

Quality assurance for polychromatic flow cytometry using a suite of calibration beads

Stephen P Perfetto, David Ambrozak, Richard Nguyen, Pratip K Chattopadhyay & Mario Roederer

Flow Cytometry Core Facility, Vaccine Research Center, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland, USA. Correspondence should be addressed to S.P.P. (sperfetto@mail.nih.gov).

Published online 8 November 2012; doi:10.1038/nprot.2012.126

This protocol is an update to *Nature Protocols* 1, 1522–1530 (2006); doi:10.1038/nprot.2006.250; published online 9 November 2006

The quality assurance program presented here provides a means to maximize and maintain the performance of individual flow cytometers in a facility. To optimize performance, we recommend performing all three steps (optimization, calibration and standardization) in this program when a new flow cytometer is installed or whenever the flow cytometer's optical path is altered (e.g., lasers, filters or detectors are replaced). The complete process requires 3–4 h. On a more frequent basis, only a subset of these procedures need to be performed as a part of daily maintenance routines. The data generated can be tracked to monitor the instrument and determine whether service is needed. In addition, the data can provide a metric for whether repairs and upgrades have improved or harmed performance, and for future instrument-to-instrument comparisons. In sum, the procedures presented here represent an updated framework for optimizing, calibrating and standardizing a flow cytometer for daily use.

INTRODUCTION

The cellular proteome is complex; a remarkable number and variety of proteins are associated with a cell's life cycle and function¹. As a means to identify and enumerate cell subsets on the basis of protein expression, flow cytometry has been used with great success in both research and clinical settings². In recent years, this technology has advanced to a great extent, allowing the measurement of up to 18 proteins simultaneously from a single cell³. Such multiplexed measurement requires the use of many different fluorochromes, each with distinct fluorescence spectra. Thus, technology using more than six colors is commonly referred to as polychromatic flow cytometry (PFC).

The success of PFC experiments depends on a number of crucial hardware factors: (i) the laser beam shape and location, (ii) the choice and quality of optical filters and (iii) the sensitivity and resolution of photoelectron detection. PFC systems with the best resolution and consistency over time show strong excitation of fluorochromes, optimal transmission and reflection of photon emission, as well as efficient collection of this energy at the detector (photomultiplier tube (PMT)). The need to optimize these elements is apparent from the literature and from observations made during routine instrument use^{4,5}. First, the position of each laser (relative to the first 'reference' laser in the instrument) can have a marked impact on signal quality, as observed during routine laser delay adjustments. Second, optical filters must be chosen for a maximal ability to reflect and transmit light. In particular, the optimal angles for transmission must be consistent with the instrument design (e.g., some instruments require the dichroic mirrors to be positioned at 11.25°, whereas others use 45°). The reflection and transmission of light can suffer markedly when the filter is reversed or its angles are not correct. Finally, the efficiency of detectors can differ substantially between PMTs; thus, more efficient PMTs should be paired with fluorochromes that release low-energy photons, such as Cy7APC.

This paper describes how these (and other) factors can be addressed in a quality assurance program. Specifically, our method optimizes and calibrates hardware and tracks performance over time (**Table 1**) from the point of installation through day-to-day

operations. It provides a structured means for detecting instrument problems and allows comparison of detectors within an instrument or across different instruments and facilities.

System optimization

Upon installation, it is important to check that critical components of the instrument are performing optimally⁶, as described in the procedures for 'System optimization' (Step 1). 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, electronic noise and synchronization of area and peak height signals (e.g., Area Scaling Factor, ASF)⁷. These procedures are largely unchanged from our previously published protocol on this topic and are provided here for convenience⁸. However, this document has been updated to incorporate new technologies that allow better calibration of the instrument.

Calibration using various bead sets

Once the system has been optimized, the procedures in the 'Calibration' section (Step 2) should be performed to measure dynamic range and photon efficiency of each PMT (e.g., detector). Bead particles are ideal for this purpose because they are reproducible over long periods of time^{9–12}. In this protocol, we use two general types of beads, hard-dyed and antibody-capture beads. Hard-dyed beads contain dyes within the bead particles that can be excited by many laser wavelengths, emitting light in multiple detectors (i.e., they are broad-spectrum dyes). These beads are available with a predefined quantity of fluorescent material, which is reported in standardized units as molecules of equivalent soluble fluorochrome (i.e., MESF units)^{9,11}. However, as beads with predefined MESF values are not easy to procure, these can be substituted with the second type of beads, which bind antibodies conjugated to the fluorochromes used experimentally.

Our previous protocol⁸ used hard-dyed Rainbow beads and antibody-capture beads; however, recently, other types of beads



PROTOCOL UPDATE

TABLE 1 | Summary and schedule of measurements in quality assurance program.

Step	Procedure	Schedule	Record
Optimization (Step 1)	Laser power check (Step 1A)	Monthly	None
	Cascade test (Step 1B)	Optional	SST
	Filter check (Step 1C)	New instrument/as needed	None
	Signal synchronization (Step 1D)	New instrument/as needed	None
	Laser delay check (Step 1E)	Daily routine	SST
	Electronic noise check (Step 1F)	New instrument/as needed	None
Calibration (Step 2)	PMT voltage scan and linearity (Step 2A)	New instrument/as needed	FlowJo
	PMT validation (Steps 2B and 2C)	New instrument/as needed	SST
Standardization (Steps 3–10)	Monitor tolerance range	Daily routine	SST
	Monitor optical background	Daily routine	SST
	Monitor M1 peak	Optional	SST

SST, spreadsheet or statistical analysis software, such as Microsoft Excel or SAS JMP.

have been developed, which have specific advantages over those used in our previous method. Therefore, we have substantially altered the calibration routines we recommend. Specifically, four types of beads should be used (**Table 2**): Quantum Simply Cellular beads (QSCBs), Cyto-Cal beads (or Duke beads), Single-peak (1×) Rainbow beads and unstained compensation beads (COMP beads). Their unique traits and the advantages conferred by those traits are presented below.

QSCBs are uniquely engineered bead particles that fluoresce with varying (but discrete; **Fig. 1a**) levels of intensity in each detector. This variation in fluorescence intensity is a function of the amount of mouse-specific IgG monoclonal capture antibody on each bead particle. Thus, when incubated with a fluorescently tagged antibody, a sample of QSCBs provides a very bright fluorescence signal (originating from the bead coated with many capture antibodies), a very dim signal (from the bead coated with very few capture antibodies) and two intermediate populations. The system also includes unlabeled beads to assess background. Because an antibody-capture system is used, the reagent can be incubated with the same fluorescently labeled antibodies that are used in the experiment, thereby calibrating

the instrument to the spectra of the experimentally relevant dyes. Moreover, the calibration is performed for bright signals, but information is provided for dim signals as well. This was not possible in the original protocol, in which a single-intensity antibody-capture dye was used alongside a broad-spectrum Rainbow bead⁸.

Cyto-Cal beads (**Fig. 1b**) also have varying levels of fluorescence (five bead populations with differing brightness, along with a negative control bead). However, these beads are more convenient than QSCBs, as no antibody capture is needed. As with the Rainbow beads used in our previous protocol⁸, the dyes used in these beads may not be fully excited by some lasers or may not fluoresce in all regions of the spectrum. For this reason, some detectors may resolve fewer peaks as compared with others showing the full complement of five peaks. Despite these limitations, Cyto-Cal beads are useful because they are better matched to the spectra of common fluorescent dyes and are manufactured more uniformly (the coefficient of variation (CV) of the fluorescence signal is lower) than Rainbow beads. For these reasons, some aspects of our calibration procedure have been modified to use these beads. However, 1× Rainbow beads (**Fig. 1c**), which emit one distinct peak of fluorescence signal in each detector, remain useful as a means to monitor instrument performance over time.

