

FlowJo for Macintosh Manual Table of Contents

FlowJo V9

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The outline of the Reference Manual is shown below. Click on any topic to learn more about it; or use the navigation buttons on the left to go to the overview of any section.



Workspaces:

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- [Polyvariate Plot](#) shows more than 2 parameters on a 2D graph. This allows the user to define complex populations of cells using one gate.

Special Analysis Platforms:

- [Creating derived parameters \(time, ratios, log <-> linear conversion\)](#)
- [Compensation](#)
- [Compensation Wizard](#) Create compensation matrices quickly and rigorously.
- [AutoCompensation Scripting](#) Find compensation samples, gate them, create the compensation matrix, and apply the matrix to experimental tubes.
- [Calibrated Parameters](#) Quantitation of absolute molecules per cell.
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Output:

- [SciBook](#)
- [Layout Editor](#) - Can be dragged (or copied) from one workspace to another.
- [Copying graphs to other programs \(generating publication-quality graphics\)](#)
- [Tables](#) - New user interface with **batching** and **formulas**.
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Menus

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Tips and Techniques:

- [Getting help](#)
- [Canceling operations that FlowJo is performing](#)
- [Using the option key](#)

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Usage Notes:

If this is your first time learning about FlowJo, you should read the pages describing the [general concepts behind FlowJo](#). You may want to run through the [tutorials](#), which will introduce you the program by analyzing a real experiment. To see how the current version is different from older versions of the program, [click here](#).

As you use the application, you will see that FlowJo is littered with **Help buttons**. Almost every FlowJo window has a help button and virtually every time FlowJo notifies you of a problem or asks you to make a decision, there will be a help button. When you click on these help buttons, FlowJo will launch a web browser (such as Netscape) and bring up a page that gives you specific information about that particular window, error message, or the decision options. You can also press the "help" key on your keyboard at any time and get information about the front-most window. Use this help feature frequently! You will find many hints about using FlowJo more efficiently. You might also want to check on the [Frequently Asked Questions](#) to get some hints once you are familiar with the program.

This online help can be downloaded in its entirety to your computer for faster access. [Click here](#) for more information.

Both the application and the help pages are dynamic: they are being continuously developed and improved. Therefore, we

want to hear any and all suggestions about how to improve them! Please report any problems, suggestions, or questions for which you can't get answers; the easiest way to do this is by [E-mail](#). [More information about [making a report](#)]

[\[Top\]](#)

Technical information:

- [Macintosh system requirements](#)
- [FlowJo memory requirements](#)
- [Reporting bugs or problems, making suggestions](#)

Type v5, v6, v7 or v8 when you enter search terms. The search results will favor the version you are using. Example: for help on compensation with version 8 type **v8 compensation** .

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Workspace Overview

FlowJo organizes all of your analyses into a "workspace". The workspace contains the following information:

- A list of all the samples that you have put into the workspace
- A list of all the groups that you have created
- All of the analyses (and results of calculations)
- All compensation matrices that you have created or loaded
- All table definitions that you created
- All layout definitions that you created

This information is at your fingertips through the workspace window.

Your workspace is like your laboratory notebook. You can save it, and then re-open it in the future and continue your analyses from where you left off. It will remember everything that you do (although you can choose to delete analyses or samples and they will be forgotten). Workspaces can become templates for batch analyses of many experiments--you can load as many samples as you wish into a single workspace.

How you organize your workspaces is up to you. We recommend that you assign each experiment a workspace. Remember that you can copy analyses between workspaces; there is no limitation imposed by maintaining multiple workspaces. Of course, all batch analyses are limited to samples within a single workspace, so you will want to keep all similarly-analyzed samples (even from different experiments) in the same workspace. You may have as many workspaces open at the same time as you wish.

For an explanation of the elements of the window, [click here](#).

wssample.gif

A rectangular box containing the text 'wssample.gif' at the top left. The rest of the box is empty, indicating a missing image or a placeholder.

Find this helpful?



Adding Samples

To analyze samples, you must first create a new workspace and then add the sample files to this workspace. Add samples by clicking on the **Add Sample** button in the workspace (leftmost function button).



You will be shown the standard Macintosh **Open File** dialog. You can add a single file by opening that file directly. If FlowJo determines that this is an FCS file, it will read it in and add it to the current group.

Alternatively, you may select a folder and click on the button **Add all files in folder**. FlowJo will then look at every file in the folder you specify. If it is an FCS file, it will be added to the workspace. In addition, FlowJo will create a new group that has the same name as the folder you selected; the files you just read in will be added to the folder. This way you can add an entire experiment at once!

Finally, if you select the button **Add all files in all folders**, FlowJo will look within the folder you select and (1) add all FCS files in that folder; (2) search within each folder contained therein for FCS files and add them; and (3) continue this search as deep as possible (i.e., looking for data in folders within folders). Each time a data file is encountered, FlowJo will create a new group with the name of the folder that the data file is in and put the data file in that group.

Note: You can also add sample files (or folders of sample files) by simply dragging and dropping the files from the finder to the workspace window.

Find this helpful?



Groups

Groups are the heart of all the powerful tools in FlowJo. A group is a collection of samples-and a mechanism by which analyses can be applied uniformly to that collection of samples. Any given sample may belong to one or more groups. FlowJo lists the groups in the upper portion of the workspace window.

There is a special case group: the "All Samples" group. It contains (by definition) all of the samples known to the workspace. The "All Samples" group can neither be renamed nor deleted.

Groups are created in one of two ways:

- (1) When you read in a folder of data files, FlowJo creates a group with the name of the folder and automatically adds the samples to the group; or
- (2) You can create a new group by clicking on the **New Group** button under the **Workspace** menu.

When you create the group, you are given the option of adding samples to the group which fit a set of criteria-this is specified by the [Group Definition dialog](#) window. In addition, you can specify that these criteria should be checked anytime new samples are added to the workspace: if the new samples meet those criteria, they are added to the group (and group-based analyses are automatically performed at once.)

You can add samples from the workspace to any group. Just click on the sample and drag it to the group. To remove a sample from a group, select the group, then select the sample and press the delete key. If the current group is **All Samples** and you delete the sample, then you will be permanently removing the sample from the workspace.

A group behaves in some ways as a "template sample" for its members. In other words, you can drag gates or statistic nodes to a group exactly the same way as you would to another sample. The only difference is that these gates, after being added to the group, are then added to every sample belonging to the group. This is one of the ways in which FlowJo performs batch analyses. For hints on creating groups to efficiently use this feature, [click here](#).

There is one unbreakable rule with regards to groups: Every sample belonging to a group must contain every analysis that has been applied to the group. Of course, if the group specifies analyses that are not applicable to the sample, then this doesn't happen. (For example, if you have created a gate based on compensated parameters, and a sample that is not compensated is added to the group, then those gates cannot be added to that sample. Once you compensate the sample, however, the gates will be automatically added.)

If a sample belongs to multiple groups, then it will have all of the analyses from all of the groups to which it belongs. Whenever you add a sample to a group (by dragging it into the group), that group's analyses are automatically applied to the sample.

Associated with group names is a color and text style. A gate that is attached to a sample through a group operation appears in the same color and style as that group. Therefore, any gate within a sample that has a group's color and style is guaranteed to be identical to the group's version of that gate. When you change a group's version of a gate (by dragging a new version of that gate onto the group or if you have the Synchronize Group's Gates option checked), then all samples with the group's version of the gate are likewise updated.

If you modify a sample's version of a group's gate (for instance, if you decide that a lymphocyte gate for one sample should be slightly different and you move it), then that gate will now appear in the workspace window in black and plain text (unlike the group's gates). This is how you can tell from the text when a gate has been modified. However, if

you checked the Synchronize Group's Gates option when you created the group, any time you modify a gate on any sample, the group's gate is automatically updated.

Note that modified gates will still behave in other ways like group gates: when you attach a subpopulation to a group's gate, it will be attached to the same gate in all samples, whether or not they are identical to the group's version of the gate.

If you have modified a gate and decide later that it should be identical to the group's version, you can select the gate and choose the **Unify analyses** option under the **Workspace** menu. Likewise, if you select a gate within a group and choose this menu option, then all sample's versions of this gate are made identical to the group's version.

How do you know what the group's version of a gate looks like? Simply open any sample which has the group's version of the gate (i.e. the node is displayed in the color and text style of the group). If you change this sample's gate (move it), then you are only changing that sample's version. To change the group's version, drag the node back onto the group after you have made the modification or if you checked the Synchronize Group's Gates option when creating the group, the update happens automatically.

If a sample belongs to multiple groups which all have an analysis of the same name, then the sample's version of the gate will be whichever one it got first.

Deleting nodes associated with groups have special consequences:

- If you delete the node in the group itself, then it is removed from the group. You are then asked if you want to remove the same nodes from all of the samples; if you choose not to remove them from the samples, they are left alone (but they are then owned by the samples-i.e., drawn in black and plain text-since they no longer belong to a group).
- You cannot delete a sample's node which is identical to the group's node, since every sample must have every analysis belonging to the groups that it is in.
- If you delete a node which is a modified version of the group's node, then FlowJo will replace it with the original, group's version of the node.
- If you delete a sample from a group, then it is removed from the group but all analyses which came from the group are still applied to the sample (but they are now owned by the sample, not the group).
- If you delete a group, then all of the group's analyses nodes are assigned to the samples.
- If you rename a sample's version of a group node, then a copy of that node is made with the new name, and the sample will retain a node with the same name as the group's node.

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Batch Analysis

The real power of FlowJo as an analysis tool becomes immediately evident when you start to do "batch" analysis; i.e., the repeated application of a set of analyses (gates, statistics, graphical outputs) to a series of samples.

Application of analyses to other samples is very easy: simply click once on the analyses that you wish to duplicate, and while holding down the mouse button - drag them to the destination sample. You can select a single gate (by clicking), or select several independent gates (by shift-clicking and dragging any of the selected gates). In addition, you can choose to take all the "children" subsets of the selected gate (i.e., the entire analysis tree) by holding down the "option" key as you drag the selected node; you can also choose to take the "parent" gates by holding down the "control" key as you drag node. For a full explanation complete with examples of these operations [click here](#).

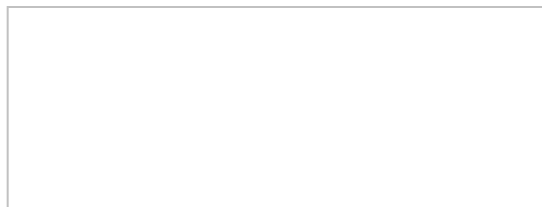
You can also apply a set of analyses to all samples within a group simultaneously. Simply drag the analyses trees onto the group. They are attached to the group itself, and then attached to each sample that belongs to the group (assuming the analysis is valid for that sample). By using these kinds of group analyses, you can assure that all samples are being analyzed identically. When you change a group's version of an analysis (by dragging a new version of a gate onto the group), then all samples belonging to the group will be automatically updated with the new copy (with the exception of those samples which have "special" versions of such gates). These operations are fully explained in the pages on [group analyses](#).

Another kind of batch analysis is the extraction of statistical information from a series of samples. This is accomplished through the table editor. Using the [table editor](#), you specify what sorts of [statistical information](#) you wish to collect for each sample. Then you create the table for the current group; FlowJo allows you to save the table to a file (which you can export into a spreadsheet), to the clipboard (so you can copy it directly into a spreadsheet), or print it out. In addition, FlowJo will save the table AND open your favorite spreadsheet program AND copy the table into this program - all with a single click! Table definitions are saved with the workspace so that you can use them again in the future.

The final type of batch analysis is the extraction of graphical displays for a series of samples. For this, you use the [layout editor](#). The layout editor allows you to arrange several graphs on a drawing board. This graphical layout is then applied to the series of samples in the current group; the result can be saved to a disk file (PICT format), copied into other applications (such as Canvas), or printed.

Template workspaces save all the analyses (gates and statistics), table definitions and layout definitions while removing the samples. Because FlowJo saves all of this information in the workspace, it is a simple matter to read new samples into the same workspace that has been saved as a template and then apply the same batch analyses to those samples.

A batch is composed of a series of tasks. The task is the unit of computation; that is the generation of each statistic or graph, sorting of a list etc. FlowJo can execute tasks asynchronously, meaning that you do not have to wait for all pending tasks to complete before interacting with the program. You can even initiate new tasks while others are being completed, so that the amount of time you have to spend waiting for the program is minimized. Because the tasks can also be sorted in such a way as to reduce the loading and unloading of files, the asynchronous processing can actually be faster than giving all of the computing resources to handling pending computations.



The list of pending tasks is managed by the Task Manager, which displays a progress window while tasks are being completed. The thermometer pane shows the percent completion, and the status flags report the current process and how many tasks are scheduled to complete it. Some tasks will schedule their own subtasks, so you are not guaranteed that the Items Remaining will steadily decrease. You can cancel operations that are in progress, or stop and restart them if you have other operations to which you need to give immediate attention.

See Also: [Groups](#), [Layout Editor](#), [Table Editor](#)

Find this helpful?



Statistics

There are several statistics that can be computed by FlowJo. Except for the **Frequency of Parent**, which is always displayed for each subpopulation once it has been gated, statistics must be added as separate nodes in the workspace window. Each statistic node holds a single value (a single statistic); it is recomputed whenever a gate that affects that subset changes. The [full list of statistics](#) includes such computations as **median, mean, or frequency within a parent population**.

Statistics can be added to populations in two ways: (1) by selecting one or more populations and clicking on the **Statistic** function button in the workspace window; this adds statistics to the selected populations, or (2) by viewing a population in a graph window and clicking on the **Statistic** button in that window. Both ways brings up the [Statistics Dialog window](#).

Statistics are displayed in the workspace window whenever they are calculated. If a statistic node in the workspace does not show its value, then you may request it to be computed. Select any node which has that statistic as a descendant (i.e., the population to which it is attached, or any parent of that population), and click the **Recalculate** button or select **Recalculate** from the **Workspace** menu. The **F5** function key can also be used to recalculate. All statistics can be hidden from view by selecting **Hide Statistics** in the Workspace menu (show them by selecting **Show Statistics** in the same menu.)

Statistics can be conveniently organized and exported by the [table editor](#). Statistics nodes can be dragged to the table definition; when the table is computed, then all statistic nodes will be updated and computed.

In addition, you can drag statistics into the [layout editor](#). This will create a text box containing the statistic description and the value; it will be updated whenever the statistic is modified (i.e., it is "live").

Now you can build a [table of statistics](#) within the Layout editor. Click the Sigma button in the Layout editor's tool palette. Drag a rectangle in the area where you want your statistical table to appear. To construct the table, [click here](#).

Find this helpful?



Summary of Drag and Drop Examples

This series of examples is based on a workspace in which there were several samples collected from different people. These samples were stained with CD4, CD8, and differentiation markers. The first sample was analyzed in detail and gates were generated to define several T cell subsets.

A series of drag operations using different modifier keys will serve to show how drag and drop can be used to replicate analyses between samples. (This information applies equally to performing operations on groups, but the resulting behavior may be somewhat different. This is discussed fully in the help on [groups](#)).

You may go through each of the examples in sequence, or jump straight to a specific example:

- [Dragging a single node](#)
- [Dragging a node with its children](#)
- [Dragging a node with its parents](#)
- [Dragging multiple nodes](#)
- [Dragging multiple nodes with children and parents](#)
- [Replacing existing nodes](#)

These examples are all based on copying analysis nodes from one sample to another. However, the same options are available for dragging within a single sample, and for dragging to the table editor or the layout editor.

Summary:

To select more than one analysis node, use the command key to select additional nodes. (Use shift key if you want to select a range of nodes that are all siblings).

To take an entire analysis tree, click on the top-most node of the set of nodes you wish to take, and drag with the **option key** down (select all children).

To take only a single analysis node with all the gates used to create it, select that node and drag with the **control key** down (select all parents).

Thus, judicious use of selection and the modifier keys allows you to drop only the portion of the original analysis tree that you want to move.

Find this helpful?



Keyword Search and Replace

Keyword Search and Replace is a tool that allows users to fix/change event annotations supplied by FCS files. This can be done in the graphical user interface or with the help of scripts. The changes are made to the FlowJo **Workspace** and the FCS data is **NOT ALTERED** in this process.

Use the GUI below to change one string at a time. If you need more complicated jobs, skip to the [Scripts](#) section further down.

□

The dialog constructs a **Search and replace** command when you choose elements from the menus. For example,

"When keyword **\$DATE** contains **01/26/2006** replace keyword value with **Thursday**."

The **Choose** drop down menu offers a list of all the keywords in the Workspace.

The **Contains** menu offers **Equals, Contains, Starts with, Ends with**.

After **Contains**, type in the text you want to replace, or...

Auto-Enter value: If you selected a keyword from the first list, this menu will display currently available values.

Replace keyword value with – this menu has two options, Replace Search String and Replace Keyword Value with.

Replace Search String will run the job on *parts of* keyword values.

Example: "When keyword "**anyKeyword**" contains **A** Replace search string with **b** will turn "\$CYT=FACSCAlibur" to "\$CYT=FbCSCblibur"

Replace Keyword Value will run the job on *whole* keyword values.

Example: "When keyword "**anyKeyword**" contains **8** Replace keyword value with **bob** will turn "\$P3S=CD**8**" to "\$P3S=**bob**"

Scripts

You can combine a series of operations like the above, and put them in a text file so FlowJo will batch-search-and-replace. This section will discuss the syntax of this file with some examples.

There are 6 commands in the current implementation:

1. Search and replace
2. Search and define
3. Copy KW value
4. Merge KW value
5. Complex Join Operations
6. Concatenate KW

1. Search and replace

Separate the brackets by typing the tab key

For each job, the syntax should be:

Search <{key, blank}>tab<{equals,contains,starts,ends}>tabtab<{Replace search},{Replace keyword}>tab

For each sample, see if the keyword (or, if blank, any keyword) has a string according to the search operator. If so, then

replace either just the search string or the entire keyword value with

Example:

Search \$P3S equals FITC Replace Keyword Fluorescein

If a sample keyword \$P3S equals FITC (exactly), then the keyword value is replaced with "Fluorescein".

Search contains 44 Replace search 45

If any keyword contains the string "44", then the "44" is replaced with "45" irrespective of where it occurs.

2. Search and define

Define tab<{equals,contains,starts,ends}>tabtabtab

For each sample, see if the keyword has a string according to the search operator (as above). If so, then set the value of keyword to be

3. Copy Keyword Value

Copy tab

For each sample, copy the value of keyword into the keyword

4. Merge KW Value

Merge tab

For each sample, copy the value of keyword into the keyword , but only if the keyword does not already have a value in it. (I.e., Copy if blank destination).

5. Complex Join Operations

This is a multiline command that begins with **Join** and ends with **EndJoin**. In between, it has any number of **Match** criteria followed by any number of **Define** criteria. It looks for samples that match certain criteria; for those samples, the defines are executed.

Format:

Join

Match tabtab ... Match tabtab

Match tabtab ... Define tabtab

Define tabtab ... EndJoin

For each sample, see if there is a matching set of keyword value pairs from the keywords {key1...KeyN} and value pairs as listed. If the Match Keywords all match the values from a single column of values (for example, for a given sample, Key1 value is Value12, Key2 value is Value22, ... KeyN value is ValueN2), the the Define commands are execute for column 2. Therefore, DKey1 is assigned DValue12, ... DKeyJ is assigned DValueJ2.

This function is essentially a "join" command for joining databases.

The Match values are used to match a sample up with a set of criteria; then the Define commands are executed for that criterion.

Example:

Join

Match \$P3S CD4 CD8 CD12

Match \$P4S CD8 CD14 CD45

Define Panel Stain1 Stain2 Stain3 EndJoin

In this Join command, if a sample has \$P3S of CD4 *and* \$P4S of CD8, then the keyword Panel is set to "Stain1". If it has \$P3S of CD8 *and* \$P4S of CD14, then the keyword Panel is set to "Stain2", and so forth.

In Complex Join Operations, between Join and EndJoin, after the Match criteria, you can also specify

Conditional tab tabtab<{contains, equals, starts, ends}>

amongst the Define commands.

This command will do the same as the Define, but ONLY for those samples which also have the keyword with the value according to the operator criteria. This lets you edit the keywords for a subset of the samples specified by the match criteria.

6. Concatenate KW

Concatenate tab<{Skey, "text"}>tab<{Skey, "text"}> ...

Creates a concatenation of all of the values and puts it into DKey. Each value can be either a keyword (take the value of the keyword) or text (enclosed in double quotes).

Example:

Concatenate DB_PatientVisit DB_SampleID ": " DB_TimePoint

When you construct your script, blank lines are ignored. Lines beginning with "/" are ignored (they can be used as comment lines)

Save your script as a plain text file. Select the **Load and Execute Changes** button at the bottom of the **Replace Keyword Values** dialog above. Point to the file you just saved. FlowJo will read the file and execute the commands, changing the Keyword values in the Workspace according to your script.

Find this helpful?



Graph Window

The **Graph** window is the window in which FlowJo displays graphical data: contour plots, histograms, dot plots, etc. From this window, you can draw gates to create subsets of the data and select a variety of different representations of the data. To create this window, double-click on any population node within the sample list in the workspace.

The graph window provides much information about the current subpopulation that it displays: cell counts and frequencies (percentage of total sample), any statistics you have requested, and any gates that were computed on the current population. There are controls to navigate the gating tree, so you can automatically go to the graph display that you used to create this subpopulation; you can also automatically display any gate that is created within this subpopulation.

You can select the default graph type for new samples through the [Preferences](#) dialog. You can change any aspect of the graph by selecting the appropriate menu item under the Graph menu; you can also open the [Options](#) disclosure triangle at the bottom of the graph window.

All graph windows have controls to switch between the next or previous sample in the workspace window list. (Yellow arrows, upper right.) When you click on one of these controls, the exact same graph for the next sample which has the same subset is shown (this may mean that intervening samples, which don't have the same gates, are skipped). These controls let you quickly cycle between different samples to compare the graphs. If you hold down the option key while clicking on this control, then all open graph windows are cycled to the next (or previous) sample. In this case, the command is applied individually to each window; thus, if different windows are pointing to different samples, then after cycling they will still point to different samples. You can also activate the next/previous command from the **Graph** menu at the top of the monitor.

Select a specific topic for more information:

- [Graph window](#)
- [Drawing gates](#)
- [Changing gates](#)
- [Manually entering a gate](#)

The following types of graphs can be generated in the graph window:

Bivariate displays

Univariate displays

[Compare bivariate display types](#)

- [Probability contour plots](#)
- [Pseudo-color density plots](#)
- [Probability density plots](#)
- [Dot plots](#)
- [Histograms](#)
- [CDF](#) (cumulative distribution function)

You may also wish to look at the pages on [copying graphics](#) to other programs and generating [publication-quality graphs](#).

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Graph Types

There are a number of different types of graphs which FlowJo can show you in a graph window. (Special graph types, such as Kinetics and Cell Cycle, can be displayed by launching the appropriate platform).

Once you have a graph window open, you can easily switch between graph types either by selecting the type (and/or options) from the "Graph" menu, or by opening the options disclosure triangle at the bottom of the graph window.

When you close a graph window, FlowJo remembers exactly what you displayed (axes, options, and graph type); the next time you open that subset for display, FlowJo will show you that graph! (The exception to this rule is if you open the graph window using the "Open Parent" (up-arrow) button in a graph window: this function opens the graph window as it looked when you created the gate for that subset.)

There are two univariate display types, and four bivariate displays. Each has several different options that can result in a wide variety of different graphics. Some are more suitable for publication in print, and some are more suitable for generating presentation slides.

The following types of graphs can be generated in the graph window:

Bivariate displays	Univariate displays
Compare bivariate display types	
<ul style="list-style-type: none">• PolyChromatic plots• Probability contour plots• Pseudo-color density plots• Gray scale density plots• Dot plots	<ul style="list-style-type: none">• Histograms• CDF (cumulative distribution function)

You may also wish to look at the pages on [copying graphics](#) to other programs and generating [publication-quality graphs](#).

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Gating

Normal Gating is the most important aspect of analysis of complex data (like flow cytometric data). "Gating" refers to the process of selecting a subset of the collected events for further analysis. You can continue to gate subsets to generate further subsets, until you have a collection of only the cells for which you want a graphic display or statistic analyzed.

The process of gating simply creates a new population. When you collect a sample and import it into the workspace, FlowJo creates a "node" to represent that set of events. When you set a gate to select a subset of the cells, FlowJo creates a new "node"--i.e., a new population. Any operation (graphic display, statistic, gating) that can be performed on the sample can also be performed on a subset of the sample--they are all just populations!

FlowJo organizes this information as "tree"--much like a family tree. The sample is the eldest generation (ancestor). When you gate to create a subset, you generate a new node which can be considered a "child" of the sample. The sample is the parent of the new subset. A second subset created on the sample would be another child of the sample; it is a "sibling" of the first subset. You can then gate on the data in a child to generate yet another generation ("grandchild")... Each new generation is indented another level in the workspace window.

Naming subpopulations is important. You cannot have siblings with the same names, lest confusion arise. In general, you should avoid using duplicate names of subpopulations within any given sample. [Click here](#) for more information on naming populations.

To create a subpopulation, you will draw any of several different kinds of gates on a graphic plot of the parent population. Once you have created the appropriate combination of gates and statistics on a sample, you can easily copy the entire analysis at once to another sample, or to an entire set of samples.

Click on one of the following topics related to drawing and manipulating gates:

- [Drawing new gates](#)
- [Editing existing gates](#)
- [Manually entering gate boundaries](#)
- [Creating Quadrant gates](#)
- [Magnetic Gates](#)
- [Copying gates](#)
- [Graph Overview](#)
- [Naming](#)

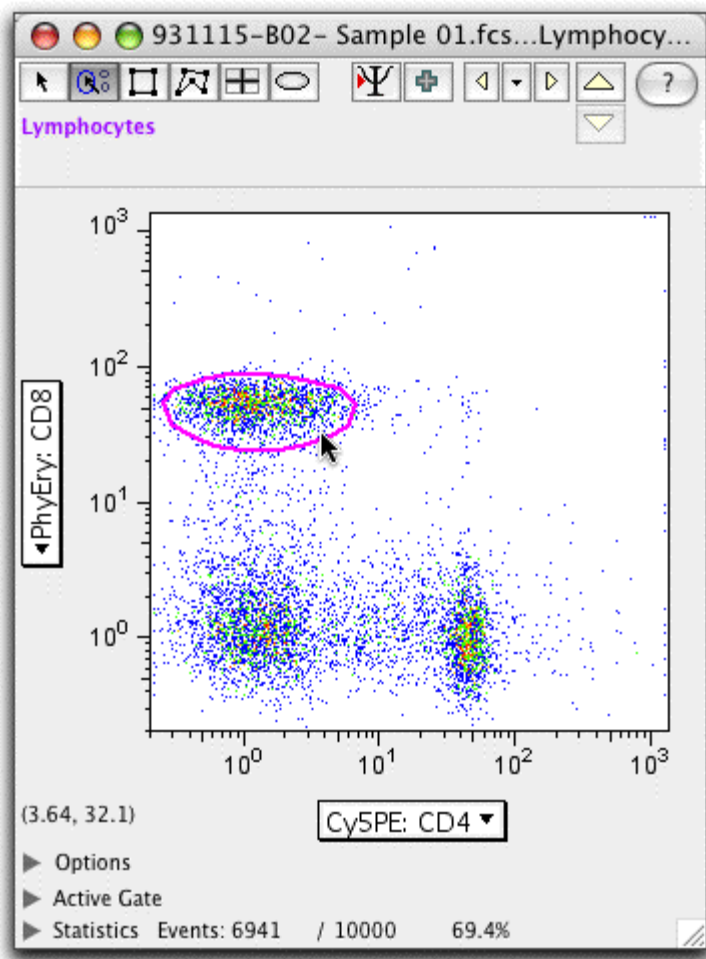
Or, [click here](#) to go the overview on graph windows. Remember to enter page title and metatags

Find this helpful?

□□

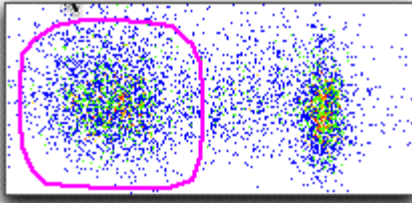
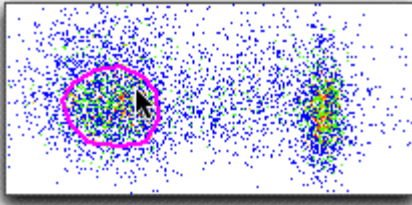
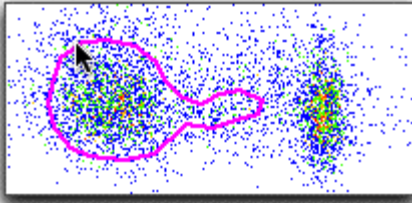
Autogating Tool

Click on the Autogate tool button on a bivariate graph window to activate the autogate feature. When moving the mouse over the graph, FlowJo will automatically find subpopulations to gate. These are shown on the graph window and change based on the position of the mouse. Simply click once to create a polygon gate when the population of interest is circled.



The region that FlowJo finds will include the region that has at least as many cells as the point under the mouse-move the mouse to a more densely populated area, and the gating region shrinks.

After you click down on the mouse, and before releasing the mouse button, you can fine-tune the gate-just move the mouse up or down to increase or decrease the size of the gate. Note in the figures below, the goal is to identify the left population using the autogating tool. Note that in the first figure, the autogate tool starts to include the right population in the gate. To prevent this, the mouse was clicked and the button held down when the gate was centered on the population of interest (middle). With the mouse button held down, the mouse can be moved up and down to change the size (not the shape) of the gate (bottom).



This polygon gate created using the autogate tool acts identically to any other polygon gate. It can be adjusted by hand. It can be dragged to other samples (note the gate on the other samples will be exactly the same as the initial gate - adjustments will not be made according to changes in the contours of the other samples). If you would like to have a gate adjust its position automatically according to the data displayed from different samples, try our [magnetic gating](#) feature. HINT: To choose the autogate tool automatically (without clicking on the button at the top of the graph window), hold down the "control" and "option" keys as you move the mouse over any bivariate graph.

Find this helpful?



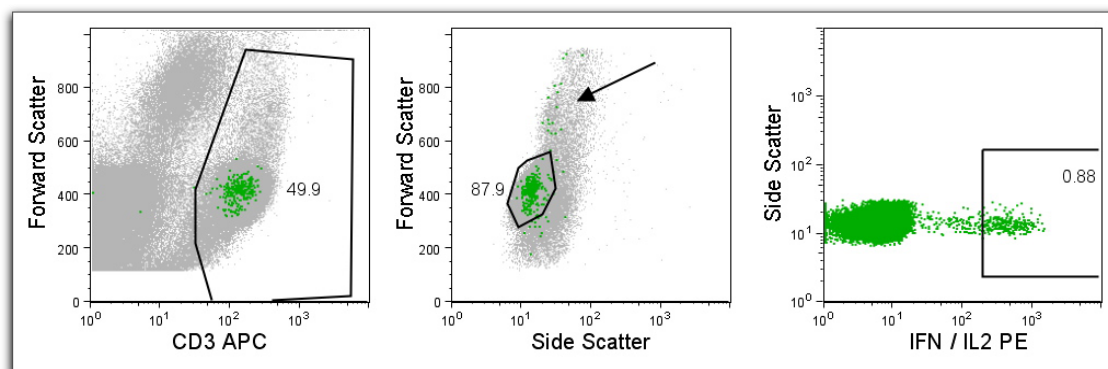
Backgating

FlowJo's backgating analysis provides a tool to view the effect of every gate in the gating tree on the final gated population.



The backgating icon shows up graph window for a subset that is gated more than once (or you can choose "Backgating Analysis." under the Graph menu).

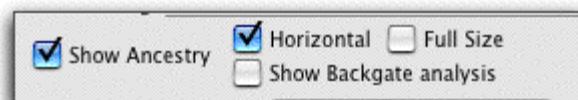
When you click this button, FlowJo shows you a window where each level in the gating hierarchy is displayed, with the final gated population overlaid at each level. This overlay is a backgating overlay: in other words, it shows what the final gated population would look like without that level's gate. Therefore, you can quickly scan to see if any one of your gates is positioned incorrectly. The backgating display can be printed, copied, or saved in the SciBook.



Above is an example of backgating analysis on an intracellular stimulation. PBMC were stimulated and stained for CD3, CD4, CD8, and cytokines. Backgating analysis helps validate gate positions. Here, a typical cytokine gating analysis shows that cytokine producing cells have a different distribution than expected for most T cells, and that the gates should have been widened in some cases to accommodate this (see arrow).

A typical gating scheme was used to identify T cells (CD3+, left), lymphocytes, then CD8+CD4- T cells, and then cytokine producing cells (far right). The backgating analysis shows how each gate affects the identification of events. In each graph, the dots shown in green are fully gated cytokine positive cells. The gray dots in each individual graph show how the final gated population (ie cytokine positives) would appear had that single gate not been applied. Thus, in the second graphic, it is apparent that some CD3+ T cells producing cytokine fall outside of the typical lymphocyte gate (particularly, some appear as "blasts" at higher FS values). This analysis shows that the researcher might benefit from using a larger lymphocyte gate. The backgating analysis is so critical because it reveals that the population of interest, which may be very small, could have a very different distribution from the main population.

Note that the Layout Editor can also show a version of the backgating analysis, by double clicking on the graph to open the Layout Item Definition. Choose the "Show Ancestry" option and select the "Show Backgate analysis" option.



Find this helpful?



PolyVariate Display

The **Polyvariate Display** function shows more than 2 parameters on a 2D graph. This allows the user to define complex populations of cells using one gate. Multiparameter populations have until now required complex boolean gates or multiple, hierarchical gates.

The gates created using this tool work as any other gates in FlowJo. You can drag/drop them just the same and the contents can be interrogated in Graph Windows or the Layout Editor.

You can launch the GUI from a Graph Window. Open a Graph Window, click **Graph** menu, and choose **PolyVariate Plot**. The Polyvariate Plot tool changes the appearance of the original graph. As you make changes in the tool, observe the corresponding changes in the graph.

There are 5 controls in this GUI. They are explained below.

1. **Add/Remove Parameters** menu.
2. **X/Y Axis Range**.
3. **Vector Controls**.
4. **Pivot Range**.
5. **Histogram Pivots**.

The Vector Controls and Pivot Range are part of the PolyVariate circle. The PV circle is a representation of the graph space. The standard 2D plot has two axes at a 90 degree angle with the 0 point in the lower left corner. The PV plot has the 0 point in the center. The vector angle determines which direction away from the 0 point the parameter will be scaled. The vector length from center controls the scaling of the parameter, where the 0 point is the minimum value and the rounded end is the max value. The pivot range circle corresponds to the pivot points on the accompanying histograms, explained below.

□

1. **Add/Remove Parameter** pulldown menu. To add one, select it from the pulldown list. To remove it, click it again from the same list. Each parameter you select will add a colored vector control line to the tool and a histogram of that parameter with the same color as the vector line. Forward and side scatter appear by default.
2. **X/Y axis range**. This is the equivalent of zoom control. The short blue lines accompanying the X and Y axes can be dragged to control the display scaling. Consider the next two screenshots as examples:

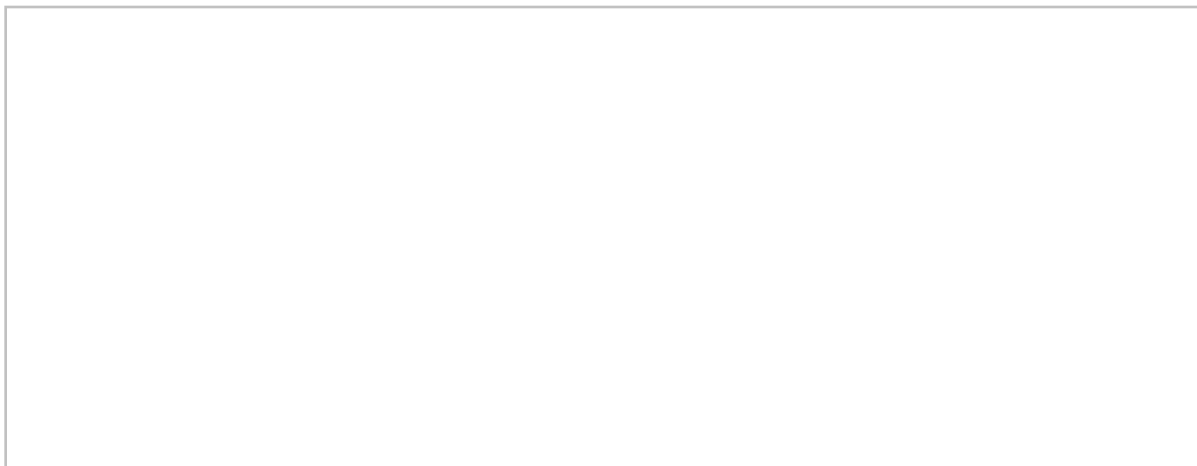
□

In the graph above, the X and Y axis zoom controls are at their default extended positions. The resulting plot is small and does not take up the whole graph space. To zoom in on it, I've adjusted all 4 of the controls to yield the plot below. Please pay attention to the positions of the blue range sliders before and after the change. Notice that they need not be moved to symmetrical positions.

□

3. **Vector Controls**. These are the colorful lines across the PV circle. You can drag the rounded end of the line to change the vector direction and magnitude.
4. **Pivot Range** – this is the light-gray inner-circle in the illustration above. It corresponds to the gray vertical lines on the histograms on the right side of the tool. The events below the grey threshold line on the histogram plot are

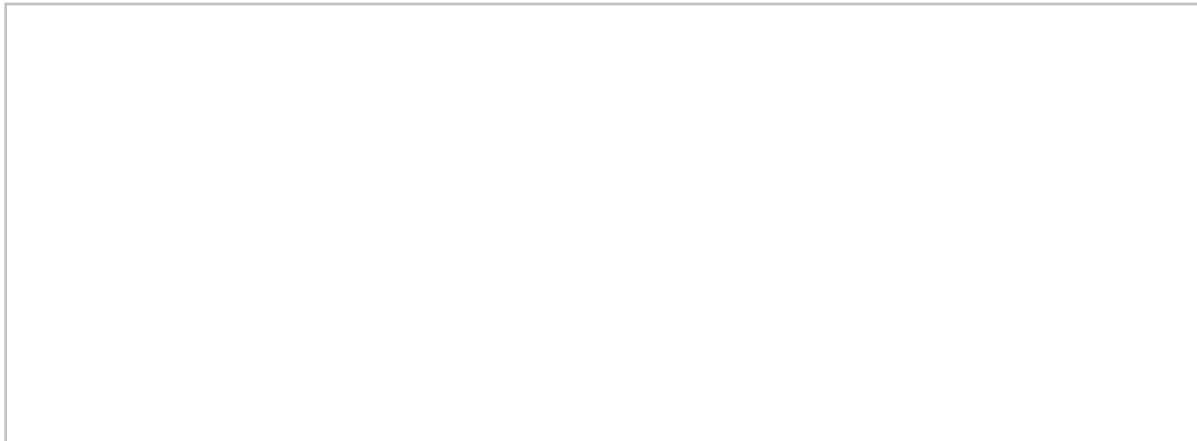
drawn from zero (the center) to the gray circle on the PolyVariate plot; the events above the gray histogram threshold line are drawn from the gray circle outward--in other words, the position of the line on the histogram plot is mapped to the position of the gray circle along the vector. You can drag this circle to change its size. Consider the next few screenshots as examples:



In the graphic above, we're looking at a cluster of CD3+ lymphocytes (lower left) with default pivots and adjusted X/Y axis ranges.

□

Above, we've adjusted the CD3 histogram pivot to accent only the brightest cells. The cluster becomes bigger after adjusting the ranges, because the bright CD3 peak is stretched into the "outer" circle.



In the screenshot above, the gray pivot range circle was dragged outward to make the CD3 cluster tighter.

- Histogram Pivots.** This control ties in with #4 to warp the parameter with the help of the Pivot Range circle. The default value is 0.25 (first quarter of your scale - imagine the length of your parameter in pixels rather than lin/log scaling, and take $\frac{1}{4}$ of that value... that's how it works)

Example 1 - A 3 Parameter exercise (FSC, SSC, CD3)

First, open a graph window for a sample stained with CD3. Select **Graph > PolyVariate Plot** from the main menu , and add CD3 from the pulldown in the PV tool:

□

The resulting graph has Forward Scatter increasing from 0 point (ZP) to vertically up, Side Scatter from ZP to

horizontally right and CD3 from ZP to diagonally right-and-up.

We know that CD3 is mostly expressed by cells with lower FSC and SSC values, so I'm inclined to point the CD3 diagonally left-and-down from the ZP. After adjusting the X/Y ranges and the histogram pivot points, the plot looks like the one below. The Forward and Side scatter pivot points were adjusted to approximately the upper value of the Lymphocyte cells (most of lymphocytes will be found to the LEFT of the FSC/SSC pivots. Most CD3 bright cells will be found to the RIGHT of the CD3 pivot):

□

Now let's draw some gates and find out what we have:

□

Draw two rectangular gates, one on the area controlled by the CD3 vector (CD3+), one on the Forward/Side scatter area. Since the center is zero, this is also the CD3 *negative* area.

Drag the ungated population from the Workspace window into the Layout Editor. Drag in a second copy and change the X axis to CD3. The Y axes on these graphs are OrthSc. Drag the CD3+ gated population onto each graph. The top two graphs above are the result. Drag two more copies of the ungated population into the Layout and adjust the X axis again on the second one.. Drop the CD3+ gated population onto each.

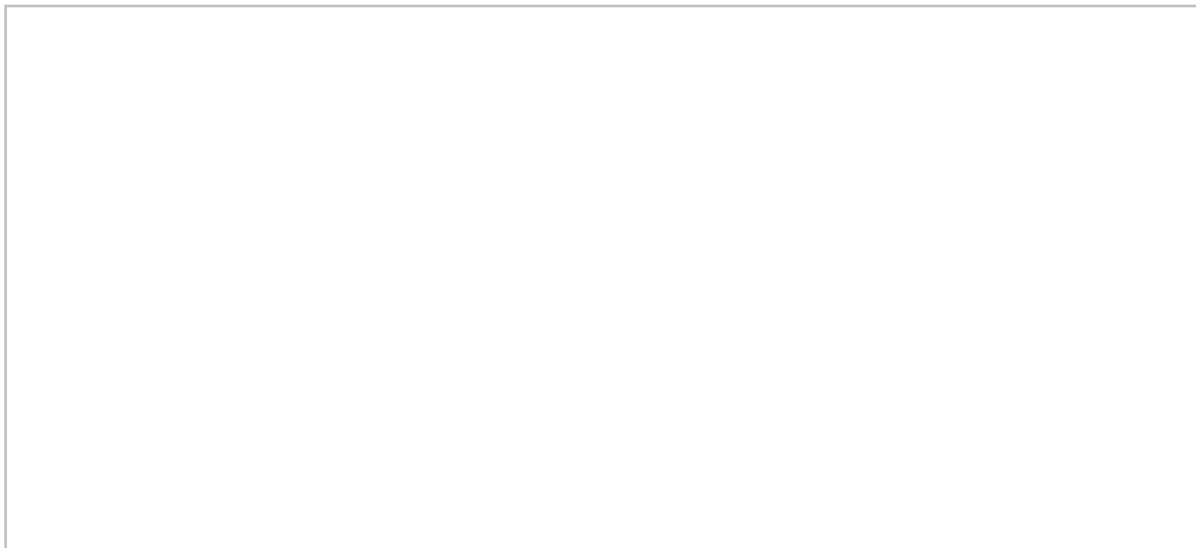
The CD3+ gate, which also includes FSC/SSC parameters, is faster to make than two separate gates on FSC/SSC then CD3. The purity and recovery is within 3% of the standard gates.

Example 2 – A 5 parameter exercise (FSC, SSC, CD3, CD4, CD8)

□

Please note, I've already adjusted pivots/vectors above. The FSC/SSC axes are both pointing up vertically - in my tests including both of them and combining them works better than using either/or.

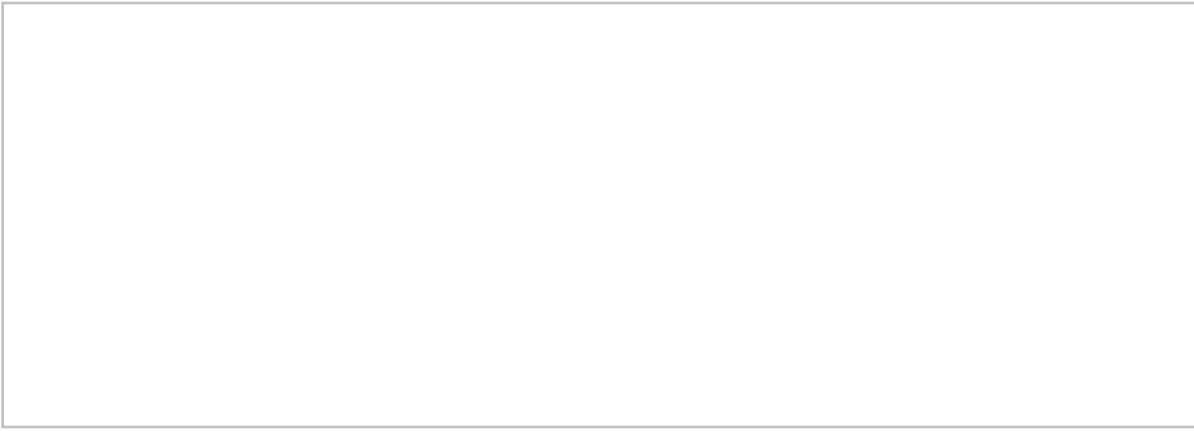
Let's inspect the gates as before, the ungated population in red, the subsets in blue:



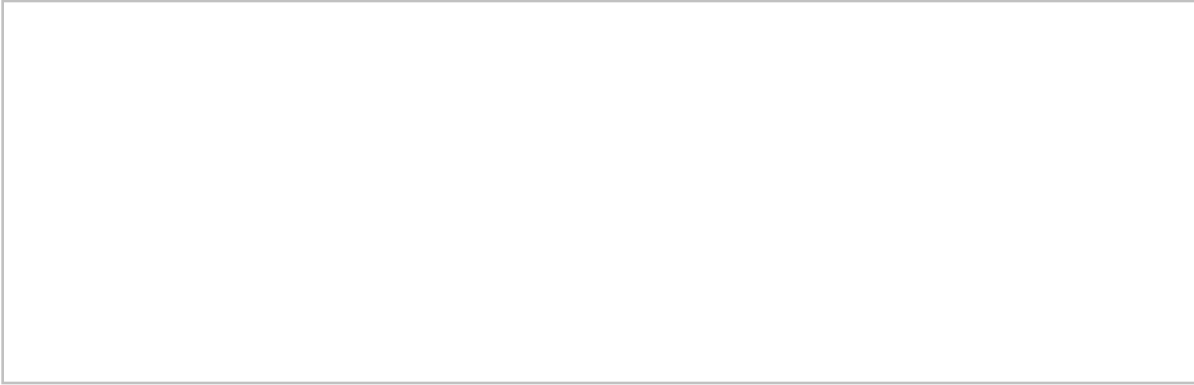
The phenotypes are as clean as they can be - you can use conventional gating to compare purity/recovery.

Example 3 - A 5 parameter exercise 2 (CD3 vs. CD4 AND CD8)

What if we wanted to create a single gate that encompasses the phenotype CD3+(CD4+ AND CD8+)? Set it up like this:



...and the gate contains...



To revert to the graph with which you began, you can click the **Clear** button at the bottom of the window, or, if you have closed the PolyVariate Plot GUI, you can use this button in the Graph Window to revert to the two parameter graph type.

Find this helpful?



Platforms

FlowJo has several analysis platforms that provide capabilities beyond the gating and statistics used for most simple data analysis. All analysis platforms are fully integrated into the FlowJo paradigm: i.e., they can be easily applied to multiple samples (usually, by using [Groups](#)), they are easy to edit, and all operations are saved with the Workspace for your permanent record.

Workspaces and Demo Data are provided for several of FlowJo's platforms so that you can try out these features yourself ([Demo Data downloads](#) page). In addition, we have several short application guides (Tech Notes) that give step-by-step instructions for FlowJo platforms and features. ([Tech Notes downloads](#) page)

FlowJo has the following analysis platforms:

[Kinetics](#)

Kinetics analyses provide you a powerful tool for computing parameter statistics as a function of time and exporting the data or graphics, or statistics computed as a function of time. If you did not collect a time parameter, FlowJo will add one for you. You can even use the Layout Editor to overlay time-series graphs to compare different samples.

[Cell Cycle Analysis](#)

FlowJo gives you a sophisticated, yet easy-to-use interface to model DNA/Cell cycle data. You can simultaneously compute several different standard models, with the ability to constrain different model parameters with the click of a mouse.

[Proliferation Studies](#)

FlowJo models proliferation data obtained using cell tracking dyes such as CFSE. FlowJo presents a graphical display as well as information about each generation in the subset. The proliferation platform also provides information about the fraction of cells from the original population that have divided, and the number of times these cells have divided. In addition the FlowJo Proliferation Platform draws gates that separate each generation.

[Population Comparison](#)

FlowJo can take two or more samples and analyze them to determine how different the distributions are. Comparisons can be univariate or multivariate, and you can rank multiple samples by their similarity to controls.

[Calibration](#)

Using this platform, you can calibrate any of your fluorescence parameters. The platform can use a calibrated bead set as a standard, a stained sample as a reference, or numbers that you enter to define the calibration manually. FlowJo creates a calibrated parameter and displays all graphs and statistics in calibrated units such as absolute number of molecules per cell.

[Compensation](#)

Sometimes your data is not properly compensated... sometimes, it is not possible to compensate your data on the machine because of the complexity of the analysis. FlowJo's compensation platform makes compensating data much easier than compensation on the machine; you are always guaranteed a correctly-compensated output!

[Derived Parameters](#)

Let FlowJo calculate a ratio of two parameters, add a time parameter (in case you want to do Kinetics analyses), or convert between log and linear scaling.

Boolean Gates

You can combine data from existing subpopulations into a gate with your choice of Boolean properties: **And, Or, And Not, Or Not.**

[Movies](#)

An analysis platform unique to FlowJo: view your data dynamically. Use the Movie Platform to generate graphs as a function of time (kinetic analyses), or to generate a graph of one or two parameters as a function of a third. This unique visualization lets you uncover subtle relationships in your data that would be impossible to see otherwise.

[Sample Validity](#)

FCS files can suffer from flaws introduced by the cytometer during acquisition. FlowJo can test the validity of selected samples in the Workspace, or of all samples as they are loaded into the workspace. You can adjust the sensitivity of the test to allow small measurement fluctuations. You can exclude parameters that are not pertinent to the current analysis.

Find this helpful?



Derived Parameters

FlowJo allows you to add new parameters to your data. These parameters are not actually added to the original data files; they are simply descriptions of new parameters that are stored with the workspace. However, they behave in other regards like parameters that were collected: you can display them, calculated statistics on them, etc.

Because they are not stored with the data file, they will not appear if you open the same sample in a different workspace--you will have to add the derived parameters again in that workspace. Currently, there is no way to copy derived parameter definitions between workspaces.

There are four derived parameter types that you can create: time, ratio, log->linear, and linear->log. (Calibrated parameters are also derived parameters, but they are created using the [Calibration Platform](#)). To add a derived parameter, select the sample in the workspace, and then select the "Define new..." menu item attached to the "Derived Parameters..." menu item, under the workspace menu. You will be presented with a [dialog window](#) that allows you to specify the parameters to define. From this dialog window, you can also remove existing derived parameters. Click here to view the special derived parameter [menu items](#).

Adding a time parameter is useful for performing [kinetics analyses](#). This is usually done when you have a response that you wish to measure (for example, calcium flux experiments); however, you might want to see how stable a sample was during collection, and simply display forward or side scatter (or fluorescence!) vs. time during collection. Time can only be added if there is not already a time parameter with the sample. When you add a time parameter, FlowJo assumes that the event rate was constant throughout collection, and distributes events evenly over time. If the start time and end time for acquisition were recorded by the program collecting the data, then FlowJo knows the time over which to distribute the events. Otherwise, FlowJo will ask you to enter how many seconds it took to collect the sample.

You can add a ratio parameter: this is the ratio of any two collected parameters. This is commonly necessary for calcium flux experiments, in which the ratio of Indo-1 fluorescence in two different channels is related to the calcium concentration. You can choose to create a ratio parameter with either linear or logarithmic scaling.

You can create a calibrated parameter. Using the [Calibration Platform](#) you can convert the scaling of any parameter (for instance, to absolute number of molecules per cell). While the calibration platform creates a derived parameter automatically, you may wish to apply such a calibration to other samples--do this by selecting the appropriate derived parameter. You can directly select a calibrated parameter (once the calibration standard exists) by selecting it under the derived parameter submenu.

Finally, you can convert any collected parameter between log and linear. FlowJo will automatically optimize the scaling of the converted parameter to show the maximum number of events.

Find this helpful?



Compensation Overview

FlowJo gives you the ability to compensate your data. This may be necessary in cases where the compensation was inappropriately set during sample collection (although if the sample was over-compensated, then there is no recourse). Also, there may be cases where the instrument is not capable of compensating between certain channels (for instance, to correct for the spillover between fluorescein and Cy5PE).

For a description of why compensation is necessary, the underlying concepts behind compensation, and some of the pitfalls of improper compensation, see the "[Compensation: A perspective](#)" by Mario Roederer (you need to be connected to a network to view this site). The remainder of the documentation below is NOT meant to teach you about compensation, simply how to use FlowJo to perform that function.

FlowJo computes the compensation matrix on control samples much the way you would manually set the compensation. To do this, you will select gates on positive and negative populations for each of these stains, and tell FlowJo to calculate the compensation matrix based on these stains.

Thus, you must collect samples that are **singly-stained** (as well as unstained) for each of the fluorescences that require compensation. Ideally, you would use a stain that only labels a portion of the sample population, so that you have an unstained set of cells in each tube. It is important to remember that the negative population and positive population must share the same autofluorescence characteristics for compensation to be valid (i.e., don't use monocytes as a negative control for a lymphocyte stain; if you are compensating a stain on fibroblasts, use an unstained fibroblast control).

Steps involved in compensation by FlowJo:

1. Define positive and negative gates for each fluorescence channel requiring compensation
2. Open the [compensation window](#); drag the positive and negative populations into the appropriate boxes or use the [Compensation Wizard](#) for extra flexibility.
3. Compute and save the matrix
4. Apply the matrix to the appropriate samples

If you need to generate another compensation matrix for other samples in the experiment, you can just repeat steps 2 through 4 as needed. This is where the [Compensation Wizard](#) will save time and effort.

You may also view an example of the [entire compensation process](#), as a tutorial.

Once you have defined a compensation matrix, it is saved with the workspace. You may subsequently apply that compensation matrix to other samples in the same workspace by selecting it from the menu. To use that matrix in other workspaces, save the matrix to a file ("Edit/Save Matrix") and load the matrix file into the new workspace ("Load matrix"). Note that a compensation matrix is generally valid only for samples collected during a single collection run! However, you can also [edit the matrix](#) using the "Edit/Save Matrix" option. You can **remove** a compensation matrix by selecting **Platform -> Compensate Sample -> Remove Compensation**.

Any sample that has been compensated is marked with a blue bar next to the sample name in the workspace window. **Each new compensation matrix is given its own color for easy recognition of which matrix has been applied to which sample(s).** Compensated samples have new parameters added to their list: for each fluorescence channel to be compensated, a new parameter is created. The parameter name is bracketed with "<>": e.g., when FITC and PE are compensated against each other, two new parameters named " " and "" will be created. Remember to select these new parameters in the graph or statistics windows!

FlowJo can transform the display of compensated data so that populations no longer are displayed squished against the axis. This transformation does not alter the data in any way, it simply changes the scale of the axis so that you can view negative numbers and therefore the entire population of cells as a whole cluster. Please visit the [display transformation](#) web pages for more information.

You may also click on the topics below to get more help on:

- [An example of Compensation](#)
- [FlowJo's Compensation menus](#)
- [The Compensation window](#)
- [The compensation matrix file](#)
- [Changing the compensation matrix manually](#)
- [Display Transformation](#) (for those pesky cells that get squished against the axis!)

Download a Compensation [Workspace with Demo Data](#) to try out this platform.

Find this helpful?



Compensation Wizard

A new interface is available to help you create compensation matrices quickly and rigorously! Launch the platform by selecting **Platforms > Compensate Sample... > Wizard..** If you select the compensation tubes (or a **Group** of compensation tubes) in the Workspace, they will be automatically loaded into the Wizard. Let it guide you through the process!

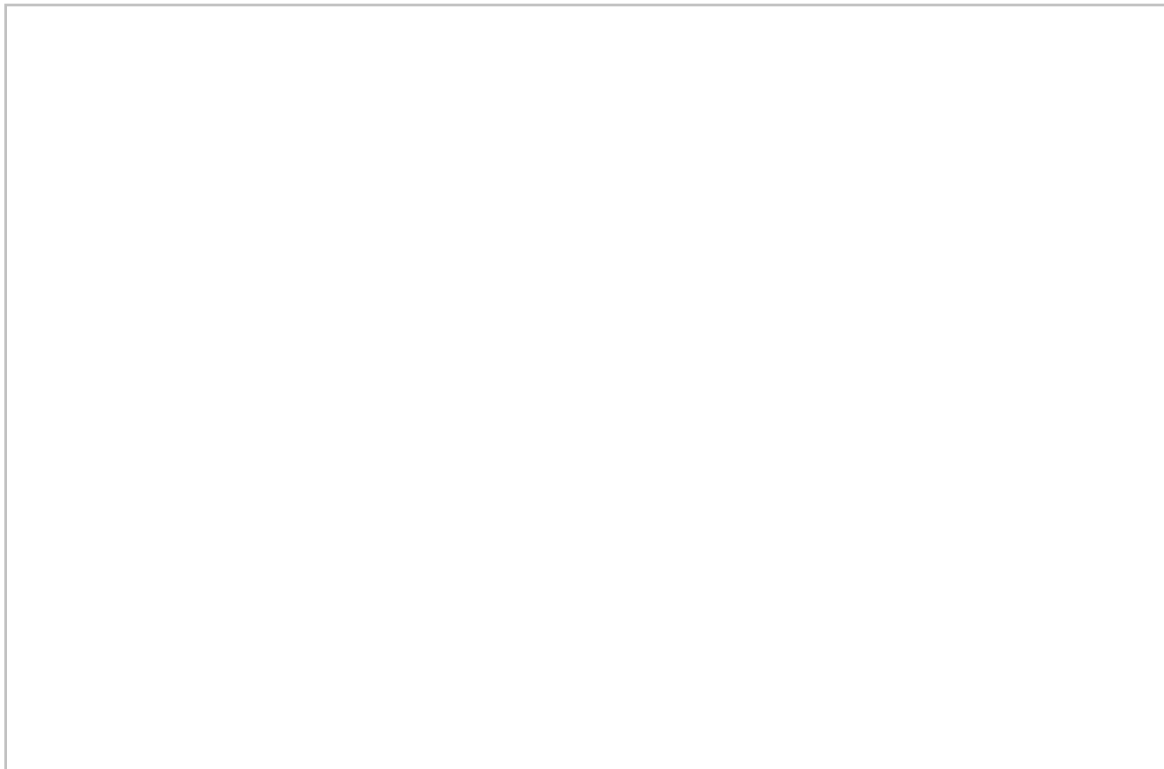
There are a number of short cuts that you can take to speed the process—specifically, create a gate on each compensation tube to identify lymphocytes or singlet beads; and, if necessary, create positive and negative gates for compensation tubes with mixed populations.

Once you have isolated the populations that will form the controls of your matrix, create a group from them, or just select them in the workspace. Open the Wizard and they will be listed as below. You can also open the Wizard and drag them in from the Workspace.



1. Click in the **Use** column to tell the Wizard which tubes will be used in the compensation matrix. This is helpful if you wish to make multiple matrices from numerous tubes.
2. For multiply stained samples, click on the disclosure triangle in the **Color** column to select the desired parameter.
3. For tubes with multiple gates, click the names in the **Positive or Negative** column to select the desired gate from a drop-down list.
4. Click the Validation Graph disclosure triangle and you will see graphic information displayed in the right pane of the Wizard as you work. The tube selected on the left will contribute graphs to this display. You can use these graphs to confirm your choice of controls in complex cases.

To quickly view a population's original graph, click the name of the positive or negative control itself, and select Open Graphic from the menu that drops down.



The Validation graphs display a gating ancestry of the selected positive and negative control populations. If you check the **Show All Fluorescence Plots** checkbox, the Wizard will also display bivariate graphs of other parameter pairs.

□

Text in the Wizard will guide you through the process until you have the right setup to create a compensation matrix. Click the **Create Matrix...** button. Give it a name (like Matrix 1) and it will appear at the bottom of the **Platforms > Compensate Sample...** menu. Select tubes in the Workspace to which you wish to apply the matrix, then select the matrix name in this menu.

Another example is available [here](#) in the pages of the [Daily Dongle](#).

Find this helpful?



AutoCompensation Scripts

1. Introduction
2. Set up the workspace/template
3. [Fill out the Script Tool](#)
4. [Use the template you have created](#)

1. Introduction -

The purpose of this functionality is to reduce the amount of time spent on compensating numerous similar experiments. You can set up a template using these instructions. When you drop a new set of samples on the template they will be compensated automatically by the script generated by this tool. This page will teach you how to set up a set of compensation controls. You can exactly duplicate the steps below using our [Compensation Demo Data](#) or you can use your own single-stained control samples.

The AutoCompensation platform specifies several important instructions to FlowJo:

1. How to identify the appropriate compensation tubes for any given experimental sample
2. Which parameters are to be compensated
3. What gates are to be applied to the compensation tubes to define the positive and negative controls

2. Setting up the Workspace/Template

There are some prerequisites that we must follow for this to be useful. We have to make a workspace that can be saved as a template for future experiments. In our example, we will be using a 4 color data set collected on a DiVa based machine.

Notice the side scatter may show up as linear unless you check the log scaling in your 32 bit file conversions dialog in Preferences.

It will help if the compensation tubes had something not common with the experimental tubes. In this example, the single stain controls were named "[channel name] comp".

The first step is to create groups - one group per compensation control. This way we can retain gates for the comp controls in the template. It is easier to make the groups if you have some keyword to differentiate the samples. Do this during acquisition, and save time later.

The four-color experiment, after setting up the gates, looks like the figure below. There is one shared Lymph gate (fsc, ssc) and the single stain tubes' lymph gates were gated on the individual control channels in histogram mode, to create the positive and negative gates for each control.

□

Four groups were then created, one for each control. I used the staining protocol definition to create the groups. For Example, for the Cy5PE group, the definition was:

□

When done with the grouping, the workspace's group pane will look like this:

□

You can see by the disclosure triangles by the groups, that some nodes are attached. In this case, each group is gated with the Lymphs followed by the positive/negative gates for each control channel.

So for the Cy5PE control, the gating would be

```
>Lymphs
>>>Cy5PE+
>>>Cy5PE-
```

Hint - make your gating "lax." If you experience variation in the position of your clusters for positive/negative region, try to come up with a broad enough gate so it works from day to day.

Once all of our control samples are properly gated and grouped, you can invoke the scripting platform.

3. Fill out the Script Tool

On the main menu click **Platforms/Compensate/AutoCompensation scripts:**

A From the top pulldown menu, click **Create new.**

The first option, **Define a rule for associating..** controls how FlowJo will differentiate the controls from the experimentals.

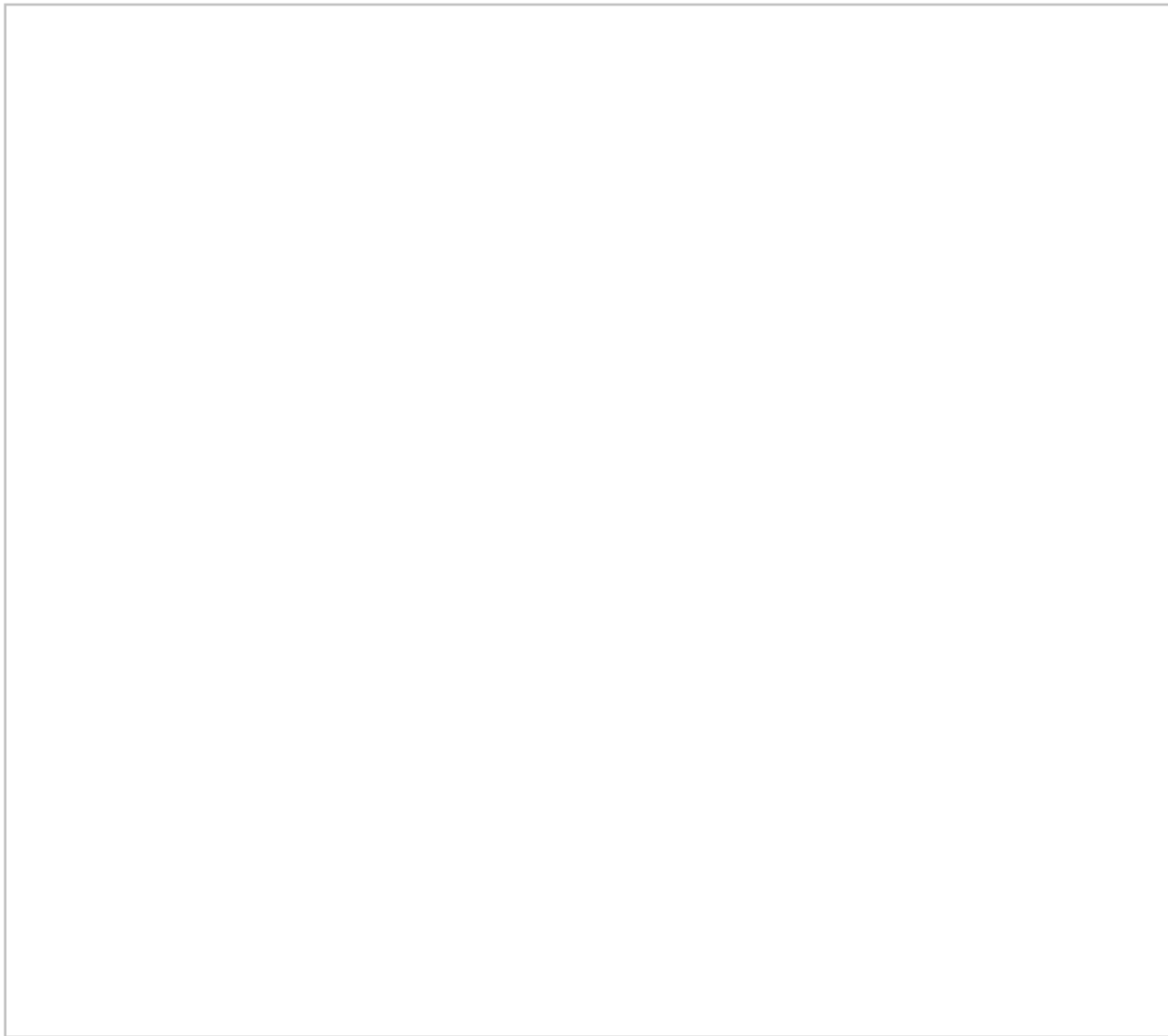
It is important to include a common keyword here. For example **\$Date**. We don't want to create comp matrices for tuesday's samples based on monday's comp controls.

You can be warned if automatically generated gates contain too few events to compensate with validity. Check the box if you wish to **Validate the compensation process.**

B Enter a threshold number depending on the nature of your samples. Check the next box if you wish to **Stop executing the script** in addition to receiving the warning.

C If some or all of the samples are already compensated and you wish to override that calculation, check the **...override existing compensation settings** box.

D Next, click **Add compensation tube for..** and from the pulldown choose the fluors one by one. Notice the table in the Parameter section will grow as you do this. Then, for each parameter choose what keyword/value we should use as identifier, and which gates (already existing in our document) should be used to gather the spillover information:



E Last step - from the **Execute current...** pulldown, choose which samples to compensate using this script. Finally, click **Done**. Now your workspace is ready to be saved as a template - do this from the **File** menu, **Save as Template**.

4. Using the template To use the template, open it, and add new, identically stained samples. Notice how they all end up in their proper groups and the purple compensation bar shows up? Your work is done.

Find this helpful?



Calibrated Parameters: Overview

FlowJo has a unique platform that allows you to calibrate any collected parameter. This gives you the ability to express any parameter's values (graphical display, any statistical calculations) in the units of your choice. Most commonly, this is used to convert the scaling into absolute number of molecules (given a standard that converts between the fluorescence intensity collected on your instrument and absolute numbers of fluorophores).

Once you have created a calibrated parameter, it is added to your sample as if it were just another parameter. You can apply the calibration standard to any other sample in your collection (and you can apply the calibration to an entire group, if you wish). You may have as many different calibration standards in a workspace as you want; in fact, you can even apply multiple calibration standards to the same sample (a new parameter will be added for each calibration that you apply).

When you graph your data, select the calibrated parameter on the axis of choice--the scale values on the axis are now drawn in your calibrated units! For example, if you calibrate to absolute number of molecules per cell, then you can display these numbers on the axis. In addition, any statistics you calculate (medians, CVs, etc.) will be expressed in units of absolute molecules. Finally, you can even enter a gate based on those units: you could, for example, specify a gate on all cells with more than 5,000 molecules of an antigen.

There are two methods by which you can calibrate a parameter: manual or reference. Use the manual mode when you want to enter the exact conversion values yourself. Use the reference when you have collected data on a standard which you can use to convert to your units (for example, a commercial bead set where each bead has a defined absolute intensity; or, a sample stained with an antibody that has a known binding capacity to the cells). When you use a reference, FlowJo automatically finds all of the peaks in the fluorescence, and asks you to enter any or all of the calibration values for those peaks. FlowJo then performs a linear (or logarithmic) least squares regression to determine the best calibration value, and creates the calibrated parameter

A "Calibration standard" is FlowJo's internal representation of the conversion of fluorescence to absolute units. Calibration standards are saved with the workspace, permanently. They are accessible as Derived parameters; thus, if you want to apply a Calibration Standard to another sample (after defining it), select its name under the "Derived Parameters" menu item. For more information on derived parameters, [click here](#).

When you select "Calibrate..." under the Workspace menu, you will be presented with the "[Define New Calibration Standard](#)" dialog window. It asks you to define which parameter you want to calibrate, and then which method to use. To see how to use either method to create a new Calibration Standard, click on [this link](#).

In addition, you can see two [examples](#) of creating Calibration Standards. Both use reference samples; one uses a bead set, the other, the expression of CD4 on lymphocytes as a defined set point. Examine these two series of pages to see how easy it is to calibrate your samples with FlowJo!

Download a [Calibration Workspace](#) with Demo Data to try out this platform.

Find this helpful?



Kinetics Overview

After reading this, you might want to look at the [kinetics question/answer](#) page for a quick introduction to kinetics analysis techniques.

Kinetics analyses are performed by selecting a population in the workspace and choosing **Kinetics...** from the **Platform** menu. If a gated population is selected, then the kinetics analyses are performed only on the cells that fall within the gated subset.

Kinetics requires that there be a parameter collected with time information; and that this parameter is named "Time". If FlowJo doesn't find a time parameter, then it asks if you would like to create a derived parameter that corresponds to time. In this case, FlowJo will assume that there was a constant event rate during collection. The time parameter is created via the [Derive Parameters](#) dialog, which you can also select directly from the **Platform** menu. Once you have defined the Time parameter, you can continue.

FlowJo begins kinetics analysis by showing the graph window for kinetics analyses, as shown below. This window shows the information and controls associated with kinetics analyses.

□

1. Click this vertical button to choose a parameter to analyze vs. time.
2. This button lets you create multiple time slices (see below) in one step.
3. Choose a function to be applied to the Y parameter for display. Adjust other parameters of the display. [More details here](#).
4. When you create time slices they are displayed here beneath All Events.
5. The **Copy** button copies the tabular data to the clipboard.
6. Start time and end time of each time slice.
7. The time of peak value within each slice.
8. The peak value itself.
9. Average value over time slice.
10. Linear least-squares within the timeslice.
11. Area under the curve.
12. The final column contains the elapsed time for each time slice, **Delta T**.

Since Time is always on the X-axis, you cannot change the X-axis. However, you may select any parameter to be displayed on the Y axis. FlowJo, by default, will display a ratio parameter (if one exists); otherwise, it will choose one of the collected parameters.

The value that is displayed in the line graph is a function of the Y parameter (the name of this function is shown directly above the graph). In this case, the line graph is the 75th percentile of fluorescence for the "Ca" parameter as a function of time. No smoothing has been applied (a **Gaussian** smoothing algorithm with a width of 3 seconds or a **Moving Average** smoothing is available.) And **Auto-scaling** is on. To change the function of the Y parameter that is displayed, use the [Kinetics Options](#) window. Activate this window by opening the triangle beside the word [Options](#) (Number 3 in the graphic above.) Refer to the [Options](#) page to see what other kinds of functions you can display.

Just below the graph is a table of statistics regarding the computed parameter. Each line in the table is a timeslice; by default, the first timeslice is **All Events**, with a begin and end time that covers the entire collection. The statistics computed for events within a time slice include the peak time and value and the slope of the line within a timeslice. The units for the peak value and the slope are the same as the units shown on the Y axis - linear scaled units for the

parameter being displayed. You can copy this information directly to a spreadsheet or word processor: click on the **Copy** button just below the left edge of the table, and a copy of the table is placed on the Macintosh clipboard. If you switch to a spreadsheet application, select EDIT/PASTE and the table will be copied in to your spreadsheet.

To create a new timeslice, simply click and drag within the graph as you would to create a histogram gate. You will then be asked to [name the timeslice](#); you may also fine-tune the start and end times in the naming dialog window. Shown below is the result of analyzing the graph above: first, the events were smoothed (via the kinetics options); then, three timeslices were defined to cover the background, response, and resolution time periods. Note that the statistics below the graph reflect these new timeslices, and pertain only to the events within the timeslices. If you wish to delete a timeslice, select it by clicking on the darker horizontal line across the time slice, and press the delete key. (Use option-delete to avoid the confirmation query).

□

Note that the statistics always reflect the smoothed data: thus, by changing the smoothing parameters, you will affect peak time and value. (The slope is generally insensitive to smoothing).

When you select the **Copy** button from the kinetics window, two different items are placed on the clipboard. One is the graph itself (hold down the option key to include the timeslices as shown in the window). This is what you will see when you subsequently paste into a drawing program. In addition, FlowJo puts the actual kinetics data itself on the clipboard. Note that these values are the smoothed values, if smoothing is selected from the [kinetics options](#) .

Kinetics nodes (shown in the workspace with a "t" icon) behave like other nodes: you can drag & drop them to copy and apply them to other populations. When you copy kinetics analyses to other nodes (in the workspace), all of the timeslices are copied as well as the specific information regarding smoothing, parameter selection, etc. You can update existing kinetics analyses for other nodes to be similar to one you have just modified by dragging the newly modified kinetics node onto the original population: FlowJo will ask you whether you want to replace the contents of the existing nodes; select **Yes**. You can apply kinetics nodes to group nodes to perform batch kinetics analysis.

See the [Tips](#) page for kinetics analyses.

Download a [Kinetics Workspace with Demo Data](#) to try out this platform.

Download the [Kinetics Tech Note](#) to print a short (four page) step-by-step guide.

Find this helpful?



