Quality assurance for polychromatic flow cytometry

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Published online 9 November 2006; doi:10.1038/nprot.2006.250

This protocol outlines a three-part quality assurance program to optimize, calibrate and monitor flow cytometers used to measure cells labeled with five or more fluorochromes (a practice known as polychromatic flow cytometry). The initial steps of this program (*system optimization*) ensure that the instrument's lasers, mirrors and filters are optimally configured for the generation and transmission of multiple fluorescent signals. To determine the sensitivity and dynamic range of each fluorescence detector, the system is then *calibrated* by measuring fluorescence over a range of photomultiplier tube (PMT) voltages by determining the PMT voltage range and linearity (Steps 2–10) and validating the PMT voltage (Steps 11–17). Finally, to ensure consistent performance, we provide procedures to *monitor* the precision, accuracy and sensitivity of fluorescence measurements over time. All three aspects of this program should be performed upon installation, or whenever changes occur along the flow cytometer's optical path. However, only a few of these procedures need to be carried out on a routine basis.

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INTRODUCTION

There is an increasing interest in using polychromatic flow cytometry (5+-color flow cytometry) in basic and translational research settings; however, there are few published descriptions of quality assurance programs for polychromatic instrumentation[1–7]. Such programs are essential, because multiple instrument components must be precisely installed and maintained in order to measure several fluorochromes with considerable spectral overlap. Moreover, data from polychromatic systems may in future be used to make clinical decisions, especially with respect to the medical management of HIV-1-infected patients[3,4,6,8–14]. This protocol describes procedures using a variety of bead particles and measurements (**Table 1**) to validate, monitor and ensure reproducible detection of fluorescent marker combinations on cells used for polychromatic analysis. The reader should use the table as a guide when following this protocol. In addition to these measurements, final validation of these practices will ultimately include the use of cells stained with markers to ensure biological significance.

Procedures described in this protocol are critical because all flow cytometers are dependent on optical filters, precision of fluorescence measurements, and laser alignment. Hence, the more efficient the detectors (e.g., PMTs) for fluorescence detection, and the more efficient the transmission and reflection properties of the optical filters and mirrors, the better the system design for polychromatic flow cytometry. For example, detectors used to measure low-energy photons (emitting in the far-red spectrum) should be matched with the most efficient PMTs. In addition, optical filters should be designed to reflect and transmit maximum light energy at instrument-specific angles as determined by the manufacturer (e.g., some instruments require the dichroic mirrors positioned at 11.25° degrees, others at 45°). Hence, the orientation of these filters is extremely important, and reversing direction or placing the filters at incorrect angles will result in unexpected reflection and transmission. Another critical component of quality assurance is the laser's stream intersection positions relative to a reference laser (typically the first laser in the series). These positions require daily monitoring to ensure overall signal quality.

A good quality control program will serve users from the point of installation through day-to-day operation. Upon installation, certain components of the instrument should be tested for optimal performance, as described in the procedures for system optimization (Step 1)[15]. These procedures examine a wide array of critical instrument parameters: the efficiency and performance of optical filters, dichroic mirror reflection and transmission, the timing of lasers (laser delay), laser power, the length of time set to collect instrument signals (e.g., window extension), amplifier linearity, and synchronization of area and peak height signals (e.g., area scaling factor)[16].

TABLE 1 | Beads and measurements for polychromatic flow cytometry quality control procedure.

		Quality control procedure[a]								
Bead	**Measurement**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
8× Rainbow bead	MFI area signal (M1 peak)				X	X				X
8× Rainbow bead	MFI area signal (M2 peak)					X				
8× Rainbow bead	CV (M1 peak)					X				
1× Rainbow bead	MFI area signal	X	X	X				X		
1× Rainbow bead	CV							X		
1× Rainbow bead	MFI height signal			X						
Unstained CompBeads	MFI area signal				X				X	
Fluorescently labeled CompBeads	MFI area signal						X			

[a]Steps of the quality control procedure: A, cascade test; B, filter transmission and reflection; C, signal synchronization; D, PMT voltage range; E, PMT linearity; F, PMT validation; G, monitor tolerance target range; H, monitor background; I, monitor M1 peak.

