Flowlogic™

Flow Cytometry Analysis Software

Developed by scientists, for scientists

User Manual

Version 7.2.1

GateLogic:

Introduction and File Analysis

Importing and Opening Files, Compensation, Boolean Gates, Scaling, Cell Cycle, Proliferation, Curve Fit, Kinetics, Overlays, Batch and Jump, Groups, Templates, Tags, Keywords, TitrateLogic, MQD Ungrouping and IndexLogic

inivai® TECHNOLOGIES

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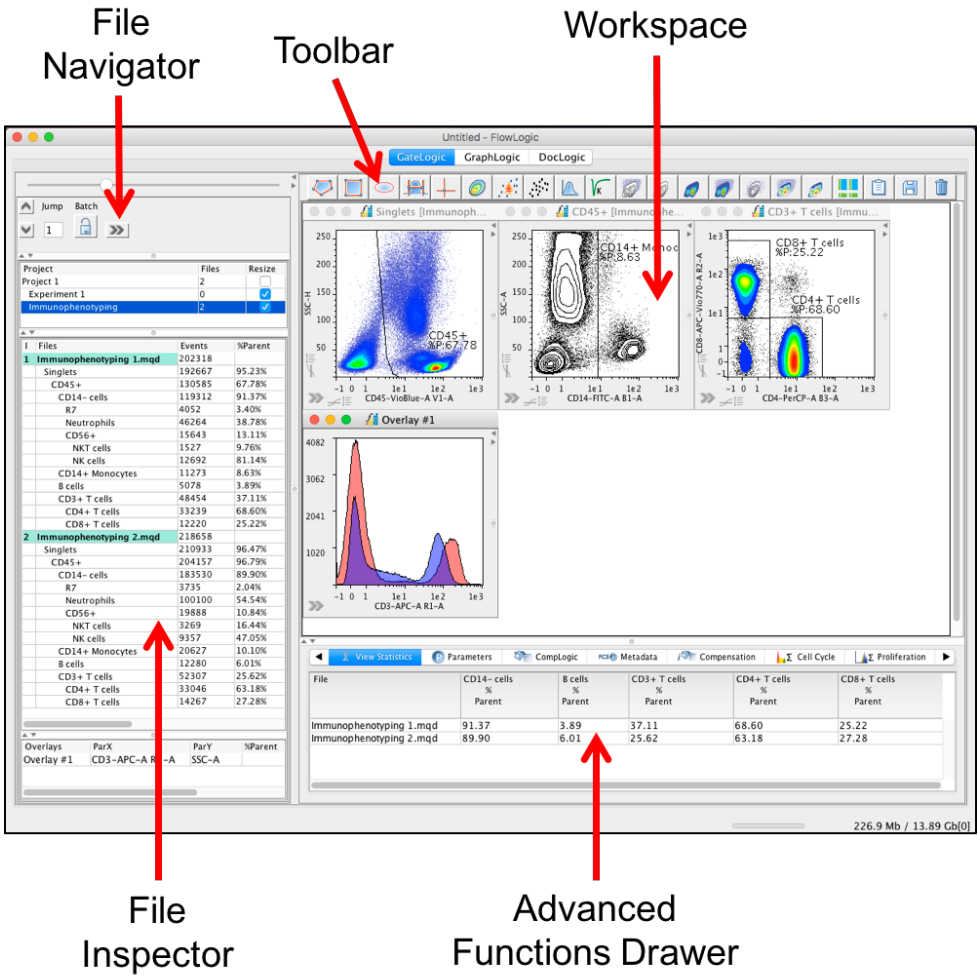
GateLogic – FCS and MQD File Analysis

GateLogic Overview

GateLogic is composed of the Workspace, Toolbar, File Inspector, File Navigator and Advanced Functions pull up drawer.

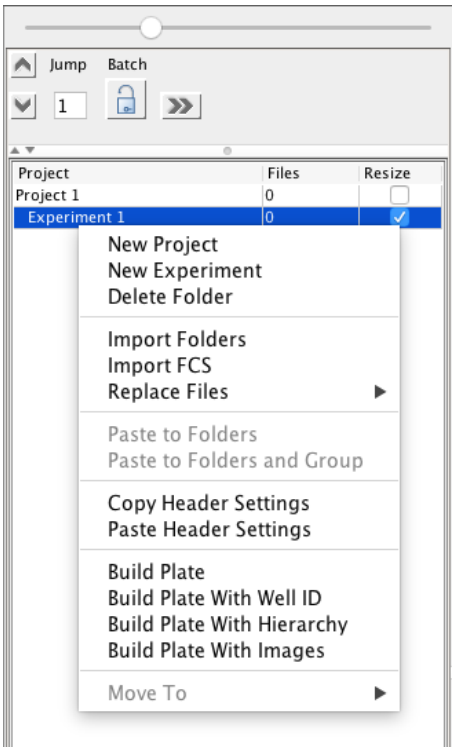


File Navigator

The File Navigator contains the 'Jump' and 'Batch' functions. The 'Jump' function allows the user to advance the plots displayed in the Workspace by a defined number of samples (the number displayed in the Jump Window). The 'Batch' function allows the user to advance one sample at a time and in the process, copy the gates from the original file to the next. The updated plots are displayed in the Workspace as the batch analysis is performed. Above the File Navigator is the plot re-sizer tool. Clicking and dragging the re-sizer button to the left or right enlarges or shrinks the plots displayed in the Workspace.

Right clicking within the File Navigator produces a menu with additional functions.

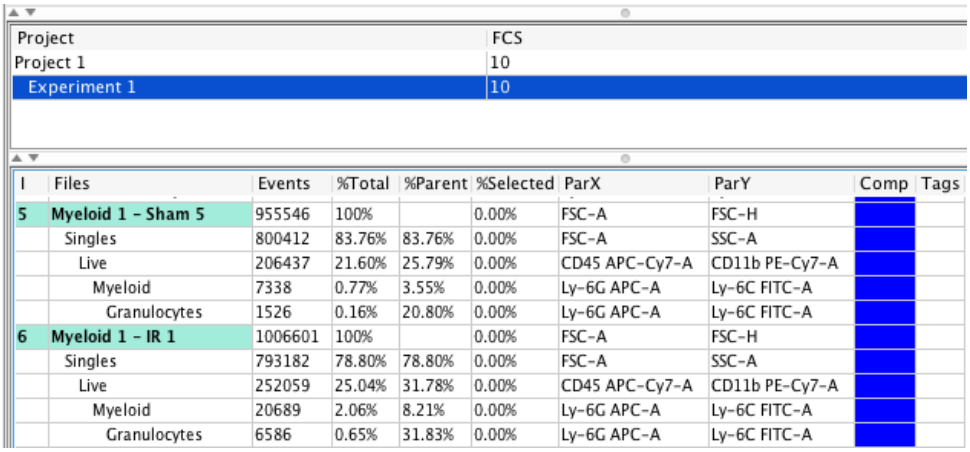
Experiment folders can be re-ordered within a Project folder or from one Project folder to another using drag-and-drop. The order of Project folders can also be changed using drag-and-drop.



Resize tick box in the File Navigator
With this option selected, the file name column in the File Inspector will resize to fit the longest file name in the list.

File Inspector
The File Inspector displays the FCS/MQD files that have been imported and any gates that have been applied to the samples. Overlays that have been created are displayed in a pull up drawer at the bottom of the File Inspector.

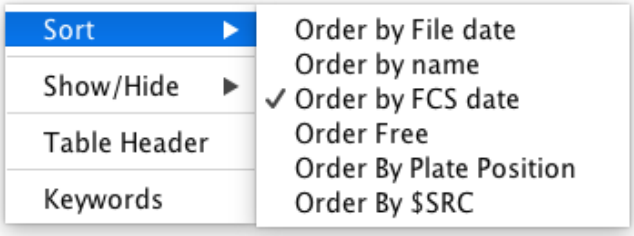
- The File Inspector also contains a number of columns containing various statistics, information on sample compensation and tags (particular sample attributes). These columns can be revealed by expanding the File Inspector by clicking and dragging the right-hand side to the window and pulling it to the right.



Project	FCS
Project 1	10
Experiment 1	10

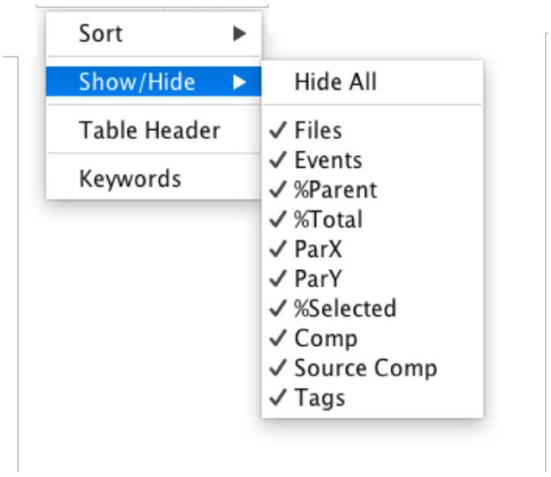
I	Files	Events	%Total	%Parent	%Selected	ParX	ParY	Comp	Tags
5	Myeloid 1 - Sham 5	955546	100%		0.00%	FSC-A	FSC-H		
	Singles	800412	83.76%	83.76%	0.00%	FSC-A	SSC-A		
	Live	206437	21.60%	25.79%	0.00%	CD45 APC-Cy7-A	CD11b PE-Cy7-A		
	Myeloid	7338	0.77%	3.55%	0.00%	Ly-6G APC-A	Ly-6C FITC-A		
	Granulocytes	1526	0.16%	20.80%	0.00%	Ly-6G APC-A	Ly-6C FITC-A		
6	Myeloid 1 - IR 1	1006601	100%		0.00%	FSC-A	FSC-H		
	Singles	793182	78.80%	78.80%	0.00%	FSC-A	SSC-A		
	Live	252059	25.04%	31.78%	0.00%	CD45 APC-Cy7-A	CD11b PE-Cy7-A		
	Myeloid	20689	2.06%	8.21%	0.00%	Ly-6G APC-A	Ly-6C FITC-A		
	Granulocytes	6586	0.65%	31.83%	0.00%	Ly-6G APC-A	Ly-6C FITC-A		

- Right clicking on the column titles opens a menu allowing the user to sort the FCS files by file date, name, FCS date (the date and time of acquisition), make them free or by plate position.

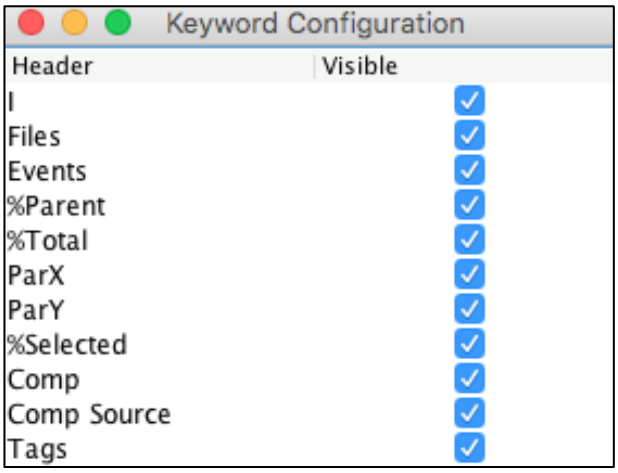


Files can also be re-ordered using drag-and-drop. Clicking and dragging the file name automatically switches the ordering to ‘Free’ and the file can be dropped in the desired position. The order of plots open in the Workspace will also be updated to reflect the order of files in the File Inspector. The files can be re-ordered by way of File Date, Name, etc., by right clicking and choosing the sort option from the list. Using drag and drop on a gate will copy and paste that gate or series of gates rather than re-ordering the files.

- Another option from this right click menu is to show or hide all or some of the statistic columns in the File Inspector. A tick next to the column indicates that the column is visible.



- Selecting Table Header from the right click menu opens a separate window where new columns can be created based on keywords contained within FCS files.



- Files can also be sorted in a numerical/alphabetical order (ascending or descending) by double clicking on the column header. For more information, see page 64.

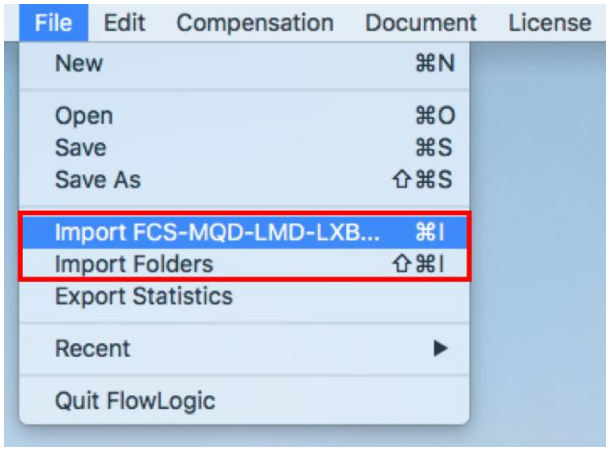
- Right clicking on a sample in the File Inspector opens a different window with a range of functions to aid in the analysis of the sample(s). See the individual sections relating to these functions for more details.

Workspace – Toolbar (summary)
The Toolbar contains various gating functions and plot display options for use on plots open in the Workspace.

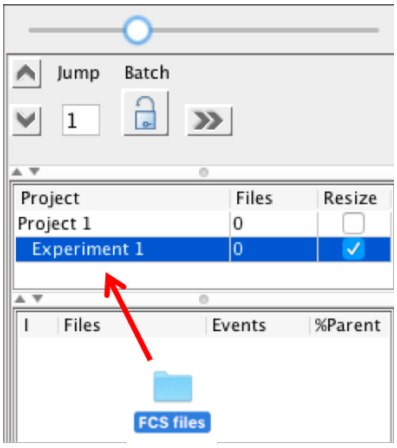
Advanced Functions Pull Up Drawer (summary)
The Advanced Functions pull up drawer contains additional analysis features to improve efficiency and aid data mining capabilities. To access the Advanced Functions, click on the top of the drawer (located at the bottom of the Workspace) and drag it up. It can be pulled up to entirely cover the Workspace, if desired. The Advanced Functions drawer consists of 11 tabs: PlateLogic, Set Statistics, View Statistics, Parameters, CompLogic, Metadata, Compensation, Cell Cycle, Proliferation, Curve Fit and Kinetics.

Importing FCS Files

To begin, click **File** on the menu bar and select 'Import FCS-MQD-LMD-LXB…' or 'Import Folders' from the drop-down menu. Locate and highlight your files and click 'Choose'.



Alternatively, right click on an Experiment Folder or drag FCS files or folders containing FCS files directly into an Experiment Folder in the **File Navigator**.

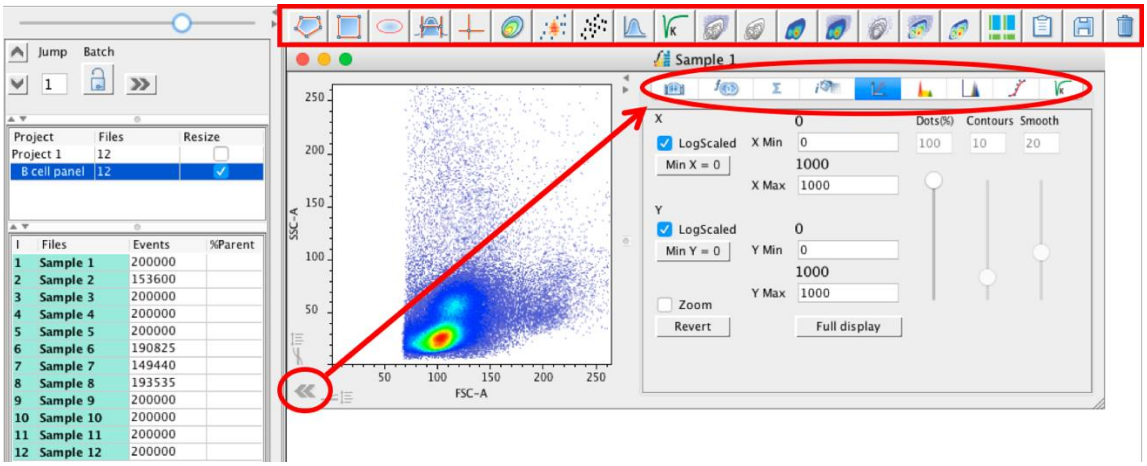


Opening Dot Plots/Histograms

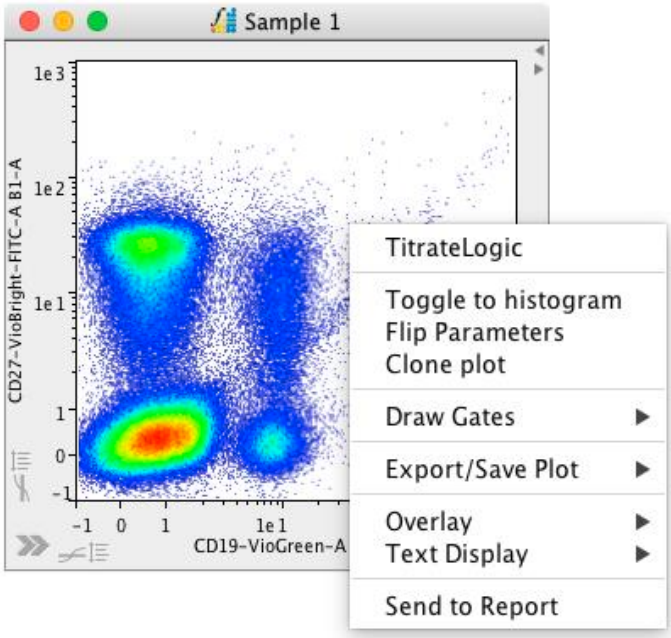
Imported FCS files appear in the File Inspector. Double click on a file or right-click→Plots→Open to open it as a dot plot.

Gating tools and plot display options, including standard dot plots, density plots, histograms, contour plots and pseudocolor plots appear in the toolbar.

Click the arrow in the bottom left hand corner of a dot plot, or press space bar, to open the side drawer for the Gate List, Boolean Gates, Statistics, Interactive Compensation, Scaling, Cell Cycle Analysis, Proliferation Analysis, Curve Fitting and Kinetics features.



Dot plot right click menu

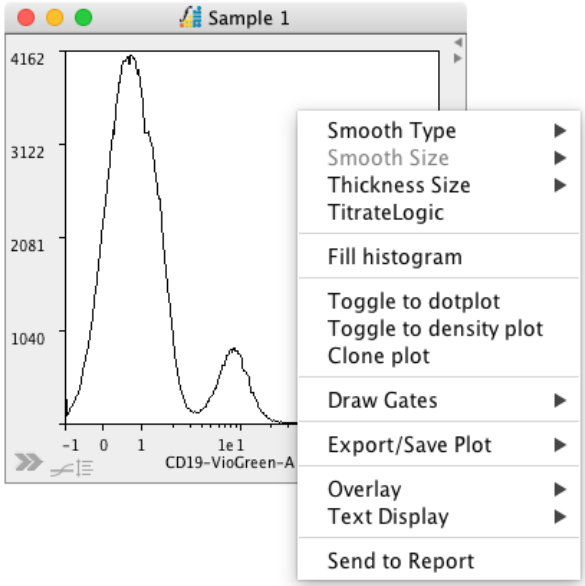


Right click within a plot to open a menu with options to:

- Perform automated titration analysis (see page 66)
- Toggle to a histogram
- Flip the parameters displayed on the x and y axes (dot plot only)
- Clone a plot (see page 15)
- Draw gates (an alternative method to selecting the gating tool from the toolbar)
- Export/Save Plots
- Create Overlays
- Format the plot text (gate label text)
- Send the plot to a report in DocLogic

A right click within a gate provides the extra option to add a gate to, or remove a gate from a group. For more on group analysis, see page 48.

Histogram right click menu



Right click within a histogram to open a menu with options to:

- Change the smoothing type and size for the histogram
- Change the thickness of the histogram line
- Perform automated titration analysis (see page 66)
- Fill (color) the histogram
- Toggle to a dot plot or density plot
- Clone a plot (see page 15)
- Draw gates
- Export/Save Plots
- Create Overlays
- Format the plot text
- Send the plot to a report in DocLogic

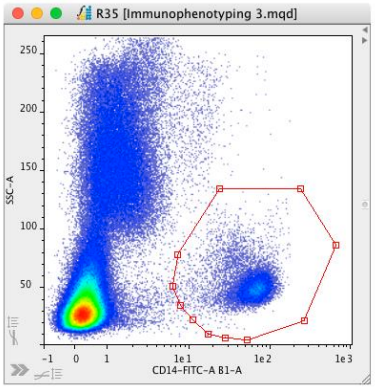
A right click within a marker provides the extra option to add a gate to, or remove a gate from a group. For more on group analysis, see page 48.

Toolbar Features

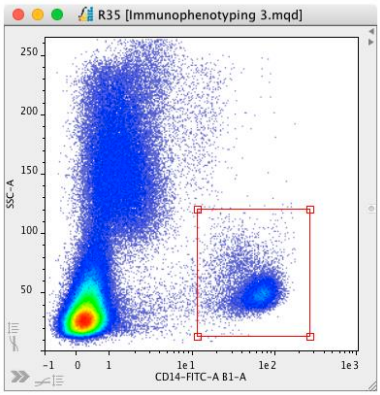
The Toolbar, located at the top of the Workspace, contains a range of gating and plot display tools. Hover the cursor over each of the buttons in the toolbar to view the description.

Gating Tools – to apply a gate to a plot that is open in the Workspace, click on the plot to select it, click on the desired gating tool and click in the plot area to draw/create the gate. The gating options are:

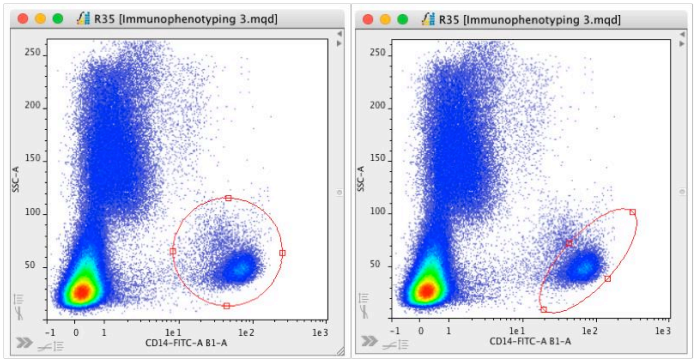
- **Create a polygon** – a polygon gate can have any number of corners. Once the polygon gating tool has been selected, click around the area on the plot that you wish to be contained within the gate. Double click or click on the original point to close the gate. Polygon gates can be moved by clicking within the plot and dragging it or by highlighting it and moving it using the arrow keys on the keyboard. Polygon gates can be adjusted by clicking and dragging a point or by clicking and dragging a side.



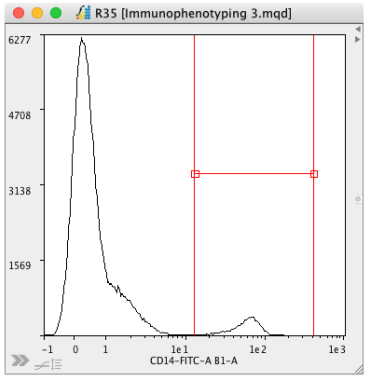
- **Create a rectangle** – a rectangle gate always has four sides, each joined at right angles. Once drawn, the size and shape of the rectangle can be adjusted by clicking and dragging a corner or by clicking and dragging a side.



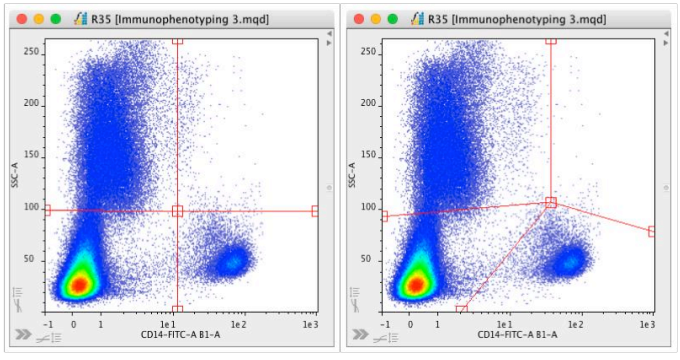
- **Create an ellipse** – an elliptical gate is created with four points (similar to the corners on a rectangle plot. Clicking and dragging these points will either adjust the width of the gate or adjust the orientation.



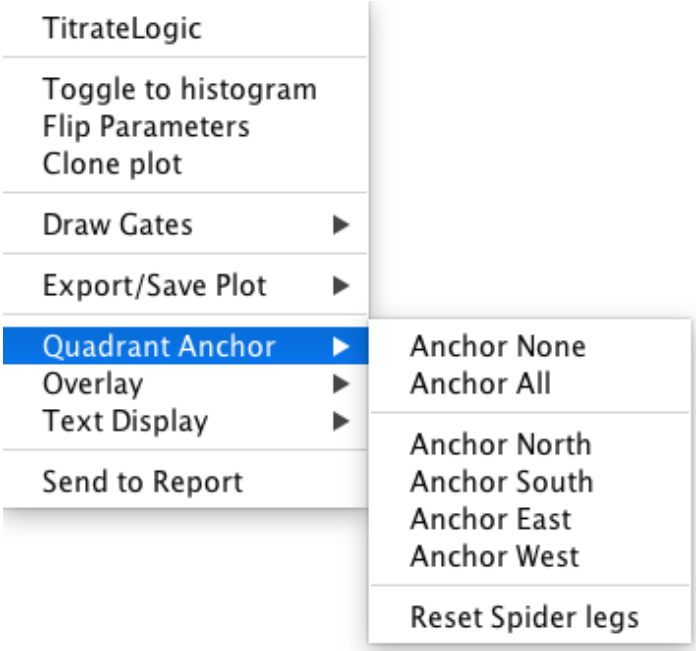
- **Create histogram marker** – a histogram marker is used to define a region on a histogram. Histogram markers can be adjusted by clicking and dragging the points at each end. The height of the cross bar can also be adjusted by clicking and dragging it down or up.



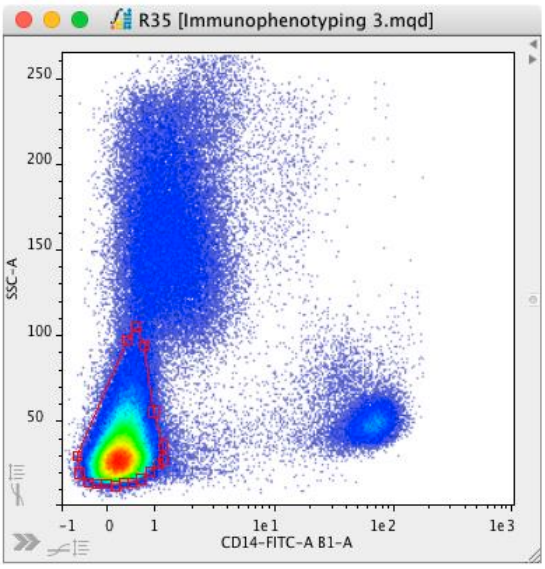
- **Create a quadrant** – a quadrant gate divides the plot into four areas. To apply a quadrant gate, highlight the plot, select the gating tool and click within the plot area. Quadrant gates can be adjusted by clicking and dragging the center point or by clicking and dragging the points at the end of each of the arms. These arms move independently of each other and do not have to form four rectangle gates.



Right clicking on a plot containing a quadrant gate provides options to anchor one, all or a selection of the quadrant gate arms to the corresponding axis. Resetting the spider legs will return all arms to a vertical or horizontal orientation, whilst maintaining any form of anchoring that has been set.

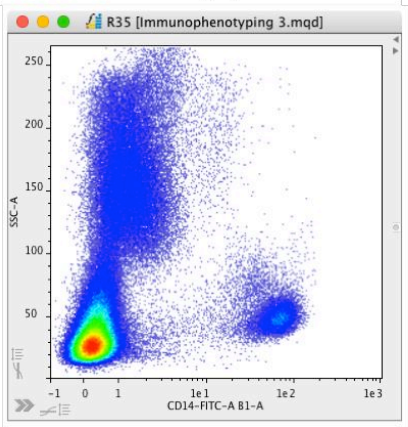


- **Autogate** – the autogating tool automatically draws a gate based on density. To use the autogating tool, highlight the plot, select the autogating tool from the toolbar and then hover over the plot. A gate will be displayed based on where the cursor is and the density of events on the plot. Without clicking, move the cursor around the plot to see how it affects where the gate is drawn. To create the gate once it is where you want it to be, simply click. This will effectively create a polygon gate with many points.