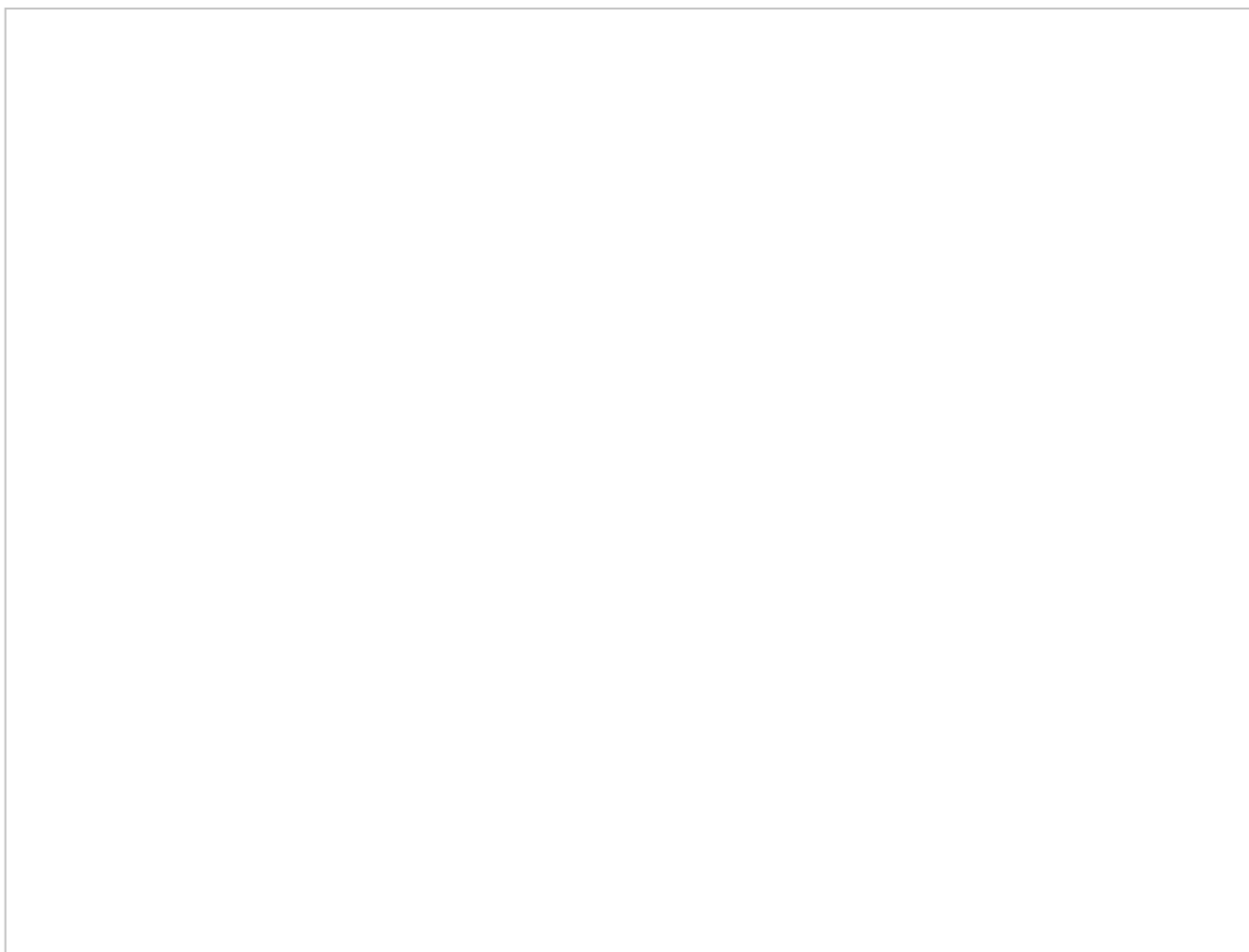
Cell Cycle

FlowJo provides a simple interface to performing fairly sophisticated DNA/Cell Cycle analysis. To launch the Cell Cycle platform, select any sample or gated population (i.e., where you have gated out debris or gated for a desired phenotype), and choose "Cell Cycle..." from the "Workspace" menu. FlowJo brings up a graph window that is specially designed for cell cycle analyses. You might want to visit the page giving [hints for Cell Cycle analysis](#) with FlowJo.

FlowJo tries to determine which parameter contains the DNA quantitation information; if it chooses the wrong one, select the correct one from the X-axis popup menu. Then click on the button "Add/Change Models" to begin your Cell Cycle analysis.

When you click on "Add/Change Models" button, FlowJo shows you the [Cell Cycle Specification Window](#). From this window, you can decide which of several models to compute for the data, and, if necessary, to constrain the fitting parameters by any number of criteria.

Once FlowJo has computed the model, it displays the fit along with statistical data in the Cell Cycle window, such as that shown below.



The statistics displayed in the table will depend on which models and options are computed, but all will include basic statistics such as the fraction of cells in G1, S, and G2, the positions of the G1 and G2 peaks (and their widths), and the

number of cells below G1 and above G2. In addition, the RMS (root mean square) error of the fit is displayed in the first column. If you change the fitting criteria, you may wish to minimize this value as a way to optimize the fit. If FlowJo fails to fit the model(s) to the data, then it will display "Invalid" in the RMS field. In this case, you will want to help FlowJo fit the data by constraining different parameters. See the "[hints](#)" page for ideas on how to proceed.

If you click and drag within the graph window, you can create "ranges". Ranges are similar to histogram gates, and can be used by the fitting criteria to constrain peak positions to within the range. In the example above, a range was defined around the G1 peak in order to help FlowJo determine the optimal fit: in the [Graph Specification Window](#), the fit was constrained such that the G1 peak must be found within the defined range.

Cell cycle analyses can be copied between subsets and between samples, and even to groups, just like every other analysis in FlowJo. In this fashion, you can compute Cell Cycle analyses on every sample in an experiment. In general, you will begin by analyzing a control sample, and use this control sample to define ranges for G1 and G2. If you have unusual distributions, constraining the fit by these ranges will help FlowJo determine the proper distribution of cells. Once you have defined the ranges and the fit, drag the analysis to other samples (or the group).

You can drag Cell Cycle analyses to the Layout Editor to generate reports that contain the graphs, the models, and the basic statistics (fraction of cells in G1, S, and G2). You can also copy the table of statistics to the clipboard by clicking on the button right above the table--and then paste into any spreadsheet or word processor for further analysis.

To learn more about applying specific models, view the page on Cell Cycle [Graph Specification](#). You may also wish to view the page on [hints](#) for performing Cell Cycle analyses.

Download a [Cell Cycle Workspace](#) with Demo Data to try out this platform.

Find this helpful?

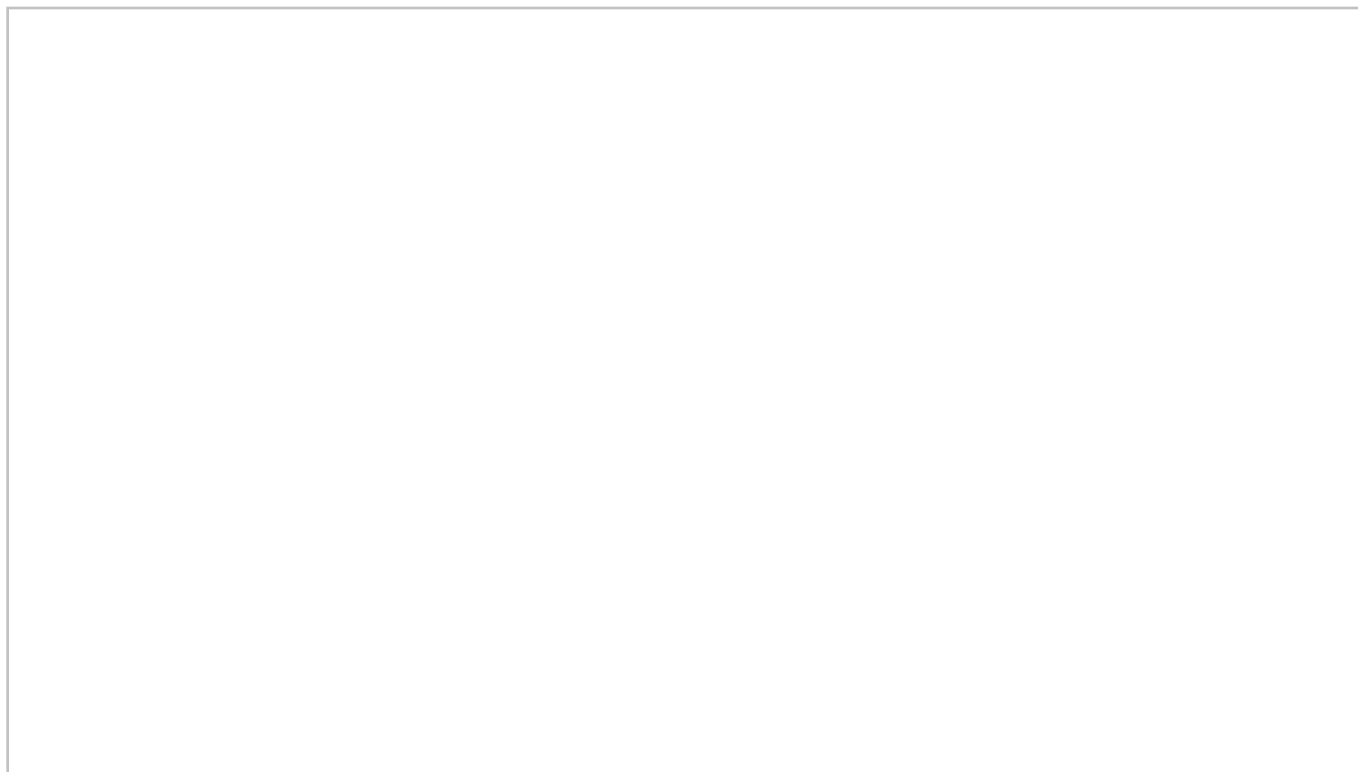
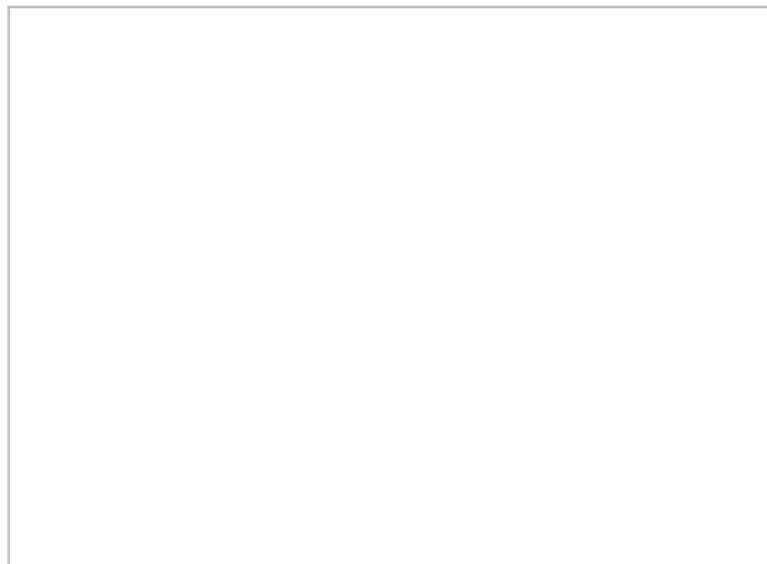


Proliferation

Using flow cytometry to **track cell generations** is now possible, thanks to the introduction of cell tracking dyes. FlowJo presents a graphical display and a table of data on each generation in the subset. This will provide you with information about how many cell divisions have occurred. In addition the FlowJo Proliferation Platform draws gates in order to separate each generation.

Start by *clicking* on the population to analyze and *selecting* **Proliferation** from the **Platform** menu.

This brings up the **Proliferation Platform**.



Select a parameter on the **X-axis**.

The default **number of peaks** is 8, although it is important that the chosen number of peaks exceed the actual number of peaks in the data.

It is useful to initially calculate the model without constraints (adding [constraints](#) as necessary to obtain the best fit).

1) **Calculate** the model by *clicking* on the **Calculate** button.



Click a **yellow arrow** to cycle through samples.

Click the **black triangle** to select a sample.

Click the **clipboard icon** to copy table to clipboard.

The **Division Index***, **Proliferation Index***, and the **%Divided*** are explained below.

The better the model fit, the lower the Root Mean Square (**RMS**).

2) **Create Gates...**Gates dividing the generations can be created by *clicking* the **Create Gates** button. (Draws a gate halfway between each peak and adds them to the Workspace.)

3) **Show Overlay:** Select this button to open the [Multigraph Overlay](#) window showing each generation's gate displayed on a bivariate plot with the parameters of your choice.

Proliferation Statistics Defined

□

Division Index is the average number of cell divisions that a cell in the original population has undergone. This is an average even for cells which never divided (i.e., includes the undivided peak).

Proliferation Index is the total number of divisions divided by the the number of cells that went into division. The proliferation index only takes into account the cells that underwent at least one division, that is, only responding cells

are reflected in the proliferation index. This is probably a more useful value to compare from sample to sample, as it considers only the fraction of responding cells.

The proliferation index more faithfully reflects what the biology of the responding system is; the division index reflects what the entire system is doing.

Another way to think about it - between the two, whichever value is smaller is the average number of divisions of all cells (including nonresponders); the larger value is therefore the average number of divisions for the responding population.

Expansion Index (Exp. Index) - determines the fold-expansion of the overall culture.

For example, if one million cells were stimulated, and EI is 3.0, then the resulting cell count at the end of the culture is three million. This statistic is therefore identical to that obtained by manual cell counting, and is predicted to be closely related to a 3H-T assay of the culture.

Replication Index(Rep. Index) - determines the fold-expansion of only the responding cells.

This reports the expansion capability of the replicating cells rather than for the entire culture (which is the *Exp. Index*).

SD (Div) - the variance of PI. It defines how much variability there is in the proliferative capacity of cells in the starting culture. It is more difficult to assign a direct biological meaning to this parameter, and therefore perhaps less useful to report on a routine basis. It can be thought of as a measure of how tightly-regulated the proliferation process will be, after the responding cells have been stimulated.

%Divided is the same as the Precursor frequency.

Peak CV is the coefficient of variation for the peaks. The same value of CV is modeled for all peaks. Typically, this value is 4-7%.

Peak ratio is the ratio of fluorescence between subsequent peaks. i.e., 0.5 implies that peak "n" has half as much fluorescence as peak "n - 1". Values > 0.5 are not biologically meaningful, and usually arise when the log amp is not very good. The value should be close to 0.5.

As an example of how Proliferation Index and Division Index would be calculated, consider the following:

G0 = 15888 G1 = 32922 G2 = 13647 G3 = 897

The number of cells at start of culture: $15888 + (32922/2) + (13647/4) + (897/8) = 35872.875$

The total number of divisions: $(32922/2)*1 + (13647/4)*2 + (897/8)*3 = 23620.875$

The number of cells that went into division: $35872.875 - 15888 = 19984.875$

Division Index: $23620.875 / 35872.875 = 0.66$

Proliferation Index: $23620.875 / 19984.875 = 1.18$

The [Model Parameter Adjustments](#) can be used to obtain a better fitting model.

As with all other platforms in FlowJo, the **Proliferation Node** can be applied to **groups** of samples by *dragging*.

Each **generation** can be analyzed separately (*double click* the generation number to open a graph).



Select the **Backgate** icon to display a backgating analysis of the generation.

□ For an in-depth look at the application of statistics to proliferation modeling, see [Interpretation of Cellular Proliferation Data: Avoid the Panglossian](#). Mario Roederer, *Cytometry Part A* • 79A: 95-101, 2011

Find this helpful?



Population Comparison - Overview

Comparing distributions of FACS data is an important goal in many applications. For example, to determine whether two samples are statistically significantly different (control vs. test sample) in order to detect a response, or to provide feedback regarding instrument stability by detecting when collected data varies significantly over time.

Comparison Algorithms

FlowJo's comparison platforms support four different comparison algorithms. Two algorithms (Overton and SED) are used to calculate the percentage of positive cells found in the sample and not the control). Two algorithms (K-S and Chi(T) / PB) are used to determine the statistical difference between samples.

The [Overton cumulative histogram subtraction](#)¹ algorithm essentially subtracts histograms on a channel-by-channel basis to provide a percent of positive cells. This method does not provide an indication of the probability with which two distributions are different; nor does it provide confidence intervals.

The Super-enhanced Dmax Subtraction (SED) is a new sophisticated algorithm by Bruce Bagwell to compute %Positives when comparing histograms.

Several algorithms can be used to compare FACS data. The Kolmogorov-Smirnov (K-S) algorithm is a commonly used method to determine the confidence interval with which one can make the assertion that two flow cytometric univariate histograms are different. Caution must be exercised with this statistic as it will erroneously report that two halves of the same population (every other cell makes up one of the halves while the cells in between make up the other half) are distinct.

A new comparison algorithm was recently developed for the comparison of distributions, called [Probability Binning \(Chi\(T\) or PB\)](#)⁽³⁻⁵⁾. The PB comparison is related to the [Cox chi-square](#)⁶ approach, but with modified binning such that it minimizes the maximal expected variance. This algorithm has been shown to detect small differences between two populations and it does so in a quantitative way.

FlowJo Population Comparison Platforms

FlowJo contains two platforms that allow the direct comparison between different populations, Population Comparison (Uni- and Multivariate) and Multi-sample Comparison. The compared populations can either be subsets of the same sample, or more commonly, equivalent populations in different samples.

Comparing Populations Using FlowJo

Method	# Parameters	Statistic	Create Gates?	Compare
Univariate	1	K-S, Overton, PB, SED Subtraction	no	Individual Populations
Multivariate	1 or more	PB	yes	
Multi-sample				Individual or aggregates of Populations

The [Univariate Comparison](#) platform compares single parameters using the Overton, SED, K-S, and Chi(T) statistics.

The [Multivariate Comparison](#) platform compares a single test sample to a single control for multivariate data using the Chi(T) statistic.

The [Multi-sample](#) platform compares either univariate or multivariate data of single samples to composites of control samples using the Chi(T) statistic.

References:

- 1) Overton WR. *Modified histogram subtraction technique for analysis of flow cytometry data*. Cytometry. 1988 Nov;9(6):619-26.
- 3) Roederer M, Treister A, Moore W, Herzenberg LA. *Probability binning comparison: A metric for quantitating univariate distribution differences*. Cytometry. 2001 Sep 1;45(1):37-46.
- 4) Roederer M, Moore W, Treister A, Hardy RR, Herzenberg LA. *Probability binning comparison: a metric for quantitating multivariate distribution differences*. Cytometry. 2001 Sep 1;45(1):47-55.
- 5) Roederer M, Hardy RR. *Frequency difference gating: A multivariate method for identifying subsets that differ between samples*. Cytometry. 2001 Sep 1;45(1):56-64.
- 6) Cox C, Reeder JE, Robinson RD, Suppes SB, Wheelless LL. *Comparison of frequency distributions in flow cytometry*. Cytometry. 1988 Jul;9(4):291-8.

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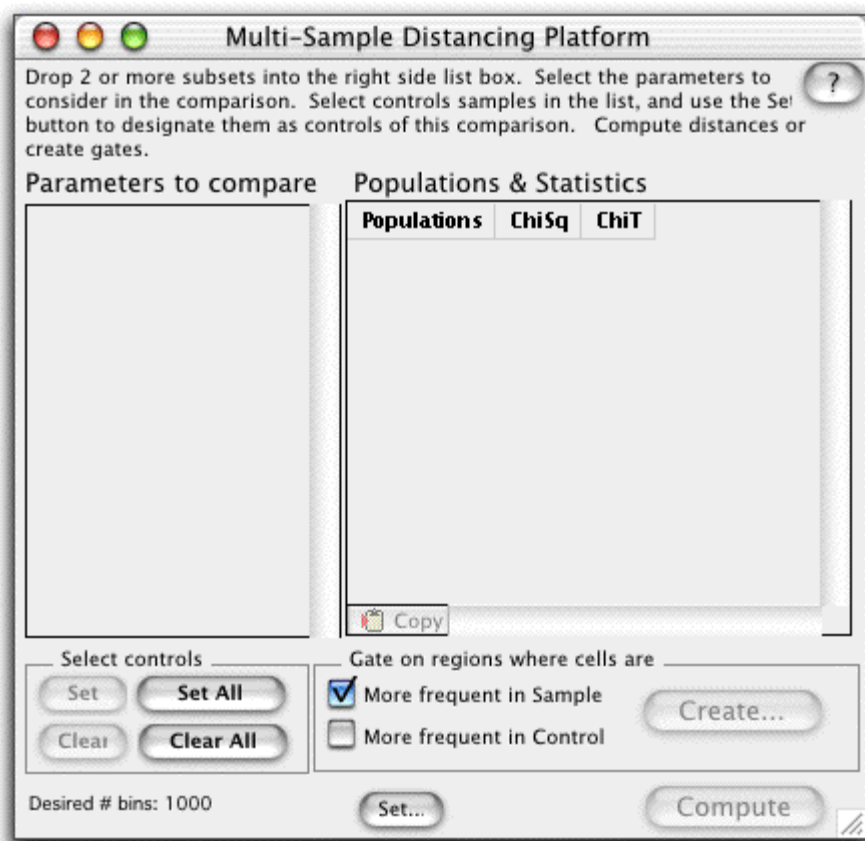


Multi-sample Population Comparison

In FlowJo, the Multi-sample Comparison platform is used to compare single or multiple parameters from a test sample to a *composite* of more than one control sample.

For example, in comparing multiple samples against each other, it is sometimes not possible (or meaningful) to assign a single sample as the control, against which all others are to be compared. In such a case, the Multi-sample Comparison platform can concatenate all of the control samples in order to use the average of all the control samples for comparison. This process mitigates the potential artifact introduced by selection of a sample as control that is actually significantly different than the expected control sample.

Determining the samples to be concatenated is best approached by an iterative process. One can concatenate all the control samples and compute the distances of each control sample to the average of all of them. Thus, those control samples which are outliers can be removed from the control set. Caution is warranted since reduction of the number of samples entered as controls can lead to sampling bias.

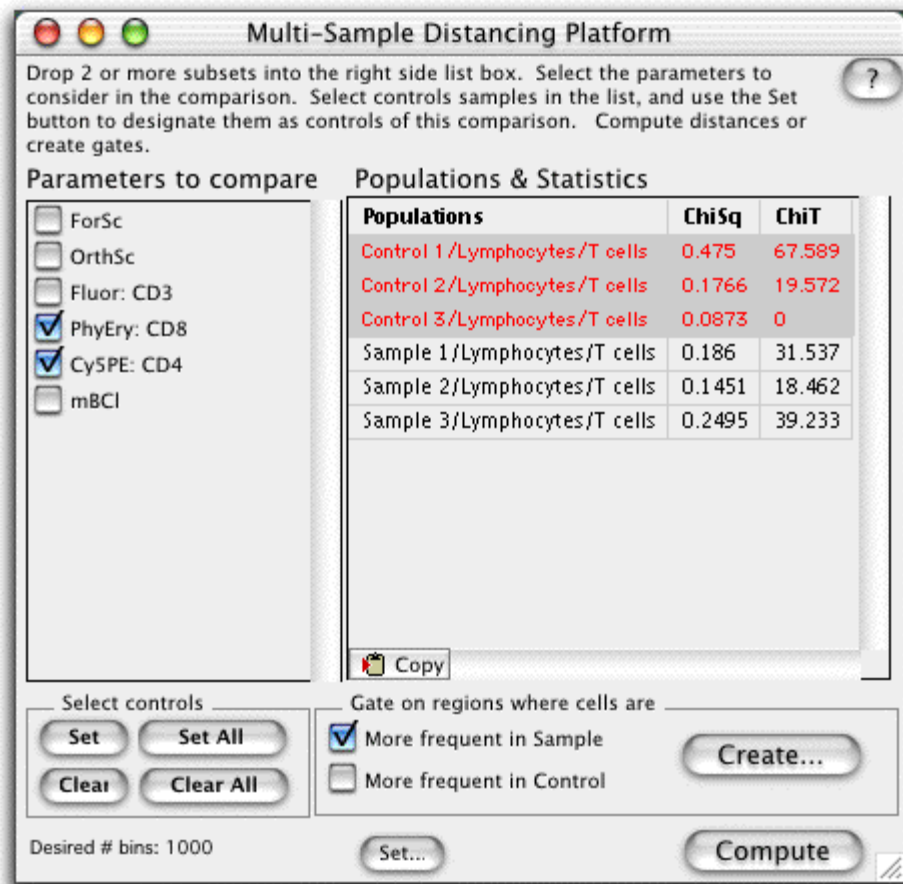


Start by selecting the **Multi-sample Comparison Platform** from the **Platforms** menu. Note that this platform does not associate with a particular population.

Drag two or more samples (or subsets of the same sample) to the right side of the platform into the Populations and Statistics box.

Select the control samples in the list by clicking (they become shaded). Click the **Set** button (under **Select controls**) to designate these samples as controls (they become red). All of the samples can be designated controls by clicking the **Set All** button.

Check the boxes on the left to choose the **Parameters to Compare**.



The [number of bins](#) can be set according to the number of events collected. Click on the **Set...** button at the bottom of the window to set the number of bins.

A gate based on the differences between the two samples can be created by clicking Create. Visit the [Gate Cut-off](#) page for information on setting the X^2 threshold.

Click the clipboard to copy this table to clipboard.

The **Chi Squared Test** divides the concatenated control sample into bins with the same number of events, divides the test sample along the same boundaries and calculates the Chi Square of the two binned data sets. The X^2 is converted into a metric ($T(X)$) that can be used to estimate the probability that each test population is different from the concatenated control population. See the [Population Comparison Overview](#) page for a complete explanation of this statistic.

When $T(X) = 0$, the two populations are indistinguishable ($p = 0.5$) and when $T(X) = 1$, the populations differ by one standard deviation, giving the probability that the two populations differ $p < 0.17$. A value $T(X) > 4$ implies that the two distributions are different with a $p < 0.01$ (99% confidence). However, the minimum value of $T(X)$ that has **biological significance** depends on the nature of the data being analyzed and therefore needs to be determined empirically. Only populations which have $T(X)$ values larger than this empirical minimum can be considered to be different.

Several populations can be compared in order to determine the minimum $T(X)$ value. Machine stability during the collection, as well as inherent variability in the FACS data are just two reasons why the comparison of a population to

itself can give a $T(X) > 0$. You can compare the same sample collected twice. Collecting one sample at the beginning and one sample end of the collection process best determines the machine stability. Or compare several different samples that have been treated with the same stimulation.

The number of bins that the test and control sample are divided into should be maximized to most easily detect small differences between populations; however, the number of bins can become limiting for this statistic (depending on the number of events collected and the number of parameters compared). Therefore, a reasonable number of bins is roughly 10% of the event count - leading to a minimum of about 10 events per bin.

As the number of parameters being compared increases, then more events may need to be collected in order to distinguish subtle variations in the populations. However, inclusion of parameters in the comparison which Do Not vary between populations does not degrade the ability to distinguish the populations.

Note that the computations in the Multi-sample Population Comparison platform are memory intensive. You may need to allocate more memory to FlowJo (more information on [memory requirements](#)).

Find this helpful?



Clustering

Adaptive Binning values

Note that adaptive binning will take a set of events, create a histogram on every parameter being clustered for those events, and then examine if any of those histograms could be divided usefully. The following parameters control how this histogram examination is performed to divide events further and further into smaller and smaller bins.

Minimum Separation Channel:

Clustering will not divide any population below this fraction of the total scale of a parameter. Thus, a value of "0.25" means don't divide any subset within the first decade (on a 4-decade log scale). Setting this value too high will not allow the platform to resolve populations near the low end of the scale. Too low, and it may create "biologically-irrelevant" populations.

Range of values: 0 - 1

Default value: 0.03 (roughly, 1/8th of a decade in a 4-decade scale).

Other useful values: 0.06, 0.125, 0.25

Histogram Resolution:

The number of channels used for histogramming. Channels are equally spaced (linear parameter), or log-spaced (log parameters), exactly as for creating displays. Setting this value too high could cause subsets to be "orphaned" during the "shaving" of empty space off of histograms during adaptive binning... this could lead to clusters that cannot be joined. In addition, the clustering algorithm will take longer with larger values. Setting this value too low will lead to an inability to distinguish clusters.

Range of values: None (However, less than 16 is probably useless, as is more than 1024)

Default value: 256.

Other useful values: 128, 64.

Minimum Shave Fraction:

During adaptive binning, one major criterion for dividing a distribution into bins is whether or not there is "empty" space at one end of the histogram. Such empty space will be "shaved off" into a separate bin from the data. Since bins that are not physically adjacent can never be re-joined into a cluster, shaving too aggressively can lead to the inability to rejoin event-containing bins into real clusters. For this clustering algorithm, the shaving step is one of the most critical.

This value specifies how much of a distribution in a given parameter must be devoid of events before the platform considers "shaving" it off. Thus, a value of "0.1" means that 10% of the distribution's width (either upper or lower end) must be completely devoid of events before shaving is considered. A value that is too small will lead to too many orphaned clusters. A value that is too large will not allow sufficient resolution to segregate otherwise close clusters.

Range of values: 0 - 1

Default value: 0.1.

Minimum Shave Channels:

See above discussion on "Minimum Shave Fraction." In addition to that criterion, the platform will never shave off any empty space if it contains less than a fixed number of channels, which is this value times the histogram resolution. In other words, this value defines the smallest empty space that can be created during binning.

Range of values: 0 - 1

Default value: 0.063 (approximately 1/4th decade on a log scale)

Other useful values: Smaller, probably as low as 0.01 might be useful.

Maximum Value Height Ratio:

During adaptive binning, the other criterion for dividing a distribution that is always checked is whether or not there is a bimodal distribution. This value determines what kind of distribution is considered bimodal. If a valley exists between two peaks, then the distribution is bimodal. A valley must be no higher than this value times the lower of the two peaks. (In other words, a value of 0.5 means that the valley must be no higher than half of the height of the lower peak). If there is no point in the distribution where this exists, then the distribution is not considered bimodal. A value that is too small will prevent the platform from finding useful peaks in a distribution. A value that is too high will divide events seemingly randomly.

Range of values: 0 - 1

Default value: 0.5

Useful value: 0.75

Separation: Valley Weighting

Separation: Even Division Weighting

If, for any given set of events, there is more than one parameter that is bimodal, then these two values determine which one better separates the events. Since adaptive binning is iterative, it probably doesn't matter which parameter is first divided; the other will be divided shortly. Nonetheless, these two parameters provide the relative weighting to compare two such divisions. A larger value on valley weighting means that emphasis is placed more on how deep a valley is between the two peaks. A larger value on the "Ev Division" means that emphasis is placed on divisions that more evenly divide the events by number.

Range of values: 0 - infinity

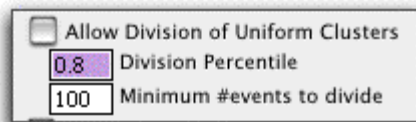
Default values: 1 and 10

Useful values: 10 and 1

Allow division of uniform clusters

If set, then adaptive binning is instructed to divide a uniform cluster of events. This occurs when no shaving can occur and no "valley" between peaks can be found in any parameter. If this option is selected, then Cluster Joining will be

required.



Division percentile

If the platform decides to divide a uniform cluster, then it will do so at this percentile of the distribution. A value of 0.5 means to divide the distribution evenly. A value of 0.8 means to divide the cluster at either the 20th or the 80th percentile, whichever gives a larger division in terms of area. Values further from 0.5 will tend to create more clusters near edges of event densities.

Range of values: 0 - 1

Default value: 0.8

Useful values: 0.5, 0.9

Minimum # of events to divide

If the number of events in a uniform cluster is less than this value, then the cluster is not divided. In general, if the uniform division is allowed, then there will be no cluster with more than this number of events. Therefore, this number effectively determines a lower bound on the number of clusters. A small value will therefore create many more clusters, and require correspondingly much greater computational time at joining.

Range of values: 1 - infinity

Default value: 100

Useful values: about 0.1 to 1.0% of the number of events in the file

Do simple Peak Find Separation

If selected, then the platform will attempt a more sophisticated peak finding algorithm to find populations (i.e., more sophisticated than the valley search performed by default). This algorithm might allow for the identification and separation of "shoulder" clusters. The algorithm functions by scanning on either side of the mode of a histogram. As it moves along either side, it computes the local slope of the histogram. By comparing the Based on the following parameters, a determination is made as to whether or not a peak has been found, and if so, what its extents are.

Chan Width of Slope Function

The number of channels over which a running slope is calculated. A large value will, in effect, use a heavily smoothed histogram to look for a peak. A small value will obviate the utility of the peak find.

Range of values: 1 - infinity

Default value: 5

Minimum Peak Height

A peak must be at least this many cells in a single channel before it is considered a true "peak". Large values will tend to prevent the algorithm from finding a peak; small values will tend to identify too many peaks.

Range of values: 1 - infinity

Default value: 10

Trigger Slope: Mode Ratio

A peak has been found when local slope (per channel) is more than this factor times the mode value.

Range of values: 1 - infinity

Default value: 0.1

Useful values: 0.01 - 0.5

Fire slope Ratio

The end of the peak is identified when the local slope is less than this factor times the maximum slope that occurred since the mode. i.e., a value of 0.1 means that the farthest reaches of the peak occurs when the slope is only 10% (or less) of the maximum slope between this point and the mode. Larger values will find more subtle shoulders in a distribution, but are more prone to simply randomly dividing the distribution. A value of 0 would require a true "valley"--i.e, for the distribution to start rising again.

Range of values: 0 - 1

Default value: 0.1

Joining Criteria:

If joining is initiated, then the algorithm attempts to join all physically adjacent bins into clusters. Bins that are not physically adjacent (tangent) are not considered; hence, the "shaving" function above cannot be too aggressive.

Minimum

Joining ceases when this many clusters remain. Since the order in which bins are joined is not well-defined, it is unlikely that this value should ever be changed from the default of 1.

Max InterClus Dist x

In order for two clusters to be joined, not only must they be tangent but the distributions of each cluster must not be significantly changed by the join. Currently, this is tested as making sure that for every parameter involved in the cluster, the distance between the centers of the two clusters is no more than this value times the width of each cluster. Therefore, larger values will tend to join clusters that are more distinct; smaller values will tend to keep clusters apart.

Range of values: 0 - infinity.

Default value: 2

Useful values: 1 - 3.

Find this helpful?



MultiGraph Overlay

FlowJo can display multiple subpopulations of a single sample overlaid on one for visual comparison. Overlaid subpopulations are distinguished by color - the color of a subset allows easy assessment of its characteristics on multiple plots. In addition to being able to display previously gated subsets of cells in overlay plots, you can start with an ungated sample and create gates directly in the **MultiGraph Overlay** platform itself.

Click once in the Workspace window on the sample you wish to analyze. Choose **MultiGraph Overlay** from the Platform menu. This brings up a window similar to the one below.



Graphical Display



From the **Configure Graphs** pulldown there are various choices (SEE graphic at right) for laying out graph sets.

With one click you can open a variety of graph setups. For example, **Fluorescence Y by Y (-Histograms)** opens a group of graphs showing each possible combination of two Y axis fluorescence parameters but does not include a graph of their histograms overlaid. **All Y by Y (+Histograms)** displays a group of graphs showing all bivariate combinations of Y axis parameters plus a graph with all their histograms overlaid.

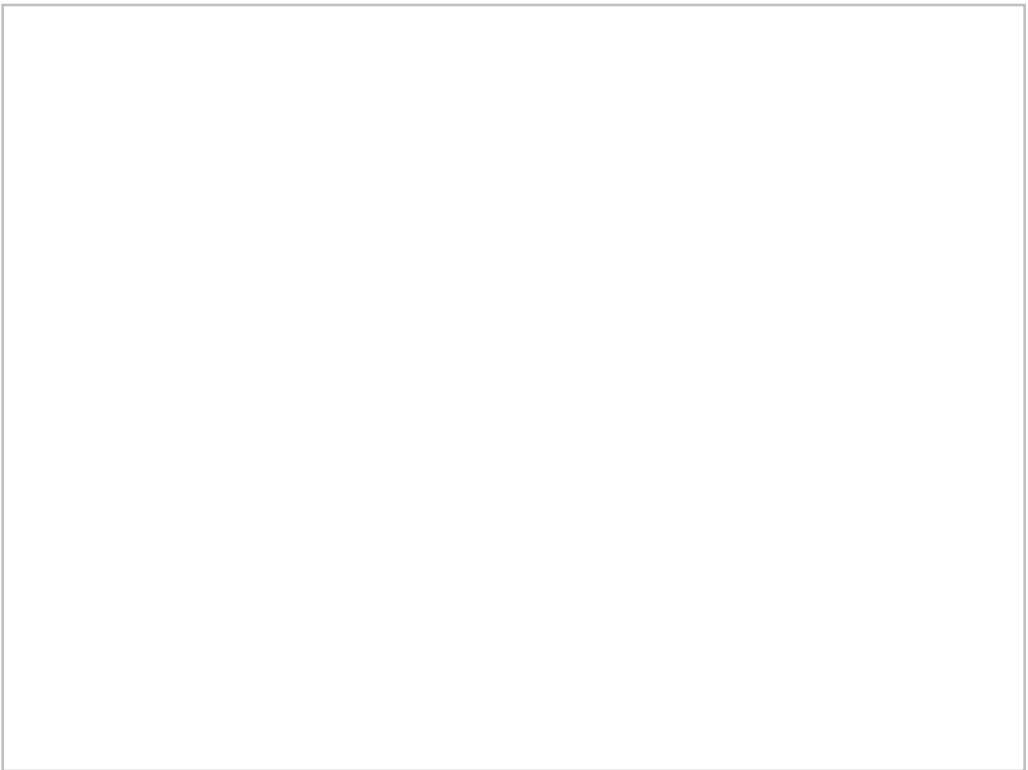
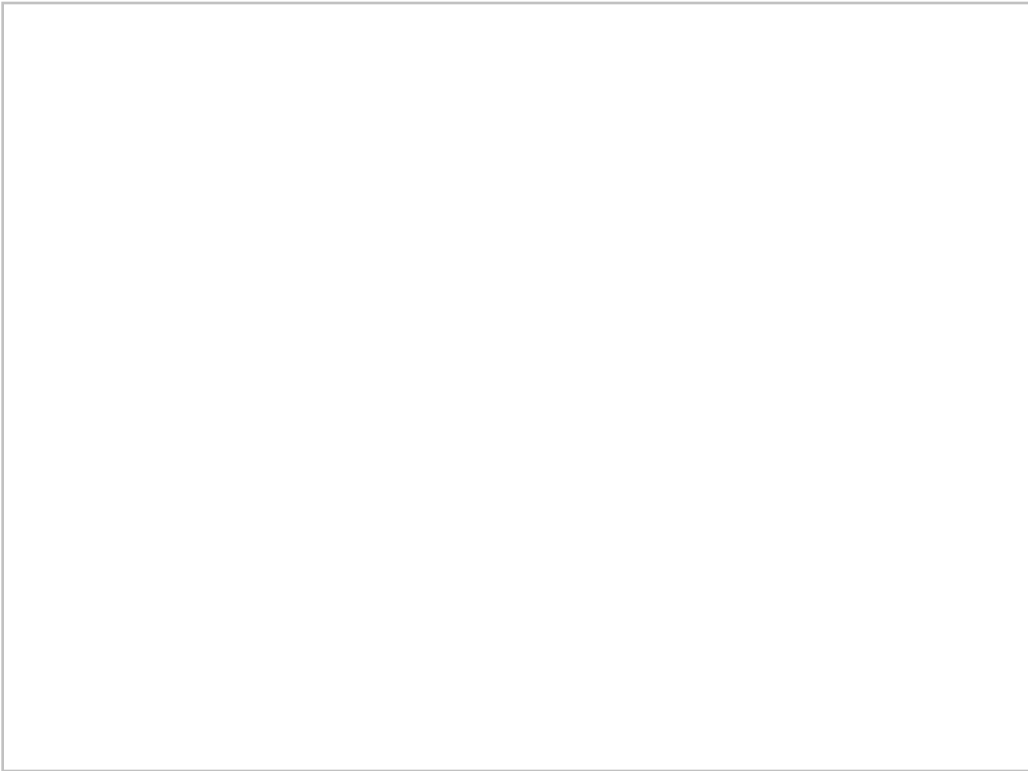
If you wish to customize the display you can:

- Add graphs (click the Add Graphs button)
- Delete graphs (click on the graph and choose delete from the popup menu)
- Change graph axes (click on the axis label)
- Move graph to end or beginning (click on the graph and choose from popup menu)
- Display a fraction of the events (change the percentage in the box at the top right)
- Change the color of the selected subpopulation or of the background of all plots

Save the customized display by choosing **Name and save this setup** from the **Configure graphs** pulldown menu. Saved setups will appear at the bottom of the **Configure graphs** pulldown menu.

Subpopulation names. The names of the gated subpopulations are displayed in the middle box of the MultiGraph Overlay window. These subpopulations can be sorted by clicking the Sort button (FlowJo will sort the subsets in a hierarchical manner with the subset containing the most events at the top of the list). In addition, you can highlight a selected subpopulation by checking the **Highlight selected** box.

Creating gates on a MultiGraph Overlay. Select a population from the list where you wish to create a gate (top center of the MultiGraph Overlay window). Click on a gating tool and create a gate in a graph window. The cells that fall in the gates subpopulation are displayed in color on all the graphs. Each subpopulation you create is displayed in the Workspace window under the sample name.



Overlaying previously gated subsets. You can also drag and drop a gated subpopulation node from the Workspace menu to the MultiGraph Overlay list. The cells that fall within this gate are now displayed in a different color.

Find this helpful?



Background Gating

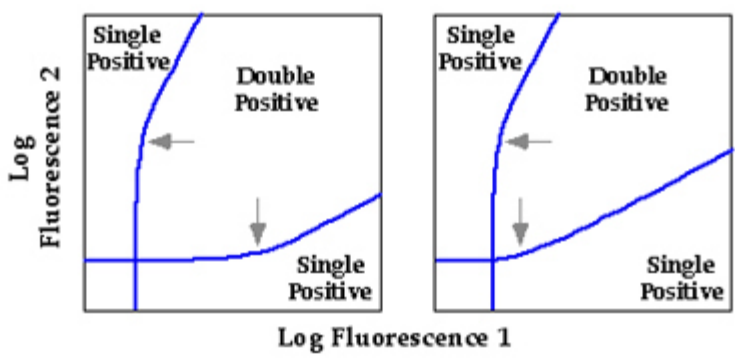
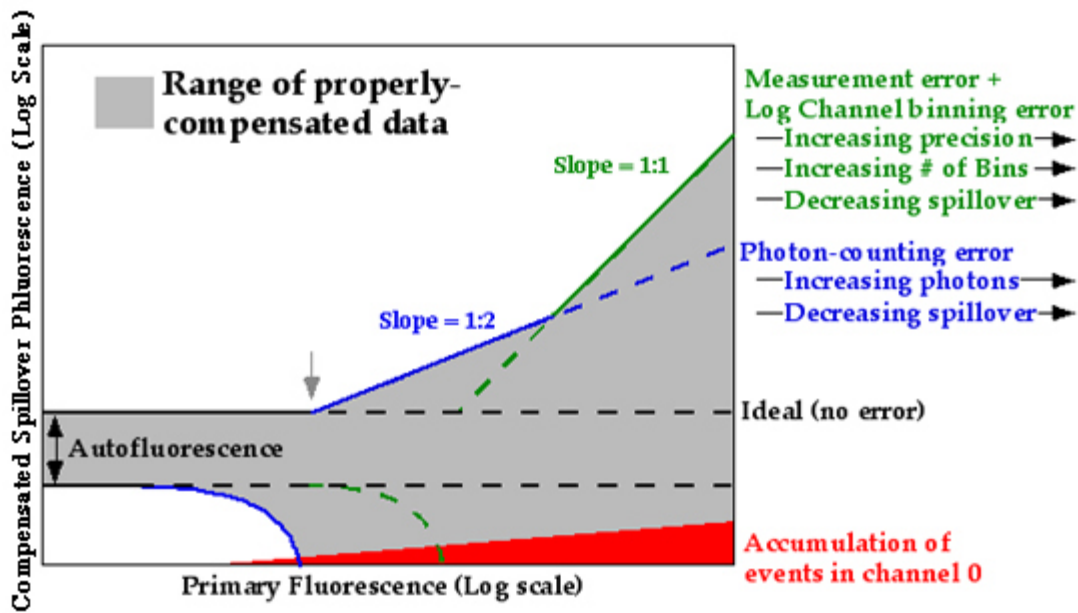
Nearly every flow cytometric experiment requires the discrimination of cells that exhibit fluorescence signal from those that do not. For many experiments, this is relatively straightforward, for example, when the signal is well-separated from negative cells. However, discrimination is not so easy when the signal is low, when the positive population does not separate well from the negative. In addition, in the multicolor world, sensitivity is often highly compromised by the post-compensation spread in autofluorescence distributions (see [Compensation An Informal Perspective](#) by Mario Roederer).

An accurate way to identify positive events is the use of an "FMO" control (Fluorescence Minus One). This is a sample that has been stained with every reagent except for the one of interest; the difference between the FMO control and the test sample identifies positive events. In multicolor experiments, compensation-caused spreading in the distribution can be calibrated by using beads singly labeled with each fluorochrome (akin to the process of assessing fluorescence spillover using compensation controls). In FlowJo, the Background Gating Platform uses the beads to compute an event-by-event background for each parameter, providing a way to discriminate positive events from those that look positive because of compensation caused spreading.

Multicolor background gates are best for identifying positive events. The negative populations will always contain a mixture of positive and negative events because of the inescapable overlap in distributions.

□

Procedure: In order to calculate the background, you need to compensate your data in FlowJo. Compensation begins by selecting the **Platform > Compensate Sample... > Define Matrix** menu item. This opens the **Compensation Definitions** dialog box shown on the right. Click on the **Record Background Gating Data** button shown on the right with a red arrow. The calculation of background works best with single color compensation beads, but can be done with stained cells.



Find this helpful?



Display Transformation Overview

FlowJo offers a useful technique called Transformation for visualizing compensated data without producing artifactual peaks or having cells squished against the axes.

Why change the display of your data?

When data is properly compensated, it is common that a large number of cells are displayed squished against the axis. The cells become piled up in the first channel (against the axis) because the fluorescence parameters are displayed on a log scale where it is not possible to display "zero" or negative values. The spreading of a population into negative compensated data values is the result of statistical error in measurement that is inherent in the data collected on flow cytometers. Even though the measurement error is the same in uncompensated samples, the variation becomes obvious when a compensated population has a low mean and therefore appears in the low regions of the log scale. This is because log scales expand the view of data in the lower regions (first decade) and compress the view of data in the upper regions (fourth decade). [Click here](#) for more details. FlowJo's display transformation displays your data on an altered scale that has a zero and a negative region. Note that the data is exactly the same, but FlowJo's display transform allows you to view the negative populations as nice symmetrical clusters instead of squished against the axis.

The four data plots in the figure below all show the same data. On the left two plots, the data is displayed using 5% contour plots with outliers; on the right, pseudocolor plots. All the plots show the data properly compensated. The median PE fluorescence is shown as the horizontal line on each plot. The top plots are the normal display and there are a large number of cells against the x-axis. It is worth noting that the cells against the axis are much more obvious on the contour plot.

The plots at the bottom have the display transformation applied. The cells that were squished against the axis on the top plots are now off of the axis and are shown as clusters of cells and it is now clear that the data is properly compensated. The B220 positive cell population spreads in both directions as the fluorescein fluorescence increases.

□

Digital Linear Data can also be transformed to provide a more interpretable view instead of the "picket fences" that occur at the low end of 5+ decade log scales. [Click here](#) for more information.

Display transformation

Having FlowJo transform the display of your data is straightforward. After the data files have been compensated using FlowJo, select a fully-stained sample (one with some signal in all fluorescence parameters) and gate it to remove dead or unwanted cells. Select this gated population and then choose "Define Transformation..." from the Compensation menu. A custom transformation is computed based on the distribution of the data in the selected population, which results in a better visualization of your data. [Click here](#) for a more detailed description of this procedure.

NOTE: this enacts a change in display and not in your data. The compensated fluorescence values for each cell are EXACTLY THE SAME as before the transformation. The display is changed, but NOT the data. Statistics computed on a particular set of cells will be the same. The new display just makes it easier to identify correct population boundaries.

Transformation Method

The display transformation process is based on a method developed by Dave Parks and Wayne Moore at Stanford

University using a generalization of the hyperbolic sine function. The display functions approach true log for high data values and approach true linear around zero. This provides smooth, near-linear display of low and negative data values.

Notes

The transformation can be used on any compensated data--but FlowJo needs to be the one to compensate the data. Therefore, it can be done on any data where you collect the comp controls and create the compensation matrix, or, alternatively, on any data files which specify their own comp matrix (currently, only BD DiVa files do this correctly).

The transformation is applied to every sample that is compensated with that compensation matrix henceforth. Therefore, if you have previously compensated data files in the workspace, the transformation will not be applied to them--UNTIL you save & close the workspace and then re-open it (at that point, all compensated files will get the transformation).

If you change the definition of a transformation for any given matrix (by selecting a different population from the original one and applying Define Transformation to it), the new transformation will automatically be applied to all samples that had the original transformation.

Find this helpful?



Output

FlowJo has a great number of output capabilities, encompassing graphics, statistics, or raw data. It supports printing from all the different views of data, as well as copying data through the clipboard or file system. FlowJo contains a special editor for the creation of Tables (statistical outputs) and Layouts (graphical outputs).

This section is divided into six parts, each discussing different aspects of generating output out of FlowJo.

A [table](#) is a text file containing one or more rows of data, that correspond to frequencies and other statistics derived from a single sample. The table editor lets you construct the set of statistics, and then generates them for every sample in the current group.

A [layout](#) is a graphical image, composed of individual elements that represent boxes, lines, text and graphs. Elements are created by dragging populations and statistics from the workspace into the canvas of the layout editor.

Both tables and layouts can perform batch output generation using the current Group as the set of data files from which to draw information. You should be familiar with the use of [groups](#) to use Tables and Layouts efficiently.

[Printing](#) is supported in all sections of FlowJo. The print command has different behaviors depending on the type of window. Specific behaviors include printing the workspace structure or single graph, as well as a set of graphs, layouts, and full report generation.

A novel feature of FlowJo is its ability to generate [movies](#) from flow data. Any graph window can add a third dimension to the view by slicing views of the events by a third parameter. You can also view any graph as a function of time or the event number during the sample collection.

You can also [export](#) raw data for analysis by other programs. You can export any gated population (or the whole sample), as its own FCS file or as tab-delimited text. You can quickly generate small subset files, optionally reducing the event count or even the parameter list. You can use this function to reformat FCS data into spreadsheet-friendly text.

Histograms (and Cell Cycle graphs and Kinetics graphs) can be exported as well. Here you will get two columns of "XY" data, where the first column is the channel number (or time for Kinetics), and the Y value is the event count or processed statistic. By default, whenever you select **Copy** (or press control-C) when viewing a histogram, cell cycle, or kinetics graph, FlowJo puts two distinct items into the clipboard: one is a graphic picture of the data (that you can paste into graphics applications); the other is a text representation of the data (that you can paste into a spreadsheet). Sometimes graphics applications will erroneously pick the text item for pasting; if this happens to you, you can ask FlowJo not to export the text data from these graphs (via the [Preferences](#) dialog).

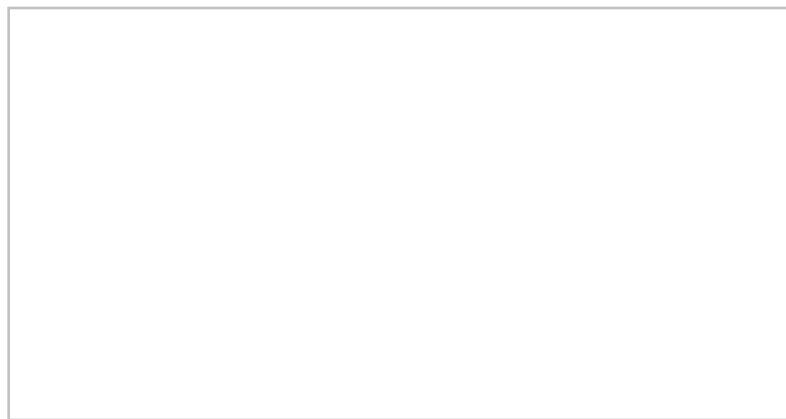
Finally, some notes on [publication quality graphics](#) out of FlowJo. Several nitty gritty details of data formats are explained in order to support post-processing of FlowJo's output. Also included is the [gallery](#) of FlowJo images, as examples of what kinds of output you can create.

Find this helpful?

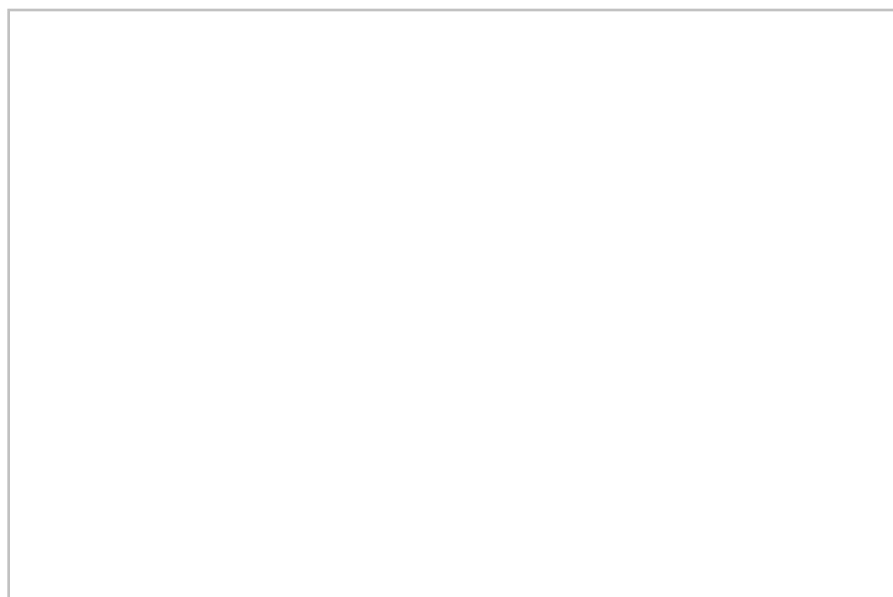


The SciBook is a place where you can store pictures, text, tables, or custom annotations. The SciBook is stored with the workspace; you can use it as a permanent record of your analyses, as a temporary holding place for outputs, or just to keep notes for yourself. It is all saved with the Workspace.

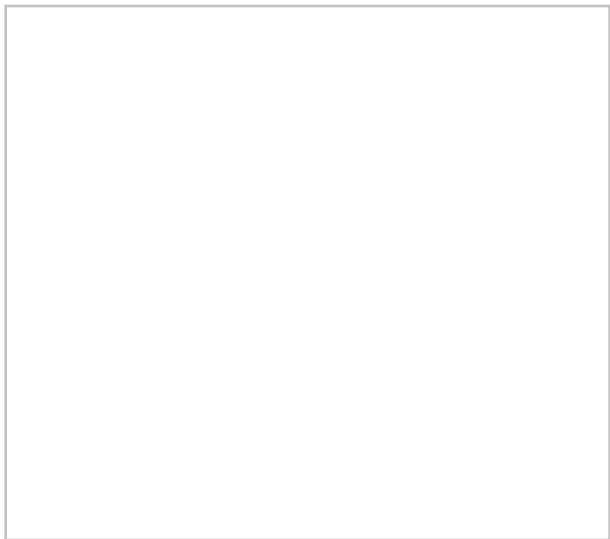
The graph window, layout editor, table outputs and the platforms all have the SciBook symbol at the top of the window. Simply click on the symbol to add the item to the SciBook. The following dialog box allows you to name the item, add comments and even open the SciBook right away (click the check box).



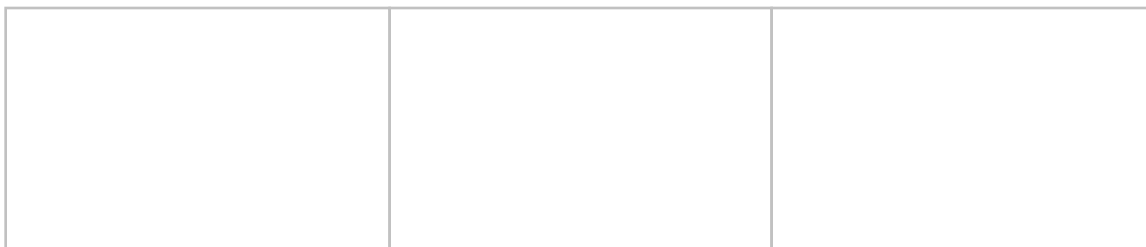
The contents in the SciBook do not change, therefore, if you change a gate, delete a sample, or make other modifications, the SciBook contents will not be modified. Once in the SciBook, you can copy into other programs, print, or simply view.



The SciBook gives you an interface to add comments about each item that you insert, and records the date and time that the item was inserted.



To view the SciBook, click on the Psi button (a symbol representing knowledge) in the Workspace window. Once open, you can click on the titles of the different items in the SciBook to easily display each one in turn.



Find this helpful?



The Layout Editor

The Layout Editor is a powerful tool in the creation of both analysis and publication graphics. It is designed to create data display layouts which can contain one or more graphical objects, such as graphs, text and lines. It works like a page layout program, but the graphs are live and will be updated to reflect any change in the data or gating. The page describing the [Layout Editor controls](#) explains the tool palette, commands and options associated with the editor. The page describing [layout elements](#) also contains useful information about FlowJo's myriad presentation capabilities.

Using the Layout Editor, you can generate graphical reports that can do any of the following:

- Show multiple different graphs from the same gated subset
- [Create a report](#) mixing graphs from different subsets or samples
- Overlay dot plots, histograms, or Kinetics analyses
- Mix statistics, text items, tables, boxes, lines, and other items with your graphs
- Generate a report for every sample in the workspace based on the layout of one sample and its analysis.

Layouts are *live*, in that the graphic window is updated whenever any of the gates or statistics change (because you moved a gate, or change an analysis, etc.). You never have to worry about whether to update the reports or not; FlowJo will do so automatically!

The Layout Editor knows how to [iterate or batch](#), that is, to create the same layout for many different samples based on the layout of one sample. It can iterate over every sample in a group, or via **Panels** where graphs from different samples are combined in a single layout.

Iterated layouts can be [previewed](#) as they will print or [animated](#) as frames in a Quicktime movie. The Layout editor can save, print or export the layout as a graphic file, as well as [save it in HTML](#) for export to the web.

Because a layout contains many different graphs and therefore can take a long time to generate, FlowJo [supports placeholders](#) to make the editor more responsive while creating the layouts.

An example can be studied in the [FlowJo advanced tutorial](#).

See Also: [Workspace](#), [Table Editor](#), [Groups](#)

Find this helpful?



Copying Graphs

You can copy and paste any graph into another Macintosh program. Simply select **Copy** from the **Edit** menu (or use command-C) when the graph window is foremost on your screen. Switch to the other application, and select **Paste**.

The graphic object is a grouped selection of multiple objects. Selecting **Ungroup** successively in the other program will ungroup these objects, which include: the axis titles, the axis notations, and the graph itself. All text is in Palatino font, size 12 by default; however, you can easily change the default font in the [Preferences dialog](#) on the **Text** tab. The format of the exported graphic depends on the type of graph as well as a preference setting.

Histograms and CDF plots are always exported as vector art. You can ungroup these and change the line style or fill pattern for the histograms.

Note also that when you copy a histogram graph, two items are placed on the clipboard: the graphic rendition as well as a text rendition of the histogram data itself. Depending on the program into which you subsequently do a paste, the correct item will be inserted. For more information, see the Webpage about [exporting raw data](#).

This is also true when you copy a graph from the kinetics window: both the graphic rendition and the spreadsheet values are copied into the clipboard. If you paste into a spreadsheet, you will get two columns: one is time, the other is the computed value as a function of time. For more information, see the section on the [kinetics platform](#).

The other types of graphs are, by default, exported as a black & white bitmap (except for density and pseudo-color plots, which are 256-color bitmaps). Bitmaps are most quickly drawn by other programs and the representation is quite good. However, bitmaps cannot be resized without risking significant distortion. If you want to export high-quality vector art for all graph types, select PICT or Quicktime format in the [Preferences dialog](#) on the **Output** tab. Now copied graphs (or layouts) will have high-quality line graphs that can be resized without loss of information. However, it may take the drawing programs considerably longer to present them to you; especially for plots that are not contour plots. See the information on making [publication quality graphics](#).


If you hold down the option key when you select **Copy** from the menu, then FlowJo will include with the graphic any gates that are drawn on the current graph. (You can change the default behavior of including gates or not through the [preferences dialog](#)).

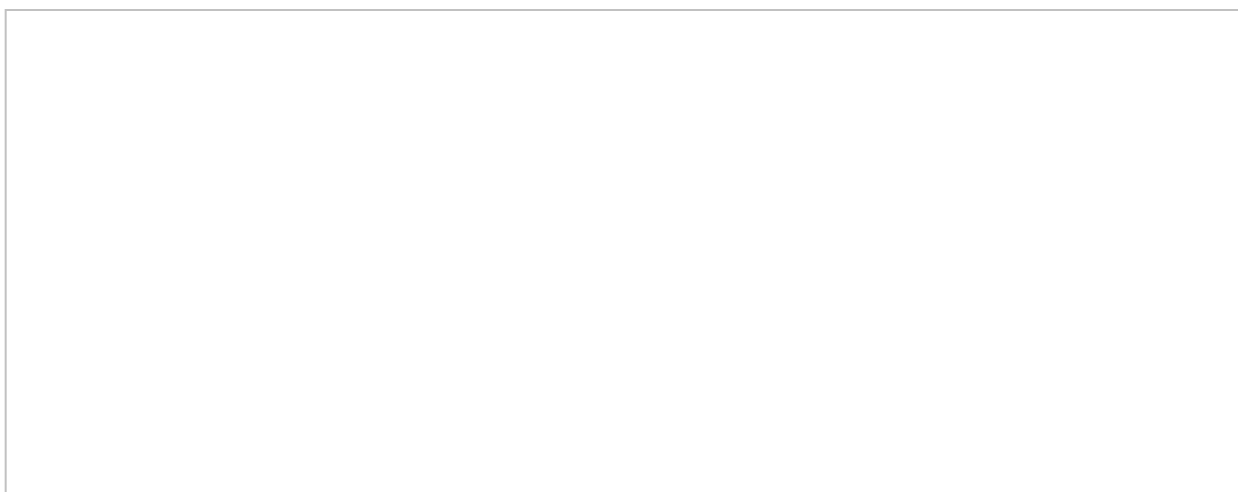
Find this helpful?




Tables

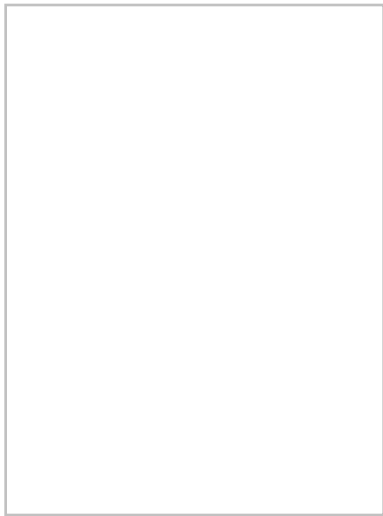
A requirement of many experiments is the ability to generate tabular output for further analysis in a spreadsheet or statistics program. The table editor is the means by which you can generate a series of statistical calculations for all samples in a group. These statistics can be any of the statistics FlowJo calculates: medians, frequencies, etc. You can also use this tool to generate [functions](#) that will display their output for every sample in a group. In order to generate a table, you will first have to generate the statistics you wish to export on at least one sample.

From the workspace window, open the table editor. This can be done in one of two ways: either click on the table editor function button  in the workspace window, or select the **Table Editor** from the **Windows** menu (command-T).



Use the Table Editor to create a new table definition. A table definition is not the output data itself. Instead it is the specification of what **columns** the table will contain, when it is generated. You can have as many table definitions in  the workspace as you wish. You can create new empty definitions, duplicate existing ones, or delete them using the button in the top left corner of the window. Each different table definition in the table editor is a different template.

To add statistics to a table definition, simply click on them in the workspace window and drag them to the definition pane on the right hand side of the table editor window. (You can use the control and option keys to modify whether or not you want to take all the parents or children, respectively; for more information, see the pages about [dragging and dropping nodes](#).)



When you drag a population node into the table editor, it assumes that you want the statistic that is set as your default in the **Preferences** on the **Tables/Layouts** tab. You can change the default choice there or in the drop-down menu shown at the right, in the Table Editor.

Each statistic in the right panel of the table editor will correspond to a column in the output table; you can change the order of these columns by clicking and dragging the statistics around. The table is created for those samples in the currently-selected group: remember to click on the appropriate group in the workspace window before you create the table!

Only drag statistics from a single sample into the table editor. The table editor will automatically calculate these statistics for each sample in the group. When you apply or create a table, each row in the table editor is applied to every sample in the current group--irrespective of which node you actually dragged into the table.

When you create a table, FlowJo will cycle through all of the samples in the currently-selected group. For each statistic node that you have dragged into the table definition, it searches for the same node in each sample. If the node does not exist, then it leaves a blank for that entry. (For instance, if the statistic you copied was the median FITC fluorescence of a Lymphocyte gate, and the Lymphocyte gate does not exist in all samples, then those samples will have blank values for this statistic). Therefore, the statistics are only gathered for those samples which have the appropriate gates and statistics already applied to them.

Keywords The table editor also supports including sample file keywords. A keyword is any attribute listed in the text section of the FCS file. Above the list of columns is a button with a picture of a key on it. Press this button to bring up a dialog which will allow you to select the keyword(s) to be included in the table. You can use shift- and command- clicking to selection multiple items from the list. Keywords are appended to the table definition. There is a list of [standard keywords](#), as defined by the FCS specification. Note that not all files will contain all keywords.

▣ **Functions** The Functions tool will construct a function column in the output table. The GUI can be triggered from the Table Editor by clicking on the fx button. It allows users to create a numerical, logical or programmatic relationship between existing Table Editor columns, much like a spreadsheet editor's "reference calculations". For details on using the [function tool, click here](#).

▣ **Define Column Attributes** You can choose to have certain statistics highlighted in the output table. FlowJo will specially format data whose value or standard deviation meets criteria you choose. For example, "I want to add a ratio of my CD4/CD8 values. If the ratio is >2, I want my values colored green. If the ratio is <2> Define Attributes tool, click here. You can also access this tool by Double-clicking on any column definition (a row in the table definition window), or choose **Special Formatting...** from the **Tables** menu.

Iteration Options Just like batching in the Layout Editor, you can select how you want the Table Editor to batch through the tubes in the current group. **Tables > Iteration Options...** in the main menu opens the [Iteration Options](#)

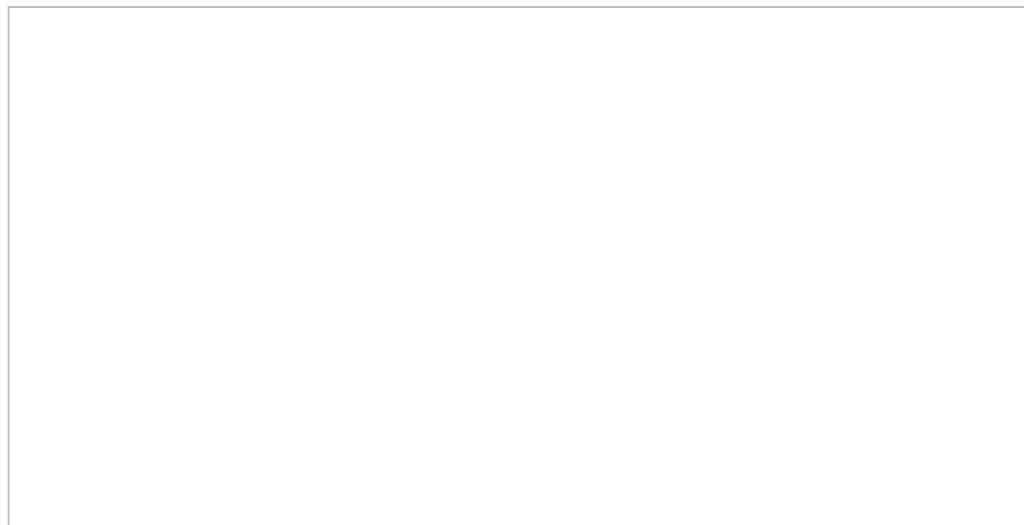
[Dialog](#). Double-clicking on the table name will also open this tool.

In generating a batch table report, each row of the table represents a unique instance of the iterator values. So, if you iterate by n th, if $n = 1$, you get one row for every sample in the group; if $n = 2$, you get one row for every other sample. If you iterate by a keyword value (for example, "Sample ID"), you get one row for every unique value of this keyword in the current group.

The discriminator tells FJ how to select each statistic from the samples which share the current iterator value. You can specify multiple discriminator keywords. A statistic is chosen for the first sample tube which has the current iterator value, as well as a matching value for all discriminator keywords as did the sample that was dragged into the table definition to begin with.

The Table Editor has a new column, **Tube ID**. This shows the value of the discriminator for the sample that was dragged in to the table definition. Thus, if we iterate by n th, and $n = 2$, then if you drag a statistic from the first (or third or fifth) sample into the table, it will show an ID of **1**; if you drag a statistic from the second (or fourth...) tube, it will show an ID of **2**. If you choose keyword for discrimination, the keyword value is shown here.

Generating the Table. Once you have put all the desired statistics and keywords into the table definition, click on the Table button in the top left corner of the definition window. This will invoke the table generation process. FlowJo will cycle through all of the samples in the current group, and calculate all possible statistics and keywords that are in the table definition. It will then create a new window containing a spreadsheet-like view of the statistics. This table can be saved to the clipboard or a file, printed or exported to another application. Each of these operations is initiated via the buttons in the top left corner of the table window. The spreadsheet application launched by the **Save and launch application** button (fourth from left) is chosen the first time you use this function and can be altered in the **Layout** pane of the [Preferences](#) window.



Sorting the Table. Many times it is useful to view the table ordered by the value of a keyword or statistic. To sort the table, double click on the header of the column by which you wish to sort. By default, sorting the table will order the rows in descending order of the values in that column. If you wish to reverse the order, and view the table with the smallest value first, then double click the column again to invert the row order.

Transfer Table to the Layout Editor. Click this button and the existing table is copied to the currently open layout in the Layout Editor. [Click here](#) for more information on editing the table once it is in the Layout Editor.

Copying Part of a Table. Normally if you copy a table, the entire table will be copied to the clipboard, regardless of the current selection. If you wish to copy just a portion of the table, you can select any number of cells, and hold down the option key while performing the copy (either via the button in that window or the Copy command in the Edit menu). If the option key is down, then only the selected cell contents will be put on the clipboard. If the selection is rectangular, then pasting the clipboard into a spreadsheet or word processor will result in output that looks the same as in did in

FlowJo's table. If you have used the command key to select an odd shape or disjoint selection, then the resulting paste may not look the same as it did in FlowJo's table. The rule is that all cells that are in the same row in FlowJo's table will come out in the same row on the clipboard (i.e., will be separated by tab characters), and cells that are on separate rows will end up on separate rows (i.e., will be separated by carriage return characters), but gaps between either rows or columns will be omitted.

Creating Groups from the Table Selection. Sometimes it is useful to be able to use the results from the tables to refine analysis in the workspace. To facilitate this, the table window has a button similar to the one in the workspace for Create Group operation. Clicking this button will create a group containing all of the samples which have one or more selected cells in the table. You don't need to select the sample name. Any cell in that row will be sufficient to include that sample in the new group.

For example, we might be interested in producing graphical output of all of the samples whose CD45 positive population is over 60% of total events. To accomplish this, you could generate a table containing the statistic for CD45+, sort the table by this column, click the first cell, shift click the last cell that has a frequency over 60%, and then click on the New Group button in order to create a group of all samples with a selected cell. Then go back to the workspace window, and select the new group, limiting the view to only the desired samples. Now when you generated the desired layout, you will only be looking at those samples that have the desired characteristics. (If this sounds overly complex, relax. It's easier to do than to explain.)

Resize Columns Click on this button to resize the table's columns to fit the text in the header.

Finally, you can choose to save the resulting table to a disk file, copy to the clipboard or print. Saving to a disk file is probably the best choice; you can then import the data into a spreadsheet. Any spreadsheet should be able to import the data; specify that the first row of the table has the column headers.

Column Names. In the output file, the first line has a tab-delimited list of column names. Each column name is a concatenation of the full name of the population (including all "parents") and the statistic name itself. For example, "Lymphocytes/CD4:Freq. of Parent" would be the name for the column with the frequencies of CD4 cells within the Lymphocyte gate. These names can become unwieldy in length... you might want to keep your subset names as short as possible. In addition, you can have FlowJo export abbreviated statistic names (in the example above, "Freq. of Parent" would be replaced by "%P"). You can set this preference in the [Preferences](#) on the **Tables/Layouts** tab.

Replace Column Names. You can type in any name you wish to replace the statistic names in the generated table. In the Table Editor, type in the new names in the **Column Name** column. When you create a table, these names will be displayed instead of FlowJo's statistic name.

Drag and Drop, Copy and Paste You can drag a table or copy it from the Layout Editor in one Workspace into the Layout Editor of another.

Find this helpful?



Iteration (Batching)

One of the best things about computers is how they handle repetitive tasks. Show the computer how to do something once, and it will do it a million times without the slightest complaint.

In FlowJo's Layout Editor, you can create a layout based on graphs and statistics from one of your samples. With a single click, you can automatically produce the same layout for each of your samples, or any subset of samples that makes sense for your experiment.



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

- A new layout in the layout editor.
- A preview window containing static tiles (data won't update if you change the original analysis.)
- A web page containing the pictures for each layout group, or
- A Quicktime movie.
- Alternatively, it can print the report directly to your printer.

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

In the simple case, each frame corresponds to one sample in a group. The batch report is made by taking each sample, applying the named gates and statistics to that sample, plotting the graphics in the format defined by the layout editor, and adding that frame to the report. This is useful in cases where the same information is desired for each tube.

Iterating in the Layout Editor

The first thing to be aware of is what is the currently active group in the Workspace window. This will be the selected item in the top half of the window. This is important to know because the current group will determine the set of samples, from which the layout will be constructed.

□

Next you should look at the popup menu in the layout. In most cases, you'll probably be iterating by sample, and the popup will appear like this:

There are a couple of terms that we use in this explanation that are important to get clear from the start. They are *attribute* and *value*. The keywords in an FCS file are organized in attribute-value pairs. The attribute is the name for the type of data recorded. The value is the data recorded for a particular tube. For example, most files will contain, in their header, a \$Date attribute, and a value containing the date on which that sample was collected. When we talk about iteration attribute we mean the attribute of the chosen tubes whose value changes in each page of the batch report that is generated.

Changing the Iteration Value

There are several ways to change the iteration value. You can use the popup menu in the layout editor window to show the layout for any value in the current group. Alternately you can use the yellow up and down arrows to move to the previous or next values. From the keyboard, you can use the home, page up, page down and end keys to move to the first, previous, next and last iteration values respectively.

Setting the Iteration Attribute

Other times, reports are created where each frame contains data from more than one tube. Examples of multi-sample reports could be several tissues from a common animal, multiple doses of a common drug, or cross patient studies using blood collected over time. In cases such as these, it is not enough to simply process successive tubes in a batch report. More sophisticated organization is required. Click **Layouts > Iteration Options...** to set more parameters for your batch operation:

□

Group tubes by Numerical Order is the easiest method of iterating. If you want to overlay two tubes and batch process the layout so that tube 2 is overlaid on tube 1 and then tube 4 overlays tube 3 etc. choose Iterate over every 2 samples (tubes).

For more complicated iteration, click **Group tubes by Keyword Value**. The popup menu in this dialog lists all the keywords found in all of the samples of the current group. Select the keyword that you want in common in all frames of your layout. The value will vary in each of the frames. From the **Iterate over...** popup menu, you can select a keyword that groups tubes into distinct units--for example, a keyword that connotes patient ID, tissue type, or experimental condition. Then you can place multiple graphs from different tubes (from different FCS files!) into the same layout and still generate a batch output.

For example, if you set the **Iterate over...** attribute to be the keyword **\$Cells**, where you have a Patient ID entered into the \$Cells keyword, then FlowJo will examine all of the tubes in the current workspace, and generate a list of the unique values of \$Cells. These are then displayed in the **Layout editor** in the **Iteration** popup menu just below the **Batch** button. By selecting any of these values from this menu, FlowJo will change the current layout view to show graphs and

statistics drawn only from tubes with that value of the Iterator. (By setting this popup menu to **Off**, you are telling FlowJo to use all of the original graphs that you dragged and dropped into the layout). This is the way in which you can build template reports which select graphs and statistics from different tubes and put them all on the same page, but retain the ability to create a new batch layout drawing from all the data in the Workspace.

Multiple sample Iteration

The Iteration Options also provide the ability to specify one or more discriminator keywords. These fields provide the value that must stay constant, while the iteration attribute changes through each of its successive values. For example, if you have a ten mouse study, and for each mouse you collect three different tissue types, then there would be thirty tubes in the experiment. You want to produce a ten page report, showing the graphs and statistics for each mouse, one per page. You've acquired the data such that the mouse identifier is stored in the FCS file's \$SMNO (sample number) field, and the tissue type information is stored in the \$CELLS field. Set the field \$SMNO to be the iteration attribute, and the keyword \$CELLS to be the discriminator.

More than one discriminator can be defined if desired. By selecting additional keywords in the popup menu, the string of discriminators will be extended. To start over, choose the second item in the menu, marked Select None. This empties the list.

The most common case is where the iteration attribute is the sample, by which we generally mean the tube that was run through the cytometer (as opposed to the sample taken from the subject.)

When you click the Batch button you see the **Make Batch Report** box at the top of this page. The **New layout** and print **Preview** reports have placement controls. You can specify the number of rows or columns you want to generate, and whether the new items are arrayed left to right or top to bottom.

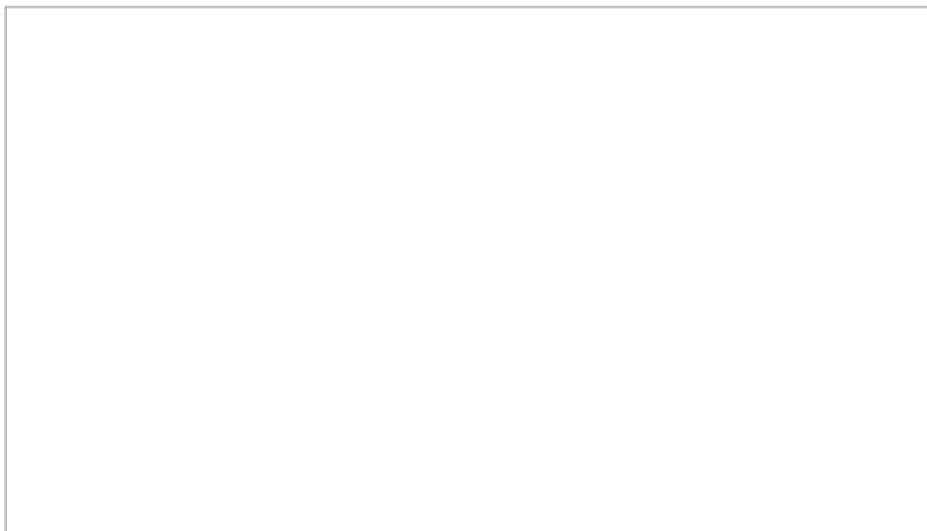
Finally, there are three additional options provided at the bottom of this dialog. **Iteration** lets you choose which group the batch process will iterate through. The option to **Save These Settings with the Original Layout**, if checked, will store all of the settings in the approved layout. In this way, it is straightforward to replicate this report many times in the future. The option **Don't Show This Again (unless Option Key is down)** gives you a means to simplify the use of the program and avoid seeing the dialog in the future. If you are always producing the same reports with the same characteristics, this is a way to prevent untrained users from producing the wrong kinds of reports. If this option is not checked, you can still avoid the dialog by holding down the option key when clicking the Batch button.

