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NIST/ISAC Standardization Study: Variability in Assignment of Intensity Values to Fluorescence Standard Beads and in Cross Calibration of Standard Beads to Hard Dyed Beads

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Abstract

Results from a standardization study cosponsored by the International Society for Advancement of Cytometry (ISAC) and the US National Institute of Standards and Technology (NIST) are reported. The study evaluated the variability of assigning intensity values to fluorophore standard beads by bead manufacturers and the variability of cross calibrating the standard beads to stained polymer beads (hard-dyed beads) using different flow cytometers. Hard dyed beads are generally not spectrally matched to the fluorophores used to stain cells, and spectral response varies among flow cytometers. Thus if hard dyed beads are used as fluorescence calibrators, one expects calibration for specific fluorophores (e.g., FITC or PE) to vary among different instruments. Using standard beads surface-stained with specific fluorophores (FITC, PE, APC, and Pacific Blue™), the study compared the measured intensity of fluorophore standard beads to that of hard dyed beads through cross calibration on 133 different flow cytometers. Using robust CV as a measure of variability, the variation of cross calibrated values was typically 20% or more for a particular hard dyed bead in a specific detection channel. The variation across different instrument models was often greater than the variation within a particular instrument model. As a separate part of the study, NIST and four bead manufacturers used a NIST supplied protocol and calibrated fluorophore solution standards to assign intensity values to the fluorophore beads. Values assigned to the reference beads by different groups varied by orders of magnitude in most cases, reflecting differences in instrumentation used to perform the calibration. The study concluded that the use of any spectrally unmatched hard dyed bead as a general fluorescence calibrator must be verified and characterized for every particular instrument model. Close interaction between bead manufacturers and NIST is recommended to have reliable and uniformly assigned fluorescence standard beads. © 2012 International Society for Advancement of Cytometry

Key terms

fluorescence; calibration; standardization; hard-dyed beads; surface-stained beads; multicolor flow cytometry; equivalent reference fluorophores (ERF)

STANDARDIZATION of flow cytometer fluorescence measurements is useful for comparison of data among laboratories and over time, improving the robustness of automated software gating algorithms, and to help determine the cause of poorly resolved populations. Standardization or calibration of fluorescence is essential when quantitative results of fluorescence staining are needed. The practical problem has been the availability of appropriate fluorescence standards.

For a few fluorochromes, there are commercially available beads surface-stained with the same fluorochromes used to tag antibodies and other probes. But the surface-stained beads have relatively short shelf lives unless the beads are freeze dried, which adds considerable expense to the product. In addition, the fluorescence intensity values vendors assign to their beads are not traceable to an authoritative body

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such as NIST so that surface-stained fluorophore standard beads from different companies can result in different calibration of the fluorescence scales on a flow cytometer.

Alternatively, hard dyed beads embedded with hydrophobic dyes or made from fluorescently tagged polymers are very stable and inexpensive, and have gained popularity as reference particles for instrument setup and for instrument performance characterization. Usually the recommendation is to use the hard dyed beads for standardization after the flow cytometer has been set up for a particular application, typically by running biological samples. The hard dyed beads are run at the same instrument settings as the reference biological samples, and on subsequent days, the hard dyed beads can be used to set up the instrument to the same condition as on the initial run. This provides reproducibility on a particular instrument. But when instruments in different laboratories, possibly in different countries, are used in a study, the ability to standardize all instruments to a biological sample can be impractical. This is particularly true for multicolor applications for which surface stained fluorescence standards are not available for all the fluorochromes used in the application. Hard dyed beads are used in these and similar situations to standardize multiple instruments to approximately the same condition. This is a practical and convenient approach.

A limitation of hard dyed reference beads is that their excitation and emission spectra generally do not match those of fluorochromes used to stain cells, which results in uncertainty as to how comparable results are from instruments that may have different excitation or detection configurations. Depending on the application, instruments may need to be standardized to within 10% of each other for quantitative results. In other situations, a two-fold variability in instrument standardization may be acceptable if fluorescent populations are all well resolved and absolute intensity is not a critical measure in the application. Without data comparing the intensities of fluorochrome reference beads and hard dyed beads, there is no way to know whether the instruments that are all standardized with hard dyed beads are measuring comparable ranges of fluorescence intensities.

This study had two aims: (1) Evaluate the variability in estimating fluorochrome intensities of hard dyed beads using surface-stained standard beads for cross calibration and (2) Evaluate the variability in ERF assignment of standard beads and cross calibrating hard dyed beads against solutions of reference fluorophores.

Materials and Methods

Cross Calibration General Procedure

Participants in the study volunteered to run samples of fluorophore standard beads and hard dyed beads on flow cytometers in their laboratories. All beads were shipped with cold packs and were kept refrigerated. For flow cytometer manufacturers, some of the data was acquired on instruments that had just been manufactured for customers but were still under factory control. Participants were provided Microsoft Excel workbooks containing spreadsheets in which information on the cytometer and mean fluorescence intensity (MFI) for each of the fluorophore standard and hard dyed beads was entered. The fluorophore standard beads were first run and detector gains adjusted to put the bead signals near the top third of the log scale (e.g., MFI of 1,000 on a scale of 1-1 10,000). The MFI for the standard beads in the detector channels used for FITC, PE, APC, and Pacific Blue were recorded. If APC (red laser excitation) or Pacific Blue (violet laser excitation) was not available on the flow cytometer, that data area was left blank in the spreadsheet. Using the same detector settings, the hard dyed beads were then run and MFIs recorded. MFIs for the fluorophore standard and hard dyed beads was then manually entered into the spreadsheets in the Excel workbook.

The Excel workbooks were emailed to the data coordinator, who electronically compiled the information from the spreadsheets into a master spreadsheet. The raw MFI data from each flow cytometer was used to calculate the ratios of hard dyed bead MFI to fluorophore standard bead MFI. The ratios were used for further analysis of the variability of cross calibration among the different flow cytometers. To allow data from all the hard dyed beads and from different detector channels to be compared, the ratio data for each detector channel and each different hard dyed bead was normalized to the median ratio of all instruments.

