Basic Tutorial

Data Analysis Software for Flow Cytometry

Windows/Mac user documentation

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ACTIVITIES

Introduction

FlowJo® is an integrated environment for viewing and analyzing flow cytometric data, called a workspace. The workspace lists all of the sample data files that you load, displays the gates, statistics, and other analyses that you apply to those samples, and provides access to tools, such as graphical layouts and statistics tables, that you can design to illustrate and export your analysis.

The workspace is saved as a document with the file name extension .wsp. When you reopen the .wsp document, you see the status of your analysis as it was when it was last saved.

This tutorial is designed to introduce you to the basics of the program. Reading through and performing the steps using the provided data and workspace will give you a feel for the workflow through FlowJo.

The tutorial data is a 9-color panel designed to interrogate phenotypic and functional responses of human T cells following stimulation with a mitogen. (See www.ncbi.nlm.nih.gov/pubmed/24464647 for more information about the tutorial data.)

Getting Started with FlowJo

Download and Install FlowJo

To begin this tutorial, you first need to install FlowJo. The easiest way to do this is to download the installer from the following link: www.flowjo.com/solutions/flowjo/downloads

- Double-click the link for your platform (Mac or PC) to download the installer, then double-click the installer, and follow the prompts.
- Detailed step-by-step installation instructions can be found at: http://docs.flowjo.com/d2/installation/installweb_exe

Launching FlowJo for the First Time

Upon launching the program, you are presented with a licensing dialog box. Agree to the end-user licensing agreement by checking the "I Agree" box. If you already have a license for FlowJo, check the appropriate box next to dongle or serial number license, and if you choose the latter option, type in the serial number provided by FlowJo or your site license administrator.

> **NOTE:** To utilize the files distributed with this this tutorial, you must have a valid FlowJo license.
> A free 30-day trial license is available using the link below. If you choose to continue without selecting a license type, FlowJo can only load specifically enabled demonstration files, such as the 8-color data files.

Free 30-Day Trial

To obtain a free 30-day trial serial number, visit: www.flowjo.com/solutions/flowjo/free-trial

Purchasing FlowJo

Send inquiries on purchasing a FlowJo license to: office@flowjo.com

Tutorial Data Download

Basic Tutorial data and 8 Color PBMC Tutorial data downloads: www.flowjo.com/solutions/flowjo/downloads/tutorials

Technical Support

If you have specific questions regarding the functions or features of FlowJo, email Technical Support: techsupport@flowjo.com

Lesson 1—The FlowJo Workspace Window

Components of the Workspace Window

When you first open FlowJo, you are presented with the Workspace window, which is your starting point in FlowJo. This window is divided into three horizontal sections. At the top is a ribbon containing action buttons, which initiate tasks and functions. Below the ribbon is a medium-sized Groups pane. Below the Groups pane is a larger Samples pane. (When there are no samples in the workspace, the Sample pane reads "Drag Samples Here.")

The Taskbar and Ribbon

At the very top left of the FlowJo Workspace window is the Taskbar, which contains a row of static buttons, starting with a FlowJo pyramid icon button at the far left, followed by right and left arrows, and so on. These buttons perform major functions within the workplace, including adding samples, creating groups, and opening the Layout Editor and Table Editor windows.

Below the Taskbar is the ribbon, which contains six tabs with the titles FlowJo, File, Edit, Workspace, Tools, and Configure. Clicking a tab gives you access to a different set of action buttons in the ribbon. Each tab is further divided into bands, each with a title at the bottom. For example, the FlowJo tab contains the Navigate, Experiment, Biology, and Help bands.

Notice that if you hover the mouse pointer over any button, a tooltip appears giving more detail about that action, along with a hotkey to initiate that action.
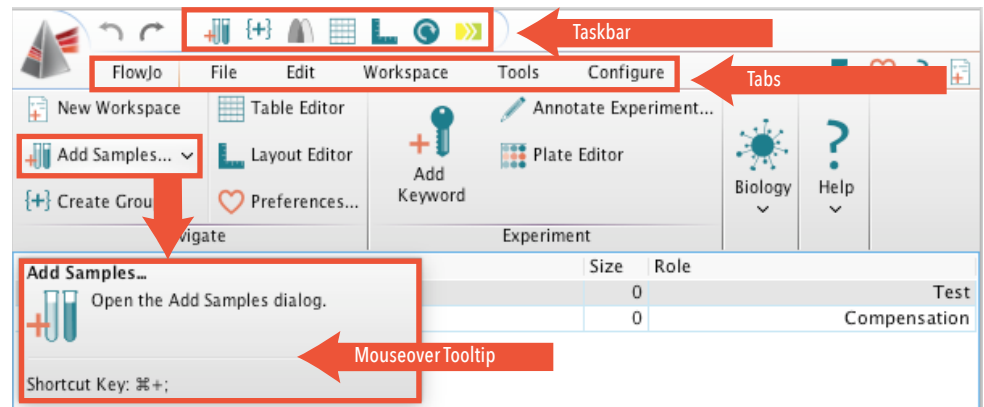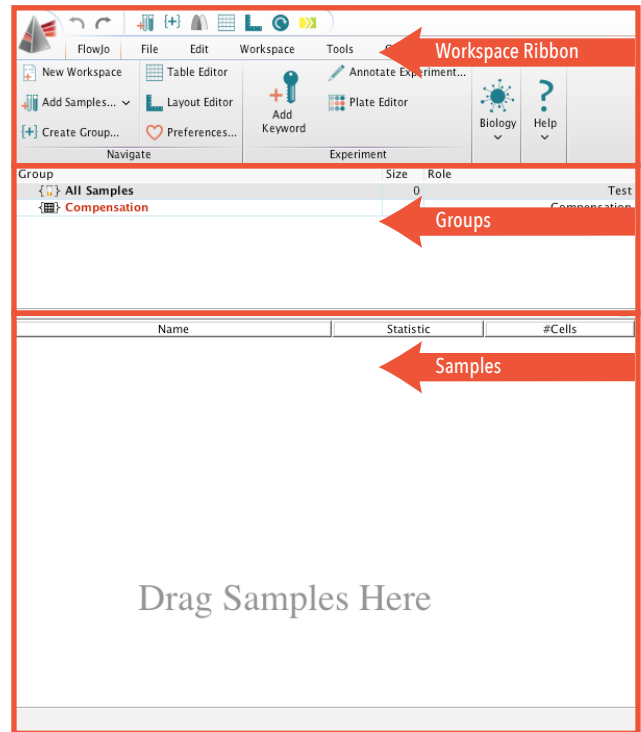
▮ ACTIVITY Add Samples to the Workspace

Let's start by adding samples to a new FlowJo workspace.

1. Find the Basic Tutorial Data folder, which you downloaded with this tutorial.
2. Simply drag and drop the Basic Tutorial Data file folder into the Samples pane.

The sample files load into the FlowJo workspace and appear as part of a new group called Basic Tutorial Data. The files also populate the "All Samples" group. Sample file names are displayed in the Name column of the Samples pane.

Alternatively, you can add samples using the Add Samples action buttons. One is located on the Taskbar, and the other is located in the Navigate band of the FlowJo tab.

1. Click Add Samples.
2. Using the file browser, navigate to the Basic Tutorial Data folder.
3. Select/highlight the Basic Tutorial Data folder, and click Choose.

In the same way, the sample files load into the FlowJo workspace and appear as part of the "All Samples" group. Note that a group with the file folder name is only created if you load at the level of a folder that contains sample files.
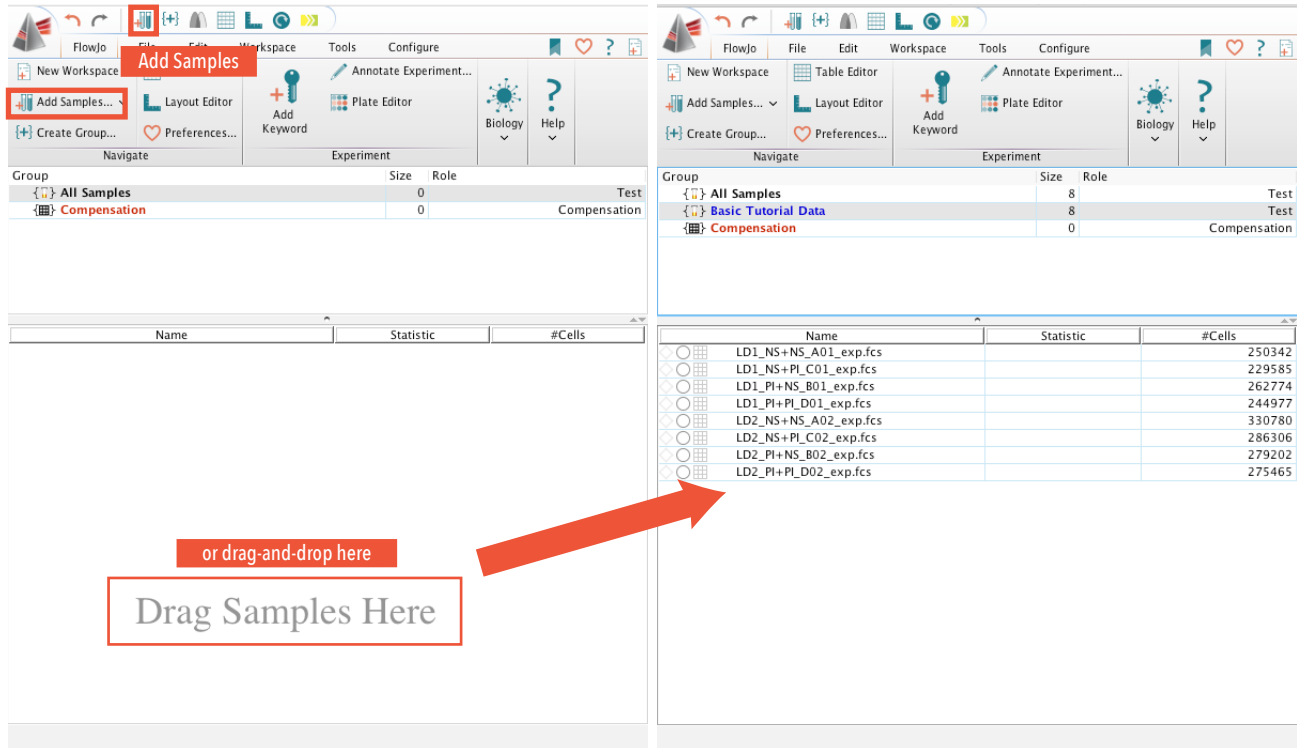
The Groups Pane

Located within the middle of the FlowJo Workspace window, the Groups pane is populated by default with two groups.

• The All Samples group, which contains all data files loaded into the workspace.
• The Compensation group, which automatically includes samples that contain the word "comp" or "unstained" in the file name.

Groups allow you to organize your sample files so that you can perform analysis functions on subsets of samples within the workspace.

Depending on your experimental setup, you can and should use groups to:

• Separate experimental all-stain samples away from single-stain compensation controls,
• Organize samples by their file keyword attributes (metadata information such as patient ID, stain reagent panel, stimulation condition, or time point),
• Apply a unified gating structure to a set of samples stained with the same reagent panel,
• Create graphical reports and tables specific to the files contained in the groups.

ACTIVITY Create a New Group

To get a feel for groups, let's create a new group.

1. On the Taskbar or in the Navigate band of the FlowJo tab, click Create Group.

2. At the top left of the window is an empty text field for entering a name for the group. In this field, type a name for the group, such as "My Group."

3. At the bottom right corner of the window, click Create Group.
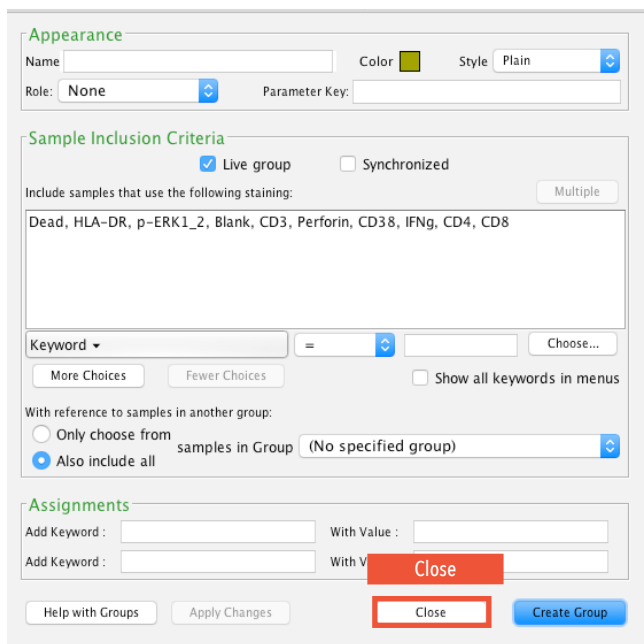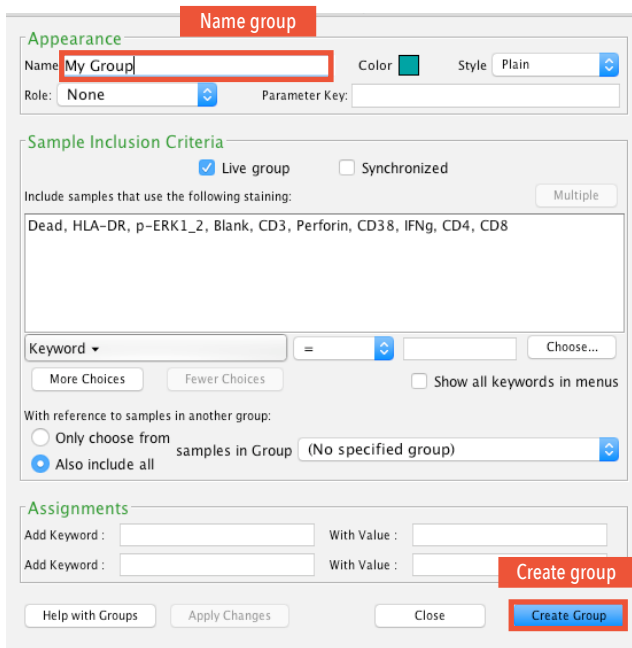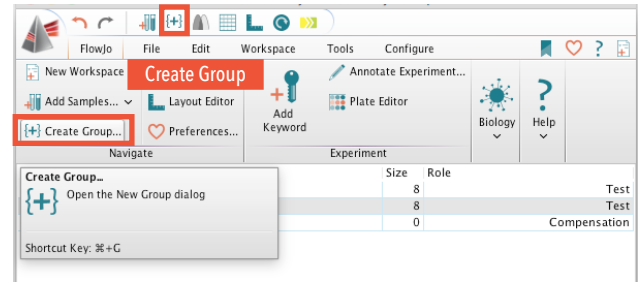
4. Click the Close button to close the dialog box.

Note that a new row appears in the Groups pane showing the group name. However, there are not yet any samples in this group, so no sample names are displayed in the Samples pane below.
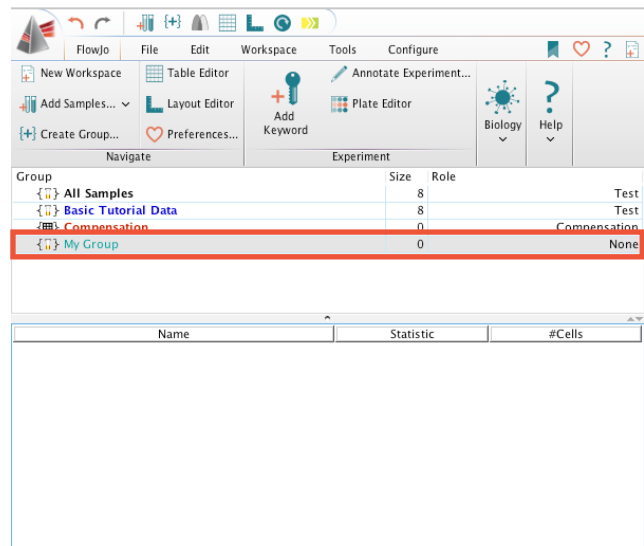
ACTIVITY Drag and Drop Samples to a Group

To manually place samples within your new group:

1. Click a group with samples in it to highlight it. This can be the All Samples group or the Basic Tutorial Data group. Select the first four samples in the Samples pane (all include LD1 in the name).

2. Drag and drop those samples onto My Group. My Group now includes those samples.

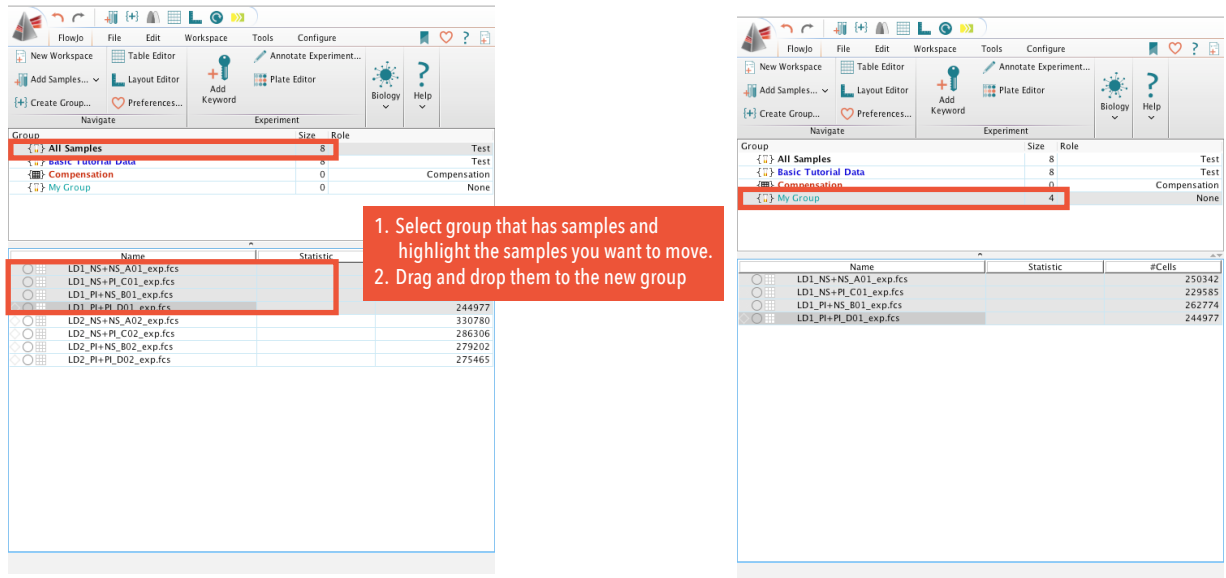Note that a single sample can be included in multiple groups (the samples you added to your new group are still present in the All Samples and Basic Tutorial Data groups). To remove a sample from a group:

1. Select the group you wish to remove samples from.

2. Select the samples you wish to remove.

3. On your keyboard, press Delete.

4. In the "Are you sure you want to remove sample(s)?" prompt, click Yes.
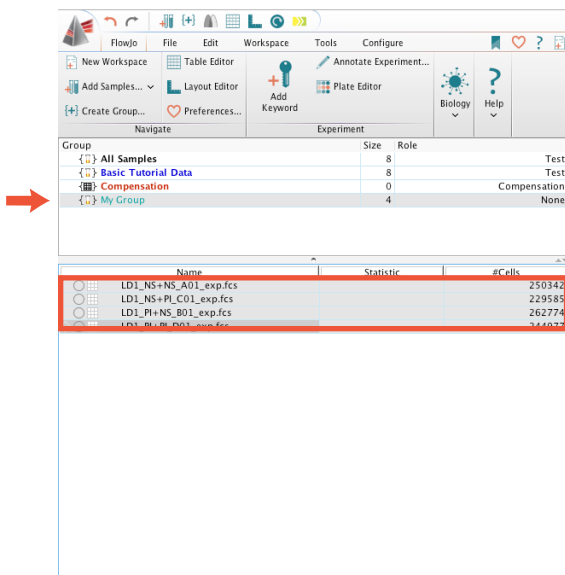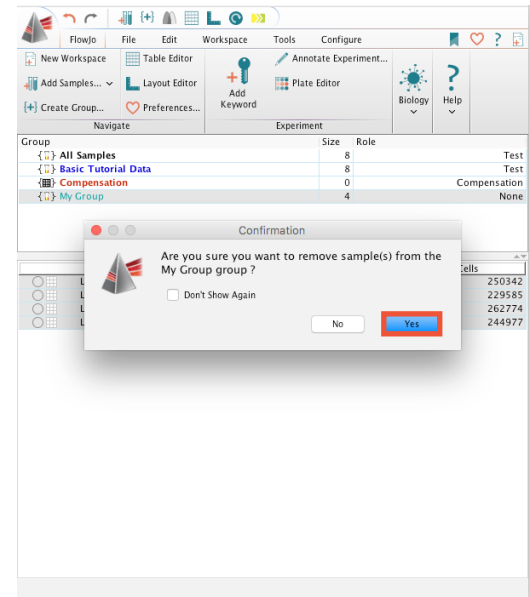
The selected samples are removed from the selected group. Selecting and deleting a sample from the All Samples group removes those samples completely from the FlowJo workspace. Deleting a sample from any group other than All Samples only removes that sample from the selected group. If you accidentally remove a sample from the workspace and want to bring it back in, simply drag and drop that sample file back into the Samples pane, or use the Add Samples action button. The sample loads back into the workspace.