The beads described above are used to calibrate positive signals. However, the identification of cell populations by flow cytometry also depends on levels of background signal, which can arise from various sources (including PMT-dependent electronic noise, stray light, component fluorescence and signals from sheath media impurities). The cleanest method for assessing background uses unstained COMP beads (**Fig. 1d**, gray peak)⁷, as described in this protocol.

On the basis of the analysis of these bead types, the ideal range of voltages (gain) can be identified. The voltages can be used to determine tolerance ranges, from which the daily gain can be set. This range is chosen such that the signal from each fluorochrome we use is maximized in the PMT assigned as the fluorochrome's primary detector. Signal from that fluorochrome should be lower in all other detectors. This is ideally tested using COMP beads, singly stained with the fluorochromes commonly used in the investigator's experiments (**Fig. 1d**, red peak). This process reduces spreading error, which in turn increases the dynamic range of cell population discrimination. We suggest that calibration routines be performed whenever filters or PMTs have been altered, to ensure that the best range of voltages has been defined.

Tracking reproducibility and reliability

Once the instrument has been optimized and calibrated, the reliability and reproducibility should be monitored daily as described below (Steps 3–10; 'Standardization'). A well-designed monitoring routine should identify both actual and potential problems. Results of daily monitoring of instrument performance should be kept in electronic logs to track trends and variations. For each parameter measured, tolerance ranges should be established that define the acceptable range of performance. To visually inspect longitudinal data for monitoring intra-instrument performance and changes in precision, sensitivity and accuracy, Levey-Jennings style charts can be used⁶. 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 **Table 3**)^{7,9–12}.

TABLE 2 | Beads and statistics used in quality assurance measurements.

Step	Bead				Statistic
	Cyto-Cal	1× Rainbow	Unstained COMP	QSCB	
Cascade test (Step 1B)		X			MFI-a, rCV
Filter check (Step 1C)		X			MFI-a
Signal synchronization (Step 1D)		X			MFI-a, MFI-h
Laser delay check (Step 1E)		X			MFI-a
Electronic noise (Step 1F)		X			90th percentile
PMT voltage scan (Step 2A)	X		X		MFI M1 peak, rCV, 90th percentile M1 90th percentile unstained
PMT linearity (Step 2A)	X				MFI M1 peak MFI M2 peak
PMT validation (Steps 2B and 2C)				X	90th percentile MFI-a
Monitor tolerance (Steps 3–10)		X			MFI-a, rCV
Monitor optical background (Steps 3–10)			X		90th percentile
Monitor M1 peak (Steps 3–10)	X				MFI-a M1 peak

MATERIALS

REAGENTS

- Well-defined cell samples and commonly used antibodies
 - Monoclonal antibody (mAb) conjugates specific for detectors
 - PBS (1× PBS, Sigma Chemical, cat. no. D-7030)
 - Formaldehyde (Tousimis Research Corporation, cat. no. 1008B; see Reagent Setup)
- Heat-inactivated newborn calf serum (Quality Biological, PAA Labs, cat. no. 110-001-101; see Reagent Setup)
 - Sodium azide (Sigma Chemical, cat. no. S-202; see Reagent Setup)
 - Single-peak (1×) Rainbow beads (Sphero Rainbow calibration particles, Spherotech, cat. no. RFP-30-5A-2; see Reagent Setup)
 - Cyto-Cal beads (Cyto-Cal Multifluor + Violet intensity calibrator, Thermo Scientific, cat. no. FC3MV; see Reagent Setup)

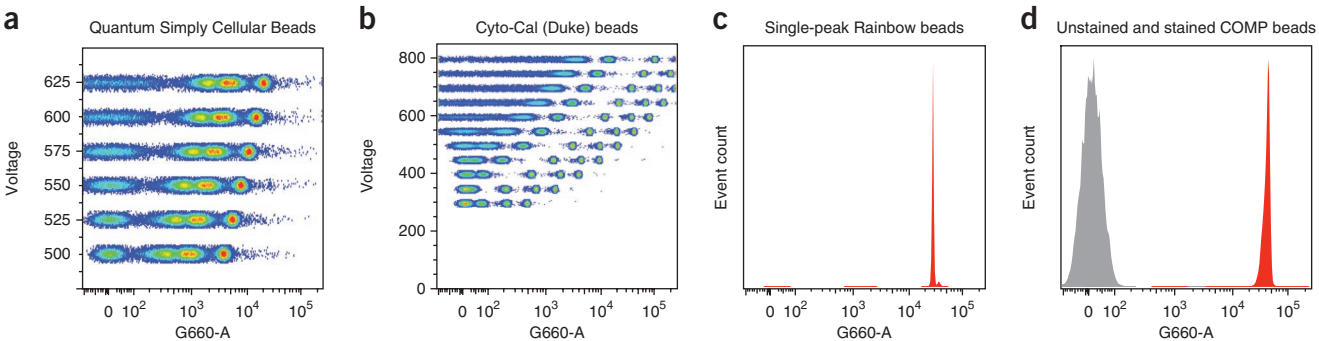


Figure 1 | Particles for a quality assurance program. Shown are examples of four beads used throughout the quality assurance procedure. **(a)** QSCBs stained with CD8-Cy55PE and shown over six voltages. **(a,b)** We used this bead type after a voltage range was defined using Cyto-Cal beads **(b)**, which contains broad-spectrum dyes at five different concentrations and one negative bead. This shows the emission in the G660 detector over a voltage scan and is useful for calibration and standardization procedures. **(c)** To monitor instrument performance on a routine schedule, we used 1× Rainbow beads containing different broad-spectrum dyes and Cyto-Cal beads with one concentration of fluorochrome. **(d)** We labeled COMP beads (positive peak, red) with specific mAb conjugates and were used in standardization procedures; these beads can be compared with unstained COMP beads (negative peak, gray) to determine the level of background noise in each detector.

PROTOCOL UPDATE

- QCSBs (Bangs Laboratories, cat. no. 815C; see Reagent Setup)
- Compensation beads (BD COMP bBeads, Becton Dickinson Immunocytometry Systems, cat. no. 552843; see Reagent Setup)

EQUIPMENT

- Flow Cytometer (LSR II, Becton Dickinson Immunocytometry Systems)
- Super-reflecting mirror (Chroma Technology, cat. no. 21010; note: designed to reflect 100% of light with specifications (diameter and filter thickness) according to the manufacturer's recommendations)
- Laser power meter (Coherent Auburn Group, cat. no. 33-1553-00; note: used for the air-cooled laser systems only)

REAGENT SETUP

Paraformaldehyde (PFA), 1% (vol/vol) (or 1% (vol/vol) formalin) Add 5 ml of 20% (vol/vol) buffered formaldehyde to 100 ml of PBS (vol/vol). Store the solution for 6 months at 4 °C.

Heat-inactivated newborn calf serum Heat the serum to 56 °C in a water bath for 1 h. Store it for 2 years at 4 °C.

