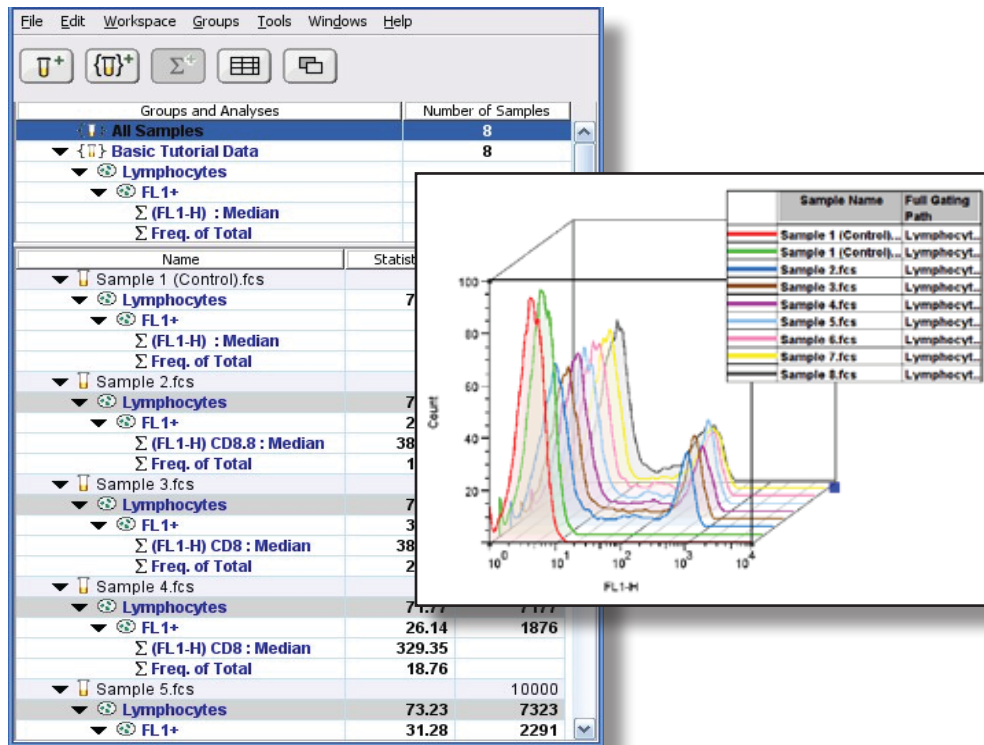


FlowJo

Basic Tutorial



FlowJo is software for the analysis of flow cytometry data. This tutorial will quickly introduce you to FlowJo using an example experiment, where a FITC anti-CD8 reagent is titrated to determine the most economical concentration for staining in future experiments.

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FlowJo is a product of Tree Star, Inc.

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FlowJo was written by Adam Treister and Mario Roederer 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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• FlowJo Basic Tutorial •
July 2009 - V.7.5
• © MMIX •

Basic Tutorial

Introduction

FlowJo software is used for the analysis of flow cytometry data. You can use FlowJo to analyze all of your flow cytometry data - regardless of the cytometer used to collect your data files. This tutorial will introduce you to FlowJo and to the 6 steps involved in analyzing a basic immunophenotyping experiment.

1. Load data into the Workspace.
2. Analyze the control tube data in detail (gate subsets and add statistics).
3. Copy gates and statistics to **All Samples**.
4. Verify gates on **All Samples**.
5. Generate a graphical report including **All Samples**.
6. Generate a table of statistics from **All Samples**.

Instructions to guide you through the analysis of a basic immunophenotyping experiment are contained in this tutorial. Follow along with the provided data files to get a feel for how FlowJo works. If you wish to skip steps, we provide pre-made Workspace files that correspond to each analysis step. The demonstration data and Workspace files can be found in the **Basic Tutorial** folder on the FlowJo CD or at: www.flowjo.com/home/tutorial.html.

Experimental Goal

The data files analyzed in this tutorial come from an experiment for titrating a newly purchased antibody reagent. This reagent consists of anti-CD8 antibody conjugated to the fluorochrome Fluorescein (FITC.) The goal of this titration experiment is to determine the concentration of the anti-CD8-FITC reagent that achieves saturating staining; i.e., maximal staining of the CD8 T cells under defined conditions (20 minutes at room temperature).

The data analysis in this experiment consists of identifying the subpopulation of cells that are stained with anti-CD8 antibody and calculating a median fluorescence intensity (MFI) statistic on this subpopulation. The computed statistics will be used to determine the most economical concentration of anti-CD8-FITC reagent to use in subsequent experiments.

*The concentration of reagents recommended by the manufacturer can be significantly in excess of the saturating concentration required for your experimental conditions.
A titration experiment like this one can conserve your reagents and save money.*

Experimental Method

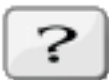
Human peripheral blood mononuclear cell preparation from a single healthy donor was divided into eight tubes. The first tube is the unstained control. The subsequent seven tubes were stained with a serial two-fold dilution of the anti-CD8-FITC reagent starting at 2 µg/ml. Cells were stained for 20 minutes, washed and resuspended in staining medium. The data files were acquired from the cytometer, with 10,000 events collected for each tube. This data can be found on the FlowJo CD in:

Sample Data Files\Basic Tutorial Data_Workspaces\Basic Tutorial Data.

You can download it from: www.flowjo.com/home/tutorial.html

In this tutorial we focus on a single color, homogenous data set, which does not need to be segmented into groups for analysis. For a more detailed example that covers more markers, and where panels mix tubes of different preparation, please continue to our Advanced Tutorial: www.flowjo.com/home/advancedtutorial.html

...



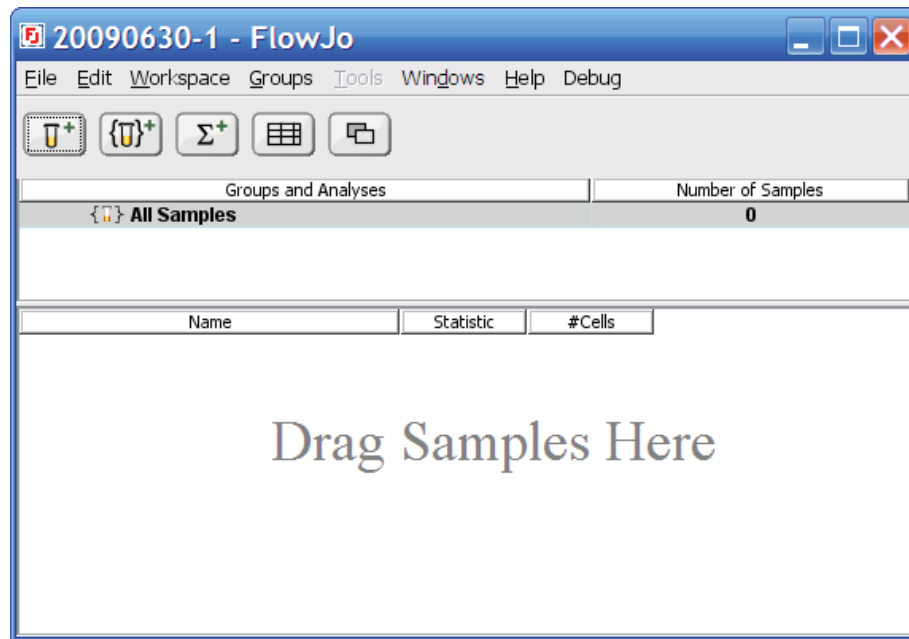
*The most important button in FlowJo is the **Help** button, found in all menu bars and dialog boxes. **Help** functions launch a web browser and takes you directly to the web page relevant to the window you are working on. To view the entire reference manual, point your web browser to:*

www.flowjo.com/home/manual.html

Step 1. Load data into the Workspace.

Create a New Workspace

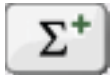
When you open FlowJo, you will see the Workspace window pictured below. The Workspace window is the main starting point for all analyses in FlowJo. It is composed of three main parts: a set of function buttons at the top, a **Group Panel**, and below it, a **Sample Panel**. When you select tubes and make a group, the group name is listed in the group panel. When you click on a group, all the samples that belong to that group can be seen in the sample panel. The **All Samples** group is created automatically and always contains all the data loaded into a Workspace.



Add Samples



Add Groups



Add Statistics



Open Table Editor



Open Layout Editor

These buttons add new elements to your workspace:
Samples are the FCS files coming from the cytometer.
Groups are sets of samples that have a common use.
Statistics give quantitative description of a population.

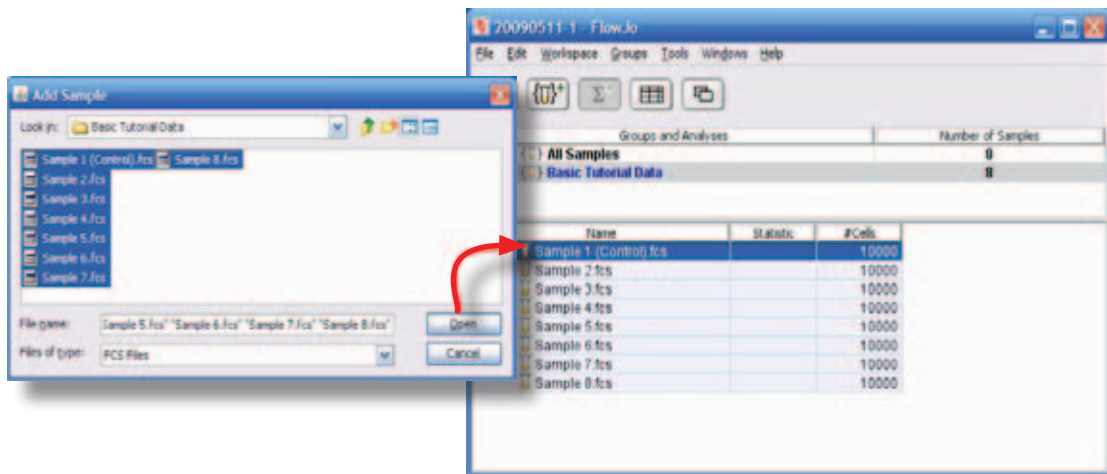
These buttons are used for constructing tabular and graphical reports of your data. Both use similar methods of defining the samples to be included in the report, and how FlowJo will *iterate* among them.



