



Data Analysis Software for Flow Cytometry
Version X User Documentation

Windows/Mac user documentation

8 Color PBMC 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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- 8 Color PBMC •
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Introduction

FlowJo is a software application with an integrated environment for viewing and analyzing flow cytometric data. The environment is presented as the Workspace which contains a list of all of loaded samples (experimental data), statistics, gates, other analyses, as well as tabular and graphical layouts. The Workspace is saved as a FlowJo document on your hard disk; when you reopen the document, you will see your analysis as it was when it was last saved.

This tutorial is designed to cover a majority of the features pf 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.

FlowJo is capable of much more that simply can't be covered in a tutorial like this (for example, there are analysis platforms for DNA/Cell Cycle analysis, Kinetics, Proliferation, etc. and tools to export raw gated data for analysis in other programs, etc.). You can learn more about FlowJo through our online help documents.

Pressing the question mark button in any window in FlowJo will launch a web browser and access a help pages relevant to that window. From this website, you can navigate the help pages to learn more about FlowJo. In addition, FlowJo.com contains a page for FlowJo FAQs, tutorial videos, and the Daily Dongle, a 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 download the most recent version of FlowJo from:

<http://www.FlowJo.com/home/windows.html>

This tutorial is designed to walk you through the data analysis process in FlowJo from adding data to producing tables of statistics and publication-quality graphics. The data is from 2 8-color immunophenotyping experiments of human peripheral blood mononuclear cells (PBMC). Keep in mind that the workflow described in this tutorial will help you in the analyses of any kind of flow cytometric data.

The tutorial is divided into 11 lessons so you may perform it stepwise. The tutorial is written for the user to perform all operations from start to finish, but we have included completed workspaces so that you can jump to any stage of the tutorial. If you would like to perform the tutorial starting with a lesson other than 1, just open the workspace from the preceding lesson and you will have all of the work completed through that lesson.

Table of Contents

<i>About FlowJo</i>	<i>i</i>
<i>Introduction</i>	<i>ii</i>
<i>Table of Contents</i>	<i>iii</i>
<i>Getting Started</i>	1
The Experimental Data	3
FlowJo Workflow	3
<i>Lesson 1: Workspaces and Basic Data Display</i>	5
Opening a New Workspace.....	5
Adding Data	6
Displaying and Editing Metadata	7
Adding a Keyword.....	9
Displaying a Graph.....	10
Graph Types	10
Three-Dimensional Polychromatic Plots	11
Adjusting the Scale	12
<i>Lesson 2: Gating and Statistics</i>	14
Creating a Polygon Gate	14
Editing a Gate.....	15
Creating a Gate on a Histogram	16
Adding Statistics	17
Other Types of Gates.....	18
Deleting Gates.....	19
<i>Lesson 3: Copying Analyses to Other Samples</i>	20
Setting up an Analysis.....	20
Copying a gate to Another File.....	20
Copying Multiple Gates.....	21
Deleting Analyses	22

Lesson 4: Groups	23
Creating a New Group	23
Modifying an Existing Group	24
Deleting a Group	24
Creating a Group Using Keywords.....	24
Adding Analyses to All members of a Group	25
Modifying Group Gates.....	26
Lesson 5: Tables and Collating Data Output	28
Creating a New Table	28
Deleting a Column	29
Ordering the Samples in a Table.....	29
Renaming the Columns in a Table	29
Adding a Keyword or Formula to a Table	30
Batching to Create a Table.....	31
Table Iteration	33
Additional Tools	33
Applying Conditional Formatting.....	33
Dynamic Updating	33
Lesson 6: Creating Simple Graphical Layouts	34
Creating a New Layout.....	34
Creating a Second Graph of a Population	35
Editing a Graphs Appearance	35
Aligning Plots.....	35
Dragging Statistics into the Layout Editor	36
Creating Formulas in the Layout Editor.....	36
Drawing Tools.....	36
Overlays	37
Ancestry and Backgating.....	38
Multigraph Overlays	39
Displaying Adjunct Histograms	40
Exporting a Layout Page.....	40

Lesson 6: Continued...

Printing out of the Layout Editor 41

Copying and Pasting Graphs from FlowJo..... 42

Lesson 7: Creating Batch Graphical Reports..... 43

Batch Plotting..... 43

Other Batch Outputs 44

Lesson 8: Generating Complex Batch Reports 46

Varying Iteration Options..... 46

Batching Overlays with Multiple Samples..... 48

Batching While Maintaining a Control Sample..... 49

Lesson 9: Creating Finished Reports 50

Importing a Graphic into FlowJo 50

Adding Text 50

Adding Plots..... 51

Using the Grid Tool..... 52

Adding a Table from the Table Editor to the Layout Editor ... 53

Lesson 10: Compensation 54

Preference settings for loading data 54

Data Compensated by the Acquisition Software..... 54

Editing an Existing Matrix 56

Creating a New Matrix..... 57

Lesson 11: Setting Preferences 60

What are preferences? 60

Getting Started

To get started using FlowJo, you will first need to install the software. The easiest way to do this is to download the most current installer which you can find on our website at: <http://www.FlowJo.com/download/index>

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 the icon to launch FlowJo.

When you launch FlowJo for the first time, the FlowJo license information window will pop up. You must select and input the type of license you have and agree to the license terms.

For the use of running our tutorials, you need only to select “Continue under free demonstration license” and click done.

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

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.



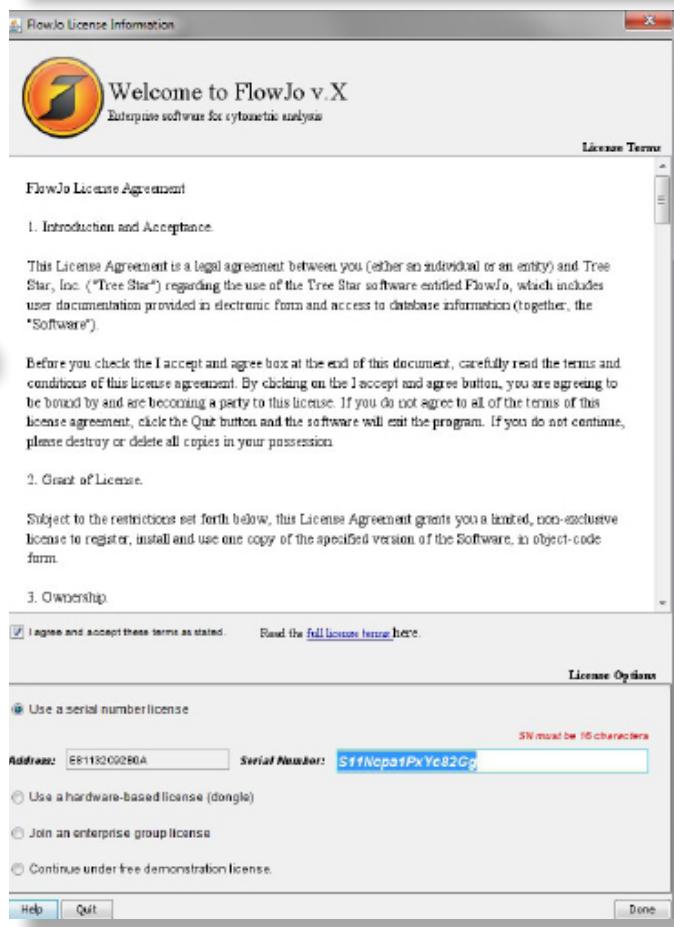
For more about dongles and for dongle support, visit:
www.flowjo.com/support/index.php?content=dongle

If you have an enterprise group license, you can configure the connection here as well.

If you have an individual license that you would like to use, input your serial number in the Serial Number field (*shown at right*).

Demonstration data and the accompanying workspaces can be acquired from our website:
<http://www.flowjo.com/home/tutorials>

Following this link will bring you to a page where you can download our Basic Tutorial and Advanced 8 Color Tutorial as .zip files 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 them on a Mac and right click to extract them on a PC.



The Experimental Data

The demonstration data set is comprised of three folders :

- A folder of compensation controls.
- A folder named Exp. 1 which contains two subfolders named Panel 1 and Panel 2 which each contain 9 samples stained with two different combinations of reagents (see table below).
- A folder named Exp. 2 which contains two subfolders named Panel 1 and Panel 2 which each contain 9 samples stained with two different combinations of reagents, similar to Experiment 1.

There are 2 experiments, 2 panels, 9 tubes for each panel, and 7 compensation control tubes for a total of 43 tubes ($2 \times 2 \times 9 + 7$). The compensation controls are provided for the user who wants to learn more about the compensation editor (described in Lesson 10). However, all the files were compensated at the time of acquisition using Becton Dickinson's Diva™ software. FlowJo reads the acquisition compensation matrix from the files and displays the compensated data by default. The stain combinations are listed by panel in the table below.

Panel No.	APC-Ax 700	APC-H7	APC-Ax 647	Viability Dye	Fitc	PE	Pe-Cy5.5	Pe-Cy7
1	CD3	CD19	2H2	7 AAD	CD32	CD86	CD209	CD14
2	CD4	CD8	2H2	7 AAD	CD56	CD11c	CD3	CD45RO

Table 1: Experimental data panels

FlowJo Workflow

When you open FlowJo, the interface that appears is referred to as a Workspace. It serves as :

- the repository for your data
- a tool for organizing the data
- a means for displaying and managing your gating strategy
- a statistical display
- a starting point for all analyses

Double-clicking on a file opens a Graph Window. This interface is used to view and set gates on individual tubes.

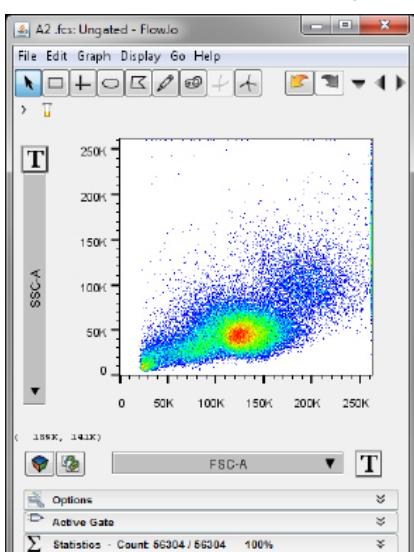


Fig. 2 - Graph window

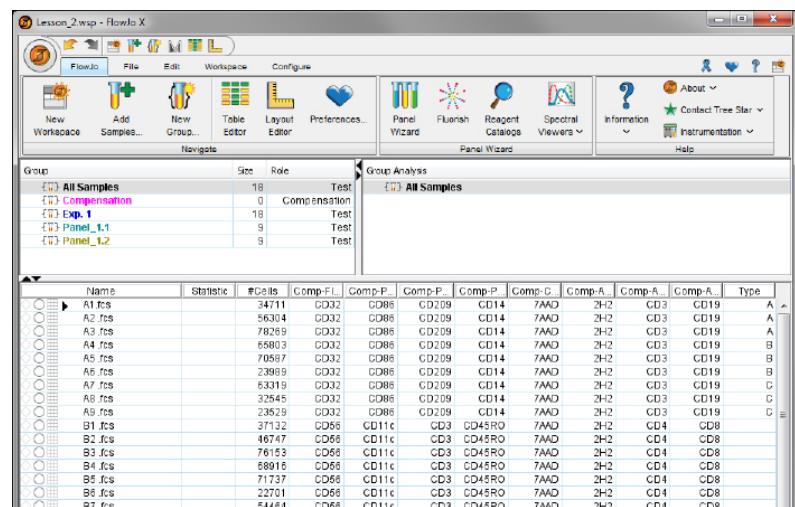


Fig. 1 - The workspace

The Table Editor is accessed through either the Table menu or through a shortcut button at the top of the workspace. But the Table Editor button is used from the populations and statistics created in the workspace (producing tabular outputs.)

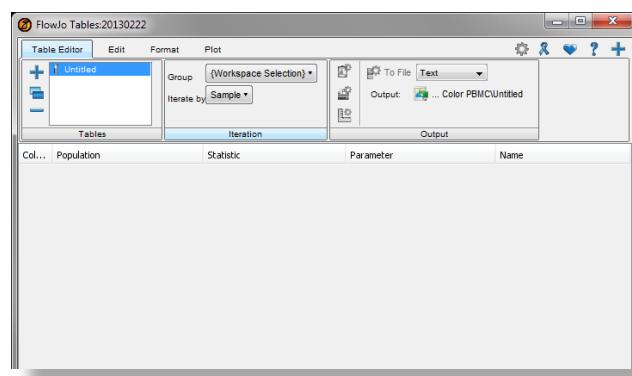


Fig. 3 - Table editor

The Layout Editor is accessed through either the Layout menu or through a shortcut button at the top of the workspace. It is used to create publication quality graphics.

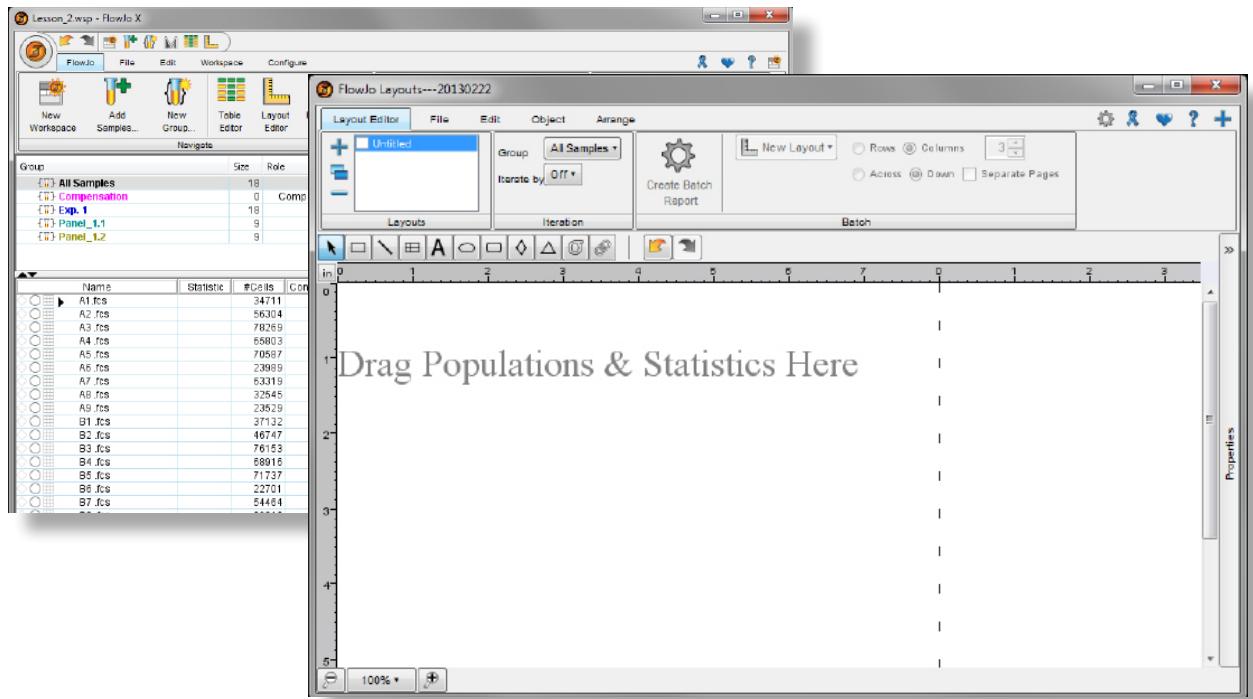


Fig. 4 - Layout editor

All of these functions are designed based on the idea that each data file is part of an experiment and has context. For that reason, many of the operations you will perform in FlowJo can be “batched”, that is, applied to a set of files that the user wishes to analyze, print, or tabulate in a common manner. Batching enables you to perform an operation once and instruct the software to repeat the action for a specified set of files, sparing the user the repetitive work. If you find yourself repeating an action frequently it's likely that a batch operation exists that will save you significant time.

Lesson 1: Workspaces and Basic Data Display

In this lesson you will learn how to start the program, load and organize your data, view graphs and other basic concepts of the workspace.

The workspace in FlowJo provides an integrated environment for the viewing and analysis of flow cytometric data. The workspace contains a list of the samples that you have loaded into FlowJo, gates applied, statistics calculated, and the tables and graphical layouts that you have designed. If you save your workspace, you will save all aspects.

The workspace does not contain the raw data. Instead, the workspace saves pointers to the loaded data, avoiding the problem of creating multiple copies of data files, which would use two or three times more memory on your computer. Because FlowJo operates in this manner, if you move the raw data files to another disk, FlowJo will prompt you to find the data the next time it needs that information. Workspaces store much of the information about samples, as well as all of the analyses (gates and statistics) that you have previously computed. The best practice is to save your analyzed workspaces in the same folder, at the same level, as the raw .fcs data files.

Think of the workspace as an experimental notebook. In general, you will create a different workspace for each experiment that you perform. Workspaces may contain multiple samples collected on various days – and can provide an efficient way to organize your data analyses.

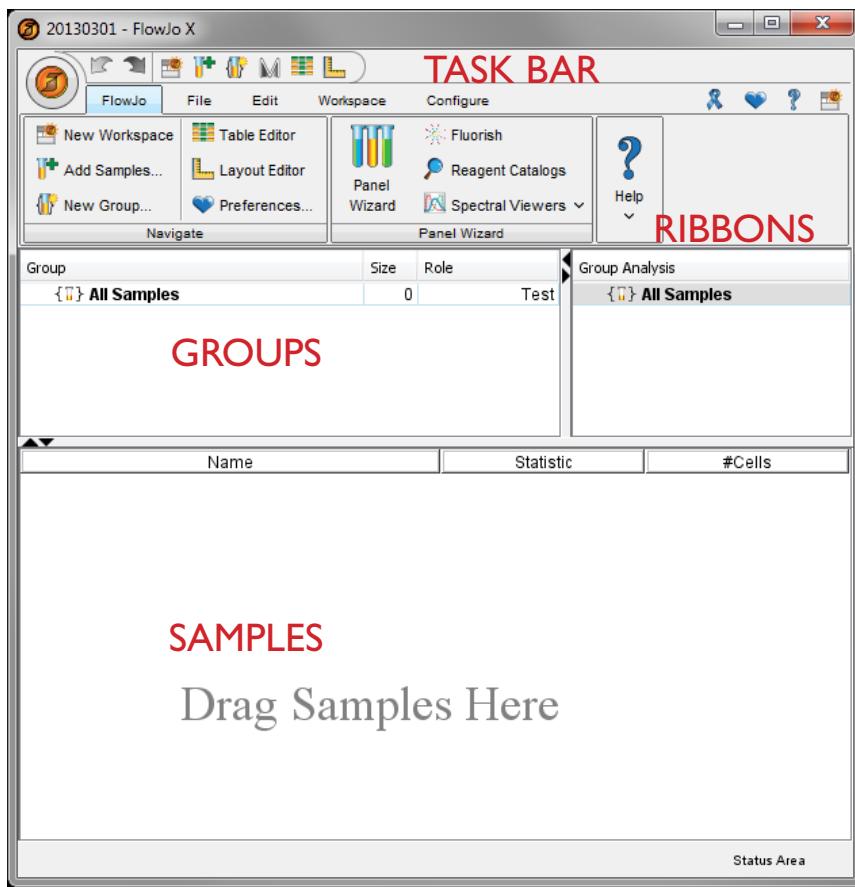
To begin analyzing data in FlowJo, you will need to create a New Workspace and add data files into it. Later, you can simply double-click on this Workspace to continue your work.

Opening a New Workspace

Launch FlowJo by double-clicking on the FlowJo shortcut icon from the desktop. Once you have launched FlowJo, Click on the FlowJo tab, you will be shown a window similar to the graphic to the right.

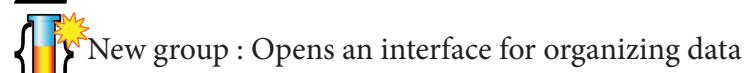
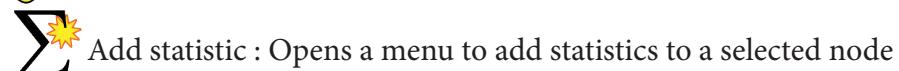
Note the Question Mark icon at the far right end of the menu choices. This is the FlowJo Help menu. When clicked, this button displays options for help in specific topics, which launch FlowJo's web browser online.

The workspace window is divided into four parts. The top portion is a taskbar. The taskbar has action buttons to let you add components to the workspace, such as samples, groups, tables, etc. As you move the mouse over an action button, FlowJo displays a description of that button's action in the tool tip.



L1 fig. 1 - Workspace

The actions associated with each button are:

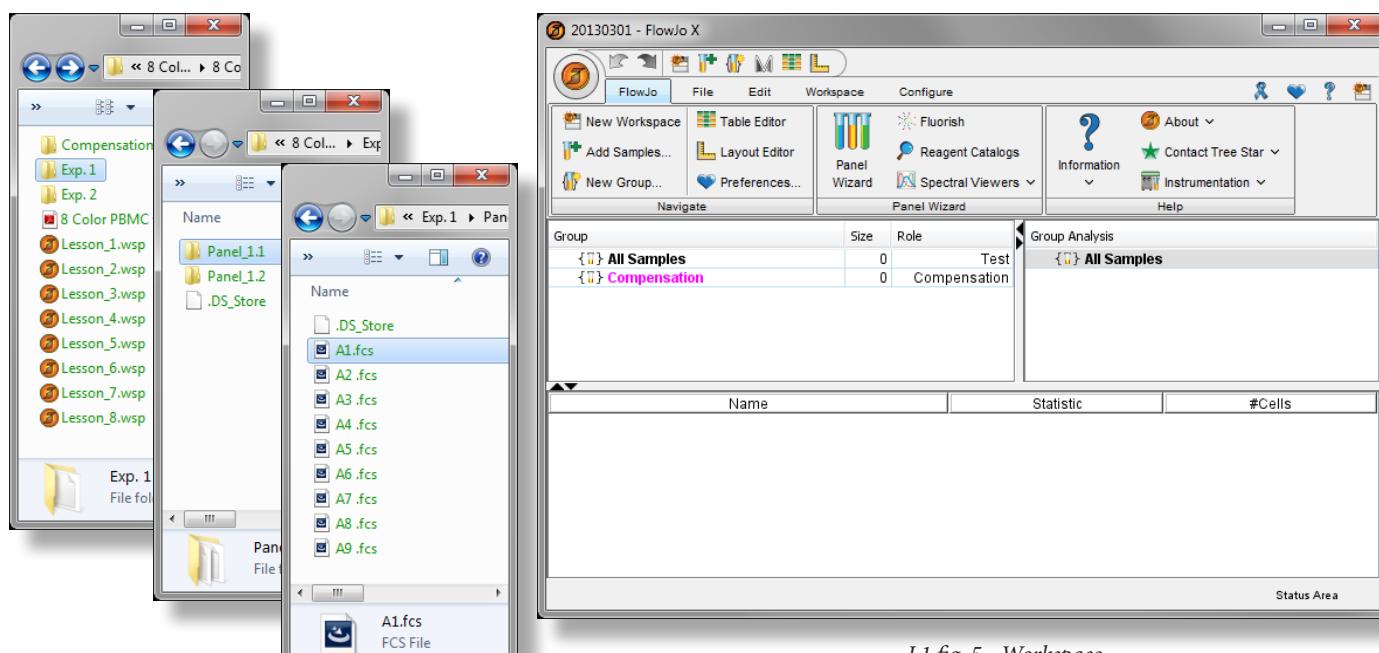


The second part of the workspace is a ribbon bar. This bar has action buttons that can be customized, which will be discussed later. As you move the mouse over each action button, FlowJo displays a description of that action. The third part of the workspace is a list of the current groups. At the moment the only groups that you will see in your workspace are the All Samples Group and a Compensation Group. These are the default groups that are always present; All Samples includes all of the data files in the workspace. Groups will be discussed in depth later in this tutorial, specifically creating new groups, editing existing groups, and using groups to facilitate batch processing.

The bottom part of the window displays individual data files. The files in a selected group are displayed here. At the moment there is no data in the workspace, so the area will be blank.

Adding Data

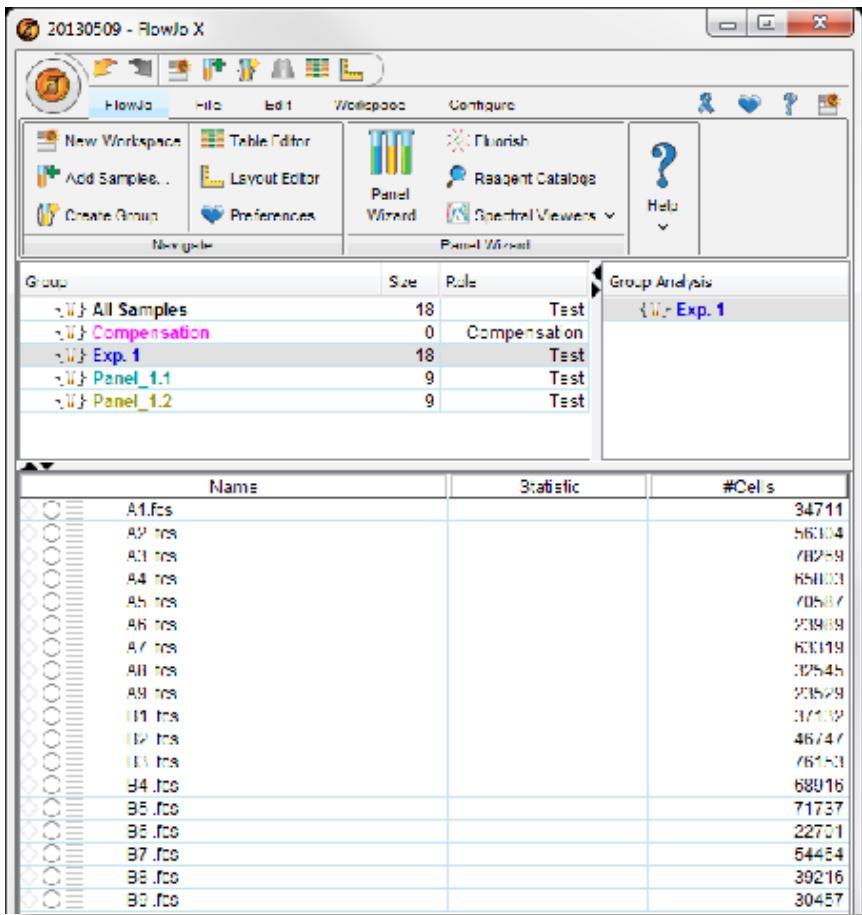
Locate the 8-Color PBMC tutorial folder that you downloaded, and double-click to open the folder. You will see the Compensation, Exp.1 and Exp. 2 folders. If you open the Exp. 1 folder you will see the Panel 1.1 and Panel 1.2 folders. If you open either of these you will see the Flow Cytometric Standard (FCS) files. The same file type a cytometer outputs. To load Experiment 1 into FlowJo, click on the “Exp.1” folder, hold down the mouse button, and then drag the folder to the bottom portion of the FlowJo Workspace. Release the mouse button.



L1 fig. 5 - Workspace

L1 fig. 2,3,4 - Samples

Your workspace will now populate with two additional groups and 18 total data files. It will look like the Workspace in figure below (fig. 6).



L1 fig. 6 - Samples

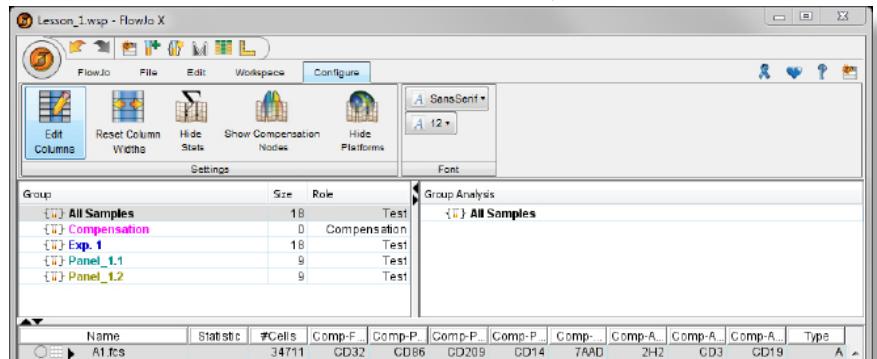
to another group, you can click on the “All Samples Group”, select a file, then drag and drop it into another group. You can also remove a file from a group by clicking on the group, locating the unwanted file, selecting it, and pressing “delete” on your keyboard. The file will not be removed from the Workspace, only that particular group. To remove a file from the Workspace entirely you must delete it from the All Samples Group.

Alternatively, to add data into the workspace you can click on the “Add Samples” shortcut button at the top of the workspace. This button opens a standard Open File dialog. At that point you must navigate to the data location and choose “Open”.

Displaying and Editing Metadata

FCS files contain a text portion with experimental information, or metadata, such as the type of cytometer on which the data was collected, date collected, etc. This information takes the form of keywords; a set of code words or abbreviations that are associated with specific information. For example, the keyword \$DATE lists the date that the data in an FCS file was collected.

Display some of these keywords in our FlowJo workspace by clicking on the “Configure” tab located at the top of the workspace, and choosing the first item in the band: “Edit Columns” (fig. 7).



L1 fig. 7 - Configure

Note that FlowJo will display the files contained in whichever group you select. Here we have selected the All Samples group, so the files in All Samples are displayed in the lower portion of the workspace. If you click on a different group, only the samples in that group will be displayed. The number of samples in each group is shown to the right of the group name.

You will note that FlowJo has preserved the file organization of the folders in its groups. A new group was created for each subfolder and the files that were stored in the subfolder were automatically added to the group of the same name. Additionally, all of the files were added to the All Samples Group.

Also note that each file is in more than one group – the All Samples Group, Exp. 1 Group, and one of the panel-based groups depending on the file. You can place a file in as many groups as you like. To add a file

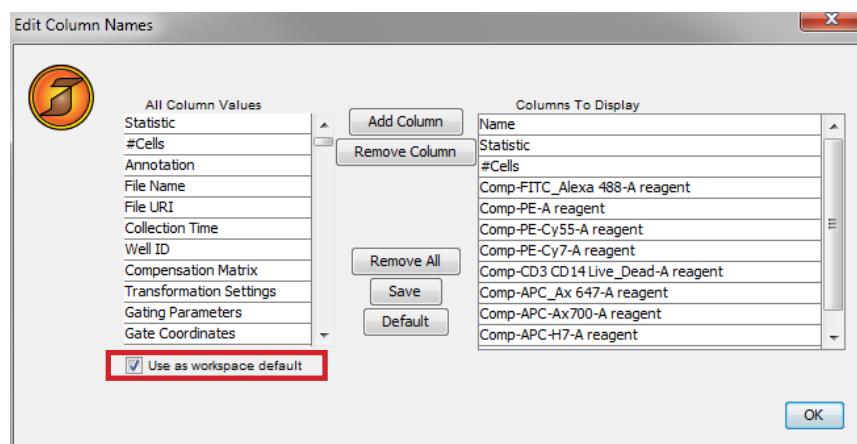
in as many groups as you like. To add a file

The window shown at right will appear (fig. 8).

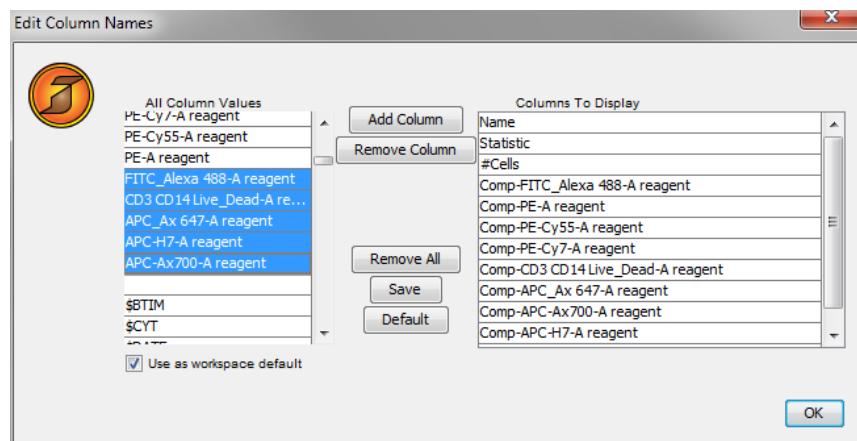
The left column in the window is a list of all the keywords contained in the FCS file. To display any of these in the workspace, click on the chosen keyword, then press the “Add Column” button. To remove a keyword, click the keyword name in the right column and select “Remove Column” from the dialogue box. Note that there is a “Default” button toward the bottom of this dialogue box that will allow you to reset the displayed columns to the default condition.

For this experiment we will display the reagents which were used to stain each sample. We would like to see the names of the compensated parameters. To display these keywords, in this case begin by scrolling down to the comp FITC_Alexa 488-A reagent. Click on this parameter, hold the shift key down to select multiple items, and press the down arrow until all of the reagents are selected (fig. 9). The last one selected will be comp APC-H7-A reagent. Now press the “Add Column” button, then press “OK” in the bottom right-hand corner. The dialogue box will close and your workspace will now be populated with eight additional columns. If you compress some of the columns by clicking on their header and dragging the edge of the column to the left, your Workspace will now look like the one shown at right (fig. 10).

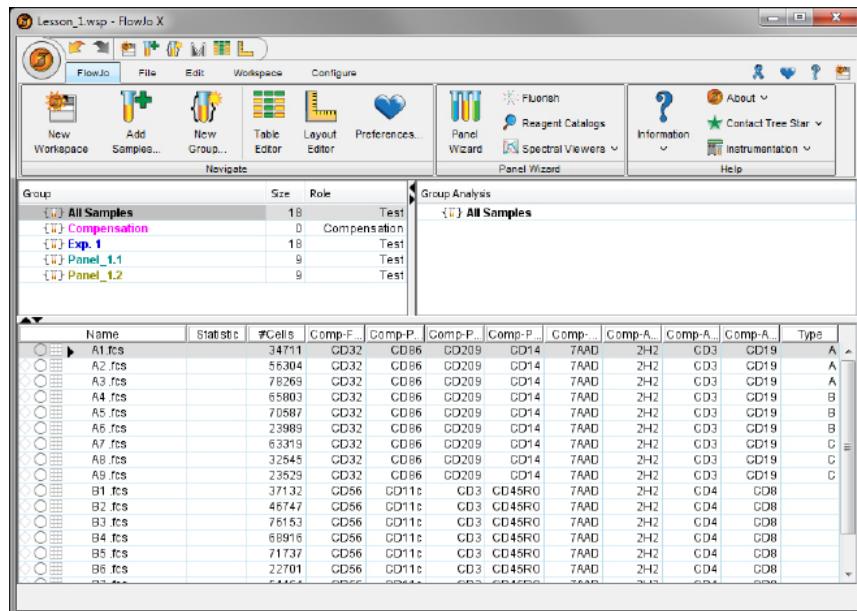
The square grid to the left of the sample name indicates that a parameter has been compensated during acquisition. The data was algorithmically compensated using BD’s FACSDiva® software. The spillover matrix is recorded into these files as the keyword \$SPIL, so that FlowJo has access to the raw data and the matrix. By default FlowJo displays the data with the compensation matrix applied.



