FlowJo for Antibody Titrations: Separation Index and Concatenation

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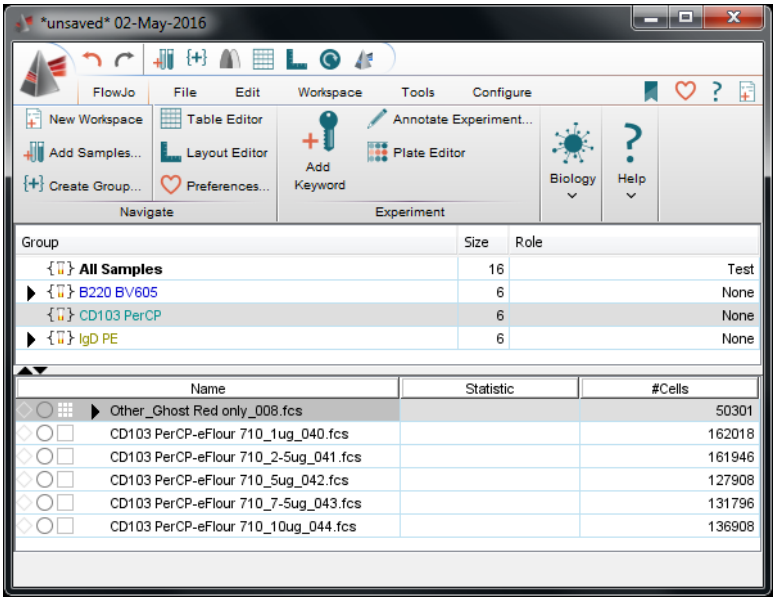
Group Samples by Antibody

After importing the FCS files into FlowJo, create a group for each antibody in the experiment. Groups can be defined with Sample Inclusion Criteria to automatically grab samples, or simply named and populated manually by dragging files from All Samples into the newly created groups. Each group may also contain the "viability only" or "no antibody" or "unstained" sample.



Sample Order

The order of samples within a group will determine the order of those samples in the final concatenated dot plot derived later in these instructions. A straightforward way to order samples is by time. Right-click on the column names and choose Edit Columns to access a window in which the Time column can be added. Then click in the new Time column to order samples based on that value.

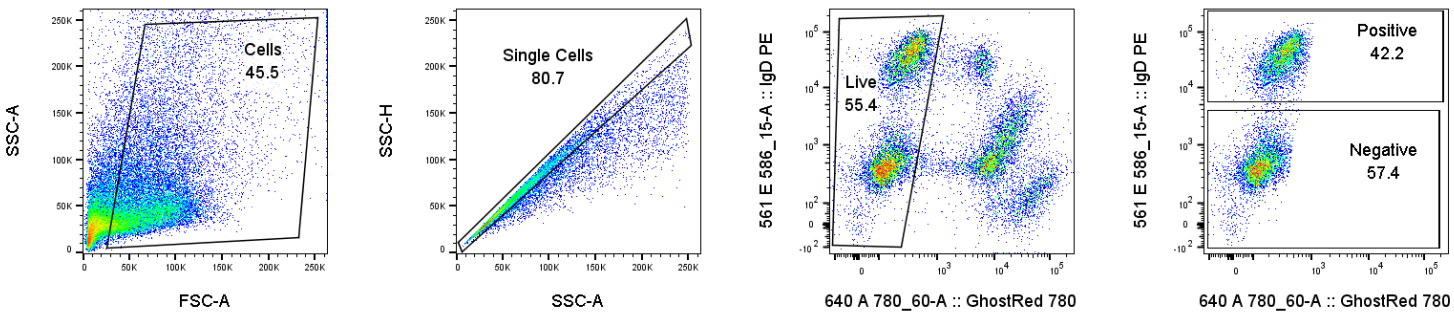
It is also possible to add a custom Keyword (not described here) to determine the order. If samples are organized by manually dragging them into groups, the listed order comes from the order in which samples are added to the group.

