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Toward Quantitative Fluorescence Measurements with Multicolor Flow Cytometry

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Abstract

A procedure is presented for calibrating the output of a multicolor flow cytometer in units of antibodies bound per cell (ABC). The procedure involves two steps. First, each of the fluorescence channels of the flow cytometer is calibrated using Ultra Rainbow beads with assigned values of equivalent number of reference fluorophores (ERF). The objective of this step is to establish a linear relation between the fluorescence signal in a given fluorescence channel of multicolor flow cytometers and the value of ERF. The second step involves a biological standard such as a lymphocyte with a known number of antibody binding sites (e.g., CD4 binding sites). The biological standard is incubated with antibodies labeled with one type of fluorophores for a particular fluorescence channel and serves to translate the ERF scale to an ABC scale. A significant part of the two-step calibration procedure involves the assignment of ERF values to the different populations of Ultra Rainbow beads. The assignment of ERF values quantifies the relative amount of embedded fluorophore mixture in each bead population. It is crucial to insure that the fluorescence signal in a given range of fluorescence emission wavelengths is related linearly to the assigned values of ERF. The biological standard has to posses a known number of binding sites for a given antibody. In addition, this antibody has to be amenable to labeling with different types of fluorophores associated with various fluorescence channels. The present work suggests that all of the requirements for a successful calibration of a multicolor flow cytometer in terms of ABC values can be fulfilled. The calibration procedure is based on firm scientific foundations so that it is easy to envision future improvements in accuracy and ease of implementation. Published 2007 Wiley-Liss, Inc.†

Key terms

multicolor cytometry; calibration; quantitation; biological standard

IN a flow cytometer measurement, cells are carried by a flowing stream across focused laser beams (1). Prior to aspiration of the cells into the sheath fluid stream of the flow cytometer, the cells are incubated with a mixture of labeled antibodies specific to various receptors on the cell surface or intracellular protein antigens. Nuclear antigens can also be detected and are often useful as proliferation markers. After the incubation, the labeled antibodies are attached to specific receptors on the surface of the cell or intracellular protein antigens. As the cells flow past the laser beams, the fluorescence of fluorophores is detected in discreet fluorescence channels (FC) via photomultipler tubes (PMT). Fluorescence signal detected in a specific fluorescence channel is representative of the expression level of a given protein antigen. Because immune cells work in a cooperative manner (2), disease diagnostics and immunotherapies are mostly carried out by monitoring expression of multiple cell receptors, such as HIV-1 infection (3,4) and chronic lymphocytic leukemia (5,6). A CLSI guideline (7) exists for fluorescence calibration and quantitation measurements, for example, with a specific FC/PMT using flow cytometry. However, a method toward quantitative fluorescence measurements with multicolor flow cytometry other than day-to-day instrument quality control and assurance (QC/QA) (8,9) is not yet

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available at this time. The need for the method has been addressed by FDA in its critical path report that solicits critical path opportunities on the basis of the identified need for innovations (10,11).

The objective of this work is to provide a calibration scheme such that the detected fluorescence signal can be presented in terms of a number of antibodies bound per cell (ABC) (7). A simplified discussion of the calibration scheme is presented below to assist the understanding of the detailed description that follows in the next section. The calibration method does not assume any special conditions that are unique to any one commercial cytometer. Therefore, the calibration method is applicable to any commercial cytometer. The calibration procedure starts with a measurement of the response in each FC due to the set of Ultra Rainbow beads. Ultra Rainbow beads are micron-sized particles that contain a mixture of fluorophores such that the beads emit fluorescence in every channel of the flow cytometer. The set of Ultra Rainbow beads consists of six different bead populations, each with a different amount of the fluorophore mix. Ideally, the proportion of the fluorophores in the mix is the same for each bead population. Each population of the Ultra Rainbow beads will have an assigned number (called equivalent reference fluorophores, ERF) that represents the relative amount of the fluorophore mix contained inside the bead. The response to the Ultra Rainbow beads in a given FC will be represented by histograms characterizing the fluorescence pulse heights from the different populations of Rainbow beads. Figure 1a shows a hypothetical calibration curve for a single FC obtained by plotting the mean pulse height associated with each bead population versus the assigned ERF value for that population. The ideal result is a straight line as shown by the solid curve in Figure 1a.

The next step is to analyze a biological standard through the cytometer. Figure 1b shows the steps taken in the preparation of the biological standard. The upper part of Figure 1b shows five containers with a population of standard cells (dotted line) at the bottom of each container. In this case, the biological standard will be cells with a known number of specific protein antigen, e.g., normal or lyophilized T lymphocytes with CD4 receptors (3,12). The biological standard is incubated with antibodies for that specific protein antigen. Prior to incubation, the antibodies are divided into several lots, and each lot is labeled with one of the fluorophores that will be detected in each FC. The symbols Y in the upper part of Figure 1b denote the antibody and the subscripts denote the label (FITC, PE, APC, etc.) corresponding to the FC. Figure 1b shows the case of five fluorescence channels; however, the number of FC can vary with the application. After the incubation, the cells stained with labeled antibodies are washed, concentrated, and pooled. The single container at the bottom of Figure 1b shows the final biological standard, which consists of the pooled antibody-stained lymphocytes. Passing the labeled biological standard through the flow cytometer leads to a response in each of the FC. The dashed arrows in Figure 1a show how the calibration line together with the response from the biological standard in that FC leads to the establishment of a scale for antibodies bound per cell (ABC) (right hand axis in Fig. 1a). Subsequent to the calibration, flow cytometer measurements on the analyte cells can be reported in terms of ABC values.