L1 Fig. 8 - Edit column



L1 fig. 9 - Reagents



L1 Fig. 10 - added columns

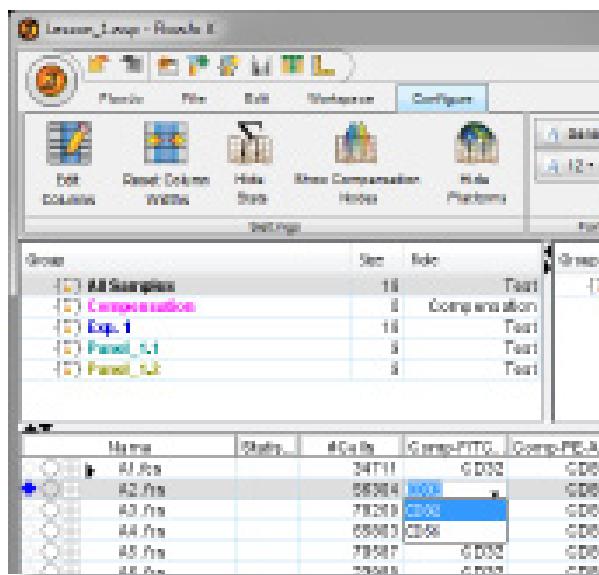
A discussion on how to create or edit compensation matrices is provided in Lesson 10: Compensation.

The workspace now displays the list of samples and reagents. The keywords are editable, so if a data entry mistake was made at the time of collection it can easily be corrected in FlowJo. Try double-clicking on one of the cells annotated with “CD32”. The box will open and highlight the text allowing you to overwrite it (*fig. 11*). Try editing the cell and then making the correction. The only keyword that cannot be edited directly here is the Name. You can however, select a different keyword to be displayed under the Name column through the preferences.

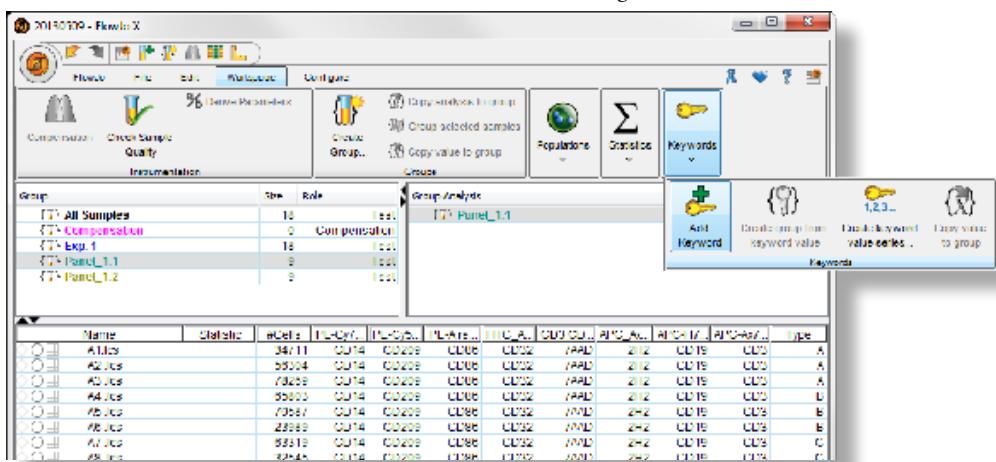
This will be explained in Lesson 11: Setting Preferences

Adding a Keyword

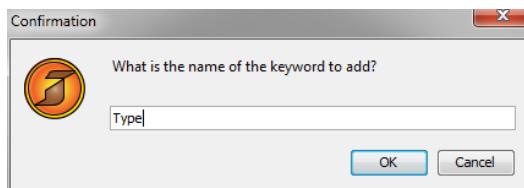
Click on the group “Panel_1”. Go to the workspace tab and from the Keywords band, select “Add Keyword”. When the dialogue box appears type “Type” into it (fig. 12). You will get a blank column under the header Type in your workspace. Double-click on the top cell and type the letter “A” into it. Data entry can be done manually into any of these cells, but there are many shortcuts. With the first T-cell selected, go to the workspace tab and select “Copy Value to Group”. Once you select this, you will notice that the entire column is now populated by the letter ‘A’ (fig. 13).



L1 Fig. 11 - edit CD32



L1 Fig. 12 - add keyword

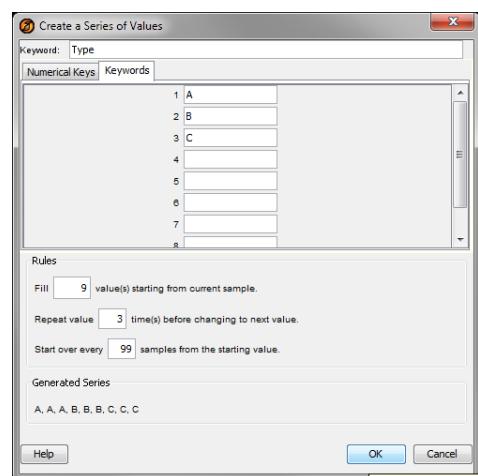


L1. Fig. 13 - keyword type

Click in the top cell in the column again, and then open the Keywords menu and select “Create Keyword Value Series”. A dialog box will appear allowing you to make a pattern of numbers in the column.

Click the “Keywords” tab, and you will see the interface change as shown to the right. (fig. 14)

This tool allows you to replace selected numbers with text to create alphanumeric patterns. Type 'A' in the box marked 1, 'B' in the box marked '2' and 'C' in the box marked '3'. Then in the bottom portion of the interface, type '3' in the box labeled "Repeat value _ times before changing to next value". The output, based on the values you have input, is displayed in the bottom portion of the window in the box labeled "Generated Series". In this case it is A,A,A,B,B,B,C,C,C. Click "OK". You will see the Type column populate with this pattern.



L1 Fig. 14 - value series

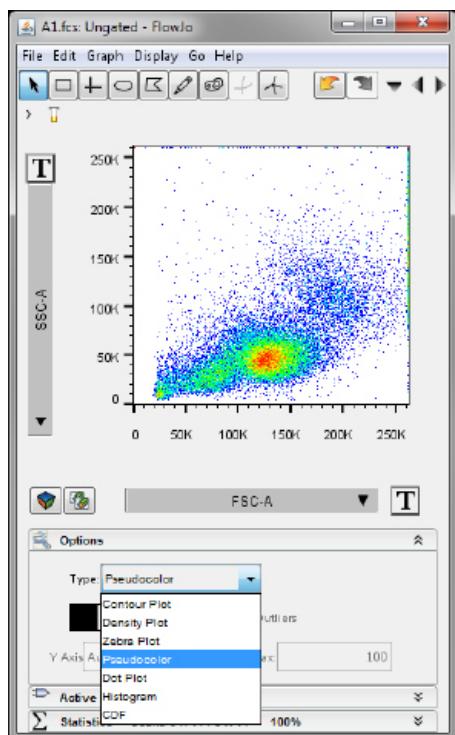
The last option for filling data into a column is through copying and pasting from a third-party program. If you copy a column of data from other software, such as Microsoft Excel, insert it into FlowJo by clicking on the top cell of the column in the workspace in FlowJo that you would like it to appear, then select “Paste” from the Edit menu.

Displaying a Graph

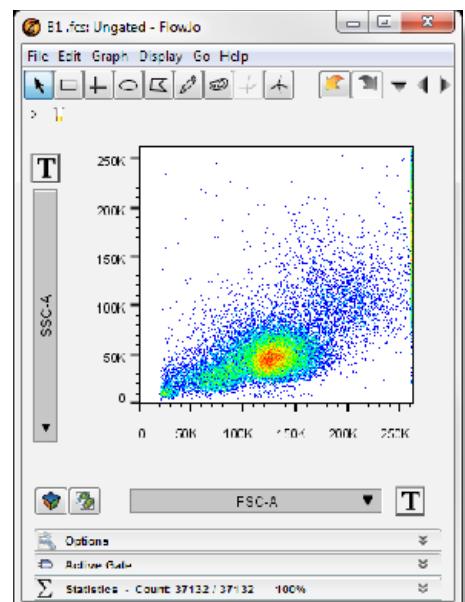
To display a graph of the data, double-click on A1.fcs and a plot will appear, as shown in the figure to the right. This is a Graph Window. There are several different kinds of plots that can be used to display flow cytometric data. The default type is the Pseudocolor Plot. The default plot type can be set using the Preferences dialog box (the blue heart icon in the upper right-hand corner of the workspace). (fig. 18)

See Lesson 11: Setting Preferences for more information.

The easiest way to change the graph type is to open the Options drop-down menu below the graph image and make personal adjustments. The drop-down menu will look like the image below. (fig. 19)



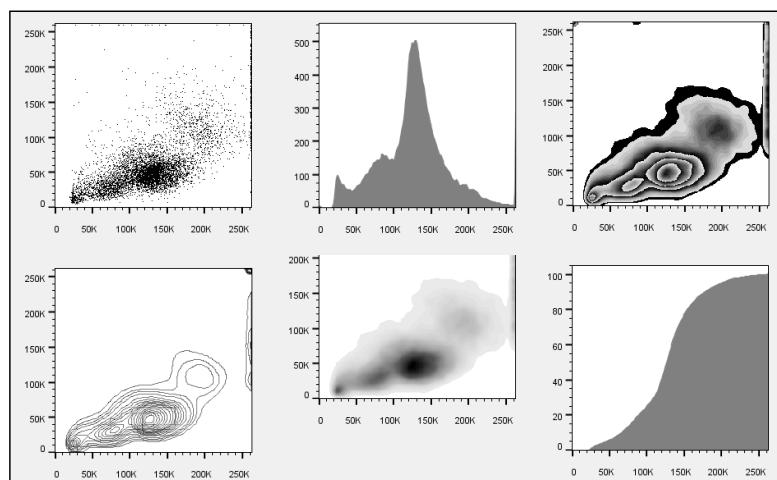
L1 fig. 19 - Graph window options



L1 fig. 18 - Graph window

Graph Types

The graph Type menu allows you to change the graph to a contour plot, density plot, zebra plot, pseudocolor, dot plot, histogram, or a cumulative distribution function (CDF). An example of each plot type is displayed below. (fig. 20)



L1 fig. 20 - Graph types

Left to right - top row : dot plot, histogram, zebra plot
Bottom row : contour plot, density plot, cdf (cumulative distribution function)

Contour, zebra, and density plots are useful in visual normalization of the number of collected events, as these plots show the distribution of the data and not individual events. Contour plots show cell distribution with lines of equivalent density that increase at user-defined intervals. Density plots do likewise with increasingly dark colors. Zebra plots show cell distribution with a combination of lines and coloring. If rare events are important to display, there is an option for “Show Outliers” that will plot individual dots for events outside of the distributions for these types of plots. Pseudocolor plots, like traditional dot plots, display all events but also add brighter colors to show increasing cell density.

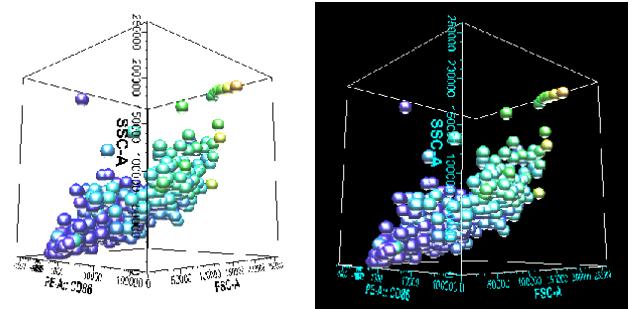
Histograms and CDF's (which display the data cumulatively versus increasing in number) are useful for displaying univariate data.

Additional features within the Options drop-down menu allow you to smooth the display, change the background or foreground colors, change from high to low resolution and make other plot-type dependent modifications.

Three-Dimensional (3D) Polychromatic Plots

To view data in three-dimensions, click on the cube icon  in the graph window.

The 3-D viewer platform will appear with a menu allowing you to display three parameters on a cubic coordinate plane. This allows for three-dimensional plots that convey information on five parameters simultaneously. (fig. 21)



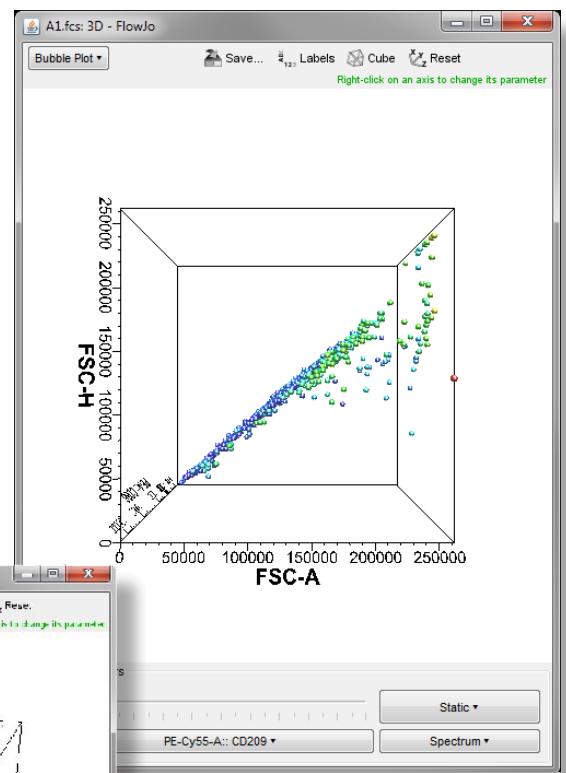
L1 Fig. 21 - 3D viewer

Using the drop-down menu in the top left portion of the window allows you to select the type of 3D plot. The default is to display each cell as a dot, or point cloud. There are also bubble plots and terrain plots. Bubble plots show the sampled cells as larger bubbles instead of dots and terrain plots are 2D Histograms with number of events extruded as the third dimension. Try each type, then select “Bubble Plot”. The figure at right shows the 3D viewer platform with a bubble plot selected. (fig. 22)

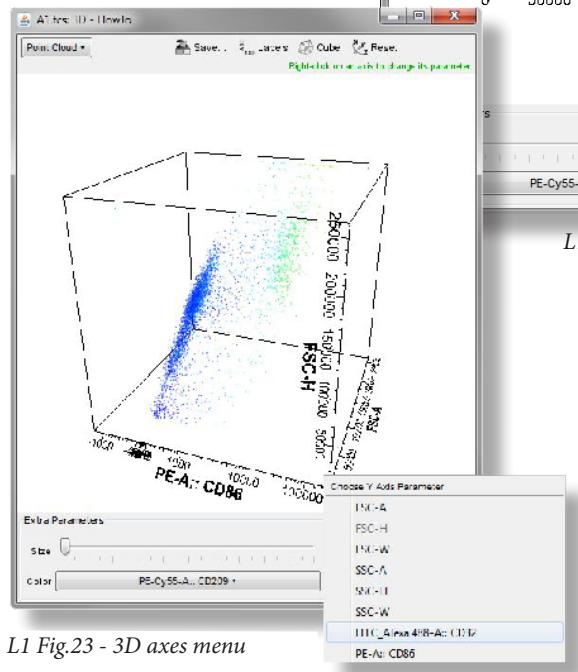
The size of the bubbles can be set to correlate to the staining intensity of a given parameter, allowing the user to display a seventh parameter. Other control buttons at the top of the screen are: Save as image, unshow axis Labels, remove the Cube and Reset the axis to the default.

The next controls, shown below, are the assignment boxes for each axis. When you right-click on one of the axes, a menu appears allowing you to assign a parameter to a specific axis. (fig. 23)

To turn the cube or move the cube around to see other populations, simply click and hold down the mouse button while you drag the mouse left and right or up and down. To set the cube in a spinning motion, click and drag quickly left or right and the cube will rotate on its own. To stop the cube from rotating, click anywhere on the cube. Close the 3D viewer to continue exploring data display.



L1 Fig.22 - 3D bubble plot



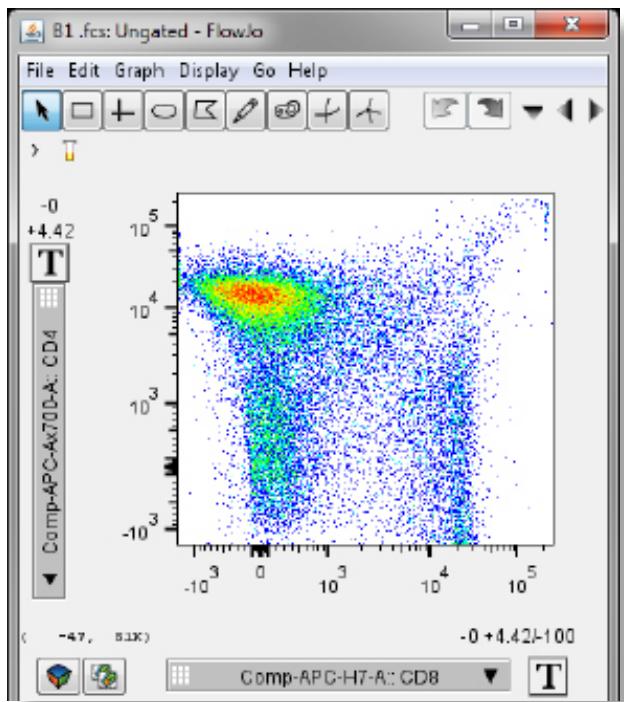
L1 Fig.23 - 3D axes menu

Adjusting the Scale

Double-click on file B1.fcs from Panel 1.2 and change the parameters displayed on the axis to CD8 versus CD4. Your plot will look like the figure to the right. (fig. 24/25) Observe that a good portion of the data is compressed onto the axis. You will want to adjust the scale to get all of the data on screen.

To do so, press the **T** button on either axis to access this menu.

Clicking the “Customize Axis” option allows you to change the axis from logarithmic to linear or biexponential. The biexponential scale is effectively a hybrid of a linear and logarithmic scale. The low end of the data is displayed with a linear scale allowing negative numbers and zero to be displayed, while the high end of the data is displayed using a logarithmic scale. This allows the very brightly stained cells to be compressed into a reasonable portion of the plot and viewed as a coherent population.

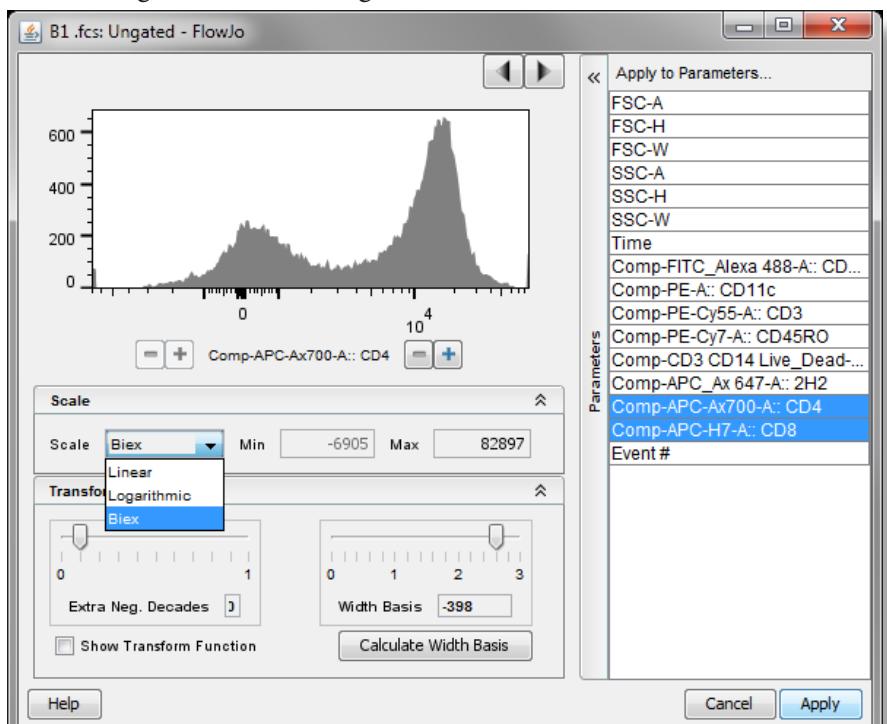


For more information on the details of the biexponential transform, visit: <http://www.flowjo.com/vX/en/gw.transform.overview.html>

This data is already shown on a biexponential scale because it was collected using a biexponential scale which FlowJo was able to gather from the FCS file. (fig. 26)

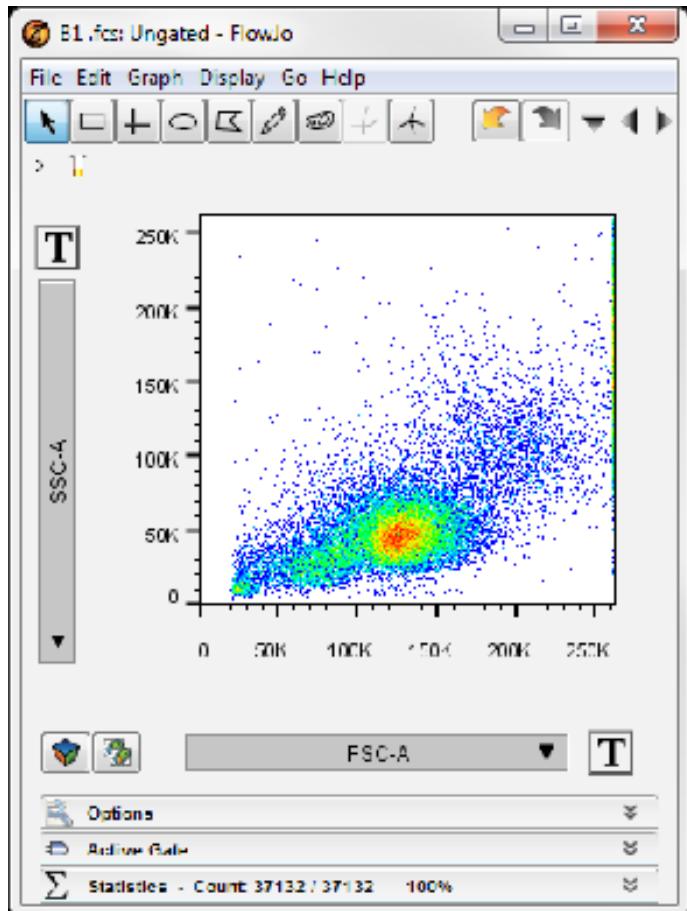
There are two sliders in this window that allow the user to modify the aspects of biexponential transform. From left to right, you can extend the width basis and change the decade range. There are also +/- buttons to control the positive decade range.

- Extra Negative Decades - On a biexponential scale, some amount of negative space is shown. If you would simply like to show more negative space, then you can move the slider toward 1, adding up to one full additional negative decade to be shown. Move the slider to see how it changes the data display and set it to one negative decade.
- Width Basis - The width basis is the amount of space shown on a linear scale, on both sides of zero. This number will be negative to make the mathematics of the biexponential transform work, but you can interpret it as a magnitude. For example, if you set the width basis to -10 then the space from -10 to +10 will be shown linearly and the space above and below that will be scaled logarithmically. Move the width basis slider up to see the effect on the data. Settle on a width basis of -398.
- Positive Decade Range (+/- buttons) - This setting allows you to control how many decades the data is displayed over. Click the buttons to see the effect. Click Apply.(for this data file, the default Positive Decades are set to 4.42)

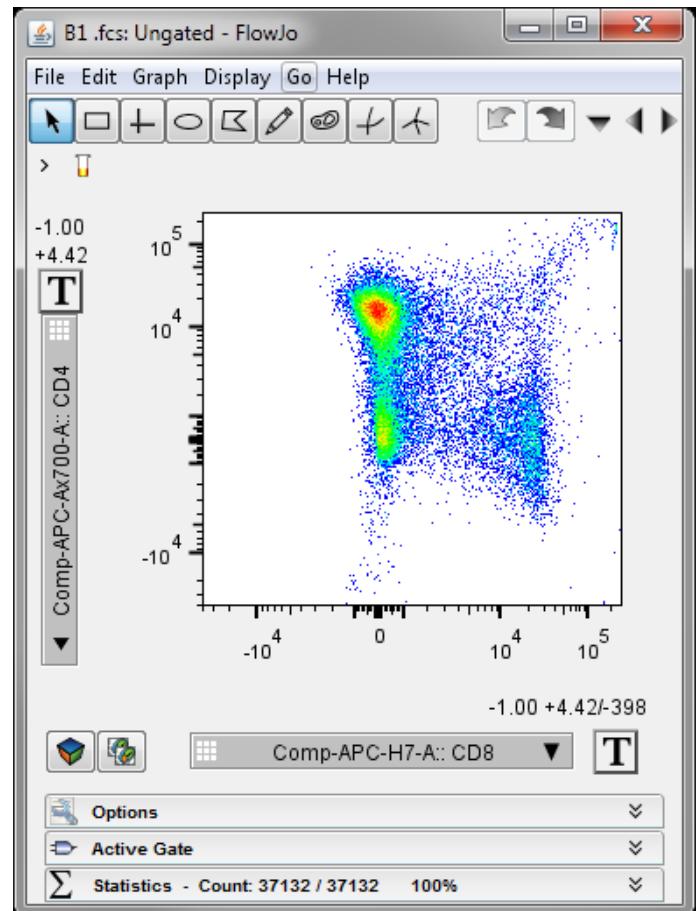


L1 fig.26 - Custom axis control

FlowJo can also help you determine the optimum width basis automatically using the “Calculate Width Basis” button. In addition, you can manually type in your width basis allowance. Once you have set your choices you can apply them to this particular parameter using the “Apply” button in the bottom right-hand corner of the platform. You can modify additional parameters by clicking the vertical “Parameters” sidebar menu. The transform you have set will be applied to each parameter selected from the list. Choose the CD8 and CD4 parameters and click “Apply” and the window will close automatically. You will notice that the scale indicator at the bottom of your axis now reads “-1.00 +4.42/-398.00”. This means that FlowJo has added an extra negative decade, expanded the display area to 4.42 decades and added a width basis of -398.00. Your plot will look like the figure displayed below on the right. (fig. 27B)



L1 fig.27A - Before parameters



L1 fig.27B - After transformation

It is important to emphasize that using a biexponential scale does not change the data. It simply changes the amount of space on the screen allotted to displaying various portions of your data. Statistics calculated on the data before and after transform will be the same. However, gates made on data before a transformation is applied may not translate between axes transformations. It is wise to look at your data, adjust the transforms so that data is visible and then begin gating.

This is the end of the lesson on Workspaces and the display in FlowJo.

To access the Workspace at this point of the analysis, open the Lesson_1.wsp file.

• • •

Lesson 2 : Gating and Statistics

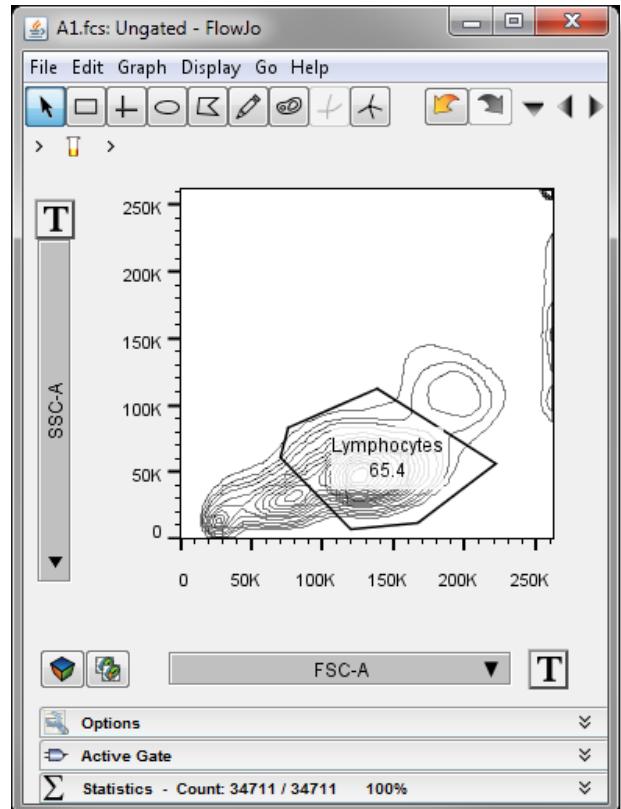
In this lesson, you will learn how to Gate your data to create subsets and isolate a given population of cells for further analysis. You will also learn how to calculate a variety of statistics. Subsets in FlowJo are exactly like subsets in biology: they represent a fraction of the entire collection with specified properties (e.g., “Lymphocytes” are those cells with low Forward and Side Scatter). You will always be asked to name a subset; the name you choose is important for organization within FlowJo. Select a name that is meaningful to you, to help keep track of your analyses.

Creating a Polygon Gate

Double-click on the first file in the workspace to open a graph window and change the graph to Forward vs. Side-Scatter (FSC-A vs. SSC-A) with a Contour Plot. You will now create a lymphocyte gate using a polygon. To do so, select the  Polygon Tool from the top of the window, click inside the graph and move the mouse. Each time you want to place a vertex and continue, click and continue to move. Attempt to create a gate similar to the figure shown on the right. If you hold down the shift key, you get horizontal, vertical, or 45° angled lines. To finish and close the gate, either click on the starting point or double-click at any time. As soon as you close the polygon, FlowJo asks you to name the subset that you have just gated. FlowJo provides a default name; however, you should choose a name that describes the population that you gated. Name this gate “Lymphocytes”. To accept the gate, click on the “OK” button. If you click on the “Display” button, FlowJo will create the gate, and open a new graph window showing only the events contained within the gate you just created.

You will note some changes to the graph window. The “Statistics” tab at the bottom of the window displays the fraction of events in the gate. “Active Gate” refers to whichever gate is selected. When you create a new gate it is automatically selected. You can tell that a gate is the active gate because the black vertices reappear on it. There is also a new entry in the workspace window - Lymphocytes as a subset of all of the events in A1.fcs as shown to the right.

This subset is represented by new row indented one level below the sample which represents the subpopulation defined by the gate. The row begins with a subset icon, followed by your subset name. To the right, you will find the frequency of these events (within the sample) and the total number of events in this gate. Note that anything that can be done to a sample can also be done to a subpopulation.



Group	Size	Role
All Samples	18	Test
Compensation	0	Compensation
Exp. 1	18	Test
Panel_1.1	9	Test
Panel_1.2	9	Test

Name	Statistic	#Cells
A1.fcs	65.4	22697
A2.fcs		56304

L2 fig.2 - Lymphocyte subpopulation

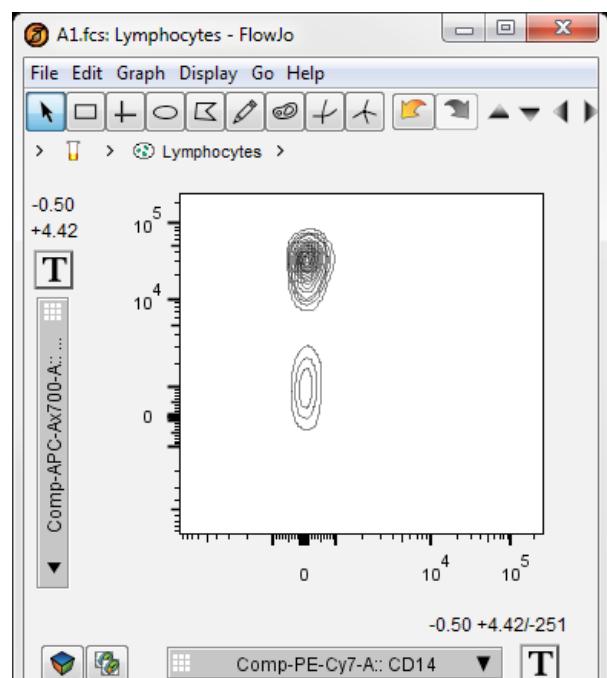
Double-click on the “Lymphocytes” subset to open its graph, which will allow you to gate within that subset. You will see the graph to the right. This is the contour of the events within the lymphocyte gate. Note that the Up arrow button is now active. Clicking on this button opens the “parent” graph, the plot you used to create this subset. The Up arrow can be used to navigate to the parent population.

Change this “child” graph to a CD14 versus CD3, and make it a Pseudocolor plot; your graph will now look like the one at the right. Note that most of the events are CD3-positive and CD14-negative (i.e. T-cells). This is exactly what we expect for Lymphocytes in PBMC. Click on the Up arrow button to make the parent population visible, and then move this window to the side so that you can see both the graph of the parent and child populations simultaneously. FlowJo uses dynamic recalculation of gates. This means that whenever you adjust a gate, FlowJo automatically updates all visible windows to reflect that change.

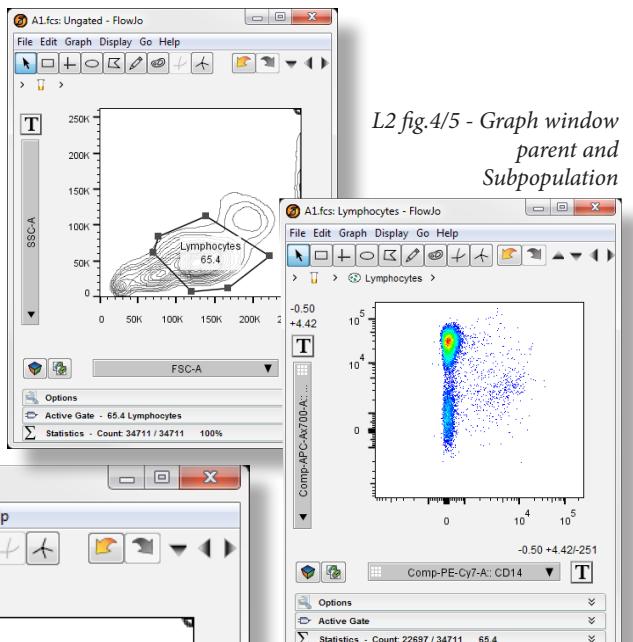
Editing a Gate

Move the mouse over the Lymphocyte gate in the parent graph window; the cursor changes to a four-way arrow. You can click and drag to move the gate. Adjust the gate to rest on the monocyte population as shown. FlowJo instantly updates the graph of the subset; that window will now look like the figure below. The new gate includes few events that are CD3-positive and a large fraction that are CD14-positive (i.e. monocytes).

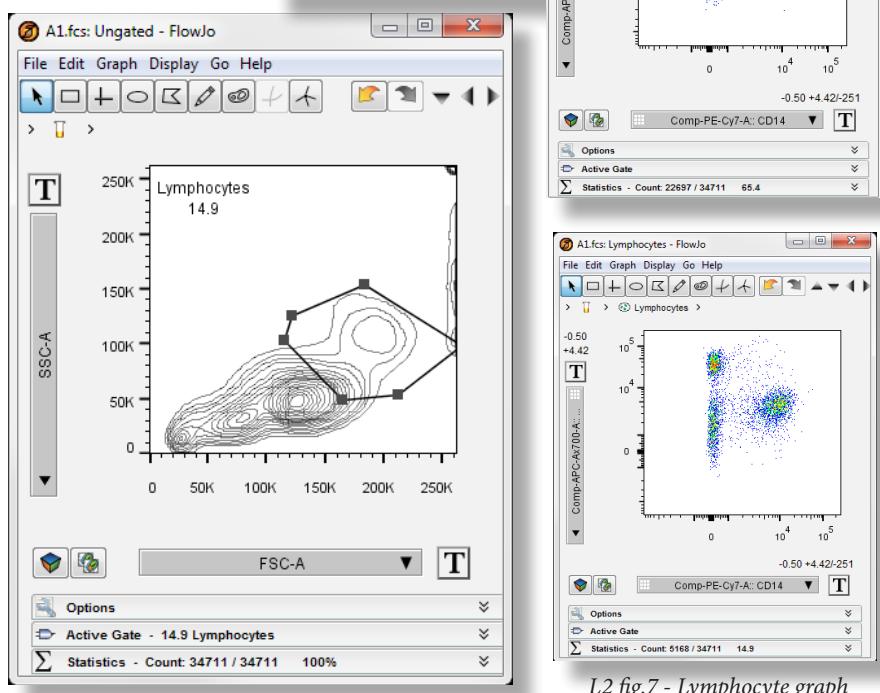
You may continue to move the gate around, and see exactly how the gating affects the subpopulations. There are a number of other ways to modify a gate. Clicking on any vertex allows you to drag the vertex to a new location and change the gate shape. Selecting a gate and pressing an arrow key allows for incremental movement. Adjustments to the parent graph will cause the child graphs to automatically update.