The Samples Pane

The bottom section of the Workspace window is the Samples pane, which displays a list of the cytometric data files within the group you have selected in the Groups pane.

The Samples pane displays three default columns:

1. **Name**—Displays the file name as written in the FCS standard ($FIL keyword attribute).

2. **Statistic**—Displays calculated statistics for the analysis we apply to the sample files.

3. **#Cells**—Displays the number of measured events contained within the sample file (or gated population once we apply an analysis gating structure).

Sample files are initially listed in alphabetical order by file name.

Sample Badges—Immediately to the left of the sample names is a series of three icons known as sample badges.

Compensation Matrix Badge (Square)

The square badge closest to the sample name is the compensation badge. This square icon presents in one of two forms:

1. An empty square, which indicates that the file does not yet have a compensation matrix applied, or

2. A filled-in latticework square, indicating that a compensation matrix has been applied to the file.

For these samples, the square is filled, because compensation was performed on the acquisition cytometer. Therefore, these data files already have a compensation matrix applied. Double-clicking the square matrix badge displays a window in which you can view the associated compensation matrix and preview its effect on the data. This window is covered in further detail in the FlowJo v10 Advanced Tutorial.

Sample Quality Badge (Circle)

The second (middle) badge indicates sample quality and provides access to sample properties.

Double-clicking this badge performs a sample quality check evaluating the stability of acquisition over time across all fluorescent parameters and brings up a sample Properties window with information on the samples' keyword attributes, acquisition cytometer instrument settings, and compensation.

The badge color indicates the stability of acquisition, ranging from blue (good quality), to green, purple, or red (poor quality). If the color is yellow or red, you might have had some clogging or other disruption in the acquisition of events during sample collection on the cytometer.

ACTIVITY Check Sample Quality and View Properties of a Single Sample

1. In the Samples pane, double-click the circle Sample Quality badge.

A properties window is displayed containing information on that sample, and a colored dot appears in the circle badge. Blue or green indicated good stability during sample acquisition.

ACTIVITY Check Sample Quality for all Samples in the Workspace

You can also perform a sample quality check on all samples in the workspace by clicking Check Sample Quality, which is located in the Cytometry band of the Tools tab.

1. In the ribbon, click the Tools tab.

2. Click Check Sample Quality.

A colored dot appears in the circle badges, indicating sample quality/ stability during acquisition.
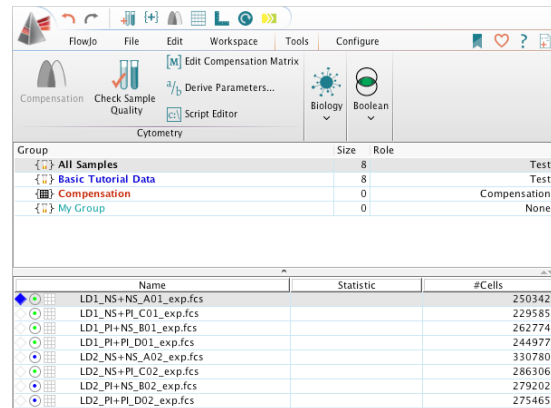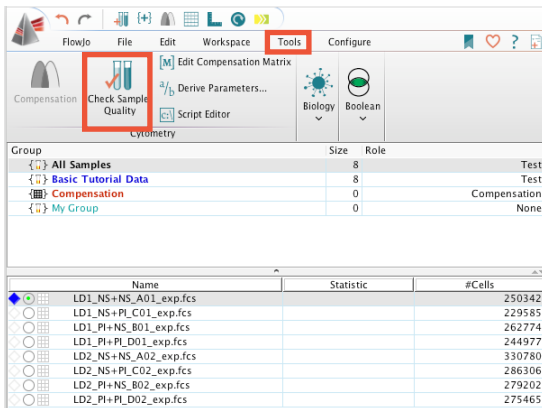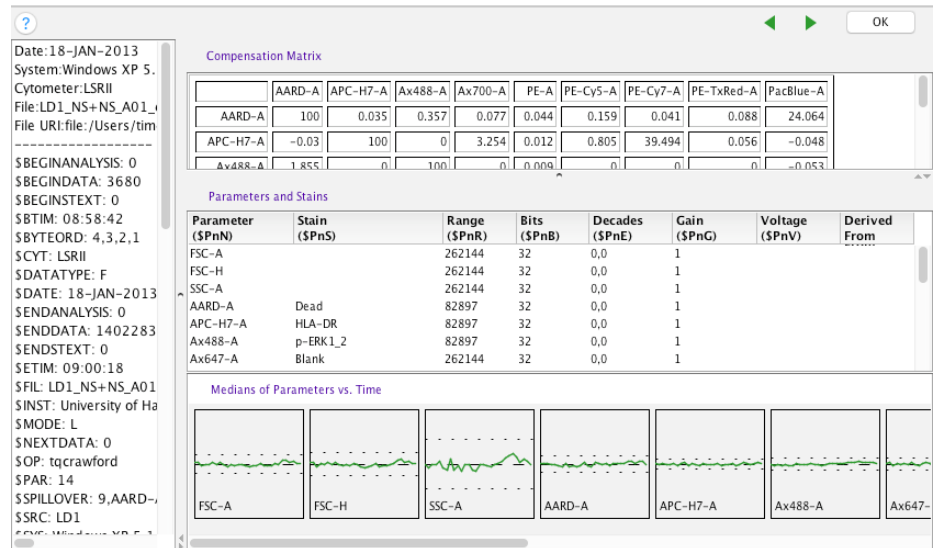
Population Marker Badge (Diamond)

The leftmost badge references which samples and populations you are currently viewing in a Sample Preview or Graph window (explained in the next section).

If you double-click a sample name in the Samples pane, a Graph window opens displaying a dot plot. The diamond marker badge next to the sample name turns blue, indicating this sample is being viewed in the active Graph window. A solid blue badge indicates that sample's Graph window is selected, and a hollow blue badge indicates that sample is displayed in a Graph window, but that Graph window is not currently selected.

<p style="text-align:center">END of Lesson 1—The FlowJo Workspace Window</p>

Lesson 2—The Graph Window and Gating
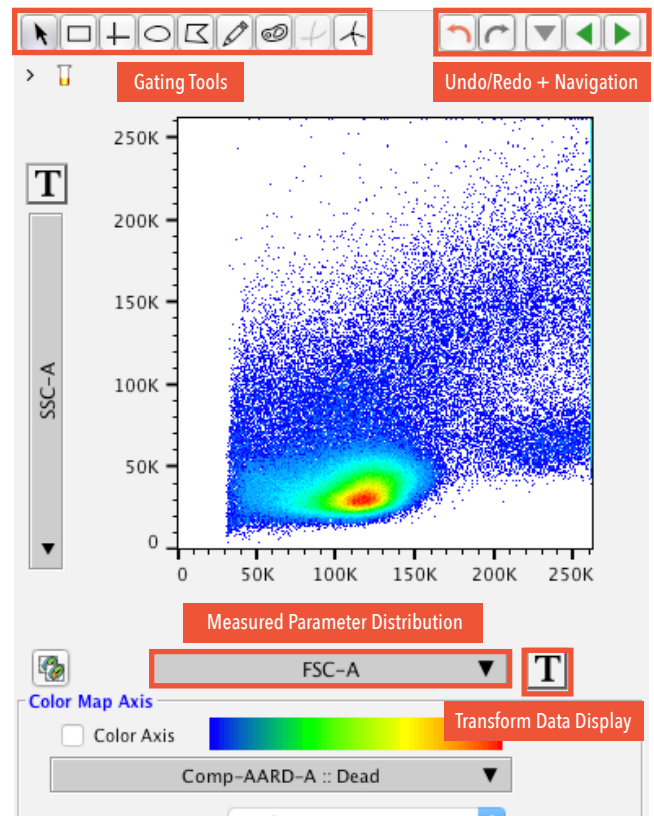
The Graph Window

The Graph window displays a graph plot representation of the data in a cytometry file or a population within it. This representation allows you to visualize the distribution of collected events (in this case human lymphocytes) along one or two measured cytometric parameters.

(Note that in flow cytometry, a 'parameter' generally refers to a specific fluorescent pulse measurement collected in a single channel on the cytometer instrument. These measurements can represent the expression levels of specific cellular proteins or represent other features of a cell, such as size, viability, DNA or membrane content, or functional processes like metabolism and signaling.)

The Graph window provides tools to draw geometric gates on the events distributions. Creating a gate isolates the events included within the gate, producing a 'child' population from the parent set of events. Creating a series of gates on different measured parameters allows you to build a gating hierarchy or gating tree, which subsets the measured events down to specific populations based on their expression of the measured parameters.

◼ ACTIVITY Open a Graph Window

1. Double-click the first sample in your workspace: LD1_ NS_NS. (Click the sample name, not any of the symbols to the left.) This opens a Graph window plotting events along forward scatter (FSC) versus side scatter (SSC) parameters.
   Notice that next to the sample name in the Workspace window, the blue diamond population marker badge fills with a solid blue color.
   Each dot on the graph plot represents one or more individual cells on which a set of cytometric measurements were taken. The density of events at a given position in the plot is color-coded, with red representing the highest number of events at that point in the graph, while yellow, green, and blue represent progressively lower event densities.

Components of the Graph Window

Gating tools are located at the top left corner of the window.

**Undo/Redo** and **Navigation** buttons can be found at the top right.

**X** and **Y axis Labels** specify which measured parameters are represented in the graph plot. Clicking the label for an axis allows you to change which parameter is displayed on that axis.

A **Transformation (T)** button is found next to each axis label, which allows you to change the X or Y axis scaling.

Additional plot and gate display **options** are located in expandable bars at the bottom of the window.

■ ACTIVITY Draw an Ellipse Gate

Let's isolate the major lymphocyte population here with an ellipse gate. The lymphocytes are the majority population in this plot, where the red, yellow, and green data points represent higher-density regions of events. To create and ellipse gate:
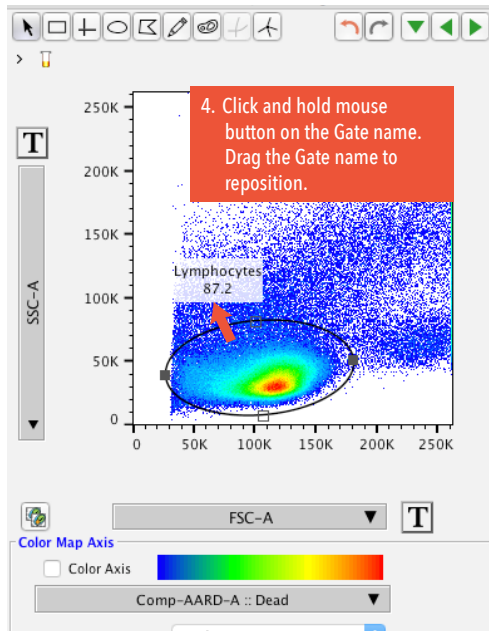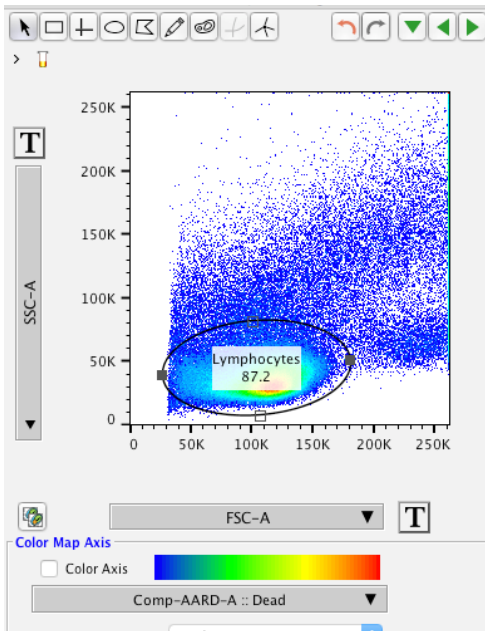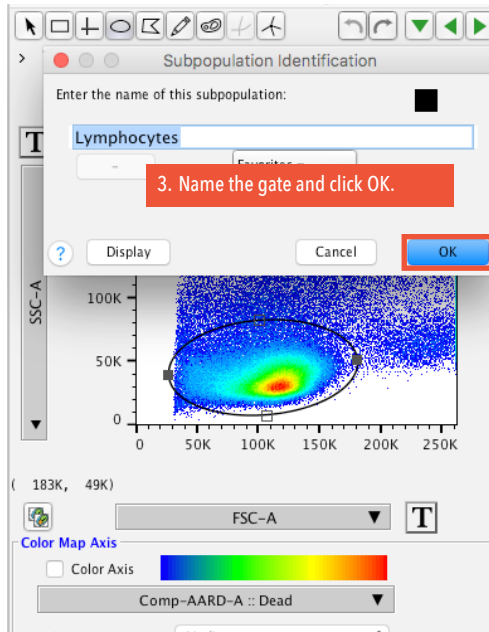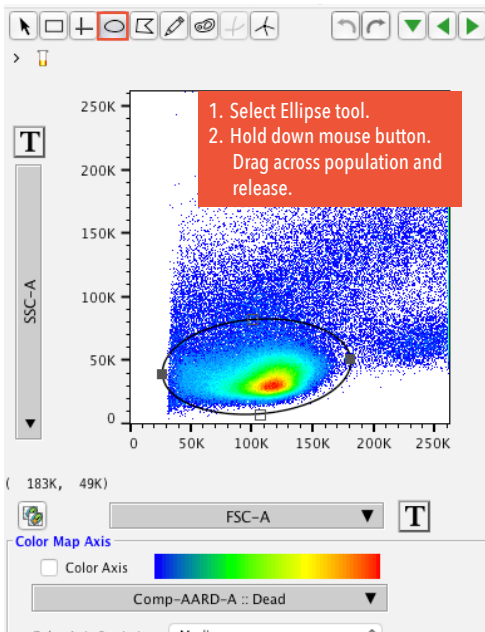
1. Click the Ellipse tool.

2. Position the mouse cursor at one edge of the population you want to isolate. Click and hold down the mouse button, drag to enclose the population, and release.

3. Name your gate or use the suggested "Lymphocytes," and press return or click OK. Your gate is now created and appears on the graph plot. Notice that the gate name and a statistic (called Frequency of Parent) appear along with the gate. This statistic represents the percentage of events from the parent population (the events displayed in the graph plot) that are contained within the child gate. Frequency of Parent is the default gating statistic.

4. Click and hold down the mouse button on the Gate label, and drag the label away from the gate.

5. Click and hold down the mouse button on the ellipse gate (not the label). Try dragging the gate to a different position in the plot, and release the mouse button. Notice how the frequency of parent statistic updates when the gate is moved.

6. Click and hold down the mouse button on one of the square gate handles along the edge of the gate, and drag the handle to modify the size or shape of the ellipse.

7. Try to resize and orient the ellipse so that it encompasses the major population.

8. When you are happy with the size and shape of your ellipse gate, double-click within the gated area. This opens a new Graph window isolating the events in that gate based on FSC and SSC parameters.
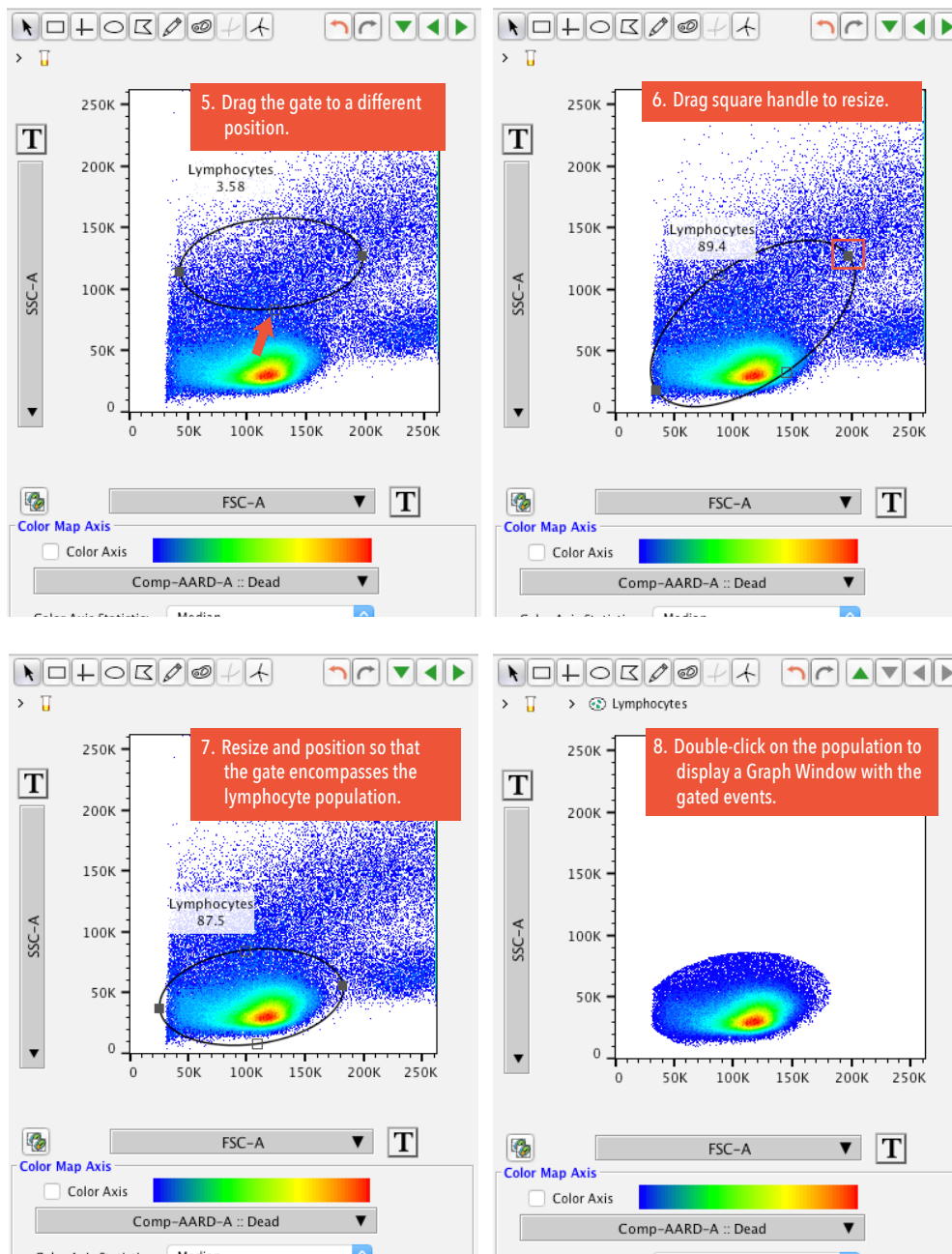
ACTIVITY Draw a Polygon Gate to Isolate Live Cells

Amine reactive dyes (ARD) are a live/dead discriminating stain reagent. Dead cells, having permeable plasma membranes, take up the dye and fluoresce brightly in the ARD channel, while live cells with intact plasma membranes exclude the dye and have a low ARD signal. Therefore, to isolate the live cells, we will gate on the ARD negative population and exclude ARD positive events.

1. First, to display the ARD parameter in the graph window, click the Y axis Label. A list of available measured parameters will be displayed. Select the *Comp-AARD-A :: Dead* parameter. The Y axis will change to display the measured fluorescence of an aqua amine reactive dye (AARD).

2. Next, select the Polygon Gate tool.

3. Click within the plot to set the points to make up the polygon gate. Make as many sides as desired. Note that holding down the Shift key modifies polygon gate side creation by limiting the angle to 45-degree increments.

4. Double-click to close the polygon and create the gate.

5. Give your gate a name such as "Live" and press OK

ACTIVITY Change Plot to a Histogram; Draw a Range Gate to Isolate CD3+ T Cells

1. Double-click within the new "Live" gate. A new Graph window appears displaying only the Live events.

2. Click the X axis parameter label. From the list of available parameters, select Comp-Ax700-A :: CD3. Click the Y axis parameter label, and select Histogram. This changes the plot to a one-dimensional histogram. Notice that the available gating tools in the top left corner change, and display either a range or bifurcation gate.

3. Select the Range Gate tool.

4. Make a gate on the right peak of the bimodal CD3 distribution: On one side of the peak, click and hold down the mouse button, move the cursor to the other side of the peak, and release the mouse button.