Gate Populations

Within each group, gate for live, single cells. Then draw broad gates for the Positive and Negative populations. It may be necessary to adjust the Positive and Negative gates for each sample if there are large shifts in the populations.

No-Antibody Control

Note that the sample with no antibody (viability dye only) will need to be gated for each group in turn. Changes made in one group will apply to that sample in all groups.



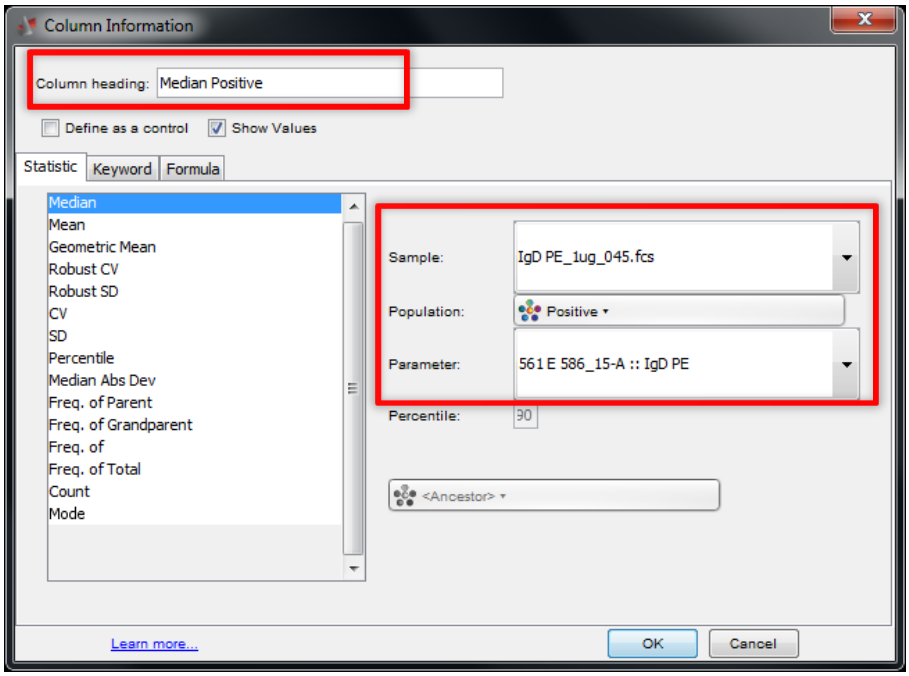
Calculating Separation Index in a Table

The Separation Index (SI), defined by the equation below, is a metric for evaluating the results of the staining. Each term in the equation refers to a statistic derived from the fluorescence intensity of the antibody staining. The higher the Separation Index value, the better the separation between positive and negative populations.

$$Separation\ Index = \frac{MedianPositive - MedianNegative}{(84\%Negative - MedianNegative)/0.995}$$