L2 fig.3 - Graph window lymphocyte subpopulation



L2 fig.4/5 - Graph window parent and Subpopulation



L2 fig.6 - Graph window monocyte gate

L2 fig.7 - Lymphocyte graph window update

Creating a Gate on a Histogram

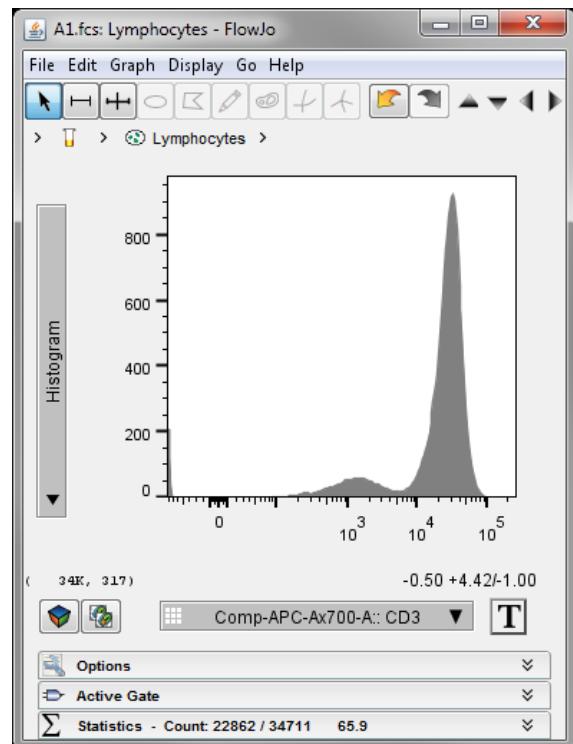
Move the gate back to the Lymphocyte population to reflect the Lymphocyte cells. Click on the down arrow to return to the Lymphocyte-only graph window. Using the Y-axis parameter menu, select “Histogram”. The graph should now appear as shown in the figure on the right.

You will notice that when you switch to a Histogram, two additional gate choices appear in the icon menu at top: a range gate and pair of range gates that bifurcate the data.

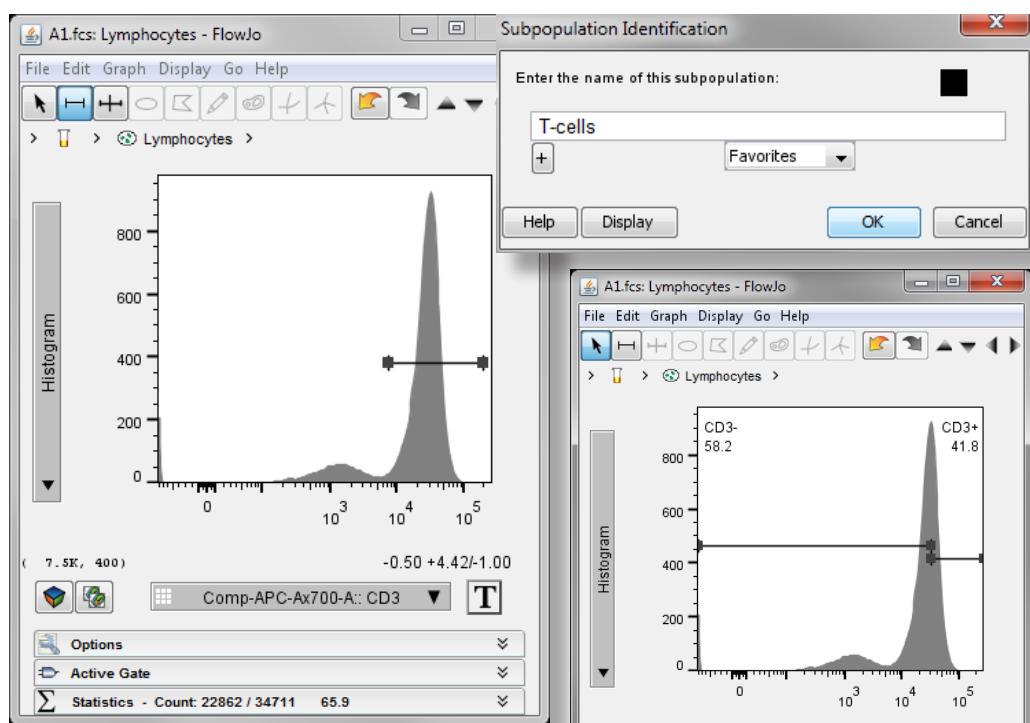
Creating a gate on a Histogram is the same as creating a gate on a 2-dimensional (bivariate) plot. Select the Range icon, then click on the Histogram image and drag in either direction. Make a gate to include only the CD3 positive cells by clicking on one side of the peak and dragging to the other side. When you let go of the mouse, FlowJo again will ask to name this new subpopulation. Type “T-cells” for the subset name.

Bifurcating using a bisector gate is useful for gating the positive and negative population simultaneously. You only need to click once on the graph window and a pair of gates will appear, with their intersection located where you clicked.

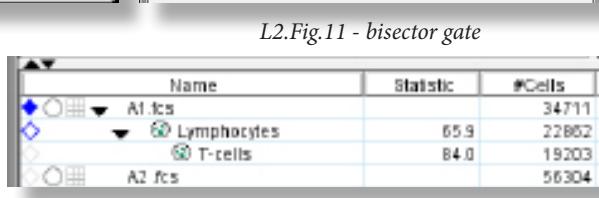
The graph window now displays the gate and the statistics within it. See figure 9 at right. The vertical placement of the gate is irrelevant; you can drag the gate horizontally or vertically by clicking on the horizontal line and moving it around. You can change the upper or lower boundaries by clicking on one of the handles and moving it. You can also move the gate name and statistics independently by clicking on them and dragging.



L2 Fig.8 - Histogram



L2 Fig.9/10 - range gate



L2.Fig.11 - bisector gate

Name	Statistic	#Cells
A1.fcs		34711
Lymphocytes		22862
T-cells		19203
A2.fcs		56304

L2 Fig.12 - workspace T-cells

The workspace window now has a new entry, shown in the example at right (fig.12).

Note that the new entry is further indented below Lymphocytes. This is because the new subset is a subset of Lymphocytes - critically, the workspace reflects this hierarchy.

The numbers at right of the subpopulations indicate the fraction of events falling within the gates. Thus, 65.9% of the sample's events fall in the Lymphocyte gate and 84.0% of the events in the Lymphocyte gate are in the T-cell gate. But what fraction of the total events in sample A1 is in the T-cell gate?

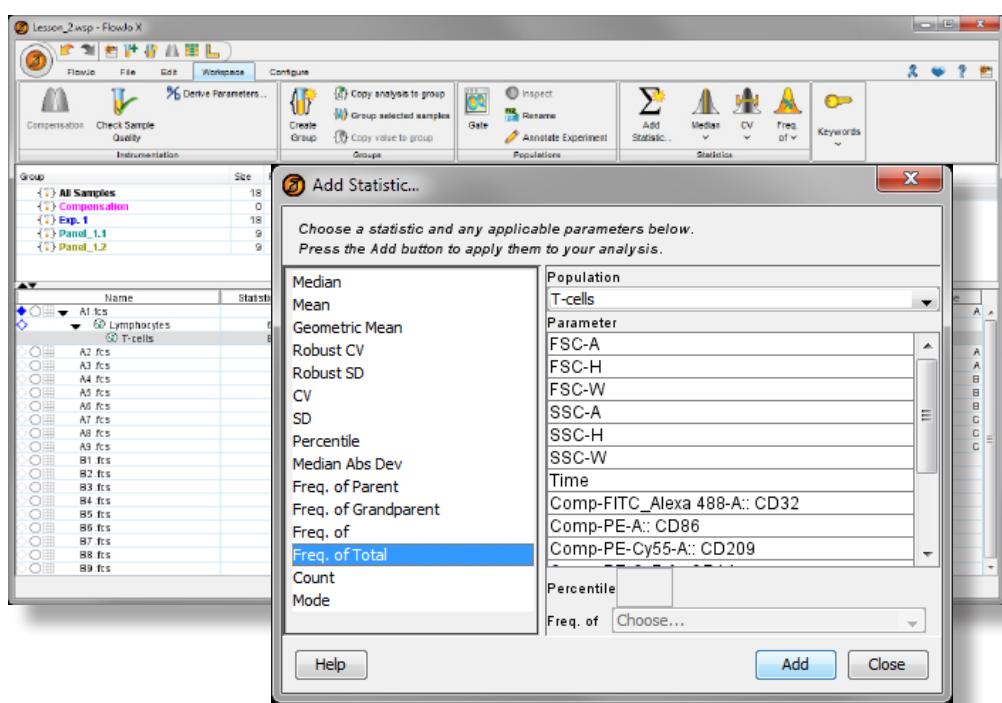


Adding Statistics

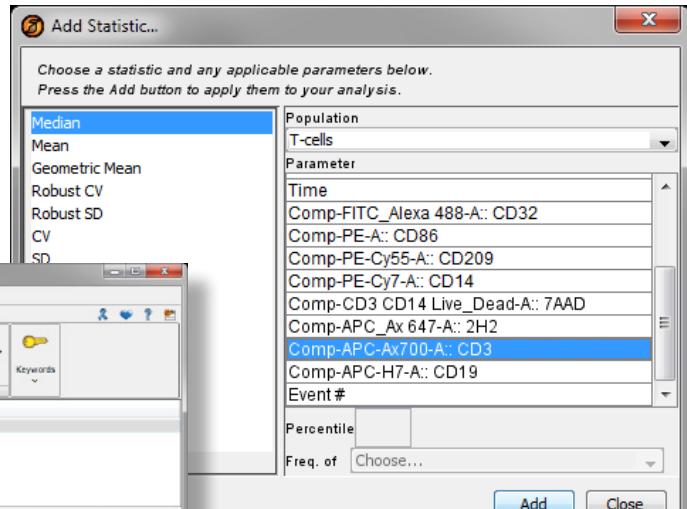
To calculate additional statistical information about your subpopulations, you can add statistics to the subsets. Click on the “T-cells” row so that it is highlighted, then click the Sigma button from the workspace tab, then click “Add Statistics”.

This indicates that you want to calculate a statistic on the currently selected subset, in this case, T-cells. You will now see the Add Statistics window, as shown to the right (fig.13).

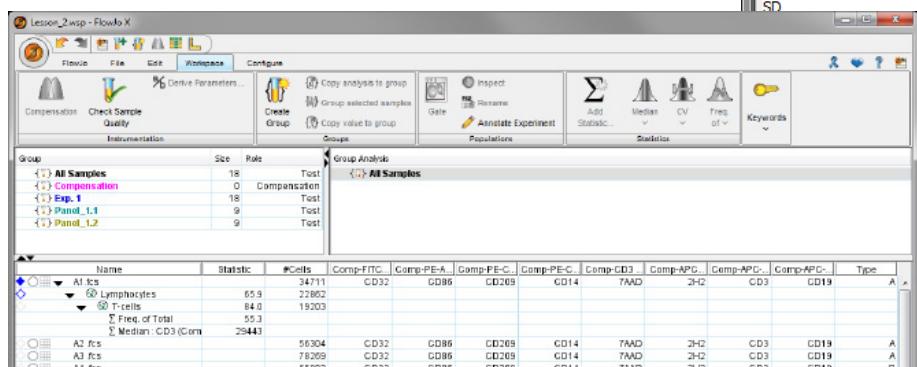
On the left side of the statistics window is a list of the available statistics. Some of these statistics require that you choose a parameter on which to calculate the statistic, for example, Median, which will calculate the median fluorescence. If you wish to compute a specific percentile for a parameter, you will type the percentile into the data entry box.



L2 fig.13/14 - Workspace add statistic



L2 fig.15 - Median freq. Of total



L2 fig.16 - Stats in workspace

Select “Freq. of Total”, and then click on “Add”. Then add the Median Fluorescence Intensity for CD3 by choosing “Median”, selecting CD3 from the parameter list, and clicking “Add”. When you have finished adding statistics to this population, close the window by pressing “Close”. Once you close the statistics window, observe the new entries in the workspace, which will resemble the figure below (fig.16).

There are also choices for All Fluorescent Parameters, All Parameters, and All Compensated Parameters. These options are available from the Quick Statistic drop-down menus to the right of the Add Statistics button (fig.16).

Other Types of Gates





FlowJo offers several other types of gates beyond polygon gates. All can be modified and interacted with as described for the polygon gates. The gating tool bar at the top of the graph window looks like this:



The Arrow Tool is used for selecting a gate. Once selected the active gate tools apply to that gate and the gate can be modified.



The Rectangle Gating Tool, and as you would expect, creates rectangular gates.

Quad Gating Tool allows the user to simultaneously create four abutting rectangular gates. This gate type is useful when you are interested in the double positive population, both single positive populations and the negatives for a pair of parameters. Select your Lymphocyte population, and then display the parameters CD19 versus CD32, two markers for B-cells. Next open the X axis transformation button and select “Customize Axis”. Set the Extra Neg. Decades to 0.5, then click “Apply”. Try putting a quad gate on your plot by selecting the quad gate icon and then clicking once on the CD19-CD32 plot. The result should look similar to the figure to the right (fig.17). To move the quad gate, click at the intersection of the quad gates hold the mouse button down and drag. The intersection point will move with your cursor.



In addition to traditional quads, FlowJo offers:



Curly Quads and Spider Gates. Both tools can be selected from the gating tool bar. Note: Curly Quad gates can be used only with compensated data.



The Elliptical Gating Tool creates elliptical gates, which similar to rectangle gates, are ideal for gating prominent populations that do not require complex shapes to identify, as they require the fewest vertices to define. The number of nodes corresponds to the amount of calculation FlowJo must do to identify a subpopulation, so fewer nodes mean faster calculations of the workspace.



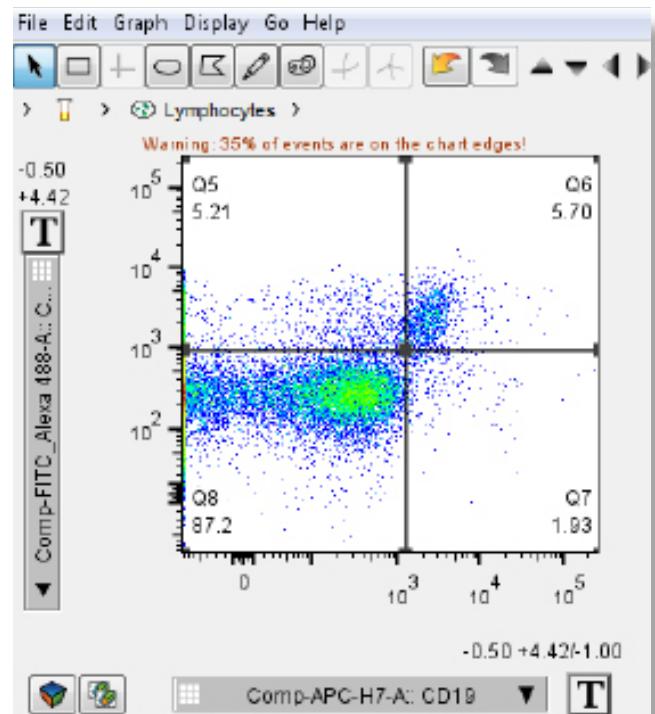
The Polygon Gating Tool allows you to place points around a population as described previously.



The Freehand Gating Tool allows you to create a gate by clicking on the plot, holding the left mouse button down, and moving the cursor anywhere. When you release the mouse button the gate will close producing an amorphous shape.



The Auto-Gating Tool. When you select this tool and drag your cursor around the graph window, FlowJo will automatically show you a gate with borders that follow a line of equal density; they will in effect follow a contour. To accept an auto gate, simply click when you are satisfied with its location. Alternatively, to finish an auto-gate you can click and hold the mouse button, drag the cursor either away or toward the center of the gate, and the gate will either expand or shrink respectively. The gate center remains fixed. You are no longer guaranteed equal density, but it allows the user some flexibility.

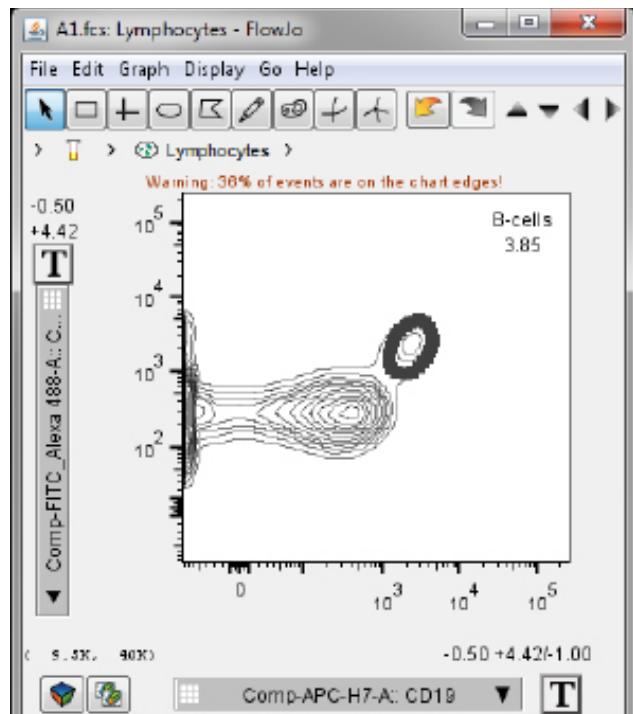


L2 fig.17 - Graph window quad gate

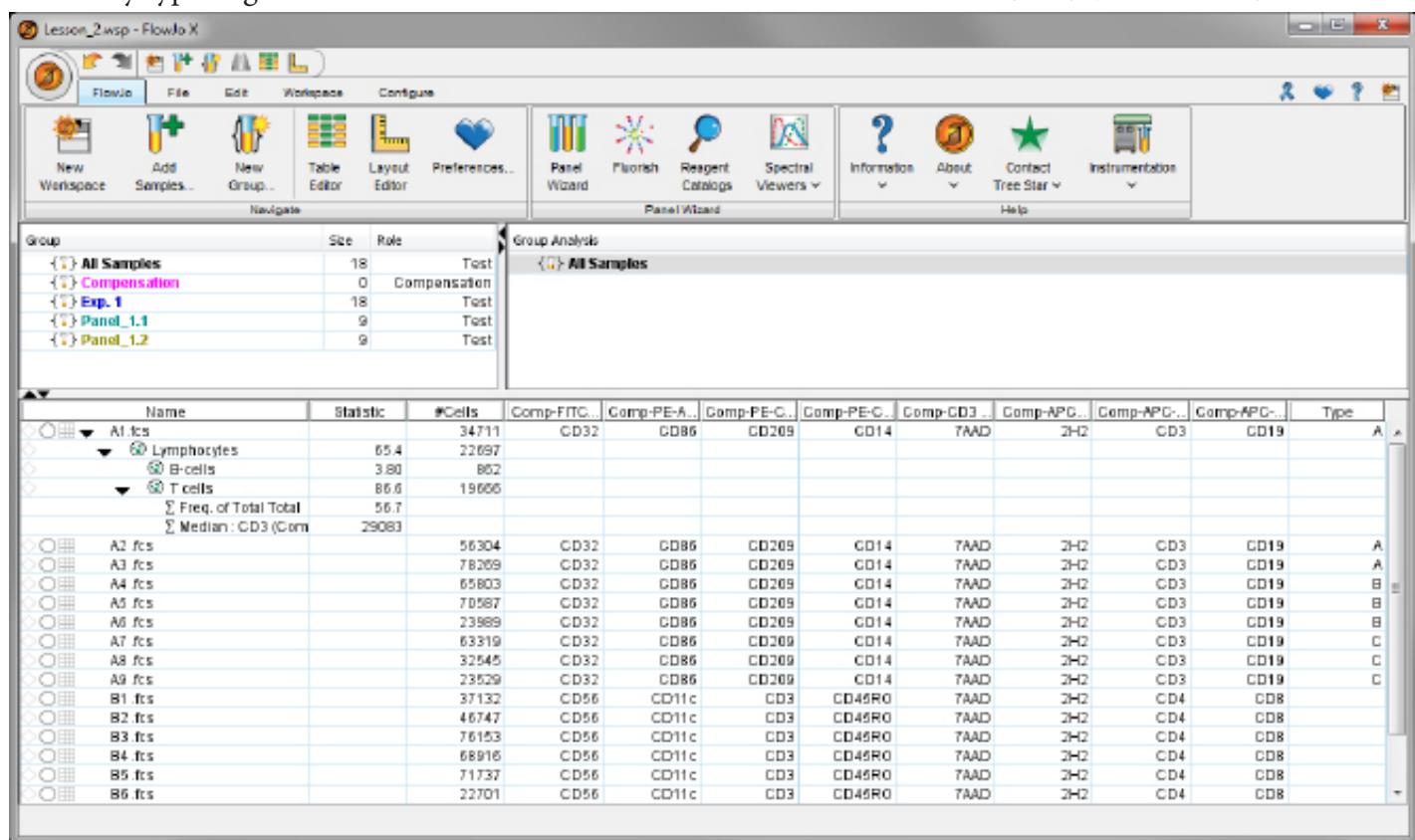
To try out this tool, choose your Lymphocyte population with the CD19 versus CD32 parameters selected. Use the “Auto Gate” tool to Gate the double positive population and name it “B-cells”. You can then switch the plot type to contour to see that the gate you have created follows the pattern of the contours.

Deleting Gates

We will now remove the quad gates. Click on a quadrant in the graph window and press the “Delete” key on your keyboard. FlowJo will bring up a dialogue box asking if you really want to delete this gate (and a shortcut checkbox asking if you would always like to show this message). Agree, and observe that the quad gates disappear from the graph window and the workspace. From the workspace window, click on the 3D view and press “Delete”. The node will disappear from the workspace list, and if it had been a gate would have disappeared from the graph window. FlowJo allows you to delete any type of gate in this manner.



L2 Fig.18 - graph window auto gate



L2 Fig.19 - workspace end of lesson 2

If you have followed all of the steps, your workspace will look like the figure below (fig.19).

To access the workspace at this point of the analysis, open the Lesson_2.wsp file.

Lesson 3: Copying Analyses to Other Samples

One of the basic principles of FlowJo is that virtually all analyses that you perform on a population of cells can be applied to other populations using the “drag-and-drop” feature. Hence, when you click on a node in the workspace (i.e a gate or statistic), and you drag it to another place in the workspace, FlowJo copies the analysis to the new location. In this lesson you will also adjust the biexponential transform for two parameters to improve the display of data.

You will now analyze a set of files with a different staining combination than A01. This lesson builds on the workspace you finished in Lesson 2. Alternatively, you can open the workspace named “Lesson_2.wsp” to get the workspace completed in Lesson 2. We will analyze files B1 and B2 stained with the second combination of reagents (Panel 1.2).

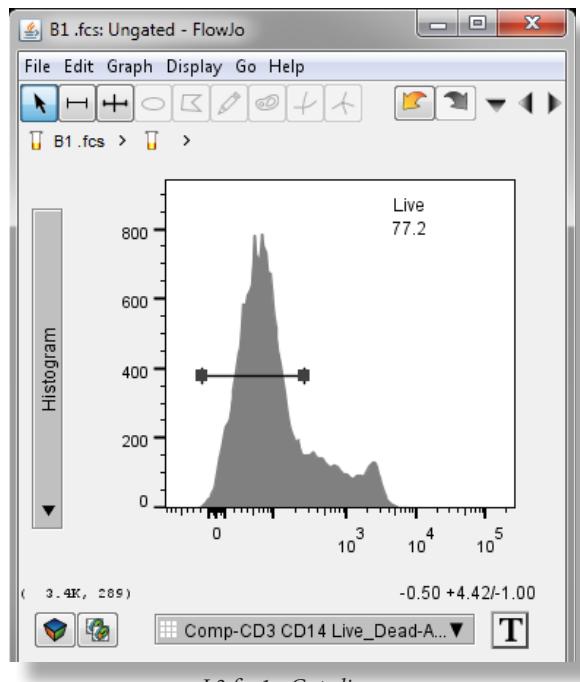
Setting up an Analysis

Start by double-clicking on file B1.fcs to open a graph window and examine a Histogram of 7-AAD by choosing 7-AAD to appear on the x-axis and selecting “Histogram” from the y-axis. Make a gate including 7-AAD negative events to isolate the live cells and name this subpopulation “Live”. Double-click on that gate to isolate those cells, and switch the parameters to FSC-A and FSC-W. Make a rectangular gate that includes the events with a consistent ratio of the two parameters as shown below to the right (fig.1), and name this population “Singlets”. Finally, let us apply a Lymphocyte gate to these events.

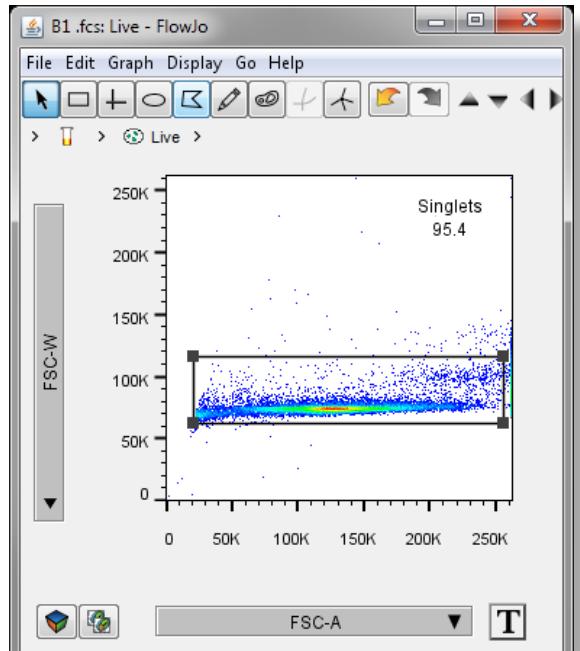
Copying a Gate to Another File

You have already made a Lymphocyte gate on file A1; presumably, this same gate should be applicable to B1. To do this, first click on “All Samples” in the group panel so that files A1 and B1 are both displayed. Then click on the “Lymphocytes” subset of A1 in the workspace window, drag it down and drop it onto the “Singlets” node of B1. FlowJo creates a new subpopulation using the same gate you had previously created (fig.2). Note that you can drop a population onto any level of the hierarchy, even across groups and to apply that gate to the selected events.

You can double-click on the “B1” Lymphocyte population and press the “Up” arrow to see how this gate lines up with the new data. Return to the Lymphocyte population and view a Histogram of CD3. You have already created a T-cell gate on sample A1, and we would also like to apply that to

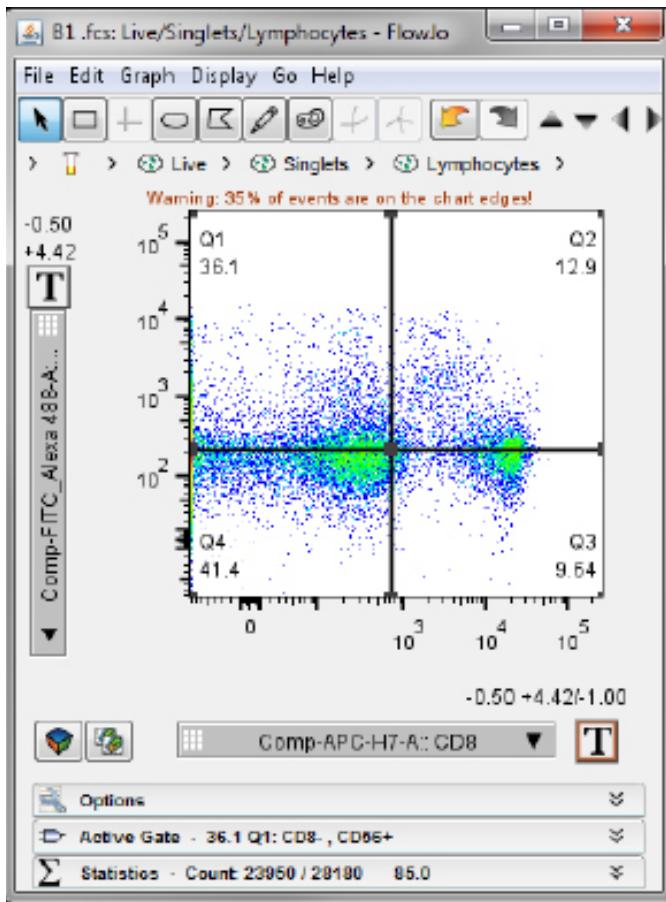


L3 fig.1 - Gate live



L3 fig.2 - Gate singlets

sample B1. However, gates are linked to the parameters so you need to draw a different T-cell gate for sample B1. As CD3 was labeled with APC-Ax700 on sample A1 and PE-Cy5.5 on sample B1, we cannot directly drag this gate between samples. For this and other reasons, it is important that you carefully enter the appropriate stain (antibody) names when you collect the samples.



L3 fig.3 - Quad gate

it becomes highlighted. Then click the “Add Statistics” Sigma button to open the dialogue window. Choose “Median”, and select “CD3” as the parameter, then click “Add”. Click on “CV” and click “Add” again to include a second statistic. Now click “Close” to close this window. The workspace now contains two additional nodes under the first quad gate.

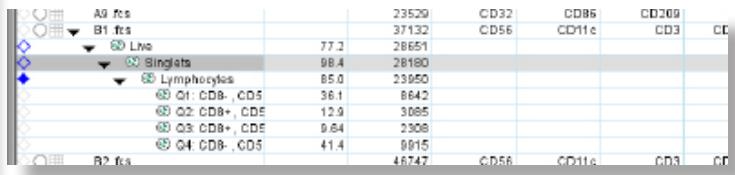
Just like gates, statistic nodes can be dragged and dropped on other subpopulations. Drag and drop both stats to the three other quad gates. Your workspace will look like the figure below (fig.5).

Copying Multiple Gates

Now, we would like to apply exactly the same gates and statistics applied to sample B1 to sample B2. We could drag each line one-by-one to B2. However, FlowJo provides a special mechanism for copying entire analysis trees. When you hold down the “Alt” key (PC, or “Command” key on Mac) and begin to drag, FlowJo will take the subpopulation you clicked and all of its descendants, including statistics. You will see this occurring via the outline that FlowJo draws, which denotes all of the analyses that you are copying.

Click on the “Live” gate, press the “Alt” key, and drag it to the “B2” row. Even if the children of the first Lymphocyte

Draw a new T-cell gate on the B1 Lymphocyte population, as shown in the figure below (fig.3). Then double-click on the T-cell population and change the parameters to CD8 versus CD4. Use a quad gate to simultaneously gate the CD8 and CD4 single positive, double positives, and double

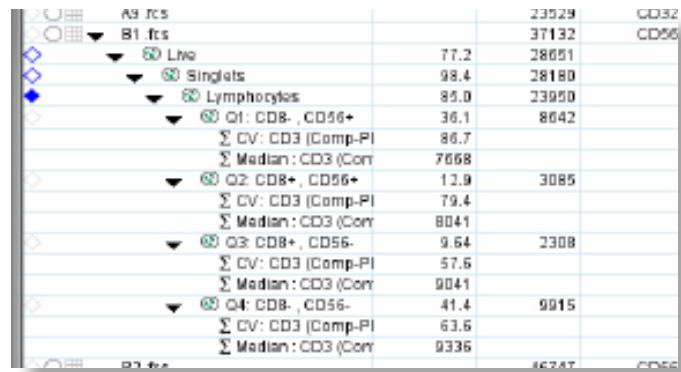


L3 fig.4 - Quad gate list

negatives.

Your workspace will look like the figure below (fig.4). Click on the disclosure triangle next to B1.fcs. These triangles open and close the views of the subset hierarchy. In the genealogical terminology that FlowJo uses, the subpopulations created by the quad gates are siblings, and are children of the “T-cell” subpopulation, and grandchildren of the “Lymphocytes” subpopulation.

Next, add a statistic to each of the four quad gates. To do so, click on the “Q1: CD8+, CD4-” subpopulation so that



L3 fig.5 - Added statistics

gate are hidden because you closed the triangle, they will still be copied. Your workspace will now reflect the fact that you copied fifteen different analyses (gates and statistics) with one operation.

There are more complex ways of selecting which analyses to copy. For instance, you can shift-click several analyses to drag multiple contiguous (within the workspace) gates simultaneously, or you can control-click to select any subsets and/or statistics to be dragged. Try selecting just the “Live”, “Singlet”, and “Lymphocyte” gates from sample B1 and dragging it to Sample B3.

Deleting Analyses

You can delete analyses by selecting them and pressing the “Delete” key. Select the “Live” subpopulation that you just added to B3, and press “Delete”. When you delete the Lymphocyte gate, all of the subsets of Lymphocytes will also be deleted. This is because those subsets have no meaning without a live gate present. Remember that every subset you name is, in reality, a subset defined by all of the ancestor populations: the parent population, grandparent population, etc.

To access the workspace at this point of the analysis, open the Lesson_3.wsp file.

Lesson 4: Groups

In this lesson, you will learn how to take advantage of sample groups. Groups are the principal functional unit within FlowJo and one of the primary mechanisms by which FlowJo allows the user to perform batch analysis; the repetitive application of analysis or generation of reports across many samples. You may create as many groups as you wish; each group may include any of the samples within the workspace. Samples may also belong to more than one group. Importantly, applying a gate or statistic to a group will be equivalent to doing the same thing to every sample in that group—the advantage is that you do it all at once.

In Lesson 3, you learned that by dragging multiple analyses or entire sets of analyses, you can accomplish the first step in batch processing: the ability to replicate multiple analyses. But that still leaves the next step: how can these analyses be applied to a whole set of samples at once? To accomplish this, FlowJo allows you to create groups of samples. A group is a list of samples from the workspace that can serve as a sample surrogate. That is, you can apply analyses to a group much as you can apply them to individual samples. There is one rule about groups and group analyses that is inviolate: every sample in a group contains every gate, statistic, or other analysis that has been applied to the group.

This lesson builds on the workspace you finished in Lesson 3. You may also open the pre-made workspace named Lesson_3.wsp.

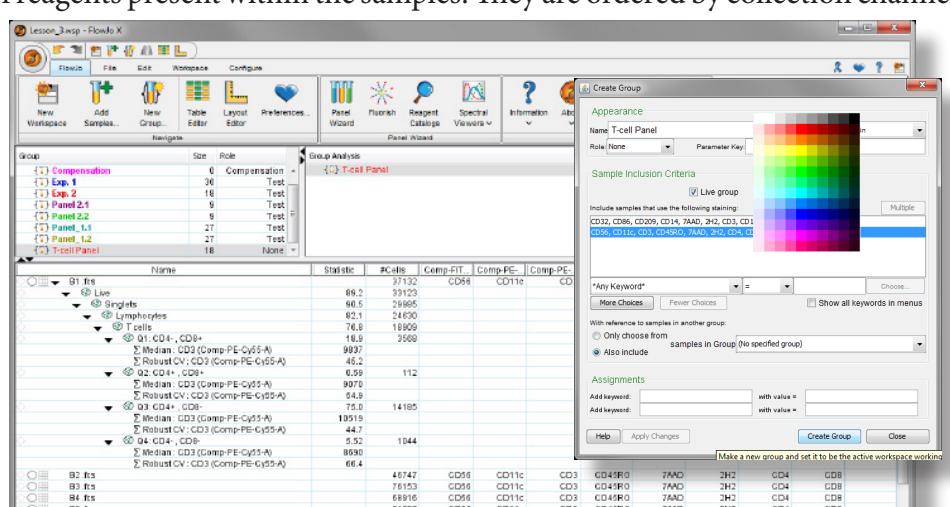
The first step is to create a group that contains a set of samples that will receive similar kinds of analyses. In this tutorial we already have a number of groups that were created by the filing structure when we loaded data into FlowJo that could be analyzed in the same way. Now you will make a new group that gathers all files with a particular staining combination, regardless of the file structure.