5. Give your new range gate a name, like CD3+, and click OK. You have now isolated the events that express CD3, an essential component of the T cell receptor.
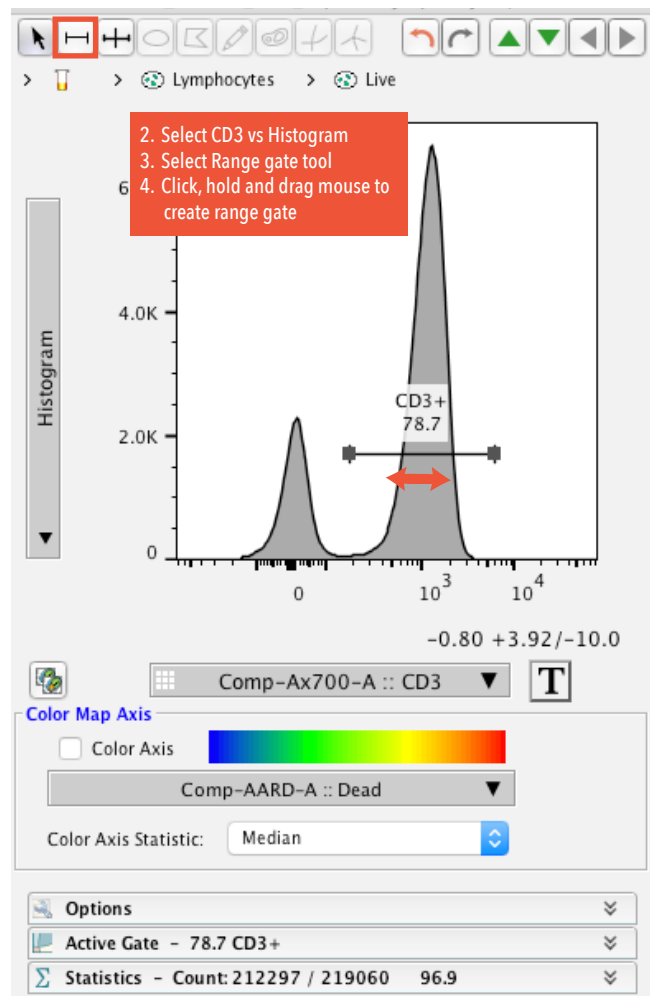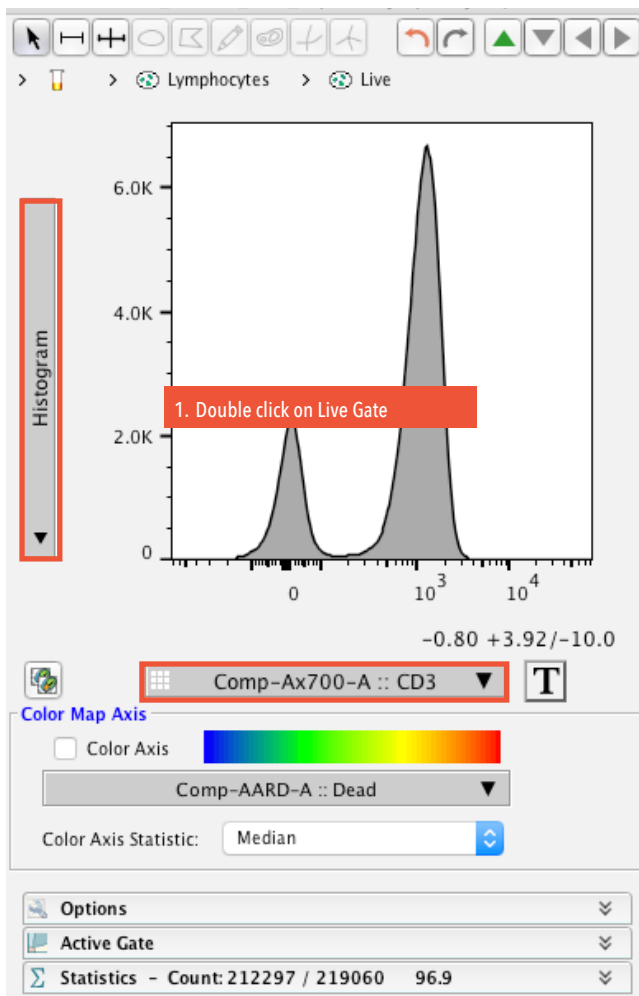
ACTIVITY Draw a Quadrant Gate to Isolate CD4+ Versus CD8+ T Cells

Now let's further subset the CD3+ cells into CD4+ T helper versus CD8+ cytotoxic T lymphocytes.

1. Double-click the CD3+ gate. A new Graph window opens containing only the CD3+ events. Change the X axis parameter to display Comp-PE-TxRed-A :: CD4. Change the Y axis parameter to display Comp-PacBlue-A :: CD8.

2. Select the Spider Quad Gate tool.

3. Position the mouse cursor at the location you want the common vertex of the quad gates. Click the mouse button once to make the quadrant gates appear. You have now created quadrant gates isolating CD4 vs. CD8 expressing T cell populations.

4. Click any of the square handles, and drag the handle to a new position. You can move the quad gate vertex and modify the angle of individual arms of the gate in this way.
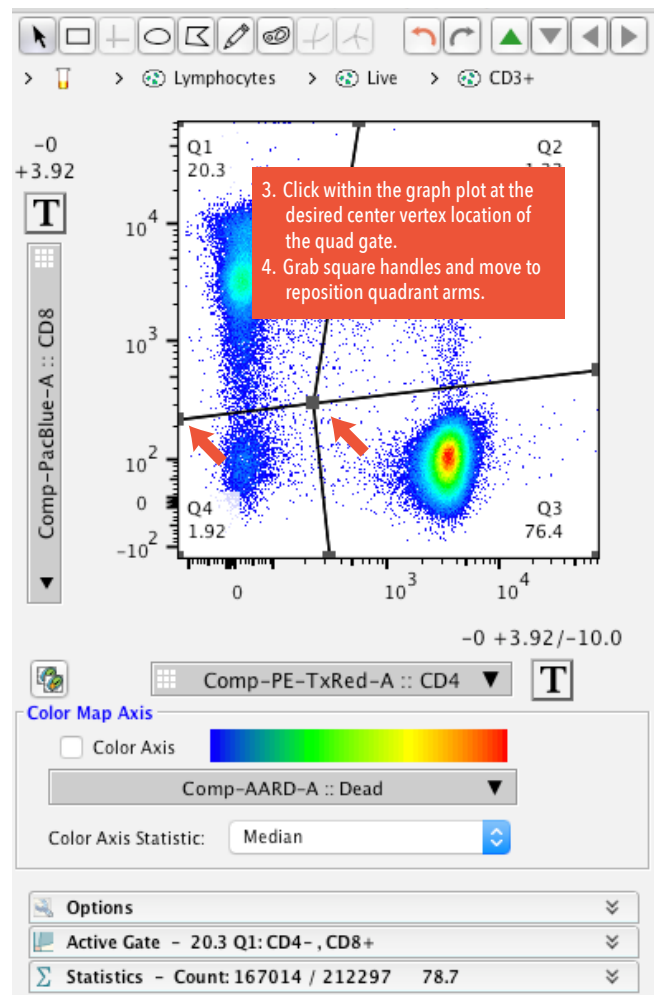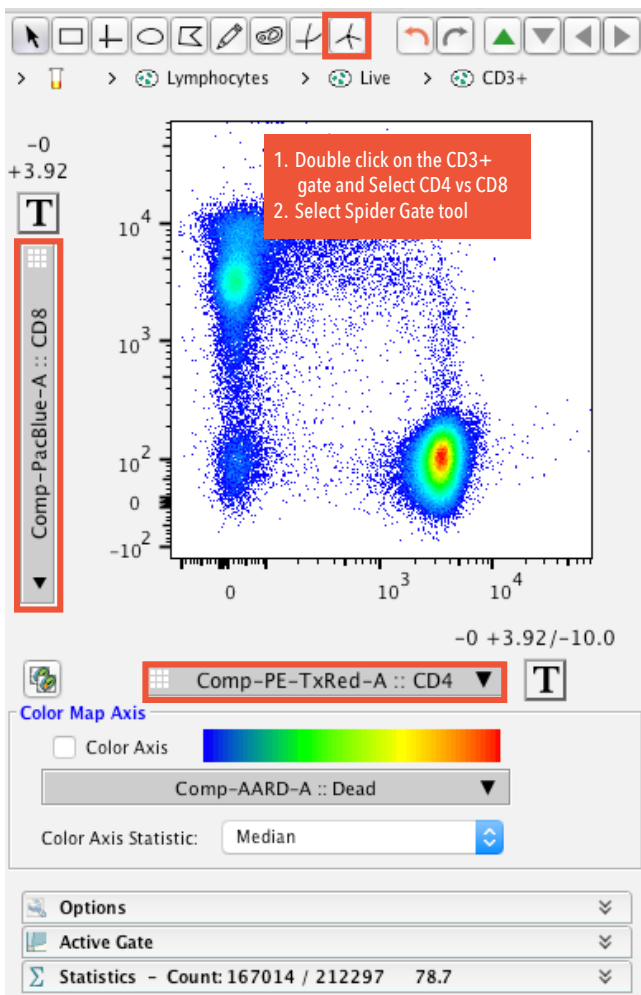
ACTIVITY Draw a Rectangle Gate to Isolate Perforin+ CD8+ Cytotoxic T Cells

Now let's focus on the CD8+ T cells and draw a gate to isolate the Perforin+ subset.

1. Double-click the Q1: CD4-,CD8+ population to open a new Graph window containing only the CD8+ T cells. Change the X axis parameter to display *Comp-PE :: Perforin*.

2. Select the Rectangle Gate tool. Position the mouse cursor where you would like one corner of the rectangle gate to be, click and hold down the mouse button and drag the cursor diagonally, then release the mouse button to create the gate.

3. Give your new gate a name, such as Perf+, and click OK. You have now isolated the Perforin+ cells within the CD8+ T cell compartment.
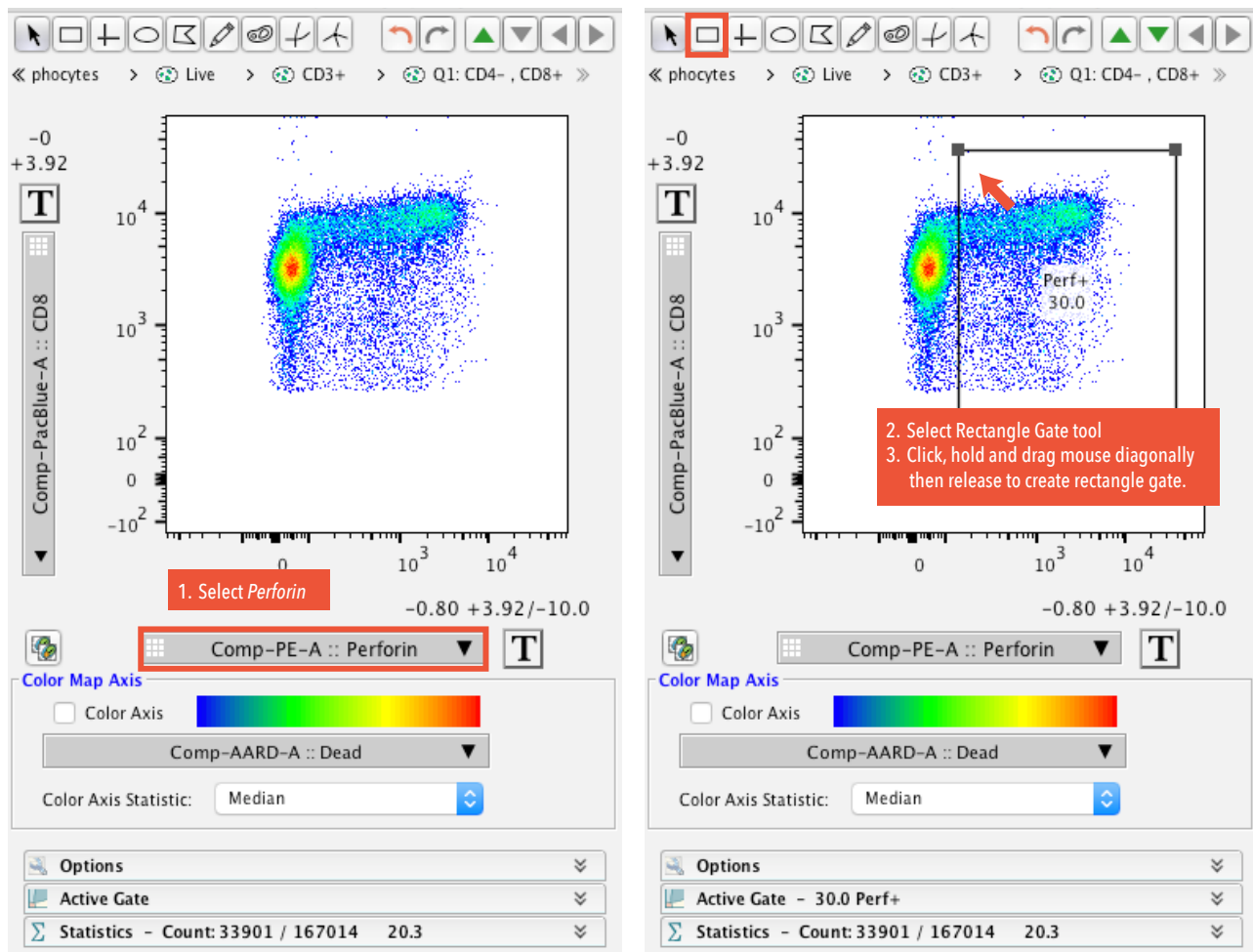
Modifying the Data Scaling and Visual Display using the T (Transformation) Button

Sometimes, depending on the type of flow cytometry file and how the acquisition software specifies the scaling of measured parameters, the visual display of data is not initially ideal for viewing the entire data distribution. Parameter scaling can be easily changed using the Transformation (T) button located next to the axis parameter label. Let's go ahead and modify the data display of the Perforin parameter and explore the scaling options available within a biexponential (Biex) transform function. Note that a biexponential transform function displays the data in a linear scale around zero and a log scale further out.

■ ACTIVITY Use the T (Transformation) Button to Modify the Graph Plot Scaling and Display

1. Click the T button next to the X axis Perforin parameter label, and select Customize Axis. This will open a Transformation window with the Perforin parameter displayed as a histogram.

2. Under the Scale options, notice that the Scale is already set to Biex by default. With Biex selected, you can:

   • Modify the maximum positive range displayed by using the -/+ buttons located to the right of the plot x axis label.

   • Add or remove extra negative range using the Extra Neg Decades slider bar.

   • Change the compression of the data around zero using the Width Basis slider bar.

3. In the Extra Neg Decades box, try moving the slider bar to the left. This shifts all the plot data to the left, giving less extra space on the low (negative) end of the scale. Try setting the Extra Neg Decades to a value of 0.

4. In the Width Basis box, try sliding the With Basis Notice that moving the slider to the right compresses the data around zero, and moving the slider to the left spreads the data out around zero. Set the Width basis back to 1 (-10).

5. On the right side of the window, highlight all parameters with a Comp- prefix.

6. Click Apply.
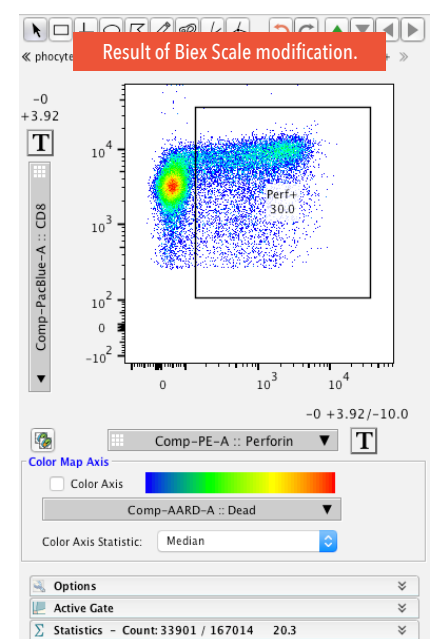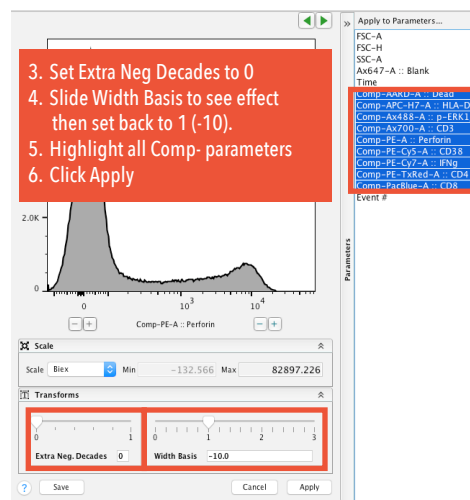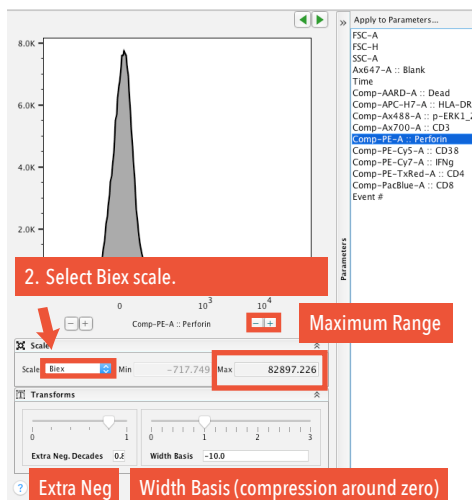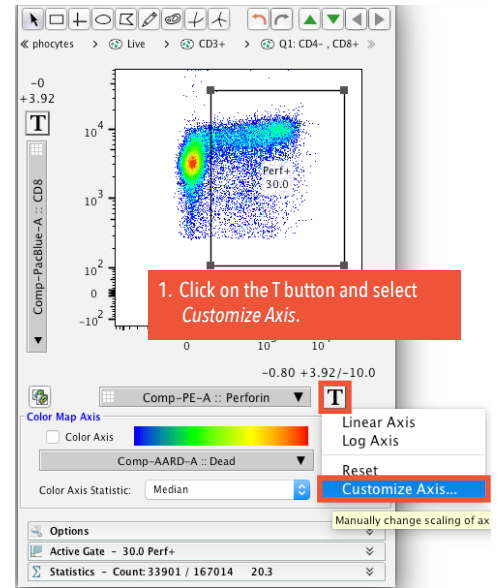
The Transformation window will close and all selected parameters will receive the scaling changes made. In this case, we're just getting rid of some of the extra unused white space on the low (negative) end of the scale by reducing the extra Neg Decades from 0.8 to 0, while keeping the same Maximum Range and Width Basis compression around zero. This gives us more visual space to view the distribution of the Perforin expression profile. Each measured parameter can be uniquely scaled to robustly visualize the distribution of events. Importantly, transformation does not change the raw data, only the way it is scaled and displayed visually in the graph plot.

Applying a Gating Hierarchy to a Group

Now that we've created a series of gates on one sample, we want to apply those gates to all samples stained with the same panel. To do this, we will apply the gates to a group of samples within the Groups pane of the FlowJo Workspace.

◼ ACTIVITY Group Apply the Gating Hierarchy

Click on the Workspace window. Notice that the gate names you created are displayed as indented populations below the sample file you have been working with in the Graph Window. The gate names are a black color and only appear below that single sample.

To apply these gates to a group:

1. Select/highlight the Basic Tutorial Data Group (which should contain all eight samples).

2. Select/highlight all the gate names listed below the single sample with gates.

3. Right-click (or Control-click) any of the highlighted rows, and select *Copy analysis to group*. The gating tree is applied to all samples within the group. Notice that the gates appear both under the samples (in the Samples pane) and under the group (in the Groups pane) Your gates now apply to the group and change from black to the color of that group.

Modifying a Group-Owned Gate

Once gates are applied to a group, they turn the color of that group to denote that they are group-owned and identical to each other. However, you can still modify a gate on a single sample.

■ **ACTIVITY** Modify a Group-Owned Gate on a Single Sample

1. Double-click the Q1 CD4- , CD8+ population node within the group-owned gating tree under a single sample in the workspace. This displays a Graph window containing those gated events displaying Perforin vs CD8 parameters.

2. Click the Perf+ Rectangle Gate tool to select it. Click on the gate and holding down the mouse button, move the gate to a slightly different position. Notice that when the gate is moved, the color of the population node within the workspace gating tree of that sample turns from the group's color back to black. The black color indicates that the gate on that sample is unique to that sample and is not the same as the other group-owned gates on the other samples in that group.

3. To apply the change you have made to the group:

    •   Select/highlight the gating group.

    •   Select/highlight only the black gate name. (You may need to click on a different gate that is not highlighted first to deselect all the group applied gates, then select the black gate name.)

    •   Right-click the selected population, and click *Copy analysis to group*.

    •   You receive a warning that the parent population already has a gate with the same name. Click Yes at the "Do you want to replace it?" prompt.

The modified Perf+ gate will apply to the group and turn the group color. Now all the samples in that group have received the modified gate and the gates are again identical.

