

# Flow Cytometry

Third Edition

*Edited by*

Michael G. Ormerod

**PRACTICAL  
APPROACH**

Ormerod

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OXFORD

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## **Third Edition**

**A Practical Approach**

Edited by

**Michael G. Ormerod**

**34 Wray Park Road, Reigate,  
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# Chapter 1

## Introduction to the principles of flow cytometry

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### 1 Introduction

Flow cytometry is a technique for making rapid measurements on particles or cells as they flow in a fluid stream one by one through a sensing point. The important feature of flow cytometric analysis is that measurements are made separately on each particle within the suspension in turn, and not just as average values for the whole population. The ability of laser- and arc lamp-based flow cytometers to measure multiple cellular parameters, based on light scatter and fluorescence, and to purify physically subpopulations of cells has led to the increasingly widespread use of this instrumentation in biology and medicine. The applications of flow cytometry and cell sorting are numerous. A wide range of fluorescent probes is available for directly estimating cellular parameters such as nucleic acid content, enzyme activity, calcium flux, membrane potential, and pH. Conjugation of fluorescent dyes to ligands and to polyclonal and monoclonal antibodies has enabled the density and distribution of cell-surface and cytoplasmic determinants and receptors to be studied, as well as allowing functional subpopulations of cells to be identified. Many of these fluorescent dyes and reagents can be used in combination to produce multiple correlated measurements. For example, it is now commonplace in immunology to measure two light-scatter and three immunofluorescence parameters on each cell.

The use of flow cytometers for cell sorting is also widespread. Applications range from the separation of large numbers of cells for functional studies or chromosomes for preparing gene libraries to the direct cloning of single, rare, transfected or hybridoma cells into each well of a tissue culture plate.

Unfortunately, flow cytometers are not simple instruments. As with all sophisticated measuring devices, it is important to possess a basic knowledge of the underlying principles of operation so that the significance and accuracy of the results can be assessed. For example, the quality of sample preparation and staining is as important in the precision of measurements as the design of the fluidic, optical, and electronic components of the instrument itself.

Within the scope of this introduction it is not possible to explain the theory of operation and design of flow cytometers in detail. However, we will try and explain in relatively simple terms the basic principles of flow cytometric practice. More detailed information can be found in refs 1-4.

## **2 Techniques for sample preparation (see also Chapter 3)**

The aim of sample preparation is to produce a suspension of single particles, stained in a specific way, which will pass through the system without disrupting the smooth flow of fluid or blocking tubes or orifices. The particles analysed may be whole cells, cell organelles, or specific clumps of tissue such as Islets of Langerhans.

Producing a suspension of individual particles from biological samples can range from being straightforward to frustratingly difficult. Body fluids, in particular blood, generally contain individual cells that can be stained and processed directly on the flow cytometer. For example, a sample of blood can be stained with Thiazole Orange orange and analysed on the flow cytometer to obtain a count of reticulocytes (see Chapter 7, Section 2). Sometimes, the sample is enriched before analysis if the cell of interest is relatively rare. For example, blood leucocytes constitute less than 0.1% of all the cells in peripheral blood and it is customary to lyse the red blood cells to obtain a sample of white blood cells for analysis.

Solid tissues present a much more difficult and varied problem for flow cytometry. A technique which successfully releases cells from one tissue can fail totally on another. For example, lymphoid tissues can usually be prepared by simple chopping and teasing of the organ followed by sieving and density-gradient centrifugation to provide a sample of separated lymphocytes. Other organs and solid tumours require enzymatic digestion before a good yield of individual cells is obtained. Detergents are often required in the preparation of organelles, such as isolated nuclei and chromosomes, where it is necessary to remove cell membranes and cytoplasm.

With all preparative methods great care must be taken to ensure that the technique itself does not bias the results. For example, density-gradient centrifugation of lymphocytes may preferentially enrich some subpopulations. Enzymatic preparative techniques can alter cell-surface antigens and affect cell viability.

Usually, cells are stained by incubation, under appropriate conditions, with a fluorescent dye or fluorescent-conjugated antibody or ligand. For accurate interpretation of results, it is important that the staining is specific for and proportional to the feature to be measured. Unfortunately, it is not unusual for fluorescent probes and even monoclonal antibodies to bind non-specifically and care must be taken to block cross-reactions. Heterogeneity in the uptake of dyes by cells also results in degraded resolution of the measured parameter, unless the heterogeneity itself is of interest.

Optical crosstalk can occur when combinations of fluorochromes with overlapping emission spectra are used together. The result of this spectral overlap is that a proportion of the fluorescence from one dye reaches the detector intended to measure the fluorescence of the second dye, and vice versa. While this effect can be easily corrected using optical filtration or electronic compensation, the inevitable compromises introduced can reduce the overall sensitivity of measurements. Energy transfer between fluorochromes in close proximity can also take place (see Chapter 2, Section 2). Here the energy absorbed by one dye is transferred to the second dye, which then fluoresces. The consequence is that fluorescence from the first dye is quenched, producing an underestimate of fluorescence intensity, while the second dye is inappropriately excited, producing an overestimate of its intensity. However, this phenomenon can be directly exploited to measure the proximity of structures in or on cells.

The problems of sample preparation and staining are easily assessed by the use of appropriate controls designed to measure the specificity and accuracy of the measurements.

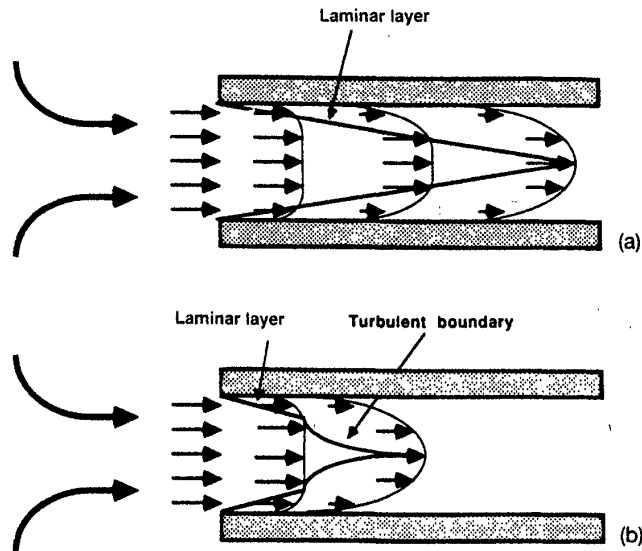
### 3 Fluidics

The fluidic system of a flow cytometer is used to deliver particles of a random three-dimensional suspension singly to a specific point in space intersected by the illuminating beam. This is generally achieved by injecting the sample suspension into the centre of an enclosed channel through which liquid is flowing. Channels have been designed so that the tightly focused core of sample remains separated from the surrounding sheath of fluid. Typical flow chambers can transport particles to the detection point with an accuracy of better than  $\pm 1$  micron.

