

R. A. Diamond
S. DeMaggio
(Eds.)

In Living Color

Protocols in Flow Cytometry
and Cell Sorting



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In Living Color

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Rochelle A. Diamond Susan DeMaggio (Eds.)

In Living Color

Protocols in Flow Cytometry and Cell Sorting

With 199 Figures and 26 Tables



Springer

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Quality Control Guidelines for Research Flow Cytometry

ROCHELLE A. DIAMOND

■ Introduction

Accurate and reproducible test results are integral to all laboratories. Quality control methods identify and minimize sources of variation in instruments and reagents. In clinical laboratories, quality control methods require rigorous documentation, certification, and monitoring for reproducibility and accuracy. This entails validation of chain of custody information (sample source, collection procedures, transportation and storage of viable samples), documented sample preparation procedures, staining conditions, reagent performance, instrument reproducibility, validated measuring conditions, meaningful data analysis, and comparative reporting of data. The bottom line for clinical cytometrists is to provide valid comparisons of clinically relevant parameters within a sample, between samples, between laboratories, over time. For an in depth review of these issues see reference one¹ which provides a good bibliography on quality control for clinical applications.

Research laboratories, unlike clinical laboratories, are not officially regulated or monitored. The responsibility falls on the shoulders of the investigators themselves and/or the flow cytometry core facility operators. The guidelines presented here are addressed solely to these research oriented laboratories and are in no way intended to give advice on clinical quality control. Research investigators, however, can certainly take the clinical guidelines set forth by regulatory agencies, consensus committees, and quality control conferences and workshops as good procedural advice in order to maintain their experimental reproducibility over time.²

Determining flow cytometric instrument accuracy has been relegated in many research laboratories to optimizing instru-

ment performance and then monitoring over time to identify trends and drifts which may need attention for preventive maintenance or repair service. There are presently no certifiable standards for monitoring absolute accuracy, although there is some movement in that direction. There are, however, many commercially available control, calibration, and standardization materials that can be used to monitor the instrument on a daily basis. These consist of a variety of cell sized plastic dye encapsulated beads or fixed cell based particles which ideally give stable values over an extended length of time (months). They come in many spectral flavors and size ranges and should be selected to match the parameters of the reagents that you will be measuring. There are particles that are generally used for setting up and monitoring instrument performance because of their broad spectral range and uniformity in fluorescence peaks and scatter profile. The first step for open optical systems that are operator aligned is to optimize the instrument for optical and electronic performance. Using these beads, optical alignment is performed which involves adjusting the components of the system for maximal signal intensity, as measured by the mean channel number, and minimal variability, as measured by the standard deviation or coefficient of variation (CV) of the bead peaks registered for each detector. This may mean aligning lasers, optical filters, focussing lenses, flow cells, adjusting sheath streams, flow rates, photodetectors, and other conditions that may influence instrument performance.

Following optical alignment, initial values must be established for the calibration particles so that they can be monitored regularly. A quality control log should be started and the values recorded daily and actively followed. There are two general procedures that can be used to monitor daily optical performance (see protocols following this text). The first protocol measures instrument performance by monitoring the reproducibility of the particle mean intensities and CVs under specified instrument conditions on a day to day basis. The second protocol monitors the reproducibility of the instrument settings needed to achieve specified mean channel numbers for the alignment particles. This second protocol gives an indication of instrument drift over time. In either protocol, a range of acceptable variation is established which defines acceptable instrument performance. For fixed optical systems, such as the FACScan, the manufacturer has speci-

fications for certain alignment products tied to specific instrument parameters and settings that define acceptability. For operator aligned optical systems, a range of acceptability can be defined by running the protocol of choice with selected calibration particles under the same defined conditions for a repetitive number of times (>20) over time (>5 days), while recording all pertinent parameters for your specific application. The range is therefore established on the observed parameter (two mean standard deviations).^{1,2,3} Be sure to test and record new lots of calibration beads in parallel with the old lot using optimized instrument settings before switching lots. Some systems come with special software or suggested protocols for instrument set-up and monitoring. Commercial programs like QC Tracker (Phoenix Flow Systems) are available for monitoring this kind of information and keeping validation records. You can also create one yourself using a spreadsheet such as Excel to plot out weekly and monthly quality control data. Good record keeping and consistent monitoring of acceptable established performance ranges are essential to maintain control of instrument performance. Trends and drifts tracked graphically can give instantaneous warning of instrument problems that need to be investigated. This can minimize down time and help to maintain high quality data output.