The protocol sent to each participant and an example of a workbook for entering data is shown in the Supporting Information S1 and S2.

Fluorochrome Standard Beads

BD Biosciences prepared the fluorophore standard beads by staining BD Comp Beads with antibodies conjugated to either FITC, PE, APC, or Pacific Blue. After staining, the beads were aliquoted to microwell plate wells (each well containing a single color standard) and freeze dried. Each microwell plate of freeze-dried fluorophore standard beads contained sufficient numbers of wells for up to eight preparations. For each use, the cover of a microwell was punctured and buffer was added to the freeze dried beads. The microwell plate with unused wells containing freeze dried beads was kept refrigerated.

Hard Dyed Beads

Beckman Coulter, BD Biosciences, and Spherotech provided a single, multifluorophore bead. Thermo Fisher Scientific provided four different intensities of multifluorophore beads, each best matched to the intensity of one of the fluorophore standard beads. Life Technologies provided four different single fluorophore beads whose emission spectrum was similar to one of the standard fluorophores (FITC, PE, APC, or Pacfic Blue). Each manufacturer provided beads in dropper bottles. Beads were diluted in buffer prior to analysis on the flow cytometers. Details of the hard dyed beads used are in Table 1.

Flow Cytometers Used in the Study

Table 2 lists the instrument models included in the study and the number of each model. The few examples of stream in

Table 1. Hard dyed beads used in the study

Beckman Coulter FlowSet Plus, multifluorophore beads, single intensity
BD Biosciences CS&T setup bead, multifluorophore, single intensity
Life Technologies spectrally similar beads, single fluorophore beads with emission spectra similar to FITC, PE, APC, and Pacific Blue. One bead for each color
Spherotech Rainbow bead, multifluorophore, single intensity
Thermo Scientific Cyto-Cal Multifluor Plus Violet Intensity Calibrators, four beads each selected to best match intensity of one of the standard beads

air sorters for which participants provided data are not included in this report since there was no way to establish variation within an instrument model.

Participants were asked to indicate whether their instrument was standard or had custom lasers or filters. This allowed nonstandard instruments to be excluded from the measured cross calibration variability of an instrument model. The standard filters for a particular instrument model varied somewhat among different instrument manufacturers, particularly the filter used for APC. Typical filters for FITC, PE, APC, and Pacific Blue are 530/30, 585/40, 660/20, and 450/50, respectively. The 488 nm laser was considered standard for measuring PE. BD Bioscience LSRIIs with green (532 nm) or yellow-green (561 nm) lasers used for PE are indicated by a –G or –Y in the *X*-axis annotation of Figures 1–4. The use of green or yellow-green lasers may also affect the FITC results due to different FITC filters being used. Some instruments used nonstandard PE and APC filters, but with standard laser excitation. In Figures 1–4 the instruments with nonstandard PE filter (570/40) used with 488-nm excitation are indicated by -B570. Instruments with nonstandard 670/40 APC filters are indicated by -670.

Data Analysis

Microsoft Excel was used for the database, to normalize the data, and to calculate medians and robust CVs for the subgroups of data. Robust statistics were used since outlier events have less effect on the result. A robust standard deviation was defined as the range of values between the 10th and 90th percentiles of the data set divided by the factor 1.29. The factor 1.29 adjusts this robust standard deviation to the standard deviation of a normal distribution. The robust CV is the robust standard deviation divided by the median. PSI-Plot (Poly Software International, Pearl River, NY) was used to make box and whisker plots using percentiles of data subgroups.

ERF Assignment to Reference Beads

Reference 2 gives the procedure for assigning ERF values to the major populations of the four fluorophore-specific reference beads used in the study—FITC, PE, APC, and Pacific Blue™. These four bead standards were provided by BD Biosciences in a stable freeze-dried format for the study, and their proper use is provided in the Supporting Information S3. Fluorescein SRM 1932, Nile Red, APC, and Courmarin 30 were used as reference fluorophores for the ERF value assignments of the four respective fluorophore-specific microsphere standards. A general ERF assignment procedure was prepared and provided to the bead manufacturers who participated in the study, and can be found in the Supporting Information S3. A short summary of this procedure is given in the following. First, a fluorometer was calibrated with a set of progressively diluted solutions of a stock solution of each reference fluorophore. The fluorometer calibrations related the fluorescence intensity from the solution of reference fluorophores to the concentration of the reference fluorophores. Next, the fluorescence intensity of a fluorophore standard bead suspension was measured. The fluorometer calibration line was used to relate the fluorescence intensity of the bead suspension to the equivalent concentration of the corresponding reference fluorophores, $C_{\text{equivalent}}$. Finally the number concentration of each microsphere suspension, $C_{\text{microspheres}}$, was measured using a multicolor flow cytometer and BD Trucount™ beads (BD Biosciences) as an internal bead counting standard. The two values, the equivalent reference fluorophore concentration and the number bead concentration, enabled the assignment of the ERF value to each fluorophore standard bead.

Results

The objective of the cross calibration study was to determine variability among different instruments when a probe-specific standard (e.g., FITC stained bead) is compared to a hard dyed bead. The ratio of the intensity of the hard dyed bead to the fluorochrome standard bead is taken for each fluorochrome and used as an arbitrary metric to compare the response of each instrument. To allow display of data for all beads on the same plot scale, the ratio data for each detector channel (e.g., FITC) was normalized to the median of the data for that dataset. The normalized ratios of mean fluorescence intensity (MFI) of hard dyed bead to fluorophore standard bead MFI are shown in Figures 1–4. Most of the plots are on a scale of 0–2.5, but in some cases the range of data for particular instruments or instrument configurations was larger and is shown on an appropriately larger scale. The plotting program

Table 2. Instruments used for the study report

INSTRUMENT MODEL	NUMBER IN STUDY
Accuri C6	10
BD FACSCalibur	12
BD FACSCanto	27
BD FACS Aria	15
BD LSR II	19
Beckman-Coulter FC500	6
Beckman-Coulter Gallios	11
Beckman-Coulter Navios	6
Life Technologies Attune	3
Miltenyi MACSQuant	17