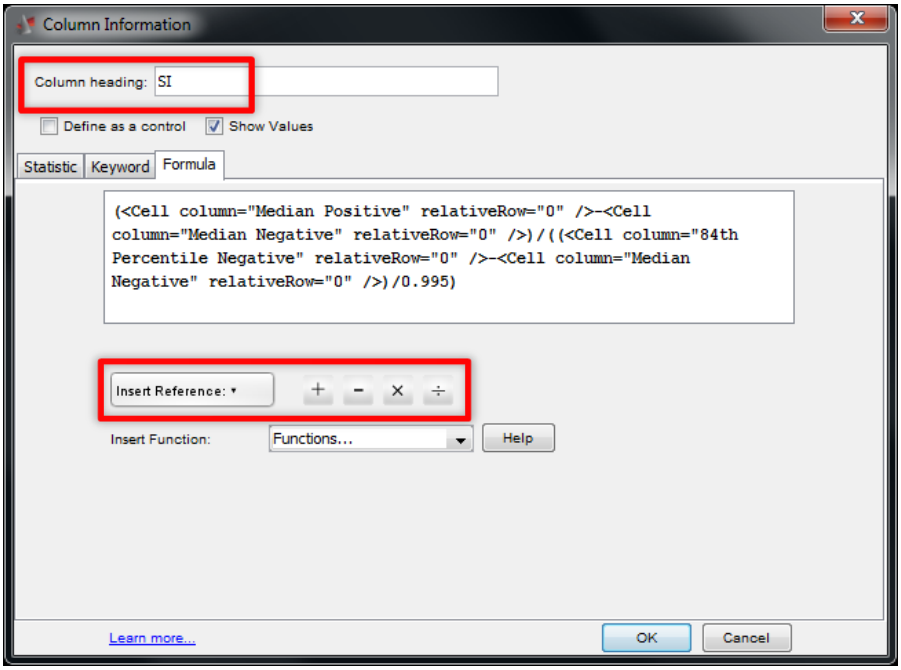
Start by adding the necessary statistics to the table:

1. Click **Table Editor** in the FlowJo ribbon to open the FlowJo Tables window
2. Double-click on the name of the table and change it to indicate the antibody in question
3. In the Edit ribbon, click **Add Column** to open the Column Information window
4. Name the column "Median Positive"
5. Choose Median from the list of available statistics
6. **Sample** can be any of the tubes from the current group (antibody) being analyzed
7. **Population** is the gate from which to calculate the statistic (e.g. Positive)
8. **Parameter** is the channel in which the antibody signal was collected
9. Repeat and adapt steps 3-8 to create "Median Negative" and "84th Percentile Negative" columns

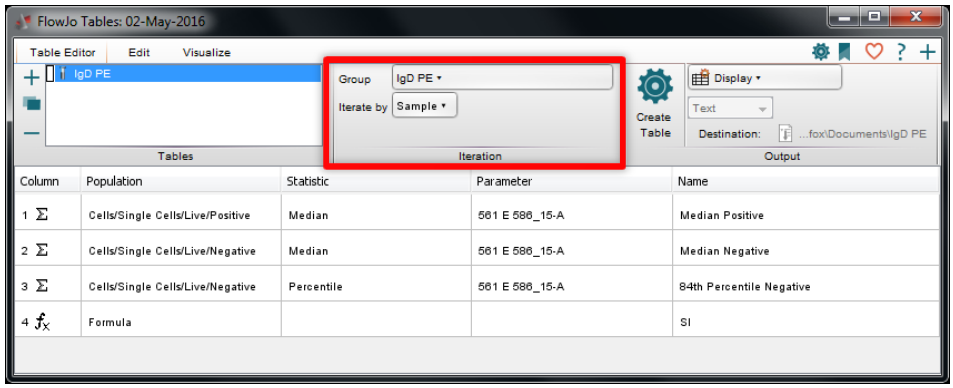


Make a fourth column to calculate the Separation Index. When entering the formula, make sure to use the menus to insert terms and functions; use the keyboard only to type parentheses and constants.

