Setting up and calibration of a flow cytometer for multicolor immunophenotyping

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(Purpose)

To obtain or maintain:
1. an optimal positioning of the window of analysis in sample space for all routinely used parameters;
2. an appropriate correction for spectral overlap between the fluorochromes used;
3. documentation of the performance of the instrument for fluorescence (FL) measurements.

(Background)

Introduction
A good quality control program for flow cytometry is designed to assess the major instrument parameters that affect the reliability and reproducibility of data and consists of 3 groups of procedures. The basis for the procedures described here consists of the concepts and pioneering work of Dr. Abe Schwartz and colleagues in this field (1, 2).

• The first group of procedures is carried out at relatively large intervals (e.g., once every 6 months) by qualified service personnel and includes examination of the efficiency and performance of the laser tube, optical filters, log and linear amplifiers, and photomultiplier tubes (PMT) of all types of flow cytometers. These procedures also include calibration of the optical alignment of flow cytometers equipped with a cuvette to assure that the brightest and tightest peaks are produced in all parameters. Most clinically used flow cytometers are of the “stream-in-cuvette” type. However, the optical alignment of the larger flow cytometers with sorting facilities, which are equipped with a nozzle (“stream-in-air” type), must be calibrated daily by the instrument operators due to its relative instability.

• The second group of procedures consists of frequent (i.e., with each “cold start” of the instrument) monitoring of instrument performance by the operators to identify both immediate and potential problems. After having establishing the proper instrument settings (i.e., instrument set-up), the mean channels for important parameters using suitable reference microbeads (see below) are recorded and the results of the day are then compared with the established tolerance limits. Trends and variations can be noted with the use of Levey-Jennings style charts that allow visual inspection of longitudinal data for monitoring instrument performance for precision and trends. If any value falls outside the tolerance limits, monitoring should be repeated and instrument settings adjusted if problems persist.

• The third group of procedures is performed at larger intervals (based on our experience only necessary when the FL PMT settings of the instrument have to be adjusted). Here the fluorescence (FL) channels are calibrated for FL intensity measurements and the minimum sensitivity of each FL channel is documented. Since FL intensity is used to distinguish “positive” from “negative” cells in immunopheno-typing assays, it is important to assure reproducibility and stability of FL intensity assessments over time. These controls are performed by calibrating the flow cytometer using a series of microbeads with at least 4 different pre-defined FL intensities, plus nonfluorescent beads. The linear regression equation between the pre-defined FL values of the fluorescent micro-beads and the instrument’s response in linear histogram channel values is computed. This algorithm yields per FL channel 4 instrument performance parameters: average residual percent, detection threshold, coefficient of response and zero channel value.

Positioning of the “window of analysis” in “sample space”

Dependent on the application, the amplifier gain or PMT settings for each parameter have to be adjusted such that the routine specimens for that application appear in the range of histogram channels of these parameters (i.e., the “window of analysis”). Changing the amplifier gain or PMT settings re-positions this window in “sample space” (i.e., the FL and light scatter characteristics of the sample which are indepen-
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dent of the instrument). For positioning of the FL windows of analysis, we recommend that, for each type of assay, unstained cells are placed in the first of 4 log decades, slightly away from the axes. With those settings, instrument noise (to be visualized with nonfluorescent beads), which typically generates signals of lower FL intensity than unstained cells due to the autofluorescence of the latter, will be partly on scale. This approach has the following advantages:

(i) The observation that nonfluorescent beads fall in a lower range of channels than unstained cells assures that the instrument’s noise does not interfere with the result of the immunophenotyping assay;

(ii) The positioning of single-stained and unstained cells slightly away from the axes of the bivariate FL allows appropriate adjustment of electronic compensation for spectral overlap.