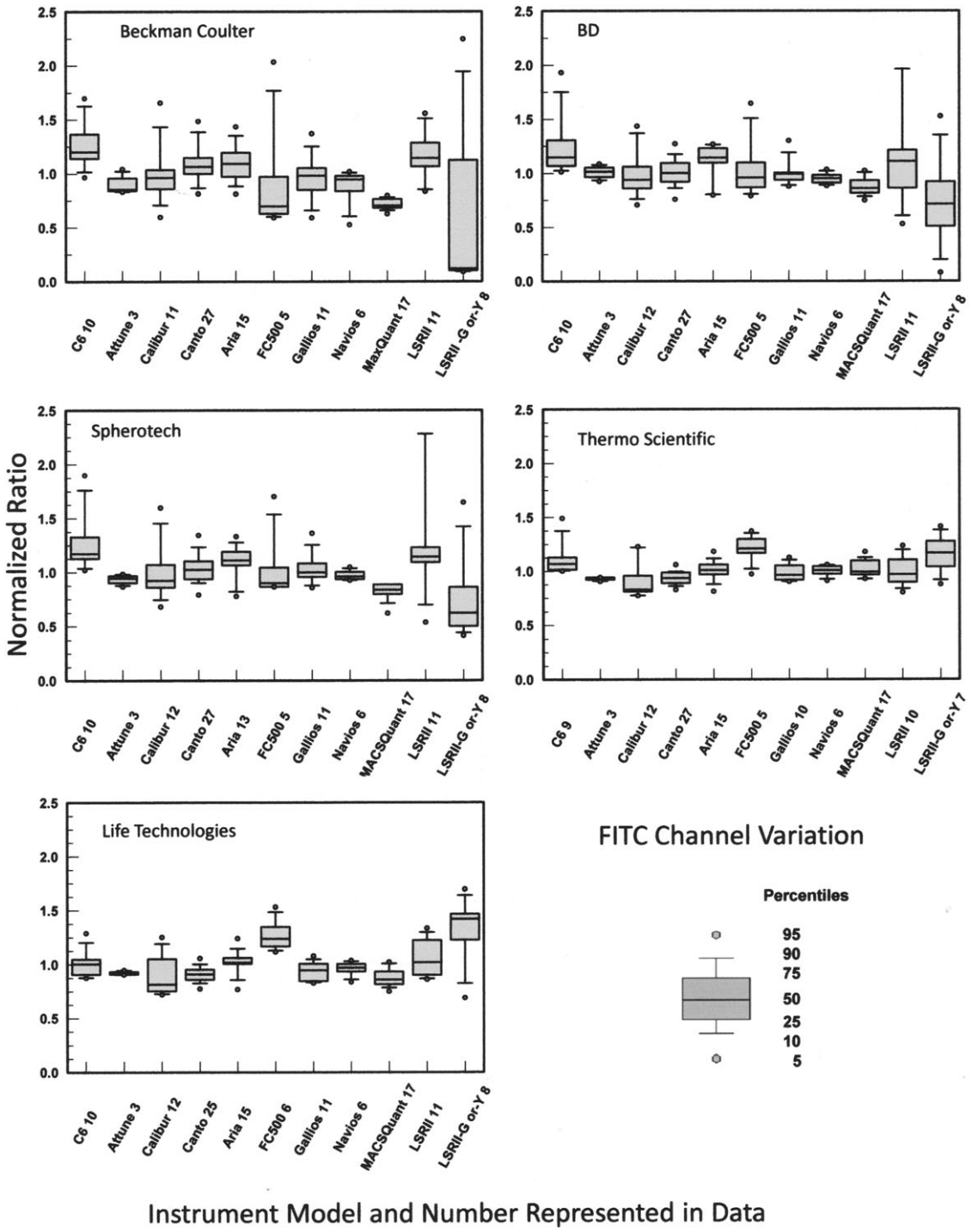

Figure 1. Box and whisker plots of the normalized ratio of the MFI of the indicated hard-dyed beads to the MFI of the FITC fluorophore standard bead for 10 different flow cytometer models. The box shows the 25th to 75th percentiles, and the line in the box indicates the median value. Horizontal bars outside the box indicate 10th and 90th percentiles and the circles indicated 5th and 95th percentiles. The percentile markers indicate the percentage of instruments for which the cross calibration was within the indicated normalized range. The number of instruments represented for each instrument model is noted after the model name on the *X*-axis of each plot.

interpolates the percentile values since the data are relatively sparse.

Data in Figures 1–4 are displayed using box plot percentiles. The box shows the 25th to 75th percentiles, and the line in the box indicates the median value. Horizontal bars outside the box indicate 10th and 90th percentiles and the circles indicated 5th and 95th percentiles. The percentile markers indicate the percentage of instruments for which the cross calibration

