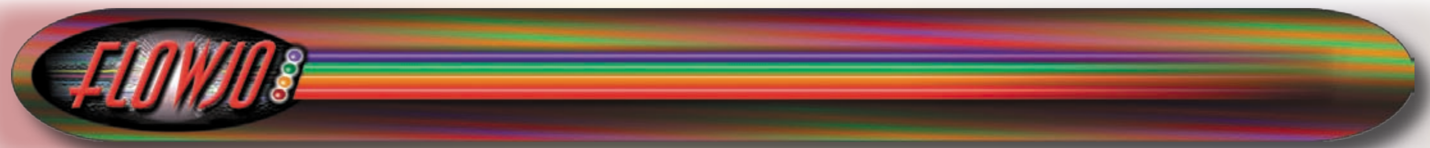


COMPENSATION

Abstract

The wide variety of new fluorochromes available increases the power of flow cytometry to discern phenotypically complex cell populations. Multi-color analysis requires compensation between most pairs of fluorochromes because their emission spectra overlap. Fluorochromes that can be excited by multiple lasers (such as tandem dyes) generate the need for cross-laser compensation that may not be possible on all instrumentation. As a result, many experiments now require post-collection software compensation.

FlowJo provides a simple interface for computing compensation based on a collection of samples each stained with a single fluorochrome (compensation controls). FlowJo uses matrix algebra to derive a compensation matrix based on a negative and positive population of cells defined for each parameter. This matrix can be applied to any or all samples within an experiment.

With the use of dyes from different manufacturers, or even different lots of dyes from the same manufacturer, it is generally necessary to have independent compensation settings for each panel of reagents used to stain the samples. FlowJo allows you to create all of the different settings you need, and then automatically remembers and applies the correct setting for each panel of stains.

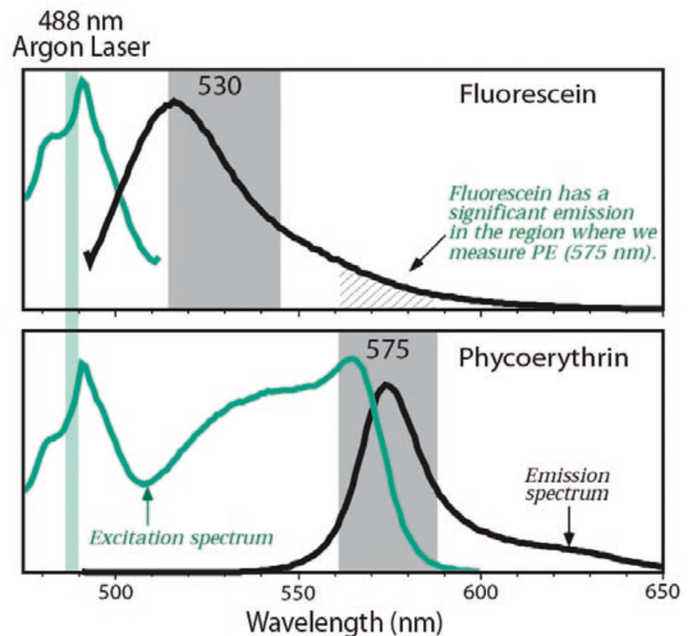
General Information

Fluorescent dyes excited by a 488 nm argon laser each emit with a characteristic spectrum: Fluorescein with an emission peak around 520 nm, Phycoerythrin (PE) at about 575 nm (right). In order to simultaneously measure these emissions, we choose optical or "bandpass" filters which collect specific wavelengths of light (shown in gray). However, it is impossible to choose filters that measure the emitted light of only a single dye. Thus, each detector actually collects light from multiple sources: principally from the desired (primary) fluorochrome, but some light from other (secondary) fluorochromes as well. The process of compensation is the correction for the light emitted by these secondary fluorochromes.

In other cases, as the number of fluorescence parameters increases, some instruments are not capable of compensating between all parameters (for instance, to correct for the spillover between fluorescein and Cy5PE).

You can compensate your data at the time you acquire data on the cytometer or you can have FlowJo calculate the compensation for you post-acquisition. In some cases, the compensation is inappropriately set during sample collection (although if the sample is over-compensated, there is no recourse). This Tech Note focuses on the mechanics of compensation using FlowJo; however, other sources exist to fully explain the underlying theory of compensation[1-3].

FlowJo computes the compensation matrix on control samples in a manner similar to manually setting the com-



pensation during collection. However, it does so automatically and completely, rather than requiring individual pair-wise settings to be selected for every pair of overlaps[4].

To do this, compensation control samples stained with each unique fluorochrome used in the experiment must be collected. Ideally, each compensation control stain labels only a portion of the cell sample, leaving an unstained population of cells in each tube.