Add Data

To add experimental data files to the FlowJo workspace click the **Add Samples** button. Use the **Open** dialog to find Basic Tutorial data files and add them to the Workspace. (**Sample Data Files\Basic Tutorial Data_Workspaces\Basic Tutorial Data**) Single data files can be added one at a time or, all the files in a folder can be selected and added to the Workspace at once by clicking the Open button.

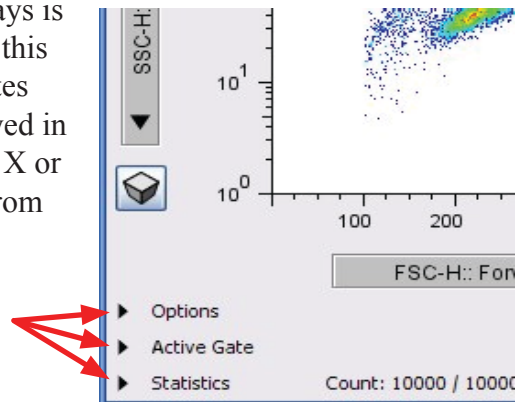
FlowJo also allows the drag and drop of files or folders from the File Manager or Finder to the Workspace window. Select the files or folders and drag them onto the Workspace. Dropping a folder of data files also adds a new group to the Groups Panel containing all the tubes from the folder.



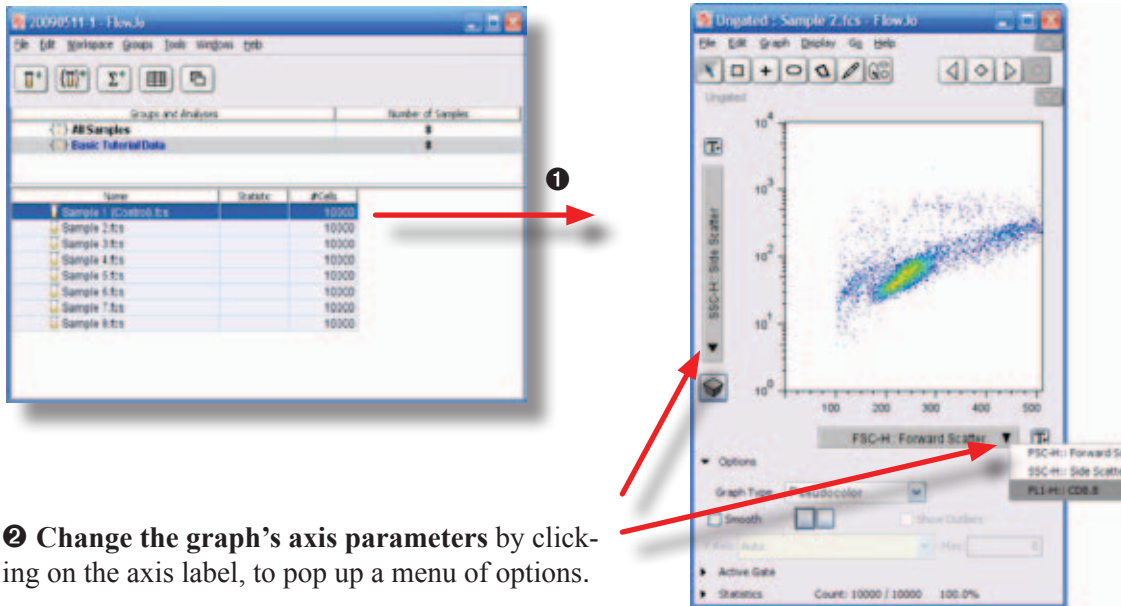
Graph Options

Display a plot of **Sample 2** by *double-clicking* on the sample in the Workspace window. FlowJo opens a Graph window (next page) displaying a bivariate dot plot of the cells in **Sample 2**. The initial graph that FlowJo displays is always a Forward vs. Side Scatter plot because this view is commonly used to identify the leukocytes subpopulations. The parameters that are displayed in the graph are easily changed by clicking on the X or Y axis labels and selecting another parameter from the pulldown menu.

The characteristics of a Graph can be altered by click-ing the **Options** disclosure triangle at the bottom of the Graph window.



① Double-click **Sample 2** to open a graph window.



② Change the graph's axis parameters by clicking on the axis label, to pop up a menu of options.

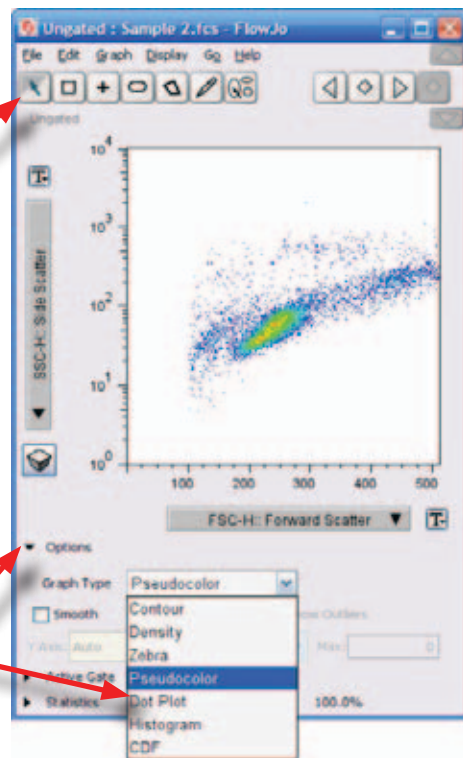
You can choose the plot type and plot options used to display the cells in your sample. Gating tools are shown at the top of the Graph window.

Gating tools

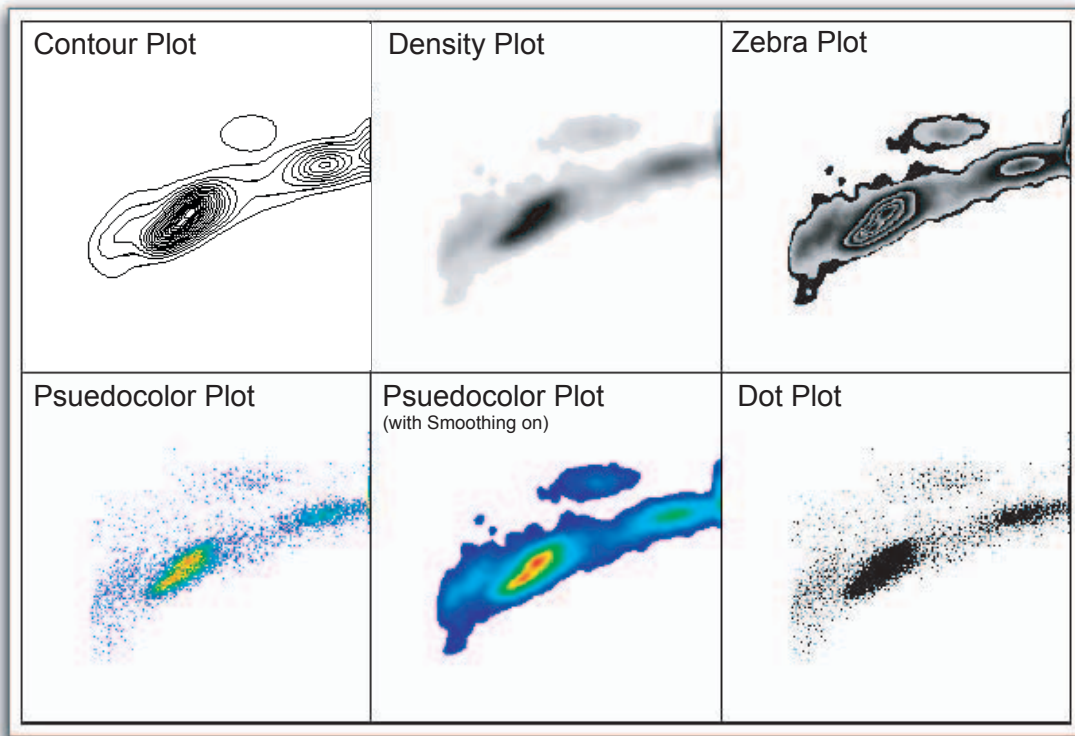
- Click the options triangle to edit graph types.
- Click the menu to choose a new graph.
- Click the **Smooth** box (only while displaying a Histogram, Pseudocolor or Density graph).
- Use Color boxes to change the foreground and background colors.

Graph options open

Graph types drop-down menu

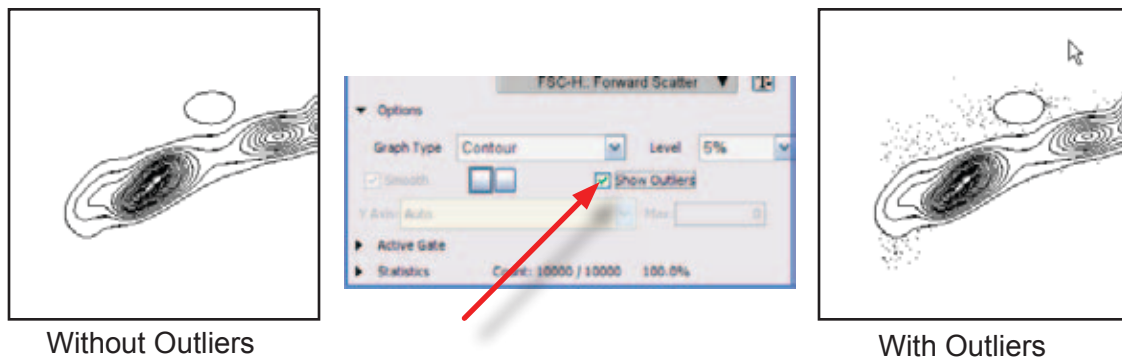


FlowJo can display many different graph formats or *plot types*.



Contour lines drawn on **Contour Plots** are similar to altitude contours on geographical maps. Five percent of cells fall between each contour line in a 5% contour plot. One drawback of contour plots is that 5% of cells fall outside of the last contour line and information about rare populations is not visible. You can add outliers to most graph types with the **Show Outliers** check box. In order not to lose information about these populations, click on the **Show Outliers** check box. Outlier Plots combine the density estimation of contours with the rare event information of a Dot Plot.

Density Plots provide density information (i.e., the number of events within an area) by using different shades of gray. **Zebra Plots** are a mix of Contour (20%) and Density Plots.




