Standardization, Calibration, and Control in Flow Cytometry

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Because flow cytometers are designed to measure particle characteristics, particles are the most common materials used to calibrate, control, and standardize the instruments. Definitions and cautions are provided for common terms to alert the reader to critical distinctions in meaning. This unit presents extensive background on particle types and cautions and describes practical aspects of methods to standardize and calibrate instruments. Procedures are provided to characterize performance in terms of optical alignment, fluorescence and light scatter resolution, and sensitivity. Finally, suggestions follow for analyzing particles used for calibration.

Keywords: standardization • calibration • control • particles • MESF (molecules of equivalent soluble fluorochrome) • ERF (equivalent number of reference fluorophores) • sensitivity • resolution

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INTRODUCTION

Standardization, control, and calibration provide different degrees of certainty about the data acquired with an instrument. Each process is aimed at ensuring that results from the instrument have the quality required for the intended purpose (Horan et al., 1990; NCCLS, 1998a; Muirhead, 1993a,b; Schwartz and Fernandez-Repollet, 1993; Owens and Loken, 1995; Schwartz et al., 1996; Owens et al., 2000). The purpose may be an individual research experiment or a clinical result that determines the longitudinal course of patient's response to treatment. In the terminology used in this commentary, an instrument is standardized at certain time points and subsequently operated under quality control conditions [see *UNITS 3.1 & 3.2* (Hurley, 1997a,b)]. These processes maintain the instrument within predetermined bounds and ensure that results will vary only within certain limits. If results are also calibrated when instruments are standardized, then future results can be objectively and quantitatively compared to future calibrated results or to calibrated results in other laboratories. Quantitation of results should be considered. Most results from flow cytometers are expressed either in terms of "percent positive" or in qualitative terms such as "dim" or "bright." These terms are relative: what is considered "negative," "dim," and "bright" in one laboratory may be quite different in another laboratory. When visualizing fluorescence using a fluorescence microscope, such relative terms are mostly sufficient, though performance benchmarking with control materials on microscopes has recently been tested (Halter et al., 2014). Flow cytometers can measure the amount of fluorescence and provide more objective criteria for expressing results.

As particles are the most common materials used to calibrate, control, and standardize the instruments, this commentary describes how various types of particles are used for these purposes. It also briefly reviews the status of standardization and quality control for flow cytometry (see Chapter 3 for further discussion of quality control). *UNIT 1.4* (Wood, 2009) covers calibration of detection system components (e.g., linear and logarithmic amplifiers) to ensure linearity of the flow cytometer response.

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The first section of this unit focuses on how the term "standard" has been used in flow cytometry (see Standards, Standardization, and Jargon). The intent is to alert readers of flow cytometry literature that they must always interpret critically how "standard" is being used in a particular context. The next section defines terms and also includes comments to put the term in context or to highlight issues (see Definitions).

After providing extensive background on particle types and cautions (see Overview of Standardization in Flow Cytometry), this unit describes practical aspects of methods to standardize and calibrate flow cytometers (e.g., in terms of optical alignment, fluorescence and light scatter resolution, and sensitivity; see Standardization and Calibration section). Finally, suggestions are given for analyzing particles used as calibrators, including how to assign to fluorescent beads a value for molecules of equivalent soluble fluorochrome (MESF) and equivalent reference fluorophores (ERF) and how to determine the inherent fluorescence coefficient of variation (CV) of a dim bead sample (see Characterizing Particles for Calibration and Control of a Flow Cytometer).

STANDARDS, STANDARDIZATION, AND JARGON

It is common in flow cytometry to combine words that describe use of a particle with the word "standard." Examples are "calibration standard" and "alignment standard" (Horan et al., 1990; Schwartz and Fernandez-Repollet, 1993; Schwartz et al., 1996; Shapiro, 2003). Rarely is there any indication of who has set the "standard" and by what authority or consensus.

There can be many levels of "standards," depending on the size and authority of the group that establishes them. For example, an individual laboratory or investigator may have standard practices or materials. A large clinical or research study may have standard practices and materials that are agreed to by all investigators involved in the study. A professional organization may establish standard methods or identify standard materials for specific purposes. If the word "standard" is not modified by a term such as "laboratory," "clinical trial," or "study XYZ," it may imply something that is generally and widely accepted by acknowledged authorities. In that authoritative sense, however, there are few "standards" in flow cytometry.

Clear and common understanding of what is meant by a term is important, especially as flow cytometry is used by increasing numbers of investigators. The verb "standardize" means to cause to be without variation. Early use of the noun "standard" in flow cytometry seems to have been in the sense of a particle used to standardize (make consistent) one instrument in one laboratory (Fulwyler, 1979). This is much different from the authoritative sense of "standard." In this commentary, other terms are used to describe more specifically what type of particle or material is being used for a particular purpose. For example, "calibration particle" or "calibrator" is used instead of "calibration standard," and "alignment particle" rather than "alignment standard."

DEFINITIONS

Concern with terminology and its evolution is not just semantics, but reflects what has been important in flow cytometer technology and how the technology has grown and changed. More precise and generally accepted terminology should clarify communication and understanding among flow cytometrists as well as scientists in other fields. Definitions related to quantitative fluorescence cytometry are provided by Henderson et al. (1998).

The definitions below should be considered a reasonable point along the way toward authoritative and broadly accepted and understood terminology. Some definitions include comments and references that may help put them in context.

Accuracy: degree to which a measurement agrees with the true or expected value.

Alignment particle: particle with uniform size, fluorescence, and light scatter characteristics that is used to check the alignment (or, in some instruments, adjust the alignment) of the excitation and emission optics in the flow cytometer. It is desirable that the alignment particle emit fluorescence in all detector channels, as this allows all channels to be checked simultaneously. Alignment of the optics is optimal when signals from the particles have maximum intensity and minimum variation or CV. The more uniform the particles, the better the degree to which small deviations from optimal alignment can be detected. Optimal alignment is most critical for measuring DNA, because of the very low inherent variation in DNA content from cell to cell.

Antibody binding capacity (ABC): number of antibodies of a particular type that can bind to a cell under saturating staining conditions. Researchers also use the term "ABC" to stand for "Antibodies Bound per Cell." This term may not always imply a requirement

for saturating staining partly due to interference caused by simultaneous staining of different kinds of antibodies on the same cell population.

Autofluorescence: inherent fluorescence from a cell or particle to which no stain or fluorochrome has been added. Manufactured particles (such as plastic beads) can be prepared to have nearly the same autofluorescence as lymphocytes.

Background (noise, fluorescence, scatter): signal present when no particles are flowing in the sample stream. Background noise is one factor that limits the sensitivity of fluorescence detection (see definitions of fluorescence sensitivity and light-scatter sensitivity below). Depending on how low the signals are that one is trying to detect in the sample, different factors are dominant contributors to the background. When no light is coming from the flow cell (e.g., lasers turned off), detector noise is the background limit. For photomultiplier tubes (PMTs), the detector background noise is called dark current and is due to random emission of electrons from the photocathode. For photodiodes and other solid-state detectors, which have no or low signal amplification, the limiting factor under best conditions is noise from the amplifier required to raise the signal to a useful level. Sources of fluorescence noise include Raman scatter from water and optical components; fluorescence from unbound fluorochrome, reagent, or contaminants in the sample or sheath stream; and fluorescence from optical components.

Calibration: process of adjusting an instrument so that the analytical result is accurately expressed in some physical measure.

Calibrator: material that has been manufactured or assayed to have known, *measured* values of one or more characteristics. The assayed values are provided with the material. Fluorescent manufactured particles can be assayed for diameter or for the amount of fluorescence they produce. A practical measure of particle fluorescence is the number of fluorochrome molecules in solution that produce the same amount of fluorescence as one bead (see definition of MESF and ERF).

Coefficient of variation (CV): statistical measurement of the broadness of a distribution of values, usually defined as $CV = \sigma/\mu$, where the standard deviation $\sigma = [\Sigma(x_i-\mu)^2/(N-1)]^{1/2}$, with the sum over N measurements of x_i (where x_i is the ith measurement of variable x), and the mean $\mu = (\Sigma\ x_i)/N$. Shapiro (2003) gives an excellent discussion of CV and other, more robust statistics for flow cytometry. Another excellent reference for statistical methods is Bevington (1969). A robust CV excludes outlier data and is typically defined using ranges of data including a specified percentage (e.g., 95%) of the data. In the case where there are no significant outliers, the robust CV is equal to the CV using the standard definition.

Control particle or material: stable material (e.g., sample of manufactured particles) that gives reproducible results when analyzed. Particles used to set up a flow cytometer are used as a control even if they do not have an assayed value assigned to a physical characteristic. Controls can be used to monitor the stability of an instrument and determine whether it is acceptably within calibration. A calibrator can be used as a control material, but a control material does not have to have an assigned value for a characteristic.

Control sample: sample prepared in the same or nearly same way as a test or unknown sample and which should give an expected, predetermined result. In immunofluorescence analysis, a positive control sample may use known cells (characterized for reactivity to a panel of antibodies) and the same antibody reagents as the test sample. A negative control sample may use the test cells but without antibody reagent or with an irrelevant antibody reagent. Also, the positive control sample can contain some negative cells which function as an internal negative cellular control. This can also be referred to as an internal isotype control.

Fluorescence sensitivity: In flow cytometry, there are two different aspects to the notion of sensitivity: threshold and resolution. The first has to do with the smallest amount of light that can be detected (Wood, 1993; Owens and Loken, 1995; Schwartz et al., 1996; Shapiro, 2003). This notion has also been given the name "detection threshold" (Schwartz et al., 1996). The second has to do with the ability to resolve dimly stained cells from unstained cells in a mixture (Brown et al., 1986; Horan et al., 1990; Shapiro, 2003). These concepts do not measure the same thing. The second notion incorporates a measure of the broadness of the fluorescence distributions for dim and unstained particles, not just the average fluorescence. Two instruments can have the same detection threshold but differ significantly in ability to resolve a dimly stained population. This is illustrated by example later (see Standardization and Calibration Section).

1. Degree to which a flow cytometer can measure dimly stained particles and distinguish them from a particle-free background (threshold). Threshold is important when the mean fluorescence of a dimly fluorescent population is measured. The greater the number of particles analyzed, the more accurately and precisely will the mean fluorescence be measured.

2. Degree to which a flow cytometer can distinguish unstained and dimly stained populations in a mixture of particles (resolution). Resolution is important for immunofluorescence analysis of subpopulations and is strongly affected by the measurement CVs for dim and unstained particles.

Inherent sample CV: actual variability in the characteristics of a sample; for example, the actual variation in the amount of fluorochrome per bead in a sample of beads. Because the measurement process is not perfect and itself adds variation, the CV of the measured fluorescence will be greater than the inherent sample CV. The inherent CV of a sample can be estimated within a small uncertainty if the measurement variability added by the flow cytometer is well characterized (see Determining Inherent Fluorescence CV of a Dim Particle Sample).

Light-scatter sensitivity: degree to which small particles can be detected above "particle-free" fluid. In practice, forward-scatter sensitivity is usually limited by optical noise caused by the excitation source, and side-scatter sensitivity is usually limited by submicron particles in the sheath fluid.

Limit of detection: the lowest amount of analyte in a sample that can be detected but not quantified as an exact value.

Limit of quantitation: the lowest amount of analyte in a sample that can be quantitatively determined with acceptable precision and accuracy under stated experimental conditions.