Begin this lesson by adding “Exp. 2” to the workspace. Locate the folder with the downloaded demo data and drag “Exp. 2” into the workspace just as you loaded Exp.1 in Lesson 1.

Creating a New Group

In the workspace, click on the “New Group”  button, which can be found in the shortcut bar at the top of the workspace, as well as the Navigate task in the FlowJo band. FlowJo brings up the Create Group window shown below (fig.1). Using this window, there are a variety of ways to make new groups. The top portion of the window controls the format of the group’s appearance in the workspace, and the middle portion allows you to set your sample inclusion criteria to control which samples will be added to the group. In sample inclusion criteria, there is a window that lists every combination of reagents present within the samples. They are ordered by collection channel. Click on the CD56, CD11c, CD3, CD45RO, 7AAD, 2H2, CD4, CD8 combination, telling FlowJo to group all samples in the workspace with this stain combination.

Type the name “T-Cell Panel” in the name box at the top, change the color to red by clicking on the “color box”, and the font style to “Plain”. Click on the “Create Group” button, and then the “Close” button to close the window and display the workspace.



L4 Fig.1 - new group and options

Each group is displayed in the group section of the workspace, in the color and font style you have selected. The number to the right of each group tells you how many samples are included in each group (fig.2). Since there were nine subjects labeled with each reagent panel for two experiments, there are now 18 samples in your T-cell group.

Modifying an Existing Group

Double-click on the name of the group you want to modify. In this case, double-click on “Panel 2.1”. Rename the group “B-Cell panel” by typing over the existing name. Because this group was created automatically when you brought in the data, there is no criteria already defined for it. Click on the other panel of regents, the panel that contains CD32, CD86, etc. Change the color to green by clicking on the color box, then click on “Apply Changes”. You will notice that the name change has taken place and there are now 18 files in the group.

Deleting a Group

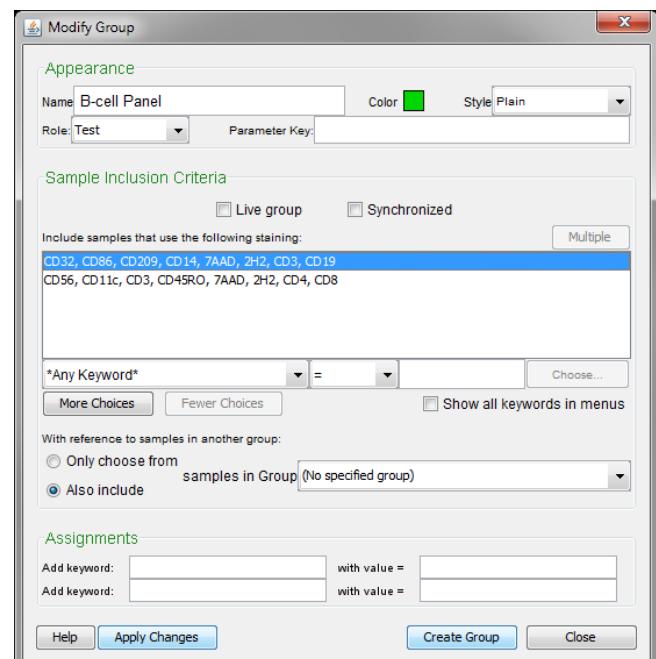
To delete a group, simply click on it and press “Delete” on your keyboard. To try this, click on the “Panel 1.1” group and delete it. Repeat for the “Panel 2.2” group.

Creating a Group Using Keywords

Display another keyword in the workspace. Right-click on the header of the “Name” column in the workspace and choose “Edit Columns”. Select the keyword “Day” from the list on the left and click on “Add Column”. It will appear in the list to the right. Now select “Day” from the list on the right and drag it to be the fourth keyword from the top under #Cells. Click “OK” to apply this change (fig.3/4).

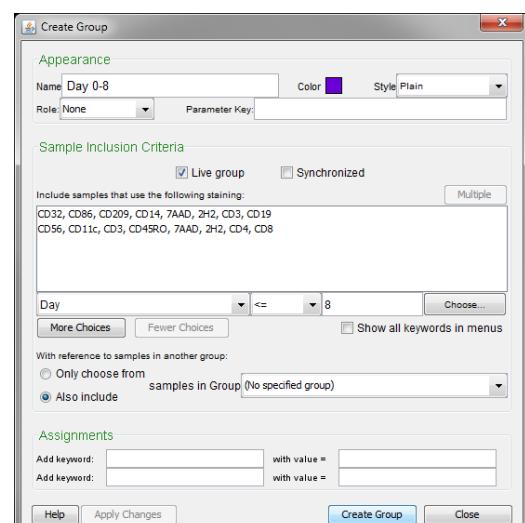
You will notice that the column “Day” has appeared numbered from 0 to 16. Using this keyword annotation, we can “isolate” the files that were stained on the first eight days of the experiment. To create a group to do so, open the “Create Group” menu again. This time you will use a keyword to define group membership. Below the staining panel window there are a set of dialogue boxes that allow you to select a keyword, a qualifier and a value. From the box initially filled with “*Any Keyword*”.

Choose the keyword “Day” from the drop down-menu. The box to the right gives you inclusion criteria. Choose “<=” (is less than or equal to). You can then either type a number into the next specification box, or click on the “Choose” value box to see what values were entered for that particular keyword within the data. Type or select “8”. Name the group “Day 0-8” and color it purple. Your



L4 fig.2 - Modify group

L4 fig.3/4 - Add “day” keyword



L4 fig.5 - Keyword grouping

group definition window will look like the window pictured to the right (fig.5).

Click “Create Group”, and then the “Close” button to close the window and display the workspace. Notice that you

Group	Size	Role
{!!} All Samples	36	Test1
{!!} B-cell Panel	18	Test1
{!!} Compensation	0	Compensation
{!!} Day 0-8	20	None
{!!} Exp. 1	36	Test1
{!!} Exp. 2	18	Test1
{!!} Panel 2.1	9	Test1
{!!} Panel_1.2	27	Test1
{!!} T-cell Panel	18	None

L4 fig.6 - Day 0-8 group

have isolated all of the files with days numbered eight or less.

Each group is displayed in the upper (group) panel, in the color and style that you have chosen. Your workspace should look like this (fig.6):

Adding Analyses to All Members of a Group

Some of the files in the groups already have gates and statistics. You will now apply those analyses to the rest of the files in each group. Remember that a group is a sample surrogate so any analyses that you apply to the group will be applied to all of the samples in that group.

Click on the “T-cell panel” group, and expand the gating tree under file B1. Click on the “Live” gate in the lower samples panel. Hold down the “Alt” key

1. on a PC (“Command” on Mac). Then with the left mouse button depressed, drag the gate and release the button on top of the “T-Cell Panel” group in the 2. group section of the workspace.
3. Notice that several things have occurred:

- the analysis tree has been

added to a row indented beneath the group name as if it were a sample

- the analyses have been added to every sample in the group

- all of the analyses appear in red, which is the same color and style that you defined for this group

Your workspace should look like the figure

L4 fig.7 - T-cell panel applied

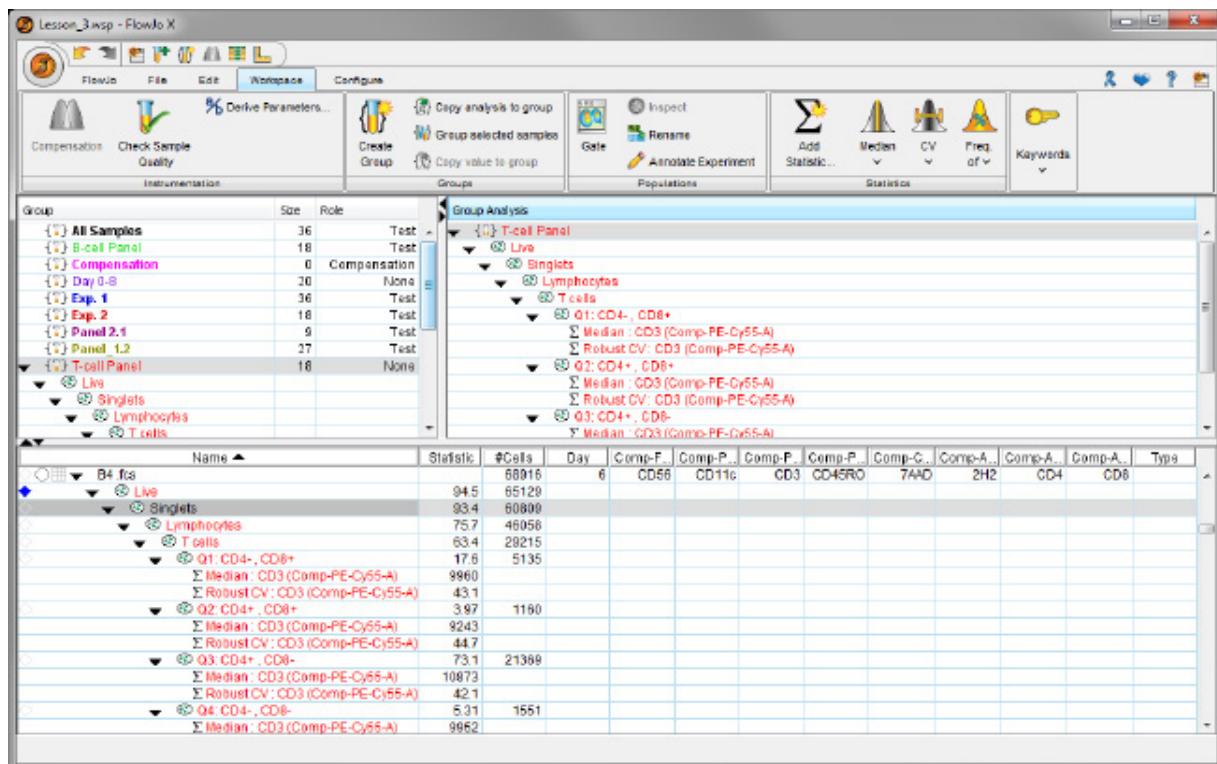
above (fig 7). The color and style of the analyses are an important cue. Any analysis that appears in the workspace in the color and style of the group is guaranteed to be exactly the same as the group's version. Thus, the gate will be in the exact same location, applied to the same parameters, etc. This is used to ensure that the exact same analyses have been done on all samples.

1.

Modifying Group Gates

2. There are two scenarios in which you may wish to modify group gates:

- Adjusting a gate on an individual sample to make it different than the rest of the



L4 Fig.8 - B-4 singlets adjusted
group

- Adjusting the gates of all samples in a group

In the first scenario, simply open the sample that you would like to modify by double-clicking on it and then adjust the gate. FlowJo will remove the font formatting from that particular gate to indicate that it no longer matches the group gates. To try this, double-click on the "Live" gate for sample B4. The graph window will display FSC-A versus FSC-W showing the gate added to identify the singlets. Click on the gate and then drag it to better match the population. If you look back at your workspace, you will see that the "Singlets" population no longer has its special formatting as shown in the figure to the below (fig 8).

In the second scenario, you have moved a gate and would like to apply that change to the whole group. To do this, drag the modified gate up to the group and drop it into the proper location in the hierarchy. Imagine that

the modified singlets gate on B4 is a better gate for the entire group. To apply this to the entire group, click on that specific “Singlets” gate in the workspace. Then drag and drop it onto the “Live” population of the T-cell group in the group portion of the workspace window. A box will prompt you to replace the previous gate name. Click “Yes”. If you use the right or left arrow keys in the graph window, you can scroll from sample to sample and see that they all reflect the change made.

This method could grow tedious if you modify many gates. A shortcut to execute rapid gate changes for entire groups is to use the “Synchronize Gates” function. Double-click on the “T-Cell Panel” group (from the group portion of the workspace). The group definition window will again appear.

Notice that there are two check-boxes near the top of the screen: one for “Live Group” and the other for “Synchronized”. The “Live” box allows the group to update as you continue to add or modify metadata. The “Synchronized” box links the group’s gates so that if you modify a gate on one sample, that gate is modified on all of the group’s samples. Check the “Synchronized” box, click “Apply Changes” and then “Close”. Now double-click the “Live” gate on file B5 and adjust the “Singlets” gate (fig.9). Notice that the gates font format in the workspace did not change, and that all of the statistics updated. This indicates that all of the gates have changed simultaneously.

To complete the analysis of this data set click on the “B-cell panel”, and then the “Lymphocyte” population of sample A1. Hold down the “Alt” (“Command” key for Mac) key and then drag the entire hierarchy to the “B-cell group” in the group panel and release the mouse button. All of the files in that group will now be gated and the statistics will be applied to all samples. Note that statistics can be applied to groups in the same manner as gates.

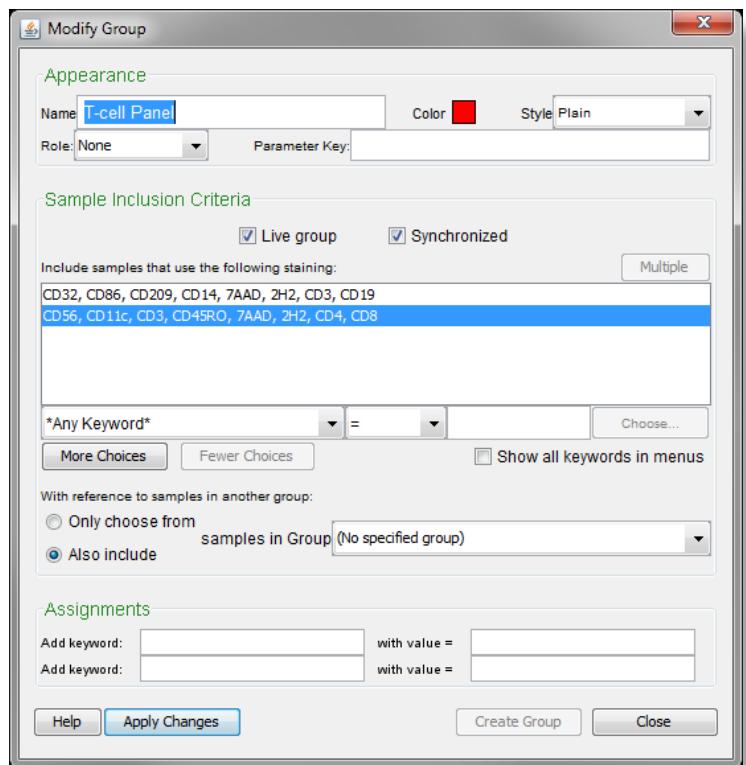
Now double-click on the “Lymphocyte” population of sample A1. Move the “B-Cells” gate in the graph window and notice that the annotation turns black in the workspace. Click on the modified gate “B-Cells” in the workspace list and press the “Delete” key on your keyboard and click “Yes” in the confirmation dialog box. You will notice that the B-cell gate is replaced by the B-cell groups’ color and that the gate position in the graph window is reset to the group default.

The work to this point will be saved as Lesson_4.wsp.

Lesson 5: Tables and Collating Data Output

In this lesson, you will learn how to transfer statistical analyses to tabular form for further analysis in FlowJo or for export to spreadsheet/statistical programs. You will be able to generate a table of statistics that brings together any set of values from any combination of samples (using groups to define the sample list).

FlowJo provides tools to collate the output from multiple analyses so that you can import them into spreadsheets or to a machine-readable format for statistical or mathematical packages. FlowJo is not a comprehensive data



L4 fig.9 - Synchronized checkbox

presentation package. For statistical analysis beyond what FlowJo provides, you will need to export your data to other programs.

Use your existing workspace from Lesson 4, or open the tutorial workspace Lesson_4.wsp.

Open the “Table Editor” by clicking on its icon in the shortcut bar at the top of the workspace or select the “Table Editor” from the FlowJo band. FlowJo shows you the Table Editor window as shown below (fig.1). You can create as many tables as you like and generate a table from as many groups as you wish.

At left in the Table Editor window you will observe the list of existing table definitions. When you press the batch for “display” button, the statistics you have selected as columns will be applied to the current group and a table will be generated in FlowJo. A table definition is simply a list of statistics, keywords, or formulas that will become the columns of a table when you batch. They are listed as rows in the table editor because vertical text is difficult to read. Each row in the template defines one  statistic to export such as frequency, mean fluorescence of FITC, etc. When you create the table, FlowJo cycles through each sample in the current group and requests the particular statistic defined in the table.

Creating a New Table

At this point it is useful to  arrange your computer screen with the workspace and the table editor side-by-side. This will allow easy addition of statistics to the table editor by directly dragging either statistics or populations from the workspace into the table editor.

Start by naming your table so that you can differentiate it from other tables you may create. In the Name box at the top of the window, double-click the table named “Untitled” and type “T-Cell table”.

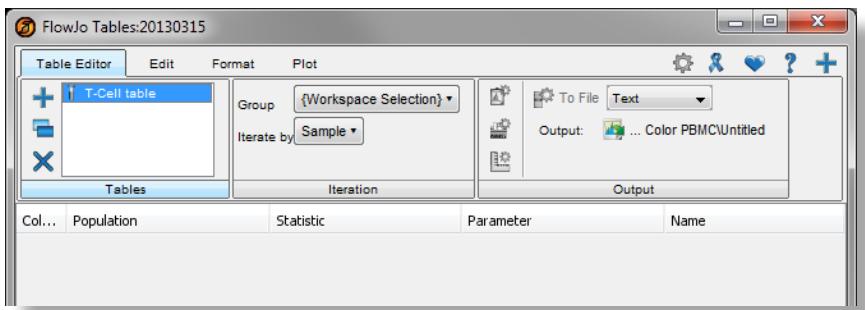
Go back to your workspace and select the “T-Cell Panel” group. We will use keywords to help define the order of the table. To do so, we will extend the keywords to the entire T-cell panel group. Go to the workspace tab, click the “Keywords” drop-down menu and select “Add Keyword”. Click on the top cell in the “Type” keyword column, then select “Create Keyword Value Series” from the Keywords menu. Enter “3” in the “Repeat Value” box. Then click on the “Keyword” tab and enter the letters “A” through “F” in the six boxes (fig.2).

Click “OK”, and you will have a value series of these letters under the keyword “Type”. The resulting pattern in the workspace is displayed in the figure below (fig.3).

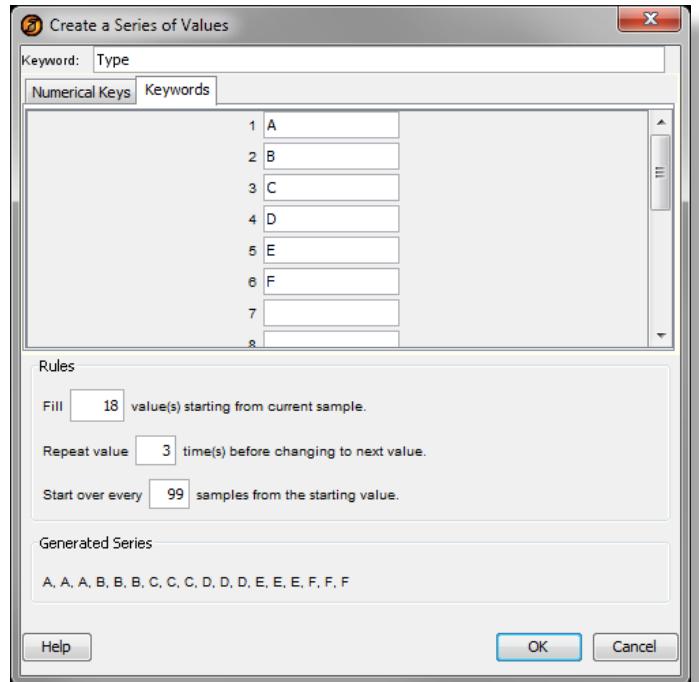
Still in the “T-Cell Panel” group, select the “Live” subpopulation from sample B1, hold down the “Alt” key (or “Command” on Mac), and drag the highlighted rows into the bottom portion of the table editor window.

The table editor will look like the figure below.

By using the Alt (or command) key, you were able to



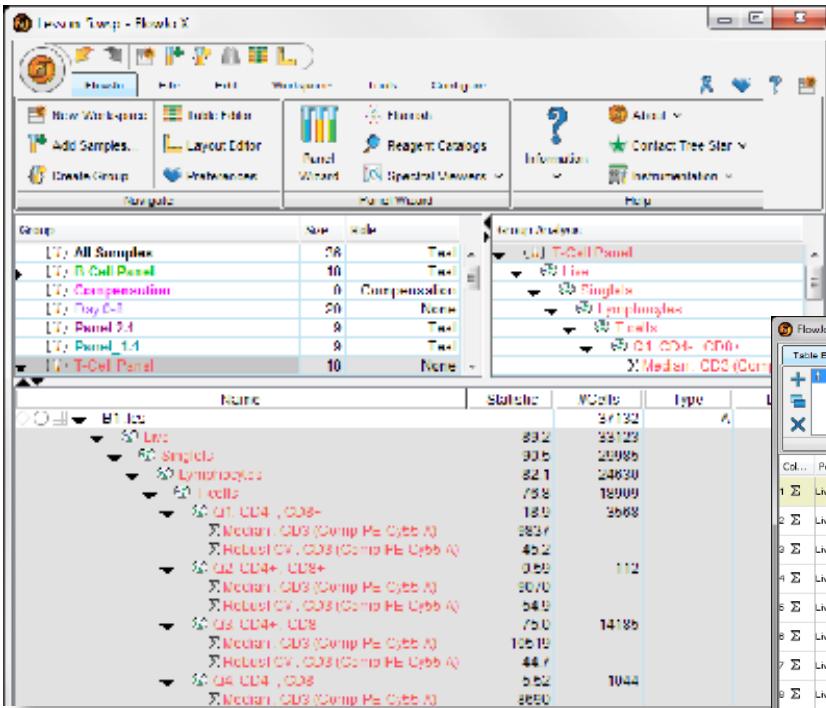
L5 fig.1 - Table editor



L5 fig.2 - Keyword value series

select all of the subpopulations and statistics downstream of the Live cells. You can of course select one population or statistic at a time if you so desire. The default is to display the frequency of the parent for each of the populations that were added. To change the statistic, double-click on the row that you would like to modify.

Once the data is dragged into the table, select the first two rows of “Live” and “Live/Singlets” and press “Delete” on your keyboard, clicking “Yes” to accept



L5 fig.3 - Workspace value series

the deletion. Next, move the “lymphocytes/T-cells” population above the Lymphocyte (Live/Singlets/Lymphocytes) row by selecting, dragging and dropping to reorder the table.

Col...	Population	Statistic	Parameter	Name
1	Live	Freq. of Parent		
2	Live/Singlets	Freq. of Parent		
3	Live/Singlets/Lymphocytes	Freq. of Parent		
4	Live/Singlets/Lymphocytes/T cells	Freq. of Parent		
5	Live/Singlets/Lymphocytes/T cells/Q1: CD4+, CD8+	Freq. of Parent		
6	Live/Singlets/Lymphocytes/T cells/Q1: CD4+, CD8+	Median : CD8	Comp PE-Cy56 A	
7	Live/Singlets/Lymphocytes/T cells/Q1: CD4+, CD8+	Robust CV : CD8	Comp PE-Cy56 A	
8	Live/Singlets/Lymphocytes/T cells/Q1: CD4+, CD8+	Freq. of Parent		
9	Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Median : CD8	Comp PE-Cy56 A	
10	Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Robust CV : CD8	Comp PE-Cy56 A	
11	Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Freq. of Parent		
12	Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Median : CD9	Comp PE-Cy56 A	
13	Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Robust CV : CD9	Comp PE-Cy56 A	
14	Live/Singlets/Lymphocytes/T cells/Q4: CD4+, CD8+	Freq. of Parent		
15	Live/Singlets/Lymphocytes/T cells/Q4: CD4+, CD8+	Median : CD9	Comp PE-Cy56 A	
16	Live/Singlets/Lymphocytes/T cells/Q4: CD4+, CD8+	Robust CV : CD9	Comp PE-Cy56 A	

With the “Lymphocyte/T-cell” row selected, click on the “Edit” tab, then the “Edit Column” button. The Column Information dialog box will appear. In the column heading, type “#T-cells”. Select the “Count” in the left hand menu, and then click “OK” (fig.5).

Now we will edit the statistic for Lymphocyte population using the Column information dialog box, as above. Click on the “Lymphocytes” row (live/singlets/lymphocytes) to edit it similarly, type “#lymphs” into the heading, and select “Count” from the statistic menu and click “OK”.

Now that we have the two starting populations, we can begin organizing the populations within the table. Select the Q1, Q2, Q3 and Q4 Frequency of Parent statistic, and drag and drop them into ordered place below one another so that they are ordered one through four.

Now we will do the same for the (robust) CV for populations Q1 through Q4. Once Lymphocytes and Frequency of Parent are organized, Median is automatically ordered below

L5 fig.5 - Table editoredit column window

the first two.

At this point, the table columns should be named so that we can keep track of our statistics. Double-click on the first empty row of the Name column, and type names into the rows as shown in the figure below (fig.6).

Adding a Keyword or Formula to a Table

In the Edit tab, click on the “Add Column” button and the column information dialog box will again appear. This time select the “Keyword” tab. When you select “Keyword”, the menu will change to show a list of all keywords. Pick “Day” from the

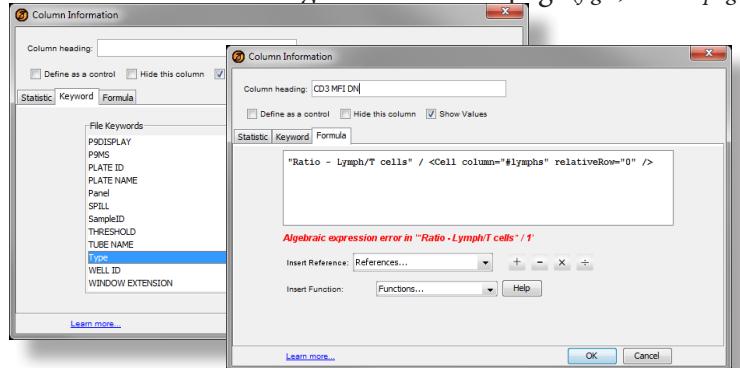
Population	Statistic	Parameter	Name
Live/Singlets/Lymphocytes/T cells	Count		#T-cells
Live/Singlets/Lymphocytes	Count		#Lymphs
Live/Singlets/Lymphocytes/T cells/Q1: CD4+, CD8+	Freq. of Parent		%Lymph CD8+
Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Freq. of Parent		%Lymph CD4+CD8+
Live/Singlets/Lymphocytes/T cells/Q3: CD4+, CD8-	Freq. of Parent		%Lymph CD4+
Live/Singlets/Lymphocytes/T cells/Q4: CD4-, CD8-	Freq. of Parent		% Double Negative
Live/Singlets/Lymphocytes/T cells/Q1: CD4-, CD8+	Robust CV : CD3	Comp-PE Cy65-A	CV CD8+
Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Robust CV : CD3	Comp-PE Cy65-A	CV CD4+CD8+
Live/Singlets/Lymphocytes/T cells/Q3: CD4+, CD8-	Robust CV : CD3	Comp-PE Cy65-A	CV CD4+
Live/Singlets/Lymphocytes/T cells/Q4: CD4-, CD8-	Robust CV : CD3	Comp-PE Cy65-A	CV DN
Live/Singlets/Lymphocytes/T cells/Q1: CD4+, CD8+	Median : CD3	Comp-PE Cy65-A	CD3 MFI CD8+
Live/Singlets/Lymphocytes/T cells/Q2: CD4+, CD8+	Median : CD3	Comp-PE Cy65-A	CD3 MFI CD4+CD8+
Live/Singlets/Lymphocytes/T cells/Q3: CD4+, CD8-	Median : CD3	Comp-PE Cy65-A	CD3 MFI CD4+
Live/Singlets/Lymphocytes/T cells/Q4: CD4-, CD8-	Median : CD3	Comp-PE Cy65-A	CD3 MFI DN

L5 fig.6 - Table editor naming list

list, then click “OK”. Repeat this process to add the keyword “Type,” the keyword that we added earlier in this lesson.

Next, we will add another column - but this time we will create a Formula by opening the “Add Column” menu and selecting the “Formula” tab instead of the Keyword tab. Name the formula in the column heading by typing “Ratio - Lymph/T-cells”. Then click on the “Insert References” drop-down menu and select “#Lymphs” (fig.7/8). Now click on the divide symbol which will create the ratio after we add the “#T-cells. See the figure on the next page (fig.9, on next page) for what your table will look like at this point.

You may use this interface to add any statistic that is not a basic MFI (median fluorescence intensity), count, etc. You can do so by choosing information that is already in the table using by the “Insert Reference” box, and modifying it with an assortment of functions selected from either the shortcut buttons or “Insert Function” box. Use this tool to create a ratio of Lymphocytes to T-cells.



L5 fig.7/8 - Adding key word or formula

Now the table is ready to be batched.

Batching to Create a Table

The screenshot shows the FlowJo Table Editor window. The main area displays a table with four columns: Population, Statistic, Parameter, and Name. The table includes rows for various cell subsets like Live/Singlets/Lymphocytes/T cells and their CD4+ and CD8+ subpopulations, along with calculated values like %T-cells, %Lymphs, and %Double Negative. It also includes rows for Comp-PE-Cy55-A CVs and MFI values. The bottom section of the table editor shows sections for Formula, Day, and Type.

Within the table editor tab, there are *L5 fig.9 - Table editor* batching options for iterations and outputs. In this case, simply press the batch to display icon at the top-right of the table editor.

The group that you will draw the samples from must be specified, as well as the iteration, which is the order and pattern in which the files will be organized. This is accomplished in the group drop-down menu from the Iteration ribbon (*fig.10*). The default selection is the group that is currently highlighted in the workspace window, which is the group from which you selected the statistics and populations. If you wish to apply the selected statistics to another group, click on the drop-down menu next to the word “Group”, and select the desired group. For this experiment, accept the default condition of T-cell panel.

Table Iteration

Iterate by Sample means that you will include every sample in the selected group. Create a table by choosing the “T-Cell Panel” as the group, and Iterate by “sample”. You will generate the table below (*fig.11*).

FlowJo cycled through every sample in the chosen group, calculated the 11 requested statistics, created one formula and included two keywords. If a sample did not have the subset or keyword requested there would be a blank entry in the table.

This screenshot shows the FlowJo Table Editor with the Iteration tab selected. The Group dropdown menu is open, showing 'T-Cell Panel' as the current selection. Other options in the dropdown include 'All Samples', 'B-Cell Panel', 'Compensation', 'Day (8)', 'Panel 2.1', 'Panel 1.1', and 'T-Cell Panel'. To the right of the dropdown, the 'Output' section is visible, showing 'Statistic' and 'Count' under 'Output'.

L5 fig.10 - Table editor groups

Iterate by Panel means that the files are organized into a panel, or set, that have some relation to each other. In this case you may only want to include a specific member of each set, and iterating by panel will allow you to do this. Once you set “Iterate by” to “Panel,” you must fill in a value for the Number of Tubes box. This number tells FlowJo to include one tube out of the entered number of tubes in your table. For example, if you change the “1” to “2”, FlowJo will include one

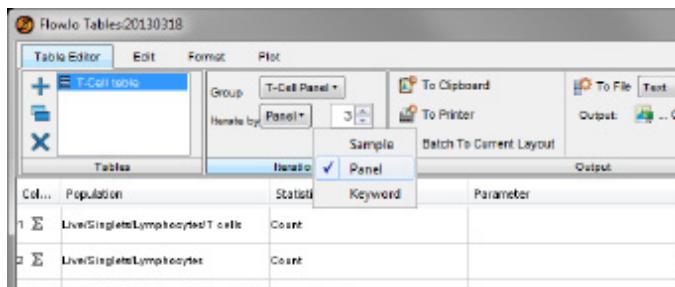
L5 fig.11- Batched table

out of every two tubes in the generated table.

Create a table that includes one file from each “Type” by selecting Iterate by Panel and entering the value “3” into the Number of Tubes / Panel data entry box (fig.12). This will create a table using only samples B1, B4, B7, D1, D4, and D7.

Iterate by Keyword means that FlowJo will use sample keywords to determine the order and inclusion of files which generate the statistics in the table. The order of the samples in the table will be based on the iteration keyword. Which files are used in the creating the table is based on the discriminator keyword. Any sample with a keyword entry matching the keyword entry of the discriminator keyword of the first sample in the chosen group is included.

In the Iteration task, choose “Iterate by keyword” and press the key icon which will open a window allowing you to



(fig.14).

L5 Fig.12- iterate by keyword

Additional Tools

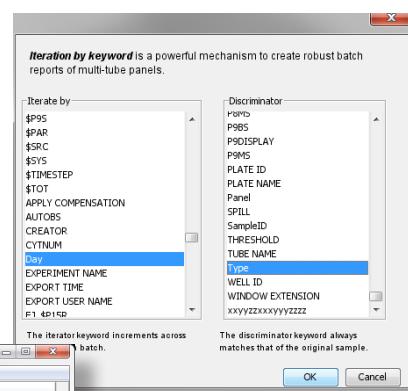
Returning to the table editor window, there are several options that have not yet been discussed. (fig.15)

The (and) buttons allow you can create new table templates, duplicate existing table templates, or delete existing table templates respectively. The

L5 fig.14 - Table results

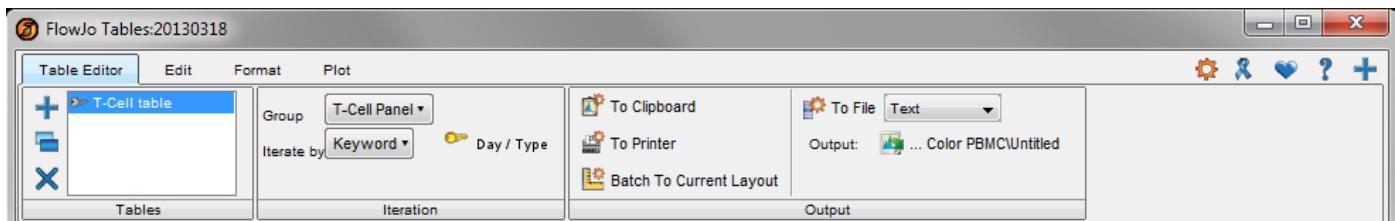
“to file” button icon allows you

to batch a table to file.



L5 fig.13- Keywords

The drop-down menus below that allow you to specify the group and the iteration options for the table you will create. In addition you can batch a table to the printer or to the current layout.



L5 fig.15 - Table editor shortcut keys

A d a second table by clicking the button in the table editor window. Type “B-Cell Table” into the name box. From the workspace, select the “B-Cell Panel” group. Open sample “A1” and select the “Lymphocyte” population. Hold down the “Alt” (or “Command”) key and drag the selected populations into the table editor. Add a formula by clicking on the “Add Column” button. Choose the “B-Cell Count”, type a “/” (division symbol), then choose the “T-Cell Count” and give the formula the name “BT Ratio”. Click “OK”. If you add custom names to the statistics and change the “Frequency of Parent” statistics to “Counts”, your table editor will look like the figure below at left (fig.16).