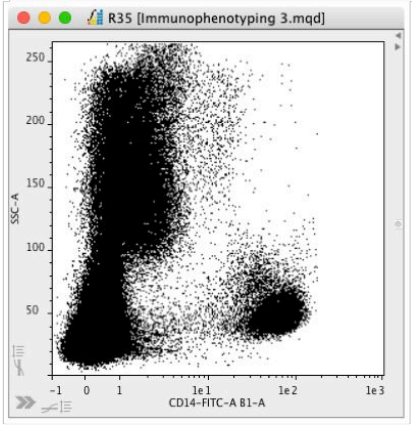


Plot Display Options – plots can be displayed in a number of different ways. Choose a plot display option to best display your populations. Plots are generally displayed as a standard dot plot (or density plot if set as a default in the Preferences). To change the plot display, click on the plot title to select it and then click on the desired plot display option. The options are:

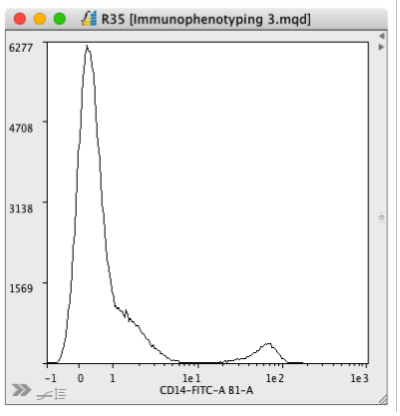
Density plot



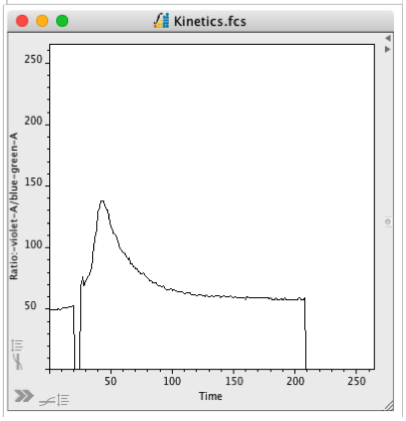
Dot plot



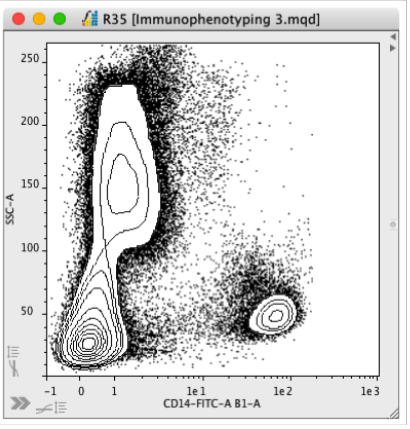
Histogram



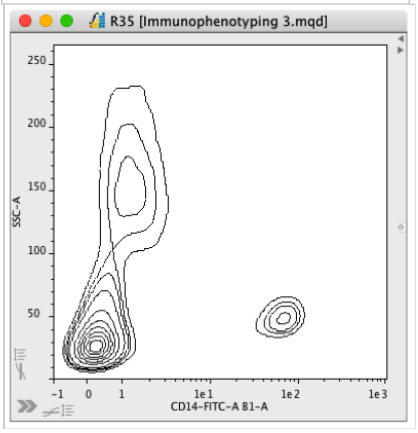
Kinetics plot



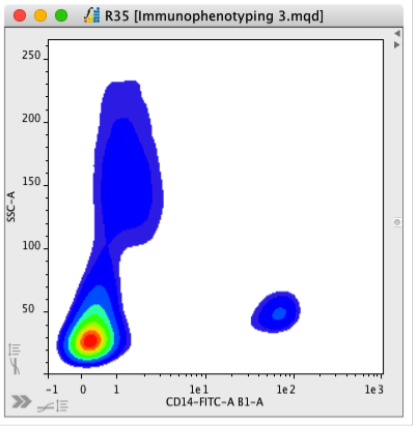
Dot plot with contours



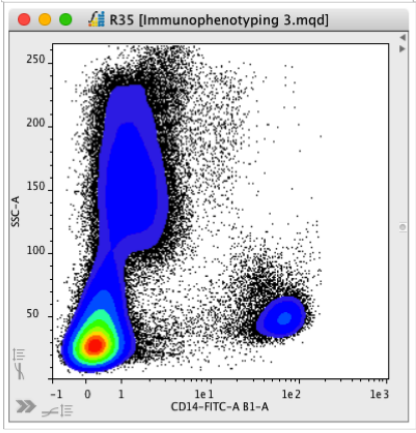
Contour plot



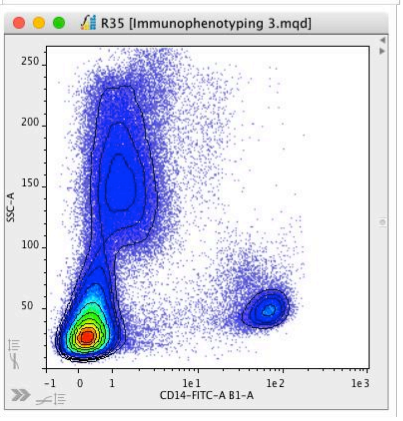
Pseudocolor plot



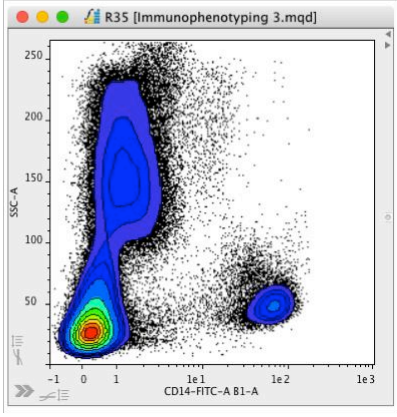
Dot plot with pseudocolor



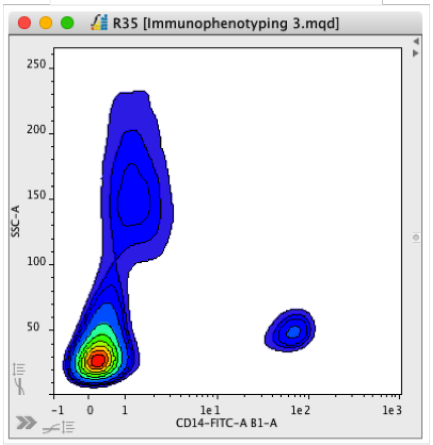
Density plot with contours



Dot plot with pseudocolor and contours



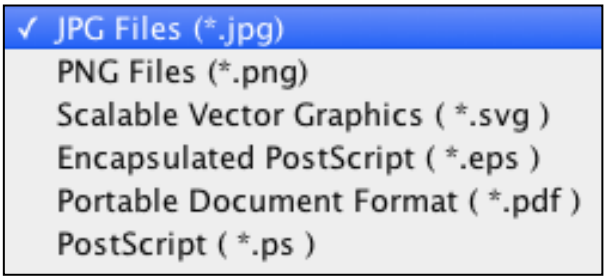
Pseudocolor plot with contours



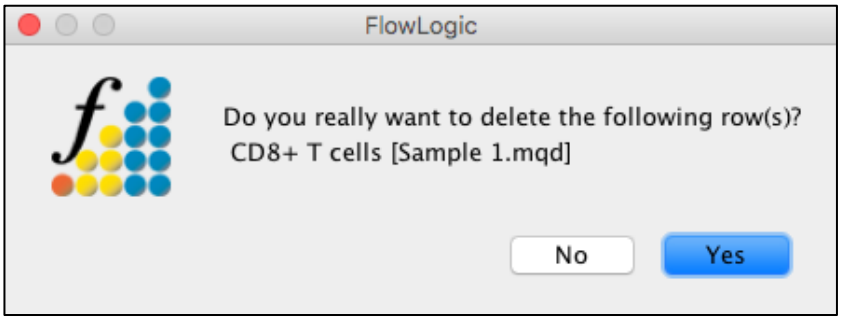
- **Clone a plot** – plots can be cloned, resulting in to identical plots at the level where the clone was created. The clone is labeled with a 'C' next to the gate name in the File Inspector. To clone a plot, highlight it on the plot or in the File Inspector, and select the toolbar button.

- **Copy plot graphic to clipboard** – this saves a plot image to the computer's clipboard to be pasted into other programs.

- **Save the graph image to disk** – one method to export a plot is to highlight it in the Workspace and click the Save the graph image to disk. An option to save the plot as one of six different files types will be presented.



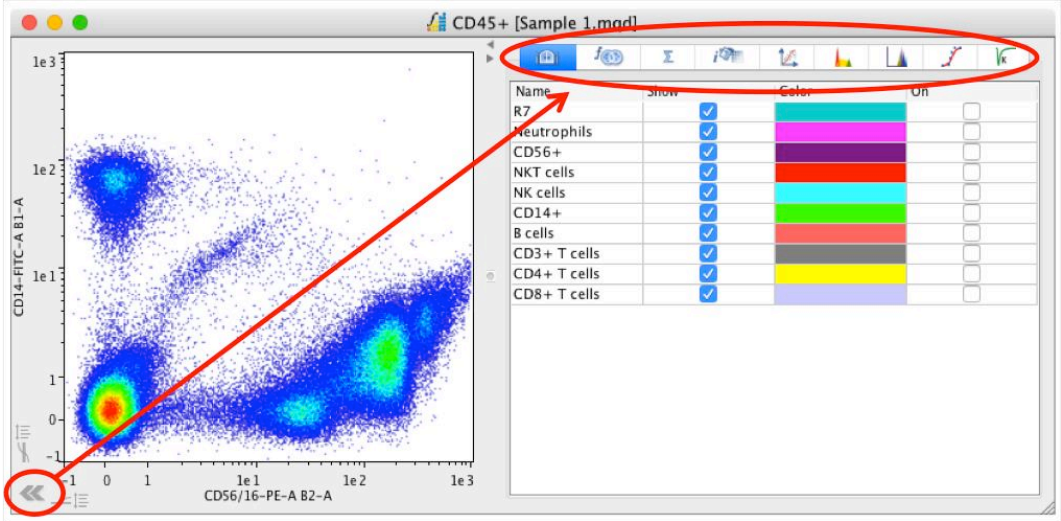
- **Delete the selected gate** – one method to delete a particular gate is to highlight it, either on the plot or from the gate lists in the File Inspector, before clicking the Delete the selected gate button in the toolbar. A window will then be displayed asking to confirm this action.



The Plot Side Drawer

Each dot plot and histogram contain a side drawer that allows the user to switch gates on and off, color gates, generate Boolean gates, view statistics, compensate samples, adjust plot scaling, reduce the percentage of dots displayed and zoom.

Open and close the side drawer by double clicking within the plot area (but not within a gate) or by using the arrow tab at the bottom left.



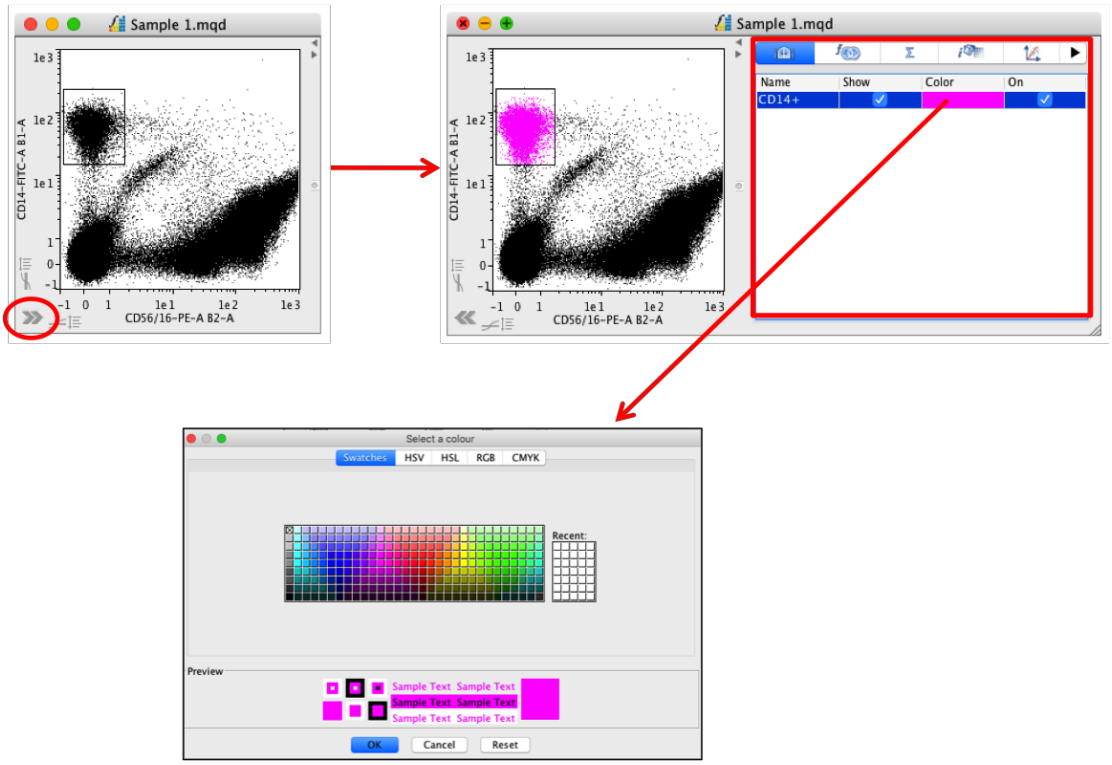
Gate List

This window has options to turn gates on and off as well as for the coloring and backgating of events within gates.

The ‘Name’ column contains the list of gates that exist on that population and all populations lower in the population hierarchy. If parameters have been changed after creating the gate, click on the gate name within this window to return to the parameters where the gate is drawn. If the gate name chosen exists at a lower level of the hierarchy, the specific parameter combination will be displayed but no gate will be visible.

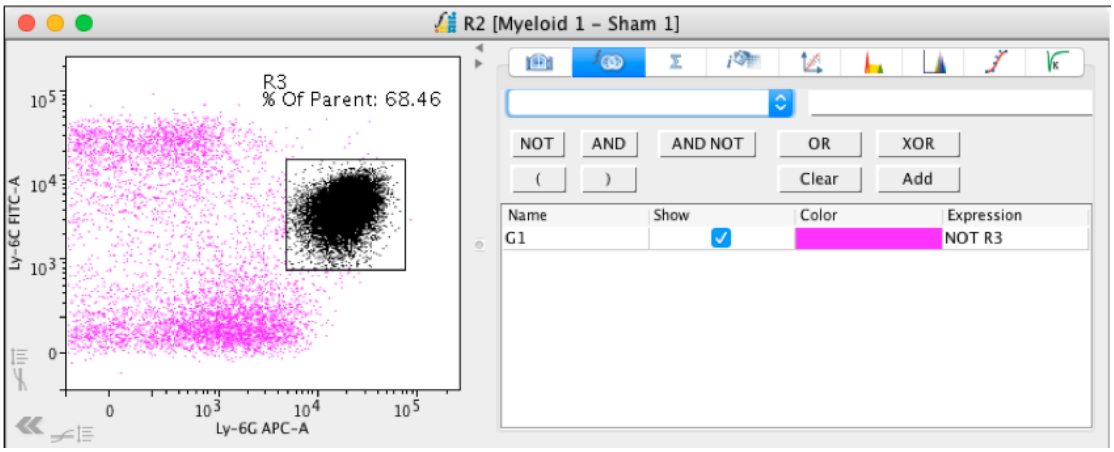
The ‘Show’ column contains a check box allowing gates to be turned off and on. This is a display option only, meaning turning off a gate only removes it from the display but the gate is not deleted and still exists. It can easily be displayed again by ticking ‘Show’.

Clicking on the color brings up a color palette. Click on a new color followed by ‘OK’ to change color of the gated population. These colored dots will be colored (backgated) on all parent plots when the box is ticked in the ‘On’ column.

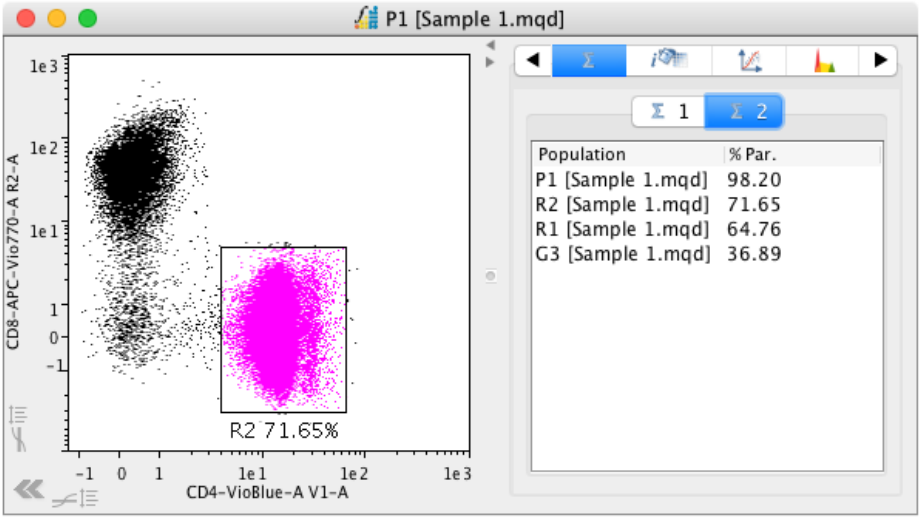


Boolean Gates

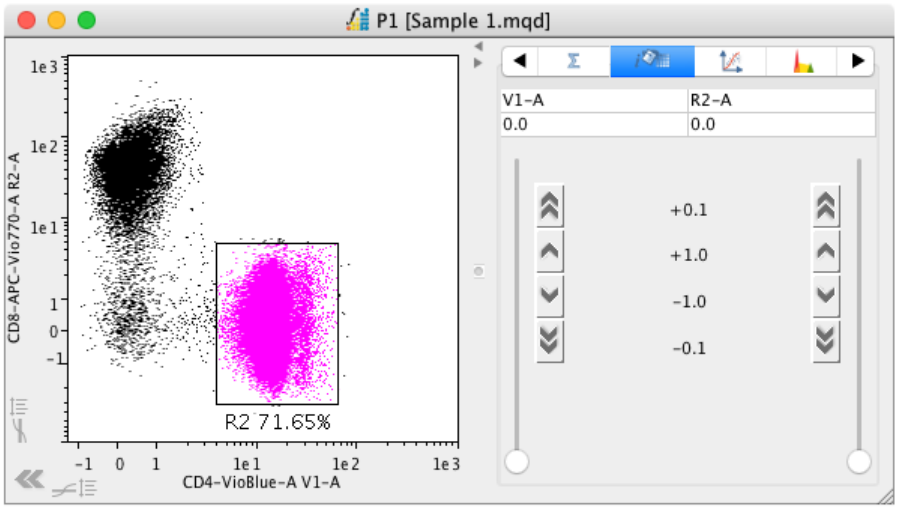
Boolean gates are created in the plot side drawer. The list of Boolean gates that exist at that particular level of the gating hierarchy will be displayed, along with options to color of the new population.



Statistics
If statistics have been calculated for a sample, they are made visible in this window. These statistics can be copied by right clicking in the side drawer.

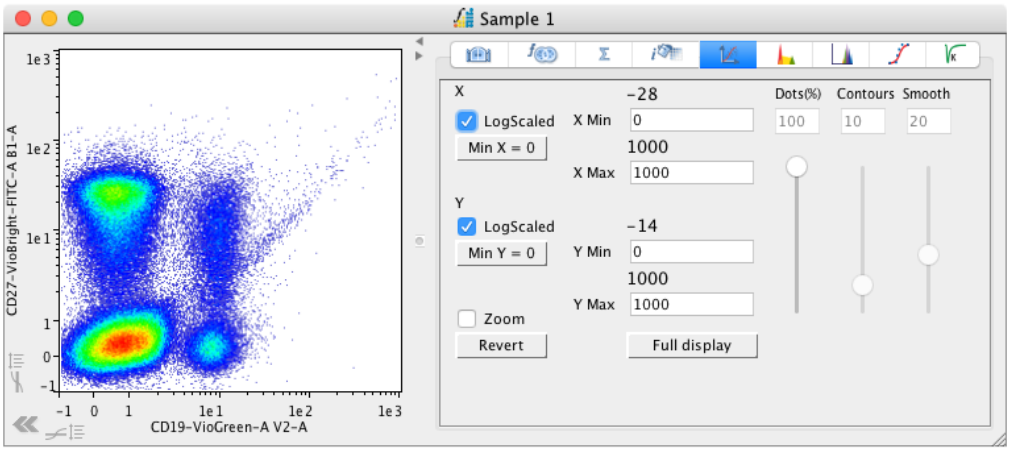


Interactive Compensation
Samples can be manually compensated using the slide bars, the up and down arrow buttons or by entering a new compensation in the text field.



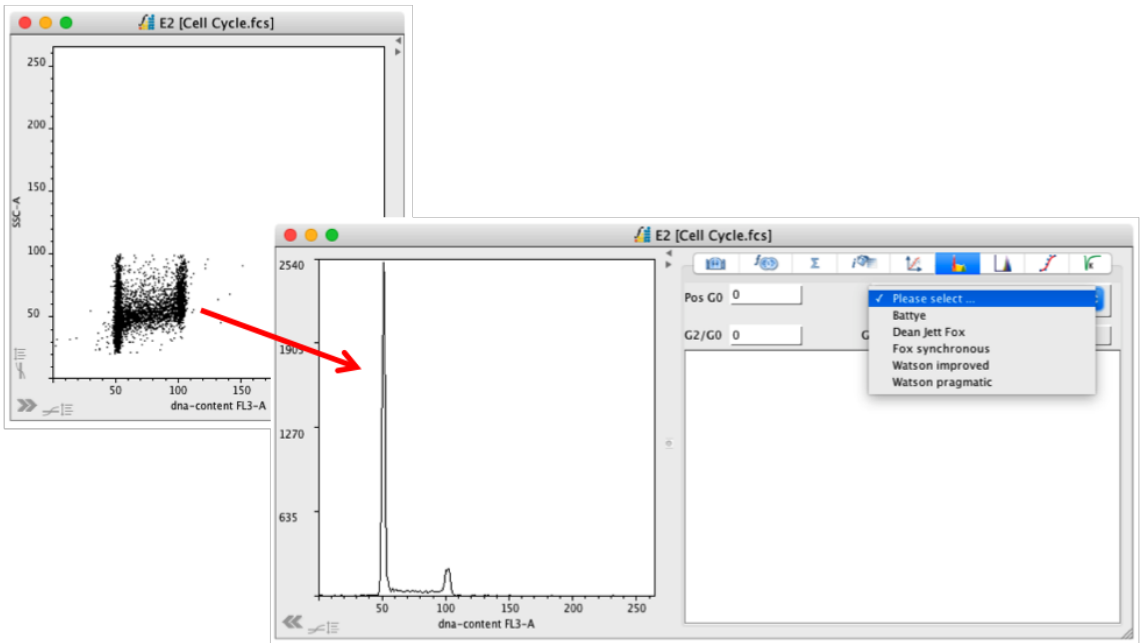
Scaling

Here, the scale used to display the dot plots can be set. The options include displaying the X- and Y-axes as ‘LogScaled’ and ‘From Zero’. Zooming is also available in this window. This requires the ‘Zoom’ box to be ticked. Clicking ‘Revert’ restores the original scale. The percentage of dots being displayed can also be reduced using the slide bar.

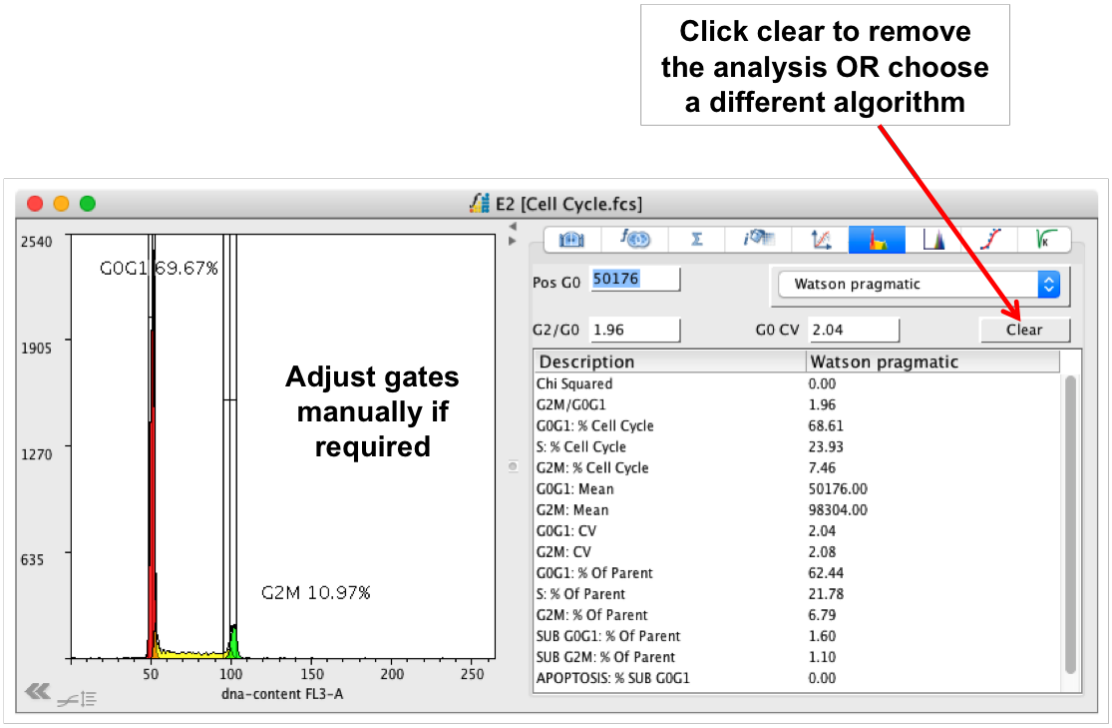


Cell Cycle

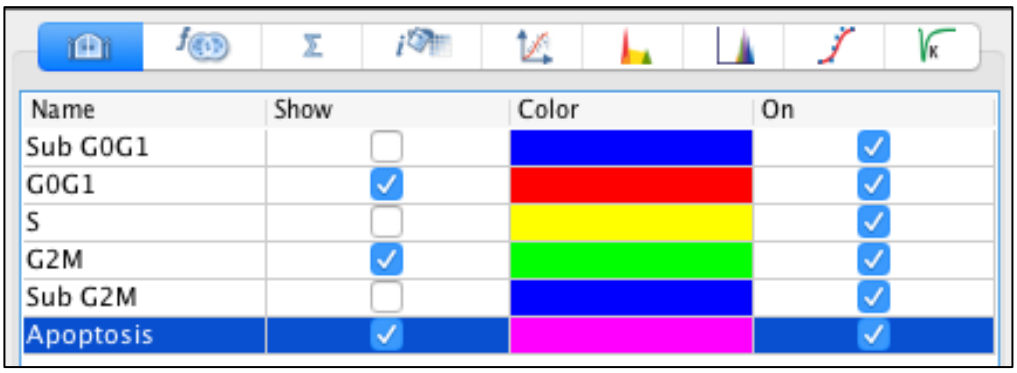
To perform cell cycle analysis, perform any pre-gating required on your DNA-content parameters before displaying it as a histogram. Then, in the Cell Cycle tab in the side drawer, choose from one of the 5 algorithms: Battye, Dean Jett Fox, Fox synchronous, Watson improved and Watson pragmatic.



The histogram is colored to represent the different phases of the cell cycle and the data relating to each phase is displayed in the plot side drawer. This data can be exported with right click → Copy Statistics.

