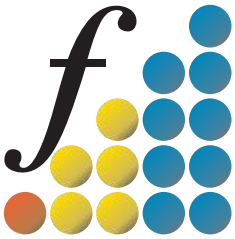


Flowlogic™

Flow Cytometry Analysis Software

Developed by scientists, for scientists

User Manual

Version 8.1

inivai TECHNOLOGIES

TABLE OF CONTENTS

FlowLogic 8 - Introduction

Layout Overview – GateLogic, GraphLogic, DocLogic and BeadLogic

FlowLogic is divided into four sections: GateLogic, GraphLogic, DocLogic and BeadLogic. These sections are accessed by clicking on the tabs located at the top of the program window.

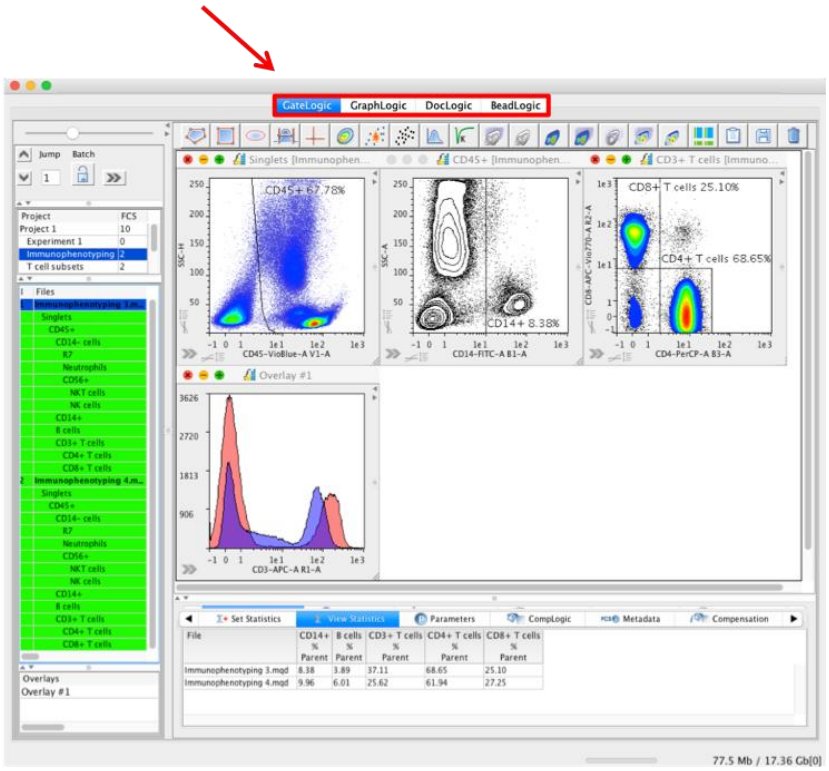
GateLogic is where FCS files are imported, gates are drawn on plots, overlays are created, data array analysis is performed and gate statistics are set and viewed.

GraphLogic is where statistical analysis is performed and graphs are created for both FCS-derived and independent data.

DocLogic is where reports and presentations are created by adding elements created in GateLogic and GraphLogic. Plots, plates and statistic tables, amongst other items, can be added to reports to be saved as PDF or .pptx files. Documents can also be annotated with a range of shapes, images and text.

BeadLogic is a multiplex data analysis tool. Initial sample gating and definition is performed in GateLogic (using PlateLogic, a plate layout and visualization tool) before being sent to BeadLogic for regression analysis, unknown sample interpolation and reporting.

GateLogic, GraphLogic and DocLogic are linked, so when a gate is adjusted, all statistics, graphs and reports are updated automatically.



FlowLogic Technical Specifications

- Cross-platform software that runs in Windows, Mac and Linux operating systems
- Operates in both 32- and 64-bit environments
    - Maximum memory allocation in 32-bit environments is 1026Mb
- Analysis files saved under one operating system can be opened using another
- Requires Java SE Development Kit (JDK) 8 and above
    - The JDK is a free download from the Oracle website
    - M1 Apple Silicon Macs require an ARM architecture specific JDK. For download instructions and further information on this topic, please contact support@inivai.com

FlowLogic Intended Operating Environment

FlowLogic is designed to operate in a local computer. This includes reading FCS/MQD files, along with saving and reading analysis files (.glf and .gatelogicexperiment files).

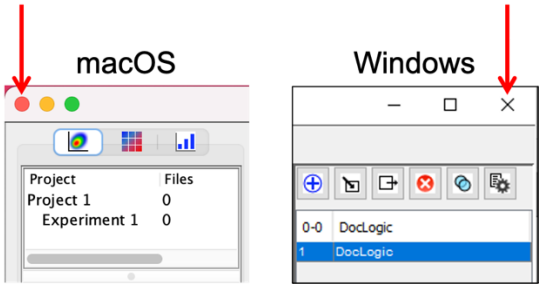
For people connected with a network drive, it is recommended to move the FCS/MQD files to the local computer where FlowLogic is installed and save the analysis files to that computer. Saved files can be synchronized to a network drive after saving.

Multi-screen mode

The GraphLogic, GateLogic and DocLogic windows to be separated and viewed simultaneously on different displays. In the GraphLogic and DocLogic toolbars, there is a ‘pop out’ icon. Clicking on this will open that particular panel in a separate window. Even when the different sections are viewed on individual displays, all elements will continue to update in real time with any adjustments to the analysis.

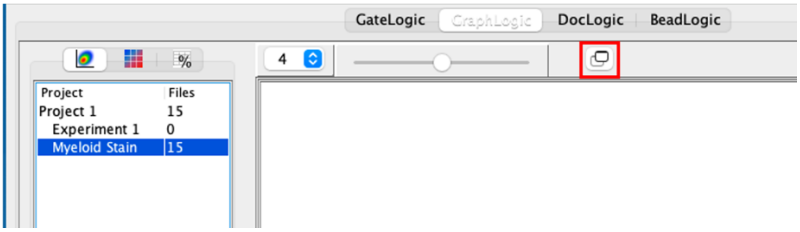
The pop out icon:

To return either GraphLogic or DocLogic to FlowLogic, simply close that individual panel:

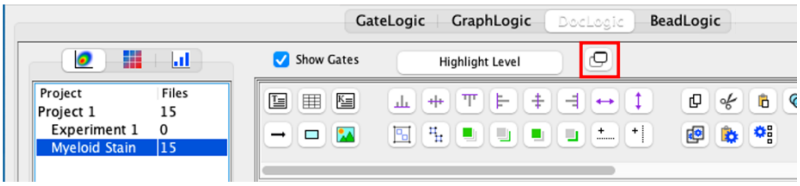


The pop out icon location in:

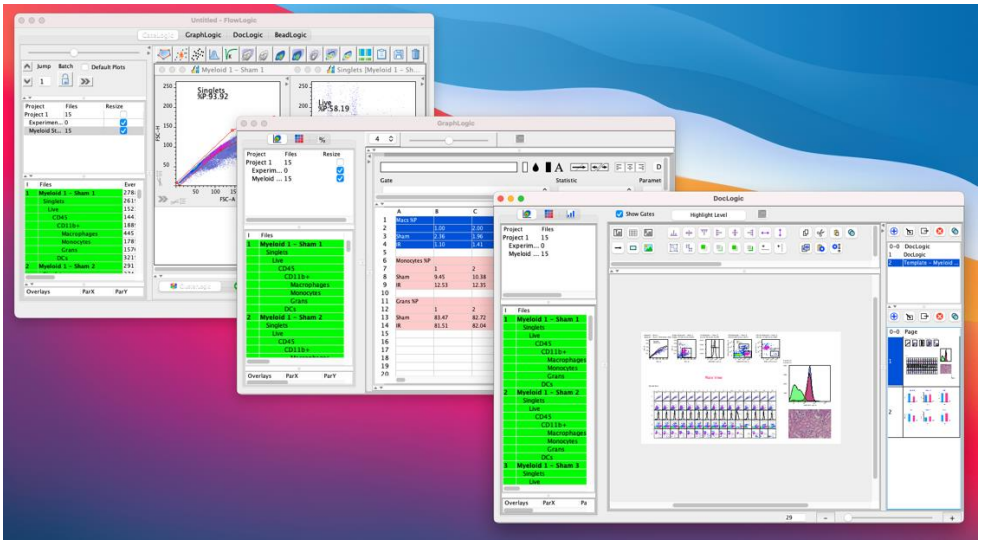
GraphLogic



DocLogic

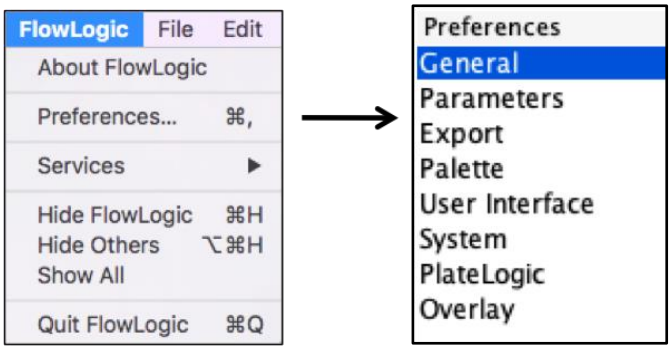


The result of separating these three components:



Preferences

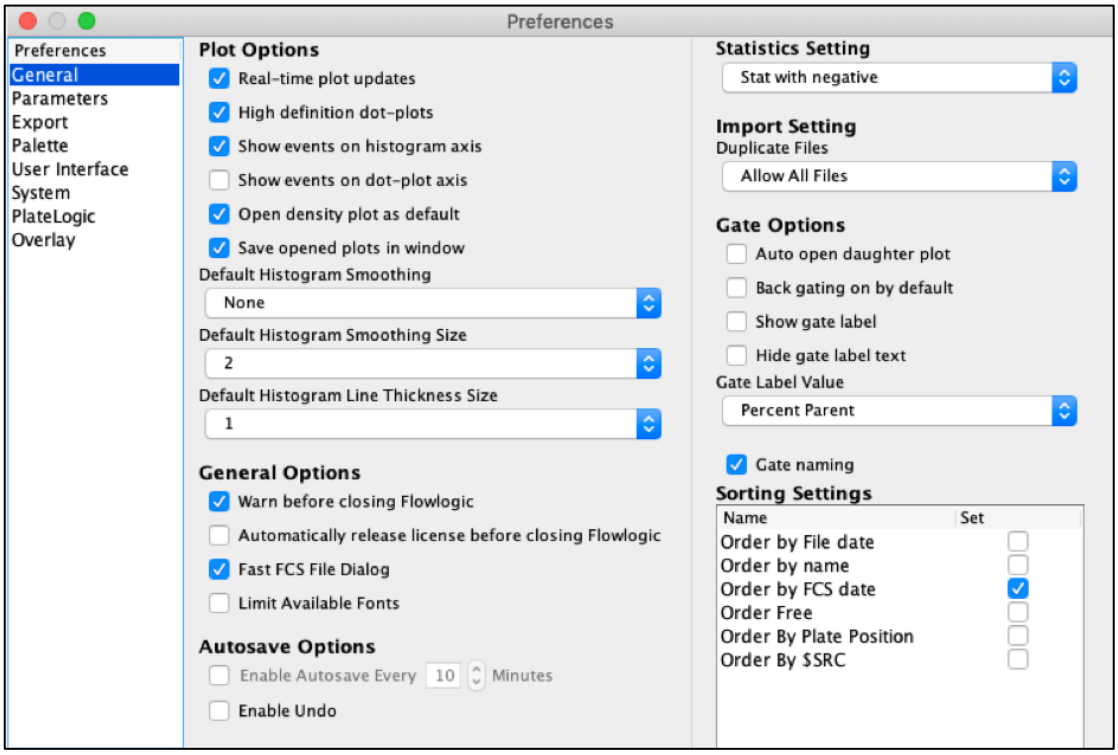
Before beginning an analysis, customize the program by setting the program preferences. From the menu bar, select ‘FlowLogic’ (macOS) or ‘Edit’ (Windows) and ‘Preferences’. Here you can change the way the plots are displayed (annotation, standard or high definition, real-time updates, etc.), choose the specific parameter labels you would like to view, set the export settings, allocate system memory, define colors used in overlay plots and more.



Select various preference menu options from the left-hand side of the Preferences window and optimize the settings using the options in the panel on the right.

Explanations of the different Preference options are as follow:

General



- **Plot Options**
  - Real-time plot updates – when selected, all plots (dot plots, histograms and overlays) are updated in real-time as gates are moved. This includes events at all levels of the population hierarchy, along with gate statistics. The coloring of events (selected in the plot side drawer in the Gate List tab) is also updated on all levels of the hierarchy as gates are moved. Deselect this option to increase program speed. When deselected, all plot and statistic updates will occur when the gate is released.

  - High definition dot plots – when selected, dot plots are displayed in the Workspace at a higher definition. Plots will also be exported and displayed in reports with whichever setting is chosen.

  - Show events on histogram axis – when selected, events existing beyond the axes will be displayed along the axes. This may affect the way the histogram is displayed, especially when viewing the histogram at 100%.

  - Show events on dot plot axis – when selected, events existing beyond the defined axes will be displayed along the axes. This will affect the plot display, especially when viewing populations based on density. Deselecting this option will cause the density of events to be calculated based on the events within the plot area only. This option does not impact statistics in any way.

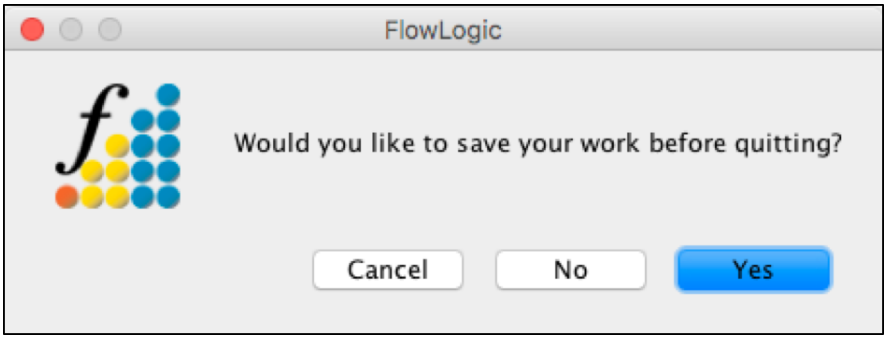
  - Open density plot as default – when selected as the default settings, all plots opened from the File Inspector or from double-clicking within a gate in the Workspace will open as a density plot. This default setting will remain for a sample once it has been opened so changing the default setting in the Preferences will apply to all newly opened plots.

  - Save opened plots in window – when selected, plots displayed in the Workspace for a given folder will be remembered so that the same plots will be displayed when switching from one folder to another and back again. This is the case for all Experiment folders in all Projects (displayed in the File Navigator). Plots displayed in the Workspace when quitting FlowLogic will be displayed upon opening a saved .glf or .gatelogicexperiment file.

  - Default histogram smoothing – histograms can be smoothed by default. This can be particularly useful in cases where there are few events. Choose either None or one of the other smoothing options to apply the relevant algorithm to the histogram: None, Traditional, Local Max, Local Min, Local Mean, Box Max, Box Min, Box Mean.

  - Default smoothing size – the amount of smoothing can also be set, ranging from 2 (least amount of smoothing) to 16 (most amount of smoothing).

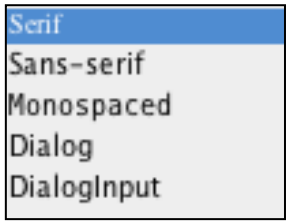
- **General Options**
  - Warn before closing FlowLogic – when selected, FlowLogic will display the following message asking whether to cancel the command, to quit or to save the current analysis before quitting:



  - Automatically release license before closing FlowLogic – when selected, FlowLogic will release the license automatically upon quitting. The license is then free to be used to run FlowLogic on a different computer. This feature is useful if the user commonly runs FlowLogic on two or more computers.

  - Fast FCS File Dialog – with this feature enabled, when searching for files to import, FlowLogic will inspect the file header to quickly identify FCS files. This will dramatically increase the speed in which FCS files can be searched and displayed.

  - Limit Available Fonts – limiting available fonts will reduce the available fonts contained in each computer to the following:



    This will ensure that saved analyses and reports, when opening on other computers, will be displayed unchanged. Limiting the available fonts avoids the risk that a font used in one computer is absent in another.

- **Autosave Options**
  - Enable Autosave Every __ Minutes – with this option, FlowLogic will automatically prompt the user to save the analysis after a defined number of minutes. Once the analysis has been saved, FlowLogic will re-save the analysis, using the same file name and type, at the defined time interval. The time interval can be set by typing the number of minutes in the field or by using the arrows to increase or decrease the displayed time in one-minute increments.

  - Enable Undo – with this setting selected, a list of the most recent actions is recorded and can be reversed by selecting Undo from the Edit menu or by selecting the action from the Undo List found in the Edit menu.

- **Statistics Setting**
  - Stat with negative – this setting means that some statistical calculations on data existing below zero may come back with an error due to the nature of the calculation.

  - Stat without negative – this setting means that all statistics can be calculated even if there is data existing below zero. This is achieved by ignoring the events that exist with a negative value.

- **Import Setting (Duplicate Files)**
  - Allow all files – with this setting, all files can be imported, even if there are two identical files.

  - Prevent duplicate names – with this setting, the importation of two files with the same file name will be prevented. This setting becomes important if the same experiment is repeated and therefore the same names are applied to the samples, although acquired at different times. Plate data will also produce multiple samples with the same name as the well name is commonly the sample name, i.e. A1, A2, etc.

  - Prevent duplicate files – with this setting, the importation of two identical files will be prevented.

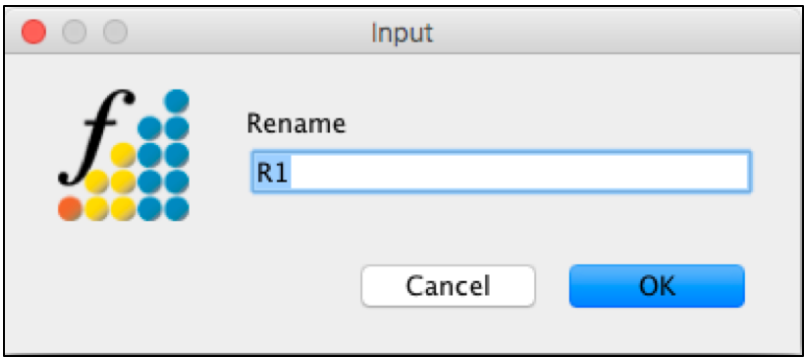
- **Gate Options**
  - Auto open daughter plots – with this option selected, the daughter population resulting from a gate is displayed in the Workspace when a gate is created.

  - Back gating on by default – with this option selected, populations are colored as a gate is created. As subsequent plots are created, the colored populations appear on all plots in the hierarchy.
  - Show gate label – the gate label is the gate name and the statistic relating to a gate. This will be displayed on the dot plot or histogram when this option is selected. The gate label can be moved around the plot area when the gate is highlighted.

- Hide gate label text – choosing to hide the gate label text will remove the gate name from the label, leaving only the associated gate statistic.

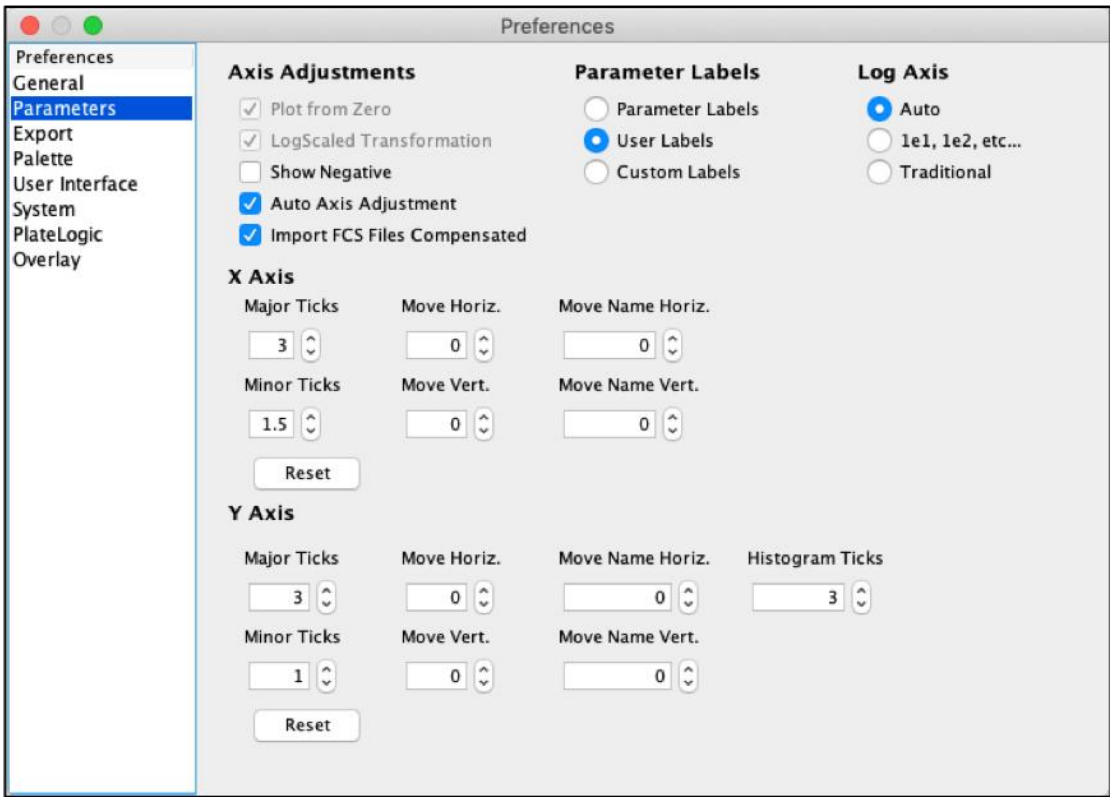
- Gate Label Value – the type of statistic displayed as part of the gate label can be chosen from the following options:
  - Event Count
  - Percentage Parent
  - Percentage Total
  - No Stat Display – choose 'No Stat Display' in combination with showing the gate label text to display the gate name alone.

- Gate Naming – the 'Gate Naming' option means that a box (as displayed below) will be displayed each time a gate is created, asking for a name to be assigned. The default name will be presented within the window each time a gate is created. If this option is not selected, then the default gate name is automatically assigned to the gate. The gate name can always be changed by right-clicking on the gate name in the File Inspector, choosing Rename and typing in the new name.



- **Sorting Settings**
  - The Sorting Settings refer to the default order in which FCS files are displayed in the File Inspector. This option includes sorting by File Date (the date the file was created), File Name (alphabetically ordered), FCS Date (the date and time of acquisition), Free, Plate Position, or $SRC keyword.

Parameters



- **Axis Adjustments**
  - Plot from zero – choose to plot from zero or to hide the first decade of events.
  - LogScaled Transformation –LogScaled axes will be displayed by default.
  - Auto Axis Adjustment – FlowLogic will determine the optimal scaling for the axes.
  - Import FCS Files Compensated – when selected, imported FCS files will load with the compensation matrix created at the time of acquisition. If not selected, the imported files will be uncompensated.

- **Parameter Labels**
  - Parameters Labels – selecting Parameter Labels displays the parameter name on the plot axis.

  - User Labels – selecting User Labels displays the parameter and the parameter name as defined at acquisition.

  - Custom Labels – selecting custom labels displays a new label that can be created in the Advanced Functions pull-up drawer in the Parameters tab. Here, all three parameter label types are displayed but only the custom labels can be changed. Double-clicking on the existing Custom Label allows the creation of a new custom label.

- **Log Axis – set the default axis labeling style**
  - Auto (displays the native format)
  - 1e1, 1e2, etc.…
  - Traditional

- **X Axis**
  - Major Ticks – increasing the number in this field, either by typing a new number or by using the arrows to increase the value, will lengthen the major axis markers. Decreasing the value shortens the major axis markers.

  - Move Horiz. – increasing this value shifts the x-axis marker values, e.g. 0, 10^3, 10^4, to the right. Decreasing this value moves the x-axis markers to the left.

  - Move Name Horiz. – increasing this value shifts the x-axis title, i.e. the parameter name, to the right. Decreasing this value moves the x-axis title to the left.

  - Minor Ticks – increasing the number in this field, either by typing a new number or by using the arrows to increase the value, will lengthen the minor axis markers. Decreasing the value shortens the minor axis markers.

  - Move Vert. – increasing this value shifts the x-axis marker values, e.g. 0, 10^3, 10^4, further from the axis (down). Decreasing this value moves the x-axis marker values closer to the axis (up).

  - Move Name Vert. – increasing this value shifts the x-axis title, i.e. the parameter name, further from the axis (down). Decreasing this value moves the x-axis title closer to the axis (up).

  - Reset – click reset to return to the default settings that were displayed before making any adjustments.

- **Y Axis**
  - Major Ticks – increasing the number in this field, either by typing a new number or by using the arrows to increase the value, will lengthen the major y-axis markers. Decreasing the value shortens the major axis markers.

  - Move Horiz. – increasing this value shifts the y-axis marker values, e.g. 0, 10^3, 10^4, to the left. Decreasing this value moves the y-axis markers to the right.

  - Move Name Horiz. – increasing this value shifts the y-axis title, i.e. the parameter name, to the left. Decreasing this value moves the y-axis title to the right.

- Histogram Ticks – increasing the number in this field, will lengthen the major y-axis markers on histograms. Decreasing the value shortens the major y-axis markers.

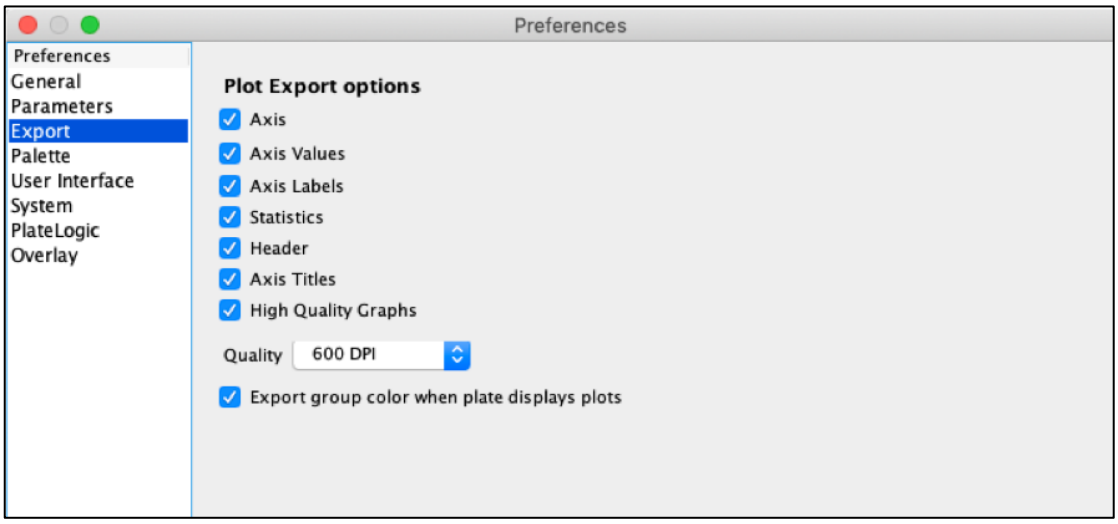
- Minor Ticks – increasing the number in this field, either by typing a new number or by using the arrows to increase the value, will lengthen the minor axis markers. Decreasing the value shortens the minor axis markers.

- Move Vert. – increasing this value shifts the y-axis marker values, e.g. 0, 10^3, 10^4, down. Decreasing this value moves the y-axis marker values up.

- Move Name Vert. – increasing this value shifts the y-axis title, i.e. the parameter name, down. Decreasing this value moves the y-axis title up.

- Reset – click reset to return to the default settings that were displayed before making any adjustments.

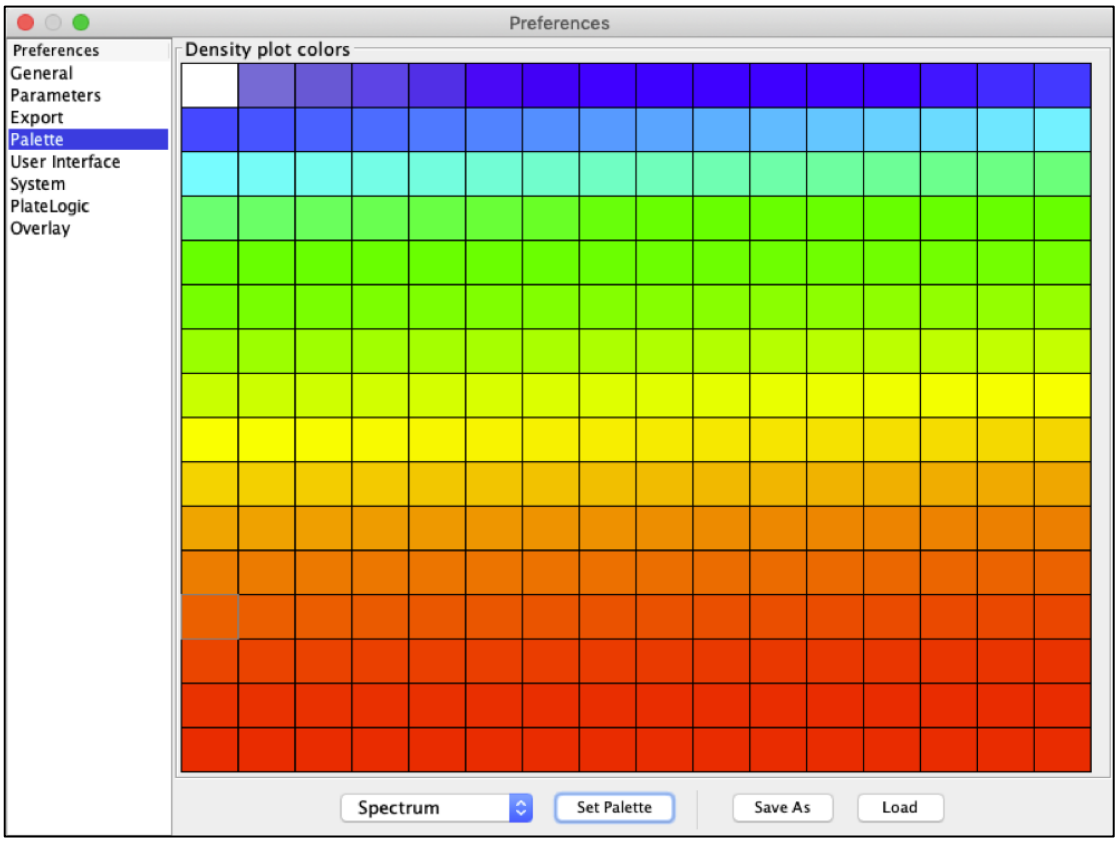
Export



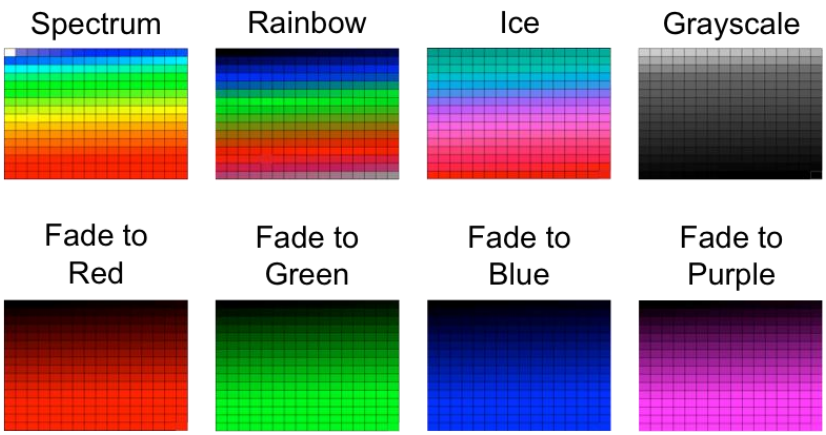
- **Plot Export Options** – selecting the following options will define the elements exported with dot plots and histograms. Some export file types can be ungrouped and edited at a later point. For file types that can't be ungrouped, define the elements that you wish to be displayed on each plot here prior to exporting.
  - Axis – this incorporates the major and minor ticks, the axis marker values e.g. 0, 10^3, 10^4 and axis titles
  - Axis Values – this is the axis marker values, e.g. 0, 10^3, 10^4
  - Axis Labels – this is the axis title, e.g. parameter name
  - Statistics – statistics relating to the parameters will be exported with the plot.

- Header – this is the sample name and associated statistics. These sit at the top left-hand corner of the exported plot.
- High Quality Graphs **(**72, 150, 300, 600, 1200 DPI) – the resolution of the exported image can be set in terms of dots per square inch.
- Export Group Color when plate displays plots – dataset colors will be exported with the plate if set to display plots and color background with dataset.

Palette

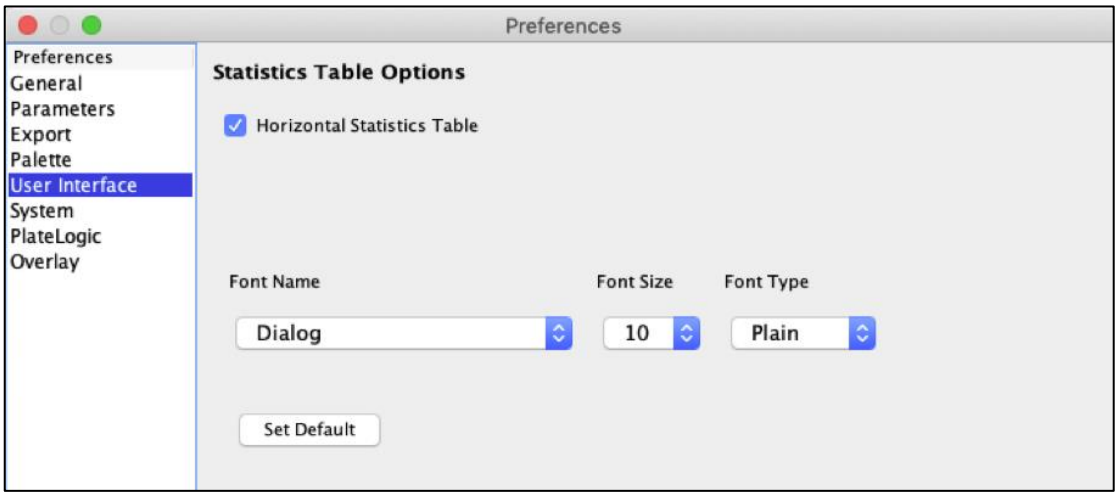


- **Density plot colors**



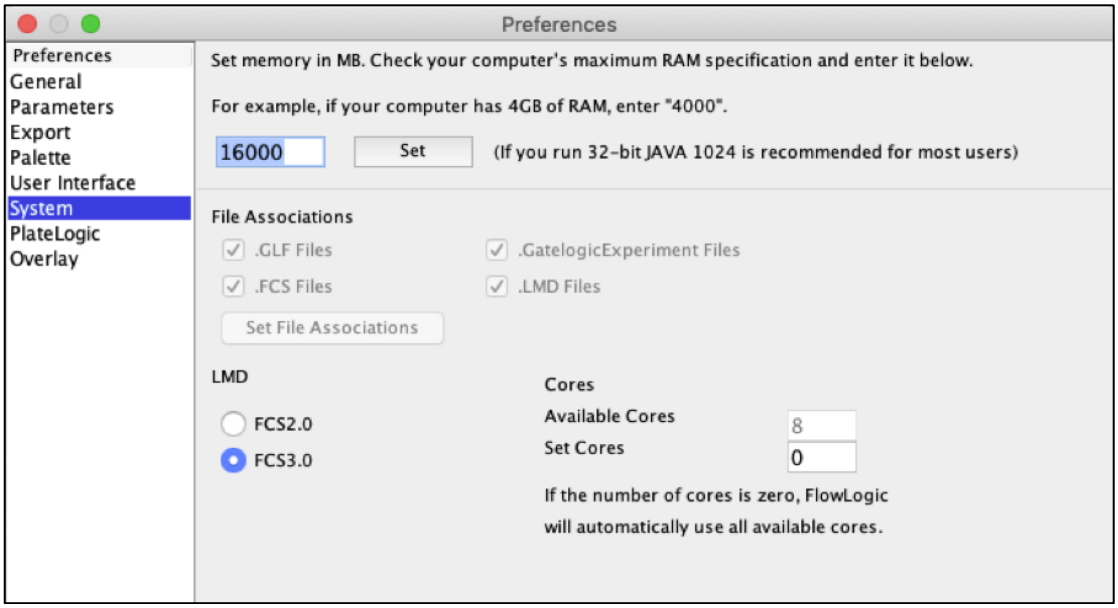
- Set Palette – to load a density plot color scheme, select it from the drop-down menu at the bottom of the Palette preferences window. Click Set Palette to confirm selection.
- Save As – new palettes can also be created. To create a palette, highlight the selected region of the density plot colors. A color chart window will then ask to choose a start color followed by an end color. The color transition will then be spread over the defined region. To save a new palette, click Save As. The program will then save a new Gatelogic Palette File.
- Load – to load a previously saved palette, click Load and locate the saved Gatelogic Palette File.

User Interface



Choose the default orientation and font settings for the statistics table in the View Statistics tab of GateLogic. Click Set Default to reset the settings.

System



- **Set Memory in MB. Check your computer's maximum RAM specification and enter it below** – maximizing the RAM dedicated to FlowLogic will improve program speed and performance.
  - Type in the available RAM available for FlowLogic in MB.
  - For example, if your computer has 4GB of RAM, enter "4000" (i.e. the amount of RAM in MB)

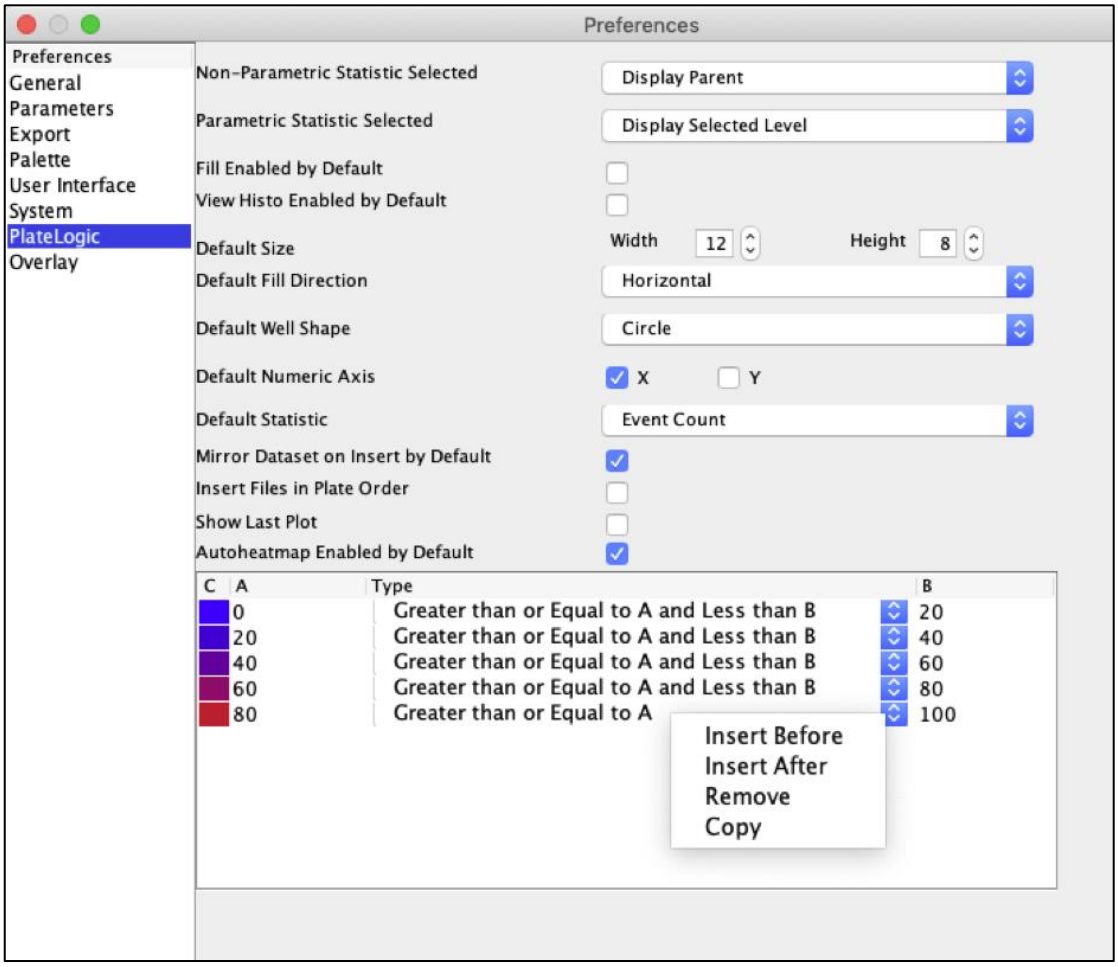
- For computers running 32-bit Java, 1024 MB is the maximum amount of memory that can be allocated.

- **File Associations** – for machines running Windows, double-clicking on the following file types will launch the program and load the files.
  - .glf Files
  - .fcs Files
  - .gatelogicexperiment Files
  - .lmd Files
  - Set File Associations

- **LMD file reading preferences –** the portion of the LMD file, FCS2.0 or FSC3.0, can be set as the default portion to read. This setting applies to all future LMD file imports. It will not change the reading of files that have already been imported.

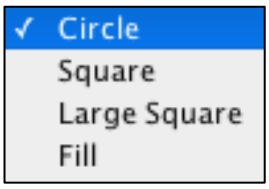
- **Cores –** the number of cores that FlowLogic uses can be set by the user. This can be useful when processing extremely large files in order to aid in memory management.

PlateLogic



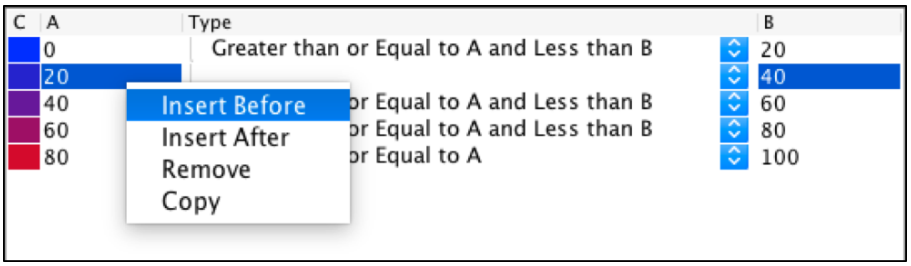
- Non-Parametric Statistic Selected
  - Display Selected Level
  - Display Parent
  - Display File
  - Parametric Statistic Selected
  - Display Selected Level
  - Display Parent
  - Display File
- Fill enabled by Default
- View histo enabled by Default
- Default Size – this is in terms of the number of wells in the horizontal direction (rows) and the number of wells in the vertical directions (columns). Change either of the numbers by clicking within the field and typing the new number or by using the up and down arrows to increase or decrease the number of wells running horizontally or vertically.
- Default Fill Direction – the fill direction relates to the placement of progressive samples added to a plate.

- Default Well Shape – the default well shape can be set as a circle, square, large square or to have the entire area filled. Click on the drop-down menu to make the selection.



- Default Numeric Axis – axes (rows and columns) can be listed either alphabetically or numerically. To label an axis numerically, tick the box next to the relevant axis. If the box is not selected, the axis will be labeled alphabetically. It is possible to list one axis numerically (and the other alphabetically), both axes numerically or both alphabetically.
- Default Statistic – choose the statistic that you wish to be displayed by default.
- Mirror dataset on Insert by Default – this feature, when enabled, creates datasets in the plate that match the groups from the File Inspector. The group color is applied to the matching dataset.
- Insert Files in Plate Order – files will be added to a plate in the order of A1, A2, A3, etc.
- Autoheatmap Enabled by Default – heatmaps can be generated automatically when samples are dragged from the File Inspector into a plate in the Data Array tab located in the Advanced Functions pull-up drawer. If the 'Autoheatmap Enabled by Default' option is selected, then FlowLogic will perform a calculation based on the range for the selected statistic. The wells in the plate will then be colored from, blue to red, over the ranges outlined in the Default Heatmap settings in the Preferences window.

- There are five ranges for the heatmap by default. However, extra ranges can be added, or existing ranges can be removed by selecting a range, rick clicking and choosing from the options provided. These are as shown below:



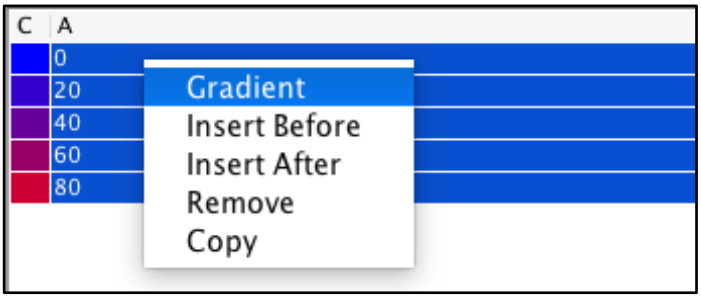
If 'Copy' is selected, a copy of the selected range will be automatically created and placed after the copied range.

- C (color) – the default color assigned for each range for the heatmap can be changed by clicking on the color itself and choosing from the options provided.
- A (bottom of range) – the default value defining the bottom end of the range can be changed by double-clicking within the filed in the column labeled ‘A’.

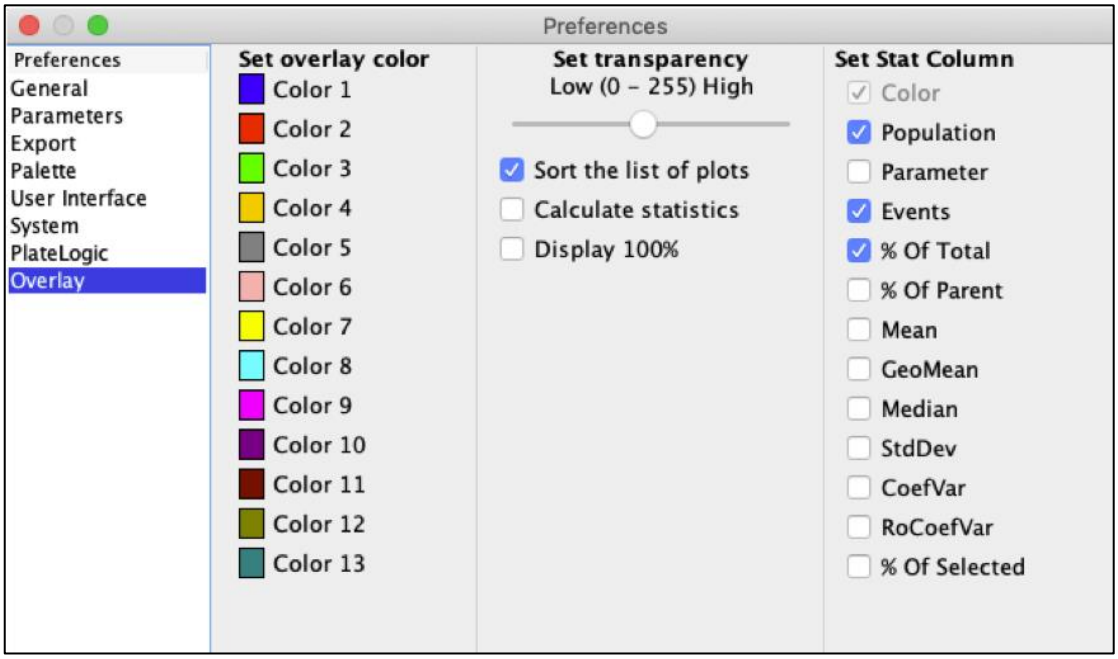
- Type – this is the rule defining each range in the heatmap, in relation to the values in columns ‘A’ and ‘B’. The type options are:
    - Less than A
    - Less than or Equal to A
    - Greater than or Equal to A
    - Equal to A
    - Greater than or Equal to A and Less than B
    - Not Greater than or Equal to A and Less than B

- B (top of range) – the default value defining the bottom end of the range can be changed by double-clicking within the filed in the column labeled ‘B’.

- The default heatmap colors can also be defined in this panel. This can be done by clicking on individual colors and making a selectin from the resulting color selection window or by applying a gradient between two colors. Do define a gradient, highlight all ranges, right-click and choose ‘Gradient’. Then select the starting color followed by the ending color. The intermediate colors will be set automatically.



Overlay



- **Set overlay color** – the default colors for the first 13 samples to be added to dot plot or histogram overlays are displayed here in the Preferences. The default color for each of these 13 samples can be set here by clicking on the color and then choosing a new one from the options provided. Overlay colors can be changed on individual overlays once it has been created.

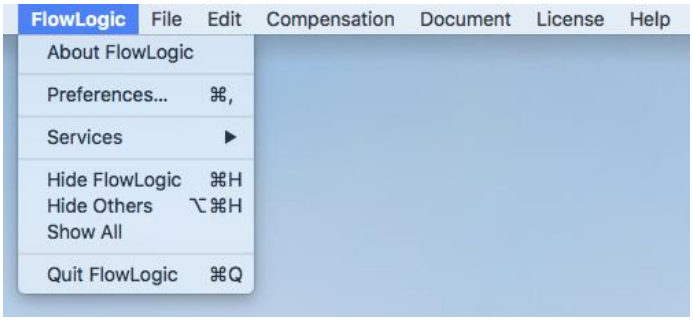
- **Set transparency**
  - Low (0-255) High – the default level of transparency can be set. This refers to the degree as to which a sample located behind another can be seen.
  - Sort the list of plots – when selected, samples will be added to an overlay with the highest density/histogram height at the back and lowest density/histogram height at the front. If 'Sort the list of plots' is not the default setting, then samples will be added in order the files are displayed in the File Inspector. These settings can be changed within the side drawer of each overlay plot by right-clicking within the Overlay Colors tab.

- Calculate statistics – statistics for all samples in the overlay can be displayed in the statistics tab in the overlay side drawer. The default selection of statistics to be displayed can be set in the Preferences window. The selection of statistics can be edited for each individual overlay within the Stat Settings tab in the overlay side drawer.
- Display 100% - for histogram overlays, the samples can be set to be displayed as either the event count or as a proportion of the highest value, which itself is at 100%. This selection determines the default display setting for all new histogram overlays. The setting can be changed on individual histogram overlays by right-clicking within the plot area and selecting Display 100%.

- **Set Stat Column** – default columns can be created in the side drawer of overlays, in the Statistics tab. Additional information may be required to define the statistic to populate these columns, which can be done in the ‘List of linked rows’, ‘Stat settings’ and ‘List of parameters’ tabs of the overlay side drawer. The different statistic columns are:
    - Color
    - Population
    - Parameter
    - Events
    - % of Total
    - % of Parent
    - Mean
    - GeoMean
    - Median
    - StdDev
    - CoefVar
    - RoCoefVar
    - % of Selected

Main Menu

The Main Menu contains several key program features and functions. In macOS, the Main Menu is set out as follows:



FlowLogic

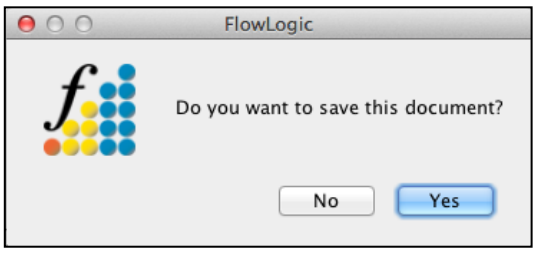
About FlowLogic – clicking About FlowLogic opens a window showing the program version, the user's system information (including the RAM and the number of cores available to FlowLogic), the license holder's username, the license type and the license expiration date.

Preferences… – clicking Preferences or using the keyboard shortcut at any time will open the program preferences. Here there are options to set default options relating to: General settings, Parameter settings, Export settings, Palette settings for density plot displays, User Interface settings, Report settings, Data Array settings and Overlay settings.

Quit FlowLogic – select quit FlowLogic or use the keyboard shortcut to exit the program. If the 'Warn before closing FlowLogic' option has been selected in the General program setting in Preferences, then a window will be displayed asking whether to save the current analysis before quitting or whether to cancel the command to quit.

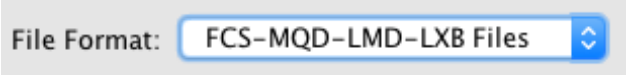
File

- New – selecting 'New' will open a new, blank analysis. Before the new analysis is created, a prompt (shown below) will ask whether to save the current analysis.

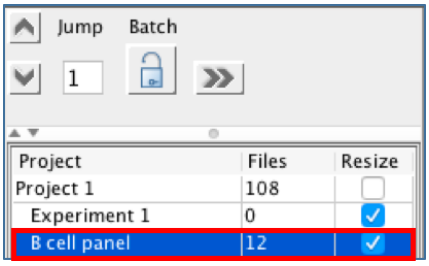


- Open – selecting 'Open' allows for the opening or loading of previously saved .glf and. .gatelogicexperiment files. Before displaying a window allowing the search for the saved analysis file, a prompt will ask whether to save the current analysis before opening a saved one.

- Open Append – this option allows a saved analysis file to be imported into the current analysis, effectively merging two analyses.
- Save – selecting Save or using the keyboard shortcut will save the current analysis. If the analysis has not been saved previously, a Save As window will open, asking to name the analysis and to choose a file type (either a .glf or a .gatelogicexperiment file). If the analysis has been saved previously, then choosing 'Save' will update the saved file, incorporating all recent changes.
- Save As – choosing Save As or using the keyboard shortcut will open a window allowing the file name and type to be set. If the current analysis has already been saved, then creating a new file name will create a new file. The original file will remain as it was when it was last saved.
- Import FCS-MQD-LMD-LXB… – choosing this file import option or using the keyboard shortcut will open a search window allowing the user to locate FCS stored on the computer or on an external drive. To import the FCS files, highlight them and select 'Choose'.



- Import Folders – selecting Import Folders or using the keyboard shortcut opens a search window where folders containing FCS files can be imported. This function imports all of the files in the folder and places them into a new Experiment folder, which takes the name of the imported folder.



- Export Statistics – selecting this option will allow you to export the statistics displayed within the View Statistics tab of the advanced functions pull-up drawer. A pop-up window will initially open, where the choice between .csv and .xlsx can be made. Following this, the file Save As window will allow the file name and location to be set.
- Recent – selecting Recent opens a window displaying the 11 most recent analysis files. Select the file by name to open it.

Edit – contained in the Edit menu are various display features and analysis tools, including copying, pasting, saving and deleting.

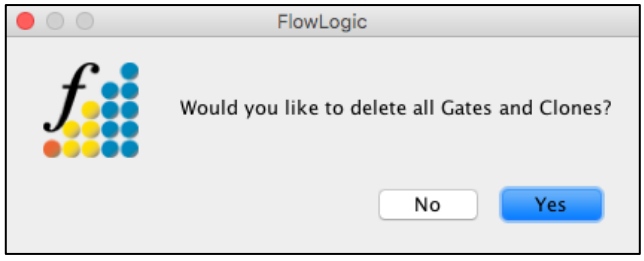
- Undo List – the Undo List shows a list of the most recent actions. This visualization allows for a particular action to be identified and by selecting it the analysis will return to the state immediately prior to this action being

performed. Note that the Undo option must be selected within the Preferences. Actions that can be undone in the undo list will be listed with black text. Actions listed with red text cannot be undone.

- Keywords – opens the Keyword Configuration window, allowing specific keywords and their values to be selected and displayed in the File Inspector.
- Plot Arrangement
  - Number of Windows (1 – 20) – the number of windows refers to the number of plots (dot plots, histograms or overlays) that are displayed horizontally across the Workspace before a plot is placed on the next row. Using this feature can greatly enhance analysis as the user can adjust the displaying of plots to fit a given screen size or to group samples in rows based on experimental groups. For example, if an experiment involves three groups, each with five samples per group, then setting up the number of windows to be displayed at 5 results in each group being displayed in a single row in the Workspace. This makes it much easier to compare differences between groups while viewing each individual sample.
  - Auto Arrangement on/off – Auto Arrangement is a feature that enables plots to keep their position in the Workspace even when individual plots are resized. For example, clicking the bottom right-hand corner of a plot and dragging it downwards will enlarge the individual plot. If there are other plots open in the Workspace, these will move in relation to the plot being resized. In this way, an enlarged plot will not obscure any other plot. Plots can also be moved around the screen by clicking the plot title and dragging it to a new location. This will result in the plot obscuring other plots if it is dragged on top of another. However, clicking and dragging the plot resizing slider at the top of the File Navigator will automatically place the plots back in their original order and make them a uniform size. The plot resizing slider can also be used to enlarge or shrink all plots in the Workspace together. Turning the Auto Arrangement off effectively makes each plot free and will all act independently when resizing and re-ordering.
- Plate Arrangement
  - Number of Windows (1 – 20) – the number of windows refers to the number of plates that are displayed horizontally across the PlateLogic workspace in the Advanced pull-up drawer before a plate is placed on the next row.
  - Auto Arrangement on/off – this feature enables plates to keep their position in the PlateLogic workspace even when individual plates are resized. For example, clicking the bottom right-hand corner of a plate and dragging it downwards will enlarge the individual plate. If there are other plates open in the Data Array Workspace, these will move in relation to the plate being resized. In this way, no plates will be obscured when one is manually enlarged. Plates can also be moved around the screen by clicking the plate title and dragging it to a new location. This will result in the plate obscuring others if it is moved on top of another. However, clicking and dragging the plate resizing slider at the bottom of the Plate List Window in the Advanced Functions drawer will automatically return the plates to their original order and make them of a uniform size. The plate resizing slider can also be used to enlarge or

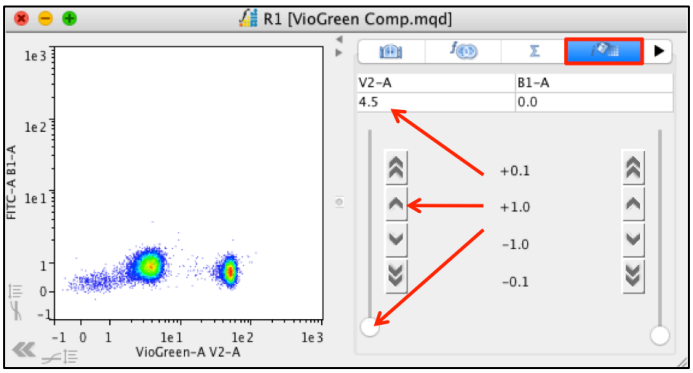
shrink all plates in the PlateLogic workspace together. Turning the Auto Arrangement off effectively makes each plate free and each will act independently when resizing and reordering.

- Copy Gates – this feature copies gates in order to paste them to another sample in the File Inspector. To copy gates, highlight them, select Edit → Copy Gates (or use the keyboard shortcut). The gates are then copied to the clipboard ready to be pasted onto new samples. The 'Copy Gates' feature can also be accessed by right-clicking on a sample and selecting Copy → Copy Gates.
- Paste Gates – once gates have been copied from a sample, highlight a new sample or samples and select Edit → Paste Gates (or use the keyboard shortcut) to paste the gates. The gates will be pasted to the sample and not appended or inserted into a selected level of the existing gating hierarchy. In effect, the gates will replace existing gates on the destination sample. In order to insert gates into a specified level of a gating hierarchy, select the level (gate) that you wish to insert the gates onto, right-click and choose Paste → Insert Gates. The 'Paste Gates' feature can also be accessed by right-clicking on a sample and selecting Paste → Paste Gates.
- Paste Gates to All – once gates have been copied, selecting any gate in the File Inspector and clicking Edit → Paste Gates to All will apply the copied gates to all samples.
- Delete Rows – rows refer to the samples and gates shown in the File Inspector. To delete one or more rows, highlight the row(s) in the File Inspector and select Edit → Delete Rows. This will delete the selected rows and any gates lower in the hierarchy. A window will open prior to the rows being deleted to confirm the action and to show the gates that will be deleted.
- Delete All Gates – select any row in the File Inspector and then click Edit → Delete All Gates to delete all gates and clones listed in the particular Experiment Folder. A window (as shown) will confirm the action to delete all gates and clones.



- Delete All Statistics – selecting Edit → Delete All Statistics will clear any statistics calculated and displayed in the Set Statistics Tab in the Advanced Functions drawer and all statistics displayed in the View Statistics Tab in the Advanced Functions drawer.
- Preferences – opens the program preferences.

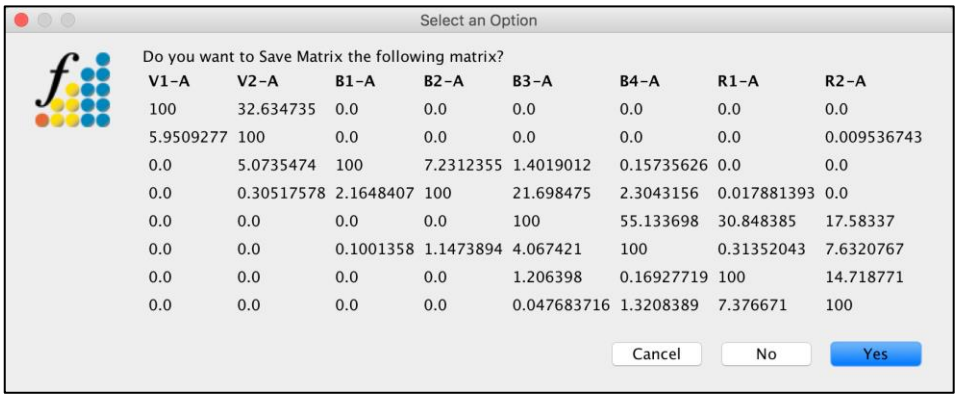
Compensation – live compensation can be performed in the side drawer of individual plots under the Interactive Compensation Matrix tab. Adjust the compensation by double-clicking in the compensation value tab and typing in a new value, by using the up and down arrows to adjust the compensation in small increments or use the slide tool to increase or decrease the compensation value:



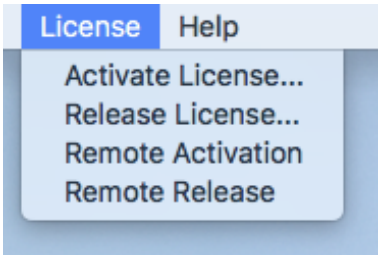
Once the compensation has been changed for one sample, there are several different options available:

- Copy New – to copy the new compensation (in order to paste it to other samples) select the sample in the File Inspector with the newly changed compensation and click Compensation → Copy New. This will save the new compensation matrix to the clipboard.
- Copy FCS Original – the compensation matrix that was created at the time of acquisition remains with the FCS file even if it is changed post acquisition. If you wish to apply the original compensation matrix to a file, copy the original FCS and paste it to any sample in the File Inspector.
- Copy Saved – a sample compensation matrix (either the original matrix or an altered one) can be saved as a TXT file and applied to samples in the File Inspector. To select a saved matrix in order to paste it to a sample(s), click Compensation → Copy Saved. A window will open allowing the user to locate the saved TXT file. The saved compensation matrix can then be pasted to selected samples in the File Inspector.
- Save New – if you wish to save an altered compensation matrix as a TXT file, highlight the sample in the File Inspector and click Compensation → Save New. A window will ask to select a destination and create a name for the saved file.
- Save FCS Original – if you wish to save an original compensation matrix (that created at the time of acquisition) as a TXT file, highlight the sample in the File Inspector and click Compensation → Save FCS Original. A window will ask to select a destination and create a name for the saved file.

- Paste – once a compensation matrix (an original matrix, a new matrix or one copied from a TXT file) has been copied to the clipboard it can be pasted to FCS files highlighted in the File Inspector. Copy the desired matrix, highlight the FCS files in the File Inspector and click Compensation → Paste. A window will open displaying the matrix to be displayed. Click Yes to paste the matrix.



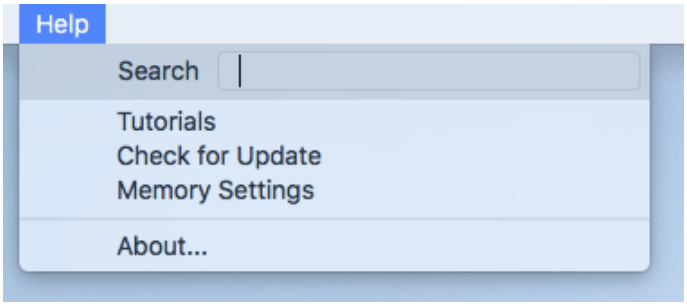
License



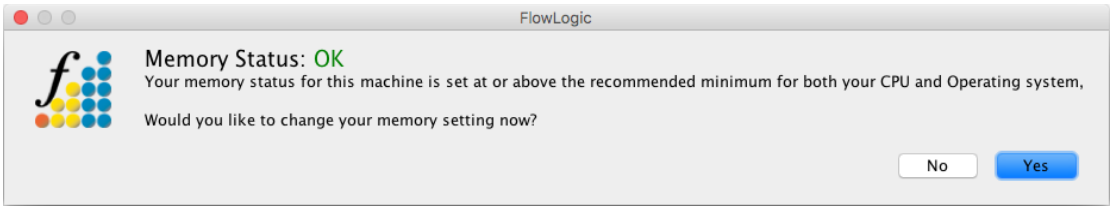
- Activate License… – to activate a license, enter the username associated with a valid license code.
- Release License… – release a license if you wish to run FlowLogic on another computer. Activate the license on the second computer when you launch FlowLogic.
- Remote Activation – remote activation can be used when there is no internet connection. When selecting this option, a window with included instructions will open enabling the generation and saving of a fingerprint. This can then be saved and transferred to a different computer with internet access. The fingerprint can be authenticated resulting in the generation of a license key. This key can be transferred back to the computer running FlowLogic in order to activate the license.
- Remote Release – remote release is also used when there is no internet connection. The process is the same as that for remote activation. After the fingerprint is generated, FlowLogic will close and will require an available username (or fingerprint if offline) in order to run.

Help

- Search – use the ‘Search’ feature under the Help menu to search for help and guidance.



- Tutorials – this option links to the FlowLogic support page on the Inivai website, where PDF guides and demonstration videos can be accessed.
- Memory Settings – clicking Help → Memory Settings will prompt FlowLogic to test the current memory settings. A window will open displaying the result and asking if you would like to change the memory settings. If you choose Yes, the program Preferences will open. From here, navigate to the System settings to change the memory settings.



Saving and Opening Analysis Files

File Menu

Once you have imported FCS files and performed an analysis you will be able to save the analysis as either a GLF or an Experiment. Both options can be found under the File menu.

GateLogic File (.GLF)

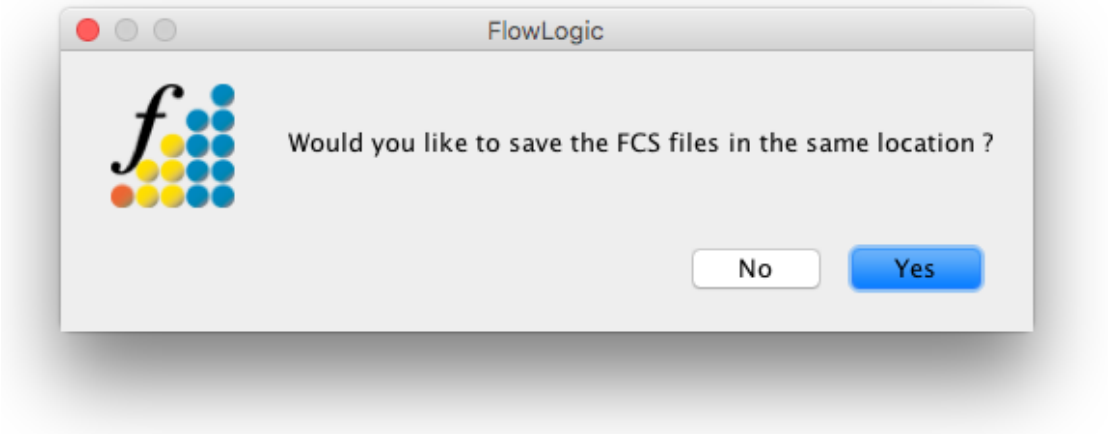
- This file type will save your analysis along with all the gates and statistics. The GLF will save the pathway for each of the imported FCS files. If the location of these files changes then FlowLogic will ask you to manually locate the file or select a folder to search in.
- To open a GLF file, start FlowLogic, choose ‘Open GLF’ from the File menu and locate the saved GFL file.
- To save a GLF, after importing FCS files, choose ‘Save GLF’ (or ‘Save As GLF’ to rename the new GLF file) from the File menu.

GateLogic Experiment (.gatelogicexperiment file)

- Saving your analysis as an Experiment will compress your analysis and FCS files together into one file. This single file can be transferred to any computer running FlowLogic.
- To open an experiment file, start FlowLogic, choose ‘Open Experiment’ from the File menu and locate the saved experiment file. When the Experiment is opened, it will create a folder containing the FCS files.

Exporting FCS files from a saved GateLogic Experiment

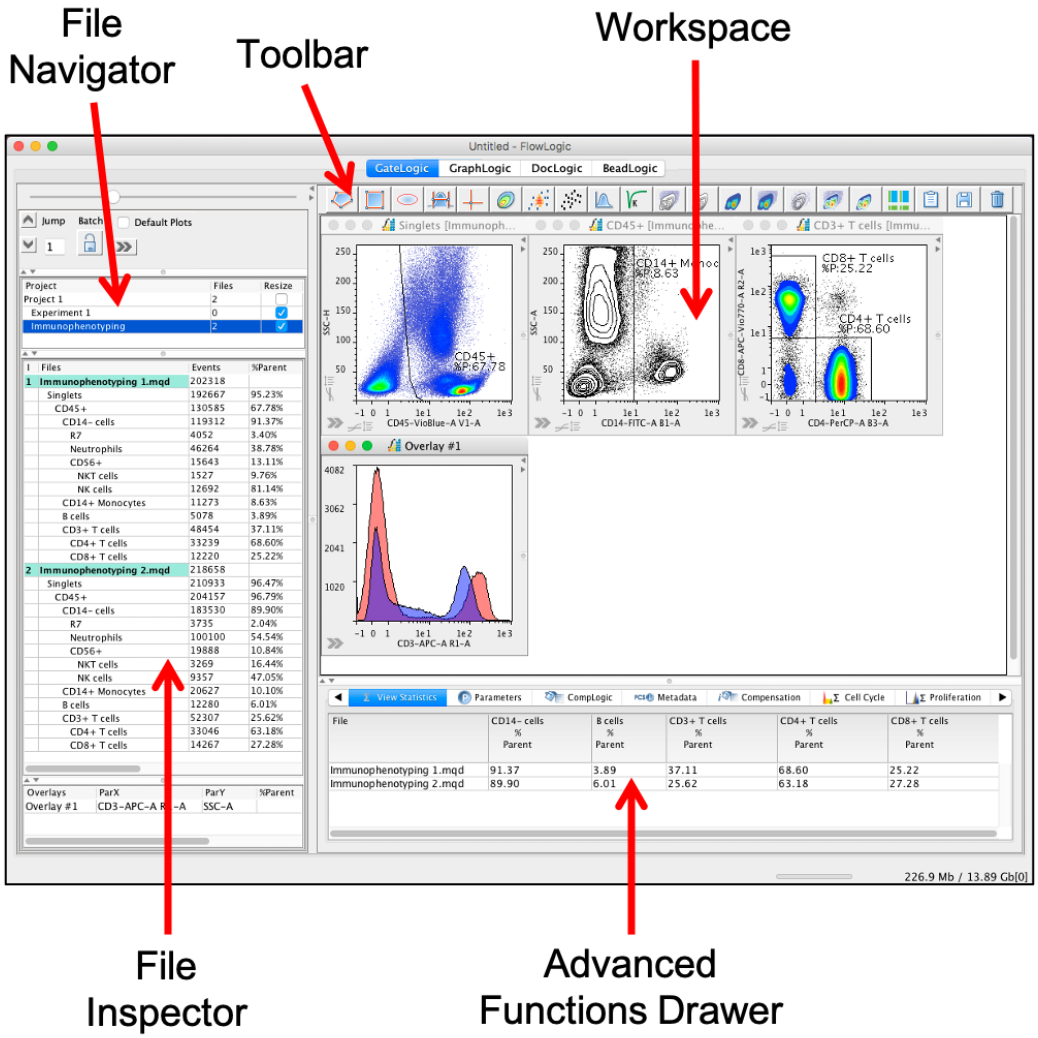
FCS files can be exported from a GateLogic Experiment (.gatelogicexperiment file) by re-saving the analysis as a .GLF. As a .glf contains the XML and not the FCS files, a prompt will ask if you would like to save the FCS files in the same location as the new .GLF. When creating the new .glf, the FCS files contained in the experiment will be extracted and placed in a new folder.



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.

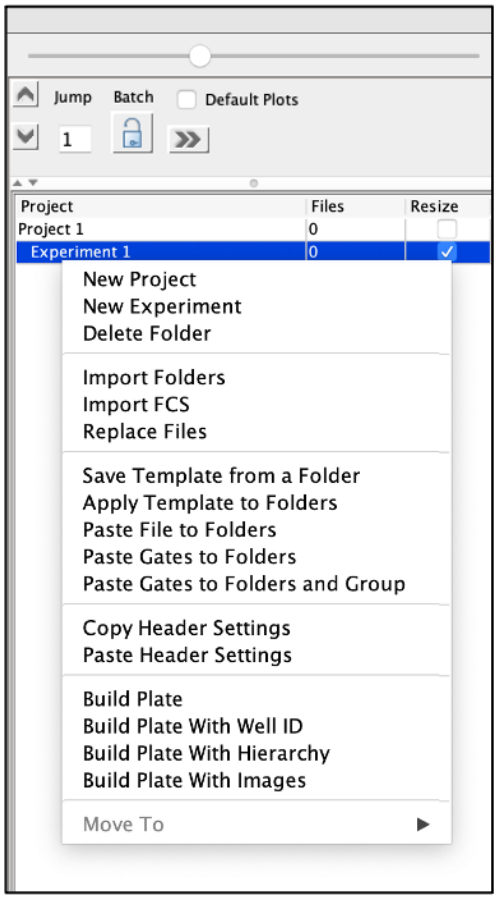


File Navigator

The File Navigator contains the ‘Jump’, ‘Batch’ and ‘Default Plots’ 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. Ticking the Default Plots options whilst plots are displayed in the screen will pin those plots to the Workspace. New plots can then be opened and when the Jump function is used, the original plots will remain in the Workspace whilst the Jump feature is applied to the more recently opened plots. 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.

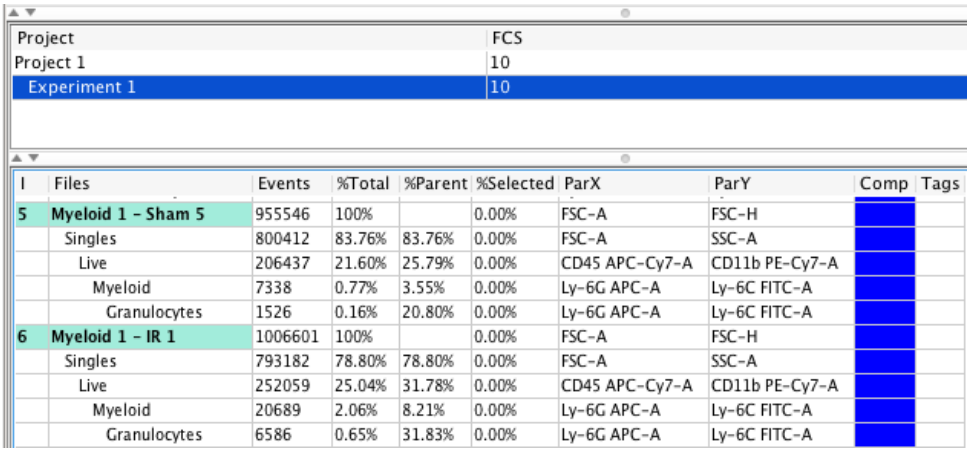
Experiment folders can be re-ordered within a Project folder or from one Project folder to another using drag-and-drop. The order of Project folders can also be changed using drag-and-drop.



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.

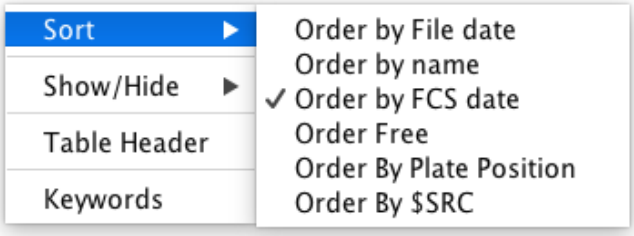
- The File Inspector also contains a number of columns containing various statistics, information on sample compensation and tags (particular sample attributes). These columns can be revealed by expanding the File Inspector by clicking and dragging the right-hand side to the window and pulling it to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



Project	FCS
Project 1	10
Experiment 1	10

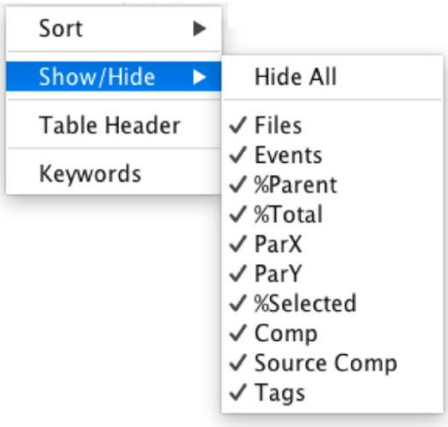
I	Files	Events	%Total	%Parent	%Selected	ParX	ParY	Comp	Tags
5	Myeloid 1 - Sham 5	955546	100%		0.00%	FSC-A	FSC-H		
	Singles	800412	83.76%	83.76%	0.00%	FSC-A	SSC-A		
	Live	206437	21.60%	25.79%	0.00%	CD45 APC-Cy7-A	CD11b PE-Cy7-A		
	Myeloid	7338	0.77%	3.55%	0.00%	Ly-6G APC-A	Ly-6C FITC-A		
	Granulocytes	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.

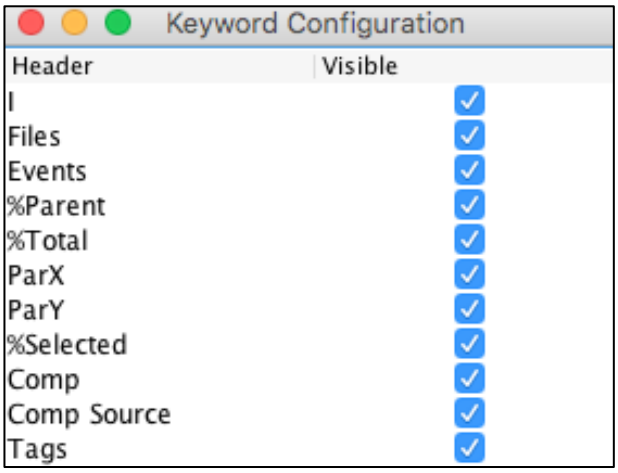


Files can also be re-ordered using drag-and-drop. Clicking and dragging the file name automatically switches the ordering to ‘Free’ and the file can be dropped in the desired position. The order of plots open in the Workspace will also be updated to reflect the order of files in the File Inspector. The 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 98.

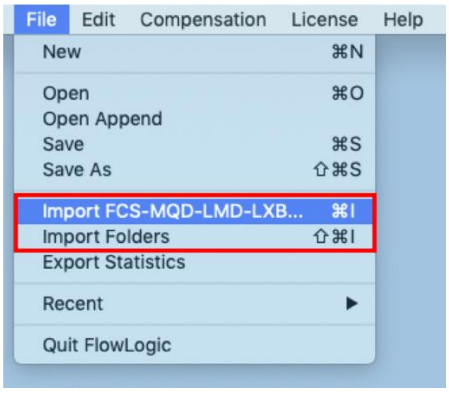
- Right-clicking on a sample in the File Inspector opens a different window with a range of functions to aid in the analysis of the sample(s). See the individual sections relating to these functions for more details.

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

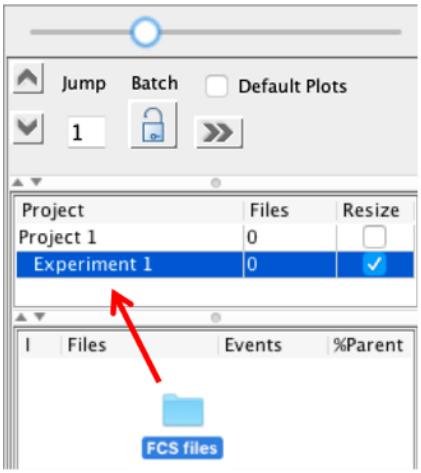
Advanced Functions pull-up drawer (summary)
The Advanced Functions pull-up drawer contains additional analysis features to improve efficiency and aid data mining capabilities. To access the Advanced Functions, click on the top of the drawer (located at the bottom of the Workspace) and drag it up. It can be pulled up to entirely cover the Workspace, if desired. The Advanced Functions drawer consists of 13 tabs: ClusterLogic, QCLogic, 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**.

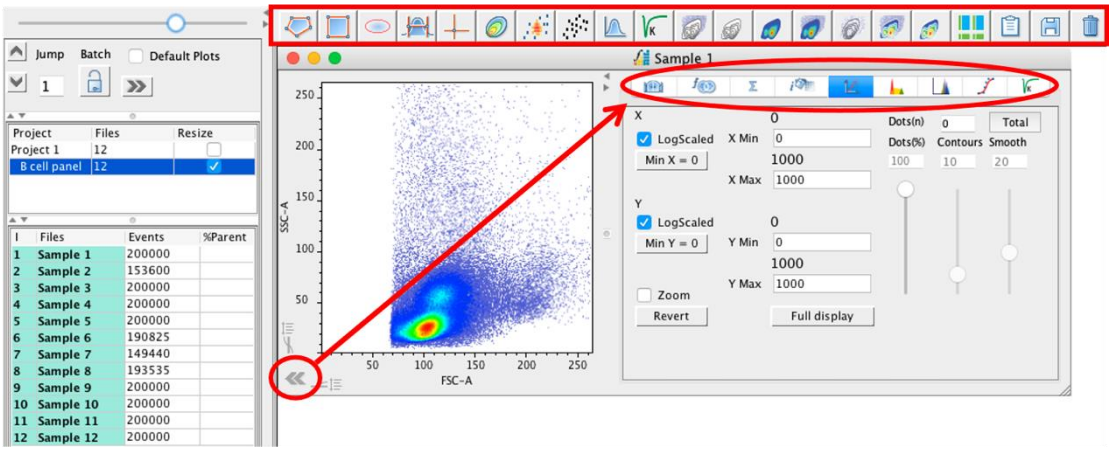


Opening Dot Plots/Histograms

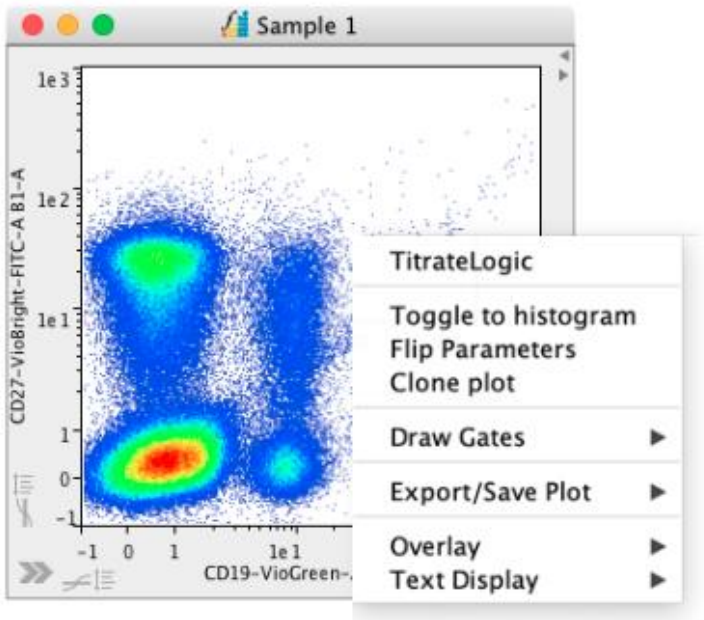
Imported FCS files appear in the File Inspector. Double-click on a file or right-click→Plots→Open to open it as a dot plot.

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 (outside gates)

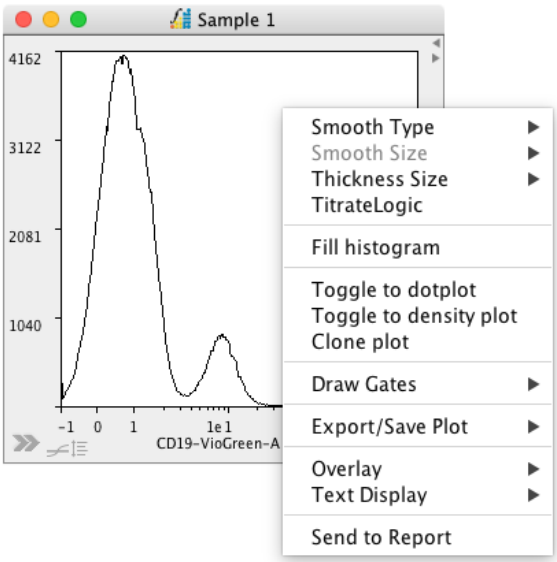


Right-click within a plot to open a menu with options to:

- Perform automated titration analysis (see page 100)
- Toggle to a histogram
- Flip the parameters displayed on the x and y axes (dot plot only)
- Clone a plot (see page 46)
- 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)

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 79.

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 100)
- Fill (color) the histogram
- Toggle to a dot plot or density plot
- Clone a plot (see page 46)
- Draw gates
- Export/Save Plots
- Create Overlays
- Format the plot text
- Send the plot to a report in DocLogic

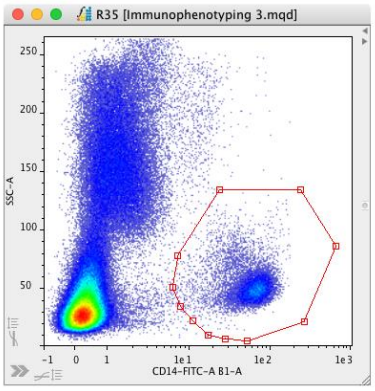
A right-click within a marker provides the extra option to add a gate to, or remove a gate from a group. For more on group analysis, see page 79.

Toolbar Features

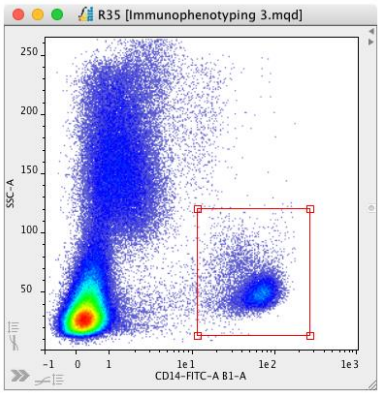
The Toolbar, located at the top of the Workspace, contains a range of gating and plot display tools. Hover the cursor over each of the buttons in the toolbar to view the description.

