Flow Cytometer Performance Characterization, Standardization, and Control

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Abstract Flow cytometry is a widely used technique for the analysis of single cells and particles. It is an essential tool for immunological research, drug and device development, clinical trials, disease diagnosis, and therapy monitoring. However, measurements made on different instrument platforms are often inconsistent, leading to variable results for the same sample on different instruments and impeding advances in biomedical research. This chapter describes methodologies to obtain key parameters for characterizing flow cytometer performance, including precision, sensitivity, background, electronic noise, and linearity. Further, various fluorescent beads, hard dyed and surface labeled, are illustrated for use in quality control, calibration, and standardization of flow cytometers. To compare instrument characteristics, fluorescence intensity units have to be standardized to mean equivalent soluble fluorochrome (MESF) or equivalent reference fluorophore (ERF) units that are traceable to the existing primary fluorophore solution standards. With suitable biological controls or orthogonal method, users will be able to quantitatively measure DNA and RNA content per cell or biomarker expression in antibodies bound per cell. Comparable, reproducible, and quantitative measurements using flow cytometers can be accomplished only upon instrument standardization through performance characterization and calibration, and use of proper biological controls.

Keywords Standard · Calibrate · Quality control · Fluorescence · Sensitivity · Linearity · Flow cytometer · MESF · ERF · ABC

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1 Introduction

1.1 Why Is It Important

This chapter covers three distinct but related activities that insure that the results from a flow cytometer will be as comparable, reliable, and accurate as possible. No two flow cytometers are exactly alike. Every instrument and instrument subsystem is made to within defined specifications, but every specification has a tolerance. Flow cytometers in current use have been developed over a period of more than 20 years. Differences among instruments are greater if they are different models or if they are made with newly available technology rather than with older technology from past decades. There are significant differences among instrument models in the linearity over their multi-decade measurement range. This affects spectral compensation accuracy, the ability to resolve dimly fluorescent particles, and the ability to resolve particle populations (especially submicron ones) by light scatter.

It is helpful and often necessary to standardize the settings on a flow cytometer by adjusting the detector gains to place signals from stable particles at specified levels. This allows results from an application to be compared to previous results on that instrument. In many cases the same particles can be used for quality control of some aspects of the instrument performance. The detector gain or PMT voltage that must be used to reach required signal levels as displayed in a dot plot or histogram can be recorded daily to monitor drifts or sudden changes that alert the user it is time to troubleshoot a problem. The CV of a bead population measured on a detector channel can show whether the sample stream is adequately aligned to the laser beams and detection optics. With the proper stable particles, it is possible to standardize groups of instruments so that populations from a biological sample would be displayed in the same locations on histograms or dot plots from all instruments in the group. But having data displayed the same on all instruments in a group does not insure that the results from each instrument would be the same. Dim populations may be resolved on some instruments but not on others. Submicron particles may be detected above background on some instruments but not on others. Compensated fluorescence plots can have artefactual positive or negative populations if the signals from the electronics are not in an adequately linear range. Information about the key performance characteristics of the instrument will help to interpret results as being truly biologically meaningful within a performance limit of the instrument.

This chapter builds on previous work and publications on standardization and flow cytometer performance characterization [1–3]. The critical issues that should be considered when using beads to standardize, calibrate, and control are discussed along with fluorescence intensity units and methods used to assign intensity units to beads. Practical approaches for characterizing instrument performance are discussed, with examples for linearity and the factors determining fluorescence

sensitivity. Standards for DNA and RNA measurements are reviewed. Different approaches to convert measured fluorescence intensity to antibodies bound per cell (ABC) are described. The chapter concludes with some thoughts on the future.

1.2 What Do Instrument Manufacturers Provide?

Most instrument manufacturers provide beads and instructions or automated software to set up instrument gains for typical applications. The beads can be used to monitor instrument performance—particularly optical alignment and for regular checks to determine whether the instrument response has stayed within an acceptable range and to alert the user when performance has changed so much that troubleshooting or service is required. A few manufacturers, e.g., BD Biosciences CS&T System, provide additional characterization of instrument performance, including measuring the range of linear response, electronic noise level, optical background noise, and detection response. If the manufacturer also sells clinical applications, there will be specific application setup conditions—sometimes with application-specific beads and software.

However, instrument manufacturers cannot anticipate every application that users will develop or every experimental condition that will be tried. So it is a good idea to know what alternatives are available for setting up instruments and evaluating and characterizing performance. This will be particularly important when instruments in a laboratory or group study are from multiple manufacturers or consist of several different models. Materials and methods used to get consistent measurement scales over a variety of different instruments may require creating an alternative set of beads and setup procedures not available from any of the instrument manufacturers.

2 Beads as Standards

2.1 Bead Characteristics

Most beads (also called microparticles) used for standardization and applications are made from polymers. Some specialty beads are made of silica and have an optical refractive index closer to that of cells. In either case, beads are available in a wide size range covering submicron to tens of microns. There are two basic approaches for making fluorescent beads. The first approach embeds fluorescent molecules within the bead, which keeps the fluorophore from contact with the suspension buffer and greatly improves the stability of the fluorophore. These beads are often referred to as “hard dyed” and have the advantage of long shelf life without loss of fluorescence. The disadvantage of hard-dyed beads is that the fluorophores used to stain cells are water soluble and not generally compatible with

the hard-dye manufacturing process. As a result, the spectral response of hard-dyed beads almost never matches well with that of fluorescently stained cells. In addition, fluorescence from hard-dyed beads and fluorophores on cells can behave differently (photobleaching, emission saturation, etc.) with respect to excitation intensity. Since the spectral responses of flow cytometers vary to some extent even among the same model, hard-dyed beads cannot be used to set up all instruments to respond exactly the same when stained biological samples are analyzed.

A second type of fluorescent bead is stained on the surface with the actual fluorophore used to stain cells. The fluorophore is in essentially the same environment as in or on a cell. In particular, fluorescent beads used to best standardize instruments for immunofluorescence are surface-stained. Unfortunately, surface-stained beads are less stable over time and can be more expensive to make. The most stable surface-stained beads are freeze-dried, which adds to the expense. So flow cytometrists need to be aware of when it is appropriate to use surface-stained, fluorophore-specific beads and when the use of hard-dyed beads will be adequate. This decision will be determined by the application and the degree to which the individual instrument needs to compare to other instruments. Figure 1 shows emission spectra from a commonly used hard-dyed bead and spectra from two common fluorophores used for immunofluorescence. It is clear from comparing the spectra that using filters with different pass bands for FITC or PE will change the relative amount of fluorescence detected from beads and the fluorophores.

To have some objective criteria for deciding when hard-dyed beads are appropriate fluorescent standards, a study was conducted on 133 instruments among 28 laboratories and instrument manufacturers [4]. Ten different instrument models were included in the study. Each instrument was first set up with stable, freeze-dried surface-stained beads, and then a variety of hard-dyed beads were analyzed at the same settings. The ratio of mean fluorescence of the hard-dyed bead to the surface-stained beads was then compared for all instruments in the study. If the hard-dyed beads gave the same fluorescence scale as the surface-stained beads (and the same mean fluorescence for stained cells), there would be no variation of this

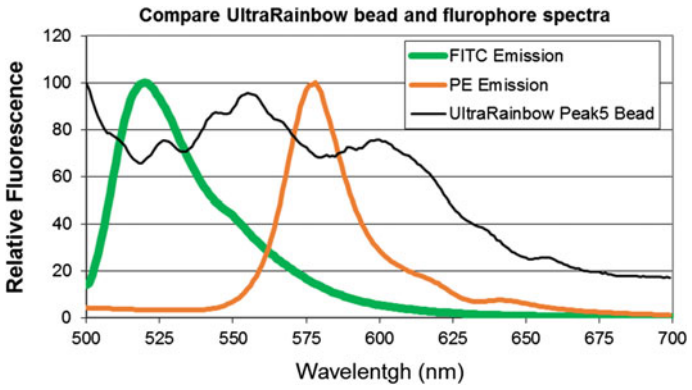


Fig. 1 Emission spectra of Spherotech Ultra Rainbow beads, FITC and PE fluorophores

ratio among different instruments. The results of the study showed just the opposite: there was considerable variation on the fluorescence scales with all the hard-dyed beads. Figure 2 shows results of the study for the PE channel.