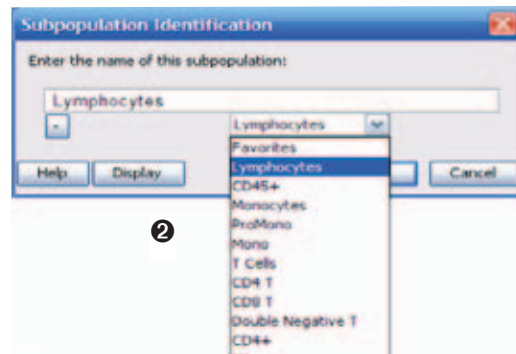
Pseudocolor Dot Plots provide benefits of a dot plot without the loss of information due to saturation. Saturation in high density areas is eliminated by drawing dots in different colors according to the cell density in that area. Turn on the **Smoothing** option for the pseudocolor plot to display a color density plot. Dot Plots display rare events well; however, these plots frequently saturate in areas of high cell density. Visual information about the cell population in the saturated area of the plot is lost. (Dot plots show only 8000 events, by default, in order to offset this problem.)

Step 2. Analyze a sample in detail.

Gating

Identifying subsets of cells is easy with FlowJo. With the **Sample 2** graph open, choose the polygon gating tool from the top of the graph window. and click anywhere in the Graph window to begin drawing a gate.

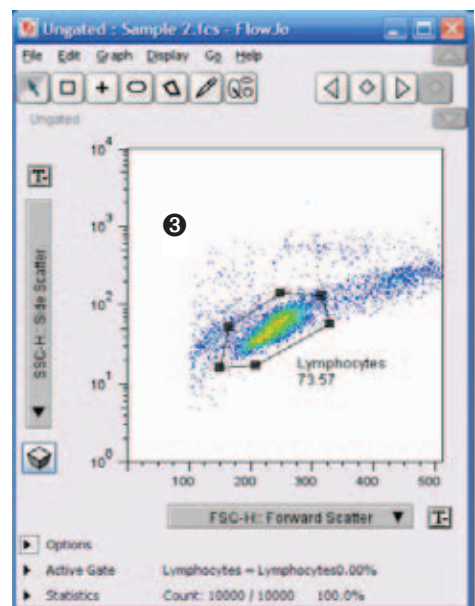
 **1 Draw a polygon gate** encompassing the lymphocyte population by clicking where you see the black squares (handles) in the image on the right. Click the first handle again to close the gate. Once you have created your gate, the box below will appear so you can name the subpopulation.

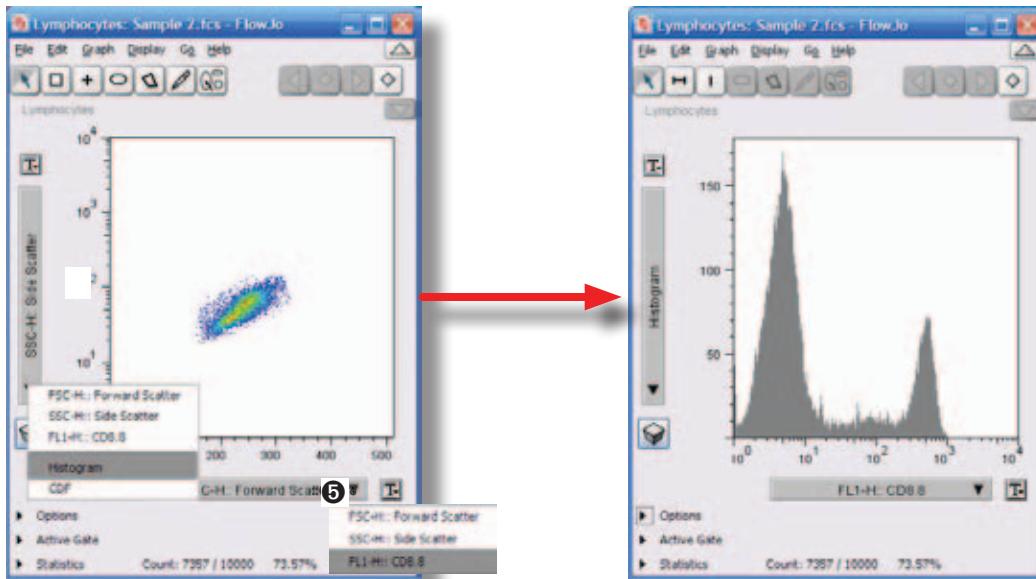


2 Name this subpopulation Lymphocytes and click OK. You can easily adjust the size of a gate, by clicking and dragging any of the handles of the polygon. Handles are only displayed on gates you have selected. To move the entire gate, hold the mouse over the gate the cursor changes. Then, drag the gate to another location.

Inside the gate FlowJo displays the frequency of the gated population. When the cursor becomes a percent sign you can move the frequency number to a new location.

3 Double-click on the Lymphocytes gate in the Graph window to open a new graph (right) displaying only the subset of cells that fall within the **Lymphocytes** gate.





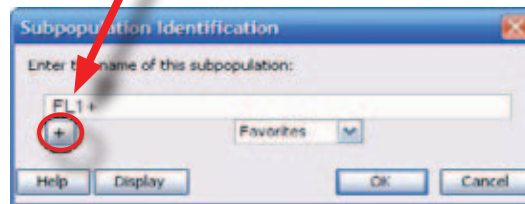
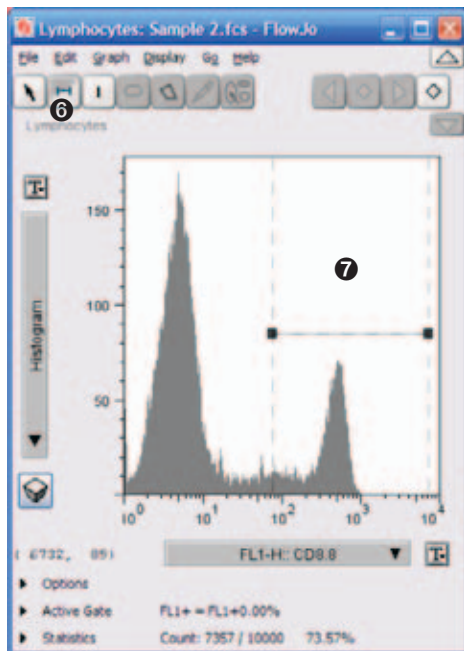
④ Click on the Y-axis label and select **Histogram** from the menu.

⑤ Click on the X-axis label and choose FL1-H from the pull-down menu.

⑥ Choose the Range Gate tool.

⑦ Drag to draw a gate to set the range of fluorescence values that will define FL1 positive cells.

⑧ Name this subset **FL1+**. Click the plus sign to add the name to the list for future use.



⑨ Click OK.

There are several gating tools for bivariate plots. Rectangle, Polygon, Quadrant or Ellipse gating tools can be selected from the top of the Graph window. Univariate (histograms) and bivariate (X-Y graphs) plots use different tools, so the buttons will change or disable, when the type of graph changes.

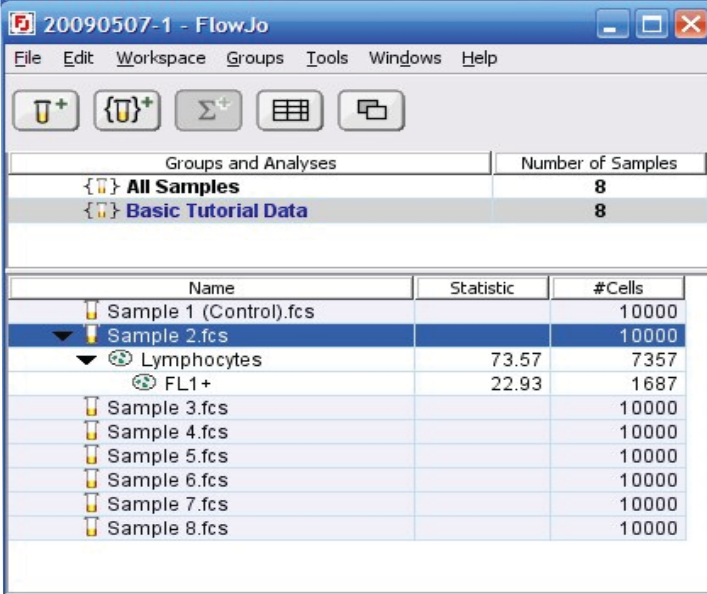
Hierarchical Description of Sample Subsets

When you create gates in a sample or subpopulation, FlowJo shows the new subsets in the Workspace in a hierarchical manner; each subpopulation is shown indented under the sample or subset that it came from. This is called a **Gating Tree**. Like a family tree, the gating tree shows Grandparent, Parent, Child and Grandchild relationships between cell populations. When you generated the Lymphocyte gate, you may have noticed a change in the Workspace window.

FlowJo placed the name of your gate, **Lymphocytes** in a new row underneath **Sample 2**. This new row represents the subpopulation of cells that fall inside the lymphocyte gate. The **Lymphocytes** name is displayed in the Workspace window indented underneath the **Sample 2** name because the lymphocyte population is a subset or child of **Sample 2**. When you generated the **FL1+** gate this gate was drawn only on the cells that fall within the lymphocyte subpopulation.

The **FL1+** gate name is written indented and underneath **Lymphocytes** as shown.

A petri dish icon  appears to indicate a subpopulation of cells.



Groups and Analyses	Number of Samples
{ } All Samples	8
{ } Basic Tutorial Data	8

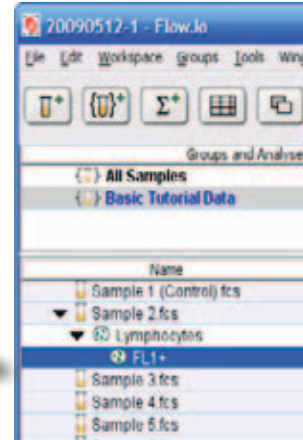
Name	Statistic	#Cells
Sample 1 (Control).fcs		10000
Sample 2.fcs		10000
Lymphocytes	73.57	7357
FL1+	22.93	1687
Sample 3.fcs		10000
Sample 4.fcs		10000
Sample 5.fcs		10000
Sample 6.fcs		10000
Sample 7.fcs		10000
Sample 8.fcs		10000

Adding Statistics

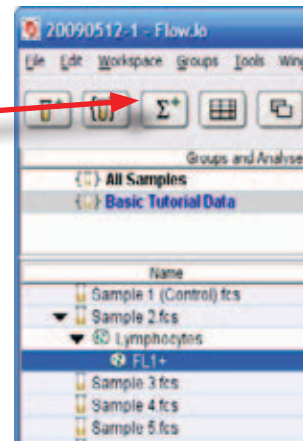
There are many statistics that can be computed and displayed by FlowJo. When you apply a statistic to a cell population it is added as a separate line in the Workspace window. If you change a population by moving a gate, statistics will be automatically recalculated. The list of statistics includes fluorescence intensity, CV or frequency within a parent population, mean, median, geometric mean and more.