Applying Conditional Formatting

Click on the heart icon in the upper right hand corner of the table editor window. This will open FlowJo preferences. Under “Tools”, click to open “Ranges”.

L5 fig.16 - Table editor updated

editor, highlight the statistic to which you would like to apply formatting. Select the “Column” tab and click “Expected Range” in the Formatting band. In the drop-down box you can select the specific population to view.

Dynamic Updating

The table window is always current. When you create a table, FlowJo goes through all the samples and makes sure they are recalculated according to the latest modifications. If you now go back and change the Lymphocyte gate and apply that change to all of the samples, that change will be immediately reflected in the table window.

The work to this point will be saved as Lesson_5.wsp.

Lesson 6: Creating Simple Graphical Layouts

clicking the button in the table editor window.

Type “B-Cell Table” into the name box. From the workspace, select the “B-Cell Panel” group. Open sample “A1” and select the “Lymphocyte” population. Hold down the “Alt” (or “Command”) key and drag the selected populations into the table editor. Add a formula by clicking on the “Add Column” button. Choose the “B-Cell Count”, type a “/” (division symbol), then choose the “T-Cell Count” and give the formula the name “BT Ratio”. Click “OK”. If you add custom names to the statistics and change the “Frequency of Parent” statistics to “Counts”, your table editor will look like the figure below at left (fig.16).

Within Ranges you are able to set any conditional formatting you would like to reference in your analysis. You are able to enter the target population with a minimum and maximum allowance (fig.17). Once all entries are complete, click “OK” to close preferences. Back in the table

Name	Min	Max
NK Cells	0.1	0.24
Lymphs	0.5	0.8
CD4/CD8 ratio	1.5	2.8
CD3+ lymphs	0.5	0.8
CD4+ lymphs	0.2	0.5
CD8+ lymphs	0.13	0.41
CD19+ lymphs	0.07	0.28

Default Values based on: Clin Vaccine Immunol January 2004 vol. 11 no. 1 168-173

L5 fig.17 - Range batch settings

This lesson describes the fundamentals of the Layout Editor. You will be able to generate layouts with multiple graphics, statistics, etc. and learn how to create overlays of graphs. In Lesson 7, you will learn how to create batch reports, where the layout can be generated automatically from all of the samples in a group. Lesson 8 demonstrates the features in the Layout Editor that allow creation of complex multi-sample layouts.

This lesson continues with the workspace document you finished in Lesson 5; alternatively, you can open the tutorial workspace named “Lesson_5.wsp”.

Creating a New Layout

To open the Layout Editor, either press “Ctrl-L” (“Command-L” on a Mac), or click on the “Layout Editor” Icon.

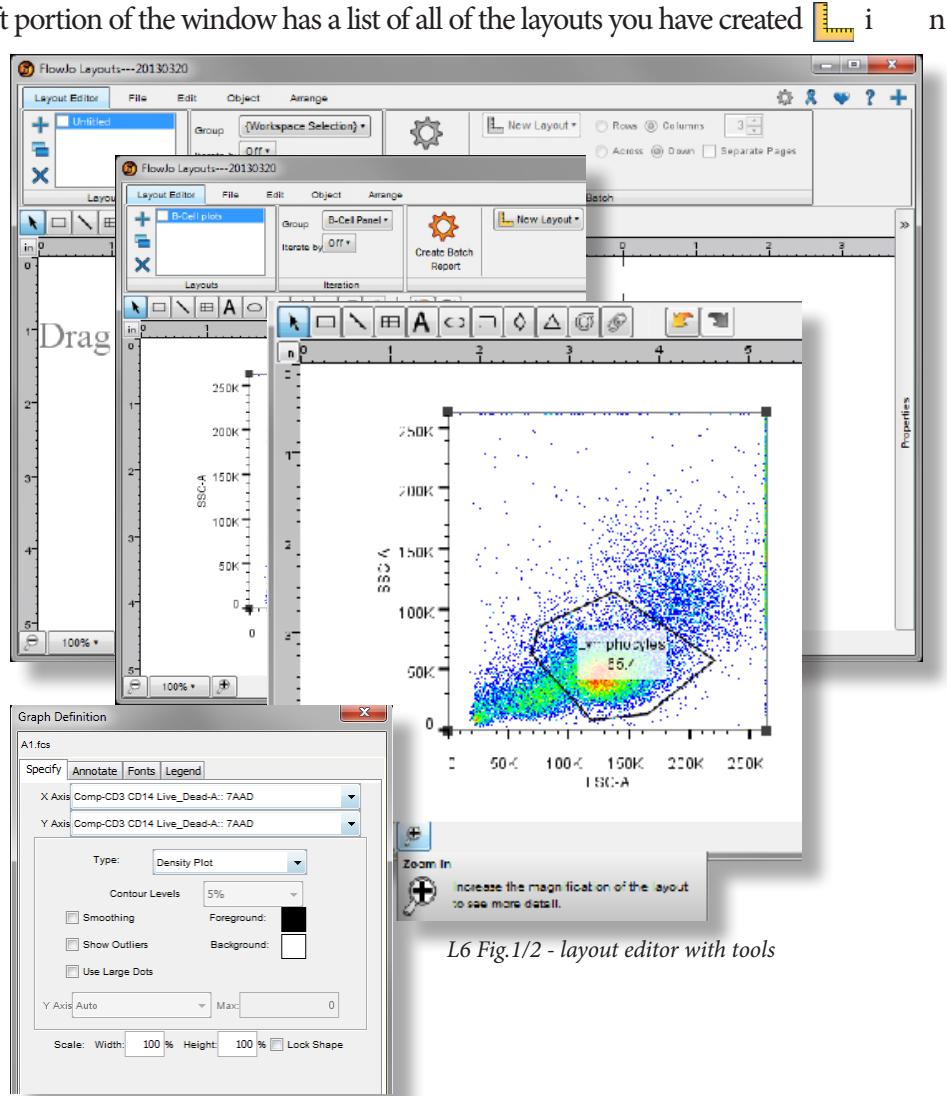
In the Layout Editor window, the upper left portion of the window has a list of all of the layouts you have created in this workspace. You can have as many layouts as you wish. This portion of the layout editor works just as in the table editor does. You can name the layout by clicking in the name field “Untitled” near the top of the window and typing a new name. Give this layout the name “B-Cell plots”.

Click on the top-level (ungated) population of sample A1 from the B-cell panel group. Hold the mouse button down and drag it into the “Layout View”. If you double-click on the plot in the layout editor, a dialog box (the Graph Definition window, *fig 3*, below right) will appear allowing you to make changes to the graph. Switch it from contour to pseudocolor. The result should look like the image to the right (*fig.1/2*). The default graph is the parameter combination and plot type that was last viewed. FlowJo also creates an annotation text box below the graphic that contains pertinent information.

Any graphic item can be resized or moved simply by clicking and dragging. To resize, click and drag on one of the four handles at each corner. You can also change the magnification of the view by clicking on the scaling tool, the magnifying glass in the bottom left hand corner of the screen.

One of the important aspects of the layout editor is that it is live. This means that any time you change or move a gate or modify an analysis in the graph window, FlowJo will automatically update the layout editor if needed. Thus, you can use the layout editor to provide instantaneous feedback for gating operations, where you can simultaneously view many different subsets (even multiple views of the same subset) while moving a gate used to define that subset.

Creating a Second Graph of a Population



L6 Fig.3 - graph definition window

L6 Fig.1/2 - layout editor with tools

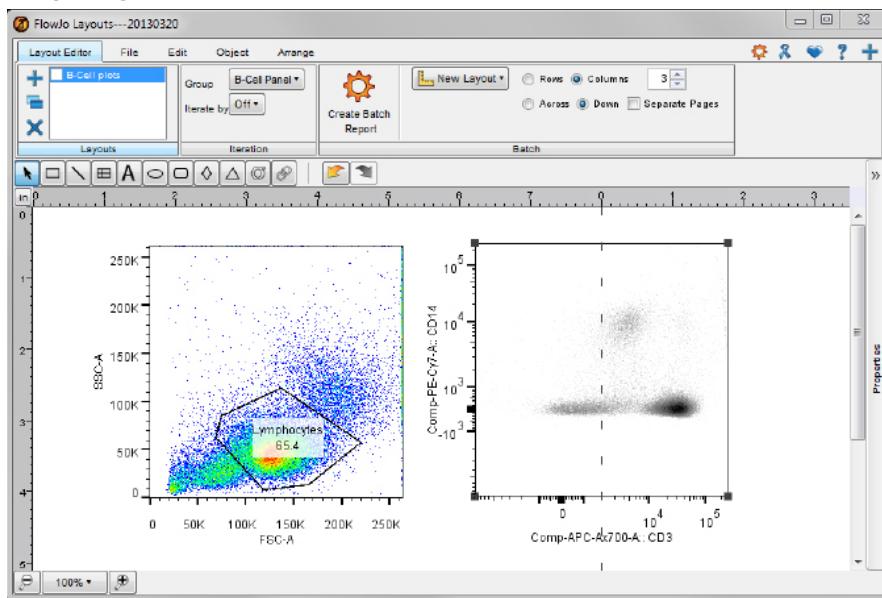
To create another view of the same subset, you have three choices: (1) drag the same subset from the workspace window into the layout editor again; (2) select the first graph in the layout editor, right click and do a “Copy” and “Paste” operation; (3) hold down “Alt” (PC, “Option” on Mac) key, click on the graph to be duplicated and drag this duplicate to a new place. For now, duplicate the first graph using the copy/paste method and place the copy next to the original.

Editing a Graphs Appearance

To change how a graph looks, double-click on it; the graph definition dialog appears, as shown to the right. From this window, you can specify exactly how you want the graphic to appear. Switch the parameters to CD3 versus CD14, from the X-Axis and Y-Axis boxes. Change the graph type to Density, and check “Show Outliers”. You will need to deselect “smoothing” if checked.

Click on the “Annotate” tab at the top of the window. This shows a different set of options that control what appears in the graphic, such as gates, frequencies, legends, adjunct Histograms, multigraph overlays, backgating, etc. Deselect the Annotation checkbox (fig.3). This will remove the annotation text below the graphic. Click “OK”, and the layout editor will look like the figure below (fig.4).

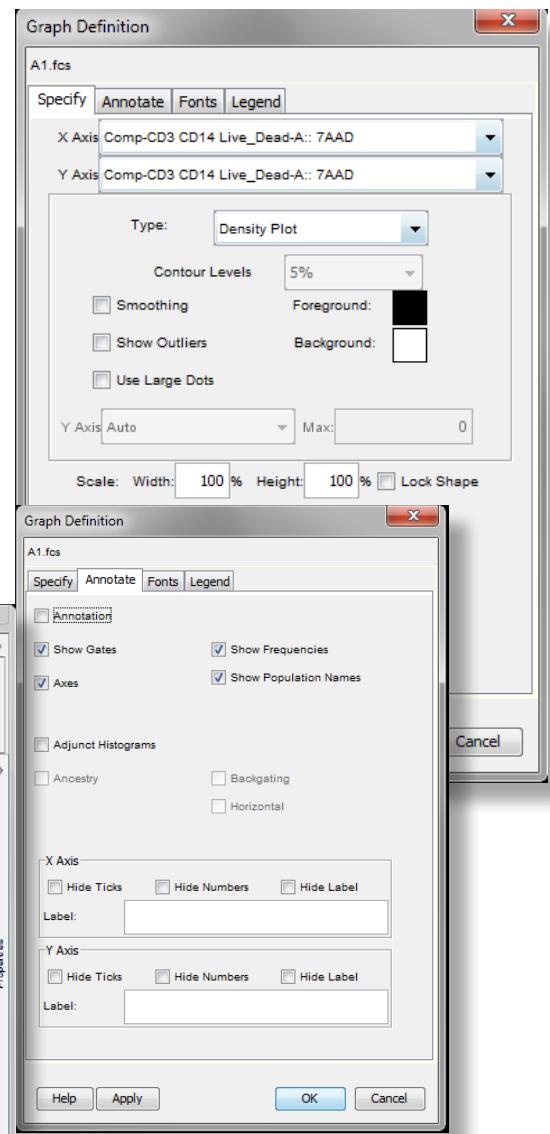
Aligning Plots



L6 fig.4 - Layout editor after changes

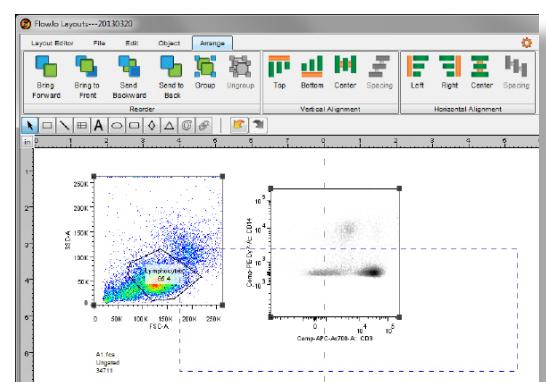
provides many features of drawing programs, such as the ability to align multiple objects. To align these graphs, left-click the mouse and with the button depressed, drag a rectangle to include both graphs. This selects the images for formatting with the Marquee Tool (fig.5). Now from the Arrange drop-down menu, click “Top” in the Vertical Alignment options. Notice that there are also selections for horizontal alignment, equal spacing and spread.

Dragging Statistics into the Layout Editor



L6 fig.3 - Graph definition window

FlowJo



L6 fig.5 - Layout editor align tools

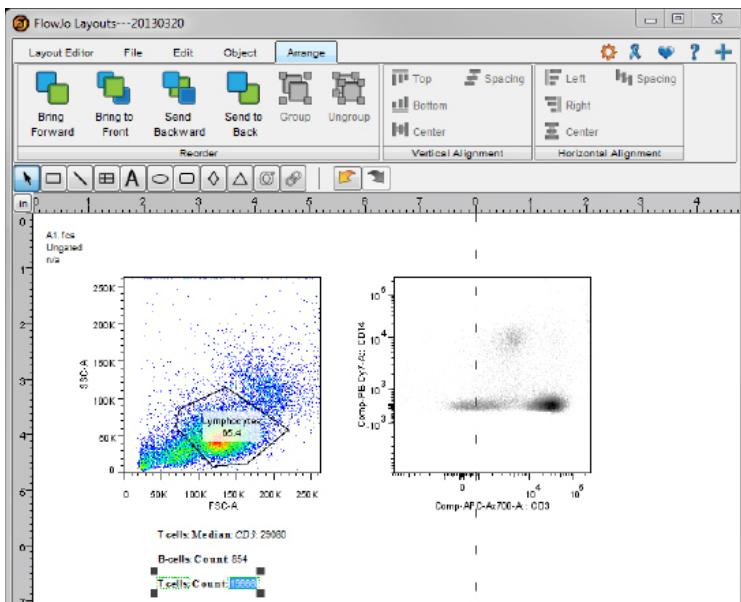
To display a statistic that you have added to the workspace, you can drag and drop it just like dragging of gated populations. To try this, click on the “Median: CD3” of the Lymphocyte population from sample A1 and drop it below your first plot. If you would like to format or edit the text, double-click on the statistic once to activate the edit, then again on either the name or number and the edit box will appear, as shown to the below (fig.6). The information within the angle brackets < > is the calculation of the statistic. You can change anything else without affecting the statistic.

Creating Formulas in the Layout Editor

In the workspace, add the statistic “Count” to both the B-Cell and T-Cell populations of Sample A1, using the Statistic band (sigma icon) within the workspace panel menu, as explained in Lesson 2. Then drag the two counts to the B-cell Panel group. Next drag the two counts separately from Sample A1 into the layout editor and place them below the figure. Double-click on the number portion of “T-Cell Count”.

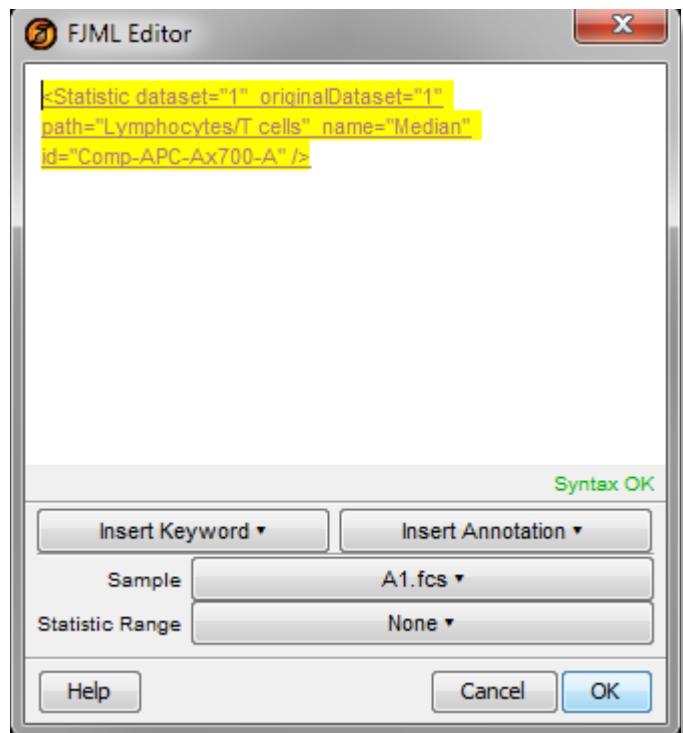
Now click “cancel” the FJML editor box.

Imagine that you want to create an additional statistic, the sum of the T-Cell and B-Cell counts. The easiest way to do so is duplicate one of the existing statistics and edit it to show the desired result. Click on the “B-Cell Count”, and duplicate it by using the keyboard shortcuts “Ctrl-C” and “Ctrl-V” to copy and paste it into the layout (fig.7).

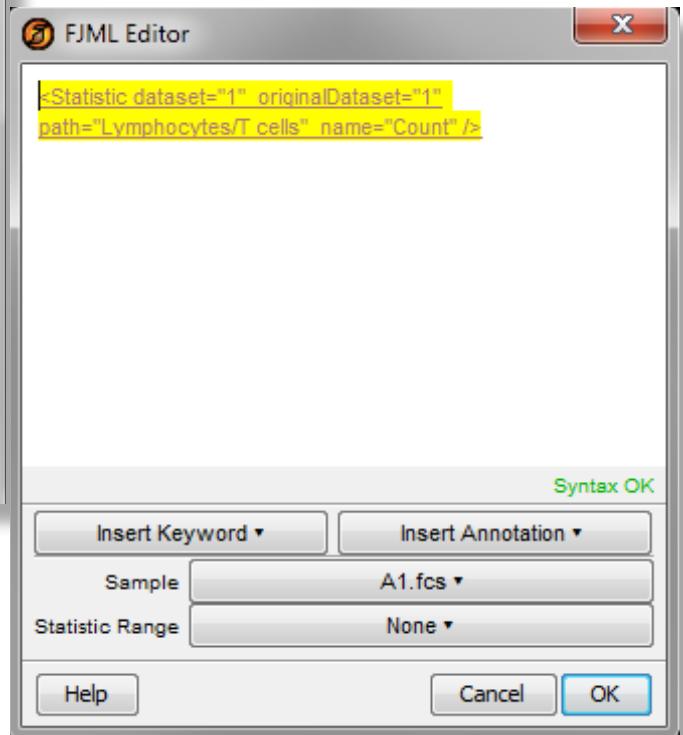


L6 fig.7 - Layout editor copy & paste

on the number portion of the “T-Cell” statistic. It will look like the text box at right (fig.8).



Next, L6 fig.6 - Layout editor text ftml box double-click



L6 fig.8 - Layout editor t-cell text edit

As you can see, “T-Cell Count” is outside of the angle brackets. This portion of the annotation is simple text that you can edit to say whatever you like. The statistic portion of the annotation is calculated only within the brackets. Information within the brackets provides FlowJo with: (1) which file the statistic is from, (2) the gating path and (3) the particular statistic to be calculated.

Highlight all of the information (from bracket to bracket) and copy it using the keyboard shortcut “Ctrl-C”. Click “OK”. Activate the FJML editor on the number portion of the duplicate “B-Cell Count”. At the end of the statistic, (outside of the angle bracket) type an addition (+) sign. Then use the keyboard shortcut “Ctrl-V” to paste in the statistic you copied from the T-Cells count after the addition sign.

To tell FlowJo the extent of the formula, type in the information in the figure to the above (fig.9).

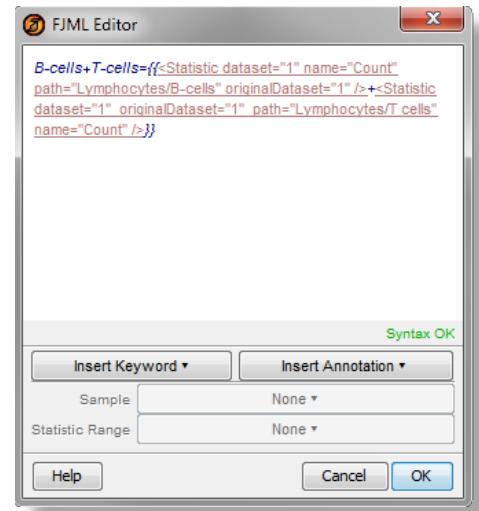
Always use double curly brackets `{} {{ statistic }}` to encapsulate the statistic you wish to apply. To add a title for the statistic, type “B-cells+T-cells =” before first curly bracket. When creating the formula, type a title outside of the angle brackets (and curly brackets if present). The title on the formula should now read Click “OK” to accept the modifications.

Drawing Tools

FlowJo’s layout editor provides a few simple drawing tools to embellish your graphical reports. These tools can be selected by clicking on one of the icons on the top-left portion of the window.

You can choose :

- The Arrow Tool is used to select existing objects. Click on an object to select it (as shown by dark handles at the corners of the object). Use the “Shift” key to select additional items. Drag items to move them. “Alt”-drag (or “Command”on Mac) to duplicate them. Start a drag in the background of the layout editor, and it will draw out a rectangle. Upon finishing the drag, the layout editor will select all objects that are enclosed by that rectangle. (Marquee selection.)
- The Rectangle tool is used for drawing simple boxes and frames. If you create the rectangle surrounding another element, and want it to serve as a background, go to the Arrange section of the ribbon, and select use the Arrange > “Send To Back” menu command to change the order of the layout elements you have selected.
- The Line Tool is used for drawing lines and arrows. Generally, you will want lines drawn on top of other elements, so draw them last or use the Arrange > “Bring To Front” command to change the order of the layout. Right-click on the line and select “Arrow Style” to add arrowheads to the line.
- The Grid Tool will create a matrix of cells within your layout. Each cell may contain text, charts, images, or other grids. The grid provides a convenient mechanism to group and align multiple elements of the layout. Grids are explained in greater detail on the Grid Tool page.
- The Text Tool is used for adding textual annotation to the layout. To create a text box, you select the text tool from the toolbar, and click once or drag out a rectangle in the layout view. When you create a new text box, a dialog will appear to help you edit the text. This is called the FJML (FlowJo Markup Language) Editor. FlowJo uses a custom markup language, similar to the HTML used in web pages, to richly express the structure of your flow analysis. Once you confirm changes via the mouse or the Enter key, or click the mouse on a different object in the layout, the editing stops, and the layout editor text is reformatted and frozen. To edit it again, double-click on the text box to return it to the edit state. Text clippings or statistics from the workspace can be dragged and dropped into a layout. In both of those cases, a text box is created automatically.



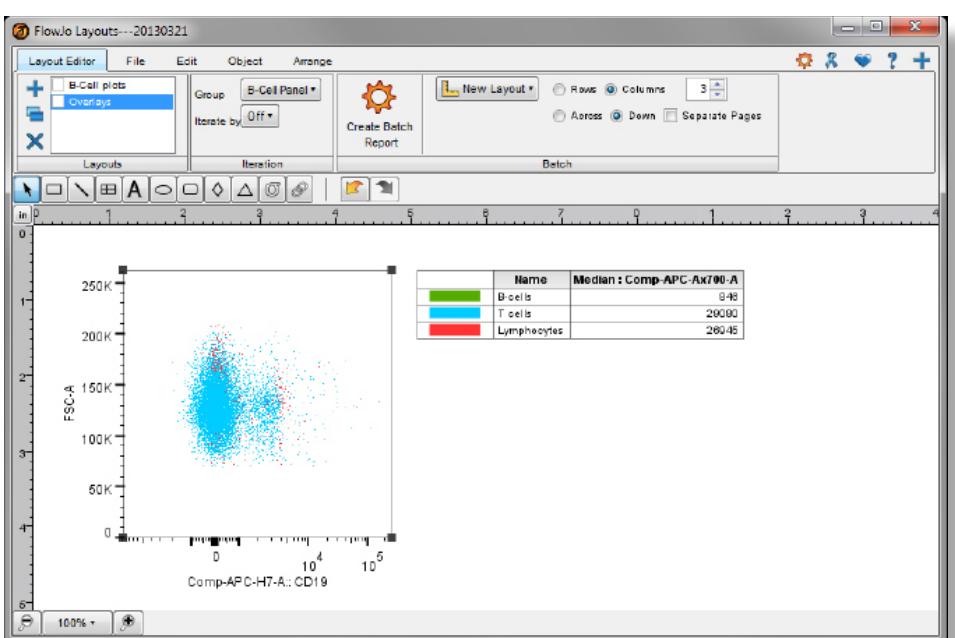
L6 fig.9 - Text editor formula

- The Ellipse tool is used for drawing simple circles and ellipses. If you create the ellipse surrounding another element, and want it to serve as a background, use the Arrange> “Send To Back” menu command to change the order of the layout.
- The rounded Rectangle tool is used for drawing simple boxes and frames. You can drag the dot to change the overall shape. If you create the rounded rectangle surrounding another element, and want it to serve as a background, use the “Send To Back” button under the Arrange menu to change the order of the layout.
- ◇ The Diamond tool is used for drawing quadrilaterals that point to the center of each bounding edge. If you create a diamond surrounding another element, and want it to serve as a background, use the “Send To Back” button under the Arrange menu to change the order of the layout.
- △ The Triangle tool is used for drawing 3-sided shapes in your layout report. If you create a triangle surrounding another element, and want it to serve as a background, use the “Send To Back” button under the Arrange menu to change the order of the layout.
- The Statistics Table Tool allows you to build a table of statistics within the Layout editor. Click the statistics button, then drag a rectangle in the area where you want to place the statistical table. A statistics table dialog box will appear allowing you to enter data.
- ⌚ The Web Box Tool creates a box with an embedded html viewer so you can display parts or all of a web page within your layout.

To add text to your plot, click on the **A** Text button and draw a rectangle that will contain the text. Type the word “Sample”, then right-click and select “Insert Keyword”. Choose the sample “A1”, then choose the keyword “Tube Name”. Click “OK”. Now with your text selected and highlighted, go to the Object tab at top of the layout editor. Change the font size to 18 using the font drop-down menu in order to make it easier to read. You will notice that the text “Sample A1” has appeared on the plot where you placed the text box. FlowJo has filled in the value for the selected keyword and placed it in line with the rest of the typed text. Using keywords instead of typing all of the text will be important in Chapter 7 when you create batched plots for all samples in a group.

Overlays

In the layout editor tab, click the  on the upper left to add a new blank layout. Title this layout “Overlays”. Select the Lymphocyte population from sample A1 and drag it into the layout editor. Any graphic item can be made into an overlay by dragging and dropping another subset onto it. You can overlay different subsets from the same sample, or overlay plots from different samples. For now, select the “T-cells” subset from the same sample and drop it on the graphic (the sample name will appear in the legend to show that it was dropped). Then drag the B-cells subset and drop it on the plot as well. You should now see the multi-color Dot Plot, shown here to the right (fig.10).



L6 fig.10 - Layout editor overlays

FlowJo draws two Dot Plots, one for each subset, in the same graph. In addition, it automatically creates a legend for the overlay, shown to the right of the graphic. To make changes to the legend, double-click on it. Click on the “Sigma” button and add the MFI of CD3 to the legend by choosing it from the pop-up menu. Once you have selected the CD3 Median, click “Add” and then “Close”. Click “OK” in the graph definition dialog box. You can change the color of any subset by clicking on the color box next to that subset in the legend.

You can change the stacking order of the colored layers by clicking on any item, and dragging it up or down in the legend list. Finally, you can delete an item from the overlay by right-clicking and selecting “Remove Layer” from the pop up menu that appears. You can overlay an almost unlimited number of different subsets on the same graph.

Note: you can choose to show the Legend box for any graphic, even if it is not an overlay, by double-clicking on a graph to open the Graph Definition Window and access Annotation options. There, you can also set color and line styles for single graphs just as you can for overlay graphs.

Once you create an overlay graphic, you can easily change its appearance (axes and plot style) – just like any other graphic. For example, double-click on the “Overlay Plot”, choose the “Specify” tab, and change the Y-axis to Histogram. Click “Apply”, then “OK”.

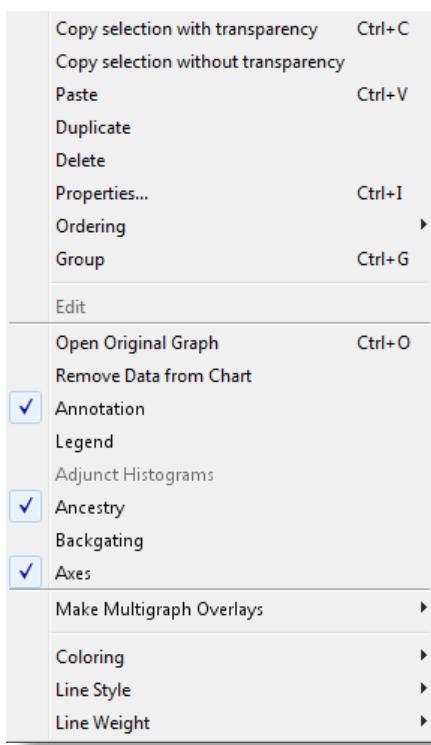
If you right-click on any sample in the legend, you can select the line weights (Hairline, Normal, Heavy, or Very Heavy) the line styles (None, Solid, Dotted, Dashed, Long-Dashed, Complex, or Dot-dashed) or if the Histogram coloring (None, Filled or Tinted). You can also remove one of the overlaid populations, or use this interface to edit what is displayed in the legend. Fill in the B-Cell layer. Now hold down the “Alt” key (“Command” on Mac), then Right click on the “T-Cell” layer and select “tinted 80%”. Notice that all layers follow the format command if the “Alt” key is depressed.

Ancestry and Backgating

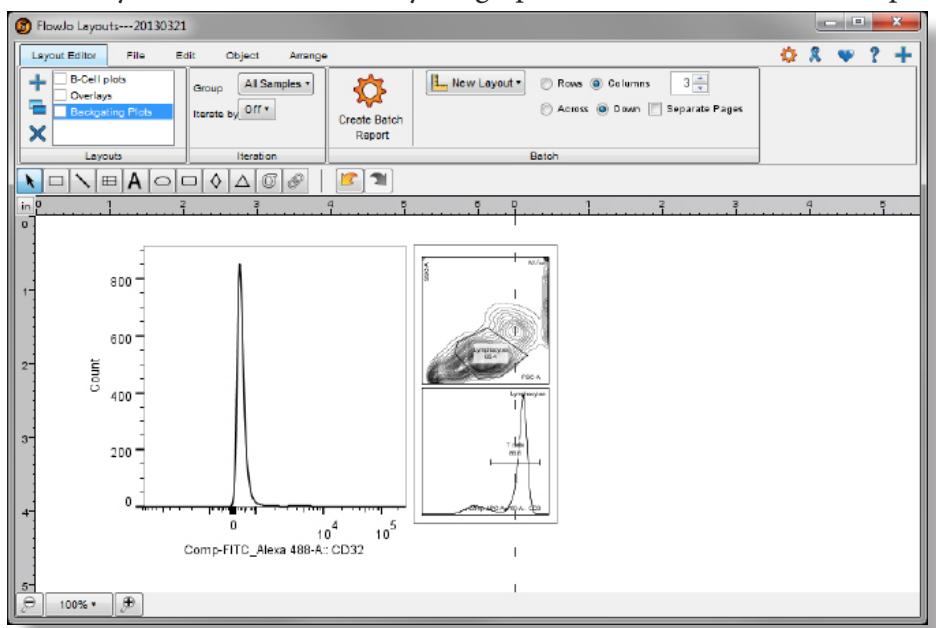


Create another new layout using the button, and call it “Backgating Plots”. This time, drag in the “T-Cell” population from sample A1. Right-click the plot and you will see the menu displayed to the below left (fig.11).

Most of the graph options can be selected from this interface. Click “Ancestry”. You will notice that your graph now includes two smaller plots



L6 Fig.11- Layout editor menu



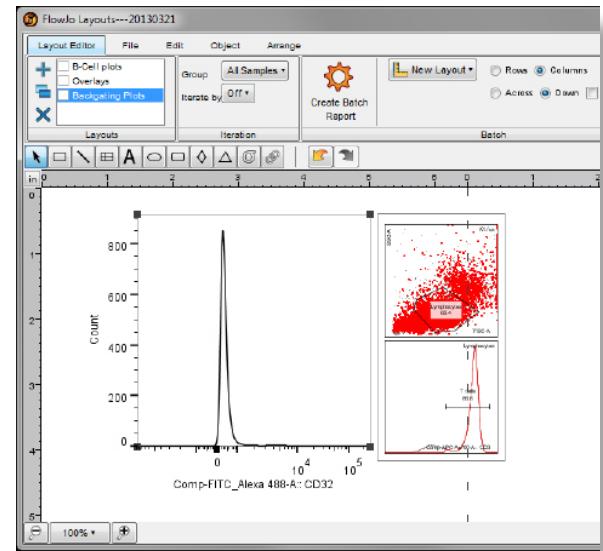
L6 fig.12- Backgating ancestry

that illustrate the strategy used to isolate the events within the T-Cell gate. The resulting plot is shown below (fig.12).

Right-click on the graph again, and select “Backgating”. The ancestry plots now show the back-propagation of events from one gate to the next. This is a tool for evaluating the effect of any gate. The result is shown in the plot on the right.

Multigraph Overlays

FlowJo offers a convenient feature for viewing multidimensional data using many parameters simultaneously called a Multigraph Overlay. Below the ancestry plot, drag the “T-Cell” population in again. Set the graph to be “CD3” versus “SSC-A” as a smoothed

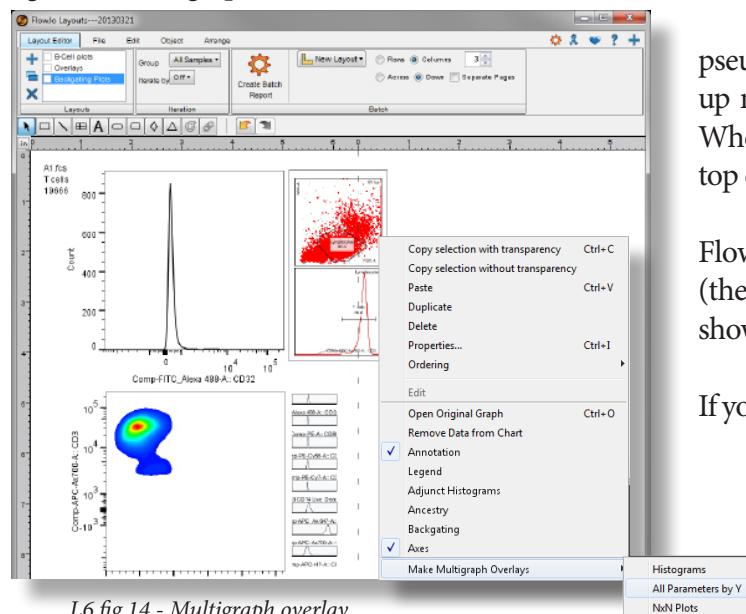


L6 fig.13- Backgating

pseudocolor plot. Right-click on the plot to open up the pop-up menu. The bottom choice is “Make Multigraph Overlays”. When moused over, it expands to show three choices. Select the top choice: “Histograms”.