#### 3.1 Principles of fluid flow and hydrodynamic focusing

There are two principles of fluid flow used in the design of flow chambers: (a) laminar flow with viscous drag; and (b) turbulent boundary drag.

- (a) In laminar flow, the velocity of the fluid front at the inlet is the same both at the walls and in the centre of the channel. As the fluid moves away from the inlet down the channel, viscous drag at the walls slows the outer layers of liquid. The fluid front changes into a parabola with the greatest velocity at the centre of the flow channel and zero velocity at the flow channel wall (see Figure 1a). This velocity gradient draws particles towards the centre in a process known as hydrodynamic focusing. Further down the flow channel the velocity front forms a stable parabola so that the particles continue to flow at the centre of the channel. The distance from the inlet to the formation of the stable velocity parabola is known as the inlet length and is approximately 50 times the channel diameter.
- (b) In turbulent boundary flow, the sample is injected into a chamber rapidly tapering to a small exit orifice. The speed of entry of the sample combined



**Figure 1** The principles of fluid flow used in flow chamber design. (a) Laminar flow with viscous drag; (b) turbulent boundary drag.

with the back pressure generated by the exit orifice results in sample turbulence. The turbulence at the interface between the sample and sheath produces a viscous boundary. The drag generated at this boundary forms a velocity parabola within a shorter distance than for laminar flow (see *Figure 1b*). Particles are again drawn towards and contained within the centre of the flow.

### 3.2 The fluidic system

A diagram of a typical fluidic system is shown in *Figure 2*. The fluidic system consists of two fluid lines feeding the flow chamber, the sheath fluid line, and the sample line. In normal operation the sheath fluid flows continuously and is controlled by regulated positive air pressure acting on the sheath reservoir. Sample flow rates are controlled by a second pressure regulator acting on the sample chamber. Differential or individual pressure gauges are used to set optimum flow conditions. An alternative approach, particularly for the accurate control of sample flow, is the use of a finely controlled syringe pump. A purge line is also often connected to the sheath inlet to allow a vacuum to be applied for clearing blockages and air bubbles.

At least one system (the Partec PAS) uses negative pressure (a vacuum) applied to the exhaust lines rather than positive pressure applied to the sheath and sample inlets to generate the fluid flow.

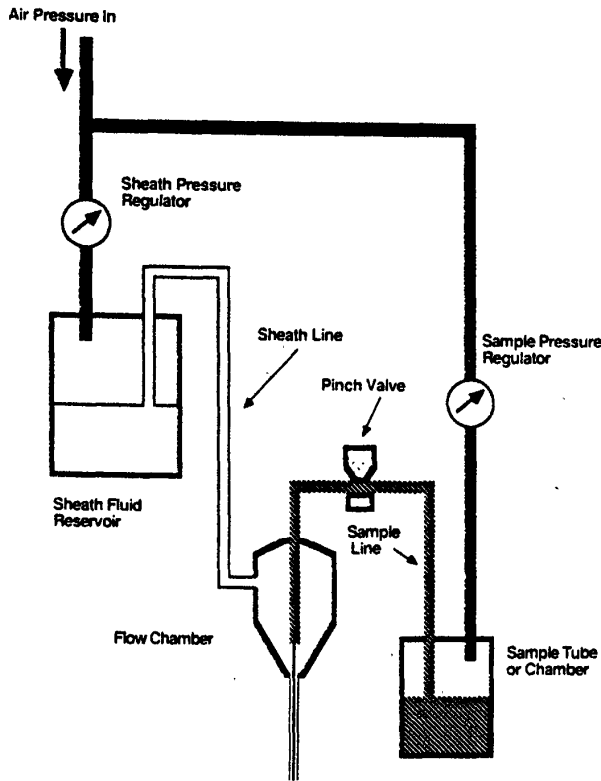


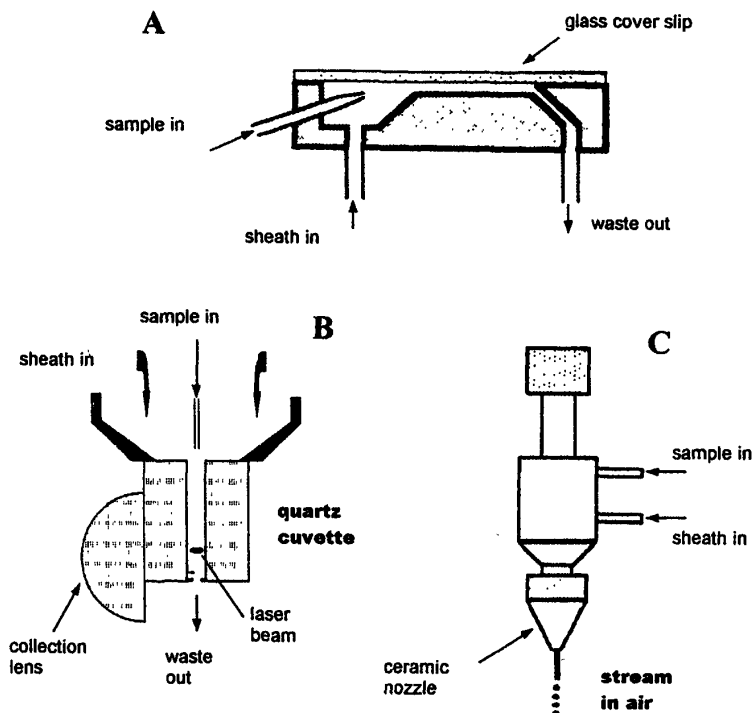
Figure 2 A typical fluidic system for a flow cytometer.

### 3.3 Flow chamber design

The general design of flow chambers can be divided into analytical and sorting chambers and into the use of laminar flow or turbulent boundary conditions. In general, flow chambers are designed with a wide inlet for sheath fluid, which tapers to either an exit orifice or a cylindrical or square-sided channel. The smaller bore tube used for sample injection is located at the centre of the wide inlet. In laminar flow designs, the tapering flow reduces the diameter of the core of sample before entry into the flow channel where the final hydrodynamic focusing of the sample takes place. In turbulent boundary designs, the tapering design itself brings about hydrodynamic focusing within a short distance of the inlet. Some typical chamber designs, as used in commercial instruments, are shown in Figure 3.