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:

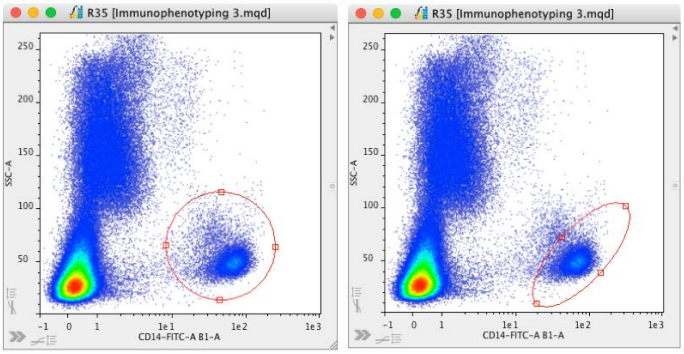
- **Create a polygon** – a polygon gate can have any number of corners. Once the polygon gating tool has been selected, click around the area on the plot that you wish to be contained within the gate. Double-click or click on the original point to close the gate. Polygon gates can be moved by clicking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



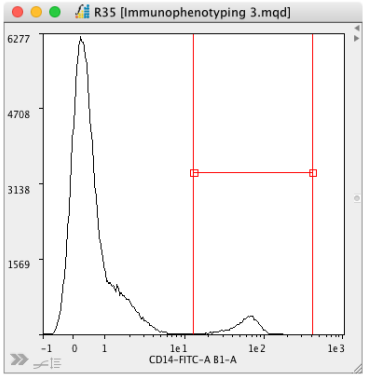
- **Create a rectangle** – a rectangle gate always has four sides, each joined at right angles. Once drawn, the size and shape of the rectangle can be adjusted by clicking and dragging a corner or by clicking and dragging a side.



- **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.

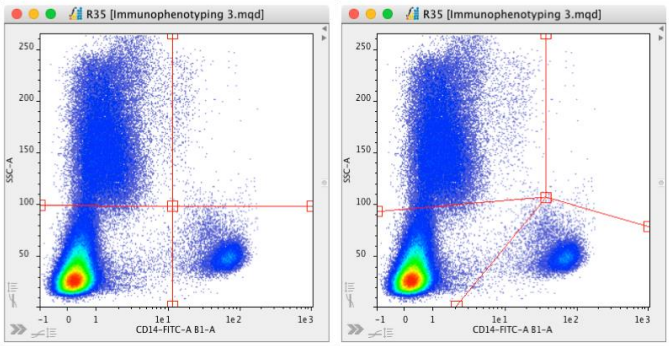


- **Create histogram marker** – a histogram marker is used to define a region on a histogram. Histogram markers can be adjusted by clicking and dragging the points at each end. The height of the cross bar can also be adjusted by clicking and dragging it down or up.

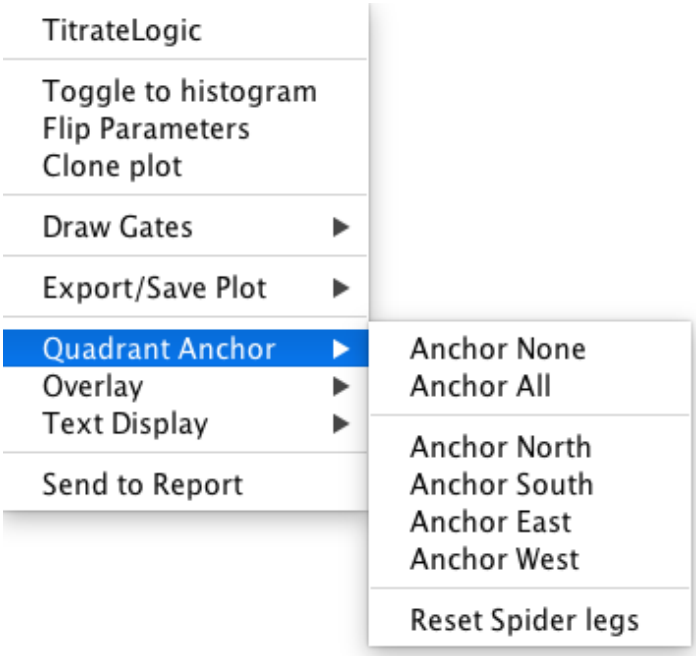


- **Create a quadrant** – a quadrant gate divides the plot into four areas. To apply a quadrant gate, highlight the plot, select the gating tool and click within the plot area. Quadrant gates can be adjusted by clicking and dragging the center point or by clicking and dragging the points at the end of each of the arms. These arms move independently of each other and do

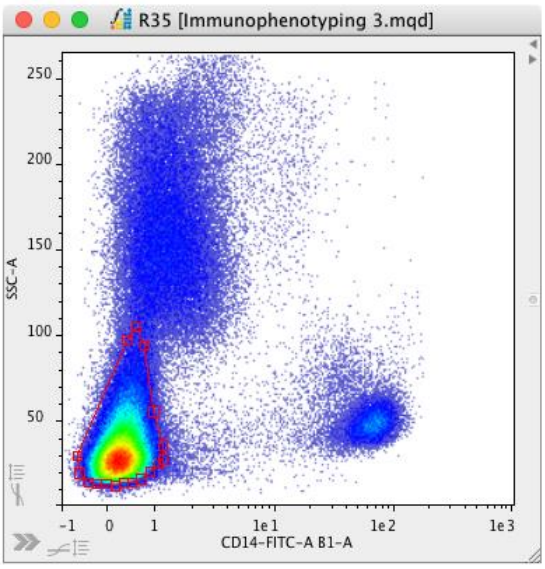
not have to form four rectangle gates.



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.

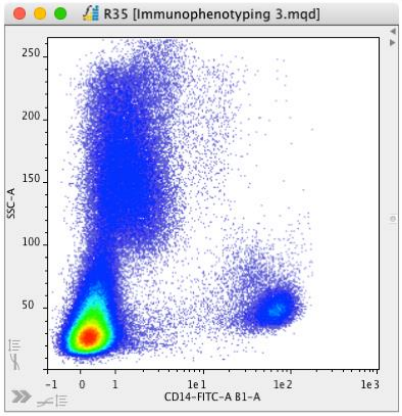


- **Autogate** – the autogating tool automatically draws a gate based on density. To use the autogating tool, highlight the plot, select the autogating tool from the toolbar and then hover over the plot. A gate will be displayed based on where the cursor is and the density of events on the plot. Without 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.

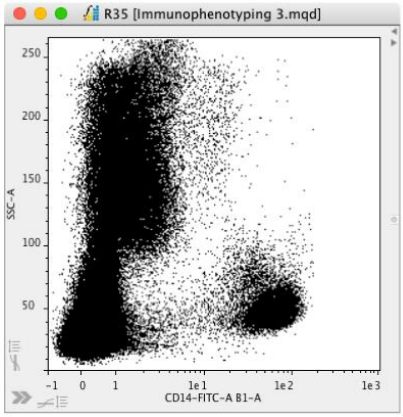


Plot Display Options – plots can be displayed in a number of different ways. Choose a plot display option to best display your populations. Plots are generally displayed as a standard dot plot (or density plot if set as a default in the Preferences). To change the plot display, click on the plot title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

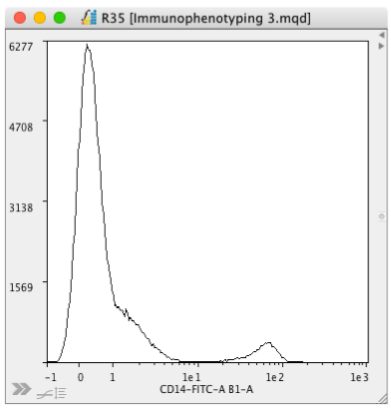
Density plot



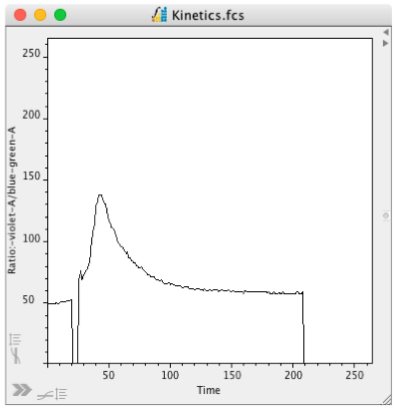
Dot plot



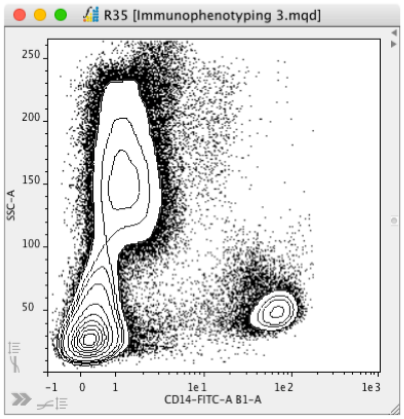
Histogram



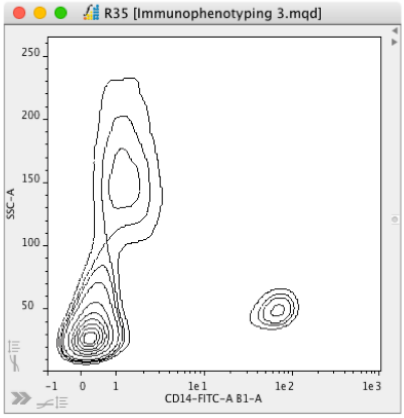
Kinetics plot



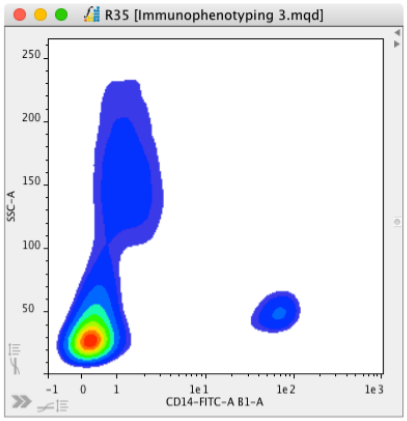
Dot plot with contours



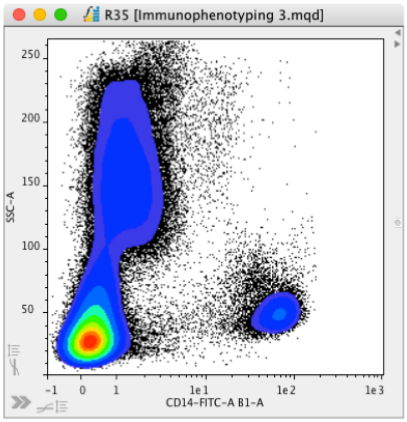
Contour plot



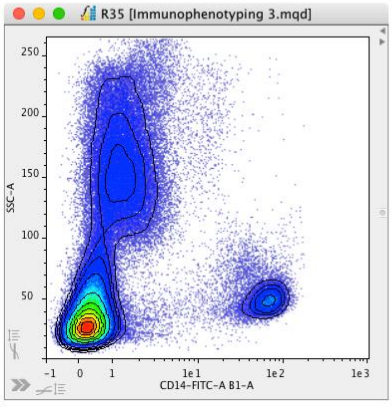
Pseudocolor plot



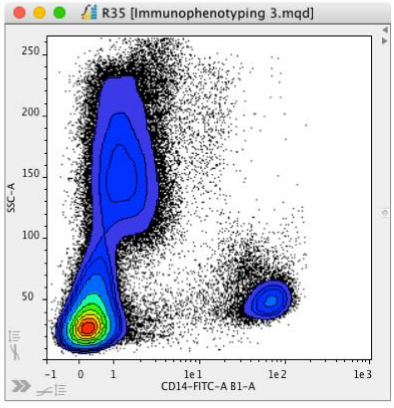
Dot plot with pseudocolor



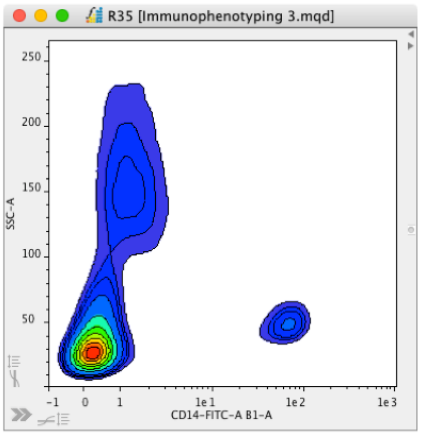
Density plot with contours



Dot plot with pseudocolor and contours



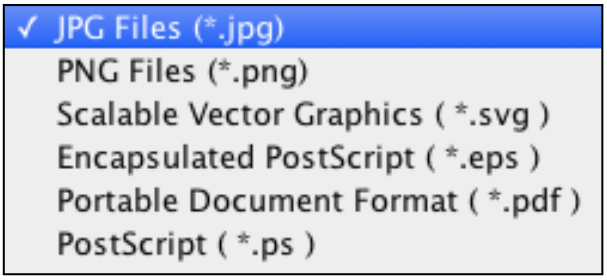
Pseudocolor plot with contours



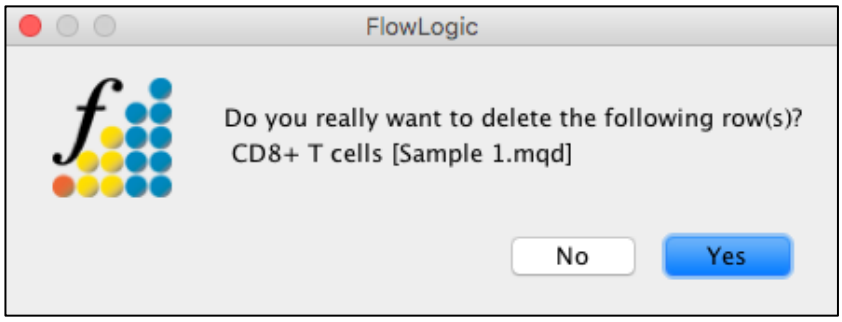
Clone a plot – plots can be cloned, resulting in to identical plots at the level where the clone was created. The clone is labeled with a 'C' next to the gate name in the File Inspector. To clone a plot, highlight it on the plot or in the File Inspector, and select the toolbar button.

- **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.



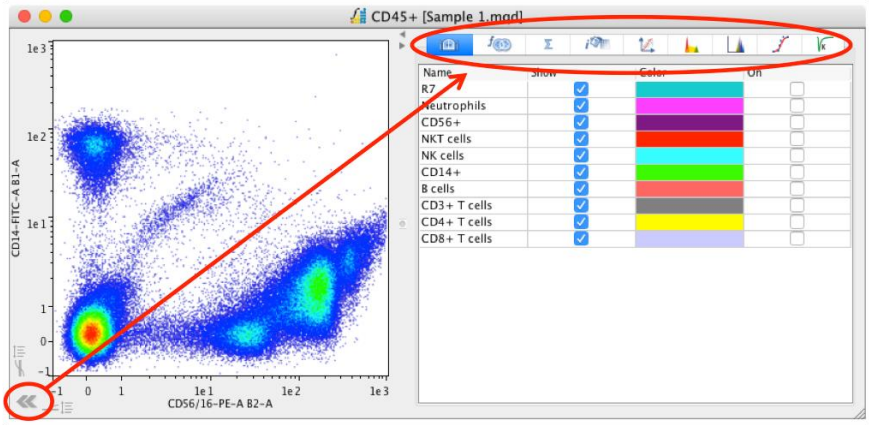
- **Delete the selected gate** – one method to delete a particular gate is to highlight it, either on the plot or from the gate lists in the File Inspector, before clicking the Delete the selected gate button in the toolbar. A window will then be displayed asking to confirm this action.



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.



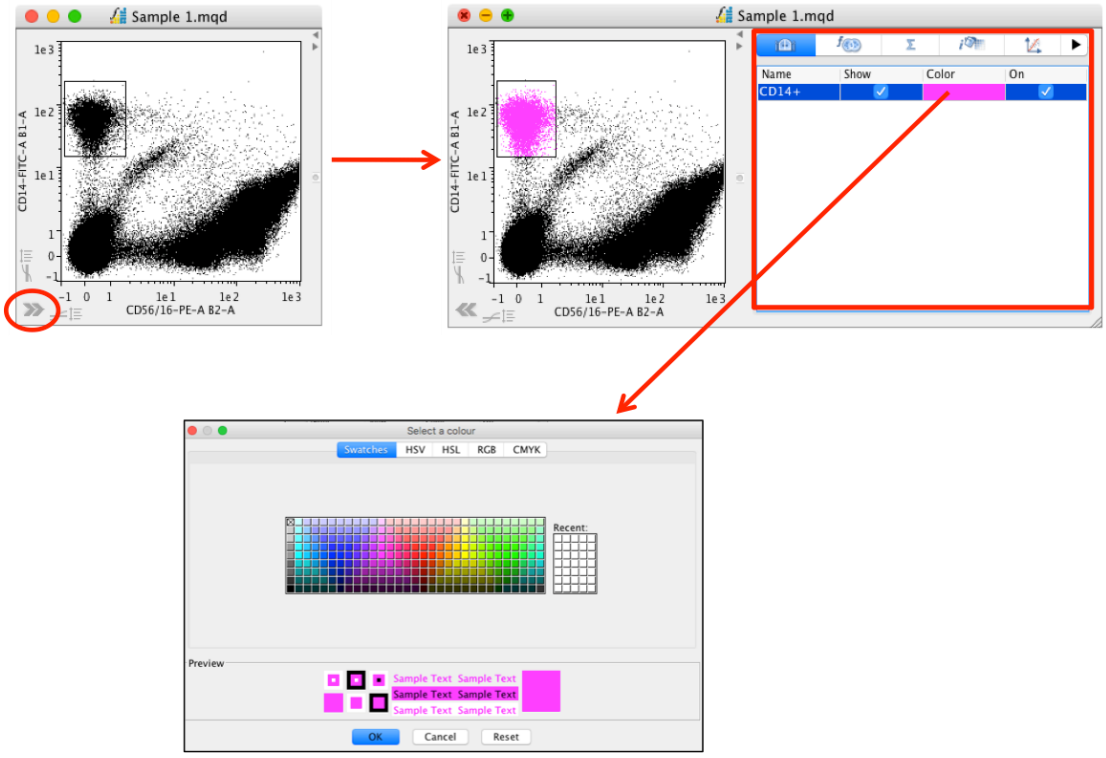
Gate List

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.

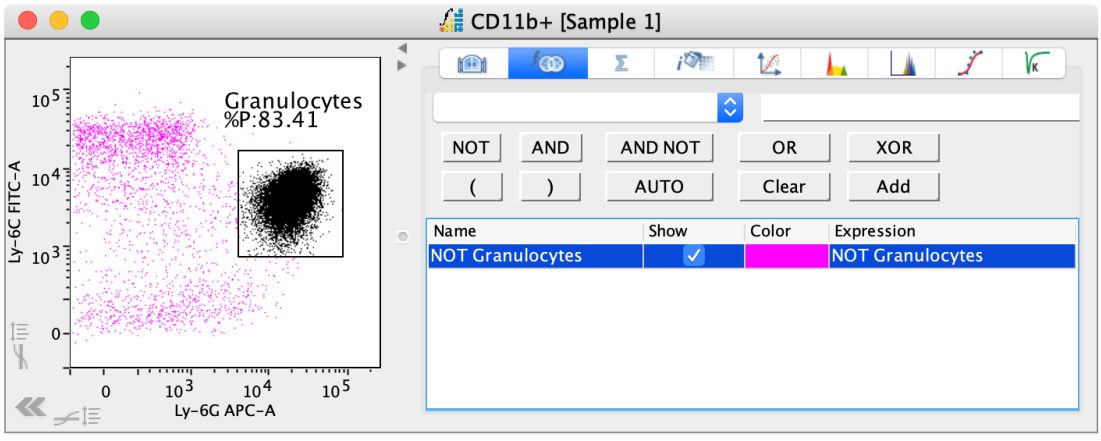
The ‘Show’ column contains a check box allowing gates to be turned off and on. This is a display option only, meaning turning off a gate only removes it from the display but the gate is not deleted and still exists. It can easily be displayed again by ticking ‘Show’.

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.

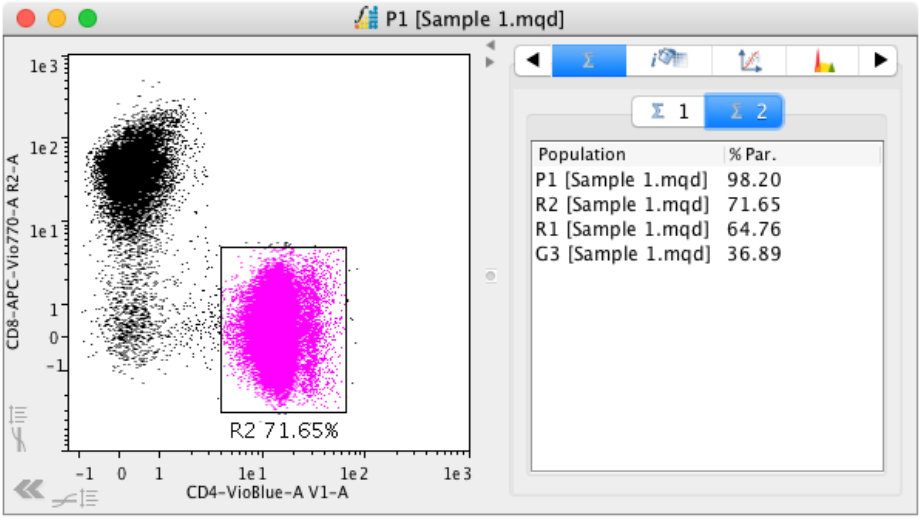


Boolean Gates

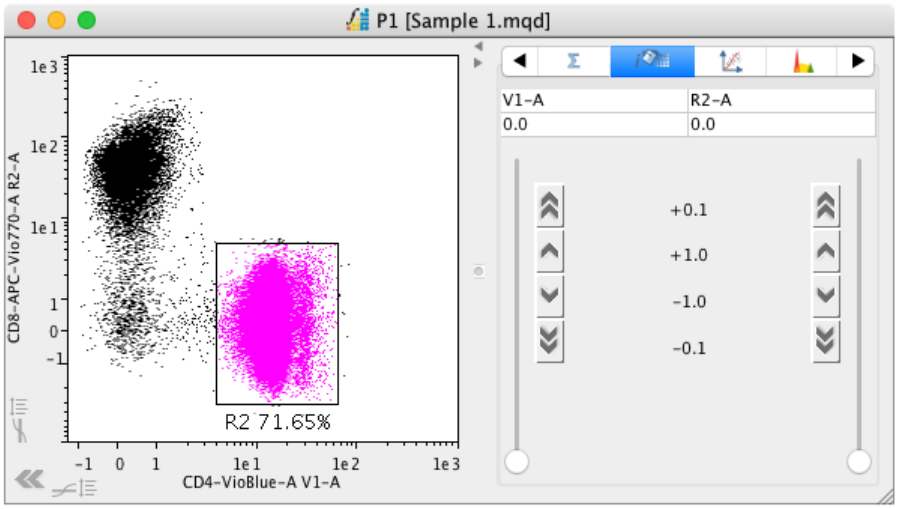
Boolean gates are created in the plot side drawer. The list of Boolean gates that exist at that particular level of the gating hierarchy will be displayed, along with options to color of the new population.



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.

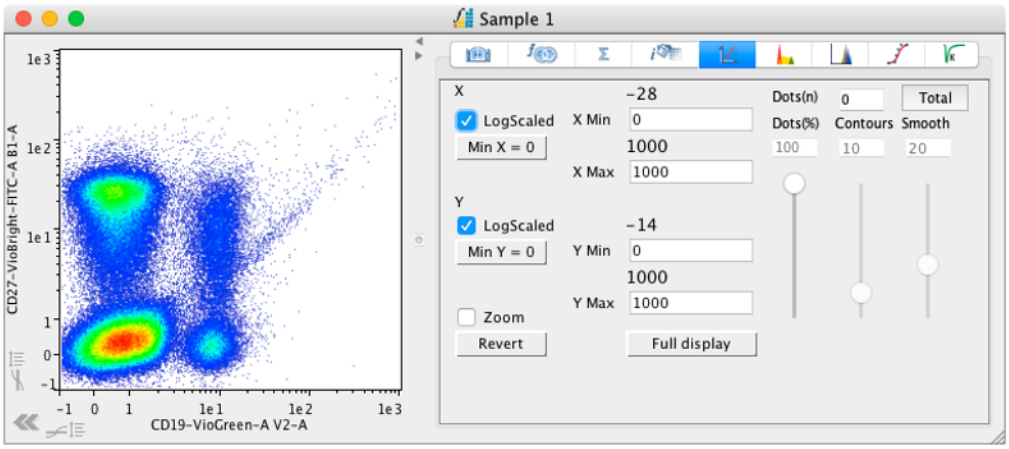


Interactive Compensation
Samples can be manually compensated using the slide bars, the up and down arrow buttons or by entering a new compensation in the text field.



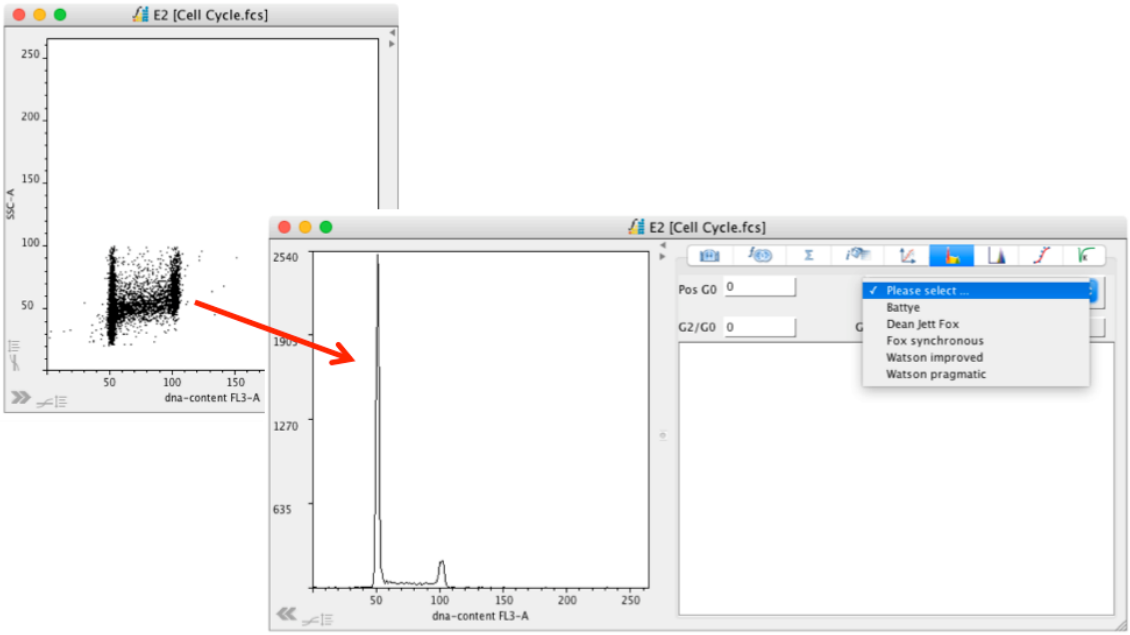
Scaling

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. A defined number of events can be displayed by typing in a value within the Dots(n) field. When using group analysis, all files within the group will display the same number of events. Return to displaying all events in the population by clicking the “Total” button.

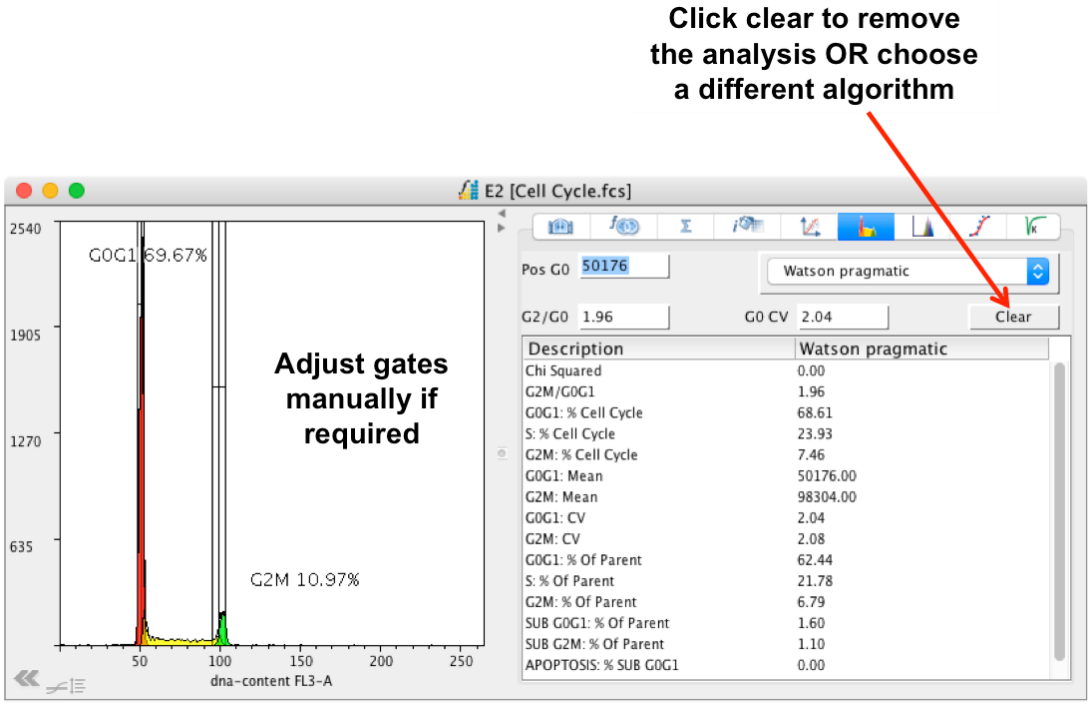


Cell Cycle

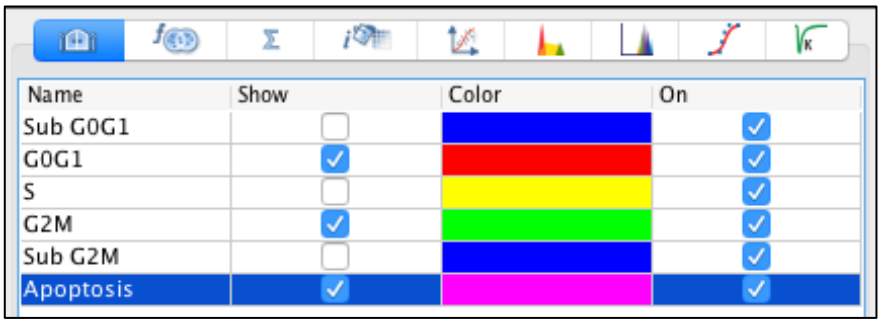
To perform cell cycle analysis, perform any pre-gating required on your DNA-content parameters before displaying it as a histogram. Then, in the Cell Cycle tab in the side drawer, choose from one of the 5 algorithms: Battye, Dean Jett Fox, Fox synchronous, Watson improved and Watson pragmatic.



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.

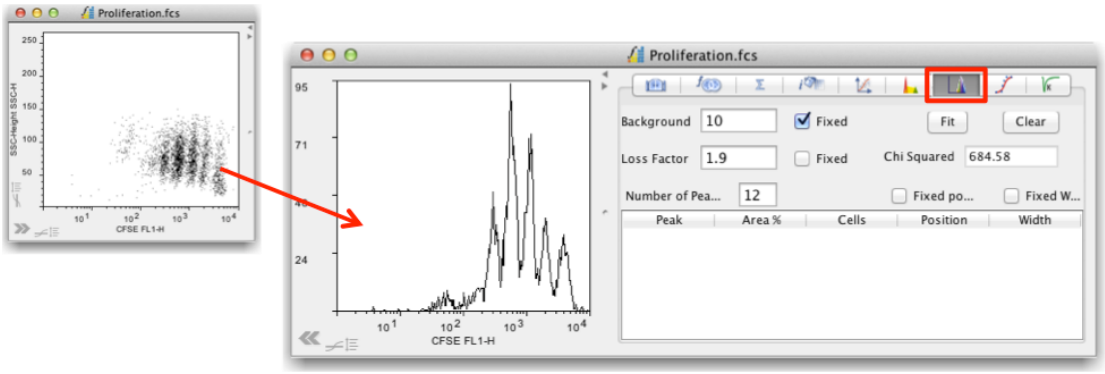


In the Gate List tab, extra gates, such as for apoptosis, can be turned on and colors can be changed.

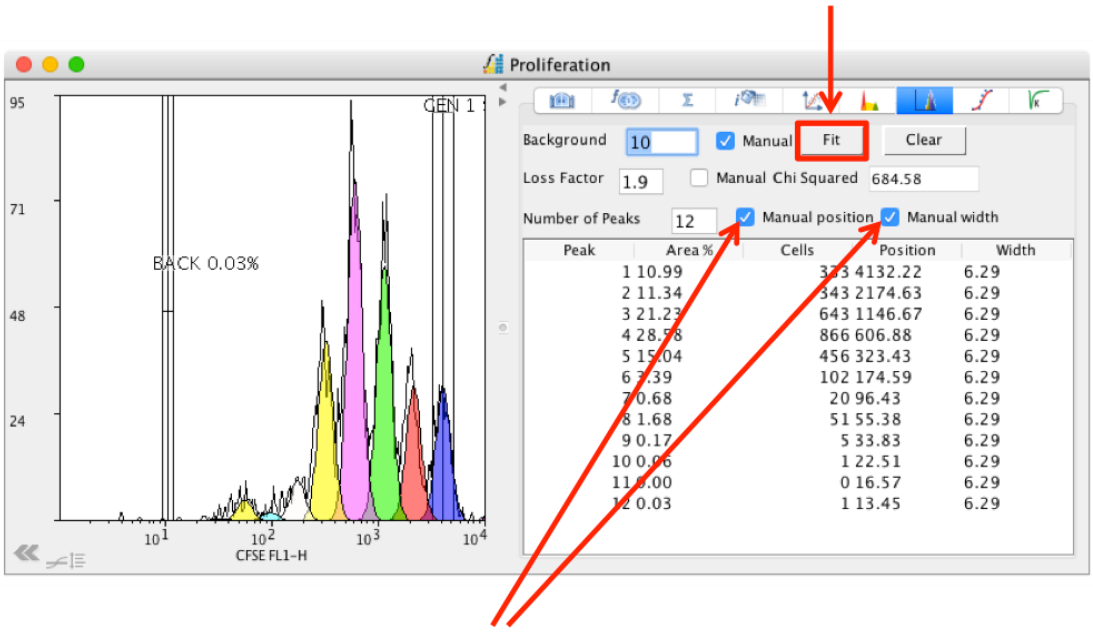


Proliferation

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.

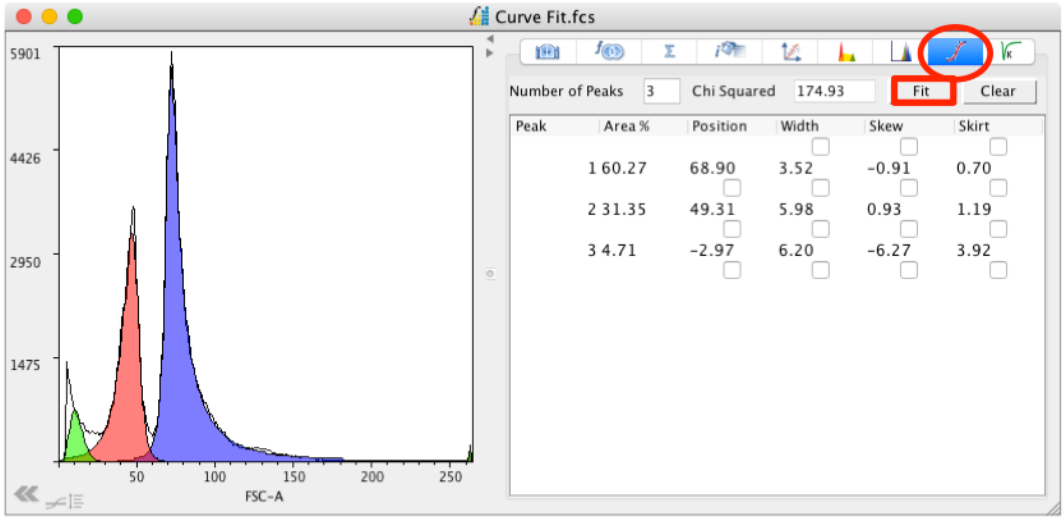
To perform proliferation analysis for multiple files, add them all to a group by selecting them in the File Inspector and choosing ‘Group’ → ‘Add to New’. Now, when clicking ‘Fit’, the curve fitting will be performed on all samples but independently from each other.

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.

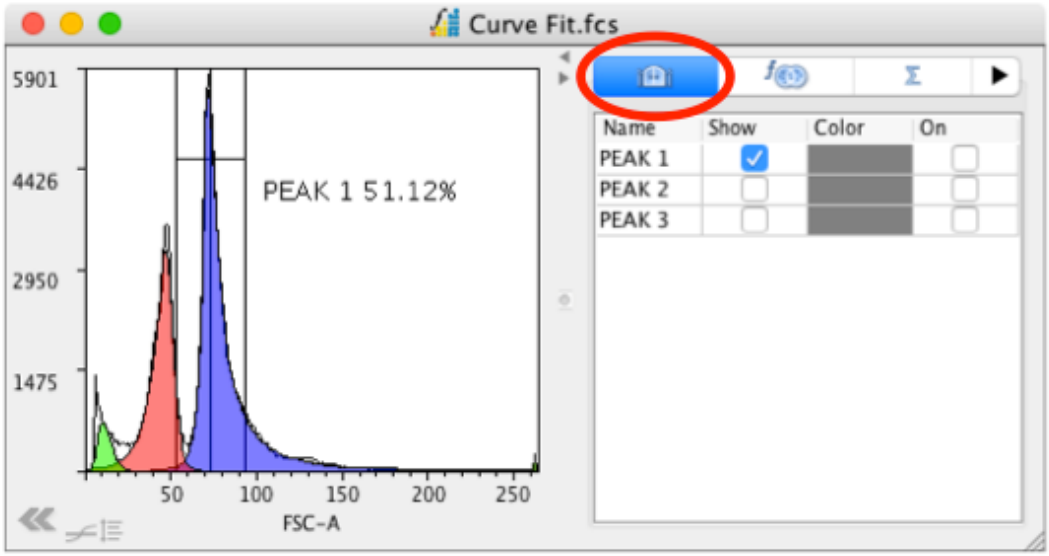
Then, tick ‘Manual position’ and ‘Manual width’ and add all of the GEN 1 gates that you wish to be governed by the master into a group. If the loss factor is not set to manual, then it will be re-calculated for each plot independently. The GEN 1 gate does not need to be displayed in order to group the 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.

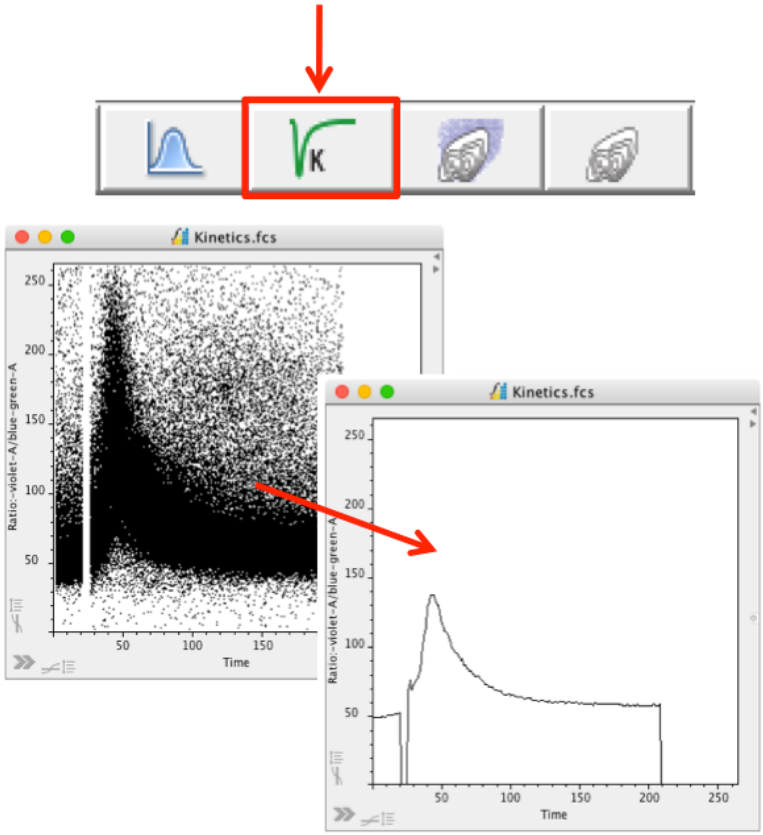


Gates relating to each peak can be turned on and off in the Gate List tab.

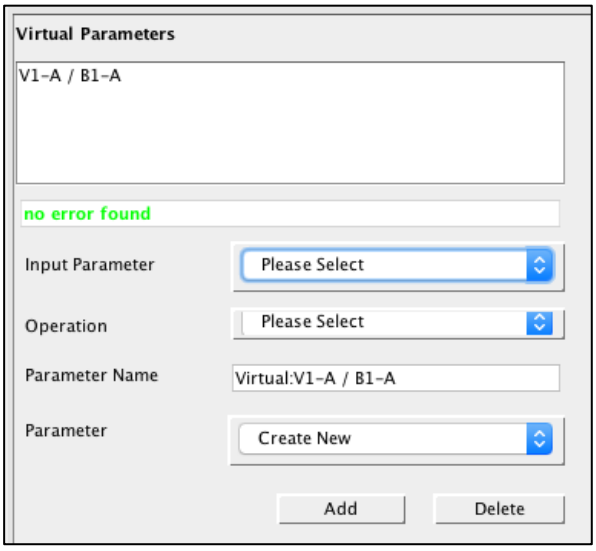


Kinetics

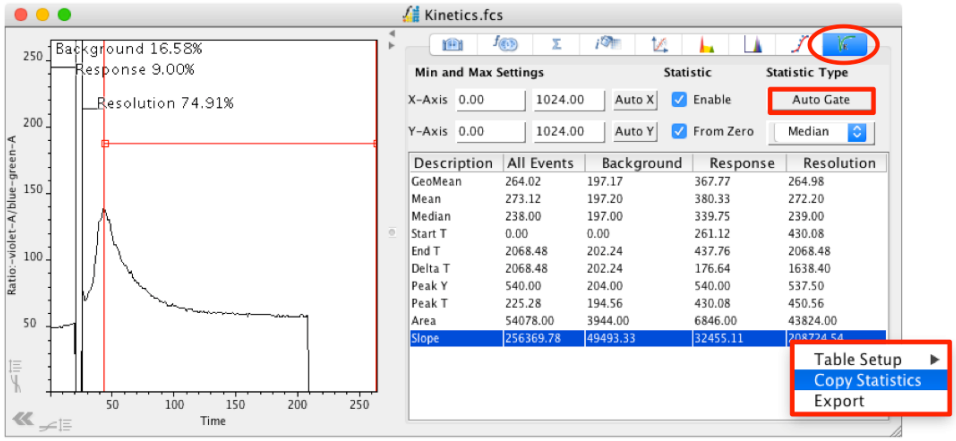
To assess cell cycle kinetics, display the data as a ratio-metric parameter versus time. Then toggle to a kinetics plot using the button in the tool bar.



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

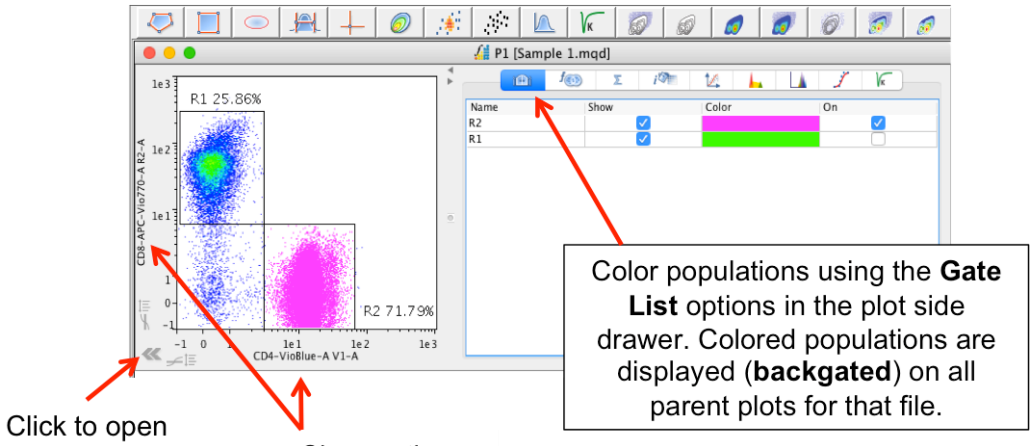


- Select the Kinetics tab in the plot side drawer and click ‘Auto Gate’. This will create Background, Response and Resolution gates. The data will be displayed in the side drawer and can be exported by right-clicking and selecting Copy Statistics.



Manipulating Dot Plots/Histograms

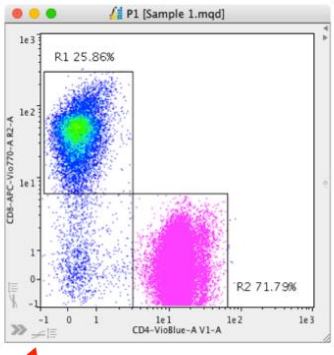
Change the graph type: dot plot, histogram, density plot, pseudocolor plot and combinations of these types



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

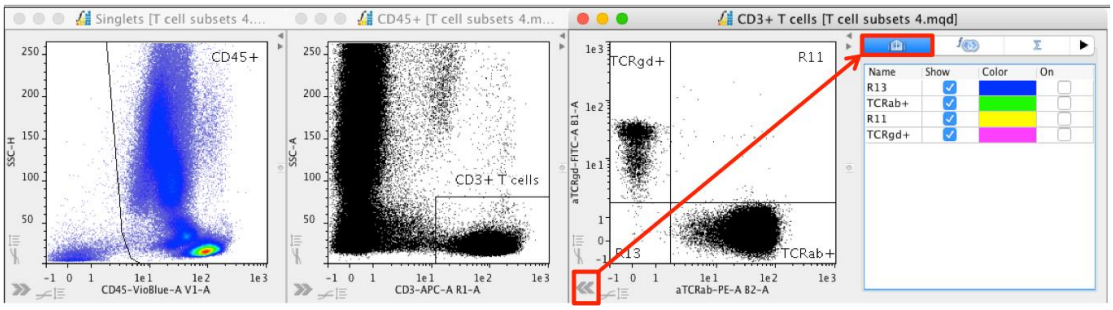
Change the parameters with a single left click on the *x* or *y* axis



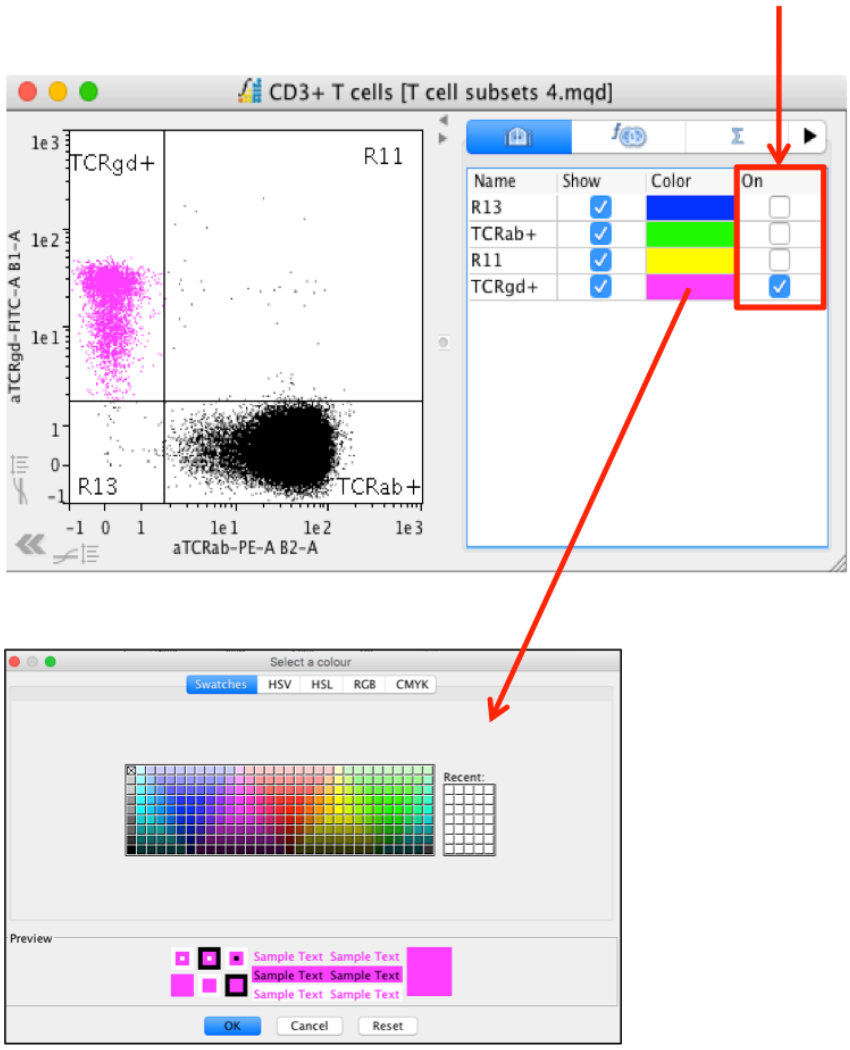
Click ≠ for log-linear transformations

Backgating

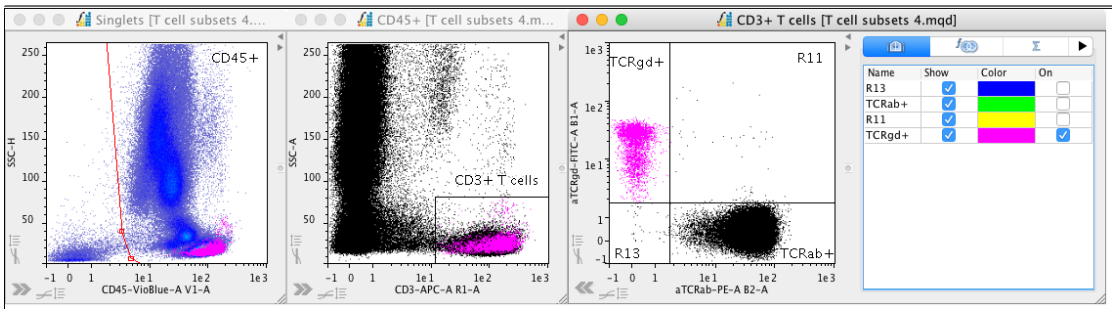
Once a gating hierarchy has been created, open the plot side drawer and choose the Gate List tab.



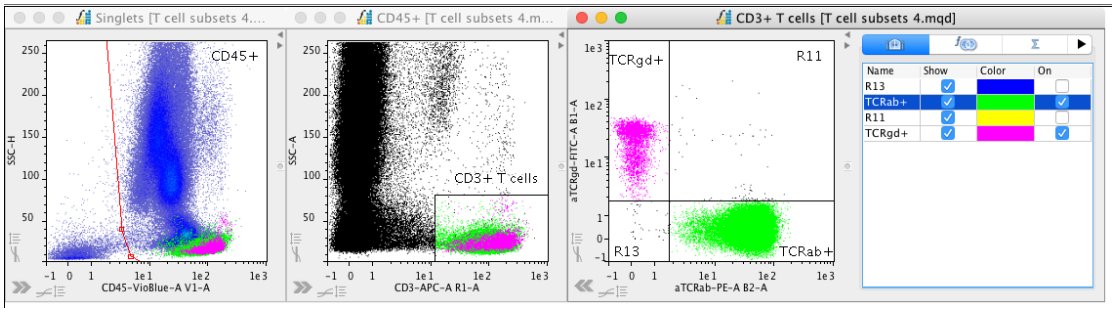
- To backgate a population, tick the box in the 'On' column. This will color the population at all higher levels in the hierarchy. To change the color, click on the color box in the side drawer and choose a new color from the selection provided.



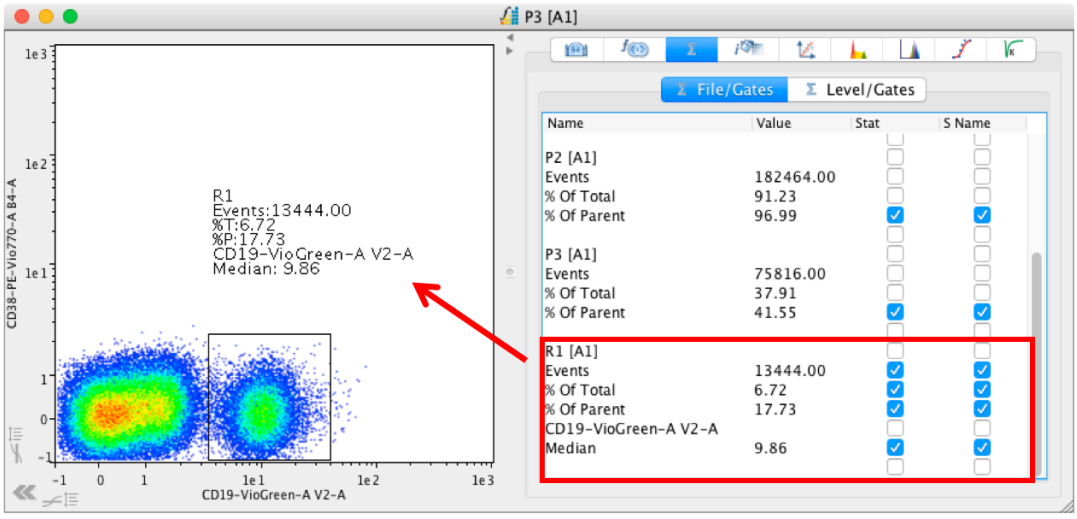
Once the gate has been selected in the side drawer, the cells are displayed at each level higher in the hierarchy. If real time update of plots has been selected in the Preferences menu, backgated events will update as the gates are moved.



Backgating can be performed on any number of populations.

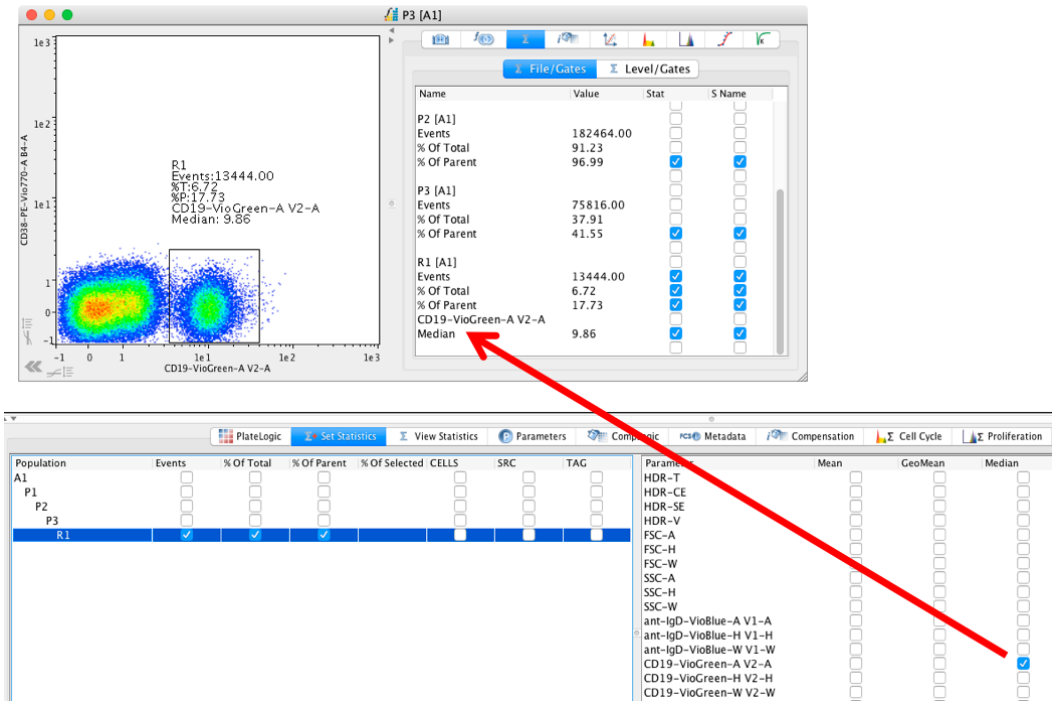


Statistics – Displaying Multiple Statistics on a Plot

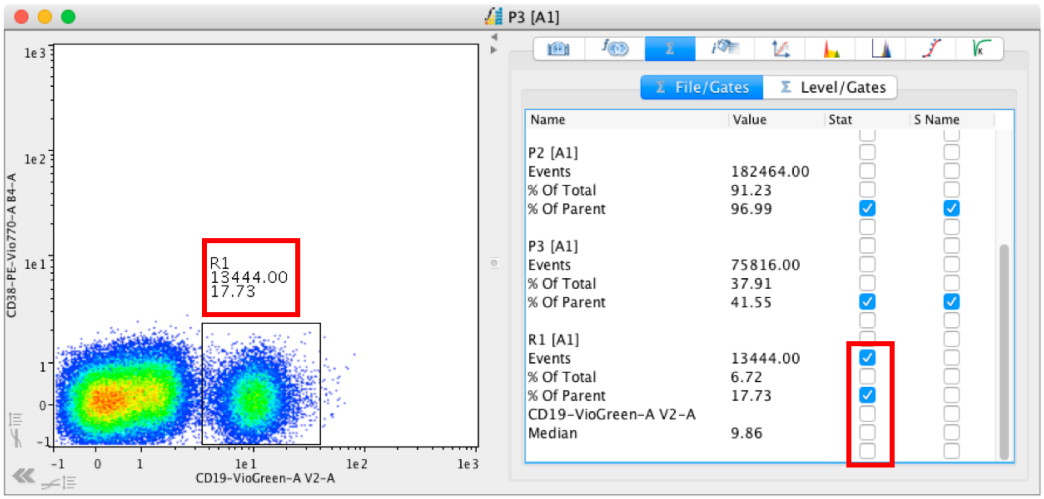


Tick the statistic (Stat) or statistic and name (S Name) relating to a specific gate to display on the plot

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.



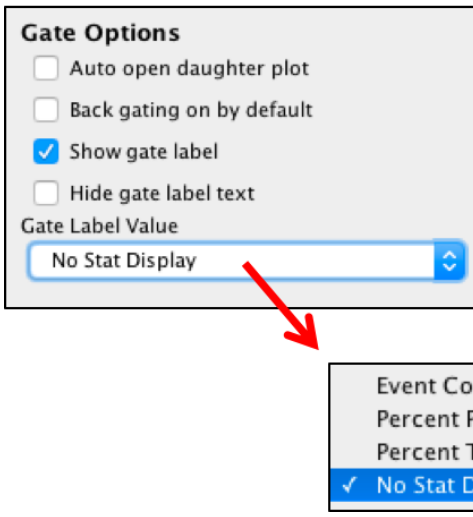
Untick the box in the 'S Name' column to display the statistic value only.



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

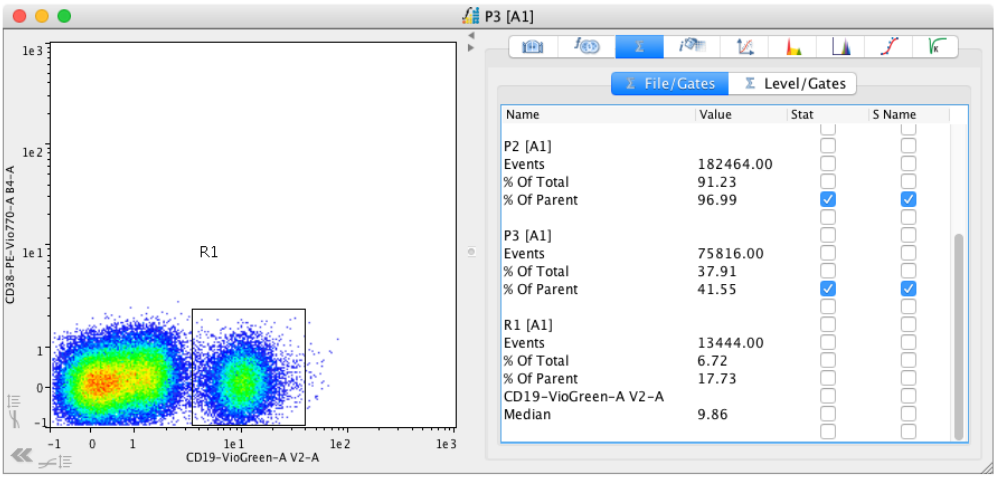
With all statistics turned off, the gate name alone will be displayed (if this is the setting in the program Preferences).

Preferences - General



Define the default settings for displaying a gate label (the gate name) along with a gate statistic (e.g. % Parent)

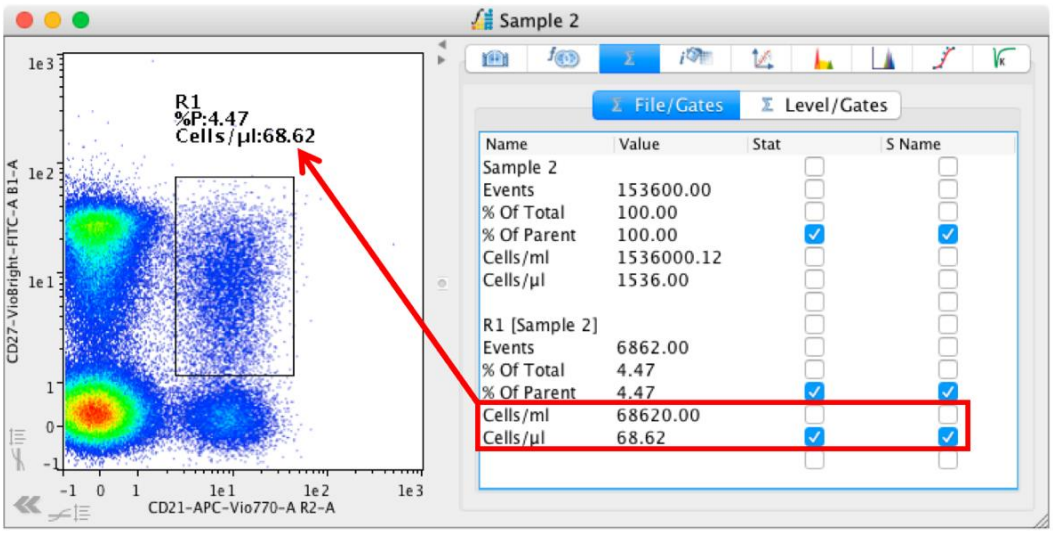
Here is the gate label (the gate name) displayed on the plot without any statistics.



MQD Volumetric Statistics – cells/volume

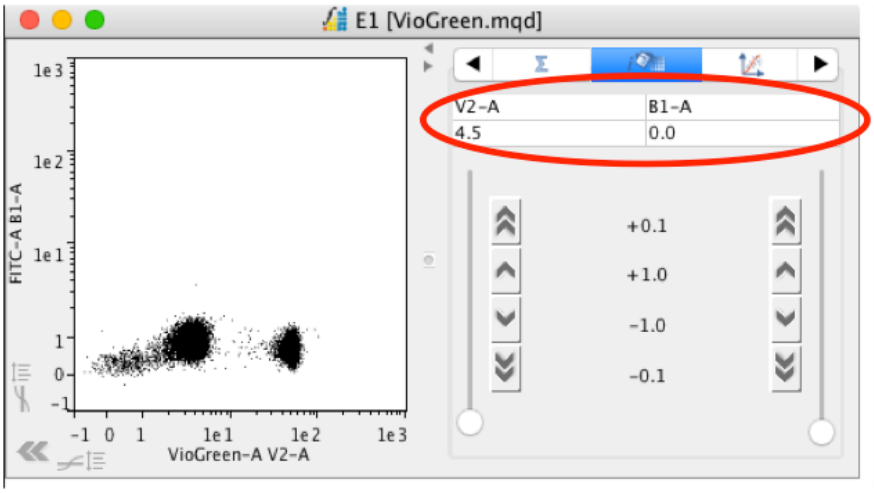
Volumetric gate statistics (cells/ml and cells/µl) can be displayed on dot plots and histograms for MQD files. These can be displayed as part of the gate label statistics from the statistics tab in the plot side drawer, as described above.

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.

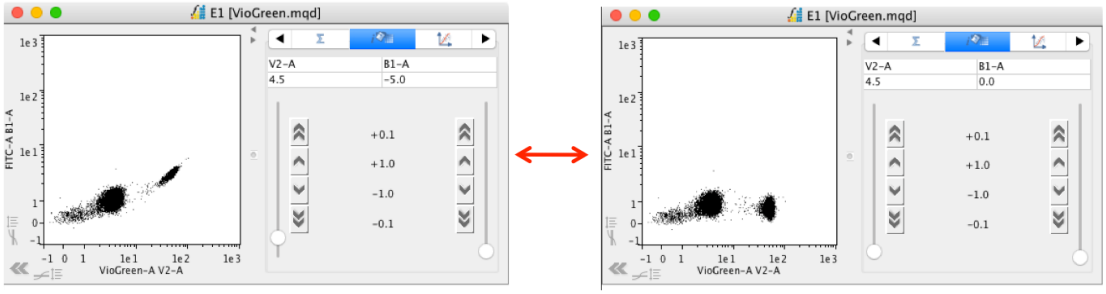


Manual Compensation

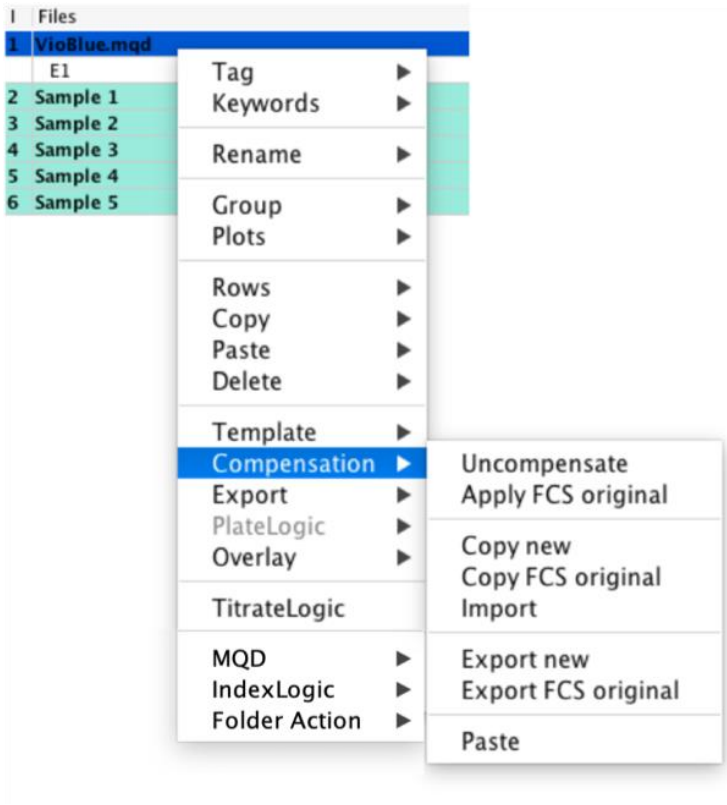
FlowLogic allows for easy, real-time compensation. To change the compensation of a file, open the plot side drawer and scroll through to the Interactive Compensation Matrix Window. Here, the two parameters shown on the plot are displayed with the compensation values (set when acquiring the sample).



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’.



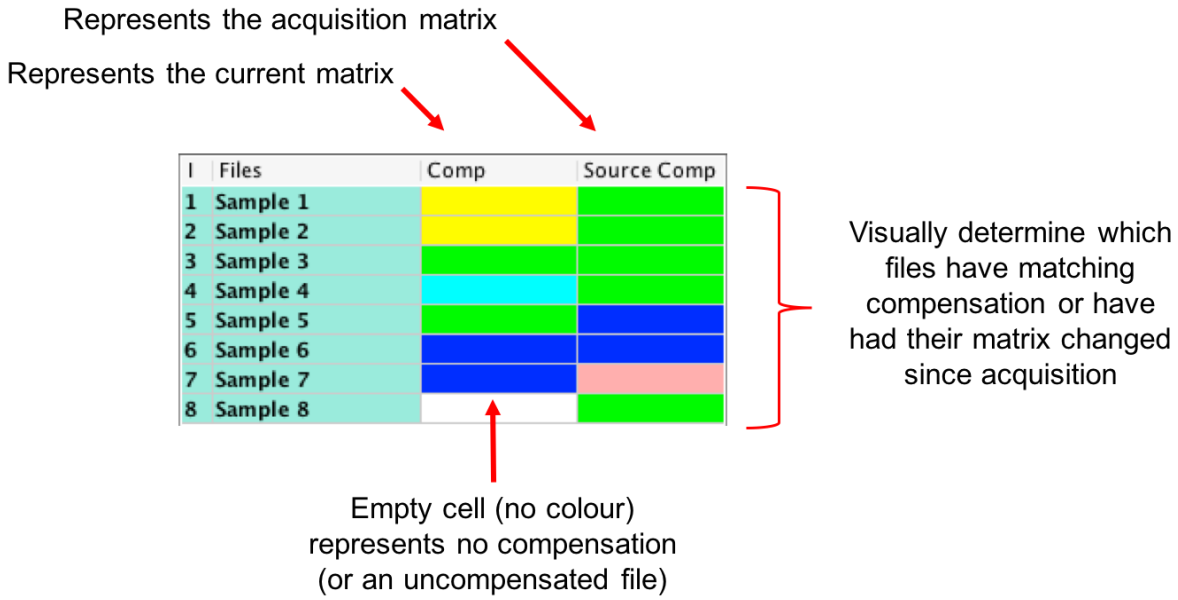
There are a number or options related to copying and importing compensation matrices:

- 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.

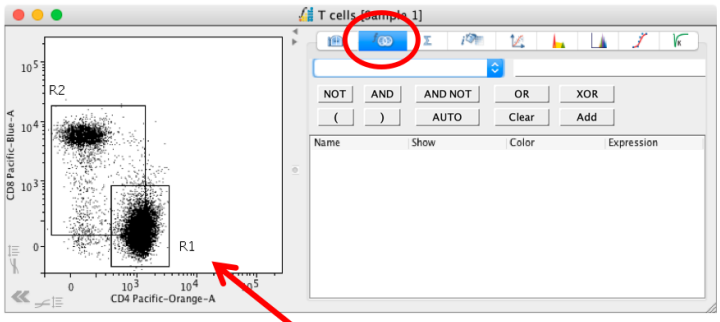


	Files	Comp	Source Comp
1	Sample 1		
2	Sample 2		
3	Sample 3		
4	Sample 4		
5	Sample 5		
6	Sample 6		
7	Sample 7		
8	Sample 8		

Boolean Gates

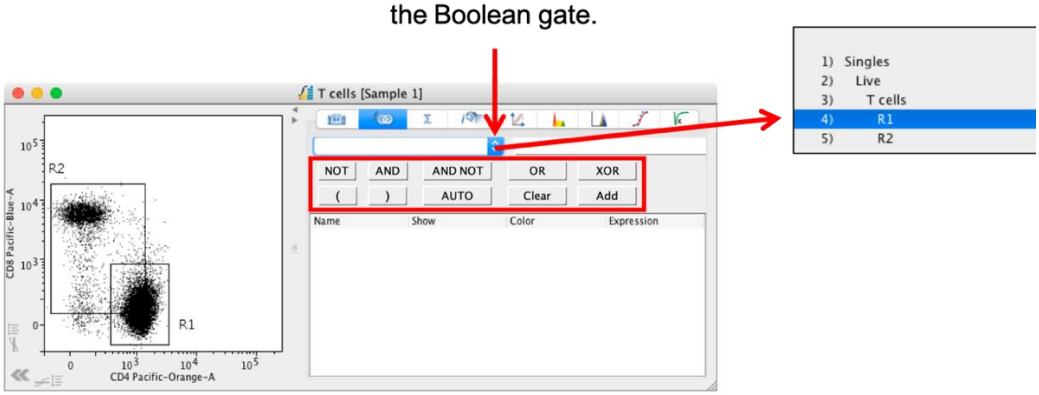
- Boolean gates allow for a more powerful form of analysis by defining how two or more gates interact.

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.

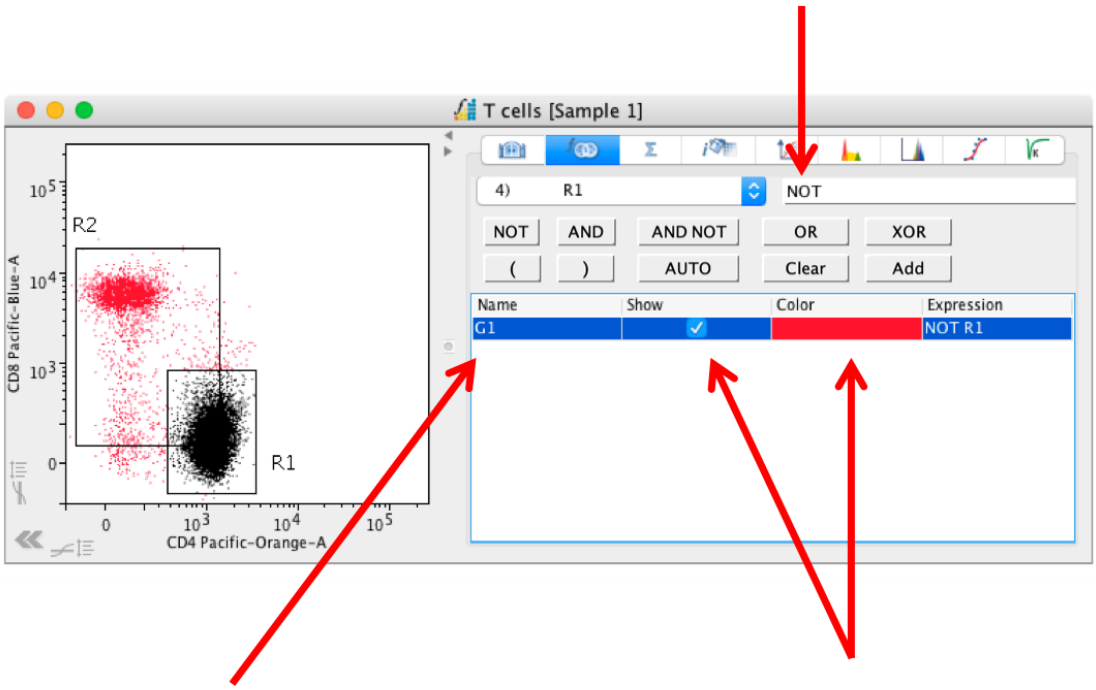
From the drop down list, select the gate that you wish to form part of the Boolean gate.



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.



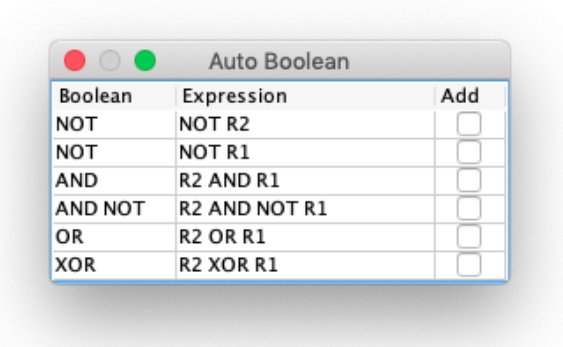
- The new Boolean gate is now called G1. The color of the Boolean gates can be changed by clicking in the color field. Tick the 'Show' option to color the newly defined population.

- 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'.

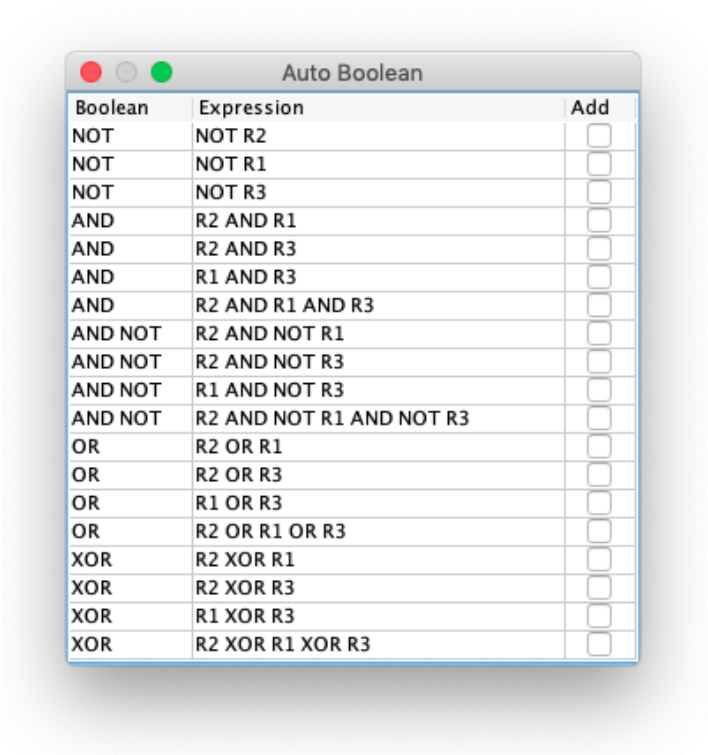
I	Files	Events	%Parent	%Total
1	**Sample 1**	326037		100%
	Singles	293752	90.10%	90.10%
	Live	173863	59.19%	53.33%
	T cells	15465	8.89%	4.74%
	G1	3296	21.31%	1.01%
	R1	12169	78.69%	3.73%
	R2	8698	56.24%	2.67%

- **Auto** – the Auto button opens a window displaying a range of available Boolean combinations given the existing gates. For the above example with gates R1 and R2 existing at this level of the hierarchy, there are six common options available in the pop-up window:



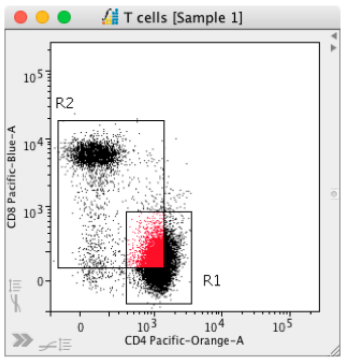
Simply tick the box in the “Add” column to automatically generate the new Boolean population.

If a third gate, R3, was added to the hierarchy, there would be many more Boolean combinations:



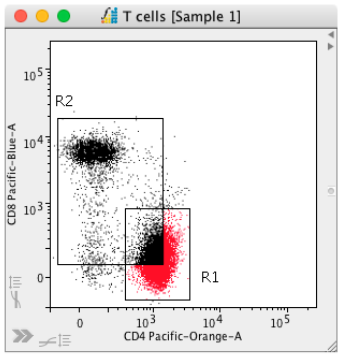
Following is an example of each of the Boolean gate types, based on the R1 and R2 gates listed above. The events within each Boolean gate are colored red.

AND
(R1 AND R2)



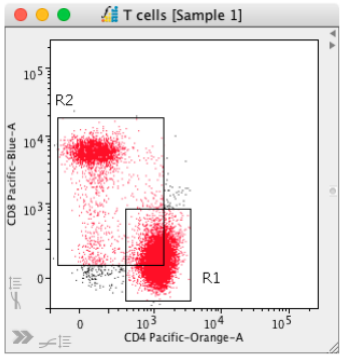
Everything in the intersection

AND NOT
(R1 AND NOT R2)



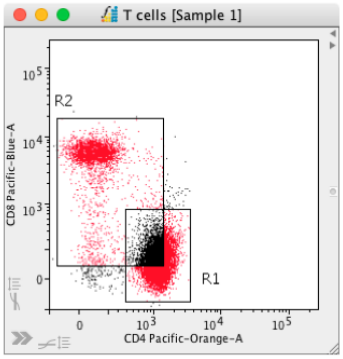
Everything in R1 minus R2

OR
(R1 OR R2)



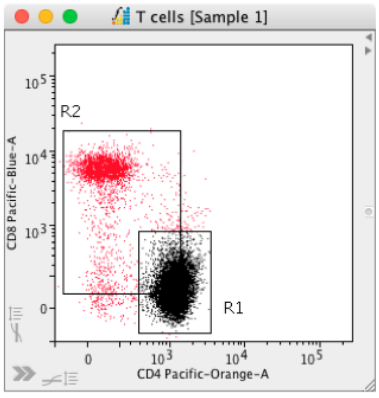
Everything in both gates

XOR
(R1 XOR R2)



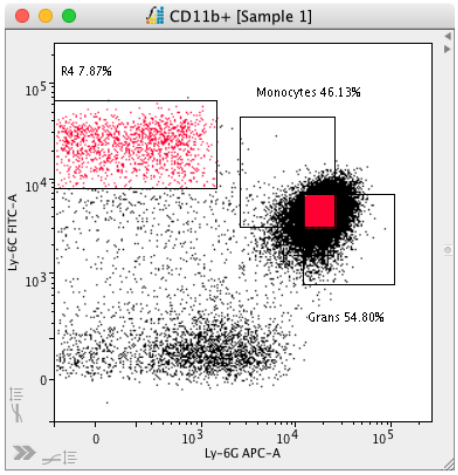
Everything within the gates minus the intersection

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.

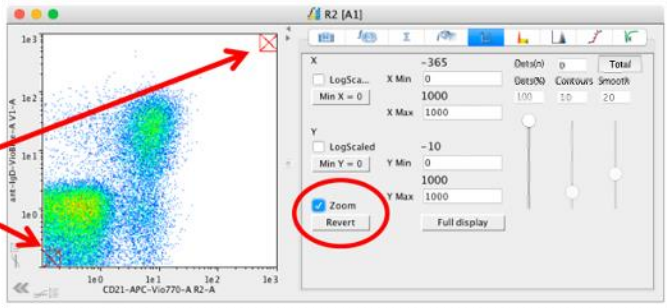
Scaling

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

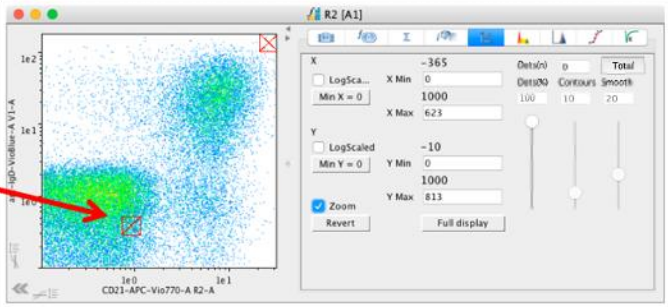
- The Scale feature can be found in the plot side drawer. Tick the Zoom box in the Scaling section to enable scaling.

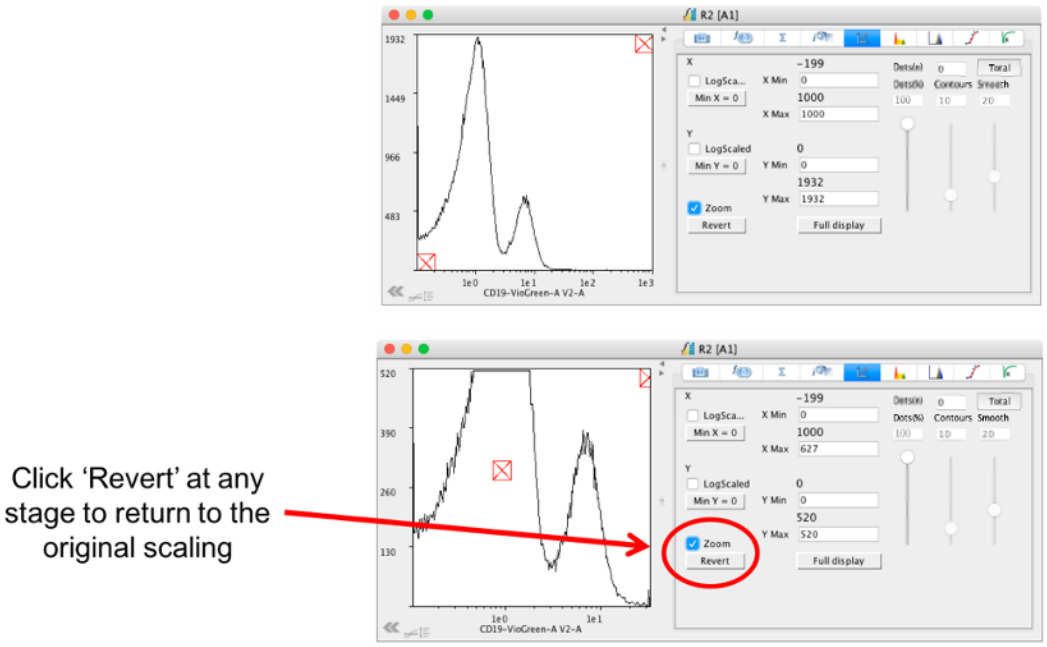
- Two red crossed squares will appear on the plot. One in the bottom left and one in the top right. To zoom, click and drag either of the red squares towards the opposite corner.

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.

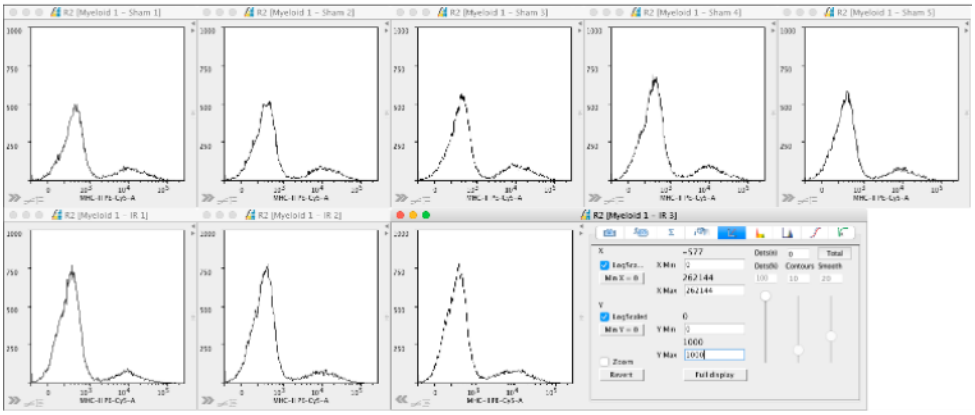
- When using the batch analysis tool (with the padlock on), the new scaling will be copied onto the subsequent plot. This can be adjusted on each plot individually if required.

- 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).