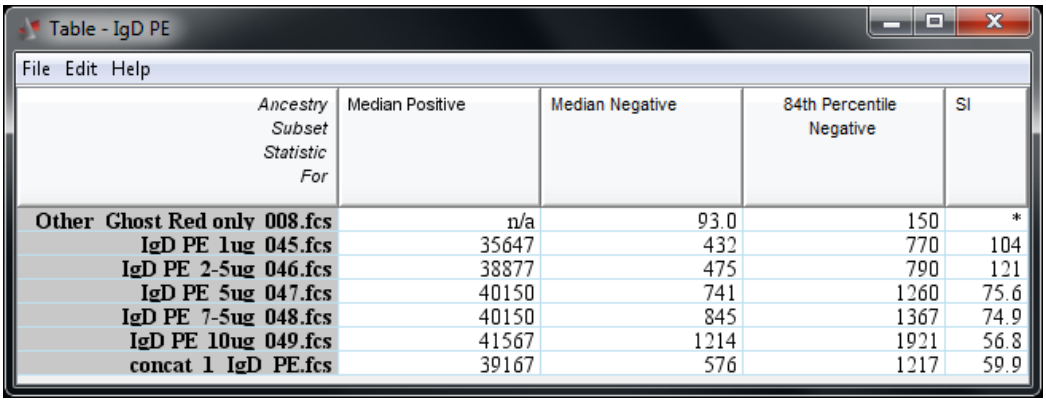
10. In the Edit ribbon, click **Add Column** to open the Column Information window
11. Name the column "SI"
12. Click the **Formula** tab
13. Click in the text box to begin creating the formula
14. Type "("
15. From the **Insert Reference** drop-down, choose Median Positive
16. Click the – button (subtraction)
17. From the **Insert Reference** drop-down, choose Median Negative
18. Click at the end of the formula and type ")"
19. Click the ÷ button (division)
20. Click at the end of the formula and type "(("
21. From the **Insert Reference** drop-down, choose 84th Percentile Negative
22. Click the – button (subtraction)
23. From the **Insert Reference** drop-down, choose Median Negative
24. Click at the end of the formula and type ")"
25. Click the ÷ button (division)
26. Click at the end of the formula and type "0.995)"
27. Click **OK**



28. In the FlowJo Tables window, in the Iteration section, set the **Group** to match the antibody in question and set **Iterate by** to Sample; note that the appearance of the Iteration section changes based on the size of the window

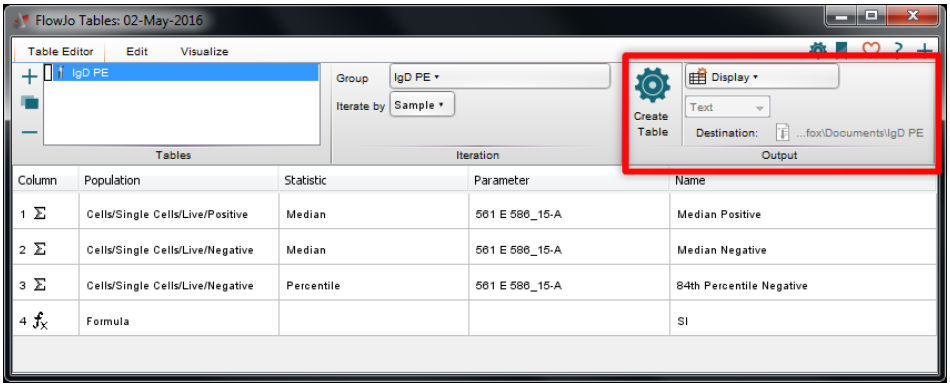


The final table definition should look like the example above, and clicking **Create Table** will generate a table like the one below.



Ancestry *Subset* *Statistic* *For*	Median Positive	Median Negative	84th Percentile Negative	SI
Other Ghost Red only 008.fcs	n/a	93.0	150	*
IgD PE 1ug 045.fcs	35647	432	770	104
IgD PE 2-5ug 046.fcs	38877	475	790	121
IgD PE 5ug 047.fcs	40150	741	1260	75.6
IgD PE 7-5ug 048.fcs	40150	845	1367	74.9
IgD PE 10ug 049.fcs	41567	1214	1921	56.8
concat 1 IgD PE.fcs	39167	576	1217	59.9

