Optimizing setup of the LSR2 system
(voltages and compensation)

The goal of optimizing your voltage settings on a digital cytometer is to have good separation between your positive and negative populations while minimizing the overlap between your parameters. A new method of instrument setup and tracking has been developed that will be recommended for all users of the flow facility. For a more in-depth description of the processes involved, the following documents will be useful:

-- BD Application note "Establishing Optimum Baseline PMT Gains to Maximize Resolution on BD Biosciences Digital Flow Cytometers"
http://www.bdbiosciences.com/cgi-bin/literature/view?part_num=23-8389-00 (also linked from the "training" section of our website)
--"Flow Cytometry Controls, Instrument Setup, and the Determination of Positivity", Cytometry Part A, 69A, 1037-1042 (2006)—there is a copy posted on the flow bulletin board in 5228 or I can email you the PDF.

The first step in this process is to determine the amount of electrical noise in the system. The documents linked above will explain how this is done, but this part of the setup will be performed as part of facility QC and the results posted by the instruments. In short, the operator runs a dimly fluorescent bead and records data files where voltages are set in 50 volt increments (all parameters at 250V, all parameters at 300V, etc, up to 800). A plot of CV vs mean will allow you to derive the Standard Deviation (SD) of the electronic noise in the system. The target mean value for your negative population should be 8-10 times this value to minimize the contribution of noise to your signal, and resolution of dim and negative particles will be optimum. (Note that beads and cells, and even different types of cells, will have different amounts of fluorescence in each channel, but the *target mean signal* for each is still the same to optimize resolution).

For most parameters with clearly defined populations, it is sufficient to set your voltages so that your unstained cells are in the first decade (mean of around 50, tail ending at around 200), but if you have a dim signal and wish to optimize the separation, set the voltage so the mean signal is 8-10 times the SD of the noise (typically resulting in a mean signal from 100-300 depending on the channel). As you increase above this setting, the negative population will also gain signal and your separation will not improve. In addition, higher voltages will mean that you will get a higher response from overlapping signal, making compensation more difficult, so it is preferable to use the lowest voltage that gives the result you want.

In the event that your positive population goes off scale at these settings, you have 2 options. Since you have separation of 3 logs or more, you may reduce the voltage to bring your bright population on scale. Alternatively, you may titer down your antibody, which would allow you to save on reagents while still getting enough separation to identify your population of interest. Another important consideration is that if your antibody is that bright in its own channel, it will also be bright in any channel it overlaps into, so titering the antibody will also aid in compensation, whereas lowering the voltage will make it harder to compensate. With something like GFP, which cannot be titered, you will have to lower the voltage and deal with any compensation issues that this causes.

Compensation

Using these voltages you have determined, you can generate a compensation matrix and see if any problems arise. When calculating compensation, it is critical to match the positive and negative particles—if you are using beads labeled with antibody, the positive population must be compared to unlabeled BEADS, while labeled cells must be compared to unlabeled cells, so that you are correcting only for the fluorescence contribution of the fluorochrome and not any difference in signal from cell to bead (or from cell to cell—Lymphocytes and monocytes have very different autofluorescence). Note that when SETTING the voltages, you want to optimize for the experimental particle you are measuring (usually cells), and then collect the compensation particles at these settings, regardless of what type they are.

It is preferable to use antibody capture beads, especially if you have a label that is dim or low frequency (<1% of the population). Beads will give you a bright, uniform signal for each reagent. If you are using antibody capture beads for all of your colors, you can simply record unlabeled beads as the unstained control, and then single stained samples for each color, and the software will automatically use the unstained control as the negative for all tubes.

For labels such as PI, CFSE, or viability dyes, which will only stain cells, you may just use cells for ALL of your controls (unless one or more of your labels is dim or low frequency—see below), and collect a sample of unlabeled cells as your unstained control. Be sure to consistently gate on the same cell population (e.g. only lymphocytes), as different cells have different levels of autofluorescence.

For viability dyes, you are typically gating out the positive events, so that the spillover of the viable cells is minimal since they have minimal signal—if you have been using labeled cells as the control and comparing them to unlabeled beads, the effect should be minimal but it is not the best way to do it.

With CFSE, where you are interested in the POSITIVE events, it becomes more problematic—for instance, there should be minimal overlap of CFSE into the Pacific Blue channel, but if you are comparing CFSE labeled cells to unlabeled beads, you may in fact see the signal in the unstained beads is higher than the CFSE stained cells in Pac Blue because the autofluorescence of beads in Pac Blue is higher than the cells. In this case it is much more important to compare to unlabeled cells to determine overlap.

