



Abstract

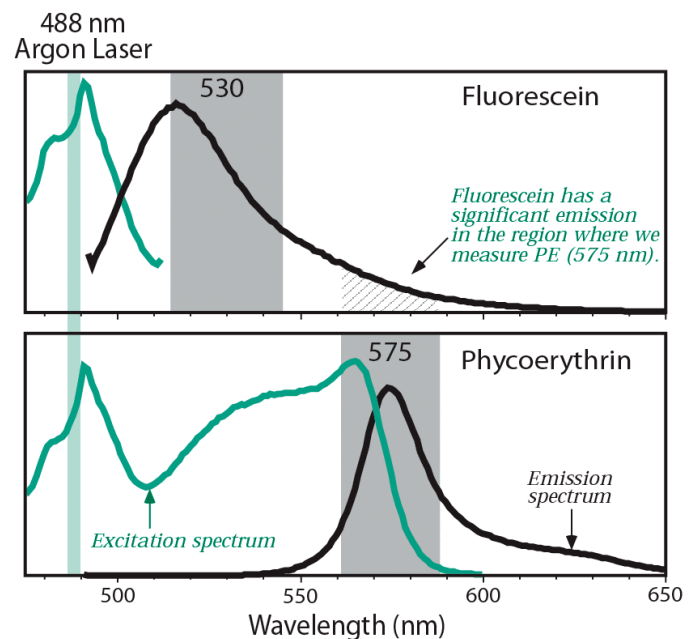
The wide variety of new fluorochromes available has increased the power of flow cytometry to discern phenotypically complex cell populations. Multicolor analysis requires compensation between most pairs of fluorochromes because of the overlap in emission spectra. Furthermore, fluorochromes that can be excited by multiple lasers (such as tandem dyes) generate the need for cross-laser compensation that may not be possible on all instruments.

FlowJo provides a simple interface for computing compensation based on a collection of control samples, each stained with a single fluorochrome (single stain 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. Post-acquisition compensation is most useful for cytometrists acquiring data on machines that allow only for manual compensation, for users who pay for time on the cytometer, or for complex analysis with multiple matrices where organization of data and matrices can be made easier in FlowJo. With the use of dyes from different manufacturers, or even different lots of tandem 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 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 a characteristic spectrum: Fluorescein (FITC) has 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 detect 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.

You can compensate your data at the time of acquisition on digital cytometers, or you can have FlowJo calculate the compensation for you post-acquisition. In some cases, the compensation is inappropriately set during sample collection (if the sample is over-compensated, there is no recourse). This Tech Note focuses on the mechanics of compensation using FlowJo; however, other sources more 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 compensation during collection. However, it does so automatically and non-heuristically, rather than requiring individual pair-wise settings to be selected for every pair of overlaps. [4]



To do this, a singly stained compensation control sample must be collected for each unique fluorochrome used in the experiment. Ideally, each compensation control stain labels only a portion of the cell sample, leaving an unstained population of cells in each tube. For any singly stained control that lacks a negative population, a sample of unstained cells can be used as a “universal negative” control.

More information on compensation can be found at:
<http://www.flowjo.com/html/compensation.html>

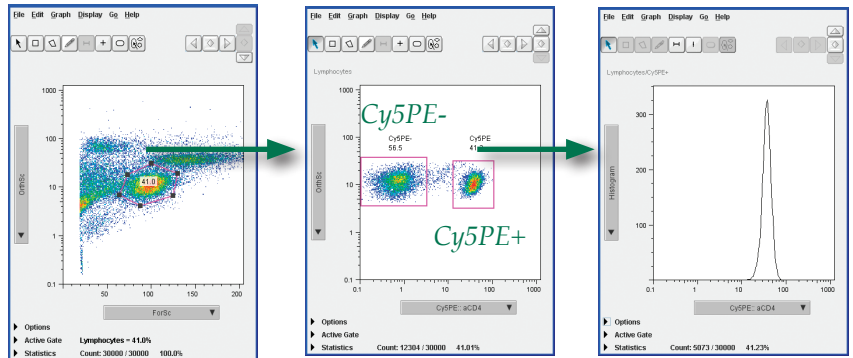


Procedure

- 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).



Name	Statistic	#Cells
3-color.fcs		50000
⊗ Lymphocytes	40.9%	20456
⊗ Cy5PE comp.fcs		30000
⊗ Lymphocytes	41.0%	12294
⊗ Cy5PE+	41.2%	5074
⊗ Cy5PE-	56.5%	6941
FITC comp.fcs		50000
⊗ Lymphocytes	39.8%	19497
⊗ CD8+	19.6%	9811
⊗ CD8-	76.5%	15216
PE comp.fcs		50000
⊗ Lymphocytes	42.1%	21031
⊗ PE+	22.8%	4797
⊗ PE-	76.9%	16117

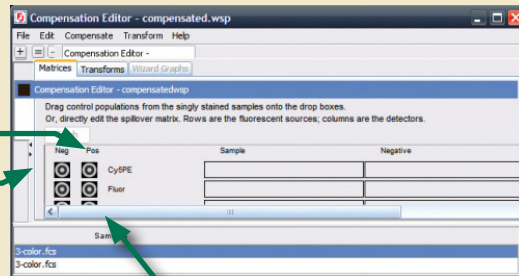
Dragging the Lymphocyte gate onto a group applies this gate to all samples in that group.

- 2 Open the Compensation Definition box.



Select **Compensation** from the **Tools** menu.

