Methods for Optimizing PMT Voltages on BD Digital Instruments

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In flow cytometry, optimal voltage settings are important for resolution sensitivity (i.e. the ability to resolve dim signals from background noise). While adjusting voltages so that unstained cells appear in the first decade may be adequate for FITC and PE, this method is not optimal for fluorochromes with longer emissions (red to far-red, ~650 nm and longer). This is because the photo-multiplier tube (PMT) detectors are less sensitive to these wavelengths. In addition, unstained cells emit little autofluorescence in this part of the spectrum. Therefore, the variance of dim signals is very high in the red and far-red channels because electronic noise makes a large contribution to these measurements.

The following protocol describes a "Minimal Noise" method for optimizing PMT voltages **for your application** (i.e. your specific cell type) to ensure that electronic noise makes only a minimal (10-20%) contribution to the measured signals. Once these optimal voltages have been set, you should check that these settings give a good dynamic range for your experimental samples and compensation controls. If needed, PMT voltages can be reduced slightly if some stained samples or compensation controls are off-scale or if they are above the linear range for that detector. Once these adjustments have been made, we recommend running fluorescent beads and recording their MFI for each parameter. By using these bead target values to set the voltages for each PMT in future experiments, you can be confident that day-to-day variations in instrument performance are accounted for to ensure that the resolution sensitivity for each parameter will be consistent from experiment to experiment.

Shortcut Alternatives

We suggest two "shortcut" alternatives for users who prefer not to manually determine the optimal voltages for their specific application as outlined below.

Shortcut Option 1:

The first option is to use the CS&T (Cytometer Setup &Tracking) voltages that were previously determined by FMCF staff using the BD CS&T software. The principles used to derive the CS&T targets are similar to those in the Minimal Noise protocol described here because both approaches determine the voltage needed to place dim particles above the level of electronic noise. However, the intrinsic CVs and optical properties of the cells are unlikely to be the same as the beads, so the CS&T PMT voltages may not be truly optimal for your application. Nonetheless, they provide reasonable starting PMT voltages for many applications.

By default when a new Experiment is created, it is automatically set to the CS&T voltages (*the purple dot beside the cytometer setting icon in the software browser window indicates cytometer settings are set to CS&T voltage settings - once you alter any of the voltages the purple dot will disappear*). To apply the

current Cytometer Setup &Tracking voltages to an existing experiment, do the following:

In the experiment, right-click the experiment-level Cytometer Settings icon and choose "Apply Current CST Settings". (If you see a warning message that the Cytometer Setup and Tracking settings have expired, click "OK" anyway.)

Please note that the CS&T voltages were determined for the filters that comprise the "New Advanced" configuration. This configuration is the default configuration and was designed for a specific set of fluorochromes in which all detectors are used simultaneously. If you would like to establish CS&T voltages for your own filter configuration please contact FMCF staff.

Shortcut Option 2:

The second option is to use the "Lymphocyte Minimum Target Values." that the FCF has established. **These target values apply only to the filters in the default ("New Advanced") configuration.** Fresh *ex vivo* lymphocytes are commonly used to optimize PMT voltages for flow cytometers because they have measurable (but dim) autofluorescence in many (though not all – see above) wavelengths. The Lymphocyte Minimum Target Values were determined using fresh *ex vivo* murine lymphocytes as described below. Adjusting each PMT voltage to achieve these bead targets will ensure that lymphocytes (and other cell types that have similar or greater amounts of autofluorescence) will be at a gain where electronic noise makes a minimal contribution to the measured signal.

In our experience, the CS&T voltages versus the Lymphocyte Minimal Targets yield very similar MFIs for unstained lymphocytes in the blue-green-yellow-orange detectors. However, we (and BD) have found that the CS&T method consistently yields much higher MFIs (often >100) for unstained lymphocytes and dim beads in the red and far-red detectors. Although both methods produce PMT voltages that provide reasonable resolution sensitivity, we prefer to use the Lymphocyte Minimum Bead Target values so that unstained cells are not placed too high on scale in the red and far-red channels.

Part I: Determining Minimal Application-Specific Optimal Voltages

Objective: Determine a minimal desirable gain using the dimmest objects to be measured (usually unstained cells) where electronic noise does not make a significant contribution to the measured signal.