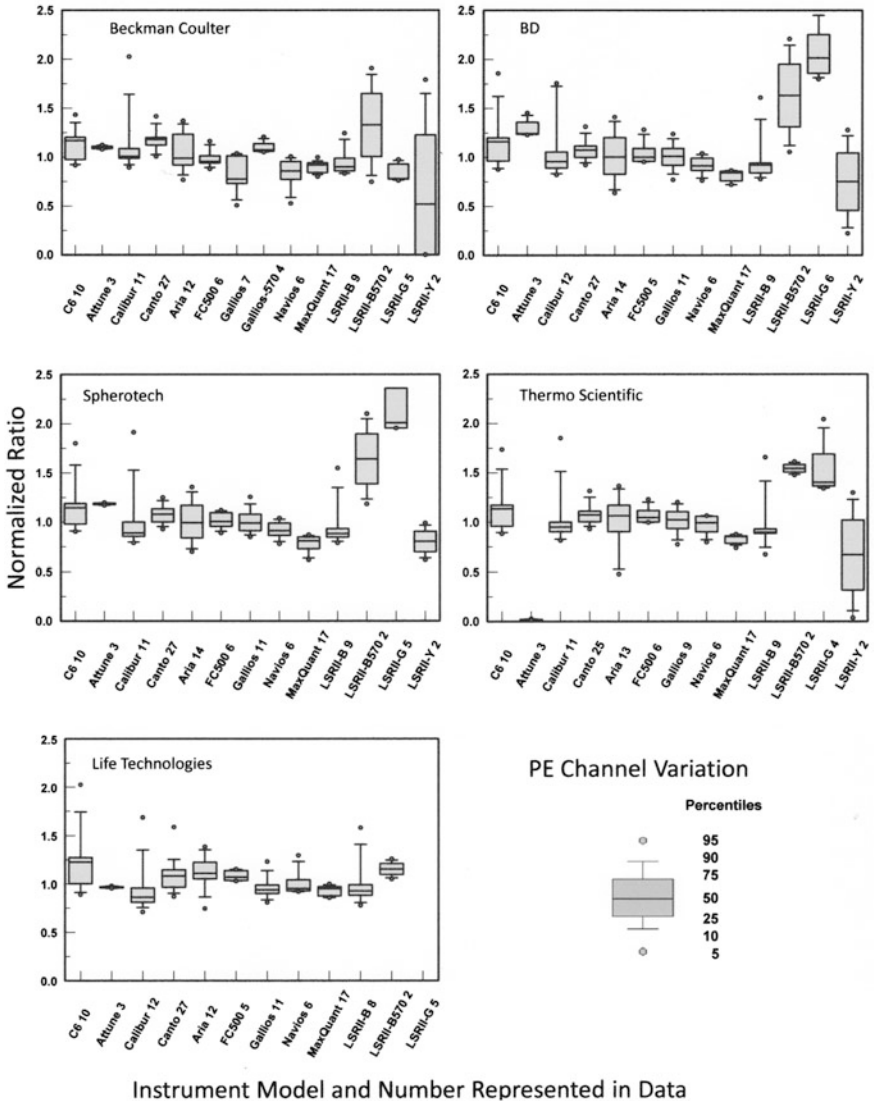


Fig. 2 Box-and-whisker plots of the normalized ratio of the MFI of the indicated hard-dyed beads to the MFI of the PE-stained fluorophore-specific standard bead for 10 different flow cytometer models. The box shows the 25–75th percentiles, and the line in the box indicates the median value. Horizontal bars outside the box indicate 10 and 90th percentiles and the circles indicate 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 reference [4] Cytometry Part A, 81A, 785)

Typical variation using hard-dyed bead fluorescence standardization even among the same instrument model was 20% or more using robust standard deviation as a measure. But the hard-dyed calibration range of 90% of the instruments varied by factors of 1.5–2 or more. If an assay, such as some clinical assays, requires a mean fluorescence to be measured within 10% accuracy, none of the hard-dyed beads would be suitable calibrators. Indeed, clinical assays that require mean fluorescence measurements with 10% accuracy use fluorophore-specific surface-stained beads for calibration. Hard-dyed beads can be a good standard to set up the fluorescence scale and verify linearity and dynamic range of the instrument among a group of study instruments. If a factor-of-2 variation in the mean fluorescence from cells can be tolerated, hard-dyed beads can be used as a standard.

2.2 *Fluorescence Intensity Units Used in Flow Cytometry*

MESF stands for molecules of equivalent soluble fluorochrome, and ERF refers to equivalent number of reference fluorophore. In both cases, the assigned number is the equivalent number of fluorophore molecules in solution that produce the same fluorescence intensity as the bead. MESF assignments use solutions of the same fluorophores used to label antibodies. MESF assignments are in units of fluorescein, PE, APC, etc. In the case of ERF unit assignment, however, the fluorophore reference solution may not be one that is used for antibody labeling. The only requirement for an ERF reference solution is that it can be excited with the same excitation wavelength and fluoresce in the wavelength range overlapping significantly with the fluorochrome associated with beads. For example, a calibration bead stained with PE can have ERF assignments in units of Nile Red. The additional requirement for an ERF assignment is that the excitation wavelength and emission wavelength range must also be specified. A complete ERF assignment for a bead labeled with PE, for example, could be equivalent to 45,000 molecules per bead of Nile Red excited with 488 nm and in the emission range 560–590 nm. In essence, MESF is a special case of ERF; both are a measure of particle fluorescence that is equivalent to the fluorescence signal from a known number of reference fluorophores in solution. The advantage to the ERF unit is that a small number of reference fluorophores can provide assignments to an unlimited number of different fluorophores used to tag antibodies, including fluorophores developed in the future. And it is practical for an authoritative body such as NIST to provide those few fluorophores as traceable Standard Reference Material. It would not be practical for such a body to provide Standard Reference Material fluorophores for all the different fluorophores used as antibody conjugates.

There are a few fluorescence intensity units defined and used by bead manufacturers for quality control of their beads. A unit of fluorescence specific for BD Biosciences is the assigned BD unit (ABD). A fluorescence intensity unit was needed for the cytometer setup and tracking (CS&T) system developed for instrument performance characterization and QC. Intensity values in ABD units

were assigned to many more detection channels than had calibrators available. In essence the ABD values for CS&T beads are tied to a gold-standard bead lot to which the initial ABD values were assigned through correlation with (not calibration to) human lymphocytes stained with CD4 conjugates tagged with a wide variety of fluorophores. Like the ERF intensity unit, the ABD unit for a particular detection channel is defined with a specific laser excitation wavelength and emission filter (emission spectral range). A fluorescent bead that is calibrated in ABD can be cross-calibrated to ERF units.

2.3 *Bead Fluorescence Assignments Vary Among Manufacturers*

Although the basic approach to assigning MESF or ERF values to beads is followed by all bead manufacturers, there seem to be differences in detail that produce differences in the assigned values. A simple comparison of commercially available beads with assigned MESF values was performed by one of the authors (RAH). With no change in the flow cytometer, calibration beads for FITC and PE from several manufacturers were run. Using the MESF values assigned by the manufacturers, the FITC and PE channels were calibrated in MESF per channel. Results are shown in Table 1. In this small sample, it appears the ratio values of MESF and MFI for FITC calibration beads are consistent within either bead type, surface-labeled beads or hard-dyed beads. There is a factor of five difference between the hard dyed and surface labeled FITC standards. However, there are large discrepancies in the ratio values for PE beads. The variations in the ratios of PE beads might likely be due to the absence of a common PE primary solution standard for bead manufacturers performing the fluorescence intensity value assignment.