Manufactured particles (beads, plastic beads, latex particles, microspheres, microbeads): particles made of synthetic polymers (plastics). Sizes range from submicron to over 100 μm, which generally covers the range of cells analyzed in flow cytometry. Most manufactured particles are made by bulk polymerization, but very uniform beads can be made employing the same droplet generation principle used for flow cytometric cell sorting (Fulwyler et al., 1973). Colored or fluorescent particles can be made by staining the beads with dyes or fluorochromes. Nonfluorescent beads, as well as many fluorescently stained beads, seem to be stable for many years. Two methods, namely, solvent (or "hard") dying and surface staining, are used to stain particles. In solvent staining, non-water-soluble dyes are mixed with the particles in an organic solvent. The particles take up the dye and are then suspended in aqueous solution. The dye is trapped in the beads, which essentially become a "hard-dyed" plastic material. In some cases, hard-dyed particles can be synthesized directly using fluorescent monomers (Rembaum, 1979). As most dyes or fluorochromes used to stain cells are water soluble, solvent staining cannot generally be used for them. When solvent staining is possible for water-soluble fluorochromes, the spectral characteristics can differ significantly from those of fluorochrome in aqueous solution. Surface staining allows many common fluorochromes—especially those used as tags on fluorescent antibodies—to be used for particle staining. In this case a chemical group on the particle surface (e.g., amino group) is covalently bound to a reactive group on the fluorochrome.

MESF (molecules of equivalent soluble fluorochrome) and ERF (equivalent number of reference fluorophores): measure of particle fluorescence in which the signal from a fluorescent particle is equal to that from a known number of molecules in solution. The ERF unit is different from MESF in that the fluorophores attached to particles and the fluorophores in solution can be very different and may have very different molar absorptivities. Hence, MESF is a special case of ERF where the labeling fluorophore and fluorophore in solution are the same. This is a practical measure because a known concentration of particles can be compared directly with a solution of fluorochrome in a spectrofluorometer (see Calibrating Particle Fluorescence in ERF).

Nonfluorescent particle: particle whose fluorescence distribution is the same as that of a particle-free sample. In practice, the concept of nonfluorescence is dependent on the sensitivity of the instrument making the measurement. A particle that is not measurably fluorescent in one instrument may be so in a more sensitive instrument. Fluorescence (or other luminescence or Raman scatter) from otherwise unstained manufactured particles depends on the material and treatment with which the beads are made. With all other factors equal, the "fluorescent" signal from microbeads will be proportional to the volume of a single bead.

Precision or reproducibility: degree to which repeated measurements of the same thing agree with each other. In flow cytometry, precision of a measurement is estimated by the CV obtained when measuring a sample of particles (biological or nonbiological) with very uniform characteristics multiple times.

Resolution: degree to which a flow cytometry measurement parameter can distinguish two populations in a mixture of particles that differ in mean signal intensity. Fluorescence sensitivity (see above) can be considered a special case of fluorescence resolution for which the signals are very dim. Note that the resolution will appear different when data are acquired and/or displayed on a logarithmic rather than linear intensity scale. Depending on the maximum number of channels into which the signal intensity is acquired (e.g., 256 or 1024), a logarithmic display of the data may not have sufficient resolution to display populations that can actually be resolved by the instrument.

Standard: 1. noun. (a) acknowledged measure of comparison for quantitative or qualitative value; (b) something recognized as correct by common consent or by those most competent to decide.

2. adj. (a) serving as a standard of measurement or value; (b) commonly used and accepted as an authority.

Standardize: (a) cause to conform to a given standard; (b) cause to be without variation.

Test pulse–triggered background fluorescence: measurement of background fluorescence in a flow cytometer by using an electronic pulse to trigger the pulse detection electronics and acquire data from the fluorescence detector(s) (see *UNIT 1.4*; Wood, 2009). As no particle is present to emit light, the fluorescence signals acquired are due only to instrument background light and noise, and thus establish the lowest signal that can be measured. The duration of a test pulse usually simulates a signal from a particle of typical size. Larger particles would have signals of longer duration and produce more background signal and noise. If equipped with a test pulse function, the flow cytometer can provide a measurement equivalent to running a sample of truly nonfluorescent particles. The background fluorescence distribution produced by a test pulse should provide a measure of the "detection threshold" described by Schwartz et al. (1996). In many instruments, the test pulse signal produces a pulse of light from a light-emitting diode that is detected and processed by only one detector. When the test pulse signal is applied only to the forward scatter detector, the response of all other detectors to background light and noise can be measured. When a sample is run under normal conditions, any signal from particles above this background and noise level actually comes from the particles. There is no guarantee, however, that the particle signal is from particle fluorescence; for example, light scattered by the particles may not be totally blocked by the optical filters, or in some cases, the light scatter may actually induce the filter to fluoresce. The possibility of scatter-induced light detected as fluorescence signal can be checked by running unstained cells and looking for a signal in the fluorescence channel. Because such a signal can also come from autofluorescence, one should also look at the side scatter versus fluorescence histogram for a strong correlation between side scatter and fluorescence from unstained cells.

OVERVIEW OF STANDARDIZATION AND PERFORMANCE CHARACTERIZATION IN FLOW CYTOMETRY

Standardization (see Definitions) is the foundation of flow cytometry and allows investigators to have confidence in instrument performance and measurements. This section surveys characteristics of particles used in flow cytometry, for example, to standardize immunofluorescence and to check alignment and measurement precision (see Types of Particles). Specific types of particles are compared. Standardization can be complicated, however, by factors other than particle type (see General Cautions for Using Particles in Standardization and Calibration; see What the Instrument Cannot Control: Sample, Reagent, and Data Analysis), but prospects for formalizing flow cytometry standards are encouraging (see Standard-Setting Organizations). The next section (see Standardization and Calibration) reviews various parameters of flow cytometers that can be standardized, such as resolution and sensitivity, and the final section (see Characterizing Particles for Calibration and Control of a Flow Cytometer) describes procedures and cautions for characterizing particles.

Where appropriate in the discussion of standardization, approaches to characterizing instrument performance will be included. Performance characterization is different from standardization in that it provides information about limitations or limits of performance.

Examples included fluorescence sensitivity for resolution of dim populations and linearity limitations of computed fluorescence compensation.

Types of Particles

Manufactured particles and biological particles may be used to standardize flow cytometers. Beads may be spectrally matched to the fluorochromes used to stain cells, or they may simply fluoresce to a useful extent in the spectral range of interest. Spectrally matched beads allow standardization or even calibration across instruments that do not have exactly the same emission filters and/or excitation wavelengths. Biological particles may be stained with the same fluorochromes used in experiments to stain cells. Examples of data for some of the more common types of particles follow. Classification schemes for various types of particles used for standardization in flow cytometry have been proposed (Schwartz et al., 1996, 1998).

Comparison of spectrally matched and unmatched fluorescent particles

Figures 1.3.1 and 1.3.2 show emission spectra from three types of particles: fluorochrome-tagged beads (CaliBRITE beads, BD Biosciences), stained with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE); broad-spectrum hard-dyed beads (Rainbow beads, Spherotech); and glutaraldehyde-fixed chicken red blood cells (gCRBC, BioSure). Figure 1.3.1A compares FITC-stained CaliBRITE beads with Rainbow beads and gCRBC, which are not spectrally matched to FITC. Figure 1.3.2A makes the same comparison with PE-stained CaliBRITE beads. Figure 1.3.1B compares quantitatively the fluorescence signal of each particle through filters of differing spectral bandwidth placed in front of PMT1, with data being normalized to the signal from FITC CaliBRITE beads for each filter. The gCRBC varied by about 35% over the range of filters used. Rainbow beads, however, varied by nearly 200% with the same filters. The differences in relative fluorescence with different filters should be considered when comparing different instruments. Even for a particular flow cytometer type or model, filters and other factors affecting spectral response vary slightly due to manufacturing tolerances. Figure 1.3.2B shows a similar comparison for the relative fluorescence with different filters placed in front of PMT2. In this case, both gCRBC and Rainbow beads vary only slightly from PE-stained CaliBRITE beads. The fluorescence could be standardized with a maximum difference of 40% with any of these particles.

A large, multi-laboratory, multi-instrument study has been performed to estimate the variability of instrument calibration and standardization using several different hard-dyed beads compared to beads surface stained with antibody conjugated to four different common fluorophores. (Hoffman et al., 2012). Across 135 different flow cytometers the fluorescence of various hard dyed beads had considerable variation compared to fluorescence of beads stained with antibodies conjugated to specific fluorophores (FITC, PE, APC, and Pacific Blue). Figure 1.3.3 serves as an example and shows the variability of hard dyed bead fluorescence in the detector channel defined as FITC channel. Within a particular instrument model, most hard-dyed beads had fluorescence variation of at least 20% referenced to fluorophore-conjugated antibody fluorescence. This study concludes that the spectrally unmatched hard-dyed beads can be used as fluorescence calibrators but have to be verified for every instrument model and bead. Calibrators that are stained with the specific fluorophore of interest, e.g., FITC, PE, etc., are more suitable for reproducible fluorescence calibration and standardization across different instrument platforms.

"Nonfluorescent" and autofluorescent particles

Figure 1.3.4 shows emission spectra for particles with very low fluorescence. Unstained CaliBRITE beads have fluorescence comparable to autofluorescence from lymphocytes. Osmium-fixed chicken red blood cells (CRBC) had no fluorescence detectable above background in the fluorometer. Such "negative" particles are useful for estimating how well low-level signals can be detected, as discussed later (see Sensitivity or Signal/Noise for Dim Fluorescence). An alternative type of "non-fluorescent" particle is submicron polystyrene beads (e.g., 0.5 μm diameter), which are easily triggered by side scatter, but, because their volume is at least 100 times less than typical particles used for standardization, have also 100 times lower autofluorescence, which (with the possible exception of using UV or violet excitation) is essentially below the detection threshold of commercial flow cytometers. As the sensitivity of instruments improves, "nonfluorescent" particles that had not previously been detectable may no longer serve as as "nonfluoresent." The

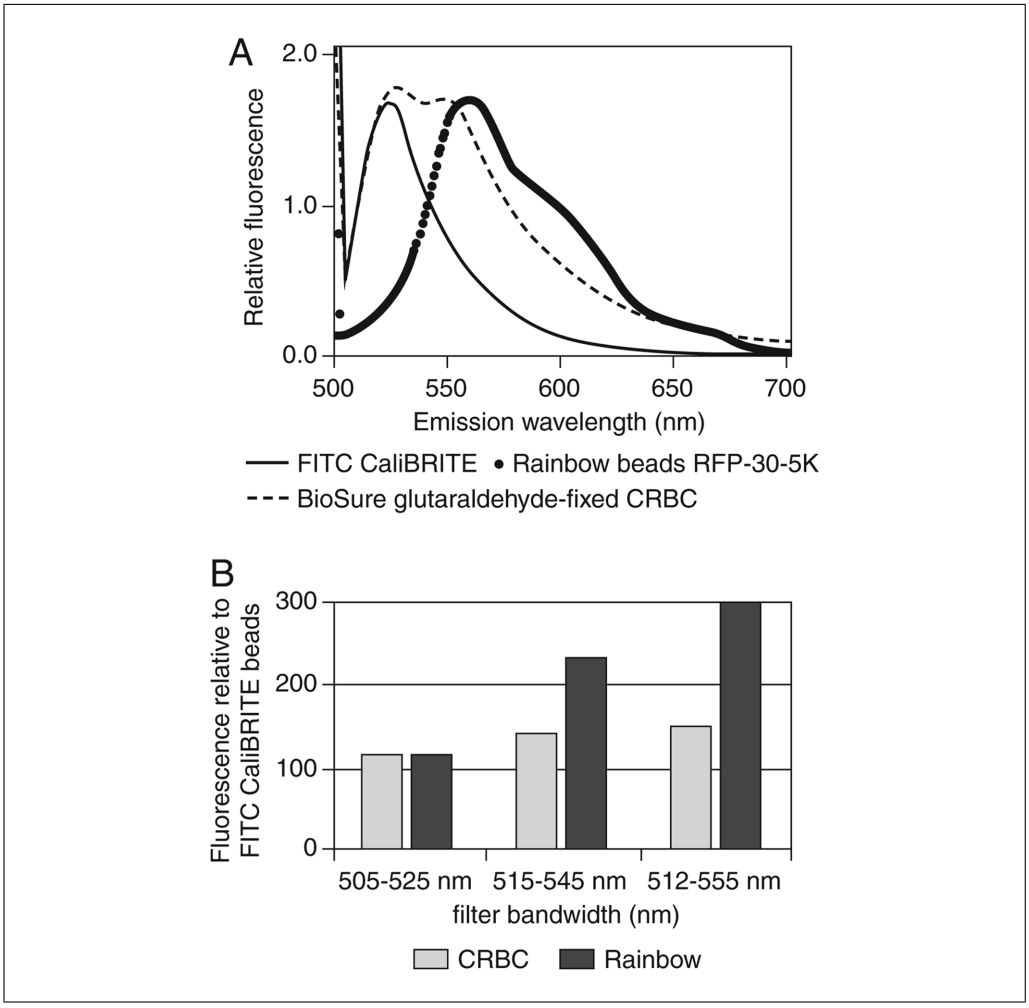

Figure 1.3.1 (**A**) Fluorescence emission spectra of FITC CaliBRITE beads (BD BioSciences), Rainbow beads RFP-30-5K (Spherotech), and glutaraldehyde-fixed chicken red blood cells (gCRBC, BioSure). Excitation at 488 nm was used. (**B**) Percentage of fluorescence signal through different optical filters for Rainbow beads and gCRBC normalized to signal from FITC CaliBRITE beads. Data in **B** are also scaled relative to the signal through the 505 to 525–nm filter.

only true nonfluorescent event is an event with no particle present, which can be achieved using an electronic or optical signal to trigger data acquisition without providing any signal to the fluorescence detector.