1. Grab and move gate position

Black gate node color = modified gate not group owned

Apply gate change to group
• Highlight the gating group
• Highlight the black gate group
• Right-click and select *Copy analysis to group*

This sequence of steps works to update the group owned gating structure with any changes to gating or statistics that you make on individual samples. However, you may want your group owned gates to remain 'synchronized,' so that any modifications made to a group owned gate automatically applied to the group. This eliminates the need to copy a gate change to the group gating hierarchy. To synchronize group owned gates:

🟧 **ACTIVITY** Synchronize Group-Owned Gates

1. In the Groups pane, double-click the gating group name (example: Basic Tutorial Data). This opens a Modify Group window.

2. Check the Synchronized box.

3. Click Apply Changes.

4. Close the modify group window. Your group gates are now synchronized so that modifying a group-owned gate on a single sample automatically applies that change to all the samples in the group.

Because we are done creating gates for now, let's go ahead and close the open Graph windows to get them out of the way.

🟧 **ACTIVITY** Close Graph Windows

1. Select a single Graph window by clicking the window once.

2. To close this single Graph window, click the Close button at the top of the window.

3. To close all open Graph windows at once, use the hot key command: Shift+Command+W for Mac, or Shift+Ctrl+W on a PC.

End of Lesson 2—The Graph Window and Gating

Lesson 3—Adding Statistics

Statistics Band

The basic gating statistic in FlowJo is Frequency of Parent (Freq of Parent). However, you can also enumerate other statistics for gated populations using the Statistics band of the Workspace tab.

Options include event count, measurements of central tendency such as median or geometric mean fluorescence intensity (MFI or GMF), coefficient of variation (CV), or frequency of any upstream population in the gating hierarchy, among others.
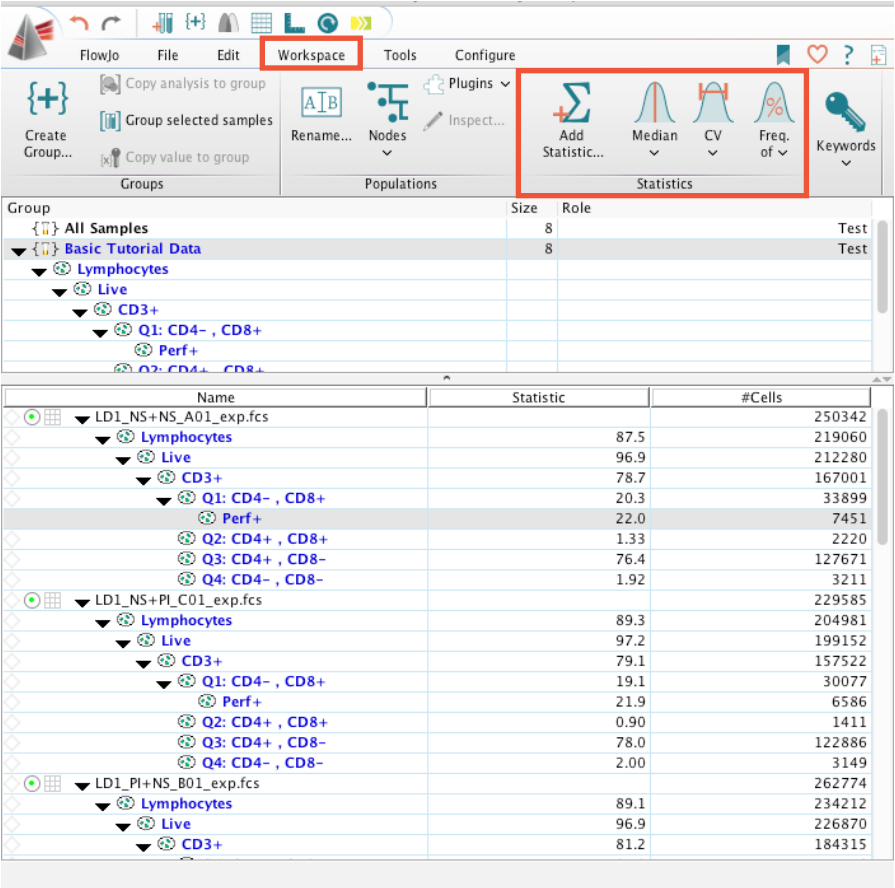
Note that if the Statistics band only shows a single icon, it is collapsed. Widen the Workspace window until the individual actions in the Statistics band are visible. If the buttons are grayed out, select a population.

Add Statistic—Brings up an Add Statistic dialog box with all statistics options.

Median—Displays a drop-down list to add a Median of any parameter to the populations selected in the workspace.

CV—Displays a drop-down list to add a CV in any measured parameter to the populations selected in the workspace.

Freq. of—Displays a drop-down list to add the Frequency of any upstream gated population.

▮ ACTIVITY Add a Median Statistic to a Gated Population, then Group Apply

1. In a workspace sample gating tree, select the Q1: CD4- , CD8+ population.

2. In the Statistics band of the Workspace tab, click the Median tool. A drop-down list of measured parameters appears.

3. Select the Median > Comp-Ax488-A :: p-ERK1_2 Parameter.
A black statistic node labeled Median : Comp-Ax488-A (p-ERK1_2) appears below the selected population.
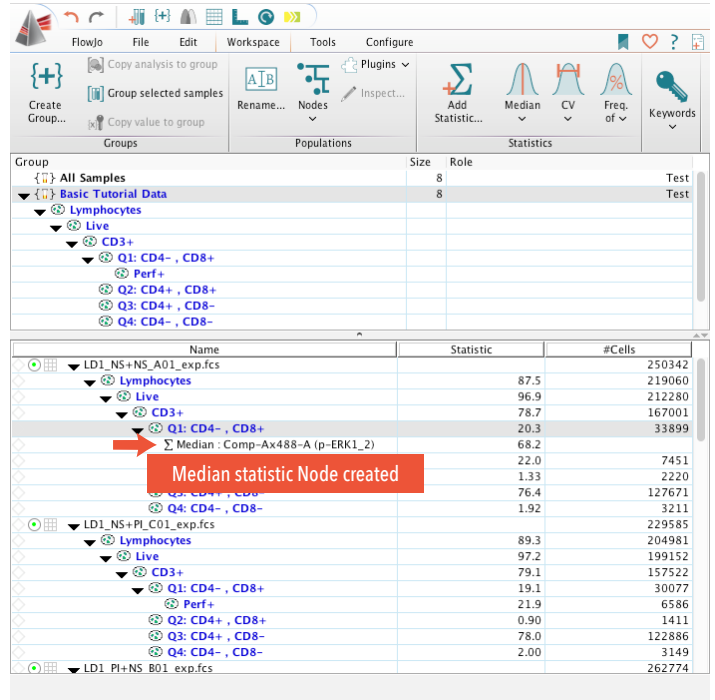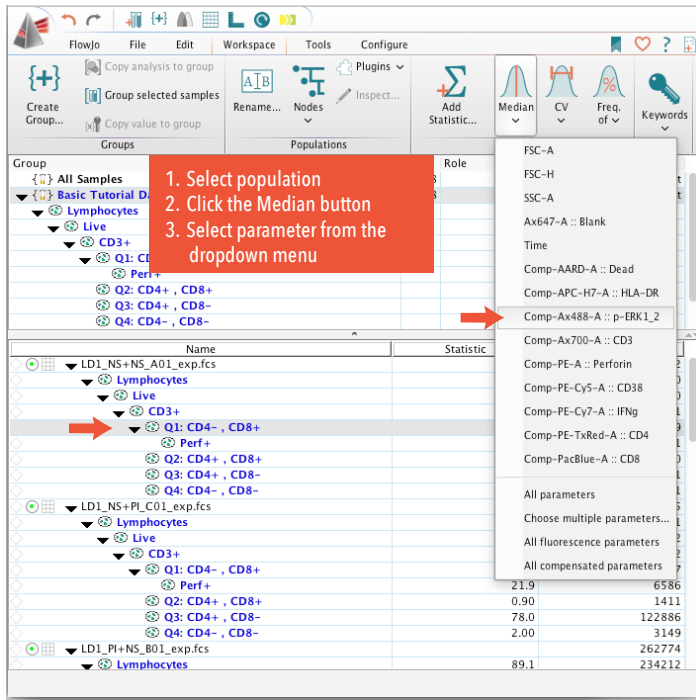
ACTIVITY Group Apply the Statistic Node

Now we want to apply that statistic node to the group gating tree. This works the in same way as applying a gate to a group.

1. Select the gating group.

2. On the single sample, select the black Median statistic node.

3. Right-click the statistic node, and select *Copy analysis to group*.

The Median statistic becomes part of the group-owned gating tree, and turns the color of your gating group. Now all samples in the group have that statistic applied.
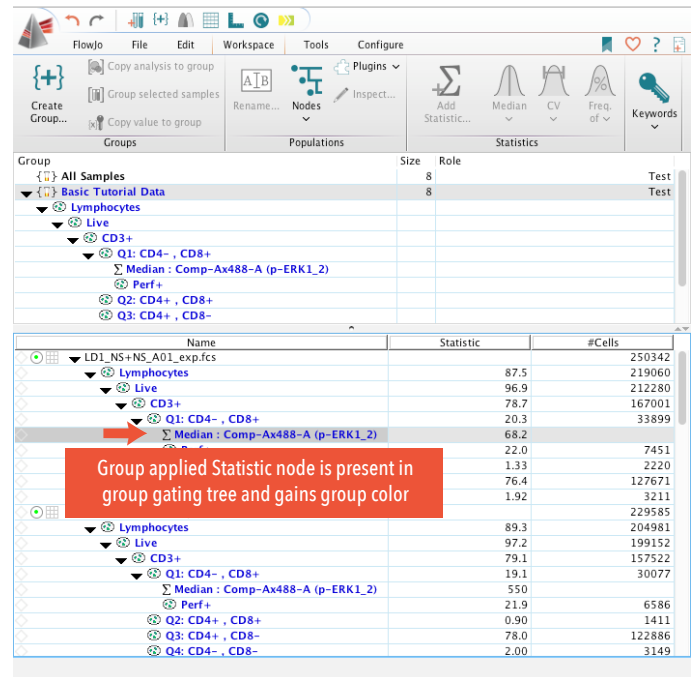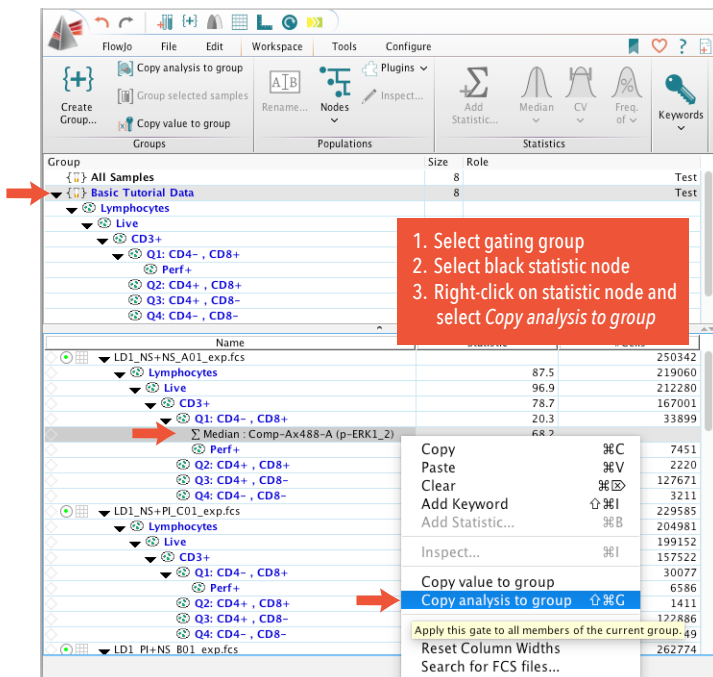
Add Statistic Window

The most versatile tool in the Statistics band is the Add Statistic option, which opens up a window allowing addition of any statistic to any population in the gating tree of a selected sample. This facilitates applying multiple statistics to different populations within a gating hierarchy in quick succession.

■ **ACTIVITY** Add Multiple Statistics using the Add Statistic Window

1. Within the gating tree of a single sample, select any population node. (Usually this is the first population you want to add a statistic to. In this example I will highlight the Q1 : CD4- , CD8+ gate.)

2. Click Add Statistic.

3. Select the Statistic you wish to add (Geometric Mean in the example below).

4. Select the Population you wish to apply the statistic to. (This field automatically populates with the population you highlighted in the sample gating tree. In this example I will first leave the default population, apply a statistic, then change to a different population and apply the same statistic (steps 7–8 below).

5. Select the Parameter you wish to enumerate (Comp-PE-A :: Perforin in the example below).

6. Click Add. This creates the new statistic in the sample gating tree, but the Add Statistic window remains open, allowing you to add additional statistics in sequence without reopening the window. Let's try adding the same statistic to a different population in the gating hierarchy.

7. Keeping the same Add Statistic window open, change the population by clicking the Population field, and select a different population from the drop-down list (example: Q3: CD4+ , CD8-).

8. Click Add. The statistic node is added to the selected population in the sample gating tree.

9. Close the Add Statistic dialog box by clicking the Close button at the top of the window.
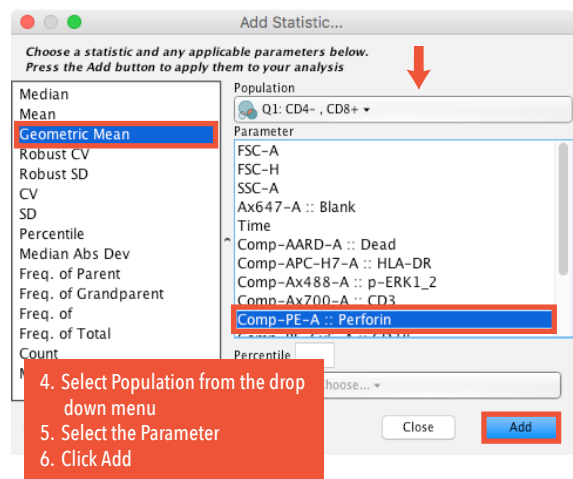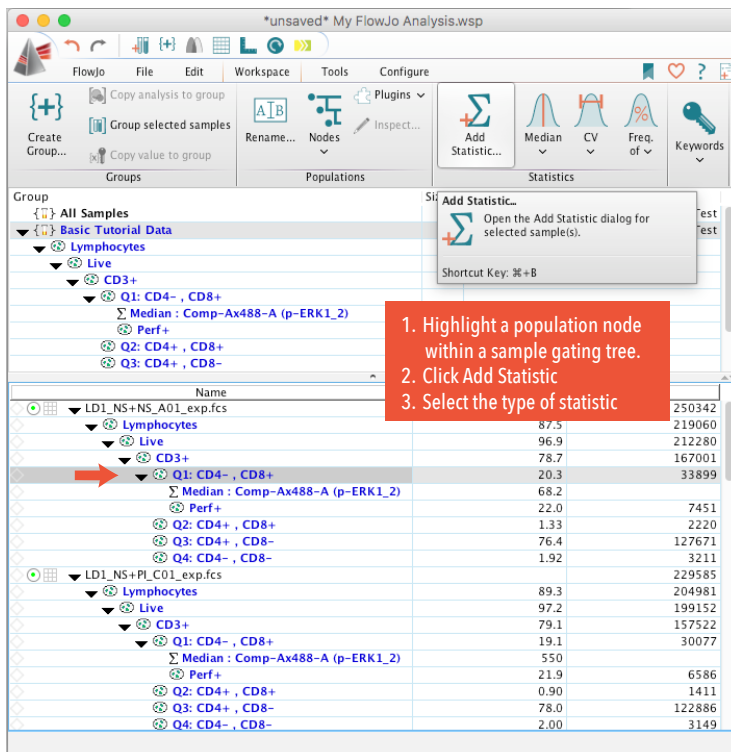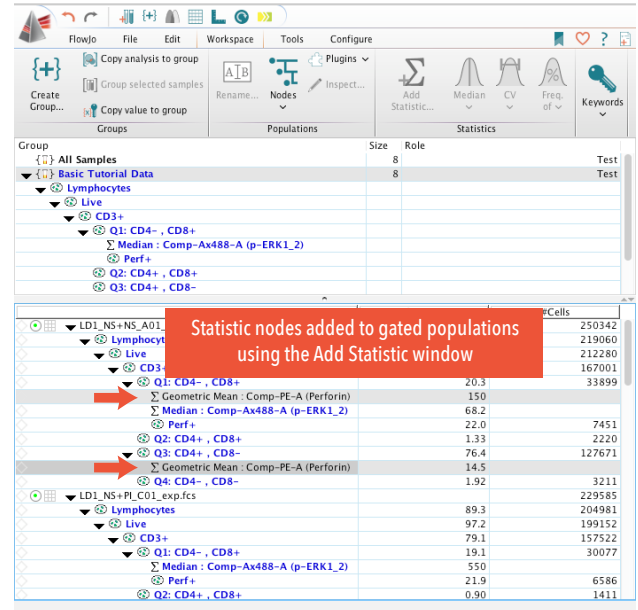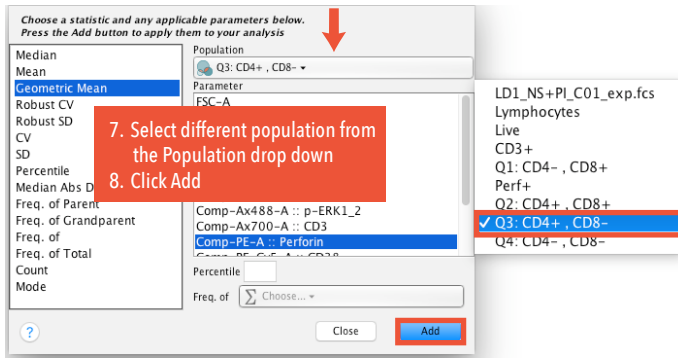
As we've done previously, you can apply the statistics nodes to the gating tree for the entire group:

1. Select the gating group.

2. Select both black statistic nodes on the single sample where they currently reside.

3. Right-click the sample, and select *Copy analysis to group*.
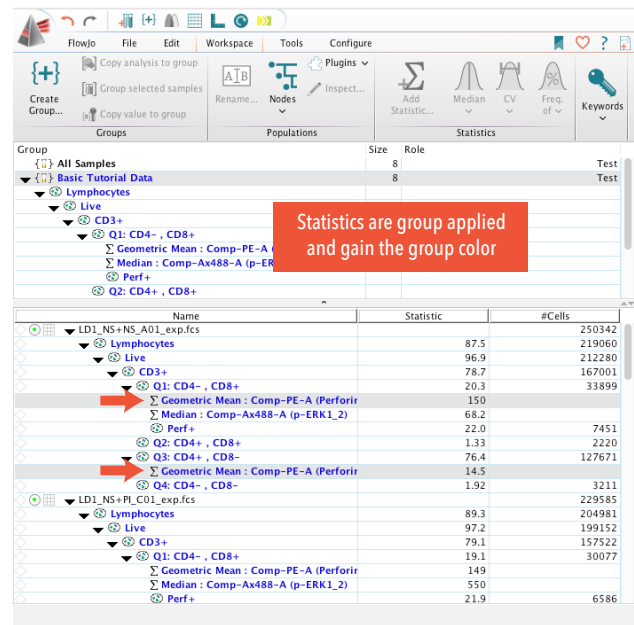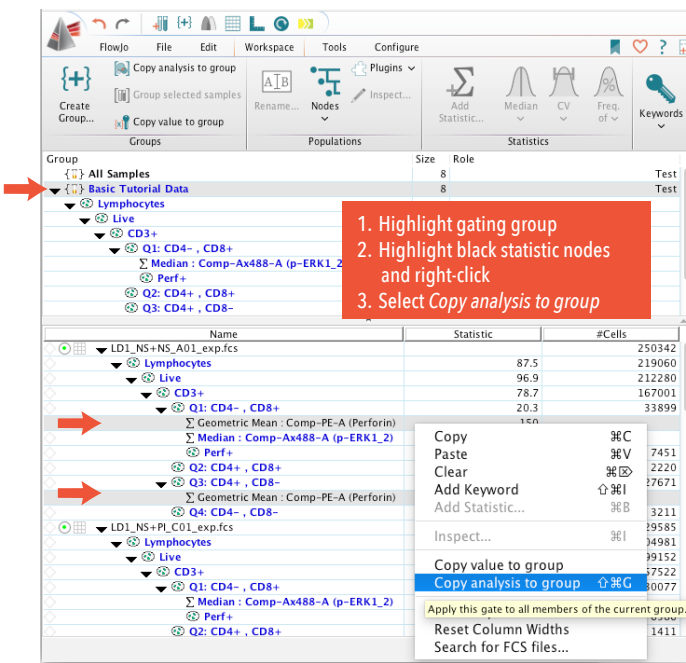

The statistic nodes are applied to the group gating tree and samples therein, at the correct position within the gating hierarchy.