Quality control is also used to establish conditions relevant to specific sample measurement protocols. Standard biological controls should be chosen to be as close to the unknown sample as possible in both scatter characteristics (size of the cells) and cell type. There are many variables in sample preparation. Positive and negative sample controls should be prepared simultaneously with the experimental sample. This kind of biological quality control establishes reagent quality and protocol validity for the assay system. The positive control should be designed to verify reagent and sample specificity and spectral compensation (see chapter this section on spectral compensation). The negative control should be designed to establish background information and report false positives (see Alice Givens chapter in section 3 on sample staining). If possible, controls for dim versus negative samples should be used to monitor sensitivity and autofluorescence. It is important to examine the literature and references provided by the various authors throughout this book to set up meaningful controls for your experiments. Clinical researchers should be aware of the literature for various consensus

conferences, workshops and committee publications³ such as the DNA Cytometry Consensus Conference⁴, the International Leukocyte Typing Workshops,⁵ and CDC MMWR recommendations and reports.⁶

■ Outline

Building a system for optimizing and monitoring quality control is individual to the laboratory and the instrumentation on hand. Still, common threads hold from lab to lab, which you can use as quality control guidelines for research flow cytometry:

- You should understand your instrument's operation, alignment, and optimization practices (consult the operator's manual and training course guide for recommended specifications and procedures).
- Minimum internal laboratory quality control procedures are important and should be clearly documented utilizing standard reagents.
- Choose relevant instrument parameters and establish initial ranges for instrument settings like photomultiplier tube voltage settings, compensation values and CV's of bead peaks.
- Monitor the above instrument conditions after alignment on a daily basis and keep a log or at least good records of the data. Tabulate or chart means and standard deviations for your parameters to generate data that makes trends and shifts easy to see. Progressive drift away from mean values or a sharp change from the values should be a cause for further evaluation, adjustment, or service call. If these procedures fail to produce expected values, common sense should prevail and the experiment delayed until the cause is tracked down and remedied.
- Use relevant biological controls to monitor your specific assay system for your instrument parameters. Establish an expected range of values for your control samples.
- Regularly review your quality control data on known control samples. Set cut-offs for acceptability or rejection of quality control data. Establish an overall monitoring frequency to evaluate the quality control system.
- Monitor your instruments before and after service calls. Document and monitor QC data for variability over the life-

time of your instrument. If you follow these guidelines you can have confidence in the reliability and reproducibility of the data generated by your flow cytometer.

Subprotocol 1

Quality Control for Standard Channel Settings

■ ■ Materials

Equipment

- Flow Cytometer

Solutions

- DNA-Check Fluorospheres - Coulter Cytometry part number 6603488

■ ■ Procedure

Clean
instrument
as needed

1. Clean instrument according to manufacturer's recommendations.

Align optics

2. Set up your instrument with optimal standard voltages and gains in linear mode. Create histograms for all parameters that you will be measuring.

Note: The following should be done daily after the instrument is warmed up.

Run
Fluorospheres

3. Run sample of alignment calibration beads. Be sure that the beads will fluoresce for all parameters to be measured. They should have reasonable scatter and fluorescence peaks. Adjust sample flow for 60-100 beads per second flow rate and let stabilize.

Note: We use DNA-check beads from Coulter Cytometry. Record lot number. Compare any new lot of beads with the current one in use before using routinely.

