



Data Analysis Software for Flow Cytometry
Version X User Documentation

Windows/Mac user documentation

Basic Tutorial



FlowJo was written by Adam Treister and Mario Roederer beginning in 1996, based on concepts developed at the Herzenberg Laboratory at Stanford. We are indebted to our active and enthusiastic users worldwide for their ideas, discussions and tireless testing of new versions.

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- Basic Tutorial •
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Revision date: 16 April 2013
version 10.0.6

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Introduction - FlowJo Basic Tutorial

FlowJo is an integrated environment for viewing and analyzing flow cytometric data, presented in the form of a Workspace. The Workspace contains a list of all of the data samples that you load, the gates, statistics and other analyses that you apply, and the table and graphical layout that you design. The Workspace is saved as a FlowJo document on your hard disk; when you reopen the document, you will see the status of your analysis as it was when it was last saved.

This tutorial is designed to introduce you to the basics of the program. Reading through it, you will learn how to operate FlowJo. Run the program as you perform the steps in the tutorial so that you can get the best feel for how the program works. As you watch FlowJo perform various operations such as creating new graphs, statistics, tables, or graphical layouts, you will see how fast and easy FlowJo is to use.

This tutorial is designed as an introduction to FlowJo. The 8-color PBMC Advanced Tutorial provides more detail on functionality. Furthermore, FlowJo is capable of much more than simply can't be covered in an introduction such as this (for example, there are analysis platforms to perform DNA/Cell Cycle analysis, Kinetics analysis, Proliferation and Population Comparison). You can learn more about FlowJo through the online help tool. Whenever you ask for help from FlowJo by pressing the question mark button in the interface of any window, it launches a web browser and accesses help pages about the part of the program you are using. You can navigate through the help pages to find out more about all of the aspects of FlowJo. In addition, FlowJo.com contains a page of FAQ's (Frequently Asked Questions), tutorial videos on many FlowJo topics, and the Daily Dongle, a searchable blog discussing all things FlowJo.

As a note, we are pleased to be able to frequently update FlowJo to provide new features and analysis capabilities. Therefore, it is possible that the graphics shown in this tutorial may not exactly match the windows that you see when you run the most recent version of FlowJo. You can always download the most recent version of FlowJo from: <http://flowjo.com/download/index.html>

Getting Started

To begin this tutorial you will first need to install FlowJo. The easiest way to do this is to download the installer from our website: <http://www.FlowJo.com/download/index.html>

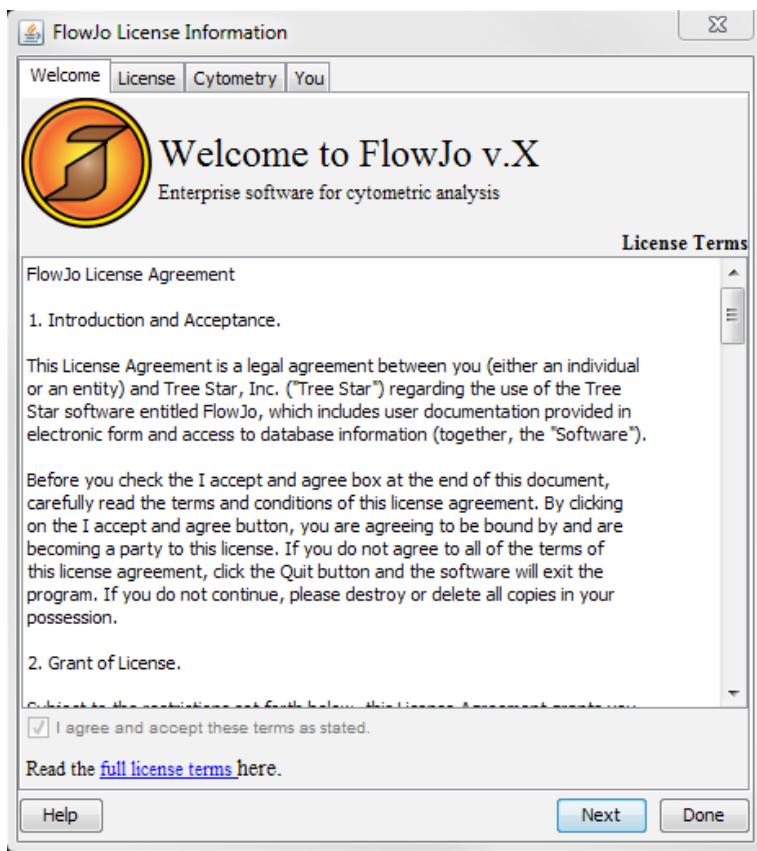
You can select your platform (Mac or PC) at the top of the page. For a PC, download the installer.exe file and open it to install the program. Choose to place a shortcut on the desktop, and when the installer finishes, double click on the FlowJo shortcut icon to launch the program.

For a Mac, download the installer.zip file and double click it to extract the program. Once the zip file is extracted, double click on the icon to launch FlowJo.

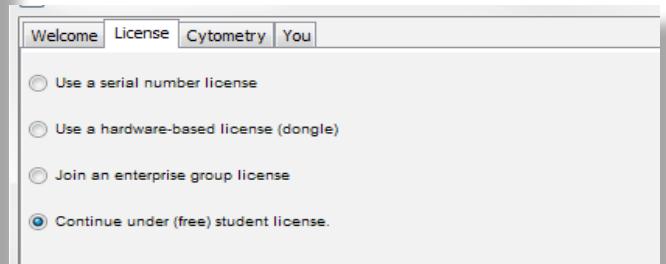
You can download the tutorial and Sample Data here : <http://flowjo.com/home/tutorials>

You have downloaded the full version of FlowJo, however, until a serial number is provided, FlowJo will only load specially enabled demonstration data files, such as those provided for this tutorial. To obtain a trial 30-day serial number so that you can try FlowJo using your own data simply fill out the form at:
<http://www.FlowJo.com/FLS/registration/trial.html>

When you launch FlowJo for the first time, the FlowJo license information window will pop up. In the welcome tab (see screenshot below), you must agree to the license terms and click next.



This takes you to the institution tab where you can select and input the type of license you have. For the purposes of performing this tutorial, you need only to select "Continue under (free) student license" and click next.



If you have an individual license that you would like to use, you can input your serial number in the window (below left). If you have a dongle (a physical thumb drive that has a license key on it) plugged into your computer, FlowJo will detect it automatically and allow you to activate the program. If you have an enterprise group license, you can configure the connection here as well (below right).

For more information about licenses, visit www.flowjo.com/home/licenseoptions.html

The screenshot shows the 'License' tab of the FlowJo interface. On the left, under 'Use a serial number license', there is a text input field containing 'EB1132C92B0A' and a dropdown menu showing 'S11Ncpa1PxYc82Gg'. A red arrow points to the 'Use Serial Number' button. Below this, a red text overlay says 'Click here to use a serial number'. On the right, there are three radio button options: 'Use a serial number license' (selected), 'Use a hardware-based license (dongle)', and 'Join an enterprise group license' (selected). Under 'Join an enterprise group license', there are fields for 'Configure your connection below or drag an institution configuration into this box.' and 'Host:Port'.

The next tab is “Cytometry” (below left), which allows you to fill out a little information about the type of experiments and analysis you do so FlowJo can automatically tune your default settings. For the purpose of this tutorial, you can leave this as is for now and click next.

The last tab titled “You” asks a little more about your FlowJo usage. Make sure ‘Experienced’ is selected under FlowJo Knowledge section so you will have access to all options in the tutorial. Click done. FlowJo has now successfully launched and you can begin the tutorial.

The screenshot shows the 'You' tab of the FlowJo interface. On the left, there are several configuration sections: 'Species' (Mouse, Human, Primate), 'Cell Types' (Leukocytes, T Cells, B Cells, Cytokines), 'Cytometer Usage' (Single instrument, Multiple instruments), and 'Primary Cytometer' (set to MacsQuant). In the center, there are three sliders: 'Log N Samples in an Experiment' (0 to 4), 'Number of fluorescent stains' (3 to 18), and 'Log N Events in a Sample' (3 to 8). Below these is a summary: 'Base Memory Requirements: 7.2MB', 'Available RAM: 954MB', and 'Percentage: 0.7%'. At the bottom are 'Help', 'Purchase', 'Next', and 'Done' buttons. On the right, there are sections for 'Number of people who share this computer' (Only One selected), 'Cytometer Usage' (I generally use FlowJo on this computer selected), 'FlowJo Knowledge' (Experienced selected), 'Data File Location' (FCS files are found on my hard drive selected), and 'Spread the Love / Meet your Colleagues' with social media icons for Fluorish, Facebook, YouTube, Google+, LinkedIn, and Twitter. At the bottom are 'Help', 'Purchase', 'Next', and 'Done' buttons.

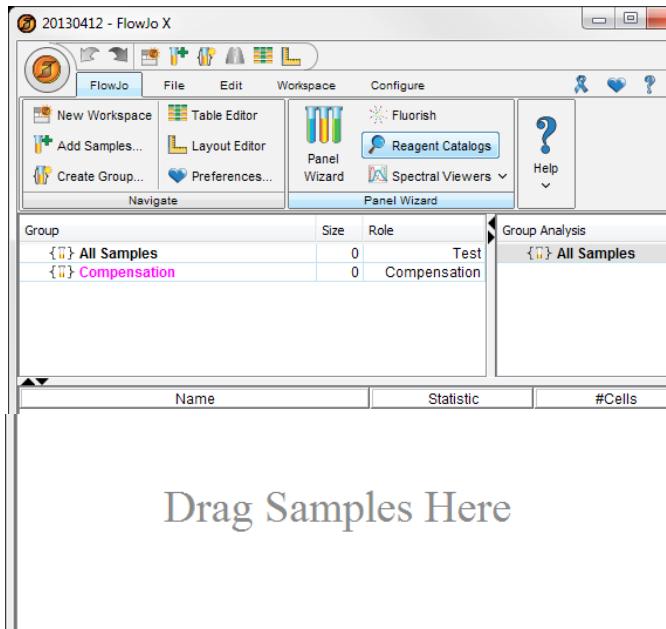
The data and accompanying workspaces can be acquired from: <http://www.flowjo.com/home/tutorials/>

Following this link will bring you to a page where you can download the Basic Tutorial zip file with FCS (flow cytometric standard) files and a PDF of the tutorial. Save these files to disk and double-click on the compressed file to extract it on a Mac and right click to extract them on a PC.

Lesson 1 - The Workspace

The data we are using for this tutorial are from a basic antibody titration experiment. There are five samples with varying dilutions of PerCP-Cy5.5 anti-CD8 antibody from 1:100 to 1:1600. When you open FlowJo, you will first be presented with the Workspace, which is your starting point in FlowJo. The ribbons contain tabbed bands, such as the FlowJo band above, and scrolling through the various bands will display the different tasks in each band. Each band can be customized with your preferred tasks which is a great way to customize any band with features you most commonly use.

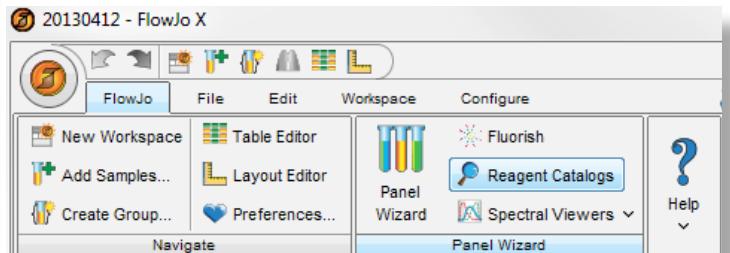
This is a great dataset, not only to explore the basic functionality of FlowJo, but to also begin to understand how to properly titrate antibodies (a necessity for ensuring proper results and preventing waste).



Lesson 1 Fig.1 - workspace

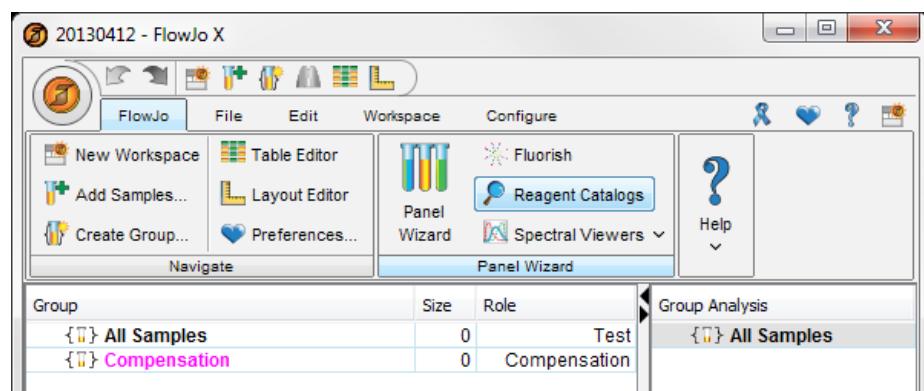
The workspace is your starting point in FlowJo. There are three major components:

- 1) The Ribbons, which organize the tasks in FlowJo at the top of the interface:



Lesson 1 Fig.2 - ribbons

To remove a task, expand the band to full view, grab it using its text label and drag it out of the ribbon.



Lesson 1 Fig.3 - bands

To add a band, click on the Ribbon Configuration icon and simply drag the icon to your ribbon.



Lesson 1 Fig.4 - ribbons menu

2) The Group section:

Groups in FlowJo allow you to organize samples for common analyses. For example, you may want to apply an analysis strategy (batch) to a group of commonly stained samples. To learn more about groups, please see the 8 color PBMC Tutorial. In this tutorial, we will not use groups for the sake of simplicity. We will discuss batching in future lessons of this tutorial.

3) The Sample section:

This section will contain a list of your samples.

In fact, let's go ahead and load the samples. Go to the Basic Tutorial folder and drag the files into the workspace:

Your workspace should now look like this:

You'll notice the files are named with the default file name keyword (\$FIL), which was written by the cytometer to the FCS sample file.