Find this helpful?



Formulas in Tables

□ If you select the **Function** button in the **Table Editor**, you will see the following...



Here you can construct a formula the output of which will appear as a column in your finished table. The calculation will be applied to each population through which the Table Editor iterates when it builds the table. It allows users to create a numerical, logical or programmatic relationship between existing Table Editor columns, much like a spreadsheet editor's "reference calculations".

The pre-requisites for this functionality are:

- one or more columns in the Table Editor
- a custom "column name" - this is used as an "alias" in creating the formulas.

You can include values appearing in other columns in your formula by including the column name in your formula. The names of other columns in the table editor are listed in the **Insert Reference to Column** drop-down box. Click on the column name you want to include in your formula. The column name will be inserted in the formula window.

A table formula can be any operation on other columns in the table (only columns which do not depend on the current column can be chosen; i.e., you can't have recursive relationships).

Column references

A column reference has the format "", where "name" is a user-defined column name. This appears in the last column of the **Table Editor** window. Before a formula can use a table column, it must be assigned a custom name. It will then show up in the **Insert Reference** list in the window above. You can type in any name you wish to label a table's columns or to replace the default statistic names in the generated table. In the Table Editor, type in the new names in the **Column Name** column. When you create a table, these names will be displayed instead of FlowJo's statistic name.

A column reference can also specify a specific row in the final spreadsheet. So a formula such as:

* 2

will generate a new column in the table whose values are always twice the value in the "MeanCD4" column. For each row in the output table, the formula computes the value of "MeanCD4" column in the current row, and multiplies by 2.

Square brackets allow you to specify a particular population, the formula:

* 2

will generate a column where all of the values are twice the first entry in the “MeanCD4” column. Let’s assume the first row in the output table is the control sample, then:

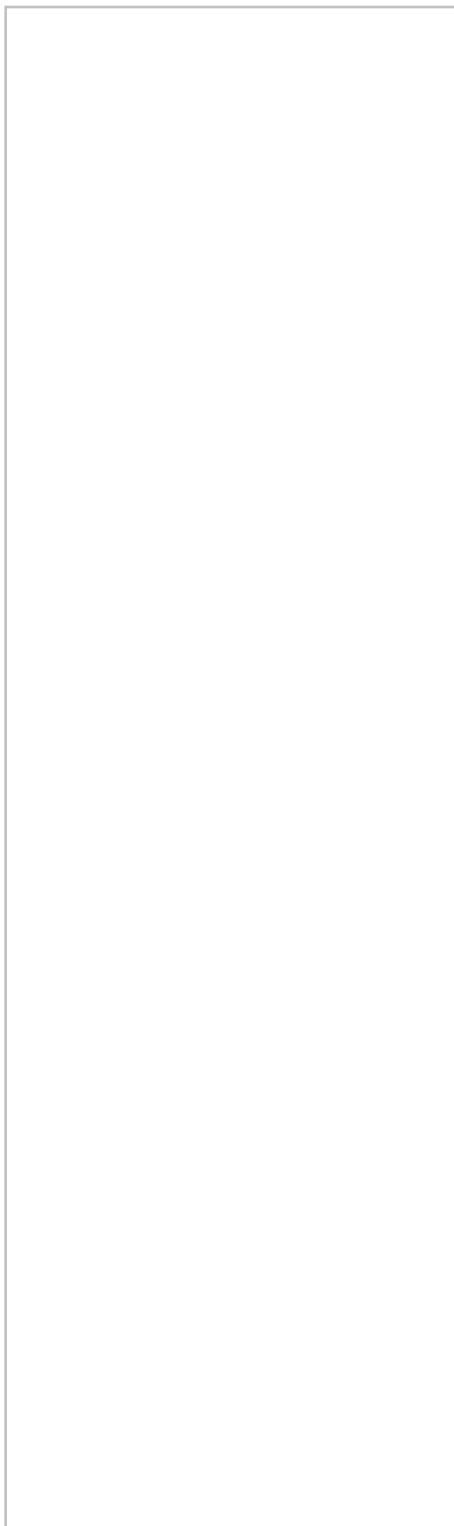
$/* 100$

Gives an output value which is the percent of control sample (first in the table).

Row references can also be “relative” to the current row. Thus:

- will generate an output column where each cell represents the difference between the current and the previous row’s values for “MeanCD4”.

- is the same, but references the subsequent row in the final table.





Insert Function

Select the **Insert Function** drop-down menu shown at the left, to add function notation to your formula. There is a wide variety of functions that can be used in FlowJo Table Editor formulas. Some of these functions operate on numbers, and some on text. FlowJo is reasonably clever at converting strings to numbers and vice versa if needed, but there are two functions that do this explicitly just in case. **char** -> converts numbers in text form to arabic numerals and **num** -> converts arabic numerals to their text form.

Simple math functions:

+, -, /, * Are self-explanatory.

% = modulus: e.g., $7 \% 2 = 1$.

^ = exponentiation: $2 ^ 3 = 8$.

Statistics functions:

avg(x1, x2, ...); **sd(x1, x2, ...)**; **cv(x1, x2, ...)**. These functions can have 2 or more parameters. Each parameter can be a column reference, a constant, or an expression. Example: “avg(, ,)”.

Numeric functions:

min(x1, x2, ...); **max(x1, x2, ...)**. These return the minimum or maximum value of the 2 or more parameters.

Abs(x): returns absolute value of x.

%: Modulus

Neg(x): returns the negative of x (or “-x”).

Transcendental functions:

sqrt(x) is the square root of x.

exp(x) is “e” raised to the power of x.

Ln(x) is the base-e log of x.

Log(x) is the base-10 log of x.

Text (String) functions:

Note that string constants must be specified in double quotes. Any text that is not in double quotes is considered to be a variable or table column reference!

+: concatenate. “Abc” + “def” = “Abcdef”

sub(x, a, b): substring of string x, starting at position a, of length b. sub(“abc”, 2, 1) = “b”

find(x, y): finds the first position of substring y in string x: find(“abce”, “c”) = 3.

Char(x): converts a number to a string: char(123) = “123”

Num(x): converts a string to a number: num(“123”) = 123

Word(x, a, b): selects word number a from string x, using b as a delimiter: word(“this is fun”, 2, “”) returns “is”.

Repl(a, b, c): replace all instances of b in a with c: repl(“ababab”, “a”, “c”) returns “cbcbcb”.

Rep1(a, b, c): replace the first instance of b in a with c: rep1(“ababab”, “a”, “c”) returns “cbabab”.

Len: returns the length of the string in number of characters

Del(a, b, c): delete from a starting at position b with length c: del(“abcd”, 2, 2) returns

Ins(a, b, c): insert b into a at position c. ins(“abcd”, “x”, 2) returns “axbcd”. “ad”.

Strt(a, b): returns 1 if a starts with b, otherwise 0. Strt(“abcd”, “ab”) = 1

End(a, b): returns 1 if a ends with b, otherwise 0.

Cont(a, b): returns 1 if a contains b

Uppr(a): returns the uppercase equivalent of a

Lowr(a): returns the lowercase equivalent of a

Trun(a, b): truncates a to a length of b characters. If b is less than zero, then delete the last “-b” characters from a. Trun(“abc”, -1) = “ab”.

Max(x1, x2, ...); Min(x1, x2, ...): return the alphabetically last or first of the parameters

Comparator functions:

All of these functions return a 1 if true or a 0 if false.

<, >, =, <=, >=,

Thus, the expression “a < b” returns 1 if a is less than b, otherwise zero.

Boolean functions:

& : and: a & b: if both a and b are nonzero, then 1, otherwise 0

| : or: a | b: if either a or b are nonzero, then 1, otherwise 0

! : not: !A: if A = 0, then 1, otherwise 0

Programmatic functions:

If(a,b,c): If the expression a evaluates to nonzero, then return expression b, otherwise expression c

For(a, b, c, d, e): Evaluate a; then evaluate b: if b returns non zero, then evaluate expression d (the main loop); then evaluate expression c; then go back to test expression b again to decide whether or not to loop. Finally, return expression e as the result. See below for example. A for loop will quit after 1000 iterations unless a variable “MaxLoops” has been defined with a different value (to prevent bad coded infinite loops)

Var(v, e): Set the variable v to have the value e.

(semicolon): use a semi-colon to separate expressions; the right-most expression value is the result. Thus: “3;4;5” returns a value of 5.

Example for loop: For(var(c,1); var(x, 0), x < 3, var(x, x+1), var(c, c*2), c)

This loop begins by evaluating the expression “var(c,1); var(x, 0)”; i.e., it defines two variables, “c” and “x”, giving

them values of 1 and 0 respectively.

It then evaluates the loop control expression, (“ $x < 3$ ”), which returns a value of 1 (since $0 < 3$)—meaning the loop should be executed.

The end-of-loop expression “ $\text{var}(c, c*2)$ ” is evaluated, which sets c to be twice the value of c , or 2.

The end of loop expression is evaluated “ $\text{var}(x, x+1)$ ”, which sets the value of x to be $x+1$, or assigns it to be 1.

Then the loop control is evaluated again—and the looping continues until x is incremented to 3, by which time c has a value of 4. Now the loop control expression tests false, and looping is terminated.

Now the last expression is evaluated; “ c ”, which returns the value of 4. Thus, the output of this loop expression is 4.

Extra functions: The following functions are available but are not shown in the menu:

Rand(x, y): generates a random number between 0 and y with uniform distribution. (y can be omitted; $\text{Rand}(x)$ is the same as $\text{Rand}(x, 1)$).

Norm(x, y): Random number with a normal distribution, between 0 and y ($\text{Norm}(x)$ is the same as $\text{Norm}(x, 1)$).

Sin, Cos, Tan, asin, acos, atan, sinh, cosh, tanh: trig functions operating on (x).

Select the **Create Formula** button to add a new column to the Table Editor. When the Table Editor creates your table, you can have results appear in bold or in color if they exceed a desired threshold. For information on this, [click here](#).

Find this helpful?



Printing

To print a single window, select the **Print** command from the **File** menu when that window is frontmost. You will see the Print dialog offering choice of printer, number of copies and so on. In addition, you can preview the page to be printed, save it as a PDF file or fax it from the Print dialog.

For example, you can print the contents of the workspace window. Select **Print** from the File menu. The printout will have the entire portion of the sample and analysis half of the window (the lower portion). All currently visible samples, analyses, and statistics nodes will appear in the printout.

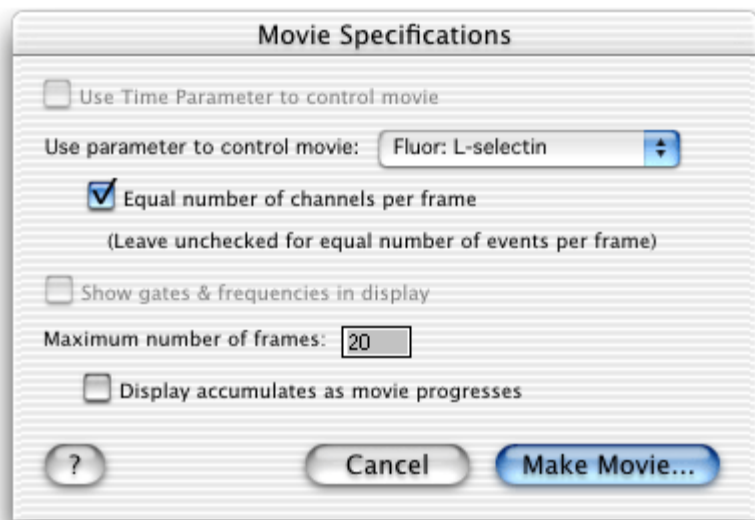
For more complex printing, you should familiarize yourself with the workings of the [Layout Editor](#). This tool provides options for easily creating reports with graphs, overlaid histograms, statistics and any amount of annotation and elaboration you desire. The reports are created by taking the first sample you set us as an example (tile) and batch processing through all the samples you wish to summarize. The resulting report can be rendered in several formats: new layout, print preview (tiled report), movie, or web report. In addition, you can print this [report directly](#).

Find this helpful?

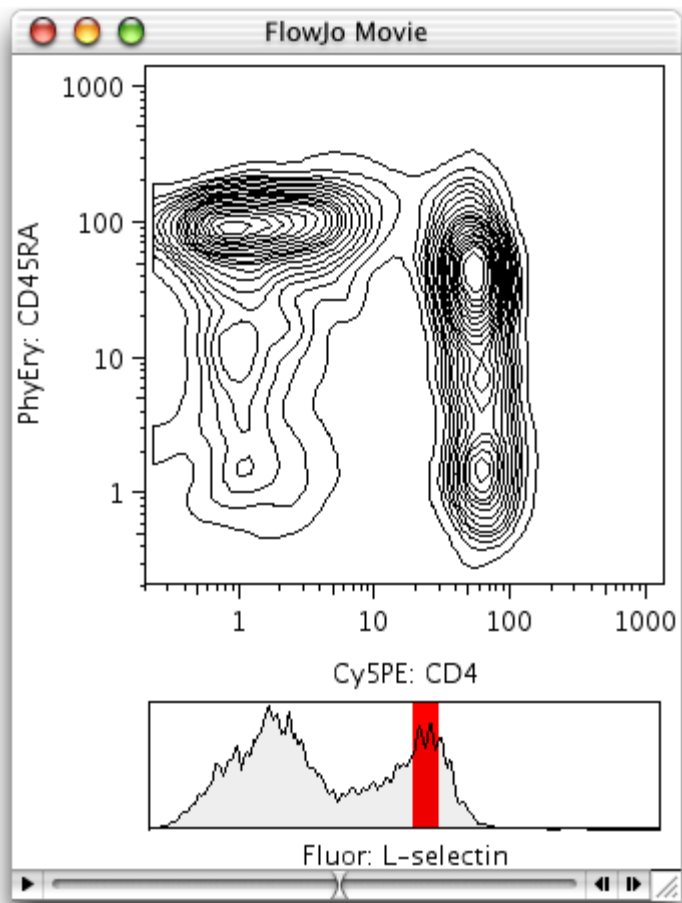


Movies

Any graph you generate in FlowJo can be made into a movie using Apple's QuickTime technology. This feature provides an entirely new way to explore data. Making a movie is simple; the movie can be viewed in FlowJo or saved to disk to view in other programs. To generate the movie, simply select **Graph > Make Movie...** while viewing a graph you would like to see animated. FlowJo shows you the following dialog window:



Here you can choose how the movie should be generated. There are several options available. The primary concern is to choose the control parameter, which is the third dimension that will be added to the graph. If time is an existing parameter in your data, then it will be the default choice as the control parameter. Otherwise, you can choose any other parameter you wish.



When generating a movie from a graph window, FlowJo will divide the events into slices that are ordered sequentially across the control parameter. You can choose whether the slices will be made with an even number of channels per slice, (check **Equal Number of Channels per Frame**) or with a variable width slice that contains an *equal number of events* in each of the slices. In the generated movie, the control parameter will be drawn in a histogram, with a red bar over the area that contains the events currently visible in the main (upper) graph. If the Equal number of channels per frame box is selected, the red bar will always have the same width as it moves across the histogram. If it not checked, the width of the red rectangle will vary so that the area under the curve inside the red bar is the same for each frame.

You can specify the **Maximum number of frames**, that the movie will contain. The larger the number of frames, the longer it will take to generate the movie, but the more sensitive it will be to trends that exist within a small range of values.

For some types of analysis, it is useful to graph events not of each individual slice, but for all of the events up to and including the current slice. The checkbox **Display accumulates as movie progresses** will cause the graph of each frame to include all of the events with a value for the control parameter of less than or equal to the frame's current value.

For more information, see these example movies:

- Time as the movie control: [Kinetic display of calcium flux](#)
- A third parameter as a movie control ([equal channels per frame](#))
- A third parameter as a movie control ([equal events per frame](#))

Find this helpful?

□□

Exporting

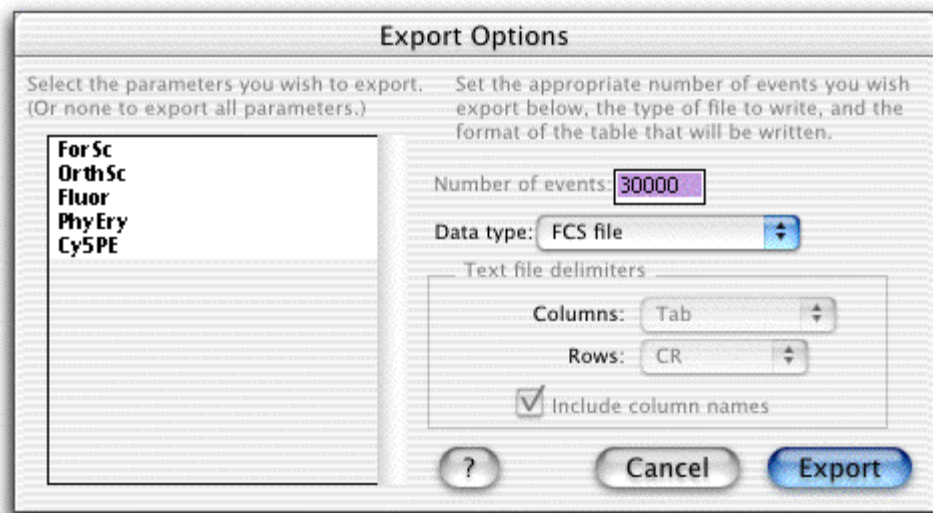
When you are displaying a population, you can choose to export the data for that gate (by selecting Export from the Workspace menu). This allows you to create a new FCS file containing only the data you want to include. Alternatively, you can create a text file that can be imported into a spreadsheet program. This spreadsheet file will contain the raw data from the sample (the event data), containing only the parameters you wish to export and can have either the channel numbers or the converted absolute intensities.

The exported FCS files can then be read in by any other application that can read FCS files. As well, you can read it into FlowJo. This may be useful if you are working with very large data files and only need to examine a small subset of the events: exporting them to a separate file and reading that in makes the program operate much more efficiently.

You can export either the raw channel numbers, or the scaled fluorescence values. The latter are the relative fluorescence intensities for each cell, taking into account logarithmic amplification or linear gain settings.

Choosing "Export..." brings up one of two dialogs. If you selected populations from only a single sample, then you will be presented with the first dialog below. This dialog allows you to export a subset of the parameters of the file (if you wish). On the other hand, if you selected populations of multiple samples (in other words, you shift-clicked nodes from more than one sample, or you chose the Export command with group populations selected), then you will be presented with the second dialog.

Exporting from a single sample:





Exporting from multiple samples (or by selecting the group and choosing Export): all the features are the same; the difference is that for all files created, every parameter will be exported to the output file. If you check the box labeled "Don't export uncompensated parameters", then FlowJo will export every parameter unless there is a compensated equivalent for that parameter. Thus, you can quickly export only compensated data.

In addition, you have the option of excluding uncompensated parameters (with a single sample export, you can select which parameters to export explicitly; hence, this option isn't shown above). When this option is selected, all parameters except those for which a compensated equivalent has been computed will be exported. This is useful for generating new files of only compensated data, saving time on future analyses (since the data will not have to be recompensated every time it is used).

Exporting Histogram Data and Kinetics Data

You can also export gated histogram data (or processed kinetics data from the [Kinetics Platform](#)) to a spreadsheet. (This is the events per channel data that is displayed in a histogram graph). You might wish to do this so that you can perform specialized analyses, such as DNA analysis (cell-cycle fitting), etc.

Exporting histogram data occurs automatically whenever you select "copy" when viewing a histogram graph. When you copy a histogram graph, two different items are placed on the clipboard: the graphic rendition of the histogram, and the text corresponding to the columnar data. Other programs know which of these you want! Thus, when you immediately follow the copy operation by pasting into a drawing package, you will see the graphic. If you paste into a spreadsheet, you will see the columnar data.

When you paste into a spreadsheet, two columns will be created. The first one has the channel numbers for the histogram (at whatever resolution the data was collected); the second has the event counts for each channel. This is the raw, unsmoothed data. If a gate was applied to view the graph, then the exported data will reflect only the gated events.

Find this helpful?



FlowJo Menus

These are pictures of the main menus in FlowJo for Macintosh. Click on a menu item in the graphics to read about each of the choices in the menu.



Some menu items change when you hold down the option key ([click here](#) for more tricks that use the [option key](#).) You can select a menu in FlowJo and press the option key to see the changes in the menu items. Both choices are explained below using a slash... **Menu Item/Menu plus Option Key**

FlowJo Menu

About FlowJo

Contains information about the current version of the program and license protection (serial number or dongle).

Serial Number...

Dialog box showing your **Hardware Address** and providing a place to enter your serial number. There is a link to TreeStar web pages for registering your hardware address in order to obtain a serial number or to obtain a temporary trial serial number.

Start / Stop Monitoring Tasks

There are many operations involved in creating complex analyses. They are scheduled to take place in the background, and not tie up the computer while you want to work. These monitor windows show you if there is work on the queue, and how much longer it will take. Use this menu item to show or hide the monitor windows.

Preferences...

Set a variety of default program behaviors. Brings up the [Preferences](#) dialog window.

Services...

General Macintosh services.

Hide FlowJo...

Hides FlowJo windows.

Hide Others...

Hides windows from other open programs.

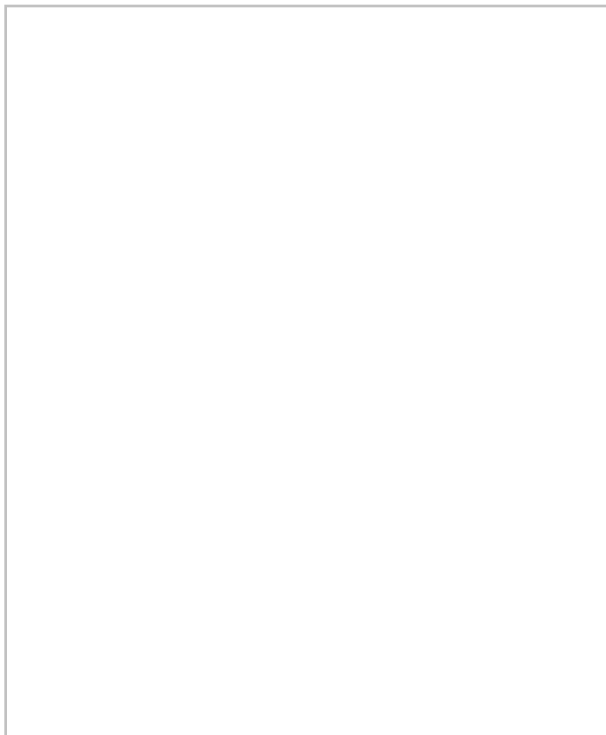
Show All...

Shows windows from all open programs.

Quit FlowJo...

Quits the program.

File Menu



New Workspace

Opens a new, empty workspace.

Open/Recover Workspace...

Open an existing workspace. Use the Macintosh standard file open dialog to select a workspace that you have previously created. In the unlikely event the Workspace won't open, use the the option key to reveal the Recover Workspace command.

Open Recent

Select from a list of the last ten Workspace files you have saved.

Close/Close All Graphs

Close the current window. If you close the workspace window, FlowJo will confirm whether or not to save any changes you have made. If you hold down the option key and a graph window is front-most, then FlowJo will close all open graph windows.

Save

Save the current workspace. If it has never been saved before, FlowJo will prompt you for a filename and directory

location.

Save as...

Save the current workspace with a different file name.

Save as Template...

Saves a version of the current workspace with all of the samples removed, so that it is easy to repeat the same analysis simply by adding new samples to the preconfigured template.

Save as XML...

Saves a Workspace in XML format. This will reopen in FlowJo for Mac or PC. Layout information is lost when opening on PC. Must also be moved with the XML file.

Revert

Discard all of the changes you have made to this workspace since saving it and reopen the last-saved version of the workspace.

Open/Recover Remote Workspace...

Enter the URL to download a Workspace file on another computer. Save the URL as a favorite. Option key lets you Recover a remote workspace that doesn't want to open.

Save As Remote...

Give the current Workspace a name and save it to the URL of another computer.

Page Setup...

Define the characteristics of printing you will do from FlowJo, page size, scale, orientation etc..

Print...

Print the current window. See the page on [Printing](#).

Quit

Quit FlowJo. If necessary, FlowJo will ask you to save any changes to current workspaces.

[\[Top\]](#)

Edit Menu

□

Undo Zoom

Returns Layout Editor to !00% scale size.

Cut

When editing text fields, or working in the layout editor, removes selected text or items and places them on the clipboard.

Copy With/Without Gates

Copy (+ Ancestors)

Used to duplicate items in the layout editor, or to export graphs from a graph window. If the current graph is a histogram, then a second item is also placed on the clipboard: the columnar histogram data in text mode, which you can paste into a spreadsheet. Also see the page on [copying graphics to other programs](#). Gates of subpopulations are included by default, excluded if you press the Option key.

Pressing the **Control** key will include the graphs of the current population's ancestors. When you paste, all the graphs are pasted. As above you will see Copy (+Ancestors) in the Edit menu.

Copy with gates (Cmd-Option-C)

This is a keyboard variation on the Copy command that also copies the selected population and its child gates to the clipboard.

Copy Samples as Text

This is another variation on the Copy command that uses the clipboard to export a textual description of the selected populations and statistics. Useful for taking the sample list out to a word processor.

Paste

Used to duplicate items in the layout editor, to add text to a layout or when editing text fields. Inserts the contents of the clipboard into the active selection.

Clear

Currently disabled, except when editing text fields.

Select All/ Equivalent Nodes

Select all items in the current window, for example, all samples in the workspace window, all gates in the graph window, or all items in the layout view. With the Option key down, Select Equivalent Nodes selects all data entries that occupy the same place in the gating hierarchy. This way you can quickly select, for example, all of the CD4 gates for all samples. The keyboard command for this type of selection is Option-Command-A. This may be a useful prelude to opening all of those graphs (select them all, then double click on one of them).

Select every....

Selects every other, third, fourth, fifth sample etc. based on the value chosen from the popup menu. This can be useful with regular protocols where, for example, every fourth tube is stained with the same reagent. Being able to select by interval is convenient for dragging several regularly spaced samples to a group.

Disable Tinting

You can choose to have new gates tinted by default in Preferences. Quickly remove the tint on a gate with this menu item. Handy if third party software renders the gates opaque after printing or pasting.

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Workspace menu

□

Show Workspace

Brings the workspace window to the front.

Show/Hide Statistics

Hides (or shows) all statistics nodes in the workspace.

Edit Workspace Columns...

Brings up a dialog to change the information displayed in the sample list of the workspace window.

Sort Sample List...

Brings up a dialog that lets you sort the sample list by a variety of keys.

Add Keyword...

Adds an additional keyword to the sample's record in the workspace, for additional annotation. Note that this does not alter the FCS file, but only creates the keyword within the scope of the current workspace. To permanently and universally add the keyword, you should use [ProJo](#).

Copy Value to Group/Create Value Series

This takes the value in the currently selected field, and applies it to all of the samples in the current group. This is useful for creating keywords with a value shared by all of the members of a group. When you select a keyword and hold down the Option key, you see **Create Value Series**. A dialog lets you set the initial keyword value, and increment it over subsequent data entries.

Make Groups From Keys... /Groups From All Values

Opens a dialog to choose a keyword as the basis for a new Workspace group. You can give the new group its own color and text style here too. Option key lets you make a group or groups that share a single value of a keyword.

Add Samples / Add Samples Into Group

Add FCS files to the current workspace. Option key lets you select a group and import new samples to it.

Add Samples From Network

Enter a URL and import samples from elsewhere.

New Group... /Put into new Group

Create a new grouping of samples. Brings up the [Group Definition](#) dialog. If you first select some samples you want to group then hold down the Option key, **Put into new Group** creates a group of those samples. The same results from the keyboard command **Option-command-G**.

Recalculate / Clear & Recalculate

This command asks FlowJo to compute all statistics and frequencies for the populations that are selected. In addition, all descendants of selected populations will also be recalculated. **Clear & Recalculate** Forces FlowJo to recompute all statistics for all populations, whether or not they have previously been computed. Keyboard equivalent is **Option-command=-**

Unify Analyses

If you have modified a gate and decide later that it should be identical to the group's version, you can select the gate and choose the **Unify Analyses** option. Likewise, if you select a group then choose a gated population within it, you can use **Unify Analyses** to apply this GROUP version of the gate to any samples whose gates have been changed. [Learn more about groups](#).

Rename

Use this command to rename whatever item you have selected in the workspace.

Get Sample Info

Opens a dialog with all the keyword information, compensation matrix, parameters and stains from the .fcs file for a selected sample.

Export...

Export the data from one or more samples and gates as .fcs files or chose to export channel or scale values as an ascii (text) file. Learn more about [exporting raw data](#).

Concatenate Samples

Combines the data from multiple samples into a single sample and saves it to disk as a .fcs file with the name of your choice.

[\[Top\]](#)

Platform menu

□

Compensate sample...

Define or apply a compensation matrix. This menu item has a submenu of [Compensation processes](#). If a group is currently selected, then all samples in that group are compensated; if a sample is selected, only that sample is compensated.

MultiGraph Overlay...

Click once in the **Workspace** window to select the sample you wish to view as a MultiGraph Overlay. Choose **MultiGraph Overlay** from the **Platform** menu. Drag and drop a gated subpopulation node from the Workspace menu or draw a new gate within the MultiGraph itself. Each addition to the MultiGraph appears in a contrasting color. [More details here](#).

Kinetics Platform

Launch the [Kinetics Platform](#) to perform time-dependent analyses.

Cell Cycle Platform

Launch the [Cell Cycle Platform](#) to perform DNA analyses.

Proliferation Platform

Launch the [Proliferation](#) platform used in conjunction with cellular markers to study the rate of cell replication.

Population Comparison Platform...

Launch the [Population Comparison](#) platform to quantitate and graph the difference between two different population distributions. The comparison utility has options for both univariate and multivariate comparisons.

Multi-sample Compare...

[Perform a comparison](#) between two or more different samples, ordering them based on an increasing difference from one sample or a composite of control samples. Creates graphs and statistical displays.

Cluster...

[Clustering](#) is the process of automatically identifying within a data collection subsets of events with similar characteristics.

Derive Parameters...

[Derive new computed parameters](#) or copy the derived parameters from a sample to other samples. You can only add derived parameters to a single sample; you can only copy derived parameters from a single sample that has them defined. From this menu item you can also delete unwanted derived parameters.

Define Calibration...

Launches the [Calibration](#) platform that you can use to convert between the fluorescence intensity collected on your instrument and absolute numbers of fluorophores.

Create Background Gate...

Boolean Gates...

Select a parent population, specify subpopulations upon which to apply **And - Or - And not - Or not...** the gated populations appear in the Workspace list.

[\[Top\]](#)

Graph menu

□

Graph Type

A hierarchical menu, from which you can select one of the following six graph types to apply to the current graph. By holding down the option key you can change all open graphs to the new graph type.

The following are the types you can select:

...Contour Plot

Makes the current graph a probability contour plot. (Holding down Option while selecting this item causes all graphs that are currently open into contour plots).

...Density Plot

Makes the current graph a density plot. (Holding down Option while selecting this item causes all graphs that are currently open into density plot graphs).

...Zebra Plot

Makes the current graph a Zebra plot. (Holding down Option while selecting this item causes all graphs that are currently open into zebra plot graphs).

...Pseudo-color Plot

Makes the current graph a pseudo-color plot. (Holding down Option while selecting this item causes all graphs that are currently open into pseudo-color plot graphs).

...Dot Plot

Makes the current graph a dot plot. (Holding down Option while selecting this item causes all graphs that are currently open into dot plot graphs).

...Histogram

Makes the current graph a histogram of whatever parameter is on the X axis.

...CDF

Makes the current graph a CDF (cumulative display function) of whatever parameter is on the X axis. A CDF is the integral of a histogram.

Graph options

A hierarchical menu, from which you can select one of the following five options to apply to the current graph. By holding down the option key, the type will be changed for all graph windows that are currently open.

The following options can be selected:

...Contour Levels...

For a contour plot, select the frequency of levels that are displayed. A submenu offers several probability settings.

...Smooth

When selected, the appearance of the graph is smoothed. Effects only contour, density, and histogram plots, does not effect underlying data.

...Show Outliers

Draws dots for all events outside of the outermost contour in contour or density plots.

...High Resolution

When on, graph is calculated at higher resolution. Requires more compute time; affects only contour, density, pseudo-color, and histogram plots.

...Invert

Reverse the color of the current plot.

Transpose axes

Switch the X and Y axes of the current plot.

Open new window

Opens a copy of the currently selected graph or if none is selected, a copy of the most recently selected graph.

Show defining gate

When the graph of a subpopulation is selected, this opens a graph window showing the parent population, with the gate that created the subpopulation.

Backgate Analysis...

Displays a series of graphs showing the [gating history](#) of a subpopulation that has been gated more than once. The final subpopulation is highlighted in each graph.

PolyVariate Plot...

Opens the controls to create a graph with multiple parameters. By adjusting these controls, subpopulations can be separated visually.

Manually enter gate...

Brings up a dialog allowing you to specify a new gate in terms of channels, absolute fluorescence, or percentile within a distribution.

Show Histograms...

Display a histogram of every channel in the current sample for the events shown in the current plot. This is a [MultiGraph Overlay](#), so you can gate on these graphs or drag subpopulations from the workspace to add to these graphs. Each graph will display each subpopulation in a contrasting color.

Open other samples...

Opens the equivalent graph of the same population for every other sample in the current group. If the windows are already open, then it changes the graph in that window to appear identical to the one you are looking at. If you hold down the option key while selecting this item, FlowJo will not ask you to confirm the operation.

Change other graphs...

Changes every other open graph to look like the one you are examining (i.e., FlowJo attempts to set the axes to the same channels, and the graph type and options identical to the current plot). If you hold down the option key while selecting this item, FlowJo will not ask you to confirm the operation.

Next Sample

Finds the next sample in the workspace sample list which has exactly the same gate as the current graph, and displays it in the current window. By holding down the option key, all open graphs will cycle in this way simultaneously. Same as Right Arrow button in the Graph window.

Previous Sample

Finds the previous sample in the workspace sample list which has exactly the same gate as the current graph, and displays it in the current window. By holding down the option key, all open graphs will cycle in this way simultaneously. Same as Left Arrow button in the Graph window.

Parent Population

When the graph of a subpopulation is selected, this opens a graph window showing the parent population, with the gate that created the subpopulation.

Child Population

Within a graph window with one or more gates, this command opens a graph of the currently selected gate. If more than one gate is selected, the most recently selected is opened.

Shrink Gate

Decreases the area of a selected gate by small increments.

Expand Gate

Increases the area of a selected gate by small increments.

Make Movie...

Turn the current graph into a QuickTime movie, using either time or another parameter to control the display of the current population. See the [Movie](#) platform for more information.

Copy Table...

Copies tabulated information from within Cell Cycle and Kinetics platform windows. The table is copied onto the clipboard, from which it can be pasted into any spreadsheet or word processor application.

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Layouts menu

□

Layout Editor...

Opens the Layout editor.

Make Batch Layout

Takes the current graphic analysis in the Layout editor and reproduces it in the layout based upon each sample in the current group.

Iteration Options.

Brings up the dialog box allowing you to change the iteration variable, to support complex multi-sample reports. More information is available in the [Layouts](#) section.

Save Layout Settings/Save Settings as Default

Stores a copy of your layout's tiling settings so that future layouts made with this template will default to the same settings. This command is only enabled for the Print Preview Window. With the **Option** key down, **Save Settings as Default** takes the current layout settings and makes them the default used when new layouts are created.

Get Item Info.

Brings up the dialog to edit the attributes of any selected object in the layout. This may be line weights and colors of an arrow or box, or the axes or smoothing algorithm in a graph.

Open Original Graph

With a graph selected in the Layout Editor window, this item opens a Graph window with the same data displayed.

Bring To Front

Operates on the selected object(s) in the layout. Causes it to be drawn on top of other items.

Move Up

Operates on the selected object(s) in the layout. Moves the selected items one step higher in the layer order.

Move Down

Operates on the selected object(s) in the layout. Moves the selected items one step lower in the layer order.

Send To Back

Operates on the selected object(s) in the layout. Moves the selected items to the back of the layer order, causing others to be drawn on top these.

Insert Picture...

Provides a dialog box for you to select a file in PICT format to be displayed in the current layout. Conversion of graphics formats GIF, TIFF, or JPEG requires either QuickTime, Clip2Gif or other plug-in translator.

Group

Multiple objects can be grouped so that they can repositioned, duplicated or deleted all at once. Select two or more items and invoke the Group command and they can be handled as a single object.

Ungroup

Divides a group into individual items again.

Put into Grid / Ungrid

Grids are an advanced form of groups which also determine the positioning and size of objects. As a grid is resized, the spacing and sizing of the elements behaves consistently across all of the cells in the grid. [More on grids.](#)

Get Grid Info...

Use **Get Grid Info...** to adjust the attributes of a grid. If a grid is currently selected, the **Get Item Info...** command will set the attributes of the *contents* of the selected cell.

Transpose Grid

The **Transpose Grid** command changes the number of rows to the number of columns and the number of columns to the number of rows. So a 3 column by 2 row grid becomes a 2 column by 3 row grid. Items are not moved among cells, but if the cell moves, the content moves with it. More at [Grids](#).

Zoom In

Increases the magnification level, so that objects are drawn larger. More controls of the layout's magnification are found in the bottom right corner of the Layout window.

Zoom Out

Decreases the magnification level, so that objects are drawn smaller. Magnification levels range from twice normal size to one eighth of normal size. More controls of the layout's magnification are found in the bottom right corner of the layout window.

Align.

Invokes the custom alignment dialog, which will facilitate building publication quality layouts. The Alignment commands are not enabled unless two or more items are currently selected in the layout. Many alignment and distribution options are available, including the relationship of objects to a grid, and the alignment of the axes of multiple graphs.

Align Horizontal

□

This item shows the submenu which contains the commands for four different ways of aligning the horizontal component of a set of layout options: by left edge, by right edge, by the center, or such that centers are equidistantly distributed between the leftmost and rightmost objects' current positions.

Align Vertical

□

Holds the submenu which contains four alignment commands governing the vertical component of a set of layout options: by top edge, by bottom edge, by the vertical center, or such that centers are spaced evenly top to bottom.

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Tables Menu

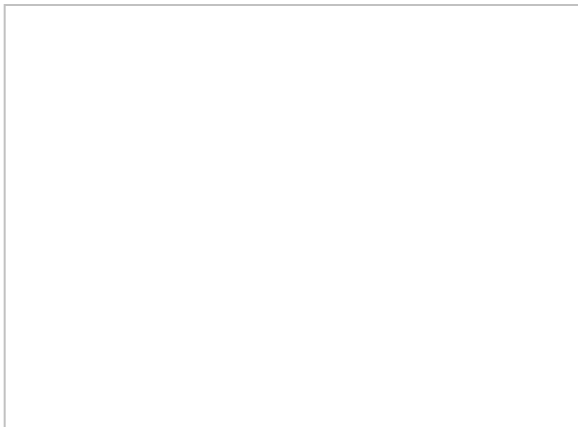


Table Editor...

Define or create a new table of statistics. Brings up the [Table Editor](#) dialog window.

Make Table

When you have chosen the contents of a new table in the table editor, this command constructs the table and opens it in a new window

Iteration Options

Opens the [Iteration Options](#) Dialog box to specify the group and the common parameter with which a batch will be

created.

Add Keyword

This menu item opens a dialog for adding a Keyword to a table. Choose a sample from which to select a keyword. A list of that sample's keywords appears.

Add/Edit Formula Column...

This opens the [Create Formula for Table](#) dialog. Defines the terms of a new formula to be applied as a column of values for each population in a table. If a formula is already selected in the Table Editor, this dialog allows you to edit it.

Special Formatting...

Opens the **Define Column Attributes** dialog. Name or rename a column, select an iteration value, choose special text formatting for values in a chosen range.

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Windows Menu

□

Table Editor...

Define or create a new table of statistics. Brings up the [Table Editor](#) dialog window.

Layout Editor...

Define or create a new layout for graphics presentation. Brings up the [Layout Editor](#) dialog window

Other Items In the Windows Menu

The names of each of your documents and work windows are added to this menu, each time a new window is created. This can be used as a navigation tool, to be able to bring you back to a desired window, after it has been covered or obscured by other windows.

Help Menu

□

FlowJo Reference Manual

Launches a web browser with the main help pages.

New Features in This Version

Launches a web browser with the list of new features.

FlowJo Tutorials

Launches a web browser with links to download our tutorials.

Check for New Version

Launches a web browser with a link to the latest version download.

Contact FlowJo Support

Launches a web browser with links to the Bug Report Form and our contact details.

Find this helpful?



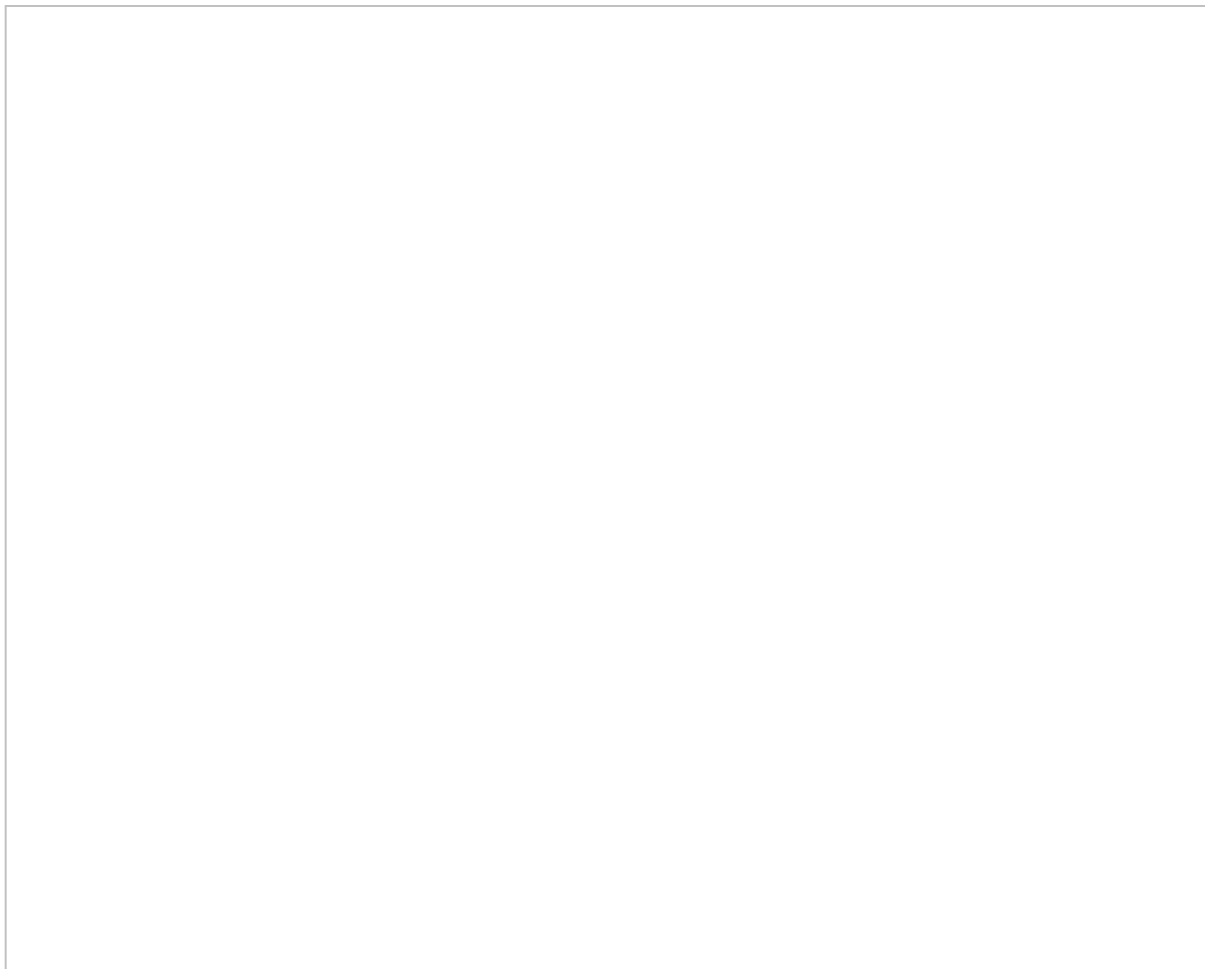
Preferences

There are a number of default behaviors of FlowJo which you can alter. These are modified through the **Preferences** dialogs, shown below. To get to these dialogs, select **Preferences** under the **Edit** menu on OS9 computers or under the **FlowJo** menu on OSX computers. Preferences are grouped by topic. To select a set of preferences, clicking on the appropriate tab at the top of the dialog.

For more information about each type of preference, click on the topic:

- [Workspace](#)
- [Graphs](#)
- [Gates](#)
- [Platforms](#)
- [Tables/Layouts](#)
- [Output](#)
- [Text](#)
- [General](#)

Workspace Preferences



The section marked **Appearance** defines preferences about the how the workspace appears. You can have FlowJo show **Elapsed Time Since Last Save** to show the amount of time that has past since you saved this workspace. The time counter is shown in the tool bar of the workspace window. Further options let you choose to have FlowJo display a light grid over the sample and group list to aid in visualization. The setting **Gate Statistic is Freq. of Parent** allows you to specify that the percentage shown in the **Statistic** column of the workspace refers to the portion of the immediate parent population. Unchecked, the number will reflect the portion of all cells in the sample tube. **Create new Workspace when launched** is checked by default. When FlowJo opens, it opens a new workspace window as well.

Just below, you can choose how FlowJo will display the data file names in the workspace window list. When you choose to display the sample's file name from the **Sample Name Keyword** - FlowJo examines the FCS keyword header information and uses the sample name keyword from the data file as the file name in the workspace.

NOTE: Options chosen when acquiring your data can cause all the samples in the workspace to be named identically (i.e., each file name is missing the suffix .001, .002 etc.). To avoid this problem, choose the next option, **Use Data File Name**. This option displays the name as it is on the system disk (i.e. with the .001 suffix) rather than using the internally-defined keyword.

An alternative file naming option is to **Specify Keywords** from the data file. For instance, displaying Sample ID or Patient ID as the file name may aid in identifying your samples. [Annotation](#) such as Sample ID or Patient ID can be entered while acquiring your data or in FlowJo.

Define Sort Order lets you choose whether data will be sorted by sample name or acquisition date and time. If you want a different sort order, return to the workspace and select **Sort...** from the bar above the tube names. There you can select multiple criteria from among the data stored in the fcs file, sorting first by \$DATE then by \$ETIM for example.

The final button in the section is to set the order and size of columns visible in the workspace. If you click on **Use As Default**, FlowJo records the columns & spacing that you have set in the current workspace, and uses that for all new workspaces that you create.

The next option is to **Remind to Save** your work. By entering a non-zero value in the box, you instruct FlowJo to remind you to save the workspace every few minutes. When this amount of time has elapsed, FlowJo puts up a dialog and lets you choose to save or not to save the workspace This dialog has a checkbox that lets you specify to always use the same answer: if you check this box and click **Yes**, then FlowJo will automatically save the workspace for you without interruption.

Save with Incrementing Version Number saves a new workspace every time FlowJo saves your work. The workspaces are named with incrementing version numbers (i.e., name.jo-1, name.jo-2 etc.) when the **Always use the same answer** box is checked upon the first save .

The popup menu for **Adding Analyses to the Workspace** controls how FlowJo causes recalculation of gates & statistics that are copied between subsets (or samples) to occur. Depending on how large your experiment is, it may become cumbersome to have FlowJo recalculate all populations affected by the action of dragging a population to a group or sample. This setting will permit you to postpone recalculation on some or all samples to maintain the responsiveness of the program. The default behavior is to compute **immediately** (which may cause FlowJo to need to load the sample data). You can also choose to have the statistics computed **only when needed** or **only if sample loaded**. The latter option will cause statistics to be computed only if the sample has already been read into memory (and still resides there). FlowJo will always compute the statistics once they are requested by the Table Editor or Layout Editor... this option is designed only to control the calculation of statistics for display in the workspace window.

The next section relates to the [Compensation Platform](#). Often, compensation causes data to be compressed onto the bottom or left axis in displays. This can be un-aesthetic... therefore you can choose to have FlowJo [transform data display](#) to improve visualization of the data. The **Allow custom visualization** checkbox allows you to apply the custom transformation from the **Compensation** menu and to choose the default values for the **Additional Negative Display Size** and the number of **Positive Decades of "log" display**.

The button at the bottom of this section, marked **Define...** goes to a separate dialog specifically for dealing with the data generated by Becton Dickinson's digital Vantage (Diva) system. This 32 bit linear data does not fit the normal data standards used by other instruments, but FlowJo has special settings to read the [DiVa acquisition files](#).

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Graph Preferences

□

The **first** section holds preferences that define how a [graph window](#) should look when you first open it.

The **Graph type** and other options are identical to those you can specify when you open the [graph options disclosure triangle](#) on the graph itself. This will be the default graph type displayed whenever you open a sample for the first time. To draw large dots, uncheck the High Resolution display option - these dots are twice as large in each dimension, and will be easier to see on slides or publications.

Forward scatter on X by default when checked, specifies that FlowJo shows Side scatter vs. Forward scatter the first time you open a sample's data, otherwise, it shows Forward vs. Side scatter.

The **Graph Sets** button opens a dialog where you can select parameters for the X and Y axes from the list of available parameters in the workspace. FlowJo will open graphs showing the combinations of the parameters you chose that are available in a selected data sample. To open graphs displaying all available graphs of the chosen parameters, Control-click a subpopulation.

Checking **Use Scientific Notation for Log Axis labels** will direct the program to draw axis labels as 100, 10¹, 10², etc. If unchecked the labels would be drawn 1, 10, 100, etc.

Check **Show uncompensated parameters in menus** when you wish to have the option to display graphs using either compensated or uncompensated data. Choose the uncompensated (without brackets) and compensated (bracketed) parameters in the axes menu pulldown lists.

The **Add event number parameter...** option adds an additional parameter to the axes pulldown menus. The event number parameter allows you to display the cells on a plot in the order they were run through the machine. For instance, if you collect 10,000 events, and display the Event Number parameter on the X-axis, the first cell will be at the left edge of the graph and the 10,000th cell will be at the right edge of the graph. This is useful to display vs. another parameter such as scatter in order to determine if changes occurred during collection.

Next you can choose what will be included in the axis labels of Layout editor's graphs. Select **parameter name, stain name** or both.

If you use **Dot Plots** (and in general, you shouldn't!), and you have more than 10,000 events in a file, you may wish to limit the number of dots drawn to speed up display or to keep it from "blacking out" completely. This can either be an absolute number, or a percentage of the total number of events in the population. If you enter 0 in this box, regardless of the radio buttons' settings, all of the events will be drawn.

If more than this percent of events is on the axis, FlowJo will open a warning dialog with an [explanation](#) or it will simply show a warning on the graph window. If you enter 100 in this box, the warnings will not be displayed.

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Gating Preferences

□

The **Gate Display** section groups preferences together that determine the behavior of gates (line weight and gate color)

and gated populations. **Show gate frequencies on plots** specifies that FlowJo should draw the frequency (within the parent gated population) of any gate drawn on a graph. The frequency is drawn in percent. The frequency is drawn on exported graphs whenever the gate is drawn, if this preference is selected. **Show gate name on plots** displays the gate name inside gate on the graph window. **Position for annotation** lets you choose where the identifying information for the gate will be displayed in relation to the gate itself.

Creating New Gates

When the **Auto-select gating tool...** option is checked, a polygon gate is started by simply clicking in the graph window. If this option is unchecked, a gating tool must be chosen from the top of the graph window before a gate will be drawn. **Choose rectangle gate by default** determines whether the polygon or rectangle is the default gating tool. **Auto-set tinting for new gates** if checked will draw the gates with a colored tint. Sometimes when exporting tinted gates, the color will become opaque and hide the data underneath the gate. Test with your presentation or graphics editing software. You can temporarily disable tinting for export or printing by choosing **Edit > Disable tinting** from the main menu.

Display other parameters than in gating plot... The graph for the new population will either have the same parameters as the graph on which you drew the gate; or, if the box is checked, FlowJo tries to intelligently select a new pair of parameters to display. The **Graph type to show** is selected by the pop-up menu. Here you can select that the new graph type is either (1) the same as the graph on which you drew the gate; (2) the same as you specified in the **Graphs** preferences or (3) a blank graph. The latter is useful when you are working with enormous data files, where you would like to specify the graph before FlowJo takes the time to calculate it for you.

Quad Gates

Show live quadrant statistics specifies that FlowJo should calculate quadrant statistics whenever the quadrant tool is selected. If unchecked, the statistics are not displayed in the graph window while you track a quadrant gate. Checking **Always ask for Quadrant gate name prefix** brings up a dialog every time you create a quad gate. This dialog asks for a prefix to attach to the quad gates - useful if you create multiple quad gates and want them to be listed together in the workspace window. **Use Q#, where...** puts Q1, Q2, Q3, as names on successive gates. **Include stain combination** adds this information to the quad gate name. At least one of these three boxes must be checked.

Finally, you can add a custom list of gate of **Gate Names** that you use frequently. If this list is defined, the gate name dialog will show a popup menu listing all of the names you have defined. You can either choose a name from this list or if you start typing the name of the population - FlowJo will automatically fill in the rest of the name. This will facilitate more consistent naming conventions, and reduce redundant typing.

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Platform Preferences

□

Choosing **Dragging to Layout...** will cause a table of statistics pertinent to the analysis to be created automatically in the Layout Editor along with the usual graphic element.

Cell Cycle - here you can declare default preferences for Cell Cycle graphs. These parameters can be changed *per graph* by opening the **Models** disclosure triangle in the graph window. **Statistics to Copy to Layouts** is a drop-down list where you can check the names of statistics you wish to be present in a table when you drag the Cell Cycle icon from the Workspace into the Layout Editor. See the **Dragging to Layout...** item just above.

Cell Cycle and Proliferation these options, also available in the graph window, will be applied to the graphs of both platforms by default.

Proliferation - Statistics to Copy to Layouts is a drop-down list where you can check the names of statistics you wish

to be present in a table when you drag the Proliferation icon from the Workspace into the Layout Editor.

Backgating - These items control the appearance of the subpopulation in each of the parent graphs when you display a backgate analysis graph group.

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Tables/Layouts Preferences

□

Within the **Layout Editor** section, you can choose the default format of batch layouts. The choices include Batch Layout, Tiled Report, Web Report, QuickTime Movie or Print Directly. You can learn more about the options [here](#).

Next you can choose how many panels (copies of your layout with new data) you want FlowJo to arrange on each page during batching of the layout. This will control how the batched layout will look when it is printed. Just below that, tell FlowJo the number of columns in which to display your batch. Then choose default foreground and background colors for new graphs. If you check the **Use Placeholders** box, you will see a simple outline with a diagonal x in place of the actual graphs in the layout.

The check boxes in the section **Use Layout Annotation** determine which information is included in the annotation of a graph added to a layout. The title, sample name, population, path, frequency and count can each be individually included in the annotation.

Options - Within the layout editor, double clicking on most items, or choosing the **Get Info...** command from the menu will edit that item's specific attributes. If multiple items are selected, then a **Multiple item settings** window comes up which contains all of the settings available. Setting the check box **Get Info always uses Multiple Items Settings dialog** will cause the larger dialog to be shown all the time. The checkbox **Draw Border** determines whether a line is drawn around the text boxes by default.

Table Editor - contains only options specific to the [Table Editor](#). Tables can optionally **show summary statistics** as additional rows in the output tables. If this is checked, additional rows for mean and standard deviation will be appended to the table. If the summary statistics are showing, values in the table will also be highlighted. If the value is greater than one standard deviation from the mean, it will be bold and italicized. If the value is greater than two standard deviations from the mean, it is also turns red.

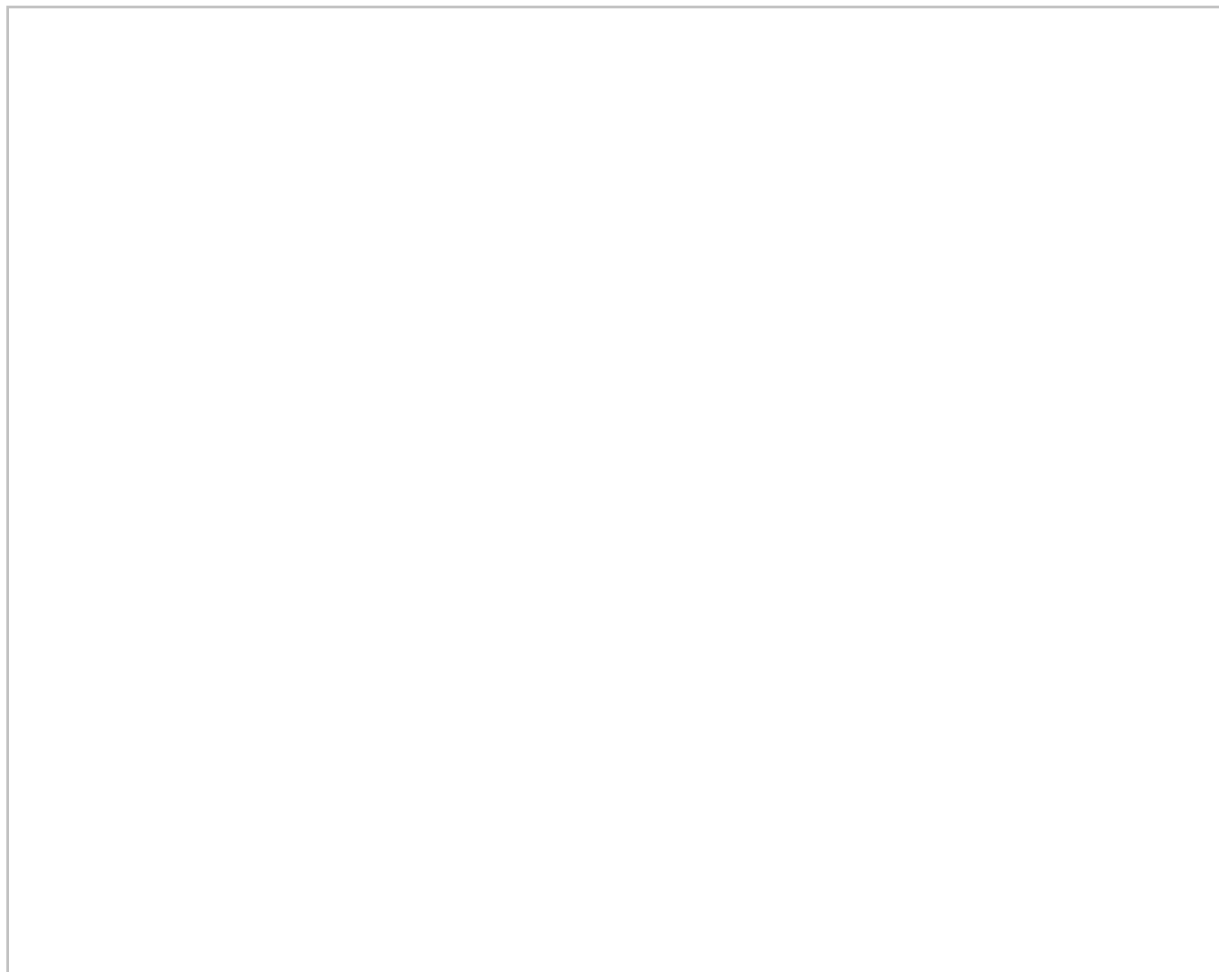
The option **Short Stat Names** causes an alternate set of statistic names to be used in the creation of tables. There is a tendency for the first row of tables, which contains the column headers describing the statistics, to become long and unwieldy. This setting will reduce that effect by substituting % for frequency, P for parent, and G for grandparent.

Helper Applications - The Layout Editor and Table Editor have buttons that will cause the current screen to be written to a file and then launched by a different program. For the table editor, you can pick a spreadsheet or statistics package as your **Helper Application**. For the layout editor, you can pick a graphics or publication program. Use the Choose... buttons here to pick the applications you wish these buttons to launch. In response to the Help button in any of the FlowJo's windows, the program will direct the Finder to launch the web Browser you have chosen in your computer's preferences, and display the HTML reference manual page specific for the context.

The **Define Legend Palette** button allows you to define the colors of the overlaid graphs in the Layout Editor. The first color by default is red and the next is blue etc. This can be changed to all black and gray scale if you do not have a color printer.

Histogram Overlay Defaults Select the line style you prefer, thicker, thinner, dashed or dotted. Tinted area without a line is also available here. Choose whether overlaid histograms each will be expressed on a common scale (checked) or each as a percentage of the maximum height of the graph (unchecked.) Finally check the last box if you wish to view overlaid histograms in a three-dimensional stack.

Output Preferences



Graph Options The first box allows you to make a gate's frequency label transparent. The next three boxes control what axis info is displayed by default.

HTML Output Here you select what format you want FlowJo to use when it saves your layouts as Web pages. Photoshop and Quicktime native formats are also available here. If you want a standard text or graphic to appear at the head and/or foot of your Web page, use the **Choose** button to show FlowJo where the files are. These files must contain the appropriate HTML tags to display properly. They may contain the logo or link information that you want included in the pages you create.

Exporting Graphics Graphs are copied without gates showing by default. Check this box to include gates when copying. Option - clicking will reverse this choice on a per graph basis. The drop-down list here lets you choose the file format of exported graphics.

Graphic Formats Options Check your preferences for PICT graphic properties. Vector objects within exported graphs can be edited separately, allowing more control of the image after it leaves FlowJo. Drawing dots as filled boxes allow you to control their size in a vector graphic program. It is efficient to display histograms as polygons, but some programs will not display the polygons correctly. Uncheck this box if your exported histograms are corrupted. Next you can choose the level of compression of exported graphics. **Lossless** compression gives the clearest display but the largest file size, **Most** compression gives the smallest file size but the least fidelity to the original appearance of the graph. Check the **Movies Back & forth Looping** box to cause quicktime movies of your batched graphs to cycle back and forth rather than jumping from the end to the beginning when they repeat.

Text Preferences

□

The **Text** tab collects the controls for all of FlowJo. Choose the text placement from the list on the left, then set the font, size, color and so forth on the right. Or, choose the settings for one area, then click the **Set All** button to have the same attributes applied everywhere.

General Preferences

□

General Appearance & Behavior Updates between versions (8.x 8.y 8.z) are free. Check this box only if you do **not** wish to know when there is a free FlowJo update available. Check the second box to be notified when Beta versions are available for testing. The third checkbox will mute FlowJo's sound feedback. If dragging windows takes too long on your computer, you can check the **Suppress Live Window Operations** checkbox. You will see a placeholder during the window moves. You can adjust how quickly pop-up windows open by specifying your preference (in seconds and tenths of seconds) in the next box. Finally, you can specify how many significant figures you want FlowJo to calculate when computing statistics.

System Management FlowJo has a task monitor window, a small status bar that displays the current number of graphs and calculations it is currently working on. Because its workload can sometimes get quite extreme, the monitor window can be useful in assessing how much longer you have to wait. FlowJo's task monitor window can be positioned in any corner of your monitor, or you can turn it off with this drop-down list. Here also, you can specify how much of your computer's RAM to use for FlowJo's operation.

Printing [This menu](#) allows you to define the headers and footers for printed pages. If you print the workspace window, a table or a layout, information such as the date and workspace name show up as [headers and footers](#) on each printed page. Click this button to type in your own text, choose from special strings (such as date and time) and even to place graphics at the footer, header or in the background of each printed page.

Confirmation/Notification Dialogs Here you can control how FlowJo handles warnings. Some choices will show you a confirmation dialog to be sure you want to perform the indicated action. If you wish, you can shut off these checks, then restart them if you need them. You can also control how long FlowJo will wait for your response before taking the action you ordered. Enter zero and it will never take the action without your confirmation.

Remote Data Transfer The settings within this box are only used by a small number of sites that provide centralized data storage for their FCS files. For some sites, FlowJo retrieves data over the internet; in these cases, you can select a maximum cache size that FlowJo uses to keep copies of the data on your Macintosh (and, if you desire, to have FlowJo delete the cache files when you quit). If files are stored remotely, FlowJo can use FTP or HTTP to download them to your Macintosh as they are needed. To speed up subsequent access to the same files, FlowJo maintains a cache on local hard drive. These settings will determine the maximum size of the cache, and whether the program should delete all of the files when it quits.

Find this helpful?



Techniques

This section discusses different techniques you can use to analyze data more efficiently with FlowJo.

FlowJo gives you the most flexibility possible with exported graphics. You can easily ungroup any graphic, and change the font attributes, line styles, etc., to make any graph look exactly the way you want! The [first page](#) in this section gives you some additional hints on exporting graphs.

The greatest strength of FlowJo is its ability to represent [batch analysis](#) in a simple and flexible structure. Don't expect to find a macro language or scripting extensions. Our approach lies in the grouping structure, within the workspaces. Create your layout, and with a single button, produce a stack. The stack can be shown as a new layout, a tiles picture or a movie. There's even a section about how to [cancel batch jobs](#) once they've been started

[Drag-and-Drop](#) is an important skill to learn if you are going to be a fluent user of this program. This section talks about the semantics of what it means to drop gates or statistics onto populations. A few modifier keys can save you all sorts of time.

Indeed, modifier keys, especially the [option key](#) are used heavily in FlowJo. Use option dragging to duplicate graphs in the layouts, or option-close to close all your windows at once. Try depressing the option key as you browse through menu items; you will find that many of the menus change to be a different command, giving you quick access to even more powerful features.

Find this helpful?



Getting Help in FlowJo

FlowJo is littered with help buttons throughout the application. Clicking on one of these launches a web browser to access our Web page describing that topic. Or, you can choose help from the Apple menu while running FlowJo to go directly to the general help page.

In addition, you can press the "help" key on your keyboard at any time. This will activate the help button on the frontmost window (if one exists); otherwise, it will provide you with general help on FlowJo.

Using the web browser, FlowJo gives you "context-sensitive help"--i.e., taking you to help information specifically regarding the operation you are currently performing.

If you are not connected to the internet, FlowJo can access help files installed on your hard drive. With the installation CD in your computer, browse to a folder called Documentation. Drag this folder into the same folder on your hard drive as the FlowJo program. Now when you click on a help button, FlowJo will access the reference manual from your computer.

[Offline Documentation](#)

You can download the latest [FlowJo's manual](#) onto your hard drive for use when you are not connected to the internet from [this page](#).

Find this helpful?



Cancelling Batch Operations

Many, but not all, of the operations that you request FlowJo to perform can be canceled while they are happening. In general, when you cancel an operation, all further calculations cease. In the case of creating tables or layouts, this will stop any output from being generated. If you select many subpopulations and accidentally double click on one of them (this directs FlowJo to open them all), it may take some time for all of the graphs to be generated. If you cancel the operation, then FlowJo will stop opening graphs (but leave the windows up for the ones it has already opened).

To cancel an operation, you can either click on the **Stop** button in the progress bar window, or press command-period (i.e., press the command (or "Apple") key and period key simultaneously). If FlowJo can cancel the operation, it will do so, although it may take a few seconds before this happens. (For instance, if FlowJo is in the middle of reading a file, you will have to wait until the file is fully loaded before the cancel can occur). In any case, FlowJo will change the message in the progress bar as soon as you attempt to cancel to let you know that it will cease its functions as soon as it can.

There are operations which cannot be canceled, in which case FlowJo will simply continue computing. Clicking on the "Stop" button or pressing cmd-period again will not help! If you wish to cancel an operation, you only need to press "Stop" or cmd-period once; if FlowJo can cancel, it will.

Find this helpful?



Option Key Techniques

There are several cases where, if you hold down the option key while performing a command, FlowJo will modify what it is doing slightly. The following is a list of these modified functions. If you are not familiar with the use of FlowJo, there is no need to learn this list; come back to it once you have familiarized yourself with the operation of the program.

If you press or release the option key while viewing FlowJo menus, some of the items may change! This quickly lets you know what different operations are accessible from the menu while holding down the option key.

- **Displaying all parameters in the Graph Window.** If you hold down the option key while clicking on the X or Y axis parameter lists, all available parameters (compensated and uncompensated) will display. By default, if a sample is compensated FlowJo will show the compensated parameters in these lists.
- **Using Next/Previous graph.** If you hold down the option key while performing these operations, every open graph window is cycled to the next or previous sample.
- **Copying a graph.** When you copy a graph from a graph window and hold down the option key, then FlowJo will also copy any gates (polygons, etc.) that are shown on the graph (this may occur anyway depending on your preference settings).
- **Creating a new gate.** When you are asked for the name of a new subpopulation (you just drew a gate on a graph), you can hold down the option key while pressing "OK" or "Select". In either case, the graph window on which you just created the new gate will be closed. (In the case of "Select", a new window containing the subpopulation you created will now be opened).
- **Navigating the gating hierarchy.** If you hold down the option key while clicking on the "Show parent" or "Show child" buttons in the graph window (the up and down arrows, respectively), then the current window is closed before the other one is opened.
- **Changing the graph characteristics.** If you hold down the option key while changing the characteristics of the graph (i.e., dot plot, contour plot, density plot, or contour levels, or smoothing, or outliers), then the same change will be applied to all open graph windows (for that workspace). This is true either while selecting the menu command (from the "Graph" menu), or while clicking on the "Apply" button in the graph tools window.
- **Closing a plot.** If you hold down the option key while clicking on the close box of a graph, then all of the open graph windows (for that workspace) will also be closed.
- **Creating a gate.** If you hold down the option key while clicking in a two-dimensional graph, then you will create a rectangular gate rather than a polygon.
- **Showing/Hiding gating hierarchy.** If you hold down the option key while clicking on a disclosure triangle in the workspace window (next to the node name), then the show or hide function will be applied to all "children" of that node (i.e., collapse/expand the entire tree). Alternatively, if you hold down the command key while clicking on a triangle, then the show or hide function is applied to all "siblings" (all other nodes at that level).
- **Opening or changing other graphs** (under the Graph Menu). Holding down the option key keeps FlowJo from confirming this option.
- **Duplicating draw objects** in the Layout Editor. Holding down the option key while dragging an objects will create a duplicate of the object in the new location.
- **Removing a layer from an overlay.** Holding the option key while passing the cursor over the legend of a multi-layer overlay shows the cursor as a trash can. Option-clicking a row in the legend will remove that layer from the plot.

Find this helpful?



Overview

This page describes some of the basic concepts underlying FlowJo; it is meant to help you familiarize yourself with some of the terminology and the features of FlowJo. As you navigate through the main documentation, you will get more details about all of these topics. In addition, you can follow links to some special topics regarding FlowJo: The all-important [Credits](#) page, where we have the opportunity to thank the many individuals who have spent much time and effort helping us make FlowJo the most sophisticated analysis package around, a [Frequently-Asked Questions](#) page (FAQ), listing some common questions and answers, a page about getting [Help](#) from within FlowJo itself, and a page detailing the history of [revisions](#) to FlowJo.

What does FlowJo do?

FlowJo should be the first step in the process of analyzing flow cytometry data. FlowJo can analyze data generated by any flow cytometer from any manufacturer. FlowJo has a number of different analysis platforms that let you not only perform standard analyses such as gating and statistics, but also specialized analyses such as DNA/Cell Cycle, Kinetics (Calcium flux), Proliferation, Calibration, and Statistical Comparison. You may find that FlowJo's sophisticated tools for generating output (graphical or tabular) are sufficient for you to generate publication-quality material.... but if not, FlowJo makes it easy for you to copy any graphs, statistics, or other information into other programs for further analysis and presentation.

Experiments and workspaces

FlowJo is a program designed to analyze flow cytometry data. The basic concept behind this analysis is that of the experiment. An experiment is a collection of samples which have a set of common attributes; for instance, there are sets of tubes stained with the same antibodies, other sets of tubes which come from the same tissue sources, etc. An experiment can be a single collection of samples, or it can stretch across multiple runs over a period of months.

With FlowJo, you will organize the samples in a [workspace](#). A Workspace is similar to a notebook: it references every sample that you are analyzing, and records the analyses (gates, statistics, graphs, tables) that you have done. You can close the workspace, and then reopen in the future and start where you left off. You can have as many workspaces as you want; the organization is up to you. We recommend that you have at least one workspace for every experiment.

Within a workspace, you can [group samples by various attributes](#). For instance, you can make a group of all samples derived from a single individual (which may have different stains); you can also make groups of all samples with the same stains (which come from different individuals). Groups are really the powerful feature of FlowJo: when you perform an operation on a group, it performs the operation on every sample belonging to that group. Thus, you can apply a gate or a statistic to a group, and that gate or calculation will be automatically performed on all samples!

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Analyzing with FlowJo

You will find that your mode of operating FlowJo will probably be very similar to the following series of steps:

1. Load the samples into a workspace.
2. Group samples for analysis with common procedures.
3. Analyze a single prototype sample in detail. Decide on gates, statistics, etc.

4. Apply the appropriate analyses to all the samples in a group.
5. Quickly check the samples in the group to make sure the gates are acceptable: they may need minor modifications to accommodate sample to sample variation.
6. Generate a [graphical layout](#) in which you can display particular graphs from all samples.
7. Generate a [table](#) in which you can generate particular statistics from all samples.

You can then save this workspace as a template... Next week, when you do another (similar) experiment, most of the work is already done for you! You simply have to import the new samples into the template workspace you created before: all of the data files will be added to the appropriate groups, which means all of your previous analyses (gates & statistics, etc.) will be automatically applied to each sample. You need only regenerate the graphical layouts and the tables.

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The Gating Hierarchy

Another fundamental concept in FlowJo is that of [the gating hierarchy](#). When you first generate a gate on a sample (for instance, a lymphocyte gate), FlowJo shows you this gate (subset) in the paradigm of a genealogical tree. In other words, the Lymphocyte subset is a child of the parent sample. It is shown in the workspace underneath the sample, indented a single level from the sample. If you were to make another gate on the sample (for instance, monocytes) FlowJo will create another new subset as another child of the sample; now, the lymphocyte and monocyte subsets (or gates) are siblings.

Any operation that you can perform on a sample can also be performed on a subset of the sample. Therefore, you can view the lymphocyte subset and create gates (subsets) of this population. If you create a T cell gate within lymphocytes, then the T cell subset becomes a child of the lymphocytes (and, by extension, a grandchild of the sample).

This paradigm is very important to the way in which FlowJo operates. When you [copy analyses](#) like gates, you can choose to copy only the single gate that you click on, or you can choose to copy it with all of its children (i.e., copy the entire analysis tree). You can copy the gate with its parents as well. Remember, any subpopulation is a single gate. When you want to recreate the same subpopulation on another sample, you need to carry all of the gates (all of the ancestors of the subset) with the subpopulation when you copy it.

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Batch operations

Batch operations (repetitive analyses performed on multiple samples) are very simple in FlowJo. In general, batch operations are performed on all samples in the currently selected group (therefore, you will want to [create sample groups](#) that will serve to help you generate batch outputs). Batch operations include copying gates and analyses, generating graphical layouts, and generating tables. In addition, there are some minor operations like opening a graph of a subset as you are viewing from all samples in the group, unifying the analyses across samples within a group, deleting analyses, and so forth.

In this way, FlowJo is a program that doesn't just analyze individual data samples. Rather, the focus is on analyzing groups of samples, experiments, or even multiple experiments at once.

Compensation

In the world of increasing fluorescence parameters, it has become impossible to fully compensate samples at the time of collection. FlowJo provides an [interface for computing the compensation matrix](#) based on the collection of singly-stained samples. You can then apply this compensation matrix to samples that are uncompensated. At this point, you can view and analyze the compensated data.

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Derived Parameters

FlowJo lets you [define new parameters](#) for samples. These include the ability to add Time as a parameter (for performing kinetics analyses), to compute the ratio of two collected parameters, or to convert between log and linear scaling. Derived parameter definitions can be copied to entire groups of samples.

Kinetics (Ca⁺⁺ flux) analyses

FlowJo provides a sophisticated platform designed to analyze [kinetics](#) data. The sample must have a parameter which corresponds to time (and is named "Time"); if it does not, FlowJo allows you to create a time parameter (assuming a constant event rate). From this platform, you can compute the maximal response time, the slope of a response, the fraction of responding cells, etc.

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Cell Cycle Analysis

The [Cell Cycle Platform](#) is easy to use, yet can view several different models simultaneously, constrain fitting parameters, and automatically calculate the percentage of cells in G1, S, or G2 peaks. Fitting can be constrained in a number of ways, letting you generate reasonable interpretations of even very unusual cell cycle distributions. All models can be copied between populations or samples, with the same easy drag and drop interface used to propagate other gates or analyses.