If one or more of your antibodies is dim or very rare (<1% of the population) AND you have a reagent that must use cells (GFP, CFSE, PI, or an antibody that does not have a compatible capture bead), you may need to use a mix of beads and cells for compensation. If this is the case, each compensation control tube will need an “internal” negative population for comparison. When you “create compensation controls”, click on the checkbox at the top of the window to turn OFF the option to create an unstained control tube, and only a single comp tube for each color in your experiment will be created. When preparing your beads, add a drop of the “negative” beads that don’t bind antibody. For cells, either mix unstained cells into your single stained sample, unless the “unstained” will pick up the label (such as with PI), in which case you can bring a tube of unstained cells and just “append” them to your data file of stained particles. You will then need to go through and draw a new interval gate on the negative population in

each of your single color histograms, and calculate compensation as usual. The software now uses the negative population defined in each tube for the comparison.

Remember that your particles will be giving off the same amount of light (unless you change the laser power, which is fixed), and adjusting the voltage changes the sensitivity of the *detector*. Compensation is determining what percentage of the "primary" signal is being detected in the other channels—if you have 30% overlap of FITC into PE and you **increase** the voltage in the FITC detector, you will increase the primary signal while the overlap signal stays the same—thus, the % of overlap will decrease. If you decrease the FITC voltage, the measured FITC signal will be lower while the overlap into PE will be the same, so you will have a higher % of overlap. Likewise, decreasing the PE voltage will decrease the amount of overlap signal detected, also resulting in lower % of overlap. However, if you decrease the PE voltage in this case, then your measured PE signal for your PE control will be lower, and your % overlap into PE-Cy5 (for example) will be greater.

If any parameters require >100% compensation (which means that the "overlapping" signal is brighter than the "measured" signal), it may be necessary to further adjust your settings. One quick trick you can use is to run (without recording) each of your single stained samples with the "unstained control" tube active—you will be able to see how much signal is generated by your particles in all of the channels, and just make sure that the brightest signal is in the histogram for the color that control is labeled with--your APC single stain control should have the brightest signal in the APC histogram, but you will see some signal in APC-Cy5.5—just make sure it is not brighter than signal in APC.

In this case, if APC-CY5.5 is brighter, you can either increase the voltage to the APC channel, or decrease the voltage in the APC-CY5.5 channel (or both), so that the APC signal is brighter. Remember that this will impact the other colors as well, so if you choose to decrease the voltage to APC-Cy5.5, then the APC-Cy5.5 sample will have lower "primary" signal, and the spillover into APC-Cy7 could be brighter—a balance must be struck to get everything to agree. Adjustments of more than 50 V in either direction from your initial settings should be made with great caution—please see me if you have any problems.

Once you have determined the "optimal settings" for your samples (good separation and minimal overlap), it is then possible to run a mid-range bead (see below) at these voltage settings and determine the mean signal levels for these beads in each of the channels. **It is critical that you use UNCOMPENSATED values for tracking your signals, as these beads fluoresce in all channels and you want to know the total fluorescence levels.** After recording the beads, click the + sign next to the tube to expand the menu, select "instrument settings" for that tube, and in the inspector click on the "compensation" tab. Click the checkbox to turn OFF compensation. If you do this in the Instrument window before collecting the tube, it will be turned off for any future tubes you collect, so be sure to turn it back ON for subsequent tubes. By running these same beads and matching these values (UNCOMPENSATED) on subsequent days or on a different instrument (by adjusting the voltages for each parameter to match the signal levels), you will ensure that the instrument is detecting the same signal levels from day to day (but compensation should still be calculated with each experiment, as tandem dyes may vary). The Application Note referenced above recommends a "mid-range bead" (BDBiosciences cat # 556298) but the signal on the UV-B (440/40) channel at typical voltages is off scale, so a different particle needs to be used for these channels. Spherotech Midrange Rainbow Fluorescent particles, RFP-30-5A, seem to work well in all channels.