Table 1 Different beads with assigned MESF values give varying fluorescence calibration in MESF/MFI

	Bead product	Bead type	MESF/MFI
FITC standards			
	1	Surface labeled	14.68
	2	Surface labeled	13.19
	3	Hard dyed	2.87
	4	Hard dyed	2.80
PE standards			
	1	Surface labeled	2.97
	2	Surface labeled	0.85
	3	Surface labeled	0.70
	4	Hard dyed	1.21
	5	Hard dyed	0.86

Table 2 ERF values assigned to the four surface-labeled microsphere reference standards by four manufacturers in addition to NIST

ERF_{major}					
Microsphere	NIST	Vendor A	Vendor B	Vendor C	Vendor D
FITC	7.74×10^4	3.08×10^4	2.19×10^7	1.33×10^7	3.11×10^5
PE	7.94×10^5	5.01×10^4	1.89×10^{10}	1.81×10^7	1.58×10^6
APC	3.21×10^4	6.12×10^3	1.93×10^8	3.62×10^7	not done*
PB	1.59×10^6	3.36×10^4	4.12×10^9	8.00×10^6	7.12×10^6

Table reproduced from Hoffman et al. [4]

To evaluate what variation might occur when different bead manufacturers assign MESF or ERF values to beads, four manufacturers and NIST used the same surface-labeled beads, reference fluorophore solutions, and protocol to assign ERF values using their own equipment and personnel. Results from this study (4) are shown in Table 2.

The study showed large differences among the different manufacturers and compared to NIST, which was considered as the reference laboratory. Partly owing to this result, NIST and ISAC organized a series of workshops that culminated in an agreement to establish an ERF assignment service at NIST available to members of a consortium described in the next section.

2.4 Authoritative, Traceable Fluorescence Intensity Assignments (NIST)

NIST has published a series of reports detailing the fundamental scientific basis and reference methods for assigning MESF or ERF values to fluorescently labeled microparticles [5–10]. Most recently, NIST has produced a primary fluorophore solution kit, Standard Reference Material 1934, that includes fluorescein, Nile Red, coumarin 30, and allophycocyanin for ERF value assignment following its published standard operating procedure [10]. NIST uses a specially designed and calibrated spectrofluorometer equipped with laser excitation and a CCD detector to perform ERF value assignment of calibration microparticles. Laser wavelengths can be selected from any commonly used in flow cytometry. This ERF value assignment service is provided to the participating members of the newly formed flow cytometry quantitation consortium [11]. 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.

2.5 Considerations Using Beads as Cell Analogs for Light Scatter

The most important particle factors that affect light scatter are size and refractive index. While size can be well controlled in microparticle production to correspond to various cell sizes, the refractive index of all polymer particles is significantly higher than that of cells. Silica particles are closer to most cells in refractive index, but are not a true analog. The use of beads to standardize light scatter is further complicated by the fact that different instrument models measure different ranges of scatter angles. Cells are also not homogeneous structures. The nucleus and other substructures have refractive indexes different from that of the more homogeneous cytoplasm. So while homogenous beads cannot reproduce the light scatter from cells, they do provide a useful standard on a particular instrument model for setting up the instrument so cells are displayed in a predetermined location on the scatter scales. Because of the difference in light scatter from the cells, beads are produced and used as an internal counting standard for measuring biological cell concentrations. The relative position of beads and cells can vary quite a lot among different instrument manufacturers and models, but is reasonably consistent for a particular instrument model.

Hydrogels are new materials that are being used to make particles that could be light-scatter standards for flow cytometry. The material allows control over refractive index in the same range as cells and also offers the possibility of heterogeneous structure more similar to nucleated cells [12].

3 Standardization, Calibration, and Quality Control/QC

3.1 How Standardization, Calibration, and Control Differ

As a generally understood term in flow cytometry, standardization is the process that assures that the response of an instrument will be set up to produce expected results when an application is run. This essentially means assuring that cell populations will appear at expected locations on the data scales such as histograms and dot plots. Hard-dyed beads are most often used to set gains or check that gains are set appropriately for the application. But for both fluorescence and light scatter there are limits to how reproducible the setup will be on different instruments, as discussed in Sect. 2.

The best standard particle for setting up a particular fluorophore channel will have the same excitation and emission spectrum as the fluorophore that will be measured in that channel. This assures that all instruments will be set up the same regardless of differences in their spectral response. If the particles have intensity units such as ERF assigned, the fluorescence scale will be calibrated. In that case, the fluorescence from cells can be reported in quantitative units rather than arbitrary

mean fluorescence intensity (MFI). Expressing fluorescence intensity measurements in calibrated units is essential in order to quantitatively compare results from different labs and over time—perhaps over decades.

Quality control of a flow cytometer requires regular monitoring of at least the stability of the detection system and alignment of the sample stream. Stability of the detection system can be monitored either by measuring the PMT voltage or detector gain required to put the scatter and fluorescence signals from stable particles at the same level each time or by measuring the signal levels at fixed PMT voltage or gain settings. The CV of a bright, uniformly fluorescent bead is used to monitor alignment. When the day-to-day change is beyond a predetermined amount, it is time to do maintenance or troubleshooting. Some instrument models or QC software such as BD's CS&T system provide additional QC tracking information based on measurements of hard-dyed beads.

3.2 Control/QC

Two ways that hard-dyed beads are particularly useful are for secondary standards and quality control. Unless components such as filters or lasers in a particular flow cytometer are changed (or change with time), one can use a fluorophore-specific surface-stained bead as the initial primary standard or calibrator and cross-calibrate a hard-dyed bead to it. This is easy to do by simply running the primary and secondary standards at the same instrument settings, preferably as successive samples. Thus occasional cross-calibration of a hard-dyed bead standard to a fluorophore-specific standard allows the hard-dyed bead to be used on a routine or daily basis owing to its superior stability. When used for quality control, the hard-dyed beads are run daily, and the instrument response is monitored for short-term and long-term change in response. For example, the beads can be used to adjust the detector gains so the bead fluorescence mean channel is the same each day and to monitor the gain required to accomplish this. When the detector gain change is more than a prescribed amount, this can alert the user to troubleshoot for a problem. If the problem requires changing an optical component or detector, the primary fluorophore-specific standard should be used to cross-calibrate the hard-dyed bead again.

Stained and fixed cells could be used as fluorophore-specific standard particles for some situations. For example, a study among a group of laboratories might send such stained, fixed cells to each lab in the study. Each lab could cross-calibrate the fluorophore-specific standard bead or cell sample to hard-dyed beads on each instrument in the study and use the hard-dyed beads as secondary standards over an extended time. Although there is no traceable fluorescence value assignment to the cells, their use would assure that all instruments in the study group were set up with identical fluorescence scales.

3.3 Standardization and Calibration

If only one flow cytometer is providing all the data and it is only important that semi-quantitative results be reported, then using a hard-dyed bead without reliable assigned intensity values to standardize the instrument setup can be sufficient. It is necessary to cross-calibrate a new lot of beads to the lot currently being used in order to maintain consistency in instrument setup.

But if fluorescence intensity results need to be compared quantitatively across labs and over time, beads for standardization should be more carefully chosen. If possible, fluorophore-specific primary standards with assigned intensity units should be used. If this is not possible, then complete description of the filters, lasers, and laser power used with the beads should be disclosed. This would allow at least the possibility to quantitatively compare fluorescence results from other instruments.

4 Standardizing and Calibrating DNA and RNA Content Per Cell

4.1 Total DNA Content

Total DNA content is one of the earliest measurements made in flow cytometry [13]. Fluorescent dyes such as propidium iodide that bind stoichiometrically to DNA are used to measure the relative amount of DNA in cells. With some sample preparations, RNA is removed by enzymes so it does not interfere. Since DNA per cell is highly controlled and conserved, the measurement of total DNA per cell requires the highest precision of any flow cytometer application—preferably with less than 2% CV in measurements of non-replicating cells. Sample preparation is critical for quantitative DNA measurements [14, 15].

As this is one of the first applications of flow cytometry, standardization and controls are well developed [16]. Several types of cells are used as standards. Chicken erythrocytes, rainbow trout erythrocytes, and human lymphocytes are well-characterized standard cell types [17, 18]. These cells may be either used as separately stained samples, or if the DNA content is sufficiently different from the test sample, mixed in and stained together with the test sample. Chicken or rainbow trout erythrocytes can be used as internal stain controls with human samples. With careful sample preparation and appropriate standards, DNA content of cells can be expressed in pg of DNA per cell. Tiersch et al. determined the DNA content of a wide variety of vertebrate cells using female human lymphocytes with 7.0 pg DNA per cell as the reference calibration [18]. In studying abnormal DNA content in malignancies, one can use normal lymphocytes from the patient or a healthy individual as an internal control with the test sample and lymphocytes prepared in the identical manner [16, 17].