Setting electronic compensation for spectral overlap (“color compensation”)

Spectral overlap of fluorochromes must be corrected to ensure accurate quantitation of cells labeled with multiple fluorochromes. Accurate compensation is accomplished by staining a control blood sample containing mutually exclusive populations of the same fluorochrome as used in the experimental samples, and by processing the control sample in an identical way to the experimental samples. In this respect we consider the use of blank and externally labeled beads inferior to the use of cells, because the spectral characteristics of fluorochromes directly bound to beads are slightly different from those of fluorochromes bound to antibodies. In addition, compensating the FL signal of labeled beads relative to that of blank beads results in slight overcompensation, because blank beads have dimmer FL signals than unstained cells due to the autofluorescence of the latter. The spectral characteristics of most fluorochromes (i.e., FITC, PE, PerCP, APC), when conjugated to different mAb, will be similar. Exception to this rule are the tandem fluorochromes; PE-Cy5 is an important case in point. The chemical composition of PE-Cy5 (i.e., the number of Cy5 molecules attached to the central PE molecule) often differs between manufacturers, between different mAb of the same manufacturer, and even for a single mAb dependent on storage conditions and time. Therefore, compensation settings defined for a given PE-Cy5-labeled mAb must always be verified for other PE-Cy5 mAb. The use of multiple PE-Cy5 mAb for an application may result in a multitude of required compensation settings for that application.

The electronic correction of spectral overlap is a function of the PMT settings involved. Therefore, each time the settings of a FL PMT are changed, compensation settings must be verified and corrected. In contrast, changing FSC photodiode or SSC PMT settings does not affect compensation.

Calibration of the instrument’s response to FL signals

Since fluorescence intensity is used to distinguish “positive” from “negative” cells in immunophenotyping assays, it is important to assure reproducibility and stability of FL intensity assessments over time. This is done by calibrating the flow cytometer using a series of beads with at least 4 different predefined levels of FL intensity, including nonfluorescent beads. The linear regression equation between the predefined FL intensity values of the fluorescent microbeads and the instrument’s response in histogram channel values is then computed. This statistic yields for each FL parameter 4 instrument performance parameters: average residual percent, detection threshold, coefficient of response and zero channel value (Tab. I).

Maintenance of appropriate instrument settings over time

Upon each “cold start” of the instrument, appropriate application-specific instrument settings must be (re-)established. Two different approaches exist in this respect:

Strategy 1: re-establish identical target channels

According to this strategy, daily adjustment of PMT settings of all FL parameters is performed to maintain a constant positioning of the window of analysis in sample space. For each FL parameter, reference beads are placed in identical target channels between runs. The daily adjustment of PMT settings implies that compensation settings must also be adjusted daily. The major instrument manufacturers have developed reagents and automated software for this purpose (i.e., calibrite beads with FACScomp software by Becton Dickinson Biosciences [BD Biosciences], San Jose, CA; Flow-Set beads with Cyto-Comp or ADC software by Beckman-Coulter [BC], Miami, FL).

Strategy 2: meeting “tolerance zones”

The appropriate positioning of the FL windows of analysis in sample space can also be monitored by checking, using reference beads, whether or not measurement of reference beads after each “cold start” fall within predefined “tolerance zones”. For this purpose, the mean FL channels of the reference beads are plotted in Levey-Jennings charts. Adjustment of PMT settings and color compensation is only necessary if beads repeatedly fall outside these tolerance zones, e.g., according to Westgard’s rules (3).

Comparison of both strategies

Both approaches (i.e., meeting the same FL target channels, and meeting the same FL channel tolerance zones over time) assure that data collected over long periods of time will be meaningfully comparable,
even if the instrument’s performance has been variable during that period, because the instrument’s responses to FL signals have been adjusted to perform relative to an independent benchmark. The advantage of strategy 1: meeting the same FL target channel over time, is its user-friendliness thanks to the use of instrument-specific software. However, without this software the approach would have been very labor-intensive. Therefore, we recommend strict adherence to the manufacturer-defined procedures when choosing this method.

The advantages of strategy 2: meeting the same FL tolerance zones over time, are: (i) its universal applicability (e.g., the BDB calibrate/FACS-comp system does not accommodate the use of PE-Cy5 or 7-AAD); (ii) labor saving if multiple application-specific instrument settings have been checked at the same time (e.g., those needed for the use of multiple PE-Cy5 conjugates, or those required for “lyse-no-wash” and “lyse-and-wash” sample preparation methods): if one of these settings is found to be appropriate, it implies that the remaining settings are also adequate at that time point. These guidelines describe in detail the approach of maintaining appropriate positioning of instrument setup by having reference FL beads meet the same FL channel tolerance zones over time.