Keywords are “metadata” contained within the file, in addition to the fluorescence values of every event. Keywords are a great way to annotate data during acquisition. (A list of commonly used keywords and use cases can be found in your workspace preferences.) Many of these keywords are recorded during data collection and every acquisition program allows you to add additional keyword values. Keywords can also be added in the FlowJo workspace.

Keyword naming convention can be changed in the workspace preferences if you prefer to have a different keyword used as your naming convention. To explore the keywords of your files, right click on a file and click Inspect.

Group	Size	Role
{} All Samples	0	Test
{} Compensation	0	Compensation

Lesson 1 Fig.5 - groups

Name	Statistic	#Cells
CD8a_1,3a,100_A01.fcs		20000
CD8a_1,3a,200_A02.fcs		20000
CD8a_1,3a,400_A03.fcs		20000
CD8a_1,3a,800_A04.fcs		20000
CD8a_1,3a,1600_A05.fcs		20000

Lesson 1 Fig.6 - adding samples to workspace

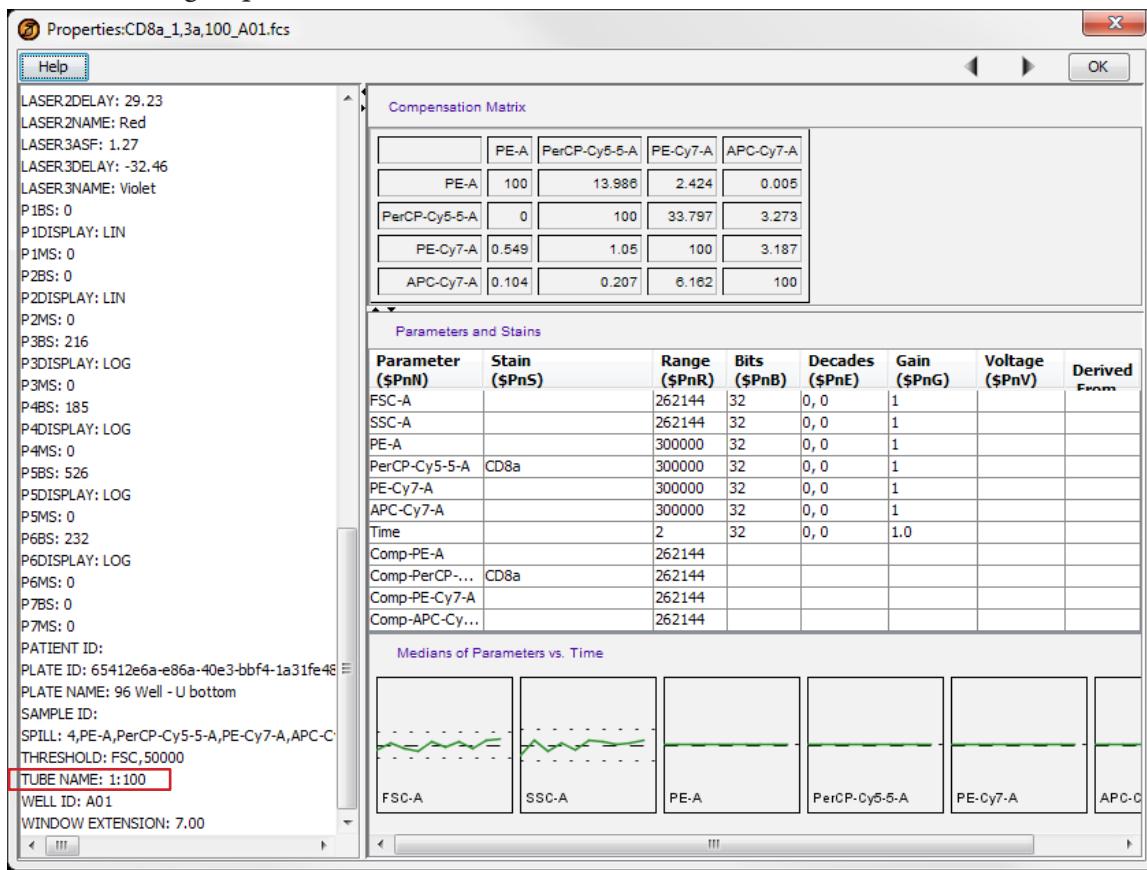
Name	Statistic	#Cells
CD8a_1,3a,100_A01.fcs		20000
CD8a_1,3a,200_A02.fcs		20000
CD8a_1,3a,400_A03.fcs		20000
CD8a_1,3a,800_A04.fcs		20000
CD8a_1,3a,1600_A05.fcs		20000

Lesson 1 Fig.7 - samples in the workspace

Copy	Ctrl+C
Paste	Ctrl+V
Rename	Ctrl+R
Inspect	Ctrl+I
Select Equivalent Nodes	Ctrl+Shift+E
Clear	Ctrl+Delete
Add Keyword	Ctrl+Shift+I
Add Statistic...	Ctrl+B
Copy value to group	
Reset Column Widths	
3D Viewer	Ctrl+3
Derive Parameters...	Ctrl+Shift+D
Export	Ctrl+E
Copy analysis to group	Ctrl+Shift+G

Lesson 1 Fig.8 - sample right click options

You can explore the keyword values of your file in this interface and we are particularly interested in a specific keyword we entered during acquisition of these data called ‘TUBE NAME’.

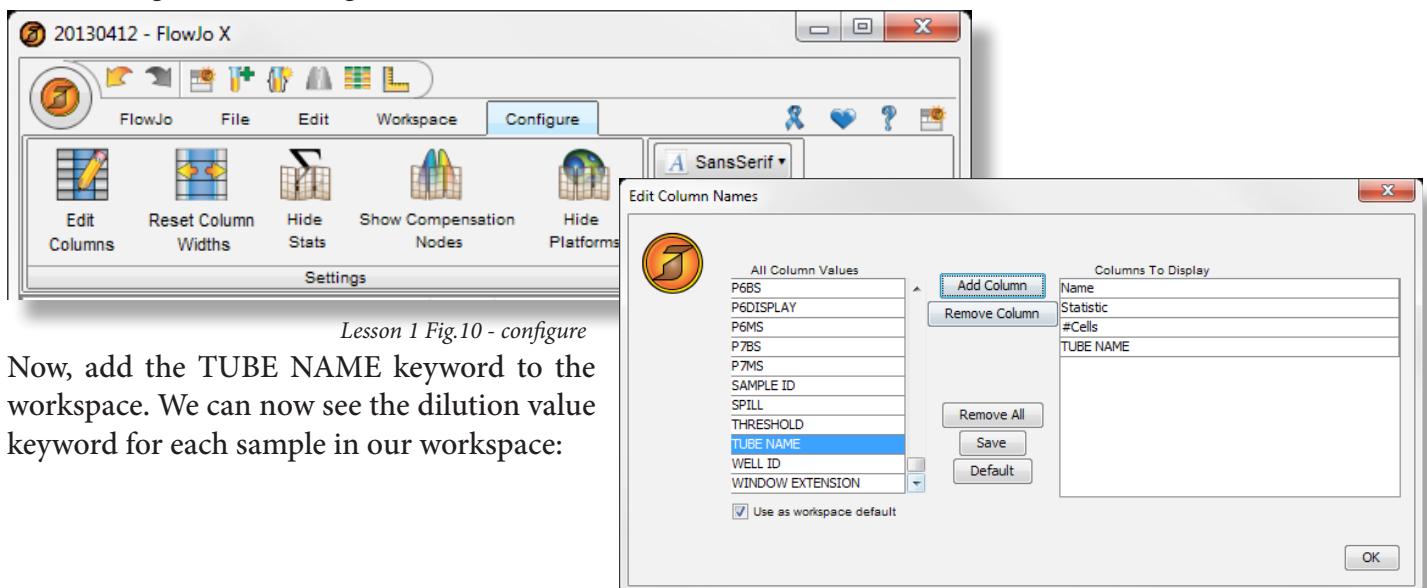


Lesson 1 Fig.9 - sample inspection window

This keyword was used to denote the dilution of antibody used in each tube. Click OK to close this window. Annotating your .fcs files during acquisition with keywords, or metadata, is a great way to quickly add important information to your analysis. We will explore these various ways to use the metadata in subsequent lessons.

Let's add this TUBE NAME keyword to our workspace so we can quickly reference the dilution used in each sample.

To do this, go to the Configure Tab and click ‘Edit Columns’:



Lesson 1 Fig.10 - configure

Now, add the TUBE NAME keyword to the workspace. We can now see the dilution value keyword for each sample in our workspace:

Lesson 1 Fig.11 - column edit dialog

Because FlowJo does not know a ratio formula as a keyword, we need to add a keyword for the antibody concentration. Click on the Workspace tab and select the Keywords band, click the button to Add Keyword.

In the Add Keyword dialog box, type “Antibody Concentration” and click ok. Another column is added to the workspace just like when we added the Tube Name keyword. Double click in the first cell and type just the dilution amount. For Sample A01, this number is 100. Do this for all samples in the workspace. When finished, your workspace will look like the one below.

Name	Statistic	#Cells	TUBE NAME
CD8a_1,3a,100_A01.fcs		20000	1:100
CD8a_1,3a,200_A02.fcs		20000	1:200
CD8a_1,3a,400_A03.fcs		20000	1:400
CD8a_1,3a,800_A04.fcs		20000	1:800
CD8a_1,3a,1600_A05.fcs		20000	1:1600

Lesson 1 Fig.12 - tube name column

The screenshot shows the FlowJo X interface with the 'Workspace' tab selected. In the top right, there is a 'Keywords' band containing several buttons: 'Add Keyword', 'Create group from keyword value', 'Create keyword value series...', and 'Copy value to group'. Below this is a table with columns: Name, Statistic, #Cells, and TUBE NAME. The data is identical to the table in Figure 12.

Name	Statistic	#Cells	TUBE NAME
CD8a_1,3a,100_A01.fcs		20000	1:100
CD8a_1,3a,200_A02.fcs		20000	1:200
CD8a_1,3a,400_A03.fcs		20000	1:400
CD8a_1,3a,800_A04.fcs		20000	1:800
CD8a_1,3a,1600_A05.fcs		20000	1:1600

Lesson 1 Fig.13 - add keyword

The screenshot shows the FlowJo X interface with the 'Workspace' tab selected. In the top right, there is a 'Keywords' band containing several buttons: 'Add Keyword', 'Create group from keyword value', 'Create keyword value series...', and 'Copy value to group'. Below this is a table with columns: Name, Statistic, #Cells, TUBE NAME, and Antibody Co... (Antibody Concentration). The data is identical to the table in Figure 14.

Name	Statistic	#Cells	TUBE NAME	Antibody Co...
CD8a_1,3a,100_A01.fcs		20000	1:100	100
CD8a_1,3a,200_A02.fcs		20000	1:200	200
CD8a_1,3a,400_A03.fcs		20000	1:400	400
CD8a_1,3a,800_A04.fcs		20000	1:800	800
CD8a_1,3a,1600_A05.fcs		20000	1:1600	1600

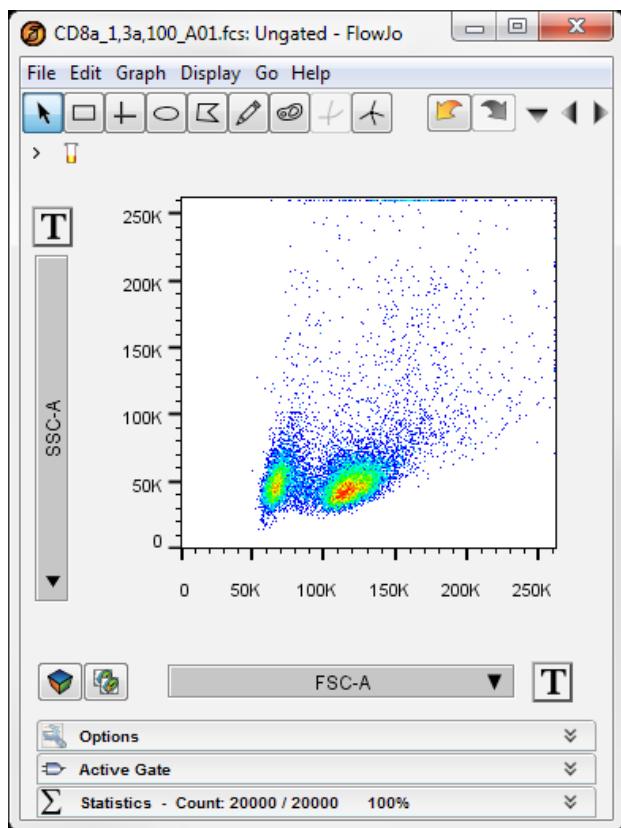
Lesson 1 Fig.14 - antibody concentration column

Lesson 2 - Gating

You can start this lesson (skipping lesson 1) by opening the Lesson 2.acs file.

Gating is the process of subsetting collected events for further analysis. Subsets can be gated to generate further subsets, until a collection has only the cells for which a graphic display or analysed statistics are desired. FlowJo offers a whole set of gating and advanced gating tools to facilitate this process.

To begin creating gates, we must first open the graph window for the first sample. To do this, double click on the sample A01. This will open the graph window:



Lesson 2 Fig.1 - graph window

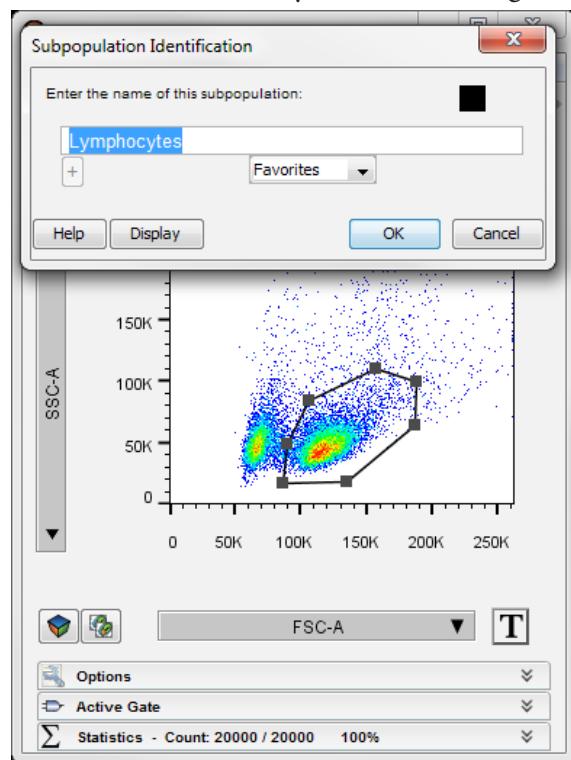
It may suggest the correct gate name, in this case Lymphocytes. You can leave this gate name or enter another name. We'll leave it as "Lymphocytes". If you click the "+" button in this dialog, your gate name will be saved for use at another time.