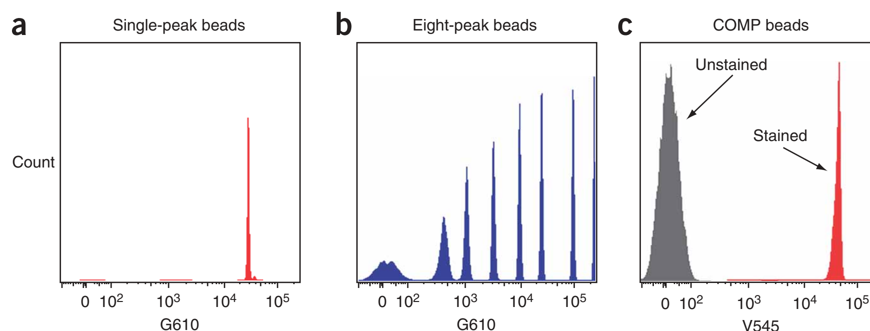

Figure 1 | Quality assurance particles. Shown are examples of three types of bead, which are used throughout the quality assurance procedure. (**a**) The single bead histogram shows the fluorescence intensity of this bead in the G610 detector (fluorescence detectors are named based on which combinations of lasers are used for excitation—B = 488 nm blue, G = 532 nm green, R = 632 nm red, V = 405 nm violet—and the central wavelength of the band-pass filter used). This bead yields reproducible results, has a broad spectrum of emission and is useful for standardization and calibration. (**b**) The eight-peak bead histogram shows the range of fluorescence intensity of all eight peaks. Many detectors cannot measure all eight peaks because of the laser excitation and dye combination within the beads. Hence, these beads are used only to determine the relative voltage range for each detector. (**c**) To measure specific fluorescence in a detector, CompBeads (positive peak) are labeled with specific mAb conjugates. These beads can be compared with unstained CompBeads (negative peak) to determine the level of background noise in each detector.

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Once optimized, the procedures in the 'Calibration' section (Steps 2–17) should be performed to measure the dynamic range and photon efficiency of specific PMT detectors. Bead particles are ideal for this purpose because their measurement values are reproducible over long periods of time[17–21]. In addition, beads are ideal for polychromatic flow cytometry applications because the dyes captured inside these particles can be excited by many laser wavelengths to emit light detectable by multiple detectors (i.e., they are broad-spectrum dyes). In the procedures described here, two such bead types are used: single- and multiple-peak Rainbow beads (**Fig. 1a,b**). The singlet-peak bead shows only one distinct peak in each detector whereas the multipeak beads can produce as many as eight distinct peaks. Importantly, the dyes used in these beads may not be fully excited by a specific laser wavelength or may not be fully measured by the optics used for a specific detector. For this reason, some detectors may resolve only a few peaks, whereas others may show the full complement of eight peaks. Note that broad-spectrum beads measure only the relative amount of light (i.e., light from the entire band-pass range from that detector). Hence, these beads cannot be used to calibrate specific fluorescence from experimentally used dyes, but are useful for measuring the dynamic range and relative PMT response of a detector.

Another uniquely engineered bead particle allows for the detection of specific fluorescence in each detector. These particles are coated with anti-mouse (κ light chain) monoclonal antibodies (mAbs). When these beads are incubated with mAbs labeled with a specific fluorochrome (e.g., anti-CD4–Qdot 545), they are captured and saturate the surface of these particles. Once labeled in this way, these beads can be used to determine specific fluorescence as measured in a specific detector (**Fig. 1c**). These particles are known as compensation beads (CompBeads). Unstained CompBeads can also be used to determine 'electronic' noise or background, which comes primarily from extraneous light scatter reaching the detector[16]. The ranges are then verified using CompBeads labeled with specific fluorochromes. Ultimately, these procedures must be validated using cells labeled with mAb conjugates for biological significance. The voltages, which are determined in this way, are used to develop tolerance ranges. These procedures should be performed periodically, especially when filters or PMTs have been altered.

Once the instrument has been optimized and calibrated, the reliability and reproducibility of data are tested daily using the monitoring procedures set forth here (Steps 18–28). A well-designed monitoring procedure should identify both immediate and potential problems. Results of daily monitoring of instrument performance should be routinely maintained in electronic logs to track trends and variations. For each parameter measured, tolerance ranges should be established which define the acceptable range of performance. To visually inspect longitudinal data for monitoring instrument performance and changes in precision, sensitivity and accuracy, Levy-Jennings style charts can be used[15]. When used in conjunction with the instrument manufacturer's recommended maintenance schedule, these procedures ensure a reliable level of performance and avoid commonly encountered instrument problems (see **? TROUBLESHOOTING**)[16–21].

MATERIALS

REAGENTS

- 1% formaldehyde (see REAGENT SETUP)
- Heat-inactivated newborn calf serum (HI-NCS; see REAGENT SETUP)
- 1× Rainbow beads (see REAGENT SETUP)
- 8× Rainbow beads (see REAGENT SETUP)
- CompBeads (see REAGENT SETUP)

EQUIPMENT

- Super-reflecting mirror (Chroma Technology, cat. no. 21010; designed to reflect 100% of light with specifications (diameter and filter thickness) according to manufacturer's recommendations)
- Laser power meter (Coherent Auburn Group, cat. no. 33-1553-00)

REAGENT SETUP

1% formaldehyde Add 5 ml of 20% buffered formaldehyde (Tousimis Research Corporation, cat. no. 1008B) to 95 ml of PBS (1× PBS, Sigma, cat. no. D-7030).

Heat-inactivated newborn calf serum (HI-NCS) Heat NCS (Quality Biological–PAA Labs, cat. no. 110-001-101) to 56 °C in a water bath for 1 h.