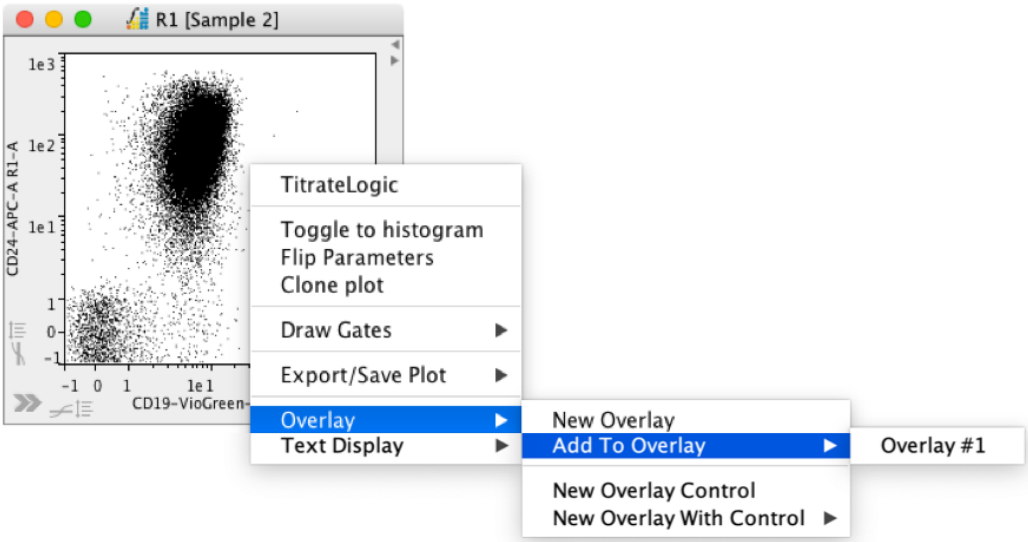
- Once the populations are selected, right-click and choose ‘New overlay’. The overlayed plot will be displayed in the Workspace.

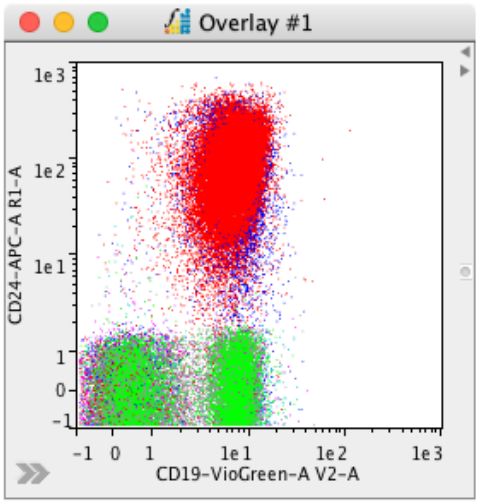
- Dot plots are saved in a separate window at the bottom of the File Inspector.

- 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’.

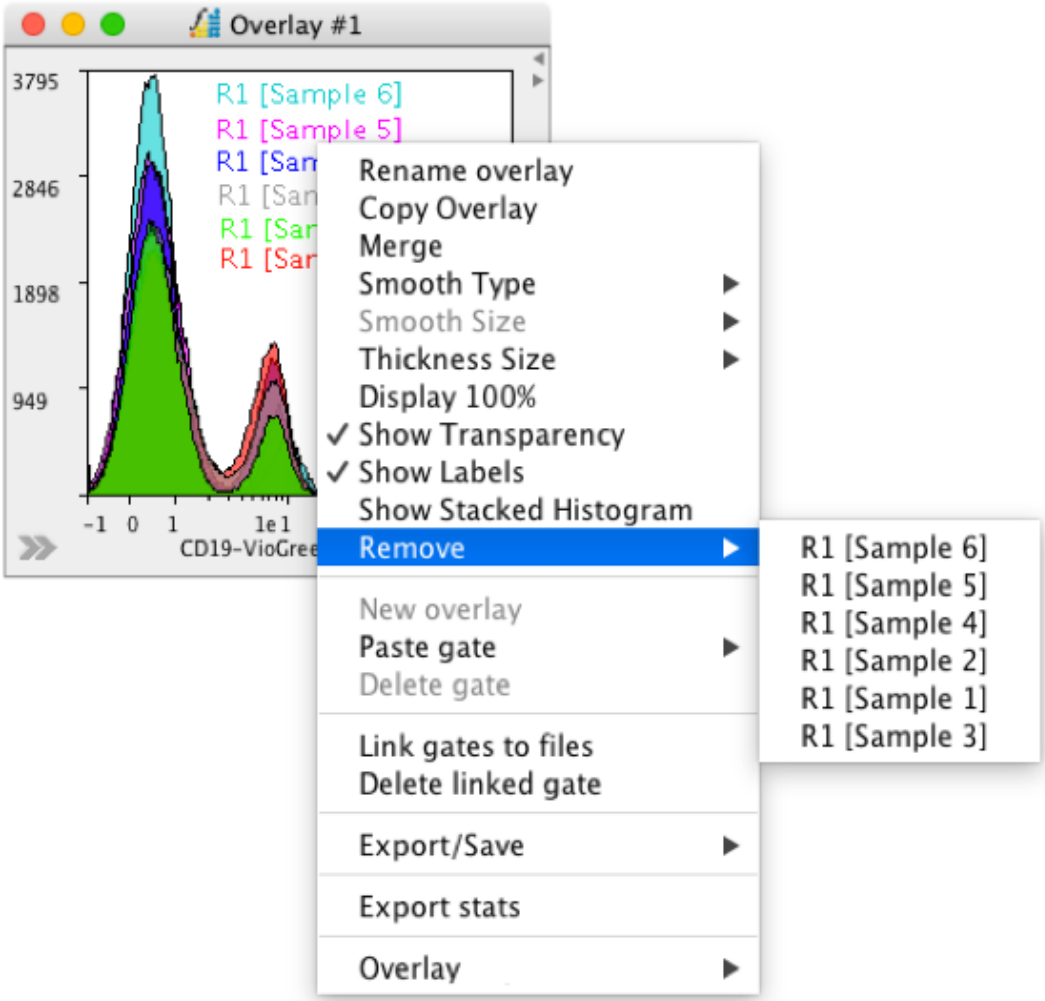
-



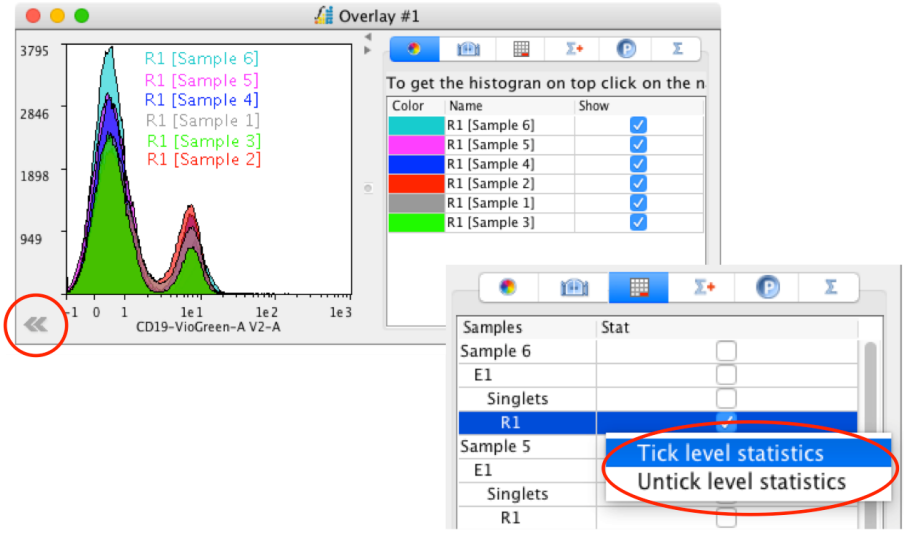


- Right-click on the overlay to bring up a range of menu options. The options include renaming, smoothing and displaying the y-axis range as percentage (Display 100%). The transparency can also be toggled on and off. Default overlay colors can be set and changed in the program Preferences. These can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

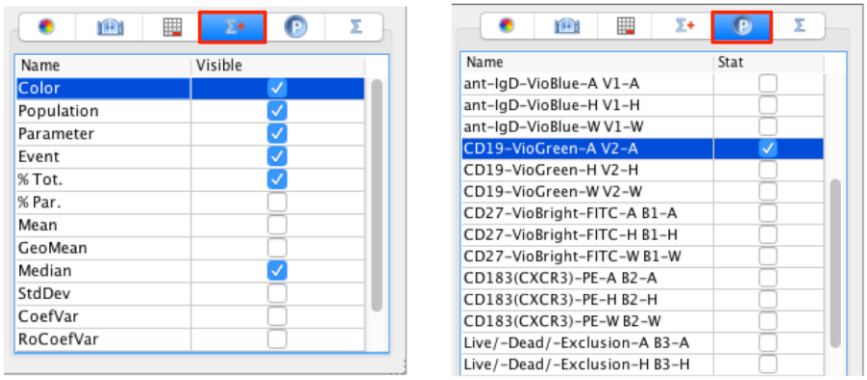
- To remove a file from the overlay, right-click on the plot area and select ‘Remove’ followed by the file name.



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

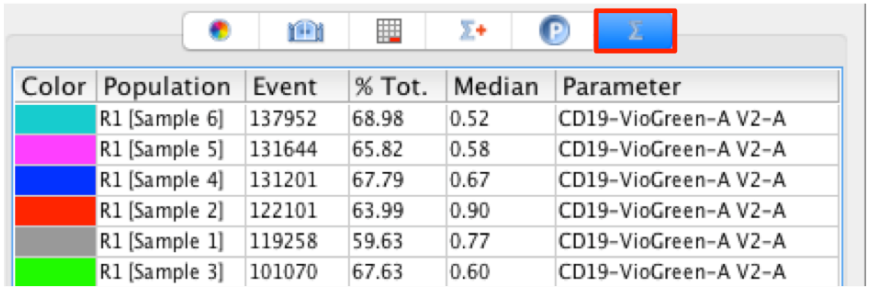


- Click in the color column to make a change.
- Re-order using drag-and-drop.
- Tick the populations to analyze. Right-click to tick all populations from the same level.



- Select the statistics to be calculated.

- Select the parameters linked to the statistics.



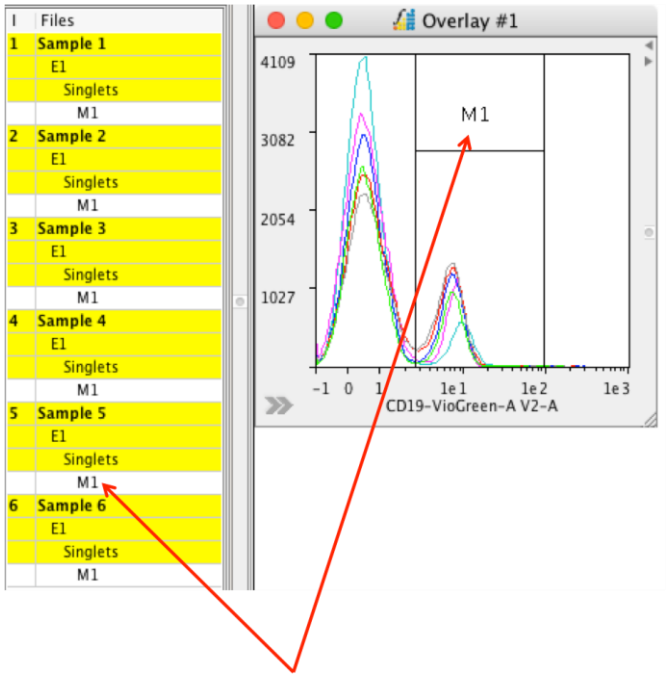
- Statistics are displayed and can be exported by right-clicking in the plot window and choosing ‘Export stats’.

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.

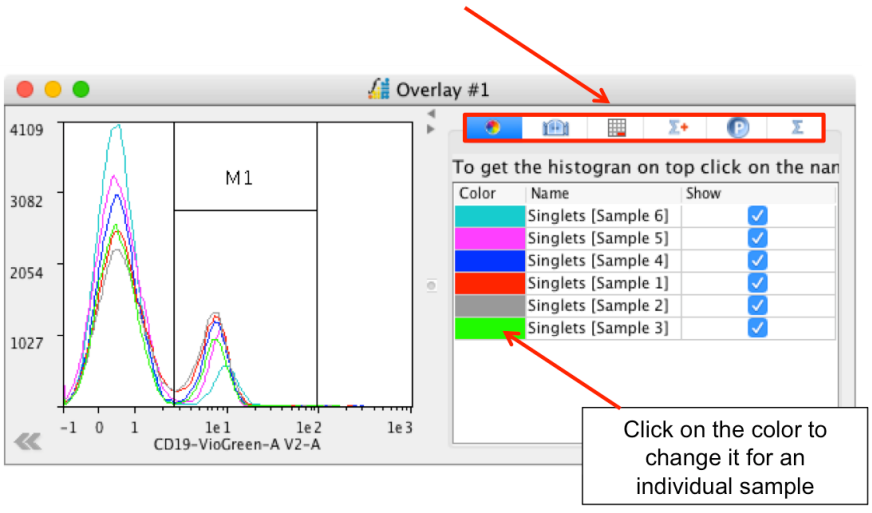
- Create either a dot plot or histogram overlay with two or more files.

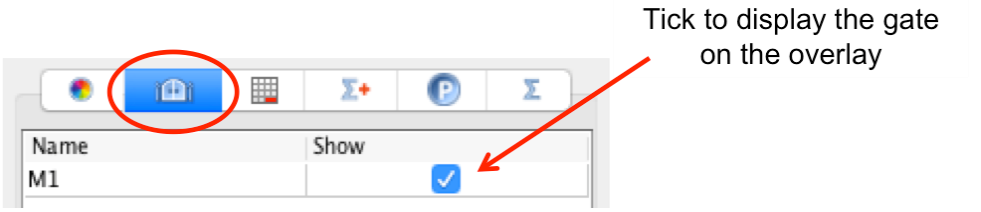
- Select an appropriate gating tool from the Workspace toolbar and draw the gate on the overlay. In this case, the gate M1 is added to all files.

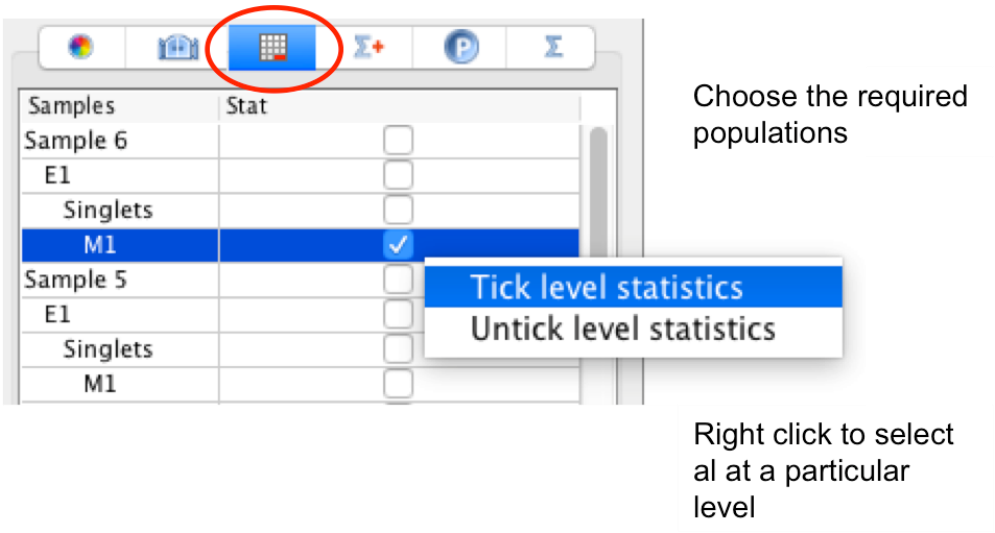


- If the gated population on the overlay is double-clicked, the daughter populations will be an overlay of the same files.

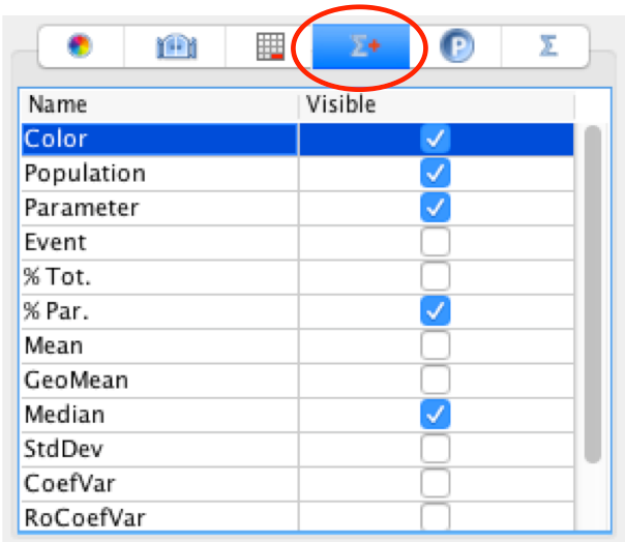
- To calculate statistics associated with the gated overlay, open the plot side drawer and sequentially click through the tabs to select the statistics to be calculated.



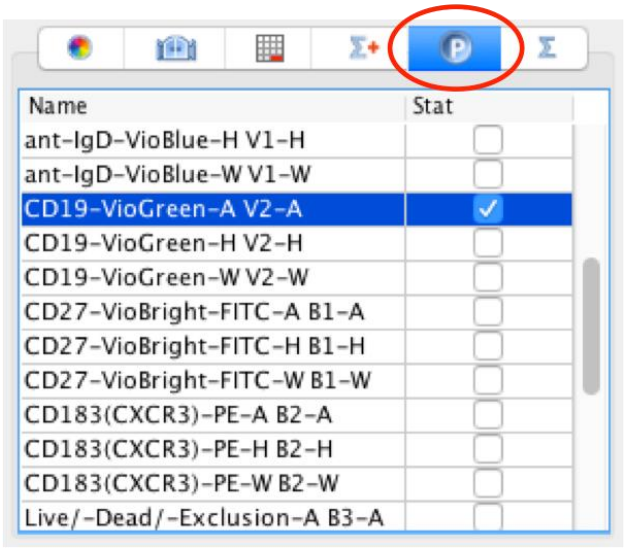




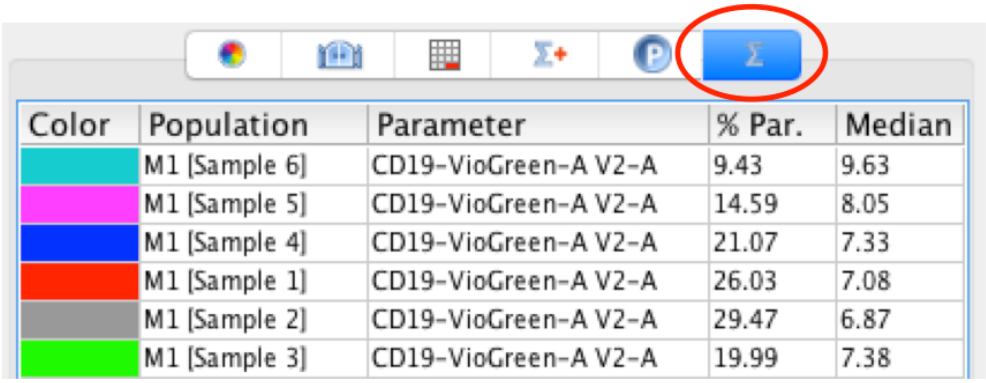
- Choose the required statistics.



- 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.

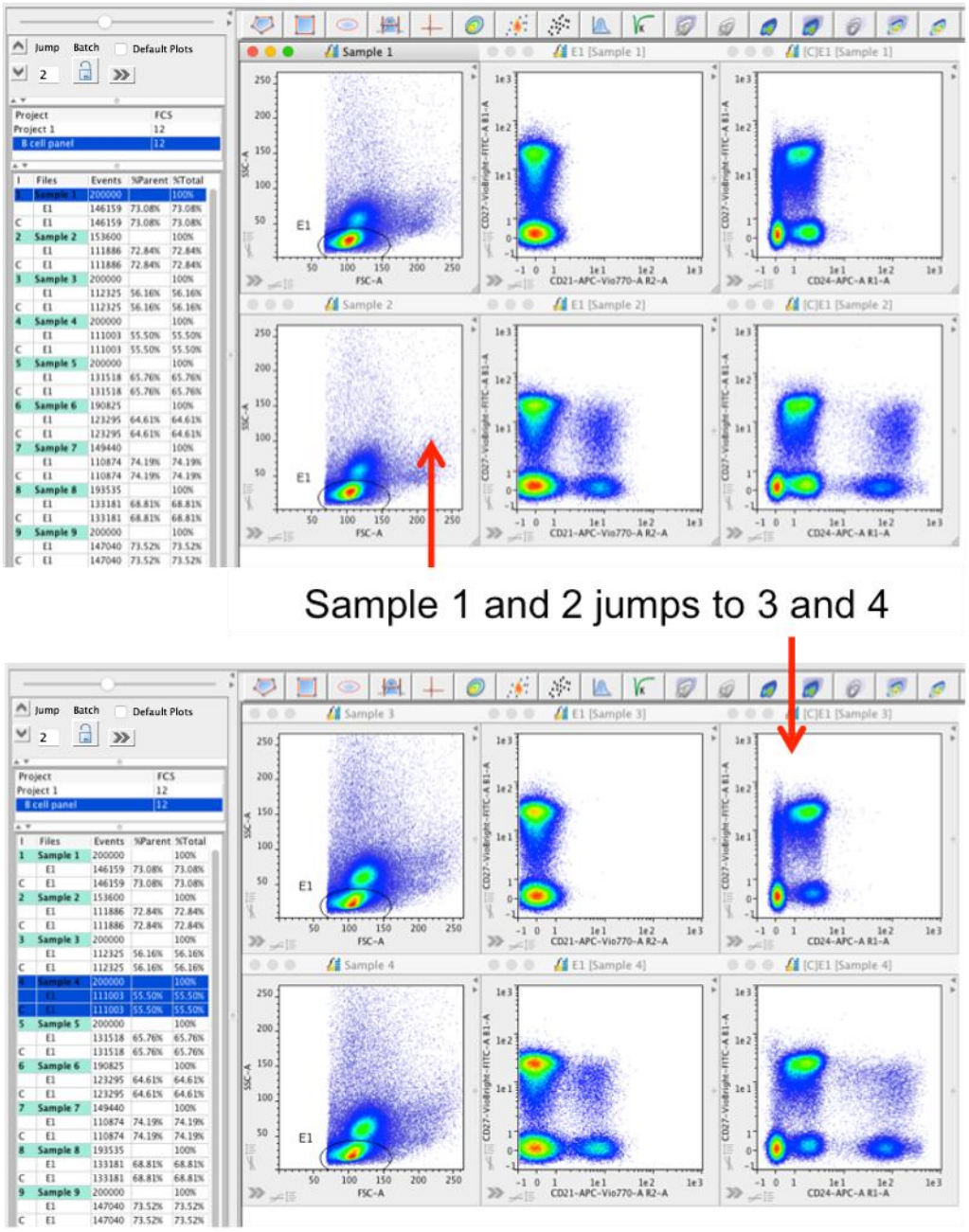
- The ‘Jump’ tool allows you to scroll through and view large data sets, multiple samples at a time.

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

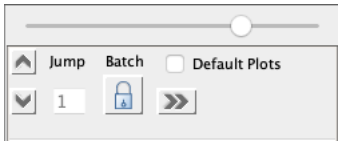
- Once you have applied gates to the FCS files, you can ‘scroll’ through your data several files at a time. For example, if you want to view 5 FCS files at a time, you can set the number in the Jump box to 5. Then when you click the up or down arrows, FlowLogic will jump through to the next 5 samples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

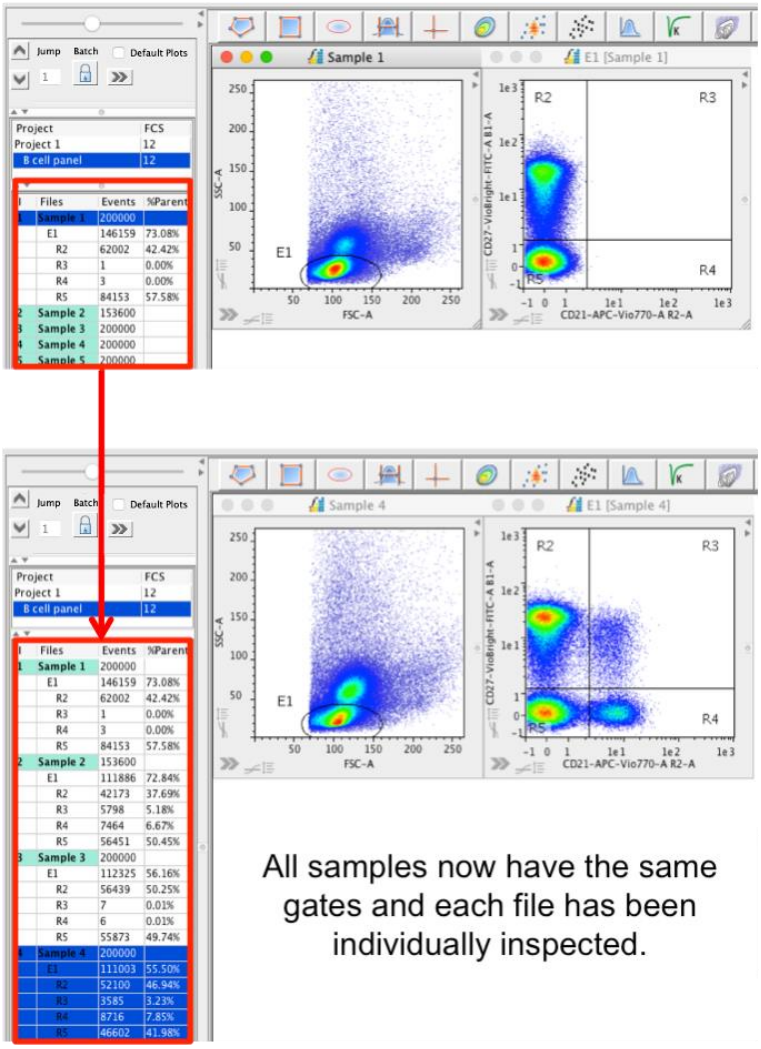
- In order to jump, click to unlock the ‘Batch’ padlock**,** set the number in the jump box and ‘Jump’ up and down using the arrow buttons.



- Ticking the Default Plots options whilst plots are displayed in the screen will pin those plots to the Workspace. New plots can then be opened and when the Jump function is used, the original plots will remain in the Workspace whilst the Jump feature is applied to the more recently opened plots. 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.
-
- 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.

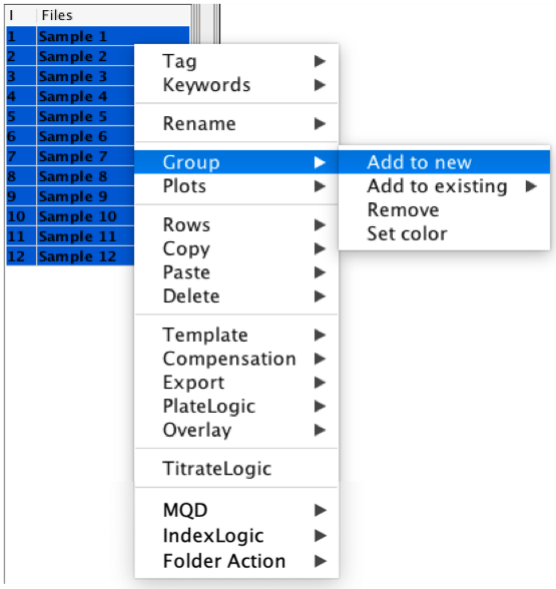


- All samples now have the same gating analysis and each file has been individually inspected. The statistics are ready to be exported.
- An alternative to batch analysis is to create Groups.

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.

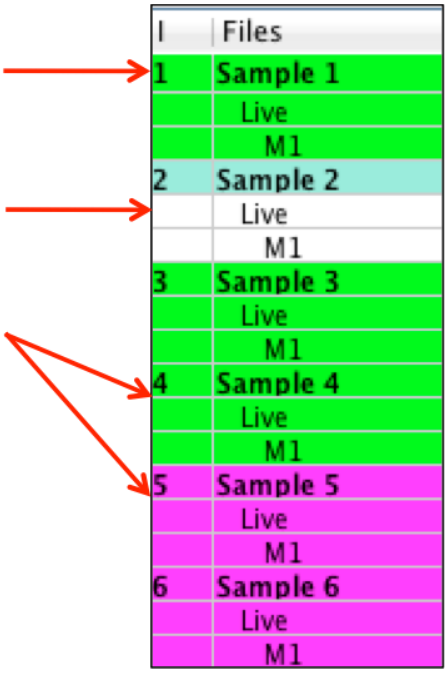
Select all files to be grouped, right-click and select Group → Add to new.



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.



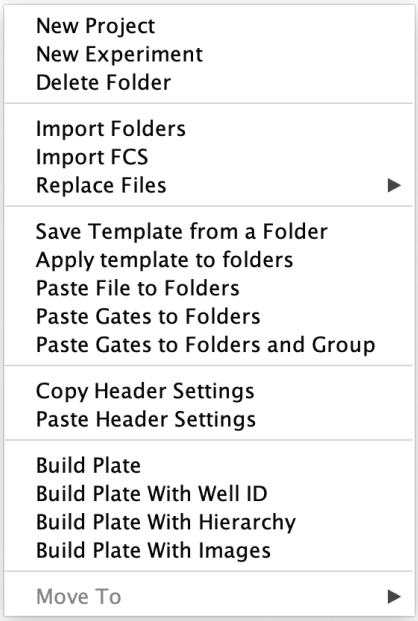
Adjustments to individual gates are automatically updated to all within the group, along with all calculated statistics.

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.

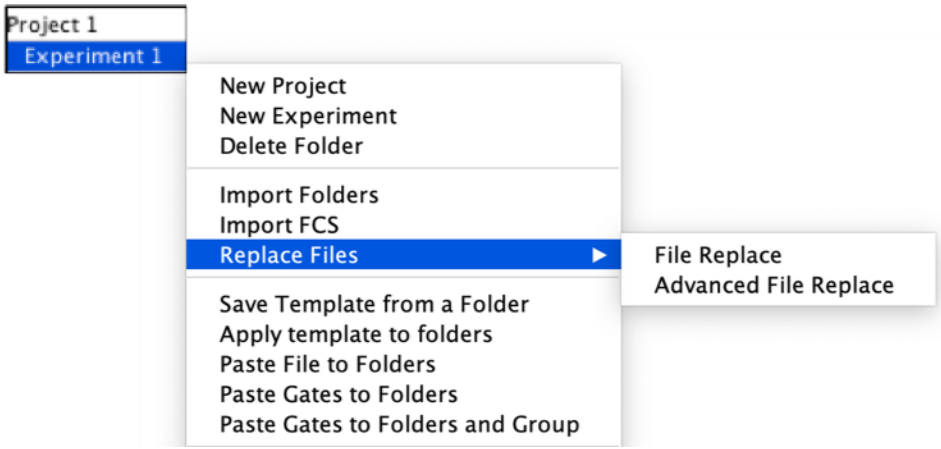
- By default, FlowLogic starts with one Experiment folder and one Project folder, as shown below. Right-clicking on these folders opens a menu with options to create new folders and delete folders. Double-clicking on the folder allows it to be renamed.

- 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 88 for more details).

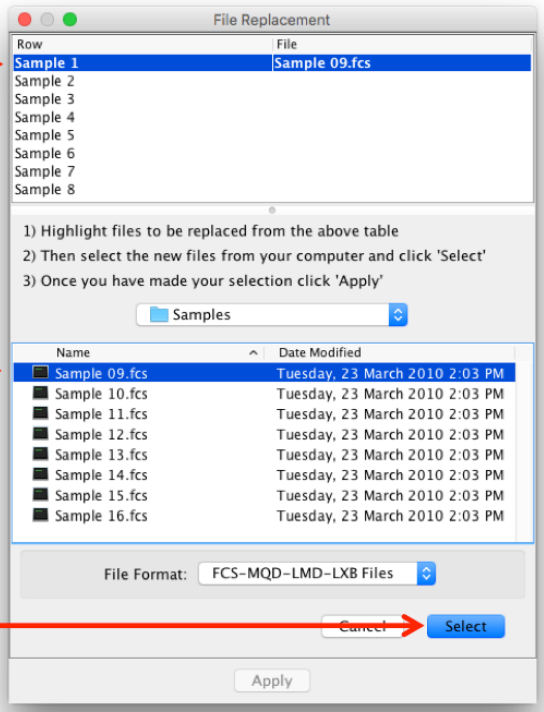
Advanced File Replace
This option allows you to specify the individual files that you wish to swap for each file in the File Inspector.

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.

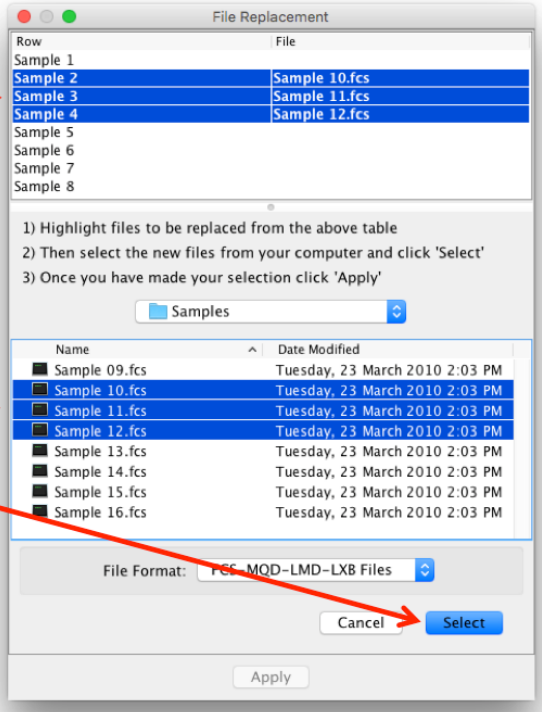
Then, select the file that you wish to replace it with.

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.

Save Template from a Folder - global analysis templates can be saved from an Experiment Folder and can contain all elements from an analysis including:

- Gating hierarchies
- Overlays
- Plates and heatmaps
- Graphs
- Statistical analyses
- Reports

These templates are very useful for repeated analyses, such as screening experiments. There are a number of rules that need to be followed when creating and saving a global analysis template. For more information, see the section on page 224.

Apply Template to Folders – to apply a global analysis template, select one or more Experiment Folders, right-click and choose “Apply Template to Folders”, locate the desired template and click “Open”. The number of files in the Experiment Folder must match the number in the template. For more information, see the section on page 226.

Paste File to Folders – one or more files can be copied from one Experiment Folder and pasted, using this option, to another Experiment Folders.

Top copy a file, highlight it in the File Inspector, right-click → Folder Action → Copy File. Then right-click on a selection of Experiment Folders, right-click → Paste File to Folders. A copy of the files will be added to the selected folders. This can be useful if you would like specific controls for different datasets.

Paste Gates 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.

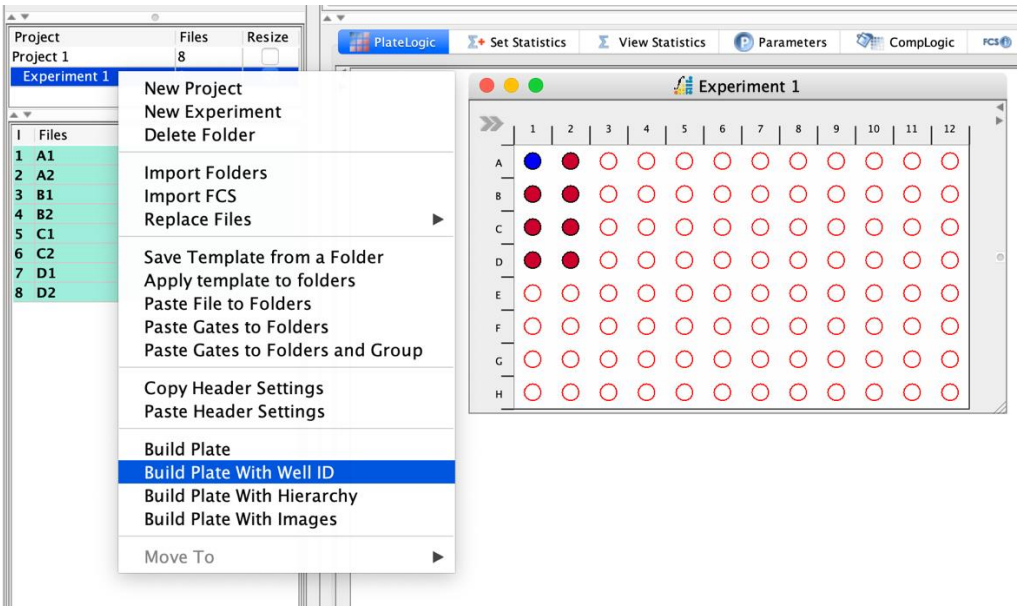
If a copied gate is part of a group, then this group setting will also be pasted to the files within the folder.

Paste Gates 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.

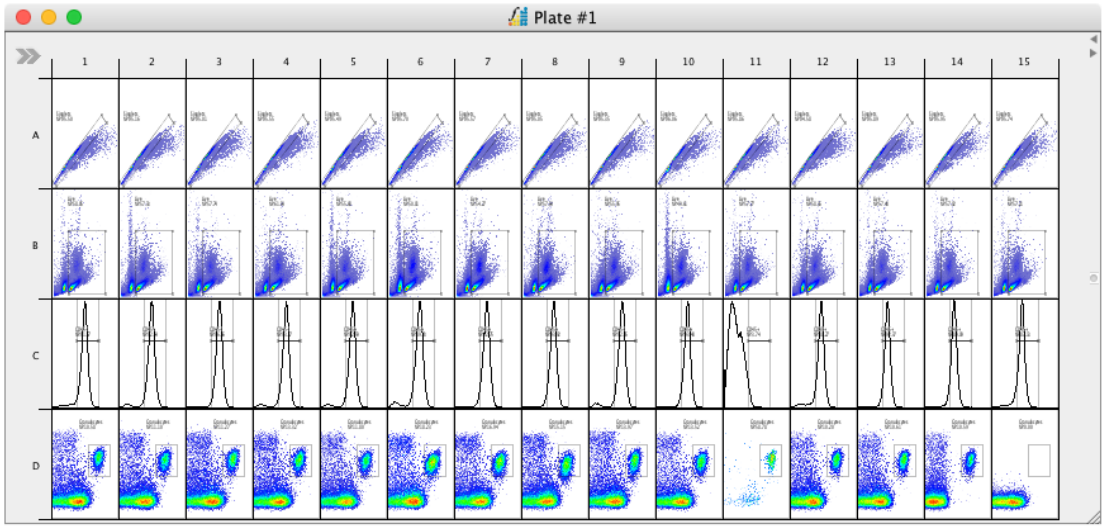
Build Plate – this creates a 96 well plate and loads the files in successive wells until the row/column is filled before starting in the next row/column.

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.

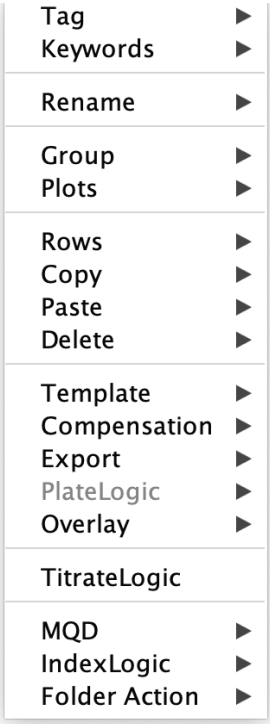


Build Plate with Hierarchy – this option will build a plate with one column per file. As gates are created, a new row will be added to the plate, displaying the subsequent daughter population and each level in the hierarchy will be assigned to a new dataset. This allows each level of the hierarchy to 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 92.

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 94.

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. For a detailed explanation, see page 100.

MQD –

- **Ungroup MQD Files** – 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.

  Split grouped MQD 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.

- **Load MQD/MQDFL gates** – gates created in MACSQuantify™ Software at acquisition can now be loaded in FlowLogic. If the MQD files contain the gate information, then right-click on the selection of files, choose 'MQD' → 'Load MQD/MQDFL'. The gating hierarchy will appear in the File Inspector and these can be manipulated like any other gate. Please note that the MQD gates when loaded in FlowLogic might not be a precise recreation due to the interpretation required by FlowLogic. However, in most cases, they should be very similar.

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.

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.

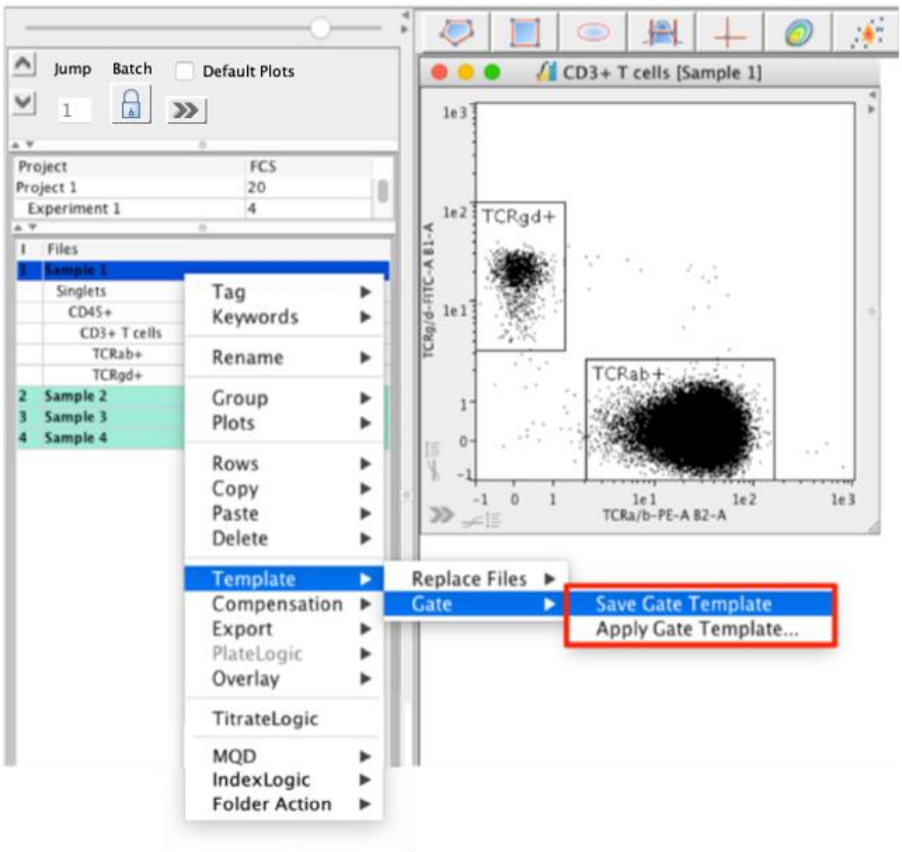
Folder Action –

- **Build One Folder per File** – this feature will take a selection of files from one Experiment Folder and place them individually into new Experiment Folders. The new Experiment Folders will be named with the name of the file added to it.
- **Copy File** – this feature copies a selection of files, which can be pasted into other Experiment Folders. To paste the selected files, select the new Experiment Folders, right-click → Paste File to Folders.

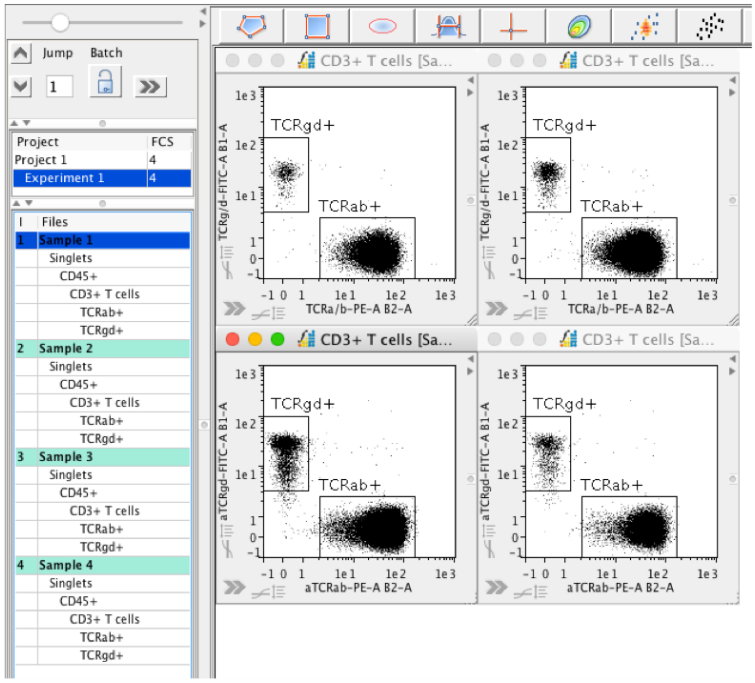
Gate Templates

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

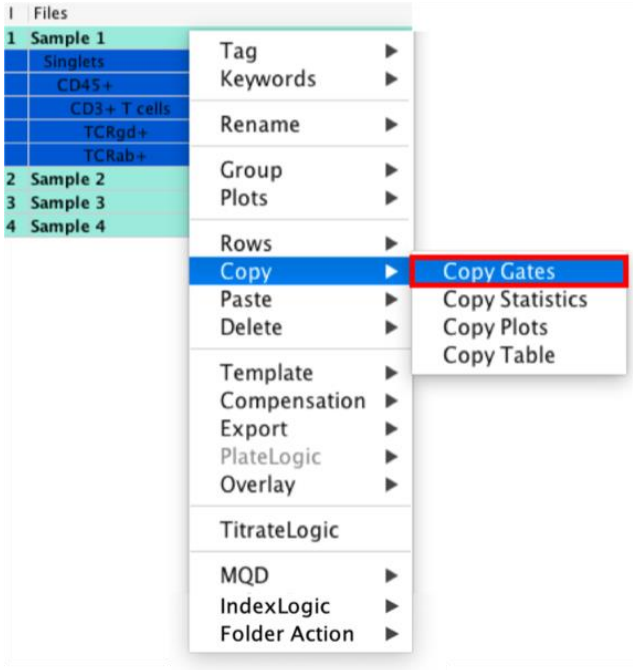
- After applying a series of gates to a file, right-click on the file name and choose Template → Gate → Save Gate Template. FlowLogic will ask you to name the template before saving it as a .GATELOGICTEMPLATE file.



- 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.



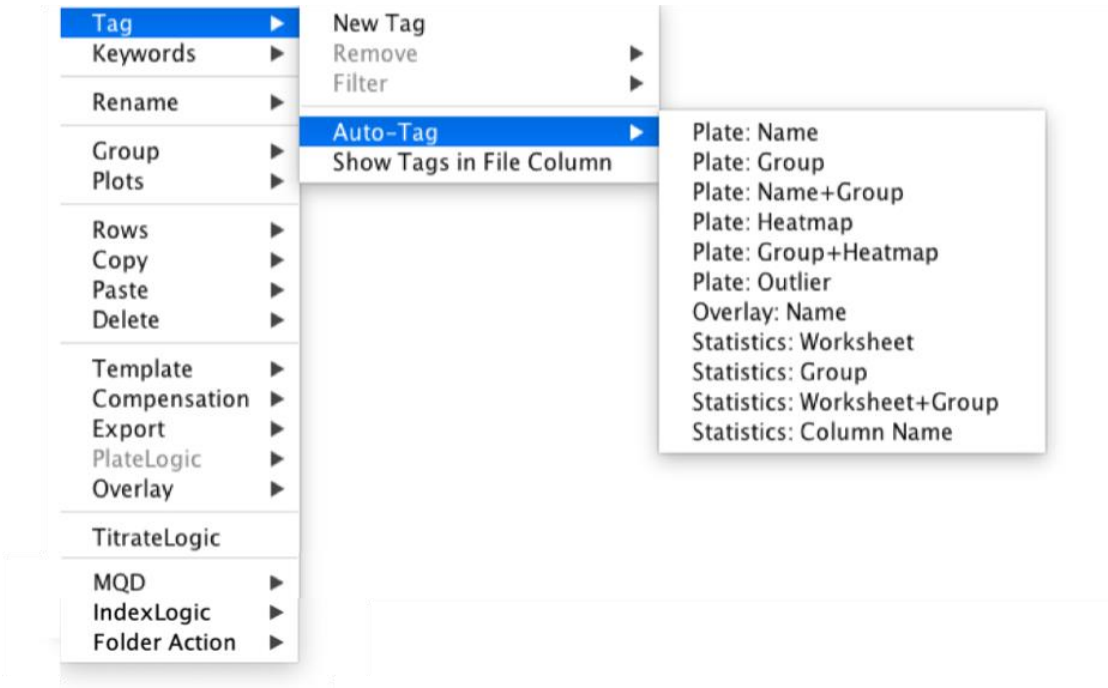
- An alternative to creating and applying templates within an experiment is to highlight the gates, right-click and select Copy → Copy Gates.
- Then, highlight the samples that you wish to apply the gates to and select Paste → Paste Gates or To All. The keyboard shortcuts Ctrl/⌘C and Ctrl/⌘V can also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



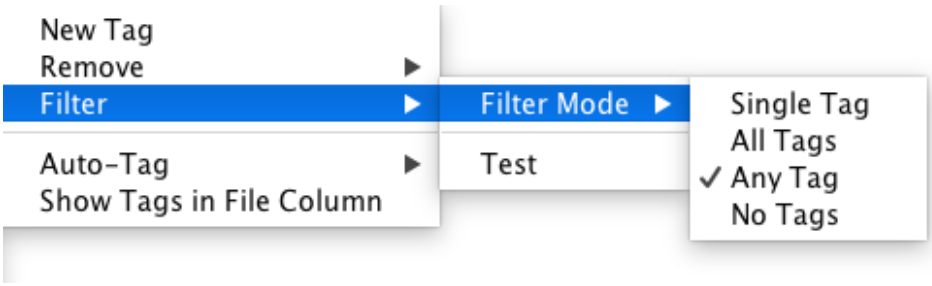
Tags

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.

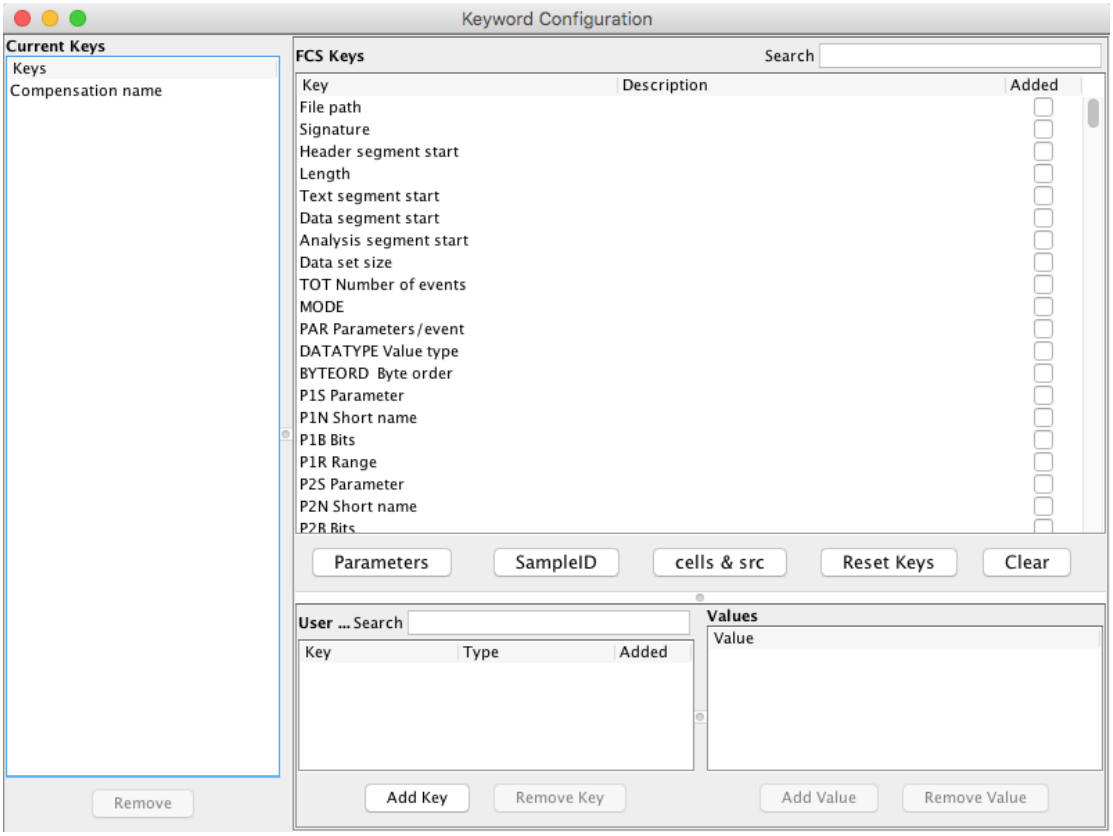


- Once tags have been assigned, right-click in the File Inspector, choose Tag → Filter. Set the Filter mode and then select the tag (in the example below, the tag is 'Test tag'. Only those samples with the specific tag will be displayed in the File Inspector.
- Auto-tags can be used effectively to display 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.

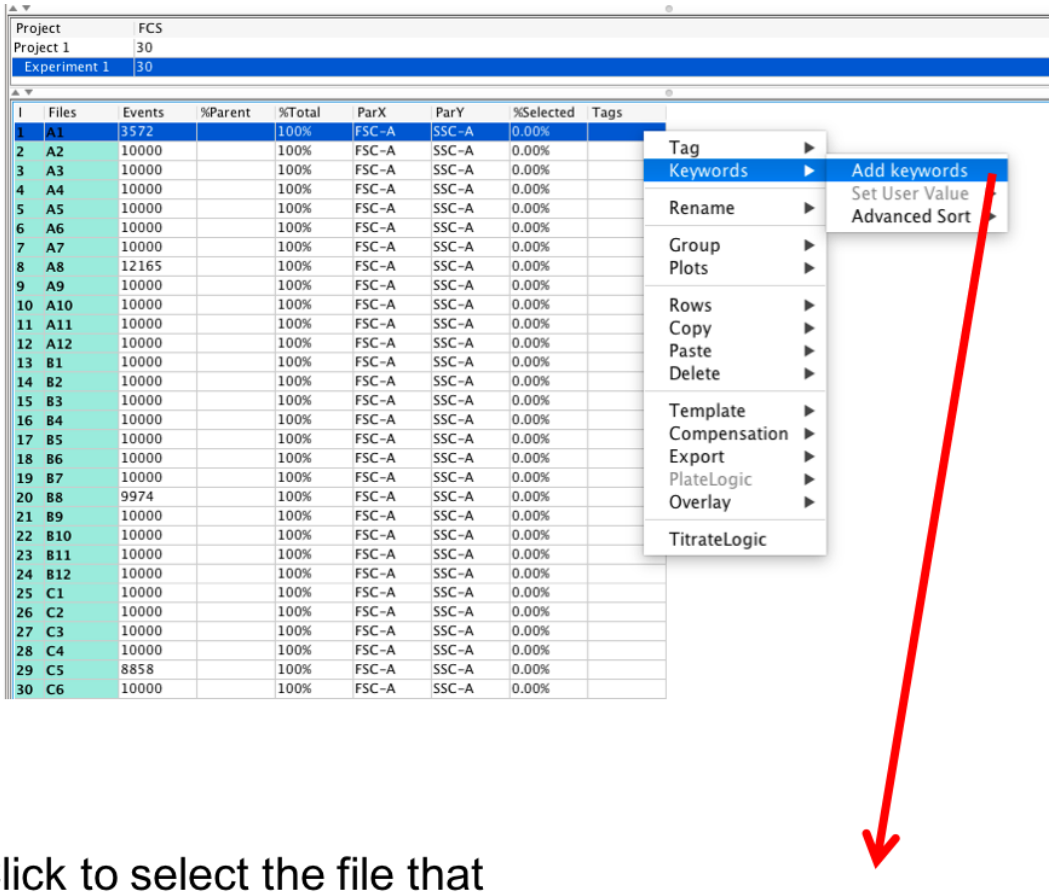


Keywords

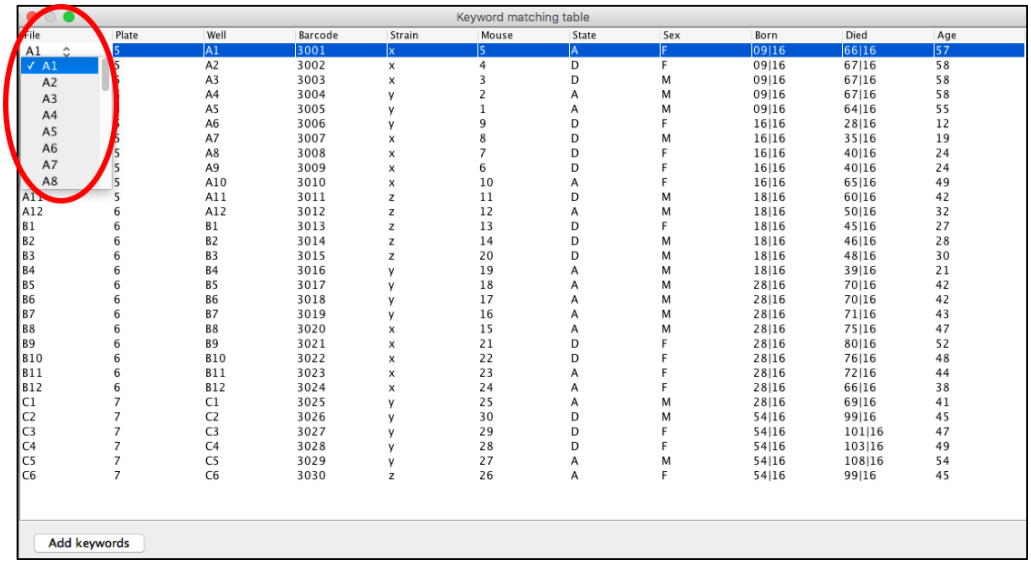
Displaying keywords with the Keyword Configuration window – keywords contained within the FCS file can be displayed in the File Inspector and subsequently used for reference or to sort samples. The Keyword Configuration window can be accessed through the Edit menu, under Keywords.



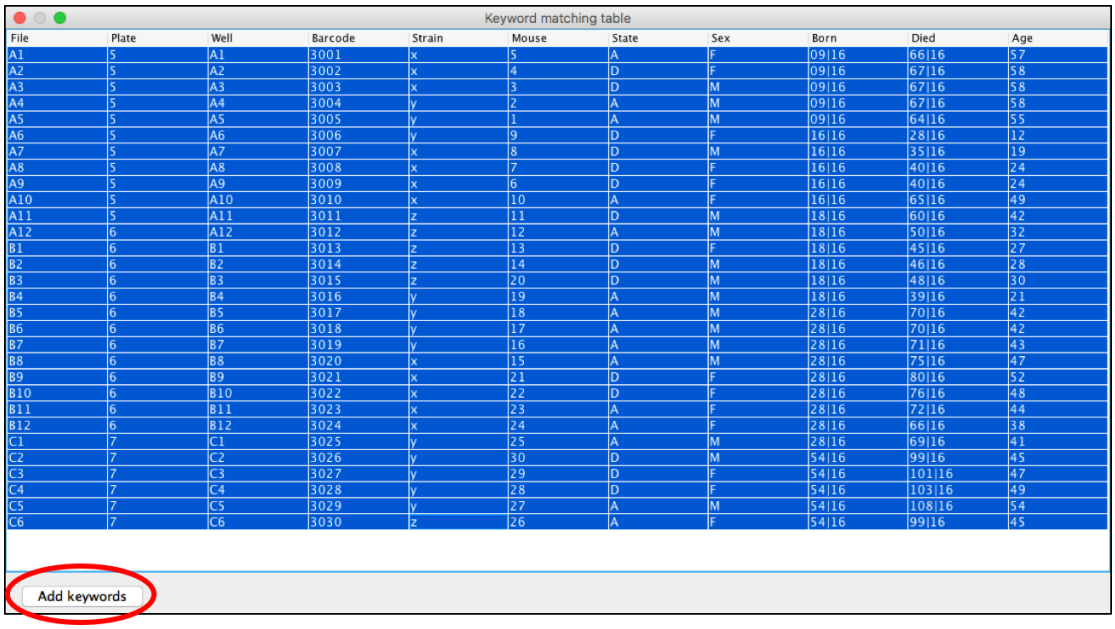
Adding keywords to files by csv – Multiple keywords contained in a csv file can be imported and assigned to one or a selection of files. By default, keywords will be matched to the files in the order that they appear in the File Inspector. Files can be sorted based on any keyword that is contained in 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.



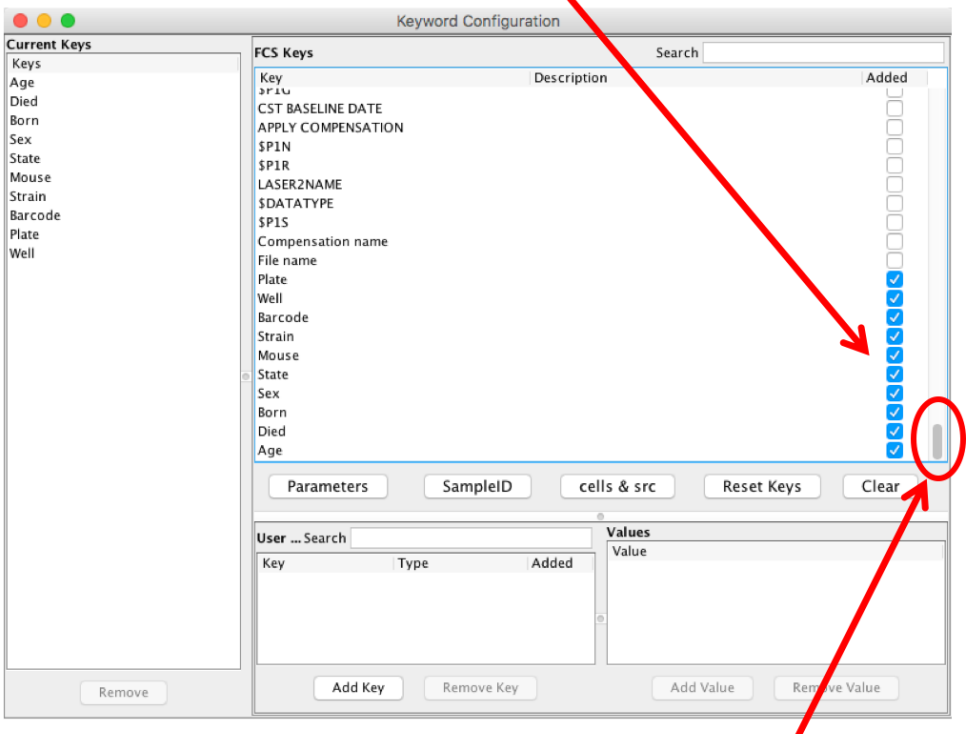
Click to select the file that matches the set of keywords



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

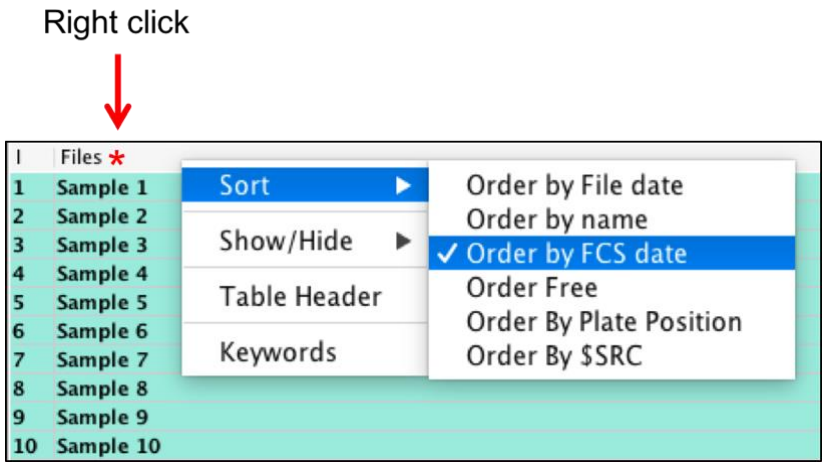
To import keywords, create the CSV file in the following format, with the first row being the keyword heading and the subsequent rows being the keyword data.

	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											

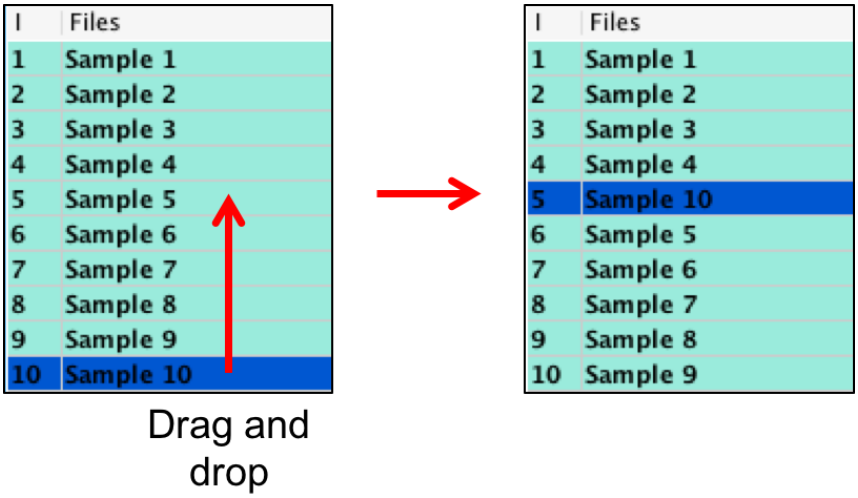
Sorting samples in the File Inspector

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

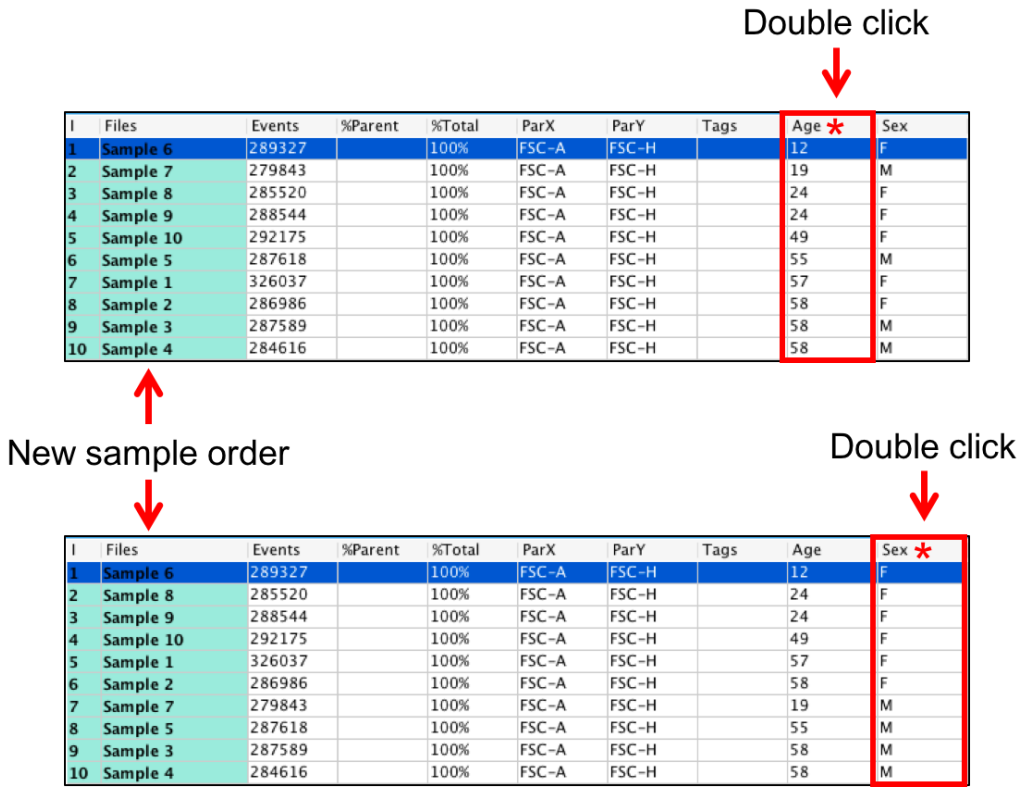
1. Right-click on column header and choose ‘Sort’ followed by a sort style:



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

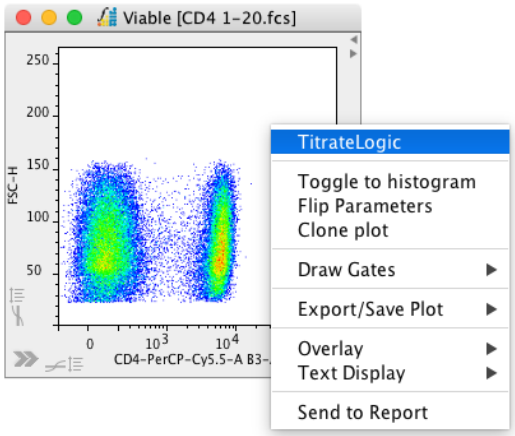
Samples in the File Inspector can be sorted based on the values or numbers in the different File Inspector columns. Double-clicking the column title will sort the samples in ascending order (numerically or alphabetically). Double-clicking again will sort them in descending order.

TitrateLogic

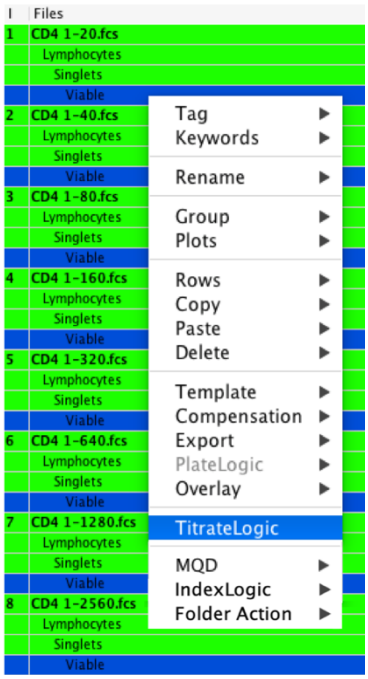
FlowLogic contains a quantitative titration analysis tool that automatically detects the positive and negative signals for a defined parameter, creating titration overlays and a report displaying all populations from the analysed samples along with graphs displaying median fluorescence intensities for 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’.

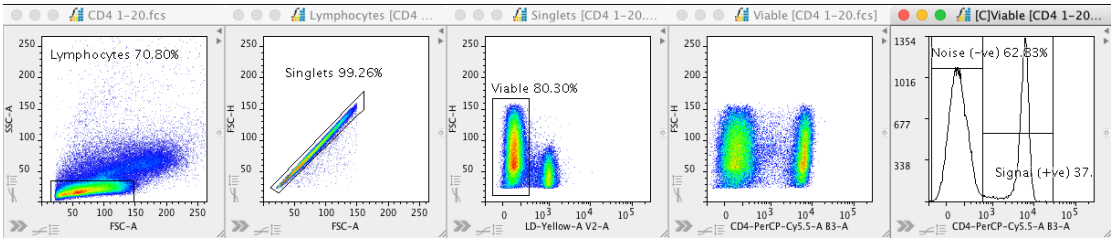


2. Highlight the populations in the File Inspector, right-click → TitrateLogic’.



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.

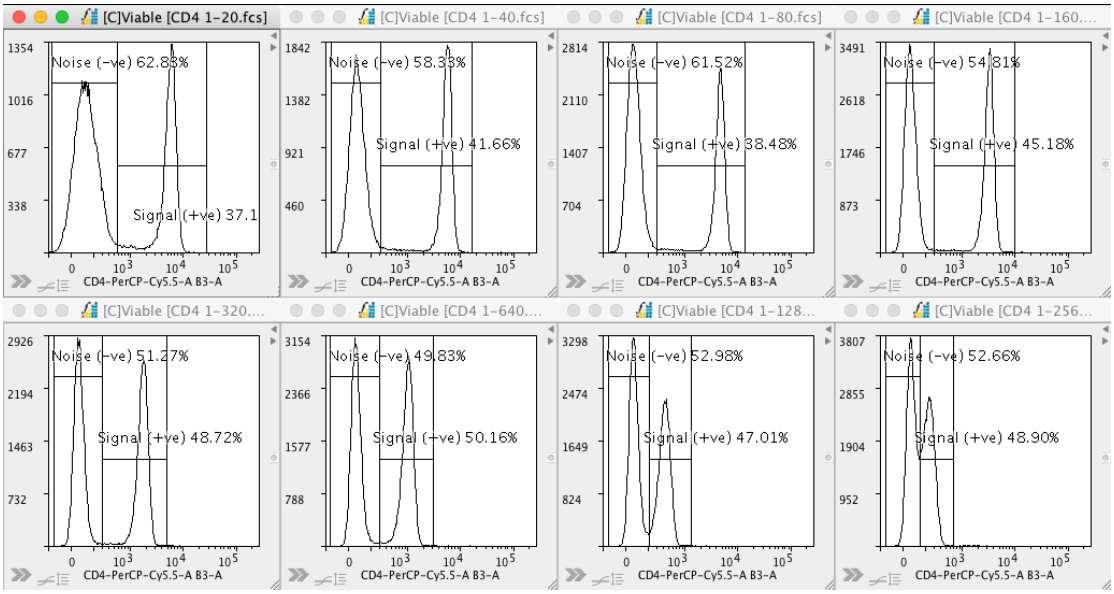
FlowLogic will then clone the population, display it as a histogram and create signal (+ve) and noise (-ve) histogram markers by automatically detecting the positive and negative peaks.



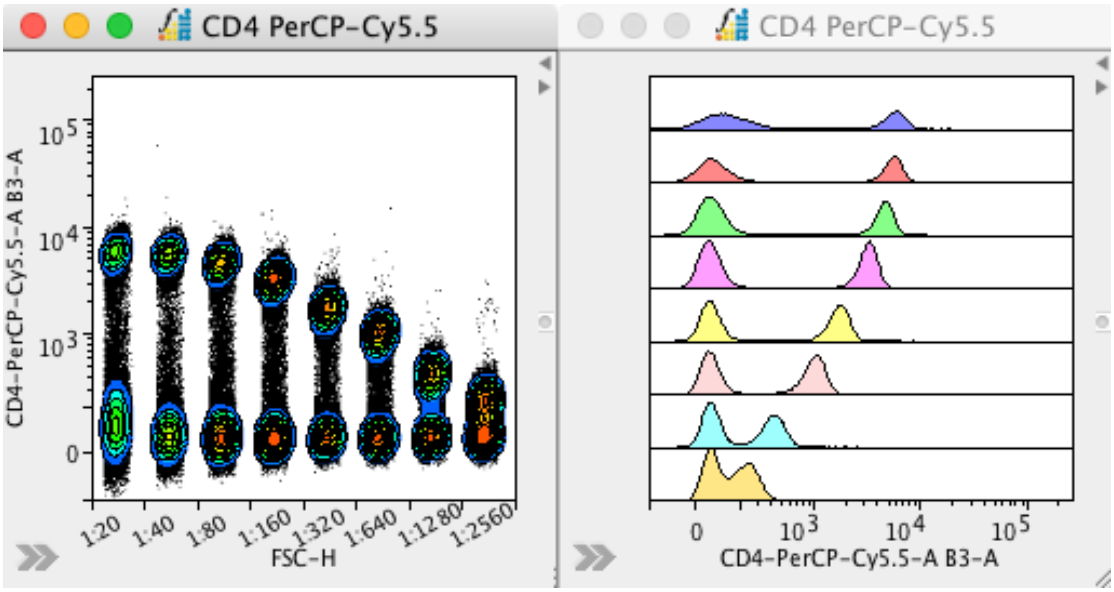
The histogram markers are not contained in the group, allowing for the adjustment on individual plots if required.

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.



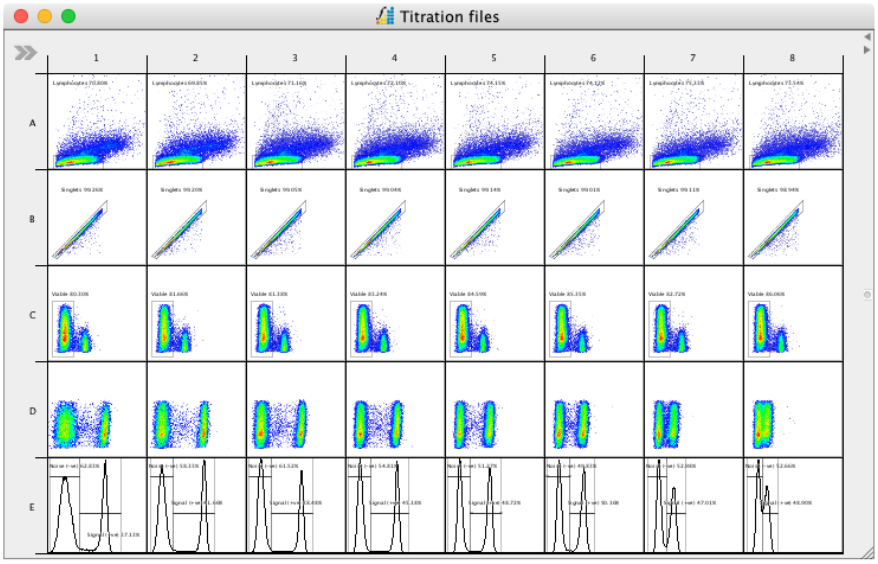
Two titration overlays are also generated and displayed in the workspace. The parameters used for these plots are taken from the plot selected to perform the analysis. The displayed parameters can be changed by clicking on the axis label and choosing a new parameter, as with any other plot.



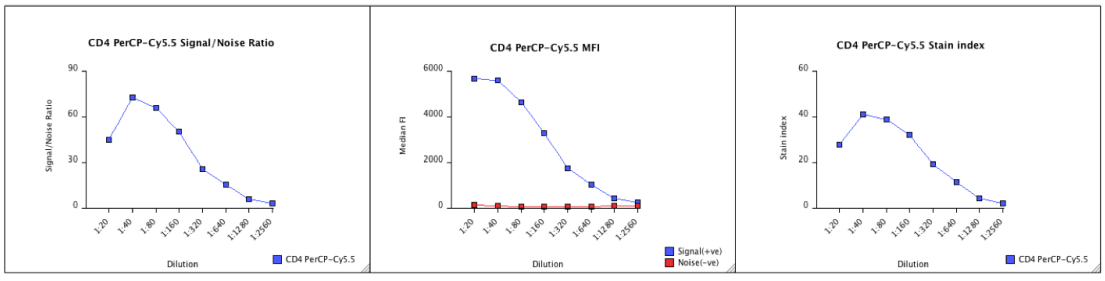
Samples can be removed and added to both plots (right-click menu) and switched on and off from the stacked histogram overlay (plot side drawer under the ‘Overlay Colors’ tab).

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).



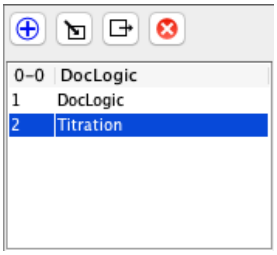
The stain index[1] is calculated using the following formula:

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

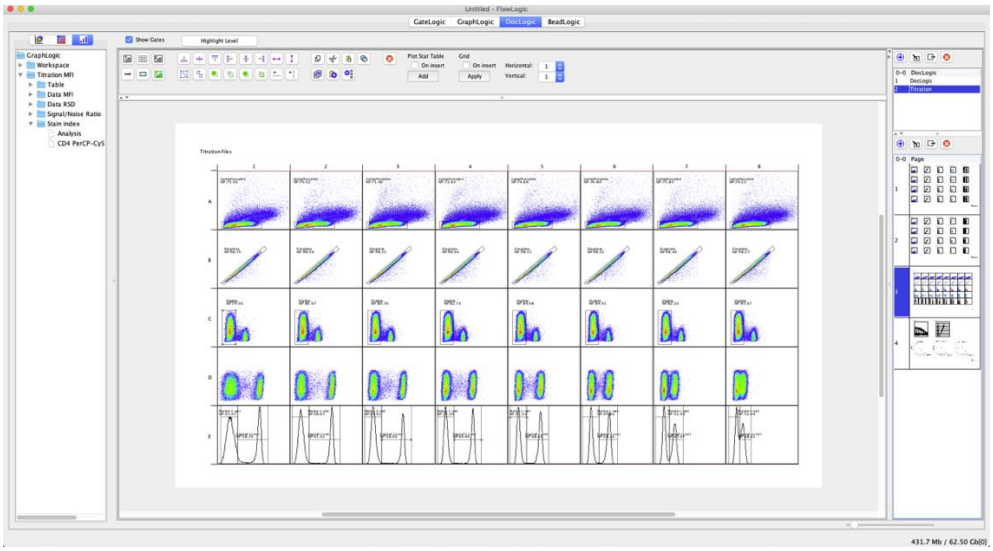
MFI = median fluorescence intensity
RSD = robust standard deviation
+ve = positive signal defined by the histogram marker
-ve = negative signal defined by the histogram marker

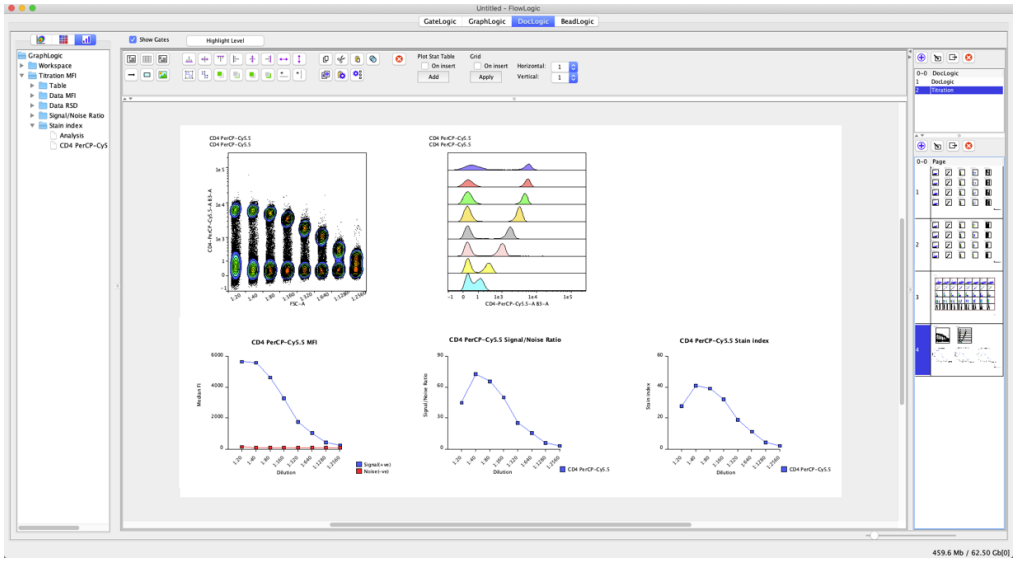
[1] Telford WG, Hawley T, Subach F, Verkhusha V, Hawley RG. Flow cytometry of fluorescent proteins. *Methods*. 2012;57:318-30. PMID: 22293036.

A report is also automatically generated from the elements in the Analysis and Graph sections. These include all dot plots and histograms, the plate and the graphs displaying median fluorescence intensity, signal-to-noise ratio and the stain index. To view the titration report, click on the titration document in the List of Documents. Double-click on the document title to rename it. The report can also be saved as a PDF using the option in the toolbar.



Samples of the final report:





Advanced Functions Pull-Up Drawer

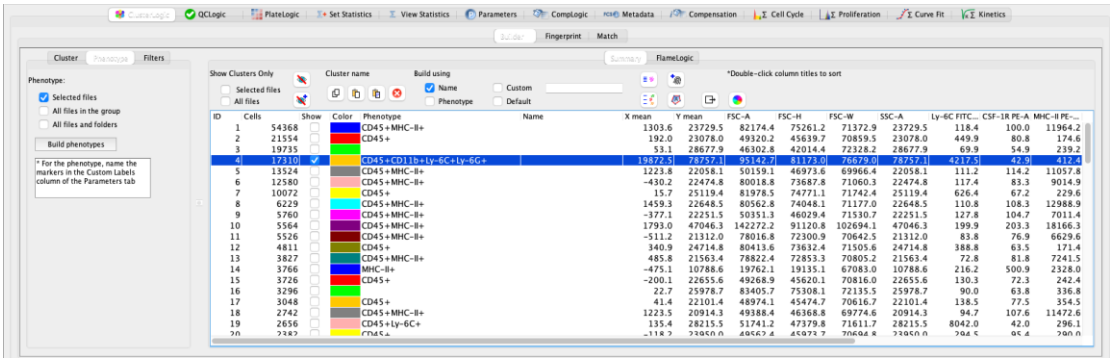
The Advanced Functions pull-up drawer contains additional analysis features to improve efficiency and increase the data mining capabilities. To access the Advanced Functions drawer, click on the top of the drawer (located at the bottom of the Workspace) and drag it up. It can be pulled up to entirely cover the Workspace, if desired. The Advanced Functions drawer consists of a number of tabs. These are PlateLogic, Set Statistics, View Statistics, Parameters, Geometric Gates, Boolean Gates, CompLogic, Metadata, Compensation, Cell Cycle, Proliferation, Curve Fit and Kinetics.

ClusterLogic Introduction

Cluster analysis in FlowLogic follows the following general workflow:

1. Generate clusters using the ClusterLogic algorithm
2. Define the clusters with the auto-phenotype feature
3. View the cluster relationships using FlameLogic
4. Find clusters in all files using a cluster fingerprint
5. Add defined clusters to the File Inspector as subpopulations
6. Validate clusters using the auto-gating feature to create a traditional gating hierarchy

There are various options available at each stage of the above workflow, where you can tailor your analysis. For example, if you have FMO controls, these can be automatically considered in naming the phenotypes. Another example, matching of a cluster fingerprint can be based on phenotype, the fluorescence signature in any combination of parameters, or both. These options will be covered in the sections below.



Generate clusters

Generate the clusters within your files using the unsupervised clustering algorithm. This step requires the user to select the parameters with which to cluster. The time required to build clusters can range from seconds to hours depending on the number of files, number of events in each file, the number of parameters and the computer's processor.

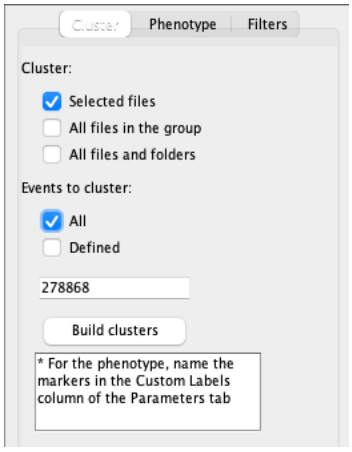
1. Import files into FlowLogic, open the Advanced Functions drawer and select the ClusterLogic tab.