4.2 DNA and RNA Measurements Using Molecular Biology Techniques

Researchers studying genetic profiles of different cell subsets by sequencing and PCR-based methods have two different technology options available to them: affinity bead-based separation and cell sorting. Both methods give more precise information than bulk analysis methods do, but still suffer from major limitations that have thus far limited clinical, therapeutic, and diagnostic advancements. With the advent of more quantitative technologies to measure isolated genomic material, improved microscopy functionality, and more powerful flow cytometry instrumentation, we are just beginning to break the barriers that previously limited us in quantitative genomic measurements. Flow cytometry allows an investigator to decisively measure genomic material within intact cells while simultaneously cross-referencing these measurements to specific cellular subsets.

Studies of simultaneous, single-cell measurement of RNA and cell-associated proteins have recently been reported [19–21]. He et al. combined florescence in situ hybridization (FISH) with flow cytometry and correlated the intracellular microRNA (miRNA) expression measurements by digital PCR from purified cell-associated miRNA [22]. Significant advancement can be further made to FISH-flow cytometry for quantitative measure of miRNA expression in terms of copy number in specific blood-cell subsets. Quantitative FISH-flow has many advantages over traditional quantitative nucleic-acid measurement techniques. Most notably it allows one to measure cell subtype–specific miRNA expression instead of averaged expression from all cell types and avoids creating artifacts introduced during RNA purification processes. The method correlation transitions the FISH-flow technology into a quantitative, single-cell measurement system.

5 Standardizing and Calibrating Antibodies Bound Per Cell

Cytometrists often use the term “ABC” to stand for “antibodies bound per cell.” This term may not always imply a saturating staining condition, which is a requirement for “antibody binding capacity,” partly due to interference caused by simultaneous staining of many different kinds of antibodies on the same cell population. An ultimate goal of immunofluorescence standardization and calibration is to express cytometry measurement results of biomarkers in terms of ABC. Four 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 avidity. Particular examples of clone variability have been noted for CD4 (Davis et al. [31]) and CD34 [23]. Therefore, if all 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 [23, 24].

One of the essential qualifications for antibody selection is the antibody binding affinity that is assessed by the affinity binding dissociation constant, K_d. However, it is challenging to understand and model the binding titration curves performed using a test antibody and cells carrying the antigen/receptor. Complications arise due to dual surface-binding interactions, cooperative effects associated with multivalent binding, and cell-surface roughness [25]. Figure 3 shows cooperative binding between anti-CD4 FITC (SK3 clone) and cryopreserved PBMCs, which is dominated by divalent binding. Presently, K_d can be estimated comparatively by fitting the linear portion of the binding titration curves [26]. The use of high-binding-affinity monoclonal antibodies, e.g., in the sub-nanomolar range, would minimize non-specific cell staining. For the same antibody clone, the values of K_d can be used for assessing the effect of fluorophore labeling to the antibody clone. Another important parameter in antibody selection is the staining index of the fluorescently labeled antibody, defined as fluorescence signal difference between positive and negative cell populations divided by 2 standard deviation of the negative population [27, 28]. The larger the staining index, the more sensitive the antigen detection would be. This parameter is extremely valuable for choosing the brightest fluorophore-conjugated antibody for the sensitive detection of dimly expressed biomarkers, in particular, in the case of multicolor antibody panel design. In essence, staining index allows the evaluation of the brightness of fluorophore-conjugated

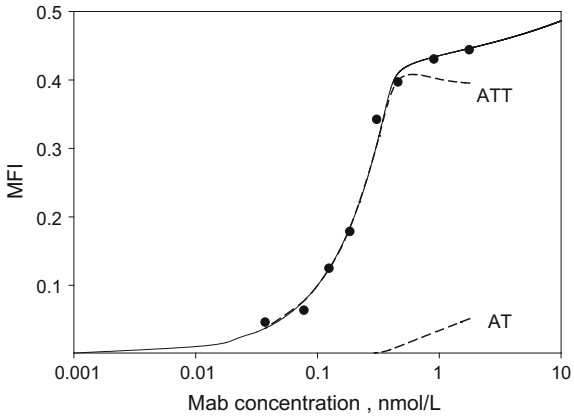


Fig. 3 Mean fluorescence intensity (MFI) measured for peripheral blood mononuclear cells (PBMCs) stained with CD4 antibodies conjugated with FITC fluorophore. The horizontal axis gives the concentration of CD4 antibodies (Mabs) used in the staining of PBMCs. The solid circles are measured values of MFI obtained from the antibody titration. The solid trace is the predicted response assuming both monovalent (trace AT) and divalent binding (trace ATT) of the CD4 antibody to CD4 receptors on the cell surface. The result suggests that CD4 antibody undergoes cooperative binding to the CD4 receptor. The binding of the first site of the CD4 antibody enhances the likelihood of the binding of the second site to another CD4 receptor

antibodies as well as non-specific cell staining. It is expected that an antibody clone with a large value of K_d should have a large value of staining index. However, owing to differences in the process of fluorophore antibody conjugation performed by different manufacturers, it is possible that antibodies with the same clone have similar K_d, but somewhat different values of staining index. It is likely the differences in the staining index are due to difference in fluorescence yield of individual antibody molecules characterized by the number of effective fluorophores per antibody molecule (effective F/P). Therefore, it is important to characterize changes in fluorescence yield induced by fluorophore conjugated to the antibody and further binding of the labeled antibody to the receptor on the cell.

The first two approaches for estimating ABC, quantitative indirect immunofluorescence (QIFI) and Quantum Simply Cellular (QSC), have recently been illustrated in detail [29]. 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. Because the fluorescence-emitting unit of the PE molecule is insulated within the protein [30], it is expected that the fluorescence yield of a single PE molecule is the same as the yield of a unimolar antibody-PE conjugate, meaning the effective F/P is equal to 1. Successful initial experiments [31, 32] ultimately led to the development and production of the Quantibrite products that include purified 1:1 PE-antibody conjugates and freeze-dried beads surface-stained with known numbers of PE molecules per bead. The Quantibrite method provides a great example of quantifying antigen expression levels in the PE channel of flow cytometers. However, the availability of unimolar PE-antibody conjugates is an issue. And although unimolar PE-antibody conjugates provide a known F/P, the effective F/P is not yet available for antibodies labeled with other fluorophores.

The QIFI and Quantibrite methods have been found to be generally comparable [23, 33] for ABC quantitation, but the QSC method frequently gives significantly different results from the other methods [23, 33]. Since the amount of CD4, CD45, and many other molecules on normal human lymphocytes is generally reproducible [34–36], these cell-surface markers may be useful as biological calibrators with a relatively small variability and uncertainty. The use of biological calibrators has become the latest method for quantifying unknowns in ABC.

A detailed protocol of quantitative flow cytometry measurements in ABC based on the human CD4 reference marker has recently been developed jointly by NIST and the FDA [37]. 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 cell (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 [26, 38]. 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 that ultimately ensures quantitative measure of target antigen expression levels independent of flow cytometers used. This approach is illustrated in Fig. 4 for determination of CD20 expression.