To compute the Median Fluorescent Intensity in our FL1+ subpopulation:

- 1 Select **FL1+** in the Workspace
(row is shaded.)

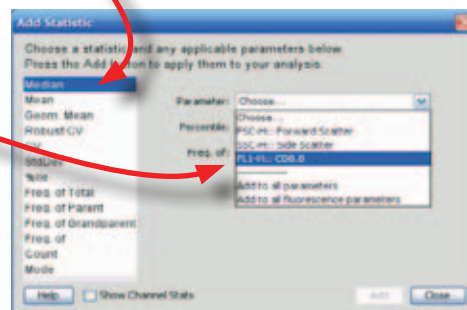


- 2 Click on the **Add Statistic** function button.
This brings up the Add Statistic dialog box.



- 3 Select **Median** in the dialog box.

- 4 Click the **Parameter** drop-down menu and choose **FL1-H** to compute the **Median** on the **FL1-H** channel.
Click the **Add** button.



Select **Frequency of Total** then click the **Add** button then **Close** to close the dialog. Statistics are added to the Workspace window as indented lines marked by a capital sigma. Σ

Notice that in the Workspace, there are 2 new rows under the **FL1+** subpopulation.

Name	Statistic	#Cells
Sample 1 (Control).fcs		10000
▼ Sample 2.fcs		10000
▼ Lymphocytes	73.57	7357
▼ FL1+	22.93	1687
Σ (FL1-H) CD8.8 : Median	425.27	
Σ Freq. of Total	16.87	
Sample 3.fcs		10000
Sample 4.fcs		10000

Median is the Median Fluorescence Intensity (MFI). It describes the brightness of the FITC parameter in the **FL1+** subset.

Name	Statistic	#Cells
Sample 1 (Control).fcs		10000
▼ Sample 2.fcs		10000
▼ Lymphocytes	73.57	7357
▼ FL1+	22.93	1687
Σ (FL1-H) CD8.8 : Median	425.27	
Σ Freq. of Total	16.87	
Sample 3.fcs		10000
Sample 4.fcs		10000

Frequency of Parent

$$73.57\% = \frac{7,357 \text{ cells}}{10,000 \text{ cells}}$$

Name	Statistic	#Cells
Sample 1 (Control).fcs		10000
▼ Sample 2.fcs		10000
▼ Lymphocytes	73.57	7357
▼ FL1+	22.93	1687
Σ (FL1-H) CD8.8 : Median	425.27	
Σ Freq. of Total	16.87	
Sample 3.fcs		10000
Sample 4.fcs		10000

Frequency of Total

$$16.87\% = \frac{1,687 \text{ cells}}{10,000 \text{ cells}}$$

The statistic **Frequency** refers to the percentage of a larger population represented by the subset. Frequency of the **FL1+** cells within **the whole sample** is known as **Frequency of Total**.

The **Frequency of Parent** statistic refers to the frequency of **FL1+** cells in the direct ancestor population. Frequency of Parent is calculated by dividing the number of cells in the subpopulation by the number of cells in its direct ancestor population.

Frequency of Parent is the default statistic for every population of cells in the Workspace. It appears in the Statistic column.

Step 3. Copy gates and statistics to All Samples.

Drag and Drop Gates and Statistics

In FlowJo, any gate or statistic can be copied to any sample or population using copy and paste or drag and drop. Dragging any gate or statistic onto the name of another population will create a new line in the Workspace with the analysis applied to that population. This is an easy way to build and expand analysis trees. The tree represents the subsets of a sample in a simpler and more intuitive way than Boolean expressions (R1 AND R2, R1 NOT R2) used in other analysis programs.

Save this Workspace, and then feel free to play with drag and drop in order to create analysis trees on other tubes or expand the gating tree on Sample 2.

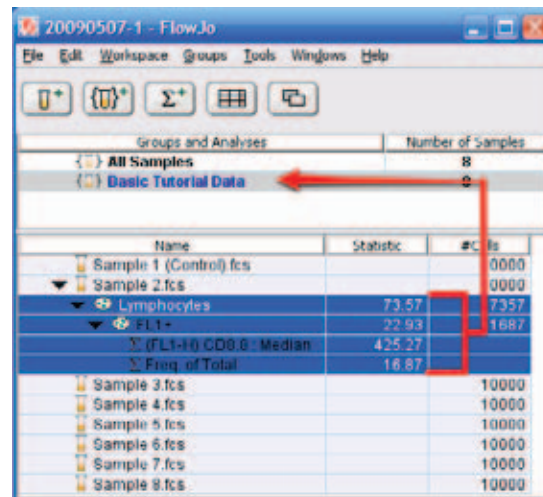
When you wish to continue with Step 3 of the Tutorial, choose **Revert** from the File menu to return to the previously saved workspace and eliminate the extra lines you have created. You should have a workspace with a gating tree applied to **Sample 2** only. If you wish you can also open the file **Basic Tutorial Workspace Step 2** to continue with the tutorial.

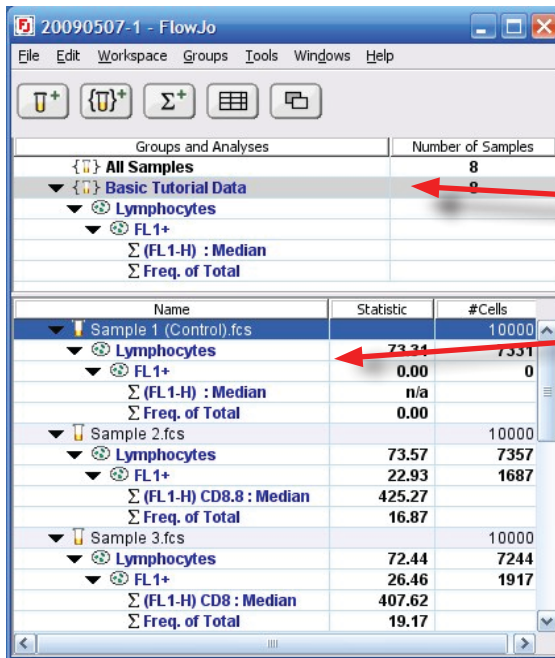
Apply the Gating Tree to Every Sample.

Analyses can be applied to every sample simultaneously by dragging the gating tree onto a group name in the upper panel of the Workspace window.

In Step 2 we analyzed **Sample 2** and now we want to apply the gating tree to the other seven tubes in the experimental data set. Select all the gates and statistics (*Shift-click*) and drag them onto the **Basic Tutorial Data** group in the upper panel of the Workspace. Release the mouse when the **Basic Tutorial Data** row becomes highlighted. Watch as the gating tree is applied to all eight tubes.

*Drag-and-drop the gating tree to the **All Samples** group.*





Gates and Statistics added to a group.

Note that the font of the **All Samples** group is **bold**.

The font of the gating tree is also **bold**.

FlowJo uses colors and font types to help you keep track of gates and groups. The bold font of the gating tree on the samples is a visual clue that these gates are identical to those that were applied to the **Basic Tutorial Data** group. This feature becomes important when you have several different groups or when altering gates on individual samples as in Step 4.

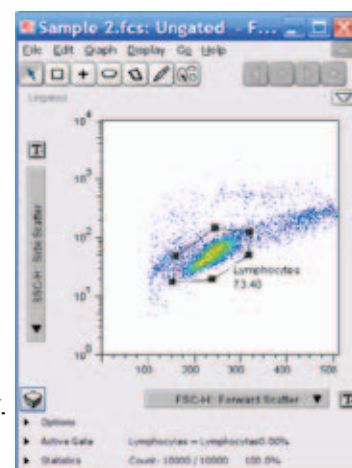
The **All Samples** group serves well for the analysis of the titration data. but, more complex experiments may consist of samples with different characteristics, making it inappropriate to apply one gating tree to every sample in the Workspace. Group your samples according to their parameter similarities to enable batch processing of only those samples with similar characteristics. See the Advanced Tutorial for a full appreciation of the power of groups and their usefulness in organizing a more complex data set. www.flowjo.com/home/tutorial.html

Step 4. Verify gates on all samples.

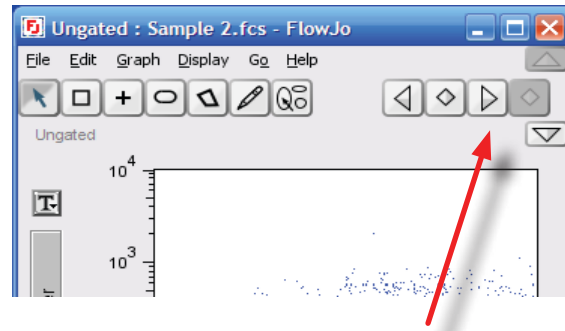
Checking Gates

Applying the gating tree to many samples at once is very easy; however, FlowJo does **NOT** check the gates on each sample for you!

To check your gates, open the graph of **Sample 1** by *double-clicking* on the sample in the **Workspace** window. Adjust it to display Forward vs. Side Scatter.



Instead of opening the graphs for each individual sample to check the gates, you can click on the forward navigation arrow at the top of the **Graph** window to cycle to the graph for the next sample. Use the navigation arrows to check the **Lymphocytes** gate on each sample.



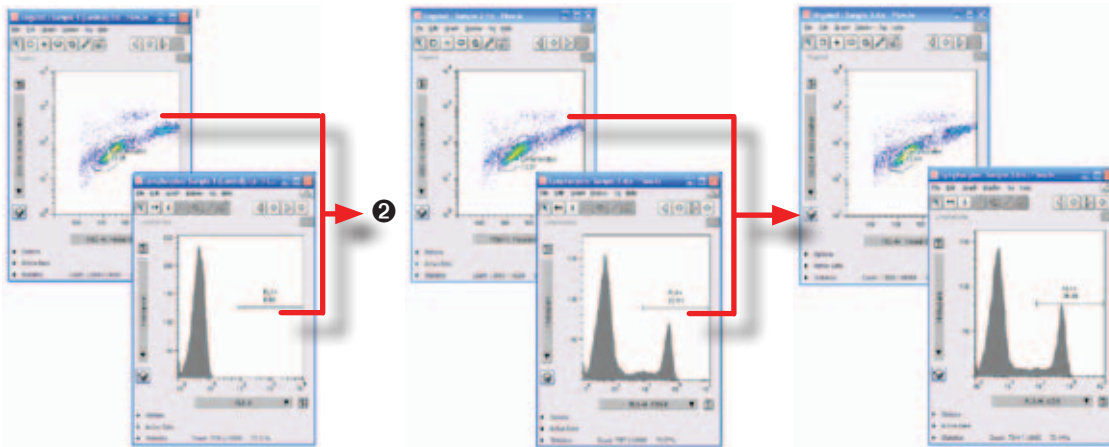
The Forward Navigation Arrow in the graph window displays a similarly configured graph of the next sample.