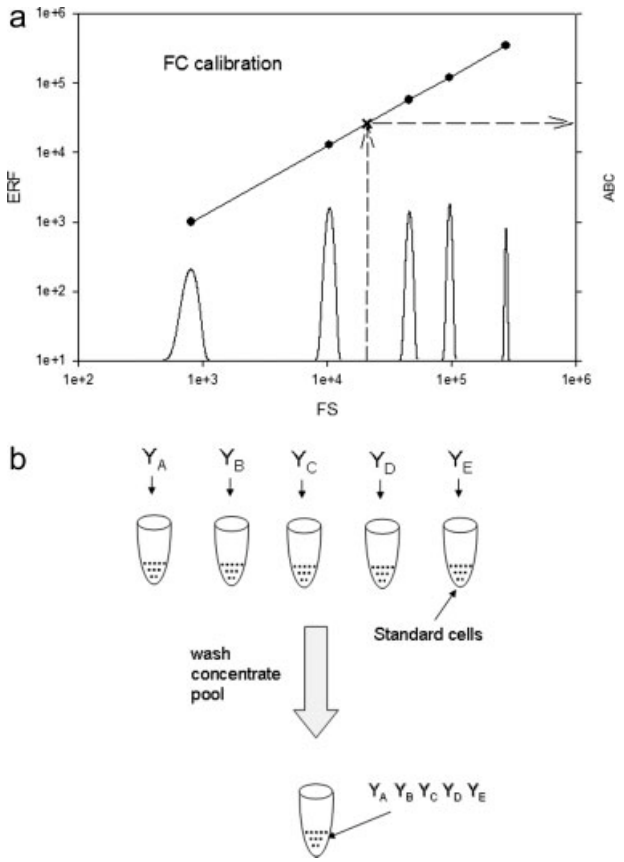

Figure 1. (**a**) The solid circles represent a plot of the hypothetical numbers of equivalent reference fluorophores (ERF) assigned to the five populations of Ultra Rainbow beads as a function of the mean pulse height associated with the five simulated peaks in the fluorescence channel (FC). The solid line is a best linear fit to the five points and constitutes a calibration of the FC. (**b**) A schematic of the process used to produce a biological standard. Standard cells are incubated with the same antibody (Y) labeled with different fluorophores (A—E). After incubation, the labeled cells are washed, concentrated and pooled. The pooled cells constitute the biological sample. The vertical dashed line in Figure 1a is drawn from the mean pulse height of the response associated with the biological standard. The point (x), where the dashed line crosses the calibration line, defines the ERF value which corresponds to the number of labeled antibodies on the biological standard. This point (as well as the zero point defined by a negative population) sets the ABC (antibodies bound per cell) scale on the right side of Figure 1a.

In the following, we develop a methodology to implement quantitative measurements in all fluorescence channels of a multicolor flow cytometer. The procedures are applicable for a given assay performed on a specific instrument. The methodology can be also applied to develop kits for quantitative measurements with specific instruments. As an example, the method will be described using a flow cytometer that has 405, 488, and 632 nm laser lines and incorporates appropriate

dichroic mirrors and band pass filters to define the fluorescence channels.

Experimental Details

Nile Red and allophycocyanin (APC) were obtained from Molecular Probes/Invitrogen (Carlsbad, CA)[1] and used as received. Coumarin 30 with a dye content of 99% was from Sigma-Aldrich (St. Louis, MO). Ultra Rainbow beads including a blank and five fluorescent populations, each embedded with a different amount of fluorophores (3.5–3.9 μm in diameter) were purchased from Spherotech (Libertyville, IL). Heparinized normal donor samples were obtained from NIH Department of Transfusion Medicine. This sample source is exemption approved by the Institutional Review Board (IRB). Monoclonal antibodies, CD4-PE unimolar conjugate (clone Leu-3a) (Catalog Number: 340586) and CD4-biotin conjugate (Catalog Number: 3,47,321), and streptavidin-labeled allophycocyanin (SA-APC) were obtained from BD Biosciences (San Jose, CA).

The procedure for whole blood staining was described previously (12). Briefly, the whole blood washed with 1× PBS was stained with labeled antibodies for 30 min at room temperature. For direct labeling with unimolar CD4-PE conjugate, the cell suspensions were subsequently lysed with 1× FACS^{TM} lysing solution (BD Biosciences). After washing twice with 1× PBS, the obtained leukocytes were resuspended in 0.5 mL of PBS. For indirect labeling with CD4-biotin conjugate, the cell suspensions with excess labeling reagent were washed once with PBS and then incubated with SA-APC for 20 min in the dark at 4°C. After lysing with 1× FACS^{TM} Lysing Solution, the cell suspensions were further washed twice with PBS and finally resuspended in 0.5 mL of PBS.

The flow cytometric measurements were carried out using a FACSCanto equipped with 405, 488, and 632 nm lasers (BD BioSciences, San Jose, CA). BD "FACSDiva" software and clinical software were utilized in the multicolor cytometer for data acquisition and analysis, respectively. For the measurements, lymphocyte populations were gated by using 2D side and forward scatter plots, and median channel numbers obtained from fluorescence histograms were used for the determination of ERF values. Back gating with CD3 versus CD4 was also used to ensure the inclusion of the total T lymphocyte population. CD45 gating was not used.

Ultra Rainbow Bead Fluorescence Spectra

Each rainbow bead contains a mix of several different fluorophores embedded in the polymeric matrix of the bead. Using the nomenclature provided by the manufacturer, bead 1 contains no fluorophores and serves as the "blank" or the negative control. Beads 2 through 6 contain increasing amounts of the fluorophore mixture embedded in the bead matrix. Figure 2 shows the fluorescence spectra of rainbow bead 4 taken with three different excitation laser lines, 405, 488, and 632 nm. The location of each excitation line is shown by the vertical bar, and the corresponding fluorescence spectrum is displayed to the right of the bar. The termination of the fluorescence emission spectrum at the higher wavelengths is due to the spectrometer settings. The actual emission spectrum continues beyond the termination wavelength. It is expected that for each excitation wavelength, the fluorescence emission spectrum will originate from a different subset of the fluorophores contained in the bead. The fluorescence spectra differed slightly for each of the bead populations. Figure 3a shows the emission spectra from beads 2 through bead 6 using 488 nm excitation, and Figure 3b illustrates the difference in the spectral shapes of the five bead populations through normalizing their emission spectra. The largest difference in the spectra occurs in the region around 600 nm. Figure 4 shows the normalized bead emission spectra for 405-nm excitation. There is a small difference between the spectrum of bead 2 and the spectra from the rest of the beads. The excitation with 632 nm resulted in similar spectra (not shown) for all bead populations. Ideally, all bead populations should display the same normalized fluorescence spectrum. Only the relative magnitude should change between the different bead populations.

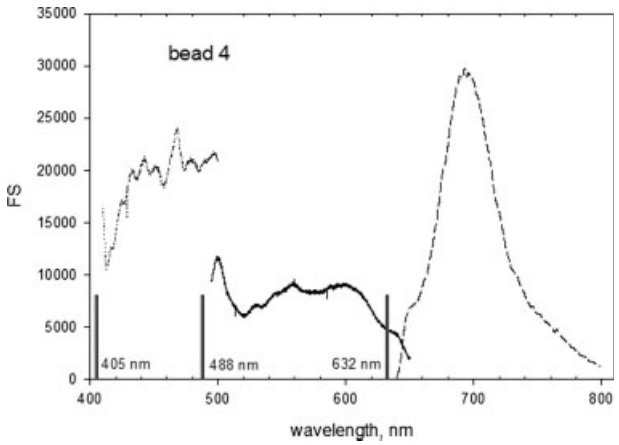

Figure 2. The fluorescence emission spectra from a suspension of Ultra Rainbow bead 4 excited with three different laser lines. The vertical bars give the location of the three laser lines, and the spectrum to the right of each line shows the corresponding emission spectrum. The spectra are not normalized or corrected for detector response.

Methodology

A set of rainbow beads containing increasing amount of a mix of fluorophores are used to calibrate the fluorescence signal linearity in each of the fluorescence channels (FC) of the cytometer. To accomplish the calibration it is necessary to assign numbers to each population of beads that will characterize the relative fluorescence signal of each population of rainbow beads in a given FC. The numbers assigned to each

[1]Certain commercial equipment, instruments, and materials are identified in this article 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. In addition, this work does not represent the official position of the Food and Drug Administration.