Calibration (Quantitation)

FlowJo has a unique [Calibration Platform](#) that allows you to calibrate any collected parameter. Most commonly, this is used to convert the scaling into absolute number of molecules (given a standard that converts between the fluorescence intensity collected on your instrument and absolute numbers of fluorophores). The platform can use a calibrated bead set as a standard, a stained sample as a reference, or numbers that you enter to define the calibration manually.

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Proliferation Analysis

The [Proliferation Platform](#) is used to model proliferation data obtained using cell tracking dyes such as CFSE. FlowJo presents a graphical display as well as information about each generation in the subset. The proliferation platform also provides information about the fraction of cells from the original population that have divided, and the number of times these cells have divided. In addition the FlowJo Proliferation Platform draws gates that separate each generation.

Population Comparison

The [Population Comparison Platforms](#) allow the direct comparison between different samples or subsets. FlowJo can take two or more samples and analyze them to determine how different the distributions are. Comparisons can be univariate or multivariate, and you can rank multiple samples by their similarity to controls. These platforms take advantage of the exciting new comparison algorithm - Probability Binning. This algorithm even allows the creation of a gate to display the difference between two populations.

Polyvariate Plots

This utility provides a unique view of several subpopulations plotted on a single graph. Using the interactive controls, you can separate numerous gated events visually.

Movies

[Movies](#). An analysis platform unique to FlowJo: view your data dynamically. Use the Movie Platform to generate graphs as a function of time (kinetic analyses), or to generate a graph of one or two parameters as a function of a third. This unique visualization lets you uncover subtle relationships in your data that would be impossible to see otherwise.

Backgating Analysis

Once your analysis contains subpopulations produced by gating within gates, it can be useful to see the source of a given subpopulation rendered graphically. Backgating analysis shows graphs of each gating operation with the final subpopulation highlighted in the graph of each stage.

Statistics and Formulas

A host of standard statistical calculations can be performed on a subpopulation then generalized to the whole experiment by batching. FlowJo will build a table of statistical analyses across groups of tubes that you designate. These tables update continuously as long as you are modifying your experiment, for example, by correcting gates. You can export these tables and work further in third party software. You can also devise and apply your own formulas within FlowJo and use its tools to complete your analysis across multiple populations without tedious duplication of effort. The tables you create can be dragged and dropped from one workspace to another.

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Version History

Listed below are the changes made to FlowJo since the release of FlowJo, version 6.4, on November 16th 2005. The changes are listed in reverse chronological order. Within each revision are listed the new features (major and minor), and any bug fixes.

Current Version is [9.4](#), posted September 19th, 2011

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[Version 9.4 \(September 19, 2011\)](#)
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[Version 6.0 \(July 5th, 2004\)](#)

[Version 4 History](#)

[Version 3 History](#)

[Version 2 History](#)

September 19th 2011, Version 9.4 released

Changes since 9.3.3

- New: FTP/HTTP completely revised and improved, for Lion compatibility
- New preference to show/hide gate tethers (magnetic or tethered gates)
- HTTP authorization preference removed - automatically done if needed
- Fix: Rare crash when drawing gates copied from now-deleted subset
- Better axis labeling for calibration of transformed parms
- Fix: Prevent crash when choosing keywords to add to pref for overlays when no WS is open
- Comp Wizard better handles already-scatter-gated controls
- Fine-tune peak identification for Calibration platform
- Better progress monitoring of FTP downloads
- Fix: Vertical histogram offset for overlays in batched outputs
- Fix crash on reading some old workspaces using 15 bit files
- Fix: Mag gate offset drawn to correct origin on resized graph windows
- Fix: Dragging mag gate annotation on resized windows

July 27th 2011, Version 9.3.3 released

Changes since 9.3.2

- Dragging WS node into LE text box inserts sample ID properly for uniterated view
- Reading LE that have Statistics Tables referencing compensated parameter positions no longer crashes
- Iterate by nth now creates "missing" graphics if not enough samples present in the list (previously would replicate last sample) -- i.e., iterate by 5th, have 8 samples in list: 8th would appear in position 9 and 10 in batch outputs
- Concatenating samples with derived parameters no longer crashes if the samples hadn't been opened first
- FLS on Lion works
- About box now shows latest downloadable FJ version

June 16th 2011, Version 9.3.2 released

Changes since 9.3.1

Note:

Workspaces saved in 9.3.2 cannot be opened with 9.2. (As usual, however, you can save a workspace as XML and open that in older versions.)

New Features:

- Parameter lists in layout "multi item edit dialog" are now sorted according to preferences
- Workspaces with large numbers of keywords operate much more efficiently
- Better refresh on some dialog windows when resizing
- New PREF for cyTOF files that allows setting uniform range for all parameters. **USE WITH CAUTION.** FJ will warn you if you are doing this for nonCyTOF file but allows. Used for CyTOF files to set uniform range for all parms... in general, set it to higher than highest of all parm ranges, use power of 2. **BE CAUTIOUS**
- Interpolate Kinetics graphs to make smoother graphs

- If you manually run Sample Validation platform, you can "Copy Detailed Report" which gives the computed value for each check, for all samples & parms. You can use this to determine appropriate ranges for thresholds for failures.

Fixes:

- Fix reading files with no \$BYTEORD (any text-fcs converted file)
- Fixed loading compensated data files when the parameter name (\$PnN) has been edited.
- Fixes bug introduced into 9.3.1 with 16 bit data (various mfrs)
- FJML Find function will now work in all uses

April 18th 2011, Version 9.3.1 released

Changes since 9.3

Note:

Workspaces saved in 9.3.1 cannot be opened with 9.2. (As usual, however, you can save a workspace as XML and open that in older versions.)

New Features:

- When resizing a Layout graph, you can hold down option key to size to nearest 10% (text appears to explain this)

Fixes:

- Properly handle remote FTP transfer of 16 bit data files
- Allow changing layout graph sizing in dialog window
- Resizing a layout graph shows feedback as to size (as it used to, long ago!)
- Transparency in SVG now supported

March 16th 2011, Version 9.3 released

Changes since 9.2

Note:

Workspaces saved in 9.3 cannot be opened with 9.2. (As usual, however, you can save a workspace as XML and open that in older versions.)

Major New Features:

- [New LE Graph statistics object](#)
- [New Clone Gates](#)

Other new Features:

- FJML Gates -- Many new features:
- FJML Gate editor supports copy/paste operations
- Gate FJML commands can be shown in workspace, TE, and LE
- New FJML command to get the center point for any quadrant gate ("Vertex_Q_parameter")
- Gates relying on FJML get special icon in workspace (circle-F)
- Gates depending on keyword values immediately change with keyword modification (yes, this means you can change gates from the Workspace!)
- Option-clicking on a workspace header will sort by that keyword and add it to the sort definition (so you can

option-click on successive column headers to do a complex sort). Option clicking on the most recently-sorted column will invert the sort order

- Improvements to Compensation Wizard when determining color for controls
- Workspace contextual menu for gate nodes adds "Copy with children" and "Copy with ancestors"
- "Recalculate" menu commands renamed as "compute" for clarity
- Preference for precision display adds a radio button to discriminate significant figures from decimal digits

Bug Fixes:

- Use all 36 colors in preferences palette for overlays
- Read workspaces with (some rare) corruptions
- Changing a keyword value immediately updates LE showing the keyword
- Templates with LE text boxes containing statistics now work after loading samples
- FJML Gate editing - when dragging gates into definition, correctly constructs FJML command referencing parameters
- Quadrant gate vertex numbers are not re-numbered when resizing so FJML gate can correctly refer to them
- Calibration of parameters using biexponential transformation fixed
- Histogram range gates appear in SVG output
- Kolmogorov-Smirnov statistics are properly annotated (titled) in comparison windows
- Properly handle \$TimeStep keyword in special cases
- Properly update some LE text boxes pointing to statistics that wouldn't invalidate when gate was modified
- Sorting workspace by \$DATE is now correct
- Quad gates created on parameters containing "slash" will now correctly replace slash with hyphen
- Table formula entry corrected for some functions (like subtraction, and less than or equal to)
- Histogram graphs obey the autoselect gate tool preference setting
- LE displays that are offset from the top left retain proper aspect ratio when printed
- Editing a compensation matrix will immediately update multigraph overlay platforms and tabular displays of the matrix in the LE
- Quit is prevented while editing a compensation matrix
- The LE graph item editor properly sets the "show legend" check box
- Changing a LE graph to/from histogram updates the overlay legend to show/hide the line style control

December 10th 2010, Version 9.2 released

Find this helpful?

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General

- Changes to layout editor comparison platform graphs are maintained
- Templates open with "All Samples" group current
- CXP correction pref only applied to files with \$SYS = CXP or \$CYT = CXP
- Changed the number of colors for layout overlays that can be set in preferences from 12 to 36
- Proliferation Platform: Added EI (expansion index), RI (replication index), & SDD stats, re
- ordered stats in graph window
- Added menu / rightclick command "Copy To LE", works on single or multiple selection.
- Don't highlight (enable) transformation menus for uncompensated samples
- Allow different transformations for uncompensated parameters with different ranges (Important for CyTOF files)
- Allow reading (and skipping) layouts that are corrupted (and read other layouts that have not been affected)
- Comparison Node: Leave enough space to display Overton Percent Positive value. Also, recalculate when switching between univariate and multivariate views to ensure updated display
- Change percentile calculation for distributions where first channel of events IS percentile (was averaging

- percentile down)
- Maintain \$PnR on floating point exports
- Fine tuning Comp Wizard for offscale parameter handling
- Calibration platform uses smoothed distributions for reference calculations
- Compensation Wizard provides helpful message when there is complete lack of shared parameters
- Handle duplicate indexed sort gate wells (gates now include all duplicated events rather than just the last one)
- Proliferation node allows setting Y axis in LE

Find this helpful?

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Bug fixes:

- Recode Gallios FCS3.0 files to correct error in assignment of parameter stain names and recode scaling.
- Template workspace textboxes retain text
- Allow concatenation of samples with different stain names when prefs are to sort parm lists by stain
- Improve Comp Wizard performance & prevent a crash
- Reopening a workspace with a magnetic gate will show the gate in the moved position upon graph open
- Fixed template creation for layouts containing groups or grids of samples
- Backgate displays always show all dots for gated subsets (rather than preference defined setting for dot displays)
- XML workspaces, when reopened, do not show deleted samples in workspace
- Selecting "Remove Compensation" when graph window is front-most works
- Graphs in Layout Grids expand to fill the cells when hiding axis information
- When selecting multiple cells in Layout Grids and moving objects, the order of the objects is not inverted.
- Boolean gates that refer to gates that have "G#" in their names (like " gate") are properly shown in the display window when they are edited
- Multigraph overlay accommodates larger axis text (from prefs)
- Moving multiple objects within grids preserves order

July 27th 2010, Version 9.1 released

Features:

Find this helpful?

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General

- FlowJo now calculates keyword values from formulas involving strings (other keywords) and the usual math operands via FJML.
- Compensation matrix identifier bar in Workspace (left edge) is wider & brighter for better visualization.
- Compensation Wizard better handles samples with off-scale scatter measurements.
- New Preference (on the General tab) to turn off sub-channel randomization of measurements during compensation - makes picket fencing happen but prevents small changes in membership of gated events on compensated parameters.
- Population Comparison: Overton statistics added to Layout Editor annotation box.
- Boolean gates that refer to missing nodes now show "RefError" in the Workspace statistic display.
- Added "?" (Help) button to Custom Transformation Window.

Compensation Wizard will use your custom scatter gate if you have pre-defined one.

Find this helpful?

□□

Bug fixes:

Fix: Disallow compensation matrices with same name (will rename them "-1", "-2" etc)

Fix: Properly scale FCS 3.0 floating point parameters with a \$PnR that is not a power of 2

Fix: Trying to load a data file for which you don't have read privileges now shows error message.

Fix: FlowJo can now handle the situation where the user sets custom transform visible decades to zero (bad value.)

March 22nd 2010, Version 9.0.2 released

Find this helpful?

□□

General

- New FJML attribute (property name: "GatePositionArea") to return the center and area of a gate (for table and layout editors). This can be used for QC purposes, by providing a column that can be scanned to identify gates that have been moved (are different).
- Fine tuned performance specifications, now more efficient.
- You can now import spreadsheet-based data files with parameter ranges that are not a power of 2 ("FJREALTEXT" format).
- New menu item to "clear & recalculate" selected items (versus all items in the current group).

Find this helpful?

□□

Bug fixes:

- Batched layouts now obey preferences properly (i.e., layout or workspace or general preferences).
- Fixed crash if you batched a new layout with an overlay graph that has a layer referring to a missing sample.
- Fixed crash if you choose to open the graph for a value in a table editor output that has only a single statistic column.
- Fixed occasional crash on some computers in the PolyChromatic Plot Controller.
- Fixed crash that occurred when you re-open a saved workspace following a cancelled relocation of a missing data file.
- Parameter names ending in "Area" (not just "-A") are considered area for scaling purposes.
- Better axis notation when few decades are shown (i.e., don't show "5x10³", partial decade values).
- Derived parameters can be computed on parameters named "P10", "P11" etc.
- FJML gates allow >255 characters in their specification.

April 19th 2010, Version 9.0 released

Features:

Find this helpful?

□ □

General

- New workspace version: workspaces created by v9 cannot be opened by older versions of FlowJo. However, you can "Save as XML" and older versions can read that file (not all options are supported).
- SVG (Scalable Vector Graphics) is a supported output format. Supports high-resolution vector graphics that can be exchanged into [Intaglio](#) (copy/paste or saved file export) or Illustrator (saved file export)
- Lists of keywords can be navigated by typing (first letter of keyword name)
- Event number parameter starts with first event = 1 (rather than 0)
- An expiring serial number is warned about only twice during any session
- Several main menus have changed: e.g., new Transformation menu; look for new menu commands

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Bug fixes:

- The correct IP address is shown in the "About" box

Find this helpful?

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Compensation & Transformation

- Compensation matrices are sorted alphabetically in the menu
- Compensation matrix elements can be added as columns in table editor
- Compensation matrix can be added as separate table in layout editor
- [Can opt to have the current workspace group to recalculate all stats \("live"\) while editing compensation \(helps determine optimal spillover value if manually adjusting\)](#)
- [When you edit an acquisition matrix, FJ automatically duplicates, assigns to all samples, and then edits the duplicate](#)
- [Can remove a compensation matrix](#)
- [New user interface to manually adjust Transformations](#)
- [Can remove a transformation](#)
- Can copy transformation settings from file to file via Adjust transformation user interface

Find this helpful?

□ □

Bug fixes:

- Custom transformation re-reads data file to accommodate large negative values
- Transformation is better computed on datasets with large negative values
- Autcompensation scripts handle secondary match keywords properly (for multiple matrices per workspace)

Find this helpful?

□□

Table Editor:

- Copying Tables with formulas to Layouts allows creation of "static" tables with all values or dynamic tables with no formulas (static tables can later be changed to dynamic)

Find this helpful?

□□

Bug fixes:

- Formulas can concatenate multiple strings (via the "+" operator)
- Statistic "Freq of." a subset works when iterating by keyword
- Fixed significant figures definition
- Copying a created table to the layout editor works for large tables
- Statistics based on gates that do not exist are shown as "i_g^{1/2}" rather than zero

Find this helpful?

□□

Layout Editor:

- Option to show or hide color and line style annotations in legend
- [Legends are drawn with a header row that can be customized](#)
- The interface to edit the legend now has an "Update Layout" button
- Can create "Y by Y" view for any given graphic (via menu or contextual menu)
- Backgating history can show fully-gated overlay (a la DiVa)
- If you drop a node onto a graph to create an overlay:

- If there are multiple graphs selected in the Layout Editor, all become overlays
- If the graph is part of a group, then all graphs in the group become overlays

Find this helpful?

□□

Bug fixes:

- Modifying iteration options for layouts in templates does not erase the discriminator
- Batched movies are properly sized (and pictures are placed properly)
- Batch to web report functions correctly
- If black background color is chosen for graph, gate frequency text color is modified to show
- Fix batched statistics tables created when iterating by keyword value
- The flag for showing or hiding the annotation or legend item is properly set in the info dialog
- Printing does not improperly scale to accomodate items that are off-view to the left
- Grid lines in overlay legends are properly drawn
- Layout-specific preferences allow specification of gate width on graphs
- Batching graphs (to printer or preview) obey Layout-specific preferences (if any)
- Current layout-specific preferences are not replaced by preferences from last layout in a list while saving
- Creating a new layout makes default preferences and batch attributes rather than copying from current layout
- Fix: Text boxes in Grids sometimes became invisible
- Multi-item attribute interface: Making overlay styles consistent works even if they overlas are part of a group

Find this helpful?



Workspaces

- Create Series (editing keyword value) allows you to create strings (e.g., "Sample 01", "Sample 02", ...), and/or replace numerical values by specified strings
- Different color bars are used to annotate samples depending on which compensation matrix they use
- When concatenating multiple samples, if they do not share a compensation matrix then compensated parameters are exported (otherwise, uncompensated parameters are exported together with the matrix)
- Exporting data:
 - Event number can be exported to floating point FCS3.0 FCS files
 - Export \$PnE keyword for floating point DiVa files
 - Can specify a maximum number of events per file when exporting many files
- Get Info on Samples: Keywords longer than 132 characters are truncated for ease of viewing
- Computing a Boolean gate referring to a non-existent gate shows a message (the non-existent gate is considered to include all events)
- Can show the data file FCS version in the workspace (and, for other places, access via the keyword, FJ_FCS-VERSION)

Find this helpful?



Bug fixes:

- Prevent Find/Replace keyword value from looping if replace string contains find string
- Allow specifying to export 1M events
- Fix cache operations supporting netowrked (remote) file transfer (i.e., permissions etc)
- When you "Create Multiple Groups" from staining panels in the Group definition, the "Live" attribute is obeyed
- Notifies with an error if trying to read a data file 2 GBytes in size

- Can now re-find data files that are moved even if their name is 32 characters long
- Deleting a group-owned gate and choosing 'Save with first sample' always works now

Find this helpful?



Gating:

- Can create FJML gates (each vertex can be a formula based on any sample statistics or other gate)
- [Can create gates based on event number](#)
- Can auto-create gates for indexed sorting data files (Supports DiVa keywords)

Find this helpful?



Bug fixes:

- Magnetic histogram gates work properly

Find this helpful?



Graph Window:

- Contextual menu to allow opening parent graph

Find this helpful?



Bug fixes:

- Gate polygon name and subset name are now always identical
- Copy CDF as text (data values) does not crash
- Polychromatic plots don't crash in some cases where sample was not yet read in
- Gating path is displayed in graph window properly

Find this helpful?



Preferences

- Define top end of log scale for 32-bit parameters

- Read only FCS2.0 portion of data files that have both FCS2.0 and 3.0 data
- [Define keyword values to auto-add to legends created in the layout editor](#)
- Backgating displays shown as fully-gated (a la DiVa) vs. true backgating
- Force authentication (username/password) when transferring remote data by HTTP protocols.

Find this helpful?

□ □

Bug fixes:

- Cache location can be successfully modified
- Backgating preferences can be properly set via Preferences user interface

Find this helpful?

□ □

Backgating platform

- Can optionally display full gated overlay (a la DiVa)

Find this helpful?

□ □

Bug fixes:

- Saving settings as preferences are applied to Global (not workspace) preferences

Find this helpful?

□ □

Multigraph overlay platform

- If you select all fluorescence channels, only the compensated channel will be shown

Find this helpful?

□ □

Derive parameter platform

- When creating linear parameters, the lower bound is explicitly set to zero (not changeable)

Find this helpful?

□ □

Cell Cycle platform

- Multiplier constraints on G2/G1 positions must be 0.25 and <4>

Find this helpful?

□ □

Cluster platform

- Histogram labels are the \$PnS rather than the full name to shorten

Find this helpful?

□ □

Bug fixes:

- Histograms are updated if the gating tree is modified
- "MinSepChannel" refers to the offset from the channel value corresponding to a scale value of "0."or the left-most channel in the display to prevent splitting autofluorescent populations spread by an inadequate biexponential transformation

Find this helpful?

□ □

Data files

- Support reading "BCI" files
- Support reading 32 bit integer data files containing log-transformed data
- Support a list of URL's as a "Summary File" import

Find this helpful?

□ □

Bug fixes:

- Properly scale values from 16-bit integer data files

February 24th 2009, Version 8.8.6 released

Features:

- "Fit all Windows on Screen" command (under "Windows" menu) repositions windows that are (partially or fully) off-screen

Bug fixes:

- Linear compensated parameters were shown with wrong axis scaling
- Canceling a file search when doing a Get Info on the sample does not lead to a dialog box that can't be dismissed
- Does not have a crash on certain corrupt layouts
- Transform all parameters does not lead to a bug when you are compensating 10-bit data files
- Polychromatic plots can now properly select compensated parameters
- Prevent crash if entering random (bad) numbers into the Print Settings dialog
- Fix storing and display of batch outputs in the SciBook

Changes:

- New workspaces are named as "YYYYMMDD Workspace" rather than "Untitled Workspace"

November 21st 2008, Version 8.8.4 released

Features:

- Contextual pop-up menu for groups (control-click or click-hold on a group node)
- "Duplicate without samples" command for groups. This is a menu item-- creates a new "shell" group that has all the gates and information from the previous group, but no samples (unless samples are auto-added). Similar to a template, but for groups.
- Sample Info Dialog (cmd-I on a sample): Keywords are sorted in a friendly way; a new popup menu lets you switch to different samples in the same dialog window
- New Preference to ignore \$PnG Keyword (under "General" tab). Can be workspace-specific. Note: this preference may apply only to samples added to the workspace afterwards

Bug fixes:

- Layout item information dialog disables the foreground color selector on overlays, since that didn't do anything; disables the "show all layers' gates" checkbox when it's not an overlay.
- Fix rare crash on reading remote (FTP) data files.
- Ancestry plots from Layout Editor are not clipped when copied and pasted into PowerPoint (before ungrouping).
- Site license access made more robust in case of server issues.

September 29th 2008, Version 8.8 released

Features:

- Sample validity platform has copy report
- Allows reading improperly-formatted CXP files (\$PnB = 10)
- F5 engages recalculation of workspace
- Moving existing quads now tracks percentage
- Spiderweb functionality: mold gates together, option-click common vertex
- Spiderweb quads: option-click quad gates to move vertices
- FlowJo Licensing Server allows 2 day-old files for servers that cache web sites
- Add vertex function (spider)

Bug fixes:

- Fixes crash on reading files when using general undo enabled
- Fixes 8.8 bug that quad gates converted to spider gates did not compute as such

- Fixes compensation picket fencing on FCS2.0 data
- Fixes disappearing dots
- Fixes clicking on a gate without moving it does not remove its group ownership
- Fixes Comp wizard, if fluorescent events off scale by scatter, fails to create gate and then crashes
- Edit overlay legend user interface- mapping Insert menu commands to proper insertion
- Preferences save text color settings
- Cell cycle remove debris for histograms on >1024 channels
- Dropping files onto a group node adds data to that group rather than current group
- Exporting file with specified parameters (bug introduced in 8.7.2)
- After Batch Layout create, could sometimes corrupt internal layout representation (LIR bug!!! - probably 10 years old!)
- Allow dragging gates onto comparison nodes to change control
- Deleting a derived parameter will not cause an exception when reopening graph that showed that DP
- Export non-FCS format (bug introduced in 8.7.2)
- Sometimes a magnetic gate would be drawn in its old position until update (always calculated right)
- Gate annotation position no longer changes when window is resized
- In setting sample sort order, if you check "Use as default" it will be set in the Preferences
- When doing Save As, mark original workspace as no longer open (wrt possible double open)
- Exporting derived parameter doesn't crash if not loaded (beta bug)
- Workspace preferences for 32 bit reading parameters are obeyed (rather than global)
- Hiding labels but not ticks -- properly exported bounds on graph

Changes:

- MUCH faster (SMP speed up for file reading and compensation)
- Relocate dialog stays up during browsing operation to see requested file
- Ctrl-drop node onto overlay reassigns sample ownership of all overlaid nodes if they come from a single sample
- Concatenate better determines if keyword value is linear or log distributed
- XML Fine tuning (SD stat, multiparameter compare parms, ws group sort order)
- Table statistics on subgroups: subgroup name is added to the statistic name in column 1
- Layout editor Overlay dot plots obey # of dots to draw
- Prefs "Use as default" from current workspace also copies sample sort criteria
- XML coding added
- Find file dialog shows much more information about lost file.

July 3rd 2008, Version 8.7.3 released

Bug fixes:

- Edit menu garbage text (undo/redo) is fixed.
- The workspace comment column text is properly added to Layout Editor Tables.
- The Group Editor dialog window doesn't show funky characters for empty parameter stain names.
- The Kinetics smoothing options are properly initialized in the kinetics window when re-opened.
- When reading DiVa 3.0 data files, the progress bar better reflects how much is left to read and compensate.
- Templates with layout editor iteration that depends on keyword iteration and discrimination now work fully.
- Templates no longer give the error that the file was not properly closed.
- Allow CellQuest data files with zero events to be added to a workspace.
- Group Edit Dialog window properly initializes the comparator menu item.
- In Layout Editor Tables that have a column for "Gate Name" -- for sample-level nodes, the sample name is shown instead of the text "Ungated."
- Make sure files made from concatenated gates of compensated data have correct number of events in FCS header.

Features:

- Keyword Edit/Replace operations allow searching for blank keywords (so you can replace blank keywords with any given replacement value)
- Layout Editor overlay graphs can optionally show gates for all overlaid layers (not just the top-most). There are new UI checkboxes in the overlay information dialog to set this.
- Layout Editor Tables can show the gate name with an "indented" option -- to make it look like the Workspace display.
- When creating a new Layout Editor Table, you can select (from the popup menu) an option to create a table that looks like the current Workspace (as much as possible); it automatically creates the appropriate columns.
- When you "concatenate" samples or gates, you can now choose to create new parameters in the resulting data file, the contents of which are equal to keyword values from each of the samples. (Use the option-key when selecting "concatenate" from the menu.) This let's you add identifier information (or other information) found in the keywords as a real parameter, on which you can gate, etc..
- Empty workspaces show "Drag Samples Here" text.
- When you drag a population onto a layout graph to create an overlay (or hold down cmd-key to reassign the graphic to that population), FlowJo will now replicate that operation to ALL layout graphs that are currently selected.
- Added Concatenate User Interface link.

Changes:

- The "Recalculate" (Immediate/When Sample Loaded/When Necessary) preference has been removed. All recalculations are now done "lazily" -- when needed. Use the "Recalculate" menu command (cmd=) to force a recalculation in the workspace display.

March 4th 2008, Version 8.7.1 released

- Fixed issue with "serial number corruption" and inability to save preferences on certain computers.
- Fixed edit menu corruption (for undo/redo).
- Fixed Group Edit dialog. It now remembers all comparator states ("does not contain" etc) properly.

Changed: CellQuest files with zero events are read into the workspace rather than ignored.

February 22nd 2008, Version 8.7 released

Known issues: Files with long names (>31 characters) will not be automatically re-found when moved to a new location.

Changes:

- You can now paste in an entire spreadsheet of keyword values into the **Workspace** for easy editing! ([Click here](#))
- You can now "Marquee" select (click and drag to select multiple items) in the Group analysis list.
- Mouse-wheel scrolling is enabled in the Group list.
- Horizontal mouse-wheel scrolling (shift-key while scrolling) is also enabled.
- FlowJo has a new **Preference** to annotate gates shown in graphs with generic FJML, allowing more flexibility on what to show (for example, statistics or annotations).
- Keyword lists show keywords in a more useful order (with user-defined keywords at the top); also, recently-selected keywords ("favorites") are shown first.
- Autocompensation Scripting Platform** has been updated: the user-interface has been improved, and features such as compensation gate validation have been added.
- Site License information (contact information etc) is available to Site Licensees in the **About FlowJo** window.
- Layouts** can be added and deleted immediately, without having to wait for the current one to finish drawing.
- Keyword Search & Replace: All instances of a search string within a single value are replaced at once.
- Keyword values are not subjected to "Significant Figures" preferences when output in Tables.
- With scientific notation set, all axis values are shown as such (not just exact log ticks.)

Bug fixes:

- Layout Editor Batch **Discriminator** and **Iterator** keyword lists are now in the same order.
- Editing a compensation matrix a second time does not falsely flag unchanged values as changed.
- You can now delete summary statistics in the dialog window with the delete key as you would expect.
- We now correctly interpret EXPO V2 non-standard files having erroneous keyword values for parameter bit lengths.
- Batch from layout direct to printer: output is properly paginated even without going through the "Custom..." dialog
- Kinetics no longer reports (incorrectly) that FJ-created time parameter is not monotonic.
- Platforms: when using arrow keys to move constraints, FlowJo no longer deselects the constraint.
- We now allow outputting of all ascii characters (like "i_{1/2}") in keyword values.
- When group gate are renamed, FlowJo prevents a "/" (slash) in the name.
- No error is generated if reading an exported data file that has compensated parameters, no uncompensated parameters, and a compensation matrix.
- Error messages are improved when there is an error reading layouts or if there are too many events on the axis.
- Robust CV calculations on biexponentially-scaled parameters are optimized.
- FlowJo allows any length boolean gate specification (It was having problems after 256 characters).
- **Save As Template** does not show file as being "currently open" (a bug from 8.7b9).
- **Cell cycle**--remove debris gates initially are set to reasonable positions for all cases.
- **Cell cycle**--immediately recalculates fit if any constraint for CV is changed.
- **Layout Editor**--cannot select pseudocolor or polychromatic plot as graph types for overlays .

November 19th 2007, Version 8.6 released

- You can now paste in an entire spreadsheet of keyword values into the Workspace for easy editing!
- You can now "Marquee" select (click and drag to select multiple items) in the Group analysis list
- Mouse-wheel scrolling enabled in the Group list
- Horizontal mouse-wheel scrolling (shift-key while scrolling) enabled
- New Preference to annotate gates shown in graphs with generic FJML, allowing more flexibility on what to show (for example, statistics or annotations)
- Keyword lists show keywords in a more useful order (with user-defined keywords at the top); also, recently-selected keywords ("favorites") are shown first
- Autocompensation Scripting Platform has been updated: the user-interface has been improved, and features such as compensation gate validation have been added
- Site License information (contact information etc) is available to Site Licensees in the "About FlowJo" window
- Layouts can be added and deleted immediately, without having to wait for the current one to finish drawing
- Keyword Search & Replace: All instances of a search string within a single value are replaced at once
- Keyword values are not subjected to "Significant Figures" preferences when output in Tables

Bug Fixes:

- Text boxes created for iterated layouts will have the correct sample ID
Changing Layout annotation item text contents immediately resizes the bounds to show the text
- Templates with gates on the "All Samples" group relying on compensated parameters will apply those gates when files are added
- Cluster platform no longer generates an error if launched on a gated population for a sample not yet read into memory
- Double-clicking on Kinetics analyses in the Group list does not give an error message
- Preference for Axis annotation (show parameter stain and/or name) is obeyed for Layouts and Workspaces
- Preferences for print headers and footers are obeyed for Layouts and Workspaces
- Create Groups and Show Graph for Table Editor works properly after Table has been sorted
- Keywords are sorted correctly in Keyword lists
Layout Editor Menu items are greyed out when Graph Windows are foremost

August 13th 2007, Version 8.5.3 released

- Open file dialogs are properly titled (e.g., "Open Workspace" or "Open Samples").
- The menu item "Create Group From Keyword Value" has been removed; a single user interface is now used for all keyword-related group creations.
- You can now drag samples from one workspace to another by dropping them on a group in the destination workspace; the samples are added directly to that group.
- For new workspaces, the Table and Layout Editor windows are by default placed just to the right of the workspace, rather than on the right edge of the screen.
- There is a new Preference for using generic FJML commands for all gate annotations in graph windows [NOTE: This requires more documentation].
- General undo preference off by default
- If general undo is slowing down too much, will warn user to turn preference off

Bug Fixes:

- Comparison Platform: The checkbox for "show difference" is properly initialized.
- SciBook: Exporting a Layout Batch View does not crash.
- Polyvariate Graphs: You can no longer select "Histogram" or "CDF" from the contextual menu or main menu.
- Table Summary Statistics: You can now immediately access the most recently-created table column; changing group names is performed correctly.
- Derived Parameters: Parameters are no longer randomly renamed on occasion.
- The Preference for "Show All Keywords in Lists" is obeyed for all keyword selection dialogs.
- The Preference for "Default number of columns" in Batch Layout creation is used correctly.
- Opening old workspace could cause undo to crash, until file was saved

June 20th 2007, Version 8.5.2 released

- Fixed crash when creating statistics table object in layout editor
- Fixed occasional debugging error message on certain operations that were OK (like dragging gates to lots of samples)
- Truncated (bad) FCS files now generate an error message rather than a crash
- Fixed crash when reading a kinetics file where the last event's time parameter value is out of range
- Fixed sorting of file names in workspace (when they had numeric components only)
- Fixed the scaling factor for linear DiVa data file that are accessed via FTP

June 4th 2007, Version 8.5 released

Note: Workspaces saved by 8.5 will not be read by 8.4. However, you can save a workspace as XML and open it in 8.4 (you will lose information regarding new 8.5 features, but everything else should be fine).

- Preference Sets: You can now store the current preferences as a "Set" (via the Preferences dialog). You can save as many different sets as you want. Later, you can open the Preferences (for the Application, Workspace, or Layout), and copy from one of the sets to engage those preferences. That way you can keep different settings for different environments. The FlowJo menu has a menu item to directly switch to a given set of Preferences (for the Application). (One application: "Style sheets" for layouts: you could designate the default fonts, graph styles, headers & footers and such for different layouts to be specific Preference sets. Then, when you create a new layout, you can copy the Preference set for the desired view for that Layout).
- Table Summary Statistics: You can now add any number of summary statistics to Tables, including statistics computed on a subset of the rows in a table. Statistics can include Mean, Median, CV, Minimum, Maximum, Range, IQR, and even T-Tests and Wilcoxon Rank tests on different subsets of data within each column. To define or change these statistics, click on the "Sigma" (Statistics) button in the Table Editor (each table can have different summary statistics).

- **Statistics:** Statistics computed on distributions (e.g., median fluorescence, mean, CV, etc) are now considered "invalid" if there are insufficient events on which to compute the statistic. By default, the minimum number of events is 1 for most statistics (or 2, for CV and SD). In addition, the statistic "Frequency of parent" (and similar) are considered invalid if there are no events in the parent subset.
- There is a new preference, under "General", where you can increase the minimum number of events on which to calculate these statistics. "Invalid" statistics are represented by a bullet ("•") in all output places. "Invalid" values are ignored by the Table Editor when computing summary statistics or formulas (a Table formula that has an invalid statistic as one of its inputs will generate an invalid output value).
- **Compensation Wizard:** Performance enhancements; better identification of positive controls.
- **Minor additions:**
 - Workspaces store a history of all versions of FlowJo that they were saved with (accessible when "Save As XML" is chosen).
 - Pseudocolor graphs obey dot size (2, 4, or 8 only) preference
 - FlowJo's file reading has been optimized to take advantage of multiprocessor machines for faster data loading
- **A number of bug fixes:**
 - Compensating DiVa data that has linear parameters now properly stores and re-reads the information when the workspace is saved.
 - A situation where layouts could be saved in a corrupted fashion has been fixed; workspaces that have this issue will now be properly read.
 - Accommodates new Coulter-generated files
 - Layout Graphics now obey Layout-specific preferences
 - Workspaces can only be opened once simultaneously
 - Statistics tables in Layout Editor obey significant figures preference
 - Allow data files with more than 128 parameters
 - Fix reading \$COMP keyword compensation matrices (e.g., Coulter data files)
 - Properly initialize Preferences on new computers
 - Fix reading FlowJo Collector data files
 - Fix: Show scientific notation preference was being re-set everytime the Preferences was launched

April 27th 2007, Version 8.4.6 released

- Fix: Behavior where files/gates would disappear from the workspace.

April 13th 2007, Version 8.4.5 released

- Fix: Bug could lead to crash while reading some workspaces.

April 12th 2007, Version 8.4.4 released

- Fixed an occasional crash on the Compensation Wizard
- Fixed a bug where (on Intel Macs) the Proliferation Platform would not compute a model for some fits
- Fixed a bug where keyword values from BioTrue-generated workspaces might have an extra character at the end

March 14th 2007, Version 8.4 released

- Table columns can now have custom significant figures assigned (in the formatting dialog for table columns)
- Compensation Wizard is significantly improved:

- Graphical display uses smaller graphs if there are a lot of compensation parameters
- Much better (if not perfect!) identification of unstained samples
- Refers to unstained as "Unstained" rather than "Universal Negative"
- You may have multiple unstained controls (for example, cells, different kinds of beads); the platform will automatically select the appropriate control based on the Forward Scatter matching
- You can choose to have the Wizard add "Clean-up" gates, which will add tight gates around the fluorescence distribution of the positive controls
- You can choose to have the Wizard add scatter gates to identify lymphocytes or singlet bead populations
- There is a menu that controls what happens when you create the matrix: by default, the matrix will automatically be applied to all samples and the Wizard finishes
- Concatenating files does not export the compensated parameters (re-compensate after concatenating)
- You can now choose to show the full data file path on the disk, as a column in the Workspace window
- A number of bug fixes:
 - Derived parameters (and Calibrated parameters) created on Intel Macs are properly saved in workspaces
 - The Calibration platform manual definition will draw only one axis scaling
 - Grouping in the Layout Editor preserves front-to-back layering
 - Intel Macs can now update purple (version 6) dongles
 - Derived parameters dialog properly enables log and linear parameter menus
 - General Undo crashing repaired
 - Table Editor summary statistics shown with desired number of significant figures
 - Table Editor formulas that referred to specific rows of a column now work
 - Boolean gates allow parentheses in the gate specification
 - Showing gate coordinates for Boolean gates in the workspace shows the full specification
 - Group gate synchronizing was fixed to handle a case where multiple gates, derived from different groups, were modified; the entire gating tree was incorrectly synchronized to one of the groups

January 9th 2007, Version 8.3 released

- Fixed: Unknown corruption leading to bad keyword values causing an error building tables with those keywords.
- Saving incrementing file name versions will tag the number before the filetype ("abc.001.jo"); will only keep most recent in "recent items" menu;
- Save as will not create extra blank file.
- Auto dialog answers will generally not store 'Cancel' answers to prevent confusion.
- Tweaked Compensation wizard auto-assignments.
- Exporting histograms includes a third column as scale fluorescence (bin / fluor / count.)
- Fixed: Edit comp matrices on intel macs wouldn't allow non-integer values.
- Long gate names are abbreviated in graph windows (abc/ad...def/fjd/).
- Fixed: Annotation items automatically resize to accommodate changing info!
- Text boxes can now be set (default) to resize automatically to text. If you resize via drag, then the flag is set off. Old textboxes do not acquire this flag.
- Fixed: reading packed data files (e.g., 9 bit files.)
- Modified: storing keychain information for servers with multiple names will store under original & modified host name.
- Preferences: changed how they are stored. Workspaces store data transform prefs with WS; not affected by global prefs.

October 13th 2006, Version 8.2 released

Known issues: Files with long names (>31 characters) will not be automatically re-found when moved to a new location.

- Linear axis values are abbreviated with "K" (like 100K) to make them shorter
- When overlaying more than 12 items in a Layout Editor graphic, the colors continue to rotate (rather than being only red)

- Cumulative records for FlowJo analysis times (under "About FlowJo") are properly incremented
- There is a new Platform, "Sample Validity Testing." This platform allows you to determine if the events collected during acquisition are uniform (i.e., unchanging over time). You can identify files which have clogs, discontinuities, or other problems. The platform can be automatically invoked for every data file through a Preference; the settings which fine tune how FlowJo identifies potential problems are set in the Platform window.
- Boolean gates that do not contain "or" operators (for example, as created by the "Create Combination" platform) now compute 30 times faster!
- In the Table Editor, there are now contextual menus (click and hold, or control-click, on table column definitions) which allow you to modify a row: move it to the top or bottom of the list; delete it; edit it; or change it to a different kind of definition.
- FlowJo will now rename duplicate parameter names (in Dako files); duplicate names led to a lot of different problems. For older workspaces that refer to such files, open them in this version, and just save them. Any error messages will now go away.
- Transformations defined on DiVa-generated compensation matrices will be applied to all files with "similar" compensation matrices. DiVa generates compensation matrices that have trivial differences that caused FlowJo to think they were actually different.
- If you copy a table with formulas to the Layout Editor, FlowJo now informs you that formulas are not supported in Layout Editor Tables.
- Linear axis values are abbreviated with "K" (like 100K) to make them shorter

Bug Fixes:

- Several stability issues were addressed.
- Proliferation statistics will now appear in Tables even if the Proliferation model has not yet computed.
- If the files were loaded in the Compensation Wizard which did not have fluorescence parameters found in other files, then the Wizard could crash. Now only parameters that are common to all files are selectable for generating compensation matrices.
- Biexponential transformation parameters are correctly initialized in preferences.
- Correctly reads workspace files that were modified on an Intel Macintosh using an early 8.0 version of FlowJo, but caused a crash when reading.
- Selecting "Save" menu when "SciBook" is open will save SciBook entry to disk; changing a SciBook comment will mark the Workspace as having been changed so it can be Saved immediately.
- When overlaying more than 12 items in a Layout Editor graphic, the colors continue to rotate (rather than being only red)
- Cumulative records for FlowJo analysis times (under "About FlowJo") are properly incremented

A number of bugs that only occurred on Intel Macintosh computers were fixed:

1. Correctly reads workspace files modified on an intel Mac using an early 8.0 version of FlowJo that caused crashes while reading
2. Does not crash when double-clicking on layout graphs that have long gating path names
3. Correctly copies table outputs to layouts
4. Properly saves statistics tables created in layouts
5. Drop-down menus in Compensation wizard work
6. Polyvariate gates are saved such that they can be read on non-Intel macintoshes
7. Linear parameter scaling does not change with each workspace save for remote-access datafiles

July 5th 2006, Version 8.1.1 released

Known issues: Files with long names (>31 characters) will not be automatically re-found when moved to a new location. Copying layouts between workspaces crashes

- Fixed: Quadrant gate statistics in tables and layouts
- Fixed: Copying Graphs from Layouts with history gating are properly cropped when pasted into other applications

- Table Editor Formula entry window is now resizable
- Derived Parameters: New "Edit" button allows you to edit a previously-defined algebraic formula.

May 17th 2006, Version 8.1.0 released

- Prevent crash in Multi-Sample Distancing Platform dialog if no sample files are available for comparison.
- Changed name of dialog box from "Layout Item Definition" to "Graph Definition", to be consistent with the Windows version.
- A "workspace" created from the BioTrue server contains translations of the original FCS file headers. If the original FCS header file contained a keyword with a blank value, the BioTrue translation of that header would confuse FlowJo, causing it to incorrectly read the header information after that value. If multiple Samples were included, this would cause some samples to have incorrect names. This condition has been corrected. FlowJo will now read the translated header correctly.
- The FlowJo Workspace window created from a BioTrue ".jo" file, is now given the name of the file instead of being an unnamed workspace.
- Added preference to enable SFTP file transfer.
- Added dialog box to ask for SFTP User name to initiate file download from BioTrue SFTP server.
- Added dialog box to ask for user, workspace, and destination information to initiate SFTP file transfer when saving a Workspace to a remote SFTP server.
- In the About Box, changed "Serial: " to be "Serial: Dongle" when authentication is via a dongle.
- Now works with V8 "purple" dongles on an Intel Mac.
- Changed the Graph Window so that the text, "Warning! More than X% of events on the axis!", appears above the graph pane, rather than touching it.

May 4th 2006, Version 8.0.3 released

- Fixed crashing bug when calculating the Kinetics Platform.
- Fixed crashing bug while attempting to quit with the Kinetics window still open.

April 28th 2006, Version 8.0.2 released

- Fixed: Cell cycle statistics overlay: axis label does not overlap with axis ticks.
- AutoCompensation scripts now generate exactly correct matrices when universal negative is used. Previously, off by a small amount for low fluorescence controls.
- MultiDimCompare: Does not crash when computing multiple chi square.
- Batch creation (to preview): Only warns on too-large layout size if ... too large!
- FlowJo properly sets picture boundaries for graphics copied from layout editor to show all axes without needing to ungroup.
- Workspace files with Derived Parameters will display correctly on an Intel-based Mac.
- Renaming a gate at the group level is properly handled so that all references to the samples' gate are also updated.
- Fixed several instances where old (PowerPC) workspace files might not open properly on Intel Macs.
- Fixed dongle-upgrade URL to paste the old dongle ID into the upgrade web page.
- Added check box to select SFTP for file download and upload. Currently disabled.
- Fixed a problem on Intel-Macs where some older workspace files would not load properly, resulting in the dreaded "You've uncovered a defect in the program" assertion-failure dialog box.
- Fixed a few more instances of handle-based I/O not being "swapped" properly on an Intel-Mac.
- Fixed problems on Intel-Macs where older workspace files would "lose track" of sample files in some instances. That is, if you load an older workspace and double-click on a sample, a Graph Window will display the expected graph, rather than asking the user to find the FCS file.

April 13th 2006, Version 8.0.1 released

- Fixed problem with LE text annotation edit dialog, which caused all menu items to be disabled, and prevented the program from responding to further command events. Changed PPob resource #4319.
- Removed second, unused "Workspace" "Rename..." menu item, #2023.

- Removed "Extended" attribute from menu items:
"Edit" "Select equivalent nodes" (#2032)
"Workspace" "Add Samples into Group..." ('AddG')
- Changed "ValidThumbDrive" to set "gDongleAttached" so that the "FlowJo" "Serial Number" menu item is disabled. This now disables the "Serial Number..." menu item when a valid thumb drive is found.
- Fixed uninitialized variable in "MultiDimCompare::MultiChiSquare", causing a crash on an Intel Box when the first sample in a Multi-Sample Compare is not selected.
- Fixed printing problem on Intel-Macs, where the Y-axis labels were rotated by other than 90 degrees.
C
- Changed FlowJo.rsrc "Miscellaneous Strings" (resource #2100), string #240 to read:
FlowJo does not support a HASP key (purple dongle) on an Intel-based Mac. Please click on "About Dongles" to view our trouble-shooting web page.
- Added version resource "8.0.0" to the SNConverter tool, so we can tell when it has been updated.
- Fixed problem in "FTableView", where the "fNodeForCell" was not being completely initialized, nor was it being indexed incorrectly. When trying to create a new Group from a list of cells in a table, via "FTableView::CreateGroup", function "FTableView::GetPopulationForCell" would loop forever looking for the last cell.
- Fixed problem where an exception would occur on an Intel-Mac when reading workspaces with Calibration tables.
- Fixed problem that would prevent FlowJo from recognizing a black dongle if another USB device with a serial number was also attached.

April 3rd 2006, Version 8.0 released

SUMMARY OF CHANGES SINCE 6.3.4

Does not support OS9.x. Minimum version is OSX.2.

Minimum workspace readable is version 20 (2.7 or beyond)...

For old Workspace, read using version 6.x and save

Need new serial number or dongle

Now built as a fat binary (runs on Intel mac and PPC Mac in native mode)

Major:

- New Table Editor: much faster, new formatting options, computed columns, iteration...
- Drag Tables and Layouts from WS to WS
- Can now define Page size (Page Setup)--saved via preferences
- Different scale, orientation for each layout, otherwise global

General:

- More performance enhancements (particularly, batch >generation)
- New Table Menu (Layout/Table menus changed)
- Keyword selection dialogs now show keyword values for any selected sample

Groups:

- Stain name mismatching is no longer considered (always allowed) [old groups--obeyed]

Compensation/Transformation:

- Menu item changed to "Define BiExponential Plot"
- Define Transformation dialog has disclosure triangle for "advanced" options
- Changing factory settings for advanced options is saved in preferences

New Graph Option:

- PolyVariate Plot: Gateable, Displayable "PolyVariate Plot"

Preferences:

- New: Obey PnDisplay keyword (DiVa only currently, FCS3.0 suggested)
- New: Size of large dots in low resolution dot plots (Great for publication)
- Removed: Stain Name mismatching in LE (always allowed)

Table Editor:

- UI has changed
- Drag Tables from WS to WS
- Building big tables is hugely faster
- Specify special formatting for any given column (including hiding it)
- Iteration like Layout Editor
- Double click table name to edit iteration attributes
- Double click column to edit formatting
- Table Editor Formulas! (NOTE: Formulas are NOT copied to Layout Editor when table is copied there)

Layout Editor:

- Drag Layouts from WS to WS
- Double-clicking layout does a get info
- Can set iteration group from get info dialog
- Can Show gate coordinates & offset vector in Layout Tables
- Cmd-E (Graph->Transpose Axes) now works for graph items in layout editor

Workspace:

- Create Group From Keyword Value--can always be chosen in menu now; can choose keyword to select from
- Show gate coordinates & offset vector in Workspace
- Sort dialog pre-loads existing sort order
- Statistics node indentation increased
- Can now rename group-based gates

Kinetics:

- Allow more than 16 time slices to have colors

Bug Fixes:

- Transformations computed on hardware-compensated data only apply to other files with same matrix
- Kinetics: Can delete multiple time slices simultaneously
- Workspace: Node icons are drawn to left of name column even if too narrow
- SciBook: Editing text entries is saved!
- XML: Save graph options re hide Ticks/Numbers/Labels in axis specification
- Groups: Membership changes affected by keyword editing happens immediately
- Group editing: disable "More choices" button when 4 sets of criteria already shown
- Fix: reading color palette preferences for layout overlays
- Fix: Deleting first level of LE overlay left "selection" above; pressing delete would cause crash
- Intermittent Site License validation problems have been corrected.
- Allowed FJ to run with a v6 or v4 Site (FLS) License.
- Fix: Badly-formatted compensation matrix in data file does not cause crash
- Fix: FlowJo doesn't crash when a workspace is closed with an open Batch Movie output
- Boolean Gates: if creating a large number of gates (which can take a long time), FlowJo provides feedback on the progress
- Statistics Window shows larger fonts and doesn't allow "Add to all compensated parameters" if no compensated parameters exist
- Intermittent Site License validation problems have been corrected.

Known issues:

Files with long names (>31 characters) will not be automatically re-found when moved to a new location.

April 13rd 2006, Version 6.4.7 released

- Fixed problem that would prevent FlowJo from recognizing a black dongle if another USB device with a serial number was also attached.
-

November 16th 2005, Version 6.4 released

SUMMARY OF CHANGES SINCE 6.3.4

Bug fixes:

- No more hanging when the Tree Star web site is impossible to reach
- Can create derived parameters on Cyan-acquired data
- Workspace statistics don't disappear when a recalculate is performed
- Read all Coulter data files
- Cell cycle: constraining peaks to gate positions is now correctly saved
- Cell cycle: option to use pattern fill for graphs is correctly saved
- Annotation items in layouts are properly filled for batch outputs
- "FS LIN" and "SS LIN" parameters are now recognized as scatter parameters
- Improved performance and stability

September 30th 2005, Version 6.3.4 released

SUMMARY OF CHANGES SINCE 6.2.1

Important Notes:

Note: workspaces created with this version cannot be opened with older versions.

New Features.

New in 6.3.4 (cumulative changes since 6.2.1):

- Significant performance improvement noticeable when compiling large layouts or tables-particularly when hundreds or thousands of samples are involved!
- Fix text issues in dialogs on some computers running OS9.2.2.
- Summit 4.x files with incorrect data location specification keywords are now read in properly.
- The compensation definition window can now be resized to allow for long parameter names.
- Fixed: Memory was slowly being used up by doing large numbers of text boxes in the Layout Editor
- Fixed: Tables with negative numbers in the statistics will now show means & standard deviations.
- A number of optimizations and bug fixes were done to prevent crashes that occur during large computational demands; stability should be much better.

General Operation:

- Site licensees are validated more efficiently Preferences:
- There is a new preference to define the default sort order for samples in the workspace Workspaces:
- Workspaces are now read in and saved much more efficiently. Note: the first time you read in an older workspace, it may be much slower; as soon as you save in the new format, it will be considerably faster!
- Workspace sort criteria are now "persistent": if you add new files (or for templates), the files will be automatically sorted according to the last selected criteria. In addition, when you define the sort criteria, you can make them the default for all new workspaces.
- Workspace sort criteria can be individually set to be increasing or decreasing values.
- Unreferenced samples are now removed from workspaces (making templates much smaller).
- XML format has been modified (more similar to PC version). This format is still being updated and is not guaranteed to be consistent with future revisions of FlowJo.

Tables:

- Tables with keyword values that have or display properly
- You can copy a series of custom column heading names (from Excel or Word, separated by returns) into any column heading name – the entire series is copied to the subsequent table columns (hit return or tab after pasting). In this way, you can edit the column names in a different program and then copy them in all at once.
- The mean and std. dev. are filled in only for valid calculations (e.g., they are left blank for text columns)

Bug fixes:

- reading newest Coulter data files
- Cell cycle models that constrain to a region--constraint is saved after closing & reopening
- Cell cycle platform option to display pattern filled models is properly saved
- Annotation items for Layout Graphs are properly built in batch reports "FS LIN" and "SS LIN" are recognized as forward and side scatter parameters
- Fixed bug that could cause crash during gate computation while other tasks were happening-improved stability!
- When directing a batch output directly to the Printer, the page information is properly initialized without having to go through the "Custom" settings button.
- Fix: Preferences correctly reads layout color palette for overlay colors
- Fix: Bug that could cause rare occasional crashes when reading files
- Fix: When choosing to sort the workspace, the current sort criteria are displayed
- Templates that refer to compensated parameters now add all gates & statistics
- Renaming a gate will no longer cause a Layout (referring to that gate) to crash
- Opening graph windows no longer occasionally reset the Layout iteration value
- Cycling graphs (next/previous) no longer cause a rare crash that happens when gate frequencies were shown
- Modifying a series of gates that are all group-synchronized no longer causes a rare crash
- \$SPILLOVER keyword values are properly supported
- Allow dragging gates from one workspace to another if the destination sample is selected
- Crash no longer occurs if changing a graph while an outlier plot is computed
- Selecting a graph from a sample not in the current group does not make that sample temporarily appear in the workspace window
- Double clicking multiple samples in the control definition list for an overlay graphic now sets all selected samples as controls
- Grid lines are displayed in histogram overlays
- Histogram overlays will not occasionally display parameter names for the Y axis label
- Boolean gate specifications are no longer case-sensitive
- The Layout batch Preview will not crash if the display is too large to show on-screen
- The line color for boxes drawn around text is correct in batched layouts
- Summit data files with badly-formatted \$SPILL keyword (Summit version 4.1) are now read in (an error message is shown) but no compensation parameters are added. These files can be compensated the usual way in FlowJo, using appropriate compensation controls.
- FlowJo does not crash if you try to delete the only layer listed in the Legend Item for a Layout Graphic.
- Batch layout generation: if Iterate by Nth ($n > 1$), the resulting layout's discriminator is properly set.

Known issues:

- Files with long names (>31 characters) will not be automatically re-found when moved to a new location.

March 4th 2005, Version 6.2.1 released

SUMMARY OF CHANGES SINCE 6.1.1

Important Notes:

The format of the workspace file has changed. Workspaces saved in 6.2b3 and beyond cannot be read by older

versions of FlowJo .

New Features

Graphs and Gating:

- There is a new preference to control the default naming scheme for new Quadrant gates (you can choose to have only "Q#", where the number increments for each new quadrant set; you can choose to add the stain names; and, as before, you can choose whether or not to ask for a prefix to the gate name).
- The Backgating analysis now shows a legend of gates applied for each graphic, to better illustrate what is being shown. In addition, the last graphic in the window is no longer a backgating analysis (as there is no useful information for that level), but rather shows a simple overlay of the gated population.
- There is a new gating tool to create two gates that bisect a histogram; while tracking with the mouse with this tool active, the graph window will show live statistics (like the quadrant tool).
- A new menu item, "Show defining gate", allows you to quickly access the graph that defined any selected subset.
- Contour plots can no longer be drawn as unsmoothed.
- Log contour plots do not allow you to select "show outliers", which was meaningless for this display.
- Kinetics analyses now show the threshold value used for calculations in the kinetics window.
- Grid lines for linear parameters are now drawn to match major axis tick marks.

Data compensation and transformations:

- Corrects Summit Compensation matrices (for all versions of Summit).
- FlowJo now supports the FCS3.1 keyword "\$SPILLOVER" as specified in the proposal.
- The compensation matrix name (if FlowJo was used to create the compensation) is now stored in a keyword ("FJ_CompMatrixName"),
- The compensation definition window has new buttons to calculate and close; to clear all positive & negative gates from the boxes; and has better selection of gates in the boxes.

General Operation:

- FlowJo keeps statistics of how many different kinds of operations you perform (number of files analyzed, gates created, events read): Choose "About FlowJo", and click on the disclosure triangle near the bottom of the window.
- All "Confirmation" dialog windows have button names that better explain what the two choices are (instead of just "Yes" or "No").

Layouts:

- When editing the attributes of platform graphics in the Layout Editor, you can now set additional options such as the colors and size of the graph.
- Scrolling large layouts is much quicker.
- You can now change fonts for individual layout items.
- For overlay legends, the cursor will change when targeting the color or line style controls to aid in identifying where to click.
- Generation of Batch Layouts was made more efficient (faster).

Tables:

- Tables can be saved as XML (for import into LIMS systems, for example). Choose "Save As XML" while viewing a completed table.

Preferences:

- Preferences reorganized (a 'Platforms' preferences panel added)
- The preference for copying the FCS Comment keyword into the workspace annotation was removed
- A new button clears all memorized auto-answers for confirmation dialog windows

New preferences to define:

- 1. Default statistics that are added to layout editor when Cell Cycle platform is dragged in (and the 'auto-create statistic table for platforms' preference is enabled).
- 2. Default statistics that are added to layout editor when Proliferation platform is dragged in (and the 'auto-create statistic table for platforms' preference is enabled).
- 3. Default columns to be created whenever a new overlay legend is created in the layout editor.
- 4. Default colors and graph types for Backgating analysis (these can be set either through the Preferences dialog, or by selecting the checkbox before closing a Backgating window).
- 5. The default scheme by which to name new Quadrant gates (you can choose any of 'Q#', where the # increments for all new quads for a subset; the stain names (e.g., CD3+CD4-); a prefix that is asked for when the quads are created).

Bug fixes.

- Correct sorting of file names that have the format 'name.#.#' (like abc.040602.001)
- Line color of histogram was sometimes forgotten when the graphic info dialog was opened in Layout Editor
- Distribute horizontally in Layout Editor works even if items are too close together
- Selecting 'About FlowJo' no longer puts application in Demonstration mode
- Layout items now correctly show cell counts after batching
- Resizing Proliferation window properly draws the model histograms
- Copy table from Multisample compare platform fixed
- Inserting keywords for specific samples in Layout Editor Text chooses correct sample (was one off)
- Occasionally, 10.e-4 would be displayed as 10.e-3 for frequency in Workspace
- Correctly displays time parameter for BD files using the 'TimeTicks' keyword
- Copying from layouts no longer clips the right or top edge of certain graphs
- Fix Copy/Paste of a single textbox in the Layout Editor creates a new text box, not a picture of a text box
- Group-synchronized gates don't crash if more than 1 is simultaneously changed (like quadrants)
- Layout text boxes with a line width of 'none' will not show the box line in printouts
- Fixed obscure bug where rarely a Layout textbox could lose the last statistic in its text
- Layouts with statistics tables won't crash during batching
- Open Graph Sets will properly tile windows (and resize if necessary) on the screen
- Holding down 'option' key while selecting a sample in the 'go to sample' menu button on graph windows will advance all open graph windows to that sample
- Fixed a memory allocation bug that could cause apparently random crashes
- Reading old layouts with legend overlays will now properly initialize the legends instead of leaving them blank
- Changing the threshold value for a Kinetics analysis forces an immediate recalculation
- Thick lines in the layout editor are better handled (selection and arrows)
- Avoid page breaks in the Layout Editor will not cause a graph and its annotation or legend to be put on different pages
- Create group window: Removing a criterion clears it; complex formulas doing comparisons on string values now work
- Clicking escape while drawing a gate now cancels the gate creation
- Selection of gate constraints in Cell Cycle platform was corrected
- Histogram gate lines in layout editor (and exported graphs) don't extend beyond edge of graph
- Adding a statistic to a group based on a parameter that has a ')' in its name no longer crashes
- Putting commas in custom table column names no longer alters the formatting of table headings
- Dragging a PICT file into the Layout Editor now refreshes the view immediately
- Tables dragged into the Layout Editor now automatically refresh statistics when gates are moved

- Double-click timing to open graphs from the workspace window restored to proper functioning
- Changing a gate does not reset the Layout Editor iteration to 'off'
- Only the 17th parameter in a file could not be compensated: now it can!
- Scrolling while entering table editor custom headings improved
- Fixed: Changing a gate does not reset the Layout Editor iteration to 'off'
- Fixed: You can again edit custom column headings in Layout Editor statistic Tables
- Fixed: Scrolling of window while editing custom column headings in Table Editor
- Fixed: You can now compensate the 17th parameter in a data file
- Fixed bugs that crept up in beta versions:
- Contextual menu for gates restored
- Group-based synchronizing multiple gates (like quads) fixed

September 10th 2004, Version 6.1 released

SUMMARY OF CHANGES SINCE 6.0

Important Notes:

Workspaces saved in FlowJo v6.1 cannot be directly opened with earlier versions of FlowJo. However, you can save a workspace as XML (File->Save As XML...); that file can then be opened in FlowJo v6.0. Please see notes on XML under "Workspace" below.

New Features:

General

- **Version Locking.** You can now "lock" a version of FlowJo on a given computer (which will apply to all users on that computer). This lock ensures that no other version of FlowJo will be used for data analysis on that computer. This feature is useful for laboratories who have validated a given version of FlowJo and want to ensure that no analysis is done by a different version. To "Lock" a given version, choose the Lock command under the file menu, enter an Administrator username and password, and then follow the instructions. Locking can be performed in a password-protected fashion (if desired). *NOTE:* "Locking" a FlowJo will not prevent versions 6.0 (or 4.x) from launching, because they cannot check for this feature. However, these older versions of FlowJo cannot read workspaces saved by this (or future) versions of FlowJo, so there is no risk of analysis by the wrong version. *NOTE:* Because the "lock" feature will apply to all users on that computer, you must have Administrator privileges. When you choose "Lock", you will be asked by the Macintosh operating system to authenticate a Username with Administration privileges.

Workspace

- **Complex Group Definitions.** Sample Groups can now be defined based on arbitrarily complex combinations of keyword information. In the Group Definition Dialog, you can convert current keyword restrictions to a formula, and continue to extend this formula to be as complex as you wish! Formulas can contain complex string manipulations, so you can search for substrings within keyword values, etc.
- **Opening sets of graphs.** Through the preferences, you can now define one or more "Graph Sets". These are predefined sets of graphs (with specific X and Y parameters defined for each). Any defined graph sets will appear in the contextual menu when you click on a sample or subset; choosing that Graph Set will cause one window for each defined parameter pair to be opened for that sample. This allows you to predefine combination of parameters to view simultaneously for each subset. (Use "option-Next" or "option-previous" to move all graph windows forward to the next or previous sample simultaneously).
- Copying **Boolean gates** will now notify you if the subsets on which they depend are not found in the destination. In addition, if you delete a subset on which a Boolean gate depends, you will also be notified that this is the case.
- FlowJo will now read badly-formatted legacy Cytomics FCS files.
- **XML Workspaces** : Versions 6.x give you the option to save as XML (and can read such workspaces as well). At this time, Tree Star does not guarantee that the XML format will be compatible with future

versions of FlowJo; we are in the process of evolving this format to be consistent with the PC version. If you have suggestions or questions regarding the FlowJo XML format and/or future plans, please contact us!

Statistics

- You can now calculate the **standard deviation** ("SD") on any parameter.
- When adding a statistic calculated on a parameter (e.g., mean, CV, SD), you can choose to add that statistic to all parameters, all fluorescence parameters, or all compensated parameters at once, by choosing those in the parameter menu of the statistics dialog.
- The order of the statistics in the Statistics Dialog window was changed to group them better.

Layouts

- **Printing:** For batched outputs (either direct to printer, or printing from the Preview output), you now have the option to use the original pagination as defined in the Layout Editor. This means that you can print batch reports where each panel crosses page boundaries—for example, if you spread the page boundaries such that each iteration of the batch is on 3 pages, you will get 3 pages times the number of samples in the group for your output. To choose this option, select "Use Layout's Pagination" where offered.
- Batched layouts are no longer grouped after being created.
- Keywords are better handled in layouts (particularly, for batched layouts and/or templates).
- When entering a keyword into a text box, you can select which sample for which to display the value (and single text boxes could have keywords from multiple different samples).
- You can now set the text style for axis labels differently from axis numbers (see Preferences); you can make the axis text as large as you wish and it will no longer be "clipped" (see Bug fixes).

Graphs

- There is a new contextual menu popup for when you click on a gate in a graph window that allows you to perform operations on the selected gate.
- The "Create Manual Gate" interface has been extended to allow you to create a series of gates that are adjacent (non-overlapping) across the entire range of the displayed parameters. The series can be specified to step by equal channel numbers, equal intensity levels, or equal percentile levels.

New Preferences

- Gates and Graphs preferences were split into separate panels.
- A preference can be specified for how FlowJo exports histograms as graphs. The default is to export as polygons, which is useful for changing their characteristics; this works well in programs like PowerPoint. Deneba's Canvas does not interpret Mac PICT polygons properly; thus, changing this preference will cause FlowJo to export histogram graphs as connected lines. This makes it show up properly in Canvas (and other programs), but the histograms cannot then be filled in those programs (exporting of filled histograms will still work from FlowJo in all cases).
- Graph Sets can be defined in order to simultaneously open multiple graphs; these sets can be chosen from the contextual menu when you click on a sample or gate in the workspace.
- The text style for layout editor graph axes labels can now be defined differently than that for the axes numbers—you can have large axes labels!
- The location of FlowJo's Cache folder for transferring data from remote servers can now be specified to be either in the User's directory (default) or in the local shared directory. The latter is preferable for computers where User directories are kept on remote computers.
- The Preference for Hiding or Moving "clipped" axes numbers has been removed (see bug fixes below).

Miscellaneous

- You can now zoom in and out of graphics in the SciBook.
- Workspaces saved with many SciBook entries now read in faster.

- A new option for Groups can be set ("Allow stain mismatch") that forces the group to add analyses and gates even if the stain names for the parameters are different than for the original sample's analyses.
- FlowJo now corrects the improperly formatted compensation matrix in Summit v4.0 data files.
- When files are moved and need to be found by FlowJo, you now have the option to ask FlowJo to perform an exhaustive search of all mounted disks for the data file (this may take several seconds).
- The text color for Print output headers and footers can be set in the Preferences.
- New quadrant gates will ignore the Preference for gate annotation position; the frequency will always be placed in the graph corners.
- The "Edit Annotation" menu item has been removed (annotations can be edited by simply double-clicking on them in the workspace).
- If you misidentify a file that has been moved, FlowJo will show you the name of the file before asking you again to locate it.
- Layouts will now show at least one additional page, horizontally or vertically, when scrolling, to allow you to better place new objects.
- A new Preferences lets you use the Bi-exponential/Logicle transformation on uncompensated height parameters ("-H") in addition to the area parameters. Click on the button: Preferences->Workspaces->Define (32 bit options).

Bug fixes.

Layout Editor

- Axes labels and numbers are no longer clipped in the Layout Editor or upon export to other programs. Finally! You can now have axes labels & numbers as large as you want!
- Layout Statistics Table tool doesn't crash if there are no samples in the workspace
- Overlaid histograms with auto-scaling have exactly correct Y axis values (previously, these "relative" numbers were off by 4%).
- Text boxes in the Layout Editor can have braces in them again "{}".
- Gate frequencies are not duplicated underneath the graph (after ungrouping an exported layout).
- Printing a specified range of pages from the Layout View now works (previously, would print all pages).

Workspace

- Double-clicking in the workspace sometimes didn't open a graph; this now works. (Although if you double-click too fast, it *may* not).
- You can now delete a keyword value in the workspace and leave it blank.
- The Time parameter is properly retained on saved and re-opened workspaces.
- Italicized text in the workspace and Table output is better displayed on-screen (not clipped).
- A crash could occur if graphs and statistics were selected in the workspace, and the contextual menu "Open graphs" was selected; this was fixed.
- Workspace mousing (click & drag, etc) is more responsive.
- Save As Template does not crash when the preference for incrementing workspace versions is selected.
- Occasional mis-sorting (or poor sorting) of samples fixed.
- No more unnecessary recalculations of subset gates that occurred in some circumstances (despite a Preferences setting of "Only recalculate when needed").
- Concatenating events from different 32-bit data files (e.g., DiVa data) is now handled properly.

Graphs & Gating

- Autogating will now place the resulting gate properly in windows that are not the standard size.
- Autogating tool is not active until the data has been loaded and displayed.
- Autogating will occur on the correct parameters after axes are changed.
- Boolean gates will immediately recalculate upon creation, if the Preferences are set for "immediate recalculate."

Boolean gates work more reliably when copied from sample to sample.

- FlowJo will not crash if you choose to create Boolean gates on a group of selected items including Statistics.
- Possible bug causing crashes during gate adjustment fixed?

Platforms

- Contextual menu for graphs & gates no longer appears for Platforms (causing a crash)
- Cell cycle and Proliferation platforms no longer crash if something happens to invalidate them during their computation (like a parent gate moving).
- *Cell Cycle and Proliferation: Copy table to text will always work now.*

Miscellaneous

- The "Application Lock" contact information and hint information are no longer switched for new Lock commands.
- After batching a layout, the graphics are properly reset to the "off" iteration state (previously, they were reset to show the first sample in the current group rather than their original state).
- Layout axes will now correctly adopt the color set by the Preferences.
- Layout overlay graphs will obey the specified background color (including transparency).
- Gate trees added by groups (i.e., when you add samples to a workspace) would not add an entire tree if any gate was based on parameters with stains that did not match the original; now only the mismatched gates are not added (unless the group option is to allow mismatches--see new feature above)
- Coulter ".HST" files are properly read and loaded.
- Cell cycle: The remove debris, remove doublets no longer crashes
- If a Boolean gate was opened without first opening the other gates, then the Boolean gate could show the wrong events.
- Fixed crash that could occur on very first use of autogate tool on first dotplot graph.
- Fixed: Opening graph sets places graph window below the menu bar.
- Fixed: Contextual menus in the workspace window show the platforms & Boolean gate options.

July 5th 2004, Version 6.0 released

SUMMARY OF CHANGES SINCE 4.6.1

Important Notes:

- Workspaces saved in version 6.0 cannot be opened with FlowJo v4.6

Major New Features ([click here](#) for more information)

Autogate tool . Point... click... gate. It shouldn't be any harder. And, with FlowJo's new Autogating tool, it can't get easier! FlowJo's autogating tool has an almost prescient knowledge of where you want the gate—an effect made possible by the instantaneous feedback of where the gate will be as you mouse over the graph! Choose the new autogating tool in the graph window, or simply hold down ctrl-option as you move the mouse over any bivariate graphic. (Note: if you hold down the mouse button as you click to create the gate, you can then track the mouse to inflate or deflate the gate to size it with even more precision!)

SciBook . The SciBook is a place where you can store pictures, text, tables, or custom annotations. The SciBook is stored with the workspace; you can use it as a permanent record of your analyses, as a temporary holding place for outputs, or just to keep notes for yourself. You can paste graphics, text, or tables from anywhere in FlowJo into the SciBook (the Graph window, Platforms windows, Layout Editor, Batch Layout View, and Table View all have buttons that will directly copy their contents into the SciBook). Contents in the SciBook do not change—therefore, if you change a gate, delete a sample, or make other modifications, the SciBook contents will not be modified. Once in the SciBook, you can copy into other programs, print, or simply just view. The SciBook gives

you an interface to add comments about each item that you insert, and records the date and time that the item was inserted.

The SciBook is meant to evolve; we look forward to your comments regarding which features you use the most, what features you would like to see in the future, and what you do (and don't) like about it!

[Backgating Analysis](#) . Validating gate positions is one of the most important aspects of multiparameter Flow Cytometry. FlowJo now provides you with a tool to view the effect of every gate on your final gated population! In any graph window for a subset that is gated more than once, you can choose “Backgating Analysis...” under the Graph menu (or, click on the Backgating button that appears near the top right of the window). FlowJo shows you a window where each level in the gating hierarchy is displayed, with the final gated population overlaid at each level. This overlay is a backgating overlay: in other words, it shows what the final gated population would look like without that level's gate! Therefore, you can quickly scan to see if any one of your gates is positioned incorrectly. The backgating display can be printed, copied, or saved in the SciBook (see below). Note that the Layout Editor can also show a version of the backgating analysis, by choosing “Show Ancestry” and selecting the “Backgating” option for a graph

[Boolean gates](#) . You can now create a gate which is a Boolean combination of any existing gates (i.e., "and", "or", and "not" combinations). Boolean gates are "live", meaning that they will reflect any changes to the gates on which they depend. FlowJo can also automatically create a series of Boolean gates that represent all combinations (plus and minus) of a set of gates. For example, if you have a sample which was stained on different parameters for IL2, TNF, and IFN γ , you can create a positive gate for each of the three cytokines (for example, a histogram gate). Then use FlowJo's Boolean Combination to create the 8 gates that represent all combinations of expression of the cytokines (e.g., IL2+TNF+IFN γ +, IL2+TNF-IFN γ -, etc.). To create or edit Boolean gates, choose the parent subset(s) and select the menu item "Boolean gates..." under the "Platforms" menu.

[Derived parameters](#) . You may now create a derived (computed) parameter that is any algebraic combination of existing parameters. FlowJo allows you to specify arbitrarily complex formulas, and includes a wide variety of mathematical functions that you can use to transform the data. This platform can be used in FRET calculations to create a parameter that reflects the efficiency of resonance energy transfer.

Layout Editor: [New Statistics Table](#). FlowJo now renders tables of statistics in a beautiful and highly customizable way in the Layout Editor. You can copy tables (either by dragging them from the Table Template Editor to use with Batch iteration, or directly from a completed table). Or, you can create them from scratch, adding your own statistics and keywords and other information. Statistics Tables in the Layout Editor come in two flavors: (1) Each row is from a single sample: this is like the output of the table editor, where statistics for subsets or the whole sample are all represented on the same row; (2) Each row can have subsets from the same sample: this allows you to view a number of statistics (e.g., MFI) on many subsets from a single sample in column format. Note that you can copy Layout Statistics Tables directly to spreadsheets (as tabular data) or to graphics programs (as their graphical representation).

Layout Editor: [Overlay legends](#) . The legends for overlays in the Layout Editor can now be customized to show statistics, keywords, or other pertinent information from each subset that is overlaid in the graphic. Simply double click on the legend to specify what should be shown.

Layout Editor: [Iterate by "nth" sample](#). Sometimes you just want to do batch iteration where the order in which you collected the samples defines how you want the output to appear—for example, every three tubes comes from a single sample, where the first of the three is a control, and the other two are two different panels. FlowJo lets you easily specify such a design by using the "Iterate by nth" option. (Choose "Iteration options" under the layout menu). In this example, you would iterate by "every 3rd sample", telling FlowJo to group tubes into samples of 3. This significantly simplifies batch operations. All new Layouts are created with the “Iteration by nth” (n = 1, i.e., every sample) by default.

[Templates: Improved functionality](#). This "Iterate by nth" Layout Editor feature also means that Template Workspaces that contain batched layouts will work well. A batched layout is now automatically defined to have

the appropriate iterator (and discriminator) values; when you batch a layout over every sample and save it as a new layout, the new layout is automatically defined to iterate by "nth", where "n" is the number of tubes in the original batch.

In addition, sample identification is significantly improved in FlowJo. This means that Template workspaces will fill out layout graphs as expected-FlowJo tries very hard to overcome any ambiguities that might arise because of multiple samples fitting the criterion for a single graphic.

Other New Features.

General

- The FlowJo icon is properly displayed on all workspace files (that have names ending in ".jo", ".ws", or ".flowjo").
- There are new "Help" menu items to provide quick links to documentation and contact information.
- Lists of FCS Keywords are sorted better, with user-defined keywords appearing first, and standard FCS keywords arranged in better order.

Workspace

- The workspace auto scrolls to show the sample corresponding to the front-most graph window.
- The number of samples in a group is now shown in the first column after the name, not aligned with the "Statistic" column.
- Template workspaces are now saved with the default extension of ".jot".
- While editing a keyword value, you can paste a selection (copied from another program) that has a series of values (tab- or carriage-return-delimited, as from Excel). FlowJo will fill out successive rows with these values.
- You can now ctrl-click (or click-and-wait) on analyses in the sample list to show a menu of actions appropriate for the selected items.
- The column resizing button has been moved from the top of the workspace window to the middle bar.
- \$PnE keywords (log/linear scaling of parameters) can now be edited.
- When sorting on keyword values, FlowJo looks to see whether a keyword value is numeric, alphabetic, or mixed, and sorts appropriately. For mixed values, FlowJo will sort "A-2" before "A-10" (even though this is not alphabetical); it recognizes the presence of both strings of letters and numbers and treats them independently when determining sort order.

Graph Window

- Graph windows now use the "ctrl-click" (or click-and-wait) to show a menu to change the graph attributes (graph type, options, etc.), similar to the Layout Editor graphs.
- Cloning a graph window ("+" button, or "Open New Window" menu) now creates a new window that has exactly the same settings.
- You can specify (for dot plots only) the number of events to display, on a graph-by-graph basis.

