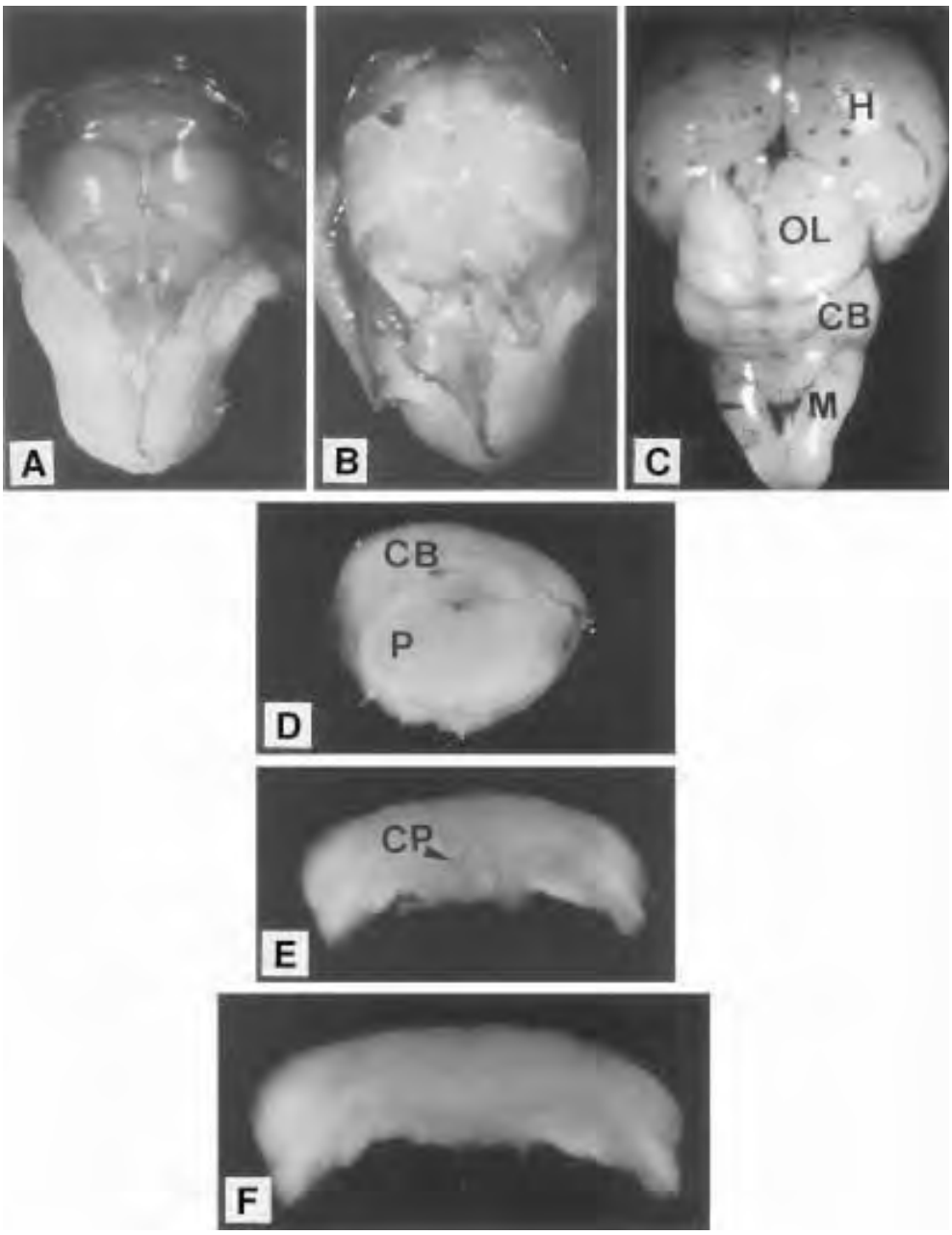

Fig. 1. Dissection of neonatal rat brain. H: Cerebral hemispheres. OL: Optic lobes. CB: Cerebellum. P: Pons. CP: Choroid plexus. M: medulla. **(A)** Removal of skin from neonatal rat. **(B)** Removal of the skull. **(C)** Neonatal rat brain removed from the skull. **(D)** Isolated cerebellum showing the pons. **(E)** Cerebellum with meninges and choroid plexus. **(F)** Isolated cerebellum with meninges and choroid plexus removed.

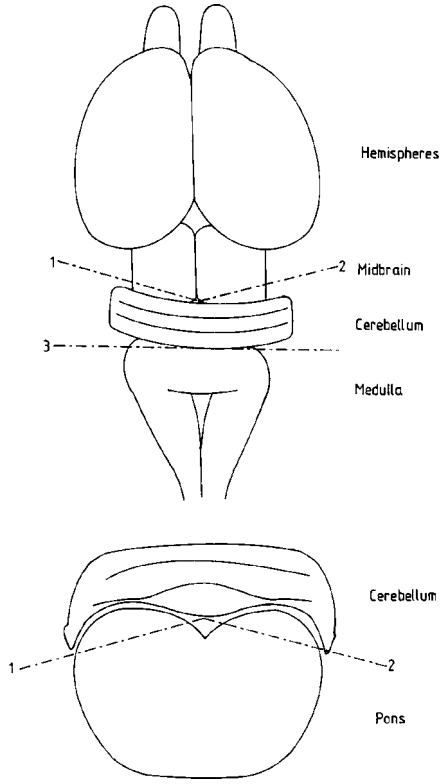

Fig. 2. Schematic representation of the dissection of the cerebellum.

Scissors (1), 10-cm, pointed, curved.
Minimum essential medium (MEM) with glucose and insulin, (MEMgI) (10 mL).
Hank's balanced salt solution (HBSS) (50 mL).
Petri dishes (2), 100-mm glass, sterile.
Petri dishes (2), 100-mm plastic, sterile.
Petri dish (1), 35-mm plastic, sterile.
95% Ethanol.
70% Ethanol.

2.2. Procedure for Gross Dissection (Figs. 1 and 2)

1. Preparation for gross dissection:
   a. Sterilization of instruments: Sterilize instruments by immersing them in 70% alcohol and drying them in air inside the laminar flow hood, or by inserting them in a hot bead sterilizer for 5–10 s at 250°C. When sterile, put the instruments into a sterile glass Petri dish, and keep the ends covered with the lid.
   b. Pipet 15 mL HBSS into one 100-mm glass Petri dish and one 100-mm plastic Petri dish. Replace covers.
   c. Pipet 1 mL MEMgI medium into a 35-mm Petri dish.
2. After sacrificing the new born rat pups by CO_2 or Metofane^{TM} inhalation, pick up and completely immerse a newborn rat into 95% ethanol. Hold the newborn rat over the empty 100-mm plastic Petri dish, and decapitate, using the large scissors. Repeat for each newborn rat.

3. While holding the head with curved forceps, use the small scissors to cut the skin. Begin at the neck, proceed along the midline, and pull the skin aside (Fig. 1A).
4. Using the scissors, cut the skull (mainly cartilage at this age) by making a Y-shaped cut (the base of the "Y" begins at the neck). Without damaging the brain, gently pull apart the flaps of the skull (Fig. 1B).
5. Remove the entire brain by using the curved forceps like a spatula, cutting all the cranial nerves. Lift the brain out of the brain case and place it in the 100-mm glass Petri dish containing 15 mL HBSS (Fig. 1C).
6. Excision of the cerebellum (Fig. 2):
   a. Place the Petri dish containing the brain under the dissecting microscope. Using the small scissors, make the following cuts:
      i. A slightly angled cut is made between the cerebellum and the midbrain on one side.
      ii. A similar, slightly angled cut is made on the other side, so that the two cuts meet at the midline, and the cerebellum is separated from the more rostral structures.
      iii. A third cut is made between the cerebellum and the medulla, separating these two structures.
   b. The "slice," containing the cerebellum (dorsal) and the pons (ventral), is now dissected (Fig. 1D). Place the isolated cerebellum into the plastic dish containing HBSS.
   c. Repeat the excision of cerebellum procedures for each remaining brain.
7. Using the fine forceps, remove the meninges and choroid plexi from each isolated cerebellum (Fig. 1E).
8. Transfer each cerebellum to one 35-mm Petri dish that contains 300–500 µL MEMg (the volume of MEMg should be the minimum in order to cover all the cerebella in a corner of the dish).

3. MICROEXPLANT CULTURES

3.1. Materials

Scissors (1), small.
MEMgI.
Erlenmeyer flasks (2), 10-mL, sterile.
Petri dishes (2), 35-mm plastic.
Centrifuge tubes (2), 15-mL, sterile.
Stoppers (2), sterile (to fit Erlenmeyer flasks).
Aluminum foil pieces (2), sterile.
Pipet (1), glass, curved, with large aperture.

3.2. Preparation of Microexplants

1. Mince the cerebella in the 35-mm Petri dish into small fragments using the sterile scissors.
2. After mincing the tissue, add MEMgI medium up to 1 mL/cerebellum (i.e., for two cerebella, add up to 2 mL).
3. Separate the microexplants from smaller clumps, single cells, or debris, as follows:
   a. Transfer the minced tissue in medium to the sterile tube. Allow the tissue to sediment for 2–3 min.
   b. Aspirate and discard the supernatant, being careful to retain the microexplants. Resuspend the pellet in fresh medium up to the original volume (1 mL/cerebellum).
   c. Repeat the sedimentation procedure (steps a and b), if necessary.

3.3. Microexplant Suspension Culture

1. Transfer the mixed microexplant suspension to a 10-mL sterile Erlenmeyer flask.
2. Gas the Erlenmeyer flask with a 5% CO_2/95% air mixture, and tightly stopper the flask. Cover the stopper with a piece of aluminum foil and place the flask on a gyratory shaker at 4.5*g* at 37°C for 24 h.

3.4. Microexplant Culture

1. After 24 h, remove the Erlenmeyer flask from the shaker.
2. Using the sterile glass, curved, large aperture pipet, transfer the microexplant suspension from the flask. Usually, not all the microexplants are recovered. Add 1 mL more of MEMgI to the Erlenmeyer flask, in order to recover the remaining microexplants. Transfer the 2 mL to a sterile 15-mL centrifuge tube. Allow the tissue to sediment, discard the supernatant, and resuspend the pellet in 1 mL MEMgI.
3. Plate the microexplant suspension from one flask into one 35-mm Petri dish coated with either poly-L-ornithine or laminin.
4. Repeat steps 1–3 for each flask prepared.
5. Incubate the microexplant cultures in a humidified 5% CO_2/95% air incubator at 37°C.
6. After 6 h, add 0.5 mL MEMgI to each 35-mm Petri dish.

Note: At 10 d, significant cell death is observed; however, a portion of the neurons will survive and can be maintained for up to several months. Four-week-old cultures are used for electrophysiological studies (Gibbs et al., 1982; MacDonald et al., 1982; Cull-Candy et al., 1987).

3.5. Comment on Substrates

On poly-L-ornithine-coated dishes, neuritic outgrowth is optimal, but outward migration of cells, including neurons, is minimal. On laminin-coated dishes, not only radial neuritic outgrowth is observed, but also outward migration of neurons and glia cells (Figs. 3 and 4); (*see* Selak et al., 1985 for further details).

4. APPENDIX

4.1. Medium

1. Eagle's MEM (Gibco-BRL, cat. no. 31095-029) formulation (Eagle, 1959):

Component	mg/L
Inorganic salts:	
$CaCl_2 \cdot 2H_2O$	264.00
KCl	400.00
$MgSO_4 \cdot 7H_2O$	200.00
NaCl	6800.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot 2H_2O$	158.00
Other components:	
D-Glucose	1000.00
Phenol red	10.00
Amino acids:	
L-Arginine·HCl	126.00
L-Cystine	24.00

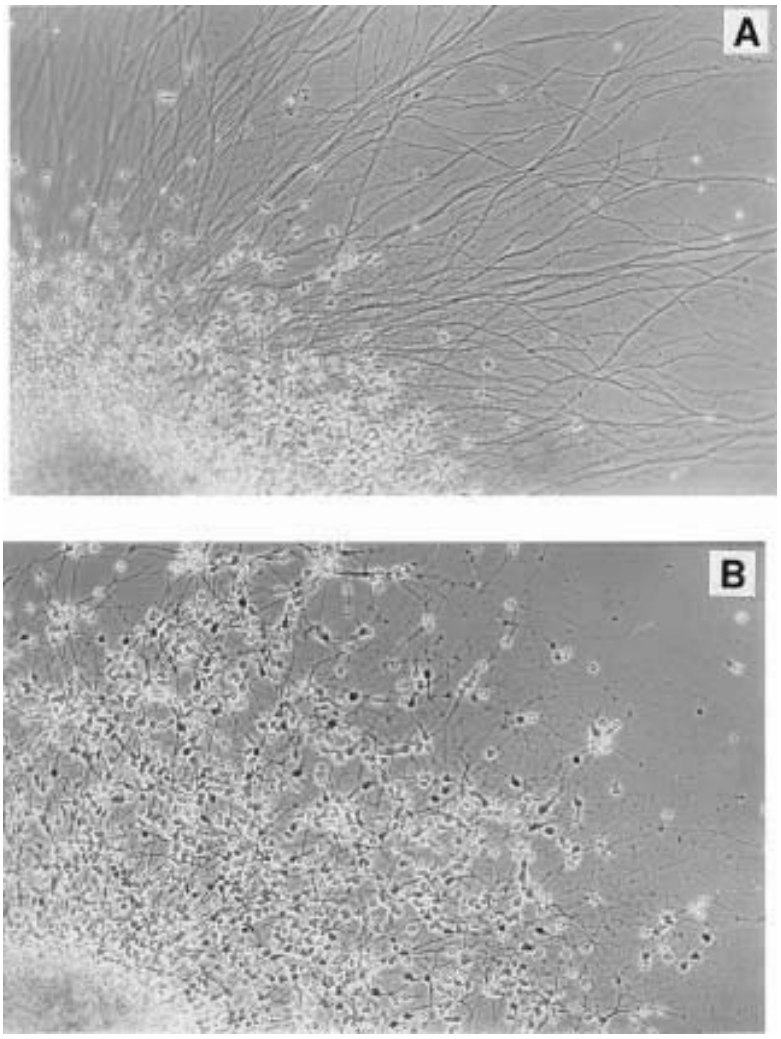

Fig. 3. Newborn rat cerebellum microexplants. Newborn rat cerebellum microexplants cultured for 4 d in MEMgI medium on **(A)** poly-L-ornithine-coated substratum and **(B)** laminin-coated substratum. In **(B)**, a more extensive outward migration of cerebellar granule cells is observed. Phase-contrast picture.

L-Glutamine	292.00
L-Histidine HCl·H_2O	42.00
L-Isoleucine	52.00
L-Leucine	52.00
L-Lysine HCl	73.00
L-Methionine	15.00
L-Phenylalanine	32.00
L-Threonine	48.00
L-Tryptophan	10.00
L-Tyrosine	36.00
L-Valine	46.00

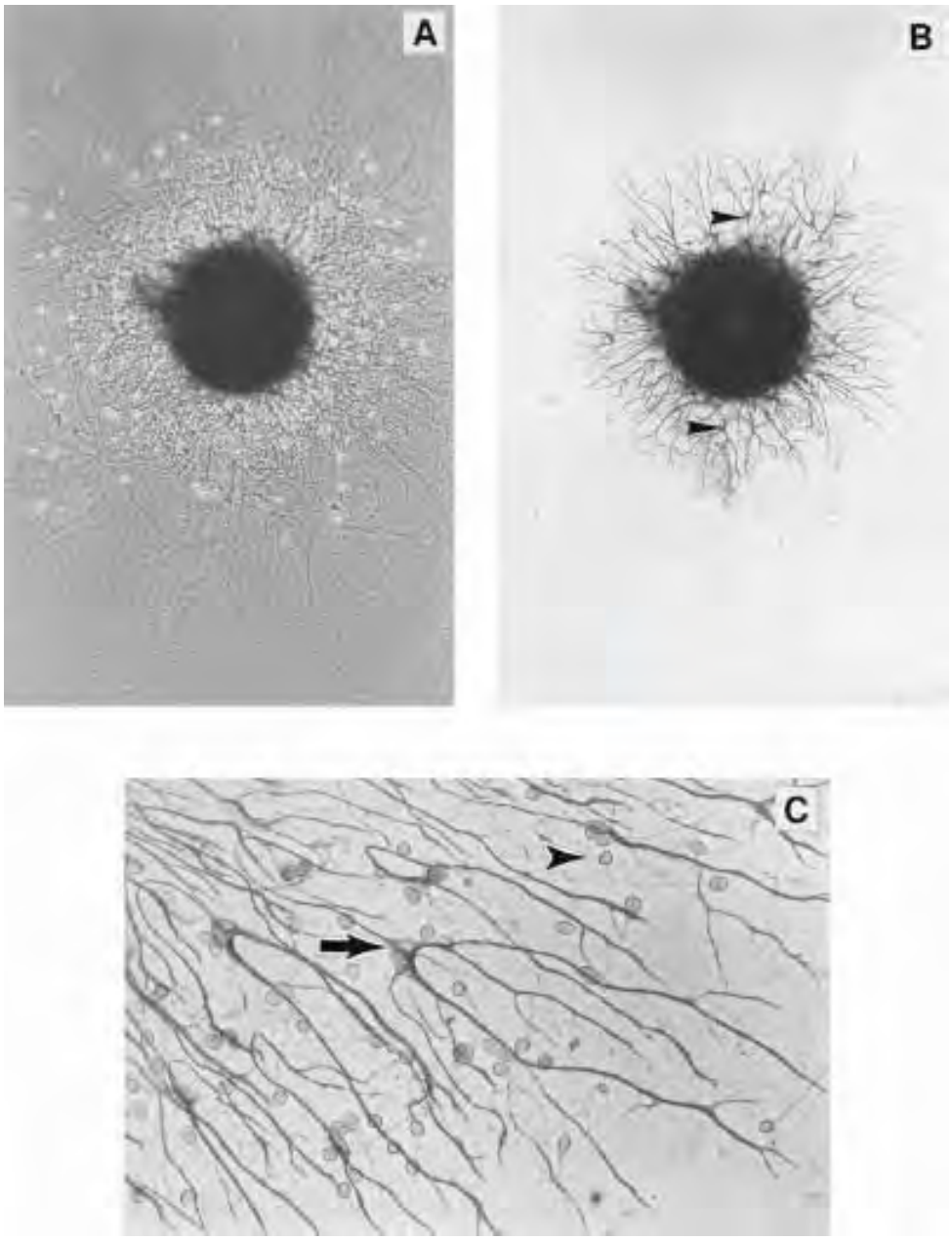

Fig. 4. Cerebellum microexplants. Cerebellum microexplants cultured for 3 d in MEMgI on a poly-L-ornithine-coated substratum and observed after immunohistochemical demonstration of the glial fibrillary acidic protein. **(A)** Low-power phase-contrast microphotograph allowing demonstration of the massive outward neuritic growth. **(B)** Low-power bright-field microphotograph showing that the glial fibrillar acidic protein (GFAP)-positive radial cells remain close to the microexplant (large arrow). **(C)** High-power phase-contrast microphotograph of an area of **(A)** that is close to the microexplant. Phase contrast was used for this photograph to demonstrate the GFAP-positive radial glial cells (large arrow), as well as the GFAP-negative granule neurons (small arrow). As can be seen, neurons remain in the vicinity of the glial process.

Vitamins:	
D-Calcium pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyroxidal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

2. MEMgI.
   a. For short-term cultures, MEM is supplemented with glucose at a final concentration of 6 g/L and bovine insulin (Sigma-Aldrich, cat. no. I-5500) at a final concentration of 5 µg/mL (MEMgI). The insulin is added just prior to use at low pH.

   Note: For long-term cultures, serum is added to the medium. MEM is supplemented with glucose at a final concentration of 6 g/L, 10% heat-inactivated (56°C, 30 min) horse serum (v/v), and 10% fetal bovine serum (v/v). Both sera are obtained from Gibco-BRL, cat. nos. 26050-039 and 102700-98 respectively.

4.2. Nonneuronal Overgrowth of Cultures

The following method may be used to avoid overgrowth of cultures by nonneuronal cells when cultures are grown in the presence of serum.

1. Culture cells in MEM supplemented with glucose at a final concentration of 6 g/L, 10% heat-inactivated (56°C, 30 min) horse serum (v/v), and 10 fetal bovine serum (v/v). Both sera are also obtained from Gibco-BRL.
2. After 4 d in culture, the culture can be treated for 48 h with the following medium: 15 µg/mL 5'-fluoro-2'-deoxyuridine (Sigma-Aldrich, cat. no. F-0503) and 35 µg/mL uridine (Sigma-Aldrich, cat. no. U-3003) in MEM, supplemented with 10% heat-inactivated horse serum.
3. After 48 h of treatment with the antimetabolite, the cells are cultured in MEM supplemented with 10% heat-inactivated horse serum.

4.3. Poly-L-Ornithine and Laminin-Coated Plates

1. Preparation of solutions:
   a. Poly-L-ornithine hydrobromide, mol wt 30,000–70,000 (Sigma, cat. no. P-3655):

   Note: Poly-L-ornithine hydrobromide should be stored desiccated at –20°C.

      i. Add 0.1 g poly-L-ornithine to 100 mL, pH 8.4, boric acid buffer, to form a stock solution of 1.0 mg/mL.
      ii. Sterilize by filtration through a 0.2-µm filter.
      iii. Store at 4°C for up to 2 mo.
      iv. For use, dilute 10 mL poly-L-ornithine stock solution to a final volume of 10 mL with sterile distilled water, to get a working solution of 0.1 mg/mL.

   b. Preparation of boric acid buffer:
      i. Make up an excess amount of 0.15 *M* boric acid buffer: 1.59 g boric acid + 0.75 g NaOH/125 mL distilled water.
      ii. Adjust pH to 8.4 by adding HCl, and testing aliquots. Do not put the pH electrode in the buffer.
   c. Mouse laminin (ICN Biomedicals, cat. no. 150027):

i. Stock solutions should be reconstituted to approx 1 mg/mL in sterile phosphate-buffered saline, pH 8.0.
ii. For use, dilute laminin to a concentration of 1 µg/mL by the addition of sterile phosphate-buffered saline.

2. Coating of plates with poly-L-ornithine and laminin:
   a. Coating plates with poly-L-ornithine:
      i. Make up stock solution of poly-L-ornithine at 1.0 mg/mL.
      ii. Dilute 10-fold with sterile distilled water, to get a working solution of 0.1 mg/mL.
      iii. Add 1 mL poly-L-ornithine solution to 35-mm tissue culture dish. Make sure the entire surface of the dish is covered.
      iv. Allow to dry at room temperature in a laminar flow hood for 1–24 h.
      v. At least 30 min before planting the cells, wash each dish twice with 2.0 mL sterile distilled water. Replace the last water wash with 1 mL medium.
      vi. Incubate 30 min in a 5% CO_2/95% air incubator at 37°C. When ready to seed cells, remove the medium from the poly-L-ornithine-coated dish; add cells and fresh medium.
   b. Coating plates with laminin:
      i. Dilute LAM in sterile water, serum-free medium, or PBS to a working concentration of 10 µg/mL.
      ii. Apply laminin to the growth surface at a concentration of 10 µg/cm^2 or at a concentration of 10 µg/mL.
      iii. Allow to stand for 2–24 h at 37°C.
      iv. Remove from incubator, and either seal in plastic bags and freeze at –20°C or remove excess laminin solution and add medium in preparation for culture.
      v. Do not allow plates to dry out.

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Chapter Five

Construction and Use of Compartmented Cultures for Studies of Cell Biology of Neurons

Robert B. Campenot and Grace Martin

1. INTRODUCTION

The compartmented culture method has numerous features that make it useful for studies of nerve fiber growth, among which are:

1. Distal axons can be exposed to a different fluid and cellular environment than cell bodies and proximal axons, which is useful for studies of trophic, ionic, and pharmacological regulation of growth.
2. Distal axons can be removed (neuritotomy), and subsequently regenerate, which permits many useful approaches to the study of axon growth and regeneration.
3. Axons can be chronically electrically stimulated during growth and regeneration.

Earlier versions of these procedures have been published (Campenot, 1979, 1992). The following protocol describes in detail the basic production of compartmented cultures and how to neuritotomize them. Figure. 1 is a schematic of compartmented cultures, and a photomicrograph of neurons in compartmented cultures is shown in Fig. 3. It is common practice to culture rat sympathetic neurons, using Leibovitz's L-15 culture media thickened with methyl cellulose and containing nerve growth factor, as described by Hawrot and Patterson (1979). Rat serum and ascorbic acid are supplied to compartments containing cell bodies, but are omitted from culture medium given to axons, because this facilitates their adhesion to the substratum, and their ability to grow in the face of medium changes (*see* Campenot, 1982). Media appropriate to the particular neurons cultured should be used, and, although MC appears to be important for the proper functioning of the compartmented system, the necessity for it has not been rigorously investigated. The authors use methyl cellulose 4000 cps (Xenex Laboratories, Coquitlam, BC, Can) at 2 g/500 mL medium. A condenser modification of an inverted-phase contrast microscope, which allows better visualization of the neurons, is useful, but its description is beyond the scope of this protocol.

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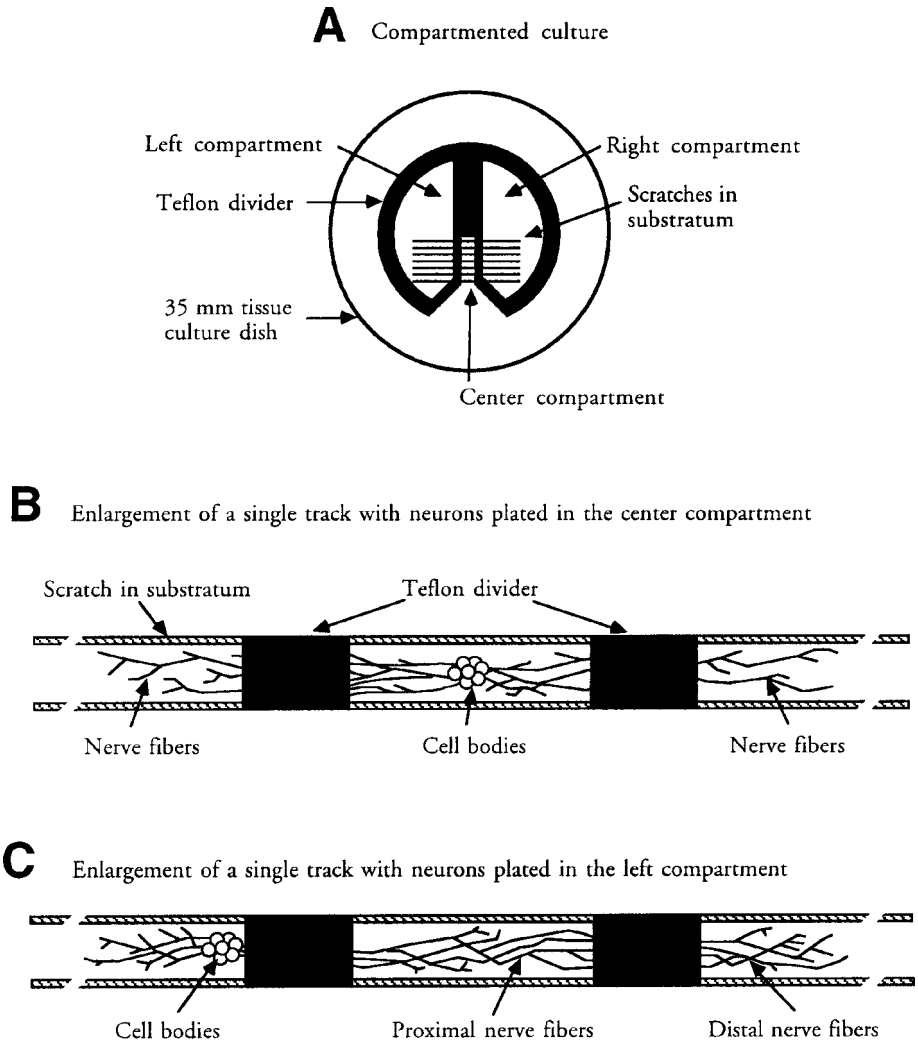

Fig. 1. Schematic drawing of a compartmented culture.

2. PREPARATION OF 35-MM TISSUE CULTURE DISHES

2.1. Materials

Rat tail collagen solution, fresh, sterile.
Tissue-culture quality distilled water (dH_2OP, sterile.
Tissue culture dishes, 35-mm, sterile plastic.
Pin rake (*see* Section 6).
Tray for 12 cultures made from black Plexiglas™ (optional).

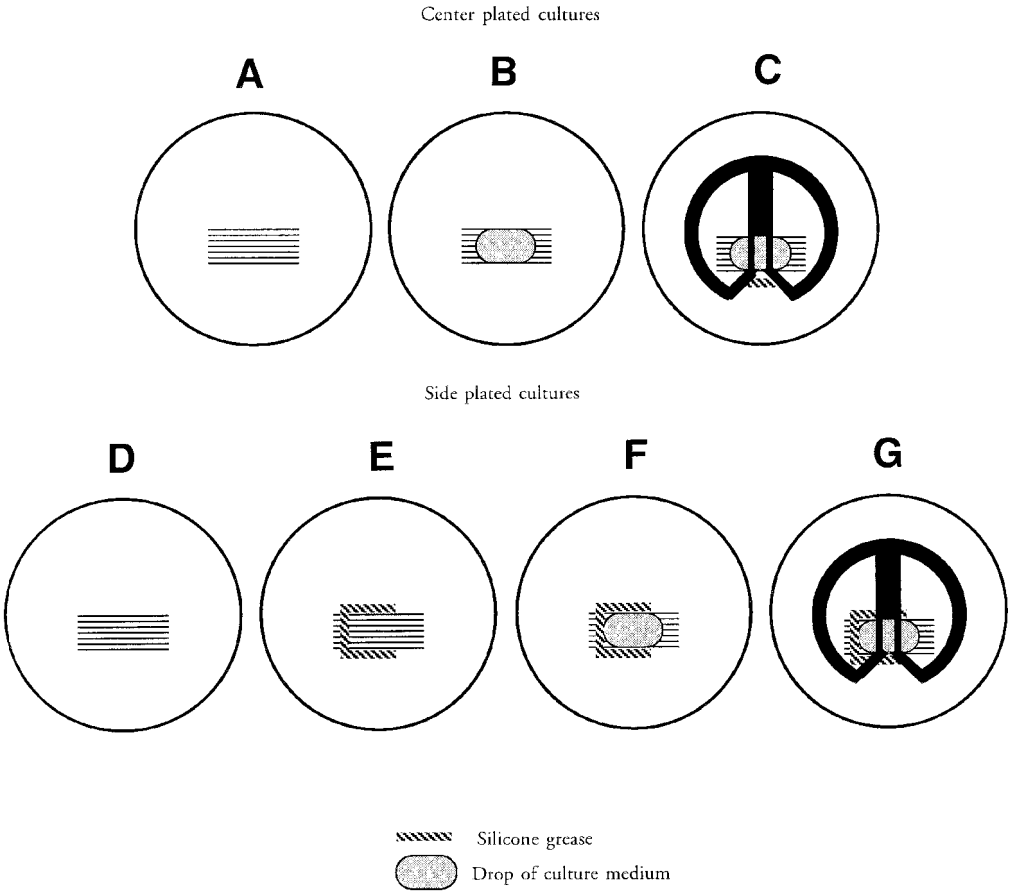

Fig. 2. Construction sequences for center- and side-plated cultures.

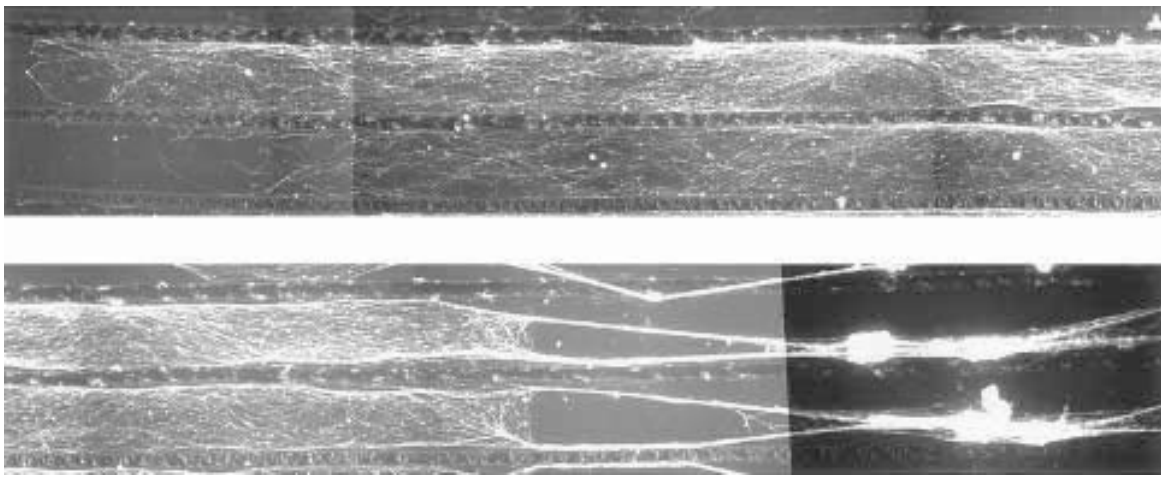

Fig. 3. One-week-old sympathetic neurons in compartment cultures stained with FM-143 (cell bodies in bottom right corner).

2.2. Collagen Coating of Dishes

Prepare a solution of rat tail collagen as described by Hawrot and Paterson (1979), using sterile technique. The shelf-life of collagen solution used for this purpose is 2 mo in the refrigerator. Collagen-coated culture dishes may be stored frozen indefinitely. "Bad" collagen will peel from tracks during scratching and produce an excessive amount of white powder. To coat culture dishes, dilute the collagen solution (four parts sterile, culture-quality dH_2O to one part collagen solution for sympathetic neuron cultures or dorsal root ganglion neuron cultures). Wet the floors of 35-mm tissue culture dishes by pouring enough solution into the first dish to fill it about half, then pouring the solution from the first dish into the second dish, and so forth. Replenish the solution as needed. This will leave behind a film sufficient to coat the floors of the dishes, then air-dry the dishes, with lids on, overnight in a laminar flow hood.

2.3. Forming Collagen Tracks

Form the collagen tracks by scoring the collagen-coated floors of 35-mm tissue culture dishes with a pin rake. It is helpful to place the dish on a black Plexiglas background, to permit visualization of the scratches as they are made. The tracks should be approx 20 mm long, with the scratches positioned as indicated in Fig. 1A.

3. TEFLON DIVIDERS

3.1. Materials

Teflon™ dividers (Fig. 1A), pin rakes, and grease syringes are commercially available (Tyler Research Instruments, Edmonton, AB, Can, E-mail:tylertech.com).

3.2. Cleaning and Sterilization

1. Remove dividers from culture dishes, with forceps, and wipe off the silicone grease with a lint-free tissue. Place dividers in a beaker reserved for acid cleaning, containing 70% ethanol, until they are to be acid-cleaned.
2. Pour off the ethanol, place the beaker in a fume hood, and add clean sulfuric acid with Nochromix™ (Godax Laboratorie, New York). Leave until dividers look pure white.

Note: Dividers can be left indefinitely in the acid, without harm.

3. Pour the acid back into its jug, catching the dividers in a funnel. Transfer them to a beaker. Rinse the dividers 5× with culture-quality dH_2O to remove the acid, then boil them in the water for 1 h. Pour off the hot water, and rinse again 5×.
4. Using clean forceps, move the dividers to clean Petri dishes, taking with them as little water as possible. Autoclave them, and dry them in an oven, if necessary.

4. ASSEMBLING THE DISH AND DIVIDER

4.1. Materials

Hemostat (1), with right-angled jaws.
Forceps (2), fine, dissection quality.
Culture medium.
Collagen-coated and scratched tissue culture dishes (*see* Section 2).
Sterile Teflon dividers (*see* Section 3).
Syringes (2), sterile, with silicone grease (*see* Section 7).

Tray for 12 cultures made from black Plexiglas (optional).
Stereo microscope set up in a sterile hood.

4.2. Procedure for Center-Plated Cultures

1. Assemble in a sterile hood equipped with a stereo microscope: a supply of sterile Teflon dividers, two pairs of forceps, a right-angle hemostat, and two sterile silicone grease syringes.
2. Under the stereo microscope, place a drop of methyl cellulose-containing culture medium on the scratched region of the floor of each dish, in order to wet the region where axons will cross under the divider (Fig. 1B). Take care not to touch the pipet to the substratum.
3. Sterilize the hemostat. Remove a Teflon divider from the Petri dish by gripping the rim of the divider with sterile forceps. Transfer the divider to the sterile hemostat, clamping the divider by the solid septum. Lay the hemostat on its back on the work surface, so that the divider is visualized under the stereo microscope. Apply silicone grease to the divider, taking care that the regions under which the axons will cross receive a neat "rope" of grease.
4. Pick up a collagen-coated prescratched culture dish, remove and set aside its lid, and invert the dish quickly, in order not to disturb the drop of medium. Bring the dish into position over the divider, and drop it in place (Fig. 1C). With a fine forceps, press on the dish bottom in the area of the solid septum, and also around the rim, to seat the grease. Do not press in the region where the axons will cross. When the dish is properly seated (a judgment developed with experience), pick up the hemostat, turn it over, and release the dish, so that it falls gently on the work surface, (placing it on the surface can disrupt the seating of the divider). Place a dab of grease at the entry of the slot (Fig. 1C), and caulk any regions around the perimeter of the divider that did not seal properly. Place a drop of culture medium in each side compartment on the scratched region of the substratum, and ensure that the full length of the scratched region is wetted with medium. This prevents drying at the edge of the original drop, without damaging the substratum. Replace the cover of the dish.
5. Repeat steps 2–4 for each dish, then store trays of 12 dishes in the incubator, as they are completed. After 1 h or more in the incubator, fill the side compartments with culture medium.

4.3. Procedure for Side-Plated Cultures

1. Assemble in a sterile hood equipped with a stereo microscope: a supply of sterile Teflon dividers, two pairs of forceps, a right-angle hemostat, two sterile silicone grease syringes, and a sheet of black Plexiglas of sufficient size to serve as a tray for about 12 cultures.
2. Arrange 12 culture dishes on the black Plexiglas sheet. Under the stereo microscope, lay down a C-shaped perimeter of silicone grease in each dish, as shown in Fig. 2E. Set the dishes in the incubator, to allow the grease to settle for about 1 h.
3. After the grease perimeter has settled, place a drop of methyl cellulose-containing culture medium on the scratched region of the floor of a dish, within the "C," in order to wet the region where axons will cross under the divider (Fig. 2F). Take care not to touch the pipet to the substratum.
4. Remove a Teflon divider from the Petri dish by gripping the rim of the divider with sterile forceps. Transfer the divider to the hemostat, clamping the divider by the solid septum. Lay the hemostat on its back on the work surface, so that the divider is visualized under the stereo microscope. Apply silicone grease to the divider, taking care that the regions under which the axons will cross receive a neat rope of grease.

5. Pick up a collagen-coated, prescratched culture dish, remove and set aside its lid, and invert the dish quickly, so as not to disturb the drop of medium. Bring the dish into position over the divider, and drop it in place. (Fig. 2G). With fine forceps, press on the dish bottom in the area of the solid septum, and also around the rim, to seat the grease. Do not press in the region where the axons will cross. When the dish is properly seated, pick up the hemostat, turn it over, and release the dish, so that it falls gently on the work surface. With a dab of grease, caulk any regions around the perimeter of the divider that did not seal properly. Place a drop of culture medium in the right compartment, and ensure that the full length of the scratched region is wetted with medium. Replace the cover of the dish.
6. Repeat steps 3–5 for each dish, then store trays of 12 dishes in the incubator as they are completed. After 1 h or more in the incubator, fill the center and distal compartments with culture medium.

5. PLATING THE NEURONS

5.1. Materials

Cell suspension.
Syringe, sterile, 1-mL, with 22-gage, 1.5-in needle (two are needed for side-plated cultures).

5.2. Procedure

1. Most commonly, sympathetic neurons dissociated from superior cervical ganglia of newborn rats are plated into the compartmented dishes; however, dorsal root ganglion neurons from rat embryos and other types of neurons have been used. Obtaining sympathetic neurons is carried out as described by Campenot and Draker (1989), modified from Hawrot and Patterson (1979). The neurons from 20 ganglia are suspended in appox 1.3 mL L-15 CO_2 medium containing methylcellulose, nerve growth factor, rat serum, cytosine arabinoside, and vitamin C. For center-plated cultures, use 0.8 mL cell suspension/12 dishes, and, for side-plated cultures, use 0.5 mL/12 dishes. Allow 0.5 mL waste.
2. Transfer cell suspension to a 60-mm plastic Petri dish, tilted so that the fluid remains on one side, and load the cell suspension into a 1-mL syringe fitted with a 22-gage, 1.5-in. needle. For center-plated cultures, inject cell suspension into the center compartment slot of each dish. For side-plated cultures, remove residual medium from within the silicone grease perimeter, which will contain the neurons (Fig. 2G), by using an empty 1-mL syringe and needle. Then inject the cell suspension with care, so it adheres to the Teflon divider. Also, with side-plated cultures, use a syringe and needle to establish confluence between the medium in the center compartment slot and the medium in the outer perimeter of the center compartment. The next day, top up the cell-containing compartments with culture medium.

6. CONSTRUCTION OF PIN RAKE

6.1. Materials

Insect pins (21), size 1 (Carolina Biological Supply, Burlington, NC, cat. no. 65-4331).
Forceps (1), fine.
Handsaw (1), small.
Aluminum sheet (1), about 3 x 3 x + in.
Rod (1), phenolic or Plexiglas, 7/16 in diameter x 6 in.

Hot plate.
Dissecting microscope.
Parafilm™.
5-Minute™ epoxy cement.

6.2. Procedure

1. A pin rake can, in principle be constructed from any type of pin. The authors typically use size 1 insect pins, which are approx 200 μm in diameter, and produce collagen tracks about 200 μm wide.
2. Cut the heads off 21 size 1 insect pins.
3. Arrange a hot plate so that its surface may be viewed with a dissecting microscope. Place a piece of 1/2 in.-thick aluminum sheet (about 3 × 3 in.) on the hot plate, and melt a piece of Parafilm (about 2 × 2 in.) onto the surface of the aluminum sheet, to produce a sticky (not liquid) surface. The amount of heat required will be easily determined empirically. Make sure the Parafilm is positioned such that it comes right to one of the edges of the aluminum sheet.
4. Hold the back end of one of the pins with fine forceps. Embed a pin in the Parafilm, sothat the point is visible under the dissecting microscope, and the back end extends over the edge of the aluminum plate. The pin may be pressed into the Parafilm with the side of the forceps. Place the next pin in the same way, and locate it against the first pin, with the points as even as possible. Continue placing pins until there are 21 pins, side by side, with their points as even as possible. Then, using the back end of the forceps as a straightedge, push against the points of the pins to move them slightly backward and, thus, even out the line of the points. In this way, the points can be made to line up almost perfectly straight. Turn off the hot plate.
5. Mix some 5-Minute epoxy cement, and put several drops on the pins at about their midsection, to cement them together. After the epoxy has set, lift the pin rake from the Parafilm.
6. A handle for the pin rake can be made from Plexiglas, or phenolic tubing or rod of about 7/16 in diameter. A slot to accommodate the pins must be sawed in the end of the rod (tube), across the diameter and about 1 1/2 in. along the length. The back ends of the pins are then placed in the slot, so that the points extend about 1/2 in. beyond the handle. Once positioned, the pins are glued in place with epoxy, and, in a series of applications of epoxy, a cone is built up around the pins, so that only about 1/8 in. of their ends extend beyond the epoxy.
7. The pin rake is cleaned and sterilized by wiping with a lint-free tissue soaked in 70% ethanol, then allowed to air-dry in the hood.

7. PREPARATION OF SILICONE GREASE SYRINGE

7.1. Materials

Syringe (1), 1-mL, glass Luer-Lok™ .
Needle (1), hypodermic, 18-gage, with a squared-off tip.
Syringe (1), disposable, 10-mL.
Dow Corning™ high-vacuum grease in 150-g tube.
Scraps of vacuum tubing and ordinary rubber tubing.
Stiff, uninsulated wire.
Pliers.

7.2. Procedure

1. Apply silicone grease to the Teflon divider, through a 1-mL, glass, Luer-Lok syringe fitted with an 18-gage needle with a squared off tip. A needle about 2 cm long works best. Because some force is required to squeeze the grease through the needle, it is beneficial to slip a 2-in. length of vacuum tubing over the barrel, and to pad the plunger by taking a $^3/_4$ in. length of rubber tubing of suitable diameter, cutting a hole in the side of the tubing midway between the ends, and slipping the head of the plunger through the hole. In this way, pressure can be applied with the first and second fingers crooked over the end of the vacuum tubing, encasing the barrel, and the thumb pressing against the padded plunger.
2. To fill the applicator with silicone grease, first squeeze the grease from its tube into a 10-mL disposable syringe with no needle.
3. Remove the needle from the applicator syringe with pliers, and, if there is grease remaining from previous applications, empty it by fully depressing the plunger. Then slowly (in order not to break the glass) withdraw the plunger completely, and set it aside.
4. Hold the 10-mL syringe by the body in one hand, nipple up, with the plunger resting on the lab bench. Hold the body of the applicator syringe in the other hand and place it over the nipple, holding it to make a tight seal. Press down with both hands, to depress the plunger of the 10-mL syringe, and fill the 1-mL syringe about three-fourths full with silicone grease. Then insert the plunger into the 1-mL syringe, and depress it until grease comes out of the nipple. Wipe with a lint-free tissue.
5. Sterilize the filled applicator syringe by dry heat, with attention to the following details:
   a. Any air bubbles trapped in the applicator syringe can expand during heating and blow out the plunger. To prevent this, wire the plunger securely to the syringe by looping some ordinary, stiff, uninsulated wire over the end of the plunger and around the barrel of the syringe below the vacuum tubing. Bubbles will then harmlessly force grease out the needle.
   b. Applicator syringes need not be wrapped for heating, but should be placed in a pan or on aluminum foil to catch the exuded grease. Store the filled applicator syringe until needed.
6. Before using the applicator syringe, squeeze out some grease, wipe it away with a lint-free tissue, then sterilize the exterior by dipping into 70% ethanol and allowing it to air-dry.

8. NEURITOTOMY

8.1. Materials

Syringe (1), sterile, 3-mL disposable, with a 1.5-in., 22-gage needle.
Serum bottle containing sterile, culture-quality dH_2O.
Dissecting microscope set up in a sterile hood.
Sterile pipets and bulbs.
Sterile Pasteur pipet set up as an aspirator.

8.2. Procedure

1. Assemble in a sterile hood equipped with a stereo microscope, a sterile 3-mL syringe with needle, and a serum bottle containing dH_2O.
2. Orient a three-compartmented culture under the dissecting microscope, so that it is rotated 180° from the ordinary orientation. Remove the lid, and aspirate the medium from the compartments to be neuritotomized. Using the 3-mL syringe, squirt the substratum bearing the axons moderately vigorously, until the compartments fill with water. Do not squirt directly

at the silicone grease barrier. Aspirate the water, and repeat squirting two more times. Then aspirate the water, and supply the desired medium to the compartments. Cover the culture dish.

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Chapter Six

Chick Spinal Somatic Motoneurons in Culture

Bernhard H. J. Juurlink

1. INTRODUCTION

Cultures enriched in spinal somatic motoneurons are useful test systems for delineating the role of trophic factors in motoneuron survival and differentiation (e.g., McManaman et al., 1989, 1990; Juurlink et al., 1991a; Henderson et al., 1994; Mettling et al., 1995; Kaal et al., 1997). They also form useful test systems for examining the roles of Fas receptor (Raoul et al., 1999) and thrombin receptor (Turgeon and Houenou, 1999) activation in motoneuron survival. Motoneurons in such cultures are obtained from embryonic spinal cord. There are basically two approaches whereby one can obtain cultures highly enriched in spinal motoneurons. One approach takes advantage of the fact that peripheral terminals of motoneurons can take up fluorescent labels and retrogradely transport these labels to the somas. Spinal cords can then be dissociated and labeled motoneurons isolated, using a fluorescence-activated cell sorter (e.g., Calof and Reichardt, 1984; Schaffner et al., 1987); this approach is labor-intensive and yields small numbers of neurons, of which only about 80% are labeled motoneurons. There are also questions concerning the effects of the tracer on the motoneurons (Smith et al., 1986). The second approach takes advantage of the fact that the buoyant density of motoneurons is significantly different from that of the other neural cell populations; here, motoneurons are separated from other cell populations by centrifuging cells through a density gradient (Schnaar and Schaffner, 1981; Dohrmann et al., 1986). This approach has the advantages that, technically, it is much simpler than the fluorescence-activated sorting method, and much larger numbers of motoneurons can be isolated. The cultures established from this latter approach, as described in this chapter, are free of nonneuronal cells, and are comprised of about 95% motoneurons as determined by calcitonin gene-related peptide immunocytochemistry (Juurlink et al., 1990).

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2. ISOLATION OF THE CHICK EMBRYOS

2.1. Materials

Fertile eggs (12), 6 d incubation (Hamilton and Hamburger [1951] stages 29–30, Carnegie stage 19 [Butler and Juurlink, 1987]).
Probe (1), sharp.
Forceps (2), sterile, 3 1/2 in.
Forceps (1), curved sterile, 4 1/2 in.
Petri dishes (3), sterile, 60-mm, containing 5 mL holding medium.
Egg carton.
Ethanol, 70%.
Box of tissue paper or small squares of gauze.
Waste receptacle.

2.2. Procedure

1. Candle eggs and select eggs with viable embryos.
2. Place eggs blunt end up (i.e., air sac end up) in an egg carton or other holder placed adjacent to the laminar flow hood. Do not remove more than six embryos at one time.
3. Using tissue paper or gauze, wash the egg surface with 70% ethanol. This cleans the shell, but does not completely sterilize it.
4. Using a sharp probe, poke a hole through the shell at the blunt end.
5. Use one pair of 3 1/2 in forceps to remove the egg shell. This is initiated by placing one tine of the forceps through the hole just made, then closing the forceps and breaking off part of the egg shell. This should be done by holding the egg at an angle over the waste receptacle, so that the broken bit of shell falls into the waste receptacle, rather than into the air space. Continue to nibble the egg shell away, until the shell surrounding the air space is completely removed. The forceps may then be resterilized by placing in 70% ethanol.
6. Use the second set of 3 1/2 in. forceps to peel away the shell membrane. This is done by grasping the membrane at the edge of the air space and gently pulling toward the other edge. The embryo, surrounded by the chorioallantoic membrane, is now exposed to view.
7. Gently pierce the chorioallantoic membrane with one tine of the curved forceps, and hook it around the neck of the embryo. Partially close the forceps, and slowly lift the embryo out of the shell. The embryo is still attached to the extraembryonic membranes (amnion, yolk sac, allantois, and chorion), and, if the embryo is slowly lifted out of the egg, these membranes will tear, thus freeing the embryo.

Note: If the embryo is lifted too quickly, the neck of the embryo will rupture.

Place the embryo into the 60-mm sterile Petri dish containing holding medium.

3. ISOLATION OF SPINAL CORDS

3.1. Materials

Forceps (1), small curved, sterile.
Forceps (2), no. 5 microdissecting.
Insect pins (3), stainless-steel, no. 1 (Polyscience, Niles, IL).
Holding medium.
Wax dissecting dish (1), sterile.

Test tube (1).
Petri dish (1), 60-mm, sterile.
Pasteur pipet (1), sterile, cotton-plugged with curved fire-polished tip.
Small rubber bulb.
Test tube rack.
Dissecting microscope.

3.2. Preparation of Materials

1. Sterile forceps can be placed on elevated surfaces on either side of the dissecting microscope. This is very convenient for dissection; however, ensure that the forceps' tines do not contact any surface.
2. Place 3 mL holding medium into the dissecting dish.
3. Place the test tube at a slant in the test tube rack situated near the dissecting microscope. Insert the rubber bulb onto end of the Pasteur pipet and wet the pipet by drawing and expelling the holding medium. Use the test tube as a holding container for the pipet.
4. Place 5 mL holding medium into a 60-mm Petri dish, and place the dish adjacent to dissecting microscope.

3.3. Removal of Spinal Cord

1. Hook one tine of the small curved forceps around the neck of the embryo, lift embryo, and place it in the wax dissecting dish.
2. Pin embryo ventral (belly) surface down.
   a. Place one pin through the hindbrain.
   b. Grab one leg with forceps, pull taut, and pin leg.
   c. Grab second leg, pull to the side, and pin. The head and the feet of the embryo should form the corners of a triangle.
3. Using the two microdissecting forceps, remove the spinal cord as follows (Fig. 1):
   a. One pair of forceps is used to steady the embryo, while a tine of the other pair is used to tear through the skin along the dorsal midline from the hindbrain to the tail. Using a tine of the forceps, free the skin from the underlying tissues along either side of the dorsal midline; this exposes the spinal cord to view.
   b. Use the tine of a pair of forceps to cut the roof of the spinal cord, exposing the central canal. This causes the entire length of the spinal cord to open up like a book.
   c. Rub a tine of the forceps along the outer surface of one side of the spinal cord, and then that of the other side. If done properly, this separates the meninges and dorsal root ganglia (DRG) from the spinal cord.
   d. Use the forceps to cut the spinal cord cranially at its junction with the hindbrain and caudally in the sacral region.
   e. The notochord is separated from the spinal cord by sweeping the ventral surface of the spinal cord with forceps. Now lift the spinal cord away from the embryo. With practice, the spinal cord can be isolated free of meninges and DRG, using this procedure. If some DRGs are still present, it is essential that these be removed before proceeding any further.
4. Use the wetted Pasteur pipet to transfer the spinal cord to the holding medium.
5. After six spinal cords have been isolated, another six embryos can be removed from the eggs, spinal cords removed, and so forth, until sufficient spinal cords have been collected.

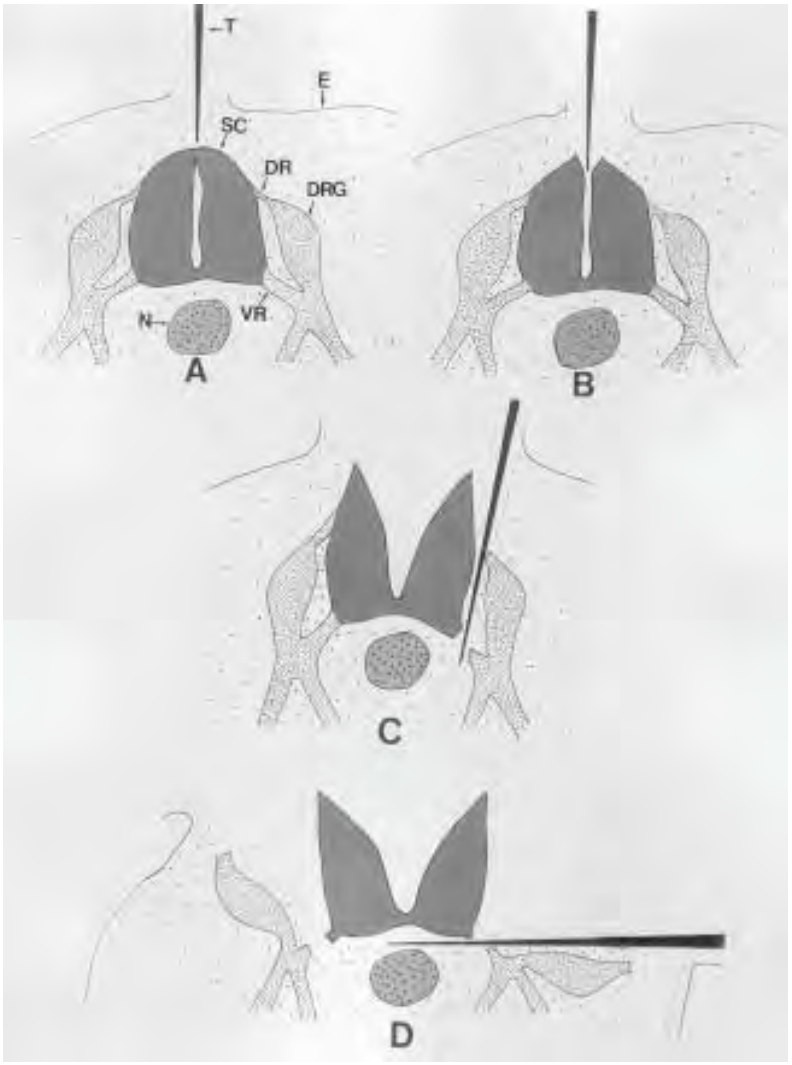

Fig. 1. Cartoon demonstrating the procedure for isolating the spinal cord from an embryo. **(A)** A tine (T) of a pair of forceps is used to cut the skin along the dorsal midline from the hindbrain to the tail, thus exposing the spinal cord to direct view. DR, dorsal root of spinal nerve; DRG, dorsal root ganglion; E, epidermis; N, notochord; SC, spinal cord; VR, ventral root of spinal nerve. **(B)** A forceps' tine is used to cut the roof of the spinal cord, causing the spinal cord to open up in the manner of a book. **(C)** The meninges and DRG are separated from the spinal cord by rubbing the forceps along the lateral surface of the spinal cord. **(D)** After cutting the spinal cord at its junction with the hindbrain, the forceps are used to separate the spinal cord from the notochord.

4. ISOLATION OF MOTONEURONS

4.1. Materials

Complete growth medium (*see* Section 7.2).
Puck's solution containing 15 m*M* sodium bicarbonate ($NaHCO_3$) and 2 m*M* Na pyruvate.

Trypsin solution (1.0%).
Deoxyribonuclease (DNase) solution (2.0%).
Bovine serum albumin (BSA) solution (3.5%).
Metrizamide solution (6.4%).
Tubes, centrifuge, polystyrene, sterile, 15-mL.
Petri dishes, tissue culture, sterile, 60-mm.
Pasteur pipets, sterile cotton plugged with fire-polished curved tips.
Test tube (1).
Serological pipets, cotton-plugged, 5-mL.
Serological pipets, cotton-plugged, 10-mL.
Stomacher Lab Blender 80™ (Seward Medical UAC House, London, UK, distributed in Canada by Baxter, Mississauga, ON, Canada), set at 160 double kicks/min.
Whirl-pak™ bag (Nasco, New Hamburg, ON, Can, distributed in Canada by Baxter), sterile, 10 cm wide, marked 8.5 cm from the bottom.
Pipetor, µL.
Refrigerated centrifuge.

4.2. Procedure

1. Place spinal cords into a 60-mm Petri dish containing 5 mL Puck's bicarbonate solution. Add 125 µL 1.0% trypsin (final trypsin concentration of 0.025%), and incubate for 30 min in an incubator containing an atmosphere of 5% CO_2 in air, and maintained at 37°C.
2. Carefully aspirate the trypsin solution, and resuspend spinal cords in 5 mL Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12). Place spinal cords and DMEM/F12 into sterile tube.

Note: From this point until cells are planted into culture, whenever possible, keep all solutions and cells at 0–4°C.

3. Allow spinal cords to settle for 5 min, and decant DMEM/F12. Add 15 mL DMEM/F12–0.02% DNase, and place spinal cords plus medium into Whirl-pak bag.
4. Place the lower 8.5 cm of the Whirl-pak bag into Stomacher Lab Blender adjusted to 160 double kicks/min. Disaggregate spinal cords for 2 min.
5. Remove cell suspension from the Whirl-pak bag, and place into test tube. Keep the test tube cold and upright. Allow fragments to settle for 10 min. Collect fragment-free cell suspension, place into centrifuge tube, and centrifuge (4°C) at 180*g* for 10 min.
6. Decant supernatant, and resuspend cell pellet in 5 mL DMEM/F12–0.02% DNase.
7. Carefully layer the cell suspension on top of 5 mL 3.5% BSA contained in a centrifuge tube. Centrifuge (4°C) for 15 min at 100*g*. This procedure results in much of the debris present in the cell suspension being trapped in the BSA cushion.
8. Decant the supernatant, and resuspend the pellet in 5 mL DMEM/F12–0.02% DNase. Carefully layer cells on top of 5 mL 6.4% metrizamide. This is done by laying the metrizamide-containing tube on ice at an angle of approx 30°. The cell suspension is taken up with a Pasteur pipet, and, with the tip of the pipet about 1 cm from the metrizamide solution surface, the cell suspension is slowly allowed to run down the tube and over the surface of the metrizamide. Once the cell suspension is layered on top of the metrizamide, the centrifuge tube is held upright, and the interface between the cell suspension and the metrizamide is very slightly intermixed, with gentle movements of the tip of a curved Pasteur pipet.
9. Place tube into centrifuge (4°C), and centrifuge at 500*g* for 30 min.
10. A band of cells is readily visible at the interface of the medium and metrizamide solution; this band consists of motoneurons. The remaining spinal cells have entered the metrizamide

solution. Remove the motoneuron fraction using a Pasteur pipet. Dilute cells in 10 mL DMEM/F12–0.02% DNase, and centrifuge at 180*g* for 10 min. Discard supernatant, and resuspend cells in 1.0 mL growth medium (*see* Section 7.2.) containing 0.02% DNase. This last procedure removes the residual metrizamide present in the motoneuron fraction.

11. Determine the cell density of this final cell suspension, using a hemocytometer. Each spinal cord should yield approx 1.5×10^5 motoneurons.

5. PLANTING OF MOTONEURONS

5.1. Materials

0.02% DNase in complete growth medium.
Petri dishes, 35-mm, sterile, coated with poly-D-lysine.
Coverslips *(optional)*, 12-mm round, glass (German glass recommended), sterile, coated with poly-D-lysine.
Microbiological Petri dishes *(optional)*, 100-mm sterile, plastic.
Petri dishes *(optional)*, 35-mm, sterile.

5.2. Establishing Motoneuron Cultures

1. Dilute cell suspension to 4×10^4 cells/mL complete growth medium containing 0.02% DNase. This results in a very low cell density in the cultures; however, cells can be planted at higher densities. Plant 1.5 mL cell suspension into each 35-mm coated Petri dish, or plant 150 µL cell suspension onto each glass coverslip (final cell densities are approx 60 cells/mm^2). Place cultures into an incubator containing a humidified atmosphere of 5% CO_2 in air.

Note: The glass coverslips should be in a hydrophobic 100-mm microbiological Petri dish, which tends to keep the medium on the coverslips.

2. After 1 h, place the coverslips into 35-mm uncoated Petri dishes containing 1.5 mL growth medium, and return to the incubator.
3. After 1 d, feed the cultures with fresh growth medium, and feed twice a week thereafter.

6. DESCRIPTION OF CULTURES

By 1 d of culture, 75% of the neurons planted are alive and putting forth neurites. Of these, half will survive to 8 d of culture (Juurlink et al., 1991b). At that time, the neurons are large cells with one long axon and numerous dendrites coming off the soma (Fig. 2). After 1 mo, 20% of the neurons are still alive. Approximately 95% of the neurons are motoneurons (Juurlink et al., 1990). If one observes glial cells in these cultures, it is because cell aggregation was not prevented in the steps between spinal cord disaggregation and cell planting; this suggests either that the cells were not kept cold during this time or that the DNase was not active.

7. APPENDIX

7.1. DMEM/F12 (Gibco-BRL, cat. no.12500-047)

Prepare per instructions that come with the medium package. This medium is buffered with 15 m*M* HEPES and 14 m*M* $NaHCO_3$. In an atmosphere of 5% CO_2, this medium attains a pH of 7.2.

7.2. Complete Growth Medium

1. Stock solutions of medium supplements:

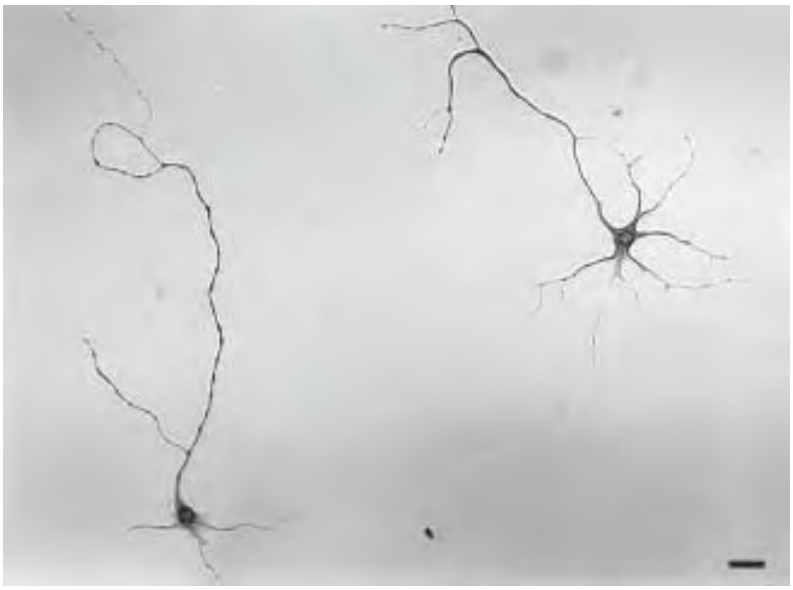

Fig. 2. Micrograph of a 12-d culture demonstrating two motoneurons stained for neurofilament protein. Bar = 40 mm.

a. Transferrin (Sigma, cat. no. T-4515), 500 μg/mL: Dissolve 50 mg transferrin in 100 mL DMEM/F12.
   Filter-sterilize and store in small aliquots at –20°C.
b. Selenium (Sigma, cat. no. S-5261), 3×10^{-6} *M*:
   i. Dissolve 5.2 mg sodium selenite in 10 mL DMEM/F12, and filter-sterilize.
   ii. Dilute 1.0 mL of this in 99 mL DMEM/F12 (final concentration is 3×10^{-6}*M*).
   iii. Store at –20°C in small aliquots.
c. Progesterone (Sigma, cat. no. P-6149), 2×10^{-6} *M*:
   i. Dissolve 3.1 mg progesterone in 10 mL 95% ethanol, and filter-sterilize.
   ii. Divide 200 μL of this in 99.8 mL DMEM/F12 (final concentration is 2×10^{-6} *M*).
d. Insulin (Sigma, cat. no. I-1882), 500 μg/mL:
   i. Dissolve 5 mg insulin in 10 mL DMEM/F12. Acidify this solution with 1 *M* HCl (the solution will be an orange-yellow), and filter-sterilize.
   ii. This solution can be stored at 4°C for up to 1 mo.
e. Pyruvate (Sigma, P-5280), 200 m*M*:
   Dissolve 0.176 g pyruvic acid in 10 mL DMEM/F12 (i.e., 200 m*M*). Filter-sterilize, and store in aliquots at –20°C.
f. Potassium chloride (Sigma, P-8041), 1 *M*:
   Dissolve 7.455 g KCl in DMEM/F12, and make up to 100 mL. Filter-sterilize, and store at 4°C.

2. Chemically defined growth medium:
   To 94 mL DMEM/F12 add:

Stock solutions	Amount	Final concentration
Progesterone	1 mL	2×10^{-8} *M*
Transferrin	1 mL	5 μg/mL

Selenium	1 mL	3×10^{-8} *M*
Insulin	1 mL	5 µg/mL
Pyruvate	1 mL	2 m*M*
KCl	1 mL	14 m*M* (additional 10 m*M* KCl)

3. Heat-inactivated horse serum:
   a. Aliquot horse serum into sterile 15-mL test tubes with screw caps. Loosen the caps, and place test tubes for 30 min in a water bath maintained at 56°C.
   b. Allow tubes to cool in a laminar flow hood, tighten caps, and store at –80°C.
4. Complete growth medium:
   To 95 mL of the above chemically defined medium, add 5 mL heat-inactivated horse serum. In addition, add muscle extract (*see* Section 7.4.) to a final concentration of 50 µg protein/mL medium.

7.3. Holding Medium

This medium consists of DMEM/F12 containing 1.0 m*M* $NaHCO_3$ and 15 m*M* HEPES, pH 7.2, as buffer plus 5% heat-inactivated horse serum. Since this medium is buffered with HEPES, it will maintain a pH of 7.2 in an air atmosphere.

7.4. Muscle Extract

1. Make extraction buffer: The extraction buffer consists of 10 m*M* Tris-HCl, pH 7.5, containing 1 m*M* ethylenediamine tetraacetic acid (EDTA), 1 m*M* *N*-ethylmaleimide, 1 m*M* benzamidine, and 2 m*M* phenylmethylsulfonyl fluoride.
   a. To 120 mL of triple-dH_2O, add 0.188 g Tris-HCl, 0.048 g EDTA, 0.025 g benzamidine-HCl, and 0.031 g *N*-ethylmaleimide.
   b. Place 98 mL of this solution in a 250-mL Erlenmeyer flask. While vortexing the solution, add 2 mL 100 m*M* phenylmethyl-sulfonyl fluoride dissolved in absolute ethanol.

   Note: Caution should be used in the preparation and use of the extraction buffer, since many of the chemicals in this buffer are extremely toxic.
2. Muscle extract is prepared as outlined by Dohrmann et al. (1986). Muscle is collected from hindlimbs of 18-d-old chick embryos and frozen at –80°C, before being homogenized with a Polytron™ in 4 vol of extraction buffer, i.e., for each gram of muscle extract, add 4 mL extraction buffer.
3. Let the homogenate stand at 4°C for 1 h, then centrifuge at 15,000*g* for 1 h, determine the protein concentration, and store in small aliquots at –80°C, until ready to be used in the culture medium.

7.5. Puck's Solution with Bicarbonate and Pyruvate

1. Puck's balanced salt solution.
   a. Composition:

Components	g/L
NaCl	8.0
KCl	0.4
KH_2PO_4	0.06
Na_2HPO_4	0.048
Glucose	1.0
Phenol red	0.02

b. Filter sterilize, aliquot, and store at 4°C.

2. To 97.5 mL Puck's solution:
   a. Add 1.5 mL 1.0 *M* $NaHCO_3$. In a 5% CO_2 atmosphere, this solution, containing 15 m*M* $NaHCO_3$ will attain a pH of 7.2.
   b. Add 1 mL 200 m*M* Na pyruvate.

7.6. Enzyme Solutions

1. Trypsin (1.0%):
   a. Add 1 g trypsin (Gibco-BRL, 1:250, cat. no. 27250) to 100 mL Puck's solution. Allow to dissolve (not all of the trypsin goes into solution).
   b. Filter through a set of filters, ranging in pore size from 5 to 0.2 μm.
   c. Aliquot, and store at –20°C.
2. DNase (2.0%):
   a. Add 200 mg crude DNase I (Sigma, cat. no. DN-25) to 10 mL DMEM/F12.
   b. Filter-sterilize, and store at –20°C in small aliquots.
3. 0.02% DNase in complete growth medium:
   Add 0.4 mL 2.0% DNase to 39.6 mL complete growth medium (final DNase concentration is 0.02%).

7.7. 3.5% BSA (Sigma, cat. no. A-2153)

Add 0.875 g BSA to the surface of 25 mL DMEM/F12. Do not stir. Allow the BSA to go into solution, then filter-sterilize. Store in aliquots at 4°C.

7.8. Metrizamide (Sigma, cat. no. M-3383)

1. Stock solution (38%):
   a. Dissolve 38 g metrizamide in 50 mL triple-dH_2O and bring volume up to 100 mL. Filter-sterilize.

   Note: Because of the varying water content of the metrizamide powder, the actual content of metrizamide may deviate from 38%; therefore, the actual density of the metrizamide solution should be determined by measuring the refractive index. The refractometer is calibrated with dH_2O and a saturated NaCl solution. The refractive index of dH_2O is 0%; saturated NaCl is 29.6% at 20°C. Appropriate instructions are provided with the refractometer.

   b. Keep this solution at 4°C in the dark.
2. Metrizamide working solution (6.4%):
   Place 1.01 mL 38% metrizamide in a sterile polystyrene centrifuge tube, add 4.99 mL DMEM/F12, and mix well. Keep the capped tube on ice.

7.9. Preparation of Culture Substrata

1. Poly-D-lysine stock solution:
   Prepare a stock solution of 1 mg/mL poly-D-lysine (Sigma, cat. no. P-6407) in triple-dH_2O, filter-sterilize, and store in small aliquots at –20°C, until ready to use.
2. Preparation of coverslips:
   Round coverslips, 12-mm in diameter, are a convenient surface for growing motoneurons. Clean such coverslips by soaking in acetone, then air-dry. Sterilize the coverslips by heating at 190°C for 3 h.

3. Poly-D-lysine-coated culture substrata.
   a. Petri dishes:
      i. Dilute stock poly-D-lysine solution in sterile triple-dH_2O to 50 µg/mL (i.e., add 1.0 mL stock solution to 19 mL water).
      ii. Place 1.5 mL of this solution in each 35-mm Petri dish. After 2 h, remove fluid, wash with sterile triple-distilled water, and allow Petri dishes to dry. Such dishes are usable for a period of several weeks. **Note**: If dishes are prepared just prior to planting cells, they should be washed with DMEM/F12.
   b. Glass coverslips:
      Place coverslips into 100-mm microbiological plastic Petri dishes and add 150 µL diluted (50 µg/mL) poly-D-lysine solution to each coverslip. After 2 h, remove the fluid, wash the coverslips with sterile triple-dH_2O, and allow to dry.

Note: Coverslips are placed into microbiological Petri dishes, because these dishes are hydrophobic, and this tends to ensure that the aqueous solution stays on the coverslip, rather than spilling onto the surface of the Petri dish.

7.10. Dissection Material

1. Instrument cleaning and sterilization:
   a. After use, soak instruments in 7X™ (ICN Biochemicals, cat. no. 76-674-95) detergent, and clean using a nylon brush. Rinse well with hot water, followed by 70% ethanol. Allow to dry and store.
   b. Before use, sterilize instruments and insect pins by placing in a plastic beaker containing 70% ethanol.
2. Dissecting dish:
   A convenient dissecting dish can be prepared by pouring a molten mixture of three parts regular blue dental inlay wax (Sybron, Romulus, MI) and one part paraffin (melting point of 56°C) into a glass 60-mm Petri dish. The dish can be sterilized for use by dry heat at 250°F for 2 h. The 60-mm dish is a convenient size, because one can manipulate the position of the dish under the dissecting microscope, using the fifth fingers, and at the same time manipulate the dissecting instruments, using the first and second fingers. The wax is not brittle, thus ensuring that the wax surface remains smooth, despite repeated pinnings of embryos. The blue color of the wax also aids in visualizing the white tissues of the embryo, when appropriate illumination is used. After use, the wax dish can be washed using 7X detergent and nylon brush. After air-drying, the wax surface can be refinished by using the flame of a Bunsen burner to melt the wax. Once cooled, the dissection dishes can be sterilized by filling with 70% ethanol. The alcohol is removed prior to use, and the dish is allowed to dry in a laminar flow hood.

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Chapter Seven

Primary Cultures of Sympathetic Ganglia

Mary I. Johnson

1. INTRODUCTION

A derivative of the neural crest, sympathetic neurons have been utilized in both in vivo and in vitro studies to approach a number of basic questions concerning the development and function of the nervous system. The superior cervical ganglia (SCG), and particularly the sympathetic chain, can provide significant numbers of neurons with relatively little effort in dissection, and, with a known and available growth factor (nerve growth factor [NGF]), can be maintained in culture for prolonged periods of time. As a result, cultures of sympathetic neurons, many times from rats or mice, but also from avian and amphibian sources, have been utilized to study factors influencing synaptic function, neurotransmitter differentiation, neurotrophic dependence, dendritic development, and axonal growth, including the structure and function of growth cones. Studies of axonal elongation, substrate requirements, and molecular interactions, underlying neurite extension and ensheathment, have utilized explants; other experiments on dendrite growth, cell death, and neurotransmitter changes have used dissociated, even isolated, single neurons, depending on the question being asked. The following paragraphs contain detailed methods for the dissection of sympathetic neurons from embryonic, perinatal, and adult rats.

2. DISSECTION OF EMBRYONIC OR PERINATAL RAT SCG

SCG from gestational d-15–21 embryos (E15–E21) or perinatal rats (postnatal d 1–4; PN1–PN4) can be used as explants or to generate dissociated neurons.

2.1. Materials

Forceps (2), fine, Dumont no. 5 (Fine Science Tools [FST], cat. no. 11252-30) or forceps, fine, Dumont no. 4 (FST, cat. no. 11241-30).
Forceps, finest (FST, cat. no. 11254-20).
Forceps (1), eye-dressing, serrated (FST, cat. no. 11051-10).
Forceps (1), Adson-Graefe tissue (FST, cat. no. 11030-12).
Scissors (1), fine iris, curved (FST, cat. no. 14061-11).
Spring scissors (1), Noyes (FST, cat. no. 15012-12).
Blade holder (FST, cat. no. 10052-11).

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Razorblade fragments: blade edge should be ~1–2 mm in length (FST, cat. no. 10050-00).
Needles (3), 25-gage.
Leibovitz's L-15 medium (Gibco-BRL, cat. no. 11415).
Petri dish, 100 × 15-mm, glass, half filled with Sylgard (*see* Appendix 1.3.).
Petri dish, 150-mm, sterile.
Petri dishes (2), 35-mm, tissue culture (each with 1–2 mL L-15) housed in a 100-mm Petri dish.

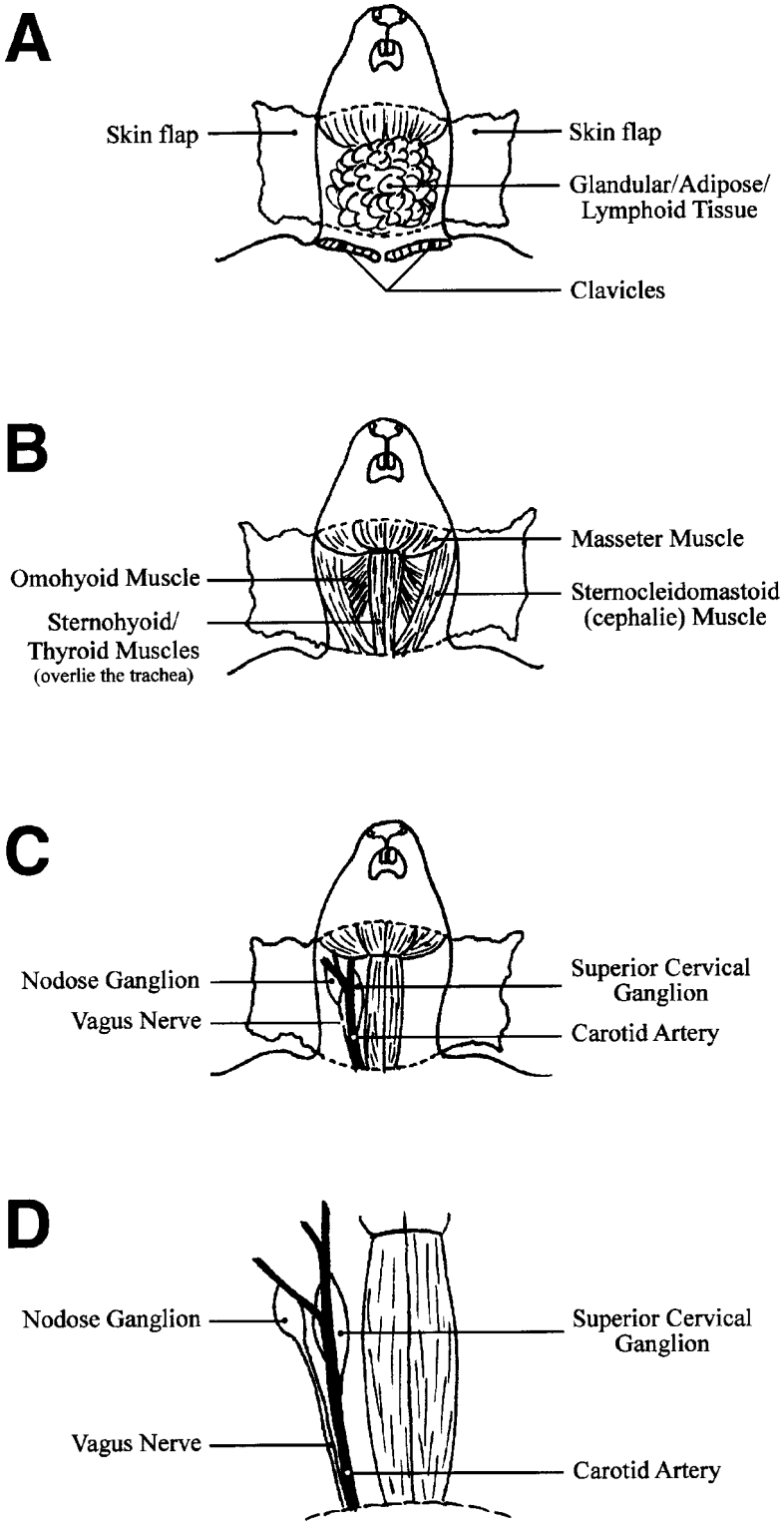
2.2. Preparation of Embryos and Perinatal Rat Pups

1. Remove embryos from pregnant rats, as detailed in Chapter 8 (Sections 2.3.–3.3.).
2. Euthanize rat pups up to 4-d-old, using CO_2 (*see* Section 5.7.), and sequentially clean them in five beakers, containing septisol, two separate rinses of water, and two separate rinses of 70% ethanol. Use cotton-tipped swabs to scrub the anterior neck, while holding the pup by the lower extremities with a sturdy forceps. Place the pups in a sterile, 150-mm Petri dish.
3. For both embryos (E15–E21) and postnatal rat pups (PN1–4), cut into the left thorax and through the heart, to assure quick exsanguination and to reduce bleeding during the dissection.

2.3. Dissection of SCG

1. Using two 25-gage needles, pin the embryos supine, by the upper extremities, onto a Petri dish half-filled with Sylgard. The Sylgard dish is sterilized by filling with 70% ethanol, pouring off the ethanol after 20 min, and allowing to dry in the hood. Use a third needle through the anterior palate to gently hyperextend the head and provide better exposure of neck. For the younger embryos (E15-17), one needle at either end of the body is sufficient to give stability.
2. Make a midline incision in the skin (fine iris scissors and Adson-Graefe tissue forceps) and lateral incisions at the level of the clavicles, as well as rostrally at the mandibles. Reflect laterally. This will expose a large mass of tissue in the midline (Fig. 1A).
3. Using spring scissors and eye dressing forceps, remove this glandular-adipose-lymphoid mass, to expose the muscle layer (Fig. 1B).
4. Reflect the sternocleidomastoid muscles laterally by incising them near the clavicle (Fig. 1B). Avoid nicking the superficial vein (external jugular).
5. Using a tip of fine forceps (no. 4 or no. 5 Dumont), lift, at its medial portion, the omohyoid muscle next to the trachea, reflecting it laterally. This is a thin muscle, so avoid grabbing too deeply with the forceps, possibly injuring the carotid sheath and SCG beneath (Fig. 1B).
6. The carotid should now be visible and, just laterally, the vagus nerve (a tough, rather substantial white band, with the nodose ganglion at the cranial end (Fig. 1C,D).

Fig. 1. Dissection of Superior Cervical Ganglion (SCG). **(A)** The initial dissection of the SCG involves a midline incision of the skin with lateral incisions, both to the left and right, at the level of the clavicles and the mandibles. The skin flaps are retracted laterally, exposing a mass of glandular, adipose, and lymphoid tissue. **(B)** The midline mass of glandular, adipose, and lymphoid tissue is removed, exposing the sternohyoid and thyroid muscles that overlie the trachea and the laterally placed sternocleidomastoid muscles. Obliquely stretching between the two are the omohyoid muscles. **(C)** The thin omohyoid muscle is carefully removed, exposing the carotid artery and the laterally placed vagus nerve. The sternocleidomastoid muscle may also be removed, allowing a better blunt dissection around and under the carotid, to expose the nodose ganglion at the cranial end of the vagus nerve and the more medially placed SCG. **(D)** A more magnified view of Figure (C). Careful dissection, using fine forceps, allows the clear definition of the SCG both cranially and caudally, removing any attachments of the ganglion posteriorly and to the carotid artery. The carotid artery may be transected and retracted back, to allow a full view of the SCG.


A
Skin flap
Skin flap
Glandular/Adipose/
Lymphoid Tissue
Clavicles
B
Masseter Muscle
Omohyoid Muscle
Sternocleidomastoid
(cephalic) Muscle
Sternohyoid/
Thyroid Muscles
(overlie the trachea)
C
Nodose Ganglion
Superior Cervical
Ganglion
Vagus Nerve
Carotid Artery
D
Nodose Ganglion
Superior Cervical
Ganglion
Vagus Nerve
Carotid Artery

7. The carotid branches and the nodose ganglion should be clearly visualized before proceeding. The area may need some freeing of loose connective tissue, fat, and lymph nodes (Figs.1D and 2).
8. Retract the carotid gently laterally with the no. 5 forceps. The SCG will be found just behind the carotid branch, on the side toward the trachea (Figs. 1D and 2).
9. Using two fine forceps (no. 4 or no. 5), blunt-dissect the carotid free from the SCG and the SCG free from the carotid connective tissue and muscle dorsal to it. This should sufficiently free the rostral end of the SCG, in order not to tear the ganglion as it is removed. Alternatively, the carotid is cut near the clavicle and retracted cranially, exposing both the SCG and the nodose attached to the vagus nerve.
10. The vagus and nodose may be removed, if desired, to avoid any possible confusion with the SCG. This is unnecessary in the older postnatal animal.
11. The SCG can now be removed, grasping it at either end, using two no. 5 forceps and lifting it out by nerve trunks, without stretching or injuring it. The younger the embryo, the more fragile the SCG.
12. Collect the ganglia in L-15 medium (in one of the 35-mm culture dishes housed within a 100-mm Petri dish).

Note: All of these steps can be accomplished in a sterile atmosphere. Remove younger embryos using two no. 5 fine forceps. For the postnatal pups and older embryos (E20 and E21), three sets of instruments are used, as described above. Care should be taken not to confuse the SCG with the more lateral nodose ganglion. After birth, the nodose ganglion becomes progressively separated from the SCG, making such confusion less likely.

2.4. Preparation of SCG Explants and Cultures

1. A well-defined capsule is present on the SCG, from approximately E18 to PN4, and its removal is facilitated by using extra-fine forceps (Biologie no. 5). For SCG from E15–E17, only thin wisps of capsule can be distinguished. Removing the capsule is desirable, if nonneuronal cell-free cultures are needed and minimal use of antimitotic agents is desirable. Removal of nerve trunks from the decapsulated ganglia will also greatly reduce the numbers of nonneuronal cells. For removal of the capsule and nerve trunks, we use the finest forceps (*see* Section 2.1).
2. The ganglia are then transferred to the second 35-mm dish containing L-15, and cut into approx 0.5–1 mm explants, using blades snapped from razorblades, and secured in a blade holder. Ganglia from E15 can be cut in half or, more often, are placed into culture intact. Older embryos yield 2–3 explants; perinatal rats, 3–4.
3. Rinse the explants in L-15 several times to remove tissue debris, remove the L-15, add media, and transfer them, in a small volume of medium, to the appropriate culture dish, with a fire-polished pipet. To promote explant attachment, the culture dishes should initially contain a small volume of medium (4–5 drops in a dish approx 2.5 cm in diameter). After 12–18 h, gently add enough medium, down the side of the dish, to cover the explants.

3. PREPARATION OF POSTNATAL GANGLIA

The dissection of the SCG from postnatal rats beyond PN4 differs only in some details from that described above for the embryonic and perinatal ganglia. For all ages, CO_2 anesthesia can be used and the dissection done after exsanguination and sterile preparation of the anterior neck. This can include shaving the neck, followed by septisol and alcohol rinses, or just vigorous septisol and alcohol scrubbing.

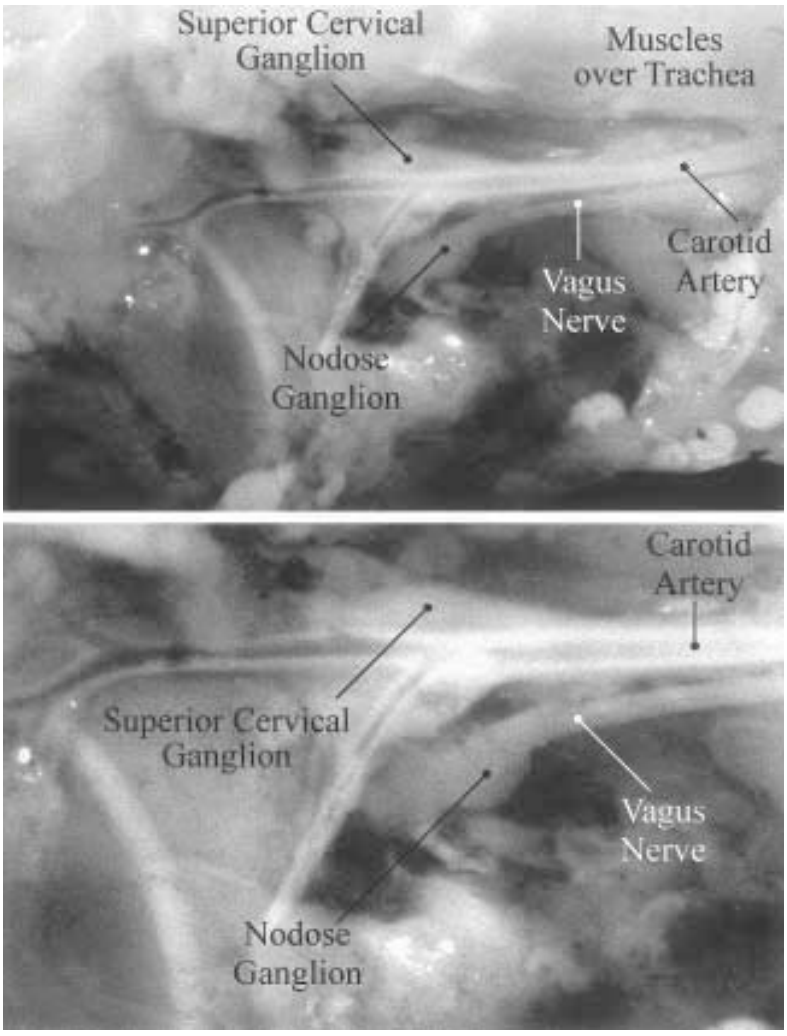


Fig. 2. Photomicrographs of SCG dissection. These views, at both low and high power, approximate Fig. 1C,D, but are horizontal in orientation. The carotid artery, as it travels cranially (to the left), divides into several branches. The internal carotid is not seen, but is traveling posteriorly between the SCG and the nodose ganglion.

1. As described above for perinatal rats up to PN4, three sets of sterile instruments are used for the dissection of skin, glands and muscles, and the SCG, to accomplish the sterile removal of the ganglia. Older postnatal animals (beyond 3–4 wk) will require bigger, sturdier forceps and scissors (4–6 in.).
2. Collect the ganglia in L-15 containing penicillin (100 U/mL) and streptomycin (100 mg/mL), and remove loose connective tissue and blood vessels. Antibiotics are used during the dissection, and sometimes for the first few days of culture, but typically not in the long-term feeding of the cultures. Use of the three sets of instruments, plus the antibiotics during the dissection, allows even prolonged culture without infection. This method was developed when concern over the effect of antibiotics on membrane properties became a concern in certain physiological studies. Antibiotics can affect the growth of nonneuronal cells (if desired) and neurons, especially in chemically defined serum-free media.

3. The ganglionic capsule can be removed from postnatal rats up to 5–7 d of age. By approximately postnatal d 10, it is impossible to remove the true capsule without damaging the ganglion.
4. Free the ganglia of nerve trunks and cut them into explants (*see* above) with a maximum diameter of 1 mm. Rinse the explants several times in the antibiotic containing L-15, before transfer to culture dishes.

4. PREPARATION OF DISSOCIATED NEURONS

Preparation of dissociated neurons from rats of all ages begins with the preparation of explants, as described above. Dissociation procedures then differ according to the age of rat under study.

4.1. Embryonic and Perinatal Neurons

Rinsed explants can be treated by two methods, to achieve disaggregation. Mechanical disaggregation, as described by Bray (1970), is preferred when exposure of the neurons to enzymes is undesirable. The cell population obtained is almost only neuronal; however, this procedure has the disadvantage of a lower yield (10–15%). Mechanical disaggregation also gives less satisfactory results for the younger embryonic SCGs (E15–E17), because these neurons seem more adherent to one another and, without enzyme treatment, remain in clumps. Enzymatic dissociation gives much higher yield (up to 90%), but nonneuronal cells will accompany the neurons in greater numbers.

1. Mechanical method:
   a. SCG are carefully stripped of capsule, all attached nerve trunks removed, and the ganglia cut into explants and rinsed. L-15 is removed and desired media added.
   b. Gently pull apart the SCG explants into small pieces, using two fine forceps.
   c. Two methods have been used to further disaggregate the ganglionic pieces:
      i. Method 1: Transfer to a test tube and agitate with a Vortex mixer. This generates a cell suspension with some remaining fragments.
      ii. Method 2: Triturate the ganglionic pieces through a small bore (0.5-mm) tip of a bent, fire-polished pipet.
   d. Filter as described below, and plate onto desired dishes or coverslips.
2. Enzymatic method:
   Enzyme dissociation utilizes 0.25% trypsin (Worthington Biochemical, Lakewood, NJ, cat. no. TRL-3 3707) made up in L-15 or Hanks' balanced salt solution (HBSS)(Gibco-BRL, cat. no. 14170), filter-sterilized, and used fresh or from a stock solution stored at –80°C.
   a. As for mechanical dissociation, the ganglia are stripped of capsule and attached nerves removed, before cutting into explants and rinsing with L-15.
   b. Remove the L-15 carefully, and incubate the explants, with gentle rotation in the trypsin solution (37°C, 30–45 min).
   c. Rinse 3× with L-15, and finally in the medium to be used for plating the cells. Serum-containing medium has a naturally occurring trypsin inhibitor. If plating in a chemically defined medium is desirable, further L-15 rinses are recommended, and both trituration and plating should be performed in defined medium with 2.5 mg/mL of bovine serum albumin (BSA).
   d. Using approx 1 mL vol, triturate the chunks against the side of the tissue culture tube, with a fire-polished pipet (0.5-mm bore).
   e. After 4–5 squirts, allow the fragments to settle, remove the cell suspension to another tube, and add more medium (0.5–1.0 mL) to the remaining chunks.

f. Repeat the trituration once or twice, combining the 2–3 suspensions. This procedure may reduce trauma to the cells released in the first or second trituration.