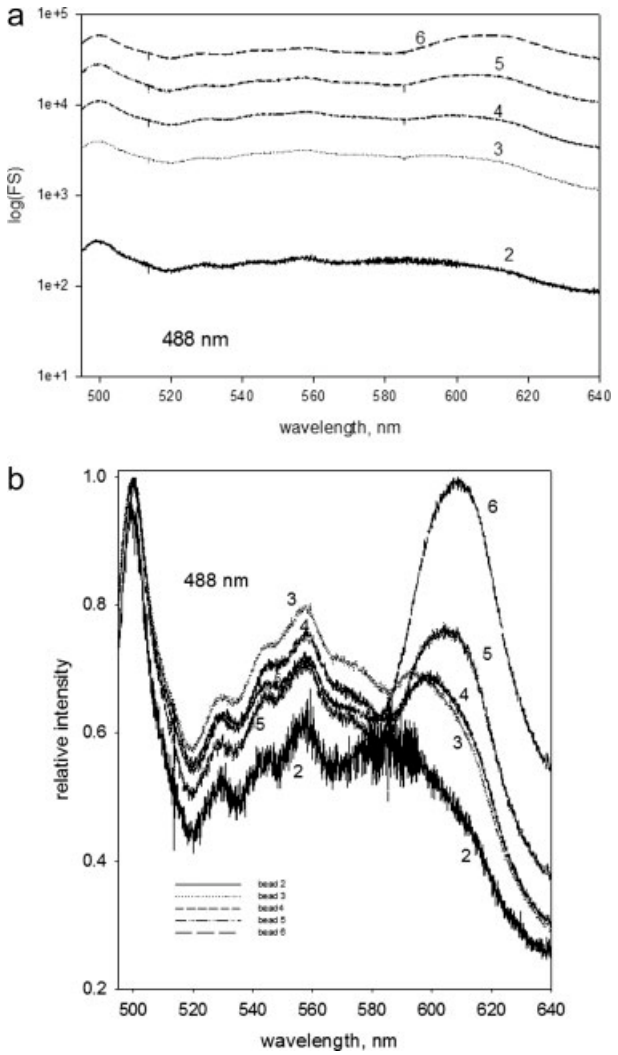

Figure 3. (**a**) The measured fluorescence signal (FS) from five populations of Ultra Rainbow beads excited with 488-nm laser line. In all cases, the signal from the blank bead population has been subtracted. Each spectrum is labeled with a number 2 through 6, which denote the bead populations with increasing dye content. As expected, the FS increases with increasing loading of dye in the Ultra Rainbow beads. (**b**) The normalized fluorescence emission spectra from Rainbow beads 2—6. The excitation wavelength was 488 nm, and the normalization was performed by dividing all of the points in the measured spectrum (a) by the maximum value in that spectrum. There is a substantial difference in the spectral shape around 610 nm.

bead population will be specific to a given excitation laser. To assign these numbers, we will compare the fluorescence signal from the suspensions of beads and solutions of selected reference fluorophores. For the 405-nm excitation, Coumarin 30 in acetonitrile will be used as a reference fluorophore. Nile red or fluorescein will be used with the 488-nm excitation and allophycocyanin with the 632-nm excitation.

Suppose that a comparison is made of fluorescence signal from a suspension of a given population of rainbow beads and the fluorescence signal from solution of reference fluorophores. The fluorescence signal is a number obtained by summing the spectrometer response over a given wavelength region. By varying the concentration of reference fluorophores it will be possible to find a solution of reference fluorophores whose fluorescence signal is the same as the fluorescence signal from the bead suspension. The equality of fluorescence signals leads to an equality of the respective products of solution and instrument properties given by Eq. (1).

$$\varepsilon_b \phi_b N_b f_b = \varepsilon_R \phi_R N_R f_R \qquad (1)$$

The subscript b denotes beads and the subscript R denotes the reference fluorophores. The symbol ε stands for molar absorption coefficient, ϕ represents the quantum yield, and N stands for the number density. It is implied in Eq. (1) that an integration of the spectrum over some wavelength range has been performed, and the parameters f_b and f_R give the fraction of the total emission spectrum that is included in the integration over the selected wavelength region (It is also implied in Eq. (1) that all measured spectra have been corrected for the relative spectral response of the instrument). In the discussion below, it will be assumed that a full integration of the spectrum from reference fluorophores is performed ($f_R = 1$). This is a practical assumption since the spectrum of the reference fluorophores extends over a reasonably small region of wavelengths (e.g., see Fig. 5) and the integration of the spectrum over this region is a consistent portion of the total spectrum. In the case of bead spectrum, Figure 2 suggests that it may be difficult to estimate a total spectrum for the beads and a practical alternative is to introduce a measure of the fraction of the bead spectrum given by f_b. A further assumption will be made that the same fraction of the spectral region will be sampled for the different bead populations.

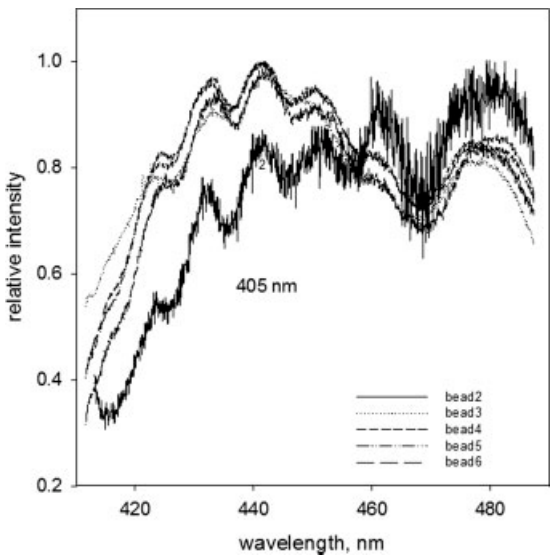

Figure 4. The normalized fluorescence emission spectra from Rainbow beads 2—6. The excitation wavelength was 405 nm, and the normalization was performed by dividing all of the points in the measured spectrum by the maximum value in that spectrum. A small difference is present between the spectral shape of bead 2 and the rest of the beads in the range of wavelengths 420—440 nm.