2. Within ClusterLogic, select the Builder tab followed by the Cluster tab on the left-hand side. Define which files to cluster and the number of events within each file to cluster.

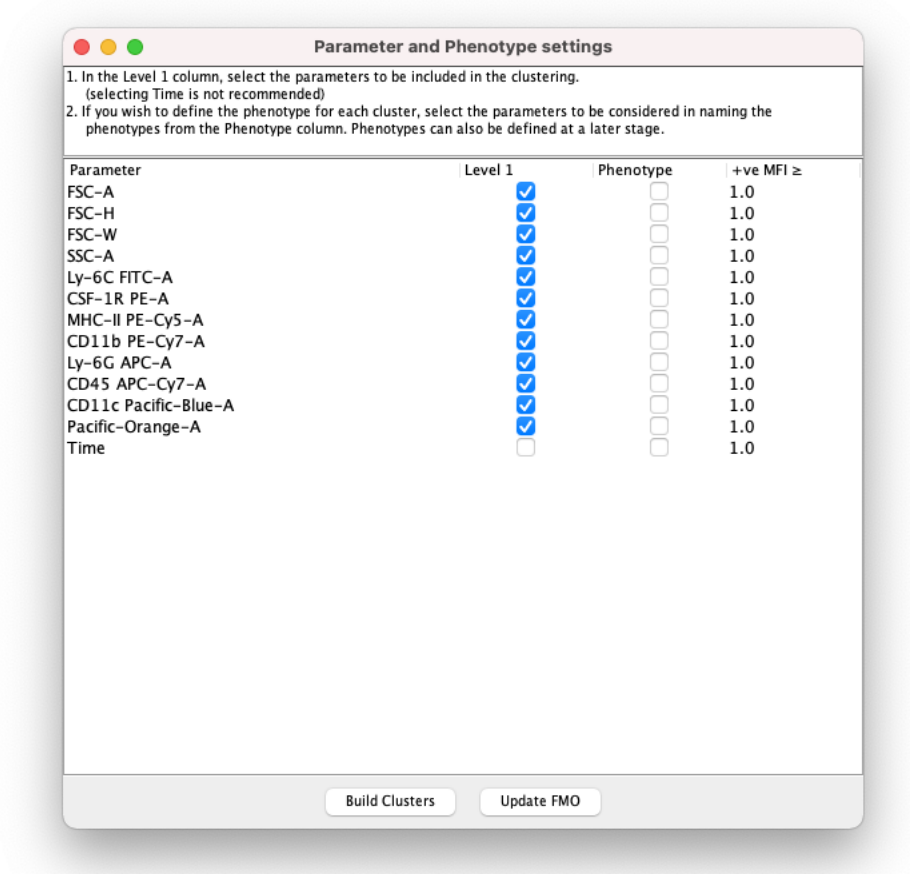
   Build clusters for:
   - Selected files: highlight the files in the File Inspector
   - All files in the group: select one file in the desired group from the File Inspector
   - All files and folders: no files need to be selected. All files in the analysis will be included.

   Events to cluster:
   - All: all events in all files will be considered
   - Define: type in the number of events to be used.

     * It is recommended to cluster with all events, although this will take longer. You may choose to cluster on a smaller number for a quick check of the identified clusters.



3. Click "Build clusters". A window will open allowing the selection of parameters to cluster with (column "Level 1"). Tick to select the desired parameters. It is recommended to select all parameters except Time. Once the selection has been made, click "Build Clusters" at the bottom of the window. A pop-up window will indicate the process has begun. This window will close automatically once the clustering is complete. If you wish to cancel the clustering at any stage, simply close this window.



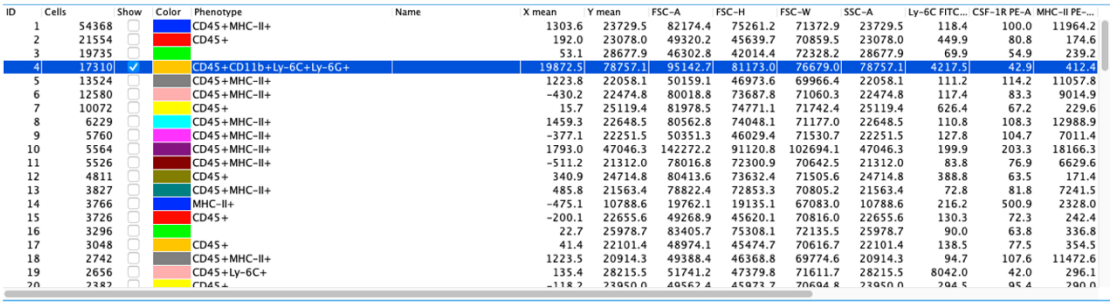
* The other options in the parameter selection window allow for the auto-phenotype naming of clusters. However, this can be done after the clusters have been generated

4. Save your analysis File -> Save As. The cluster information will be saved with your .glf or .gatelogicexperiment files and will be instantly accessible when the file is re-opened, meaning the clustering only needs to be performed once.

The Cluster Summary window

After the cluster process has finished, all clusters derived from a selected file will be displayed in the cluster Summary window of the ClusterLogic Builder tab. The summary window contains a number of attributes:

Summary table



ID	Cells	Show	Color	Phenotype	Name	X mean	Y mean	FSC-A	FSC-H	FSC-W	SSC-A	Ly-6C FITC...	CSF-1R PE-A	MHC-II PE-...
1	54368	☐		CD45+MHC-II+		1303.6	23729.5	82174.4	75261.2	71372.9	23729.5	118.4	100.0	11964.2
2	21554	☐		CD45+		192.0	23078.0	49320.2	45639.7	70859.5	23078.0	449.9	80.8	174.6
3	19735	☐				53.1	28677.9	46302.8	42014.4	72328.2	28677.9	69.9	54.9	239.2
4	17310	☑		CD45+CD11b+Ly-6C+Ly-6G+		19872.5	78757.1	95142.7	81173.0	76679.0	78757.1	4217.3	42.9	412.4
5	13524	☐		CD45+MHC-II+		1223.8	22058.1	50159.1	46973.6	69966.4	22058.1	111.2	114.2	11057.8
6	12580	☐		CD45+MHC-II+		-430.2	22474.8	80018.8	73687.8	71060.3	22474.8	117.4	83.3	9014.9
7	10072	☐		CD45+		15.7	25119.4	81978.5	74771.1	71742.4	25119.4	626.4	67.2	229.6
8	6229	☐		CD45+MHC-II+		1459.3	22648.5	80562.8	74048.1	71177.0	22648.5	110.8	108.3	12988.9
9	5760	☐		CD45+MHC-II+		-377.1	22251.5	50351.3	46029.4	71530.7	22251.5	127.8	104.7	7011.4
10	5564	☐		CD45+MHC-II+		1793.0	47046.3	142272.2	91120.8	102694.1	47046.3	199.9	203.3	18166.3
11	5526	☐		CD45+MHC-II+		-511.2	21312.0	78016.8	72300.9	70642.5	21312.0	83.8	76.9	6629.6
12	4811	☐		CD45+		340.9	24714.8	80413.6	73632.4	71505.6	24714.8	388.8	63.5	171.4
13	3827	☐		CD45+MHC-II+		485.8	21563.4	78822.4	72853.3	70805.2	21563.4	72.8	81.8	7241.5
14	3766	☐		MHC-II+		-475.1	10788.6	19762.1	19135.1	67083.0	10788.6	216.2	500.9	2328.0
15	3726	☐		CD45+		-200.1	22655.6	49268.9	45620.1	70816.0	22655.6	130.3	72.3	242.4
16	3296	☐				22.7	25978.7	83405.7	75308.1	72135.5	25978.7	90.0	63.8	336.8
17	3048	☐		CD45+		41.4	22101.4	48974.1	45474.7	70616.7	22101.4	138.5	77.5	354.5
18	2742	☐		CD45+MHC-II+		1223.5	20914.3	49388.4	46368.8	69774.6	20914.3	94.7	107.6	11472.6
19	2656	☐		CD45+Ly-6C+		135.4	28215.5	51741.2	47379.8	71611.7	28215.5	8042.0	42.0	296.1
20	2382	☐		CD45+		-118.2	23950.0	49562.4	45973.7	70694.8	23950.0	294.5	95.4	290.0

ID – this is the cluster rank, based on the cluster size (number of events in the cluster). ID1 is the largest, ID2 is the second largest, etc.

Cells – this is the number of events in the cluster. If all events in the file were involved in the clustering, then all events will be assigned a cluster ID. However, many events will not exist in true clusters and these will be listed in the table as clusters with 1 event.

Show – tick the box in this column to color the events in the cluster on the dot plot.

Color – this is the color assigned to the cluster when displayed on the dot plot. Click on the solid color to select a new one from the available options.

Phenotype – this is the cluster phenotype as created using the auto-phenotype feature.

Name – this is the cluster/population name as defined by the user. This is editable.

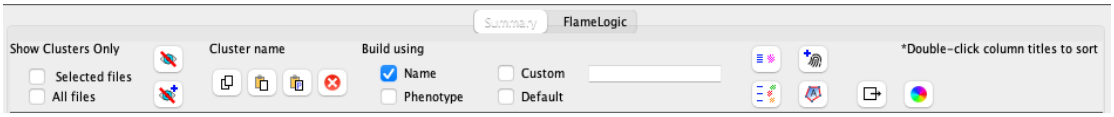
X mean – this is the mean fluorescence value of the cluster for the parameter displayed on the x axis of the dot plot. This value will change if the parameter selection on the dot plot is changed.

Y mean – this is the mean fluorescence value of the cluster for the parameter displayed on the y axis of the dot plot. This value will change if the parameter selection on the dot plot is changed.

Parameters – the MFI for each cluster will be displayed for each parameter saved in the file. You can scroll to the right to scan all of the values.

The columns of the summary table can be re-ordered by clicking and dragging the column heading to a new position. Each column (except for the parameters-labelled MFI columns) can be sorted by double clicking the column title. Double-click once to sort high to low. Double-click again to sort low to high.

Summary window toolbar



The toolbar contains a number of different functions

Show clusters only:

Selected files - if clusters are colored on dot plots, this option will hide all other events in the dot plot except for the chosen clusters but only for the files highlighted in the File Inspector

All files – this will hide all events in all files except for those in shown clusters.

Cluster name:

Copy name – after typing in a population/cluster name, select the cluster row and click the icon to copy the name to the clipboard

Paste to selected – a copied cluster name can be pasted to a selection of clusters

Paste to matching phenotype – a cluster name copied from a cluster containing a phenotype can be pasted to all cluster with the same phenotype without having to select them.

Build using:

Name – when adding a cluster/s to the file in the File Inspector, selecting this option will rename it with the cluster name

Phenotype – when adding a cluster/s to the file in the File Inspector, selecting this option will rename it with the cluster phenotype

Custom – when selecting this option, the name typed in the text field will be applied to all selected clusters. This is particularly useful when combining clusters with different phenotypes, such as building a parent population made up of various subpopulations.

Default – this will apply a default cluster name, following the sequence C1, C2, C3, etc.

Build one cluster gate with selected – multiple clusters can be combined into one population and added to the file in the File Inspector. Select the clusters you wish to combine and select this button to build the cluster.

Build one cluster gate for each selected – each selected cluster will be added to the file in the File Inspector as an individual population.

Add all selected clusters to the fingerprint table – this creates a new fingerprint for each selected cluster and adds it to the fingerprint table. These fingerprints contain the MFI in each parameter for the specific cluster. These can then be used to search through other files for matching clusters.

Auto-gate via a cluster – clicking this icon after selecting a cluster will open the parameter and phenotype settings for auto-gating window. From here, auto-gating with hierarchy generation can be executed in order to validate a cluster.

Export – export the cluster summary table as a .xlsx or .csv file

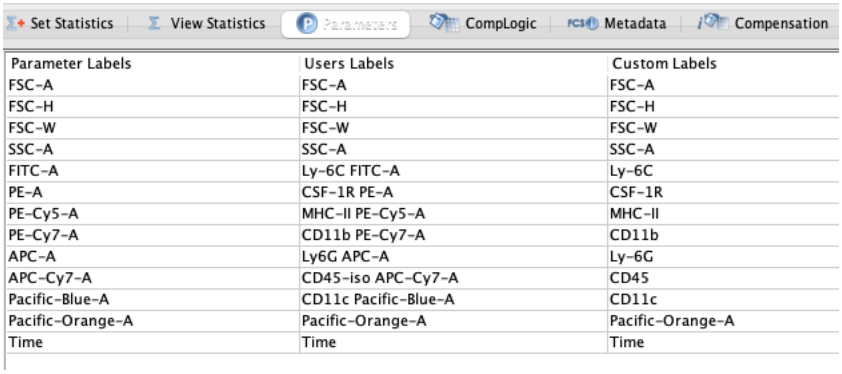
Color wheel – set the color for a selection of clusters

Auto-phenotype and cluster naming

FlowLogic can automatically define the cluster phenotype given two pieces of information:

- The marker names
- The fluorescence value for each parameter above which is considered positive fluorescence

1. Name the markers – in the Advanced Functions drawer, select the Parameters tab. In the Custom Labels column, double-click within a row to edit the name. Type the marker name that corresponds to the relevant parameter, as listed under the Parameter Labels and User Labels columns.

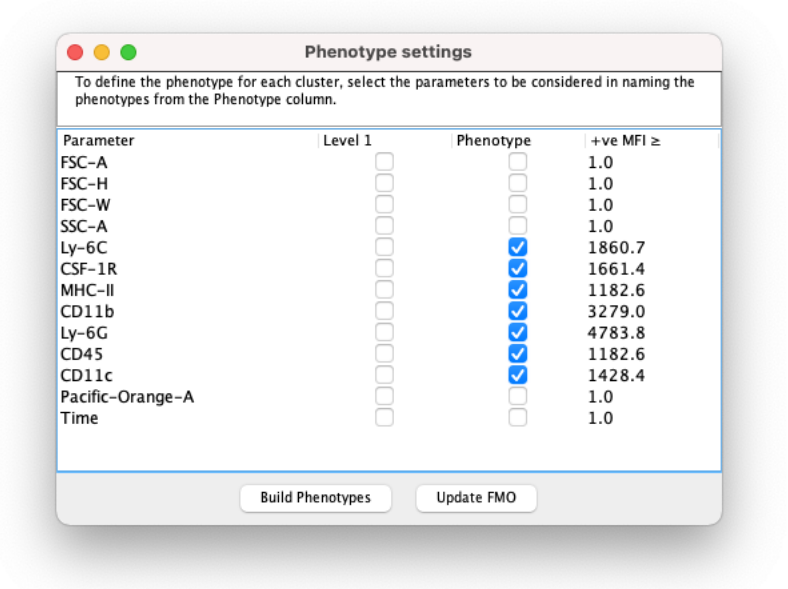


Parameter Labels	Users Labels	Custom Labels
FSC-A	FSC-A	FSC-A
FSC-H	FSC-H	FSC-H
FSC-W	FSC-W	FSC-W
SSC-A	SSC-A	SSC-A
FITC-A	Ly-6C FITC-A	Ly-6C
PE-A	CSF-1R PE-A	CSF-1R
PE-Cy5-A	MHC-II PE-Cy5-A	MHC-II
PE-Cy7-A	CD11b PE-Cy7-A	CD11b
APC-A	Ly6G APC-A	Ly-6G
APC-Cy7-A	CD45-iso APC-Cy7-A	CD45
Pacific-Blue-A	CD11c Pacific-Blue-A	CD11c
Pacific-Orange-A	Pacific-Orange-A	Pacific-Orange-A
Time	Time	Time

2. Within ClusterLogic, select the Builder tab followed by the Phenotype tab on the left-hand side. Define the files to have the phenotype added to the list of clusters.

   Phenotype:
   - Selected files: highlight the files in the File Inspector.
   - All files in the group: select one file in the desired group from the File Inspector.
   - All files and folders: no files need to be selected. All files in the analysis will be included.

   Click “Build phenotype”.
   - The Phenotype Settings window will open. In the Phenotype column, tick to select the parameters you wish to be considered when naming the phenotypes (not all markers have to be used).
   - In the +ve MFI ≥ column, double-click to type the fluorescence value for which the marker expression is considered to be positive (each marker can have a different value).



* If you have FMO controls, these can be used to automatically determine the fluorescence value. See the explanation in the section “Using FMOs”

Once all fluorescence values are set, click the “Build Phenotypes” button at the bottom of the Phenotype Settings window.

3. To view the phenotypes, click on an individual file in the File Inspector. The list of clusters including the phenotype will be displayed in the Summary table of the ClusterLogic Builder tab.

ID	Cells	Show	Color	Phenotype	Name	X mean	Y mean
1	43616			CD45+B220+	B cells	81962.8359	24162.4238
2	37850					53686.2031	33810.9180
3	12384			CD45+		100560.4453	67564.4609
4	11939			CD45+		100482.4219	72038.0547
5	8615			CD45+CD4+TCR-B+		48931.3164	22678.7188
6	8123			CD45+B220+	B cells	81779.6484	23852.5898
7	7535			CD45+CD4+TCR-B+		80237.7734	24927.3027
8	7456			CD45+TCR-B+CD8+		49311.7266	25393.8008
9	7139			CD45+B220+	B cells	78066.4062	21777.2734
10	7122			CD45+B220+	B cells	79169.6328	22314.5059
11	6511			CD45+CD4+TCR-B+		48528.6055	22839.9004
12	5423			CD45+B220+	B cells	84987.1953	26035.8418
13	5244			CD45+B220+	B cells	142374.3281	49268.1172

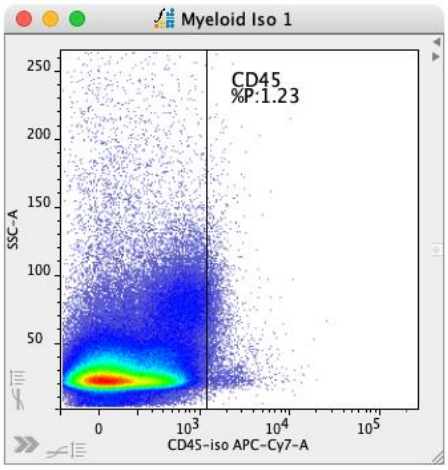
Using FMOs to name the phenotypes

Auto-phenotyping requires a fluorescence value to be added for each parameter in order to define where positive marker expression begins. If FMO controls have been acquired, FlowLogic can automatically determine the fluorescence value from a simple rectangle gate.

Not all markers are required to have an FMO in order to use this feature and the same control can be used to set the fluorescence value for two different parameters.

To derive fluorescence values from FMOs:

1. Open the FMO as a dot plot with the x-axis showing the parameter without the antibody/stain.
2. Add a rectangle gate beginning at the point of positive marker expression.
3. Name this gate with the marker name, as defined in the Parameters tab.



Then, when performing the auto-phenotyping (as described on page 111):

4. When the Phenotype Settings window opens, highlight all of the gates on the FMOs in the File Inspector and then click “Update FMO” in the Phenotype Settings window.

The fluorescence values will appear in the +ve MFI ≥ column. These values can be edited by double-clicking on the cell and typing in a new number.

The values for samples without associated FMOs can also be added by double-clicking on the cell and typing number.

Gates added to multiple parameters from one file can be used to update the fluorescence values for multiple markers. Just be sure to rename the gates with the marker name and select it before clicking the “Update FMO” button.

Viewing and naming clusters

Viewing

After clusters have been built, then can be coloured and viewed on the dot plots. This is generally easier to do after defining the phenotypes, as these provide an indication as to which parameters to choose for best viewing.

To colour a cluster, open a dot plot and tick the 'Show' box related to a specific cluster. This will colour the events comprising that cluster. To change the cluster color, click on the solid color in the Color column and select a new one from the options available.

Multiple clusters can be colored on a dot plot and the coloring will be maintained as parameter combinations on the plot are changed.

Naming

In addition to the cluster phenotype, names can be assigned to different populations. To add a name, double-click in the corresponding cell in the Name column of the cluster Summary table. Then, type the new name.

ID	Cells	X mean	Y mean	Show	Color	Name	Phenotype
1	43616	81962.8359	24162.4238	☐		B cells	CD45+B220+
2	37850	53686.2031	33810.9180	☐			
3	12384	100560.4453	67564.4609	☐			CD45+
4	11939	100482.4219	72038.0547	☐			CD45+
5	8615	48931.3164	22678.7188	☐			CD45+TCR-B+CD4+
6	8123	81779.6484	23852.5898	☐		B cells	CD45+B220+
7	7535	80237.7734	24927.3027	☐			CD45+TCR-B+CD4+
8	7456	49311.7266	25393.8008	☐			CD45+TCR-B+CD8+
9	7139	78066.4062	21777.2734	☐		B cells	CD45+B220+
10	7122	79169.6328	22314.5059	☐		B cells	CD45+B220+
11	6511	48528.6055	22839.9004	☐			CD45+TCR-B+CD4+
12	5423	84987.1953	26035.8418	☐		B cells	CD45+B220+
13	5244	142374.3281	49268.1172	☐		B cells	CD45+B220+

Icons in the toolbar can be used to copy a name and paste it to a selection of clusters, or you can choose to paste a copied name to all clusters with a matching phenotype, by using the paste to matching phenotype icon.

When adding clusters to the File Inspector, there is a toolbar option to build the clusters and rename with either the population Name or phenotype. This will save you from renaming the cluster once it is added to the file in the File Inspector.

Filters

There are four categories of filters withing ClusterLogic: Cluster ID, cluster size, X and Y MFI and phenotype. These filters can be accessed under the Filter tab in the panel on the left-hand side of the Builder section of ClusterLogic. A modified version is available under the Match tab, where the MFI options have been removed.

Clusters – Clusters are ranked in order of size (number of cells within the cluster). Clusters are then assigned a Cluster ID, with the largest being ID 1, followed by ID 2, etc. This filter can be used to select clusters from a defined range of IDs. The most common would be to select the *x* number of biggest clusters, such as the biggest 50. However, it is possible to display the second largest 50 clusters (ID 51-100) if desired. To use this filter, double-click to define the Cluster ID in the From and To text fields. Then, tick the box in the ON column. The range can be edited at any stage.

Cells – similar to filtering on Cluster ID, clusters can be filtered on a range of sizes (number of cells within the cluster). The difference here, is that the exact number of events is entered into the From and TO columns. For example, choose to show only clusters with a size of 20 events to 1,000,000 events. This excludes all clusters with fewer than 20 events. To use this filter, double-click to define the Cluster ID in the From and TO text fields. Then, tick the box in the ON column. The range can be edited at any stage.

X and Y MFI – This option refers to the plot displayed in the Workspace. Each cluster has an MFI for each parameter and when a plot is displayed, the parameter on the X and Y parameter MFIs are displayed in the Summary table. MFI ranges can be entered for the X, Y or both displayed parameters to isolate clusters in a specific portion of the plot. Tick the box in the ON column once the MFI values have been added. These values can be edited at any stage.

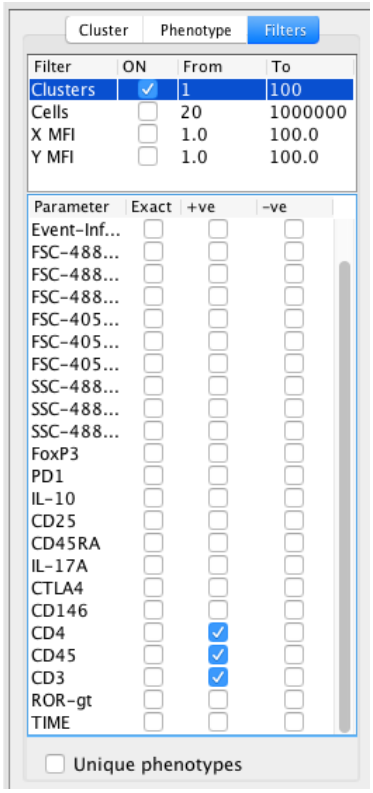
Phenotype – Once auto-phenotyping has been performed, clusters can be filtered on marker expression. At the bottom of the Filter panel is a list of all parameters in the file (this list mirrors the list in the Custom Labels column of the Parameter tab). This list contains all of the markers included in the phenotype definitions.

Phenotypes can be filtered by an exact phenotype by ticking the positively expressed markers in the Exact column. For example, ticking CD45 and CD3 will show only clusters that are exactly CD45+CD3+ and none of the subpopulations.

In order to display clusters that are at least positive and negative for defined parameters, tick the appropriate boxes in the +ve and -ve columns. For example, if you tick CD45 in the +ve column, all clusters that contain CD45 will be displayed, even if they also positively express other markers. So, this selection will display all CD45+ populations. In this case, all clusters can be selected and built in the File Inspector, effectively extracting the full CD45+ population. Be aware that all filtering options are considered, so if you have

chosen to display the top 50 clusters, there may be additional CD45+ clusters that are not part of this extraction.

The -ve selection column is used to exclude clusters displaying a particular marker. For example, CD4 T cells could be identified by ticking the CD45 and CD3 boxes in the +ve column and the CD8 box in the -ve column.



At the bottom of the Filter panel is an option to display Unique phenotypes. When clusters are identified, there will often be many with a given phenotype. By ticking the Unique phenotypes option, only the largest example of each different phenotype will be displayed. This is useful when generating fingerprints for a range of cell types in a given family. The fingerprints can then be used to search for matching clusters in a range of files and different parent and sub-populations can be extracted (using the filtering options) from the results in the Match tab.

FlameLogic

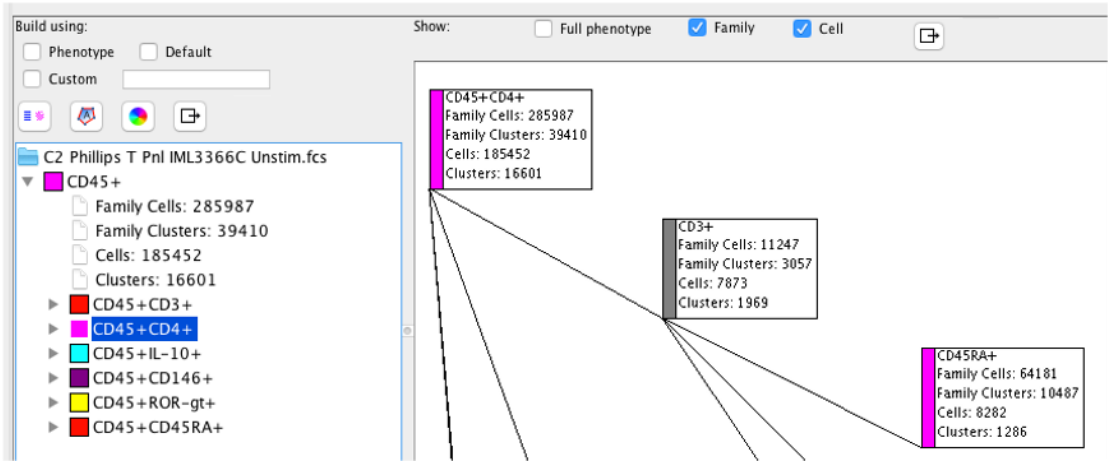
FlameLogic provides a schematic overview that depicts the relationships between different cluster families. It shows the most abundantly expressed markers at the top of the tree, with the various subpopulations displayed below.

The FlameLogic panel is divided in two section: a collapsible folder hierarchy and an interactive diagram. Both sections are able to be filtered using the options in the Filter window.

Information is displayed within each box showing:

- The cluster family phenotype (as defined by the auto-phenotyping process)
- Family cells – the total number of cells from all clusters that are linked by containing at least the markers listed in the phenotype at that level of the tree (includes subpopulations)
- Family clusters - the total number of cells in all clusters that are linked by containing at least the markers listed in the phenotype (the cell count from a particular level of the tree and all subpopulations)
- Cells – the total number of cells from all clusters that have the exact same phenotype
- Clusters – the total number of clusters possessing the exact same phenotype

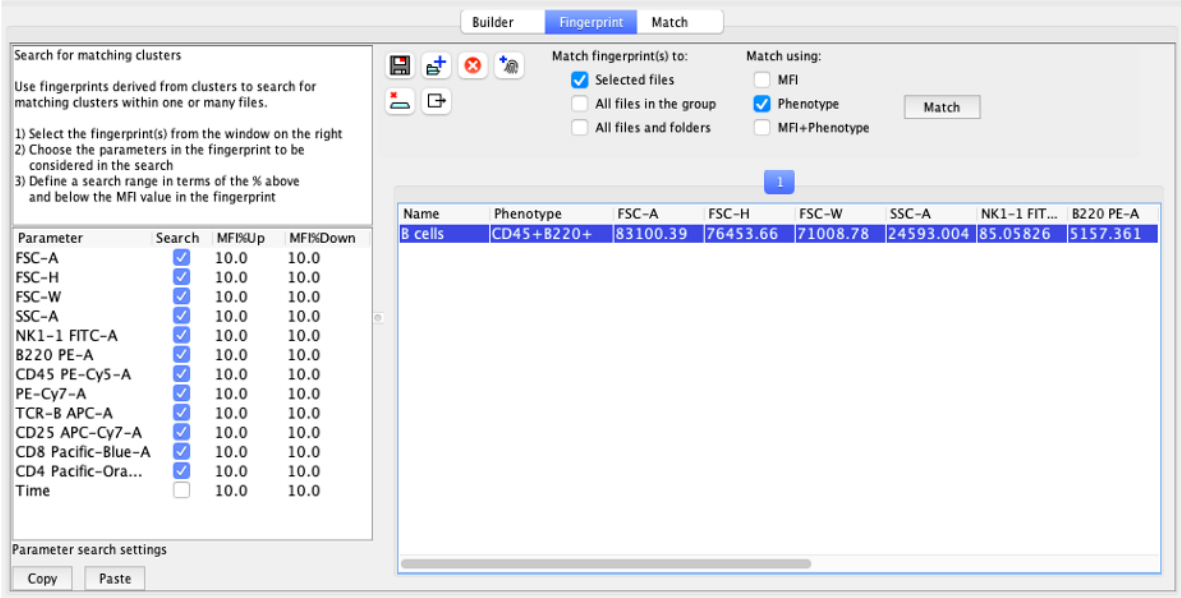
The type of information can be toggled on and off using the options in the toolbar. It is also recommended to filter the number of clusters, e.g., 20. This will clarify the table by excluding non-specific clusters, such as those with only one event.



Auto-gating for cluster validation can be performed by double clicking on a box in FlameLogic and following the steps as described in the auto-gate validation section of this manual.

Fingerprint matching

After a cluster fingerprint has been created, it will be listed in the table under the Fingerprint tab.



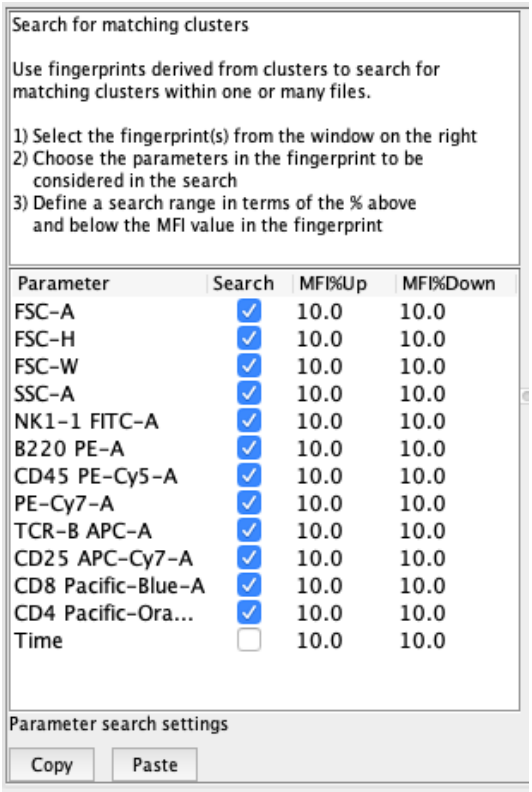
To search other files (or within the same file) for clusters with a matching fingerprint:

1. Highlight the fingerprint in the table
2. Choose which files to search in by selecting the appropriate option in the toolbar:
   - Selected files
   - All files in the group
   - All files and folders
3. Choose the type of matching:
   - MFI (see following section for setting the MFI range)
   - Phenotype
   - MFI + Phenotype
4. If searching based on MFI, update the MFI range window to the left of the fingerprint window (instructions detailed below)
5. Click ‘Match’ in the fingerprint toolbar

The resulting matches will be displayed in the ‘Match’ tab.

Setting the MFI range table

A cluster fingerprint contains the cluster phenotype (if defined) and the MFI for each parameter. The phenotype and any number of MFI values can be used to search for similar clusters. And for each selected parameter, an MFI range in terms of %above and %below the cluster MFI can be defined.

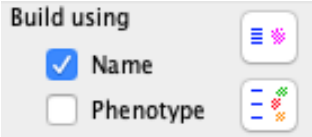


1. In the search column, tick the parameters that you wish to be considered in the matching of the fingerprint.
2. Double-click on a value in either the MFI%Up or MFI%Down column to enter a new value.
3. If you are using the same values for the MFI range in other parameters, highlight on row, click Copy at the bottom of the window, then select the other parameters before clicking Paste.

The settings in this table will be used when searching for matching clusters when using the MFI options in the fingerprint toolbar.

Add clusters to the File Inspector

From either the Builder or the Match windows, selected clusters can be added to the File Inspector. They will be listed with each file in the same way as a gated population.



To add clusters, highlight them in the table and click the icon to either:

- Build one cluster gate with selected
- Build one cluster gate for each selected

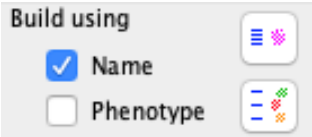
The first option will combine all selected clusters and generate a single population in the File Inspector. The second option will generate one population for each selected cluster.

In addition, the generated clusters can be named automatically, by ticking the option in the toolbar to name with the description in the Name column or the Phenotype column.

Auto-Gate Validation

ClusterLogic contains an auto-gate feature that generates a traditional gating hierarchy for a selected cluster. The gated population can be compared to the cluster and used for validation. Statistics from the two populations can be analyzed, whilst the populations can be overlaid and viewed on all parameters to identify any subtle differences.

To perform the auto-gating, select one or more clusters and add the populations to the File Inspector using one of the two following toolbar icons:

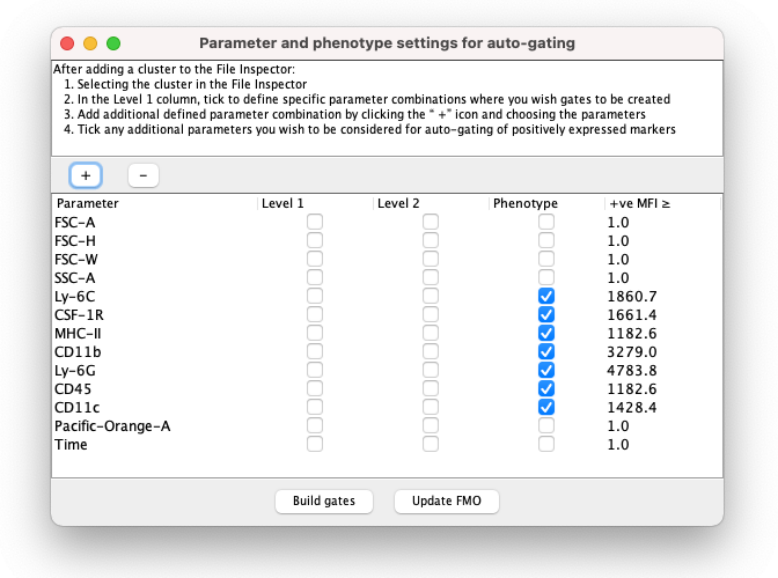


Once the cluster exists on the File Inspector, click on the auto-gate via cluster icon in the toolbar.

Clicking on the icon will open the Parameter and Phenotype settings for auto-gating window. This is where you can define the nature of the gating hierarchy, of which there are three options:

1. Choose the parameter combinations for each level of the hierarchy
2. Select the markers to be considered in the gating hierarchy and allow ClusterLogic to determine the combinations
3. A combination of user defined and automatic parameter combinations.

Option 3 is the most likely. This allows the user to create a ‘Singlets’ gate on FSC-A vs FSC-H and then let ClusterLogic determine the remaining combinations.



To define the combinations, tick two parameters in the ‘Level 1’ column. To add another level of defined parameters, click the “+” at the top of the window. This will add a ‘Level 2’ column.

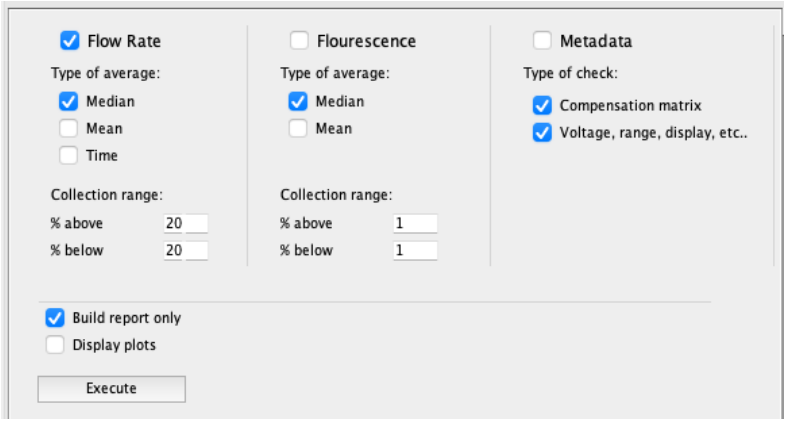
In order for ClusterLogic to determine the combinations, tick all of the parameters you wish to be considered when creating the gating hierarchy in the ‘Parameters’ column. When all selections have been made, click the ‘Build Gates’ button at the bottom of the window.

QCLogic

QCLogic provides options to check and compare files to identify and remove abnormalities. A report is created, which can be exported as a .txt file.

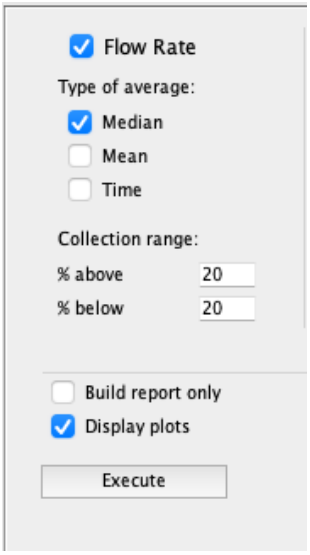
Assessment can be performed on the flow rate, fluorescence signal over the time of acquisition and the metadata, which consists of attributes such as the compensation matrix and PMT voltages.

QCLogic can be accessed from the pull up drawer at the bottom of the GateLogic window. The selection panel appears as follows:



Flow Rate

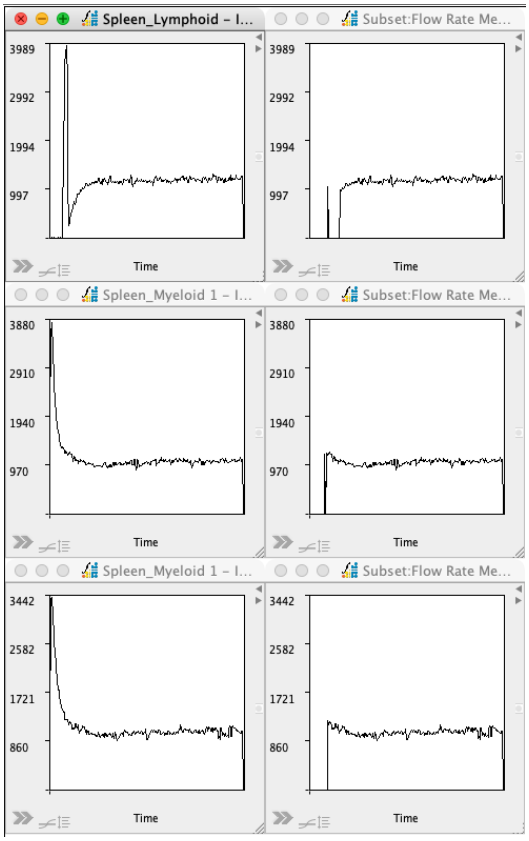
FlowLogic can assess the flow rate of a sample and extract events that fall within a user-defined range. The extracted events effective form a subpopulation, meaning the original dataset is preserved in the File Inspector. It is also possible to run an assessment, producing a written report, without performing the event extraction.



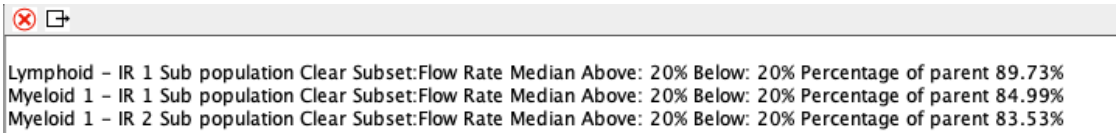
To assess the flow rate:

- Tick the Flow Rate option within QCLogic
- Select the type of average, being Median, Mean or Time
- Define the collection range as a percentage above and below the average
- Choose to “Build report only” to create a statistical report without performing the actual extraction and generation of the subpopulation
- Choose to “Display plots” to open the assessed plots in the workspace upon executing

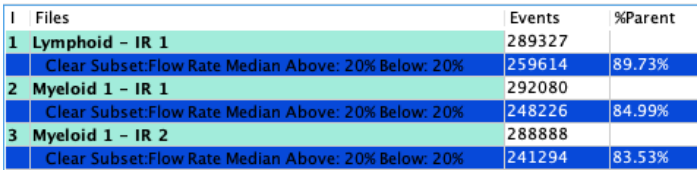
Here is the result of the extraction of events within 20% of the median flow rate, showing the original and extracted data in pairs.



And here is the corresponding flow rate extraction report, located to the right of the QCLogic settings. The text can be cleared by clicking on the red 'Clear text' icon or exported as a .txt file using the 'Export text' icon at the top of the report panel. If another check is performed without clearing the previous text, the new results will be appended. The full report can then be exported as a single .txt file.



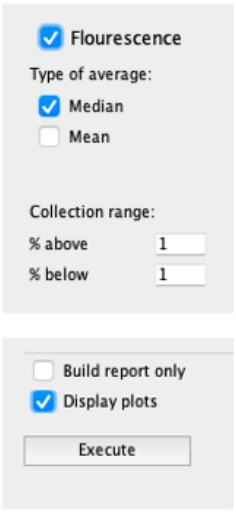
The extracted events appear as a subpopulation in the File Inspector, immediately below the population that the extraction was performed on. These can be treated as a normal gated population. Expanding the File Inspector will also reveal a column containing the flow rate for each sample.



I	Files	Events	%Parent
1	Lymphoid – IR 1	289327	
	Clear Subset:Flow Rate Median Above: 20% Below: 20%	259614	89.73%
2	Myeloid 1 – IR 1	292080	
	Clear Subset:Flow Rate Median Above: 20% Below: 20%	248226	84.99%
3	Myeloid 1 – IR 2	288888	
	Clear Subset:Flow Rate Median Above: 20% Below: 20%	241294	83.53%

Fluorescence

The process for assessing the fluorescence signal over time is similar to that of the flowrate assessment. Note that FlowLogic will assess all fluorescent parameters together.

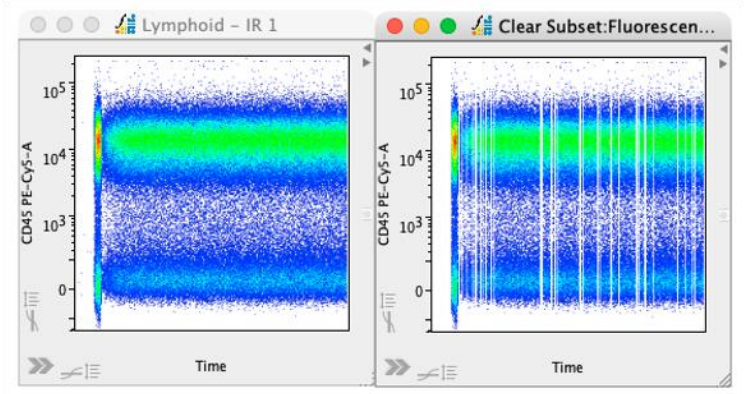


To assess the flow rate:

- Tick the Fluorescence option within QCLogic
- Select the type of average, being Median or Mean
- Define the collection range as a percentage above and below the average
- Choose to “Build report only” to create a statistical report without performing the actual extraction and generation of the subpopulation
- Choose to “Display plots” to open the assessed plots in the workspace upon executing

Here is the result of the extraction of events within 0.2% of the median flow rate (the very small range was used as an example in order to show the removal of some events), showing the original and extracted data in pairs.

As with the flow rate check, the results are displayed in the QCLogic report panel, where the text can be cleared or exported using the icons in the toolbar. The percentage of parent and total statistics for the extracted population can also be viewed in the File Inspector.

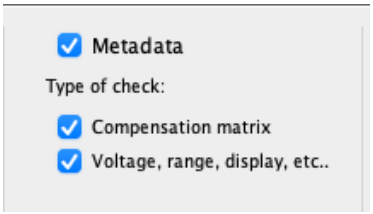


Lymphoid – IR 1 Parameter FITC-A Percentage of parent 100.00%
Lymphoid – IR 1 Parameter PE-A Percentage of parent 100.00%
Lymphoid – IR 1 Parameter PE-Cy5-A Percentage of parent 85.97%
Lymphoid – IR 1 Parameter PE-Cy7-A Percentage of parent 100.00%
Lymphoid – IR 1 Parameter APC-A Percentage of parent 100.00%
Lymphoid – IR 1 Parameter APC-Cy7-A Percentage of parent 100.00%
Lymphoid – IR 1 Parameter Pacific-Blue-A Percentage of parent 100.00%
Lymphoid – IR 1 Parameter Pacific-Orange-A Percentage of parent 100.00%
Lymphoid – IR 1 Sub population Clear Subset:Fluorescence Median Above: .2% Below: .2% Percentage of parent 85.97%

	Files	Events	%Parent
1	Lymphoid – IR 1	289327	
	Clear Subset:Fluorescence Median Above: .2% Below: .2%	248738	85.97%

Metadata

The metadata within a selection of files can be compared to check if the files match appropriately in order to be analyzed together. For example, the compensation can be assessed to check if any files possess a different matrix. In addition, any differences in the PMT voltages can be identified and reported to the user.



To assess the metadata:

- In the File Inspector, highlight the files to assess
- Within QCLogic, tick the Metadata option
- Select the checks to be performed
- Choose to “Build report only” to create a statistical report without performing the actual extraction and generation of the subpopulation
- Choose to “Display plots” to open the assessed plots in the workspace upon executing

Here is one result showing files with different PMT voltages, along with the reported flow rate. Results are shown as differences to the first file as listed in the File Inspector.

```
File Kidney_Myeloid 1 - a Sham 1.fcs diff compensation matrix from Spleen_Myeloid 1 - IR 1.fcs
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key $P12V value 360 value 470
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key $P11V value 320 value 400
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key $P5V value 400 value 460
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key $P6V value 340 value 390
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key $P7V value 510 value 580
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key $P8V value 640 value 660
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key $P9V value 510 value 660
File Kidney_Myeloid 1 - a Sham 1.fcs diff value Spleen_Myeloid 1 - IR 1.fcs meta key Flow Rate value 3756 value 2517
Please check the File Inspector.
```

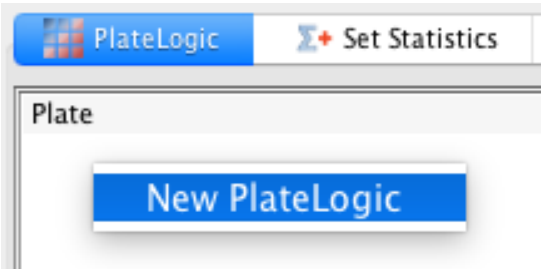
In addition to the readout in the QCLogic report panel, the identified differences are also added to the expandable window of the File Inspector, with the different attributes grouped by column with the keyword as the column title. The information contained within the File Inspector can be managed by right-clicking on the column titles and choosing from the menu items (see page 35 for more details).

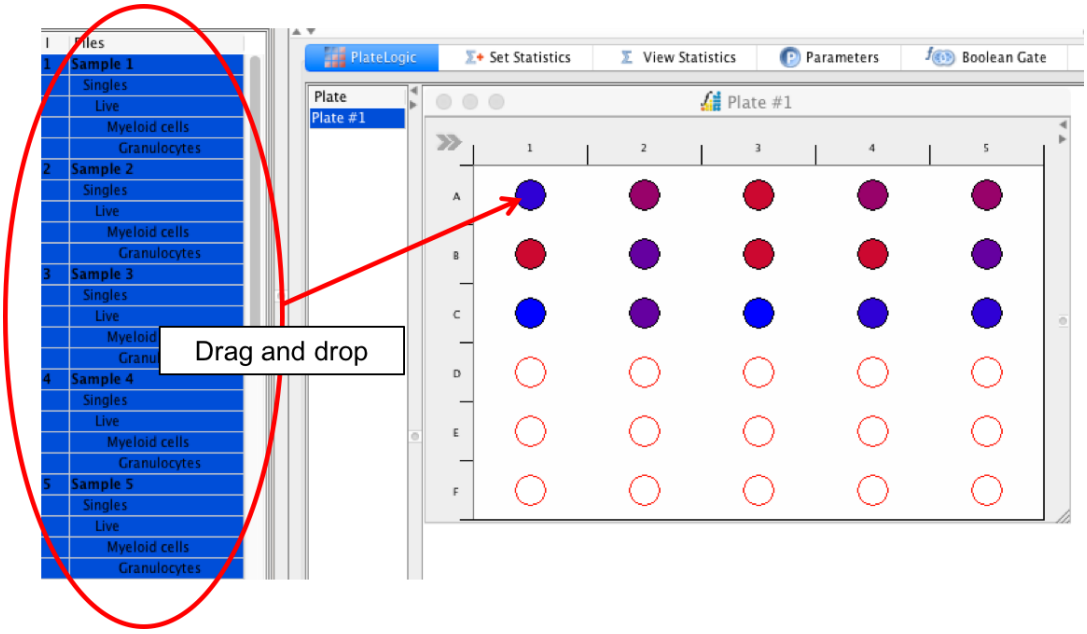
Files	$P12V	$P11V	$P5V	$P6V	$P7V	$P8V	$P9V
Spleen_Myeloid Iso 1.fcs	470	400	460	390	580	660	660
Spleen_Myeloid 1 - IR 3.fcs	470	400	460	390	580	660	660
Spleen_Myeloid 1 - IR 2.fcs	470	400	460	390	580	660	660
Spleen_Myeloid 1 - IR 1.fcs	470	400	460	390	580	660	660
Spleen_Lymphoid - IR 1.fcs	470	400	460	390	580	660	660
Kidney_Myeloid 1 - a Sham 1.fcs	360	320	400	340	510	640	510

PlateLogic – data array analysis

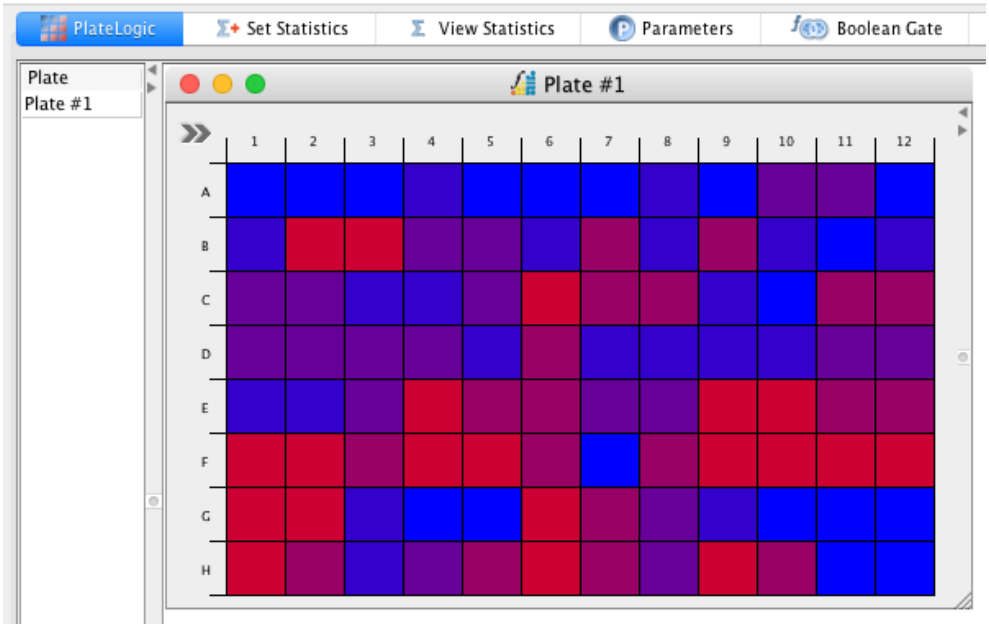
PlateLogic has been designed to assess large amounts of data, such as plate data, utilizing a plate layout. Highlighted FCS files can be dragged from the File Inspector and added to a plate, where a heatmap is automatically generated. Various statistics can be calculated, and tags can be applied to the analyzed files, allowing for the easy identification of samples with a specific characteristic. In addition, FCS data can be passed directly to GraphLogic in the format that they are arranged in a plate. This process does not rely on the selection of a gate or statistic in PlateLogic as the whole process is performed in GraphLogic.

To create a plate, click in the plate window and select New PlateLogic. To fill the plate, highlight a group of files in the File Inspector and drag them into the desired well in the plate. Continue filling the plate with all groups of files. It does not matter which gate or level of the gating hierarchy is selected and dragged into the plate as only the sample is recorded. Once in the plate, the specific subpopulation (gates) can be set and analyzed. It is also possible to add the same sample to multiple wells. If the same sample is added multiple times, each copy can be assigned to a different data set, with each dataset set to display a different level of the gating hierarchy.





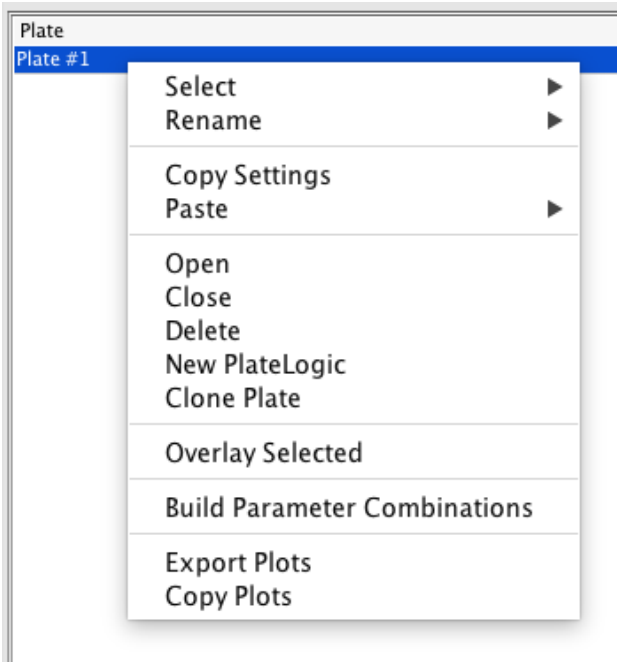
Once all files have been added to the plate, use the functions in the Plate Side Drawer to help analyze the samples.



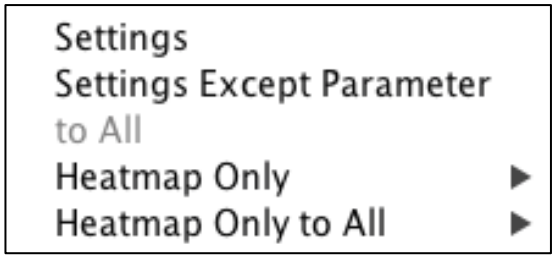
Here is an explanation of the features and elements located in the Data Array tab:

Plate list window – the Plate List window, located to the left of the plate workspace, displays all plates that have been created. To create a new plate, right-click within the window (but not on an existing plate) and choose New PlateLogic.

- Right-clicking on an existing plate name opens a different menu, with features to help manage the plates.



- Select – the select option is used to highlight specific plates in the Plate List window. The two selection options are All and Inverse. Clicking Select → All will highlight all the plates in the window. Select → Inverse will select all plates that were not highlighted, while deselecting all that were.
- Rename – select 'Rename' to access a field where a new plate name can be entered.
- Copy Settings – once a plate has been setup to suit the specific analysis, the settings from that plate can be copied and pasted onto another plate. Simply highlight the plate name, right-click and choose Copy Settings. This copies the settings to the clipboard.
- Paste – once a plate's settings have been copied, right-click the new plate and click Paste, followed by one of the following options:

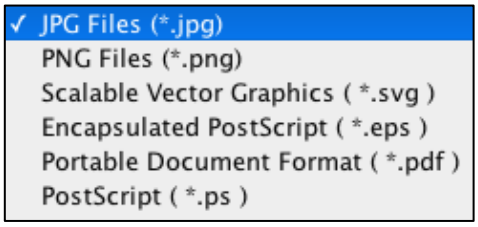


  - Settings – will paste the plate settings to the selected plate.
  - Settings Except Parameter – will paste the plate settings to another plate except for changes made in the Parameter field of the Heatmap Settings tab.

- To All – will paste the settings copied from one plate to all listed plates
- Heatmap Only – will only paste settings associated with the heatmap.
- Heatmap Only to All – will paste the settings associated with the heatmap to all other plates.

- Open – highlighting a plate or plates in the Plate List window, right-clicking and selecting Open will open the plates in the plate workspace.
- Close – highlighting a plate or plates in the Plate List window, right-clicking and selecting Close will close all plates in the plate workspace.
- Delete – highlighting a plate or plates, right-clicking and selecting Delete will open a window asking if you wish to delete the specified plate. If confirmed, the plate will be deleted.

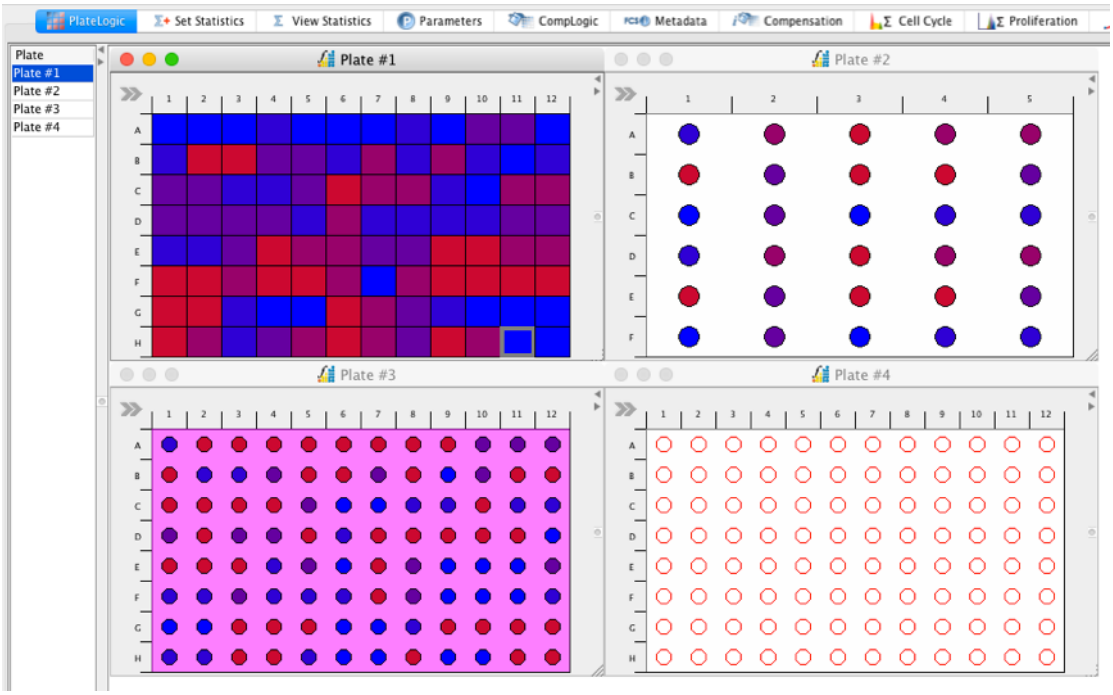
- New PlateLogic – this will create a new plate in the same way as selecting New PlateLogic after right-clicking in the Plate List window.
- Clone Plate – Highlighting a plate, right-clicking and selecting Clone Plate will create a copy of the selected plate and assign it the next available name.
- Overlay Selected – if two or more plates exist with FCS files that match (parameters and gates), then overlay plots can be created for all corresponding wells. So, the samples existing in well A1 will all be overlaid on one plot, while the next will be an overlay of all samples in well A2, etc.
- Build Parameter Combinations – highlighting a plate and selecting Build Parameter Combinations will result in a plate being created for every parameter contained within the FCS file. The parameter will then be set in the heatmap settings tab. If a parameter-dependent statistic is selected, then each plate will have a different result displayed in the statistics tab in the plate side drawer.
- Export Plots – highlighting a plate in the Plate List tab and selecting Export Plots will open a window allowing the heatmap (plate view) to be saved as one of six different file types, as shown:



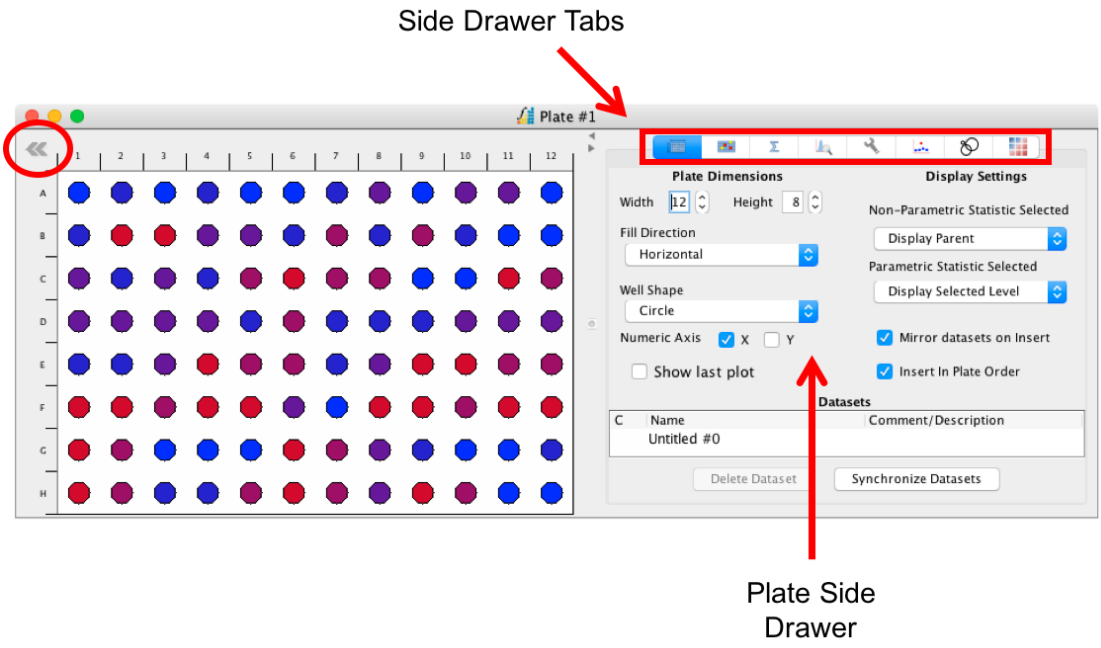
If multiple plates are highlighted when Export Plots is clicked, then each heatmap will be saved once after another.

- Copy Plots – in computers running Microsoft Windows, the plate image will be copied to the clipboard.
- Re-sizer – the re-sizer tool, located below the Plate List window, can be used to increase and decrease size of all plots open in the Data Array Workspace together. If one or more plates have been re-seized manually by clicking and dragging the bottom right-hand corner of the plate, the re-sizer tool will return all plates back to the same size.

- Plate display window – plates are displayed in the Plate display window in a similar fashion to plots in the Workspace. The arrangement of plates in the display window can be optimized by clicking Edit → Plate Arrangement (see the topic about the Edit menu for more details).



- Plate side drawer – each plate has a plate side drawer, which can be open by clicking the button in the top left-hand corner of the plate or by selecting the plate and pressing the space bar. Tabs are located at the top of the side drawer, containing a range of different display and analysis functions.

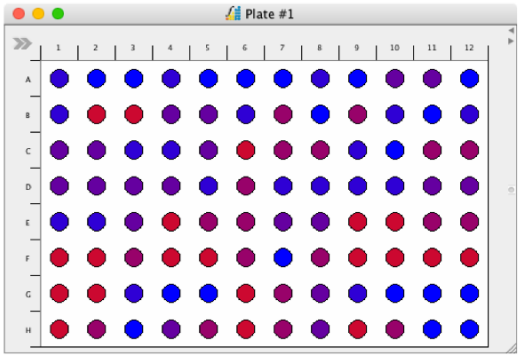


The plate side drawer tabs are as follow:

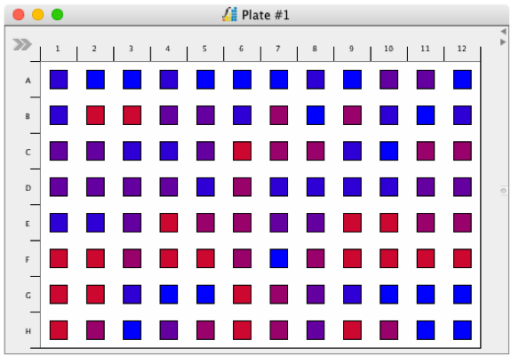
Plate Setup – set up the plate to best represent the design of your experiment. By default, the setup represents a 96 well plate.

- **Plate Dimensions** – set the width and height of the plate (number of wells) by typing in a number into the field or by using the up and down arrows. The plate will update in real time.
  - Width – the width determines the number of columns.
  - Height – the height determines the number of rows.
  - Fill Direction – the fill direction determines where samples will be automatically placed when either imported one after the other or when a selection is imported at once.
  - Horizontal – horizontal fill direction means that samples will continue to fill up an entire row before moving to the second. E.g. sample one will be placed in well A1 and sample two in A2, etc.
  - Vertical – vertical fill direction means that samples will continue to fill up an entire column before moving to the second. E.g. sample one will be placed in well A1 and sample two in B1, etc.
  - Well Shape – choose to display the wells as circles, squares, large squares or fill.

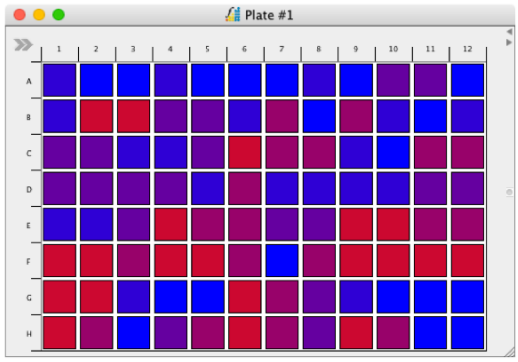
Circle



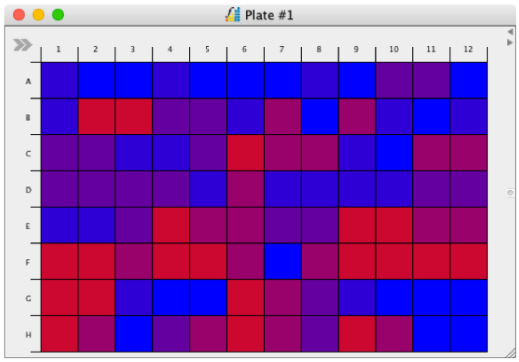
Square



Large Square



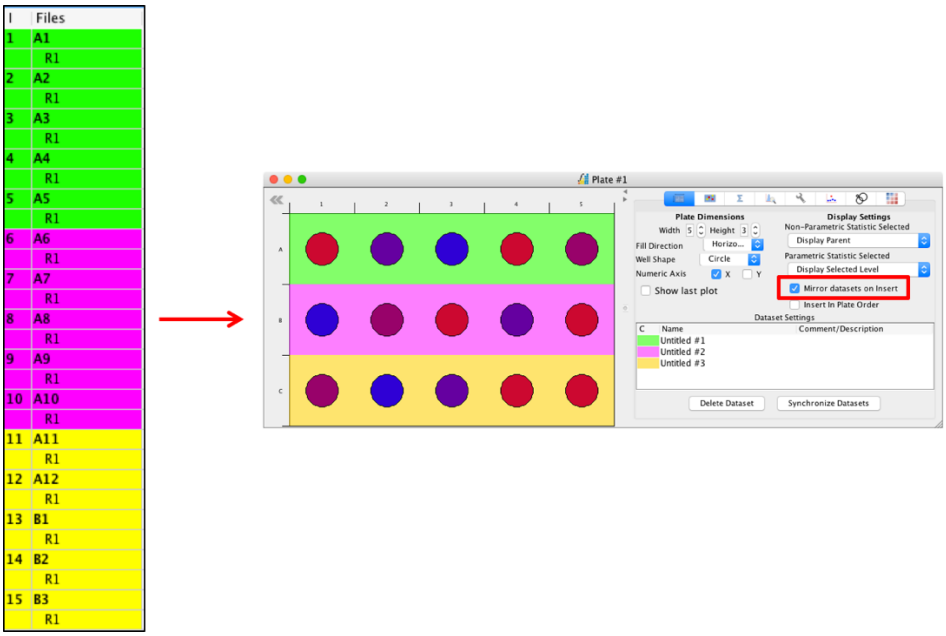
Fill



- Numeric Axis – choose to have either of the axes labeled numerically. If the numerical option is not selected, the wells will be labeled alphabetically. In most cases, it is best to have one axis labeled numerically and the other alphabetically.

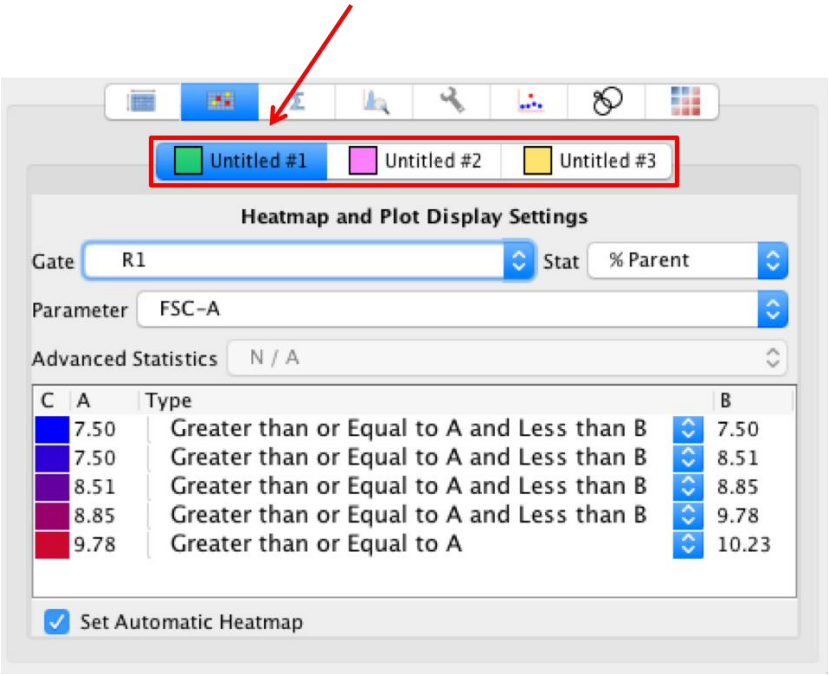
- **Display Settings** – the display setting options allow the viewing of different levels of the hierarchy, depending on the type of statistic that is being viewed.
  - Non-Parametric Statistic Selected – these are the statistics that are not related to the parameters where the gates have been created, such as event count and % parent. In many instances, it may be desired to view the statistics in relation to the parent plot. That is, if a gate is drawn, viewing the gate on the parent plot rather than the resulting daughter plot provides more information. The three different options are:
    - Display Selected Level
    - Display Parent
    - Display File
  - Parametric Statistic Selected – these are the statistics that are related to the parameters where the gates have been created, such as the mean fluorescence. In many instances, it may be desired to view the statistics of the populations resulting from a gate. The three different options are:
    - Display Selected Level
    - Display Parent
    - Display File

- Mirror datasets on Insert – this feature, when enabled, creates datasets in the plate that match groups from the File Inspector. The group color is applied to the matching dataset. Datasets are groups of wells/samples. Individual datasets can be modified without impacting other wells in the plate. For example, the same 5 files can be added to a plate three times, each in its own dataset. If there are a few gates associated with the files, then each dataset can be set to a different level of the hierarchy and/or statistic.



- Insert In Plate Order – samples are added to the plate in the order of A1, A2, A3, etc.
- Dataset Settings – the datasets can each be modified in terms of representative color and name. Comments or descriptions can also be added for each dataset.
  - C (color) – to change the dataset color, click on the color in the 'C' column and choose a new one from the options provided.
  - Name – double-click on the dataset name to type in a new one.
  - Comment/Description – double-click in the comment/description field to add a note relating to the dataset.
- Delete Dataset – to delete a dataset, highlight the dataset by clicking on its name and click Delete Dataset. This will delete the dataset but not the files in the wells.
- Synchronize Datasets – if FCS files are placed in groups in the File Inspector after they have been added to the plate, synchronizing the samples will create datasets based on the groups in the File Inspector. The result is the same as with mirrored datasets.

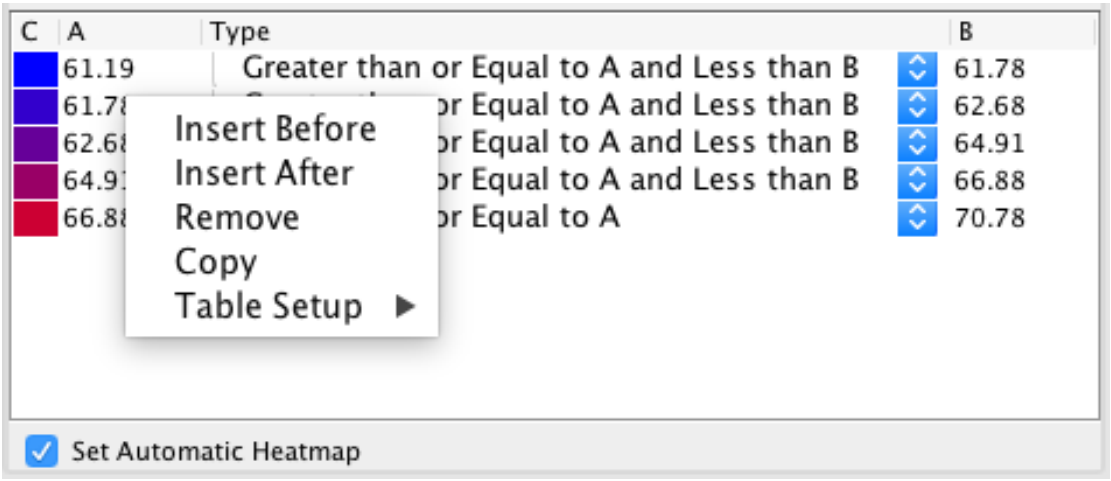
- **Heatmap Settings** – the heatmaps generated in the plate can be set up in the heatmap settings tab. Heatmaps are modified for each dataset individually.
  - Dataset name – defined datasets are listed at the top of the Heatmap Settings tab in the side drawer. To edit the heatmap settings for a dataset, click on the dataset name prior to changing the settings.



  - Heatmap and Plot Display Settings
    - Gate – choose the population that you wish the data array feature to take the settings from to create the heatmap. Click on the Gate drop-down menu to select the population by gate name.
    - Stat – click the statistic drop-down menu to choose the statistic that you wish the heatmap to be generated for. The statistics available are: Mean, GeoMean, StdDev, CoefVar, RCoefVar, %Total, %Parent, Event Count, %Selected, RStdDev, Cells/ml, Cells/µ.
    - Parameter – for statistics that refer to a particular parameter, such as the mean, choose the parameter from the parameter drop-down menu.
    - Advanced Statistics
      - C – the color for each range in the heatmap can be changed by clicking on the color in the in the column titled ‘C’.
      - A (bottom of range) – this column shows the value defining the bottom end of the range and can be changed by double-clicking within the filed in the column labeled ‘A’ and typing a new one.
      - Type – this is the rule defining each particular range in the heatmap, in relation to the values in columns ‘A’ and ‘B’. The type options are:

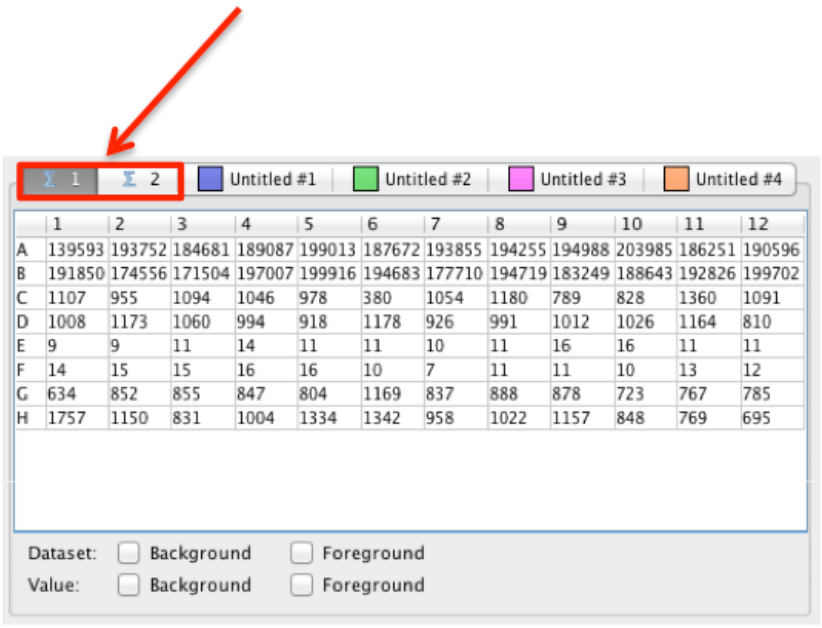
- Less than A
- Less than or Equal to A
- Greater than or Equal to A
- Equal to A
- Greater than or Equal to A and Less than B
- Not Greater than or Equal to A and Less than B
- B (top of range) – this column shows the value defining the top end of the range. The value can be changed by double-clicking within the filed in the column labeled ‘B’ and typing a new one.

- Right-click on a heatmap range to open a window with options to add a new range by inserting it before the selected range, after a selected range, delete a range or copy a range. Selecting copy range will add a copy of the selected range immediately after the copied range. If Set Automatic Heatmap is enabled, the range associated with the new copy will be recalculated. If Set Automatic Heatmap is not enabled, the range associated with the new copy will be the same as the range that it was copied from. To apply changes made to the heatmap settings for one dataset to another, click Apply to Dataset and select the dataset from the options. Click Table Setup to change the font and number of decimal places displayed in the heatmap setting table.
-



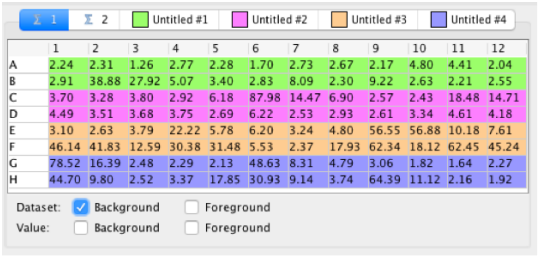
- Set Automatic Heatmap – when this option is selected, a heatmap will automatically be generate when samples are imported into a plate. The values for columns A and B cannot be edited if Set Automatic Heatmap is enabled.

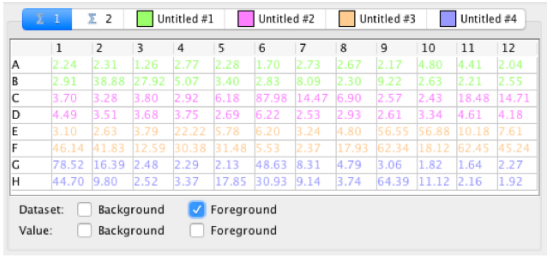
- **Statistics** – the statistics tab displays statistics that have been created in the Heatmap Settings tab. There are two table formats:
  - Statistic Table 1: table matches that in the plate setup tab.

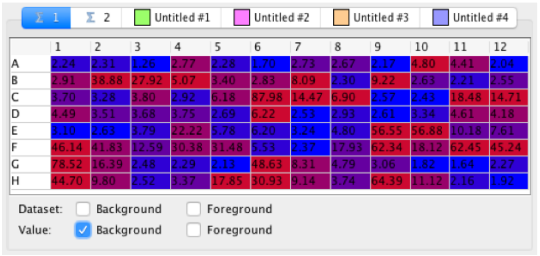


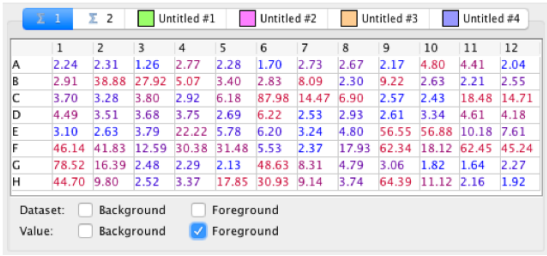
	1	2	3	4	5	6	7	8	9	10	11	12
A	139593	193752	184681	189087	199013	187672	193855	194255	194988	203985	186251	190596
B	191850	174556	171504	197007	199916	194683	177710	194719	183249	188643	192826	199702
C	1107	955	1094	1046	978	380	1054	1180	789	828	1360	1091
D	1008	1173	1060	994	918	1178	926	991	1012	1026	1164	810
E	9	9	11	14	11	11	10	11	16	16	11	11
F	14	15	15	16	16	10	7	11	11	10	13	12
G	634	852	855	847	804	1169	837	888	878	723	767	785
H	1757	1150	831	1004	1334	1342	958	1022	1157	848	769	695

  - Options at the bottom of the window allow the statistics to be colored based on the dataset or on their value where the colors match those from the heatmap. Both color options can be applied to the background (coloring the cell, not the text), the foreground (coloring the text, not the cell) or the background for one and the foreground for the other.



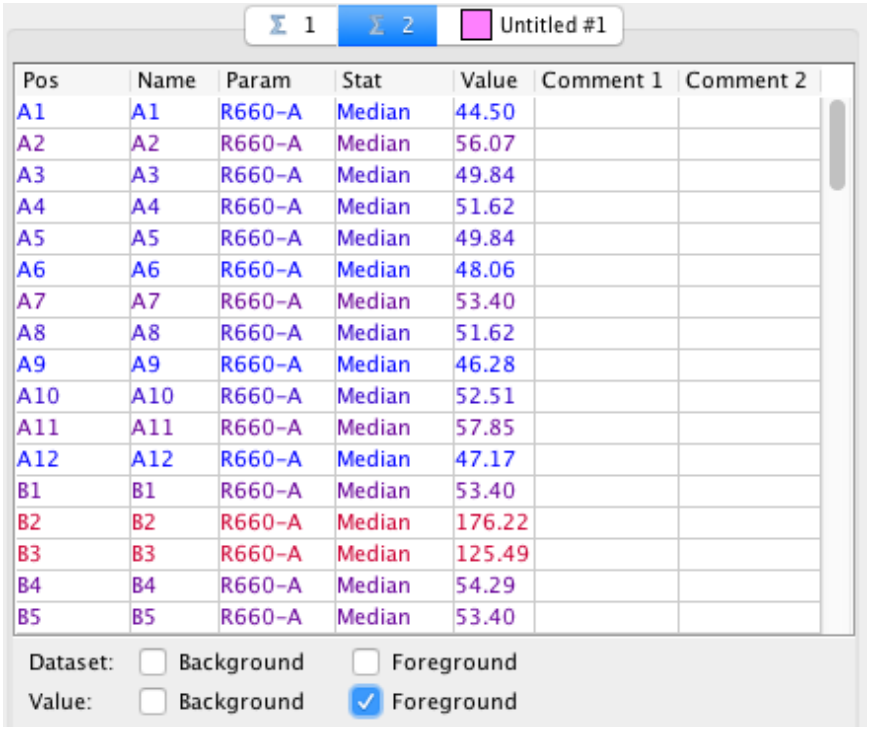




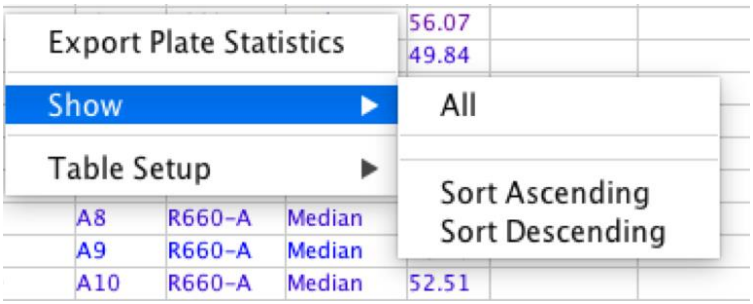


  - Right-click on the table opens a window allowing the statistics to be saved as a CSV file.
  - Selecting Table Setup allows for the font and number of decimal places to be changed.

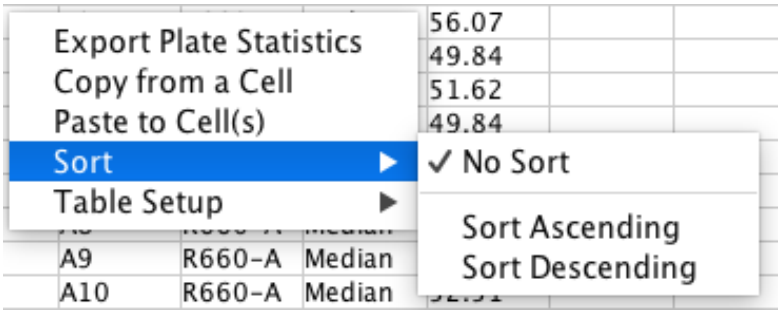
- Statistic Table 2: the data is set out in columns. The columns in Statistic Table 2 are Pos (plate position), Name, Param (parameter), Stat (statistic), Value, Comment 1 and Comment 2. Double-click in the Comments columns to make notes related to specific samples.
- The same dataset and coloring options are available for Statistic Table 2 as described for Statistic Table 1.