Comparison of particles for standardizing immunofluorescence analysis

Figure 1.3.5 shows light-scatter dot plots (panel A) and green (515 to 545 nm) fluorescence histograms (panels B-F) for several types of particles used to standardize flow cytometers for immunofluorescence analysis. Fluorescence from the stained particles is in the range observed for immunofluorescence from cell-surface markers. All data were acquired using the same instrument settings, and panels A-D were obtained from the same sample acquisition of a mixture containing (1) unstained (autofluorescence) and FITC-stained CaliBRITE beads, shown in region R1 in panel A; (2) a combination of unstained and multiple levels of stained Rainbow beads, shown in region R2 in panel A; (3) gCRBC, shown in region R3; and (4) forward-scatter (FS) test pulses (no particle, R4 in panel A). Fluorescence histograms in Figure 1.3.5 are from unstained and FITC CaliBRITE beads (panel B); Rainbow beads (panel C); FS test pulses and gCRBC (panel D); Quantum 24 beads (Flow Cytometry Standards; panel E); and osmium- and glutaraldehyde-fixed CRBC (panel F). Panels B, D, and F illustrate different pairs of particles or signals at the low and high ranges of a scale for immunofluorescence. The Quantum 24 beads shown in Figure 1.3.5E had calibration values for the stained beads (upper four peaks in the histogram) of 4,201, 16,936, 37,466, and

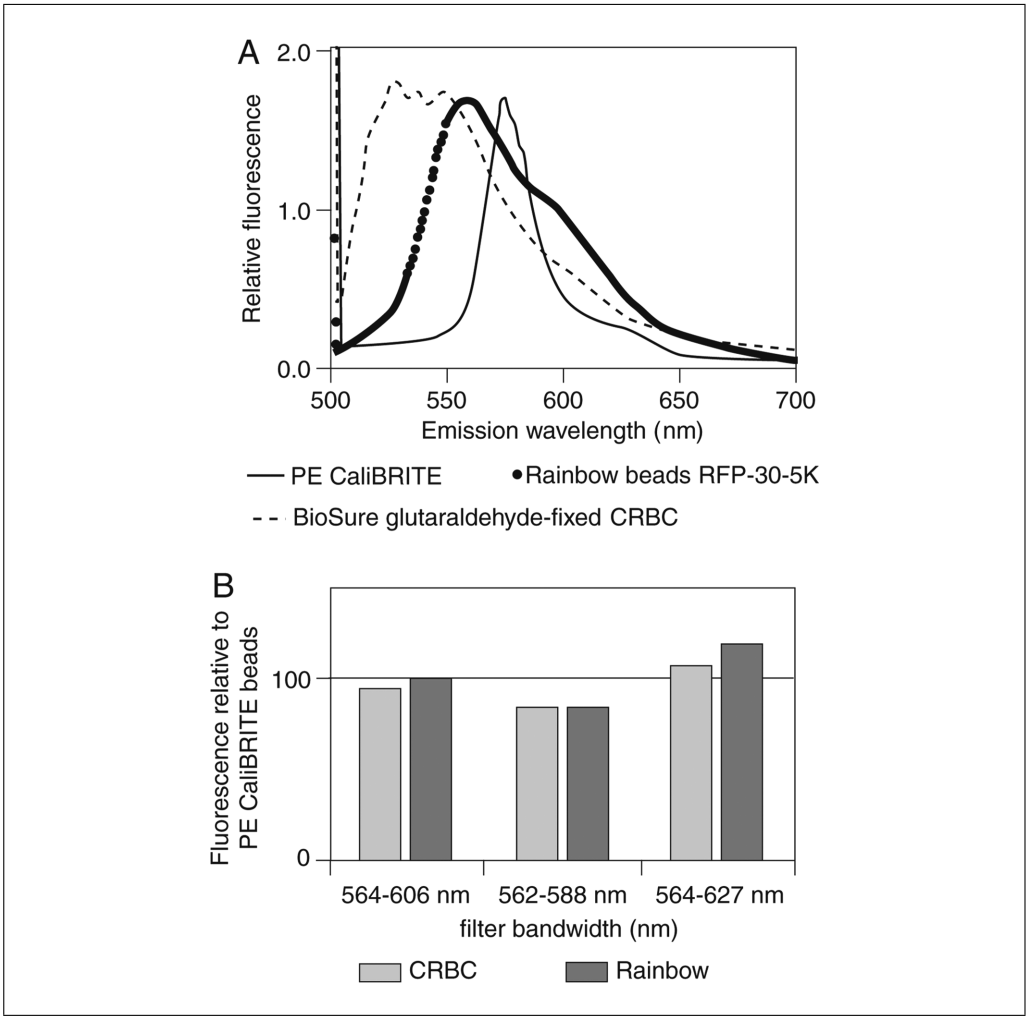

Figure 1.3.2 (**A**) Fluorescence emission spectra of PE CaliBRITE beads (BD Biosciences), Rainbow beads RFP-30-5K (Spherotech), and glutaraldehyde-fixed chicken red blood cells (gCRBC, BioSure). Excitation at 488 nm was used. (**B**) Percentage of fluorescence signal through different optical filters for Rainbow beads and gCRBC normalized to signal from PE CaliBRITE beads. Data in **B** are also scaled relative to the signal through the 564- to 606-nm filter.

65,797 fluorescein MESF (molecules of equivalent soluble fluorochrome) provided by the vendor.

In the same multi-laboratory, multi-instrument study whose results are illustrated in Figure 1.3.3, four different bead manufacturers used identical protocols and materials to assign ERF values to beads. Considerable variation in results was found among manufacturers compared to values measured by the National Institute of Standards and Technology (NIST; Hoffman et al., 2012). To provide reproducible and traceable fluorescence intensity assignments to beads, NIST has established a flow cytometry quantitation consortium under which a service of the ERF intensity value assignment to beads will be provided to bead manufacturers.

Particles for aligning and checking measurement precision

Figure 1.3.6 shows scatter and fluorescence data for a uniform 2.49-μm-diameter fluorescent bead that is useful for checking or adjusting optical alignment. All fluorescence CVs were <2%. A low fluorescence CV is an important performance characterization, particularly for DNA content measurements where for non-replicating cells the biological variation is essentially zero.

General Cautions for Using Particles in Standardization and Calibration

There are two important factors to remember when using manufactured particles rather than cells in a flow cytometer. First, beads are not cells and do not necessarily scatter light

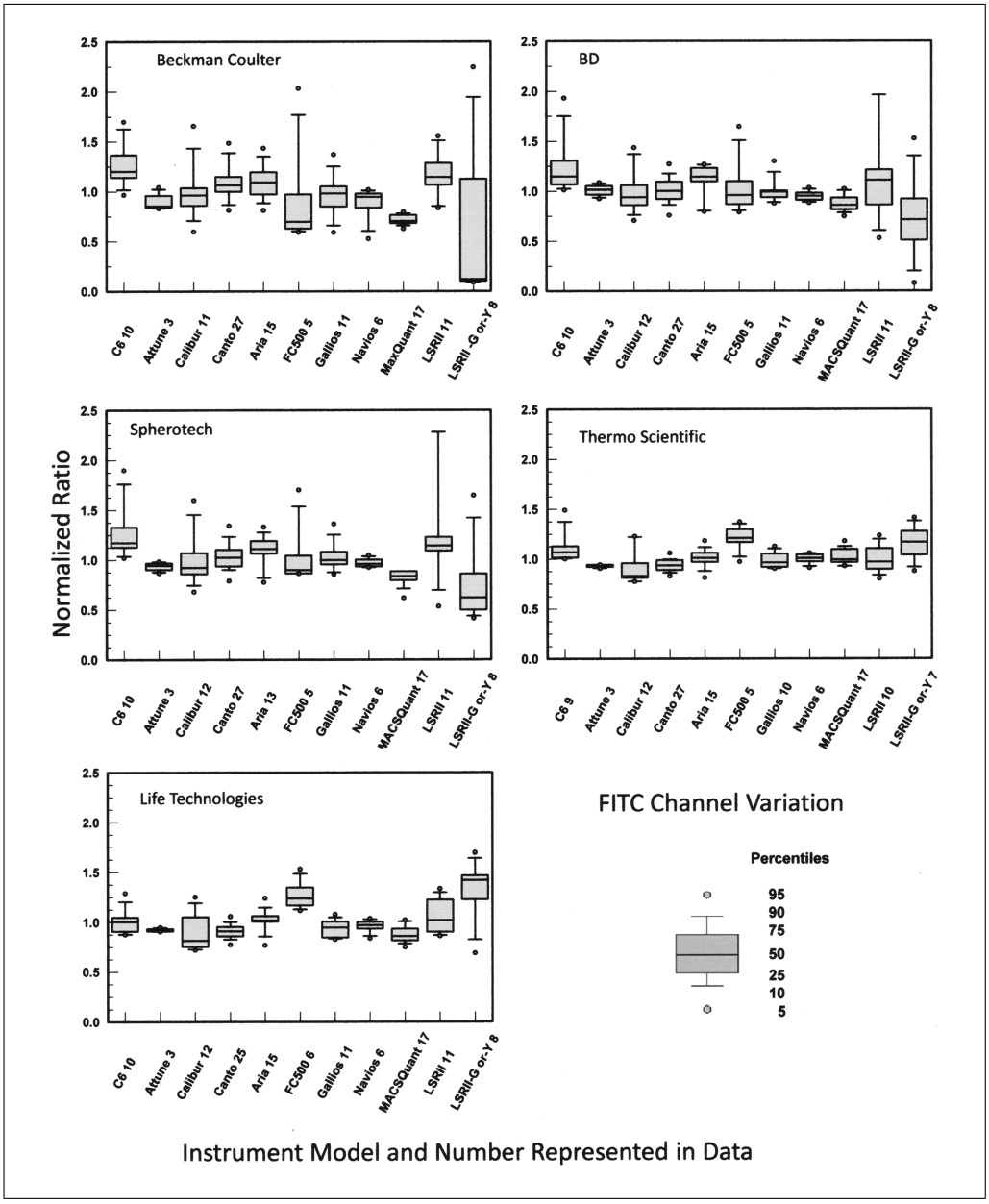

Figure 1.3.3 Box and whisker plots of the normalized ratio of the MFI of the indicated hard-dyed beads to the MFI of the FITC fluorophore standard bead for 10 different flow cytometer models. The box shows the 25th to 75th percentiles, and the line in the box indicates the median value. Horizontal bars outside the box indicate 10th and 90th percentiles and the circles indicated 5th and 95th percentiles. The percentile markers indicate the percentage of instruments for which the cross calibration was within the indicated normalized range. The number of instruments represented for each instrument model is noted after the model name on the *x* axis of each plot. (This figure is from Hoffman et al., 2012, Cytometry Part A, 81A, 785).

as cells do. Second, fluorescence from a bead may be similar to that from a cell stained with a particular dye, but it is almost never identical.