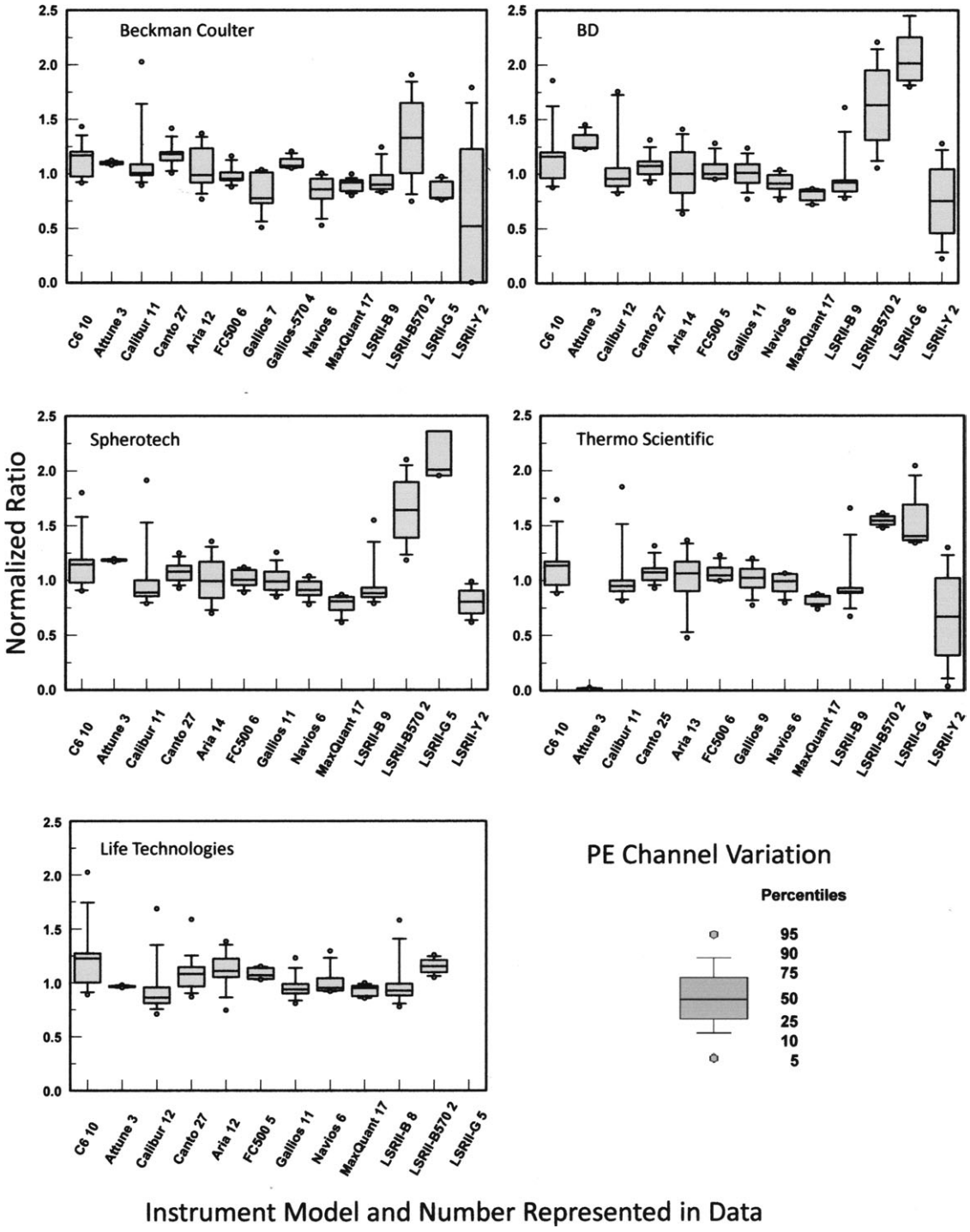

Figure 2. Box and whisker plots of the normalized ratio of the MFI of the indicated hard-dyed beads to the MFI of the PE fluorophore standard bead for 10 different flow cytometer models. Details are the same as for Figure 1.

was within the indicated normalized range. Extreme outliers in the data are excluded from analysis. The number of instruments represented for each instrument model is noted after the model name on the *X*-axis of each plot. When data was different for instruments using nonstandard lasers or filters, the nonstandard instruments are shown as a separate model. When variation across instrument models was very large, data are displayed on a log scale. The data for The Life Technologies PE-like bead using a green laser based LSRII are off scale with a normalized median 13.6 times the norm.

An overall instrument model-dependent statistical measure, rCV, is shown in Table 3 for instrument models that had