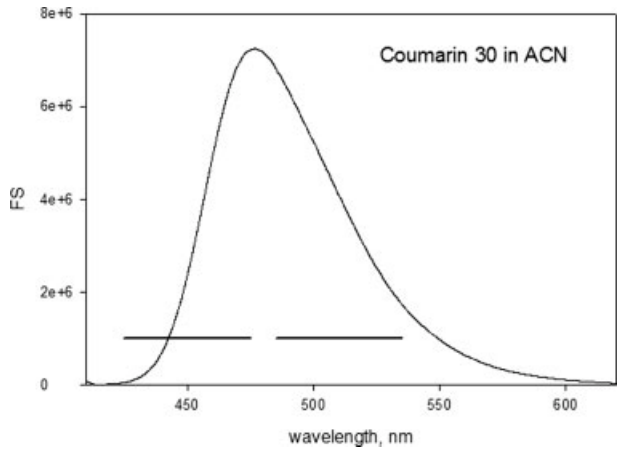

Figure 5. The emission spectrum from a solution of Coumarin 30 in acetonitrile (ACN). The excitation was a 405-nm laser line. The horizontal lines show the two wavelength ranges defining the two fluorescence channels, FC450 and FC510. During the assignment of the number of equivalent reference fluorophores to the Ultra Rainbow beads, the entire Coumarin spectrum was integrated. The emission spectra from the beads were integrated over the indicated channel range of wavelengths.

Equation 1 provides an equivalence relation between the number of beads in the suspension and the number of reference fluorophores in the solution. For specified solution conditions (e.g., pH and solvent), the values of ε and ϕ are fixed for the reference solution and the bead suspension. However, the fraction of the total spectrum, f_b, that is included in the bead fluorescence signal is a property of the instrument and may be different for different instruments. Equation 1 suggests that depending on the value of f_b, the concentration of reference fluorophores that is needed to give an equal fluorescence signal (FS) has to be adjusted up or down. To obtain an instrument-independent quantity, we define a value of "equivalent reference fluorophores" (ERF_b), which is assigned to each bead population by Eq. (2).

$$ERF_b = \frac{6.022 \times 10^{23}}{1000} \frac{C_{eq}^b}{N_b f_b} \tag{2}$$

C_{eq}^b (mol/L) is the molar concentration of fluorophores in the reference solution which gives the same fluorescence signal as a suspension of beads with a number concentration N_b (1/mL). The numerical factor in Eq. (2) is equal to Avogadro number divided by the conversion factor 1,000 mL/L. The factor $1/f_b$ takes into account the fact that only a fraction of the bead emission spectrum was used in acquiring the fluorescence signal. Equation 2 defines ERF_b for a given excitation wavelength.

Equation 2 can be used with all of the different populations of Ultra Rainbow beads to assign a number of equivalent reference fluorophores to each population of beads. It is assumed that the molecular absorbance coefficient and quantum yield is the same for all bead populations. Therefore the number of equivalent reference fluorophores, ERF, assigned to the bead populations using Eq. (2), should reflect the number of fluorophores embedded in the different bead populations. However, this is strictly true only if all of the bead populations are characterized by the same fluorescence emission spectrum. If the spectrum for the beads changes from a bead population to another then Eq. (2) suggests the following relation between the number of equivalent reference fluorophores assigned to each bead population and the corresponding equivalent reference fluorophore concentration.

$$\frac{ERF_{b2}}{ERF_{b3}} = \frac{f_{b3}}{f_{b2}} \frac{C_{eq}^{b2}}{C_{eq}^{b3}} \tag{3}$$

The integers in the subscripts refer to different bead populations, where we have chosen 3 and 2 just to be specific. Equation 3 implies that in the case where the spectrum of the different bead populations is the same, the ratio of the f_b coefficients drops out, and the ratio of ERF values is equal to the ratio of corresponding concentrations of reference fluorophores. Thus, the relative ERF values will be proportionate to the relative concentration of the reference fluorophore and the accurate measurement of the reference fluorophore concentrations will provide a linear relationship between the assigned ERF values. In other words, in the case where the fluorescence emission spectra of the different bead populations are the same, the value of the instrumental factor, f_b, does not play a role in preserving the linearity of the assigned ERF values. We will make a simplification and assign ERF values to the beads using Eq. (4) which is the same as Eq. (2) except that the factor f_b has been set to 1.

$$ERF_b = \frac{6.022 \times 10^{23}}{1000} \frac{C_{eq}^b}{N_b} \tag{4}$$

This procedure is consistent with the ultimate role of the ERF values, which is to establish a linear relation between the FS of different bead populations and the ERF values. ERF values are not meant to provide an interpretation of the absolute FS of the different bead populations. If the fluorescence spectra of different bead populations are different, then filters associated with different fluorescence channels will yield slightly different relative fluorescence signals. This will introduce a small, instrument-dependent, nonlinearity in the calibration that uses ERF values based on Eq. (4).

Assignment of ERF Values to the Ultra Rainbow Beads

The values of ERF were assigned for three excitations using three different reference fluorophores. In the following, to keep track of which reference fluorophore is used, we replace the letter R in ERF with the letter specifying the reference fluorophore used in the assignment. If Coumarin 30 is the reference fluorophore then ERF is changed to ECF. For fluorescein ERF becomes EFF, and for Nile Red and alophycocyanin ERF become ENRF and EAF respectively. Table 1 gives the definitions of the fluorescence channels (column 2) with the associated excitation wavelength (column 1) and the range of

Table 1. Definition of fluorescence channels

EXCITATION	FLUORESCENCE CHANNEL NAME	WAVELENGTH RANGE (nm)
405 nm	FC450	425–475
	FC510	486–535
488 nm	FC530	515–545
	FC585	564–606
	FC660	630–690
	FC760	730–800
632 nm	FC660R	650–670
	FC760R	730–800

wavelengths defined by filters and detected by cytometer (column 3).

Excitation with 405 nm. The fluorescence channels associated with the 405-nm excitation are FC 450 and FC 510. The wavelength regions defining the detected spectral range are from 425 to 475 nm for FC 450, and from 486 to 535 nm for FC 510, respectively. Figure 4 suggests that for FC450 there is a small change in the emission spectrum of bead 2 relative to the other beads. The change in spectral shape is negligible for FC510 (data not shown). The reference fluorophore is Coumarin 30 dissolved in acetonitrile (ACN). The stock solution of Coumarin 30 was prepared by combining gravimetrically determined masses of Coumarin 30 and ACN. Using a molecular mass of 347.41 g/mol for Coumarin 30 and a density of 0.77588 g/mL for ACN, the concentration of the stock solution was found to be 90.85×10^{-6} mol/L. Serial dilutions of the stock solution were used to calibrate the response of the fluorimeter. In all cases the fluorescence signal (FS) was determined by integrating the entire spectrum of Coumarin 30 as shown in Figure 5. The horizontal lines in Figure 5 show the spectral regions defining FC450 and FC510. Figure 6a shows the calibration of the fluorimeter obtained using the serial dilutions of the Coumarin 30 stock solution. The slope (0.9711) is close to 1 indicating a linear relationship. The fluorescence signal associated with the five fluorescent bead populations was obtained by integrating the measured spectrum (with contribution from blank beads subtracted) in the defined channel range. The fluorescence signal was then used to determine the equivalent concentration of Coumarin 30 fluorophores according to the calibration curve in Figure 6a. The equivalent concentration and the measured bead concentration by Multisizer 3 (13) were used in Eq. (4) to obtain the number of equivalent Coumarin 30 fluorophores (ECF_{450} and ECF_{510}) for each bead population. The results are shown in Table 2, columns 2 and 3. Figure 6b shows the correlation between ECF values obtained for FC450 (ECF_{450}) and FC510 (ECF_{510}). The slope of 1.021 indicates a linear relationship, while the intercept of -0.482 gives a proportionality constant of about 0.33 which is related to the ratio of the fraction of entire fluorescence spectrum sampled by each fluorescence channel. The linear relationship between the two sets of ERF values suggests that in practice only one set of ERF values may be sufficient to characterize the linear scale in both channels.