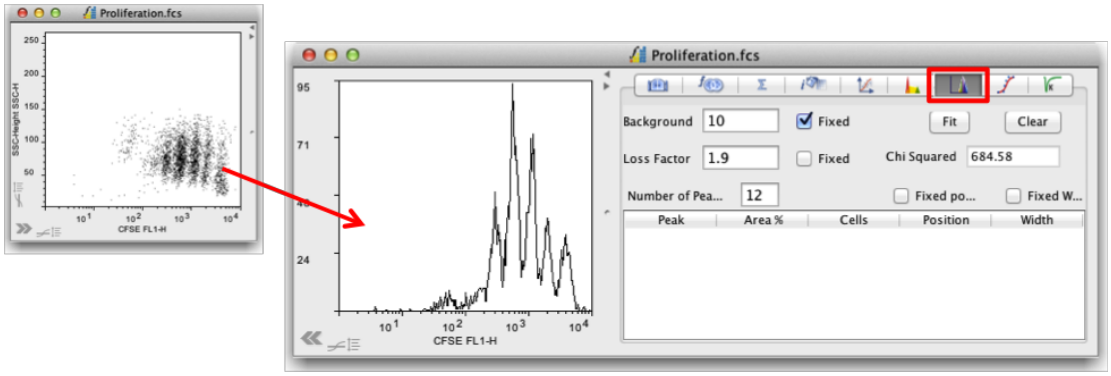


In the Gate List tab, extra gates, such as for apoptosis, can be turned on and colors can be changed.

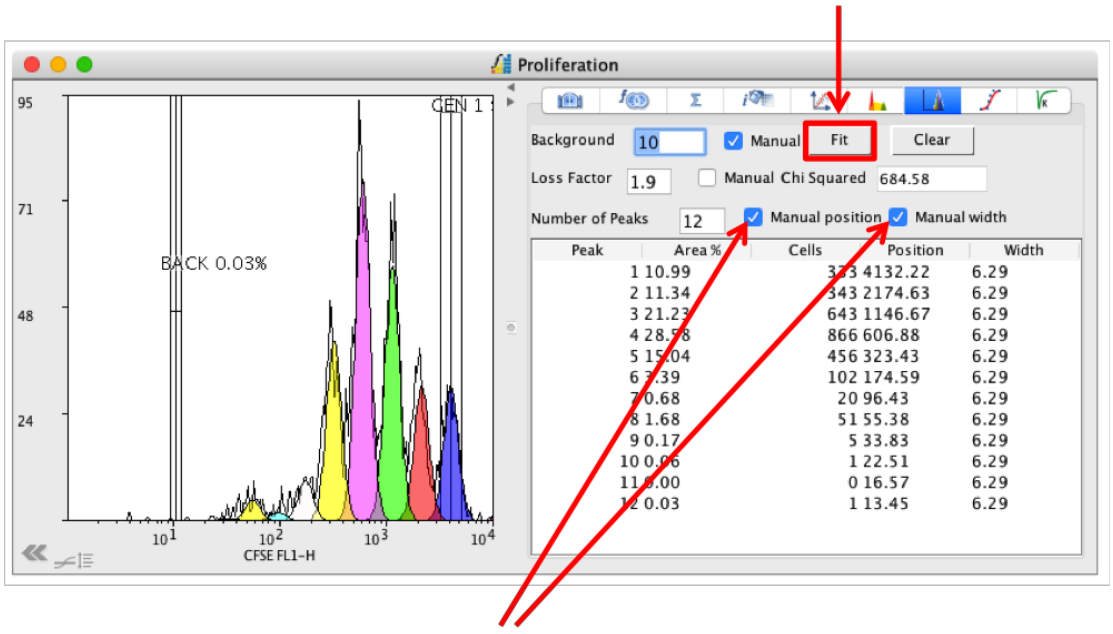


Proliferation

To assess proliferation, convert the data to a histogram displaying the appropriate parameter and select the Proliferation tab from the plot side drawer.



Click ‘Fit’ to perform the curve fitting. The curves created for each peak will be colored on the plot and the related statistics will be displayed in the side drawer. The statistics can be exported by right clicking and choosing to copy or export.



The fitting of the curves can be adjusted manually by displaying the GEN 1 gate from the Gate List tab in the side drawer. Then, from the Proliferation tab, tick ‘Manual position’ and/or Manual width’. With these selected the position and/or width of the GEN 1 gate can be adjusted. As this gate is moved, the curves fitting the data will update in real time.

If the loss factor is not set to manual, then FlowLogic will determine the loss factor when fitting the curves. If the loss factor is set to manual, then the curves will be drawn in accordance with the user defined loss factor value.

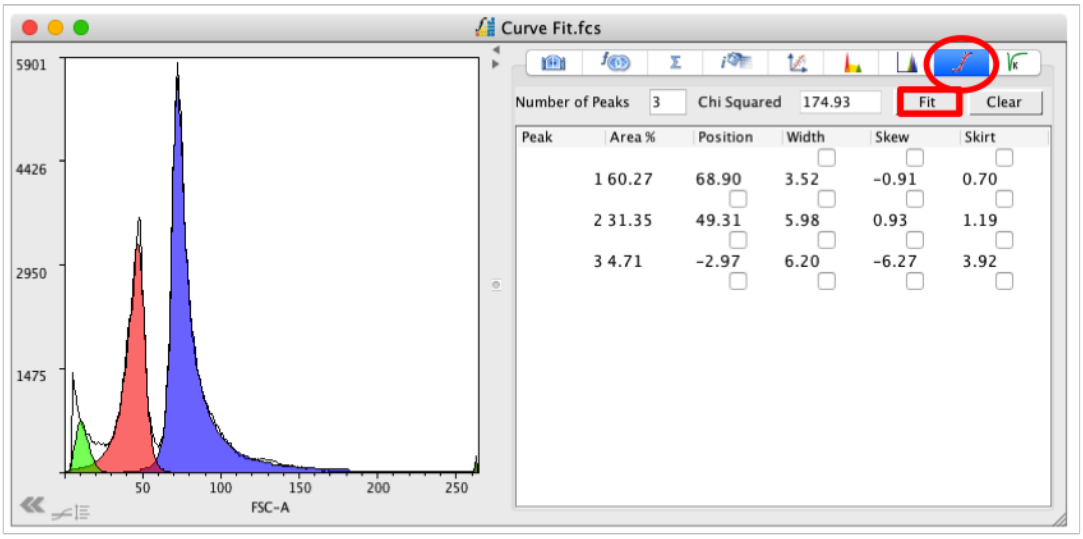
To perform proliferation analysis for multiple files, add them all to a group by selecting them in the File Inspector and choosing 'Group' → 'Add to New'. Now, when clicking 'Fit', the curve fitting will be performed on all samples but independently from each other.

It is possible to set a control or master GEN 1 gate for a set of files. This is useful if there is no longer any fluorescence in the position where the first peak should exist. This setting of a master gate uses FlowLogic's group analysis feature. In relation to proliferation analysis, this involves grouping the GEN 1 gates for all samples in the File Inspector. As the file that is listed at the top of the list of files in the File Inspector dictates the setting of a gate for all other files in a group, drag-and-drop the file that you wish to be the master file to the top of the list.

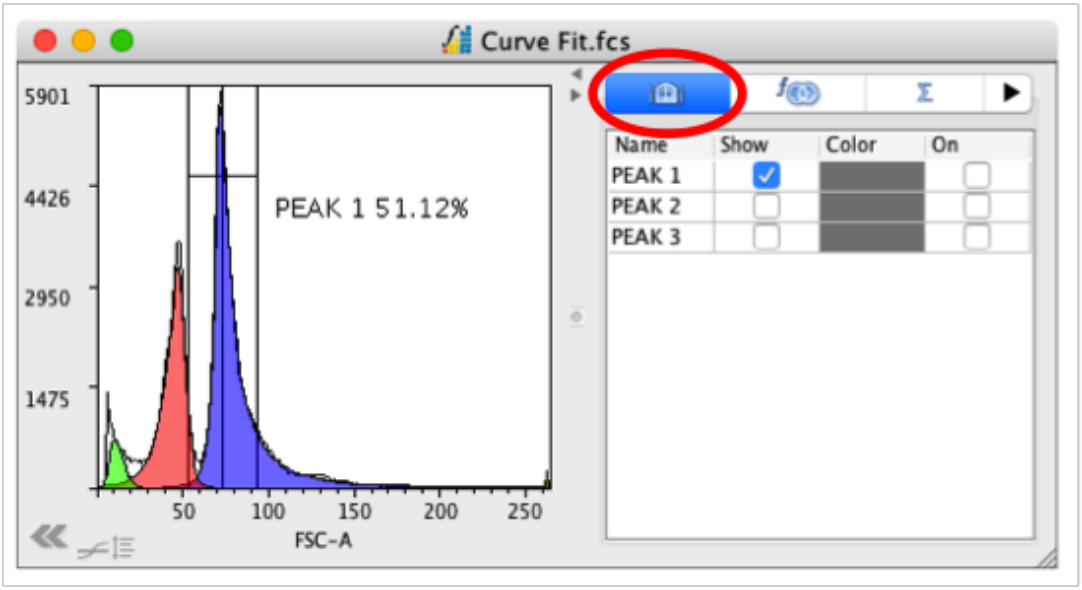
Then, tick 'Manual position' and 'Manual width' and add all of the GEN 1 gates that you wish to be governed by the master into a group. If the loss factor is not set to manual, then it will be re-calculated for each plot independently. The GEN 1 gate does not need to be displayed in order to group the files and set a master file. However, once the GEN 1 gates are grouped, they can be displayed on any plot and adjusted to any position or width. This will update for the entire group regardless of which file in the list the adjustment was made on.

Curve Fit

Curve fitting analysis can be performed on suitable histograms displaying a number of peaks. In the plot side drawer, select the Curve Fit tab and click Fit. The different peaks will be colored and the statistics relating to each peak, such as the area under the curve, will be displayed in the side drawer.

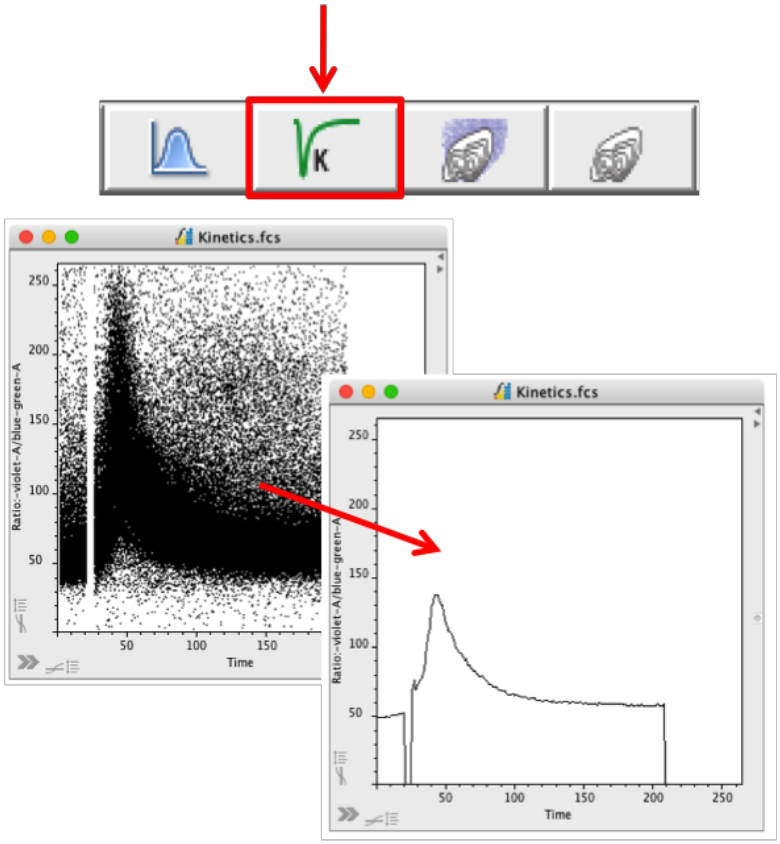


Gates relating to each peak can be turned on and off in the Gate List tab.

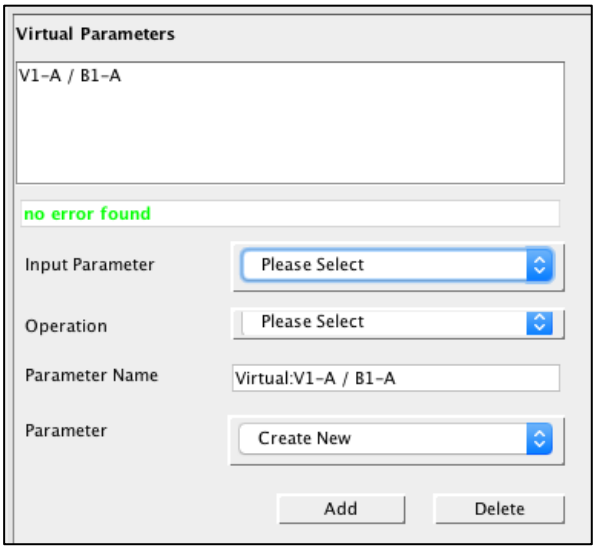


Kinetics

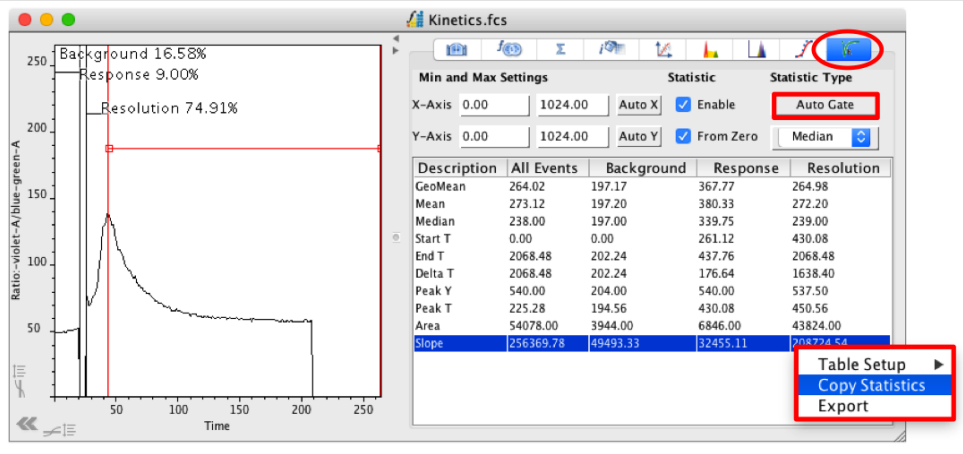
To assess cell cycle kinetics, display the data as a ratiometric parameter versus time. Then toggle to a kinetics plot using the button in the tool bar.



- Ratiometric parameters can be created in the Parameters tab in the Advanced Functions drawer if they were not created at the time of acquisition.

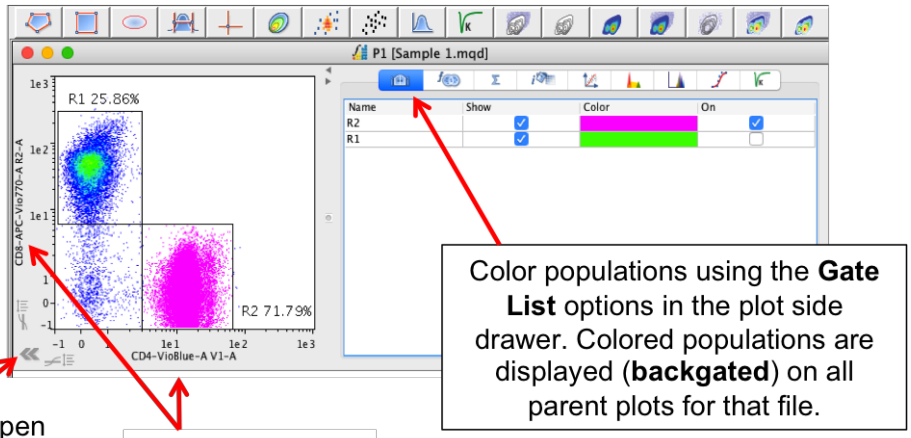


- Select the Kinetics tab in the plot side drawer and click ‘Auto Gate’. This will create Background, Response and Resolution gates. The data will be displayed in the side drawer and can be exported by right clicking and selecting Copy Statistics.



Manipulating Dot Plots/Histograms

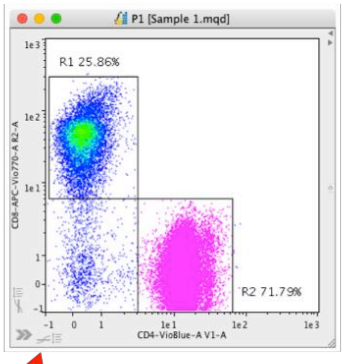
Change the graph type: dot plot, histogram, density plot, pseudocolor plot and combinations of these types



Color populations using the **Gate List** options in the plot side drawer. Colored populations are displayed (**backgated**) on all parent plots for that file.

Click to open plot side drawer

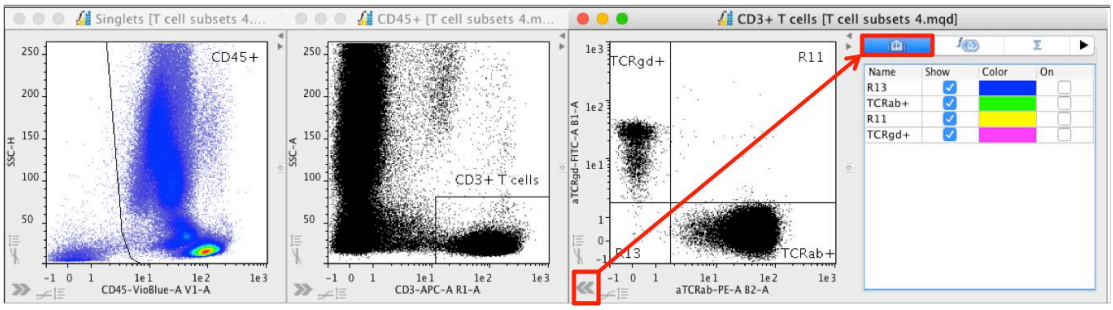
Change the parameters with a single left click on the *x* or *y* axis



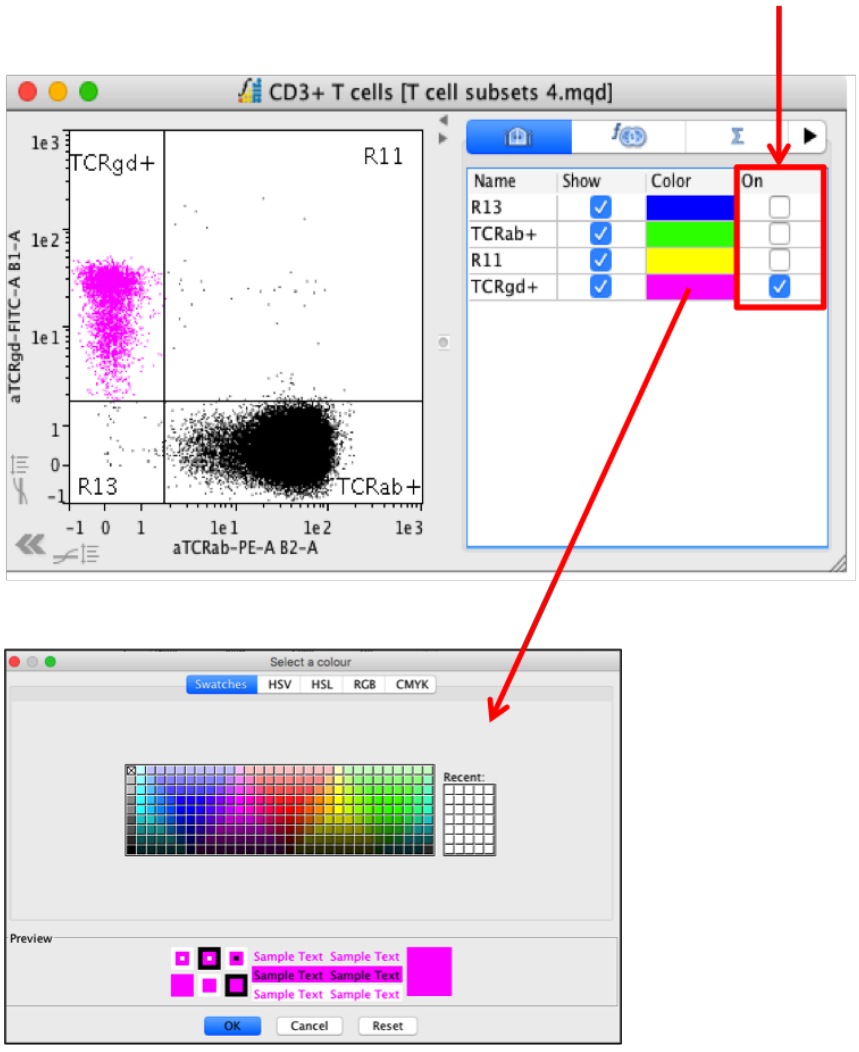
Click ≠ for log-linear transformations

Backgating

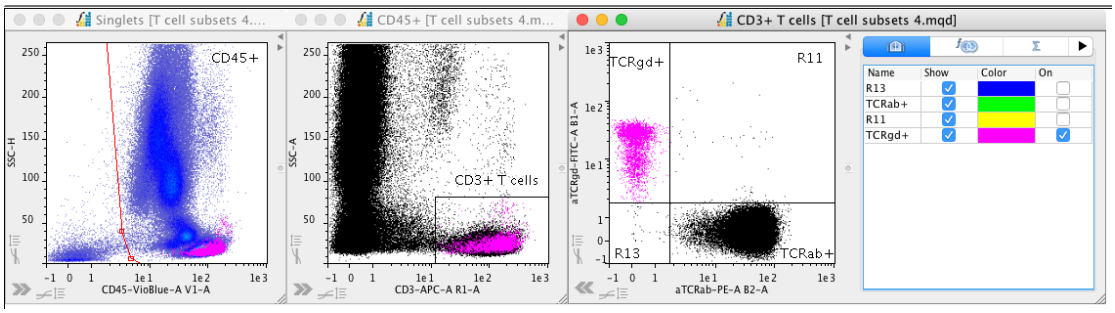
Once a gating hierarchy has been created, open the plot side drawer and choose the Gate List tab.



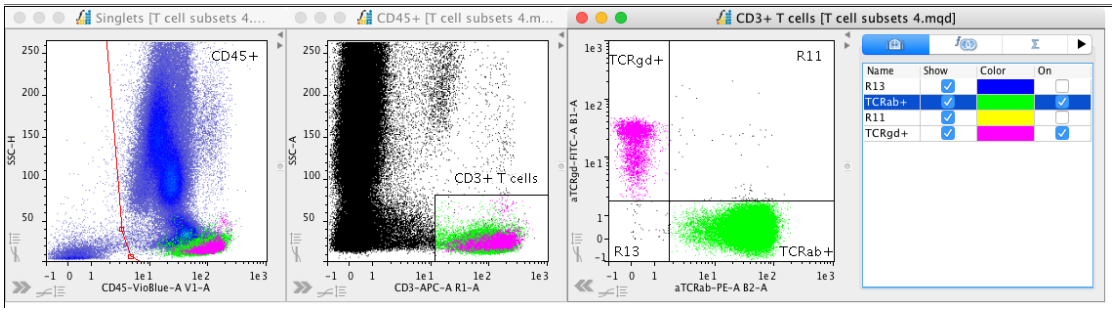
- To backgate a population, tick the box in the 'On' column. This will color the population at all higher levels in the hierarchy. To change the color, click on the color box in the side drawer and choose a new color from the selection provided.



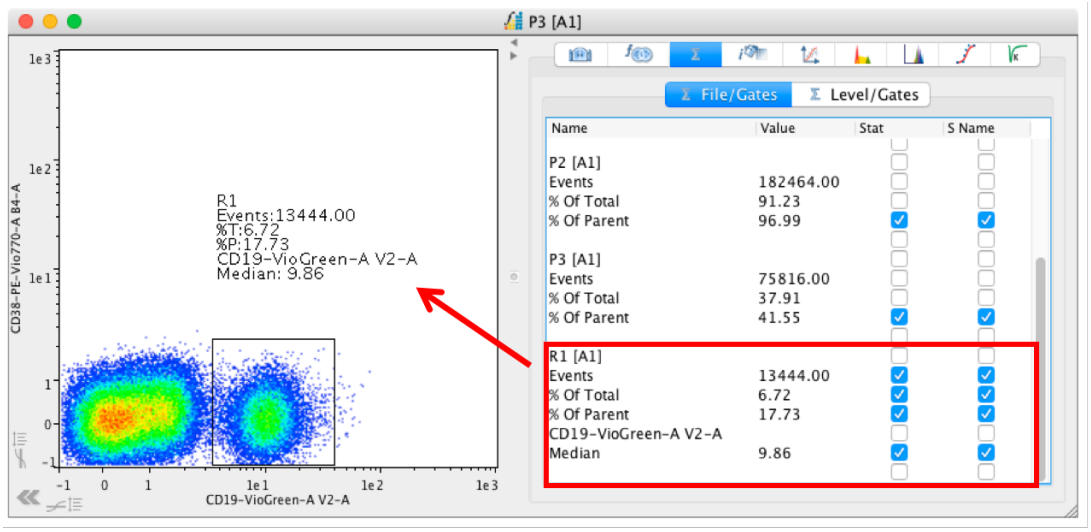
Once the gate has been selected in the side drawer, the cells are displayed at each level higher in the hierarchy. If real time update of plots has been selected in the Preferences menu, backgated events will update as the gates are moved.



Backgating can be performed on any number of populations.

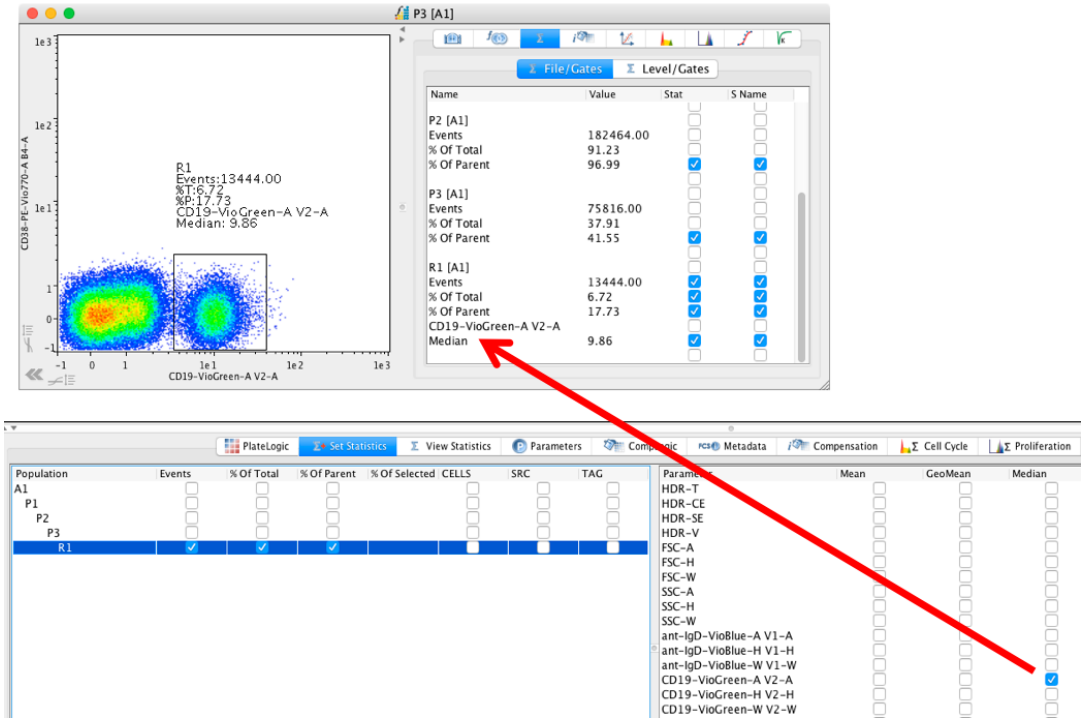


Statistics – Displaying Multiple Statistics on a Plot

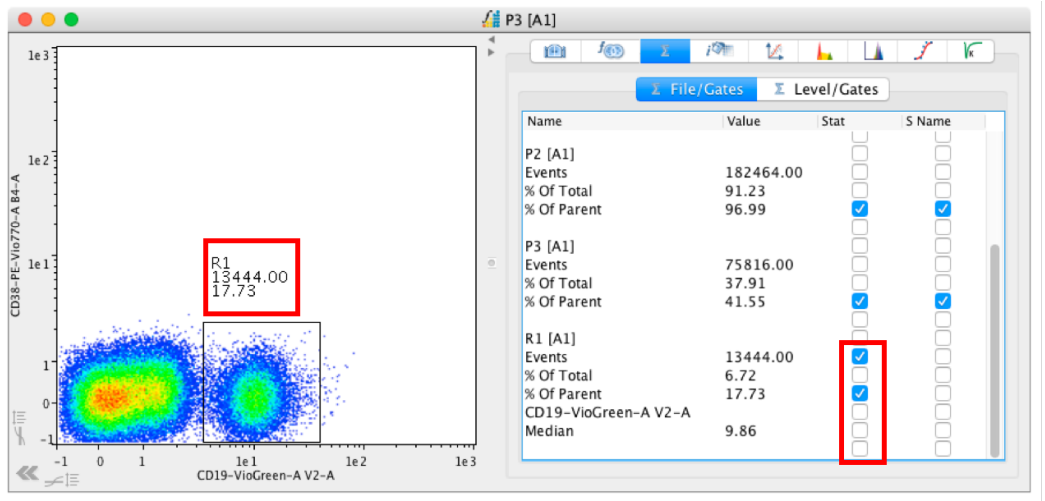


Tick the statistic (Stat) or statistic and name (S Name) relating to a specific gate to display on the plot

By default, the event count, '% of Total' and '% of Parent' are displayed for each gate in the hierarchy. To add additional statistics to the plot, open the Advanced Functions drawer and tick the specific statistic in the Set Statistics window. The new statistic will be calculated and be displayed in the plot side drawer and can be ticked to be displayed on the plot. The following example shows the calculation and display of the median fluorescence intensity for the R1 gate for the V2-A parameter.

