DHVI Flow Facility Newsletter

Winter 2018-2019

Say hello to our little friend!

Tired of counting cells on a hemocytometer? Wish there was a simple and inexpensive cytometer to do Annexin V or cell cycle analysis? Look no further! We recently acquired a Muse Cell Analyzer from EMD Millipore. The Muse is a 3-parameter flow cytometer specialized for accurate and quantitative analysis of both suspension and adherent cells of 2-60 µm in diameter using ready-made reagents and protocols. Convenient "Mix-and-Read" assays are optimized for accuracy and reproducibility, and cover a broad range of cellular assays, including kits for:

- Cell Count and Viability
- Apoptosis: Annexin V, ATM/H2A.X DNA damage, Mitopotential, Caspase 3/7
- Phospho Signaling: Bcl-2, PI3K/MAPK, EGFR-RTK
- Cell Phenotyping: Human B cells (CD19), CD8 T cells, CD4 T cells, CD25 cells
- Activation/Proliferation: Human CD69, Ki67
- Cell cycle
- Nitric oxide
- Oxidative stress
- Autophagy: LC3, RFP-LC3 reporter
  *For a complete list of Muse kits, visit https://www.luminexcorp.com/flow-cytometry-kits-and-reagents/

The kits range from $150-$450 and typically contain 50-100 tests. We are currently setting the instrument up for 24/7 independent use in RP105, with training to be provided by facility staff. We do not yet have an official hourly rate for this instrument, but it will be substantially lower than the rate for the LSRIIs and Fortessa. Stay tuned for more information as we bring this instrument on-line!

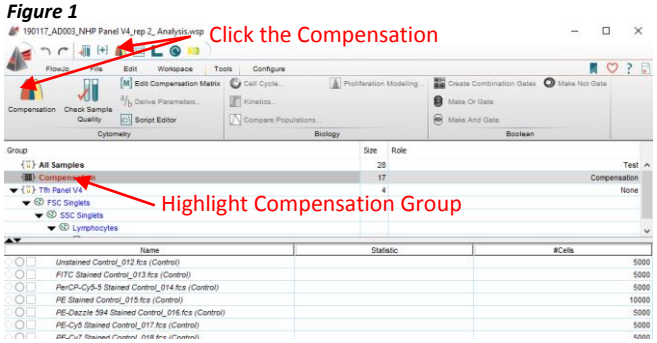
FlowJo Tips & Tricks: Compensation

By Ariel Arus-Altuz

In this article, I will walk through the compensation wizard in FlowJo version 10. Whether setting compensation from scratch or tweaking values from a matrix generated at the cytometer, this skill will ensure that your flow cytometry data are interpreted as accurately as possible.

1. Drag the FCS files of your compensation controls into the "Compensation" group in the workspace. With the group highlighted, click on the compensation button located either on the static toolbar or in the 'Tools' menu (Fig. 1).

Figure 1

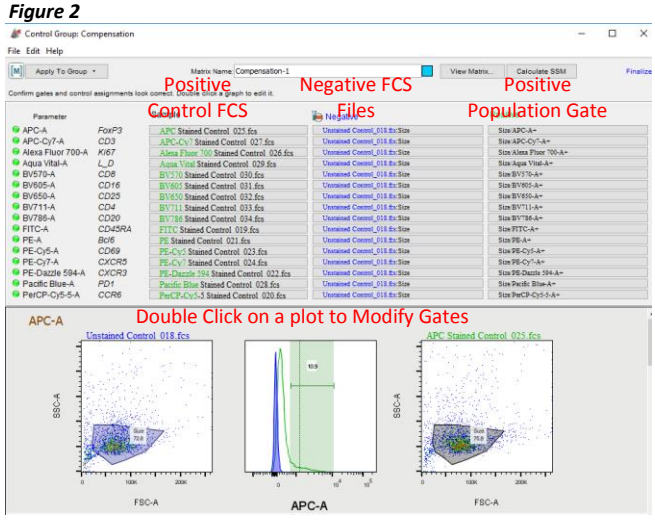


2. Choose the parameters you wish to include in your compensation matrix.

3. In the following window, you are presented with four columns (Fig. 2). The "Parameter" column lists fluorochromes and associated markers. The second column ("Sample") contains FCS files that FlowJo "thinks" are the single-color samples corresponding to the fluor in the first column. The third column ("Negative") lists the FCS file and gate for a negative sample. The fourth column ("Positive") lists the gates generated by the wizard for the FCS file in column 2 that identifies the positive events in that sample.