Platforms

- Compensation: When editing a compensation matrix, you can now copy it (to the clipboard) or print it.
- Cell cycle: There is a new preference to choose a Pulse Area parameter as the default for new analyses, and new preferences to control the default modeling characteristics.
- Cell cycle: The cursor coordinates are now shown in the window.
- Proliferation: The modeling algorithm has been improved to better find good fits. (The formula for the model has not changed, only the approach to the fit).
- Derived Parameters: Older derived parameters for Ratios, Gain/Offset are now automatically converted to Algebraic derived parameters with the correct formula shown.
- Derived Parameters: You can double click a previously-defined algebraic parameter to edit it.

Layouts

- Overlay Legends: You can now click to select any row in the legend; press the "delete" key to remove it from the overlay; drag to re-order the overlay.
- Show ancestry plots: You can choose to make all ancestry graphs show at equivalent size, rather than scaling them to fit in a single row or column.
- Text boxes: If you select a text box and choose "Copy", then the contents of the text box are put on the clipboard so you can paste them into other applications.
- Arrows: If you create a line with arrow heads, you can now specify the shape of the arrow head to customize its appearance.
- Graphs: You can now specify the number of events to display (dot plots only) on a graph-by-graph basis.
- Graph Item Annotation: The dialog window in which you can specify custom contents for a graphic item annotation box now has a menu to choose keywords to insert into the annotation text. Also, graphic item annotations can be edited for multiple graphs simultaneously from the "multi-item attribute editor" window.
- Batch Iteration Dialog: The dialog window that appears when you create a new Batch output has been made easier to understand; the iteration controls have been removed from this window (to specify iteration options, you must choose them via the "Iteration options" menu item).
- Text boxes, annotations: In addition to specifying FJML commands for items like statistics ("") and Keywords (""), you can also specify information about any given subset using the "Property" command. Properties that can be displayed include: "Comment" (any comment in the workspace for that subset), "GateName" (the name of the subset), "SampleName" (the name of the sample), and "Path" (the full gating path for that subset). For example, "" will be translated into the subset's sample name.

Tables

- Editing column names: While editing a column name, you can paste a selection (copied from another program) that has a series of values (tab- or carriage-return-delimited, as from Excel). FlowJo will fill out successive rows with these values.
- Hold down the option key while clicking on the "Copy to clipboard" button and a transposed version of the table is copied onto the clipboard.

New Preferences

- Pulse-area parameter should be the default for Cell cycle analyses.
- Newly-created histogram overlays in the Layout Editor should be scaled to a fixed value by default (rather than scale-to-max).
- Specify the default line styles for histogram overlays.
- Default graph window size for newly-created graphs (brought back from Version 3.x of FlowJo!).
- Text traits for gate frequencies shown in graphs in the Layout Editor
- Text traits for headers and footers in printouts
- Specify the default statistic that is added to the Table Editor when a gate is dragged to the Table.
- Specify whether axis numbers, on graphs that are near the edge and would appear clipped as a consequence, should be moved slightly or hidden.
- Cell cycle platform preferences can be defined.
- Proliferation platform model display preferences can be defined.

Bug fixes

- Suggested output file names are chosen better.
- Sample Information window can be resized to show keyword values better.
- Deleting a sample from the workspace will close its graph window.
- Create Value Series (while editing a keyword value) now obeys the maximum number of values to fill in, and better remembers the previous values you used for it.
- Cell cycle and Proliferation analyses immediately recompute a model if a parent gate changes.

- Proliferation: If create a constraining gate to position the undivided peak, you can now select it without having to close the window first.
- Layout graph items with oversized text (from the preferences) no longer randomly resize themselves. However, large text will still be clipped if it is too big. (You can choose to hide ticks or axis numbers to provide additional space for label text).
- Cell cycle: Remove apparent constraints that allowed positioning of peaks by absolute values (this was never a possibility).
- Cell cycle: Fixed synchronous peak constraints for DJF model.
- Cell cycle: copying analyses to groups or other samples retains the preferences for how models are displayed
- Workspace: Fixed editing a keyword value for workspaces with >1000 samples.
- Kinetics: Fixed an issue that could cause a crash for files with corrupt data at the end of the time series.
- Layout Editor: Multiple graphs dropped in from the workspace are now positioned better (so as not to overlap).
- Workspace column resizing no longer occasionally resizes the window height.

Find this helpful?



Techniques

This section discusses different techniques you can use to analyze data more efficiently with FlowJo.

FlowJo gives you the most flexibility possible with exported graphics. You can easily ungroup any graphic, and change the font attributes, line styles, etc., to make any graph look exactly the way you want! The [first page](#) in this section gives you some additional hints on exporting graphs.

The greatest strength of FlowJo is its ability to represent [batch analysis](#) in a simple and flexible structure. Don't expect to find a macro language or scripting extensions. Our approach lies in the grouping structure, within the workspaces. Create your layout, and with a single button, produce a stack. The stack can be shown as a new layout, a tiles picture or a movie. There's even a section about how to [cancel batch jobs](#) once they've been started

[Drag-and-Drop](#) is an important skill to learn if you are going to be a fluent user of this program. This section talks about the semantics of what it means to drop gates or statistics onto populations. A few modifier keys can save you all sorts of time.

Indeed, modifier keys, especially the [option key](#) are used heavily in FlowJo. Use option dragging to duplicate graphs in the layouts, or option-close to close all your windows at once. Try depressing the option key as you browse through menu items; you will find that many of the menus change to be a different command, giving you quick access to even more powerful features.

Find this helpful?



Mac and Memory

1GB of RAM is a good starting place for version 8. The more RAM, the faster FlowJo will work. FlowJo does its own memory management; generally, you should have at least 2.5x as much memory as the largest file that you want to read. If you do lots of gates, then you should increase this. OS X responds very favorably to additional memory.

Find this helpful?



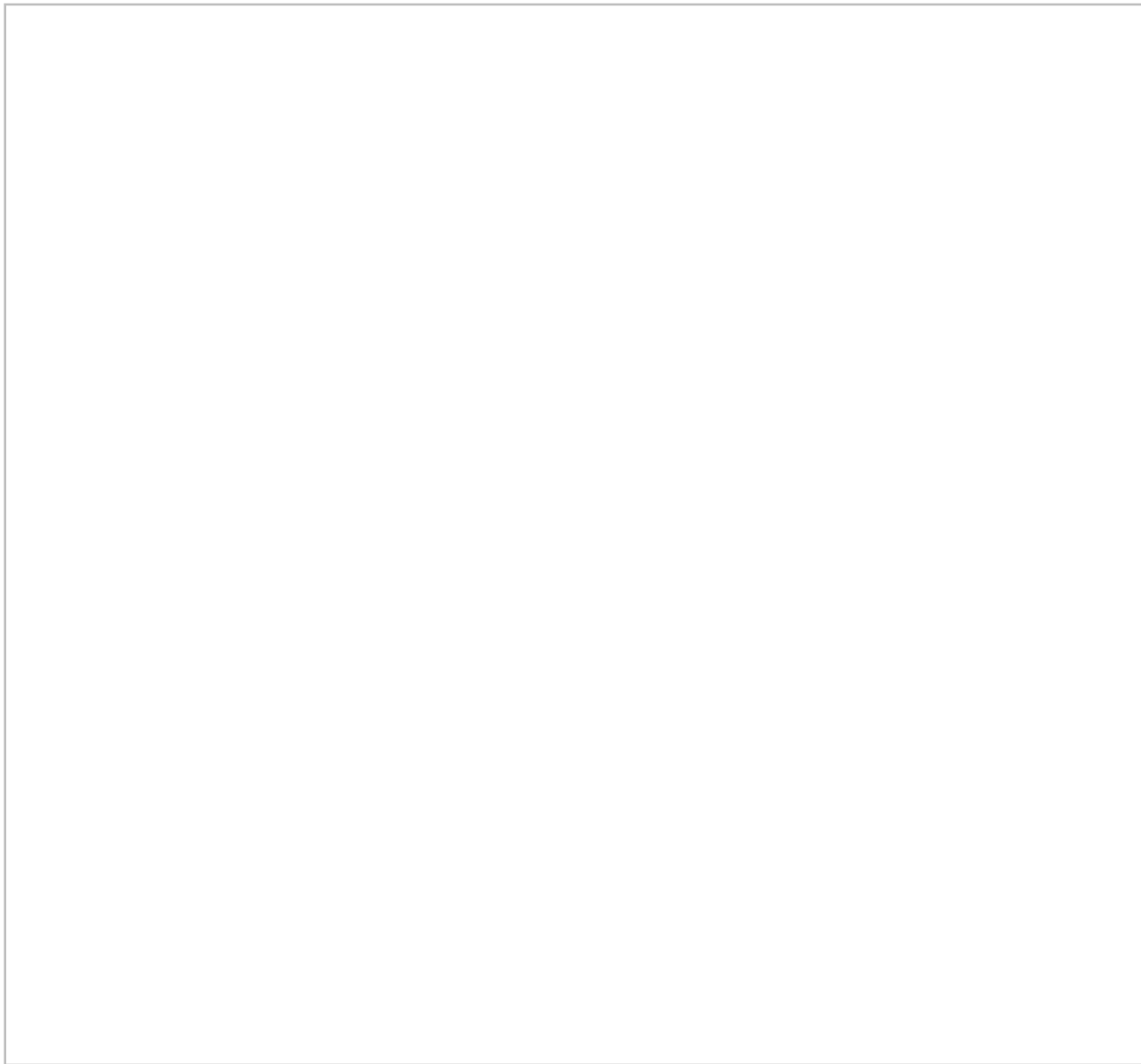
Workspace Window

The workspace window is the main launching point for all analyses in FlowJo. It is composed of three main parts: a set of function buttons at the top, a "group" pane, and a "sample analysis" pane. The group pane contains a list of all of the groups which have been created. It always contains the "All Samples" group, to which all samples in the workspace belong. Any new group that you create will be listed here as well.

The sample pane contains all of the samples (and their analyses) which belong to the selected group(s). See the graphic below for examples of what sorts of actions you can take from the workspace window and an explanation of the information presented in the workspace window. In addition, the standard Macintosh window operations (such as the close window box, the zoom box, scroll boxes) function as expected. The "disclosure triangles" (to the left of populations which have subsets or other analyses attached) function like those in the finder: pressing the option key while clicking on the triangle opens (or collapses) all of the subsets belonging to that population; pressing the command key applies the same operation to all "siblings" (other nodes at the same level).

You can use the up-arrow and down-arrow keys to navigate the workspace window (i.e., to move between lines of the sample or group list). You can use the Page Up, Page Down, Home and End keys to scroll the workspace.

The header bar between the group and sample lists defines what information is shown in the workspace. You can resize the columns; you can delete columns or add new columns by double clicking on this bar (or by selecting the appropriate menu item). You can also sort the sample list by double clicking on the "Sort..." button in this bar or by selecting the "Sort samples..." menu item.



See the following topics for more information:

- [Sorting the sample list](#)
- [Changing workspace information display](#)
- [Editing sample parameters](#)
- [Groups](#)
- [Statistics](#)
- [Batch analysis](#)
- [Dragging and dropping nodes](#) (copying gates, statistics or other analyses)

Find this helpful?



Creating and Editing Groups

When you create a new group, you are presented with the dialog window below. This window also appears when you double-click on a group in order to edit the criteria for membership.

□

FlowJo examines all data files in your workspace to build a list of the different staining panels. These are shown in the list box in the middle. You can select one or more of these to state that only samples stained with the particular combination of reagents should be added to the group.

If you select multiple stains, you can choose the **Create Multiple** button to make a separate group for each selected reagent panel.

You can also specify one or more criteria based on FCS keyword values. Select an FCS keyword, a comparison, and the value to compare against; FlowJo will add samples only if they meet the selected criteria.

The **Formula** button translates the criteria chosen above to a formula.

Finally, you can specify that the group take its samples only from another group (or to use all samples in another group)--essentially providing you with the ability to make subgroups. Samples must fit all criteria specified in this window in order to be added to the group.

You may manually add samples to a group by dragging and dropping them in the workspace window. They will automatically become members of the group irrespective of the criteria you have established for the group. You may also remove samples from a group by selecting and deleting them (they will not be deleted from the workspace as long as they are members of the **All Samples** group).

If you select the **"Live" group** checkbox at the upper left, FlowJo will examine any new data files that you bring into the workspace to see if they fit the criteria for this group. If they do, then they are automatically added to the group, and any group-based analyses are performed on them as well. If you are constructing "template" workspaces for future use by yourself or others, make sure that your groups are **"Live" groups**.

If you choose to **Synchronize Group's Gates**, FlowJo will automatically update the gates for all the samples in a group as soon as you adjust a gate on one sample. This option saves time by applying newly adjusted gates automatically to all the samples in the group; skipping the step of dragging the newly adjusted gate up onto the group name. However, it does not allow between-sample-variation of gates. All the gates will be the same as the last adjusted gate on any sample in the group.

The color and style apply not only to the nodes of the group in the group list window, but also to any node in the samples which was created by the group AND is still identical to the group's version of the gate. This is how you can tell if a gate has been modified from the group's version: it will not appear in the same color/style as the group's version, but rather in black and plain text. Therefore, you should avoid having group nodes be shown in black with plain text: as you would be unable to distinguish between gates which belong to the sample, and gates which belong to the group.

You can reopen this dialog and change group attributes such as the name, style, and color of a group as well as automatic sample selection criteria by double-clicking the group name in the Workspace window.

If you want to create more than one group, check the **Keep this window open to create more groups** box and click the **Create Group** button.

Find this helpful?

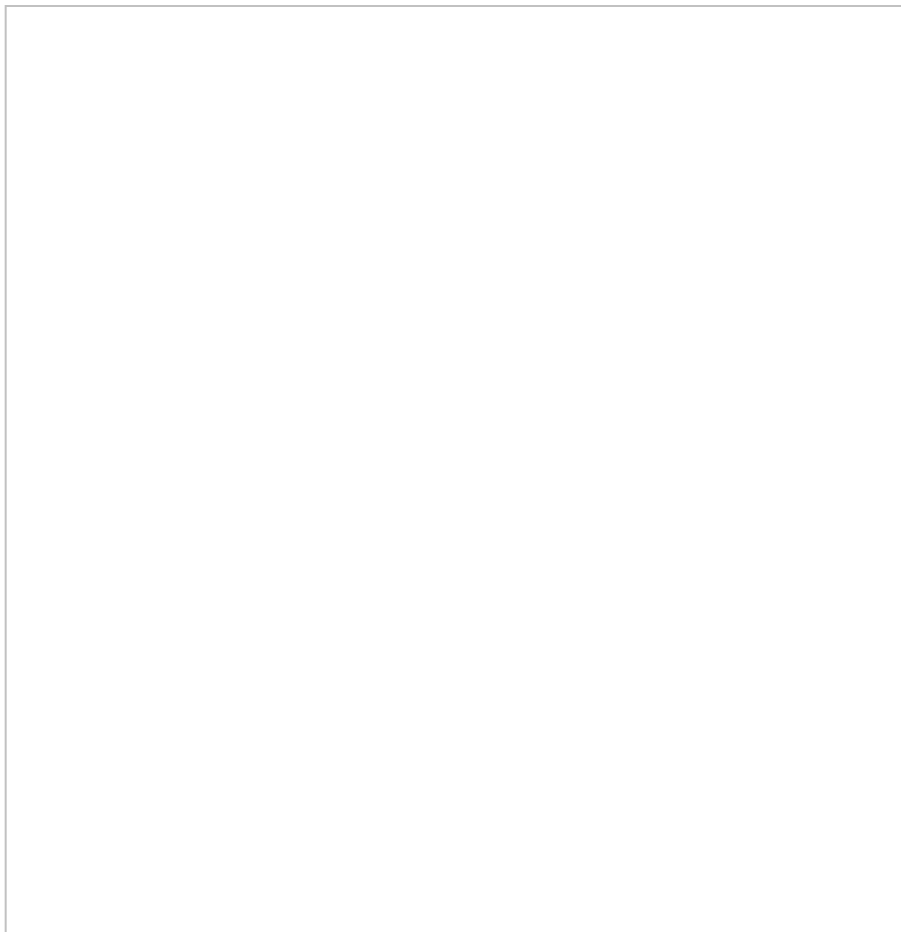


Example 1

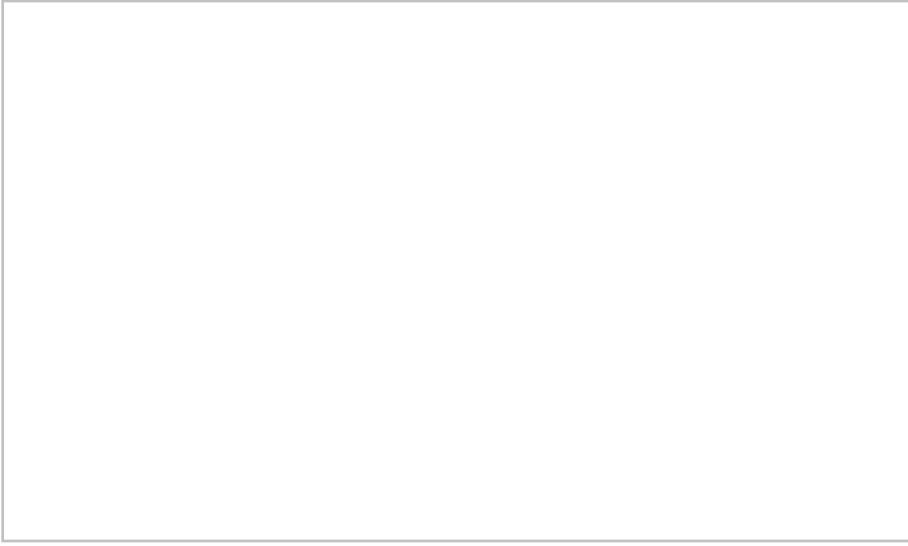
This example of group operations uses an experiment in which 8 samples of human PBMC were stained with two combinations of antibodies. The first combination is CD45, CD16, and CD14 (for enumeration of leukocyte subsets); the second combination is CD3, CD4, and CD8 (for enumeration of T cell subsets).

In this example, a lymphocyte gate will be defined on a single tube. This lymphocyte gate is applied to all samples. Then, the samples stained for the T cell antigens will be examined, and gates defining CD4 and CD8 T cells will be applied. Finally, the frequencies of the populations will be calculated.

The first panel below shows the workspace after its inception and the creation of two groups. There are three groups listed because there is always an All Samples group in the workspace. The first group that was created is named "Leukocyte subsets". The color chosen for this group is red, and the style is italic and this group contains all of the tubes stained with CD45, CD16 and CD14. The final group was named "T cell subsets" and was created to contain only the samples that were stained with CD3, CD8 and CD4. The color chosen for this group is blue and bold. These groups were created via the [Group Creation Dialog](#) window, selecting staining combinations to automatically add samples to the groups.



The panel below shows the sample list after clicking on the "T cell subsets" group.



Find this helpful?



Definition of Statistics

This page details the statistics that can be selected from the [Statistics Dialog window](#) and appear in both the workspace and the graph window.

Units

Relative intensity values are in arbitrary units and can be expressed as scale values or channel numbers. The scale values take into account scaling factors such as the gain setting for the parameter. Channel numbers ignore the scaling factors, and use an integer scale for relative intensity ranging from 0 to the maximum value defined by the data collection program (i.e 255, 1023, 4095). To get the options for channel statistics, hold down the option key while clicking the statistic button in the FlowJo workspace.

If you use the [Calibration Platform](#) to calibrate a fluorescence parameter to absolute units, then all statistics computed on the calibrated platform are given in terms of those absolute units.

Definition of statistics

Mean - The arithmetic mean. For a [normal distribution](#), the mean = median = mode.

Median - The median is the relative intensity value below which 50% of the events are found; i.e., it is the 50th percentile. In general, the median is a more robust estimator of the central tendency of a population than the mean.

Mode - The relative intensity value which is most frequently found for a given parameter. This is the same intensity value at which the highest point on a histogram is found.

Geom. Mean - The [geometric mean](#). Can be a more applicable metric for a [log-normal distribution](#). It is always less than or equal to the arithmetic mean.

SD - The Standard Deviation is a measure of the spread of the dataset. Lower values indicate the data points are closer to the mean and give higher confidence to the mean value.

CV - The Coefficient of Variation is a normalized Standard Deviation. $CV = SD/Mean$. In FlowJo, the CV statistic is displayed in percent (i.e. a CV of 0.15 is displayed as 15). $1/CV$ is a common way to define the Signal to Noise Ratio.

Robust CV - Equals $100 * 1/2(Intensity[at 84.13 percentile] - Intensity [at 15.87 percentile]) / Median$. The robust CV is not as skewed by outlying values as the CV.

%ile - Percentile. This is the relative intensity below which n% of the events are found, where n is the selected value. n=50 is equivalent to the median.

Frequency - The percentage of events (cells) in this gate out of the total number of events in the sample.

Freq. of Parent - The percentage of events (cells) in this gate out of the parent gate (one level up).

Freq. of Grandparent - The percentage of events (cells) in this gate out of the gate two levels up.

Count - The absolute number of events (cells) in a gate.

Note- A common question is "Which statistic should I use?". There are many reasons to choose a mean over a median

over a mode, or vice versa. It really depends on how your cells are expressing the markers that you use. Also, when using the term "MFI", it is a good idea to clearly define it in your context.

More on means, median, geometric means, and modes can be found in this [DD article](#).

[Total Fluorescence](#) of a gate (integrated area of a histogram)

Find this helpful?



Adding Statistics

Shown below is the statistics definitions window. This window appears when you select the **Statistics** button in the [workspace window](#), the [graph window](#) or in the [Layout editor](#) when you click the table tool. The statistic that will be computed is added to whatever subpopulations are currently selected (workspace window) or displayed (in the graph window). Select multiple populations in the workspace to add the same statistics to all of them at once.

Adding statistics to a population is performed in up to four steps, as shown below. Add all of the different statistics to a single sample at once by repeating steps 1-4 for each statistic.

statwindow.gif



Once you have added statistics to a node, the easiest way to compute these statistics for other samples or subpopulations is to drag the nodes and drop them onto the desired destination. You may also drag statistic nodes into the [Layout Editor](#) to create a text box with the statistic value, that is updated whenever the statistic itself changes value (for example, in response to your moving a gate). Statistics are most conveniently collected and exported using the [Table Editor](#). Now you can build a [table of statistics](#) within the Layout editor. Click the Sigma button in the Layout editor's tool palette. Drag a rectangle in the area where you want your statistical table to appear. To construct the table, [click here](#).

You may view a complete [Definition of these statistics](#).

Find this helpful?



Layout Controls

In many ways, the Layout Editor is like a simple page layout program. It can draw boxes and lines, type text and move the items around with a familiar interface. It has some additional aspects as well: it supports multiple pages each with its own layout defined; it has controls to apply the same layout commands to many different datafiles; and most importantly, it has the ability to reference live data in the workspace so that graphs are updated when the underlying gating hierarchy changes.



Use the layout editor to create a new layout definition. A layout definition is not the output data itself. Instead it is the specification of which graphs will be placed at what locations, when the layout graphic is generated. Graphs, gates and statistics will continue to be updated in the Layout editor until your analysis is complete. You can have as many layout definitions in the workspace as you wish. You can create new empty definitions or delete them using the buttons in the top left corner of the window. The **Plus** sign opens a new blank layout, the **Equals** sign opens a copy of the current layout, the **Minus** sign deletes the currently visible layout.



This window has a large number of controls, which are organized in groups. At right is an enlargement of the top right corner of the layout window, showing the groups of buttons in the window. Clicking on on the red numbers will scroll you to the section of this page that describes that group of controls.

Tool Palette

Tucked into the top of the vertical scroll bar is a miniature tool palette, Number One at the right. It contains an **Arrow** tool, a **Line** tool, a **Rectangle** tool, a **Text** tool, a **Grid** creation tool, and a **Statistics Table** tool. Only one tool is active at a time, and the active tool will return to the arrow after any new object is created.

The Arrow Tool



The Arrow Tool is used to select existing objects. Click on an object to select it (as shown by dark handles at the corners of the object). Use the shift key to select additional items. Drag items to move them. Option-drag to duplicate them. Start a drag in the background of the layout editor, and it will draw out a rectangle. Upon finishing the drag, the layout editor will select all objects that are enclosed by that rectangle. (Marquee selection.)

The Rectangle tool



The Rectangle tool is used for drawing simple boxes and frames. If you create the rectangle surrounding another element, and want it to serve as a background, use the **Layouts > Send To Back** menu command to change the order of the layout. Double click on the rectangle or use the **Get Info** command from the **Layout** menu to edit the properties of the rectangle.

The Line tool



The Line Tool is used for drawing lines and arrows. Generally, you will want lines drawn on top of other elements, so draw them last or use the **Layouts > Bring To Front** command to change the order of the layout. Double click on the line or use the **Layouts > Get Info** command to edit its properties. Properties supported for lines include which end has an arrowhead, line weight, and dashing pattern.

The Text Tool

□

The Text Tool is used for adding textual annotation to the layout. To create a text box, you select the text tool from the tool bar, and click once or drag out a rectangle in the layout view. When you create a new text box, a dialog will appear to help you edit the text. This is called the **FJML Editor**. FlowJo uses a custom markup language, not unlike the HTML used in web pages, to richly express the structure of your flow analysis. Once you confirm changes via the mouse or the Enter key, or click the mouse on a different object in the layout, the editing stops, and the layout editor text is reformatted and frozen. To edit it again, double click on the text box to return it to the edit state. Text clippings or statistics from the workspace can be dragged and dropped into a layout. In both of those cases, a text box is created automatically.

The Grid Tool



The Grid Tool will create a matrix of cells within your layout. Each cell may contain text, charts, images, or other grids. The grids provides a convenient mechanism to group and align multiple elements of the layout. Grids are explained in greater detail on the [Grid Tool](#) page.

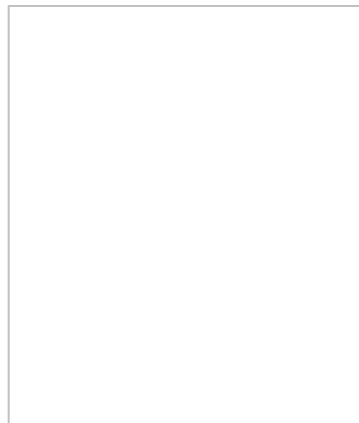
The Statistics Table Tool

□

Now you can build a table of statistics within the Layout editor. Click the Sigma button in the Layout editor's tool palette. Drag a rectangle in the area where you want your statistical table to appear. The following dialog opens within which you can construct your table.

□

Drag-and-drop samples or subpopulations from the Workspace into the upper left box. This list will label the rows of your new table. Drag-and-drop statistics or other information into the upper right box. These will label the columns of your table. When you click the **Update Layout** button, your table will appear in the Layout editor with the calculations in place.



Use the **Choose Column Value** (Number One above) drop-down list to add columns that don't appear in the current

layout. Possible values are shown at right.

Click the Sigma button (Number Two above) to add a new statistic just as you might do in the Workspace. Use the checkbox **Add Statistics to Subsets If Missing** (also next to the Two) to tell FlowJo to calculate the statistical values for populations in the new table that have not yet been calculated in the Workspace.

Opening the disclosure triangle labeled **Appearance** shows the usual tools for controlling the style of the text and boxes in the new table.

Click **Update Layout** to view your changes in the Layout Editor, click **OK** to accept the current choices and close the **Edit Statistics Table** dialog box.

Inserting Pictures

You can add graphics from other sources into FlowJo layouts. If the graphic is in PICT format (a Mac standard) then insert graphics simply by pasting them from the clipboard. To include GIF, JPEG or TIFF files, use the **Insert Picture...** command in the Layout menu. This is a way to include institution or laboratory logos or backgrounds from other sources into your FlowJo layouts. Like boxes and arrows, the pictures are replicated in each frame of an iteration.

Direct Output Commands

□

Across the top of the layout window are five buttons, describing the functions of adding data to your SciBook, copying to the clipboard, saving to disk, printing, and launching another program.

The [SciBook](#) button, Number One, will copy the current layout and place it on a page of the SciBook. For more information on using the [SciBook](#) to collect information pertinent to the current experiment, [click here](#).

The **Copy** command, Number Two, puts the layout onto the Clipboard, and from which it can be transferred to your favorite graphics package. Note that certain settings of FlowJo can cause it to make a picture where every dot in a dot plot is a separate element in the picture. In particularly large files, this has been known to overtax other programs' ability to paste the contents of the clipboard. If you are having trouble pasting pictures copied from FlowJo, you should go to the [Preferences](#) and turn off the setting **Export Graphics as Vectors** under the **General** Tab.

The **Save** operation, Number Three, will create a graphics file of the current layout at its current iteration value. Depending on the setting in the [Export preferences](#) dialog, this may be written as a standard Macintosh PICT file, or a GIF, TIFF or JPEG file for special purpose exports. The PICT file is the recommended format, as it contains additional grouping information that can be useful in further editing. PICT files are readable by virtually all graphics programs on the Macintosh, and most of the popular Windows editing packages.

The **Print** button, Number Four, will cause the current version of the layout to be sent to your chosen printer. If you want to print the current layout from several different values or samples, you should use the Batch button and choose one of three options, Direct to Printer, Preview or New Layout. The second option builds a tiled report which you can then print. The third option builds a report in the style of a layout (i.e., it is fully editable) which can be printed after adjusting the page breaks.

The **Launch Helper Application** function, Number Five, will activate your favorite graphic program and transfer the data to it. To do this, FlowJo must save the data as a file, so the first thing you will see is a request for a file name and location. Once you have saved the file, FlowJo will attempt to transfer control to the application you have listed in the bottom of the [Layout Preferences](#) dialog. If you have not set a preference for graphic or spreadsheet helper applications, a dialog will open that directs you to choose one.

Iteration Controls

□

Across the top right corner of the window, near the Direct Output controls, is a row of controls used to change the sample or samples currently viewed in layout. It is easy to take a layout viewing one of your samples and its associated gates and statistics, and see the same displays applied to other data.

The largest and leftmost control, Number One , is a pop-up menu containing all values of the current attribute that exist in the current group. If the current attribute is sample identifier, the label next to the popup is **Sample #**, and all of the samples in the current group will be listed in the menu. If another keyword has been set to be the iteration attribute via the [Iteration Options](#) dialog, then all values of that attribute will be listed. In a patient study, this may be a patient id, a date or therapy for which multiple samples have been drawn and collected.

The **Batch** button, Number Two, is the Iterate command button. Pressing this will take you to FlowJo's [Make Batch Report...](#) dialog. It will prompt you to choose from one of four report types: making a new layout, preview (tiled report), web page with graphic, or Quicktime movie. In addition, you can choose to [directly print](#) the layout.

The rightmost control, **Number Three** , the **Up and Down Buttons**, will reset the iteration attribute to its next or previous value. This is convenient for quickly looking through multiple layouts, without generating the entire set of them. You can also use the page up and page down keys to move through the iteration.

Page Setup And Magnification



At the bottom of the layout window are additional controls that govern the scaling of the layout, either on the printed page (**Page Setup**) or on the screen. The Page Setup button will bring up the Mac's standard Page Setup dialog, allowing you to set options such as the printer defaults, the paper size, the paper orientation and the scaling. You will notice that the layout editor draws light gray lines signifying where the page breaks would be if you were to print the layout. Changing the Page Setup options will generally cause the page breaks to be redrawn to reflect the new page size and scale. In addition to changing the scaling from the Page Setup dialog, you can also place the mouse over the intersection of any of the gray pagination lines in the layout and rescale the magnification just by dragging the page size larger and smaller.

The page size can also be changed automatically using the **Print Scale** popup menu or manually by dragging the corners of the page outlines in the layout editor. The **Print Scale** popup will provide commands to Scale to Page Width or Scale To Page Height. These will measure the size of the layout, and then rescale the page to make the desired dimension fit. The option Scale to Page chooses between Scale to Page Width and Scale To Page Height, finding the option that leaves the images as large as possible, while still fitting them on the page.

Those settings set the way the layout is scaled to fit the printed page. The settings on the right side of the control strip govern how the layout is scaled to be displayed on the screen. The smaller buttons showing distant and closer mountains zoom in and out, that is, they reduce and increase the magnification within the layout window. The popup menu allows you to set the magnification directly. Scales from 12.5% to 200% of the original size are available. The keyboard commands Cmd-[and Cmd-] will also zoom in and out, respectively.

The [Use Placeholders](#) button is discussed later in its own page.

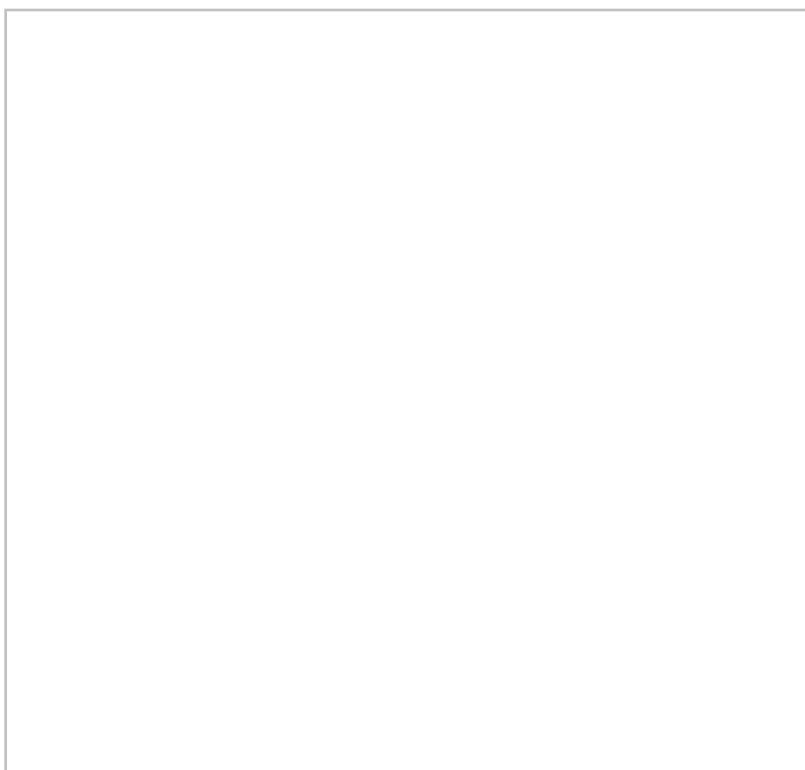
Find this helpful?



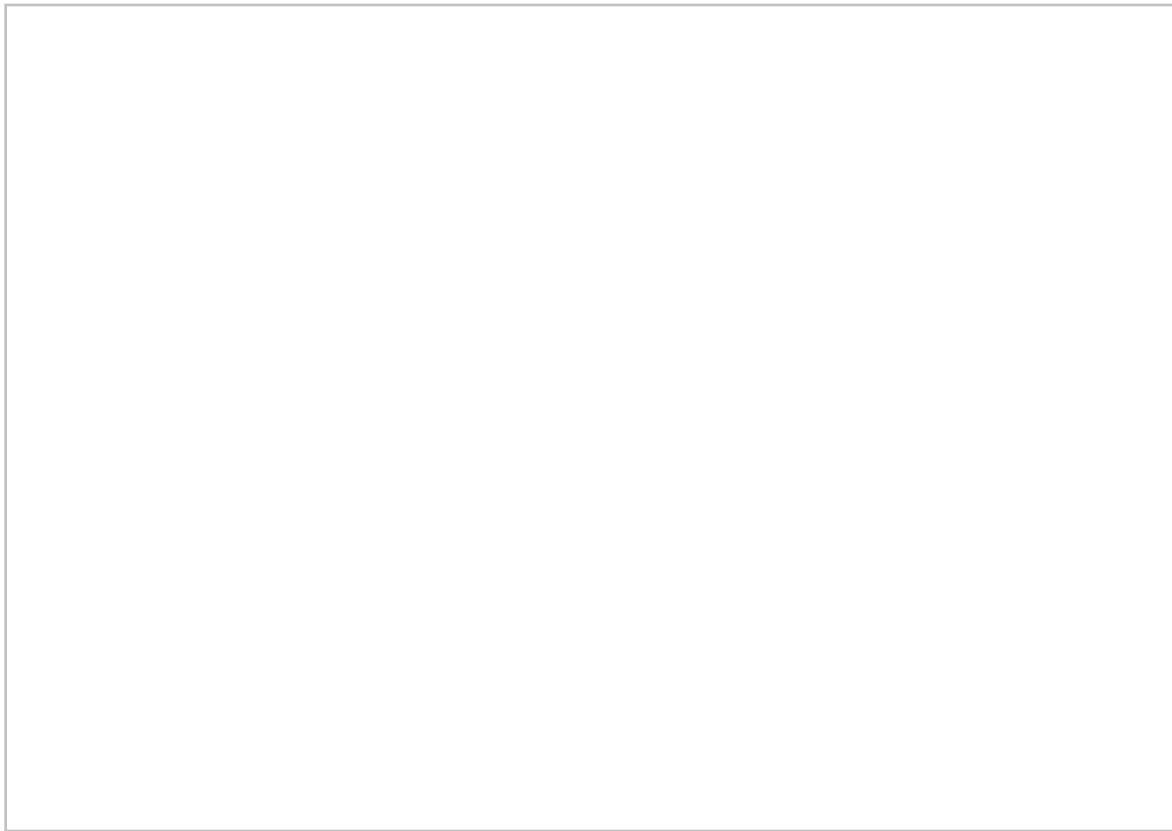
Dragging a Node's Children

In this example, an entire "tree" of gates is applied to a node. Here, only the CD4+ node is selected, but when the drag is begun (i.e., at the moment you start to move the mouse), the "option" key is depressed. This results in FlowJo dragging all of the children of the CD4+ node (and all of their children, etc.) along with the CD4+ node itself.

The first graphic shows what the screen looks like when the drag has begun. Note the dotted outline which identifies the set of nodes which are being dragged.



The second graphic shows what happens at the end of the drag. The mouse is released when the "CD3+" gate in the second sample was highlighted.



The entire "CD4+" tree has been copied from the first patient to the second.

Go on to the [next example](#).

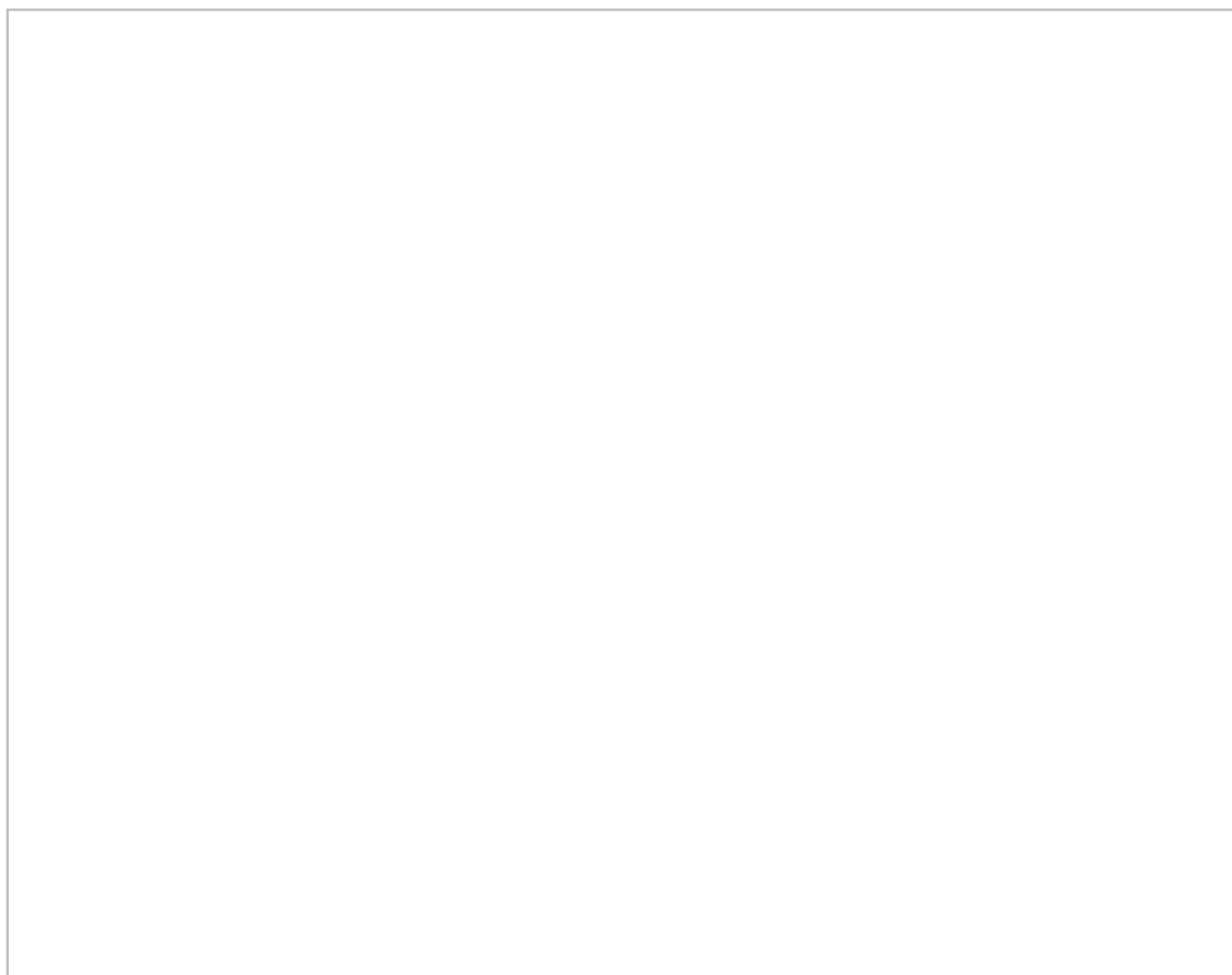
Find this helpful?



Dragging a Node's Parents

In this example, you wish to copy a single final analysis from one sample to another: that is, the frequency statistic under the Naive, CD8+, CD3+ gates. You could simply select the "CD3+" node and drag it with all of its children (using the option key) and drop it on the sample. However, this reproduces the entire tree; here you only want particular analyses. To do this, select the analysis you want to copy, and depress the control key while dragging. This tells FlowJo to select only the portion of the tree necessary to create your specific analysis request.

The first graphic shows what the screen looks like when the drag has begun. Note the dotted outline which identifies the four nodes being dragged: the statistic node, which you wanted, and all of the parent gates ("Naive", "CD8+", and "CD3+") needed to specify the exact subpopulation.



The second graphic shows what happens at the end of the drag.



Note that only the part of the tree required to exactly construct the statistic has been copied.

Go on to the [next example](#).

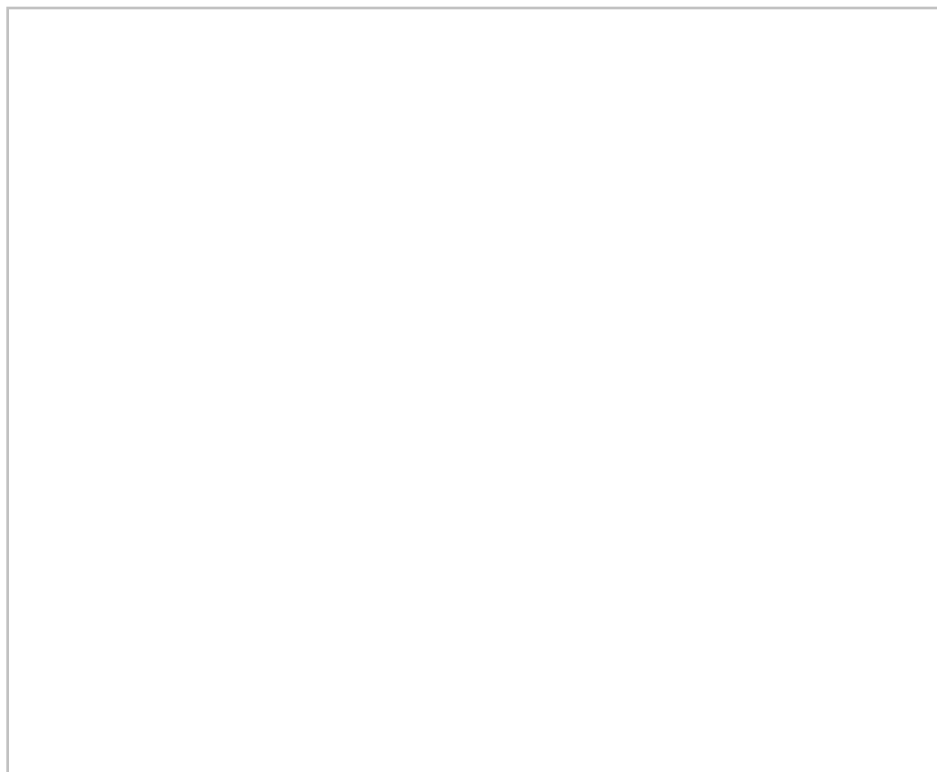
Find this helpful?



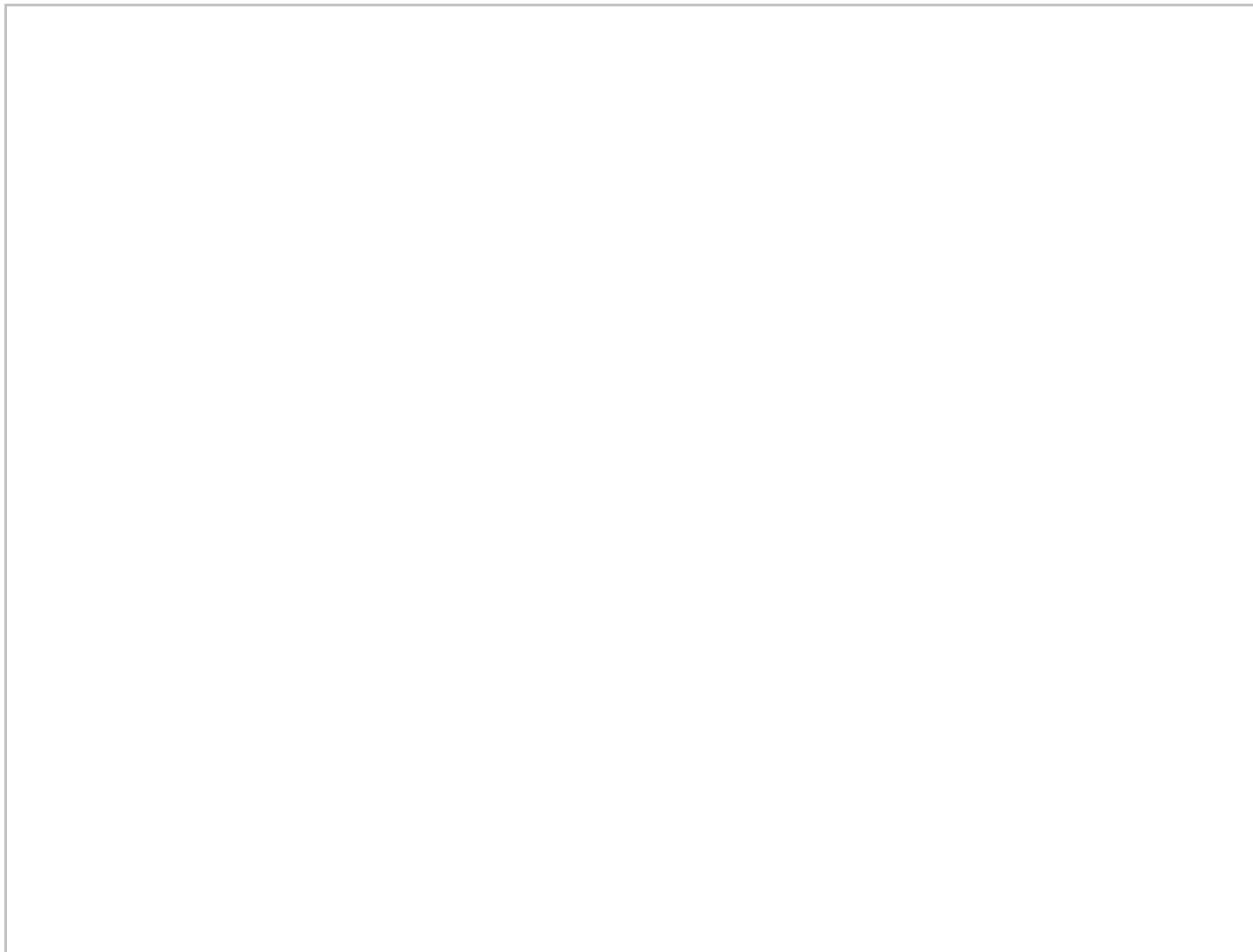
Dragging Multiple Nodes

In this example, you wish to copy multiple gates from one analysis to another. Here, you decide that you only want the CD4+ gate, the M11a+ gate, and the M3 gate. You have selected these three nodes by holding the command (apple) key while clicking on them.

The first graphic shows what the screen looks like when the drag has begun. Note the dotted outline which identifies that the three nodes being dragged. Since neither option nor control keys are depressed, no children nor parent information is being taken.



The second graphic shows what happens at the end of the drag. Look closely at the difference in the original and destination trees!



Note how each node was separately added to the CD3+ node even the M11a+ node. This is because dragging multiple nodes selected by shift- or command-click does exactly the same thing as dragging each node one at a time. Dragging the M11a+ node to the CD3+ node in D01 means that you want the M11a+ gate to be applied to the CD3+ gate which is exactly what happened here. If you wanted the CD8+ gate to be applied in-between CD3+ and M11a+, then you would have had to select parents (press "control" during the drag) and dropped the entire set onto the sample level "D01".

Go on to the [next example](#).

Find this helpful?



Complex Drags

In this example, a complex drag is executed. Three different nodes have been selected, and both the option key ("take children") as well as the control key ("take parents") were depressed.

The first graphic shows what the screen looks like when the drag has begun. Note the dotted outline which identifies the nodes being dragged. Since "take parents" was selected, all nodes up to the CD3+ gate are chosen; since "take children" was selected, all of the children of "CD4+" are taken (the other two nodes have no children). Since these multiple nodes have a common ancestor ("CD3+"), you can think of this as taking a single analysis tree based at "CD3+", but only take a subset of its children.



The second graphic shows what happens at the end of the drag.



The desired subset of nodes is applied to the destination sample E01: Pt:E(PMA). In general, when you select "take parents", you will drop onto a sample node rather than a subpopulation node, since the original top gate was applied to a sample as well. However, you could drop the tree onto a subpopulation node. For instance, had you created a "Live gate" node on the sample to exclude dead cells, you would have dropped the tree onto this gate so that the new tree is a child of "Live gate".

Go back to the [summary](#) of drag and drop examples.

Find this helpful?



Dragging Multiple Nodes

When you drag a node onto another sample, then FlowJo checks to see if the destination node already has a gate with the name of the node that you are dragging.

Thus, if you are dragging a Lymphocyte gate onto a sample which already has a lymphocyte gate, your options are three-fold. These options are presented to you via the [node replacement dialog window](#).

- (1) You may choose to **Replace** the existing gate with the version that you are dragging. Do this when you want to make the destination sample's gate the same as the one you are dragging.
- (2) You may choose to **Duplicate** the existing gate. It will be added to the sample, but its name will be modified by appending a version number to the name (for instance, the new gate will be named "Lymphocyte-2" in this example).
- (3) You may choose to **Retain** the gate in the destination sample. Initially, this option may sound like it is useless, but in fact it is one of the most important features of FlowJo, so it is important to recognize how it works. With this option, FlowJo will not alter any of the gates in the destination sample; their specific versions of any particular gate will remain as they are.

If you are dragging only a single population, then the **Retain** option causes nothing to happen; i.e., it is not useful for this case.

But consider the case where you are dragging a whole tree of analyses: now you can use this option to add only the specific analyses or gates that the destination doesn't have, without changing the gates that already existed in the destination (because they may be slightly different than they are in the source sample). For example, let's assume that you have gated two samples for lymphocytes, but the gates are slightly different. You gave the gates the same name in each sample (Lymphocytes), because they identify the same subset of cells. In the first sample, you then generate additional gates to identify subsets of lymphocytes, like CD4 and CD8 T cells.

By clicking on the first sample's lymphocyte population while holding down the **option** key, you will drag the entire tree (all of the subsets below lymphocytes); this is how you duplicate all analyses quickly across samples. When you drag this whole tree to the second sample, you want only the new analyses to be added in the appropriate locations (i.e., the CD4 and CD8 gates to be attached to the existing lymphocyte gate). Here is where you would choose the "retain" option: now as FlowJo adds the dragged tree onto the sample, it will retain all of the old gates that have the same name (Lymphocytes). It then adds the CD4 and CD8 gates to this gate.

If you had chosen the **Replace** option, then the lymphocyte gate itself would have been changed to be identical to first one, and then the remaining nodes (CD4 and CD8 gates) would be added to it. Choose this option if you want all of the analyses and gates in both the source and the destination to be identical.

Again, the **Retain** option allows you to copy analyses (and populations) without changing any

specific changes you made for a specific sample.

One final note: when you drag nodes to a group, then you are not presented with the dialog giving you the options. Rather, FlowJo assumes that you wish to **Replace** the versions of the group nodes with the ones you are dragging. This means that all samples which have the group's version of the nodes you are dragging will have their nodes updated as well. For more information, see the pages on [Groups](#).

Find this helpful?



Specifying the Graph View

When you open the **Options** disclosure triangle at the bottom of the graph window, the options below are shown. From this window, you can modify the attributes of the current graph (or currently-selected gate). The first time you are shown this window, the bottom three sections are hidden; click on the black triangles to reveal them.



1. Select the type of graph you wish to display... Contour, Dot Plot etc.
2. The gate can be inclusionary (checked) or exclusionary (unchecked.)
3. Check here to make the gate adjust automatically to the density of events.
4. Check here to add a transparent overlay to the gated events.
5. Click this box to choose a color for the transparent overlay.
6. Above the white box is the number of events gated, the total population, and the Frequency of total. Inside the box are any statistics already applied to the population.



Use the gating tools (top) when you want to create a new gate of a particular kind. The **Quadrant tool** is a special case: it creates four gates ([click here](#) for more information). The next section lets you select which parameters to view; the third section defines the graph type and options. The fourth section is disabled unless the current graph is a histogram or CDF, in which case you could select to change the vertical (Y-axis) scaling for the plot. Finally, the bottom section is enabled whenever you have a single gate selected in the graph window. Here you can choose to gate for events inside or outside the gate, and choose to draw the gate in a different color than the default. Tinting the area inside the gate only alters the drawing of the gate; it does not change the colors of any events (dots) on any of the graphs.

Magnetic gates automatically move to accommodate the maximum event density. For more information, [click here](#).

The preferences dialog specifies whether you must click on a gate tool to create a new gate; or whether FlowJo will auto-select a gate tool when you click in a graph window.

To make selected changes, you must click on the **Apply** button, which becomes active once the attributes of this window do not reflect the current graph. If you hold down the **option** key while pressing Apply, then the graph specification is applied to ALL open windows (at least, to those where it can be applied).

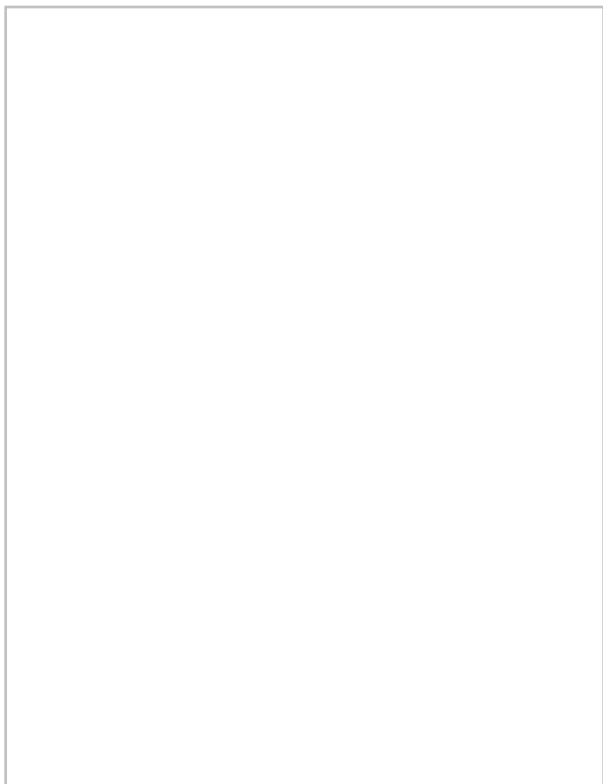
You do not have to close this window; you can leave it in one corner of your screen. It automatically updates itself to reflect any new graph window that you open.

Find this helpful?

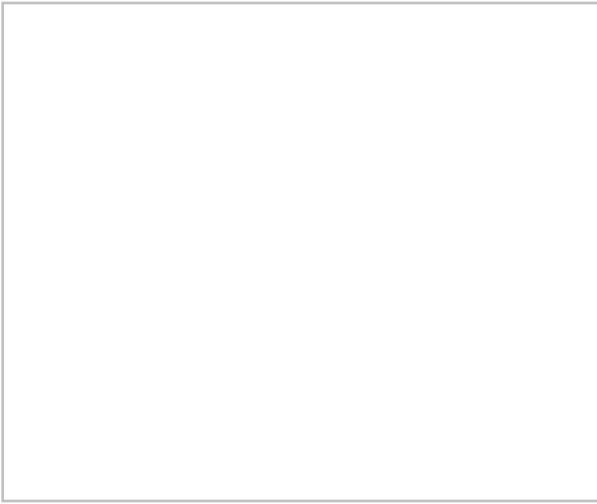


Graph Window

When you double-click a sample in the **Workspace** window, a **Graph window** opens. It's features are described below.



1. The name of the population displayed.
2. Gating tools.
3. The [SciBook](#) tool, lets you add the graph to a notebook that is saved with the workspace. You can annotate or just track pictures, text, tables etc. If you update a gate or statistic, the SciBook is updated too.
4. Opens a second copy of this graph.
5. Shows the same type of graph of the previous or next sample in the Workspace list. Hold down the Option key and all open graph windows will cycle to the adjacent sample.
6. The Up arrow displays the graph used to gate the current population. The Down arrow displays the graph of the subpopulation in a currently selected gate.
7. Click inside any gate to select it.
Click and drag a selected gate to move the gate independent of the population inside it. (Cursor is a hand.)
Click and drag the black square handles (red arrow) to adjust the shape of the gate. (Cursor is a crosshair.)
8. Three black **Disclosure Triangles** open when clicked to reveal more tools. (Below)



1. Here you can choose the graph type and adjust properties like color, resolution and scaling. Options are active if they can be applied to the current graph type.
2. If a **gate** is selected, its name and **Frequency of Total** will appear here. Uncheck Gate Events Inside to gate the events outside the shape. Check **Magnetic Gate** if you want the gate to adjust to event density when applied to a new population. **Draw Tinted** adds a transparent tint to the inside of the gate. Click on the **N** to choose what color to use for the tint.
3. This box contains a list of statistics applied to the population in the graph. Next to the triangle is the number of events in the graph followed by the number of events in the parent population.

Go back to the overview of the [Graph Window](#).

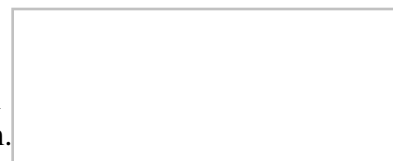
Find this helpful?



Drawing Gates

Gates are the way by which you can specify a subset of the data for further analysis. You can create several kinds of gates in FlowJo. FlowJo represents this new population by creating a new **node** in the workspace analysis tree. The node is inset below its parent, which designates that the node is a subpopulation of the parent population.

You can create gates by using a gate tools in the graph window. Gate tools are shown at the top of the graph window, and include a **range tool** for histogram plots (right), as well as 2D tools such as **rectangles, polygons, ellipses, and quadrants** (below). The selection tool (arrow) is used to select gates before moving them, deleting them, or modifying them. Selected gates are indicated with square black handles.



Depending on how you have set the preferences, FlowJo may automatically select a gating tool when you click in a graph window. If your preference is to have FlowJo automatically select a gating tool, you can choose to have the polygons or rectangles tool as your default for bivariate displays. Note that you can hold down the option key while

clicking, to toggle between the rectangle and polygon gating tool. To draw other types of gates, select the appropriate tool.

Once you have created a gate (except when you use the quadrant tool), FlowJo asks you to name it. FlowJo supplies a default name based on the parameters used to draw the gate; you may type in any name you wish. Note that gate names must be different than any other "sibling" of that gate; i.e., a population cannot have two gates with the same name (see information on [naming subpopulations](#)).

You can [change a gate](#) by moving it or moving one of its vertices; the gate is automatically recomputed as you change it. You can delete a gate by pressing the delete key when a gate is selected.

Note that you can [manually](#) enter in the boundaries of a gate if you wish; you can specify the gate boundaries in terms of channel number, absolute fluorescence, or percentile within the subset.

Types of Gating Tools

To draw a gate on a one-dimensional plot (on a [histogram](#) or [CDF](#) plots), simply point the cursor in the graph window where you wish to have the upper or lower bounds, click on the mouse, and while holding the mouse down, drag to the other extent of the gate. You can click on the line to move it right or left (changing the bounds of the gate), or up or down (which does not change the gate itself). Alternatively, you can move the upper or lower bound to extend the gate.

The other types of gates are drawn on any bivariate plots. A polygon gate with any number of vertices is started by clicking within the graph window. Move the cursor to the next vertex; click to generate another point in the polygon. Holding down the shift key will force the edge of the gate to be horizontal or vertical. You may close the polygon either by clicking on the originating point, or by double-clicking on the next-to-last point. (You can cancel the generation of a polygon by hitting the "esc" key on the keyboard at any time). You can press the "delete" key to erase the last vertex you created for any polygon.

To automatically create a polygon gate that fits your data clusters, use the [Autogate](#) feature.

A rectangle gate is started by clicking the rectangle tool (or holding the option key while clicking in the graph window). Move the mouse to create the appropriate sized rectangle. Holding down the shift key will force rectangle to have equal sides (ie, to be a square).

An ellipse gate can be created by selecting the ellipse tool. Ellipses can be rotated by clicking on and moving the left or right handles of the ellipse. The width of the ellipse is changed by moving the top and bottom handles.

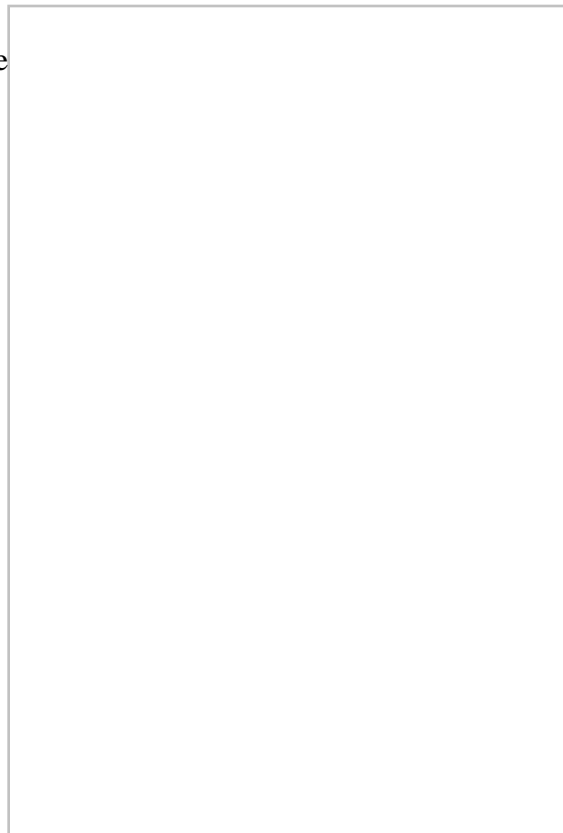
Use the quadrant tool to create four non-overlapping rectangular gates. For more information on creating and using quadrant gates, [click here](#).

Link Adjacent Gates Together

You can glue adjacent gates together, or more accurately, link their vertices.

- Drag gates together so that their vertices line up.
- Press CMD+Opt+M to join the adjacent vertices
- Opt+click to jointly move the shared vertices

When you deselect the group and select and move an individual gate it, the gates are once again independent.



Find this helpful?



Editing Gates

There are several ways to modify an existing gate. To change one of the vertices, select a gate, and then simply click on the vertex and (while holding the mouse button down), drag it to a new location:

If you want to move the entire gate, click anywhere in the middle of a selected gate (the mouse will look like a hand), and drag it to the new location. For finer movement, select a gate and use the arrow keys on the keyboard to move the gate one pixel in the direction of the arrow key.

Note that you can select multiple gates (by shift-clicking on several gates) and move them altogether--either by dragging them or using the arrow keys. You can also select a gate that is entirely underneath another gate by command-clicking (hold down the shift key while clicking in the area of the desired gate, and click until the gate you want is selected).



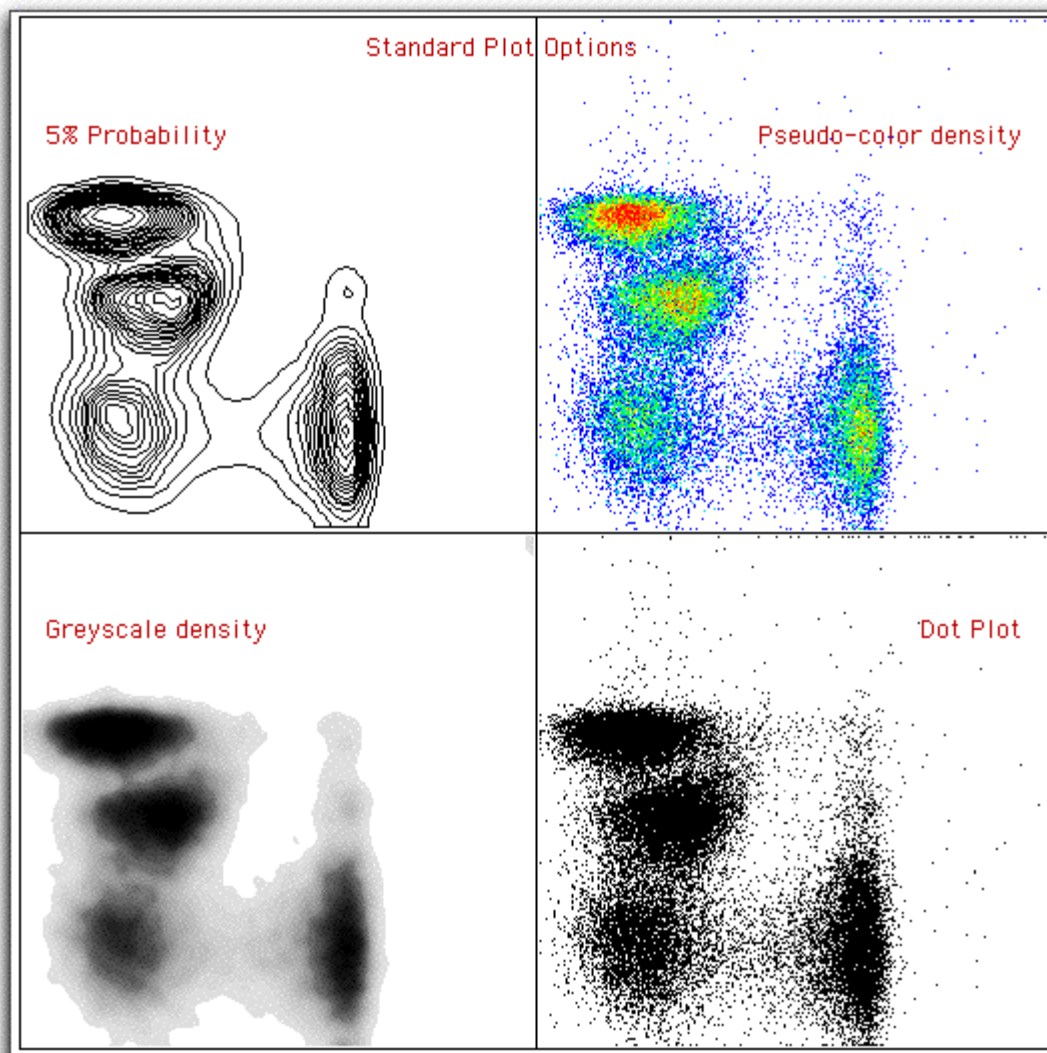
As soon as a gate is moved, FlowJo automatically recomputes the number of events in the gate. In addition, any graphs that rely on the gate you have moved (for instance, if you have a different window that displays the data for that gate) will be automatically updated as you move the gate.

Find this helpful?



Display Comparison

Four ways of displaying exactly the same data are shown below.



Of these, the Dot Plot is the least desirable: it conveys the least information as to the density of events (because in areas with a lot of events, the dots are saturated, and it becomes impossible to note any more events). Also, unless you choose to display all events as dots (in the [preferences dialog](#)), then not all events will be shown.

The density plots have many options for changing their looks; the pseudo-color and grey-scale plots are essentially the same with the exception of the colors chosen. (The two shown here have different options selected). See the page on [density plots](#) for more information.

The contour plots give the most balanced view of the data. However, rare events may be missed because they are outside the contours. This deficit can be overcome by selecting to display outlier events. See the page on [contour plots](#) for more information on these options.

Find this helpful?



Probability Contour Plots

FlowJo draws only one type of contour plot, using equal probability contouring. This algorithm generates, in general, graphs which are most accurately interpreted by our brains, in terms of relative frequencies of subpopulations. All contouring algorithms have advantages and disadvantages, but probability contouring is probably the most informative. Combined with the display of outliers, this generates an extremely informative graphic that does not over-emphasize narrow peaks nor hide low-frequency information.

There are several options available in drawing the plots (available by clicking the **Options** disclosure triangle in the [Graph window](#)). These include the option of drawing "2%", "5%", "10%", or "Logarithmic" contour plots, "smoothed" or not, and "outliers" or not. Contrary to the belief of many people, smoothing does not alter the data nor does it distort it. Thus, it is by default left on. Note that turning smoothing off can cause the program to take much longer to draw the plots. "Show Outliers" controls whether events falling outside of the lowest contour are drawn with dots--thus, combining the best advantages of dot plots (revealing low-frequency data) with the best of contour plots (allow high-frequency data to be accurately displayed). 2%, 5% and 10% plots have 50, 20, and 10 contour lines (respectively) that are distributed so that the same number of cells fall between each pair of contour lines. Logarithmic plots distribute the contour levels logarithmically in height; each contour line encloses twice as many events as the previous.

The first set of graphs below compares the same data using different contouring thresholds. The second set of graphs compares 5% probability plots with different options.



You may view a comparison of all the [bivariate displays](#) for this same data.

Find this helpful?



Density Plots

Bivariate density plots are computed exactly the same way as are contour plots (equal probability contouring). This algorithm generates, in general, graphs which are most accurately interpreted by our brains, in terms of relatively frequencies of subpopulations. All contouring algorithms have advantages and disadvantages, but probability contours have the fewest disadvantages.

There are two types of density plots: grey-scale and pseudo-color. They are identical, except the color scheme is different. Grey-scale may be more acceptable for publication; pseudo-color may be more acceptable for slide presentations.

There are two options that apply to density plots: smoothing and high resolution. The graphs below (shown for pseudo-color plots) show the effect of these options. Note that with smoothing off, every event gets a dot; thus, this plot is much better than dot plots, since it allows the simultaneous information of rare events (dots) and high-frequency areas (dots of a different color).

You might choose low resolution to make data easier to see in presentations: the small dots can be hard to see on slides.

□

You may view a comparison of all the [bivariate displays](#) for this same data.

Find this helpful?



Dot Plots

In a dot plot display, FlowJo draws dots for events. The default number of dots to draw is 8000.. You may change the number of events drawn in a dot plot via the Preferences dialog. You can also have FlowJo draw "large" dots, which are twice the size in both dimensions as small dots. This may make the display easier to see in publication or slides. This option is set via the [Preferences](#) dialog.

Dot plots also suffer from the under-emphasis of populations that are tightly-grouped. A more balanced representation is found with [contour plots](#), especially with outliers displayed.

Shown below is a typical dot plot graph. Axes are removed for simplicity.

□

You may view a comparison of all the [bivariate displays](#) for this same data.

Find this helpful?



Histograms

Histograms display a frequency distribution of the data, as a function of fluorescence intensity. The histograms can be smoothed (by default) or unsmoothed; this option has little effect when more than a few thousand events are in the gate. Shown below are the same dataset shown with and without smoothing turned on.

The Y-axis for histograms is the number of cells falling within each "bin" of the histogram; there are 256 bins for each histogram.

□

You can manually set the lower and upper limits on the Y axis; the default is to have FlowJo determine the optimal limits. To set these limits, use the [Graph Specifications Dialog](#), by opening the **Options** triangle in the graph window.

When you copy a histogram graph, two items are placed on the clipboard. One is the graphic rendition (such as shown above); the other is a text item containing two columns of text data that represent the histogram (channel numbers & channel counts). When you paste them into a spreadsheet, you will create the two new columns of the histogram data. (Note: a few graphics programs will preferentially paste a text item (the column data) instead of a graphic item from the clipboard. If you encounter this problem, set the Preference such that FlowJo only copies the graphic item onto the clipboard.)

See also the page on [CDF displays](#).

Find this helpful?



Generating Publication-Quality Output

FlowJo's sophisticated [Layout Editor](#) generates graphics that are publication-ready (or presentation-ready, for slides). However, you can also easily export the graphics to other programs to use their more complex tools for generating presentation graphics.

After reading the information on this page, you should [view some examples](#) of graphs that were easily created in [Canvas](#) from plots generated in FlowJo. One example is a contour plot with outliers; the other is an example overlaying histograms.

You can export graphics into other programs in two main ways: (1) simply [copy graphs](#) from any graph window and paste them directly into the drawing package; or (2) generate a layout, and either save the output to the clipboard (and paste it directly into a drawing package), or save the output to a disk file and read it from the drawing package. The discussion below applies to both of these export functions.

When you paste a graph into a drawing package (or read it from a disk file saved by a layout), it is a group of objects. You can ungroup the objects and access each one individually. All text is formatted as 12-point Palatino. All lines are one-point wide (with the exception of high-resolution contour plots; see below). The graph of the events themselves (internal to the axes), is either a line-drawing or a bitmap.

All histograms and CDF plots are copied as line drawings. This means that when you ungroup the plot, you can click on the histogram and change its line style (bold, dashed, color) and/or fill pattern (the default fill pattern is "No fill") and color. This allows you to overlay histograms and/or CDF plots easily (simply paste them on top of each other). (Note that you can also use the [Layout Editor](#) to overlay graphics for you).

The other plot styles (contour plots, dot plots, density plots, and pseudo-color plots) are copied as bitmaps by default. A bitmap is a single "paint" object: it does not consist of lines and polygons, but rather of pixels of a particular color. Dot plots and contour plots are "1-bit" bitmaps (black & white); the others are 8-bit depth to allow for color/grey-scale gradations.

Bitmaps have the advantage of being easily and quickly manipulated by drawing programs, and hence this is the default output style. As well, you will find that for most presentations or publications, the bitmap style is adequate. The disadvantage of bitmaps is that they cannot be resized without risking serious distortion problems. This makes the generation of complex figures somewhat more difficult.

Thus, FlowJo gives you the option of setting the default output of these kinds of graphs as draw objects ("high resolution PICT"). This setting is defined in the [preferences dialog](#). When you export graphs as draw objects, then they are easily resized without distortion. In addition, for contour plots, the contour lines are exported as "hairlines", which appear nicer on high-resolution printouts. Because each contour line is a polygon, you have the option to edit the contour lines to be different color or fill patterns to make your plot look exactly how you want.

The drawback of exporting draw objects is that it can be much more difficult for the drawing package to handle. This is especially true for dot plots, density plots and pseudo-color plots! You may find these to take a long time to draw or resize, because each dot (and each "pixel" in the density plots) is actually a separate object. Thus, a dot plot with 10,000 dots has 10,000 objects that the drawing program has to keep track of.

You may want to experiment with different output styles to decide which suits your needs the best.

Find this helpful?