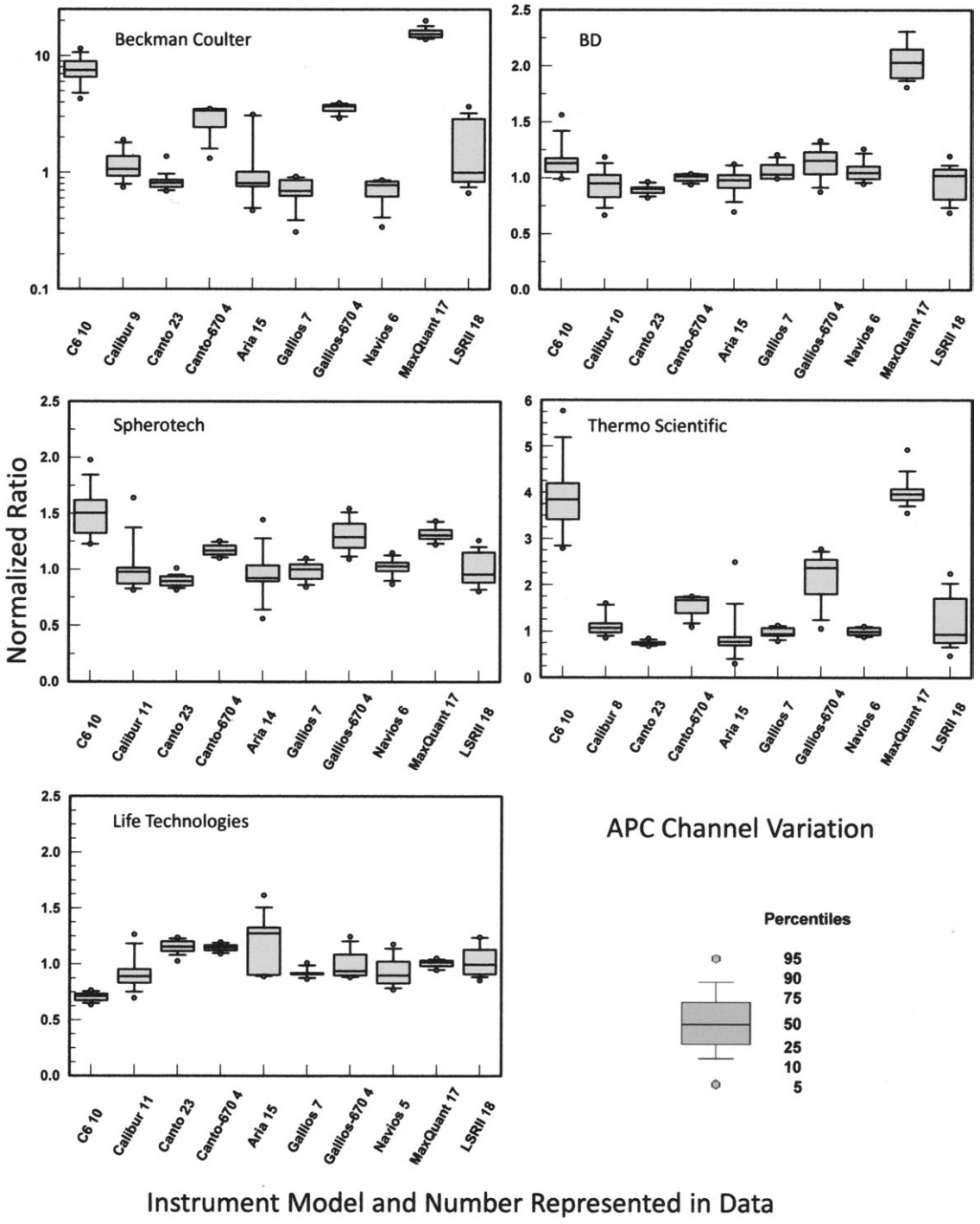

Figure 3. Box and whisker plots of the normalized ratio of the MFI of the indicated hard-dyed beads to the MFI of the APC fluorophore standard bead for 10 different flow cytometer models. Details are the same as for Figure 1.

at least 10 instruments represented. The rCV calculation excluded extreme outlier data and gives a measure of the spread in the data. Because there was considerable variation in configurations for LSR II, that model is not included. PE and APC data are excluded for 4 of 11 Beckman Coulter Gallios instruments and 4 of 27 BD FACSCantos because nonstandard filters were used. Of the more than 1,800 data points representing instruments in Table 3, 11 data points were exclude as outliers. As an example for interpreting the results, an rCV of 20% means that 68% of instruments had a cross calibrated value with a particular hard dyed bead that was within ±20% of the median cross calibrated value for that instrument model.

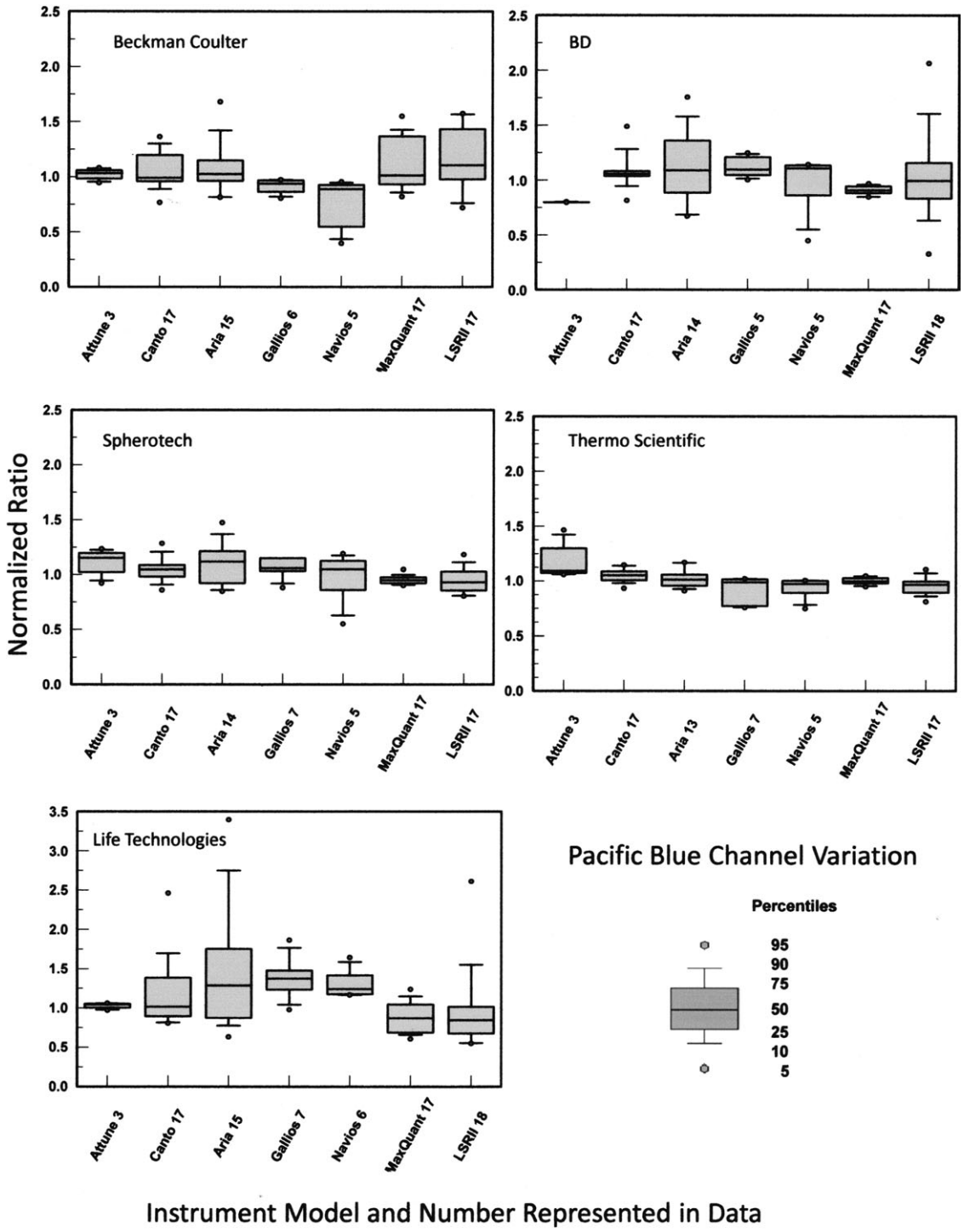

Figure 4. Box and whisker plots of the normalized ratio of the MFI of the indicated hard-dyed beads to the MFI of the Pacific Blue fluorophore standard bead for 10 different flow cytometer models. Details are the same as for Figure 1.