Choosing the appropriate beads for (monitoring of) instrument set-up and calibration of FL channels

Overview of beads
Reference 2 provides a detailed discussion of a useful classification system for calibration beads, developed

<table>
<thead>
<tr>
<th>TABLE I - INSTRUMENT PERFORMANCE PARAMETERS FOR QUANTITATIVE ASSESSMENTS OF FLUORESCENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average residual percent</strong></td>
</tr>
<tr>
<td><strong>Definition</strong> The absolute average percent that the calibration regression line between a series of calibration beads labeled with different FL signals of predefined intensities, and the instrument’s response (in histogram channels) deviates from the actual data points</td>
</tr>
<tr>
<td><strong>Significance</strong> Indicates:</td>
</tr>
<tr>
<td>• the linearity or goodness of fit of the calibration line</td>
</tr>
<tr>
<td>• the confidence of the instrument’s response across the FL intensity range</td>
</tr>
<tr>
<td><strong>Tolerance zone</strong> (arbitrary) &lt;5%</td>
</tr>
<tr>
<td><strong>Detection threshold</strong></td>
</tr>
<tr>
<td><strong>Definition</strong> Value, in arbitrary units of FL intensity, of the histogram channel at the right-hand foot of the peak of nonfluorescent standards</td>
</tr>
<tr>
<td><strong>Significance</strong> Indicates:</td>
</tr>
<tr>
<td>• the lowest level of fluorochrome that can be detected by the instrument</td>
</tr>
<tr>
<td><strong>Tolerance zone</strong> To be defined by user</td>
</tr>
<tr>
<td><strong>Coefficient of response</strong></td>
</tr>
<tr>
<td><strong>Definition</strong> The slope of the calibration regression line (see under “Average Residual Percent”) calculated using a linear scale of 256 histogram channels. Indicates a theoretical value for the number of histogram channels per decade of log amplification (e.g., 4-log decade, 256/4 = 64.0; 3-log decade, 256/3 = 85.3)</td>
</tr>
<tr>
<td><strong>Significance</strong> Indicates:</td>
</tr>
<tr>
<td>• range of sample space covered by the window of analysis</td>
</tr>
<tr>
<td>• deviation from the theoretical value indicates suboptimal instrument performance</td>
</tr>
<tr>
<td><strong>Tolerance zone</strong> (arbitrary) 59 - 69 (for 4 decades log amplification)</td>
</tr>
<tr>
<td><strong>AUF value of histogram channel 0</strong></td>
</tr>
<tr>
<td><strong>Definition</strong> Intercept of the calibration line with the axis on which units of FL intensity have been plotted</td>
</tr>
<tr>
<td><strong>Significance</strong> Indicates:</td>
</tr>
<tr>
<td>• theoretical number of units of FL intensity corresponding with histogram channel 0; hence, it defines the left-hand boundary of the FL window of analysis</td>
</tr>
<tr>
<td><strong>Tolerance zone</strong> To be defined by user</td>
</tr>
</tbody>
</table>
by Schwartz et al, to which we refer in this and the following sections.

For instrument set-up and monitoring of the position of the FL window of analysis in sample space, we recommend the use of a single bead with FL intensity around the middle of the range of intensities routinely monitored. Such a bead should have high stability of FL signals in order to prevent drift of the position of the FL window of analysis over time, due to decay of the fluorochrome on the bead. The Type II A beads (internally or “hard-dyed”) meet this criterion, but Type II B beads (externally labeled with the same fluorochromes as used for immunophenotyping) do not (see further below).

For monitoring instrument noise in each FL channel, a nonfluorescent bead (Type 0, a.k.a. a “zero fluorescence” bead) is used. The combined results of the Type 0 and IIA beads is a parameter to monitor the instrument’s sensitivity for FL measurements.