Double click within the lymphocytes gate in the Graph Window to isolate just the lymphocytes.



Gating options are in the top left hand corner. We are interested in examining lymphocytes, so lets create a polygon gate on the lymphocytes. Select the polygon gating tool:

Click the mouse to make a gate node and then continue clicking to draw a gate around the lymphocytes. Double click to close the gate. FlowJo will then ask you to name the gate.



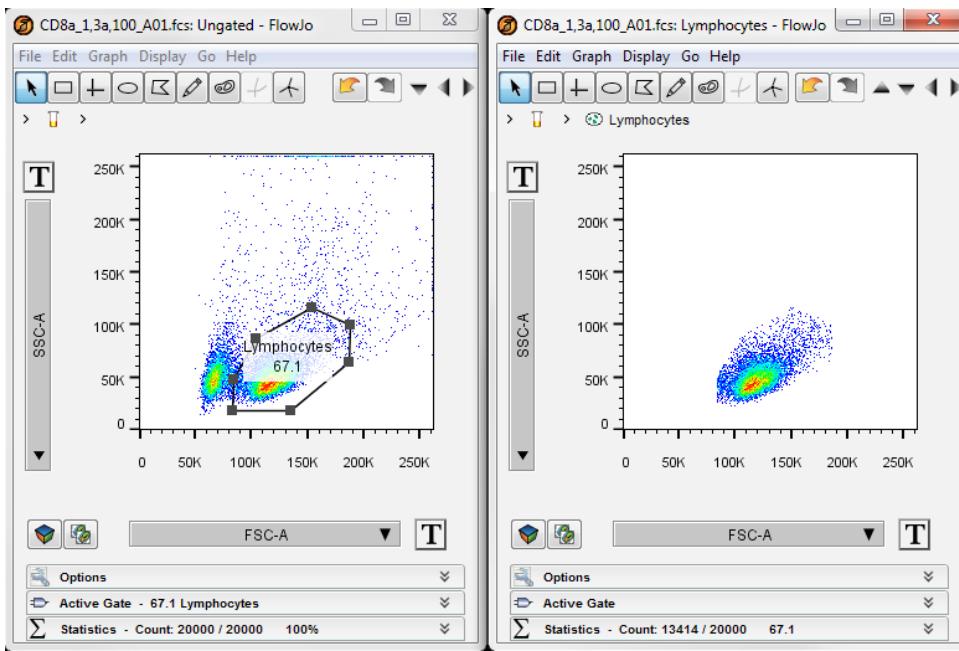
Lesson 2 Fig.2 - gate naming

Notice how the gated population is created in the workspace as well below and indented from the total sample. This is FlowJo's revolutionary hierarchical gating which immediately indicates the gating strategy and derivation to the user.

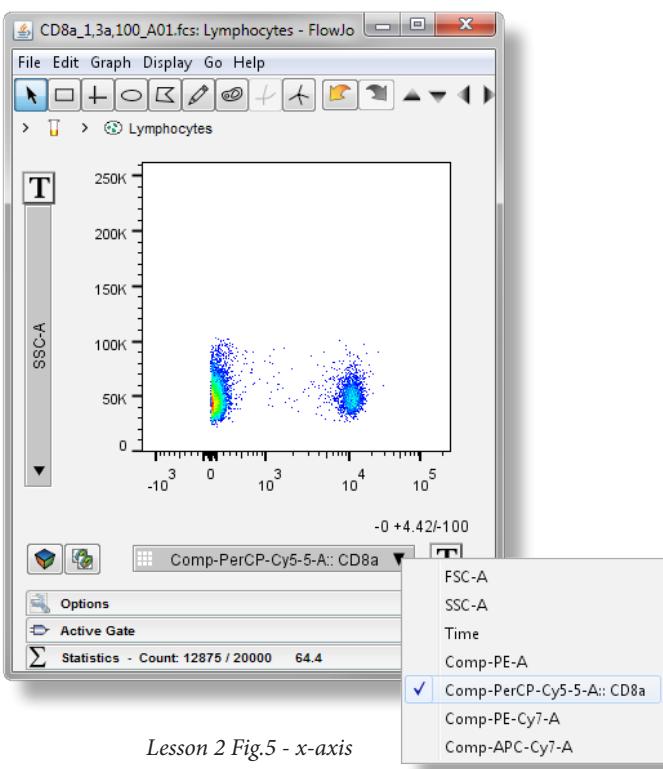
Name	Statistic	#Cells	TUBE NAME	Antibody Co...
CD8a_1,3a,100_A01.fcs		20000	1:100	100
Lymphocytes	67.1	13414		
CD8a_1,3a,200_A02.fcs		20000	1:200	200
CD8a_1,3a,400_A03.fcs		20000	1:400	400
CD8a_1,3a,800_A04.fcs		20000	1:800	800
CD8a_1,3a,1600_A05.fcs		20000	1:1600	1600

Lesson 2 Fig.3 - sample analysis hierarchy

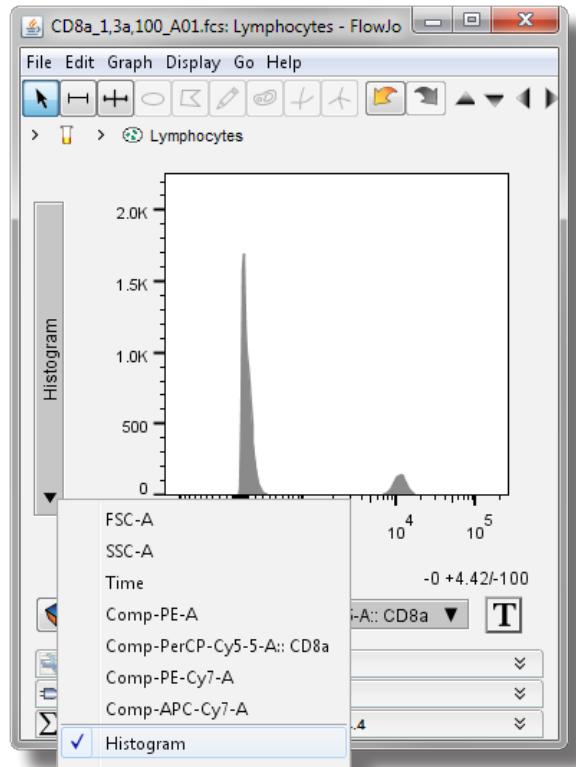
Change x and y axis parameters to match the picture below on the right (fig.4).



Lesson 2 Fig.4 - lymphocyte gate



Lesson 2 Fig.5 - x-axis



Lesson 2 Fig.6 - y-axis

There are a couple of things to note about data display in the Graph Window:

*FlowJo applies a biexponential transformation automatically to digital (fcs3.0) data. There is a preference to turn this off, but it is actually a preferable way to display digital data. The T button also allows you to customize the axis to adjust the transform or convert the axis to log or linear.

*The parameters say comp-parameter name because these are digital data (fcs3.0) files that were acquired with a compensation matrix. FlowJo applies the compensation matrix generated by acquisition software automatically upon loading data. If you did not compensate on the machine or use fcs2.0 data, the parameters will not have the comp-prefix. Compensation is described in more detail in the 8 color PBMC tutorial.

You'll notice that we have isolated the lymphocytes. Since we are doing a titration on CD8a, we will now change the x-axis of the lymphocyte population to CD8a and the y-axis to histogram. Do this by clicking on the currently displayed parameter label. It will drop down a list of all additional parameters:

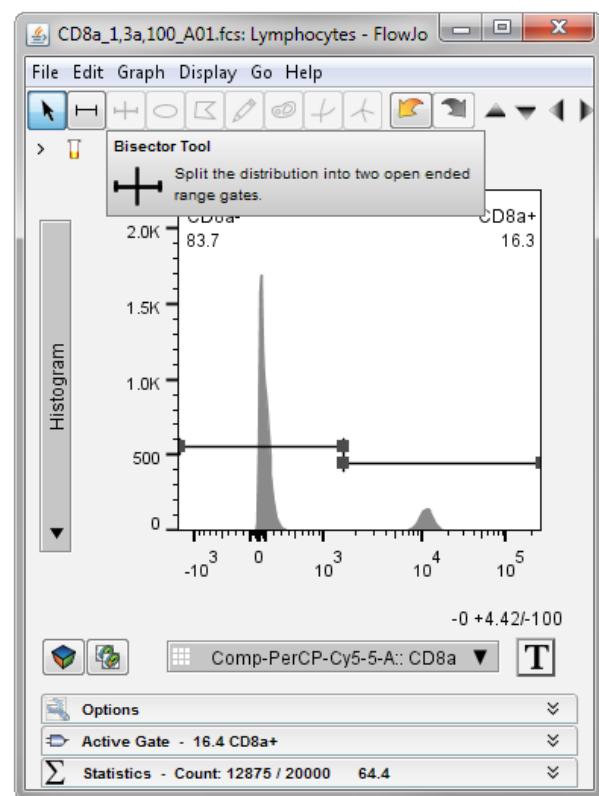
To set a gate on the negative and positive populations, let's select the bisector gate tool and draw a range gate on the negative and positive populations. FlowJo will name each gate accordingly.

Name	Statistic	#Cells	TUBE NAME	Antibody Co...
CD8a_1,3a,100_A01.fcs		20000	1:100	100
↳ Lymphocytes	67.1	13414		
↳ CD8a+	16.3	2181		
↳ CD8a-	83.7	11233		
CD8a_1,3a,200_A02.fcs		20000	1:200	200
CD8a_1,3a,400_A03.fcs		20000	1:400	400
CD8a_1,3a,800_A04.fcs		20000	1:800	800
CD8a_1,3a,1600_A05.fcs		20000	1:1600	1600

Lesson 2 Fig.7 - samples

*Typically we would gate on a control (unstained, Fluorescence Minus One or Biological control) to properly set gate boundaries. However, with a titration experiment, this isn't always necessary. Therefore, please consider this an exercise in gate creation and not in theory on how to properly set gate boundaries.

This concludes Lesson 2. You can always find more information on gating and gating options at flowjo.com.

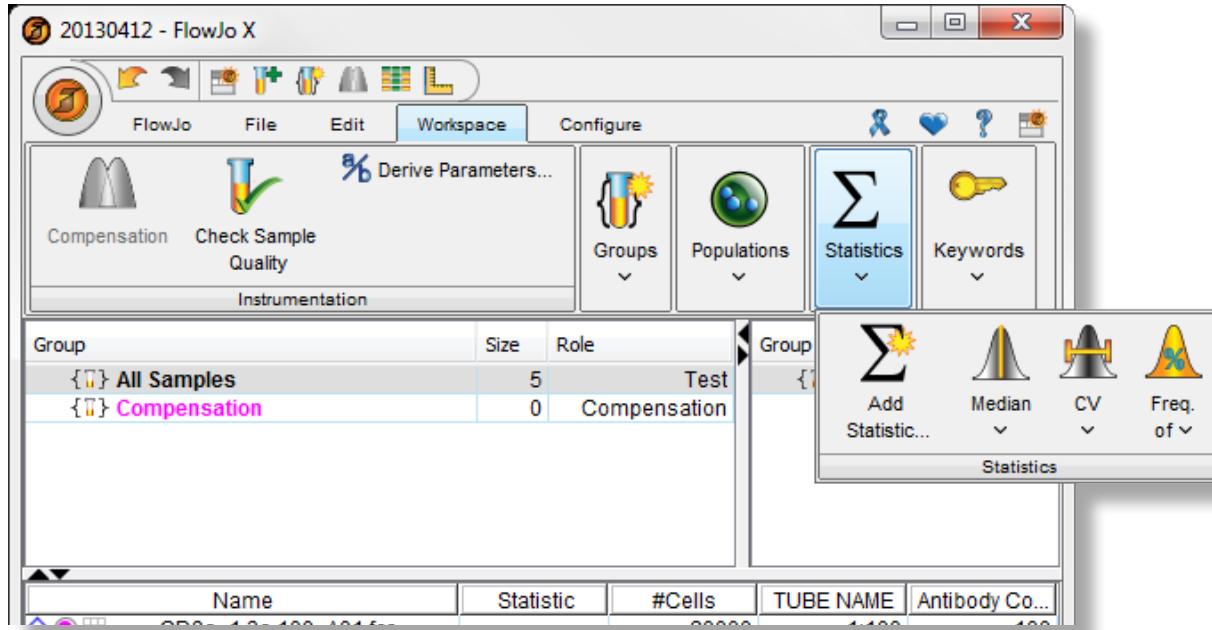


Lesson 2 Fig.8 - bisector gating tool

Lesson 3 - Statistics

You can start this lesson (skipping lessons 1 and 2) by opening the Lesson 3.acs file.

In version 10 of FlowJo, there is now a 'quick statistic' option to calculate the median, CV and Freq. of from the Statistics band. Since flow cytometry data is typically NOT normally distributed, we refer to the Median as a MFI.



Lesson 3 Fig.1 - adding statistics

To add the MFI (Median Fluorescence Intensity), CV (Coefficient of Variation) or Freq. of ... (Frequency of a population), these 'quick statistic' options can be used.

MFI technically stands for Mean Fluorescence Intensity, but a mean assumes a normal distribution. Since flow cytometry data is typically NOT normally distributed, in flow cytometry, we refer to the Median as a MFI.