Excitation with 488 nm. The procedure described for the 405-nm excitation was repeated for 488-nm excitation with fluorescein SRM 1932 and Nile Red as the reference solutions. Four fluorescence channels are defined for the 488-nm excitation: FC530, FC585, FC660, and FC760. The spectra shown in Figure 3b suggest that the ERF values for FC585 may be influenced by the difference in the spectra between the bead populations. However, the largest differences are outside the range of wavelengths defining the channel and may not be critical. For FC660 and FC760, the bead spectra are similar (data not shown). The emission spectrum of fluorescein is strong below 580 nm and was used to assign EFF values for FC530 and FC585. Nile Red, excited by 488 nm, has a stronger emission spectrum above 580 nm, and it was used to assign ENRF values to channels FC660 and FC760. Columns 4 and 5 in Table 2 give the assigned EFF values for the Rainbow beads for each of the defined FC. The two sets of EFF values correlated

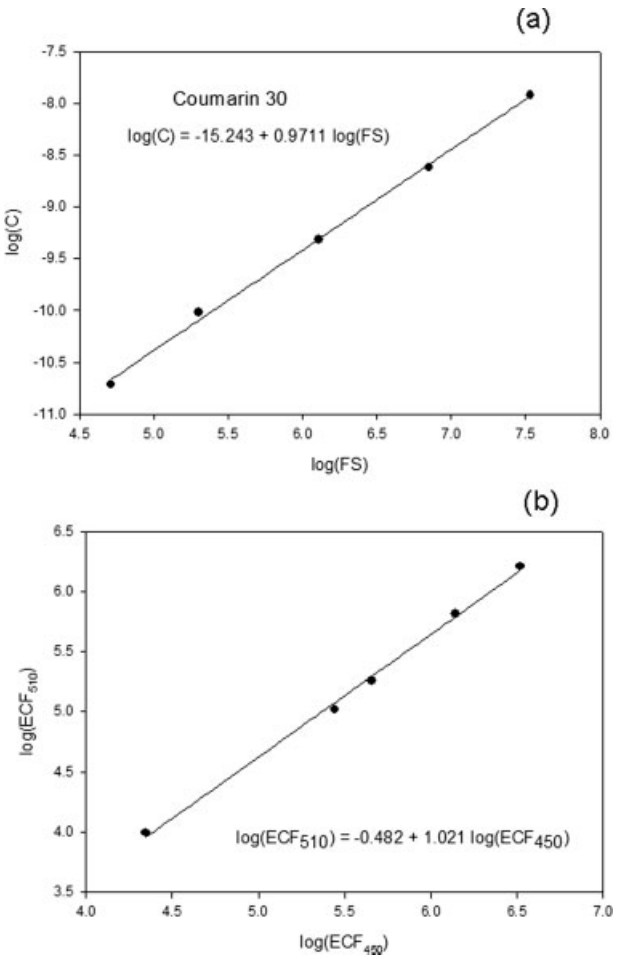

Figure 6. (**a**) The solid circles show the calibration of the fluorometer using serial dilutions of the stock solution of Coumarin 30 in ACN. The slope of the linear curve fitted to the points is 0.9711 suggesting good linearity. (**b**) The solid circles show the correlation of the Ultra Rainbow bead values of ECF (equivalent coumarin fluorophores) for each of the two fluorescence channels. The slope of the best straight line fit suggests a linear relation of the assignments for the two channels.

Table 2. Assigned values of equivalent reference fluorophores

	EXCITATION							
	405 nm		488 nm				632 nm	
BEAD NUMBER	FC450	FC510	FC530	FC585	FC660	FC760	FC660R	FC760R
2	2.21×10^4	9.88×10^3	6.09×10^3	8.13×10^3	5.53×10^3	–	3.10×10^3	4.65×10^3
3	2.78×10^5	1.04×10^5	5.06×10^4	7.01×10^4	6.21×10^4	8.70×10^3	1.74×10^4	2.56×10^4
4	4.54×10^5	1.80×10^5	1.16×10^5	1.65×10^5	1.71×10^5	3.75×10^4	3.55×10^4	5.73×10^4
5	1.39×10^6	6.46×10^5	2.46×10^5	3.72×10^5	6.14×10^5	1.66×10^5	9.99×10^4	1.75×10^5
6	3.31×10^6	1.62×10^6	5.23×10^5	8.64×10^5	1.78×10^6	6.71×10^5	1.89×10^5	3.87×10^5

Using repeated measurements of selected cases, the uncertainties are estimated to be about 20% of the values in the table.

linearly, $EFF_{585} = 0.88\ EFF_{530}$. Following the procedure described above we assigned values of equivalent Nile Red fluorophores to the beads. Columns 6 and 7 in Table 2 show the resulting values of ENRF in FC 660 and FC 760 for the five bead populations. Nile Red could also be used to assign ENRF values to FC580 though Nile Red emits less in this channel than fluorescein. The second column of Table 3 gives the ENRF values for FC585 while the third column of Table 3 reproduces the values of equivalent fluorescein fluorophores given in the 5th column of Table 2. As expected the magnitudes of the values of ENRF and EFF are different for the FC585. However, the two sets of assigned values correlate linearly. The linear relation between FC530 and FC585 and between ENRF and EFF values for FC585 suggests that, just as in the case of 405-nm excitation, a single set of ERF values may be sufficient for all of the channels associated with 488-nm excitation. This requires that the reference fluorophore chosen has to emit strongly in only one channel associated with the 488-nm excitation wavelength.

Excitation with 632 nm. Finally the assignment of ERF values was carried out for the 632-nm excitation using allophycocyanin (APC) as the reference fluorophore. There are two fluorescence channels defined for the 632-nm excitation: FC660R and FC760R. APC was obtained from Invitrogen. Three samples of APC were hydrolyzed for 22 h at 110°C in 6 N HCl containing liquefied phenol (4 g/L). The hydrolyzed samples were analyzed on a Hitachi 66000 amino acid analyzer. APC is known to consist of 3 α chains and 3 β chains (14,15). The expected numbers of amino acid residues were obtained from the known sequence of α and β chains of APC. Only those amino acids that were considered to be well recovered were used to calculate the concentration (aspartic acid, glutamic acid, glycine, alanine, leucine, lysine, and arginine). The average concentration of the three APC samples was $(33.4 \pm 2.7) \times 10^{-6}$ mol/L. The stock solution of APC was diluted serially and the diluted solutions were used to calibrate the fluorimeter. Following the procedure described above we obtained EAF values for the five bead populations shown in Columns 8 and 9 in Table 2.