You can check *multiple* graphs of any sample simultaneously.

❶ **Open a new graph** displaying the **FL1+** gate. (Double-click on the **Sample 1 Lymphocytes** in the **Workspace** window.)

Name	Statistic	#Cells
Sample 1 (Control).fcs		10000
Lymphocytes	73.31	7331
FL1+	❶ 0.00	0
Σ (FL1-H) : Median	n/a	
Σ Freq. of Total	0.00	
Sample 2.fcs		10000
Lymphocytes	73.57	7357

❷ In order to advance the gates in both graphs at once, **Shift-click the arrow**. All open graphs of sample 1 cycle to graphs of the next sample.



Sample 1 Graphs

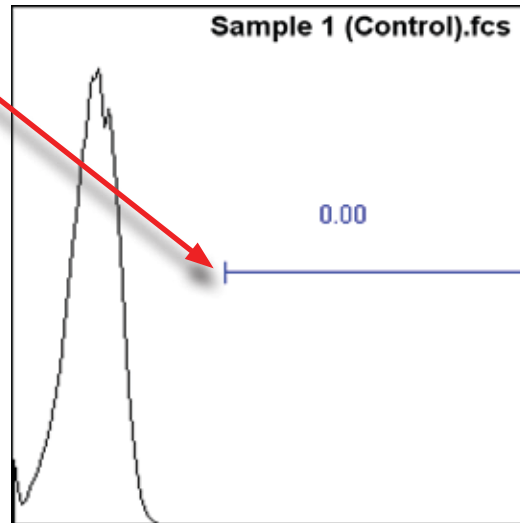
Sample 2 Graphs

Sample 3 Graphs

Altering Gates

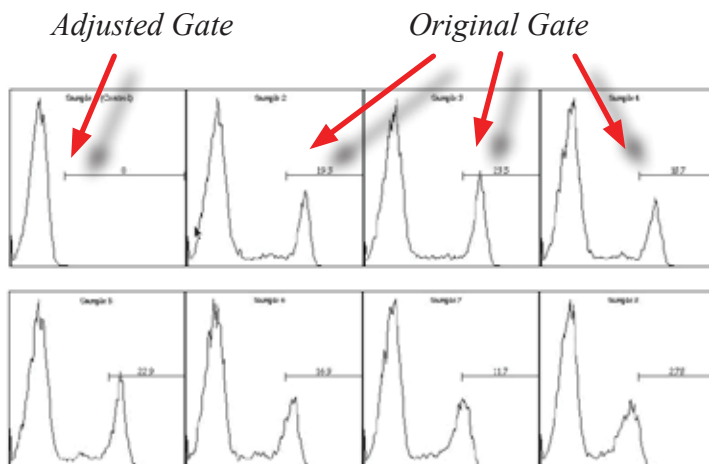
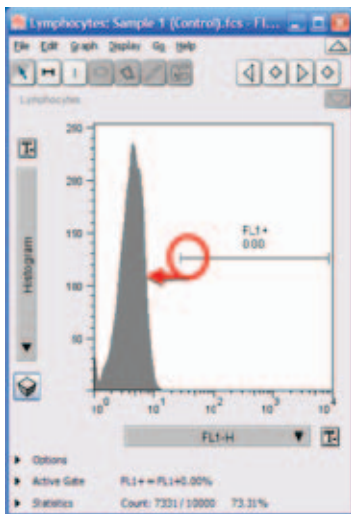
While checking the gates on each sample you may find that adjustments need to be made. Gates could be altered on each individual sample but its easier to adjust one gate and apply the new region to all the samples by drag-and-drop. The histograms show that the range gate we drew in Step 2 does not include all the **FL1+** subpopulation.

The fluorescence cutoff is too high.



❶ Double-click **Lymphocytes** in the Workspace window to open the graph window displaying the **FL1+** gate for Sample 1.

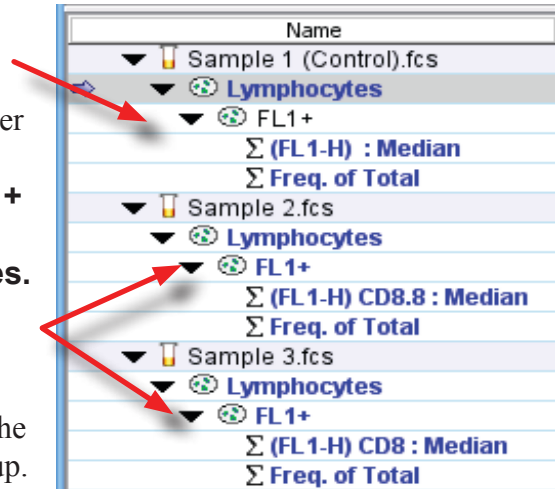
❷ **Adjust the FL1+ gate** by clicking on the left handle of the histogram gate and dragging to the left. We want to adjust the **FL1+** gate to start at a lower fluorescence intensity and include a larger portion of the histogram as shown below.



Notice in the **Workspace** window, that the FL1+ gate name is no longer in bold type.

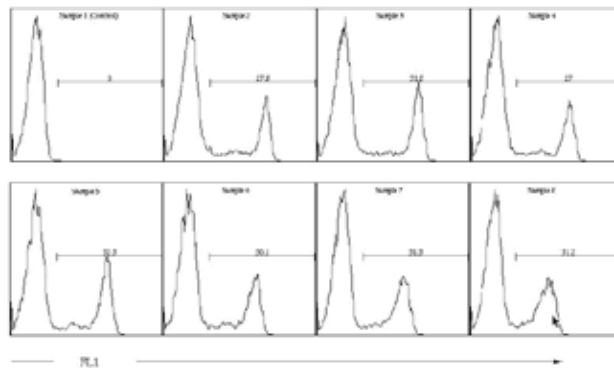
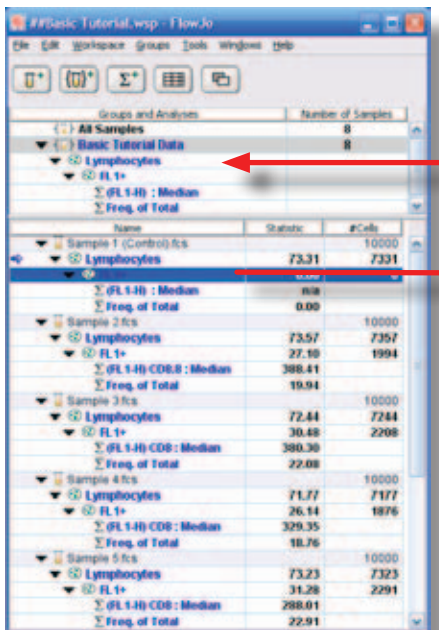
This indicates that the FL1+ gate is no longer the same as the gate that was dragged onto the **All Samples** group. Changing the **FL1+** gate on one sample DOES NOT automatically change the **FL1+** gate on **All Samples**. The **FL1+** gate on the other samples remains **bold**.

That's because these gates are the same as the gate that was initially dragged onto the group.



You now have two options; either leave the new gate on Sample 1 and the original gate on the rest of the samples or apply the new gate to all the samples. Let's apply the new **FL1+** gate to all the samples by dragging the **FL1+** gate from **Sample 1** up onto the **All Samples** group. Click once to select the **FL1+** gate under **Sample 1**. Click and drag it up to the **All Samples** group. Release the mouse when you are over the **Lymphocytes** name (it will become highlighted) in the **All Samples** group. Notice that all of the graphs now show the new FL1+ gate.

*Drag and drop the **FL1+** gate onto the **Lymphocytes** in the Group Panel.*



*The new gate is applied to **all** of the samples in the group.*

The preceding exercise illustrates **three** powerful features of FlowJo:

First, FlowJo gives you the ability to make sure that all of your samples are analyzed identically, that they have the exact same gates, statistics, and other analyses, in order to limit subjectivity in the experiment.

Second, FlowJo gives you the ability to make sample-specific modifications. There are times when the Lymphocyte gate (or any other gate) must be moved slightly to accommodate a specific sample. FlowJo lets you move the gate or change the analysis for individual samples without affecting all the rest.

Third, the workspace window provides feedback that this has occurred, and allows you to proceed with your batch analyses and report generation using the sample-specific modifications for those samples where it is necessary.

With these three features, FlowJo provides an analysis environment that can be as rigorous or as flexible as you need.

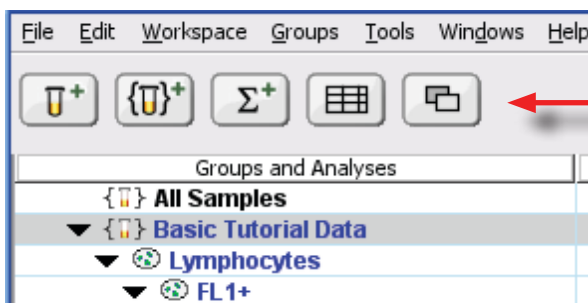
Step 5. Generate a graphical report.

Now that we have drawn gates to divide each sample into subpopulations, added statistics, and checked our gates, it is time to make a graphical summary report of this experiment. The Layout Editor and Table Editor are used for making graphical and statistical reports to summarize all the samples. The Table and Layout Editors work similarly; each button opens a new window that is saved with the Workspace. In these editing windows, you define which statistics or graphs to display in your report by drag-and-drop. FlowJo automatically builds graphs and tables that include all the samples in your experiment. The repetitive tasks are done for you.