In this experiment, we want to know the CD8a MFI, or CD8a expression level, of the CD8a+ and CD8a- populations. Therefore, all you need to do is select the CD8a+ population in your workspace.

The MFI is a measure of the expression profile of a particular parameter. Typically, MFI is used when looking at up- or down-regulation of a marker, or fluorophore expression like GFP. In this experiment, we use the MFI to calculate the separation/staining/sensitivity index. There are many formulas to calculate these different indices, but a very simple way to do it is to take the MFI of the positive and divide it by the MFI of the negative. For titration experiments, this basic formula is sufficient and will be described in more detail in a later lesson.

We will now go to the quick statistic interface and choose Median>Comp-PerCP-Cy5.5:: CD8a

Lesson 3 Fig.3 - median

This will add the MFI (CD8a expression level) to your CD8a+ population.

Name	Statistic	#Cells	TUBE NAME	Antibody Co...
CD8a_1,3a,100_A01.fcs	20000	13414	1:100	
Lymphocytes	67.1	2181		
CD8a+	16.3	2181		
CD8a-	83.7	11233		
CD8a_1,3a,200_A02.fcs	20000	1:200		
CD8a_1,3a,400_A03.fcs	20000	1:400		
CD8a_1,3a,800_A04.fcs	20000	1:800		
CD8a_1,3a,1600_A05.fcs	20000	1:1600		

Name	Statistic	#Cells	TUBE NAME	Antibody Co...
CD8a_1,3a,100_A01.fcs	20000	1:100	100	
Lymphocytes	67.1	13414		
CD8a+	16.3	2181		
Σ Median : CD8a (Con)	11184			
CD8a-	83.7	11233		
Σ Median : CD8a (Con)	43.9			
CD8a_1,3a,200_A02.fcs	20000	1:200	200	
CD8a_1,3a,400_A03.fcs	20000	1:400	100	
CD8a_1,3a,800_A04.fcs	20000	1:800	100	
CD8a_1,3a,1600_A05.fcs	20000	1:1600	100	

Now, do the same thing to the CD8a-population. You can also drag and drop the MFI for CD8a from the CD8a+ to the CD8a- too.

Additional statistics are available through the 'Add Statistic...' interface such as Mean, Standard Deviation (SD), Percentile, and Count.

Lesson 3 Fig.4 - median statistic applied to CD8a+ population

Name	Statistic	#Cells	TUBE NAME	Antibody Co...
CD8a_1,3a,100_A01.fcs	20000	1:100	100	
Lymphocytes	67.1	13414		
CD8a+	16.3	2181		
Σ Median : CD8a (Con)	11184			
CD8a-	83.7	11233		
Σ Median : CD8a (Con)	43.9			
CD8a_1,3a,200_A02.fcs	20000	1:200	200	
CD8a_1,3a,400_A03.fcs	20000	1:400	100	
CD8a_1,3a,800_A04.fcs	20000	1:800	100	
CD8a_1,3a,1600_A05.fcs	20000	1:1600	100	

Lesson 3 Fig.5 - median statistic applied to CD8a- population

Count is a bit redundant since it is in the workspace already, but if looking to export the count of population(s) out of FlowJo, adding the statistic to the workspace allows for drag and drop to the Table or Layout Editor.

Lesson 4 - Batching in the Workspace

To skip to lesson 4, open the Lesson 4.acs file.

Batching is a process that performs an operation automatically for a group of files, rather than having to manipulate one file (or sample) at a time. Almost everything in FlowJo can be batched because batching saves you time! As FlowJo is developed by leaders in the field, any feature to save time has likely been implemented. In this Lesson, we will discuss batching in the workspace before we discuss batching to outputs using the Table Editor (Lesson 5) and Layout Editor (Lesson 6). In Lesson 4, we'll discuss batching in the workspace.

A single gate, a single statistic or an entire gating tree can be batched. In this experiment, we want to batch our entire tree to the group so that the gates and statistics we added to sample 1 are present in every sample. To do this, select the Lymphocytes population in the workspace, hold down shift on your keyboard and select the bottom statistic. You should have the entire gating tree highlighted now:

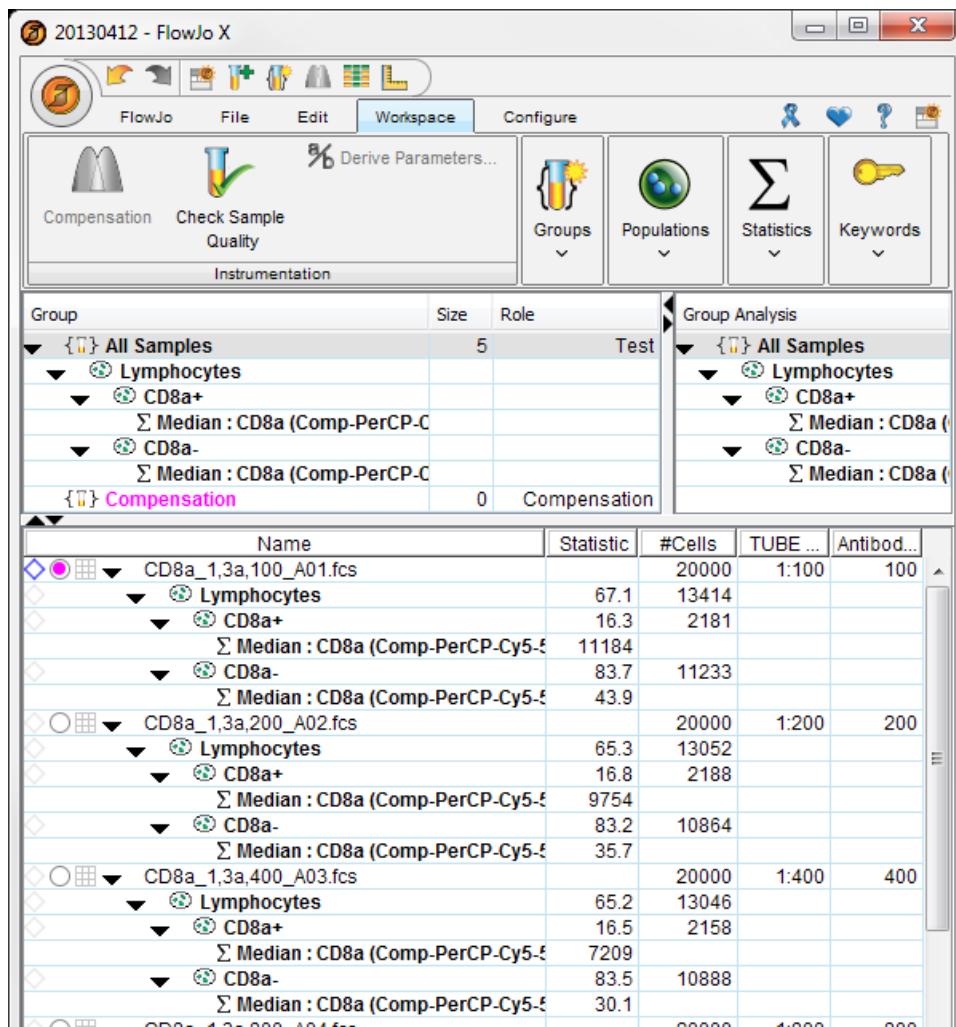
You can drag and drop this to any group. Go ahead and drag this gating tree to the All Samples group.

What you'll notice is that the gates and statistics have now been applied to every sample in the group. The initial gate location for every sample is based on Sample 1. FlowJo allows you to go through each sample and adjust the gates on a per sample basis. FlowJo will not propagate a change made in any one sample to all samples unless you drag the altered gate to the group. This allows you to control each sample's gates and choose when you want to re-batch any gate to the whole set of samples.

On the other hand, if you change a gate in one sample and want it to revert to the original group applied gate, just select it in the workspace and click delete (backspace) on the keyboard. The altered gate will reset to the group-owned gate.

Name	Statistic	#Cells	TUBE N...	Antibody...
CD8a_1,3a,100_A01.fcs		20000	1:100	100
Lymphocytes	67.1	13414		
CD8a+	16.3	2181		
Σ Median : CD8a (Comp-PerCP-Cy5-5)	11184			
CD8a-	83.7	11233		
Σ Median : CD8a (Comp-PerCP-Cy5-5)	43.9			
CD8a_1,3a,200_A02.fcs		20000	1:200	200

Lesson 4 Fig.1 - selected analysis



Lesson 4 Fig.2 - selection applied to all samples

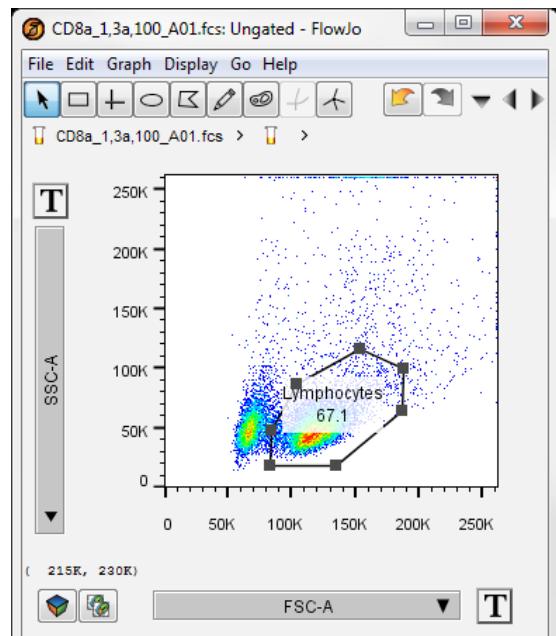
Use the arrow keys in the graph window to scroll through plots and check gates.



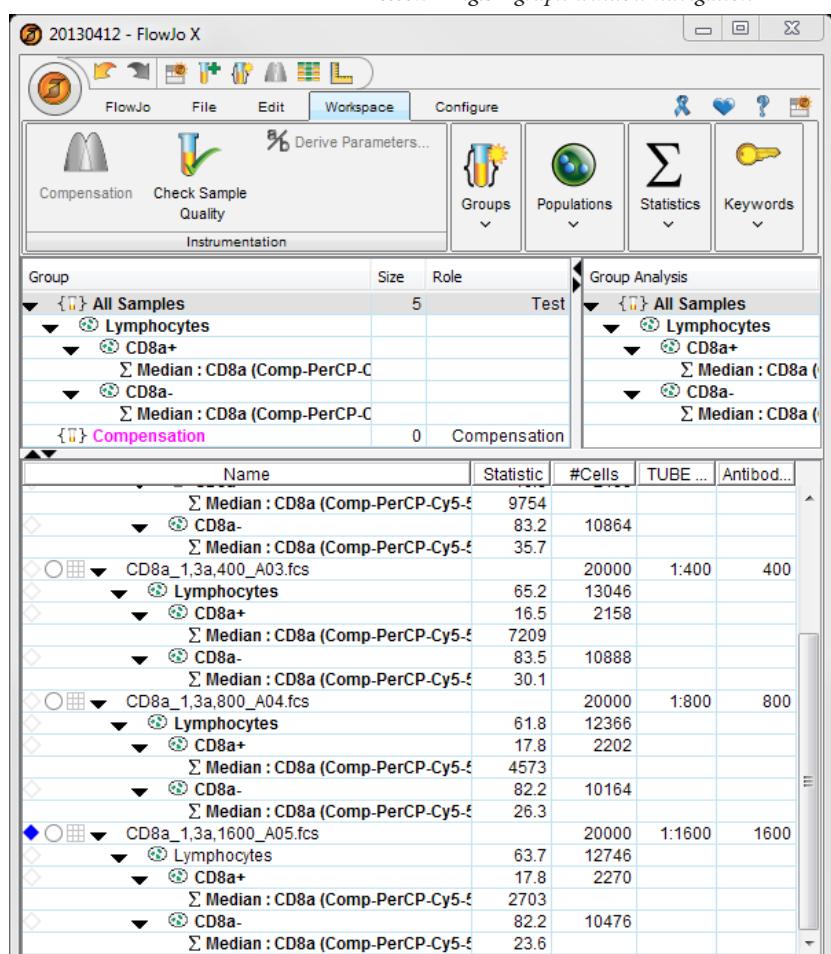
Alternately, with two or more graph windows open, you can hold down the shift key on the PC or the control key on the Mac and scroll through more than one Graph Window at once. This allows you to check multiple gates simultaneously.

Any gate that is modified will turn black and unbold. Group owned gates/statistics receive the formatting of the group to which they have been applied. Here you'll notice all gates/statistics are black and bold because the 'All Samples' group is black and bold. If the group was a different color, the group-applied gates/statistics would inherit that different color. Modifying gates after they have been applied to a group is acceptable in many circumstances depending on your analysis. FlowJo notifies you of the change by turning the population black and unbold.

As stated above, select a modified gate and click delete on the keyboard to have it reset to the group-applied gate or drag it to the group to apply the altered gate to every sample in the group.



Lesson 4 Fig.3 - graph window navigation



Lesson 4 Fig.4 - group applied analysis

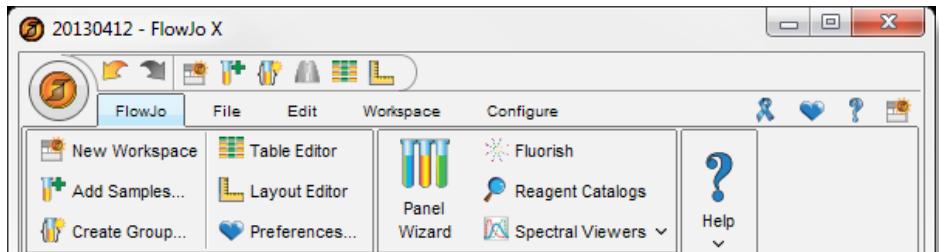
Lesson 5 - Basic Table Editor Functionality

To skip to lesson 5, open the Lesson 5.acs file.