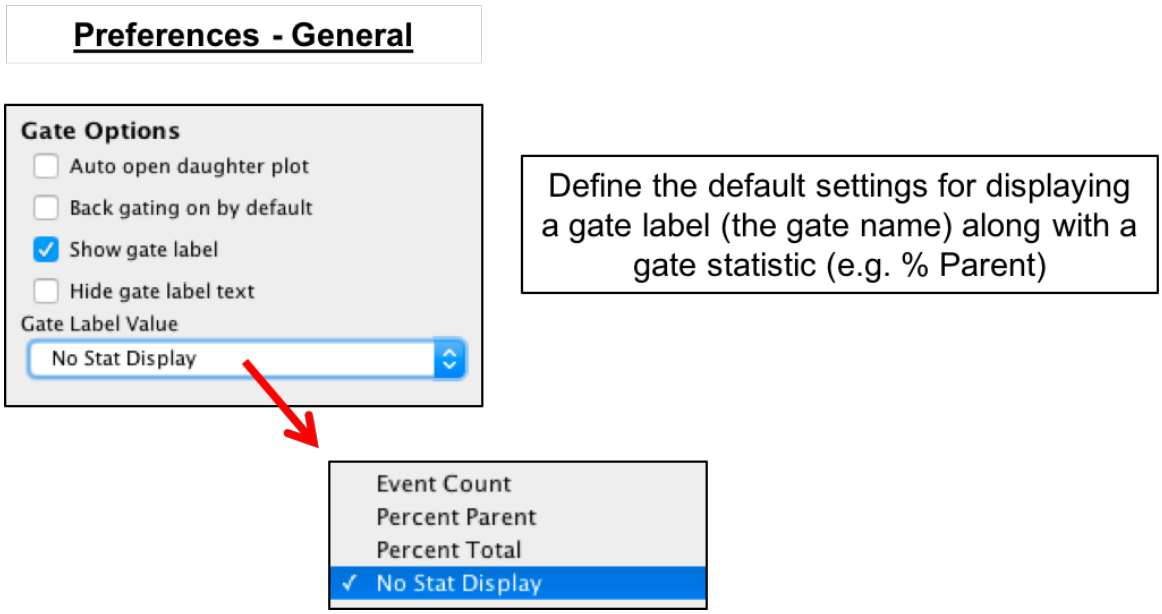


Untick the box in the 'S Name' column to display the statistic value only.

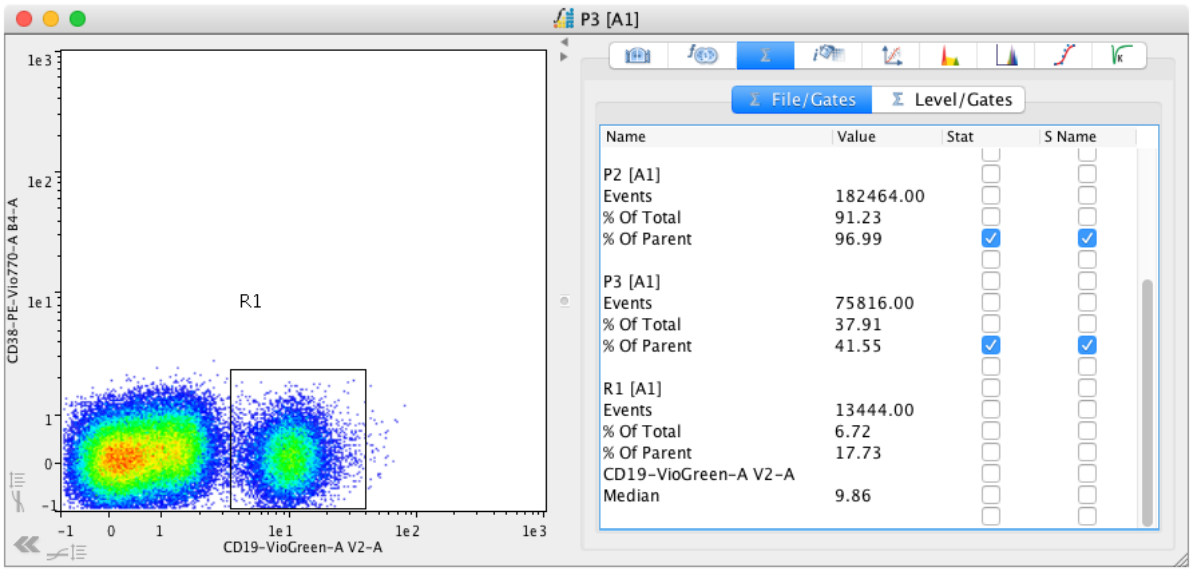


Selecting just the statistic (and not the name) will display the statistic value only

With all statistics turned off, the gate name alone will be displayed (if this is the setting in the program Preferences).



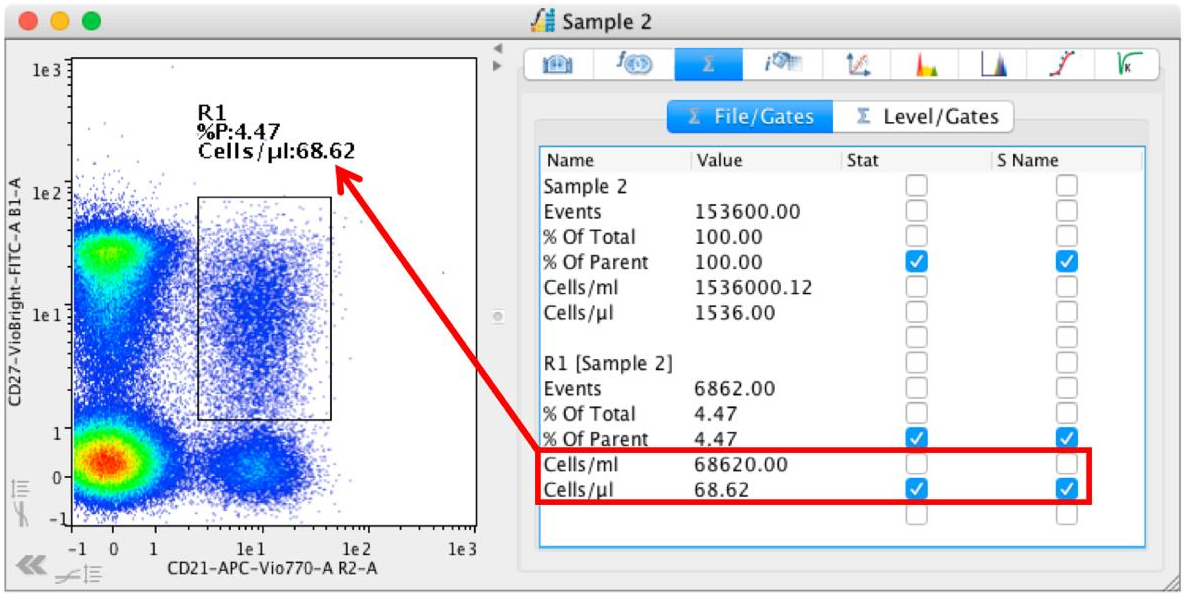
Here is the gate label (the gate name) displayed on the plot without any statistics.



MQD Volumetric Statistics – cells/volume

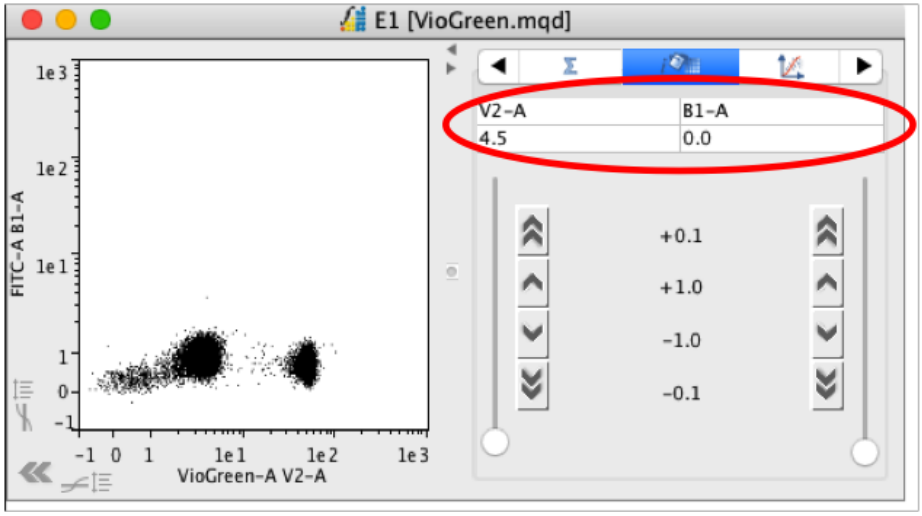
Volumetric gate statistics (cells/ml and cells/µl) can be displayed on dot plots and histograms for MQD files. These can be displayed as part of the gate label statistics from the statistics tab in the plot side drawer, as described above.

These same statistics can be calculated for a selection of files and displayed alongside all other statistics in a table in the Advanced Functions drawer. They can also be accessed in PlateLogic and GraphLogic.

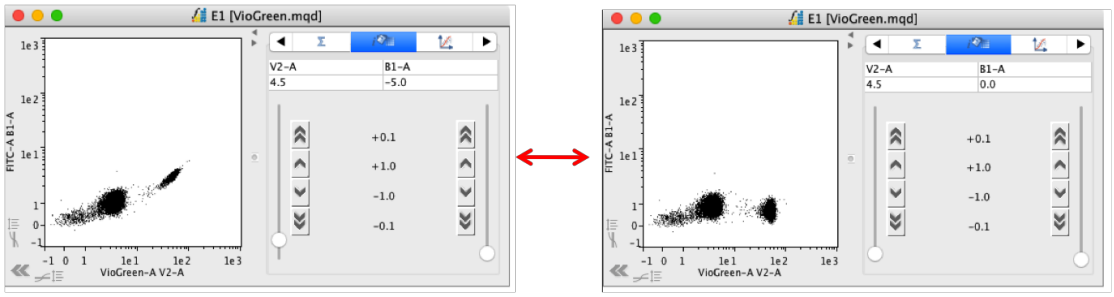


Manual Compensation

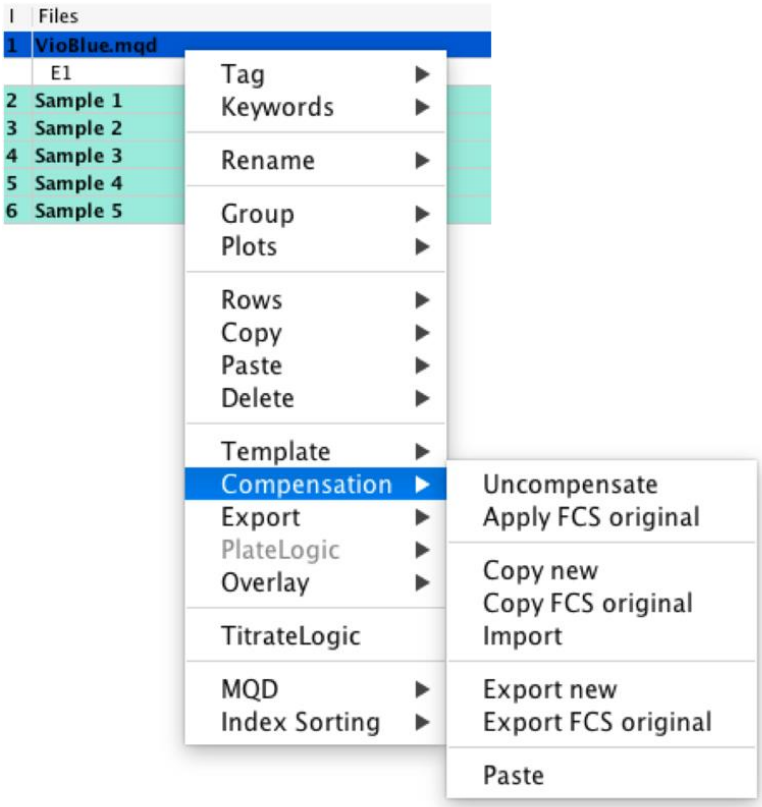
FlowLogic allows for easy, real-time compensation. To change the compensation of a file, open the plot side drawer and scroll through to the Interactive Compensation Matrix Window. Here, the two parameters shown on the plot are displayed with the compensation values (set when acquiring the sample).



The compensation can be changed with the slide bar, by stepping the value up and down (either by 1.0 or by 0.1 at a time) or by clicking on the compensation value itself and entering a new value. Changes in the compensation are updated in real time.



Once the desired compensation has been set, the new compensation matrix can be saved as a TXT file or copied and pasted onto another FCS file. Options relating to the saving and applying of compensation matrices can be found by right clicking on a file in the File Inspector and choosing ‘Compensation’.

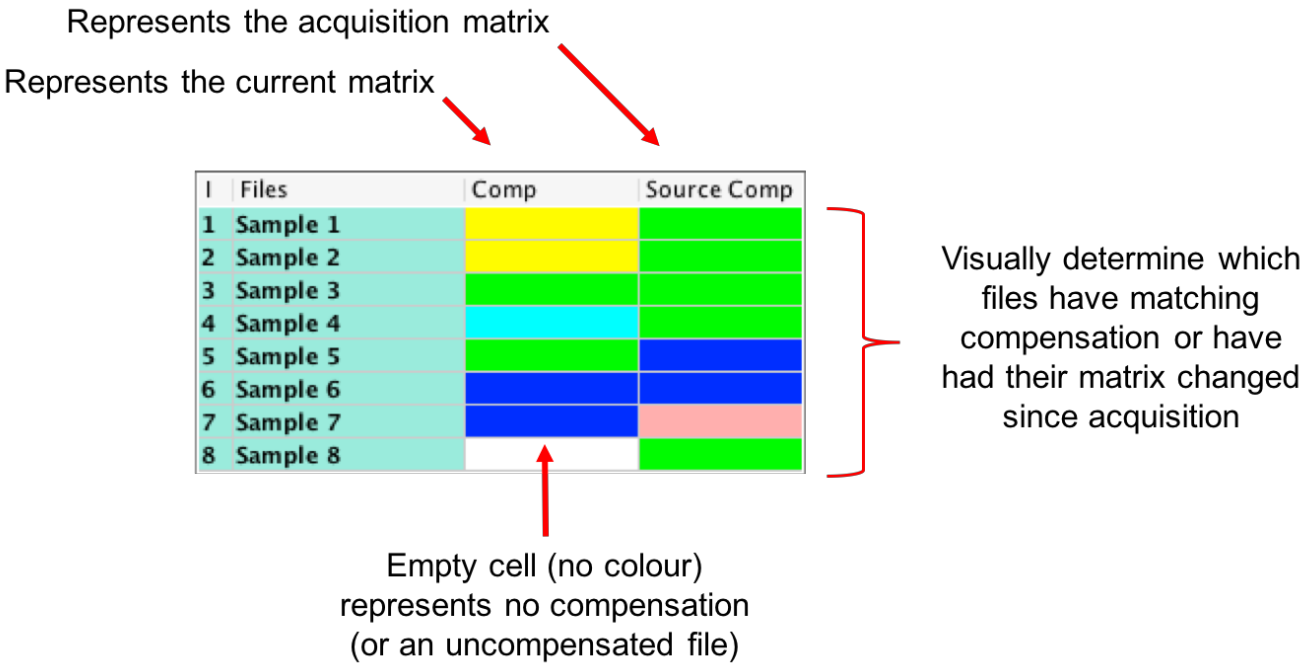


There are a number or options related to copying and importing compensation matrices:

- Uncompensate – removes all compensation.
- Apply FCS original – applies the compensation matrix created at the time of acquisition.
- Copy new – this copies the matrix after it has been changed in FlowLogic. This matrix can then be pasted onto other files.
- Copy FCS original – this copies the original matrix created when the file was acquired. The original matrix always remains with the FCS file so if a change is made that is unwanted, the original settings can always be restored.
- Import – this imports a matrix that has been saved as a TXT file (a matrix altered in FlowLogic post acquisition or saved from an original file).
- Export new – if the compensation is changed within FlowLogic, the new matrix can be saved as a TXT file to be applied at any future time.
- Export FCS original - this saves the original matrix created at the time of acquisition as a TXT file. This can then be pasted onto files in future analyses.
- Paste – once matrices have been copied or imported, they can be pasted onto other files. To do this, highlight the files and from the Compensation menu select ‘Paste’.

Located in the File Inspector, the ‘Comp’ and ‘Source Comp’ columns use color to display information about the current and original compensation matrices associated with each file.

Colors are assigned to each unique matrix. So, files that were saved at acquisition with a particular matrix will have matching colors in the ‘Source Comp’ column. Visual comparisons can also be made between the acquisition compensation matrix (Source Comp) associated with an individual file and the current compensation matrix (Comp), created in FlowLogic. A ‘Comp’ color that differs to the ‘Source Comp’ color indicates that the compensation has been adjusted within FlowLogic.

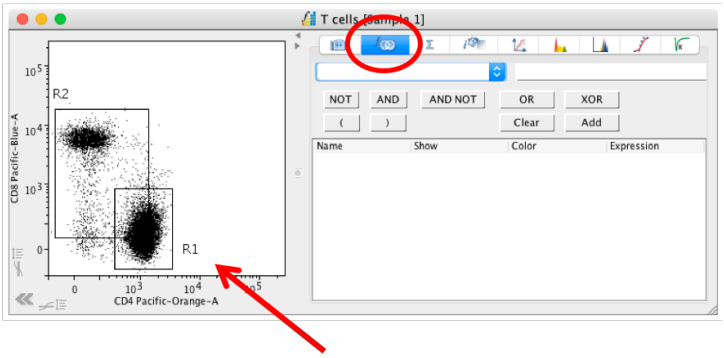


I	Files	Comp	Source Comp
1	Sample 1		
2	Sample 2		
3	Sample 3		
4	Sample 4		
5	Sample 5		
6	Sample 6		
7	Sample 7		
8	Sample 8		

Boolean Gates

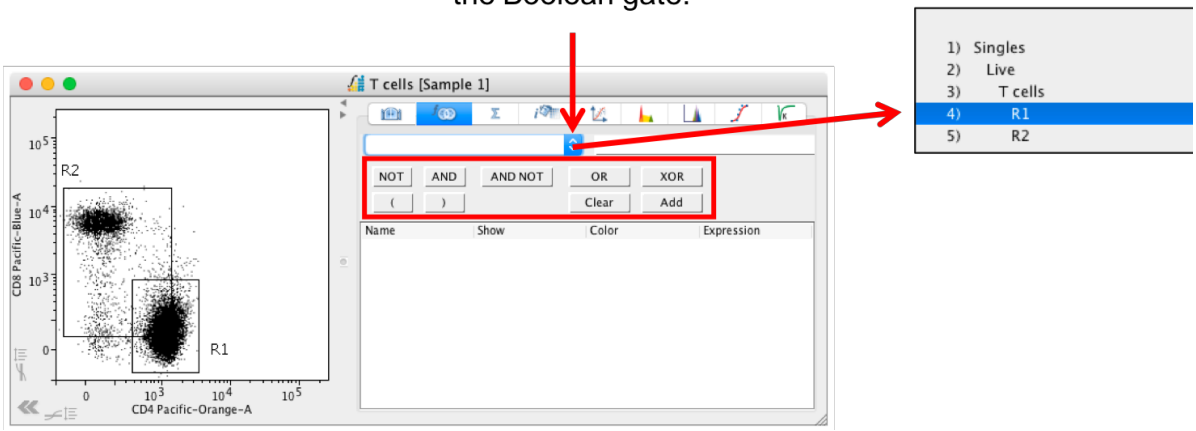
- Boolean gates allow for a more powerful form of analysis by defining how two or more gates interact.

The Boolean gate options are located in the plot side drawer.



To demonstrate the different Boolean gates, two intersecting gates (R1 and R2) have been created.

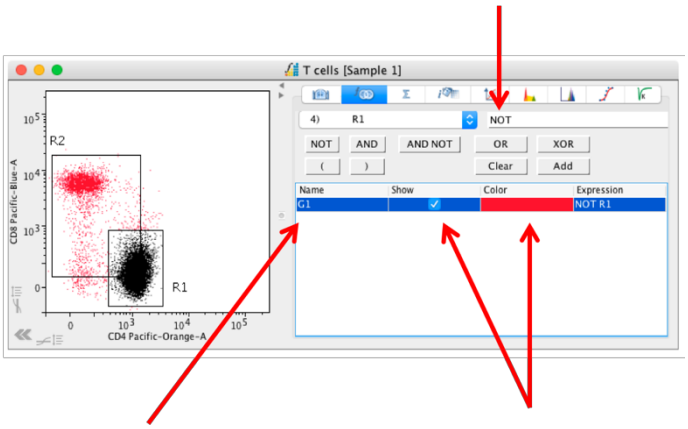
From the drop down list, select the gate that you wish to form part of the Boolean gate.



Then define the Boolean function from the options 'NOT', 'AND', 'AND NOT', 'OR' and 'XOR' that you wish to apply to the gate. Finally, select another gate from the drop-down menu and click 'Add' to create the Boolean gate.

- **AND** – This displays all events in the intersection.
- **AND NOT** – This displays the events located in the first gate minus the second.
- **OR** – This displays the events located in both gates.
- **XOR** – This displays all events within the gates but outside the intersection.

- **Inverse Gate** – This displays all events outside a single gate. To create an inverse gate, choose or type **'NOT'** followed by the gate name, e.g. NOT R1 (in capital letters), into the expression field.



- The new Boolean gate is now called G1. The color of the Boolean gates can be changed by clicking in the color field. Tick the 'Show' option to color the newly defined population.

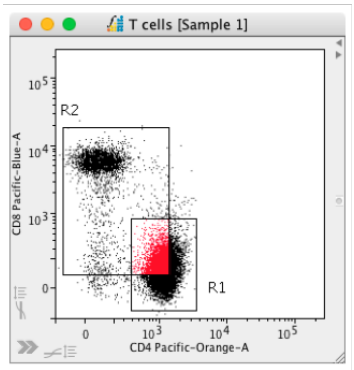
- The Boolean gate will also be listed in the File Inspector.

- To delete a Boolean gate, select the gate in the File inspector, right click and select 'Delete' → 'Rows'.

I	Files	Events	%Parent	%Total
1	Sample 1	326037		100%
	Singles	293752	90.10%	90.10%
	Live	173863	59.19%	53.33%
	T cells	15465	8.89%	4.74%
	G1	3296	21.31%	1.01%
	R1	12169	78.69%	3.73%
	R2	8698	56.24%	2.67%

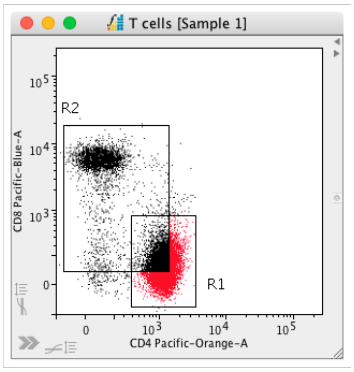
Following is an example of each of the Boolean gate types, based on the R1 and R2 gates listed above. The events within each Boolean gate are colored red.

AND
(R1 AND R2)



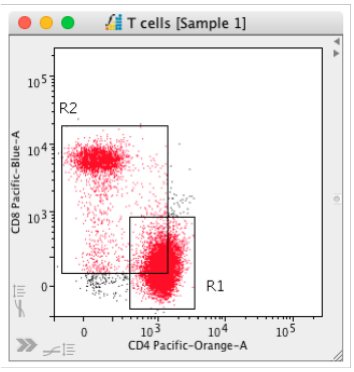
Everything in the intersection

AND NOT
(R1 AND NOT R2)



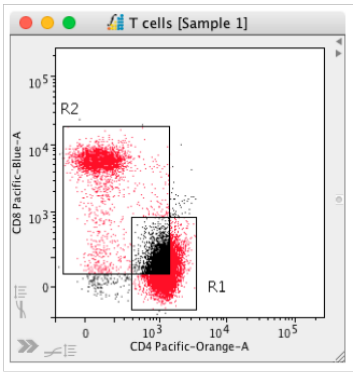
Everything in R1 minus R2

OR
(R1 OR R2)



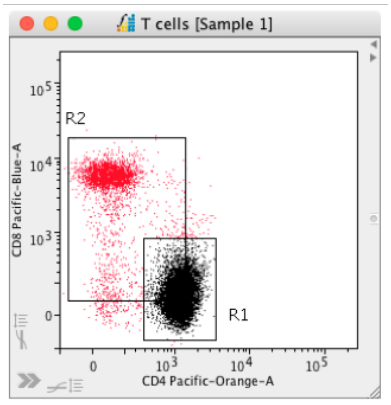
Everything in both gates

XOR
(R1 XOR R2)



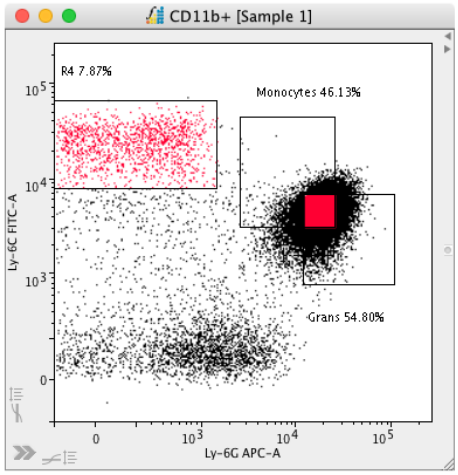
Everything within the gates minus the intersection

NOT
(NOT R1)



Everything outside R1

Complex Boolean gates can also be created by adding to the gate definition in the Expression field. For example, to create the intersection between two gates and the events from a third gate, create the Boolean gate as: **Monocytes AND Grans OR R4**



If an invalid expression is entered, an error message will be displayed.

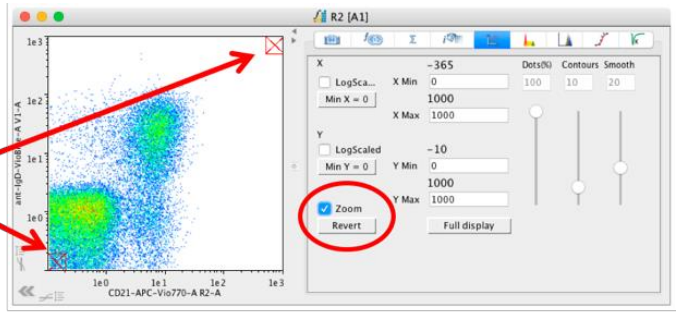
Scaling

FlowLogic can Scale or Zoom plots, allowing you to view plots optimally.