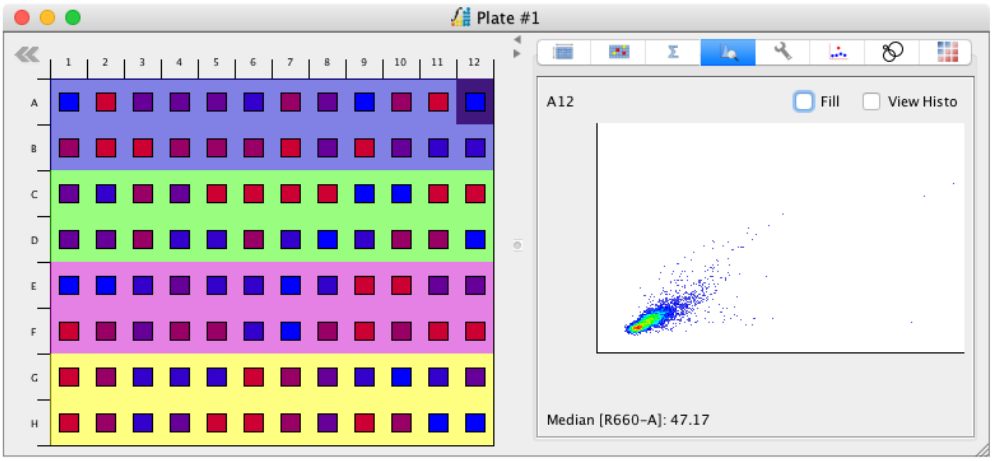
- Clicking on the dataset names above the table will display the results for the individual datasets in the same format as Statistic Table 2.
- The results in Statistic Table 2 can also be set to show an individual dataset by right-clicking on the table, selecting Show and selecting the desired dataset. Right-clicking, selecting Show and All will display all of the datasets.
- The results in Statistics Table 2 can also be sorted into ascending or descending numerical order by right-clicking in the table, selecting Show and Sort Ascending or Sort Descending. Selecting No Sort (when a table has been sorted) will return the results back the sample order.

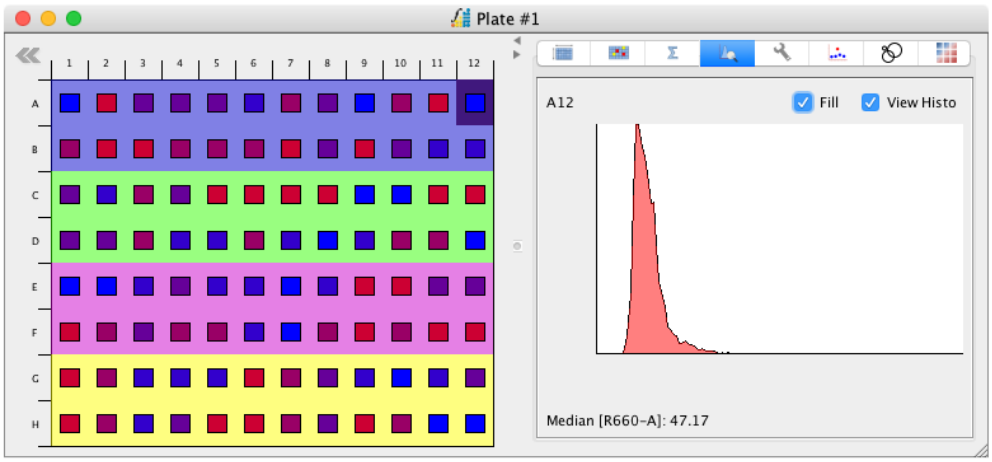


- Right-click in the dataset table to:
  - Export Plate Statistics - opens a window allowing the statistics to be saved as a .csv file.
  - Copy from a Cell
  - Paste to Cell(s)
  - Sort: No Sort, Sort Ascending, Sort Descending
  - Table Setup: Change Font, choose number of decimal places

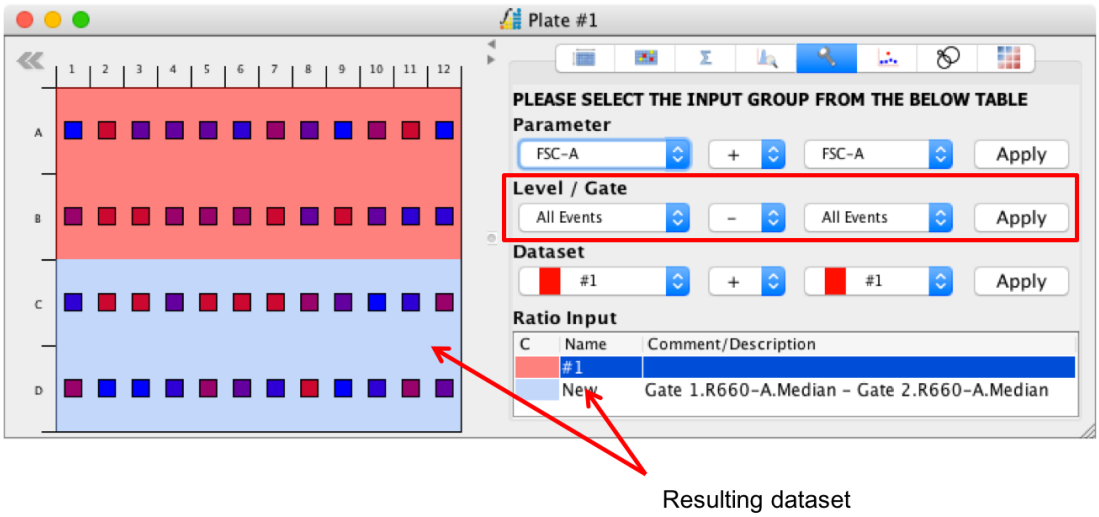


- **Plot Viewer** – the Plot Viewer tab allows the histogram or dot plot for each individual well to be inspected. Simply hover over the well in the plate to view the dot plot in the side drawer. The population displayed is determined by the setting in the Plate Setup tab under the Display Settings. The parameter to be displayed is determined by the settings in the Heatmap Settings tab.
  - Right-click: Remove Control – samples can be set as controls for data arrays by right-clicking on the samples in the File Inspector and choosing Set as control. This will overlay the control in the plate when viewing the plots in the Plot Viewer tab in the plate side drawer. Right-click → Remove Control removes control from the Plot Viewer tab when inspecting the wells.
  - View Histo – with this option selected, hovering over the wells will display a histogram.
  - Fill – with this option selected, a displayed histogram will be filled.
  - The dot plot display option will mirror what is set when the dot plot is open in the Workspace. To change this, double-click on the well to open the plot in the Workspace. Then, choose from a new plot display option from the Toolbar. The dot plot in the plate side drawer will be updated to reflect the selection.

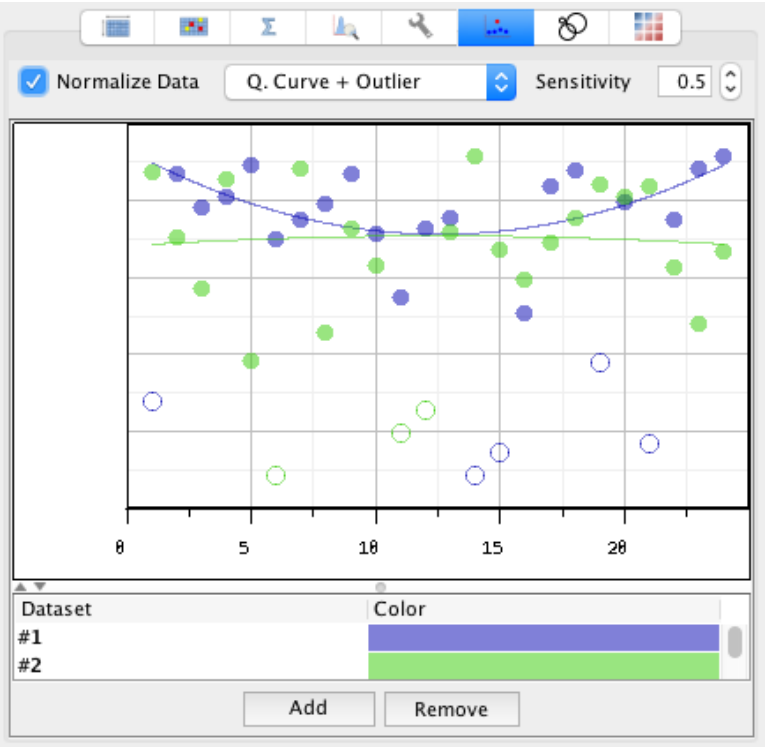


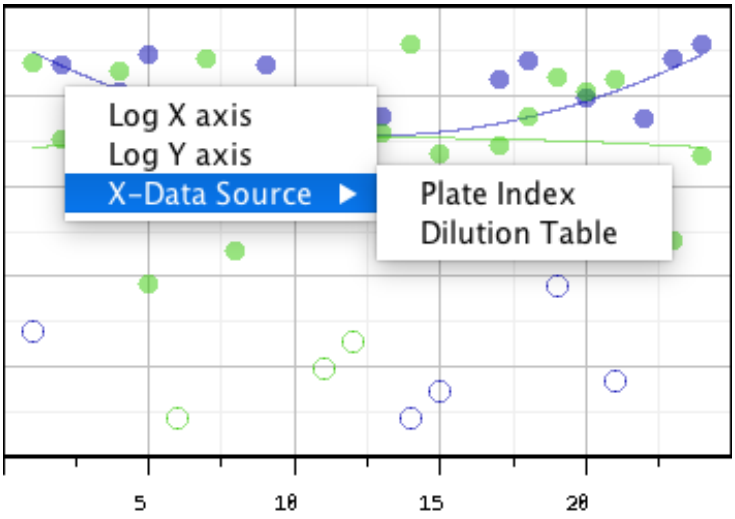


- **Advanced Functions** – the Advanced Functions tab allows for comparisons between different parameters, gates and datasets. Choose an option to compare, select the two statistics and choose the action to be performed. The row should read like an equation, e.g. *Gate 1 – Gate 2*. When the equation is set, select the input dataset from the Ratio Input window and click Apply. This will create a new dataset with the result. The new data set will also be displayed in the plate itself.

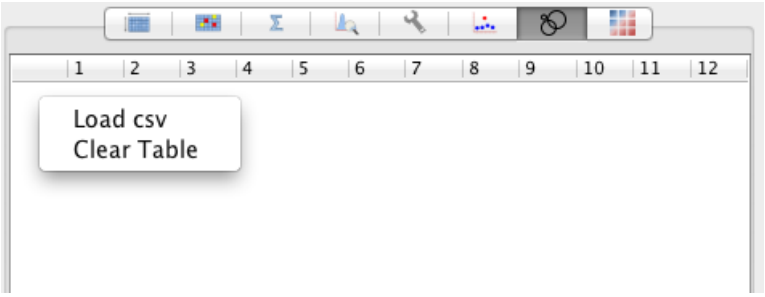


- **Plate Outlier Detection**
  - Data can be viewed in relation to a quadratic curve (Q. Curve), linear line or an asymmetrical 5PL curve, with outliers represented by a hollow circle. Double-click on a circle to open the dot plot in the Workspace.
  - If multiple datasets are displayed on the same graph, normalize the data to so that they can all be viewed.
  - Pull up the drawer at the bottom of the graph for options to Add or Remove datasets.

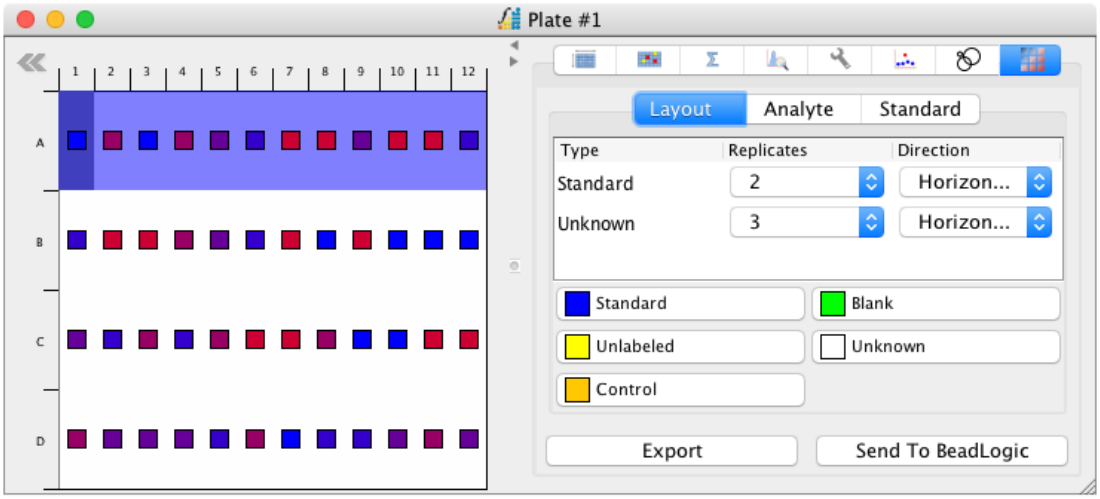




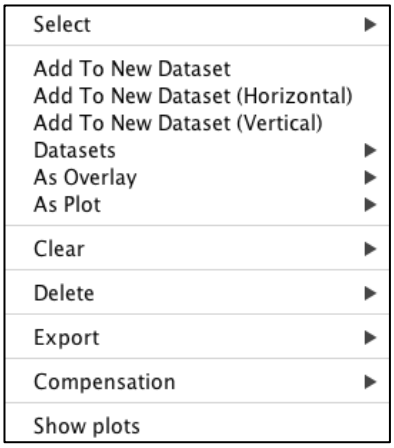
- **Dilution Table** – a dilution table saved as a .csv can be imported by right-clicking in the side drawer under the dilution table tab and selecting 'Load csv'.



- **BeadLogic Export** – wells can be defined as Standards, Blanks, Unlabeled, Unknowns and Controls before exporting to Beadlogic for multiplex analysis.



- **Plate options** – right-click on a well



- **Select** → allows the quick selection of groups of wells in a plate:
    - All – right-click anywhere in a plate and choose 'Select' → 'All' to highlight all wells.
    - Extend Horizontally – highlight a well from one or more rows, right-click and choose 'Select' → 'Extend Horizontally' to select all wells in the highlighted rows.
    - Extend Vertically – highlight a well from one or more columns, right-click and choose 'Select' → 'Extend Vertically' to select all wells in the highlighted columns.
    - Dataset → to select all samples from a particular dataset, right-click anywhere in the plate and choose 'Select' → 'Dataset' → and choose the dataset by name.
    - Inverse – an inverse selection is useful if you wish to exclude a relatively small number of wells. To make an inverse selection, highlight the wells to be excluded (using the options described above or by selecting them with the mouse), then choose 'Select' → 'Inverse'.
    - None – to clear all selections, right-click anywhere in the plate and choose 'Select' → 'None'.

- **Datasets** – individual datasets can be manipulated separately, allowing the same group of samples to be imported multiple times into a single plate but analysed independently (e.g., different gates or stats of the same files in the one plate):
    - Add to new dataset – adds the selections to a new dataset.
    - Add to new dataset (horizontally) – creates a new dataset for each row in the plate.
    - Add to new dataset (vertically) – creates a new dataset for each column in the plate.
    - Dataset (set/delete) – deletes the dataset but samples remain in the plate.

- **As Overlay** will create overlays based on the specific selection of wells:
    - Selection – creates a dot plot overlay of the samples highlighted in the plate.
    - Selection (Horizontal) –creates an overlay containing all samples that exist in the same row as the selected well.
    - Selection (Vertical) – creates an overlay containing all samples that exist in the same column as the selected well.
    - Dataset → creates an overlay containing all samples that exist in the same dataset as the selected well.

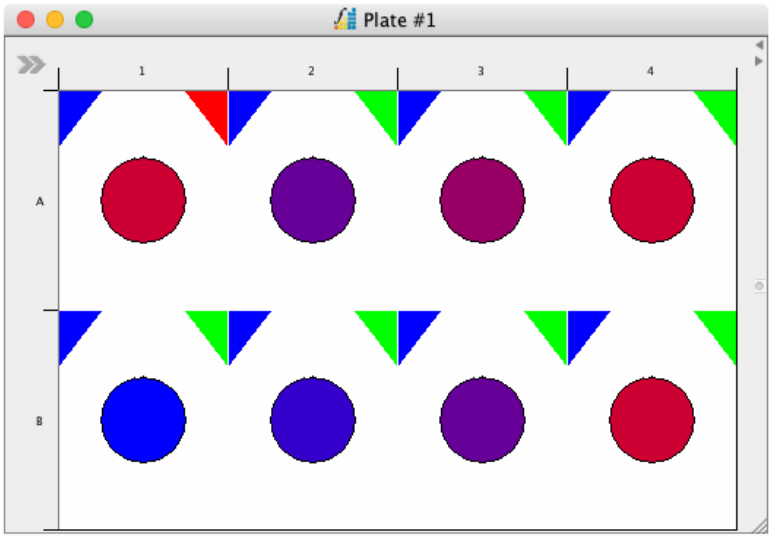
- **As Plot** opens a dataset or selection of files as dot plots in the Workspace, either from the selection of individual wells or datasets.

- **Clear** will clear all files from within selected wells. This does not delete the dataset:
    - All – deletes all samples from the plate
    - Selection – deletes the selected samples from the plate
    - Dataset → deletes the samples contained within a dataset.

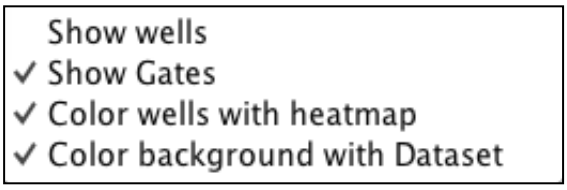
- **Delete** will delete an entire row or column from the plate.
    - Selected Row(s) – deletes the selected row or rows and all data contained within them.
    - Selected Column(s) – deletes the selected column or columns and all data contained within them.

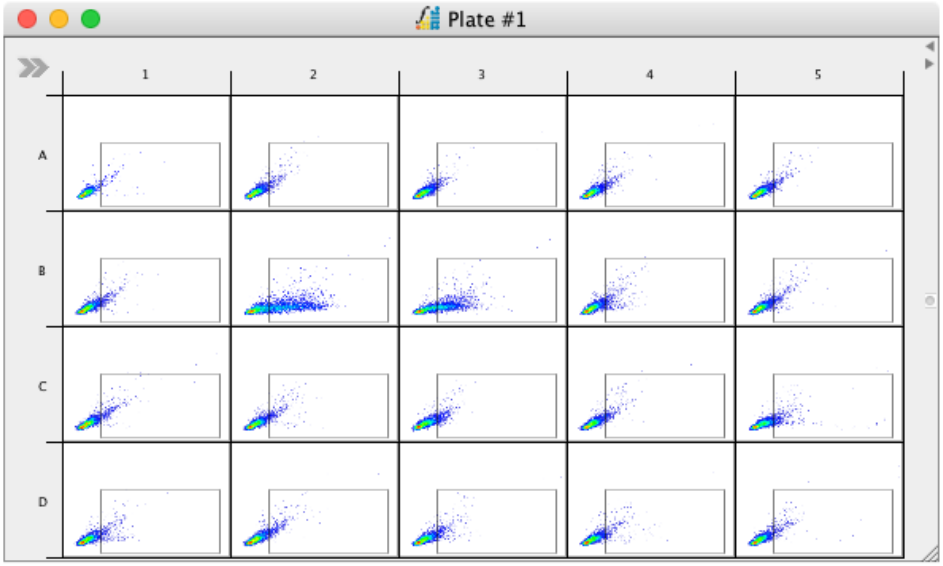
- **Export** a plate by either saving it as a file or copying it to the computer's clipboard.
    - To File – opens a window allowing the plate to be saved as one of a number of image file types.
    - To Clipboard – copies the image of the plate to the computer's clipboard to be pasted elsewhere.

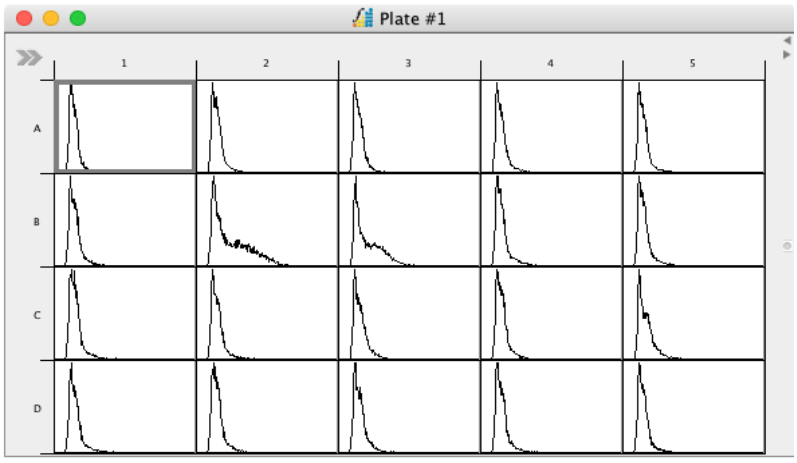
- **Compensation** displays differences in compensation matrices. The top corners of each well are colored. The left-colored corner represents the original compensation matrix. If this is the same in multiple wells then they all have the same original matrix. The right corner represents a post-acquisition change in compensation. If the two corners of a well are different it indicates that the compensation has been changed. Compensation matrices can be copied from one file in the plate and pasted onto another.
    - Show Comp Matrix – colors the corners to depict the applied compensation
    - Copy – copies the compensation matrix from a selected well/sample.
    - Paste → All/Selection – applies the copied compensation matrix to a selection of samples or to all within a dataset.

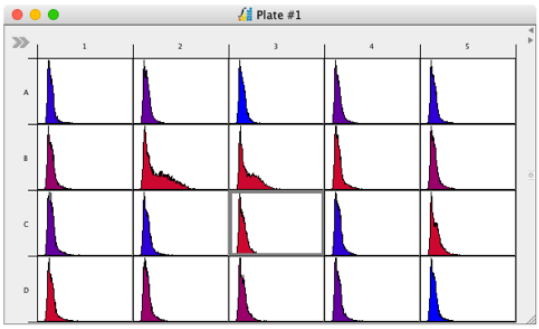


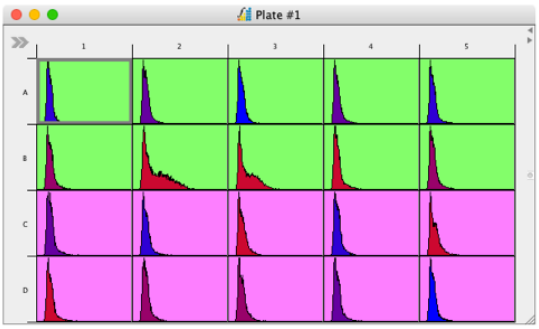
- **Show plots** displays each file as a dot plot. There are additional options to color the dots to match the heatmap colors, to color the background to reflect the different datasets and a combination of both.











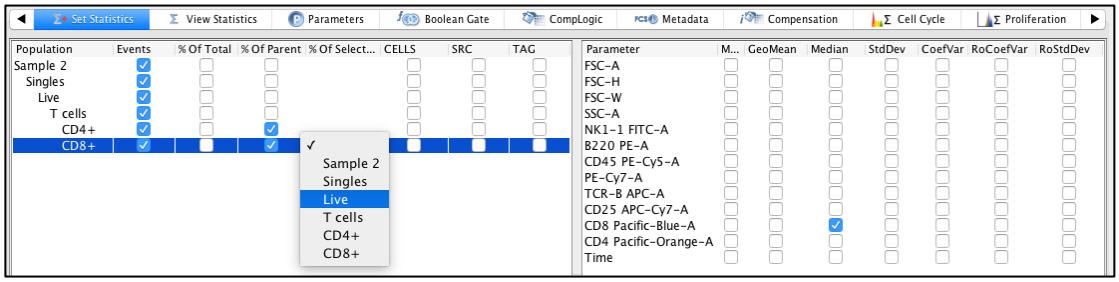
Building Plates from the File Navigator
Plates (data arrays) can be created directly from the File Navigator for all files contained in an experiment folder. This is particularly useful if you wish to display each gated population, as depicted in the population hierarchy) as a separate dataset. This enables all plots in the experiment to be displayed in the plate view.

Set Statistics

The set statistics tab is in the Advanced Functions is where the type of statistics to be calculated is set to display in the View Statistics tab.

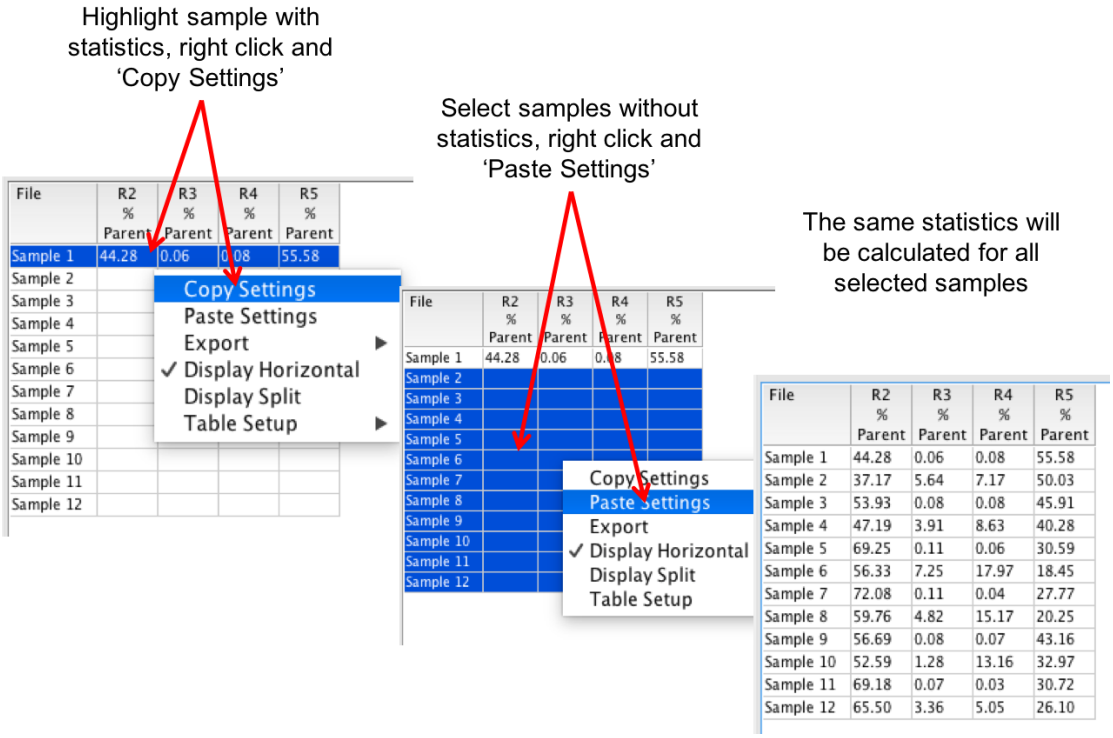
- Highlight the files in the File Inspector for which to calculate statistics. If files are contained in a group, selecting one of the grouped files will result in statistics being calculated for all files in the group.
- Open the Advanced Functions drawer and tick the boxes that correspond to the populations/gates. The statistics are separated into two windows. The left-hand window displays statistics that are linked to the selected files in the File Inspector. The right-hand window lists parameter-specific statistics that are linked to populations highlighted in the Populations column in the left-hand window. In the example that follows, the mean fluorescence will be calculated for the Pacific-Blue-A and Pacific-Orange-A parameters on the CD8+ population only. If multiple populations are highlighted in the Populations column prior to selecting a statistic in the right-hand window, then the parameter-specific statistic will be calculated for all selected populations.

- Population statistics include Number of Events, % Of Total, % Of Parent, % Of Selected, along with data contained in the keywords: CELLS, SRC and TAGs.
- % Of Selected means that a specific population can be shown as a percentage of any other population higher in the hierarchy, not just the parent (one level higher in the hierarchy) or total (the top level in the hierarchy). To define the selected population, click in the column in the row that corresponds with the gate of interest. Then, from the drop-down menu, choose the selected population.
- The parameter statistics include the mean fluorescence (Mean), geometric mean fluorescence (GeoMean), median fluorescence (Median), standard deviation (StdDev), coefficient of variation (CoefVar), robust coefficient of variation (RoCoefVar) and robust standard deviation (RoStdDev).
- Double-clicking on the column title will tick all boxes in that column.
    - The columns in both windows can be reordered by dragging the header
- Double-clicking on the parameter title in the right-hand window will tick all statistics for that parameter.
- To view the calculated statistics, click on the View Statistics tab in the Advanced Functions drawer.

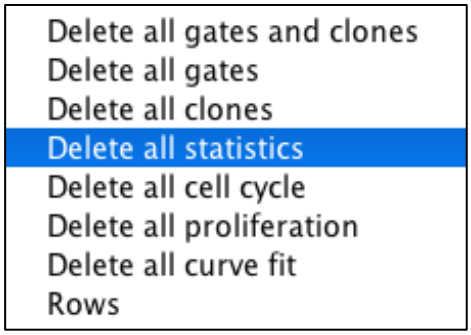


View Statistics

- Once statistics have been set in the Set Statistics tab, click on the View Statistics tab to display the statistics tables.
- Right-click on the table to copy, export or change the table setup.
    - Within the View Statistics table, the settings for specific statistics can be copied and pasted from one file to another (or multiple). For example, if statistics for one file have been calculated, highlight that file in the table, right-click → Copy Statistic Settings, highlight remaining files in the View Statistics table, right-click → Paste Statistics Settings. All statistics calculated for the first file will then be calculated for all remaining files.

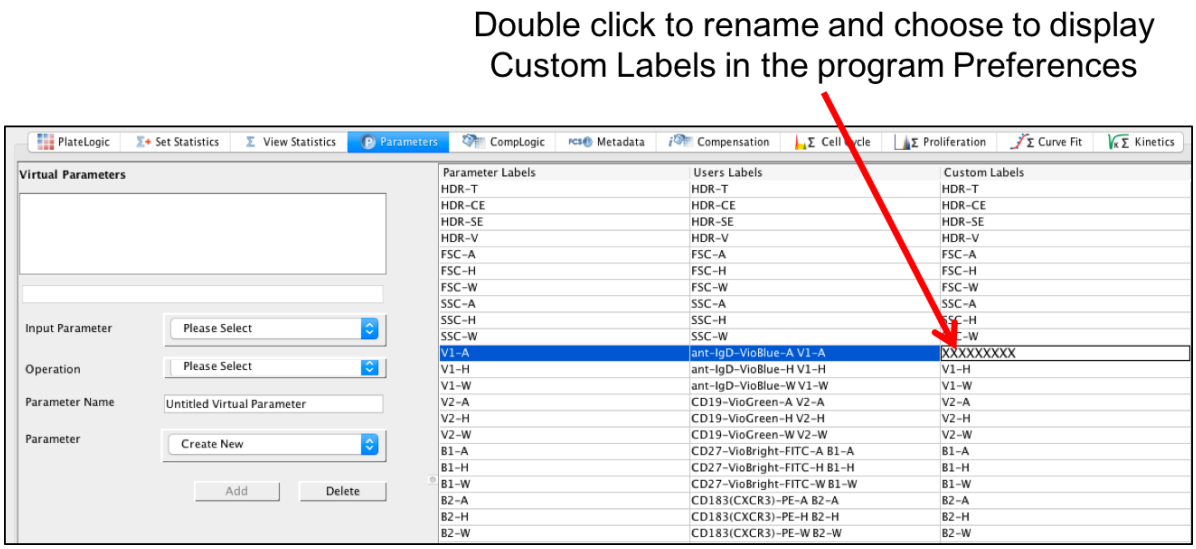


- To clear the statistics, right-click in the File Inspector and choose Delete → Delete all statistics.

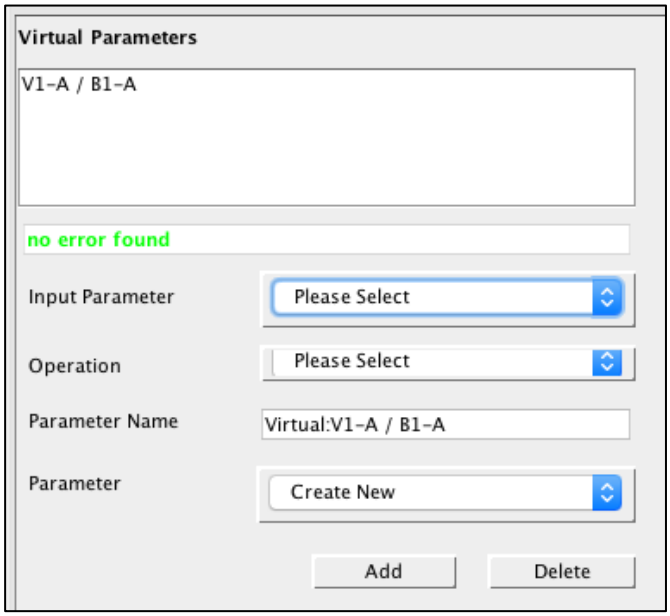


Parameters

- All parameters are listed in the Parameters tab.
- Custom Labels can be changed by double-clicking in the field and typing the new name. Display the custom labels on plots by selecting to display Custom Labels in the program preferences.



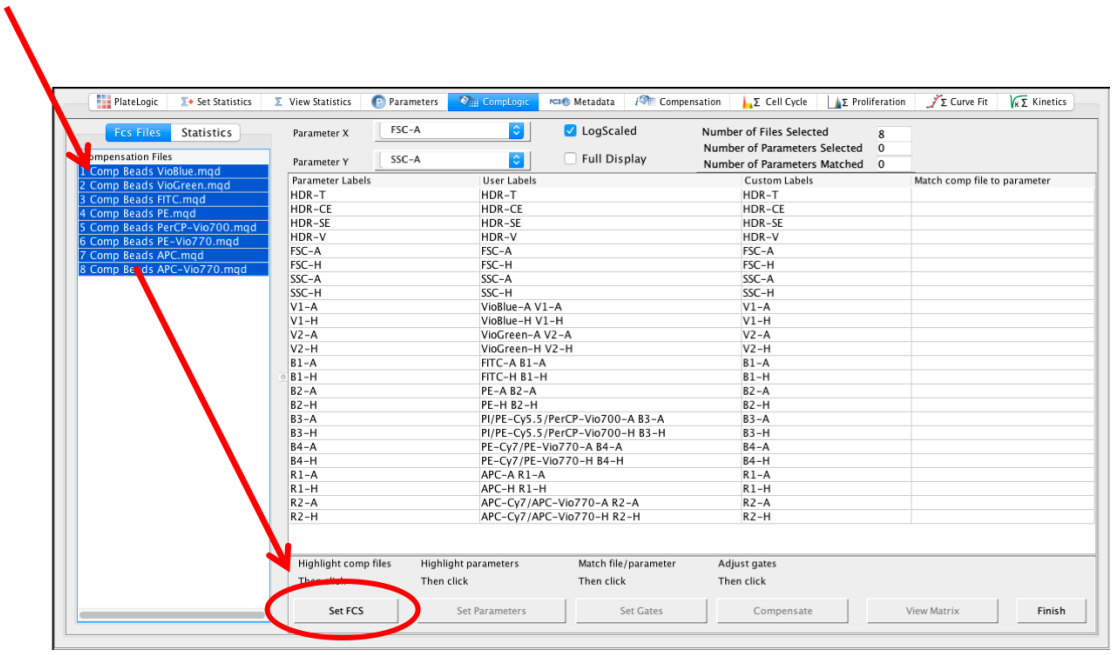
Virtual parameters can be created by selecting an input parameter followed by an operation and another input parameter or value. Click ‘Add’ to create the new parameter. To delete a virtual parameter, select the parameter in the ‘Parameter’ drop-down list and click ‘Delete’.



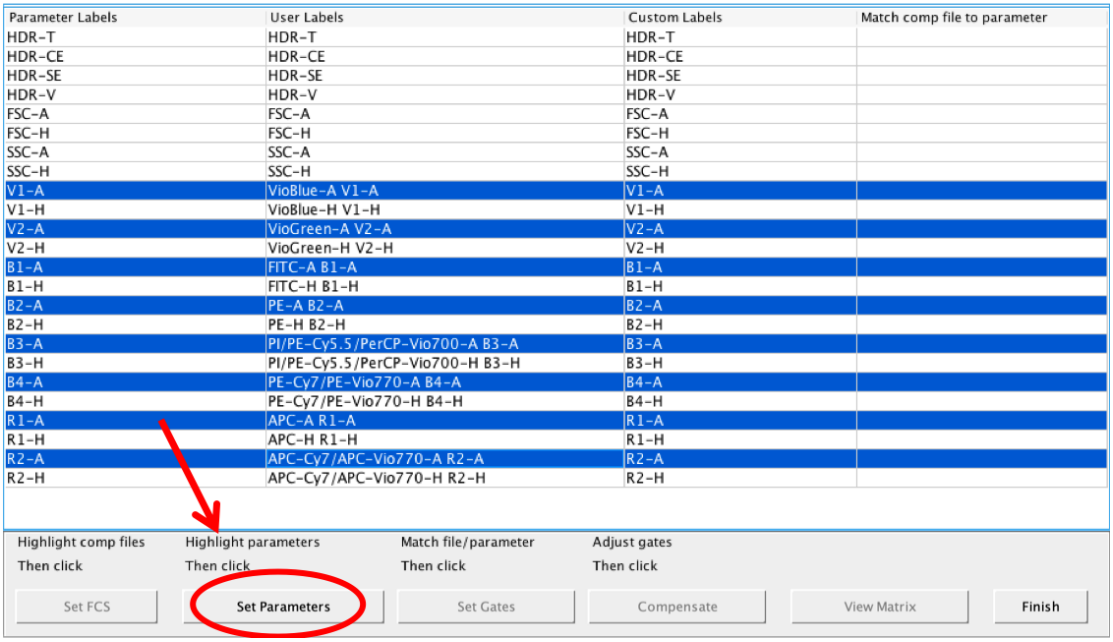
CompLogic – Auto-compensation

FlowLogic can perform auto-compensation given appropriate single-color controls.

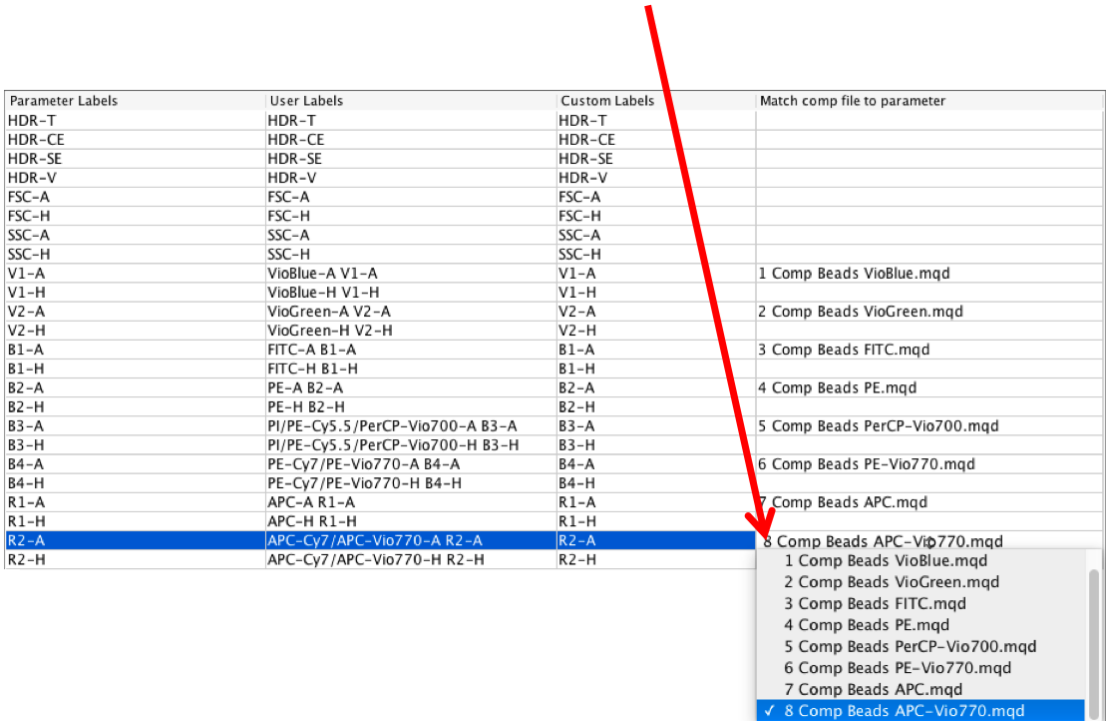
- From the Advanced Functions drawer, select the CompLogic tab. From the samples list on the left of the drawer, select your single-color controls then click ‘Set FCS’.
- In the parameters box highlight the parameters that you want to compensate and click ‘Set Parameters’. The boxes in the ‘Auto’ column will be ticked.



- In the parameters box highlight the parameters that you want to compensate and click ‘Set Parameters’. The boxes in the Auto column will be ticked.

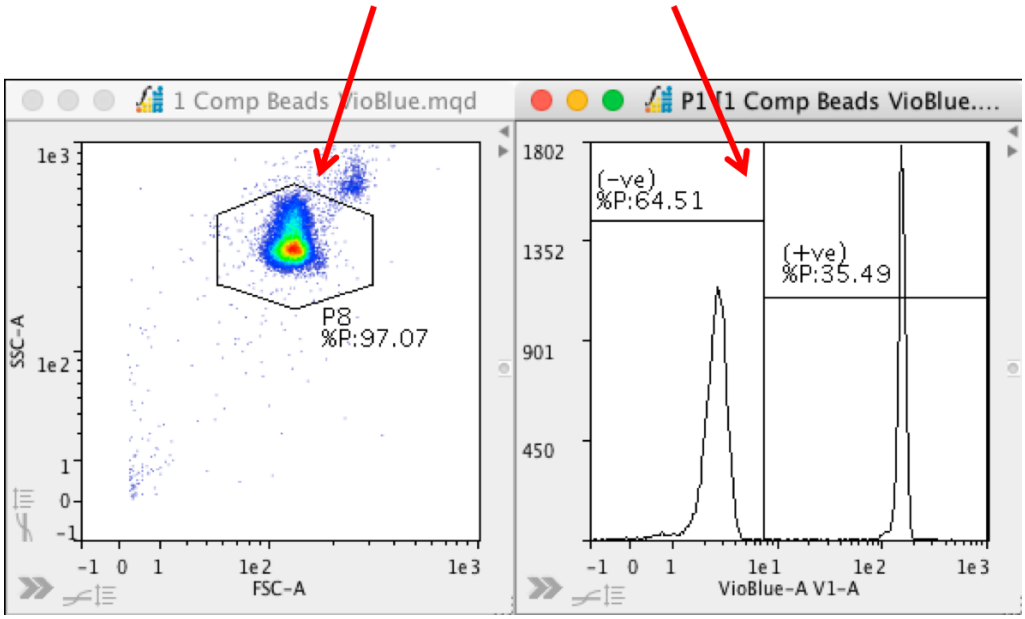


- Verify that the suggested file listed in the FCS Files column matches the parameter as shown in the Parameters column. If not, click on the FCS file title and choose the matching FCS file from the available options.



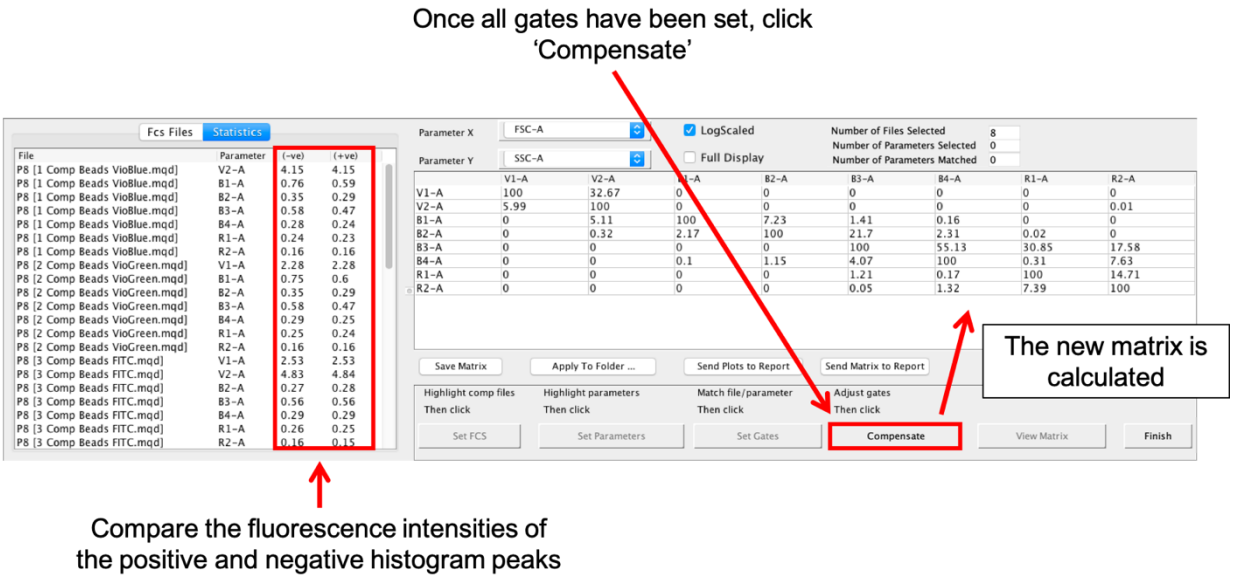
- When you have matched the FCS files and parameters click ‘Set Gates’. FlowLogic will create a FSC-A vs. SSC-A plot with a polygon gate and the daughter population histogram for each parameter. The polygon gate itself, will automatically detect the population of greatest density. Adjust the polygon and histogram markers to select the negative and positive populations.

- To make viewing easier, change the number of windows displayed per row to two. Click Edit → Plot Arrangement → Number of Windows → 2.



- When all gates have been corrected, click Compensate. FlowLogic will then calculate the compensation matrix.

- The newly calculated compensation matrix will be displayed. You can now save the compensation matrix or apply it to all samples within a folder with one click.

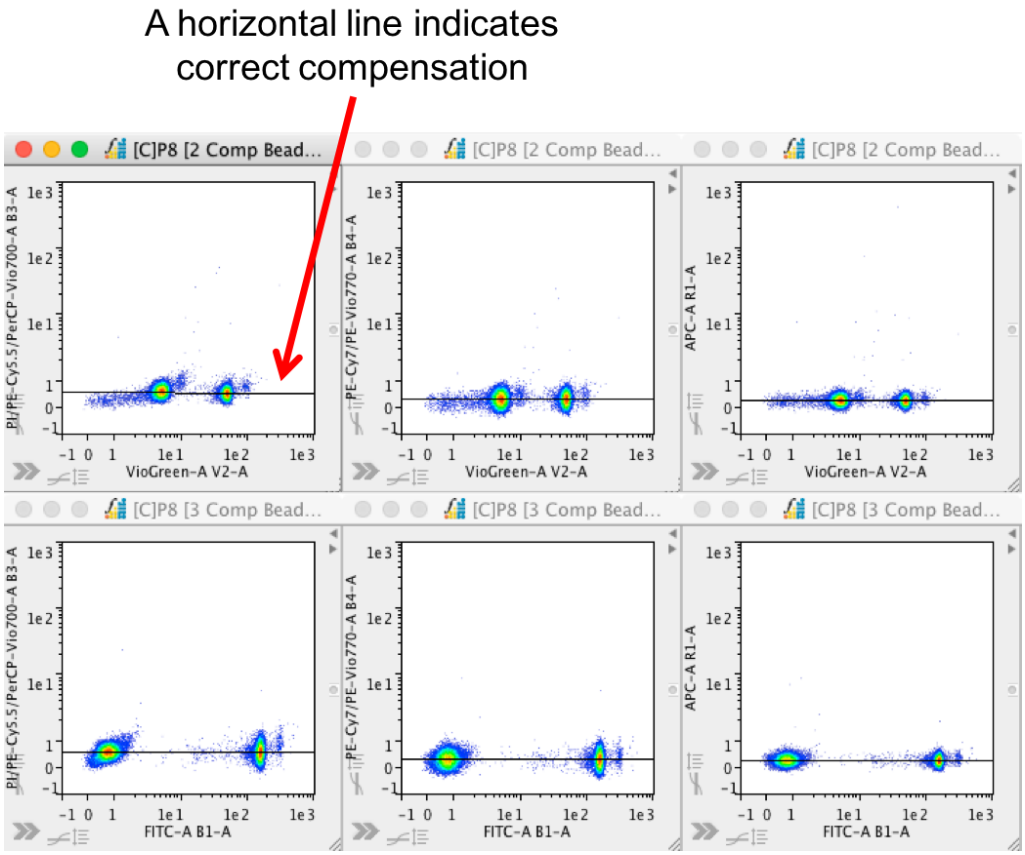


- The MFI is calculated for the positive and negative populations in all spillover channels. A line is subsequently drawn connecting these two values. A horizontal line indicates correct compensation.

- The original compensation created at acquisition remains saved with the file and can be re-applied at any time.

- Auto-compensation can be performed as many times as you wish.

- The compensation for each individual file can be checked by opening the appropriate dot plots. Fine adjustments can be made in the plot side drawer and the new matrix saved and applied to all other files.

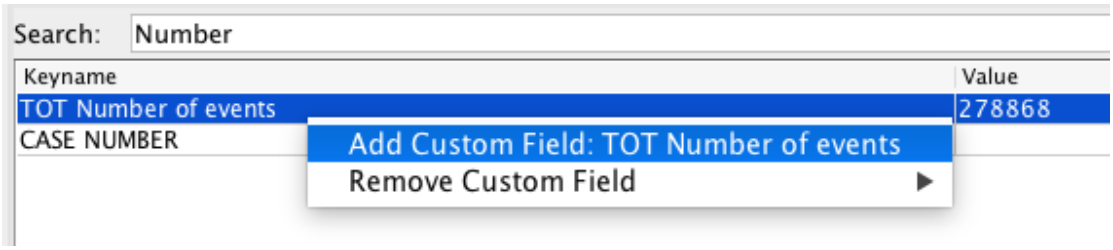


In the CompLogic window, tick ‘Full Display’ to view all events on each plot. Unticking ‘LogScaled’ will revert the axis to a logarithmic scale for all plots.

Metadata

Each FCS file has associated metadata that can be viewed in this tab.

- A specific metadata keyword and its associated value can be added to the File Inspector table for each sample. To add the keyword to the File Inspector, right-click on the specific row and choose ‘Add Custom Field’.
- To remove a keyword from the File Inspector, right-click in the Metadata table and select ‘Remove Custom Field →’.
- Use the Search field at the top of the pull-up drawer to search for specific items within the metadata.



Compensation

The compensation matrix for each FCS/MQD file can be viewed in this tab.

- To view a compensation matrix, highlight the FCS/MQD file in the File Inspector. The matrix will automatically be displayed.
- Right-clicking on the matrix will provide an option to export the matrix as a TXT file.

Cell Cycle, Proliferation, Curve Fit and Kinetics Tabs

These tabs provide statistical summary tables from the Cell Cycle, Proliferation, Curve Fit and Kinetics features. Statistics can be exported from the tables by right-clicking within the table and choosing the export option. This data can also be imported directly into GraphLogic by right-clicking in the Workspace and choosing to add the table related to the specific analysis type.

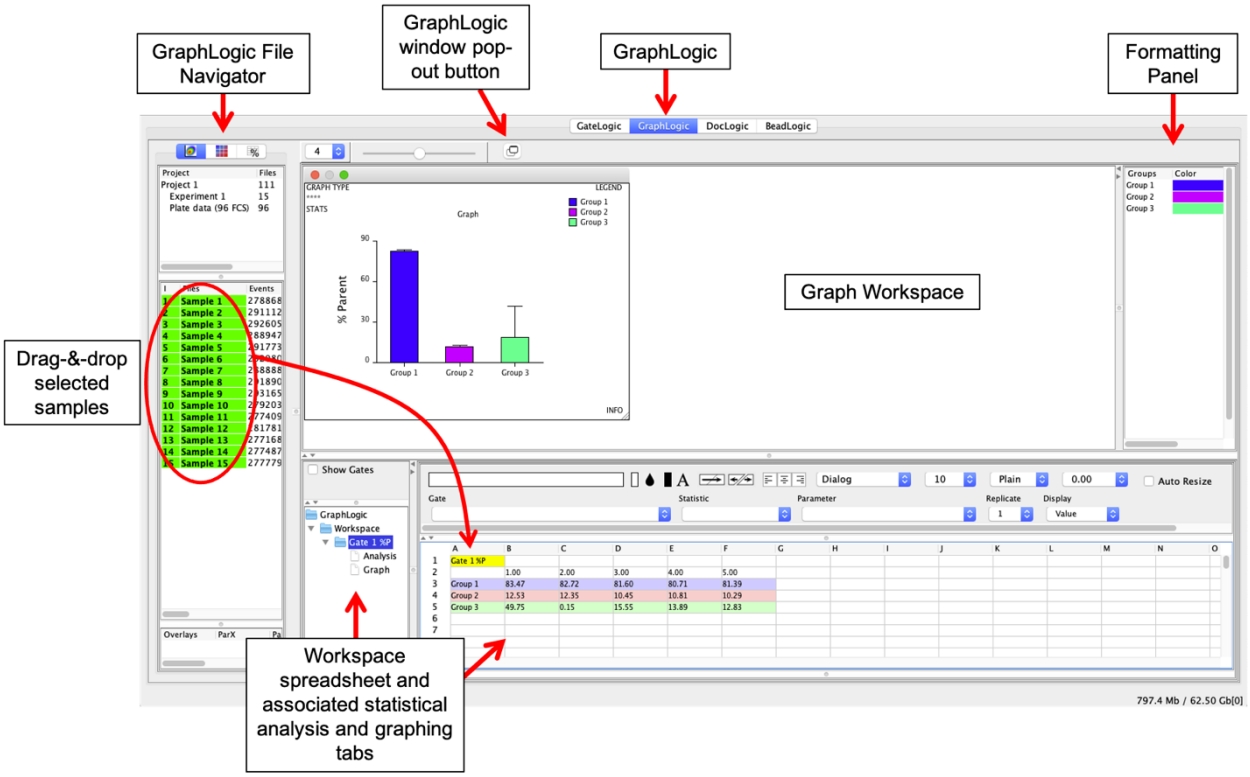
GraphLogic – Graphing and Statistical Analysis

Overview

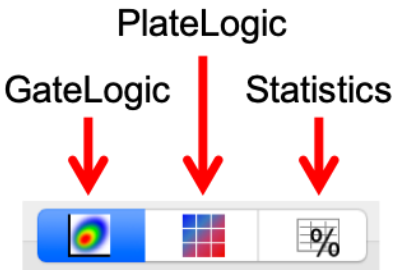
GraphLogic enables the graphing and statistical analysis of data derived from FCS/MQD files. All sections in FlowLogic are linked, so any adjustment to gates in GateLogic will automatically update the graphs and statistics tables in GraphLogic.

Below is an overview of the GraphLogic window.

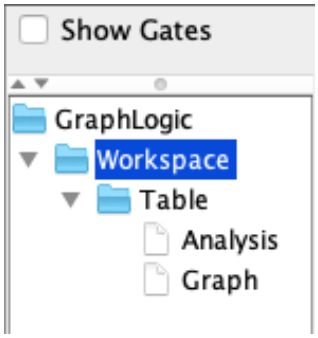
- The panel on the left contains the File Navigator with three tabs: GateLogic (dot plots, histograms and overlays), PlateLogic (plates/heatmaps) and statistics (the table as exists in the 'View Statistics' tab of the Advanced Functions pull-up drawer).
- Selected elements from the File Navigator can be dragged directly into the Workspace Spreadsheet at the bottom of the window. After adding files to the spreadsheet, the gate/population and related statistic can be defined. Data tables can be dragged into Table folders to the left of the spreadsheet. From here, graphs can be displayed in the Graph Workspace at the top of the window and statistical analyses can be performed in the analysis pull up window.
- Clicking on specific parts of a graph (bars, axes, labels, titles, etc.) displays the corresponding Formatting Panel on the right of the screen.



The GraphLogic File Navigator



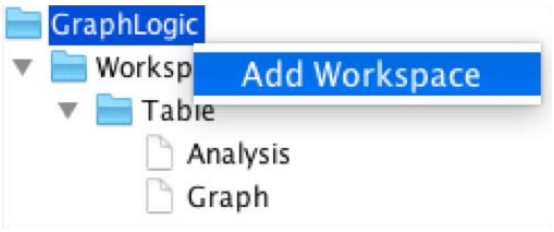
Selecting the GateLogic tab will display all of the FCS/MQD files. The appearance is similar to the File Inspector in GateLogic, except that the gates/subpopulations are hidden and there is no right-click menu. This is because files are added to the spreadsheet where the population and statistic is defined. It is possible to display the gates by ticking “Show Gates” above the Workspace folders.



For more detail about adding files to the spreadsheet, see page 159.

Show Gates – by default, gates are hidden and only the file name is displayed. In GraphLogic, it is generally only necessary to view the files themselves, with the ability to show gates only necessary to be reminded of the gating hierarchy without having to click back to GraphLogic.

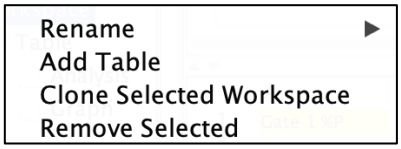
Workspace Folders



Data existing in the Workspace arranged within graph templates (see page 159) can be dragged and dropped into Table folders from which graphs are automatically generated and displayed in the Graph Workspace. Statistical tests can also be performed on data in the graph table folder in a few easy steps. As the FCS derived data in the tables remains linked to GraphLogic, all graphs and statistical analyses are updated automatically. Data can also be imported from CSV files or typed directly into the spreadsheet. Data contained in the Workspace can also be exported as a CSV file or as a MS Excel (.xlxs) file, with the latter exporting with all color and formatting.

Right-click menu for the GraphLogic folder:
The GraphLogic folder contains all subfolders. By default, one Workspace folder containing a Table folder with the associated Analysis and Graph sheets are created. Additional Workspace folders (with a table, analysis and graph) can be created by right-clicking on the GraphLogic folder, followed by 'Add Workspace'.

Right-click menu for the Workspace folder:



Workspace folders can be renamed using the first option in the right-click menu. Additionally, new Table folders can be added to a single Workspace folder. In this way, multiple graphs can be generated, and statistics test performed from all of the data in one Workspace spreadsheet.

There is also an option to clone a given Workspace and all of its subfolders. This can be very useful if you have a lot of formatting and calculations in one Workspace that you wish to duplicate and then replace the data. Once a Workspace has been cloned, the data can be deleted and new files or values can be imported.

Finally, a Workspace can be deleted or removed using the last option, 'Remove Selected', in the right-click menu.

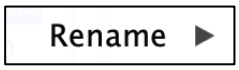
Right-click menu for the Table folder:

When FCS files are dragged into the Workspace, the naming template is automatically created around the data. When this same data is dragged into a Table, the Table folder name will take the name in the 'Table' cell form the Workspace. However, if you are importing non-FCS data, you have removed the 'Table' name from the Workspace or you wish to change it, this can be done from the 'Rename' option in the right-click menu.

Tables can also be cloned. This is useful if you have formatted graphs and performed particular statistical tests. The easiest way to replicate this is to clone a Table and then drag the new data into the clone. This will then update all current graphs and statistics.

As with Workspaces, individual Table folders can be deleted by right-clicking on the desired folder and choosing 'Remove Selected' from the menu.

Right-click menu for the Analysis and Graph icon:

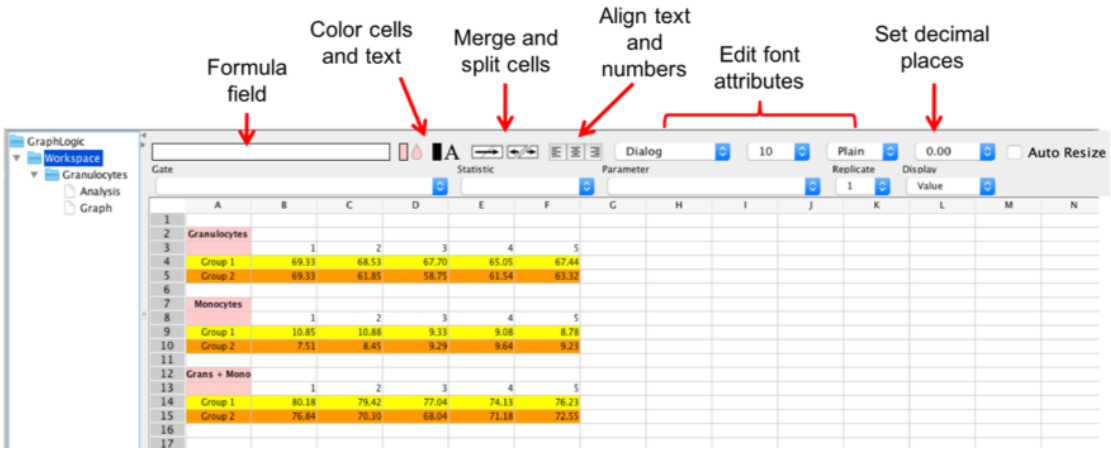


The analysis and graph can be renamed to help with identification. Renaming is particularly useful when adding elements to a DocLogic report.

Workspace Spreadsheet

The Workspace spreadsheet can accept FCS data, which remains linked to the original gates so that any adjustments in GateLogic are automatically updated in the GraphLogic spreadsheet. In addition, non-FCS data can be imported from CSV files, and numbers and text can be typed directly into the cells.

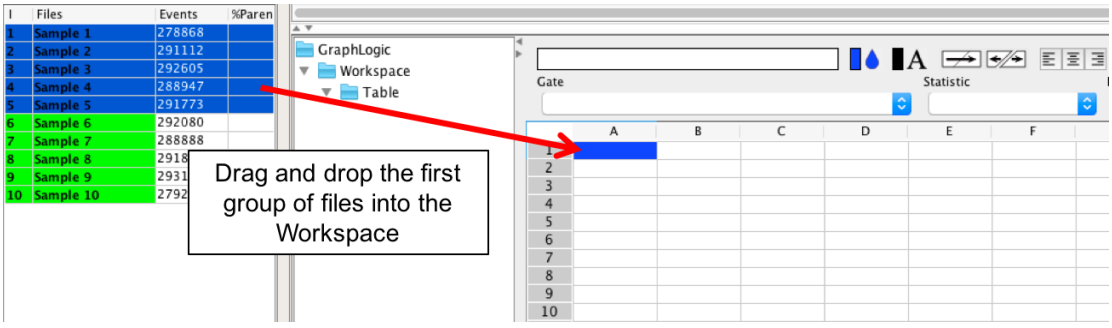
Common editing features exist, allowing cells and text to be colored, the merging and splitting of cells, changing the font type, style and size, changing text and number alignment within cells and selecting the number of decimal places to be displayed. A detailed description of each of these features can be found from page 169.

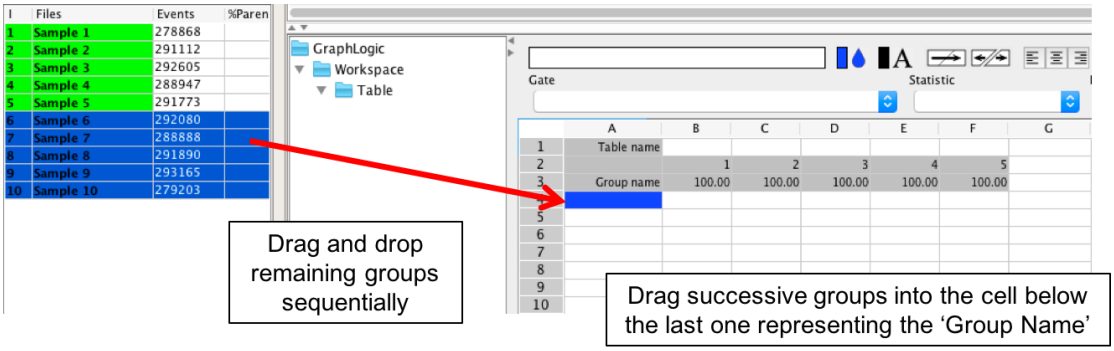


Mathematical functions can be applied to any data in the Workspace, such as adding, subtracting, multiplying or dividing one cell by another. The equation defining the calculation can be created in the formula field above the Workspace or directly in the spreadsheet. For example, to add the values in cells A1 and A2, type “=A1+A2” and then press enter.

Adding FCS-linked Data

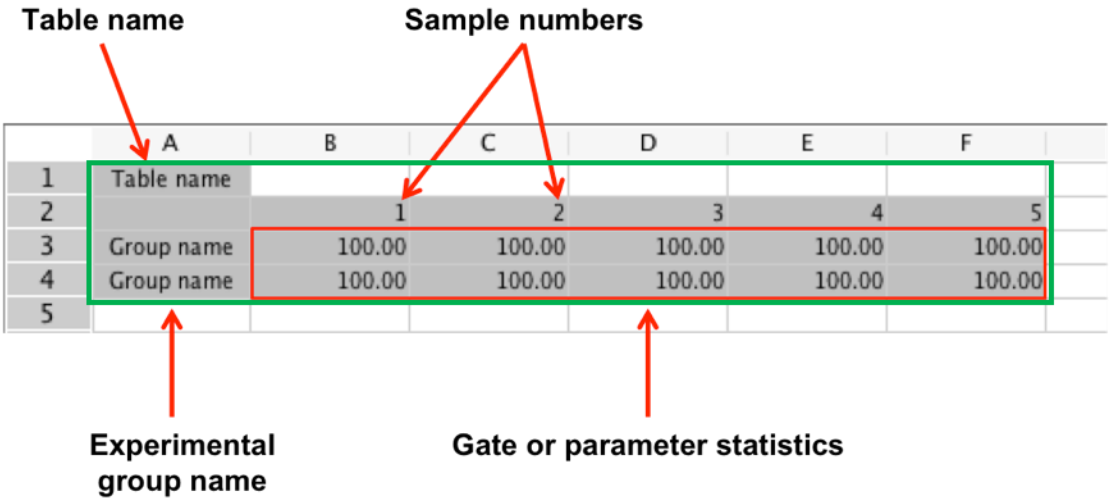
The feature that makes the GraphLogic Workspace so useful is the ability to drag in FCS files, or import files contained in a plate, and then display any gate or parameter statistic using the drop-down menus at the top of the Workspace. Data can then be dragged into a Workspace Table, which automatically generates a graph. Statistical analyses can also be performed with a few clicks. In addition, FCS-linked data in the spreadsheet can be duplicated and set to a different gate or parameter statistic whilst maintaining all other generated graphs and tables.





When FCS files are dragged into the Workspace, a template is automatically created with titles for the Table (dataset) name, the experimental group names and the sample count.

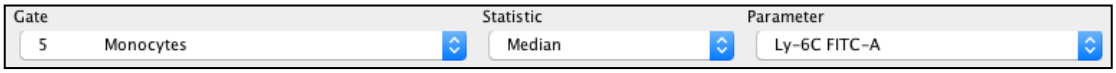
- Table name: the table name refers to the table containing the dataset that the associated graph is derived from. For example, this could be Lymphocytes %Parent.
- Group names: the group name relate to the experimental groups, which are reflected as separate bars in a bar graph, for example. This is unrelated to the groups created for group analysis. In the same example, there might be two groups: treatment and control.
- Sample number: this refers to individual samples within each experimental group, or the *n* value.



The linked data exists in the red border above. Highlight these cells and choose a population and statistic from the toolbar above the Workspace. Graphs can be created by highlighting the entire data table (within the green border) and dragging it directly to a Table folder in the window to the left. The table name and group names will automatically be applied to the Table folder and graph, respectively. Updating these names in the Workspace after the data has been added to the table will update the table folder and the groups in the graph.

Selecting gate and parameter statistics
When FCS files are dragged into the Workspace, by default the value for each sample will be 100.00, relating to the %Parent, All Events.

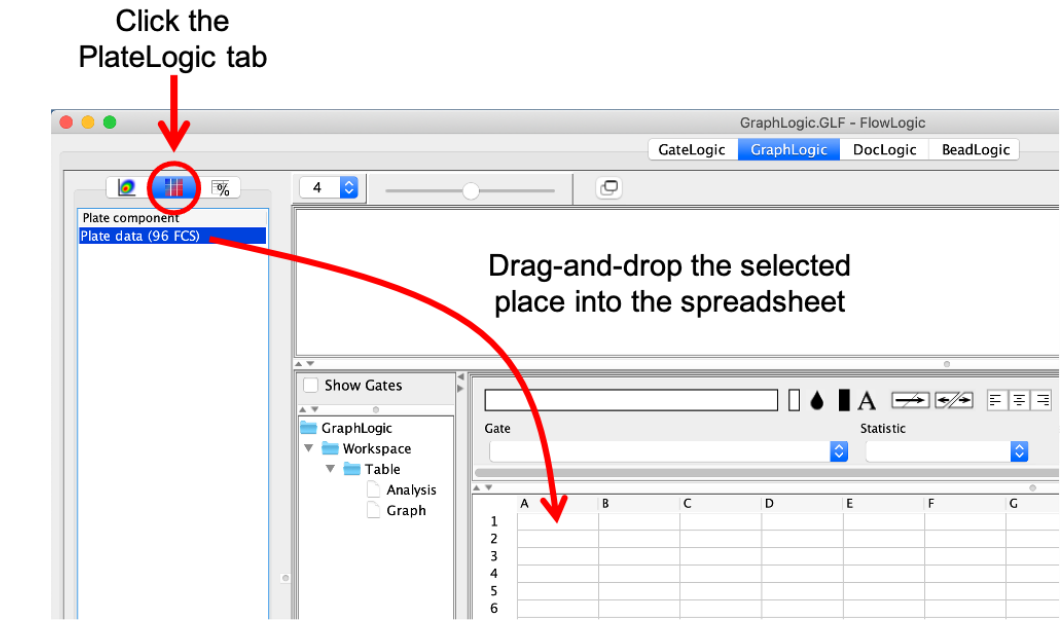
Use the drop-down windows above the table to select the population (gate) and the type of statistic (event count, %parent, %total, mean, etc.) to graph. Finally, you can specify the number of replicates performed.

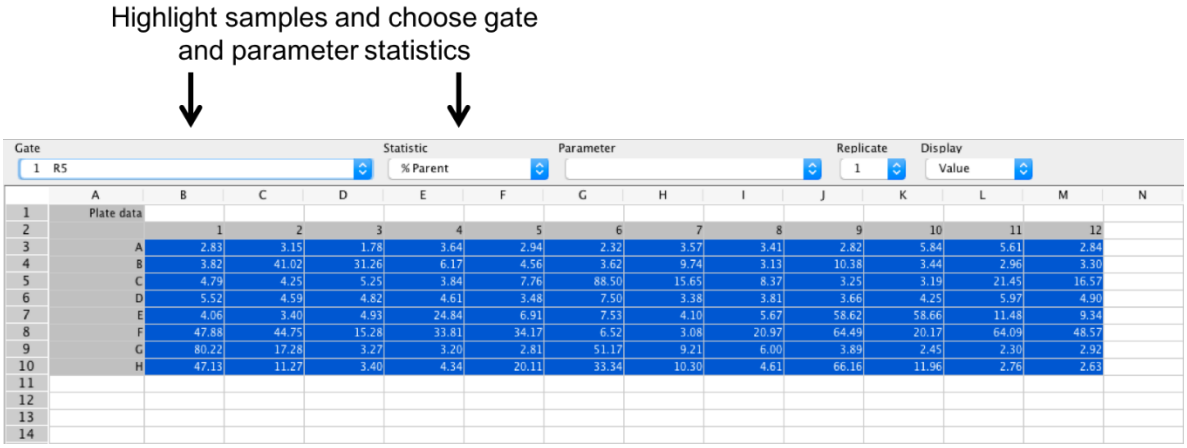


Importing PlateLogic Data
An alternative method for adding FCS-derived data into a GraphLogic Workspace is to import files that are already arranged in plates within PlateLogic. Files that have been imported into a plate in PlateLogic can be added directly to the Worksheet in GraphLogic by selecting the desired plate from the list of plates under the PlateLogic tab in the File Navigator. The data will be imported into the Worksheet in the same arrangement as the files exist in the plate. Files can be added to a plate before gating or at any time after gates have been created.

There is no need to define the statistics in PlateLogic as the selection can be made in GraphLogic. The data remains linked to the gates in GraphLogic and so will update automatically if a gate is adjusted. The same ‘plate’ can be imported multiple times, or an imported dataset can be copied and pasted elsewhere in the Workspace. The data from either form of duplication can be changed using the drop-down menus to define a different population or statistic. When importing plate data, the table structure with a Table name, Group names and Sample counts will automatically be created in preparation for generating a graph.

Once any of these datasets have been imported into a GraphLogic Workspace, the data can be rearranged for graphing and statistical analysis. Data imported via PlateLogic remains linked to the gates and so any adjustment to gates in GateLogic will update the data in GraphLogic.





Importing Statistics Tables

In a similar way that PlateLogic data can be imported into a GraphLogic spreadsheet, other statistics tables can be selected from the statistics tables tab in the File Navigator and imported using drag-and-drop. The types of tables include:

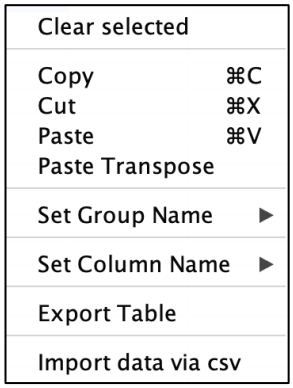
- The statistic table displayed under the View Statistics tab in the Advanced Functions pull-up drawer
- The statistics tables in the PlateLogic side drawer
- Cell cycle, proliferation, curve fitting and kinetics analyses statistics under the View Statistics tab in the Advanced Functions pull-up drawer

If an adjustment is made to the gates used to derive the cycle, proliferation, curve fitting and kinetics analyses after importing into GraphLogic, re-import the data to display the updated values.

Add Cell Cycle, Proliferation, Curve Fit and Kinetics table data

Data derived from Cell Cycle, Proliferation, Curve Fit and Kinetics analyses can be added to the GraphLogic Workspace by selecting the desired statistic table from the list of tables under the Statistics tab in the File Navigator, as described above.

The Workspace Right-Click Menu



Clear Selected

Selecting ‘Clear Selected’ cells from the right-click menu after highlighting in the Workspace will clear all data, formulae, font formatting, cell alignment and color settings.

Copy

Cells can be copied, by highlighting and selecting ‘Copy’ from the right-click menu, and be pasted elsewhere in the same Workspace, in a different Workspace or into a Table folder. This process copies all data, formulae, font formatting, cell alignment and color settings. The original, copied data will remain after replicated data is pasted to another position in the Workspace.

Cut

The Cut function removes the data in the selected cells, along with all formulae, font formatting, cell alignment and color settings and pastes it to the specified destination. When pasted, the Cut data will overwrite all data and formatting in the location where they are pasted.

Paste

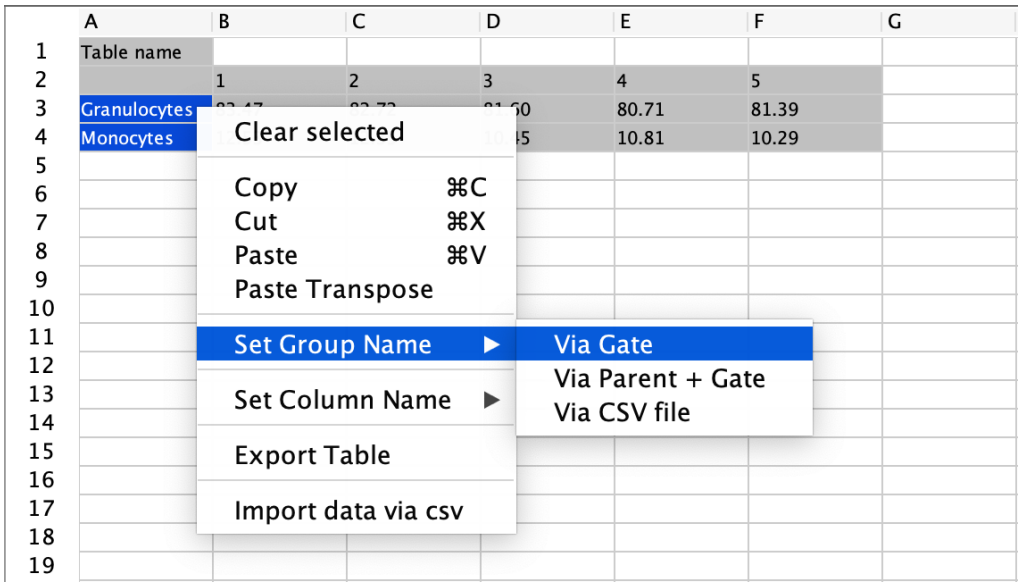
The Paste function adds the Copied or Cut data to a specified location in the same orientation.

Paste Transpose

The Paste Transpose function flips the orientation so that Row 1 becomes Column 1, Row 2 becomes Column 2, etc. Data can only be transposed within the Workspace and not into a Table folder.

Set Group Name →
The group name for a data set can be set using several different references. This is achieved by selecting the cells containing the group titles to be renamed, right-clicking and selecting 'Set Group Name' → and one of:

- *Via Gate*
- *Via Parent + Gate*
- *Via CSV file*



Via Gate – This function is for data derived from FCS files that are dragged into the Workspace from the File Inspector or imported via a plate. To rename the Group, highlight the Group Name cells, right-click and select Set Group Name → Via Gate. The Group name will then be updated with the gate name, which is displayed it the gate drop-down menu in the toolbar.

Via Parent + Gate – This function is also for data derived from FCS files that are dragged into the Workspace from the File Inspector or imported via a plate. It functions in the same way as the 'Via Gate' option but adds the parent gate name before the population gate name.

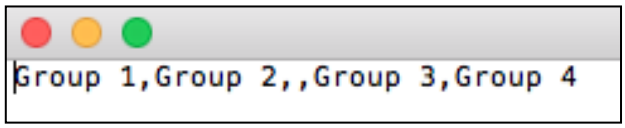
Via CSV file – This function allows groups to be named from a .csv file. Multiple Group Name cells can be named at once, as long as they are highlighted prior to naming. This feature can be very useful if the same experimental groups are used consistently over a number of analyses or experiments.

It is important, however, to set up the .csv file in the correct format. The names in the .csv file need to be listed horizontally, with one name per cell if created in a spreadsheet or having each name separated by a comma if created with a text editor. However, FlowLogic will apply these names vertically, assuming each experimental group is displayed one on top of the next. In addition, only the selected number of Group name cells will be named, even if the list in the .csv file is longer. For example, if the names in the .csv file are 'Group A', 'Group B', Group C', Group D' and Group E' but only two cells are highlighted in the

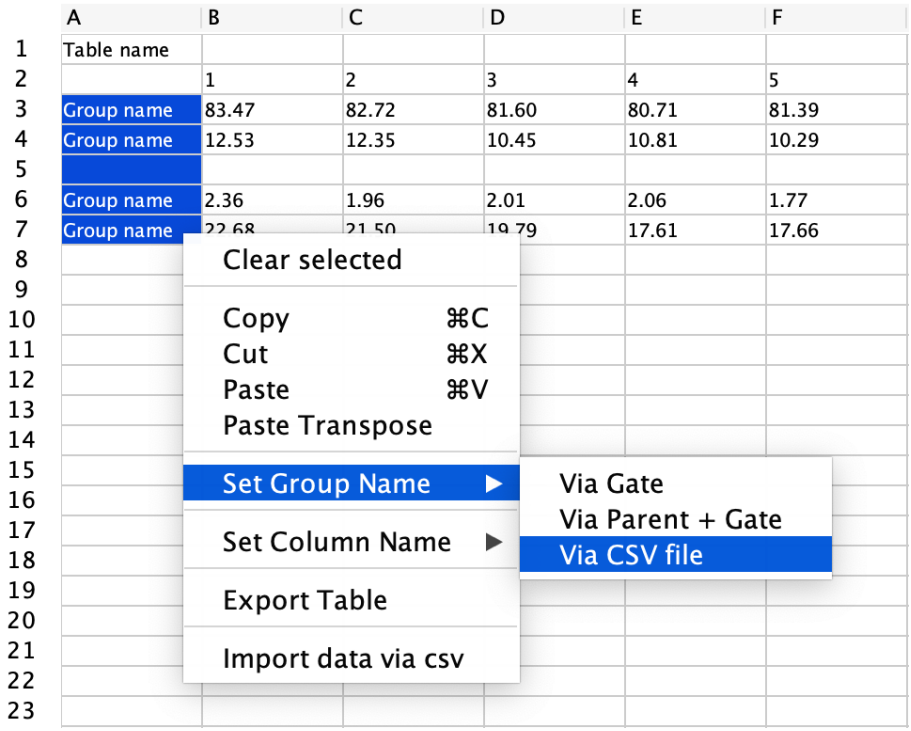
Workspace, only the names ‘Group A’ and ‘Group B’ will be applied. Similarly, if more cells are highlighted than names exist in the CSV file, not all cells will be renamed.

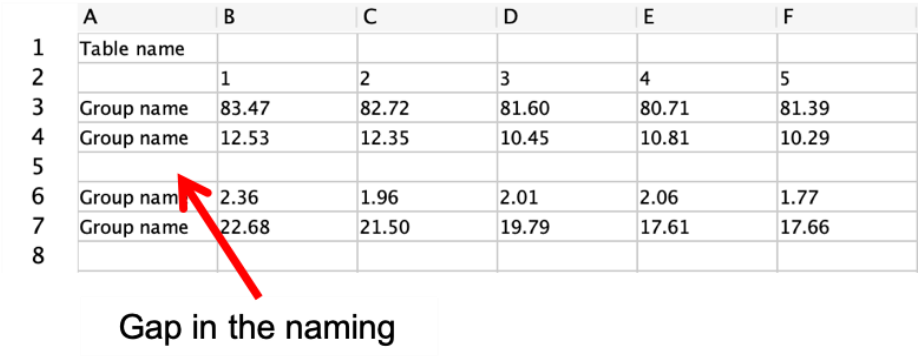
It is also possible to set up a .csv file with spaces between the names. For example, here is a .csv file with a gap between ‘Group 2’ and ‘Group 3’:

Group 1	Group 2		Group 3	Group 4

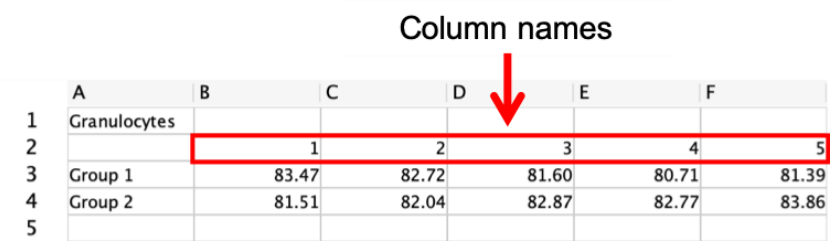


When cells A3 to A7 are highlighted and this .csv file is imported, the third name, corresponding to cell A5, is left blank.





Set Column Name →
The column name for a data set can be set using several different references. The column name refers to the title above each sample, which are numbered by default.



Select the cells containing the group titles to be renamed, right-clicking and selecting 'Set Column Name' → and one of:

- *Via Count*
- *Via $SRC*
- *Via $CELLS*
- *Via TAG*
- *Via CSV file*

Via $SRC – This function allows columns to be named from the $SRC keyword contained in the FCS file. This keyword can differ from cytometer to cytometer and can be defined at the time of acquisition with certain acquisition programs.

Via $CELLS – This is another keyword contained in the FCS file.

Via TAG – tags applied to a sample can be used to name the column titles. If multiple tags have been applied to a sample, these will all be listed in the column name.

Via CSV file – naming columns via a .csv file works in a similar way to naming the groups. Select the column titles to be named, right-click and choose Set Column Name →Via CSV file. The names in the .csv file need to be listed horizontally and any spaces in the .csv file will result in a cell being missed in the Workspace.

Exporting Table

The Workspace table can be exported as either a .csv file or Excel XML Workbooks (*.xlsx) file. The .csv export option will record all text and numbers in the displayed table format without any formatting. Formulae will not be saved in the .csv file, only the resulting value displayed in the cell. The .xlsx file will save all the text and numbers along with text and cell formatting, such as coloring, alignment within cells, font style/type/size and formulae.

Importing Data from CSV Files

Data saved in the .csv file format can be imported into the Workspace by right-clicking in the destination cell and selecting ‘Import data via CSV’.

Manually Adding Data

Data can also be added to the Workspace by selecting a cell and typing text and numbers with the keyboard.

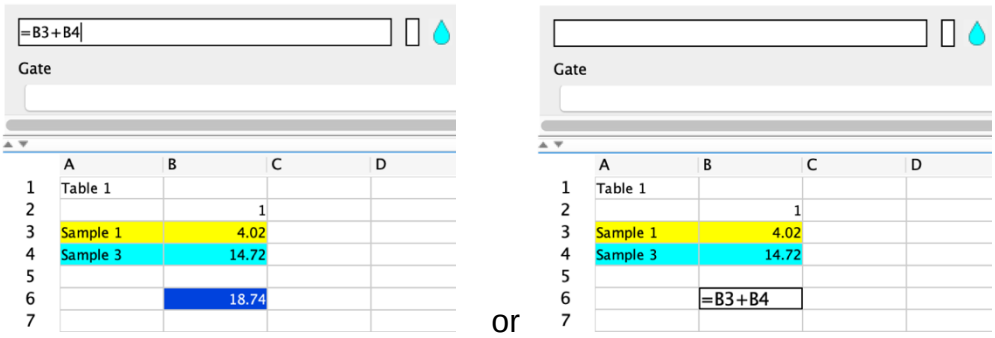
Workspace Toolbar

The Formula Field

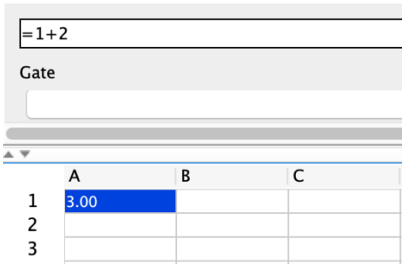
Formulae can be created to apply mathematical functions to selected cells. Addition, subtraction, multiplication and division of cells, typed values or a combination of both can be performed by defining the equation in the formula field in the toolbar above the Workspace.

Examples of each of these situations are as follow:

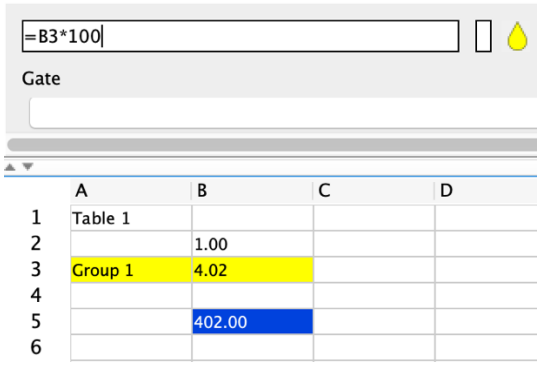
- To add the values in cells B3 and B4, select a destination cell (B6 in this example), then type “=B3+B4” and press Enter. Alternatively, type “=B3+B4” directly into the cell where the answer is to be displayed.



- To add a series of numbers and display the result in one cell, select a destination cell, then click in the formula field and type the equation, e.g. “=1+2”. Then press enter on the keyboard and the result (3) will be displayed in the destination cell.



- To multiple a cell by a number (e.g. B3*100), select a destination cell (B5 in this example), then click in the formula field, click cell B3 and in the formula field type “*100”. Finish by pressing enter on the keyboard. The final value will be calculated and displayed in the destination cell.