Table 3. Equivalent Nile Red and fluorescein fluorophores for FC 585

BEAD NUMBER	$ENRF_{585}$	EFF_{585}
2	1.36×10^4	8.13×10^3
3	1.19×10^5	7.01×10^4
4	3.37×10^5	1.65×10^5
5	9.34×10^5	3.72×10^5
6	2.14×10^6	8.64×10^5

The uncertainties are about 20% of the values in the table.

Correlation between ERF assignments of fluorescence channels. Figure 7 shows the linear correlation between EFF_{530} (solid circles), EAF_{760} (open circles), and ECF_{450}. The three sets of ERF values were obtained using three different excitation wavelengths. The slopes of the two straight lines (solid and dashed curves) are 0.88 suggesting some non linearity in the dependence. There is also some deviation of the measured points from a linear fit. Since the slopes of the two correlation curves are very similar and the discrepancies are relatively small, it may be possible to have a common assignment of ERF values to all FC using one excitation wavelength. Such a

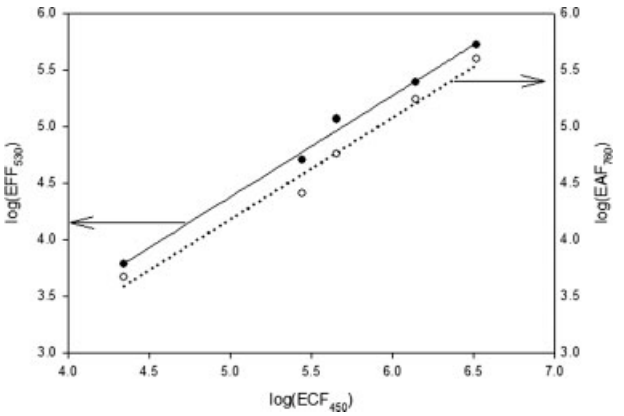

Figure 7. The open circles show the correlation between the values of EAF (equivalent allophycoerythrin fluorophores per bead) and the values of ECF_{450} (equivalent coumarin fluorophores per bead). The solid circles show the correlation between EFF_{530} (equivalent fluorescein fluorophores per bead) and the values of ECF_{450} (equivalent coumarin fluorophores per bead). The slope of 0.88 suggests a nonlinear correlation. However, the nearly linear correlations suggest that it may be possible to assign a single value of ERF (equivalent reference fluorophores) for all excitation wavelengths and all fluorescence channels.

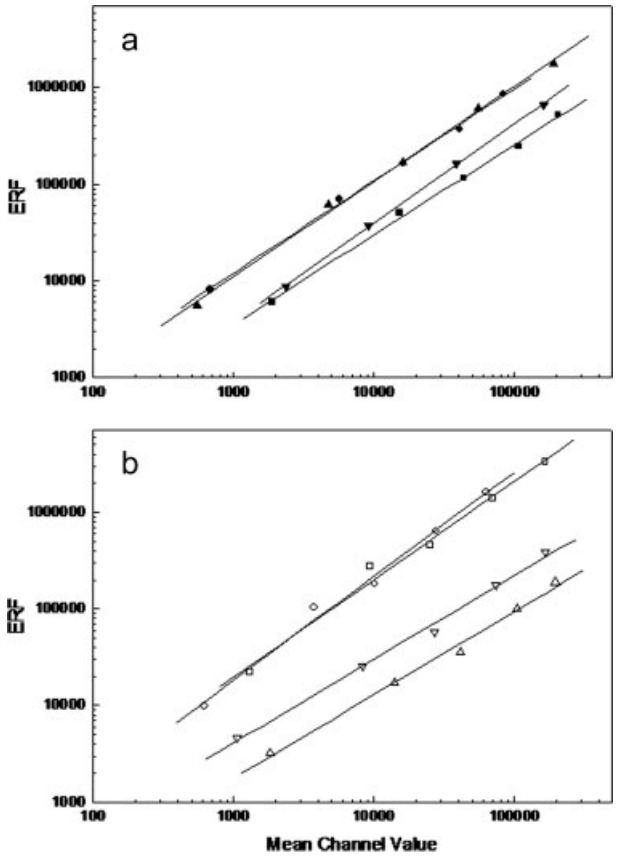

Figure 8. The calibration standard curve of the assigned values of EFF (FC530, ■ and FC585, ●) and ENRF (FC660, ▲ and FC760, ▲) (**a**) and of ECF (FC450, □ and FC510, ○) and EAF (FC660R, △ and FC760R, ▽) (**b**), respectively, as a function of the corresponding mean channel value from each bead population, ensuring a linear response of the detector of each fluorescence channel.

procedure would be very attractive since it would save material and time. However, as shown by the discrepancies in Figure 7, the validity of this procedure requires further investigation in order to estimate the errors. An alternate path to enhance the accuracy of the calibration is to develop a non linear calibration model. The biological standard will change the scale of the entire calibration curve irrespective of whether it is linear or non linear.

Cytometer Linearity Calibration

Subsequent to the assignment of ECF, EFF, ENRF, and EAF values, the bead populations were mixed and passed through a cytometer to yield a calibration curve for each fluorescence channel of the multicolor cytometer. When the mixture is passed through a cytometer, (after the compensation is performed) each fluorescence channel that is associated with a specific excitation exhibits peaks associated with the different populations of rainbow beads. Figures 8a and 8b show the calibration standard curve of the assigned values of EFF and ENRF and of ECF and EAF, respectively, as a function of the corresponding mean channel value of the peak, giving a linear relation between the equivalent reference fluorophores (ERF) in the bead and the resulting FS. The slope of the calibration curves falls in the range between 0.9 and 1.1. The cytometer fluorescence signal is obtained by either finding the peak of the fluorescence pulse or by estimating the area under the fluorescence pulse. Pulse peak measurement is only an accurate measure of fluorescence if the particles are significantly smaller than the size of the laser beam. On the other hand, peak area method is more resilient to the size variation of the particles. For a given set of measurements on a given cytometer, the same fluorescence signal characterization should be used for the calibration and the measurement. In that case, there will be no problems associated with the different methods of extracting the fluorescence signal.

Cytometer Biological Signal Calibration

The final step is to convert the fluorescence signal in each channel into a number of labeled antibodies bound on the cell surface (ABC). This is achieved by using a biological standard such as normal blood lymphocytes or lyophilized lymphocytes which have a known number of CD4 surface antigens (3,16–19). The standard cells are reacted with CD4 antibodies with different fluorescent labels yielding populations of lymphocytes with different labels. These lymphocytes are mixed and analyzed by the cytometer. The resulting FS in each fluorescence channel converts the linearity calibration scale into the antibodies bound per cell scale for that particular fluorescence channel (see the discussion of Fig. 1a). This procedure allows conversion of the standard calibration curve in ERF scale to ABC scale.