#### 3.3.1 Analytical chambers

These chambers generally use laminar flow conditions in their design and fall into two groups. The first group of chambers is used in laser-based flow systems



**Figure 3** Typical flow chamber designs. (A) PAS II flow chamber (microscope-based). (B) Quartz cuvette (Beckman Coulter). (C) FACStar flow chamber (Becton Dickinson).

where fluorescence is measured at right angles to the illuminating beam. Most systems use a flat-sided cuvette designed to minimize unwanted light reflections and are usually positioned vertically to the laser beam. In analytical systems, the flow of fluid is usually upwards to allow air bubbles to be easily removed from the chamber. Scatter measurements can be made both at right angles to the excitation beam and in the forward direction. However, in the forward direction it is necessary to use a blocker bar to eliminate unscattered laser light.

The second group of chambers is used in microscope-based flow systems where fluorescence is measured in line with the optical path. Chamber design is constrained by the limitations of this optical system, with the chamber replacing the horizontal microscope stage (e.g. see Figure 3a). The top surface of these chambers is usually a glass coverslip, so that immersion objectives of high numerical aperture can be used. Scatter measurements are restricted to within the direct optical path and are generally difficult to obtain. Some systems do not use an enclosed channel but simply squirt the hydrodynamically focused sample at a low angle across a microscope slide to be aspirated by a vacuum waste line.

Analytical chambers typically use a minimum channel bore of 250  $\mu\text{m}$  to help prevent blockages and unwanted reflection from the walls. Unrestricted, the flow through such a channel would require large volumes of sheath fluid and would be difficult to control. To reduce the flow and to obtain control over operating pressures, a restriction in the exhaust from the chamber is used. Many designs use a coiled length of narrow-bore tubing to provide this resistance to the flow.

### 3.3.2 Flow chambers for cell sorters

The most common principle used for cell sorting is the electrostatic charging of droplets as applied to laser-based flow systems. The flow chamber design incorporates an exit orifice, usually a watchmaker's jewel or a precision drilled hole, which produces a jet of fluid. Stable droplet formation takes place under the influence of an applied oscillation. The exit orifice also provides the required resistance to control sheath flow rates.

There are two basic designs of droplet sorting chambers in general use. The first type (see *Figure 3b*) uses laminar flow conditions in a square channel cuvette where cells intersect the laser beam above the exit orifice. The second type (see *Figure 3c*) uses turbulent boundary conditions, and cells intersect the laser beam immediately below the exit orifice in the exhaust jet. This second type of analysis and sorting design is known as 'stream in air' or 'jet in air'.

Scatter and fluorescence measurements are made in the same way as analytical, laser-based, flow systems with the exception that a second blocker bar is required for 'stream in air' analysis and sorting. This blocker bar is placed in front of the right-angle collection lens to block laser light reflected by the cylindrical surfaces of the jet.

Microscope-based flow cytometers are less well suited for cell sorting. However, Partec PAS systems achieve sorting by the use of a piezoelectric fluidic valve operating on one arm of a 'Y'-shaped flow channel. This principle does not have the particle size restrictions imposed by jet and droplet formation and has been used for sorting large particles such as whole Islets of Langerhans. Another approach has been adopted in the FACSort and FACSCalibur instruments. Here a small collector arm is moved into the sample stream to selectively intercept cells of choice (see Chapter 4, *Figure 3*).

## 4 Detection and measurement

The flow cytometers, which are the subject of this book, make measurements based on light as the source of excitation. Intense illumination is required because cells are small and pass through the detection point rapidly. In addition, the light source must be capable of producing specific wavelengths that can be used to excite fluorescent dyes. The scattered and fluorescent light generated by cells passing through the illuminating beam is collected by photodetectors which convert the photon pulses into electronic signals. Further electronic and computational processing results in graphic display and statistical analysis of the

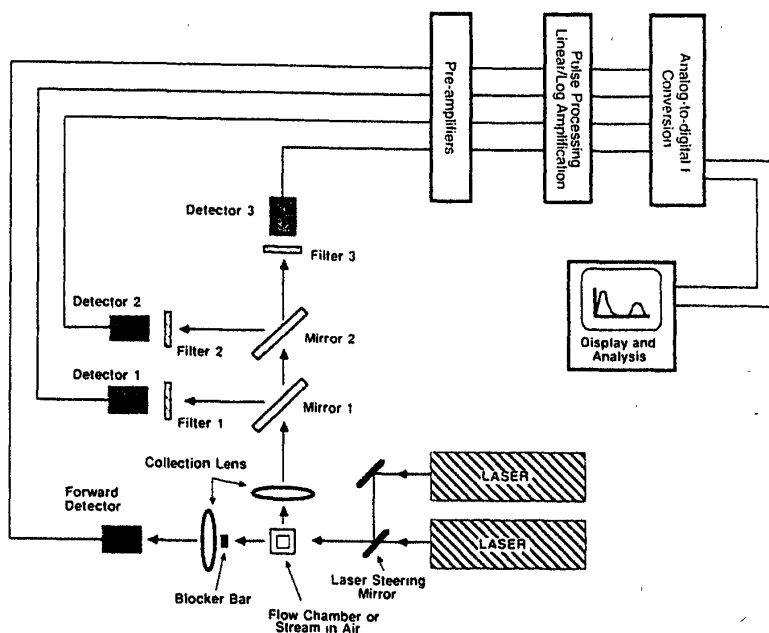


Figure 4 A generalized flow cytometer system.

measurements being made. A generalized flow cytometer system is shown in Figure 4.

#### 4.1 Illumination and beam shaping

The two types of light source commonly used in flow cytometers are arc lamps and lasers. An arc lamp is a glass envelope containing a gas or vapour at high pressure. An initial, high-voltage spark between two electrodes within the envelope forms a luminous plasma arc which is maintained by the application of a high current at low voltage. The arc is intrinsically unstable and is prone to flicker and wander. Flow cytometers using epi-illumination of the stream are able to use the spot of highest intensity formed at the cathode as the focal point for the lamp condenser lens. Even illumination is then obtained by using Köhler optics where the lamp condenser lens acts as a uniformly illuminated disc. (For a description of Köhler illumination, see *Light microscopy in biology: a practical approach*, ed. A. J. Lacey, this series.) The average life of arc lamps is short and is at best only a few hundred hours.

The name laser is an acronym for 'light amplification by stimulated emission of radiation'. The laser produces a coherent, plane-polarized, intense, narrow beam of light at specific selectable wavelengths. A typical gas laser is shown schematically in Figure 5. Mirrors, positioned at each end of the resonator, form an optical cavity within which the plasma tube is located. The plasma tube

## INTRODUCTION TO THE PRINCIPLES OF FLOW CYTOMETRY

contains a gas at a critical pressure which fluoresces under the application of a current, emitting light in all directions. The applied current raises electrons of the gas atoms into higher energy orbits. Light is produced when these electrons in high-energy orbits spontaneously decay to the ground state. The wavelength of the released photon is dependent upon the energy difference produced by the transition of the electron from the high- to the low-energy orbit.