Bead medium Prepare PBS (1× PBS, Sigma, cat. no. D-7030) containing 1% sodium azide (Sigma, cat. no. S-202) and 1% HI-NCS.

1× Rainbow beads Add one drop (20 µl) of Sphero Rainbow Calibration Particles (second peak from the eight-peak bead set, Spherotech Inc., cat. no. RFP-30-5A-2) to 1 ml of bead medium.

8× Rainbow beads Add one drop (20 μl) of Sphero Rainbow Calibration Particles (eight peaks, Spherotech Inc., cat. no. RFP-30-5A) to 1 ml of bead medium.
Unstained CompBeads To a 12 × 75–mm tube, add 40 μl of CompBeads stock (BD CompBeads, Becton Dickinson Immunocytometry Systems, cat. no. 552843) to 60 μl of bead medium. Add 100 μl of 1% PFA and incubate an additional 10 min. If less than 60% of the total population is within the singlet bead gate, sonicate the beads for 5 min.

Stained CompBeads To a 12 × 75–mm tube, add 40 μl of CompBeads stock (Becton Dickinson Immunocytometry Systems, cat. no. 552843), 60 μl of bead medium and a titrated volume (predetermined from cell titrations) of an anti-mouse (κ light chain) mAb conjugated to a fluorochrome of a wavelength suitable for the detector. Incubate 20 min at room temperature. Add 100 μl of 1% PFA and incubate an additional 10 min. Repeat for all detectors using the specific fluorochromes measured in each detector.

PROCEDURE

System optimization

1| Several calibrations must be carried out when initially configuring an instrument, including calibrating the laser power and electronic pulse window (A), determining photoelectron efficiency (B), determining filter transmission and reflection (C), synchronizing the signals (D) and setting the laser delay (E). Once this initial calibration is done, specific calibrations from among these five should be done as needed.

(A) Monitoring laser power and setting electronic pulse window (e.g., window extension)

(i) Laser power on most diode-pumped solid-state (DPSS) lasers is fixed but should be monitored using a laser power meter at least monthly according to the manufacturer's instructions. The electronic pulse window is the timing window, which allows signals to be processed within a specified frame of time. Usually this is set at a constant value (according to the manufacturer's instructions) during acquisition. However, when adjusting the laser delays (see Step 1E), this window is adjusted to zero to observe the fastest change in signal.
(ii) Adjust laser power meter to the wavelength of the first laser to be measured. Hold the meter at the head of the laser and measure the laser power according to the manufacturer's instructions.
(iii) Record result and measure power for each laser.
(iv) Take 10–15 independent meter readings to establish an average and a range. Generally, a 25-mW laser should be within 10% of the maximum power or 25 mW ± 2.5 mW (±1 s.d.).
(v) Repeat Steps 1A(ii)–(iii) for the remaining lasers.

(B) Cascade test to determine photoelectron efficiency

(i) Perform this procedure at the time of instrument installation or if the optical path is altered. Following is an example of a five-detector (PMT) series consisting of detectors A–E, where, typically, the longer wavelengths are measured in the first part of the detector series (starting at detector A) and the shorter wavelengths are measured in the last part of the series (ending at detector E). Note that some instruments do not have a dichroic filter in the last detector position; in this case place a super-reflective mirror (100% reflection filter) in the second-to-last detector. Once this is in place, the last detector in the series can be measured.
(ii) Adjust all voltages on all detectors to 500 V.
(iii) Remove all filters in the detector system.
(iv) Place the last set of filters (the dichroic mirror and the band-pass filter, which are referred to as the 'test filter set') from detector E into the first detector (A).
(v) Run 1× Rainbow beads and measure the coefficient of variation (CV; equal to s.d./mean) in detector A.
(vi) Remove the test filter set from detector A and move these to detector B. Replace original filters into detector A.
(vii) Run 1× Rainbow beads and measure the CV in detector B.
(viii) Repeat this cascade procedure (Steps 1B(ii)–(vii)) through the entire detector series.
(ix) Using the formula: $1/(n)^2$, where $n = \text{CV}/100$, the *statistical photoelectron estimate* can be determined for each detector.
(x) Typically, efficiencies can average 800 ± 200 photoelectrons (±1 s.d.). The higher the statistical photoelectron estimate, the better the detector efficiency. Values below 500 photoelectrons are not acceptable. Higher-efficiency detectors should be moved to the detector assigned to measure the far-red end of the spectrum. (Note: each detector and laser system can produce different efficiencies. The numbers reported here were collected using a 532-nm laser in a five-detector system using a 600LP dichroic filter and a 610/20 band-pass filter.)