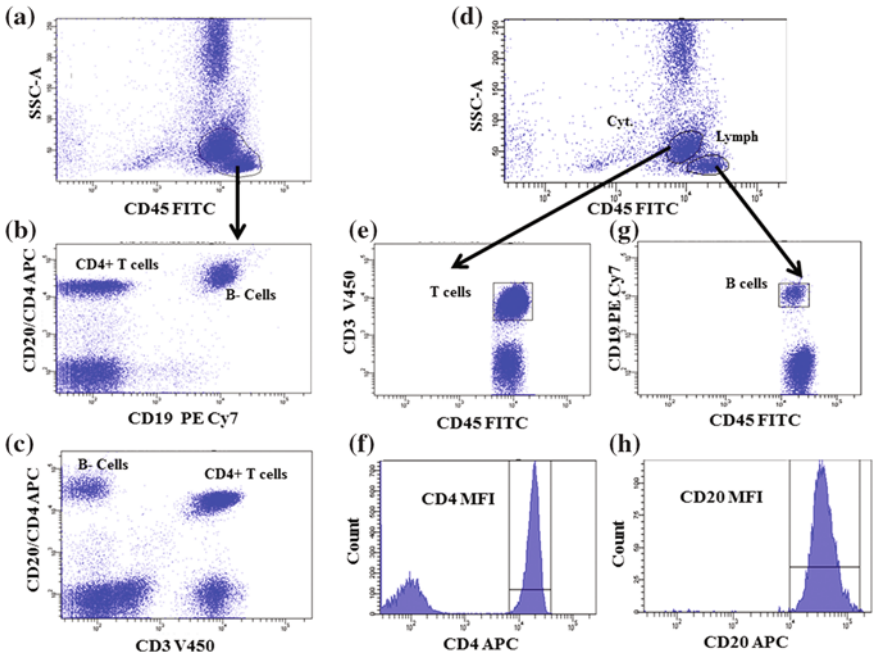


Fig. 4 Quantifying CD20 expression level in ABC units based on a known CD4 expression level on T helper cells from Cyto-Trol control cells, both stained in APC. The unknown whole-blood sample was stained with CD45 FITC, CD19 PE-Cy7, and CD20 APC, and Cyto-Trol was stained with CD45 FITC, CD3 V450, and CD4 APC, in two separate sample tubes. After staining and washing, the two samples were combined in a single tube and run on a linearity-calibrated flow cytometer. Two different gating strategies are shown. Gating strategy I: **a** a large lymphocyte gate (CD45+ and low SSC) was drawn in CD45 FITC versus SSC-A; **b** gated on lymphocytes, CD4+ T cells and CD19+CD20+ B cells were identified in a dot plot of CD19 PE-Cy7 versus CD20/CD4 APC; **c** alternatively, CD4+ T cells and CD20+ B cells can also be identified in a dot plot of CD3 V450 versus CD20/CD4 APC. The MFI values of CD20 and CD4 can then be obtained from a CD20/CD4 histogram under the respective CD20+ B-cell gate and CD4+ T-cell gate. Gating strategy II: **d** two individual lymphocyte gates (CD45+ and low SSC) were drawn as 'Cyt' for Cyto-Trol cells and 'Lymph' for unknown whole blood sample in CD45 FITC versus SSC-A; **e** gated on 'Cyt,' T cells were identified in a dot plot of CD45 FITC versus CD3 V450; **f** under T-cell gate, CD4 histogram shows the positive CD4+ gate, which was used to obtain the respective MFI value of CD4; **g** gated on 'Lymph,' B cells were identified in a dot plot of CD45 FITC versus CD19 PE-Cy7; **h** gated on B cells, CD20 histogram shows the positive CD20+ gate that was used to obtain the MFI value of CD20. With measured MFI values of CD20 and CD4, CD20 expression in ABC can be determined on the basis of the CD4 expression level from Cyto-Trol

Biological-cell reference materials have been gaining momentum as phenotypic benchmarks for quantitative and reproducible measure of patient characteristics in longitudinal studies and/or across locations. High-quality measurement data generated for patients on drug treatments will fill the gap between drug/therapy treatment and clinical treatment outcome. Currently, three different dried or lyophilized human PBMCs are commercially available: FACSCyto PBMC from BD Biosciences, Cyto-Trol Control Cells from Beckman Coulter, and Veri-Cells PBMC from Biolegend. Proper characterization of these cell reference materials would enhance their utility in clinical trials, disease diagnosis, immune-cell manufacturing, and therapy monitoring, drug, and device development.

The biological reference approach relies on antibody conjugates with a particular fluorophore having essentially the same fluorescence intensity per antibody independent of the antibody specificity. One approach to determine the relative fluorescence per antibody relies on measuring the fluorescence from beads that capture antibody. If different antibodies are captured identically at saturation staining levels, then the relative fluorescence per antibody can be determined from the mean fluorescence of the beads. This approach has been problematic, however, since various factors can affect the binding of antibodies to capture beads and affect the degree of fluorescence quenching at near saturated staining levels. Kantor et al. [39] propose an improved approach to determine the relative fluorescence per antibody molecule that does not depend on the saturated staining level. Instead, the approach measures the fluorescence from two antibodies, conjugated to two different fluorophores, which together saturate the binding sites of an antibody capture bead. The antibody conjugated to a first fluorophore (the Test antibody) is used in several dilutions to load the capture beads with a range of antibody levels. After washing the Test-stained beads, the second (Fill antibody) conjugated to a second fluorophore is added to the Test samples in adequate amount to fill the remaining capture sites on the beads. If staining were ideal, the relationship between fluorescence of the Fill and Test antibodies would be linear, with decreasing fluorescence of the Fill antibody as the beads captured more of the Test antibody. To account for possible non-linear behavior near saturation, the method by Kantor et al. fits the data with a quadratic function and uses the linear term of the fit to estimate relative brightness at low antibody density. Unless the relationship between Test and Fill reagents is highly non-linear, this approach gives quantitative measures of the relative brightness among different antibodies conjugated to the same fluorophore. If the relationship is highly non-linear, the Test reagent is considered unsuitable for quantitative measurements. If a fluorophore conjugate with a known quantitative relationship between fluorescence and antibodies bound is used, the system can be calibrated to give fluorescence per antibody conjugate of any fluorophore. Kantor et al. use antibody conjugated to exactly 1 PE molecule and beads with known numbers of PE molecules per bead to make this quantitative step.

An ideal simulated situation is illustrated in Fig. 5, where the Fill antibody is conjugated to FITC and Test antibodies are conjugated to either CY5 or PE, with the PE conjugate highly purified with exactly 1 PE molecule per antibody. Panel A illustrates how the relative brightness of two different CY5 antibody conjugates is

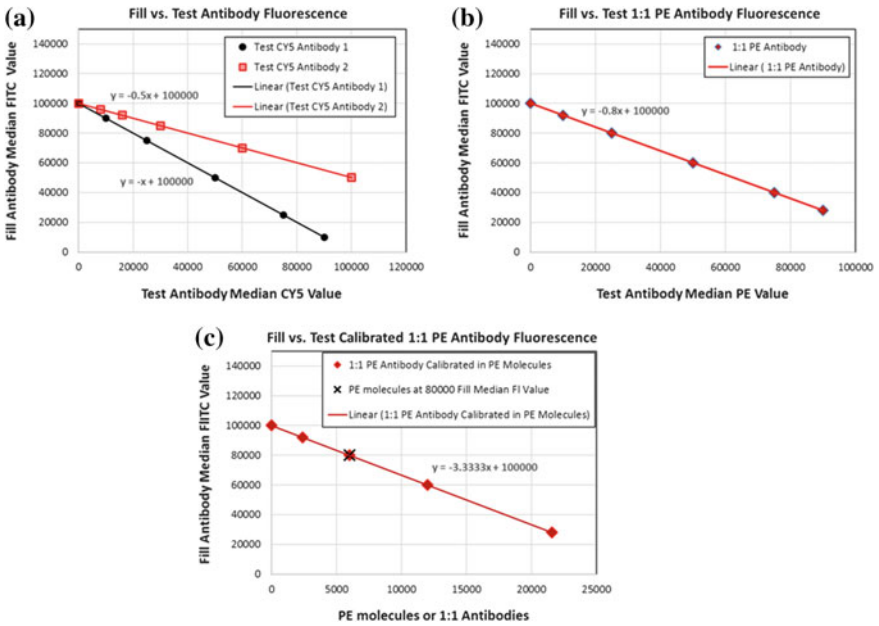


Fig. 5 Simulated example of the Test-Fill method to compare (panel A) and calibrate (panels B and C) fluorescent antibody conjugates. **a** Antibody capture beads are first stained with various amounts of a Test antibody conjugated to one fluorophore (CY5 in this example) and then stained with sufficient Fill antibody conjugated to a different fluorophore (FITC in this example) to saturate all the antibody binding sites on the beads. If fluorescence is proportional to the amount of antibody on the bead, there is a linear relationship of capture bead fluorescence between the two fluorophores. The greater the fluorescence of the Test reagent at a particular level of Fill reagent fluorescence, the brighter the Test reagent. Two different CY5-labeled Test antibody conjugates are compared. **b** A 1:1 PE conjugate is used as Test reagent. **c** If the PE scale is calibrated in PE molecules, equivalent to antibody molecules for a 1:1 conjugate, the Fill axis is calibrated in fluorescence intensity per antibody molecule

determined. The relative amount of test antibody is indicated by the reduction in Fill antibody from the saturation level (zero Test antibody added). In this example, antibody conjugate 2 is brighter because it has a smaller slope, indicating less Test antibody is on the beads at any level of Test antibody fluorescence. In this case CY5 antibody 2 is twice as bright as CY5 antibody 1. Panel B illustrates the relationship when a highly purified PE conjugate with exactly 1 PE molecule per antibody (such as BD Quantibrite reagents) is used. If the PE fluorescence axis is calibrated in PE molecules (for example with BD Quantibrite PE beads), then the relationship between reduced Fill fluorescence and the number of PE molecules is obtained.