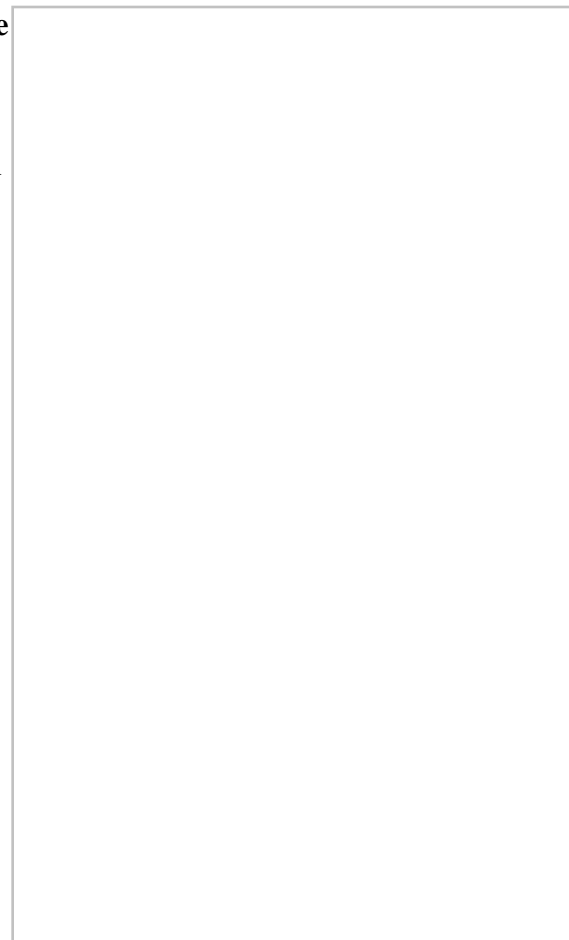
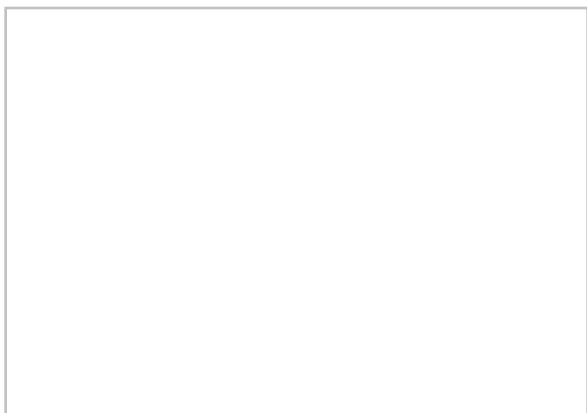


The PolyChromatic Plot

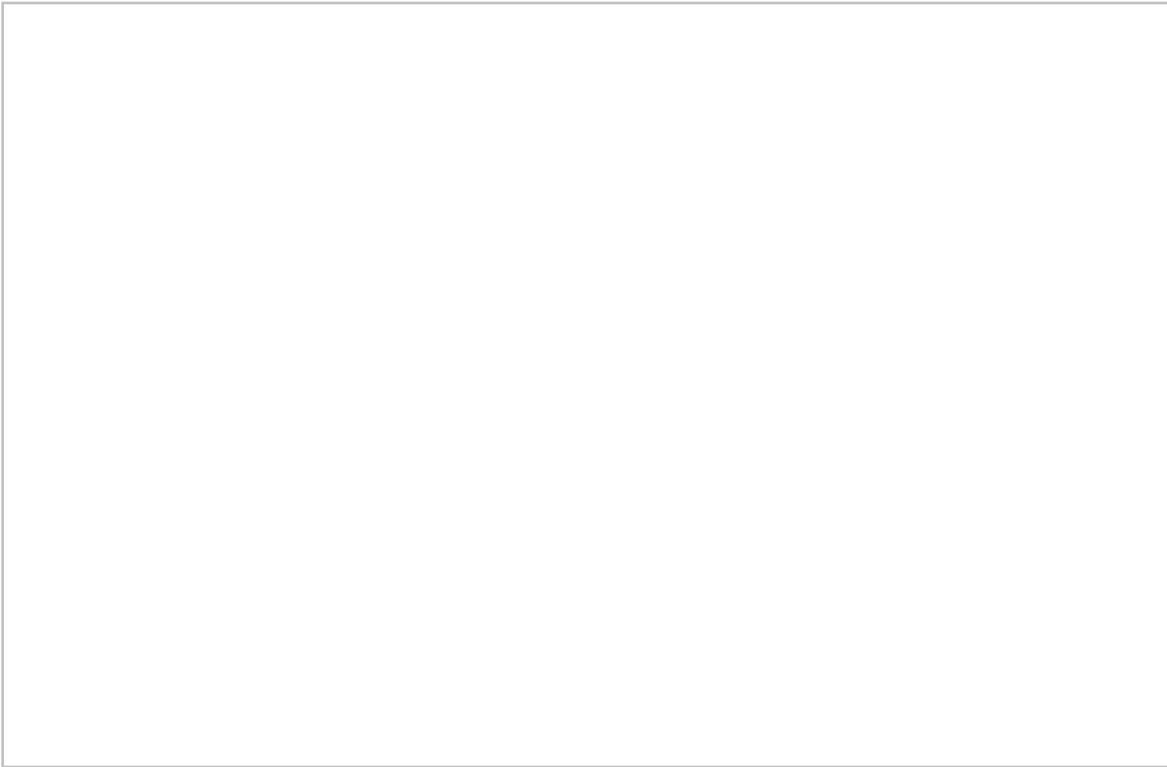
You can use color to assist in visualizing a sample's staining and intensity. For maximum contrast, the example at right has the **Background** color set to black and the **High Resolution** box checked.

Open a graph window and select **PolyChromatic Plot** from the **Graph type** menu in **Options**. This choice is also available under FlowJo's main **Graph** menu.

This will open a dialog window shown below, where you can specify which parameters are associated with which colors. Then you can [refine the color display](#) to maximize its clarity.



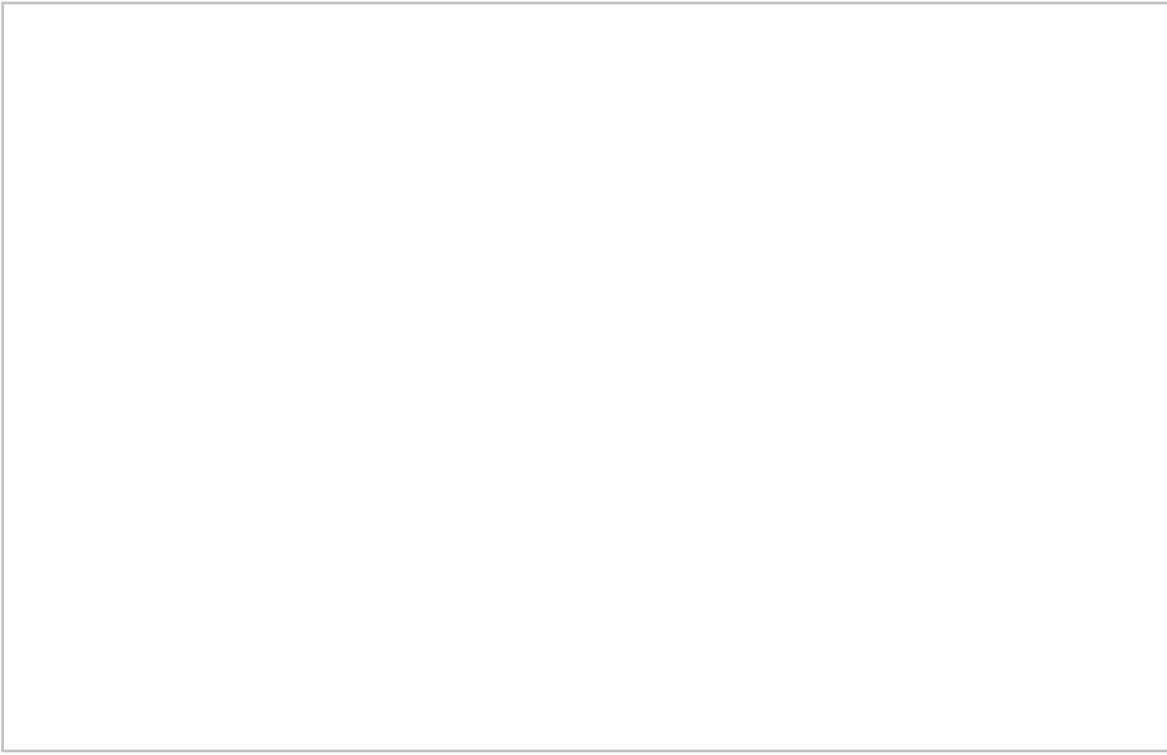
With only the first parameter assigned to a color, the display looks like this:



Assign a second parameter to a second color, the color spectrum changes as follows...



With a third parameter assigned to a color, the display reveals those events by color.



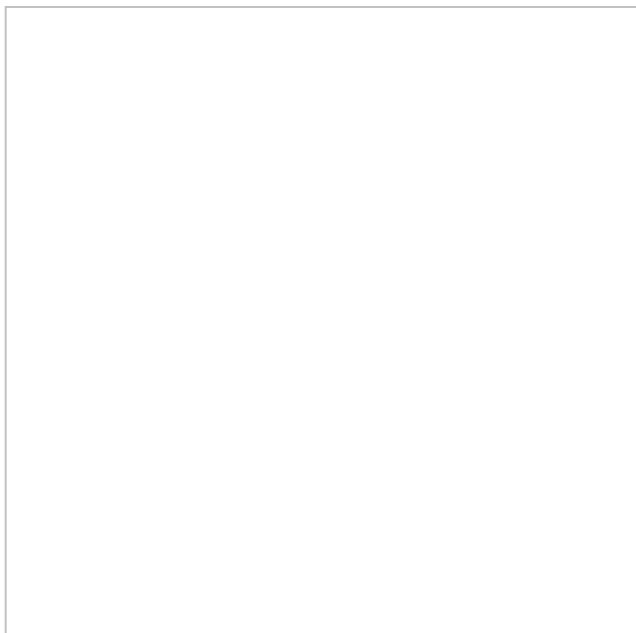
For information on the additional settings, [click here](#).

Find this helpful?

□□

Cumulative Distribution Function

CDF, or **Cumulative Distribution Function** plots display exactly the same information as do histograms. The difference is that the histogram values are summed as the fluorescence intensity increases; thus, the CDF begins at 0% (left axis) and ends at 100% (right axis). You can change the vertical scaling (to expand a portion of the CDF plot) by setting the appropriate fields in the [Graph Specification Dialog](#) (click **i** on the graph window to show this dialog). CDF plots are extremely useful for quickly finding the fluorescence of a distribution corresponding to any given percentile (such as the median). The sample plot below shows how to find the median (50th percentile), 75th, and 90th percentiles for this data. Note that unlike the histogram, the Y-axis is labeled in units of relative frequency. In this example, the median fluorescence of the population is about 6.0 fluorescence units.



The plot below overlays the CDF and the histogram for the same data. (This overlay was done by copying the graphs from FlowJo into Canvas and overlaying them in that program).

□

See also the page on [histograms](#).

Find this helpful?



Naming Nodes

FlowJo identifies populations (samples, or gated subsets of samples) by a name that you assign. Naming populations is very important for the way in which FlowJo works... especially for [batch operations](#). The rules for naming gates are similar to what we expect for rules for naming people in families: siblings cannot have the same name; however, different generations can have gates with the same name.

When you specify a kind of analysis (for instance, a statistical measurement, or a gate selecting a subset of cells), it is applied to a population. During batch operations, you can specify that this analysis be applied to all samples within a group. FlowJo decides how to do this by looking at the names of the gates.

For instance, you may have 3 samples in a workspace. You first decide on a gate to exclude dead cells and clumps of cells; you apply this gate to a group containing all three samples (and call it "Live cells"). After looking at the 3 samples, you find that you have to alter the position of the gate slightly for each one--therefore, the gates are no longer identical.

Now you create a statistic that tells you the median forward scatter signal for "Live cells." Thus, you create a new node (analysis) attached to the "Live cells" gate of the first sample. When you now drop this node on the group's version of "Live cells", the node is attached to each sample's version of its own "Live cells" gate. Even though each gate is slightly different, FlowJo assumes that the gate represents the same population and thus you want the same operations applied to that gate.

This is why a gate cannot have the same name as another gate of the same population: FlowJo would not be able to decide which gate you want analyses applied to. Note that FlowJo decides which gate you mean by not only name, but generation. Thus, if you have a sample which has a "Live Cells" gate as a child of a gate you named "Lymphocytes", this is NOT the same gate as "Live Cells" attached directly to the sample--and, in the example above, would not have your statistical analysis applied.

Realize that the complete name of any population is actually the concatenation of all of its ancestors' names with its own name. Thus, in a sample, if you have created a lymphocyte gate, and within that a CD3 gate, then that subpopulations "true" name is "Lymphocyte/CD3". FlowJo only shows you the "CD3" portion of the name, as the "Lymphocyte" portion is implied by the indentation in the workspace. You could subset the "CD3" population and also call it CD3. This is legal, because its full name would be "Lymphocyte/CD3/CD3" which is distinct from "Lymphocyte/CD3". However, it could be confusing--so try to avoid duplicate gate names at any level.

If you wish, you may get help on how to create [new subpopulations](#) by gating.

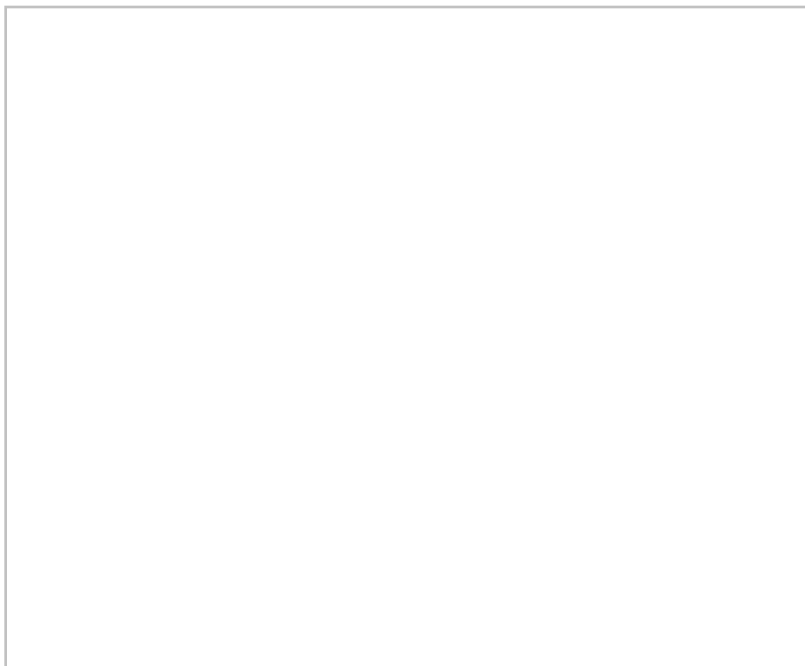
Find this helpful?



Manual Gates

To manually enter a gate, display the graph on which you wish to enter a gate. For instance, to manually type in a lymphocyte gate, you might display Forward vs. Side scatter as below. (If you display a histogram or CDF, then you will be entering a one-dimensional gate.)

From the **Graph** menu, select **Manually enter gate**. You will be presented with the following dialog box. Enter the lower and upper limits of the gate for each axis...



From the **Channels** pop-up menus, choose whether to use units of channels, units of absolute intensity or fluorescence intensity percentile. FlowJo tells you the minimum and maximum acceptable values for the gate.

When you select **percentile**, FlowJo creates a gate based on the percentiles that you enter. For instance, if you select a gate that has a lower bound of 50 and upper bound of 100 (for percentile), then FlowJo constructs a gate which includes the brightest half of all of the events. Likewise, a percentile gate of 0 to 5 results in a gate for the dimmest five percent of the events.

When you make a new gate based on the fluorescence distribution (percentile), the gate is stored in terms of the channel numbers. Thus, when you copy the gate to another sample, it is copied with regard to its position in the window. This way you can make a gate on the brightest 5% of events, copy it to other samples and see what fraction of events are above that value in those samples.

If you select the **Make Quad Gates** check box, you can enter the X and Y coordinates for the center point of the four quadrant gates.

You may also choose to manually divide a graph into a grid of contiguous gates by checking the **Make a series of gates** button.



FlowJo shows the numerical range of the data for each axis, in this case, 0-100 for percentile measurement. The X Axis will be divided every 25th percentile, so it will be divided into four equal parts. The Y Axis will be divided every 25th percentile, so it too will be divided into four equal parts. The graph then will be divided into a 4 by 4 grid of 16 gates. FlowJo will display 16 new gates when you click the **Finish** button.

Find this helpful?



Quadrant Tool

Use the quadrant tool in the **Graph** window to create **quadrant gates**. FlowJo implements quadrant statistics by creating four adjacent but nonoverlapping rectangular gates.

When the quadrant tool is selected, and if you have not deselected the **Show live quadrant statistics** preference in the preferences dialog (**Preferences/ Gates/ Quad Gates**) then FlowJo will track a cross hair over the graph window. It will also continuously update the frequencies of events within each quadrant (these frequencies are the percentage of events falling within each quadrant, with respect to the events that fall in the current subset. Thus, the four frequencies add up to 100%).

The live quadrant statistics are also displayed when dragging to reposition the crosshairs defining the quadrants. Once you click to select the quadrant position, FlowJo will create four rectangular gates as subsets of the current population. FlowJo names the gates according to the parameters and each quadrant position. A quadrant gate is therefore named as "Q: parmX± parmY±", where parmX and parmY are the X axis and Y axis parameters, respectively, and "±" will reflect whether the quadrant is the higher (+) or lower (-) quadrant for that parameter. (If necessary, FlowJo may truncate the names of the parameter stains such that the overall gate names are not too long).

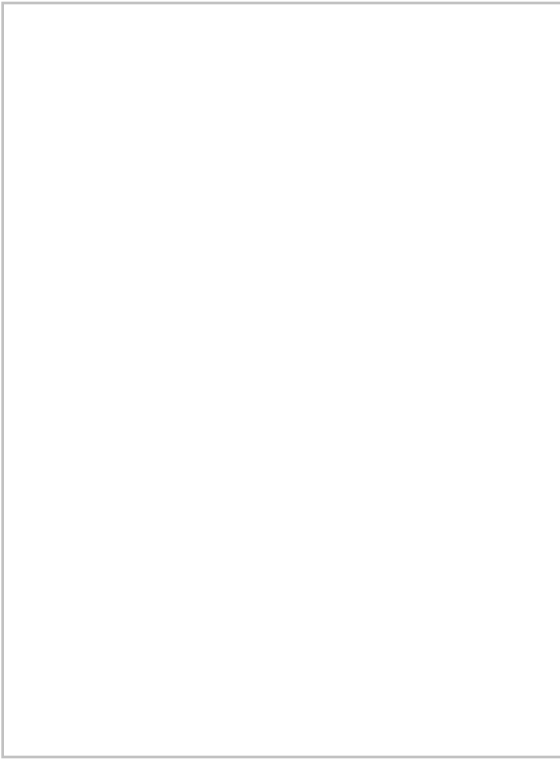
Once you have created the quadrant gates, each one behaves as a separate subset gate. You can independently move any single quadrant gate (note that then you will no longer necessarily cover all events in the plot!). If you want to move all four quadrant gates together, shift-select each of the quadrants in the graph window. Now you can move them to a new position. You may also click on the central, shared vertex of quadrants to move all four simultaneously.

When you have quadrant gates on a graph, they cover the entire area of the graph at all times. This means that other gates may be "below" these gates. To select other gates, press the command-key while clicking in an area of the graph: this will select the next gate in the same quadrant occupied by the mouse.

Spider Gates

Opt-clicking the vertex unlocks the right-angle constraint of quads, creating Spider Gates. Like quad gates you can move the spider gates independently, or Opt-click to reposition their common vertex.

To reposition the vertices at the edges of the plot, select all with CTL+A and drag anywhere within the enclosed area to move the gates as a group.



Find this helpful?



Magnetic Gates

Draw any gate in the Graph Window and you can make it *magnetic* by clicking on the appropriate option in the Graph Tools Window. Once you make a gate magnetic, FlowJo moves it until it accommodates the maximum number of events--i.e., it relocates to where the events are. When you copy a magnetic gate from one sample to another, it is automatically readjusted. If a parent gate is modified, then the magnetic gate is again recalculated to accommodate the maximum number of events.

This is useful for instances where the population may move slightly from sample to sample and you don't want to have to individually adjust each sample's gate.

When FlowJo moves a magnetic gate, it draws a black arrow in the graph. The arrow is drawn from where the gate was originally set to where the gate has been moved by FlowJo. If the gate only moves a short distance, you may have to drag aside the white *Frequency of Parent* label to see the arrow. Gates which move a long distance should be checked for correct positioning!

Magnetic gates are, in every other way, exactly like normal gates: you can create subsets and add statistics to populations based on magnetic gates. They are supported in both univariate (histogram) and bivariate gating tools.

The workspace displays the presence of magnetic gates by changing the icon to a magnet, shown here in the Lymphocytes ->



Find this helpful?



Copying Gates

Copying analyses (gates, statistics, kinetics, cell cycle, etc.) from one sample to another is very easy: simply click on the analysis (a gate, statistic, or population node), and drag it to the destination. The node is added on to the destination node as a "child" of the destination (i.e., it is applied to only those cells which fall within the population you drop the analysis on).

You can drag entire trees around this way, selecting children, parents, or multiple nodes. There are specific rules for how these are taken around when you drag; see the pages on [dragging and dropping](#).

Note that if you drag a subpopulation onto a population which already has a subpopulation with the same name as that you are copying, then FlowJo asks what you would like to do: you can replace the existing subpopulation, you can retain the existing subpopulation, or you can add the current subpopulation with a change in its name. (The reason for all of this is that FlowJo does not allow you to have two subpopulations with the same name at the same level of analysis). These options are more fully described in the pages on [replacing nodes](#).

You can automate the process of applying analyses to samples by using [groups](#). You can add analyses to a group; then every sample which belongs to the group or is later added to the group gets those analyses. This is one of the powerful batch analysis features of FlowJo. You may view an example of [group-based analysis](#).

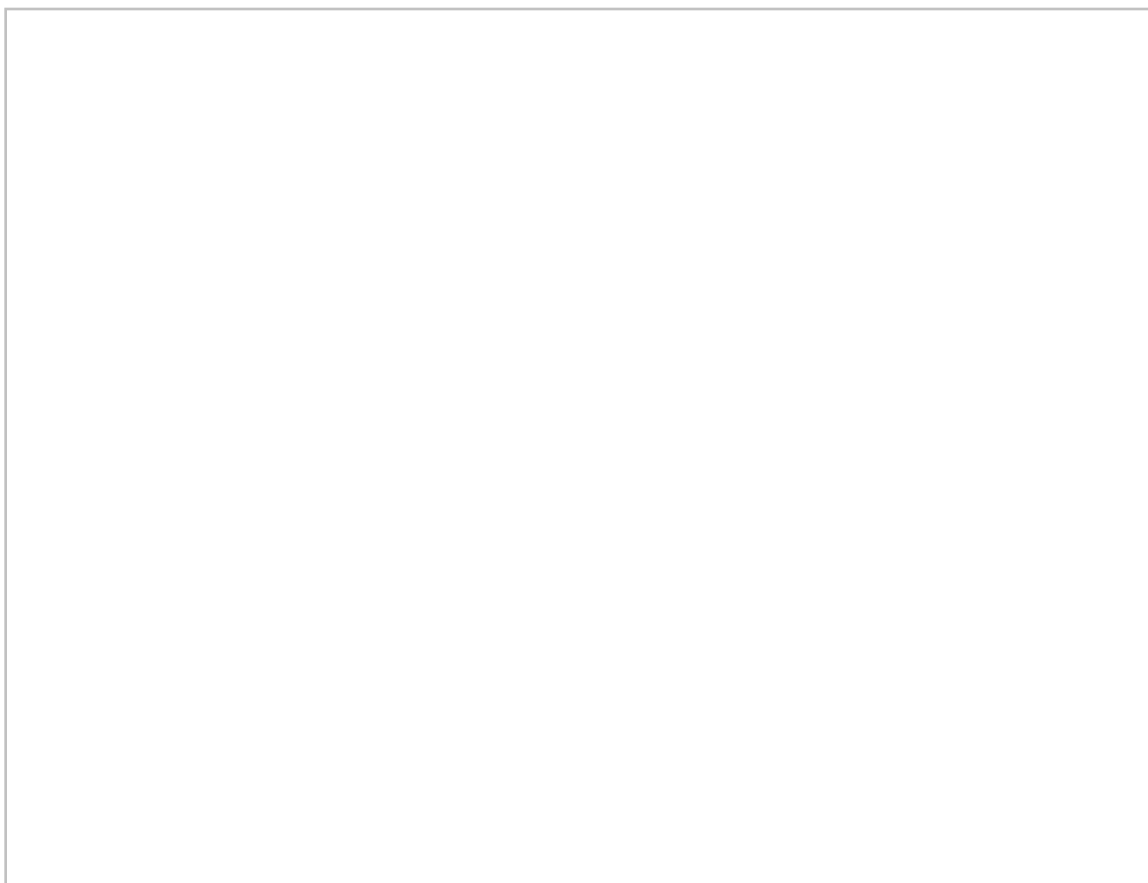
You can drag analyses across workspaces; in other words, you can drag an analysis node from one workspace and drop it onto a sample or a group in a different workspace. You need to have both workspace windows open to do this; simply drag from one to the other.

Find this helpful?



Sample Validity Platform

FCS files can suffer from flaws introduced by the cytometer during acquisition. FlowJo can test the validity of selected samples in the Workspace, or of all samples as they are loaded into the workspace. You can adjust the sensitivity of the test to allow small measurement fluctuations. You can exclude parameters that are not pertinent to the current analysis.



Select samples of suspect validity in the Workspace. Open the utility from **Platforms > Verify Sample Validity**.

If you wish to test every subsequent sample, check the box in the upper left (red circle above.)

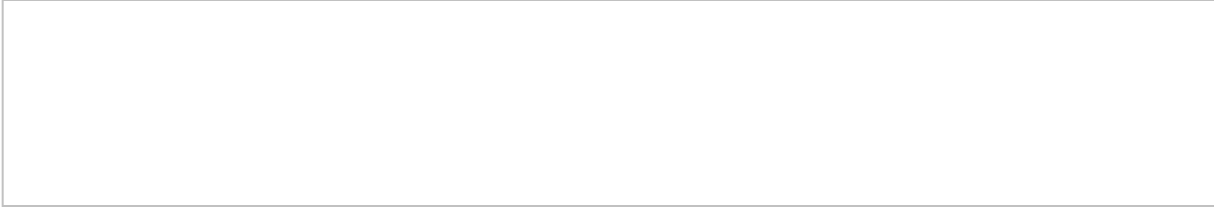
The samples you selected will be listed on the left. Sample 1 in the example above was highlighted, then checked individually by pressing the **Check Sample Validity Now** button. The black dot in the **Chk?** column indicates that it has been checked. Had it failed, there would also be a dot in the **Fail?** column.

Sample 3 is highlighted and ready to be checked by the same procedure.

- You can exclude from checking any files with too few events. Enter the minimum number of events to consider in the text box 1.
- You can choose to exclude any parameters by selecting them from the **Add to list...** drop-down menu 2.
- You can narrow the scope of your validity check by removing check marks from any of the subtests... **Value Spikes, Measurement Drift, Event Rate Uniformity**.
- You can also change the sensitivity of the test to be more or less tolerant of mechanically induced error. Next to each sensitivity text box is a suggested range of adjustment. In each case, a lower sensitivity setting rejects more

samples.

- All selected samples may be checked simultaneously by clicking **Check All Samples**.
- Using the **Copy Report** button 3, the text of the validity check can be copied as a list of tab separated values, and pasted into another application. Here's an example pasted into Excel.



Clicking **Show Time Plots** opens the window below. For more on this tool, [click here](#).



Find this helpful?

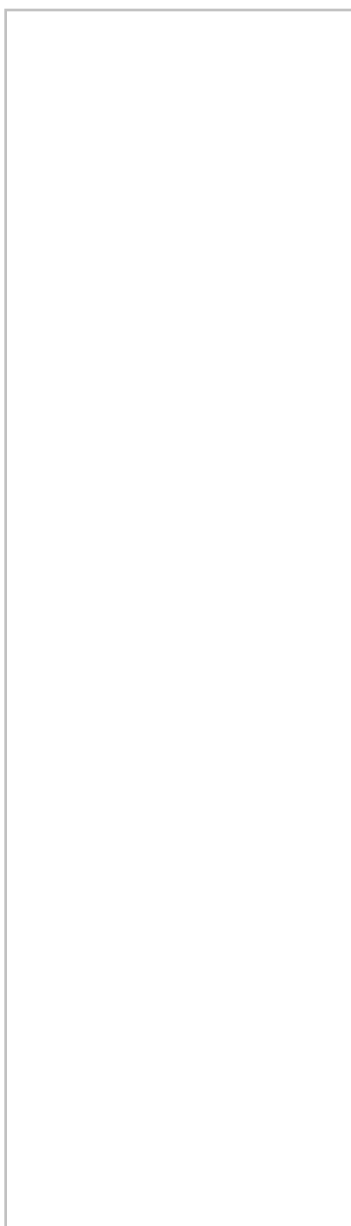


Derived Parameters

When you choose to add new or edit derived parameters, you will be shown the dialog window below:



From this window you will manage all derived parameters for a sample. You can add any algebraic parameter as well as time, ratio, or log/linear conversions). The algebraic parameters can be created by using the pulldown **Insert Parameter** list (as seen above) and the **Insert Function** list just to its right. As you can see below - there is no limit to the functions you can choose from!



You can have as many derived parameters as you wish (but only one time parameter can be defined). In all cases except when you add a linear ratio parameter with a defined scale, FlowJo computes the optimal scaling factors to display the most events onscale. In some cases, this means that the same derived parameter for different samples may have different

scaling. Thus, you should be careful if you copy gates based on such derived parameters between samples to ensure that the gates are appropriately placed.

Use the **Show Range of Computed Values** button to display the upper and lower limits of the parameter's values. You can then manually enter values for the highest and lowest number you want to display on the axis. If you wish your derived parameter to be displayed on a linear scale, check the **Display With Linear Scale** checkbox.

Each newly-defined derived parameter is given a parameter name combining the type of function with the parameters of the function (e.g., "FL4/FL5" for ratio). Because parameter names for any given sample must be unique, adding a second derived parameter of exactly the same type (& parameters) will result in the new name being appended with a number (e.g., "FL4/FL5 -2").

The bottom of the dialog window shows currently defined derived parameters; you can choose to delete any or all of those parameters. Of course, if you have any analyses in the workspace that are based on those derived parameters, then they too will be deleted when you delete the parameters.

These additional functions can be added manually:

- sin (sine)
- cos (cosine)
- acos (arccosine)
- atan (arctangent)
- sinh (hyperbolic sine)
- tanh (hyperbolic tangent)
- norm (Normal random)

Go back to the overview on [Derived Parameters](#).

Find this helpful?



Derive Parameters Submenu

The **Derive Parameters** menus are a sub-menu below the **Platform** menu.



Define new or change...

This menu item is only available if a single sample is selected in the workspace. It brings up the dialog window to [define new derived parameters](#), or edit existing parameters for the sample.

Copy to group's samples

This menu item is only available if a single sample is selected in the workspace, and that sample has derived parameters attached to it. It copies the definitions for derived parameters in the current sample to all other samples in the current group. Note that these derived parameters are appended to the list of derived parameters already existing in other samples. If you copy a ratio parameter to another sample that already has a ratio parameter (with the same numerator and denominator), then FlowJo will rename the new parameter by appending a version number (e.g., "-2") in order to maintain unique parameter names. This can cause potential confusion when gates are copied, since gates are defined on parameter names. FlowJo will notify you if any of the derived parameter names had to be modified to perform the copy.

Delete derived parms...

Delete any derived parameters for all selected samples, or (if a group was last selected) for all samples in the current group.

Calibration Standards

If you have created calibration standards via the [Calibration Platform](#), then these will be listed individually under the Derived parameter submenu. (Like the one named Absolute shown at the bottom of the menu above.) You can select one of these directly to apply it to the selected samples (or group of samples) without going through the derived parameter dialog.

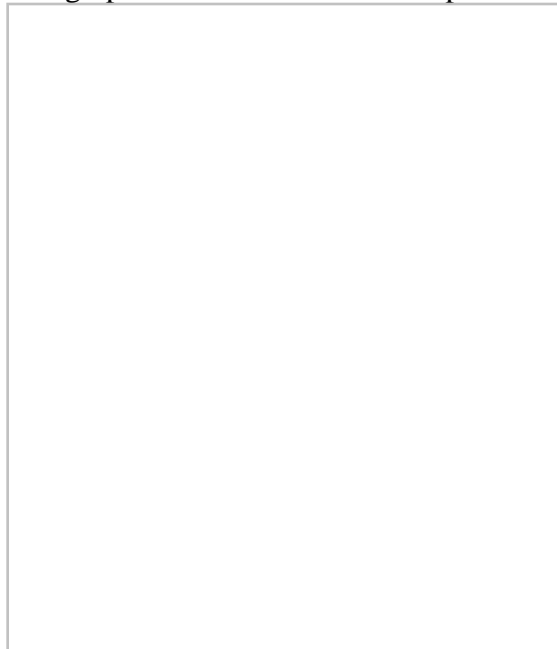
Go back to the overview on [derived parameters](#).

Find this helpful?



Compensation Interface

The graphic below shows the compensation definition window and its controls.



There are several ways to apply the positive and negative populations you have defined into the comp matrix dialog boxes. The simplest is to define a positive and negative population on each single stained comp control sample and drag each of these to the boxes beside the appropriate parameter (see **a** below.) However, you can have a *universal* negative that can be used for all compensation controls that have only positive populations. For example, when using antibody capture beads to compensate, each sample has beads that capture one color (with no negatives). Another sample contains unstained beads and is used as the negative population for each parameter (see **d** below.)

a) Drag the positive and negative subsets from the workspace to the boxes beside each parameter in the Comp Definitions window.

OR

b) Drag a subset that has one or two gated subsets to the Auto Assign Button. FlowJo looks at each subset you drop on it, and looks for child gates. If there is only one child gate, it assumes that this represents a positive comp control population. If there are two child gates, it assumes that there is a positive and a negative.

OR

c) Drag a sample that contains a mix of singly stained comp control bead samples to the PolyComp Button. FlowJo automatically finds the positive populations for each parameter. Define the negative population by dropping the gated unstained bead sample onto the universal negative button.

OR

d) Drag an unstained sample or negative subset to the Universal Negative button. FlowJo puts this negative population into all the negative boxes for each parameter. In general, you should drop the universal negative on the comp window first--because if you do it second, it will replace any existing negative populations you have already added.

If you have dragged the incorrect population into one of the boxes, simply select it and hit the delete key to remove it from the box. Or, you can drag and drop the correct population onto the box, replacing the existing one. You can know what population is in a box by selecting that box (clicking on it); the name of the population that you dragged into the box will be shown in the lower left corner of the window.

Multicolor background gates are best for identifying positive events. The “negative” populations will always contain a mixture of positive and negative events because of the inescapable overlap in distributions.

Procedure. In order to calculate the background in FlowJo, you need to compensate your data in FlowJo. Click on the **Record Background Gating Data** option . The calculation of background works best with single color compensation beads, but can be done with stained cells.

Note that some flow cytometers collect a fluorescence parameter in the SSC-W channel. This channel will therefore be displayed in the compensation dialog window when sample files in the workspace contain this parameter.

Go back to the [overview of compensation](#).

Find this helpful?



Compensation Example

This is a simple example showing how to do three-color compensation in FlowJo. There are several steps involved.

1. Set a gate for the positive and negative populations for each singly-stained sample.
2. Bring up the [Compensation Definition dialog](#) window, or [Compensation Wizard](#).
3. Drag each of the positive and negative populations from the workspace window into the appropriate box in the compensation dialog window.
4. Compute the matrix, save it onto disk.
5. Apply the compensation matrix to the appropriate samples.
6. Display the compensated parameter (in brackets $\langle \rangle$) on the graph axes.

You can follow this example on your own computer by downloading the [Compensation Demo Data](#).

In some cases, you will need multiple compensation matrices for the same experiment. This is true, for instance, when you are using different lots of tandem dyes (like Cy5PE). Different Cy5PE reagents (even from the same manufacturer) have different spectra and thus require different compensations. You will need to collect a singly-stained compensation control sample for each different Cy5PE reagent, and then construct a different matrix for each of these.

When making multiple matrices like this, you only need to repeat the drags of the successive Cy5PE populations followed by steps 4 and 5, saving a different matrix for each set of reagents.

The next page begins [the tutorial](#).

Go back to the [overview of compensation](#).

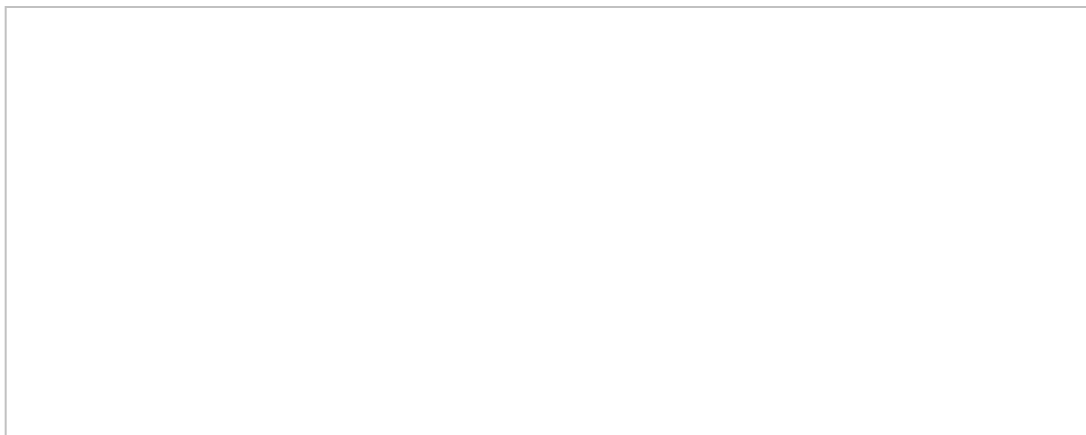
Find this helpful?



Editing the Compensation Matrix

If you are not familiar with the format of the [Compensation Matrix](#), read that page first.

Starting with FlowJo version 4.5, you can easily change the compensation matrix values. When you create a new compensation matrix, FlowJo no longer automatically saves it to a file. You can edit the compensation matrices directly from FlowJo, using the **Platform-> Compensate Sample-> Edit/Save Matrix** command (from the **Platform -> Compensate Sample** menu, you can also **Remove Compensation**).



From this interface, you can save matrices to disk (so that you can import them into other workspaces), you can create an entirely new matrix from scratch, or you can modify an existing matrix. (FlowJo will always show you which values you modified.) If you have graphs open, they will automatically update to show you the changes you are making. If you check the **Immediately recalculate Workspace statistics in current group with changes** checkbox, your statistics will update live to reflect changes made to a matrix.

□

Note that you can edit a hardware compensation matrix by choosing to create a new matrix using the acquisition matrix as the source. The acquisition matrix is created when you set your compensation on a new generation instrument such as the DiVa, FC500, or CyAn.

Warning! Manual alteration of the spillover coefficient matrices is not trivial, especially when more than a few parameters are involved. If you find such alterations necessary, then perhaps you should consider the quality of the compensation controls.

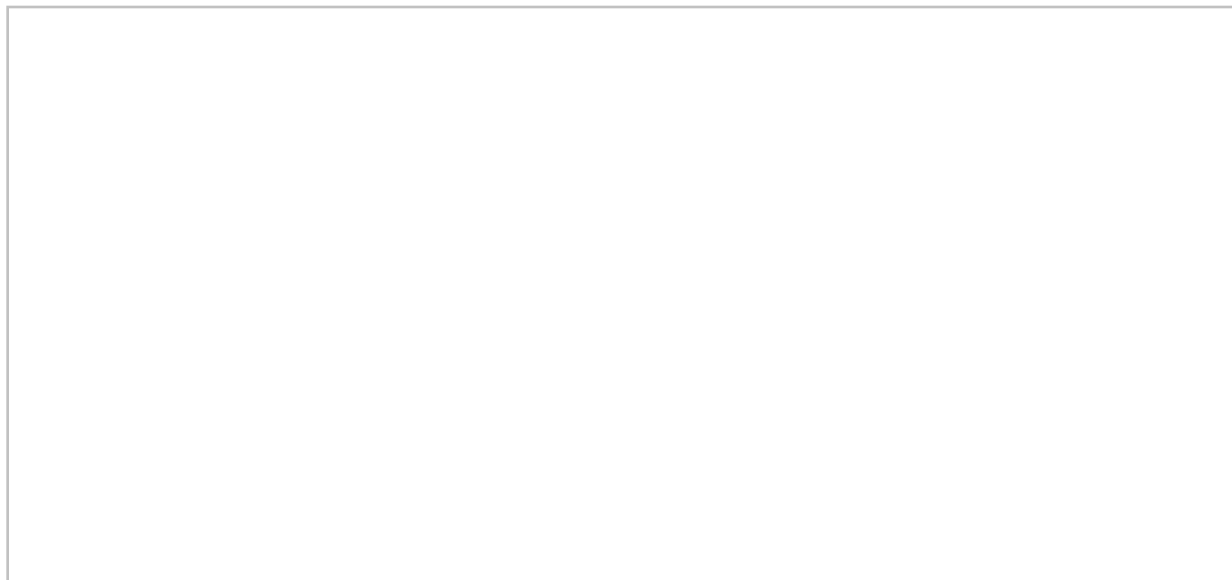
Go back to the [Overview of Compensation](#).

Find this helpful?



Compensation Menus

The compensation menus are a sub-menu below the **Platform** menu.



Wizard...

Brings up the [Compensation Wizard](#) Window to begin the process of defining a new compensation matrix. To use this menu item, select control tubes or drag them from the workspace window. Give your Matrix a name.

[AutoCompensation Scripts...](#)

This tool helps you define a script that will automatically select a set of compensation controls from new data.

Define New Matrix ...

This is the familiar Compensation Interface window.

[Edit/Save Matrix...](#)

You can [edit the compensation matrix](#) and you can save the matrix to be used in another workspace.

Load Matrix from File...

Asks you to open up a previously-created and saved compensation matrix file and load it into the workspace. To save a matrix file from another workspace, use the Edit/Save matrix option.

Import Matrix from Other Workspace...

Lets you grab a matrix that is resident in another Workspace open on your desktop.

[Define BiExponential Transformation...](#)

If a lot of cells are squished against the axis after compensation, you can change the display. This allows a more intuitive display of the data by changing the scale of the graphs using [Bi-Exponential scaling](#).

Acquisition Matrix/Reapply Acquisition Matrix

If you compensated your data during acquisition on a new generation of instruments (DiVa, FC500 or the CyAn), the acquisition matrix will be available for editing. If you have applied another matrix, you can return to the Acquisition Matrix with this command.

Apply Your Matrix

If you have created or loaded matrices, the names will appear below **Acquisition Matrix** (like "Matrix1") on the above menu. Click on a name to apply the matrix to selected tubes.

Go back to the [overview of compensation](#).

Find this helpful?



Compensation Matrix File

When the "Compute" button in the compensation matrix dialog window is clicked, FlowJo generates a compensation matrix. If you save the matrix to a file (using the "Edit/Save Matrix" option), the file looks like this:

CompMatrix

< >

Fluor	PhyEry	Cy5PE
1.0000	0.1803	0.0404
0.0091	1.0000	0.2595
0.0045	0.0473	1.0000

The first line has the title of the compensation matrix. The second line has the names of the fluorescence channels that are being compensated. Channels that are not in this list are not compensated; channels in this list that are not in the sample are ignored. The names must match exactly; use the names that appear in the \$PnN keywords of the FCS file. The names are separated by tabs.

The subsequent lines are the spillover coefficients, corresponding to each of the channels named. The rows are the spectra of each channel; the columns are the contributions of each fluor into the channels. Thus, 0.1803 is the spillover coefficient between FITC and PE, or the amount of FITC signal in the PE channel for a FITC-stained cell; 0.0091 is the amount of the PE signal in the FITC channel for a PE-stained cell. The coefficients are tab-delimited. In very rough terms, the 0.1803 corresponds to approximately 18% spillover (PE-%FITC).

[Click here](#) for instructions on modifying the matrix. In version 4.5 and after, the matrix can be modified right in FlowJo using the "Edit/Save Matrix" menu option. When you modify the matrix, the numbers are converted to give an estimation of the amount (percentage) of spillover into the other parameter.

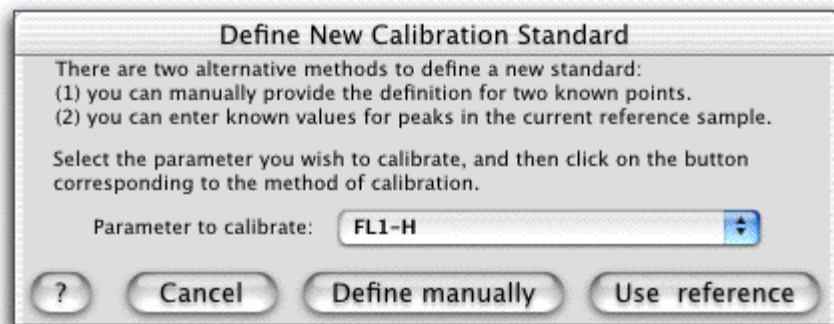
Go back to the [overview of compensation](#).

Find this helpful?



Define Calibration Standard

When you select the "Calibrate..." command from the workspace menu, you are shown the following dialog window:



First choose which parameter you wish to calibrate from the pop-up menu. By default, FlowJo searches for a parameter that most likely corresponds to phycoerythrin staining, since this is the most commonly calibrated parameter.

You can then choose either [Manual Calibration](#), where you will enter values for one or two points to calibrate the parameter of choice, or, you can choose to use the current gated sample as a [Reference Standard](#). If you wish to use a reference standard, make sure that you have selected the appropriate gate in the sample list of the workspace! FlowJo will use that gated sample for generating the reference peaks.

Go to the overview of [Calibrated Parameters](#) (which also has links to examples of using the Calibration Platform).

Find this helpful?



Calibration Examples

Below are links to two different examples using a reference standard to create a calibrated parameter. The first uses an 8-bead set, and shows you how FlowJo creates different kinds of regressions depending on what options you choose. The second uses CD4 expression as a reference standard, and then shows you how graphs and statistics are computed based on the calibrated parameter.

In both cases, you should first gate on the original collected sample to "clean" it up. In the case of beads, you should gate on "singlets" based on Forward and Side scatter (as shown below.)



This aids FlowJo in identifying the peak positions corresponding to each bead. Select the gated subpopulation in the Workspace window, and then choose "Calibrate..." under the Workspace menu.

For the CD4 calibration, you would gate on lymphocytes then select the lymphocyte gate as a calibration. Then select the appropriate menu item.

See the [bead calibration](#) example, or the [CD4 calibration](#) example.

Go to the [overview of Calibrated Parameters](#).

Find this helpful?



Kinetics Tips

Here are some quick answers for how to perform certain kinetics analyses. This list is not comprehensive; you should read the main [Kinetics help pages](#) to understand how the platform works before you examine these tips.

How can I determine the fraction of responding cells?

The fraction of responding cells is defined as the percentage of events with a fluorescence (or ratio) above a certain threshold value. You can set this value manually; choose to display **% of events over threshold** under **Function** in the Kinetics **Options** box, then click on **Set New Threshold**. Enter the exact value you want to use as a threshold.

□

Another strategy might be to define a certain time period of the analysis as the "background" time. This might be the first 30 seconds of collection; perhaps it is the end of the collection period. Create a timeslice corresponding to the background period. Then, after clicking on **Set threshold** in the Kinetics Options box, select that time slice in the popup menu. Now choose which percentile of the events in that time slice to set as the background. For example, if you choose the 95th percentile, you ask FlowJo to define the threshold as the 95th percentile of events in your timeslice (thus, the background time has 5% responding cells.) You might also choose the 98th percentile (to limit to 2% responding cells during the background).

The advantage of the second strategy is that when you copy the kinetics analysis to other samples, you don't have to adjust the threshold manually for each samplerather, FlowJo calculates it with respect to the particular time-period you define as background.

I have determined that only 40% of my cells respond; how do I display the fluorescence of only the responding cells?

Here you have two options. First, realize that the median fluorescence of the top 40% of a population is the 80th percentile of the entire population. Thus, you could display the 80th percentile as a function of time, and you will get a plot that gives you, essentially, the median fluorescence of the responding population as a function of time.

Alternatively, you could define a response threshold (see above), and then choose to display the mean or median of events above this threshold.

I wish to perform more complex statistical analyses on the kinetics data using other programs. How can I get the kinetics data?

Simply copy from the graph window and paste into your spreadsheet. Note that if you have selected smoothing, then smoothed values are copied. In other words, whatever values were used to plot your graph will be copied to the spreadsheet. (Note that there is a **Preference** that controls whether or not the data values are copied to the clipboard in addition to the graphic; the default setting is to copy both.)

Find this helpful?



Kinetics Graph Specification

When you click the Options disclosure triangle in the kinetics window, you are shown the following options. They modify the attributes of the current graph (or currently-selected gate.)



1. Select a function for the Y parameter.
2. Select a smoothing function.
3. For percentile functions, select the percentile value here.
4. If checked, graph axes are scaled to the data.
5. If Auto is not checked, enter Y Axis limits here.

The functions that can be displayed are each calculated on the Y parameter for each time interval across the entire graph. FlowJo selects an appropriate time interval over which to calculate the function values (usually, this value is 1 channel of the time parameter). After the function is computed, any smoothing that has been selected is applied; the result is graphed in the window. The Y axis scaling is also defined in this window: either automatic (FlowJo scales the Y axis so that the minimum and maximum values of the graph will be onscale) or, you can set the minimum and maximum Y axis values manually.

The possible functions that can be displayed are:

- Median: the median fluorescence of the events in each time interval.
- Mean: the mean fluorescence of the events in each time interval.
- Geometric mean: Same as mean for linear Y parameters; otherwise, it is average of the channel values converted to the scale of the Y parameter.
- Percentile: An arbitrary percentile of the events in each time interval. Entering 50 in the percentile value generates the same graph as Median.

The remaining functions in the drop-down list are computed based on a threshold value. The threshold is a scale value of the Y axis parameter (i.e., not the channel value, but the scaled linear fluorescence value). The threshold can either be an absolute value, or a relative value based on time slices that you have defined (see information on [defining thresholds](#)).

The first threshold function is just the fraction of events that are above the threshold for each time interval. If you were to define a certain threshold fluorescence value as the minimum for a cell to be considered a "responding" cell, then the resulting graph would show you the fraction of cells responding as a function of time. Each of the other functions gives you the appropriate statistic calculated only on the cells above the threshold value. Thus, you could, for example, plot the median fluorescence of responding cells to eliminate the averaging in of non-responding cells.

The two possible smoothing functions are **Moving Average** and **Gaussian**. They are similar; the Gaussian smoothing places less weight on values more distant from the center. The smoothing width defines approximately how many time intervals are averaged for each displayed time interval; a smoothing width of 1 implies 3 time intervals are averaged for each point.

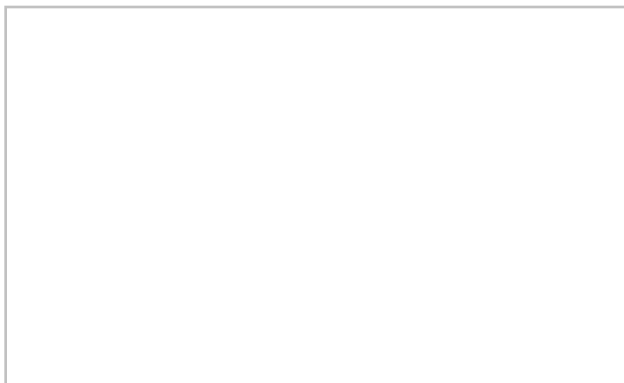
Go to the overview of [Kinetics](#).

Find this helpful?



Naming Timeslices

When you create a new time slice (by clicking and dragging an interval in the kinetics window), or when you want to modify a time slice (by double clicking on the row in the kinetics table corresponding to that time slice), you are shown the window below:



Enter the name for the time slice (it must be unique with respect to existing time slices in this analysis). In addition, you can modify the exact start and end times if you wish. When you are finished, click OK to accept the values, or Cancel to leave the kinetics analysis unchanged.

Go to the [Kinetics overview](#).

Find this helpful?



Cell Cycle Hints

FlowJo uses two different computational models to find the peak and S-phase populations from a univariate distribution curve. For more information on the algorithms used in the software, please see:

Watson, Chambers, & Smith: A Pragmatic Approach to the Analysis of DNA Histograms with a Definable G1 Peak [Cytometry 8:1-8 (1987)]

Fox: A Model for the Computer Analysis of Synchronous DNA Distributions Obtained by Flow Cytometry [Cytometry 1:71-80 (1980)]

Unfortunately, no algorithms are consistently reliable in fitting all the distributions you may encounter. You may have to experiment with different options and constraints in finding your best results.

You should first apply the models of choice to the data without any parameter constraints. For most distributions, FlowJo will be able to locate the G1 and G2 peaks, and thereby accurately fit the distribution with the model. (Of course, some distributions are not easily fit by some of the models; you may have to select an alternative model or an option to the model to obtain a better fit.)

When you encounter a difficult-to-fit distribution, you will want to begin constraining parameters. The first parameter to constrain is usually the G2 peak position (if the G2 peak is hard to find), or the G1 peak position (if the G1 peak is hard to find). Here you may wish to analyze a control sample that has a good distribution, and set constraining ranges for the peak(s) based on that sample. When you copy the Cell Cycle analysis to the difficult sample (by drag-and-drop), the ranges will automatically be applied. FlowJo now constrains the peak positions; this may be sufficient to help it fit the data.

Also consider constraining the peak positions relative to each other. For example, you can define the G2 peak position to be 2x the G1 peak, or the G1 peak to be 0.5x the G2 peak. By constraining this particular parameter, you make it easier for FlowJo to fit the data.

If the fit still looks awful, consider constraining the CV of the ill-defined peak. If the CV's for the two peaks are significantly different, then you should probably perform this constraint anyway. For most analyses, the G2 CV should be equal to the G1 CV; you can set either one to be equal to the other, relieving that parameter from the fitting model.

By judicious use of ranges to constrain peak positions, and relative or absolute values for the peak widths, you should be able to fit nearly any distribution.

Note that the "2-populations" model is very difficult to use. There are 24 different parameters that are fit in the unconstrained model; therefore, it is difficult for FlowJo to generate a well-behaved model without guidance. If you need to use this model, you should probably constrain at least the positions of the two G1 peaks, and the relative positions of the G2 peak(s), as well as the relative CVs of the G2 peaks.

Find this helpful?



Change Graph and Models for Cell Cycle Platform

Open the disclosure triangles at the bottom of the Cell Cycle platform window. You can choose the model you wish to apply, change any of the fitting criteria with the click of a button, apply them immediately, and continue to change them until you have satisfactorily fit the data.

There are three principal models that FlowJo can (simultaneously) compute for any given distribution. These include the "Watson Pragmatic" model, the "Dean-Jett-Fox" model, and the "2-populations" model. The 2-populations model is based on the "Dean-Jett-Fox" model, but simultaneously fits two different dividing populations, each with a single Dean-Jett-Fox model.

The Watson model makes no assumptions about the shape of the S-Phase distribution; it (by definition) fits the S-phase exactly. The DJF model assumes that the S-phase is can be modeled by a second degree polynomial (that is convoluted with gaussian distributions of varying width throughout S-phase). You can also choose to have a synchronized peak within the S-phase as an option to this model.

Once you select one or more models and apply them, you can also choose to constrain the fits. Each gaussian peak (G1, G2, or the synchronous population) can be constrained by absolute position within a defined range (that you have created in the Graph Window), or by relative position to the other peak. For example, if the G2 peak position is twice the intensity of the G1 peak position, you may wish to constrain the fit such that this is always true. This is useful if the G2 peak is difficult to ascertain.

In addition, you can constrain the widths (CV) of each of the Gaussians. Again, you can constrain them either to an absolute value, or you can set one equal to the other. If your G2 peak is difficult to ascertain, you might want to make the assumption that the G1 CV and the G2 CV are equal, and therefore constrain the G2 CV to be equal to the G1 CV.

More hints on fitting the data can be found on the [next page](#).

Find this helpful?



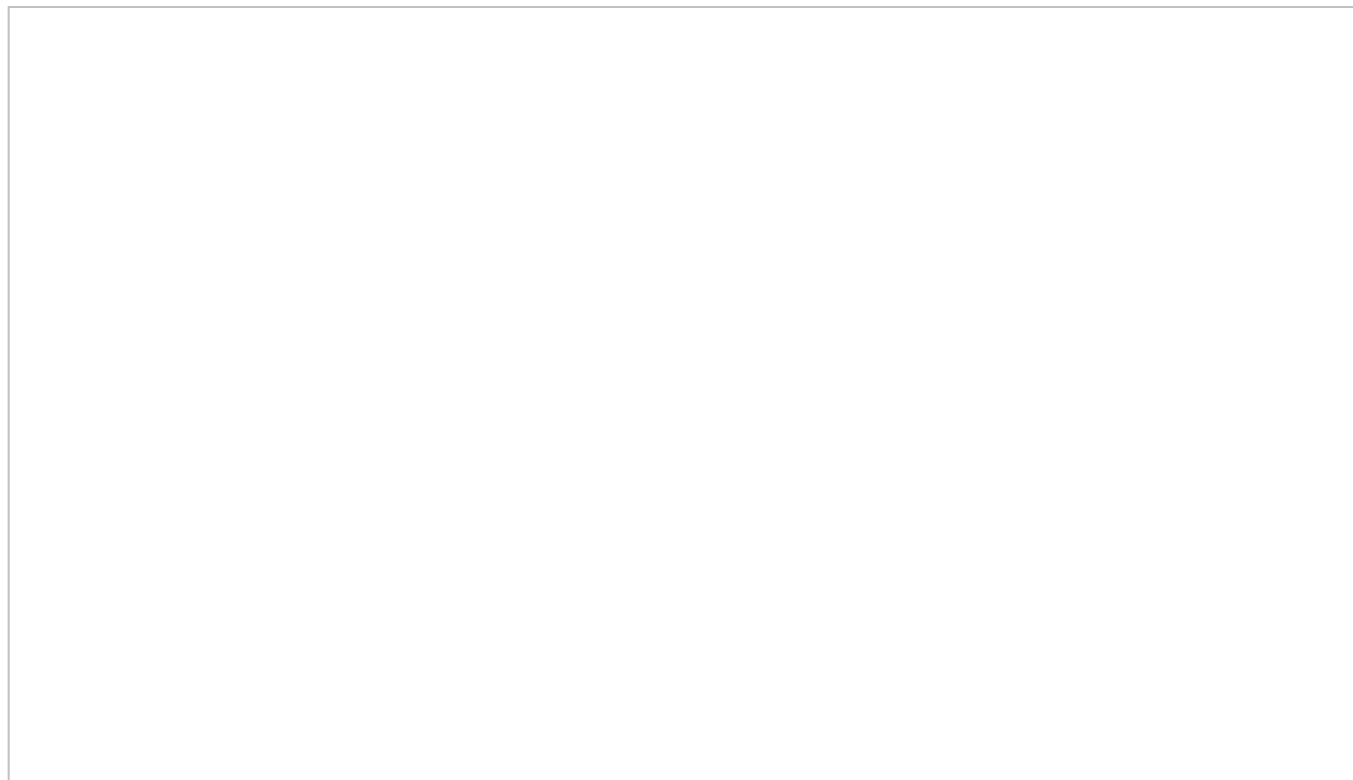
Proliferation

Model Parameter Adjustment

To obtain a better fitting model, you can fix the location of the **undivided population**. Simply *click and drag* to draw a gate over the undivided population.

Other options to constrain the model for an optimal model fit are:

- Fix the **ratio** of fluorescence between successive peaks
- Define the **CV** (width) of each peak
- Fix the **background fluorescence** (in units of absolute fluorescence)
- **Constrain** the model to just above the first peak and just under the last peak



Hints

If you have difficulty fitting a model, choose the sample with the best data and determine the Ratio, CV, and Background parameters. These parameters can then be fixed (by *checking* the boxes) and applied to the difficult to fit sample by *dragging* the **Proliferation Node** (indicated by a dividing cell icon in the Workspace) to the other samples.

The background fluorescence can be determined empirically from the median fluorescence of the unstained control sample (*i.e.*, cells that have undergone the same treatment, but were not stained with CFSE).

Find this helpful?

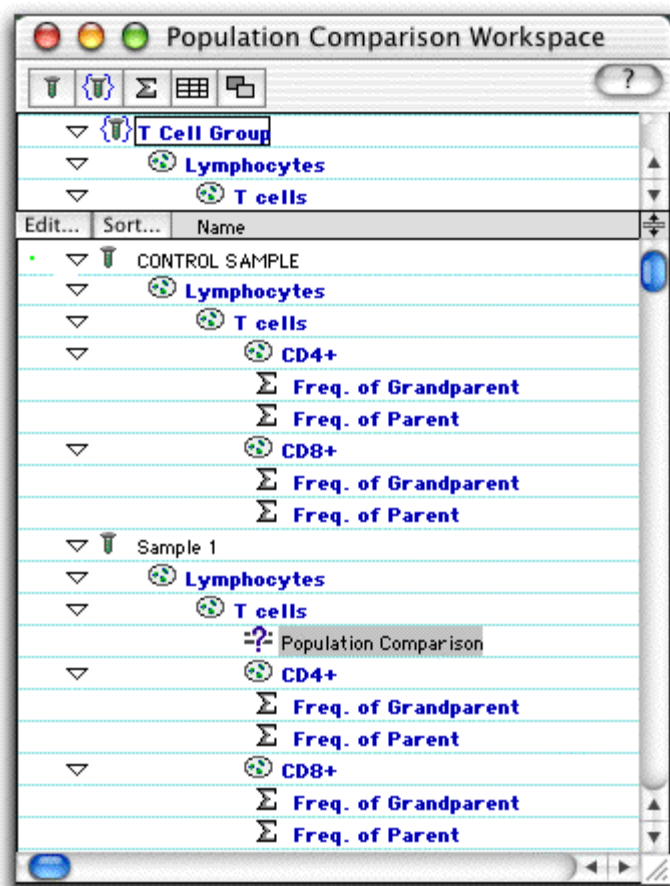


Population Comparison

Univariate Population Comparison

Start by clicking on the population to analyze (the test population) and selecting the Population Comparison platform from the Workspace menu.

This brings up the **Population Comparison platform**.

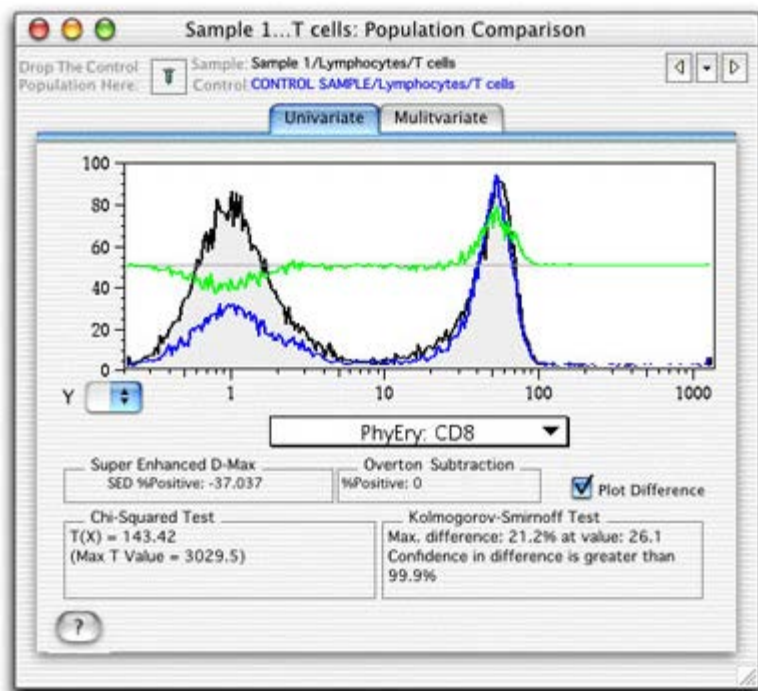
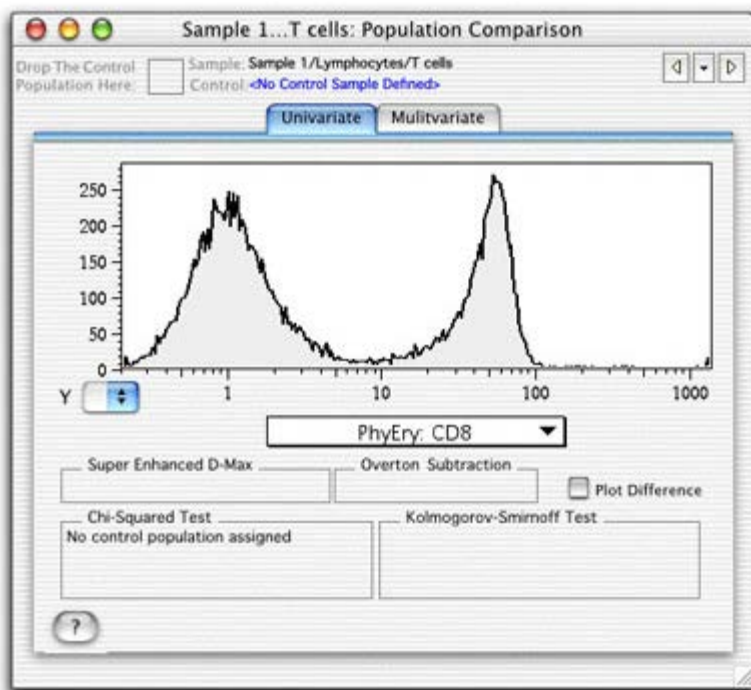


Select a parameter to analyze on the X-axis.

To define the control sample or population, drag it from the workspace window and deposit it in the box in the top left hand corner of the platform's window. ([Defining Controls](#)).

Click a yellow arrow to cycle through samples.

Click the black disclosure triangle between the arrows to select a sample.



Check the **Plot Difference** box to superimpose a plot (green line) of the difference between the test and control populations on the histogram.

For any channels in which the test sample has a greater portion of the events, the green line will be above the midline and visa versa. (**Note** that it will be scaled independently of the histogram).

The **Super-enhanced Dmax Subtraction** and **Overton Subtraction** provide the percentage of events that are positive compared to the control. The **K-S statistic** states a confidence interval for the assertion that the two populations are NOT drawn from a common distribution.

The **Chi Squared Test** divides the control sample into bins with the same number of events, divides the test sample along the same boundaries and calculates the Chi Square of the two binned data sets. The X^2 is converted into a metric ($T(X)$) that can be used to estimate the probability that a test population is different from a control population. See the [Population Comparison Overview](#) page for a complete explanation of these statistics.

When $T(X) = 0$, the two histograms are indistinguishable ($p = 0.5$) and when $T(X) = 1$, the populations differ by one standard deviation, giving the probability that the two populations differ $p < 0.17$. A value $T(X) > 4$ implies that the two distributions are different with a $p < 0.01$ (99% confidence). However, the minimum value of $T(X)$ that has biological significance depends on the nature of the data being analyzed and therefore needs to be determined empirically. Only populations which have $T(X)$ values larger than this empirical minimum can be considered to be different.

Several populations can be compared in order to determine the minimum $T(X)$ value because machine stability during the collection, as well as inherent variability in the FACS data are just two reasons why the comparison of a population to itself can give a $T(X) > 0$. You can compare a population to itself by opening the Population Comparison platform on a sample and dragging the same sample to the control box. FlowJo compares the two halves of this population (one half made up of every other cell while the other half is made up of the cells in between). You can also compare the same sample collected twice (at the beginning and end of the sample collection best determines the machine stability). You can also compare several different samples that have been treated with the same stimulation.

As with all other platforms in FlowJo, the **Population Comparison** Node can be applied to groups of samples by dragging.

Find this helpful?

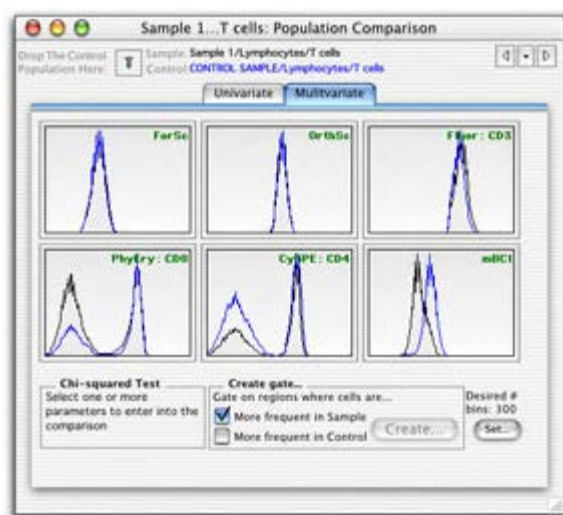


Population Comparison

Multivariate Population Comparison

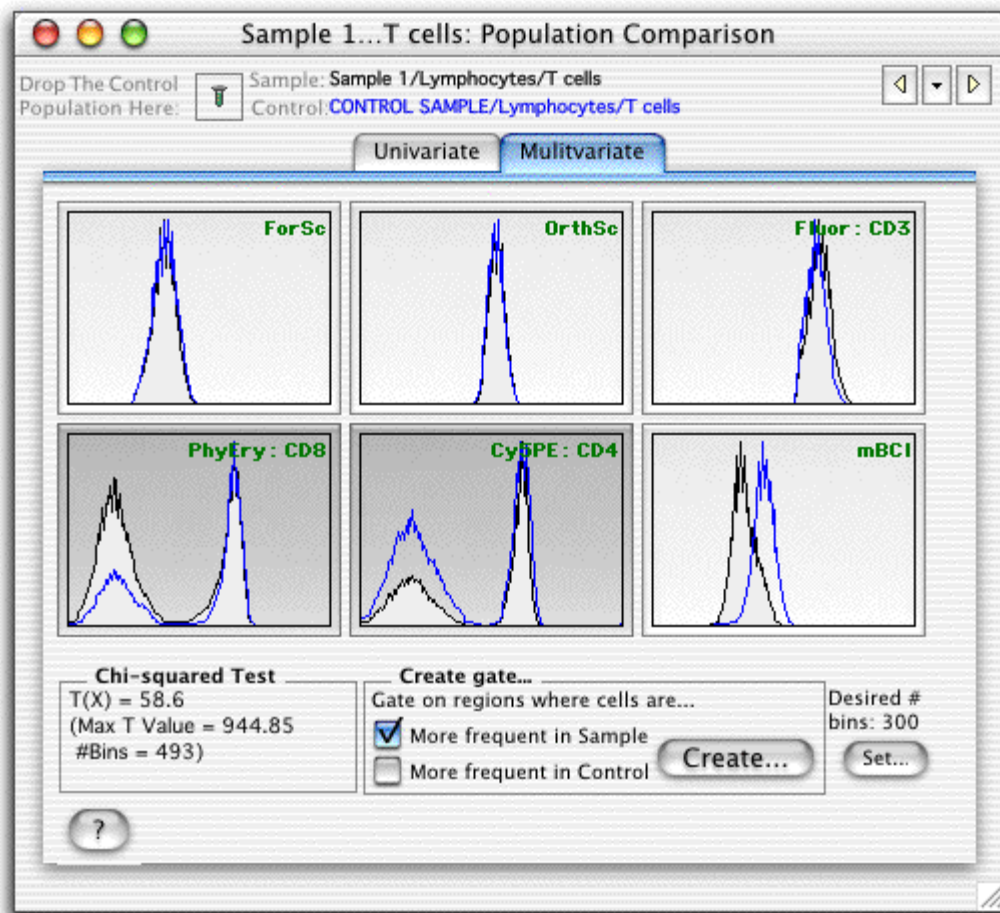
Start by clicking on the population to analyze (the test population) and selecting the Population Comparison Platform from the Platform menu.

In order to compare multiple parameters, click on the "Multivariate" tab.



As described for the [Univariate comparison](#), you define the control sample or population by dragging it from the workspace window to the box in the top left corner of the window. (Or by dragging the control population onto the comparison icon in the workspace.) ([Defining Controls](#)).

Click on the parameters that you want to compare (they will become shaded).



Click a yellow arrow to cycle through samples

Click on the black disclosure triangle between the arrows to select a sample.

The [number of bins can be set](#)* according to the number of events collected.

A gate based on the differences between the two samples can be created by clicking Create. Click [here](#) for information on the Gate Cut-off and setting the X2 threshold.

The Chi Squared Test divides the control sample into bins with the same number of events, divides the test sample along the same boundaries and calculates the Chi Square of the two binned data sets. The X2 is converted into a metric ($T(X)$) that can be used to estimate the probability that a test population is different from a control population. See the Population Comparison Overview page for a complete explanation of this statistic.

When $T(X) = 0$, the two populations are indistinguishable ($p = 0.5$) and when $T(X) = 1$, the populations differ by one standard deviation, giving the probability that the two populations differ $p < 0.17$. A value $T(X) > 4$ implies that the two distributions are different with a $p < 0.01$ (99% confidence). However, the minimum value of $T(X)$ that has biological significance depends on the nature of the data being analyzed and therefore needs to be determined empirically. Only populations which have $T(X)$ values larger than this empirical minimum can be considered to be different.

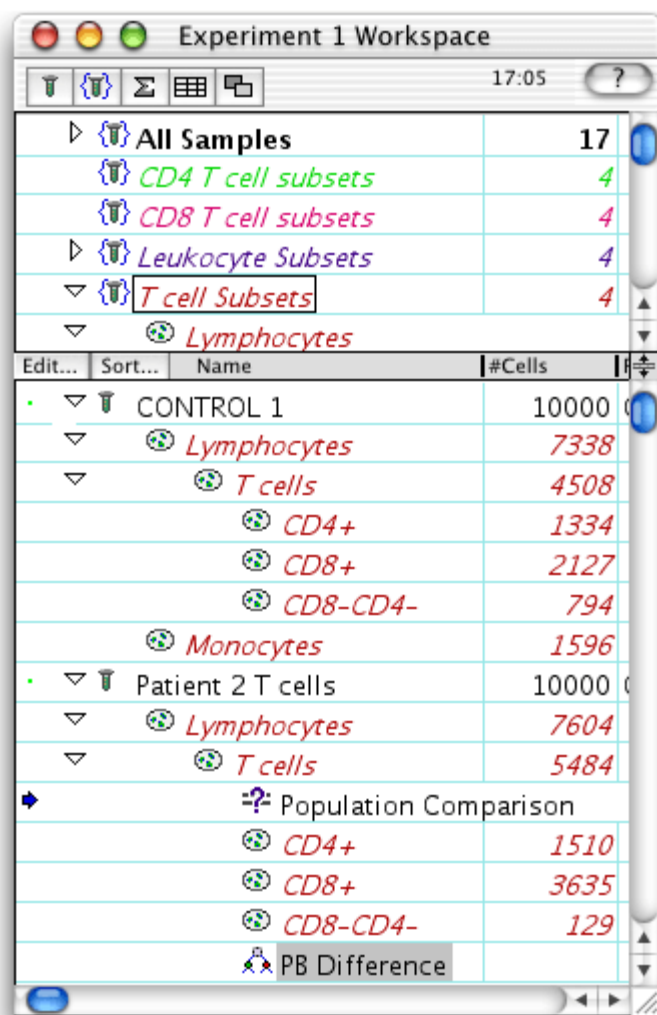
Several populations can be compared in order to determine the minimum $T(X)$ value. Machine stability during the collection, as well as inherent variability in the FACS data are just two reasons why the comparison of a population to itself can give a $T(X) > 0$. You can compare a population to itself by opening the Population Comparison platform on a sample and dragging the same sample to the control box. FlowJo compares the two halves of this population (one half made up of every other cell while the other half is made up of the cells in between). You can also compare the same sample collected twice (at the beginning and end of the sample collection best determines the machine stability). One could also compare several different samples that have been treated with the same stimulation.

* The number of bins that the test and control sample are divided into should be maximized to most easily detect small differences between populations; however, the number of bins can become limiting for this statistic (depending on the number of events collected and the number of parameters compared). Therefore, a reasonable number of bins is roughly 10% of the event count - leading to a minimum of about 10 events per bin.

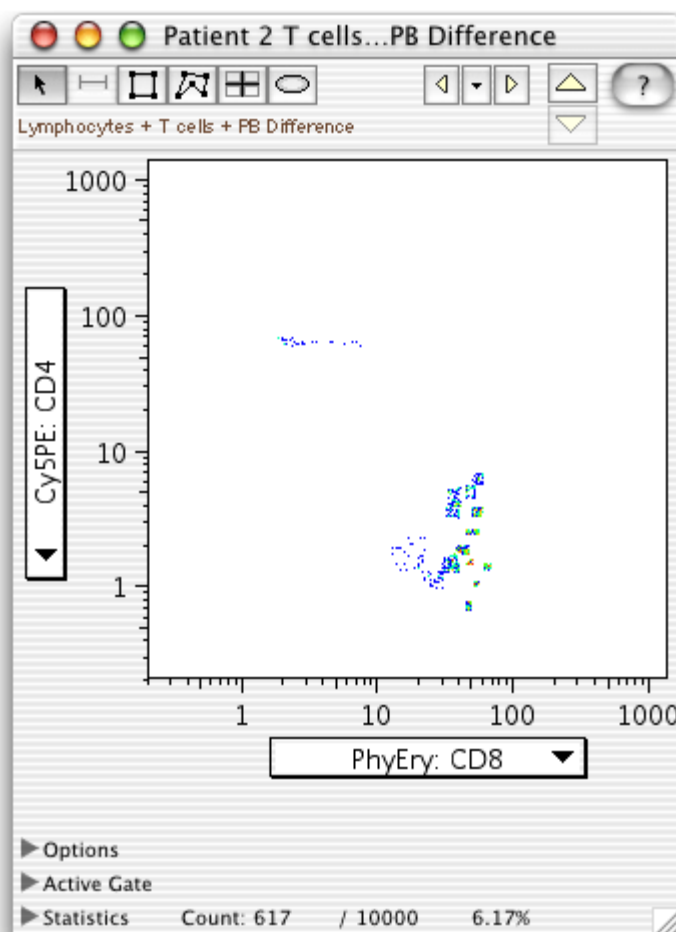
As the number of parameters being compared increases, then more events may need to be collected in order to distinguish subtle variations in the populations. However, inclusion of parameters in the comparison which **do not** vary between populations does not degrade the ability to distinguish the populations.

Note that the computations in the Multivariate Population Comparison platform can be time and memory intensive. You may need to allocate more memory to FlowJo ([more information on memory requirements](#)).

As with all other platforms in FlowJo, the Population Comparison Node can be applied to groups of samples by dragging.



The PB Difference population can be viewed (double click to open the graph). Note that the parent population cannot be viewed because it is based on multi-dimensional bins.