<div align="center">End of Lesson 3—Adding Statistics</div>

Lesson 4—The Layout Editor

The Layout Editor is a platform with tools for displaying multiple graph plots and statistics in a graphical report, which resides in its own window separate from the Workspace window. Layout Editor reports can be exported as images or 'batched' across samples to generate customized external reports.
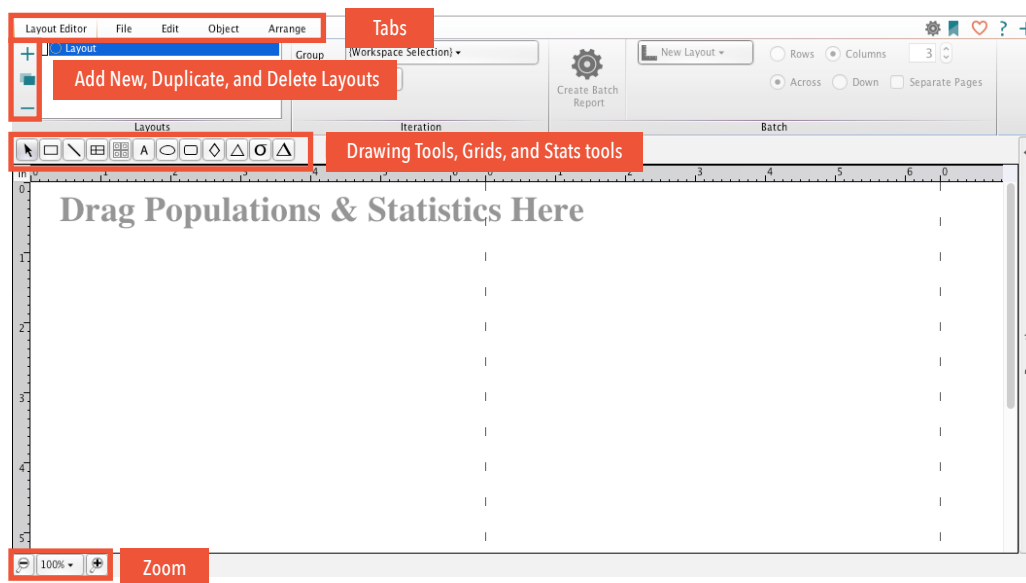
Components of the Layout Editor

Ribbon—The Layout Editor has its own ribbon with a series of five tabs to access the formatting, layout, and other functions.

Add New, Duplicate, and Delete Layouts—The default tab, called Layout Editor, presents a list of layouts. You can create, duplicate, or delete existing layouts using the three buttons to the left of the Layouts list. To rename a layout, double-click the layout name.

Drawing Tools, Grids, and Stats tools—A static toolbar with basic drawing tools, grids, and a plate statistic tool.

Zoom—Zoom functions are found at the bottom left corner.

> ■ **ACTIVITY** Drag a Gated Population to the Layout Editor

Let's bring a population into the current layout and view the graph.

1. On the workspace toolbar, click the L button to open the Layout Editor. Position the Workspace window and Layout Editor side-by-side.

2. From the gating tree of a sample in the Workspace window, drag a population (example: Q1: CD4- , CD8+) into the Layout Editor.

A graph plot displaying the gated events appears in the Layout Editor. Basic information, including the sample name, gate name, and event count, are displayed in a text box below.
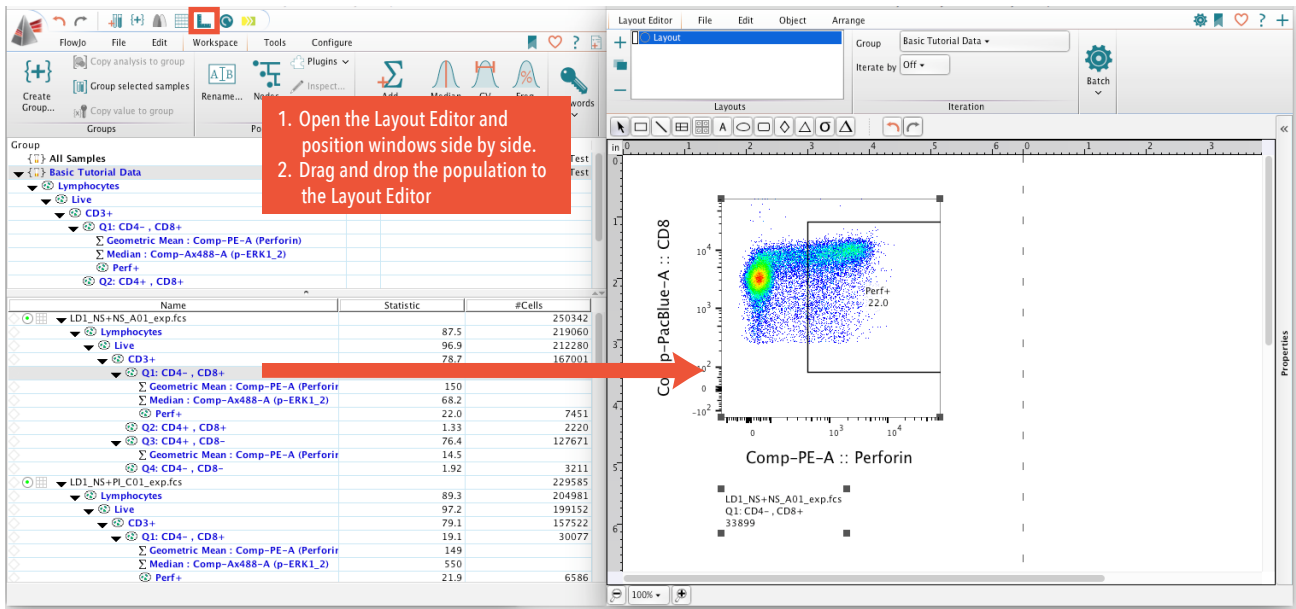
ACTIVITY Show the Gating Hierarchy Upstream of a Graph Plot Population

1. In the Layout Editor, right-click the graph plot.

2. In the drop-down list, select *Ancestry*.
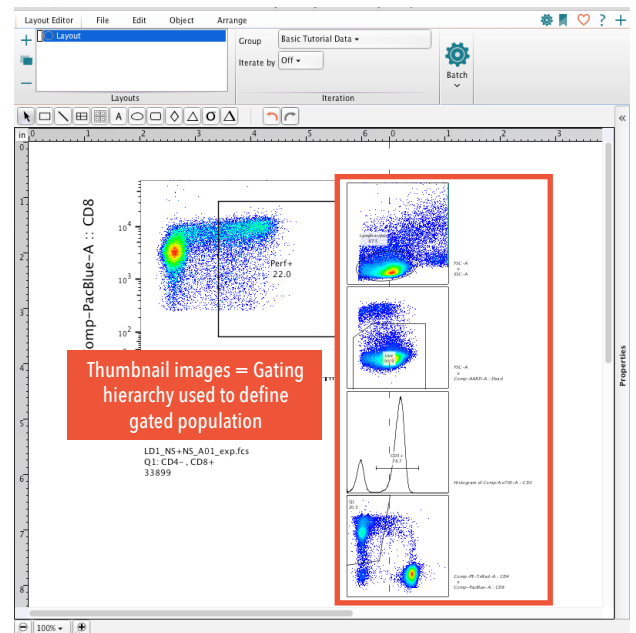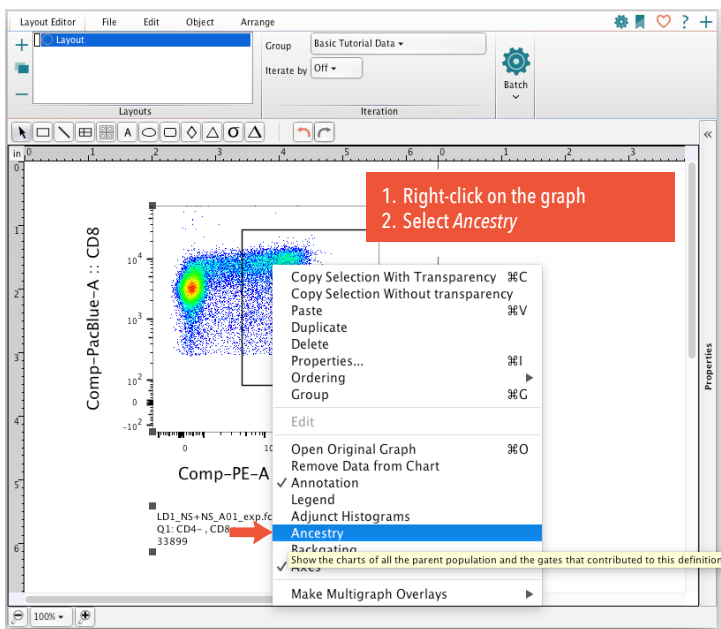
Thumbnail images are displayed to the right of the original plot showing the gating hierarchy used to define the population of events in that original larger plot.

Note: There are a number of useful actions you can access by right-clicking the graph plot, but you might need to resize the Layout Editor window, or use t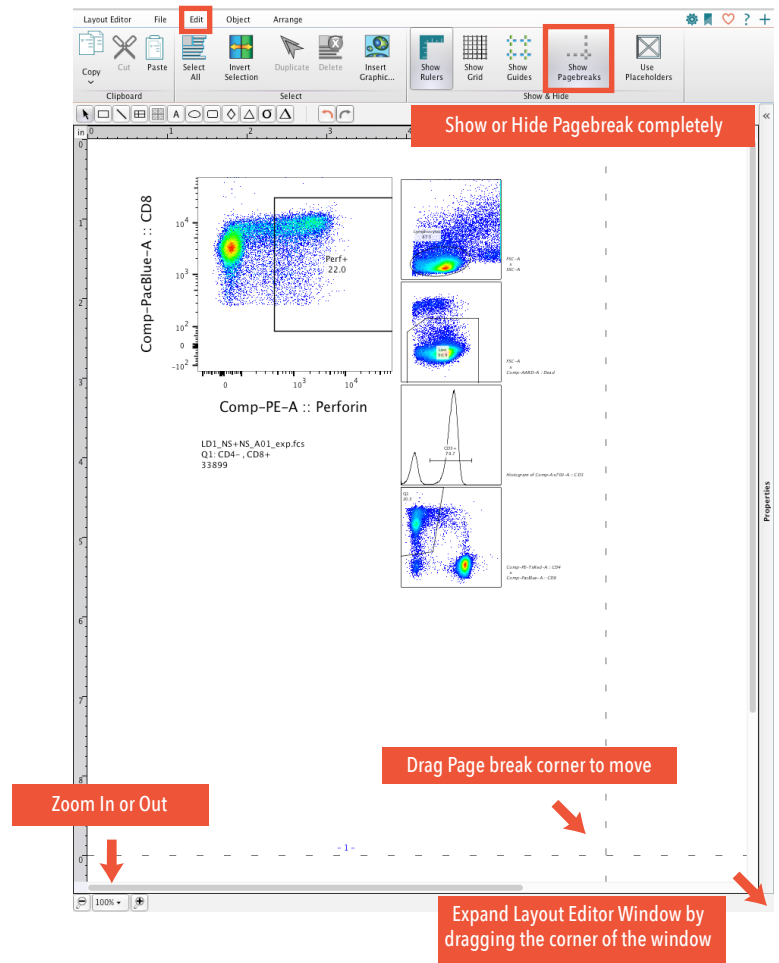he Zoom options, in order to see the entire series of thumbnail plots or other features you choose to display. You can resize and reorient the pages by dragging the intersection points of the dashed page break lines. The page break lines can also be hidden completely by toggling off by clicking the Show/Hide Pagebreaks action button (located under the Edit tab).

Show or Hide Pagebreak completely

Drag Page break corner to move

Zoom In or Out

Expand Layout Editor Window by dragging the corner of the window

ACTIVITY Change the Graph Plot Axis

1. Right-click the graph axis label annotating the currently displayed parameter (example: Comp-PE-A :: Perforin).

2. Select a different parameter (example: Comp-Ax488-A :: p-ERK1_2) from the drop-down list.

1. Right-click on the axis label
2. Select Parameter


Comp-Ax488-A :: p-ERK1_2

New selected parameter is displayed
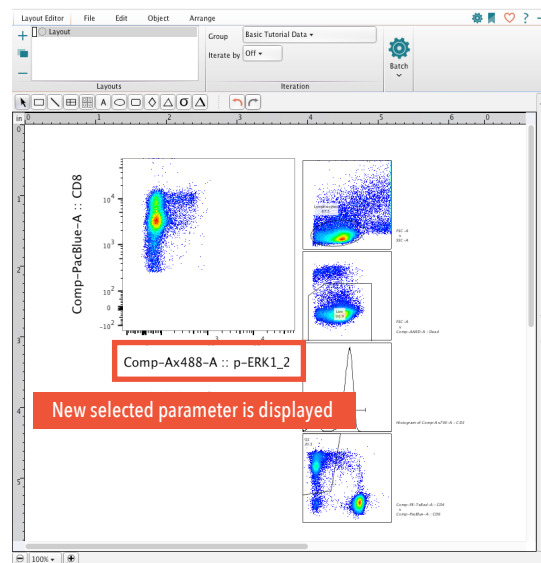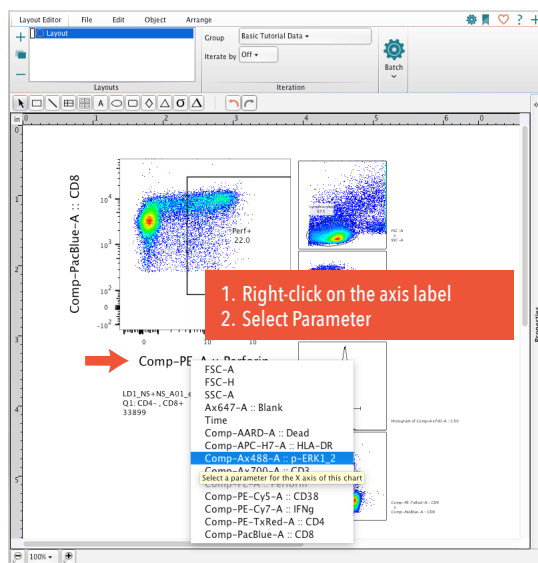
The selected parameter is displayed in the Layout Editor plot. This capability allows you to quickly modify displayed parameter information.

■ ACTIVITY Rename a Layout

Try renaming the layout to something more specific.

1. Double-click the layout's name, and give it a more descriptive name based on the population you brought into the layout and are now observing the phenotypic and functional properties of (example: CD8+).

Note that in this example, we have defined single-cell events that are lymphocytes based on scatter profile and express both CD3 and CD8 on their surface. Therefore, by definition they are CD8+ cytotoxic T cells.
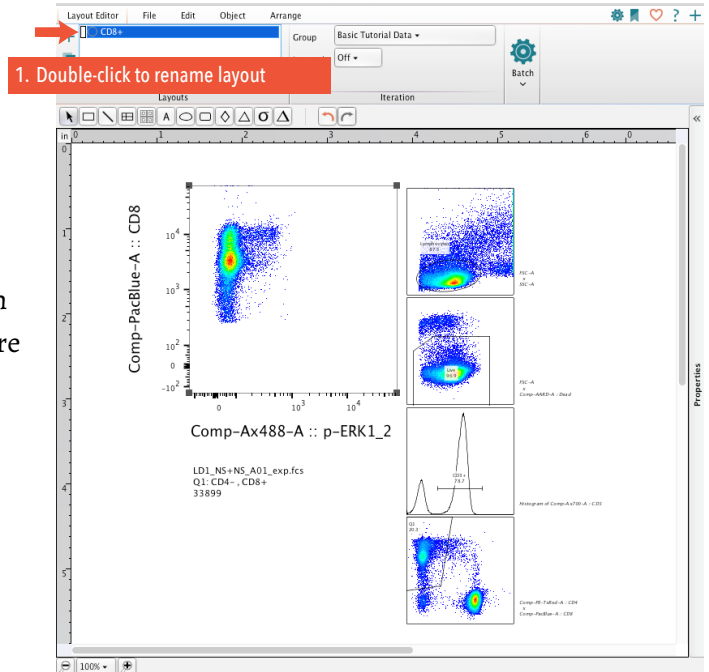
■ ACTIVITY Open the Graph Definition Properties

Additional options for graph plot formatting and display can be accessed through the Graph Definition (aka Properties) window. To open this window:

1. Right-click the graph plot, and select *Properties*.
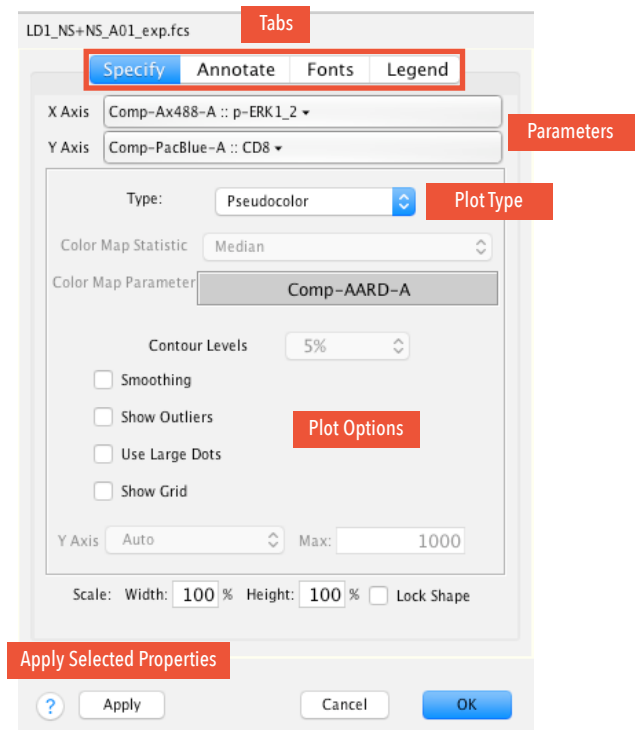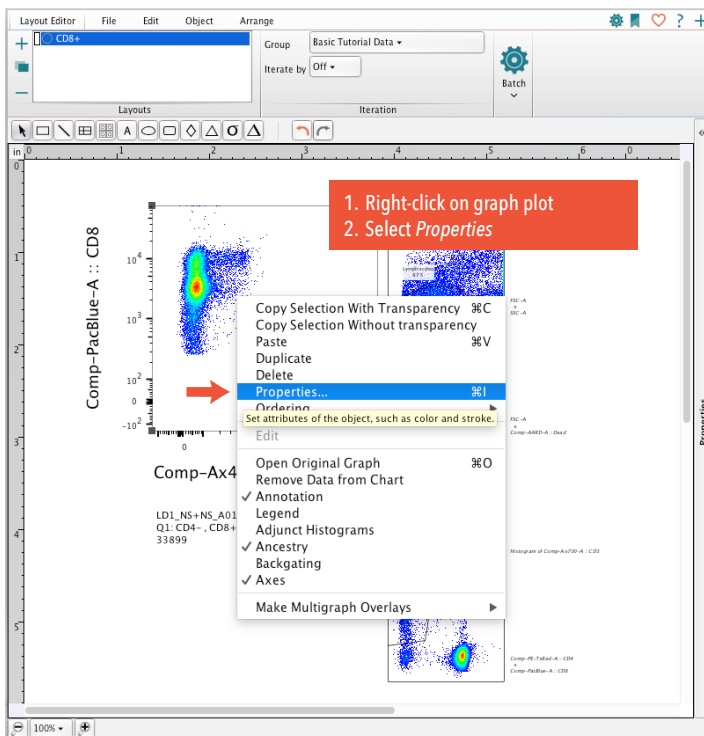
A Graph Definition window appears. This window has four tabs with formatting and legend options for the Layout Editor graph plot. With the default Specify tab selected, you can change the displayed parameters, type of plot, and display options.