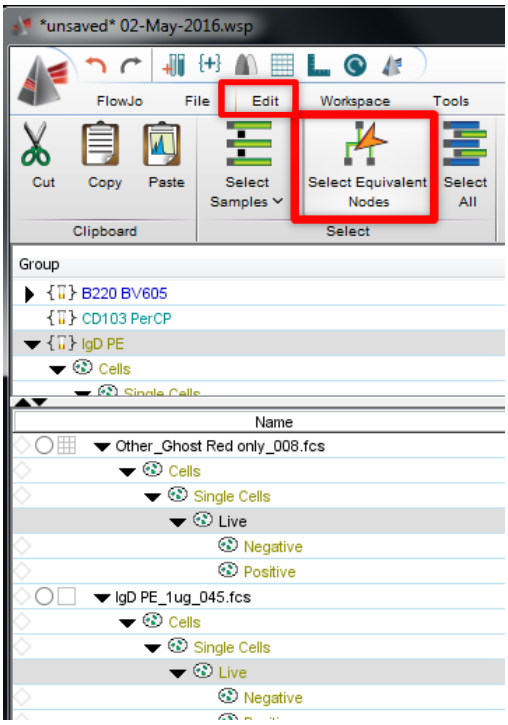
The table can be generated for display in FlowJo (as above), sent to the current FlowJo Layout, exported as a File, etc., depending on the selections made in the Output section of the FlowJo Tables window.

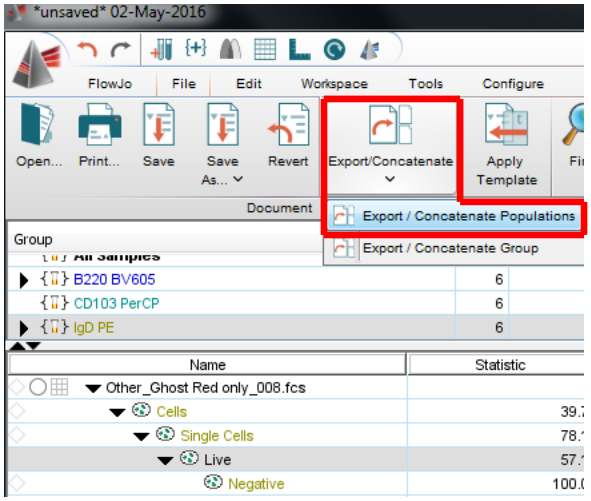


Concatenating Populations for Visualization

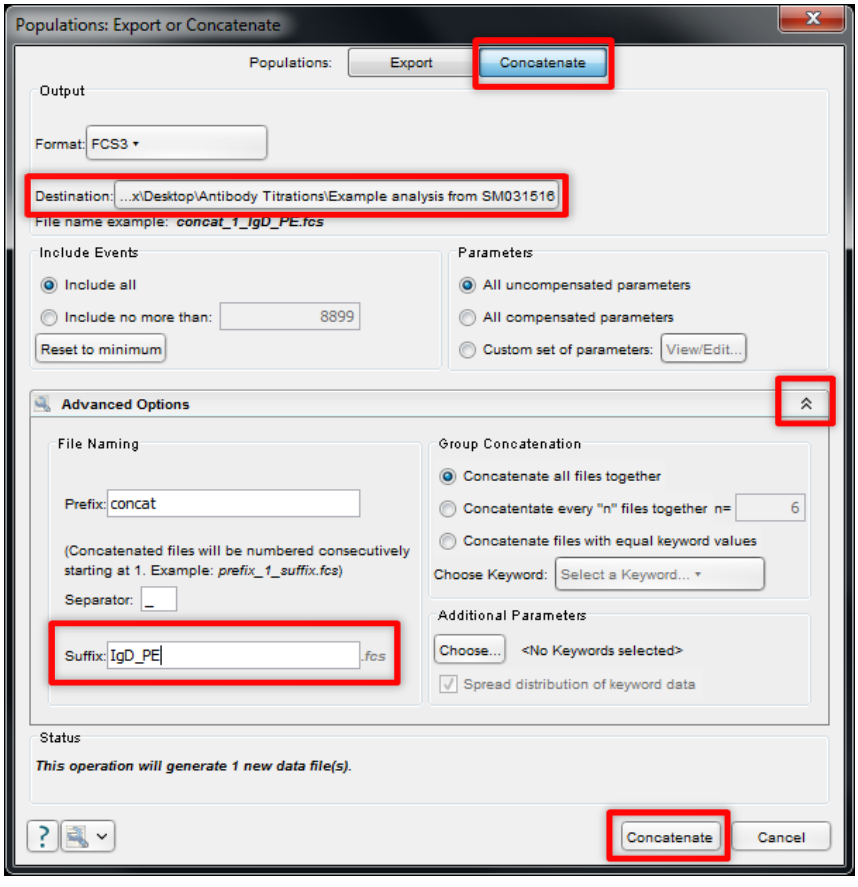
Combining all the live, single cells from each tube of the titration can help with visualizing the data.