Find this helpful?



Population Comparison

Multi-sample Population Comparison

Chi Square Cut-off Value Selection

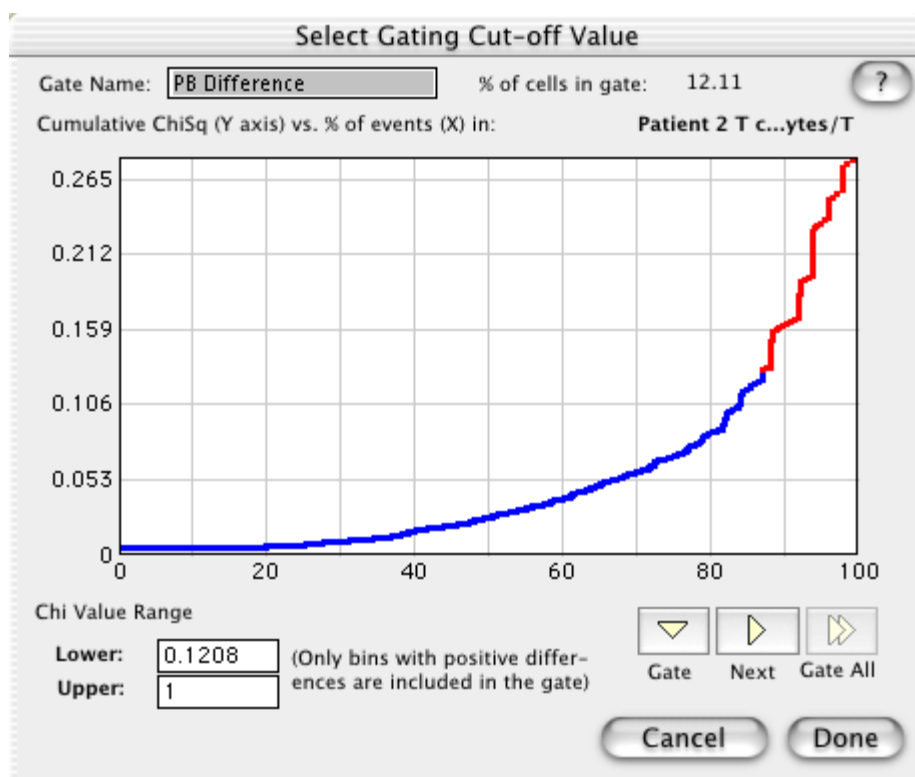
Use the [Multi-Sample Distancing Platform](#) to reach this stage of your analysis...

In order to create gates based on the differences between the control and the test sample(s), the **threshold/cut-off Chi square** value must be selected. When computing the [Probability Binning](#) algorithm, a X^2 value is computed for each bin. The union of the bins with the largest X^2 values becomes the "difference gate". The higher the Chi square value, the more the control and test samples differ.

The distribution of X^2 scores for each bin are graphed versus the percentage of cells that would fall into the gate if that X^2 value is chosen as the cut-off value. This Cut-off graph shows the bins sorted according to their X^2 value--i.e., those bins where the frequencies of events between the test and control population are the least different are shown to the left. Thus, the bins from the right edge represent those bins with the greatest difference. The default threshold is the Chi square value corresponding to those bins that are at least two standard deviations above random variation; i.e., $T(X) = 2$. The bins that fall above this X^2 cut-off value are shown by **red dots**.

The threshold/cut-off can be altered by drawing a new **gate** (click and drag) or by entering the upper and lower X^2 values in the boxes.

Clicking on the boxes in the lower right corner enable you to chose to **create a gate** on this sample, gate next sample, or gate all samples using these Chi X^2 values for the remaining samples.



Find this helpful?



Why do you need to transform the data display?

When data is properly compensated, it is common that a large number of cells are displayed squished against the axis. The cells become piled up in the first channel (against the axis) because the fluorescence parameters are displayed on a log scale where it is not possible to display "zero" or negative values. These negative values occur as a result of measurement error (not as a result of compensation). The spreading of a population into the negative is the result of measurement error that is inherent in the data we collect on flow cytometers. Even though the measurement error is the same in uncompensated samples, the error becomes obvious in the low regions of the log scale because log scales expand the view of data in the lower regions (first decade) and compress the view of data in the upper regions (fourth decade).

Find this helpful?



BiExponential Transformation - Instructions

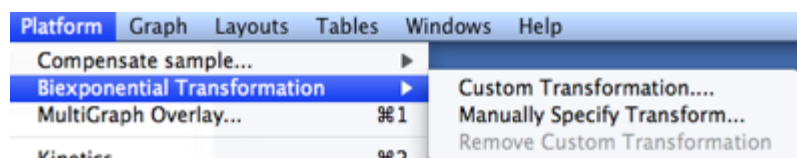
The display transformation can be used on any compensated data--but FlowJo needs to be the one to compensate the data. Therefore, it can be done on any data where you collect the single stained compensation controls and create the compensation matrix, or, alternatively, on any data files which specify their own comp matrix (currently, only BD DiVa files do this correctly). [Click here](#) for more information about visualizing digital data.

Software Compensation

To determine how to create a compensation matrix in FlowJo [click here](#) or [download](#) the [Compensation Tech Note](#). Briefly, define a positive and negative population on each single stained compensation control. Open the Define Matrix compensation dialog box (under the Platform menu) and drag the positive and negative populations from the workspace window onto the appropriate boxes in the dialog for each parameter. Click compute matrix. Apply this matrix to the experimental samples.

Procedure

(1) After creating the compensation matrix, click on a [FULLY stained sample](#) (it may help to click on a gated subset of the sample that has the autofluorescence characteristics of the cells you wish to analyze), and choose **Platform > BiExponential Transformation**



(2) In the dialog box that appears, make sure the Compensation Matrix shown in the popup menu is correct for your data file, and click on **Create Transformation**. Clicking the disclosure triangle reveals **Custom Visualization Options** explained below.

(3) Voilà! The visualization of the compensated parameters has now been adjusted to display the negative values. Open any data file that has the same comp matrix applied and the data will be transformed.

(4) To remove a transform, use **Platform->Biexponential Transform->Remove Custom Transformation**.

Remember, you have to do this for each matrix that you create in a workspace. The custom transformation will be applied to all samples that are compensated the same way in the workspace; hence it only need be computed once for each unique compensation matrix (and when computed for other matrices, requires a sample compensated with that matrix as an input to the customization.)

Options

Parameters in the transformation definition:

- (i) **Additional negative display size.** Controls, to a large extent, how much visual space to devote to events that have values less than zero.
- (ii) **Positive decades of display.** Controls, to a large extent, how many decades of dynamic range is shown for events

greater than zero. By default, this is 4.5--even if you export 5.5 decade data, use 4-4.5, otherwise too much visual space is devoted to the lowest (meaningless) decade.

Tips

Choose a sample which has been stained with as many of the colors you are analyzing as possible. If you did not acquire a data file that has been stained with all the colors you are analyzing in the experiment, it is possible to create a file by concatenation of all the compensation controls. [Click here](#) for instructions on how to use FlowJo to concatenate data files so that you have one data file that is stained in all colors. Another (and perhaps better options is to collect a sample which is a mixture of all your comp controls - i.e., mix the single stained comp controls before acquiring data.

If too many events are showing up on the axis, increase "extra" display. If the margin (empty space) between the events and the left axis is too large on all parameters, then decrease "extra" display parameter.

To enact the transformation, first make sure that you check the box in your Preferences (under **General**) to **Allow custom transformations**. The Preferences are under the **FlowJo** menu in OSX and the **Edit** menu in OS9.

Find this helpful?



Graph Gallery

The examples shown on this web page were created using Canvas from Deneba software; however, any drawing program can perform these operations. Each plot took only a couple of minutes to create; the steps performed are listed below each plot.

Example 1: Contour plot with outliers.

□

To generate this plot, the preference "Export all graphs as high resolution PICTs" was checked in the [preferences dialog](#). A contour plot (with outliers) was displayed on the screen, and the graph was copied (cmd-C, or "Copy" under the "Edit" menu). In Canvas, the graph was pasted into the document window. Ungroup the objects once to separate the internal graph objects from everything else. For the graph contents, select a foreground color of yellow and a background color of black. The contents were ungrouped again: each contour level is grouped: the outermost level was selected, and the line pattern was changed to 2-pt width, dashed line, and red. The fifth, 10th, and 15th contour lines were changed to red. At this point, all outlier points are still grouped; select the outliers, change the color to cyan and a 2-pt penwidth.

Note that the axes, axis labels, and parameter labels are all grouped in logical order so that you could easily change the text font, size, style, or color easily.

Example 2: Overlaid histograms.

□

This plot was generated in FlowJo's Layout Editor. A single node was dragged from the Workspace window to the Layout Editor. Five other nodes were then dragged onto the existing graph in the Layout Editor. Colors and line/fill options were chosen from the legend for each histogram. By double-clicking on the graph, you can choose to "Offset Overlaid Histograms" and remove the axes.

Return to the general information on [generating publication quality graphs](#).

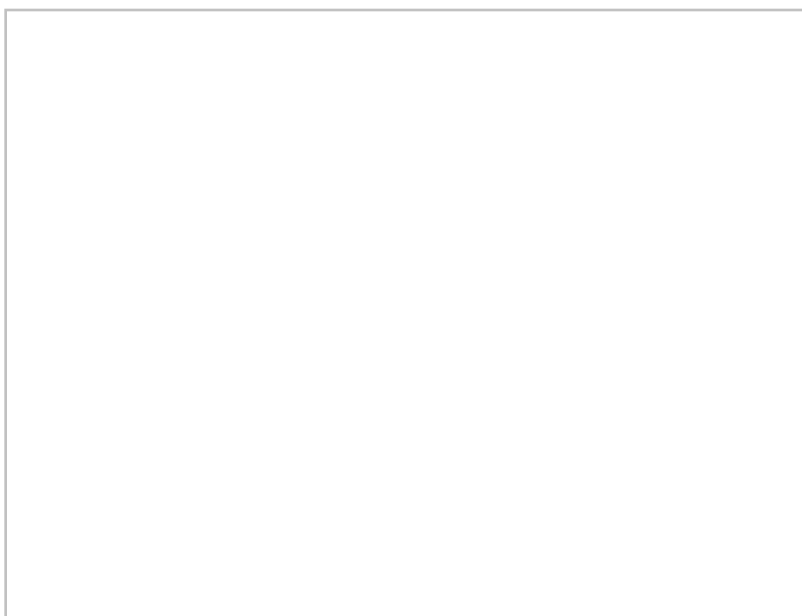
Find this helpful?



Layout Objects

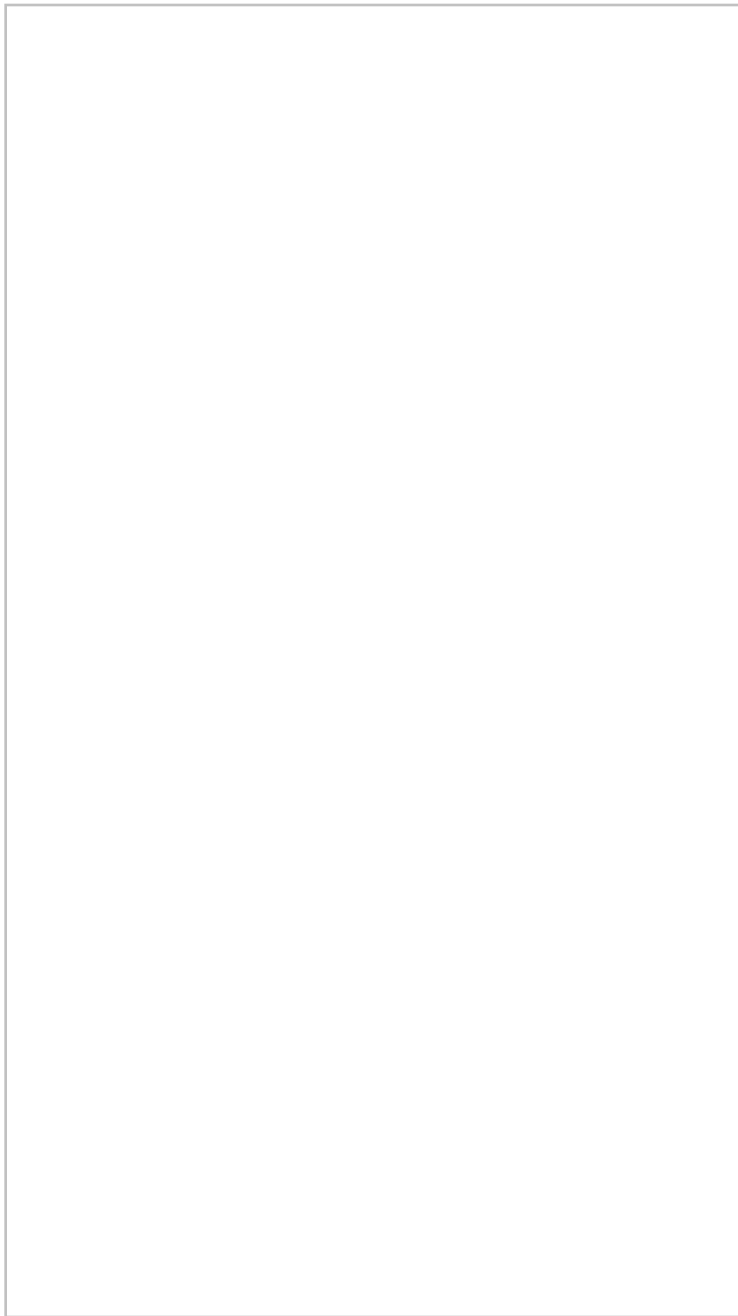
A layout is created by adding objects to a blank canvas. The objects may be rectangles, lines, text or graphs. Once you have placed objects onto the canvas, you can move them (by clicking and dragging one or more selected items), resize them (by clicking and dragging on the "handles" of a selected item), or align them to each other (by selecting "Alignment..." from the "Layout" menu). The Alignment tool is a powerful tool that even lets you put multiple graphs right next to each other (i.e., abutting axes) to create a compressed view!

Rectangles, lines and text can be added via the tool palette, found above the vertical scrollbar on the right edge of the layout window. Select the desired tool, and drag within the editor's view in order to create a new object. [More on Layout Tools]



To edit the color, line weight, text styles, or other information about the object, select the object and choose **Get Info.** from the **Layout** menu. (As a shortcut, you can also double click on the object.) This will bring up one of the **Layout Item Definition** dialog boxes, which provides the user interface for editing any of the graphical characteristics of the object. The content of the dialog is dependent on the type of the object. At right is an example of the dialog that comes up when you edit the definition of a text box.

Graph Objects



To add a graph to a layout, drag the node representing the population or sample from the Workspace window and drop it into the layout. This will create a new graph object in the layout editor. As with other objects, you can use the **Get Info.** command (or a double click) to edit the characteristics of the graph. If you select multiple items in the layout editor (ie, several graphs or a graph and a box...), the multiple items window will allow you to change all attributes at once.

This dialog is divided into two panels, **Specify** and **Annotate**. **Specify** is shown at the left. There are popup menus listing the available X and Y axis parameters, as well as others to set the graph type and the optional parameters that govern the appearance of the resulting plot.

In the case of histograms, it is also possible to fix the minimum and maximum values on the Y axis. This will enable you to prevent the axis from being recalculated for each frame in the layout, and make it easier to compare successive frames of a multi-sample layout.

More description on these items can be found in the Graph Window documentation of the [Graph Specification](#) floating palette.

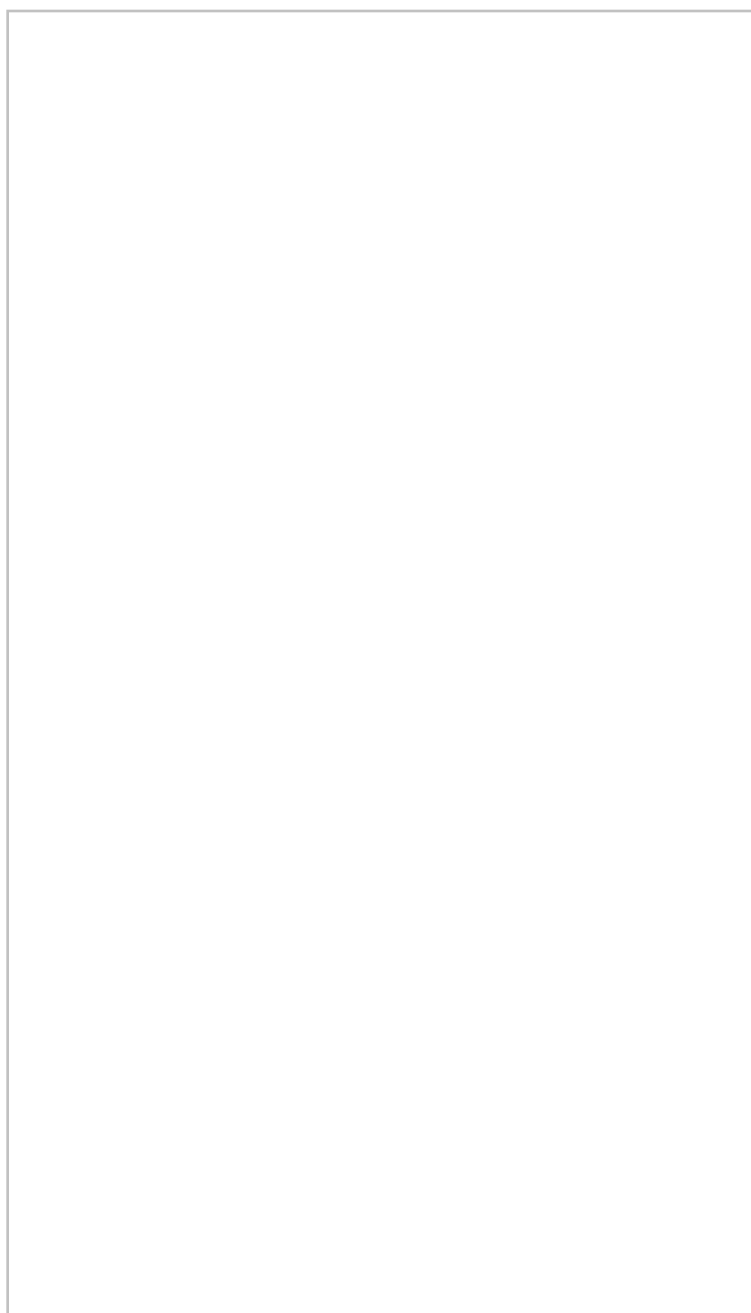
An additional item found in this dialog is the **Controls** text box. This will let you specify a graph, or layer in an overlay

graph, to be a control. Control graphs remain the same while others in the layout are changed as the batch process iterates through the other samples and statistics in the current group. This provides you a way to create layouts that show multiple samples, all compared to an original or control sample.

You can request the graph to **Show Ancestry**, meaning that all the parent populations and gates used to define this population will be shown as part of this graph. If this option is chosen, then you can also choose whether to show the ancestry horizontally across the top of the graph, or vertically along the left of the main view. You can also choose whether gates that are defined on this population should be shown.

Histogram overlays have the additional option of adding an offset that produces a 3D-like plot of the multiple histograms. This separates the lines and makes it easier to compare several curves on the same graph. Any value between 0 and 100% can be used as the **Amount** to offset. This puts a distance between the histograms on the Z Axis. The **Angle** setting rotates the origin of the baseline up and away from the viewer. Values of five to twenty give useful results, but *experiment*.

The second configuration panel appears when you click **Annotate**. It governs the additional annotation that (optionally) accompanies a graph in a layout.



The setting **Show Annotation** determines whether the annotation is desired at all. If you uncheck this box, FlowJo will not generate a text box associated with this graph. Assuming that it is checked, you can optionally have the annotation include the graph's title, the name of the sample, then name of all the gates leading to this population (the full name or full path), as well as statistics showing the frequency and/or the event count. There is also an additional text box that will let you include any extra information you may want to display in the layout.

There is a setting to **Show Legend** for a graph. By default legends are only created for overlay graphs, where the color is used to distinguish the different data sets. But because the legend is the means to set the color and line style of histograms and dot plots, it can be useful to override the default and show legends for single data series graphs. Conversely, there may be times when the legend is not necessary, even with multiple data sets, or you are making your own legend. In those cases, you can tell the layout editor to suppress drawing of any graph's legend.

Other settings available in this dialog include the ability to suppress either axis. This is useful if you are producing a layout with a large number of graphs and you want to save space by compressing the graphs close together and only label the outside axes.

Graphs can be resized in the layouts by dragging any of the corners. The mouse will show the percentage of magnification as you resize. It is also possible to use the dialog to resize graphs to a known size or aspect ratio. Typing the values manually gives more precise control of the scaling.

To navigate between a graph that appears in a layout, and the graph window where its gates are defined, there is a command **Open Original Graph** in the **Layout** menu. This opens the Graph Window containing the population selected in the layout. From there it is easy to navigate up the gating hierarchy to see all of the gates that define this population. There are shortcuts for this command:

- from the keyboard: Cmd-R, or
- with the mouse, Option-Cmd-click on the graph.

Find this helpful?



Print Preview

Once you have created a layout in the editor, you can direct FlowJo to produce a batch analysis. That is, to create the same picture, using each of the samples or values of the iteration attribute in the current group.

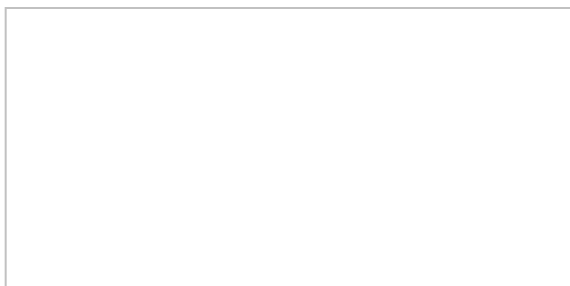
□

[Iteration](#) is a process of repeating analyses for all members of a group. [Other pages](#) describe it in detail, the current page describes what you can do with the results.

There are several potentially interesting things you might want to do with the results of a batch/iteration process:

- Print it, one layout per page, as a report.
- Print it at a minimum readable size for summary analysis.
- View it as tiled graphs onscreen.
- View it as a movie onscreen.
- Save a standard Pict file to disk for further processing / publishing.
- Create a Webpage showing all images.
- Create a Webpage displaying the movie.

Print Preview Report



There are several output types for batched reports: a new layout, a movie, a web page, and a **Print Preview** (tiled) report. In addition, you can [directly print](#) a report without first viewing the results of the batching. The Print Preview is used to display a tiled report on your screen. As the layout editor iterates, it builds a series of frames. The collection of all the frames is called a stack.

The preview report displays the stack of frames in two different ways:

- [Print Preview \(Tiled\) Frames](#): a grid of images that can be printed.
- [Animated Frames](#): a movie that can quickly display a batch report one page at a time using the QuickTime player.

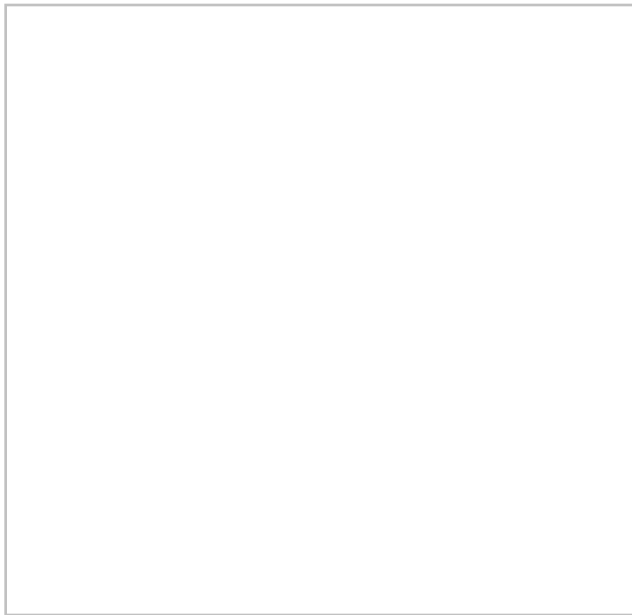
Both views support output of the graphic display to disk, clipboard, or printer. These functions can be accessed by main menu commands (under the **File** menu), the command-key equivalents (command-S, command-C, and command-P, respectively), or by clicking on the appropriate icons near the top right of the Print Preview window.

Below is a screenshot of a Print Preview in Tiled Report mode:



Tiling Options

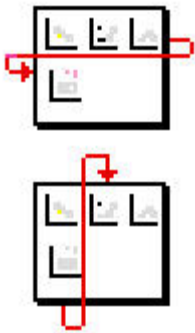
The tiling pattern on the screen is controlled by the dropdown menu in the upper left. You can choose to lay out your frames by the number of columns you desire. One up, Two up, Three up and Four Up are offered, and should cover most cases. When you click on this button, the final item in the dropdown menu, **Custom...** will bring up the following dialog containing more options and information.



The **Custom Layout Options** dialog handles special case scenarios where you want to determine the layout from a fixed number of rows instead of a fixed number of columns. It allows you to order the graphs from top to bottom instead of from left to right.

You can enter a value for either rows or columns, and the other dimension will be automatically recalculated to accommodate the number of iterations in your batch.

Order successive samples is a way to determine how the frames are sequenced, as illustrated below.



Order **Across**: Each frame is placed to the right of the one before it, until the number of columns you have chosen is reached, and then a new row is started.

Order **Down**: Each frame is placed below the one preceding it, until the number of rows you have entered is reached, at which point a new column is started.

Printing

One of the most annoying aspects of large analyses is having to manually piece pages back together because the layout program can't conform to the printer's page breaks. FlowJo avoids these problems by printing a specific number of tiles per page. When you click the printer button from a Tiled report, the [Choose Print Layout Options](#) dialog will open and allow you to choose the number of tiles per page and many [other printing options](#).

Animated Frames

In addition to having the ability to tile frames for efficient printing (shown above) FlowJo also has a unique tool for visualizing this large accumulation of information. Click the **Animation** button and each page of your batch will become a frame in a QuickTime movie.

□

The human eye and brain are better suited for finding patterns changing through time than they are in juxtaposing patterns in a two dimensional grid, or in constructing them in a three dimensional space. If you are searching through a large number of samples looking for patterns, similarities or trends, this animated view may give you new insight.

Since the data for the animated frames and tiled frames are based on the same graphs, you can quickly switch between the views without have to recompute extensive numbers of graphs.

Once your movie appears on screen, you can save it to disk or embed it in a Web page. The spider web button at the top of the Print Preview window will create a folder with your Webpage and movie in it.

Across the bottom of this window are the standard controls used to play a quicktime movie. The leftmost arrow is the play button. The right side controls move to the previous or next frame in the movie.

There are also keyboard shortcuts for each of these actions: The space bar (or double clicking the frame) will play the movie. The left and right arrow keys move to previous and next frame.

It is possible to export movies to other Mac programs or web sites by dragging the image from the window to the Finder. Shift click on the thumb of the scroller to select a subrange for export.

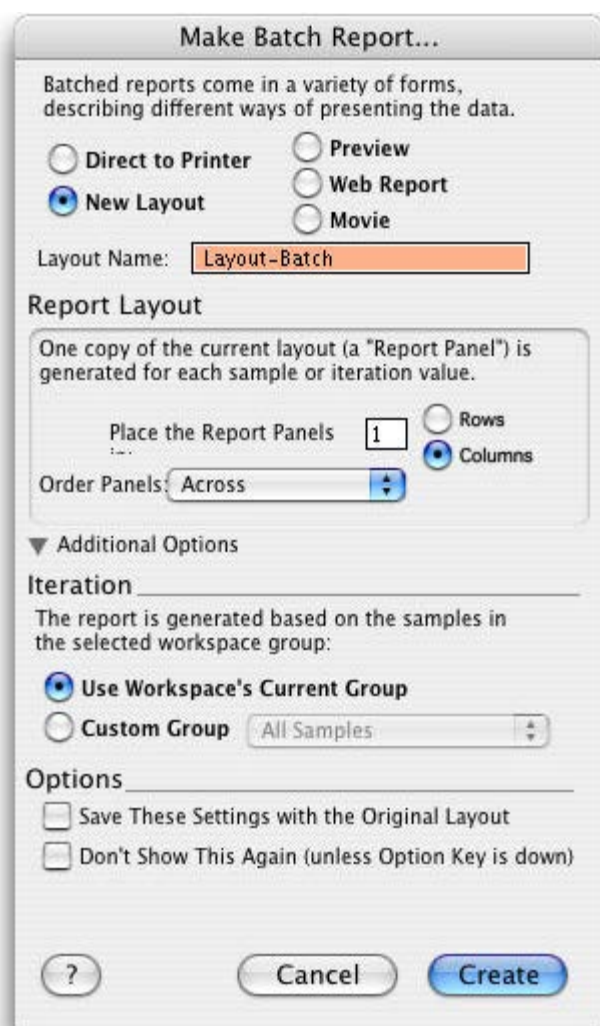
Movies can be generated from any graph in FlowJo, not just from a batch operation. [Click](#) for more information on the scope of the [Movie tools](#).

Find this helpful?



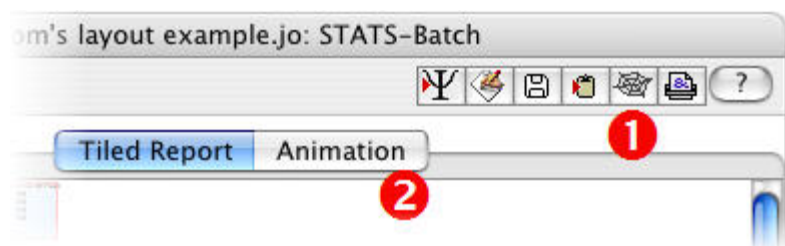
HTML Output - Batch to Web

Although printing will remain the dominant way to summarize analyses for some time to come, there are times when being able to write direct to web pages can make it easier to share, publish or archive your experimental results. At the very least, this gives you the opportunity to check a lot of data, and determine what is worth printing. And the trend seems undeniable; over time, more and more of what today is printed will tomorrow be posted.



When your layout is complete and you are ready to iterate through the current group, click the **Batch** button at the top right of the Layout editor. This opens the **Make Batch Report** dialog shown at right. Select the **Web Report** button then the **Create** button at the bottom. You will be asked for a name and location for your Web page. You can create a new folder or FlowJo will make one using the name of the Layout. It will contain the HTML file and the graphic files that the page will display. Navigate to the new folder, click on index.html and your browser will display the batch report.

You can also make a batch report Web page from the Print Preview Dialog. As before, click the **Batch** button in the layout editor. On the **Make Batch Report** dialog select **Preview** instead of Web Report.



Once your Print Preview is generated, select the **spider web** button (Number One) to create a Web page batch report like the one above. You will be prompted to give the name and location of a folder which will hold the HTML text file and the JPEG image files which combine to form the web page.

The Print Preview window will create a different format of web page depending on what mode it is in. In the **Tiled Report** mode, it will produce a web page containing an embedded image for each frame of the batch. If you select the **Animation** button (Number Two) the index.html file will contain an embedded QuickTime movie. The generated pages are editable in text processors or web authoring programs if further customization is required.

Find this helpful?



Using Placeholders in the Layout Editor

Placeholders are a way of letting FlowJo work with graphs inside a layout without the computation-intensive task of recalculating all the image information whenever you update a graph, gate or textbox.

The time to calculate a graph of a large data set can be several seconds. In a layout, which may contain several to dozens of graphs, this recalculation can be very time consuming. Therefore you are able to tell FlowJo to defer calculation until later, and to represent the graph as simply a box with diagonals running through it.

Use the **Placeholders** checkbox in the lower right border of the layout editor to tell FlowJo whether to use placeholders or not while you are working on the layout. Note that this setting only applies to the layout editor itself. Whenever you produce a batch report, the graphs are fully calculated regardless of this setting.

There are a couple other times when placeholders may be drawn in the layout editor, even when you don't have the Placeholders box checked. If the layout is zoomed way out (less than 25% magnification) then text boxes will be turned into placeholders. Also, if any of the graphs in the layout are based upon parameters which are not available within the current iteration value, or it is otherwise impossible to draw a graph based on the data available to FlowJo, then it will simply draw the placeholder instead.

In the main menu, **FlowJo > Preferences > Tables/Layouts**, there is a **Use Placeholders** checkbox. Check this box if you wish to replace all graphs with placeholder boxes.

Find this helpful?



Drag and Drop

Many of the operations you will perform in FlowJo are executed by "dragging and dropping." For instance, to copy an analysis gate to another sample, simply click on the subpopulation gate, and while holding the mouse button down, drag until the desired destination sample (or subpopulation) is highlighted; then let go of the mouse button. FlowJo applies the gate to that sample, creates a new node and displays it in the workspace.

Likewise, analysis nodes can be dragged to the [table editor](#) in order to create tables of statistics, or to the layout editor to create graphical representations. Analyses nodes can be dragged to different workspaces (that you have also opened) in order to apply them to samples in that workspace.

When you click on a second node, the first node is unhighlighted and the second node becomes highlighted. However, if you command-click a second node, then both nodes are highlighted. You can shift-click to select a series of nodes (this only works if the nodes are siblings; otherwise shift-click is equivalent to command-click). Any drag operation applies to all selected nodes.

When you drag a node, the default action is that node itself will be moved; none of its descendants or parents go along. However, if you hold down the "option" key when you start the drag, then all children of the node become part of the drag; if you hold down the "control" key, then all parent nodes of the selected node become part of the drag. Holding down both keys takes parents and children. The inclusion of children or parents applies to all selected nodes.

Whenever you start a drag operation, FlowJo creates a "drag outline" which represents all of the nodes that you are dragging. This way, you will have a visual feedback of what is happening.

When you finally drop your selection onto a node, all of the selected nodes are applied to the destination node: i.e., they all become children of the destination node. (Note that if you selected parents with the node (control key), then the highest-most parent is the one that becomes the immediate child of the destination node. Generally, you will only do this operation when you drag onto the sample-level).

If the nodes you are dragging already exist in the destination, then you will be asked what to do: you can replace the existing nodes with the ones you are copying, you can duplicate the nodes you are dragging (the duplicate nodes will have a "-2" appended to their name), or you can elect to retain the nodes in the destination sample. The latter is useful when you want to copy a whole tree with new analyses onto a sample which already has some of the gates, but you want to change the sample's versions of the gates. These options are more fully discussed in the pages on [replacing existing nodes](#).

All of this is rather complex to describe... an [example of the different kinds](#) of operations will better help you understand the ramifications of the various dragging and dropping operations.

Find this helpful?



FCS Keyword List

This page contains a list of the special keywords defined as part of the FCS 3.0 Standard. Scientists can use their own keyword identifiers, but they may not start with \$, unless it is defined in the specification document. The official ISAC description is [here](#).

Required Keywords	
\$BEGINANALYSIS	Byte-offset to the beginning of the ANALYSIS segment.
\$BEGINDATA	Byte-offset to the beginning of the DATA segment.
\$BEGINTEXT	Byte-offset to the beginning of a supplemental TEXT segment.
\$BYTEORD	Byte order for data acquisition computer.
\$DATATYPE	Type of data in DATA segment (ASCII, integer, floating point).
\$ENDANALYSIS	Byte-offset to the end of the ANALYSIS segment.
\$ENDDATA	Byte-offset to the end of the DATA segment.
\$ENDSTEXT	Byte-offset to the end of a supplemental TEXT segment.
\$MODE	Data mode (list mode, histogram).
\$NEXTDATA	Byte offset to next data set in the file.
\$PAR	Number of parameters in an event.
\$PnB	Number of bits reserved for parameter number n.
\$PnE	Amplification type for parameter n.
\$PnR	Range for parameter number n.
\$TOT	Total number of events in the data set.
Optional Keywords	
\$ABRT	Events lost due to data acquisition electronic coincidence.
\$BTIM	Clock time at beginning of data acquisition.
\$CELLS	Description of objects measured.
\$COM	Comment.
\$COMP	Fluorescence compensation matrix.
\$CSMODE	Cell subset mode, number of subsets to which an object may belong.
\$CSVBITS	Number of bits used to encode a cell subset identifier.
\$CSVnFLAG	The bit set as a flag for subset n.
\$CYT	Type of flow cytometer.
\$CYTSN	Flow cytometer serial number.
\$DATE	Date of data set acquisition.

\$ETIM	Clock time at end of data acquisition.
\$EXP	Name of investigator initiating the experiment.
\$FIL	Name of the data file containing the data set.
\$GATE	Number of gating parameters.
\$GATING	Specifies region combinations used for gating.
\$GnE	Amplification type for gating parameter number n.
\$GnF	Optical filter used for gating parameter number n.
\$GnN	Name of gating parameter number n.
\$GnP	Percent of emitted light collected by gating parameter n.
\$GnR	Range of gating parameter n.
\$GnN	Name used for gating parameter n.
\$GnT	Detector type for gating parameter n.
\$GnV	Detector voltage for gating parameter n.
\$INST	Institution at which data acquired.
\$LOST	Number of events lost due to computer busy.
\$OP	Name of flow cytometry operator.
\$Pkn	Peak channel number of univariate histogram for parameter n.
\$PKNn	Count in peak channel of univariate histogram for parameter n.
\$PnF	Name of optical filter for parameter n.
\$PnG	Amplifier gain used for acquisition of parameter n.
\$PnL	Excitation wavelength for parameter n.
\$PnN	Short name for parameter n.
\$PnO	Excitation power for parameter n.
\$PnP	Percent of emitted light collected by parameter n.
\$PnS	Name used for parameter n.
\$PnT	Detector type for parameter n.
\$PnV	Detector voltage for parameter n.
\$PROJ	Name of the experiment project.
\$RnI	Gating region for parameter number n.
\$RnW	Window settings for gating region n.
\$SMNO	Specimen (tube or well) label.
\$SRC	Source of the specimen (patient name, cell types)
\$SYS	Type of computer and its operating system.
\$TIMESTEP	Time step for time parameter.
\$TR	Trigger parameter and its threshold.
\$UNICODE	UNICODE code page for string type keyword values.

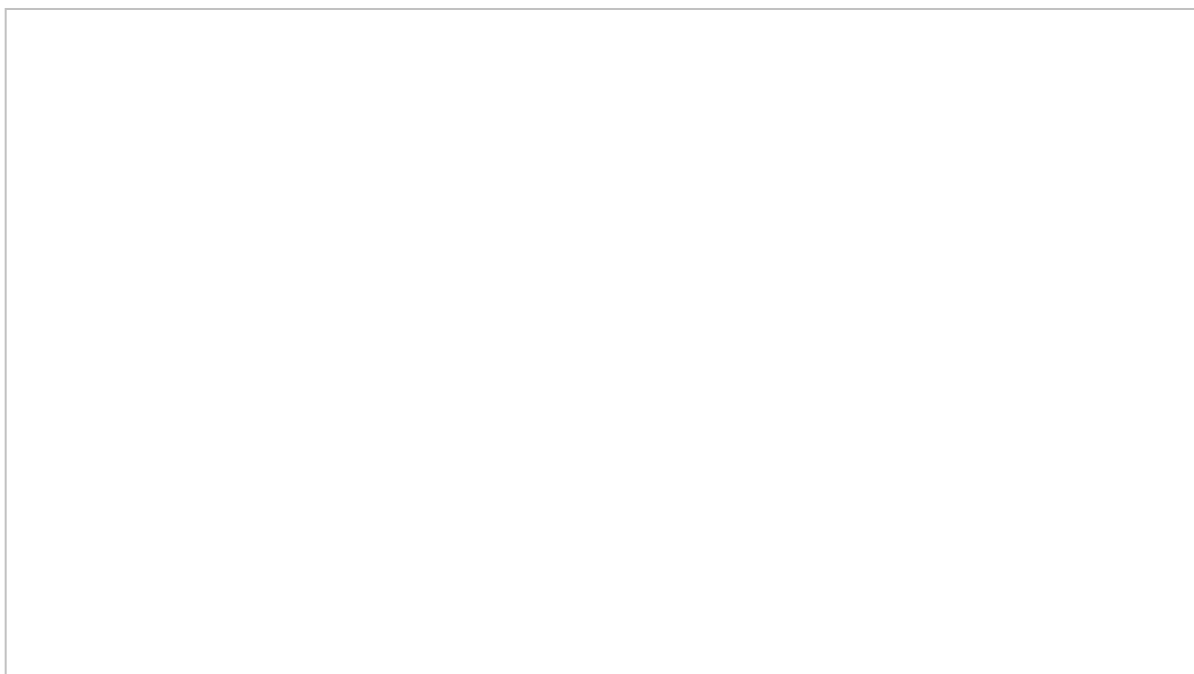
Find this helpful?



Defining Column Attributes

You may want to highlight certain results when you create a table with the [Table Editor](#). FlowJo lets you change the formatting of table values based on their relationship to standard deviation or a numerical value. Select a row in the Table Editor and click on the **Info** button. This brings up the tool below...

Info
Button



In the **Define a custom name...** box you can create a name for the column when it appears in the finished table, or override the name it was given by default in the Table Editor.

Check the **Define as a control value...** box if you do NOT wish this column to be batched when the table is created.

Check **Hide this column in output table** to perform the indicated calculation but to prevent it being displayed in the final table.

Next note the blue text reading, "**When the table value matches this criterion... use this formatting.**" Choose your criteria on the left, and values that satisfy them will be formatted according to your selections on the right. By default, FlowJo's tables will make $2 > SD > 1$ values bold, red and italic, and $SD > 1$ values bold and black.


Values in the selected column will be checked against the criteria you list here, in the order that you list them. As soon as a criterion is satisfied, the corresponding formatting is applied and the next value in the column is examined.

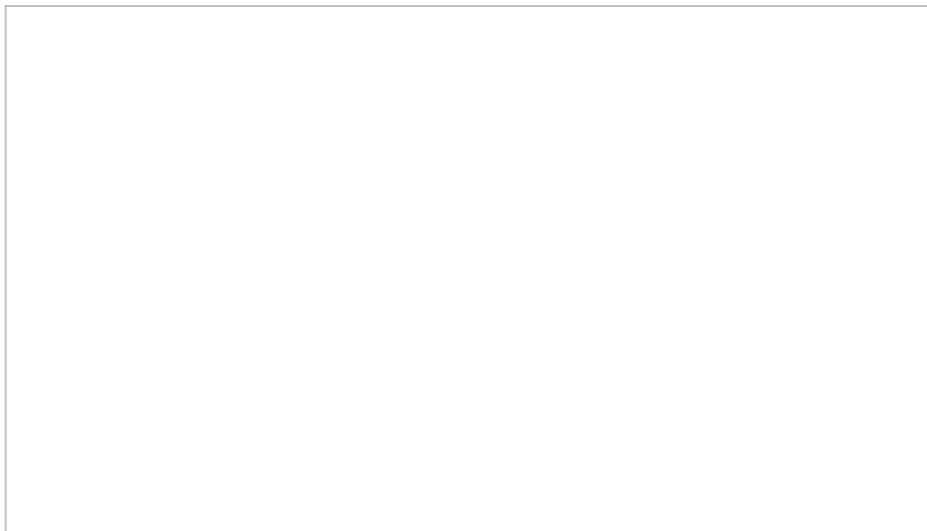
□

Example: "I want to add a ratio of my CD4/CD8 values to the table. If the ratio is > 2 , I want the values in my table to be green. If the ratio is < 2 " First, we'll need some kind of gating in the workspace. In this example, I used the Workspace at the right.

Next, drag the nodes that correspond to CD4/8 T cells to the Table Editor. Notice the Column Name field is filled in:

□

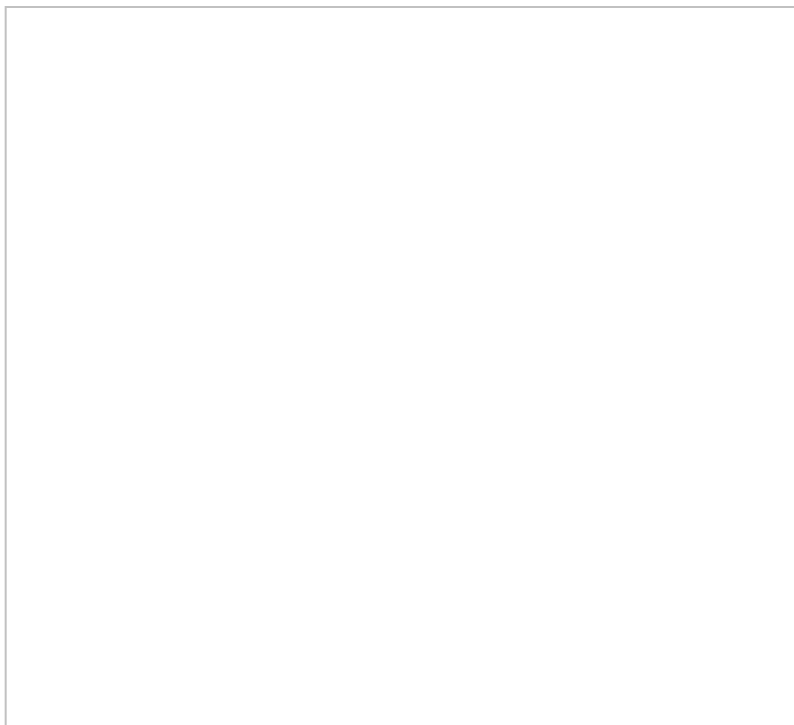
Next, click the Function button  in the Table Editor and define a ratio of CD4 to CD8. Select the blue **Create Formula** button shown below. For explicit instructions on functions [click here](#).



Now, double click the new row in the Table Editor to style the output:

□

Click the **Create Table** button in the Table Editor. If the ratio is >2 , the values in the table will be bold and green. If the ratio is <2

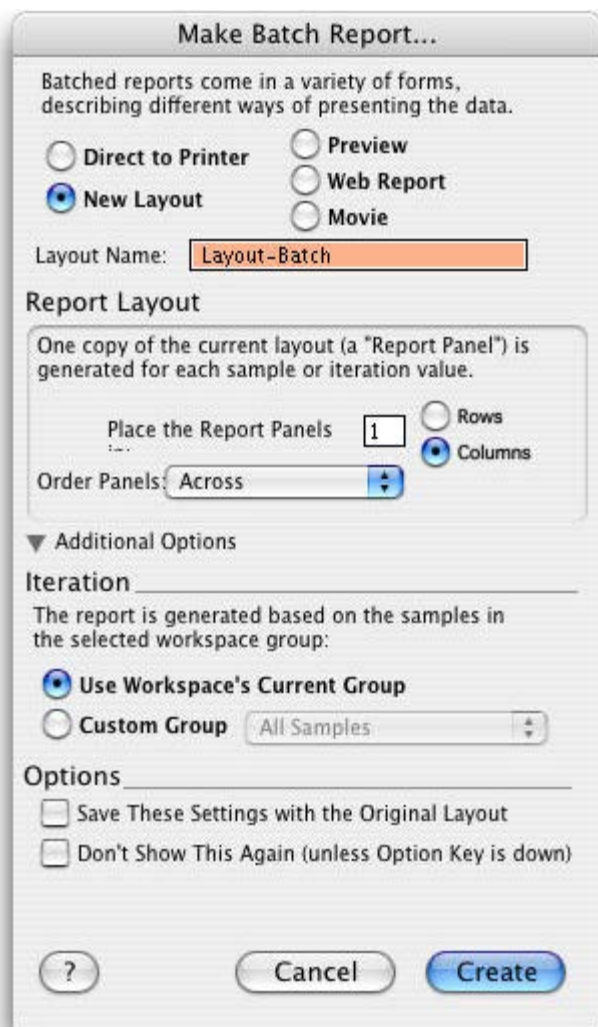


Find this helpful?



Set Up Printing

You can create several types of reports using the **Batch** button at the top right of the [Layout Editor](#) window or you can print reports directly. In order to create a Batch Report, place in the layout editor the graphs, statistics, annotation and extras (boxes, lines, text boxes etc.) you would like to see in your report. Do this from ONE data file. Once you have defined the tile or panel you would like to see from each of the data files, click on the **Batch** button at the top right corner of the Layout Editor.



When FlowJo makes a batch report, it gathers the information in the tile (graphs etc.) from all the data files in order to summarize all the data files in the selected group. The type of report you choose determines the printing method. If you create a **New Layout**, FlowJo creates a report with all the tiles arranged in a single display. This report can be printed directly from the new layout window after editing and adjusting page breaks. If you create a **Preview** or choose the **Direct to Printer** option, FlowJo creates a report and prints it using the method shown below.

Direct to Printer

When you choose **Direct to Printer** in the Make Batch Report dialog, this report is printed directly to your selected

printer. The **Report Layout** section of the Make Batch Report dialog determines the number of data files or tiles to place on each page.

Printing a Preview Report

When you choose to create a [Preview](#) report, FlowJo gathers the graphs (tiles) from all the samples and displays them in a tiled print preview report. This can be printed by clicking the printer button at the top right corner of the report. This opens the **Choose Print Layout Options** dialog box shown below.



Choose Print Layout Options

You can choose how many panels (tiles/data files) per page and the right part of the window will display the tiles as they will be laid out on the page. The panel arrangement area allows you to choose the number of columns to place the tiles across or rows to place the tiles down. In addition, the graph size, spacing and centering of the tiles can be customized. Automatic page orientation chooses the Portrait or Landscape that is best suited to the panel arrangement options you have chosen.

The header and footer information can be hidden or edited. Clicking the Edit button brings up the [Edit Printed Page Layout Information](#) dialog box. This box is also available by clicking the **Headers, Footers and Print Info** button in preferences on the **General** tab. With it you can add text and graphic information that will be repeated on every page of your printed report.

Find this helpful?



Kinetics Movie

This time series is from a calcium-flux experiment. In this experiment, peripheral T cells were stained with anti-CD markers to identify subsets and loaded with Indo-1. The ratio of Indo-1 for a gated subset of CD4 T cells is shown as a function of time. Approximately 30 seconds into the collection, anti-CD3 was added to stimulate the cells. The time in seconds since the beginning of the acquisition is shown in the upper left hand corner.

□

You will notice that about 230 seconds the first indications of calcium mobilization can be seen by the appearance of a peak of high Indo-1 ratio (Ca, X axis). Within 50 seconds after that a peak of high calcium concentration appears (at about 2 on the Ca scale), which quickly resolves into the main, intermediate peak (about 0.7 on the Ca scale), lasting to the end of the collection.

In FlowJo, or when you view the movie using a third-party application, you can view the movie frame-by-frame, or use the mouse to scan back and forth over a small time period: this gives you the ability to view small time-slices with high precision so you won't miss anything.

View the [next sample movie](#), or go back to the [movies overview](#).

Find this helpful?



Parameter Movie 1

This data is from a 3-color immunophenotyping experiment using CD4, CD45RA, and CD62L (Leu8) to stain human PBMC. The sample was gated for lymphocytes using forward and side scatter, and then for CD4 T cells. A pseudocolor plot of CD45RA vs. CD62L is shown as a function of the CD4 intensity (below).

□

The bottom graph shows the slice of CD4 intensity that is used to gate the current frame of the movie. For this movie, equal channel size was selected; thus, each frame can have different number of events. (To see how this movie would look with equal event framing, [click here](#).)

You will notice several features about the graph as the CD4 intensity is varied. First, the upper left population (CD45RA+CD62L-) only appears at the very lowest CD4 intensities (this is an activated T cell population that has likely downregulated expression of CD4 itself). As the CD4 intensity increases, you will also note that the amount of CD62L (Leu8) per cell for the CD62L+ cells increases as well.

View the [Kinetics sample movie](#), or go back to the [movies overview](#).

Find this helpful?



Parameter Movie 2

This data is from a 3-color immunophenotyping experiment using CD4, CD45RA, and CD62L (Leu8) to stain human PBMC. The sample was gated for lymphocytes using forward and side scatter, and then for CD4 T cells. A pseudocolor plot of CD45RA vs. CD62L is shown as a function of the CD4 intensity (below).

□

The bottom graph shows the slice of CD4 intensity that is used to gate the current frame of the movie. For this movie, equal channel number of events was selected; thus, each frame can have different ranges of CD4 intensity; this is why the bottom and top slices are particularly wide. (To see how this movie would look with equal channel framing, [click here](#).)

You will notice several features about the graph as the CD4 intensity is varied. First, the upper left population (CD45RA+CD62L-) only appears at the very lowest CD4 intensities (this is an activated T cell population that has likely downregulated expression of CD4 itself). As the CD4 intensity increases, you will also note that the amount of CD62L (Leu8) per cell for the CD62L+ cells increases as well.

View the [Kinetics sample movie](#), or go back to the [movies overview](#).

Find this helpful?



Grids

FlowJo's Layout editor contains a tool for creating grids inside your layouts. In the right margin tool bar, click the grid icon.

Drag out a rectangle where you want your grid to be. FlowJo will respond with a dialog asking for the dimensions of this grid:

Fill in the dimensions and click OK, you will see a grid appear in the layout editor:

A grid is made up of cells, aligned in rows and columns. You can drop any text, chart or picture into a cell. You can even embed grids inside of other grids. Below is a grid containing six different graphs. The grid container makes it easier to align graphs and to work with them collectively. When you drag a grid to a new location in your layout, all of the contents of the cells move with the grid as a group.

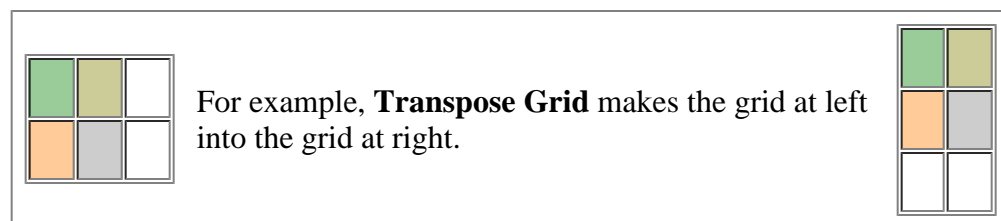
Mouse click behavior

If nothing is selected in the layout, then the first click anywhere in the grid will select the entire grid. If the grid is selected, then mouse clicks are passed down into the cell. To drag a grid to a different location, you must deselect it (by clicking in the background of the layout, or on a different item) and then click and drag the grid in one step. If the grid is already selected, then a click-and-drag motion will select a cell and drag its contents.

There are additional grid commands in the **Layout** menu. **Put Into Grid** places selected layout elements into a one row grid with each item in its own column.

Other commands are available when a grid or its contents are selected:

The **Transpose Grid** command changes the number of rows to the number of columns and the number of columns to the number of rows. So a 3 column by 2 row grid becomes a 2 column by 3 row grid. Items are not moved among cells, but if the cell moves, the item moves with it.



Also in the **Layout** menu is the **Get Grid Info** command. Clicking this command opens the **Grid Attributes** window shown at the right. You can also reach this dialog by double clicking on a grid's border, or select any part of the grid then select **Get Grid Info** from the **Layout** menu.

The first items dictate the dimensions of the grid; the number of **rows** and **columns** it contains. Set the desired lengths here to change the size of the grid. Note that eliminating cells can cause cell data to be lost.

The **Fill Color** and **Pen Color** will determine the colors used in drawing the background of the cells and the lines in the grid. Note that graphs you have placed in the grid are drawn on either a white or a black background (depending on the *Inverted* flag for that graph), and are not affected by changing the fill color. The area under the labels and surrounding the graph is affected by changing the fill color. Here you can choose line weight, colors etc. for your grid. You can also choose to have the grid adjust to the size of the items inside, or to have the graphs and other items resize themselves within the available grid space.

The **Line Weight** option determines how thick the lines in the grid will be. The **Inside dashing** options lets you choose solid lines, or different lengths of dashed lines between the cells in the grid. The outside edge of the grid will always be solid, and is unaffected by this setting.

The settings **First row contains headers**, and **First column contains headers** are provided so that FlowJo can resize grids more intelligently. When the program knows to treat the first row or column separately, then it can avoid scaling all cells proportionally during a resize. This reserves more of the space for the actual content, and produces a more aesthetically pleasing graphic.

The group of items called **Layout** will determine how the cell content is positioned in the cell rectangles, and what will happen to them if the grid is resized. If the **Force contents to resize with grid** checkbox is set, then the full content of the graph or pictures will be rendered in the grid, regardless of the cell size. If this setting is not checked, then the chart or graphic will remain its original size, and if the cell size is smaller than the graphic, then only the top left corner of the image will be visible. This setting is available for the grid as a whole, and not for each cell individually. To accomplish independent scalings you must rescale graphics outside of the grid, and then drag them into it at the desired size.

Deleting Cells, Rows & Columns from a Grid

To delete elements of the table, click on a cell and hit the delete key. You will see a dialog box requesting information about what aspect of the table you want to delete. You can choose among removing just the currently selected cell contents, or removing the entire row or column that includes the selected cells. Using the command and shift keys, it is possible to select multiple cells of the table and perform several deletions in one step. You may also drag an element out of the grid and delete it with the delete key in the usual way.

Shortcut:

Hold down the Option key and double click in a cell to add a textbox, and activate the [FJML editor](#). This tool will create text annotations of statistics that remain linked to the FlowJo workspace. When you update gates or otherwise alter details of the analysis, these annotations update themselves to match. [Click here](#) for instructions on use of this tool.

Find this helpful?

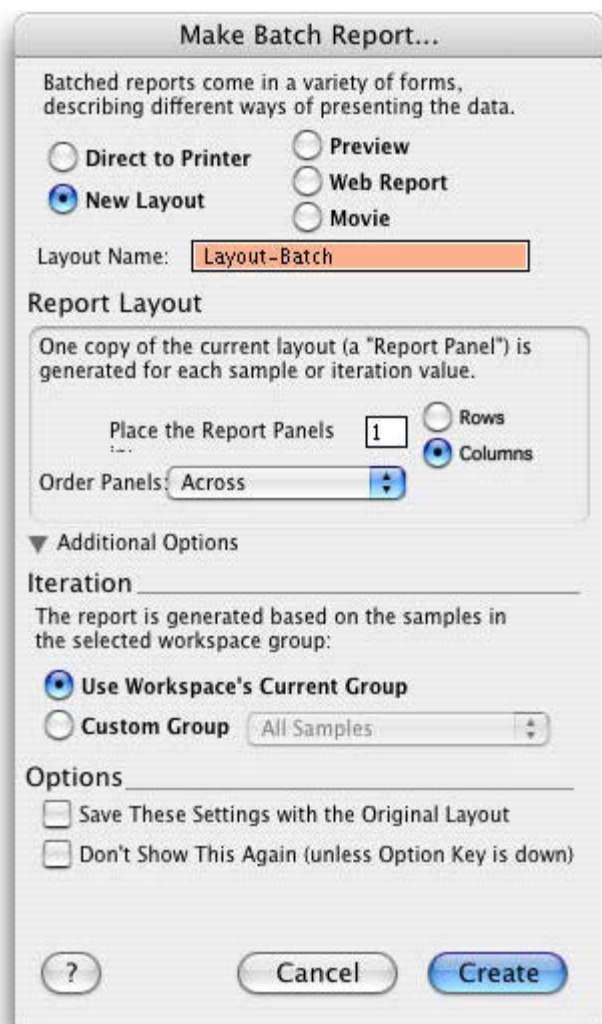


Iteration (Batching)

Build from <http://www.flowjo.com/v6/html/leiteration.html> instead of lebatch

One of the best things about computers is how they handle repetitive tasks. Show the computer how to do something once, and it will do it a million times without the slightest complaint.

In FlowJo's Layout Editor, you can create a layout based on graphs and statistics from one of your samples. With a single click, you can automatically produce the same layout for each of your samples, or any subset of samples that makes sense for your experiment.



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

- A new layout in the layout editor.
- A preview window containing static tiles (data won't update if you change the original analysis.)
- A web page containing the pictures for each layout group, or

- A Quicktime movie.
- Alternatively, it can print the report directly to your printer.

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

In the simple case, each frame corresponds to one sample in a group. The batch report is made by taking each sample, applying the named gates and statistics to that sample, plotting the graphics in the format defined by the layout editor, and adding that frame to the report. This is useful in cases where the same information is desired for each tube.

Other times, reports are created where each frame contains data from more than one tube. Examples of multi-sample reports could be several tissues from a common animal, multiple doses of a common drug, or cross patient studies using blood collected over time. In cases such as these, it is not enough to simply process successive tubes in a batch report. More sophisticated organization is required. Click **Layouts > Iteration Options...** to set more parameters for your batch operation:

□

There are a couple of terms that we use in this explanation that are important to get clear from the start. They are *attribute* and *value*. The keywords in an FCS file are organized in attribute-value pairs. The attribute is the name for the type of data recorded. The value is the data recorded for a particular tube. For example, most files will contain, in their header, a \$Date attribute, and a value containing the date on which that sample was collected. When we talk about iteration attribute we mean the attribute that is changed for each frame of the stack that is generated, for instance the date of the collection.

In advanced reporting, there is an additional feature known as the discriminator. This field is the mechanism for finding the correct sample, when more than one sample is collected with the same iteration value. This is described in more detail at the bottom of this page.

From the **Iterate over...** drop-down box you can select a keyword that groups tubes into distinct units--for example, a keyword that connotes patient ID, tissue type, or experimental condition. Then you can place multiple graphs from different tubes (from different FCS files!) into the same layout and still generate a batch output.

For example, if you set the iteration attribute to be the keyword "\$Cells", where you have a Patient ID entered into the \$Cells keyword, then FlowJo will examine all of the tubes in the current workspace, and generate a list of the unique values of \$Cells. These are then displayed in the "Iteration" popup menu. By selecting any of these values from this menu, FlowJo will change the current layout view to show graphs & statistics drawn only from tubes with that value of the Iterator. (By setting this popup menu to "Off", you are telling FlowJo to use all of the original graphs that dragged and dropped into the layout). This is the way in which you can build template reports which select graphs and statistics from different tubes and put them all on the same page, but retain the ability to batch the outputs.

The most common case remains that where the iteration attribute is the sample (by which we generally mean the tube run through the cytometer, as opposed to the sample taken from the subject).

The layout and preview (tile) report formats have parameters to regulate the geometry of the output. You can specify the number of rows or columns you want to generate, and whether the frames are placed in a row-major (where the second frame is to the right of the first) or a column major (where it is below the first) order.

Finally, there are three additional options provide at the bottom of this dialog. The option to Save These Settings with the Original Layout, if checked, will store all of the settings in the approved layout. In this way, it is straightforward to replicate this report many times in the future. The option Don't Show This Again (unless Option Key is down) gives you a means to simplify the use of the program and avoid seeing the dialog in the future. If you are always producing the same reports with the same characteristics, this is a way to prevent untrained users from producing the wrong kinds of reports. If this option is not checked, you can still avoid the dialog by holding down the option key when clicking the Batch button.

Find this helpful?



Editing Keywords in the Workspace


When flow data is collected at the cytometer a data file is created in a specific format called FCS. Each FCS data file has two essential components, the description of the conditions at the time of collection, and the sets of numbers measured for each event. Whereas the cytometer formats all of the numerical data automatically, it is up to the operator to correctly specify the conditions. Keywords organize the non-numeric information in FCS files and each keyword is designated with a [\\$three letter code](#).

The names of the reagents used to stain each tube of cells is very useful information to have when analyzing you data. In the case that the sample was not properly annotated during collected, some or all of this information may be missing or incorrect. It is very common that data will be collected with labels FL1, FL2, FL3, etc., but missing the staining reagent - making it difficult to understand the nature of the experiment. FlowJo also uses this information in the creation of groups, so it will be hindered in your attempts to, for instance, apply the same gates to all samples stained with CD3 & CD4.

To edit the keywords, you must first be [displaying those keywords](#) in the workspace.

Double click on the keyword you wish to edit (even if the box is blank). As in the figure below, you will see that the information is redrawn with a box around it, and a popup triangle to offer choices of existing values of the keyword.

At this point you may type in a new value for this keyword. Alternately, if you click on the triangle, a menu will appear containing all of the values used for that keyword in the current workspace. Select any of these choices to change the existing keyword to that value. Choosing the item out of the list is the recommended method if possible, because it guarantees that typographical errors will not yield different samples which are labeled with minor differences that may not be noticed by the user, but still prevent FlowJo's batch processors from handling the data in the same way.

 Note that the changes you make to the keywords are only saved within the current workspace. If you change a keyword, and then add that file to a different workspace, the change will not be seen in the second context. In order to make persistent changes to the keyword values in the FCS files themselves, you should use [ProJo](#), a companion utility created explicitly for this purpose. If you need to make changes to large numbers of files, ProJo will also be more convenient for the task.

See the following topics for more information:

- [The Workspace Window](#)
- [Changing workspace information display](#)
- [Groups](#)

- [Statistics](#)
- [Batch analysis](#)
- [Dragging and dropping nodes](#) (copying gates, statistics or other analyses)

Find this helpful?



Reading FACSDiVa Data Files

Export from DiVa

BD FACS DiVaT version 4.01: - export as Experiment - This format allows for the best transformed displays of uncompensated parameters; however, very little annotation (stain names, acquisition time, etc) is included in the file. If these are important, then export as FCS 3.0, 18 bit resolution (262,144 channels). Uncompensated data will not be transformed (but compensated will be). BD recommends that you upgrade to version 4.0, and has indicated that this is a free upgrade.

BD FACS DiVaT version 4: - Export as FCS 3.0. FCS 3.0 data files from BD FACSDiVaT Software v4.0 are in floating-point format and are fully annotated.

Import to FlowJo

Compensation

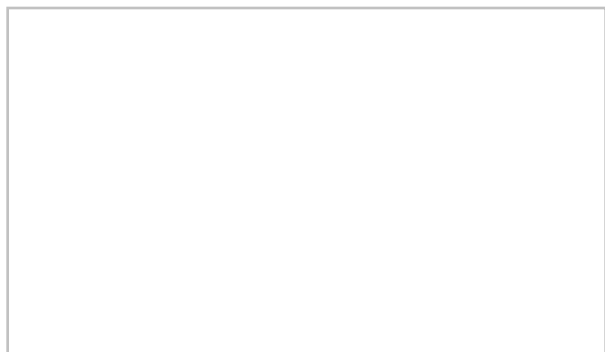
All data exported from the BD FACSDiVaT software (except FCS 2 files) is saved in **linear, uncompensated** format. If the data was compensated during acquisition, this compensation matrix is included in the data file (in FlowJo, it is referred to as the **Acquisition matrix**).

FlowJo will apply the Acquisition matrix to the data files. Therefore, when you add the data files to a FlowJo workspace, they show up with a red bar beside them (indicating they have been compensated). To look at the parameters uncompensated, hold the option key and click on the graph window axis pulldown lists and choose parameters without brackets < >.

The Acquisition matrix can be edited or an entirely new matrix can be created and applied to the data files. In order to replace the previous matrix, simply apply the new one to the data file. To edit the Acquisition matrix, you must first create a new matrix that is a copy of the acquisition matrix, and then edit that.

Linear or Log Scaling

Since data exported from the BD FACSDiVaT software (except FCS 2 files) is saved in linear format, the user must tell FlowJo in which format to display the data (log or linear.) The factory default FlowJo Preference is to show all fluorescence parameters in log and both side scatter and forward scatter in linear format.



To change these Preferences, go to the menu **FlowJo > Preferences**. on the **Workspace** tab you will see **Reading DiVa**

and other 32 bit Data Files. (Shown at right) Click on **Define**.

This dialog is then displayed by FlowJo:

□

In the upper-most area, you can select which parameters should be shown with logarithmic scaling by default. In the box to the right, you can type the names of selected parameters to always display as linear (for example, when doing Cell Cycle analysis, these parameters should be linear). If you have a workspace open, then the popup menu **Add to list** will show parameters collected in the files in that workspace.

If you do not wish to use FlowJo's visualization aid, you can remove the check mark from **Enable Transformation**. You can then make your own display choices in the area called **Standard Log Conversion Settings**.

How Many Decades to Display

The BD FACSDiVaT software has an option to only show the top 4 decades on the plots - in order to avoid showing the picket fencing in the lower region. However, FlowJo shows all the data and transforms the way the data is displayed using Logicle scaling. This will add a small amount of negative to each axis in order to remove the picket fencing and bring the negative populations up off the axis. Note the Preference above, set to "Enable Transformation".

Display Transformation (Bi-exponential Scaling)

FlowJo can display data in order to show all the cells that are normally squished against the axis. A minor transform is applied to the uncompensated data when users export as directed above from the BD FACSDiVaT because this export generates data files that can have negative values for Area parameters. FlowJo uses a single automatic transformation for these parameters.

When you then compensate these parameters (i.e., those that are already transformed), the compensated parameters by default will get the same minor transformation. This might be enough transformation--but often isn't. For this case (and whenever you compensate data), you can still choose to create a custom transformation for your data.

For this purpose, you should select a fully-stained sample (that has fluorescence in all channels), gated to remove dead or unwanted cells (i.e., lymphocyte gate), and then choose "Define Transformation..." from the Compensation menu (under Platforms). A custom transformation is computed based on the distribution of the data, which results in a better visualization.

The custom transformation will be applied to all samples that are compensated the same way in the workspace; hence it only need be computed once for each unique compensation matrix. The transformation can be re-calculated based on a different stained sample (or gate) if necessary.

Note that if gates are drawn on a sample before it is transformed, then the positions of the gates should be verified. This is because the lower edges of the original gates may only extend down to small positive values (the original log scale)-after transformation, many events may be in the negative value area and thus no longer within the gate.

Find this helpful?



Cells Squished Against the Axis

Cells on the axis

There are a large number of cells squished against one or both axes. This is not necessarily incorrect, as properly compensated data sometimes has a large number of cells against the axis. [Click here](#) for more information on why the occurs and how to know if your data is correctly compensated. If you have compensated using FlowJo, the data will be properly compensated. If you compensate when acquiring on the flow cytometer, the data may be overcompensated with too many cells squished against the axis (unless you were careful to make sure that the medians of the positive and negative populations are the same).

□

This warning was implemented for two reasons.

- 1) When you have overcompensated your data, too many cells are squished against the axis.
- 2) When drawing gates to define populations of cells that are negative for one or both of the parameters (i.e., the double negatives or the single positives on a bivariate plot), make sure you include the cells on the axis in your gate. Because a large number of cells may be on the axis, you must draw the gate on the axis to include all the cells in that population.

You can make changes to the warning under the Graphs and Gates tab in the Preferences. To turn the warning off, set the percentage boxes (right) to 100%.

Find this helpful?



Printing

Header and footer information printed at the top of each batch report page can be added or edited using the Edit Printed Page Layout Information dialog box. When creating a Preview report and then printing or when Printing Directly, the header and footer options can be edited directly as shown below. For other types of reports such as a New Layout, the header and footer information can be edited from the Preferences dialog box.

Create a Preview report and print it.

1) In the **Layout Editor** click the **Batch** button.



2) In the batch report layout dialog, click the **Preview** button.



3) Once the batch preview is complete, click the **Print** icon.



4) In the **Layout Options** dialog click the **Edit** button.



Clicking the Edit button brings up the following dialog box where the information shown at the top and bottom of each printed page can be edited. Type in the box any text that you would like printed at the top or bottom of each page in the report. In order to incorporate information from the workspace into the header or footer of the report, use the special purpose strings. The options are listed out in the center of the dialog box below and begin with a # sign. Special purpose strings information (such as the group name) will be interpreted from the workspace at the time of printing.



Alternatively, choose **Direct to Printer** in step 2 and click **Customize** under the Geometry area of the **Make Batch Report** dialog.

Find this helpful?



Credits

FlowJo was primarily written by Adam Treister and Mario Roederer, based on concepts developed with David Parks, Martin Bigos, and Wayne Moore.

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We are indebted to the Herzenberg laboratory at Stanford and our tireless beta testers for comments and suggestions.

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Find this helpful?



The Layout Editor Gate Statistics Legend

The Layout Gate Statistics Legend allows for the assignment of multiple statistics, or keywords, per gate level on plots in the Layout Editor, nicely organized in a tabular form. Like all layout objects, the statistics table can be batched across a data set or experiment.

To enable the table object, double click on a plot in the Layout Editor, select the **Annotate** tab in the **Graph Definition Window** and then check the **Show Gate Statistics Legend** checkbox.

□

When you return to your layout you will see the legend. By default, the legend will appear with just the Gate Name and Frequency of Parent columns and will be populated with gate level information for those columns. The Statistics Legend updates live, so any additional gates created on a population will update in the Statistics Legend for a plot in the Layout Editor.

Double-click on the Statistics Legend to open the legend options.

□

Form the Statistics Legend dialog, you can add additional statistics from the Workspace via drag-and-drop to the Table Columns area of the dialog (upper right area), as well as choose additional keywords to display, or formatting options.

□

Once the Statistics Legend has been formatted to your liking, you can batch the plot(s) across datasets, and a legend will be generated for each plot.

Find this helpful?



Cloning Gates

Please note: this functionality requires FlowJo for Mac 9.3b5 or later.

Introduction

The use case for this feature came from our clinically inclined users who wanted one version of a gate propagated to different points in a hierarchy. This used to be done by one of two ways, neither of which is optimal in terms of workflow:

- Create a version of the gate at a top level, and at each point in the tree, insert a boolean gate that refers solely to that top gate
- Create the gate; copies of the gate are FJML where each vertex refers to the vertex of the original gate

We implemented a new gate type, a "clone". Once you create a gate, you can choose to "clone" it, and apply it to different points in the sample hierarchy. Both the original and all copies are then designated as a "clones". Whenever a clone is modified, all of its clones are also modified (including renaming it). Thus, a clone can be modified at any point in the tree, and all of its versions will update appropriately.

Detailed Description

- Any gate can be defined as a "clone". This is just a property of a gate. A clone can be unique in a sample, or there may be multiple clones of the gate in different places in the hierarchy. All clones in a sample will be identical; modify any one of them and all update. To clone a gate, select it, and choose Workspace->Clone Gate. It can be un-cloned via the same menu operation.
- A clone gate is designated by a special additional icon in front of the gate name (3 identical green circles) :
 -
- There can be no other gates with the same name as a "clone" gate in the sample. If you specify that "CD4" is a clone (in the /Lymphs/CD3 gate), then you can't have any other gate named "CD4" anywhere else in the hierarchy. Once you've defined "CD4" as a clone, you can drag it to other places and clone it. (Alternatively, if you specify that "CD4" is a clone and there are other "CD4" gates, the program could allow you to force them to all become clones, I think that's reasonable).

Note: If you drag a clone (e.g. "CD4") into a destination sample, then ALL "CD4" gates in that destination sample will automatically become clones.

Brief Example

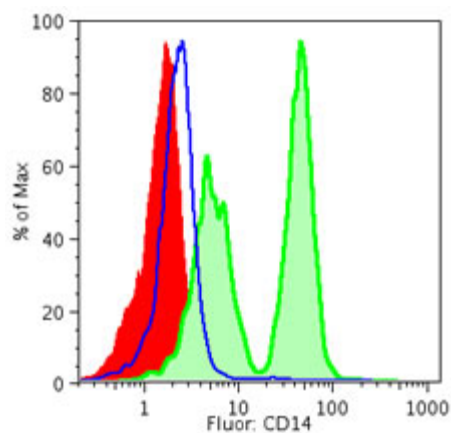
Let's link and display quad gates on all levels of a T cell analysis to show effect of different gates on contents of quads.

- First, take a regular analysis with quads attached only in the relevant spot:
 -
- Select the quads and “clone” them from the Workspace menu:
 -
- Next, copy the clones to other points in the hierarchy:
 -
- And finally display them in the layout:
 -
- Now adjusting the quads in any graph window that displays this set, will update it for all 4 plots.

Find this helpful?



Graph Overlays and Legends






Often it is useful to plot two or more data sets or population on the same axes. This makes it easy to compare the data, using differing colors to show multiple populations at the same time.

Overlays may be created on either univariate (right) or bivariate displays (below). Within the **Layout Editor** you can edit the color and order of the items, to customize the look of the overlay graph that best highlights your data. Once an overlay has been defined, the layout editor can create the same graph for many different samples or sets of samples, by iterating over a group in the workspace.

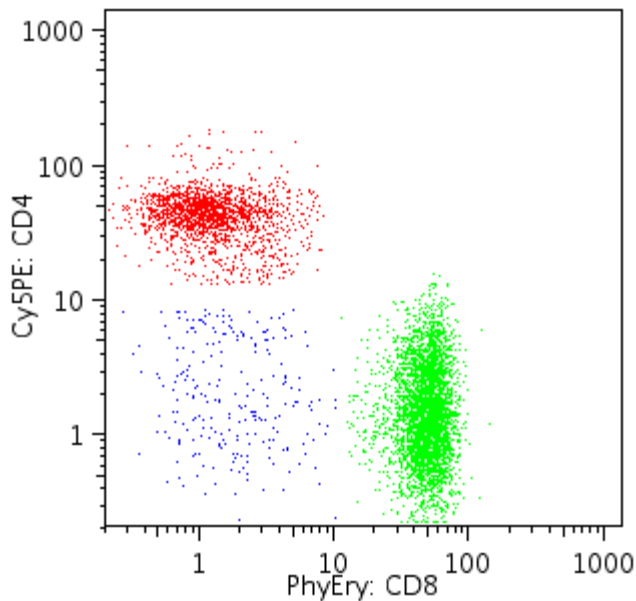
To create an overlay, drag a graph into the Layout Editor. Then drag a second on top of the first one. As the mouse moves into the original graph item, you'll see that it highlights its borders to signify that it will accept the contents of the drag. Drop the second graph (or more, if you had multiple nodes selected in the workspace) on top of the original, and an overlay is automatically created.