Figure 9 gives two examples of the calibration scale conversion from ERF to ABC value. A light scatter dot plot (forward scattering channel vs. side scattering channel) is routinely used to gate the total lymphocyte populations in normal whole blood samples (magenta circle in the top figure). In the multicolor cytometer, this gating allows displaying single parameter histograms for multiple fluorescence channels of interest, for instance, PE channel (FC585, middle panel in Fig. 9) and APC channel (FC660R, bottom panel in Fig. 9). The left axis in each of the two lower panels shows the ERF scale of the calibration curves generated using Ultra Rainbow beads. Using the FS associated with a known value of 48,000 receptors for normal $CD4^+$ T cells (the value was also confirmed by performing QuantiBRITE PE calibration), the calibration curves in ERF scale are converted to the standard calibration curve in ABC scale (right axis). Finally, patient blood samples, stained with labeled antibodies for various protein antigens, are passed through the cytometer. The labels on the antibodies are chosen to be visible in specific FC of the cytometer. It is important to ascertain that these antibodies give similar fluorescence signals as the labels on CD4 antibodies which served as the biological standards. Using the calibrated cytometer, the ABC values for these protein antigens can be directly obtained. These ABC values are useful for immunophenotyping of various cell receptors and are of direct biological interest. Furthermore the results are independent of instrument setting, and hence, make interlaboratory data comparison feasible. Some preliminary steps

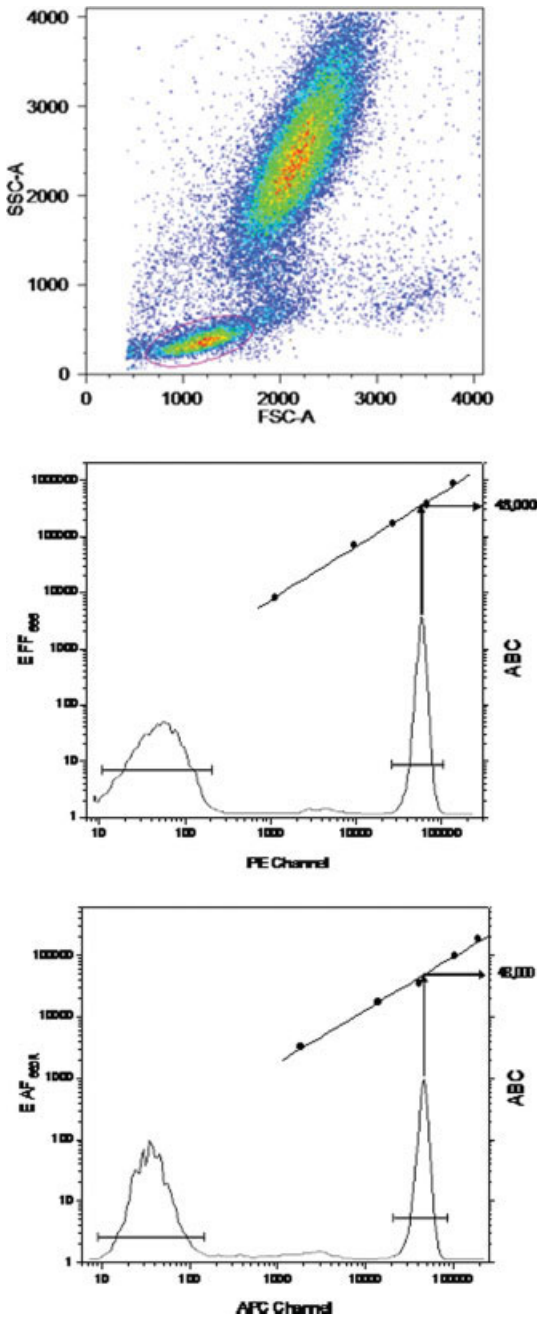

Figure 9. Top panel: A light scatter (forward scattering channel vs. side scattering channel) dot plot showing the gating of the total lymphocytes from a normal blood donor (magenta). Middle and bottom panels show single parameter histograms for the same blood donor stained directly with unimolar CD4-PE conjugate (middle) or indirectly with CD4-biotin conjugate and then SA-APC (bottom). With a known ABC value of 48,000 for normal $CD4^+$ T cells, the linearity calibration curves in ERF scale (left scale in middle and bottom panels) generated using Ultra Rainbow beads for PE channel (FC585) and APC channel (FC660R) are converted to the standard calibration curve in ABC scale (right scale in middle and bottom panels). The EAF_{660R} value for CD4 staining is very close to EFF_{585} value obtained with unimolar CD4-PE, suggesting there is one APC per CD4 antibody in this case.

have been undertaken by BD toward quantitative multicolor flow cytometry, and are discussed below.

BD's Cytometer Setup and Tracking Beads

BD Biosciences recently introduced a system for Cytometer Setup and Tracking (CS & T) that includes a set of three multifluorophore beads. The beads are stained at levels that are dim, moderate and brightly fluorescent in at least 18 different fluorescent colors commonly used for immunofluorescence. Among instrument characteristics that are measured with CS & T on BD's digital flow cytometers are linearity, detection efficiency (Q) and optical background (B) (20). Linearity is measured as deviation from strict proportionality using the ratio of median fluorescence of the bright bead to moderate bead. PMT voltages are varied to vary the median channel. Q and B are measured essentially by the increased CV of the moderate bead (for Q) and dim bead (for B) corrected for factors unrelated to photoelectron statistics. The intrinsic fluorescence CV's of the beads are measured during manufacture and are used as necessary to correct CV's measured by the flow cytometer in order to determine the CV contribution due to the instrument. The bright bead also provides an alignment check at an intensity level where photoelectron statistics are not a significant contribution to the total CV. CS & T also measures the background electronics noise and recommends optimal PMT voltages for each detector channel.

Q and B are conventionally expressed in terms of MESF, but MESF standards do not exist for most fluorochromes. It was also desirable to have some type of fluorescence intensity unit assigned to the bright bead so that lot-to-lot variations can assigned quantitatively. Since standards for most fluorochromes do not exist, BD established an arbitrary fluorescence intensity unit, the **A**rbitrary **BD** unit (ABD). ABD units are assigned in many different fluorescence spectral ranges and for UV, violet, blue and red lasers. ABD units are assigned only to the bright bead in the set since slightly different spectral responses of PMTs and optical filters among instruments of the same design cause some variation in the ratio of the moderate and dim bead fluorescence to the bright bead fluorescence.

With the CS & T approach, fluorescence scales are calibrated in ABD units using the bright bead, and Q and B are calculated from this calibrated scale. To indicate that Q and B are not measured in MESF units, the CS & T notation uses Qr and Br since Q and B are relative to ABD units.