Light emitted from the ends of the plasma tube is reflected by the mirrors back into and along the tube. When these reflected photons strike an atom in an excited state, a second photon is produced, which is of the same wavelength and phase as the stimulating photon and travels in the same direction along the plasma tube. These photons stimulate further photon release in a chain reaction that produces light amplification. The magnitude of the amplification increases with the length of the plasma tube, and is controlled by the rate at which electrons are pumped to higher energy orbits by the applied current. Flat, orthogonal windows at the ends of the plasma tube would reflect too much light to enable lasing to take place. Therefore, the windows are cut to Brewster's angle at which reflection is at a minimum. A consequence of using these Brewster windows is that the laser beam is plane-polarized.

Between 95% and 99% of the light produced within the plasma tube is required to maintain lasing, so that only between 1% and 5% of the laser light can be used. The front mirror is designed to transmit the appropriate percentage of light to form the usable laser beam.

The light produced by the laser is a mixture of the specific wavelengths (laser lines) defined by the finite energy levels that the electron orbits can achieve. Selection of specific lines is produced by the use of mirror coatings which efficiently reflect only the desired wavelength. Alternatively, a prism can be placed in front of the high reflector (see Figure 5) to separate physically the laser emission. Light dispersed off the optical axis by the prism is not reflected back into the plasma tube and cannot stimulate lasing. Rotating the prism will alter the wavelength that will lie on the optical path, so enabling one from the range of possible lasing lines to be selected.

The lasers used in flow cytometers are atomic (e.g. helium-neon), ionic (e.g. argon-ion ion or krypton), molecular (e.g. helium-cadmium), or liquid (e.g. dye

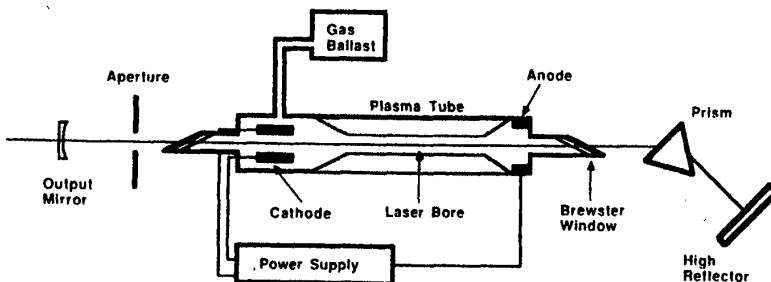


Figure 5 A typical gas laser.

lasers). Solid-state lasers are becoming available in a form suitable for flow cytometry and have the potential to be the laser of choice in the future.

The ideal laser beam shape would be a line orthogonal to and wider than the flow core, but whose thickness is less than the diameter of the smallest particle to be measured. This beam shape would maximize optical resolution of the particle passing through the spot while minimizing the positional sensitivity of the particle trajectory. In practice, a beam of this ideal shape is difficult to achieve and is usually approximated by the use of lenses producing ellipsoid or simple circular beams. Ellipsoid beams are produced by the use of crossed, cylindrical, paired lenses or by the use of spherical-cylindrical lens combinations. For example, one of the crossed, cylindrical, paired lenses used in the Epics Elite (Beckman Coulter) produces an ellipsoid focal spot of approximately 6 by 80 microns from a laser beam of 0.9 mm diameter and a wavelength of 488 nm.

'Stream in air' cytometers provide additional optical design considerations, as the stream itself acts as a half cylindrical lens in the illumination of the particle. In addition, the illuminating beam must be of a low numerical aperture to minimize the spherical aberration of the stream acting as a lens in the light path of the image. A beam wider than the stream would also produce unacceptable noise at the scatter detectors due to the stream perturbations introduced by the use of the transducer during sorting (see Chapter 4). 'Stream-in-air' cytometers use crossed, cylindrical or spherical-cylindrical lenses producing spot sizes of typically  $20 \times 60$  microns. Alternatively, simple long focal length lenses producing circular spots of typically 30-50 microns in diameter are used to simplify the optical design at the expense of resolution.

## 4.2 Collection optics

For routine analysis, the forward collection lens gathers scattered light from approximately 1 to 20 degrees off the laser beam axis. The exact angle depends on the geometry of the system and is different for different systems. Some systems allow the user to change the angle of light collection. This angle of light minimizes the effect of refractive index changes on forward scatter measurements, so maximizing the dependence on particle size. A lens of a numerical aperture of at least 0.3 is required to collect this cone of light, a specification achieved by a simple, long working distance lens.

For the greatest sensitivity in the measurement of fluorescence, the right-angle lens is designed with a high numerical aperture to collect light over the greatest cone possible. However, as the numerical aperture increases, the working distance of the lens (the distance from the front surface of the lens to the object plane) decreases. The physical dimensions of flow chambers limit the minimum working distance and so the maximum numerical aperture which can be used. The design of most commercial sorting flow chambers limits the numerical aperture of the collection lens to 0.6 or less. However, flow cytometers using non-sorting glass cuvettes or coverslip flow chambers are able to use lenses with numerical apertures of 1 or more. The light-gathering efficiency

with these chambers can be increased further by the use of immersion objectives, which eliminate light loss due to refraction at the glass-air interface. In some designs of cell sorter, which use a cuvette flow chamber, a small lens is glued to the side of the cuvette, again to maximize light collection (see Figure 3b).

## 4.3 Optical filtration

### 4.3.1 Introduction

In cytometers employing arc lamps, filters are used to select the correct wavelength of exciting light. However, most instruments use lasers which give monochromatic light so that further filtration is unnecessary. On the output side, filters are needed to separate the mixture of scattered and fluorescent light collected from stained particles so that specific independent but correlated measurements can be made. The separation of different wavelengths is achieved by the use of dichroic mirrors and interference and absorption filters.

There are two types of glass filters in use: coloured glass and interference filters. The details of their design can be found in the manufacturers' catalogues, which also give representative graphs of the spectral properties of their filters. An individual spectrophotometer trace should also be supplied with each filter.

Chapter 2 gives information about the excitation and emission wavelengths of different fluorophores, and this should be used as a guide to the selection of sets of filters. An example of a typical arrangement is given in Section 4.4 below.

A useful discussion on the selection and use of optical filters together with further references will be found in ref. 5.

### 4.3.2 Coloured glass filters

The most useful of these are the long-pass filters which transmit light above a given wavelength and absorb light of lower wavelengths. They can be obtained with a cut-off between 300 and 700 nm. The amount of light absorbed will increase if the filter is tilted since it presents a greater thickness to the light beam but, apart from this, the optical properties are unchanged. Some coloured glass filters fluoresce slightly under UV light.

Bandpass filters in coloured glass seldom have sufficient discrimination to be of use in flow cytometry.