For a more elaborate calibration of the instrument’s response to FL signals, Type III or calibration beads are used. These consist of a series of beads, each with different predefined FL intensities. At a minimum, such calibration kit consists of 4 fluorescent beads and 1 nonfluorescent bead. The FL intensities of these beads should cover that part of the FL scale in which a linear response of the instrument to FL signals can be expected, i.e., the 2nd, 3rd and the left-hand half of the 4th log decade. Again, high stability of the beads’ FL signals is preferred in order to guarantee consistent and meaningfully comparable data over time. Importantly, the assignment of FL intensity values to these beads must be consistent between batches in order to avoid conflicting results.

When is spectral matching between beads and experimental samples needed?

“Spectral matching” means that the fluorochromes used for internal staining have the same spectral characteristics to those routinely used for immunophenotyping. In practice, this is achieved by externally labeling beads with e.g. FITC, PE, PE-Cy5 or APC. Type A beads are very stable (at least 1 year after opening of the vial), relatively cheap but are not spectrally matched. Type B beads are less stable (3-6 months after opening of the vial), relatively expensive but are spectrally matched with routinely used fluorochromes. EWGCCA has balanced the advantages and disadvantages in practice, and has chosen to recommend Type A beads in these guidelines.

The advantages of spectral matching between reference or calibration beads and experimental samples are the following:

(i) In the setting of studies involving multiple instruments, spectral matching enables optimal standardization of results between instruments. Spectral matching allows correction for differences in the optical system between the instruments, due to which each instrument sees the routinely used fluorochromes slightly different than the others do.

(ii) Based on (i), spectral matching allows the expression of FL intensity in instrument-independent units. The unit of measure for FL intensity is MESF (Molecules of Equivalent Soluble Fluorochrome). The number of MESF units on a given calibration bead is defined as the equivalent number of free fluorochrome molecules in solution, assessed spectrophotometrically, that have the same emission intensity as that calibration bead. For quantitation of intensity measurements, any fluorochrome requires a set of calibration beads labeled with that particular fluorochrome.

Calibration of the FL scale using beads that are not spectrally matched implies that any quantitation of FL intensity is done in instrument-dependent units, which are in fact equivalent to histogram channel numbers. This situation precludes meaningful comparison of quantitative results of FL intensity measurements between instruments, but still allows to compare the results within each instrument over time, as long as the optical characteristics remain unchanged (i.e., no change of filter and mirror sets).

Reasons for recommending Type A rather than Type B beads

Our experience over the past 10 years is:

(i) For standardization of clinical cell analysis at (inter)national levels, small differences between instruments in positioning the window of analysis in sample space (e.g., up to 50 channels on a linear scale of 1,024 histogram channel scale) does not impact on the standardization of results between centers in terms of the end product, e.g., a CD34+ cell or lymphocyte subset count, a leukemia/lymphoma immunophenotype, or even a HLA-B27 typing result.

(ii) We have monitored our instruments, using the “tolerance zone” method with Type IIB beads over a 1-year time period, and ran Type IIA beads in parallel. We observed a downward trend in FL channel numbers obtained with the Type IIB beads, whereas the channel numbers obtained with the Type IIA beads remained stable. Eventually, the decay of the Type IIB beads yielded “out-of-range” results, which would have required re-positioning of the FL windows of analysis and adjustment of color compensation, whilst a comprehensive check of the instrument’s performance using Type IIIA beads yielded no “out-of-range” results. In conclusion:

(a) the Type IIA bead results suggested that the positioning of the FL windows of analysis were still “within specs”;

(b) we had no evidence that instrument performance had deteriorated;

(c) adapting of the positioning of the FL windows of analysis based on the Type IIB bead results was inappropriate.
In conclusion, these arguments do not warrant to recommend the more expensive Type B beads for routine purposes of instrument set-up, monitoring and calibration and, therefore, we recommend the use of Type A beads for these purposes.