Bead medium Mix 1% (vol/vol) sodium azide and 1% (vol/vol) heat-inactivated newborn calf serum in PBS. Store the medium for 6 months at 4 °C.

Single-peak (1×) Rainbow beads Add one drop (20 µl) of particles into 1 ml of bead medium in a 12 × 75 mm tube. Store the beads for 4 months at 4 °C.

Cyto-Cal beads Add one drop (20 µl) of particles into 1 ml of bead medium in a 12 × 75 mm tube. Store the beads for 4 months at 4 °C.

Unstained COMP beads Add 40 µl of COMP beads stock into 60 µl of bead medium into a 12 × 75 mm tube. Store the beads for 4 months at 4 °C. Note that these beads should be sonicated if <60% of the total population is within the singlet bead gate. Wash twice in bead medium and decant the supernatant. To the bead pellet, add 100 µl of 1% (vol/vol) PFA and incubate it for an additional 10 min at room temperature (25 °C). This material should be discarded after use.

Stained COMP beads Add 40 µl of COMP beads stock, 60 µl of bead medium and a titrated mAb conjugate volume (predetermined from cell titrations) into a 12 × 75 mm tube. Incubate the mixture for 20 min at room temperature (note that time and temperatures can be different for different mAb conjugates and should be maintained as predetermined from cell titration experiments). Wash twice in bead medium and decant the supernatant (centrifuge at 256g for 5 min at 23 °C). To the bead pellet, add 100 µl of 1% (vol/vol) PFA and incubate it for an additional 10 min at room temperature. Repeat for all detectors using the specific fluorochrome measured in each detector. Store the beads for 4 months at 4 °C.

QSCBs Add four or five drops (80 µl of each level (i.e., corresponding to the fluorescence intensity for each bead)) of QSCBs stock into a 12 × 75 mm tube and wash once in 100 µl of bead medium (centrifuge at 256g for 5 min at 23 °C). Decant all of the medium and add 100 µl of bead medium containing a titrated mAb conjugate volume (predetermined from cell titration experiments). Incubate the mixture for 20–30 min at room temperature (note that time and temperatures can be different for different mAb conjugates and should be maintained as predetermined from cell titration experiments). Wash twice in bead medium and decant the supernatant (centrifuge at 256g for 5 min at 23 °C). To the bead pellet, add 100 µl of 1% (vol/vol) PFA and incubate it for an additional 10 min at room temperature. For convenience in acquisition, all separately stained tubes can be centrifuged, resuspended (20 µl of PBS per tube) and combined into a single tube (i.e., level I + level II + and so on) at each predetermined voltage setting (see Step 2). Repeat for all detectors using the specific fluorochrome measured in each detector. **▲ CRITICAL** Freshly prepare for each experiment.

PROCEDURE

System optimization

1| To optimize the system, follow options A–F.

▲ CRITICAL STEP Upon installation, new instruments can be optimized using the following procedural options: check laser power and adjust the electronic pulse window (option A), determine photoelectron efficiency (option B), test filter transmission and reflection (option C), synchronize signals (option D), set the laser delay (option E) and electronic noise check (option F). These procedures should be followed in order when performed for the first time. Once initial optimization is complete, individual options can be performed whenever the optical path is altered (e.g., new lasers, filters, detectors).

(A) Checking laser power and electronic pulse window (e.g., window extension) ● TIMING 1–2 min per laser

- Hold the laser power meter at the head of the laser. Measure the laser power according to the manufacturer's instructions.

▲ CRITICAL STEP Set the electronic pulse window at a constant during acquisition. In order to minimize signal noise, this value must not be >5 µs.

- Record the result and measure each laser.

▲ CRITICAL STEP The meter must be adjusted to each laser wavelength before measuring.

- Repeat Step 1A(i) and Step 1A(ii) until 10–15 independent meter readings are obtained. The range should be within ± 1 s.d., for example, generally a 25-mW laser should be within 10% of the maximum power or $25 \text{ mW} \pm 2.5 \text{ mW}$.

▲ CRITICAL STEP Laser power on most diode-pumped solid-state lasers is fixed but should be monitored at least monthly.

? TROUBLESHOOTING

(B) Cascade test to determine photoelectron efficiency ● TIMING 15 min per detector series

- Adjust voltages on all detectors to 500 V.
- Remove all filters in the detector system.

▲ CRITICAL STEP This procedure describes the cascade test for a five-detector (PMT) series (A through E), in which the longer wavelengths are measured first (starting at detector A) and the shorter wavelengths are measured in the last part of the series (ending at detector E). The principles described here can be adapted for a series with more detectors.

- Move 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 to detector A.

- (iv) Run 1× Rainbow beads and measure the CV (s.d./mean) in detector A.
▲ CRITICAL STEP Use a minimum amount of 10,000 beads for 1× Rainbow beads and all other beads.
- (v) Move the test filter set from detector A to detector B. Replace the original filters in detector A.
- (vi) Run 1× Rainbow beads and measure the CV in detector B.
- (vii) Repeat Step1B(v) and Step1B(vi) for the entire detector series.
▲ CRITICAL STEP Some instruments do not have a dichroic filter in the last detector position. In this case, a super-reflective mirror (100% reflection filter) should be placed in the second-to-last detector. This mirror prevents signal loss in the last detector and allows measurement of bead CV in the last detector.
- (viii) By using the formula $1/(n)$ (ref. 2), where $n = CV/100$, calculate the statistical photoelectron estimate for each detector; this represents an estimate of the efficiency of photon detection. Typically, efficiencies can average 800 ± 200 photoelectrons (± 1 s.d.). The higher the photoelectron estimate, the better the detector efficiency. Values of <500 photoelectrons are not acceptable. Higher-efficiency detectors should be assigned to measure the far-red end of the spectrum, as low-energy fluorescence emissions require optimal sensitivity.
▲ CRITICAL STEP Repeat the cascade test if the optical path is altered.

? TROUBLESHOOTING

(C) Filter transmission and reflection ● TIMING 5 min per detector

- (i) Check each filter for correct location and correct labeling. All filters must be in their correct locations in the detector series. If possible, lot numbers should be matched with optical density curves and transmission curves to verify proper detection.
- (ii) Adjust the voltages on all detectors to 500 V.
▲ CRITICAL STEP This procedure describes a qualitative measurement; precise voltage selection is not necessary.
- (iii) Run 1× Rainbow beads and measure the median fluorescence intensity (MFI) of the signal in each detector of the series.
- (iv) If large differences between detectors are observed, or if signals are completely absent from detectors, examine the dichroic mirrors. In some cases, light may not be transmitted to a particular detector, whereas in other circumstances light may not be reflected to downstream detectors. The degree of fluorescence is not important; investigators should merely determine whether the signal is positive (above the first-decade log scale) or negative. Thus, there are three potential results for measurements between detectors (for example) A, B, C and D. First, when a large loss in signal is observed in detector B, but there is no change in detector A, C or D, filter transmission is faulty and the dichroic mirror in detector B must be replaced. Second, if there is no signal loss in either detectors A or B, but there is a large drop in MFI for each subsequent detector (C and D), filter reflection is faulty, and the filter in detector B must be replaced. Third, if a filter is replaced and the problems described above are not solved, the angle of the mirror is not correct and the manufacturer should be contacted to correct the problem (this is quite rare).
▲ CRITICAL STEP Repeat this procedure each time new filters are installed.