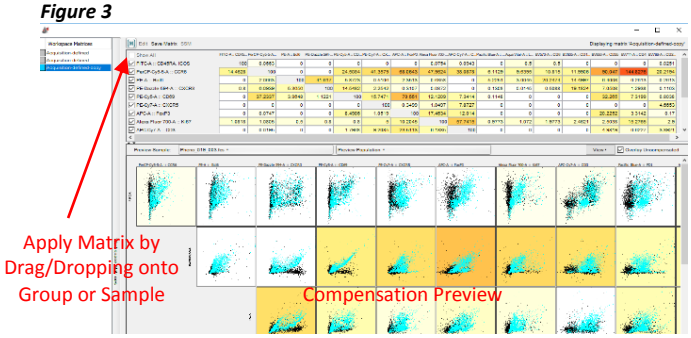
4. The lower half of the wizard allows you to view and adjust FlowJo-generated gates so that positive and negative events are properly gated for each parameter (Fig. 2). Three plots are shown for each parameter: a FSC vs. SSC plot with the "Size" gate on the negative sample (left), a FSC vs. SSC plot with a "Size" gate on the positive sample (right), and an overlaid histogram of negative (blue) and positive (green) fluorescence values for the two Size gates (middle). If needed, double-click on any of the FSC/SSC plots to adjust Size gates, and/or double click on the histogram to ensure positive events are correctly gated. Once satisfied with the setup, click "View Matrix" at the top.

Figure 2



5. The compensation matrix is divided into three sections: a list of color-coded matrices (left), a heat-mapped NxN matrix (top right), and a dot plot preview window where you can see the effect of the compensation on any sample and gated population (bottom right) (Fig. 3).

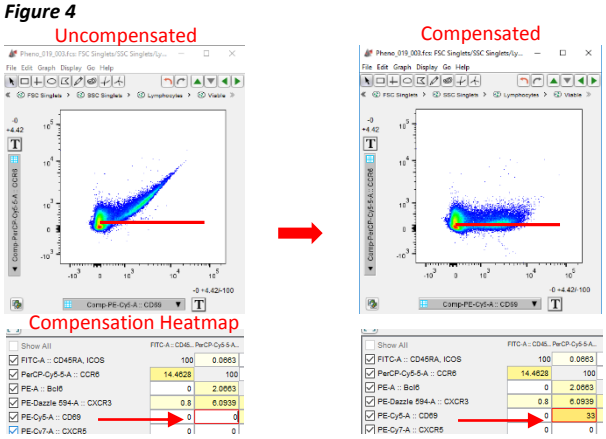
Figure 3



6. To adjust compensation, click "Edit" at the top of the window, which copies values into a new matrix. Adjust values in the new matrix, and the effects are visible in the preview window (Fig. 4). To apply a matrix to samples, drag the Matrix Icon [M] (Fig. 3) onto a sample or group in the workspace. You are finished!