More information on the entire compensation process can be found at:

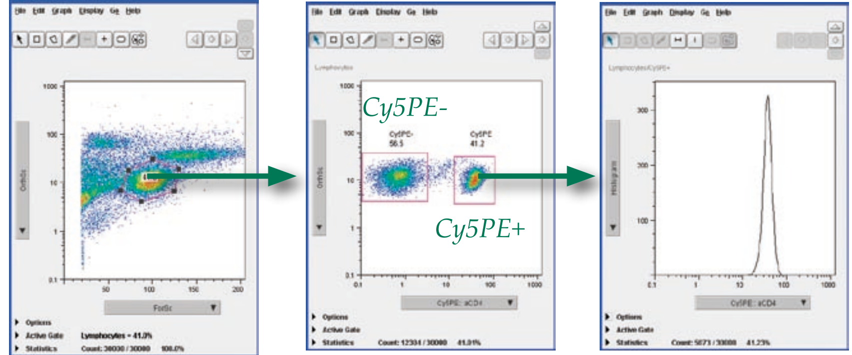
http://www.flowjo.com/v7/html/compensation.html

Procedure

1 Define a positive (stained) and negative (unstained) population for each singly stained compensation control sample.

Hint

The negative and positive population must share the same autofluorescence characteristics (e.g., Don't use a monocyte gate to compensate lymphocytes.)



Dragging the Lymphocyte gate onto a group applies this gate to all samples in that group.

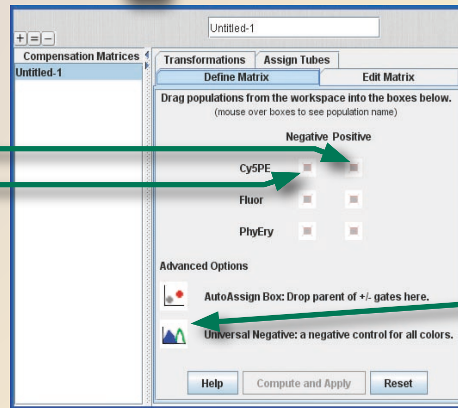
Group Name	Number of Samples
All Samples	
Lymphocytes	

Name	Statistic	#Cells
3-color.fcs		50000
Lymphocytes	41.0	20486
Cy5PE comp.fcs		30000
Lymphocytes	41.0	12304
Cy5PE	41.2	5073
Cy5PE-	56.5	6950
FITC comp.fcs		50000
Lymphocytes	39.9	19935
PE comp.fcs		50000
Lymphocytes	42.1	21051

2 Open the **Compensation Definition** box.

Select Open Compensation Editor from the Windows menu.

Platform ➔ Compensate sample... ➔ Define new matrix...



Drag the positive and negative subsets from the workspace to the Comp Definitions window.

Hint

Drag an unstained sample or negatively gated population to the Universal Negative *button. FlowJo puts this negative population into all the negative boxes.*

Drag the parent population (in this case Lymphocytes) to the Auto Assign *button. FlowJo automatically puts the "daughter" positive and negative populations into the correct boxes.*

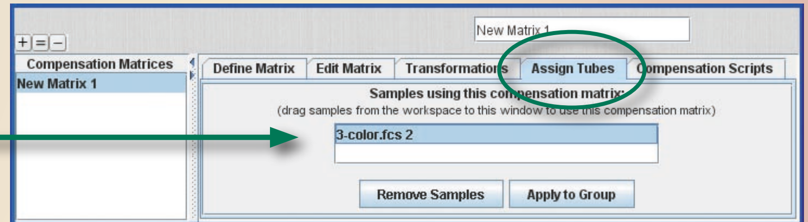
3 **Repeat Step 2 for each parameter that needs to be compensated.** Drag the pair of gated populations (positive and negative) to the Compensation Definition dialog window for each remaining parameter.

4 **Compute the matrix and save.** This matrix can be applied to other workspaces or be modified; however, a compensation matrix is generally valid only for the data files collected at the same time.

5 Apply the Compensation matrix to each sample or those in a group.

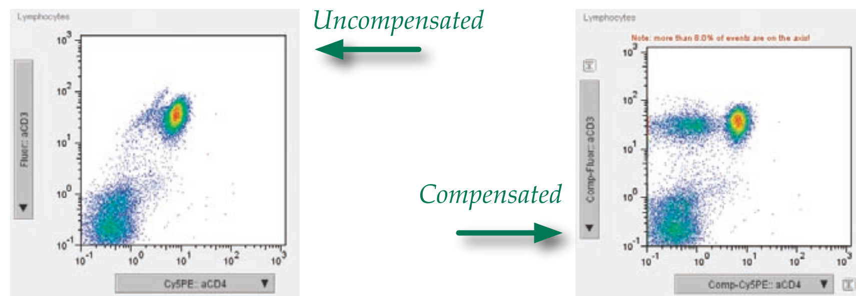
With the matrix selected in the list, and the Assign Tubes tab open: Drag files to which you want to apply the matrix into the white box on the Assign Tubes tab.