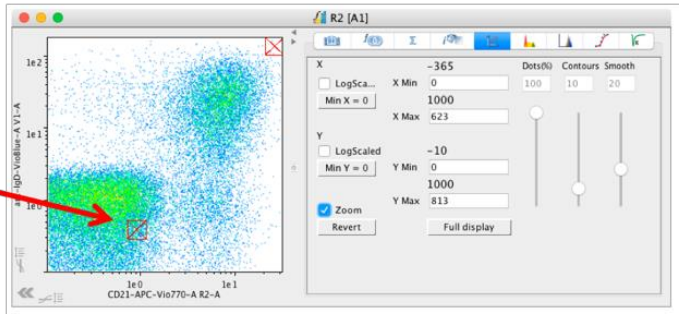
- The Scale feature can be found in the plot side drawer. Tick the Zoom box in the Scaling section to enable scaling.

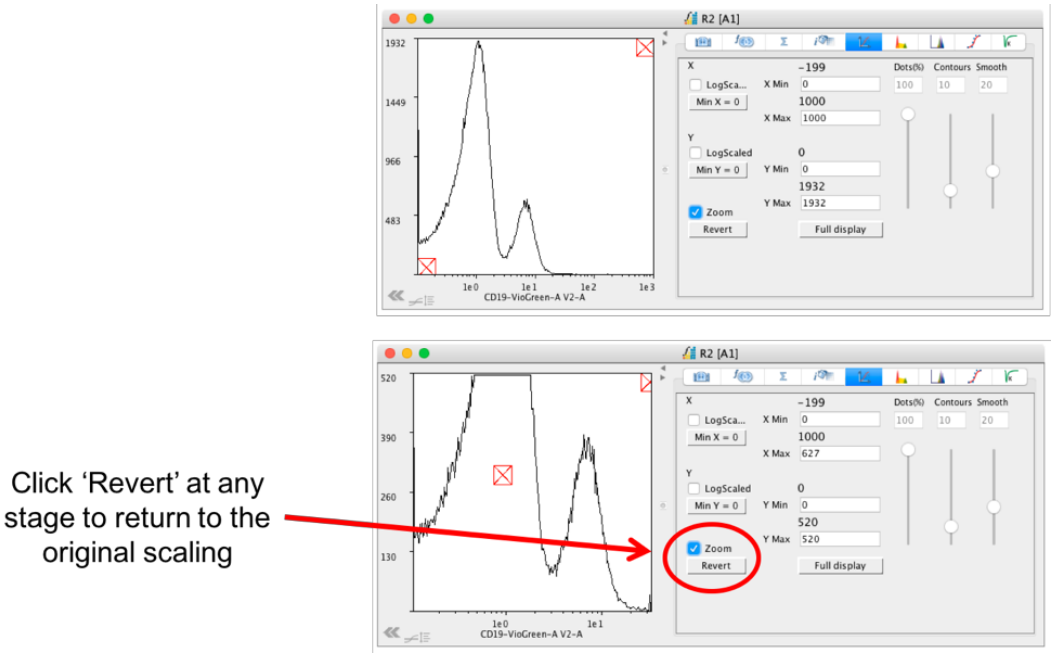
- Two red, crossed squares will appear on the plot. One in the bottom left and one in the top right. To zoom, click and drag either of the red squares towards the opposite corner.

Tick the 'Zoom' box to display the red squares in two of the corners



Drag the red squares away from the axes to zoom the scaling





Features associated with scaling:

- When the plot has been re-scaled, unselect the check box. This will allow gating tools to be selected as well as allowing access to the right click menu within the plot.

- By clicking the ‘Revert’ button, the original scaling will be restored. The scaling can be reset as many times as you wish.

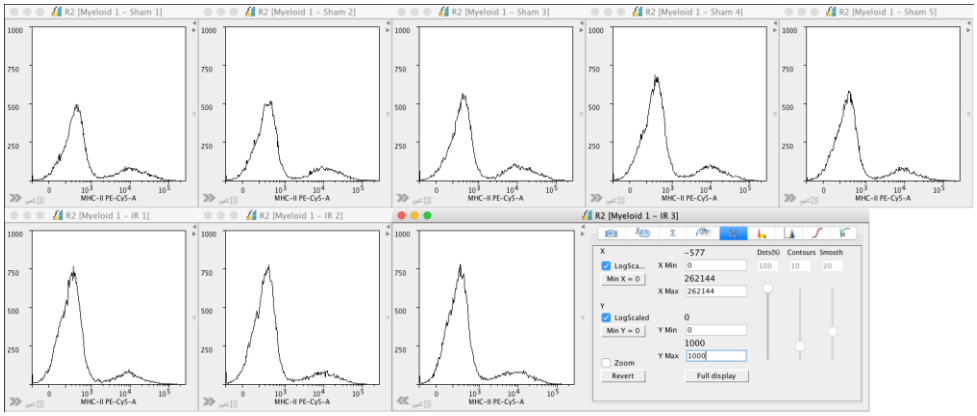
- When using the batch analysis tool (with the padlock on), the new scaling will be copied onto the subsequent plot. This can be adjusted on each plot individually if required.

- Clicking ‘Full Display’ will display the entire dataset, which can contain events below zero on both axes.

- Untick the ‘Logscaled’ viewing option in order to compress the scaling, i.e. drag the red box in the top right towards the left-hand side.

The axis range for dot plots and histograms can easily be set by typing a new value into the min and max fields for the x and y axes in the Scaling tab of the plot side drawer. When files are grouped, the newly defined range will be applied to all files. To alter the min settings, the ‘LogScaled’ setting needs to be unselected, due to the nature of the compression.

This feature is particularly useful when viewing histograms as the range on the y axis can be uniformly set for all sample in the group and therefore, the height of the histograms can be compared directly. The adjustment is also applied to plots displayed in PlateLogic.



Overlays

- Dot plot and histogram overlays are easy to create. In addition, gates can be applied to overlays. This results in the gate being added to every file contained in the overlay. If the gate is moved it is automatically updated on each file. Once you have built your gating hierarchy, the fastest way to create an overlay is to add all your populations directly from the File Inspector.

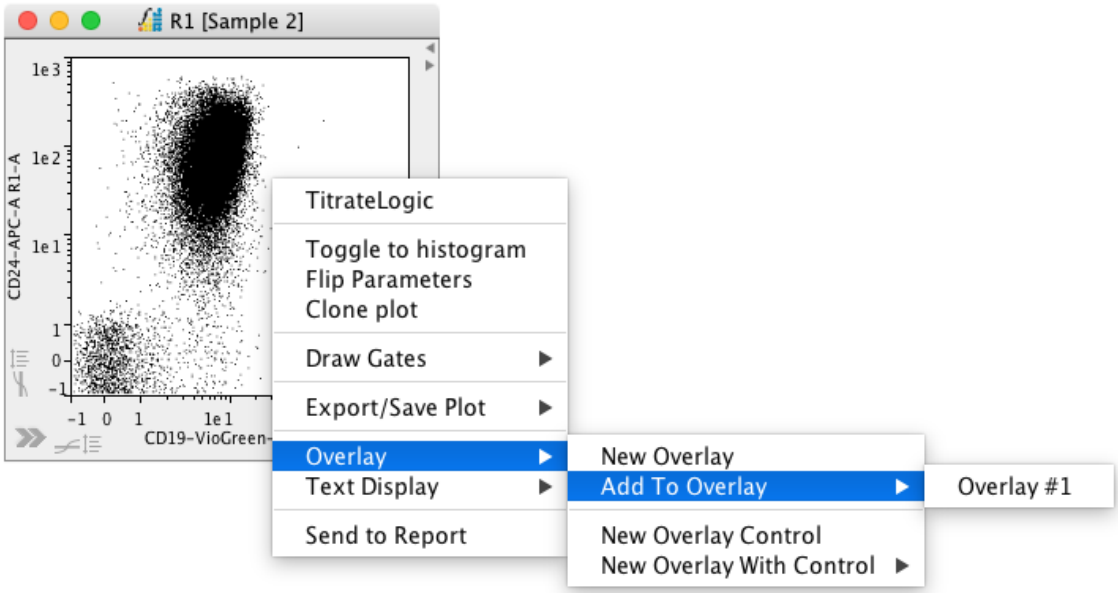
- Highlight all the populations to be overlayed. Adding all files at a particular level can be done by right clicking on the appropriate sample and choosing Rows → Highlight Level (or 'Highlight level within group' for grouped samples).

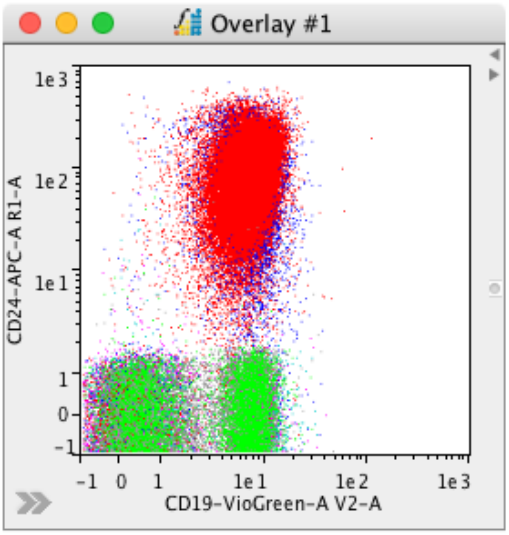
- Once the populations are selected, right click and choose ‘New overlay’. The overlayed plot will be displayed in the Workspace.

- Dot plots are saved in a separate window at the bottom of the File Inspector.

- If the individual populations have already been drawn as dot plots, then the resulting overlay will also be a dot plot. Overlays can be toggled between dot plots and histograms using the toolbar. The parameters displayed can also be changed.

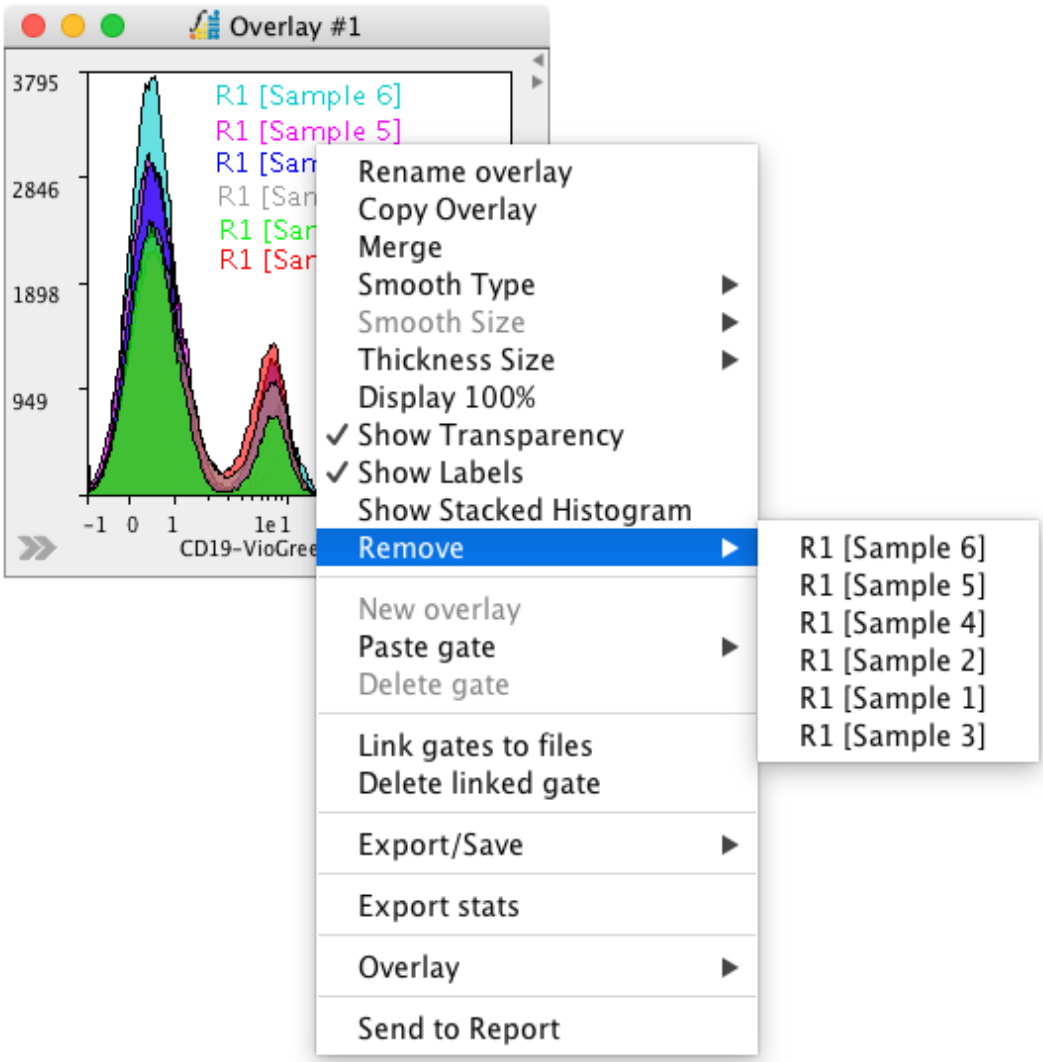
- Plots that are displayed in the Workspace can be used to create a new overlay or can be added to existing overlays. Right click on the plot and select ‘Add To Overlay’. Choose new or an existing overlay. Continue to add more plots to the overlay by right clicking on them in the workspace or by right clicking on the population in the file navigator and choose ‘Add to overlay’.



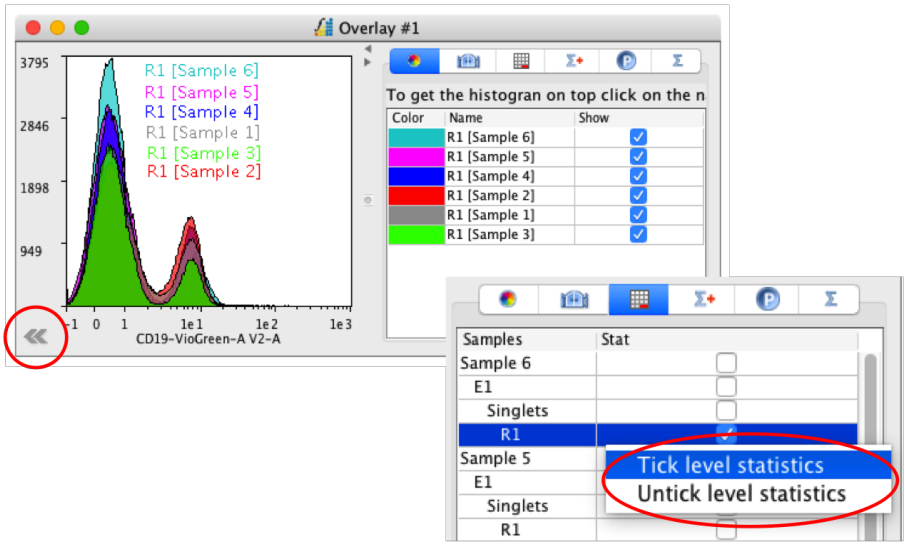


- Right click on the overlay to bring up a range of menu options. The options include renaming, smoothing and displaying the y-axis range as percentage (Display 100%). The transparency can also be toggled on and off. Default overlay colors can be set and changed in the program Preferences. These can be modified in the overlay plot side drawer.

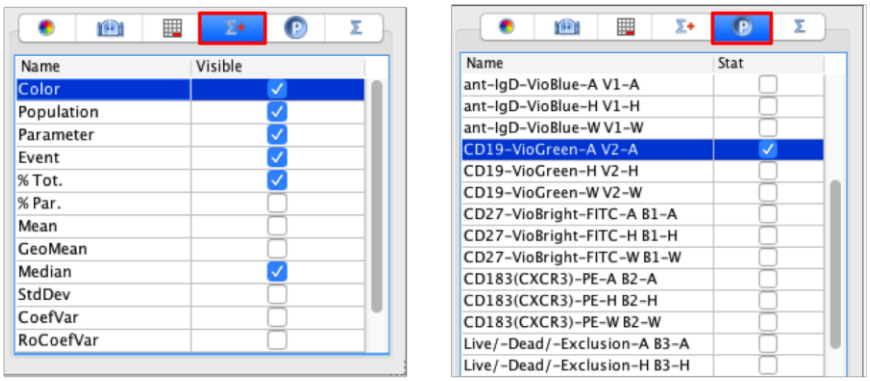
- To remove a file from the overlay, right click on the plot area and select 'Remove' followed by the file name.



- In the plot side drawer, sequentially click through the tabs to change colors and calculate or view statistics.

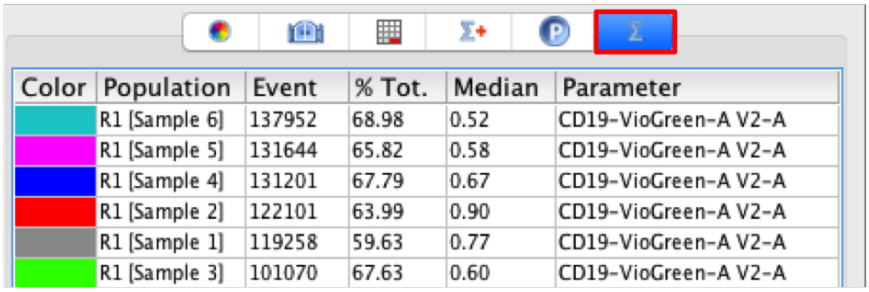


- Click in the color column to make a change.
- Re-order using drag-and-drop.
- Tick the populations to analyze. Right click to tick all populations from the same level.



- Select the statistics to be calculated.

- Select the parameters linked to the statistics.



Color	Population	Event	% Tot.	Median	Parameter
	R1 [Sample 6]	137952	68.98	0.52	CD19-VioGreen-A V2-A
	R1 [Sample 5]	131644	65.82	0.58	CD19-VioGreen-A V2-A
	R1 [Sample 4]	131201	67.79	0.67	CD19-VioGreen-A V2-A
	R1 [Sample 2]	122101	63.99	0.90	CD19-VioGreen-A V2-A
	R1 [Sample 1]	119258	59.63	0.77	CD19-VioGreen-A V2-A
	R1 [Sample 3]	101070	67.63	0.60	CD19-VioGreen-A V2-A

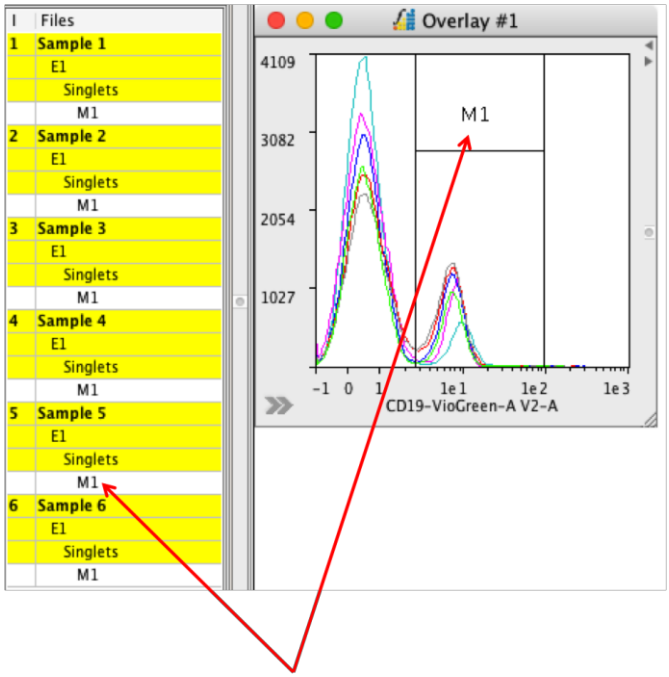
- Statistics are displayed and can be exported by right clicking in the plot window and choosing ‘Export stats’.

Gating on Overlays

- FlowLogic not only enables the easy generation of overlays but also allows for gates to be drawn on overlays. This feature adds a gate to all files in the overlay and calculates statistics in the side drawer, which can be directly exported.

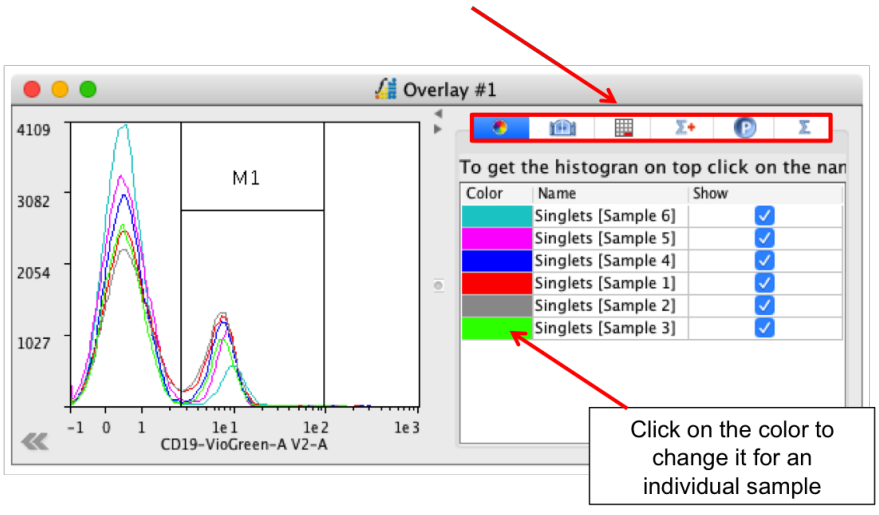
- Create either a dot plot or histogram overlay with two or more files.

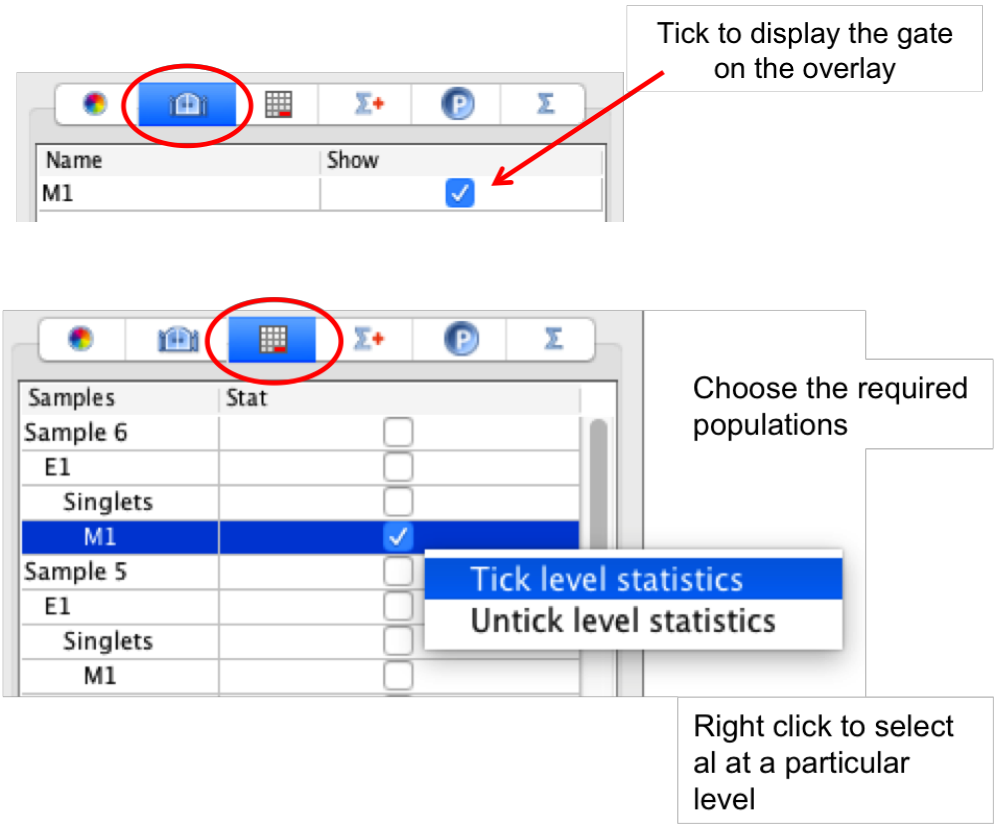
- Select an appropriate gating tool from the Workspace toolbar and draw the gate on the overlay. In this case, the gate M1 is added to all files.



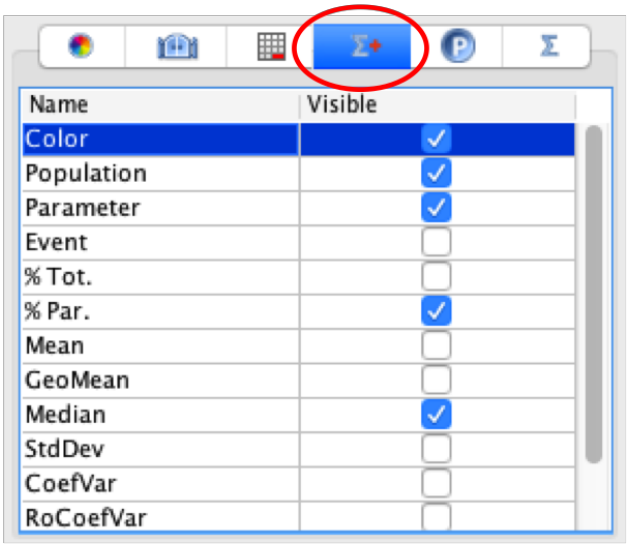
- If the gated population on the overlay is double clicked, the daughter populations will be an overlay of the same files.

- To calculate statistics associated with the gated overlay, open the plot side drawer and sequentially click through the tabs to select the statistics to be calculated.

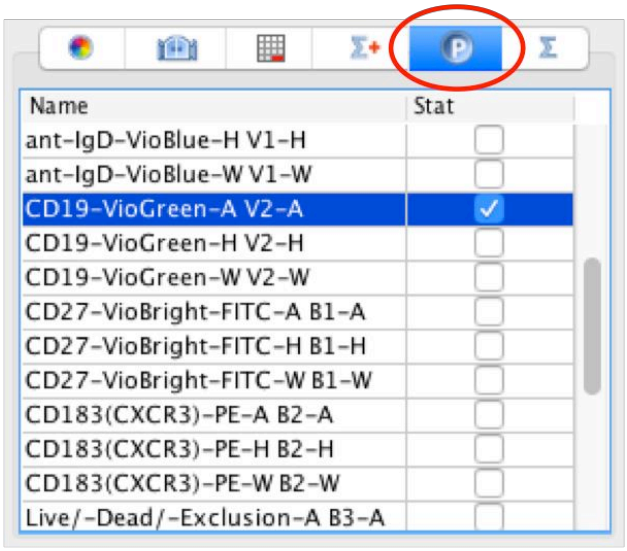




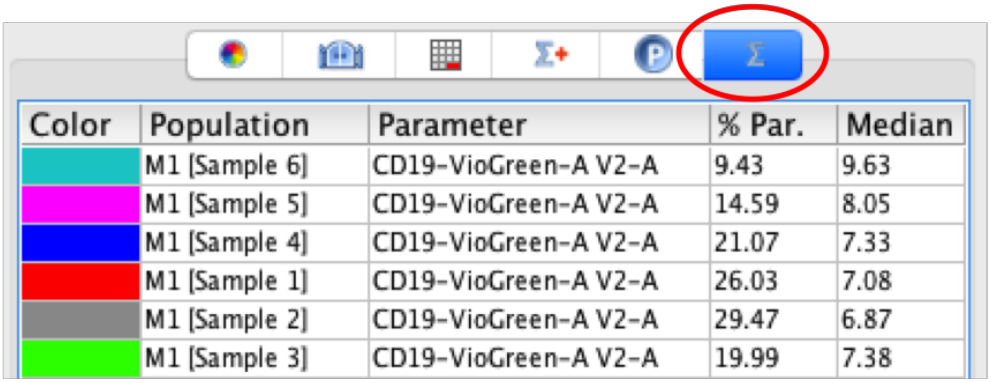
- Choose the required statistics.



- Ensure the parameter associated with the gate is selected.



- View the statistics in the final tab.