3. Preparation for plating suspended cells.
   a. The cell suspension (generated by either mechanical or enzyme dissociation) may be filtered through a nylon mesh (Tetko, Des Plaines, IL, pore size 15-μm, Nitex, cat. no. HD3-15) to remove cell aggregates and other tissue fragments. This step may reduce the yield, and may be eliminated if small fragments are not a problem for the experimental design. Alternatively, the cell suspensions can just be allowed to stand in a centrifuge tube for 3–5 min, to allow larger clumps or fragments containing more connective tissue to settle. The supernatant containing the suspended cells is then carefully removed.
   b. Estimate the cell numbers using a hemacytometer, and adjust the volume to give desired numbers of neurons per drop for plating.
   c. Plate filtered suspension onto the prepared culture dishes. To promote uniform plating, the suspension should be agitated frequently, and only 4–5 dishes seeded between agitations.

Note: If collagen is being used as the substrate for optimum attachment and growth, the air-dried layer of collagen should be applied to the dishes and air-dried just shortly (approx 1 h to allow for drying time) before plating the cells. Under these conditions, the cell suspension can be applied as several drops to the center of the dish. The neurons will be confined by surface tension to the area of the initial drop, facilitating the counting of all cells on the dish at a later date. After 18–24 h, further drops of media are added to completely cover the culture dish bottom.

4.2. Postnatal Rat SCG Neurons

The disaggregation procedure using trypsin for embryonic rats can be used for the SCGs from young rats up to 3–4 d postnatal, with similar results. Dissociated neurons can still be obtained from rats up to 10–12 d of life, but with reduced yields. With trypsin alone, it is virtually impossible to obtain substantial numbers of single neurons from rats over 2–3 wk of age. The best disaggregation and subsequent survival (10%) the author has obtained uses the following procedure.

1. Incubate SCG explants in 0.25% collagenase (Worthington, cat. no. CLSPA 5273) in HBSS (Gibco-BRL, cat. no. 24020-125), with gentle agitation, in a 35-mm Petri dish in a humidified atmosphere (37°C, pH 7.4).
2. After 45–60 min, the explants are sticky and clumped. Tease them apart, and gently stretch each one out, using two fine forceps.
3. Remove the collagenase solution, and add fresh collagenase-containing solution. Continue incubation for another 45–60 min. If the tissue is incubated for 90–120 min continuously in collagenase, without being teased apart, the yield is considerably reduced.
4. Rinse the chunks once in L-15, and incubate (45–60 min) in 0.25% pronase (Calbiochem-Novabiochem, La Jolla, CA, cat. no. 53702) in L-15, pH 7.4. Enzyme solutions should be prepared and filter-sterilized shortly before use.

Note: Empirically, it has been found that not all lot numbers of either enzyme (collagenase or pronase) give the same results. For pronase, in particular, some lots give few, if any, viable neurons. Therefore, check several lots and order a supply of the lot that gives satisfactory results. The enzymes store well as powders at –20°C.

5. Transfer chunks to a test tube, and rinse 4× (3 mL each) in L-15 and once in medium. Because no specific inhibitors of collagenase and pronase are available as for trypsin, careful rinsing is particularly important.

6. Trituration is carried out as for embryonic neurons. A two- or even three-step procedure, with subsequent combining of supernatants, is particularly important in avoiding unnecessary trauma to the neurons released during the initial one or two triturations. Usually, very few tissue fragments remain, but they can be allowed to settle, and the supernatant is removed. Filtration through Nitex is omitted.
7. Plating is done as described above for embryonic neurons. A thicker collagen substratum (i.e., 3–4 drops for the air-dried layer vs 1–2 drops on 2.5-cm dishes) promotes initial attachment of the neurons, and avoids breakdown of the substrate after a few days of culture, secondary to residual collagenase.

5. APPENDIX

5.1. Dishes

The Maximow double-coverslip assembly had been successfully used for a number of years, but was cumbersome in its maintenance, therefore limiting the number of cultures available for experimentation. A major need was flexibility; therefore, in 1970, a small plastic "minidish," with raised edges to contain the culture medium, was developed (Bunge and Wood, 1973) for the following reasons:

- Cultures were sometimes maintained for extended periods. Ease of handling, for refeeding and any treatments or manipulations, was important.
- Cultured nervous tissue may benefit from a degree of self-conditioning of its medium.
- Some cultures appear to develop more satisfactorily with a shallow overlay of medium, presumably because of better oxygen exchange with the atmosphere.
- Media components, expensive drugs, or chemicals could be applied in a small volume.
- Ease of handling allowed more variables per experiment and more cultures per variable.
- Several types of tissue could be combined in the same dish.
- Observation at high magnifications with the compound microscope was still possible.

1. Aclar dishes:
   Aclar dishes are molded from inert plastic with a properly machined aluminum punch and die (Honeywell, Pottsville, PA; cat. no. Aclar 22C). The punch is heated on a hot plate and the plastic (roughly cut to size) is molded by applying pressure with the cold die and the edges of the small Aclar dishes are trimmed. The more common size used is a "bottle cap" (2.5-cm); smaller (~10-mm diameter) dishes have also been used, and reduce the needed volume of media even more. The dishes are cleaned in 70% nitric acid, rinsed first in running tap water, then in distilled water, and finally soaked overnight in tissue culture grade water. They are then sterilized in 70% ethanol, dried upside down, and coated with the desired substrate.
   The Aclar dishes are often housed in groups of six in 100 ¥ 10-mm glass or plastic tissue culture dishes, and are monitored with an inverted compound microscope. The Aclar dish provides the advantages listed above, and has, in addition, the feature that its bottom can be cut out after fixation and used as a coverslip to carry the cultures through various staining procedures, with subsequent mounting, tissue side down, onto a standard microscope slide. The Aclar dishes also allow fixation and embedding for electron microscopy directly in the dish. The inert plastic can then be peeled off the polymerized embedding resin. The flat (2–3 mm thick) disk of polymerized resin allows the review of the tissue and marking of specific regions of the culture (even specific myelin segments or single neurons) for electron microscopic analysis.

2. Tissue culture plastic:
   Depending on the specific needs of an experiment, standard plastic tissue culture dishes (35- or 60-mm) or multiwell plates can also be used usually after collagen, laminin (LAM), or polylysine coating (*see* Appendix 3).
   Tissue culture plastic has been treated to facilitate cell attachment and growth, and is usually radiation sterilized. Sources include Falcon, Nunc, Costar, Greiner, Corning, and so on. Ordinary plastic may release fumes that are toxic to the cells. This can be a particular problem if the dishes are incubated in the closed system with desiccators (Mithen et al., 1982), in which the volatile toxic agents accumulate.
3. Glass coverslips:
   Glass coverslips in a variety of sizes, with a variety of substrates, can be housed inside plastic tissue culture dishes or multiwell plates.
   The only suitable glass coverslips for the dissociated SCG neurons system are the German-made Assistant Brand borosilicate glass available from Carolina Biological Supply (12-mm round; Burlington, NC; cat. no. AA-63-3009). Other suppliers may treat or coat the coverglasses so that dissociated neurons will not adhere. The coating is not removed by ordinary washing procedures.
   a. Preparation for washing: Before use, the coverslips are washed. The coverslips are singularly oriented in porcelain staining racks (Thomas Scientific, Swedesboro, NJ, cat. no. 8542-E40) to allow for free flow of the washing solutions.
   b. Procedure.
      i. 1 h in 0.1 *M* HCl.
      ii. Rinse 3× with distilled water.
      iii. Soak 1 h in each of three changes of ethanol.
      iv. Rinse 3× with distilled water.
      v. Cover with tissue culture-grade water, and autoclave 20 min.
      vi. Immediately after removing from autoclave, pour off hot water and rinse with tissue culture-grade water. Pour off rinse. The coverslips are now ready to sterilize and use.

5.2. Incubation Conditions

CO_2 incubators can be used for the cultures; for cultures using defined media, however, a higher concentration of CO_2 is needed (8%), and may be more susceptible to evaporative losses. We have therefore employed a system of segregating groups of cultures in sealed desiccators which may also help prevent spread of mold and fungus infections. Of note is the fact that no antibiotics are used in the feeds, except in some cases (adult or postnatal rat) during the dissection and for the first days of culture. The desiccators (Corning Glass Works, Corning, NY, 160-mm Pyrex, cat. no. 3118) are thoroughly cleaned with alcohol, and autoclaved. Sterile water (300 mL) is added to the bottom reservoir to provide a humidified atmosphere. Dow Corning high-vacuum grease (Fisher Scientific, cat. no. 14-635-5D) is used to seal ground glass surfaces (sparingly, to prevent contamination of culture glassware). Depending on the type of medium in use (*see* below), CO_2 is added to maintain pH of approx 7.3–7.4. This amount is approx 100 mL for the serum-containing medium and approx 160 mL for the defined medium. The desiccator jars are then placed in an incubator at 37°C.

5.3. Sylgard Dish for Dissection

For dissections, Sylgard has proven easier to use and to clean than wax-filled Petri dishes. (Dow Corning 184 silicone elastomer kit, cat. no. SYLG184, available from World Precision Instruments, Sarasota, FL 34240). Store kit in refrigerator.

1. By weight, combine 1 part curing agent and 10 parts silicone elastomer.
2. Procedure:
   a. Cover balance with Parafilm or other plastic, to protect from drips.
   b. Tare glass Petri dish.
   c. Pour silicone elastomer into 100-mm glass Petri dish until within 1/4 in. from top (or a little more, to allow room for curing agent). For 150-mm dishes, 1/4 in. from top.
   d. With Pasteur pipet, add curing agent (e.g., for 10 g elastomer, add 1.0 g curing agent).
   e. Mix well with pipet or other disposable item.
   f. Let sit on FLAT surface overnight, to get rid of bubbles.
   g. Place in drying oven at 65°C for 1–2 d to cure.
   h. To sterilize for dissection: 70% ethanol for 20 min, pour off ethanol, and dry in hood.

5.4. Media

A variety of media have been developed over the years for a variety of tissue types and questions to be asked. The development of defined media, and modification from that originally used for neuroblastoma cells (Bottenstein and Sato, 1979), resulted in culture systems that provided several advantages, particularly for studies on myelination and the development of neuronal morphology. Cultures of dorsal root ganglia co-cultured with Schwann cells could be "poised," allowing proliferation of the Schwann cells before being transferred to media that induced myelination. SCG neurons, dissociated and maintained without glia in defined media, were found to develop only an axon, thus setting up a system to allow the methodical study of factors involved in dendritic development (Bruckenstein and Higgins, 1988a,b).

In the following sections detailed components of basal media, then formulations of various media used for specific culture types, are given.

1. Basal Media
   a. L-15 medium (Leibovitz, 1963)(Gibco-BRL, powder, cat. no. 41300; liquid, cat. no. 11415):

Note: The powder form of basal media may generally be cheaper, but needs to be filtered. Because filters may be expensive, the liquid form may be advantageous, depending on the amount of media used.

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	140.00
KCl	400.00
KH_2PO_4	60.00
$MgCl_2$ (anhydrous)	94.00
$MgSO_4$ (anhydrous)	98.00
NaCl	8000.00
Na_2HPO_4 (anhydrous)	190.00
Other components:	
D(+)-galactose	900.00
Phenol red	10.00
Sodium pyruvate	550.00
Amino acids:	
L-Alanine	225.00
L-Arginine (free-base)	500.00
L-Asparagine	250.00

L-Cysteine (free-base)	120.00
L-Glutamine	300.00
Glycine	200.00
L-Histidine (free-base)	250.00
L-Isoleucine	250.00
L-Leucine	125.00
L-Lysine (free-base)	75.00
L-Methionine	75.00
L-Phenylalanine	125.00
L-Serine	200.00
L-Threonine	300.00
L-Tryptophan	20.00
L-Tyrosine	300.00
L-Valine	100.00
Vitamins:	
D-Calcium pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-Inositol	2.00
Niacinamide	1.00
Pyridoxine HCl	1.00
Riboflavin-5'-phosphate, Na	0.10
Thiamine monophosphate	1.00

b. Minimum essential medium with Earle's salts (Eagle's; EMEM) (Eagle, 1959) (Gibco-BRL, liquid, cat. no. 11095):

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	200.00
KCl	400.00
$MgSO_4 \cdot 7H_2O$	98.00
NaCl	6800.00
$NaHCO_3$	2200.00
$NaH_2PO_4 \cdot H_2O$	140.00
Other components:	
D-Glucose	1000.00
Phenol red	10.00
Amino acids:	
L-Arginine HCl	126.00
L-Cystine 2HCl	31.00
L-Glutamine	292.00
L-Histidine HCl $\cdot H_2O$	42.00
L-Isoleucine	52.00
L-Leucine	52.00
L-Lysine HCl	73.00
L-Methionine	15.00
L-Phenylalanine	32.00
L-Threonine	48.00
L-Tryptophan	10.00

L-Tyrosine (disodium salt)	52.00
L-Valine	46.00
Vitamins:	
D-Calcium pantothenate	1.00
Choline chloride	1.00
Folic acid	1.00
i-Inositol	2.00
Niacinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
Thiamine HCl	1.00

c. Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, liquid) cat. no. 10316):

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	200.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.10
KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6400.00
$NaHCO_3$	3700.00
$NaH_2PO_4 \cdot H_2O$	125.00
Other components:	
D-Glucose	1000.00
Phenol red	15.00
Sodium pyruvate	220.00
Amino acids:	
L-Arginine HCl	84.00
L-Cystine 2HCl	63.00
Glycine	30.00
L-Histidine HCl $\cdot H_2O$	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophan	16.00
L-Tyrosine 2Na $\cdot 2H_2O$	104.00
L-Valine	94.00
Vitamins:	
D-Ca pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i-Inositol	7.20
Niacinamide	4.00
Riboflavin	0.40
Thiamine HCl	4.00
Pyridoxine HCl	4.00

d. Ham's nutrient mixture F-12 (Gibco-BRL, liquid, cat. no. 11765):

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	33.20
$CuSO_4 \cdot 5H_2O$	0.0025
$FeSO_4 \cdot 7H_2O$	0.83
KCl	223.60
$MgCl_2$ (anhydrous)	57.22
NaCl	7599.00
$NaHCO_3$	1176.00
Na_2HPO_4 (anhydrous)	142.00
$ZnSO_4 \cdot 7H_2O$	0.86
Other Components:	
D-Glucose	1802.00
Hypoxanthine Na	4.77
Linoleic acid	0.08
Lipoic acid	0.21
Phenol red	1.20
Putrescine 2HCl	0.161
Sodium pyruvate	110.00
Thymidine	0.70
Amino acids:	
L-Alanine	8.90
L-Arginine HCl	211.00
L-Asparagine H_2O	15.00
L-Aspartic acid	13.00
L-Cysteine HCl $\cdot H_2O$	35.00
L-Glutamic acid	14.70
L-Glutamine	146.00
Glycine	7.50
L-Histidine HCl $\cdot H_2O$	21.00
L-Isoleucine	4.00
L-Leucine	13.00
L-Lysine HCl	36.50
L-Methionine	4.50
L-Phenylalanine	5.00
L-Proline	34.50
L-Serine	10.50
L-Threonine	12.00
L-Tryptophan	2.00
L-Tyrosine 2Na $\cdot 2H_2O$	7.80
L-Valine	11.70
Vitamins:	
Biotin	0.007
D-Calcium pantothenate	0.50
Choline chloride	14.00
Folic acid	1.30
i-Inositol	18.00

Niacinamide	0.04
Pyridoxine HCl	0.06
Riboflavin	0.04
Thiamine HCl	0.30
Vitamin B_{12}	1.40

e. HBSS, calcium-, magnesium-free (Gibco-BRL, liquid, cat. no. 14170):

Components	mg/L
Inorganic salts:	
KCl	400.00
KH_2PO_4	60.00
NaCl	8000.00
$NaHCO_3$	350.00
Na_2HPO_4	48.00
Other components:	
D-Glucose	1000.00
Phenol red	10.00

f. Neurobasal (Gibco-BRL, cat. no. 21103-031):

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	200.0
$Fe(NO_3)_3 \cdot 9H_2O$	0.1
KCl	400.0
$MgCl_2$ (anhydrous)[a]	77.3
NaCl[a]	3000.0
$NaHCO_3$[a]	2200.0
$NaH_2PO_4 \cdot H_2O$	125.0
Other components:	
D-Glucose	4500.0
Phenol red[a]	8.1
HEPES[a]	2600.0
Sodium pyruvate[a]	25.0
Amino acids:	
L-Alanine[b]	2.0
L-Arginine HCl	84.0
L-Asparagine H_2O[a]	0.83
L-Cysteine[a]	1.21
L-Glutamine[a, b]	73.5
Glycine	30.0
L-Histidine HCl $\cdot H_2O$	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine HCl	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Proline[a]	7.76
L-Serine	42.0
L-Threonine	95.0
L-Tryptophan	16.0

L-Tyrosine	72.0
L-Valine	94.0
Vitamins:	
D-Calcium pantothenate	4.0
Choline chloride	4.0
Folic acid	4.0
i-Inositol	7.2
Niacinamide	4.0
Pyridoxal HCl	4.0
Riboflavin	0.4
Thiamine HCl	4.0
Vitamin B_{12}	0.34

[a]Changed from DMEM.
[b]Not supplied in liquid medium; needs to be added.

g. B27 Serum-free supplement (Gibco-BRL, cat. no.17504-010): The B27 supplement contains the following components. The concentrations are not published.
   Biotin
   L-Carnitine
   Corticosterone
   Ethanolamine
   D(+)-Galactose
   Glutathione (reduced)
   Linoleic acid
   Linolenic acid
   Progesterone
   Putrescine
   Retinyl acetate
   Selenium
   T_3 (triodo-L-thyronine)
   DL-α-tocopherol (vitamin E)
   DL-α-tocopherol acetate
   Proteins
   Albumin, bovine
   Catalase
   Insulin
   Superoxide dismutase
   Transferrin

2. Media for Growth and Maintenance
   a. Defined medium: The formula for the defined medium we have used is modified from that designated "N2" by Bottenstein and Sato (1979). Unlike the initial N2, no HEPES, antibiotics, or bicarbonate is added. Added components include 1.4 m*M* of L-glutamine and 100 ng/mL of purified 2.5 S NGF. Finally, the cultures are grown in an 8% CO_2 atmosphere (vs 5% for standard medium), to maintain the pH at approx 7.4. Since the pH of this medium drifts upward rapidly under ambient air, any handling and feeding of the cultures should be done expeditiously. Plastic flasks (Corning, cat. no. 25600) are used to mix the components, to reduce binding, particularly of NGF, which is more problematic with glass. In addition, BSA is added before either NGF or insulin, to further reduce their adsorption to the plastic. Pipets used for NGF, particularly, are also

rinsed with BSA-containing media. Finally, F12 is added last because its high concentration of cysteine, especially in the absence of serum, may reduce the activity of insulin (e.g., disulfide bonds). The media therefore should be immediately aliquoted and frozen at –80°C. Stability is maintained for >1 yr (up to 3–4 yr). Freeze-thaw cycles should be limited to two. Combine the components in the following order

Note: The order of combining the components is crucial.

Components	Volume/200 mL	Final Conc.
i. DMEM (Gibco-BRL, cat. no.10316-024 and glucose [4.5 g/L] without Na pyruvate).	95 mL	
ii. BSA (Calbiochem, cat. no. 126609, Fraction V, fatty-acid poor; nuclease- and protease-free) 20 mg/mL DMEM.	5.0 mL	0.5 μg/mL
iii. Glutamine (Gibco-BRL/Life Technologies, cat. no. 25030-081; 200 m*M*).	3.4 mL	3.9 m*M*
iv. Transferrin* (Chrompure Rat Transferrin, Jackson Immunoresearch Labs, cat. no. 012-000-050) 2 mg/mL sterile tissue culture water; store desiccated 0–5°C.	1.0 mL	10 μg/mL
v. Insulin (bovine, crystalline, Sigma, cat. no. I-5500) 2.5 mg/mL 10 m*M* HCl; store desiccated below –20°C.	0.8 mL	10 μg/mL
vi. Sodium selenite (Sigma, cat. no. S-1382) 6 μ*M* in DMEM).	1.0 mL	30 n*M*
vii. NGF, 2.5S (Harlan Bioproducts, cat. no. 005017) 100 μg/mL 1 mg/mL BSA in 0.9% NaCl.	200 μL	100 ng/mL
viii. F-12 nutrient medium (F-12) (Gibco-BRL, cat. no. 21700, with L-glutamine).	95 mL	50% (v/v)

*The original formula called for human transferrin, but, because of concern about the danger of concentrated human blood products, the author shifted to the use of rat transferrin.

b. Maintenance medium:

Component	mL
EMEM containing 2 m*M* L-glutamine	90.00
Glucose (200 mg/mL solution)	1.15
Crude NGF (50 ng/mL)[a]	0.05
Serum[b]	10.00

Aliquots in tissue culture tubes are stored at –80°C.

[a] Purified NGF (100 μg/mL; *see* Defined Medium above) may also be used at 100 μL/100 mL of feed, to give a final concentration of 100 ng/mL).

[b] Early studies utilized human placental serum; more recently, for a variety of reasons (availability, use of human products, and so on) fetal bovine, horse, and rat serum have been used.

c. Antimitotic medium:
Maintenance medium as above, with the following added:
Fluorodeoxyuridine to 10^{-5} *M*
Uridine to 10^{-5} *M*

d. Myelination media:

Component	mL
EMEM containing 2 m*M* L-glutamine	85.00
Glucose (200 mg/mL solution)	1.15
Crude NGF (50 ng/mL)	0.05
Human placental serum	15.00
L-Ascorbic acid (5 mg/mL)	1.00

Note: L-Ascorbic acid is filter-sterilized immediately prior to use. This ingredient is known to replace embryo extract in meeting the requirements for Schwann cell myelination (Eldridge et al., 1987).

e. Other media for dissection and maintenance of Schwann cell cultures:

i. D-10.

Component	100 mL
DMEM, high-glucose (Gibco-BRL, cat. no. 12800-058), 4°C.	90 mL
Fetal bovine serum (FBS) (–80°C)	10 mL

Note: FBS is stored originally at –20°C in original glass bottles; when aliquoted into tissue culture tubes, it is stored at –80°C.

Penicillin–streptomycin (–80°C)	1 mL

ii. D-15:

DMEM (4°C)	85 mL
FBS (–80°C)	15 mL

iii. D-10-Mitogen (2 μ*M* forskolin/20 μg/mL pituitary extract):

Component	100 mL
D-10	100 mL
Forskolin (2 μ*M*)	13.3 μL
Bovine pituitary extract (Collaborative Biomedical Products, cat. no. 40123) (1 mg/mL, –20°C)	5 mL
Or	
Pituitary extract, 12.5 mg/mL (–20°C)	160 μL

iv. 10% Rat serum:

Component	50 mL
DMEM (4°C)	45 mL
Rat serum (–80°C)	5 mL

v. Trypsin ethylenediamine tetraacetic acid (EDTA) in HBSS:

Component	50 mL
Trypsin/EDTA (–20°C)(10X)	5 mL
1X HBSS (room temperature)	45 mL

vi. 10X Dispase/collagenase (12.5 U/mL dispase/0.5% collagenase):

DMEM (4°C)	5 mL
Dispase (4°C)(12.5 U/mL)	125 mg
Collagenase CLS (–20°C)(0.5%)	25 mg

d. "Old Reliable" medium:

100 mL of the standard medium includes 61 mL EMEM, 25 mL human placental serum, 10 mL 9-d chick embryo extract (50% in balanced salt solution), 3 mL 20% glucose, and

0.7 mL 200 m*M* glutamine and NGF. The usual concentration of NGF is approx 25 biological U/mL medium (s*ee* below for discussion on serum).

e. Comments concerning medium components:
   i. Antimitotic agents: Antimitotic agents are generally regarded as having little effect on the nondividing neuronal population. However, recent studies (unpublished) have shown a decrease in the rate of neurite extension from explants, when fluorodeoxyuridine is continuously present in the medium. This effect is most dramatic when neurons are derived from early embryonic or older postnatal rats.
   ii. Serum: Studies with a variety of media formulations have shown the importance of the concentration of certain components. Thus, the concentration of human placental serum or embryo extract can affect the development of choline acetyltransferase activity. Elevated K^+ levels are known to increase survival for some neuronal types, but, in SCG cultures, they will also affect neurotransmitter synthesis of the neurons (for review, *see* Johnson and Argiro, 1983). Because of this, hemolyzed serum must be used with caution, since the K^+ concentrations may be increased. We have used human placental serum almost exclusively in the past, because it yields consistent results, and was available from an affiliate hospital. Other sera (horse serum, human adult serum, fetal calf serum) may be used, but from our experience, we recommend that the effects of any serum be well characterized before routine use. In particular, commercially available sera may vary considerably from lot to lot.

5.5 Substrata

1. Collagen

We continue to produce rat tail collagen to provide a stable substratum for long-term cultures (myelination studies; axonal growth, dendrite development), which are carried for many weeks. Many substrata (polylysine or laminin) may lose their adhesiveness after several weeks in culture, and the cells are lost before the experiment is finished. Commercial collagen preparations should be tested for stability before use in long-term experiments.

a. Collagen preparation:

Collagen is prepared by a modification of the method of Bornstein (1958). Because standardization of this procedure has proven critical for obtaining consistent culture results, it is given here in detail. Tails from 6-mo to 1-yr old male rats are used, because those from younger males or female rats give unsatisfactory preparations. Each tail is thoroughly scrubbed with antiseptic soap, rinsed once each in 70% alcohol and tap-distilled water, placed in a filter-paper-lined Petri dish, and frozen for 24 h or longer at –20°C. The tail is sterilized in 70% alcohol for 20 min, and dried in a large Petri dish with filter paper. With the tail held at 1–1.5 cm from the small end with two hemostats, the vertebrae of the tail are fractured without cutting the tendons that run from the pelvis muscles all along the tail and attach to each vertebrae. If the vertebra is cracked free and pulled laterally to separate it from the rest of the tail, the attached tendons are pulled out with it, then hang free from the detached vertebral fragment. The tendons are cut free with fine scissors, and placed in a culture dish with sterile tissue-grade water. Moving progressively, 1.5 cm at a time, toward the larger end of the tail, the procedure is repeated until the last of the tail is used. The tendons in the tissue culture-grade water are teased apart with small forceps (no. 5) to loosen up clumps, and at the same time removing blood vessels and any other adherent nontendon connective tissue.

The tendons are extracted using 150 mL sterile 0.1% acetic acid for each gram of tendons for 5 d at 4°C, with daily agitation. The dissolved tendons are centrifuged in

sterile, screw-capped Oak Ridge centrifuge tubes (Sorvall SS-34 Rotor; 12,000*g*; 1 h), and the supernatant harvested. The acid extract is dialyzed (dialysis tubing, Fisher Scientific, cat. no. 8-667D) against 50 vol of sterile tissue culture grade water for 18 h at 4°C. The collagen is removed from the dialysis bags, aliquoted into sterile containers, tested for sterility in soy broth, and stored at 4°C.

b. Application of collagen to dishes:
The detailed procedure for collagen-coating Aclar dishes is given below; appropriate modifications can be made for other culture dishes. Aclar dishes (Bunge and Wood, 1973) are sterilized in 70% alcohol, dried, and transferred to a Petri dish. Two to three drops (on a dish approx 2.5-cm in diameter) of dialyzed collagen are spread evenly over the bottom, using a sterile disposable Pasteur pipet flamed to close the tips and bent to the shape of a hockey stick. Avoiding delay, the freshly spread collagen is exposed to ammonia vapor for 2 min. The dishes are rinsed once or twice with sterile tissue culture grade water, drained, and allowed to dry. Because the collagen may be in fact more adhesive without these rinses, both methods should be tried. When thus dried, the dishes have the advantage of being able to be used to seed cells in a confined region, because the dried collagen is not easily wettable. Thus, a drop of feed will stand up as a bubble confining the neurons to the collagen area beneath. Utilizing this technique, cultures are obtained that are intermediate between disaggregated neuronal culture and explant cultures. Thus, several thousand neurons can be deposited in the disaggregated state, but confined to the center of the dish. The next day, the entire dish surface is made wet and the culture refed. Over the next few days, the neurons partially reaggregate and develop a neuritic outgrowth that eventually fills the remainder of the dish. This preparation thus has both a disaggregated neuronal and a purely neuritic domain in the same culture dish.

If a particularly adhesive collagen surface is desired, then a second layer of collagen is added after drying the first ammoniated layer, as described above. Another 1–2 drops collagen are then added, gently spread, and allowed to dry. Suspensions of disaggregated cells may be added directly to the air-dried layer, without prior wetting (*see* above). For explants, wet the dishes first, using L-15, which is then removed and replaced by 3–4 drops of the desired medium.

The double-layered collagen substratum has been very useful in the culture of either disaggregated or explanted SCG neurons. Disaggregated neurons may not attach or grow well on the ammoniated collagen alone. Although explants will grow on ammoniated collagen, their rate of growth is slower, and, after several weeks, they tend to detach from the substrate. Furthermore, neurite growth and nonneuronal cell migration from explants are not only age-dependent, but also substrate-dependent. Thus, E15 sympathetic ganglia explants on ammoniated collagen have neurites with accompanying nonneuronal cells. On air-dried collagen (double-layered, as described above), the neurites have but a few nonneuronal cells, and grow slower. These results, plus those of others (for review, *see* Roufa et al., 1986), serve to alert an investigator to the possible effects of both substratum and neuronal age on neuronal growth, glial cells, and their differentiation.

2. Laminin
Laminin (Collaborative Research, cat. no. 40232; Fisher, cat. no. CB-40232):

Note: On rare occasions, there is a lot number of laminin that will not allow neurite outgrowth from neurons. The company may be willing to replace those vials.

a. Store 50-µg aliquots frozen (–80°C) on receipt from company. Avoid freeze-thaw cycles. One day before use, thaw in refrigerator. It may gel if thawed too quickly. On day of use, add 50-µg aliquot to 2.5 mL sterile carbonate buffer.

b. Preparation of carbonate buffer.
   i. 0.05 *M* $NaHCO_3$ = 0.42 g/100 mL tissue culture-grade water.
   0.05 *M* Na carbonate = 0.53 g/100 mL tissue culture-grade water.
   ii. Pour up to 10 mL bicarbonate solution into beaker with stirbar. Adjust to pH 9.6 with carbonate solution.
   iii. Sterilize by filtering through 0.2-μm Gelman Acrodisc (VWR, cat. no. 28144-040).
   iv. Measure 2.5 mL into another tube, and add 50 μg laminin.

c. Laminin application:
   i. Sterile 12-mm glass coverslips are placed on sterile "hats" (microcentrifuge caps), with a drop of sterile water in hat to hold coverslip in place. Eight to ten "hats" plus coverslips are housed within a sterile 100-mm Petri dish.
   ii. Add laminin to cover the coverslip.
   iii. Place in desiccator (with water reservoir for humidity) overnight (37°C).
   iv. Drain liquid off coverslip, and place coverslip into multiwell plate with 1.5 mL L-15.
   v. Rinse 5× with L-15.
   vi. Remove L-15, and add (3–4 drops for SCGs, 10 drops for SCG neurons) desired media.

3. Poly-L-lysine (PLL) (Sigma, cat. no. P-2636)
Coating of glass coverslips:

a. Materials: Make stock solution of 1 mg PLL/mL of tissue culture-grade water. Store in aliquots at –80°C. The stock solution is thawed and diluted in 0.1 *M* boric acid/NaOH buffer, pH 8.4 (filter-sterilized), at a final concentration of 100 μg/mL.

b. Methods:
   i. Sterile coverslips are placed on sterile hats (inverted microcentrifuge caps [*see* above]), with a drop of sterile water in the hats to hold the coverslips in place.
   ii. Polylysine is added, dropwise, to cover the coverslips.
   iii. The coverslips are left in a humidified chamber at 4°C overnight.
   iv. On the day of the dissection, the coverslips are placed in a multiwell plate, and are rinsed 5× with sterile water.
   v. Water is removed, and serum-free medium (or other desired media) is added.

Coating of plastic tissue-culture dishes (100-mm).

a. Materials:
   Petri dishes, 100-mm.
   PLL, 200 μg/mL H_2O.

Note: The higher concentration of PLL is used in this protocol, because the dishes are incubated for only 1 h (*see* below).

   Gibco tissue culture water (Gibco-BRL, cat. no. 15230).

b. Methods:
   i. Put 7 mL PLL into the dishes to be coated.
   ii. Allow them to sit at room temperature in a hood for 1 h.
   iii. Remove the PLL, and rinse the dishes 2× with Gibco water.
   iv. The dishes are now ready to use.

5.6 Freezing of Schwann Cells

1. Materials
   D-10 Medium.
   D-15 Medium.

Dimethyl sulfoxide (DMSO).
Tubes.
Pasteur pipets, glass.
Pipets, graduated.
Cyrovials, sterile.
Freezing container.

2. Methods
   a. Rinse the dishes containing the cells 2× with HBSS.
   b. Add 0.5 mL trypsin–EDTA to each dish, and wait for 3–5 min, until the cells start to come off the dish.
   c. Remove the cells from the dish, and put into a tube containing D-10 (1 mL/dish), then rinse the dish with 3 mL D-10, and put this into the tube containing the cells (tube size is determined by the number of dishes being split: 1 dish, 14-mL snap cap; 2–5 dishes, 50-mL conical).
   d. Centrifuge the cells at ~500*g* for 10 min at 4°C.
   e. Remove the supernatant, and resuspend the cells in 2–3 mL D-10 with a small-bore pipet, then add D-10 to a final volume of 5 mL in a small tube and 10 mL in large tube.
   f. Count the cells using a hemacytometer. Count the number of cells in one of the large blocks. Calculation:
      Number of cells × 10^4 = number of cells/mL
      Number of cells/mL × number mL = Total number of cells
      Note: Freeze about 1.5 million cells/mL/cryovial.
   g. Centrifuge the cells at ~500*g* for 5 min at 4°C.
   h. Resuspend the cells in the appropriate amount of D-15 needed, as determined from the counting. When the cells are well suspended, add the DMSO, to get a concentration of 6%.
   i. Quickly aliquot the suspension into the cryovials, and put in the Nalgene Cryo 1°C (cat. no. 5100-0001) freezing container at –80°C. The DMSO can kill the cells, and should not be allowed to be in contact with the cells, without freezing for any length of time.
   j. The next day, the frozen cells should be put in the liquid nitrogen container.

5.7 Animal Care and Handling

The specifics of animal anesthesia or euthanasia and preparation are given in appropriate sections in this and the following chapter. All animal protocols are detailed and approved by the institutional animal care committee. When possible, animals, specifically embryonic and perinatal ages, are used for more than one project, e.g., PN1 rat pups for both SCG cultures and sciatic nerves for Schwann cell cultures.

Over the years, a variety of methods for anesthesia or, more often, euthanasia have been used. Investigators should be aware that the use of certain anesthetic agents or procedures could possibly affect results. The CO_2 chamber has the advantage of not using any volatile organic agent (e.g., ether), and, to the author, by direct observation of the animals, has been the least irritating and most humane. This, followed by exsanguination (cutting through the thorax, heart, great vessels), assures quick euthanasia, and is used for most experiments.

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Chapter Eight

Primary Cell Cultures for the Study of Myelination

Mary I. Johnson, Richard P. Bunge, and Patrick M. Wood

DEDICATION

This chapter is dedicated to Richard P. Bunge (1932–1997), whose contributions were innumerable to the study of the cell of Schwann and both peripheral and central myelination.

1. INTRODUCTION

Tissue culture allows the separate establishment of neurons and glia under a variety of conditions of substrate and media. By subsequent recombination of relatively pure cell populations, a number of questions of neuronal development and neuronal–glia interactions, specifically myelination, may be studied. In vivo, axons of dorsal root ganglion (DRG) neurons course through both the central nervous system (CNS) and peripheral nerve trunks, and induce myelination by both central (oligodendrocytes) and peripheral (Schwann cells) neuroglia. For this reason, and because dissociated DRG neurons are long-lived in vitro when nerve growth factor (NGF) is included in the medium, co-cultures of purified DRG neurons and purified myelinating cells (i.e., Schwann cells or oligodendrocytes) are ideal for the study of myelination. In culture, either peripheral nervous system (PNS) or CNS glial cells are added to the networks of nonneuronal cell-free disaggregated DRG neurons. When these co-cultures are provided with suitable medium, the glia expand in number; given another media, myelination occurs in several weeks.

The following sections detail methods for preparing cultures for in vitro study of myelination. General methods, such as tissue culture dish preparation, incubation conditions, and media formulation, are detailed in Chapter 7, Section 5.

2. REMOVAL OF RAT EMBRYOS

2.1. Materials

Pregnant rat, timed pregnancy of desired embryonic age.

Note: The day after insemination is considered d 0; DRG for neuronal cultures are best dissected from embryos at 15 d of gestation, i.e., at E15).

Protocols for Neural Cell Culture, 3rd Ed. • Ed.: S. Fedoroff and A. Richardson • Humana Press, Inc., Totowa, NJ

Scissors (3), 6–8 in. (Fine Science Tools, cat. no. 14010–15).
Forceps (3), 6–8 in. (Fine Science Tools, cat. no. 11000–18).
Kelly clamps (2), 6–8 in. (Fire Sciencr Tools, cat. no. 13018–14).
Petri dishes (2), 150-mm, sterile, glass or nontissue culture plastic.
Ethanol (70%).
Antiseptic handwashing liquid, e.g., Septisol.
Gauze squares (4), 4 × 4 in.
Cork board (Styrofoam works well also) with pins or no. 20-gage needles.
Shaver.

2.2. Sterilization of Instruments

Sterilize forceps and scissors by immersing them in 70% alcohol (made from 100% absolute alcohol and tissue culture-grade water) for a minimum of 20 min, and drying them inside the laminar flow hood. When dry, put the instruments into a sterile 150-mm, plastic or glass Petri dish, and keep the ends covered with the lid.

2.3. Removal of Uterine Horns

1. The pregnant rat is euthanized by exposure to CO_2, before immobilizing, ventral side up, with pins or twine to the four distal extremities on a cork (styrofoam) board.
2. Preparation for embryo removal.
   a. Shave abdomen.
   Note: This step may be omitted for those who may be particularly sensitive to rat fur and dander. If shaving is omitted, thoroughly soak and scrub the abdominal wall and lower chest with antiseptic liquid soap solution, using gauze pads, followed by several rinses of 70% ethanol.
   b. Squeeze Septisol solution onto the shaved abdomen of the rat, and scrub with gauze. Repeat once.
   c. Wash the shaved area with 70% ethanol, cover the abdomen with gauze, and drench gauze with 70% ethanol.
3. Three sets of scissors and forceps are used for removing the uterus. With the first set of sterile scissors and forceps, make a lateral cut across the lower abdomen just anterior to the vaginal orifice. Cut just through the skin of the rat, and not into the underlying muscle. Continue the incision up the left side of the animal and back across the lower sternum.
4. Retract the skin to the right side, and pin it to the board.
5. With the second set of fresh sterile scissors and forceps, cut through the muscle layer (a 3–4 in. incision) in the midline, avoiding lacerating bowel or bladder. A second person can aid by using Kelly clamps to secure and elevate, up and laterally, both the right and left cut edges of the muscle layer.
6. With the third set of sterile scissors and forceps, taking care to avoid contacting the maternal skin or muscle, free each horn of the uterus, and remove it to a sterile 150-mm Petri dish.
7. Exsanguinate the mother rat by opening the thoracic cavity and cutting the aorta.

3. DRG DISSECTION

Note: All subsequent procedures are performed in the laminar flow hood.

3.1. Materials

E15 rat embryos within uterine horns in 150-mm Petri dish (*see* Section 2.3.).
Forceps (1), 4 in., Biologie (Fine Science Tools, cat. no. 11252–20).
Scissors (1), fine (Fine Science Tools, cat. no. 14091–09).
Spring scissors (1) (Fine Science Tools, cat. no. 15370–26).
Forceps (1), small (Fine Science Tools, cat. no. 11051–10).
Forceps (2), Dumont no. 5 forceps (Fine Science Tools, cat. no. 11251–30).
Leibovitz's L-15 medium, 25 mL (Gibco-BRL, cat. no. 11415).
Petri dishes (2–3), tissue culture, 100-mm (Corning, cat. no. 25020).
Petri dishes (2), tissue culture, 100-mm, each containing a 35-mm tissue culture dish bottom (Corning, cat. no. 25000).
Dissecting microscope.
Ethanol (70%).

3.2. Preparation for Removal of Rat Embryos

1. Sterilization of instruments: Sterilize forceps and scissors by immersing them in 70% alcohol (20 min), and drying them in air inside the laminar flow hood. When sterile, put the instruments into a sterile 100-mm Petri dish, and keep the ends covered with the lid.
2. Preparation:
   a. Pipet 15 mL L-15 medium into a 100-mm plastic tissue culture dish.
   b. Pipet 2–3 mL L-15 medium into a 100-mm plastic tissue culture dish.
   c. Pipet 2 mL L-15 medium into each of two 35-mm plastic tissue culture dish bottoms contained in a 100-mm Petri dish.

3.3. Removal of Rat Embryos from Uterus, and Initial Dissection

1. With sterile fine scissors and forceps, make a cut throughout the length of the uterine horn.
2. Remove each embryo, along with the disk-shaped placenta, and transfer (with or without amniotic membranes) to a 100-mm plastic Petri dish containing 15 mL L-15 medium.
3. Dissection of embryo:
   a. Remove membranes, if they are still intact, and transfer the first embryo to a 100-mm Petri dish with 2–3 mL L-15.
   b. Before proceeding with the dissection, check the age of the embryo (Long and Burlingame, 1938). At E15, the vertex of the head is elevated by the mesencephalon (by d 17, it will be smooth). The digits on forelimbs are partly defined; those of the hindlimbs are paddle-like. The eye is well developed (and open) at this point.

   Note: E15 embryos are at a good age to ensure that DRGs are pulled out with the spinal cord as it is removed from the vertebral column. The vertebral bodies are soft enough to easily cut through. The body of the embryo as a whole is fragile, and should be handled with care. DRGs mature in a rostral to caudal direction; therefore, at E15, the cervical DRGs have longer rootlets, are better separated from the spinal cord, and are easier to pluck. E15 DRGs contain fewer fibroblasts and Schwann cells, making it easier to obtain purified neurons. E16 ganglia are preferable for the preparation of pure Schwann cell cultures; however, fewer DRGs can be recovered from E16 embryos.

   c. Remove the head at the cervical flexure.
   d. Remove viscera and the limbs by cutting with fine sterile spring scissors on each side of the body from tail to head, just anterior to the vertebral column.

e. Lay the remaining tissue on the dorsal surface, so that the ventral surface is uppermost.
f. Move the Petri dish under the dissecting microscope for the following steps.

3.4. Removal of Spinal Cord

Note: Use a fine spring scissors and a no. 5 forceps or two fine no. 5 Biologie forceps.

1. Clean the ventral surface of the embryo and, specifically, the spinal column of any remaining viscera, such as the aorta.
2. Make a ventral cut, in a rostral (from where the head was removed) to caudal direction, through the vertebral bodies, to expose the cord throughout its length. Alternatively, the vertebral pedicles can be cut on each side, and the strip of vertebral bodies removed *en bloc*. Fine spring scissors or forceps pinching the vertebrae, 2–3 at a time, can be used.
3. Using the medulla as a handle, pull straight up (toward the microscope) on the cord, freeing it from the vertebral canal. Try to minimize side-to-side movement of the cord, in order to ensure that DRGs will remain attached to the cord (~50 or so from E15 embryos).
4. Place dissected cord with attached DRGs in one of the two 35-mm Petri dishes filled with L-15 medium.
5. Repeat Section 3.3.3., steps a.–e., for each embryo, and Section 3.4., steps 1– 5 for each spinal cord.

3.5. Removal of Ganglia

1. Using two fine forceps (one # 5 and one # 5 Biologie), the first to stabilize the cord and the latter to grasp the dorsal roots, pinch off ganglia from the spinal cord, and at the same time try to minimize the amount of meninges that may come off with the ganglia. Place ganglia in 35-mm dish containing L-15.

Note: The particular advantage in using the E15 rat embryos is that the ganglia come out of the vertebral column with the cord, and can be easily plucked off the cord. With practice, one can obtain approx 40 or more ganglia/embryo. If the litter has 15 embryos, it is therefore possible to obtain 600 ganglia (in approx 3 h). With a yield of about 5000 neurons/ganglion, this can give $3 \times 10^{\circ}$ neurons.

2. Repeat above steps to remove the DRG from all spinal cords.

Note: If possible (or necessary), discard ganglia that have too much of the meninges attached, to avoid introducing more nonneuronal cells.

4. PREPARATION OF DISSOCIATED DRG CULTURES

4.1. Materials

35-mm tissue culture dish containing DRG ganglia, housed in 100-mm dish (*see* Section 3.5.).
Neurobasal (NB) medium (100 mL) (*see* Appendix 14.1).
L-15 containing 10% heat-inactivated fetal bovine serum (L-15/10% HI-FBS) (10 mL).
Trypsin (Worthington, cat. no. 3707) solution, 0.25% in Hanks' calcium/magnesium-free balanced salt solution (CMF-HBSS) (Gibco-BRL, cat. no. 141700). Filter-sterilized.
1 mL HI-FBS (Hyclone, cat. no. SH30070–0). Heat-inactivated at 56°C for 30 min.
Tube, polypropylene, sterile, 14-mL (Falcon, cat. no. 2006).
Petri dishes (3), tissue culture, plastic, 35-mm, collagen-coated. (*See* Chapter 7, Section 5.5 for collagen-coating procedure). Alternatively, precoated 35-mm dishes are available from Becton-Dickinson (cat. no. 354456).
Pasteur pipet, sterile, with tip fire-polished and constricted to 0.5-mm.

4.2. Trypsin Digestion

1. After all the ganglia are collected (up to a maximum of 150 ganglia/35-mm dish), gather the ganglia into the center of the dish by swirling it gently in a circular motion. Carefully pipet off the L-15 medium.
2. Add 2 mL 0.25% trypsin solution to the 35-mm dish containing the DRGs. Incubate at 37°C for 40 min on a rotary shaker. If a shaker is not available, incubate the DRGs with the trypsin solution for 1 h at 37°C.

Note: Do not use a CO_2 incubator for this step, because the CO_2 will make the HBSS medium very acidic.

3. Remove the dish from the incubator, and add 10 drops of HI-FBS to inactivate the enzyme.

4.3. DRG Neuron Disaggregation and Plating

1. Using a bent tipped, cotton-plugged pipet, transfer the ganglia–trypsin mixture to a sterile 14-mL tube.
2. Centrifuge the ganglia at 500*g* at room temperature for 5 min.
3. Completely remove and discard the supernatant without disturbing the pellet. Add 2 mL L-15/10% HI-FBS. Triturate with a fire-polished, small-bore tipped (0.5-mm), disposable Pasteur pipet, until no cell clumps are visible. Increase volume to 5.0 mL (using L15/10% HI-FBS), and mix.
4. Centrifuge the cell suspensions at 500*g* at room temperature for 5 min. Completely remove and discard the supernatant.
5. Repeat steps 3 and 4 (to give an extra rinse). Resuspend cells in maintenance medium (NB medium) to obtain a concentration of 1–1.5 ganglia per drop of medium.
6. Plate 1 drop ganglia cell suspension into each collagen-coated 35-mm dish.
7. Incubate at 37°C in a 5% CO_2 incubator.

Note: Because the initial drop remains in a confined spot on the collagen, the neurons and other cells attach to the central area of the dish (~5000–7000 neurons/dish).

8. After 24 h, the culture dish is flooded with 1.0 mL NB medium, to wet the entire dish surface (*see* next section), but the cells remain attached in the center.

5. CULTURES OF PURIFIED DRG NEURONS

After the cultures are established as dissociated cells, they contain a mixture of neurons, fibroblasts, Schwann cells, phagocytes, and various other cell types. The cells that are capable of division (essentially all other cell types, except neurons) must be removed. The neurons will survive treatment with antimitotic agents, because almost all of the DRG neuronal population has finished its proliferative phase prior to the time the tissue is taken. The object of the antimitotic medium is to drive proliferation of the dividing cells in the presence of a drug that is lethal for dividing cells. This is accomplished by the following protocol:

Treatments	Day
NB	1
Antimitotic medium (*see* Section 14.3)	2–4
NB	5–8 (two feedings)
Antimitotic medium	9–11
NB	12–15 (two feedings)
Antimitotic medium	16–18
NB	19–26

Note: The final 1 wk in maintenance feed removes any residual fluorodeoxyuridine prior to addition of any glial cells.

During the first 7–10 d, considerable cell death is observed, because the nonneuronal cells die, and cell debris is evident. By the end of the third week, the neurons will have extended their axons from the initial drop area well into the periphery of the dish. Both the outgrowth area and the central area of the drop should be essentially free of nonneuronal cells, and exhibit bare axonal growth.

6. MYELINATION BY OLIGODENDROCYTES

Cultures of dissociated populations of DRG neurons can be used to support the formation of myelin by both Schwann cells and oligodendrocytes. Oligodendrocyte myelination in DRG neuron cultures was first seen when unseparated cell suspensions, derived from dissociated embryonic spinal cord, were added to disaggregated DRG cultures, after treatment of the DRG cultures to eliminate endogenous Schwann cells and fibroblasts (*see* Section 5; Wood and Williams, 1984). In such co-culture preparations, the new myelinating cells are generated from oligodendrocyte precursors, rather than from pre-existing oligodendrocytes. To prepare the mixed cell suspensions containing oligodendrocyte precursors, as described below, in Method 1, cervical spinal cord is dissected from rat embryos on d 14–16 of gestation. Myelination may also be obtained when cells derived from the dissociation of adult spinal cord or purified adult oligodendrocytes are added to the DRG neuron cultures. The preparation of adult oligodendrocytes is described below, in Section 8, Method 2.

7. METHOD 1. EMBRYONIC OLIGODENDROCYTE PRECURSORS

7.1. Materials

DRG neuron cultures (*see* Section 5).
O-medium (*see* Section 14.3)
L-15/10% HI-FBS (10 mL).
HI-FBS (1 mL).
Tubes, polypropylene, sterile, 14-mL (Falcon, cat. no. 2006).
Pasteur pipet, sterile, with tip fire-polished and constricted to 0.5–mm.

7.2. Preparation of Mixed Embryonic Spinal Cord Cell Suspension

1. Remove spinal cords from E14–E18 embryos (*see* Sections 2–3.4. above).
2. Dissect off the meninges and the dorsal half of the cord, and discard. Leave the two ventral halves connected by the ventral commissure.
3. Cut ventral cord halves into approx 1-mm length fragments.
4. Dissociate (without enzyme treatment), by triturating gently 12–24× in maintenance medium (O-medium, *see* Section 14.3).
5. For a moderate glial cell density after plating, disaggregate the tissue fragments from each cervical ventral cord in 3–4 drops of O-medium.
6. Filter through a nylon filter with 20-μm pores (Small Parts, Miami Lakes, FL; cat. no. A-CMN-20).
7. Add one drop of this suspension, containing $3–10 \times 10^4$ cells (counted on hemocytometer), to the center of each culture of dissociated DRG neurons (*see* Section 5 above).
8. Refeed the cultures with O-medium on alternate days for 3–4 wk. Myelin sheaths begin to appear during the fourth week after the addition of glial cells.

8. METHOD 2. ADULT SPINAL CORD OLIGODENDROCYTES

8.1. Materials

Rats, 2–3 mo old, female Sprague-Dawley.
Note: Male rats can be used, but present more technical problems secondary to size and bone thickness, making the dissection more difficult. Three-month-old female or male mice can also be used.
Scissors (2), large, 6-in. (*see* Section 2.1).
Scissors (1), large, pointed (Fine Science Tools, cat. no. 14002–14).
Spring scissors (1), small (Fine Science Tools, cat. no. 15006–09).
Forceps (2), fine, no. 5 - standard (Dumont: *see* Section 3.1.).
Forceps (2), large (Fine Science Tools, cat. no.11000–18).
Forceps (1), large, with teeth (Fine Science Tools, cat. no. 11021–15).
Scalpel handles (2), no. 3; scalpel blades (2), no. 11 (Sterisharp, cat. no.77–2110).
L-15 medium.
O-medium (*see* Section 14.3.).
Heat-inactivated fetal bovine serum.
Trypsin (Worthington, cat. no. TRL3, LS003707).
Deoxyribonuclease (DNase) I, Type II (Sigma, cat. no. D-4527).
Petri dishes, 100-mm tissue culture, with 20 mL L-15 medium.
Petri dish, 100-mm tissue culture, containing a 60-mm tissue culture dish (Corning, cat. no. 25010) with 5.0 mL L-15.
90% Percoll (Sigma, cat. no. P-1644) in HBSS.
Nitex, filter (2), 20-μm pore size (Small Parts, cat. no. A-CMN-20).
Tubes, Oak Ridge centrifuge, with caps, 10-mL, polycarbonate (VWR, cat. no. 21009–310).
70% Alcohol.

8.2. Oligodendrocyte Preparation: Adult Rat or Mouse

Note: Most preparations include one or two rat cords. One adult rat cord is the equivalent in cell number of four adult mouse cords; the total cell yield from one rat cord is 3–5 million cells.

1. Euthanize animal in a CO_2 chamber. Shave back from top of skull to proximal tail and a limited area of the left chest. Wash chest with 70% alcohol. Using large scissors, open left thoracic cavity and exsanguinate the animal by cutting the aorta. This exsanguination procedure ensures euthanasia, and prevents complications resulting from bleeding, later, during the cord dissection. Shave all hair from the back of the animal. Place animal prone, dorsal side up, and secure all four extremities. Clean back with antiseptic scrubbing soap and 70% alcohol.
2. Dissection of whole spinal cord: Reflect to one side a flap of skin stretching from the back of the head to the caudal end of the spine. With pointed scissors, cut through the vertebral column, including through the spinal cord and brainstem junction between the skull and the first vertebra. Beginning at this site, cut with the same pointed scissors along the vertebral column on both sides just dorsal to the vertebral bodies. As these cuts are made, the dorsal portion of the column is lifted away from the vertebral bodies using the forceps with teeth to hold the dorsal portion; the spinal cord is at the same time lifted out in association with this bony strip. After reaching the sacral region of the spinal column, a transverse cut is made

with the scissors, to sever any remaining roots. The entire spinal cord is then easily removed intact, by pulling with a blunt forceps grasping the meninges at the cervical end of the cord. Place the cord in a 100-mm tissue culture dish containing cold L-15 medium; store in the refrigerator until all cords are removed.

3. Remove the meninges: To do this, cut through the meninges with a small spring scissors, along the ventral midline from the rostral to caudal end of the cord. Using finer forceps, gently free the meninges from underlying tracts at the rostral end of the cord. Grasp the cord and, with a second pair of forceps, peel back the meninges along the entire length of the cord. This operation should remove the meninges in one piece, along with associated roots and blood vessels. The cord surface should appear smooth, white, and shiny.
4. Transfer each cord to a 60-mm dish containing 4.0 mL 0.22% trypsin plus 50 μg/mL DNase I in Earle's BSS. The cord is separated into two lateral hemisections. Slice or mince the cord into 0.5 mm (or smaller) pieces. DNase is required to prevent the slices from sticking together. Incubate in a 5% CO_2 atmosphere at 35–37°C for 1 h on a rotating shaker or shake by hand every 10 min, if a shaker is not available.
5. Stop the trypsin by the addition of 1.0 mL HI-FBS. Transfer tissue and medium to a tube, and centrifuge at 400–500*g* for 5 min at 4°C. Discard the supernatant.
6. Place the tube in ice, and add 2.5 mL L-15 medium containing 10% HI-FBS and 50 μg/mL DNase. Triturate the cord fragments by aspirating in and out of a reduced-tip pipet, until a single-cell suspension is obtained; this should require no more than 30 cycles of trituration. Increase the volume by adding 2.5 mL more L-15 medium (plus 10% HI-FBS and 50 μg/mL DNase). Transfer the suspension to an Oak Ridge tube (10–mL size) containing 3.0 mL percoll suspension (i.e., 90% Percoll in HBSS, prepared by mixing 9 parts Percoll and 1 part 10X HBSS). Mix the cell–Percoll mixture thoroughly. Centrifuge at 30,000*g* for 30 min at 4°C.
7. Oligodendrocytes will be found in the cloudy zone floating just above the red blood cells at the bottom of the gradient. Discard the myelin band and the clear band below the myelin layer of the gradient. Transfer the cell fraction (containing oligodendroglia, microglia, and other cells) into a second polypropylene tube containing 8.5 mL L-15 plus 2% HI-FBS. Mix well, and centrifuge at 500*g* for 10 min at 4°C. Discard the supernatant.
8. The pellet should contain about 3 million oligodendrocytes at 50% purity (rat) or 70% purity (mouse). This yield is obtained with one rat cord or four mouse cords. Resuspend this pellet in O-medium, for plating onto neuronal cultures. Before use in experiments, the cell suspension should be filtered through a 20-μm pore size filter (nylon). For myelination experiments, it is recommended that the oligodendrocytes from adult rat or mouse be further purified by negative immunopanning or fluorescence-activated cell sorting (*see* Sections 9.1. and 9.2.).

9. METHODS TO PURIFY OLIGODENDROCYTES

9.1. Materials

Goat antimouse immunoglobulin G (IgG), -A, -M, affinity-purified, Cappel (ICN, cat. no. 55486).

Monoclonal antibody(mAb) OX42 (Sera-Lab; Harlan Bioproducts for Science, cat. no. MAS370cf).

mAb A2B5 (hybridoma-conditioned medium) (American Type Culture Collection; hybridoma, cat. no. CRL-1520).

mAb Thy 1.1 (hybridoma-conditioned medium) (American Type Culture Collection, hybridoma, cat. no. TIB103).
mAb 01 (hybridoma-conditioned medium).

Note: Hybridoma is not commercially available, obtain from Dr. Melitta Schachner, University of Hamburg, Hamburg, Germany.

Fluoroscein-conjugated goat antimouse IgM, affinity-purified, Cappel (ICN, cat. no. 55519).
L-15 (2–3 L), sheath fluid for cell sorter.
HI-FBS.
Nitex filter, 20-μm pore size (Small Parts, cat. no. A-CMN-20).
Petri dishes (2), nontissue culture Petri dishes, 100–mm (Falcon, cat. no. 351029).

9.2. Purification by Negative Immunopanning

1. 1–2 d before preparing oligodendrocytes, prepare two 100-mm sterile nontissue culture dishes to be used for panning.
   a. Treat each dish with 10 mL filter-sterilized 0.05 *M* Tris, pH 9.5, containing 10 μg/mL affinity-purified goat antimouse IgG, IgA, IgM. Allow dishes to sit overnight in the refrigerator.
   b. Rinse both dishes 3× with L-15 medium or phosphate-buffered saline (PBS).
   c. OX-42 Pan: Treat one of the dishes with OX-42 mAb (Sera-Lab) diluted 1:50 in L-15 or PBS containing 0.05% bovine serum albumin (BSA). Use 5.0 mL per dish. Leave in fridge 2–24 h.
   d. A2B5–Thy-1.1 Pan: Treat the other dish with a mixture of A2B5 and Thy-1.1 mAbs. Hybridomas (from ATCC) are maintained in the laboratory and are used to generate conditioned media, which are used at a 1:4 dilution in L-15 with 2.5% HI-FBS or PBS with 0.1% BSA. Use 8 mL total volume per dish. Leave in refrigerator 2–24 h.
2. Harvest crude preparation (pellet) of spinal cord cells from the Percoll gradient, as described in the previous section (*see* Sections 8.2., steps 7 and 8), and rinse.
3. Resuspend pellet in 10 mL O-medium (*see* Section 14.3.). This suspension contains 40–50% oligodendrocytes.
4. Plate the cell suspension onto the OX-42 pan. Incubate for 1 h at 37°C in a CO_2 incubator. The cells stuck on the OX-42 pan are mostly microglia.
5. Shake dish gently to dislodge loosely adhering cells, and replate the suspension on the A2B5–Thy-1.1 pan. Incubate for 1 h in the CO_2 incubator at 37°C.
6. Shake dish gently to dislodge loosely adhering cells consisting mostly of oligodendrocytes. Recover oligodendrocytes from the supernatant by centrifugation. Resuspend cells in medium, as required for experiment. The A2B5 pan will retain O-2A progenitors and other cells.
7. This procedure should provide 90% oligodendrocytes, at a yield of about 500,000 per cord (or 500,000 per 0.5 g white matter tissue).

9.3. Purification by Cell Sorting

1. Harvest crude cell pellet from the Percoll gradient (*see* Section 8.2., steps 7 and 8).
2. Resuspend in O1 antibody (e.g., 0.5 mL O1 hybridoma culture supernatant diluted 1:5 with L-15/2% HI-FBS).
3. Incubate for 30 min at 4°C (on ice). Mix every 10 min.
4. Add 20 λ sterile, affinity-purified, fluorescein-conjugated goat antimouse IgM (Cappel, 1 mg/mL) to the cell suspension. Mix well.

5. Incubate an additional 30 min on ice. Mix every 10 min.
6. Dilute cells to 2.0×10^6 cells/mL.
7. Filter suspension through a Nitex filter with 20 μm pore size. This step is critical, to avoid frequent clogging of the flow cytometer.
8. Sort cells on a flow cytometer. The fluorescein-positive cells are the oligodendrocytes. Purities in excess of 99% oligodendrocytes can be obtained by cell sorting. The yield at this purity is about 250,000 oligodendrocytes per spinal cord.

9.4. Myelination in Oligodendrocyte/Neuron Co-Cultures

Oligodendrocytes (or fetal oligodendrocyte precursors), prepared by the methods described in Sections 7–9, can be added to cultures of purified DRG neurons to generate myelinating cultures. This is accomplished simply by replacing the medium from the DRG neuron cultures with homogeneous suspensions of oligodendroglia in a volume large enough to ensure an even distribution of the added cells over the culture. For DRG neuron cultures in 35-mm dishes, a volume of 2.5 mL is recommended for each culture. If the volume is too low, most of the added cells will attach in the periphery of the culture, where there are few, if any, axons with which they can interact. An alternative is to add a single drop of more concentrated cell suspension into the center of DRG neuron cultures already containing about 2.5 mL medium. Using this alternative approach, more of the added cells attach on the axons, but with an uneven distribution; myelination is most reliably obtained if at least 20,000 oligodendrocytes are added to each culture. After addition of the oligodendrocytes, the co-cultures are maintained in O-medium throughout the remainder of the experiment. Myelination is preceded by a period during which the added cells proliferate. The addition of basic fibroblast growth factor (10 ng/mL, R&D Systems, cat. no. 233–FB-025), during the first week following oligodendrocyte addition, enhances the proliferation of adult oligodendrocytes, but will prevent myelination if kept in the medium throughout the experiment. Many adult-derived oligodendrocytes undergo dedifferentiation, as well as proliferation, prior to redifferentiation into myelinating cells. In general, myelination begins by the third week after adding the cells, and progresses for 3–5 wk. Despite the fact that the cultures are initially completely dissociated and the density of added oligodendrocytes is low, most myelin is formed in regions of the culture where the cell density is high.

10. MYELINATION BY SCHWANN CELLS

Several protocols can be used to generate pure populations of Schwann cells. Either rat fetal DRG (*see* Sections 3.5 and 4) or rat neonatal sciatic nerve can be used as the source of the Schwann cells. Last and more recently, Schwann cells have been obtained from adult rat (Morrissey, Kleitman, and Bunge, 1991) and human peripheral nerves (Levi, et al., 1995). Only the adult rat preparation will be described here, because only minimal myelination by adult human Schwann cells has yet been achieved in co-cultures with DRG neurons (Morrissey, Kleitman, and Bunge, 1995).

10.1. The Wood Method

The Wood (1976) approach is to establish fetal DRG cultures as explants, and to utilize an intermediate level of treatment with antimitotic agents (compared to that used for generating isolated neurons). The explant can then be cut out of the culture, leaving the desired pure Schwann cell population, following degeneration of the axons they had ensheathed. Success here depends on the fibroblasts being more susceptible to antimitotic treatment than are the Schwann cells. Although the treatment does kill many Schwann cells, those that survive subsequently proliferate substantially, to repopulate neuritic outgrowth, and are fully functional in that they are capable of myelinating

axons. The transplantation procedure expedites the repopulation of the outgrowth, because the remaining Schwann cells proliferate vigorously in response to a new neuritic outgrowth. An advantage of this approach is that Schwann cell expansion is driven by their natural mitogenic stimulus. The disadvantage of this procedure is that it is somewhat laborious.

1. Preparation of Schwann cell progenitor cultures:
   a. Materials (*see* Section 4.1. above): In addition, for this procedure, different maintenance (CH medium) and antimitotic (CHF medium) media are required (*see* Section 14.2.).
   b. A pregnant rat at gestational day E16 is euthanized, and the uterine horns removed (*see* Sections 2.1.–2.3.).
   c. Remove embryos from the uterine horns, as previously described in dissociated neuron preparation (*see* Sections 3.1.–3.3.).
   d. Remove spinal cord and associated DRG by the same method as described for the E15 disaggregated neuron preparation (*see* Sections 3.4. and 3.5.). In embryos of this age, however, only cervical and lumbar DRG will remain firmly attached to the cord during its removal.
   e. Once DRGs have been removed from the spinal cord, dissect and remove remaining remnants of the roots and any connective or meningeal tissue, into a dish of L-15 containing 10% FBS.
   f. Transfer clean DRG with a Pasteur pipet, which has been wetted with L-15/10% FBS, to a fresh dish of L-15/10% FBS (serum prevents ganglia from sticking to the pipet surface).
   g. Separate ganglia into groups of 3–4. Transfer each of these groups of ganglia, with a serum-rinsed pipet, to a 35–mm dish that has been coated with dialyzed collagen, dried, and wet again with 20 drops (~1.0 mL) of antimitotic medium (CHF).
   h. With sterile forceps, arrange ganglia so that they are an equal distance from each other, and are centrally located within the dish. Take care not to nick the collagen surface of the dish with forceps, or to poke or squeeze the ganglion in the process of arranging it in the dish.
   i. Once the ganglia are properly arranged in the dish, remove 3–5 drops of the medium from the dish, to prevent ganglia from floating. Do not remove too much of the medium, because the tissue will dry out.
   j. These progenitor cultures receive alternating 2 d pulses of maintenance and antimitotic medium (CH and CHF) for a period of 2 wk, to eliminate fibroblasts, and are then transplanted.
2. Transplantation procedure:
   a. Materials:
      Sterilized blade holders (Fine Scientific Tools, cat. no. 10052–11).
      Razorblade pieces, ~1 mm cutting edge (Fine Scientific Tools, cat. no. 11050–100).
      Forceps, sterilized, Dumont no. 5.
      Forceps, sterilized, no. 5 Biologie.
      N2 medium.
      Petri dishes (2), 35-mm (Corning, cat. no. 25000) filled with L-15/10% FBS.
      Petri dishes, tissue culture, collagen-coated, 35-mm.
   b. With small pieces of razorblade held in blade holders, cut around the central cluster of neurons of the original ganglia.
   c. With forceps, lift the explant of neurons by the edges (without squeezing the explant), and transfer to a dish of L-15/10% FBS.

d. When all of the explanted neurons have been removed in this manner, carefully strip off the collagen surface from the bottom of the explants, taking care not to poke or squeeze the explant with the forceps. The collagen is readily distinguished from the granular neuronal surface, under transillumination, in the scope by its shiny appearance.
e. Once all of the collagen has been removed from the explants, separate into small groupings of explants in the number desired for each culture to be established. Transfer the explants to the collagen-coated (*see* Chapter 7, Section 5.5) 35-mm dishes already filled with 1.0 mL N2 medium.
f. Transplanted cultures are fed 1.0 mL N2 medium per 35–mm dish 3× per week for a period of 2–3 wk, until the axons growing from the explants are fully repopulated with Schwann cells. Repopulated cultures may then provide Schwann cells for disaggregated neuron cultures.

Note: A problem, sometimes experienced using this method, is that the regrowing axons are not populated with Schwann cells. If this occurs, fewer pulses of antimitotic medium should be used. Growth in N2 media will curtail the proliferation of any contaminating fibroblasts.

10.2. THE BROCKES METHOD

This procedure has been described in detail by Brockes et al. (1979), and modified (Porter, et al., 1986) to obtain the considerable number of Schwann cells present in the neonatal rat sciatic nerve trunk, at a time in development when the nerve contains relatively few fibroblasts. One or two pulses of antimitotic treatment with cytosine arabinoside are utilized to reduce fibroblast number, and, subsequently, as the cells are passaged, they are treated while in suspension with an antibody to the Thy-1 antigen, which is expressed on fibroblasts, but not on Schwann cells, and with rabbit complement. The Schwann cell populations obtained can be expanded and subsequently used for transfer onto DRG neurons, where full functional expression is observed (Porter et al., 1986).

1. Materials:
   Rat pups, 1 d-old rat pups, anesthetized with CO_2, decapitated, cleaned with 70% alcohol, and placed prone, with hind limbs pinned out.
   Forceps, no. 5 fine.
   Scissors, fine.
   D-10 Medium: Dulbecco's modified Eagles' medium (DMEM) + 10% HI-FBS.
   D-10 Mitogen medium: D-10 medium plus bovine pituitary extract (20 μg/mL, Biomedical Technologies, cat. no. BT 215) and 2 μ*M* forskolin.
2. Removal of sciatic nerves:
   a. Incise and retract skin over upper hind limbs.
   b. Dissect and remove ~1 cm length of sciatic nerve as it runs posterior and parallel to femur.
3. Dissociation:
   a. Collect nerves in L-15 within a 60-mm tissue culture dish.
   b. Remove L-15, and replace with 0.1% collagenase (Worthington cat. no. CLSS-3 LS004206) in L-15. Incubate 30 min at 37°C.
   c. Remove collagenase medium, and replace with L-15 containing both 0.25% trypsin (Worthington, cat. no. TRL3, LS003707) and 0.1% collagenase. Incubate 30 min at 37°C.
   d. Transfer nerves to tube (14-mL); after nerves settle, remove medium carefully.
   e. Add L-15/HI-FBS to inactivate trypsin, and mix gently to rinse nerves.
   f. Centrifuge at 500*g* for 5 min at 4°C; remove supernatant completely.

g. Resuspend pellet in 2 mL D-10; triturate 20–40× to generate a single-cell suspension; centrifuge, and remove supernatant.
h. Repeat step 7; resuspend the pellet in 1 mL D-10, and count cell numbers in a hemacytometer.

Note: The yield from 20 sciatic nerves is ~2 million cells.

i. Plate cells onto one uncoated 60-mm Corning tissueculture dish prewetted with 2 mL D-10 to ensure even distribution of cells.
j. Following day: Replace medium with fresh D-10 containing 10^{-5} *M* cytosine arabinoside (Sigma, cat. no. C-1768). After 3 d, remove and replace with fresh cytosine arabinside-containing media.
k. After 2 d, remaining fibroblasts are eliminated, using Thy-1.1 Ab/complement mediated lysis.
l. Rinse the 60-mm dish twice with CMF HBSS; remove cells from culture dish with 0.05% trypsin and 0.02% ethylenediamine tetraacetric acid (EDTA) (trypsin/EDTA, Gibco-BRL, cat. no. 15400–054) in CMF HBSS. Incubate 5 min at room temperature.
m. Add HI-FBS to stop trypsin action.
n. Transfer cells and medium to tube (Falcon, cat. no. 2006); rinse dish, and add the rinse to the tube; centrifuge at 500*g* for 5 min.
o. Resuspend cells in 2 mL L-15–HI-FBS; centrifuge again for 5 min; resuspend pellet in 1 mL solution of anti-Thy-1.1 (hybridoma supernatant; American Type Culture Collection, cat. no. TIB-103); triturate gently; leaving the cap of the tube loose, incubate in 6% CO_2 incubator for 30 min at 37°C; close tube, centrifuge, remove supernatant.
p. Resuspend in 1 mL rabbit complement (ICN, cat. no. 642822); incubate in 6% CO2 incubation 30 min at 37°C; centrifuge to collect cells.
q. Dilute in 1 mL D-10 mitogen medium.
r. Plate cells onto poly-L-lysine (PLL)-coated Petri dish in D10 mitogen medium (20 μg/mL bovine pituitary extract). For polylysine coating, use polylysine of mol wt 30,000–70,000 at 0.5 mg/mL in distilled water for 30 min (Sigma, cat. no. P-2636).

11. ADULT RAT (OR HUMAN) SCHWANN CELLS

The method was modified by Morrissey et al. (1991), from a method originally developed by Scarpini et al. (1988), and yields large numbers of highly purified Schwann cells that can either be used in myelination studies or for transplantation into experimental paradigms in vivo. It involves dissection of intraneural fascicles from within the epineurial sheaths, and initial culture (4 wk), during which the nerve fragments are moved through a series of new uncoated culture dishes. By serial replanting of the explants, the fibroblasts, which readily attach to the uncoated plastic, migrate out of, and are eventually depleted from, the explants. The explants are then dissociated and the Schwann cells are cultured in much the same way as the Brockes cells. Human Schwann cells can be obtained by the same method, but do not proliferate well in culture, unless purified heregulin or glial growth factor is used in the medium. Reliable, extensive myelination has not yet been achieved with adult human Schwann cells in vitro.

11.1. Preparation of Sciatic Nerve Explants

1. Materials.
   Rats, about 2-mo-old.
   Forceps, fine, sterile.
   Scissors (2) (Fine Scientific Tools, cat. no. 14091–09), to cut through skin and muscle.

Forceps (2) (Fine Scientific Tools, cat. no. 11050–10 or 11051–10), to cut through skin and muscle.
Forceps (1), small (Fine Scientific Tools, cat. no. 11051–10), to remove nerves.
Scissors (1), small, to remove nerves.
Spring scissors (2) (Fine Scientific Tools, cat. no. 15370–26).
Forceps (2), no. 5 Biologie (Fine Scientific Tools, cat. no. 11252–20), to pull fascicles.
D-10 medium.
L-15.
Petri dishes, 100-mm, tissue culture.
Petri dishes, 60-mm, tissue culture.
Pipets, glass.
Graduated pipets.
Gauze pads.

2. Prepare the hood for the procedure:
   a. Set up two 100–mm tissue culture dishes, and put one 60-mm tissue culture dish within a 100-mm dish for every two animals.
   b. Put 30 mL L-15 in each of the two 100-mm dishes, and 3 mL L-15 in the 60-mm dishes.
   c. Take out all the instruments needed; remember to take forceps out of alcohol to let them dry.
3. Anesthetize rats in CO_2 chamber. Exsanguinate. Pin out prone, fixing the lower extremities, positioned laterally, with no. 20 gage needles. Shave the hair from thigh area, and clean with antiseptic soap and 70% alcohol, using gauze pads.
4. Cut out the sciatic nerves from all the rats, and put them all into one of the 100-mm dishes containing L-15. Use a different set of forceps and scissor for each layer of tissue that is cut through (skin, muscle, and nerve).
5. Remove any excess tissue from the nerves:
   a. Using fine (Biologie) forceps, remove any muscle, fat, or blood vessels that are attached to the nerves.
   b. Put the cleaned nerves into a new 100-mm dish with L-15.
6. Using one fine (Dumont no. 5) forceps, grasp the epineurial sheath at the end of the nerve most proximal to the spinal cord. With a second pair of Dumont no. 5 or Biologie forceps, grasp the tufted end of the perineurium-enclosed endoneurial fascicles, and pull gently until the fascicle is removed from within the epineurium. The clean nerve fascicle will appear smooth and white, and exhibit a discernible banding pattern. Place the fascicle into a 60-mm culture dish.
7. Cut the cleaned fascicles into approx 1-mm-long fragments or explants, using spring scissors or scalpels with no. 11 blades. Each 60-mm dish should have 10 1-mm nerve fragments.
8. Carefully remove the L-15 medium from the 60-mm dishes with the explants, and add 3 mL D-10.
9. These cultures are fed 2× a week with D-10. The sciatic nerve explants are transferred, approximately every week, using fine no. 5 Biologie forceps to fresh 60-mm dishes containing fresh media. They should be transferred when a large amount of outgrowth from the explant is seen. The explants will be ready to dissociate in about 4 wk.

11.2. Dissociation of Explants

1. Materials:
   D-10 Medium.
   D-10 Mitogen medium: contains 20 μg/mL bovine pituitary extract plus 2 μ*M* forskolin in D-10.
   Dispase–collagenase in D-10: 2.5 mg/mL dispase (Boehringer Mannheim, cat. no. 165–859) and 0.5 mg/mL collagenase (Worthington cat. no. CLSS-3 LS004206).
   Petri dish, 100-mm.
   Petri dish, 60-mm.
   Petri dishes, tissue culture, 100-mm, PLL-coated (*see* Chapter 7, Section 5.5.).
   Pipets, glass.
   Pipets, graduated.
2. Day 1:
   a. Set up a 60-mm dish in a 100-mm dish.
   b. Transfer all the explants from one set (all the explants from one prep) into the 60-mm dish, then add 3 mL dispase–collagenase solution.
   c. Put the explants in enzyme in the 6% CO_2 incubator at 37°C overnight.
3. Day 2:
   a. PLL-coat dishes for plating out the cells at the end of the procedure (*see* Chapter 7, Section 5.5.).
   b. Transfer the solution containing the explants into a 14-mL tube containing 1 mL D-10. Rinse the dish that had the explants with 2 mL D-10, until there is nothing left on the dish. Add the rinse to the tube.
   c. Centrifuge the mixture at 500*g* for 10 min at 4°C.
   d. Remove the supernatant, triturate the cells in D-10 (2 mL) with a small-bore pipet, until the explants are dissociated into a single cell suspension, then add more D-10, to make a total of 5 mL.
   e. Centrifuge the cell suspension at 500*g* for 5 min at 4°C. Discard supernatant.
   f. Repeat steps d. and e. (2–3×), until the supernatant looks clear. The cells are then ready to be plated out.
   g. Resuspend the cells for plating out in D-10 mitogen medium, use enough medium to put 2 mL/100-mm dish (*see* step h. below). Mix well.
   h. Plate cells out into the PLL- coated dishes, which already contain 5 mL D-10 mitogen medium.
   i. Put cultures into the CO_2 incubator.
   j. Feed cultures 2×/wk with D-10/mitogen, 7 mL/100-mm dish. When feeding the cultures, make sure to remove the media from the side of the dish, and gently add the media to the side of the dish, to avoid dislodging the cells. The Schwann cells can be used for myelination studies or for any other protocols requiring them.

11.3. Thy-1.1 Treatment of Schwann Cells

Further treatment may be used to eliminate fibroblasts, if present. The procedure is the same as that described for Schwann cells generated from neonatal rat pups (*see* Section 10.2.). This procedure will not work if fibroblast contamination is high.

11.4. Splitting of Cells

1. Materials:
   Petri dishes, PLL-coated.
   D-10 Medium.
   D-10 Mitogen medium.
   CMF HBSS.
   Trypsin/EDTA (0.05% trypsin/0.02% EDTA, prepared from 10X stock solution, Gibco-BRL, cat. no. 15400–054) in CMF HBSS.
   Tubes.
2. Method:
   a. PLL-coat a number of dishes, equal to twice the number of dishes that are to be split.
   b. Rinse the dishes containing the cells 2× with HBSS.
   c. Add 3 mL trypsin/EDTA to each dish, and wait for 2–5 min, until the cells start to come off the dish.
   d. Remove the cells from the dish, and put in a tube containing 1 mL/dish D-10, then rinse the dish with 3 mL D-10, and put this into the tube containing the cells. Tube size is determined by the number of dishes being split; 1 dish/14-mL tube, 2–5 dishes/50-mL conical tube.
   e. Centrifuge the cells at 500*g* for 10 min at 4°C.
   f. Remove the supernatant, and resuspend the cells in 2–3 mL D-10, with a small-bore pipet, then add D-10 to a final volume of 5 mL in a small tube and 10 mL in a large tube (50-mL conical).
   g. Centrifuge the cells at 500*g* for 5 min at 4°C.
   h. Remove the supernatant, and resuspend the cells in 2–3 mL D-10 mitogen medium; then add more medium to a final volume equal to 2 mL, multiplied by the number of new PLL-coated dishes.
   i. Plate 2 mL cells into each PLL-coated dish to which 5 mL D-10 mitogen medium was previously added.
   j. Put dishes into the CO_2 incubator. Feed twice weekly with 7.0 mL D-10 mitogen medium.

12. OBTAINING MYELINATION BY SCHWANN CELLS

The objective now is to transfer the pure populations of Schwann cells, obtained by one of the three methods described above, onto the pure population of DRG or sympathetic neurons (*see* Chapter 7). This system can then be used as a model to study Schwann cell–neuronal interaction, including either myelination or ensheathment of axons. Schwann cells prepared by the Wood method are grown on collagen, and must be removed by enzymatic treatment. Schwann cells can also be transferred to certain plastic culture dishes, and removed by scraping, prior to transferring to the neuronal cultures, if it is desired to avoid a trypsinization step prior to seeding.

12.1. Seeding Dissociated DRG Neuronal Cultures with Schwann Cells Prepared by the Wood Method

1. Rinse progenitor culture 1–2× with Earle's BSS (Gibco-BRL, cat. no. 24010–068) to remove medium.
2. With blade (*see* Section 10.1.2.), cut around the ganglion and remove neurons from the dish.

Note: The extirpated ganglia can be transplanted to fresh dishes to produce new cultures.

3. On average, each culture now without the ganglion yields approx 2×10^5 Schwann cells.
4. Add 1 mL 0.05% collagenase solution to every 5–6 layers of collagen to be digested, making sure none of the layers is stuck to the side walls of the tube.
5. Place in desiccator, and gas with 150 cm^3 CO_2.
6. Shake at approx 80 rpm on rotatory shaker for 30 min (37°C). The Schwann cells will form clumps.
7. Pick up clumps with forceps and transfer to a tube. Rinse clumps with 5.0 mL CMF HBSS. Centrifuge for 5 min at 500*g*.
8. Remove supernatant. Add 0.25% trypsin (2.5 mg/1.0 mL HBSS CMF; filter-sterilized).
9. Place on rotator (approx 80 rpm) for 30 min (37°C).
10. Add 0.2 mL HI-FBS to quench the trypsin reaction. Centrifuge 5 min at 500*g*, and discard supernatant.
11. Rinse the Schwann cells 4× with CMF HBSS. Use 2.0 mL for each rinse, and centrifuge at 500*g* for 5 min. Discard supernatant completely after each spin.
12. Resuspend cells in approx 2 mL myelination medium. The choice of the myelination medium is discussed in "Conditions for Myelination" below, or *see* Chapter 7, Section 5.4. Triturate until clumps are dissociated, and count the cells in suspension.
13. Seed DRG neuron cultures (*see* Section 5) with approx 0.5×10^4 cells/35-mm dish in a volume of at least 2.0 mL maintenance medium. Alternatively, fill the 35-mm dish (containing the DRG neurons) with 2.0 mL medium, then add 5×10^4 Schwann cells in 50 µL, directly into the center of the culture dish. Handle dish carefully, to avoid dispersion of cells.
14. Incubate 18–24 h, without disturbing.

12.2. Seeding Dissociated DRG with Schwann Cells Prepared by Brockes Method

1. Rinse dishes containing Schwann cells (*see* Section 11.) with CMF HBSS media, after removing culture media.
2. Add trypsin/EDTA solution (Gibco-BRL, cat. no. 15400–054) diluted from 10× to 1× in CMF HBSS.
3. Agitate culture dish during incubation (1–2 min). Observe under microscope as Schwann cells lift off.
4. Stop trypsin treatment with 10% FBS in DMEM, as soon as cells have lifted off the culture dish.
5. Centrifuge in tube (Falcon, cat. no. 2006). Rinse cells 1× with L-15/10% HI-FBS.
6. Resuspend in desired medium, and plate cells as described above in Section 12.1., step 13.

12.3. Seeding Dissociated DRG Cultures with Schwann Cells Obtained from Adult Rat Sciatic Nerves

Follow the same procedure used for Brockes Schwann cells (*see* Section 12.2.).

13. CONDITIONS FOR MYELINATION

Depending on the experimental objectives, a variety of culture conditions are supportive of myelination. Earlier studies relied on the use of complex, undefined media containing sera, but, more recently, extensive Schwann cell myelination has been obtained when the cultures are maintained in serum-free, completely defined media. The use of defined media are particularly advantageous when the cultures may contain some fibroblasts, since serum causes the fibroblast to overgrow, and this will often cause the cultures to deteriorate. If serum can be tolerated, abundant myelination

will occur in DRG neuron–Schwann cell co-cultures in DMEM/F12 medium with 15% serum, NGF, and 50 μg/mL ascorbic acid.

Note: Medium containing ascorbic acid should always be stored frozen at –80°C, because the ascorbic acid is rapidly oxidized in solution.

Abundant myelination will also occur if the cultures are maintained in N2 medium (Bottenstein and Sato, 1979) containing laminin (50 μg/mL) and ascorbic acid (50 μg/mL), as described in Guenard et al. (1995); this medium is completely defined. An alternative, completely defined medium that can be used is NB (Gibco) medium supplemented with the B27 supplement (Gibco), NGF, and ascorbic acid. The substrate may also be varied, because myelination has been obtained in cultures that were grown on either collagen-coated dishes or laminin-coated glass coverslips (Fernandez-Valle, et al., 1993). The latter substrate is preferred when the cultures are to be analyzed by *in situ* hybridization techniques. It is important to note that the co-cultures should be well populated with Schwann cells prior to beginning myelination feeding. The initial Schwann cell proliferation period will require 1–2 wk, depending on how many Schwann cells are added to each DRG neuron culture. After beginning to feed with myelination medium, at least 1 wk of further culture is required, before myelin sheaths are formed. Fairly extensive myelination should occur by the second to third week after initiating myelination conditions. Thus, overall, 4–5 wk of co-culture will be required for extensive myelination.

14. APPENDIX

14.1. NB Medium and NB Antimitotic Medium

1. NB Medium:

NB medium (Gibco-BRL, cat. no. 21103–31)	98 mL
B-27 (Gibco-BRL, cat. no. 17504–44)	2 mL
NGF (Boehringer-Mannheim, cat. no. 100700)	50 ng/mL

2. NB Antimitotic medium: NB medium plus 10^{-5} *M* fluorodeoxyuridine and 10^{-5} *M* uridine.

14.2. CH Medium and CHF Medium

1. CH Medium:

Eagle's minimum essential medium	88 mL
Human placental serum (heat-inactivated)	10 mL
20% glucose	2 mL
NGF	5 μg

2. CHF Medium: CH medium with 10^{-5} *M* fluorodeoxyuridine and 10^{-5} *M* uridine.
3. Comment: There is no commercial source of human placental serum; however, umbilical cord blood can sometimes be obtained through an arrangement with a local hospital maternity clinic, especially one affiliated with a university. If human placental serum is used, precautions must be taken to minimize the risk of exposure to bloodborne pathogens, such as HIV and hepatitis viruses.

14.3. O-Medium

DMEM:F12 (1:1) (Gibco-BRL, cat. no. 11320–033) supplemented with:

HI-FBS	2.5%
Rat transferrin (Jackson Immunoresearch, cat. no. 012–000–050)	10 μg/mL
Insulin (Sigma, cat. no. P-7507)	5 μg/mL
Sodium selenite (Gibco-BRL, cat. no. 13012–018)	30 n*M*
NGF (Gibco-BRL, cat. no. 13257–019)	10–100 ng/mL

14.4. N2 Medium

DME:F12 (1:1) (Gibco-BRL, cat. no. 11320–033) supplemented with:	
Rat transferrin (*see* Section 14.3.)	10 µg/mL
Insulin (*see* Section 14.3.)	5 µg/mL
Putrescine (Sigma, cat. no. P-7505)	100 n*M*
Progesterone (Sigma, cat. no. P-0130)	20 n*M*
Selenium (Gibco-BRL, cat. no. 13257–019)	30 n*M*
NGF (Gibco-BRL, cat. no. 13257–019)	50 ng/mL

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Chapter Nine

Preparation of Astrocyte, Oligodendrocyte, and Microglia Cultures from Primary Rat Cerebral Cultures

Ruth Cole and Jean de Vellis

1. INTRODUCTION

The study of glial cell development and function has been considerably enhanced by the development of methods to culture oligodendrocytes and astrocytes from central nervous system tissue. A primary mixed glial culture, composed mostly of astrocytes, oligodendrocytes, and microglia, is obtained when newborn disaggregated cerebral brain cells from rat are plated at high cell density ($2 \times 10^5/cm^2$) in serum-supplemented medium (McCarthy and de Vellis, 1980).

At low cell density (e.g., 5×10^4 cells/cm^2), few oligodendrocytes develop, and the culture consists mostly of astrocytes. At high cell density, phase-dark, process-bearing cells appear by 4 d, and stratify into clusters and individual cells above the bedlayer of cells. The bed layer consists of astrocytes rich in glial filaments. This observation led to the development of the shaking procedure, which results in selective removal of the process-bearing cells from the underlying astrocytes (McCarthy and de Vellis, 1980). Thus, highly purified cultures of astrocytes and oligodendrocytes can be obtained from the same piece of brain tissue (Fig. 1).

The microglia (Giulian and Baker, 1986) can be harvested from the stationary cultures by harvesting the medium on d 6 and 7, when they can be microscopically observed to be suspended in the medium. The remaining microglia and loosely adhering astrocytes are then removed from the mixed culture by a 6-h preshake, before the oligodendrocyte lineage cells are removed. The microglia cultures are about 95% pure, as characterized with immunocytochemistry using the microglia marker, ED-1 (Liva et al., 1999).

At the time of harvesting the process-bearing cells from the 7–9–d-old cultures, the cells are mostly immature, containing progenitor cells and immature oligodendrocytes (Holmes et al., 1988). If the process-bearing cells are placed in a chemically defined serum-free medium, <4% of the cells express astrocyte markers, such as glial fibrillary acidic protein (Saneto and de Vellis, 1985). In fetal-bovine-serum (FBS)-supplemented medium, 25–35% of the cells express glial fibrillary acidic protein, the so called type II astrocyte. It has now been determined that type II astrocytes are an in vitro phenomenon with no in vivo counterpart (Espinosa et al. 1993). The bed layer cells can

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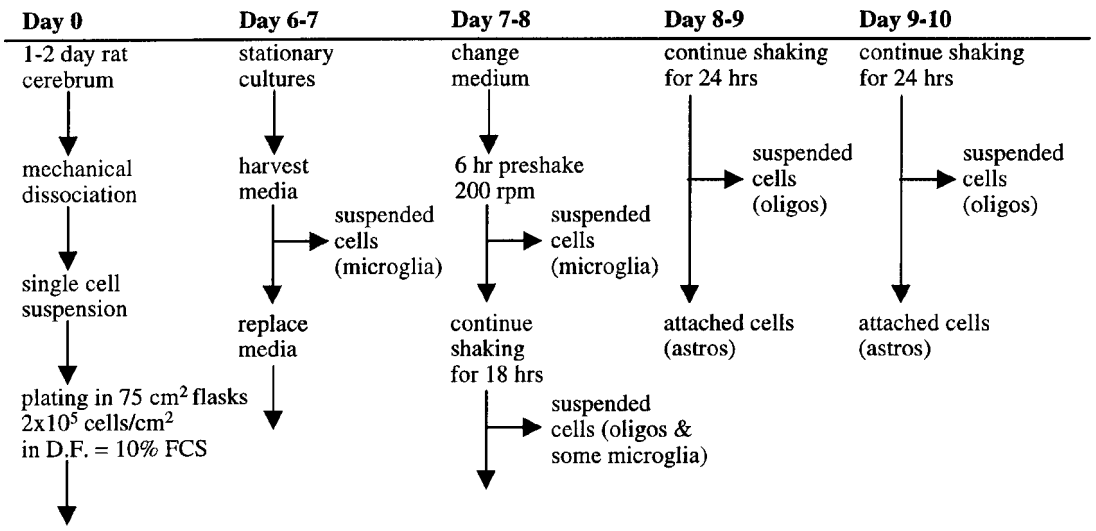

Fig. 1. Diagram for the preparation and isolation of oligodendrocytes, microglia, and astrocytes, from cultures of cerebral hemispheres from early postnatal rats.

be maintained as pure astrocyte cultures by keeping the flasks shaking slowly, to keep removing dividing oligodendrocyte progenitors and microglia. This primary culture of astrocytes can be used to set up pure secondary astrocyte cultures in serum or serum-free medium (Morrison and de Vellis, 1981, 1984). Astroglial and oligodendroglial cell lines have been developed from the cultures described above (Bressler et al., 1982; Bressler and de Vellis, 1985).