### 4.3.3 Interference filters

These consist of dielectric layers deposited *in vacuo* on a glass substrate. Depending on the thicknesses of the layers and the wavelength of the light, the internally reflected beams interfere with one another either destructively or constructively allowing some wavelengths to pass through the filter while others are reflected.

When an interference filter is viewed, one surface has a metallic appearance;

this should be placed towards the light source. In this way the amount of heat absorbed in the filter is minimized (this is particularly important when a filter is placed in the primary light beam) and any fluorescence from an associated coloured glass filter is also minimized.

Interference filters can deteriorate with time and their properties should occasionally be checked in a spectrophotometer to ensure that they perform to their original specification. Spectral properties should always be measured at the orientation of use since they depend on the angle of the filter in the light beam; tilting a filter shifts its spectral properties to shorter wavelengths.

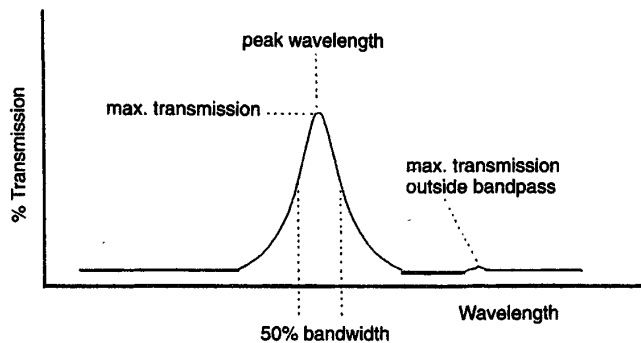
Interference filters come in two forms—bandpass and edge filters.

Bandpass filters transmit light of the desired wavelength over a narrow band (see *Figure 6*). In their simplest form they consist of two reflecting layers separated by a dielectric layer of exactly one half-wavelength thickness. This is referred to as a cavity—commercial filters generally have from one to four cavities. The basic interference filter is often combined with a blocking filter to cut out transmission from sidebands.

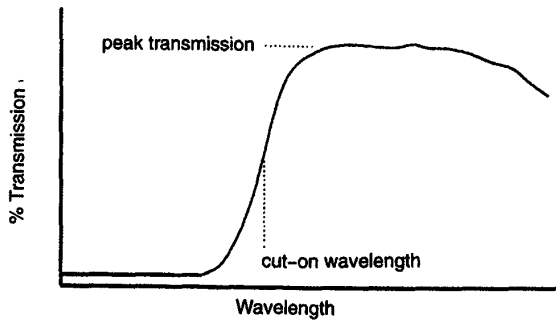
The major parameters of bandpass filters are the peak wavelength of transmission, the percentage of light transmitted at the peak wavelength (peak transmission), the bandwidth (usually measured as the separation in nanometres of the 50% transmission points), and the maximum transmission outside the bandpass. The shape of the transmission curve is also important and this can be estimated by comparing the bandwidths at 50% and 10% transmissions. The greater the number of cavities, the sharper the cut-off at either side of the central band.

Edge filters are either short wavelength pass (SWP) filters, which transmit light below a given wavelength and reflect light of a longer wavelength, or long wavelength pass (LWP) filters, which work in the reverse way (*Figure 7*). They are generally used in the flow cytometer as dichroic mirrors (often called beam splitters) at an angle of 45° to the light beam. For the reason given above, their spectral properties should always be measured at this angle.

The major parameters of edge filters are the cut-off (for LWP) or the cut-on (for SWP) wavelength (the wavelength for 50% transmission), the peak trans-



**Figure 6** The properties of a bandpass filter.



**Figure 7** The properties of an edge filter.

mission, and the slope. The latter parameter defines sharpness of the cut-off and is measured between the wavelengths for 80% and 5% transmission.

The cut-off for interference filters is far sharper than that found with coloured glass.

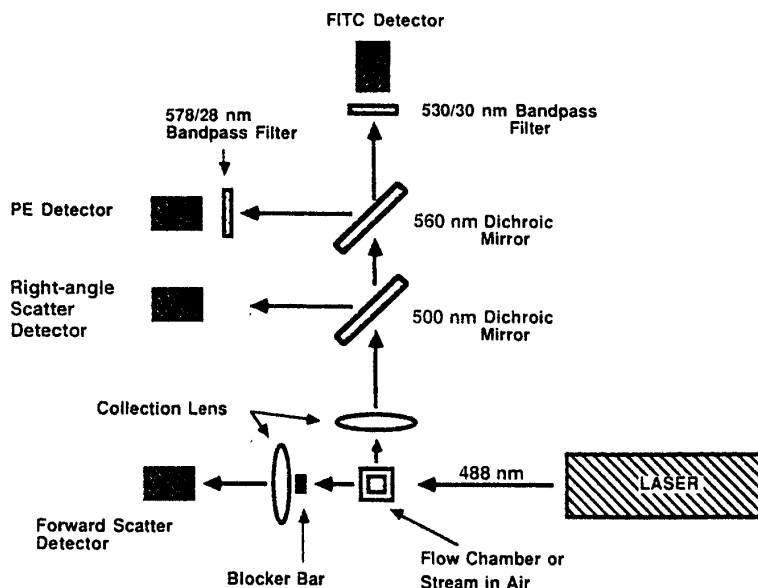
#### 4.3.4 Optical configuration

A typical optical configuration that could be used for the measurement of scattered light, fluorescein (FITC—green) and phycoerythrin (PE—orange) immunofluorescence is shown in Figure 8. Dichroic mirrors are placed at 45° to the incident beam while the absorption and interference filters are orthogonal. The first mirror in the optical path at right angles to the laser beam is a 500 nm long-pass dichroic filter, which reflects wavelengths shorter than 500 nm (the 488 nm scattered laser light) towards the right-angle scatter detector. Longer wavelengths pass on towards the second mirror, a 560 nm short-pass dichroic filter. Wavelengths greater than 560 nm are reflected towards the PE fluorescence detector and through a filter centred at 578 nm with a 28 nm half-peak bandpass. The shorter wavelengths between 500 and 560 nm incident at the second mirror pass on towards the FITC fluorescence detector and through a 530 nm filter with a 30 nm half-peak bandpass. More colours could be measured by introducing further mirror, filters, and detectors.