FlowJo will display the Histogram of the plotted cell population (the T-cells in this case) for all parameters. The resulting plot is shown above (fig.13).

If you right-click on the plot again, you can hide the multigraph



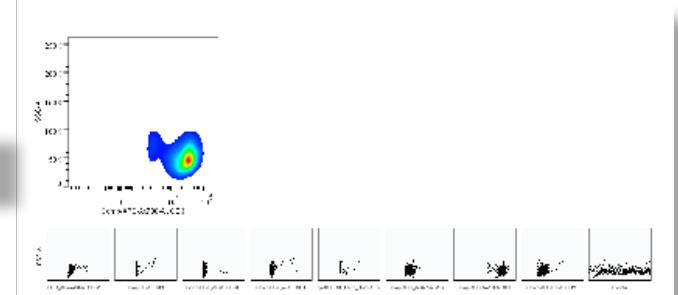
L6 fig.14 - Multigraph overlay

overlay, and then select the second option (“All Parameters by Y”) to show all plots by the parameter on the Y-axis, in this case SSC-A (fig.14). Select this and you will get a plot with SSC-A on the Y-axis, and the rest of the parameters shown on the X-axis one at a time. A portion of the resulting figure is shown below (fig.15).

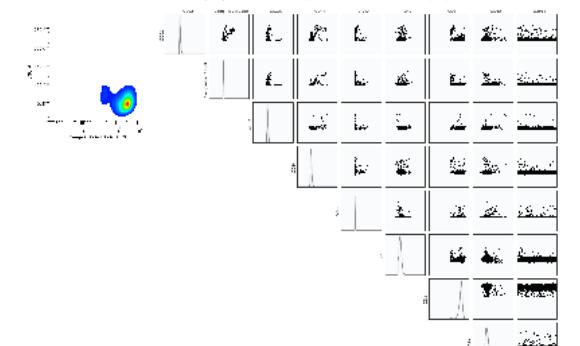
If you hide the multigraph overlay again, then switch to the final choice. NxN, FlowJo will make a grid of all possible two-parameter combinations. Additionally, if you overlay another population on the original graph, the additional population (or populations) will be overlaid in all of the NxN plots. Try dragging the “B-Cell” population onto the original T-Cell plot. The resulting figure is shown to the right (fig.16).

Displaying Adjunct Histograms

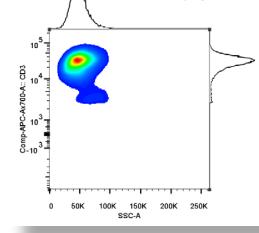
Choose to hide the multigraph overlay. Right-click



L6 fig.15 - All parameters by y

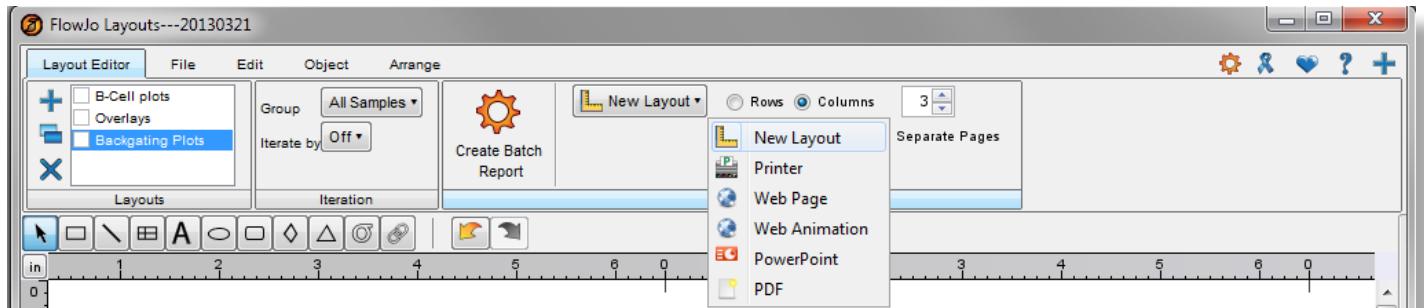


L6 fig.16 - NxN plots/combination



L6fig.17 - Adjust histograms

on the plot again and this time select “Adjunct Histograms” from the pop-up menu. This selection will add a Histogram of each of the component parameters in a two-dimensional plot to the axes. If you have an overlay (as we do in the case), FlowJo will display the histogram of each population overlaid in the histograms as well. The resulting graphic is shown to the right (fig.17).



L6 fig.18 - Export options

Exporting a Layout Page

The tools at the far right-side of the toolbar, shown below (fig.18), allow you to export a layout page to some other format. They apply to the layout page that is open.

The result of a batch report can be viewed in several different ways. Clicking the “Batch” button can create:

- A new layout in the layout editor.
- Alternatively, it can print the report directly to your printer.
- A web page containing the pictures for each layout group
- With web animation, an HTML file will be created that contains an embedded Quicktime movie with options.
- With animation, each of your batches will become a continuous frame in a movie format.
- Each page of your batch will become a slide in PowerPoint.
- A preview window containing static tiles-PDF (data will not update if you change the original analysis).

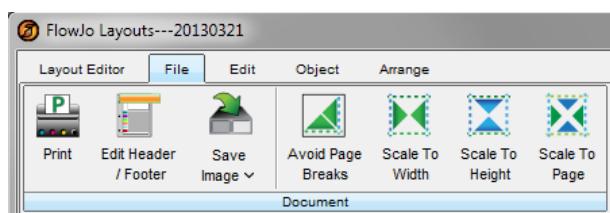
In every case, FlowJo will produce a series of frames, each one containing graphs and statistics from one or more tubes.

Printing from the Layout Editor

The printer icon in the File tab opens a menu allowing you to choose a printer to print a hardcopy of the current layout. You will notice that FlowJo draws gray dotted lines on the Layout Editor pages: these correspond to page breaks in a printed document. (Select a small magnification, like 12.5%, to see many pages at once). Note that as you change the viewing magnification, the relative scaling of the graphs to the page boundaries do not change –

you are not changing the print magnification! You can change the relative scaling of all graphs by clicking and holding on an intersection point of the gray lines, then either dragging away from the origin point to stretch all of the pages, or dragging toward the origin point to shrink all of the pages. By using this method to scale plots, all plots will always remain the same size. Additionally, the orientation of the pages can be toggled between landscape and portrait, by grabbing an intersection point and dragging upward diagonally.

There are also shortcuts that allow you to specify that FlowJo automatically scale all plots to fit on one page to be a



L6 fig.19 - File panel options

41

certain width, height, or one full page. This is done by selecting “Scale to Page Width”, “Scale to Page Height”, or “Scale To Page” from the File menu at the top of the layout editor (fig.19).

If you select “Avoid Page Breaks” from the same menu, FlowJo uses your paper type/shape but places tiles on the page such that tiles will not cross page boundaries.

Once you have arranged the tiles exactly the way you like, you can print them – you will know exactly how they will appear on the pages.

Copying and Pasting Graphs from FlowJo

In addition to exporting, you can take an individual graph, or set of graphs out of FlowJo and into a presentation

or publishing software like Powerpoint or Word. Do this by selecting the desired graphics, choosing “Copy” from the Edit menu and then pasting the figure into the destination document.

To access the workspace at this point of the analysis, open the Lesson_6.wsp file.

Lesson 7: Creating Batch Graphical Reports

In this lesson, you will continue with what you learned in Lesson 6 and generate graphical reports for entire experiments. You will learn how to cycle the layout through different samples in the workspace and how to create a combined output for printing or exporting of graphs for every sample.

This lesson builds on the workspace you finished from Lesson 6; alternatively, you can open the workspace named “Lesson_6.wsp”.

One of the strengths of computers is how they handle repetitive tasks. Show the computer how you want something done once, it can repeat the task a million times without complaint. Flow cytometry often requires application of the same analysis to any number of samples, and FlowJo’s ability to batch through a set of samples to produce a graphical report is the heart of FlowJo’s Layout Editor. At the same time, computers are very literal and so it is a

1. Build a Prototype tile
2. Know the type of batch you are looking to perform
3. Double check key “successful batch” iteration controllers
4. Batch the prototype

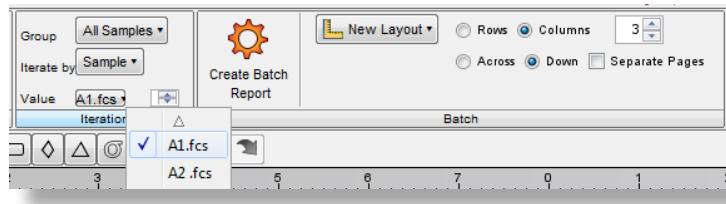
good idea to know exactly what information FlowJo needs available in order to batch successfully.



In every batch FlowJo will produce a series of tiles, each one containing graphs and statistics and/or other elements from one or more tubes.

The overall process of producing a batch report is as follows:

To begin examining how to set up a batch in FlowJo, open the layout editor by clicking on the layout editor icon in the workspace. When you drag items into the layout view, FlowJo by default shows you the desired graph for the sample from which you dragged the subset. FlowJo can also show



L7 fig.1 - Iterate by sample buttons

1. The sample has the parameters that are displayed in the graph (like Forward Scatter, FL3, FL4, etc.).
2. The sample has a gating tree that has exactly the same subset as what is desired in the graphic (i.e., if the graph is generated from a CD3 subset of Lymphocytes, then FlowJo looks for a CD3 subset of Lymphocytes in the current sample).

you the corresponding graph or graphs from any sample in the current group. To do this, return to the layout titled “B-Cell Plots”, and change the iteration box from “Off” to “Sample” (fig.1). Choose one of the samples from the list that appears next to the iteration box once a sample has been selected. The up and down arrows allow you to scroll from file to file.

Batch Plotting

FlowJo will generate a graph for any given layout item for all samples in a group during batch processing if the samples meet all of the following criteria:

At this point you have only looked at the layout report for individual samples, one-by-one. To look at graphs for all samples at once, click on the “Create Batch Report” button in the center of the layout editor ribbon.

You can set the output format of the report, the iteration style, geometry of the rows and columns and the order in which plots will be placed as either going down or across. The set of plots, graphics, statistics, text and anything else included in the layout page for one sample is called a tile. Set the batch to place the tiles in 1 column as shown in the image at right above (fig 2) and select “Down”.

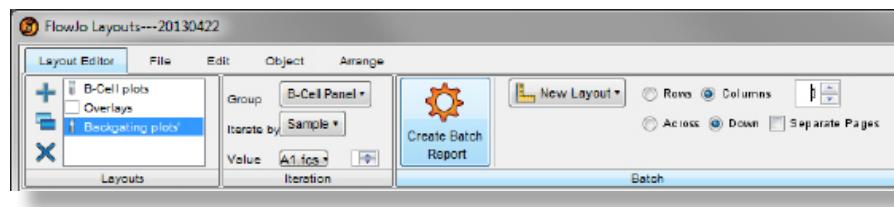
Leave the default output “New Layout” selected and click “Create Batch Report”. The result will look like the figure to the right (fig 2b).

Observe that a new layout page has been created named “B-Cell plots-Batch-1”. FlowJo has created the same set of plots, stats, and text that you created for sample A1 for the rest of the samples in the B-cell group. Since there are 18 samples in the group, there are now eighteen sets of two plots, a statistic and a text box.

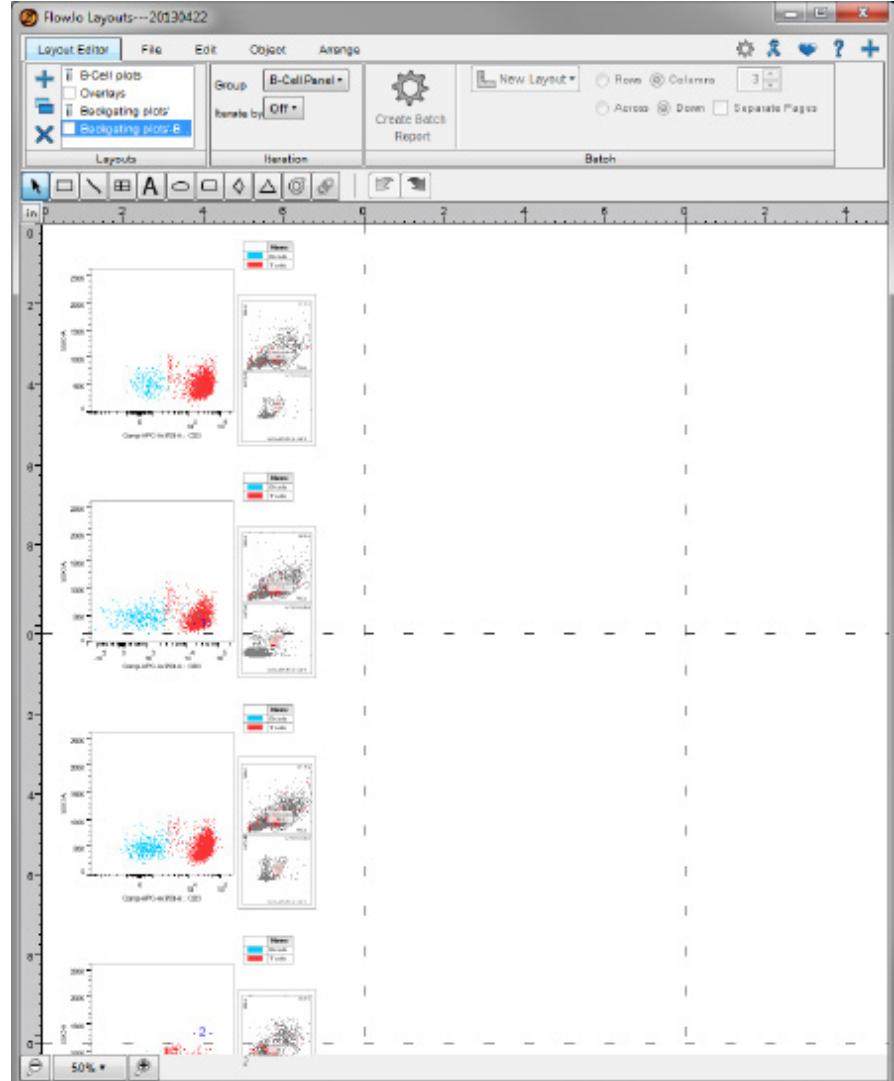
Other Batch Outputs

In the image below (fig 3) you will see that if we return to the original layout and press the “Batch” button again, the batch layouts can be generated in six different modes, each accompanied with an icon.

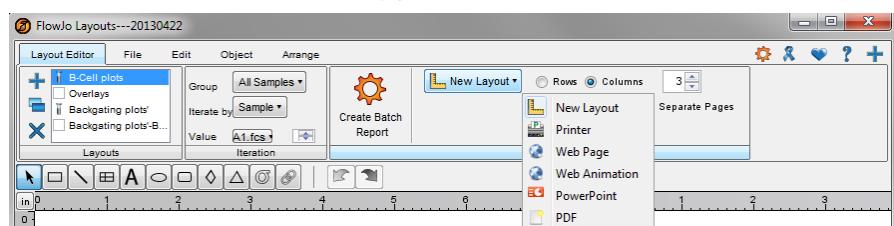
The first item listed is “New Layout.” We used this previously to create a new layout in the layout editor, with all of the iterated samples shown. This is the most flexible way to generate a report, as you can edit the layout further



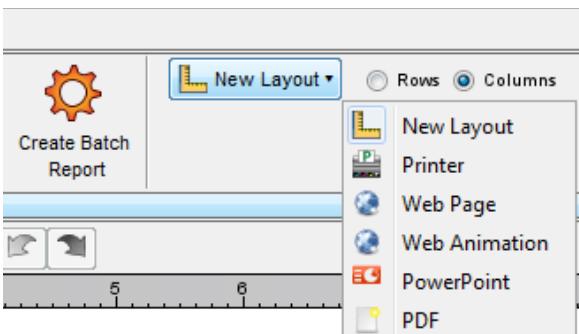
L7 fig.2 - Batch settings



L7 fig.2B - batched column



L7 fig.3 - Batch options



L7fig.3(detail) - Batch options

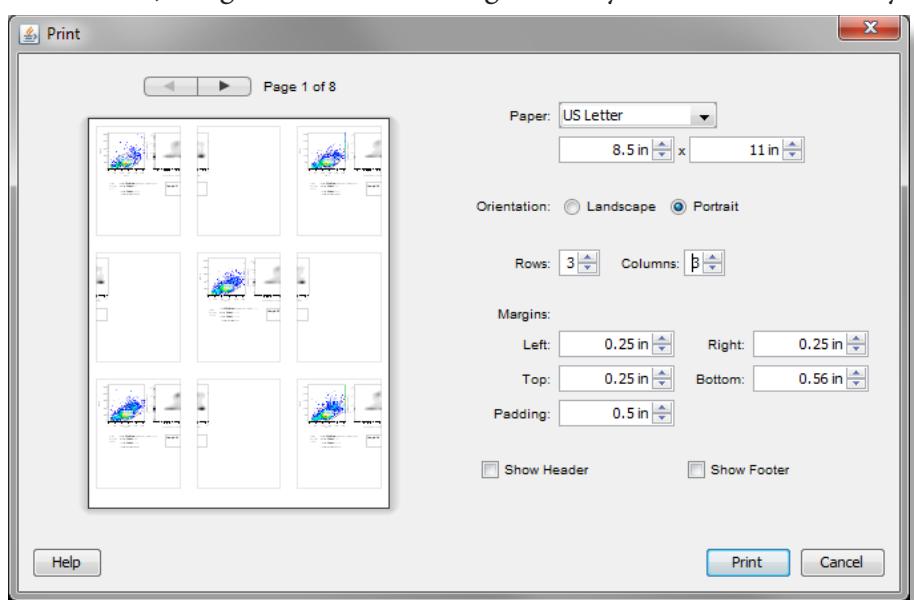
click on the “Printer” option and then click “Create Batch Report”, you will see the dialogue box below appear (fig 4).

From this dialogue you can select the paper size, orientation and how many tiles will appear on each page by increasing the number of rows and columns. In the following figure, three rows and three columns have been selected. It is important to remember that when batching directly to the printer, FlowJo uses the pages as specified by the page breaks to determine the tiling. So

by adding titles or annotations to specific graphs, or by removing unnecessary graphs. Unlike other batch exports, batching to a new layout has the advantage that the resultant layout will update “live” and reflect changes made in the workspace.

The PowerPoint option produces a pptx file with one slide for every tile created by the batch option. In the B-cell group we have nine files, so batching to PowerPoint button will create a PowerPoint file with 18 slides, each with the two graphs, one statistic and one text box.

The Printer option allows you to print all of the batched plots. FlowJo aligns the tiles, ensuring that they are the same size. If you



L7fig.4 - Batch printer dialogue

you should choose the Scale to Page function in the File tab of the layout editor before selecting this batch option. One last important piece of information; the Layout Editor view is live in that whenever you change a gate or analysis, the view is automatically updated to reflect the change. However, Web Reports, any PDF you may generate, and Movies are NOT live. If you create one of these reports and then change a gate, the report is NOT UPDATED, because the destination is outside of FlowJo. In order for these media types to reflect changes, you will need to regenerate the batch output.

Try batching to each type of output.

The iteration band contains the iteration controls. This is the same set of options as provided when batching from the Table Editor. Lesson 8 will discuss in detail how these options apply to layouts.

To access the workspace at this point of the analysis, open the Lesson_7.wsp file.

Lesson 8: Generating Complex Batch Analysis

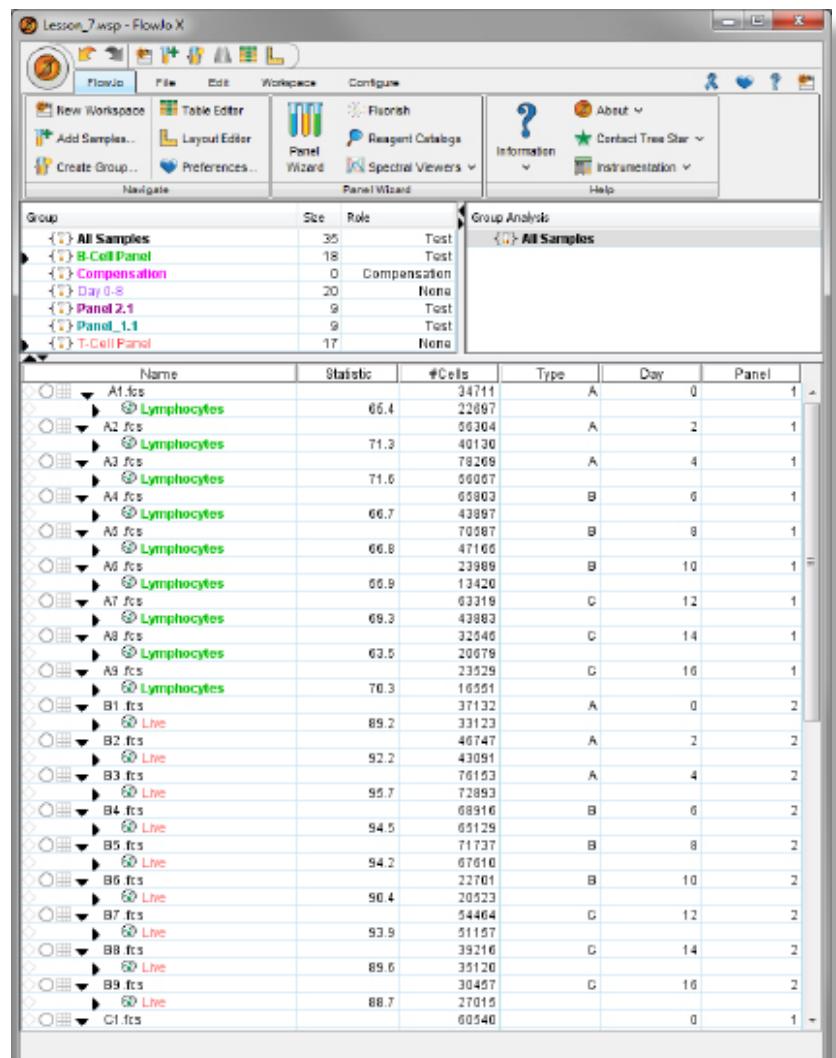
In this lesson, you will build on what you learned in Lesson 7 to generate graphical reports that iterate the samples included in a batch in a variety of ways.

This lesson builds on the workspace you have finished from Chapter 7; alternatively, you can open the workspace named “Lesson_7.wsp”.

Varying Iteration Options

In Chapter 7, you created a layout with several plots all from the same sample then batched the layout with the iteration option set to “Sample”, which forces all graphs to be derived from the same group during iteration. To create a batch output, FlowJo iterates sample by sample through the current group creating the selected plots for each sample but for only one sample per tile.

However, you may want to generate graphical reports wherein each tile derives graphics from multiple samples. For example, you may want to generate one figure for a panel collected on a particular day, in which case you could iterate by day. Alternatively, you may want to overlay plots from multiple samples for comparison against each other and repeat that pattern for all samples. In these cases, you would like to iterate not over samples, but over time point or panel. FlowJo gives you this ability if your FCS data has keywords that contain such information. This kind of data can be added at the time of collection on the cytometer or afterwards in FlowJo, as described in Lesson 1.



L8 fig.1 - Workspace

This tutorial data has a keyword field, “Day”, which lists the experimental time point in days that the blood samples were drawn. For example, samples A1 and B1 were both collected on Day 0. There is also a keyword field “Panel” that

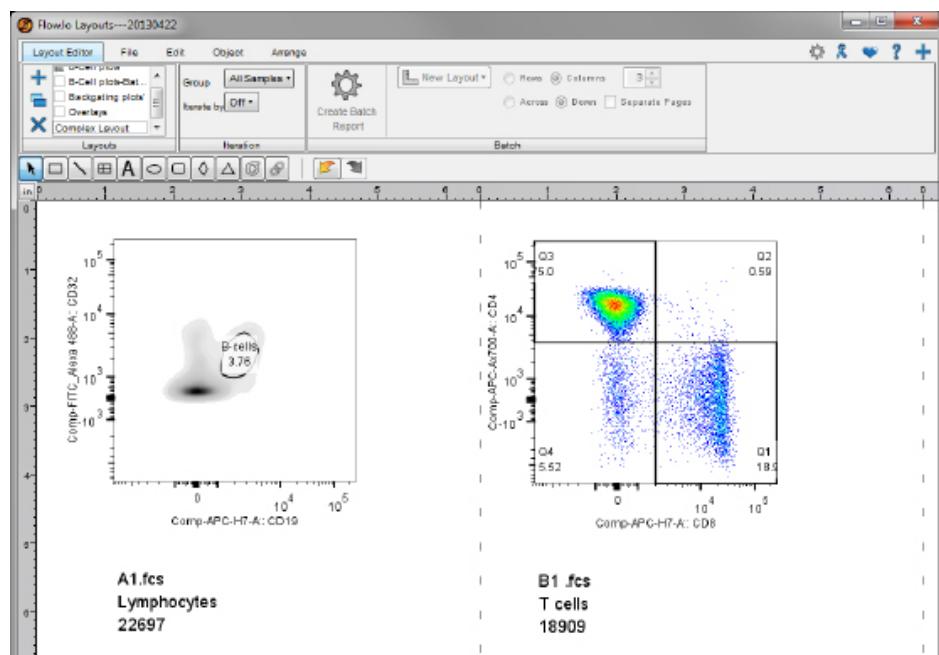
lists which panel of antibodies was used to stain the PBMC samples. Right-click on any column header and select “Edit Columns” on the right-click menu. This will open the Edit Column Names dialog box. Select “Panel” from the dialog menu, then press the “Add Column” button, then “OK”.

This will display these two keywords in the workspace. Now click on the “All Samples” group to display all of the files. The workspace will look like the image above (fig 1).

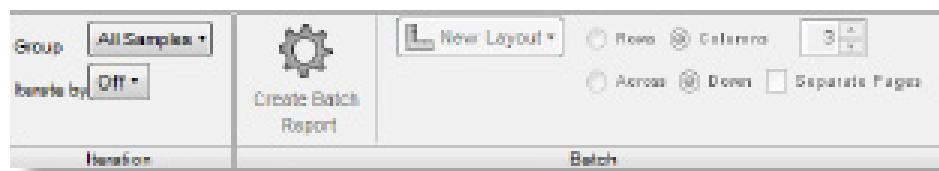
Create a new layout and call it “Complex Layout”. Drag the “Lymphocyte” subpopulation from sample A1 into the Layout Editor, and then drag in the “T-cell” subpopulation from sample B1. Double-click on the “Lymphocyte” population and display the graph as a density plot of CD32 versus CD19. Double-click on the “T-cell” plot and make it a pseudocolor plot of CD8 versus CD4. To increase the font size, select the the annotation, then the “Object” tab. Here you will see the text ribbon and font size options. Select “18” from the font size drop-down menu. Your plots will look like the figure here (Fig.2).

Notice that the batch button is grayed out. These plots come from two different samples, so iteration is automatically set to “Off” and they cannot be batched without instructing the software how to proceed. This is because it is unclear how to proceed with the default iteration setting of “By Sample” when there are two different samples to choose from.

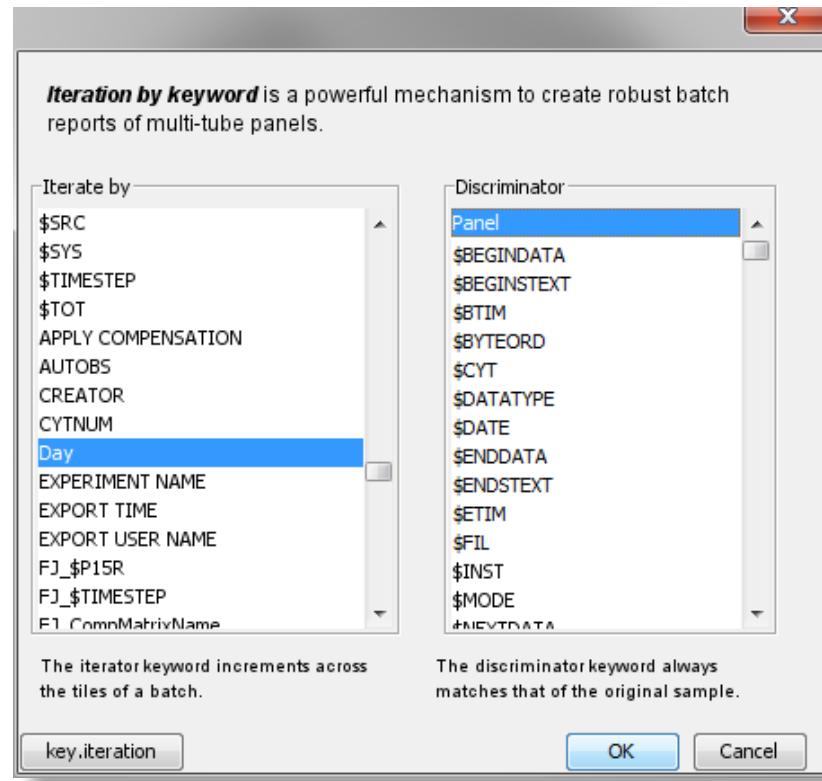
At this point we want to tell FlowJo to iterate by the keyword. From the drop-down box that currently says “Off”, select the “Keyword”. Then press the key icon and select “Day” (this we will iterate over). The Iteration by Keyword dialog box allows you to choose a value for the starting point and



L8 fig.2 - Plots with changes



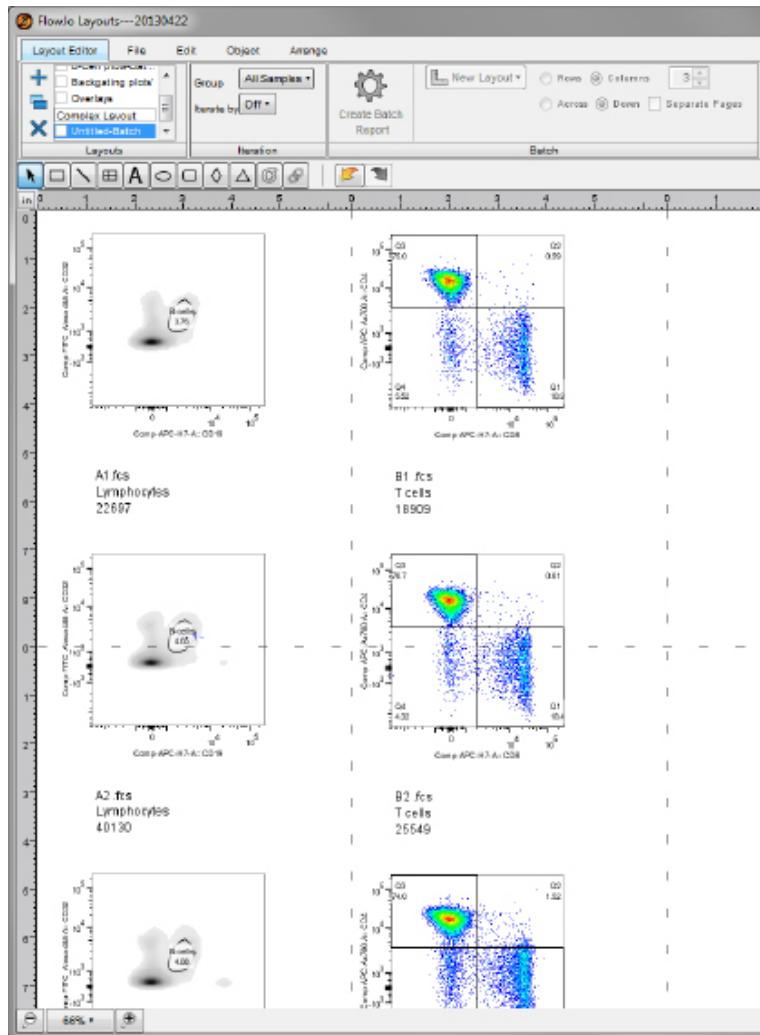
L8 fig.3 - Iteration options



L8 fig.4 - Keywords

is what

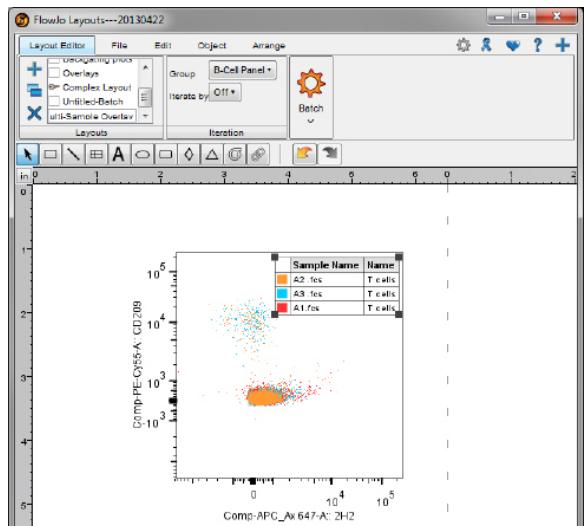
a discriminator. Choose “Panel” in the discriminator then “Okay” to close the dialog box. By selecting “Panel” as a discriminator you’ve set up the batch to only include files that have the same value for Panel as the sample you have used to make the original layout. Now from the Value drop-down tab menu, select “0” as the Day starting point. When you have made these selections, “Batch” will become enabled and the selection boxes will look like the illustration to the right (Fig.4).



L8 fig.5 - Batched plots

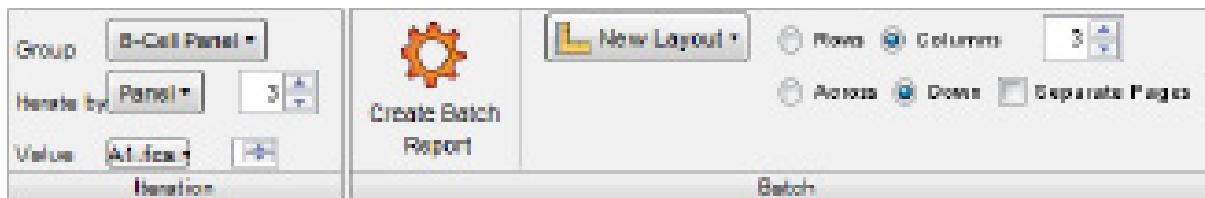
Press the “Create Batch Report” button. The batch menu will appear as usual, allowing you to select the output. Accept the default output of “New Layout”, and change columns setting to “1”. Then press “Create Batch Report”. You will produce a new layout that looks like the one below (fig 5). Notice that you have created a batch of tiles that contain different samples within them, with each tile containing samples from the same day, and the subsequent tiles iterate to the next time point, but are of the same panel as the picture directly above it.

Batching Overlays with Multiple Samples



L8 fig.6 - Overlayed dot plots

Create a new layout called “Multi-Sample Overlay”. In the workspace, select the “B-Cell Panel” group, then drag the T-cell population from sample A1 to layout editor.



L8 fig.7 - Iteration settings

Double-click on “T-cell” plot in the layout editor and create a dot plot displaying 2H2, a marker for Dengue fever and CD209, a dendritic cell marker. Then drag the same population from samples A2 and A3 on to that plot, creating the overlay image below. You will notice that FlowJo automatically changes the format of the graph to match the existing population. Drag the legend onto the plot to save space.