2. PREPARATION OF NEWBORN RATS FOR CULTURING

2.1. Materials

Newborn rats, P 1–2 d.
Scissors (1), $5^1/_2$ in., straight, operating.
Scissors (2), 4 in., straight, microdissecting.
Forceps (2), microdissecting.
Forceps (1), $5^1/_2$ in., tissue.
Forceps (1), curved, fine.
Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12), serum-free (10 mL).
Petri dishes (5), 35–mm, tissue culture.
Sponges (6), 4 × 4 in., sterile.
Disposable towels (3), double-thickness, sterile.
Ethanol (70%).
Containers (2), $4^1/_2$-oz (Falcon, cat. no. 4014), containing 80 mL 70% ethanol.
Plastic bag.

2.2. Procedure for Dissection

1. Clean the area for dissection with 70% ethanol, and set up instruments in an array that logically follows the dissection procedure (Fig. 2):
   a. Place a $4^1/_2$-oz container filled with 70% ethanol next to container of pups.

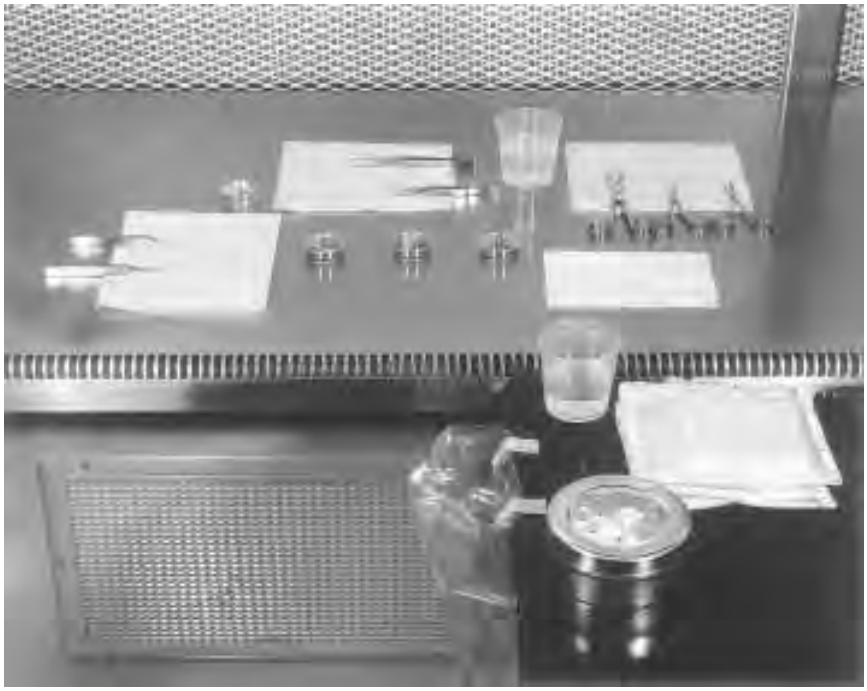

Fig. 2. Arrangement of work space for dissection of cerebral hemispheres from newborn rat.

b. Place sterilized towels in an area next to a plastic bag, for disposal of beheaded pups.
c. Place the three sterile scissors on sterile towels next to another $4^1/_2$-oz container with 70% ethanol.
d. Also, near this container of ethanol, place tissue forceps and fine curved forceps on sterile toweling.
e. Place fine microdissecting forceps on sterile gauze.
f. Fill three 35-mm Petri dishes with 2.5 mL DMEM/F12 (serum-free). One empty 35-mm Petri dish is used to dissect both cerebra and one for disposal of the remaining brain tissue.

2. Removal of the brain:
   a. Immediately after euthanizing the newborn pup by CO_2 inhalation, dip it into 70% ethanol, allowing the thumb and forefinger also to be immersed.
   b. Using a pair of sterile operating scissors, and holding the body of the rat, decapitate the head onto a sterile towel. The scissors are dipped in ethanol and returned to the sterile towel with the other scissors. The rat's body is disposed of in the plastic bag.
   c. With the cut portion of the head facing you, and with the head lying flat on the surface of the sterile towel, use a pair of microdissecting scissors to cut the skin at the midline of the head. Begin cutting from the base of the head, and cut to the mid-eye area. The scissors are dipped in ethanol and returned to the sterile towel with the other scissors. These scissors are used only for procedures b. and c. The skin is folded back by pulling the loose skin forward, using the index finger and thumb, and held open.
   d. The second pair of microdissecting scissors is utilized to cut the skull at the midline fissure. The scissors are dipped in ethanol and returned to the sterile towel.
   e. Using the thumb and forefinger, hold and apply slight pressure to the skull. Remove the raised portion of the skull with sterile tissue forceps.

f. Remove the brain by running the curved forceps along the bottom and sides of the brain calvarium, from the olfactory lobes to the posterior of the midbrain. Place the freed brain into a 35-mm Petri dish containing 2.5 mL DMEM/F12 SFM.
g. Repeat step 2, a.–f., for each pup, placing all the brains into the same Petri dish.

3. Removal of the cerebral hemispheres:
   a. Remove one brain from the 35-mm Petri dish to the inverted lid of a 35-mm Petri dish, and remove the cerebral hemispheres as follows:
      i. Using one pair of the microdissecting forceps, steady the brain at the median fissure. Using the second pair of microdissecting forceps, gently cut the tissue along the median fissure, and pull one cerebral hemisphere away from the rest of the brain.
      ii. Remove the cerebral hemisphere by pinching it against the Petri dish surface. Remove the other hemisphere in a similar manner. Make sure that the olfactory lobes are no longer attached.
      iii. Immediately immerse the cerebral hemispheres in SFM contained in another sterile 35-mm Petri dish. Repeat step a., i.–iii. for each pup.

   Note: Hemispheres from 15–20 pups are usually collected.

   b. Use the same microdissecting forceps, which were used to remove cortices, to remove meninges. Place a hemisphere onto the inverted lid of a second Petri dish. While using one pair of forceps to hold the hemisphere steady, pull off the meninges with the second pair of microdissecting forceps. The meninges can be easily teased away from the hemisphere by beginning with an exposed corner of the membrane on the outer surface, and gently pulling the meninges off. The removed meninges are blotted onto a piece of sterile gauze.
   c. Place the hemisphere piece into a fresh Petri dish containing serum-free medium. Repeat this process (steps b. and c.) until all the cerebral hemisphere pieces are free of meninges and blood vessels.
   d. Dip both forceps in ethanol, allow to drain, and place on sterile towels.

3. PREPARATION OF GLIAL CELL CULTURES: NITEX™ BAG METHOD

The Nitex bag method was developed by Lu et al. (1980). The procedure is done in a laminar flow hood.

3.1. Materials

Forceps (2).
DMEM/F12 containing 10% FBS (DMEM/F12–10% FBS) (75 mL).
Hank's balanced salt solution (HBSS) (25 mL).
Petri dish, plastic, 100–mm.
Flask, 75-cm^2.
Flask, 25-cm^2.
Nitex (No. 210) bag, $1^1/_2 \times 3$ in., sterile.
Glass rod, $7 \times {}^5/_{32}$ in., sterile.
Beaker (1), covered with 130-μm Nitex mesh (or Cellector™ sieve with a 140–μm mesh (no.100) and a sterile $4^1/_2$-oz container).
Beaker (1), covered with 230-μm Nitex mesh (or Cellector sieve with a 230–μm mesh (no. 60) and a sterile $4^1/_2$-oz container).
Centrifuge tube, 50-mL, sterile.
Hemocytometer.

Pasteur pipet.
Hand-held digital counter.
Microscope.

3.2. Disaggregation of Brain Tissue

1. Add 15 mL DMEM/F12–10% FBS to a 100-mm sterile Petri dish. Place the Nitex bag in this dish, and hold the mouth of the bag open with sterile forceps.
2. Pour or pipet the tissue pieces into the bag. Close the bag with sterile forceps, and immerse it in the medium in the Petri dish.
3. Use the sterile glass rod to tease the tissue through the mesh of the Nitex bag. Light strokes are used to prevent cell damage.
4. When teasing is completed, hold the closed bag upright with forceps, above the Petri dish containing the screened cell suspension, and rinse 10 mL medium down the side of the bag. The combined cell suspension is then removed and filtered through the Nitex 230-μm covered beaker (or Cellector sieve, 230 μm, no. 60), which has first been wetted with 1 mL HBSS to allow the fluids to flow through properly.
5. Next, filter the cell suspension through the Nitex 130-μm covered beaker (or Cellector sieve, 140 μm, no. 100). Collect the cell suspension in a 50-mL centrifuge tube.
6. Centrifuge the resulting filtrate at 200*g* for 5 min at room temperature. Resuspend the pellet in 10 mL culture medium.
7. Determination of cell number:
   a. Prepare a sample from the cell suspension for counting with a hemocytometer.
   b. Dilute 0.1 mL of the suspension with 1.9 mL HBSS. This is a cell dilution of 1:20. Because this is a total cell count, no trypan blue dye is required.
   c. Fill the hemocytometer with the diluted cell suspension, using a Pasteur pipet.
   d. Count the number of cells in at least one hemocytometer chamber (four squares).
8. Dilute the cell suspension in DMEM/F12–10% FBS. Plate 15×10^6 cells/75 cm^2 culture flask in a total volume of 10 mL, or 5×10^6 cells/25 cm^2 culture flask in a total volume of 5.0 mL.
9. Gas the flask with 5% CO_2 and incubate at 37°C, with the lid tightly closed, or incubate in a 5% CO_2/95% air incubator, without gassing procedure and lid loosened.

Note: Care should be taken not to disturb the flasks for 2 d. Movement of flasks during this initial 2 d period reduces cellular attachment. Culture medium is changed after d 2, and every other day thereafter.

4. ISOLATION OF ASTROCYTES AND OLIGODENDROCYTES

4.1. Introduction

The initial cortical cell cultures should be cultured for 7–9 d. Periods shorter than this are not sufficient for stratification of astrocytes and oligodendrocytes. Periods longer than this can result in clustering of astrocytes above the bed layer. On subsequent isolation of oligodendrocytes, the clustered astrocytes break up and contaminate the oligodendrocyte preparation. We have found that the oligodendrocytes tend to adhere more strongly, the longer they remain in mixed culture, making the shaking process less efficient.

4.2. Materials

DMEM/F12–10% FBS.
HBSS.

Puck's BSS (15 mL).
Versene (ethylenediamine tetraacetic acid [EDTA]) solution (10 mL).
2.5% Trypsin solution (2 mL).
Petri dishes (4), tissue culture, 35-mm.
Beaker (1), covered with 30-μm Nitex mesh (or Cellector sieve, 25-μm, no. 500).
Centrifuge tubes (2), 15-mL.
Lab-Line Junior orbit shaker in a dry 37°C incubator.
Note: A layer of packing foam will help insulate the flasks from the heat generated by the shaker.

4.3. Method for Isolation of Microglia, Oligodendrocytes, and Astrocytes

1. Microglia: At the end of the 6–7 d culture period, microglia can be microscopically observed floating in the medium of the stationary cultures. Harvest the medium on these days, and either centrifuge at 80*g* for 5 min, to obtain a pellet of nearly pure microglia, or plate directly into the desired culture dishes.
2. Oligodendrocytes:
   a. At the end of the 7–9 d culture period, phase-dark, process-bearing cells are observed to approximate a confluent phase-gray bed layer of cells. Change the culture medium, close and tighten the plastic culture lids, and place the flasks on a rotary shaker.
   b. Shake flasks at 200 rpm with a 1.5-in. stroke diameter at 37°C for 6 h.
   c. Remove flasks from the rotary shaker, change medium (the discarded medium usually contains dividing astrocytes and macrophages), and return them to the rotary shaker for 18 h.
   d. After 18 h, remove the cell suspension and filter through a Nitex 30-μm mesh covered beaker (Cellector sieve, 25 μm, no. 500). Collect the filtrate in a tube for counting.
   e. Determine the cell number by enumerating the cells, using a hemocytometer (0.1 mL cell suspension plus 1.9 mL diluent for a 1:20 dilution). Plate 4×10^5 cells in a volume of 1.5 mL/35-mm Petri dish.
   f. Additional oligodendrocytes can be harvested by replenishing the culture medium and shaking the flasks for an additional 24 and 48 h, with fewer cells, but more purified oligodendrocytes being harvested at each interval.
3. Astrocytes:
   a. After the oligodendrocytes have been harvested, replenish the medium, and place the flasks on a rotary shaker at 100 rpm, until fewer than 10 phase-dark cells per microscope field (×100) are observed. Replenish the medium every 2 d.
   b. Purified cultures can subsequently be subcultured by utilizing a versene–trypsin wash. Remove all medium from the flasks, and wash the cells with 5 mL versene (EDTA) solution (*see* Section 5.4.). Pour off, and wash the cells with 2.0 mL/flask of a 2.5% trypsin solution, making sure that all the cells have been bathed. Pour off, and incubate the flasks at 37°C, until the cells are completely disaggregated and run freely when the flask is inverted, usually 5–10 min.
   c. Resuspend the cells in 8 mL DMEM/F12–10% FBS. Remove cells to a centrifuge tube, and centrifuge for 5 min at 80*g*. Aspirate off old medium, and discard. Resuspend the pellet in 10 mL fresh culture medium.
   d. Determine the cell number by hemocytometry, and plate 4×10^5 cells/35-mm Petri dish.

Note: As cells are passaged in culture, they change in biochemical and immunological properties, and we do not use astrocytes past the initial passage.

5. APPENDIX

5.1. DMEM with High Glucose F12

DMEM/F12 is prepared by mixing DMEM and F12 in a 1:1 ratio (v/v). Then add 1.2 g/L $NaHCO_3$ and 15 m*M* HEPES. Filter through a 0.2-μm filter. The medium is completed with 10% FBS.

1. DMEM formulation (Dulbecco and Freeman, 1959; Morton, 1970):

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	200.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.10
KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6400.00
$NaH_2PO_4 \cdot H_2O$	125.00
Other components:	
D-Glucose	4500.00
Phenol red	15.00
Amino acids:	
L-Arginine HCl	84.00
L-Cystine 2HCl	62.57
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl $\cdot H_2O$	42.00
L-Isoleucine	05.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophan	16.00
L-Tyrosine (disodium salt)	104.20
L-Valine	94.00
Vitamins:	
D-Calcium pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i-Inositol	7.20
Nicotinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00

2. F-12 Nutrient mixture formulation (Ham, 1965):

Components	mg/L
Inorganic salts:	
$CaCl_2 \cdot 2H_2O$	44.00
$CuSO_4 \cdot 5H_2O$	0.00249

$FeSO_4 \cdot 7H_2O$	0.834
KCl	223.60
$MgCl_2 \cdot 6H_2O$	122.00
NaCl	7599.00
$NaHCO_3$	1176.00
$Na_2HPO_4 \cdot 7H_2O$	268.00
$ZnSO_4 \cdot 7H_2O$	0.863
Other components:	
D-Glucose	1802.00
Hypoxanthine	4.10
Linoleic acid	0.084
Lipoic acid	0.21
Phenol red	1.20
Putrescine 2HCl	0.161
Na pyruvate	110.00
Thymidine	0.73
Amino acids:	
L-Alanine	8.90
L-Arginine HCl	211.00
L-Asparagine H_2O	15.01
L-Aspartic acid	13.30
L-Cystine HCl $\cdot H_2O$	35.12
L-Glutamic acid	14.70
L-Glutamine	146.00
Glycine	7.50
L-Histidine HCl $\cdot H_2O$	20.96
L-Isoleucine	3.94
L-Leucine	13.10
L-Lysine HCl	36.50
L-Methionine	4.48
L-Phenylalanine	4.96
L-Proline	34.50
L-Serine	10.50
L-Threonine	11.90
L-Tryptophan	2.04
L-Tyrosine	5.40
L-Valine	11.70
Vitamins:	
Biotin	0.0073
D-Ca pantothenate	0.4800
Choline chloride	13.9600
Folic acid	1.300
i-Inositol	18.000
Niacinamide	0.0370
Pyridoxine HCl	0.0620
Riboflavin	0.0380
Thiamine HCl	0.3400
Vitamin B_{12}	1.3600

5.2. Nitex Mesh-Covered Beakers

1. Suppliers of Nitex mesh:
   a. B. & S. H. Thompson, 140 Midwest Road, Scarborough, ON, M1P 3B3 Can.
   b. B. & S. H. Thompson, 8148 Chemin Devonshire, Ville Mont Royal, PQ, H4P 2K3 Can.
2. Preparation of mesh: Nitex mesh is soaked in a large container of triple-distilled water. The water is changed 3×. The mesh is then coiled into a large beaker, and dried in a drying oven at 65°C.
3. Preparation of Nitex mesh-covered beakers:
   a. Materials.
      Beaker (1), 50-mL.
      Nitex, mesh (1), 7-cm square.
      Aluminum foil (2), 8-cm squares.
      Tape, masking and autoclave.
   b. Procedure:
      i. Place the square of Nitex mesh on the beaker, and tape all four corners down, using masking tape.
      ii. Cut autoclave tape to about 1-cm wide, and long enough to encircle the beaker.
      iii. Wrap the tape securely over the mesh, just under the lip of the beaker. The mesh should not be very taut. It is preferable to avoid taping the mesh to the beaker, since the tape is difficult to remove for washing.
      iv. Remove the masking tape from the corners of the mesh.
      v. Cover the beaker with two layers of aluminum foil.
      vi. Place a small piece of autoclave tape in the center of the foil cover. Autoclave at 30 min, wrapped setting.

5.3. Cellector Tissue Sieves

For the filtration of cell suspensions, Cellector tissue sieves may be used in place of Nitex mesh-prepared beakers. The sieve is first sterilized with the selected screen in place, then is placed into a sterile cup ($4^1/_2$-oz, Falcon, cat. no. 4014), and the tissue suspension is poured into it, allowing the liquid to flow through by gravity only. Forcing the suspension through the sieve can result in damage to cell membranes. Any remaining cells are rinsed into the sterile container with medium or a balanced salt solution. The Cellector, complete, including 85–mL pan, glass pestle, key, and nine screens (cat. no. 1985–85000) with various replacement screens, is available from Bellco Glass (Vineland, NJ).

5.4. Versene Solution

Component	Amount
NaCl	8.0 g
KH_2PO_4	0.2 g
KCl	0.2 g
Na_2HPO_4	1.15 g
EDTA (disodium salt)	0.2 g

1. Dissolve components in 1000 mL distilled water.
2. Dispense in convenient amounts and sterilize by autoclaving at 120°C for 15 min.

5.5. Alternate Disaggregation Method: Stomacher Blender (Stomacher Blender Tekmar, available from VWR CanLab)

1. Materials:
   DMEM/F12–10% FBS (60 mL).
   Tubes, centrifuge, 50-mL.
   Lab-blender bag with 80-mL capacity.
   Cellector sieve with 230-μm screen (no. 60).
   Cellector sieve with 140-μm screen (no. 100).
   Containers (2), $4^1/_2$-oz, sterile (Falcon, cat. no. 4014).
   Stomacher blender attached to a variable transfer set at 80 V.
2. Disaggregation:
   a. Carefully pipet the tissue–medium suspension into a sterile blender bag, and add enough medium to bring the total amount in the bag to 50–60 mL.
   b. Place the bag into the Stomacher blender, with about 2 cm of the bag visible above the closed door.
   c. The blender should be attached to a variable transformer that is set at 80 V. This setting controls the speed of the blender paddles, and prevents the disruption of the cell membranes.
   d. Blend the tissue for 2.5 min, and remove the bag with the cell suspension to the hood.
   e. Pour the cell suspension into the no. 60 sieve, allowing it to filter by gravity only.
   f. Pour this filtrate into the no. 100 sieve, again allowing the cells to flow by gravity only. Wash the adhering cells through the screen with 10 mL complete medium.
   g. Centrifuge the cell suspension at 80*g* for 5 min.
   h. Pour off the supernatants, resuspend the cells in DMEM/F12 (approx 100 mL/10 brains), and prepare a 1:100 dilution of cells for counting in a hemocytometer.
   i. Dilute the cell suspension in DMEM/F12–10% FBS, and plate at 15×10^6 cells/75-cm^2 culture flask, in a total volume of 10–11 mL.
   j. Incubate at 37°C in a humidified 5% CO_2 incubator, with the lid tightly closed.

5.6. Optimal Plating Densities

1. The optimal plating density for oligodendrocytes is as follows:
   a. 5×10^6 cells/75–cm^2 flask.
   b. 1×10^5 cells/well in 2.0-cm^2, 24-well culture plate.
   c. 4×10^5 cells/35-mm Petri dish.
   d. 4×10^4 cells/well in 0.32-cm^2, 96-well culture plate.
2. The optimal plating density for astrocytes is as follows:
   a. 1×10^6 cells/75-cm^2 culture flask.
   b. 2×10^4 cells/2.0 cm^2, 24-well culture plate.
   c. 4×10^5 cells/35-mm Petri dish.

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Chapter Ten

Culture of Glial Cells from Human Brain Biopsies

V. Wee Yong and Jack P. Antel

1. INTRODUCTION

Surgical resections of selected human brain areas. to ameliorate intractable epilepsy, provide opportunities to isolate, maintain, and examine nonmalignant human neural cells in vitro. Because these specimens tend to be from patients of early adulthood or older, neurons do not survive the isolation process; the cells extracted are thus of glial origin, and include oligodendrocytes, astrocytes, and microglial cells. In the Yong or Antel laboratories, although the biopsy materials (frontal or temporal lobes and corpus callosum) are mostly from subjects undergoing surgery to treat intractable epilepsy, we have not found any differences in properties of cells from such surgery, when compared to cells from other types of resections, such as those used to treat cerebral cardiovascular diseases or brain trauma. In the frontal and temporal lobe resections to treat epilepsy, tissues are removed either *en bloc* or by Cavitron ultrasonic aspiration (CUSA), which fragments the tissue into cubes of 2 mm^3 on average. Corpus callosum tissue is always removed by CUSA.

Biopsy-derived human brain specimens offer several advantages over autopsy-derived materials. Being relatively fresh, the cell viability and yield are high (5–10 million cells/gram wet wt of tissue); cell yield for autopsy specimens is at least an order of magnitude less. The success rate of bulk-isolating viable cells from biopsy specimens has been in the over 90% range. Another advantage offered by biopsy specimens is that blood samples, and the associated mononuclear cells, can be easily procured from living patients and tested on cultured neural cells, to assess autologous or heterologous immune reactivity. Nonetheless, we shall include a small section in this chapter that describes experience with autopsied brain material.

2. PREPARATION OF CULTURED CELLS

2.1. Materials

Human brain tissue.
Scalpels (2).
Forceps (2).
Feeding medium (*see* below).

Protocols for Neural Cell Culture, 3rd Ed. • Ed.: S. Fedoroff and A. Richardson • Humana Press, Inc., Totowa, NJ

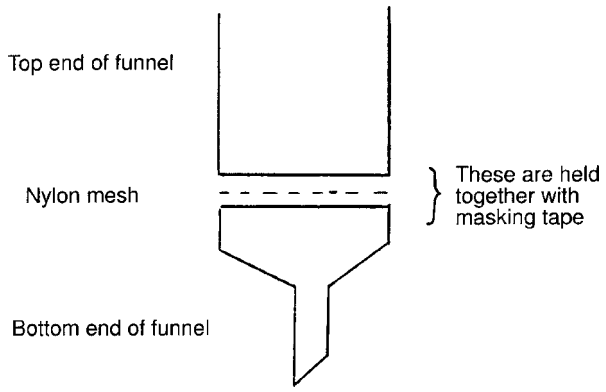

Fig. 1. Buchnel funnel—nylon mesh assembly. The Buchnel funnel is a two-piece Nalgene polypropylene funnel (Fisher, 10-362 B or C) with the perforated cutout filter. This assembly is useful, because the funnel can sit comfortably in 50-mL tubes during the filtration process.

Fetal bovine serum (FBS) (Gibco-BRL, cat. no. 10437-028).
Phosphate-buffered saline (PBS), pH 7.4 (Unipath, Basingstoke, Hampshire).
Trypsin (2.5% stock solution, Gibco-BRL, cat. no. 15090-046).
Deoxyribonuclease (DNase) type I, 1 mg/mL stock (Boehringer, Mannheim cat. no. 85020521).
Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, cat. no. 17-0891-01).
Coverslips, 12-mm diameter glass coverslips, coated with poly-L-lysine (PLL), 10 μg/mL (Sigma, cat. no. P-1524).
Lab-tek slides, 16-well (Nalge Nunc, cat. no. 178599).
Flasks, 25-cm^3, uncoated or PLL-coated (Falcon).
Petri dishes, 60-mm, plastic.
Petri dish (1), 100-mm, glass.
Pipets (5), disposable, plastic, with wide mouth, 10-mL.
Pasteur pipets (2), glass, 2-mL.
Tubes, polycarbonate tubes, 40-mL.
Tubes, sterile, plastic, conical, 50-mL.
Glass bottle (1), 100-mL, with cap.
Buchner funnel (1) with perforated plate removed, and containing a 130-μm nylon mesh (B. and S. H. Thompson, Montreal, PQ, Canada) (Fig. 1).
Magnetic stir bar (1).
Water bath set at 37°C, and placed on a magnetic plate.

2.2. Sterilization of Instruments

1. Sterilize scalpels, forceps, and magnetic stir bar prior to use, by immersing in 95% ethanol. The excess ethanol is then air-dried off. Flaming of instruments after ethanol immersion may be employed, but this can quickly dull the forceps.
2. Autoclave Buchner funnel–nylon mesh, glass Petri dishes and bottle, Pasteur pipets, and polycarbonate tubes, which have previously been wrapped in autoclave bags. All other sterile, disposable materials are purchased from manufacturers.

2.3. Bulk Isolation of Mixed Human Glial Cells

1. CUSA bag specimens:
   a. Cut the top end of the CUSA bag with scalpel, to expose the specimen.
   b. Using a 10-mL pipet, decant blood, and transfer tissues either into 50-mL plastic tubes or, if the bulk of the specimen is over 2 mm^3, into the 100-mm glass dish, for further dicing with scalpels. After dicing, these samples are then transferred into 50-mL plastic tubes.

   Note: We prefer to have approx 20 mL tissue suspension in each 50-mL tube, so that subsequent PBS washes can be more effective.
2. *En bloc* specimens:
   a. Remove the blood vessels with forceps.
   b. Using scalpels, cut the tissue into chunks of <2 mm^3 dimensions. Transfer these cubes into 50-mL tubes.

   Note: We prefer to have approx 20 mL tissue suspension in each 50-mL tube, so that subsequent PBS washes can be more effective.
3. Washing the tissue:
   a. Add enough PBS to the 50-mL tube to make volume up to 45 mL. Allow chunks to settle (10–20 s), then remove the supernatant, using a 10-mL wide-mouth pipet.
   b. Wash tissue several times, as above, to remove contaminating blood. During these washes, use a glass Pasteur pipet to fish off visible capillaries and blood vessel.
   c. For an bloody preparation, even after the PBS washes, overlay the specimen in PBS with a 50/30% Percoll gradient.
      i. Percoll gradient, from top to bottom, consists of 20 mL brain suspension, 5 mL 30% Percoll, and 5 mL 50% Percoll.
      ii. Centrifuge the gradient at 1700*g* for 20 min.

      Note: The brain chunks remain above the 30% Percoll layer: blood contaminants are trapped between the 30 and 50% Percoll layers.
      iii. Remove the brain suspension, dilute with PBS, and centrifuge at 160*g* for 10 min.
      iv. Repeat this wash twice, to remove the Percoll.
4. Trypsinization of the tissue:
   a. Place the brain pieces into a 100-mL glass bottle, to which is added PBS, to make a total volume of 68 mL.
   b. Add 8 mL trypsin (2.5% stock solution) to achieve a final concentration of 0.25%.
   c. Add 4 mL DNase stock, for a final concentration of 50 μg/mL DNase.
   d. Place a stir bar in the solution, and cap the bottle.
   e. Incubate the enzyme brain suspension at 37°C in a water bath on a magnetic plate. The speed of the stirrer should be slow enough so that a conical vortex does not form at the top of the solution. Incubation times can range from 30 min to 1 h, depending on the rate at which tissues are fragmented during this process.
5. Preparation of cell suspension:

Note: Pipeting should be done gently, to ensure high cell viability. Squirting of air bubbles into the cell suspension should be discouraged.

   a. Pass the cell suspension through the Buchner funnel–nylon mesh, into one of six 50-mL plastic tubes, each containing 1 mL of FBS to inactivate the trypsin.
   b. Add 20 mL PBS to the filter, and mechanically disrupt the fragments retained on the filter with a 10-mL pipet (horizontal motion of pipet on the filter), in order to yield further disaggregated cells. Repeat this process several times.

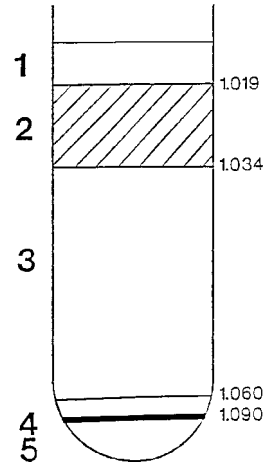

Fig. 2. Gradients following Percoll centrifugation. The numbers on the right of the diagram show the specific gravity's of the gradient. The left panels are the following: Layer 1: PBS; layer 2: myelin and cellular debris; layer 3: viable cell layer; layer 4: red blood cells; layer 5: PBS.

c. After further disaggregation of the tissue, completely mix the cell suspension in the 50-mL tube with the serum, using a fresh 10-mL pipet (three strokes).

Note: If large amounts of chunks are retained on the filter, these are collected and retrypsinized for 30 min (steps 5 and 6).

d. Centrifuge the filtrate at 300*g* for 10 min.
e. Aspirate off most of the supernatant, leaving behind 2 mL fluid. Resuspend the pellet, using a 10-mL wide-mouth pipet in this medium.
f. When the cells are suspended, add 5 pellet vol of PBS.
g. Gradients (Fig. 2):
   i. Add 21 mL suspension to one of several 40-mL polycarbonate tubes, each containing 9 mL undiluted Percoll. The final concentration of Percoll is thus 30%.
   ii. Centrifuge the suspensions at 15,000 rpm (31,000*g*) for 30 min, in order to allow gradients to form.
   iii. Aspirate off the cell debris–myelin layer. Using a glass Pasteur pipet, transfer the viable cell layer to clean 50-mL plastic tubes. Discard the red cell layer.
h. Transfer 15–20 mL of the viable cell layer to each 50-mL tube. Add enough PBS to make a total volume of 45 mL. Mix the suspension, using a 10-mL pipet. Cap the tubes, and centrifuge at 800*g* for 10 min.

Note: A common mistake here is that the viable cell layer collected from the Percoll gradient is centrifuged at 800*g*, without at least a 1:1 dilution with PBS. If so, no viable cells will be pelleted by the 800*g* centrifugation.

i. Aspirate the supernatant, leaving 1 mL. Using a glass Pasteur pipet (the finer bore of glass pipets allows better resuspension of live cells at this stage), resuspend the pellet in the 1 mL supernatant.
j. Cell suspensions from several tubes can be pooled into a single 50-mL plastic tube. Add 2 mL feeding medium into each tube that contains the cell suspension, twice, so that cells can be transferred with maximal yield into the pool.
k. Add enough feeding medium to make a total volume of 45 mL. Cap the tube, and centrifuge at 300*g*, 10 min.

l. Again, aspirate the supernatant leaving 1 mL. Then resuspend the pellet with a glass Pasteur pipet. Add feeding medium (25 mL) and centrifuge at 160*g*, 10 min. Repeat this process twice.

Note: The lower centrifugation speed at this point allows the majority of viable cells to settle, while keeping cellular debris in the supernatant.

6. Cultures.
   a. Resuspend the cell pellet in feeding medium, to an appropriate density. Plate these cells directly onto 10 μg/mL PLL-coated glass coverslips (contained within 60-mm dish) or 16-well Lab-tek slides at 50,000 cellscoverslip/well, or into uncoated Falcon flasks (10 million/flask in 5 mL), for further separation of cell types.

   Note: For cultures seeded onto coverslips, a good density is 50,000 cells in a droplet volume of 75 μL. We routinely flood these after 24–48 h; flooding consists of the addition of 3 mL feeding medium into each 60-mm dish, so that all coverslips are entirely submerged in feeding medium. 16-well Lab-tek slides receive 50,000 cells in 100 μL/well; there is no need for further flooding until the medium change a week later.

   b. Incubate the cultures in humidified incubators at 37°C, pulsed with an atmosphere of 5% CO_2 and 95% air.

3. SEPARATION OF OLIGODENDROCYTE, ASTROCYTE, AND MICROGLIA POPULATIONS

3.1. Enriched Oligodendrocyte Preparation

Oligodendrocytes derived from adult brains adhere relatively poorly to tissue culture plastic, compared to astrocytes or microglia cells (the poor adherence increases with advancing age of the human subjects). Thus, 24 h after initial cell plating in uncoated Falcon flasks, floating cells, most of which are oligodendrocytes, can be removed with glass Pasteur pipets. At this stage, the proportion of oligodendrocytes tends to be around 70–80%. These cells are centrifuged (160*g*) for 10 min, resuspended, and plated on PLL-coated coverslips at seeding density of 50,000 cells/coverslip. Alternatively, to further enrich for oligodendrocytes, the floating cells removed can be replated onto another set of 25-cm^3 uncoated Falcon flasks for another 24 h (5 million/flask, in 5 mL medium). The floating cells are then collected to give oligodendrocyte preparations of about 90% purity.

3.2. Microglia and Astrocyte Mixed Populations

Following removal of oligodendrocytes, add 5 mL feeding medium to each flask. The adherent cells (microglia and astrocytes) are kept undisturbed in the 37°C incubator, to allow them to develop morphologically. These cells can be removed from the flask at any stage during the incubation, to yield a mixed astrocyte and microglia preparation.

1. Removal from flasks:
   a. Aspirate off the feeding medium, then effect four changes of PBS, to completely remove trace amounts of feeding medium.
   b. Then incubate the cells with 0.25% trypsin in 4 mL PBS at 37°C for 15 min.

   Note: Although astrocytes will be dislodged after the trypsin treatment, most microglia cells remain adherent. To dislodge all cells, the sealed flask is knocked strongly against the palm of the hand, once.

c. 100 μL FBS is then added to each flask to inactivate the trypsin. The floating cells are collected into a 50-mL plastic tube with glass pipet.
d. Each flask is washed twice with PBS, to maximally remove cells, and the preparation is centrifuged at 300*g* for 10 min. The pellet is resuspended with feeding medium, and centrifuged at 160*g*, 10 min. This process is repeated twice.

2. Cells are then seeded on PLL-coverslips or 16-well Lab-tek slides at densities, of 5000–50,000 cells/coverslip/well.

3.3. Separation of Microglia and Astrocyte Populations

1. Following removal of oligodendrocytes, adherent cells are left for 1–2 wk, to allow cells to develop morphologically. To further separate microglia from astrocytes, another differential adhesion protocol is employed; this takes advantage of the observation that the microglia cells have very strong adherence to plastic, compared to astrocytes. The following procedure allows separation of the microglia and astrocyte populations:
   a. Remove the feeding medium, and add 5 mL fresh medium.
   b. Tightly seal the caps of the Falcon flasks, and place them on a rotary shaker, 150 rpm, at room temperature, for 5 h.

   Note: During this process, most astrocytes and some microglia cells are floated off.

   c. Remove the floating cells, and wash the flask twice with feeding medium, to maximize removal of floating cells.
   d. Centrifuge the cell suspension at 160*g* for 10 min, and replate the cells.
2. At best, such preparations contain up to 70% astrocytes, the rest being microglia cells. We presently cannot purify astrocytes beyond 70% purity, even though magnetic separation (allowing the rapidly phagocytosing microglia cells to ingest magnetic beads) and chemical treatments for eliminating microglia from rodent glia cultures (leucine-methyl ester and silica ingestion) (Giulian and Baker, 1986) were used.
3. For microglia enrichment, the adherent cells following floatation are retrypsinized as described above. Such preparations routinely yield microglia purity of over 90% (Williams et al., 1992).

4. APPENDIX

4.1. Notes on Cultured Human Adult Glial Cells

1. Feeding medium: An adequate feeding medium for adult human glial cells in culture is Eagle's minimum essential medium (Gibco-BRL, cat. no. 330-1430AJ) supplemented with 5% FBS, 1 mg/mL dextrose, and 20 μg/mL gentamicin (all from Gibco-BRL, NY). This medium can allow some cells to survive for up to 8 mo in vitro (the longest time period examined). Higher amounts of serum in the feeding medium have not conferred any advantage to cell viability or development, in our experience. Cells need only to be refed once a week.
2. Cell yield from biopsy specimens: Total cell yield is between 5 to 10 million/g wet wt of tissue. This is considerably higher than that from freshly sacrificed adult rodent brains using the same isolation process in which cell yield ranges from 1 to 3 million/g wet wt.
3. Autopsy human brains: Cells can also be isolated from human brains at autopsy. In this case, we dissect out the corpus callosum, and process chunks of this structure in an identical manner to that described above for *en bloc* biopsy specimens. Cell yield is low, and averages

0.5 million/g wet wt of tissue. For reasons that are not entirely clear, not all autopsy specimens will yield healthy cells for tissue culture study. Thus, rather than being confident that nearly all biopsy preparations will yield viable cells, our experience has been that one in three autopsy specimens will yield some cells for analyses. In general, specimens should have a death-to-trypsinization interval of 12 h or less.

4. Cell composition from biopsy specimens: Of the cells bulk-isolated from human brain biopsies, approx 25% tend to be oligodendrocytes. The rest is a mixture of microglia, and astrocytes; this ratio is extremely variable, and can be as low as 10% for microglia or as high as 95%. Presently, we have not been able to correlate the amount of microglia cells to patient diagnosis, the site of brain resection, the amount of gliosis in the resected specimen, or the age of the patient.
5. Plating requirements:
   a. When plated on 12-mm coverslips or 16-well Lab-tek slides, astrocytes and microglia cells require relatively low seeding density for survival, or to develop morphologically. A seeding density of as low as 5000 is sufficient, and we routinely employ a plating density of 10,000 cells/coverslip or well.
   b. Seeding requirement for oligodendrocytes is more stringent. At a seeding density below 10,000/coverslip or well, survival is poor. At 10,000 or 25,000 plating density, morphological differentiation (process formation) is slow (Oh and Yong, 1996). We routinely plate oligodendrocytes at a seeding density of at least 50,000 cells/coverslip.
6. Immunohistochemical characterization of cultures (Fig. 3).
   We employ routine immunofluorescence to characterize the types of cells present in culture (McLaurin et al., 1995). For oligodendrocytes, the expression of galactocerebroside on the cell surface is used. Microglia can be identified by the surface expression of Leu-M5 (CD11b), a monocyte marker, or by the cytoplasmic expression of anti-CD68. An antibody to glial fibrillary acidic protein (GFAP) following fixation of cells, is employed to label astrocytes.
7. What about fibroblasts?
   Unlike the adult rodent brain, fibroblasts do not become a contaminating problem for adult human brain cells isolated by trypsin digestion, except at longer-term cultures. We do not observe fibroblasts (cytoplasmic fibronectin immunoreactivity) in cultures until about 3–4 wk postisolation; no explanation is readily apparent for the inability of human brain fibroblasts to thrive in the early culture periods following trypsin dissociation, compared to cultures from the adult rodent brain.
8. Chemically defined serum-free medium for cells: It is sometimes necessary to culture cells in a serum-free medium. The following culture components and final concentrations (Boutros et al., 1997) are appropriate for the survival of cells in the absence of serum.

   DMEM/F12 (Gibco-BRL, cat. no. 11330-032) containing:

Dextrose (Sigma, cat. no. G-7528)	1 mg/mL
Gentamicin (Sigma, cat. no. G-1397)	20 μg/mL
Insulin (Gibco-BRL, cat. no. 13007-018)	0.5 U/mL
Hydrocortisone (Sigma, cat. no. H-0396)	50 n*M*
Human transferrin (Gibco-BRL, cat. no. 11108-018)	50 μg/mL
Selenium (Gibco-BRL, cat. no. 13012-018)	30 n*M*
Tri-iodo-L-thyronine (Sigma, cat. no. T-5516)	30 n*M*
Sodium pyruvate	1 m*M*
MEM nonessential amino acids (Gibco, cat. no. 320-1430AJ)	100 μ*M*

9. Characteristics of adult human glial cells in culture:
   a. Oligodendrocytes:

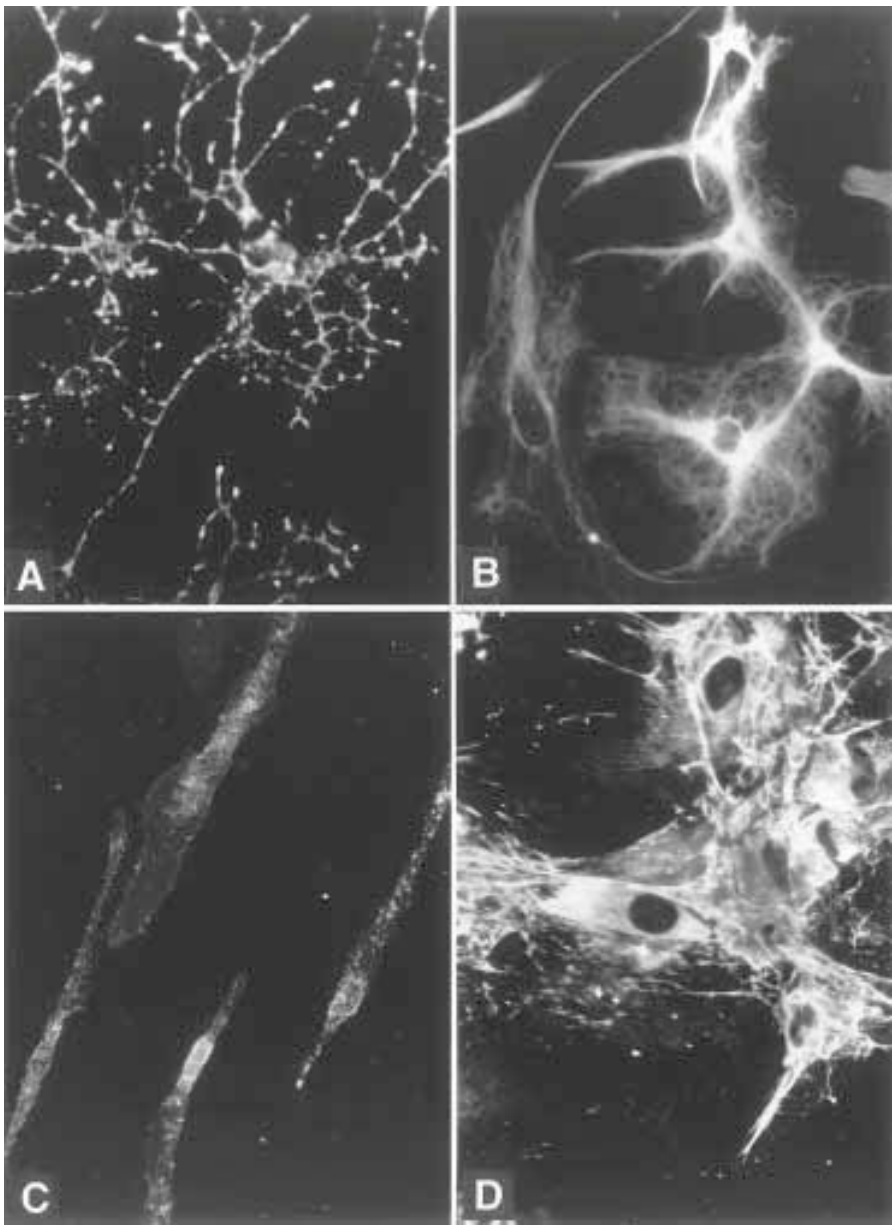

Fig. 3. Immunofluorescence characterization of cells form cultures of adult human brain tissue. **(A)** Galactocerebroside-positive oligodendrocytes. **(B)** GFAP-positive astrocytes. **(C)** Leu-M5-positive microglia cells. **(D)** Fibronectin-positive fibroblasts.

Under phase inverted microscopy, these cells have a phase bright round cell body of approx 8–15 μm in diameter. When examined for possible capability to proliferate, it has been found that galactocerebroside-positive cells do not incorporate bromodeoxyuridine (a thymidine analog and an index of proliferation) under any conditions.

We have assessed the rate of process formation by these cells, and have noted that this is dependent on seeding density, as mentioned. However, this is variable between different preparations, even when seeded at the same density; it is influenced mainly, but not exclusively, by the age of the human subjects: The younger the patient, the better the process-extending capability of the oligodendrocytes. The extent of process formation can be enhanced by treatment of these cells with biologically active phorbol esters (4α-phorbol-12, 13-dibutyrate, and phorbol-12-myristate-13-acetate) (Yong et al., 1994; Oh and Yong, 1996); again, although the majority of oligodendrocytes will enhance process formation after phorbol ester treatment, there is variability in the rate between preparations.

Recently, we showed that the matrix metalloproteinase member, MMP-9, is a regulator of the extension of processes (Oh et al., 1999). Besides galactocerebroside, adult human oligodendrocytes express the following markers as detected by immunohistochemistry: 2',3'-cyclic nucleotide phosphohydrolase; myelin basic protein; proteolipid protein; and myelin-associated glycoprotein.

b. Astrocytes:
Unlike the well-described flat and process-bearing morphology of rodent astrocytes, adult human astrocytes from biopsy materials are heterogenous in morphology. These range from a flat-fibroblastic form to process-bearing cells with little soma cytoplasm; the majority of astrocytes are intermediate in form (Yong et al., 1990).

Adult human astrocytes can undergo proliferation, but the extent is comparatively low. When pulsed with bromodeoxyuridine for 48 h in the 5% FBS-containing feeding medium, approx 2% of adult human astrocytes will incorporate bromodeoxyuridine. In contrast, approx 20% of adult rat astrocytes will incorporate the proliferation label under the same conditions.

c. Microglia:
The morphology of adult human microglia tends to be bipolar upon morphological differentiation, although some preparations of human biopsies will present with microglia that are round and amoeboid-like. Microglia cells can be activated, and, in this regard, γ-interferon and lipopolysaccharide can both elicit the release of interleukin-1 or tumor necrosis factor-α from microglia (Williams et al., 1995). Another good activator of microglia is T-lymphocytes, through an antigen-independent mechanism (Chabot et al., 1997; 1998). We are convinced that microglia cells from human brain biopsies are not merely monocytes from blood contaminants during the cell isolation process. In studies in which the properties of microglia cells are compared with monocytes isolated from peripheral human blood (Williams et al., 1992), we have observed that, although microglia cells have high survival rates and plating efficiency on PLL coverslips, monocytes do not. In addition, monocytes tend to remain rounded in morphology, except at longer-term culture (after at least 2 wk), when some can assume a bipolar form. In contrast, the majority of microglia cells undergo morphological differentiation within 4 d in vitro. The high yield of microglia cells from biopsy specimens also cannot be accounted for by the relatively lower amounts of contaminating blood or vessels.

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Chapter Eleven

Cultures of Astroglia and Microglia from Primary Cultures of Mouse Neopallium

Sergey Fedoroff and Arleen Richardson

1. INTRODUCTION

Brains of newborn rats or mice are generally used as the source of tissue for glial cultures. Only about 1% of cells survive the cell disaggregation process and culture environment, and neurons that survive die within the first few days of culturing. Such cultures contain progenitor cells and glia cells in different stages of differentiation (Alliot et al.,1991; Fedoroff et al., 1997; *see also* Chapter 14). Terminally differentiated glia cells, which can no longer divide, are overgrown by the proliferating, immature cells. How the cultures will develop, and which cell types (astroglia, oligodendroglia, ependymal cells, or microglia) will predominate, depend on the culture medium and the physical conditions under which the cells are grown (*see also* Chapters 12 and 14). The medium can be modified either by changing its chemically defined components or by adding or deleting serum. It is also possible to add growth factors and/or cytokines, either to the culture medium in pure recombinant form, or as medium conditioned by cells that produce and secrete growth factors/cytokines into the medium (the latter is considerably cheaper). The addition of cytokines to cultures can have a dramatic effect on the morphology and function of cells. It should be noted that cytokines and growth factors may affect more than one cell type, and may initiate variable effects in different cell types. The growth factors/cytokines may interact with other factors in the medium synergistically, additively, or in an inhibitory way. It is important to remember that the half-life of cytokines is short. However, the cytokines/growth factors can be added to the culture in microcapsules, which release the factors at a constant rate over a long period of time, thus assuring a constant concentration of a given factor to culture medium over long periods of time (Maysinger et al., 1992; 1996a,b).

This chapter outlines a procedure for initiation of neopallial cell cultures and their development into astroglia-enriched cultures or nearly pure microglia cultures. The next chapter describes a procedure that uses neopallial cell cultures to produce oligodendroglia. Neopallium (cerebral cortex and underlying white matter) yields progenitor cells that, when cultured in medium containing blood serum, produce highly enriched cultures of astroglia. The same progenitor cells, when stimulated with colony stimulating factor-1 (CSF-1), differentiate into microglia (Fedoroff et al., 1999). CSF-1 is required for the development, survival, and differentiation of microglia, and it is mainly

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produced in the central nervous system by astroglia. In cultures, astroglia secrete CSF-1 into the medium (Hao et al., 1992). Therefore, a simple protocol is described, originally developed by Hao et al., (1991), which utilizes CSF-1-secreting astroglia cultures for production of better than 99% pure cultures of microglia.

2. ASTROGLIA CULTURES

2.1. Preparation of Newborn Mouse for Dissection of Neopallium

1. Materials:
   Mouse, newborn.
   Forceps (2), curved, 12 cm.
   Forceps (1), straight, blunt, 14 cm.
   Forceps (2), Irex no. 5.
   Forceps (1), fine, curved, 10 cm.
   Scissors (1), large, 15 cm.
   Scissors (1), straight, 10 cm.
   Scissors (1), curved, 10 cm.
   Needles, 25-gage, sterile.
   High-sucrose phosphate buffer.
   Wax dissecting dish (1), sterile.
   Petri dishes (3), glass, sterile, 100-mm.
   Ethanol, 70% in Coplin jar.
2. Preparation of materials:
   a. Place several discrete drops of high-sucrose phosphate buffer around the periphery of the Petri dish (one cerebral hemisphere will be placed in each drop). In the center of the Petri dish, place a number of drops of high-sucrose phosphate buffer, in which the neopallia will be collected.
   b. Sterilize all instruments. When the instruments have been sterilized, place them into two sterile glass Petri dishes, and keep the ends covered with a lid.
3. Preparation of newborn mouse:
   a. Deeply anesthetize a newborn mouse with Halothane™ or Metofane™.
   b. Using large straight forceps, pick up the mouse, and briefly submerge it in the container of 70% alcohol.
   c. Using the large scissors, decapitate the mouse, so that the head falls onto the wax dissecting dish.
   d. Using a sterile needle, pin the head through the nose, dorsal side up, to the wax dish (Fig. 1A).

2.2. Dissection of the Cerebral Hemispheres

1. Using sterile curved forceps and curved scissors, remove the skin over the dorsal surface of the skull. This is easily accomplished by beginning the cut ventral to the ears, continuing toward the snout, then across the head just above the eyes, and finally back toward the neck, and, at the same time, by gently pulling the skin from the skull with forceps (Fig. 1A).
2. Using straight sterile scissors, divide the skull and cerebral hemispheres from posterior to anterior, i.e., by bringing the bottom blade of the scissors up through the brain. Do not cut down initially through the skull (Fig. 1A), because the pressure will mutilate the soft brain tissue.

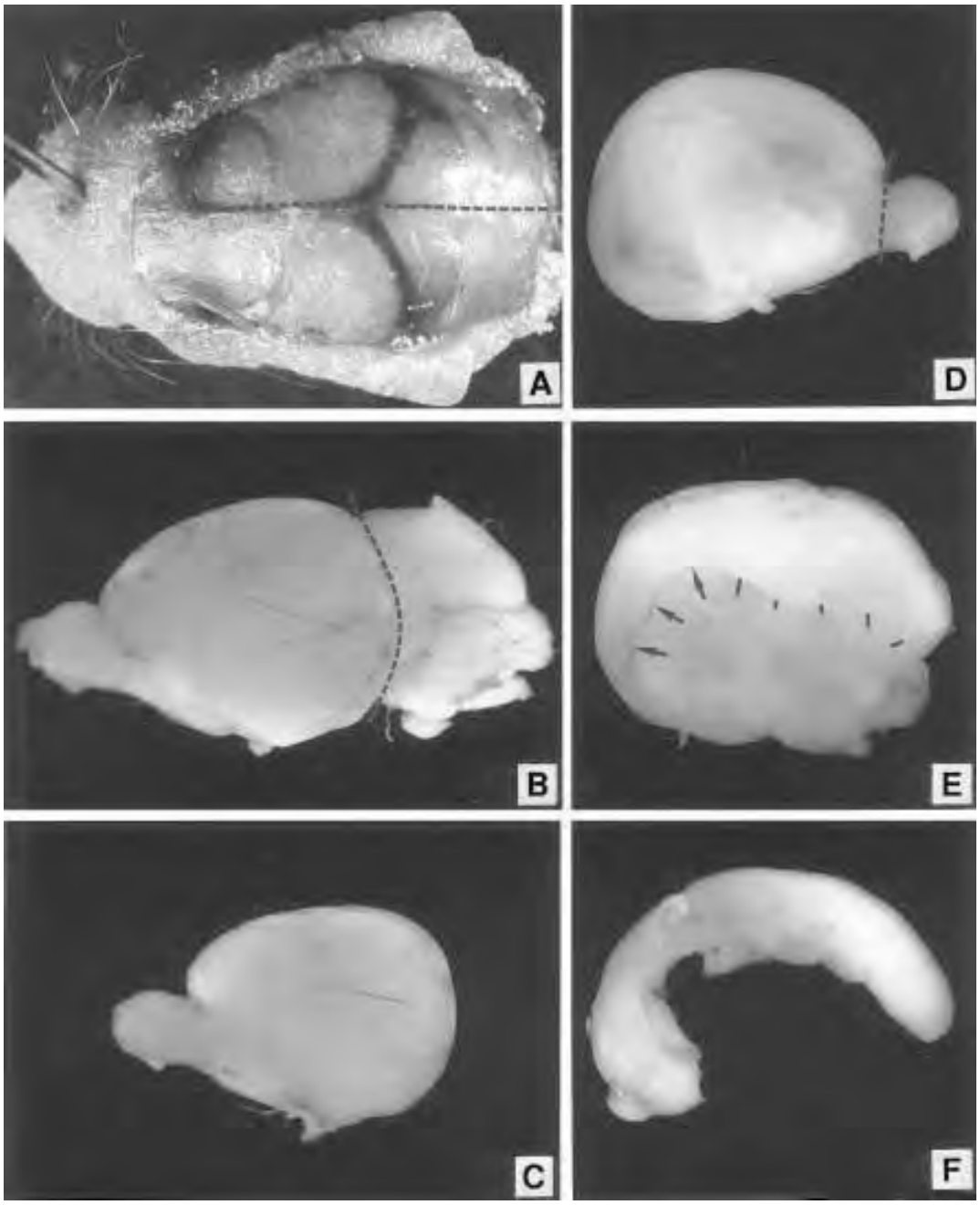

Fig. 1. Dissection of newborn mouse neopallium. **(A)** Head of mouse pinned through snout, using sterile needle, skin removed. Dotted line indicates midline cut through skull and brain. **(B)** Half of brain removed from skull, showing cerebral hemisphere, cerebellum, and olfactory bulb. Dotted line indicates cut for removal of cerebellum. **(C)** One cerebral hemisphere after removal of cerebellum (dorsal view). **(D)** The same cerebral hemisphere seen in **(C)**, with dotted line indicating cut for removal of olfactory bulb. **(E)** Bow-shaped hemisphere after the removal of basal ganglia. Arrows depict the site of dissection to remove hippocampus. **(F)** Isolated neopallium.

3. Remove the cerebral hemispheres:
   a. Using a pair of sterile curved forceps, grasp the muzzle of the mouse and squeeze gently. This serves to hold the head in place, as well as to open the skull.
   b. Next, using the fine curved forceps, lift the brain out of the skull and place each hemisphere into a drop of medium in the sterile Petri dish.
   c. Using the Irex forceps, remove the delicate, almost transparent meninges. Begin at the olfactory bulbs and peel meninges off, by pulling gently posteriorly. Try to avoid tearing the meninges.
   d. Pinch off the cerebellum (Fig. 1B) and olfactory bulb from the cerebral hemisphere (Fig. 1C,D), using the Irex forceps.

2.3. Dissection of the Neopallium

1. Place the cerebral hemisphere with the ventral surface facing up (Fig. 1D). Scoop out the basal ganglia, using the fine forceps. With the removal of the basal ganglia, a bowl-shaped hemisphere is left (Fig. 1E).
2. Remove the hippocampus, using the Irex forceps (Fig. 1E). The remaining part of the hemisphere is neopallium (Fig. 1F).
3. Transfer each neopallium into the drops in the center of the Petri dish.
4. Cut each neopallium into two or three fragments.

2.4. Preparation of Cultures

1. Materials:
   Puck's balanced salt solution (Puck's BSS).
   Medium (30 mL), modified Eagle's minimum essential medium (mMEM) containing 5% horse serum (HS).
   Tissue culture flask (1), 75-cm^2.
   Beaker (1), covered with 75-μm Nitex™ mesh.
   Centrifuge tube (1), 50-mL, sterile.
   Pasteur pipet (1), sterile, with bent tip.
   Rubber bulb (1), to fit Pasteur pipet.
2. Disaggregation of neopallia:
   a. Place fragments of neopallia on top of 75-μm Nitex mesh, which is stretched over a beaker.
   b. Place a few drops of growth medium over the neopallia to keep them moist.
   c. Gently roll the fragments of neopallia over the mesh, using the back side of a curved Pasteur pipet and, at the same time, add medium that has been taken up in the curved Pasteur pipet. Avoid adding air bubbles. Add 5 mL medium/brain to the mesh, and continue rolling the fragments until the neopallia are completely disaggregated and the cells have passed through the mesh. Rinse the mesh with the medium.
   d. Remove the mesh and transfer the cells in the beaker to a centrifuge tube. Use a pipet to resuspend the cells.
   e. Determine the cell number:
      i. Prepare a sample from the cell suspension for counting in a hemocytometer. Use 0.05 or 0.1 mL 0.3% Nigrosin as a viability indicator (viable cells exclude the dye) and 0.2 or 0.4 mL cell suspension, respectively.
      ii. Fill the hemocytometer, and count the number of viable cells (unstained) in at least four large corner squares of one chamber.

iii. Calculate the number of viable cells/mL (*see* Chapter 26).

Note: Usually, the neopallium of a newborn mouse should yield 2–3 × 10^6 viable cells.

3. Preparation of astroglia cultures:
   a. Before plating, make sure that the cells are well suspended. Plate 3–5 × 10^6 viable cells in a 75-cm^2 tissue culture flask, in a final volume of 12 mL mMEM with 5% HS.
   b. Incubate culture flasks in a 37°C highly humidified atmosphere containing 5% CO_2 in air.
   c. Feed cultures every 2–3 d for 10 d.

2.5. Comments

1. Instead of flasks, Petri dishes of various sizes can be used. A convenient way to prepare astroglia cultures is to place coverslips into a nonculture Petri dish, and to plant cells as described above. The coverslips can be removed from the Petri dish at any stage of culturing and used for sequential analyses. Cells grown on coverslips are convenient for immunocytochemistry, histochemistry, morphometry, or electron microscopy.
2. When large numbers of astroglia are required for chemical analysis or for conditioning medium, large-sized flasks, roller bottles, or large flasks filled with beads can be used to increase the surface area.

3. MICROGLIA CULTURES

3.1. Materials

Culture medium, mMEM with 5% HS.
LM cell-conditioned medium.
Hank's balanced salt solution (HBSS).

3.2. Preparation of Highly Enriched Microglia Cultures

1. Prepare astroglia cultures as described in Section 2.4., and incubate 10–12 d.
2. Examine the cultures under the microscope, to ensure that they have reached confluency, but are not too dense.
3. Wash and refeed the cultures, and incubate for another 10–12 d without medium change.
4. Remove the medium, and wash cultures with HBSS. Repeat the washing until all dead and floating cells have been removed.
5. Refeed the cultures with mMEM with 5% HS, and add 20% LM cell-conditioned medium, which contains CSF-1, or add 1000 IU/mL recombinant CSF-1.
6. Incubate cultures at 37°C in a highly humidified atmosphere containing 5% CO_2 in air.

3.3. Comment

The first indication of the formation of microglia is the appearance of phase-dark cells with poorly defined, thick, cytoplasmic processes abutting the underlying astroglia. A few days later, the phase-dark cells show cytoplasmic vacuoles and become attached to the substratum. Subsequently, they assume a morphology characteristic of ameboid cells (vacuoles, irregular shape, and pseudopodia), and eventually form process-bearing cells that resemble parenchymal, ramified microglia. In the presence of CSF-1, microglia proliferate (Hao et al., 1990).

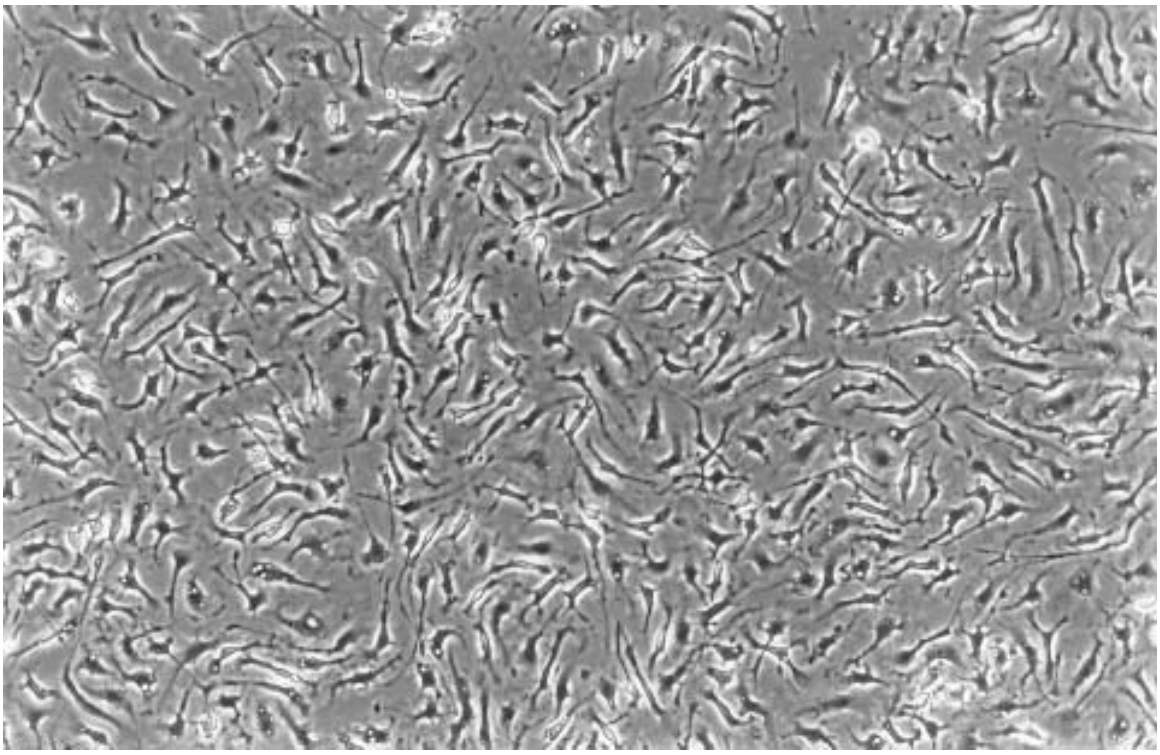

Fig. 2. Microglia from neonatal mouse astroglia cultures.

3.4. Subculturing of Microglia

The cultures of microglia (Fig. 2) can be used directly, or subcultured. Microglia attach firmly to the substratum.

1. Trypsin: To remove them from the substratum, microglia can be treated with 0.25% trypsin for 10 min at room temperature. The trypsin can then be inhibited by the addition of medium containing serum or soy bean trypsin inhibitor (*see* Chapter 27). Transfer the resulting disaggregated cell suspension into another culture vessel.
2. Cold versene: A more gentle and more effective way to remove microglia from the substratum, without altering their cell surface antigenic characteristics, is the following procedure:
   a. Refrigerate a culture of microglia at 4°C for 10 min.

   Note: Microglia round up when subjected to low temperature, thus decreasing the area of the cell surface attached to the plastic substratum.

   b. Remove medium, and add cold (4°C) 0.01% versene.
   c. Incubate culture for 10 min with versene at 4°C.
   d. Gently triturate culture 30× with a Pasteur pipet.
   e. Transfer the cell suspension into a test tube, and centrifuge at 200*g* for 5 min.
   f. Remove the versene solution, and resuspend microglia in fresh medium.
   g. Transfer the resulting disaggregated cell suspension into another culture vessel.

4. APPENDIX

4.1. Comments

1. Gebicke-Haerter et al. (1989) reported that bacterial lipopolysaccharide (LPS), present in culture medium, inhibits the formation of brain macrophages. It should be noted that most commercially available fetal bovine serum contains some LPS (Northoff et al., 1986a,b), and thus may inhibit the formation of microglia. It is therefore advisable to use horse serum, which normally has no detectable LPS. Moreover, it is advisable to use the embryos of C_3H/HeJ mice, which are resistant to LPS (Sultzer et al., 1993).
2. This method for isolation of microglia from mouse neopallia–astroglia cultures can also be used to obtain microglia from rats or humans.
3. To preserve high viability of cells, it is important to work quickly, and thus prevent evaporation of medium, which increases its osmolarity and pH change. To avoid pH change, use media in which the buffer calibrates with the atmospheric air CO_2, e.g., high sucrose phosphate buffer, HBSS, or Hibernate A medium (*see* Chapter 19).

4.2. Solutions

1. Medium: MEM is modified to contain a fourfold concentration of vitamins, a double concentration of amino acids except glutamine, and 7.5 m*M* glucose (Gibco-BRL, cat. no. 90-5175). Before using the modified MEM for culturing, 5.2 mL 1 *M* sodium bicarbonate and 2.5 mL 200 m*M* glutamine are added to 200 mL mMEM, and the pH is adjusted to 7.2 by bubbling 5% CO_2 into the medium. The prepared modified MEM is supplemented with 5% HS.
2. Puck's BSS:
   a. Composition:

NaCl	8.00 g/L
KCl	0.40 g/L
$Na_2HPO_4 \cdot 2H_2O$	0.06 g/L
KH_2PO_4	0.06 g/L
Glucose	1.00 g/L
1% Phenol red	2.00 mL

   b. Procedure for making 1 L Puck's BSS:
      i. Dissolve all components, in order (except Phenol red), in a beaker containing 400 mL triple-distilled water.
      ii. Add phenol red to a 1 L volumetric flask. Add other components to the flask. Rinse beaker used for solutions with triple-distilled water, and add to the volumetric flask. Add enough water to make the volume up to 1 L.
      iii. Stir on magnetic stirrer for 30 min (minimum time).
      iv. Filter through 0.22-μm filter.
      v. Label, and store stock solution at 4°C.
3. High-sucrose phosphate buffer: This maintenance medium is for freshly dissected parts of the brain. It contains physiological ion concentrations (Na^+ and K^+), some buffering action, and an energy source. Sucrose is the best compound for adjustment of osmolality, because it is neither taken up by the cells nor extracellularly degraded.

Component	g/L
NaCl	8.00
KCl	0.40

Na_2HPO_4	0.024
KH_2PO_4	0.03
Glucose	0.90
Sucrose	20.0

a. Dissolve chemicals in 1 L high-quality distilled water.
b. Sterilize by filtration through a 0.22-μm filter, aliquot, and store at 4°C.

4.3. Preparation of LM Cell-Conditioned Medium

For the preparation of conditioned medium containing CSF-1, LM cells (American Type Culture Collection, cat. no. CCL-1.2) are used. The advantage of using LM cells is that they can grow well in suspension and in serum-free medium. By using suspension flasks, a large amount of conditioned medium can easily be prepared. LM cells can also be grown in large stationary flasks or roller bottles. The cells are grown in Medium 199 with 0.5% Bacto-peptone, without the addition of serum. Suspension cultures are usually inoculated with 6×10^5 cells/mL, and grown for a week or 10 d without feeding, or until they reach about 2×10^6 cells/mL. By then, the culture medium appears orange in color and cloudy, because of the high cell concentration. The cells are allowed to settle, and the supernatant is removed and centrifuged at 200*g* for 15 min. The supernatant medium is then filtered through a stack of 1.2, 0.8, and 0.45-μm pore-size filters, and sterilized by filtration through a 0.22-μm pore-size filter. The sterile conditioned medium is aliquoted, labeled, and stored at –80°C. The conditioned medium can also be concentrated, using a stirred ultrafiltration unit and a YM-10 (>10,000 mol wt) filter (Amicon).

It is important that an aliquot of the conditioned medium be used for determination of the CSF-1 content in the medium, using the radioreceptor assay (Das et al., 1980, as modified by Hao et al., 1990). The biological activity of CSF-1 can be determined by using the sodium XTT colorimetric population growth assay of 5/10.14 and DA1.K cells (Branch and Guilbert, 1986; *see also* Chapters 20 and 21).

4.4. Nitex Mesh-Covered Beakers

See Chapter 9, Section 5.2., for suppliers of Nitex mesh, and for the preparation of Nitex mesh-covered beakers.

4.5. Wax Dissecting Dishes

1. Preparation:
   a. Break nine sticks of Kerr Brand Blue Inlay Casting Wax, Regular Type II (Sybron, Emeryville, CA) into pieces. Place wax pieces in 100-mm Petri dishes.
   b. Place the dishes containing the wax in a pan, and sterilize by dry heat in an oven for 2 h at 250°F.
   c. The dishes may be allowed to cool overnight in the oven. If removed while hot, be careful not to tilt the dishes so that wax reaches the cover of the Petri dish.
   d. When cool, wrap wax dishes in aluminum foil, and store until needed.
2. Cleaning used wax dissecting dishes:
   a. Clean the wax dissecting dishes by rinsing with water. Dry with a soft, lint-free tissue.
   b. Wash the wax dishes with 70% ethanol, and dry with a soft, lint-free tissue. Close the lids, and sterilize as above for new wax dishes.

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Chapter Twelve

Cultures of Oligodendroblasts from Primary Cultures of Rat Neopallium

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1. INTRODUCTION

Several distinct stages of differentiation have been described for the oligodendroglial lineage in vitro (Gard and Pfeiffer, 1990; Gard et al., 1995). These include the actively proliferating bipolar or tripolar oligodendroglial precursor cell, characterized by the presence of GQ1c and GD3 gangliosides in the plasmalemma. The oligodendroglial precursor cell differentiates into the multipolar oligodendroblast, a proliferative cell that has sulfatide, but no galactocerebroside, in its plasmalemma. The oligodendroblast differentiates into the mitotically quiescent oligodendrocyte, a cell characterized by the presence of galactocerebroside in its plasmalemma. These oligodendrocytes also express other myelin-associated proteins, such as myelin basic protein. When transplanted into the central nervous system (CNS) of hypomyelinating hosts, oligodendrocyte precursors migrate over considerable distances, and give rise to large numbers of myelinating oligodendrocytes; oligodendroblasts migrate only short distances, and give rise to far fewer myelinating oligodendrocytes (Warrington et al., 1993). When transplanted into the CNS, the mature oligodendrocyte will also myelinate axons (Duncan et al., 1992).

Dissection of cellular interactions that occur during myelination (or remyelination) of CNS axons requires access to large numbers of oligodendroglial cells. Currently, several methods are described in the literature for isolation of large numbers of oligodendroglial cells from the CNS. One approach is to isolate mature oligodendrocytes directly from adult brain tissue, using enzymatic dissociation of the nervous tissue, followed by isolation of the oligodendrocytes through the use of differential centrifugation (Szuchet et al., 1980; *see also* Chapter 10), or differential centrifugation followed by fluorescence-activated cell sorting (Duncan et al., 1992). A second approach is to isolate oligodendroglial precursor from the immature brain and allow them to differentiate into mature oligodendrocytes in vitro. Oligodendroglial precursors can be separated from other brain cells, using fluorescence-activated cell sorting (Behar et al., 1988) or immunopanning (Gard et al., 1993). A third approach takes advantage of the fact that primary glial cultures, established from the immature brain, are composed of a mixture of cell types, including precursor cells. Thus, oligoden-

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droglial and astroglial cells develop in primary cultures established from the newborn rat cerebrum; the oligodendroglial cells can be separated from the astroglial cells by using shearing forces (McCarthy and de Vellis, 1980; *see also* Chapter 9).

The approach used in this laboratory is a modification of a procedure initially described by Hunter and Bottenstein (1989, 1990; Louis et al., 1993), and takes advantage of the observation that conditioned medium obtained from B104 neuroblastoma (NB) cells contains one or more factors that will cause proliferation of oligodendroglial precursor cells. This chapter demonstrates a technically simple procedure for directly isolating large numbers of oligodendroglial precursor cells from primary cultures of newborn rat neopallial cells. The predominant cell population isolated in the procedure described below is the oligodendrocyte precursor cell; this precursor can be induced to differentiate into the oligodendroblast, then into the oligodendrocyte.

2. ESTABLISHMENT OF PRIMARY RAT NEOPALLIAL CULTURES

2.1. Materials

Newborn rat (Wistar) pups (P1–P4).
Stainless steel no. 1 insect pins (Polyscience, Niles, IL) sterilized in 70% alcohol.
Scissors (1), 3 1/2 in., sterile.
Forceps (2), 3 1/2 in., sterile.
Forceps (1), 3 1/2 in., curved, sterile.
Watchmaker's forceps (4), sterile.
Scalpels (2), sterile.
Primary culture growth medium (PC-GM).
Tissue storage medium.
Beakers (5), plastic.
Beaker (1), 50-mL, sterile, covered with nylon mesh (75 μm).
Petri dishes (4), 100-mm tissue culture (Falcon, cat. no. 3003, obtained from VWR Scientific, Toronto, ON).
Wax dissecting dish, sterile.
Beaker containing 2% iodine in 70% alcohol.
Beaker containing 70% alcohol.
Metafane (methoxyfluorane) anesthetic.
Small chamber with lid for anesthesia.
Dissecting microscope.

2.2. Procedure

1. After euthanizing neonatal rats by overdosing with methoxyfluorane, sterilize the heads by dipping them into iodine solution, followed by a rinse in 70% alcohol. Cut off the head of the rat pup, and place into an empty sterile dish, ventral surface down. Examining Fig. 1 in Chapter 11 will facilitate following the description of the dissection outlined below.
2. Pin the head, ventral surface down, onto a wax dish, using sterile stainless steel insect pins. Place pins through the snout, and through the occipital bones on either side of the caudal part of the skull, making sure that the pins do not pierce the cerebrum. Slant the pins away from the head, which gives more room to maneuver during the dissection.
3. With two pairs of sterile 3 1/2 in. forceps, peel the skin off the head; make sure that the skin is well away from the roof and sides of the skull. The forceps can be returned to a beaker of 70% alcohol. Underneath the thin bones of the skull is seen the Y-shape of the dural venous sinuses, with the stem of the "Y" going to the nasal region (*see* Fig. 1A in Chapter 11).

4. With two pairs of fine watchmaker's forceps, remove the parietal bones on either side of the stem part of the Y-shaped dural venous sinus. Do this by inserting the tips of one pair of forceps into the sutural joint separating the two halves of the skull, then, by a gentle stroking movement, separate the two bones; the other pair of forceps is used to steady the head by bracing the exposed skull bones. Do the same for the sutures overlying the forked part of the Y-shaped dural venous sinus, then separate the parietal bones from the posterior skull bones. Reflect the parietal bones to the sides. These forceps can be returned to the beaker of 70% alcohol, and reused.
5. With the remaining pair of sterile 3 1/2 in. forceps, gently stroke the cerebral hemispheres anteriorly at their junction with the olfactory bulbs, thus separating the bulbs from the hemispheres; make sure that force is not used, because one may enter the oral cavity very easily from this direction. With the same forceps, gently stroke through the midbrain, thus freeing the cerebral hemispheres from the hindbrain. Using the tines of the curved forceps to cradle the ventral surface of the forebrain, lift the forebrain and place into a Petri dish containing tissue storage medium.
6. With the second pair of sterile watchmaker's forceps, separate the two hemispheres of the cerebrum, by opening the tips of the forceps, then divide the brain into the two hemispheres. Using the tips of the forceps as scissors, remove all noncerebral tissue, such as the midbrain and thalamus. The hippocampus, which forms the most medial part of the cerebrum, is removed, then the neopallium is separated from the basal ganglia. Remove the meninges, the connective tissue coverings of the brain, from the neopallium. Place the neopallium into the PC-GM. The neopallium can be transferred using forceps, by spreading the tines of the forceps slightly, and using surface tension to hold the neopallium between the tines, or by using a wetted Pasteur pipet.
7. Dice the neopallium into small fragments of about 1 mm^3, using sterile scalpel blades. Then gently force these fragments through nylon mesh covering the top of a beaker; this is done by stroking with the side of a sterile pipet, and occasionally washing the cells through the mesh with growth medium. Plant the cells into culture vessels, and place into an incubator containing a humidified atmosphere of 5% CO_2 in air, and maintained at 37°C. We routinely plant the cells obtained from the neopallia of one rat pup into four 100-mm tissue culture Petri dishes (Falcon no. 3003, obtained from VWR), i.e., about 800,000 viable cells/100-mm Petri dish. Total volume of the PC-GM used per 100-mm Petri dish is 8 mL. Cultures are fed with PC-GM after 2 d.

3. PREPARATION OF OLIGODENDROCYTE PRECURSOR CELL CULTURES

3.1. Materials

Oligodendroglial precursor medium with glucose (OPM-G).
Oligodendroglial precursor medium with no glucose (OPM).
Oligodendrocyte differentiation medium (ODM).
Cell harvest medium (HM).
Primary oligodendroglial planting medium (PO-PM).
Puck's balanced salt solution (Puck's BSS).
Petri dishes, tissue culture, polylysine-coated.
Cell filtration tubes containing 15-, 35-, and 50-μm sterile nylon meshes.
Cotton-plugged, sterile Pasteur pipets.
Hemocytometer.

3.2. Procedure

1. After 5 d, primary cultures are placed on OPM-G and fed every 2 d with this same medium.
2. After 10–14 d of culture, the majority of cells are oligodendroglial precursors, and cultures are ready for harvest (*see* Section 5.). The day before harvest, feed the cultures with OPM-G. The following day, cultures are washed twice with Puck's BSS, then placed in HM and maintained at 37°C in air (i.e., not in a CO_2 incubator).
3. After 15–30 min, cell processes can be seen to retract. Use the Pasteur pipet to gently swish medium over the entire surface of the dish; this should remove all adherent cells.
4. Filter cell suspension sequentially through 50-, 35-, and 15-µm nylon filters. This removes cell clumps, which contain the majority of surviving astrocytes.
5. Centrifuge cells at 180*g* for 15 min at room temperature, and resuspend in a small volume (1–5 mL) of PO-PM. Do a cell count, and plant cells in PO-PM into culture vessels coated with polylysine. We routinely plant 50,000 cells into 35-mm Petri dishes and 400,000 cells into 100-mm Petri dishes.
6. After the cells have attached, the medium is replaced with the glucose-free OPM, a medium that contains lactate as the primary energy-yielding substrate.

Note: This medium acts as a selection medium for oligodendroglial precursors, since this is the only cell type that preferentially survives and proliferates with lactate as the primary energy source.

7. Cultures can be repeatedly subcultured, as long as cells remain at the oligodendroglial precursor stage (*see* Section 5.); however, if cultures become too dense, the cells differentiate into oligodendroblasts, then into mature oligodendrocytes. One day before subculturing, cultures should be put on the glucose-containing OPM-G, then cells can be harvested using HM. Because the cultures should be composed of oligodendroglial precursor cells, there is no necessity to filter the harvested cells through nylon-mesh filters. Cells can be centrifuged, resuspended in PO-PM, and planted into polylysine-coated culture vessels. After cells have attached, the medium can be replaced with OPM-G (if cultures are not contaminated with nonoligodendroglial cells) or with OPM (if cultures have a significant nonoligodendroglial contamination).
8. Oligodendroglial precursor cell cultures can be placed in ODM, and induced to differentiate into mature oligodendrocytes.

4. LONG-TERM STORAGE OF CELLS

4.1. Materials

Dulbecco's modified essential medium (DMEM) containing 2 m*M* pyruvate.
Fetal bovine serum (FBS).
Dimethyl sulfoxide (DMSO).
Hemocytometer.
Cryogenic vials (Nalgene, obtained from VWR Scientific).
Cryo 1°C freezing container (Nalgene, obtained from VWR Scientific).
Freezer, –80°C.
Cryobiological storage container (VWR Scientific).

4.2. Procedure

1. After harvesting oligodendroglial precursors, resuspend in pyruvate-containing DMEM and count cells.

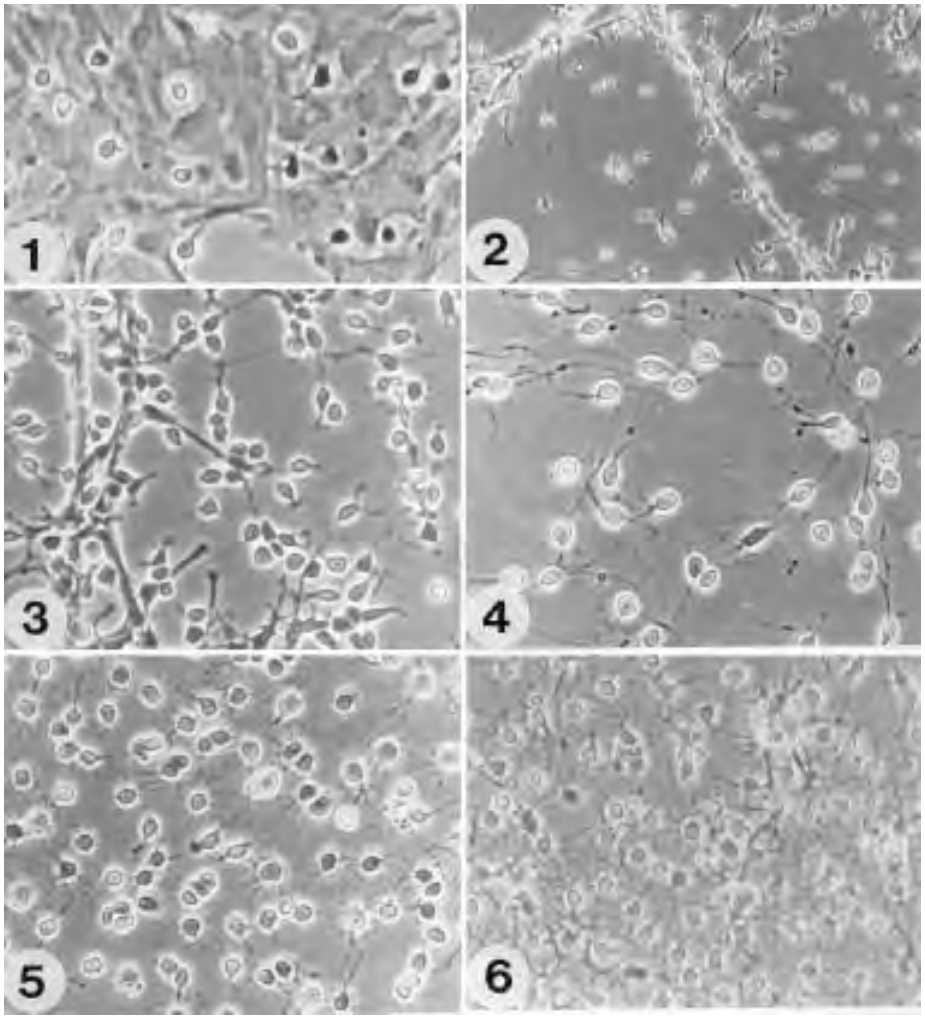

Plate. Phase-contrast micrographs (Figs. 1–6) of living glial cells in cultures derived from the newborn rat neopallium. The diameters of the white number-bearing disks represent 48 μm, except for Fig. 2, in which the diameter of the disk represents 96 μm.

Fig. 1. Five-day-old culture, grown using primary culture growth medium, demonstrating that the predominant cell populations are large, flat astrocytes and small process-bearing oligodendrocyte precursor cells.

Fig. 2. Ten-day-old primary culture grown for the last 5 d on OPM-G. The astroglial cells are detaching from the substratum and forming cable-like structures. From these cables, oligodendrocyte precursor cells migrate over the substratum.

Fig. 3. Twelve-day-old primary culture demonstrating that OPM-G causes proliferation of oligodendrocyte precursor cells.

Fig. 4. Secondary culture demonstrating oligodendrocyte precursor cells proliferating in OPM.

Fig. 5. Dense tertiary culture established from oligodendrocyte precursor cells. These cells have been placed on ODM for 2 d, and exhibit the typical multipolar morphology of oligodendroblasts, and express sulfatide.

Fig. 6. This is a sister culture to that in Fig. 5. These cells have been placed on ODM for the last 5 d, and exhibit the complex morphology of oligodendrocytes. Such cells express oligodendrocyte-specific antigens, such as galactocerebroside, myelin basic protein, and proteolipid protein.

2. Dilute cells to 4×10^6 cells/mL, using pyruvate-containing DMEM containing FBS, so that the final concentration of FBS is 10% (v/v).
3. Slowly add DMSO to the cell suspension, to a final DMSO concentration of 10% (v/v). Place the cell suspension into cryogenic vials.
4. Place vials into the Cryo 1°C freezing container, then into the –80°C freezer. Although the cells can be maintained for several months at –80°C, for long-term storage, it is best that the vials be transferred to liquid nitrogen in the cryobiological storage container.
5. To prepare cultures from frozen cells, thaw vials rapidly at 37°C, dilute the cell suspension with PO-PM, do a cell count, and plant cells in the usual way.

5. CULTURE DESCRIPTION

By 5 d after planting of cells into primary cultures, several distinct cell populations are evident when viewed with the phase-contrast microscope, including a larger flat-cell population and a smaller process-bearing cell population (Fig. 1). The flat cells contain glial fibrillary acidic protein, and are, thus, astrocytic in nature. The majority of the process-bearing cells express the GQ1c and GD3 gangliosides on their cell membranes (Fig. 2), and thus are oligodendroglial precursor cells. If these cultures were allowed to develop in PC-GM for an additional 5–10 d (e.g., Husain and Juurlink, 1995), they would be the same culture preparation as that described in Chapter 9.

Several changes soon become apparent, once the cultures are placed on OPM-G. The astrocytic cell population tends to become detached from the substratum and form long cable-like structures that are in intimate association with the oligodendroglial lineage of cells (Fig. 2). This is followed by proliferation of the oligodendroglial lineage of cells, with subsequent migration of these cells over the Petri dish surface. Subsequent to this, the astrocytic cell population tends to become detached and float into the medium, but there is rapid proliferation of the oligodendroglial lineage of cells (Fig. 3).

To obtain highly enriched cultures of oligodendroglial lineage cells requires harvesting and filtering out cellular aggregates, to end up with a single-cell suspension of cells. The cell aggregates are composed mainly of oligodendroglial precursors and astroglia. The majority of cells in the single-cell suspension are oligodendroglial precursor cells; however, there are a few astroglia and microglia present. These latter two cell populations require glucose in the OPM for their survival; thus, they can be selectively eliminated by leaving glucose out of the medium, and having lactate as the principal energy substrate source. Harvesting results in damage of some cells, resulting in the release of DNA from the injured cells. DNA is sticky and causes cells to aggregate; hence, it is important to have deoxyribonuclease (DNase) in the HM, to obtain as high a yield as possible of single cells following filtration.

After 1 wk, a typical moderate density secondary culture (Fig. 4) is minimally contaminated by astroglia, being comprised of $0.9 \pm 0.7\%$ glial fibrillary acidic protein-positive cells. The remaining cells are at early stages of differentiation in the oligodendroglial lineage; thus, $91.9 \pm 4.1\%$ of the cells express the GQ1c ganglioside, and $8.6 \pm 2.4\%$ of the cells express sulfatide, but no cells express galactocerebroside. Such secondary cultures can readily be subcultured, giving rise to tertiary, quartenary, and other cultures.

When cultures become more dense, an increasing proportion of the cells exhibit a multipolar morphology typical of the oligodendroblasts (Fig. 5); these cells express sulfatide. Mature oligodendrocytes can also be seen expressing galactocerebroside in such cultures. When these cultures are placed on the medium described by Gard and Pfeiffer (1995), more than 95% of the cells differentiate into oligodendrocytes (Fig. 6) and express galactocerebroside, myelin basic protein, and other oligodendrocyte-specific macromolecules.

Two features of this procedure facilitate obtaining large numbers of oligodendroglial cells. One feature takes advantage of factor(s), the number of which is unknown, present in medium conditioned by B104 neuroblastoma cells. They actively promote the proliferation of oligodendrocyte precursor cells, while interfering with the survival and/or attachment of astroglial cells. The second feature of the procedure, which results in obtaining cultures highly enriched in oligodendrocyte precursor cells, is the substitution of glucose with lactate. Although oligodendrocytes and astrocytes require glucose for survival, oligodendrocyte precursors can survive and proliferate, using lactate as their primary energy source. This suggests that the oligodendrocyte precursors are exclusively dependent on mitochondrial respiration for production of adenosine triphosphate. Metabolic studies are in agreement with this, demonstrating that oligodendrocyte precursors produce more CO_2 from lactate than do oligodendrocytes and astrocytes, and that anoxia does not increase glucose uptake in oligodendrocyte precursors; there is an increase in glucose uptake during anoxia in astrocytes and mature oligodendrocytes (B. H. J. Juurlink and L. Hertz, unpublished observations).

If there is no glucose in the medium, oligodendrocyte precursor cells are readily damaged during subculturing, resulting in low cell survival. This increased fragility is probably related to lack of proper glycosylation of membrane proteins and lipids; therefore, it is critical to feed cultures with glucose-containing medium 1 d prior to subculturing. Even if glucose is in the medium prior to harvesting the cells, we find much better survival of the cells if they are initially planted in a serum-containing medium (i.e., primary oligodendroglial planting medium). As soon as the cells are attached, they can be placed on OPM.

6. APPENDIX

6.1. Media

1. Stock Dulbecco's DMEM: We recommend Gibco-BRL for all media, because of problems with powdered media obtained from other sources. Purchase the glucose-free, sodium bicarbonate ($NaHCO_3$)-free, and glutamine-free form of DMEM (Gibco-BRL, cat. no. 23800-048), since this gives one greater flexibility in controlling medium composition. Prepare as recommended by the manufacturer.
2. Working DMEM: Add glutamine, to a final concentration of 2 m*M*. We buffer this medium with 14 m*M* $NaHCO_3$. In an atmosphere of 5% CO_2, this medium attains a pH of 7.2.
3. Primary culture growth medium: To 79 mL working DMEM, add 0.75 mL 1.0 *M* glucose stock solution and 20 mL horse serum (Hyclone, Logan, UT).
4. OPM-G: To 82 mL working DMEM, add, in the following order:

1.0 *M* Glucose stock solution	0.75 mL
Lactate stock solution	1.0 mL
Transferrin-biotin-selenium (TBS stock solution)	1.0 mL
B104 conditioned medium	15.0 mL
5 mg/mL Insulin stock solution	100.0 µL

5. OPM: To 83 mL working DMEM, add, in the following order:

Lactate stock solution	1.0 mL
TBS stock supplement solution	1.0 mL
B104 conditioned medium	15.0 mL
5 mg/mL Insulin stock solution	100.0 µL

6. PO-PM: To 93 mL working DMEM, add:

Glucose	0.75 mL
Pyruvate	1.0 mL

FBS	5.0 mL
5 mg/mL Insulin stock solution	100.0 µL

7. DMEM/F12 Medium (Gibco-BRL, cat. no. 12500-047): Prepare according to manufacturer's instructions. This medium is buffered with 15 m*M* HEPES and 14 m*M* $NaHCO_3$. In an atmosphere of 5% CO_2, this medium attains a pH of 7.2.
8. ODM: To 98 mL DMEM/F12, add 1.0 mL heat-inactivated FBS and 1.0 mL oligodendrocyte conversion stock supplements.
9. B104 neuroblastoma medium: To 99 mL DMEM/F12, add 1.0 mL transferrin–putresine–progesterone–selenium (TPPS) supplements and 100 µL 5 mg/mL insulin stock solution.
10. Medium supplements:
    a. Glucose (Sigma, St. Louis, MO, cat. no. G-7021): Dissolve glucose in triple-distilled water (dH_2O) to a final concentration of 1.0 *M*, filter-sterilize, and store stock solution at 4°C.
    b. Pyruvate (Sigma, cat. no. P-5280): Dissolve pyruvate in stock DMEM, and filter-sterilize. Pyruvate is unstable, particularly in the presence of oxygen; therefore, store pyruvate solution in aliquots at –20°C, and, when thawed, use within 1 wk.
    c. Glutamine (Sigma, cat. no. G-5763): Dissolve glutamine in triple-dH_2O to a final concentration of 200 m*M*, filter-sterilize, and store in aliquots at –20°C.
    d. Lactate (Sigma, cat. no. L-4338): Dissolve L(+)-lactate in stock DMEM, to a final concentration of 0.5 *M*, filter-sterilize, and store at 4°C.
    e. TBS solution:

Note: Any proteins (e.g., transferrin, insulin) added to serum-free medium will tend to adhere to glass, therefore, always store protein-containing solutions in plastic containers.

    i. Prepare a 100X stock solution containing 100 µg/mL transferrin (Sigma, cat. no. T-1283), 3 µ*M* Na selenite (Sigma, cat. no. S-5261), and 1 µg/mL D-biotin (i.e., 40 n*M*; Sigma, cat. no. B-4639) in stock DMEM.
    ii. Filter-sterilize, and aliquot in 5 mL vol, and store at –20°C. Once thawed, the stock solution is maintained at 4°C.

    f. TPPS solution:
    i. Prepare progesterone (Sigma, cat. no. P-6149) initially as a 2 m*M* stock in ethanol.
    ii. For preparation of 100 mL 100X TPPS stock solution, add the following to 99 mL stock DMEM:

Transferrin	100 µg/mL
Putrescine (Sigma, cat. no. P-5780)	10 m*M*
Na selenite	3 µ*M*
2 m*M* Progesterone (*see* above)	1.0 mL

    iii. Filter-sterilize, and store in appropriate (~5 mL) aliquots at –20°C. Once thawed, the stock solution is maintained at 4°C.

    g. Oligodendrocyte conversion supplements:
    i. Prepare a 1.5 m*M* stock of triiodothyronine (T_3) in alkaline stock DMEM. Add 100 µL stock T_3 to 100 mL 100X stock solution of oligodendrocyte conversion supplements.
    ii. To stock DMEM, add the following:

T_3 (Sigma, cat no. T-5516)	1.5 µ*M*
Transferrin	100.0 µg/mL
Na selenite	3.0 µ*M*)
D-Biotin	1.0 µ*M*

Na pyruvate	200.0 m*M*
Hydrocortisone hemisuccinate (Sigma cat. no. H-2270)	1.0 m*M*

iii. Filter-sterilize, and freeze in 1-mL aliquots.

h. Insulin (Sigma, cat. no. I-1882): Prepare insulin, as indicated, in an acidified fluid, to a final concentration of 10 mg/mL, and freeze in 0.5 mL aliquots. Thaw only once, and dilute in acidified Puck's fluid (phenol red having a yellow color), to final concentration of 5 mg/mL.

i. HEPES (Sigma, cat. no. H-9136) buffer, pH 7.2:
   i. Dissolve 23.83 g *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonate, Na salt in 50 mL triple-DW.
   ii. Adjust pH to 7.2 with 1*M* HCl. Make up to 100 mL, and filter-sterilize. Store in aliquots at 4°C, protected from the light.