The other tabs (Annotate, Fonts, and Legend) each contain additional options to modify the graph plot formatting and information displayed.
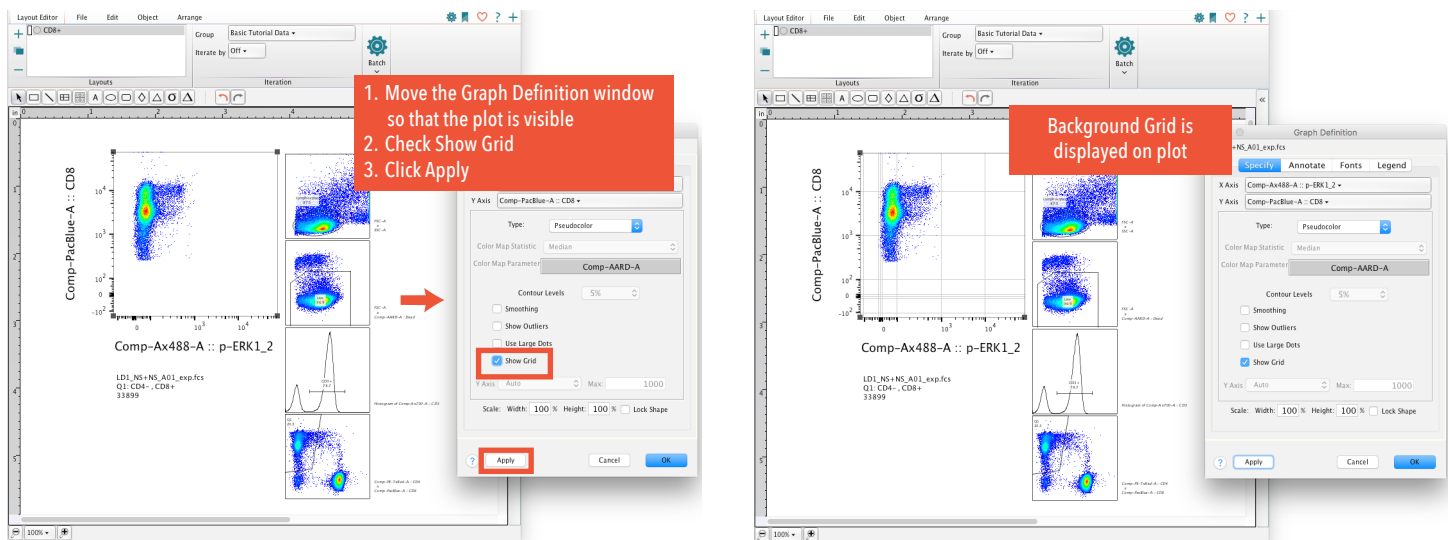
Let's modify a couple of the formatting options to get a feel for the process.

ACTIVITY Show a Background Grid in the Layout Editor Graph Plot

1. Position the Properties window to the right of the graph plot displayed in the Layout Editor.

2. Within the Graph Definition window, check the Show Grid box.

3. Click Apply.

ACTIVITY Increase Font Size for Plot Axis Labels

1. In the Graph Definition window, select the Fonts tab.

2. Under Axis Labels, select Size, and pick a larger font size (example: 18 pt).

3. Click the Color box, and choose your favorite color (example: purple).

4. Click Apply. The font size and color selected within the Fonts tab of the Graph Definition window are applied to the Layout Editor plot.

5. Close the Graph Definition window by clicking the Close button at the top of the window.

1. Select Fonts tab
2. Pick a font size
3. Pick a font color
4. Click Apply

Axis label font size modified
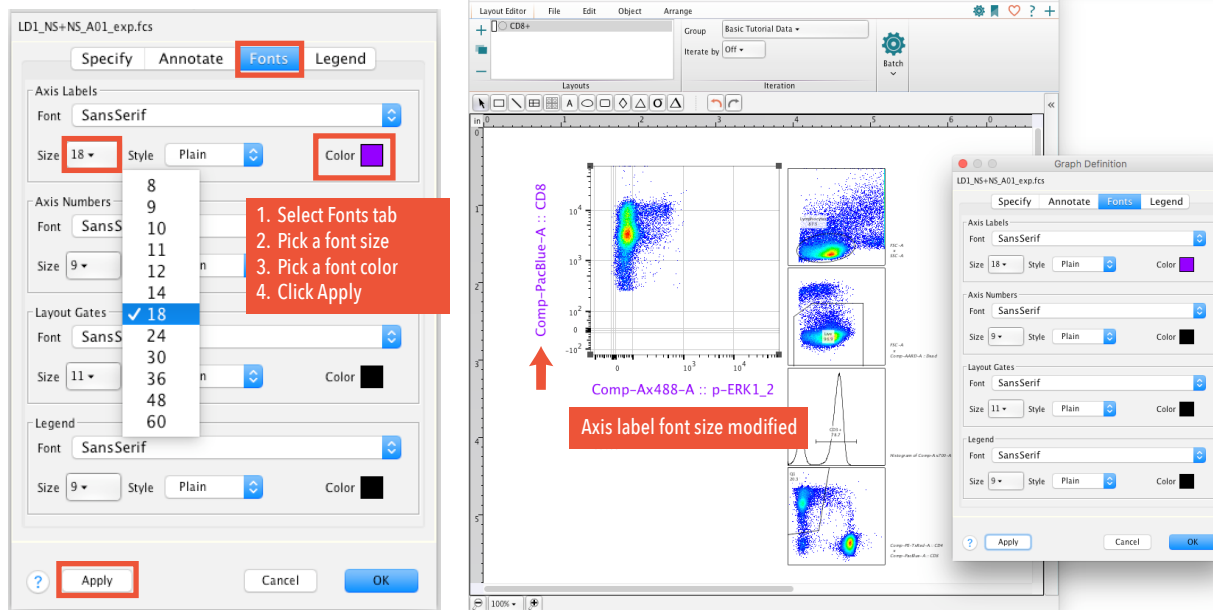
Now that we know where the graph properties and formatting options are, let's move on to reviewing data using the iteration functions.
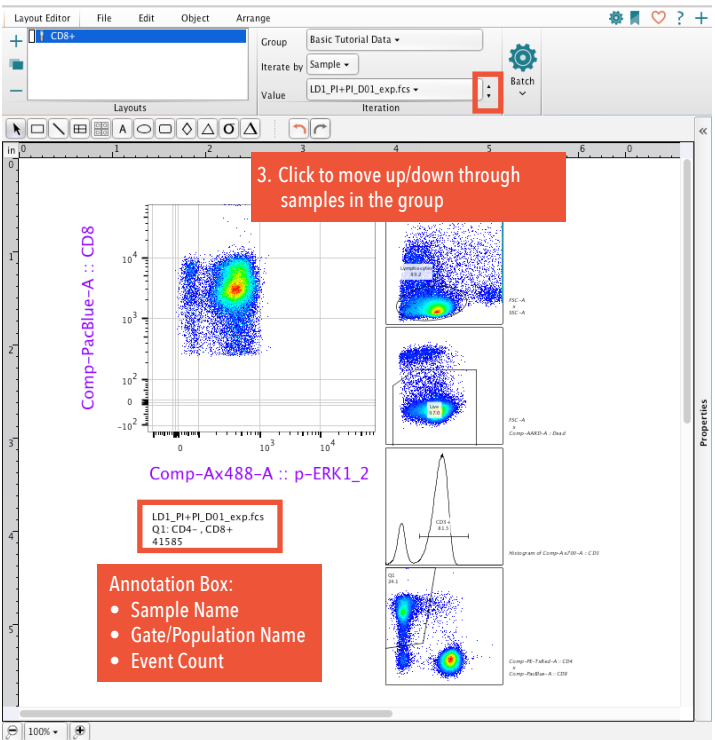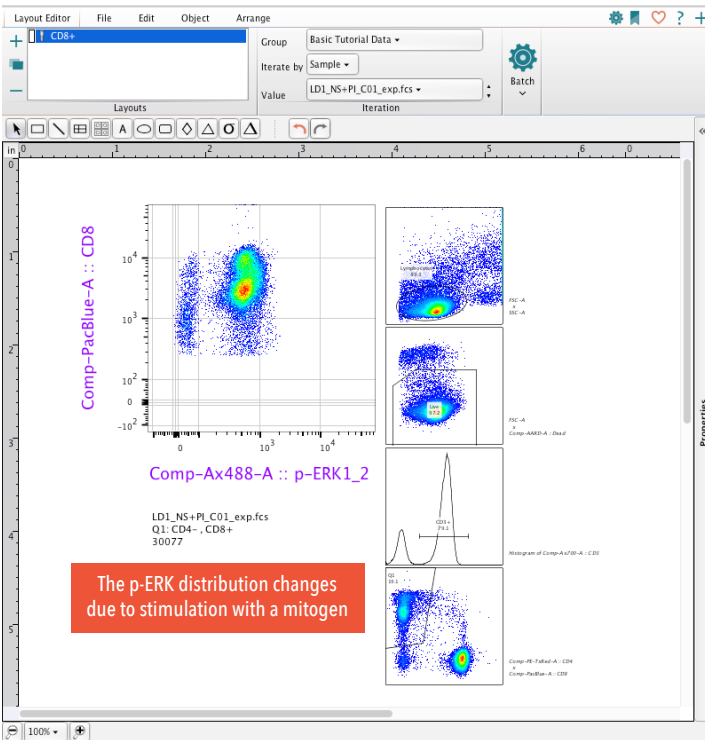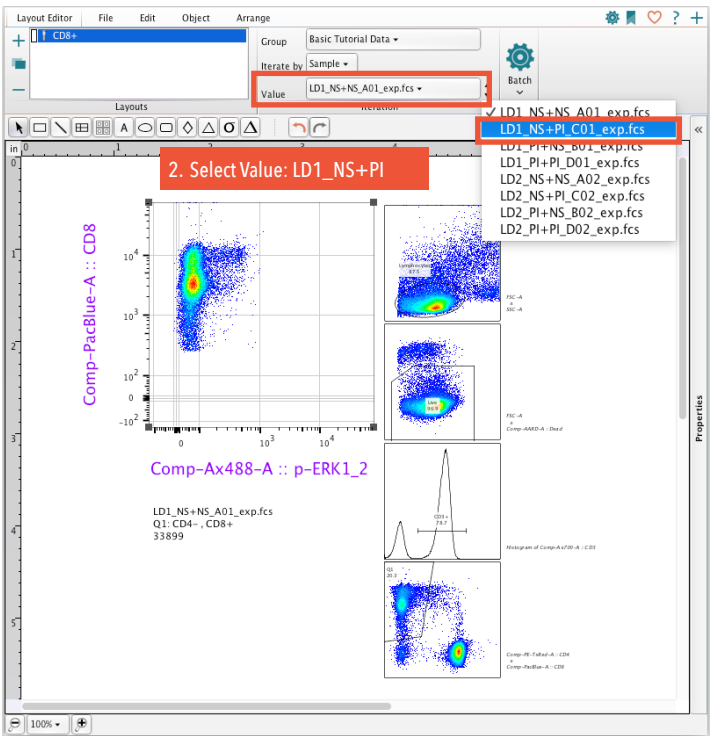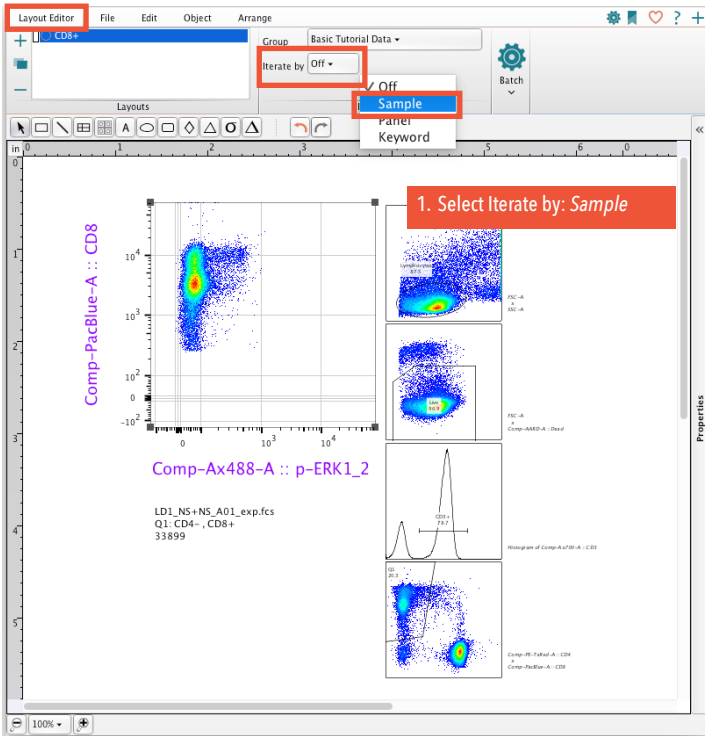
Iteration

Iteration functions are located in the Layout Editor tab, next to your list of Layouts. By default, you see two drop-down menus: 1) Group and 2) Iterate by.

The Group menu allows for selection of a workspace group containing the samples you wish to focus on for review and reporting, while the Iterate by menu controls the units over which you want to look at the samples within the selected group.

To get an idea of how this works, let's start by changing the Iterate by option from Off to Sample and review the samples in a group.

⬛ ACTIVITY Iterate by Sample and Review Samples in the Layout Editor

1. Click Iterate by, and select *Sample* from the menu. A new Value selection menu appears below the Iterate by menu.

2. Click Value, and select a different sample (example: LD1_NS+PI_C01_exp.fcs). Notice that the distribution of events along the Comp-Ax488-A :: p-ERK1_2 axis changes. We are now viewing a different sample that has been stimulated with a mitogen, as opposed to the LD1_NS+NS, which is a no-stimulation control.

3. Next to the Value menu, click the Down arrow , then click the Up arrow. Notice that these buttons allow you to scroll up and down through files in the selected group.

1. Select Iterate by: *Sample*

2. Select Value: LD1_NS+PI

The p-ERK distribution changes due to stimulation with a mitogen

3. Click to move up/down through samples in the group

Annotation Box:
• Sample Name
• Gate/Population Name
• Event Count

Batch Reporting

Batch reporting, or 'batching,' in the Layout Editor enables the application of a layout (which can contain many plots and statistics with user-defined formatting) to the selected group of samples across the selected Iterate by criteria.

For example, with the Iterate by setting set to Sample, the batch process applies the layout to each sample in the specified group, displaying data from each sample one at a time.

Let's give batching a try.

▮ ACTIVITY Generate a Batch Report

1. Widen out the Layout Editor window until the Batch band expands.

2. Click Create Batch Report.
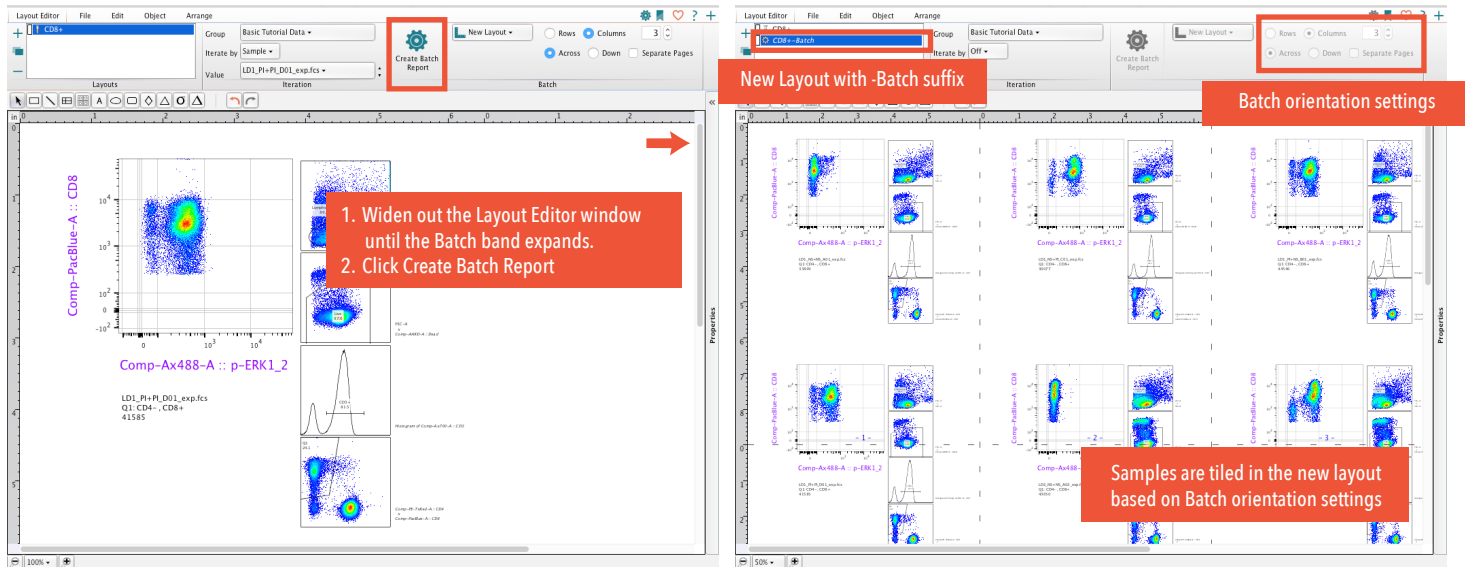
A layout is created with the suffix -Batch in the name. This layout contains plots and information for each sample in the specified group, and individual sample data is tiled across the page with the orientation specified in the Batch options (Rows or Columns and Up or Down). In this case we left the default settings so the layout and batched samples with three columns across. Instead, let's try the batch again, but change the orientation options to Two Rows Across.

▮ ACTIVITY Modify Orientation of a Sample Iteration Batch Report

1. Select the original layout (with just one sample displayed).

2. Under Batch options, select the Rows button and set the number of rows to 2.

3. Select the Across button.

4. Click Create Batch Report.
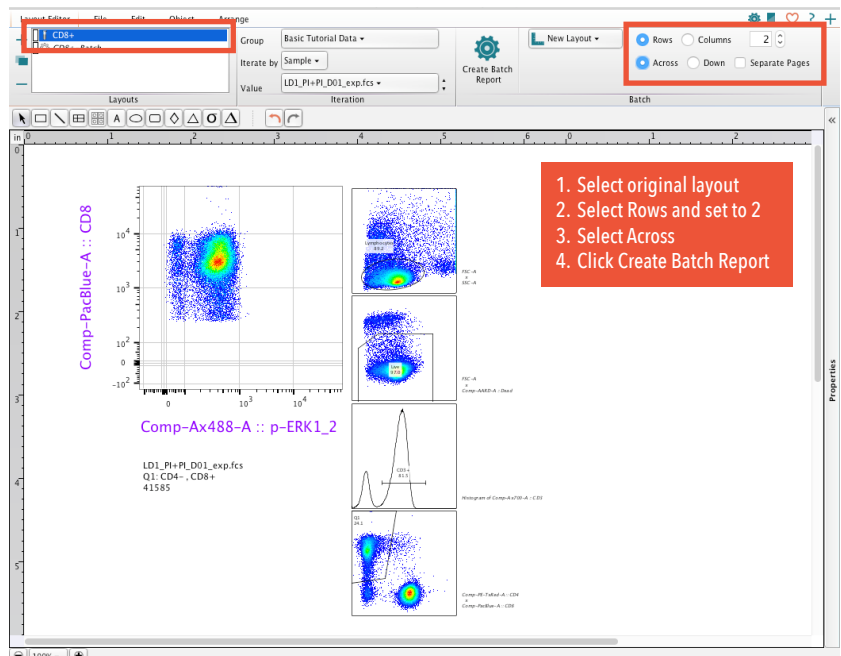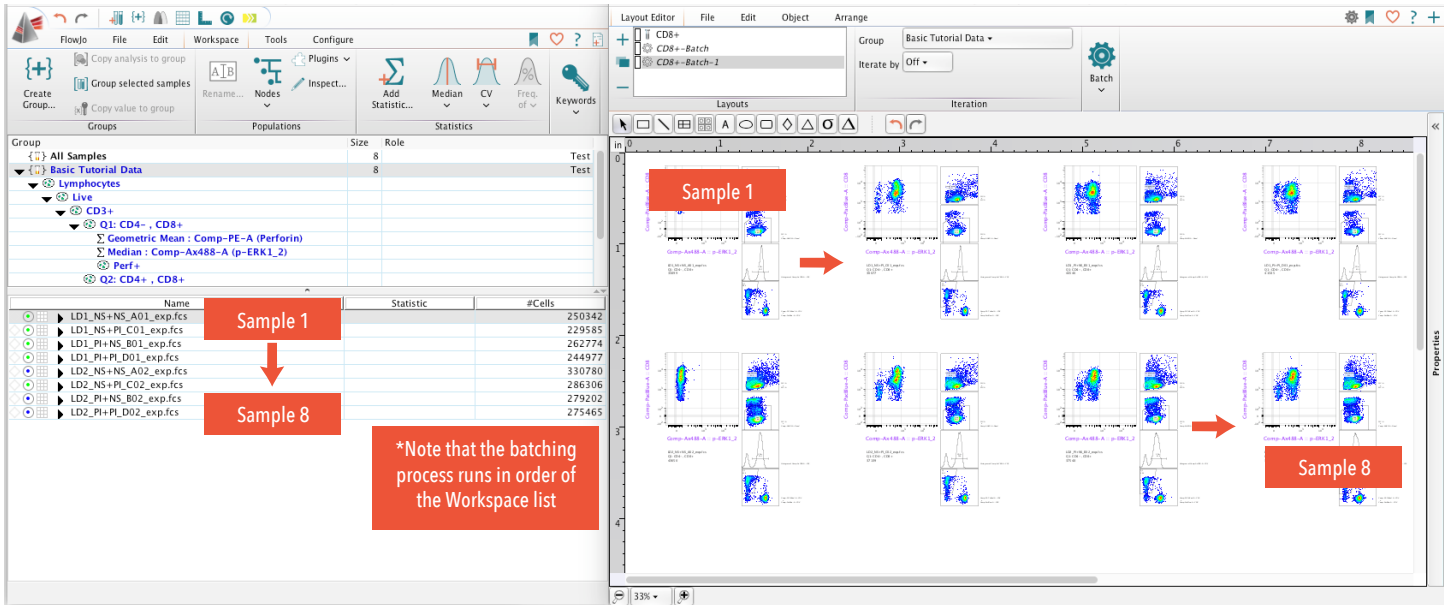
The sample tiles of the resulting two-rows-across batch report are oriented 4x2 because there are eight samples in the Basic Tutorial Data group. Note that the batching process runs in order of the samples as they are listed in the Samples pane.