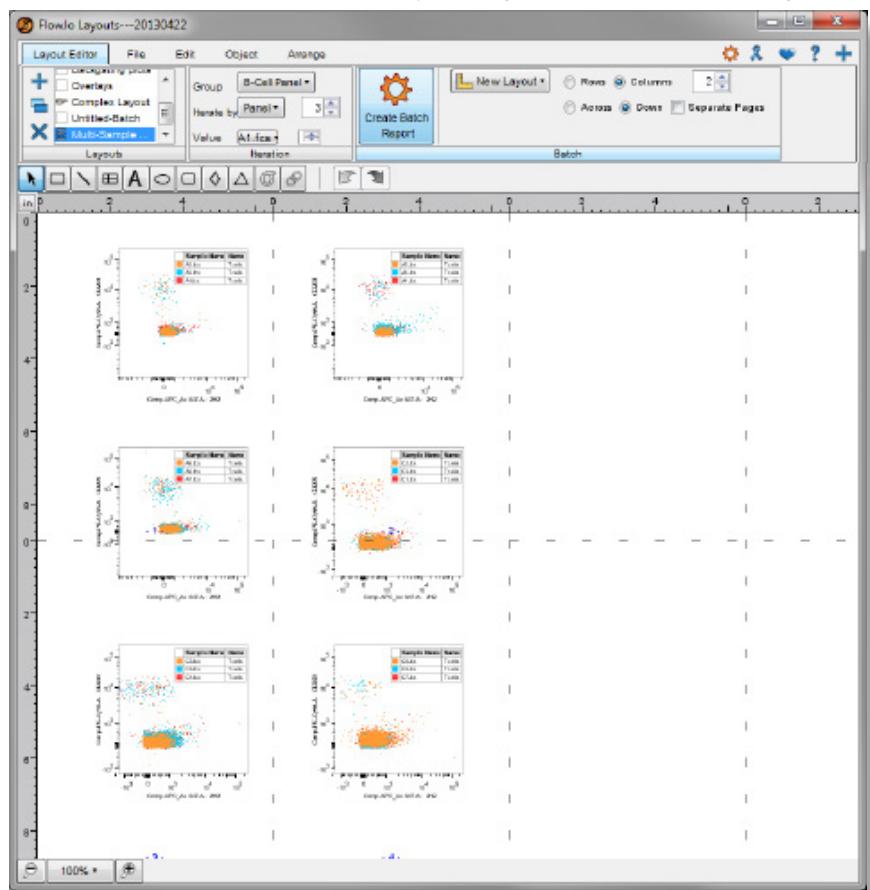
Iteration is again in the default “Off” setting. This time, select “Panel” from the iteration drop-down menu and set the iteration to every “3” samples as shown in the image to the below.

Now click “Create Batch Report”. Notice again that the iteration settings match what was selected in the workspace. Return to the Multiple Sample Overlay Layout and this time change the “3” columns to “2” columns, and press “Create batch report”. The result is six plots containing three consecutive samples, as shown below (fig 8).

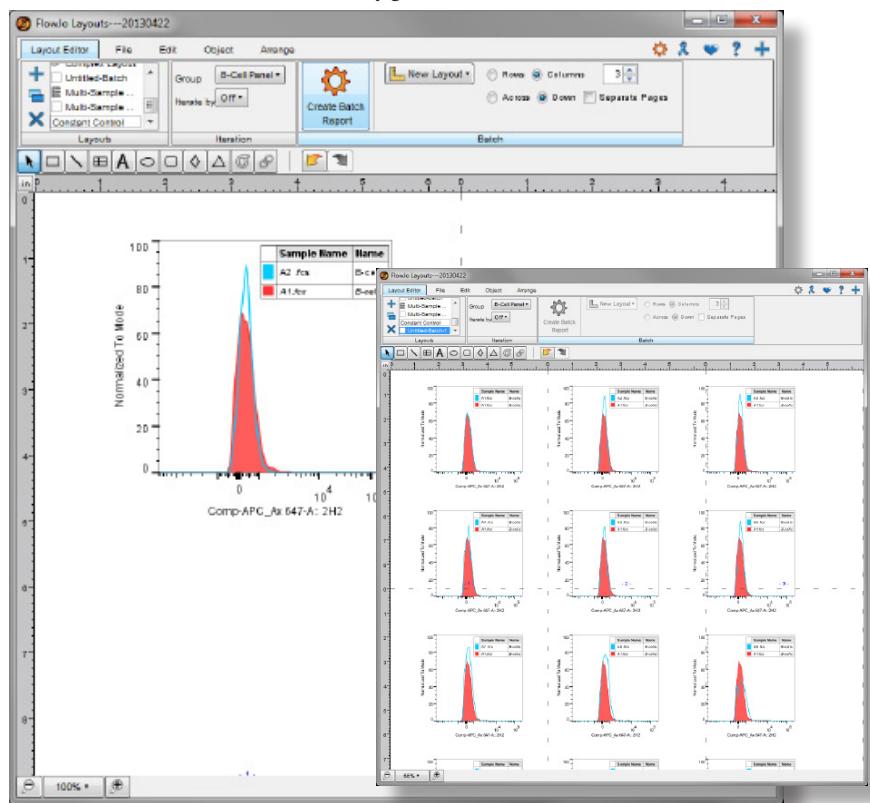
Batching While Maintaining a Control Sample

Assume sample A1 is a control of some kind that we would like to compare to the other samples one at a time. To do this, create a new layout page called “Constant Control”. Drag sample A1’s “B-cell” subpopulation into the Layout Editor and create a Histogram of 2H2. Then drag the matching population from sample A2 in and overlay it by dropping it directly on top of the existing plot. Double-click on the plot and the Graph Definition window will appear. To set a sample to remain constant through batching, click on the “Legend” tab. Double-click on the “A1.fcs...” item in the box that specifies which sample should remain locked. You will note that the text has become italicized. You can select as many samples as you wish to remain constant. At the moment we will select only this one. Now click “OK”.

Notice that in the legend of the plot, the sample that will remain fixed is now



L8 fig.8 - Iteration band



L8 fig.9/10 - Final batch

listed in italics as well. For emphasis you can use the format box of in the legend to tint the sample that will remain constant. Your figure will look like the image to the right at this point. Click “Create Batch Report” and set a three-column layout. The result will be a series of 18 plots; each sample plotted with sample A1, as shown.

In the next Chapter, you will learn some additional Layout Editor techniques, including creating Grids and placing background graphics underneath the reports.

To access the workspace at this point of the analysis, open the Lesson_8.wsp file.

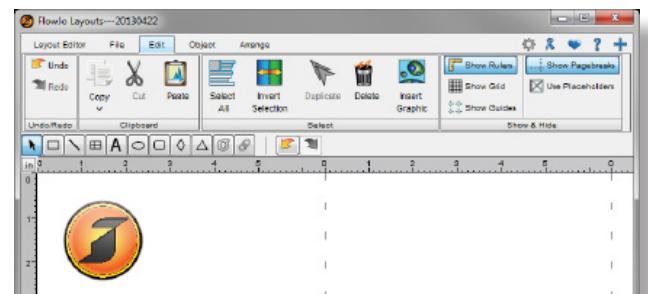
Lesson 9: Creating Finished Reports

- 1 This lesson describes the advanced features of FlowJo’s layout editor which are used in creating finished reports.
- 2 You will learn how to create live text objects containing sample-specific information and statistics, to put in a backdrop containing (for example) logos or specialized forms, and to manipulate Layout Grids – specialized tabular elements that can contain text, tables, graphs, or any other items.
- 3
- 4
- 5

This lesson builds on the workspace you finished in Lesson 8; alternatively, you can open the workspace named Lesson_8.wsp.

The report that we will generate includes five separate elements:

- an image to use either as a background or a header
- a text box containing information regarding the sample
- a series of graphs demonstrating the gating strategy to achieve a target population
- a grid containing the target population for each of nine samples
- a table displaying the statistics for all

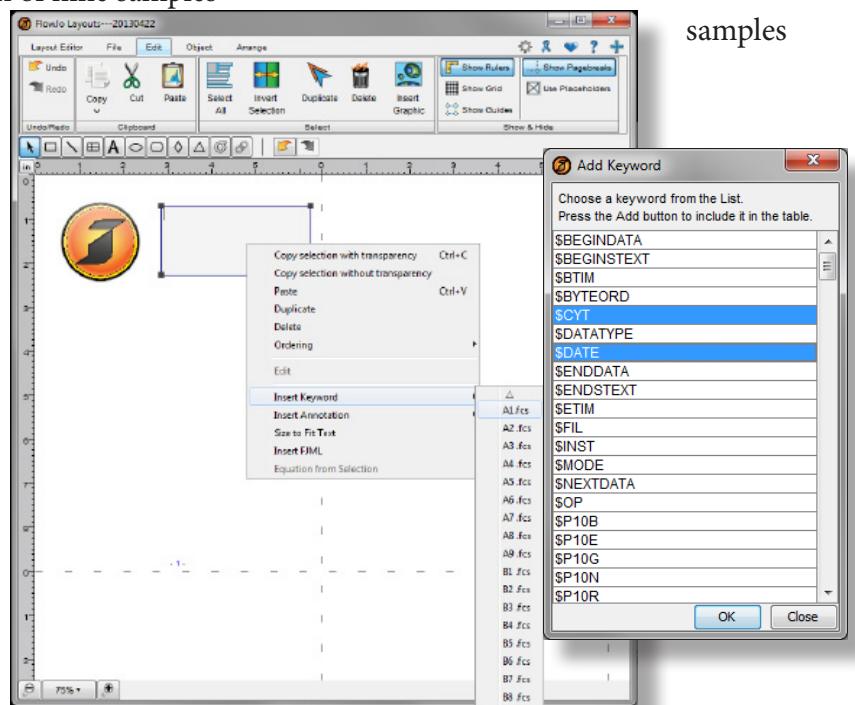


L9 Fig.1 - logo in layout editor

Importing a Graphic

T

Open the layout editor, and create a New Layout named “Final Report.” From the Edit Menu, select “Insert Graphic” and choose an image file of the logo of the institution where you work. We will include a FlowJo icon in the top left corner. Alternatively, you could select a larger image; stretch it to cover the entire layout editor and drop plots and text boxes onto it, thus making the image a background.



L9 Fig.2/3- text box options

samples

Adding Text

Click on the Text Box tool then click-and-drag a rectangular area that will fit to the right of the image that you inserted. click in the text box to edit. Right-clicking on the text box opens a drop-down menu with several options. Select

D o u b l e -

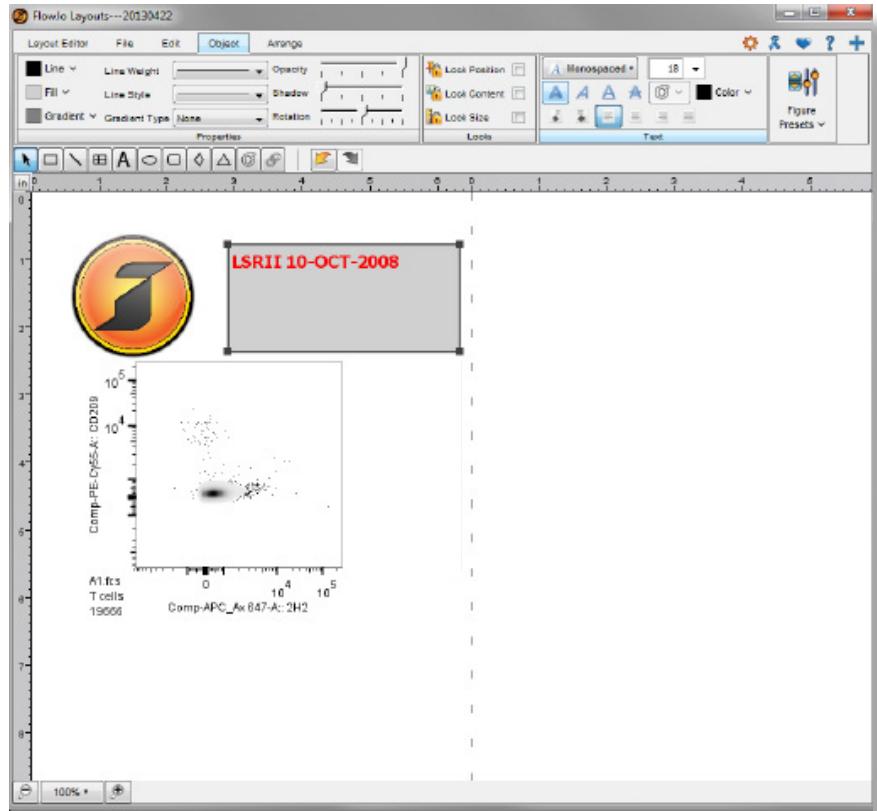
“Insert Keyword”. Here you will select several keywords that contain sample-specific information. To insert a keyword value, click on sample A1.fcs then select “\$DATE” (date of sample collection) from the Add Keyword dialog box. Depress the “Ctrl” button on the keyboard (“Command” on Mac) and select “\$CYT” (cytometer used for collection) as well. Click “OK”. In your own datasets, you may have other keywords pertinent to your research which you can add using these rich text elements.

If you double-click on either keyword in the text box, you will note that the keyword command is bracketed in green “[...]. Do not make edits within these brackets. However, you may add text between sets of brackets. For example, you may add explanatory text (and hit return to create line breaks) to format the text box, shown in the following image. From the Object tab, set the Fill Color (background color) to gray, the text color (called simply “Color”) to red, and the Style to bold. When you are finished, click “OK,” and magnify the view to 100%. The layout editor should look similar to the figure below right (fig 4).

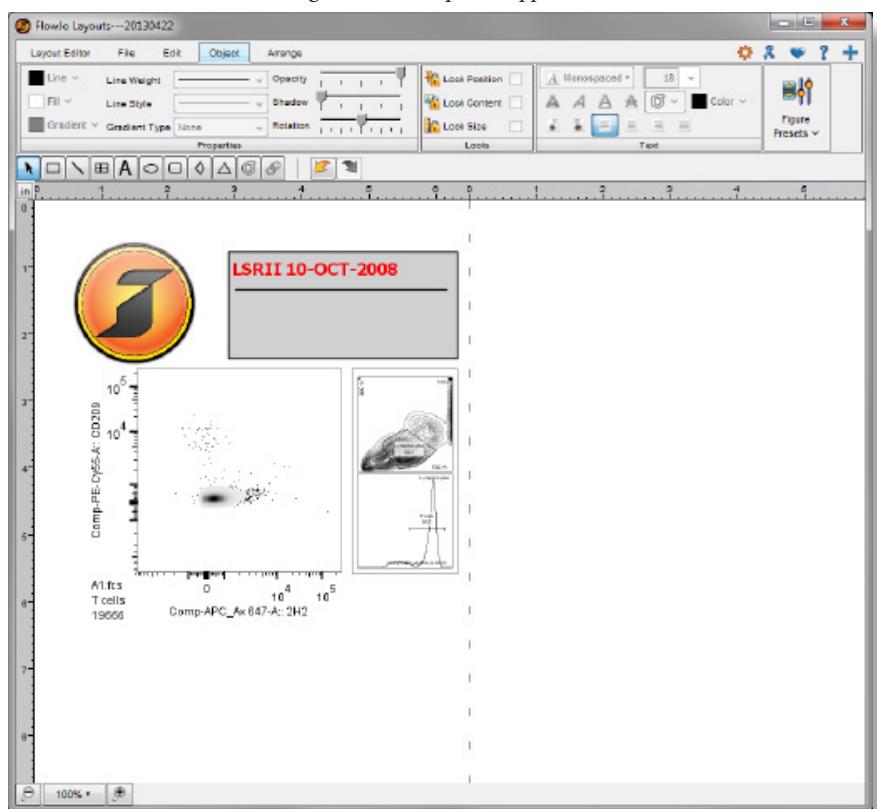
This rich text and formatting allows you to highlight your results and add sample annotation alongside graphical objects. If you wished to have a particular keyword value in a different font or color, you would have to make a separate text box for it and format it accordingly. You can further demarcate the text from the rest of the layout by drawing a line using the Line icon, also in the tools of the layout editor. Double-click on the line itself to make edits for color or thickness in the object tab.

Adding Plots

The next task is to add plots to your layout, showing the gating strategy used for each antibody panel. This is similar to the layouts created in previous

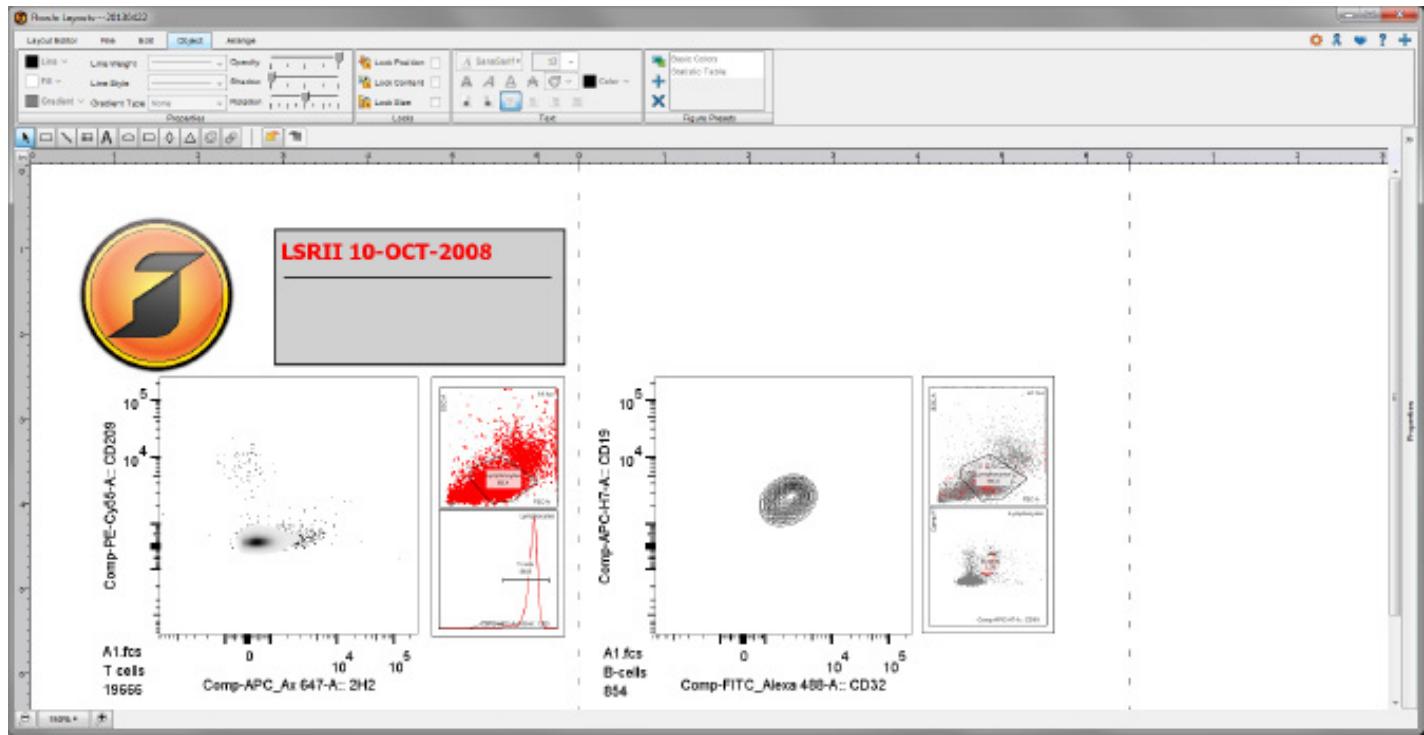


L.9 Fig.4- Text box options applied



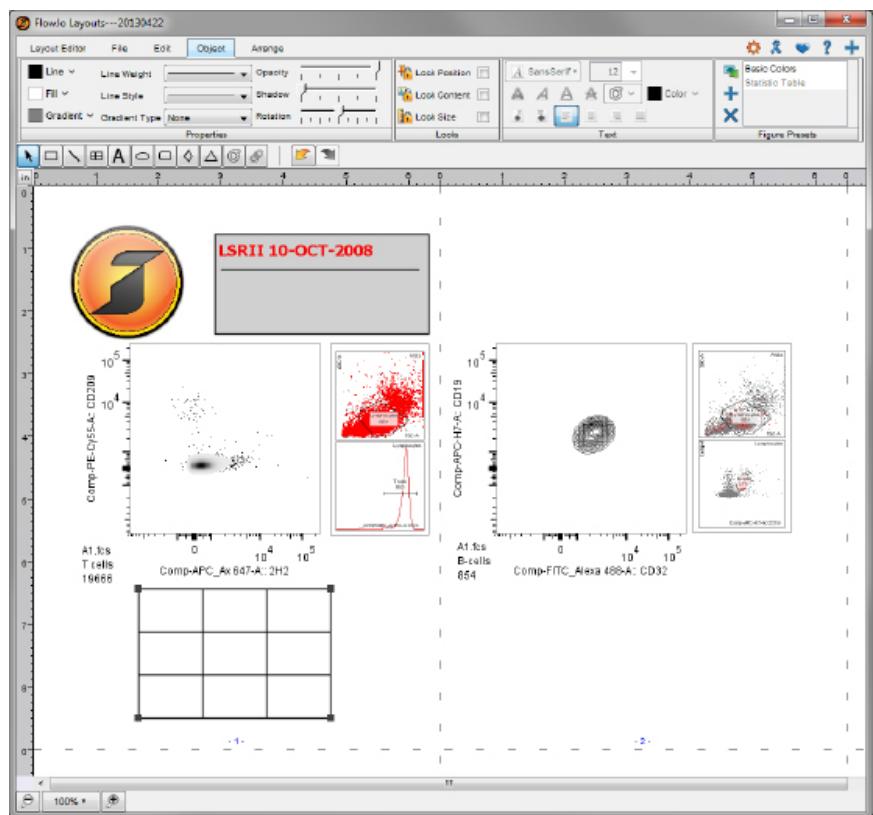
L9 fig.5- Annotated plots

chapters. First, drag the “T-cell” subpopulation from sample A1 and drop it below the graphic inserted previously. Double-click on the plot to open the graph definition window and change its attributes to a density plot of CD209 versus 2H2, with outliers displayed. Your graph will look like the plot to the right. Click “Apply”. To display the gating strategy, click on the “Annotate” tab in the Graph Definition window and click the “Ancestry” check-box. Click “OK”. You will see two smaller plots appear to the right of the original showing the gates used to get to the target subpopulation.



L9 fig.6 - Annotated plots with B-cells

Next, drag in the “B-Cell” subpopulation from the same sample (A1) and place it next to the “T-Cell” plot. Double-click on this plot and change it to a Contour plot of CD32 versus CD19 and click the check-box for “Backgating.” Click “OK.” In the layout editor two plots will appear again, as shown below. All events will be colored grey with any cells that would be included in the downstream gate (regardless of whether they were gated out in an earlier step) are back propagated onto the preceding



L9 fig.7 - Grid tool

plot and colored red. This is a tool to assess the effect of a gating strategy, as gates may restrict what you analyze in a subpopulation.

Using the Grid Tool

Now we will add a grid to your layout. Choose the grid tool from the toolbar above your layout in the Layout Editor. Click, drag, and release within the layout editor to draw a grid. Right-click, choose cell dimensions to set the number of rows and columns to 3 rows and 3 columns. You can now drag populations directly into the grid box and they will snap to the size of the box. You can also drag text boxes or statistics in to create tables or annotation.



Create a text box, and type in it a sentence or two stating that the grid will hold the B-cell population for samples A1 – A9. Then, drag and drop that text box into the upper left grid box. Next, drag and drop the B-cell population from each of those files into the grid. A quick way to do this is to select the “B-cell” population in the workspace for sample A1 from the “B-Cell Panel” group, then open the “Edit” menu and click on “Select Equivalent Nodes”. You will notice that this will highlight all of the B-cell populations. Click

Col...	Population	Statistic
1 Σ	Lymphocytes	Lymphocytes
2 Σ	Lymphocytes/B-cells	B-cells
3 Σ	Lymphocytes/T cells	T cells
4 Σ	Lymphocytes/T cells	Freq. of Total
5 Σ	Lymphocytes/T cells	Median : Comp-APC-Ax700-A
6 fx	Formula	

on a “B-Cell” population and drag-and-drop it into the upper right grid box. The selected populations will fill out the rest of the

L9 fig.8/9 - Final layout with table

grid boxes.

Adding a Table from the Table Editor to the Layout Editor

Finally, we will add a table of statistics. We can reopen the Table Editor by clicking on the table editor shortcut button from the workspace. Then choose the “B-Cell Table” and select the batch to current layout icon. The table will appear in the currently open layout page, which is the Final Report page. You can scale the table by grabbing a corner and dragging it larger or smaller. Your layout should now look like the following figure.

*You may need to adjust the paper size in the print dialogue box to allow for your table to fit your layout.

From here you can print a report, publish to the web, or generate a movie or pdf. These options are explained at the end of lesson 6.

To access the workspace at this point of the analysis, open the Lesson_9.wsp file.

Lesson 10: Compensation

Lesson 10 does not build on the previous lessons. Rather it is an independent tutorial on the compensation tools within FlowJo, using the data set that you have become familiar with through the first nine lessons.

This tutorial also does not serve as a comprehensive guide to what compensation is and why we do it. There are many good references that deal exclusively with the subject. A tutorial that we recommend is:

M. Roederer. Compensation: An informal perspective. May 2000. Web 18 April 2013. www.drmr.com/compensation

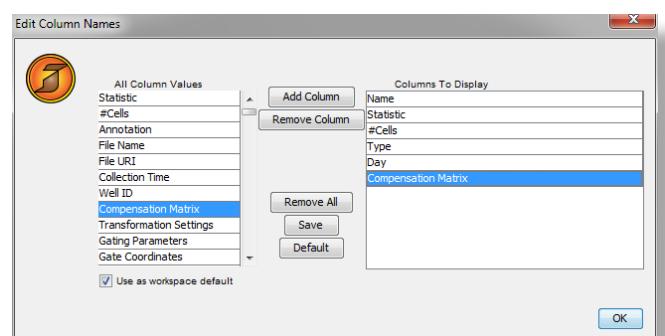
For the purpose of this tutorial it will suffice to explain that when more than one fluorescent probe is used in a cytometric experiment, the emitted fluorescence spectral outputs overlap, adding a “noise” component to the measured intensity of all parameters. The contribution of the noise component can be determined by collecting tubes containing cells stained with a single fluorescent color. These single stain controls allow us to measure the intensity produced across all colors that is due to the single color used in that control tube. All cells produce some amount of autofluorescence so we do not assume that median fluorescent intensity (MFI) for a single stain control in channels other than its collection channel should be zero. Rather, we assume that the MFI of these parameters should be equal to that of the background.

Compensation is the process of correcting the spectral overlap that occurs during multicolor flow cytometry. This is done by quantifying each dye with which a particular cell is labeled, and subtracting the amount of intensity that adjusts MFI of single stained cells for all parameters they were not stained with to the background MFI.

Compensation is applied in one of three ways:

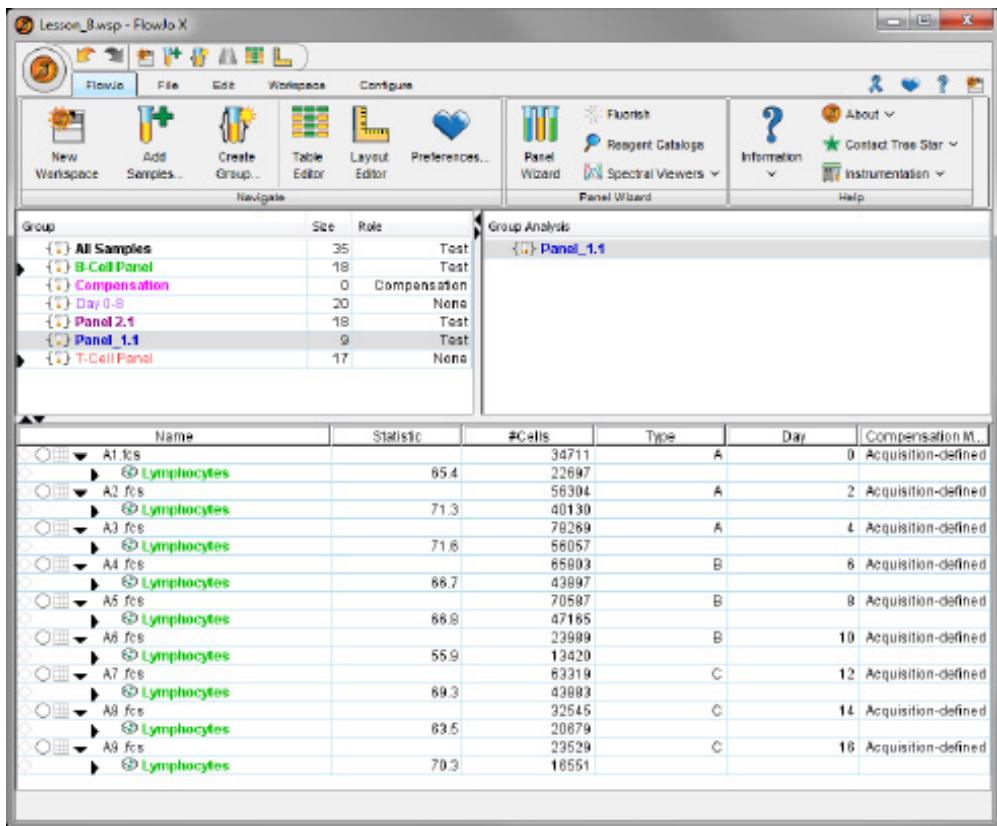
1. Using an algorithm on the acquisition software before FCS files exported and loaded into FlowJo
2. Post-acquisition in FlowJo using an algorithm
3. Manually on the cytometer

In the last case, the record of the compensation performed, the matrix created, and the raw (non-compensated)

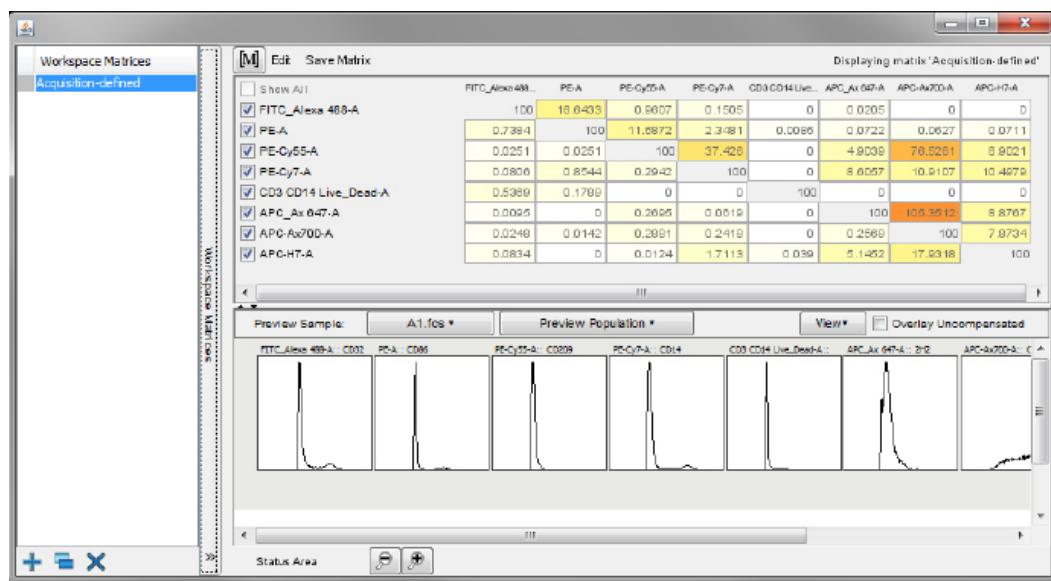


L10 fig.1 - Add comp matrix column

data are not stored in the FCS file. (Most software compensation algorithms will produce very similar results,



making algorithmic compensation more robust than manual.) Therefore, you will not be able to edit the matrix or examine uncompensated parameters, though



L10 fig.3 - Matrix editor

the data can be analyzed. In this tutorial, we will thus focus on the first two cases.

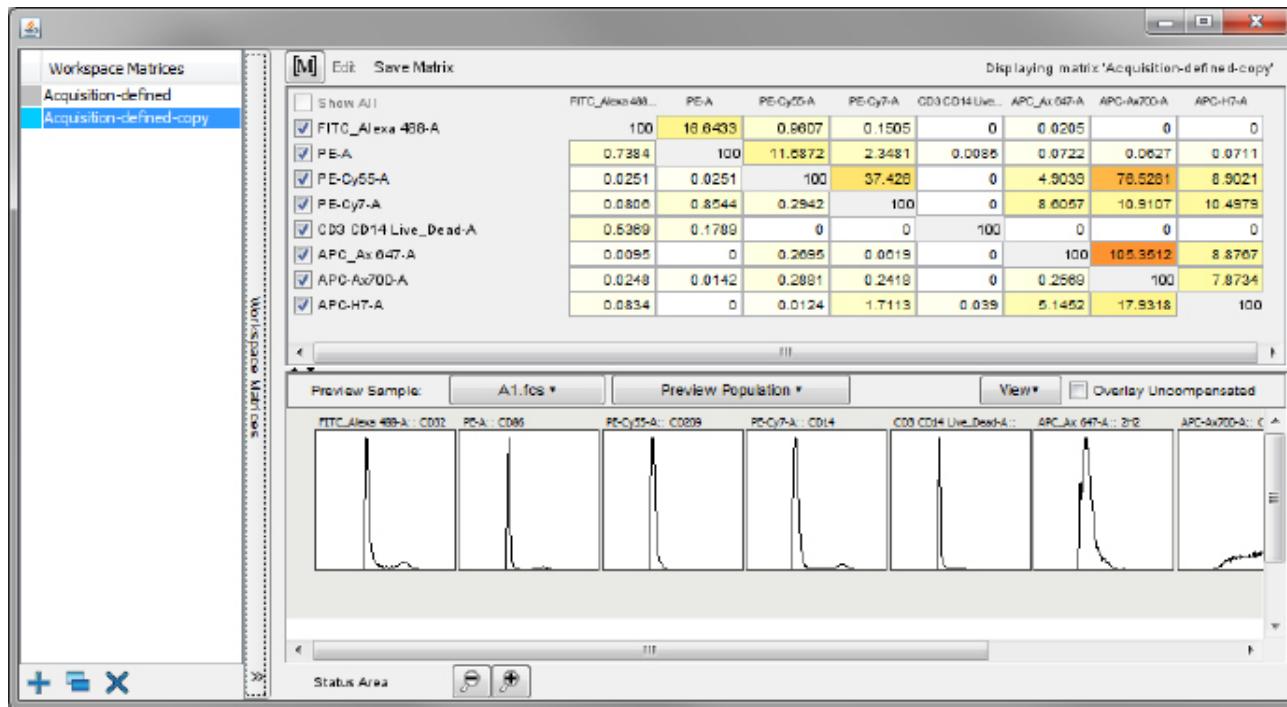
Data Compensated by the Acquisition Software

Load in the folder Exp. 1, Panel_1.1. The grid displayed next to each of these samples indicates that there is a compensation matrix associated with these samples. There are two ways to see which matrix is associated with which sample. First, go to the workspace and right-click on a column header. Click "Edit Columns" and select the

keyword “Compensation Matrix”, then “Add Column”. Click “OK”. (fig 1)

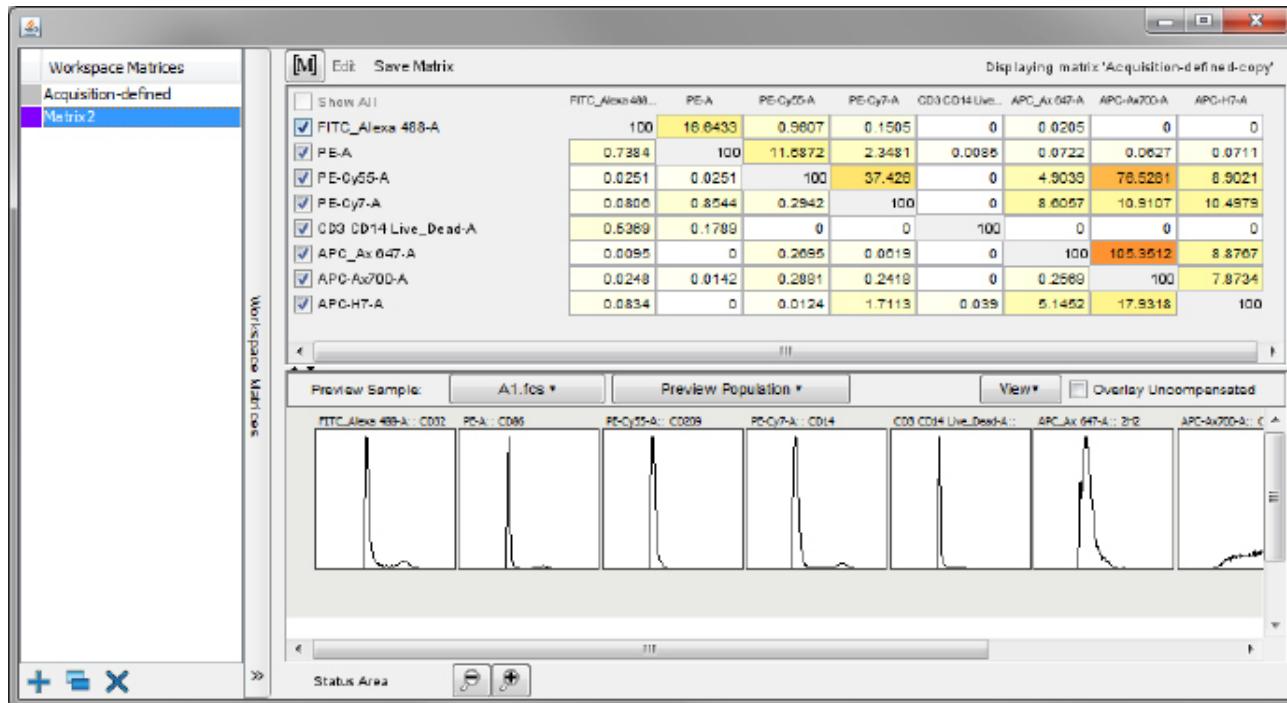
The workspace will look like the figure below, showing that all files were compensated with the Acquisition-defined matrix.