Regarding the first point, light scatter from beads usually differs greatly from scatter from cells of the same size. This is primarily due to differences in the optical refractive indexes of beads and cells. Beads with high water content and consequently low refractive index, such as Sephadex chromatography beads, give light scatter results similar to those from cells (Sharpless et al., 1977). However, such commercially available beads have a wide distribution of diameters and are difficult to use in standardizing an instrument.

Regarding the second point, fluorochromes used in hard-dyed beads are rarely the same as those used to stain cells. Even when the

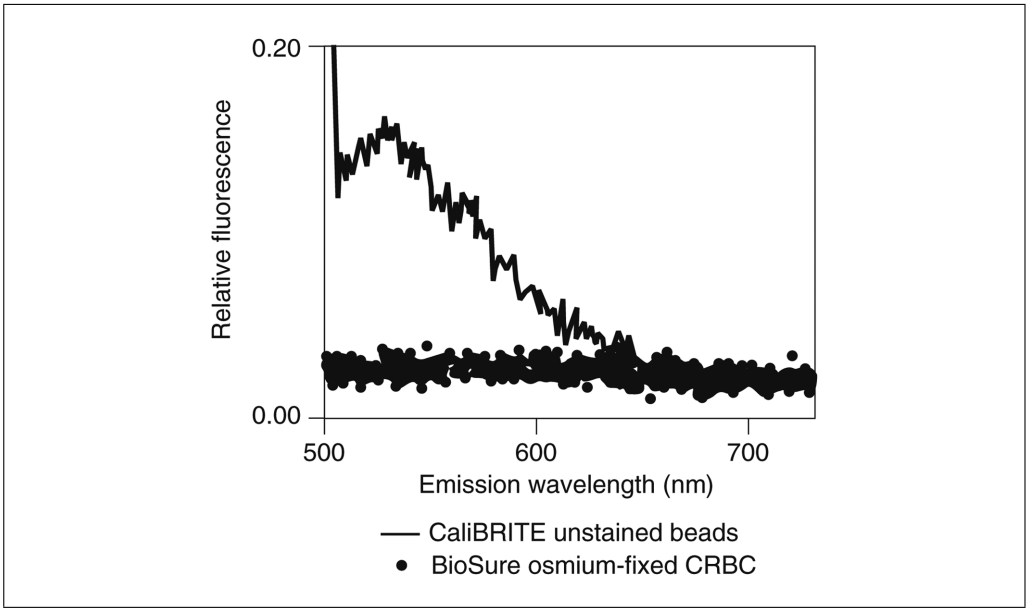

Figure 1.3.4 Fluorescence emission spectra of unstained CaliBRITE beads (BD Biosciences) and osmium-fixed chicken red blood cells (CRBC, BioSure). Excitation at 488 nm was used, and concentrations of the two types of particles were the same. No fluorescence from the osmium-fixed CRBC could be measured above background noise in the spectrofluorometer.

fluorescence emission spectra of a hard-dyed bead and a fluorescently stained cell are closely matched, it will be rare that the excitation spectra also match unless bead and cell are stained with the same fluorochrome. Also, one needs to be aware of a possible nonlinear relationship between the fluorescence signal and the intensity of light used to illuminate the particles [*UNIT 1.4* (Wood, 2009) discusses system linearity]. The relative fluorescence of two different fluorochromes can differ considerably with the intensity of excitation light (Bohmer et al., 1985).

Fluorochromes such as FITC and PE, which are used to tag antibodies, are available on surface-stained beads, and such beads closely match the fluorescence characteristics of cells stained with tagged antibodies. The surface-stained beads have the same excitation spectrum and sensitivity to excitation light intensity as do cells stained with the same fluorochromes. However, surface-stained beads in suspensions are less stable compared to hard-dyed beads, and, hence, have limited shelf life. Freeze-dried surface-stained beads have a longer shelf life.

What the Instrument Cannot Control: Sample, Reagent, and Data Analysis

It is important to keep in mind the factors that affect results but are beyond the control of the flow cytometer. A well-calibrated instrument and careful quality control cannot correct for samples and reagents that are not properly maintained, prepared, and used (see Chapter 4 for information on molecular and cellular probes; see Chapter 5 for specimen handling, storage, and preparation). Good data produced by the instrument cannot guarantee correct results if data analysis is wrong (see Chapter 10 on data processing and analysis). The flow cytometer hardware is only one part of the system that must work correctly to give good results. Owens and Loken (1995) provide an excellent and instructive introduction to the entire range of factors that affect results of flow cytometric analyses commonly performed in clinical laboratories.

Standard-Setting Organizations

The Clinical and Laboratory Standards Institute, an international clinical laboratory standards–setting organization, has established several guidelines specifically for flow cytometry or that apply to flow cytometry. The list (at the time of this writing) includes H42-A2 (Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline- Second Edition); H43-A2 (Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline- Second Edition); and H52-A2 (Red Blood Cell Diagnostic Testing; Approved Guideline- Second Edition).

The CLSI guideline H42-A2 (CLSI, 2008) takes a conservative approach in stating "There

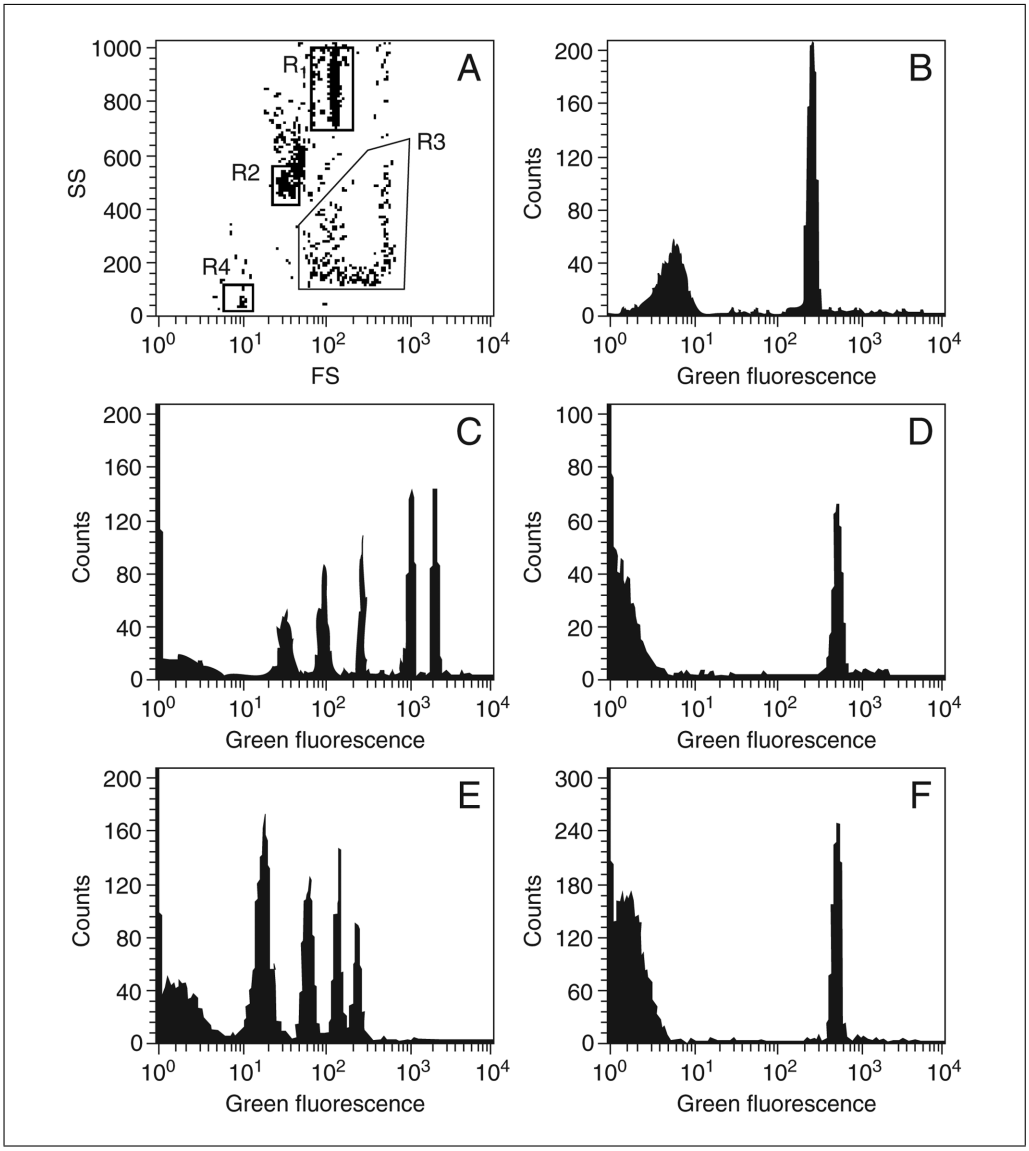

Figure 1.3.5 Light scatter and green fluorescence distributions from several types of standardization particles or sources. All data were acquired with identical instrument settings, and pulse height was measured. (**A**) Forward scatter (FS) versus side scatter (SS) dot plot of mixture of beads and test pulse signals. Each type of particle or the test pulse is enclosed by a region in the dot plot: region R1 contains unstained and FITC CaliBRITE beads or Quantum 24 beads; R2 contains the Rainbow bead mixture RCP-30-5K; R3 contains glutaraldehyde-fixed chicken red blood cells (gCRBC). Region 4 contains forward-scatter test pulses, which allow background noise from all other parameters to be measured. (**B**) Green fluorescence histogram of unstained and FITC CaliBRITE beads. Unstained beads have nearly the same autofluorescence as lymphocytes. (**C**) Green fluorescence histogram of Rainbow bead mixture containing unstained beads and five levels of stained beads. (**D**) Green fluorescence histogram of background noise from test pulse–triggered acquisition (region R4 in panel A) and gCRBC (region R3 in panel A). (**E**) Green fluorescence from mixture of Quantum 24 FITC beads from Flow Cytometry Standards. (**F**) Green fluorescence histogram of osmium-fixed CRBC (low population) and gCRBC (high population).

are at present no standards which can be used to check the accuracy of flow cytometric test results. Hence, verifying reproducibility of instrument performance is an essential element of daily quality assurance for the flow cytometry laboratory. Instrument performance must be monitored under the same conditions as are used to run test samples." The guideline proposes a two-step procedure for instrument quality assurance. First, establish that the instrument performance is acceptable at a particular point in time. Then, monitor performance with stable materials under test-specific instrument conditions. No specific criteria for

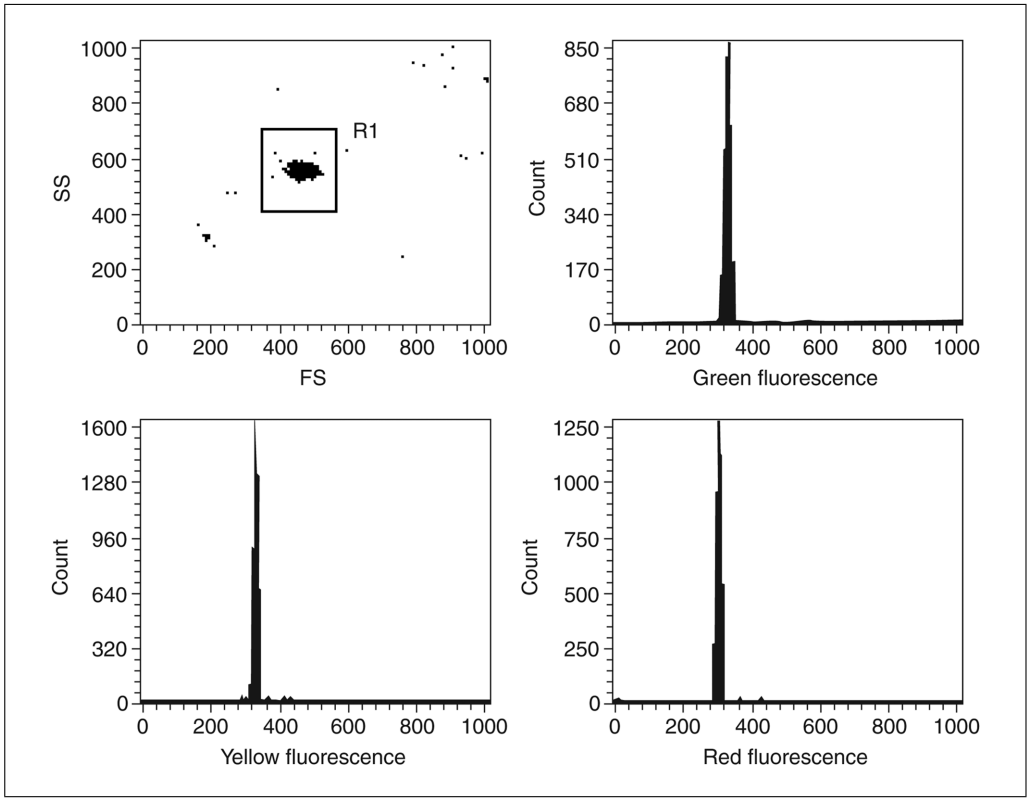

Figure 1.3.6 Scatter (FS and SS) dot plot (**A**) and fluorescence histograms (**B-D**) of 2.49-μm-diameter beads stained with Nile red.