SOP for LSR Optimization

I. Determine control samples required

a. You will need one tube of unstained cells (for the initial run at least).
b. You will need one single-stained control per color in the experiment.
c. Antibody capture beads should be used for your single stained controls unless any of the following apply:
    i. If you are using PI, CFSE, or Invitrogen amine-reactive viability dyes*, these reagents will ONLY stain cells, so cells must be used as control for this color. Note: *Bangs Labs make an amine-tagged bead that may be used with the amine reactive viability dyes instead of cells—but unlabeled bead from Bangs Labs may be different from BD comp beads!
    ii. If you are using an antibody that is NOT from mouse, rat, or hamster, BD's antibody capture beads will not bind them and you will need to use cells as your controls for at least those colors.
d. For single fluorochrome labels (FITC, PE, APC, Pacific Blue, etc), if the signal for your antibody is dim (no defined population can be resolved) or frequency is low (<1% of population) and you wish to use cells, you may substitute another antibody of the same color with a more robust response (CD8, CD4, etc) for the purposes of compensation. If using beads, they will give a bright uniform signal so it doesn't matter how the antibody stains cells.
e. If you are using a tandem dye (PE-Cy5, APC-Cy7, etc), you MUST use the same exact reagent as in the experiment—if it is dim or low %, capture beads should be used.
f. If you are using mixed controls (some cells, some beads), each sample tube must have a negative population in it. Compensation must be done comparing the same particle so that differences in autofluorescence are not skewing the calculation. If you have mixed particles, you cannot collect a "universal negative" for all of them to be compared to. This means for beads, you must include a drop of the "negative" beads (that do not bind antibody) in your tube. For cells, if 100% of the cells get labeled and if the staining won't bleed over, then you can add some unstained cells to the stained cells (as long as the stained cells have been washed, etc). Otherwise (like with PI) you can use the tube of unstained cells (will explain below).

II. Initial setup

a. You will need unstained cells, unstained beads (only if all comp tubes are labeled beads), single stained compensation tubes, FMOs if needed, and midrange rainbow beads (Speherotech RFP-30-5A).
b. Create new experiment. Select "instrument settings" and select desired colors in the "inspector" window (magnifying glass icon).
c. Go to Instrument pulldown menu>instrument setup>create compensation controls.

i. Activate “unstained control” tube and load unstained cells.
ii. Adjust voltages for FSC/SSC to get events on scale, and for fluorescent channels so that:
    1. for clearly defined populations, the edge of your negative population should be around 200 (tick after 10^2, mean~50)
    2. If low signal is expected/experienced in that channel, then mean of negatives should be between 100 and 300.
iii. If using only cells, record unstained cells as unstained control.
iv. If using only beads, record unstained beads as unstained control.
v. If using mixed beads and cells, go to Instrument pulldown menu, “modify compensation controls”, and turn off “Unstained control tube” checkbox at the top. Click OK. This will remove the “unstained control” tube from the Comp Control Specimen.
vi. Continue on recording each of your single stained controls. If comp controls are mixed particles, each tube should have a negative population in it. If necessary, to create a “negative” population in a cell sample, you can collect the stained sample and then load the unstained cells and “append” to the file (click Record again, and choose “append”).
vii. Go through each of your worksheets and make sure the positive population is properly gated. If you do not have an unstained control tube, you must also draw new interval gates on the negative population in each histogram.
viii. Instrument pulldown menu>Intrument setup>calculate compensation.

d. With experiment name highlighted, click on syringe button to create a new specimen.
e. Expand specimen to view tubes.
f. Activate first tube, rename as “rainbow beads”
    i. Load and run midrange rainbow beads, record.
    ii. Expand rainbow beads tube, and select “instrument settings” that is revealed. These will show the settings used for collecting that tube in the inspector. Click on the “compensation” tab, and click on the checkbox to disable compensation.
    iii. Select the green “worksheet” button in the browser to create a new global worksheet. If it is greyed out, click on an element in your experiment and it should become activated.
    iv. Draw FSC/SSC plot on worksheet, and histogram for each color. Draw a region around your single bead population. This should be P1.
    v. Right-click on border of any plot, and select “create statistics view”. Right click on statistics view and select “edit statistics view”
    vi. In the “header” tab, turn off all checkmarks. In the Populations tab, turn off all checkmarks except for the one under “Show Population” that is next to your FSC/SSC population (P1). In “Statistics” tab, make sure only “mean” for all fluorescence parameters are checked. Click OK.
    vii. The values displayed indicate the fluorescence levels of the beads for each of the channels you measured. Make note of these values.
g. Return to the main global sheet, draw your plots for gating, and record your sample tubes. To add tubes to your specimen, you may either click the tube button at the top of the browser, or click “next” in the acquisition controls.

III. Future experiments

a. If duplicating your experiment layout, go to the Global Sheet 2 with the rainbow bead plots. Otherwise, re-create it.
b. Load a tube of your Mid Range Rainbow beads (same lot number as before).
c. Adjust the voltages so that the mean signals detected for each parameter match the values detected during the initial setup (within reasonable limits, i.e. +/- 5% or so). This should be done with compensation turned OFF, and will result in your detectors having the same response to the same fluorescence as in your initial setup experiment.
d. Run compensation tubes as usual, calculate compensation, and run sample tubes.