1. In the list of samples for the current antibody group, click to select the gating level for live, single cells from one sample
2. In the Edit ribbon, click the **Select Equivalent Nodes** button to select the same gating level for all samples in the group
3. In the File ribbon, click **Export/Concatenate** and Choose Export/Concatenate Populations

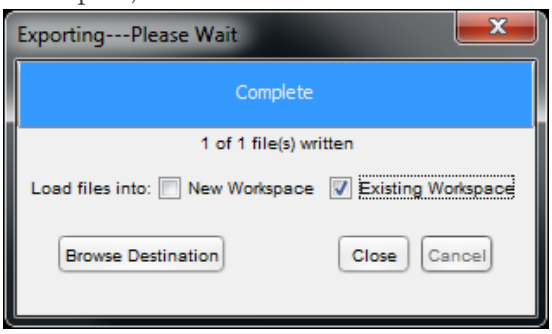




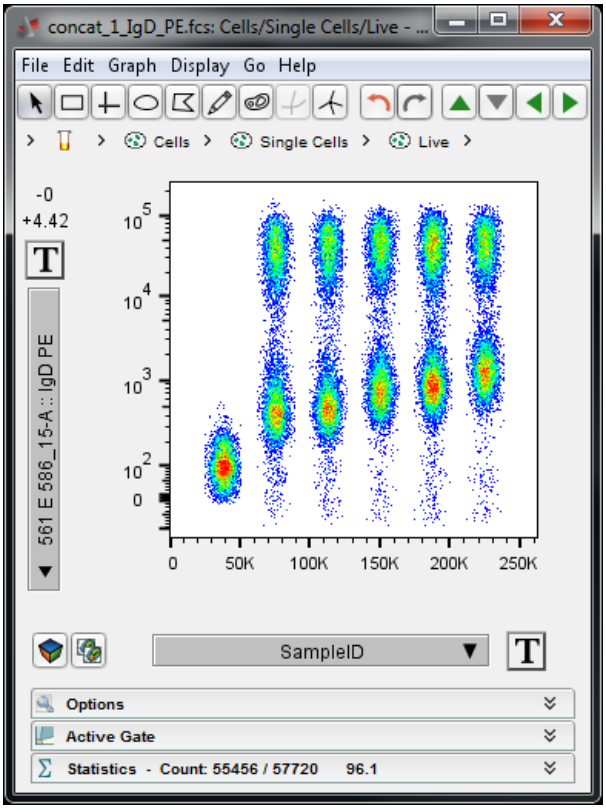
4. Choose the **Concatenate** option at the top of the window
5. Click in the **Destination** box to choose a location for the concatenated file
6. Expand the Advanced Options section and add a suffix indicating the antibody
7. Click the **Concatenate** button at the bottom of the window



8. After the new file is written, choose whether to open it in a New or Existing Workspace, then close the windows used to configure the concatenated file



9. In the main FlowJo workspace window, find the new concatenated file and open a plot of live, single cells
10. Change the axes to display the antibody channel on the Y axis and the Sample ID on the X axis



Sample order in this plot is determined by the order in the concatenated file, described in the first section of these instructions.