4. Align the optics so that the beads are optimally displayed for the standard voltages on your fluorescence detectors and for standard gains on both of your scatter detectors according to your manufacturer's instructions. **Adjust optics**
- Note:** Adjust for the brightest and tightest peaks possible for scatter signals and fluorescence signals according to manufacturer instructions (some machines cannot be aligned by the user/operator - ask for service if not meeting specifications).
5. Place the alignment /calibration beads in a specific channel for each detector that is standard on a daily basis by adjusting the flow cytometer voltages as necessary. **Assign beads to particular channel numbers**
6. Acquire data for the beads at these settings. **Acquire data for beads**
7. Record photomultiplier voltages and positions for each peak - mean, median, and CV **Record voltages and bead statistics**
8. Monitor the daily standardizations to identify any changes in performance by plotting the values over time. **Monitor daily and plot over time**
9. Compensate for spectral overlap daily with single color biological samples labeled with fluorochromes of interest respectively. Monitor changes for fluorochrome usage. **Compensate for spectral overlap and record settings**
10. Run relevant QC biological control samples that are negative, dim, and bright to determine sensitivity for each parameter to monitor. **Establish minimum expected range of biological control values**
11. Establish minimum acceptable distances between negative and dim peaks. Record values. Monitor for acceptability before performing subsequent assays. **Monitor and document QC data for variability over time**

Subprotocol 2 Quality Control for Specified Voltage Settings

■ ■ Materials

Equipment

- Flow Cytometer

Solutions

- DNA-Check Fluorospheres - Coulter Cytometry part number 6603488

■ ■ Procedure

- | | |
|----------------------------|---|
| Clean instrument as needed | 1. Clean instrument according to manufacturer's recommendations. |
| Align optics | 2. Set up your instrument with optimal standard voltages and gains in linear mode. Create histograms for all parameters that you will be measuring.

Note: The following should be done daily after the instrument is warmed up. |
| Run Fluorospheres | 3. Run sample of alignment calibration beads. Be sure that the beads will fluoresce for all parameters to be measured. They should have reasonable scatter and fluorescence peaks. Adjust sample flow for 60-100 beads per second flow rate and let stabilize.

Note: We use DNA-check beads from Coulter Cytometry. Record lot number. Compare any new lot of beads with the current one in use before using routinely. |
| Adjust optics | 4. Align the optics so that the beads are optimally displayed for the standard voltages on your fluorescence detectors and for standard gains on both of your scatter detectors according to your manufacturer's instructions.

Note: Adjust for the brightest and tightest peaks possible for scatter signals and fluorescence signals according to manufacturer |

instructions (some machines cannot be aligned by the user/operator – ask for service if not meeting specifications).

- | | |
|--|--------------------------------------|
| 5. Acquire 10,000 events. | Acquire bead information |
| 6. Analyze the histograms for half peak coefficient of variation (CV) and mean fluorescence of the bead peaks. | Analyze histogram statistics |
| 7. If CV's are greater than 2.0 then readjust the optical alignment until the values are under 2.0. The bead peaks should ideally be close to midrange of the histogram. | |
| 8. Record CV and mean values with the instrument settings in your records. | Record values and plot for variation |

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In Living Color

The goal of this book is to make this increasingly valuable technology accessible to researchers, including the students, post-doctoral scholars, and technicians who labor in the fields harvesting the grains of knowledge inherent in this integration between analysis and physical isolation/purification methodologies. A step by step approach to the methodology for measuring various cellular attributes demonstrated in the particular cells of interest is provided. In addition a myriad of resources to fuel the curiosity and answer questions of both the new and adept user is presented. The vision for this book stems from the editors' desire to encourage young investigators, researchers in emerging fields, and core facility operators to explore the use of flow cytometric and cell sorting techniques. The editors' experience and expertise draw from a combined total of 54 years in Laboratory Science, 30 years specifically in Flow Cytometry. They currently manage Core Facilities in 2 major Southern California Universities, servicing researchers in physiology, immunology, developmental, cellular, neural, and molecular biology disciplines.

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