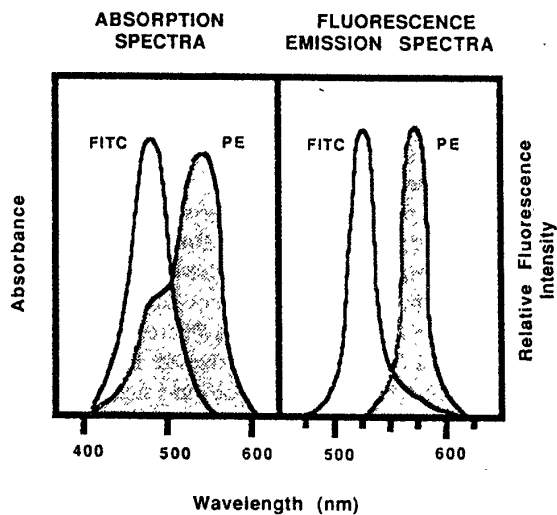
The emission spectra of FITC and PE and the spectral characteristics of the two bandpass filters are shown in Figures 9 and 10. It can be seen from Figure 9 that, although the bandpass filters select separate wavebands, the emissions from the two fluorochromes overlap such that some fluorescence from one fluorochrome will pass to the detector intended to measure the fluorescence from the other and vice versa. This spectral overlap can be corrected during signal processing (see Section 4.5). Most modern instruments can measure at least four colours. The problem of spectral overlap then becomes more acute.

#### 4.4 Detection devices

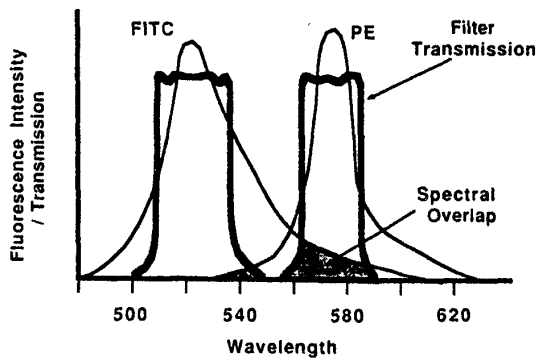
The two types of devices used in flow cytometers to detect scatter and fluorescence are PIN diodes and photomultiplier tubes (PMTs). PIN diodes are cheap



**Figure 8** An optical configuration for the simultaneous measurement of forward scatter, right-angle scatter, fluorescein (FITC) and phycoerythrin (PE) fluorescence.



**Figure 9** The absorption and emission spectra of fluorescein (FITC) and phycoerythrin (PE).



**Figure 10** Filter transmission characteristics for the separation of fluorescein (FITC) and phycoerythrin (PE) fluorescence.

solid-state detectors of relatively low sensitivity but wide spectral characteristics and fast response. The lack of sensitivity of PIN diodes limits their usefulness and they are generally restricted to the measurement of forward scattered light. Photomultipliers are photosensitive electron tubes with a more restricted spectral response, but with high gain and good signal-to-noise characteristics suitable for the detection of weak fluorescence. The spectral sensitivity of PMTs is determined by the composition of the light-sensitive photocathode and this is an important factor in determining the sensitivity of a flow cytometer.

#### 4.5 Signal processing

Light falling on the photodetector surface generates a current that is fed into a filtering pre-amplifier. The output of the amplifiers is a smoothed voltage pulse usually of between 0 and 10 volts. The amplitude of this pulse is proportional to the number of photons reaching the photodetector. Pulse shape is determined by the size and speed of the particle, the width of the illuminating beam, and, in the case of fluorescence, the distribution of the fluorochrome within the particle. The measurement of small particles using narrow illuminating beams or in 'stream in air' flow chambers will produce pulses with very fast rise times. The pre-amplifiers are designed to follow both these rapid pulses as well as the slower pulses from larger particles without distortion.

The output from the pre-amplifier will inevitably contain some background noise. It is not desirable for further signal-processing circuits to process this noise, which may be at a high frequency and could mask true pulses. This is prevented by the use of a system threshold so that further processing only takes place when the input voltage rises above a pre-set value. Usually the threshold is set on a single signal that triggers all other measuring circuits. Forward scatter is often used as the system trigger pulse, particularly when fluorescence measurements are being made. If fluorescence was used as the trigger, negative cells might not produce signals above threshold and so would not be detected or

measured. An exception is in the measurement of DNA, in which case the DNA-dye fluorescence is used as the trigger since only particles containing DNA are of interest. The thresholding parameter is often referred to as the discriminator.

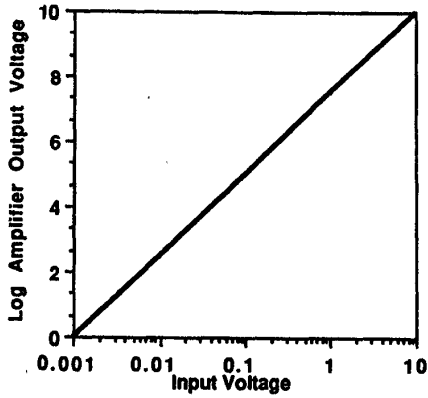
The pulses' output by the pre-amplifiers are generally too fast to allow measurement and display circuits to function properly. Therefore, the pulse processing circuitry provides an analog voltage memory of the signal which is held long enough for further processing to take place. The sustained voltage output from this 'sample and hold' circuit can be derived from the input signal in three ways—the output voltage can be made proportional to the height (peak height), the area (integral), or the width (time of flight) of the pulse.

Sample-and-hold circuits maintain their voltage output for a fixed period, usually between 15 and 120  $\mu$ sec, and are triggered when the signal voltage rises above the pre-set threshold. During the period while the sample-and-hold circuit is operating, the electronics are unavailable for the processing of other pulses. This period is known as the 'system dead time' and events occurring during this processing period pass undetected. A large system dead time has less consequence for analysis than for sorting. The cells undetected during the system dead time will be taken randomly from the population of cells being analysed and no bias of the final results will take place. However, the time taken to acquire a specific number of events will be increased. A large system dead time during sorting will not only reduce the maximum sorting rate but will also increase the chance of undetected cells being sorted coincidentally, thus compromising sorting purity.

The various pulse-processing modes enable different measurements to be made from the same signal. Pulse width is related to the size of the particle or area of fluorochrome staining. The width of the pulse is composed of the width of the illuminating beam plus the width of the particle (and minus the small triggering and de-triggering widths). The constant offset due to the beam width can be electronically removed to produce a measurement directly proportional to the particle size.

Generally, a linear relationship between the measured fluorescence intensity and the number of fluorochrome molecules is required. If the width of the illuminating beam is less than the particle width, only a fraction of the fluorochrome molecules will be excited at one time and pulse peak measurements will only reflect the highest fluorochrome concentration and not the total content. In this case, pulse-area processing will integrate the fluorescence as the particle passes through the narrow illuminating beam so that the output becomes proportional to the total dye content. The difference between either pulse peak and area or pulse width and area can be utilized to discriminate doublet particles as applied in cell-cycle analysis (see Chapter 6).