Layout Editor

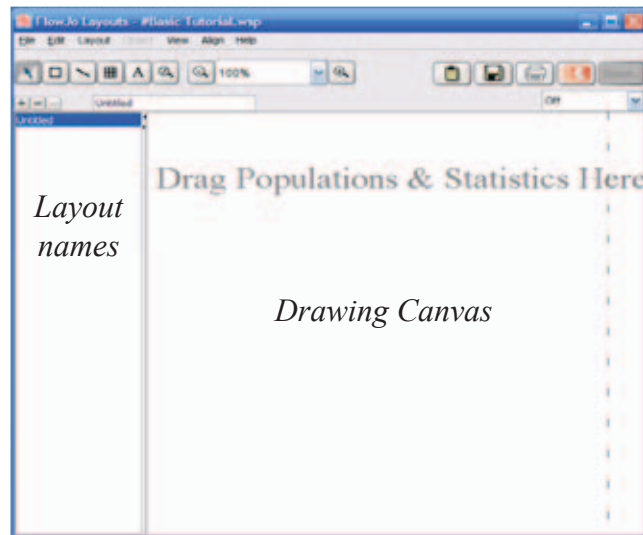
Open the Layout Editor via the Layout button at the top of the Workspace window.



Open the Layout Editor

The left panel of the Layout Editor window shows the names of the layouts you make, the right panel is a drawing board or canvas.

In the canvas, you will create a print layout for a single sample. From the example of one sample, FlowJo will then create graphs for all of the samples in the whole group (a **Batch Report**.) FlowJo *iterates* step by step through each of the samples in active group, applying the same instructions to each data sample and retrieves the matching graphs for each sample.

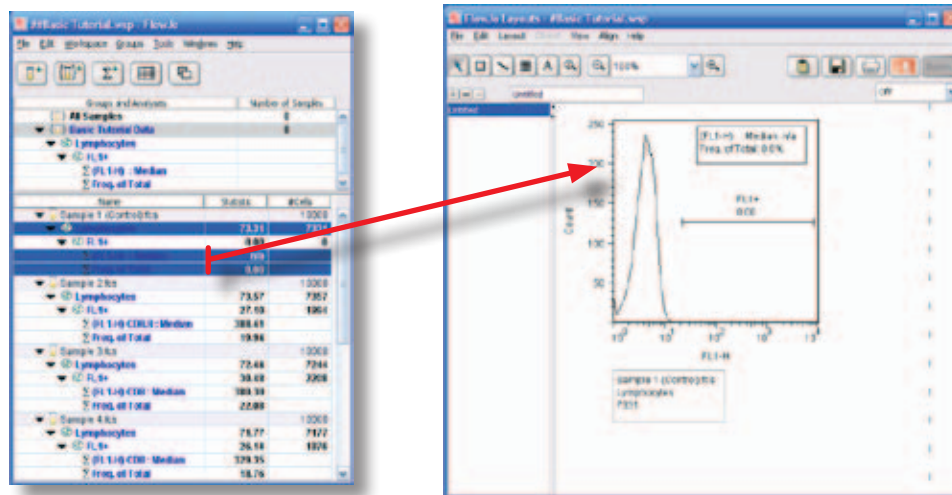


You can display graphs from every sample in a **Batch Report**, or overlay multiple histograms for comparison. It is also possible to combine the overlay and batch report by overlaying each sample in turn onto the **Control histogram**.

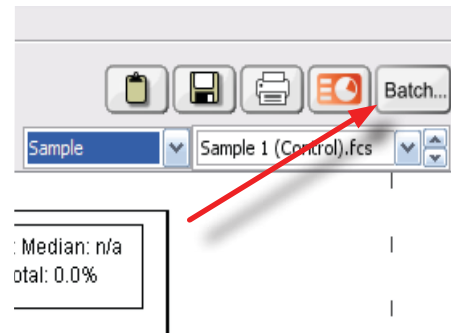
Batch Report

❶ In the **Workspace Window**, drag the **Lymphocytes** population from **Sample 1** to the **Layout Editor**. A histogram of the FL-1 parameter will appear. (*Double-click* on the graph in the Layout Editor to edit parameters displayed in the graph.)

❷ In the **Workspace Window**, *shift-click* to select the two statistics, **Median** and **Frequency**, under **Sample 1** and drag them to the **Layout Editor**.

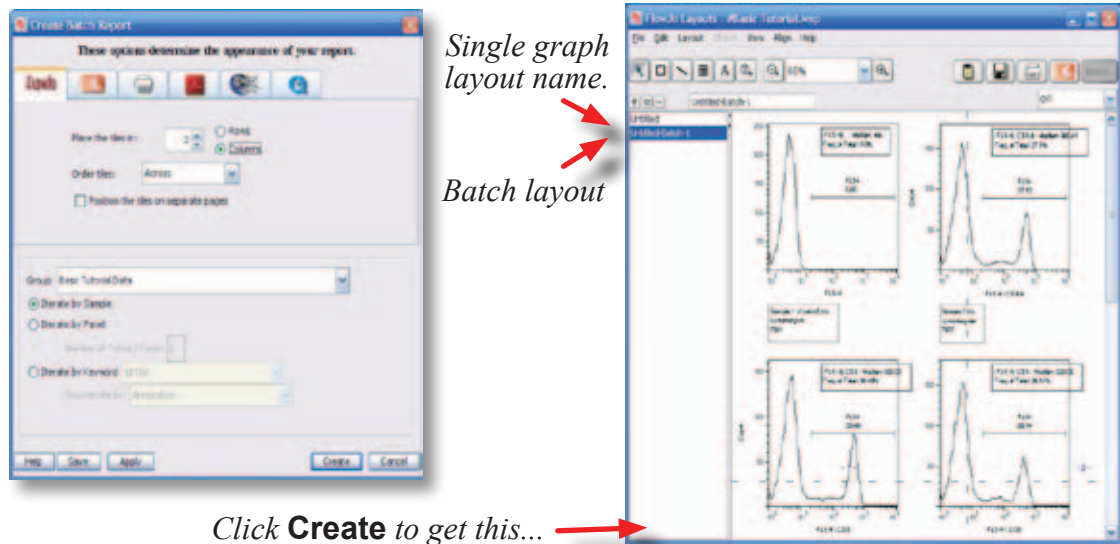


③ **Make a batch report:** Click the drop down box and select **Sample**. Then click on the **Batch** button in the upper right corner of the **Layout Editor**. This opens a dialog where you can name and customize the report.



Click **Create** in the dialog window. A new layout is created, named **Untitled-1 Batch**. The name is listed on the left panel of the Layout Editor.

To create this new layout, FlowJo iterated through each sample in the **Workspace** and retrieved an FL-1 histogram and the requested statistics for each sample.



Exporting Graphics: *The layouts can be saved to disk or copied and pasted into any drawing program. Select output choices in the Preferences tool. Save and Export to App use the format set in Preferences. Copy/Paste uses a bitmap format. Bitmaps are transferred at screen resolution. Graphics saved in the .SVG format can be imported into drawing programs where each graph can be ungrouped into individual objects. This gives you the ability to adjust axis labels and line weights.*

Printing: *You can print from the Workspace window, Table or Table Editor, Graph window or Layout Editor window. In the Layout Editor dotted lines show the size and shape of the pages FlowJo will print. To adjust the page scale in order to fit the graphs on a page, click and drag on the intersection of two dotted lines.*

Histogram Overlay

Create a new layout by clicking the **New Layout** button (+ sign) in the Layout Editor. In this layout, you will overlay a histogram of the **Lymphocytes** subpopulation from every sample.

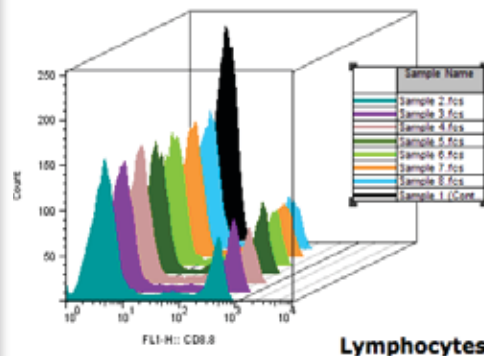
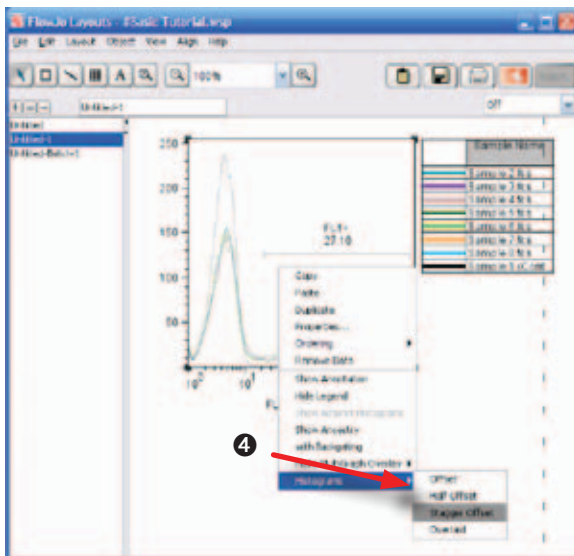
❶ **Drag the Lymphocytes of Sample 1** from the Workspace to the Layout Editor. Adjust if necessary to display a histogram of the FL-1 parameter. (Double-click on the graph if you need to make adjustments.)

❷ **Control-click the Lymphocytes** from Samples 2 through 8 and drag them all on top of the graph already in the Layout Editor. When you release the mouse button, the **Lymphocytes** subpopulation from every sample will be overlaid in one plot. To quickly select the lymphocyte populations from among the other samples choose **Select Equivalent Nodes** from the **Edit** menu, or, alternatively control-click each of the desired lymphocyte populations to add it to the selection.

When a large number of histograms are overlaid, viewing the individual histograms can become difficult. To address this problem, you can offset the histograms.

❸ **Right-click on the graph** to open the menu that adjusts the graph's features.

❹ **Select the Staggered Offset option.** Your graph will look like the one below. To change the color of a histogram, click on the color box in the legend. To change the line style, coloring of a histogram, *right-click* the sample name. To change the fonts or appearance of the legend itself, double click the legend.