6.2. Preparation of Solutions Used in Harvesting Cells

1. Tissue storage medium: To 9.35 mL stock DMEM, add 0.075 mL 1.0 *M* glucose stock solution, 0.5 mL horse serum, and 0.15 mL 1.0 *M* HEPES buffer, pH 7.2.
2. Puck's BSS, a calcium- and magnesium-free BSS:
   a. Stock solution, 10X Puck's BSS:

NaCl	80.0 g
KCl	4.0 g
$Na_2HPO_4 \cdot 7H_2O$	0.9 g
KH_2PO_4	0.6 g
Glucose	10.0 g

   Dissolve in 1000 mL triple-dH_2O, and filter-sterilize.

Note: 20 mL 1% aqueous phenol red solution may also be added, before making up to 1000 mL. Phenol red addition has the advantage that one has a visual indicator of solution pH.

   b. Working solution, 1X Puck's BSS. Dilute stock solution 1:10 with sterile triple-dH_2O.
3. Versene solution: Dissolve 0.2 g ethylenediamine tetraacetic acid, disodium salt (EDTA or versene) in 1000 mL Puck's BSS (i.e., 0.6 m*M*), filter-sterilize, and store at 4°C in 50-mL aliquots.
4. DNase stock solution: Dissolve 4 g DNase (Sigma, cat. no. DN-25) in 100 mL Puck's BSS (4% DNase stock solution), filter-sterilize, and store in 1-mL aliquots at –20°C.
5. Harvest medium: Mix 10 mL Puck's BSS with 10 mL versene solution. To this add:

1.0 *M* HEPES stock buffer solution, pH 7.2	0.2 mL
200 m*M* stock pyruvate solution	0.2 mL
5 mg/mL Insulin stock solution	20.0 µL
4% DNase stock solution	0.1 mL

   Note: Antibiotics are not necessary for successful culture, and are therefore not recommended; however, cultures are most easily contaminated when cells are harvested, therefore, one may wish to add antibiotics to the harvest medium.

6.3. B104 Conditioned Medium

1. Grow B104 neuroblastoma cells (Schubert et al., 1974) to confluency using DMEM/F12 containing 10% FBS (v/v) as growth medium. Convenient culture vessels are 100-mm tissue culture Petri dishes (Falcon, cat. no. 3003, VWR Scientific) or expanded surface roller bottles (Falcon, cat. no. 3079, obtained from VWR Scientific). The roller bottles have a

1500-cm^2 surface area available for cell growth; the 100-mm Petri dishes have a usable surface area of 75 cm^2. For the Petri dishes, use 8 mL medium, and, for the roller bottles, use 100 mL medium.

2. Once cultures are confluent, cultures are washed with Puck's BSS and fed with B104 neuroblastoma medium. To 100-mm Petri dishes, add 8 mL medium, and, to roller bottles, add 150 mL medium.
3. After 4 d, the B104 conditioned medium is removed, and cultures are fed with a fresh lot of B104 medium.
4. Preparation of conditioned medium:
   a. Phenylmethylsulfonyl fluoride (PMSF) (Sigma, cat. no. P-7626):
      i. Prepare a 1 mg/mL stock solution of PMSF in absolute ethanol.
      ii. Store in aliquots at –20°C.

      Note: Use caution in handling. because the compound is a potent protease inhibitor, and is highly toxic if ingested.
   b. PMSF stock solution is rapidly mixed into the B104 neuroblastoma conditioned medium, to a final concentration of 1 μg/mL.
   c. The B104 neuroblastoma-conditioned medium is centrifuged at 2000*g* for 30 min, filter-sterilized, and stored frozen in aliquots. Protein content of this B104 neuroblastoma-conditioned medium ranges from 3.5 to 4.5 mg/mL. For short-term storage, use -20°C, and, for long-term storage, use –80°C.

   Note: We routinely maintain B104 neuroblastoma cultures in 100-mm dishes, as a source of cells for the preparation of new B104 neuroblastoma cultures for the purposes of collection of conditioned medium. These maintenance cultures are harvested using harvest medium.

6.4. Preparation of Cell Filtration Apparati

1. Beakers: Nylon meshes of differing pore sizes can be obtained from L. and S. H. Thompson (Montreal, PQ, Can). Cut nylon mesh of 75-μm pore size into squares that fit over the mouths of 50-mL beakers. Use masking tape to fix the mesh in place. Enclose beaker in aluminum foil, and autoclave. These meshed beakers are used in the preparation of primary cultures.
2. Filtration tubes: The apparatus consists of a polypropylene tube, open at the top and covered by a nylon mesh at the bottom. Remove the bottom of a 50-mL polypropylene centrifuge tube (VWR Scientific, cat. no. 21008-667), using a hot knife. Remove the cap from the tube, and, using a hot cork borer, remove central disk of the cap. Cut nylon mesh into 4-cm squares, cover the top of the tube with the Nitex, fix nylon in place by screwing on the cap, place in an autoclave bag, and autoclave. The original top of the tube now becomes the bottom of the filtration device. Prepare filtration devices with nylon meshes of the following pore sizes: 50, 35, and 15 μm.

6.5. Preparation of Culture Substrata

1. Poly-D-lysine stock solution: Prepare a stock solution of 1 mg/mL poly-D-lysine (Sigma, cat. no. P-6407) in triple-dH_2O, filter-sterilize, and store in aliquots at –20°C.
2. Wash coverslips made of German glass (Fisher Scientific, Nepean, ON, Can, e.g., cat. no. 12-546) by soaking in acetone overnight, air-drying, and sterilizing by heating at 190°C for 3 h.
3. Poly-D-lysine-coated culture substrata.
   Note: We prepare these on the same day as used.

a. Petri dishes:
   i. Dilute stock poly-D-lysine in sterile triple-dH_2O to 20 µg/mL.
   ii. Place 1.5 mL of this solution in each 35-mm Petri dish, or 7 mL solution into each 100-mm Petri dish.
   iii. After 2 h, wash dishes with Puck's BSS.
b. Coverslips:
   i. Place coverslips into microbiological plastic Petri dishes. These dishes are convenient for preparing poly-D-lysine-coated coverslips, because they are hydrophobic, which tends to ensure that the aqueous solution stays on the coverslip, rather than spilling onto the surface of the Petri dish. Add poly-D-lysine to the surface of each coverslip.
   ii. After 2 h, wash coverslips with Puck's BSS, then plant cells directly onto the coverslips. After cells have attached, the coverslips can be placed into Petri dishes that are suitable for purposed experiments.

6.6. Dissection Material

1. Instrument cleaning and sterilization:
   a. After use, soak instruments in 7X™ detergent (ICN Biomedicals, Aurora, OH), and clean, using a nylon brush. Rinse well with hot water, followed by 70% ethanol. Allow to dry and store.
   b. Before use, sterilize instruments and insect pins, by placing in a plastic beaker containing 70% ethanol.
2. Dissecting dish: Pour a molten mixture of three parts regular blue dental inlay wax (Sybron, Romulus, MI) and one part paraffin (melting point 56°C) into a glass 60-mm Petri dish. The dish can be sterilized for use by dry heat at 121°C (250°F) for 2 h. The 60-mm dish is a convenient size, because one can manipulate the position of the dish, using the fifth digits, and at the same time manipulate the dissecting instruments, using the first and second digits. The wax is not brittle, thus ensuring that the wax surface remains smooth, despite repeated pinnings. After use, the wax dish can be washed using 7X detergent and a nylon brush. After rinsing in water, air-dry, and refinish the wax surface, melting the surface using the flame of a Bunsen burner. Once cooled, the dissection dishes can be sterilized by filling with 70% ethanol. The alcohol is removed prior to use, and the dish is allowed to air-dry in a laminar flow hood.

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Chapter Thirteen

Olfactory Ensheathing Cell Cultures

Ronald Doucette

1. INTRODUCTION

Over the past several years, neuroscientists have developed a considerable interest in a glial cell found only in the first cranial nerve. These glial cells, which are referred to as "olfactory ensheathing cells," provide ensheathment for the unmyelinated axons of the olfactory nerve (Doucette, 1984, 1986, 1993a; Raisman, 1985). Two major reasons why these cells have become so popular are their ability, first, to promote the long-distance growth of regenerating axons in the adult mammalian central nervous system (CNS) (Li et al., 1997; Perez-Bouza et al., 1998; Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto et al., 1998; Smale et al., 1996), and second, to remyelinate spinal cord axons in adult rats (Franklin et al., 1996; Imaizumi et al., 1998). For an overview of the cell biology of these glial cells, the reader is referred to several reviews on the subject (Doucette, 1990, 1995; Franklin and Barnett, 1997; Ramon-Cueto and Avila, 1998; Ramon-Cueto and Valverde, 1995). The present chapter chiefly addresses those cell biological aspects that are pertinent to obtaining purified cell cultures of ensheathing cells from the fetal rat olfactory bulb.

Cell cultures of olfactory ensheathing cells have been successfully initiated from the olfactory bulbs of fetal (E18) (Devon and Doucette, 1992, 1995; Doucette, 1993b; Doucette and Devon, 1994, 1995), newborn (Chuah and Au, 1993, 1994; Franceschini and Barnett, 1996), and adult rats (Gudinocabrera and Nieto-Sampedro, 1996; Ramon-Cueto and Nieto-Sampedro, 1992; Ramon-Cueto et al., 1993). The cells in these diverse sets of cultures share, in general, a common phenotype, and have all been shown to support the regeneration of axons in the adult mammalian CNS (Li et al., 1998; Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto et al., 1998; Smale et al., 1996). The cell culture technique described in this chapter was used to obtain fetal olfactory ensheathing cells, which were used to provide the first evidence that these cells possessed the ability to assemble a myelin sheath around appropriately sized axons (Devon and Doucette, 1992, 1995). The in vivo remyelinating ability of these cells was demonstrated using cells obtained from newborn rats (Franklin et al., 1996; Imaizumi et al., 1998).

This chapter describes the technique, in use in my laboratory for more than 10 yr, for initiating cell cultures of ensheathing cells from the olfactory bulbs of fetal rats and mice. Those individuals interested in using newborn or adult tissues for setting up their ensheathing cell cultures are referred to the references in the previous paragraph, or can direct their browser to the Ensheathing Cell Homepage at http://duke.usask.ca/~rondouc/.

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2. PREPARATION OF RAT FETUSES

For this dissection, it is important to have access to timed pregnancies. The dissections are easiest to do using Theiler stage 23 fetuses (Theiler, 1972), which approximately corresponds to an E18 rat fetus (the day after mating is designated as E1). Developmental staging (Butler and Juurlink, 1987) of the animals, prior to use, ensures that all ensheathing cells are obtained from fetuses of a similar stage of development. It also ensures a cleaner and easier dissection, because it is more difficult to cleanly dissect the nerve fiber layer (NFL) of the olfactory bulb at both younger and older developmental stages.

2.1. Materials

Pregnant rat (E18; d after mating designated as E1).
Forceps (1), tissue forceps, 11 cm, 1 × 2 teeth.
Forceps (2), Dumont, AA.
Forceps (2), Dumont, 5A (off center).
Scalpel (1), with no. 11 blade.
Scissors (2), $4^1/_2$ in.
Scissors (1), $5^1/_2$ in.
Pins (4).
Dulbecco's minimum essential medium (DMEM) (Sigma, cat. no. D-5648)/Hams nutrient mixture F-12 (F12) (Sigma, cat. no. N-6760) containing 10% fetal bovine serum (FBS) (Summit Biotechnology, cat. no. FP-100-05), sterile.
Hank's balanced salt solution (HBSS), sterile.
Petri dishes (1), 60-mm tissue culture (Falcon, cat. no. 353004).
Petri dishes (4), 100-mm glass.
Gauze sponges, 5 × 5 cm.
Styrofoam.
Tinfoil.
95% alcohol.
Dissecting microscope.
Steri 350 sterilizer (Inotech Biosystems, Lansing, MI).

2.2. Preparation for Dissection

1. Sterilization of materials and instruments:
   a. Sterilize tissue forceps, Dumont forceps (AA), and the three pairs of scissors, by immersion in 95% alcohol.
   b. Autoclave glass Petri dishes.
   c. Disposable Petri dishes are purchased sterile.
   d. Using the Steri 350 sterilizer, sterilize the Dumont forceps (5A) and scalpel.
2. Prepare a dissecting board by covering a piece of styrofoam with aluminum foil. Other material, which can support pinning, may also be used for the board.
3. Add sterile HBSS to one 100-mm glass Petri dish. Add DMEM/F12-10% FBS to 60-mm Petri dish.

2.3. Dissection of Uterine Horns

1. Euthanize the pregnant rat by overdose of approved anesthetic. Place rat ventral side up, and immobilize by pinning limbs.

2. Sterilization of abdominal wall:
   a. Moisten a gauze sponge with 95% alcohol, and swab the abdominal wall.
   b. Always wipe from the rib cage down, toward the pubic area, to avoid bringing contamination onto the abdominal wall from the genital area.
3. Dissection of skin and subcutaneous tissue:
   a. Using tissue forceps (11 cm), pinch a small fold of skin in the midline at the lower portion of the abdominal wall.
   b. Using the $5^1/_2$ in. scissors, make an incision and proceed to cut a flap of skin and subcutaneous tissue by proceeding up the right and left sides of the abdominal wall.
   c. Leave the flap of tissue attached at its upper end, and flip it over (i.e., subcutaneous tissue out) on the surface of the chest.
4. Opening the abdominal wall:
   a. Moisten a gauze sponge with 95% alcohol, and swab the superficial surface of the abdominal muscles (as in step 2), to remove any stray hairs.
   b. Using a pair of Dumont forceps (AA), pinch a small fold of muscle in the midline at the lower portion of the abdominal wall.
   c. Using a pair of $4^1/_2$ in. scissors, make an incision, and proceed to cut open the abdominal wall, thus exposing the contents of the peritoneal cavity, by proceeding up the right and left sides of the abdominal wall.
   d. Leave the flap of muscle attached at its upper end, and flip it over (i.e., serous membrane out) on the surface of the chest.
5. Removal of uterine horns:
   a. Using a new pair of Dumont forceps (AA) and $4^1/_2$ in. scissors, grab hold of the uterus in the midline, and cut it free of the underlying cervix. Cut laterally (both the right and the left sides) to free the uterine horns from their attachments.
   b. Transfer the uterine horns to a 100-mm glass Petri dish containing sterile HBSS.
   c. Cut each uterine horn into several pieces, by cutting between each swollen portion, each of which contains a single fetus.
   d. Place the cover on the glass Petri dish and set it beside the dissecting microscope.
6. Removal of fetuses from uterine horns:
   a. Using sterile Dumont forceps (5A), transfer each piece of uterine horn into a new 100-mm glass Petri dish containing sterile HBSS.
   b. Using a pair of sterile Dumont forceps (5A), dissect each fetus from the uterine horns, determine the developmental stage, and decapitate (using the scalpel with no. 11 blade).
   c. The remaining two sterile 100-mm glass Petri dishes may be used to store the Dumont forceps or scalpel during this part of the dissection.
   d. Transfer each head to a 60-mm Petri dish containing sterile DMEM/F12/10%–FBS. Place the cover on the dish after all the heads have been transferred. Leave it sitting beside the dissecting microscope as the next part of the dissection is prepared.

3. DISSECTION OF OLFACTORY BULBS

3.1. Materials

Forceps (1), Dumont, 5A (off center).
Scissors (1), angled spring.
Scalpel (1) with no. 11 blade.
DMEM/F12–10% FBS.
Petri dish (1), 60-mm.

Dissecting microscope.
Steri 350 sterilizer (Inotech Biosystems).

3.2. Preparation for Dissection

1. Sterilize the Dumont forceps (5A), scalpel and spring scissors, using the Steri 350 sterilizer.
2. Add DMEM/F12–10% FBS to 60-mm Petri dish.

3.3. Dissection of Olfactory Bulbs

1. Transfer one head at a time to a 60-mm Petri dish containing sterile DMEM/F12–10% FBS, and place the dish under a dissecting microscope.
2. Stand the head upright, and stick the prongs of a pair of Dumont Forceps (5A) into the tissue lying to either side of the nose; these forceps will keep the head from moving during the subsequent dissection.
3. Using the angled spring scissors, cut through the skin, subcutaneous tissue, and fibrous connective tissue of the cranium, in the median plane, beginning posteriorly at the level of the presumptive cerebellum, and continuing as far forward as the presumptive nasal bones of the skull.
4. Move the prongs of the Dumont Forceps, so that they now lie to either side of the presumptive cerebellum.
5. Using the scalpel, proceed to separate the head into right and left halves, by cutting in the sagittal plane.
6. Carefully dissect the olfactory bulbs out of each half of the head (Fig. 1).
   a. To facilitate the removal of an intact olfactory bulb, first cut the olfactory nerves along the entire ventral surface of the bulb, using a pair of spring scissors. This is the area of maximal attachment of the NFL to the floor of the cranial cavity, and is one of the major impediments to its easy removal.

   Note: The NFL is only loosely adherent to the surface of the olfactory bulb at this developmental stage. This situation both facilitates its easy removal from the olfactory bulb and increases the risk of it being left behind in the skull when the olfactory bulb is dissected out of its position in the anterior cranial fossa.

   b. Using the angled scissors, next cut the olfactory peduncle, and let the olfactory bulb fall out of the skull into the growth medium.
   c. Discard the rest of the head, leaving only the two olfactory bulbs behind in the 60-mm dish.
   d. Repeat for each fetus.

4. ISOLATION AND PLATING OF ENSHEATHING CELLS

4.1. Materials

Forceps (2), Dumont, 5A.
Flasks, 25-cm^2 (Falcon, cat. no. 353108).
Petri dish (1), 35-mm tissue culture (Falcon, cat. no 353001) containing DMEM/F12–10% FBS.
Chamber slides (Nunc, cat. no. 177402).
Glass coverslips in 35-mm tissue culture Petri dishes.
Tube, conical, 50-mL, sterile (Falcon, cat. no. 2070).
Pasteur pipet (1), cotton-plugged with angled tip.
Beaker (1), 50-mL, covered with 75-μm nylon mesh.

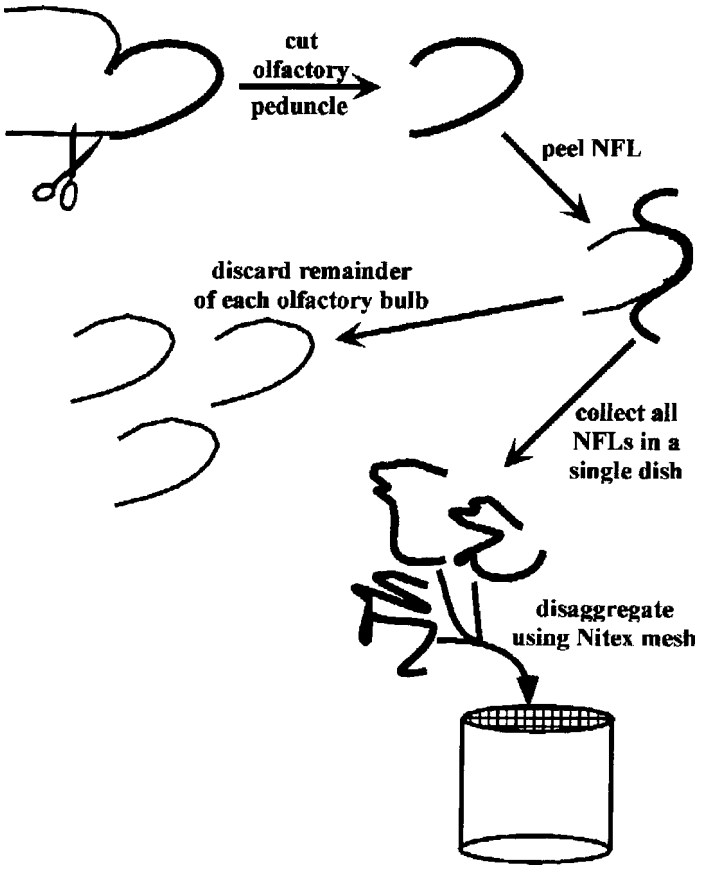

Fig. 1. A drawing depicting the setting up of nerve fiber layer (NFL) cell cultures from the olfactory bulbs of Theiler stage 23 rat/mouse embryos. After cutting the olfactory peduncle, the NFL, which is the outermost layer of the olfactory bulb, is peeled away from the deeper layers. The tissue of the NFL is then mechanically disaggregated and plated inside dishes or flasks.

4.2. Preparation for Dissection

1. Sterilize forceps, using the Steri 350 sterilizer.
2. Autoclave Pasteur pipet, glass coverslips, and the 50-mL beaker with nylon mesh.
3. Disposable Petri dishes, conical tubes, flasks, and chamber slides are purchased sterile.

4.3. Isolation of Ensheathing Cells

1. Peel the NFL off each olfactory bulb (*see* Fig. 2).
   a. The prongs of one pair of forceps are placed inside the cut end of the bulb, where the bulb had previously been attached to the olfactory peduncle.
   b. The other pair of forceps are used to grasp the free edge of the NFL along its posterior edge.

   Note: Posteriorly, the NFL has often started to lift away from the underlying bulb, thus simplifying this part of the dissection.

   c. The NFL is then gently peeled off the bulb, a procedure that normally removes this layer from the entire circumference of the bulb, with the exception of an occasional small tag

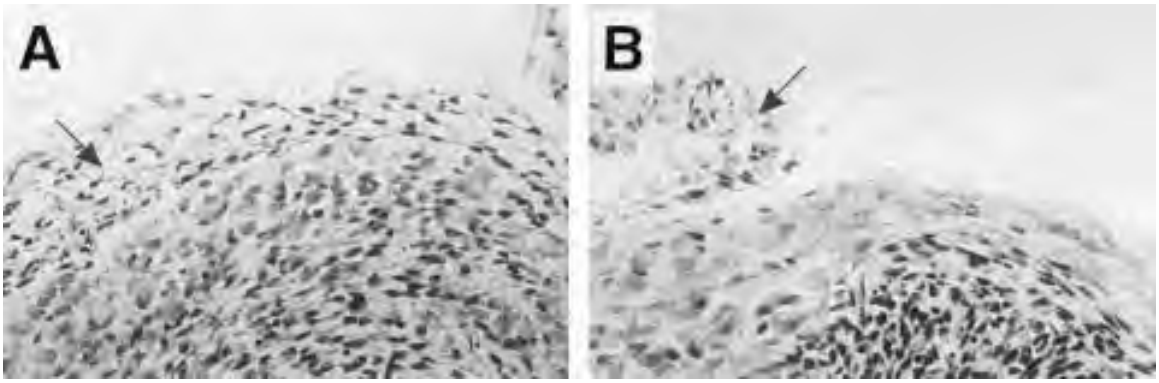

Fig. 2. Sagittal sections through the olfactory bulbs of Theiler stage 23 mouse embryos. The rostral pole of each bulb points toward the top of each micrograph. **(A)** Section through a whole olfactory bulb. The nerve fiber layer lies external to the marginal zone of the bulb (arrow). **(B)** An olfactory bulb in which the nerve fiber layer (arrow) had been partially peeled away from the underlying marginal zone. The arrow points to the tissue that is used to initiate ensheathing cell cultures.

of tissue that may be left behind. The NFL should easily peel off the underlying bulb, much like peeling the skin off an onion.

d. Transfer each NFL to a 35-mm Petri dish containing DMEM/F12–10% FBS.

2. The dissected NFLs, obtained from all of the olfactory bulbs of a single litter of rat fetuses, are mechanically disaggregated by gently forcing the tissues in medium through a Nitex mesh (75-μm pore size), using a cotton-plugged Pasteur pipet.
3. The disaggregated NFLs, obtained from 20–24 rat olfactory bulbs, are diluted to a final volume of 40 mL, in a 50-mL conical tube. If fewer NFLs are obtained, either because there are fewer fetuses or because of loss of NFLs during the dissection, then adjust the final volume appropriately.

4.4. Plating the Ensheathing Cells

1. Substrata:
   a. Bulk isolation:
      When the purpose of the cell culture is to obtain sufficient numbers of cells for use in a secondary cell culture, for grafting purposes, or for molecular analyses, then the primary cell cultures should be set up in plastic tissue culture flasks. We routinely use 25-cm^2 flasks, in each of which 5 mL cell suspension is plated at the time of the initial dissection. The growing surface of these flasks is not coated with any substrate, thus not facilitating fibroblast attachment or proliferation. The cell cultures are fed with mG5 and maintenance media, as described below.
   b. Immunohistochemical studies:
      For some experiments, immunohistochemical (IHC) techniques are used to examine the expression of specific molecules by ensheathing cells, or *in situ* hybridization techniques are used to study gene expression. For these experiments, the NFL cell suspension should be plated onto glass coverslips (inside 35- or 60-mm plastic Petri dishes) or into the wells of chamber slides. The glass surfaces of the coverslips or the chamber slides may be coated with a substrate to make them more adhesive.

Note: The reader should be aware that very little is known regarding how the substrate influences gene or protein expression in these cells.

c. Electron microscopy: For experiments requiring the embedding of ensheathing cells in a resin suitable for cutting on an ultramicrotome, it is advisable to grow the cells directly on the plastic of a Petri dish. To study the myelinating ability of ensheathing cells in dorsal root ganglion–glial cell co-cultures, the bottom of the dish should be precoated with collagen type I. For the general procedure involved in the preparation and processing of ensheathing cell cultures for electron microscopy, the reader is referred to the papers of Devon and Doucette (1992, 1995).

2. Plating the ensheathing cells: Following disaggregation of the cells from the tissue of the NFL, the cells are plated at a very low density in plating medium consisting of DMEM/F12 (1:1) containing 10% FBS. The disaggregated cell suspension obtained from the olfactory bulbs of one litter of rat fetuses (typically 10–12 fetuses) is diluted with plating medium to a final volume of 40 mL. For smaller litters, the final volume should be decreased in proportion to the lower number of fetuses. The olfactory ensheathing cells of mouse fetuses (8–10/litter on average) are not as viable in cell culture as those of the rat, and therefore cannot be plated at such a low cell dilution. For mouse cultures, we routinely dilute the cell suspension obtained from each litter (~20 NFLs) to a final volume of no more than 6–10 mL plating medium.

5. FEEDING CELL CULTURES

5.1. Serum-Free Medium (mG5)

At the first feed, usually after 3–4 d in vitro, the growth medium is changed to a serum-free medium. Removal of serum ensures that any meningeal fibroblasts finding their way into the sparse cell cultures will not grow very well. On the other hand, with the serum-free medium in use in this lab for the past several years, the ensheathing cells continue to proliferate (albeit slowly). The medium used is a modification of the G5 medium (mG5) of Bottenstein (1985). Since we found that neither epidermal growth factor nor fibroblast growth factor were mitogens for fetal olfactory ensheathing cells, Bottenstein's mG5 was modified by deleting these two growth factors from the recipe. The mG5 contains DMEM as the basal medium, to which is added selenium, 30 n*M*; transferrin, 50 µg/mL (Sigma, cat. no. T-1428); insulin, 5 µg/mL (Sigma, cat. no. I-1882); hydrocortisone, 10 n*M* (Sigma, cat. no. H-0135); and biotin, 0.04 µ*M* (Boehringer Mannheim, cat. no. 732-516).

5.2. Maintenance Medium

After 7–10 d in mG5, the cells in these cultures are ~98% S100-positive. They are then ready for more rapid expansion, using a serum-containing medium. The medium routinely used in this laboratory is DMEM/F12 (1:1)–10% FBS. It usually takes at least another 2–3 wk for the cell cultures to reach confluency, when grown inside 25 cm^2 flasks.

6. PHENOTYPE OF OLFACTORY ENSHEATHING CELLS IN VITRO

6.1. Morphology

In the serum-containing plating medium, many ensheathing cells possess a flat, phase-dark morphology. However, numerous bipolar and tripolar cells may also be seen in ensheathing cell cultures grown in this medium. After withdrawal of serum, e.g., in cell cultures fed with mG5 for 7–

10 d, bipolar, tripolar, and multipolar (stellate) cells make up the predominant morphologies assumed by the ensheathing cells. When the cell cultures are fed once again with serum-containing medium (i.e., the maintenance medium), the cells once again assume a more flattened morphology.

6.2. Immunohistochemistry

The best immunohistochemical marker for ensheathing cells in vitro is an antibody to S100β. If the cell cultures are prepared as described above, then >95% of the cells should be S100-positive. Ensheathing cells also express other phenotypic markers, such as the p75 neurotrophin receptor and intermediate filament proteins (such as glial fibrillary protein and nestin), and can be stained with the O4 monoclonal antibody. However, these immunohistochemical markers only stain a subpopulation of ensheathing cells, and cannot be used to check the purity of the cell cultures. For a complete listing of the immunohistochemical phenotype of ensheathing cells, the reader is referred to several recent reviews (Doucette, 1993b; Ramon-Cueto and Avila, 1998; Ramon-Cueto and Valverde, 1995).

7. APPENDIX

7.1. Notes on Different Techniques for Obtaining Ensheathing Cells

With the cell culture technique described in this chapter, the investigator can reliably obtain a sufficient number of cells in primary culture to address a wide array of questions. The technique is not selective for any particular subpopulation of ensheathing cells, which are known to express a heterogeneous phenotype in vivo (Ramon-Cueto and Avila, 1998), as well as in vitro (Ramon-Cueto and Nieto-Sampedro, 1992). At the present time, it is not known whether some of the phenotypic differences observed in ensheathing cell cultures initiated by the several different protocols may be caused by the manner in which the cell cultures are initiated and the unwanted cells eliminated from the culture.

Ensheathing cell cultures have also been initiated from the olfactory bulbs of newborn rats. This technique, which was developed by Barnett (Franceschini and Barnett, 1996), uses a fluorescence-activated cell sorter (FACS) and the O4 monoclonal antibody (Sommer and Schachner, 1981) to pull the ensheathing cells out of the dissected tissue. The NFL cannot be cleanly dissected from the newborn rodent olfactory bulb, thus necessitating an additional purification step (i.e., FACS) in the protocol. In the process, any ensheathing cell not expressing the O4 epitope consequently will not be included in the cell suspension that is used to initiate the cell culture. In the fetal cell cultures we use, only about one-half of the cells can be stained with the O4 antibody (unpublished observations). It is not known what percentage of ensheathing cells in the newborn NFL in vivo express the O4 epitope. Nevertheless, the work of Barnett et al. has significantly advanced understanding of the cell biology of ensheathing cells, particularly in demonstrating that they can remyelinate CNS axons in vivo (Franklin et al., 1996).

The work of Ramon-Cueto et al. (Ramon-Cueto and Avila, 1998; Ramon-Cueto and Nieto-Sampedro, 1992; Ramon-Cueto et al., 1993; Ramon-Cueto and Valverde, 1995) has also contributed to a better understanding of the cell biology of ensheathing cells, and, in addition, has also demonstrated that cells from adult animals are just as effective as those from younger sources in supporting the growth of CNS axons (Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto et al., 1998). Their cell culture protocol requires the investigator to dissect the NFL off the olfactory bulb, an even harder job to do cleanly than when using the neonatal bulb. The cell cultures then undergo multiple passages to eliminate unwanted cells, so that in the end the experiments must be done on at least a tertiary cell culture. In the process, the procedure may well select those cells that best respond to the combination of adhesive substrates and growth factor supplements used to purify the cell population comprising the cell culture.

7.2. Notes on Determining Purity of Cell Cultures

As mentioned in Section 6.2, the calcium-binding protein, S100β, is the best immunohistochemical marker for identifying ensheathing cells in vitro. The expression of other markers, such as glial fibrillary acidic protein, p75, or the O4 epitope, is influenced by cell culture conditions. Therefore, variable numbers of ensheathing cells will express these markers, depending on the substratum, the growth medium, and whether other cells (e.g., dorsal root ganglion neurons) are included in the cell culture.

A time-consuming, though effective, method used in my laboratory is to count the number of S100-positive cells growing on coverslips, and to express the number as a percentage of all cells counted. We typically count all the cells (both S100-positive and -negative) in every third field, at approx 400× magnification, with a minimum of 200 cells being counted per coverslip. These counts are usually done after the cells have been in mG5 medium for at least 5–7 d. By this time, 95–98% of the cells are S100-positive.

7.3. The Theiler Staging System

Theiler's staging system (Theiler, 1972) was developed on the basis of a combination of external and internal morphological features. Usage of this staging system, or that of Carnegie (Butler and Juurlink, 1987), is more accurate than gestational age alone in ensuring uniformity of embryo and fetal age. The following are the main external morphological features that are present on a Theiler stage 23 rat/mouse fetus. For comparison purposes, a description of Theiler stage 22 and 24 fetuses is also provided.

1. Theiler stage 22: These embryos are characterized by almost complete separation of the fingers of the forelimb footplate, and by initial separation of the toes of the developing hindlimb. Their skin has numerous hair follicles, except for that of the head region. The growing pinna is turned forward and covers about one-half of the external auditory meatus. The umbilical hernia is conspicuous. It is possible to identify early and late Theiler stage 22 embryos by noting the degree to which the hindlimb footplate has developed. Separation of the distal phalanges of this footplate only occurs near the end of this developmental stage.
2. Theiler stage 23: Characteristic features of this developmental stage are complete separation of the digits in both limbs, distinct flexures at the elbow and knee, and the presence of well-defined eyelids that are about to close. Although the digits are separated, they are still divergent, and will not become parallel until much later in development. The pinna covers more than half of the external auditory meatus, and hair follicles can be seen all over the body.
3. Theiler stage 24: By this stage, the eyelids have usually fused, and the pinna has almost completely covered the external auditory meatus. The umbilical hernia is disappearing, and the skin is becoming wrinkled. The fingers of the forelimb are becoming more parallel, while the toes of the hindlimb are still divergent.

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Chapter Fourteen

Cultures of Stem Cells of the Central Nervous System

Angela Gritti, Rossella Galli, and Angelo Luigi Vescovi

1. INTRODUCTION

Over the past few years, the view that little or no cell turnover or replacement takes place within the adult central nervous system (CNS) has changed dramatically. The adult brain of both rodents and primates has been shown to embody undifferentiated, mitotically active precursor cells that are multipotential in nature and may contribute new, differentiated neurons and glia to specific regions of the mature brain, such as the olfactory bulb (Hinds, 1968a,b; Bayer, 1983; Corotto et al., 1993; Lois and Alvarez-Buylla, 1994), the hippocampus (Altman and Das, 1965; Kaplan and Bell, 1984; Kuhn et al., 1996) and the cortex (Kaplan, 1981; Huang and Lim, 1990; Gould, et al., 1999). Although this clearly suggests the presence of stem cells in the adult CNS in vivo, testing the proliferation, self-renewal, and differentiation capacity of "putative" CNS stem cells relies on the development of methodologies that allow for their extensive propagation and expansion in vitro.

Stem cells, or staminal cells, are undifferentiated elements that function as a reservoir of undifferentiated tissue precursors. They play an essential homeostatic role by replacing differentiated tissue cells worn off by physiological turnover or lost to injury or disease. In the absence of any identifying antigenic markers, some specific functional attributes provide the only basis for a reliable identification of stem cells, also in the CNS. The most widely accepted definition, as provided in the seminal review by Loeffler and Potten (1997), identifies stem cells as undifferentiated cells that lack markers of differentiated tissue-specific cells, are capable of proliferation, and, more importantly, that possess self-renewal capacity, are able to generate functional differentiated progeny, and are able to regenerate the tissue after injury. A certain degree of flexibility in the use of these options is also considered a stem cell property.

It is impossible to assess simultaneously all the functional characteristics listed in the stem cell definition given above. Thus, some terms are given greater weight in identifying a candidate stem cell: self-renewal, or the capacity to generate a wide array of differentiated progeny, or the ability to regenerate a tissue may be accepted, even alone, to identify a stem cell (Morrison et al., 1997). To clearly point out the difference between stem cells and transiently dividing progenitors that can also be found in the brain, the definition of CNS stem cells is herein applied to neural precursors

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that have been shown to self-renew extensively, and can be propagated for months, displaying a steady capacity to generate neurons, astrocytes, and oligodendrocytes (i.e., are multipotential), as determined by a clonogenic assay. The term "progenitor: cells is used to indicate undifferentiated cells possessing limited proliferative capacity and more restricted developmental potential.

What follows is a detailed discussion of some methods developed and/or improved by the authors' group for the isolation of neural stem cells, their extensive culturing, the assessment of their full developmental potential (the ability to differentiate into the three major CNS cell types), and the essential issue of their clonogenic capacity (*see* below). Because embryonic and adult stem cells display different growth requirements, and because rodent and human stem cells possess overtly different functional characteristics, this chapter has been divided into specific subheadings**.**

The appropriate implementation of the procedures described here also allows the establishment of a renewable source of undifferentiated CNS precursors, which may be maintained as such, or differentiated by altering the culture conditions. A simple model for studying the epigenetic cues that control the genesis of mature neurons and glia from undifferentiated CNS precursors, under fully defined conditions, is therefore available. The use of stem cells isolated from transgenic or null mutant mice also allows the study of the influence of specific genes in neural development, as well as in neuronal survival and differentiation. Finally, this technique is particularly important for human studies, since neural stem cells from human embryos deriving from spontaneous abortion, also carrying specific genetic defects, can provide an ideal alternative to the use of human tissue. For example, as little as 10^4–10^5 human embryonic cells can be expanded exponentially for up to 3 yr, generating over 10^{30} cells. These can be used for both in vitro and transplantation experiments, without using any further primary human tissue throughout this period of time.

2. BASIC CONCEPTS IN CULTURING NEURAL STEM CELLS

Despite the significant number of recent publications in this area, the technical subtleties and pitfalls related to the culturing of neural stem cells have not been discussed in detail. Although neural stem cells have been isolated by means of oncogene-mediated immortalization (Vescovi and Snyder, 1999) or in the presence of serum (Gage, et al., 1995), the methodology described in this chapter refers to the isolation, culturing, and long-term expansion of neural stem cells under fully chemically defined, serum-free conditions. As such, this kind of culture possesses some peculiar characteristics that need to be discussed, to avoid misinterpretation of the results obtained with this system.

The most important concept is that serum-free CNS stem cell cultures represent a selective system in which most primary differentiated CNS cells are eliminated soon after having been put in culture, while, conversely, the undifferentiated stem cells enter into an active proliferation state. Four conditions should be satisfied for the neural stem cells to become the main cell type in these cultures: low cell density (approx 5×10^4 cells/cm^2); absence of serum; addition of the appropriate growth factors, i.e., epidermal growth factor (EGF) and/or fibroblast growth factor (FGF-2); and absence of a strong cell adhesion substrate (poly-L-lysine or polyornithine may be used). Under these conditions, cells from a freshly dissociated brain attach loosely to the substrate, and most of them die in 2–3 d. During this time, a very small fraction of undifferentiated precursors become hypertrophic, round up, and begin to proliferate, while remaining attached to the plate. The progeny of the proliferating cells do not migrate away, but preferentially adhere to each other and form spherical clusters. These clusters, because of their increasing mass, eventually lift off the substrate, float in suspension, and have been called neurospheres. It is important to understand that not all the progeny that are found in a sphere is represented by stem cells. Indeed, only a fraction of this progeny, ranging from 10 to 50% of the total cells generated, retain stem cell features; the remain-

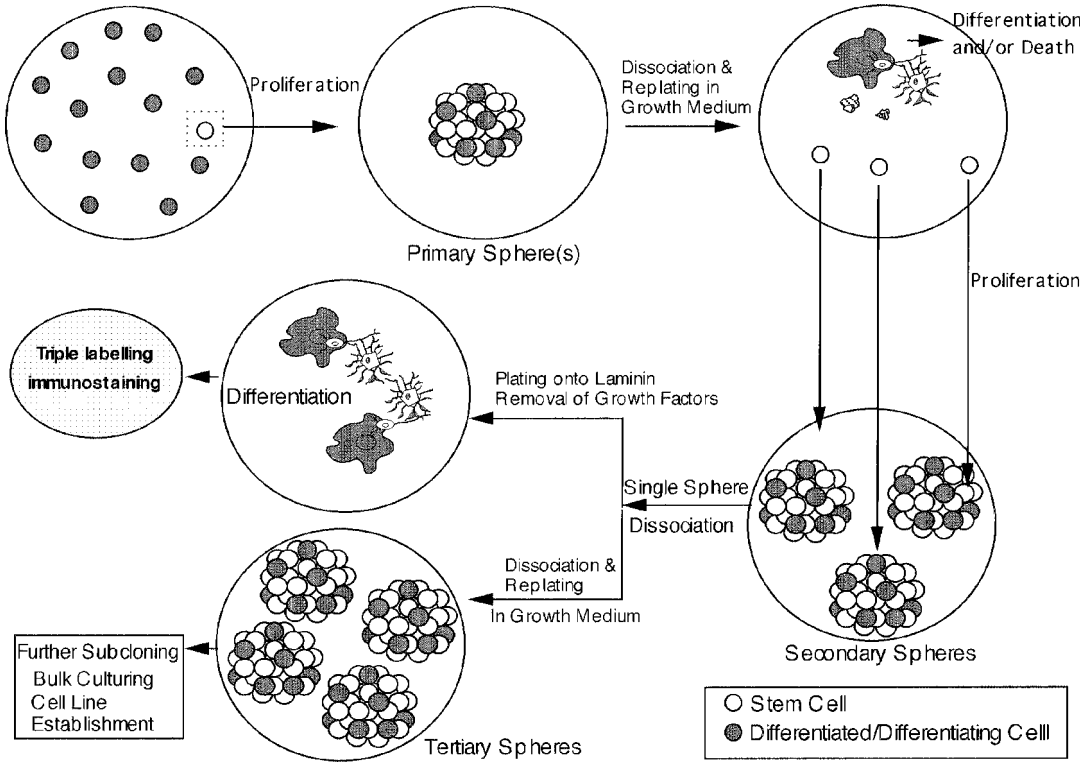

Fig. 1. A schematic diagram, illustrating the general procedure for culturing and expanding CNS stem cells under serum-free conditions, as described throughout this chapter.

der of the cells undergoes spontaneous differentiation. In fact, a neurosphere is a mixture of stem cells, differentiating progenitors, and even terminally differentiated neurons and glia. This is the reason why neurospheres are subcultured by harvesting, mechanically dissociated, and replated under the same growth conditions. As in the primary culture, differentiating/differentiated cells rapidly die, but the neural stem cells continue to proliferate, giving rise to secondary spheres that can then be further subcultured. This procedure can be sequentially repeated several times. Because each stem cell gives rise to many stem cells by the time a sphere is formed, the result is an exponential increase in the number of stem cells in the culture. Figure 1 schematically illustrates this phenomenon.

This underlies a counterintuitive notion that has seldom been spelled out, i.e., that, in a good neural stem cell culture system, many of the cells generated by the stem cells selectively die. One of the most common mistakes made when culturing neural stem cells in this serum-free system is the use of excessively high cell densities, particularly in primary cultures. Under such conditions, cells of different types, including neurons, glial cells, and differentiating progenitors, rapidly aggregate to form a neurosphere-like cluster, even in the absence of significant cell proliferation. The interpretation that the formation of these spheres indicates the presence of stem cell proliferation in the culture is, at best, far-fetched. At these high cell densities, and with tightly clustered cells, many lineage-restricted progenitors, such as specific neuronal precursors, can survive for a significant time in culture, and, if subculturing is carried out under the same conditions, they can survive for the first few passages, being eliminated only gradually. This often leads to the mistaken

interpretation that certain neural stem cells lose the capacity to generate specific neurotransmitter phenotypes over serial subculturing, but it is in fact a contaminating cell population with limited proliferation capacity that is being negatively selected. As explained below, it is recommended that this selection take place at the stage of primary culture or after the earliest passages.

3. RODENT AND HUMAN EMBRYONIC CULTURES

Embryonic neural stem cells have been isolated from different CNS regions, i.e., striatum, cortex, spinal cord (SC), thalamus, ventral mesencephalon, of both human (Vescovi et al., 1999) and rodent embryos (Davis and Temple 1994; Kilpatrick and Bartlett 1995; Reynolds and Weiss 1996; Qian et al., 1997) at different stages of development. The general protocol reported here can be used to isolate and expand embryonic neural stem cells from both species, beginning with the tenth embryonic day or with the sixth week postconception, in mouse or human, respectively. According to this protocol, dissociation of embryonic tissues does not normally require an enzymatic predigestion. It should be emphasized that some differences, in terms of growth characteristics, do exist between human and rodent cells, and even between mouse and rat embryonic neural stem cells. The reader should refer to Section 3.5 for a detailed discussion of this issue.

3.1. Collection of Embryonic Tissue(s)

Rodents, usually mice, are mated overnight, separated the next morning (E0), and checked for the presence of a gestational plug. Pregnant animals can also be purchased at the desired gestational age, from specialized animal care facilities. Sacrifice of pregnant animals and removal of embryos are performed outside the laminar flow hood. Particular caution should be exercised during these steps to avoid contamination of tissue(s).

Human tissue is collected in complete, ice-cold growth medium containing 5 μg/mL gentamicin (Gibco-BRL, cat. no. 15750-037) and 0.2 mg/mL kynurenic acid (Sigma, cat. no. K-3375). The brain tissue should be separated from the remainder of the fetal material as soon as possible, yet in accordance with bylaws and rules dictated by the local ethics committees.

3.2. Dissection Procedure

1. Materials:
   Scissors (1), large.
   Scissors (2), small, pointed.
   Scissors (1), fine, curved.
   Microscissors (1).
   Forceps (1), large.
   Forceps (1), small.
   Forceps (1), small, curved.
   Forceps (1), fine, curved.
   Phosphate-buffered saline (PBS), cold, sterile.
   Petri dishes, 100-mm, sterile, plastic.
   Petri dishes, 35-mm, sterile, plastic.
   Beakers (2), lined with gauze and filled with 70% ethanol.
   70% Ethanol.
2. Preparation for dissection:

Note: It is important to have all the materials and instrumentation ready before starting the dissection.

   a. Add cold, sterile PBS to:

i. Two 100-mm Petri dishes to hold embryos/tissue.
ii. Several 100-mm Petri dishes to wash embryos/tissue, and to hold mouse embryo head and brains.
iii. Several 35-mm Petri dishes to hold dissected tissues.

b. Dissection tools can be sterilized in a hot bead sterilizer, in a preheated oven (250°C for 2 h) or by autoclaving (120°C for 20 min).
c. For removal of uteri from pregnant rodents, place the large scissors, small pointed scissors, large forceps and small curved forceps in a beaker lined with gauze and filled with 70% ethanol.

Note: Use a gauze-lined beaker to avoid spoiling the tips of the forceps and scissors.

d. For removal of rodent fetuses from uteri, and for fine dissection of brain, place small forceps, curved fine forceps, small scissors, and curved fine scissors in a second beaker lined with gauze and filled with 70% ethanol.
e. Arrange the dissection tools for the removal of the uterus, one 100-mm Petri dish containing PBS, 70% ethanol, and absorbent toweling in an area for gross dissection (outside of the laminar flow hood).
f. Arrange the dissecting microscope, the dishes containing PBS, and the dissection tools in the laminar flow hood. Keep some sterile Petri dishes and PBS ready at hand.

3. Removal and collection of embryonic brain tissue:
   a. For human tissue, go to step e.

Note: The following steps are performed outside the laminar flow hood.

   b. Removal of uterus:
      i. Anesthetize pregnant mice by intraperitoneal injection of pentobarbital (120 mg/kg) or approved anesthetic, and sacrifice by cervical dislocation.

Note: Assume an average of 8–12 fetuses/animal; 2–3 animals are usually required to start a bulk culture.

      ii. Lay the pregnant animal on its back on an absorbent towel, and rinse the abdominal area with 70% ethanol.
      iii. Using the large forceps, grasp the skin above the genitalia, and, with large scissors, cut through the skin and fascia to open the entire peritoneal cavity.
      iv. Remove the uteri, using small forceps and scissors, and transfer them into a 100-mm dish containing PBS. During these steps, rinse tools frequently in ethanol, to exclude fur that may be a source of bacterial contamination.

Note: The following steps are performed in the tissue culture laminar flow hood. From this point on, use aseptic technique.

   c. Removal of embryos from the uterus:
      i. To reduce the chance of contamination, wash uteri once or twice by subsequently transferring them to new 100-mm Petri dishes containing sterile PBS.
      ii. Using small scissors, open the uterine horns, and, with small forceps, transfer embryos to a new 100-mm dish containing PBS.
      iii. Check all the embryos, and discard those that appear malformed, abnormal, or too small with respect to their gestational age, since they could be a source of dead tissue.
   d. Collection of embryonic brain tissue:

Note: Dissection should be carried out as quickly as possible (possibly within 2 h). Over time, tissue becomes soft and sticky, and may be difficult to dissect. If the estimate is that more than 2 h are required, remove and dissect 8–10 brains at a time, keeping the remaining embryos at 4°C.

i. Cut off heads just below the cervical SC region, and, with fine forceps, transfer them to a new 100-mm dish containing PBS, taking care to not damage the brain.
ii. Place dish under dissecting microscope (×10 magnification). To quickly remove the brain, position the head side up, and hold it from the caudal side at the ears, using fine curved forceps. Use microscissors to cut a horizontal opening above the eyes and tease brain out of the opening by gently pushing on the head from the side opposite to the cut.

e. At high magnification (×25), dissect out the desired brain region(s) to be used for establishing the culture. Refer to human or rodent brain atlas for details on how to dissect the specific areas.
f. Place tissue from each region into a 35-mm dish containing ice-cold PBS. Make sure to label each dish with a region-specific code.

3.3. Dissociation of Brain Tissue and Primary Culture

1. Materials:
   Culture medium, sterile, prewarmed to 37°C.
   Tissue culture vessels (as desired).
   Tubes, 15-mL, conical centrifuge.
   Pasteur pipets (2), sterile, glass, fire-polished, cotton-plugged.
   Trypan blue.
   Hemocytometer.
   70% ethanol.
2. Preparation for dissociation:
   a. Set up the laminar flow hood for the dissociation procedure. If using the same hood used for the dissection procedure, remove everything, and thoroughly clean and disinfect the working surface with 70% ethanol.
   b. Label sterile, plastic, conical 15-mL tubes (one tube for each brain region), and add 3 mL culture medium to each.
3. Dissociation protocol:
   a. Transfer tissue pieces into tubes, using a sterile, fire-polished, cotton-plugged glass Pasteur pipet. Before transferring tissue, prewet the pipet with fresh medium to prevent tissue from sticking to the glass wall.
   b. Dissociate tissue by triturating, until the suspension looks cloudy and only small pieces of tissue are left. Normally, 20–30 strokes are sufficient.
   c. Allow enough time for the undissociated pieces to settle to the bottom of the tube.
   d. Transfer the top cell suspension to a clean, labeled plastic conical 15-mL, tube leaving about 1 mL containing the undissociated pieces. To the undissociated pieces, add 2 mL culture medium, using a new fire-polished, cotton-plugged glass Pasteur pipet, and repeat step 2 until the cell suspension looks homogeneous, with no undissociated pieces left. During triturating, always avoid foaming and bubbling.
   e. Transfer all the volume, except 200–300 μL, to the tube containing the first round of dissociate cells.
   f. Pellet the cells by centrifugation at 75*g* for 10 min.
   g. Discard supernatant, and resuspend cells in 1 mL culture medium. Dilute a 10-μL aliquot from each sample in trypan blue, and count in a hemocytometer.

   Note: To provide an example, the approximate number of cells yielded by this protocol, using each single E14 mouse brain, is: striatum, 5×10^5 cells; cortex, 2.5×10^6 cells; thalamus, 10^6 cells; mesencephalon, 10^5 cells; and spinal cord, 3×10^5 cells.

h. Seed cells at a density of 5×10^4 viable cells/cm^2 in growth medium, in the desired tissue culture vessels. Use approx 6 mL for a 25-cm^2 flask or 10 mL for a 75-cm^2 flask.
i. Incubate at 37°C, in a humidified atmosphere of 5% CO_2/95% air.

3.4. Cultures

1. Single cells should proliferate to form spherical clusters (primary neurospheres), which eventually lift off as they grow larger. The time in which primary neurospheres should be ready for subculturing depends on the growth factors used, the developmental stage of the original tissue, the area and species of origin of the cells, and ranges between 3 and 21 d after plating (*see also* Section 5.).
2. After a few days in culture, cell aggregates may be observed, which can be mistaken for primary spheres. This is particularly evident if debris and dead cells are present in excess in the cultures. Cell aggregates are normally of large size, but the cells that make them up are rather small, phase-dark, and irregularly shaped. Bona fide, primary neurospheres appear like small clusters of round, phase-bright cells, which may often be covered, and sometimes completely hidden, by debris and dead cells. The early subculturing passages are critical for the selection of proliferating stem cells, while getting rid of cell aggregates, debris, dead cells and short-term dividing precursors at the same time (*see also* Section 5.).
3. Striatum and cortex are expected to give clean neurosphere cultures from the beginning. However, in primary cultures from spinal cord and ventral mesencephalon, debris and adherent cells are present, but both should disappear after a few subculturing passages.

3.5. Differences Between Human and Rodent CNS Stem Cell Cultures

Although the basic protocol given above allows the culturing of both human and rodent neural stem cells, some differences do exist between these species.

With few exceptions, embryonic, but also adult rodent, stem cells, can be expanded in the presence of either EGF or FGF-2 alone (Reynolds and Weiss 1992, 1996; Davis and Temple 1994; Kilpatrick and Bartlett 1995; Gritti et al., 1996); the combined use of these growth factors (GFs) results in a faster growth rate (Gritti, et al., 1999) (*see* Fig. 2). Conversely, human embryonic CNS stem cells require the simultaneous exposure to both EGF and FGF-2 to grow and expand in vitro (Vescovi and Snyder 1999).

The growth rate of human cells is significantly slower than their mouse counterpart. Under the best culture conditions, human embryonic cells display a doubling time ranging between 5 and 12 d, compared to 1–2 d for their mouse counterpart.

Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12) is the basal medium originally used for culturing neural stem cells from rodents. But DMEM/F12 should be replaced with NS-A basal medium (refer to the formulation of the growth medium in Section 10 for details), because the former does not allow for the efficient growth of human cells.

The use of NS-A in place of DMEM/F12 increases the rate of growth of rodent neural stem cells by two- to fourfold.

4. ADULT MURINE CULTURES

De novo neurogenesis occurs within discrete areas of the adult brain, namely the olfactory bulb (Hinds 1968a,b; Bayer 1983; Corott et al., 1993; Lois and Alvarez-Buylla 1994), the hippocampus (Altman and Das 1965; Kaplan and Bell 1984; Kuhn et al., 1996), and the cortex (Kaplan 1981; Huang and Lim 1990; Gould et al., 1999). Adult hippocampal precursor cells can be isolated and cultured in vitro by means of FGF-2 and serum (Richards et al., 1992; Suhonen et al., 1996). The source of cell precursors accounting for olfactory bulb neurogenesis is the periventricular region,

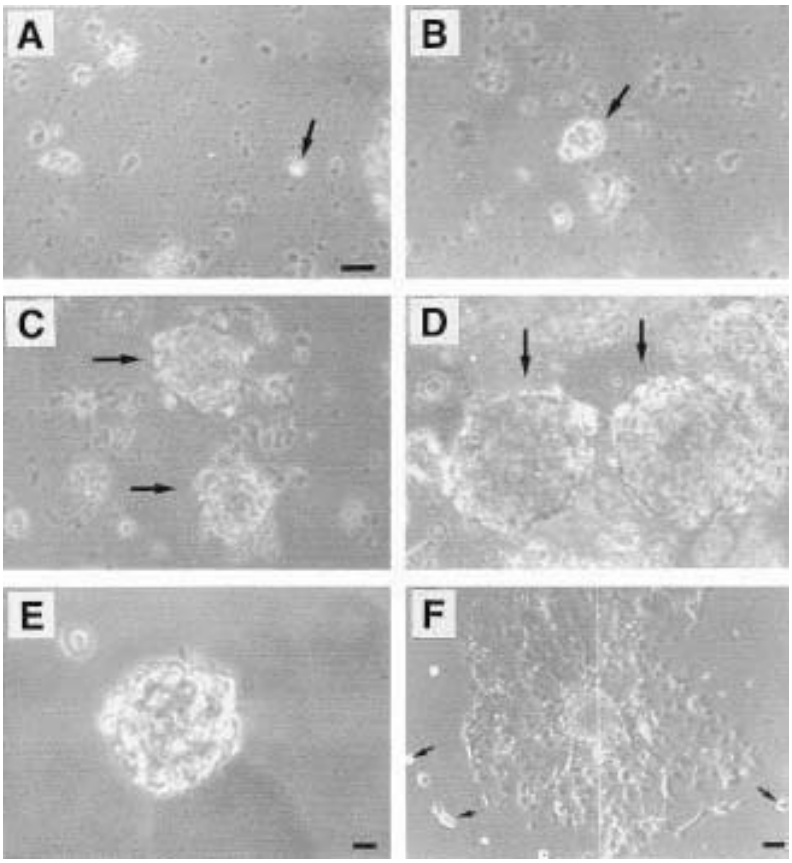

Fig. 2. Examples of adult CNS stem cells at various stages, in culture. **(A)** Representative field of a primary culture from the periventricular region of the forebrain of an adult mouse, 24 h after plating. Single phase-bright cells, like the one shown in A (arrow) will proliferate (**B**, arrow, 5 d after plating), and will give rise to small spheres after approx 7 d (**C**, arrows); cultures should be harvested and passaged at this stage. **(D)** Example of neurospheres that have overgrown (10 d, arrows); subculturing at this stage will result in low replating efficiency. **(E)** Secondary neurosphere, 7 d after subculturing; note the very hypertrophic, phase-bright cells within the sphere, as well as the absence of debris in the plate, which was clearly evident in the primary culture dishes (A–D). A representative example of a single sphere, 3 d after having been plated onto a laminin-coated glass coverslip for differentiation, is shown in **F**. Cells are migrating away from the sphere, and are beginning to differentiate. Yet, at this stage, a few cells that are undergoing mitosis can still be observed (arrows). If spheres are dissociated prior to plating for differentiation, cultures will be very similar to the primary neuronal cultures described in this book. Bars: A–D, 20 μm, bar in A; E, 25 μm; F, 30 μm.

including the ependymal and subependymal layers of the forebrain lateral ventricles. Among these precursors are the multipotent stem cells that have been isolated and cultured by means of EGF and FGF-2, alone or in combination (Reynolds and Weiss 1996; Gritti et al., 1996, 1999). Recently, stem cells with specific requirements for proliferation have also been described in the adult mammalian spinal cord, along the ventricular neuroaxis, and, occasionally, from normally quiescent adult brain regions (Palmer et al., 1995; Weiss et al., 1996). Described here is a protocol to isolate adult murine neural stem cells, and to establish continuous stem cell lines by means of GF stimulation. This protocol can also be applied to rats, and implies the use of enzymatic predigestion,

prior to mechanical dissociation. Stem cells isolated from different mice strains display similar general features, although differences regarding their growth rate and differentiation capacity may be observed.

4.1. Dissection Procedure

1. Materials:
   Scissors (1), large.
   Scissors (2), small, pointed.
   Scissors (1), curved, fine.
   Forceps (1), large.
   Forceps (2), small, curved, fine.
   Forceps (1), small.
   Spatula(1), small.
   Scalpel (1).
   Culture medium.
   Earle's balanced salt solution (EBSS) (Gibco-BRL, cat. no. 24010-043).
   PBS, sterile.
   Papain (Worthington DBA, cat. no. 3119).
   L-Cysteine (Sigma, cat. no. C-8277).
   Ethylenediamine tetraacetic acid (EDTA) (Sigma, cat no. E-6511).
   Ovomucoid (Sigma, cat. no. T-9128).
   Petri dishes, 100-mm sterile plastic.
   Petri dishes, 60-mm sterile plastic.
   Petri dishes, 35-mm sterile plastic.
   Tube (1), 50-mL plastic, sterile.
   Tube (1), 15-mL plastic, sterile.
   Beakers (2) lined with gauze and filled with 70% ethanol.
   Rocking platform (SpeciMix, PBI, cat. no. 14380).
   70% Ethanol.
2. Preparation for dissection.

Note: Have all the materials and instrumentation ready before starting the dissection procedure.

   a. For 50 mL digestion solution, weigh out 47.2 mg papain, 9 mg cysteine, and 9 mg EDTA, and transfer them into a sterile 50-mL plastic tube. Keep the tube at 4°C until the end of the dissection procedure.
   b. Weigh out about 10 mg ovomucoid (enzyme inhibitor), and transfer to a plastic sterile 15-mL tube. Keep the tube at 4°C until the end of the enzymatic incubation period.
   c. Add cold PBS to sterile plastic Petri dishes: one or two 100-mm dishes to hold tissue; several 60-mm dishes to wash tissues; some 35-mm dishes to hold dissected tissues.
   d. Dissection instruments:
      i. Dissection tools can be sterilized in a hot-bead sterilizer, in a preheated oven (250°C) for 2 h, or by autoclaving (120°C for 20 min).
      ii. Select tools needed to remove brain and spinal cord (large scissors, small pointed scissors, large forceps, small curved forceps, and a small spatula). Immerse in gauze-lined beaker filled with 70% ethanol.

      Note: Use a gauze-lined beaker to avoid spoiling the tips of the microforceps and scissors.

iii. Select tools for tissue dissection (small forceps, curved fine forceps, small scissors, curved fine scissors, scalpel). Immerse in 70% ethanol in the second gauze-lined beaker.

e. Arrange, and check the rocking platform.
f. Warm culture medium to 37°C in a thermostatic water bath.
g. Warm EBSS to room temperature.
h. Just prior to beginning the dissection procedure, add 30 mL EBSS to the tube containing papain–cystein–EDTA. Vortex until the solution is clear. Add an additional 20 mL EBSS, and aerate with 95% O_2/5% CO_2 for 30 min.

Note: Thirty minutes is the average time required for an experienced person to dissect tissues from two mice.

3. Removal and dissection of the brain and the spinal cord.

Note: Sacrifice of animals, and removal and dissection of brain and/or spinal cord are performed outside the laminar flow hood. Particular caution should be exercised to avoid contamination.

a. Anesthetize mice by intraperitoneal injection of pentobarbital (120 mg/kg), or use other approved anesthetic, and sacrifice them by cervical dislocation.

Note: Tissues from two or three mice (age, from 2–8 mo) are generally pooled to start a culture.

b. Removal of brain:
   i. Using large scissors cut off the head just above the cervical spinal cord region. Rinse the head with 70% ethanol.
   ii. Using small pointed scissors, make a medial caudal-rostral cut, and remove the skin of the head. Rinse the skull with sterile PBS.
   iii. Using the small scissors, make a longitudinal cut through the skull along the sagittal suture. Be careful not to damage the brain.
   iv. Using curved, pointed forceps, grasp and peel the skull of the right hemisphere outward to expose the brain. Repeat for the left hemisphere.
   v. Using a small curved spatula, scoop the brain out and place in a Petri dish containing PBS.
c. Removal of spinal cord:
   i. To remove spinal cord, cut the dorsal muscles to isolate the whole backbone.
   ii. Using small pointed scissors, cut the vertebral disks on the lateral sides, to expose the cord. Cut the spinal nerves, remove the spinal cord and transfer it to a Petri dish containing PBS.
d. Wash brain and spinal cord twice by subsequently transferring them to new Petri dishes containing PBS.
e. Dissection of forebrain subventricular region:
   i. To dissect the forebrain subventricular region, place the dish containing the brain under the dissecting microscope (×10 magnification). Position the brain flat on its ventral surface, and hold it from the caudal side, using fine curved forceps.
   ii. Using the scalpel, make a coronal cut just behind the olfactory bulbs. Make a second coronal cut to dissect an approx 2-mm-thick slice embodying the lateral ventricles (Fig. 3).
   iii. Discard the remaining pieces of the brain, and keep the slice.
   iv. Shift to a ×25 magnification. Using fine curved microscissors, cut the thin layer of tissue surrounding the ventricles, excluding the striatal parenchyma and the corpus

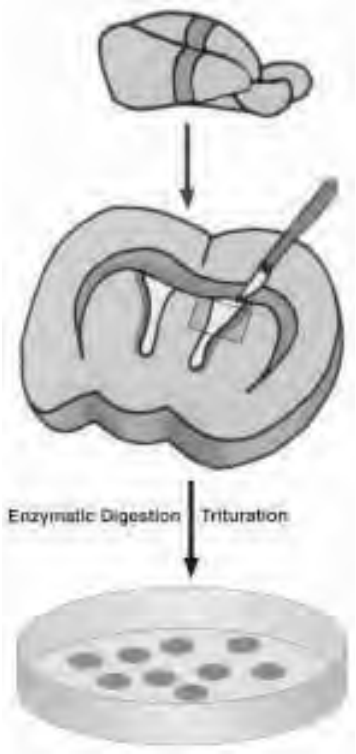

Fig. 3. This figure schematically illustrates the dissection of the periventricular region from the forebrain of an adult mouse or rat. Note that multiple slices may be dissected all along the longitudinal axis of the ventricles, to maximize the harvesting of the periventricular tissue.

callosum (Fig. 3). Make sure not to contaminate the tissue with meninges. Place dissected tissue into labeled 35-mm Petri dishes containing sterile PBS.

f. Dissection of the spinal cord:
   i. Place the dish containing spinal cord under the dissecting microscope (×10 magnification). Carefully remove spinal nerves and meninges.
   ii. Cut a 1-mm-thick coronal section, and dissect out the region surrounding the ependymal canal, placing it into labeled 35-mm Petri dishes containing PBS.

Note: The following steps are performed in the tissue culture laminar flow hood. From this point on, use aseptic technique.

4.2. Dissociation of Brain Tissue and Primary Culture

1. Materials:
   Scissors (1), fine.
   Culture medium.
   0.1% Deoxyribonuclease (Dnase) stock.
   Tubes, 15-mL, sterile, plastic, conical.
   Pasteur pipets (2), glass, sterile, fire-polished, cotton-plugged.
   Filters (2) for sterilization.

Pipetman, 200-μL, and appropriate tips.
Trypan blue.
Hemocytometer.
Rocking platform placed in 37°C chamber.

2. Preparation for dissociation:
   a. Using fine scissors, cut dissected tissue into small pieces.
   b. Stop aerating the papain solution and add 0.5 mL 0.1% DNase stock to it, vortex, and filter-sterilize.

   Note: The 14-mL volume of papain solution is sufficient for a good digestion of tissue from 2–3 mice. Pooling tissues from more than three animals may require a larger volume of papain solution, in order to avoid poor enzymatic digestion.

   c. Label sterile, plastic, conical 15-mL tubes (one tube for each brain region), and add 14 mL papain–DNase solution to each.
3. Dissociation protocol:
   a. Transfer the pieces of tissues into the 15-mL tubes containing the papain solution.
   b. Transfer the tubes to the rocking platform. Incubate at 37°C for 30–60 min, depending on the amount of tissue and on its consistence.

   Note: Generally spinal cord requires longer incubation than the subventricular region.

   c. At the end of the enzymatic incubation, pellet tissues by centrifugation at 110*g* for 10 min.
   d. In the meantime, add culture medium to the tube containing ovomucoid, to obtain 0.7 mg/mL solution (10 mg/14 mL). Filter-sterilize.
   e. Remove almost all the supernatant overlaying the pellets (do not use the vacuum, since there is a substantial risk of sucking out the tissue, as well). Add 3 mL ovomucoid solution. Dissociate by triturating 20–30× using a sterile, fire-polished, cotton-plugged glass Pasteur pipet.

   Note: To prevent tissue and/or cells from sticking to the walls of the Pasteur pipet, rinse the pipet several times with medium, before every dissociation step, or when a new pipet is used. During trituration, always avoid foaming and bubbles.

   Let the suspension settle down for 3–4 min. If many undissociated pieces of tissue are left, reremove cell suspension to a clean, labeled tube, leaving about 1 mL behind. To the latter, add 2 mL fresh ovomucoid solution, using a new fire-polished, cotton-plugged glass Pasteur pipet, triturate again 20–30×, until almost no undissociated pieces are left. Let the suspension settle down for 3–4 min. Transfer almost all the volume of this tube to the labeled tube, thus pooling the cells from both trituration steps.
   f. Pellet the cells by centrifugation at 110*g* for 10 min.

   Note: In primary cultures from adult CNS, debris is normally present, particularly in spinal cord cultures, together with adherent cells. To reduce debris, steps e. and f. my be repeated, increasing centrifugation time to 20–30 min. Generally, debris and adherent cells are eliminated after a couple of passages.

   g. Remove the supernatant, leaving behind about 200 μL. Using a pipetman with the volume set at 180–200 μl, gently dissociate the pellet 20–25×.
   h. Add 5 mL culture medium and pellet cells by centrifugation at 15*g* for 15 min.
   i. Discard supernatant, and resuspend cells in 0.5 mL culture medium. Dilute a 10-μL aliquot from each sample in trypan blue, and count in a hemocytometer (initially try a 1:2 dilution).

   Note: Counting cells is sometimes difficult because of the presence of debris and the small number of cells that can be isolated. In the authors' experience, this protocol

should yield about 5×10^4 cells from the subventricular region of one brain. Thus, if no careful quantification of the primary neural stem cell number has to be carried out, cell suspension derived from two mice may be plated in 4 wells of a 6-multiwell tissue culture plate, yielding an approximate final cell density of about 3500 cells/cm^2, or, in one 25-cm^2 tissue culture flask, obtaining a final density of about 4000 cells/cm^2.

j. Seed cells at a density of 3500 viable cells/cm^2 in culture medium, in untreated 6-multiwell tissue culture plates (2 mL volume), or 25-cm^2 tissue culture flasks (6 mL volume).
k. Incubate at 37°C, 5% CO_2, in a humidified incubator.

4.3. Cultures

Cells should proliferate to form spherical clusters, which eventually lift off as they grow larger. These primary spheres should be ready for subculturing 5–10 d after plating, depending on the GF(s) used.

5. CULTURE PROPAGATION: SUBCULTURING PROTOCOLS

Subculturing is one of the most critical steps in neural stem cell culturing. As discussed earlier, one stem cell can give rise to several daughter stem cells, as well as to differentiating progeny, during the formation of the spheres that eventually undergo subculturing. The number of stem cells found in a sphere varies from different regions, age, or species. but is normally higher in embryonic than in adult rodent spheres, and lower in human embryonic cultures. This is the first parameter that, together with the length of the cell cycle, determines the rate of amplification and the growth curve of neural stem cells in culture. Yet, upon subculturing, not all stem cells survive and generate secondary spheres. Thus, the subculturing efficiency is an additional parameter that needs to be taken into consideration when expanding neural stem cells in culture. For instance, let's imagine a low growing stem cell population in which the stem cell number increases by 50% at each passage. If the subculturing procedure has not been optimized, and the efficiency is <50%, the number of stem cells in cultures will decrease, rather than increase, over time. The conclusion that these cultures do not contain stem cells would, therefore, be misleading. Many parameters may influence subculturing efficiency. Among these, failing to harvest all the cells/spheres, but, more important, loss of cells because of poor or, conversely, excessively harsh mechanical dissociation, wrong pH of the medium, and excessive sticking of cells to the vessels, play a prominent role in decreasing the subculturing efficiency. The protocol described below is essentially identical for all kinds of neural stem cell cultures. If properly implemented, even single spheres can be subcultured.

5.1. Protocol

1. Tap sides of flasks to be passaged, to dislodge spheres and transfer content of the flask to 15-mL sterile, plastic, conical tubes, using a sterile plastic pipet. Use 5 mL fresh medium to rinse flask, and add rinse to the tube.
2. Pellet cell suspension by centrifugation at 110*g* for 10 min.
3. Remove the supernatant, leaving behind approx 200 µL. Using a sterilized p200 pipetman set to a volume of 180–200 µL, rinse the tip with medium, to avoid cell sticking inside the tip. Gently triturate pellet: 25–30× for embryonic rodent cells; 40–50× for adult rodent cells; up to 150× for human cells. Rinse down sides of tube periodically, to dislodge undissociated spheres. Slightly tilt the pipetman, and press tip against the bottom of the tube to generate a fair amount of resistance. For murine cells, go to step 6.

4. For human cultures only (use also for murine cultures, if debris or dead cells are present in excess): add 5 mL culture medium, and centrifuge once more at 15*g* for 15 min. Remove supernatant, and gently dissociate 10–20×to desegregate pellet.
5. Count viable cells by trypan blue exclusion, and seed cells at the appropriate density in culture medium in untreated tissue flasks: 1×10^4 cells/cm^2 for murine cells; 1×10^3 cells/cm^2 for human cells.
6. Subculture when the spheres start to lift off and float in suspension. This will require approx 3–7 d for embryonic murine cells, 5–10 d for adult murine cells, and 7–21 d for embryonic human cells.
7. Depending on both the culture conditions and the region of origin of the cells, the total cell number should increase at each passage 2–10-fold for embryonic murine cells, 2–5-fold for adult murine cells, and two-fold for embryonic human cells.

5.2. Comments

1. Each type of stem cell culture described above has different subculturing times; those indicated here are only indicative, and should be adjusted, based on the good quality of handling and culturing. As a general rule, spheres should be passaged when they are sufficiently large (approx 100 µm diameter; Fig. 2C,E). If spheres are too small when passaged (Fig. 2B), the yield will be low; if spheres are allowed to grow too much before being passaged (Fig. 2D), the number of dead cells inside the spheres will be high, trituration will be difficult, and viability of the culture will be very low. Should this situation be protracted for some subculturing steps, the total number of cells in the culture will progressively decrease, and the culture will be lost.
2. Viability (number of live cells/total number of cells) after dissociation should never fall below 50–60%.
3. Always avoid foaming and bubbles.
4. Feed cells with fresh medium (replace about 25–50% of the total volume in the flask) every 3–5 d.

6. DIFFERENTIATION OF STEM CELLS

Stem cells grow and expand in culture in an undifferentiated state. When growing under the culture conditions described above, cells inside the sphere rarely display a differentiated morphology or antigenic properties typical of neuronal or glial cells. The vast majority of the cells are immunoreactive for nestin, an intermediate filament characteristic of undifferentiated neuroepithelial cells (Hockfield and McKay, 1985; Grenschwind and Hockfield, 1989; Reynolds and Weiss, 1992; Tohyama et al., 1992; Gritti, et al., 1996). Nonetheless, plating stem cell progeny onto a good adhesion substrate, such as laminin or Matrigel, and removal of GFs is generally sufficient to promote a spontaneous differentiation process, resulting in the production of neuronal and glial cells. The procedure described below is a basic one, in which cells are differentiated in serum-free medium, according to the same protocol used to establish enriched neuronal cultures from the E14 striatum. This approach provides the culture conditions against which one can test the effect of specific molecules on the differentiation of stem cells and their neuronal and glial progeny. Some considerations on neural stem cell differentiation are worth considering:

1. *Survival of stem cell progeny upon removal of GFs.* Like all neural stem cells, human stem cells differentiate into the three major CNS cell types, following GFs removal. Differentiated cultures can survive for up to 4 wk in culture. Sometimes, the survival of rodent stem cells, in the absence of GFs, is poor: Adult rodent cells differentiate, but cultures start to die

after 3–4 d, and embryonic cultures may even die within 24 h, upon removal of GFs. To overcome this problem, murine stem cells can be plated onto the adhesion substrate in the presence of FGF-2, and shifted to GF-free medium a few days after plating. As far as the substrates are concerned, cell survival is much better on laminin and matrigel than on polyornithine.

2. *Conditions allowing stem cell progeny to display full differentiation potential.* Differentiation of stem cell progeny follows a time profile remarkably similar to that observed within most brain regions in vivo. Upon GF removal, neurons are generated first, followed by astroglia, and eventually by oligodendrocytes. The main consequence is that the result of a given differentiation experiment may vary with the times at which differentiation is assessed. If one looks too early, only neurons and a few glial cells may be seen, but, if the analysis is performed too late, the neurons may have died, and their actual number may thus be underestimated. Further, the differentiation patterns, and particularly neuronal survival, are also strongly influenced by the plating density. It is also possible that neuronal and glial progenitors fail to express differentiation markers in culture (or toexpress them to a level that is too low to be detected by standard immunohistochemistry), unless specific epigenetic factors are added (or removed). For example, FGF-2 prevents the differentiation/maturation process of glial and neuronal–glial mouse precursors in serum-free medium (Perraud et al., 1988; Vescovi et al., 1993). Generally, removal of FGF-2 and an eventual addition of low concentration of serum (2%), improve neuronal maturation and allow the detection of glial antigens. These observations should all be taken into account when evaluating data from differentiation experiments on neural stem cell progeny.
3. *Enrichment of differentiated cultures in one specific cell type.* Stem cell progeny spontaneously differentiate into neuronal–glial mixed cultures. Seven d after plating, the neuronal–astroglial–oligodendroglial ratio is approx 15:75:1, with the protocol described here. Yet, this ratio can be altered by changing the differentiation conditions. For instance, in both rodent (Johe et al., 1996) and human cells (Galli et al., 2000), the final outcome of the differentiation process can be varied, so that almost one-half of the differentiated progeny acquires a neuronal fate by addition of platelet-derived growth factor or leukemia inhibitory factor, respectively.

The following represent basic differentiation protocols for rodent and human cells. Suggestions are given to match the points considered above.

6.1. DIFFERENTIATION PROTOCOLS

1. Preparation of materials:
   a. Immerse round-glass coverslips in 70% ethanol. Dry with thin adsorbent paper. Place in a glass Petri dish, and sterilize in preheated oven (250°C for 2 h).
   b. Using sterilized fine forceps, add one coverslip to each well of a 48- or 24-multiwell plate. Alternatively, glass or plastic chamber slides can be used.
   c. Prepare adhesive substrate:
      i. Dilute polyornithine stock (1:10 in sterile water), laminin, or Matrigel (1:100 of the stock solution in water or in GF-free medium [control medium], respectively).
      ii. Add 125 µL of the chosen substrate, for each square centimeter of surface to each well. Incubate at 37°C for at least 2 h. Rinse thoroughly with sterile PBS.
   d. Warm control medium to 37°C, and add it to each well (0.25 mL for 48-multiwell plates and 0.5 mL for 24-multiwell plates). Equilibrate plates at 37°C in a humidified atmosphere of 5% CO_2/95% air.

2. Protocols:
   a. Tap sides of flasks to dislodge spheres.
   b. Remove content of the flask to 15-mL sterile, plastic, conical tubes, using a sterile plastic pipet, and centrifuge at 110*g* for 10 min.
   c. To wash cells from growth factors, remove supernatant and resuspend cells gently, with a large-bore plastic pipet, in 10 mL control medium. Spin at 110*g* for 10 min.
   d. Remove the supernatant, leaving behind about 200 µL. Using a sterilized p200 pipetman set at 180–200 µL, gently triturate pellet (100–150× for human cells; 50–60× for adult mouse cells; and 30–40× for embryonic mouse cells).
   e. Centrifuge cells at 15*g* for 15 min.
   f. Remove supernatant, and resuspend pellet in 0.5 mL control medium (*see* also notes below); count viable cells by trypan blue exclusion. Resuspend cells in the appropriate volume of control medium, so that the final number of cells to be plated per well is contained in 0.25 mL for 48-multiwell plates or 0.5 mL for 24-multiwell plates. Agitate plate to distribute cells evenly on coverslip.

   Note: Plating cell densities: human cells, 8×10^4–10^5 cells/cm^2; rodent embryonic or adult cells, 5×10^4 to 7×10^4 cells/cm^2.

   g. Incubate at 37°C in a humidified atmosphere of 5% CO_2/95% air.

6.2. Culture Conditions

The following suggestions are only indicative, and need to be adapted to one's own conditions.

1. Neuronal cells can be detected as early as 1–2 d after plating. Still, detection of astrocytes and oligodendrocytes by immunostaining may be difficult, but can be facilitated by the addition of fetal bovine serum (Gibco-BRL, cat. no. 10106-151), at a final concentration of 2%, 3–4 d after plating. Under these conditions, simultaneous detection of the three cell phenotypes is usually successful at 7 d after plating. For immunostaining, one may use the same protocols used for staining primary CNS cultures (Reynolds and Weiss 1992; Gritti et al., 1996; Vescovi et al., 1999).
2. To prevent the massive cell death that may occur upon removal of GFs, particularly with embryonic cells, CNS stem cells may be plated in FGF-2-containing medium (10 ng/mL final concentration). In this case, because proliferation of FGF-2-responsive progenitors occurs, the lower plating density should be used. In the presence of FGF-2, neuronal and astroglial differentiation is delayed by 1–2 d, and astrocytes cannot be detected at all, unless fetal bovine serum (2% final concentration) is added to the cultures 3–4 d after plating. Replacement of FGF-2-containing medium with control medium, at the same time, may be advisable.

6.3. Comments

1. Adhesion to a substrate, and complete removal of GFs, may determine up to 50% cell loss. Use a large number of cells to start with, and increase cell density, if substantial cell death is observed upon plating.
2. Quantitative analysis of the relative proportion of neurons, astrocytes, and oligodendrocytes is the easiest method for comparing the effects of different protocols. Variability between experiments is perhaps the most critical and limiting step, for this type of analysis to be rigorously accomplished, and the first source of this variability is the heterogeneity of the starting CNS stem cell population. To increase homogeneity in the cell population:
   a. Use spheres that have been subcultured at least twice, so that, in the starting cultures, short-term dividing precursors are absent. Generally, an increase in subculturing pas-

sages does not affect the proportion of the different cell types produced by stem cell progeny, upon differentiation.
   b. In order to have a good cell yield do not use spheres that have grown too much (*see also* Section 5.).
   c. Take into account the growth rate of the cell line(s), and use spheres after a fixed number of days after the last subculture passage.
   d. If dissociation has been efficient, almost the totality of the cells plated after subculturing should be single cells, which eventually proliferate in response to GF(s). If cultures aare harvested after cells have made only one or two proliferation cycles (when one sees mostly doublets or very small clusters), culture will be highly enriched in stem-cell elements and very undifferentiated precursors.
3. The protocol described for dissociated cells can be applied to differentiate single spheres as a whole, which can be plated onto the growth substrate without being previously triturated.

7. CLONAL ANALYSIS

Clonal analysis and serial subcloning are probably the most difficult and time-consuming tasks in neural stem cells culturing. Yet these analyses are essential, to demonstrate the self-renewal capacity and the multipotency (i.e., the ability to generate neurons and both glial cell types) of the candidate cell, and to prove its stem cell nature. In practical terms, if the progeny of an individual clone-founder cell contains cells that give rise to neurons and glia, and, more importantly, contains one or more cells identical to itself (i.e., is able to proliferate producing multipotent progeny), it can be concluded that the founder cell displays stem cell features. Alternative techniques are available to perform clonal analysis. Described here are three of these, all of which are based on the direct observation of the clone formation, starting from an individual cell.

7.1. Limiting Dilution

1. Preparation for limiting dilution:
   a. Warm culture medium to 37°C.
   b. Prepare a humidified chamber (a glass or plastic chamber with wet gauze on bottom) to hold 96-multiwell plates.
2. Limiting dilution procedure:
   a. Tap sides of flask to dislodge spheres.
   b. Remove contents of the flask to 15-mL sterile, plastic, conical tubes, using a sterile plastic pipet, and centrifuge at 110*g* for 10 min.
   c. Remove the supernatant, leaving behind about 200 µL. Using a sterilized p200 pipetman set at 180–200 µL, vigorously dissociate the pellet to a single-cell suspension (100–150× for human cells, 50–60× for adult mouse cells, and 30–40× for embryonic mouse cells).
   d. Add 10 mL fresh culture medium, and centrifuge cells at 15*g* for 15 min.
   e. Remove supernatant, and resuspend pellet in 0.5 mL culture medium. Dilute a 10-µL aliquot in trypan blue, and count in a hemocytometer.
   f. Resuspend cells in culture medium at a cell density of 5–10 cells/mL. Use a dispenser to add 100 µL of this cell suspension to each well, frequently resuspending the cell solution. To prepare three 96-multiwell plates, 300 cells/30 mL culture medium as needed.
   g. Incubate cells in the humidified chamber at 37°C, in a humidified atmosphere of 5% CO_2/95% air.

h. Carefully score plates, under an inverted microscope, to unequivocally identify and mark wells containing single cells. Make sure to use high magnification to assess that a cell is indeed single. Wells containing two cells or more should not be further considered for the clonal analysis. Take pictures of each single cell if you deem that photographic documentation will be necessary.
i. Score the plate once a week; make sure the pH of the medium does not change excessively. Take pictures as needed, to follow the fate of single cells over time. Many of them will die, and some will differentiate. Only a small percentage will proliferate to form a clonal sphere, which could undergo further subcloning. This will require 10–30 d, depending both on the type of cells and on culture conditions.
j. Either differentiate cells by transferring the sphere onto a laminin- or Matrigel-coated coverslip, and proceed as described in Section 6, or perform serial subcloning as described in Sections 7.3 or 7.4.

7.2. Manipulation of Individual Cells

1. Preparation:
   a. Warm culture medium to 37°C.
   b. Prepare a humidified chamber (a glass or plastic chamber with wet gauze on bottom) to hold 96-multiwell plates.
2. Cloning procedure:
   a. Tap sides of flask to dislodge spheres.
   b. Remove contents of the flask to 15-mL sterile, plastic, conical tubes, using a sterile, plastic pipet, and centrifuge at 110*g* for 10 min.
   c. Remove the supernatant, leaving behind about 200 µL. Using a sterilized p200 pipetman set at 180–200 µL, vigorously dissociate pellet to a single-cell suspension (100–150× for human cells, 50–60× for adult mouse cells, and 30–40× for embryonic mouse cells).
   d. Add 10 mL fresh culture medium, and centrifuge cells at 15*g* for 15 min.
   e. Remove supernatant, and resuspend pellet in 0.5 mL culture medium. Dilute a 10-µL aliquot in trypan blue, and count in a hemocytometer.
   f. Resuspend cells in culture medium, and plate in 35-mm Petri dishes in 2 mL culture medium, at a density of 50 cells/cm^2.
   g. After 2–6 h, choose viable cells on the basis of round shape, phase-brightness, hypertrophic appearance, and lack of processes. Transfer to a 96-multiwell plate (1 cell/well), using a heat-polished glass microelectrode pipet (internal tip diameter 40–70 µm) connected by silicon tubing to a screw-driven 500-µL syringe.
   h. Follow the fate of single cells over time, also by time-lapse microphotography. Many of them will die, and some will differentiate. Only a small percentage (<5%, in the authors' hands) will proliferate to form a clonal sphere. This will require 10–30 d, depending on both the type of cells and culture conditions. Clonally derived spheres could be differentiated as described in Section 6, or may undergo further subcloning, either as shown here, or by methylcellulose (MC) assay (*see* Section 7.3), or as proposed in Section 7.4.

7.3. Methylcellulose Assay

1. Preparation:
   a. Warm culture medium to 37°C.
   b. Prepare a humidified chamber (a glass or plastic chamber with wet gauze on bottom) to hold plates.

c. Prepare methylcellulose (Dow, Methocel A4M, premium grade) gel matrix (4% final concentration) in DMEM/F12 or NS-A growth medium (*see* Section 10.1).

2. Cloning procedure:
   a. Tap sides of flask to dislodge spheres.
   b. Remove contents of the flask to 15-mL sterile, plastic, conical tubes, using a sterile plastic pipet, and centrifuge at 110*g* for 10 min.
   c. Remove the supernatant, leaving behind about 200 µL. Using a sterilized p200 pipetman set at 180–200 µL, vigorously dissociate pellet to a single-cell suspension (100–150× for human cells, 50–60× for adult mouse cells, and 30–40× for embryonic mouse cells).
   d. Add 10 mL fresh culture medium, and centrifuge cells at 15*g* for 15 min.
   e. Remove supernatant, and resuspend pellet in 0.5 mL culture medium.

   Note: Make sure that the vast majority of the cells are single cells, by withdrawing an aliquot and checking it under the microscope. Repeat dissociation if necessary, until only single cells are present in the suspension.

   f. Dilute a 10-µL aliquot in trypan blue- and count in a hemocytometer.
   g. Resuspend cells in growth medium containing 40 ng/mL EGF and 20 ng/mL FGF-2. Final cell concentration should be less than 200 cells/mL.
   h. Aspirate 2.5 mL cell suspension into a 5-mL syringe.
   i. Aspirate 2.5 mL methylcellulose gel matrix into the same syringe.
   j. Gently inject the mixture of cells and methylcellulose gel matrix into a 60-mm Petri dish, avoiding bubbling and foaming.
   k. Using the same syringe, resuspend the mixture several times, until a semisolid homogeneous gel has formed and the single cells are thoroughly dispersed.
   l. The day after plating, score the plate, to identify single hypertrophic cells.
   m. Mark their position on the plate with a fine marker, and take microphotographs over time. If the appropriate setup is available, use tissue culture flasks, instead of Petri dishes, seal tightly, and use time-lapse cinematography to monitor clone formation.
   n. Individual clonal spheres can be subcloned by this same procedure, or as described in Section 7.4., or can be differentiated as described in Section 6.

7.4. Subcloning Procedure

1. Transfer individual clonal spheres to 5-mL microcentrifuge tubes containing 1 mL appropriate medium (1 sphere/tube), using a sterilized p200 pipetman set at 180 µL. Rinse tip with medium, first, to avoid cells sticking to the tip walls. To generate a clonal cell line, use the limiting dilution protocol (*see* Section 7.1.), then dissociate single clonal spheres inside their own dish, without transferring them, then proceed to step 5.
2. Centrifuge at 110*g* for 10 min. Remove supernatant, leaving behind about 200 µL medium.
3. Using a sterilized p200 pipetman set at 180 µL, dissociate spheres by trituration to a single cell suspension (100–150× for human cells, 50–60× for adult mouse cells, and 30–40× for embryonic mouse cells). Rinse tip with medium, first, to avoid cells sticking to the tip walls. Press the tip to the bottom of the well to generate a fair amount of resistance. Avoid foaming and bubbles.
4. Plate all the cell suspension in a clean dish of a 48-, 24-, or 12-multiwell plate (depending on the number of viable cells), and incubate cells at 37°C in the humidified chamber, in a humidified atmosphere of 5% CO_2/95% air. Embedding cells into MC prior to plating, as described in Section 7.3, is strongly recommended, in order to avoid cell aggregation.
5. Within 1 h of plating, count the total number of cells obtained by dissociation of each clone, under the microscope. A subset of these cells will proliferate, giving rise to secondary clones.

The cloning efficiency can be calculated normalizing the number of secondary clones by the total number of cells initially present in the well, as assessed by direct observation at the time of plating.

6. Individual secondary clones can either be differentiated to assess their multipotentiality (*see* Section 6.), or can be transferred to 5-mL microcentrifuge tubes (1 sphere/tube), to undergo further subcloning (*see also* Fig. 1).
7. If a clonal cell line must be generated, secondary spheres, derived from a single primary sphere, can be pooled, mechanically dissociated to a single cell suspension, and plated at a cell density of 1×10^4 cells/cm^2 in the appropriate medium. Subculture until a bulk culture is established (*see also* Section 5.).

7.5. Comments

1. Feed cells with fresh prewarmed medium every 4–5 d. For the first feeding, add 100 µL; if further feeding is needed, carefully replace 100 µL medium in the well with 100 µL fresh medium.
2. Because small volumes of medium are used in 96-multiwell plates, evaporation of medium is very critical: Always keep plates in humidified chambers.
3. Cloning is a critical procedure. The use of NS-A, rather than DMEM/F12-based growth medium, is recommended.