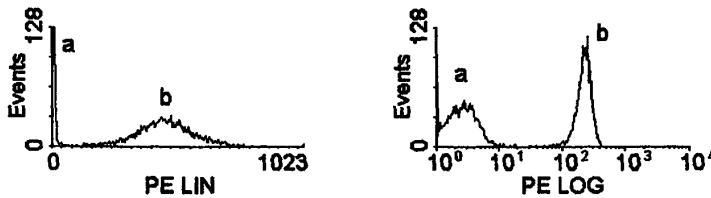
Signals can be processed directly or after passage through a logarithmic amplifier. The relationship between the input and output voltages of a four-decade logarithmic amplifier is shown in *Figure 11*. An input voltage of 10 millivolts produces a 2.5 volt output, while a 10-fold increase in the input voltage to



**Figure 11.** The relationship between input and output voltages for a 4-decade logarithmic amplifier.

100 millivolts only doubles the output to 5 volts. In this way logarithmic amplification amplifies weak signals and compresses large signals. Logarithmic amplification not only increases the resolution of weak signals but also increases the dynamic range of the measurements so that both weak and strong signals can be displayed on the same scale. The use of 10-bit analog-to-digital converters with 1024-channel resolution is now universal. With linear amplification, a 1000-fold difference in signal intensity can be displayed. However, the use of a four-decade logarithmic amplifier allows signals with a 10 000-fold difference to be displayed on the 1024-channel scale. An example of the same sample analysed using both linear and logarithmic amplification is shown in *Figure 12*.

Spectral overlap of the emission from two fluorochromes can also be corrected during signal processing. The spectral overlap produces a small signal from one fluorochrome at the detector intended to measure the fluorescence from the second fluorochrome, and vice versa. Simple circuitry can be used to electronically subtract the proportion of the fluorescence due to spectral overlap from each pulse. Examples of two-colour fluorescence measurements before and after spectral overlap correction are shown in Chapter 5, *Figure 3*.



**Figure 12** Linear and logarithmic displays of the immunofluorescence of a phycoerythrin-labelled (PE) monoclonal antibody to the CD4 antigen. Panel A, linear; panel B, logarithmic amplification (a, negative cells; b, CD4+ cells).

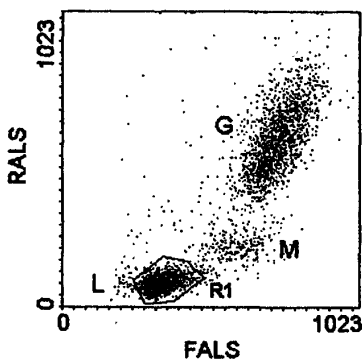
#### 4.6 Digital conversion

For analysis and display by computer systems or pulse-height analysers the held voltages from the analog circuitry are digitized. The analog-to-digital converter (ADC) translates the continuous voltage analog range into a discrete scale which can be represented by a binary number. The resolution of the measurements is dependent upon the scale interval of the conversion. ADCs providing 10-bit resolution will divide the scale into 1024 elements (10 mV per division) with voltages being represented by a binary number between 0 and 1023.

#### 4.7 Analysis and display

The result of the voltage pulses is a stream of numbers which need to be processed by a computer to display meaningful data. The most common and useful forms of display are the frequency histogram and the dual-parameter correlated plot, often known as a cytogram or dot plot. The frequency histogram is a direct graphical representation of the number of events occurring for each channel of the ADC (i.e. counts against intensity). The cytogram or dot plot is a two-dimensional extension of the frequency histogram. In this case, the locations in memory correspond to a two-dimensional array of the channels of one ADC correlated against the channels of a second. Each location within the array is incremented according to the digitized values produced by the two ADCs. The memory can then be read on to the screen to produce a square plot where each cell is represented at the co-ordinates appropriate to the measured values.

A typical frequency histogram is shown in *Figure 12*. A typical cytogram is shown in *Figure 13*. This display shows the correlated measurements of forward light scatter plotted against right-angle light scatter for a sample of peripheral blood leucocytes. Here, three main clusters can be seen which relate to the three major cell types, namely: lymphocytes, monocytes, and polymorphonuclear granulocytes.



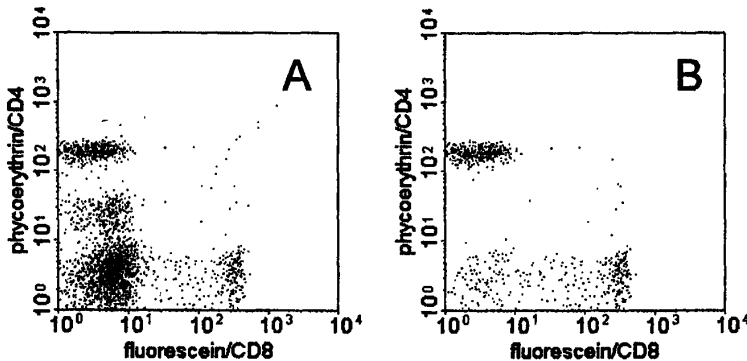
**Figure 13** A cytogram or dot plot. A sample of human peripheral blood leucocytes. The intensity of right-angle light scatter (RALS) is plotted against the intensity of forward-angle light scatter (FALS) for each cell. L, lymphocyte cluster; M, monocyte cluster; G, granulocyte cluster; R1, region of interest encircling the lymphocyte cluster.

## INTRODUCTION TO THE PRINCIPLES OF FLOW CYTOMETRY

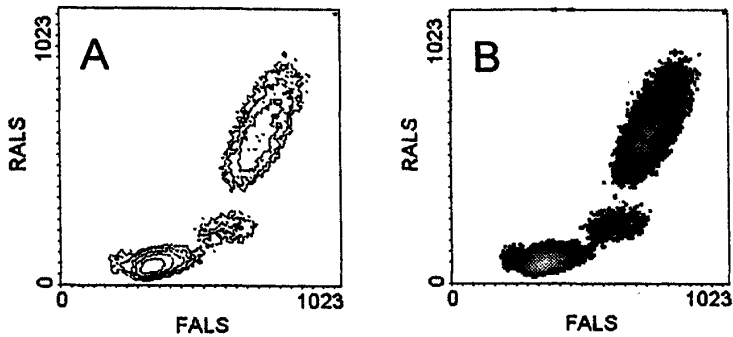
Statistical analysis of these displays is a straightforward task for the computer. Markers can be set at specific channels of a histogram and the percentage of total, mean value, and many other statistics generated for these selected events. Cytograms are usually analysed by setting boxes or polygons around areas of interest (called regions).