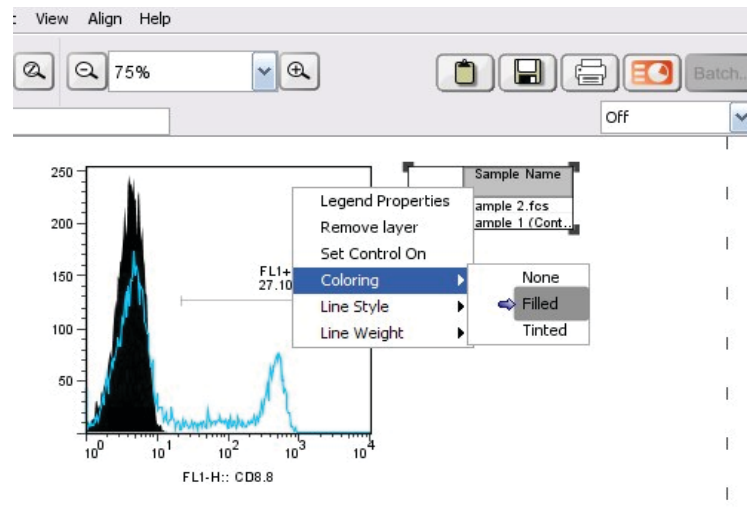
Setting a Control Sample

Many experiments, including this one, are made up of one or more control samples and several test samples. Our final report style will overlay each test sample by itself onto the unstained control sample for direct comparison. To do this you will overlay **Sample 2** onto **Sample 1** (the unstained control), define sample one as the control, and then create a batch report showing each sample overlaid one by one on the graph of the control sample.

- ❶ **Create Overlay:** Click the plus sign button at the top left of the **Layout** Window to create a new layout.
- ❷ From the Workspace, **drag the Lymphocytes from Sample 1** to the Layout Editor.
- ❸ **Drag the Lymphocytes from Sample 2** onto the graph in the Layout Editor to create an overlay.

To make the **Sample 1** histogram a solid color:

- ❹ **Right-click on Sample 1 in the legend.** In the menu, under Coloring select **Filled**.



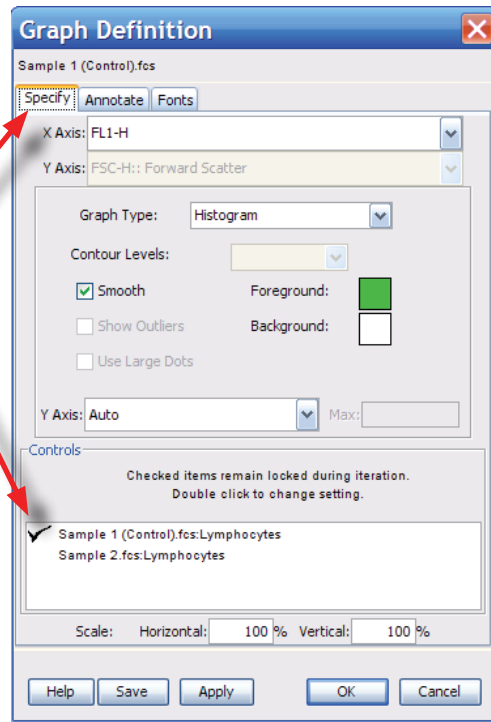
Define which sample is the control. It will appear overlaid with each of the other samples.

⑤ **Double-click on the graph** in the Layout Editor and click on the **Specify** tab in the dialog window that pops up.

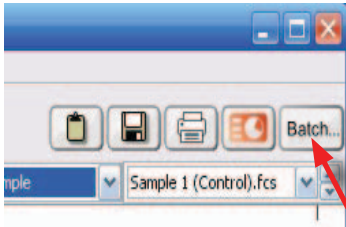
Under the **Specify** tab, there is a section for **Controls**, which contains a list of the populations comprising the layers in your chart.

⑥ **Double-click on the control tube.** A check mark will appear beside it.

⑦ **Click the OK button.** Now **Sample 1** will not vary when you create a batch report of all the samples.

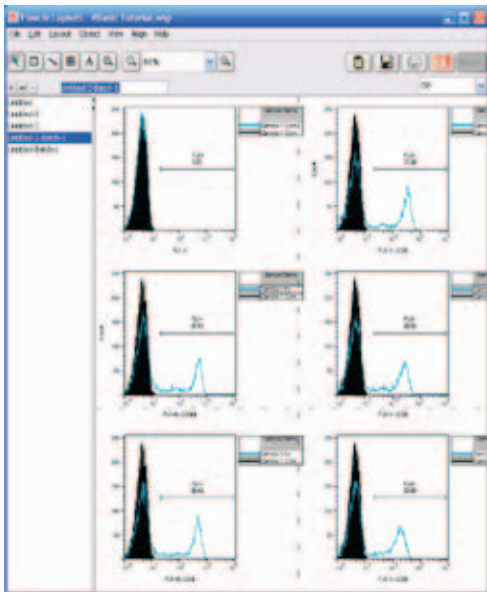
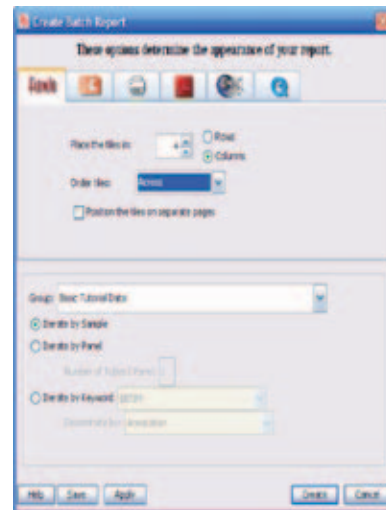


Generate a Batch Report.



⑧ **Click on the drop-down box** below the batch button and set iteration to Sample. Click the **Batch** button in the Layout Window.

⑨ **Click the Create** button in the dialog.

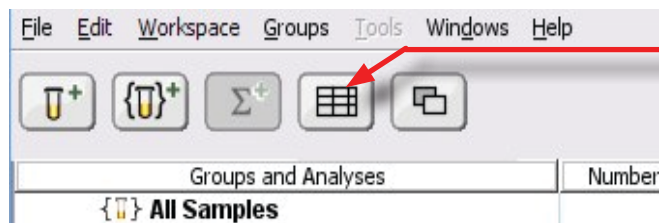


As a result, FlowJo creates a new layout displaying each individual sample overlaid on the **Sample 1** histogram.

Step 6. Generate a table of statistics.

Table Editor

The **Table Editor** works in a manner similar to the Layout Editor. You can define which statistics to display in your table by dragging them from the workspace and dropping them in the Table Editor. FlowJo takes those statistics from *each* sample and builds a new table. You may build multiple tables from the same experimental data. Their names will appear in the left panel of the Table Editor.

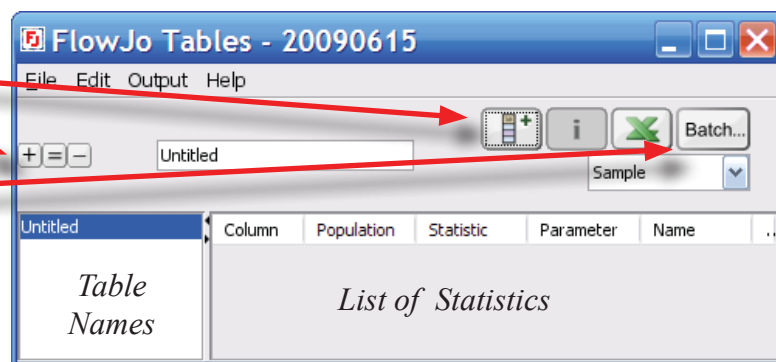


① Open the Table Editor by clicking the grid icon.

Column Editing

Table Administration

Batch Creation



Button Functions



Add Column. Create columns that reference keywords or that calculate relationships between other parameters.

Edit Column. Gives access to the parameters of a selected column.

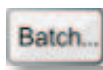
Export. Write the table's data in the format set in preferences for display in a spreadsheet or external statistics program.



Add Table. Each table setup has its own name and column list.

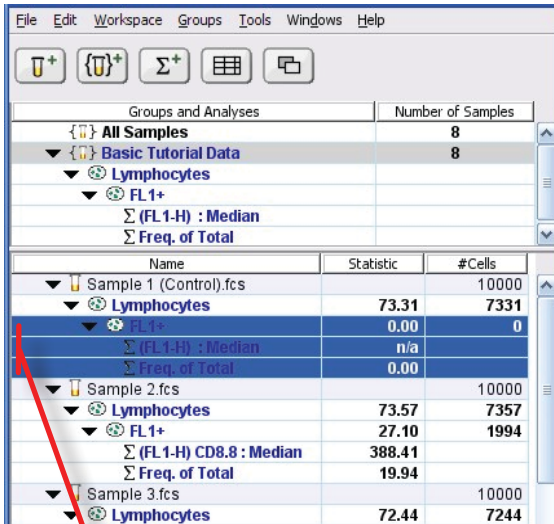
Copy Table. Duplicates an existing table and keeps the original.

Delete Table. Clear the Table Editor of unwanted table definitions.



Batch. Creates the table, using current group and iteration, as described on page 26.

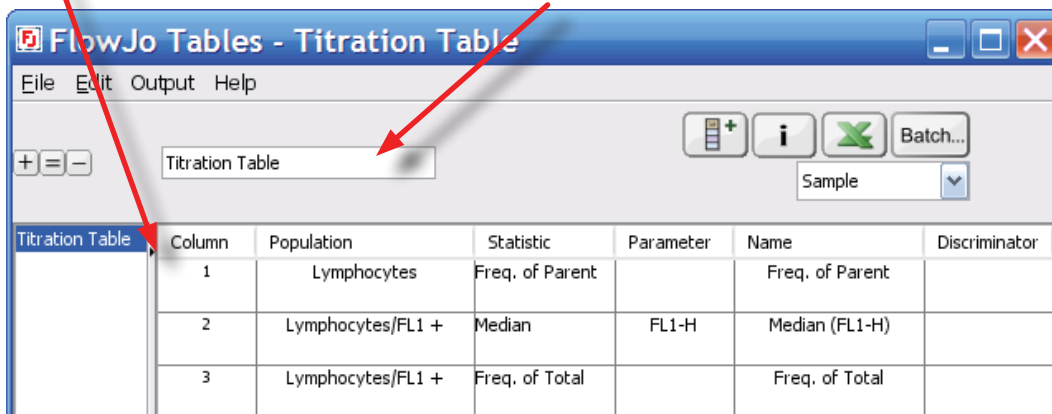
② In the **Workspace Window**, shift-click the **FL1+** subpopulation and **Frequency** and **Median** statistics from **Sample 1**.



③ Drag them to the right panel of the **Table Editor** as shown below.