For many recently introduced instruments with standard, fixed filter sets (e.g., Gallios/Navios, MACSQuant), within-model CVs were as low as 20% or lower for most hard dyed beads and fluorescence channels, while instruments with a larger age range and/or customizable filter sets (FACSCalibur, FACSAria, LSR) tended to have the highest within-model CVs (Table 3). Across instruments and for all hard-dyed beads, models with fixed filter sets tended to have the most consistent ratios, with 10th–90th percentile ranges generally within a factor of two and often much better. Again, instruments with customizable filter sets exhibited much higher deviation.

Table 3. Robust CV's (expressed as percentage) of normalized ratio for selected instrument models

BEAD	INSTRUMENT	FITC RCV	PE RCV	APC RCV	BLUE PACIFIC RCV
BD CS&T Setup	Accuri C6	30.6	25.5	15.2	
	BD FACSAria	24.0	47.6	19.9	49.1
	BD FACSCanto	17.9	18.7	9.3	16.6
	BD FACSCalibur	37.6	17.6	19.4	
	Gallios	13.0	13.4	11.5	12.2
	MACSQuant	16.2	12.7	13.0	8.3
Beckman Coulter Flow Set Plus	Accuri C6	27.9	20.1	40.5	
	BD FACSAria	23.8	33.2	174.4	34.6
	BD FACSCanto	31.6	15.6	21.1	26.8
	BD FACSCalibur	22.5	18.3	54.4	
	Gallios	29.4	31.5	39.5	8.4
	MACSQuant	10.6	9.8	16.8	38.5
Spherotech Rainbow	Accuri C6	33.3	22.7	21.8	
	BD FACSAria	23.1	38.5	33.3	28.4
	BD FACSCanto	20.4	16.0	9.2	15.0
	BD FACSCalibur	38.6	19.5	23.4	
	Gallios	15.8	11.4	9.0	56.9
	MACSQuant	18.7	18.4	8.5	6.0
Thermo Fisher Cyto-cal	Accuri C6	12.8	23.1	28.9	
Selected multifluor intensity for each standard	BD FACSAria	11.7	48.2	44.1	15.0
	BD FACSCanto	10.5	17.8	11.5	10.6
	BD FACSCalibur	38.3	17.8	40.3	
	Gallios	13.3	13.1	16.8	15.6
	MACSQuant	20.4	8.4	12.0	6.3
Life Technologies	Accuri C6	15.2	24.7	7.8	
Standard specific	BD FACSAria	11.4	22.0	30.7	92.4
	BD FACSCanto	12.7	20.5	21.5	46.9
	BD FACSCalibur	33.9	15.7	9.6	
	Gallios	13.9	16.8	4.7	18.2
	MACSQuant	7.0	9.5	5.8	38.4

The LSR II PE data for instruments with green lasers (Fig. 2) are the extreme example of model dependent variation. The APC channel showed the most variation among instrument models (Fig. 3). This reflects the significant number of different choices of APC filters that are used in these models. The Beckman Coulter Flowset Pro beads are not designed for use in the APC channel, so it is not surprising that those beads show the greatest variation among different instrument models.

To evaluate whether there was more variability of results in end user labs compared to testing that was done at instrument manufacturer's facilities, we separately analyzed the end user and BD in-house data for the BD FACSCanto II. In this case, 13 instruments were tested in-house at BD and 14 in seven different end user labs, including a lab in Europe. Results are shown in Table 4. For most beads and fluorescence channels, the median ratio of hard dyed to reference bead MFI varied 5% or less between instruments measured in-house at BD and in the seven different end user labs.

Although there are a few combinations of hard dyed bead, detector channel, and instrument model that have an rCV <10% (indicating 68% of instruments in the subgroup vary in cross calibration by $<\pm 10\%$), most of the instruments show much greater variation. BD LSR II have particularly large variation in most cases. This is not surprising since most of these instruments are sold with nonstandard laser power and/or filters. Where different laser wavelengths were used for a particular fluorochrome (e.g., 532 or 561 laser for PE), these instruments are shown as a separate model in Figures 1–4. The hard dyed bead that shows the most variation is the Beckman Coulter Flow Set Pro in the APC channel. But this bead was not designed for APC and a separate hard dyed bead for APC is sold to complement the Flow Set Pro. It was somewhat surprising that the Life Technologies beads stained with a single fluorophore that had emission spectra intended to be similar to the fluorophores defining the detector channels of cytometers, i.e., FITC and PE had about the same variability as multifluorophore beads.

Table 5 gives the estimated values of the equivalent concentration of reference fluorophore ($C_{\text{equivalent}}$, mol L^{-1}), microsphere concentration ($C_{\text{microspheres}}$, mL^{-1}), and equivalent reference fluorophore (ERF_{major}) for four surface labeled standard beads measured at NIST using the reference fluorophores shown in the last column. A JY Horiba Fluorolog 3

Table 4. Median normalized ratios for BD FACSCanto determined in house at BD or at end user labs. 13 instruments tested at BD and 14 at end user labs