8. CRYOPRESERVATION OF NEUROSPHERES

Once established, stem cell lines can be effectively expanded to obtain a large number of cells, which can be cryopreserved. This allows for the establishment of a reservoir of cells at early subculture passages, which can be further expanded to create a homogeneous stock of cells for future experiments. Repeated cycles of freezing and thawing do not affect the CNS stem cell functional properties. More importantly, tissue from which the cell lines have been established can also be cryopreserved and stored, if the need to re-establish a specific cell line should arise. This is not particularly relevant for murine cell lines, but it is of fundamental importance for human neural stem cell lines.

8.1. Cryopreservation Using Controlled-Rate Freezing Containers

1. Preparation:
   a. Ensure that freezing jar (Criostep, Nalgene, PBI, cat. no. 5100-0001) is at room temperature and filled with isopropanol.
   b. Make freezing medium, namely, culture medium containing 10% dimethyl sulfoxide. In our experience, glycerol yields poor results when freezing neural stem cells.
   c. Label cryovials with date, cell type, and passage number.
2. Freezing neurospheres:
   a. Collect spheres by gentle pipeting, and pellet them by centrifugation at 110*g* for 10 min.
   b. Wash the pellet once with fresh medium, and resuspend them in 1.5 mL freezing medium. Swirl gently to resuspend spheres.
   c. Transfer cells into labeled 2-mL cryogenic vial(s).
   d. Let cells equilibrate at room temperature for 15 min.
   e. Transfer vials into the freezing jar containing isopropyl alcohol.
   f. Leave the jar at –80°C for a minimum of 4 h, to allow a slow and reproducible decrease in temperature (–1°C/min).
   g. Transfer vials into a liquid nitrogen tank for long-term storage.

3. Thawing of cryopreserved neurospheres:
   a. Warm culture medium and a water bath to 37°C.
   b. Quickly transfer cryovial(s) from liquid nitrogen to 37°C water bath, and leave until thawed. Swirl the vial to favor thawing.
   c. Wipe entire cryovial with 70% ethanol.
   d. Slowly transfer cell suspension from cryovial to 15-mL plastic tube containing 5 mL warm culture medium.
   e. Spin cell suspension for 8 min at 110*g*, and remove most of the supernatant.
   f. Gently resuspend pellet in fresh medium and plate in flask(s) of appropriate size.

8.2. Comments

1. Do not let spheres grow too large before harvesting for cryopreservation (Fig. 2D), and do not mechanically dissociate spheres before freezing. This increases the number of dead cells, and viability of the culture upon thawing will be very low.
2. This same freeze–thaw protocol can also be applied to finely chopped embryonic human tissue. To start a CNS stem cell culture from frozen human tissue: Upon thawing (steps a.–e. above), follow protocol for dissociation and primary culture described in Section 3.3. (embryonic tissue).

9. FINAL REMARKS

Neural stem cell cultures are very sensitive to pH changes. The pH of the culture medium should be around 7.4, which is indicated by a dark orange color. If the color is close to violet, leave the medium to equilibrate in the incubator. If the color is light orange/yellow, check the composition, and prepare fresh medium if necessary.

Seeding cells at the appropriate cell density is a critical step to obtain viable and well-characterized cultures. The dilutions suggested in the protocols described above are based on our experience. The optimal dilution for cell counts could vary from time to time and must be found empirically. Consider that, for accuracy in the count, 100–200 cells/square are needed..

As mentioned above, NS-A basal medium can be used to replace DMEM/F12 in growing stem cell cultures. Although this is necessary for obtaining a satisfactory growth rate in human stem cell cultures, DMEM/F12 is sufficient for the growth of murine cells. Nevertheless, the latter can grow 2–4× faster in this medium, which greatly facilitates neural stem cells cloning.

10. APPENDIX

10.1. Solutions

Note: These cultures are extremely sensitive to contaminants present in water or on glassware. Distilled sterile nonpyrogenic water should be used (before use, filter-sterilize in sterile-disposable plastic bottles). Otherwise, purchase ultrapure cell culture tissue grade water (Gibco-BRL, cat. no. 15230-147).

A set of glassware, to be used only for tissue cultures, should be prepared. Bottles, cylinders, beakers, and so on, should be accurately rinsed several times with distilled water, before being sterilized in an autoclave that is used for tissue culture purposes only. We suggest that media and all stock solutions be prepared only in sterile, disposable tubes and/or bottles.

1. 30% glucose (Sigma, cat. no. G-7021):
   a. Mix 30 g glucose in 100 mL water.
   b. Filter-sterilize. Store at 4°C.

2. 7.5% sodium bicarbonate ($NaHCO_3$)(Sigma, cat. no. S-5761):
   a. Mix 7.5 g $NaHCO_3$ in 100 mL water.
   b. Filter-sterilize. Store at 4°C.
3. 1 *M* HEPES (Sigma, cat. no. H-9136):
   a. Mix 23.8 g HEPES in 80 mL water; bring volume up to 100 mL with water.
   b. Filter-sterilize. Store at 4°C.
4. PBS:
   a. Mix 430 mL water, 50 mL 10X PBS (without calcium, without magnesium, Gibco-BRL, cat. no. 14200-067), 10 mL 30% glucose, and 10 mL penicillin/streptomycin (Gibco-BRL, cat. no. 15140-114).
   b. Filter-sterilize. Store at 4°C.
5. 0.2% heparin (sodium salt, grade 1A, Sigma, cat. no. H-3149):
   a. Mix 100 mg heparin in 50 mL water.
   b. Filter-sterilize. Store at 4°C.
6. 3 m*M* Na selenite (Sigma, cat. no. S-9133):
   a. Add 1.93 mL water to a 1-mg vial of Na selenite, and mix.
   b. Aliquot into sterile tubes, and store at –20°C.
7. 2 m*M* progesterone (Sigma, cat. no. P-6149):
   a. Add 1.59 mL 95% ethanol to a 1-mg vial of progesterone, and mix.
   b. Aliquot into sterile tubes, and store at –20°C.
8. 0.1% DNase stock (Sigma, cat. no. D-4513):
   a. Add 10 mL water to 10 mg DNase. Mix well, and filter-sterilize.
   b. Aliquot in sterile tubes (0.5 mL/aliquot), and store at –20°C.

10.2. 10× Stock Hormone Mix

1. Combine the following:

Components	mL
10X DMEM/F12	40
30% glucose	8
7.5% $NaHCO_3$	6
1 *M* HEPES	2
Pure water	300

2. Add 400 mg apo-transferrin (Sigma, cat. no. T-2252).
3. Dissolve 100 mg insulin (bovine, Roche, cat. no. 977 420) in 4 mL sterile 0.1 *N* HCl. Mix in 36 mL water, and add all to the hormone mix solution.
4. Dissolve 38.6 mg putrescine (Sigma, cat. no. P-7505) in 40 mL water, and add to hormone mix solution.
5. Add 40 µL 2 m*M* progesterone and 40 µL 3 m*M* Na selenite to hormone mix solution.
6. Mix well, and filter-sterilize. Aliquot in sterile tubes, and store at –20°C.

10.3. Stock Growth Factors

Reconstitute EGF (human recombinant, Peprotech, Rocky Hill, NJ, cat. no. 100-15) and FGF-2 (human recombinant, Peprotech, cat. no. 100-18B), in order to have a 500-µg/mL stock of each. Aliquot into sterile tubes, and store at –20°C.

10.4. Media

1. 10X stock solution of DMEM/F12. Dissolve 5-L packages each of DMEM (DMEM: high-glucose, with L-glutamine, without $NaHCO_3$ bicarbonate or sodium piruvate; Gibco-BRL,

cat. no. 52100-021) and F-12 (F-12: with L-glutamine, without $NaHCO_3$; Gibco-BRL, cat. no. 21700-109) powder in 1 L of water, under gentle continuous stirring. Filter-sterilize. Store at 4°C.

2. DMEM/F12-based growth media (500 mL): Mix together the following:

Pure water	375 mL
10X DMEM/F12	50 mL
30% Glucose	10 mL
7.5% $NaHCO_3$	7.5 mL
1 *M* HEPES	2.5 mL
200 m*M* Glutamine (Gibco-BRL, cat. no. 25030-024)	5 mL
10X Hormone mix	50 mL
0.2% Heparin	1 mL
EGF stock	20 µL
or/and FGF-2 stock	10 µL

Note: Final concentration: 20 ng/mL EGF and 10 ng/mL FGF-2.

3. NS-A-based growth media (500 mL): Mix together the following:

NS-A*	445 mL
200 m*M* Glutamine (Gibco-BRL, cat. no. 25030-024)	5 mL
50 mL 10X hormone mix	50 mL
EGF Stock	20 µL
and/or FGF-2 stock	10 µL

Note: Final concentration: 20 ng/mL EGF and 10 ng/mL FGF-2.

* NS-A: Euroclone, UK, (e-mail: headquarters@euroclone.net) cat. no. ECM-0833.

10.5. Substrates

1. 10X stock solution of polyornithine (Sigma, cat. no. P-3655):
   a. Transfer the content of a 50-mg vial of polyornithine into a bottle containing 333.3 mL pure water.
   b. Rinse vial twice with the solution, add rinse to solution. Mix well by swirling.
   c. Sterilize by autoclaving at 120°C for 20 min.
   d. Aliquot in sterile tubes, and store at –20°C.
2. 100X stock solution of Matrigel (GF-reduced, Becton Dickinson, cat. no. 40230):
   a. Thaw a vial of matrigel overnight at 4°C.
   b. Aliquot into sterile tubes (0.5 mL/aliquot), using refrigerated plastic pipets.
   c. Store at –20°C.
3. 100X stock solution of laminin (Roche, cat. no. 1 243 217):
   a. Thaw a vial of laminin solution (1 mg/1 mL) at room temperature.
   b. Aliquot into sterile tubes (20 µL/aliquot).
   c. Store at –20°C.

10.6. Plasticware

The following is a list of the plasticware the authors commonly use in ther lab.

1. Flasks:
   a. 25 cm^2 Nunclon-Delta surface, 0.2-µm vented filter cap, Nunc, cat. no. 136196.
   b. 75-cm^2, 0.2-µm vented filter cap, Costar, cat. no. 3376.
2. Multiwell plates, Petri dishes: Nunclon-Delta surface.

3. Chamber slides: Lab Tek, Nalge Nunc, Permanox slide, cat. no. 177 347.
4. Filters:
   a. Bottle-top filters: low protein-binding, 0.22-μm Corning.
   b. Syringe filters: cellulose acetate 25-mm, 0.22-μm, Nalgene, cat. no. 1902520.
5. Bottles and pipets: Costar.

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Chapter Fifteen

Aggregating Neural Cell Cultures

Paul Honegger and Florianne Monnet-Tschudi

1. INTRODUCTION

Aggregating brain cell cultures are primary, three-dimensional cell cultures consisting of even-sized, spherical structures that are maintained in suspension by constant gyratory agitation. Because of the avidity of freshly dissociated embryonic cells to attach to their counterparts, cell aggregates form spontaneously and rapidly under appropriate culture conditions. The reaggregated cells are able to migrate within the formed structures, and to interact with each other by direct cell–cell contact, as well as through exchange of nutritional and signaling factors. This tissue-specific environment enables aggregating neural cells to differentiate, and to develop specialized structures (e.g., synapses, myelinated axons) resembling those of brain tissue *in situ*. Aggregating cell cultures are therefore classified as organotypic cultures (Doyle et al., 1994).

The basic methodology of rotation-mediated aggregating brain cell culture was introduced by Moscona (1961), and was subsequently applied to cells of the central nervous system (CNS) for morphological and biochemical investigations (e.g., De Long and Sidman, 1970; Seeds, 1971). The original culture procedure was subsequently modified by using a mechanical sieving technique for dissociation, instead of the enzymatic procedure (Honegger and Richelson, 1976), and by developing an appropriate chemically defined culture medium (Honegger et al., 1979). These modifications greatly simplified the procedure for the culture preparation, and provided a means to grow large numbers of highly reproducible replicate cultures for multidisciplinary studies. Furthermore, the availability of a chemically defined medium greatly facilitated the study of the role of hormones and endogenous messengers in the brain. (For protocols that use enzymatic tissue dissociation for aggregating neural cell cultures, *see* Choi et al., 1993).

2. PREPARATION OF AGGREGATING NEURAL CELL CULTURES

It is important to start with immature brain tissue of an appropriate, well-defined developmental stage. The optimal gestational period for culture preparation depends on both the species and the brain region. The protocols presented here use embryonic rat brain tissues, because of the relative ease in obtaining well-timed and large numbers of fetuses from this species. The cellular composition and developmental potential of the cultures are influenced greatly by the properties of the starting brain tissue (Honegger and Richelson, 1976). Using rat tissue for culture preparation, the

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spinal cord should be taken not later than at gestational d 14; whole brain not later than gestational d 15; and the telencephalon not later than gestational d 16. The use of mice has some advantage, because of the ever-increasing number of mutant and transgenic mice available. There is no fundamental difference between mouse and rat cells in the preparation and handling of aggregate cultures, nor in their developmental characteristics.

Rats are usually mated overnight, then separated the next morning. The day of separation is counted here as d 0. As a rule of thumb, with an average of 12 fetuses/animal, it would take about seven timed-pregnant rats to prepare 50 replicate cultures of 16-d embryonic rat telencephalon cells; or 10 animals for 50 replicate cultures of 15-d embryonic rat telencephalon cells. Ideally, 2–3 persons should participate, setting up a large number of replicate cultures: One person can kill the timed-pregnant animals and remove the embryos, the other(s) can do the dissection in a sterile hood with horizontal air flow. During the entire dissection, the biological material is kept cold (close to 4°C).

3. PREPARATION FOR DISSECTIONS

3.1. Materials

Puck's salt solution D and gentamicin-sulfate (Puck's D-GS) (*see* Section 11.3., items 1 and 2).
Tube (1), plastic, sterile, 50-mL.
Petri dishes (2), plastic, 60-mm.
Ice bucket and ice.

3.2. Preparation

1. Add 40 mL Puck's D-GS to a 50-mL plastic tube (for the storage of the dissected tissue).
2. Add 25 mL Puck's D-GS to additional 50-mL plastic tubes (one tube for each pregnant animal, for the reception and transfer of the fetuses); place the tubes in ice.
3. Set up the sterile hood for the dissection (Fig. 1A).
4. Add 8 mL Puck's D-GS to two 60-mm plastic Petri dishes, and place them on the dissecting block (or on ice, if no dissecting block is available).
5. Set up the lab for the decapitation and the excision of the whole uterus containing the embryos.

4. REMOVAL OF RAT EMBRYOS

Prior to the removal of fetuses from the pregnant rat, assemble all required instruments and solutions in the laminar flow hood.

4.1. Materials

Scissors (1), large.
Scissors (1), small, pointed.
Forceps (1), large.
Forceps (1), small, curved.
Plastic 50-mL tube containing 25 mL ice-cold Puck's D-GS (1/pregnant animal).
95% Ethanol denatured with 2% ketone.
Gauze squares (4), 2 × 2-in.
Absorbent toweling.

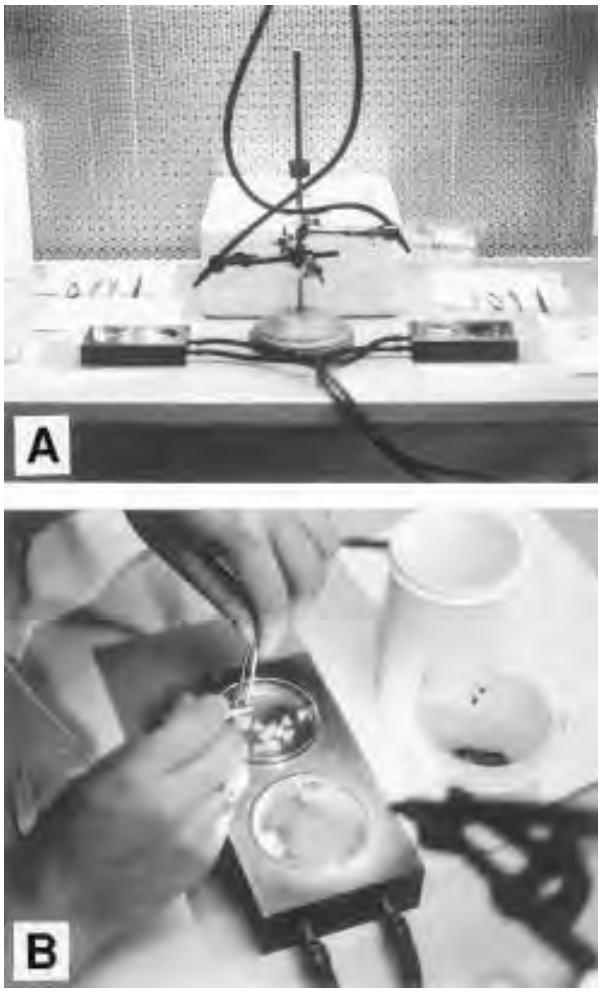

Fig. 1. Dissection of embryonic neural tissue. **(A)** Setup of the sterile hood for dissection (for two persons). The dissecting blocks are perfused with an ice-cold liquid and illuminated by a lamp with fiber optics. The dissection instruments lie on sterile pads. A styrofoam box filled with ice is used to store the tubes and Puck's D-GS. **(B)** Close-up of a dissecting step: extraction of the embryonic brain.

4.2. Dissecting Out Embryos

Note: The following procedure is performed outside of the laminar flow hood; extra caution should be exercised to prevent contamination.

1. Sterilize instruments using the hot-bead sterilizer. When the temperature reaches 250°C, insert dry and clean instruments for at least 5–10 s, depending on their size. Instruments may also be sterilized by autoclaving prior to needing them, or by immersing them in 70% ethanol for 5–10 min and drying them inside the laminar flow hood.
2. Sacrifice the pregnant rat by decapitation. (Make sure to avoid all unnecessary stress to the animal, and to follow the ethical guidelines).

3. Lay the decapitated animal on its back on an adsorbent towel, and, using the gauze, swab down the abdomen with 95% alcohol.
4. Using the large forceps, grasp the skin about 1 cm above the genitalia. With the large scissors, cut through the skin and fascia, and extend the cut on both sides of the abdomen, until the entire peritoneal cavity lies open.
5. Remove the uterine horns containing the fetuses, using small curved forceps and small pointed scissors, and transfer them to the 50-mL plastic tube containing 25 mL ice-cold Puck's D-GS.
   Note: All subsequent procedures are performed in the laminar flow hood.

5. DISSECTION OF EMBRYONIC BRAIN TISSUE

5.1. Materials

Scissors (1), curved Vannas.
Forceps (1), fine curved.
Forceps (1), extrafine, Dumont no. 5.
Scalpel holder and no. 11 blade.
Cold Puck's D-GS (1–2 bottles of 500 mL) (*see* Section 11.3., items 1 and 2).
Plastic 50-mL tube (1) filled with 40 mL Puck's D-GS.
Petri dishes, plastic, 60-mm, fitting on a dissecting block or on ice (3/pregnant rat).
Ice bucket and ice.

5.2. Dissection of Embryonic Brain Tissue

1. Sterilize instruments using the hot-bead sterilizer. When the temperature reaches 250°C, insert dry and clean instruments for at least 5–10 s, depending on their size. Instruments may also be sterilized by autoclaving prior to needing them, or by immersing them in 70% ethanol for 5–10 min and drying them inside the laminar flow hood.
2. Transfer the uterine horns to one of the Petri dishes placed on the cold dissecting block.

Note: Make sure that during the entire dissection procedure (until step 10), the biological material is always immersed in cold Puck's D-GS.

3. Open the uterine horns with the Vannas scissors, and, using small forceps, transfer the embryos to a new Petri dish.
4. Using the Vannas scissors and small forceps, separate the embryos from their amniotic sacs and placentae.
5. Transfer one embryo to a new Petri dish containing Puck's D-GS, and dissect the brain.
   a. Hold the head of the embryo fixed in a lateral position with the aid of the Dumont forceps (Fig. 1B).
   b. Make an extended lateral incision at the base of the brain using the Vannas scissors, by cutting skin and cartilage from the region of the brain stem to the most frontal point.
   c. Extract the entire brain by lifting it through this opening, using the blunt side of the closed Vannas scissors. Keep the brain, and discard the rest of the embryo.
6. Repeat step 5 for the rest of the fetuses from the same animal.
7. Using the scalpel, dissect the region(s) to be selected for preparation of cultures.
8. Transfer the dissected brain tissue to the 50-mL plastic tube filled with 40 mL Puck's D-GS.

Note: During dissection, replace the supernatant of this tube several times with fresh, cold Puck's D-GS.

9. Repeat steps 3 and 4 of Sections 4.2. and 5.2. for each pregnant rat.

Note: The duration of the dissection should not exceed 2 h. An experienced person can dissect 40–60 embryos/h.

6. MECHANICAL DISSOCIATION OF EMBRYONIC BRAIN TISSUE

To dissociate the dissected and collected brain tissue, a sieving procedure, according to Rose (1965), Schrier (1973), and Varon and Raiborn (1969), is used in a slightly modified form (Honegger and Richelson, 1976).

6.1. Materials

Chemically defined medium (*see* Section 11.1.).
Cold Puck's D-GS (500 mL) (*see* Section 11.3., items 1 and 2).
Nylon-mesh bag (1–2), 200-μm pore size, fitted around a glass funnel.
Nylon-mesh bag (1), 115-μm pore size, fitted around a glass funnel and attached with tape (*see* Section 11.2., item 3).
Glass rods (1–2), 0.6-cm diameter, 20-cm long, with blunt, fire-polished ends.
Centrifuge tubes (two/batch), plastic, 50-mL conical.
Plastic culture flask of appropriate size to hold the total culture volume.
Nigrosin or trypan blue for cell viability tests.
Hemocytometer (or electronic cell counter).
Ice buckets (2), filled with ice.

6.2. Preparation

1. Set up the laminar flow hood for the dissociation procedure.
2. If necessary, divide the pooled brain tissue into batches of not more than 50 whole brains (or 100 brain parts) per batch.
3. Rinse each batch of tissue 3× with about 40 mL cold Puck's D-GS.
4. Add 25 mL cold Puck's D-GS to the 50-mL plastic tubes (1/batch), and place them in ice, together with the empty 50-mL tubes (1/batch).

6.3. Dissociation Protocol

1. Rinse the collected brain tissue 3× with cold Puck's D-GS.
2. Place the 200-μm mesh bag (with a glass funnel fitted in the inside) in a 50-mL conical plastic tube containing 25 mL Puck's D-GS. Keep on ice.
3. Pour the pooled brain tissue of one batch into the bag. Rinse the funnel, if necessary, with 2 mL Puck's D-GS.
4. Remove the funnel, close the bag, and hold its upper ends against the outer side of the tube wall, *making sure that the tissue remains immersed.*
5. Using the glass rod, gently stroke downward, from the outside of the bag, to squeeze the tissue through the mesh into the surrounding solution.
6. After this first dissociation step, place the second nylon bag (115-μm mesh), attached with tape to a short glass funnel, on top of an empty 50-mL conical plastic tube.
7. Using a 5-mL serological plastic pipet, transfer the cell/tissue suspension to the nylon bag, and let the suspension pass through the filter by gravity flow into the 50-mL tube. Toward the end of the filtration, add Puck's D-GS up to a final volume of 50 mL.

Note: If there is a second batch, repeat steps 1–7.

Table 1. Outline of Preparation of Aggregating Neural Cell Cultures

Day 0	Day 1	Day 2
Fetal CNS tissue, pooled and rinsed	Orbital shaker: 73 rpm (morning) addition of 4 mL medium	Transfer of aggregates to 50-mL Erlenmeyer flasks
Mechanical dissociation in Puck's D-GS Passage through 200-μm nylon mesh Filtration through 115-μm nylon mesh Centrifugation (300*g*, 15 min, 4°C) Resuspension of pellet (trituration) Cell count Centrifugation (300*g*, 15 min, 4°C)	74 rpm (evening)	Orbital shaker: 77 rpm
Resuspension of pellet (trituration) in cold serum-free medium Final cell density: 7.5×10^6 cells/mL		
Incubation (CO_2 incubator, 37°C) Aliquots (4-mL) in 25-mL Erlenmeyer Orbital shaker: 68 rpm (at start) 70 rpm (evening)		

8. Centrifuge the resulting filtrate (300*g*, 15 min at 4°C, with slow acceleration and deceleration).
9. After centrifugation, place the tube(s) on ice:
   a. Discard the supernatant, using a pipet.
   b. Add 2.5 mL fresh, cold Puck's D-GS, and resuspend the pellet by trituration (5–6 strokes up and down, using a 5-mL plastic pipet, but *avoid foaming*).
   c. Bring the volume of this suspension to 50 mL with cold Puck's D-G, and take a 0.1-mL aliquot for cell counting.
10. Centrifuge the cell suspension(s) (300*g*, 15 min at 4°C, as above).
11. Count the cells (from step 9c):
    a. Determine the total cell number in each batch. An aliquot of 0.1 mL cell suspension is mixed with 50 mL Puck's D-GS (dilution 1:500), and the total cell number is determined by the use of either a hemocytometer or an electronic cell counter.
    b. Viability: It may be useful (particularly for beginners) to also examine cell viability (determination of the percentage of cells that exclude a test dye, such as nigrosin or trypan blue). Usually, this test shows a viability of about 50%.
12. At the end of the second centrifugation, discard the supernatant, and resuspend the pellet by trituration (as described for step 9b).
13. Fill the plastic culture flask with the appropriate amount of cold culture medium, to obtain a final cell density of 7.5×10^6 total cells/mL. Add the cell suspension to the medium, and mix well.

Note: The final cell density should be increased if tissue of a more advanced developmental stage is used for dissociation.

7. INITIATION AND MAINTENANCE OF AGGREGATING NEURAL CELL CULTURES

Culture initiation is the most critical step in the entire procedure, because in order to obtain truly replicate cultures, it is crucial that all steps in the procedure are rigorously adhered to (Table 1). This is particularly important for the phase of cell reaggregation under gyratory agitation, since a balance between attracting and shearing forces must be established. For the importance of uniformity of culture flasks, *see* Section 11.2., item 2.

Cell aggregation starts immediately after the cell suspension has been placed in the incubator. However, during the period of warming up, the pH of the culture medium will rise above physiological values.
In order to keep this critical period as short as possible, two people should work together during culture initiation, one who pipets the aliquots of the cell suspension, and one who transfers the flasks to the incubator.

7.1. Materials

Erlenmeyer flasks, 25-mL (*see* Section 11.2., item 2).
Erlenmeyer flasks, 50-mL (*see* Section 11.2., item 2).
Gas-permeable plastic caps for Erlenmeyer flasks (Bellco Biotechnology, Vineland, NJ).
Gyratory shaker, 25-mm shaking diameter (*see* Section 11.2., item 1).

7.2. Protocol for Culture Initiation and Maintenance

1. Prepare the required number of 25-mL Erlenmeyer flasks (Schott Duran, modified, as described in Section 11.2.), and loosen the gas-permeable plastic caps.
2. Transfer 4-mL aliquots of the final cell suspension to the 25-mL Erlenmeyer flasks.
3. Place the flasks onto the platform of a rotating gyratory shaker in the CO_2 incubator (Fig. 2) at 37°C, with a CO_2 atmosphere in accordance with the bicarbonate concentration in the medium (e.g., 10% CO_2 and 90% humidified air, with medium containing 3.7 g bicarbonate/L). The initial frequency of agitation is 68 rpm.
4. On the evening of the day of culture preparation (d 0), increase the agitation frequency to 70 rpm.
5. The next day, increase agitation to 73 rpm (in the morning), then to 74 rpm (in the evening).
6. At d 2, transfer the cultures to 50-mL modified Erlenmeyer flasks equipped with gas-permeable plastic caps. Pour the content of each flask carefully along the glass wall of the new flask, and repeat exactly the same movement by adding an additional 4 mL fresh, prewarmed medium, with which the original flask was rinsed. Ideally, *no aggregates should remain attached on the wall of the new culture vessel.*

Note: Again, this procedure will cause a rise in the pH of the medium (as at culture initiation [*see above*]). Therefore, care should be taken to keep this operation as short as possible (not exceeding 30 min/series). Allow to equilibrate for 1–2 h, before a further series is transferred.

7. After completion of the transfer, increase the frequency of agitation to 77 rpm, and, thereafter, increase it every day by 1 rpm, until the maximal rotation speed (80 rpm) is attained at d 5.
8. At d 5, culture medium is replenished for the first time:
   a. Warm the required amount of fresh culture medium in a water bath.

Fig. 2. View inside an incubator containing a gyratory shaker. The culture flasks (here 50-mL Erlenmeyer flasks with gas-permeable caps) are kept in ranges of 10 (max load 100 flasks). Attached to the shaker platform is a thermometer to show the local temperature (kept between 36.5 and 37°C).

b. Put the culture flasks, in groups of five, on a slanted support, and allow the aggregates to settle.
c. With a 5-mL pipet, remove 5 mL medium, and add an equal volume of fresh medium.

Note: To avoid contamination, always use a fresh pipet with fresh medium.

9. The medium is replenished every third day until d 14, and every other day thereafter.
10. At 20 d, because of the greatly increased metabolic rate of the cultures, the content of each flask must be divided into two separate cultures ("splitting"):
    a. Use a 2-mL plastic pipet to split the cultures. Tilt the flask containing the aggregates, quickly resuspend the aggregates by short aspiration and brisk expulsion of the supernatant medium, then quickly transfer a 2-mL aliquot to a fresh flask containing 4 mL medium.
    b. Transfer a second 2-mL aliquot in exactly the same way.
    c. Replace the missing volume in the original flask with 4 mL fresh medium.

    Note: The new culture flasks, containing 4 mL of fresh medium, must be equilibrated in the incubator for at least 1 h, before splitting.
11. A second split may be necessary at d 30.

8. BRAIN CELL AGGREGATES ENRICHED WITH EITHER NEURONS OR GLIAL CELLS

It may sometimes be necessary to compare the regular aggregate cultures with aggregate cultures enriched with either neurons or glial cells. The preparation of aggregates from cells enriched previously in monolayer culture was found to be tedious, and the resulting cultures difficult to grow (Guentert-Lauber et al., 1985). Therefore, a pharmacological approach was taken (Honegger and Werffeli, 1988; Corthésy-Theulaz et al., 1990), to eliminate either the glial cells by an early treatment with cytosine arabinoside or the neurons by a single treatment with cholera toxin.

8.1. Preparation of Neuron-Enriched Aggregate Cultures

In aggregate cultures of embryonic rat telencephalon, the majority of glial cells proliferate during the first week. Therefore, cultures treated during this early stage with cytosine arabinoside (Sigma, cat. no. C-1768) contain mostly neurons, and only few glial cells. Cytosine arabinoside, 0.4 μ*M* final concentration, is given twice during the first week, i.e., at d 2 and 4, to cultures derived from 15-d embryonic telencephalon, and, at d 1 and 2, to cultures of 16-d embryonic telencephalon cells. However, the virtual absence of glial cells greatly affects neuronal survival and maturation. Therefore, supportive measures are required. This is best achieved by regularly replenishing the culture media with conditioned medium obtained from mixed-cell parallel cultures, and diluted 1:1 with fresh medium (Honegger and Pardo, 1999). Alternatively, cultures may be maintained in depolarizing concentrations of KCl (30 m*M* final concentration) from d 7 onward (Riederer et al., 1992).

8.2. Preparation of Glial Cell-Enriched Aggregate Cultures

Aggregate cultures derived from 15-d embryonic rat telencephalon are treated at d 7 (i.e., 24 h prior to the regular medium change) with a single dose of cholera toxin (100 n*M*). During the following 2 wk, the neurons progressively disappear from the aggregates, giving rise to glial cell-enriched aggregate cultures.

Note: The commercially available cholera toxin (*Vibrio cholerae* type Inaba 569B from Gibco-BRL or Calbiochem) usually contains NaN_3, which is cytotoxic (Calbiochem now offers an azide-free preparation for tissue culture). To remove the azide, cholera toxin is dialyzed against 0.9 % NaCl, buffered with HEPES (0.5 m*M*, pH 7.4), and subsequently sterilized by filtration (0.2 μm). It should be kept in mind that cholera toxin is a very labile compound; thus, solutions of cholera toxin should not be frozen and thawed repeatedly.

9. ANALYSIS OF AGGREGATING NEURAL CELL CULTURES

Initially, aggregating cell cultures were used exclusively for light microscopic morphological studies (e.g., Moscona, 1961; Delong and Sidman, 1970). The application of biochemical criteria was introduced by Seeds (1971), and thereafter has been considerably extended, and combined with the techniques of molecular biology. Furthermore, morphological studies, at the electron microscopic level, and cytochemical evaluations have been introduced.

For the different analytical approaches, aggregates are washed with phosphate-buffered saline (PBS) (*see* Section 11.3., item 3). For morphological analyses, the PBS is prewarmed; for biochemical and molecular biological techniques, the PBS is used ice-cold. In stationary suspensions, the sedimentation of aggregates by gravity is sufficiently rapid; only very early cultures (in vitro d 1–2) require gentle centrifugation (75*g*, 1 min) for washing.

9.1. Morphological Analyses

Aggregating neural cell cultures, in contrast to monolayer cell cultures, do not permit direct microscopic observation; they require an approach similar to that used for tissue fragments. However, all basic light microscopic and electron microscopic techniques can be applied.

1. Fixation and embedding:
   Specific fixation and embedding procedures are used for immunocytochemistry (ICC), histochemistry, and electron microscopy. For immunocytochemistry, the best preservation of antigenicity is obtained by omitting tissue fixation. Therefore, cryostat sections are rou-

tinely used. In certain cases, immunostaining can be obtained on paraffin sections, after a classical deparaffination.

a. For cryostat sections (10 μm), the PBS-washed aggregates are transferred to a gelatin capsule (Feton International, Brussels, no. 00), by using a 1-mL plastic pipet. The supernatant is then removed with a Pasteur pipet, and the aggregates are covered with cryoform (Cryomatrix; Shandon Scientific, Runcorn, Cheshire, UK). The capsule is immediately immersed in isopentane cooled in liquid nitrogen. The temperature of the isopentane is critical for good tissue preservation. It is optimal when the first white spots of solid isopentane appear at the bottom of the container. Frozen capsules are transferred to a plastic tube kept in dry ice, to prevent accidental fracturing. They are stored at –80°C. The day before cutting on the cryostat, the frozen capsules are transferred to –20°C. This procedure was found advantageous for work with antibodies directed against glial fibrillary acidic protein (GFAP), vimentin, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein, and neurofilaments.
b. For cryostat sections after fixation, aggregates are washed in PBS and fixed for 30 min at room temperature (RT) in a mixture containing 4% paraformaldehyde, 15% of a saturated aqueous solution of picric acid, and 0.08% glutaraldehyde. The aggregates are washed in sucrose solutions of increasing concentrations (successively in 10, 20, and 30%, for 30 min at each concentration). They are then processed for cryostat section (*see* a. above). This procedure is used for staining with antibodies directed against choline acetyltransferase (ChAT), glutamic acid decarboxylase (GAD) and the neuronal glutamate transporter excitatory amino acid carrier 1. These stainings permit the localization of cholinergic, GABAergic, and glutamatergic neuronal subtypes.
c. For paraffin sections, the aggregates are fixed either in 4% paraformaldehyde or in Carnoy solution (for immunocytochemistry, histochemistry, and regular light microscopy). For paraformaldehyde fixation, the PBS-washed aggregates are immersed in PBS containing 4% paraformaldehyde, for 1 h at 4°C. For Carnoy fixation, the aggregates are transferred to glass tubes. The supernatants are discarded, and freshly prepared Carnoy solution (for formula, *see* Section 11.3., item 4.) is added for 1 h at room temperature. The Carnoy solution is changed once, after 15 min of fixation. The aggregates are then dehydrated by sequential passage to 100% ethanol (2×15 min), ethanol–toluol (15 min), and toluol (15 min). The fixed aggregates are transferred to gelatin capsules (Feton, 000), and placed on a prewarmed support in an oven kept at 60°C. The aggregates are covered with melted paraplast, using a prewarmed 1-mL disposable plastic pipet. Thirty minutes later, the paraffin is removed and replaced by a fresh bath. After two more baths of 30 min each, the support with the capsules, is taken out of the oven. Once room temperature is attained, the gelatin capsules are removed, and the paraffin cylinders are embedded in paraffin blocks. This procedure is used for staining with antibodies directed against GFAP, microtubule-associated protein 2 (MAP2), and neurofilaments.
d. For electron microscopy, the PBS-washed aggregates are fixed for 1 h at room temperature in PBS containing 1.5% glutaraldehyde and 0.5% paraformaldehyde. Thereafter, they are rinsed in PBS for 1 h, then transferred to 1.5-mL Eppendorf tubes. The aggregates are postfixed for 1 h in 2% osmium tetroxide, and dehydrated in ethanol (successively in 30, 70, 90, and 100%, for 10 min at each concentration), and finally in propylene oxide for 5 min. The aggregates are then embedded in TAAB 812™, either in bean capsules (8-mm) or in gelatin capsules (Feton, no. 0). Between each step of the dehydration and embedding protocol, the aggregates are sedimented by centrifugation (500*g*, 5 min).

2. Immunocytochemistry:
   For cryostat sections, the pre-equilibrated (–20°C) block is attached to the cryostat support with cryoform, and the gelatin capsule is removed with the use of a razorblade. Ten-µm cryostat sections are transferred to glass slides coated twice with undiluted polylysine (slide adhesion solution, Sigma, cat. no. P-8920) and kept at room temperature. The sections are fixed in acetone (10 min at room temperature), then kept overnight at 4°C, until staining. For best results, staining is always done on freshly cut sections.
   a. Immunofluorescence staining: For immunofluorescence staining, the sections are rehydrated in PBS for 10 min, the unspecific sites blocked with serum (normal rabbit serum, Dako, cat. no. X-0902, if monoclonal antibodies are used; normal swine serum, Dako, cat. no. X-0901, with polyclonal antibodies; both diluted 1:25 in PBS), then exposed to the primary antiserum (overnight at 4°C in a moisture chamber). Thereafter, they are washed 3× for 20 min in PBS, then exposed (30 min) to the fluorescent secondary antibody (Dako, 1:50 in PBS, filtered just before use through Gelman Acrodisc PF, cat. no. 4187, 0.8/0.2 µm). Then the sections are washed again in PBS (20 min), and mounted in a semipermanent medium, according to Lennette (1978). The polymerization of the medium requires 1–2 h at 4°C. The sections are stored at 4°C.
   b. Peroxidase staining: For peroxidase staining, the sections are first treated for 30 min with methanol containing 0.3% H_2O_2, then with 95% ethanol, and finally dried in the air. The dried sections are encircled with a delimiting pen (Dako, cat. no. S-2002). After rehydration with PBS, the same procedure is used as described above for the immunofluorescence.

      Note: Using cryostat sections of fixed aggregates, antigenic sites must be exposed prior to the traditional staining procedure. This is achieved by exposing cryostat sections immersed in 10 m*M* citrate buffer, pH 6.0, to microwaves for 5 min at 800 W. During the 5 min, the heating procedure is interrupted 3× in order to add buffer solution. The sections are cooled for 20 min at room temperature, then washed 3× in PBS.

      Biotinylated immunoglobulins and avidin–biotin bound to peroxidase are obtained from Vector Laboratories. After avidin–biotin (30 min), sections are sequentially rinsed, first in PBS (15 min), followed by Tris-HCl, pH 7.6 (5 min); Tris-HCl containing 0.2% nickel sulfate (10 min); 0.05% diaminobenzidine/0.2% nickel sulfate (10 min); and finally in 0.05% diaminobenzidine/0.003% H_2O_2/0.2% Ni sulfate (5 min). This Ni enhancement is according to Adams (1981). After two washes in Tris-HCl, the sections are counterstained with 0.5 % toluidine blue (30 s), differentiated, dehydrated, and mounted in Pertex (Histolab, Göteborg, Sweden).
3. Histochemistry: Histochemical staining is used for the detection of microglial cells in rat brain cell aggregate cultures (Monnet-Tschudi et al., 1995). Microglial cells express a surface glycoprotein recognized specifically by the isolectin B4 isolated from *Griffonia simplicifolia*. This staining technique was first described by Streit and Kreutzberg (1987), and Ashwell (1990). In brief, the 5-µm paraffin sections are deparaffinized and rehydrated, then blocked with H_2O_2 (0.3% in methanol), followed by ethanol 100 and 96%. The dried sections are encircled with a delimiting pen (Dako) and rehydrated in Tris saline (*see* Section 11.3., item 5). They are incubated overnight at 4°C in isolectin B4 from *G. simplicifolia* conjugated with peroxidase (Sigma L-5391; 12.5 mg dissolved in 1 mL Tris saline containing 1% Triton X-100). For the peroxidase staining with nickel enhancement, the same procedure is used as described above for immunocytochemistry.

4. Autoradiography: For autoradiography, both cryostat sections and paraffin sections can be used. The dried sections are dipped in Ilford L4 emulsion and exposed for 4 wk. They are developed in phenizol (Ilford no. 34 Do 57; dilution 1:4 in water), dried, and, finally, counterstained with toluidine blue, as described in Section 9.1., step 2b.
   Note: For combined Ab staining/autoradiography, the Ni enhancement procedure, described in Section 9.1., step 2b, is omitted.

9.2. Biochemical Analyses

Because of the high reproducibility of aggregate cultures, and the possibility of preparing numerous replicate cultures that contain relatively large amounts of material, this culture system is particularly suitable for multidisciplinary studies. Biochemical analyses are most convenient for quantitative routine assays. For example, by measuring the activities of neuronal and glial marker enzymes (point 1), developmental events and changes in the cellular composition can be easily monitored. Metabolic labeling studies are available to monitor cell proliferation (point 2), synthesis of proteins and neurotransmitters (Honegger and Richelson, 1979), or changes in metabolic activity (point 3). Furthermore, the expression of specific proteins can be studied at both the transcriptional and the protein level, following standard protocols (point 4). For more detailed studies, subcellular extraction protocols can be applied, e.g., for the quantitative extraction of synaptosomes or myelin (point 5). In most cases, it is sufficient to use three parallel cultures for quantitative analysis. Often, only a fraction of one flask is necessary for a series of assays. It is also possible to perform sequential assays with aggregates from the same flasks, e.g., for sampling at different time points. Examples of some tested applications are given below:

1. Enzymatic activities, total protein, and DNA content:
   The aggregates are washed twice in 5 mL ice-cold PBS and homogenized in 2X 250 µL 2 m*M* potassium phosphate containing 1-m*M* glass homogenizers (Bellco). The homogenates are briefly sonicated (two 2-s pulses of 30 W, using a microtip), divided into aliquots for the different assays, and stored at –80°C for analysis. The activities of cell type-specific enzymes, such as ChAT, GAD, tyrosine hydroxylase (all neuron-specific), glutamine synthetase (representing astrocytes), and 2',3'-cyclic nucleotide 3'-phosphohydrolase (typically oligodendroglial), are determined using either radiometric or colorimetric assays (Seeds, 1971; Wilson et al., 1972; Honegger and Richelson, 1976; Honegger and Schilter, 1992). Specific enzymatic activities are expressed as a function of either the protein or the DNA content.

   Total protein is determined either by the Folin phenol method (method of Lowry et al., modified by Wilson et al., 1972) or by the Coomassie blue dye binding method of Bradford (1976), using the Bio-Rad dye reagent (cat. no. 500-0006), according to the protocol of the supplier. The DNA content is measured by a fluorimetric assay (Downs and Wilfinger, 1983).
2. Mitotic activity:
   DNA synthesis is determined by measuring the incorporation of [^{14}C]thymidine into a macromolecular fraction (Lenoir and Honegger, 1983). The cultures are incubated for 4 h in regular medium containing [^{14}C-methyl]thymidine (0.5 µCi/flask, specific activity 61 mCi/mmol). Aggregates are then washed 3× with ice-cold PBS, homogenized in 0.4 mL 0.05% (v/v) Triton X-100, using conical glass–glass homogenizers (Bellco), and sonicated briefly. Aliquots (20 and 40 µL) of the homogenate are placed on separate glass-fiber filter disks (Whatman GF/A 2.5 cm), and dried under an infrared heat lamp. The filters are then transferred to supports on a filtration manifold, and washed once with 5 mL 10% (w/v) trichloroacetic acid and 3× with 5 mL 5% trichloroacetic acid. Thereafter, they are rinsed in 200 mL of ethanol–ether (1:1), and subsequently in 200 mL ether. The filters are then transferred to

glass scintillation vials, air-dried, and incubated overnight with 0.5 mL NCS tissue solubilizer (Amersham). After neutralization of the digest with 17 µL glacial acetic acid, 10 mL scintillation cocktail (Optifluor, Packard) is added for counting.

3. Uptake of 2-deoxyglucose:
   To measure the uptake of radiolabeled 2-deoxyglucose (Honegger and Pardo, 1999), replicate aggregate cultures are used, corresponding to one-sixth to one-fourth of the amount of aggregates present in the original flask. Aggregates from several flasks are pooled, and their medium replaced by fresh growth medium containing 5.5 m*M* of D-glucose. For the aliquoting and transfer of the aggregates, 2-mL plastic pipets are used. Aliquots (4 mL) of the aggregate suspension are distributed to 25-mL culture flasks and equilibrated for 1 h under normal culture conditions before initiation of the 2-deoxyglucose uptake. The uptake measurement is started by the addition of [1,2-^{3}H]2-deoxy-D-glucose (specific activity 25 Ci/mmol, final concentration 125 nCi/mL; DuPont/NEN). After incubation for 30 min under normal culture conditions, the aggregates are transferred to 15-mL conical plastic tubes in ice, and washed 4× with 4 mL ice-cold PBS. The sedimented aggregates are then resuspended in 0.5 mL 50 m*M* NaOH and subjected to a freeze–thaw cycle with subsequent sonication. Of the resulting homogenates, 0.4-mL aliquots are taken for scintillation counting after addition of 7 mL Pico-Aqua (Packard) scintillation fluid. Furthermore, 20-µL aliquots are taken for protein determination by the Lowry method. Background levels of radioactivity are determined in replicate cultures maintained at 4°C.
4. Protein expression:
   a. Total cellular RNA:
      Total cellular RNA can be extracted from the cultures by using standard protocols, e.g., according to Chomczynski and Sacchi (1987). From the aggregates of each flask, 100–200 µg total RNA can be obtained, depending on the developmental stage of the cultures. Isolated total RNA, or mRNA (obtained by further purification), will serve for specific applications, such as Northern blot analysis, polymerase chain reaction, and cDNA cloning (Corthésy-Theulaz et al., 1990; Bardoscia et al., 1992; Taylor et al., 1994; Appel et al., 1995).
   b. Relative rate of protein synthesis:
      The relative rate of protein synthesis can be examined by metabolic labeling, using [^{35}S]-methionine as a radioactive tracer in serum-free medium lacking unlabeled methionine (Corthésy-Theulaz et al., 1990). The labeled cultures are washed in ice-cold PBS containing 5 µg/mL cycloheximide, and solubilized in 0.4% deoxycholic acid, 1% Nonidet P-40, 60 m*M* EDTA, and 10 m*M* Tris-HCl, pH 7.4, containing protease inhibitors (10 µg/mL each of leupeptin [Sigma, cat. no. L-0649], antipain [Sigma, cat. no. A-6191], and pepstatin [Sigma, cat. no. P-4265]); 5 µg/mL E-64 (Sigma, cat. no. E-3132); and 1 m*M* aminoethylbenzene-sulfonyl fluoride (Sigma, cat. no. A-5938). The nonsolubilized material is eliminated by centrifugation (2 min, 8000*g*). The resulting supernatant is used for further purification steps, including immunoprecipitation, and for final analyses. Inhibition of protein synthesis at the transcriptional and translational levels can be achieved according to Pan and Price (1984), using actinomycin D (0.1 µ*M* final concentration) and cycloheximide (1 µ*M* final concentration), respectively.
   c. Western blot analysis:
      For Western blot analysis (Riederer et al., 1992; Monnet-Tschudi et al., 1995), PBS-washed aggregates are homogenized in 2 m*M* phosphate buffer, pH 6.8, in the presence of protease inhibitors (10 µg/mL each of leupeptin, antipain, and pepstatin); 5 µg/mL E-64 (Sigma, cat. no. E3132) and 1 m*M* aminoethylbenzene-sulfonylfluoride, as described

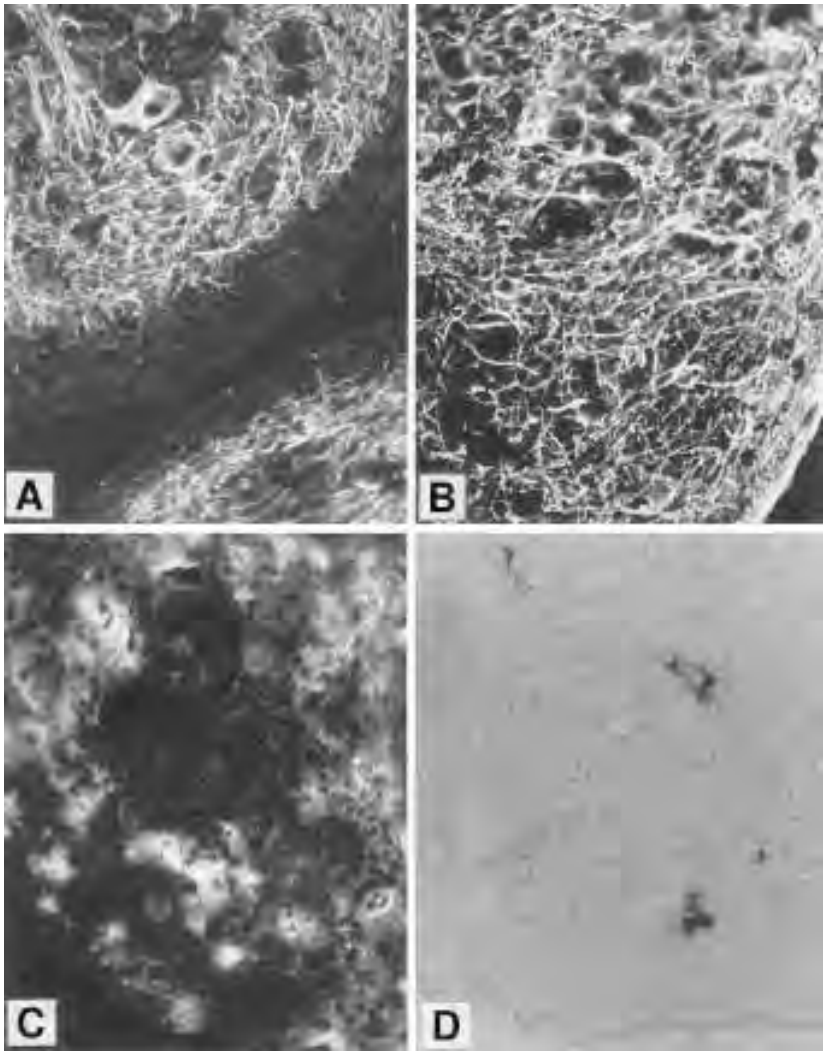

Fig. 3. Examples of morphological analyses of aggregating cell cultures of fetal rat telencephalon. **(A)** Immunostaining for neurofilament protein (NF-M); **(B)** immunostaining for glial fibrillary acidic protein (GFAP); **(C)** immunostaining for myelin basic protein (MBP); **(D)** histochemical staining for microglial cells, using isolectin B4 from *Griffonia simplicifolia*. (A–C) Cryostat sections of aggregates grown for 35 d in chemically defined medium. (D) Paraffin section of 15-d aggregate fixed in Carnoy solution. Magnification: +400.

above. Proteins (10–50 μg/slot) are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels are taken for the electrophoretic transfer of proteins to nitrocellulose sheets (BA-S 85; Schleicher and Schuell). Specific proteins are probed by incubation overnight with specific antibodies. Blots are revealed either by the peroxidase-conjugated antibody technique, using peroxidase-conjugated rabbit antimouse IgG (Dako, cat. no. P-0260) for monoclonal antibodies, or goat antirabbit IgG (Nordic) for the polyclonal antibodies, both diluted $1:10^3$, and either the chromogen chloro-1-naphthol or the chemiluminescence kit of Boehringer Mannheim. The nitrocellulose sheets

are exposed to hyperfilm MP (Amersham), which is then developed in a Curix 60 (Agfa) developing apparatus.

d. Enzyme-linked immunosorbent assay and radioimmunoassay:
For the quantitation of specific proteins by enzyme-linked immunosorbent assay or by radioimmunoassay, total culture homogenates can be used, e.g., to measure MBP content by radioimmunoassay (Kerlero de Rosbo et al., 1990).

5. Subcellular fractions:
Pooled aggregates from a minimum of five flasks, taken at an advanced developmental stage, can be used for the quantitative extraction of myelin (Matthieu et al., 1979), or for the preparation of synaptosomal fraction (Monnet-Tschudi et al., 1995).

10. CHARACTERISTICS OF SERUM-FREE AGGREGATING NEURAL CELL CULTURES

Aggregating cell cultures can be grown in up to 150 replicate cultures per batch, each flask containing more than 1000 individual aggregates. It is important to obtain even-sized aggregates with a final diameter of 300–400 μm. At the outset, the dissociated cells appear to reaggregate in a random fashion, but, in the formed aggregates, the cells reorganize in a tissue-specific way (Fig. 3). Thus, the neurons tend to be localized more toward the center of the aggregate, and oligodendrocytes and astrocytes are more concentrated toward the periphery. A small population of microglial cells is found scattered throughout the aggregates. No fibroblasts can be found in these cultures.

During the first 2 wk in vitro, most of the glial cells proliferate. Thereafter, little mitotic activity is observed. The differentiation of neurons and glial cells progresses for several weeks. Myelination of axons starts in the third week, and attains a maximum after 4 wk of culture. The neuronal maturation and synapse formation may persist for 2 mo or more. With the progression of maturation, the metabolic rate increases dramatically, and the neurons exhibit spontaneous electrical activity.

11. APPENDIX

11.1. Chemically Defined Culture Medium

Because aggregating neural cell cultures form tissue-like structures, many endogenous factors necessary for cell survival, development, and maintenance are provided by cell–cell interactions within the aggregates. It is probably for this reason that they were among the first neural cell cultures to grow in a serum-free, chemically defined medium (Honegger et al., 1979). The original defined medium has been modified only slightly through the years, and should be considered as a minimal growth medium. An increasing number of growth and maturation factors, which can be added to the chemically defined media described here, are able to enhance the development of neurons and glial cells. The protocol for the preparation of 10 L serum-free culture medium is as follows:

1. Purchase Dulbecco's Modified Eagle's Medium powder mix for 10 L (Gibco-BRL, cat. no. 52100-039), containing high glucose (4.5 g/L) and L-glutamine, but no bicarbonate and no pyruvate.
2. Fill a glass jar (Pyrex) with 8 L ultrapure water, and dissolve the powder under gentle continued stirring (use large magnetic stirring bar).
3. Add the following supplements:
Choline chloride (Sigma, cat. no. C-7527) 1.35 g

L-Carnitine (Fluka, Buchs, Switz, cat. no. 22018)	20 mg
Lipoic acid (Sigma, cat. no. T-5625)	2 mg
Vitamin B12 (Fluka, cat. no. 95190)	13.6 mg

4. Add 1 mL of each of the following 10^4-fold concentrated stock solutions of trace elements (stored frozen at –20°C):

$CdSO_4 \cdot 8H_2O$ (Merck, Darmstadt, Germany, cat. no. 2028)	50 µ*M*
$CuSO_4 \cdot 5H_2O$ (Merck, cat. no. 2790)	100 µ*M*
$MnCl_3 \cdot 4H_2O$ (Merck, cat. no. 5927)	50 µ*M*
Na_2SeO_3 (Serva, Heidelberg, Ger, cat. no. 30210)	150 µ*M*
$NaSiO_3 \cdot 5H_2O$ (Fluka, cat. no. 71746)	2.5 m*M*
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (Sigma, cat. no. M-0878)	5 µ*M*
$NiSO_4 \cdot 6H_2O$ (Merck, cat. no. 6727)	2.5 µ*M*
$SnCl_2 \cdot 2H_2O$ (Merck, cat. no. 7815)	2.5 µ*M*
$ZnSO_4 \cdot 7H_2O$ (Merck, cat. no. 8883)	50 µ*M*

5. Adjust the pH of the medium close to the final pH with NaOH (1 *N*) (*see* below).
6. Add sodium bicarbonate according to the CO_2 atmosphere used in the incubator (e.g., 3.7 g/L, if cultures are kept at 10% CO_2), and immediately gas with 10% CO_2 in air, until the pH is adjusted to 7.4.
7. Adjust the osmolarity of the medium to 340 ± 2 mOsm, by adding the required volume of water.
8. Sterilize the medium by filtration using MediaKap-10 hollow fiber filters (Microgon, 0.2 µm) and a peristaltic pump. Store the medium in 500-mL bottles (Pyrex) with gas-tight closures, at 4°C in the dark.
9. Just before use, complete the medium by the following additions:
   a. Basal Medium (Eagle) [BME] vitamins (Gibco-BRL, cat. no. 21040-035) from a 100-fold concentrated stock solution.
   b. Vitamin A alcohol and vitamin E (trace concentrations; *see* Note below).
   c. Transferrin (Sigma, cat. no. T-2252; 1 mg/L final concentration).
   d. Triiodothyronine (sodium salt, Sigma, cat. no. T-2752; 30 n*M* final concentration).
   e. Insulin (Sigma, cat. no. I-5500; 5 mg/L final concentration).
   f. Hydrocortisone-21-phosphate (Sigma, cat. no. H-4251; 20 n*M* final concentration).
   g. Linoleic acid (sodium salt; Sigma, cat. no. L-8134; 3 mg/L final concentration).
   h. Gentamicin sulfate (Sigma, cat. no. G-1264; 25 mg/L final concentration) as antibiotic.

Note: The stock solutions of transferrin, linoleic acid, triiodothyronine, insulin, hydrocortisone-21-phosphate are prepared as 10^3-fold concentrated aqueous solutions, and stored at –20°C. The gentamicin sulfate stock solution, 500-fold concentrated, is also stored at –20°C.

Cultures older than 20 d may be given albumin-bound lipids (Albumax II; Gibco-BRL, cat. no. 11021-011; final concentration 0.1% w/v), instead of linoleic acid. The Albumax II stock solution (stored at 4°C) is prepared 100-fold concentrated (10% w/v), and its pH adjusted to 7.4 with 0.2 *N* NaOH, using phenol red (15 mg/L final concentration, from a 50-fold concentrated sterile stock solution) as an indicator.

Preparation of the stock solution for vitamins A and E: 114 mg vitamin A alcohol (Fluka, cat. no. 95144) is dissolved in 200 µL absolute alcohol, mixed with 2.0 mL of α-tocopherol (Sigma, cat. no. T-3251), and stored at –20°C. Of this mixture, 50 µL is transferred to 10 mL of medium, sonicated, and added to one bottle (500 mL) of medium by sterile filtration (Gelman, Acrodisc PF, cat. no. 4187; 0.8/0.2 µm).

11.2. Materials

1. Gyratory shaker: Besides the standard equipment of a tissue culture laboratory, aggregating cell cultures require a gyratory shaker that fits inside a CO_2 incubator. Care should be taken to purchase a heavy-duty-type gyratory shaker built to function at 100% humidity. It should be driven by magnetic induction rather than by a driving belt, and it should not create too much heat in the incubator. The incubator, on the other hand, should be able to tolerate the minimal heat produced by the shaker.
2. Culture flasks: Uniform Erlenmeyer-type culture flasks are required for aggregating cell cultures. It is difficult to find a large batch of uniform so-called DeLong flasks on the market (between batches, there are enormous variations in the geometry of the flasks). Therefore, it is best to purchase a large batch of standard Erlenmeyer flasks directly from the factory (e.g., Schott Duran 25- and 50-mL Erlenmeyer flasks), if possible with unfinished necks, and to have the necks modified (short-straight neck, outside diameter 18 mm) suitable for a gas-permeable plastic cap (Belco). To adjust for an optimal vortex, it is best to keep the shaking protocol (*see* Section 7.), and to vary, if necessary, the volume of the culture medium in the flask.
3. Nylon-mesh filter sacs: The nylon mesh (nybolt, nylon monofilament, 200/360 and 11P-115, Swiss Silk Bolting Cloth, Zurich) can be purchased in large sheets. Suppliers for this material in Canada and the United States: B. & S. H. Thompson, PQ, and Tetko, Elmsford, NY. The filter sacs can be produced from double sheets by cutting with a suitable soldering iron (40 W for 200-µm mesh and 30 W for 115-µm mesh), so that the sacs are cut and sealed at once.

   The filter sacs and appropriate funnels are prepared in the following sizes:
   a. The 200-µm-mesh sacs are 3.5 cm large and 14 cm long; the appropriate funnels have a 12-cm-long stem and outside diameters of 2.5 cm (top) and 1.6 cm (bottom).
   b. The 115-µm-mesh sacs are 5.0 cm large and 5.5 cm long; the appropriate funnels have a 4-cm-long stem, and outside diameters of 4.5 cm (top) and 2.5 cm (bottom).

11.3. Solutions

1. Puck's solution D (Puck's D), according to Wilson et al., 1972:

NaCl	8 g/L
KCl	400 mg/L
$Na_2HPO_4 \cdot 7H_2O$	45 mg/L
KH_2PO_4	30 mg/L
D-Glucose	1 g/L
Sucrose, pH 7.4, 340 mOsm	20 g/L

   Store at 4°C
2. Puck's D-GS: Just *before use*, add to Puck's D 25 mg/L gentamicin-sulfate (Sigma, cat. no. G-1264). Adjust the pH to 7.4 with 0.2 *N* NaOH (approx 0.5 mL/500 mL Puck's D).
3. Phosphate-buffered saline:

NaCl	6.43 g/L
KCl	395.00 mg/L
$Na_2HPO_4 \cdot 7H_2O$	540.00 mg/L
D-Glucose	4.50 g/L
Sucrose	24.00 g/L
$MgCl_2 \cdot 6H_2O$	160.00 mg/L

Table 2. Useful Antibodies for ICCl Studies in Neural Cell Aggregates

Antigen recognized	Designation[a]	Dilution	Source
Choline acetyltransferase	ChAT (p)	1:500	Chemikon, AB144
Galactocerebroside	GalC (m)	1:50	Boehringer, no. 1351 621
Ganglioside GD3	GD3 (m)	1:10	Dr. Pallmann AG, München
Glial fibrillary acidic protein	GFAP (m)	1:800	BioMako/Sigma, no. 6077
Glial hyaluronate protein	GHAP (m)	1:40	Bignami and Ashner (1992)
Glutamate decarboxylase	GAD (p)	1:250	Chemikon, AB108
Glutamate transporter	EAAC1 (p)	1:800	Chemikon, AB1520
Glutamine synthetase	GS (p)	1:50	Juurlink et al. (1981)
Microtubule-associated protein 2	MAP2 (m)	1:10	Huber and Matus (1984)
Microtubule-associated protein 5	MAP5 (m)	1:10	Riederer et al. (1986)
Myelin basic protein	MBP (m)	1:500	Boehringer,1118 099
Myelin oligodendrocyte glycoprotein	MOG (m)	1:50	Linington et al. (1984)
Neurofilament 200 kDa	NF H (m)	1:100	Boehringer, no. 814 342
Neurofilament 160 kDa	NF M (m)	1:200	Boehringer, no. 814 334
Neurofilament 60 kDa	NF L (m)	1:10	Riederer et al. (1993)
Synaptophysin	SYN (m)	1:10	Boehringer, no. 902 314
Vimentin	Vim (m)	1:10	Dako, no. M-0725

[a] m and p indicate monoclonal and polyclonal Abs.

$CaCl_2$	200.00 mg/L
Gentamicin-sulfate, pH 7.3, 340 mOsm.	25.00 mg/L

Store at 4°C.

Note: Neutralize the solution before adding the Mg^{2+} and Ca^{2+} salts. Gentamicin-sulfate is purchased from Sigma (cat. no. G-1264)

4. Carnoy solution: Ethanol (100%):chloroform:glacial acetic acid, 30:15:5. Protect solution from light.
5. Tris saline for histochemistry:

Trizma-base	1.94 g/L
Trizma-HCl	13.22 g/L
NaCl	9.00 g/L

Dissolve in H_2O, final pH 7.4.

11.4. Washing the Glassware

The detergents for washing the culture glassware should be devoid of organic additives. We recommend using a product line like Neodisher® (Chem. Fabrik Dr. Weigert, Hamburg). The culture glassware should never be allowed to dry before it is cleaned. Used glassware is stored immersed in a basic detergent solution (e.g., Neodisher LM-10; 5% v/v). After washing with special detergent (e.g., Neodisher FT), the glassware is rinsed with cold tap water, then with a citric acid solution (e.g., Neodisher Z) to remove traces of detergent. Thereafter, the glassware is rinsed several times with deionized water, and finally with ultrapure water. After drying in an oven, the glassware is sterilized.

11.5. Sterilization Procedures

The culture flasks, with gas-permeable plastic caps in place, are sterilized in an autoclave (120°C) with pulsed vapor. After the first sterilization, they are wrapped in a layer of aluminum foil and

sterilized once more. The nylon filter bags, each with their glass funnel inside, are loosely wrapped in aluminum foil and autoclaved in a vapor-permeable box at 120°C. Cotton-plugged glass pipets are sterilized in an oven for 4 h at 180°C. For the sterilization of dissecting instruments, *see* Sections 4.2. and 5.2. Table 2 lists some useful antibodies for immunocytochemical studies in neural cell aggregates.

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Chapter Sixteen

Neural Cell Lines

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1. INTRODUCTION

The availability, in the past three decades, of well-characterized and immortalized neural cell lines has led to a rapid expansion of knowledge in many aspects of neurobiology. The major advantages of cell lines are that they are capable of long-term or indefinite growth, and generally represent a single cell type, providing a degree of reproducibility and simplicity in an otherwise complicated biological field. With these advantages come disadvantages as well, but careful experimental design, and an appreciation of the limits of the approach, allows the investigator using neural cell lines a powerful tool in answering precise questions with a minimum of interpretational complications. Furthermore, the use of cell lines eliminates the considerable cost and other concerns associated with animals as a source of experimental material. This chapter addresses the usefulness, limitations, and availability of neural cell lines. Because methodology of routine cell culture is covered in other chapters of this volume, general technical aspects of laboratory manipulations of the lines are not covered here.

2. EXPERIMENTAL ADVANTAGES AND LIMITATIONS OF NEURAL CELL LINES

The ability to clone and genetically manipulate immortalized cells in culture opens opportunities to ask precise questions regarding mechanisms of action for a variety of molecular phenomena. Techniques are well established for introducing heterologous DNA, in plasmid form, into cells, allowing expression of chosen genes. A subset of this approach involves the production of in vitro knockouts, in which exogenously introduced, altered genes express dominant negative proteins that specifically interfere at chosen points in biochemical and regulatory pathways.

A powerful approach, which has recently become popular, is the reintroduction into animals of genetically marked or otherwise manipulated cells from culture. These experiments allow in vivo assessments of cellular function, and have obvious potential for future treatments of neural injury or disease, in addition to contributions to basic understanding of neural processes. The availability

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of a single cell type in large-scale culture also provides advantages for purification of natural or recombinant products, including neuroendocrine factors present in tiny amounts in the whole animal. Thus, it is not surprising that neural cell cultures have found applications in every aspect of neurobiology, including studies of differentiation, migration, myelination, cell–substratum and cell–environmental interactions, pharmacology, toxicology, and cellular signaling, as well as basic cell biology, biochemistry, and molecular biology.

Other in vitro techniques covered elsewhere in this volume, including slice and explant cultures, provide in some ways a more reliable representation of the in vivo environment than do cell lines. Certainly, it is easier to approximate the highly complicated mixture of cell types and interactions in a neural tissue, with a slice or mixed explant culture, than with a cell line. However, the failure of most of the cells to survive and function for more than short periods under these conditions is a limitation to the approach. Such cultures represent, to some degree, cells that are deteriorating from the time of removal from the in vivo environment. In this sense, the culture conditions represent a constantly changing environment as the cells respond to the removal from the organism, influence their surroundings, and vice versa.

Primary or early passage cultures, under conditions that meet the environmental requirements of the cell types, provide an alternative to slice cultures. However, these also are complicated, because the cell population, even if healthy, is changing in cell type, cell number, and phenotypic expression over time in culture, sometimes dramatically so. Neural stem cell cultures provide another approach to generating neural cell types under conditions in which the health of the cells may be reasonably assured. However, precise control of the state and degree of differentiation among the population in these cultures has not been achieved thus far, and these cultures, for most purposes, should be considered mixed cell types, similar in some ways, to primary cultures from early embryos.

Although providing convenience in experimental design and interpretation, the reliability and relevance of results with cell lines, compared to the in vivo situation, must be approached carefully. Nearly all available, immortalized neural cell culture models are genomically abnormal, and many were derived from tumors. The genomic abnormality of these cultures often is reflective of a more pervasive genomic fluidity or infidelity characteristic of tumor cells in general, so that the genotype of cells in the cultures may be constantly changing. At the same time, genetic variants that may be generated in culture are subject to the selective pressures of the in vitro culture conditions. Because of this, long-term maintenance of a cell line represents a dynamic process, with both genotypic and phenotypic variations occurring spontaneously within the population. Recently, progress has been made toward the development of neural stem cell lines that do not show the grossly abnormal genotype of tumor-derived cells, while maintaining the capability for indefinite growth and expression of neural cell function in vitro (Loo et al., 1987; Sakai et al., 1990).

Functional limitations in the use of neural cell lines also exist. Often associated with neoplastic transformation is a reversion to a more fetal-like cell type, with metabolic emphasis on growth and proliferation, rather than function. For this reason, many established cell lines exhibit only a subset of the differentiated functions associated with their cell type of origin. The incompleteness of function exhibited by many existing neural cell lines in vitro is further compounded by the lack of cell lines representing in vitro cell culture models for many of the specialized cell types present in the nervous system in vivo. Finally, the cell culture environment itself may contribute to abnormal phenomena. The cell culture medium is at best a crude approximation of the environment in vivo, and may adversely affect cell function in vivo in unpredictable ways. Following is a discussion of a number of useful neural cell lines. Data regarding some of these lines are summarized in Table 1.

Table 1. Neural Cell Lines

Cell line	Species	Tissue source	Culture medium
Neuronal			
PC12	Rat	Pheochromocytoma	F12K/15% FCS
NG108	Rat/mouse	Hybrid NB-glioma	DMEM/10% FCS
N1E	Mouse	NB	DMEM/10% FCS
NB41A3	Mouse	NB	F10/2.5% FCS/15% HS
Neuro2a	Mouse	NB	EMEM/10%MFCS
SKNBE2	Human	NB metastasis	EMEM/F12—10% FCS
CHP212	Human	NB	EMEM/F12—10% FCS
SKNDZ	Human	NB metastasis	DMEM/10% FCS
IMR32	Human	NB	EMEM/10% FCS
SKNAS	Human	NB metastasis	DMEM/10% FCS
SKNFI	Human	NB metastasis	DMEM/10% FCS
D341	Human	MB	EMEM/20% FCS
D283	Human	MB metastasis	EMEM/10% FCS
Daoy	Human	Desmoplastic MB	EMEM/10% FCS
PFSK1	Human	Neuroectodermal tumor	RPMI/10% FCS
TE671	Human	MB	DMEM/10% CS
Glial			
C6	Rat	Glioma	F12K/2.5% FBS/15% HS
F98	Rat	Glioma	DMEM/10% FCS
DITNC1	Rat	SV40-Transformed astrocyte	DMEM/10% FCS
M059K	Human	Glioma	DMEM/F12—10% FCS
Hs683	Human	Glioma	DMEM/F12/10% FCS
A172	Human	Glioblastoma	DMEM/10% FCS
DBTRG05MG	Human	Glioblastoma	RPMI/10% FCS
Others			
P19	Mouse	Embryonal carcinoma	EMEM/7.5% CS/2.5% FCS
Hs294T	Human	Melanoma	DMEM/10% FCS

Abbreviations: F12K, Ham's F12 K modification; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; CS, calf serum; HS, horse serum; MB, medulloblastoma; NB, neuroblastoma; RPMI. The lines listed can be obtained from the American Type Culture Collection, Manassas, VA. Citations to cell lines can be found in the text.

3. CELL LINES OF NEURONAL ORIGIN

3.1. PC12 Rat Pheochromocytoma Cell Line

Perhaps the most widely used model of neuronal cell function and differentiation is the PC12 cell line, a clonal derivative from a transplantable rat pheochromocytoma (Greene and Tischler, 1976). These cells initially were of particular interest because they demonstrated a reversible differentiated response to nerve growth factor, the prototype signaling molecule for neural differentiation. The cells also synthesize catecholamines, dopamine, and norepinephrine, and express tyrosine hydroxylase and choline acetyltransferase, but do not synthesize epinephrine. Like many neuronal cell types, PC12 cells do not stick well to plastic, and may be better anchored by plating on a collagen-coated culture vessel. The cells have been used extensively to identify nerve growth factor-responsive genes and other molecular phenomena associated with neuronal differentiation, and can be induced to differentiate by alteration of cyclic adenosine monophosphate (cAMP) levels (Levi et al., 1985).

Beyond these uses, the range of phenomena that have been explored using P12 cells is tremendous, including neurotoxicology, neuronal–substratum interactions, hypoxia and oxidative stress responses, neurotransmitter synthesis and storage, apoptosis, pharmacology related to a variety of diseases, and neural receptor interactions (Berkemeier et al., 1991; Javanovic et al., 1996; Sherwood et al., 1997; Kruttgen et al., 1998; Fu et al., 1999; Kippenberger et al., 1999; Kobayashi and Millhorn, 1999; Nie et al., 1999: Walowitz and Roth, 1999). The cells have also been used in transplants in vivo into other species, including primates, as a potential disease treatment (Yoshida et al., 1999), and provide a manipulatable system for transient or stable expression of heterologous genes (Li et al., 1999). Clonally derived PC12 genetic variants, altered in specific properties, have been isolated as tools for mechanistic studies (Pance et al., 1999). Another, perhaps less well known use of PC12 cells is as a substrate for the growth of neurotrophic viruses (Su et al., 1998).

3.2. Rodent Neuroblastoma Cell Lines

Other rat neuronal model cell types include several isolated from experimentally induced neuroblastomas after transplacental exposure to nitrosoethyl urea (Schubert et al., 1974). A variety of treatments have been used to influence neuronal expression in these lines, including bromodeoxyuridine and treatments that increase cAMP levels. Unlike the variable results in attempts to culture human tumor tissue, cultures derived from experimental rat neuroblastomas rarely fail, and the lines available generally represent the most "neuron-like" clones derived from the initial tumor cultures.

A somewhat older model, the C1300 mouse neuroblastoma, was derived from a tumor of the spinal cord, and was successfully cultured by Augusti-Tocco and Sato (1969). The cultures contained neurite-like cells, with various clones exhibiting somewhat different properties. As with rat neuroblastoma models, bromodeoxyuridine and treatments that increase cAMP levels induce neuronal expression. These cells express neural markers, including choline acetylase, tyrosine hydroxylase, and acetylcholine esterase, and have been used in both electrophysiology and studies of neural development. In particular, the N1E clone has been useful in both biochemical and electrophysiological studies (Amano et al., 1972; Richelson et al., 1973). The N1E line expresses receptors for 14 neurotransmitters, as well as tyrosine hydroxylase and acetylcholine esterase, but not acetyltransferase. Receptors expressed include those for adenosine, enkephalin, serotonin, and the muscarinic acetylcholine receptor. C1300 cell lines also have provided a useful model to identify and study both receptors for neuroendocrine signaling molecules and synthesis of these molecules by the cells themselves.

Other mouse neuroblastoma cell lines are also available, notably the Neuro-2a, which has been used, among others, in the study of prion-related diseases (Kaneko et al., 1997). The NG108 hybrid cell line represents a specialized line useful in studying synapse formation under culture conditions that induce differentiation. It originated as a hybridization of rat C6 glioma and a mouse C1300 neuroblastoma clone (Christian et al., 1977).

3.3. Human Neuroblastoma and Medulloblastoma Cell Lines

Although a variety of cultures have been derived as potential neuronal models from neuroblastomas of humans, the use of these has not been wide, and the impact on the field has not been as significant as that of rodent models. Many of these lines were established from metastases, rather than from the primary tumor (Table 1), and may therefore be less representative of the tissue of origin (Biedler et al., 1978; El-Badry et al., 1989; Iavarone et al., 1993). Furthermore, because many of the lines were derived from patients who had undergone extensive chemotherapy and radiotherapy, additional genomic changes may exist in these cells beyond those related to tumori-

genicity. Some of the lines exhibit differentiative responses to treatments previously described for rodent cells, including bromodeoxyuridine, or treatments that increase cAMP levels or retinoic acid, but others do not (Tumilowicz et al., 1970). As with rodent neuroblastoma lines, the human cells often adhere poorly to the culture vessel, and may grow as detached aggregates. The lines have not been used extensively in electrophysiological studies.

Another class of human neural-derived tumors from which useful cell lines have been established are primitive neuroectodermal tumors or cerebellar medulloblastomas (Table 1). These lines often express markers of neural stem cells, and do not exhibit clear or consistent differentiated properties of either neurons or glia. Some available lines are established from metastases, but others are derived from the primary tumor, and adherence to the culture vessel substratum is variable from line to line. Although these lines may be useful for the study of differentiated neuronal function, they have found primary utility in understanding of the role of ligand-activated tyrosine kinase membrane receptors, such as trk's, and receptors for insulin-like growth factor and fibroblast growth factor in neural cell development (Syapin et al., 1982; Friedman et al., 1985; Chin et al., 1996; Rostomily et al., 1997; Derrington et al., 1998; Chiappa et al., 1999). Finally, attempts are also being made to produce human neuronal cell culture models by conditional transformation, through the introduction of oncogenes under the direction of controlled promoters (Raymond et al., 1999).

4. CELL LINES OF GLIAL ORIGIN

4.1. Rodent Cell Lines of Glial Origin

The most widely used glial-derived cell lines are, like the neuronal cell models, derived from tumors, most commonly astrocytomas. These may be either spontaneous or chemically induced. Such lines have not only provided in vitro models for the study of glial cell development and function, but also have provided a source of growth factors (GFs) active on neurons. Probably the most widely used glial cell line is the C6 rat glioma line derived from a *N*-nitrosomethylurea-induced tumor (Benda et al., 1968). These cells produce the marker S-100 protein, and it was the culture of these cells that first established which neural cell type was responsible for S-100 synthesis. Various subclones and genetic modifications exist, such as the thymidine kinase-negative clone used to produce the NG108 cell line described above (Christian et al., 1977). Another example of a genetic modification of the C6 cell line is the introduction of the β-galactosidase marker gene by replication-deficient retroviral transfection, allowing identification of the cells when injected in vivo into animals as a model of tumorigenic invasion (Lampson et al., 1993). Like PC12 cells, C6 cells also have been used as a substrate for the growth of neurotrophic viruses (Su et al., 1998). Another, similarly derived Fischer rat glioma line, F98, has been used primarily as a model for experimental therapies for brain tumors (Barth, 1998).

As another approach, the DITNC1 rat astrocyte cell line was derived by IV transformation of a neonatal rat brain culture with the SV40 T-antigen oncogene. The cells continue to express glial fibrillary acidic protein and high-affinity γ-aminobutyric acid uptake, and appear phenotypically to be type 1 astrocytes (Radany et al., 1992). Rat Schwann cell lines also have been established from normal adult or embryonic sources, without oncogenic transformation (Li, 1999). Derivation of these lines depends on the use of recombinant GFs recently identified as stimulatory to these and other cell types.

4.2. Human Cell Lines of Glial Origin

Human glia-derived cell lines, primarily from astrocytomas and oligodendrogliomas, are also available (Giard et al., 1973; Owens et al., 1976; Kruse et al., 1992; Allalunis-Turner et al., 1993).

In general, only the more malignant human tumors will give rise to indefinitely proliferating cell lines, and the success rate is about 5%. Most of these lines are astrocytomas, but a few unusual human lines also exist, including a line derived from a pediatric ependymoma, an oligodendroglioma cell line producing myelin basic protein, and an oligodendrocyte-type 2 astrocyte progenitor cell line (Westphal and Meissner, 1999).

5. OTHER CELL LINES RELEVANT TO EXPERIMENTAL NEUROBIOLOGY

It has been recognized for some time that embryonal carcinoma or embryonal stem cells, which have the capability to differentiate into a variety of cell types, including neurons, could provide a useful model of neural cell development. However, the major problem in this area still remains the difficulty in predictably inducing mass differentiation of these cultures toward a particular developmental pathway. In vitro treatments include retinoic acid or hexamethylene-bisacetamide, along with modulators of cAMP levels, as well as specific trophic factors. The P19 mouse embryonal carcinoma is an example of a useful cell line of this type (McBurney et al., 1982; Bain and Gottlieb, 1998). The line, derived from a tumor of a C3H mouse, can be cloned easily, and is pluripotent. Retinoic acid treatment leads to differentiation into both neuronal and glial-like cells, with subpopulations expressing specific neurotrophin receptors. Other treatments, such as dimethyl sulfoxide, favor muscle differentiation over neural development.

Embryonal stem cells, the more normal counterpart to embryonal carcinoma cells, also can be induced to differentiate into neural cell types in vitro by treatments similar to those used with embryonal carcinoma cells. Success has been achieved with embryonal stem cell-like cultures from primates and zebrafish, as well as rodents (Sun et al., 1995; Bain and Gottlieb, 1998; Thomson et al., 1998; Brustle et al., 1999). These models hold promise for the eventual use of in vitro-derived material for treatment of neural injury and disease. However, fundamental differences exist across species. For instance, fibroblast GF, a strong neural inducer in mammalian embryos, is an inhibitor of early neural crest-derived cell differentiation in zebrafish embryos (Bradford et al., 1994, Singh et al., 1999). Although not routinely used as a neural cell model, melanocyte or melanoma cultures also are neural crest-derived, and some respond to neurotrophins, such as nerve GF. Cell lines from rodents and humans are available (Fabricant et al., 1977).

Neural stem cells as models are covered in a separate chapter of this volume. However, it is worthwhile to point out that at lease one neural stem cell model, SFME, is also an indefinitely proliferating neural cell line. The serum-free mouse embryo (SFME) line, derived in serum-free medium, maintains a normal karyotype, is dependent on epidermal growth factor (EGF) for survival, and is growth-inhibited by serum (Loo et al., 1987). SFME cells, and similar cells of human origin, express astrocyte markers in the presence of transforming growth factor β (Sakai et al., 1990; Loo et al., 1994, 1995), and several TGF-β-responsive mRNAs have been identified and sequenced from these cells (Solem et al., 1990; Nishiyama et al., 1993; Weiss et al., 1993). The cells in the undifferentiated state also express nestin, a marker of neural stem cells (Loo et al., 1994). SFME cells enter an apoptotic cell death mode in the absence of EGF, and this effect is prevented by expression of some exogenously introduced oncogenes (Rawson et al., 1991; Loo et al., 1998, Slinskey et al., 1999).

6. PERSPECTIVES

Several challenges still await those interested in addressing the limitations of currently available neural cell lines. These problems have been recognized for decades, yet progress remains slow. It is clear from the information given above that the majority of neural cell lines are derived from

abnormal tissues. The potential for genotypic and phenotypic abnormalities influencing experimental results will continue to compromise in vitro research with these lines. The next step, hopefully, will be the derivation of cultures from normal cells that can be routinely propagated on a long-term basis.

A second problem is the lack of knowledge concerning factors controlling differentiation and proliferation of neural cells in culture. This is a complicated problem that may be best solved through the techniques of molecular biology, in concert with cell biology. Finally, there is the issue of relevance of the in vitro situation to the in vivo one, even under the best of conditions, when the "normalcy" of the cells in culture is maximized. These issues may best be addressed by transplantation experiments in which cultured cells are returned to the in vivo environment and functionally assessed. Recent work in this area represents a much-needed means to validate results obtained with cell cultures.

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Chapter Seventeen

Virus Vectors for Gene Therapy of the Nervous System

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1. OVERVIEW

Advances in molecular virology, and in understanding the molecular basis of human disease, has led to intensive efforts to use viruses for delivering therapeutic genes to cells. Although gene therapy (GT) to treat disease is still in its infancy, the techniques developed for gene delivery to cells have become more sophisticated, and are useful in a variety of experimental settings. This chapter focuses on two viral systems: adenovirus (AD) and herpes simplex virus (HSV)-based vectors. AD represents a nonenveloped DNA virus with a well-characterized genome. Vectors have been developed that are reasonably nontoxic and easy to manipulate in the laboratory. HSV vectors are enveloped viruses with a large and well-characterized DNA genome. Nontoxic variants of this virus have also been developed. Although Ad and HSV vectors share some basic features, there are also significant differences in the types of cells that they infect. This chapter focuses on HSV amplicons, which are essentially plasmids packaged into a HSV shell, and on replication-defective Ad.

2. HSV VECTORS

2.1. Introduction

Two general strategies are used to construct HSV vectors. In one strategy, the gene of interest is inserted into the genome of the vector, either wild-type virus or, more often, virus with deletions in genes essential or nonessential for viral replication and growth. This chapter focuses on a second strategy, which uses HSV genes resident on a separate "helper" vector, to package an HSV amplicon. An amplicon is a plasmid that contains the gene of interest, the HSV replication origin, and the HSV packaging signal ("a" sequence). Helper functions resident on a bacterial artificial chromosome (HSV-BAC), and amplicon DNA can be co-transfected into cells. The HSV genes expressed from the HSV-BAC direct the replication of the amplicon and the packaging of amplicon concatemers into viral capsids. By using a specially modified HSV-BAC lacking DNA packaging signals, the resulting amplicon stocks are "helper-free," and therefore relatively nontoxic.

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Procedures for working with HSV are described below. Gloves and eye protection should be used whenever viral stocks are being handled.

Note: Remember that HSV is a human pathogen. Exercise care at all times.

2.2. Generation of Recombinant HSV

Recent advances in the development of BAC containing a complete copy of the infectious HSV genome have made it possible to exploit this molecule for generating amplicon vectors for GT applications. Detailed descriptions of generating HSV-BACs are now published, and can be used to generate HSV-BAC vectors with the desired properties (Horsburgh et al., 1999). HSV-BAC constructs suitable for amplicon vector production can be generated by deleting both *cis*-acting HSV *pac* sites from HSV-BAC. The deletions can then be confirmed by Southern blotting, polymerase chain reaction, and restriction analysis (Horsburgh et al., 1999).

Note: HSV-BAC DNA is infectious, i.e., it can direct the formation of infectious virus following transfection into mammalian cells. Appropriate institutional guidelines for the handling and disposal of biohazardous materials should be followed.

1. Materials:
   HSV-BAC bacterial cultures (e.g., *pac*-deleted p45-25; refer to Horsburgh et al., 1999).
   Amplicon plasmid bacterial cultures.
   Tris-ethylenediamine tetraacetic acid (TE) buffer, 10 m*M*, pH 8.0.
   Qiagen plasmid purification kit (Qiagen, cat. no. 12163).
2. Purification of HSV-BACΔa_2 and amplicon plasmid DNA:
   a. Grow 2-L cultures of bacteria harboring HSV-BAC and amplicon plasmid DNA at 37°C for 18 h, and shaking at 250 rpm.
   Note: Amplicon DNA can be pHSVlac or amplicon plasmids containing a gene of interest and the *pac* sequence. pHSVlac contains *lacZ* under the HSV IE4/5 promoter.
   b. Pellet bacteria by centrifugation.
   c. Isolate DNA, using a Qiagen plasmid purification kit or equivalent.
   d. For added purity, DNA can be banded twice on cesium chloride (CsCl) gradients.
   e. Suspend purified DNA in 500 μL TE buffer (10 m*M*, pH 8.0).

2.3. Generation of Infectious HSV Amplicon Particles

1. Materials:
   2-2 cells, vero cells, or similar cell line.
   HSV-BACΔa_2.
   Amplicon DNA.
   Lipofectamine reagent (Gibco-BRL, cat. no. 18324-012).
   Opti-MEM I (Gibco-BRL, cat. no. 31985-070).
   Recovery medium.
   Petri dishes, sterile, 100-mm.
   Tubes, sterile.
2. Packaging amplicons with BACs:
   a. On the *day before transfection*, plate 3×10^6 2-2 cells per 100-mm dish.
   b. On the *day of transfection*, prepare two sets of tubes:

Tube 1	HSV-BACΔa_2	6 μg
	Amplicon DNA	2 μg
	Opti-MEM I	to a final volume of 300 μL
Tube 2	Lipofectamine	45 μL
	Opti-MEM I	255 μL

c. Mix each tube gently, then transfer tube 2 to tube 1, and incubate for 1 h at room temperature (RT).
d. Aspirate media, and wash plate with 6 mL Opti-MEM I.
e. Add 4 mL Opti-MEM I to plate, and add DNA–Lipofectamine–Opti-MEM I mixture to plate, dropwise. Rock plate gently to distribute DNA precipitate.
f. Incubate for 6 h (37°C, 5% CO_2); rock the plates 2–3× every hour.
g. After incubation, wash the cells 3× with 10 mL Opti-MEM I.
h. After the last wash, add 9 mL recovery medium, and incubate for 60 h at 37°C, 5% CO_2.

2.4. Harvesting Amplicon Stock

1. Materials:
   Phosphate-buffered saline (PBS).
   Freezing vials, 1–2 mL.
   Cell scraper or rubber policeman.
   Tubes, centrifuge, disposable plastic.
2. Procedure for harvest and storage of virus stock:

Note: At 60 h posttransfection of 2-2 cells with HSV-BAC and amplicon plasmid DNA, most cells will be round in appearance. At this point, the amplicon stock can be harvested.

a. Scrape cells off the dish with a rubber policeman or a disposable cell scraper. Collect growth medium and cells in one disposable centrifuge tube.
b. Pellet cells at 1000*g* for 10 min at 4°C. Remove most of the supernatant, and set aside. Suspend cells in remaining supernatant or in 5 mL PBS.
c. Quickly freeze cells in a dry-ice bath, and thaw in a 37°C water bath. Repeat once. This will allow for the release of cell-associated virus into the growth medium.
d. Centrifuge cells at about 1500*g* for 10 min to pellet cell debris. Collect supernatant. This can be pooled with the supernatant removed at step b. This is the amplicon stock. Concentrate the amplicon stock at this step (*see* Section 2.6.).
e. Titer amplicons (*see* Section 2.6.).
f. Amplicons should be stored at –70°C, or below, in 1-mL aliquots in cryovials. When needed, thaw by placing the vial in a water bath at 37°C.

2.5. Preparation of Purified Amplicon Particles

This procedure allows for the purification and concentration of amplicon stocks. This may be required to ensure the delivery of high-titer amplicons for in vivo work:

1. Materials:
   30% Sucrose solution.
   PBS *or* Tris-HCl buffer, pH 7.8, low-molarity (<50 m*M*) containing 50 m*M* NaCl.
   Centrifuge tubes, ultracentrifuge.
   Cryotubes.
2. Preparation of Virus Stocks:
   a. Prepare a 30% (w/w) sucrose solution in PBS or a low-molarity Tris buffer. Filter-sterilize. Chill on ice.
   b. Chill amplicon stock on ice.
   c. Gently layer the amplicon stock onto a 1.5-mL 30% sucrose cushion in an ultracentrifuge tube. Centrifuge for 2–3 h at 288,000*g*, 4°C (Beckman SW 41 rotor). Following

centrifugation, remove the supernatant, being careful not to lose the amplicon pellet at the bottom of the tube.

d. Add 100 µL cold PBS or a low-molarity Tris-HCl buffer to the tube, to cover the pellet. Do not attempt to resuspend the pellet at this time.

Note: Resuspend the pellet in a smaller volume of PBS or Tris-HCl buffer, if higher titers are required. However, ensure that the volume of PBS is sufficient to cover the viral pellet.

e. Store amplicon stock overnight at 4°C. Next day, gently mix, to dissolve pellet, and transfer to cryotubes for storage at –70°C. If additional purity is desired, the suspension can be layered over a 30-mL continuous gradient of 5–45% sucrose in the same buffer and centrifuged at 288,000*g* (SW 41 Beckman rotor) for 1 h. Amplicons will appear as a white band in the gradient.

2.6. Determination of Amplicon and Virus Titer

Using this method, it is possible to determine the number of contaminating replication-competent particles in an amplicon stock. Although it is rare, helper-free amplicon stocks can contain a mixture of replication-competent and amplicon particles, and the ratio of the two types of particles may alter the usefulness of the stock. The ratio of total particles to the number of infectious particle plaque-forming units (PFUs) is called the particle:PFU ratio. Although it is rarely necessary to do so, the number of particles can be determined by counting negatively stained particles in the electron microscope. The number of PFU/mL (the titer) is a more important determinant of the usefulness of an amplicon stock.

The contaminating virus titer is determined by infecting monolayers of host cells with serial dilutions of amplicon stock. Infected cells are detected by allowing the virus to spread to adjacent cells to form a plaque. A plaque is usually a clear zone or a zone of dead cells amid the monolayer of healthy cells. To prevent virus from forming satellite plaques in the monolayer, the monolayer is normally overlayered with 0.8% agarose (2× agarose is combined with 2× Dulbecco's modified essential medium (DMEM)–4% fetal bovine serum (FBS), which is then used to overlay the culture). A good alternative when working with HSV is to overlay the cultures with 0.1% pooled human immunoglobulin-G in culture medium. Pooled human immunoglobulin-G contains anti-HSV antibodies (Abs) that inactivate virus particles as soon as they are released from infected cells. Plaques can still form, because most viruses, including HSV, can spread to adjacent cells by direct cell-to-cell contact. Antibodies do not inhibit this type of spread.