? TROUBLESHOOTING

(D) Signal synchronization (e.g., ASF, BD instruments only) ● TIMING 2 min

- (i) Adjust the voltages on all detectors to 500 V.
- (ii) Run 1× Rainbow beads and measure the median fluorescence intensity (MFI) of each detector in a series.
▲ CRITICAL STEP The ASF is dependent on the laser beam width; therefore, in some cases the use of cells or larger particles (as compared with smaller beads) is recommended when setting this parameter. Consult the manufacturer's guidelines before setting this instrument parameter.
- (iii) Collect both height and area signals on one detector from each laser and the forward scatter detector.
▲ CRITICAL STEP On some instruments, the forward scatter detector has a separate adjustment for signal synchronization that can be altered using the procedure described here.
- (iv) Adjust the ASF until the height and area signals have equal median values on a log-scale histogram. On one detector for each laser, the peak height MFI signal should be equivalent to the area MFI signal on either a log scale or a linear scale. If the height signal is greater than the area, then area measurements may occur with off-scale heights. However, if the height is less than the area, it is generally not an issue unless the difference is excessive ($>3\times$), in which case only a fraction of the full signal range will remain on scale. In this case, large-cell measurements are most affected.
▲ CRITICAL STEP Repeat this procedure when the optic path of the lasers is changed, for example, if the dichroic steering mirrors are altered or the lasers are replaced.

? TROUBLESHOOTING

(E) Laser delay settings ● TIMING 2 min

- (i) Adjust the voltages on all detectors to 500 V or use the tolerance ranges defined in the calibration procedure (Steps 2 and 3).
- (ii) Decrease the electronic window (e.g., window extension) or gate to the lowest setting, usually zero. This setting will ensure the quickest and most sensitive change in signal delay settings.

- (iii) Run 1× Rainbow beads and measure the MFI of each detector.
- (iv) While observing one detector from each laser, adjust the delay settings in increments of one unit, first up and then down, and observe the rise and fall of the MFI.
- (v) Select the delay setting that yields the highest MFI from a representative 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).
- (vi) Adjust the electronic window according to the manufacturer's recommended settings. Optimal settings will ensure the maximum fluorescence detection for each laser optimized. If the settings are outside of the optimal detection range, an overall loss of fluorescence will be observed in all detectors for a given laser.

▲ **CRITICAL STEP** Repeat this procedure daily to verify the peak signal location relative to the reference laser.

? TROUBLESHOOTING

(F) Electronic noise check ● TIMING 10 min

- (i) Adjust all gain (voltage) values to zero.
 - (ii) Run 1× Rainbow beads, collecting 20,000 events.
 - (iii) Calculate the 90th percentile of the fluorescence signal from each detector.
- ▲ **CRITICAL STEP** Call a service engineer if this value exceeds channel 25.

Calibration

2| Ensure that all filters are in the correct locations for the detector series, as outlined in Step 1C of the optimization procedures (5 min). Next, calibrate the instrument following options A–E as described below.

▲ **CRITICAL STEP** Perform this and subsequent steps only after system optimization procedures are completed. Optional steps in the system optimization procedures (**Table 2**) need not be completed before calibrating the instrument.

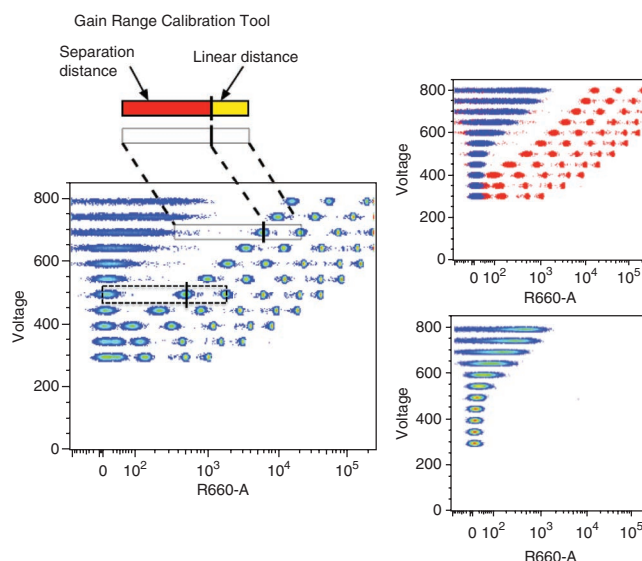
(A) Determination of PMT voltage range and linearity ● TIMING 60 min

- (i) Set voltages for all detectors to 350 V.
- (ii) Run Cyto-Cal beads (as prepared in Reagent Setup) in each detector. Gate on one of the top three brightest particles of the visible peaks, provided it remains on scale throughout the voltage scan in Step 2A(iii) and Step 2A(iv).

? TROUBLESHOOTING

- (iii) Increase the voltage by 50 V and repeat Step 2A(ii) until measurements for a voltage range between 350 and 800 V have been collected for each detector (**Fig. 2**).
- (iv) Repeat Step 2A(i–iii) using unstained COMP beads and measure the 90th percentile of each detector (defined as general background). The Cyto-Cal reagent will show the location of background, but in some detectors the negative beads may not resolve. In these cases, the unstained COMP beads can be useful to determine the correct degree of background.
- (v) By using the Gain Range Calibration Tool (**Fig. 2**), determine both the linearity and the initial gain range. The tool can be dragged across the voltages (from highest to lowest gain settings) to determine linearity and separation of positive peaks from the background. The correct location (gain or voltage) is where the linearity is unchanged (between two upper beads) and the separation distance between the negative bead and the first detectable positive bead is maximal. Note that COMP beads (**Fig. 2**, right) provide an independent indication of the background, which is distinct from the Cyto-Cal beads measured at the same gain setting. In addition, as the Gain Range Calibration Tool is moved from voltage to voltage, some settings show increased background. The gain setting at which we found that background was elevated in the R660

Figure 2 | Determining a gain (voltage) range using the Gain Range Calibration Tool. The Gain Range Calibration Tool can be created from a histogram showing the fluorescence signal of Cyto-Cal beads in one detector (e.g., R660) on one axis versus the range of voltages on the other axis. Select one of the upper voltages (i.e., 700 V scan), and draw two boxes (by using the appropriate tool from FlowJo or graphics software). The first box will delineate the separation distance (red), whereas the second box will be used to test linearity (yellow). The box used for separation should stretch from right above the negative population to the first separated bead. The box used for linearity should stretch from the first separated bead to the very next separated bead, as illustrated. On the right, negative beads are run separately and overlaid (lower right) or combined with the Cyto-Cal beads (upper right). In this example, the optimal voltage range for the instrument was 550–600 V.



(allophycocyanin (APC)) detector was 500 V (**Fig. 2**, left). Therefore, the gain range for the R660 detector that should be tested with the next bead set (QSCBs) would be 550 ± 25 V. In some cases, more than three gain measurements may be selected, but in general only three settings are necessary at 25 V-increments around the suspected 'ideal' setting.

▲ **CRITICAL STEP** When assessing background, it is important to use only one unstained bead type. The plastic material used in COMP beads has a higher intrinsic fluorescence when excited by violet lasers; therefore, unstained Cyto-Cal beads are necessary for this calibration routine.

▲ **CRITICAL STEP** Repeat when a new laser, detector or filter is installed.

▲ **CRITICAL STEP** Once linear PMT voltage ranges for each detector have been determined, move to Step 2B.