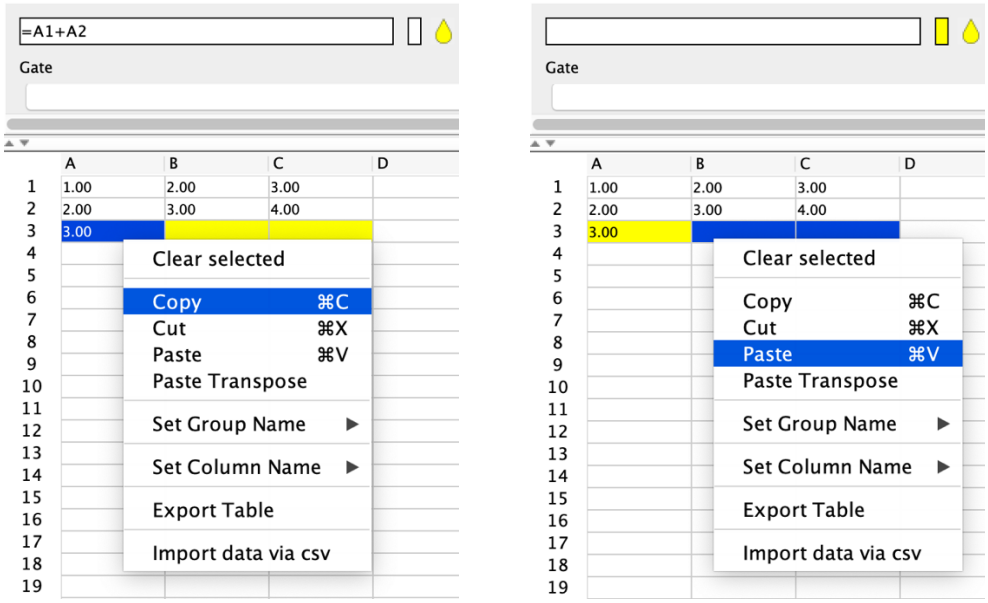


Much more complex formulae can be created using all combinations of mathematical functions (+, -, *, /).

If a formula has been created based on the relationship between a specific selection of cells and numbers, it can be copied and pasted to a new selection of cells, with the formula in the new cells referring to cells with the same spatial relationship. To copy a cell, right-click in the cell and select ‘Copy’. Then, select the destination cells, right-click and select ‘Paste’.

For example, if the formula in A3 is A1+A2, then copying A3 to B3 and C3 will result in:

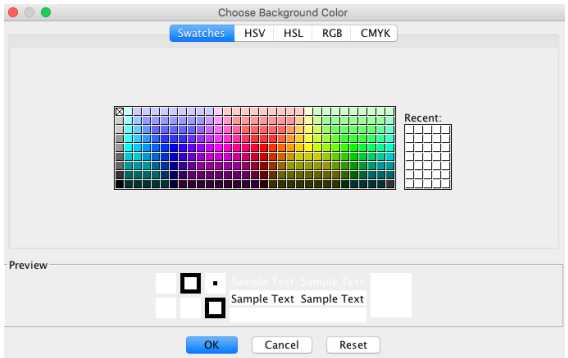
- A3=A1+A2
- B3=B1+B2
- C3=C1+C2

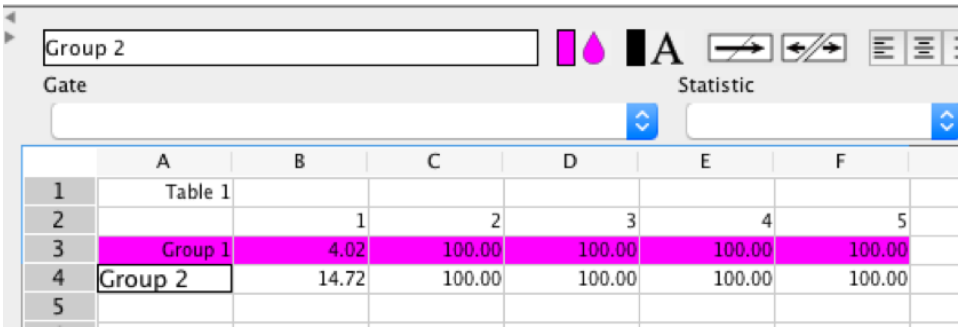


Coloring Text and Cells

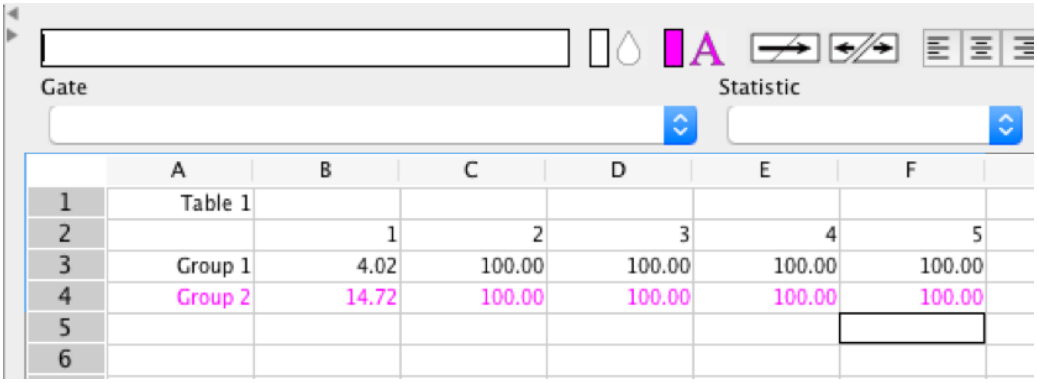
Color can be applied to cells and text in the Workspace spreadsheet. This can be achieved using the icons in the toolbar. Each function (background color and foreground/text color) has two associated icons: select color (the colored rectangle icon) and apply color (drop icon for cells and 'A' icon for text).

To color the cell (background), highlight the cells to be colored and click the background color selection icon. This will open a color palette. Click to choose the desired color and then click 'OK'. This will apply the chosen color to the highlighted cells. You will notice that the Select Color and Apply Color icons have taken the most recent color choice. To continue filling cells with the same color, simply highlight the cells and click the Drop icon.





The same process applies to coloring text within cells. Text is colored black by default but to change the text color, highlight the desired cells and click the select color icon next to the ‘A’ icon. Choose a color and click ‘OK’. The ‘A’ icon is now colored with the most recent selection. To continue to color text with the selected color, highlight the desired cells and click the ‘A’ icon.



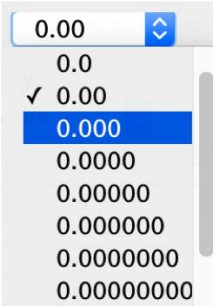
Split and Merge Cells
Multiple cells in the Workspace can be merged to form a single larger cell and merged cells can be split back into their original components. To merge or split cells, highlight them in the Workspace and then click the appropriate button in the toolbar.

Cells running horizontally, vertically or a block spanning a number of columns and rows can be merged (are subsequently split).

Text Alignment
Text can be aligned to either the left, centre or right of a cell using the three icons in the toolbar. To change the alignment, select the cell(s) and click on the appropriate icon.

Font Settings
Three font setting options can be accessed in the toolbar, being the font type, size and style. To change the font settings, highlight the cells in the Workspace and select from the different options in the font settings drop-down menus.

Decimal Places
The number of decimal places that are displayed in individual cells can be set by selecting the cell(s) and choosing an option ranging from no decimal places through to ten. Individual cells within the same Workspace can be set to display different numbers of decimal places.



Population Statistic Menus
The population statistics menu allows for the selection of specific gate and parameter statistics to be displayed for a single FCS sample, or a group of FCS samples, that have been dragged into the Workspace.

To define a statistic, highlight the cell or cells containing data derived from an imported FCS file and select the desired gate, statistic and parameter (if applicable). The value in the cell will update to reflect the defined statistic and can be changed to any other statistic without having to re-import the sample. The statistics will also update following any changes or adjustments to the defining gates in GateLogic.

The Population Statistic Menus can also be used to verify the statistic in a selected cell. Clicking on any cell containing FCS-derived data will prompt the three population statistic menus to display the gate, statistic and parameter for that cell. If multiple cells containing different statistics are selected, the display in the menus will reflect the first cell in the selection.

For more information regarding importing and displaying FCS-derived data, see page 159.

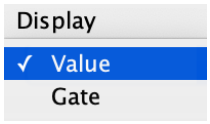
Defining Replicates

If values from all technical replicates have been imported, the number of replicates can be defined with the average of the technical replicates being displayed in merged cells.

Gate/Value Display

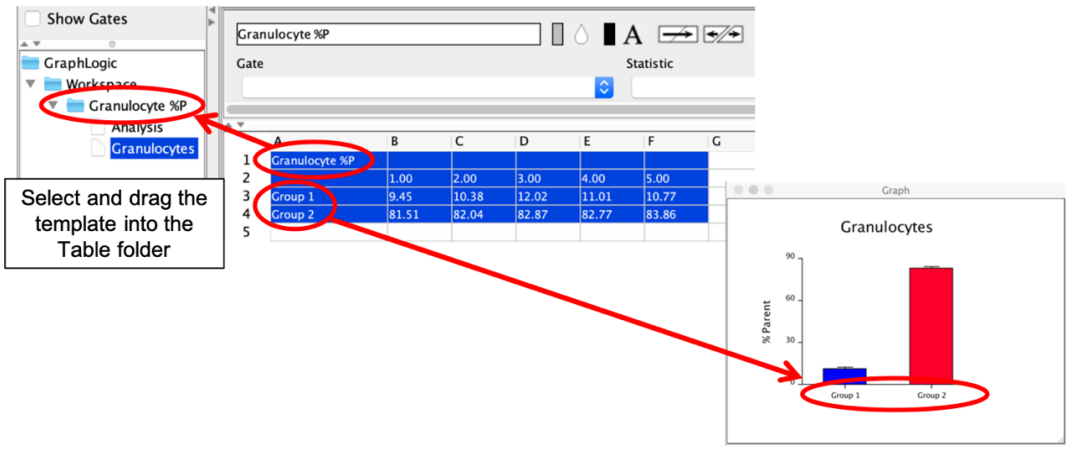
The Gate/Value Display function allows the for the gate name linked to the gate/parameter statistics to be displayed within the cells instead of the statistic itself. This can be useful if you wish to check the origin of a selection of cells without having to click on the cells one-by-one to view the gate in the Population Statistic 'Gate' menu.

To change the information displayed in the cells, select them in the Workspace and choose the desired option in the Display drop-down menu.



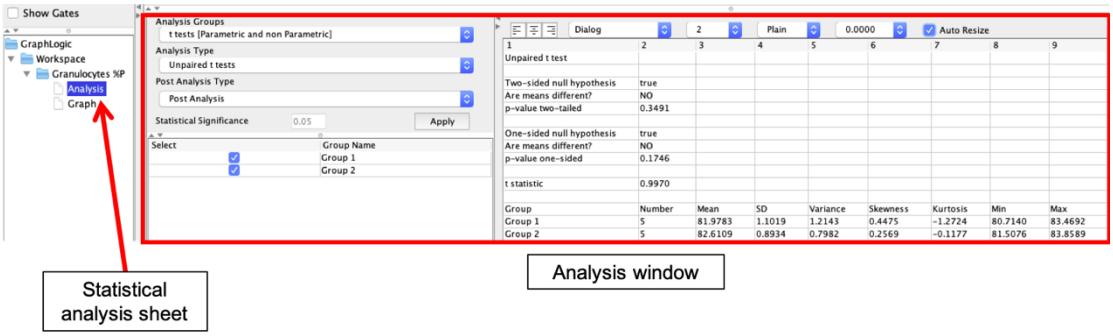
Adding Data to a Table Folder for Statistical Analysis and Graphing

When data exists in the Workspace within the data template as shown below, it is very easy to add it to a Table folder in order to perform statistical analyses and graph. Simply select the data (not the Group or column names) and drag it into a Table folder. When you release the data on top of the table folder, choose either to 'Replace' the existing data (even if the table folder is empty) or 'Overlay', which allows for overlaying of regression curves.



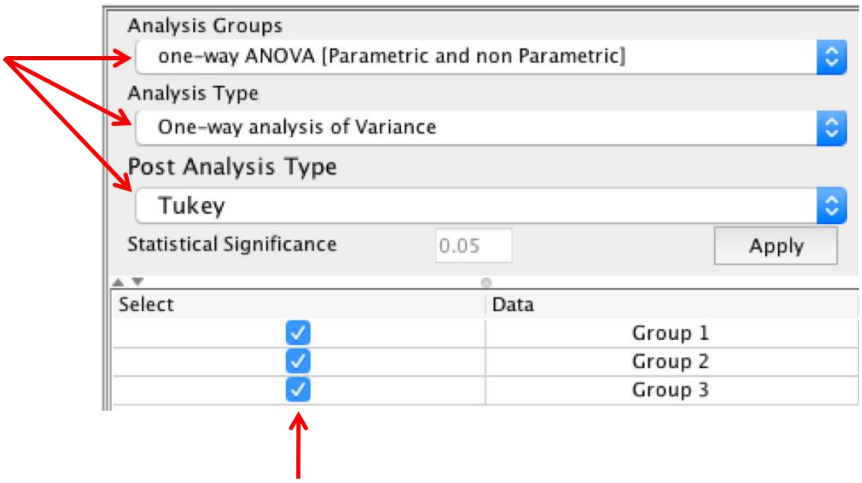
The Table folder will be automatically renamed the dataset title and the groups in the graph and statistical analyses will be named automatically renamed from the group names in the Workspace.

Performing Statistical Analyses



- After data has been added to the data table (see page 159), select a statistical analysis sheet. This opens the Analysis window. The workspace from where the data originates can be viewed by selecting the Workspace in the Graph Data window or by clicking on the Table folder associated with the particular analysis sheet.

- To perform a statistical test, select the experimental groups to analyse and the specific statistical analysis test (and post-test if applicable) from the drop-down menus.



- The result will be displayed in the Description and Analysis columns adjacent.

Description	Analysis
One Way Anova	
Number of groups	3.00
F Value	13.42
P Value	0.00
Null hypothesis	false
Are means different?	YES
Significance level	0.05
Number	12.00
Mean	35.15
SD	11.78
Variance	138.86
Skewness	0.29
Kurtosis	-0.94
Min	18.45
Max	55.58
Tukey	
Groups Group 1 Vs Group 2	
Mean Diff.	23.69
Standesized Error	3.26
Critical Value	3.95
Lower	10.80
Upper	36.57
Sig Diff?	NO

Analysis Groups

- t test (parametric and non-parametric)
- ANOVA (parametric and non-parametric)
- Simple statistics
- Regression analysis

Analysis Type

- Unpaired t tests with Welch-Satterthwaite approximation
- Unpaired t test
- Unpaired t test equal variance
- Paired t test
- Mann-Whitney

- One-way analysis of variance
- Kruskal-Wallis test
- Two-way analysis of variance

- Linear regression
- 4 PL
- 5PL

Post Analysis Type

- Bonferroni
- Tukey

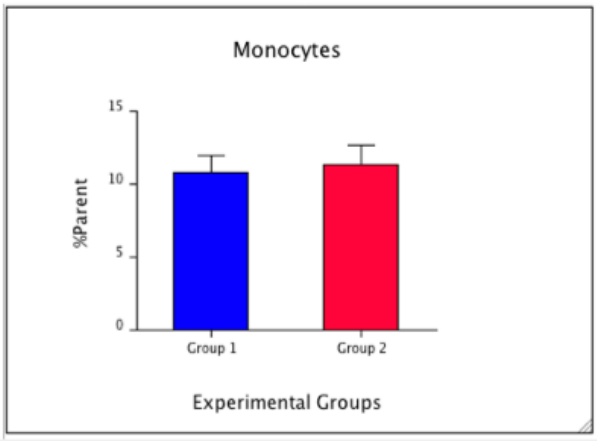
Two-way ANOVA and Automatic Stars of Significance

GraphLogic can also display graphs with two independent variables. This requires merging the cells above the column counts to define the second variable. In addition, stars of significance and be automatically added to these graphs after performing a two-way ANOVA with a Bonferroni multiple comparisons test.

Data Table Structure for the Two-way ANOVA

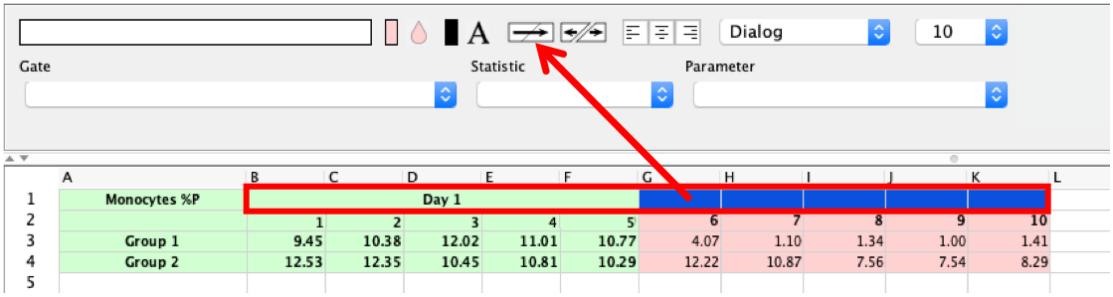
When dragging files into the GraphLogic workspace, experimental groups are defined by rows. However, it is also possible to define a second independent variable within a row by merging the cells above the column count for an entire experimental group. The resulting table can be analysed using a two-way ANOVA.

The example below shows two groups from one timepoint.



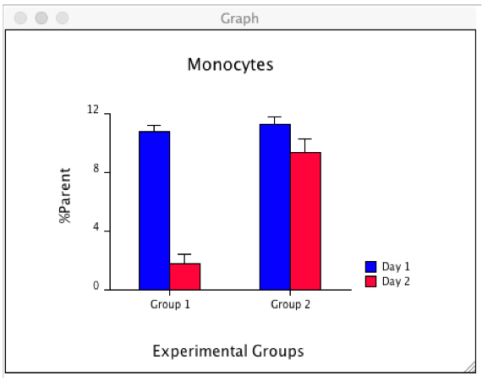
	A	B	C	D	E	F	G
1	Monocytes %P – Day 1						
2		1	2	3	4	5	
3	Group 1	9.45	10.38	12.02	11.01	10.77	
4	Group 2	12.53	12.35	10.45	10.81	10.29	
5							
6							

When there is a second independent variable, add the data immediately to the right of the existing data. Then, merge the cells above the column count for the groups by highlighting the cells and clicking the Merge Cells icon in the toolbar. Type the group name into the merged cells. Finally, select the whole table and drag it into a Table Folder.



	A	B	C	D	E	F	G	H	I	J	K	L
1	Monocytes %P	Day 1										
2		1	2	3	4	5	6	7	8	9	10	
3	Group 1	9.45	10.38	12.02	11.01	10.77	4.07	1.10	1.34	1.00	1.41	
4	Group 2	12.53	12.35	10.45	10.81	10.29	12.22	10.87	7.56	7.54	8.29	
5												

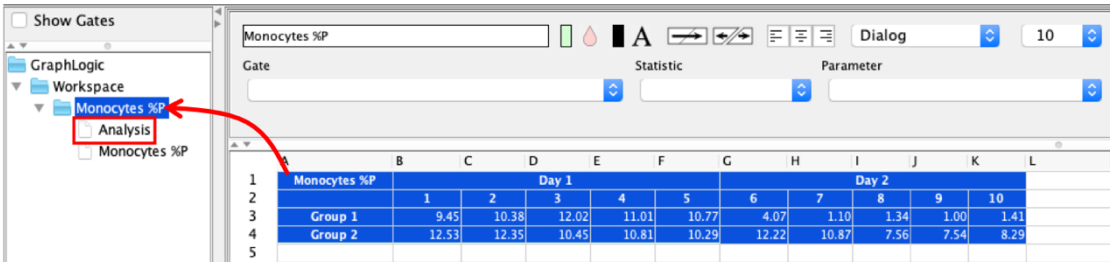
The final table and resulting graph are as follows:



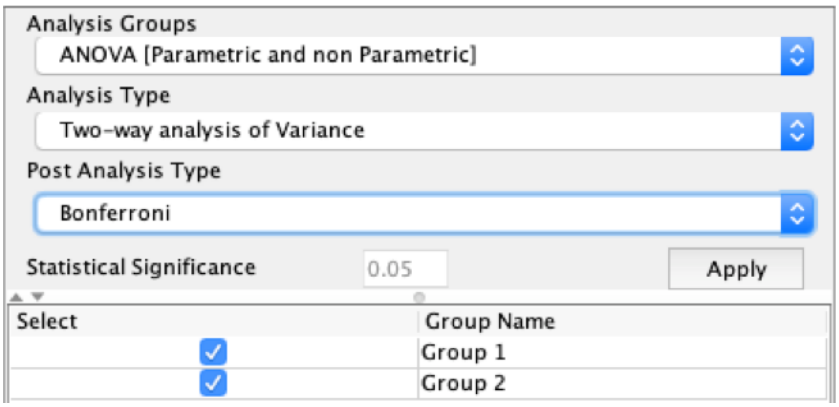
	A	B	C	D	E	F	G	H	I	J	K	L
1	Monocytes %P	Day 1					Day 2					
2		1	2	3	4	5	6	7	8	9	10	
3	Group 1	9.45	10.38	12.02	11.01	10.77	4.07	1.10	1.34	1.00	1.41	
4	Group 2	12.53	12.35	10.45	10.81	10.29	12.22	10.87	7.56	7.54	8.29	
5												

Performing a Two-way ANOVA

With the data organized as described in the section above, drag the entire data table from the spreadsheet into a Table Folder, then select the Analysis tab.



From the drop-down menus, select ANOVA and Two-way Analysis of Variance. There is also the option for a Bonferroni or Tukey multiple comparisons post. Test. Finally, tick the groups to be compared.



The statistical analysis summary will be shown in the panel to the right.

Dialog | 2 | Plain | 0.0000 | Auto Resize

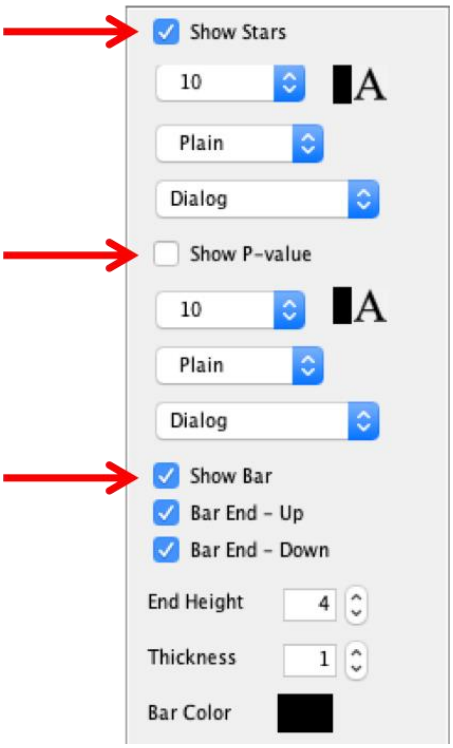
1	2	3	4	5	6	7	8	9
Two Way Anova								
Source	SS	DF	MS	F	P			
Column Factor	149.32138	1	149.32138	72.61657	0	****		
Row Factor	81.47695	1	81.47695	39.62311	0.00001			
Interaction	60.44332	1	60.44332	29.39423	0.00006			
Residual	32.90078	16	2.0563					
Total	324.14243	19						
Bonferroni								
Groups	Mean Diff.	Critical Value	Lower	Upper	Sig Diff?	P-value	Stars	Show
Day 1-Group 1 Vs Day 2-Group 1	8.9417	2.72834	6.21335	11.67004	YES	0	****	ON
Day 1-Group 1 Vs Day 1-Group 2	-0.55988	2.72834	-3.28822	2.16846	NO	1		OFF
Day 1-Group 1 Vs Day 2-Group 2	1.42807	2.72834	-1.30028	4.15641	NO	0.80945		OFF
Day 2-Group 1 Vs Day 1-Group 2	-9.50158	2.72834	-12.22992	-6.77323	YES	0	****	ON
Day 2-Group 1 Vs Day 2-Group 2	-7.51363	2.72834	-10.24197	-4.78529	YES	0	****	ON
Day 1-Group 2 Vs Day 2-Group 2	1.98795	2.72834	-0.7404	4.71629	NO	0.26112		OFF

Automatic Stars of Significance

Stars of significance can be added to graphs automatically following a two-way ANOVA with a Bonferroni multiple comparisons test (as described above).

If there is a significant difference, the P-value and corresponding stars of significance will be displayed in the last two columns. These can be added in the graph in the correct position between the two groups being compared.

To add the stars, select the relevant graph and click on the “****” in the top right to display the relevant formatting panel. Tick the elements to be displayed, such as stars, P-values and connecting bars. Then in the statistical analysis summary table, click the cell in the “Show” column next to the stars that you wish to add to the graph. The work “OFF” will switch to “ON”.



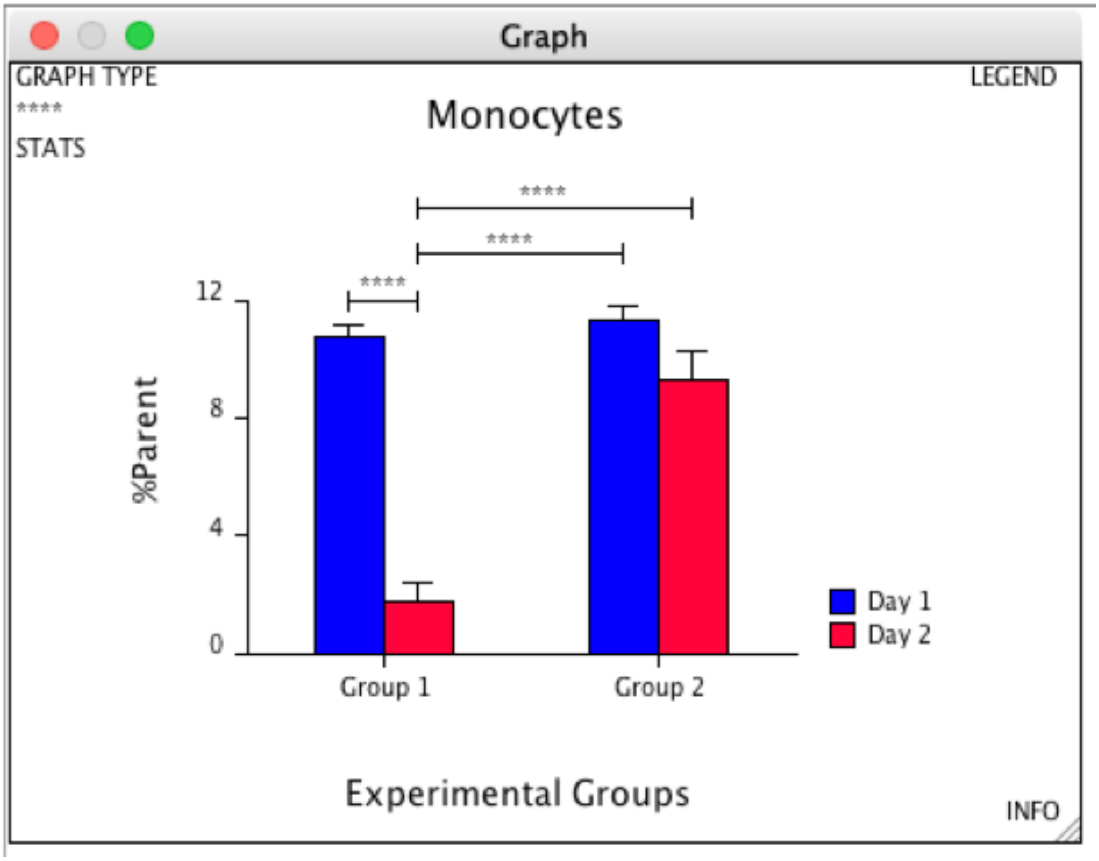
Other formatting option allow the font size, color style and type to be set for the stars and P-values.

Bar ends (up and down) can be added to the horizontal bar connecting the two groups.

The bar end height, bar thickness and color can also be set.

Sig Diff?	P-value	Stars	Show
YES	0	****	ON
NO	1		OFF
NO	0.80945		OFF
YES	0	****	ON
YES	0	****	ON
NO	0.26112		OFF

The stars, P-values and bars will be added to the graph once set to “ON”. The bars will be positioned between the two groups the statistics describe. These bars can only be moved up and down, so will always be connecting the correct groups. The stars and P-value can be moved independently of the bar and positioned where you wish.



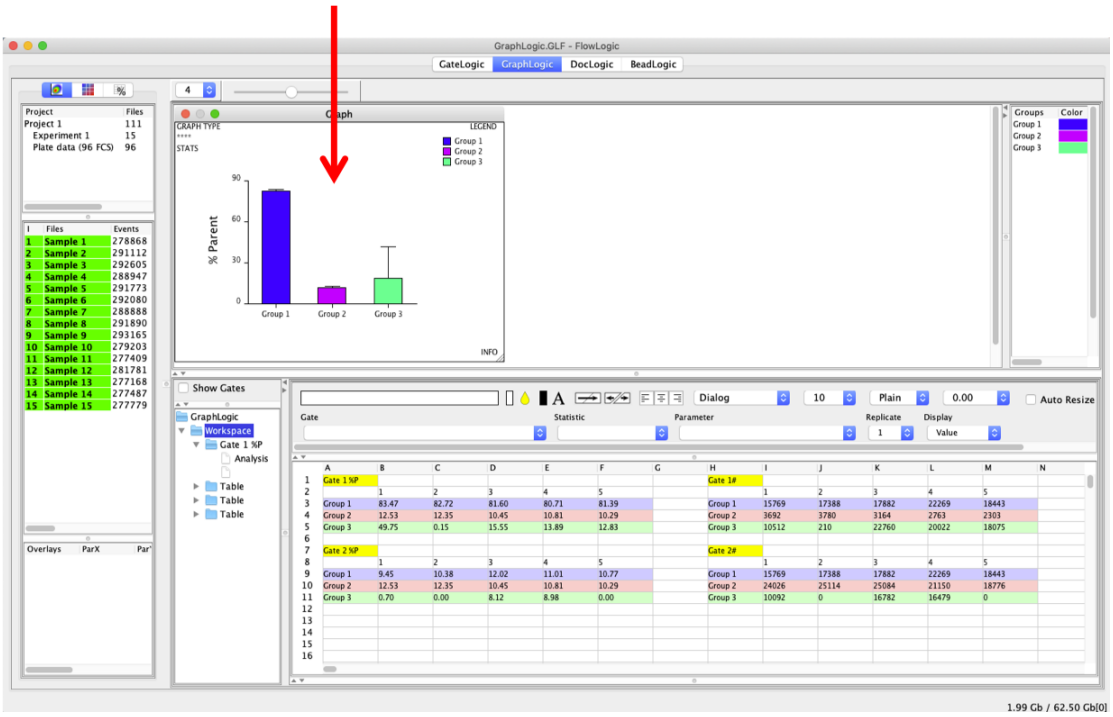
As with all other data in GraphLogic, the stars of significance on graphs will update if gates are adjusted in GateLogic.

If more space is required above the graph, consider adjusting the margins. The margin formatting panel can be displayed by clicking in the area within the graph window surrounding the graph. For more information, see page 186.

Creating a Graph

To graph your data, select the GraphLogic window at the top of the program and select a Workspace folder to the left of the spreadsheet. Then, highlight the data within a workspace template and drag it into a Table folder (see page 172).

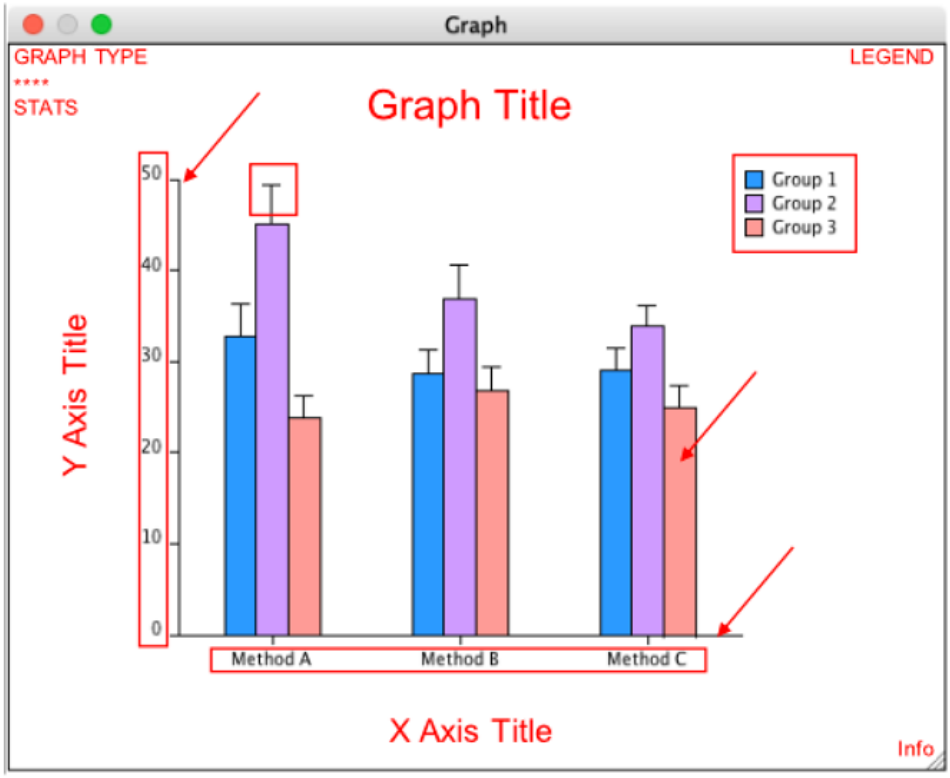
Click *Graph* in the Data Folder window to create a bar graph of the imported data. The graph appears in the Graph Window.



Formatting Graphs

Graphs can be formatted using the formatting panel on the right-hand side of the screen. Specific formatting panels can be displayed by clicking on the part of the graph that you wish to edit. For example, click on the x axis to display the options to change the x axis scaling, the tick thickness and length, and to turn on a vertical grid. Or click on the graph title to change the text and font colour, size, style and type. Some formatting panels are represented by words positioned in the corners of a selected graph. These include the *Graph Type*, items associated with statistical analyses (represented by ****), *STATS* for the type of average and error bars, the *Legend* and an *INFO* window.

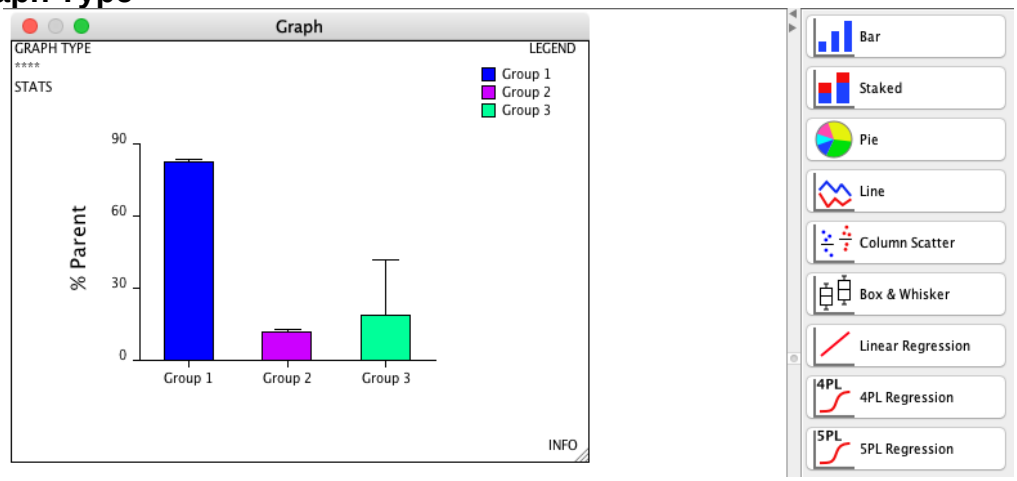
The different parts of the graph that can be specifically clicked and formatted are shown below. This information window can also be displayed by clicking on the “Info” within each graph.



Graph Type – choose a different type of graph
**** – stars of significance, P-values and bars
Stats – displayed statistic and error bars
Legend – drag the legend to the desired position
Colors – click on a bar to set the group color
X & Y axis – click on the x or y axis for scaling options
Axis labels – format the labels on each axis
Titles – edit and format the text for each title

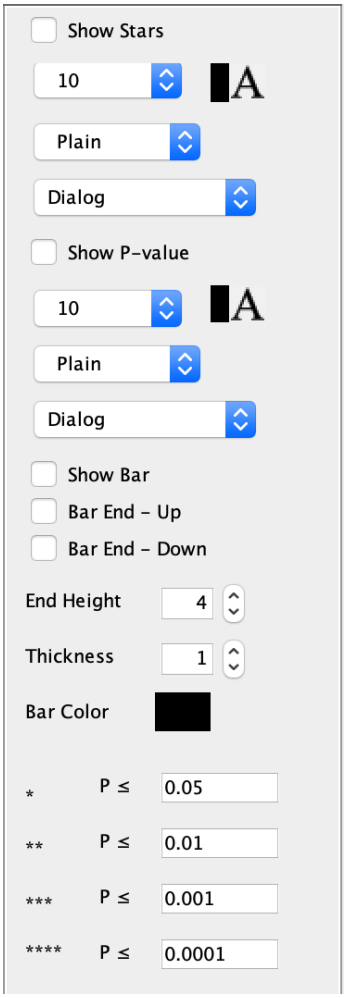
The following is a description of each of the formatting panels:

Graph Type



Click on the text “GRAPH TYPE” in the top left corner of the graph to display the list of graph types in the formatting panel. Choose from a range of graph types, including Bar, Stacked Bar, Pie, Line, Column Scatter, Box and Whisker, Linear Regression, 4PL Regression, 5PL Regression.

****



Clicking the four stars (****) in the top left corner of a selected graph will display the formatting panel associated with statistical analysis tests.

If a Bonferroni multiple comparisons post-test has been performed following an ANOVA, it is possible to automatically display the resulting stars of significance on the graph. This is done by ticking the “Show” option next to each set of compared groups in the statistical analysis summary table (see page 177 for more detail).

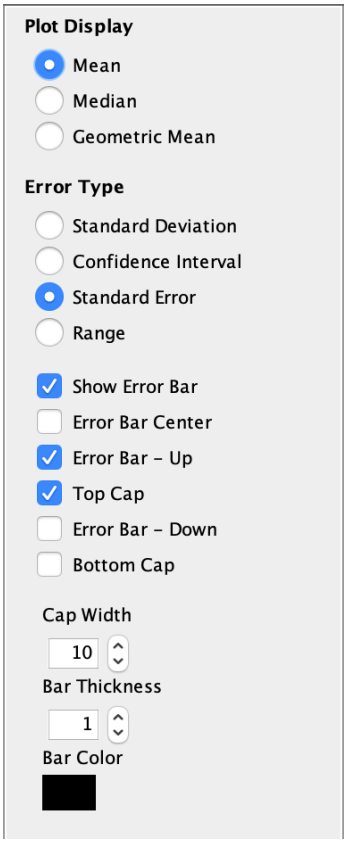
Items that can be formatted include the stars themselves, which can be hidden or changed in terms of the font size, color, style and type.

The calculated P-value can also be displayed and formatted in terms of the font size, color, style and type.

The horizontal bar connecting the two compared groups can also be displayed, along with vertical bar ends that extend up and/or down. The color, thickness and bar end heights can also be changed.

Finally, the number of stars associated with each range of P-values can be defined at the bottom of this panel.

Stats



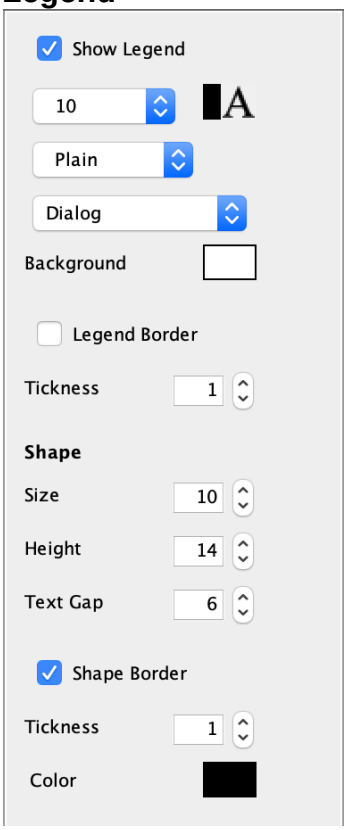
The stats formatting panel provides options to set the type of average to be displayed on the graph (mean median or geometric mean).

The type of error displayed by the error bars can be chosen from standard deviation, confidence intervals, standard error of the mean and the range.

The error bar itself can be displayed and hidden, displayed above and/or below the average and with/without top and bottom caps. The centre can also be denoted with a line.

Finally, the cap width, bar thickness and color can also be defined.

Legend



Each graph has a legend. However, this can be hidden if desired, such as when the x axis labels depict the groups.

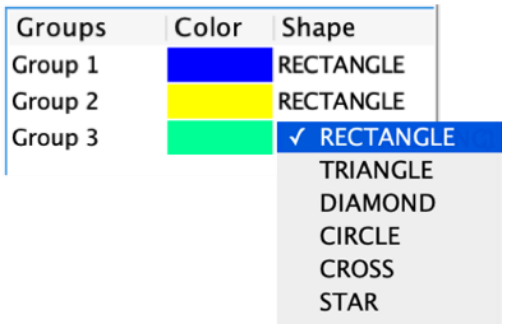
When displayed, the legend font size, color, style and type can be set.

A border around the legend can be displayed and the border thickness set. A background color can also be chosen.

Each group has a shape assigned to it. This is set in the color panel, by clicking on the bar or data point. Within the legend formatting panel, the shape size, height and gap between the shape and the text can be defined.

Finally, a border around the shape can be turned on, the border thickness increased, and the border color changed.

Colors



The color for each group can be set by clicking on the data, such as within a bar in a bar graph or on the data point in a line graph. To change the color, click on the color displayed next to the group name and choose a new one form the pop-up window.

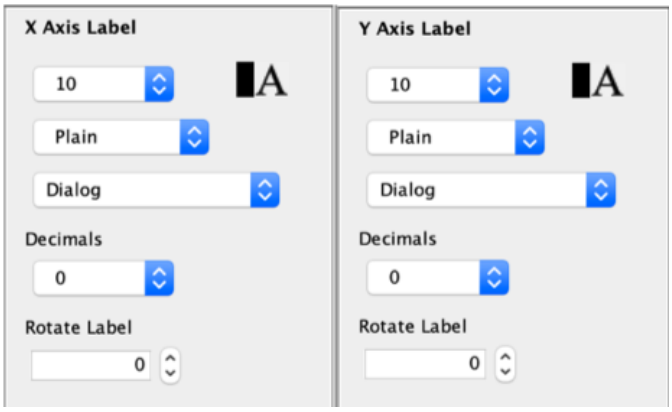
Each group has a shape assigned to it, which is displayed on line graphs and in the graph legend. The shape for each group can be changed by clicking on the shape name and choosing a new one from the drop-down list.

X & Y Axis

The x and y axes can be formatted individually. Clicking on each axis displays an identical formatting panel. By default, each axis is auto scaled, depending on the data. However, this setting can be turned off and the minimum and maximum range values can be entered manually.

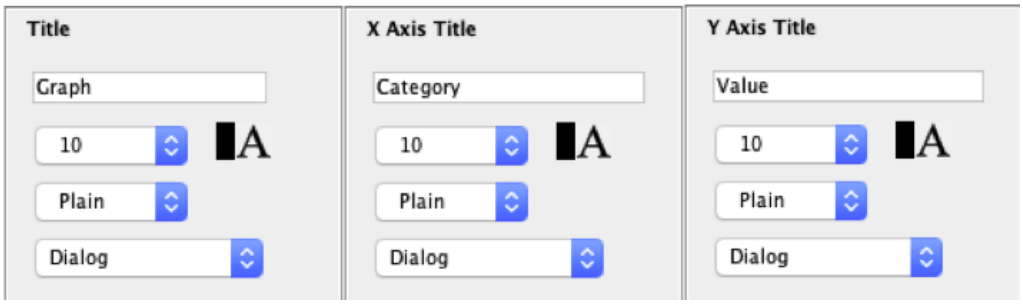
The interval tick thickness, length and color can be set and a grid extending from each tick across the entire graph can be displayed. The color for these grid lines can also be defined.

Axis Labels



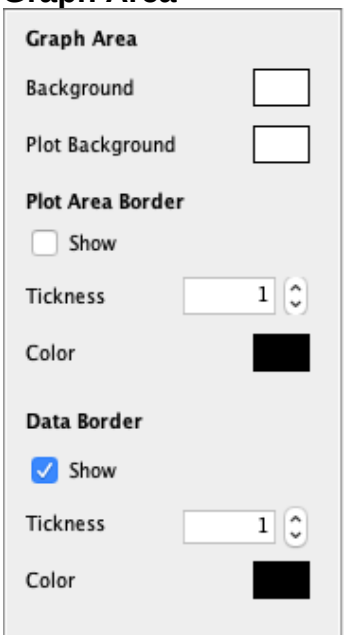
The axis label elements that can be formatted include the font size, color, style and type. The number of decimal places can be set, and the labels can be rotated by a specified number of degrees.

Titles



Clicking on the graph title or either of the axis titles displays a formatting panel with options to enter new text and change the font size, color, size, style and type.

Graph Area



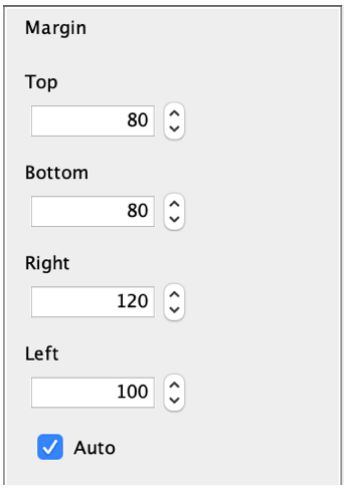
The graph area formatting panel is displayed by clicking in the area defined by the axes but not on a bar, line or data point.

Here, the background (area outside the graph area) and the plot background (within the graph area) can be set.

A plot area border can be displayed with options to set its thickness and color.

Data borders for bars and shapes (line graphs) can be shown/hidden, their thickness increased and color changed.

Margins



The margins formatting panel is displayed by clicking in the space surrounding the graph but within the selected graph window. The left, right, top and bottom margins can be independently set. These settings will be transferred to graphs added to reports in DocLogic.

Regression Analysis

Linear, 4PL and 5PL regression analyses can be performed. Individual curves from one data set can be displayed and analysed by themselves or multiple datasets can be analysed together, with the curves overlaid on a single graph.

For 4PL and 5PL regressions, the EC50 (or any defined percentage) can be calculated and displayed on the graph, individual datapoints can be weighted or hidden, and in the case of an overlay of many curves, the sample with the lowest EC50 value can be identified as the 'most potent'.

The table name can be replaced with either the analyte name or a title that describes the comparison in the case of regression overlays.

Regression data

Regression data can be imported into GraphLogic as linked data from GateLogic analyses, or by typing or importing directly into GraphLogic. The latter will not update automatically as it is not linked to any other source.

The data is arranged in the GraphLogic spreadsheet in a similar manner to that mentioned in the 'Adding FCS-linked data' section of this manual but with one difference. Instead of the default column count values of 1, 2, 3, etc., replace these with the corresponding sample concentration. It may help to rename the second row in the table with "Conc.", "Concentration", and with the relevant units.

	A	B	C	D	E	F	G	H	I	J
1	Table Name									
2	Conc. pg/ml	0.64	3.20	16.00	100.00	400.00	2000.00	10000.00	50000.00	
3	IL-12	1.19	1.20	1.19	2.08	6.15	23.93	90.93	207.64	
4										

Generate regression graphs

To generate a regression graph, follow the steps below:

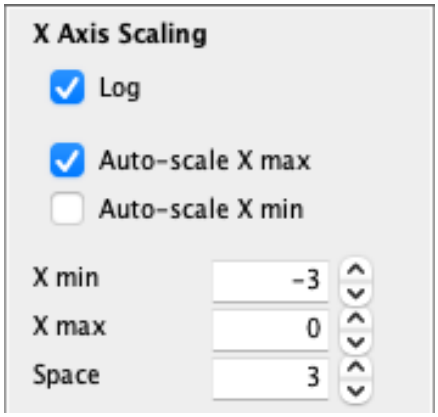
- Arrange data in the GraphLogic spreadsheet by dragging in linked files and choosing to display the relevant statistic from the toolbar options (e.g., MFI for a given gate/analyte) or by importing/typing in the data.
- Replace the sample column count with the concentration values that correspond to each of the samples.
- Rename to the table (the default name is 'Table Name') to make it easily identifiable and to help when auto-naming the Table Folder when the data is added.
- Highlight the complete data table and drag into a Table folder in the panel to the left (the Table will automatically be renamed with the table name as exists in the spreadsheet).
- Upon dropping the table into the Table Folder, select either 'Replace' (even if there is no existing data) or Overlay in order to plot multiple curves on the one graph.

- Click on the Graph tab to open the graph in the workspace.
- To change the graph type to a regression plot, select the graph and click on the ‘GRAPH TYPE’ keywords in the top left of the graph window. This will open the corresponding formatting panel to the right of the workspace. From here, choose one of Linear Regression, 4PL Regression or 5PL Regression.

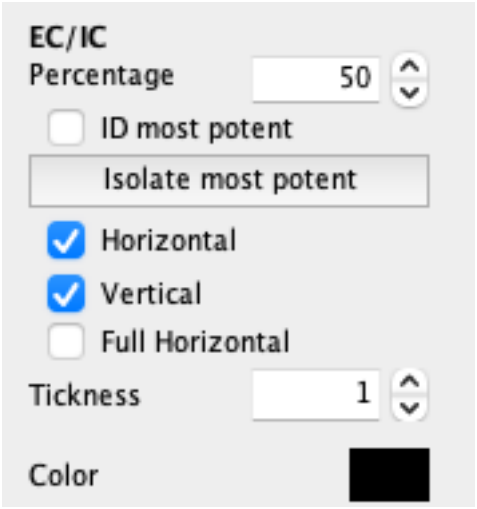
Regression graph formatting panel
The regression plots can be formatted using the range of options in the graph formatting panels. Options specific to different parts of the graph can be viewed by clicking on the relevant sections of the graph itself.

Some useful formatting options include:

- Click on the x-axis to display the X Axis Scaling panel. Change the scale to a logarithmic scale using the ‘Log’ option.
- Additional decades above zero can be added by deselecting ‘Auto-scale X min and reducing the value in the X min field. With the log scale displayed, the values within the X min field represents the exponent to base 10.



- Click within the graph space to display the Graph Area formatting panel. This will present some options specific to the 4PL and 5PL regression plots, shown in the EC/IC section

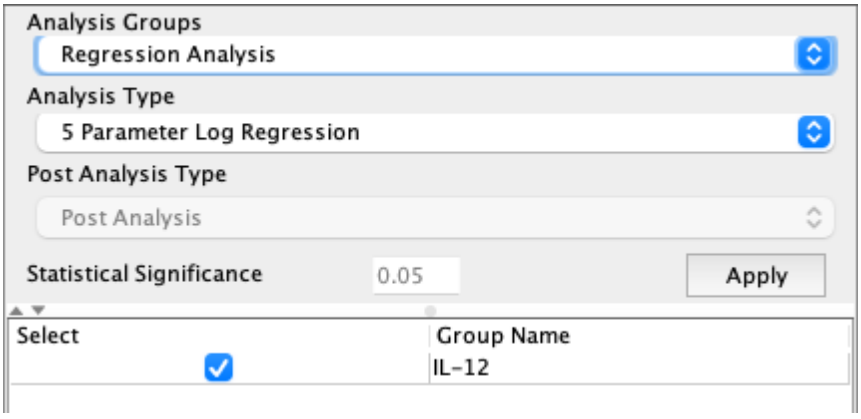


- Define the EC/IC percentage (e.g., 50 to calculate EC50/IC50)
- Mark the defined EC/IC value on the graph using a combination of horizontal and vertical lines. These begin at the relevant axis and finish at the point on the regression curve. The 'Full Horizontal' option spans the entire width of the graph.
- The thickness of the lines marking the EC/IC value can be increased and decreased
- The colour of the lines can also be changed

Performing regression statistical test

To a regression analysis, click on the Analysis tab under the corresponding Table Folder. This will switch the display from the spreadsheet to the statistical analysis window. From here:

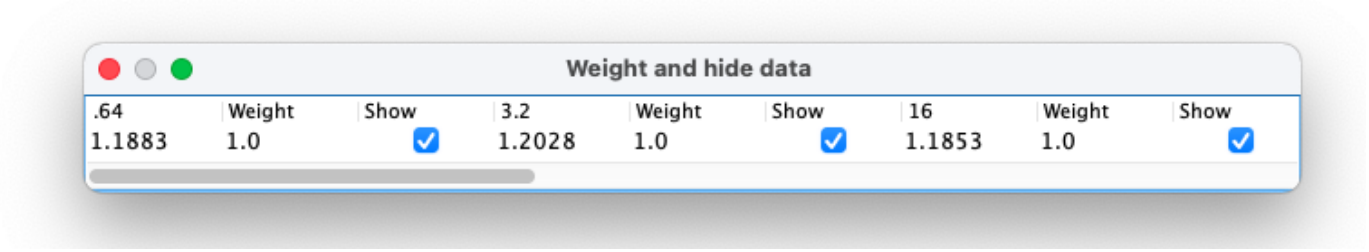
- From the Analysis Groups, choose Regression Analysis
- From the Analysis Type, choose one of linear, 4PL or 5PL regression
- From the list of groups/analytes to be included in the calculations. The results will be displayed in the panel to the right



Auto-updating of graphs to changes in data
If the data represented in a regression analysis is derived from gates within GateLogic, then the any adjustment to the gates will automatically update the graphs and statistical test results.

The graphs and statistical test results will also update to reflect new data when performing a File Replacement or when applying an Analysis Template derived from a regression analysis.

Weighting and Hiding Data
To change the weighting of data and to hide data points on the graph, highlight the table folder in the spreadsheet, right-click and select Weight and hide data. A pop-up window will open showing the values for each concentration, a weighting value and a 'Show' tick box.



For any given data point, type in a new value and press Enter. The graph and results will update automatically. A weighting value of zero, will mean the data point is completely ignored. By default, all values are given an equal weighting of one.

Replicating and overlaying regression curves
There are two methods for overlaying two curves on the one graph: overlaying different data tables in one table folder or combing data for multiple analytes in one large data table.

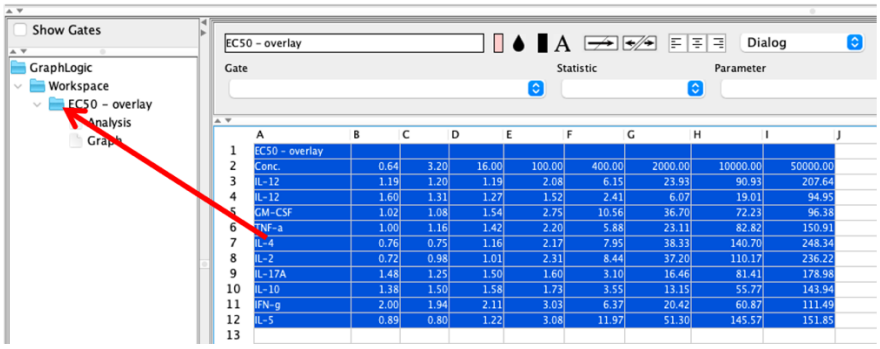
To overlay multiple data tables, generate one regression table as described above and then drag a second data table into the same Table Folder. Upon releasing the data, select 'Overlay' form the pop-up menu. Click the Graph tab to update the graph.

To create a regression overlay from one large data table, append the initial data table by adding additional datasets to the rows immediately beneath the initial dataset. Then drag the entire data table into a table folder.

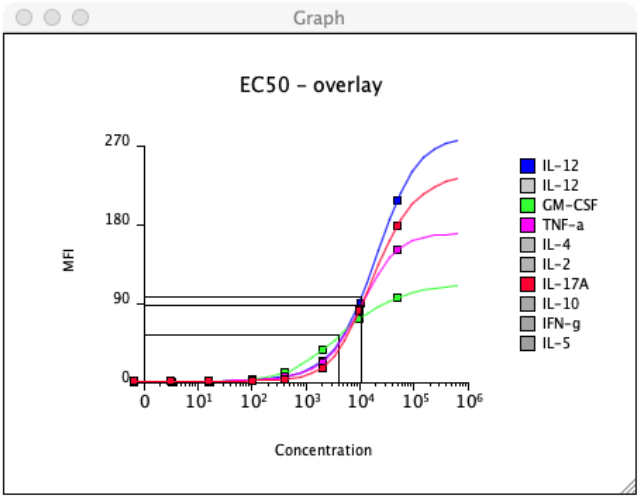
Data table format

EC50 – overlay								
Conc.	0.64	3.20	16.00	100.00	400.00	2000.00	10000.00	50000.00
IL-12	1.19	1.20	1.19	2.08	6.15	23.93	90.93	207.64
IL-12	1.60	1.31	1.27	1.52	2.41	6.07	19.01	94.95
GM-CSF	1.02	1.08	1.54	2.75	10.56	36.70	72.23	96.38
TNF-a	1.00	1.16	1.42	2.20	5.88	23.11	82.82	150.91
IL-4	0.76	0.75	1.16	2.17	7.95	38.33	140.70	248.34
IL-2	0.72	0.98	1.01	2.31	8.44	37.20	110.17	236.22
IL-17A	1.48	1.25	1.50	1.60	3.10	16.46	81.41	178.98
IL-10	1.38	1.50	1.58	1.73	3.55	13.15	55.77	143.94
IFN-g	2.00	1.94	2.11	3.03	6.37	20.42	60.87	111.49
IL-5	0.89	0.80	1.22	3.08	11.97	51.30	145.57	151.85

Select and drag into a Table Folder



Resulting Graph for this dataset with some curves hidden for simplicity

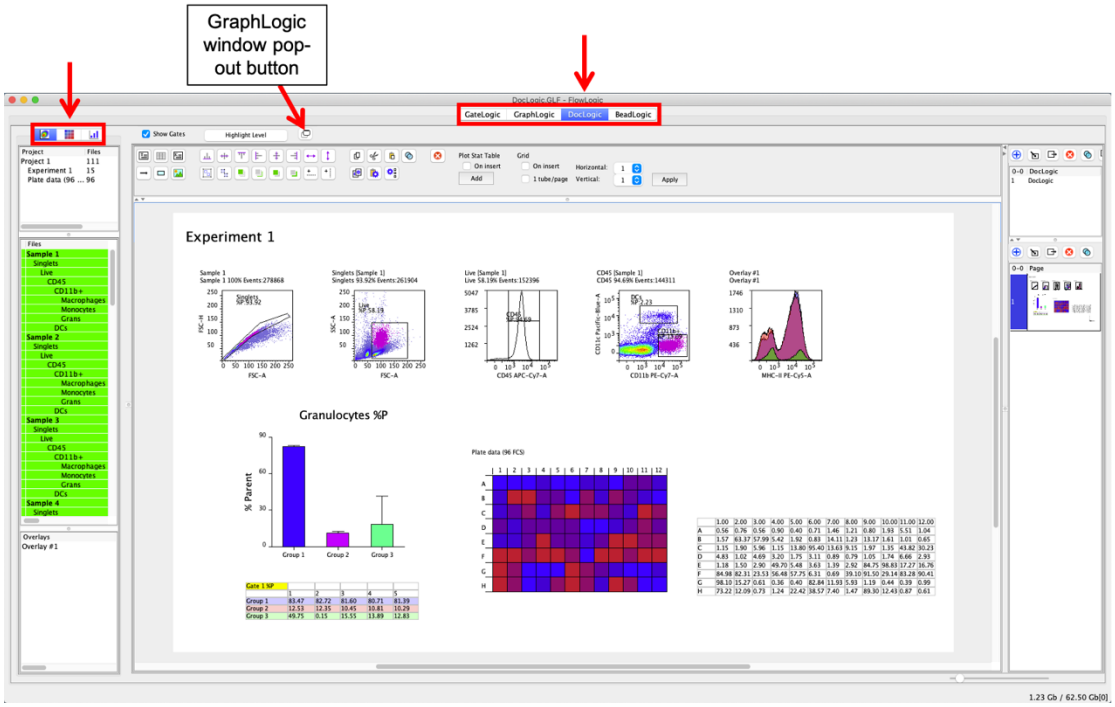


One tip when creating this type of table is to name the analytes with the gate name they are derived from is by highlighting the names in the spreadsheet, right-clicking and selecting Set Group Name → Via Gate.

DocLogic – Reports and Presentations

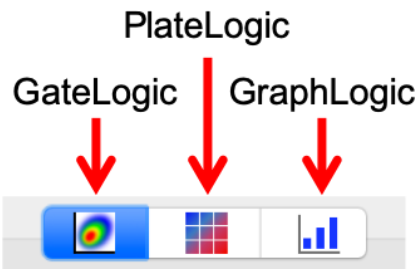
DocLogic is the document and report building function in FlowLogic. It allows you to arrange and annotate plots, graphs, plates, heatmaps, tables and more. DocLogic is also linked to GateLogic and GraphLogic, so, any changes made to the gates or graphs will automatically update in the report.

To create a report, select the DocLogic tab at the top of the program window. In the centre of the screen is the report itself, displaying one page at a time. Above the report is the formatting toolbar. On the left of the screen is the File Navigator, where tabs allow you to drag elements from GateLogic, PlateLogic and GraphLogic into a report. On the right of the screen is the list of documents, page preview and the element formatting panels.



The DocLogic File Navigator

Click on the GateLogic, PlateLogic and GraphLogic tabs at the top of the panel on the left to view the elements from these sections. Click-and-drag selected elements directly to the page.



Dot plots, histograms and overlays can be added to a report from within the ‘GateLogic’ panel. Once selected, individual or multiple plots can to the using drag-and-drop. Plates/heatmaps and corresponding statistic and summary tables can be added to a report from the ‘PlateLogic’ panel, whilst graphs, statistical analysis tables and data tables can be added from the GraphLogic panel.

Document and Page Preview

A page preview window exists to the right of the report window. Within the window are thumbnails for each page in the document. Clicking on a page thumbnail will display that particular page of the document.

The Page Preview window can be resized by clicking and dragging the window border or turned off using the ‘Display Page Preview icon in the toolbar.

DocLogic Toolbar

The DocLogic toolbar contains a variety of formatting functions. Below is a description of the toolbar buttons:

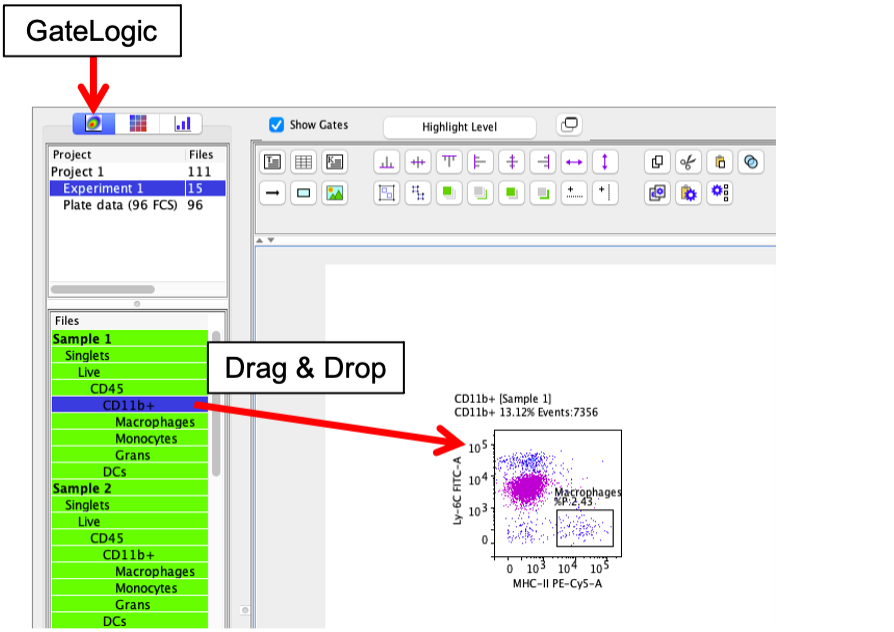
Insert Text Box		Align Horizontal – Bottom, Middle, Top		Bring to Front	
Insert Table		Align Vertical – Left, Middle, Right		Send to Back	
Insert Keyword Text Box		Distribute Horizontally/Vertically		Bring Forward	
Insert Arrow		Group		Send Backward	
Insert Rectangle		Ungroup		Horizontal Guide	
Insert Picture				Vertical Guide	

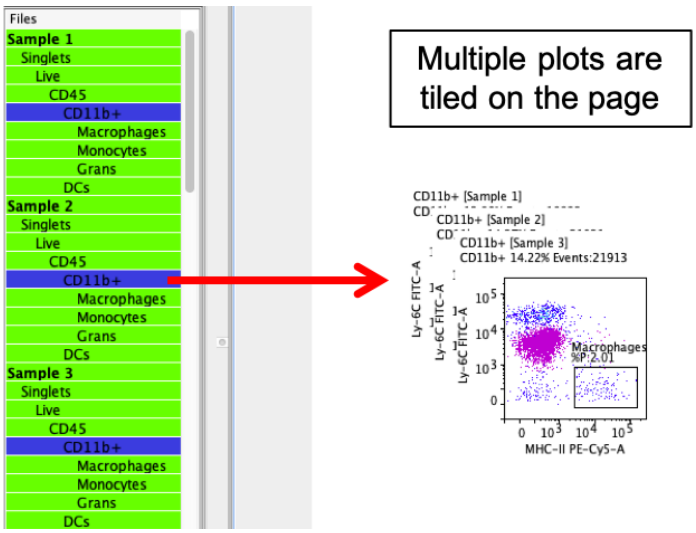
Copy Selected		Copy Selected Settings	
Cut Selected		Paste Settings to Selected	
Paste		Paste Settings to Selected Docs	
Clone Selected		Delete Selected	

Adding dot plots, histograms and overlays

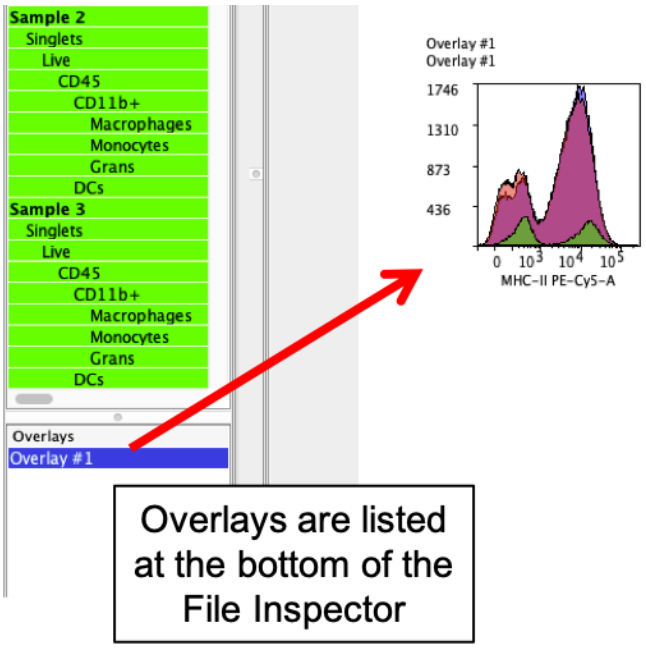
Dot plots, histograms and overlays can be added to a report by selecting them in the File Inspector and dragging them onto the page. The GateLogic File Inspector can be displayed by clicking the GateLogic tab at the top left of the screen. If a single plot is selected, it will be placed at the position where the mouse is released on the page. If multiple populations are added at once, the plots will be tiled. Individually plots can then be dragged to other areas on the page.

Plots will be displayed in DocLogic as they were defined in GateLogic. So, to switch a dot plot to a histogram or to change the color of backgated populations, make the changes in GateLogic. The report will subsequently be updated to reflect the changes.

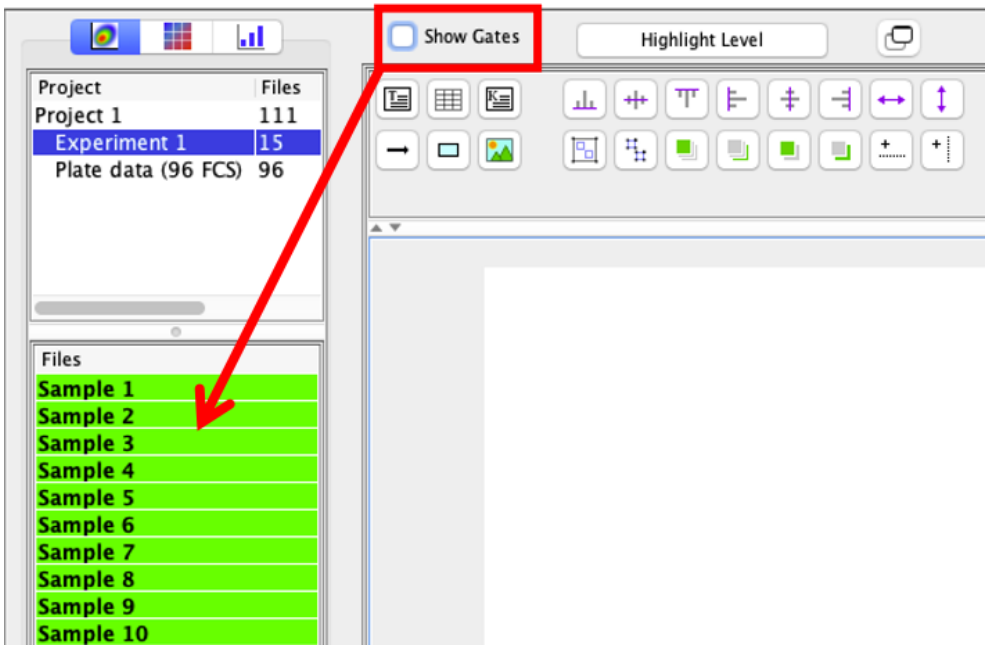




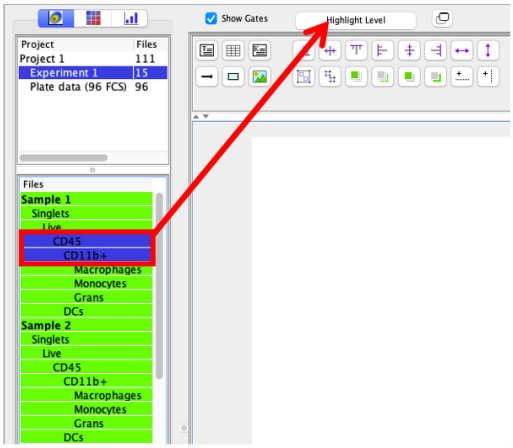
The list of overlays is displayed at the bottom of the File Inspector, as they are in GateLogic.

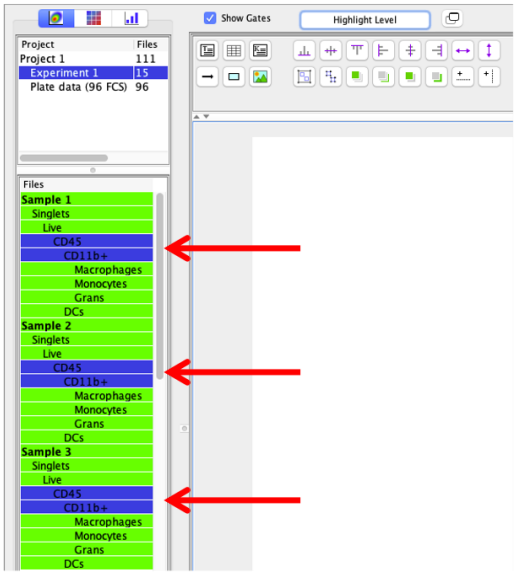


By default, all populations (gates) are displayed in the DocLogic File Inspector. However, the gates can be hidden by unticking “Show Gates” above the toolbar.



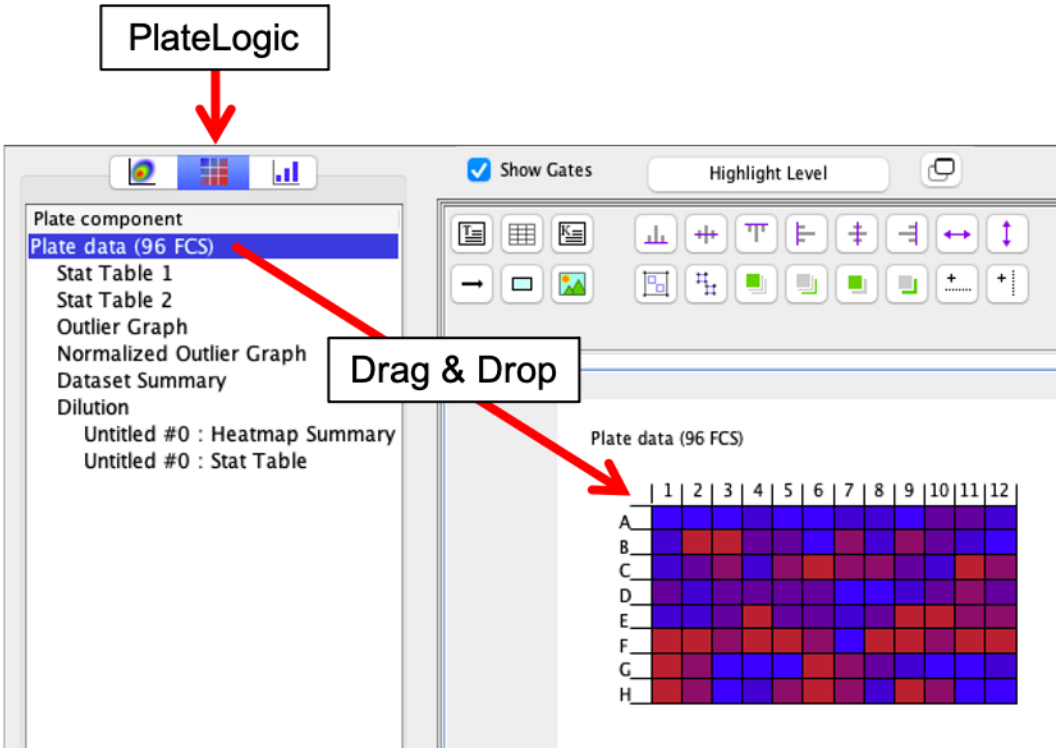
If you wish to select the same populations in all samples in the folder, make the selection on one sample and then click the Highlight Level button above the toolbar. These selected populations can then be dragged into a report together.





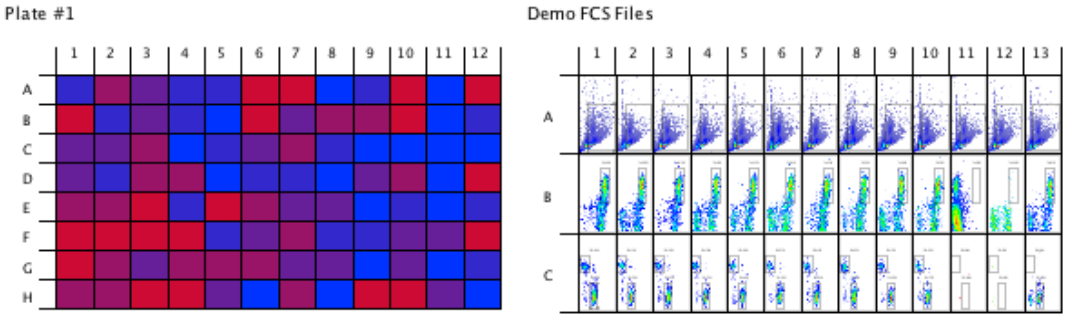
Adding plates/heatmaps and associated statistics tables

PlateLogic elements, including plates/heatmaps, statistics tables, outlier graphs and summary tables can be added to a report by selecting them from the PlateLogic tab in the File Navigator and dragging them onto the page. These elements will be displayed in the report as they appear in PlateLogic.



Elements in the PlateLogic panel

- Plate # - Plates will be listed with the name that is assigned in the data array tab in the GraphLogic Advanced Functions drawer.



Listed under each plate in the DocLogic Components tab are a variety of statistics and data tables, as follow:

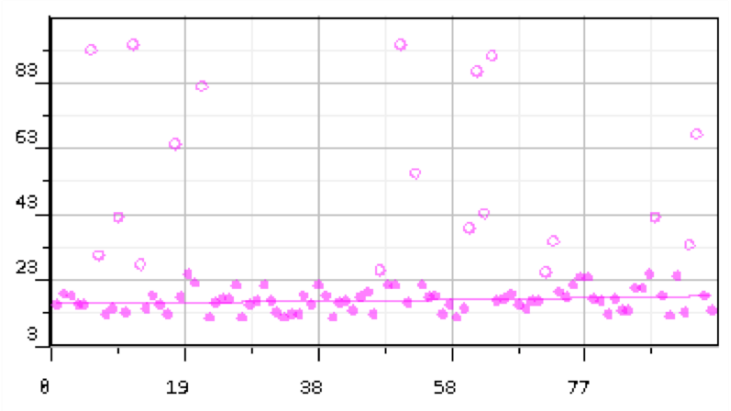
- **Stat Table 1** – this table shows the statistics defined in the plate side drawer and can be colored with the heatmap colors (foreground or background) as defined in the plate side drawer.

	1	2	3	4	5	6	7	8	9	10	11	12
A	15.77	19.09	18.41	15.94	15.91	93.59	31.02	12.75	14.44	42.75	13.52	95.30
B	28.00	14.88	18.25	16.02	12.92	65.01	18.06	24.97	22.31	82.44	11.90	16.11
C	17.50	17.54	21.81	11.87	15.65	17.05	21.61	16.64	13.67	11.70	13.02	12.76
D	18.47	15.60	21.61	18.73	11.61	16.36	16.72	14.09	18.07	19.69	12.73	26.48
E	21.61	21.98	95.69	16.22	55.95	21.99	18.24	18.38	12.76	15.50	12.12	14.64
F	39.14	87.37	43.88	91.85	16.68	17.58	18.89	15.99	14.74	16.81	16.87	25.91
G	35.49	19.90	18.09	21.76	23.95	24.28	17.18	17.03	13.01	17.16	13.86	14.27
H	20.79	20.94	25.17	42.34	18.40	12.61	24.68	13.73	34.11	68.17	18.35	14.02

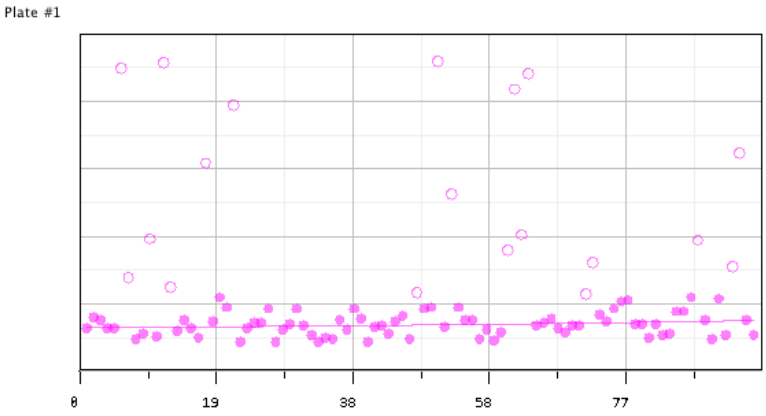
- **Stat Table 2** – this table shows the same data in a different layout and with the addition of two comments columns. Comments can be added from within the plate side drawer in GraphLogic.

Pos	Name	Param	Stat	Value	Comment 1	Comment 2
A1	A1		% Parent	15.77		
A2	A2		% Parent	19.09		
A3	A3		% Parent	18.41		
A4	A4		% Parent	15.94		
A5	A5		% Parent	15.91		
A6	A6		% Parent	93.59		
A7	A7		% Parent	31.02		
A8	A8		% Parent	12.75		
A9	A9		% Parent	14.44		
A10	A10		% Parent	42.75		
A11	A11		% Parent	13.52		
A12	A12		% Parent	95.30		
B1	B1		% Parent	28.00		
B2	B2		% Parent	14.88		
B3	B3		% Parent	18.25		
B4	B4		% Parent	16.02		
B5	B5		% Parent	12.92		
B6	B6		% Parent	65.01		

- **Outlier Graph –** see page 140



- **Normalized Outlier Graph**



- **Dataset Summary –** datasets can be used to separate different samples or populations within a plate. This can be matched to the different groups created for group analysis. Alternatively, the same files could be added multiple times, labeled as different datasets and then analysed

independently. The datasets are defined by a color and a name and the dataset summary table provides this information. More information on datasets can be found on page 133.

C	Name	Setting (Plate #1)
	Untitled #1	R5.FSC-A.% Parent

- **Heatmap Summary**

C	Condition(Plate #1:Untitled #1)
	11.61 >= x > 14.27
	14.27 >= x > 16.81
	16.81 >= x > 18.73
	18.73 >= x > 25.17
	>= 25.17

Adding graphs, statistics tables and statistical analysis results

The elements in the GraphLogic panel are displayed in a tree, as they are in GraphLogic. For each Workspace (the GraphLogic spreadsheet) there can be multiple Tables where data can be added in GraphLogic. Associated with each Table is a statistical analysis table and a graph. These elements, once created and customised in GraphLogic, can be added to a report in DocLogic. If the Table, Analysis or Graph have been renamed, then they can be identified by the new name. Here is a description of the four elements:

- **Workspace** – the Workspace heading is for reference only and cannot be added to the report

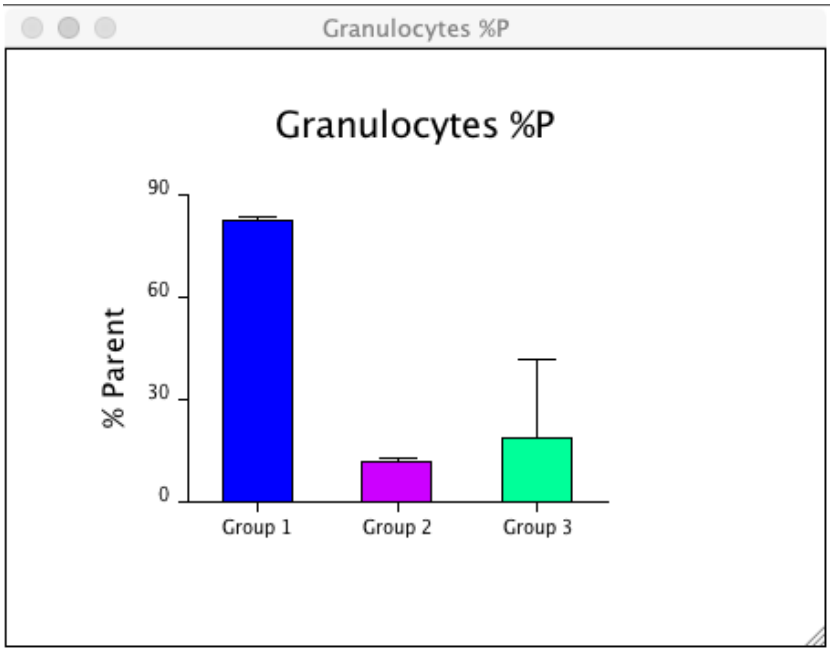
- **Table** – this is the data set used to build the associated graph

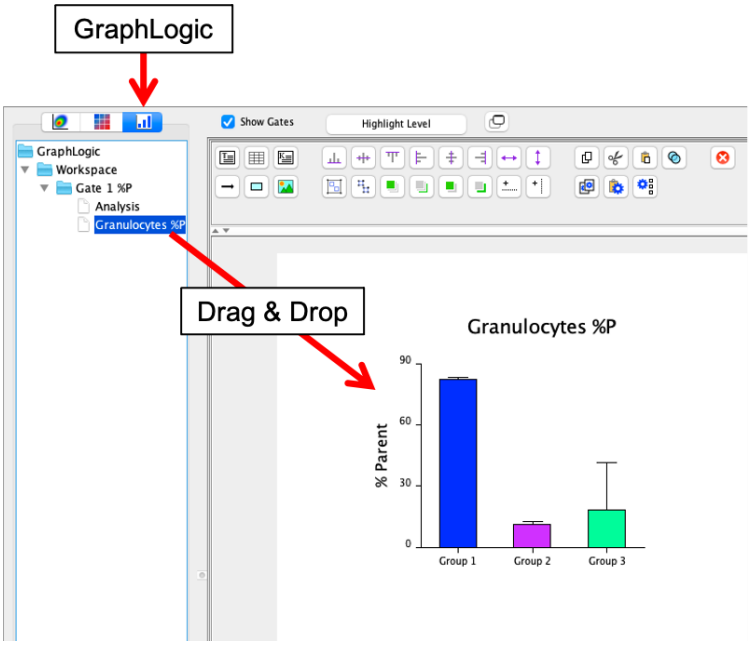
Gate 1 %P					
	1.00	2.00	3.00	4.00	5.00
Group 1	83.47	82.72	81.60	80.71	81.39
Group 2	12.53	12.35	10.45	10.81	10.29
Group 3	49.75	0.15	15.55	13.89	12.83

- **Analysis** – this is the resulting statistical analysis summary

1	2	3	4	5	6	7
One Way Anova						
Number of groups	3					
F Value	65.60254					
P Value	0	****				
Null hypothesis	false					
Are means different?	YES					
Significance level	0.05					
Source	DF	SS	MS	F	P	
Groups	2	15144.01077	7572.00539	65.60254	0	****
Errors	12	1385.06933	115.42244			
Total	14	16529.0801				
Tukey						
Groups	Mean Diff.	Critical Value	Lower	Upper	Sig Diff?	
Group 1 Vs Group 2	70.69313	3.773	52.56526	88.82101	YES	
Group 1 Vs Group 3	63.54345	3.773	45.41557	81.67132	YES	
Group 2 Vs Group 3	-7.14969	3.773	-25.27756	10.97819	NO	

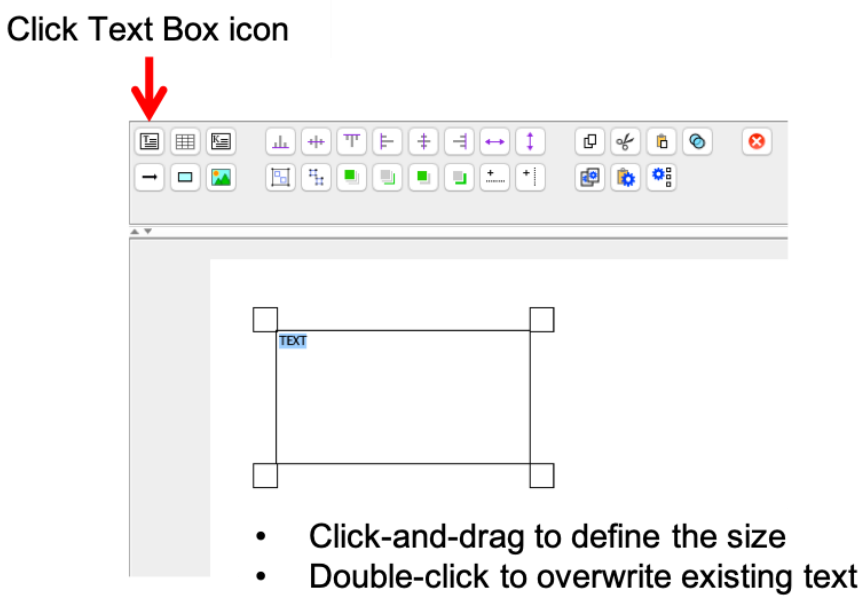
- **Graph** – this is the graph, as viewed in GraphLogic, derived from the data set in the associated Table. When imported into a report in DocLogic, it will retain all of the formatting. However, the same formatting panels form GraphLogic exist in DocLogic, so the graph can continue to be edited in DocLogic.