calibration or control materials are given. The guideline instead provides a process that a laboratory can follow by using materials recommended or supplied by the instrument manufacturer or by establishing independent criteria and materials. [Further discussion of the principles of quality control is given in *UNIT 3.1* (Hurley, 1997a); applications of quality assurance in phenotyping and in nucleic acid analysis are covered in *UNIT 6.1* (Hurley, 1997c) and *UNIT 7.2* (Darzynkiewicz, 2011)

A guideline for validation of clinical tests developed in-house by clinical laboratories was written by a working group sponsored by the International Clinical Cytometry Society (ICCS) and the International Council for Standardization of Haematology (ICSH). This guideline addresses instrument and analytical issues (Tanqri et al., 2013) and assay performance criteria (Wood et al., 2013).

NIST has developed a method for determining particle MESF (Gaigalas et al., 2001; Schwartz et al., 2002) and has produced a standard fluorescein solution (Standard Reference Material 1932). Practical issues to consider in using MESF in quantitating fluorescence have been addressed in detail by NIST (Wang et al., 2002). Because of the increasing demand for multiparameter flow cytometric measurements, e.g., 12 color clinical cytometry assays, NIST has further developed a new standard reference material, SRM 1934, to support the calibration of microspheres in the units of ERF. The SRM 1934 includes four fluorophore solutions or suspension, Fluorescein, Nile Red, Coumarin 30, and Allophycocyanin for ERF value assignment performed with the three laser excitations most commonly used in commercial flow cytometers, 405 nm, 488 nm, and 633 nm. A standard operating procedure for ERF value assignment using this SRM 1934 has been documented recently by NIST (Wang et al., 2016a). Uniformed ERF value assignment to calibration microspheres ensures the traceability of the value assignment and enables the standardization of the fluorescence intensity scale of flow cytometers in quantitative ERF unit.

INSTRUMENT PERFORMANCE CHARACTERIZATION, STANDARDIZATION AND CALIBRATION

A flow cytometer may be characterized or standardized by running samples, such as manufactured particles, that have some known properties. Controlling the parameters and characteristics provides consistent results

over time from one instrument but, unless the instrument was calibrated, does not necessarily allow results to be quantitatively compared with those from other instruments. Performance characterization gives detailed quantitative information about the analytical capabilities of a flow cytometer such as linearity of the measurement scale, sensitivity to measure dim fluorescence, resolution of small differences in DNA content, or an estimate of the smallest size of particles that can be detected by light scatter.

Flow cytometers have many parameters that can be standardized and controlled (e.g., see Optical Alignment; see Light Scatter and Particle Sizing; see Fluorescence and Light Scatter Resolution; see Measurement Response and Logarithmic Calibration; see Sensitivity or Signal/Noise for Dim Fluorescence; and see Spectral Overlap Compensation). This section also discusses aspects of standardization relating to specific applications (see DNA Measurements; see Sorting Purity and Recovery; see Standardization with a Particle in the Analysis Sample; and see Particle Concentration). The final subsection provides a summary (see What Measurements Can Be Calibrated, and How Frequently Is Calibration Necessary?). Actual procedures and guidelines for characterizing beads follow (see Characterizing Particles for Calibration and Control of a Flow Cytometer).

Optical Alignment

Optical alignment is most critically assessed and most easily optimized using particles with very uniform scatter and fluorescence. The objective of optical alignment is to center the sample stream in the light beam and simultaneously image the intersection of sample stream and light beam through the detection optics. At optimal alignment, signal pulses will have maximum amplitude and minimum width, and be most reproducible. In a histogram of the fluorescence or scatter, the distribution will be at its narrowest with highest amplitude. The most uniform particles are generally smaller (1 to 3 μm) than typical cells. Although much more difficult to make, larger uniform particles are available. Fluorescence CVs <2% can be expected for alignment particles (see Fig. 1.3.6). Other than cell sorters, most modern flow cytometers have a fixed alignment that is not intended to be adjusted by the user. The objective then is to evaluate the alignment using the CV of the uniform fluorescent particles. Instrument manufacturers provide expected and maximum CV's from defined alignment particles. There will also typically be expected CVs for light scatter. Some manufacturers give specifications for low sample flow rate (minimum sample stream diameter) as well as for high sample flow rate (maximum sample stream diameter) which will have a larger CV due to wider range of particle position within the excitation laser beam.

Light Scatter and Particle Sizing

Scatter

Light scatter is a difficult parameter to standardize because it depends critically on the scatter angles measured and the geometry of the collection optics. The scattering of particles has strong nonlinear dependence on the angles measured and the refractive index of the particle, as well as particle size, internal structure, and content (Salzman et al., 1990; Doornbos et al., 1994; Shapiro, 2003). It is even possible at certain scatter angles to have a smaller signal from larger particles. The refractive index of most manufactured particles is much larger than that of cells, and beads and cells of the same size have different scatter intensities. For these reasons, it is not advisable to use scatter for quantitative cell sizing. Scatter is primarily useful for discriminating cells based on relative scatter properties (e.g., discrimination of lymphocytes, monocytes, and granulocytes is a common application).

Although it is difficult to standardize light scatter across instrument types using manufactured particles, it is possible to standardize within a particular instrument type and to monitor the relative performance of an instrument. The relative and absolute scatter intensities of two different-sized particles provide one way to standardize scatter. The ellipsoidal shape of fixed chicken red blood cells (CRBC) produces a characteristic light scatter pattern with two peaks in the forward scatter distribution. Region R3 in Figure 1.3.5A shows an example of the light scatter distribution for CRBC. Although the scatter pattern for CRBC may vary among different instrument designs, it may be useful for monitoring a particular instrument (Horan and Loken, 1985; Horan et al., 1990). At the present time, however, the most reliable approach is to use a biological sample of the same or similar type that is to be used for the flow cytometric analysis (Owens and Loken, 1995; CLSI, 2008).

Pulse width

Pulse widths of fluorescence or scatter signals depend on both the height of the laser

beam (in the direction of flow) and the size of the particle passing through the beam. When the particle diameter is at least as large as the beam height, it is possible to measure particle diameter accurately (Sharpless and Melamed, 1976; Sharpless et al., 1977; Eisert and Nezel, 1978; Leary et al., 1979; Shapiro, 2003). A mixture of two or more sizes of particles of known, calibrated diameter can be used to calibrate this measurement. For a detailed protocol *UNIT 1.23* (Hoffman, 2009).

Electronic cell volume

Electronic cell volume (Schwartz et al., 1983; Kachel, 1990; Shapiro, 2003) is provided as a parameter in some commercially available flow cytometers. Microbeads are an excellent material for standardization and calibration of electronic cell volume. Accurate measurement of particle volume requires the particles to be electrically nonconductive. Thus, caution should be used when comparing results with fixed cells in which the plasma membrane has become permeable and electrically conductive.

Fluorescence and Light Scatter Resolution

Resolution is usually estimated by running a sample of uniform particles and measuring the CV. For fluorescence measurements, CV is a good estimate of resolution because fluorescence signals are generally proportional to the amount of fluorochrome on the particle [i.e., the measurement and the characteristic of the particle are linearly related; see *UNIT 1.4* (Wood, 2009) on system linearity]. Resolution of dimly stained particles is considered a special case (see Sensitivity or Signal/Noise for Dim Fluorescence). Resolution of uniform particles characterizes the alignment of the optics and should not be greater than the instrument manufacturer specifications for the specified beads.

For light-scatter measurements, the CV obtained with one particle may not be a good measure of how well the cytometer may be able to resolve particles of different sizes, since the relationship between light-scatter signal and particle size is usually not linear.

Measurement Response and Logarithmic Calibration

The measurement response of a flow cytometer can be determined in relative or absolute terms. Evaluating the relative response can be as simple as determining whether two identical particles stuck together give twice the signal as one. Alternatively, it may be as complex as determining the response over a range of four or more decades. Absolute response calibrates the measurement (e.g., channel number in a histogram) in units such as molecules of equivalent soluble fluorochrome (MESF), equivalent number of reference fluorophores (ERF), or antibody binding capacity (ABC).

Knowing how well the displayed data actually follow a linear response (or logarithmic response for instruments that use log amplifiers) is particularly important when the data are used to compute compensation. Small deviations in linearity can cause large absolute errors when a fraction of one number is subtracted from another. With the exception of the BD FACSCalibur, which does fluorescence compensation using analog amplifiers, compensation is done using the linear data (or linearized data from log amplifiers) by mathematical calculation that assumes the data are perfectly linear. It is easy to demonstrate situations in which a deviation from linearity of 3% in a large number (say in the last decade of a histogram) causes a truly negative population to be displayed and then assumed to be positive following correctly computed compensation.

Linear and logarithmic response

Testing the relative linearity of a measurement on a limited range of a linear scale is conveniently done using small beads or other stained particles such as cell nuclei that contain aggregates. Measurement of the sample should then give histogram distribution mean channels that are multiples of the mean channel of a single particle.

Measuring the response of a nominally logarithmic scale can be done in two ways. The first uses a known linear scale as reference and compares measurements on the linear and logarithmic scales (Muirhead et al., 1983; Horan et al., 1990). The second approach uses a mixture of particles with known, different intensities. The log response is determined by measuring the separation of the peaks on the log scale as the signal level is varied by changing the PMT voltage (Schmid et al., 1988). A more convenient version of the second approach uses a bead mixture containing a wide range of fluorescence levels whose relative intensities are known (e.g., see Fig. 1.3.5C). If the beads in the mixture are calibrated in terms of MESF or ERF, then the absolute log response may be determined (Schwartz and Fernandez-Repollet, 1993; Schwartz et al., 1996).

Another standard method for measuring the linearity (or logarithmic) response of an optical detector is to use pulses from a light emitting diode (LED) that alternate at two different intensities, and vary the amount of light emitted into the detector without changing the ratio of the input intensities. If the detection system is linear, the ratio of the measured signals will be constant and independent of the absolute magnitudes of the two LED pulses. A simple way to change the intensity of the LED light without changing the inputs to the LED is to move the LED further from the detector or introduce neutral density filters or apertures that reduce the amount of light reaching the detector. By measuring the output of the system as means of populations in a flow cytometer, one can determine any deviation from linearity by noting where the ratio of the measured means deviates from the expected ratio. A somewhat less rigorous method can use the intensities of two fluorescent particles and vary the input to the detection electronics by changing the voltage on a PMT while measuring the ratio of the mean signals from the two beads. A high degree of linearity (e.g., no more than 2% deviation from linearity) is required in instruments that calculate fluorescence compensation rather than use analog electronics to perform compensation. Most currently available flow cytometers use calculated compensation, with the BD FACSCalibur being the exception. A seemingly small (e.g., 5%) percentage deviations from linearity can cause large (order of magnitude) errors in the absolute calculated compensation value. Thorough discussion of establishing and maintaining system linearity appears in *UNIT 1.4* (Wood, 2009).