More complex analyses use the ability of the program to set gates or windows on defining parameters. Only cells which fall within the gates are analysed further. An example is shown in Figure 14. Peripheral blood leucocytes were stained with a monoclonal antibody specific for CD4-positive lymphocytes. The immunofluorescence profile generated without gating is shown in Figure 14A. Ungated, 10.7% of the cells display positive staining. However, if a gate is placed around the lymphocyte cluster on the forward- against right-angle scatter cytogram (region of interest 1, Figure 13) the profile shown in Figure 14B is generated; in this case 39.0% of the gated cells are positive. Both of these results are valid but represent different analyses of the same sample. The ungated data generates the number of CD4-positive cells expressed as a percentage of all leucocytes (lymphocytes, monocytes, and granulocytes). Gating enables the number of CD4-positive cells to be expressed as a percentage of lymphocytes only. Gating techniques are very powerful and allow sophisticated questions about cell and organelle subpopulations to be studied.

The histogram and the cytogram are the most used form of data display and can be updated by the computer in real time while a sample is being analysed. For publications, the data can be presented in other forms such as the contour plot, density plot, and the isometric plot. The contour plot is a cytogram where elements of similar event frequency are joined by contours (Figure 15A) while, in the density plot, a similar effect is achieved using different colours or grey



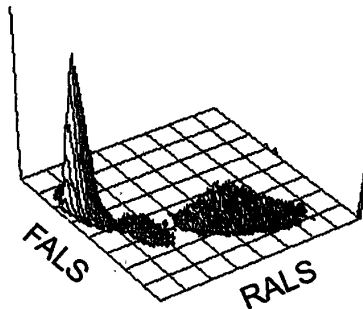
**Figure 14** Scatter gating in the analysis of cell subpopulations. Panel A shows the immunofluorescence profile of the human peripheral blood leucocytes (see Figure 13) stained with fluorescein-labelled anti-CD8 and phycoerythrin-labelled anti-CD4 monoclonal antibodies. All cells, including lymphocytes, monocytes, and granulocytes, contribute to this profile. Panel B shows the profile obtained using the gate displayed as region of interest R1 in Figure 13. Only cells with scatter characteristics falling within this gate (i.e. the lymphocytes) contribute to the profile displayed in Panel B.



**Figure 15** (A) A contour plot and (B) a density plot (data as in *Figure 13*).

scales (*Figure 15B*). The isometric plot uses the Z axis to display the frequency of correlated events and has the attractive feature of variable rotational and tilt viewing angles (see *Figure 16*).

Data can be processed immediately into histograms and cytograms (real-time analysis) or the individual correlated measurements stored on disc in time sequence (list mode). List-mode data has the advantage that the data may be re-processed, gated, and displayed as often as required. This feature is very useful when it is not clear where to set the gates and a more considered analysis is required, although real-time analysis is the most efficient approach for rapid results. Flow cytometers generate vast amounts of data. Analysing cells at 5000 per second and measuring six parameters on each cell will eventually fill up a hard disc. It is unusual to find researchers willing to delete data, valuable or not, so it is important to have as great a capacity for data storage as is possible. High-capacity tape drives, optical discs, or writable CD-ROMS are ideal for removable storage. It should also be remembered that magnetic media is not permanent and can easily be deleted in error or by physical accident (e.g. storage on top of laser transformers!). Back-up copies of important data and system software should always be kept in a safe place against these eventualities.



**Figure 16** An isometric plot (data as in *Figure 13*).

#### 4.8 Interpretation of acquired data

The results from any instrumentation will always be subject to a degree of variation. Flow cytometric analysis, although capable of measurements with small variance, is also prone to the introduction of systematic errors. Poor sample preparation and staining and improper operation of the instrument leads to inaccurate results. Immunofluorescence measurements become inaccurate in the presence of large numbers of dead cells as these cells take up the antibody non-specifically. If staining is not specific and homogeneous, variation will be introduced that may be larger than the effect to be measured. Furthermore, the emission from fluorochromes may change with pH and temperature. Care must be taken to buffer the pH and to allow the temperature to stabilize before measurements are made. It should also be remembered that scatter measurements are proportional to the size of particles only if the refractive index of the particles is the same.

Measurements are degraded by both optical and electronic noise. Scratched or incorrectly chosen filters will lead to optical noise and poor signal discrimination. Generally, the degree of electronic noise is not within the control of the cytometer user, but it should be monitored by the routine use of a standard, e.g. fluorescent microspheres. PMTs will only produce a linear response within a defined voltage range. Below about 300 volts the response is non-linear and unnecessarily high voltages produce excessive noise. If the PMT becomes saturated with light then non-linearity of response can occur. PMT saturation is prevented by the use of neutral-density filters or control of the power output from the laser. The laser itself is a source of noise. Even the best regulated lasers will retain a small degree of ripple on the light output. Operating the laser at high power outputs reduces the contribution of this constant ripple on the output. Laser light fluctuations and ripple are reduced further if a power control circuit is used. A small proportion of the laser output is monitored and the plasma current regulated by negative feedback to maintain a constant light output.

Any degradation in the accuracy of measurements is detected rapidly if standard particles are used routinely. Fluorescent microspheres are ideal for this purpose, enabling the optical alignment and parameter settings to be reproduced each day. Samples should be analysed only if the standards display acceptable values. However, microspheres are artificial particles and the best settings for these may be inappropriate for biological specimens. If possible, known biological standards or fixed samples should also be run routinely.

A more detailed discussion of quality control will be found in Chapter 8.

#### 5 Cell sorting (see Chapter 4)

The ability of some flow cytometers to separate physically (sort) cells or organelles specifically identified during analysis adds a further dimension to the capability of these instruments. A simple, but very useful, application of flow sorting is the sorting of selected cells on to microscope slides for morphological

staining and light microscopy. Important information about the cell types in a complex mixture, particularly when the sample has not previously been analysed, can be gained in this way.

Although it is possible to analyse many cellular parameters directly, it may be necessary to determine cell function by other assay systems. Cell sub-populations can be identified and sorted using flow cytometry before their use in function assays. Flow sorters are particularly adept at identifying and purifying rare events, and have been used for the selection and direct cloning of transfected cells and hybridomas and for the isolation of fetal cells from maternal blood. The purification of specific chromosomes is an important use of flow sorting, and the DNA libraries generated from this material have had a major role in the Human Genome Project.

While other sorting systems have been developed—such as panning or magnetic microbead separation—these are generally single parameter methods, i.e. positively stained cells can be separated from negatively stained cells. Flow sorting has the major advantage that any combination of any analytical parameters can be used to set the criteria for sorting. For example, it is straightforward to define a population of cells which possess the forward and orthogonal scattering characteristics of lymphocytes and are bright for one antigen, dim for a second antigen, and negative for a third and to sort these cells into a tube as a highly purified fraction. It is also possible to generate even more sophisticated sorting gates by the application of other Boolean operators to combinations of sorting gates. For example, a population of cells could be sorted which is dim for one antigen OR is bright for a second antigen but does NOT possess the scattering characteristic of monocytes. By the combination of analytical gates in this way extremely specific sorting criteria can be generated.

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