Now let's try batching to a Web Page report outside FlowJo.

![] ACTIVITY Batch Report as Web Page

1. Select the original layout (with just one sample displayed).

2. In the Batch band, select the Web Page option.

3. Click Destination, and select a location to save the web page link (.html file).

4. Click Create Batch Report.
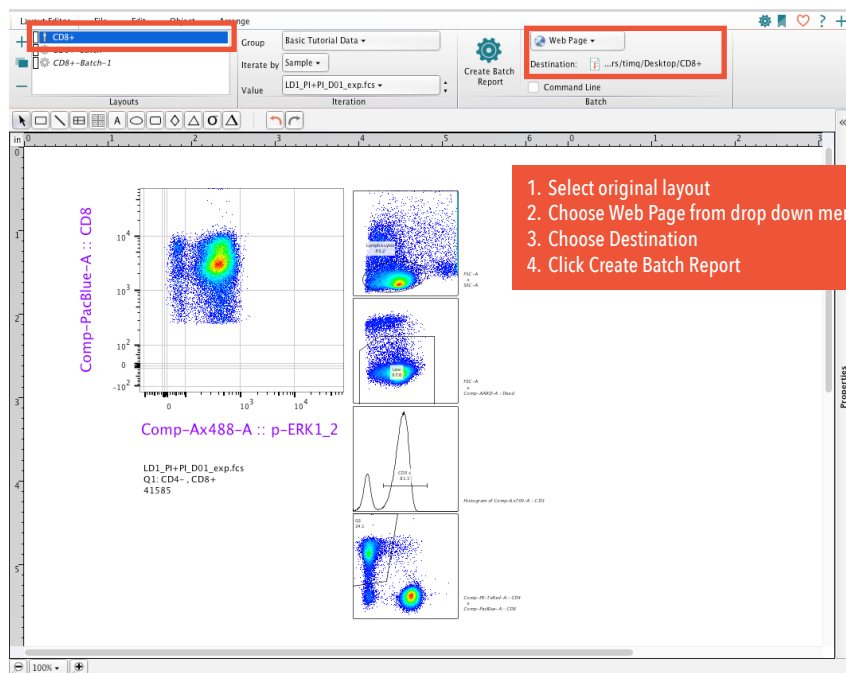
A web page .html file is saved to the destination selected, PNG images for each sample are saved to the same location within a folder called FlowJo Web Files, and the images open in your default web browser. Try scrolling down through the layout plots to view each individual sample. If you close the web browser tab and wish to view the images again in your browser, simply double-click .html file. Note that there are also options for batching directly to a Printer, PDF or PowerPoint file.
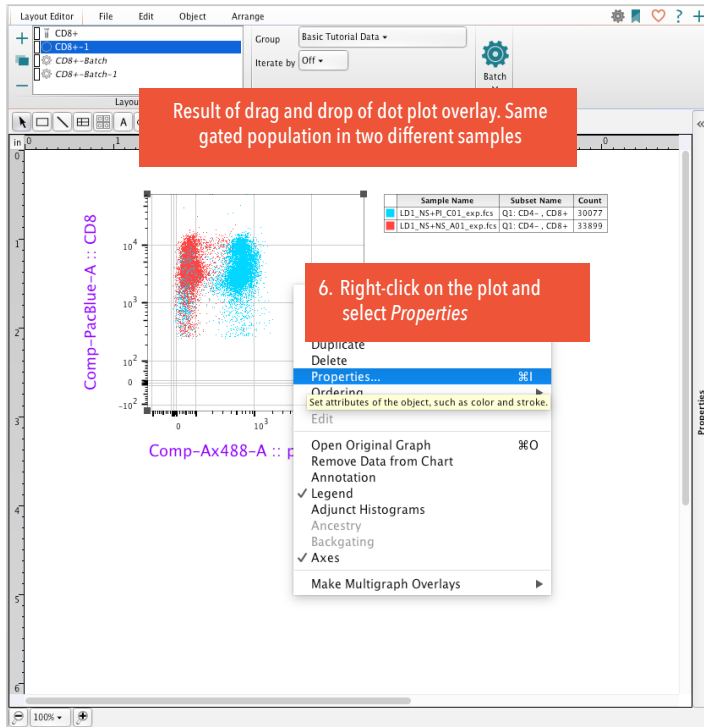
Overlays

Now, we will create an overlay comparing multiple samples in the same graph plot.

🟥 **ACTIVITY** Create a Dot Plot Overlay and Convert it to a Histogram

1. In the Layouts list, select the original layout (example: CD8+).

2. To the left of the Layouts list, click the Duplicate Layout button.

3. Within the gating hierarchy of the second sample in the Workspace window's samples list (example: LD1_NS+PI_ C01), select the Q1: CD4- , CD8+ population node.

4. Drag and drop this population onto the same plot in the Layout Editor. A dot plot overlay is created.

5. If you are prompted with an iteration mismatch warning, select the Turn Off Iteration option. The layout plot converts to a dot plot overlay, and the ancestry thumbnails disappear and are replaced by a color-coded legend.

6. Right-click the dot plot overlay, and select Properties, bringing up the Graph Definition window.

7. In the Graph Definition window, click the Specify tab.

8. Using the Type drop-down list, change the plot from Dot Plot to Histogram.

9. Further down in the window, use the Y axis drop-down menu to change the Y Axis style from Auto to Modal.

10. Click OK. The plot converts to an overlaid histogram with the peak heights normalized to mode (the peaks look the same height even though there are may be different numbers of events in the two populations).

11. Finally, right-click the plot, scroll to the very bottom of the drop-down list, and select Histograms » Offset. The plot displays each histogram stacked independently on the Y axis.

Result of drag and drop of dot plot overlay. Same gated population in two different samples

Sample Name	Subset Name	Count
LD1_NS+PI_C01_exp.fcs	Q1: CD4- , CD8+	30077
LD1_NS+NS_A01_exp.fcs	Q1: CD4- , CD8+	33899

6. Right-click on the plot and select _Properties_

7. Select Specify tab

8. Set graph type to Histogram

9. Set Y Axis to Modal

10. Click OK

11. Right-click on the plot and select _Histograms » Offset_

Offset Histogram view

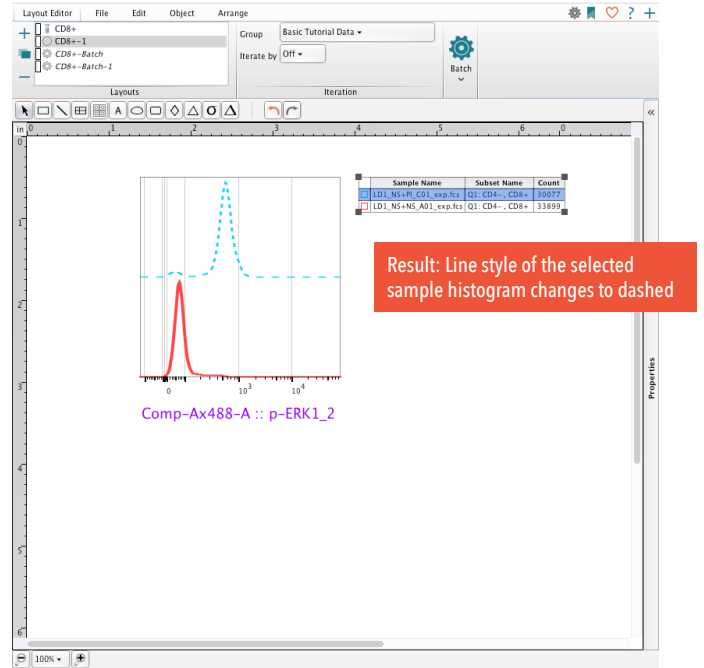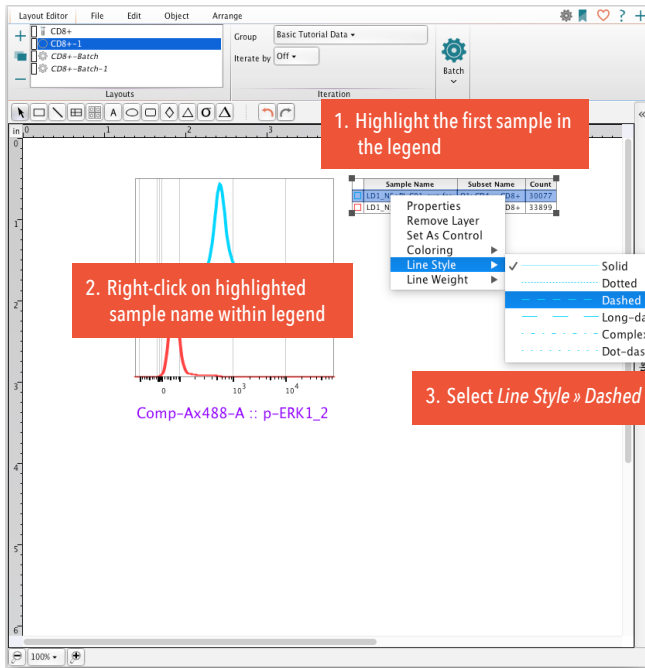| Sample Name	Subset Name	Count
LD1_NS+PI_C01_exp.fcs	Q1: CD4- , CD8+	30077
LD1_NS+NS_A01_exp.fcs	Q1: CD4- , CD8+	33899
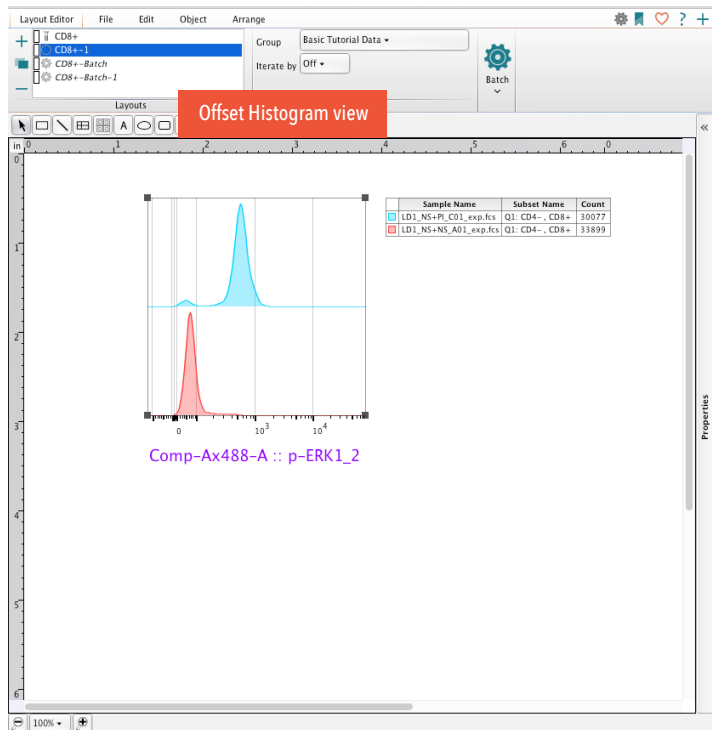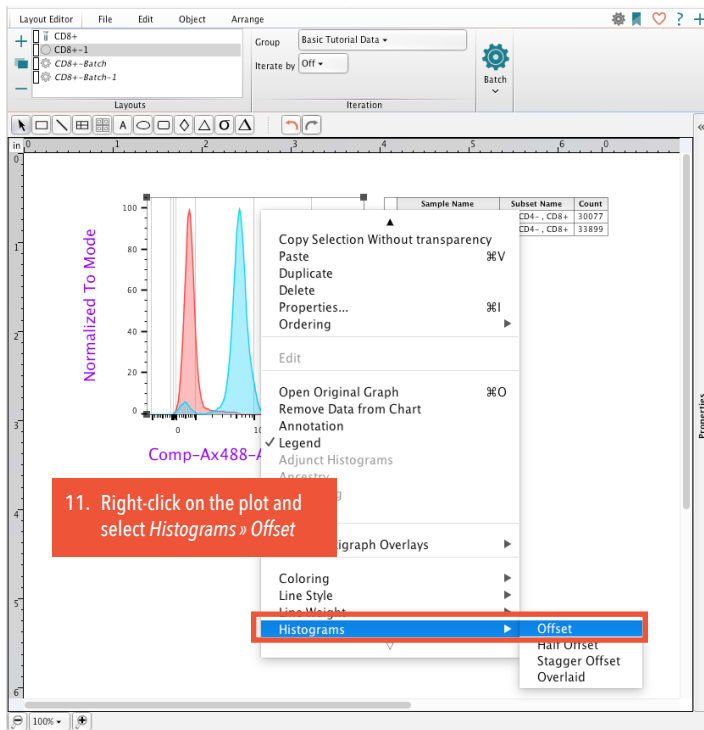
▌ ACTIVITY Modify the Histogram Formatting

If you don't like the default line thickness, style, or shading options, you can easily change them, either globally for all populations in the overlay, or individually for each specimen in the overlay. Let's try a global change to make the line weight heavier and remove the tint color.

1. Right-click the histogram overlay plot. Near the bottom of the list are options for Coloring, Line Style, and Line Weight.

2. Choose *Line Weight » Heavy*. This changes the line thickness for all populations represented in the plot, making the lines Heavy and easier to see.

3. Right-click the histogram overlay again.

4. Choose *Coloring » None*. The tint coloring is removed from all histograms in the overlay plot.
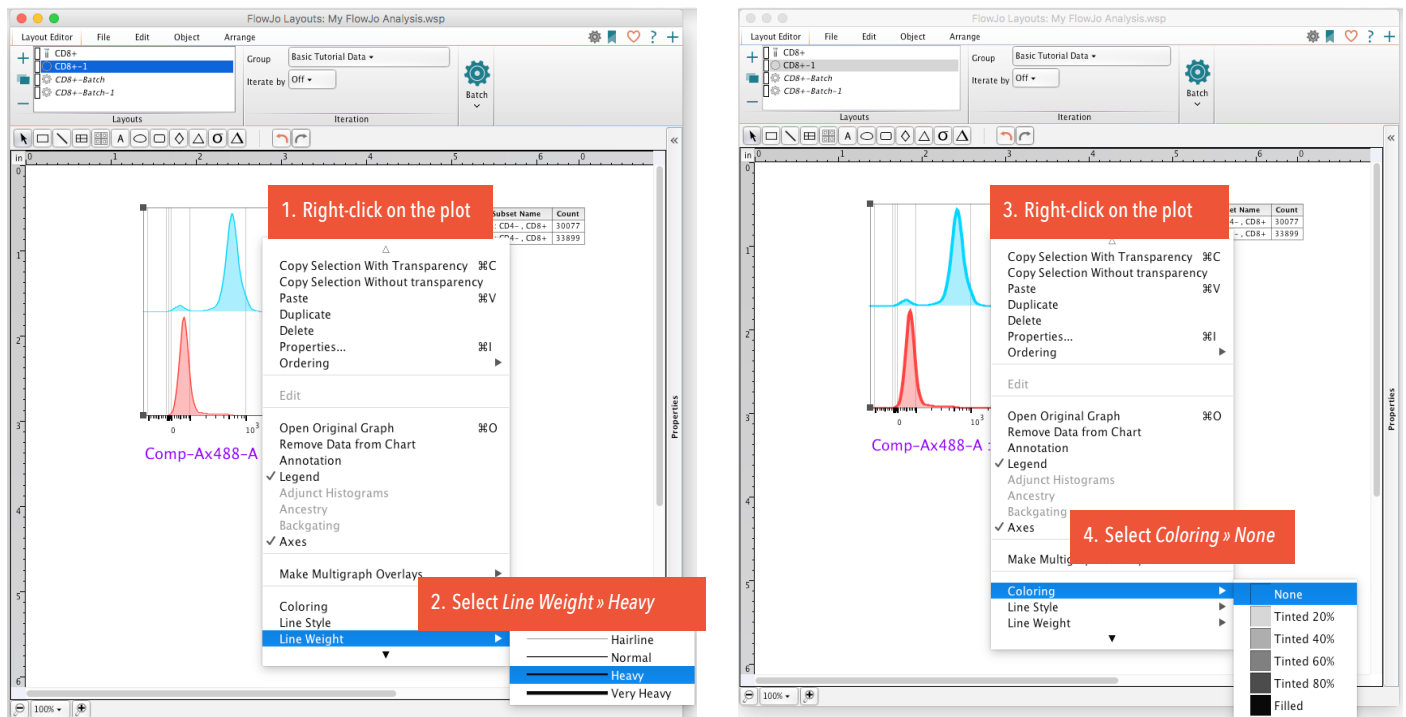
## ▌ ACTIVITY Modify the Line Style of a Single Histogram in the Overlay

1. Select the name of a single sample within the legend of the histogram overlay.

2. Right-click the sample name.

3. Choose *Line Style » Dashed Line*. The line style of the selected sample histogram changes to a dashed line while all other lines remain solid.

1. Highlight the first sample in the legend
2. Right-click on highlighted sample name within legend
3. Select *Line Style » Dashed*

Result: Line style of the selected sample histogram changes to dashed

ACTIVITY Modify the Histogram Overlay Colors

If you don't like the default colors of each individual histogram, this can also be modified. To do so:

1. Select the legend (corner handles should become visible), and click the square color box next to a sample name.

2. Select a different color from the pop-up color palette. The histogram color for that sample will change to your selected color.

1. Select the legend and click on a square color box next to sample name
2. Select a different color from the palette

Result: Color of the histogram for that sample changes to the selected color
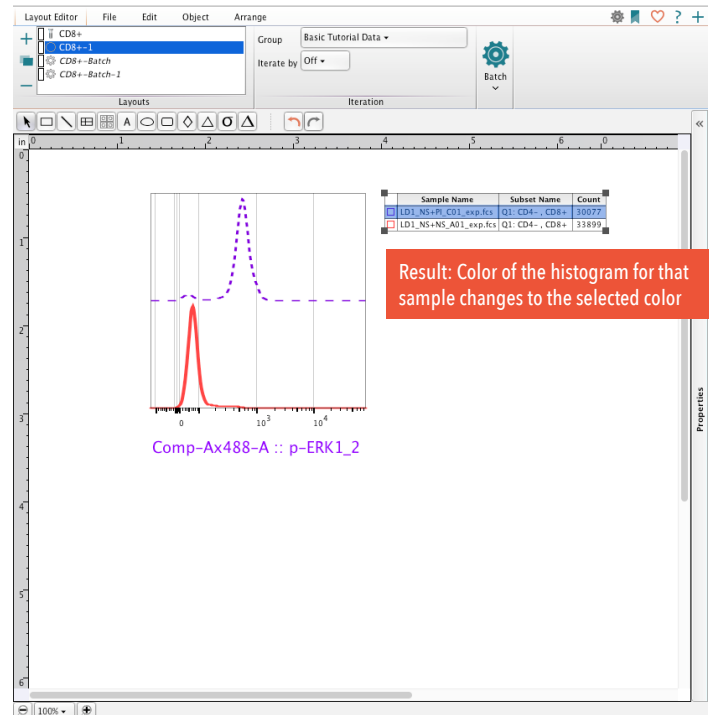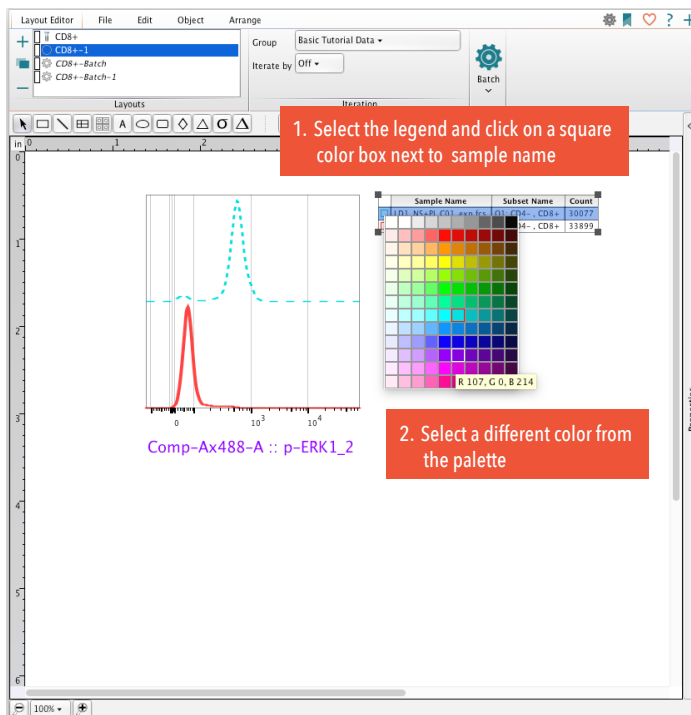
▮ **ACTIVITY** Change the Layering of Histograms in the Overlay

If you don't like the default layering of the histogram overlay (which sample appears at the top versus bottom), this can also be modified.