Sidenote: The 3 Cardinal Rules of Compensation
1. Comp controls should be as bright or brighter than samples.
2. Background fluorescence should be the same for comp control and negative control.
3. Comp controls must match fluorochrome in samples. (i.e., don't use FITC to comp for GFP)

Figure 4



Additional Notes:

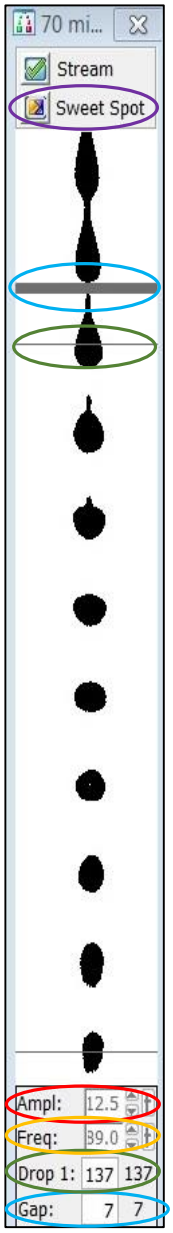
- Note the small square to the left of the sample name in the workspace. If the square is empty, there is no compensation matrix. If the square is a 3x3 grid, then a matrix has been applied. The grid icon is color-coded based on the matrix applied (see the Workspace matrices on the left side of Fig. 3).
- To adjust the compensation matrix generated on the cytometer, double-click on the grid icon next to the sample in the FlowJo workspace. This will open up the matrix, which you can adjust as in step 6.
- If cells were used for compensation controls (rather than beads), you will probably have to make adjustments to the FlowJo-generated Size and/or Positive gates in the Compensation Wizard (Fig. 2).

A Guide to the Sort Stream, Part 1

By Steve Slater

In cytometry, the **Sort Stream** is the continuous column of sheath fluid (containing particles) that is broken into small, identically-sized droplets through the actions of a piezoelectric transducer.

The "magic" of electrostatic sorting takes place around the moment a new droplet breaks off from the stream: when a droplet containing a desired cell is about to break off, the cytometer applies a positive or negative charge to the *entire* stream. Immediately after the droplet breaks off from the stream, the cytometer removes the charge potential from the stream, *but the newly formed droplet retains the charge*. The droplet then falls through an electrical field generated by the cytometer's deflection plates, which redirects the path of the charged droplet so that it (along with the desired cell) lands in a collection tube; uncharged droplets fall straight through the electrical field into the waste.
So, a good sort stream is key to a successful sort. Instrument settings control the characteristics of the sort stream. To the right is the sort stream window from BD's Diva software, which is a digital image of the sort stream. The black blobs are droplets that have broken off from the stream (note the two droplets at the top of the window are still connected).



Here is a quick description of the key settings that control the droplet formation:

- **Freq** (yellow circle): The frequency, in kHz, at which droplets are formed. At a setting of 89.0, the cytometer generates 89,000 droplets per second. The frequency is affected by pressure and nozzle size; the lower the sheath pressure, the lower the drop frequency.
- **Ampl** (red circle): the voltage applied to the transducer to shake the stream into droplets.
- **Gap** (blue circles): the distance between the stream and the first broken droplet, as measured in pixels. Each nozzle size has a different ideal Gap value (for a 70 um nozzle, the idea Gap is between 5 and 8). If set incorrectly, the sort stream may be unstable, compromising the sort.
- **Drop 1** (green circles): the distance between the top of the sort stream window to the center of the first droplet, measured in pixels. *During a sort, the Gap and Drop 1 values must remain constant!*
- **Sweet Spot** (purple): A computer algorithm that, when enabled, automatically adjusts the frequency to ensure that every droplet breaks off at the same place (i.e., maintains a consistent Gap and Drop 1 distances). Think of it as cruise control for the cytometer.

Establishing and maintaining a good sort stream is key to the efficient recovery of highly pure cells. In the next newsletter, I'll address more advanced topics involving the sort stream.

Got a burning question about cytometry that you'd like us to address in a future newsletter? Send your question or topic request to DHVIflo@dm.duke.edu.

Please acknowledge the DHVI Flow Facility in your publications:
"Flow cytometry was performed in the Duke Human Vaccine Institute Flow Cytometry Facility (Durham, NC)."
Email a PDF of your paper acknowledging the DHVI Flow Facility to dhviflo@dm.duke.edu to earn a free hour of flow time!