A reasonable minimal gain is one in which the variance of electronic noise (en) is not more than 20% of the total variance. To set the voltage, also known as gain, where the variance of electronic noise (en) is not more than 20% of the total variance we use a target of between 10% and 20% for unstained cells (i.e. 15%).

Since variance = SD^2, we therefore want to set the gain where
$SD^2_{en} = (0.15)*(SD^2_{target})$

Therefore $SD_{target} = \dfrac{SD_{en}}{\sqrt{0.15}}$

*Note: rSD_{en} = **robust** standard deviation of electronic noise (this metric excludes the skewing effect of outliers)*

The following three steps were performed to derive the rSD targets and the calculated values are posted beside each instrument.

    i.   Obtained rSD_{en} from Baseline Report for each PMT.

    ii. Calculated target rSD for each PMT ($rSD_{target} = \dfrac{rSD_{en}}{\sqrt{0.15}}$).

    iii. Calculated upper and lower limits: $rSD_{upper} = \dfrac{rSD_{en}}{\sqrt{0.10}}$ and $rSD_{lower} = \dfrac{rSD_{en}}{\sqrt{0.20}}$

The protocol below will guide you through a procedure in which you will adjust the gain until the rSD of your unstained cells equals the rSD_{target}.

1. Using the rSD target values from the posted chart, run unstained cells and adjust voltage until the rSD of the unstained peak roughly meets the rSD target and is within the rSD lower and rSD upper limits. If you have more than one cell type in your experiment, use the ones that you expect to have the lowest levels of autofluorescence for this step.

2. Check dynamic range.

   Put on a **fully** stained sample at (minimal) target gain and choose the samples that you expect to have the brightest total signal (i.e., autofluorescence plus fluorochrome signal) for this step.

   a) Ensure the positives are on scale in the linear range of the detector and not greater than ≈ 100,000 – 120,000. If they are at or above 200,000, lower the voltage until positives are ≈ 100,000 – 120,000. This will leave some "wiggle room" at the top end of the scale in case some samples are even brighter.
   b) Run compensation samples to ensure that they are also within the linear range of the detector (may exceed 100,000 but not into a nonlinear region) and staining is greater than or equal to staining levels of cells. If it's not then there isn't anything that you can do at this point.

   Note: The linear range for each detector can be found on the baseline report.

Part 2: Capturing bead target values for future experiments

Objective: To capture bead target values for use in future experiments. This will allow you to set the instrument in a consistent manner from experiment to experiment.

1. Create a worksheet template with histogram plots for each parameter.

2. Run SPHERO Rainbow Calibration particles (Spherotech, cat# RCP-30-5A-4) to capture bead target values (i.e. the MFI of the beads for each channel). These are beads that contain a mixture of fluorophores that fluoresce in all channels.

3. Create a gate around the peak on each histogram and note MFIs. You can export statistics as a CSV file that can be viewed in or printed out from Excel. Save worksheet template for use in subsequent experiments.

4. Export and save the cytometer instrument settings by right-clicking the cytometer settings icon in the browser and choose "Export".

5. For subsequent experiments, right-click an open experiment in the browser and choose "Import Cytometer Settings". Click "Yes" to overwrite the current settings. Select the settings file you want to import and click "Import".

    Note: Cytometer settings include PMT voltages, compensation, threshold and ratio values.

6. Run beads and adjust voltages so that they meet the bead target values established in step 3.

7. Proceed with experiment.

    For cells whose autofluorescence will change from experiment to experiment (e.g. tumour cells, cultured cells etc.):
    Using the Lymphocyte Bead Target Values, highly autofluorescent cells will often sit in the second decade or higher in the Pacific Blue, FITC, PE or PE-TR channels. The amount of autofluorescence is much lower in the red and far-red channels. It is recommended that you DO NOT arbitrarily lower the PMT voltages to bring the "background" down to the first or second decade. This autofluorescence is real fluorescence and should be recorded as such as long as your stained cells are well on-scale. However, if your stained cells are off-scale on the upper end, then lower the voltages just enough to bring their MFI to 100,000-200,000. After that, capture bead target values for future experiments as described above.