The amplicon titer can be determined easily if it expresses a reporter gene, such as *lacZ*, or other gene for which a suitable detection reagent is available.

1. Materials:
   Vero cell (or other suitable cell line) grown as monolayers in 6-well tissue culture plates.
   Virus stock to be tested.
   DMEM (Gibco-BRL, cat no. 11995-065).
   Plaquing medium.
   Phosphate-buffered saline.
   PBS containing 1% bovine serum albumin (BSA) (fraction V; Sigma, cat. no. A-3311).
   X-Gal (final concentration) in X-gal buffer (0.1%).
   Formaldehyde or paraformaldehyde in PBS (4%).
   5% methylene blue in 70% methanol.
   Tubes, snap-cap, disposable, sterile, 15 mL.
   Pasteur pipets.

Plastic pipets, 1- and 10-mL sterile.
Container filled with bleach.

2. Determination of virus titer:
   a. Immerse virus stock in a 37°C bath to quickly thaw the virus.
   b. Remove the vial from the bath, and rinse with 70% ethanol.
   c. Open the vial in the hood and remove 1 mL to a 15-mL snap-cap tube. Label this tube "A."

   Note: Discard the virus stock vial in the bleach container. All pipet tips and pipets used in subsequent steps should be discarded into bleach containers, to inactivate virus.

   d. Put 900 μL MEM into each of two sterile tubes. Label the tubes B and C.
   e. Transfer 100 μL virus from tube A into tube B, to make a 10-fold dilution.
   f. Transfer 100 μL virus from tube B into tube C. Continue making serial dilutions in the same manner. We normally do serial dilutions of 10^{-2}–10^{-7}.
   g. Using a Pasteur pipet, remove the growth medium from each well of the 6-well plate.
   h. Using a 1-mL pipet, transfer 100 μL virus from each dilution tube into an individual cell monolayer growing in a 6-well plate. Repeat this procedure for each virus dilution.
   i. Incubate the plates at 37°C, in an atmosphere of 5% CO_2, for 1 h. During this time, rock the plates every 10 min, to redistribute the virus onto the monolayers. Do not let any part of the monolayer dry during this period.
   j. After 1 h, remove the liquid from each well, using a Pasteur pipet, and rinse each well with 3 mL PBS or growth medium.
   k. Add 2 mL plaquing medium to each well, and incubate at 37°C in a humidified atmosphere of 5% CO_2 for 3–4 d, by which time plaques will be visible.
   l. After plaques develop, remove medium from the monolayers. Wash the monolayers once with PBS, and cover cells with 2 mL methylene blue in 70% methanol. This will inactivate the virus.

   Note: Sterile technique is no longer required.

   m. Incubate at room temperature for 15–30 min. Pour off methylene blue stain, and wash cells under tap water to remove excess stain.

   Note: Wash with enough force to remove loosely adherent cells, so that they leave white spots among the uninfected blue cells. Some cell types are less adherent than others are, so do not wash too hard.

   n. Allow the dishes to dry. Count the plaques, if any, and determine the titer as follows:

$$\text{Titer} = \text{PFU/mL}$$

$$\text{Aliquot size} = 100\ \mu\text{L in above procedure}$$

$$\text{PFU/mL} = \frac{\text{No. of plaques}}{\text{Aliquot size}} \times 1000 \times \text{reciprical dilution factor}$$

3. Determination of amplicon titer: Many vector constructs contain the bacteriophage *lacZ* gene, which encodes β-galactosidase. β-galactosidase can be detected directly, using a substrate, or by immunohistochemical detection, using an Ab. The substrate method is described below, and is often used to determine the titer of vectors expressing *lacZ* or other suitable genes.
   a. To titer using β-galactosidase, follow the protocol in step 2., a–j, above, then proceed with the following protocol.
   b. Add 2 mL DMEM–4% FBS–0.1% IgG to each well. Incubate at 37°C in an atmosphere of 5% CO_2 for 10 h.
   c. Remove medium from an infected cell monolayer, and rinse cell monolayers with PBS.

d. Add 4% paraformaldehyde in PBS to each well, so that the cells are covered (1 mL). Incubate at room temperature for 10 min to fix cells. Sterile technique is no longer needed, because this procedure inactivates virus.
e. Remove fixative, and rinse once with PBS containing 1% BSA.
f. Add 2 mL 0.1% X-gal solution. Incubate monolayers at 37°C until blue cells appear. This can take up to 24 h, but may be visible within 1 h. Count the number of blue cells, and determine the titer of blue cells/mL vector stock (according to formula in step 2).

2.7. Immunohistochemistry

Immunohistochemistry can be used to detect other expressed gene products, and to determine their localization. Following fixation, cells should be permeabilized by incubating the cell monolayers with 0.02% Triton X-100 for 3 min at room temperature. The monolayers should be rinsed with copious amounts of PBS + 1% BSA to completely remove the Triton X-100 solution. Cells can then be incubated with antibodies diluted in PBS + 1% BSA. Alternatively, following fixation, the primary antibody can be diluted in PBS–1% BSA–0.25% saponin, and incubated. Antibody detection, using a secondary antibody conjugated to alkaline phosphatase or horseradish peroxidase, can then be performed according to the manufacturer's instructions.

3. AD VECTORS

3.1. Introduction

In this section, protocols are described for transferring a foreign gene from an Ad E1 or E3 shuttle plasmid to an infectious Ad5 vector, and for amplifying and purifying that vector. First, a method is described whereby a foreign gene is rescued into the virus by co-transfecting the shuttle plasmid with a noninfectious "genomic" plasmid. Recombinants capable of forming plaques on the E1-complementing cell line 293 are then selected. After expansion in 293 cultures, these plaques are screened by restriction analysis. The correct recombinants are then plaque-purified and amplified in 293 monolayer cultures. Since most of the virus is cell-associated until very late in infection, high-titer stocks can be prepared easily by collecting infected cells by centrifugation, followed by disruption of the concentrated cell suspension to release the virus. After plaque titration, this crude lysate can be used for most in vitro experiments, with little interference from 293 cellular proteins and DNA. For animal experiments, or in cases in which interference has been a problem, further purification of the virus by CsCl banding is required. These procedures were directly adapted from Hitt et al. (1995), Graham (1987), and Hardy et al. (1997) and are all described in detail below.

Cre/lox recombination is a powerful tool for genetic manipulation of ads. Constructing new E1-substituted adenoviruses is an efficient alternative to the existing overlap recombination techniques and detailed descriptions of generating E1-substituted or gutless adenovirus vectors are now published (Graham, 1987). By this method, the viruses are almost pure, without resorting to plaque purification. Thus, a working stock of virus can be produced for initial experiments in 10 d. In addition, Cre/lox recombination enables the use of a small shuttle vector that is easy to manipulate (Hardy et al., 1997). Recombinant virus construction is facilitated by a recombinant adenovirus ψ5. The negative selection is based on the observation that deletion of a sequence from 194 to 358 bp, in the left end of adenovirus prevents the viral chromosome from being packaged into capsids (Zabner et al., 1993; Crystal et al., 1994). To make a conditional version of such a deletion, the packaging site in ψ5 is flanked by repeated *lox*P recognition sites. In CRE8 cells, 293 cells that express CRE recombinase, the packaging signal is efficiently excised, thus producing an unpackageable chromosome. To make an E1-substituted virus, ψ5 is used as a donor virus to sup-

ply the viral backbone. In addition to supplying negative selective pressure, CRE recombinase can catalyze recombination between ψ5 and a shuttle plasmid with a single *lox*P site, providing an efficient means to construct recombinants. Selective growth of the recombinant viruses is ensured by installing a normal packaging sequence on the shuttle plasmid.

3.2. Growth and Purification of Viral Recombinants

1. Materials:
   293 cell line (American Type Culture Collection [ATCC], cat. no. CRL 1573).
   Shuttle plasmid DNA (refer to Hitt et al., 1995).
   Circular Ad genome plasmid (refer to Hitt et al., 1995).
   pFG140 plasmid (refer to Graham, 1984).
   Carrier DNA solution, salmon sperm.
   Joklik's modified MEM (Gibco-BRL, cat. no. 22300) plus 10% newborn bovine serum (NBS).
   Joklik's modified MEM–agarose overlay.
   Citric saline (1X).
   Calcium chloride solution, 2.5 *M*.
   HEPES-buffered saline (HBS).
   Petri dishes, 60-mm tissue culture, sterile.
   Tubes, sterile, plastic, clear, 5 mL.
2. Co-transfection for rescue of recombinant Ad vectors:
   a. Preparation for cotransfection:
      i. Set up low-passage (>p40) 293 cells in 60-mm dishes, so that they will be about 70–80% confluent at the time of use.

      Note: 293 monolayers are maintained in Joklik's modified MEM + 10% NBS. Split cells 1:2 or 1:3 at 90% confluence, by removing the medium, washing each dish twice with 10 mL 1X citric saline, then incubating for a maximum of 15 min at room temperature in 2 mL 1X citric saline, to detach cells.

      ii. Make HBS–carrier DNA by adding 0.005 vol 2 mg/mL salmon sperm carrier DNA to 1X HBS. Prepare 6 mL per virus to be rescued. Vortex for 1 min.
      iii. For each virus to be rescued, add 2 mL HBS–carrier DNA into each of three sterile clear plastic tubes. Two mL HBS–carrier DNA is sufficient for four dishes.
      iv. To these tubes, add shuttle plasmid DNA and the circular Ad genome plasmid with which it will recombine, in the following amounts:

Shuttle DNA	Ad plasmid
20 μg	20 μg
20 μg	40 μg
40 μg	40 μg

      Note: Infectious pFG140 is a good positive control for transfection efficiency (Graham, 1984).

      v. Shake each tube gently, then slowly add 100 μL 2.5 *M* $CaCl_2$, dropwise, to each tube, with gentle mixing.
      vi. Let stand at room temperature for 15–30 min. A fine precipitate will form, producing a DNA suspension.
   b. Without removing the growth medium, add 0.5 mL DNA suspension to each dish of cells, then incubate at 37°C for 4–5 h or overnight.
   c. Remove the medium, and add to each dish 10 mL Joklik's modified MEM agarose overlay. After the agarose solidifies, incubate at 37°C. Plaques should appear, after about 1 wk, as zones of clearing in the cell monolayer.

3.3. Small-Scale Preparation of Viral Stocks

1. Materials:
   293 cell line (ATCC, cat. no. CRL 1573).
   Joklik's modified MEM plus 5% horse serum (HS).
   Tissue culture dishes, 150-mm.
   Centrifuge tubes, 50-mL.
2. Set up five 150-mm dishes of 293 cells, so that they will be 90% confluent at the time of infection.
3. Remove the medium from the 293 cells, and infect at an multiplicity of infection of 1–10 PFU/cell (1 mL virus suspension per 150-mm dish). To minimize the probability of rearrangement of the recombinant virus, prepare subsequent high-titer stocks from the same viral screening sample.
4. Adsorb for 30 min, then add 25 mL complete Joklik's modified MEM + 5% HS to each dish. Incubate at 37°C, and examine daily for cytopathic effect.
5. When cytopathic effect is nearly complete, i.e., most cells are rounded but not all are detached, remove supernatant, place in a centrifuge, and set aside. Scrape the cells off the dish, and combine with supernatant. Keep cells from each 150-mm plate separate.
6. At this point, proceed with steps for crude stocks or those for Cs purification of viral stocks.
7. Crude stocks:
   a. Materials:
      PBS^{2+} + 10% glycerol.
      Dry ice/ethanol bath.
      37°C water bath.
   b. Centrifuge at 800*g* for 15 min, to pellet the cells. Remove the medium, and resuspend the cell pellet in 2 mL PBS^{2+} + 10% glycerol/150-mm dish.
   c. Freeze in a dry ice/ethanol bath and thaw in a 37°C water bath, 3×.
   d. Centrifuge at 1500*g* for 10 min, to remove cell debris.
   e. Titer and store aliquots at –70°C.
8. Cs purification:
   a. Materials:
      Tris buffer, 10 m*M*.
      CsCl solution, 1.25 g/mL.
      CsCl solution, 1.4 g/mL.
      CsCl solution, 1.34 g/mL.
      30% glycerol–PBS^{2+}.
      PDIO column (Pharmacia, prepacked G25 column).
      Centrifuge tubes for SW41 Beckman rotor.
      Syringes (2), 10-mL, and needles.
      Tubes, sterile (for column samples).
      Dry ice/ethanol bath.
      37°C water bath.
   b. Centrifuge at 800*g* for 15 min, to pellet the cells. Remove the medium, and resuspend the cell pellet in 6 mL 10 m*M* Tris buffer.
   c. Freeze in a dry ice/ethanol bath, and thaw in a 37°C water bath, 3×.
   d. Centrifuge at 1500*g* for 10 min, to remove cell debris. Save the supernatant.
   e. Prepare and run the following step gradient in a Beckman SW41 tube:
      1.4 g/mL CsCl solution 2.5 mL

1.25 g/mL CsCl solution	2.5 mL
Viral supernatant	7 mL

i. Centrifuge for 1 h at 210,000*g* in a Beckman SW41 rotor at 4°C.
ii. Remove the viral layer at the interface between the 1.4 and 1.25 g/mL CsCl layers, by puncturing the side of the tube with needle on a 10-mL syringe. Virus is white and fluffy looking.

f. Prepare and run an equilibrium gradient of 1.34 g/mL CsCl:
   i. Place the viral layer, obtained from the step gradient, onto the equilibrium gradient.
   ii. Centrifuge in a vertical or fixed-angle rotor at 287,000–510,000*g* (e.g., Beckman VTi 90 rotor). At 80,000 rpm, centrifuge for 4 h, or centrifuge at 60,000 rpm overnight.
   iii. Remove the viral layer, which is the thick, fluffy, white band in the middle of the tube, by puncturing the side of the tube with a needle on a 10-mL syringe.

g. Prepare and run a PDIO column:
   i. In a laminar flow hood, equilibrate a PDIO column with a 30% glycerol solution in 50 mL PBS^{2+}.
   ii. Load viral sample (1–2 mL) obtained from the equilibrium gradient.
   iii. Collect 500-µL samples into sterile tubes. Regulate the flow by adding buffer to the column.
   iv. The virus should be in fractions 6–9. These fractions will appear milky. Make a 1:10 dilution of each fraction, and read at OD_{260} in a spectrophotometer. Pool the fractions that have the highest concentrations (and highest OD).

h. Store fractions at –70°C.

Note: **1 OD_{260}/cm = 1 × 10^{12} particles/mL**
20 particles is approx 1 PFU on HeLa cells.

3.4. Large-Scale Preparation of Viral Stocks

Recombinant Ads can be purified from crude lysates of either monolayer or suspension cultures. Because of the greater ease of handling suspension cultures (one 4-L suspension culture is equivalent in cell number to approx 80 150-mm dishes), this source is preferable for the preparation of purified high-titer viral stocks.

1. Materials:
   293N3S cells (Graham, 1987) (*for replication-defective viruses*).
   Joklik's modified MEM + 10% NBS.
   or
   KB cell line (ATCC, cat. no. CCL 17) (*for replication-competent viruses*).
   Joklik's modified MEM + 10% HS.
   Tris-HCl buffer, 0.1 *M*, pH 8.0.
   Sodium (Na) deoxycholate, 5%.
   Magnesium chloride ($MgCl_2$) solution, 2 *M*.
   Deoxyribonuclease (DNase) I solution.
   Ribonuclease (RNase) A solution.
   CsCl solution, saturated.
   PBS^{2+} + 10% glycerol.
   Na citrate, 1%.
   Carnoy's fixative.
   Orcein.

Spinner flasks.
Tissue culture flasks.
Tubes, centrifuge, 15-mL.
Bottles, centrifuge, sterile, 1-L.
Tubes, centrifuge, sterile, 50-mL.
Beckman 50Ti Ultraclear Quickseal tubes.
Beckman 50Ti rotor.
Needle.
Dialysis tubing.
Microscope slides and coverglasses.

2. For infection with replication-defective viruses (E1 insertion recombinants), grow 293N3S (Graham, 1987) cells in spinner culture to a density of $2–4 \times 10^5$ cells/mL in 4 L complete Joklik's modified MEM + 10% HS. For infection with replication-competent viruses, 4 L KB cells are grown to a density of $5–6 \times 10^5$ cells/mL in complete Joklik's modified MEM + 10% NBS. Centrifuge the cell suspension at 750*g* for 20 min, saving half of the conditioned medium. Resuspend the cell pellet in 0.1 vol fresh medium.
3. Add the virus at an multiplication of infection of 10–20, and stir gently at 37°C for 1 h. Add 2 L conditioned medium and 2 L fresh medium, and continue stirring at 37°C.
4. Monitor the infection twice daily by inclusion body staining as follows:
   a. Remove a 5-mL aliquot from the infected spinner culture. Centrifuge for 10 min at 750*g*. Resuspend the pellet in 0.5 mL 1% Na citrate.
   b. Incubate at room temperature for 10 min, then add 0.5 mL Carnoy's fixative, and let stand for 10 min at room temperature.
   c. Add 1 mL 1% Na citrate, and centrifuge for 10 min at 750*g*. Resuspend the pellet in a few drops of 1% Na citrate.
   d. Add one drop of fixed cells to a slide, and let air-dry for at least 1 h; then add one drop of orcein and a coverglass. Under the microscope, inclusion bodies appear as densely staining nuclear structures resulting from an accumulation of large amounts of virus and viral products at late times in infection. Use uninfected cells as a negative control.
5. When inclusion bodies are visible in 80–90% of the cells (36–72 h), harvest by centrifugation at 750*g* for 20 min in sterile 1-L bottles. Combine the pellets in a small volume of medium, transfer to sterile 50-mL tubes, and spin again. Resuspend the pellet in 20–30 mL 0.1 *M* Tris-HCl, pH 8.0. Store at –70°C until use.
6. Thaw the frozen crude stock, and add 0.1 vol 5% Na deoxycholate. Mix well, and incubate at room temperature for 30 min. This disrupts the cells without disrupting the virions, resulting in a relatively clear, highly viscous suspension.
7. Add 0.01 vol 2 *M* $MgCl_2$, 0.005 vol DNase I solution, and 0.005 vol RNase A solution, then mix thoroughly. Incubate at 37°C for 30–45 min, mixing well every 10 min.
8. Add 1.8 mL saturated CsCl solution (equilibrated to room temperature) for each 3.1 mL virus suspension. The CsCl concentration is critical, so volumes must be accurately determined. The final density should be 1.35 g/mL.
9. Transfer the virus to Beckman 50Ti Ultraclear Quickseal tubes and centrifuge at 96,000*g* in a precooled Beckman 50Ti rotor for 16–20 h at 4°C.
10. Collect the viral bands in a small volume, and pool. This can be collected by puncturing the top of the tube with a hot needle, then puncturing the bottom, and controlling the flow of solution out the bottom with a gloved finger over the top hole.
11. Centrifuge the pooled virus in a precooled Beckman 50Ti rotor at 96,000*g*, at 4°C for 16–20 h.

12. Collect the virus band in a small volume and dialyze at 4°C against two changes of 500 vol PBS^{2+} + 10% glycerol for at least 4 h each change. It is important, especially for in vivo work, to remove all traces of CsCl. Precipitation of the virus is occasionally observed, when the virus concentration is very high.

3.5. Plaque Assays for Purification and Titration of Adenoviruses

1. Materials:
   293 cell line (ATCC, cat. no. CRL 1573).
   Viral stock.
   *Hin*dIII.
   PBS^{2+}.
   10% glycerol + PBS^{2+}.
   Joklik's modified MEM + 5% HS.
   Joklik's modified MEM–agarose overlay.
   Glycerol, sterile.
   Buffer-saturated phenol.
   0.1X standard sodium citrate (SSC).
   Pronase–sodium dodecyl sulfate (SDS) solution.
   Petri dishes, 60-mm.
   Pasteur pipets, sterile.
   Cryovials, 5-mL.
   Tubes, microcentrifuge.
   96% Ethanol.
2. Plaque assay:
   a. Set up 60-mm dishes of 293 cells to be confluent at the time of infection.
   b. Remove the medium from the dishes. Spread 1 mL virus dilution (dilute agar plug suspension appropriately in PBS^{2+}, if plaque-purification is desired, or dilute stock for titration) evenly over each dish. Adsorb the virus for 60 min at room temperature with rocking every 15 min. Add 10 mL Joklik's modified MEM–agarose overlay, cool, then incubate at 37°C.
   c. Plaques should be visible within 4–5 d, and should be counted for titration at 7 d, and again at 10 d.
3. Screening Ad plaque isolates:
   The following protocol describes the amplification of plaque isolates by growth in 293 cells. The conditioned medium containing released virus can be stored for further purification, the cell monolayer, with which most of the virus remains, is harvested for viral DNA analysis.
   a. Pick well-isolated plaques from transfected cultures (above), by punching out agar plugs with a sterile Pasteur pipet. Store agar plugs in 0.5 mL sterile PBS^{2+} + 10% glycerol at –70°C until use.
   b. Set up 60-mm dishes (one dish per plaque) of 293 cells to be 80–90% confluent at the time of infection.
   c. Remove the medium from the cells, and add 0.2 mL virus (agar plug suspension). Rock dishes once, and adsorb at room temperature for 30 min. Add 5 mL complete Joklik's modified MEM + 5% HS, and incubate at 37°C.
   d. Viruses are ready to harvest when all cells are rounded and most have detached from the dish (usually 3–4 d).

e. To permit collection of the medium, while retaining the majority of the infected cells, leave the dishes undisturbed in the tissue culture hood for 30 min. Gently remove 4 mL medium, and add to a sterile vial containing 0.5 mL sterile glycerol. Store these candidate viruses at –70°C. Slowly aspirate any remaining medium from the plate. If this is done carefully, the majority of the cells will remain in the dish.
f. To extract DNA from the infected cells, add 0.5 mL pronase-SDS solution to each dish, and incubate at 37°C for 4–18 h.
g. Transfer the viscous lysate to a microcentrifuge tube, and extract once with buffer-saturated phenol. Centrifuge for 10 min. Collect the aqueous phase (top), and transfer it to a fresh tube.
h. Add 1 mL 96% ethanol to precipitate the DNA. Mix by inversion; a fibrous precipitate should be easily visible. Centrifuge for 10 min at 11,000*g*. Wash the pellet twice with 96% ethanol, and air-dry.
i. Dissolve the DNA pellet in 100 µL 0.1X SSC (complete solubilization may take several hours), and digest 10 µL with *Hin*dIII (one U overnight is usually sufficient for complete digestion).
j. Analyze the digests on a 1% agarose gel with appropriate markers (a *Hin*dIII digest of wild-type Ad5 being one convenient marker). If the CPE was complete, viral DNA bands should be easily visible above a background smear of cellular DNA.

Note: In *Hin*dIII digests of human DNA, there will be a band of repetitive cellular DNA at 1.8 kb, not to be confused with viral DNA (Grable and Hearing, 1992).

k. Verify candidate recombinants, using other diagnostic restriction enzymes. Correct recombinants should be further purified by at least one round of plaque purification and screening, prior to the preparation of high-titer stocks.

4. APPENDIX

4.1. Medium

1. Medium:
   a. Recovery medium:
      i. DMEM (Gibco-BRL, cat. no. 11995-065) with HEPES and high glucose.
      ii. Add 2 m*M* glutamate (Gibco-BRL, cat. no. 25030-032), filter-sterilize, and aliquot.
      iii. Prior to use add 6% FBS.
   b. Plaquing medium: Prior to use, add 4% FBS and 0.1% pooled human immunoglobulinG (ICN Biomedicals, cat. no. 823102) to DMEM.
   c. Joklik's modified MEM (Gibco-BRL, cat. co. 22330-024).
      i. Prior to use, add the following to Joklik's modified MEM containing 2 m*M* L-glutamine:
         100 U/mL penicillin
         0.1 mg/mL streptomycin
         2.5 µg /mL Fungizone
      ii. Store at 4°C for up to 2 wk.
      iii. Add heat-inactivated newborn bovine serum (NBS) or horse serum (HS) prior to use.

4.2. Buffers

1. 10X PBS solution:
   $Na_2HPO_4 \cdot H_2O$ 14.2 g

NaCl	80 g
KCl	2 g
KH_2PO_4	2 g

Make up to 1 L with H_2O. Dilute before use.

2. PBS^{2+}: To sterile 1× PBS, add 0.01 vol sterile 68 m*M* $CaCl_2$ and 0.01 vol 50 m*M* sterile $MgCl_2$.
3. TE buffer pH, 8.0:
   a. 1 *M* Tris buffer, pH 8.0:
      i. Dissolve 121.1 g Tris base in 800 mL distilled water (dH_2O).
      ii. Adjust the pH to 8.0. Sterilize by autoclaving.
   b. 0.5 *M* EDTA:
      i. Add 186.1 g of disodium EDTA $2H_2O$ to 800 mL dH_2O.
      ii. Adjust the pH to 8.0 with NaOH, and sterilize.
   c. 10 m*M* TE buffer:
      i. Mix 0.5 mL 1 *M* Tris-HCl, pH 8.0, with 0.1 mL 0.5 *M* EDTA.
      ii. Bring the volume to 50 mL with dH_2O. Final concentration is 10 m*M* Tris, 1 m*M* EDTA.
   d. 100 m*M* TE buffer, pH 8.0:
      i. Mix 5 mL 1 *M* Tris-HCl, pH 8.0, with 0.1 mL 0.5 *M* EDTA.
      ii. Bring the volume to 50 mL with dH_2O. Final concentration is 100 m*M* Tris, 1 m*M* EDTA.
5. X-gal buffer:

Potassium ferrocyanide	10 m*M*
Potassium ferricyanide	10 m*M*
$MgCl_2$	2 m*M*

Dissolve in PBS.

6. HEPES-buffered saline:
   a. Composition:

HEPES	21 m*M*
NaCl	0.137 *M*
KCl	5 m*M*
Na_2HPO_4	0.7 m*M*
Glucose	5.5 m*M*

   b. Adjust pH to 7.1.
   c. Aliquot into glass bottles, autoclave, and store at 4°C.

4.3. CsCl Solutions

1. Saturated CsCl solution:
   a. At room temperature, add sufficient CsCl to 10 m*M* Tris-HCl, pH 8.0, and 1 m*M* EDTA, to saturate the buffer.
   b. Store at 4°C, but bring to room temperature before use.
2. CsCl solutions for gradients:
   a. 1.4 g/mL CsCl: 53 g CsCl in 10 m*M* EDTA; 100 m*M* Tris, pH 8.0.
   b. 1.25 g/mL CsCl: 32.3 g CsCl in 10 m*M* EDTA; 100 m*M* Tris, pH 8.0.
   c. 1.34 g/mL CsCl: 45.5 g CsCl in 10 m*M* EDTA; 100 m*M* Tris, pH 8.0.

4.4. Miscellaneous Solutions

1. Citric saline:
   a. 10X Stock citric saline:
      i. Make a solution of 1.35 *M* KCl and 0.15 *M* Na citrate.
      ii. Autoclave, and store at 4°C.
   b. Dilute 1:10 in sterile water, to prepare 1X citric saline.
2. Na citrate, 1%:
   a. 1 g Na citrate to 100 mL final volume with dH_2O.
   b. Autoclave, and store at 4°C.
2. SSC:
   a. 10X SSC:
      i. Make a solution of 1.5 *M* NaCl and 0.15 *M* Na citrate. Adjust pH to 7.0. Autoclave and store at room temperature.
      ii. Prepare 0.1X SSC by diluting 10X SSC, followed by autoclaving.
3. 2.5 *M* $CaCl_2$:
   a. 36.8 g $CaCl_2$ to 100 mL final volume with dH_2O.
   b. Sterilize by filtration, and store in small plastic tubes at 4°C.
4. 2 *M* $MgCl_2$:
   a. 50.8 g $MgCl_2$ to 100 mL final volume with dH_2O.
   b. Autoclave, and store at 4°C.

4.5. Enzyme Solutions

1. Pronase:
   a. Stock solution:
      i. 0.5% (w/v) pronase in 10 m*M* Tris-HCl, pH 7.5.
      ii. Heat at 56°C for 15 min, then incubate at 37°C for 1 h.
      iii. Aliquot, and store at –20°C.
   b. Working pronase–SDS solution.
      0.05% pronase stock solution in 10 m*M* Tris-HCl, pH 7.5, containing 10 m*M* EDTA and 0.5% (w/v) SDS.
2. DNase I solution:
   a. Dissolve 100 mg pancreatic DNase in 10 mL 20 m*M* Tris-HCl, pH 7.4, containing 50 m*M* NaCl, 1 m*M* dithiothreitol, 0.1 mg/mL BSA, and 50% glycerol.
   b. Store in small aliquots at –20°C.
3. RNase A solution:
   a. Dissolve 100 mg RNase A in 10 mL 10 m*M* Tris-HCl, pH 7.4, containing 15 m*M* NaCl.
   b. Store in small aliquots at –20°C.
4. Na deoxycholate, 5%:
   a. 5 g Na deoxycholate to 100 mL final volume with dH_2O.
   b. Filter sterilize, and store at room temperature.

4.6. Overlays

1. 2× Joklik's modified MEM for overlay:

Yeast extract	0.2%
Horse serum	10%
Glutamine	4 m*M*
Penicillin	200 U/mL

Streptomycin	0.2 mg/mL
Fungizone	5 μg/mL

in 2× Joklik's modified MEM. Store at 4°C.

2. Joklik's modified MEM–agarose overlay:
   a. Warm 2× Joklik's modified MEM for overlay to 37°C.
   b. Autoclave 1 g agarose/100 mL water, then cool to 44°C.
   c. Combine equal volumes of agarose and 2× Joklik's modified MEM immediately before use.

4.7. Salmon Sperm DNA, 2 mg/mL

1. Dissolve 100 mg salmon sperm DNA in 50 mL sterile 0.1X SSC by stirring overnight at room temperature. Determine the concentration by reading the OD at 260 nm.
2. Store in small aliquots at –20°C.

4.8. Carnoy's Fixative

Methanol:glacial acetic acid (3:1).

4.9. Orcein

1. Dissolve 2% (w/v) orcein dye in 50% (v/v) acetic acid.
2. Filter through Whatman No. 1 paper.

4.10. Safe Handling of Virus

All viruses used for gene therapy, including adenovirus, herpesvirus, and retroviruses, are potentially dangerous human pathogens. Although they have been debilitated, in many instances, they are capable of replicating under certain conditions. Experimentation with these vectors should be carried out in at least a P2 containment facility, in accordance with relevant regulations. The following guidelines should be followed carefully to reduce the risk of accidental exposure:

1. Syringes with needles should be used with viruses only when absolutely necessary. In general, syringes should not be used in the routine transfer of virus, except when animals are to be infected. Thick gloves should be worn to reduce the risk of stabs.
2. Latex gloves should be worn at all times when handling virus. Double gloves are useful, so that the first pair can be removed without touching them with bare hands.
3. Bleach inactivates most enveloped viruses quickly. The bleach should be relatively fresh, however, and pipets and tubes containing virus should be fully immersed, to inactivate all traces of virus prior to discard. Pipets can be safely removed from bleach after a day or so, and discarded in biohazardous waste containers.
4. Always place a sign in the work area to indicate that human pathogens are being used.
5. If contamination accidentally occurs, by getting liquid containing herpesvirus on one's skin, wash immediately with copious amounts of warm, soapy water. This will rapidly inactivate the virus.

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Chapter Eighteen

Preparation of Substrata for In Vitro Culture of Neurons

Paul C. Letourneau

1. INTRODUCTION

Cell adhesion, cell contact, and intercellular interactions are critical to neuronal function. Neuronal cell surfaces are closely associated with the surfaces of other cells, including the specialized contacts of synapses, and the close appositions of astrocytes, oligodendrocytes, satellite cells, and Schwann cells with neuronal perikarya, axons, and dendrites (Peters et al., 1990). The importance of these interactions to neuronal metabolism and function is emphasized by recent identification of the receptors and ligands that mediate intercellular contacts and the cytoplasmic regulatory signaling that is activated by receptor–ligand binding (Zigmond et al., 1999). These complex associations are disrupted when neurons are placed into tissue culture, and the effects of these disruptions of cellular associations on neuronal function are not well understood. Still, these disruptions of cellular associations are necessary to ask many experimental questions, although one should remember that in vitro results are obtained from neurons placed into a highly altered environment. Because neurons require a substratum to which they can adhere in order to survive and form axons and dendrites, one must consider how to prepare substrata for culturing neurons.

This chapter discusses the preparation of substrata for supporting the survival and function of neurons in vitro. The history of neuronal cell culture dates to 1910, when Harrison (1910) conducted the first in vitro studies and explanted amphibian spinal cords into plasma clots, to observe axonal outgrowth. Until recently, the development and modification of methods for preparing substrata for culturing neurons has involved mostly guesswork and empirical observations. The understanding of receptor-ligand interactions, which mediate neuronal contacts and subsequent cytoplasmic signaling, has allowed more refined modifications and choices for preparing in vitro substrata. Yet, because of the diversity of neurons and their intercellular associations, empirical approaches will remain important in developing new methods of preparing in vitro substrata.

This discussion of substrata for neuronal culture is separated into three sections; the underlying support surface or substratum, adhesion-promoting molecules that are bound to the support surface, and methods of enhancing the binding of molecules to in vitro substrata. A final section mentions methods used to control the pattern of binding or deposition of molecules to substrata.

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2. SUPPORTING SUBSTRATUM

Most in vitro studies of neurons involve standard light microscopic observations, and thus, require a transparent substratum. Polystyrene (PS) plastic and glass slides or coverslips are the most widely used substrata. Plastic tissue culture dishes, flasks, and bottles come in a wide variety of commercially available sizes and shapes, and they are conveniently used without cleaning or sterilization. The PS of these in vitro vessels is treated to be hydrophilic, and is negatively charged. Untreated, bacteriological grade plastic is more hydrophobic, and is not suitable as a substratum for adhesion of most cells.

Despite their convenience, plastic dishes are not suitable for studies on inverted microscopes that require high magnification and detailed resolution of cells and their components. The plastic dish bottoms are too thick, birefringent, and sometimes fluorescent, to allow microscopy with high magnification, short working-distance objectives, and DIC or fluorescence optics. Thus, many researchers use glass coverslips as substrata for neuronal culture. If cells are to be fixed and stained before observation, the coverslips can be placed loose within culture dishes, and, at the end of the culture period, the coverslips are subsequently removed, processed, and mounted on glass slides. For observations of living cells, there are several types of commercially available chambers that have a bottom consisting of a glass slide or coverslip, and plastic sides and covers that can be removed later (e.g., Nalge Nunc). Many researchers prepare their own dishes for observing live cells, by drilling a hole in the bottom of a plastic dish and gluing a glass coverslip over the hole with aquarium silicone cement or other nontoxic adhesive (Griffin and Letourneau, 1980). Alternatively, cells can be cultured first on glass coverslips, then assembled into commercially available (often sophisticated) or homemade chambers for observation.

2.1. Preparation of Coverglass-Bottomed Dishes for Videomicroscopy

1. Drill a 15- to 20-mm hole in the bottom of a 35–60-mm bacteriological Petri dish with an electric cork borer. Remove plastic burrs with a sharp edge.
2. Glue a precleaned glass coverslip over the hole with aquarium or medical-grade silicone sealant (e.g., Dow-Corning). The coverslip can be glued into the dish or below the dish.
3. Air-dry dishes for several hours to cure the sealant.
4. Rinse dish thoroughly with sterile PBS, then sterile water.

2.2. Comments

Unlike tissue culture plastic, glass coverslips are neither clean nor sterile, and can vary in the glass composition. Most commonly, glass coverslips are cleaned by immersion in concentrated sulfuric or nitric acid for several hours, followed by extensive rinsing in distilled water. Coverslips can be sterilized by dry heat (at least 160°C for at least 1 h). If the coverslips are glued into a plastic dish, they can be sterilized by ultraviolet (UV) irradiation after assembling the dishes. Sterilization with alcohol can be done, but this may leave residues in the dish. Working with concentrated acids to clean coverslips can be bothersome, and we find that glass coverslips can be alternatively cleaned by overnight baking in a hot oven at about 250°C.

3. SUBSTRATUM-ASSOCIATED MOLECULES

The charged surfaces of PS substrata and glass coverslips are poor alternatives for the cell surface molecules that mediate neuronal contacts in vivo. Neurons do not attach well to these substrata, if they are not further treated. Thus, the addition of substratum-associated molecules to in vitro surfaces has been a longstanding and evolving concern of neuroscientists who study neurons

in vitro. This activity involves the direct treatment of substrata with solutions containing different molecules that adsorb to the substratum, before adding neurons and their culture media. As the culture progresses, the substrata become further modified by binding molecules released from cell surfaces, or that are released from cells. In addition, molecules from the culture medium may also bind to the in vitro substratum. This is especially true when serum is present in the culture medium.

Undiluted serum contains about 60 mg/mL serum albumin, which binds to substrata and can interfere with neuronal adhesion, as well as the binding of adhesion-mediating molecules in the serum, like fibronectin (FN) and vitronectin, to the substratum. Further, growth factors may bind to the substratum, because many growth factors are positively charged and bind to negatively charged groups on the substratum or substratum-associated molecules. Thus, a worker must be aware that the nature of the in vitro substratum becomes modified by the adsorption of molecules derived from the cells and from the culture medium, especially if serum or tissue extracts are included.

Finally, in considering a choice of substratum-attached molecules, whether by pretreating a dish before adding cells, or by adsorbing molecules from the culture medium and serum or molecules released from cells, a worker must be aware that these substratum-attached molecules regulate cell signaling and metabolism (Giancotti and Ruoslahti, 1999). For example, the signaling pathways that involve integrin receptors for extracellular matrix (ECM) molecules are complex, and knowledge of them is incomplete. Recent papers emphasize the role of neuron–substratum interactions in regulating axonal and dendritic growth (Lein and Higgins, 1989; Esch et al., 1999; Hopker et al., 1999).

3.1. Polylysine and Polyornithine

Neuronal adhesion to PS and glass surfaces is poor, because PS and glass surfaces have a net negative charge, and neuronal surfaces contain many glycoproteins (GP) and glycolipids with many anionic groups that are repelled by the negative surface charge. In the 1970s workers began to improve cell adhesion to glass and plastic surfaces by pretreating them with solutions of synthetic basic polypeptides, polylysine and polyornithine, which adsorb to the substratum (Macieira-Coelho and Avrameas, 1972; Yavin and Yavin, 1974; Letourneau, 1975a). Neuronal adhesion, survival, and growth are greatly improved by this treatment, and today this remains a widely used and simple method for improving neuronal attachment (especially central nervous system neurons) to in vitro surfaces.

The basic polymers probably improve neuron–substratum adhesion, not simply by allowing ionic bonding between neuronal surface components and the substratum, but also because the positively charged substratum binds adhesion-promoting molecules from serum, and that are shed or released from cells. In fact, one of the best all-around and simple methods for preparing substrata for in vitro culture of many neurons is to first treat plastic or glass surfaces with polylysine or polyornithine followed by treatment with a solution of laminin or other ECM component. The binding of ECM components to the substratum is enhanced by the additional presence of the basic polymers.

1. Treating substrata with polylysine or polyornithine:
   a. Polylysine or polyornithine can be purchased from a variety of suppliers (e.g., Sigma,). A range of mol wt from 3000 to 70,000 daltons, and either Poly-D or poly-L forms, can be used.
   b. Dissolve peptides in borate buffer (pH 8.4) or phosphate-buffered saline (PBS) (pH 7.4) at concentrations from 10 μg/mL to 1 mg/mL, then filter-sterilize the solution.

   Note: If glass coverslips are to be treated, they should be cleaned and sterilized first.

c. Sufficient solution is added to completely cover the substratum, and is left on the substratum at room temperature, or at 4°C for 15 min to 24 h.
d. It is important to thoroughly rinse and wash off any unbound material, by several rinses and a final soak of at least 15 min in PBS.

2. Comments: The varying concentrations and times of treatment with these basic polypeptides result in different amounts of binding, and a worker must determine which method is optimal for their experimental system. These basic polypeptides unbind from glass or plastic substrata after several days, and this may be evident by loss of neuronal adhesion and viability. If this factor is important, the worker should consider methods of covalent linkage or stronger binding of the polypeptides to the substratum.

3.2. ECM Components

Among the most important participants in cellular adhesions and contacts in vivo are components of the ECMs, which are arrayed in distinct layers, in fibrils and fibers, and are bound to cell surfaces (Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999). ECM components are found throughout the body, although, in the mature central nervous system they appear to be present at lower levels than elsewhere. These macromolecules, e.g., the collagens, laminins, fibronectins, thrombospondins, tenascins, and proteoglycans, comprise large, complex proteins and protein complexes, with a great diversity of binding domains that mediate interactions with other ECM components, and with cell surface molecules, including receptors. Ideas about the roles of cell adhesion to ECM components were revolutionized by the realization that the interactions of ECM components with their cell surface receptors mediate not just cell adhesion, but directly regulate metabolic pathways, cellular differentiation, and gene expression (Letoureau et al., 1994). Thus, it is not surprising that ECM components are often important in preparing in vitro substrata for neurons, especially peripheral nervous system neurons.

Collagen is the most abundant protein in human bodies, and actually comprises a multigene family of ECM components. In 1958, fibrillar type I collagen was first reported for use in culturing neural tissues (Bornstein, 1958), and its use still remains common, especially for peripheral nervous system neurons and neural cell lines. Originally, type I collagen solutions were prepared in the laboratory by extracting rat tail tendons in acetic acid, as described by Bornstein (1958). Recently, several commercial preparations of bovine dermal collagen have been made available (e.g., Vitrogen 100, Collagen, Celtrix). These contain types I and III collagen, and the reagents come with protocols for applying the collagen solutions to substrata. The easiest approach is to cover a substratum with a small amount of collagen solution, and let the water evaporate in a sterile hood, drying the collagen onto the substratum. This approach is adequate for many uses. Alternatively, more natural collagen gels can be prepared, as described in commercial products or in several papers (*see* Elsdale and Bard, 1972, for methods and uses). Neurons can be applied to the tops of collagen gels or incorporated into the collagen solution before it gels. Neuronal growth and function, in association with these gels, can be robust, including demonstrations of chemotactic growth of axons (Guthrie and Lumsden, 1994), and additional uses of ECM gels will come in the future.

After the earlier development of methods for using type I collagen on substrata for neuronal culture, fibronectin and laminin became the two major ECM components that are used for in vitro substrata for neurons (Carbonetto et al., 1983; Manthorpe et al., 1983; Rogers et al., 1983). These large multifunctional GPs are not closely related evolutionarily, but they are generally similar in having multiple binding domains for interacting with ECM components and cells. Both have multiple cell surface receptors, including several integrins and other proteins, and the two GPs initiate distinct cellular signaling pathways. Different cell types in vitro exhibit different activities when placed on fibronectin- vs laminin-treated substrata (Letourneau et al., 1994). Neurons generally

prefer laminin for growth and neurite extension. The nonneuronal cells (glia, Schwann cells, fibroblasts) that are often present in cultures from nervous tissues deposit ECM components, including fibronectin and laminin, on the in vitro substrata.

Fibronectin and laminin are commercially available from several sources at a variety of costs (and probably purities; e.g., Sigma, Becton-Dickinson). They are usually diluted in PBS or minimum essential medium at concentrations of 10–100 μg/mL, and placed in contact with the sterile substratum for several hours, followed by rinsing. Because these ECM components are expensive, workers should be empirical in determining how much ECM component to apply to their substrata. We find that chick embryo sensory neurons adhere and extend neurites in culture dishes coated with 10 μg/mL laminin or 20 μg/mL fibronectin.

Care must be taken in handling solutions of these large adhesive glycoproteins, because they can permanently aggregate and lose most of their activity in coating the substratum. Solutions should not be refrozen. Directions from the commercial source should be followed in storing and preparing solutions of fibronectin and laminin. Laminin's activity is reduced by UV illumination, so that sterilizing the substratum after applying ECM components may be harmful (Hammarback et al., 1985).

Another ECM preparation that has been shown to promote neuronal growth and development goes by the commercial name of Matrigel (Becton-Dickinson). This complex mixture is an extract of ECM components from the EHS sarcoma cell line. Major components of Matrigel that contribute to its biological properties are laminin, collagen type IV, nidogen, and heparan sulfate proteoglycan. As mentioned above, neuronal growth on substrata containing these ECM components may be further improved by adsorbing a basic polypeptide to the substratum, before applying the ECM molecules.

Another approach, which involves deposition of ECM molecules to promote neuronal growth on in vitro substrata, is an old method of producing a microexudate from a cell monolayer previously grown on a plastic or glass substratum (Orr and Smith, 1988):

1. Preparation of cellular microexudate substratum:
   a. A monolayer of glia, fibroblasts, or Schwann cells is grown on a substratum for several days.
   b. Remove the cells by addition of a solution, such as 1 *M* urea, 5 m*M* ethylenediamine tetraacetic acid, 10 m*M* Tris at pH 8.3, which will lyse the cells and extract the cytoplasmic contents, leaving ECM and other cell surface components deposited on the substratum.
   c. After 30 min, gently pipet the solution up and down to remove the cells, then rinse the substratum with PBS, and store at 4°C.
2. Comment: This method was developed before purified ECM components were available. It can be time-consuming, and produces a heterogeneous substratum, but it may be a substratum that promotes neuronal growth.

3.3. Other Methods of Preparing Substrata

The use of polylysine, polyornithine, and the ECM components, collagen, fibronectin and laminin are the most commonly used and readily available methods for preparing substrata for neuronal culture. Other approaches have been used. An approach that can achieve strong neuron–substratum adhesion is to bind lectins, carbohydrate-binding proteins, to the in vitro surface (Lochter and Schachner, 1993; Masuda-Nakagawa and Nicholls, 1991). The lectin, concanavalin A, binds mannose residues that are present in many polysaccharide chains on neuronal surfaces, and neurons adhere strongly to concanavalin A-treated substrata. Workers should be aware, before using lectins,

however, that neuronal surface activities may be altered by the binding to the lectin, and, perhaps, immobilization of many cell surface glycoproteins by the substratum-bound lectin. Another approach has been to bind to substrata, antibodies against specific neuronal surface antigens (Leifer et al., 1984). A heterogeneous suspension of cells is applied to this substratum for several hours, followed by several rinses to remove cells that have not bound to the antibody-containing substratum. This would potentially allow the selective adhesion and growth of only the cell type(s) that express the antigen recognized by the substratum-bound antibody (Leifert et al., 1984). Papers are published that use this method, but a worker would expect to develop this approach for a specific application.

3.4. Cell Surface Adhesion Molecules

As previously mentioned, it was important to recognize that cell adhesion molecules are signaling molecules that contribute to the regulation of cellular metabolism and gene expression. Thus, after the identification and cloning of several neuronal adhesion molecules, they became candidates as substratum-bound molecules for the in vitro investigation of neuronal growth and metabolism. Several adhesion molecules (NCAM, L1, and *N*-cadherin) have been demonstrated to be excellent substratum-bound molecules for promoting neuronal growth and development (Bixby and Jhabvala, 1990; Burden-Gulley and Lemmon, 1996; Drazba et al., 1997). An interesting result from some experiments indicates that there are differences among these ECM and cell surface molecules in promoting the outgrowth of axons vs dendrites from neurons (Esch et al., 1999). These cell surface adhesion molecules are not as large as fibronectin and laminin, and they do not have binding domains that facilitate their adsorption to glass or plastic surfaces. This introduces the next section on methods for improving the binding of molecules to in vitro substrata.

3.5. Other Methods of Binding Molecules to Substrata

The above methods of applying macromolecules to in vitro substrata often involve adsorption or drying of molecules from an aqueous solution onto the substratum, usually glass or plastic. This process involves biophysical properties and interactions that are beyond the scope of this chapter, but it is clear that this approach is limited in the variety of molecules that can be applied to in vitro substrata. Lagenaur and Lemmon (1987), who purified the neuronal adhesion molecule, L1, were able to bind it to in vitro substrata by first applying a solution of nitrocellulose dissolved in methanol. This dried to a thin film of nitrocellulose on the substratum, which avidly bound L1 or any protein from solution onto the substratum. The method described in Lemmon's paper is reproduced below. Some experimentation is needed to adjust the amount of dissolved nitrocellulose applied to the substratum, so that the layer is thin enough to allow microscopic observations of the neurons. The nitrocellulose film remains well adherent to plastic dishes, but it can annoyingly become released from a glass coverslip. Pretreatment of the coverslip with polylysine improves retention of the nitrocellulose film on the glass coverslip. This nitrocellulose approach allows the application of many proteins or polypeptides that could not previously be effectively bound to in vitro substrata. This can lead to new applications and in vitro investigations of neurons. My laboratory has bound neurotrophins to nitrocellulose-treated substrata and observed growth and differentiation of neurotrophin-dependent neurons, without adding neurotrophins to the culture medium. Neurotrophins bound to the nitrocellulose substratum were not released into the medium over a period of at least 3 d in vitro (G. Gallo, personal communication).

1. Protein binding to nitrocellulose-treated substrata (from Lagenaur and Lemmon, 1987).
   a. Dissolve 5 cm^2 nitrocellulose in 6 mL methanol.

   b. Rapidly spread 0.5 mL solution over the surface of a 60-mm Petri dish, and dry in sterile hood.
   c. Apply protein solution to nitrocellulose film for a few minutes, then aspirate off.
   d. Block unbound sites by washing twice with a solution of 10 mg/mL bovine serum albumin.
2. Nitrocellulose treatment of glass coverslips (from G. Gallo, personal communication).
   a. Dissolve 5 cm^2 nitrocellulose in 12 mL methanol.
   b. Apply 100 µL of solution to 22×22 coverslip.
   c. Tilt the coverslip to completely cover surface with the nitrocellulose solution. Rapidly aspirate excess solution.
   d. Dry coverslip in a sterile hood before applying proteins, as described above.

Another limitation of applying molecules to an in vitro substratum by adsorption is that the binding is not permanent, and both basic polypeptides and ECM components may slowly unbind from the substratum, which can result in decreased neuronal adhesion and viability in cultures that are several days old. In addition, many proteins and smaller peptides do not effectively adsorb to glass or plastic. This problem can be addressed by a method developed to covalently couple proteins to glass coverslips (Aplin and Hughes, 1981; Jousimaa et al., 1984). The method detailed below is straightforward. Limitations to the applicability of this technique may arise, because the glutaraldehyde linkage of a protein to the silane-treated glass may change the protein's conformation, and reduce its activity. Whether this is a problem in the application will have to be determined empirically.

Covalent coupling of proteins to glass (from Jousimaa et al., 1984):

1. Clean coverslips overnight in 20% sulfuric acid, rinse in distilled water and 0.1 *M* sodium hydroxide, then blot them dry and place in glass dishes.
2. Apply 3-aminopropyltriethoxysilane to one side of each coverslip, for 4 min at room temperature. Rinse off silane with excess distilled water, then PBS.
3. Apply 0.25% glutaraldehyde in PBS to the top of each coverslip for 30 min. Rinse off glutaraldehyde in PBS.
4. Remove excess PBS from coverslips, and apply 250 µL of the desired protein solution for 1 h at room temperature.
5. Wash coverslips 3× with PBS, and apply 250 µL of a 100 µg/mL solution of bovine serum albumin for 1 h. This treatment may be unnecessary.
6. Rinse coverslips 3× with PBS and store in PBS at 4°C.
7. It may be optional to reduce unreacted aldehyde residues by treating coverslips with 100 $NaBH_4$ in 50 m*M* borate buffer, pH 9.0, for 30 min, followed by rinsing in PBS.

3.6. Controlled Deposition of Molecules to In Vitro Substrata

Certain investigations of neuronal growth and activity may be enhanced by the ability to deposit materials on a substratum in a controlled pattern. The preference of elongating axons for particular surfaces was investigated by using electron microscope grids as masks placed on a plastic or glass surface, to pattern the deposition of palladium medal in the chamber of a vacuum evaporator (Letourneau, 1975b). Depending on the treatment of the substratum before the patterned deposition of palladium, the preferences of axonal growth cones for palladium vs another surface were examined.

In another study, electron microscope grids were used as masks to protect substratum-bound laminin from inactivation by UV irradiation (Hammarback et al., 1985). Axons of sensory neurons

plated on these patterned substrata preferred to elongate on the laminin that was protected from UV-irradiation. Methods for the sequential deposition of membrane and ECM components in alternating stripes were developed in the laboratory of Bonhoeffer (Vielmetter et al., 1990), and this approach was used by Gomez to deposit fibronectin and laminin in alternating, nonoverlapping stripes on plastic or glass substrata (Gomez and Letourneau, 1994). The method involved gently pressing thin strips of silicone onto a substratum, then covering the substratum with a solution of a molecule to be adsorbed onto the surface. The surface under the silicone strip was protected from the protein solution. Then, after several hours, the surface was rinsed and incubated with a solution of PBS containing 5 mg/mL bovine serum albumin, which would occupy all remaining binding sites on the surface not protected by the silicone strips. Then, after several hours, the albumin solution was rinsed off, the silicone strips were removed from the substratum, and a solution of the second molecule to be adsorbed to the substratum was added. This molecule will only adsorb where the silicone strips had formerly been contacting the surface, because the adsorbed albumin occupies all other binding sites.

Recently, methods of photolithography and material science have been used to produce "designer" substrata, employing methods of organic chemistry and physics to link proteins, and peptides that mimic protein functions to in vitro substrata on a microscopic scale that is almost subcellular (Herbert et al., 1997; Kleinfeld et al., 1988). These emerging collaborations of nerve cell biologists with physical scientists and engineers will continue to allow the production of precisely defined in vitro substrata, which will lead to novel experimental investigations into the cellular and molecular mechanisms of neuronal growth, differentiation, and function.

4. CONCLUDING REMARKS

In vitro culture is an exciting and powerful approach for investigating fundamental issues of neuronal development, metabolism, and physiology. Neuronal function in vitro depends on the ability of the neuron to make appropriate adhesive contacts with a substratum. It is a basic requirement for in vitro work, as is culture medium. The realization of the signaling and regulatory functions of cell adhesion molecules and ECM components highlight the fundamental importance of neuronal adhesion.

Much trial and error, over a 90-yr history has led to the development of a variety of methods for preparing in vitro substrata for neurons. The immense diversity of neuronal types suggests that the best in vitro solutions may differ for different neurons, so that an empirical approach will still be important for each worker. However, general considerations in preparing in vitro substrata for most uses include the proper cleaning of glass coverslips; the adsorption of polylysine or polyornithine to promote neuronal adhesion and the binding of macromolecules from the culture medium, either serum or cell-derived; and the adsorption of ECM components.

Despite the great increase in what is known about cell adhesion, ECM molecules and growth factors, and how to apply them to substrata, workers are often dependent on adding serum to culture media in order to achieve the best neuronal function. An unknown number of macromolecules bind from serum to the in vitro substrata, including serum albumin, which can interfere with neuron–substratum adhesion. If neurons survive and function in a defined medium, a worker can avoid the unknowns and variability that arise from using serum. As more and more is learned about growth factors and the individual molecules that mediate neuronal contacts and interactions, the preparation of in vitro substrata for culturing neurons will become more rational, and require less guesswork.

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Chapter Nineteen

Serum-Free Media for Neural Cell Cultures

Adult and Embryonic

Paul J. Price and Gregory J. Brewer

1. INTRODUCTION

The ability to grow primary neurons under serum-free conditions is facilitating better control in studies of neuronal development, mechanisms of neuronal signaling, electrophysiology, pharmacology, plasticity, in vitro growth requirements, gene expression, and neurotoxicity. Some of the new commercially available media combinations allow for the growth of sparse populations of neurons, which in turn allow for the study of individual neurons and synapses. This has not been possible using serum-supplemented media without a feeder layer of glial cells. In serum-supplemented media, glial cells continue to multiply, necessitating the use of cytotoxic mitotic inhibitors (Wallace and Johnson, 1989). Serum also contains unknown and variable levels of growth factors, hormones, vitamins, and proteins.

Based on earlier studies (Romijn et al., 1984, 1988; Romijn, 1988; Brewer and Cotman, 1989), Brewer et al. (1993) developed a basal medium formulation optimized for the survival of primary rat embryonic neurons after 4 d in culture, together with a supplement, B27 (B27 is a proprietary formula of Life Technologies). The basal medium, Neurobasal™ (Table 1) is a modified Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12), in which the osmolality and the concentration of several amino acids have been reduced and ferrous sulfate eliminated. The supplement, B27, resulted from an optimization of 20 components of an earlier supplement called B18 (Brewer and Cotman, 1989; Table 2). The combination of Neurobasal with B27 allows for the long-term growth of primary embryonic hippocampal and other brain neurons at both low and high seeding densities, and the growth of numerous cell lines of neuronal origin. Brewer also showed that neurons from different areas of the brain maintain their individual characteristics under the same culture conditions (Brewer, 1995).

A subset of components from B27 was developed earlier, as N2 (Table 3). N2 is also used with Neurobasal, or with other basal formulations, such as Eagle's minimal essential medium, DMEM, or DMEM/F12, but survival is inferior to B27, unless a high initial plating density is used (Brewer et al., 1994). Because N2 supplements are chemically defined, studies are possible in the absence of unknown factors found in serum or serum components, such as serum albumin, which may influence growth and differentiation, or introduce neurotoxins (Bottenstein and Sato, 1976;

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Table 1. Composition of Neurobasal Medium

Component	Final mg/L
Inorganic salts	
$CaCl_2$ (anhydrous)	200.0
$Fe(NO_3)_3 \cdot 9H_2O$	0.1
KCl	400.0
$MgCl_2$ (anhydrous)[b]	77.3
NaCl[b]	3000.0
$NaHCO_3$[b]	2200.0
$NaH_2PO_4 \cdot H_2O$	125.0
$ZnSO_4 \cdot 7H_2O$	0.19
Other components	
D-Glucose	4500.0
HEPES[b]	2600.0
Phenol red[b]	8.1
Na pyruvate[b]	25.0
Amino acids	
L-Alanine[b]	2.0
L-Arginine · HCl	84.0
L-Asparagine · H_2O[b]	0.83
L-Cysteine[b]	1.21
L-Glutamine[a,b]	73.5
L-Glutamate[c]	
Glycine	30.0
L-Histidine HCl · H_2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine HCl	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Proline[b]	7.76
L-Serine	42.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	94.0
Vitamins	
D-Calcium pantothenate	4.0
Choline chloride	4.0
Folic acid	4.0
i-Inositol	7.2
Niacinamide	4.0
Pyridoxal HCl	4.0
Riboflavin	0.4
Thiamine HCl	4.0
Vitamin B_{12}	0.34

[a]Not supplied in liquid medium; needs to be added.
[b]Changed from DMEM.
[c]Glutamate at 3.7 μg/mL (25 μ*M*) should be added to start primary embryonic HC neurons.

Table 2. Composition of B18 Supplement and Ingredients of B27

Component	Final mg/L
Amino acids	
L-Alanine[a,b]	2.0
L-Glutamate[b]	3.7
L-Glutamine[b]	441.0
L-Proline[a,b]	7.76
Vitamins	
Biotin[b]	0.10
Vitamin B_{12}[a,b]	0.34
Hormones	
Corticosterone[c]	0.02
Progesterone[c]	0.0063
Retinol, all trans (vit. A)	0.1
Retinol, acetate[c]	0.1
Insulin[a,c]	4.0
T_3 (triodo-L-thyronine)[c]	0.002
Antioxidants	
Na pyruvate[a,b]	25.0
Lipoic acid (thioctic acid)[a]	0.047
D,L-α-Tocopherol (vit. E)[c]	1.0
D,L-α-Tocopherol acetate[c]	1.0
Catalase[a,c]	2.5
Glutathione (reduced)[c]	1.0
Superoxide dismutase[a,c]	2.5
Other	
L-Carnitine[c]	2.0
Ethanolamine[c]	1.0
D(+)-Galactose[c]	15.0
HEPES[a,b]	2600.0
Putrescine[c]	16.1
Penicillin	50 IU/mL
Streptomycin	0.5
Selenium[a,c]	0.016
Zinc sulfate[a,b]	0.194
Linoleic acid[c]	1.0
Linolenic acid[c]	1.0
Albumin, bovine[c]	2500.0
Transferrin[a,c]	5.0

[a]Changed from the original formulation of Romijn et al. (1984).
[b]Not needed when used with Neurobasal.
[c]Included in B27, but concentrations optimized for 4 d survival of hippocampal

Table 3. Composition of the N2 Supplement

Component	Final mg/L
Insulin (bovine)	5 mg/L
Progesterone	20 n*M*
Putrescine	100 µ*M*
Na selenite	30 n*M*
Transferrin (human)	100 mg/L

Bottenstein, 1983, 1984). However, for some studies, fetal bovine serum or fibronectin is used as an attachment step, and some laboratories further supplement N2 with bovine serum albumin at 1 mg/L (e.g., Pongrac and Rylett, 1998). Aggregates of neural cells can also be grown under serum-free conditions (Honegger et al., 1979).

For the growth of postnatal and adult neurons, Brewer has optimized some modifications to Neurobasal and the isolation protocol (Brewer, 1997). The modified Neurobasal or Neurobasal-A is identical to Neurobasal, except for the osmolality. For embryonic neurons the optimal osmolality was shown to be in the range of 235 ± 10 mOsm, and, for postnatal and adult neurons, the optimal osmolality is in the range of 260 ± 10 mOsm. In addition to an optimized medium, the growth of adult central nervous system neurons also requires gentle proteolytic separation of their numerous connections, a density gradient for the separation of oligodendrocytes and debris and enrichment of neurons. When all of these requirements are met, neurons regenerate neuron-like morphology, electrophysiology (Evans et al., 1998; Collings et al., 1999), and can be maintained in culture for several weeks. The addition of fibroblast growth factor (FGF-β) enhances viability at least three-fold, independent of the age of the animal, without affecting the length of the processes. These advances also enable recovery of live neurons for several hours postmortem at room temperature (McManus and Brewer, 1997), or up to 24 h on ice (Viel et al., 2000).

Levels of CO_2 in mammals and cell culture incubators are 20–50-fold above the ambient level of 0.2%. Neurobasal, which was optimized for growth of embryonic rat hippocampal neurons, contains 26 m*M* bicarbonate as a physiologic buffer of pH, like many culture media. When transferred to ambient CO_2, neurons die within hours in this or other such high bicarbonate buffers, as the medium pH rises to 8.1. A CO_2-independent modification of Neurobasal, Hibernate™-E, when supplemented with B27, can maintain neuron viability for at least 2 d in ambient CO_2 (Brewer and Price, 1996). Another version of Hibernate with higher osmolarity, Hibernate-A, is useful in longer procedures outside the incubator, such as isolation of adult neurons. Hibernate contains common salts, a buffer, glucose, pyruvate, amino acids, and vitamins. This same medium can also be used to store viable brain tissue for up to 1 mo, with refrigeration (Brewer and Price, 1996). Therefore, brain tissue can be inventoried and transported with little loss of viability. BrainBits is a service that can supply rat brain tissue through express mail, so that investigators can start isolated neuron cultures within 20 min of receipt of tissue (gbrewer@siumed.edu). Thus, BrainBits replaces the need for a certified animal facility and a trained neuroanatomy technician to dissect specific brain regions.

2. NEURONS FROM EMBRYONIC TISSUE

2.1. Isolation of Cells

1. Materials:
   Timed pregnancy rats:
   18 d gestation for hippocampus or septum.

16 d gestation for cortex or striatum.
Note: For cerebellum, use postnatal d 8.
Neurobasal medium (Gibco-BRL, cat. no. 21103).
Hibernate-E (Gibco-BRL, cat. no. 10741).
B27 (Gibco-BRL, cat. no. 17504).
Hank's balanced salt solution (HBSS) (Gibco-BRL, cat. no. 14025).
HBSS without Ca^{2+}, Mg^{2+} (Gibco-BRL, cat. no. 14175).
Papain (Worthington, cat. no. 3119).
Sodium bicarbonate ($NaHCO_3$) (Gibco-BRL, cat. no. 25080).
Na pyruvate (Gibco-BRL, cat. no. 11840).
Trypan blue, 0.4% (Gibco-BRL, cat. no. 15250).
Pasteur pipet, 9-in. siliconized (2% dichlorosilane in $CHCl_3$, Sigma), fire polished tip to 0.7–0.9-mm.
Tubes, centrifuge, 15-mL, polystyrene (not polypropylene) (available from Fisher, Corning, or VWR).

2. Protocol for cell isolation from embryonic tissue:
   a. Dissection:
      i. Recover embryos by C-section under Nembutal anesthetic.
      ii. Dissect desired brain tissue (typically, six HCs are dissected from three brains).
   b. Isolate cells by trituration 10+ in 1.0 mL HBSS without Ca^{2+}, Mg^{2+}, 0.035% $NaHCO_3$, 1 m*M* Na pyruvate, 10 m*M* HEPES, pH 7.4, through a 9-in. siliconized Pasteur pipet, with the tip barely fire-polished.

   Note: For a higher yield or tissue more than 1 d postmitotic (e.g., E18 cortex), dissected regions are treated with papain at 2 mg/mL in solution, given in step b, for 20 min at 30°C. Tissue is then transferred to 1 mL Hibernate-E/26B27 and triturated 10–15+ through the fire-polished pipet.

   c. Restore divalent cations by adding 2.0 mL HBSS containing 0.035% $NaHCO_3$, 1 m*M* Na pyruvate, and 10 m*M* HEPES, pH 7.4.
   d. Allow nondispersed tissue to settle for 3 min.
   e. Draw off and transfer supernatant to a 15-mL centrifuge tube, and centrifuge for 1 min at 200*g*.
   f. Gently resuspend pellet in 1 mL Neurobasal/2% B27 containing 0.5 m*M* glutamine and 25 μ*M* glutamate per brain.
   g. Determine viable cell count by trypan blue exclusion, by mixing an equal volume of cell suspension with 0.04% trypan blue, and counting live and dead cells in a hemacytometer (Tolnai, 1975).
   h. Reconstitute the cells at the desired plating concentration in Neurobasal, with 2% B27 containing 0.5 m*M* glutamine and 25 μ*M* glutamate.

   Note: Yield of neurons is about 1 million cells/HC or 6 million cells/cortex.

2.2. Growth of Embryonic Neurons

1. Materials:
   Neurobasal medium (Gibco-BRL, cat. no. 21103).
   B27 (Gibco-BRL, cat. no. 17504).
   N2 (Gibco-BRL, cat. no.17502).
   L-Glutamine (Gibco-BRL, cat. no. 25030).
   Glutamic acid (Sigma, cat. no. G-1626).
   Poly-D-lysine (mol wt 30,000–70,000) (Sigma, cat. no. P-7280), 1 mg/mL sterile water, store

2 mL aliquots frozen in 15-mL polystyrene tubes.

Note: Polylysine stored in polycarbonate or polypropylene tubes does not support the reproducible culture of neurons without added proteins.

Human Fibronectin (Gibco-BRL, cat. no. 33016). To prepare human fibronectin, reconstitute 5 mg in 6.75 mL sterile distilled water for a 740 μg/mL stock.

Bovine vitronectin (Gibco-BRL, cat. no. 12172).

Tubes, centrifuge, 15-mL, polystyrene (*see above*).

2. Protocol for growth of embryonic neurons:
   a. Poly-D-lysine substrata:
      i. Coat surface of glass coverslips, plastic well, or flask with a 0.05 mg/mL solution of poly-D-lysine at 0.15 mL/cm^2 surface area and incubate for 1 h or overnight.
      ii. Flasks or dishes coated with poly-D-lysine are washed with sterile distilled water. Remove water, and let substrate dry.
   b. To Neurobasal medium, add 0.5 m*M* L-glutamine, 25 μ*M* glutamate and either 2% B27 or 1% N2 supplement.
      i. For tissues more than 1 d past the start of differentiation, such as E18 cortex or striatum, leave out the glutamate.

Note: For tissues harvested at the time of differentiation, such as E18 hippocampus, glutamate improves survival (Brewer et al., 1994).

      ii. If using N2 supplement, prior to adding the cells, add human plasma fibronectin at a final concentration of 5–10 μg/mL directly to the medium (add 50 μL stock per 5 mL culture medium, for a final concentration of 7.4 μg/mL), or substitute bovine vitronectin for fibronectin at 0.5–1.0 mg/mL.
   c. Cells are added to the wells of flasks at 160 cells/mm^2, or other desired concentrations, if using B27 as the supplement, or at 800 cells/mm^2 or higher, if using N2 as the supplement. Tilt vessel immediately, to ensure even distribution.
   d. Cultures maintained more than 4 d should have half of the medium changed at 4 d, and, thereafter, once per week. The fresh medium should not contain glutamate.
   e. Cultures are maintained at 37°C in a humidified atmosphere of 5% CO_2, 9% O_2 (Forma, model 3130, Marietta, OH).

Note: Incubators without control of oxygen give satisfactory results in most applications.

3. POSTNATAL AND ADULT BRAIN NEURONS

3.1. Materials

Neurobasal-A (Gibco-BRL, cat. no. 10888).

B27 (Gibco-BRL, cat. no. 17504).

Hibernate-A (Gibco-BRL, cat. no. 10740).

OptiPrep™ (Gibco-BRL, cat. no. 103-0061) diluted 0.505–0.495 (v/v) with 145 m*M* NaCl, 10 m*M* 3-[*N*-morpholino] propanesulfonic acid (MOPS), pH 7.4, to produce a density of 1.15.

Note: OptiPrep, adjusted to a density of 1.15, substitutes for the originally specified NycoPrep, which is not available in North America.

FGF-β (Gibco-BRL, cat. no. 13256).

L-Glutamine (Gibco-BRL, cat. no. 25030).

Papain (Worthington, cat. no. 3119).

Gentamycin, 10 mg/mL (Gibco-BRL, cat. no. 15710).

Poly-D-lysine (Sigma, cat. no. P-7280).

Petri dishes, 35-mm, tissue culture (e.g., Falcon, cat. no. 3001).
Petri dish, 100-mm, sterile, plastic (e.g., Falcon, cat. no. 1029).
Tubes, centrifuge, 15-mL polystyrene (*see* Section 2.2., step 1.).
Pasteur pipet, 9 in., siliconized (*see* Section 2.1., step 1.).
Coverslips, 12-mm, glass (Carolina Biologicals, Assistant Brand, cat. no. AA-63-3029).
Paper, sterile, 7-cm (Whatman no. 3).
MacIlwain tissue chopper (Brinkman, Westbury, NY).
Orbital shaker, water bath (Labline, Melrose Park, IL, model 3535).

3.2. Isolation of Tissue

Note: Volumes are indicated for two hippocampi, about 150 mg tissue. Larger masses of tissue should use larger volumes.

1. Hippocampi, other brain regions, or spinal cord are rapidly dissected from the brain in 2 mL Hibernate-A, supplemented with B27 and 0.5 m*M* L-glutamine, at 4°C in a 35-mm dish.
2. Meninges and excess white matter are removed in the same medium to a second dish at 4°C.
3. Hippocampi are transferred to sterile paper prewet with the same medium on the cooled stage of a MacIlwain tissue chopper.
4. 0.5 mm thickness slices are made perpendicular to the long axes of the hippocampi and transferred to a tube at 4°C of the same medium.
5. After shaking for 8 min at 30°C, slices are transferred, with a wide-bore pipet, to another tube at 30°C containing papain.

3.3. Digestion of Tissue

1. Papain (15–23 U/mg protein, and not activated by cysteine) is prepared by dissolving 12 mg in 6 mL Hibernate-A, warming for 5 min at 37°C. (Dissolution is not complete.) The solution is filter-sterilized, and stored at 4°C. The papain solution should be used within 3 h.
2. Slices are incubated for 30 min in a 30°C water bath, with a platform rotating at a speed sufficient to suspend the slices (e.g., 170 rpm).
3. Slices are transferred to a 15-mL tube containing 2 mL Hibernate-A/B27 at 30°C, and allowed to sit for 5 min at room temperature.
4. Slices are triturated 10 (in about 30 s) with a siliconized 9-in. Pasteur pipet with the tip fire-polished.
5. The pieces are allowed to settle for 2 min, and the supernatant is transferred to another tube.
6. The sediment from the first tube is resuspended in 2 mL Hibernate-A/B27, and the procedure (steps 4–5) repeated 2× more.
7. The supernatants from each trituration are combined giving 6 mL suspension (Fig. 1A).

3.4. Gradient Isolation

1. The cell suspension is carefully applied to the top of a gradient of OptiPrep 1.15 g/cm^3 in a 15-mL centrifuge tube. The OptiPrep gradient is made in four 1.0-mL steps of 35, 25, 20, and 15% OptiPrep in Hibernate-A/B27.
2. The suspension is centrifuged at 800*g* for 15 min.
3. The top 6 mL, containing debris, is discarded.
4. Desired fractions are collected with the trituration pipet into 5 mL Hibernate-A/B27.

Note: Fraction 1 is the top 2 mL, and is enriched for oligodendrocytes. Fraction 2 contains the most dense band of fragments, neurons, and other cells. Below this band is fraction

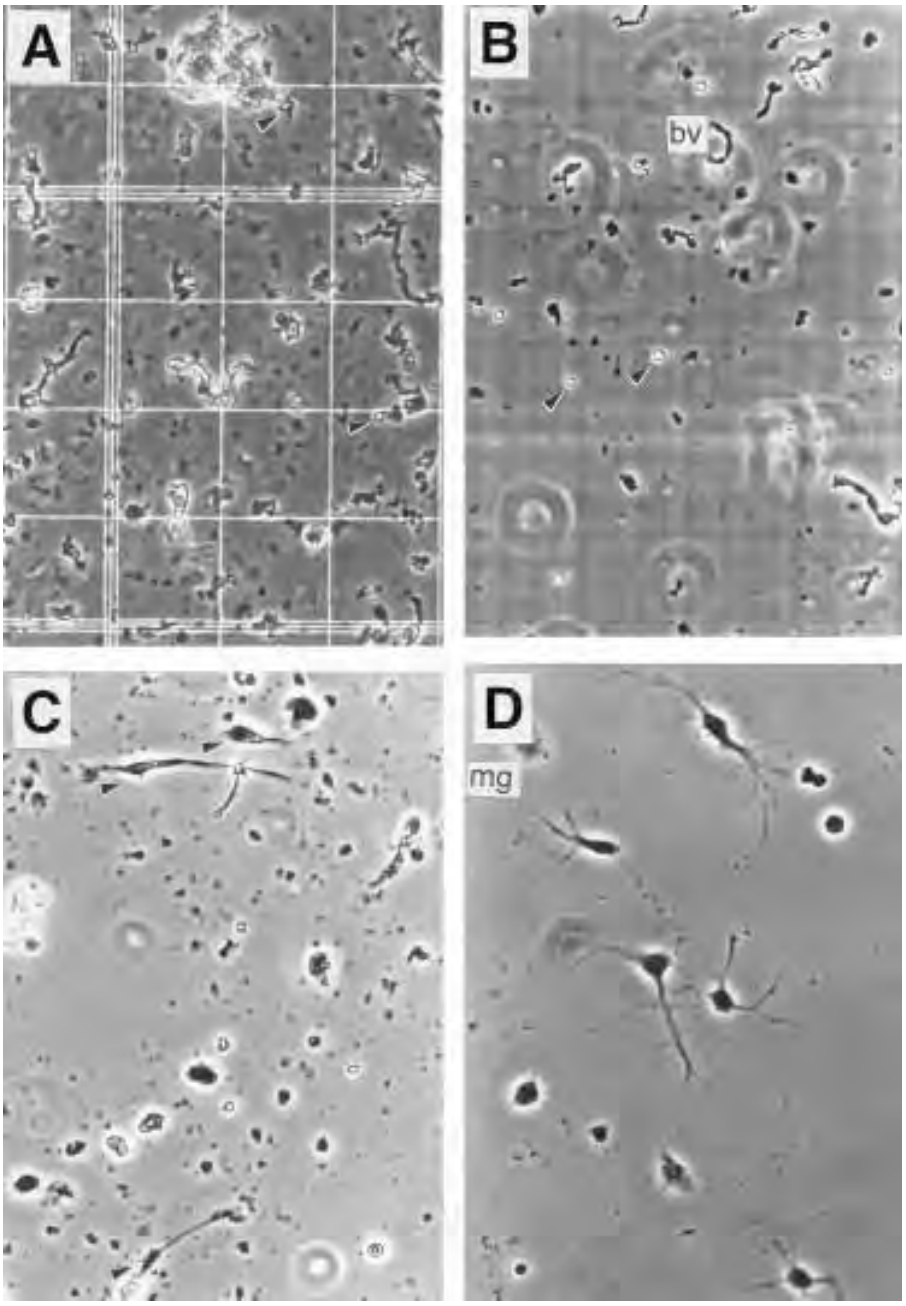

Fig. 1. **(A)** Phase contrast images of isolated adult hippocampal neurons. Arrowheads indicate phase-bright live cells. Debris in the background results from sheared axons, dendrites, and blood vessels. The grid pattern is from the hemacytometer with 50 μm squares (same magnification on all panels). **(B)** After gradient isolation (Section 3.3., step 8). Arrowheads indicate phase-bright live cells. A curved remnant of a blood vessel is identified (bv). **(C)** Three hours after plating (Section 3.4., step 4). Arrowheads indicate neurons that have sprouted. **(D)** After 4 d of culture, a majority of cells have neuron-like neurites and growth cones. A microglial cell is identified (mg).

3, which is enriched for neurons. The pellet (fraction 4) is enriched for microglia, and is resuspended in 2 mL Neurobasal-A/B27, with 0.5 m*M* L-glutamine.

5. The fractions are centrifuged for 1 min at 200*g*, and the pellets resuspended in 1–3 mL Neurobasal-A/B27 with L-glutamine.
6. Expected yield for hippocampus is about 250,000 cells/hippocampus, about 60% of which survive and regenerate as neurons (Fig. 1B).

3.5. Cell Plating

1. Cells are plated at the desired concentration (i.e., 90–320 cells/mm^2) in 60–150 µL Neurobasal-A/B27, onto autoclaved glass previously coated with 50 µg/mL poly-D-lysine in water (135 kDa), distributed in a 100-mm sterile plastic dish.

Note: The polylysine can be applied overnight, aspirated, rinsed once with water, and allowed to dry 1 h.

2. One h after plating and incubation (ambient oxygen with 5% CO_2 is acceptable, but 9% oxygen with 5% CO_2 is preferable, and more physiological), the coverslip is quickly picked up, allowed to drain, and transferred to 0.4 mL Neurobasal-A/B27 in a 24-well plate at 37°C.
3. The medium is aspirated, the coverslips rinsed once with warm Hibernate-A, and the cells refed with Neurobasal-A, supplemented with B27, 0.5 m*M* L-glutamine, 10 µg/mL gentamycin, and 5 ng/mL FGF-β (Fig. 1C).
4. When culturing the cells for longer than 4 d, one-half of the medium is removed on d 3 or 4, and replaced with an equal volume of medium now containing 5 ng/mL FGF-β (Fig. 1D).

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Chapter Twenty

Development of Multiple-Well Plate Biological Assays

John W. Harbell

1. INTRODUCTION

This chapter focuses on the application of multiple-well plate technology to biological assays for neuroactive or other agents. It does not attempt to address specific cell types, culture systems, or individual assays for neurotoxicity, neural cell differentiation, or other applications of neuronal cells in culture. These topics are far too broad. Other chapters in this book provide that information. Rather, this chapter provides points to consider in developing the bioassay of interest in the multiple-well plate format. It highlights many of the issues that must be addressed both to reduce the size of the individual cultures and to use end point assays, which can readily be instrument-scored. The term "end point assay" refers to the specific method used to measure the induced change in the cells. Multiple-well plates of 96 or more wells per plate are not new, nor are the bioassays based on them. However, the physical dimensions of the wells, the need for uniformity among wells, and the frequent focus on spectrophotometric or fluorometric end points require special attention in the experimental design.

Assessing the action of neuroactive agents often involves a multifactorial experimental design. Dose, time of exposure, cell type, and end point measures of action may all be dependent variables in a matrix design. The multiple-well plate is ideal for this purpose. It allows one to handle large numbers of individual cultures, easily maintain their spatial relationships through complex manipulations, and automate data collection and analysis.

Perhaps the most common type of multiple-well assay measures the impact of an active agent as a function of dose on a population or several populations of cells. Relatively small quantities of agent are required, given the small volumes of medium used in each well. For screening purposes, doses can be spaced at intervals to cover the range of possible active concentrations. Using the screening data, subsequent assays can be directed at the appropriate dose ranges for comparing active agents. Time course assays are also common, in which several plates are used to compare the effects of dose over a range of exposure times. Time-matched controls are contained on each plate in the series. In the same way, multiple cell types may be compared to evaluate the specificity of an active agent. For example, the chemical's action on cells expressing a receptor of interest may be compared to a similar cell without the receptor.

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In most cases, one is measuring the impact of the agent on the whole population of cells within a well, rather than on individual cells within the populations, because the standard end point assays measure the change for the whole population within the well. In contrast to the visual end points (i.e., changes in morphology or immunohistochemical staining) that might be used in other format assays, the 96-well plate is well suited to "population" end points. These end point assays include measures of cell viability, adenosine triphosphate (ATP) levels, ion fluxes, and expression of signal molecules (by enzyme-linked immunosorbant assay). Cell viability might be measured by one or more end points (assays), such as vital dye binding and cytoplasmic enzyme release. Many, if not most, of the end points employed are indirect measures of the desired marker of effect. For example, metabolism of the tetrazolium dye, 3·(4,5-dimethylthiazol-z-yl)2,5-diphenyltetrazolium bromide (MTT), is often used as a measure of the number of viable cells within a treated population, compared to the number of viable cells within the control population. This is an indirect measure of viable cell number, in contrast to the direct measure provided by viable cell counts. More complex indirect measures may be used to predict induction of differentiation or other functional changes within the population. To this end, one is often looking for a measurable "upstream" event/change that is predictive of the "downstream" change of interest. Selection of appropriate and measurable upstream events, which accurately predict the subsequent downstream events, is one of the greatest challenges in developing cell-based bioassay.

2. EXAMINING THE CELL CULTURE SYSTEM

For the purposes of this chapter, it is assumed that the multiple-well bioassay will be based on a cell culture system that is already described. Other chapters in this text contain many systems that would be amenable to such bioassays. Evaluation of the standard culture system should be the first step in designing the bioassay. The basic cell system may be fashioned with primary cultures or cell lines. Cell lines are ideal, since they can be better characterized and expanded to provide cells on a convenient basis for seeding into the multiple-well plates. On the other hand, the use of primary cultures or pluripotent stem cells may be warranted when a cell line cannot model the specific activity of interest. Human cells may be obtained from several commercial sources. Even if the cells have a limited functional life-span in culture, they provide the species-specific receptor and metabolic profile that may be needed for some studies.

After selecting the cell type, the other parameters of the culture system should be evaluated. All of these factors will impact the design of the bioassay system.

1. Substrate type: What type of substrate is required to achieve the desired cell attachment, growth, and/or differentiation? Note the brand of plastic on which the cells have been successfully cultured. Are special coatings or substrates required?
2. Medium: What is the standard medium, and does it contain serum?
3. Cell seeding: What are the cell seeding density and seeding efficiencies?
4. Incubation conditions: What are the incubation conditions? What is the ratio of substrate surface area to volume of medium? How often must the medium be renewed to maintain the desired conditions?
5. Cell proliferation: Do the cells proliferate, and, if so, how rapidly do they approach a cell density that reduces the proliferation rate?

3. THE CRITICAL NEED FOR UNIFORMITY

A requirement of multiple-well bioassays is that there be uniformity across the wells within a plate, the plates within an assay, and the assays over time. Experience has shown that consistency must be designed into all aspects of the assay; from the maintenance of the stock cell cultures (or

isolation of the primary cultures), to the assay end point determinations. The first critical step is to develop the methods to produce a reproducible cell population for seeding into the assay plates. This cell population must have the following qualities. The cell line must be free of adventitious agents, such as mycoplasma; the purity of the culture should be known; and it should be the intended cell type (tissue and species). These questions must be addressed in the beginning. Once the cell line is characterized, it is advisable to prepare a frozen cell bank from which stock cultures can be prepared at intervals. This cell bank provides a reserve, in case the stock cultures are lost, and it helps to maintain uniformity in the cultures over time. This uniformity is especially important if the bioassay is to be used for an extended period.

The procedures for maintaining stock cultures will depend on the cell type. However, several general principles should be followed:

1. If possible, stock cultures should be maintained in the absence of antibiotics, which can mask an occult infection (e.g., mycoplasma) introduced by a breach in aseptic technique or a contaminated reagent. Ideally, one would want to be able to see contamination manifested as a macroscopic overgrowth or other clear changes in the cultures. The contaminated culture can be identified and replaced before erroneous data are produced.
2. The length of time that a stock culture can be maintained and still have the cells behave in the desired fashion should be established. Cultures show genetic drift over time, and many normal cells have finite life-spans. Even cell lines may exhibit unexpected changes as a function of genetic drift. Cell function may degrade as the end of that life-span is approached.
3. The schedule for routine feeding and passaging is also critical. If the cells are proliferating, they should generally be kept at subconfluent densities. Overconfluence will depress proliferation rates and markedly change cell-seeding efficiency.

Experience has shown that it is important to establish and maintain a strict schedule for routine maintenance of the stock cells, and, especially, for preparing cells for seeding into the 96-well plates. For example, in preparation for cell seeding, one might routinely passage the stock cultures 3 days before use, and refeed them the day before use. Consistent seeding efficiency and subsequent proliferation/differentiation in the 96-well plate is the goal of setting and maintaining a fixed schedule.

4. SELECTING THE MULTIPLE-WELL PLATE

As the number of cells in a culture decreases, the uniformity of each culture becomes more critical. The 96-well plate format allows only 0.32 cm^2 growing area per well, compared to 1.8 cm^2 for a 24-well plate or 9.5 cm^2 for a 35-mm dish. Small changes in the absolute number of cells will be manifested in greater differences in the measured bioassay end points. The proprietary treatments, used to place a charge on the growing surfaces of polystyrene plasticware, yield slightly different charge characteristics and densities. Once cells are adapted to a certain surface treatment, they may not attach or replicate as well on another. It is generally best to start by using the 96-well plates from the same manufacturer as the plasticware used for routine culture. The plastic substrate is especially critical in the absence of serum or additional coating. However, plate-molding techniques and/or inadequate quality control measures may render the 96-well plate less acceptable than the manufacturer's larger-format vessels. It may be necessary to test several brands of plates to find one that will provide the required well-to-well uniformity across the full plate.

The end point assay will impact the selection of the plate, as well. For most applications, the standard flat-bottomed clear plate is acceptable and the easiest to use. It also allows for the best visual inspection of the cells across the bottom of each well. Spectrophotometric end points (e.g., vital dye viability assays) are performed directly in the clear plate. Some fluorometric and all

A

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		t1c1	t1c1	t1c1	t1c1	ctrl	t2c1	t2c1	t2c1	t2c1	ctrl	
C		t1c2	t1c2	t1c2	t1c2	ctrl	t2c2	t2c2	t2c2	t2c2	ctrl	
D		t1c3	t1c3	t1c3	t1c3	ctrl	t2c3	t2c3	t2c3	t2c3	ctrl	
E	B	t1c4	t1c4	t1c4	t1c4	ctrl	t2c4	t2c4	t2c4	t2c4	ctrl	
F	B	t1c5	t1c5	t1c5	t1c5	ctrl	t2c5	t2c5	t2c5	t2c5	ctrl	
G		t1c6	t1c6	t1c6	t1c6	ctrl	t2c6	t2c6	t2c6	t2c6	ctrl	
H												

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	std1	std1	t1c1	t1c1	t1c1	t1c1	ctrl	t3c1	t3c1	t3c1	t3c1	ctrl
B	std2	std2	t1c2	t1c2	t1c2	t1c2	ctrl	t3c2	t3c2	t3c2	t3c2	ctrl
C	std3	std3	t1c3	t1c3	t1c3	t1c3	ctrl	t3c3	t3c3	t3c3	t3c3	ctrl
D	std4	std4	t1c4	t1c4	t1c4	t1c4	ctrl	t3c4	t3c4	t3c4	t3c4	ctrl
E	std5	std5	t2c1	t2c1	t2c1	t2c1	ctrl	t4c1	t4c1	t4c1	t4c1	ctrl
F	std6	std6	t2c2	t2c2	t2c2	t2c2	ctrl	t4c2	t4c2	t4c2	t4c2	ctrl
G	std7	std7	t2c3	t2c3	t2c3	t2c3	ctrl	t4c3	t4c3	t4c3	t4c3	ctrl
H	B	B	t2c4	t2c4	t2c4	t2c4	ctrl	t4c4	t4c4	t4c4	t4c4	ctrl

Fig. 1. Examples of plate maps for the 96-well plate bioassays. **(A)** Plate map showing the outer well empty or filled with sterile water or saline, to reduce desiccation. **(B)** Plate map prepared to show cell seeding and wells left empty for reference standards to be used in the end point assay. B, Blank (may be seeded with cells, or not, as appropriate, for the end point assay); ctrl, negative/solvent control; t1, test material 1; t2, test material 2; c1–6, test material concentrations; std1–6, standards (added during the end point assay).

bioluminescent (e.g., ATP assays) end points require plates that do not allow light to pass between wells. Black-walled wells with clear bottoms are best when visual observations will be performed on the cells, in addition to the other end points, and when the fluorometer or luminometer reads from the bottom of the plate. White opaque plates are available for top-reading instruments.

Adhesion and/or differentiation of certain cell types require specific substrates. These are applied to the plastic before the cells are seeded. If the cells in the bioassay require such a substrate, it should be applied to the 96-well plates, as well. Although this may be tedious, the use of a multichannel repeater pipetor will greatly facilitate the operation. Confining the coating to the bottom of the well, as much as possible, will help maintain a uniform growth area in each well. The coating may be applied directly to the well bottom, by holding the pipet tips vertically in the wells. If the coating process requires that the excess coating be removed, use a Pasteur pipet attached to a vacuum to aspirate the excess, rather than inverting the plate to pour it off. Most coatings will impact the end point assays by dye binding and/or altering the light transmission through the well. Therefore, control and blank wells must also be coated, so that assay values can be adjusted for these factors.

5. PREPARING THE PLATE MAP

The plate map is the plan or plate layout used to seed, treat, and assay the plate. The layout of the plate will depend on the assay end point(s), the number of treatment groups, the replicate wells within each treatment group, and the controls, blank wells, and standards needed. Figure 1 shows two examples of plate maps. There are many designs possible, but certain features are helpful in designing a plate map:

1. Try to develop a standard plate map that can be used for most, if not all, of the testing to be performed. This will greatly facilitate data reduction after the end point assays are completed. It will also reduce errors in seeding and treatment.
2. Group wells that will be seeded with cells, so that the multichannel repeater pipet can be used most effectively.
3. Group treatments to facilitate dosing.
4. Place control wells so that they are representative of the plate as a whole. In the examples shown, the controls run vertically across the plate, and are located in the middle and end columns. If the plate is seeded from left to right, the uniformity of the plate can be judged by comparing the center with the last column. Similarly, the middle column will show any differences between the outer wells (rows A and B or G and H) and the center wells (rows D and E).
5. Blank wells are used to determine background absorbance or fluorescence in the end point assay. Depending on the specific end point assay and cell type, the blank cells should be seeded with cells or left unseeded.
6. Wells may also remain unseeded, to be used to hold reference standards in the end point assay. For example, reference standards, containing specific concentrations of ATP, might be added to the plate just before the ATP bioluminescence assay is performed.

6. PREPARING PLATES FOR CELL SEEDING

Several steps should be completed for preparation of the plates prior to cell seeding:

1. Each plate should be numbered (both cover and base), and other appropriate information added, so that there is no chance of confusion after the cells are seeded. Many researchers find it convenient to outline the basic plate map on the cover, especially if more than one design is to be seeded at once.
2. Cell density within the well of a 96-well plate can be markedly affected by static electricity, particularly in serum-free medium. The well wall, with a circumference of ~1.9 cm, provides a large surface area, compared to the bottom of the well. Because the cells themselves carry a charge, the static charge on the plate may draw most cells to the outer edge of the well, leaving the center mostly empty. The charge can be developed during the unwrapping of each plate, or during other steps in handling. Winter dryness may exacerbate the problem. The author's laboratory uses an antistatic mat (similar to those used for handling electronic components) and a ^{210}polonium (^{210}Po) ion generator (also used on balances to reduce static electricity) to reduce the static charge, so that cells settle more evenly. The open plates are passed over the ion generator, recovered, and held on the antistatic mat for seeding.

   Laboratory personnel and their clothing are also major sources of static electricity. Working in a well-grounded biological safety cabinet allows the operator to dissipate some of the charge to the cabinet. All this effort may not be necessary in every situation. However, if one is experiencing poor seeding uniformity within the wells, then static electricity control may be necessary to correct the problem.

7. SEEDING THE PLATES

The number of cells to seed in each well depends on the cell type and assay end point. Nonproliferating cells must be seeded so that the desired final density is reached, after accounting for the plating efficiency. Proliferating cells are seeded so that the cells can attach and begin log-phase asynchronous proliferation before treatment. The cell density at the end of the assay, when the end point assays are performed, is also important. Proliferation assays require that the control wells be at subconfluent density, so that these cells are proliferating normally. If the control wells become too dense, their proliferation rate will slow, allowing the treated populations, which experience minimal toxicity, to reach similar cell densities. Thus, the cell-seeding density and density at treatment should allow for constant cell proliferation in the control wells. On the other hand, the end point assay will require a minimum number of cells to provide a strong signal relative to the background noise of the system. If the cells do not proliferate or if they must differentiate before treatment, the cell seeding and treatment densities will be driven by the needs of the end point assay. It is advisable to perform one or more trial seedings at various densities, to determine the optimal cell number.