? TROUBLESHOOTING

(B) Validation of PMT voltages using QSCBs ● TIMING 30 min

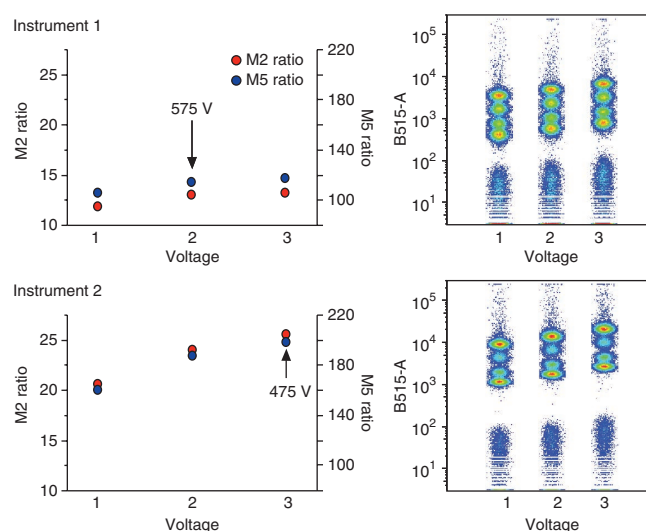
- Stain QSCBs with a mAb conjugate specific for each detector.
- Run these beads across the range of the gain settings defined in Step 2A in order to determine linear range of PMT voltage.
- Repeat Step 2B(ii) for all detectors.
- Once all detectors have been run, calculate the M2 ratio (dimmiest bead separation) and the M5 ratio (brightest bead separation). These ratios are defined as follows: M2 ratio = MFI of the lowest positive peak/90th percentile of the negative bead, and the M5 ratio = MFI of the highest positive peak/90th percentile of the fluorescence signal from the negative bead. **Figure 3** shows an example of the B515 detector and QSCBs stained with CD4-APC on two separate instruments. The top image depicts an example of the bead separation over three selected voltages (550, 575 and 600) of instrument 1, and the bottom image shows instrument 2 with selected voltages (425, 450 and 475). When examining the beads' separation in the histogram (right side), it is not obvious that any one voltage is ideal. However, when calculating the M2 and M5 ratios, we determined that the gain setting of 575 V was best for instrument 1 and 475 V for instrument 2 (arrow, left). Note that higher gain setting in instrument 1 does not provide increased separation, but separation is increased for instrument 2. The following rules have been developed for this analysis: a gain setting with the best M2 separation should be selected over a better M5 separation, and if there are no differences in ratios over three gain settings the middle gain setting is selected. These settings can be different from the Cyto-Cal beads (Step 2A) because of detector efficiency and spectral differences in the individually stained mAb-conjugated QSCBs.

? TROUBLESHOOTING

(C) Validation of PMT voltages using a biological control ● TIMING 30 min

- Set ideal gain settings as defined in Step 2A and Step 2B.
- Obtain a cell sample that contains known, well-defined populations of cells, and stain it with a panel of fluorescently tagged antibodies against those cell populations. For example, peripheral blood mononuclear cells from healthy donors contain high frequencies of CD3⁺, CD4⁺ and CD8⁺ T cells, CD14⁺ monocytes and CD20⁺ B cells; a panel of antibodies against these CD markers could be developed for this test. Alternatively, a cell sample and antibody panel routinely used in the laboratory could be used here. Prepare appropriate compensation controls using COMP beads.
- Collect and save at least 800,000 peripheral blood mononuclear cell counts in a separate file.
- Compare collected data with known biological tolerance ranges for a given sample. In some cases, cell populations can be compared with known published normal ranges or other reference instruments. As an example (**Fig. 4a**), CD8-PB versus CD4-ECD should show four distinct populations with percentages comparable to published normal ranges. In addition, it is important to examine the compensation plots for lot-to-lot mAb conjugate consistencies; we expect the MFI and the degree of spread into secondary detectors to be similar. Nevertheless, it is important to examine the compensation matrix values to check that spillover values are <100%. Although values of >100% do

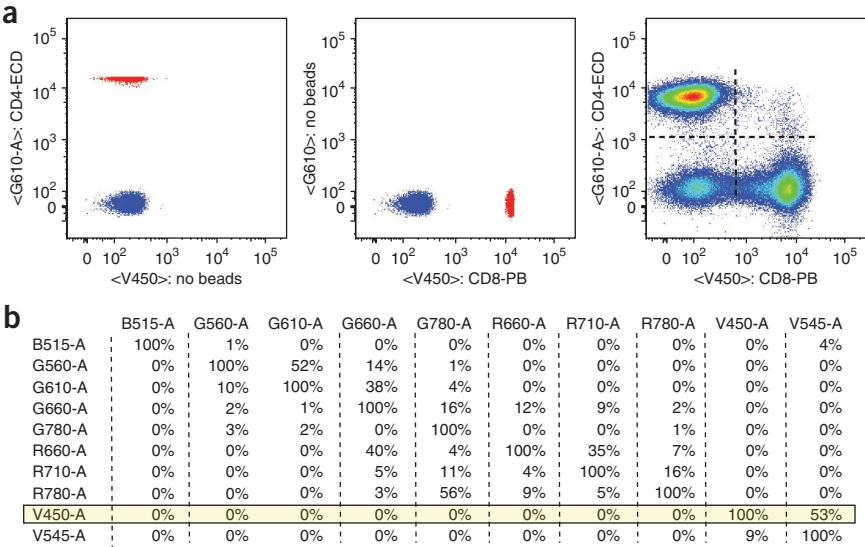
Figure 3 | Determining the optimal PMT voltage using QSCBs. This graphic illustrates how to select the final PMT voltage by measuring stained QSCBs. These are antibody-capture beads with four different intensities and a negative bead. In this example, we stained beads with CD3-FITC (right). Each population is named M1 through M5 (left), where M1 is defined as the negative bead population and M5 is the brightest bead population. The M2 ratio (dimmiest bead separation) and the M5 ratio (brightest bead separation) were calculated as follows: M2 ratio = MFI of the M2 bead/90th percentile of the M1 bead, M5 ratio = MFI of M3 bead/90th percentile of the M1 bead. If these ratios are unchanged over the voltage range, as in instrument 1 (top), then the middle voltage is selected (e.g., 575 V). However, if these ratios continue to increase, as in instrument 2 (bottom), then the highest voltage in the range (for that particular instrument/detector) is selected (e.g., 475 V).