1. Select the legend (the corner handles should become visible), then click and hold down the mouse button on the name of the sample you want to move.

2. Drag that sample name up or down through the legend.
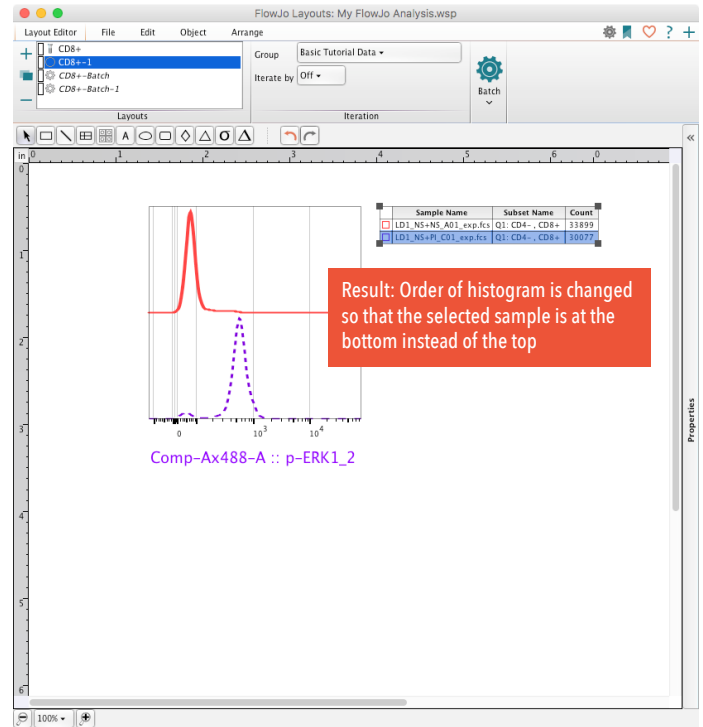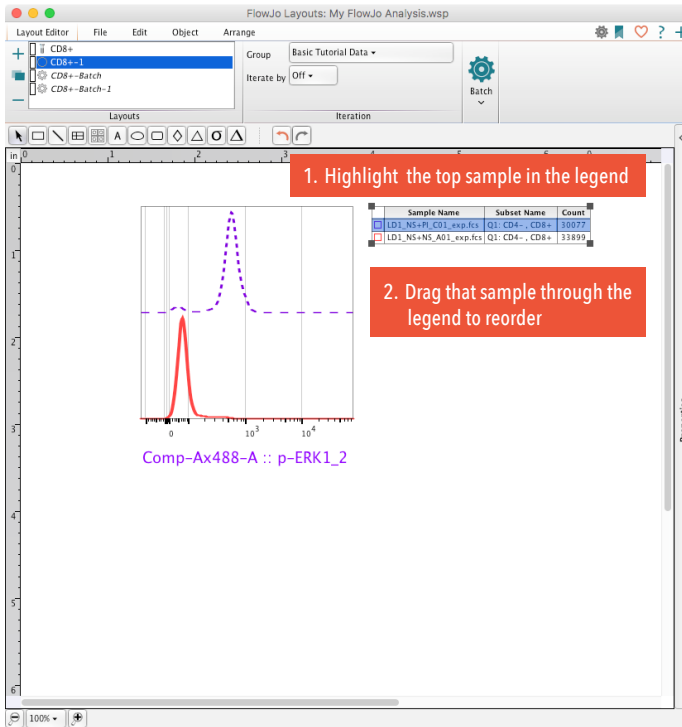
▮ **ACTIVITY** Export Image

The Export Image function (located under the File tab in the Layout Editor ribbon) provides single-image export options for the currently displayed layout, saving the layout to an external file. Let's save the current layout as a simple portable network graphics (PNG) image.

1. Select the File tab.

2. In the Document band, click Export Image.

3. Click the type of image file you wish to export (example: PNG). A save dialog box opens prompting you to select a save location.

4. In the save dialog box, give the image a name, or leave the default, which includes both the workspace and layout names (example: Mu FlowJo analysis-CD8+.png).

5. Select your preferred save location (example: Desktop).

6. Click Save.
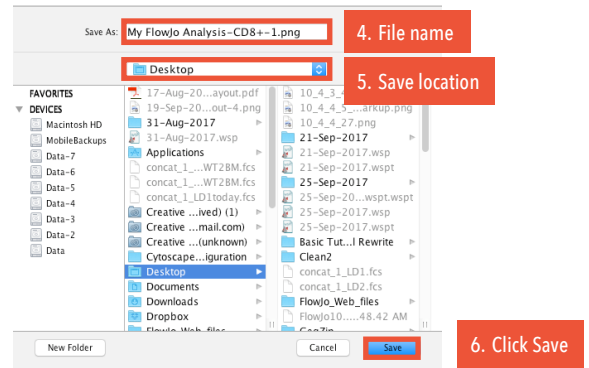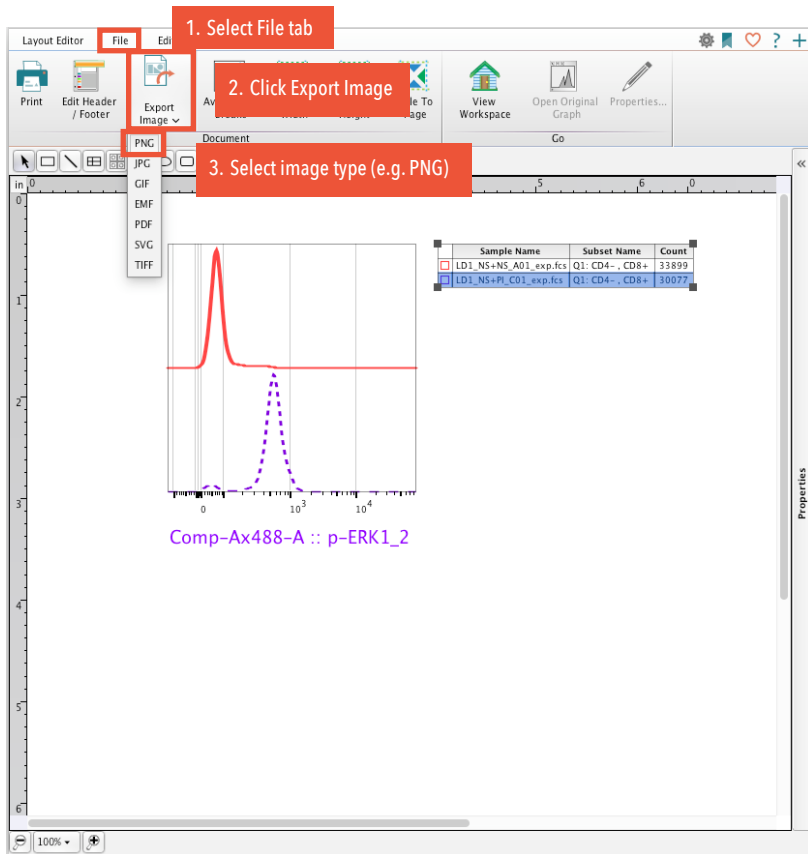
Try opening your saved PNG image.

Because we are done creating layouts for now, close the Layout editor window by clicking the Close button at the top of the window.

End of Lesson 4—The Layout Editor

Lesson 5–The Table Editor

The Table Editor is a platform for visualizing and exporting the statistics enumerated from gated populations. Like the Layout Editor, the Table Editor is provided in a separate window.

Table Editor reports can be displayed for visual inspection and exported as text, CSV, Excel, and SQL database files. The process of saving a report from the Table Editor is similar to the batch reporting process in the Layout Editor.

Components of the Table Editor

Ribbon—The Table Editor has its own ribbon with three tabs.

Table Editor—Add, duplicate, and delete tables, and set options for Iteration and output.

Edit—Add and edit columns, add keywords, and change Header/Footer options.

Visualize—Set heatmap, table formatting, and plot options.

■ **ACTIVITY** Add Populations to the Table Editor and Generate a Table to Display

Let's bring some populations and statistics nodes into the Table Editor and generate a table to display.

1. In the Workspace window's toolbar, click the latticework button to open the Table Editor, and position the Workspace window and Table Editor side-by-side.

2. Select one or more populations and/or statistics nodes in the gating tree of a single sample in the workspace.

3. Drag and drop the selected nodes into the Table Editor. For each selected node, a row appears within the Table Editor displaying the node gating hierarchy and type of statistic that is enumerated when the table is created.

4. In the Output band, click Create Table. The table is displayed in a new window below the Table Editor. Notice the orientation of the output table relative to the Table Editor list: Each row in the Table Editor becomes a column in the output table. Rows in the output table represent individual samples from the workspace group specified in the Iteration options.

ACTIVITY Rename a Row in the Table Editor

Sometimes a gating tree can get lengthy, and it is convenient to rename the population node in the Table Editor with a shortened name.

1. Within a single row in the Table Editor, double-click the Name field.

2. Enter a unique name (example CD8 %).

3. Click Create Table. A new table is created using the name you specified.

1. Double-click on the name column cell
2. Enter a unique name
3. Click Create Table

Result: Table output column header has a unique name

ACTIVITY Add Heat-Mapping to the Table Output

To more readily visualize the range of enumerated statistics, apply visual formatting to the table output columns.

1. In the Table Editor, select all statistic rows.

2. In the Table Editor's ribbon, select the Visualize tab.

3. Click Heat Map. A fire icon appears within the population column of each highlighted row in the Table Editor.

4. In the Table Editor's ribbon, select the Table Editor tab.

5. Click Create Table.

A new output table displays. Each column in the table output is heat-mapped from blue to yellow, representing low to high values for the statistic in that column.

1. Highlight statistics rows
2. Select visualize tab
3. Click Heat Map

4. Select Table Editor tab
5. Click Create Table

Result: Each statistic column in the output is independently heatmapped

■ ACTIVITY Save the Table as a Text File

Let's export the data table, and save it to a file outside of the FlowJo application.

1. In the Output band, click the Display field. From the drop-down list, select To File. The file type field directly below is populated by Text, indicating that a text file will be saved when we create the table. Leave this default for now, but additional options are available, including CSV, Excel, HTML, and SQL files.

2. Click the Destination field, select a location to save the text file (example: Desktop), and click Save.

3. Click Create Table.
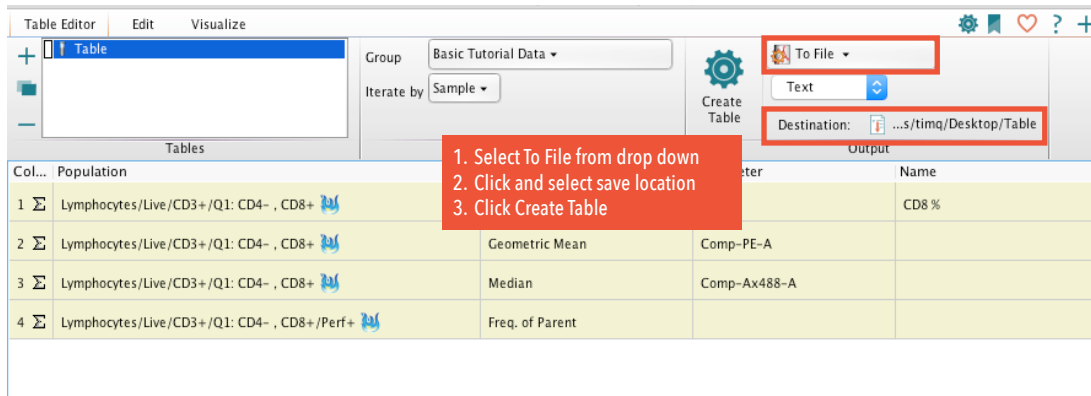
The table is saved in the destination specified, and can be opened with any text editor or spreadsheet program. Note that you can also copy and paste the text from a displayed table to a spreadsheet to transfer information.

Also, if you wish to retain the heat map formatting, try saving the table as an HTML file. The HTML file opens as an image in a web browser, including any visualization formatting.

Because we are done creating tables for now, close all the open Table Editor windows to get them out of the way.

End of Lesson 5—The Table Editor

Lesson 6—Saving the Analysis

The analysis structure created within a FlowJo workspace can be saved at any time by using the Save functions, which are located within the File tab of the Workspace window's ribbon.

File Formats

There are several different ways to save your analysis, depending on the need.

Workspace (WSP)—Saves the analysis and references to the data files. When you open the saved workspace, all the analysis and data files are present. The data is not duplicated, but instead is pulled in from wherever the files are located on a storage drive.

Archived Cytometry Standard (ACS)—Saves both the analysis and data files together in a zipped archive file. The data and analysis are included, and stay with the file if it is moved.

Workspace Template (WSPT)—Saves the analysis structure of your workspace without data files. The analysis structure of your workspace template can be applied to data files acquired in different experimental runs, as long as the measured parameters are identical.

■ ACTIVITY Save the Analysis as a Workspace (.wsp) File

1. In the Workspace window's File tab, in the Document band, click Save As.

2. Select *Save as Workspace (WSP)*.

3. Enter a name for the workspace file.

4. Click Save.
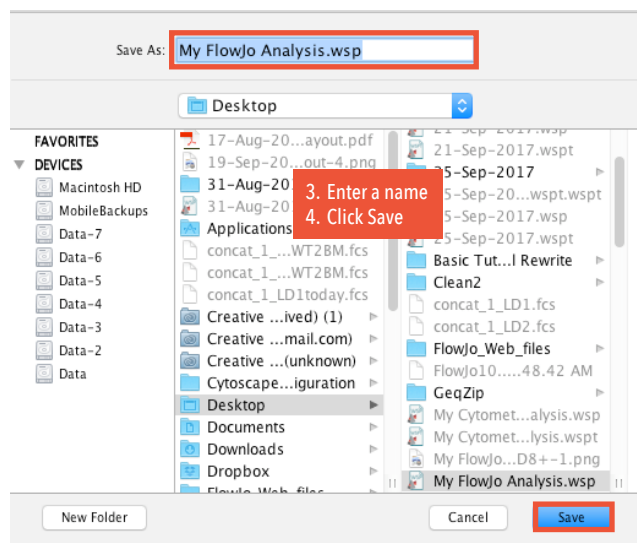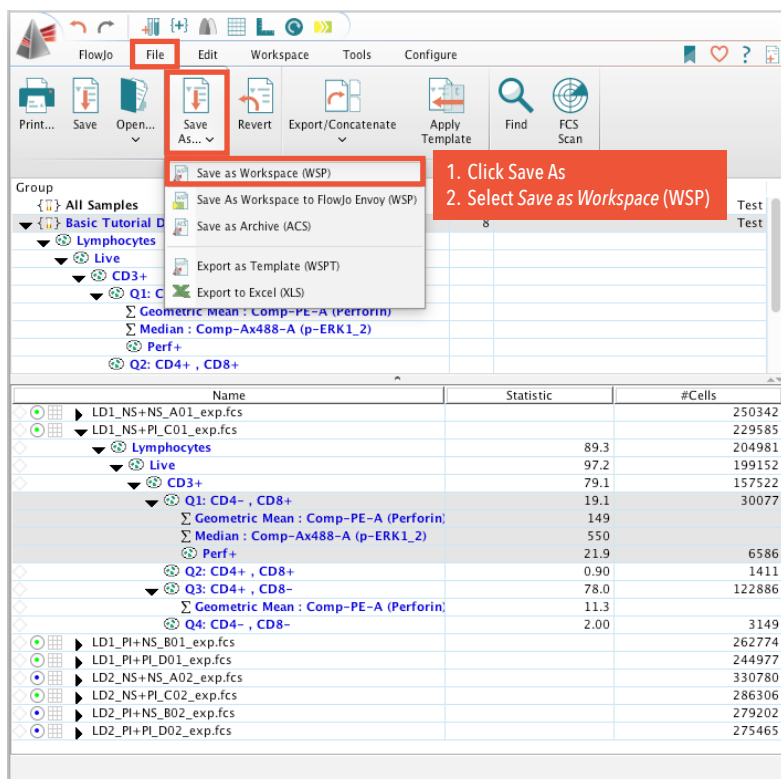
Reconnecting to Data

The workspace is saved in the specified directory location. When you reopen the workspace, all the analysis is retained, and the data files load from their saved location.

Note that a regular workspace save does not make a duplicate copy of the data, but instead references the data files based on their directory location. If these files are moved, the workspace will open with red crosses to the left of the data files (in the Sample Quality badge), indicating that the raw data could not be found.

To reconnect the workspace with the raw data, right-click a sample quality badge with the red X, and choose *Search for FCS files*. In the file browser, find and select the file folder containing the raw data. Note that it is enough to find the folder containing the raw data; it is not necessary to find the individual files.
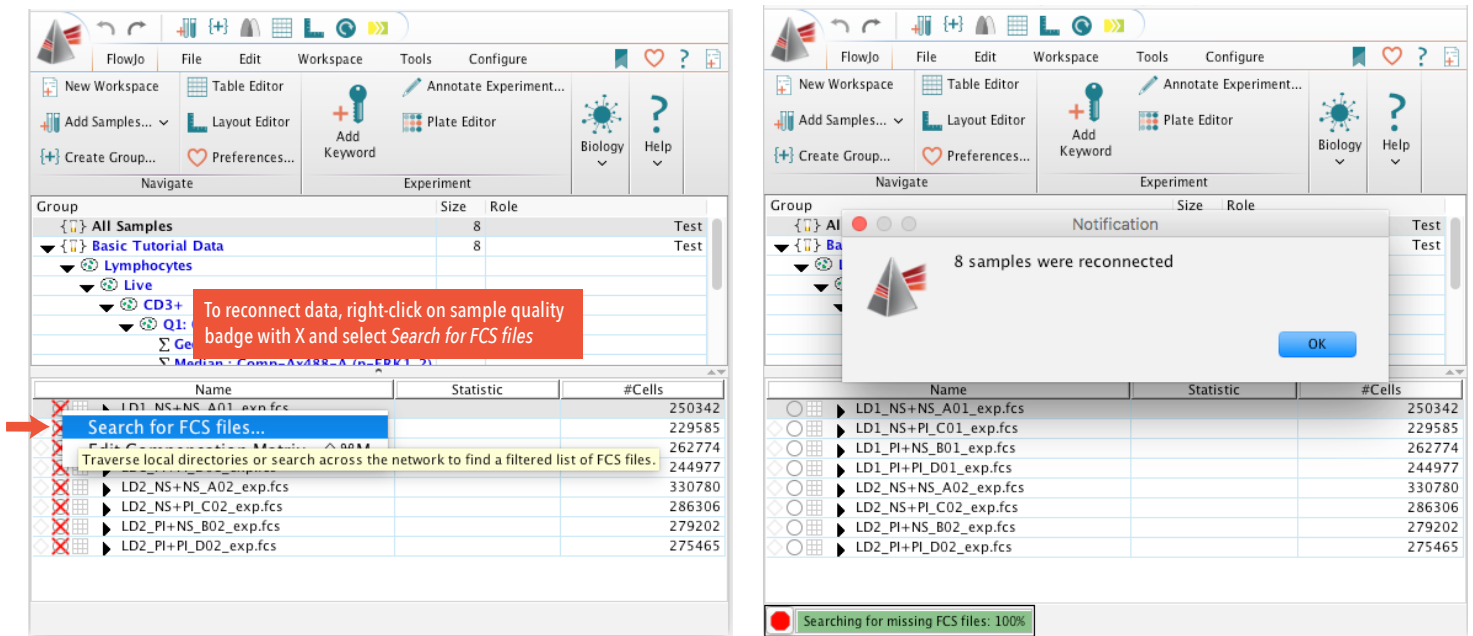
Saving your analysis as an archive (ACS) file should prevent you from ever needing to reconnect the data to that workspace in future, because the file includes copies of the source data.

End of Lesson 6—Saving the Analysis

Conclusion

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

Compensation—Corrects for overlap from the emission spectra of fluorochromes. As your experiments become more complex, the importance of accurate and automated compensation becomes more pronounced.

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.

Kinetic Analysis—Measures 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 (for example: 7AAD, PI, DAPI).

Proliferation Studies—Models the frequency of successive generations, and gate cells by the number of divisions undergone, as measured by intensity of CFSE, or other intracellular dye.

Dimensionality Reduction—Reduces multidimensional data into a 2D plot using t-Distributed Stochastic Neighbor Embedding (t-SNE).

Help links from any FlowJo window display the documentation page describing that topic, giving you context-sensitive help.

Learn More:

Product information: https://www.flowjo.com/solutions/flowjo

Complete official documentation: http://docs.flowjo.com/d2

FlowJo University's product training resources: https://www.flowjo.com/learn/flowjo-university

Technical Support: https://www.flowjo.com/support