Adding Elements from the DocLogic Toolbar

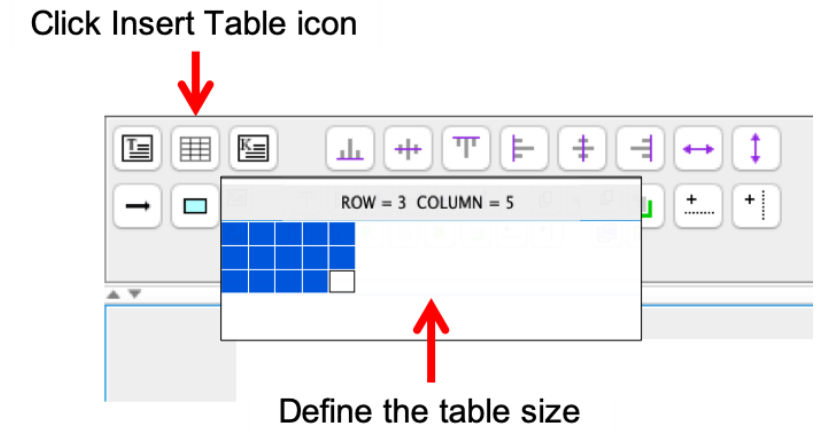
Text – a text box can be added to a report by clicking the Insert Text Box icon in the toolbar and then using click-and-drag in the report to define the size of the text box. By default, the word “TEXT” is added to new text boxes. Click twice in the new text box to display a flashing cursor and then start typing or double-click to highlight any text. Typing when text is highlighted will overwrite with the new text.

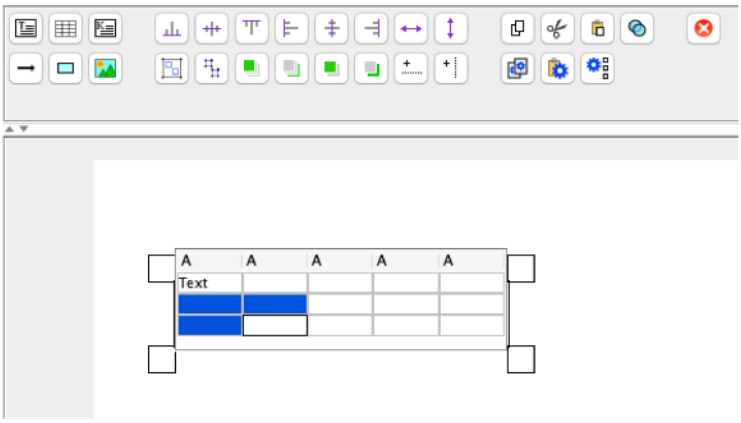


Tables – empty tables can be added to a report by clicking the Insert Table Icon and then defining the dimensions of the new table form the drop-down menu. The new table will be automatically added to the report.

Single-click and drag the table to a new position or click and release to select the table. Once selected, double-click in a cell to enter text or numbers highlight one or more cells in order to copy, paste or format the text or numbers in those cells. Click-and-drag a column header to resize individual columns.

Resize the entire table by single-clicking to select the table and then dragging a corner or a corner node.



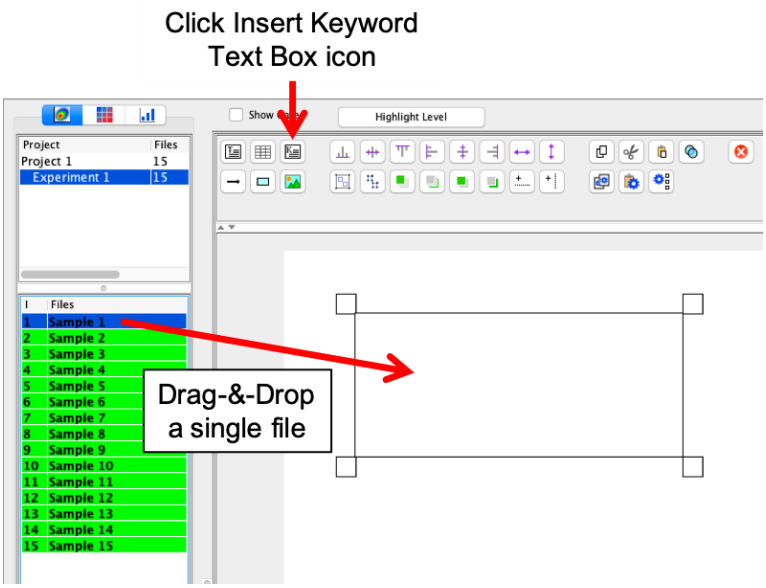


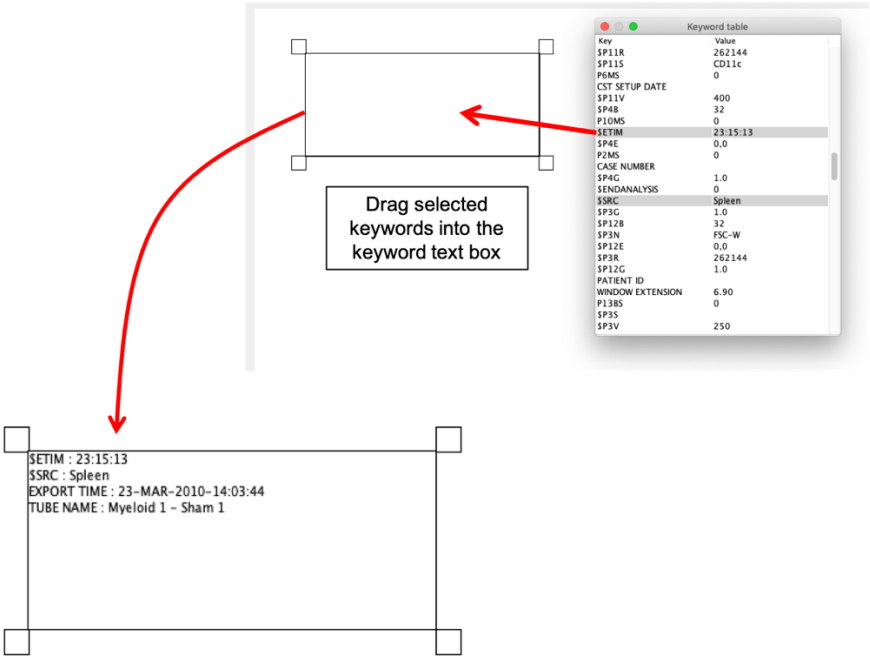
- Click-and-drag to move the table
- Click a cell on a selected table to enter text and numbers
- Click-and-drag in a selected table to highlight cells for copying or formatting
- Click-and-drag a corner node to re-size a table
- Click-and-drag the border of a column header to resize individual columns

Metadata – Metadata derived form a specific file can be added to a report in the form of a keyword text box. These appear like a standard textbox except text cannot be entered using the keyboard.

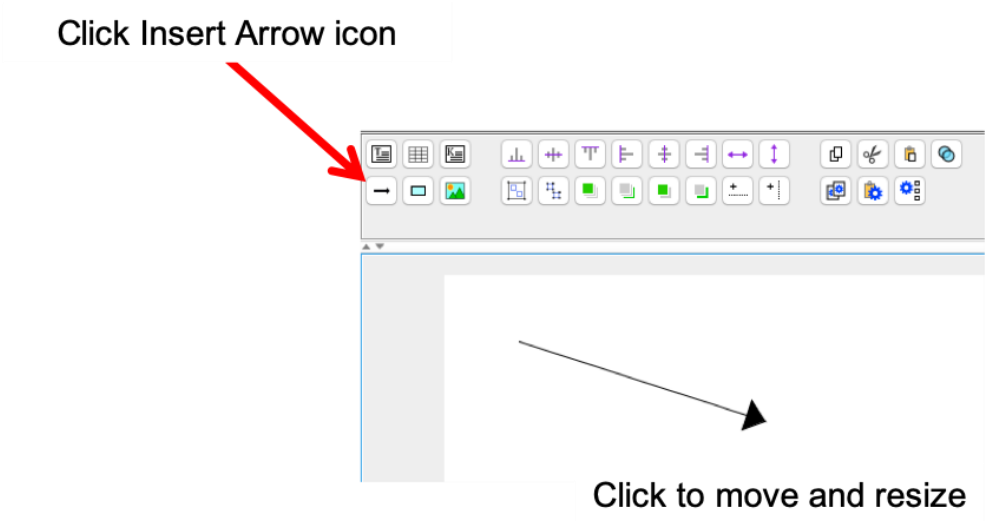
To create a keyword text box, click the Add Keyword Text Box icon and then click in the report. This creates the keyword text box. Then, select a specific file from the File Inspector (under the GateLogic tab) and drag it into the keyword text box. The Keyword Table window, containing all of the file specific keywords and values, will appear. Select one or more keywords (hold control/command for multi-selection) and then drag the selection into the keyword text box.

Multiple keyword text boxes can be created for one or many files. The text can be formatted in the same way as the standard text box.

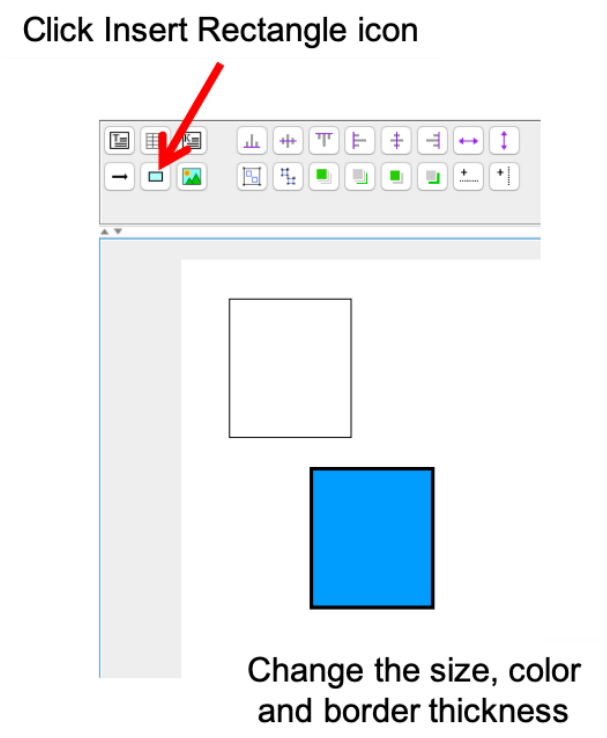




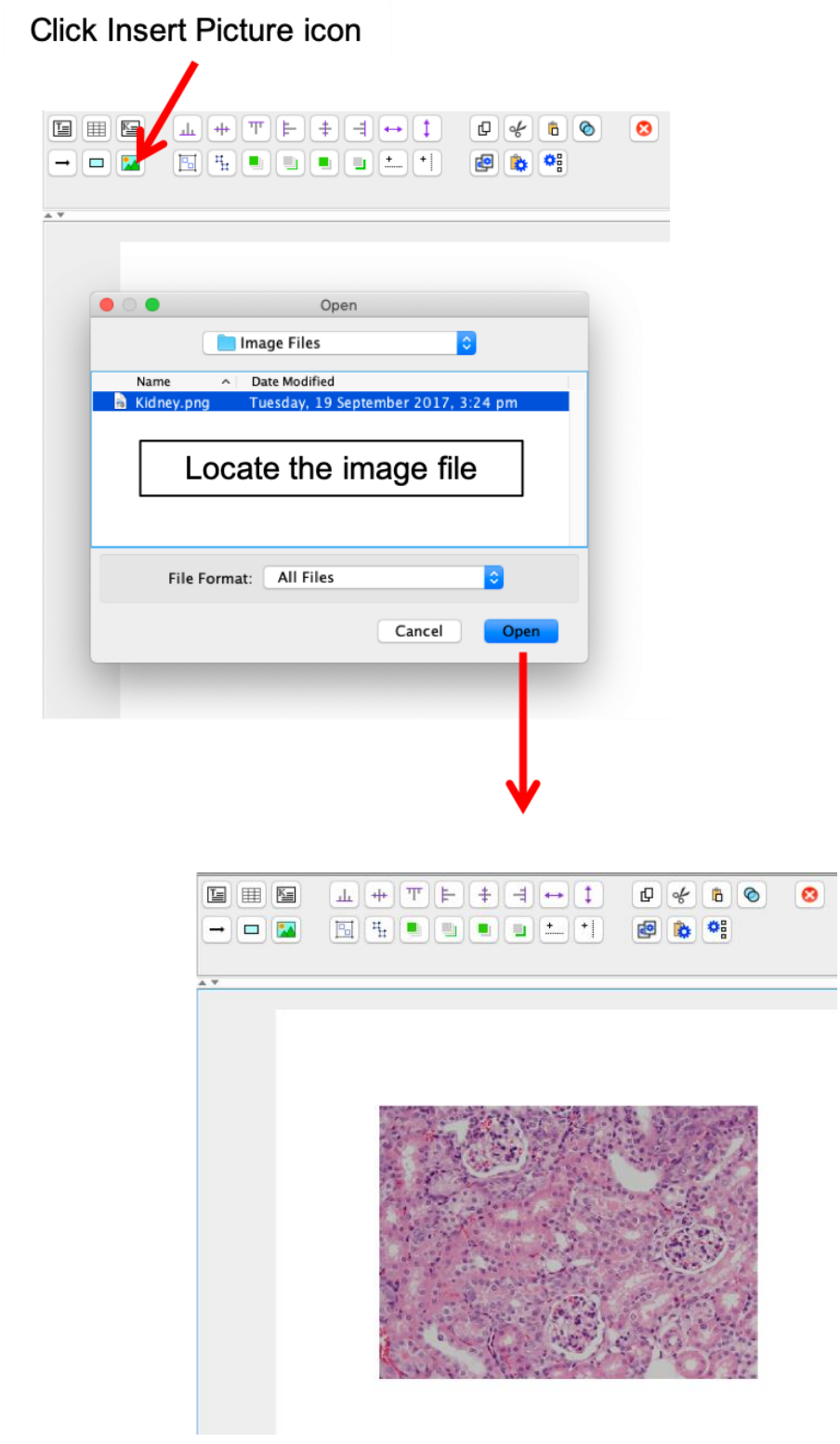
Arrows – click the Insert Arrow icon and then click-and-drag in the report to draw an arrow of a defined length. Click-and -drag the arrow to move it or click-and-drag a node to resize it.



Rectangles – click the Insert Rectangle icon and then click-and-drag in the report to define the size of the new rectangle. Click-and-drag to move a rectangle on the page or select and drag a corner node to resize the shape. When the rectangle is selected, the corresponding formatting panel is displayed on the right side of the screen. Here, the aspect ratio can be locked to resize proportionally, or the height and length can be set manually. The border and background colours can also be formatted here.



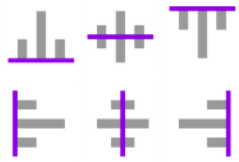
Images – PNG and JPG image files can be imported into a report by clicking the ‘Insert Picture’ icon in the toolbar and selecting the image by its file location and name. Imported images can be resized and positioned anywhere in the document.



Aligning, Distributing, Grouping and Reordering

There are a range of new tools in the DocLogic toolbar to help arrange elements added to a report. These include the ability to align and distribute a selection of items, group and ungroup, reorder overlapping items and adding vertical and horizontal guides to visually align plots, graphs, plates, shapes and text boxes.

Aligning Items



Align Horizontal – Bottom, Middle, Top

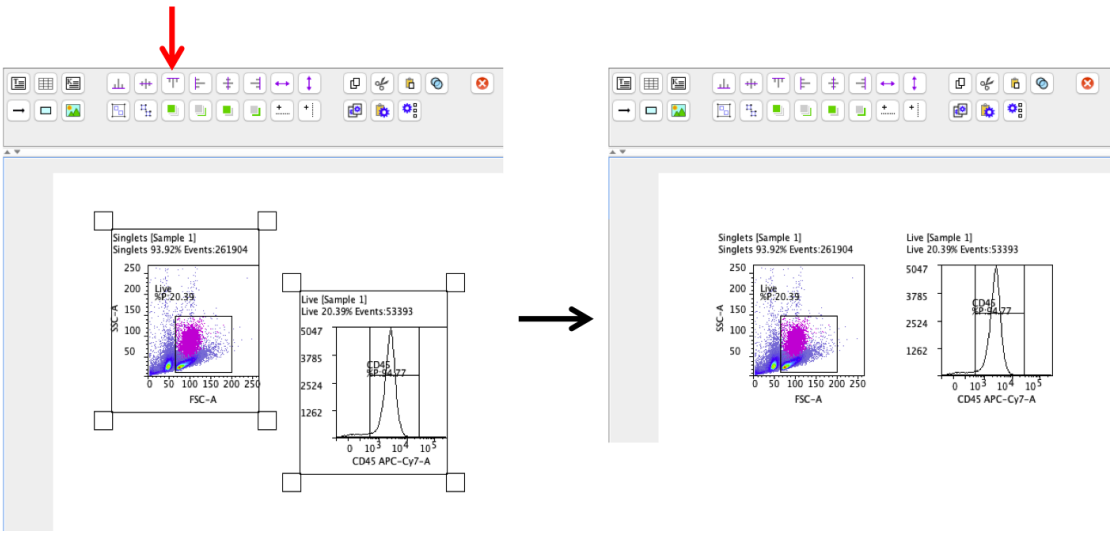
Align Vertical – Left, Middle, Right

Items in a report can be automatically aligned using one of six icons in the toolbar. To align items, select them using either click-and-drag (in the direction from top-left to bottom-right) or click to select while holding control/command, then click to align horizontally or vertically.

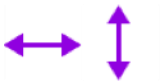
Horizontal alignment can be by the bottom of the lowest side of any selected item, the middle (halfway between the highest and lowest side) or the highest side.

Vertical alignment can be by the side furthest to the left, the middle (halfway between the furthest left and right sides) or the side furthest to the right.

Once items have been aligned, they remain selected and can be repositioned all together by clicking and dragging.



Distributing Items

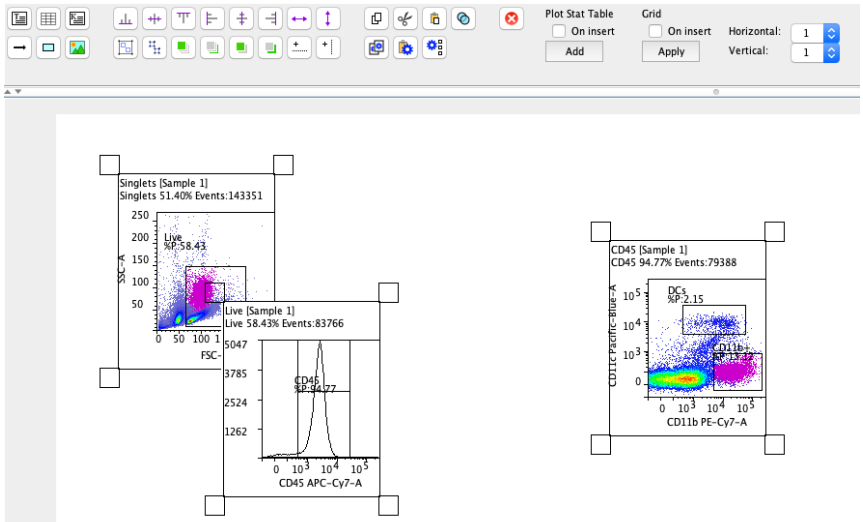


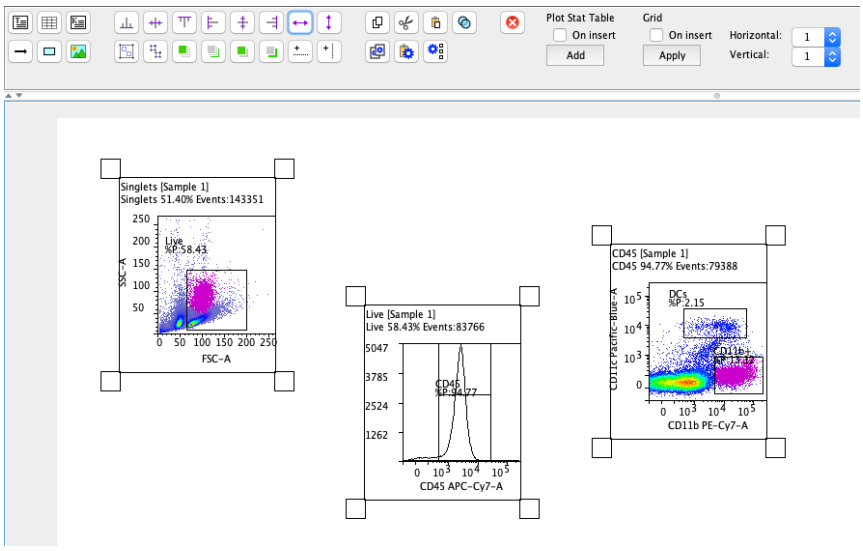
Distribute Horizontally/Vertically

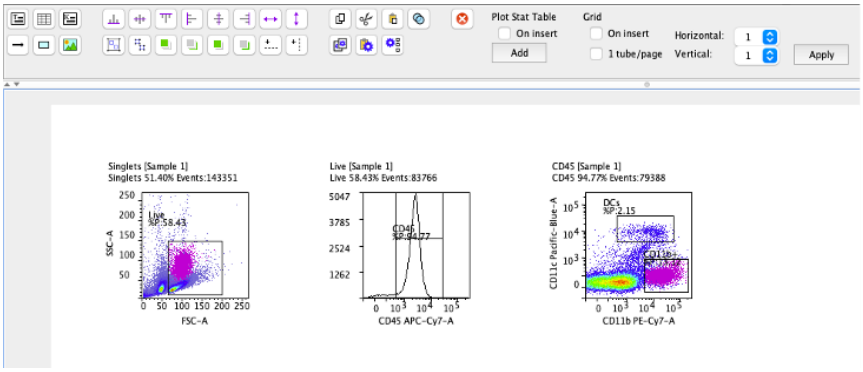
Items can be distributed evenly both horizontally and vertically. To distribute items, select them using either click-and-drag (in the direction from top-left to bottom-right) or click to select while holding control/command, then click to distribute horizontally or vertically. This spaces the items evenly between the first and last in the selection.

Items remain selected after distribution so can be moved together or aligned horizontally, as described above.

In the following example, three dot plots are distributed horizontally and then aligned to the top.







Grouping and Ungrouping

Group

Ungroup

A collection of items can be grouped into a single element. Select the items to group and click the icon in the toolbar. Grouped items can be moved and resized as a single item. Grouped items can be copy/pasted or cloned as a single item. Additional elements can be grouped with already grouped items.

Ungrouping items will ungroup everything in the group, even if some items were grouped into an existing group. To ungroup, select the group of items and click the ungroup icon in the toolbar.

Reordering

Bring to Front

Send to Back

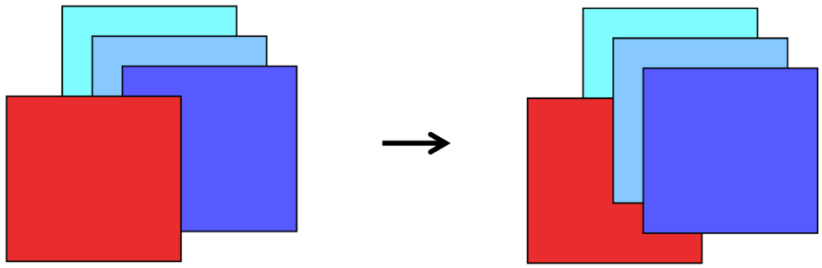
Bring Forward

Send Backward

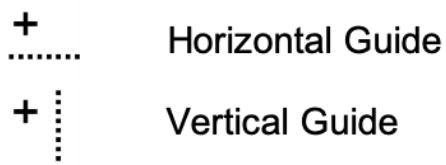
Overlapping items are displayed in the order of creation, with the original item being on the bottom and the newest on top. The order can be changed using the reordering options in the toolbar.

To reorder, select an item and choose to bring it forward one place or to the front, or send it backward one place or to the very back. Items don't have to be overlapping in order to change their order on the screen.

In the example below, the red square is sent backwards 2 places.



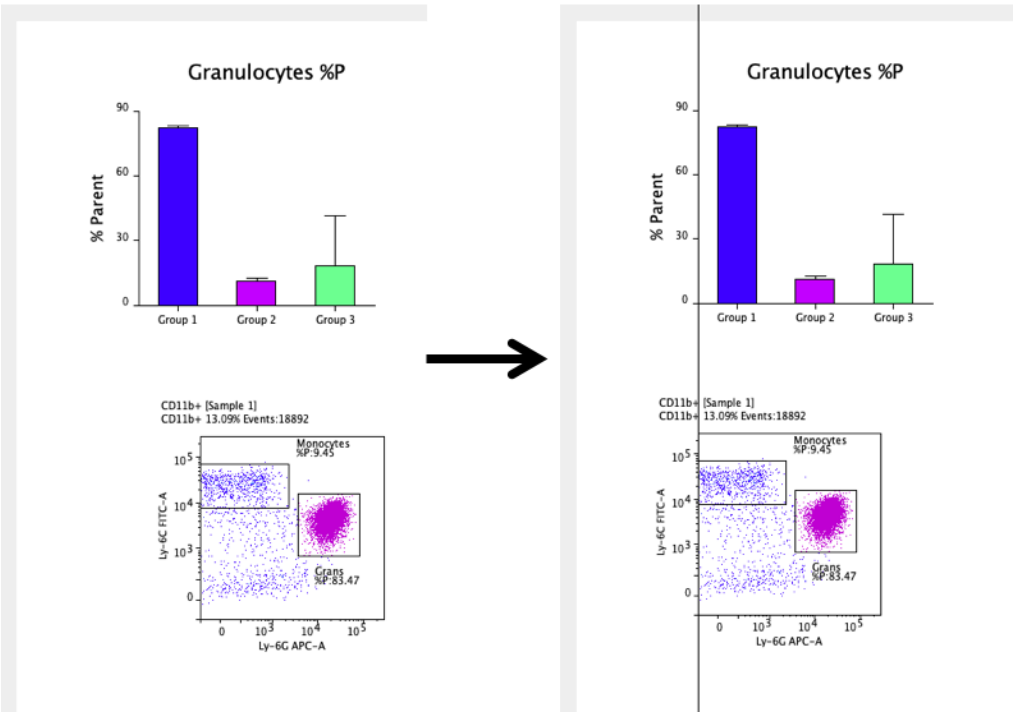
Adding Horizontal and Vertical Guides



Horizontal and vertical guides can be added to a page to help align different objects. While the aligning buttons can align the edge of an item, guides can help align elements within an item, such as an axis on a graph.

To add a guide, click the icon and then click on the page. Move a guide by clicking and dragging. Delete a guide by clicking on the guide and then clicking delete on the keyboard.

Multiple guides can be added to a page and guides will remain in the same position as you view different pages within a document.



Copy, Paste and Clone Items

Copy Selected

Cut Selected

Paste

Clone Selected

Items can be duplicated using the range of copy and paste icons. Select one or more items and click the Copy Selected icon. This copies the items(s) to the clipboard. Then click the Paste icon to paste the copy. If the item is pasted to the same page, then the copy will be offset from the original. However, if pasting to a different page, the items will be pasted to the same relative position as the original.

Clicking Cut Selected will remove the original item(s) when the Paste icon is clicked. This effectively moves the selected item(s).

Choosing Clone Selected will create a copy of the selected item(s) and offset it to the original. The result is effectively the same as choosing Copy Selected and Paste.

Copy and Paste Item Settings

Copy Selected Settings

Paste Settings to Selected

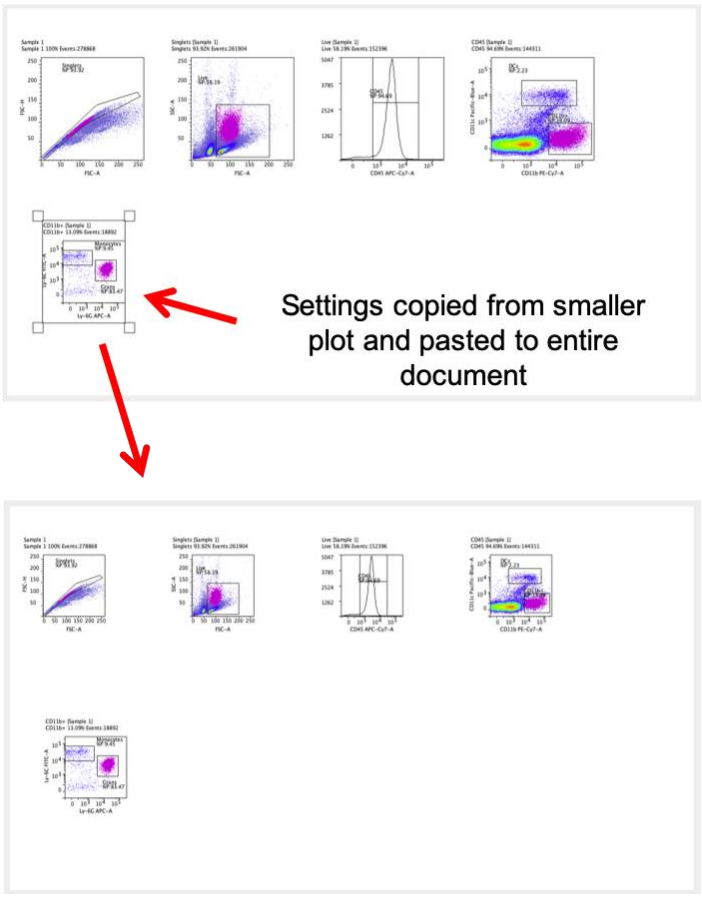
Paste Settings to Selected Docs

In the same way that objects can be copied and pasted, object settings can also be copied form one item and pasted to another. Examples of settings that can be copied include the size of an object and the graph type.

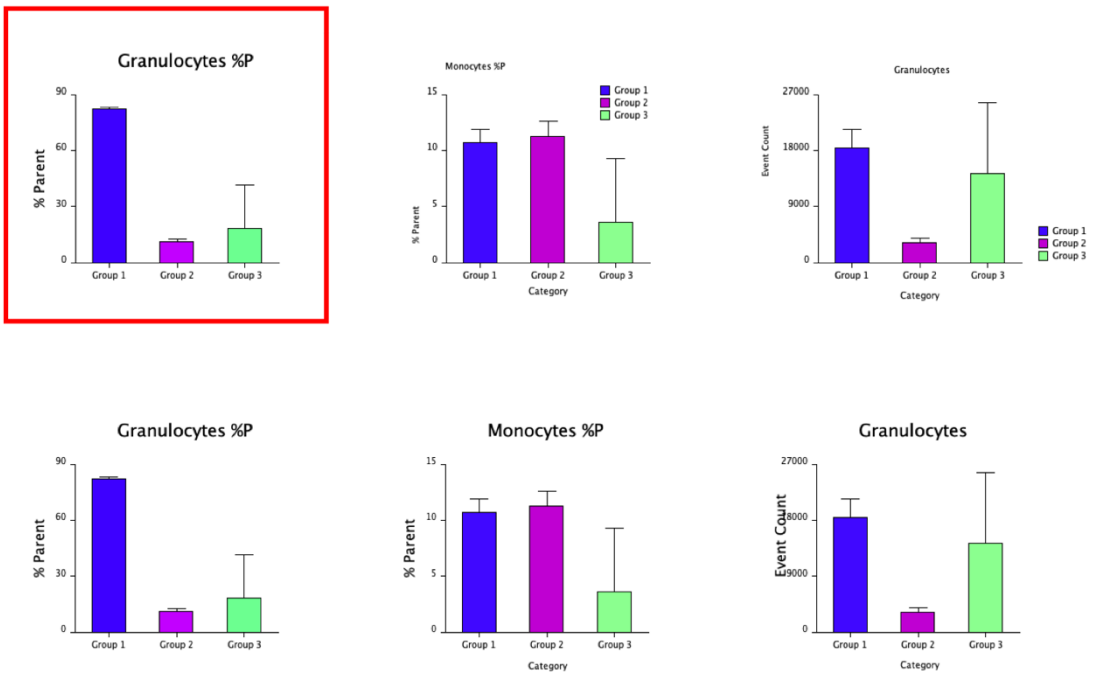
To copy settings, select an item and click the Copy Selected Settings item in the toolbar. Then, choose one or more similar items and click the Paste Settings to Selected.

Copied settings can also be pasted to an entire document or a selection of documents. After copying the settings from an item, select one or more documents and then click the Paste Settings to Selected Docs icon.

Example 1 – resizing multiple dot plots:



Example 2 – copying and pasting different graph settings. In the first row of three graphs, the settings from the graph marked in red were pasted to the two graphs to its right. In the second row, all graphs have the same title text size and position, and the legend has been hidden.



All graph settings are copied except for the titles themselves (it would be rare that all graphs would have the same title) and the group color. The group color is not copied to allow for settings to be copied and pasted to graphs containing different numbers of groups.

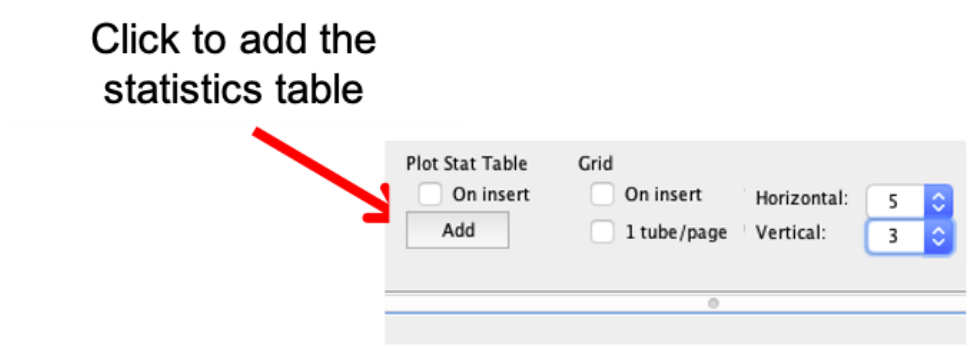
Deleting Items from a Report

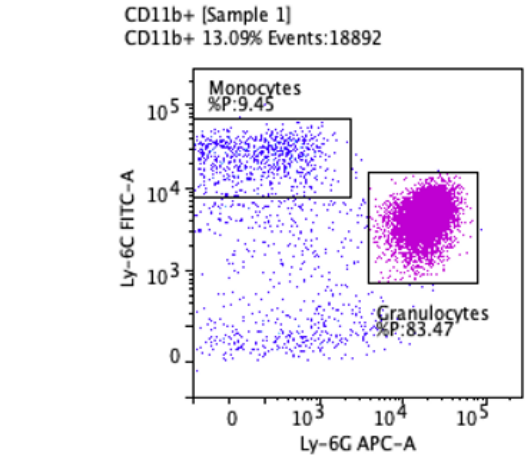
Delete Selected

To delete one or more objects from a report, select them and click the Delete Selected icon from the toolbar or ‘delete’ on the keyboard.

Adding Plot Statistics Tables

Statistics calculated in the plot side drawer in GateLogic can be added to a report in DocLogic. To add the table after the plot has been added to a report, select the dot plot and then click “Add” under Plot Stat Table in the toolbar. The table is a separate element and can be resized and formatted if required.

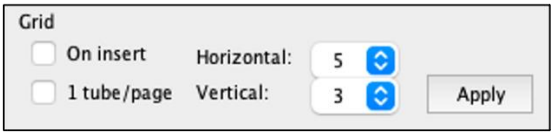




Population	% Par.
CD11b+ [Sample 1]	13.09
Macrophages [Sample 1]	2.36
Monocytes [Sample 1]	9.45
Granulocytes [Sample 1]	83.47

To add the table automatically when importing plots to the report, tick the “On Insert” option above the “Add” button. This results in the plot and table being grouped. Therefore, to format or move either element, independently from the other, select the plot and click the Ungroup icon in the toolbar.

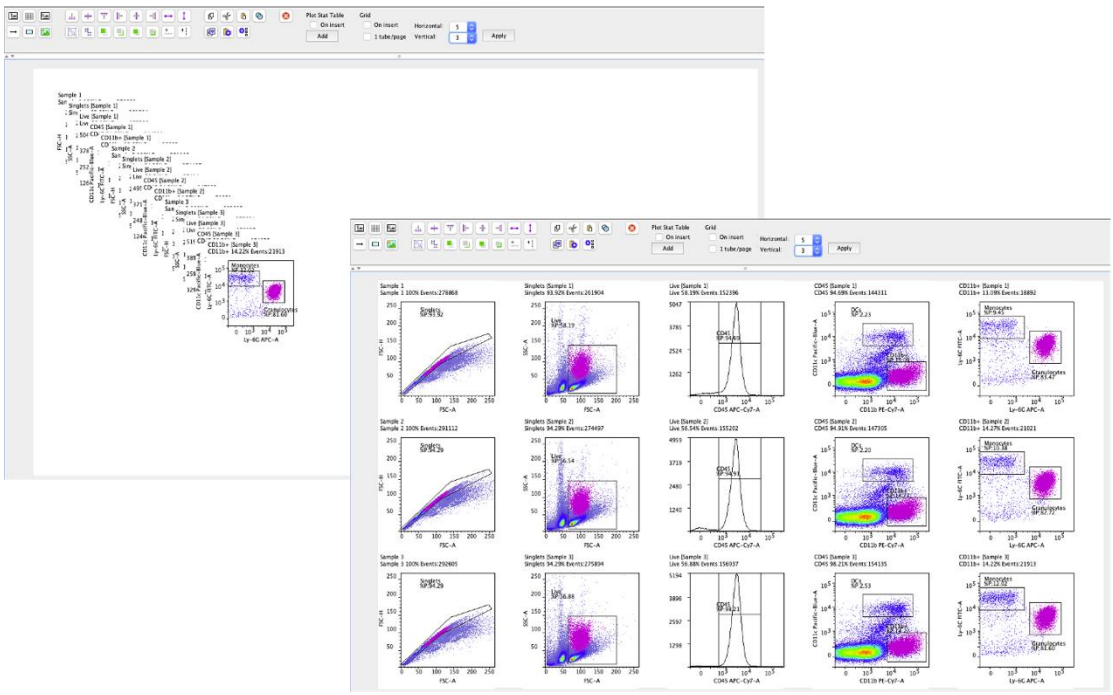
Applying Grid Settings to Reports



Items existing on a page can be placed into a grid using the options in the toolbar. To apply the grid to a page, set the number of rows (vertical value) and columns (horizontal value) and then click “Apply”.

Alternatively, by ticking “On insert” will add objects directly into a grid automatically when they are added to the page.

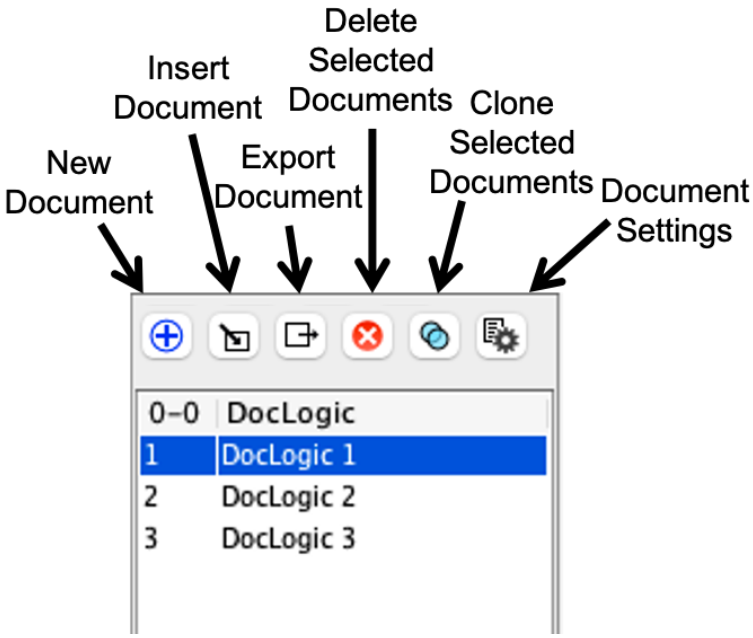
For example, if 15 plots were added to a page at once, they will be tiled. Setting a 5 x 3 grid and then clicking “Apply” will resize and arrange the plots into 3 rows of 5 plots.



If there are more objects than can fit on one page, additional pages will be added to the report. The items that don’t fit on the original page will be placed into a grid with the same dimensions on the successive pages. However, if the grid dimensions are changed and there are fewer items than grid positions, the additional pages remain and the items are arranged on the pages that they exist on.

When the ‘1 tube/page’ option is selected when applying a grid setting, all plots belonging to a file will fill the defined grid on the initial page and a new page will be started for each subsequent file. In this way, the analysis will be arranged as 1 sample (or tube) per page.

The List of Documents

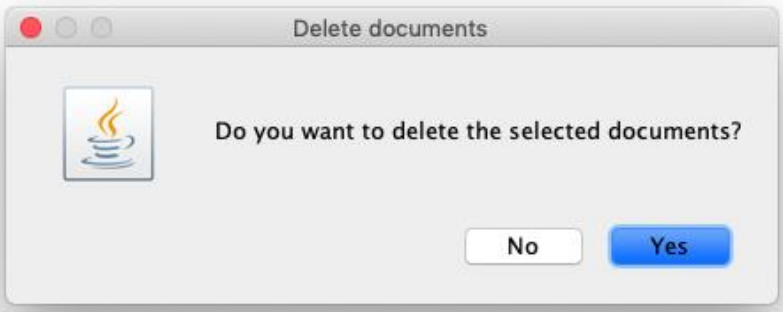


Multiple documents can be created in one FlowLogic analysis. The list of documents is located on the right-hand side of the DocLogic window. Above the list of documents is a small toolbar with icons to create a New Document, insert a document between existing documents, export selected documents delete selected documents, clone selected documents and change the document settings (page size and orientation).

Documents can be renamed by double-clicking on the existing name and typing in a new one.

When documents are exported, the new file will be named with the document name.

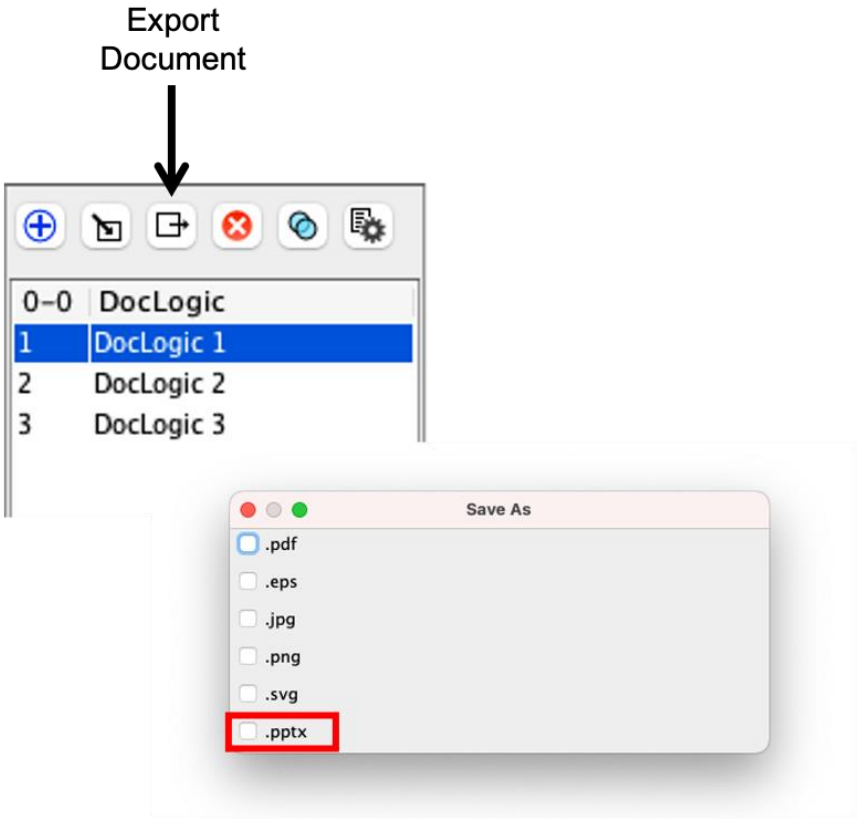
To delete one or more documents, select them and click the delete button in the toolbar. A warning window will open asking to confirm the action.



Exporting Documents as .pptx files

In addition to the formatting tools available in the DocLogic toolbar, it is also possible to export a report as a .pptx file, which can be opened in Microsoft PowerPoint. In addition, elements can be further ungrouped for further editing and report generation.

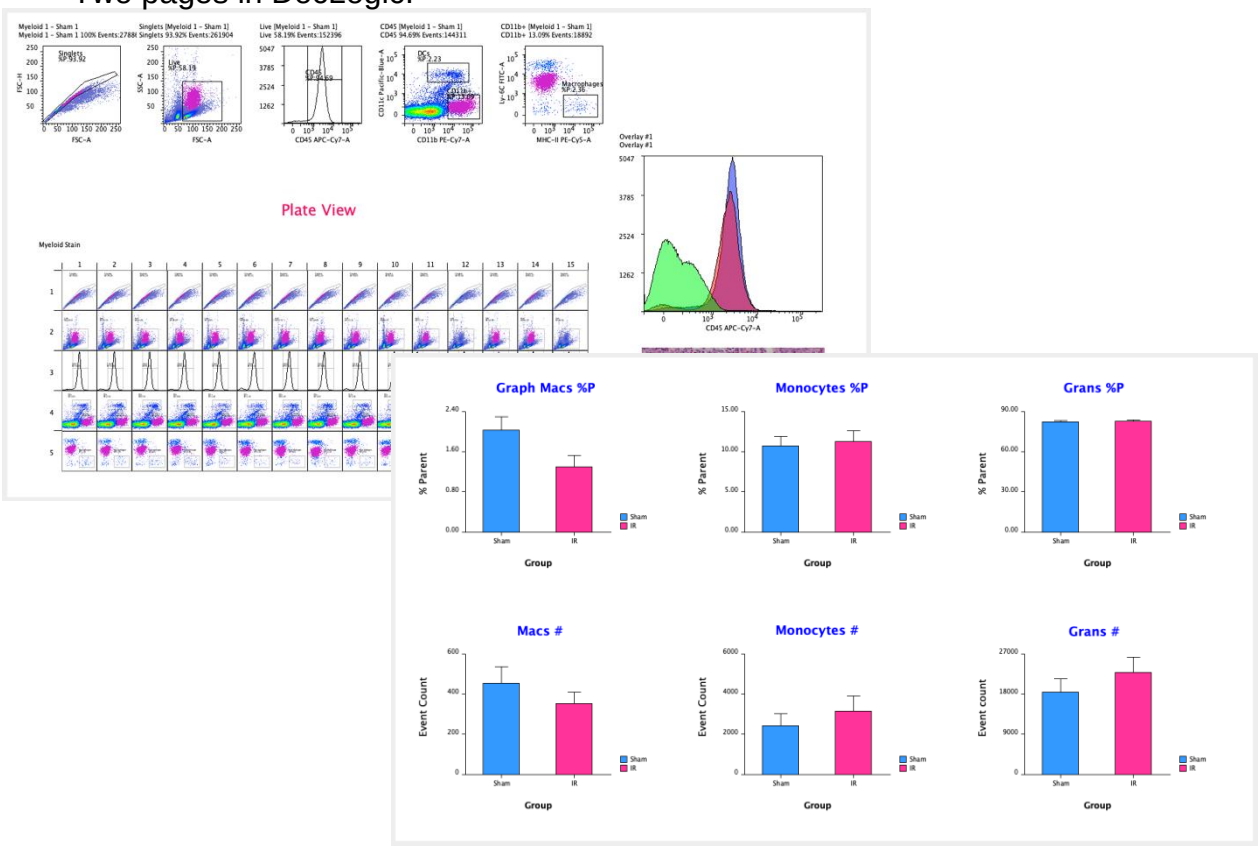
To export a report as a .pptx file, select the document and then choose the Export Document option in List of Documents toolbar. Select .pptx form the list of files pop-up window before defining the file name and location.



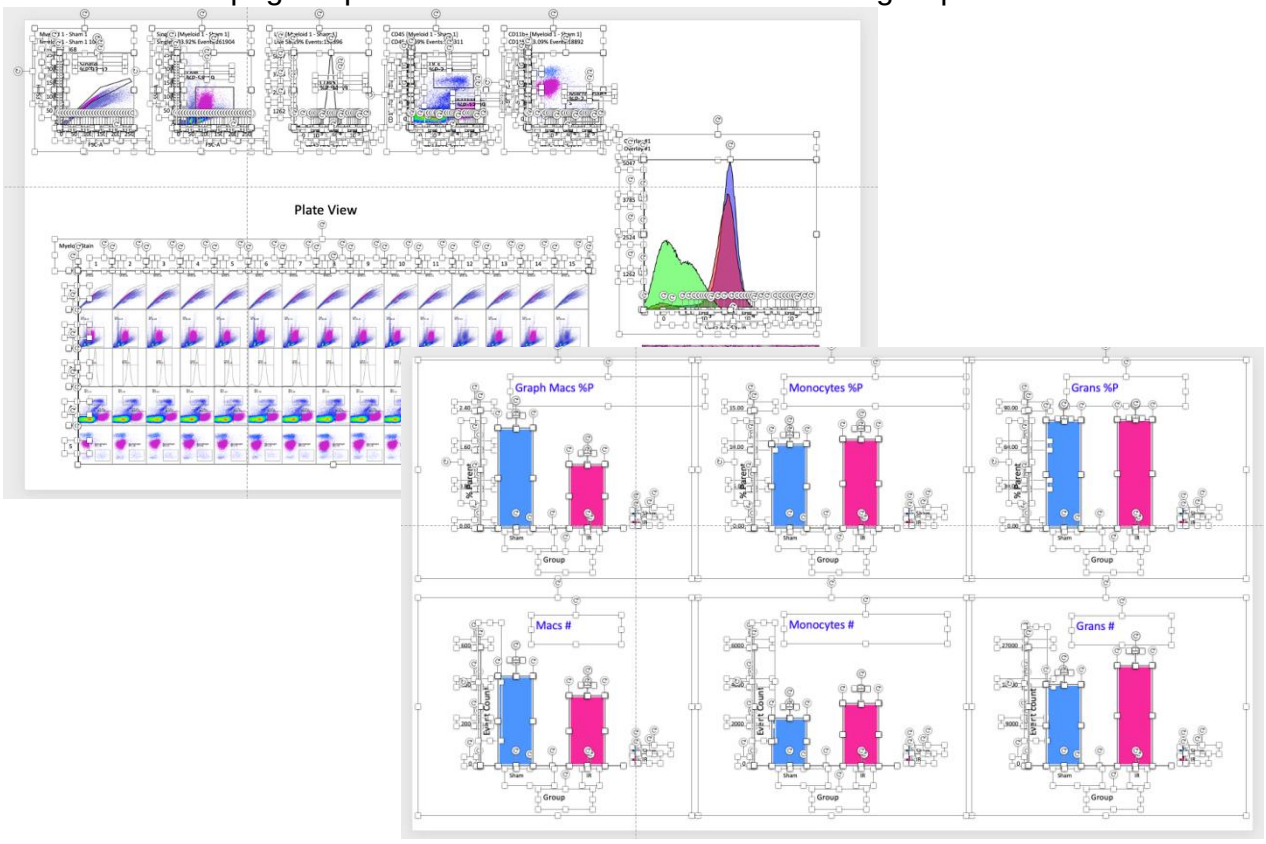
When you open the file in PowerPoint, right-click and choose to ungroup individual elements or a selection. It may be that not all elements are converted to the .pptx file in the exact same format and location but the tools in PowerPoint can aid in easily formatting and arranging the items precisely.

Below is an example of the extent of the ungrouping in PowerPoint. The images show the extent to which further editing and formatting can be achieved.

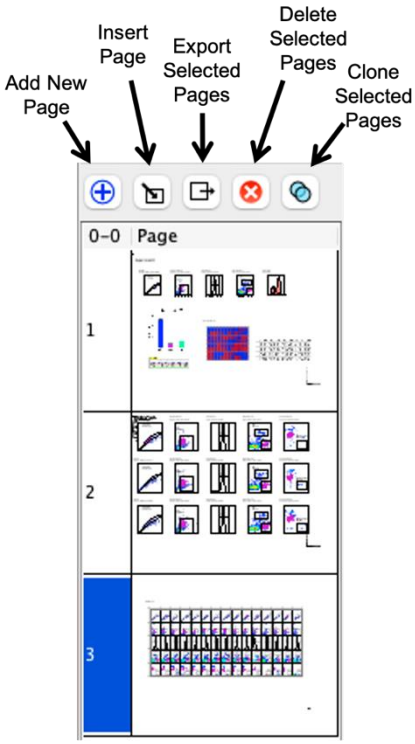
Two pages in DocLogic:



Same two pages opened in PowerPoint with all items ungrouped:

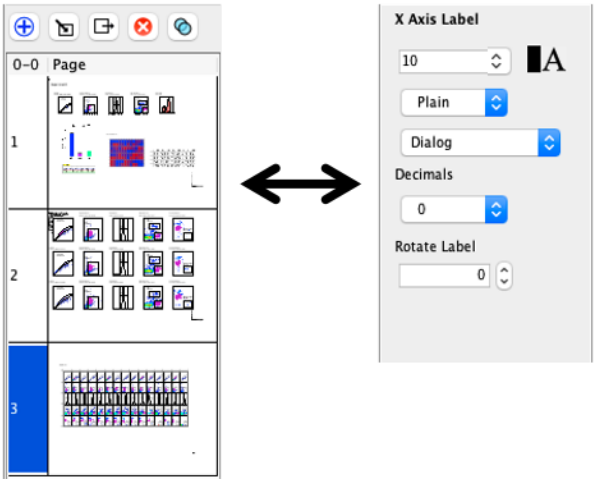


The Page Preview



The page preview is located beneath the list of documents window. Click on a page in the preview to display it. Above the page preview is a small toolbar with icons to Add a New Page, Insert Page, Export Selected Pages, Delete Selected Pages and Clone Selected Pages.

The page preview window is shared with the formatting panel. If an item in the report is clicked, the corresponding formatting panel is displayed. However, clicking on an empty part of the page or outside the page margins will return the panel to page preview.



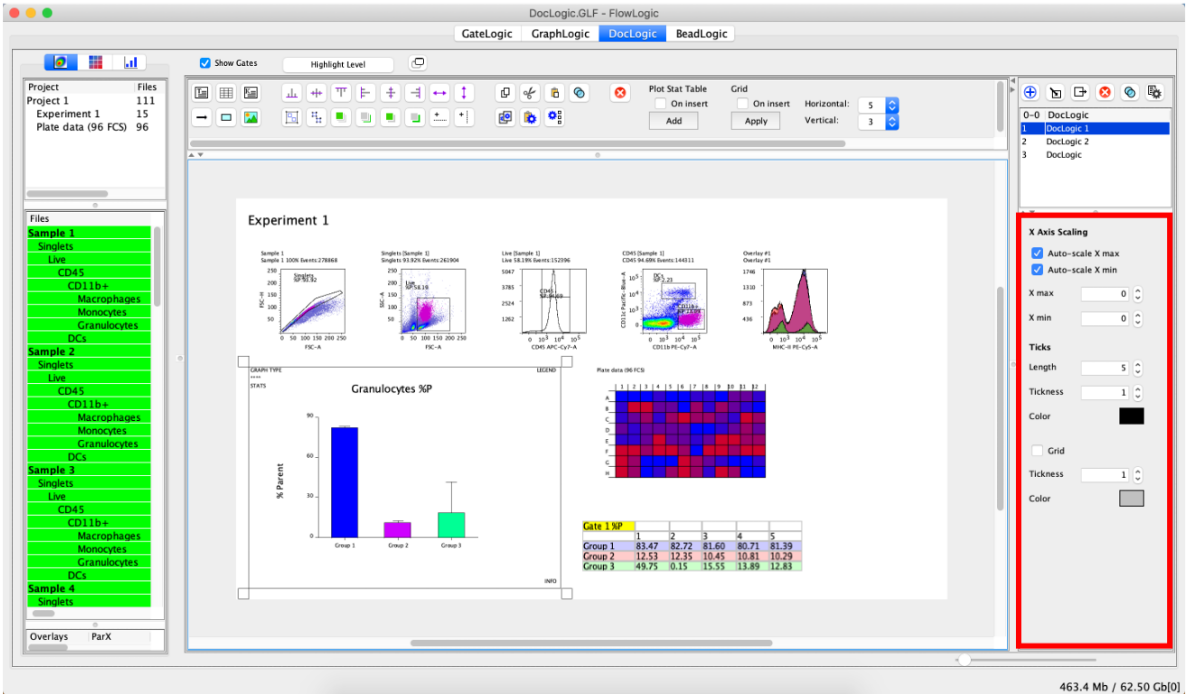
If a page has been zoomed, click on the particular page on the page preview to fit the page to the computer screen.

The DocLogic Formatting Panel

Items added to a report, including dot plots, heatmaps, graphs and text, can be formatted in the formatting panel on the right side of the DocLogic window.

The formatting panel shares the same space as the page preview and is only made visible when clicking on the item that you wish to edit. Each item has a unique formatting panel based on its attributes.

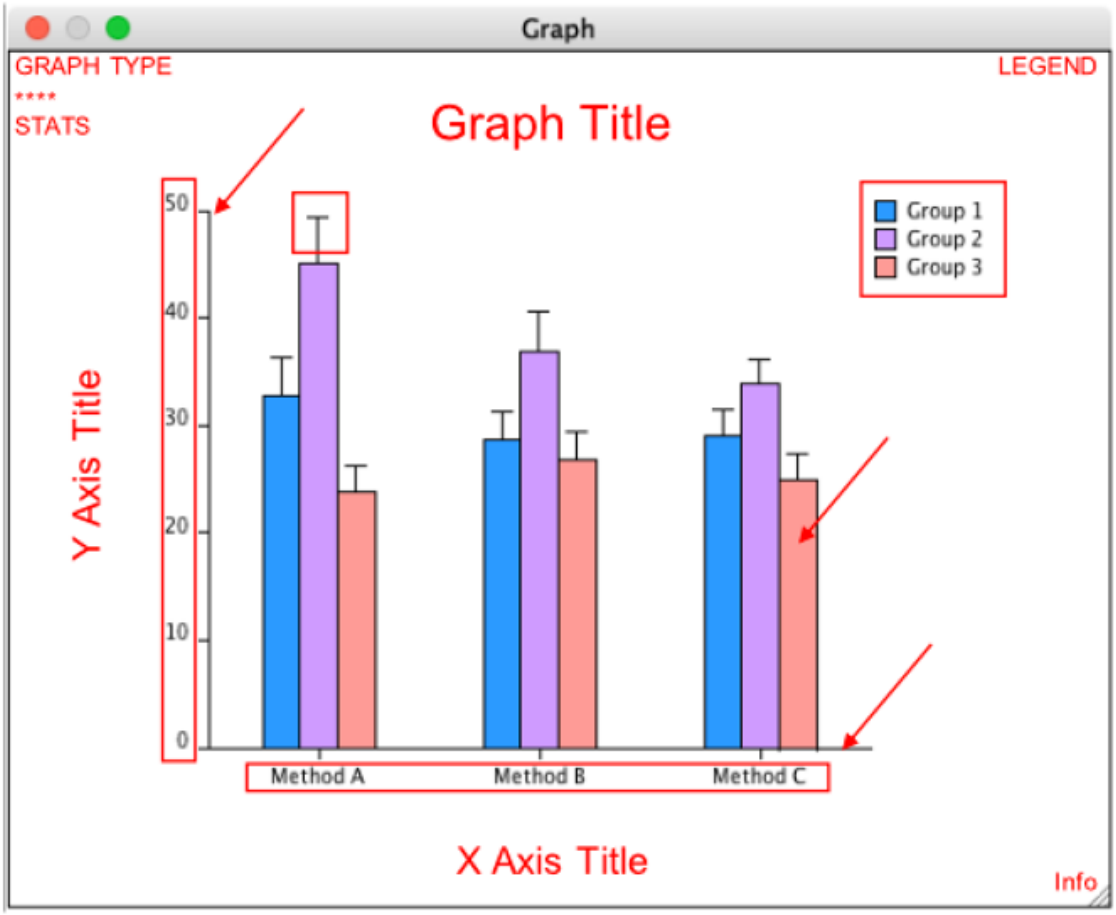
Below, the DocLogic formatting panel is marked in red.



Formatting Graphs

Graphs generated in GraphLogic have the greatest variety of formatting panels, as all aspects from the graph type, group colors, labels, fonts, axes and legends can be adjusted.

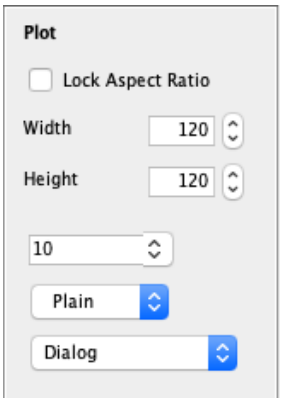
Below is an overview of the different regions of a graph that can be clicked (marked in red) to display unique formatting panels. For a detailed description of these, see page 180.



Graph Type	– choose a different type of graph
****	– stars of significance, P-values and bars
Stats	– displayed statistic and error bars
Legend	– drag the legend to the desired position
Colors	– click on a bar to set the group color
X & Y axis	– click on the x or y axis for scaling options
Axis labels	– format the labels on each axis
Titles	– edit and format the text for each title

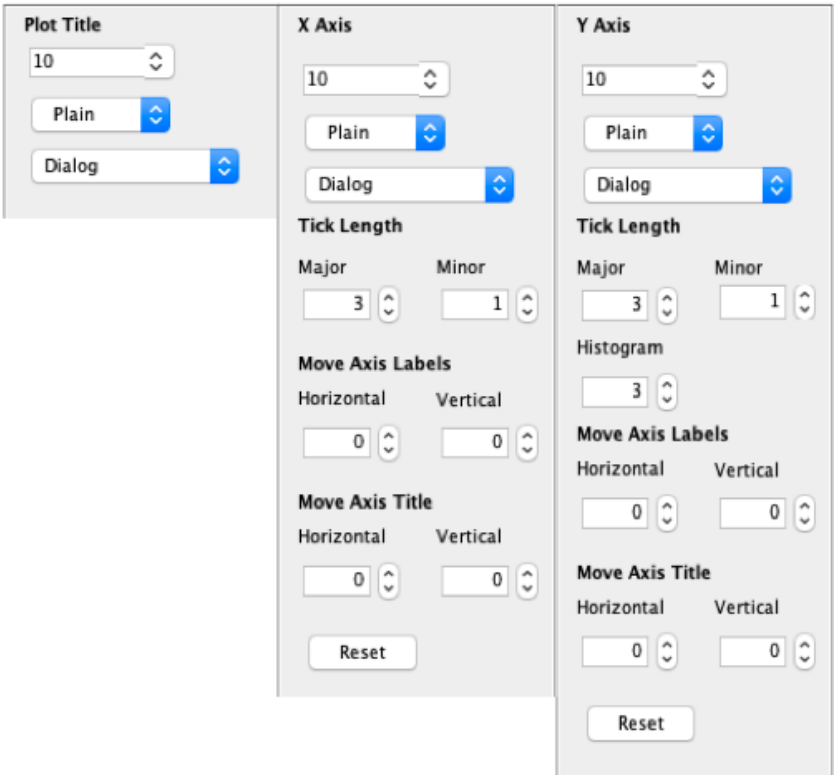
Formatting Dot Plots and Histograms

There are four formatting panels relating to dot plots: the plot size and gate label font, plot title, y axis and x axis.



Click inside the plot area to display the size and gate label font formatting panel. The aspect ratio can be locked to maintain the relative shape when resizing by dragging a corner of the plot or by changing the width and height values directly in the panel.

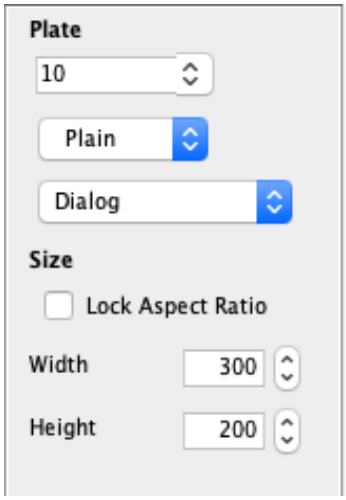
The gate label font size, style and type can also be changed.



Click on the plot title to change the font size, style and type.

Click outside the x or y axis to change the axis title and label font size, style and type. Additionally, change the major and minor tick lengths and move the axis titles vertically and horizontally. The y axis panel also has an option to adjust the tick length for histograms. Click “Reset” to return all lengths to the default values.

Formatting Plates

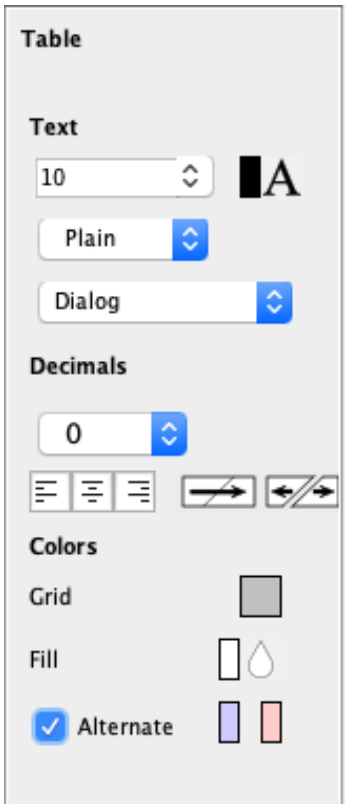


Click anywhere in a plate to display the Plate formatting panel.

Here, the title font size, style and type can be defined.

Choose to lock the aspect ratio and enter values to set the width and height dimensions.

Formatting Tables



There is one formatting panel for tables generated form the Insert Table icon in the DocLogic toolbar.

* For tables generated in GraphLogic, edit the attributes in GraphLogic. As the two sections are linked, all formatting will be automatically updated in DocLogic.

The font size, color, style and type can be set for text in individual cells. To format the font, highlight the desired cells and then change the attribute in the panel.

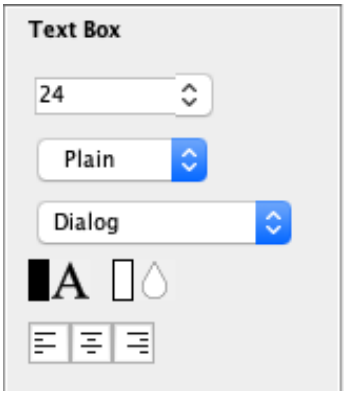
The number of decimal places can be defined for individual cells.

The alignment of text within individual cells can be set to the left, middle or right.

A selection of multiple cells horizontally or vertically adjacent can be merged and merged cells can be separated.

The grid color can be defined along with the cell fill color. Additionally, an alternating color scheme can be set by ticking the “Alternate” box and choosing two new colors by clicking on each of the color boxes.

Formatting Text Boxes



The text box formatting panel has options to change the font size, style, type and color.

A background color can be set for the text box.

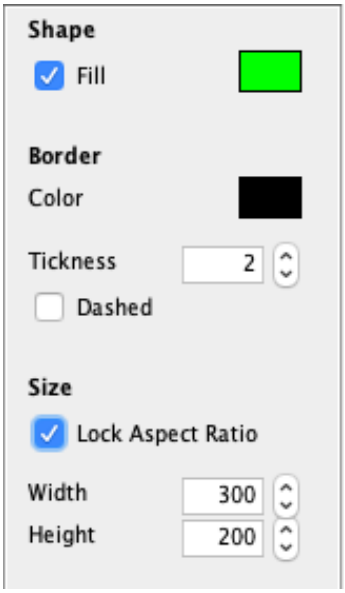
Text can be aligned to the left, middle and right of the text box.

The setting described here are for the entire text box and cannot be applied to a selection of text only.

Formatting Pictures

Lock the aspect ratio and enter values to set the width and height dimensions.

Formatting Rectangles



Choose a fill color for rectangles.

Change the border color, thickness and style.

Lock the aspect ratio and enter values to set the width and height dimensions.

Global Analysis Templates

Global analysis templates can be saved from an Experiment Folder and can contain all elements from an analysis including:

- Gating hierarchies
- Overlays
- Plates and heatmaps
- Graphs
- Statistical analyses
- Reports

So, if an analysis is regularly repeated on the same type of files, a template can be created and simply applied to successive datasets. This can automatically generate every aspect of the analysis through to the report. After a global analysis template has been applied, the analysis is 'live', and can be edited and refined, such as adjusting gates on individual files. Whilst a template is saved from a single Experiment Folder, it can be applied to multiple Experiment Folders at once.

There are some rules regarding global analysis templates:

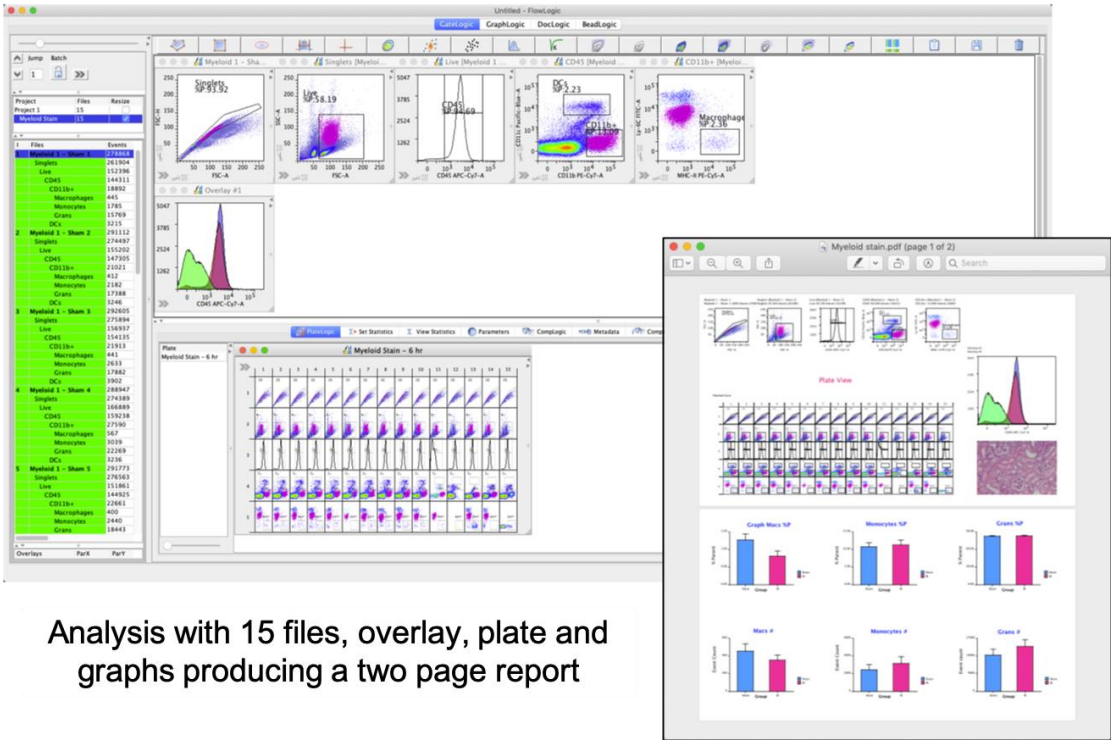
- A template can only be saved from one Experiment Folder and only one Experiment Folder can exist in the analysis at the time of saving
- The number of files in the Experiment Folder that the template is being applied to needs to match the number in Experiment Folder when the template was saved
- The files in the Experiment Folder that the template is being applied to must match the files in the template i.e. the same parameters

A template can:

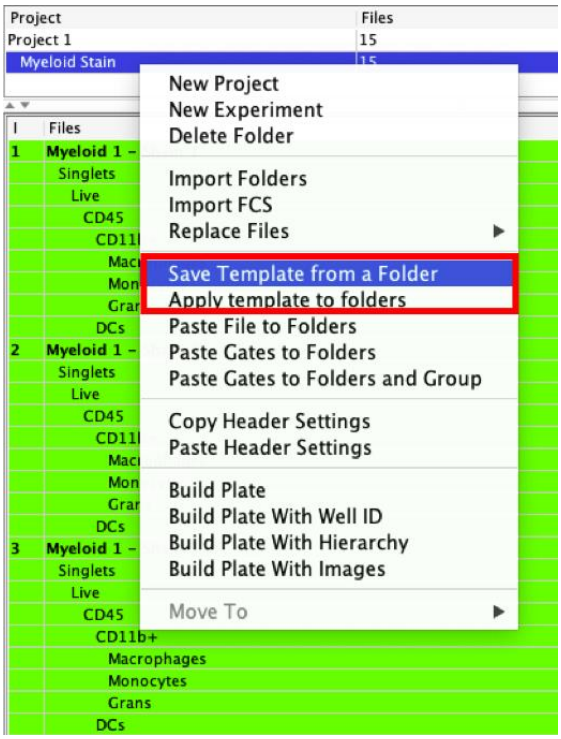
- Be applied to multiple GateLogic Experiment Folders at once
- Contain multiple GraphLogic Workspaces
- Contain multiple DocLogic reports
    - Different reports can contain the same files
    - Reports can have any number of pages

There are additional tools that can aid in creating and applying global analysis templates. Firstly, saving gate templates can be a quick way to reproduce all or part of a gating hierarchy (see page 90). Secondly, when applying global analysis templates, the right-click menu in the File Inspect has features to split a selection of files into individual Experiment Folders and copy a selection of files and paste copies to all Experiment Folders, such as global controls (see "Folder Action" on page 90). Finally, saved analysis files (.GLF and .gatelogicexperiment) can be imported through File → Open Append (see page 27 for more detail). This can be useful to combine multiple analyses and generate new graphs or reports from the combined data.

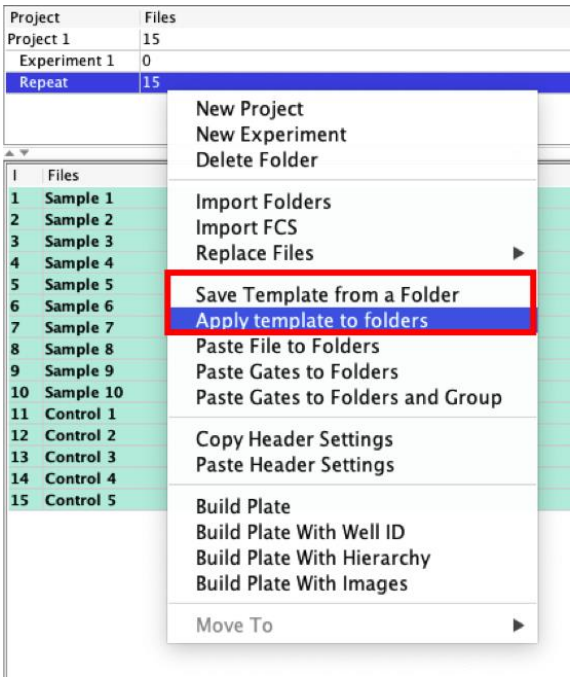
Saving and Applying a Global Analysis Template



Analysis with 15 files, overlay, plate and graphs producing a two page report

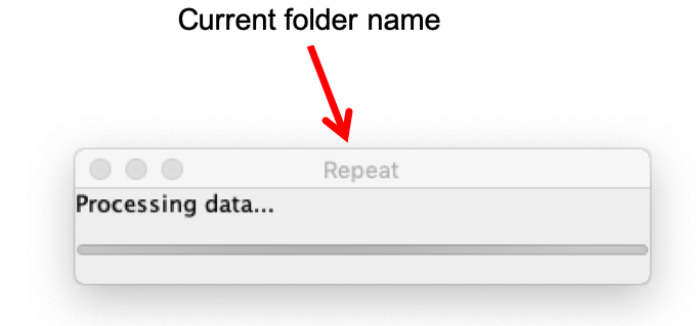


To save the global analysis template, right-click on the single Experiment Folder in GateLogic and choose “Save Template from a Folder.



To apply a global analysis template, select one or more Experiment Folders, right-click and choose “Apply Template to Folders”. Then, locate the desired template and click “Open”.

The process of applying folders can take some time depending on the number of folders and files within each folder. A pop-up window will indicate that the system is processing data. The Experiment Folder name in the pop-up window indicates the progress.



Tips for creating a useful global analysis template

- As the number of files in the Experiment Folder must match the number in the template, if the number of files in each repeated experiment might vary, create the template with the maximum number that is likely. It is easier to delete files from an analysis after applying a template than incorporating them afterwards. E.g., if performing plate screenings, create a template with 96 files. If the next run has 90 files, use 6 files from the original dataset and then delete the original files from the analysis.
- File replacement can be a useful tool if you want to swap a selection of files, rather than applying a template to a complete dataset (for more information see page 81).

BeadLogic - Introduction

BeadLogic is a multiplex data analysis tool. Initial sample gating and definition is performed in GateLogic (using PlateLogic, for plate layout and visualization) before being sent to BeadLogic for regression analysis, unknown sample interpolation and reporting.

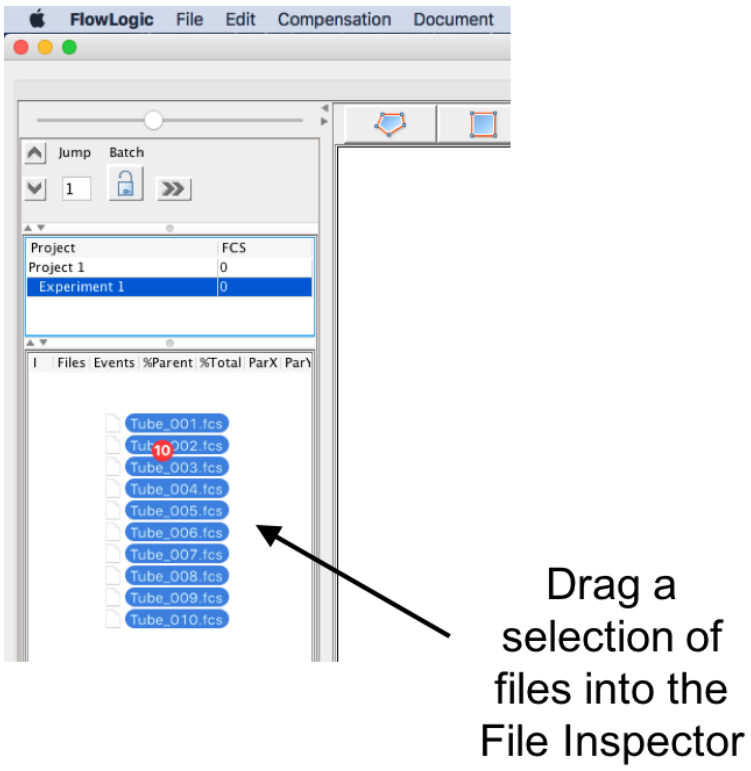
BeadLogic - FCS/MQD analysis and plate setup in FlowLogic

Data acquired on a flow cytometer can be analyzed in FlowLogic flow cytometry analysis software and exported to BeadLogic with all plate setup parameters defined.

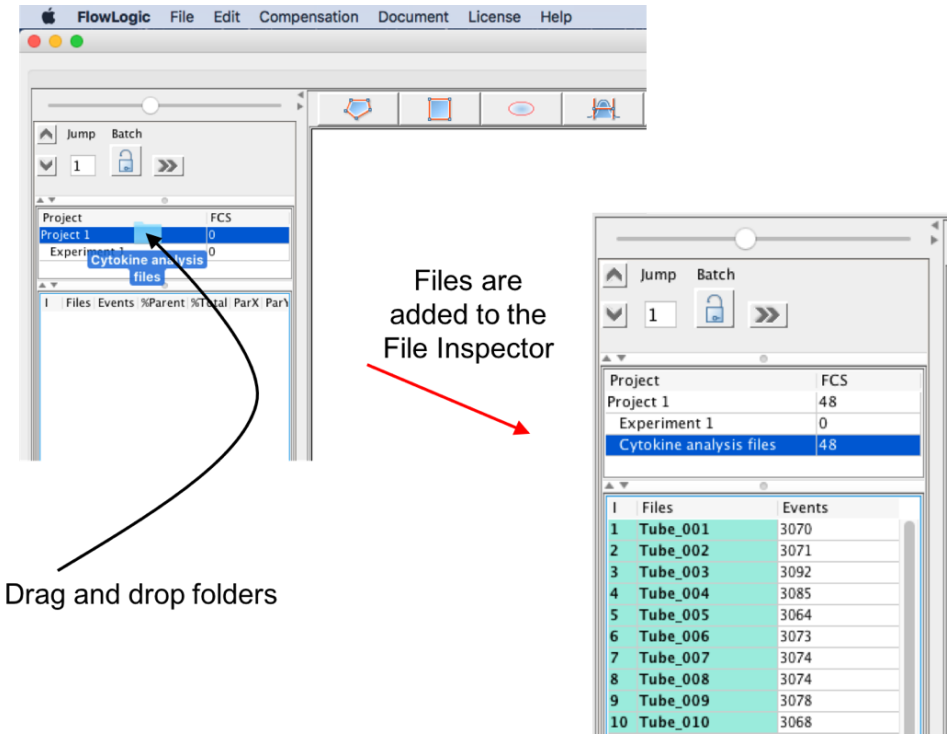
Here is a step-by-step description on how to perform this initial part of the sample analysis in FlowLogic. For a detailed explanation of the features FlowLogic, please consult the FlowLogic manual and online video guides.

Importing FCS/MQD files

Files can be imported into FlowLogic by dragging a selection of FCS or MQD files directly into the Files Inspector. A folder containing these files can also be dragged into the File Navigator. Alternatively, from the main menu choose File→Import FCS-MQD-LMD-LXB… or Import Folders and select the files by their location.



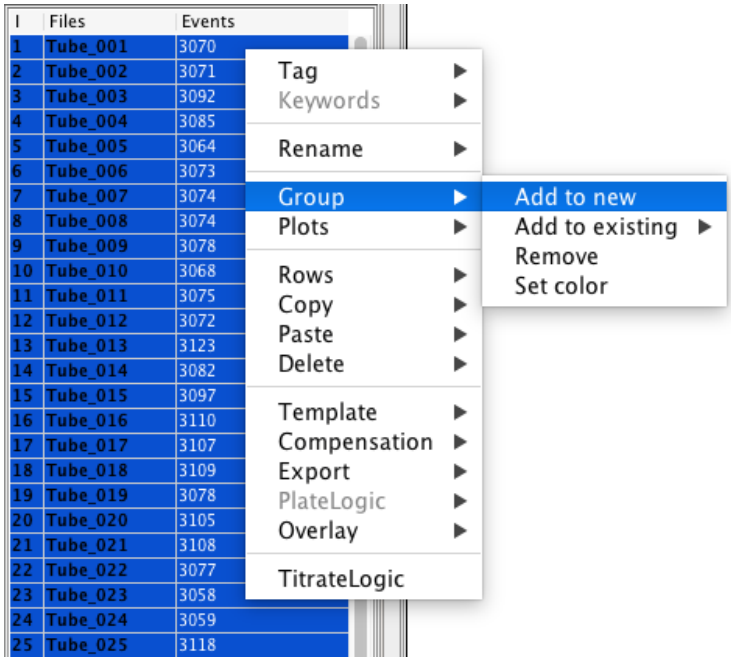
OR

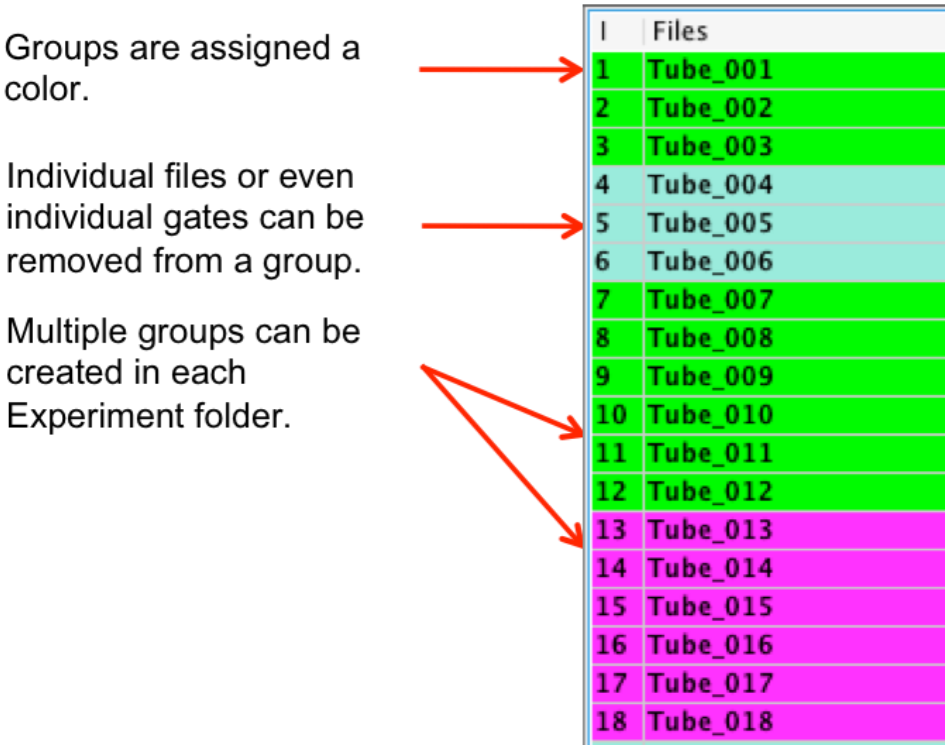


Grouping files and gating on populations

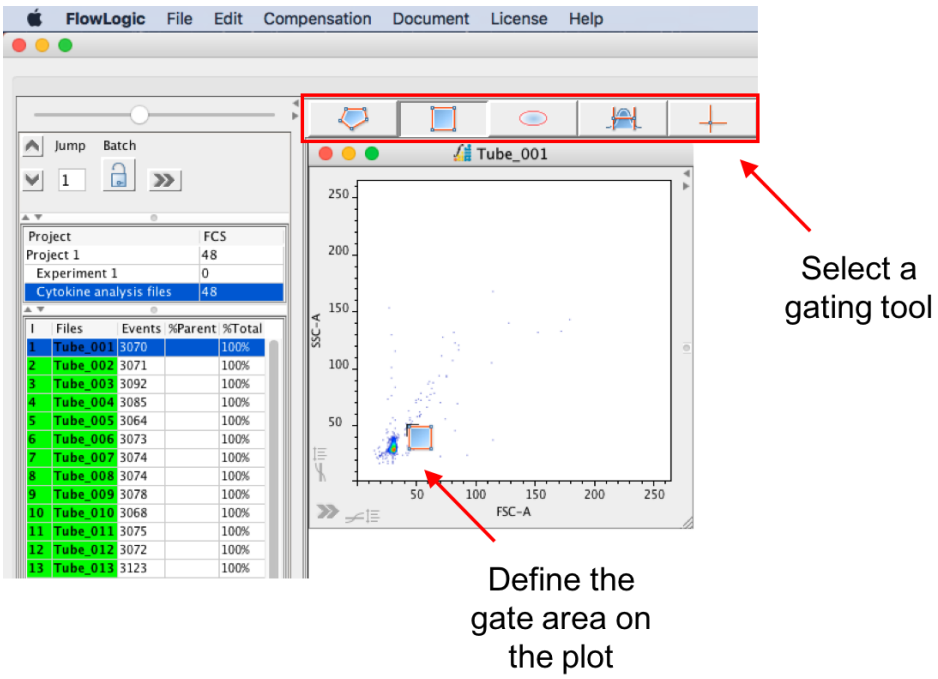
Creating groups can make analysis of multiple samples very quick and easy. If a gate is applied to one file in a group, it is automatically applied to the same parameters on all other files in the group.

