Advanced Flow Cytometry Training – Getting the most of out the Cytometer

When setting voltages it should be understood that all photomultiplier tubes (PMTs) operate optimally within certain ranges. Factors influencing detector performance can include but are not limited to: systemic electronic, detector quantum efficiency, optical filter quality, laser alignment, detection wavelengths, and the voltage applied to the PMTs.

Since all cells are variable with respect to their background autofluorescence there is no "one size fits all" group of instrument settings that can be universally applied to the PMTs on a particular cytometer. Moreover, instrument settings for negative populations should not be arbitrarily assigned to low fluorescence values (as was done in older systems; it is known that insufficient gains (voltage) can decrease resolutions between negative and positive populations, whereas high gain values can push very bright populations off scale making them less easy to quantify. In order to limit the influence of all these factors when establishing instrument settings the following guidelines should be followed. In addition, when these procedures are followed, cytometer performance will be traceable over the long term, as is often necessary when repeating the same experiment many times or in clinical trials.

What you will need:

- Unstained cells at a concentration of 3 – 5 million cells/mL for 10 – 20 measurements.
- A spreadsheet with the extracted Cytometer baseline data for the current bead lot with an additional column for 2.5 rSDen.
- Retrieve the baseline data as follows…
    - CYTOMETER MENU →CST → Find the Baseline for the current bead lot →Right click on the file name and select Export
    - Relabel the file with your name and experiment e.g. Susan_Lymphocyte Panel Nov 2017

Table 1: Example Baseline Data for BD Cytometer Set-up and Tracking Beads

Laser	Detector	Parameter	Linearity Min Channel	Linearity Max Channel	Slope	Intercept	Electronic Noise Robust SD (rSDen)	Qr	Br	2.5 x rSDen
Blue	B	B_525/50	372	185098	7.4097	-15.4	13.4	0.1331	1005	33.5
Blue	A	B_710/50	146	242502	7.6454	-16.5	13.3	0.0153	220	33.25
Red	C	R_670/30	21	240116	7.6136	-16.6	13.9	0.0318	150	34.75
Red	B	R_730/45	74	242590	7.4731	-15.4	13.9	0.016	311	34.75
Red	A	R_780/60	36	246210	7.5514	-15.7	15.1	0.0133	350	37.75
Violet	D	V_450/50	19	235409	7.9471	-17.2	17.8	0.0301	1156	44.5
Violet	C	V_525/50	17	262143	7.7039	-16.1	17.7	0.0308	1690	44.25
Violet	B	V_610/20	87	256619	7.6416	-16.6	17.1	0.347	37	42.75
Violet	A	V_780/60	16	243282	7.6842	-16.2	18.4	0.2471	16	46
Yel Gr	E	YG_586/15	55	235424	7.5749	-16.5	18.7	0.341	553	46.75
Yel Gr	D	YG_610/20	24	234776	7.3944	-15.6	16	2.3434	5518	40
Yel Gr	B	YG_710/50	81	239048	7.4875	-15.8	20	0.0838	271	50
Yel Gr	A	YG_780/60	30	235937	7.492	-15.8	17.4	0.0699	3	43.5

Part 1: Rules for Setting Cytometer Voltages

Rule 1: Minimize Electronic Noise in the Data.

The minimum PMT voltage setting for each detector is determined so that the resulting robust standard deviation (rSD) of the unstained cells is approximately 2.5x the robust standard deviation of the electronic noise (2.5 $rSD_{en)}$.

Purpose: To ensure that electronic noise does not interfere with measurements with dim fluorescence/negative events and that you are aware of these limits should it be necessary to decrease the voltages lower than these values in **Rule 2** (below).

The short way: Use the application settings features of FACS DIVA (grey plot regions) to help place your negative cells at 2.5 rSD_{en}.

- o   Refer to your training notes for instructions on how to set this up.

The long way:

- Create a plot of FSC and SSC and gate the cells of interest.
- Create a Statistics Box and select the FSC x SSC population (P1) and the rSD values from the stats tab for every parameter.
- Sequentially record data files of at least 5000 events starting from the CST voltages, reducing the voltage in increments of 25 until the rSD of the unstained cells comes into line with the values calculated in the in the last column of the baseline report spreadsheet (refer to Table 1's example).
- Make a note of these voltages on a worksheet – these are the minimum voltages that may be used to ensure that no more than 10 – 20% of electronic noise contributes to the biological background of the sample.
    - Data from a statistics box can be exported to an excel worksheet with the desired information.
    - The minimum acceptable voltage can be extrapolated by plotting rSD_{en} vs. voltage on a graph.

Rule 2: **Ensure that the brightest populations of the stained cells remain in the linear range of the detectors.**

The Short Way: Acquire data for a fully stained tube of cells and reduce the voltages for any detectors where data is off scale positively.

- Ensure to leave room at the top of the scale so that unexpectedly bright positives can still be captured on scale.

The Long Way:

- Refer to the **Linearity Max Channel** column of the extracted baseline report; the intensity (MFI) of your fluorescence should not exceed this value.
- Create a statistics box with the MEDIANS for each fluorescent parameter and acquire data for **cells** stained with one colour only (or with colours that are well separated and have no spectral overlap).
    - Note: The measured fluorescence must be biologically relevant therefore beads cannot be used in this process.

- If there are populations with a larger spread (wide %CV's) decrease the voltage so that the brightest events remain within the linear range of the detector.
- There should always be some extra room at the top of the scale to accommodate events that have unexpectedly high fluorescence.
- Compensation controls should also fall within the linear range of the detector.
  - If the compensation controls are too bright, it is best to remake the samples with lower antibody concentration as opposed to decreasing voltages to accommodate the bead fluorescence.