(C) Testing of filter transmission and reflection

(i) Perform this procedure after the installation of new filters. Note that mean fluorescence intensity (MFI) between a series of detectors can be expected to vary as much as two- to fourfold. There are a number of reasons for this variation. First, the collection filters may not be in the optimal detection range of the dye emitting photons contained within the beads. Second, reflected light can be lost from one dichroic mirror to the next. Third, PMT manufacturing is inconsistent enough to cause large differences in detection efficiency, particularly in the far-red region of the spectrum.
(ii) Place all filters in their correct locations in the detector series.
(iii) Adjust the voltages on all detectors to 500 V.
(iv) Run single-peak Rainbow beads and measure the MFI of each detector in a series.

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(v) There are three possible results. First, and most typically, the MFI between detectors may have as much as a fourfold difference, as stated above. Larger differences can define three potential problems and conditions. As an example, consider the measurements between four detectors A, B, C and D. When there is a large loss in signal in detector B, but no change in detector A, C or D, this indicates that filter transmission is faulty and the dichroic mirror in the B detector must be replaced. Second, if there is no change between detectors A and B, but there is a large drop in MFI for each subsequent detector (C and D), this indicates faulty filter reflection and the filter in detector B must be replaced. Third, if the filter is replaced with no change as described above, it is possible that the angle of the mirror is incorrect, and the manufacturer should be contacted to correct the problem.

(D) Signal synchronization (e.g., area scaling factor (ASF), Becton Dickinson instruments only)

(i) Perform this procedure when the optical path of the lasers is changed: for example, if the dichroic steering mirrors are altered, the lasers are replaced, etc. Note that the ASF is dependent on the laser beam width; therefore, in some cases the use of cells or larger particles (as compared to smaller beads) is recommended when setting this parameter. Consult the manufacturer's guidelines before setting this instrument parameter. Additionally, on some instruments the forward scatter detector has a separate adjustment for signal synchronization that can be altered using the same procedure as described in this step.

(ii) Adjust the voltages on all detectors to 500 V.

(iii) Run single-peak Rainbow beads and measure the MFI of each detector in a series.

(iv) Collect both height and area signals on one detector from each laser.

(v) Adjust the ASF until the height and area signals are equal on a log-scale histogram.

(vi) On one detector for each laser, the peak height MFI signal is equivalent to the area MFI signal on either a log or linear scale.

(E) Testing of laser delay settings

(i) Perform this procedure daily to verify the peak signal location relative to the reference laser.

(ii) Adjust the voltages on all detectors to 500 V or use the voltages to obtain the target values as described later in this protocol (Step 16) for the calibration procedure.

(iii) Decrease the electronic window (e.g., window extension) or gate to the lowest setting, usually zero.

(iv) Run single-peak Rainbow beads and measure the MFI of each detector in a series.

(v) While observing one detector from each laser, adjust the delay settings in increments of 1, first up and then down, and observe the rise and fall of the MFI.

(vi) Select the delay setting to yield the highest MFI from one detector associated with each laser. Repeat for each laser except for the reference laser (typically the 488-nm laser is the reference laser as defined by the manufacturer).

(vii) Adjust the electronic window according to the manufacturer's recommended setting.

Calibration: Determination of PMT voltage range and linearity

2| Perform this procedure only after system optimization is completed. Repeat when a new laser, detector (PMT) or filter is installed.

3| Place all filters in their correct locations in the detector series.

4| Set voltages for all detectors to 350 V.

5| Run eight-peak Rainbow beads and measure the MFI and the CV of each detector. Select two of the peaks, which are on scale throughout the voltage scan described in Step 4. The MFI of the lower bead peak is defined as M1 and that of the upper bead peak is defined as M2.

6| Increase voltage by 50 V and repeat Step 5 until measurements for a voltage range between 350 and 750 V have been collected for each detector (**Fig. 2**).