Alternatively, you can double-click the grid to open the Matrix Editor. Click on the matrix next to sample A1.



L10 fig.4 - New matrix

The box on the top shows the compensation matrix (fig 3). If you look at a box in the grid, denoting the intersection of two parameters, the grid can be read as “X.XX %” of the measured intensity of the row parameter is being subtracted



L10 fig.5 - Matrix renamed

from the column parameter. So in the figure above, for example, 0.7384% of the PE-A intensity is being subtracted from the FITC-Alexa intensity. The bottom panel shows Histograms of compensated data for the selected population in the selected sample, and allows one to compare this to uncompensated data. The left panel shows the matrices that are available in the current workspace and allows you to edit their color formatting and name.

Editing an Existing Matrix

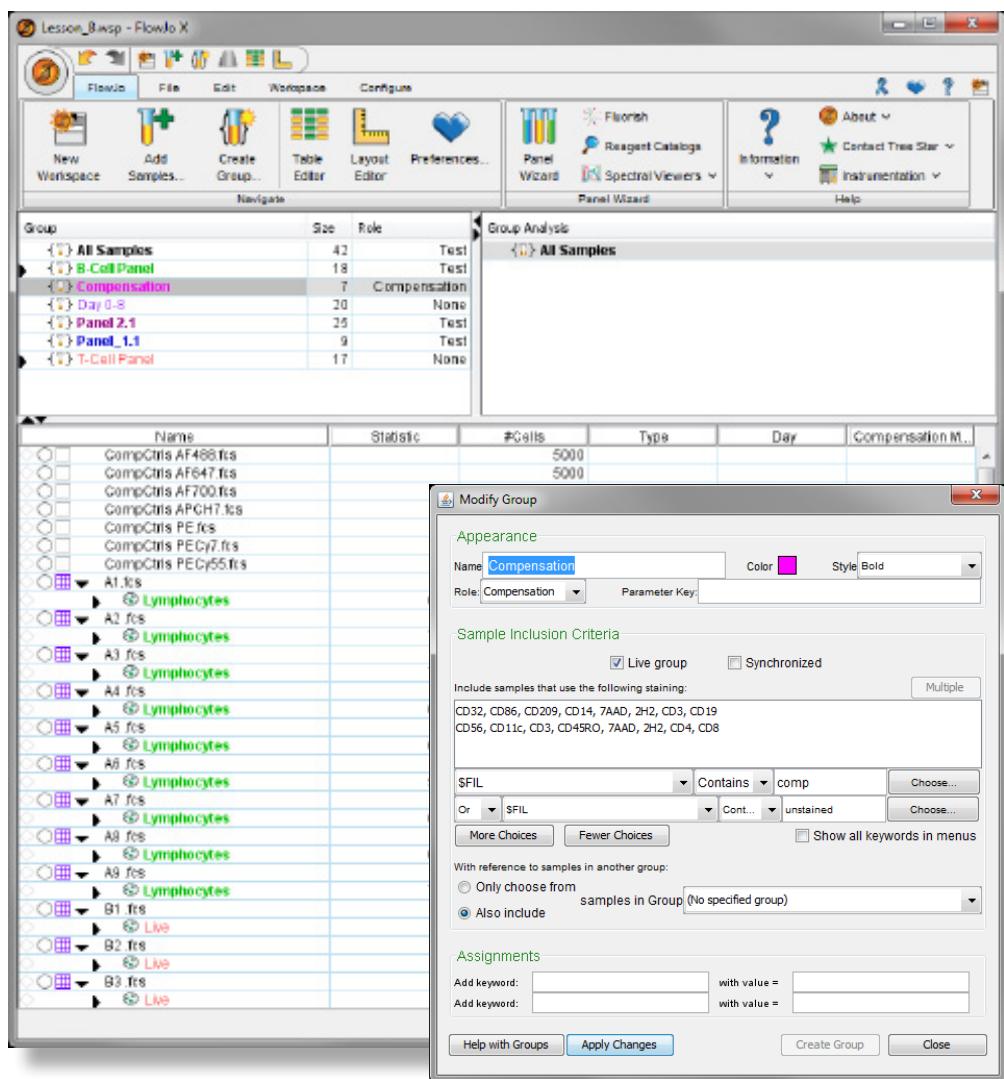
FlowJo will not allow you to modify your raw data files, nor the compensation matrix. However, if you would like to modify your compensation matrix in this FlowJo workspace, click “Edit” at the top of the matrix, a confirmation window will pop up, click ok and then you can click in any cell of the matrix to edit it, type in the new number, and then press the “Enter” key. By default, editing a matrix creates a new matrix, visible in the matrix navigator (fig 4).

Rename the edited matrix from “Acquisition-defined-copy” to “Matrix2” by clicking on the text in the left panel and choose purple for its color formatting (fig 5).

By default, you will see that this matrix has been applied to the Panel_1.1 group. To change the compensation matrix that is applied to a sample or a group, simply drag-and drop the [M] icon onto either a sample in the bottom part of the workspace or a group in the group analysis pane, respectively. Finally, right-clicking on the matrix badge in the workspace allows you to edit the matrix, to re-apply the acquisition matrix, or re-apply the acquisition matrix to the group.

A word of warning - we discourage random matrix editing. The algorithmically calculated values are the correct values for the compensation controls that were presented. However, it is possible that inappropriate controls were used and correction is needed. The best way to accomplish this is to find a population “positive” for a single parameter and gate on this positive population. Then modify the compensation matrix and check that there is a nearly equal MFI of background

and MFI of the parameters for which



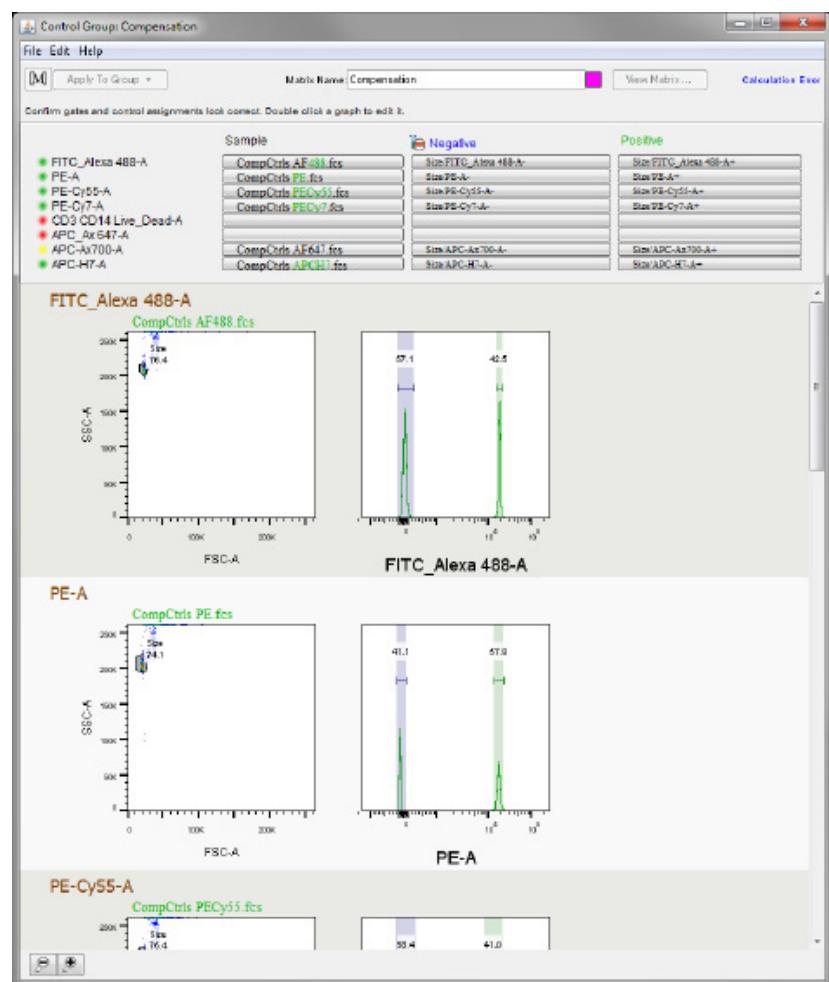
L10 fig.6/7 - Group definition for matrix editor

you expect the population to be negative.

Creating a New Matrix

If you have single stain control sample data, FlowJo has the ability to calculate a new compensation matrix, even if the data files were compensated previously by the acquisition software. In order to create a new matrix in FlowJo with single stain controls, load the compensation controls folder into the workspace. Once loaded the workspace will look like the figure below.

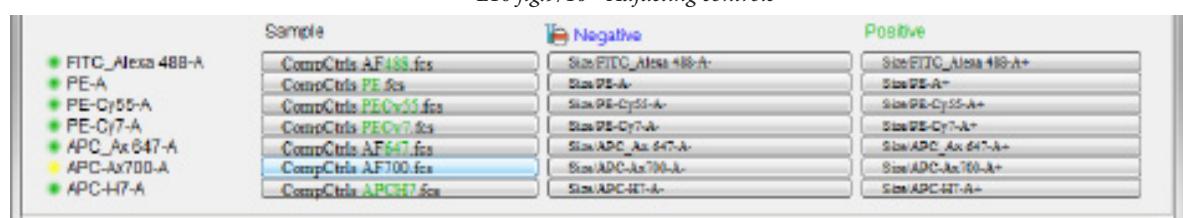
You will note that the pink Compensation group has populated with 7 single stain sample files - there is a single stain control for every color except 7-AAD, which this experimenter chose not to compensate. These samples have been automatically loaded into the compensation group, based on its group definition (fig 6/7). To examine this group definition, double-click on the compensation group, and you will note that this group has been programmed to look for file names (\$FIL) that contain the text "comp" or "unstained."



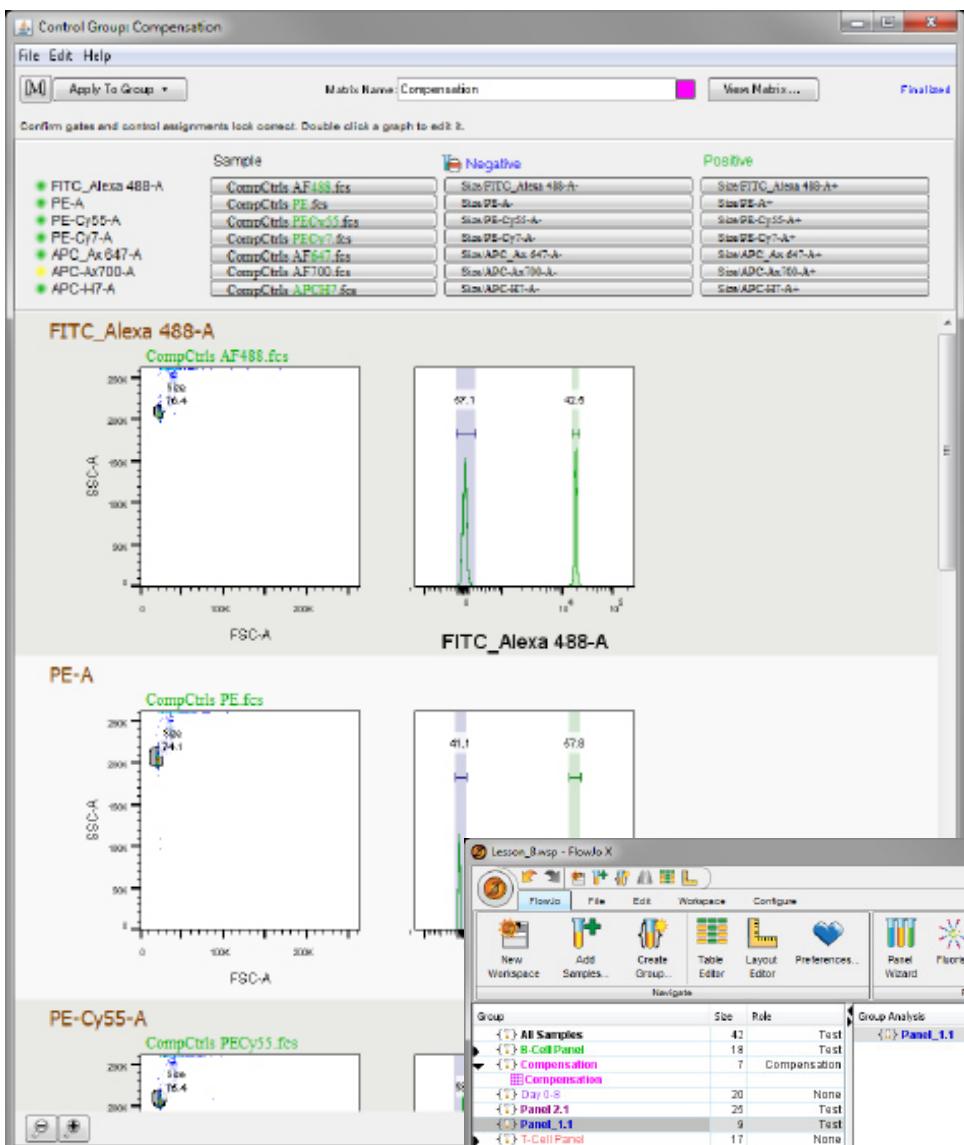
L10 fig.8 - Single stain controls in compensation editor



L10 fig.9/10 - Adjusting controls



L10 fig.11 - Adjusting controls



L10 fig.12 - Compensation control

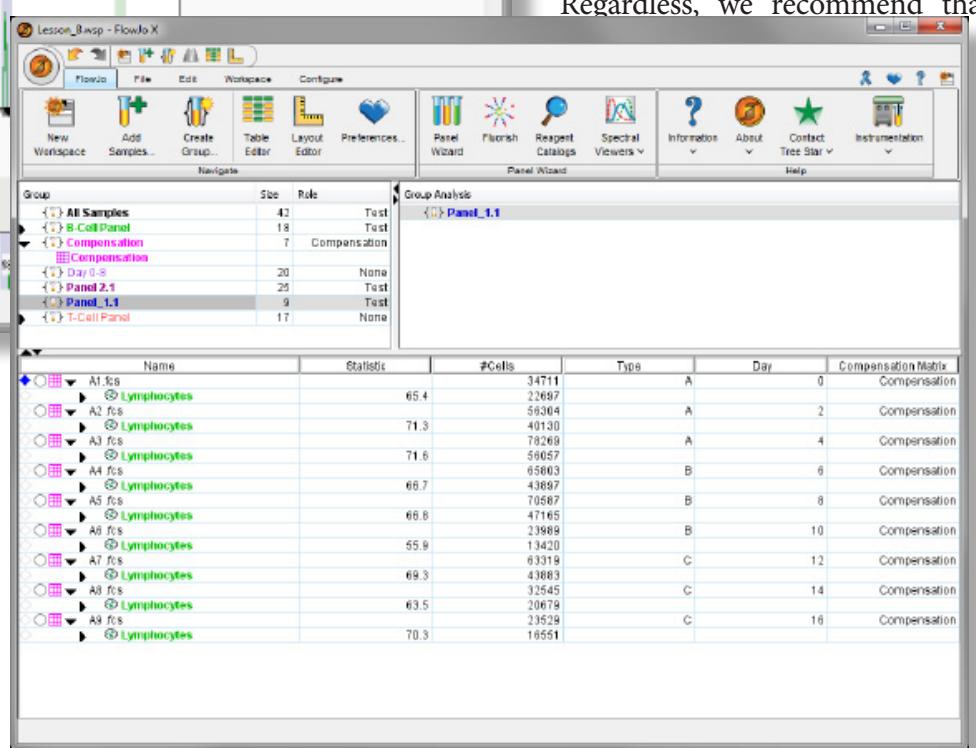
you check the file names against the parameters to make sure FlowJo has done the assignment properly. In this case, three

Close this window, and we will create a new matrix using the Compensation Editor.

With the compensation group selected, the compensation editor icon will become live - press this to open the editor.

FlowJo will automatically attempt to match the controls samples to the proper parameter using peak finding - that is, FlowJo scans the data and attempts to identify which tube has a “positive” (*fluorescence above background intensity*) population for a parameter. Since these are single stain controls, there should only be one parameter per file with a positive peak. (fig 8)

Regardless, we recommend that



L10 fig.13 - Compensation complete

adjustments must be made so that the matrix calculation can be finalized:

Since no compensation control was acquired for 7AAD, click on the sample drop-down menu next to 7AAD and select “Remove This Parameter”. For APC_Ax 647-A (2H2), select the proper single stain control using the sample drop-down menu to “CompCtrls AF647.fcs”. (fig 9/10)

Correct the sample selection for APC_Ax700-A (CD4) to “CompCtrls AF700.fcs”. FlowJo will automatically set gates for positive and negative populations on this sample. (fig 11)

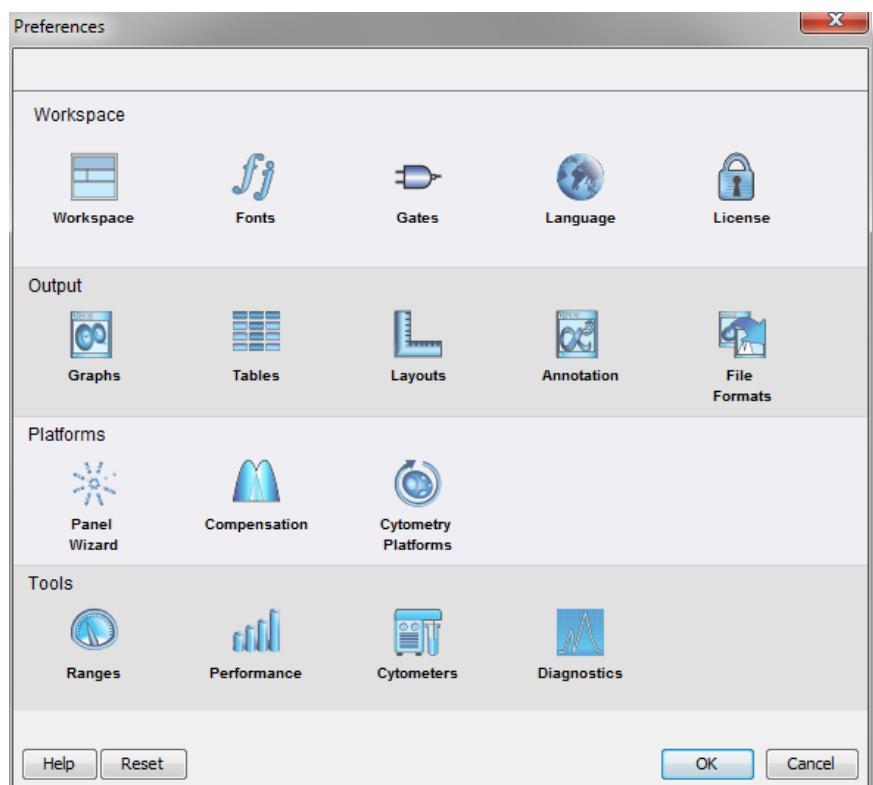
The compensation editor should now look like the screenshot below (fig 12):

A compensation matrix has now been calculated, and the status in the upper right-hand corner of the compensation editor should now read “Finalized.” In order to apply this matrix, you may either drag and drop the [M] button onto a sample or group, or alternatively, use the “Apply to Group” drop-down menu at the top left-hand corner of the compensation editor. Go ahead and apply this new matrix to the Panel_1.1 group, and you will observe that the compensation badges next to the samples in this group indicate that the FlowJo-calculated matrix has been applied to these samples (compare the matrix color under the Compensation group with the badges next to the samples). (fig 13)

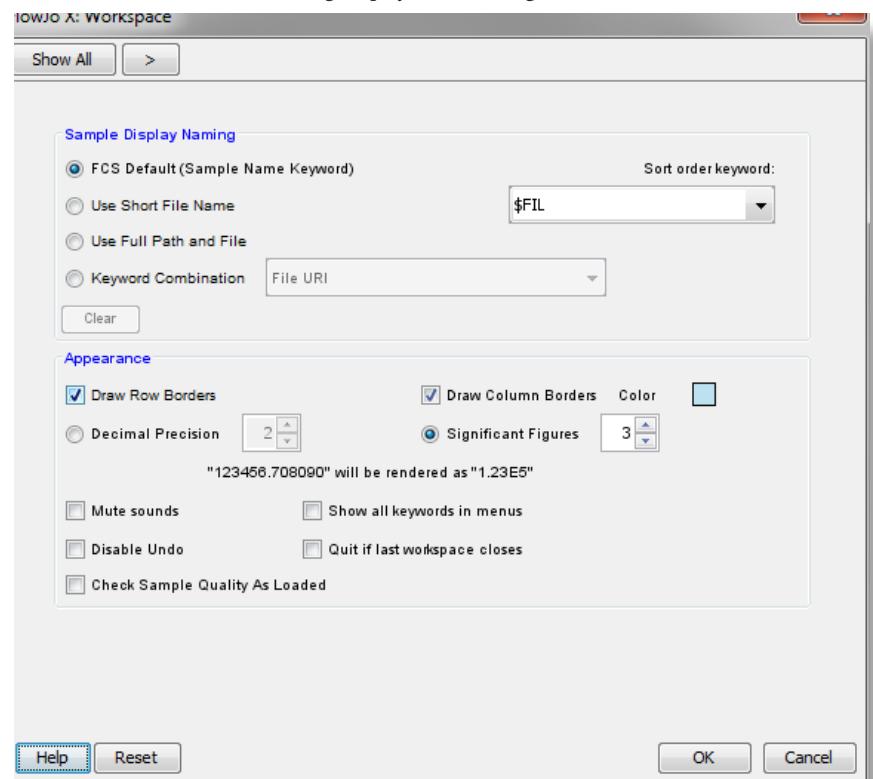
This concludes the compensation tutorial. This work is saved as Lesson_10.wsp.

Lesson 11: Setting the Preferences

Lesson 10 is an easy finish; there is no work to do here. This chapter is simply

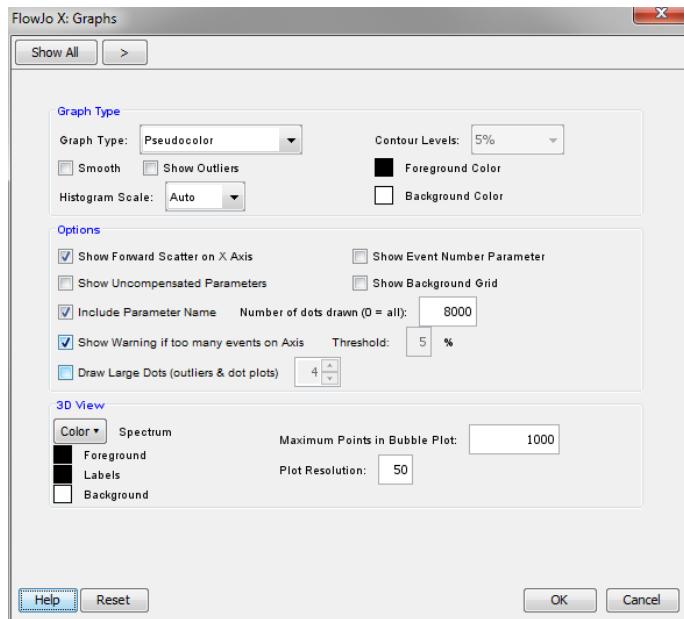


L11 Fig.1 - preferences dialogue



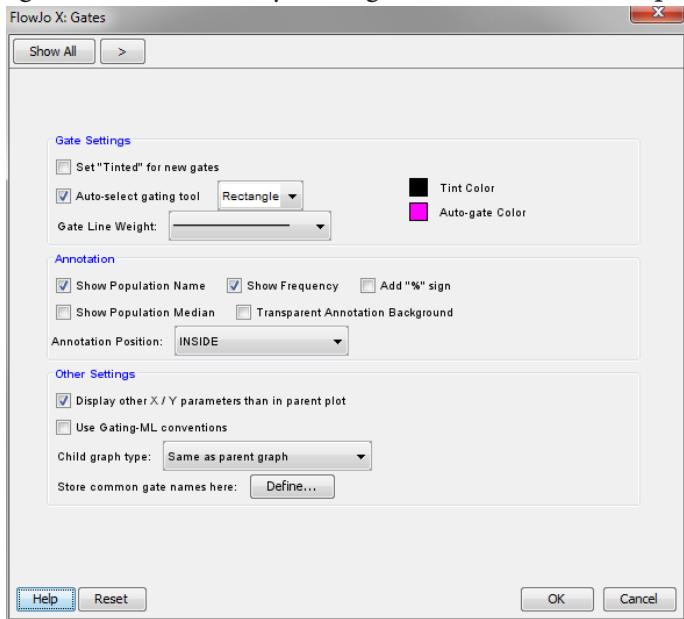
L11 Fig.2 - workspace preference

aimed at highlighting some of the more commonly used preference settings in FlowJo to help you start customizing the software to your needs and to emphasize that most aspects of FlowJo are customizable.



L11 Fig.3 - graph preferences

Preferences are the customizable set of defaults that FlowJo uses throughout the program. If you find yourself repeating an action over and over again, consider that you might be able to set the program to do what you like by default. The preferences are accessed through the Edit menu. When you click on the preferences icon, the interface shown to the right will be displayed. (fig 1)

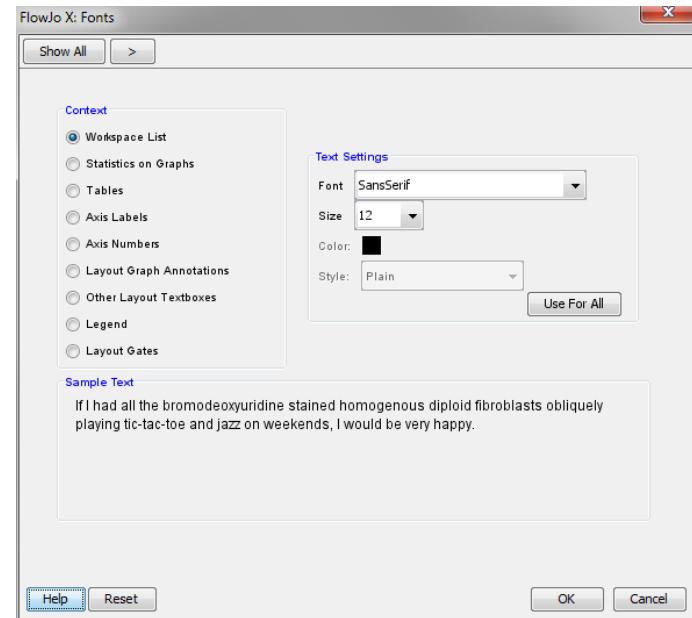


L11 Fig.5 - gate preferences

FlowJo.

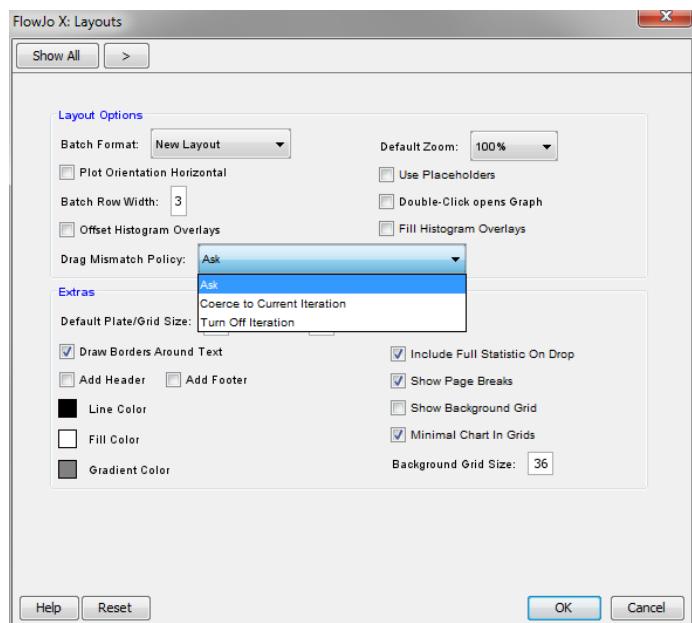
The workspace menu (fig 2) allows you to control the workspace appearance. There are controls for borders, decimal precision, file sort order, and of special interest, the choice of which keyword is displayed under the "Name" column in the workspace. That

What are preferences?



L11 Fig.4 - font preferences

Each icon controls the settings for the listed aspect of

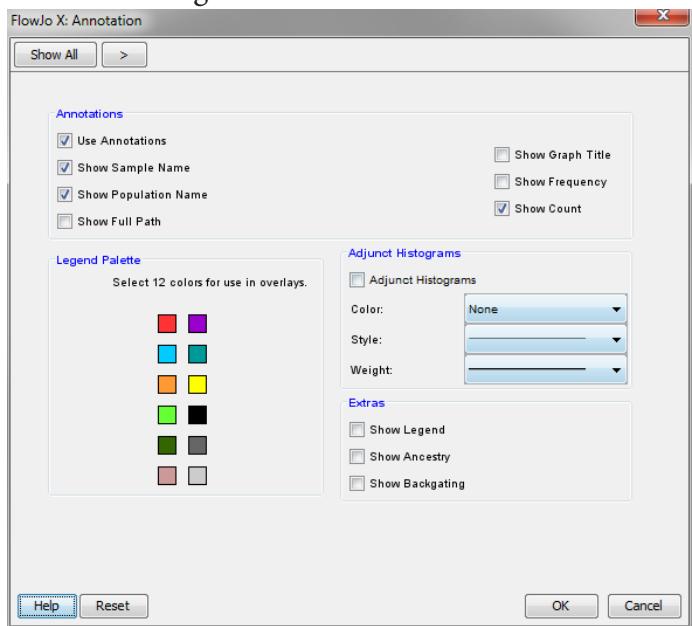


L11 Fig.6 - layout preferences

interface is shown below.

FlowJo does not allow you to directly double-click and rename a file in the workspace, but from this interface you can control what appears by selecting the keyword or combination of keywords that contains the information for identification of each file. If there are no existing keywords that display the information you need, you can create your own keyword and then choose it from this menu.

The Graphs menu (*fig 3*) allows you to control the default appearance of graphs. The type of graph and its options can be set using the interface below.



L11 fig.7 - Preferences annotation

The Gates menu allows you to control the appearance of gates, the annotations that appear on screen when a new gate is created, and what to display when you double-click on a gated population. The entire menu is shown in the figure below at left (*fig 5*).

The Layouts menu (*fig 6*) allows you to control what happens in many aspects of the Layout Editor. One of the most important preference in this menu is the “Drag Mismatch Policy” drop-down menu settings.

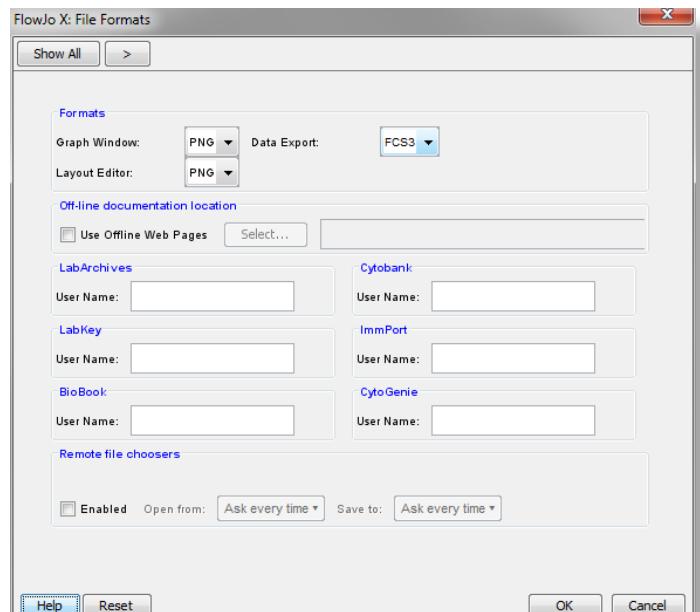
Drag mismatch tells FlowJo what to do when you drag plots from two different samples into the layout editor. “Turn Off Iteration” will allow you to place any plot into the layout. Batching will then become disabled, as FlowJo will not know what sample to iterate. Therefore if you want to batch, you will have to specify a keyword by which to iterate and discriminate (see Lesson 8 for details).

“Coerce to Current Iteration” forces all of the plots to come from one sample, so FlowJo will change what you drag in. “Reset Iteration Value” will change the iteration value to match the selected samples, if this is possible. “Ask” will cause FlowJo to bring up a pop up window asking you to specify what you would like done every time you drag different samples into one layout.

The Annotation menu allows you to specify which keywords are automatically displayed when a sample is dragged into the layout editor. It also allows you to define the color order for overlays and define what special features appear on plots by default. The main portion of this menu is shown below left (*fig 7*).

The File Formats menu (*fig 8*) allows you to specify what the default file output will be for exporting from the graph

The Fonts menu (*fig 4*) allows you to set the font characteristics for nine different places in FlowJo, or to unify them.



L11 fig.8 - Preferences file types