		NORMALIZED RATIO			
BEAD	WHERE TESTED	FITC	PE	APC	PACIFIC BLUE
BD CS&T Setup	At BD	0.99	1.09	0.92	1.08
	End User	1.03	1.07	0.90	1.06
Beckman Coulter Flow Set Pro	At BD	1.03	1.20	0.84	0.98
	End User	1.13	1.19	0.79	1.06
Spherotech Rainbow	At BD	0.98	1.07	0.94	0.96
	End User	1.05	1.10	0.89	1.05
Thermo Fisher FITC intensity	At BD	0.92	0.99	0.83	1.03
	End User	0.95	1.01	0.72	1.08
Life Technologies FITC Like	At BD	0.91			
	End User	0.91			
Life Technologies PE Like	At BD		1.12		
	End User		1.04		
Life Technologies APC-Like	At BD			1.17	
	End User			1.15	
Life Technologies PacificBlue-Like	At BD				1.14
	End User				1.00

commercial fluorescence spectrometer, which was modified to allow excitation with an external laser: a 405 nm laser for Pacific Blue bead standard, a 488 nm laser for FITC and PE bead standards and a 633 nm laser for APC bead standard (1). The corrected fluorescence emission spectrum from reference fluorophore solutions and microsphere standards is provided in the Supporting Information S4 (after subtraction of the spectrum for the medium used to dilute the reference fluorophore and the microspheres). Calibration lines obtained for APC and PE are also shown in the Supporting Information S5. The fluorescence intensity and concentration are given on the horizontal axis and the vertical axis, respectively. The solid circles show the results from a series of diluted APC or Nile Red solutions and the solid line is a result of a linear fit to log of data described explicitly in Ref. 2. The nearest calibration point marked with an arrow was used for deriving the equivalent reference fluorophore concentration for microsphere standard (2nd column in Table 5) marked by a "X" with averaged fluorescence intensity with three replicates displayed on the horizontal axis.

The microsphere concentrations obtained for the four surface-labeled reference beads are given in the third column of Table 5. With known $C_{\text{equivalent}}$ and $C_{\text{microspheres}}$, ERF value for each microsphere standard was calculated using Eq. (17) in Ref. 2. After measuring the percentage of the major bead population within each reference bead, ERF value was obtained for the major bead population. The values of $\text{ERF}_{\text{major}}$ are provided in the fourth column of Table 5. The uncertainties in the ERF values are about 8% of the actual value, and were obtained from the uncertainties of the values of $C_{\text{equivalent}}$ and $C_{\text{microspheres}}$. Additional systematic uncertainties are introduced by the choice of the nearest calibration point algorithm.

Table 6 shows the median ERF values cross calibrated to the hard dyed beads on selected instruments. The ERF value was calculated by multiplying the group median ratio (hard dyed bead MFI divided reference bead MFI) times the ERF value of the reference bead. The NIST assigned ERF values from Table 5 were used for the reference beads.

Table 7 provides ERF values assigned to the major population of surface-labeled bead standards by four manufacturers in comparison to the values obtained at NIST. There are large discrepancies among the assigned ERF values. Assuming that the vendors performed ERF value assignments according to

Table 5. Estimated values of the equivalent concentration of reference fluorophore (mol L^{-1}), microsphere concentration (mL^{-1}), and equivalent reference fluorophore for four surface-labeled microsphere reference standards performed at NIST using four respective reference fluorophores

MICROSPHERE	$C_{\text{EQUIVALENT}}$	$C_{\text{MICROSPHERES}}$	$\text{ERF}_{\text{MAJOR}}$	REFERENCE FLUOROPHORE
FITC	$(4.33 \pm 0.46)10^{-11}$	$(3.11 \pm 0.21)10^{5}$	$(7.74 \pm 0.91)10^{4}$	Fluorescein SRM 1932
PE	$(8.32 \pm 0.49)10^{-10}$	$(5.89 \pm 0.41)10^{5}$	$(7.94 \pm 0.91)10^{5}$	Nile Red
APC	$(2.58 \pm 0.20)10^{-11}$	$(4.54 \pm 0.31)10^{5}$	$(3.21 \pm 0.40)10^{4}$	APC
PB	$(1.58 \pm 0.14)10^{-9}$	$(5.61 \pm 0.50)10^{5}$	$(1.59 \pm 0.20)10^{6}$	Coumarin 30

Table 6. Median ERF values cross calibrated to hard dyed beads for selected instrument models

BEAD	INSTRUMENT	FITC	PE	APC	PACIFIC BLUE
BD CS&T Setup	Accuri C6	116,966	488,777	169,284	
	BD FACSAria	116,474	423,024	146,826	8,081,378
	BD FACSCanto	103,356	452,925	135,050	7,850,117
	BD FACSCalibur	95,686	398,720	129,941	
	Gallios	100,470	401,660	154,347	8,135,475
	MACSQuant	87,612	355,006	304,739	6,738,919
Beckman Coulter Flow Set Plus	Accuri C6	302,237	1,531,079	56,644	
	BD FACSAria	274,489	1,273,130	6,079	10,945,116
	BD FACSCanto	267,736	1,549,748	6,192	10,600,779
	BD FACSCalibur	242,009	1,296,178	7,727	
	Gallios	248,340	1,149,053	5,168	10,317,193
	MACSQuant	176,958	1,205,597	116,049	10,829,891
Spherotech Rainbow	Accuri C6	14,185	70,970	48,662	
	BD FACSAria	13,349	61,538	29,800	1,752,394
	BD FACSCanto	12,404	66,841	28,903	1,640,149
	BD FACSCalibur	11,509	55,397	31,434	
	Gallios	12,044	59,226	32,526	1,675,605
	MACSQuant	10,134	50,057	42,286	1,485,969
Thermo Fisher Cyto-cal Selected multifluor intensity for each standard	Accuri C6	6,504	802,703	124,333	
	BD FACSAria	6,139	762,611	25,244	165,158
	BD FACSCanto	5,679	753,135	24,361	168,785
	BD FACSCalibur	5,064	686,793	34,538	
	Gallios	5,859	704,789	31,583	154,518
	MACSQuant	6,516	602,169	128,166	158,564
Life Technologies Standard specific	Accuri C6	380,835	1,063,967	22,012	
	BD FACSAria	385,956	963,024	39,384	18,265
	BD FACSCanto	343,780	950,114	35,634	14,407
	BD FACSCalibur	303,424	779,426	28,995	
	Gallios	360,547	830,427	28,393	19,866
	MACSQuant	463,023	825,081	31,477	12,345

the assignment procedure provided, a reasonable step forward would be to identify sources of these discrepancies.