PROTOCOL UPDATE

Figure 4 | Biological validation and COMP bead matrix. (a) To validate the calibration process, we stained COMP beads (left and middle images) and peripheral blood mononuclear cells (PBMCs; right image) with a well-characterized panel of mAb conjugates and reviewed them for correct staining patterns and lineage population percentages. (b) After we applied compensation, we examined the COMP bead matrix to ensure that spillover percentages were consistent with expectations. Thus, fluorochrome pairs with great spectral overlap should have higher spillover percentages than pairs that are spectrally distinct. Here, for example, CD8-PB (V450 detector) requires little compensation with CD4-ECD (G610 detector), as expected on the basis of their distinct fluorescence spectra. PB has a much greater spectral overlap with Aqua-Blue; thus, the spillover is high (53%). Where spillover values are >100%, the optimal PMT voltage is not set properly, as this indicates that the signal from a particular fluorochrome is not being detected primarily in the detector set for it. PB, Pacific Blue.



not present compensation problems in and of themselves, such high values indicate that the fluorescence signal from a given dye is not being detected optimally in the primary detector. Higher signals in secondary detectors cause confusion in experimental troubleshooting and can increase spreading error (thereby decreasing the effective dynamic range of the detector). For example (Fig. 4b), CD8-PB-stained COMP beads show no spillover in the G610 detector and 53% spillover into the V545 detector. Note that each entry in Figure 4b in a given row or column denotes the specific spillover (percent) from that row's fluorochrome into the column's detector. Thus, each row specifies how much a detector's primary reagent spills over into the other detector. In some cases in which a secondary detector is >100% relative to the primary detector, the voltage of the secondary detector can be lowered to within 25 V as long as the QSCB ratios remain the same as the selected 'ideal' voltage of the secondary detector. In the event the secondary detector's ideal voltage cannot be altered (i.e., highest ratios are only at this voltage setting), the primary detector voltage can be lowered no more than 25 V as long as the QSCB ratios remain the same as the selected ideal voltage of the primary detector. If the problem is still not solved, a new detector may be needed or a replacement filter may be required.

? TROUBLESHOOTING

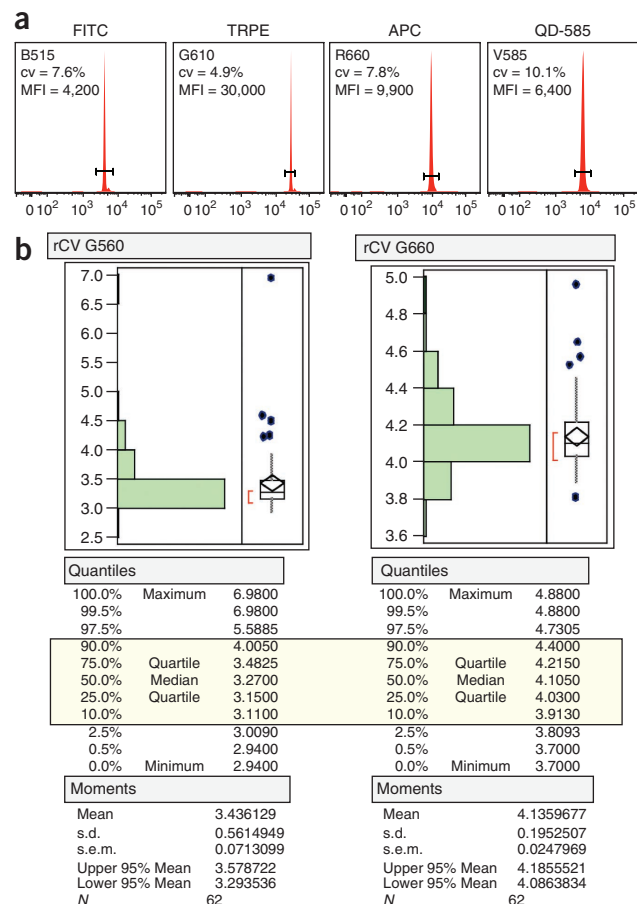
Standardization ● TIMING ~30 min

- Set the gain settings for each detector on the basis of the calibration routine (Step 2).
- Run the 1× Rainbow beads.
- By using the tolerance target range (Step 2A), adjust the voltages on each PMT until the MFI is within this range. Use these voltages with every experiment run on the same day.
- Collect and save at least 10,000 counts in a separate file.
- Run 40 µl of unstained COMP beads, and then resuspend them in 300 µl of buffer.

? TROUBLESHOOTING

- Collect and save at least 10,000 counts in a separate file.
- ▲ CRITICAL STEP** Before adjusting the PMT voltages, check the laser delay setting as defined in the System optimization section (Step 1E).
- Repeat Steps 4–8 until at least 20 measurements have been made using 1× Rainbow beads. The measurements should be made at the voltages established in the calibration routine, in order to define relevant tolerance target ranges for the robust CV (rCV) and MFI for each detector. In daily use, voltages can be adjusted slightly to remain within the tolerance target range of MFI. Figure 5a shows an example histogram of four detectors used in a single collection; the values represent the rCV and the MFI. Once at least 20 measurements have been collected, a tolerance range can be defined.

Figure 5 | Tolerance ranges for 1× Rainbow beads over time. (a) CV for 1× Rainbow bead measurement in various detectors. (b) Measurements of 1× Rainbow beads over time ($n = 62$ d). The 10th and 90th percentile values for all daily 1× Rainbow bead data can be calculated for the G560 and G660 detectors (3.1–4% and 3.9–4.4%, respectively). TRPE, Texas Red PE.



We set this to be between the 10th and the 90th percentiles. **Figure 5b** shows the tolerance range of rCV in the G560 and G660 detectors (after 62 measurements).

10 | Once the tolerance ranges are determined, collect data and use it to determine whether the instrument is currently operating properly (real-time tolerance criteria) or whether there is a possibility of hardware failure in the future (prognostic tolerance criteria). Real-time tolerance criteria consist of voltage and rCV readings. When voltages must be changed by >5% to remain within the tolerance target values for MFI (established in the calibration procedure), the instrument should not be used until it is serviced. The same is true for rCV values >10%. Prognostic tolerance criteria define the instrument's performance over time, and can warn of impending hardware problems. The factors tracked give an indication of accuracy, precision and sensitivity. Resolution can be tracked by graphing rCV (1× Rainbow beads) as a function of time (**Fig. 6**) within a defined MFI range. The variation should not exceed ± 2 s.d. or 5% from the mean ($n = 20$), but these tolerances should be re-evaluated after collecting more data ($n > 60$). In this example, the rCVs above the red line indicate that the detector is associated with poor resolution (**Fig. 6**). Precision or reproducibility is the degree of voltage spread to achieve the MFI tolerance range of a single detector. In the present example, voltage variation should not exceed ± 2 s.d. or 5% from the mean ($n = 20$) to be considered reproducible. Note that detectors can be reproducible but lack resolution; hence, resolution is the most important parameter for validation (**Fig. 6**). Sensitivity can be tested, for a sample with negative and positive populations (or peaks), by examining background signal present over time, while the same MFI tolerance range is maintained. The measurement of negative populations also gives an indication of optical noise in each detector. The lower right image in **Figure 6** shows how background varies no more than ± 2 s.d. or 5% from the mean ($n = 20$) over the many days that the MFI is held within the tolerance target range. Occasional outliers (points outside of the red line) should be investigated or repeated. All instruments show similar total background, ± 2 s.d. or 5% from the mean ($n = 20$). It can be informative to plot these data for all instruments in a facility, as shown in **Figure 7**.