The Overlay Legend

	Lymphocytes/T cells/CD4 T
	Lymphocytes/T cells/DN T
	Lymphocytes/T cells/CD8 T

As soon as you release the second node on the first, the graph will change into an overlay. Overlays are recognizable by the existence of a legend on the right side of the graph. The number of the rows in the legend shows the number of layers in the overlay. The graphs often change from black and white to color, as each data set gets its own color within the overlay.

The legend provides the user interface to edit several aspects of the overlay. Click the mouse once on the legend to select it. Then pass the mouse over different areas of the legend to see to change the cursor to signify the different editing operation that are available.



When the mouse passes over an edge of the legend, the cursor will become a hand. Clicking the mouse on the outside edge of the legend lets you move or resize the legend.

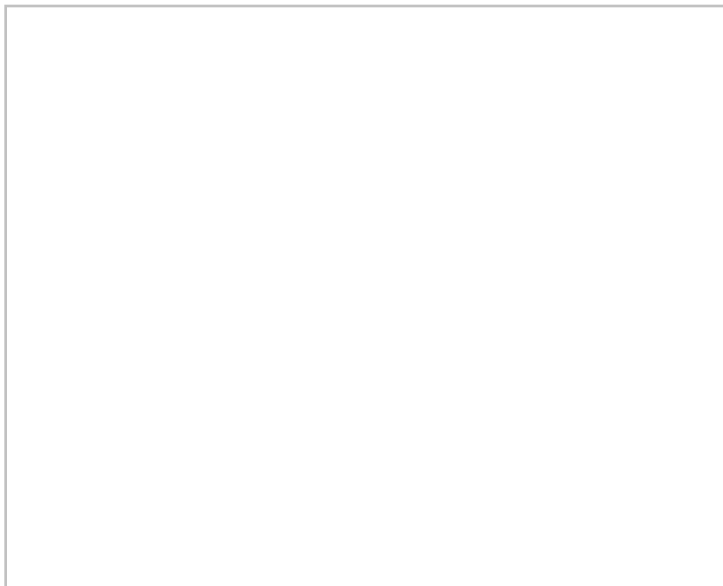
If you move the mouse over the text description of the layers, you'll see the cursor become a pair of bars with vertical arrows sticking out of it. This tool allows you to reorder the legend. The order of the lines in the legend mirrors the order of the graphs displayed. Moving a graph's name to the top of the list moves the graph to the top of the overlay stack. Because events in front can obscure others, you may want to have the smaller populations on top of the larger ones, though this depends greatly on the context of your analysis. Dragging the order of the layers will cause the graph to be redrawn, so you can quickly see which layers you want to feature in the top positions. If you hold down the option key, you'll see that the cursor changes from a vertical reordering tool into a trash can. This signifies that option-clicking a layer in the legend of a graph will delete that layer from the graph.

Clicking the mouse in the colored box at the left side of the legend will pop up a rainbow palette, to let you change the colors of the layers in the graph.

If the graph is a univariate (a histogram or cumulative distribution function) the line weight and fill style of the graphs are also editable from a dropdown menu under the line style box. (Right) This is the gray and white square just to the right of the color box. It is only visible in the legend of a univariate graph.

The content of the legend itself can be edited by double-clicking on the text of the legend. This produces the **Edit Overlay Legend** dialog shown at the left. Here you can select color and style for text, box and background. You can add column header names by clicking in the cells underneath the **Heading** column of legend **Edit Overlay Legend** window. You can also choose to display more information using the **Insert** button. To preview the legend before accepting changes click on the **Update Layout** button.

Choose Column Value displays a dropdown list. Selecting a value here places it in the **Columns to Show in Legend** box. When you select **OK** the graph will display the new column and its values for the populations in the overlay graphs. All keywords available in your data files, as well as **Workspace Display Columns** and all statistics are available to place into the legend.



Just below the **Insert** button is the **Sigma** button, which opens the **Add Statistics** dialog window. Here you can choose from a list of statistics that will be added as columns to the Overlay Legend. Several statistics are repeated with **(channel#)** appended. These statistics will be reported for their values in channel space, the others in scale space.

□

Select the channel to which your statistic should be applied from the **Channel** dropdown list. Note the additional options:

- Add for all parameters
- Add for all fluorescence parameters
- Add for all compensated parameters

To save time, you can set your preference for the keywords you would like FlowJo to display in the legend by default. Go to **FlowJo -> Preferences** and click on the **Tables and Layouts** tab. There you can select the **Default Columns in Legend**.

□

Using these options will save you the tedious job of selecting and adding each channel individually. Unwanted channels can be deleted from the Legend by control-clicking.

The legend is used to control all of the attributes of the graphs appearance. By default the legend is visible if there are multiple items in an overlay, and hidden for single histograms or 2D plots. If you want to edit the color, line weight, or dashing of a single graph, you can double click on the graph, and set the Show Legend attribute of the Annotation panel.

□

Histograms can optionally be offset when they are overlaid, so as to distinguish the curves better. The feature, as well as how much of an offset is used are specified in the [Annotate panel](#) of the [Layout Item Definition](#) dialog, summoned by double clicking the graph (or selecting it and choosing **Get Info...** from the **Layout** menu).

Find this helpful?



Event Gates

To manually enter a gate that is created by event number, select **Event Number Gate** from the **Platform** menu. You will be presented with the following dialog box. Enter the first and last event that you would like FlowJo to use as the bounds for the gate. Provide a name for the new gate and then press **Create Gate**.



FlowJo will display the total number of events that can be gated on next to the **First** and **Last** fields and uses a time parameter to select which events to include/exclude when making the gate.

Find this helpful?



Editing Gates

Boolean Gates

You can now create a gate that is a Boolean combination of any existing gates using all the Boolean functions, including OR. To create or edit Boolean gates, select the parent subset in the Workspace and then select the menu item **Boolean gates...** under the **Platforms** menu.

Edit...	Sort...	Name	Statistic	#C
		Sample1.fcs		
		Lymphocytes	39.8	
		T Cells	63.5	
		CD4+	67.8	
		CD8+	31.4	

In this example, we have chosen the T Cells subpopulation within the Lymphocytes gate as the parent. When the tool pictured below opens, give a name to the new subset you are creating...

Create Boolean Gate

Gated events will be selected from the subset: ?
 Sample1.fcs/Lymphocytes/T Cells

Name the new subset:

Specify the gate as a Boolean combination of existing subsets:
 1

2

Insert subset specified relative to this gate:

Insert subset specified relative to sample:

Insert function: 3

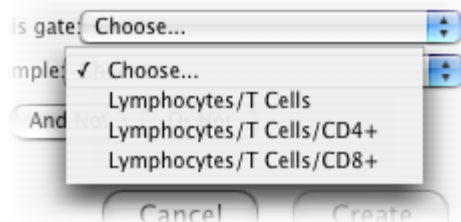
4

1. The specification of the new gate will be created in this window.
2. Select the first subset of the boolean gate from a drop-down list including positions **relative** to the chosen subset in the gating hierarchy. That list looks like this:



OR...

3. Select the first subset in relation to the population at the **sample** level, its **absolute** position. That list looks like this:



4. Choose the boolean function by clicking one of the **Insert function...** buttons.

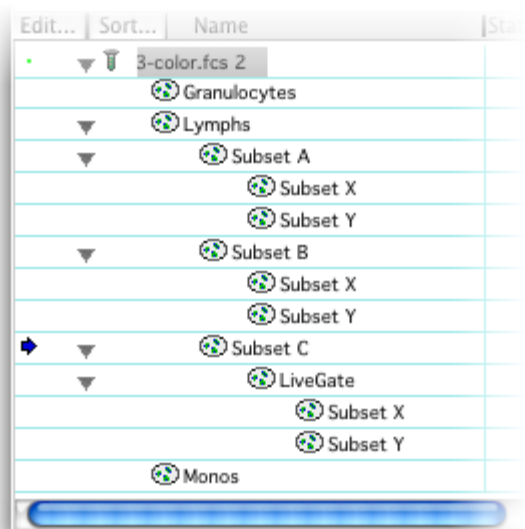
Finally, select a second subset to complete the specification of the gate as shown at #1 above.

Click **Create**. The Boolean gate will be displayed in the workspace window as part of the gating hierarchy.

dit...	Sort...	Name	Statistic	#(
▼		Sample1.fcs		
▼		Lymphocytes	39.8	
▼		T Cells	63.5	
		Boolean Gate	99.2	
		CD4+	67.8	
		CD8+	31.4	

Relative vs. Absolute Gate Specification

You can choose the gate's position from either of the two drop-down menus. The results are the same for this particular gate. But if you wish to distribute this gate to other populations, it is essential that you select the appropriate parent for the gate. With relative boolean gates, the gates to which the boolean operators refer will be different after dragging and dropping -- FlowJo now looks for new gates in the destination hierarchy. On the other hand, with absolute references, the gates referred to don't change. Here's an example:



If you add a boolean gate to Subset A which is **Subset X OR Subset Y** with relative paths, and then drag that boolean gate to Subset B, the boolean gate will now change to refer to the gates Subset X and Subset Y that are under the Subset B tree. With absolute reference, they would still refer to the ones under the Subset A gate--which is probably not what you wanted.

But there are times when you want absolute references. Consider a gate that you want to add to Subset A which is something like **Subset X AND Subset Y AND NOT Monos** (i.e., you want events that are both in Subset X and Subset Y but want to exclude Monos). In relative terms, this is specified as **/Subset X AND /Subset Y AND NOT ///Monos** -- i.e., have to go up three levels to get to Monos. If you now dragged this gate to the **LiveGate** under Subset C, then the references to **Subset X AND Subset Y** work just fine (because those exist at the relative level under Subset C--but it won't find the Monos gate because that one is now one more level up the hierarchy. Here, what you would want to do is to specify **SubsetX AND SubsetY** as relative, and **Monos** as absolute path--so that the Monos references would be correct no matter where in the hierarchy the Boolean gate is dragged.

So, use absolute specification if you need to move the boolean gates and they refer to gates that are at different levels. Otherwise, you would almost always want to use relative specification.

Boolean gates are "live", meaning that they will reflect any changes you make to the gates on which they depend. Boolean expressions can be arbitrarily complex; you can use parentheses to associate the expression properly. For example: **Live AND (CD4 or CD8) AND NOTGammaDelta** will create a gate that includes cells in the predefined Live gate, GammaDelta gate, and either the CD4 or CD8 gates.

Note that the Boolean expression you specify is applied to the cells in the subset to which you are adding the Boolean gate. For example, if you add a Boolean expression to the Lymphocyte gate, then only lymphocytes can be part of the gate (no matter what the Boolean expression is you can't include a Monocytes gate)-if you specify **NOT Lymphocytes** in the expression then you would get no cells! In this case, you probably want to add the Boolean gate at the sample level.

Because all parent gates are automatically part of the final gate (from FlowJo's standard hierarchical approach), they will not appear in the popup menu of possible subsets to include in the expression.

FlowJo can also automatically create a series of Boolean gates that represent all combinations (plus and minus) of a set of gates. For example, if you have a sample which was stained for CD8 and CD4, you can automatically create 8 gates that represent all combinations of expression of the cytokines (e.g., CD4+CD8+, CD4+CD8-, CD4-CD8-) using FlowJo's [Boolean Combination](#) option.

Find this helpful?



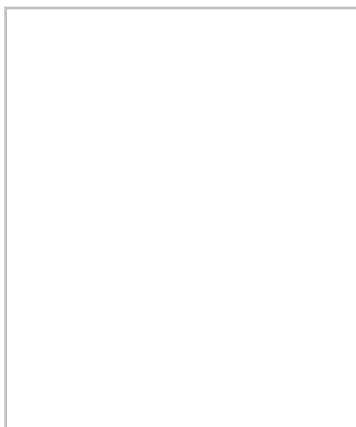
Statistics Table Tool



Now you can build or edit a table of statistics within the Layout editor. To edit an existing table, double click on it to produce the dialog box below. To create a new table, click the **Sigma** button in the Layout editor's tool palette. Drag a rectangle in the area where you want your statistical table to appear. The following dialog opens within which you can construct your table.



Drag-and-drop samples or subpopulations from the Workspace into the upper left box. This list will label the rows of your new table. Drag-and-drop statistics or other information into the upper right box. These will label the columns of your table. When you click the **Update Layout** button, your table will appear in the Layout editor with the calculations in place.



Use the **Choose Column Value** (Number One above) drop-down list to add columns that don't appear in the current layout. Possible values are shown at right.

Click the **Sigma** button (Number Two above) to add a new statistic just as you might do in the Workspace. Use the checkbox **Add Statistics to Subsets If Missing** (also next to the Two) to tell FlowJo to calculate the statistical values for populations in the new table that have not yet been calculated in the Workspace.

Opening the disclosure triangle labeled **Appearance** shows the usual tools for controlling the style of the text and boxes in the new table.

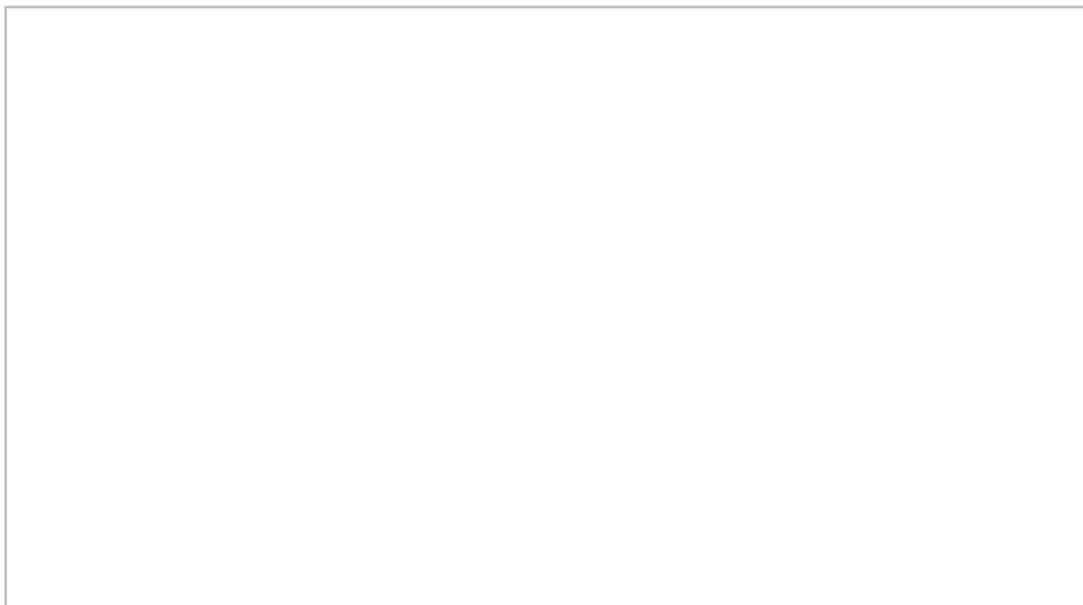
Click **Update Layout** to view your changes in the Layout Editor, click **OK** to accept the current choices and close the **Edit Statistics Table** dialog box.

Find this helpful?



Sorting the Sample List

To sort the sample list, either choose the **Sort Sample List...** item under the **Workspace** menu, or double click in the first column of the header bar (between the group and sample lists). This brings up the dialog shown below.



1. Add selected keys from the left hand list of **Possible** sort values to the **Active** list.
2. Remove selected keys from the **Active** list.
3. Remove *all* keys from the **Active** list.
4. Restore the default keys.
5. Toggle the selected **Active** sort value from ascending to descending and back.

From this dialog, you can choose from a variety of different keywords on which to sort. If you select more than one keyword, then the list is first sorted on the first key; then, for all samples that have the same value of that key, they are sorted on the basis of the second key; and so forth. The sample list is sorted identically for all groups.

The arrow button lets you choose whether to sort in ascending or descending order, the **Use as Default** button lets you save your sort order for future use.

If you choose **Sort**, the sample list is sorted according to the criteria you select. Note that if you later add samples to the workspace, the list is *no longer sorted!* To keep the sample list sorted, go through this procedure again after adding samples to the workspace. If you checked the **Use as Default** button, just click the **Sort** button and you're ready.

Note that you do not have to display the keyword values in the workspace to sort on them.

For more information about the possible keywords that you might see in the selection list, [click here](#).

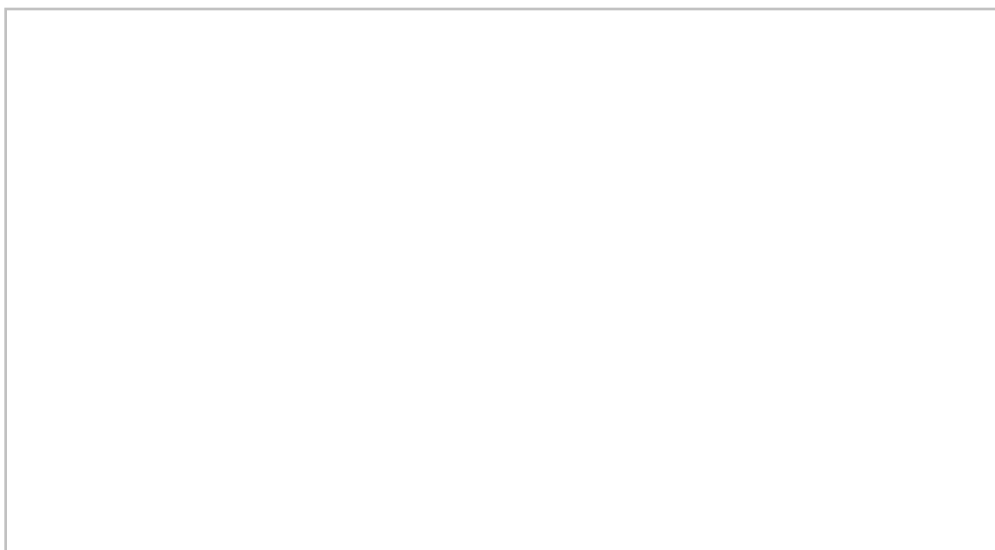
For more information about changing or adding annotation after sample collection, [click here](#).

Find this helpful?



Customizing Workspace Display

When you double click on the header bar (between the sample and group lists), or if you select **Edit Workspace Columns...** from the **Workspace** menu, then the dialog below is shown. From this dialog, you can change what information FlowJo shows you about the samples in your workspace.



From this dialog, you can choose from a variety of different keywords whose values you wish to display in the workspace. Each FCS data file has two essential components, the description of the conditions at the time of collection, and the sets of numbers measured for each event. It is up to the operator to correctly annotate the conditions using Keywords to organize this information in each FCS file. Each keyword is designated with a \$abc (non-standard keywords do not have a \$). For more information about the possible keywords that you might see in **Possible Column Values**, [click here](#).

The order in which the Keywords are shown in the workspace is the same as the order in the list on the right (drag the keywords to change the order). The **Name** column must always be displayed. If you select a keyword for which a sample has no value, then the column is left blank for that sample. For information on annotating your data-post acquisition, [click here](#).

If you choose to display **File name**, then FlowJo will add a column which shows the actual file name (on the disk). This can be different from the keyword value **\$FIL**, which has the file name with which the data was originally saved.

Find this helpful?



Graph Window Sizes

There are three different size options for graph windows... the windows that are created when you double-click on a subset to view a graph of the data within that subset. The graphs will differ in the amount of space that they require on the screen, and, perforce, the amount of information that each can display.

You can choose to have FlowJo open graphs in Normal, Medium or Large size by default. Each graph has the same data displayed. Each graph is resizeable to fit the space on your monitor.

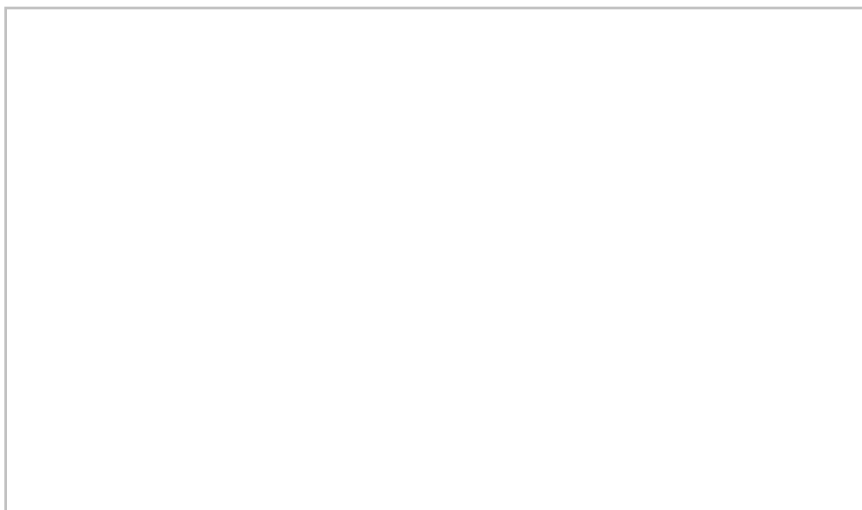
The size of any newly-created graph window is determined by the preference that you have set. If you want to change the default size, open [Preferences](#) under the **FlowJo** menu item. Choose **Graphs** and under **New Graph Windows** choose your preferred size from the **Size of new window** drop down list. Click the save button and you're done.

Find this helpful?



Replace Node Dialog

When you drag a node (or a tree) onto another sample which has a node with the same name, then you are presented with the dialog shown below. (Note that if you are dragging a node with its parents (holding the control key down during the drag), then it is the name of the top-level parent which is compared to nodes underneath the destination node.) Choose one of the four options by clicking on the buttons.



The **Cancel** button will discontinue any more copying of analyses to the destination. The **Rename** option will cause the nodes you are dragging to be added to the destination with its name modified by a version tag (e.g., "-2" is added to the name). **Retain** tells FlowJo that you only wish nodes that you are dragging to be added to the existing tree if they do not already exist--i.e., the existing gates in the destination sample will be unaltered. **Replace** tells FlowJo to replace the gates (which have the same names as the gates you are copying) with the versions of the gates that you are dragging.

These options are more fully described in the page on [node replacement](#).

Find this helpful?



The PolyChromatic Plot - Refine Settings

The PolyChromatic Plot uses color to assist in visualizing a sample's staining and intensity. For basic configuration, [click here](#). To learn about the other settings, click the following links, or click on the tool itself.

[Color Scaling](#)

[Color Display Priority](#)

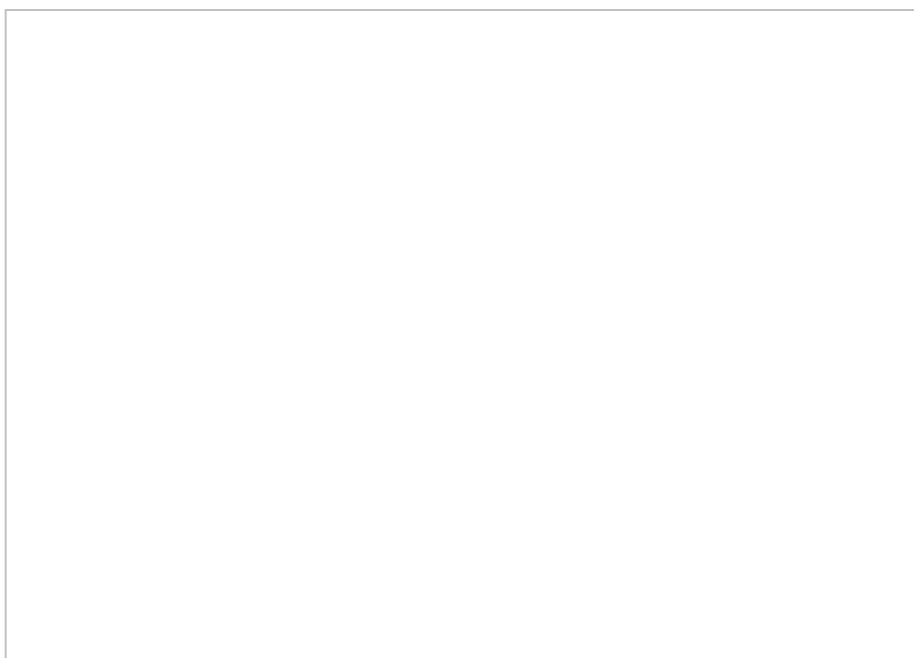
[Explore Priorities](#)

[Explore Scaling](#)

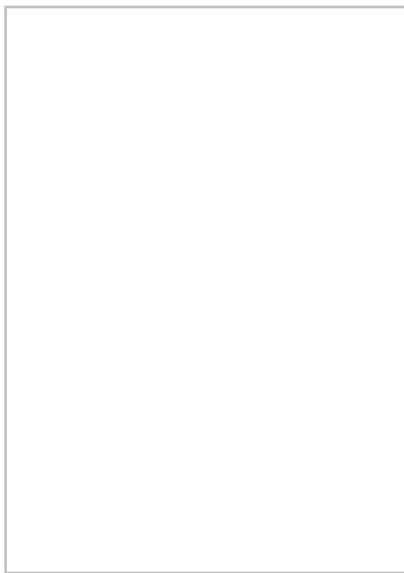
[Copy/Paste Settings](#)

[Revert - Copy to Clipboard](#)

When you are using the PolyChromatic plot, you can open and adjust its settings by clicking the triangular spectrum icon in the **Graph** window: Adjustments to the color settings of each plot are independent of the other plots, whether you are working in the **Graph** window or the **Layout** editor.



Color Scaling is controlled by three drop-down menus circled above. Scaling is how FlowJo maps fluorescence intensity to color intensity. (You can most easily see this by choosing only one parameter to control color, and changing its **Option**. The changes are most dramatic when used on a population that is *bimodal* in the parameter used to control color). Chose **Uniform** (the default), **By Percentile** or **Clustered**.

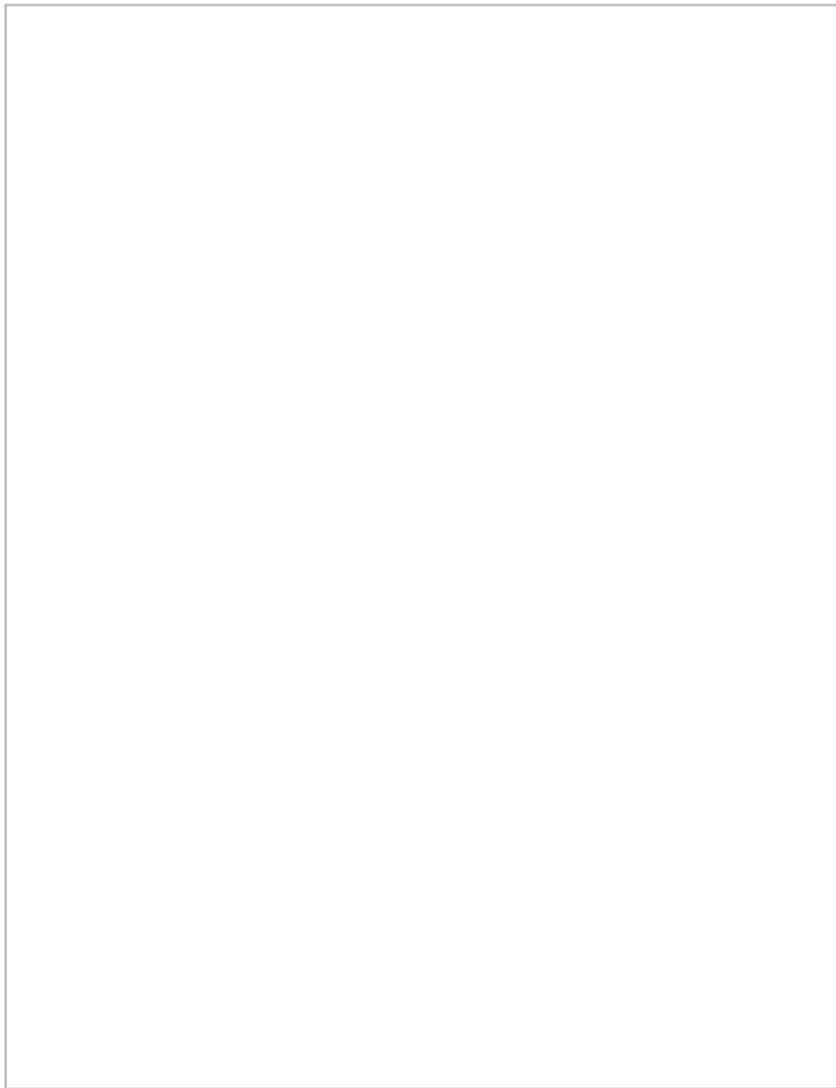


With the **Uniform** scaling, the color range (Black to Red, for example) scales uniformly as the fluorescence intensity changes from the dimmest to the brightest, irrespective of the actual distribution of events in the reference population (although the reference population will define what is dimmest and what is brightest). So if the dimmest event happens one third of the way across the graph, and the brightest event occurs two thirds of the way across the graph, then the color for the events will scale linearly (black to red) as the intensity varies from the 1/3rd point to the 2/3rds point.

The other scaling choices will change this mapping from a straight line. The **Percentile** scaling means the intensity (black to red) maps linearly with the percentile of the distribution. Thus, at the median, the color is halfway between black and red; at the 75th percentile, the color is 3/4ths red; at the maximum fluorescence, the color is fully red. This scaling tends to make the color differentiate within a subset -- i.e., if you have a uniform cluster, the color will change as you go across that subset.

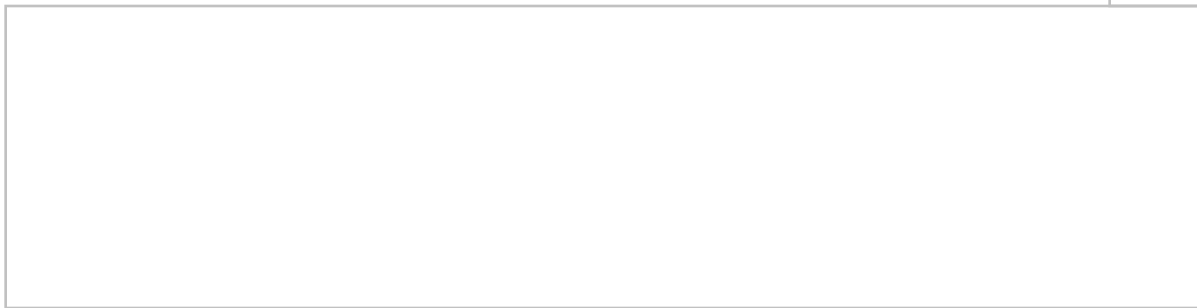
The **Clustered** scaling does the opposite -- the color intensity changes the fastest between subsets, and tries to remain relatively constant as you go across a population.

Explore Scaling... This button opens a new window with scaling settings displayed in an array. The results of all combinations of **Percentile**, **Uniform** and **Clustered** settings are shown for all three colors. You can double click on any thumbnail in this view to change the original graph to that new combination.

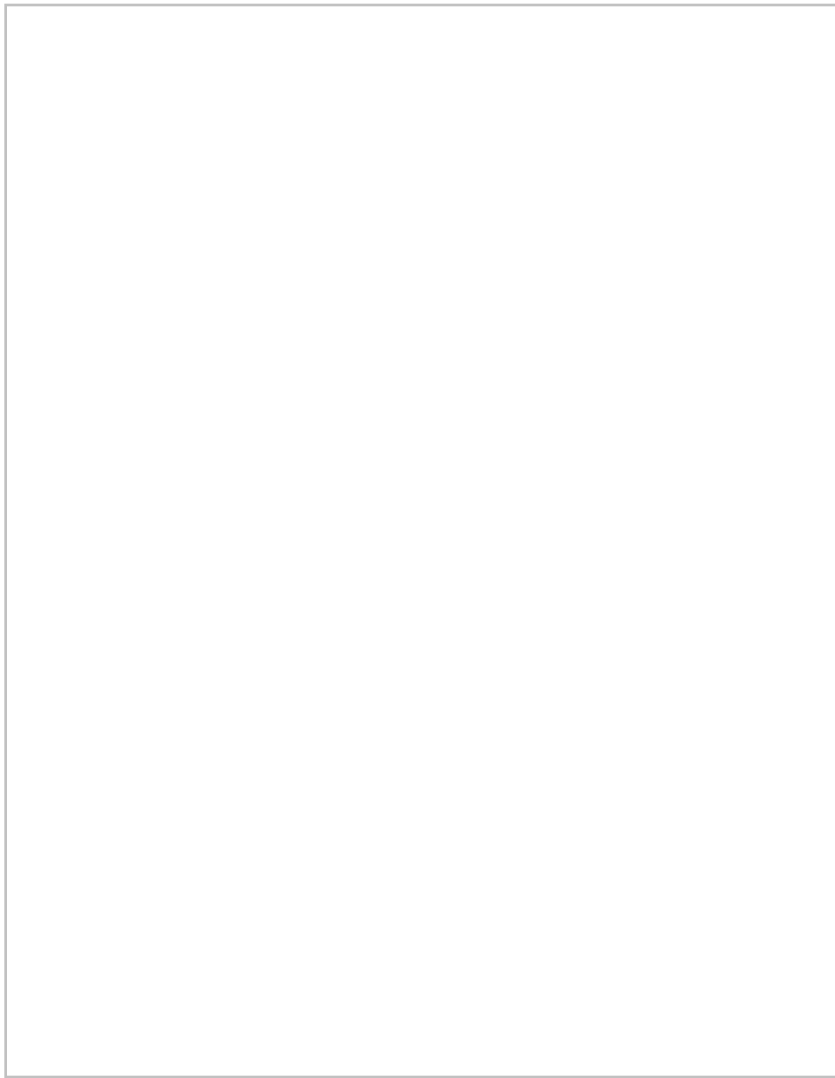


Color Display Priority: this controls how dots are drawn in the dot plot. As you give blue more priority, then dots with more blue color will "float" to the top and be over-emphasized. Likewise, if you give red less priority, those dots with more red color will float down and be hidden. This enables you to reveal rare populations by giving them priority.

Here, each color in turn was set to 100 with the others at 0 (neutral).



Explore Priorities... This button opens a new window with an array similar to the [Explore Scaling](#) array above. All combinations of the three priority settings for three values (-100, 0 and +100) are shown. Like the Scaling array, you can double click any image and transfer those settings to the current graph.



Copy/Paste Settings... You can move the PolyChromatic settings from one sample's graph to another sample with the same staining by clicking **Copy Settings** then move to another PolyChromatic plot and click **Paste Settings**.

Revert returns the settings to what they were when you first opened the Settings dialog... or what they were when you changed the graph window to a new population and the Settings tool pointed to this new graph with its existing settings).

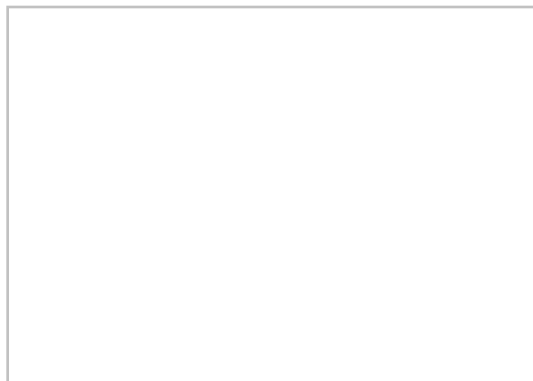
Copy to Clipboard puts a copy of the spectrum image on the clipboard, from whence it can be copied into the Layout editor or elsewhere.

Find this helpful?

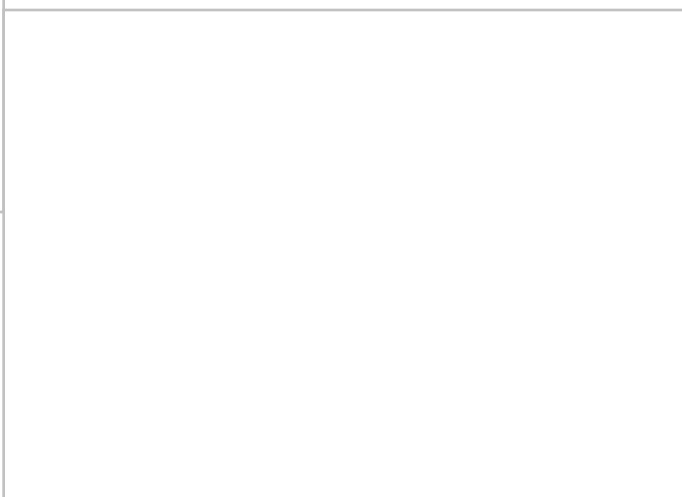


Compensation Example: Step 1

As a simple example, we will look at compensating a workspace which has five tubes collected: three compensation controls (one each for FITC, PE, and Cy5PE), and two PBMC samples that were stained with all three reagents. Our workspace shows these five samples, and the group that was created to hold this experiment:



For organization, we create a new group to hold just the compensation samples, and drag the three compensation samples into the group. Selecting this group results in only those samples being displayed as in the window below.



The [next step](#) is to define the gates for each of the singly-stained subpopulations.

Go back to the [overview of compensation](#).

Find this helpful?



Manual Calibration

If you choose the manual calibration method for calibrating a parameter, you will be shown the following window (with the exception that the "Change Relative Scaling" checkbox is not selected, and the second calibration point entry box is grayed out).

Define Manual Calibration Standard

You can define the calibration standard by defining a single known value, or by entering two values (which may change the resulting dynamic range).

PhyEry Channel values range from 0 to 512

Scale conversion: Reference
 (Define known value) Scale value:
 = -or- Channel:

Change Relative Scaling

Rescaling
 (Define Background) Scale value:
 One Unit = -or- Channel:

Enter values for all fields.

? Cancel Define Parameter

For a simple calibration, you can just enter one pair of values in the "Reference" box. Enter a known absolute fluorescence in the leftmost box, and then enter the corresponding channel number OR the corresponding scale value (of the original parameter) in the boxes on the right. If this is all you enter, then FlowJo simply rescales the original parameter by an appropriate multiplier so that the reference position is correct.

The scale value (or channel value) that you enter might be derived from a gating or calculation on the parameter from this or another sample that you have performed manually. For example, you may have stained cells with anti-CD4, and determined that the mean fluorescence of the CD4 positive cells is 25.6 scale units. You would then enter 25.6 in the "Scale Value" box, and 50,000 in the known value box (there are 50,000 molecules of CD4 per CD4 T cell), and then click on "Define Parameter". FlowJo creates a new parameter which is absolute molecules based on the conversion you entered.

Note that the one-point standard curve is the same as fitting a straight line through the origin (on a linear scale parameter), or maintaining the same dynamic range as the original parameter (for a log scale parameter). In the latter case, this means that the number of decades for the scaled parameter is identical to the number of decades for the original collected parameter.

If you wish to perform a 2-point standard curve, you can check the box "Change Relative Scaling". For linear parameters, you will then enter the scale value (or channel number) corresponding to Zero absolute units. For log parameters, in which there can be no zero, you will enter the scale value (or channel number) corresponding to 1 absolute unit. The net effect of this is a complete rescaling of the parameter. For linear parameters, this may "move" the

events left or right on the graph, because zero absolute units will always be the left-most value on the graph.

For log parameters, rescaling means that you may change the number of decades in the parameter (in order to fit both known points). This means that the absolute values may no longer scale with fluorescence intensity, assuming that the original parameter was a reasonable estimate of that intensity.

The effect of 2-point calibrations is too complex for the scope of this help documentation. If you need more help understanding 2 point calibrations, please [contact the FlowJo administrators](#) for more information.

Find this helpful?



Reference Standard Calibration

If you choose to use a reference standard to create a Calibrated Parameter, FlowJo shows a window similar to that below. (The graph will be a histogram of the parameter you are calibrating, gated according to how you have created the subpopulation). For this example, an 8-bead set was collected; singlets were gated by Forward and Side Scatter. (For detailed information, see the [examples](#) for using the Calibration Platform).

□

In this example, FlowJo identified the 8 peaks (shown in the histogram). To the right, you are asked to enter the absolute calibration points for one or more of these peaks. As you enter values, FlowJo continually performs regressions and graphs the data points and the regression overlaid on the histogram. Further, the final scaling of the calibrated parameter will be shown as a vertical axis on the histogram.

Once you have entered more than one data point, FlowJo offers to force the use of the original dynamic range or to fit that value as well. (By the time you enter a third calibration point, FlowJo automatically switches to fit this value by default; you can switch it back by selecting the checkbox).

When you choose to use the original dynamic range, then FlowJo assumes the calibrated parameter will have the same number of decades of fluorescence as the original parameter (in this example, 4 decades); it only fits a scalar that is used as a multiplier. (For linear parameters, the option given is to force the linear fit through the origin (i.e., that channel 0 is zero absolute units) or to fit an intercept as well). If you choose not to use the original dynamic range, FlowJo uses the data you enter to determine what the actual dynamic range of the calibrated parameter will be. This will be shown on the left axis; as you enter values, FlowJo draws a line as the regression that lets you see how the original parameter will be converted into the calibrated parameter. In this case, FlowJo will calculate and display the correlation coefficient of the fit.

Once you are satisfied with the fit, click on "Define Parameter".

For examples of how to use this platform, [click here](#).

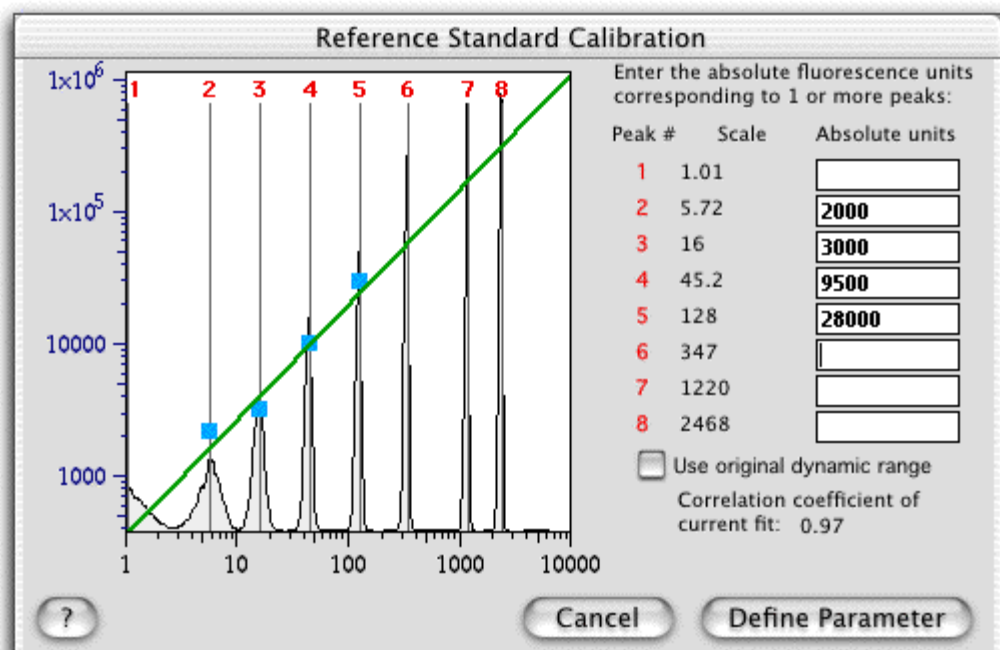
Go to the overview of [Calibrated Parameters](#).

Find this helpful?

□□

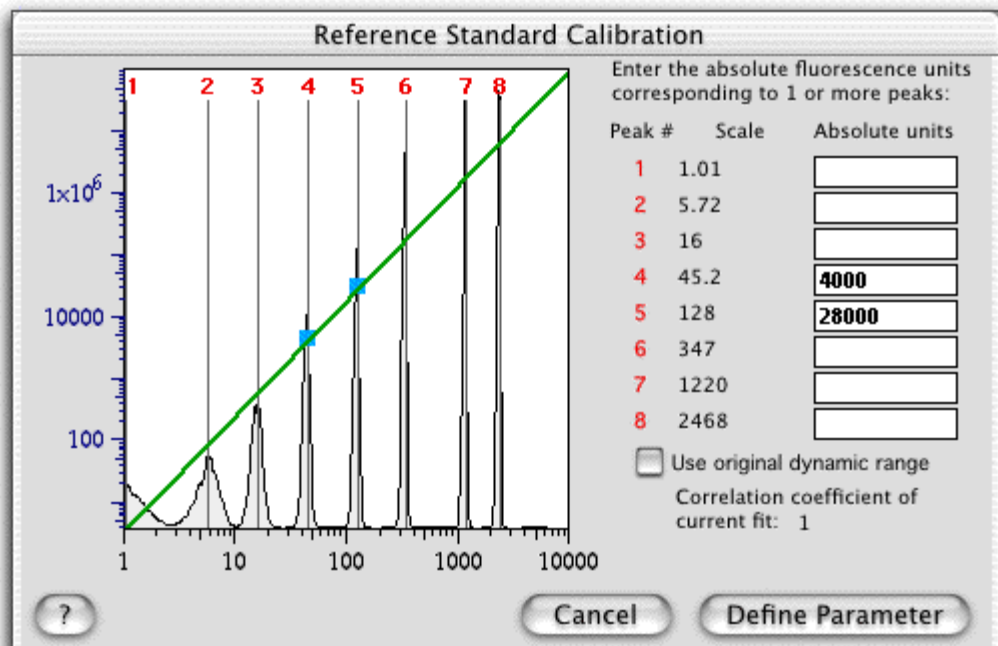
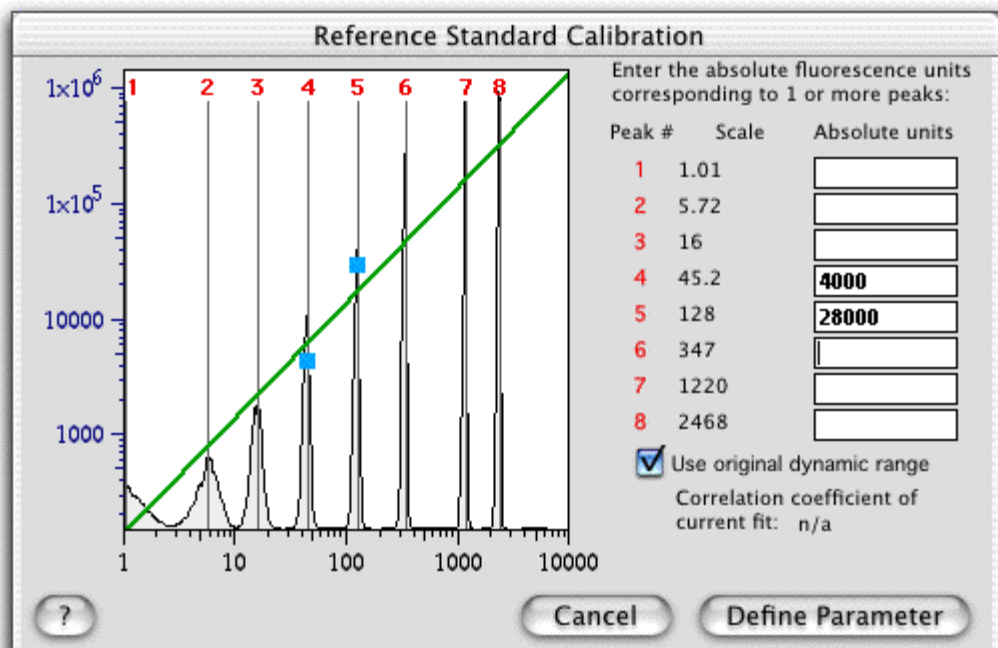
Example Bead Calibration

Once you have gated on bead singlets, and chosen the Calibrate menu item, you might see a window as shown below (this is for an 8-bead set). In this example, four values were entered to define the peak positions of bead numbers 2 through 5:



Note that FlowJo has drawn a light blue point corresponding to each of the values you entered. The green line shows the semilogarithmic least-squares regression to the data. On the left is a vertical axis (drawn in blue) which shows you the scaling of the calibrated parameter. Because the option to "Use original dynamic range" is unchecked, FlowJo is fitting the number of decades in addition to the scaling. In this case, the number of decades of fluorescence turns out to be slightly less than 4, whereas the original collected data assumed there were 4 decades of dynamic range.

In the examples below, only 2 data points are entered, and the dynamic range checkbox is either set or not. Compare these carefully to identify the differences.



Note in the first case, the calibrated parameter will have four decades of dynamic range (the axis ranges from 90 to 90,000). Because the dynamic range is fixed, the two data points cannot be fit exactly on a line, so FlowJo adopts the best fit given the constraint on the dynamic range. In the lower graph, the dynamic range is a free variable. Thus, the two points can be fit exactly. Here, however, there are now nearly 7 decades of range (every other decade is labeled for clarity)! This is because the values entered differ by a factor of 7, but the scale values only differ by a factor of just under 3: thus, FlowJo is forced to increase the number of decades to account for this geometric relationship.

You should be careful when you let FlowJo fit the dynamic range as a free variable. While FlowJo automatically starts fitting this value once you have entered three known values, you can still force it to use the original dynamic range at any time. Of course, with only one value, FlowJo can only fit a single variable and thus fixes the dynamic range at the same level as the original parameter.

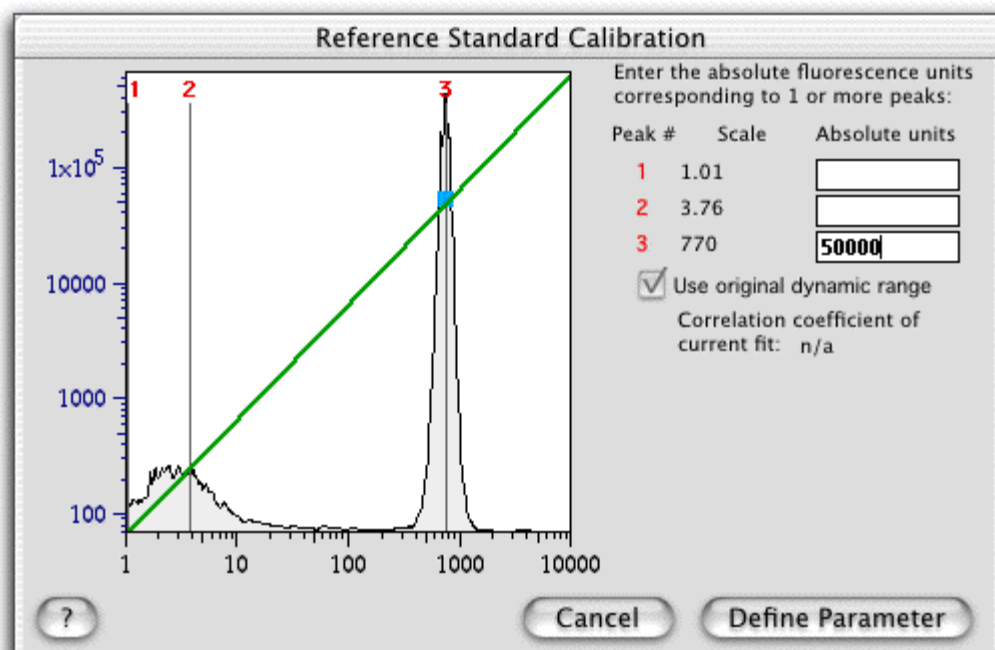
Go to the overview of [Calibrated Parameters](#).

Find this helpful?

□□

CD4 Calibration Example

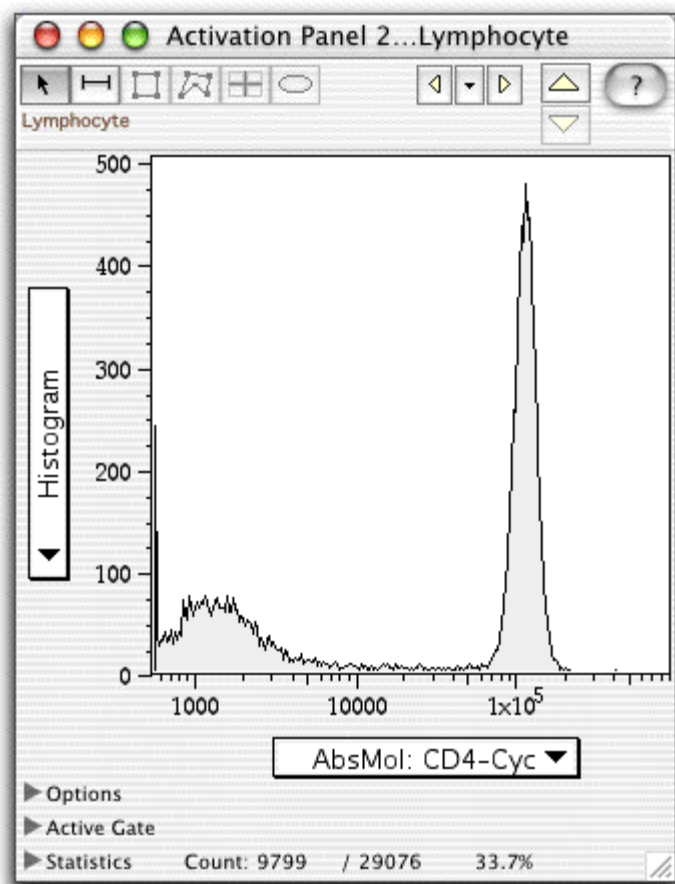
This example is based on the assumption that CD4 T cells express 50,000 molecules of CD4, and that a well-defined antibody reagent is used for detection. First, lymphocytes were gated in the whole sample; the lymphocyte population was then used as the reference standard. The graph in the window below is for the CD4 parameter:



In this case, only a single reference value needs to be entered: 50,000 (molecules) corresponding to peak number 3. FlowJo will assume that the calibrated parameter has the same dynamic range (same number of decades) as the original parameter. In the absence of a much better calibration standard, like a multi-bead set, this assumption must be made.

Once you are satisfied, you can click on "Define Parameter"; in the dialog window (right), the name for this standard is "Absolute Molecules".

At this point, the sample has the "Absolute Molecules" parameter, corresponding to absolute numbers of molecules, already added. If you open the lymphocyte gate and display a histogram of this parameter, you will see the graph:

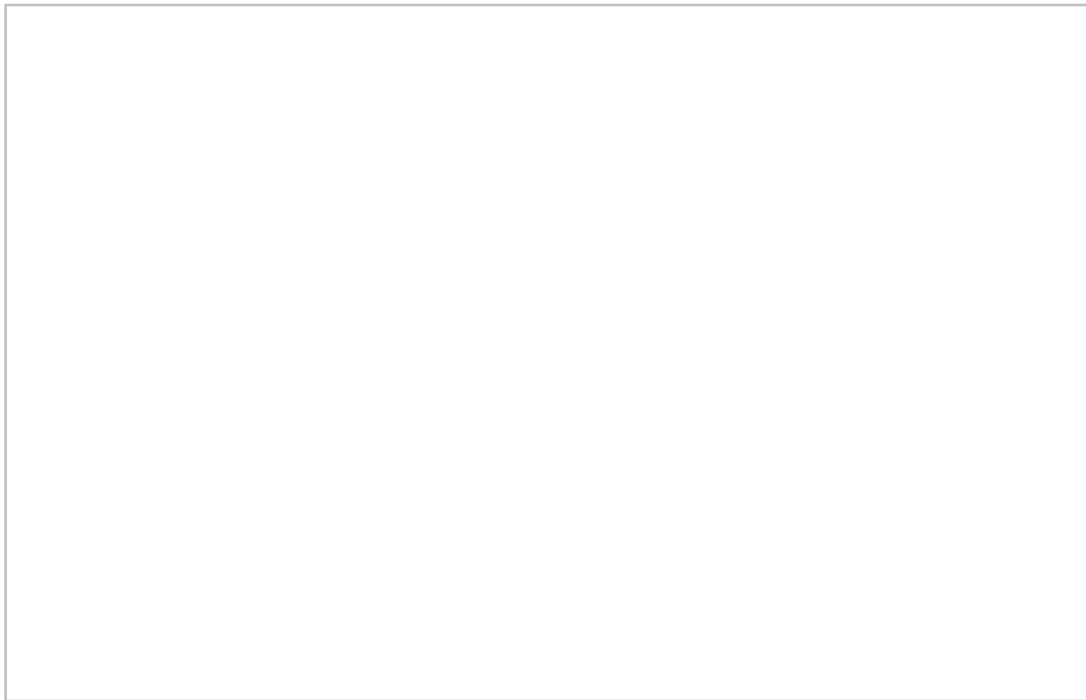


Note that the X-axis scaling is such that the CD4 peak is now right at 50,000. Here, you could choose to manually define a gate (Select "Manually enter gate" under the Graph menu) to gate on cells which have, for example, more than 10,000 molecules of CD4.

At this point, you would like to apply this calibration standard to another sample. Select the other sample, and from the "Derived Parameters..." menu, select the submenu that corresponds to your new standard:

□

Now the other sample has this parameter as well. In our case, we applied the absolute scaling to a sample stained with CD8. At this point, gates were defined on CD4 and CD8 positive cells in the two samples, and the median fluorescence was calculated for all populations. The workspace is shown below:



In this workspace, you can see that CD4 T cells have 50,186 molecules per cell. The "Others" gate was for negative (autofluorescent) cells; in this experiment, autofluorescence of lymphocyte corresponds to less than 200 molecules. The CD8 stain shows up with 66,107 molecules. This is expected, since CD8 is expressed at higher levels than CD4. Again, the negative cells show up with less than 200 molecules of background fluorescence.

Go to the overview of [Calibrated Parameters](#).

Find this helpful?



Kinetics Thresholds

Some of the functions that can be computed from the kinetics Options box take a threshold as a parameter. These functions compute statistics on the events with a Y parameter value greater than the given threshold. The units of the threshold value are the same as the scaled value on the Y parameter (i.e., the same as the units shown on the axis when the Y parameter is displayed in a graph).

Clicking on the "Set Threshold" button in the kinetics Options brings up the window shown below.

□

If you have not defined time slices, then there will be no menu items selectable for the second option.

You can either select the first option and manually enter the threshold value, or the second option to compute a threshold. In the latter case, you will select a previously-defined time-slice and a percentile of the events in that time slice. FlowJo will compute the threshold based on these values. This threshold is then used in the computation of the graph.

In the example, the 95th percentile of the events in the **Background** time slice is defined as the threshold. If you then plot **% of events over threshold**, the graph will average 5% over the time intervals within the **Background** time slice.

Go to the [Kinetics overview](#).

Find this helpful?



Population Comparison

Controls

The **control population** is a different population (either another sample or another population within the same sample) with which the test population is compared. The test population is the one with the Population Comparison platform icon under it.

To define the control population, drag it from the workspace window and deposit it in the box in the top left corner of the platform's window OR drag the control population onto the Population Comparison platform icon in the workspace.

If you copy the Population Comparison platform from one sample to another (or to a group), the same control population is used for this sample, unless the control and test populations are different populations from the same sample. In that case, the control population will change with each new sample to ensure that the test and control populations continue to be derived from the same sample (i.e., the control population is an internal control for each sample). For Example:

1. To compare many samples to a single control - create the platform node for a single sample (that is not the control sample). Drag the control sample to the platform (it is now designated as the control). Finally, drag the platform node to the group.
2. To automate the comparison of different subpopulations within each sample - create the platform for a subpopulation of one sample. Drag another subpopulation from the same sample. Finally, drag the platform node to the group.

You can change the control sample for the whole group by dragging it to the platform node that is in the group panel. However, this won't work for example #2, because it automatically makes the comparison an intersample comparison.

Find this helpful?



Editing Statistics in the Layout

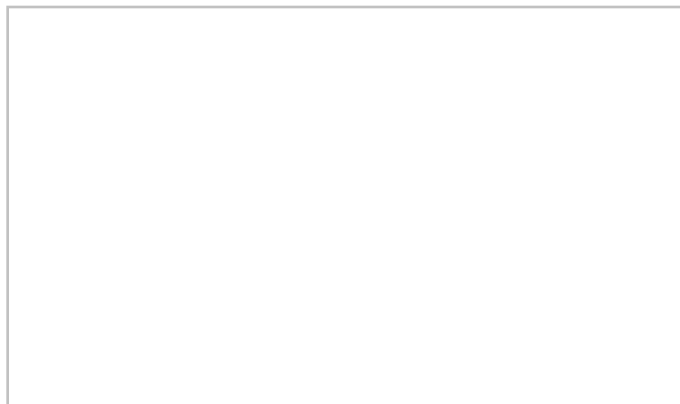
FlowJo has a sophisticated mechanism that allows you to create graphical and textual layouts which can both be edited to fit your particular circumstances, yet can also retain links to the workspace, such that statistics and graphs can be recalculated whenever their underlying data or analysis parameters change. The power of this approach does have something of a cost, in that the process of editing the statistics is somewhat more complex than it would be if the statistics were simply static text.

Drag any statistic from the workspace to the Layout Editor. Double click this statistic. You will see a dialog box containing a special purpose editor.

You will notice that some text in the upper window is outside of brackets and some is inside the brackets. The text outside of the brackets is the *human readable* description of the statistic. The part inside the brackets is a command that is able to direct FlowJo to derive the desired statistic.

If you try to edit the text you will see that the human readable portion *is* editable. Outside the brackets, you can make changes.

Click the Update Layout button to show your changes in the layout window.



Often you'll want to produce reports which reference some sample keyword information (such as Sample ID, Patient ID, etc.) in the layouts. This is can be accomplished by selecting the desired keyword with the **Insert value for keyword** button.

You will also find that typing the string inside angle brackets e.g. will accomplish the identical result.

It is actually possible to edit the FJML tags. The checkbox Edit FJML Directly, will change the editor such that editing the text inside of the brackets is allowed. We don't recommend this practice, as a minor typo can render the field meaningless.

You can drag more statistics from the workspace into this editor window and insert many keywords to make the statistic arbitrarily complex. More information on FJML.

Find this helpful?



Getting Help in FlowJo

If you find a bug in FlowJo, please [report it!](#)

Phone: 800-366-6045

Intl: 650-591-2854

Fax: 541-482-3153

E-mail: flowjo@treestar.com

Post: Tree Star, Inc.

340 A Street #203

Ashland, Oregon 97520

Tree Star, Inc. is a Oregon Corporation, and classified as a Small Business.

FEIN: 77-0232733

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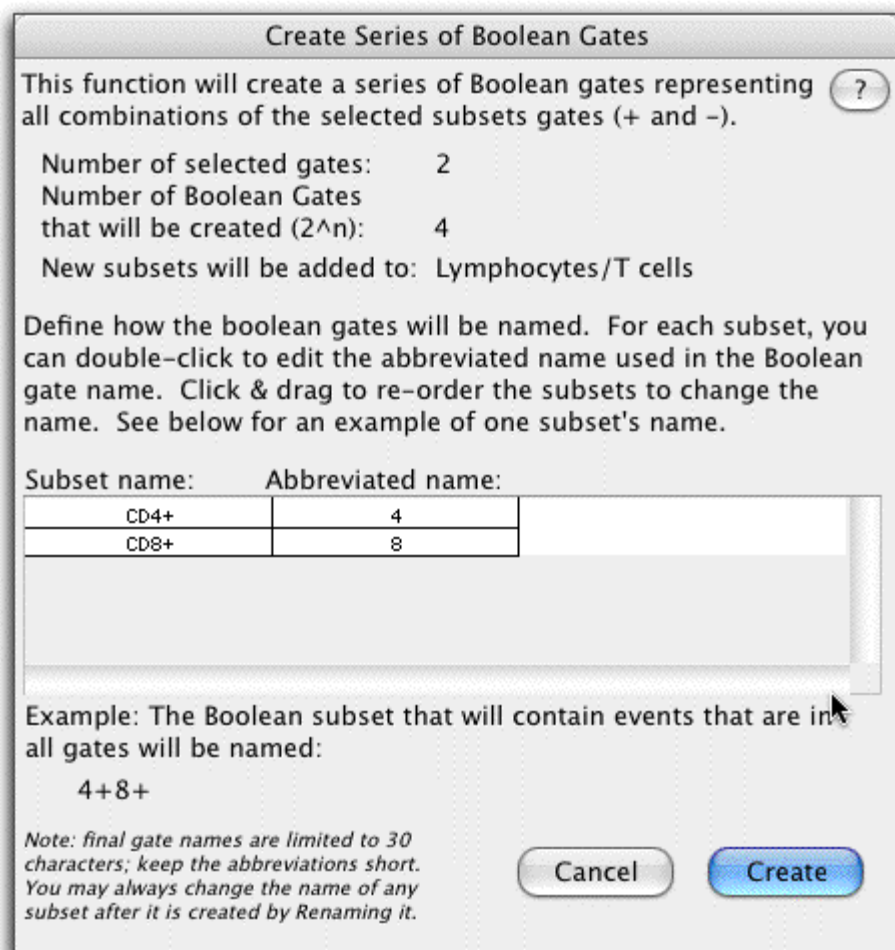
Editing Gates

Boolean Combination Gates

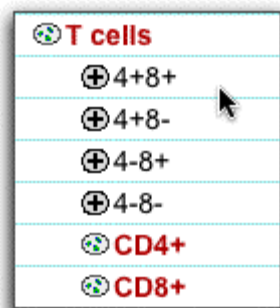
You can now create a gate that is a Boolean combination of any existing gates using all the Boolean function, including "OR". For a description of how to create simple Boolean gates, [click here](#).

FlowJo can also automatically create a series of Boolean gates that represent all combinations (plus and minus) of a set of gates. For example, if you have a sample which was stained on different parameters for CD8 and CD4, you can create a positive gate for each of the antigens. Then use FlowJo's Boolean Combination to create the 8 gates that represent all combinations of expression of the markers (e.g., CD4+CD8+, CD4+CD8-, CD4-CD8+, CD4-CD8-).

Select the individual gates you would like included in the combination gates (ie, CD4 and CD8) in the workspace window. To create the combinations of Boolean gates, select the menu item "Boolean gates..." under the "Platforms" menu and choose "Create Combination of Gates".



This will create subpopulations in the workspace as seen here.



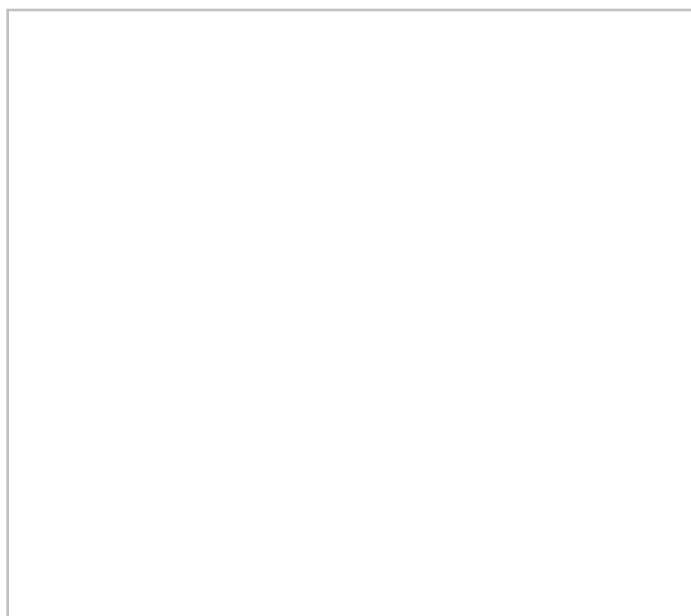
Find this helpful?



Compensation Example: Step 2

We must now define the populations which represent the positive (stained) and negative (nonstaining) cells for each of the singly-stained compensation samples. First of all, we will open the first sample (FITC Comp sample), in order to make a lymphocyte gate. Proper compensation requires that the positive and negative populations be gated on similar cell types (i.e., that have the same autofluorescence when unstained). Since PBMC are a mixture of monocytes and lymphocytes, which have very different autofluorescence, and the compensation stains we used only stain lymphocytes, we first make a lymphocyte gate.

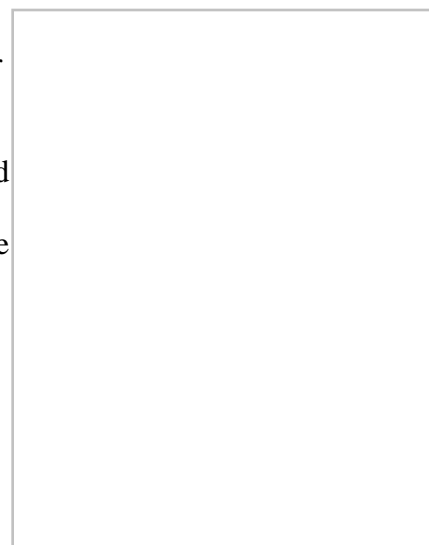
The gate was drawn around the lymphocyte population, defined by forward and side scatter. In the workspace, this resulted in the lymphocyte population being added to the sample tree.



So that all of the compensation calculations are performed identically, this same gate is dragged and dropped onto the Comp Samples group, adding it to all of the compensation samples.

For each of the three compensation control samples, a positive and negative gate are drawn around stained and unstained cells. (Note- it is not critical that the cells be completely unstained; any two populations representing a widely disparate staining are enough.)

For simplicity, the gates are named by their stain and whether they represent stained or unstained/dim cells. In the graph above, the last population defined was the FITC-negative population, which is 36.6% of the total cells in the sample.



The [next step](#) is to bring up the compensation definition dialog window or the [Compensation Wizard](#), and to drag the populations you just defined into the appropriate boxes.

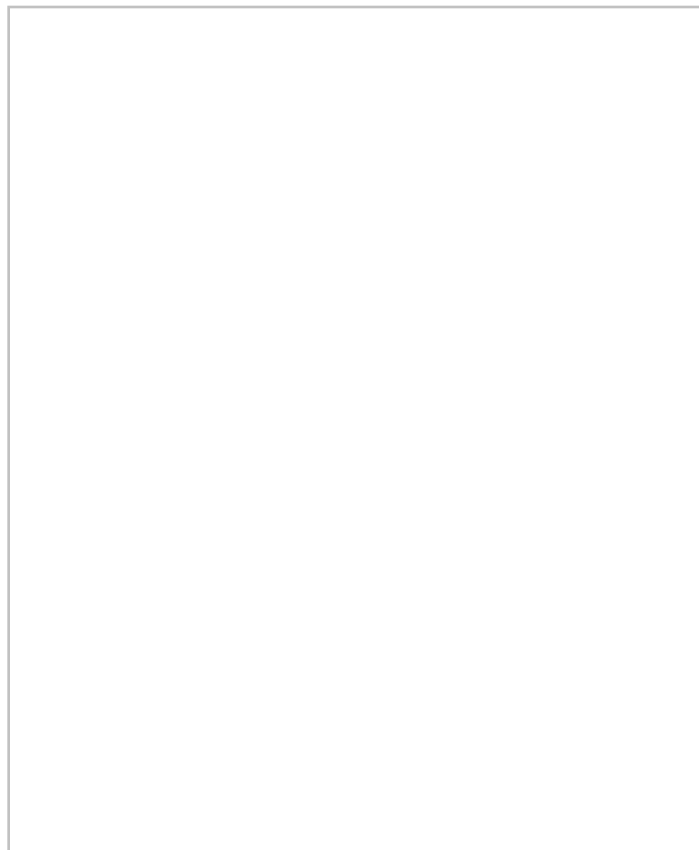
Go back to the [overview of compensation](#).

Find this helpful?



Compensation Example: Step 3

Once you have all positive and negative populations defined for each of the compensation control samples, your workspace will look roughly as follows:



At this point, it is time to bring up the [compensation definition dialog](#) window shown below, or the [Compensation Wizard](#). The Wizard will assist you in making multiple matrices with minimum effort, and display the ancestry of your control populations for visual verification. [Click here](#) for more information.

In the Compensation Definitions Dialog, select any sample with the appropriate parameters (the sample will not at this time be compensated; this selection is merely to tell FlowJo which parameters you are dealing with). For instance, you could select the first compensation sample. Then, under the Experiment menu, you will find a Compensation... menu with an associated sub-menu Define Matrix. Select this submenu, and you will be presented with a dialog window showing all parameters in the sample, and two boxes for each parameter.

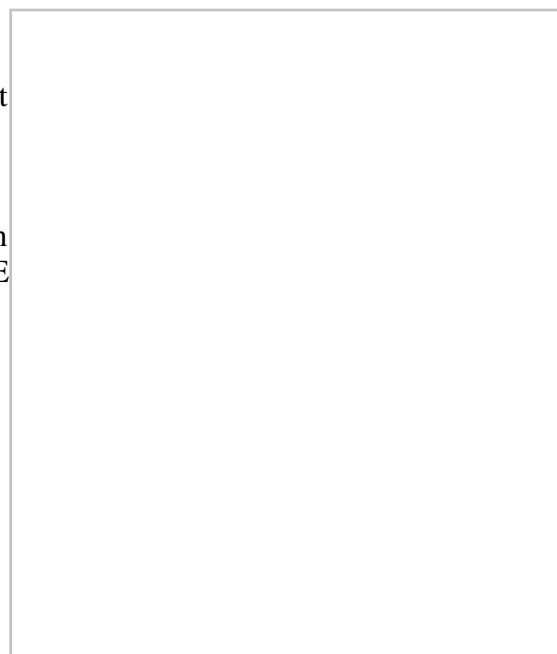
Click on one of the gated populations you generated and drag it to the appropriate box in the compensation dialog window. For example, click on the FITC-negative population, and drag it to the **Fluor** (the machine's name for FITC) box on the left (Low). An icon appears in the box denoting that a population has been assigned; the population's name appears above.

If you make a mistake and drop the population into the wrong box, just select it, and press delete on the keyboard to remove it.

Continue to fill in all of the boxes for the parameters which require compensation (the scatter parameters, of course, do not require compensation in this case). In our example, all six boxes for Fluor, PhyEry (PE) and Cy5PE will have different populations. You can click on any box to check which population is being used (the name of the population appears in red near the lower left edge).

The [next step](#) is to compute the matrix and save it.

Go back to the [overview of compensation](#).

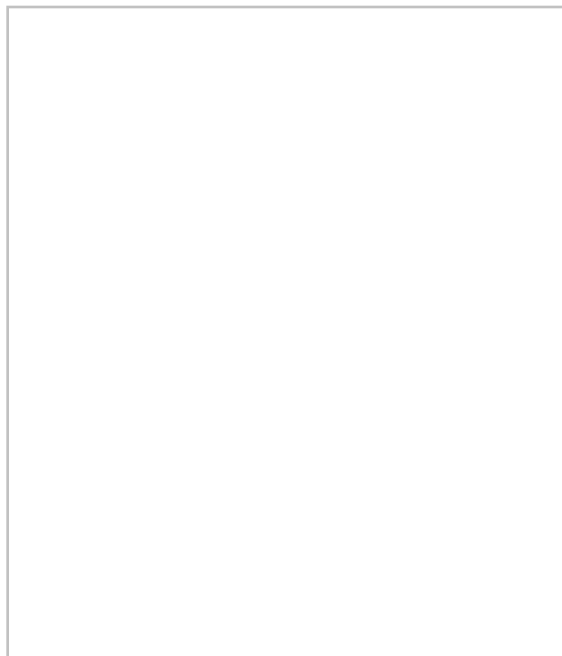


Find this helpful?



Compensation Example: Step 4

Once you have a fully-filled compensation dialog window as shown left, simply click on the **Compute** or **Compute and Close** button.



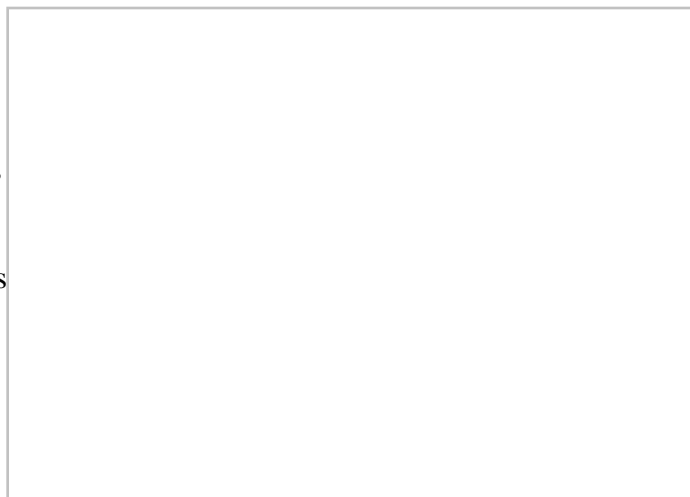
FlowJo now asks you for a name for the compensation matrix. FlowJo also automatically saves the compensation matrix with the workspace itself. You can save the matrix to a file because you may wish to use this matrix for a different workspace (to do this, select Load Matrix from the **Compensate Sample... > Load Matrix from File...** submenu. In addition, you can [modify the compensation matrix](#) yourself .

Once you have computed a matrix, it will appear in the **Compensate Sample...** menu under its own name. To apply it to one or more samples, select the sample(s) in the Workspace, then select the matrix from the menu. You can apply it to all samples in a group by selecting the group followed by selecting the matrix from the menu.

In our example, the matrix we computed is applicable to all

samples in the workspace; thus, we will select the All Samples group and then select the new compensation matrix. FlowJo lets us know that the samples are compensated by putting a colored bar next to the sample nodes.

If we now open a compensated test sample, we can see the effects of compensation. First, you will note that there are three new parameters to choose from in all statistics and graphics. *These three have the fluorescence channel names surrounded by brackets (<>) to denote that they are compensated; the stain names (reagent names) are the same as for the uncompensated channels.* See below for a comparison of the uncompensated and compensated parameters for the test sample (a lymphocyte gate has been applied already):





One last thing: remember, when you draw gates on compensated parameters, you can't drag them to an uncompensated sample (because the parameters don't exist yet!). Remember to compensate all of your samples before you proceed in your analysis.

That's all there is to doing compensation in FlowJo! There is some additional information about the [compensation matrix](#) (its format, etc.), and on [manually changing the matrix](#).

Otherwise, you should return to the [compensation overview](#).