Secondary calibrators

Cross-calibration of gCRBC or hard-dyed particles to surface-labeled beads calibrated in MESF or ERF can generally be done for any *one* instrument (Schwartz et al., 1996; see Characterizing Particles for Calibration and Control of a Flow Cytometer). To ensure that the secondary calibrator on an instrument is reliable, however, the emission filters and the wavelength and intensity of the excitation light must remain unchanged. See Figures 1.3.1 and 1.3.2 and cautions given by Schwartz et al. (1996). (Also see Types of Particles and discussion of comparison of spectrally matched and unmatched particles.)

Antibody binding capacity (ABC)

A further step in immunofluorescence standardization and calibration is to express measurement results in terms of antibody binding capacity or antibodies bound per cell (see Definitions). Three approaches have been used to estimate ABC. Each approach has different critical technical requirements and potential sources of error. Although not, strictly speaking, a source of error, it must be kept in mind that different antibody clones with the same cluster designation (CD) can have different binding affinity and capacity. Particular examples of clone variability have been noted for CD4 (Davis et al., 1998) and CD34 (Serke et al., 1998). Therefore, if the three approaches to quantitative ABC are to be compared, they should be compared with the same clone or with clones that are demonstrated to give the same ABC. In addition, the sample preparation method can affect the antibody binding and must be taken into consideration (Islam et al., 1995; Serke et al., 1998).

The earliest approach (quantitative indirect immunofluorescence, or QIFI) uses a calibrated anti-mouse fluorescent second-step reagent (Poncelet and Carayon, 1985; Bikoue et al., 1996). The anti-mouse reagent can be calibrated using particles coated with known amounts of mouse immunoglobulin G (IgG). Polyvalent reagents are used in this method, however, and their reactivity with certain monoclonal mouse antibodies may produce artifacts. Bikoue et al. (1996) observed that different monoclonal antibodies to a molecule (e.g., CD8) can give different ABC values on the same cells. Whether this is due to differences in binding of the primary antibody to cells or to differences in binding of the secondary antibody to the primary antibody is not clear. Altered reactivity of the second-step reagent with fluorochrome-conjugated mouse monoclonal antibody is another possible variable.

A second approach (Quantum Simply Cellular or QSC) uses particles coated with known amounts of polyvalent anti-mouse antibody (Schwartz and Fernandez-Repollet, 1993; Schwartz et al., 1996). The QSC method is designed to capture quantitatively any mouse monoclonal antibody independent of fluorochrome conjugation or IgG isotype. However, conjugates of the same monoclonal antibody with different fluorophores give different ABC values when calibrated with QSC beads, and different antibody clones directed against the same molecule can give differing results with QSC beads (Lenkei and Andersson, 1995). The QSC beads could, however, be calibrated to a specific monoclonal antibody reagent whose production is carefully

controlled (Lenkei and Andersson, 1995; A. Schwartz, pers. comm.).

A third method uses antibody conjugates that have been prepared with a known MESF/antibody ratio and a flow cytometer that has been calibrated in MESF. Phycoerythrin is an attractive fluorochrome for this approach since antibody conjugates can be prepared with exactly one PE molecule per antibody. Initial experiments with this approach were promising (Davis et al., 1998; Iyer et al., 1998). In fact, it was this system that led to the development of the QuantiBRITE products of purified 1:1 PE-antibody conjugates and freeze-dried beads surface-stained with known numbers of PE molecules per bead.

The QIFI and QuantiBRITE methods have been found to be generally comparable (Lenkei et al., 1998; Serke et al., 1998) for ABC quantitation, but the QSC method frequently gives significantly different results from the other methods (Lenkei et al., 1998; Serke et al., 1998). Since the QuantiBRITE method uses direct fluorescence staining, it can be easily used in a multicolor staining protocol. The general recommendation for comparing ABC across laboratories or over time is to use a single method along with the manufacturers' recommended reagents.

Certain cell-surface markers may be useful as biological calibrators with a relatively small variability and uncertainty. Although not as reproducible as the amount of DNA per cell, the amount of CD4, CD45, and many other molecules on normal human lymphocytes is generally reproducible (Brown et al., 1986; Poncelet et al., 1991; Bikoue et al., 1996); thus, CD4 content may be a useful biological calibrator for immunofluorescence analogous to the use of normal lymphocytes or other defined nucleated cells as biological calibrators for DNA quantitation (Hultin et al., 1998).

A protocol of quantitative flow cytometry measurements in ABC based on human CD4 reference marker has been recently developed jointly by NIST and FDA (*UNIT 1.29*; Wang et al., 2016b). The reference marker, CD4 receptor protein on human T helper cells, can come from either whole blood of normal healthy individuals or Cyto-Trol Control Cells, a commercially available peripheral blood mononuclear cells (PBMC) preparation, depending on the preference of users and the accessibility of normal individual whole blood samples. The CD4 expression levels in ABC are approximately 45,000 for fixed normal whole blood samples and approximately 40,000 for Cyto-Trol cells, respectively. These CD4 expression levels have been verified by orthogonal measurement methods, quantitative flow cytometry and mass cytometry, using a well characterized anti-human CD4 monoclonal antibody (SK3 clone from BD Biosciences) as well as quantitative mass spectrometry using an isotope-labeled, full-length recombinant CD4 receptor protein as the internal quantification standard. The known reference CD4 expression enables the translation of a linear fluorescence intensity scale to the ABC scale, which ultimately ensures quantitative measure of target antigen expression levels independent of flow cytometers used.

Separately, another method for estimating how many bound antibodies are required to produce a particular measured signal and thereby antibodies bound per cell has been developed by Kantor and colleagues (*UNIT 1.30*; Kantor et al., 2016). This method, so called the Test-Fill method, is based on complementary binding of Test and Fill antibody reagents to antibody capture microspheres. Quantitative values for the reference reagents are anchored using QuantiBRITE PE beads to calibrate the PE channel scale and a 1:1 antibody-PE test reagent in complementation bead stains with the Fill reagents. While replacing antibody capture microspheres with biological cells, and implementing the Test-Fill method on the reference flow cytometer with known statistical photoelectron scales under the same operational conditions, estimation of ABC values for the Test antibody reagents can be carried out in the same manner as on the antibody capture microspheres.

Sensitivity or Signal/Noise for Dim Fluorescence

The practical notion of fluorescence sensitivity involves the ability to resolve populations of dimly fluorescent particles. The factors affecting the ability to reliably detect dim fluorescence include electronic noise, the amount of fluorescent light collected, the efficiency with which the fluorescent light is converted into electrons in the detector, and the amount of background light that is present. Characterization of an instrument's detection capability should include the effect of these factors. An overly simplified method for characterizing fluorescence sensitivity can give misleading results, as illustrated by the following example.

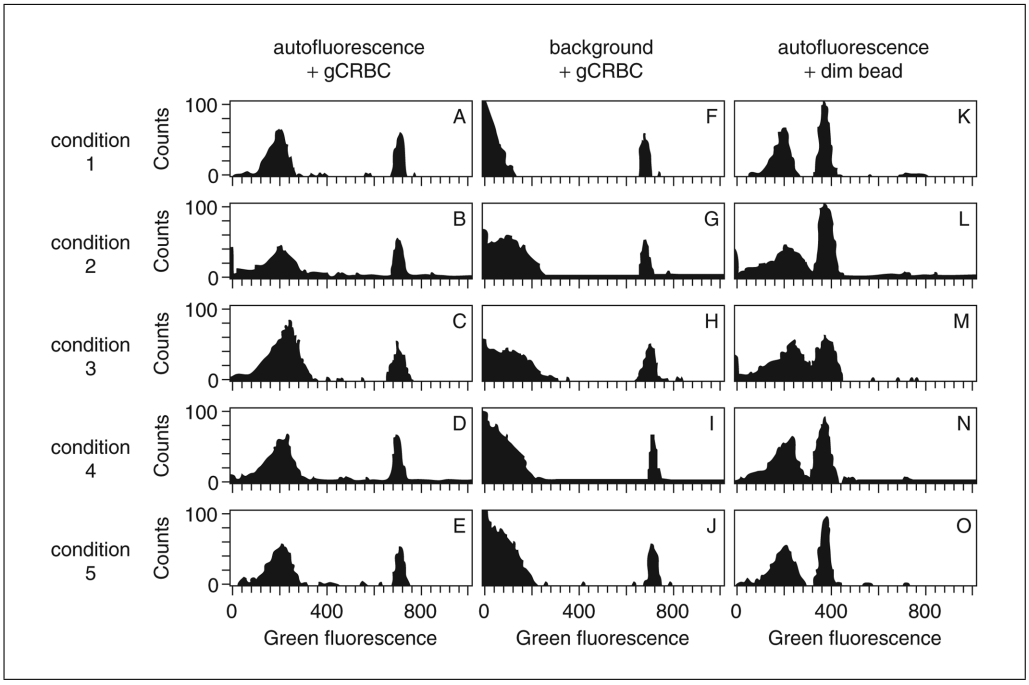

Figure 1.3.7 Comparison of three methods for estimating green fluorescence sensitivity. Histograms are four-decade log with 256 channels per decade. Each column of histograms represents a different measure of fluorescence sensitivity. (**A-E**) Histograms of autofluorescent unstained CaliBRITE beads (low peak) and gCRBC (high peak). (**F-J**) Histograms of background noise from a test pulse trigger (low peak) and gCRBC (high peak). (**K-O**) Histograms of autofluorescent unstained CaliBRITE beads and dim Rainbow beads. Each row of histograms shows results for a different condition or perturbation on the optical system of the instrument (see Sensitivity or Signal/Noise for Dim Fluorescence).

Resolution of dimly stained from unstained particles

Three methods for assessing fluorescence sensitivity are illustrated in Figure 1.3.7. Data for each row of panels were obtained with exactly the same instrument conditions with a single sample that contained all the particle and test pulse events. The fluorescence histograms were gated to contain the events of interest by setting regions in a scatter dot plot, as in Figure 1.3.5A. The left column in Figure 1.3.7 (panels A-E) are histograms of unstained "autofluorescence" beads and glutaraldehyde-fixed CRBC (gCRBC). The middle column (panels F-J) shows histograms of background light (from test pulse–triggered fluorescence) and the same gCRBC. The right column (panels K-O) shows histograms of autofluorescence beads and dimly stained beads. The instrument condition for each row of histograms was varied by adding background light or reducing the amount of fluorescent light that reached the PMT.

Delta channel and detection threshold methods: traditional and not very sensitive

The "delta channel" approach measures the mean or median channel of autofluorescent or nonfluorescent particles (or test pulse–triggered background) and that of relatively bright particles. This approach is illustrated in Figure 1.3.7A-E, where the negative population is a bead with about the same autofluorescence as unstained lymphocytes and the bright population is gCRBC. With autofluorescence as the negative reference point, there is almost no difference in mean channel or delta channel for the different instrument conditions. There are considerable differences, however, in the ability of the instrument to resolve autofluorescent beads from dimly stained beads (Fig. 1.3.7K-O). For very dim populations, the delta channel method does not critically assess the ability to resolve unstained from dimly stained particles. This is primarily because the method does not take into account the broadness of the fluorescence distributions; rather, it

considers only the mean or median channels. Resolution of the dimly stained population, however, is critically dependent on the broadness of the distributions, which is determined by background fluorescence and the amount of fluorescence signal reaching the PMT.

If nonfluorescent particles or test pulse–triggered fluorescence is used as the negative population, one obtains additional information about sensitivity. For Figure 1.3.7 F-J, the instrument was configured to have the same response for bright particles, but different amounts of background signal. In the terminology used by Schwartz et al. (1996), the instrument had the same "window of analysis" for data in Figure 1.3.7F-J. Comparing panels F-J with panels K-O in Figure 1.3.7, one sees an improved ability of this method to predict whether dimly stained particles will be resolved from unstained particles. The method is not perfect, however. Panels L and M have the same amount of background signal or, as expressed by Schwartz et al. (1996), the same "detection threshold," but they differ noticeably in resolution of the autofluorescent and dimly stained particles. The same considerations hold for panels N and O of Figure 1.3.7, which have the same, intermediate amount of background signal. So, the detection threshold method also fails to reliably measure differences in the ability to resolve dim populations from the unstained population.