Rule 3: Rule 2 takes precedence over Rule 1

If the positives are so far up the scale using the minimum acceptable voltage that they are in the nonlinear range or going off scale, the voltage must be lowered to properly view the positive data regardless of where the negatives reside.

Rule 4: Verify good resolution for dim populations at the selected voltages.

Placing negative cells at 2.5rSDen does not always result in the best resolution between the negative and positive fluorescence. For example, at the minimum voltage, the separation between negative and positives may be reduced when compared to voltages closer to the CST values - this can negatively impact the detection of weakly expressed antigens. Alternatively, when too much voltage is applied, negative populations can spread/broaden, especially in channels where there is little to no detectable autofluorescence (red emissions). It is therefore important to optimize the resolution of all stains, paying particular attention to dim fluors and weakly expressed markers, to ensure their correct identification by calculating the Stain Index over a range of voltages.
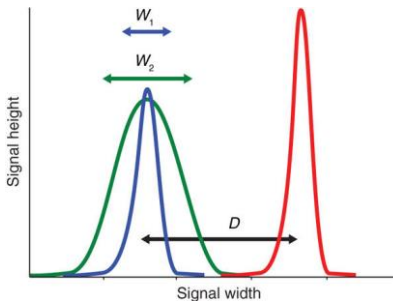

Figure 1: The effective brightness of a reagent depends on the difference (*D*) between the positive (red) and the negative (blue, green) populations and the spread of the negative population (*W*). The stain index is a useful metric for normalized signal over background.

The following method will help to minimize the spread of the negatives while optimizing the resolution of the stains for each detector.

Procedure: Acquire data for the single stained control cells, over a range of voltages to determine where the best resolution is obtained. Use this voltage instead of the rSD_{en} as your starting voltage.

Calculating Stain Index:

$$\text{Stain Index} = [\text{MFI}_{pos} - \text{MFI}_{neg}]/ (2.5 \times \text{SD}_{neg})$$

- Draw histograms for each parameter and draw positive and negative interval gates on the plots
- Create a statistics box with the MFI_{poS}, MFI_{neg} and SD_{neg} for each parameter.
- Create a spreadsheet on which you can input data directly from DIVA statistics to calculate Stain Index as you change voltages. You may also export the statistics from FACS DIVA and format the resulting table for this calculation.
- For populations that smear (no easily identified negative/positive boundary) keep some unstained aside so that each time you change the voltage data is acquired for the negative cells alone in addition to the stained cells,
    - Use the negative cells alone in the stain index calculation (MFI_{neg}).

Part 2: Create Software Application Settings

FACS DIVA based "Application Settings" are used to standardize your experiments across time and between instruments.   If the CST program has changed the system voltages to separate the DIM bead population apart from noise, the software will automatically adjust your specific application settings, to a proportional degree, so that populations will appear in the same position on the scale each time the CST values are updated (daily).

When to create application settings:

1. After fully optimizing your experiments.
2. After new baseline measurements are recorded, for example, when new bead lots are used.
3. When transferring experiments between different instruments.

Procedure:

- At the experiment level, right click on the Cytometer Settings, scroll down to application settings> SAVE
    - Name the settings as follows: Your Name (First)_ Experiment Name_ Date

Create Rainbow Bead Reference Targets:

- Create a new global worksheet: In the browser, right click on the global worksheet icon in the experiment and select "new Global worksheet" from the drop down menu.
    - Create Histograms for each fluorescence parameter plus FSC and SSC
    - Place a tight gate around the main RB population on FSC and SSC
    - Create an interval gates on the histograms
    - Create a statistics box for each histogram and edit the stats to include the MEDIAN for the fluorescent parameter and interval gate with which it is associated.
    - Create a text box that displays the medians for each detector
- Record an *uncompensated* data file for the Rainbow Beads
    - Label the tube with the bead type and Lot number, for example: RCP – 38 – 3A Lot AF02
- Create a text box and record the medians for each channel in your experiment (so it will be there for every run)
    - If using multi-peak rainbow beads, make sure to identify which peak was gated (so the same peak is always targeted).
    - Record bead targets in your lab book (for an offline reference).

- When repeating experiments or transferring to a new cytometer, use the Rainbow Targets to adjust the bead MFI's to the same values +/- 5%

- If transferring to a new cytometer:

    - Save new application settings).
    - Save the experiment template so the bead target worksheet is accessible for future runs.

IMPORTANT: Rainbow targets will verify that the application settings have been set correctly - These readouts are Bead Lot dependent.

- When lots change, data files that correlate old targets from earlier lots to the new lot (acquired on the same day, with the same voltages) must be recorded.
    - The MFIs of the fluors may be different between lots therefore it is important to note this on your template by updating the reference values.

Recommended Bead Products:

Rainbow beads may be purchased directly from the manufacturer (Spherotech.com or Cedarlane Laboratories).

Product Name: Ultra Rainbow Calibration Particles, Kit

RCP Manufacturer: Spherotech, Catalogue No: URCP-38-2K

There are six peaks of fluorescence in these beads; choose which peak best suits your data and then must match your MFI's with same peak per detector each day

Recommended for experiments with many red detection parameters and UV excitations.

Product Name: Sphero Rainbow Fluorescent Particles (Mid-Range) 3.05 uM, 5mL.

Manufacturer: Spherotech, Catalogue No: RFP- 30-5A

These beads have poor red/far-red fluorescence and do not work for UV excited fluors.

Best for FITC/PE/PE-Cy5 and violet excitations

References:

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