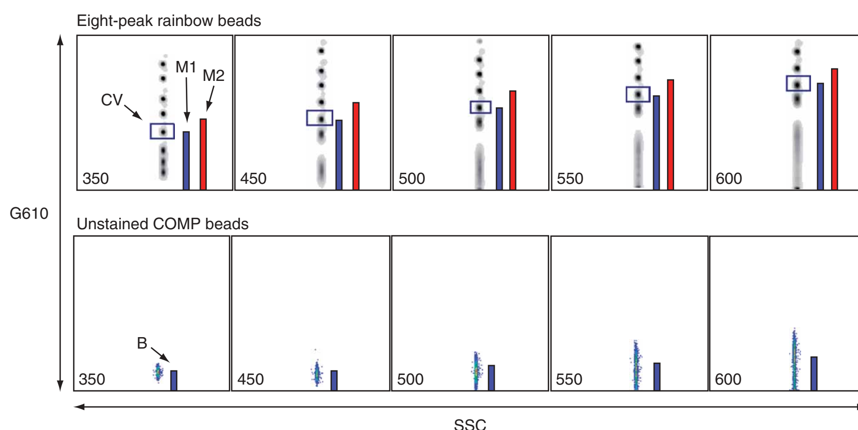

Figure 2 | Voltage series applied to eight-peak Rainbow beads and unlabeled CompBeads. Eight-peak Rainbow beads (top) and unlabeled CompBeads (bottom) were collected over a series of voltages in the G610 detector. In this example, MFI is displayed over the voltage range of 350–600 V. Measurements include the MFI (blue bars, referred to as M1) and the CV (rectangular gate) of the M1 bead. An additional measurement of intensity (red bar, referred to as M2) is used to calculate PMT linearity (M2 – M1/M1). The lower panel shows the increasing amount of background (B) with increasing voltage. The MFIs of unlabeled beads (blue bars, bottom) are used to calculate the signal-to-background ratio. As an example, at 500 V the CV = 5.29%, M1 = 4,500, M2 = 14,000, MFI = 35. The linearity was calculated to be 2.11 (14,000–4,500/4,500) and the M1/B ratio was calculated to be 129 (4,500/35).

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7| Repeat procedure using the unstained CompBeads. In this case measure only the MFI of each detector (this is defined as background).

8| Calculate the signal-to-background ratio by dividing the M1 through the voltage series by the background MFI of the unstained CompBeads.

9| Calculate the PMT linearity as follows: PMT linearity = (M2 – M1)/M1 (**Figs. 2** and **3**).

10| Graph CV, signal-to-background ratio and PMT linearity on one graphic (**Fig. 3**). Using this graphic produced for each detector, determine the range of voltages that yields the largest ratio and lowest CV at the lowest PMT voltage range. **Figure 4** shows examples of two typical graphics. In the first plot (**Fig. 4a**), the PMT voltage range can be defined as the voltage point showing the highest signal-to-background ratio at the lowest CV and a point on the linearity curve where the slope is zero. Taken together, the range from 450 to 550 V is defined as the *PMT voltage range*. In the second plot (**Fig. 4b**) all curves flatten out starting at 500 V and at 700 V the PMT no longer appears linear. In this example, the PMT voltage range can be defined to be from 500 to 700 V.

Calibration: Validation of PMT voltages

11| Perform this procedure after determining linear PMT voltage ranges as described in the previous section.

12| Place all filters in their correct locations in the detector series.

13| Using the voltage ranges determined with the previous procedure (see eight-peak bead procedure, Steps 2–10), initially set all voltages to the middle value of the range corresponding to that detector.

14| Adjust the voltage until a value is found where the highest primary fluorescence (defined as the fluorochrome on the bead) is attained, with minimal MFI in all of the other secondary detectors (defined as the detectors not specific for the fluorochrome on the CompBeads).

15| Repeat Steps 13 and 14 for all detectors.

16| Once completed, run the single-peak Rainbow beads and measure the MFI from each detector. This channel value (MFI) is known as the *target value*.

17| **Figure 5** shows an example of eight CompBeads profiles. The arrows point to the peak of the primary detector (e.g.,CD8-FITC–labeled CompBeads, CD8-PE–labeled CompBeads, etc.). As expected, detectors closest to the emission of the primary detectors will have the closest MFI values relative to the primary detector. In order to define the *target value range*, repeated values were collected ($n = 20$) and either ±1 s.d. or ±10% from the mean was used. These same values can be used to define the upper CV value for each PMT. Hence, the target value range for a CV is defined as any value *less than* the highest value obtained within all values collected (e.g., V605 detector has a CV range ≤6.5%).

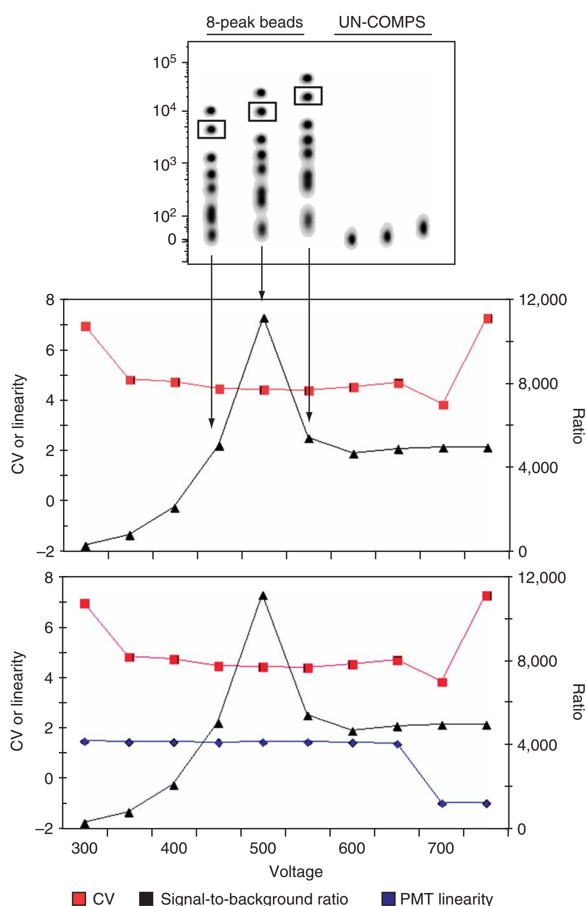

Figure 3 | Determining the *linear PMT voltage range* using a plot of CV, PMT linearity and ratio. This graphic illustrates how to plot three curves from the eight-peak Rainbow beads and the unlabeled CompBeads (UN-COMPS) collected over a series of voltages. Ratio is determined by dividing the MFI (M1) by the MFI of the unlabeled CompBeads (B). In this example, the highest ratio was determined to be at 500 V. Notice that within the range of 450–550 V, the CV of the M1 bead (rectangular gate) is decreased or unchanged, while at the same time the PMT linearity curve is flat, suggesting that the PMT is linear. This range is defined as the linear PMT voltage range.