Resolution of dimly stained from unstained particles

The most direct measure of the ability to resolve unstained from dimly stained particles is simply to run a mixture of the particles and see if they are resolved. Panels K-O in Figure 1.3.7 illustrate this approach. This method at least guarantees unambiguously that the instrument is able to resolve a certain low level of fluorescence. Making this approach quantitative requires not only attention to the relative numbers of unstained and dimly stained particles, but also some limits on the inherent particle fluorescence CV. By calibrating the unstained and dimly stained particles in MESF or ERF, one could have a method that would standardize and calibrate sensitivity.

The Q and B method to characterize instrument sensitivity

To address the quantitative characterization of fluorescence sensitivity, the underlying physics of optical detectors needs to be taken into account (Wood and Hoffman, 1998). A theoretical model for the CV of dimly fluorescent particles (Gaucher et al., 1988; Steen, 1992) can be developed based on optical detection efficiency, Q (number of photoelectrons per fluorescence intensity of fluorochrome molecule analyzed), and background light, B. B can be expressed in units of the equivalent number of fluorochrome molecules that would produce that background. The studies by Gaucher et al. (1988) and Steen (1992) used flashes from a light-emitting diode (LED) to produce dim signals to a flow cytometer detector and used the CVs of the resulting signals to determine Q. The contribution to the CV from background light was determined by comparing CVs that were obtained from LED flashes with the laser shining on the sample stream to those obtained with the laser blocked. A fluorescence intensity standard bead was used to calibrate the flow cytometer in MESF and ERF units. Interest in using LED pulses as the ideal signal for determining Q and B has encouraged development of at least one commercial LED test unit specifically for use on flow cytometers, e.g., quantiFlash from Angewandte Physik & Elektronik GmbH in Germany.

As a generally practical approach, it has been shown that sets of beads with uniform but dim fluorescence could be used instead of LED flashes to determine Q and B (Chase and Hoffman, 1998; Wood, 1998). The intrinsic CVs of the dim beads could be determined by comparing their measured bead CVs with CVs of LED light flashes and CVs of identical but brightly stained beads (Chase and Hoffman, 1998). Thus, a set of beads stained at varying levels from dim to bright could be characterized for intrinsic properties and then used to measure Q and B in a flow cytometer. Q and B can then be determined by using a fluorescence intensity standard to calibrate the flow cytometer in terms of MESF/ERF and measuring the CVs (or SDs) of the dimly and brightly fluorescent beads. There are several ways to analyze the resulting bead data to determine Q and B (Chase and Hoffman, 1998; Wood, 1998).

A robust method for determining Q is to correct the standard deviations of the beads for the illumination uniformity contribution to SD (this is determined from the CV of a bright bead). A plot of standard deviation squared (SD^2) versus the mean bead intensity in MESF units gives a straight line whose slope is $1/Q$. An estimate of B can be determined from the intercept of the line with the SD^2 axis. The intercept is B/Q. Alternatively, and probably more accurately, B can be determined from

the SD of a blank bead or noise distribution (Chase and Hoffman, 1998).

UNIT 1.20 (Hoffman and Wood, 2007) provides a detailed protocol for determining Q and B using the SD^2 approach and simple linear data fitting. The degree to which the data fit a straight line at every point is an indication of how well assumptions used in the method are met—particularly the assumption that all beads in the set used have the same intrinsic CV and same uniformity of illumination, which is accounted for by the brightest bead in the set. An alternative approach uses a quadratic fitting function that estimates the contribution of intrinsic and illumination uniformity from the fit parameters rather than directly measured by the brightest bead in the set. Another alternative is used in BD's automated CS&T system, which has beads with only three different brightness levels, but includes data for the important intrinsic CV differences. All these approaches to measuring Q generally give somewhat similar results.

An important reason for characterizing sensitivity in terms of Q and B is that these values can be used to predict the resolution of dimly fluorescent populations of cells (Chase and Hoffman, 1998). Q and B can also be used to determine the effect of background contributions from unbound fluorescent antibody or from spectral overlap on the resolution of populations.

Spectral Overlap Compensation

Measurement of fluorescence in a particular spectral range is not the same as measuring the fluorescence from a fluorochrome. One usually measures fluorescence in a spectral range that contains the emission peak of the fluorochrome, but emission from other fluorochromes used simultaneously to stain the cells may also overlap into the desired spectral region. In Figures 1.3.1 and 1.3.2, the spectral overlap of FITC fluorescence extends into the range where PE has maximum emission at about 575 nm. Similarly, PE has a small amount of fluorescence in the range where FITC has peak emission. The effect of the spectral overlap is illustrated in Figure 1.3.8A, which shows a dot plot of yellow and green fluorescence from a mixture of unstained, FITC-stained, and PE-stained beads.

To make the dot-plot axes read in units of FITC and PE fluorescence rather than green and yellow fluorescence, the amount of spectral overlap from each fluorochrome can be subtracted (Bagwell and Adams, 1993). The method used to accomplish the subtraction is instrument dependent; ask the manufacturer for details. Since the percentage of FITC fluorescence in the yellow detector is always a constant fraction of the amount of FITC fluorescence in the green detector, the same percentage of green FITC fluorescence can be subtracted from the yellow signal no matter what the FITC signal is. For example, PE fluorescence = yellow fluorescence $- f \times$ green fluorescence, where the fraction f is a constant.

Figure 1.3.8B shows the same sample as in Figure 1.3.8A but with spectral compensation applied. Spectral overlap compensation simply transforms the readout of the fluorescence from green and yellow to something more directly related to the analysis results: the amount of FITC and PE fluorescence. Correctly adjusting the fraction of compensation requires care, however, since small differences in the emission spectrum of the particle can have a large effect on the amount of compensation (Schwartz and Fernandez-Repollet, 1993; Schwartz et al., 1996). Manufactured particles stained with fluorochromes such as FITC or PE may not have exactly the same emission spectrum as cells labeled with the same fluorochromes. If beads are used to adjust compensation, it is always advisable to check compensation with labeled cells at least once for each new batch of beads (Schwartz and Fernandez-Repollet, 1993; Owens and Loken, 1995).

Antibody capture beads are an alternative to using fluorochrome-stained beads or stained cells for compensation. It is advisable to stain the antibody capture beads with the same antibody conjugates that will be used to stain the cells. Antibody capture beads are especially useful as compensation control samples for multicolor analysis, since the unstained reference particle is identical for all fluorochromes, and all fluorochromes are controlled with a stained population that is relatively uniform and bright.

When more than two fluorochromes are used simultaneously to stain cells, compensation becomes significantly more complex. If the third and fourth fluorochromes in the sample have little or no spectral overlap, compensation can be set manually. However, if three or more fluorochromes all have significant spectral overlap among one another, correct compensation requires a matrix calculation that is practical only using software (Bagwell and Adams, 1993; Roederer, 2001). In instruments that use log amplifiers, a combination of hardware and software compensation may give the most accurate results (Baumgarth and Roederer, 2000). With compensation performed by

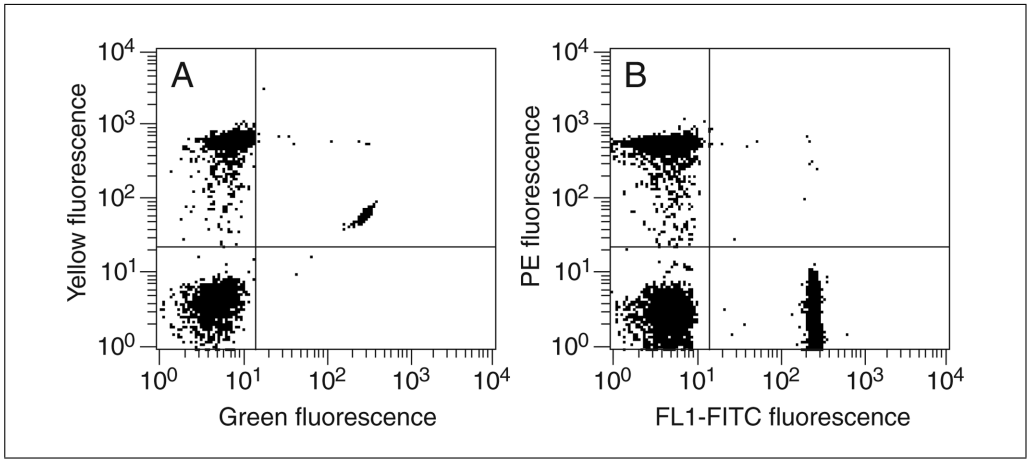

Figure 1.3.8 Example of spectral overlap with FITC- and PE-stained beads. Three populations are shown in each dot plot: unstained beads, FITC-stained beads, and PE-stained beads, with unstained beads appearing in the lower left quadrant. (**A**) Uncompensated data with FITC-stained beads in the upper right quadrant with about 300 units of green fluorescence and 80 units of yellow fluorescence. PE-stained beads are in the upper left quadrant with about 8 units of green fluorescence and 800 units of yellow fluorescence. Unstained beads have about 6 units of green and 5 units of yellow fluorescence. (**B**) Same data after compensating for spectral overlap to make the dot-plot axes represent fluorescence from a specific fluorochrome rather than a color of fluorescence signal. Note that the FITC-stained beads in the lower right quadrant of B have the same mean "PE" fluorescence as the unstained beads, and the PE-stained beads in the upper left quadrant have the same amount of "FITC" fluorescence as unstained beads. FITC beads have no more PE signal than unstained beads, and PE beads have no more FITC signal than unstained beads.

software and with proper controls, accurately compensated results are possible and practical with ten or more fluorochromes (Baumgarth and Roederer, 2000).

DNA Measurements

For DNA measurements, fluorescence linearity and resolution must be assured. Sample preparation and data analysis must also be carefully controlled. Reviews of standardization issues for DNA analysis include those by Dressler (1990), Bauer (1993), Darzynkiewicz (1993), and Wheeless (1993); also see Chapter 7 for nucleic acid analysis. Approaches to performing instrument standardizations were discussed earlier (see Optical Alignment, see Fluorescence, and see Light Scatter Resolution, and Measurement Response and Logarithmic Calibration: Linear and Logarithmic Response). Either fluorescent beads (see Fig. 1.3.6) or stained cells or nuclei can be used. For determination of abnormal DNA content it is important to use an internal staining control in the sample. Chicken erythrocytes, trout erythrocytes, and normal human cells have been used for internal controls. Calibrated measurement of the amount of DNA per cell has been reported (see Shapiro, 2003,for a brief review), but sensitivity of the staining to chromatin structure should caution against overinterpretation of the results (Darzynkiewicz, 1993).

Sorting Purity and Recovery

Various manufactured particles can be used to determine purity and recovery for sorting. A mixture of beads with different fluorochromes is typically used, but using only two types of stained particles can give results that are overly optimistic. This is because a mixture of two differently stained beads has "built-in" doublet detection. For example, if two different stained beads with primary fluorescence in fluorescence channels 1 and 2, respectively, are measured together (coincident in time), the event is easily discriminated in a dot plot of channel 1 versus channel 2 fluorescence. This coincident event appears as doubly stained with signal in both fluorescence channels 1 and 2. A sort gate that was set only for a singly stained bead (i.e., in channel 1 only) would exclude these coincidences. If the real sample to be sorted has unstained cells that produce undetectable coincidences with stained cells, the sort electronics will either (1) detect the coincidence, abort the event, and reduce yield; or (2) miss the coincidence, sort the coincidence, and give lower purity. If one of the beads is unstained, however, it is not possible to detect a

coincidence in a dot plot of channel 1 versus channel 2.