EQUIPMENT and MATERIALS

1. Becton Dickinson Biosciences (BD Biosciences, San Jose, CA, USA) FACS analyzer or Beckman-Coulter (Miami, FL, USA Flow Cytometer
2. Adjustable pipettes
3. Disposable pipette tips
4. Disposable polypropylene tubes
5. Table centrifuge (500 x g)
7. Vortex mixer

REAGENTS

1. Type 0 (blank) bead: Spherotech BCP-60-1 (6.0 - 6.4 µm);
2. Type IIA (reference) bead: Spherotech RFP-60-1 (6.0 - 6.4 µm); for Beckman-Coulter flow cytometers, Flow-Set fluorospheres (Beckman-Coulter # 6607007) can be used as an alternative;
3. Type IIIA (calibration) beads: Spherotech RCP-30-1L (3 low intensity peaks) and Spherotech RCP-30-1H (4 high intensity peaks; both 3.0 - 3.4 µm);
4. Only for 4-color measurements on a BD Biosciences dual laser flow cytometer: Type IIB (reference) bead: Calibrite APC (BD Biosciences # 340487);
5. Monoclonal antibodies (mAb) according to Table IIA or IIB. All mAb must be used in appropriate concentrations, i.e., the same as used for routine applications.

SPECIMEN

Normal peripheral blood sample (i.e., leukocyte count ranging between 4 and 10 X 10^9/L, with 20% to 40% lymphocytes).

SPECIAL SAFETY

All cell suspensions must be considered as infectious unless fixed in media containing 1% PFA. mAb and calibration standards are supplied in NaN_3-containing media in most cases; waste-containing NaN_3 must be discarded with ample running water. Special safety precautions for flow cytometers are limited to those instruments equipped with high-powered laser tubes (i.e., 3 to 5 W).

PROCEDURE

Note: All histogram channels referred to in this and subsequent sections are expressed using a linear scale of 1,024 histogram channels.

A. Preparation of bead suspensions
1. Gently vortex the vials immediately prior to use.
2. Type 0, IIA or IIB beads: Add 1 drop of bead suspension to 0.5 mL PBS or sheath fluid.
3. Type IIIA beads: Add 1 drop of RCP-30-1L and 1 drop of RCP-30-1H beads to 0.5 mL PBS or sheath fluid.

B. Preparation of stained and unstained lymphocytes

Use a normal peripheral blood and stain it with mAb, using routine sample preparation technique, according to Table IIA (4-color) or Table IIB (3-color immunofluorescence assays). All mAb must be used in appropriate concentrations, i.e., the same as used for routine applications.

Note: This procedure is performed: (i) if the instrument is used for the first time for measurements using a given application; (ii) after each major service by qualified personnel (e.g., biannual maintenance controls involving cleaning of the optical and fluidics systems; replacement or alignment of the laser tubes); (iii) each time when 1 or more FL PMT settings are out of their acceptable range(s).

C. Setting up the flow cytometer

1. Switch on the flow cytometer and rinse the fluidics according to the manufacturer’s instructions.
2. Time delay calibration (only for 4-color BDIS instruments): Perform this procedure as per the manufacturer’s instructions.
3. Define amplification modes and threshold: FSC and SSC parameters, linear amplification; all FL parameters, logarithmic amplification; set FSC threshold in channel 52.
4. Set all compensation settings to zero.
5. Positioning of the FSC and SSC windows of analysis: Run a representative cell suspension for the relevant assay. Position the FSC and SSC windows of analysis such that all relevant populations fall on scale (for example, see Figure 1, upper left panel). This positioning is performed by adjusting the FSC photodiode amplifier gain and the SSC PMT settings.
6. Positioning of each FL window of analysis: run the unstained blood sample (see Procedure, paragraph B). Select the lymphocytes by placing a FSC, SSC window on this population (Fig. 1, upper left panel). Adjust the PMT settings of each FL parameter such that the unstained lymphocytes are placed in the first log decade, slightly away from the axes (Fig. 1, upper middle and right panels).
7. Recording FL application-specific initial target channels: Run the Spherotech reference bead (RFP-60-1) under the obtained FL PMT settings (Fig. 2; left panels). Acquire 5,000 singlet beads. All compensation settings are still zero. Record the mean FL1, FL2, FL3 (and FL4) channels in a
database. These channels represent the “application-specific initial target channels” for subsequent instrument set-up procedures.