Color	Population	Parameter	% Par.	Median
	M1 [Sample 6]	CD19-VioGreen-A V2-A	9.43	9.63
	M1 [Sample 5]	CD19-VioGreen-A V2-A	14.59	8.05
	M1 [Sample 4]	CD19-VioGreen-A V2-A	21.07	7.33
	M1 [Sample 1]	CD19-VioGreen-A V2-A	26.03	7.08
	M1 [Sample 2]	CD19-VioGreen-A V2-A	29.47	6.87
	M1 [Sample 3]	CD19-VioGreen-A V2-A	19.99	7.38

- Right click on the overlay plot and select ‘Export stats’ to save the statistics as a CSV file.

Batch and Jump

- The FlowLogic File Navigator is comprised of two main tools. Jump and Batch.

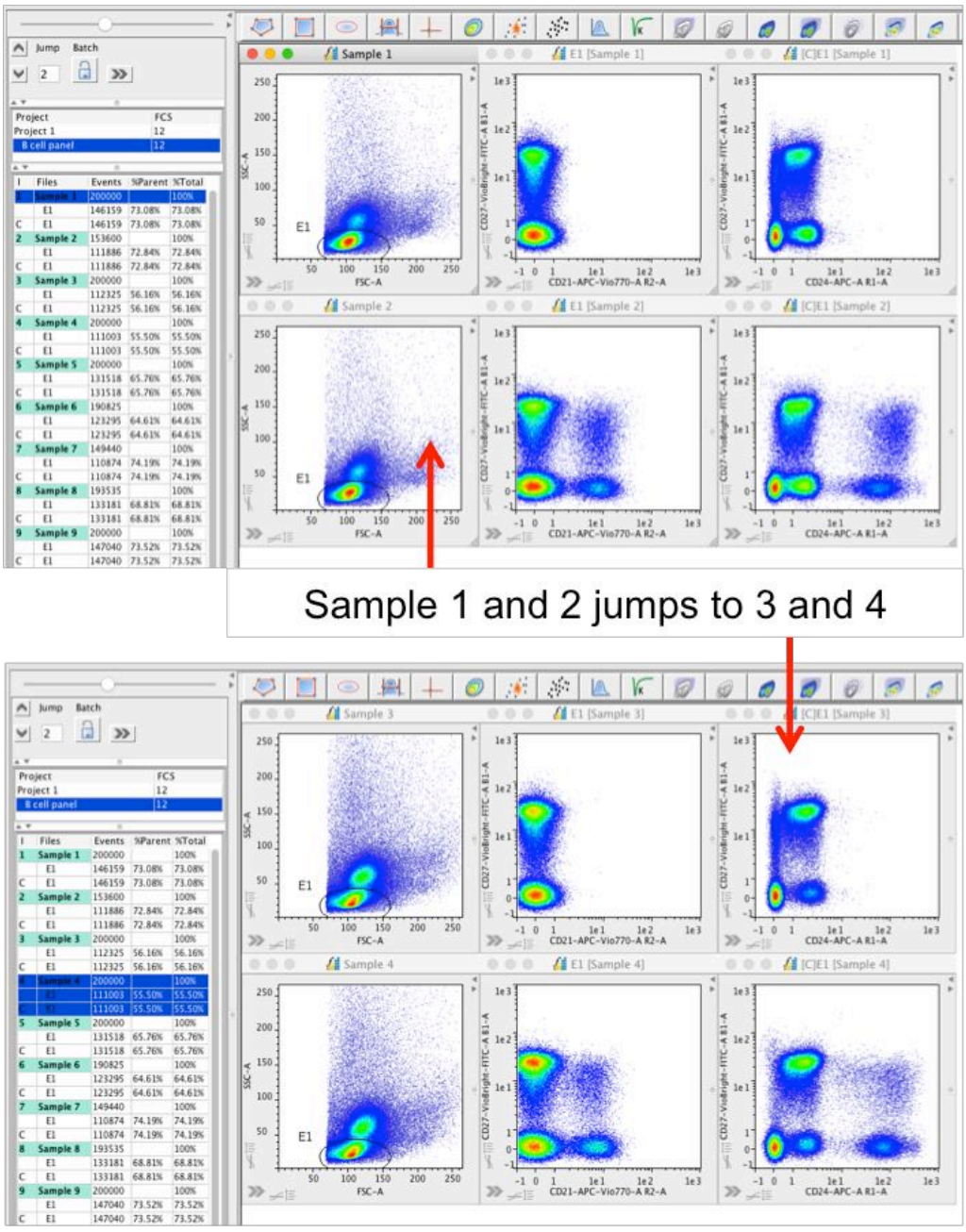
- The ‘Jump’ tool allows you to scroll through and view large data sets, multiple samples at a time.

- Batching can be a very useful tool when you want to apply a set of gates to multiple samples one by one.

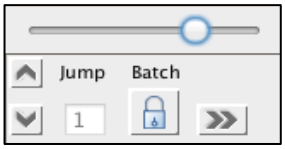
- Once you have applied gates to the FCS files, you can ‘scroll’ through your data several files at a time. For example, if you want to view 5 FCS files at a time, you can set the number in the Jump box to 5. Then when you click the up or down arrows, FlowLogic will jump through to the next 5 samples in order.

- To make the most of this feature set the numbers of windows in the edit menu to the number of plots in your gating strategy (i.e. the example below shows three dot plots with the number of windows set to three).

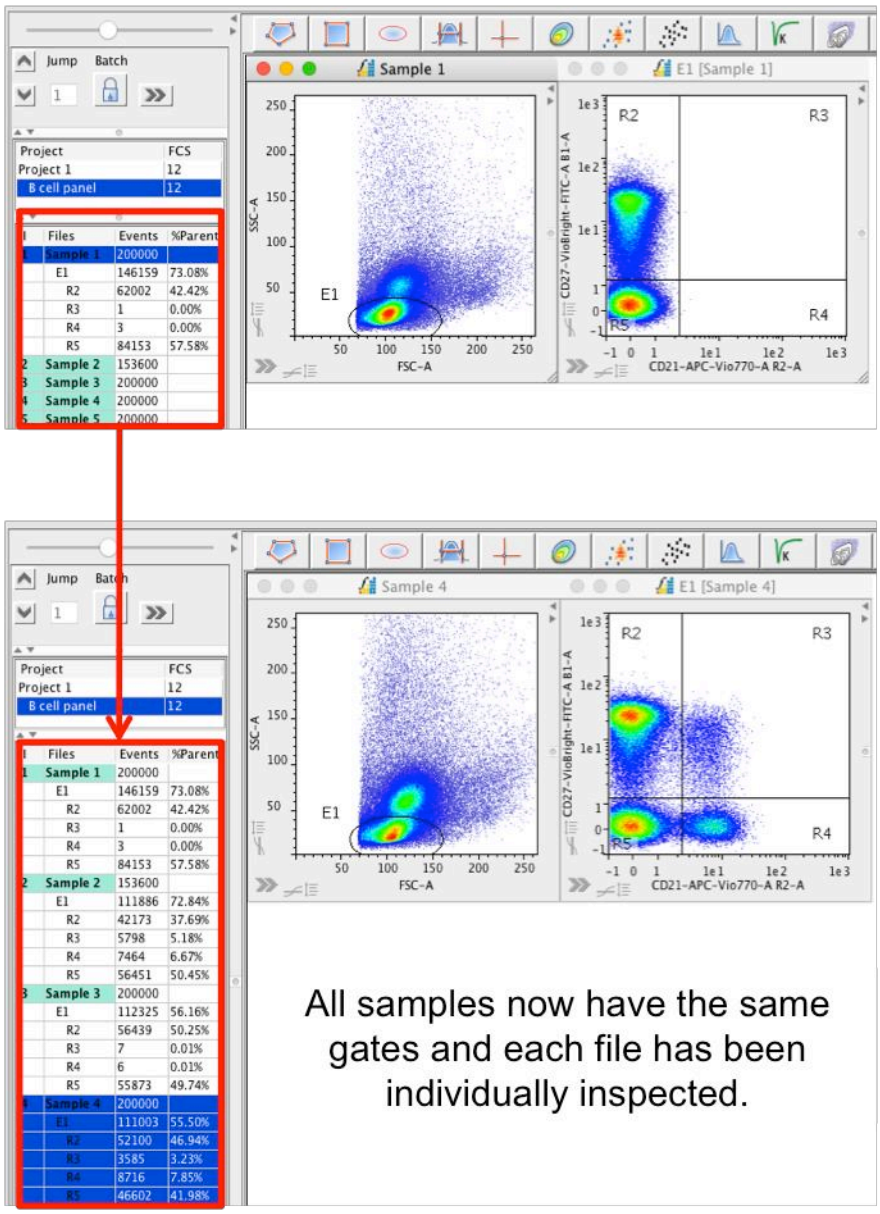
- In order to jump, click to unlock the ‘Batch’ padlock**,** set the number in the jump box and ‘Jump’ up and down using the arrow buttons.



- Batching is performed when the Batch padlock is closed. This will prevent you from using the Jump function.



- Once gates are applied to a file, click the padlock to close it and click the down arrow. All gates and statistics are automatically copied to the next sample, which will then be displayed in the Workspace in place of the previous file. Any modification you make to the gates will then be transferred as you click to the next file.

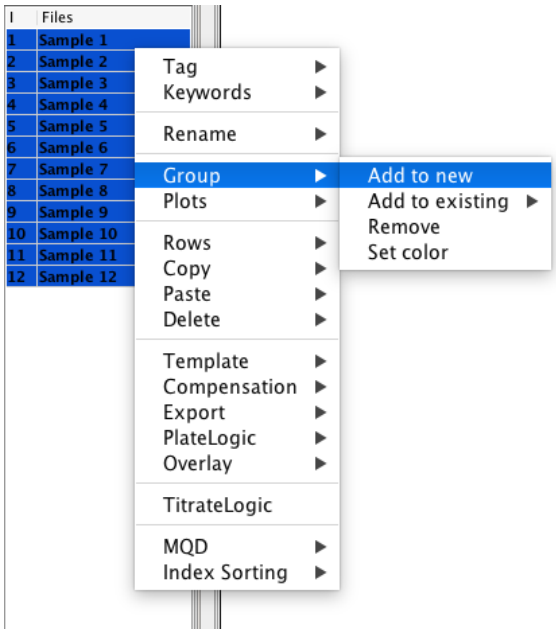


- All samples now have the same gating analysis and each file has been individually inspected. The statistics are ready to be exported.
- An alternative to batch analysis is to create Groups.

Group Analysis

Creating groups can make analysis of multiple samples very quick and easy. If a gate is applied to one file in a group, it is automatically applied to the same parameters on all other files in the group.

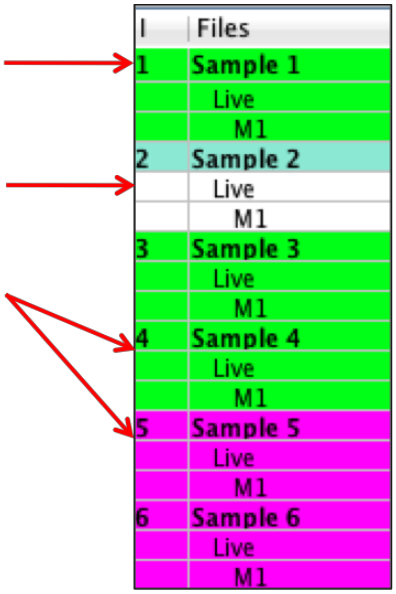
Select all files to be grouped, right click and select Group → Add to new.



Groups are assigned a color.

Individual files or even individual gates can be removed from a group.

Multiple groups can be created in each Experiment folder.



Adjustments to individual gates are automatically updated to all within the group, along with all calculated statistics.

If copied gates are inserted into a grouped hierarchy on one file, all of the gates for that file will be removed from the group. This new hierarchy can then be copied and pasted to all other samples (or a selection) and then re-grouped.

Right Click Menu in the File Navigator and File Inspector

Several options to aid with your analysis are available by right clicking on the FCS samples or the Experiment and Project folders in the File Inspector.

File Navigator right click menu:

Experiment and Project Folders

The File Navigator has both Experiment folders with Project folders contained within them. This allows for the easy organisation and analysis of FCS files.

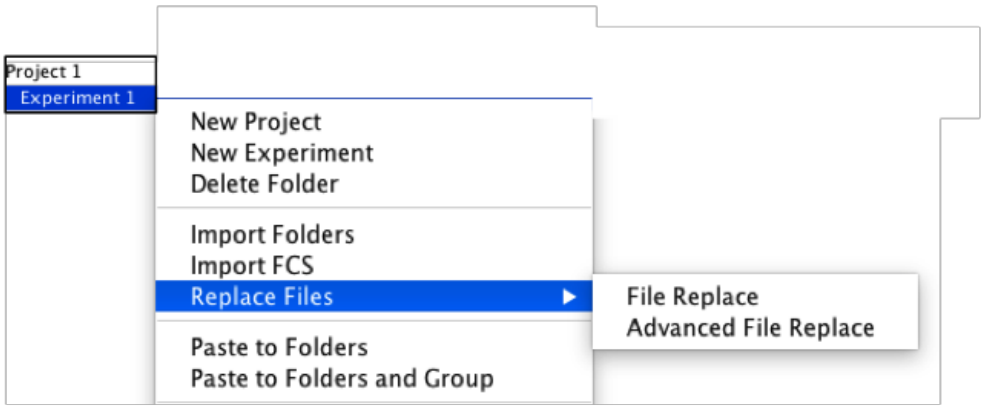
- By default, FlowLogic starts with one Experiment folder and one Project folder, as shown below. Right clicking on these folders opens a menu with options to create new folders and delete folders. Double clicking on the folder allows it to be renamed.

- Right click on the Experiment or Project folders to create, add to or delete folders.

- Re-order the Project and Experiment folders by drag-and-drop.

- FCS files can be dragged and dropped into the Experiment folders or imported by selecting '**Import FCS**…' from the **File** menu.

- The **Replace File** function allows you to swap the files used in one analysis with a new set of files, whilst maintaining all gates, statistical analyses, graphs and reports. This can be very useful if you are doing repeat experiments and wish to apply the same analysis. There are two options under the **Replace Files** menu: **File Replace** and **Advanced File Replace**.



File Replace

This option allows you to swap an equal number of files as exist in the File Inspector. When selected, you will be prompted to select the files to replace the current collection. If you choose fewer files than already exist in the File Inspector, FlowLogic will ask if you want to delete the files that are not being swapped. In effect, you will end up with only the files that you choose to swap into FlowLogic.

This option does not allow you to swap a greater number of files into the File Inspector than are already there. Individual files can be replaced by selecting the files in the File Inspector and choosing Template → Replace Files (see page 56 for more details).

Advanced File Replace

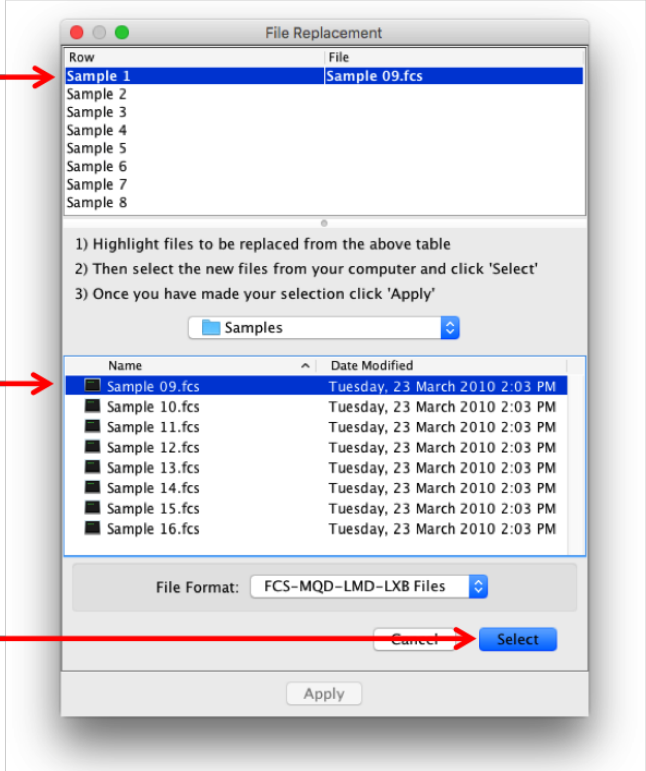
This option allows you to specify the individual files that you wish to swap for each file in the File Inspector.

If you choose the **Advanced File Replace** option, you will receive a list of the files in your current Experiment Folder.

Select a file in the Row column.

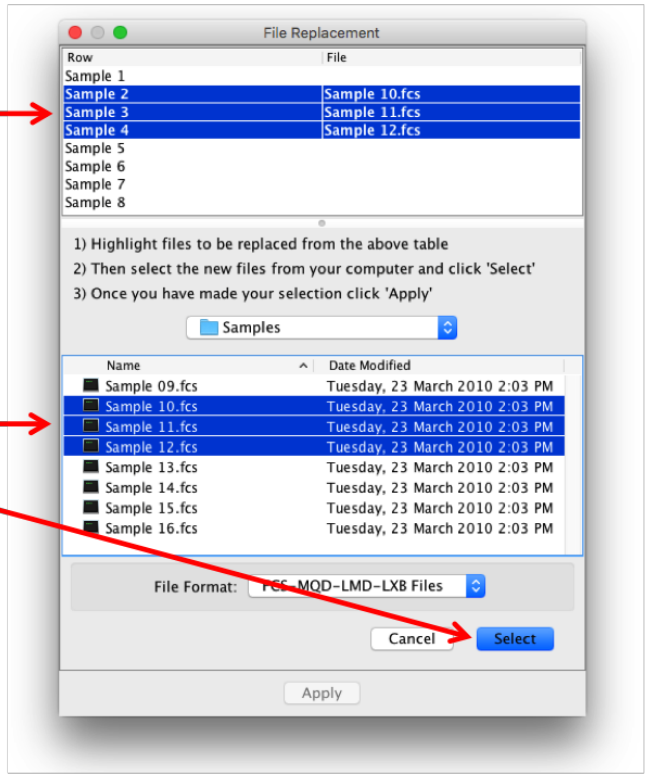
Then, select the file that you wish to replace it with.

To confirm the swap, click '**Select**'. You can now choose a new file from the Row column to swap



To replace multiple files at a time, highlight all of the files you wish to swap.

Then, select the equivalent number of new files and click '**Select**'. In this case the new files will replace the old files in the order that they are displayed.



Once all files have been matched with their replacements, click '**Apply**' to perform the replacement.

Paste to Folders – Gates copied from a single file in the File Inspector can be pasted to an Experiment Folder in the File Navigator. This will paste the gates to all compatible files in that folder. Gates can also be pasted to multiple folders at once. To do this, highlight the desired folders before choosing 'Paste to Folders' from the right click menu.

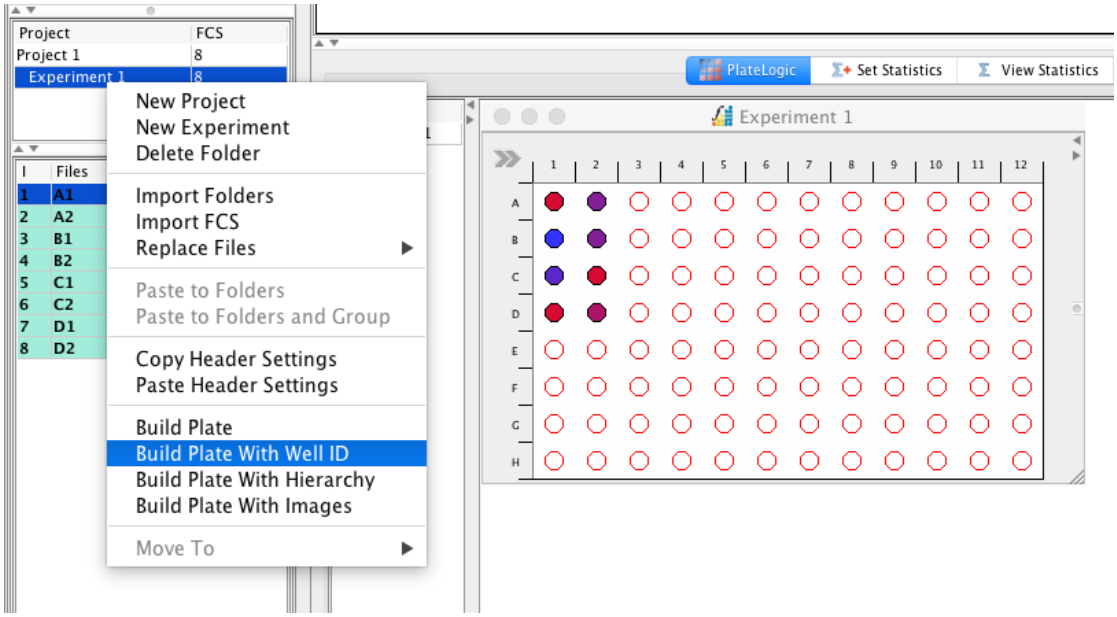
If a copied gate is part of a group, then this group setting will also be pasted to the files within the folder.

Paste to Folders and Group – when selecting this option when pasting a copied gate(s) to a folder, all compatible files within the folder will be added to a group. If a copied gate is pasted to multiple folders at once, files in each folder will be added to a new group.

Copy/Paste Header Settings – if the order of the columns in the File Inspector has been changed, then these settings can be copied from one Experiment folder and pasted to others.

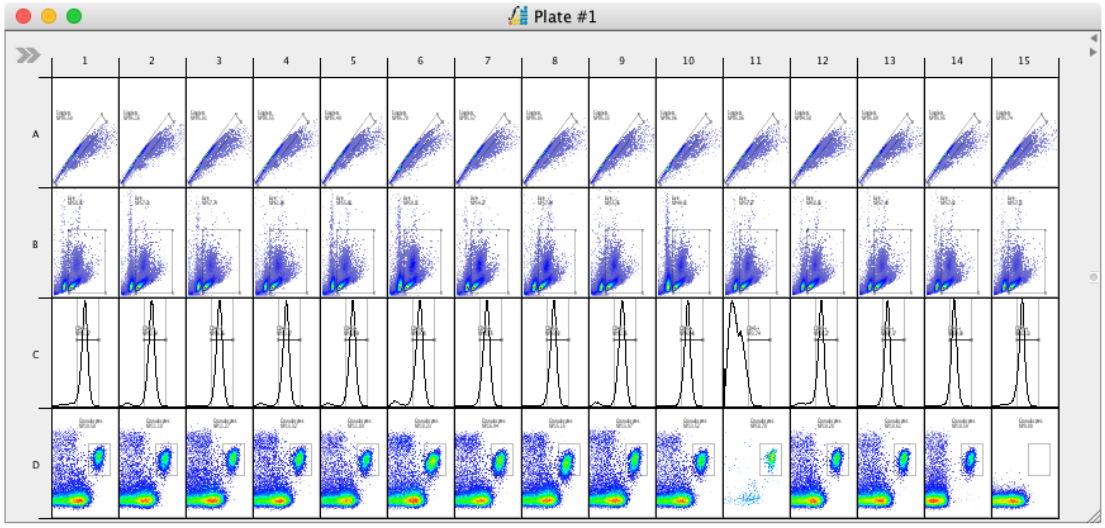
Build Plate – this creates a 96 well plate and loads the files in successive wells until the row/column is filled before starting in the next row/column.

Build Plate With Well ID – if the files name is the Well ID, then selecting this option will load the files in the wells that correspond to their names/Well ID. If the Well ID is outside the standard dimensions of a 96 well plate, the plate dimensions will automatically be adjusted to incorporate the files in their correct position. The Well ID has to be set as the naming option prior to creating the plate. Once the files have been loaded into a plate, the name can be changed from the Well ID to another format whilst maintaining the position in the plate.



Build Plate With Hierarchy – this option will build a plate with one column per file. As gates are created, a new row will be added to the plate, displaying the subsequent daughter population and each level in the hierarchy will be assigned to a new dataset. This allows each level of the hierarchy to be treated independently, such as in the type of statistic calculated. This type of plate can be built at any stage of the analysis as it will grow as new gates are created.

Build Plate With Images – this option also builds a plate with a column for each sample and a row for each level of the hierarchy, although the rows will not be separated into different datasets. In effect, only the dot plot image is displayed.



FCS File Menu – right click on the FCS file or gate name

Tag – choose to add tags (user defined or a range of auto-tags) to highlighted samples. Once samples have been tagged, use the filter option in the tag menu to display a select number of samples based on their tags. For more information, see page 59.

Keywords – additional information contained within FCS files can be displayed in the File Inspector. There is also the option to import additional keywords contained within a CSV file. The configuration of the keywords can be defined by selecting 'Edit' → 'Keywords'. For more information, see page 60.

Rename – rename individual files by inputting a new name or choose from the original FCS file name or sample name created during acquisition.

Group – FCS files can be placed into groups. This means that gates added to one file in a group are automatically added to the rest. Statistics are also applied to the entire group. To add files to a group, highlight them in the File Navigator and select 'Group' → 'Add' to new.

Plots – choosing 'Plots' → 'Open' will open all highlighted rows in the File Inspector as either dot plots or histograms. This is the fastest way to open multiple plots at once. Choosing 'Plots' → 'Close' will close all plots and histograms open in the workspace. This is the fastest way to clear the Workspace.

Rows – each file and gate name in the File Inspector represents one row. Use the Rows menu options to help select and delete single or multiple rows at a time.

- Show/Hide Gates – Hiding gates results in only the file name being displayed. Showing gates reveals all levels of the gating hierarchy.
- Select All/Inverse – Select all highlights every row. The same result can be achieved with the keyboard shortcut 'Ctrl/⌘A'. This is useful if you want to open every gate as a dot plot/histogram or if you would like to delete everything. Select Inverse highlights all but the files that are selected before clicking 'Inverse'.
- Delete – deletes all rows that are highlighted. It may be useful to use the different select features in conjunction with the delete option. FlowLogic will warn you before deleting the rows.
- Highlight Level – select a gate on one file. Clicking 'Highlight Level' results in the same gate being highlighted on every file in the File Inspector. This is very useful if you wish to open plots for a particular subpopulation for many samples or for creating overlays. After highlighting an entire level, choose to create an overlay or add the files to an existing overlay.
- Highlight Level within a Group will only select the rows from files in the same group as the initial sample.

Copy

- Copy Gates – copies the highlighted gates to paste onto other files. If you wish to paste gates, take note that the gate is pasted to the file and not to another gate. If statistics have been calculated for a sample before its gates are copied, the statistics will also be calculated for the new samples when the gates are pasted.

- Copy Statistics – if statistics have been calculated for one sample, the selection can be copied and pasted onto other samples, resulting in the same statistics being calculated for the selected samples. This is relevant when not working in Groups.

- Copy Plots – highlighted dot plots/histograms/overlays can be added to the computer's clipboard and subsequently pasted into other programs. In Windows, multiple files can be added to the clipboard at once. These are pasted as .svg images. In macOS, one file at a time can be added to the clipboard. This image is pasted as a .pdf.

- Copy Table – you can copy the data displayed in the columns of the File Inspector for individual files.

Paste – paste copied gates either to selected files or to all files at once. Choosing 'Insert Gates' will paste the selected gates into the selected level of the gating hierarchy.

Delete – delete all gates in the experiment, all statistics or individually selected rows. Rows refer to the different levels or gates in a hierarchy, so specific gates can be deleted, or the samples/files if the top level of the hierarchy is selected.

Template –

- **Replace Files** – this function works similarly to that described previously in this section. Find Replace swaps an equal number of highlighted files with new files. Advanced File Replace allows you to specify individual pairs of files to swap. All gates, statistics and reports remain but are updated to match the replacement files.
- **Gate** – gating templates can be saved and applied within the current experiment or loaded onto files in other experiments.

Compensation – compensation matrices can be copied, saved and applied to other files within an experiment or to files in other experiments.

Export –

- Plots – single or multiple plots can be exported in .jpg, .png, .svg, .eps, .pdf and .ps file formats. Plots can also be copied and exported by right clicking on the plot in the Workspace
- Statistics – this refers to any statistics calculated in the Advanced Functions drawer for the selected files
- Raw FCS data can also be exported as a .csv file. This is the data without considering compensation
- Real FCS data is the data with compensation applied
- Table – this is the complete table displayed in the File Inspector

PlateLogic – if a plate has been created in PlateLogic, additional data can be added to existing plates by highlighting the files and choosing **Data Array Insert** followed by the plate name. These samples will be added to the plate in the first available wells, in the direction defined in the plate side drawer. To choose the location of the inserted samples, highlight the empty wells in the plate before selecting the files and inserting them via the right click menu.