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Standardization

18| Perform this procedure (Steps 18–28) daily to obtain the correct voltage used to define the *tolerance target range* for each PMT. Before adjusting the PMT voltages, check the laser delay setting as defined in Step 1.

19| Place all filters in their correct locations in the detector series.

20| Run the single-peak Rainbow beads.

21| Using the target value ranges as determined from the calibration procedures (Steps 2–17), adjust the voltages on each PMT until the MFI is within this range. Use these voltages with every experiment run on the same day.

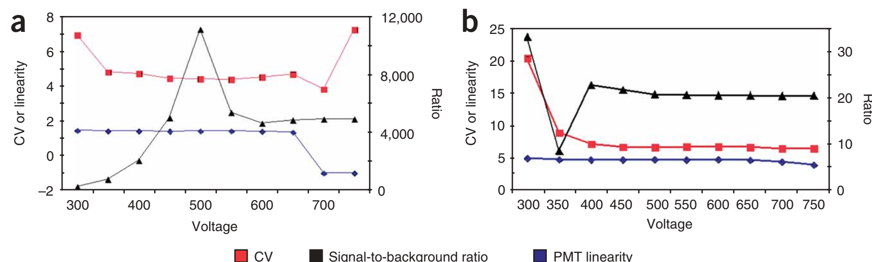

Figure 4 | Sample graphic illustrating CV, PMT linearity and signal-to-background ratio. Two typical graphs are depicted that highlight the possible differences that can occur in plots of CV, PMT linearity and ratio. (**a**) Here the PMT voltage range can be defined as the voltage point showing the highest signal-to-background ratio co-occurring with the lowest CV and a slope of zero on the linearity curve. In this case, the range from 450 to 550 V is defined as the PMT voltage range. (**b**) Here all curves flatten out starting at 500 V and are nonlinear at 700 V; in this example, the voltage range lies between 500 and 700 V.

22| Collect and save at least 10,000 counts in a separate file.

23| Run the eight-peak beads.

24| Collect and save at least 10,000 counts in a separate file.

25| Run the unstained CompBeads.

26| Collect and save at least 10,000 counts in a separate file.

27| Data is collected and examined for real-time tolerance criteria and prognostic tolerance criteria. Real-time tolerances are established to determine whether a test procedure can be measured correctly just before acquisition and therefore can rule out instrument error. Voltages and CVs of the single-peak Rainbow beads are examples of real-time criteria. Voltages must not exceed a 5% variation of the target value as defined in the calibration procedures (Step 17)and the CVs must be below the predefined range.

28| Prognostic tolerance criteria can determine potential instrument error and defines the accuracy, precision and sensitivity of the instrument. *Accuracy* is defined in a graphic showing voltage (1× Rainbow bead) as a function of time (**Fig. 6a**). The variation should not be greater than ±2 s.d. or 5% from the mean ($n = 20$). In the present example, the instrument was accurately measuring the correct amount of fluorescence at similar voltages over time. *Precision* is defined in a graphic showing CV (1× Rainbow bead) as a function of time (**Fig. 6b**). The variation should not be greater than ±2 s.d. or 5% from the mean ($n = 20$). *Sensitivity* is defined in a graphic showing the signal-to-noise ratio (8× Rainbow bead MFI/unstained CompBeads) as

