

Forward & Side Scatter Optimization Protocol

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Objective: To determine optimal voltage settings for forward scatter (FSC) and side scatter (SSC) parameters and to use beads to set these voltage parameters consistently from experiment to experiment.

Optimal voltage settings will allow you to view the populations of interest on scale and to maximally resolve populations that differ in these parameters.

This protocol uses unlabeled BD Calibrite beads (cat# 349502) and 10 micron size beads from Invitrogen's Flow Cytometry Size Calibration Kit (cat# F-13838) to set voltages for lymphocytes, however a different bead type could be used if your cells are substantially different in size or refractive index from normal lymphocytes. The goal is to use a standardized particle that will appear on scale with the populations of interest. That being said, beads often exhibit a much higher side scatter property than cells of the same size because they have different refractive indices. A particle with a refractive index that is very different from that of the surrounding medium will scatter light more. While the beads mentioned above are on scale on the forward scatter parameter the beads are off scale on side scatter. The side scatter voltage was therefore lowered to record the beads.

1. Run cells and adjust FSC and SSC voltages so that all populations of interest are on scale and roughly centred. Ensure that populations with different scatter properties are sufficiently resolved from each other. (e.g., lymphocytes and macrophages/granulocytes in murine bone marrow or human peripheral blood). Also consider whether you anticipate running several different cell types of substantially different sizes in the same experiment. In that case make sure to choose FSC/SSC settings that will keep both the smallest and largest cells on scale. In some cases this may require log scaling for FSC or SSC or both parameters. Note that although you can view the acquired data with linear or log scaling in FlowJo after data collection, the optimal voltage settings for placing the cells at the low-mid range of the log scale will likely be very different than those for optimal placement on a linear scale. Therefore, if log or bi-exponential scaling is desired, carry out the voltage optimization while viewing the live events.
2. Create a worksheet template with histogram plots for FSC and SSC parameters.
3. Run beads to capture bead target values (i.e. the MFI of the beads for each parameter). Please note that the side scatter voltage may need to be

lowered to view the beads on scale. If so, note the difference in the voltage setting.

4. 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.
5. Export and save cytometer instrument settings by right-clicking the Cytometer Settings icon in the Browser and choose "Export".
6. 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.

7. Run beads and adjust voltages so that they meet the bead target values established in step 4.
8. If the side scatter voltage was adjusted to bring the beads on scale adjust the voltage back the same amount before running samples.
9. Proceed with experiment.