Overlay – overlays can be created by highlighting a gate and selecting Overlay. If an overlay already exists, individual or multiple gates of the same level can be added by highlighting them and selecting Overlay, Add To Overlay and the particular overlay.

TitrateLogic – Automatic titration analysis can be performed on specific populations from a selected number of files. Once gating has identified the population/parameter to be analyzed, display it along the x-axis, highlight the populations/rows in the File Inspector, right click and choose TitrateLogic. This will create histograms and histogram markers for the positive and negative peaks, a data array view showing all populations, and two titration overlays. A report is automatically generated and includes graphs showing the median

fluorescence intensities for the positive and negative signals, signal versus noise ratio and the stain index. For a more detailed description of the titration analysis, see the relevant section in this manual.

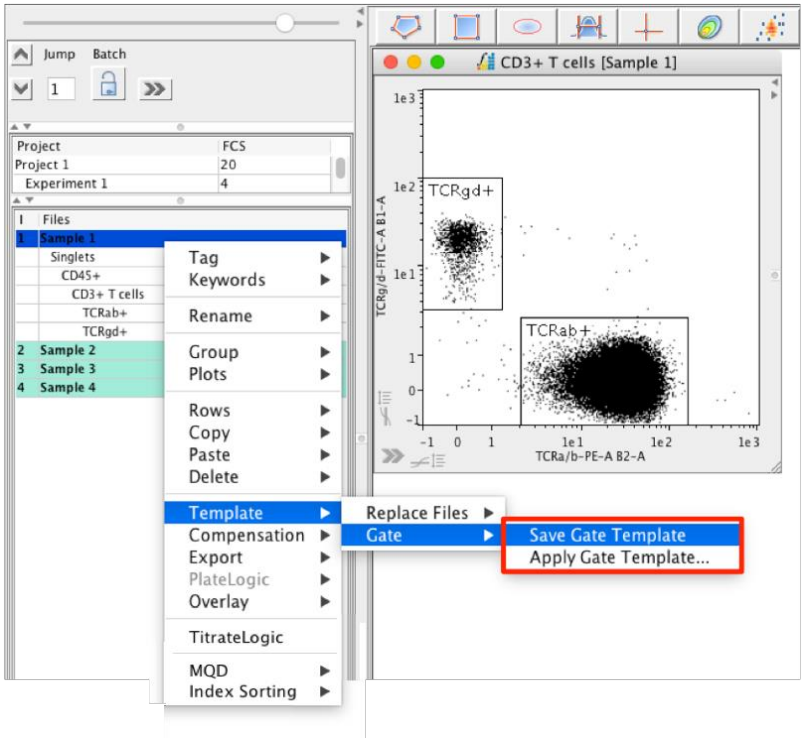
MQD – FlowLogic can split an MQD file that was grouped in Miltenyi Biotec MACSQuantify™ Software and display the individual files. These split files are exported/saved in a defined folder and can be re-loaded into MACSQuantify™ Software.

Index Sorting – This feature displays data compiled from an index sort, in PlateLogic, with the corresponding plate position reflecting the sort 'Well ID'. Gated events from the original sort file can display in a new plate, again in the true well position. This feature is optimized for BD FACSAria™ index sort files.

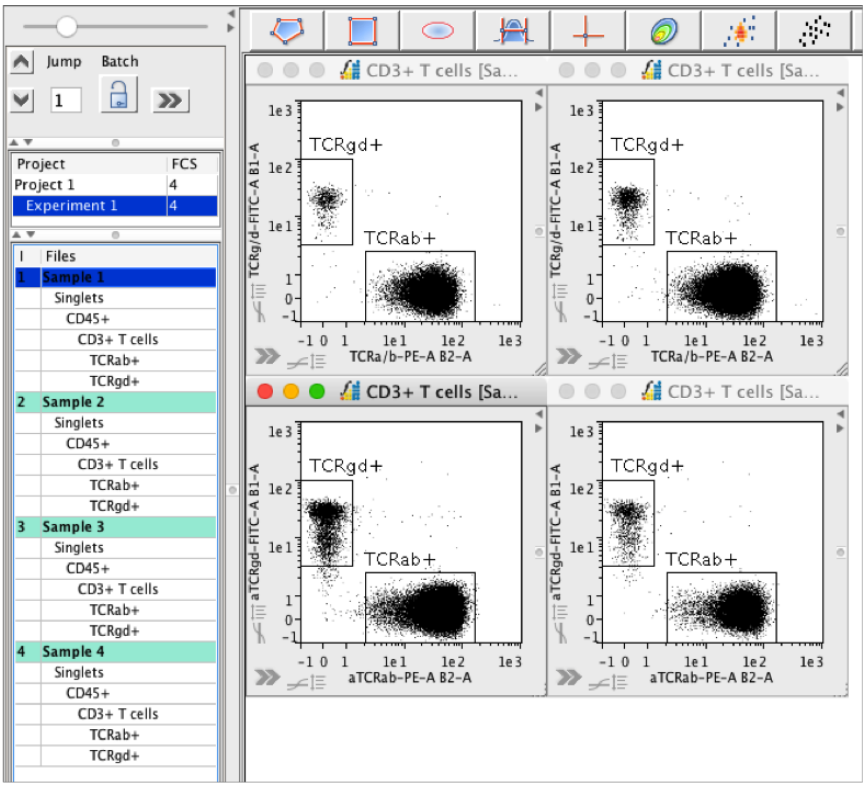
Templates

Gating analyses can be saved as a template to be applied to other files in the same experiment or to files in other experiments.

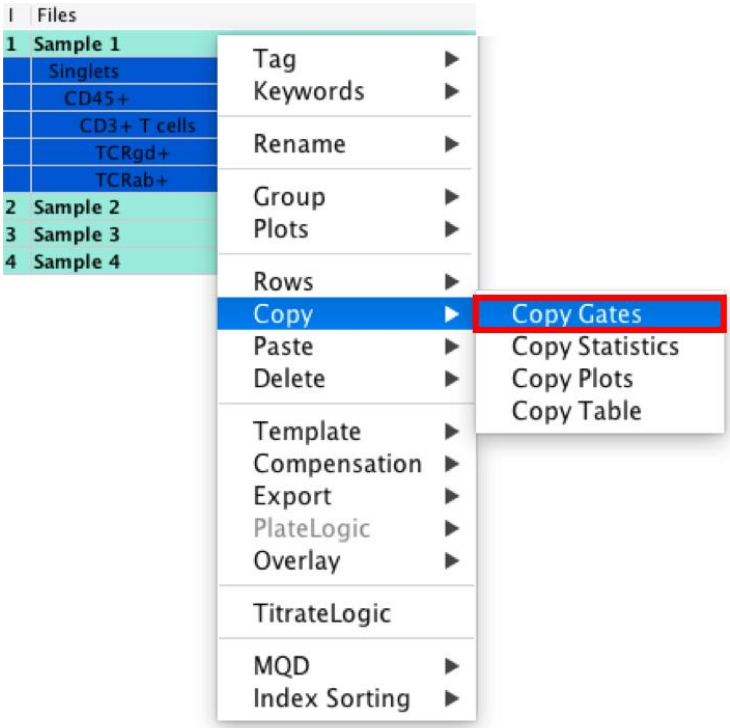
- After applying a series of gates to a file, right click on the file name and choose Template → Gate → Save Gate Template. FlowLogic will ask you to name the template before saving it as a .GATELOGICTEMPLATE file.



- To apply a saved template, highlight one or more samples, right click and select Template → Gate → Apply Gate Template… Select the template and click Open. FlowLogic will then apply the gates to all selected files. Statistics such as %Parent and %Total, located in the File Inspector, will be updated when the sample is opened in the Workspace.



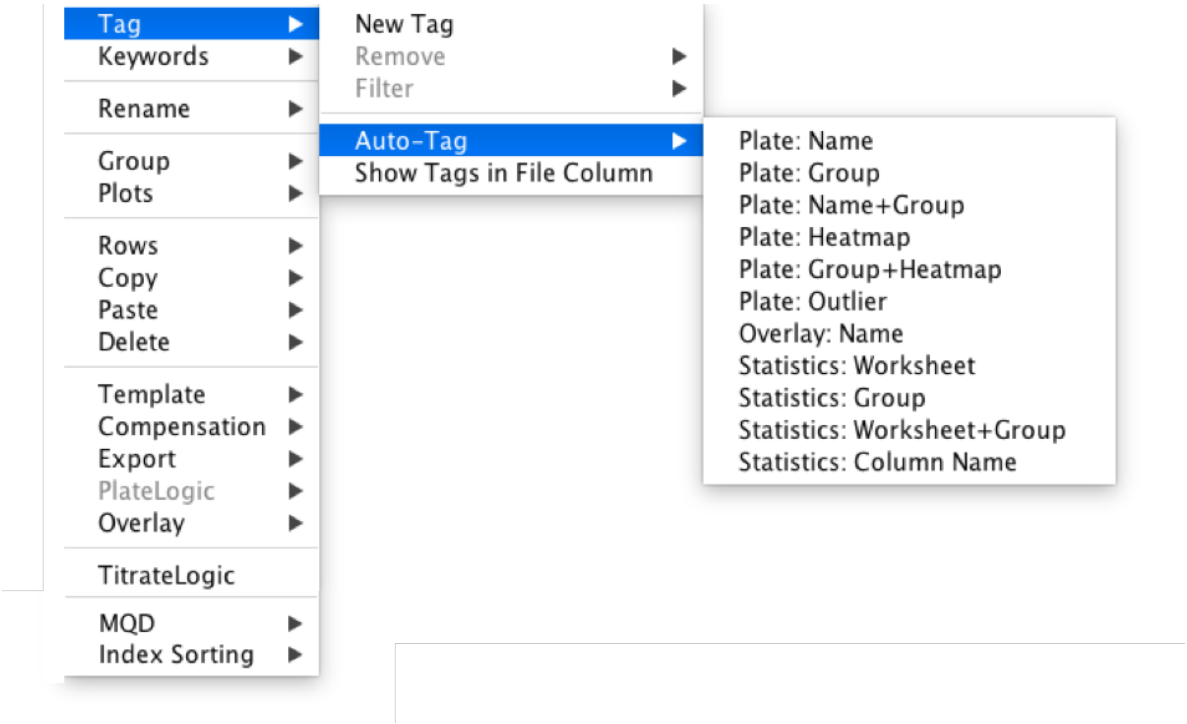
- An alternative to creating and applying templates within an experiment is to highlight the gates, right click and select Copy → Copy Gates.
- Then, highlight the samples that you wish to apply the gates to and select Paste → Paste Gates or To All. The keyboard shortcuts Ctrl/⌘C and Ctrl/⌘V can also be used to copy and paste gates.



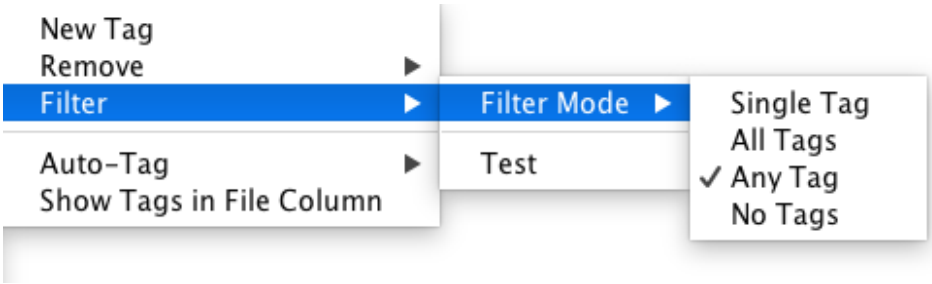
Tags

Tags can be applied to samples in order to help search through large datasets or to filter results. Tags can be user defined or chosen from a selection of automatic tags.

- To create a tag, right click on a sample in the File Inspector and select Tag.
- To create a user defined tag, select New Tag and type in the keyword.
- To create an automatic tag, select Auto-Tag and choose an option from the list.

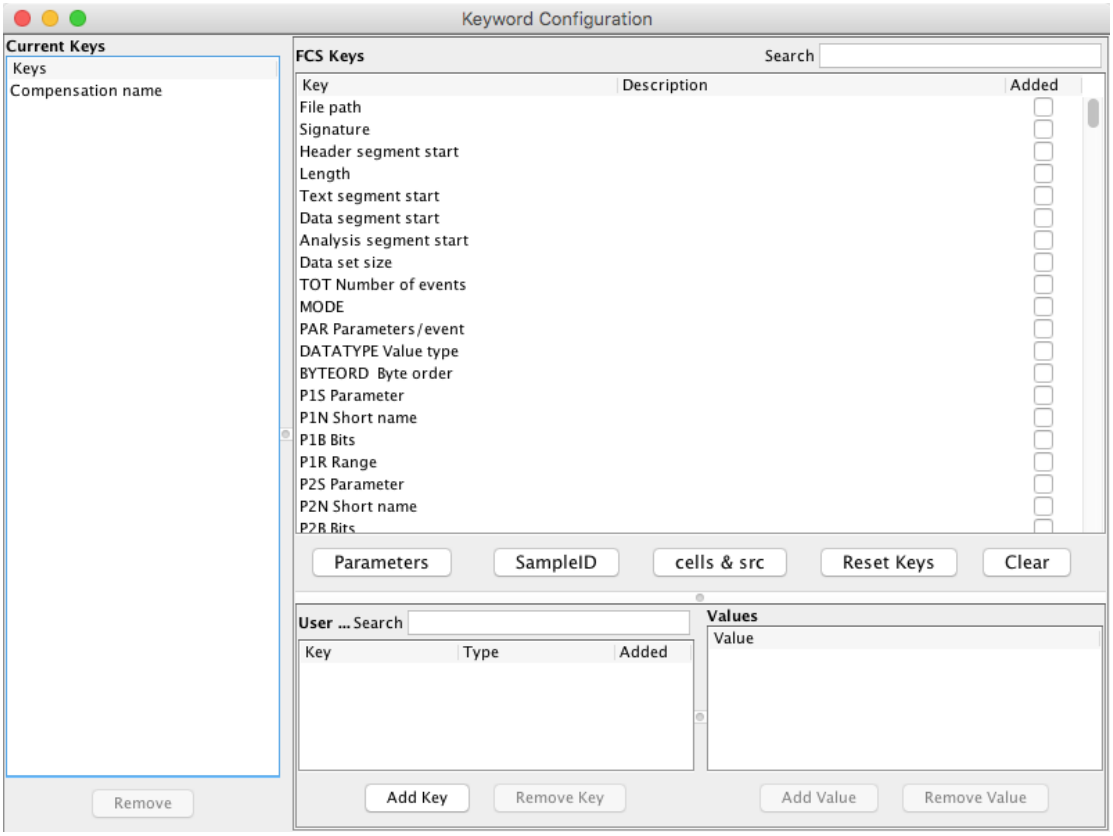


- Once tags have been assigned, right click in the File Inspector, choose Tag → Filter. Set the Filter mode and then select the tag (in the example below, the tag is 'Test tag'. Only those samples with the specific tag will be displayed in the File Inspector.
- Auto-tags can be used effectively to display plate data that fall in a specific range in a heatmap. To create this type of tag, choose Plate:Heatmap from the auto-tag list, then filter on the specific heatmap color.

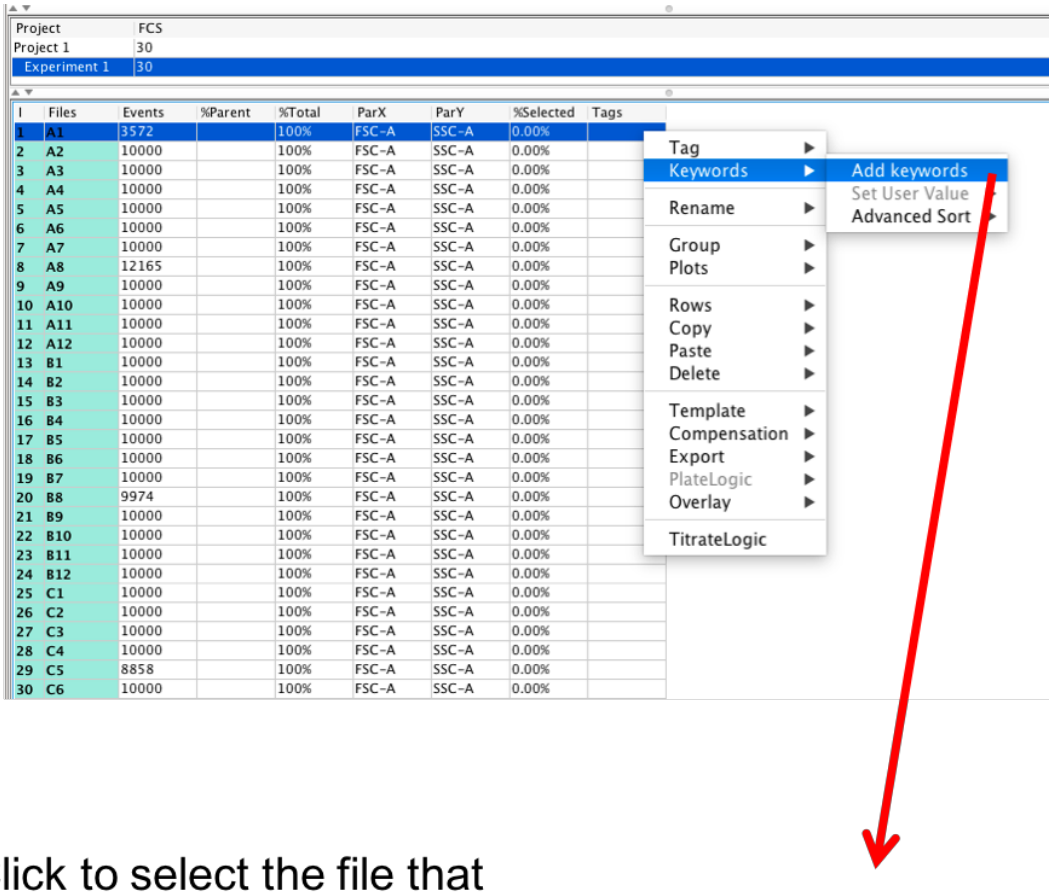


Keywords

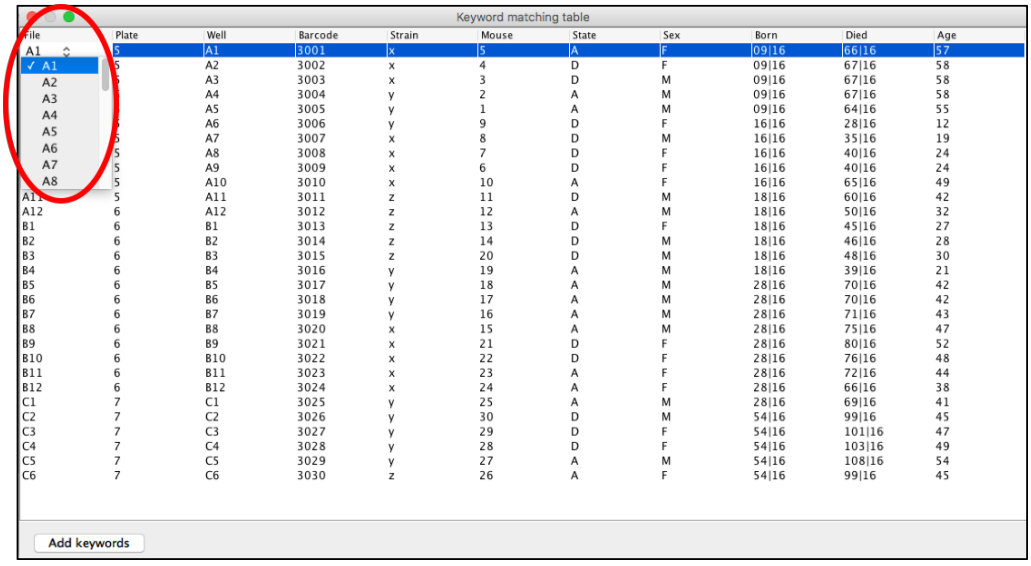
Displaying keywords with the Keyword Configuration window – keywords contained within the FCS file can be displayed in the File Inspector and subsequently used for reference or to sort samples. The Keyword Configuration window can be accessed through the Edit menu, under Keywords.



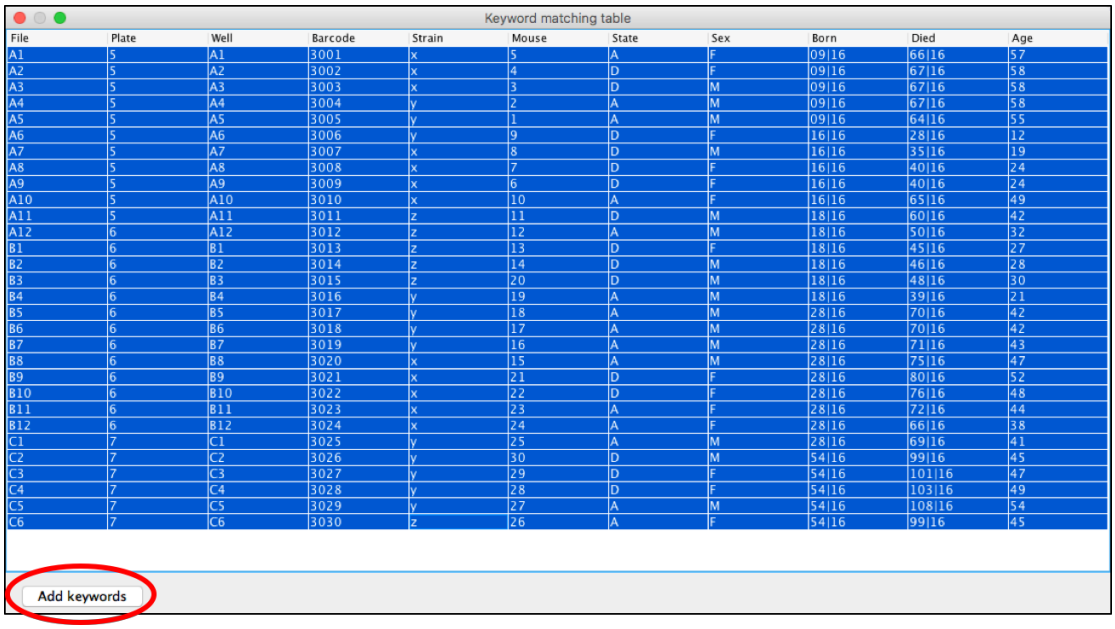
Adding keywords to files by csv – Multiple keywords contained in a csv file can be imported and assigned to one or a selection of files. By default, keywords will be matched to the files in the order that they appear in the File Inspector. Files can be sorted based on any keyword that is contained in the FCS/MQD/LMD file or from imported keywords. Samples can be sorted either ascending or descending by double clicking on the column title in the File Inspector.



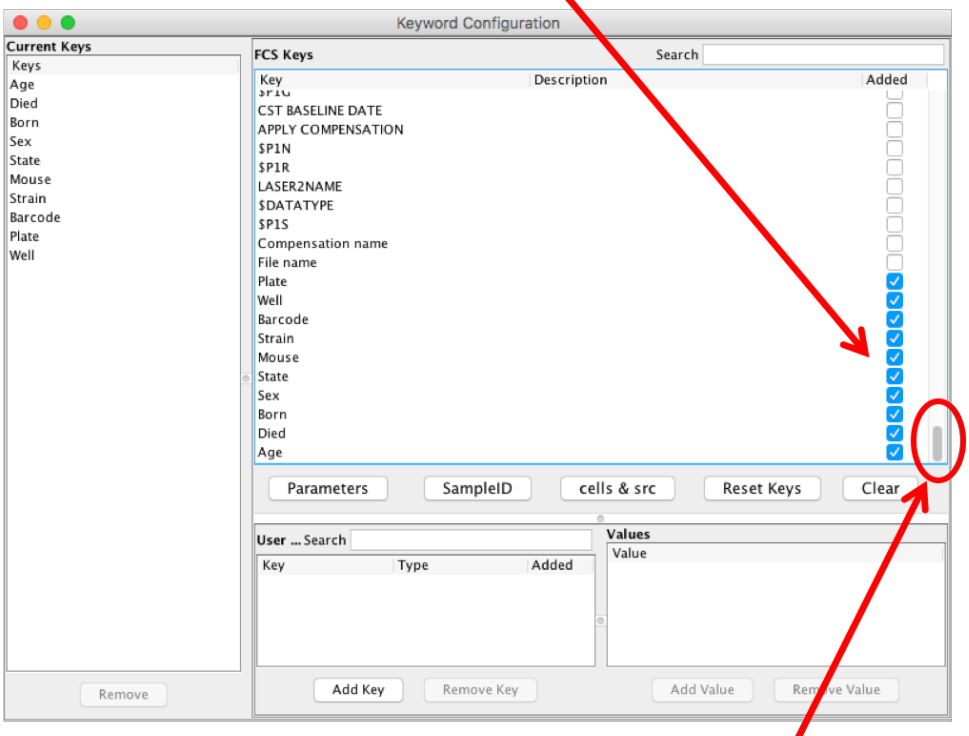
Click to select the file that matches the set of keywords



Highlight the selection of keywords matched to the files that you wish to import and click ‘Add keywords’. The Keyword Configuration window will then open.



Ticking the box will add the keywords and the data to the File Inspector



Imported keywords are listed at the end of the list of FCS keywords

The new keywords assigned to the selected files will now be displayed in the File Inspector:

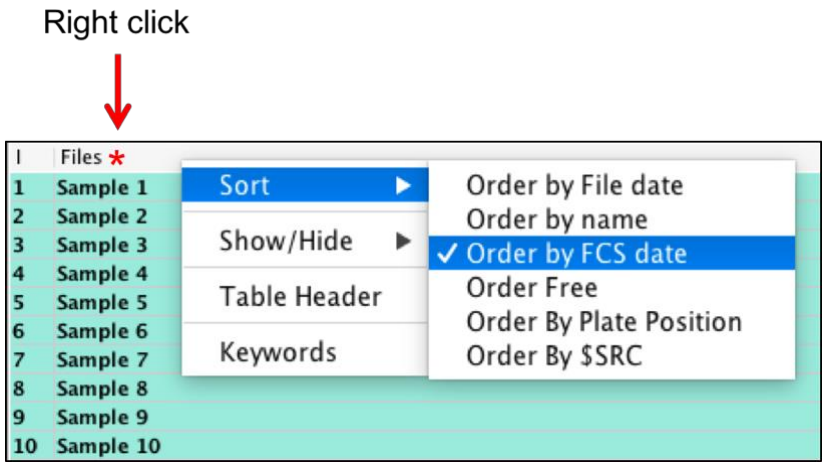
	Files	Events	%Parent	%Total	ParX	ParY	%Selected	Tags	Age	Died	Born	Sex	State	Mouse	Strain	Barcode	Plate	Well
1	A1	3572		100%	FSC-A	SSC-A	0.00%		57	66|16	09|16	F	A	5	x	3001	5	A1
2	A2	10000		100%	FSC-A	SSC-A	0.00%		58	67|16	09|16	F	D	4	x	3002	5	A2
3	A3	10000		100%	FSC-A	SSC-A	0.00%		58	67|16	09|16	M	D	3	x	3003	5	A3
4	A4	10000		100%	FSC-A	SSC-A	0.00%		58	67|16	09|16	M	A	2	y	3004	5	A4
5	A5	10000		100%	FSC-A	SSC-A	0.00%		55	64|16	09|16	M	A	1	y	3005	5	A5
6	A6	10000		100%	FSC-A	SSC-A	0.00%		12	28|16	16|16	F	D	9	y	3006	5	A6
7	A7	10000		100%	FSC-A	SSC-A	0.00%		19	35|16	16|16	M	D	8	x	3007	5	A7
8	A8	12165		100%	FSC-A	SSC-A	0.00%		24	40|16	16|16	F	D	7	x	3008	5	A8
9	A9	10000		100%	FSC-A	SSC-A	0.00%		24	40|16	16|16	F	D	6	x	3009	5	A9
10	A10	10000		100%	FSC-A	SSC-A	0.00%		49	65|16	16|16	F	A	10	x	3010	5	A10
11	A11	10000		100%	FSC-A	SSC-A	0.00%		42	60|16	18|16	M	D	11	z	3011	5	A11
12	A12	10000		100%	FSC-A	SSC-A	0.00%		32	50|16	18|16	M	A	12	z	3012	6	A12
13	B1	10000		100%	FSC-A	SSC-A	0.00%		27	45|16	18|16	F	D	13	z	3013	6	B1
14	B2	10000		100%	FSC-A	SSC-A	0.00%		28	46|16	18|16	M	D	14	z	3014	6	B2
15	B3	10000		100%	FSC-A	SSC-A	0.00%		30	48|16	18|16	M	D	20	z	3015	6	B3
16	B4	10000		100%	FSC-A	SSC-A	0.00%		21	39|16	18|16	M	A	19	y	3016	6	B4
17	B5	10000		100%	FSC-A	SSC-A	0.00%		42	70|16	28|16	M	A	18	y	3017	6	B5
18	B6	10000		100%	FSC-A	SSC-A	0.00%		42	70|16	28|16	M	A	17	y	3018	6	B6
19	B7	10000		100%	FSC-A	SSC-A	0.00%		43	71|16	28|16	M	A	16	y	3019	6	B7
20	B8	9974		100%	FSC-A	SSC-A	0.00%		47	75|16	28|16	M	A	15	x	3020	6	B8
21	B9	10000		100%	FSC-A	SSC-A	0.00%		52	80|16	28|16	F	D	21	x	3021	6	B9
22	B10	10000		100%	FSC-A	SSC-A	0.00%		48	76|16	28|16	F	D	22	x	3022	6	B10
23	B11	10000		100%	FSC-A	SSC-A	0.00%		44	72|16	28|16	F	A	23	x	3023	6	B11
24	B12	10000		100%	FSC-A	SSC-A	0.00%		38	66|16	28|16	F	A	24	x	3024	6	B12
25	C1	10000		100%	FSC-A	SSC-A	0.00%		41	69|16	28|16	M	A	25	y	3025	7	C1
26	C2	10000		100%	FSC-A	SSC-A	0.00%		45	99|16	54|16	M	D	30	y	3026	7	C2
27	C3	10000		100%	FSC-A	SSC-A	0.00%		47	101|16	54|16	F	D	29	y	3027	7	C3
28	C4	10000		100%	FSC-A	SSC-A	0.00%		49	103|16	54|16	F	D	28	y	3028	7	C4
29	C5	8858		100%	FSC-A	SSC-A	0.00%		54	108|16	54|16	M	A	27	y	3029	7	C5
30	C6	10000		100%	FSC-A	SSC-A	0.00%		45	99|16	54|16	F	A	26	z	3030	7	C6

To import keywords, create the CSV file in the following format, with the first row being the keyword heading and the subsequent rows being the keyword data.