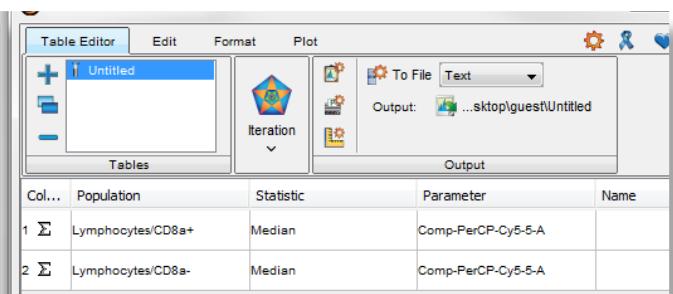
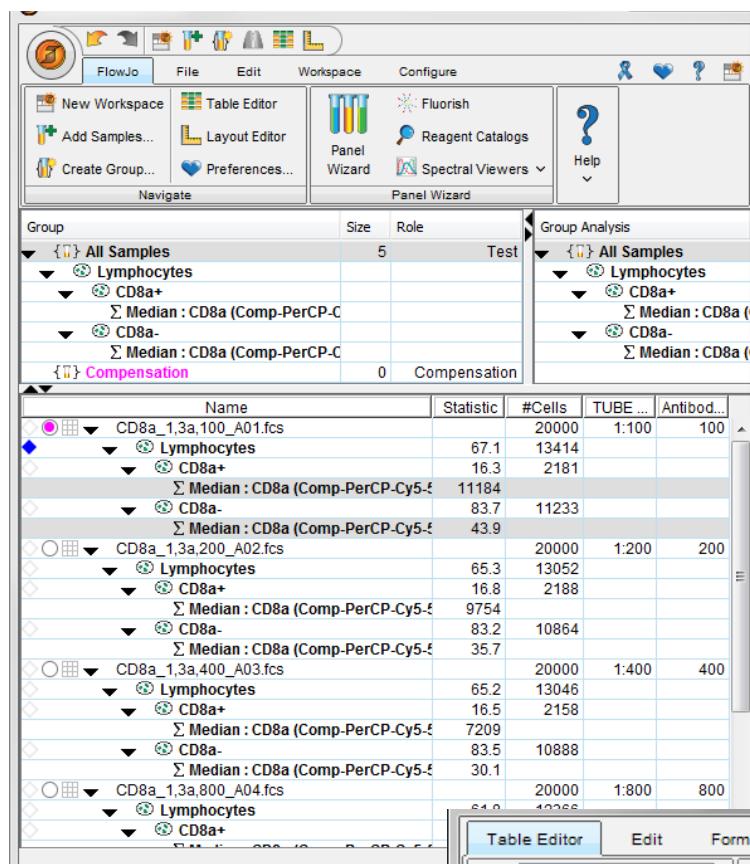
The Table Editor in FlowJo allows you to quickly create tables for export into a spreadsheet program like Excel or to a database. The Table Editor is solely for creating statistic tables and does not manage graphics. Exporting graphics will be discussed in Lesson 6.

To open the Table Editor, click on the grid icon button  in the toolbar or the Table Editor button in the FlowJo band.

When you open the Table Editor, the first interface is blank. Here, we will drag both Median statistics from Sample 1 to the Table Editor.



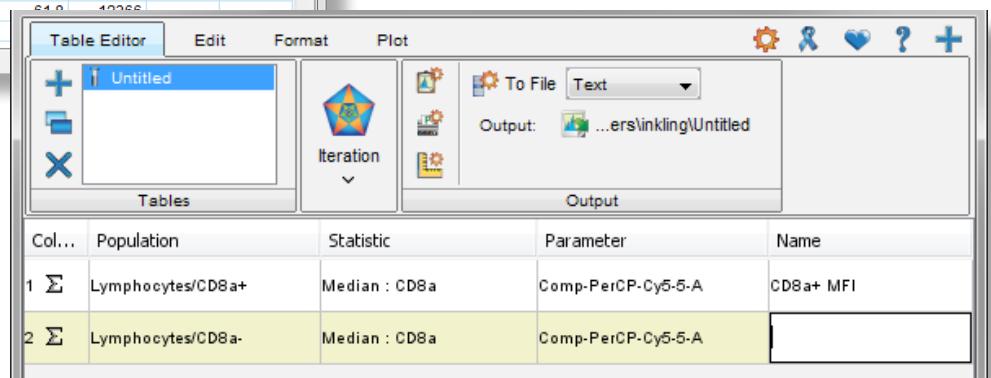
Lesson 5 Fig.1 - table editor button locations



Lesson 5 Fig.2 - adding statistics

Statistics only have to be placed into the Table Editor from one sample in the group. When the table is created, it will batch automatically for all samples in the group.

We now have our CD8a MFI, or expression intensity, for the CD8a+ population and CD8a- population in our Table Editor. To change the name of the column header, just click in the rightmost cell for each statistic. Add custom names for both statistics as depicted in the figure below (fig.3).



Lesson 5 Fig.3 - custom named columns

Next, we want to add a keyword to the table, click on the Edit tab then click on the Add Column button. Next, the Column Information dialog window will appear. Here you will select the Keyword Tab, and scroll down until you find the Antibody Concentration Keyword from the column on the left. Now type Antibody Concentration in the column heading field and click OK. This adds a keyword to your table so you have the concentration for each sample upon batching and creating a line plot.

The screenshot shows the FlowJo Tables interface. On the left is the Table Editor window with two rows of data. The first row has Population 'Lymphocytes/CD8a+' and Statistic 'Median : CD8a'. The second row has Population 'Lymphocytes/CD8a-' and Statistic 'Median : CD8a'. Below the table is the caption 'Lesson 5 Fig.4 - adding a column'. On the right is the 'Column Information' dialog box. In the 'Column heading' field, 'Antibody Concentration' is typed. The 'Keyword' tab is selected. A list of keywords is shown, with 'Antibody Concentration' highlighted. The 'OK' button is at the bottom right of the dialog.

Your table should look like this:

Col...	Population	Statistic	Parameter	Name
1 Σ	Lymphocytes/CD8a+	Median : CD8a	Comp-PerCP-Cy5	
2 Σ	Lymphocytes/CD8a-	Median : CD8a	Comp-PerCP-Cy5	
3 🌐	Antibody Concentration			Antibody Concentration

Lesson 5 Fig.5 - new column

We've added statistics and keywords. Now, we can also add equations in the Table Editor as well. Calculations can be done in Excel upon export but you can also do them in the Table Editor before batching.

To create a formula in the Table Editor, go to the Edit tab and click Add Column.

In the resulting interface, click on the 'Formula' tab:

The screenshot shows the FlowJo Tables interface. On the left is the Table Editor window. On the right is the 'Column Information' dialog box. The 'Formula' tab is selected. In the 'Column heading' field, 'Ratio MFI' is typed. The formula editor contains '<Cell column="CD8a+ MFI" relativeRow="0" />'. A red error message 'Algebraic expression error in '1'' is displayed. The 'Insert Reference:' dropdown shows 'CD8a+ MFI' selected. The 'OK' button is at the bottom right of the dialog.

Now, for a titration experiment, we want to calculate the ratio of the expression between positive and negative. Therefore, we will create a ratio of CD8a+ MFI / CD8a- MFI, with a column heading name "Ratio MFI".

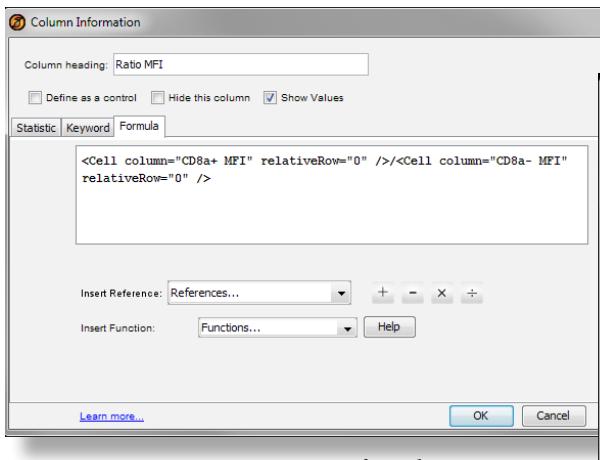
Enter the Column Heading and then select the Reference CD8a+ MFI.

Now, insert the divide function and insert the reference for CD8a- MFI.

The screenshot shows the 'Column Information' dialog box. The 'Formula' tab is selected. In the 'Column heading' field, 'Ratio MFI' is typed. The formula editor contains '<Cell column="CD8a+ MFI" relativeRow="0" />'. A red error message 'Algebraic expression error in '1'' is displayed. The 'Insert Reference:' dropdown shows 'CD8a+ MFI' selected. The 'Insert Function:' dropdown shows 'CD8a- MFI' selected. The 'OK' button is at the bottom right of the dialog.

Lesson 5 Fig.7 - column information dialog

Your formula should look like this:



Lesson 5 Fig.8 - formula

The Table Editor window displays a table with four rows. Row 1: Col... Population Statistic Parameter Name (Σ Lymphocytes/CD8a+ Median : CD8a Comp-PerCP-Cy5-5-A CD8a+ MFI). Row 2: Σ Lymphocytes/CD8a- Median : CD8a Comp-PerCP-Cy5-5-A CD8a- MFI. Row 3: Antibody Concentration. Row 4: f_x Formula. The table has tabs for Table Editor, Edit, Format, Plot, and a toolbar with icons for Add Column, Edit Column..., Select All, Clear, and Edit Header / Footer.

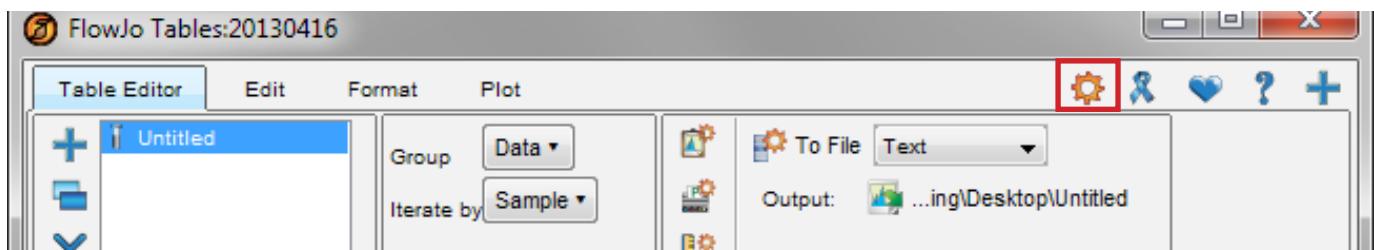
Lesson 5 Fig.9 - table editor

You have set up the Table Editor with statistics, keywords and a formula. Many users only place statistics in the Table Editor, but using keywords and formulas can save you work down the line.

You can batch now to generate the table for every sample in your group or you can explore the data a bit more.

To set your batch output, go to the Table Editor tab. You can batch to clipboard, to printer, directly to the Layout Editor, or to a file. The option most users prefer is to batch to FlowJo first. Batching to FlowJo first will allow you to check the table over before export and then, from the created table, you have all the options of where to export the table. So, there is no disadvantage to batching to FlowJo first.

Click the orange sprocket icon in the upper right hand corner to start the batch:



Lesson 5 Fig.10 - batch

Here is the resulting table:

Ancestry Subset Statistic For	CD8a+ MFI	CD8a- MFI	Antibody Concentration	Ratio MFI
CD8a 1,3a,...	11205	42.9	100	261
CD8a 1,3a,...	9769	34.9	200	280
CD8a 1,3a,...	7216	29.5	400	245
CD8a 1,3a,...	4581	25.9	800	177
CD8a 1,3a,...	2717	22.8	1600	119
Mean	7098	31.2	620	216
SD	3519	7.94	610	66.9

Lesson 5 Fig.11 - batched table

You may choose to “export” or “save” your table data to Excel and write and apply your formulas there. That is fine, but hopefully you now understand how to do it in FlowJo as well!

	Antibody Concentration	Ratio MFI
CD8a 1,3a,....	3.9	100
CD8a 1,3a,....	5.7	200
CD8a 1,3a,....	0.1	400
CD8a 1,3a,....	5.3	800
Mean	3.1	1600
SD	1.8	620
	3516	212
	8.22	64.9

Lesson 5 Fig.12 - output menu

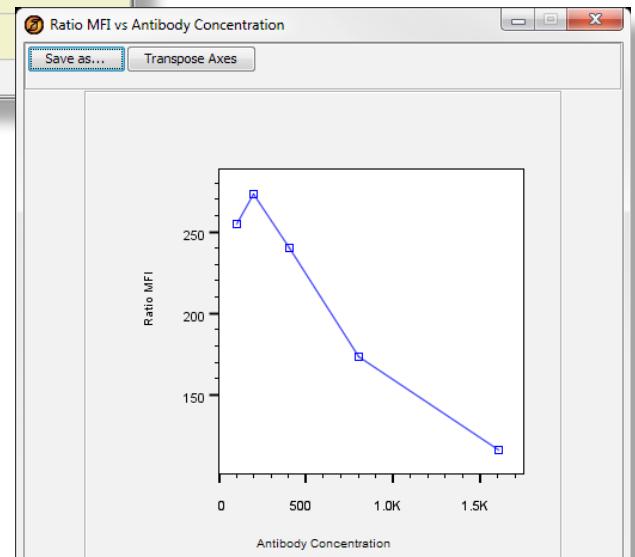
To find the saturation point in FlowJo, plot the concentration versus the ratio. Select the Antibody Concentration row in the Table Editor, hold down the shift key and also select the Ratio MFI row, so both are now highlighted. Click on the Plots tab in the Table Editor and select Line Plot.

Col...	Population	Statistic	Parameter	Name
1 Σ	Lymphocytes/CDBa+	Median : CDBa	Comp-PerCP-Cy5-5-A	CDBa+ MFI
2 Σ	Lymphocytes/CDBa-	Median : CDBa	Comp-PerCP-Cy5-5-A	CDBa- MFI
3 ⚡	Antibody Concentration			Antibody Concentration
4 fx	Formula			Ratio MFI

Lesson 5 Fig.13 - line plot button

Here we find that the proper dilution for this antibody is 1:200 because this gives us the greatest difference (separation) between positive and background staining.