8. Setting color compensation (BC instrument users may use the ADC Wizard in Expo or RXP software to run step 8 and 9): Run the single-color stained blood samples (Tab. II or III). Acquire list mode data on 5,000 lymphocytes per file. Select the lymphocytes by FSC, SSC gating. Adjust the parameters for electronic compensation such that, for each FL parameter to be compensated for, the mean FL channels of the CD8<sup>neg</sup> and the CD8<sup>bright</sup> lymphocyte populations are identical ±10 channels, as detailed in Tables II and III (Fig. 1, middle and lower panels).

9. Verification of color compensation: Re-run the single-color stained blood samples (Tab. II or III). Acquire list mode data on 5,000 lymphocytes per file. Select the lymphocytes by FSC, SSC. Verify, and if necessary adjust, the parameters for electronic compensation such that, for each FL parameter to be compensated for, the mean FL channels of the CD8<sup>neg</sup> and the CD8<sup>bright</sup> lymphocyte populations are still identical ±10 channels (Fig. 1, middle and lower panels).

10. Store the obtained instrument settings so that these can be recalled prior to subsequent experiments.

11. Document the obtained FL1, FL2, FL3 (and FL4) PMT settings, and the compensation settings

### TABLE II - APPLICATION FOR 4-COLOR IMMUNOFLUORESCENCE; FL1 (FITC), FL2 (PE), FL3 (X) AND FL4 (APC)

<table>
<thead>
<tr>
<th>Staining Parameter</th>
<th>Dotplot (x,y)</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unstained cells</td>
<td>Detectors/ Amplifier</td>
<td>FSC, SCC FL1, FL2 FL3, FL4</td>
</tr>
<tr>
<td>2 CD8 FITC</td>
<td>FL2-%FL1</td>
<td>FL1, FL2</td>
</tr>
<tr>
<td>3 CD8 PE</td>
<td>FL1-%FL2</td>
<td>FL1, FL2</td>
</tr>
<tr>
<td>4 CD8 X&lt;sup&gt;1&lt;/sup&gt;</td>
<td>FL2-%FL3</td>
<td>FL3, FL2</td>
</tr>
<tr>
<td>5 CD8 APC</td>
<td>FL3-%FL4</td>
<td>FL3, FL4</td>
</tr>
<tr>
<td>6 Control&lt;sup&gt;3&lt;/sup&gt;</td>
<td>FL2-%FL1</td>
<td>FL1, FL2</td>
</tr>
<tr>
<td>CD19 PE</td>
<td>FL3-%FL2</td>
<td>FL1, FL2</td>
</tr>
<tr>
<td>CD4 X&lt;sup&gt;1&lt;/sup&gt;</td>
<td>FL4-%FL3</td>
<td>FL3, FL4</td>
</tr>
<tr>
<td>CD8 APC</td>
<td>FL3-%FL4</td>
<td>FL3, FL4</td>
</tr>
</tbody>
</table>

<sup>1</sup> X = ECD, PerCP, PerCP-Cy5.5, PE-Cy5 or 7-AAD

<sup>2</sup> Expressed on a scale of 1,024 linear channels. Conversion formulas:
- 4-decade relative linear channels (RL: 1 - 10,000) → 1024 linear: 10log(RL) x 256
- 4-decade relative linear channels (RL: 0.1 - 1,000) → 1024 linear: 10log(RLx10) x 256