Platform → **Compensate sample...** → **Define new matrix...**



Drag the positive and negative subsets from the workspace to the Compensation window.

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

Hint

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.

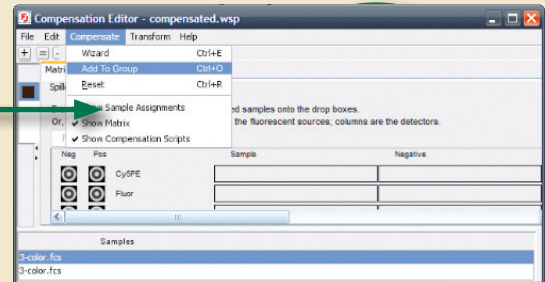
- 3 Repeat Step 2 for each parameter that needs to be compensated. Drag the pair of gated populations (positive and negative) to the Compensation Definition dialog window for each remaining parameter.

- 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 to samples in a group.

Group	Size	% of Group	# of Cells
All Samples	4		
Lymphocytes			
3-color.fcs	50000		
Lymphocytes	40.9%	20486	
Cy5PE comp.fcs	30000		
Lymphocytes	41.3%	12294	
Cy5PE+	5074		
Cy5PE-	6941		
FITC comp.fcs	50000		
Lymphocytes	39.8%	19897	
CD8+	19.6%	3901	
CD8-	76.5%	15216	
PE comp.fcs	50000		
Lymphocytes	42.1%	21031	
PE+	22.8%	4797	
PE-	76.9%	16177	

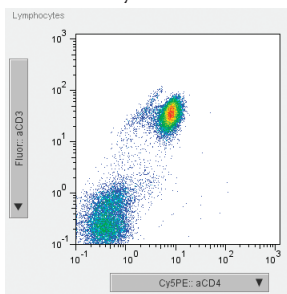
With the matrix selected in the list, and the **Assign Tubes** tab open: Drag files to which you want to apply the matrix into the white box on the **Assign Tubes** tab.



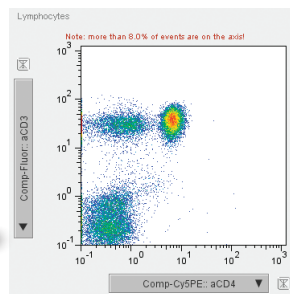
A colored matrix next to the sample denotes that the sample has been compensated.

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

To view compensated and uncompensated parameters:



Uncompensated



Compensated



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.

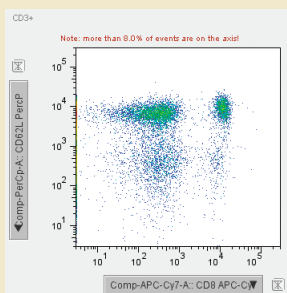
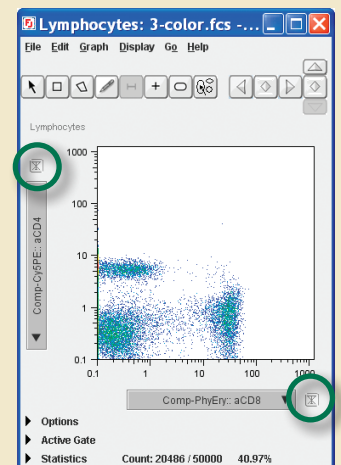
6 To display Transformation for compensated parameters:



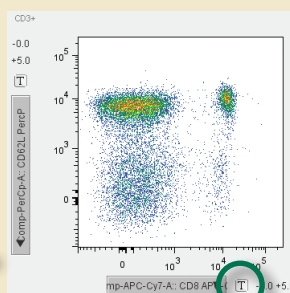
Select a compensated parameter in the graph's axis label drop-down list. Click the Transform button on the graph window.



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 to be properly compensated. Because the fluorescence parameters are displayed on a log scale (on which it is not possible to display zero or negative values), the accumulation of cells on the axis makes it impossible to judge whether compensation is set correctly. You can display these 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.

- 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, such as 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 and then loaded into another Workspace. Because the instrument settings may be different, exercise caution in applying a compensation matrix file to a different experiment.
- 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 often the result of compensating by eye.

For more on Biexponential Transformation: <http://www.flowjo.com/vX/en/displaytransformfaq.html>

For specifics regarding 32-bit data files : <http://www.flowjo.com/vX/en/displaytransformdigital.html>

Links and References

1. Roederer M. *Compensation: A Perspective*. www.drmmr.com
2. Roederer M. (1999). *Compensation*. In: Current Protocols in Cytometry. Robinson JP, Darzynkiewicz Z, Dean PN, Dressler LG, Rabinovitch PS, Stewart CC, Tanke, HJ and Wheelless, LL (ed.), John Wiley & Sons, Inc., New York.
3. Roederer M. *Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats*. Cytometry 2001 Nov 1:45(3):194-205.
4. Bagwell CB, Adams EG. *Fluorescence spectral overlap compensation for any number of flow cytometry parameters*. Annual N Y Academy of Sciences 1993; 677:167-184.
5. Parks R, Roederer M, Moore W., *A new "logicle" display method avoids deceptive effects of logarithmic scaling*. <http://herzenberg.stanford.edu/Publications/Reprints/LAH517.pdf>
6. Herzenberg, L. et al. *Interpreting flow cytometry data: A guide for the perplexed*. <http://herzenberg.stanford.edu/Publications/Reprints/LAH516.pdf>