The fluorescence spectrum of Pacific Blue reference microspheres in the Supporting Information S4 shows several different emission modes when compared to other three microsphere standards. However, its fluorescence intensity was significantly higher than the medium background, though the medium gave a substantial background signal with 405-nm excitation compared to the other two laser excitations. From the detailed ERF assignment data submitted by one vendor, the fluorescence spectra of both FITC and Pacific Blue microspheres are heavily contaminated by scattering signals from beads. It is difficult to eliminate the scattering signals and obtain the true fluorescence intensity values of the microspheres, in particular for Pacific Blue microspheres. A holographic notch filter in front of the detector used in the value assignment at NIST is crucial to minimize the scattering from microsphere suspension. Furthermore, the use of laser excita-

Table 7. 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[a]
PB	1.59×10^6	3.36×10^4	4.12×10^9	8.00×10^6	7.12×10^6

[a] The assignment was not carried out due to the expiration of APC reference solution.

tion helps to achieve higher signal to noise ratios of microsphere standards.

Discussion

Wang et al. (2) have proposed using multifluorophore hard dyed beads for fluorescence calibration when the excitation wavelength and emission band are specified. To distinguish this approach from MESF, the fluorescence intensity unit used for assignment is called equivalent number of reference fluorophores (ERF) (2). As long as this is restricted to a particular instrument model, the data in this report indicates the approach can be used if variation of 10% or more is acceptable. For many applications this will be appropriate, but in each case an experimental evaluation such as in this report should be done to establish the range of variability.

Data has not previously been published on the variation of standardization when hard dyed beads are used as intensity calibrators. A study similar to the one reported here was performed comparing different FITC samples (surface stained beads, stained fixed thymocyte nuclei, and stained fixed cells) (3). The authors of that report on an older generation of flow cytometers found that the cross calibration of FITC surface-stained beads to fixed, FITC-stained cells varied by about 30%. Depending on the local conditions around the FITC molecule, the FITC emission spectrum can vary even in the same buffer solution. This is probably the cause for the differences those authors observed between FITC beads and cells stained with FITC-conjugated antibody. The FITC beads used in this study should have spectra more closely matching FITC-stained antibodies, since FITC-conjugated antibodies were used to stain the fluorophore standard beads used here.

While some bead manufacturers in the past have recommended their beads as fluorescence intensity calibrators with assigned values that is no longer the case. Hard dyed beads are usually sold as instrument controls to give consistent setups on a particular instrument. It still seems to be common practice, however, for flow cytometrists to use the beads to try to standardize groups of instruments to common operating conditions.

One systematic approach is the use of BD CS&T setup beads, which are intended to set certain instrument models to within a predefined range of operating conditions. Internal studies at BD establish acceptable ranges of interinstrument variability for this approach, but there has not yet been published documentation of these studies. The BD CS&T beads are also used to establish reference fluorescence intensities for measuring instrument performance such as sensitivity.

Another widely used bead product used to assess fluorescence sensitivity is the Rainbow Bead 8-peak bead set from Spherotech. Instrument manufacturers frequently use histograms of this bead set to demonstrate resolution of the dim populations in the set. They have also been used in research publications to demonstrate performance of new lasers or optical detectors. As seen in the data in this report, the cross calibrated value to specific fluorophores depends by at least a factor of 2 on the particular instrument that these beads are analyzed on.

The Thermo Scientific Cyto-Cal Multifluor Plus Violet Intensity Calibrator beads have assigned intensity units for specific filter bandwidths common on various flow cytometer models. This could provide better standardization for different instrument models with these beads, but this was not evaluated in our study.

Assigned units of MESF or ERF for the beads used in this study are shown in Table 5. The reference fluorophores for FITC and APC beads used in the study were FITC and APC, so those assignments are in terms of MESF. For PE and Pacific Blue, the reference fluorophores were nile red and Coumarin 30, and intensity assignments were in terms of ERF. Surface-stained beads intended as MESF fluorescence calibrators for the most frequently used immunofluorescence fluorochromes are available from BD, Spherotech and Bangs Laboratories. But since there are no fluorescent bead reference standards from an authoritative body such as NIST, one can expect some variation in the values that different manufacturers assign as shown in Table 7 from this study. If fluorochromes-specific standards from different manufacturers are used within a laboratory or a multisite study, it is important to cross calibrate those standards to insure internal consistency. The MESF values of surface-stained beads from one manufacturer can be referenced through cross calibration to the values assigned by another manufacturer.

For any laboratory with multiple flow cytometers or for study groups using flow cytometers in multiple laboratories, cross calibration of multifluorophore hard dyed beads to stable fluorochrome-specific standard particles should be a straightforward and practical process. For multiple instruments in one laboratory, the standard particles could be stained cells that are fixed or known to be stable over the time needed to run the standard particles and hard dyed beads on all the flow cytometers in the laboratory. The cross calibrated values of all the instruments can then be normalized to a standard intensity unit, the intensity of the standard particles. This does not provide a universal calibration that can be used in other laboratories, but it assures that each instrument in that laboratory will produce the same results.

To establish standardization among laboratories, particularly if those laboratories have different models of flow cytometers, standard particles that remain stable during shipping are required. If one laboratory is sending the standard particles to the other labs, the stability of the standards after shipping can be tested by having each laboratory resend the particles back to the originating lab for comparison with retained samples. The standard particles could be surface-stained beads or fixed cell samples. Ideally, these standard particles will have fluorescence values assigned by an authoritative body, such as NIST.

Conclusion

Hard dyed beads are useful for monitoring performance of flow cytometers and for setting up individual instruments to predetermined conditions. But the use of any spectrally

unmatched hard dyed bead as a general fluorescence calibrator must be verified and characterized for every particular instrument model, and ideally for each individual instrument. Additionally, the comparison of the results at each step of ERF assignment as determined at NIST and by bead manufacturers participated in the ISAC/NIST study can help reveal any limitations due to (1) use of lamp-based fluorometers without holographic filters for microsphere measurements, (2) importance of detector spectral response calibration, and (3) differences in other steps in the procedure due to differences in flow cytometers and data analysis. This exercise in the assignment of ERF values points out the need for a consensus on how best to achieve standards for quantitative flow measurements.

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