Click File > Save As... to choose the format to save the file as an image file.



Lesson 5 Fig.14 - line plot graph

Lesson 6 - Basic Layout Editor Functionality

To skip to Lesson 6, open the Lesson 6.acs file.

The Layout Editor in FlowJo is one of the most feature rich areas in the program. In version 10, it has much of the functionality that any graphics program has, such as rich text, shading, opacity, rotation, gradient fill, and various shapes.

In this lesson, we will only cover:

- 1) Making a report, editing it and creating a simple batch.
- 2) Overlaying.

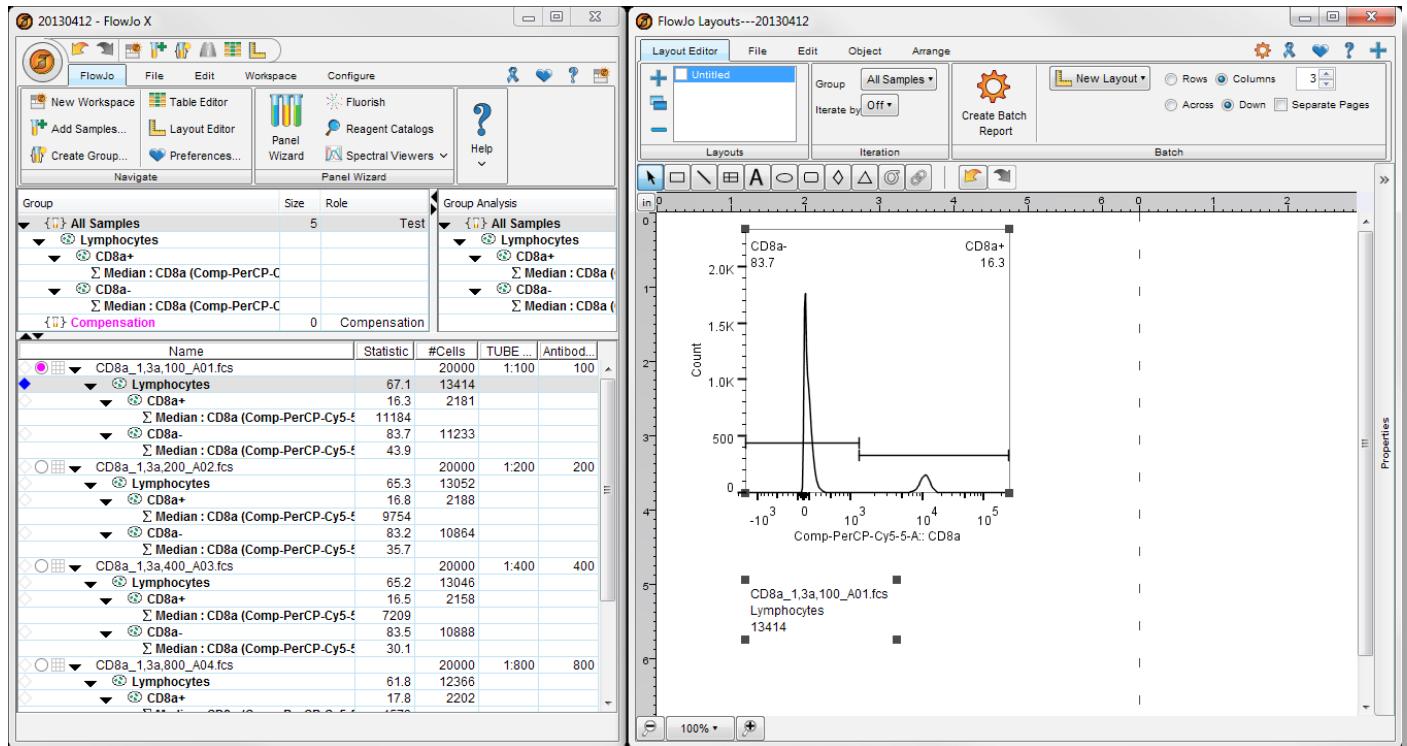
The 8 color PBMC tutorial goes into further detail on the Layout Editor and there is a Batching and Iteration Tutorial available for advanced batching in the Layout Editor.

For more details on the Layout Editor, please visit: <http://docs.flowjo.com/vx/graphical-reports>

To make a report in the Layout Editor, you need only to create a report for one sample and then when you batch it, the same layout of the initial report will be generated for every sample in the group.

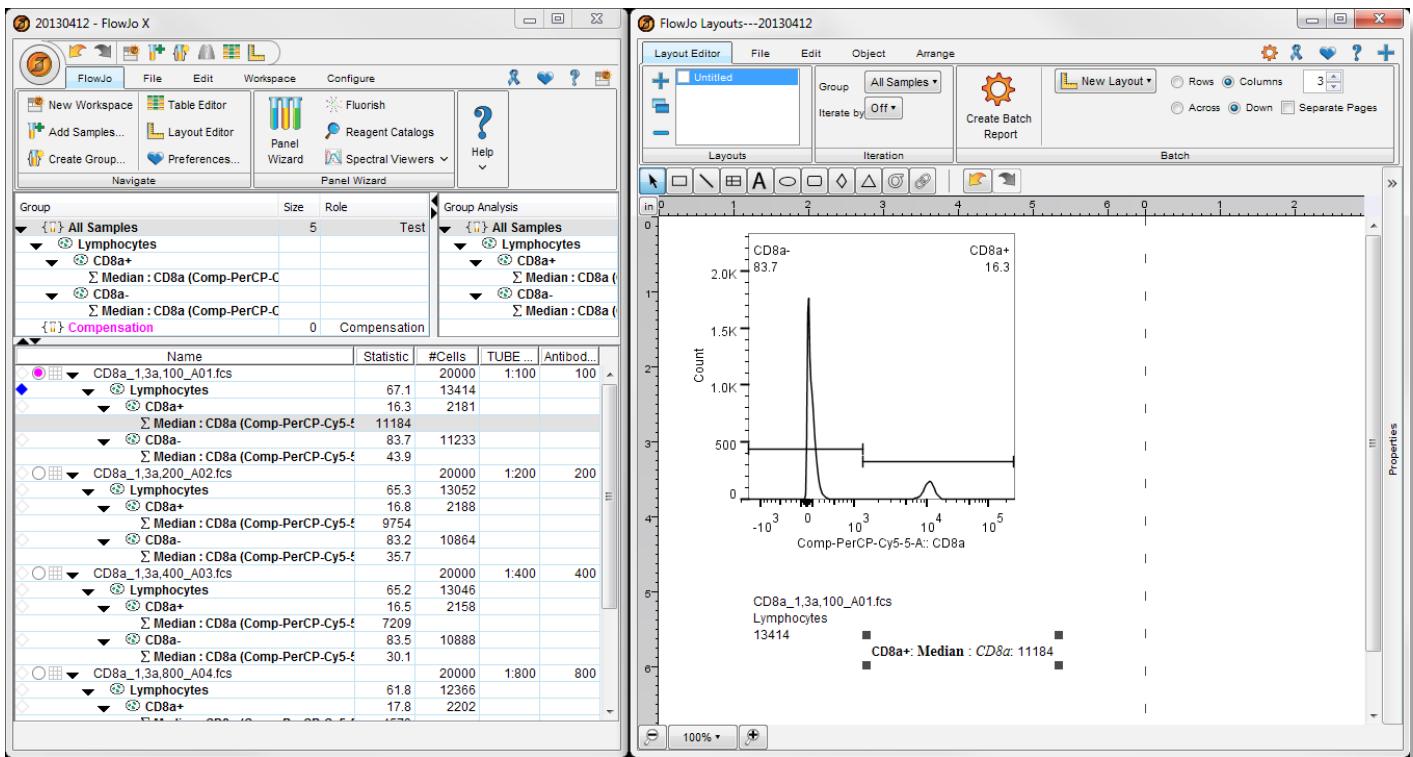
To begin making a report for our first sample, open the Layout Editor and drag the Lymphocytes gate from the first sample from the workspace to the Layout Editor.

When you drag a population to the Layout Editor, you will get a graph:

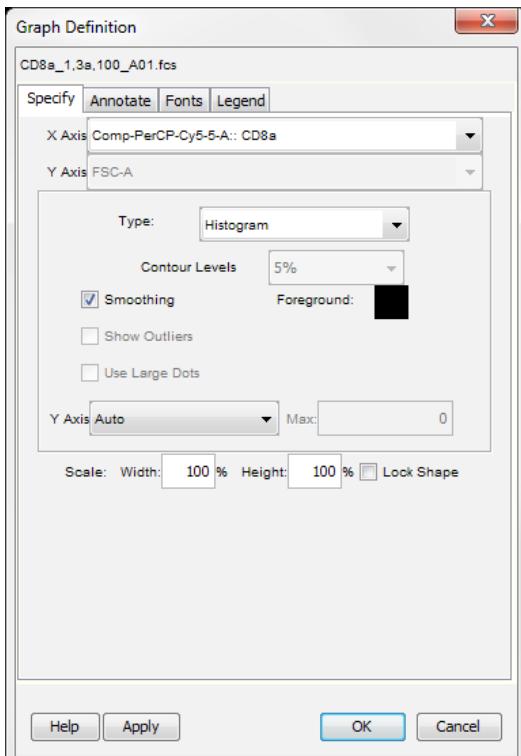


Lesson 6 Fig.1 - layout editor

When you drag a statistic to the Layout Editor, you will get a text box of that statistic. Drag the Median CD8a statistic from the CD8a+ population of sample A01 to the Layout Editor Workspace.



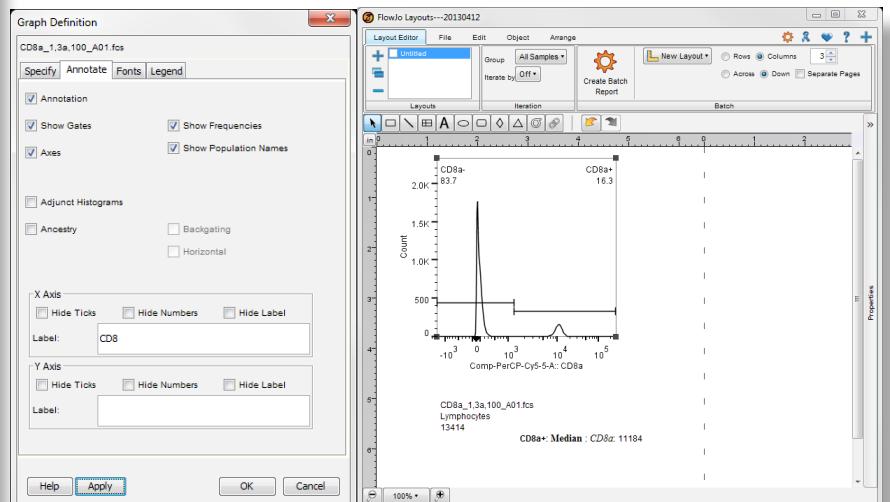
Lesson 6 Fig.2 - stats in layout editor



Lesson 6 Fig.3 - graph definition window

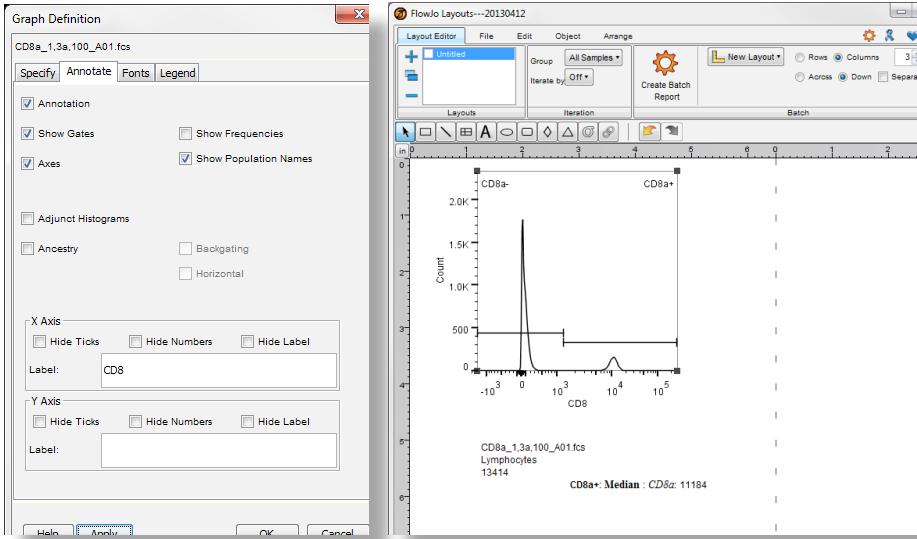
To edit the graph, simply double click on it. The resulting graph definition window will allow you to edit any of the properties of the graph in the Layout Editor.

For example, lets edit the x-axis. Go to the Annotate tab of the 'Graph Definition' window and under the 'X Axis' section, enter CD8a as the Label.



Lesson 6 Fig.4/5 - editing layout

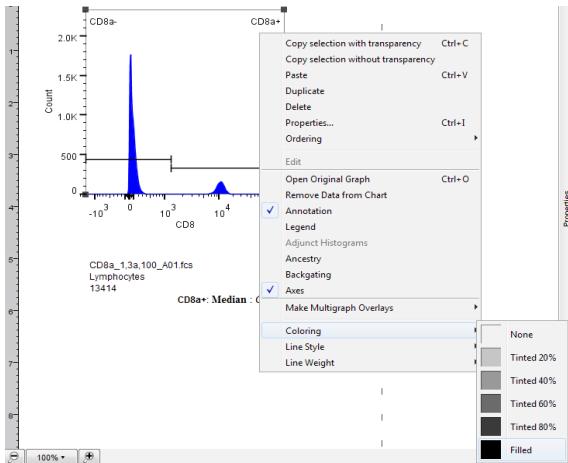
Uncheck show frequencies, then click Apply and you will observe that the label and annotation change:



Lesson 6 Fig.6/7 graph definition layout options

Click ok, notice that the histogram lines change color in the Layout Editor. In the Layout Editor, right click on the histogram. At the bottom of this drop-down menu, there are options for line style, weight and coloring. Here, you are going to make the histogram solid by selecting Coloring and the Filled option.