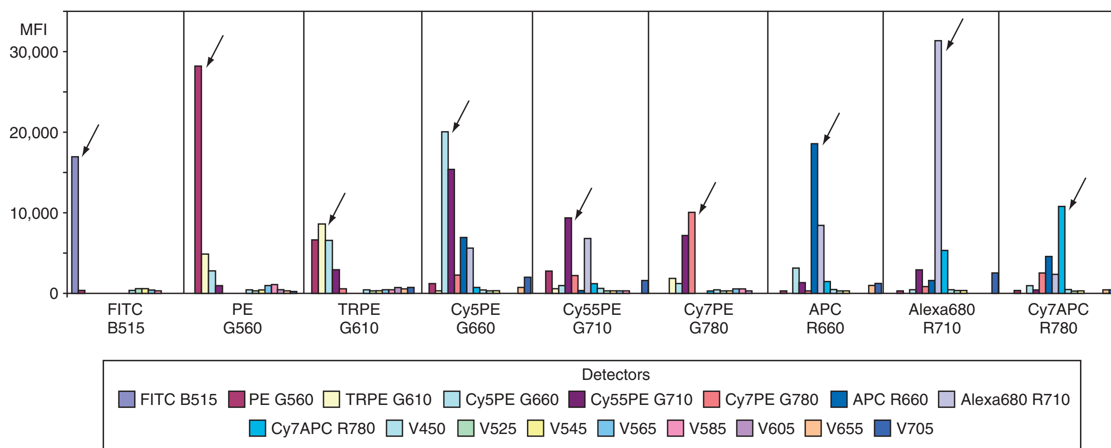

Figure 5 | Compensation profiles of a selected group of detectors. Typically, after calibration of PMT detectors, CompBeads labeled individually with specific fluorochromes are measured. Here each detector voltage was adjusted until the primary detector MFI was larger than that of any of the secondary detectors (see arrows). Some detectors will show only small differences (G610, G660, G710, G780 and R780 detectors) while others will show larger differences as indicated in the B515 (FITC), G560 (PE), R660 (APC) and R710 (Alexa 680) detectors. The objective is to achieve this type of separation at the lowest possible PMT voltage within the dynamic range as determined during the calibration procedure. Letters before numbers refer to the excitation laser (V = violet laser at 408 nm, B = blue laser at 488 nm, G = green laser at 532 nm and R = red laser at 638 nm). All numbers following the laser designations refer to the emission peak.

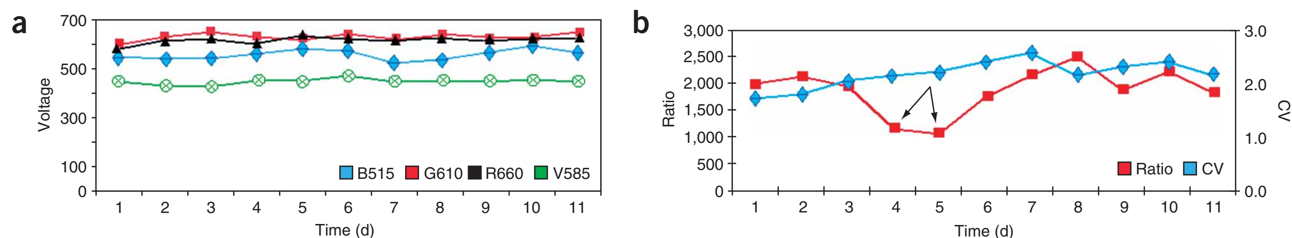

Figure 6 | Time plots of accuracy, precision and sensitivity. (**a**) Monitoring voltage as a function of time illustrates the accuracy of obtaining the same median fluorescence intensity (defined as the target value) at a voltage with a 5% variation. In this example, all voltages are within acceptable variance. (**b**) Monitoring signal-to-background ratio indicates the sensitivity of each detector and monitoring CV as a function of time provides a measurement of instrument precision. In this case (arrows), on days 4 and 5 a loss of instrument sensitivity occurred but the instrument precision (CV) is within the expected range. Here background noise increased as a result of a faulty filter allowing contaminated light into that detector (R660), but at the same time, the voltage needed to obtain the correct MFI was not affected.

a function of time (**Fig. 6b**). The variation should not be greater than ±2 s.d. or 5% from the mean ($n = 20$). In some cases precision can be within expected range while the ratio (sensitivity) is decreased (arrows in **Fig. 6b**). In the illustration, the background in the detector was increased as a result of poor alignment.

● TIMING

Step 1A: 1–2 min per laser.
Step 1B: 15 min per detector series.
Step 1C: 5 min per detector.
Step 1D: 2 min
Step 1E: 2 min
Steps 2–10: 60 min
Steps 10–17: 30 min
Steps 18–28: 10 min

? TROUBLESHOOTING

See **Table 2** for troubleshooting advice.

TABLE 2 | Troubleshooting table.

Problem	Possible cause	Solution
System optimization		
PMT detector sensitivity is below 500 photoelectrons (see Step 1B for details).	The detector is faulty or poorly aligned.	Replace PMT or adjust PMT alignment.
	Dirty optics are reducing light transmission and/or reflection.	Inspect and clean filter.
MFI signals appear dim in the far-red detectors of a single laser source.	Low-sensitive PMTs were used in these detectors.	Replace PMT with far-red sensitive detectors (defined as a PMT with a high statistical photoelectron estimate value).
MFI signals in all detectors of one laser are reduced.	The laser delay setting is incorrect.	Check and adjust timing delay.
	A narrow timing window was set.	Check and set electronic window.
	Peak and area signals are not synchronized.	Check and properly set signal synchronization.
	There is a dirty filter in the optical path.	Clean filter using methanol.
One detector in a series shows a very low MFI value. All other detectors appear normal.	The dichroic mirror is a poor transmitter or the dichroic filter is reversed.	Replace the dichroic mirror and check it is correctly positioned.
The first two filters in a series have high MFI values and all downstream filters have very low MFI values.	The angle of reflection is incorrect or the dichroic mirror is a poor reflector.	If possible, correct the angle of the dichroic mirror.
	An incorrect band-pass filter was used.	Replace this dichroic mirror or contact the manufacturer

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TABLE 2 | Troubleshooting table (continued).