While ABD units are considered arbitrary, they do have a practical association with antibodies bound per cell (ABC). The ABD assignments (with the exception of the PE channel) were made by comparing the bright CS & T bead fluorescence to CD4 staining of normal human blood lymphocytes with appropriate fluorochrome conjugates. Staining was done with commercial BD antibody conjugates if available. In some cases custom conjugates were used. Cells were fixed prior to analysis, which causes a reduction in CD4 binding, and the number of CD4 antibodies bound per cell was estimated to be 40,000 (18). CS & T beads were analyzed under the same conditions as the CD4 cells, and the fluorescence scale in ABD units was

defined by assigning the median ABD of CD4 lymphocytes to be 40,000. ABD units of the CS & T beads were derived from this scale. For the PE channel, CS & T beads were compared to Quantibrite beads, and ABD units for the PE channel are PE molecules. The cross comparison of CS & T beads was performed on a relatively small number of blood samples and small number of instruments (FACSCanto and LSR II). So the ABD units should be considered only approximately correlated to antibodies bound per cell (ABC), not a calibration.

Conclusion

The objective of this work was to provide a calibration scheme such that the detected fluorescence signals in various fluorescence channels of a multicolor flow cytometer could be presented in terms of a number of antibodies bound per cell (ABC).

A set of Ultra Rainbow beads containing increasing amount of a mix of fluorophores were used to calibrate the fluorescence signal linearity in each of the fluorescence channels (FC) of the cytometer. Each population of beads was assigned a number of effective reference fluorophores (ERF) that characterizes the relative fluorescence signal of each population of rainbow beads in a given FC. The values of ERF were assigned using different reference fluorophores appropriate for the different excitation wavelengths. It may be possible to have a common assignment of ERF values to all FC using one excitation wavelength. Such a procedure would be very attractive since it would save material and time. However, the validity of this procedure requires further investigation.

Normal blood lymphocytes or lyophilized lymphocytes, which have a known number of CD4 surface antigens, where used as biological standards to convert the ERF scale in each FC to a scale of antibodies bound per cell (ABC). These ABC values are useful for immunophenotyping of various cell receptors and intracellular proteins and are of direct biological interest. This work suggests that the proposed calibration procedure for multicolor flow cytometers is feasible. This calibration procedure is based on firm scientific foundations so that it is easy to envisage future improvements in accuracy and ease of implementation. Indeed, a first step has been taken by BD toward quantitative flow cytometry. We hope this work serves as an initial impetus for developing a widely accepted method for this objective.

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Literature Cited

1. Shapiro HM. Practical Flow Cytometry, 3rd ed. New York: Wiley-Liss; 1995.
2. Janeway CA, Travers P, Hunt S, Walport M. Immunobiology: The Immune System in Health and Disease, 3rd ed. Current Biology. New York: Garland Publishing; 1997.
3. Hultin LE, Matud JL, Giorgi JV. Quantitation of CD38 activation antigen expression on $CD8^+$ T cells in HIV-1 infection using CD4 expression on $CD4^+$ T lymphocytes as a biological calibrator. Cytometry 1998;33:123–132.
4. Yang OO, Ferbas JJ, Hausner MA, Hultin LE, Hultin PM, McFadden D, Sawicki M, Detels R, Majchrowicz M, Matud JL, Giorgi JV, Jamieson BD. Effects of HIV-1 infection on lymphocyte phenotypes in blood versus lymph nodes. J Acquir Immune Defic Syndr 2005;39:507–518.
5. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, Marcé S, López-Guillermo A, Campo E, Montserrat E. Zap-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003;348:1764–1775.
6. Chen Y, Peterson LC, Dittmann D, Evens A, Rosen S, Khoong A, Vincent Shankey T, Forman M, Gupta R, Goolsby CL. Comparative analysis of flow cytometric techniques in assessment of Zap-70 expression in relation to IgVH mutational status in chronic lymphocytic leukemia. Am J Clin Pathol 2007;127:182–191.
7. CLSI. Fluorescence Calibration and Quantitation Measurements of Fluorescence Intensity, Approved Guideline I/LA24-A. CLSI: Pennsylvania; 2004.
8. Lambert C, Cristina I, Christian G. Enumeration of peripheral lymphocyte subsets using 6 vs. 4 color staining: A clinical evaluation of a new flowcytometer. Cytometry Part B: Clin Cytometry 2005;70B:29–38.
9. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. Quality assurance for polychromatic flow cytometry. Nat Protocols 2006;1:1522–1530.
10. Innovation/Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products. Available at http://www.fda.gov/oc/initiatives/criticalpath/whitepaper.html.
11. Bloom ET, Epstein SL, Simek SL. Critical path workshop on the development of cellular and gene therapy products. Mol Ther 2005;12:5–8.
12. Wang L, Abbasi F, Gaigalas AK, Vogt RF, Marti GE. Comparison of fluorescein and phycoerythrin conjugates for quantifying CD20 expression on normal and leukemic B-cells. Cytometry Part B: Clin Cytometry 2006;70B:410–415.
13. Gaigalas AK, Wang L, Schwartz A, Marti GE, Vogt RF. Quantitating fluorescence intensity from fluorophore: Assignment of MESF values. J Res Natl Inst Stand Technol 2005;110:101–114.
14. Brejc K, Ficner R, Huber R, Steinbacher S. Isolation, crystallization, crystal structure analysis and refinement of allophycocyanin from the cyanobacterium *Spirulina platensis* at 2.3 Å resolution. J Mol Biol 1995;249:424–440.
15. Liu JY, Liang T, Zhang JP, Liang DC. Crystal structure of allophycocyanin from red algae *Porphyra yezoensis* at 2.2 Å resolution. J Biol Chem 1999;274:16945–16952.
16. Poncelet P, Carayon P. Cytofluorometric quantification of cell surface antigens by indirect immunofluorescence using monoclonal antibodies. J Immunol Methods 1985;85:65–74.
17. Poncelet P, Poinas G, Corbeau P, Devaux C, Tubiana N, Muloko N, Tamalet C, Chermann JC, Kourilsky F, Sampol J. Surface CD4 density remains constant on lymphocytes of HIV-infected patients in the progression of disease. Res Immunol 1991;142:291–298.
18. Davis KA, Abrams B, Iyer SB, Hoffman RA, Bishop JE. Determination of CD4 antigen density on cells: Role of antibody valency, avidity, clones, and conjugation. Cytometry 1998;33:197–205.
19. Wang L, Abbasi F, Gaigalas AK, Hoffman RA, Flagler D, Marti GE. Discrepancy in measuring CD4 expression on T lymphocytes using fluorescein conjugates in comparison with unimolar CD4-phycoerythrin conjugates. Cytometry Part B: Clin Cytometry 2007;72B:442–449.
20. Chase ES, Hoffman RA. Resolution of dimly fluorescent particles: A practical measure of fluorescence sensitivity. Cytometry 1998;33:267–279.