Standardization with a Particle in the Analysis Sample

Adding the particle used for standardization or control to the sample to be analyzed gives an extra level of confidence to a flow cytometric analysis. Fluorescent beads are generally used for "standardization in tube" methods (Horan and Loken, 1985; Horan et al., 1990). The standardization or control particle must be sufficiently different in some measurement parameters to be distinguished from the cells in the sample. For example, the particles can be chosen to have lower forward scatter but much higher fluorescence than the cells in the sample. If a known number of particles is added to a known volume of sample, one can also use the particles to measure the concentration of cells (cells per volume) in the sample (see Particle Concentration). One commercial and clinical assay kit, Leuko64, for instance, uses an internal FITC bead control for assay value assignment of activated granulocytes.

Particle Concentration

Particle concentration (i.e., the number of particles per unit volume) is becoming a more widely used measurement. This is largely motivated by the clinical need to measure the concentration of CD4 cells in HIV-positive individuals and in AIDS patients. In this context, the concept of "absolute count" has been used instead of cell concentration. The count is "absolute" in numbers per microliter of the original blood sample, as calculated from the concentration of cells (e.g., CD4 T cells) for the sample analyzed and the known dilution of blood used in preparing the sample. Reference samples of known particle concentration can be used to standardize particle concentration measurements on a flow cytometer. For standardizing and calibrating the absolute count, a controlled or calibrated dilution of the original sample must be made.

Some instruments are capable of measuring particle concentration directly, as they measure a fixed volume of sample. An alternative approach uses a known number, N, of reference particles added to a sample. The ratio, r, of sample particle events (e.g., CD4 T cells) to reference particle events is measured in the flow cytometer. Then, the number of sample particles is computed from the product $r \times N$ (Stewart and Steinkamp, 2005; Stebbings et al., 2015).

What Measurements Can Be Calibrated, and How Frequently Is Calibration Necessary?

Flow cytometry measurements that currently can be calibrated in at least some commercially available instruments are particle diameter or volume, numbers of fluorochromes (or MESF, ERF or ABD) per particle, antibody binding capacity/antibodies bound per cell (ABC), and particle concentration (e.g., particles per microliter). Calibration need not be a daily practice, but the instrument must be quality controlled daily prior to calibration. Monitoring measurements with control material ensure that the instrument is still in calibration as long as the measurements do not exceed acceptance limits determined by the application. If controls are out of range, it will be necessary to recalibrate. If the instrument is changed or serviced, it is usually advisable to recalibrate.

CHARACTERIZING PARTICLES FOR CALIBRATION AND CONTROL OF A FLOW CYTOMETER

This section gives suggestions for how to assign fluorescence values to test beads (see Calibrating Particle Fluorescence in MESF and ERF) and for assignment and use of inherent fluorescence CV of a particle (see Determining Inherent Fluorescence CV of a Dim Particle Sample and see Measuring Signal to Noise from Dim Particles).

Calibrating Particle Fluorescence in MESF/ERF

Assigning intensity values to fluorescent particles

Relatively bright beads or other particles of known concentration can be measured in terms of molecules of equivalent soluble fluorochrome (MESF) and equivalent number of reference fluorophores (ERF) with a spectrofluorometer using a solution of fluorochrome as reference (Brown et al., 1986; Schwartz and Fernandez-Repollet, 1993). The spectrofluorometer is adjusted for the excitation and emission of the fluorochrome to be measured. Depending on how close the fluorescence excitation and emission wavelengths are, it may be necessary to use a band-pass filter in the excitation light path and a high-pass or band-pass filter in the emission path to adequately reduce light scatter from the particles. The signal from a reference concentration of

fluorochrome is measured to calibrate the responses from a spectrofluorometer, and then a suspension of the particles to be calibrated is measured under exactly the same conditions as the fluorochrome solution. The fluorescence of the particle suspension is expressed in terms of equivalent molecules of fluorochrome by comparing it with the reference fluorochrome solution. The particle concentration (particles/ml) is determined, correcting for doublets or aggregates if necessary. The MESF or ERF per particle is calculated as the fluorochrome concentration equivalent of the bead suspension divided by the particle concentration.

The U.S. National Institute of Standards and Technology (NIST) has published a series of papers (Gaigalas et al., 2001; Schwartz et al., 2002; Wang et al., 2002; Wang and Gaigalas, 2011; Wang et al., 2016a) detailing the fundamental scientific basis and reference methods for assigning MESF or ERF values to particles. NIST has recently developed a standard fluorescein solution kit, Standard Reference Material 1934, that includes fluorescein, Nile Red, coumarin 30, and allophycocyanin for ERF value assignment. Compared to the use of a commercial spectrofluorometer for fluorescence value assignment, a spectral response–calibrated fluorometer equipped with laser excitations such as 405 nm, 488 nm, and 633 nm, commonly used in flow cytometers, and a CCD detector, is used by NIST to perform ERF value assignment of calibration particles. The use of SRM 1934 establishes the traceability of the ERF value assignment and ultimately enables the standardization of the fluorescence intensity scale of flow cytometers in quantitative ERF units.

At low-particle MESF or ERF, the spectrofluorometer may become inaccurate or lack sensitivity because of the measurement performed in suspension. If the log response for the fluorescence channel of the flow cytometer has been carefully calibrated [see Measurement Response and Logarithmic Calibration; also see *UNIT 1.4* (Wood 2009)], then the brighter particles can be used to calibrate the upper range of the fluorescence channel in MESF per linear fluorescence unit on the histogram. Alternatively, the same process can be done using linear amplification. Dimmer particles can now be assayed on the calibrated flow cytometer and assigned MESF or ERF values.

A different approach to establishing intensity values is essentially arbitrary assignment of values to a reference lot of beads, and using the reference lot to assign values to future lots of beads. BD Biosciences has used this approach for Assigned BD units (ABD units) of fluorescence intensity to the CS&T beads used in their instrument performance characterization and tracking system. The creation of ABD units was necessary at the time due to the absence of traceable fluorescence standards for the many fluorophores that are in routine use. Hopefully, the concept of ERF will allow a universal, traceable fluorescence intensity unit. When ERF standards are available, alternative fluorescence intensity units such as MESF and ABD can be cross calibrated to ERF.

Assigning relative intensity values to hard-dyed beads

A flow cytometer can also be used to measure and assign accurate *relative* fluorescence intensity values to hard-dyed particles such as Rainbow beads from Spherotech. For example, a flow cytometer with calibrated logarithmic or linear amplifier could be used to measure fluorescence from each peak in a fluorescence histogram for a mixture of particles. Figure 1.3.5C shows data for Spherotech Rainbow beads.

Using hard-dyed particles as a secondary calibrator on one flow cytometer

A mixture of stable, hard-dyed particles with a range of intensity levels and a sample of fluorochrome-labeled particles with known MESF values are used to calibrate a flow cytometer as follows:

1. Calibrate or verify calibration of the electronic response of the data acquisition electronics. Alternatively, a mixture of fluorochrome-labeled beads (e.g., labeled with FITC) of varying, known MESF can be used to calibrate the intensity scale (Schwartz et al., 1996).

2. Adjust the PMT voltage so the fluorochrome-labeled bead(s) of known MESF is in an appropriate histogram channel(s). This calibrates the histogram scale in MESF.

3. Run the hard-dyed bead mixture at the same PMT voltage as the fluorochrome-labeled beads.

4. Use the histogram calibrated in MESF to assign MESF values to each of the hard-dyed bead populations. The hard-dyed beads are now a calibrator or "secondary standard" for this instrument and only this instrument. On subsequent days and at other PMT voltage settings the hard-dyed beads can be used to calibrate the fluorescence histogram in MESF.

Cautions using hard-dyed particles as MESF calibrators

Because hard-dyed particles do not have exactly the same excitation and emission spectra as a fluorochrome used to stain cells, they will not necessarily have the same fluorescence intensity relative to that fluorochrome on another instrument. Caution and skepticism should be used in trying to assign to the hard-dyed beads a "global" MESF value that is valid for calibration in terms of MESF on all instruments (Schwartz et al., 1996). For further information, see Figures 1.3.1, 1.3.2, and 1.3.4, as well as the discussion in the section above on Comparison of Spectrally Matched and Unmatched Fluorescent Particles.

Determining Inherent Fluorescence CV of a Dim Particle Sample

The inherent fluorescence coefficient of variation (CV; see Definitions, "Coefficient of variation") of a particle sample should be due only to variation in the amount of fluorochrome in the particles. Measurement limitations or noise in the flow cytometer broaden the measured CV (see Steen, 1992 and Chase et al., 1998, for more detailed discussion). The contribution to the CV from variation in illumination can be estimated by running a very bright, uniform "alignment" particle. For dimly fluorescent particles, background noise and photoelectron statistics become dominant contributors to the CV. To determine the inherent CV of a dim particle, where the total CV may be $\geq 10\%$, one must measure the background noise and photoelectron statistics. This can be done by using dim light flashes from a light-emitting diode (LED). Dim signals are created by using filters to attenuate the light from the LED or by simply holding the LED far from the detector. The inherent CV of the dim particles is found by subtracting (in quadrature) the noise CV from the total CV (Steen, 1992).

The inherent CV of dim, hard-dyed beads can be determined in this way, effectively calibrating the particles in terms of population CV. When the particles are subsequently analyzed on any flow cytometer, the noise contribution from the measurement can be determined by the broadening of the CV. Essentially one works in reverse from what was explained above: the noise CV is determined by subtracting (in quadrature) the inherent particle CV from the total measured CV. For dim signals, this gives a measure of the fluorescence sensitivity in terms of conventional signal/noise.

Measuring Signal to Noise from Dim Particles

In engineering, a standard definition of minimum resolvable signal is that for which the signal (S) and noise (N) are equal, that is, S/N = 1. In flow cytometry, it is customary to use CV rather than S/N, but the simple relation CV = N/S can be used to translate between the two measures.

If the inherent CV of a dim particle is known (see Determining Inherent Fluorescence CV of a Dim Particle Sample), the system noise can be determined. The fluorescence of the dim particle is measured on the flow cytometer, and the CV of the resulting distribution is determined. Although a range of particle intensities might be required to measure system noise accurately, a single particle can be used to determine whether S/N at a particular MESF is above a required minimum. Noise can also be expressed in MESF, and the MESF level at which S/N = 1 or some other predetermined number is a measure of sensitivity.

The instrument noise determined with the dim particle sample correlates with the ability to resolve dim particles from unstained particles. The other important factor is how large a signal is produced by unstained particles or cells. A convenient measure of the response to truly nonfluorescent cells is provided by the test pulse mode available on many flow cytometers. The pulse detection electronics can be triggered by an electronically generated signal to a nonfluorescence parameter such as forward scatter. The fluorescence channels then measure the response only to background light and other noise sources.

CONCLUDING REMARKS

Flow cytometry has had rapid growth since the mid 1980s. It has moved from a technology platform that only a few hundred "initiated" experts understood and could use to become both a common laboratory tool and clinical diagnostic system. To name a few applications, flow cytometry is essential for accurate measurement of $CD4^+$ cell counts for ensuring that patients receive the appropriate antiretroviral treatment for HIV/AIDS monitoring. Counting the number of viable $CD34^+$ cells using flow cytometry for reconstitution of the hematopoietic immune system of patients after chemotherapy has become a gold-standard clinical practice. Multiplexed flow cytometry assays (≥ 12 fluorescence parameters) are routinely used in clinics for disease diagnosis and therapies. This is clearly seen in the clinical

flow cytometric analysis of hematologic malignancies. Moreover, it has also become an essential clearance tool for the production of protein and cell therapeutics. All these applications essentially require that comparable and reproducible results can be generated using different flow cytometer platforms at different locations and times. The consistency of the measurements can only be accomplished with the use of multiple controls, e.g., particles for instrument standardization and calibration and biological cell reference materials in the measurement process. Without proper use of these process controls, the value of this information-rich instrument will not be realized nor will further advancement be made into new biological and clinical applications.

DISCLAIMER

Certain commercial equipment, instruments, and materials are identified in this paper to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment are necessarily the best available for the purpose. Definitions provided in this unit are well accepted in the field of flow cytometry, and a few may be slightly different from those provided by the International Vocabulary of Metrology (VIM) under the International Bureau of Weights and Measures (BIPM).

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