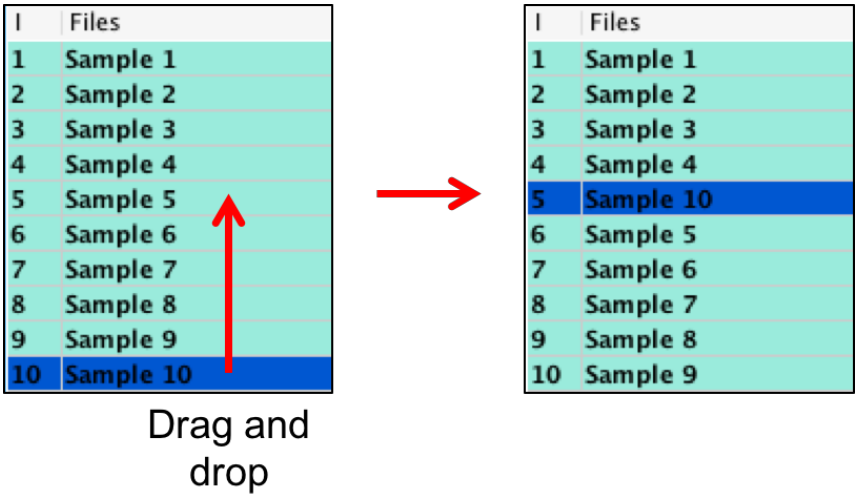
	A	B	C	D	E	F	G	H	I	J	K
1	Plate	Well	Barcode	Strain	Mouse	State	Sex	Born	Died	Age	
2	5	A1	3001	x	5	A	F	09|16	66|16	57	
3	5	A2	3002	x	4	D	F	09|16	67|16	58	
4	5	A3	3003	x	3	D	M	09|16	67|16	58	
5	5	A4	3004	y	2	A	M	09|16	67|16	58	
6	5	A5	3005	y	1	A	M	09|16	64|16	55	
7	5	A6	3006	y	9	D	F	16|16	28|16	12	
8	5	A7	3007	x	8	D	M	16|16	35|16	19	
9	5	A8	3008	x	7	D	F	16|16	40|16	24	
10	5	A9	3009	x	6	D	F	16|16	40|16	24	
11	5	A10	3010	x	10	A	F	16|16	65|16	49	
12	5	A11	3011	z	11	D	M	18|16	60|16	42	
13	6	A12	3012	z	12	A	M	18|16	50|16	32	
14	6	B1	3013	z	13	D	F	18|16	45|16	27	
15	6	B2	3014	z	14	D	M	18|16	46|16	28	
16	6	B3	3015	z	20	D	M	18|16	48|16	30	
17	6	B4	3016	y	19	A	M	18|16	39|16	21	
18	6	B5	3017	y	18	A	M	28|16	70|16	42	
19	6	B6	3018	y	17	A	M	28|16	70|16	42	
20	6	B7	3019	y	16	A	M	28|16	71|16	43	
21	6	B8	3020	x	15	A	M	28|16	75|16	47	
22	6	B9	3021	x	21	D	F	28|16	80|16	52	
23	6	B10	3022	x	22	D	F	28|16	76|16	48	
24	6	B11	3023	x	23	A	F	28|16	72|16	44	
25	6	B12	3024	x	24	A	F	28|16	66|16	38	
26	7	C1	3025	y	25	A	M	28|16	69|16	41	
27	7	C2	3026	y	30	D	M	54|16	99|16	45	
28	7	C3	3027	y	29	D	F	54|16	101|16	47	
29	7	C4	3028	y	28	D	F	54|16	103|16	49	
30	7	C5	3029	y	27	A	M	54|16	108|16	54	
31	7	C6	3030	z	26	A	F	54|16	99|16	45	
32											

Sorting samples in the File Inspector
Samples in the File Inspector can be sorted (re-ordered) in several different ways.

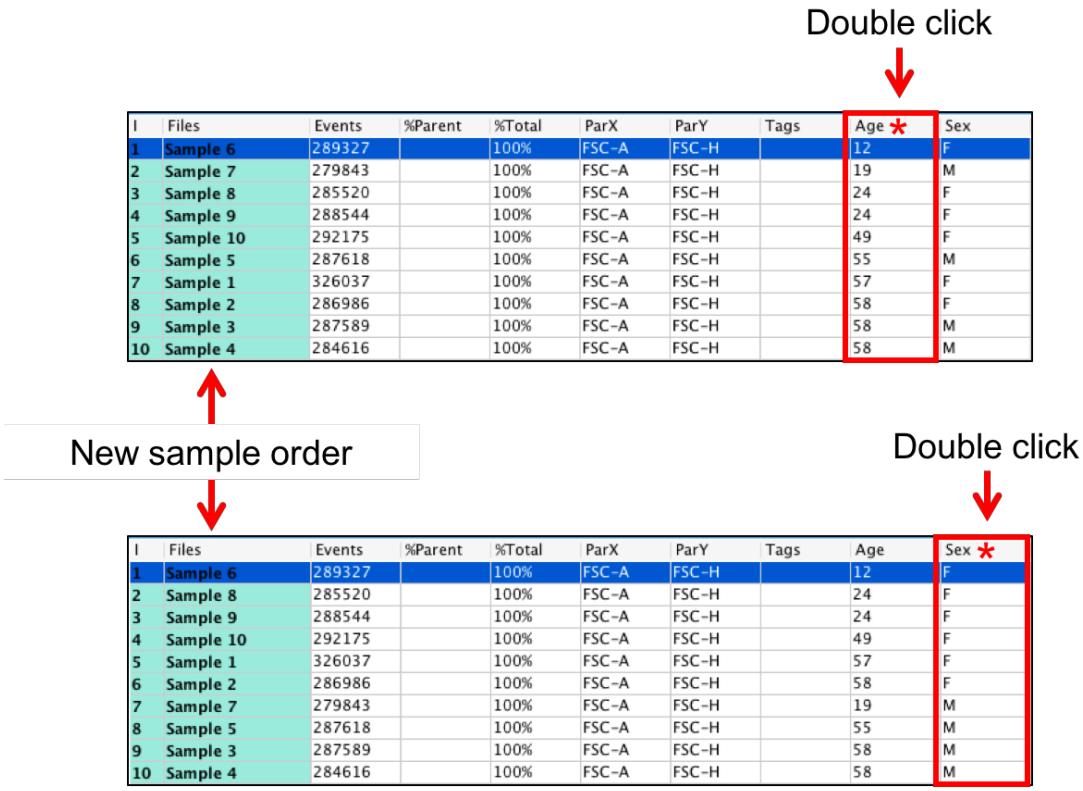
1. Right click on column header and choose ‘Sort’ followed by a sort style:



2. Click on one or a selection of samples and drag them to a new position. This will automatically set the Sort style to ‘Free’:



3. Double click on the column headers to sort in numerical/alphabetical order:



I	Files	Events	%Parent	%Total	ParX	ParY	Tags	Age *	Sex
1	Sample 6	289327		100%	FSC-A	FSC-H		12	F
2	Sample 7	279843		100%	FSC-A	FSC-H		19	M
3	Sample 8	285520		100%	FSC-A	FSC-H		24	F
4	Sample 9	288544		100%	FSC-A	FSC-H		24	F
5	Sample 10	292175		100%	FSC-A	FSC-H		49	F
6	Sample 5	287618		100%	FSC-A	FSC-H		55	M
7	Sample 1	326037		100%	FSC-A	FSC-H		57	F
8	Sample 2	286986		100%	FSC-A	FSC-H		58	F
9	Sample 3	287589		100%	FSC-A	FSC-H		58	M
10	Sample 4	284616		100%	FSC-A	FSC-H		58	M

I	Files	Events	%Parent	%Total	ParX	ParY	Tags	Age	Sex *
1	Sample 6	289327		100%	FSC-A	FSC-H		12	F
2	Sample 8	285520		100%	FSC-A	FSC-H		24	F
3	Sample 9	288544		100%	FSC-A	FSC-H		24	F
4	Sample 10	292175		100%	FSC-A	FSC-H		49	F
5	Sample 1	326037		100%	FSC-A	FSC-H		57	F
6	Sample 2	286986		100%	FSC-A	FSC-H		58	F
7	Sample 7	279843		100%	FSC-A	FSC-H		19	M
8	Sample 5	287618		100%	FSC-A	FSC-H		55	M
9	Sample 3	287589		100%	FSC-A	FSC-H		58	M
10	Sample 4	284616		100%	FSC-A	FSC-H		58	M

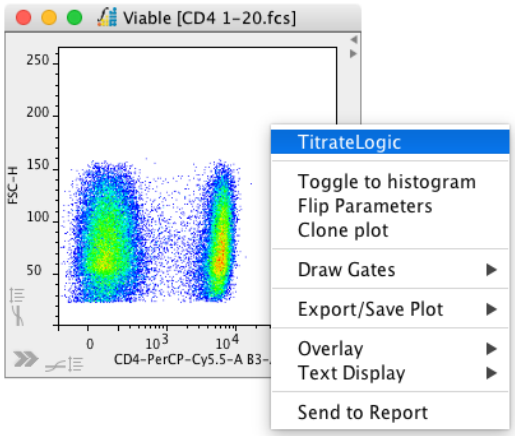
Samples in the File Inspector can be sorted based on the values or numbers in the different File Inspector columns. Double clicking the column title will sort the samples in ascending order (numerically or alphabetically). Double clicking again will sort them in descending order.

TitrateLogic

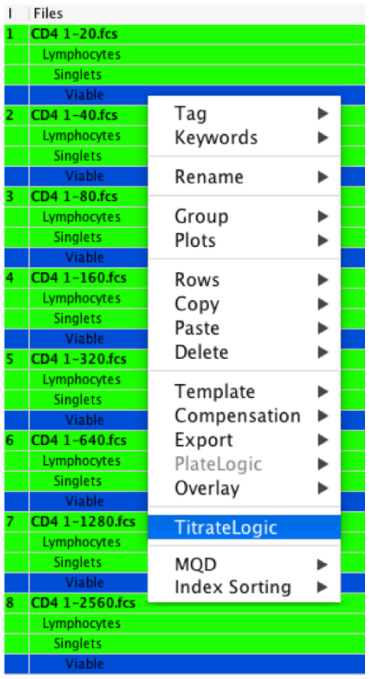
FlowLogic contains a quantitative titration analysis tool that automatically detects the positive and negative signals for a defined parameter, creating titration overlays and a report displaying all populations from the analysed samples along with graphs displaying median fluorescence intensities for the positive (signal) and negative (noise) populations, the signal to noise ratio and the stain index.

Titration analysis is generally faster and easier when all files are grouped but grouping is not necessary. The first step is to apply the relevant gates to identify the population and marker to be analysed. Display this parameter on the x-axis of the dot plot. From this point there are two ways to perform the analysis:

1. Right click on the dot plot and from the options choose 'TitrateLogic'.

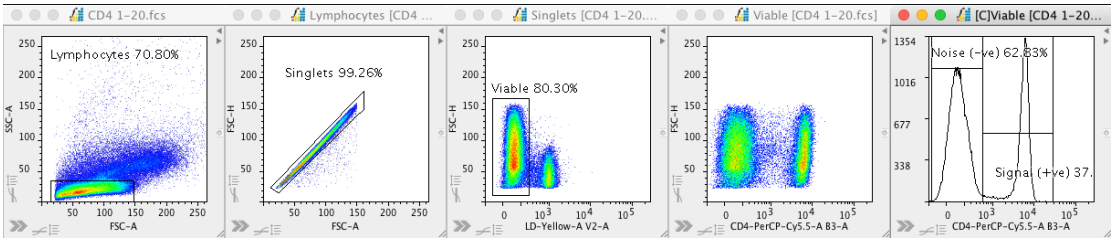


2. Highlight the populations in the File Inspector, right click → TitrateLogic'.



The first option will perform the analysis for all files in the Experiment folder that contain the matching gates. The second option will only include the selected populations in the analysis.

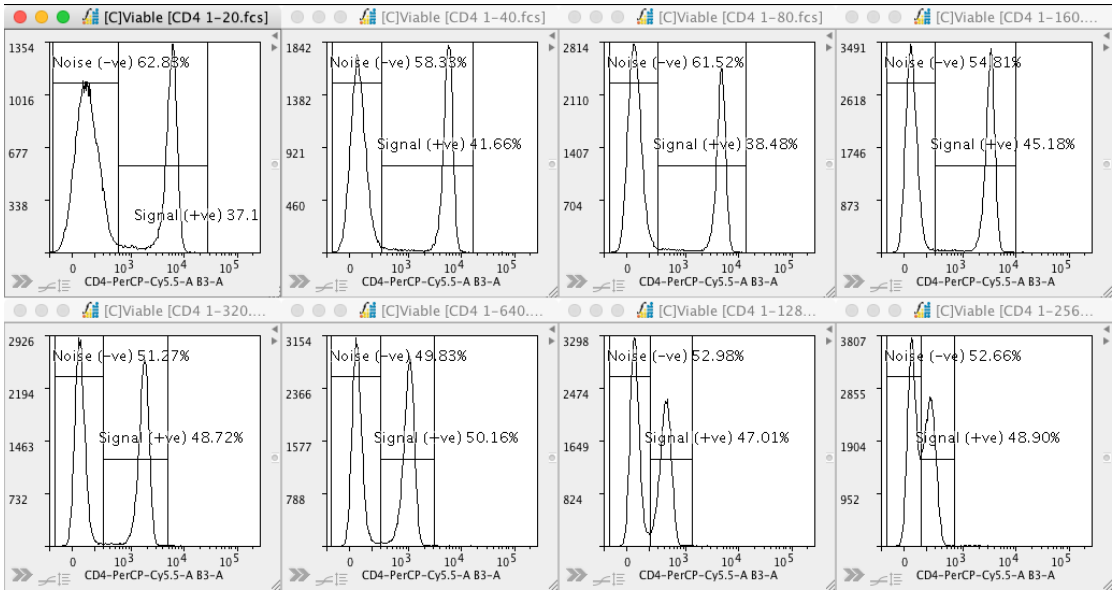
FlowLogic will then clone the population, display it as a histogram and create signal (+ve) and noise (-ve) histogram markers by automatically detecting the positive and negative peaks.



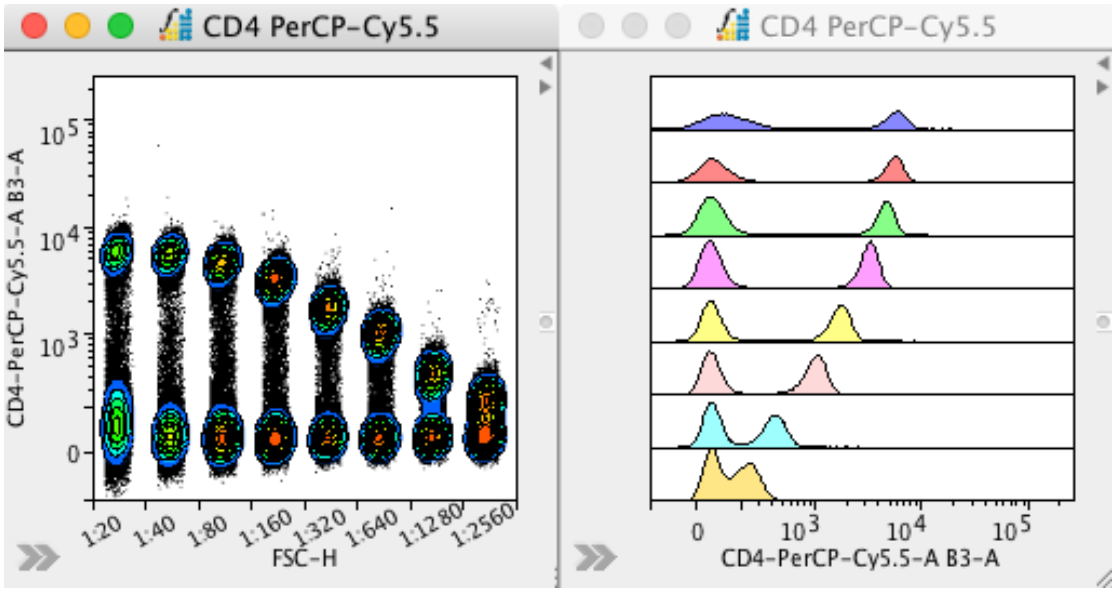
The histogram markers are not contained in the group, allowing for the adjustment on individual plots if required.

I	Files
1	CD4 1-20.fcs
	Lymphocytes
	Singlets
	Viable
C	Viable
	Signal (+ve)
	Noise (-ve)
2	CD4 1-40.fcs
	Lymphocytes
	Singlets
	Viable
C	Viable
	Signal (+ve)
	Noise (-ve)

The following image shows the automatic creation of histogram markers for the signal and noise peaks.



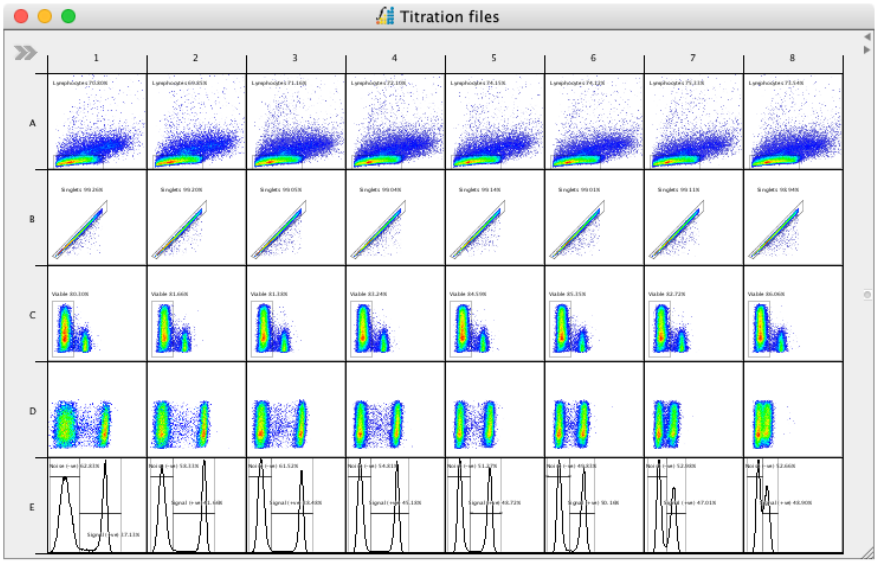
Two titration overlays are also generated and displayed in the workspace. The parameters used for these plots are taken from the plot selected to perform the analysis. The displayed parameters can be changed by clicking on the axis label and choosing a new parameter, as with any other plot.



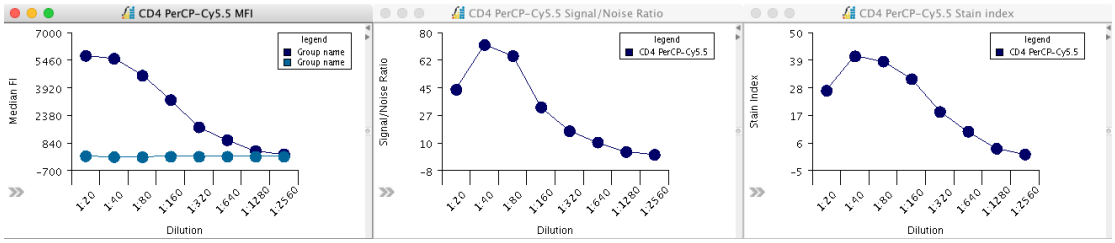
Samples can be removed and added to both plots (right click menu) and switched on and off from the stacked histogram overlay (plot side drawer under the ‘Overlay Colors’ tab).

The titration dot plot overlay can be viewed using any of the plot display options from the toolbar above the workspace.

All plots involved in the analysis are also displayed in a plate under the Data Array tab in the Advanced Functions drawer. This plate can be saved/exported by right clicking and choosing Export → To File.



In the Graph section, the data derived from the histogram markers are used to calculate and display median fluorescence intensity (signal versus noise), the robust standard deviation for the noise (-ve) marker, signal-to-noise ratio, the difference in median fluorescence intensity (+ve minus –ve) and the stain index. In theory, the highest value on the stain index graph equals the optimum saturating titration. If there are too many antibodies, sensitivity is reduced by increasing noise (SI is decreased). If there are too few antibodies, sensitivity is reduced by decreasing the positive signal (SI is decreased).



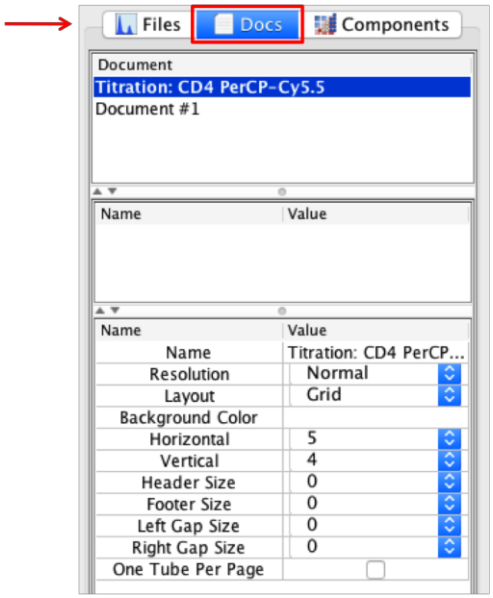
The stain index[1] is calculated using the following formula:

$$\text{Stain index} = \frac{\left[MFI_{+ve} - MFI_{-ve}\right]}{2 \times RSD_{-ve}}$$

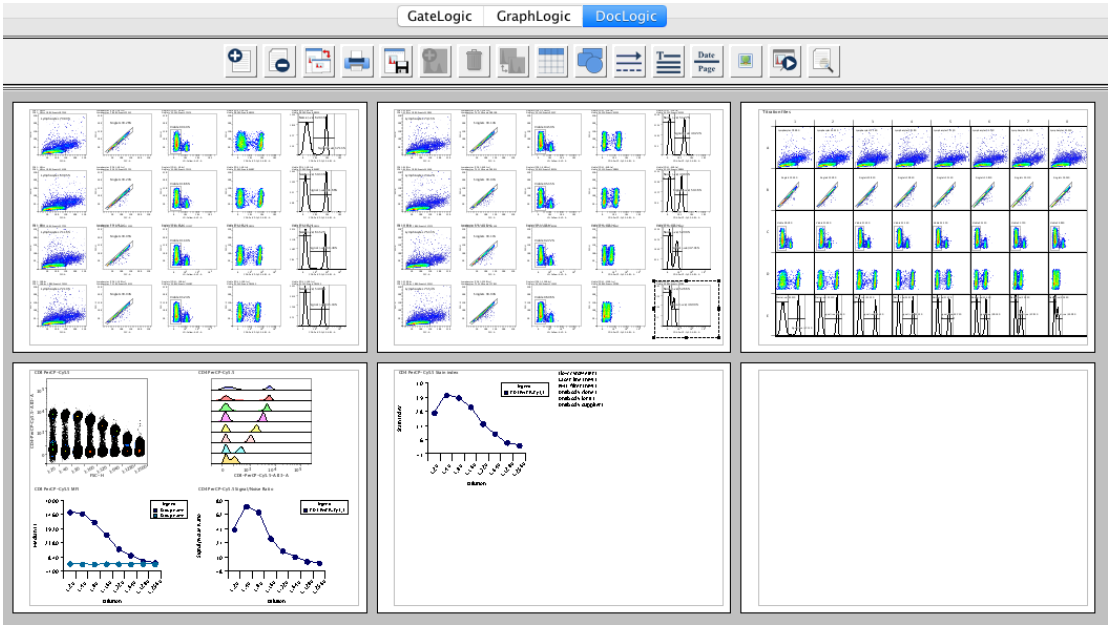
MFI = median fluorescence intensity
RSD = robust standard deviation
+ve = positive signal defined by the histogram marker
-ve = negative signal defined by the histogram marker

[1] Telford WG, Hawley T, Subach F, Verkhusha V, Hawley RG. Flow cytometry of fluorescent proteins. *Methods*. 2012;57:318-30. PMID: 22293036.

A report is also automatically generated from the elements in the Analysis and Graph sections. These include all dot plots and histograms, the data array plate and the graphs displaying median fluorescence intensity, signal-to-noise ration and the stain index. To view the titration report, click on the List of Documents tab and choose the titration document, which is titled per the parameter being analysed. This report can be renamed in the document setting section window below. It can also be saved as a PDF using the option in the toolbar.



The full report:



MQD Ungrouping

Grouped MQD files can be split into their individual files by right clicking on the file in the File Inspector and choosing 'MQD' → 'Ungroup MQD Files'. When choosing this option, FlowLogic will ask to select a folder on the computer where the extracted files can be saved. The files will also be automatically loaded into the File Inspector, underneath the original grouped MQD file.

IndexLogic

IndexLogic enables the analysis of files resulting from an index sort, whereby samples are displayed in a plate corresponding to the cell well IDs. To export the individual event files and load them into the corresponding position in a plate, right click on the file in the File Inspector and choose 'Index Sorting' → 'Export Files'. Then choose a location where the individual event files will be saved. These individual files will then be loaded into the File Inspector.

Once the files have been exported, events in the original index sort file can be gated and matched to the plate in PlateLogic. To do this, gate on the desired events in the original file, right click on the gate name in the File Inspector and select 'Index Sorting' → 'Match Cells in a Plate', followed by the plate name. A new plate will be created showing just those samples defined by the gate. Additional gates can be created on the original file and matched to either the plate containing all events or subsequent plates containing gated events.