What volume of medium should be used to seed the cells into each well? The multichannel repeater pipet delivers 1.1–1.2 mL per fill, which is sufficient to seed 11 columns with 100 μL/well. One hundred μL is a good minimum volume, since the pipetor has a 2–4% error. If there is a desire to seed cells in a larger volume (e.g., 200 μL/well), one may also dispense 100 μL/well filling from left to right, then a second 100 μL/well, starting from right to left. The total volume of medium that is needed in each well will also depend on the amount of time the cells are to remain in the plate before the assay is performed. For comparison, a culture flask with 25 cm^2 cell growth area may receive 5 mL medium. This volume will provide 0.2 mL/cm^2 of cells on the growth surface. A 96-well plate containing 100 μL medium in a well will provide 0.31 mL/cm^2. If the medium volume is 200 μL/well, the ratio of medium to growth area is 0.62 mL/cm^2. Compare the cell density in the flask with the density in the well, to determine the medium volume for a comparable number of cells.

Preparation of the single-cell suspension would be performed in accordance with the standard procedure for the cell type being used. Attention should be focused on preparing the cells (rinses, trypsin treatments, inactivation of the trypsin, and so on) in a consistent and reproducible (i.e., documented) manner each time. Of particular importance will be the accuracy of the cell counts performed in order to dilute the suspension to the required final cell density. Ideally, a calibrated particle counter would be used, so that several samples of 500 μL each could be counted to generate the final count. If a hemocytometer is used, both sides should be loaded and counted. In no case should the plates be seeded without a count to adjust the cell density accurately.

To seed the prelabeled plates, the cell suspension is transferred to a sterile solution basin, from which the repeater pipetor is loaded. The cell suspension should be gently mixed between each loading, to maintain a uniform suspension. Because the cells will also settle in the pipetor tips, one should load the plate with dispatch. Some prior practice may be helpful (water and a blank plate). Rack-mounted sterile tips are recommended. Racks may be conveniently preloaded with 6–8 tips across, depending on the plate map being seeded. The pipetor should be maintained in a slightly upright (vertical) position between plates, so that medium does not flow to the nonsterile pipetor barrel at the top of the tip. Blank wells containing cells may be seeded with a single channel pipetor.

If desiccation is a concern, and the outer wells are not seeded with cells, sterile tissue culture-grade water or saline is added to these wells. This technique is often used when several days of postseeding and/or posttreatment incubation are required. For the most uniform cell attachment

and growth, the 96-well plates should be placed into the incubator as a single layer on the shelf, rather than stacking the plates. Some cooling will have taken place during seeding; therefore, each plate should be allowed to warm and equilibrate without stacking.

If the cultures need to be refed or changed to a special medium, to induce cell differentiation before treatment, proceed as indicated below for refeeding before treatment.

8. REFEEDING MULTIPLE-WELL CULTURES

Depending on the length of time the cultures have been incubated before treatment, changing the medium may be important to prepare the cells for expression of the end point(s) under study. If the cells are attached to the substrate, the refeeding can be relatively easy. Using a tray to collect the spent medium, remove the cover and invert the plate with a slight shake, to dislodge the medium. Gently blot the inverted plate on sterile paper towels or other sterile absorbent material, to wick away the medium that will collect on the end of the wells. Refeed immediately with fresh medium, using the multichannel repeater pipet. Refill the outer wells with water or saline, if appropriate. If the cells are weakly attached to the substrate, the pipeting action must be very gentle, to avoid stripping the cells from the bottom of the well. If the cells are being refed just before treatment, use half the final volume of medium, so that the addition of the 2X dosing solution will bring the final volume to the desired level. Assays with very short-term end points (i.e., immediate ion flux studies) may not require refeeding. In that case, the dosing volume should be adjusted to keep the total volume in the desired level and still allow for accurate pipeting. Before refeeding or dosing, representative wells should be examined with an inverted microscope, to assure that the cells are in the desired state and free of contamination. Because repeater pipets are used for refeeding and dosing, even a few contaminated wells will be sufficient to eventually contaminate the whole plate.

9. TREATING CULTURES

Water-soluble chemicals may be diluted directly in culture medium, then serially diluted to prepare the dosing solutions. Poorly water-soluble chemicals will require an intermediate step with an organic solvent. Dilution in the intermediate solvent will produce a true solution, and allow accurate dilutions to be performed. Solvents, such as ethanol, acetone, or dimethyl sulfoxide, are often used for this purpose. Some texts suggest that the final concentration of these solvents should be kept at 1% or less.

Many stromal cells grown in the presence of 10% serum will tolerate 1% solvent, at least for 24 h. However, neuronal cells, especially those grown in the absence of serum, may be adversely effected by considerably less solvent. The goal should be to reduce the solvent concentration as much as possible (consistent with the requirements of the test chemicals), to minimize solvent effects on the cells.

Solvent controls are tested, in parallel with untreated controls, to evaluate the impact of the solvent. One major design flaw seen in many assays is the failure to maintain a uniform intermediate solvent concentration in all treatment groups (including the solvent control). For example, if the final intermediate solvent concentration is to be 0.2%, then the initial dilutions of the test chemical(s) should be made in the solvent at 500× the final concentration desired in the medium. A complete dilution series will be prepared in the solvent first, then an appropriate volume of each dilution transferred to an appropriate volume of medium, to prepare the final dosing solutions.

As discussed below, a half-volume of fresh medium may be added to each well to prevent desiccation. Therefore, a second half-volume of dosing solution will be added. This dosing solution should be 2+ the desired final test chemical concentration. Assume that one wants to assay a poorly water-soluble steroid, for its action over a concentration range of 10^{-6}–10^{-10} *M* (in one-log steps), in

a cell system that would tolerate only 0.2% ethanol (a final dilution of 1:500 in medium). For this example, the final dosing volume in each well would be 100 µL. To achieve this, the medium from the wells is removed, and 50 µL fresh medium is added to the wells before the 50 µL dosing solution is added. A 5×10^{-4} *M* solution of the steroid in ethanol would be prepared (a 500X stock), then serially diluted in ethanol (1:10) (i.e., 100 µL solution added to 900 µL ethanol, and mixed).

Once all the dilutions were made in ethanol, the dilutions of the ethanol stocks into culture medium would be made, to make the 2X dosing solutions. The 2X dosing solutions are made, because the wells already contain 50 µL medium, and, when 50 µL 2X dosing solution is added, the desired final concentration is achieved in 0.2% ethanol solvent. To allow an accurate measurement, one may add 20 µL 500X ethanol stock to 4.98 mL medium (mix well). Be certain to prepare the solvent control (20 µL ethanol into 4.98 mL medium). Then 50 µL of each 2X dosing solution would be added to the appropriate wells of the plate to yield the 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} *M* doses with 0.2% ethanol and the ethanol control.

The effective dose to the cells may depend not only on the concentration of test substance in the medium, but on the volume and composition of the medium. Lipid-soluble chemicals can partition into the cell membrane and be stripped from the medium. Thus, the larger the volume of medium over the cells, the greater the effective dose. Serum proteins (and some substrate proteins) may bind test substances, and thus reduce the effective dose to the cells. Effective concentrations of some chemicals may differ by 10-fold in the presence of 10% serum (Shopsis and Eng, 1985). Therefore, several assay culture systems have been modified for use with low-serum or serum-free medium, at least during the treatment phase of the assay.

Since the stability of test materials in intermediate solvent or culture medium may not be known, the common practice is to prepare the dosing solutions as close to treatment time as is practical.

Test chemicals prepared directly in medium may carry a certain bioburden, which can lead to overt contamination of the cultures before the bioassay is completed. One solution may be to filter-sterilize the highest dose of test chemical, before preparing the remaining dilutions. However, certain test materials may adhere to filter membranes (particularly proteins), and some media will also lose components during filtration. Care should be taken to use low-protein binding filter membranes, such as polyethersulfone or cellulose acetate. Alternatively, a broad-spectrum antibiotic may be employed in the assay medium. The impact of the antibiotic, at the selected concentration, should be assessed in advance.

10. END POINT ASSAYS

A period of time is required for the cell population to express the potential impact of the test chemical that is to be measured in the end point assay. Some changes may be rapid (minutes, i.e., change in ion flux), but others may require several hours or days. Cytotoxicity assays should allow sufficient time for both immediate and delayed cell death to be manifested. Studies of nondividing populations, or those in which apoptotic cell death is expected, may require several days of incubation between initial treatment and the assessment of population viability.

There are too many possible end point assays to discuss in this chapter. However, cytotoxicity assays are one of the more common cell culture bioassays, and they illustrate several features worthy of consideration in other types of assays, as well. With the focus of the multiple well plate bioassay on instrument scoring, the measures of cell/population viability are necessarily indirect. These assays often rely on the incorporation or biotransformation of a vital dye to provide a measure of the number of viable cells in each well. Another approach to measuring cell death has been to measure the disruption of the cytoplasmic membrane as indicated by the release of cytoplasmic enzymes (i.e., lactate dehydrogenase [LDH]) or ^{51}Cr preloaded into the cell. More recently, combi-

nations of DNA-binding dyes have been used to differentiate between cells with and without intact membranes (Alagarsamy et al., 1997). The combination of calcein AM and ethidium homodimer-1 is an example of one such dye pair. Ethidium homodimer-1 is not membrane-permeable, and so it stains only the nuclei of dead cells with disrupted cytoplasmic membranes (red); the calcein AM penetrates the intact cytoplasmic membrane, is cleaved to the fluorescent form, and stains viable cells (green). The plate is read twice (using a fluorometric plate reader), and the ratio of red to green is determined.

In all cytotoxicity assays, the wells should be assessed visually before the end point assay is begun. These visual observations provide a check for contamination or precipitated test material, which may interfere with the dye end point, as well as grossly providing a confirmation for the indirect end point assay results.

Among the most commonly used vital dyes are the tetrazolium derivatives. MTT was one of the first in this series to enjoy wide popularity (Mosmann, 1983). Although initially believed to measure mitochondrial metabolism (succinate dehydrogenase), it is now believed to reflect pyridine nucleotide (reduced nicotinamide adenine dinuclotide [+phosphate][NADH and NADPH]) production (Marshall et al., 1995). Oxidized MTT (yellow) is moderately water-soluble, and can enter the cell, where it serves as an artificial electron acceptor. Cytoplasmic enzymes in several compartments (mitochondrial, microsomal, and cytoplasmic) act to reduce the dye to its insoluble, blue-black state. Live cells will regenerate reduced NADH as part of normal glucose metabolism, so that dye reduction will continue throughout the incubation.

Water-soluble forms of the tetrazolium dyes, such as sodium 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), use an intermediate electron acceptor, phenazine methosulfate, to facilitate dye reduction (Berridge et al., 1996). As a result, the metabolic pathways responsible for dye reduction and overall reduction kinetics are somewhat different. In general, the rate of dye reduction is proportional to the rate of glucose utilization. Thus, glucose utilization rates in the treated and control populations are being compared

The tetrazolium dyes have several advantages for routine cytotoxicity assays. The oxidized dyes (starting material) do not appreciably absorb at the wavelengths of 540–570 nm used to read the reduced MTT absorbance. Therefore, dye binding to extracellular components, such as protein coating, is not a problem. MTT can be used with almost any cell type, with certain caveats (discussed below). The absorbance of the dyes is determined in solution, so that only a single reading is necessary on each well. In contrast, cell-bound markers require several readings in each well to obtain a representative value. The reduced MTT dye is not readily soluble in aqueous medium, and remains precipitated within the cells. For attached cells, this is an advantage, as the medium is poured off and the dye extracted from the cells into solution with isopropanol. In the original publication, Mosmann called for mild acidification of the isopropanol to facilitate extraction, but the acid will decrease the color, and therefore should be avoided. Use a plate shaker to aid in the extraction and mixing of the dye.

Blank wells are important as controls for the absorbance of the solvent and plate. For the MTT dye, the blank wells should contain cells, but not be treated with MTT. Isopropanol is added after the medium is poured from the wells. This controls for the absorbance of the plate, cell residue, and solvent. The soluble dyes, such as XTT, should have blank wells treated with the dye solution, but prepared without cells. In this case, one is controlling for the spontaneous reduction of the dye in solution as well as absorbance by other components in the medium. Dye reduction data are expressed as a percentage of the control dye reduction (percent control viability). The background (blank) value is subtracted from both the treated and control values before performing the calculations.

There are several points that need to be addressed in using the tetrazolium dyes as measures of cell viability. The cells require an adequate supply of glucose (and physiological pH in their surrounding medium) to maintain their metabolic rate. To this end, the wells may be refed with fresh medium containing the dye. This assures that all wells begin with sufficient glucose and a uniform pH, as well as removing test materials that may interfere with the dye. Reducing agents can directly reduce the dye, causing the cells to appear more "alive" than they are. Chemical reduction is of greater concern when the dye is added to the cells in the presence of the test material (such as with cells in suspension).

A cell-free control should be performed, using the highest concentration of the test material, in order to rule out or account for interference. Certain classes of transmitters, receptor agonists, excitotoxins (e.g., kanic acid), and surface-active agents will raise the cells' metabolic rate. The increase may be two- to three-fold over the resting state (McConnel et al., 1992). In cells treated with sublethal concentrations of such materials, it is reasonable to observe dye absorbances well above those of the controls. Such data can be useful in understanding a chemical's mode of action.

Changes in dye reduction generally precede the loss of membrane integrity (frequently measured by LDH release). If one compares the two end points as a function of dose, the LDH curve will be displaced toward the higher doses. Apoptotic cell death will show only limited enzyme release, because the cytoplasmic membrane remains intact until very late in the process. LDH is sensitive to inactivation by some chemicals and by freezing.

Because the LDH samples will also contain the test material, an interference control may be necessary. Medium containing 0.1% Triton X-100 can be used to lyse the cells and release the enzyme, to determine the total LDH available in a control well. This way, LDH values can be expressed as a percentage of the LDH released by the controls and total LDH available in the cells. Medium samples for LDH assays should be refrigerated at 2–6°C, and tested within 24 h of harvest, if possible.

11. POSITIVE CONTROLS

The positive control has a number of functions in the development and routine execution of a bioassay. The positive control is a material selected because it is expected to induce the effect of interest. Initially, it allows the developer to optimize the assay and measure its performance against a known standard. In routine execution, the ability to assess the performance of the assay quantitatively over time is essential. The positive control (and, in certain ways, the negative control) provides a direct measure of the overall assay performance. It should be tested, in parallel, each time the assay is performed. It monitors cell culture quality, treatment, and the end point(s) assays, to provide reassurance that all are performing as expected. This assurance allows data from the unknown test materials to be interpreted within the historical body of data from the bioassay.

Limits are placed on the acceptable values for the positive control responses. These limits (acceptance criteria) may be established as a function of the mean and standard deviation of the positive control responses over time. In our own laboratory, the limits are set as the 95% confidence limits around the mean (mean ± 2 standard deviations). The positive control results are evaluated within the context of these limits, to assure data integrity and to identify sources of variation to improve assay quality.

12. APPENDIX

In addition to the standard equipment found in a cell culture laboratory, the following equipment will be useful in performing multiple well plate assays:

Antistatic mat with ground wire (electronics/computer supply store).

Polonium ion generator, unit number 2U500 (NRD, Grand Island, NY).

Note: Polonium ion generators can be purchased from scientific supply houses or directly from the manufacturer. Because ^{210}Po has a half-life of 138 d, the units should be replaced every 12–18 mo.

96-well plate reader (spectrophotometric and fluorometric).
Orbital plate shaker.
Pipetors:
Eppendorf Repeater Plus/8 Pipettor with eight-channel head (or similar instrument).
Eight-channel, variable-volume pipetor.
Single-channel, variable-volume pipetor (10–50, 20–200, 100–1000 μL).
Pipet tips:
Sterile, rack-mounted, disposable tips (1.25-mL).
Sterile, rack-mounted disposable tips (0.2-mL).
Reagent reservoirs or solution basins, sterile, polystyrene.
Sterile paper towels.

FURTHER READING

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Chapter Twenty-One

Neural Cell Counting

Cell Number Determination in Microplate Cultures

Marston Manthorpe

1. INTRODUCTION

The purpose of this chapter is to introduce the reader to standard procedures for quantifying the number of neural cells in microcultures. The chick embryo was chosen as the source of neural cells, because embryos can be conveniently grown in an inexpensive laboratory incubator, in large, replicate numbers with defined developmental stages. Also, chicken embryos are used by many investigators as a routine source of peripheral and central nervous system tissue. Nevertheless, the procedure outlined can be adapted to neural cells from any source.

At one time, quantitative assays of viable neural cell numbers involved microscopic counts of surviving cells. These assays required the determination of the number of individual surviving cells within a defined fractional area of each culture, and calculation of total cell number per culture. The disadvantages of such assays are that they are time-consuming (requiring about 1 h to count the viable cells in 96 wells of a microtiter plate), somewhat subjective (in scoring certain cells as "surviving" vs "dead"), and tedious.

Mosmann (1983) reported a rapid colorimetric assay for determining lymphocyte cell number in 96-well microplate cultures. This assay involves supplying the cultures with a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which is reduced to an insoluble blue formazan product by living cells, but not by dying cells or their lytic debris. The blue formazan product accumulates in the cells, eventually killing them. The blue product is solubilized by the addition of an alcoholic solution, and color intensity is measured using a multiwell scanning spectrophotometer (enzyme-linked immunosorbant assay plate reader). The optical density (OD) of the solution in the microwell is directly proportional to the number of viable lymphocytes present.

Mosmann used the assay for the determination of cell numbers in the range of 100–100,000 cells/culture. Here, the author has modified this technique for use in determining chick forebrain cell numbers per culture well. The modifications involve using 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT). The XTT reagent is a derivative of MTT, but the living cells convert XTT into a soluble, nontoxic red product secreted into the culture medium. Thus, one can sample the culture OD periodically, then remove the XTT and red product, and still use the same cultures for additional experiments.

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2. PREPARATION OF CHICK EMBRYOS FOR CULTURING

2.1. Materials

Fertile eggs (approx 8 d incubation), Hamburger and Hamilton (1951) stage 34.
Forceps (2), utility, 15-cm.
Forceps (1), curved, 10-cm.
Hanks' balanced salt solution (HBSS), 75 mL.
Petri dishes (2), sterile, 100-mm plastic.
Petri dish (1), sterile, 100-mm glass.
Beaker, 600-mL with double-lined, plastic, disposable bag.
Ethanol, 70%, in squeeze bottle.
Ethanol, 70%, in beaker.

2.2. Procedure

1. Candle the eggs to select viable embryos. If there is no spontaneous movement of the embryo or no clearly discernable blood vessels, discard the egg.
2. Clean the egg shell surface as follows:
   a. Stand eggs in the test tube holder with the air sac up (blunt end up).
   b. Using the 70% ethanol in a squeeze bottle, wash the surface of the egg.

   Note: The shell is considered cleaned, but not sterile. Therefore, avoid contact between shell and embryo.
3. Sterilize instruments: Sterilize two pairs of utility forceps and one pair of curved forceps, by either autoclaving them or immersing them in 70% alcohol, and drying them in air inside the laminar flow hood. When dry, put the instruments into a sterile glass Petri dish, and keep the working parts of the forceps covered with the lid.
4. Add approx 15–20 mL HBSS to two plastic 100-mm Petri dishes.
5. Remove embryos from the eggs:
   a. With the sterile utility forceps, crack the shell at the top of the egg. Hold the egg over the 600-mL beaker, and peel off the broken shell to expose the air sac and its underlying chorioallantoic membrane. Take care not to rupture the membrane. Carefully remove any pieces of shell that fall on the membrane.
   b. With the second pair of sterile utility forceps, remove the chorioallantoic membrane. Start at the edge and move around the circumference, trying to avoid contact between any small pieces of shell and the adjacent vascular membrane. With sterile curved forceps, break the vascular connections between the embryo and the membrane. Using one tine of the forceps, hook the embryo under the neck and lift it gently into a sterile plastic Petri dish containing HBSS.

   Note: Stage the embryos according to Hamburger and Hamilton (1951). Use only stage 34 embryos.
   c. Decapitate the embryo by using the curved forceps, and transfer the head to the second plastic Petri dish containing HBSS. Repeat for each embryo and combine up to 10 heads in one dish.
6. Clean work area with 70% alcohol before proceeding further.

3. REMOVAL OF FOREBRAIN

3.1. Materials

Forceps (1), small eye.
Forceps (2), fine watchmaker's, angled.
Scalpel handle (1) and blade (1).
HBSS.
Sterile capped plastic test tube containing 5–8 mL Puck's balanced salt solution (Puck's BSS).
Petri dish (1), sterile, 100-mm, plastic.
Petri dish (1), sterile, 100-mm, glass.
Pasteur pipet (1), cotton-plugged, siliconized, sterile, with fire-polished tip.
Ethanol, 95%.
Binocular dissecting microscope.

3.2. Procedure

1. Preparation:
   a. Add approx 15–20 mL HBSS to the 100-mm plastic Petri dish.
   b. Sterilize instruments by either autoclaving them or immersing them in 70% alcohol (*see* Section 2.2., step 3.). When sterile, put the instruments into a sterile glass Petri dish, and keep the working ends covered with the lid.
2. Place the 100-mm plastic Petri dish, containing the chick embryo heads, on the stage of the dissecting microscope. Identify the structures. The forebrain (telencephalon) is the clearly visible, slightly flattened bilobed structure anterior to the larger, more easily visible bilobed optic lobes (which are immediately behind the eyes).
3. Using angled watchmaker's forceps, with the curved side down, insert the forceps under the eye, close the forceps, and remove the eye.
4. Carefully cut between the forebrain and optic lobes with the tips of the fine forceps. Cut in front of the forebrain, through the fine ridge of cartilage in the forehead area. Cut under the lobes to free the forebrain from the orbits.
5. Remove the skull from the forebrain.
6. Carefully peel away and discard the meninges (containing many branching blood vessels) and other contaminating tissues that connect the two forebrain lobes together.
7. Using a scalpel, cut the brain into about eight pieces per lobe.
8. Carefully transfer the tissue pieces from only one forebrain (the one that one believes was dissected most properly), with the fine forceps or Pasteur pipet (without crushing the tissue), into the capped plastic test tube containing 5–8 mL Puck's BSS.

4. DISAGGREGATION OF FOREBRAIN

4.1. Materials

Culture medium (Eagle's basal medium with supplements [HEBM]-penicillin/streptomycin [P/G]-N1–1.0% fetal bovine serum [FBS]; *see* Section 8.2.) (25 mL).
0.08% Trypsin in Puck's BSS (6 mL).
HBSS.
Pasteur pipets (3), cotton-plugged, siliconized, sterile, tips fire-polished.
Pasteur pipet (1), cotton-plugged, sterile, siliconized, tips flame-constricted to 1.0-mm inner diameter.

Test tube (1), 15-mL, plastic, capped, sterile.
0.4% Trypan blue.
Hemocytometer and coverslip.
Hand-held digital counter.
Water bath, 37°C.
Phase-contrast microscope.

4.2. Procedure

1. Incubate fragments in 5–8 mL Puck's BSS for 10 min in a 37°C water bath.
2. Carefully aspirate off the Puck's BSS, and replace it with 5 mL 0.08% trypsin solution. Incubate the minced tissue in trypsin solution for 20 min in a 37°C water bath.
3. Remove trypsin by careful aspiration with a Pasteur pipet, and wash tissue 3× with 1.5 mL culture medium, by allowing the tissue to settle and carefully aspirating off the medium. Leave the tissue in the last wash of 1.5 mL culture medium.
4. Using the siliconized constricted Pasteur pipet, pass the tissue and the near-entire 1.5 mL culture medium through the pipet repeatedly, until there are no intact tissue pieces visible (about 10–20×).

Note: Do not create air bubbles during this process. Air bubbles can be avoided by not expelling the last microliters from the tip of the Pasteur pipet, and by not drawing air bubbles into the tip during aspirations.

5. Determination of cell number:
   a. Prepare a sample from the cell suspension for counting with a hemocytometer. Dilute a 0.1-mL aliquot of the cell suspension with 1.7 mL HBSS. Add 0.2 mL trypan blue solution. This makes a cell dilution of 1:20.
   b. Fill the hemocytometer with the diluted cell suspension, using a Pasteur pipet.
   c. Count the number of viable cells in at least one hemocytometer chamber (four squares). The use of trypan blue as a viability indicator is based on the ability of live, intact cells, or viable cells, to exclude the dye. Therefore, viable cells will appear unstained.
   d. Record the cell counts, and calculate the number of cells/mL in the remaining 1.4 mL cell suspension.
6. Dilute the cell suspension to 2.4×10^5 cells/mL (6×10^3 cells/25 µL) in culture medium in a volume of 10 mL.

5. MICROCULTURES

5.1. Materials

Culture medium (HEBM-P/G-N1–1.0% FBS).
Test agent.
A/2 96-well microplates (2) (Costar, cat. no. 3696) coated with poly-L-ornithine and laminin.
Multichannel microliter pipet.
Reagent reservoir, sterile.
Plastic tips, sterile.
Microplate template record sheets (2).

5.2. Procedure

1. Add 25 µL culture medium to all wells in both plates. The wells in column 1 then receive 25 µL test agent in culture medium, to make a total of 50 µL. Then 25 µL is transferred from

each well of column 1 to the corresponding wells of column 2, and the contents are mixed. This serial twofold titration is repeated for each set of wells, up to column 11, and the last 25 µL is discarded. Thus, the wells in the last column (12) are controls without added test substance.

2. Mix the diluted cell suspension (2.4×10^5 cells/mL), and pipet 25 µL into each well of each microplate using a multichannel pipetor.
3. Place the two plates in a humidified 5% CO_2/95% air incubator at 37°C for 4 d.

6. QUANTIFICATION OF VIABLE CELL NUMBERS

6.1. Materials

XTT dye (Sigma, cat. no. X 4251) made to 1 mg/mL in HEBM, or already solubilized in kit form (Boehringer Mannheim, cat. no. 1-465-015) at 1 mg/mL in RPMI medium).
Logarithmic graph paper, four cycles.
Microplate spectrophotometer.
Standard inverted light microscope.

6.2. Selective Staining of Viable Cells with Vital Dye

1. Four h before the end of the selected culture period, add 5 µL of the vital dye, XTT, to each well of one 96-well microplate.
2. Incubate the culture for an additional 4 h.
3. Determine the OD of each well by using the microplate spectrophotometer, set at a test wavelength of 492 nm, reference wavelength at 690 nm.

6.3. Evaluation of Cell Number by Multiwell Scanning Spectrophotometer

Instructions for operating the computerized microplate reader are given in the manuals accompanying the reader. Consult the manual for specific procedures.

6.4. Data Analysis

Plot the sample dilution on the logarithmic abscissa (*x* axis) and the average ODs of the eight corresponding wells on the linear ordinate (*y* axis).

7. SELECTIVE IMMUNOSTAINING OF NEURONS IN MICROCULTURES

7.1. Materials

Primary antibody (Ab) to neurofilament protein in blocking buffer.
Peroxidase-conjugated secondary Ab in blocking buffer.
Fixing solution.
Blocking buffer.
Wash solution.
Saline.
Water.
Chloronaphthol solution covered, and stored in the dark.
Peroxide solution.
Plastic gloves.
Multichannel pipetor.
Reagent troughs for pipets.

7.2. Fixation and Staining of Microculture

1. Remove the XTT solution from each well, being careful not to remove attached cells.
2. Add 100 µL fixation solution to each well of the replicate plate.

Note: During all subsequent steps, never squirt solutions directly onto the well bottom because this tends to detach the cells. Rather, let the fluids run slowly down the well edges.

Let the plate fix for 20 min at room temperature, remove the fixative by matting (flip the plate upside down into the waste bucket or sink, and shake the solution out, then tamp the plate upside down onto a thick bed of clean paper towels), and replace with another 100 µL fixation solution. Let fix another 20 min.

3. Remove the fixative by matting, and add 100 µL blocking buffer. Remove by matting, and wash with 100 µL blocking buffer twice more. Allow the culture to incubate in the blocking buffer for 30 min, to permeabilize the cells.
4. Remove the blocking buffer, and add 50 µL primary antibody to neurofilament. Incubate the plate at 37°C for 1 h.
5. Remove the primary antibody and wash the wells 3× with 100 µL washing solution.
6. Add 50 µL of secondary antibody and incubate at 37°C for 1 h.
7. Remove the secondary antibody and wash the wells as before, 3× with 100 µL washing solution. Wash a fourth time with saline.
8. Add peroxide to the stock chloronaphthol solution (*see* Section 8.5.), then add 100 µL chloronaphthol peroxide solution to each well. Store the plate in the dark at room temperature for 30 min. Replace the chloronaphthol with fresh chloronaphthol,and observe under the microscope. Keep the plate in the dark, taking it out only occasionally to observe it under the microscope. When the neurons are at the desired staining intensity, replace the chloronaphthol with water, wash the culture with water a few times, and finally discard the water and allow the wells to dry. The neurons, their axons and growth cones, but not any contaminating glial cells and fibroblasts, should be stained dark brown.

7.3. Results

These stained plates can be used for counting the number of individual surviving neurons within a defined area, and the results used for calculation of the total neuronal number per culture. The total neuronal number of both the control cultures and those treated with test substances should be determined.

8. APPENDIX

8.1. Solutions

1. HBSS and Puck's BSS. 10X stock solution:

Component	HBSS (g/L)	Puck's BSS (g/L)
$CaCl_2$	1.4	0
KCl	4.0	4.0
KH_2PO_4	0.6	0.6
$MgCl_2 \cdot 6H_2O$	1.0	0
$MgSO_4 \cdot 7H_2O$	1.0	0
NaCl	80.0	80.0
$NaHCO_3$ *(See note)*		
$Na_2HPO_4 \cdot 7H_2O$	0.9	0.9

Glucose	10.0	10.0
Phenol red	0.1	0.1 (10 mL 1% solution)

Note: Sodium bicarbonate ($NaHCO_3$) is prepared as a solution containing 1.4 g $NaHCO_3$ in 100 mL triple-distilled water (dH_2O). It is sterilized by filtration through a 0.2-μm filter. Approximately 2.5 mL (0.35 g/L) of this sterile solution is added after dilution, and before use to Puck's BSS or HBSS.

a. Weigh out each chemical (except phenol red and $NaHCO_3$). Dissolve in 990 mL triple-dH_2O.
b. Add 10 mL 1% phenol red (1 g/100 mL triple-dH_2O), and mix.
c. Sterilize by filtration through a 0.2-μm filter.
d. For use, dilute 1:10 with sterile water, or dilute and sterilize by filtration through 0.2-μm filter.

2. Phosphate-buffered saline (PBS): Make up a solution of 154 m*M* sodium chloride in 10 m*M* Na phosphate buffer.

8.2. Eagle's Basal Medium (EBM) with Supplements (Eagle, 1955)

1. HEBM:
   a. Formulation:

Components	mg/L
Inorganic salts:	
$CaCl_2$ (anhydrous)	200.00
KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6800.00
$NaH_2PO_4 \cdot H_2O$	140.00
Other components:	
D-Glucose	1000.00
Phenol red	10.00
Amino acids:	
L-Arginine · HCl	21.00
L-Cystine · 2HCl	15.65
L-Glutamine	292.00
L-Histidine	8.00
L-Isoleucine	26.00
L-Leucine	26.00
L-Lysine · HCl	36.47
L-Methionine	7.50
L-Phenylalanine	16.50
L-Threonine	24.00
L-Tryptophan	4.00
L-Tyrosine (disodium salt)	26.00
L-Valine	23.50
Vitamins:	
Biotin	1.00
D-Calcium pantothenate	1.00
Choline chloride	1.00

Folic acid	1.00
i-Inositol	2.00
Nicotinamide	1.00
Pyridoxal · HCl	1.00
Riboflavin	0.10
Thiamine · HCl	1.00

b. To the above formulation of EBM, add 5.0 g/L D-glucose and 2.2 g/L $NaHCO_3$. In addition, it is supplemented with 1.0% FBS, and, as indicated below, with penicillin, glutamine, N1 mixture, and insulin (HEBM-P/G-N1–1% FBS).

2. Insulin:
   a. Weigh out 25 mg insulin.
   b. Add insulin to 10 mL HEBM containing P/G.
   c. Acidify the insulin solution with drops of 1 *N* HCl, until the suspension is dissolved. Solution will be clear yellow.
   d. Sterilize by filtration through a 0.2-μm filter.
   e. Aliquot 0.5 mL/12 × 75-mm plastic tube.
   f. Store in the refrigerator at 4°C for up to 4 wk. **Do not freeze**.
3. Penicillin/Glutamine:
   The solutions of penicillin and glutamine are made up so that each are 100× the final concentration. They are then mixed together equally, so that they are in solution at 50× the final concentration.
   a. Penicillin "G" potassium or penicillin "G" Na:

Final concentration:	100 U/mL
Stock solution:	10,000 U/mL
Stock solution:	1,000,000 U/mL
Note: 1585 U/mg = 1,000,000 U/*x*;	***x* = 630.9 mg**

   b. L-Glutamine (mol wt 146.15):

Final concentration:	2 m*M*
Stock solution:	200 m*M*
Stock solution:	2.92 g/100 mL

   c. Procedure:
      i. The above solutions are diluted with isotonic saline (0.85%), which is made with ddH_2O.
      ii. Mix the two solutions together thoroughly, for a total volume of 200 mL.
      iii. Sterilize by filtration through a 0.2-μm filter.
      iv. Aliquot 5.2 mL P/G per 17 × 100-mm sterile opaque tube.
      v. Store by freezing at –20°C.
      Note: The volume of 5.2 mL P/G is sufficient for a 250-mL bottle of medium.
4. N1 Components (serum substitute):
   a. Preparation of N1 components: This volume is sufficient for 18–20 tubes of N1 mixture as prepared below. A larger quantity may be made by increasing each component proportionally.

Chemical	Wt (mg)	Vol (mL)	Solvent
Transferrin (Sigma, cat. no. T 4515)	25.00	5	HEBM-P/G
Putrescine (Sigma, cat. no. P 7505)	80.55	5	HEBM-P/G
Progesterone (Sigma, cat. no. P 0130)	3.145	5	95% E/TOH
Note: Dilute progesterone further: 50 μL/10 mL HEBM-PG.			
Na selenite (ICN, cat. no. 201657)	5.19	100	HEBM-P/G

Note: Dilute Na selenite further: 0.5 mL/5 mL HEBM-PG.

b. Final N1 mixture (minus insulin): 25 mL:

Transferrin	5 mL
Putrescine	5 mL
Progesterone	10 mL
Selenium	5 mL

c. Procedure:
   i. Prepare chemical solutions as indicated in step a.
   ii. Combine chemical solutions as indicated in step b.
   iii. Sterilize by filtration through a 0.2-µm filter.
   iv. Aliquot 1.25 mL N1 mixture/12 × 75-mm tube.
   v. Store by freezing at –20°C for up to 2–3 wk.

d. Preparation of HEBM-P/G-N1:
   i. Thaw one aliquot of N1, and add the 1.25 mL N1 to 250 mL medium containing PG.
   ii. Add 0.5 mL insulin solution to the N1, medium, P/G solution.
   iii. Store at 4°C for up to 3–4 d, but no longer than 5 d.

f. Final concentrations of each component in medium:

Transferrin	6.25×10^{-8} *M*
Putrescine	1.00×10^{-4} *M*
Progesterone	2.00×10^{-8} *M*
Selenium	3.00×10^{-8} *M*
Insulin	8.30×10^{-7} *M*

5. FBS (1.0%): Add 0.1 mL to each 9 mL basal medium containing penicillin, glutamine, insulin, and N1.

8.3. Poly-L-Ornithine and Laminin-Coated Plates

1. Preparation of solutions:
   a. Poly-L-ornithine hydrobromide, mol wt 30–70 kDa (Sigma, catalog no. P3655), should be stored desiccated at –20°C.
      i. 0.1 g poly-L-ornithine is added to 100 mL, pH 8.4, boric acid buffer, to form a stock solution of 1.0 mg/mL.
      ii. Sterilize by filtration through a 0.2-µm filter.
      iii. Store at 4°C for up to 2 mo.
      iv. For use, dilute 10 mL poly-L-ornithine stock solution to a final volume of 100 mL with sterile dH_2O, to obtain a working solution of 0.1 mg/mL.
   b. Preparation of boric acid buffer:
      i. Make up an excess amount of 0.15 *M* boric acid buffer: 1.159 g boric acid plus 0.75 g NaOH/125 mL dH_2O.
      ii. Adjust pH to 8.4.

   Note: Test aliquots of the buffer for pH. Do not put the pH electrode into the entire buffer.

   c. Mouse laminin (EY Laboratories, San Mateo, CA, cat. no. 2404).
      i. Stock solutions should be reconstituted to approx 1 mg/mL in sterile PBS, pH 8.0. Store in 50-µL aliquots at 4 or -20°C, depending on manufacturer's instructions.
      ii. Dilute laminin immediately before use, to a concentration of 10 µg/mL, by the addition of sterile PBS.
2. Coating of 96-well A/2 microplates with poly-L-ornithine and mouse laminin.

a. Add 25 µL working solution of poly-L-ornithine/microwell.
b. Leave on for approx 30 min at room temperature.
c. Remove poly-L-ornithine, and add 25 µL sterile dH_2O water/well.
d. Remove all the water with a suction pipet.
e. Add 25 µL laminin–PBS solution/well.
f. Incubate overnight at 37°C.
g. Remove from incubator, and freeze with laminin–PBS solution.
h. Store in Zip-Lock™ bags at –20°C.

3. Preparation of coated plates for culturing:
   a. Remove plate(s) from freezer, and thaw in 37°C incubator.
   b. Remove laminin–PBS solution, and wash wells one time with media (optional).

Note: Do not allow the laminin-coated surface to dry out.

8.4. Staining and Fixation for Quantification

1. XTT, Sigma, cat. no. X4251 or Boehringer Mannheim XTT kit comes premade for use (cat. no. 1-465-015):
   a. Working concentration is 1 mg/mL in culture medium (HEBM-P/G-N1–1.0% FBS). To make 50 2-mL aliquots at 1 mg/mL:
      i. Weigh out 100 mg XTT.

      Note: Weigh out quickly, but carefully. This chemical is light-sensitive.

      ii. Add XTT to 100 mL medium prewarmed to 37°C, and stir until the XTT is completely dissolved (10–15 min). Keep covered with aluminum foil to protect from light, while the solution is being stirred.
      iii. Sterilize by filtration through a 0.2-µm filter, in a hood with the lights out.
      iv. Aliquot 2 mL/12 × 75-mm polypropylene tube. Wrap tubes in foil or keep in light-tight box.
      v. Freeze at –20°C.
   b. Thaw by placing tube in a 37°C water bath.

8.5. Selective Immunostaining of Neurons in Microcultures

1. Fixing solution: Add 4 g paraformaldehyde to 100 mL PBS, and add three drops 5 *N* NaOH. Heat with stirring to dissolve. Cool to room temperature. Adjust the pH to 7.0–7.4 with 5 *N* NaOH. Store at room temperature no longer than 2 d.
2. Blocking buffer. Add 1 g Tween–20 and 1 g bovine serum albumin (Sigma, Fraction V) to 1 L PBS, and mix to dissolve. Store at 4 or –20°C. Use at room temperature.
3. Wash solution: Dissolve 1 g Tween–20 and 9 g NaCl in 1 L water. Use at room temperature.
4. Saline: Dissolve 9 g NaCl in 1 L water. Store, and use at RT.
5. Chloronaphthol solution: Add 50 mg 4-chloro-L-naphthol (Sigma, cat. no. C 8890) to 0.5 mL methanol, to dissolve. Add the methanol solution to a stirring solution of 100 mL saline (0.9 g NaCl/100 mL water). Cover, and stir in the dark 1 h. Filter the saturated solution through a standard 0.2- or 0.4-µm filter. Keep in the dark at room temperature until used. Immediately before use, add 20 µL H_2O_2 (30%; Fisher, cat. no. 325). Make sure that the peroxide is relatively fresh, because this reagent will deteriorate on prolonged storage.
6. Antibodies:
   a. Mouse ascites fluid, containing monoclonal antibody RT97 to neurofilament protein, is diluted 1:100 in blocking buffer.

b. Peroxidase-conjugated goat antimouse IgG-antibody (Cappel, Durham, NC, cat. no. 3211-0231) diluted 1:200 in blocking buffer.

Note: The optimal concentration for use of each antibody should be determined by using a range of dilutions.

8.6. Method for Siliconizing Pasteur Pipets

1. Use a tall beaker to accommodate 9-in. Pasteur pipets (e.g., a pipet soaker).
2. Place pipet tips up in a pipet basket with holes in the bottom.
3. Dilute Prosil–28™ (American Scientific Products) 1:100 with water.
4. Submerge the pipets in the solution, making sure the pipets are fully coated. Flush the basket up and down to eliminate bubbles in the tips.
5. Remove the basket to a siphon-type pipet washer, and flush with deionized water several times, making sure the tips are not blocked by bubbles.
6. Place the pipet tips up in foil-lined baskets, and dry thoroughly in a drying oven.
7. Plug pipets with cotton balls (no. 1 size is available from dental supply houses), and autoclave in glass or paper holders for later use in the disaggregation of tissues.

8.7. Comments

1. Never let tissues dry out during dissection. Always keep them immersed in a BSS.
2. Minimize mechanical trauma:
   a. Teasing tissues apart damages cells.
   b. Cutting tissues with very sharp instruments tends to break only cells contacting the instruments.
   c. For cutting, a tungsten needle in one hand and an iridectomy knife in the other make a good combination, provided they are sharp, and that they are used for cutting and not for teasing.
   d. A Pasteur pipet "charged" with dissecting medium is less traumatic than forceps for transferring tissues from one dish to another. Pipets break easily, and care must be taken to:
      i. Charge the pipet with 1 mL or less of dissecting medium.
      ii. Introduce the tip into the solution containing the tissue.
      iii. Expel a small amount of buffer.
      iv. Aspirate the tissue into the pipet tip, and release the bulb completely, before taking the tip out of the solution.

   Note: This procedure prevents bubble formation. If you are transferring tissue to a solid substrate (agar, plasma clot, and so on) you can control the position of the tissue within the pipet (close to the tip), thus reducing the amount of fluid that you add to the substrate.
3. If one has to dissect more than one animal and pool the tissues, there is no way to have all the tissues treated in exactly the same way: Either some will wait longer before being taken out of the animal, or they will spend more time in BSS after being taken out of the animal. The procedure must be standardized. In routine work, one must determine exactly how long the dissection will take, then depend on this same time for the experiments. Successive experiments must always use tissues and cells prepared as identically as possible.
4. Sometimes, a concave surface is more practical than a flat one, especially if small organs, such as ganglia, must be collected. A depression slide or watch glass, contained within a Petri dish, can be used.

Table 1 . Differences in Size of Available Vessels for Monolayer Cultures

Type of vessel	Diameter (mm)	Total surface area (mm^2)	Relative area (mm^2)	Seeding/well at 100 cells/mm^2
Roller bottle (250-mL)	N/A	85,000	6071	8,500,000
Roller bottle (144-mL)	N/A	49,000	3500	4,900,000
Tissue culture (TC) flask (1000-mL)	N/A	15,500	1071	1,500,000
TC flask (250-mL)	N/A	7500	536	750,000
TC dish (100-mL)	84	5541	396	554,000
TC flask (30-mm)	N/A	2500	179	250,000
TC dish (60-mm)	52	2124	152	212,400
TC dish (35-mm)	32	804	57.4	80,400
TC plates (24-well)	16	201	14.4	20,100
Glass rings (10-mm)	10	78	5.6	7800
TC microtiter plates (96-wells)	6	28	2.0	2800
A/2 plates (96-well)	4.5	14	1.0	1400

5. Mistakes made during dissection frequently explain erratic experimental results:
   a. Before beginning an experiment, spend some time precisely defining the landmarks to be used during dissection. For instance, if one plans to use segments of spinal cord, remember that the different levels have a different structure and contain different cell types: Be sure those levels can be recognized. External landmarks, such as somites, vertebrae, ribs, and so forth, can be of help. If ganglia are collected, be sure to cut the nerve roots at the same level each time.
   b. The tissue that one plans to use will frequently be contaminated with unwanted tissues. Be sure to remove these contaminants. Sometimes, further dissection will suffice (such as removing meninges from the spinal cord), but sometimes a brief enzyme treatment will be necessary.
   c. Blood left in tissues may alter the meaning of the cell counts, and hemoglobin can be toxic to some cultured cells. Warm dissecting medium can help remove blood from the dissected tissue.
   d. Discard any piece of tissue the identity of which is not absolutely certain. Remember this especially when under the pressure of such circumstances as not having as many embryos as one would wish. A smaller, yet meaningful, experiment is better than a larger, useless one.

8.8. Tissue Culture Vessels and Cell Seeding Levels

Table 1 illustrates the differences in size of available vessels for monolayer cultures. Also shown are the cell plating requirements needed to obtain a modest cell density of 100 cells/mm^2. Although, theoretically, one should be able to generate equivalent cultures in different vessels plated with the same cell density (e.g., 100 cells/mm^2), other properties of the culture, such as medium volume, depth, and movements, as well as differences in the culture plastics, may tend to make the cultures unequivalent.

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Chapter Twenty-Two

Assay for Neuronal Cell Migration

Toby N. Behar

1. INTRODUCTION

Chemoattractant-induced neuronal migration can be directly and quantitatively evaluated in vitro, using a microchemotaxis assay. This assay has been used to quantitate the chemotropic responses of several migratory cell types, including neutrophils (Harvath et al., 1980; Geiser et al., 1993), monocytes (Reinisch et al., 1993), melanoma cells (Stracke et al., 1989), and smooth muscle cells (Higashiyama et al., 1993). In general, relatively low concentrations of attractants (n*M*–f*M*) typically stimulate migratory responses in the greatest number of cells (Stracke et al., 1989; Yao et al., 1990; Grant et al., 1992; Hendey et al., 1992; Rot et al., 1992; Shure et al., 1992; Geiser et al., 1993; Higashiyama et al., 1993; Reinisch et al., 1993). In this assay, cells are acutely dissociated from the developing nervous system, and resuspended in buffer. The suspension of dissociated cells is placed in the upper half of a microchemotaxis chamber, soluble chemoattractants are placed in the bottom half of the chamber, and the cells are separated from the chemoattractants by a membrane containing 8-μm pores. Cells induced to migrate squeeze through the pores, then adhere to the underside of the membrane, which is precoated with substrates to promote cell adhesion.

Following incubation in the presence of attractants, cells that migrated through the pores and adhered to the underside of the membrane are then fixed, stained, and counted (Fig. 1). At the end of the migration assay, immunocytochemistry, using antibodies directed against neuronal markers, can be used to identify the migrated cells as neurons. One advantage of the in vitro chemotaxis assay is that the type of migratory response can be readily characterized. By observing the migration of cells in the presence or absence of a concentration gradient of attractant, chemotaxis (directed migration along a concentration gradient of attractant) can be distinguished from chemokinesis (increased rate of random cell movement) (Fig. 2).

The microchemotaxis assay also provides a system to assess the types of receptors involved in the migratory response and the intracellular signaling mechanisms associated with neuronal motility. The use of receptor agonists and antagonists can provide insight into the nature of the receptors that mediate an attractant's motility signals, pharmacological studies, using modulators of intracellular signaling mechanisms, can help to elucidate the signal transduction pathways that are involved in mediating motility responses to attractants. Thus, the in vitro assay on dissociated nerve cells is a useful method for screening and characterizing chemotropic responses of neuronal populations to exogenously applied presumptive attractants.

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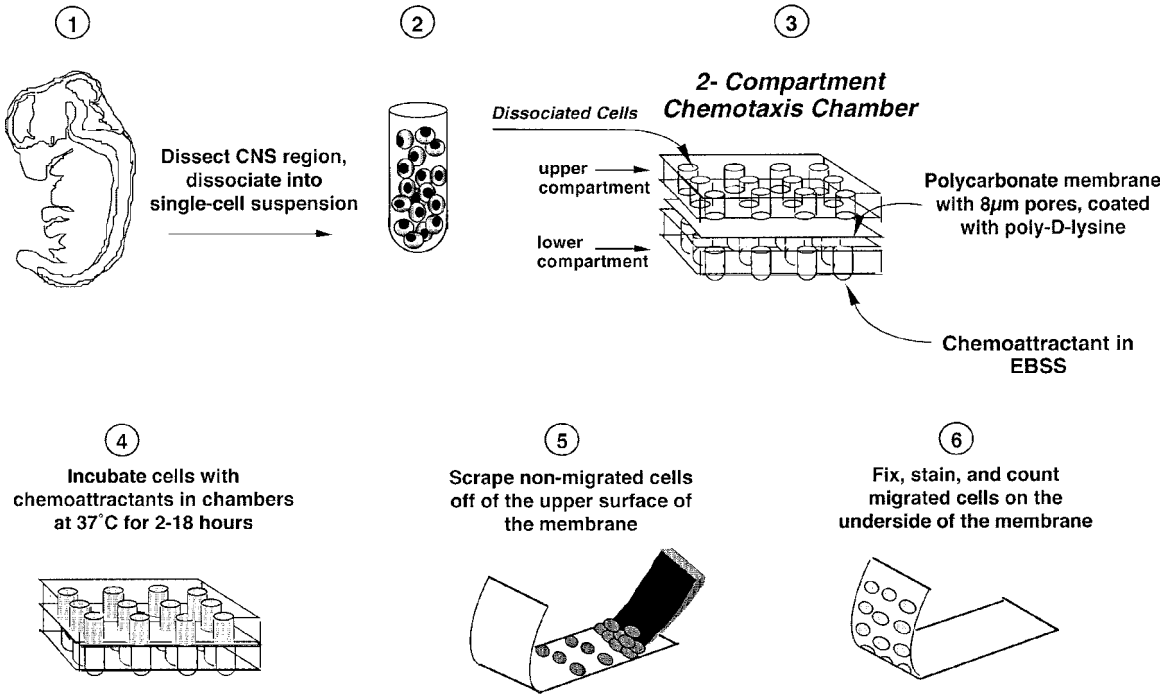

Fig. 1. Schematic drawing depicting the in vitro chemotaxis assay for embryonic neuronal migration. **(1)** CNS tissue is dissected from embryos. **(2)** Tissue is dissociated into a single-cell suspension. **(3)** Dissociated cells are placed in the upper compartment of the chemotaxis chamber. Attractants are placed in the lower compartment of the chamber. Cells are separated from the attractants by a membrane containing 8-μm pores. Prior to assembly, the membrane is precoated with poly-D-lysine, to promote cell adhesion. **(4)** Cells are incubated in the presence of attractants at 37°C. **(5)** At the end of the assay, the chamber is disassembled, and the cells on top of the membrane, which failed to migrate, are scraped off. **(6)** The cells that migrated through the pores of the filter, toward the attractants, are fixed, stained, and counted.

2. REMOVAL OF UTERUS

2.1. Materials

Pregnant rat (E13–E20 embryos/fetuses).
Scissors (1), large.
Scissors (1), medium.
Forceps (1), curved.
Leibovitz's-15 (L-15) medium (Gibco-BRL Life Technologies, cat. no. 11415-064) (15 mL).
Gentamycin (GENT), 50 μg/mL (BioWhittaker, Walkersville, MD, cat. no. 17-518z) (0.015 mL).
Petri dish (1), 150-mm, tissue culture grade.
Sterile gauze pad.
70% Ethanol in beaker.
Absorbent, plastic-backed paper.
Micropipetor.
Micropipetor tips.

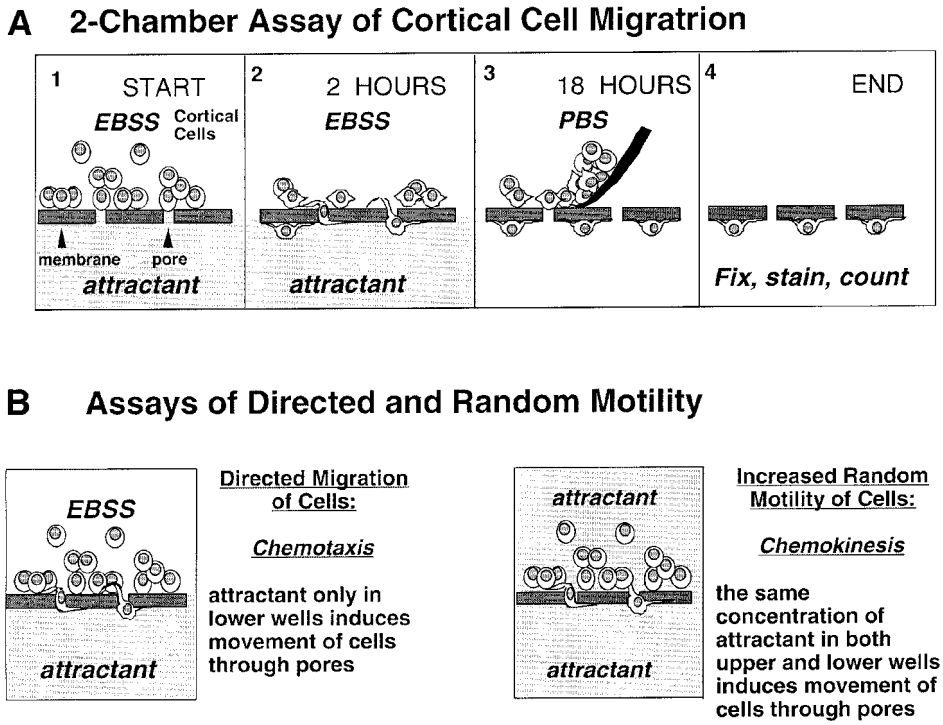

Fig. 2. Schematic drawings depict the movement of cells inside the chambers. **(A1)** Initially, cells settle onto the membrane surface. In the process, some cells settle over pores. **(A2)** Cells induced to migrate squeeze through the pores and adhere to the underside of the membrane as they come through. **(A3)** At the end of the assay, the cells on top of the membrane, which failed to migrate, are scraped off. **(A4)** The cells that migrated through the pores, and adhered to the underside of the membrane, are fixed, stained, and counted. **(B)** Assays to discriminate between directed and random migration. To test for directed migration (chemotaxis), cells resuspended in buffer are placed in opposite wells containing attractants. Thus, cells with receptors for attractants sense a concentration gradient; one pole of the cell binds attractant, but the opposite pole does not. To test for increased rate of random migration (chemokinesis), cells are resuspended in the same concentration of attractant that is encountered in the lower wells, thereby abolishing the concentration gradient.

2.2. Procedure

1. Preparation for dissection:
   a. Soak the scissors and forceps in 70% ethanol for 10 min. Remove the instruments, and allow them to air dry on sterile gauze.
   b. Add 0.015 mL gentamycin to the 15 mL L-15 medium. Place L-15 medium plus gentamycin into the 150-mm Petri dish. Cover and set aside.
   c. Euthanize the pregnant rat, using CO_2 followed by cervical dislocation.
   d. Lay the rat on a sheet of the absorbent, plastic-backed paper, ventral side up.
   e. Prepare the abdominal surface of the rat by soaking it with 70% ethanol.
2. Dissection:
   a. Using the large scissors and curved forceps, make a small incision laterally across the center of the abdomen. Using the medium scissors, cut vertically down the center of the abdomen, toward the vaginal orifice.

Note: Use caution to avoid cutting the intestines, because this is a major source of bacterial contamination.

b. Then cut vertically towards the thoracic cavity. At the level of the diaphragm, make lateral incisions parallel to the ribs, forming two flaps. Pull the skin, muscle, and peritoneal lining aside by using the curved forceps and the tips of the scissors.
c. Using forceps, grab one horn of the uterus. Dissect it from the abdominal cavity, and transfer the intact uterus to the 150-mm Petri dish containing the L-15 medium supplemented with gentamycin.

3. REMOVAL OF EMBRYOS/FETUSES

3.1. Materials

Scissors (1), small.
Forceps (1), medium, curved.
L-15 medium (10 mL).
Gentamycin, 50 μg/mL (0.01 mL).
Petri dish (1), 100-mm, tissue culture grade.
Sterile gauze pads.
70% Ethanol in beaker.

3.2. Procedure

1. Preparation for dissection:
   a. Prepare instruments by presoaking them in 70% ethanol for 10 min. Allow them to air-dry on sterile gauze.
   b. Add 0.01 mL gentamycin to the 10 mL L-15 medium. Place 10 mL of the L-15 medium with gentamycin into a 100-mm Petri dish.
2. Dissection:
   a. Remove each fetus by grasping the edge of a uterine horn with the curved forceps, then gently make an incision along the uterine wall, using the small scissors. Exercise care not to cut the fetus.
   b. Gently push the fetus through the incision, using the curved forceps.
   c. Transfer the fetus to the clean, 100-mm Petri dish containing the 10 mL L-15 medium with gentamycin. Collect several embryos into one dish.

4. DISSECTION OF CEREBRAL CORTEX AND SPINAL CORD

Dissection of the fetuses involves removing the brain or spinal cord, and cleaning it of meninges. For cortical dissociates, the cortex is isolated by removing extraneous brain tissue.

Note: These procedures take place outside of the laminar flow hood, thus caution should be exercised to prevent contamination.

4.1. Cerebral Cortex

1. Materials:
   Scissors (1), small.
   Forceps (2), Dumont no. 5.
   L-15 medium (20 mL).
   Gentamycin, 50 μg/mL (0.02 mL).
   Petri dishes (2), 100-mm tissue culture grade.
   Sterile gauze pads.
   70% Ethanol in beaker.

2. Procedure:
   a. Prepare instruments by presoaking them in 70% ethanol for 10 min. Allow them to air-dry on sterile gauze.
   b. Add 0.02 mL gentamycin to 20 mL of L-15 medium. Place 10 mL of the L-15 medium with gentamycin into each of two 100-mm Petri dishes.
   c. Decapitate several embryos, using the small scissors, and transfer the heads to one of the clean 100-mm Petri dishes containing L-15 medium with gentamycin.
   d. Under the dissecting microscope, use the Dumont forceps to remove the skin and skull. Gently lift the brain, and transfer it to a clean Petri dish with L-15 medium and gentamycin. Continue to collect all of the brains into the same dish.
   e. Under the dissecting scope, separate the left and and right cerebral hemispheres. Using the Dumont forceps, gently peel off the meninges. Turn each hemisphere ventral side up.
   f. Dissect away the striatum, using the Dumont forceps. For embryos >E16, the hippocampus can be removed by gently trimming it away, using the Dumont forceps.

4.2. Spinal Cord

1. Materials:
   Scissors (1), small.
   Scissors (1), micro (iris), angled.
   Forceps (2), Dumont no. 5.
   Forceps (1), curved medium.
   L-15 medium (20 mL).
   Gentamycin, 50 μg/mL (0.02 mL).
   Petri dishes (2), 100-mm, tissue culture grade.
   Sterile gauze pads.
   70% Ethanol in beaker.
2. Procedure:
   a. Prepare instruments by presoaking them in 70% ethanol for 10 min. Allow them to air-dry on sterile gauze.
   b. Add 0.02 mL gentamycin to 20 mL L-15 medium. Place 10 mL of the L-15 medium with gentamycin into each of two 100-mm Petri dishes.
   c. Decapitate several embryos, using the small scissors; discard the heads. Transfer the bodies to one of the clean dishes (100-mm) containing the L-15 medium with gentamycin.
   d. Under the dissecting microscope, lay the body dorsal side up, and use the angled iris scissors to make an incision from the neck to the base of the tail. Slip the tip of the curved forceps underneath the cord at the cervical end. Gently slide the forceps toward the tail to ream the cord away from the surrounding tissue.
   e. Grasp the cervical end of the cord with the curved forceps, and remove it from the body, by gently lifting it and pulling it toward the tail. Transfer the cord to a clean Petri dish containing L-15 medium and gentamycin. Continue collecting all of the cords into the same dish.
   f. Under the dissecting scope, gently peel off the meninges, using the Dumont forceps. Mince the cords into small tissue chunks, using the Dumont forceps.

5. ENZYMATIC DISSOCIATION OF TISSUE

The dissected tissue (cord or cortex) will be enzymatically dissociated into a single-cell suspension with papain, layered over a density gradient to remove cell debris, and the final cell concentration will be adjusted for use in the migration assays.

5.1. Preparation for Dissociation

1. Materials:
   Earle's balanced salt solution+ (EBSS+) (5 mL).
   Deoxyribonuclease (DNase), 0.1% (0.5 mL).
   EBSS (5 mL).
   10:10 solution (0.5 mL).
   Papain (Worthington, tissue culture grade).
   Syringes, 6 cc.
   Syringe filter units, 0.22 μm.
2 Procedure:
   a. Prepare 1:1 solution:
      i. To 4.3 mL EBSS, add 0.5 mL 10:10 solution.
      ii. Add 0.25 mL 0.1% DNase stock. Mix well.
      Note: Make the 1:1 solution fresh before each use.
   b. Prepare EBSS+/papain solution:
      i. To 5 mL EBSS+, add 0.25 mL 0.1% DNase.
      ii. Add papain, to give a final concentration of 20 U/mL.
      Note: Each lot of papain has a different number of U/mg protein; therefore, the appropriate dilution to yield 20 U/mL must be calculated. It generally ranges between 90 and 110 μL/5 mL EBSS+.
      iii. The EBSS+/papain solution will be cloudy. Warm the EBSS+/papain solution at 37°C, until it appears clear.
      iv. Filter-sterilize the EBSS+/papain solution, and transfer the sterile EBSS+/papain solution to a 15 mL conical centrifuge tube.
      Note: Make up the papain solution fresh before each use.

5.2. Dissociation of Tissue

1. Materials:
   EBSS+/papain (5 mL).
   1:1 Solution (3 mL).
   10:10 Solution (5 mL).
   EBSS/glucose (50 mL).
   Centrifuge tubes (2), 15-mL, conical.
   Pipets, 5 mL.
   Hemocytometer.
   Platform rocker.
2. Procedure:
   a. Transfer the dissected tissue from Petri dishes into the 15-mL conical centrifuge tube containing 5 mL sterile EBSS+/papain solution. Cap the tube, and place it on a platform rocker in a 37°C incubator. Incubate, with gentle rocking, for 10 min.
   b. Triturate the tissue gently with a 5-mL pipet, until most of the tissue clumps disappear.
   c. Centrifuge the cell suspension at 300*g* for 5 min. Decant the supernatant, and resuspend the pellet of cells in 3 mL 1:1 solution.
   d. Place 5 mL 10:10 solution into a 15-mL conical centrifuge tube. Using a 5-mL pipet, gently layer the cells on top of the 5 mL 10:10 solution, by letting the cells run slowly down the inside surface of the tube. If layered properly, there should be a sharp interface

between the cell layer and the 10:10 solution. Centrifuge the cells over the density gradient at 80*g* for 7 min. Decant the supernatant.

e. Resuspend the cells, in 3–5 mL EBSS/glucose, triturating the pellet until the clumps disappear.

f. Count the cells, using a hemocytometer. Adjust the cells to a final concentration of 1×10^6 cells/mL in EBSS/glucose.

6. MICROCHEMOTAXIS ASSAY

Place 50,000 cells in each well in the upper half of the microchemotaxis chamber. Presumptive attractants are placed in the bottom wells of the chamber, directly opposite the cells. The cells are separated from the attractants by a membrane containing randomly spaced, 8-μm pores. Prior to chamber assembly, the membrane is precoated on both sides with poly-D-lysine (PDL), to promote cell adhesion. The cells and attractants are incubated in the chambers at 37°C in a mixture of 10% CO_2, 90% air, for 2–18 h. Cells in the upper wells, which are induced to migrate, pass through the pores, then adhere to the underside of the membrane. After the incubation period, the cells on top of the membrane, which failed to migrate, are scraped off. The migrated cells on the underside of the membrane are fixed, stained, and counted.

6.1. Materials

Forceps (2), curved, medium.
Scissors (1), small.
EBSS/glucose (100 mL).
Chemoattractants in high concentration (m*M*) stock solutions.
PDL, 2 mg/mL stock solution (0.1 mL).
Water, tissue culture grade (7.4 mL).
Microchemotaxis chamber, 48-well (Neuroprobe, Cabin John, MD, cat. no. AP48).
Polycarbonate membranes, polyvinylpyrrolidone-free, with 8-μm pores (Neuroprobe, cat. no. PFB8-50) (25 ×80 mm).
Petri dish (1), 100-mm.
Petri dish (1), 150-mm.
Pipet tips.

6.2. Preparation of Chemoattractants

Dilute the chemoattractants in the EBSS/glucose to working concentrations. Mix the chemoattractants thoroughly using a vortex mixer immediately before use.

6.3. Preparation of the Membrane

1. Marking for orientation:
   a. The membrane will be marked, for orientation purposes, by clipping off one corner. One side of the membrane is glossy, the other side is dull. To mark the membrane for use as an orientation guide, place the membrane with the glossy side facing up.
   b. Using the small scissors, clip 2–3 mm off the upper left-hand corner of the membrane.
2. Coating with PDL:
   a. Add 0.1 mL PDL stock (2 mg/mL) to 7.4 mL tissue culture-grade water. Mix well.

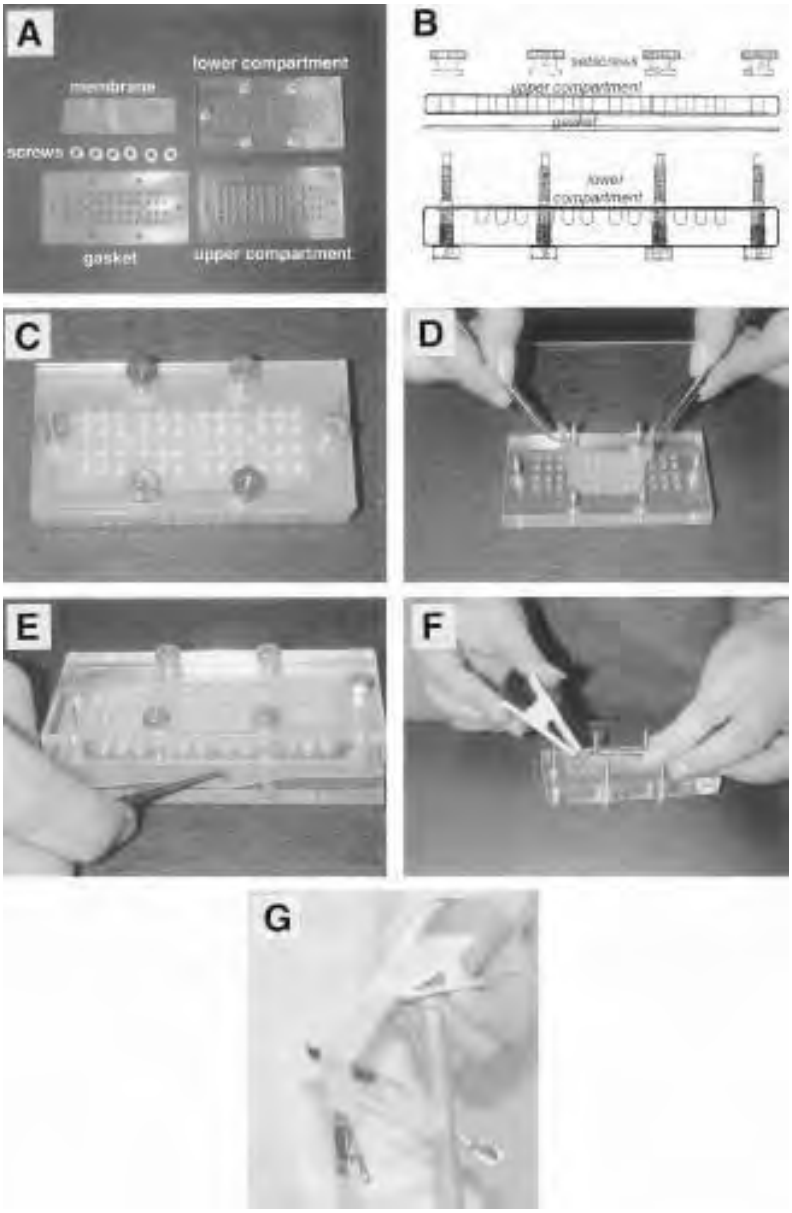

Fig. 3. Key steps in the chemotaxis assay. **(A)** The disassembled chamber consists of the membrane, six set screws, the gasket, the lower compartment, and the upper compartment. **(B)** Schematic diagram depicting the assembly of the chamber. From bottom to top: the membrane covers the wells of the lower compartment. The gasket is placed on top of the membrane. The upper compartment sits on top of the gasket. The set screws fasten the upper compartment to the lower compartment. **(C)** Photograph of assembled chamber. **(D)** During assembly, as the membrane is applied to the lower half of the chamber, it contacts the center of the chamber first, then each side is carefully lowered. **(E)** At the end of the assay, the chamber is inverted. Forceps are used to gently push the gasket down, away from the lower chamber compartment (which is now on top). **(F)** The membrane is removed by grasping the edge with forceps, and attaching the large clamp to the short side of the membrane. **(G)** To remove the nonmigrated cells, the weight of the small clamp pulls the membrane taut, as the membrane is drawn across the edge of the scraper.

b. Put the 7.5 mL diluted PDL into a 100-mm Petri dish. Grasp one end of the membrane with curved forceps, and transfer it to the dish with PDL. Make sure that the membrane is lying flat on top of the PDL.
c. Incubate the membrane in PDL for a minimum of 30 min at 37°C. Use the curved forceps to turn the membrane over, so that the other side is exposed to the PDL. Incubate the second side of the membrane in PDL for a minimum of 30 min at 37°C.

6.4. Filling the Chamber with Chemoattractants

1. Disassemble the chamber into its component parts. There should be six set screws, the upper half of the chamber (with open-bottom wells), the gasket, and the lower half of the chamber with round-bottomed wells (Fig. 3A).
2. To run a negative control for assessing spontaneous cellular motility, set aside one set of three wells to be filled with buffer only (EBSS–glucose).
3. Place 30 µL chemoattractant into each of the round-bottomed wells in the lower half of the chamber.
   Note: Once the lower wells are filled with buffer or attractant, each well should exhibit a slight positive (convex) meniscus. This is essential for forming a tight seal, once all of the chamber components are assembled.

6.5. Adding Membrane to Chamber Assembly

1. Remove the membrane from the Petri dish containing PDL, by grasping one short side with curved forceps. Allow the excess PDL to drain off the surface of the membrane, by holding the membrane against the inside vertical wall of the Petri dish. Lift the membrane, and grasp the other short side with a second pair of curved forceps. Hold the membrane horizontally flat, and oriented so that the clipped corner is in the upper left position.
2. With the membrane suspended over the chemoattractants, center it so that, when it is lowered, it will completely cover all of the wells.
3. Bring your hands, holding each short side of the membrane, together slightly, such that the center of the membrane sags. Slowly lower the membrane on top of the attractants, allowing the sagging center of the membrane to contact the chamber first (Fig. 3D). Next, carefully lower each side of the membrane onto the attractants. This will ensure that a tight seal is formed. Avoid allowing air pockets to form between the membrane and the surface of the chamber. The initial alignment of the membrane, when it is suspended over the chamber, is critical for the proper membrane placement during assembly.
   Note: Once the membrane touches the bottom chamber, it should not be moved, since this could mix attractants, or allow air pockets to form under the membrane.

6.6. Assemble the Chamber

1. Place the gasket on top of the membrane. When the chamber is assembled, the engraved "logos" on the upper and lower halves of the chamber should be superimposed. Place the upper half of the chamber on top of the gasket, orienting it so that the engraved logo on the upper compartment aligns with the engraved logo on the lower compartment.
2. Press down firmly and evenly across the top of the chamber, maintaining pressure as the set screws are tightened.
   Note: Be careful not to release pressure on the chamber top while tightening the screws; this will ensure that air pockets do not form under the membrane.

6.7. Addition of Cells

Add 50,000 cells in 50 µL EBSS/glucose to each well. Avoid bubbles as the cells are added (the suspension should just barely fill the well), and there should be no meniscus (the air–surface interface should be flat). This can be accomplished by filling each well rapidly, using a micropipetor.

6.8. Culture Conditions

1. Make a humidified compartment for the microchemotaxis chamber by placing 15–20 mL water in the bottom of a 150-mm Petri dish. Place the assembled chemotaxis chamber in the dish, and set the Petri dish cover on top of the chemotaxis chamber, to keep in moisture.
2. Incubate the chamber in the humidified compartment in an incubator set at 37°C, with 10% CO_2 and 90% air. The cells should be incubated for a minimum of 2 h, but may need to be incubated for up to 18 h, depending on the age and region of origin of the cells.

Note: The 10% CO_2 is critical for maintaining proper pH, when using EBSS as the buffer in this assay.

3. Most cortical cells from early developmental stages (gestational d 13–15) will migrate within the first 2 h. Cells derived from older ages (gestational d 16–21) should be incubated in the chambers for at least 4 h. Similarly, spinal cord cells from early developmental stages (gestational d 12–14) will migrate within the first 2 h; however, cells from older cords should be incubated for at least 4 h. For convenience, the cortical or spinal cord cells can be incubated overnight in the chambers with the attractants for up to 18 h, without a significant reduction in the number of migrated cells.

7. FIXATION AND STAINING

The chamber will be disassembled, the nonmigrated cells will be washed and scraped off, and the cells that migrated through the pores will be fixed and stained with cresyl violet.

7.1. Materials

Forceps (2), curved, medium.
Large filter clamp (1) (Neuroprobe, cat. no. P48CL).
Small filter clamp (1) (Neuroprobe, cat. no. P48cs).
Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}, pH 7.2 (Quality Biologicals, Gaithersburg, MD, cat. no.114-059-100) (50 mL).
Fixative (8 mL).
Cresyl violet solution (2 mL).
Cell scraper (Neuroprobe, cat. no. P48W).
Petri dish (1), 100-mm.
Petri dish (1), 150-mm.
Glass slides, 2 × 3 in.
Immersion oil.
Parafilm.
Plastic container with water for soaking the chamber.

7.2. Preparation

1. Place fixative into a 100-mm Petri dish.
2. Put 15–20 mL PBS into a 150-mm Petri dish, for washing the membrane.

7.3. Disassembling the Chamber

1. Remove the chemotaxis chamber from the incubator, and take it out of the humidified compartment. Align the chamber so that the clipped corner of the membrane is in the upper left position.
2. Remove the set screws, and turn the chamber upside down by inverting it toward yourself.

Note: The clipped corner should now be in the lower left position: The cells that migrated to the underside of the membrane are now facing up.

3. Using curved forceps, gently push down the upper chamber compartment (which is now on the bottom) with the gasket and membrane adhering to it (Fig. 3E). Lift off the bottom half of the chamber (which is now on top) that held the chemoattractants, and place it into a plastic container with water, to soak.

7.4. Removing the Membrane

At this point, the assembly should consist of the membrane lying on top of the gasket, which is now lying on top of the upper half of the chamber. The assembly should be oriented so that the lower left corner of the membrane is clipped. In this position, the cells that migrated through the pores are facing upwards.

1. Using curved forceps, gently lift the right side of the membrane, and grasp the membrane edge with the large clamp (Fig. 3F).
2. Holding the membrane by the large clamp, peel the membrane off the gasket, and turn it so that the migrated cells face toward you.

7.5. Washing and Fixing the Membrane

1. Use the small clamp to grab the opposite edge of the membrane. Hold the membrane by the clamps, and turn it so that it is held horizontally flat, and the migrated cells are facing up.
2. Gently dip the underside of the membrane (the nonmigrated cells) in PBS in a 150-mm Petri dish.

Note: Be careful not to get PBS on the top of the membrane (on the side with the migrated cells).

3. Drop the small clamp that is holding the left side of the membrane, so that the weight of the clamp makes the membrane hang vertically. Drag the underside (the rinsed, nonmigrated cells) across the rubber edge of the scraper, to remove the nonmigrated cells (Fig. 3G).
4. Repeat the PBS rinse and scraping 2× more. Work quickly, so that the side of the membrane containing the migrated cells does not dry out.
5. Invert the membrane, and place it, migrated-cell side down, onto the fixative in the 100-mm dish. The membrane should be situated so that the clipped corner is now in the upper left position.
6. Incubate the cells in the fixative for 30 min (maximum) at room temperature. Rinse off the fixative, by transferring the membrane to a 150-mm Petri dish containing PBS. Allow the membrane to float briefly in the PBS (cell side down).

7.6. Staining and Mounting the Migrated Cells

1. Place 2 mL cresyl violet solution onto a sheet of parafilm, approx 3 in. square. The dye should form a bubble on the parafilm.

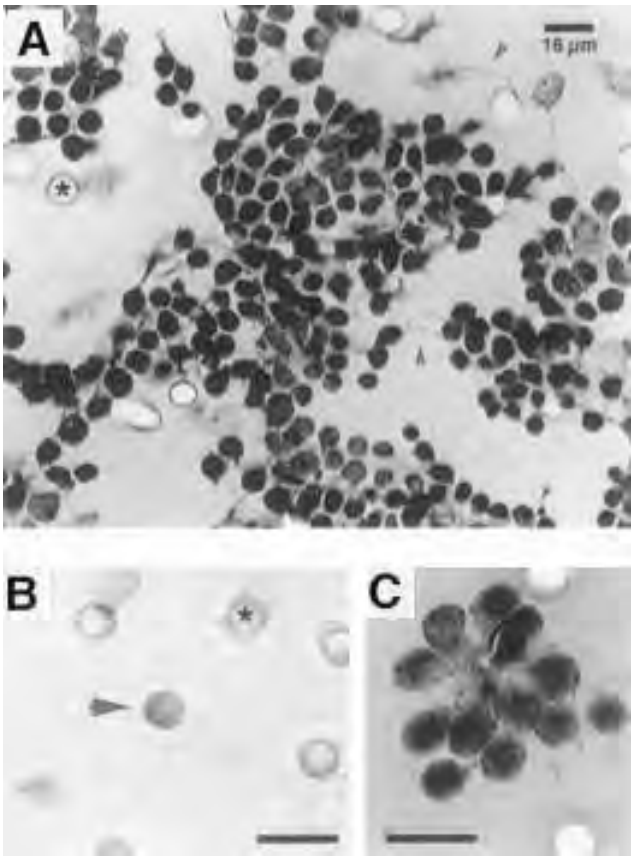

Fig. 4. Photomicrographs of migrated cells. **(A)** Low-power magnification of a field of embryonic murine cortical neurons that migrated through the pores of the membrane toward the attractant, glutamate. Some migrated cells have extended processes (arrowheads). Bar = 16 µm. **(B)** Example of a negative migratory response. High-power magnification shows the cytoplasm of a cell that filled the pore, but failed to migrate completely through (arrow). Only cells that pass through the pores and migrate onto the surface of the membrane are counted as positive. **(C)** High magnification of a group of embryonic murine cortical neurons migrating through a single pore, toward the attractant, glutamate. Bars (B,C) = 20 µm. Asterisks denote pores in the membranes. All cells (A–C) are stained with cresyl violet.

2. Invert the membrane so that it is cell side down (upper left corner is clipped), onto the dye. Make sure that all of the membrane is contacting the dye. Add more dye under the membrane by capillary action, if needed.
3. Incubate the cells on the bubble of dye for 5 min (not longer). Rinse the membrane gently in a 150-mm Petri dish containing PBS.
4. Mount the membrane, cell side up, onto glass slides (clipped corner is in the lower left position). Allow the membrane to air-dry, then immediately overlay it with a thin layer of immersion oil, taking care not to disturb the cells on the membrane surface.

8. DATA ANALYSIS

The wells in the 48-well chemotaxis chamber are arranged in four columns. Each column contains four rows of three wells; therefore, it is convenient to design experiments so that each experimental condition is replicated in one row of three adjacent wells. Only cells that pass completely through the pores, and migrate out onto the underside surface of the membrane, are counted as migrated cells (Fig. 4A,C). In some instances, the stained cytoplasm of a cell, which laid within a pore, but failed to migrate completely through, is evident. Cells that fail to pass completely through the pores are not included in the counted population (Fig. 4B).

For quantitative analysis, cells are counted using oil immersion ×16 or ×40 objectives and bright-field optics. Each chemoattractant condition is run in triplicate wells. Count five fields of stained cells for each well. The average number of migrated cells/mm^2 for each chemoattractant condition is calculated. Significance of data is assessed using analysis of variance, followed by a *post hoc* test, such as Fischer's protected least significant difference test. It is noteworthy that cells characteristically exhibit bell-shaped dose-response curves to chemoattractants in these assays (Stracke et al., 1989; Yao et al., 1990; Grant et al., 1992; Rot et al., 1992; Shure et al., 1992; Geiser et al.,1993; Higashiyama et al., 1993; Reinisch et al., 1993).

To discriminate between chemotaxis (the directed migration of cells toward regions of higher concentrations of a chemical attractant) and chemokinesis (stimulation of increased random cell motility), the migration of cells resuspended in EBSS can be compared to the migration of cells resuspended in the same concentration of attractant encountered in the lower wells (thereby abolishing a chemical concentration gradient). Migration, in the absence of a chemical concentration gradient, is taken as an indicator of chemokinesis (Fig. 2). At the end of the assay, migrated cells may be phenotypically characterized, using immunocytochemistry with antibodies directed against neuronal markers, such as neurofilament (Behar et al., 1994). When immunostaining is used, it is preferable to use an enzymatic end point, such as peroxidase and diaminobenzidine substrate, because the membranes autofluoresce under wavelengths of light used to visualize rhodamine or fluoresceine, rendering immunofluorescence problematic. When immunoperoxidase staining is used, the cresyl violet staining enables the visualization of total cells; the immunostaining appears as a brown immunoreaction product accumulated in the soma and/or neurites. By counting the number of immunolabeled cells that migrated, vs the total number of cells that migrated, the relative number of neurons in the responding (migrating) population can be calculated.

9. APPENDIX

9.1. Solutions

1. EBSS+ solution:
   a. Add 1 mL 50 m*M* ethylenediamine tetraacetic acid (EDTA) solution to 100 mL EBSS (Gibco-BRL, cat. no. 24010-68).
   b. Dissolve 0.0176 g L-cysteine (JRH Biosciences, Lenexa, KS, cat. no. 90-109-100) into the EBSS–EDTA solution.
   c. Filter the solution through a 0.22-μm filter unit, and keep it sterile. The solution may be stored at 4°C for several weeks.
2. 0.1% DNase I stock solution:
   a. Dissolve 20 mg DNase (Boehringer Mannheim, cat. no. 104-159) into 20 mL PBS without Ca^{2+} and Mg^{2+}, pH 7.2 (Quality Biological, Gaithersburg, MD, cat. no. 114-057-100).

   b. Filter the DNase solution through a 0.22-μm filter unit, distribute the solution into aliquots (0.5 mL–1 mL), and store the aliquots at –20°C.
3. Stock 10:10 solution:
   a. To 40 mL EBSS (Gibco-BRL, cat. no. 24010-68), add 400 mg ovomucoid trypsin inhibitor (Sigma, cat. no. T-9253) and 400 mg bovine serum albumin, fraction V (Sigma, cat. no. A-9647).
   b. Mix the solution, and allow it to warm at 37°C, until dissolved.
   c. Filter the solution using a 0.22-μm filter unit. Stock 10:10 solution can be stored at 4°C for several weeks.
4. EBSS/Glucose solution:
   a. To 100 mL EBSS (Gibco-BRL, cat. no. 24010-68), add 1 mL 30% glucose solution.
   b. Filter using a 0.22 μm filtration unit.
5. 2X PBS with Ca^{2+} and Mg^{2+}, pH 7.2: Dilute 10X PBS (Quality Biologicals, cat. no. 119-069-100), pH 7.4, 1:5 with tissue culture-grade water.

9.2. Fixative

1. Preparation of 4% paraformaldehyde:
   a. Dilute 10 mL 16% paraformaldehyde (EM grade, Polysciences) in 10 mL tissue culture-grade water.
   b. Add 20 mL 2X PBS with Ca^{2+} and Mg^{2+} (final concentration is 4% paraformaldehyde in 1X PBS).
   c. Adjust the pH to 7.2 by adding the 0.1 *N* NaOH (tissue culture-grade, Sigma), dropwise, checking the pH after each addition.
   d. The 4% paraformaldehyde solution can be stored up to 2 wk at 4°C.
2. Making the fixative: Add 0.1 mL glutaraldehyde to 7.9 mL 4% paraformaldehyde. Mix well.

9.3. Cresyl Violet Stain

1. Add 0.75 g cresyl violet acetate (Sigma, cat. no. C-1791) to 500 mL water. Add 3 mL glacial acetic acid.
2. Place the solution on a magnetic stirrer, and allow the powder to dissolve completely. This may take several hours. Filter the cresyl violet solution, using a 500-mL capacity, 0.22-μm filter unit.
3. The dye solution may be stored at room temperature for several months, but may need to be refiltered periodically before use.

9.4. Poly-D-Lysine (mol wt >300,000)

1. Dissolve 20 mg PDL (Collaborative Biomedical, cat. no. 40210) in 10 mL tissue culture grade water (final concentration = 2 mg/mL).
2. Filter the PDL, using a 0.22-μm filter unit. The 2 mg/mL PDL stock may be stored up to 2 wk at 4°C.

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Chapter Twenty-Three

Colony Cultures

Plating Efficiency Assay and Cloning

Sergey Fedoroff and Arleen Richardson

1. INTRODUCTION

The premise of colony culturing is the assumption that single viable cells can attach to the substratum, divide, and form a progeny of cells that constitute a cell colony or clone. Colony cultures can be initiated either from a disaggregated cell suspension made directly from animal tissue, or from primary or secondary cultures or cell lines. Colonies, especially ones initiated directly from tissues, are not all identical. They vary in the morphology of their cells, and in their size and compactness. The morphology and size of the colony depends on the kinds of cells plated, interactions between cells, the degree of their differentiation, the cell generation time, the composition of the medium, the type of substratum, and the physical conditions to which the cells are subjected.

In practice, cells in graded dilutions are plated in dishes and cultured for a specific time, depending on the cell type and its population doubling time. The cells are plated in small enough numbers that viable cells can attach to the substratum as single cells and form discrete colonies. The colony cultures are stained at specified times, and the colonies are counted and sized manually or by using an automatic colony counter.

Disaggregated tissue usually yields a variety of cells of varying degrees of maturity, resulting in heterogeneity in colony size and morphology. Only a small percentage of the cells from disaggregated tissue attach to a substratum and form progeny; the older the tissue, the smaller the percentage of cells that form colonies. This method preferentially selects proliferative progenitor cells, which form colonies, if planted in small numbers, and therefore such cells are referred to as "colony-forming cells."

A special advantage of colony cultures is that they can be used to estimate the fraction of viable cells in a given cell suspension and the proliferative efficiency of the cells in developing progeny. Cell colonies are much more sensitive to the environment than cells in dense cultures; therefore, colony cultures are often used for cell nutrition studies, and for testing the effects of growth factors or cytotoxic agents. Colony cultures can also be effectively used for cell genetic studies: isolation of single cells, regulatory mechanisms of cell lineages, and isolation of cell mutants or cells secreting a product at high level.

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This chapter describes colony cultures initiated directly from newborn mouse neopallium, the use of such cultures for determination of colony-forming efficiency (CFE), or, as it is often referred to, as "plating efficiency" (PE), and a procedure for isolation of single cells and developing clones from single cells.

2. PREPARATION OF COLONY CULTURES

2.1. Materials

Disaggregated cells from mouse neopallium (*see* Chapter 11).
Modified Eagle's Minimum Essential Medium (mMEM) containing 5% horse serum (HS), 30 mL.
Petri dishes, tissue culture, sterile, 100-mm.

2.2. Procedure

1. Determine the cell number present in the cell suspension obtained from disaggregating mouse neopallium. Calculate the number of viable cells/mL (*see* Chapter 26).
2. Add 2 mL mMEM to each 100-mm Petri dish, and spread the medium to coat the entire floor of the dish.
3. Dilute the cell suspension to 1×10^5 cells/mL.

Note: It is important to resuspend the cells, before making any dilutions. If they are not properly resuspended, reproducibility of the colony cultures will not be possible.

4. Before plating, make sure the cells are well-suspended. Plate four dilutions of cell densities: 0.5×10^5, 1.0×10^5, 2.0×10^5 and 4.0×10^5. Adjust the amount of medium to 6 mL per dish. The cell density dilutions to be used in this assay must be determined experimentally. They may vary between the regions of the brain and the strains of mice or species used.

Note: It is important that the cells are randomly distributed within the culture vessel. To achieve this, place a hand flat on the covered Petri dish, and move the dish forward and backward several times, then the same number of times from side to side. Never use a circular motion, to avoid centrifugal distribution of the cells.

5. Incubate the culture dishes in a 37°C highly humidified atmosphere, containing 5% CO_2/95% air. After 3 d incubation, wash out the cell debris and nonattached cells, using fresh medium at 37°C. Incubate cultures for 10 d, without disturbance.

Note: Cultures may be incubated without medium change, because of the small cell inoculum, which does not deplete the medium of required nutrients.

6. Select for analysis the dishes that have no overlapping cell colonies, and in which individual colonies have more than 50 cells.

2.3. Comments

1. The first 24–48 h of culturing are critical for colony cultures. During this time, small cell aggregates may form, especially if the inoculum is too large, or if the culture vessels are disturbed. Sometimes, the vibration of the incubator fan or vibrations in the building are enough to upset the random distribution of cells. Aggregates are also more likely to form in cultures derived from young embryos than from those of older embryos or neonatal animals. Thus, it is important that, for at least the first 3 d, the cultures should not be disturbed.
2. The colony cultures are much more sensitive to culture conditions than are cultures initiated from a large inoculum. In colony cultures, the ratio between cells and the volume of medium is very great. It is therefore important to provide conditions under which the pH and osmo-

larity of the medium are as constant as possible during the culture period. The pH can be maintained by careful control of the CO_2 in air atmosphere and the bicarbonate concentration in the medium. Providing sufficient humidity to prevent evaporation of the medium prevents changes in osmolarity. All the components used in the medium must be optimized to obtain the highest possible PE for the neopallial cells.

3. The compactness of the colony can vary greatly from species to species. For example, mouse and rat glial cells form compact colonies, but human glial cells do not. We have found that the best colonies for the assay are produced by neopallial cells of C_3H/HeJ mice.
4. The frequency of occurrence of colonies of various morphologies is related to the age of the donor embryo. For example, cells from mouse embryos at E16–E18 produce colonies in which the centers are formed by epithelial-type immature cells (glioblasts). Toward the periphery, various stages of astroglial differentiation can be identified, i.e., proastroblasts, which are vimentin-positive and glial fibrillary acidic protein (GFAP)-negative; astroblasts, which are vimentin-positive and GFAP-positive; and small stellate astroglia, which are vimentin-positive and GFAP-positive, and contain little actin (Fedoroff et al., 1990).
5. Colony cultures initiated from perinatal mouse or rat brain tissue are plastic, and cell fate can be affected by the composition of the culture medium. In regular medium containing 10–15% serum, astroglial cell colonies will predominate (*see* Fedoroff and Doering, 1980). When colony stimulating factor-1 is added to this medium, the number of astroglia colonies decreases, and the number of microglia colonies increases (Fedoroff et al., 1991). In serum-free medium, the number of oligodendroglia colonies will increase. The addition of platelet-derived growth factor to the serum-free medium will further increase the number of oligodendroglial colonies (Noble et al., 1988; *see also* Chapters 11 and 12).
6. To obtain clearly defined colonies that do not overlap, and a sufficient number of colonies per dish to permit quantification, it is advisable to use 100-mm tissue culture dishes. In 100-mm dishes, as many as 300–400 good-sized colonies can form without overlapping.
7. Not all colonies formed are progeny of a single cell; some are formed by two or more cells. To obtain reproducible results, it is important that all parameters of the assay be carefully optimized. Therefore, colony is not equivalent to clone, unless it is proven that the colony began from a single cell. To obtain clonal cultures, *see* Section 4.
8. When colonies are to be grown on coverslips (*see* Section 5.2.) for immunocytochemical studies, it is advisable to use round coverslips placed in nontissue culture Petri dishes. These dishes have hydrophobic surfaces, thus ensuring that the growth of the cells will be limited to the coverslips. The colonies accommodate better on round coverslips than on square ones.

3. PLATING EFFICIENCY ASSAY

There is a direct relationship between the number of cells planted in cultures and the number of cell colonies formed. This relationship has been exploited for development of a PE assay. The PE of a given cell population provides a convenient way to estimate the number of viable cells present (colony number) and their proliferative efficiency (colony size), and is therefore an excellent growth or cytotoxicity assay.

PE, or CFE, is the percentage of individual cells in the cell suspension that gives rise to cell colonies, when inoculated into culture vessels. The total number of cells in the inoculum, type of culture vessel, the environmental conditions (medium, temperature, atmosphere), and the source of cells used in the assay should always be stated when reporting PE.

Determination of PE is a very sensitive assay. It can be used not only for determining the effect of the immediate environment on cell CFE, but also for determining the effect of a drug *in situ*. For

example, it has been used for determination of the effects of alcohol on mouse fetal brain development. Alcohol given to a pregnant mouse dramatically affects the PE of neopallial cells of the embryo (Blakley and Fedoroff 1985).

3.1. Determination of PE

1. Materials:
   Single-cell suspension from neopallium of newborn mice (*see* Chapter 11).
   Petri dishes, tissue culture, 60-mm, sterile.
   mMEM supplemented with 5% HS.
2. Procedure:
   a. Determine the cell number present in the cell suspension obtained from disaggregating mouse neopallium. Calculate the number of viable cells/mL (*see* Chapter 26).
   b. Add 1 mL mMEM to each 60-mm Petri dish, and spread the medium to coat the entire floor of the dish.
   c. Dilute the cell suspension to 1×10^5 cells/mL.