Now, to finish this report, place the CD8a- MFI statistic in the Layout Editor as well and create a ratio formula right in the Layout Editor.



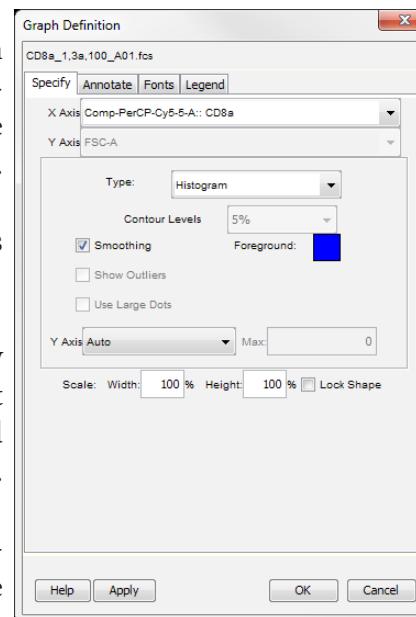
Lesson 6 Fig.9 - fill menu

It is important to copy/paste the string as this is the link to the statistic. Simply entering in 11184/43.9 will create the formula properly for this sample, but when batching, will simply batch 11184/43.9 for every sample. We will want to actually batch the ratio, so we want the statistic string that you get with copy/paste.

You can also adjust the font size or style.

*If you find that you have to adjust the font size/style often, simply go to the Fonts section of Preferences where there is a font preference set for axis labels in the Layout Editor. Changing it here will cause all new plots in any subsequent layout to automatically receive the preference set attributes.

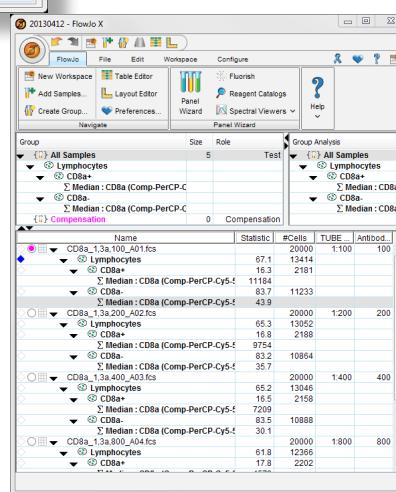
Also, set the color to blue by going to the Specify Tab and selecting blue as the Foreground Color.



Lesson 6 Fig.8 - color options

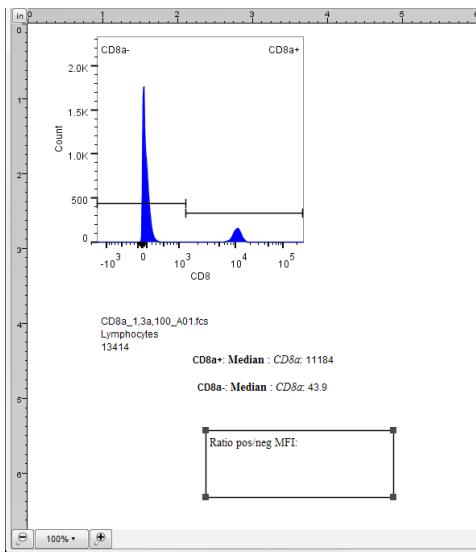
To make the ratio, create a new text box and double click to put the text box in edit mode and enter in “Ratio of pos/neg MFI:”.

Now, double click the CD8a+ MFI text box and copy/paste the 11184 string from the CD8a+ text box to the new text box. Do the same for the CD8a- 43.9 string. Separate them with a divided by or /.

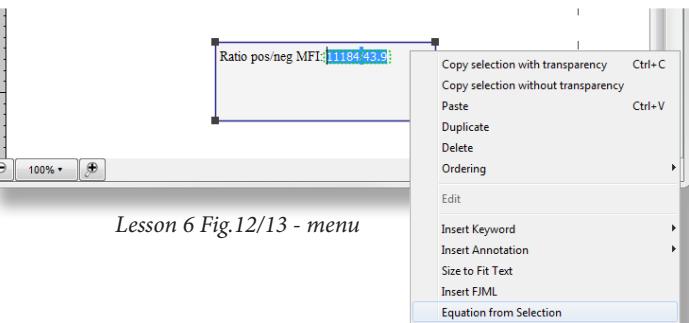


Lesson 6 Fig.10 - resulting layout

To create the equation, highlight the text, right click and select “Equation from Selection”



Lesson 6 Fig.11 - equation



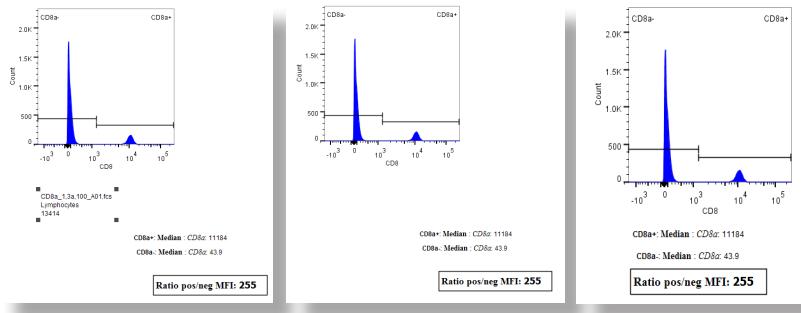
The text box should now convert to this:

Ratio pos/neg MFI: 255

Then change the line style formatting to bold and text formatting size to 18 point in the Layout Editor to get this:

*Text within a text box must be highlighted for changes to take effect.

One last thing we may want to do is to get rid of the sample annotation. To do this, you can simply select it and hit delete.

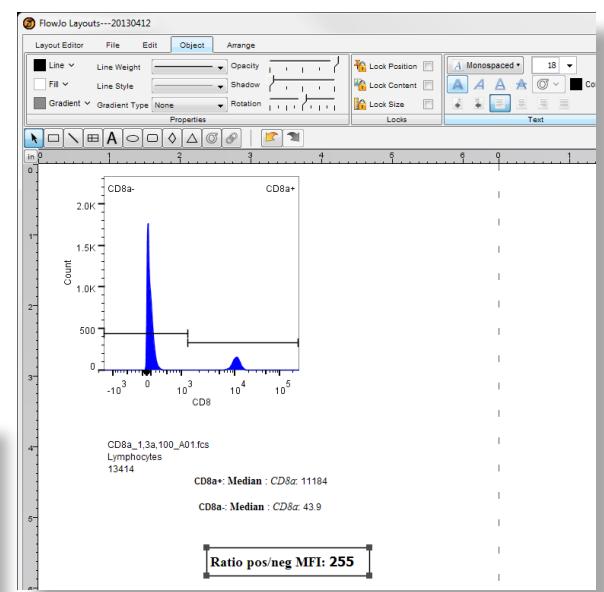


Lesson 6 Fig.15/16/17 - layout

You are able to reorganize the layout to how you prefer it and then we'll batch!

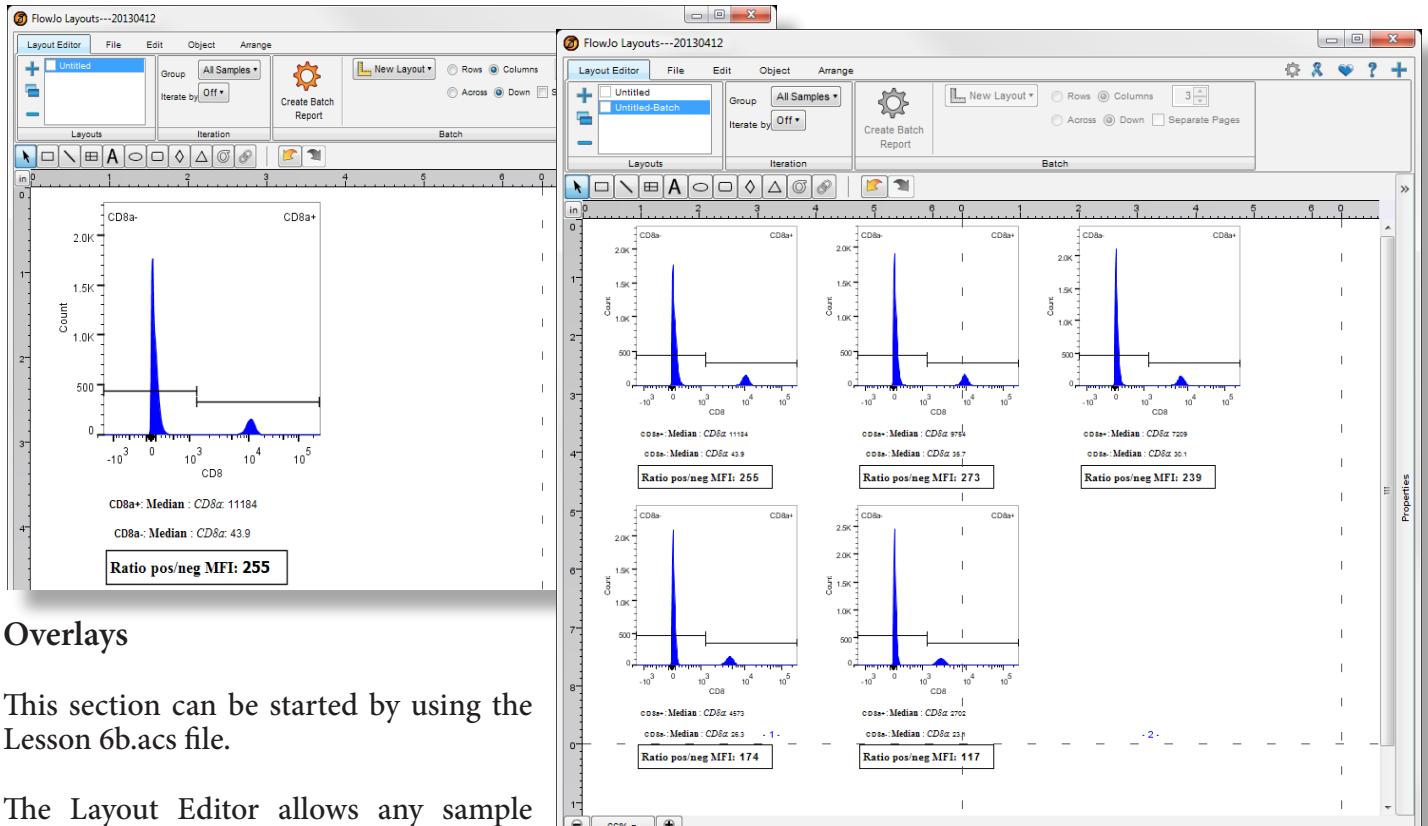
- 1) Return to the Layout Editor Tab to batch these graphs and statistics, you can select any group you want, but in this experiment, you want group All Samples.
- 2) The iterator is off, so it will batch by sample by default. This is what we want. It could also be set to sample with the same effect. Advanced iteration can be learned in the Batching and Iteration tutorial.
- 3) We will batch to a new layout, but many other options are available.
- 4) Set batch orientation to 3 columns going down the Layout Editor.
- 5) Click the sprocket icon  to generate the batch report!

FlowJo will generate the batch report for all the samples in the selected group. Arrange them on five pages so you can see the results in this document (Obviously, you can also see the results on your screen).



Lesson 6 Fig.14 - ready to batch

As you can see, everything batches. Graphs, statistics, text boxes or other objects will all batch. Batching can save you an extraordinary amount of time. By setting up the prototypical report for your first sample, you can quickly generate a report and get every object present for ALL samples by batching.

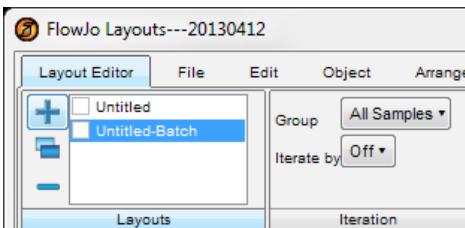


Lesson 6 Fig.18 - batched layout

This section

This section can be started by using the Lesson 6b.acs file.

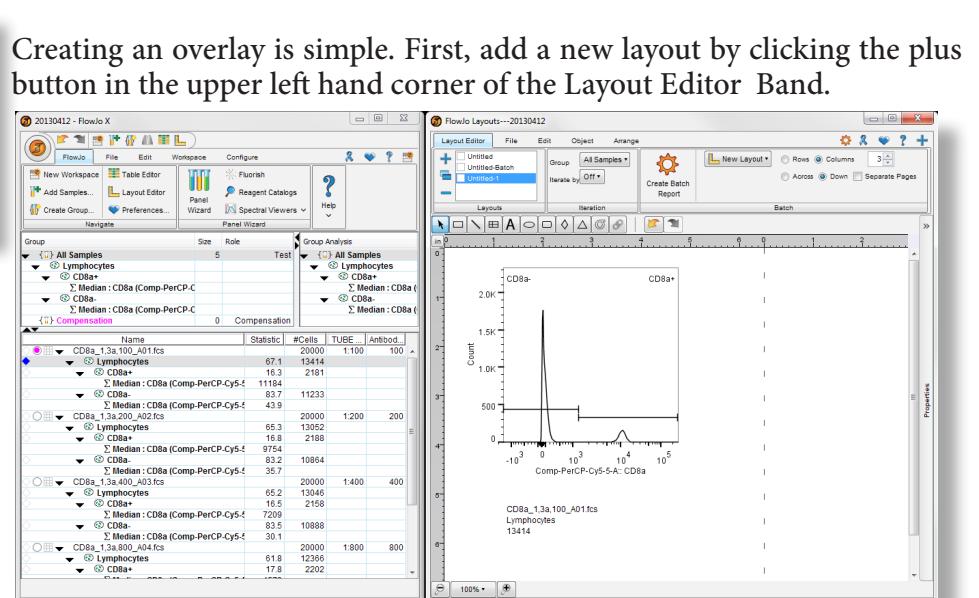
The Layout Editor allows any sample or population to be overlaid on any existing graph of a sample or population.