Once the Fill fluorescence scale has been calibrated in antibody molecules per fluorescent unit for a particular Fill reagent, the relative relationship between antibody brightnesses can be translated to absolute fluorescence per antibody for any Test reagent conjugated to a fluorophore other than the one used for the Fill

reagent. In the illustration of Panel C, 6000 PE molecules or equivalently 6000 antibody molecules conjugated to exactly 1 PE molecule cause a reduction of 20,000 units of fluorescence in the Fill reagent. Or equivalently 20,000 units of fluorescence from the Fill reagent is equal to 6000 antibody molecules or 6000/20,000 ABC/FITC FlUnit, or 0.3 ABC/FITC FlUnit. With this additional information the CY5 fluorescence scale can be translated to ABC for each of the CY5 conjugates. With CY5 Antibody 1, which has a slope of 0.5, the scale translates to (0.3 ABC/FITC FlUnit)x(0.5 FITC FlUnit/CY5 FlUnit) = 0.15 ABC/CY5 FlUnit. With CY5 Antibody 2, which has slope of 1, the CY5 scale for this antibody would translate to 0.3 ABC/CY5 FlUnit.

6 Fluorescence Performance Characterization

When controls are run regularly and quality control is practiced, a flow cytometer will provide reproducible results. But this does not guarantee that the results will be adequate for all applications. The performance of flow cytometers varies among different instrument models. Even different instruments of the same model will have different levels of performance, particularly regarding fluorescence. Performance can degrade over time as well. It is best if a flow cytometrist has objective and measurable criteria for instrument performance. This is particularly important when data from multiple instruments are used in a study.

A sample of multilevel beads such as the Spherotech Rainbow beads shown in Fig. 6 tells much about instrument performance. Such mixtures of beads stained at different levels are made from the same batch of unstained beads and all have nearly the same intrinsic CV. The brightest beads in the mixture are used to assess optical

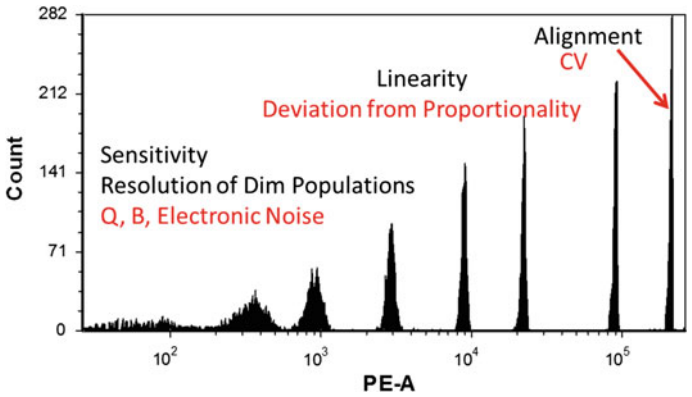


Fig. 6 Histogram of Spherotech 8-peak Rainbow beads (catalog number RCP-30-5A). MFIs and CVs of the seven stained populations can be applied in different ways to characterize performance of the fluorescence detection system

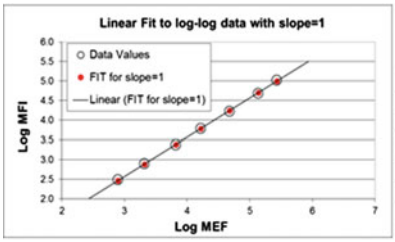
alignment—the smaller the CV, the better the alignment of the sample stream to the focused laser beam. Comparing the measured mean fluorescence intensity (MFI) to the intensity value of each bead population provided by the manufacturer gives information about the linearity of the instrument response. The broadening of the populations as the beads have less fluorescence is not due primarily to the beads themselves but rather to the decreasing number of photoelectrons generated in the detector (usually a PMT) and the effect of background light (such as Raman scatter from water) and the fixed level of electronic noise that is present in the amplifier and digitizing electronics. For most practical purposes the CV of the brightest bead can usually be treated as having the same intrinsic CV as all the other beads, and broadening of the dimmer bead populations is due to the other factors detailed later in this chapter. In some very sensitive instruments, the dimmest stained bead in the Rainbow bead set has a small but measurable increase in intrinsic CV compared to the brighter beads, but the dominant contribution to broadening of the populations are instrument related.

6.1 Linearity

Before measuring the contributions to population broadening of dim particles, however, it is important to know the range over which the measurements are linear [40]. An underappreciated effect of nonlinearity is the significant error that can be introduced into the calculation of spectral overlap compensation, which assumes that the measured signal is strictly proportional to the input optical signal. Under some conditions, nonlinearity of a few percent at the top of the scale in one fluorescence channel can cause an order-of-magnitude error in compensated values of a double-stained population at the low end of the scale in another channel. For clear data interpretation and quantitative measurements, a maximum deviation from linearity of 2% or less is recommended. Significant nonlinearity at the low end of the scale will cause errors in measured CVs that affect characterizing detection of dim fluorescence.

A set of multi-intensity beads such as the Spherotech Rainbow beads shown in Fig. 6 can provide a limited test of linearity using the manufacturer's assigned intensity values for each population. Figure 7 shows the result of such a test, where the MFI is plotted versus the assigned intensity units (MEF) for the FITC channel on a flow cytometer. Data are plotted on a log-log scale and fitted with a linear function of slope 1, which assumes that the MFI is proportional to the assigned MEF. The visual plot indicates a good fit, but the result shows deviations from proportionality of up to 4% at some parts of the scale. This instrument was also tested for linearity by an alternative method described next.

A better way to test for proportionality (strict linearity) is to compare the measured ratio of two output signals whose relative input values (ratio) are known. If the electronics are strictly linear, the ratio of the two measured signals will be the same as the ratio of the two input signals. The standard manufacturer's specification



Rainbow Bead population	Assigned MEF	Measured MFI	linear % diff from fit
Peak8	271647	104057	3.87
Peak7	137049	48441	-4.18
Peak 6	47497	16793	-4.15
Peak 5	16471	6024	-0.69
Peak 4	6588	2375	-2.15
Peak 3	2079	788	2.84
Peak2	792	304	4.06
		Average %residual	2.46

Fig. 7 Evaluation of linearity in the FITC channel of a flow cytometer using comparison of MFI to manufacturer-assigned fluorescein intensities per bead (MEF) of Spherotech 8-peak Rainbow beads. Data were acquired on the same instrument used for data shown in Fig. 8

for flow cytometer linearity (if it is specified at all), is that the ratio of the MFIs of doublet and singlet chicken erythrocyte nuclei stained with a DNA dye will be 2.00 ± tolerance. For example, the doublet to singlet ratio will be 1.95–2.05. While this ratio approach is useful at one point on the scale, it does not give any information about other points on the scale, which can range over four to seven orders of magnitude.