   Note: It is important to resuspend the cells, before making any dilutions. If they are not properly resuspended, reproducibility of the colony cultures will not be possible.

   d. Before plating, make sure the cells are well-suspended. Plate four dilutions of cell densities: 0.5×10^4, 1.0×10^4, 2.0×10^4 and 4.0×10^4. Plate triplicates of each dilution. Adjust the amount of medium to 3 mL per dish.

   Note: It is important that the cells are randomly distributed within the culture vessel. To achieve this, place a hand flat on the covered Petri dish and move the dish forward and backward several times, then the same number of times from side to side. Never use a circular motion, to avoid centrifugal distribution of the cells.

   e. Incubate the culture dishes in a 37°C highly humidified atmosphere, containing 5% CO_2/95% air. After 3 d incubation, wash out the cell debris and nonattached cells, using fresh warm medium. Thereafter, do not feed cultures, and incubate them for 10–12 d without disturbance.
   d. After 10–12 d incubation, fix and stain cultures.

3.2. Fixation and Staining

1. Materials:
   Hanks' balanced salt solution (HBBS).
   Absolute methanol (–20°C).
   Distilled water.
   0.25% Coomassie brilliant blue R-250 (C. I. 42660).
2. Fixation of colony cultures. Wash the cultures 3× with HBSS, and fix with absolute methanol at –20°C for 10 min. At this point, cells may be left overnight at room temperature, with sufficient methanol to allow for evaporation. Cells should not be allowed to dry.
3. Staining the cultures:
   a. Dilute the methanol in the culture dish with distilled water.
   b. Decant and add distilled water.
   c. Decant the water, add 0.25% Coomassie brilliant blue R-250, and stain for 1–2 min.
   d. Decant the stain, and wash the cultures in tap water.
   e. Dry the cultures in air.

   Note: If cultures are stained too dark, washing with methyl alcohol can destain them. If the stain is not dark enough, the staining procedure can be repeated (repeat step c.).

4. Count and size colonies, using an automatic colony counter (*see* Appendix). It is also possible to count colonies manually, and size them by microscopic examination.

3.3. Calculation of PE

$$\text{PE (\%)} = \frac{\text{Number of colonies counted per culture}}{\text{Number of viable cells plated per culture}} \times 100$$

3.4. Comments

1. PE for different cell types may vary from nearly zero to nearly 100%. A cell type with a high PE will require plating of a lower concentration of cells, to achieve discrete colonies, than a cell type with a low PE. To determine the best concentrations for use for PE assays, a wide range of concentrations of cells should be plated initially. Neopallial cells, isolated directly from mice, have about a 1% PE.
2. To determine the PE, it is important to plate a graded number of cells. Theoretically, the PE for each concentration of cells plated should be the same, as illustrated by the following example:

No. of viable cells plated $\times 10^{-4}$	No. of colonies formed	PE
2.0	200	1%
1.0	100	1%
0.5	50	1%

If the density of cells is too low for the culture vessel, an erroneously low PE may result. If the density of cells is too high for the culture vessel, colonies may overlap, which may also result in a low PE. In practice, 4–5 dilutions of cell densities are used, and, on examination of stained culture dishes, dishes with too low or too high numbers of colonies are eliminated. For this assay to be valid it is essential to have at least three different dilutions of the cell densities, in order that a straight-line relationship between the number of cells plated and the number of cell colonies formed can be determined.

In 60-mm Petri dishes, 50–200 colonies per dish are required to make an accurate estimate; in 100-mm Petri dishes, 200–400 colonies per dish are optimal. The colony to be counted should have at least 50 cells. Colonies with less than 50 cells, or very large colonies, should be eliminated from counting. The assay is valid if there is linearity of colonies formed vs cells plated.

Note: In a PE assay, it is common practice to stack the 60-mm Petri dishes in the incubator. We, however, recommend that stacking be avoided, in order to decrease the effect of variability caused by environmental conditions in the incubator, particularly temperature.

4. CLONING

The ultimate goal of the colony culture is to clone the cells, i.e., to produce a cell population that can be verified as originating from an individual cell. The early attempts to achieve this were to pick up single cells directly from a culture, and to allow them to propagate in a minienvironment. Puck and Marcus (1955) developed serial dilution procedures that are the basis of colony culture techniques, and have been dealt with in this chapter. Application of the limiting dilution method to cloning mammalian cells in cultures made the process considerably easier. The limiting dilution

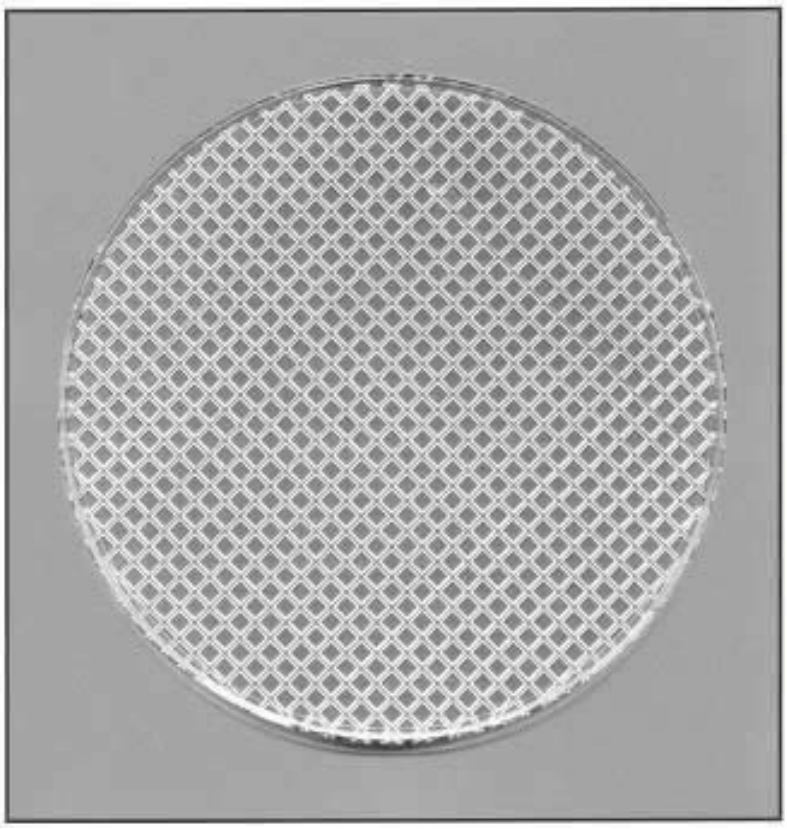

Fig. 1. Greiner's hybridoma tissue culture dish (94-mm) subdivided into about 700 wells, 0.04 cm^2 each. The wells can be viewed under an inverted phase-contrast microscope with ×20 and ×40 objectives.

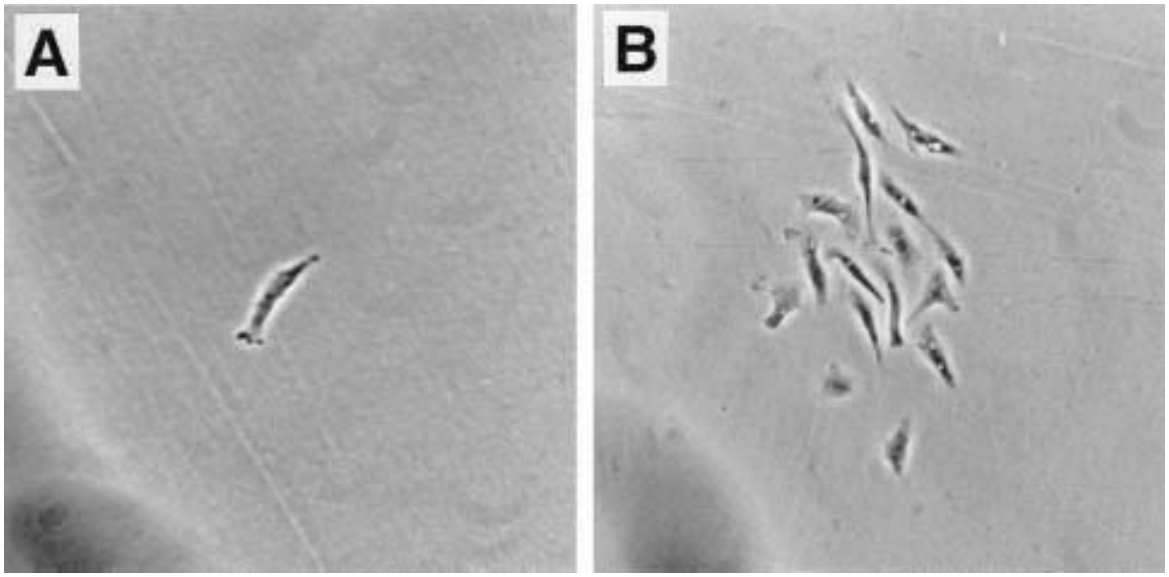

Fig. 2. **(A)** Single neopallia cell in a well of the Greimer hybridoma culture dish 49 h after plating. **(B)** A clone of cells after 8 d of culturing.

method is based on the principle of Poisson distribution, i.e., a single cell suspension is planted into multiwell plates; the cells will assort randomly and independently, so that some culture wells will have no cells, others a single cell, and the rest, two or more. (Consult Lefkovits and Waldmann, 1979, for limiting dilution analysis using the Poisson formula).

In Chapter 14 of this volume, Gritti et al. describe three methods for cloning cells from neurospheres, and for directly observing the formation of the clones from a single cell. This chapter describes a method for cloning cells directly from mouse neopallium, and for directly observing formation of the clones from single cells under a phase microscope. In this procedure, we use Greiner's hybridoma tissue culture dishes (Greiner Labortechnik, Bellco Glass, Vineland, NJ), the limiting dilution method, and direct phase contrast observation of clone formation from single cells. The 94-mm Greiner's hybridoma dishes are subdivided into about 700 wells, 0.04 cm^2 each (Fig. 1). The dishes are designed so that the clones from one well in the dish cannot spread to the neighboring well. The dishes are planted with 5×10^4 disaggregated neonatal mouse neopallial cells per dish. At this cell density, the cells assort randomly and independently, so that some culture wells have no cells, others have a single cell, and the rest have two or more cells. The culture medium contains mMEM (Gibco-BRL, Gaitharsburg, MD, cat. no. 90-5175), 5% HS and 5% mouse embryonic fibroblast STO cell (American Type Culture Collection, cat. no. CRL 1503) conditioned medium. After 48 h incubation at 37°C, in a highly humidified atmosphere of 5% CO_2/95% air, the medium is carefully changed, and dishes are examined under a phase-contrast microscope at magnification of ×100. Wells that contain only one cell are marked with a diamond marker. The formation of the colony can be observed and photographed throughout the subsequent 10-d incubation, during which time most of the single cells develop into clones, then the clones can be used for further analyses (Fig. 2).

5. APPENDIX

5.1. Solutions

1. mMEM (Gibco-BRL, cat. no. 90-5175, special order).
   MEM is modified to contain a fourfold concentration of vitamins, a double concentration of amino acids except glutamine, and 7.5 m*M* glucose. Before using the mMEM for culturing, 5.2 mL 1 *M* sodium bicarbonate and 2.5 mL 200 m*M* glutamine are added to 200 mL mMEM, and the pH is adjusted to 7.2 by bubbling 5% CO_2 into the medium. The prepared mMEM is supplemented with 5% HS.
2. 0.25% Coomassie brilliant blue stain:
   a. Mix together the following:

Coomassie brilliant blue R-250	1.1 g
Absolute methanol	200.0 mL
Glacial acetic acid	40.0 mL
Triple-dH_2O	200.0 mL

   b. Filter the stain through Whatman paper into amber-colored bottles. Store tightly sealed at 4°C.

5.2. Preparation of Coverslips

Occasionally, it is an advantage to put several coverslips in a 100-mm nontissue culture dish. When cells are planted, they attach and grow only on the coverslips. Such coverslip cultures are in an identical environment, and single or replicate coverslip cultures can be removed from the dish when required, without affecting the remaining cultures.

The composition of the glass is important for the attachment of neural cells. Manufacturers may change the composition of glass without notice. We have found that coverslips made from German glass are consistently satisfactory.

1. Metso (sodium metasilicate): Dissolve 40 g Calgon and 360 g Metso in 3785 mL dH_2O. Let stand overnight. Filter.
2. Using forceps, place coverslips in staining rack (cradle) (Arthur H. Thomas, staining rack cat. no. 8542-E40).
3. Add filled rack to a boiling mixture of 20 mL stock Metso/1000 mL highly purified water. Boil for 20 min.

Note: Glass boiling beads (marbles) should be added to the boiling mixture, prior to adding the rack of coverslips.

4. Remove rack from the hot solution. Immediately rinse it through two changes of tap water, two changes of dH_2O, and two changes of highly purified water. Each change of water should contain 500–1000 mL.
5. Dry coverslips in a drying oven.
6. When completely dry, place coverslips in glass Petri dishes, and sterilize in a preheated oven at 375°C for 3 h.

5.3. Automatic Colony Counter

The automatic cell/colony counter (e.g., Artek, Imaging Products, Chantilly, VA) is a highly sensitive video scanning instrument that counts and sizes micro- and macrosize objects. In cell/colony counting, accuracy depends on the ability to see colonies distinctly, whether by eye or electronically. In both methods, it is advantageous to employ plating procedures that enhance the visibility of colonies, that is, a uniform distribution, distinct morphology, and good contrast. In manual colony counting, statistical reliability decreases with time and operator fatigue. In general, 50–200 colonies/60-mm dishes and 300–400 colonies/100-mm dishes provide the most accurate counts, when comparing manual and automatic counts. The automatic counts tend to be slightly lower than the manual, however, because of masking of colonies around the periphery of the dish and overlapping or clustered colonies that are not fully counted. A single percentage calibration factor determined experimentally can provide machine counts that will correlate well with precise manual counts.

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Chapter Twenty-Four

Hybridoma Cultures for Production of Antibodies

Purification and Specificity of Antibodies

J. Denry Sato

1. INTRODUCTION

During the process of characterizing the structure and function of novel antigens, it is usually necessary to create new monoclonal or polyclonal antibody reagents. However, once validated, these antibodies can be put to a multitude of experimental uses, such as detecting and quantitating antigens in cell and tissue extracts or biological fluids, purifying proteins for structural analyses, studying protein–protein interactions, and monitoring cellular differentiation. Although many well-characterized monoclonal antibodies (mAbs) to antigens of general interest are commercially available, mAbs to specialty antigens may need to be individually purified from hybridomas obtained from academic sources, or from not-for-profit cell repositories, such as the American Type Culture Collection (www.atcc.org). This chapter describes protocols for producing, purifying, and verifying murine mAbs from pre-existing hybridomas.

2. PRODUCTION OF MABS

mAbs can be collected from hybridomas cultured either in vitro or in the peritoneal cavities of major histocompatibility complex-compatible or immunodeficient strains of mice. Although mAbs can be recovered at high concentrations in ascites fluid, those antibodies are contaminated with an unknown proportion of irrelevant antibodies from the host mice. Because there are applications in which accurate estimates of antibody concentrations are important, we prefer to collect mAbs in serum-free cell culture medium. The medium formulation provided below is a modification of a serum-free medium developed for NS-1-Ag4-1 and related mouse myeloma cell lines (Kawamoto et al., 1986; Sato et al., 1987; Myoken et al., 1989).

2.1. Materials

RPMI-1640 DMEM nutrient medium plus five factors (RD+5F).
50X BSA-oleic acid stock solution.

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Roller bottles, 2-L.
Pipets, disposable, plastic.
Tubes, conical, polypropylene, sterile, 50-mL.
95% air/5% CO_2, sterile.

2.2. Procedure

1. Add 250 mL RD+5F medium to each 2-L roller bottle. Add BSA-oleic acid stock solution (1X-1 mg/mL BSA and 10 µg/mL oleic acid).
2. Collect hybridoma cells in 50-mL conical tubes, and centrifuge for 5 min at 200*g*. Add the cells to the roller bottles at a final density of 1–5 + 10^5 cells/mL medium.
3. Gas the medium with sterile 95% air/5% CO_2 to prevent the pH from increasing, and tighten the roller bottle caps, to prevent gas exchange.
4. Incubate the roller bottles at 37°C. The bottles can be rolled at 1 rpm, but rolling is not necessary, and it may decrease cell viability.
5. Harvest the conditioned medium when the phenol red has turned orange, and clarify the medium by centrifugation at 200*g* for 10 min. Store the medium at 4°C.
6. Split the hybridomas at a ratio from 1:4 to 1:10, in fresh medium, to continue producing antibodies.

3. PURIFICATION OF MONOCLONAL ANTIBODIES (mABs)

For some applications, such as immunoadsorption and Western blotting, pure mAbs are not necessary, and hybridoma-conditioned medium or diluted ascites fluid may suffice. However, pure antibodies are required for applications such as immunohistochemistry and the determination of antibody affinity for antigen. There is no single method that allows for the purification of all species of mAbs. The most convenient method of purifying murine mAbs of immunoglobulin G (IgG) isotypes is chromatography on immobilized protein A (Ey et al., 1978) or protein G (Akerstrom et al., 1985). This method both concentrates and purifies the antibodies in a single step. However, this method cannot be used to purify IgM antibodies, and the low-pH buffers used to elute bound antibodies may have a deleterious effect on the activity of some antibodies.

3.1. Materials

Protein A-agarose (Pierce, cat. no. 20333) or protein G-agarose (Pierce, cat. no. 20398) slurry.
Phosphate-buffered saline (PBS), Ca^{2+}–Mg^{2+}-free.
Wash buffer: PBS, pH 7.4; or 3 *M* NaCl/50 m*M* Tris-HCl (pH 8.9) (High-salt buffer).
Elution buffer: 0.1 *M* glycine-HCl (pH 2.5).
Neutralization buffer: 1 *M* Tris-HCl (pH 7.5).
Columns (Bio-Rad, cat. no. 731-1550), 10-mL, disposable polypropylene.
Dialysis membrane with a mol wt cutoff of less than 100 kDa (Slide-A-Lyzer dialysis casette, Pierce, cat. no. 66407).

3.2. Procedure

1. Add 1–5 mL protein A- or protein G-agarose slurry to a polypropylene column. Wash the column with 5 column volumes of elution buffer, followed by 5 column volumes of wash buffer. Use high-salt buffer, if purifying IgG_1 on protein A (*see* Section 3.1. above).
2. Run the clarified antibody supernatant (conditioned medium) over the column, using gravity feed or a peristaltic pump at a flow rate of 1 mL/min.

3. Wash the column with 50–100 column volumes of wash buffer. High-salt buffer is only necessary if washing IgG_1 bound to protein A.
4. Elute bound antibody in 1-mL fractions, with at least 5 column volumes of elution buffer.
5. Add 0.2 mL 1 *M* Tris-HCl (pH 7.5), to neutralize each fraction of eluate.
6. Pool fractions containing antibodies, as determined by absorbance at 280 nm, a serological assay, or sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Dialyze the pooled antibodies against PBS or distilled water.
7. Determine the antibody concentration spectrophotometrically by absorbance at 280 nm (1 mg/mL IgG = 1.4 OD_{280} with a 1-cm light path).
8. Monitor the purity of the antibody preparation by SDS-PAGE. The heavy and light chains of reduced IgG run as 50 and 25 kDa bands, respectively.

3.3. Comments

1. Murine IgG_1 binds poorly to protein A, but this interaction can be substantially improved by adding NaCl to the antibody solution, to a final concentration of 3 *M*, and by raising the pH of the antibody solution to 8.9. Alternatively, murine IgG_1, binds with high affinity to protein G at physiological salt concentrations.
2. If the antigen-binding activity of the antibody of interest is adversely affected by the low-pH buffer used to elute antibodies from protein A or protein G, it may be possible to raise the pH of the elution buffer by up to 1 pH unit or more to reduce this effect, while still recovering antibody from the column. If this method is unsatisfactory, chromatography on a solid support, coupled to diethylaminoethyl (DEAE) and the dye, Cibacron blue F3GA (DEAE-Affigel Blue, Bio-Rad), can be used to purify all IgG isotypes at a pH closer to 7.4 (Bruck et al., 1982). The drawbacks of this method are:
   a. Antibodies do not bind to this matrix in 0.15 *M* NaCl, and therefore they are not concentrated.
   b. The final antibody preparation will contain transferrin.
3. IgM mAbs cannot be purified on protein A or protein G. However, IgM precipitates in low-ionic-strength solvent, so that can be purified from conditioned medium by dialysis against distilled water (Heide and Schwick, 1978). The precipitate is collected by centrifugation and redissolved in PBS. If necessary, IgM can be further purified by gel filtration chromatography on Sephadex G-200 or similar gel filtration medium.

4. VERIFYING ANTIBODY ACTIVITY

Once a mAb has been purified, it is important to verify that it has retained its antigen-binding activity, before it is used in further experiments. The most convenient methods for assessing antigen-binding activity are Western blotting using purified target antigen or a complex cell or tissue fraction, and immunoadsorption coupled with SDS-PAGE. A protocol for immunoadsorption is provided below.

4.1. Materials

Protein sample containing the target antigen.
mAb solution.
Protein A-agarose or protein G-agarose slurry.
Immunoprecipitation buffer.
3X SDS-PAGE sample buffer (reducing).
Eppendorf tubes, 1.5-mL.

Microcentrifuge.
Minigel apparatus.

4.2. Immunoadsorption

1. Solubilize antigen in 50–100 µL immunoprecipitation buffer in a 1.5-mL Eppendorf tube. Centrifuge the solution at 13,000*g* in a microcentrifuge, and transfer the supernatant to a second tube.
2. Add 1–10 µg mAb, and incubate the antigen-antibody mixture at 4°C for at least 1 h.
3. Add 50 µL protein A-agarose or protein G-agarose in immunoprecipitation buffer, and incubate at 4°C for 1–2 h.
4. Pellet the beads by centrifugation at 13,000*g* in a microcentrifuge, and wash the antibody-antigen complexes, adsorbed to the protein A or protein G beads, with 0.5 mL immunoprecipitation buffer, 5×. Collect the beads by centrifugation after each wash.
5. Wash the beads a final time with distilled water, to reduce the amount of salt in the sample.
6. Add an appropriate volume of 1X SDS-PAGE sample buffer, heat the sample for 5 min at 95°C, then put it on ice.
7. Electrophorese the sample in an SDS-polyacrylamide minigel of appropriate concentration. Stain the gel with Coomassie brilliant blue or silver, to detect the protein antigen.

4.3. Comments

1. In Western blotting, if epitope reactivity is sensitive to reducing agents, omit 2-mercaptoethanol from the electrophoresis sample buffer.
2. mAbs raised against native protein antigens may not be suitable for use in Western blotting. Immunoadsorption is likely to be a more suitable application for these antibodies. Conversely, mAbs raised against denatured protein antigens may be most suited for use in Western blotting.
3. Murine mAbs that do not bind protein A or protein G can be used in immunoadsorption assays, by precoating the protein A or protein G beads with secondary polyclonal antibodies such as rabbit antimouse Ig. If this method is used, it is important to include a negative control omitting the mAb.
4. Complex protein antigens such as cell or tissue lysates, may be preferable to pure antigen in Western blotting or immunoadsorption assays of mAbs, because they will provide additional information about antibody crossreactivities.
5. An additional method by which mAbs can be verified is antibody typing. By identifying antibody heavy and light chains, this assay provides supporting evidence that the antibody that has been purified is indeed correct. Enzyme-linked immunosorbant assay-based typing kits are available from a number of commercial sources.

5. APPENDIX

5.1. Media and Supplements

1. RD nutrient medium (a 1:1 mixture of RPMI-1640 and Dulbecco's modified Eagle's medium):
   a. Dulbecco's modified Eagle's medium with high glucose (Gibco-BRL, cat. no. 12100-046). Formulation:

Inorganic Salts:	mg/L
$CaCl_2$ (anhydrous)	200.00
$Fe(NO_3) \cdot 9H_2O$	0.10

KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6400.00
$NaH_2PO_4 \cdot H_2O$	125.00
Other Components:	
D-Glucose	4500.00
Phenol red	15.00
Amino Acids:	
L-Arginine · HCl	84.00
L-Cysteine 2HCl	63.00
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl · H_2O	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophan	16.00
L-Tyrosine 2Na · $2H_2O$	104.00
L-Valine	94.00
Vitamins:	
D-Calcium pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i-Inositol	7.20
Niacinamide	4.00
Riboflavin	0.40
Thiamine HCl	4.00
Pyridoxine HCl	4.00

b. RPMI-1640 formulation:

Inorganic salts	mg/L
$Ca(NO_3)_2 \cdot 4H_2O$	100.00
KCl	400.00
$MgSO_4$ (anhyd.)	48.84
NaCl	6000.00
Na_2HPO_4 (anhyd.)	800.00
Other Components:	
D-Glucose	2000.00
Glutathione (reduced)	1.00
Phenol red	5.00
Amino Acids:	
L-Arginine	200.00
L-Asparagine (free base)	50.00
L-Aspartic acid	20.00
L-Cystine 2HCl	65.00
L-Glutamic acid	20.00

L-Glutamine	300.00
Glycine	10.00
L-Histidine (free base)	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine · HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine 2Na · $2H_2O$	29.00
L-Valine	20.00
Vitamins:	
Biotin	0.20
D-Calcium pantothenate	0.25
Choline chloride	3.00
Folic acid	1.00
I-Inositol	35.00
Niacinamide	1.00
Para-aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B_{12}	0.005

c. Reconstitute equivalent amounts of powdered RPMI 1640 (Gibco-BRL, cat. no. 31800-022) and DMEM with high glucose with Milli-Q water according to the manufacturer's instructions to give a final 1:1 mixture (by volume).

d. Stir in the following reagents to the final concentrations:

Component	Final concentration
HEPES (Research Organics, Cleveland, OH, cat no. 6003H)	15 m*M*
L-Glutamine (Sigma, cat. no. G-3126)	2 m*M*
Na pyruvate (Sigma, cat. no. P-2256)	0.01%
$NaHCO_3$ (J.T. Baker, cat. no. 3506-01)	2.0 g/L
Penicillin (Sigma, cat. no. P-3032)	100 IU/mL
Streptomycin (Sigma, cat. no. P-3032)	50 μg/mL

e. Adjust the pH to 7.5, and sterilize the medium by filtration through a 0.22-μm filter.

2. Medium supplements, 200X stock solutions:

a. Make the following stock solutions:

i. Insulin (Sigma, cat. no. I-5500), 200X stock.
2 mg/mL insulin in 10 m*M* HCl.

ii. Transferrin (Sigma, cat. no. T-2252), 200X stock.
2 mg/mL human transferrin in PBS.

iii. Ethanolamine (Sigma, cat. no. E-9508), 200X stock.

2 m*M* ethanolamine in Milli-Q water.

iv. 2-Mercaptoethanol (Sigma, cat. no. M-6250), 200X.
2 m*M* 2-mercaptoethanol in Milli-Q water.

v. Na selenite (Sigma, cat. no. S-1382), 200X.
2 μ*M* Na selenite in Milli-Q water.

b. Sterilize stock solutions by filtration through 0.22-μm filters.

c. Keep supplements at –20°C for long-term storage.

3. Fatty acid-free bovine serum albumin (BSA) (Bayer, cat. no. 82-002-2) conjugated with oleic acid (Sigma, cat. no. O-1008) (50X Stock solution).
   a. Make a 50-mg/mL solution of fatty acid-free Fraction V BSA in PBS. Filter-sterilize through 0.22-μm filter.
   b. Make a 20-mg/mL solution of oleic acid in 100% ethanol.
   c. For each mL BSA solution, add, dropwise, with constant stirring at 37°C, 25 μL 20 mg/mL solution of oleic acid (final concentration 500 μg/mL). If the solution is turbid, incubate it in a 37°C water bath until it clears.

   Note: A commercial BSA–oleic acid conjugate is available from Sigma (cat. no. O-3008).

   d. Store the 50X stock solution at 4°C in a light-tight container.
4. RD+5F (Factor) medium:
   a. Add appropriate volumes of the 200X stock solutions of insulin, transferrin, ethanolamine, 2-mercaptoethanol, and Na selenite to RD nutrient medium.
   b. Supplement the medium with BSA–oleic acid to final concentrations of 1 mg/mL BSA, which corresponds to 10 μg/mL oleic acid.

5.2. PBS, Ca^{2+}-Mg^{2+}-Free

NaCl	8.00 g/L
KCl	0.20 g/L
KH_2PO_4	0.20 g/L
$NaHPO_4$	1.15 g/L

Adjust pH to 7.4.

5.3. Immunoadsorption Buffers

1. Immunoprecipitation buffer:
   PBS (*see* 5.2), adjusted to 0.5 *M* NaCl (pH 7.4).
   0.1% SDS (Sigma, cat. no. L-4509).
   1% NP-40 (Sigma, cat. no. N-6507).
   0.5% Na deoxycholate (Sigma, cat. no. D-6750).
2. 3X SDS-PAGE sample buffer (reducing):

0.5 *M* Tris-HCl (pH 6.8)/0.4% SDS	1 mL
25% SDS in H_2O	0.8 mL
2-Mercaptoethanol (Sigma, cat. no. M-6250)	0.5 mL
Glycerol (J.T. Baker, cat. no. 2140-01)	1 mL
Bromophenol blue (Bio-Rad, cat. no. 161-0404)	0.05%

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Chapter Twenty-Five

Elimination of Cell Types from Mixed Neural Cell Cultures

Richard M. Devon

1. INTRODUCTION

The isolation and purification, or enrichment, of neuronal or glial cell types from central nervous system (CNS) tissues require the application of one of several strategies. It is possible to separate a particular cell type, based on its cell mass or presence of specific cell surface antigens. Differential gradients and centrifugal force (Pretlow and Pretlow 1983, *see also* Chapter 10), binding a variety of ligands, including magnets, to specific cell surface antigens can be utilized to isolate a cell type before the cells have been planted in cell cultures. Another approach is to plant a mixed cell population in culture, then proceed with purification of the culture, either by elimination of a certain cell type, or by encouraging others to proliferate and overgrow a culture. Selection of a cell type in culture requires the addition of compounds or substitution of specific nutrients to the growth medium.

The nervous system contains a mixture of cell types in different stages of differentiation and function, including a variety of neurons, Schwann cells (in peripheral nervous system [PNS]), astroglia, oligodendroglia, ependymal cells, meningeal cells, fibroblasts, and microglia, as well as endothelial cells. The following sections highlight some procedures for purification of neural cell populations in cultures initiated from primary disaggregated nervous tissue.

2. CULTURES OF NEURONS

The age at which specific populations of neurons may be available for cell or tissue culture depends on their regional location in the CNS and the stage of their differentiation. It is customary to use embryonic nervous tissue at a stage when neurons have stopped their proliferation. In mouse cerebrum, this occurs at embryonic d 15–16 (E15–E16). Some populations of neurons, however, such as cerebellar granule neurons, are still dividing postnatally, and therefore provide an excellent source of neurons for cell cultures. Understanding of the developmental timing of neural cells is critical. In establishing neural cell cultures, the task is to maintain viability of the postmitotic neurons, while reducing the population of dividing glia and other nonneuronal cells.

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2.1. Elimination of Dividing Cells

1. Use of antimitotics: A procedure that appears well suited for selection of neurons from a mixed cell culture of either PNS or CNS tissue is the use of antimitotics in the culture medium. Fluorodeoxyuridine (FudR), at concentrations of 10–50 μ*M*, is the most commonly used antimitotic agent. It is incorporated into DNA of cells that are still capable of DNA synthesis, and interferes with cell proliferation and subsequently kills them. Treatment of cells with FudR, coupled with low-density plating (7×10^4 cells/cm^2) on appropriate adhesion substrates, and medium containing trophic factors, is most effective. The use of FudR, in combination with uridine at a concentration of 50 μ*M*, results in the elimination of most nonneuronal cells within a 2-wk period. The presence of uridine in the culture medium prevents the inhibition of RNA synthesis in the nondividing neurons.

   Nonneuronal cell proliferation may be enhanced by the introduction of conditioned medium or specific growth factors (such as fibroblast growth factor) or serum, to produce high rates of DNA synthesis during the incubation in the antimitotic-containing medium, thereby increasing the incorporation of the antimitotic agent into DNA of the dividing cells. To achieve purified neuronal cultures, a 2-d incubation in the antimitotic-containing medium is followed by a 2-d return to regular growth medium. This routine may be repeated several times over the 2-wk period, to purify the neurons of any contaminating glia or fibroblasts. Use of chemically defined medium for purified neuronal cultures should be considered (*see* Chapter 19), since astrocytes and fibroblasts require serum for proliferation.

   Once the antimitotic agent has eliminated the glial cells, the neurons may die, unless appropriate trophic support is provided. Medium conditioned by glial cells is often used as a source of trophic factors. Collagen is used as substrate for PNS neurons and polyornithine or poly-L-lysine for CNS. If neurons are cultured on tissue culture plastic substratum, they may begin to detach from the substratum and die.

   Note: Polymers of the D-isomer form of polyornithine or poly-L-lysine are preferred by some as a substratum, because these are not subject to breakdown by proteases, which cultured cells often release. Poly-L-lysine may be toxic to certain developing neuronal cell populations, i.e., fetal dorsal root ganglion (DRG) neurons.

   It should be recognized, however, that most antimitotics could also be toxic to neurons. For example, cytosine arabinoside (ara-C), at concentrations as low as 10^{-8} *M*, has been shown to be toxic to postmitotic cerebellar granule neurons (Martin et al., 1990; Seil et al., 1992) and to parasympathetic neurons (Banker and Goslin, 1992). It is also implicated in the demise of neurons that are still mitotically active during the first 5 d in aggregate cultures, such as GABAergic neurons (Honneger and Werfflei, 1988).

   Note: ara-C acts on neurons by interfering with deoxycytidine metabolism, a pathway distinct from interference with DNA biosynthesis. Inclusion of deoxycytidine in the growth medium, at concentrations 1000-fold less than that of ara-C prevents this neurotoxicity.

   Usually DRG neurons are maintained in the antimitotic medium for only 2 d to minimize neurotoxic effects of the antimitotic. However, Martin et al. (1990) reported that antimitotics, such as adenine arabinoside, thymidine arabinoside, hydroxyurea, and FudR, can be used at doses of 1 m*M* for up to 5 d, without observable adverse effects on the neurons.

   Purity of neuronal populations in cultures can be assessed by use of cell surface markers, such as anti-Thy-1.1 (anti-Thy-1.2 for BalbC and C57 mice), anti-microtubule associated protein (anti-MAP), anti-Tau, antineuron-specific enolase, or antineurofilament protein, on fixed coverslip samples of the culture. Anti-Thy-1.1 also recognizes fibroblasts, but these can be recognized, compared to neurons, by their spindle-shaped morphology.

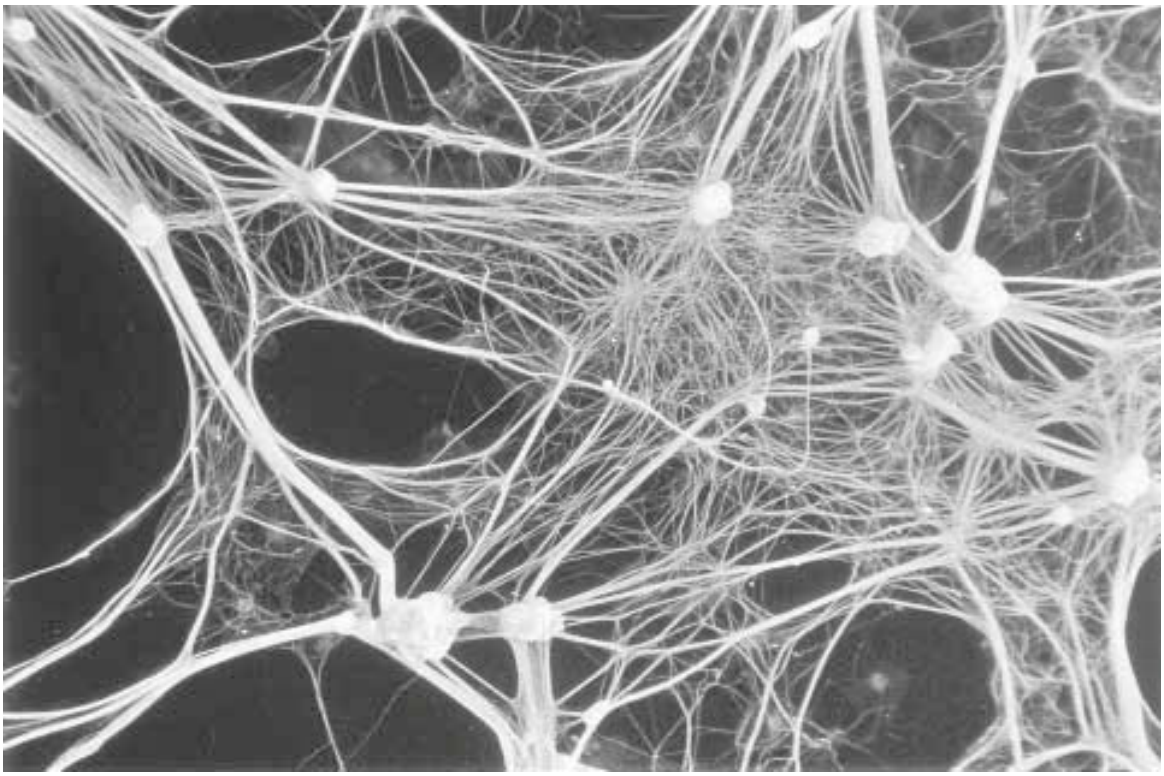

Fig. 1. Fluorescent light micrograph showing extent of neuritic growth and fasciculation between neurites in a purified, 2-wk-old DRG neuronal culture immunostained with antineurofilament protein antibodies.

2. Photo-induced killing of dividing cells: In those instances in which a population of slowly dividing nonneuronal cells remains, it may be necessary to employ a photo-induced killing procedure to remove contaminating glial cells (Shine, 1989).
   a. Incubate cultures for 24 h in the thymidine analog 5-bromodeoxyuridine (5-BUdR) at a concentration of 10^{-5} *M*. This leads to BUdR incorporation into the recently synthesized DNA of the dividing cells.
   b. Then incubate cultures in which the dividing cells have incorporated 5-BUdR into their DNA, for 2–3 h, in the presence of the fluorescent dye, Hoechst 33258, at a concentration of 2.5 μg/mL. This treatment renders the dividing cells with incorporated BUdR uniquely sensitive to light.
   c. Expose cultures in a light box (a modified X-ray box), at room temperature, to bright or fluorescent light for 45 min in 5% CO_2/95% air. It is important that, during the exposure to light, the pH of the culture medium is kept constant, thus increasing the effectiveness of the treatment, as well as the selectivity of the cells that are killed.
   d. After the exposure to light, maintain the neuronal cultures either by incomplete media changes or less regular feeding, to enable the neurons to condition their culture medium and promote cell survival. After such treatment, the neurons will begin to establish an elaborate and extensive network of neurites on the culture substrate (Fig. 1).

      Note: This technique should be used with caution, because 5-BUdR may be toxic to certain types of neurons. Determine the appropriate photo killing conditions for

the culture setup. In exposing the cells to the light source, ensure that the temperature of the cultures does not exceed 37°C.

3. CULTURES OF ASTROGLIA

Pure astroglia cultures may be routinely derived from mixed cell cultures that contain neurons, fibroblasts, oligodendrocytes, and microglia. Astroglia can proliferate in serum-containing medium, and readily grow to confluence. The culture can be subsequently purified through the elimination of the other contaminating cells.

3.1. Elimination of Fibroblasts

Fibroblasts may contaminate astroglia cultures when cultures are started from adult animals and trypsin is used in the isolation process, or when the meninges are not entirely removed from the brain surface prior to disaggregation of the tissue. The fibroblast contamination can be reduced or eliminated by the following procedures:

1. Avoid the use of trypsin in the isolation process and remove all meninges. Plate the cells at high density.
2. Employ complement-mediated cell lysis, using anti-Thy-1.1 antibody to specifically eliminate fibroblasts. Astroglia do not express Thy-1.1 antigen on their surfaces, but fibroblasts do.
3. Fibroblasts can also be eliminated from astroglia cultures by substitution of D-valine for L-valine in the culture medium (Estin and Vernadakis, 1989). Fibroblasts lack D-amino oxidase, and therefore are unable to convert D-valine into L-valine (Cholewinski et al., 1989), and will die. If the culture medium includes serum, the serum must be dialyzed to remove all traces of L-valine, otherwise, fibroblasts will persist.

3.2. Elimination of Microglia

Contamination by microglia may present a problem in long-term astroglia cultures, and becomes readily apparent when astroglia cultures are not fed frequently.

1. Microglia can be eliminated from astroglia cultures by addition of low concentrations of L-leucine methyl ester (1–10 m*M*) to the medium, which preferentially kill the microglia (Theile and Lipsky, 1985). Microglia in L-leucine-containing medium commence the formation of large vacuoles within 15–30 min of incubation, and subsequently die following rupture of the cell membrane.

Note: Astroglia viability and growth can also be affected at higher doses of L-leucine methyl ester (>10 m*M*).

2. Reduction of microglial cell numbers in culture can also be accomplished by preventing the proliferation and differentiation of microglia progenitor cells. These progenitors require the presence of colony stimulating factor-1 (CSF-1) to differentiate into microglia. Astroglia produce and secrete CSF-1 into culture medium (Hao et al., 1990). Therefore, reducing CSF-1 accumulation in the medium, by frequent feeding (i.e., every second day), effectively limits the proliferation and differentiation of microglia progenitor cells.
3. Complement-mediated cell lysis, using antibodies directed against the CR3 surface receptor on microglial cell surfaces, reduces the number of microglia in culture.

3.3. Elimination of Neurons and Oligodendroglia

Possible contaminating cells in astroglia cultures, other than microglia, are neurons and oligodendroglia. Neurons, a minor contaminating cell population, are readily distinguishable in cultures. Oligodendroglia are small, rounded cells, and grow on top of astroglia.

Many laboratories use the preferential adhesion of astroglia to tissue culture plastic substrates as an initial step of purification. Cells that remain attached to the astroglia cell layer may be removed from the cultures by the shaking method (*see* Chapter 9). Oligodendrocytes that remain after the shaking may be preferentially eliminated from cultures by growth in medium containing an increased concentration of serum. If any oligodendrocytes still remain in culture, complement-mediated cell lysis, using, e.g., antibody to cell surface galactocerebroside, in the presence of complement, can be used.

3.4. Selective Medium for Purification of Astroglia Cultures

Astroglia do not require glucose or ketone bodies for respiration, because they possess appropriate enzymes for oxidizing fatty acids (i.e., glycogen phosphorylase, aldose reductase, and sorbitol dehydrogenase). It is possible, therefore, to selectively destroy oligodendrocytes, neurons, and microglia, which require glucose for respiration, by growing cells in a glucose-free medium containing both sorbitol and serum (Wiesinger et al., 1991).

Glial cultures are first established by using glucose- and serum-containing medium. After 2–7 d of incubation, the glucose- and serum-containing medium is changed to glucose-free medium containing 25 m*M* sorbitol and serum. The cultures grown for an additional 14 d in sorbitol-containing medium develop into a homogeneous glial fibrillary acidic protein-positive population of astroglia, free of microglia, oligodendroglia, and neurons (Wiesinger et al., 1991). In glia cultures started in glucose-free medium containing sorbitol, cells do not attach to the substratum, and they die. Therefore, it is important that cultures are first started in glucose-containing medium. Astroglia grown in sorbitol-containing medium, upon re-exposure to glucose, exhibit properties that are not distinguishable from those of cells fed with glucose exclusively (Wiesinger et al., 1991).

Note: It should be stressed that, even in these "pure" cultures, several subpopulations of astroglia may exist (for review, *see* Levison and McCarthy, 1989). The degree of astroglia heterogeneity depends on the age of the animal, the region chosen for the starting tissue, and the cell plating density.

4. CULTURES OF OLIGODENDROGLIA

Starting material for purified oligodendroglia cultures contains neurons and other glia cells. In establishing purified oligodendroglia cultures, astroglia are eliminated by preferential adhesion. The cells that shake off from the astroglia cultures are bipotential glial progenitor cells, oligodendroglia, neurons, and microglia. The cell suspension can be purified before the cells are planted into cultures, using immunoselection. The immunoselection can be directed toward A2B5 antigen to select for retention of oligodendrocyte progenitors, antigalactocerebroside, to select for more mature oligodendrocytes; or CR3 antigen for elimination of microglia (Woodroofe et al., 1995).

4.1. Elimination of Microglia from Oligodendroglia Cultures

If microglia survive in these cultures, a preadherence step can facilitate the elimination of microglia prior to planting the cell suspension into a culture. Microglia are very adhesive cells, and will generally adhere to tissue culture substrata within 30 min, leaving oligodendroglia suspended in the medium. The preadherence step is not always 100% effective.

A CSF-1-free culture environment will control the proliferation of any remaining microglia and their progenitor cells (Section 2.1., step 2; *see* also Chapter 11). If some microglia still remain, then complement-mediated immunocytoxicity to CR3 cell surface antigen will eliminate them.

4.2. Selective Media for Oligodendrocyte Purification

1. In studying oligodendrogliogenesis, it has become obvious that serum is to be avoided in culturing these cells, since serum contains both inducers and repressors of oligodendroglia differentiation. Also, by excluding serum from the medium, neurons, astroglia, and microglia do not survive, leaving cultures that are highly enriched with developing oligodendroglia.
2. Bottenstein et al. (1988) have reported that the addition of medium conditioned by B104 cells dose-dependently selects a population of bipotential glial progenitor cells, whose progeny proliferate and differentiate (*see* also Chapter 12); the proliferation of astroglia is inhibited. However, more morphologically differentiated astroglia develop under these growth conditions.
3. Serum-free medium has been formulated for the selection and maintenance of mature oligodendroglia in long-term (3–4-wk) cultures (Espinosa de los Monteros et al., 1988). It should be noted, however, that when this procedure is used, a small number (<2%) of astroglia may remain.

4.3. Comments

1. In the presence of fetal bovine serum, bipotential glial progenitor cells differentiate in culture into glial fibrillary acidic protein-expressing, process-bearing astroglia.
2. Neuronal contamination is seldom a problem in these cultures, but when it does occur, care should be taken to remove the neuronal cells. When tetanus toxin is used for this purpose, special care must be taken, because immature oligodendrocyte cells contain several gangliosides that can readily bind cholera toxin or weakly bind tetanus toxin, leading to their subsequent demise (Fields, 1985).
3. It is possible to select a population of pro-oligodendroblasts that differentiate into antigenically identifiable oligodendrocyte progenitors and mature oligodendrocytes (Hardy and Reynolds, 1991). Recently, it has been demonstrated that these cultures of pro-oligodendroblasts can also be purified by substituting lactate for glucose in the medium. Growth of cells in the lactate-containing medium, after establishing the cultures in glucose-containing medium, results in a purified population of maturing oligodendrocytes, which grow to cover the surface of the tissue culture flask. Switching to a differentiation medium (*see* Chapter 12) results in the oligodendrocytes expressing myelin basic protein (MBP), galactocerebroside and other oligodendrocytic markers within 1 wk.

5. CULTURES OF MICROGLIA

As stated above, the starting population of mixed cells contains many different cell types that need to be eliminated to obtain purified cultures of microglia. This can be achieved by utilizing the protocols to remove astroglia, oligodendrocytes, and neurons, as previously outlined in this chapter.

5.1. Purification of Microglia

Purification of microglia is accomplished by exploiting the adhesiveness of this cell type in the shaking protocol for the isolation of microglia (*see* Chapter 9) and the immunopanning step previ-

ously outlined. In the initial preadherence step, following removal of surface cells from the underlying astroglia cells, the microglia preferentially adhere to the tissue plastic within the first 30 min of culture. The main problem with this approach is that there are generally low yields of microglia.

5.2. Contamination by Astroglia

Astroglia that contaminate microglial cultures can be effectively eliminated by not feeding the cultures, thereby reducing the amounts of nutrients (Neuhaus and Fedoroff, 1994; *see also* Chapter 11) that astroglia require, and accumulating microglia growth factor CSF-1 in the medium. After 10 d of incubation, astroglia die. At this stage, cultures must be fed, and CSF-1 must be added to the medium, to facilitate microglial proliferation (*see also* Chapter 11).

5.3. Contamination by Macrophages

Circulating macrophages may contaminate isolates of microglia because of the expression of common markers, but it has been suggested that perfusion of the animal prior to the removal of the brain will reduce the number of contaminating macrophages (Woodroofe et al., 1995).

In the case of fetuses, it is advisable to make a cut to the heart, and to adsorb the blood with an absorbent pad before removal of the brain, thus considerably reducing the amount of blood in the brain.

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Chapter Twenty-Six

Quantification of Cells in Culture

Arleen Richardson and Sergey Fedoroff

1. HEMOCYTOMETRY AND DETERMINATION OF NUMBER OF VIABLE CELLS BY DYE EXCLUSION

Cell enumeration using the hemocytometer is applicable when determining the number of cells in a suspension, and when the number of samples to be analyzed is relatively small. Hemocytometry is also useful for determining the proportion of singly dispersed cells in a suspension, and for estimating the frequency of viable cells.

To prepare a cell suspension, it is important to base dilution on the number of viable cells, rather than on the total number of cells. The number of viable cells in any given cell suspension varies, depending on the age and species of the animal from which the cells are isolated, the type of tissue, and the procedure for cell disaggregation. In suspensions prepared from cell cultures, the number of viable cells present depends on whether the cells in the culture were in the logarithmic or stationary phase of growth.

The average error in counting cells, using the hemocytometer, approaches 15–20% but it may be kept as low as 5–8%. Errors inherent in cell enumeration by this method are caused by inadequate suspension of cells, inaccurate dilution, overfilling the hemocytomter chambers, too few or too many cells in the sample to be counted, and inaccurate counting. The optimal number of cells to be counted is 1×10^5 cells/mL.

1.1. Determination of Number of Viable Cells by Dye Exclusion

1. Materials:
   Trypan blue (0.4%) or nigrosin (0.3%).
   Note: Caution should be used with trypan blue, because it is a cancer suspect agent and teratogen.
   Test tube (1).
   Capillary or Pasteur pipet.
   Serological pipets, sterile, 1-mL.
   Improved Neubauer hemocytometer and coverslip.
   Ethanol, 95%.
   Soft lint-free gauze or tissue.

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2. Preparation for counting cells:
   a. Cleaning the hemocytometer:
      i. Using a lint-free gauze or tissue, clean the surfaces of the counting chamber of the hemocytometer and the coverglass with water. Do not scratch the counting surfaces.
      ii. Repeat the cleaning, using 95% ethanol. Completely dry surfaces and coverglass.
   b. Mount the coverslip over the two chambers (Fig. 1).

   Note: To ensure accuracy in the depth of the chamber, it is advisable to moisten the edges of the coverslip, then gently slip the coverslip over the chamber. If the coverslip is properly placed, an interference pattern (rainbow color rings) should appear where the coverslip touches the hemocytometer.

   c. Prepare an aliquot of the cell sample for counting cells:
      i. Add 0.2 mL nigrosin to a test tube.
      ii. Mix the cell suspension thoroughly by triturating, and immediately add 0.8 mL cell suspension to a test tube with nigrosine.

      Note: Smaller amounts can be used, but the proportion of nigrosine to cell suspension must be maintained.

      iii. Mix the contents of the tube by gentle agitation by hand.

      Note: If the cells remain in the dye solution too long, the cells may settle out, or may be injured. Such suspensions will give wrong estimates of viable cell numbers.

      iv. Gently mix the cell suspension with the pipet, and immediately fill a capillary pipet or tip of a Pasteur pipet.
   d. Fill both halves of the counting chamber: Place the tip of the pipet on the edge of the hemocytometer chamber, being careful not to move the coverglass, and allow the cell suspension to fill the chamber by capillary action. The rate of flow can be regulated by placing a finger over the top of the pipet. A micropipet can also be used to fill the chamber (approx 20 μL cell suspension is necessary to fill one chamber). Fill the other half of the chamber.

   Note: This step should be done quickly to avoid settling of the cells in the pipet, which would cause uneven distribution of cells in the hemocytometer.

   Note: Be careful not to overfill the chambers, because this will cause counting errors. If the chamber is filled improperly, clean the hemocytometer and coverglass and repeat the procedure.

3. Counting viable and dead cells: Using a 10× microscope objective, count all unstained cells (viable cells) and all stained cells (nonviable cells) in the four large corner squares in both counting chambers (Fig. 1).

Note: Trypan blue and nigrosin are vital dyes, and they do not penetrate the cell membrane. However, the cell membrane of injured or dead cells is permeable, and the dyes enter the cells and stain them. Therefore, in the dye exclusion test, the viable cells are unstained and nonviable cells are stained.

4. Determining the cell number:
   a. The hemocytometer is divided into nine 1-mm^2 (large) squares (Fig. 1). The height of the chamber is 0.1 mm, and the volume over one large corner square is 0.1 mm^3 (0.0001 mL). Using the 10× microscope objective, count all cells in the four large (1-mm^2) corner squares in both halves of the counting chamber (total of eight large corner squares). Count cells that touch the outer left and upper lines (if the chamber has triple outside lines, count the cells that touch the middle of the three outside lines on these two sides), and disregard those touching the right and lower outer lines.

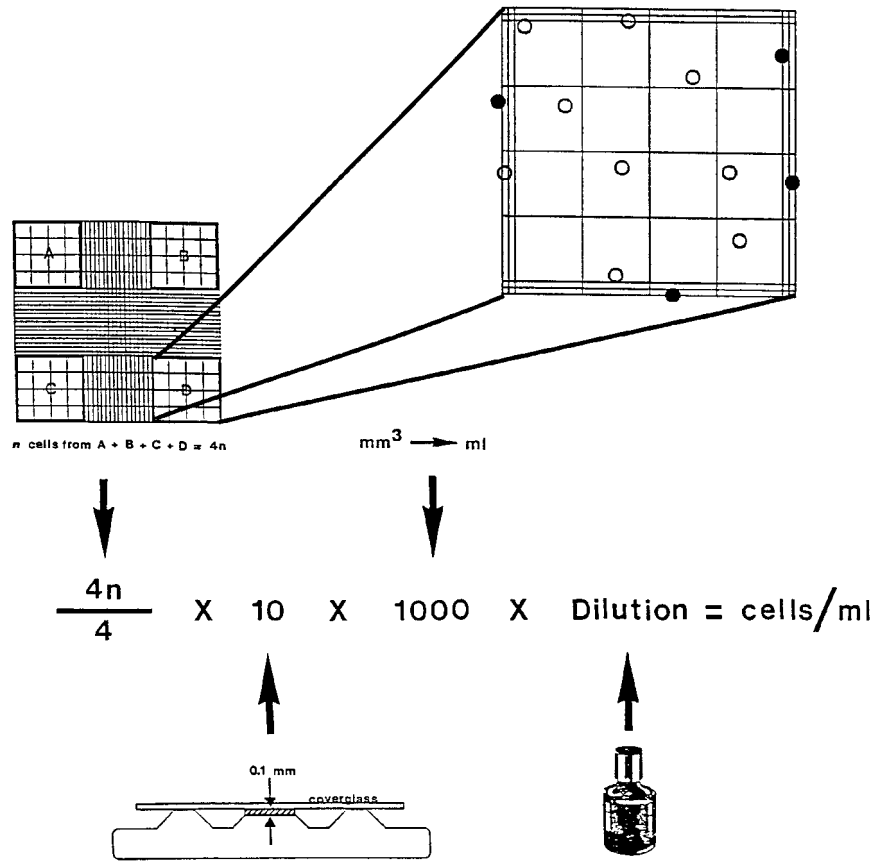

Fig. 1. Calculation of cell number by using the hemocytometer and the dye exclusion method. One large corner square of the hemocytometer is enlarged, to illustrate the counting of cells. Count all cells within the area bounded by the triple lines, and, in addition, count the cells that touch the middle of the outer triple boundary lines on the top and on the left side (open circles). Do not count cells that touch the middle outer boundary lines on the bottom and right side of the square, nor any on the other sides that do not touch the middle outer boundary line (filled circles).

b. If the cell counts in the two chambers differ by more than 20% of the mean, clean and refill the chambers, and repeat the counts.
c. Remove the coverslip, and place both the coverslip and the hemocytometer into a container of distilled water. Clean and dry the hemocytometer and coverslip.

1.2. Calculation of Cell Number

1. Add up the number of viable and nonviable cells in the eight large squares, and divide by eight to obtain the mean number of the cells in one large corner square.
2. Multiply the mean number of cells by 10 to obtain the number of cells in 1 mm^3 (0.001 mL).
3. Multiply by 1000 to convert from 1 mm^3 (0.001 mL) to 1 mL.
4. Finally, to determine the number of cells in 1 mL of the original cell suspension, multiply by the factor of cell dilution used to prepare the cell-dye suspension. In the procedure described, the dilution factor is 10:8.

5. Equation for calculating total number of cells/mL:

$$\frac{\text{Number of cells in 8 squares}}{8} \times 10 \times 100 \times \frac{10}{8} = \text{Total number of cells / mL}$$

1.3. Calculation of Frequency of Viable Cells

1. The percentage of viable cells in suspension is a good indicator of the effect of treatment on the viability of cells and variability in the procedures used, and is useful in standardizing procedures.
2. Equation for calculating frequency of viable cells:

$$\%\ \text{of viable cells} = \frac{\text{viable cells counted}}{\text{total cells counted}} \times 100$$

2. ELECTRONIC CELL COUNTER

Counters operating on the gating principle, introduced by Coulter in 1956, have an aperture through which electric current flows. Cells suspended in an electrolyte solution are drawn in a measured amount by vacuum through the aperture. When a cell passes through the aperture, the flow of current through the aperture changes because of increased resistance, causing a voltage pulse. This voltage pulse is directly proportional to the volume (size) of the cell. Therefore, when cells pass through the aperture, there is a voltage pulse for each cell. The pulses are recorded on a counter, and the relationship between the number and the size of the cells can be visualized on the oscilloscope.

Interpretation of oscilloscope patterns can be of valuable assistance in setting up the counter to determine aperture current and amplification settings, to detect blockage of the aperture, and to get an overview of distribution of the cells in the sample. An upper and lower threshold can be set in such a way that no particles smaller than the lower threshold settings, and no particles larger than the upper threshold settings, are counted. This excludes counting cell debris or cell clumps.

The electronic cell counter is particularly useful when a large number of samples must be counted. The procedure is fast, the counts are reproducible, and, when operated properly, yield single counts with an error of no more than 10% and multiple counts with an error as low as 1–2%. As mentioned, in addition, counters are capable of providing information about the size of cells. For accurate cell enumeration, optimum instrument settings should be determined for each cell type, the aperture should be examined for blockage, the cell suspension should be examined for cell debris and cell aggregates that may be indiscriminately counted, and sampling errors, resulting from improper mixing, should be eliminated. Most electronic cell counters cannot distinguish between viable and nonviable cells, and therefore the percentage of viable cells is best estimated using the dye exclusion technique and hemocytometry.

3. COUNTING CELL NUCLEI IN CULTURES

It is often of great advantage to be able to estimate the number of cells in a culture, especially to determine how many cells are present in a colony or a clone in colony cultures. The Hoechst 33258 fluorescent stain (Sigma, cat. no. B 1155) is a benzimidazole derivative that intercalates in the A-T region of DNA, and makes cell nuclei visible. Staining cells with Hoechst 33258 is rapid, easy, and gives sensitivity of 10 ng/mL of intact double-stranded DNA. An additional advantage is that the Hoechst 33258 fluorochrome is readily distinguishable from fluorescein and rhodamine. There-

fore, the cells can be double- or triple-immunostained, in addition to the Hoechst staining, thereby allowing determination of the frequency of different cell types in the culture. Hoechst 33258 exhibits fluorescent excitation at 350–365 nm, and emission over 450 nm. If the cultures are densely populated, it is possible to count only predetermined areas in the culture, then to extrapolate the number obtained to the whole culture.

Hoechst 33258 can also be used to detect contamination in cultures. In noncontaminated cultures, only cell nuclei are stained. Cells infected with mycoplasma have punctate or filamentous staining in the cytoplasm or adsorbed to the cell membrane. Bacteria and fungi also stain brightly, and are easy to recognize. Care should be taken that cell nuclear debris are not confused with the contaminating organisms.

3.1. Procedure

The dye is nontoxic to cells, except in the presence of light, when the cells have incorporated 5-bromodeoxyuridine (*see* Chapter 25).

1. Materials:
   Hoechst 33258 working solution of 1 μg/mL.
   Dulbecco's phosphate-buffered saline (DPBS) (Gibco-BRL, cat. No. 21600-010).
2. Procedure:
   a. Staining with Hoechst 33258 dye can be done alone or in conjunction with other immunocytochemical procedures that use probes labeled with fluorescein, rhodamine, or Texas red. When used in conjunction with other immunostains, use Hoechst staining as the last step.
   b. Stain cells with Hoechst 33258 dye for 5 min at room temperature.
   c. Wash cells twice with DPBS for 5 min.
   d. Mount and observe cultures with the fluorescent microscope, equipped with excitation filter 350–365 nm and emission filter 450 nm.
3. Solutions:
   a. Hoechst 33258 stock solution.

   Note: Hoechst 33258 is toxic. Wear rubber gloves, and make up solution under a fume hood.

      i. Dissolve 1 mg Hoechst 33258 in 10 mL DPBS. This stock solution of 100 μg/mL is 100× more concentrated than the working solution.
      ii. The bottle of stock solution should be wrapped in aluminum foil and stored in the dark at 0–5°C. The solution is stable for approx 2 wk under these conditions. The stock solution may also be frozen in aliquots at –20°C. At this temperature, the stock may be stored indefinitely.

   b. Hoechst 33258 working solution: Dilute 0.1 mL Hoechst 33258 stock with 9.9 mL DPBS. This gives a working solution of 1 μg/mL.

4. GROWTH ASSAYS

The following growth assay is rapid, sensitive, reproducible, avoids the use of enzymes, and makes possible the processing of large numbers of cultures. A known number of cells (as a single-cell suspension) are plated in multiwell culture plates, and the agent to be tested is added in serial dilutions. The effects on growth or cytotoxicity effects can be evaluated by direct determinations in cell cultures, and values obtained are read from standard curves. Precautions should be taken to avoid increased evaporation in edge wells in multiwell plates during incubation (*see* Chapter 20).

4.1. [^{3}H]-Thymidine Incorporation Assay

Tritiated thymidine is incorporated into cells that are synthesizing DNA during the S phase of the cell cycle. This assay therefore measures the degree of DNA synthesis that has occurred during the time the cells have been incubated with tritiated thymidine. The assay does not determine the number of cells present in the culture at the end of the experiment.

1. Materials:
   96-well plate containing cells at a suitable stage of growth.
   [^{3}H]-thymidine solution.
   Scintillation solution.
   Scintillation vials.
   Multichannel pipet.
   Absorbent glass-filter paper.
   Cell harvester.
2. Procedure:
   a. Add 20 µL [^{3}H]-thymidine solution to each well of the multiwell plate, using a multichannel pipet.
   b. Incubate the multiwell plate at 37°C for 4 h.
   c. Harvesting of cells:
      i. Nonadherent cells: Nonadherent cells may be harvested directly onto absorbent glass-filter paper, using a cell harvester.
      ii. Adherent cells: Add 100 µL 0.1% Triton X-100 to each well of the culture plate, gently agitate the multiwell plate for 2 min, and harvest onto absorbent glass-filter paper, using a cell harvester.
   d. Dry the absorbent glass-filter paper containing the cells for 30 min in a drying oven (or air-dry overnight).
   e. Cut circles containing the cells from the glass-filter paper. Place each circle of glass-filter paper into a scintillation vial.
   f. Add 1 mL liquid scintillation solution.
   g. Measure the radioactivity of each sample by using a scintillation counter.
3. [^{3}H]-thymidine solution:
   a. Stock solution: 1 µCi [^{3}H]-thymidine (New England Nuclear, Boston, MA) with a specific activity of 20 Ci/mmol.
   b. Dilute stock solution 1:19 in culture medium for use.
      Note: It may be necessary to increase the amount of [^{3}H]-thymidine when using medium that contains thymidine as a nutrient.

5. OTHER ASSAYS

This chapter describes four ways to enumerate cell numbers in culture: hemocytometry, electron cell counting, fluorescent staining of cells in cultures, and [^{3}H]-thymidine incorporation. Additional assays are described in this volume. Chapter 15 describes the use of aggregate neural cell cultures in biochemical and molecular biological assays; Chapter 20 deals with the use of multiple well plates in biological assays; Chapter 21 deals with cell number determination in microplate culture, and Chapter 23, the use of colony cultures for determination of plating efficiency.

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Chapter Twenty-Seven

Tips for Tissue Culture

Arleen Richardson and Sergey Fedoroff

1. SUBCULTURING CELLS

Cells attach to the culture substratum with varying degrees of adherence (*see* Chapter 18). Cells that must attach to the substratum are referred to as "adhering cells" (anchorage-dependent cells). Cells that do not adhere and grow as cell suspensions in the medium are referred to as" nonadhering cells" (nonanchorage-dependent cells).

When cells proliferate in culture, they eventually completely populate the culture vessel and must be subcultured (passaged). To keep cells growing logarithmically, it is important to know how the culture should be split, i.e., how many new culture flasks can be initiated from a culture that has become confluent. Slow-growing cell cultures are split twice, i.e., cells from one confluent culture are passed to two new cultures. Rapidly growing cell cultures may require splitting as many as 6–8×. It is convenient to arrange cell subculturing for every 7–10 d, and to feed cultures every 3–4 d. When cells are sensitive to subculturing, it may be necessary to combine several flasks of cultures, which ensures that the new culture begins with a sufficient number of cells to make it viable.

It is easy to subculture cells of nonadhering cell types, because they grow in suspension. An aliquot containing a defined number of viable cells may simply be used to inoculate fresh flasks containing medium. Subculture of adherent cells requires a number of strategies to remove the cells from the substratum with the least damage, and to prepare a cell suspension for subculturing into fresh culture vessels. Two approaches are used: mechanical, or chemical separation from the substratum.

1.1. Mechanical Separation

1. Shaking: When the culture contains only loosely adherent cells, a portion of the medium is removed, and the cells in the small volume of remaining medium are dislodged from the substratum by sharply rapping the culture vessel against the hand, or by using a mechanical shaker. Creation of air bubbles or frothing of the medium should be avoided.
2. Washing: When the cells are not firmly attached to the substratum, they may be removed by repeated washing of the culture with medium flushed from a Pasteur pipet. Washing should be started at the top of the flask, proceeding toward the base, holding the flask at a slant. The creation of air bubbles or frothing of the medium should be avoided.

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3. Scraping: A soft rubber policeman may be used to remove more adherent cells by gentle scraping. The most efficient type of rubber policeman has the end sealed flat and cut on an angle. A rubber policeman can be made, using a short piece of tissue culture-grade tubing (approx $1^1/_2$–$2^1/_2$ cm long), with half of the tubing removed lengthwise. This piece is then inserted into the short bend of a glass rod bent at a 45° angle. Such a device is particularly useful for scraping the cells off the flask's surface; it may be autoclaved, and can be made to any length and degree of bend.

   A number of disposable cell scrapers and lifters are available commercially. Cell scrapers usually have blades that swivel and variable length handles, angled for easy access into flasks (Falcon, Corning-Costar). The lifters are designed for use on cells grown in dishes (Corning-Costar). The wing-type rubber policeman can also be used, but it is difficult to insert into the neck of a small flask.

1.2. Chemical Separation

1. EDTA (Versene): The disodium salt of ethylenediamine tetraacetic acid (EDTA), or versene, in a Ca^{2+}- and Mg^{2+}-free medium, may be used to disaggregate cells by chelating calcium ions. This procedure is often used in conjunction with enzymatic digestion. It should not be used with enzymes that require calcium for activation, such as collagenase, dispase, pronase, and deoxyribonuclease.

   Cultures should be washed with Ca^{2+}- and Mg^{2+}- free basic salt solution (BSS), such as Puck's BSS, before use of EDTA. Otherwise, EDTA in the medium will be quickly neutralized by the high concentration of divalent ions present in the growth medium. The lowest possible effective concentration of EDTA should be used.

   When used together with trypsin, EDTA reduces the trypsin activity (to about 80%). However, the combination of EDTA and trypsin reduces the aggressive nature of trypsin, and may improve the viability of the released cells.
2. Enzymes: Enzymes are used to digest the extracellular matrix proteins and the proteins of cell junctions responsible for cell adhesion to each other, and to the substratum. Trypsin is most commonly used for this purpose, because it is active within the physiological pH range, and can easily be inactivated.

   Crude trypsin contains a number of other enzymes: proteases, polysaccharidases, nucleases, and lipases, as well as impurities. It is very effective in releasing cells from the intercellular matrix of the tissues. However, batches of trypsin vary greatly in activity and amount and type of impurities. Crude trypsin may be cytotoxic. Therefore, each batch must be pretested before it is acceptable for use in a laboratory. In contrast, crystalline trypsin has no impurities and has less variability, but it is not as efficient in disaggregating cells in tissues, and is more expensive. It can be used effectively in combination with elastase, hyaluronidase, pronase, and/or collagenase. Crystalline trypsin very effectively releases cells from the substrate in continuous layer cultures.

   Generally, enzymes are supplied in a lyophilized powder form in sealed ampules, and are stored at 2–8°C. The ampules must to be brought to room temperature before opening. Open vials must be stored in desiccators, because the powder is very hydrophilic. Proteolytic enzymes diluted in medium or phosphate-buffered saline (PBS) are unstable and should be used as soon as possible, or frozen at –20°C. Trypsin does not dissolve well in PBS. It should first be reconstituted in 0.001 *N* HCl, then added to the medium or PBS. To minimize damage to the cells, the pH of the trypsin solution must be adjusted by gassing with CO_2 /air or O_2 mixture. Crude trypsin may contain viruses, mycoplasma, and various bacteria, and

therefore must be sterilized by filtration through 0.22-μm pore size membranes. Even though the trypsin may be sterile, it may contain variable amounts of endotoxins, which is good reason for using crystalline trypsin when possible.

Cells grown in a serum-containing medium should first be rinsed with a BSS to remove all traces of serum, before the addition of the enzyme. A Ca^{2+}- and Mg^{2+}-free BSS, such as Puck's BSS, is used for this step, when trypsin is used. Other enzymes, such as collagenase, dispase, and pronase, require the presence of calcium for their activity, and should be rinsed with a BSS containing calcium and magnesium, e.g., Hanks' basic salt solution (HBSS).

Commonly, crude trypsin is used to disaggregate cells in cultures at concentrations of 0.025–0.05 (w/v), and crystalline trypsin at concentrations of 0.01–0.05 (w/v). A more precise way is to to use the enzymes on an activity basis, i.e., U/mL.

Note: In general, the smallest possible volume (activity) of enzyme and the shortest possible exposure time should be used to disaggregate cells.

The most effective way to determine the end point of trypsinization is by observing the culture under the inverted phase-contrast microscope. At the point when the cells round up and begin to come off the substrate, enzyme activity should be inhibited. When possible, trypsinization should be done at 4°C. At that temperature, trypsin does not enter the cell, and the viability of cells is improved.

Trypsin activity can be inhibited by adding an equal volume of medium containing 5–10% serum to the cell–trypsin solution. In serum-free medium and medium low in serum, soybean trypsin inhibitor (1 mg/mL) may be added to the medium to neutralize the trypsin. Excess of the antitryptic agent should be avoided, because it is slightly inhibitory to cell growth. Crude trypsin contains a variety of enzymes, and the soybean trypsin inhibitor does not neutralize all the enzymes. Therefore, either crystalline trypsin should be used in this procedure, or, after trypsinization with crude trypsin, the cells should be centrifuged, washed, and resuspended.

To further disaggregate cell clumps following incubation with proteolytic enzyme, the cell suspension is triturated. This is a very crucial step in cell disaggregation. It has been recommended that a 10-mL pipet be filled and emptied at a rate of about 3.0 mL/s, without causing bubbles to form in the cell suspension.

Note: To avoid bubbles when triturating, do not pull the tip of the pipet out of the medium. Fill and empty the pipet while it is submerged in the cell suspension.

Enzymes other than trypsin may be used for disaggregation of cells from intercellular matrix or cell culture substrate. Enzymes, such as pronase, dispase, and collagenase, or a combination of the two, may be used. These enzymes require the activation of calcium and are not, or are only partially, neutralized by serum. Therefore, these enzymes should be removed by centrifugation.

2. STERILITY CONTROL

2.1. Laboratory Environment

The tissue culture laboratory may be a single room designated for cell culture, or may simply be space in a larger multipurpose room. It is usually defined by the presence of a laminar flow cabinet and an incubator. A laminar flow hood is advantageous for maintaining a sterile environment for preparation of cultures, and provides personnel and/or product protection. However, a laminar flow hood is not an absolute requirement for tissue culture. A desk top that is clean and in a dust-free environment can be used for tissue culture purposes, if aseptic techniques are meticulously followed.

It is important to be familiar with the environment of the room in terms of air flow, traffic patterns, and possible sources of contamination. The placement of bacteriological plates throughout, the room and at different times of day and night, can aid in determining the changes needed to optimize the area for cell culture. The plates should be exposed for 30 min–1 h, then incubated as usual. Bacterial (sheep blood agar, tryptic soy agar, and so on) and fungal (Saboraud dextrose agar and others) plates are placed in front of all air vents and around doors entering the room. Plates are also placed on and around major equipment in the room (centrifuges, refrigerators, ice machines, and the like), on and around the laminar flow hood and incubator, and areas of traffic (including the floor) that are near the working space. The growth of bacteria or fungi on the plates will pinpoint problem areas, and appropriate steps can then be taken to eliminate or minimize the sources of contamination.

2.2. Autoclaving

Autoclaves are effective sterilizers only if materials to be autoclaved are properly wrapped and placed in such a way that the steam has access to them. The autoclave should not be overloaded. The autoclave must be operating properly, maintaining both temperature and pressure (121°C, 15 psi).

To ensure that sterilization has occurred, commercial indicators can be used. The best indicators of sterilization take into account the parameters of time, temperature, and steam penetration. Indicators, such as Steriolmeter-plus® (VWR CanLab, Mississauga, ON, Canada), can be placed in the center of a pack, inside capped tubes, and so on, and only when the temperature is reached (121°C), and 12–16 min have elapsed, do the indicators change color. It should be noted that the end point for most indicators is variable, and that the minimum autoclaving time is 15 min.

Autoclave tapes are available to attach to the outside of packs. They change color during autoclaving: Most do not indicate that the product is sterilized, but simply indicate that items have been exposed to autoclaving.

Nontoxic materials should be used to wrap items to be autoclaved. There are many commercially available wrappers (Dualpeel® Tubing, Baxter Healthcare, IL; SteriLine® white paper bags, Thomas Scientific, Swedesboro, NJ) designed for use in autoclaves. In addition, unbleached cotton muslin may be used for bagging large glassware, and envelopes of various sizes can be constructed from 27-lb uncoated vegetable parchment paper (available from restaurant supply companies).

Steam used for sterilization in autoclaves may contain many impurities, including the contaminants present in the feedwater and additives that prevent scale formation and corrosion. These contaminants are deposited on the sterilized material, which then may be dissolved in solutions used in cell culture. The addition of a steam filter (Balston Inc., Lexington, MA), placed as close to the steam inlet as possible, eliminates impurities in the steam, before it enters the autoclave. The steam filters should be changed on a regular basis.

2.3. Medium and Other Cell Reagents

As an integral part of a quality control program, it is important to monitor the sterility of media and other cell reagents. Media may be tested for bacteria and fungi by inoculating fluid thioglycolate broth, trypticase soy broth, Sabouraud dextrose broth, and blood agar plates. A bacteria and fungi detection kit may be purchased from the American Type Culture Collection (ATCC), Manassas, VA.

To ensure sterility of large volumes of media, it is important that adequate sampling is done. In our laboratory, a sample of the medium is removed at the beginning and end of filtration, and, in addition, a 25-mL sample is removed after filtering every liter of medium. All bottles of filtered

medium and samples are numbered in sequence, so that problems that occurred during filtration can be readily identified. These samples are incubated at room temperature, until the entire batch of medium is used. Any samples that show signs of microbial contamination are matched to the numbers on medium bottles. Those bottles of medium are discarded.

Media are also tested for growth-promoting qualities on primary cultures or cell lines that are routinely used in the laboratory. In addition, media can also be tested using a plating efficiency assay (*see* Chapter 23).

2.4. Incubators

1. Carbon dioxide: For control of pH during culturing, incubators are used in which the amount of CO_2 in the atmosphere can be controlled. For proper pH control, the CO_2 concentration in the incubator should correspond to the concentration of the bicarbonate buffer in the medium. Purchased media with specified concentrations of bicarbonate buffer require specified concentrations of CO_2. For example, Eagle's Minimum Essential Medium (EMEM) with HBSS does not require additional CO_2 in air. Such a medium is designed for a regular atmospheric environment. MEM with Earle's salts requires an atmosphere of 5% CO_2 in air and Dulbecco's modified Eagle's medium requires an atmosphere containing 10% CO_2.

   The gas mixtures fed into the incubator from pressurized tanks may contain impurities that may be cytotoxic. Therefore, an in-line hydrophobic filter should always be inserted between the gas cylinder and the incubator.
2. Cleaning incubators: Incubators that have high humidity and operate at 37°C provide an excellent environment for growth of molds and yeasts. Therefore, incubators should be cleaned on a regular basis (every 2–3 mo), or at the first sign of mold or yeast contamination. We use the following method to clean our incubators:
   a. The incubator is turned off and unplugged.
   b. All removable parts (shelves, and so on) are taken out, washed, rinsed with good-quality water, and autoclaved.
   c. The incubator is dried with lint-free towels, and CO_2 sensors are covered with plastic. The incubator is then sprayed with a good-quality disinfectant (not bleach) (e.g., Super Bacterole, Magic White Western, SK), paying particular attention to corners, seam lines, and so on. The incubator is closed and left for approx 2 h.
   d. The incubator is rinsed with distilled water until all traces of the disinfectant are removed. It is then dried with lint-free towels.
   e. The incubator is sprayed with 70% ethanol, again paying particular attention to corners and seam lines. A pan of 70% ethanol is placed in the incubator. The incubator is closed and left for approx 24 h.
   f. After 24 h, the 70% ethanol is removed, all surfaces are dried, and the incubator is reassembled.

3. BIOLOGICAL CONTAMINATION

3.1. Cell Lines

In laboratories that carry several cell lines, the risk of cross-contamination among cell lines is very high. "Thus, only one cell line should be permitted inside a safety cabinet at any one time, while separate supplies of culture medium, and so forth, should be dedicated to each cell line." (MacLeod et al., 1999).

To safeguard against cell line cross-contamination, the laboratory should have a record of the DNA profile and cytogenetic analysis of each cell line. When any change in the cell line behavior

is observed, authentication of the cell line is mandatory. Cell line authentication can be done by a cell repository, such as ATCC, or by commercial organizations specializing in cell line characterization (e.g., BioReliance, Rockville, MD; Stirling, Scotland; Heidelberg, Germany; and Tokyo, Japan).

3.2. Bacterial Contamination

Bacterial infection in cultures is manifested by cell debris, a decrease in pH of the medium, and turbidity of the medium. Bacteria in a culture can be seen under the microscope at high power. A low level of bacterial contamination or slow-growing bacteria may go undetected; however, the cells in the culture will exhibit slowed and uncharacteristic growth.

Cultures containing bacteria, as well as any reagents used with those cultures, must be autoclaved and discarded. In exceptional cases, an attempt can be made to save irreplaceable cultures by extensive washing, treatment with high concentrations of antibiotics, and subculturing the cells into as many cultures as possible, to enhance the probability that some will be free of contamination. If the use of antibiotics is unacceptable, extensive washing (10× or more) of the culture and subculturing into 96-well plates may produce some cultures free from bacterial contamination.

1. Procedure for decontamination:
   a. Prepare a solution of antibiotics in HBSS. We use a highly concentrated penicillin and streptomycin mixture, which has a broad bactericidal spectrum against Gram-positive and Gram-negative bacteria. The solution is made up at 50× the recommended working concentration.
   b. Wash the culture with HBSS at least 5×, or more if heavily infected.
   c. Treat with the antibiotic solution for 1–3 h. Examine the cultures about every 20 min. As soon as the morphology of the cells begins to change (round up), remove the antibiotic solution, and replace with fresh medium.

   Note: The antibiotics at 50× the recommended concentration can be cytotoxic to cells; therefore, it is important to remove the solution as soon as the cells show any sign of change in normal morphology.

   d. Incubate cultures (with no antibiotics) for 24 h at 37°C.
   e. Examine the cultures for the presence of bacteria. If bacteria are present, the cultures may either be treated with gentamycin (50 μg/mL) or may be washed, and split into many small cultures, using 96-well plates.
2. Solutions:
   a. Penicillin and streptomycin solution: The recommended concentrations of antibiotics for prophylactic use in cell culture are 100 U/mL penicillin G and 100 μg/mL streptomycin. However, the concentration of antibiotics tolerated by cells will vary according to the cell type. The high concentrations of antibiotics in HBSS that we use for decontamination are 5000 U/mL penicillin G and 5000 μg/mL streptomycin.
   b. Gentamycin sulfate: Gentamycin is a broad-spectrum antibiotic widely used to treat persistent contaminations. It is bactericidal in vitro, and is effective against Gram-negative and some Gram-positive bacteria, some penicillin-resistant strains of bacteria, and some mycoplasmas. The effective prophylactic concentration is 50 μg/mL.
   c. HBSS:

Component	g/L
NaCl	8.0
KCl	0.4
$Na_2HPO_4 \cdot 2H_2O$	0.06

KH_2PO_4	0.06
$CaCl_2$	0.14
$MgSO_4 \cdot 7H_2O$	0.2
$NaHCO_3$	0.35
Phenol red	0.02
Glucose	1.0 g/L

3.3. Fungal Contamination

Common fungi in cultures grow as single cells, such as yeast, or as multicellular mycelium (molds). Mold infections of cultures may be unnoticed until they have grown large enough to be readily apparent (many long, intertwined, branching filaments in one section of the culture dish). A change in pH and turbidity of the medium may indicate the presence of yeast. Yeast is usually noticed first when the culture is examined under the microscope. Yeast appears as refractile ovals in chains or branches, or shows evidence of budding, and is usually found throughout the culture. The common sources of fungal contamination in the tissue culture laboratory are humidified incubators, cardboard, and the tissue culture personnel.

Cultures containing fungi must be autoclaved and discarded. In addition, the incubators in which the cultures were grown must be thoroughly cleaned and disinfected, and all reagents used with the contaminated cultures discarded.

1. Antifungal agents commonly used in tissue culture include amphotericin B (Fungizone®) and nystatin (Mycostatin®). The prophylactic concentration of amphotericin B is 2.5 μg/mL, and that of nystatin is 50 μg/mL or 100 U/mL.
2. Amphotericin B, depending on the concentration used and the susceptibility of the fungal contamination, is either fungistatic or fungicidal. Nystatin is fungistatic. Therefore, if these antibiotics are used prophylactically, they should be used continuously throughout the experiment, because removal of the antibiotic may allow any fungi present to proliferate.

3.4. Mycoplasmal Contamination

1. Mycoplasmas have many effects on cell cultures, e.g., changes in growth rate and metabolism of cells, induction of chromosomal aberrations, depletion of arginine in culture medium, and so on, but are difficult to detect. The major sources of mycoplasma in the tissue culture laboratory are cross-contamination from infected cell cultures, serum and nonautoclavable medium additives, primary tissue (gastrointestinal and respiratory tract), and laboratory personnel.
2. Since contamination of cell cultures by mycoplasma is difficult to detect, periodic screening of cultures is recommended. Although many screening methods are available (*see* ATCC Quality Control Methods for Cell Lines, 1992), it is usually more accurate, less time-consuming, and therefore cheaper to send samples to a laboratory that specializes in testing for mycoplasma contamination (ATCC; Bioreliance, Rockville, MD).
3. Cultures infected with mycoplasmas, and all reagents used with them, must be autoclaved and discarded.
   Note: Mycoplasmas are not removed by conventional 0.22 μm porosity filters.

3.5. Endotoxin Contamination

Endotoxins (lipopolysaccharides) from the membranes of Gram-negative bacteria are common contaminants in tissue culture. They have many and varied effects on cells, even when present in

nanogram quantities. Endotoxins are released during the growth of bacteria, when the membranes are sloughed off, or when the bacteria die and lyse. Bacterial contamination in the water purification system, or during preparation of culture media, will result in contamination with endotoxins, even if the bacteria are removed by filtration. Water is the major source of endotoxin contamination, but endotoxins are also present in sera and other biological materials, such as soybean trypsin inhibitor, bovine serum albumin, growth factors, and so on.

1. The *Limulus* Amebocyte Lysate test is recommended for the quantification of endotoxin in water, and for monitoring levels in reagents and in tissue culture media and additives. Kinetic turbidimetric and gel clot method kits are available (Associates of Cape Cod, MA), both of which are quick and easy to use.
2. It is difficult to remove endotoxins from serum or other complex cell culture solutions. Use of such methods as ultrafiltration, charcoal and solvent extraction results in protein denaturation and the loss of serum quality. Recently, solid-phase, endotoxin-adsorbing reagents (Acticlean Etox, Sterogene Bioseparations, Carlsbad, CA), which have high specific affinity for endotoxins, have been developed. The reagents remove endotoxin from serum and other biological solutions, without loss of activity in tissue culture.
3. Serum used in tissue culture medium should be selected for its low endotoxin level.

3.6. Use of Antibiotics

Good aseptic technique is usually superior to the use of antibiotics in tissue culture, but in certain cases the prophylactic use of antibiotics is necessary. Antibiotics should be used when the starting material for primary cultures is not sterile, e.g., tissue obtained from the abattoir, human tissue obtained during surgery or postmortem, and tissue from nasal passages, gastrointestinal and urogenital tract, and skin. In such cases, the tissue should be bathed in a solution containing a high concentration of antibiotics, before initiating cultures. Antibiotics can also be used in the cultures, but for as short a time as possible, and not more than 1 or 2 d. Antibiotics can also be used prophylactically, when cultures are to be terminated at the end of an experiment. In exceptional cases, antibiotics may be used to decontaminate irreplaceable cultures *(see above*).

4. WATER

The importance of high-quality water in cell culture cannot be overemphasized. High-quality water is essential not only as a solvent for culture medium and reagents, but also as the final rinsing step used in the preparation of glassware for tissue culture. Water quality is especially critical when cells are grown in serum-free medium.

4.1. Water Purification

Water can be purified by distillation, ion exchange (deionization), carbon adsorption, microporous membrane filters, ultrafiltration, and reverse osmosis. Each of these procedures has certain advantages and disadvantages, but no one procedure by itself is satisfactory for obtaining water of a quality high enough for tissue culture purposes. The solution is to combine a number of the procedures in sequence. Systems are available that use a number of replaceable cartridges for carbon adsorption, ion exchange, ultrafiltration, and, finally, a 0.22-μm porosity filter. Such a system can remove, by ultrafiltration, particles with a mol wt of more than 10 kDa. Most endotoxins (lipopolysaccharides) are larger than 20 kDa, and therefore are removed by ultrafiltration. Sometimes, it is necessary to prepurify the water, before it is fed into the system, to extend the life of the cartridges.

Using equipment composed of several purification steps, it is possible to obtain large amounts of ultrapure, type I reagent-grade water, as defined by the College of American Pathologists, the American Society for Testing and Materials, and the National Committee for Clinical Laboratory Standards. Water acceptable for tissue culture use must be free of endotoxins and organic contaminants, be essentially ion free, and have a resistivity close to 18 MΩ/cm at 25°C (resistivity of chemically pure water has been calculated to be 18.3 MΩ/cm).

The water content of inorganic and ionizable solids, organic contaminants, and particulate matter varies from one geographical region to another, and, therefore, the requirements for purification of water may vary. The best way to determine the quality of the water is to send a sample of the water for analysis to Barnstead/Thermolyne, IA, or to Millipore, MA, or to analyze the quality of the water by a resistivity monitor (purity meter), and by high performance liquid chromatography. The water purification system to be used depends greatly on the amount of water required and the geographical location.

1. Ultrapure water should be used within a day of its production, and should not be stored longer, since the highly purified water has the capability of leaching out impurities from the storage container (Pyrex™ or plastic). These contaminants then become part of the medium and tissue culture solutions, or are deposited on glassware.
2. All water purification systems require monitoring, proper maintenance, and cleaning, to ensure continued water quality. All tubing in contact with the water purification system should be changed frequently, to prevent microbial and algae growth. Bacterial growth can occur in resin beds, storage vessels, distillation apparatus, and so on, and, therefore, maintenance should be carried out on a regular basis.

5. VISIBLE FLUORESCENT LIGHT

Visible fluorescent light is detrimental to media, sera, and cells in culture, because of the generation of free radicals in the media. Light affects tryptophan and tyrosine, with riboflavin acting as a photosensitizer. Wavelengths up to 450 nm are responsible for the deleterious effects, and, to eliminate them, certain precautions must be taken.

1. Fluorescent tubes in the light fixtures in the laminar flow hoods and in the tissue culture room can be replaced by yellow fluorescent tubes (Westinghouse 40 Watt, F 4OGO Gold; General Electric Gold). This will reduce the formation of free radicals in media.
2. Sera, especially fetal bovine and calf serum, which have low levels of catalase activity, should be stored in the dark and protected from light by the use of yellow plastic bags.
3. HEPES-containing media are particularly sensitive to the effects of visible fluorescent light. Bottles containing media with HEPES as a buffering agent should always be wrapped in aluminum foil.
4. As a rule, all media should be stored, and cultures grown, in the dark. Incubators with glass doors should have the glass covered, to prevent any light from entering the incubator.

6. DISSECTING INSTRUMENTS

The quality of dissecting instruments depends on whether they are made of stainless steel, alloy, or titanium. Good instruments are corrosion-resistant, nonmagnetic, heat-resistant (e.g., titanium resists temperatures up to 440°C, and Dumostar alloy up to 550°C), stainless, and of adequate hardness.

Most importantly, forceps must have fine tips. The tips can be examined under 40× magnification, and should close flatly. The greater the distance the tips are closed flat, the better the forceps.

The tips of the best forceps close flat together for about 3 mm, and do not open under excess pressure.

Note: Dissecting instruments are precision-made, expensive, and require special care. They should be the responsibility of the individual, and should not be given to others to use. Instruments should be used only for the purposes for which they are designed.

Proper cleaning of the instruments is important. After the instruments have been used, they should be put in a tray lined with soft material and soaked in distilled water, then the instruments should be cleaned carefully, one at a time, with gauze, clean cloth, or a soft brush, to remove any tissue remnants or blood. A very dilute nontoxic soap solution may be used for cleaning, but careful rinsing with hot tap water, followed by distilled water, is necessary. Instruments should be thoroughly dry before sterilization.

The instruments can also be cleaned in ultrasonic devices. The manufacturers provide recommendations for the cycle time. To begin, the instruments, in the open position, are placed into the device and completely submerged. After ultrasonic cleaning, instruments are rinsed carefully in warm tap water, followed by distilled water, to completely remove cleaning solution. It is advisable that instruments of similar metal content should be cleaned together.

Care of the instruments after use and cleaning is also important. Instruments used for tissue culture are not to be oiled, because this may create problems when the oil mixes with the dissected tissues or becomes burned to the steel. Scissors with spring handles should be stored in the open position, to prevent tiring of the spring. Particular care must be given to the tips of fine forceps and scissors. Short lengths of flexible tubing may be used as tip protectors during storage.

Every instrument should be examined under a dissecting microscope before sterilization. The ends of forceps and scissors should be of equal length, the tips should meet perfectly, and the shearing edges should be sharp. Instruments should be firmly secured within the instrument box, so that they do not move when the box is handled. Gauze may be placed between layers of instruments.

The instruments can be sterilized by alcohols, autoclaving, or glass-bead sterilizers. Methanol, ethanol, and isopropanol are used as bactericidal agents at concentrations of 70–80% alcohol in water. Alcohols leave no residues on surfaces, and are effective agents for sterilization and disinfection. Instruments can be immersed in 70% alcohol, and allowed to dry in air inside the laminar flow hood; this procedure should be repeated 3×. Another method is to dip the instrument in alcohol, then pass it through the flame of a gas burner, repeating the procedure 3×. Scissors should be in the open position while flaming.

Note: Make sure the flame is completely out before reimmersing the instrument in the alcohol. If the alcohol does catch on fire, immediately cover with a metal lid.

Note: Instruments may be damaged by high heat, depending on the alloy used and the degree of hardness. Do not keep the instrument in the gas flame while the alcohol is burning, just pass it through the flame to ignite the alcohol. Do not heat the instruments to red-hot heat.

Sterilization of instruments by autoclaving is rapid and efficient; however, instruments that are not rust-resistant will be damaged, unless they are completely dried.

Glass-bead sterilizers (Inotech Biosystems l, Lansing, MI) are convenient and portable, and the sterilization process is rapid. Instruments can be sterilized at the work place. The procedure is simple: insert dry, clean instruments, for at least 5–10 s, in the bed of beads heated to 250°C, then place instruments in a sterile glass Petri dish, and allow them to cool before use.

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The Essential Guide for Every Neuroscientist!

Protocols for Neural Cell Culture

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Edited by
Sergey Fedoroff and **Arleen Richardson**
Department of Anatomy and Cell Biology
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