<sup>3</sup> When using PE-Cy5 conjugated mAbs, compensation settings must be defined for each individual PE-Cy5 labeled mAb. Thus, it may be necessary to adapt the choice of mAb for control staining 6
<table>
<thead>
<tr>
<th>Staining</th>
<th>Parameter</th>
<th>Dotplot (x,y)</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unstained cells</td>
<td>Detectors/ Amplifier</td>
<td>FSC, SCC, FL1, FL2, FL3, FL4</td>
<td>Position FSC Amplifier and SSC, FL1, FL2, FL3 and FL4 Detectors according to section C.5 and C.6 in the procedure</td>
</tr>
<tr>
<td>2 CD8 FITC</td>
<td>FL2-%FL1</td>
<td>FL1, FL2</td>
<td>FL2 Mean CD8 FITC&lt;sup&gt;pos&lt;/sup&gt; = FL2 Mean CD8 FITC&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>3 CD8 PE</td>
<td>FL1-%FL2</td>
<td>FL1, FL2</td>
<td>FL1 Mean CD8 PE&lt;sup&gt;pos&lt;/sup&gt; = FL1 Mean CD8 PE&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>3 CD8 PE</td>
<td>FL3-%FL2</td>
<td>FL3, FL2</td>
<td>FL3 Mean CD8 PE&lt;sup&gt;pos&lt;/sup&gt; = FL3 Mean CD8 PE&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>4 CD8 Y&lt;sup&gt;1&lt;/sup&gt;</td>
<td>FL2-%FL3</td>
<td>FL3, FL2</td>
<td>FL2 Mean CD8 Y&lt;sup&gt;pos&lt;/sup&gt; = FL2 Mean CD8 Y&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Control&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 CD4 FITC +</td>
<td>FL2-%FL1</td>
<td>FL1, FL2</td>
<td>FL2 Mean CD4 FITC&lt;sup&gt;pos&lt;/sup&gt; = FL2 Mean CD4 FITC&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>FL1-%FL2</td>
<td>FL1, FL2</td>
<td>FL1 Mean CD4 PE&lt;sup&gt;pos&lt;/sup&gt; = FL1 Mean CD4 PE&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>CD 8 PE +</td>
<td>FL3-%FL2</td>
<td>FL3, FL2</td>
<td>FL3 Mean CD8 PE&lt;sup&gt;pos&lt;/sup&gt; = FL3 Mean CD8 PE&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>CD45 Y&lt;sup&gt;1&lt;/sup&gt;</td>
<td>FL2-%FL3</td>
<td>FL3, FL2</td>
<td>FL2 Mean CD45 Y&lt;sup&gt;pos&lt;/sup&gt; = FL2 Mean CD45 Y&lt;sup&gt;neg&lt;/sup&gt; (±10 channels&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Y = ECD, PerCP, PerCP-Cy5.5, PE-Cy5 or 7-AAD

<sup>2</sup> Expressed on a scale of 1,024 linear channels. Conversion formulas:
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- 4-decade relative linear channels (RL; 0.1 - 1,000) → 1024 linear: 10<sup>log</sup>(RLx10) x 256
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12. Recording application- and instrument-specific target channels: Run the Type IIA (reference) beads with the obtained instrument settings (activated compensation settings!). Acquire 5,000 singlet beads. Record the mean FL1, FL2, FL3 (and FL4) channels of both types of beads in a database. For the Type IIA beads, these channels represent the “application- and instrument-specific target channels” for subsequent controls of the positioning of the FL windows of analysis.

13. For each FL channel:
   (i) the “narrow-margin tolerance zone” is defined as: the “application- and instrument-specific target channel” ± 10 histogram channels;
   (ii) the “wide-margin tolerance zone” is defined as: the “application- and instrument-specific target channel” ± 20 histogram channels.

14. Recording the instrument’s sensitivity level: Run the Type 0 (blank) beads with the same instrument settings. Acquire 5,000 singlet beads. Record the mean FL1, FL2, FL3 (and FL4) channels of both types of beads in a database. These values represent the noise level of the instrument for each FL channel.

15. For each FL channel:
   (i) the “narrow-margin tolerance zone” for the instrument’s sensitivity level is defined as: (application- and instrument-specific target channel - noise level) ± 20 histogram channels;
   (ii) the “wide-margin tolerance zone” for the instrument’s sensitivity level is defined as:
(application- and instrument-specific target channel - noise level) ± 40 histogram channels.

D. Calibration of the fluorescence channels

*Calibration of the instrument's response to FL signals*: Reset all FL compensation settings to zero. Run the Type IIA (calibration) beads with the obtained instrument settings (compensation settings reset to zero!). Acquire 5,000 singlet beads (Fig. 2, upper right panel). Record the mean channels of each peak for each FL channel in a database (Fig. 2, lower 4 right panels). Calculate the calibration regression line for each parameter (Appendix 1) and evaluate the instrument performance parameters relative to the “acceptable ranges” as defined so far (Tab. I).