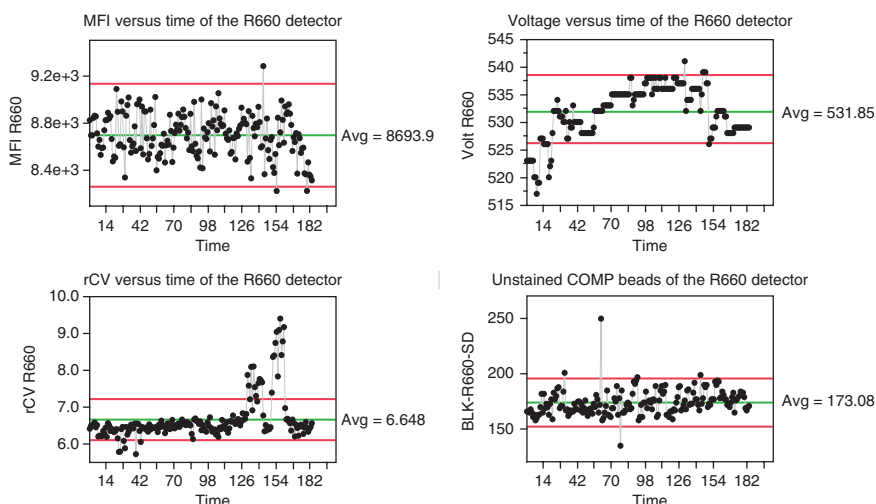


Figure 6 | Monitoring of the R660 detector over time across five instruments. Monitoring voltage as a function of time increases the likelihood that median fluorescence intensity (within the tolerance target range) will be stable across experiments, from day to day. In this example, all voltages are within acceptable variance. Slight variation in the microfluidic environment of the flow cell reveals an increase in the rCV at and around time point 154. After correction of the steering mirrors, the rCV reduced to the normal tolerance range. All other parameters measured were not affected and were within normal tolerance ranges for that same time period. We found that the single spike in the unstained COMP beads was due to a contaminated vial of beads, either from APC or Cy5PE conjugates.

PROTOCOL UPDATE

Figure 7 | Intra-instrument quality control data for the G560 detector. Voltages required to maintain the same degree of MFI are very different within each instrument. **(a)** In addition, the voltage settings for the LSR-E instrument appear to be split into high and low values (blue dots, upper right), yet the MFI values remain consistent. **(b)** These values did not track with high or low rCV values, thus indicating a defective PMT. In another example, LSR-A showed some out-of-range high rCV values (a, green dots, lower left). **(c)** A closer evaluation showed that these values were found early in the testing period, indicating that a change in dichroic mirror alignment was required to bring these measurements into the proper tolerance range. Error bars are placed 1.5× the interquartile data range.

▲ CRITICAL STEP Plotting these data for all instruments in a facility (**Fig. 7**) shows how voltage and MFI values can vary markedly from instrument to instrument, even when resolution is standardized across instruments with this (or similar) quality assurance routines. This arises from the substantial differences between PMTs in their ability to detect the same number of photons. In addition, this analysis can identify instruments/detectors with poor resolution. LSR instrument E (LSR-E), shown in the blue dots (upper right, **Fig. 7a**), maintained consistent MFI but required marked changes in G560 (PE) detector voltages to do this. **Figure 7b** shows that the higher voltage values were coincident with higher rCV values (a marker of inconsistent resolution). Once the PE detector was replaced, rCV returned to consistently low values (<4%). In another case, LSR-A showed rCV values outside the tolerance range (green dots, lower left, **Fig. 7a**), and this was solved (purple dots, **Fig. 7c**) by realignment of the dichroic mirror with the laser light path. In fact, these mirrors are often the source of out-of-tolerance rCV, and thus they should be checked first when problems are identified. Generally, disturbances in rCV occur before problems with MFI; thus, the former is a more sensitive marker of instrument performance.

TROUBLESHOOTING

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

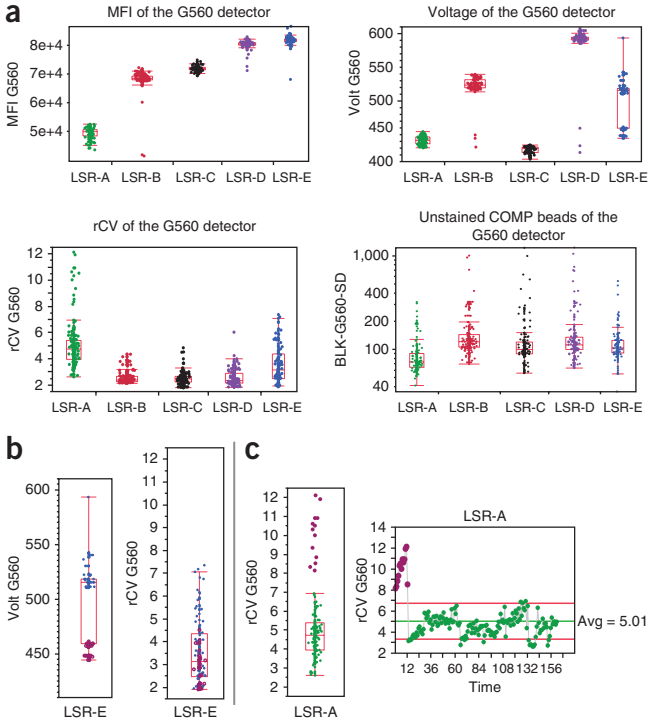


TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A–E	MFI signals in all detectors of one laser are reduced	Laser power is lost; incorrect laser delay setting	Check laser power and adjust timing delay
		Narrow timing window set	Check and set electronic window
		Peak and area signals are not synchronized	Check and set proper signal synchronization
1B(viii)	PMT detector sensitivity is found to be below 500 photons	Faulty detector or poor alignment	Replace PMT or adjust PMT alignment
		Dirty optics reducing light transmission and/or reflection	Inspect and clean the filter
1B, 1C	MFI signals appear dim in the far-red detectors of a single laser source	Low-sensitivity PMTs are used in these parameters	Replace the PMT with far-red sensitive detectors (defined as a PMT with a high statistical photo-electron estimate value)

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	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 the correct position
1C	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
		Incorrect band-pass filter	Replace the dichroic mirror or contact the manufacturer
2A(ii)	Cyto-Cal beads show no separation for any scanned voltage used	Detector filter issue, incorrect voltage selected or faulty PMT	Correct the filter position; determine the correct voltage for the scan used; replace the PMT or electronics
2A(v)	The gain range tool shows that the PMT linearity is out of expected range	PMT is not linear	Replace the PMT
2B(iv)	Stained QCSBs or COMP beads are negative for specific fluorochrome-conjugate tested	The mAb used is not compatible with the bead (anti- κ mAb will not bind to a λ -mAb or anti-mouse mAb will not bind to rat antibodies); PMT is faulty	Use the correct mAb conjugate for beads to capture the mAb. If this fails, check the PMT and replace it if necessary
2C(iv)	Fluorochrome-conjugate-labeled COMP beads do not show the highest MFI value compared with secondary detectors	Voltage on the primary detector is too low or the secondary detector voltage is too high	Increase the voltage on the primary detector to within the expected range. Alternatively, decrease the voltage of the secondary detector to within the expected range. (See 'Calibration', Step 2)
7	Unstained COMP beads are positive in one detector; there is a decreasing signal-to-background ratio with consistent or no change on voltage (e.g., decrease in sensitivity)	Contaminated COMP beads stained with mAb-conjugated. Increasing background resulting in poor resolution on dim cell markers	Use a new vial of COMP beads and repeat. Examine filters for possible light contamination from incorrect or faulty filters. Change band-pass filters to block out the Raman scatter effect ⁴ . Replace the PMT
	COMP beads light scatter gate have a low percentage (<60%)	COMP beads are aggregated	Sonicate the COMP beads for 5 min, or use new beads
10	Robust CVs on the 1 $\times$ Rainbow beads are greater for all detection in a laser series (e.g., decrease in resolution)	Dichroic steering mirror is out of alignment; low or altered laser power	Adjust steering mirror for the laser line until the robust CVs are as low as possible
	An increasing PMT voltage trend as a function of time, either in a single detector or in multiple detectors. Robust CVs on the 1 $\times$ Rainbow beads are greater in one detection series (one laser) as compared with another series (e.g., decrease in precision)	Faulty PMT detector	Replace the PMT or detector alignment
		Dichroic steering mirror is out of alignment	Adjust the dichroic steering mirror
		Low laser power	Replace the laser
		Dirty filter in the optical path	Inspect and clean the filter