The reference method for testing the linearity of an optical detection system exposes the detector to flashes of light from a light-emitting diode (LED), with alternating flashes of light at two different but consistent levels. While the electrical drive to the LED is not changed, the amount of light reaching the detector (e.g., PMT) is varied by positioning the LED closer to or further from the detector or by using neutral density filters to reduce the intensity. If the detector is linear (i.e., output proportional to the input light) the ratio of the two output signals will be constant no matter how much of the LED light reaches the detector. Deviation of the output ratio from the expected value is an indication and measure of non-linearity. This approach is easy to do at an engineering level but is not usually practical for routine use in most flow cytometer labs. An alternative by Bagwell et al. [41] used the ratio of florescence intensities of two different beads to evaluate the linearity of detector system electronics by varying the PMT voltage to cause the signals to the electronics to cover the entire measurement scale. One of the authors (RAH) extensively compared this approach using PMT voltage to vary the input signal to the reference LED ratio method during the development of the BD CS&T system at BD Biosciences. The two approaches gave equivalent measures of detector system linearity, and the PMT voltage variation approach was integrated into the CS&T system to measure linearity.

Table 3 shows the results of this ratio method from the same instrument used for the data in Fig. 7. Two of the Rainbow bead populations were used and the ratio of their MFI determined over the entire measurement scale by varying the PMT voltage for the FITC channel. The ratio method indicated a much higher degree of linearity than suggested by the comparison with manufacturer-assigned intensity values. This method to evaluate linearity is easy to do and takes only a short time.

Table 3 Example of electronics linearity test using the ratio of means of two bead populations as PMT voltage is varied to place beads along a histogram scale of 0–262544

PMT voltage	Bead 1 median	Bead 2 median	Bead2/Bead1	% deviation from average ratio
300	26	65	2.50	−1.75
350	77	200	2.60	2.07
400	203	514	2.53	−0.50
500	1033	2636	2.55	0.28
600	4040	10309	2.55	0.28
700	12730	32474	2.55	0.25
800	36441	92438	2.54	−0.31
900	98468	245714	2.50	−1.94

The same instrument was used for the linearity test shown in Fig. 7

6.2 Noise Contributions Broaden Measured Populations

In simplest terms, the CV or variance of a population is the sum of the CVs or variances intrinsic to the sample itself and the added variance from the measurement process in the instrument. The contributions to measurement variation from the instrument are due to a constant level of electronic noise in the electronics, optical background light, statistical variation in the number of photoelectrons generated by a light pulse, excitation variation (or laser noise), and variation in how uniformly each particle is illuminated and the fluorescence collected on the detector. The total instrument contribution to the standard deviation (SD) is calculated from the squares of individual contributions. SD^2 is also called the variance.

$$SD^2_{Instrument} = SD^2_{Photoelectron} + SD^2_{Backgnd} + SD^2_{LaserNoise} + SD^2_{Position} + SD^2_{ElectronicNoise} \quad (1)$$

For bright signals, the variability of particle illumination and detection based on particle position in the sample stream (grouped in the contribution $SD_{Position}$) and laser noise are dominant, but for lower signals, the statistical nature of the photon detection process adds variance along with variance from added non-signal photoelectrons from background light. Variance due to the limited number of signal photoelectrons is determined by the detection efficiency, Q, which is described more fully in Sect. 6.3 below. Conceptually, Q is the equivalent number of photoelectrons generated in the detector by a fluorophore molecule passing through the laser beam. At the low end of the measurement scale (independent of PMT voltage), a contribution from electronic noise can be expected.

Quantitative relationships for the various factors are:

$SD^2_{Photoelecton} = \frac{F}{Q}$, where F is fluorochrome per particle measured in intensity units

$SD^2_{Backgnd} = \frac{B}{Q}$, where B is equivalent background fluorochrome

$SD^2_{LaserNoise} = [n^*(Signal + Background)]^2$, where n is fractional laser noise

$SD^2_{Position} = (Signal^* CV_{particle})^2$

$SD^2_{ElectronicNoise} = Constant.$

Electronic noise does not change with PMT gain and can be measured in several ways. If accurate measurements around zero signal can be made, as in most recent BD flow cytometers other than FACSCalibur, the SD due to electronic noise can be measured by turning the PMT voltage to zero and measuring the SD of the resulting noise signal. Alternatively, one can monitor the SD of a bead with relatively bright, uniform fluorescence as the PMT voltage is reduced to successively lower values. The distribution on the histogram will broaden as electronic noise becomes a significant factor of the total variance, and the SD will tend toward a stable number no matter how bright the initial bead fluorescence. An example is shown in Fig. 8.

The SD approached by all the beads at low signal levels is the electronic noise, which is always present but becomes insignificant at sufficiently higher signal levels. For best resolution of dim signals, the gain should be set so the CV of the unstained cell population is not significantly broadened by electronic noise. For

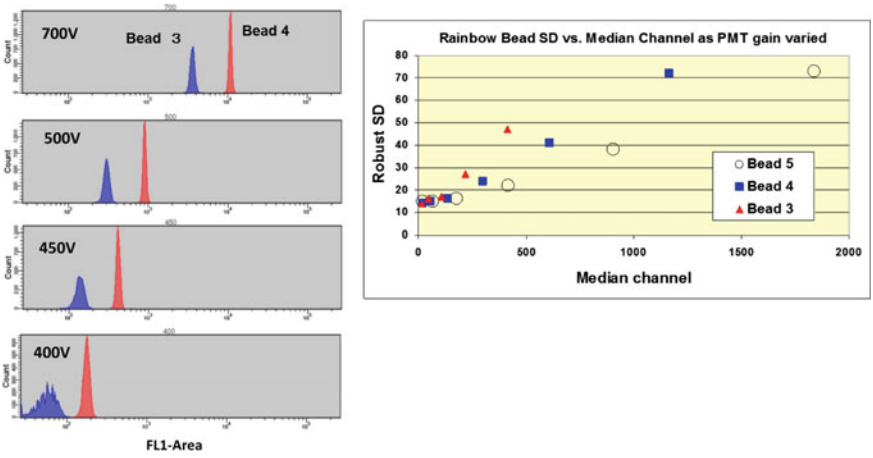


Fig. 8 Histogram of gated populations of Spherotech Rainbow beads at various PMT gains and robust standard deviation of the bead populations versus median fluorescence intensity at different PMT gains

example, if the electronic noise SD is 15, and the median channel of a population is 300, the electronic noise contribution to the measured CV is only 5%.

Since all the contributions to measured CV except $SD^2_{ElectonicNoise}$ do not vary with PMT voltage, one can also measure electronic noise by plotting measured fluorescence CV^2 versus $1/Mean^2$ of a particle over a range of PMT voltages. The slope of the plot is $SD^2_{ElectonicNoise}$, which is expected from the following relationships from dividing the SD^2 factors by $Mean^2$ to put the relationships in terms of CV rather than SD.

$$CV^2_{Instrument} = CV^2_{Photoelectron} + CV^2_{Backgnd} + CV^2_{LaserNoise} + CV^2_{Position} + \frac{1}{Mean^2} * SD^2_{ElectronicNoise} \tag{2}$$

$$CV^2_{Instrument} = Constant + \frac{1}{Mean^2} * SD^2_{ElectronicNoise} \tag{3}$$

6.3 Detection Efficiency, Q, and Background Light

If both signal and background light contributions are considered together, the variance in photoelectron contribution is the sum of both variances. Background, B, is expressed as the amount of fluorophore units that would produce the background light. When measured under conditions where signals are detected well above electronic noise and with flashes from an LED, one has [42]

$$SD^2_{TotalPhotoelectron} = f \cdot \frac{1}{Q} + \frac{B}{Q} \tag{4}$$

f = calibrated particle signal intensity in fluorescence units, Q = statistical photoelectrons per fluorescence unit, B = background in fluorescence units.

The best way to measure the instrument contributions to variance is to use light flashes from a light-emitting diode (LED) to simulate signals from a sample with zero intrinsic CV [43]. To make this performance characterization broadly available, Chase and Hoffman showed that sets of beads stained at varying levels could adequately replace LED flashes when the intrinsic CV and instrument broadening of the brightest bead in the set are taken into account [44]. They proposed the term Q as the measure of photoelectrons generated per particle fluorescence unit (e.g., MESF or ERF) and B as the constant background light always present when particles are measured. The variation due to the statistical nature of photon conversion to photoelectrons is increased slightly in a PMT owing to the amplification process. If there were no added noise in amplification, the SD of photoelectrons would be the square root of the average number created by repetition of identical light pulses. The concept of statistical photoelectrons is a measure of that variance

and has been given the symbol S_{pe} [45]. So Q is more properly described as S_{pe} per particle fluorescence unit. B is also measured in the same fluorescence units as Q.