A colored bar next to the sample denotes that the sample has been compensated.



Select the target sample or group, go to Platform ➔ Compensate sample... and select the name of the matrix.

Each parameter (reagent) can be displayed as compensated or uncompensated. Compensated parameters are displayed in the graph window by default.



Uncompensated

Compensated

To view both compensated and uncompensated parameters:

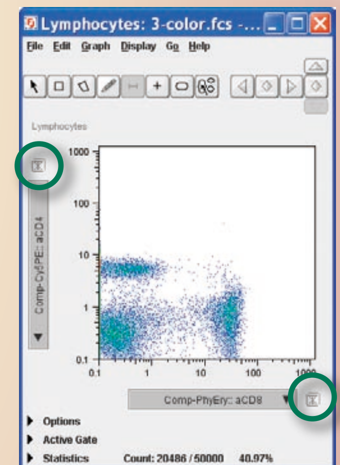
Check Show Uncompensated Parameters in the Display menu.

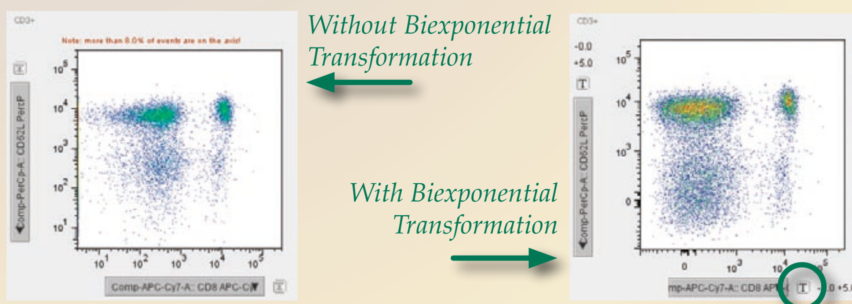
Hold down the Option key and select the drop-down menu on the axis label. Parameter names with brackets (< >) are compensated. Names without brackets are uncompensated.

6 To display Transformation for compensated parameters:

Select a compensated parameter in the graph's axis label drop down list. Click the transform button on the graph window.

Select Platforms ➔ Compensate Sample ➔ Define Transformation ➔ Compute Transformation





Without Biexponential Transformation

With Biexponential Transformation

Right click the transform button to access these options:

- Change transform values
- Auto-calculate extra negative decades
- Apply to all Comp-FITC-A
- Apply to all compensated parameters

Biexponential Display

Data from multicolor experiments may not appear properly compensated because the fluorescence parameters are displayed on a log scale where it is not possible to display zero or negative values. The resulting accumulation of cells on the axis makes it impossible to judge whether compensation is set correctly. You can display this data in a more intuitive manner by applying Biexponential transformation. Picket fencing on digital data can be visually smoothed using FlowJo's display transformation feature. More options exist to tailor the display. Additional negative decades can be added to the axis scale to accommodate large event clusters. Additional positive decades can be added to reduce the amount of the display devoted to negative events.
To emulate the display seen in DiVa software, open the Compensation Editor (Windows ➔ Open Compensation Editor ➔ Transformations tab) Change the width basis to 100.

For full details on Biexponential Transformation in FlowJo, visit **http://www.flowjo.com/v8/html/displaytransformfaq.html** For specifics regarding 32 bit data files visit **http://www.flowjo.com/v8/html/displaytransformdigital.html**

- The positive population stain for each compensation control should be at least as bright as the brightest stain in the samples.
- In some cases, you will need multiple compensation matrices for the same experiment. Different lots of tandem dyes, like Cy7PE, can have different spectra and thus require different compensation. You will need to collect a single stain control for each reagent and construct separate matrices.
- Once a matrix is computed, it will appear in the Compensation Editor under its own name. To apply this matrix to another Workspace, the matrix file must first be saved (Windows ➔ Open Compensation Editor ➔ Edit Matrix ➔ Save to File) then loaded into another Workspace (Windows ➔ Open Compensation Editor ➔ Edit Matrix ➔ Load From File). Excercise caution in applying a compensation matrix file to a different experiment because the instrument changes between experiments.
- A sample's compensation matrix can be changed by applying a new matrix (with a new name) to that sample. This replaces the old matrix.
- You can edit the compensation matrix within FlowJo. (Windows ➔ Open Compensation Editor ➔ Edit Matrix). It is not recommended! Overcompensating is a common result of compensating by eye.

Hints

- Compensation is a property of the fluorochromes and not the cell type! The matrix is valid for all cell types as long as the PMT voltages are held constant throughout the experiment.
- A single unstained sample can be used for all negatives: drag the unstained sample onto the Universal Negative box in the Compensation Definition Dialog.

FlowJo is a product of **Tree Star, Inc.**

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Links and References

FlowJo compensation information:
www.flowjo.com/reference.html

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