Lesson 6 Fig.19 - new layout

This can also be done in the Table Editor to create multiple tables of statistics. There is no limit to the amount of layouts or tables you can make in FlowJo.

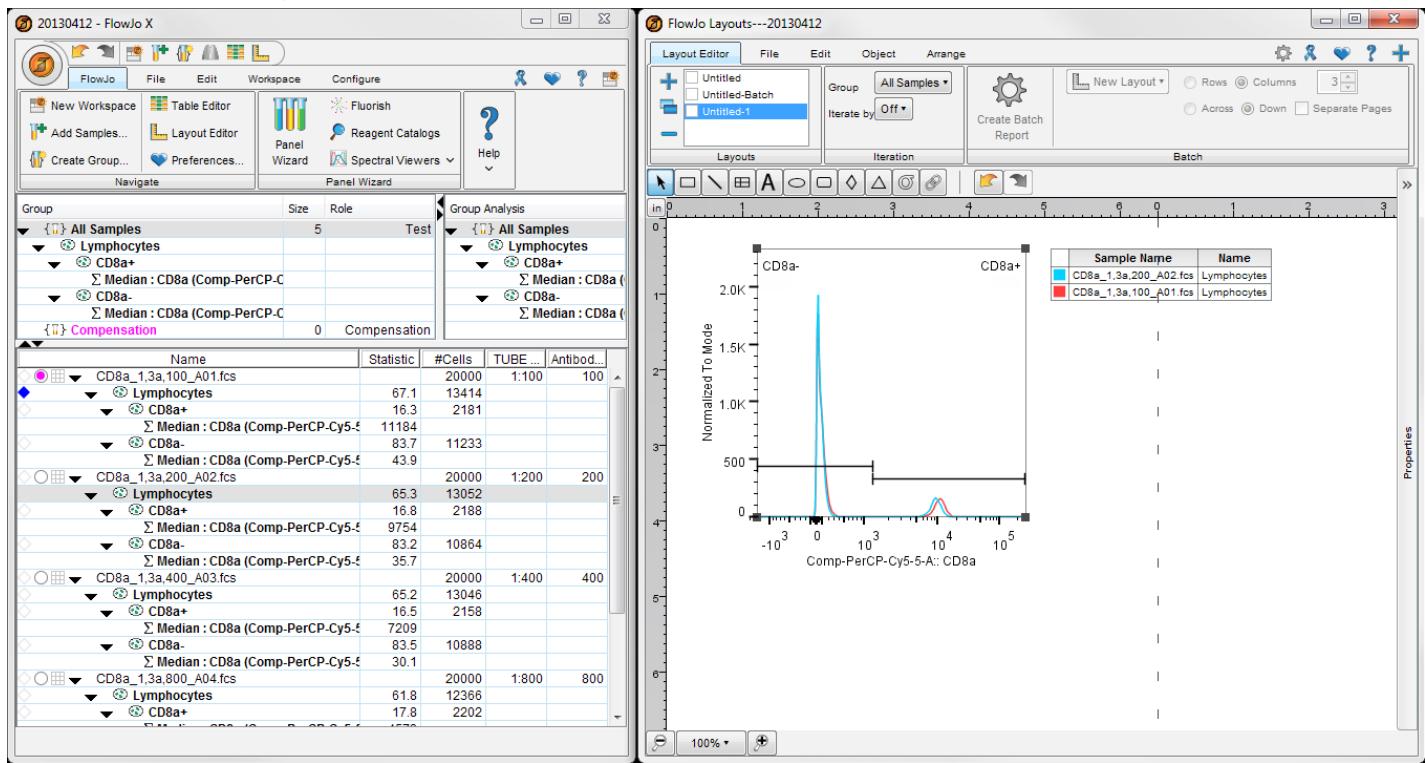
In this experiment, we will drag the lymphocyte population from Sample A01 into the Layout Editor.



Lesson 6 Fig.20 - new batch overlay

(We have removed the frequency, as we did in the first batch):

Now, drag the lymphocyte population from sample A02 onto the existing graph of Sample A01 in the Layout Editor. When you do that and drop the population, an overlay will be created. You will notice that the graph border also turns blue, this is to let you know that you are creating an overlay. When you do that and drop the population, an overlay will be created.

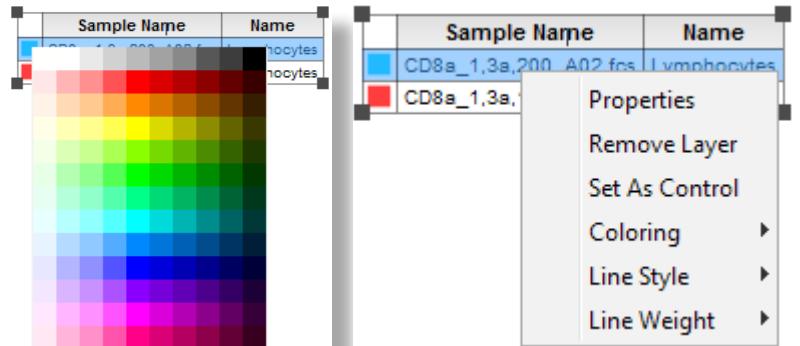


Lesson 6 Fig.21 - overlaid sample

A legend of the overlay will also be present. Left clicking on the color boxes will provide a color palette to change colors of selected populations.

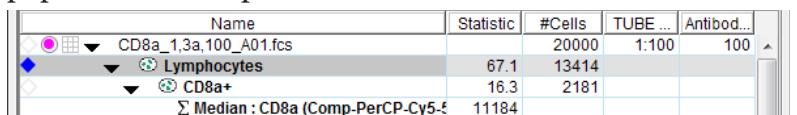
With histogram overlays, you can also right click on the color box to change the line weight, style or fill.

Lets overlay all lymphocyte populations from all samples onto this overlay now.



Lesson 6 Fig.22/23 - menus

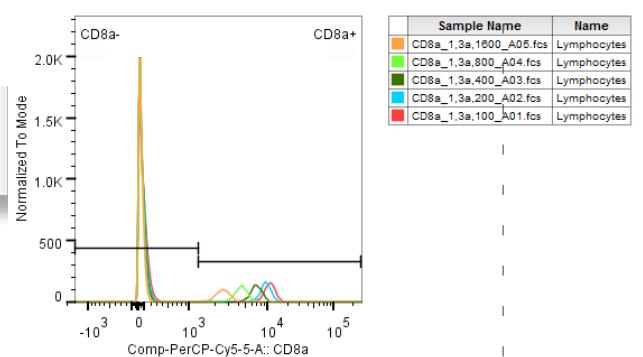
Go back to the workspace and select the lymphocyte population in sample A01.



Lesson 6 Fig.24 - selected node

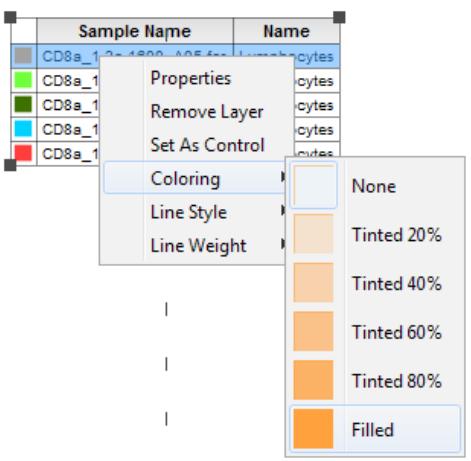
Now, click 'Edit' tab and 'Select Equivalent Nodes':

This will select all the lymphocyte populations in every sample in the workspace. You can now drag them all over with one motion onto the existing plot to overlay every lymphocyte population.



Lesson 6 Fig.25 - all samples overlaid

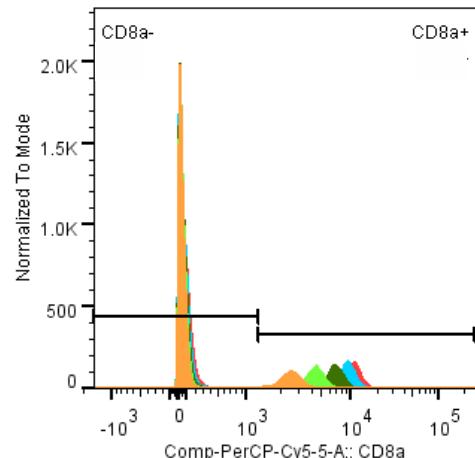
Now, hold down the alt key on the keyboard, right click on the color boxes of the legend and select 'filled'.



Lesson 6 Fig.26 - coloring option

Now all the layers of the overlay should be filled.

You can change the order of the populations in the overlay by clicking any row in the legend and dragging it up or down.



Sample Name	Name
CD8a_1,3a,1600_A05.fcs	Lymphocytes
CD8a_1,3a,800_A04.fcs	Lymphocytes
CD8a_1,3a,400_A03.fcs	Lymphocytes
CD8a_1,3a,200_A02.fcs	Lymphocytes
CD8a_1,3a,100_A01.fcs	Lymphocytes

Lesson 6 Fig.27 - all samples filled

Histogram overlays can also be offset. Right click on the histogram overlay and choose offset. Select the 'Stagger Offset' option here:

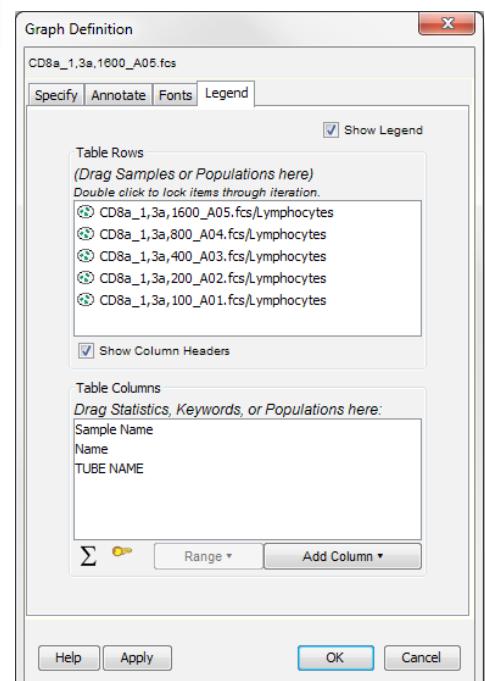
Lesson 6 Fig.28 - menu options

Lesson 6 Fig.29 - stagger offset

By grabbing the back corner of the staggered offset overlay, you can change the perspective.

To edit the legend, double click on it.

In the Legend tab of the Graph Definitions dialog, you can add additional statistics or keywords. Add the TUBE NAME keyword by clicking on the key icon.



Lesson 6 Fig.30 - graph definitions

Saving

There are several ways to save your analysis. Click on the FlowJo icon in the top left corner of your workspace to access these options.

- 1) Always save your analysis as a Workspace (.WSP) or Archive File (.ACS). The workspace file will require your data in order to function and only saves the link to the files. Hence, if your files move independently of the workspace, you have to relink every file back to the workspace. We suggest always saving your workspace in the same folder as your FCS files.
- 2) A better way to save the workspace is as an Archive (.ACS) file. This file type will save the analysis AND the data file information in one big container file (like a .ZIP file).



Lesson 6 Fig.31 - saving options

**If hard drive space is a concern, these files will take up a lot more memory due to the fact that the file is duplicating the .fcs file information. Most of the time this is not a concern, but if these files are 100MB each and you only have 10GB of space on your hard drive, you can see how this could be a problem! Luckily, most people have 500GB or more and don't work with such large data sets.*

- 3) The other option is to save as a Template (.WSPT). If you will run this experiment again, save the analysis as a template so all of the analysis is ready for the next experiment. A template will preserve your analysis structure, but remove the data files. When you run your new data, open the template, drop in your new files and presto, all of your new files are analyzed! You may have to adjust the gates a little, but everything else will be ready for you.

Thank you for taking the time to learn the fundamentals of FlowJo. Our goal is to produce software that is easy to use, easy to learn and scientifically accurate. This tutorial has only scratched the surface of FlowJo's powerful features and functions. Here are a few more things FlowJo can do, which were not covered in this tutorial:

- Compensation - Corrects for overlap from the emission spectra of fluorochromes. As your experiments become more complex, the importance of accurate and automated compensation becomes more pronounced.
- Transformation - Uses custom biexponential functions to include zero and negative value events on scale, maintaining a logarithmic distribution of positive cells.
- Derived Parameters - Supports the creation of new parameters through the algebraic combination of others, including a wide variety of functions and formula structures, analogous to computed columns in a spreadsheet.
- Calibrated Parameters - Calculate the quantitation of absolute molecules per cell (MESF) using reference markers with known calibration values.
- Kinetic Analysis - Measure the change in signal of dyes, or ratios of dyes, over time. Generate statistics and subpopulations based on the subset of cells that have responded to stimulation.
- Cell Cycle Analysis - Computes the percentage of cells in each phase of the cell cycle, based on a dye measuring the amount of DNA present in the cells (eg. 7AAD, PI, DAPI) FlowJo is the first package to integrate multidimensional cell cycle analysis, using BrdU or similar markers to differentiate S phase cells.
- Proliferation Studies - Model the frequency of successive generations, and gate cells by the number of divisions undergone, as measured by intensity of CFSE, or other intracellular dye.

Help menus from any FlowJo window launch a web browser to access a web page describing that topic, giving you context sensitive help.

You can learn more about FlowJo at the links below.

View the entire Reference Manual:
www.flowjo.com/home/manual.html

For a more in depth tutorial, download the 8 Color PBMC Analysis at:
<http://www.flowjo.com/home/tutorials/eightcolor.html>

Application tech notes:
<http://www.flowjo.com/home/tutorials/>

We welcome your feedback! flowjo@treestar.com

Revision date: 16 April 2013
version 10.0.6