The approach used by Chase and Hoffman [44] estimated B at zero signal level and separately measured Q at a sufficiently high signal so that electronic noise and background light were negligible. The measured CVs were corrected for the CV of the brightest bead in the set. Hoffman and Wood [46] used a linear fit to Eq. 4 to determine Q and B, where the slope is 1/Q and intercept of the fit is B/Q. Again in this case measured CVs were corrected for the CV of the brightest bead in the set to calculate the SD due to photoelectron statistics. Figure 9 is an example of a spread sheet using this approach. The data for the Q and B measurement should be obtained using linear rather than logarithmic amplifiers for instruments such as BD FACSCalibur where both options are available.

Rather than estimate the intrinsic measurement CV from the CV of the brightest bead in a set, Parks et al. [45] improved the fitting for Q and B determination by adding to Eq. 4 a term $C*f^2$ that includes the intrinsic measurement variance. The data are then fit with a quadratic function that gives best estimates for 1/Q, B/Q and the "intrinsic" CV of the measurement when the particle is so bright that factors other than photoelectron statistics are dominant. The quadratic fitting approach was applied to both LED flashes and multi-level bead sets and found to work well. Beads used in the study and LED flashes generally gave equivalent results on an instrument. The exceptions were when the instrument used log amplifiers, which affected accuracy of the measurements, and when instruments had particularly high

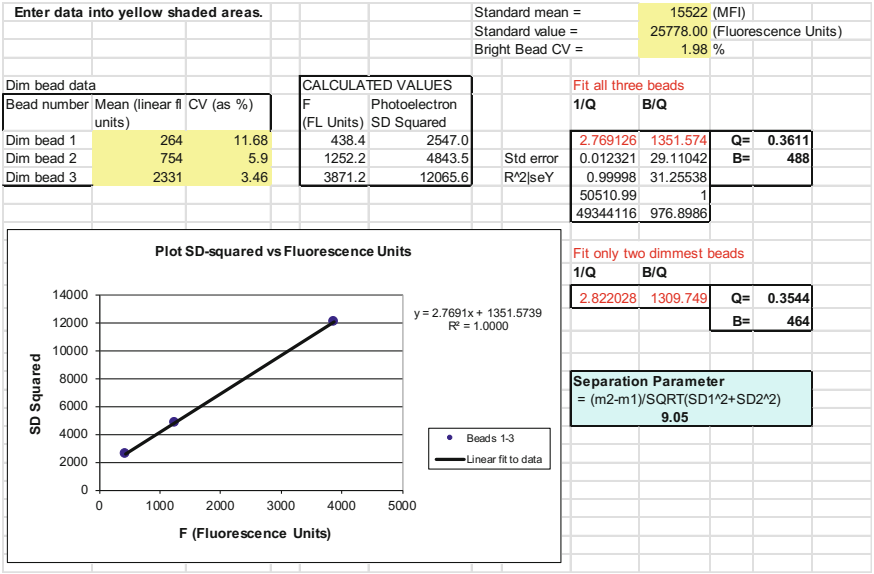


Fig. 9 Example of Q and B determination for the PE channel on a flow cytometer. BD Quantibrite beads were used as the standard and Spherotech 8-peak Rainbow beads as the test sample. See Fig. 6 for an example of a histogram of these beads

sensitivity (high Q), in which cases the assumption that all beads in the set had the same intrinsic CV seemed to not apply to the dimmest beads in the set.

All the performance characteristics—optical alignment, linearity, electronic noise, background light, and detection efficiency—are straightforward to assess. BD Biosciences' CS&T system conveniently performs these tests automatically with proprietary beads and software. In whatever way the performance is objectively measured, the results can be used to predict performance of biological applications. See Chase and Hoffman [44] for a simple example. In the future, this may be the most valuable use of this information. At least it can be used to set the minimum instrument performance requirements for important research or clinical assays. Furthermore, when these critical measures of instrument performance, Q and B, are standardized with traceable fluorescence units of MESF and ERF, users can take into account the difference in the performance of various cytometer platforms and design the most sensitive assays in the multisite studies.

6.4 Buyer Beware

Historically, simpler approaches to characterizing fluorescence sensitivity have been proposed that reduce this performance measure to a single number. Unfortunately, a simple but non-informative method has become the industry standard used in marketing literature and technical specifications. It is easy to show how any attempt to do this (e.g., detection threshold or delta channel) allows two instruments with the identical single-number "sensitivity" measure to have significantly different ability to resolve dim populations [47]. It is disappointing that as this chapter is being written, all instrument manufacturers still use the extrapolated intercept of a plot of Spherotech-assigned intensity values versus MFI of Rainbow or Ultra Rainbow beads to advertise "sensitivity" in terms of "molecules of equivalent soluble fluorochrome." This is no longer a scientifically justifiable measure and in fact has been refuted by presentations at international cytometry meetings. BD Biosciences continues to use the non-informative "molecules of equivalent soluble fluorochrome" specification in marketing literature for instruments that provide users with BD's rigorous CS&T performance characterization system. This measure of "sensitivity" has been around so long that a brochure or technical specification sheet apparently must have "molecules of equivalent soluble fluorochrome" in order to show how the instrument compares to the competition. Perhaps if enough customers ask serious questions about fluorescence sensitivity, technical data sheets will eventually have scientifically meaningful specifications for Q, B, and electronic noise.

7 Future Possibilities

Flow cytometry has had rapid growth since the middle of 1980. It has moved from a technology platform that only a few hundred "initiated" experts understood and could use to become an essential immunological tool for research, drug and device development, clinical trials, disease diagnostics, and immune-cell manufacturing and therapy monitoring. Flow cytometry is essential for accurate measurement of CD4+ cell counts for ensuring that patients receive the appropriate antiretroviral treatment for HIV/AIDS monitoring. A validated reference standard has been developed for quality control of clinical CD4+ cell counting following the World Health Organization's call for establishing an external quality assessment program [48]. At present, multiplexed flow cytometry assays are routinely used in clinics for disease diagnosis and therapies [49–51]. Moreover, flow cytometry has also become an essential clearance tool for the production of protein and cell therapeutics [52, 53]. All these applications essentially require that comparable and reproducible results can be generated using different flow cytometer platforms at different locations and times.

Consistency of flow cytometry measurements can only be accomplished using proper controls and standards, 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.

In an ideal world, a flow cytometrist would be able to compare flow cytometer performance requirements that have been previously determined and recorded in a newly published journal article, check those requirements against the performance of the necessary light scatter and fluorescence channels on the instrument in their lab, and know in advance whether the new application should run successfully. If the lab's instrument is capable of successfully performing the application, the flow cytometrist would run controls that check whether the instrument is still performing as well as previously. If the controls indicate performance is still good enough for the application, beads would be run to standardize or "set up" the instrument for the application by setting the detector gains so populations of cells will be in the expected range on the detector channels. If it is not automated by the software, the user may have to run separate samples to set spectral compensation.

The only part of this idealized scenario that is not yet commonly done is the publication of instrument performance requirements that are necessary to assure that an application will give adequate results. It is now possible to predict and model multicolor flow cytometry data once the fluorescence characteristics of the sample and critical performance characteristics of the flow cytometer are known. If engineering support is available, it is also possible to intentionally detune and degrade aspects of instrument performance until an assay is just still giving reliable results. With either approach it is possible to define the minimum performance required of critical instrument characteristics such as those affecting fluorescence sensitivity. The tools are available to do this. Now they need to be used.

Acknowledgements The authors would like to thank Adolfas Gaigalas of NIST for his pioneering work and continued engagement in developing traceable fluorescence standards. They would also like to thank David Parks of Stanford for reviewing and clarifying the chapter section on the TEST-FILL method.

Disclaimer Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately 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.

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