Three statistics are now listed on the right side of the **Table Editor**. All three statistics are calculated on the **FL1+** population. When you drag a subset, for example, the **FL1+** subpopulation to the Table Editor, the **Frequency of Parent** is calculated

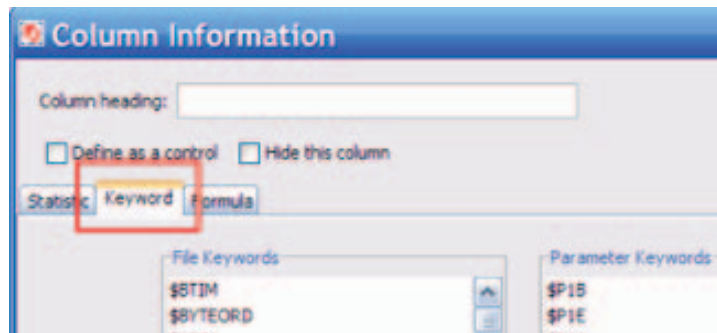
④ Type a name for your table in the text box at the top of the Table Editor Window.




Your cytometry data may contain keywords like **SAMPLE ID** or sample date (**\$DATE**). You tell FlowJo to include these in your table by clicking the Column Information Button, then the Keyword tab.

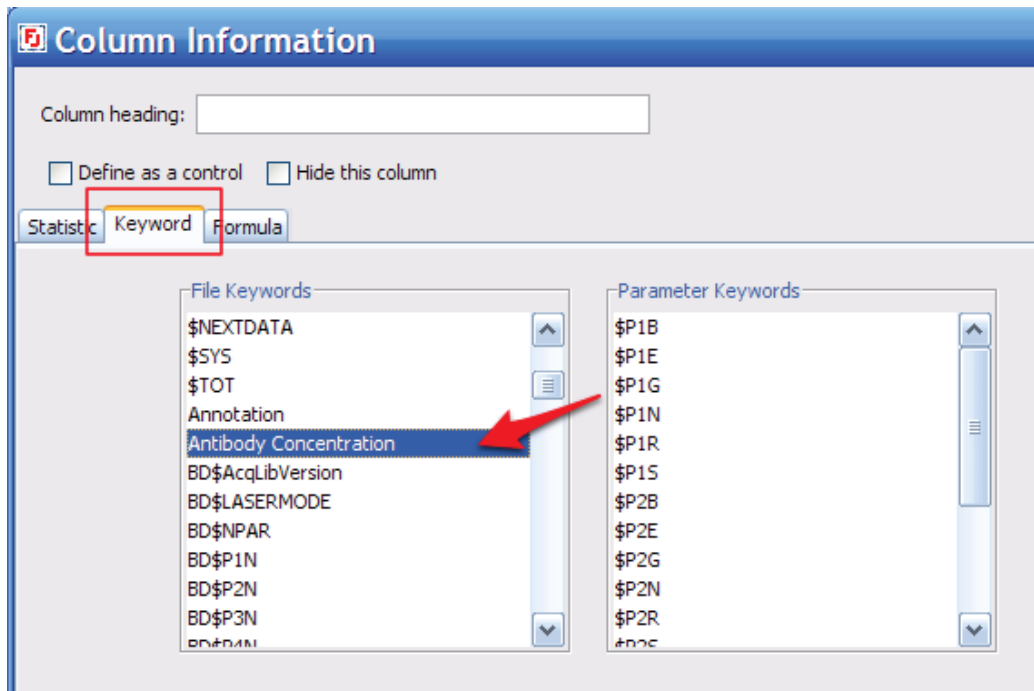


In our example data set, the **Antibody Concentration** keyword was used to note the concentration of antibody used in sample preparation.

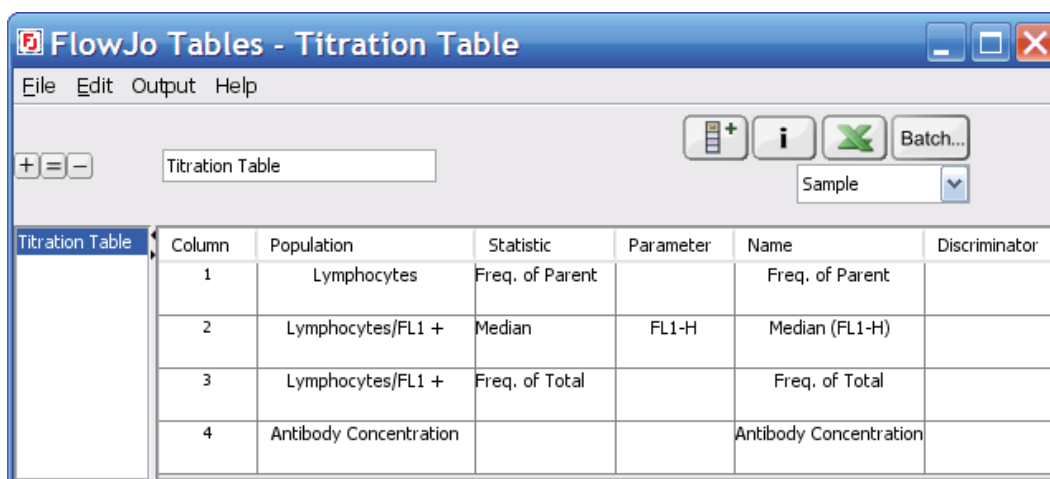


5 Click the column info button to open the Column dialog. 

6 Select the **Keyword** tab and click **Antibody Concentration** in the list.



7 Click **OK** to add the keyword to the Table Editor.



Titration Table	Column	Population	Statistic	Parameter	Name	Discriminator
	1	Lymphocytes	Freq. of Parent		Freq. of Parent	
	2	Lymphocytes/FL1 +	Median	FL1-H	Median (FL1-H)	
	3	Lymphocytes/FL1 +	Freq. of Total		Freq. of Total	
	4	Antibody Concentration			Antibody Concentration	

Create the table by clicking the **Batch** button in the upper right of the **Table Editor**. Each *statistic* listed in the Table Editor will become a column in the table and each *sample* open in the **Workspace** will become a row. If you have other groups to display (with the same populations and statistics), you can switch groups in the Workspace or in the table batch dialog.

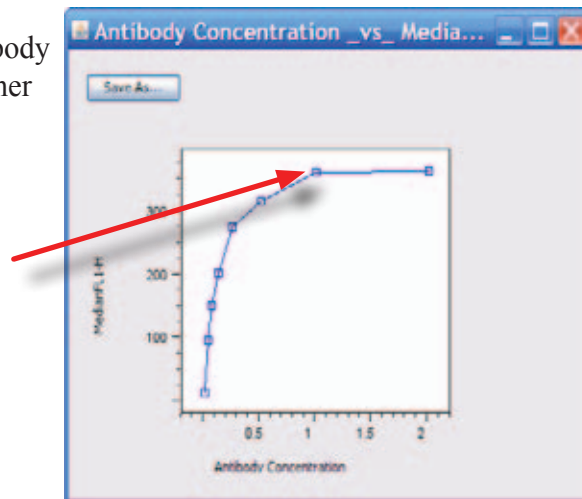


The screenshot shows two windows from FlowJo. The top window is 'FlowJo Tables - Titration Table' with a menu bar (File, Edit, Output, Help) and a toolbar containing a 'Batch...' button. Below the toolbar is a table editor with columns: Column, Population, Statistic, Parameter, and Name. The bottom window is 'Table - Titration Table' with a menu bar (File, Edit, FlowJo, Help) and a data table.

Samples	Lymphocytes Freq. of Parent	Lymphocytes/FL1 + Median (FL1-H)	Lymphocytes/FL1 + Freq. of Total	Antibody Concentration
Sample 1 (Control).fcs	75.62%	15.12	0.10%	
Sample 2.fcs	76.03%	363.45	22.42%	2
Sample 3.fcs	74.80%	360.62	24.32%	1
Sample 4.fcs	74.14%	315.49	21.08%	.5
Sample 5.fcs	75.74%	275.75	24.37%	25
Sample 6.fcs	76.04%	202.13	23.16%	.125
Sample 7.fcs	75.26%	151.82	23.47%	.0625
Sample 8.fcs	75.46%	96.70	23.35%	.03125
Mean	75.39%	222.61	20.28%	0.57
Standard Deviation	0.65%	128.01	8.22%	0.72

Right-click the header in the Antibody Concentration column to see a pop-up menu of the other numerical columns. Select any column from the menu to plot the correlation between the column you right-clicked to the column chosen from the menu. In this case, we are more interested in the trend than the correlation. Hold down the **Alt-Key** (option key on Macintosh) to plot concentration vs. Median FL1 as a line graph. The saturating concentration of this reagent is 1 $\mu\text{g/ml}$. Using higher concentrations of reagent will not increase fluorescence intensity. Therefore this CD8-FITC antibody reagent should be used at 1 $\mu\text{g/ml}$ in further experiments as long as the same staining conditions are maintained.

Saturation is reached at 1 $\mu\text{g/ml}$



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

- **Data Compensation** - corrects for overlap from the emission spectra of neighboring fluorochromes. As your experiments become ever more complex, the importance of accurate and automated compensation becomes more pronounced.
- **Transformation** - uses custom biexponential functions to include zero and negative value events on scale, maintaining a logarithmic distribution of the positive cells.
- **Derived Parameters** - supports the creation of new parameters through the algebraic combination of others, including a wide variety of functions and formula structures, analogous to computed columns in a spreadsheet.
- **Calibrated Parameters** - calculate the quantitation of absolute molecules per cell (MESF) using reference markers with known calibration values.
- **Kinetic Analysis** - measure the change in signal of dyes, or ratios of dyes, over time. Generate statistics and subpopulations based on the subset of cells that have responded to stimulation.
- **Cell Cycle Analysis** - computes the percentage of cells in each phase of the cell cycle, based on a dye measuring the amount of DNA present in the cells (eg. 7AAD, PI, DAPI) FlowJo is the first package to integrate multidimensional cell cycle analysis, using BrdU or similar markers to differentiate S phase cells.
- **Proliferation Studies** - model the frequency of successive generations, and gate cells by the number of divisions undergone, as measured by intensity of CFSE, or other intracellular dye.
- **Templates** - provide the way to repeat and automate experimental processes. Drag new data into the template and it is analyzed and displayed just like the first run.

You can learn more about the program at the links below.

Next Steps...

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

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

*For a more in depth tutorial, download the Advanced Tutorial at:
http://offsite.treestar.com/downloads/AdvancedTutorial06_09.zip*

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

We seek your feedback! flowjo@treestar.com