**Note 1:** *The fluorochromes used in the Type IIA beads have very different spectral characteristics from FITC, PE, PE-Cy5, PerCP or APC. Hence, it is inappropriate to acquire these beads with activated compensation circuits.*

**Note 2:** *For the detection threshold and AUF value of histogram 0, tolerance zones have to be defined for each instrument. This situation is caused by the fact that the Type IIA beads do not spectrally match with routinely used fluorochromes and hence, no universally applicable units are available to quantify these tolerance zones. We recommend that for each instrument, Levey-Jenning plots are constructed for detection threshold and AUF value of histogram 0, and that remedial actions are taken in case of outlying results or deviating trends.*

E. Verification of the positioning of the FL windows of analysis and instrument sensitivity for FL measurements

**Note:** *This procedure is performed prior to each “cold start” of the instrument*  

1. Switch on the flow cytometer and rinse the fluidics according to the manufacturer’s instructions.
2. *Time delay calibration (only BDIS for 4-color instruments)*: Perform this procedure as per the manufacturer’s instructions.
3. Recall one of the relevant instrument settings obtained using step C.
4. *Control of the positioning of the FL windows of analysis*: Run the Type IIA (reference) beads with the recalled instrument settings (i.e., with activated compensation settings!). Acquire 5,000 singlet beads. Record the mean FL1, FL2, FL3 (and FL4) channels of the beads in a database. Check whether or not these values meet their respective tolerance zones. If not, rinse the fluidic system and repeat step 4.
5. *Control of instrument sensitivity for FL measurements*: Run the Type 0 (blank) beads with the same instrument settings. Acquire 5,000 singlet beads. Record the mean FL1, FL2, FL3 (and FL4) channels of the beads in a spread sheet. Check or not whether for each FL parameter the instrument’s sensitivity levels meet their respective tolerance zones.
6. If not, rinse the fluidic system and repeat steps 4 and 5.

**INTERPRETATION**

**Rejection of instrument settings:**

1. If, after repeated rinsing, a Type IIA bead result for any FL parameter is outside the “wide-margin” tolerance zone on a single occasion;
2. If a Type IIA bead result for any FL parameter is outside the lower limit of the “narrow-margin” tolerance zone on 3 consecutive occasions;
3. If a Type IIA bead result for any FL parameter is outside the upper limit of the “narrow-margin” tolerance zone on 3 consecutive occasions.
APPENDIX 1: CALIBRATION CURVE FL1 (ARBITRARY UNITS OF FLUORESCENCE)

<table>
<thead>
<tr>
<th>Bead</th>
<th>Relative linear channel (1-10,000)</th>
<th>AUF</th>
<th>Linear channel (256 scale)</th>
<th>Calculated AUF</th>
<th>Residual%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.28</td>
<td>7</td>
<td>0</td>
<td>155</td>
<td>4.6%</td>
</tr>
<tr>
<td>2</td>
<td>3.89</td>
<td>600</td>
<td>38</td>
<td>629</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>26.77</td>
<td>4700</td>
<td>91</td>
<td>4578</td>
<td>2.7%</td>
</tr>
<tr>
<td>4</td>
<td>76.91</td>
<td>15000</td>
<td>121</td>
<td>13560</td>
<td>10.6%</td>
</tr>
<tr>
<td>5</td>
<td>231.03</td>
<td>40000</td>
<td>151</td>
<td>42051</td>
<td>4.9%</td>
</tr>
<tr>
<td>6</td>
<td>775.85</td>
<td>140000</td>
<td>185</td>
<td>146255</td>
<td>4.3%</td>
</tr>
<tr>
<td>7</td>
<td>1688.21</td>
<td>330000</td>
<td>207</td>
<td>325486</td>
<td>1.4%</td>
</tr>
</tbody>
</table>

Graph statistics

- $R^2 = 0.9987$
- Zero channel value = 155
- Max channel value = 1,956,104
- Abs. average residual = 4.7%
- Detection threshold = 200
- Coef of response = 62.16
- Log Apm decases = 4.1
REFERENCES