Problem	Possible cause	Solution
Calibration		
CVs on either the 1× or 8× Rainbow beads are greater in one detection series (one laser) as compared to another series.	The dichroic steering mirror is out of alignment.	Adjust steering mirror for that laser line until the CVs are as low as possible.
PMT linearity curve does not have a plateau.	PMT is not linear.	Replace the PMT.
The CVs on the 8× Rainbow beads increases with increasing voltage.	PMT is faulty.	Replace the PMT.
Stained CompBeads are negative for specific fluorochrome conjugate tested.	The mAb used is incompatible with the bead: e.g., anti-κ mAb will not bind a λ mAb and anti-mouse mAb will not bind rat antibodies.	Use correct mAb conjugate for CompBeads capture mAb.
Fluorochrome-conjugate labeled CompBeads do not show the highest MFI value compared to secondary detectors.	Voltage of that PMT is too low.	Increase PMT voltage on that specific detector to within the tolerance range.
Standardization		
CVs on the 1× Rainbow beads are greater in one detection series (one laser) than in another series (i.e., decrease in precision).	The dichroic steering mirror is out of alignment.	Adjust steering mirror for that laser line until the CVs are as low as possible.
An increasing PMT voltage trend is seen as a function of time, either in a single detector or in multiple detectors.	The PMT detector is faulty.	Replace PMT or detector alignment.
	The steering mirror is out of alignment.	Adjust dichroic steering mirror.
	The laser power is low.	Replace laser.
	There is a dirty filter in the optical path.	Inspect and clean filter.
A decreasing PMT voltage trend is seen as a function of time, either in a single detector or in multiple detectors (i.e., decrease in accuracy).	There is a problem with flow stream alignment or pressure.	Clean flow cell and check pressure system.
A decreasing signal-to-background ratio is seen with consistent or no change in voltage (i.e., a decrease in sensitivity)	Increasing background is resulting in poor resolution on dim cell markers.	Examine filters for possible light contamination due to incorrect or faulty filters.
		Change band-pass filters to block out the Raman scatter effect[16].
		Replace PMT.
Unstained CompBeads produce a positive result in one detector.	Contaminated CompBeads were stained with mAb conjugate.	Use a new vial of CompBeads.
CompBeads light scatter gate shows a low percentage (<60%).	CompBeads are aggregated.	Sonicate CompBeads for 5 min.

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ANTICIPATED RESULTS

A critical component in the operation of complex instrumentation is the consistent and reproducible monitoring of the standardization results. Deviation in any of these parameters should be considered out of tolerance because it may compromise sample analysis. In these situations no data should be collected. Therefore, it is recommended that tolerance ranges be checked before every experiment. This provides a real-time standardization to assure users that their results were obtained under optimal instrument conditions. Voltages obtained during the initial calibration procedure are used throughout the daily experimental runs. Trends in voltages over time for each detector can be monitored for changes in detector sensitivity and/or changes in filter reflection or transmission. If changes occur in the optical path during the lifetime of an instrument (new laser installation, new filter configuration, etc.), the system optimization and calibration procedures will need to be repeated. Finally, many instrument components are expected to fail with time, especially those related to the pressure systems and component value assemblies. Therefore, it is highly recommended that an active preventive maintenance program be maintained.

A future goal is to determine the correspondence between median fluorescence intensity and the presence of a standard amount of molecules of equivalent soluble fluorochrome (MESF) measured by a given detector. This measurement has been described previously as the 'Q' value. Therefore, each detector can be standardized against this reference material and voltages can be adjusted on a PMT to yield an 'ideal' target value. As previously reported, values are generated with knowledge of the instrument background, known as the 'B' value[22]. Once these measurements are determined, instrument-to-instrument variation and standardization can become more efficient, hence improving instrument performance[21]. However, such standards are not yet available for all of the detectors used in polychromatic flow cytometry. We must therefore rely on 'relative' units of fluorescence without the knowledge of the Q value or the exact determination of the B value.

Described here is an approach to standardize the practice of polychromatic flow cytometry, focused on three areas of quality assurance: (i) system optimization, (ii) calibration and (iii) standardization. Although most procedures are considered routine in nature, many are not and will require some practice in understanding the operation of the complex instruments. Notably, we are working on further developing these procedures with the hope of simplifying the process and providing useful quality control tools for future instrumentation.

ACKNOWLEDGMENTS We would like to thank D. Parks (Stanford University School of Medicine) for his expert advice and knowledge and J. Trotter (BD Biosciences) for his conceptual work in quality controls and technical support.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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