(continued)

PROTOCOL UPDATE

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Dichroic steering mirror is out of alignment	Adjust the steering mirror for that laser line until the rCVs are as low as possible
	A decreasing PMT voltage trend as a function of time, either in a single detector or in multiple detectors (e.g., decrease in resolution). An increasing PMT voltage trend as a function of time, either in a single detector or in multiple detectors	Flow stream alignment or pressure issue	Clean the flow cell and check the pressure system
		Faulty PMT detector	Replace the PMT or detector alignment
		Steering mirror is out of alignment	Adjust the dichroic steering mirror
		Low laser power	Replace the laser
		Dirty filter in the optical path	Inspect and clean the filter
	A decreasing signal to background ratio with consistent or no change on voltage (e.g., decrease in sensitivity). A decreasing PMT voltage trend as a function of time, either in a single detector or in multiple detectors (e.g., decrease in resolution)	Increasing background resulting in poor resolution on dim cell markers; flow stream alignment or pressure issue	Examine the filters for possible light contamination due to incorrect or faulty filters. Change the band-pass filters to block out Raman scatter effect ⁴ . Replace the PMT. Clean the flow cell and check the pressure

● TIMING

- Step 1A, checking laser power and electronic pulse window: 1–2 min per laser
- Step 1B, cascade test to determine photoelectron efficiency: 15 min per detector series
- Step 1C, filter transmission and reflection: 5 min per detector
- Step 1D, signal synchronization: 2 min
- Step 1E, laser delay settings: 2 min
- Step 1F, electronic noise check: 10 min
- Step 2A, determination of PMT voltage range and linearity: 60 min
- Step 2B, validation of PMT voltages using QSCBs: 30 min
- Step 2C, validation of PMT voltages using a biological control: 30 min
- Steps 3–10, standardization: ~30 min

ANTICIPATED RESULTS

This quality assurance protocol generates data that can be monitored to ensure that photons are optimally detected, and that results are reproducible. Data collected over weeks or months can be used to establish tolerance ranges, which can then be used to define when the instrument is out of specification. When signals fall outside of the tolerance ranges established, experimental results may become inconsistent and design of new PFC staining panels will be difficult. Therefore, it is strongly recommended to check that values are within tolerance ranges before every experiment. This standardizes the instrument in real time to assure users that data were collected under optimal instrument conditions. Voltages obtained during the initial calibration procedure are used throughout the daily experimental runs. If changes occur in the optical path during the lifetime of an instrument (new laser installation, new filter configuration and so on), 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, we highly recommended maintaining an active preventive maintenance program.

In the future, these procedures will also standardize instrument-to-instrument variations (i.e., inter-instrument quality assurance) in order to compare detector differences and ultimately to predict panel success on the basis of an instrument's resolution and sensitivity. Such efforts will provide a better basis for standardizing and comparing results across multicenter clinical trials. In this regard, systems that relate the MFI of a flow cytometer's signal to the number of fluorochrome molecules are being developed. In addition, software companies are developing faster and more efficient tools to take advantage of new bead reagents. Ultimately, systems will be developed to calculate a Q value that defines the number of photoelectrons generated per molecule of excited fluorochrome. This value, which is currently largely theoretical or estimated, defines the resolution of a detection system. With better measures of Q , each detector can be standardized against a reference material and voltages can be adjusted on a PMT to yield an ideal target value. Similarly, instrument sensitivity is reflected by the level of optical background, known as the B value¹³. Once Q and B can be accurately measured, instrument-to-instrument variation and standardization could become more efficient¹¹. However, until such standards are available for all of the detectors used in PFC experiments, we must rely on relative units of fluorescence used in this protocol, without inclusion of precise Q and B values.

In conclusion, this document updates our earlier protocol⁸ describing a systematic approach to standardizing flow cytometry instrumentation and ultimately PFC experiments. It focuses on updated methodology for performing optimization, calibration and standardization that is possible because of the availability of new beads. It is unique for its integration of optimization, calibration and standardization into a single program, and in its applicability to any panel of fluorescent antibodies an investigator plans to use (other methods must be rerun for each staining panel). Although some of the procedures described are straightforward, some require careful consideration to perform, analyze and interpret. Notably, these procedures are evolving with the hope of simplifying the process and to providing useful quality control tools for future instrumentation.

AUTHOR CONTRIBUTIONS S.P.P. conceived and defined experiments, analyzed data and wrote the manuscript. D.A. and R.N. performed experiments and analyzed data. P.K.C. and M.R. designed experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Published online at <http://www.nature.com/doifinder/10.1038/nprot.2012.126>. Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

1. Bendall, S. *et al.* A deep profiler's guide to cytometry. *Trends Immunol.* **33**, 323–332 (2012).
2. Chattopadhyay, P.K. *et al.* Cytometry: today's technology and tomorrow's horizons. *Methods* **57**, 251–258 (2012).
3. Chattopadhyay, P.K. *et al.* Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat. Med.* **12**, 972–977 (2006).
4. Le Meur, N. *et al.* Data quality assessment of ungated flow cytometry data in high throughput experiments. *Cytometry A* **71**, 393–403 (2007).
5. Harvath, L. Quality control in clinical flow cytometry. *Pathol. Immunopathol. Res.* **7**, 338–344 (1988).
6. Horan, P.K., Muirhead, K.A. & Slezak, S.E. Standards and controls in flow cytometry. in *Flow Cytometry and Sorting*, 2nd edn. (eds. Melamed, M.R., Lindmo, T. & Mendelsohn M.L.) 397–414 (Wiley-Liss, 1990).
7. Shapiro, H.M. *Practical Flow Cytometry* (Wiley-Liss, 2003).
8. Perfetto, S.P., Ambrozak, D., Nguyen, R., Chattopadhyay, P. & Roederer, M. Quality assurance for polychromatic flow cytometry. *Nat. Protoc.* **1**, 1522–1530 (2006).
9. Schwartz, A., Marti, G.E., Poon, R., Gratama, J.W. & Fernandez-Repollet, E. Standardizing flow cytometry: a classification system of fluorescence standards used for flow cytometry. *Cytometry* **33**, 106–114 (1998).
10. Schwartz, A., Fernandez Repollet, E., Vogt, R. & Gratama, J.W. Standardizing flow cytometry: construction of a standardized fluorescence calibration plot using matching spectral calibrators. *Cytometry* **26**, 22–31 (1996).
11. Schwartz, A. *et al.* Formalization of the MESF unit of fluorescence intensity. *Cytometry B Clin. Cytom.* **57**, 1–6 (2004).
12. Zenger, V.E., Vogt, R., Mandy, F., Schwartz, A. & Marti, G.E. Quantitative flow cytometry: inter-laboratory variation. *Cytometry* **33**, 138–145 (1998).
13. Chase, E.S. & Hoffman, R.A. Resolution of dimly fluorescent particles: a practical measure of fluorescence sensitivity. *Cytometry* **33**, 267–279 (1998).