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

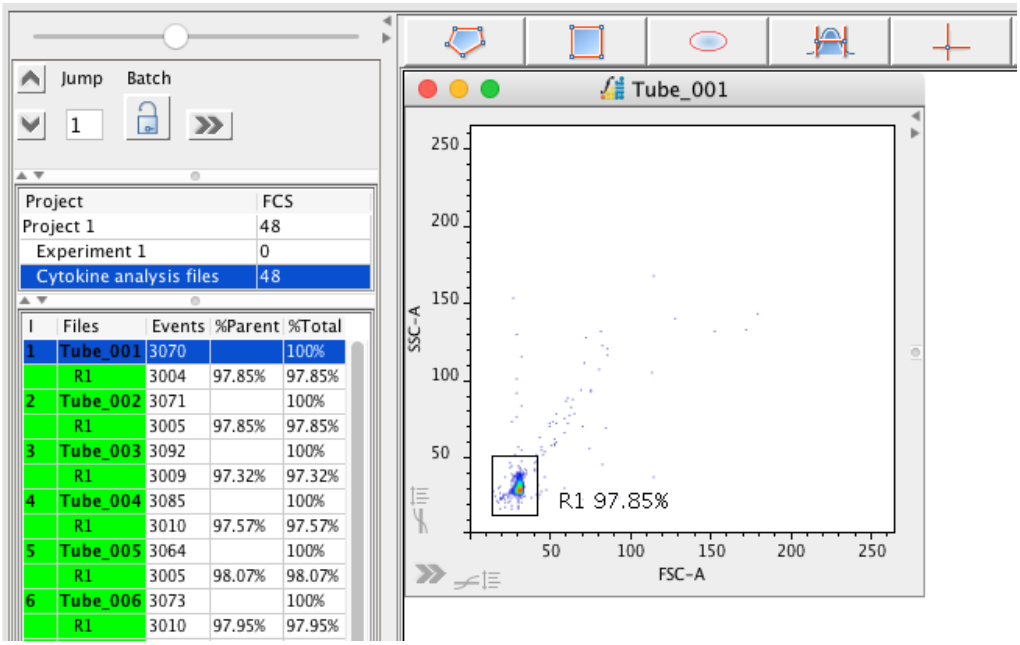




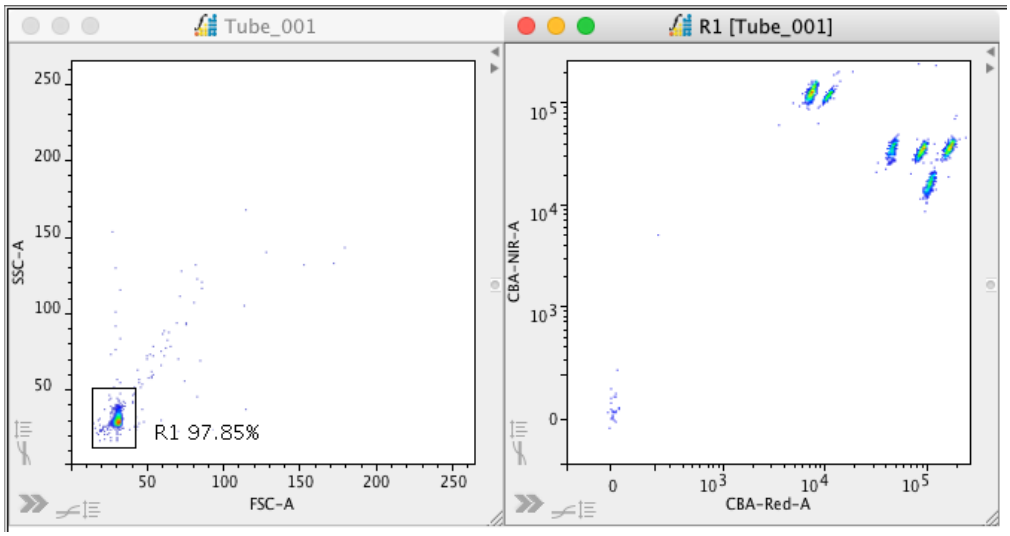
Double-click on a file in the File Inspector to open the plot in the Workspace. Click on the parameter labels on the plot to change them. Gate on populations by selecting the plot in the Workspace, clicking a gating tool from the Toolbar and then use the cursor in the plot to define the gate.



After a gate has been created, it will be listed beneath the sample in the File Inspector (in this case, R1). The gate name can be changed by right-clicking on the gate in the File Inspector. To open the subpopulation, double-click on the population in the File Inspector or double-click within the gate on the plot.

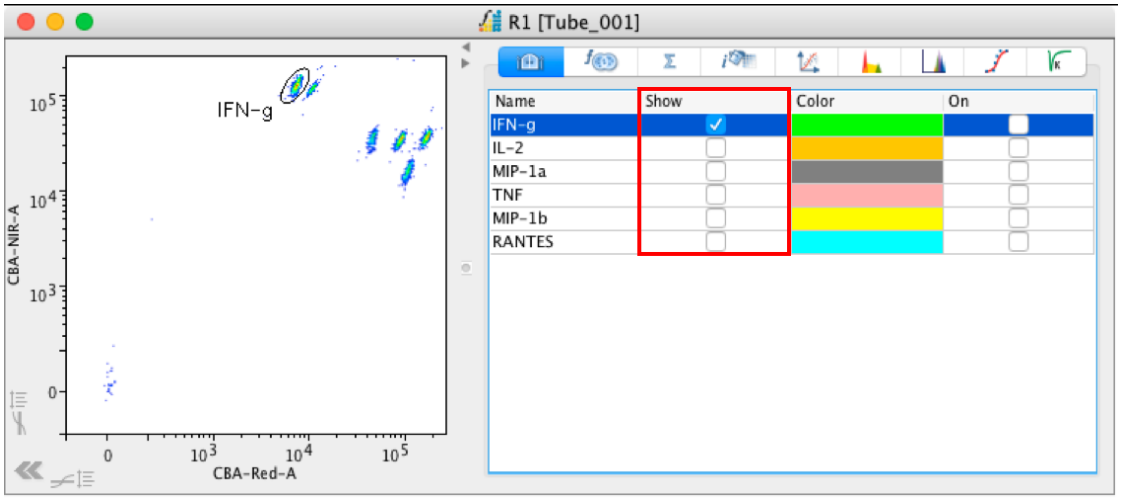


I	Files	Events	%Parent	%Total
1	Tube_001	3070		100%
	R1	3004	97.85%	97.85%
2	Tube_002	3071		100%
	R1	3005	97.85%	97.85%
3	Tube_003	3092		100%
	R1	3009	97.32%	97.32%
4	Tube_004	3085		100%
	R1	3010	97.57%	97.57%
5	Tube_005	3064		100%
	R1	3005	98.07%	98.07%
6	Tube_006	3073		100%
	R1	3010	97.95%	97.95%



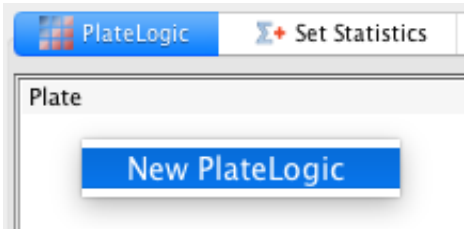
If the files have been grouped, as in the above Adjustments to individual gates are automatically updated to all within the group. If not, highlight the gates in the File Inspector, right-click and choose Copy→Copy Gates. Then, highlight the files to which the gates need to be pasted to, right-click and choose Paste→Gates.

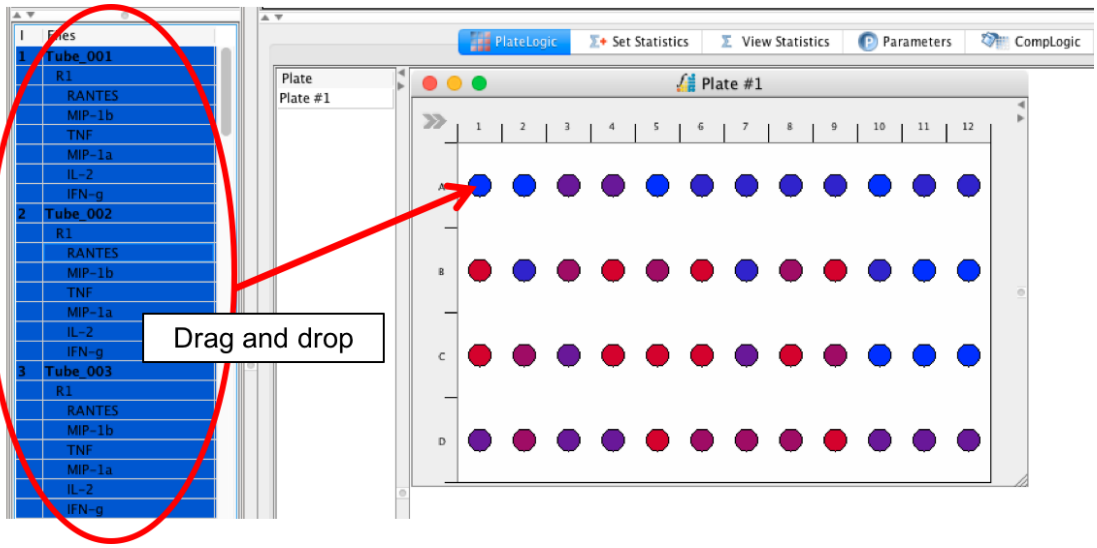
Continue gating on the specific bead populations that correspond to the different analytes. Naming the populations with the analyte name as the gates are created makes assessing the results in BeadLogic much easier. As the different populations can be relatively close together on the plot, a useful trick is to turn a gate off after it has been drawn. This is done by opening the plot side drawer by clicking on the button in the lower left of the plot window (or double-clicking within the plot but not within a gate) and selecting the first tab called the Gate List tab. Untick the box in the column titled ‘Show’ to turn the gate off. This does not delete the gate, it just hides it from view and makes drawing new gates easier. Once all gates have been created, tick all of the boxes in the ‘Show’ column to make them visible again.



Adding samples to a plate in PlateLogic

After gating and naming the bead populations, add all of the samples to a plate in PlateLogic. PlateLogic is located in the Advanced Functions drawer located below the Workspace.
To create a plate, click in the plate window and select ‘New PlateLogic’. To fill the plate, highlight a group of files in the File Inspector and drag them into the desired well in the plate. It does not matter which gate or level of the gating hierarchy is selected and dragged into the plate as only the sample is recorded. Once in the plate, the specific subpopulation (gate) can be set and analyzed.



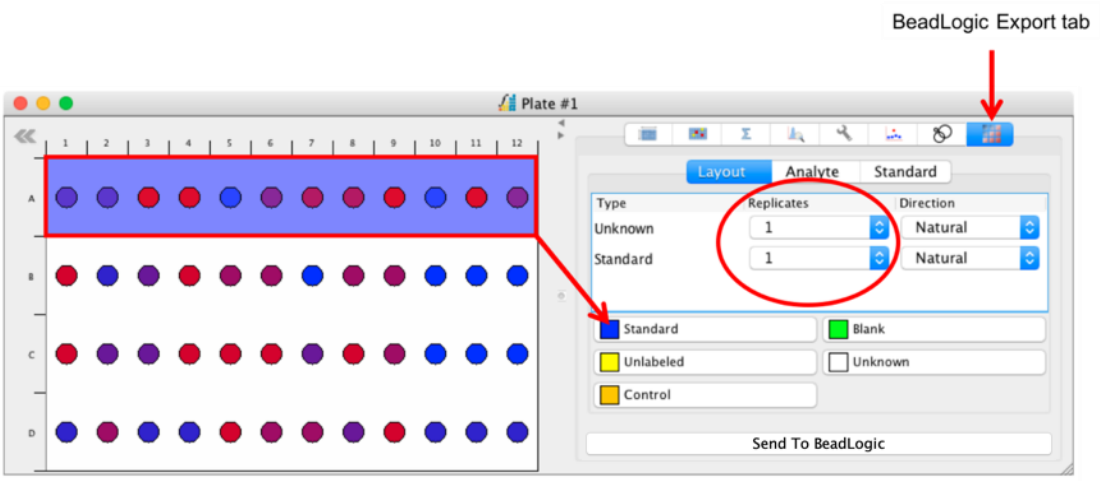


Once all files have been added to the plate, use the functions in the Plate Side Drawer to help analyze the samples. Specifically, the BeadLogic Export tab.

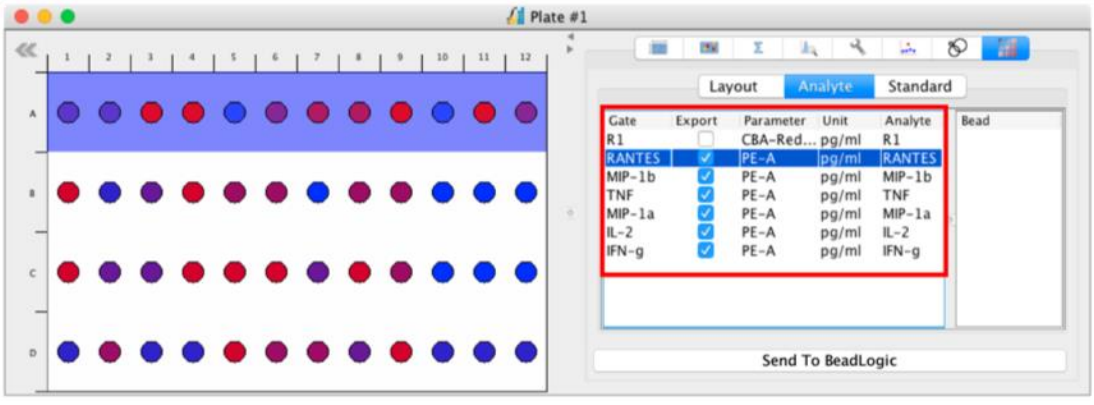
Plate setup

In the ‘Layout tab’, define each of the samples by highlighting them in the wells and clicking on one of the sample type buttons. This will color the background of the wells to reflect the different sample types, being Standards, Blanks, Unlabeled, Unknown or Controls.

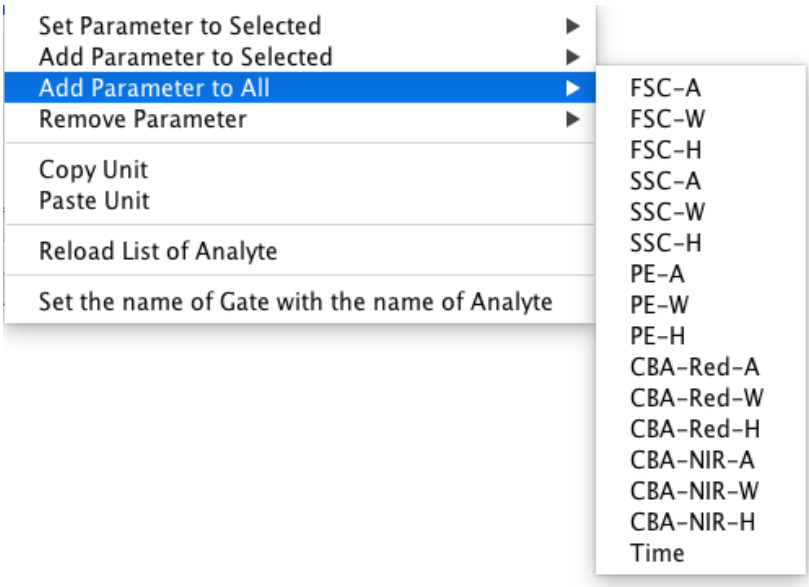
Also, define if technical replicates were used. To do this, choose a replicate number for the corresponding sample type. This will result in an average value for the replicates being used in BeadLogic.



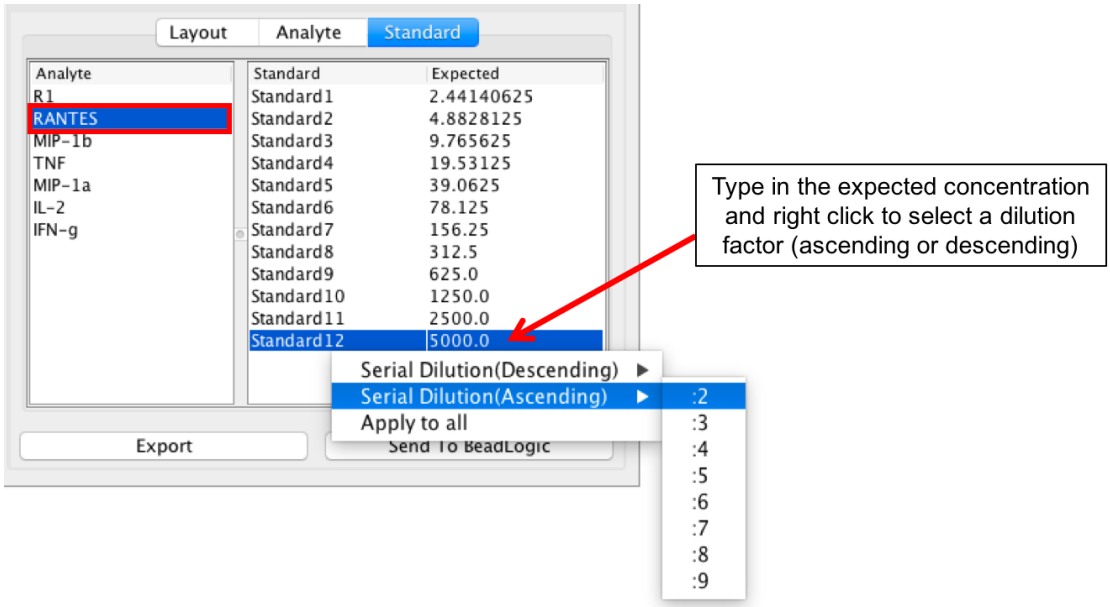
In the ‘Analyte tab’, choose the analytes to export, set the reporting parameter, define the units and name the analyte. These functions can be aided using the right-click menu.



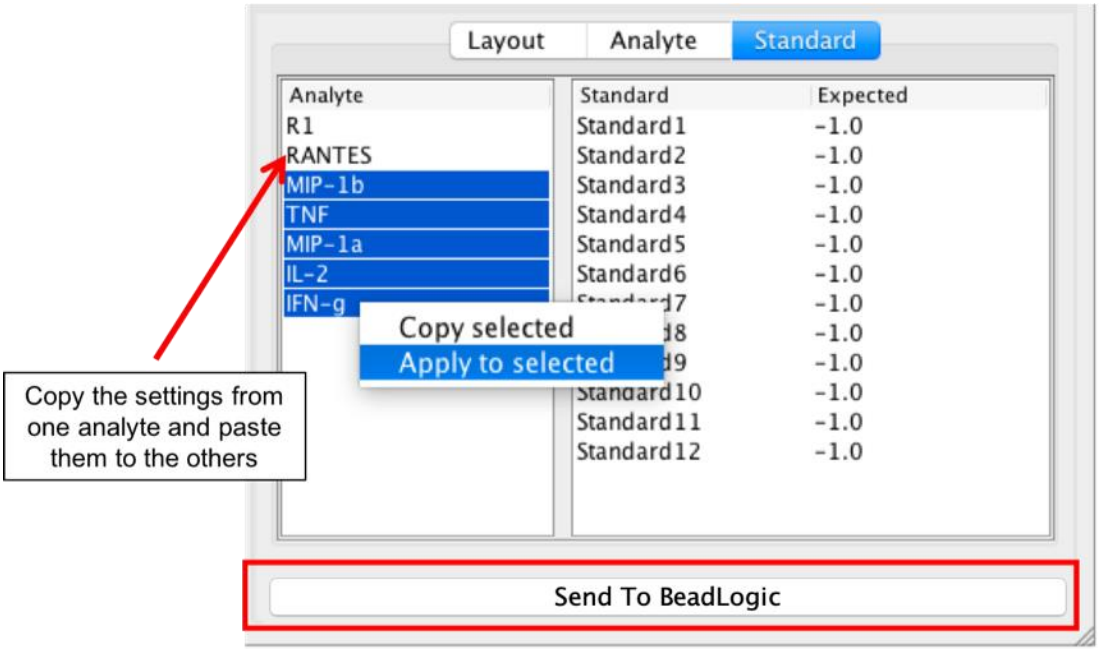
Right-click within the ‘Analyte’ window for the following menu options:



In the ‘Standard’ window, define the expected concentrations for the standards. Highlight one standard and type a value in the ‘Expected’ column and right-click to choose a serial dilution factor in an ascending or descending order, depending on which standard you begin with.



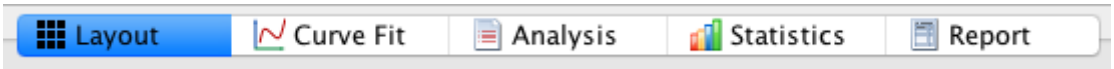
Once the expected values have been defined for one analyte, right-click and choose ‘Copy selected’ to copy the settings. Then, highlight other analytes, right-click and choose ‘Apply to selected’ to paste the same values to the highlighted analytes.



Once all settings have been made, click the ‘Send to BeadLogic’ button. Then navigate to BeadLogic to continue with the regression analysis.

BeadLogic - Layout

After importing new files or loading a saved experiment, BeadLogic will load with the following toolbar displayed at the top of the screen:

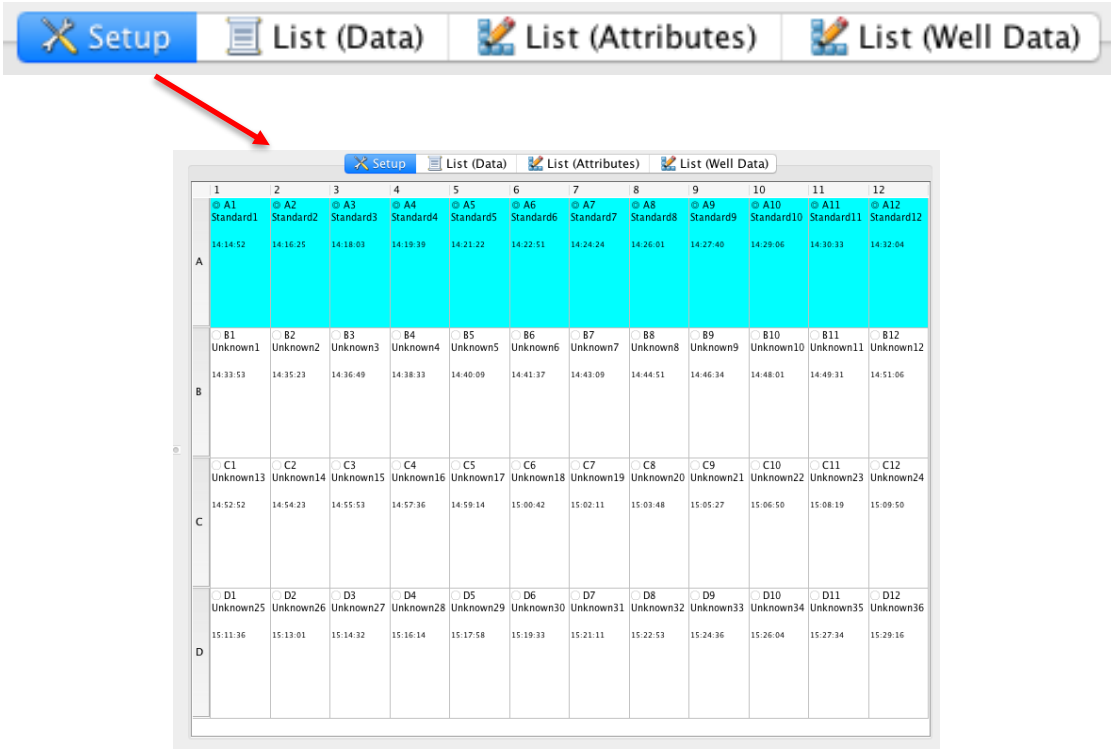


To follow a basic workflow, use the functions located under each of the tabs, working from left to right, starting with **Layout**.

When **Layout** is selected, a range of Plate Setup features is located on the left-hand side of the screen. Click on each of these to access options to select between plates, define the sample configuration (plate layout), define replicates, assign attributes to samples, define dilution factors, display the set of analytes tested and to view sample metadata.

In most cases, selecting one or more samples from the diagram to the right of the Plate Setup section will enable the various plate setup options to be accessed and changed.

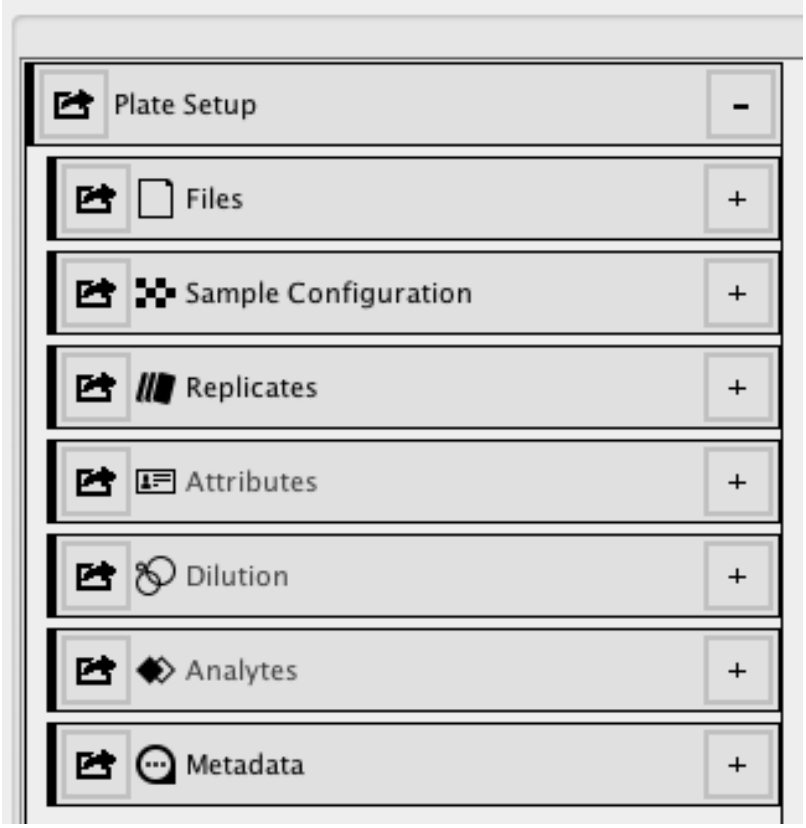
To view the Plate Layout, select **Setup** from the sub toolbar under the **Layout** tab.



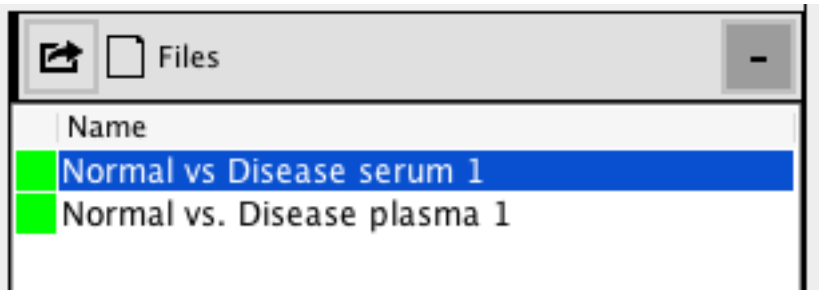
From the plate **Setup** tab, you can highlight wells and apply the settings from the menus from the left-hand side.

Right-click in the plate layout and choose ‘Discontinuous Selection’ to make a selection of wells from across the plate (not just connected horizontally or vertically.

Plate Setup Features



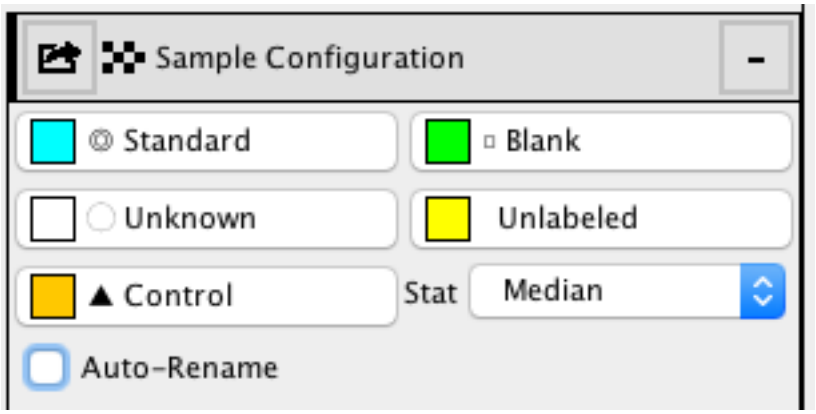
Files



Listed in the **Files** window is a list of all 'plate' files, which have been imported into BeadLogic. A green square beside the plate name indicates that appropriate standards have been defined in the plate layout. A red square indicates that appropriate standards have not been defined in the plate layout.

When a plate is highlighted in this panel, it is considered to be 'in focus' and the rest of the system will display its related properties and settings.

Sample Configuration



This panel allows for wells to be defined as a standard, blank, unknown, unlabeled or a control. One or more wells have to be highlighted in the plate layout for the options in this panel to use.

Standard: a well that is used for the calculation of the standard curve.
Unknown: a well with an 'Unknown' value (an experimental sample being analyzed).

Control: a 'Control' well, such as a high control or low control. These have a separate display on standard curves and output tables.

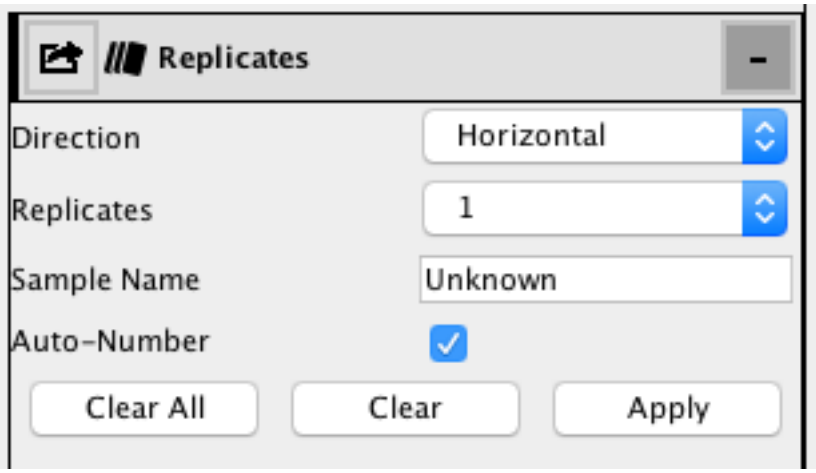
Blank: a well that is used to record background or non-specific readings. The values from 'blank' wells are subtracted from all fluorescence intensity (FI) readings.

Unlabeled: a well that is ignored from all output and reporting.

Stat: select the desired statistic for use in the selected plate. Some file formats do not provide any options while others may give a large number of possible statistics to choose from.

Auto-Rename: this will allow you to automatically apply replicate settings when assigning well types.

Replicates



Direction: allows for the 'direction' of replicates to be set. The options are:

- Horizontal - the next replicate in the series will be to the right of the previous one. Once the series of replicates is filled, the next series will start to the right of the previous series. When the end of the selection is reached horizontally, the allocation will continue from the next row.
- Vertical - the next replicate in the series will be below the previous. Once the series of replicates is filled, the next series will start below the previous series. Once the end of selection is reached vertically, the allocation will continue from the next column.
- Natural - the next replicate in the series will be to the right of the previous one (horizontal allocation). Once the series of replicates is filled, the next series of will start below the previous one until the end of the selection is reached vertically. The setting will then continue from the next column and allocate the wells horizontally.

Replicates: this drop-down menu allows you to set the number of replicates in a given series.

Sample Name: this text field allows you to set the 'sample name prefix' for wells prior to replication. A number is appended to the name to signal, which wells belong to which series.

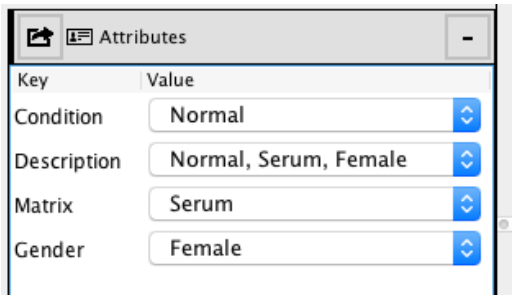
Auto-Number – if the name and replicate number is defined for a selection of wells, the auto-number feature will append each name with a number in ascending order.

Clear All: resets all wells in the current plate, renaming them with their well ID.

Clear: resets all selected wells, renaming them with their well ID.

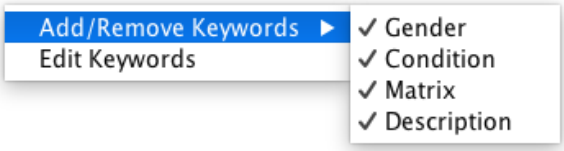
Apply: applies the settings to the selected wells.

Attributes

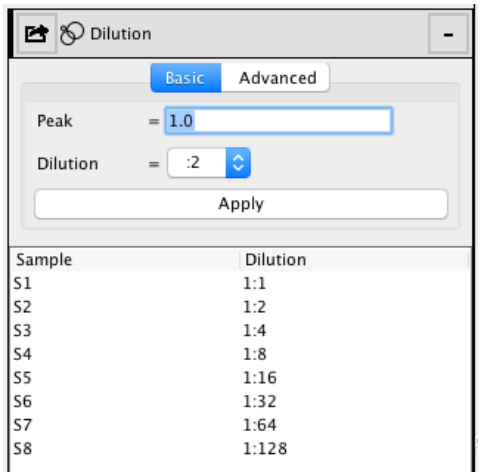


This window provides a list of ‘attributes’ or keywords for highlighted wells. A drop-down box populated with ‘*’ indicates that the selected wells have different values for the given attributes.

Right-click within this window to access options to add or remove keywords from the current plate as well as edit the global attributes list.



Dilution



This panel can be used to apply dilution factors to wells. If dilution factors have been applied, BeadLogic can signal if there is a difference in dilution between different wells. Dilution factors are also used in conjunction with the Curve Fit section (accessed in the main toolbar) to define serial dilutions of standards without having to define expected values manually.

Dilutions can be defined using the features under either the **Basic** or **Advanced** tabs.

Basic:

- Peak: the ‘Initial’ or ‘Neat’ concentration.
- Dilution: the serial dilution ratio of subsequent wells.

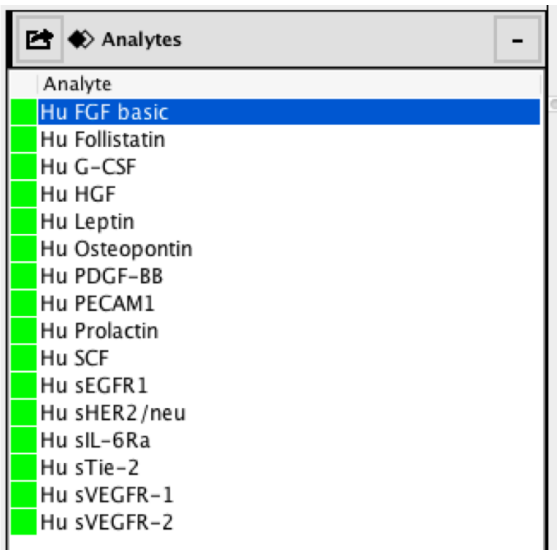
Advanced:

- y[0]: the ‘Initial’ or ‘Neat’ concentration.
- y[n+1]: an area where an equation can be entered for the calculation of the next value in the dilution series.

Apply: when clicked, the Basic or Advanced settings will auto-populate the selected wells with the appropriate dilutions.

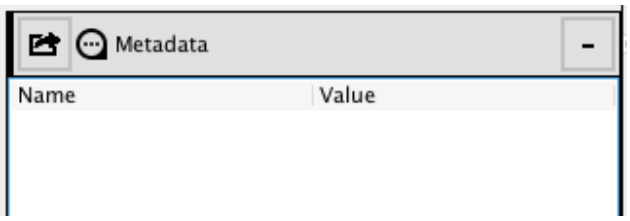
Dilution Table: a table where dilutions can be both viewed and manually entered on a sample-by-sample basis.

Analytes



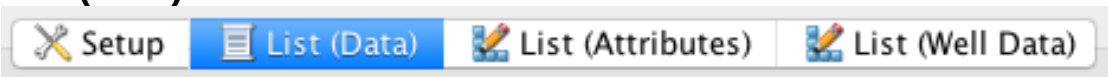
The analytes being analysed are displayed in this window. A green square next to the analytes indicates that all of the standards for that analytes fit within a defined acceptable error range. A red square indicates that at least one standard falls outside of the defined acceptable error range. To automatically fit the curves to the data, right-click on an analyte and select Auto-Fit.

Metadata



The metadata window displays all of metadata associated with an imported file. Metadata can be exported by right-clicking in the metadata window and choosing **Export Table.** Choose to export the metadata as either a CSV or an Excel XML Workbooks file. Note: some file formats do not provide metadata.

List (Data)



The **List (Data)** tab provides a table display of the current data. To export the data, right-click within the table and select **Export Table**.

Above the table are a number of table format settings. These are:

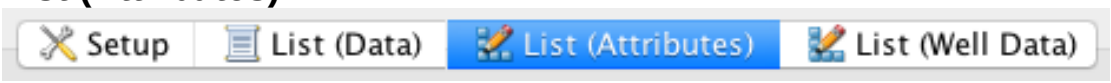
- Table Value: select the type of value to be displayed in the table for the current plate
    - FI (Fluorescence Intensity): when selected the statistic for the table is the raw fluorescence intensity.
    - Observed: this is the observed (regressed) value without any markings whether the value is OOR or not
    - Observed (limit): this is the observed value, although if a value is considered to be OOR, it will be limited to the relevant upper or lower limit of quantization.
    - Observed (removed): this is the observed value but when it is considered OOR, it will be marked OOR> or OOR< appropriately.
    - Bead count: this is the recorded bead count for the sample. Note: some file formats do not provide bead count data.

- Table Statistic: the aggregate statistic for the replicates used for the table.
    - Mean: the mean of all the selected ‘Table Values’ for the appropriate sample.
    - CV%: the Coefficient of Variation of the selected ‘Table Values’ for each sample.
    - SD: the Standard Deviation of the selected ‘Table Values’ for each sample.
    - Min: the minimum value of the selected ‘Table Values’ for each sample.
    - Max: the maximum value of the selected ‘Table Values’ for each sample.
    - Sum: the sum of the ‘Table Values’ for each selected sample.

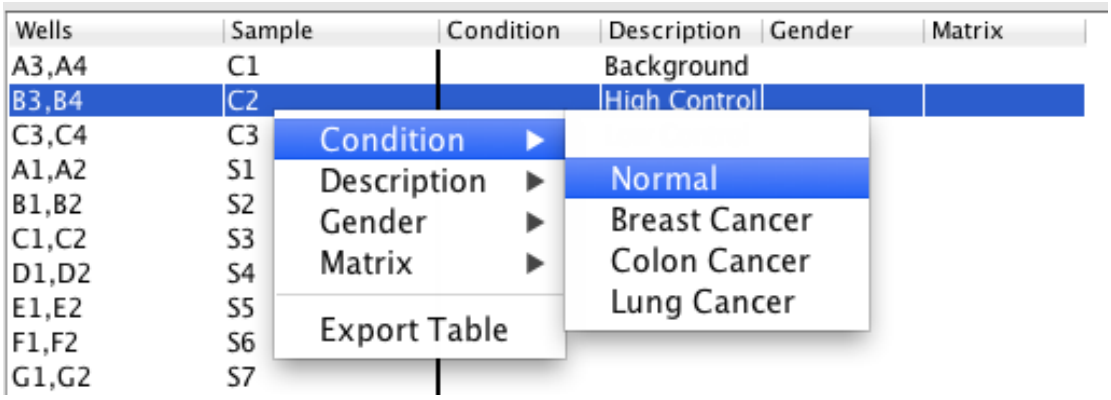
- Highlight OOR: if enabled then the ‘Out of Range’ samples will be highlighted in red.

- Background Color: provides the rules for the background color of the given wells.
    - No Background: If selected then there is no background color coloring
    - CV FI: if selected then the cell background will be colored yellow when the CV of the fluorescence intensity is outside the threshold
    - CV Obs: if selected then the cell background is colored yellow when the CV of the observed values of the given cells is outside of the threshold
- CV% Thresh: The threshold that is applied to the background color settings.

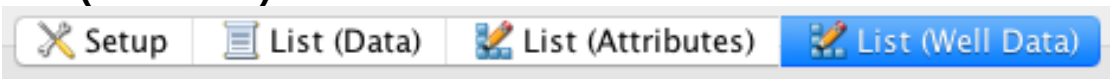
List (Attributes)



The **List (Attributes)** tab provides a list of all samples and the Attributes/Keywords that have been applied to them. Right-click to export the entire table or to apply keywords to selected samples.

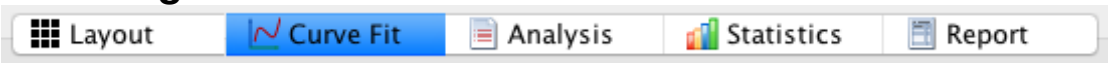


List (Well Data)



The **List (Well Data)** provides a quick view of all the raw data in the table not separated by sample.

BeadLogic - Curve Fit

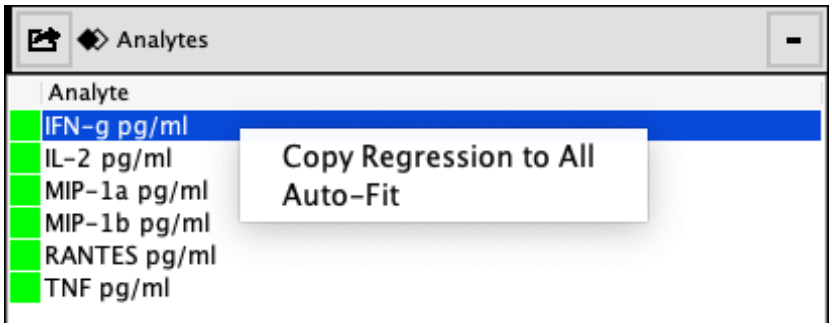


In the Curve Fit tab, curve-fitting functions can be performed manually or automatically. Manual adjustments (enabling and disabling standards) are performed individually, on a per analyte basis.

Within the **Curve Fit** tab there are five windows. On the left-hand side are the Files and Analytes windows and in the workspace on the right-hand side are the Regression Curve, Regression Tornado and Regression Table windows. Click on the window headings to expand and collapse them.

Files
See Layout → Files for a more detailed explanation.

Analytes



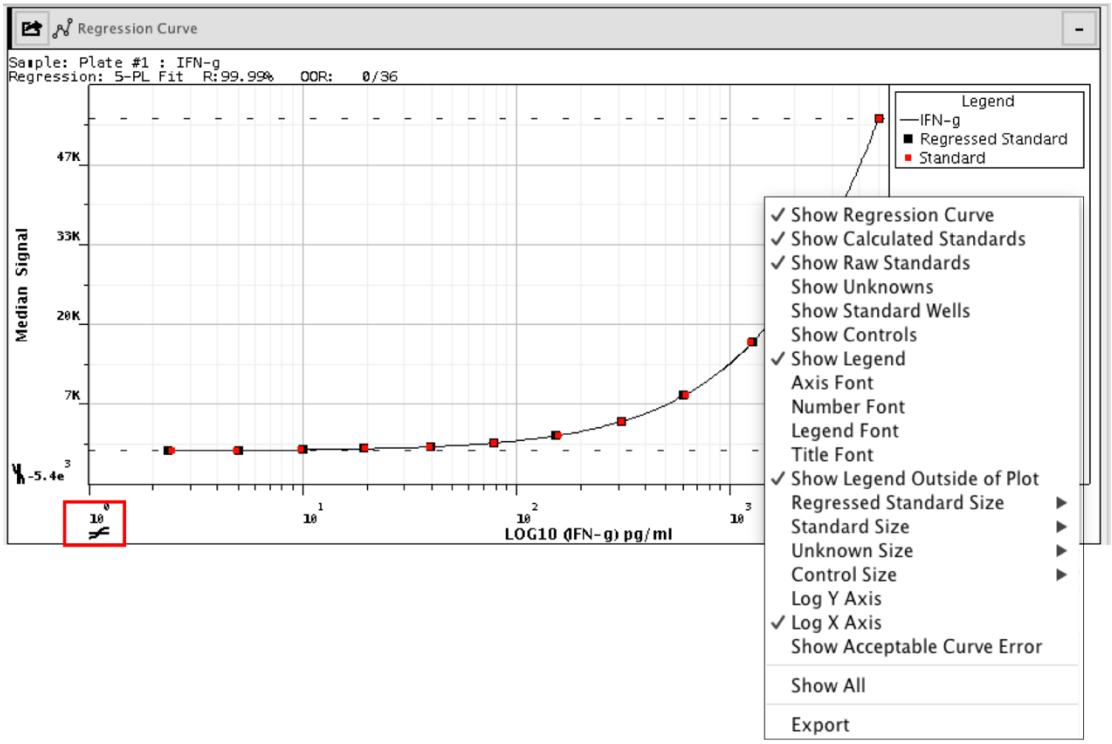
The analytes in the current plate are displayed in this window. When an analyte is selected within this window the output in the Regression Curve, Regression Tornado and Regression Table windows will update to display the result for the specific analyte.

A red square next to the analyte name signals that it has at least one standard with an error outside of the acceptable recovery range. If a given plate provides 'bead Id' information, this can be used to apply a compatible kit info to the plate, which will pre-fill expected concentrations of standards.

The right-click menu is as follows:

- Copy Regression to All: copy the Acceptable Recovery Range and regression settings and apply it to all analytes in the plate.
- Auto-Fit: runs the 'Auto-Fit' algorithm for all analytes in the plate. The results can then be checked visually. This algorithm progressively disables standards at the upper and lower limits of the range until all standards sit within the acceptable recovery range.

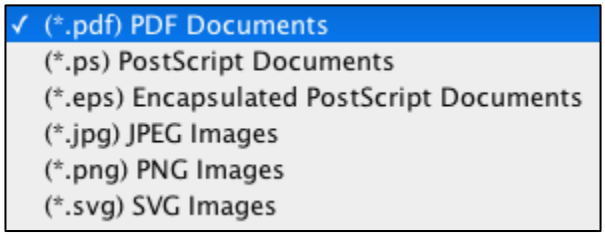
Regression Curve



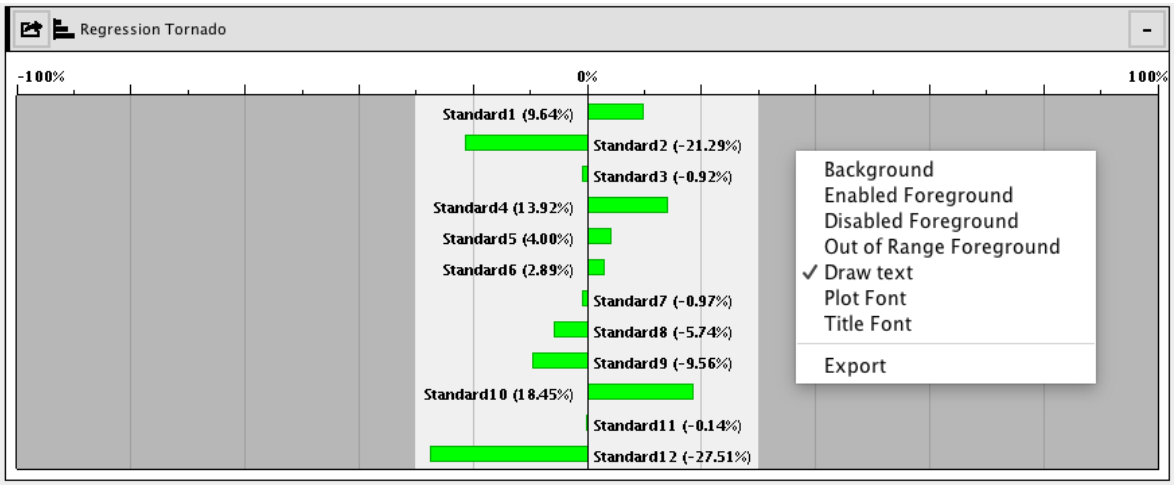
The Regression Curve window displays the standard regression curve. There is one 'Log → Lin' button on each axis to allow for the switching of axis transformations. This display is configurable from the right-click menu:

- Show regression Curve: enables/disables the regression curve from the display.
- Show Calculated Standards: enables/disables the 'calculated' standard values.
- Show Raw Standards: enables/disables the 'Raw' standard values (non-regressed).
- Show Unknowns: enables/disables display of unknowns on the curve
- Show Standard Wells: enables/disables the actual 'wells' that make up each standard sample. All wells are included in the calculation for a quick visual display of CV within a standard sample.
- Show Controls: enables/disables the display of controls from the standard curve.
- Show Legend: enables/disables the display of the Legend on the regression curve display.
- Axis Font: allows the axis label font to be changed.
- Number Font: allows the numeric axis label font to be changed.
- Legend Font: allows legend font to be changed.
- Title Font: allows regression plot title font to be changed.
- Show Legend Outside of Plot: allows the location of the legend to be changed:
    - outer right-hand side of the plot area
    - top left-hand side inside the plot

- Regressed Standard Size: allows the size of the 'dots' for the regressed standards to be changed.
- Standard Size: allows the size of the 'dots' for the raw standards to be changed.
- Unknown Size: allows the size of the 'dots' for the unknowns to be changed.
- Control Size: allows the size of the 'dots' for the controls to be changed.
- Show All: displays all plot items for the standard curve.
- Export: export the standard curve in one of a number of external file formats:



Regression Tornado

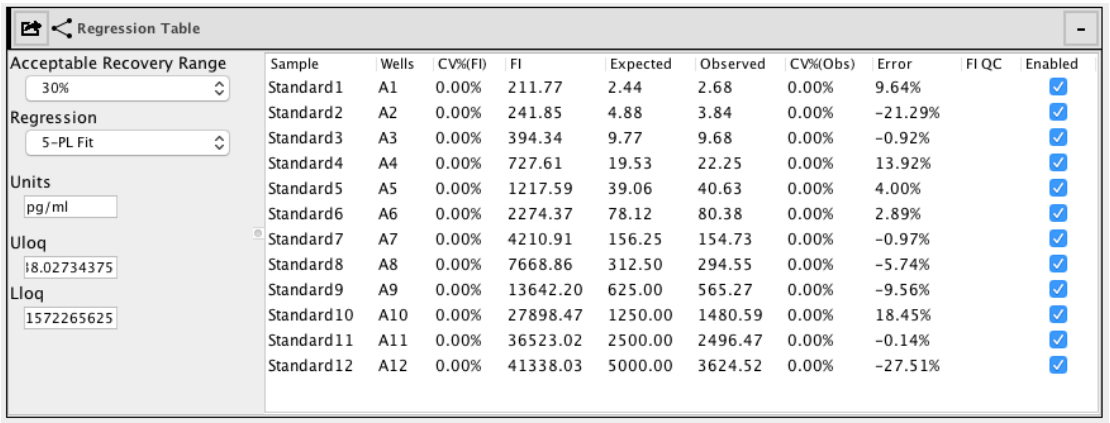


The Regression Tornado window provides a simple display to view the difference between expected and actual values for the standards.

The plot is configurable via the right-click menu:

- Background: change the background color for the plot.
- Enabled Foreground: set the foreground color for a standard that is both enabled and within the acceptable recovery range.
- Disabled Foreground: change the foreground color for a standard that has been disabled.
- Out of Range foreground: set the foreground color for a standard that is both enabled and out of range.
- Draw Text: if enabled, text appears on the inside of the tornado plot.
- Plot Font: change the font inside the plot.
- Title Font: change the font for the title (axis).

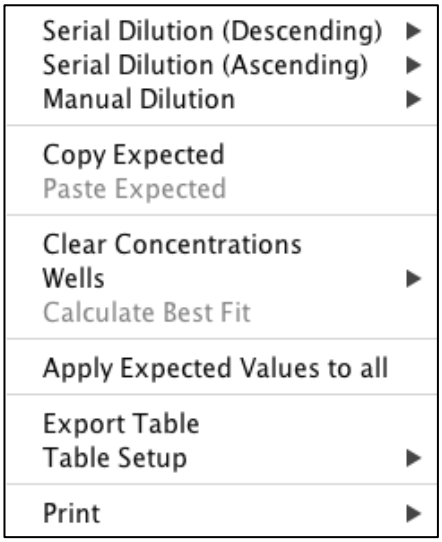
Regression Table



Sample	Wells	CV%(FI)	FI	Expected	Observed	CV%(Obs)	Error	FI QC	Enabled
Standard1	A1	0.00%	211.77	2.44	2.68	0.00%	9.64%		☑
Standard2	A2	0.00%	241.85	4.88	3.84	0.00%	-21.29%		☑
Standard3	A3	0.00%	394.34	9.77	9.68	0.00%	-0.92%		☑
Standard4	A4	0.00%	727.61	19.53	22.25	0.00%	13.92%		☑
Standard5	A5	0.00%	1217.59	39.06	40.63	0.00%	4.00%		☑
Standard6	A6	0.00%	2274.37	78.12	80.38	0.00%	2.89%		☑
Standard7	A7	0.00%	4210.91	156.25	154.73	0.00%	-0.97%		☑
Standard8	A8	0.00%	7668.86	312.50	294.55	0.00%	-5.74%		☑
Standard9	A9	0.00%	13642.20	625.00	565.27	0.00%	-9.56%		☑
Standard10	A10	0.00%	27898.47	1250.00	1480.59	0.00%	18.45%		☑
Standard11	A11	0.00%	36523.02	2500.00	2496.47	0.00%	-0.14%		☑
Standard12	A12	0.00%	41338.03	5000.00	3624.52	0.00%	-27.51%		☑

Within the Regression Table, standards can be enabled and disabled. The manual entry of expected concentrations can also be performed in this window.

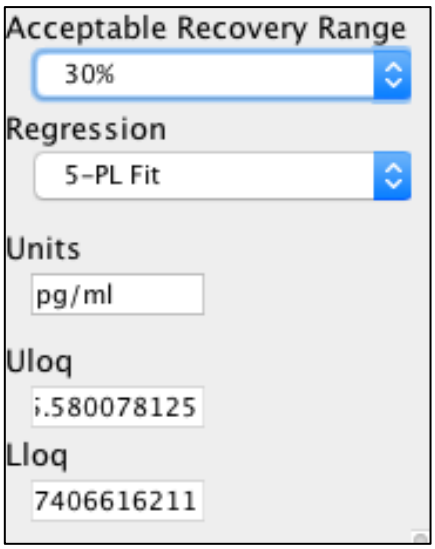
Right-click to display options to configure the table:



- Serial Dilution (Descending and Ascending): allows for the setting of expected concentrations via a serial dilution (starting at the value of Standard 1).
- Manual Dilution: set the defined dilution factor using the format: *x (where x is the dilution factor)
- Copy Expected: once the dilution factor has been defined, the settings determining the Expected Values can be copied from one analyte and pasted into the Regression Table of other analytes.
- Paste Expected: this option becomes available after copying the dilution settings from one analyte. This option allows the settings to be pasted into the Regression Table of other analytes.
- Clear Concentrations: clears all concentration settings in the Expected column.

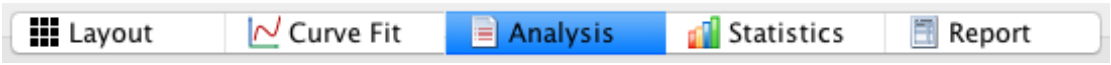
- Wells: displays the well ID and FI for an individual sample.
- Calculate best fit: performs the best fit algorithm for the standards.
- Apply Expected Values to All: this applies the Expected values from one analyte to all others in the analysis.
- Export Table: export the table as either a CSV file or an Excel XML Workbooks file.
- Table Setup: define the number of decimal places and the text font used in the table.
- Print: allows for the printing of the Regression Table.

Within the Regression Table panel are settings to define the Acceptable Recovery Range, Regression type and the concentration units. These are as follow:



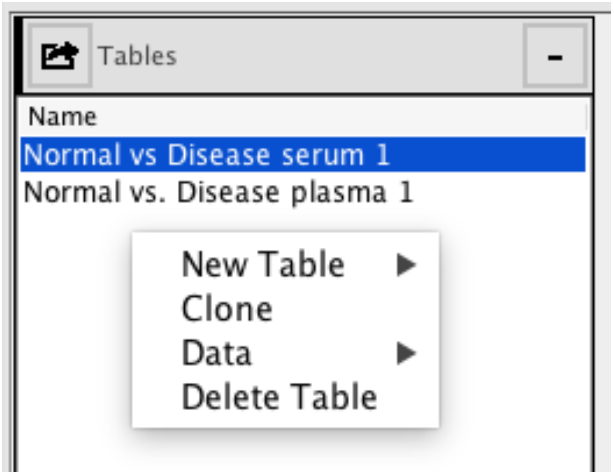
- Acceptable Recovery Range: the percentage for which a given standard's expected value can fall in based on the regressed value.
- Regression: use to set the current regression type. The options are:
    - Linear
    - Linear (Point to Point)
    - 4-PL Fit
    - 5-PL Fit
    - Cubic Polynomial
- Units: the units to be displayed in reports and tables for a given analyte
- Uloq and Lloq: the upper and lower limits of quantification. These values represent the highest and lowest fluorescence intensity values from the standards.

BeadLogic - Analysis



The core of the analysis area is data mining and preparation for statistical analysis and reporting.

Tables



This is the primary list of tables, which are used for data mining and analysis. When a plate is imported a table is automatically created but a table should not be mistaken for a plate. Tables can contain the data from many plates. Rename a table by double-clicking on its name and typing the new name.

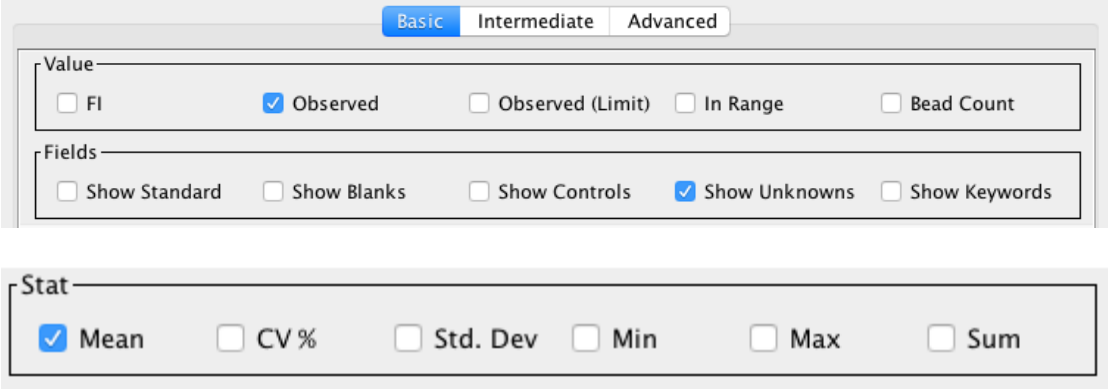
A right-click within the table window brings up a menu with the following features:

- New Table: creates a new table from a plate containing data.
- Clone: creates an identical copy of the highlighted table.
- Data: allows data from individual plates to be enabled/disabled.
- Delete Table: deletes the highlighted table.

Table Setup

Three levels of control are available for data mining: Basic, Intermediate and Advanced.

Basic



This is the simplest form of table generation. It does not provide any mining or aggregation tools, although it does make it simple to quickly produce a table of results. There is a number of buttons that enable/disable columns and rows in the data table. These are located in different subsections.

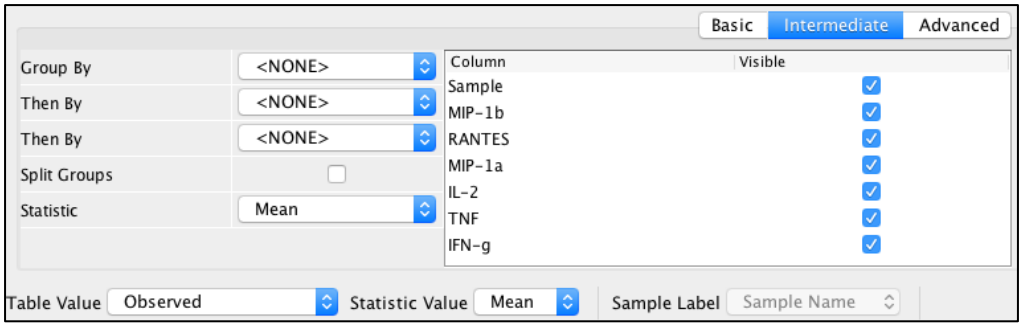
- Value
  - FL: displays the Fluorescence Intensity columns in the table for each analyte.
  - Observed: displays the Observed value columns for each analyte.
  - Observed (Limit): displays the Observed (Limit) columns for each analyte.
  - In Range: displays the In Range columns for each analyte.
  - Bead Count: displays the Bead Count columns for each analyte.

- Fields
  - Show Standard: displays all rows that where wells have been marked as standards.
  - Show Blanks: displays all rows where wells have been marked as blanks.
  - Show Controls: displays all the rows where samples have been marked as controls.
  - Show Unknowns: displays all rows where wells have been marked as unknowns.
  - Show Keywords: displays all the ‘Text’ columns for a given table.
- Stat
  - Mean: displays the mean of the replicates for each displayed column.
  - CV %: displays the coefficient of variation between replicates for each displayed column.
  - Std. Dev.: displays the standard deviation between replicates for each displayed column.
  - Min: displays the minimum value of the replicates for each sample for the displayed columns.
  - Max: displays the maximum value of the replicates for each sample for the displayed columns.

- Sum: displays the sum of replicate values for each given sample for the displayed columns.

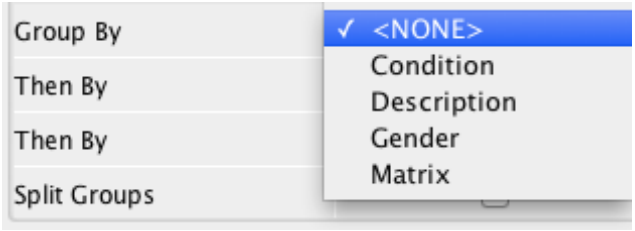
- Sample Label: choose between displaying the sample name or other sample descriptions as defined in the Attributes panel of the Layout tab, through the right-click menu and the Add/Remove Keywords option.

Intermediate

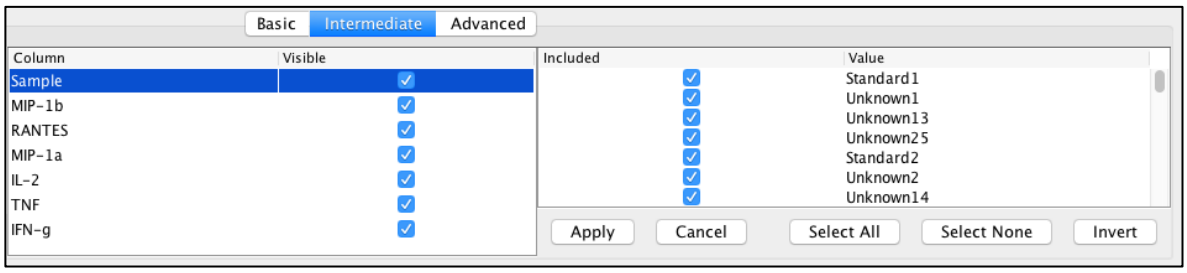


The intermediate table setup allows for both data aggregation as well as data filtering and grouping. The features contained within the Intermediate window include:

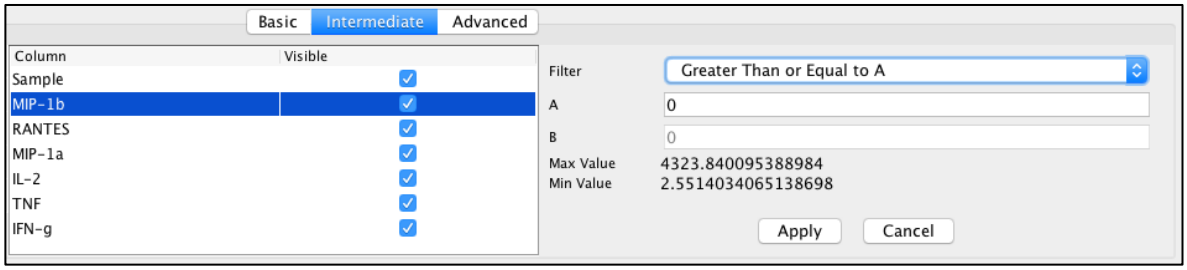
- Group By: creates an initial level of grouping. Once a column is selected as available for grouping, all of the rows will be 'merged' with the selected aggregation statistic.



- Then By: provides the second and third levels of grouping.
- Split Groups: when enabled, the sorting is kept but the groups are split back into their samples. This can be useful for a quick view of the source data without having to modify grouping settings.
- Statistic: allows for the selection of the aggregation statistic for grouped data.
- Table Value: allows for the selection of the source statistic for each sample.
- Statistic Value: allows for the selection of the statistic calculation used to generate a single sample value.
- Sample Label: allows the Value in the 'sample' column to be changed.
- Visible (column): allows data columns to be made visible/invisible.
- Sample Filter: Click on 'Sample' under 'Column' in the table to view the entire sample list to the right. Make a selection using the check boxes in the 'Included' column, or by using the buttons below the panel, and click apply. The resulting sample list and statistics will be displayed in the Data Table below the Table Setup window.

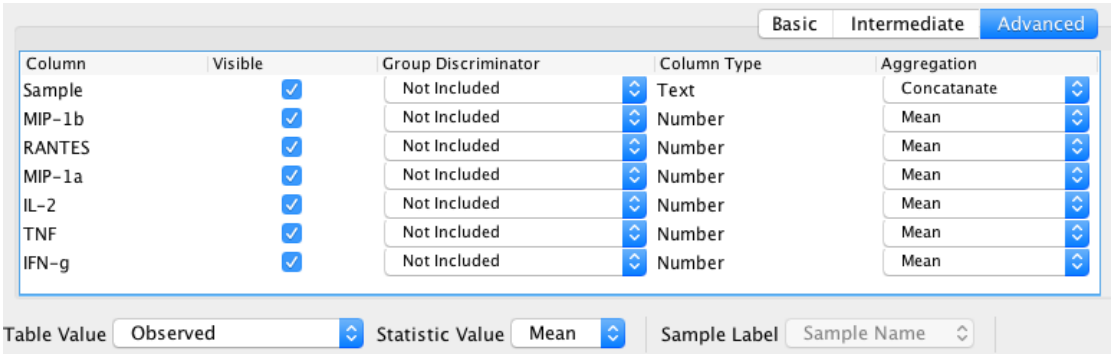


- Numeric Filter: use the numeric filter to remove rows that do not meet certain criteria. The numeric filter will appear when a row is selected in the table.
-



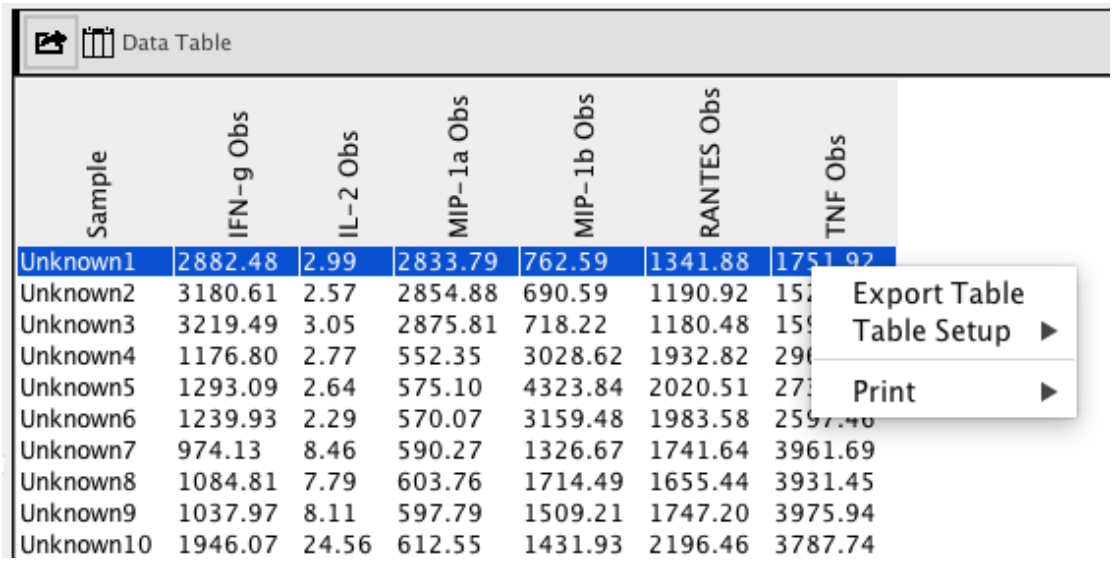
- List table: once the setup is complete, click the ‘Apply’ button to apply the filter. There are a number of filter options available.
    - None: no filtering is performed.
    - Equals A: when a value from the given column matches the value entered in the text box for ‘A’ it will be included in the output.
    - Not Equals A: when a value from the given column does not match the value entered in the text box for ‘A’ it will be included in the output.
    - Less than A: when the value for the given column is less than A it will be included in the output.
    - Greater than A: when the value for the given column is greater than A it will be included in the output.
    - Less than or Equal to A: when the value for the given column is less than or equal to A it will be included in the output.
    - Greater than or equal to A: when the value for the given column is greater than or equal to A it will be included in the output.

Advanced



The advanced table setup is similar to the intermediate table with the exception of having a full grouping window where the grouping level of any given analyte can be set from the main editor.

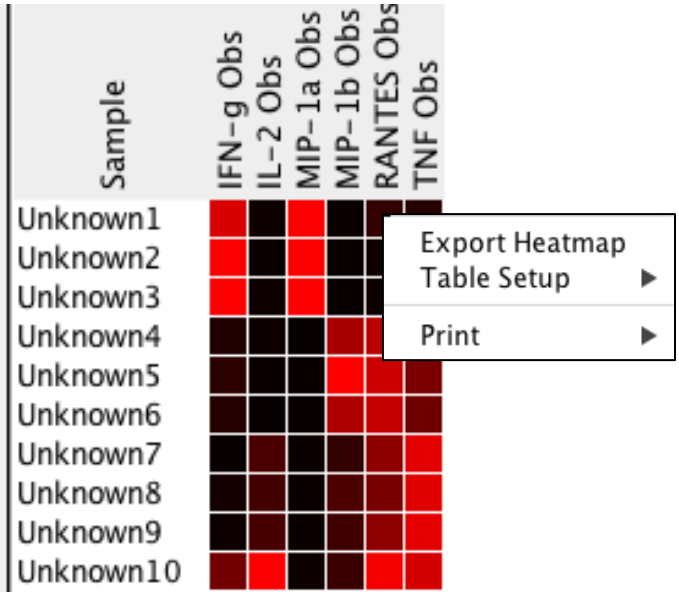
Data Table



Right-click in the table and select from the options to Export the data, define the number of decimal places, change the font and print.

By clicking on the table column headers, you can sort the rows by a given column's values in either ascending or descending order.

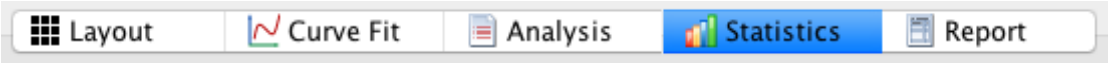
Quick Heatmap



The quick heatmap has all of the basic functionality of the 'Data Table'. Each column's values are separate in regard to the heatmap calculation and therefore the color, which is defined as the 'maximum' for that particular statistic. The heatmap is updated as the selections made in the Table Setup tab are changed.

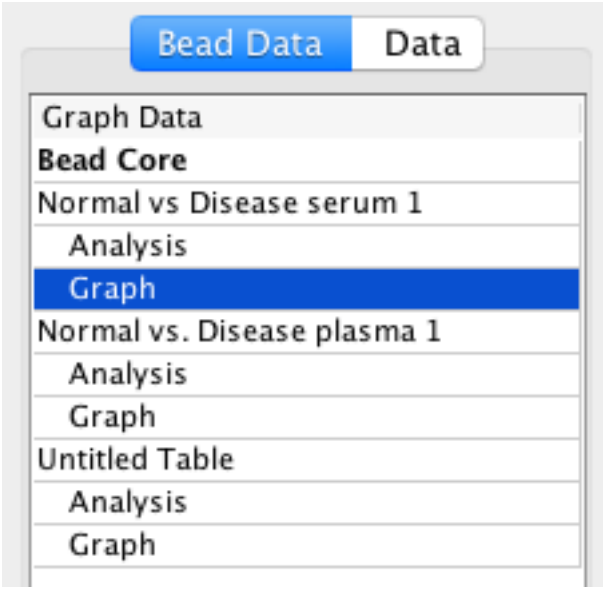
Color Scheme: this drop-down box different color options for the heatmap. The different color options are:

BeadLogic - Statistics

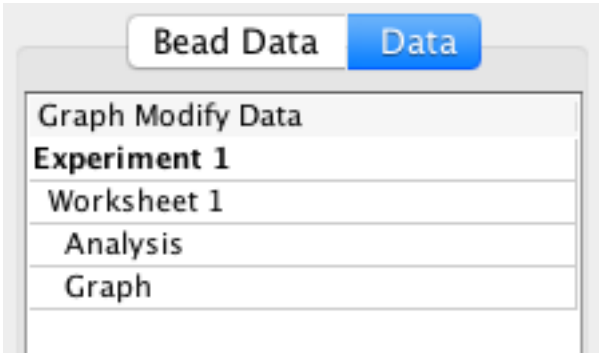


Within the Statistics tab, values displayed in the Data Table of the Analysis tab can be graphed and analyzed.

Bead Data: located to the left of the worksheets, the Bead Data window contains a list of all the 'tables' that have been built in the Analysis section. A default graph and analysis are created for each table. Double-click the graph name to open it in the workspace above the worksheet. Right-click on the graph name for options to rename it (along with adding a new experiment, opening a graph in the graph workspace and deleted the highlighted row).



Data: the data section allows for mathematical manipulation and concatenation of data tables.

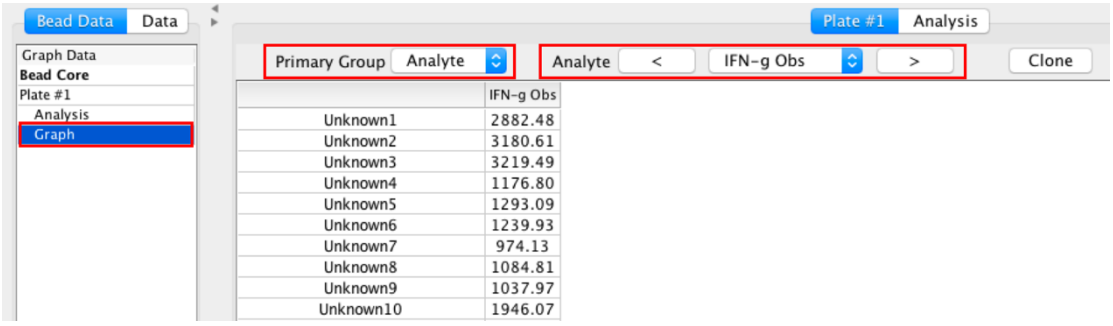


Graphing data

Display all of the relevant data in the Data Table of the Analysis tab and choose the specific samples that you wish to be included in a graph. Note that the different values and specific analytes can be chosen in the Statistics tab but the selections of samples cannot.

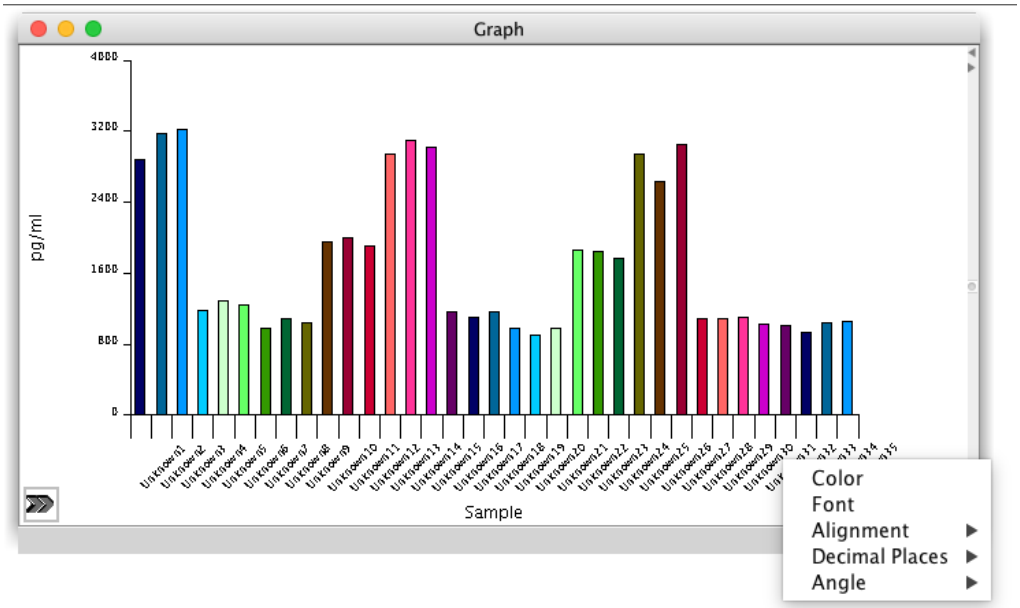
Once all desired data and the correct samples selection has been performed, return to the Statistics section. Choose the specific plate in the Bead Data window and the same data displayed in the Data Table will be displayed in the data window to the right.

Select populations from the drop-down menus for the Primary Group and the Analyte. The table will automatically update to show the defined data. To open a graph for the defined data, double-click or right-click → Open Graph the 'Graph' tab in the Bead Data tab. The graph will be displayed in the graph workspace above the data tables.

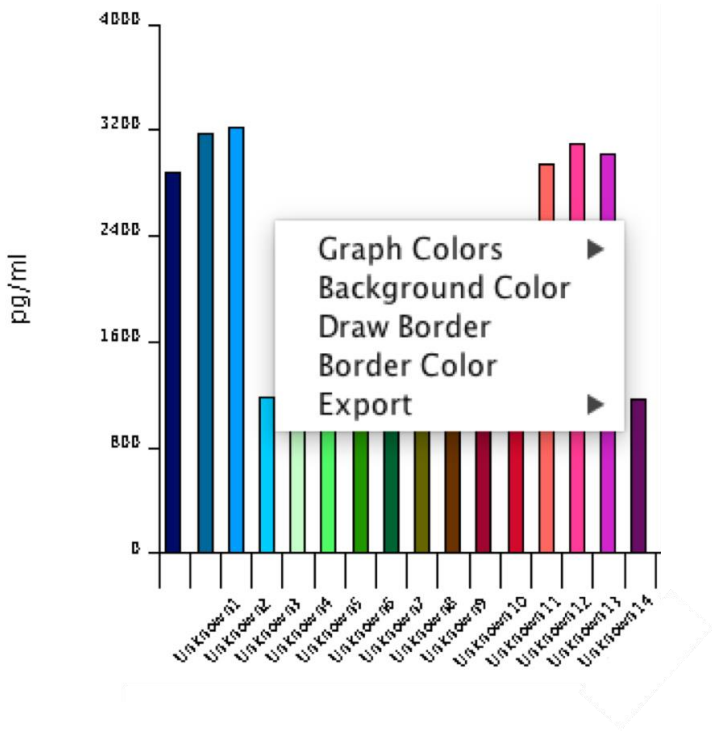


	IFN-g Obs
Unknown1	2882.48
Unknown2	3180.61
Unknown3	3219.49
Unknown4	1176.80
Unknown5	1293.09
Unknown6	1239.93
Unknown7	974.13
Unknown8	1084.81
Unknown9	1037.97
Unknown10	1946.07

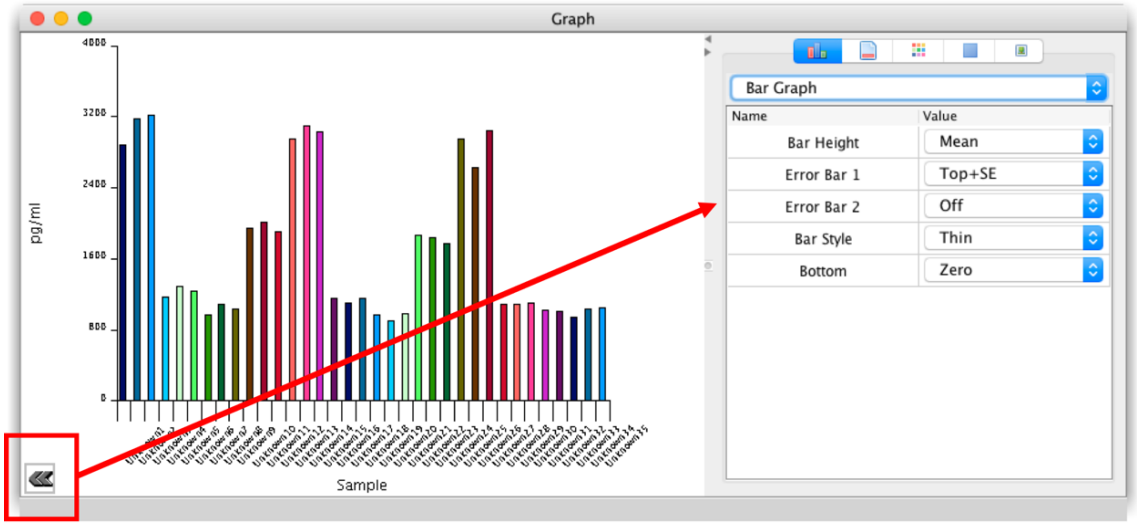
Right-click on each axis of the resulting graph to display options to set the font type, color and alignment, define the number of decimal places and change the angle of the text.



Right-click within the graph area to display a different menu with options to change the color of individual samples, the background color, turn the graph border on or off, set the border color and export the graph.

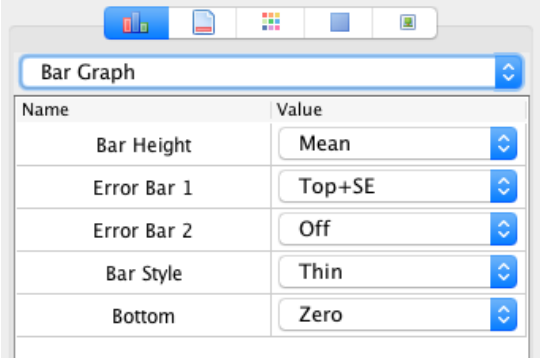


Each graph has a side drawer containing a range of formatting options. This can be opened by clicking on the double arrows at the bottom right of the graph.

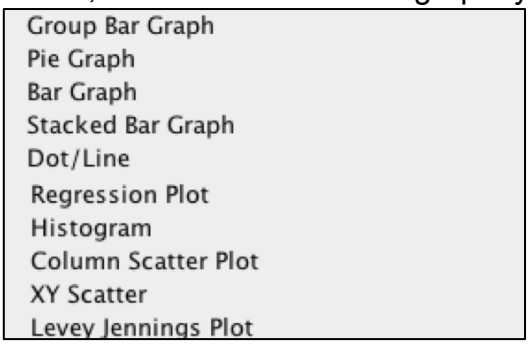


The side drawer is divided into five tabs: Graph Settings, Graph Axis Settings, Graph Color Settings, Graph Legend Settings and Graph Border and Background.

Graph Settings



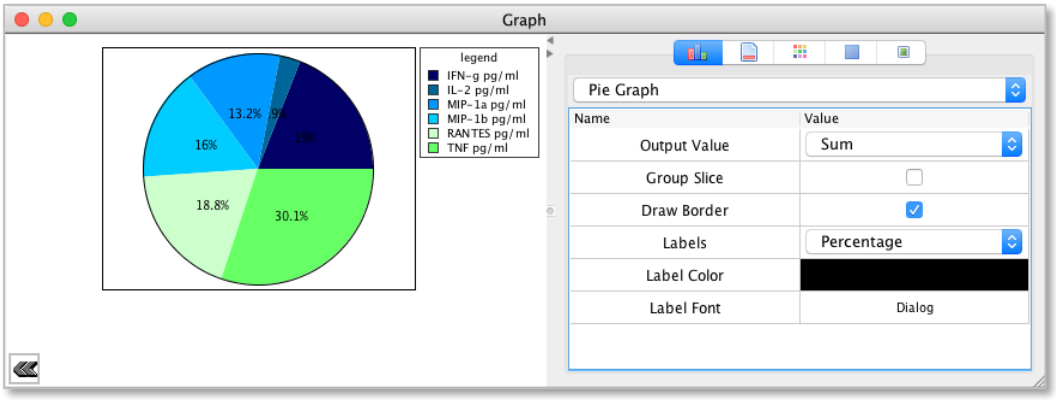
From the drop-down menu, choose from different graph types:



Different graph formatting options will appear in the window depending the type of graph chosen.

Graph types:

Pie Graph

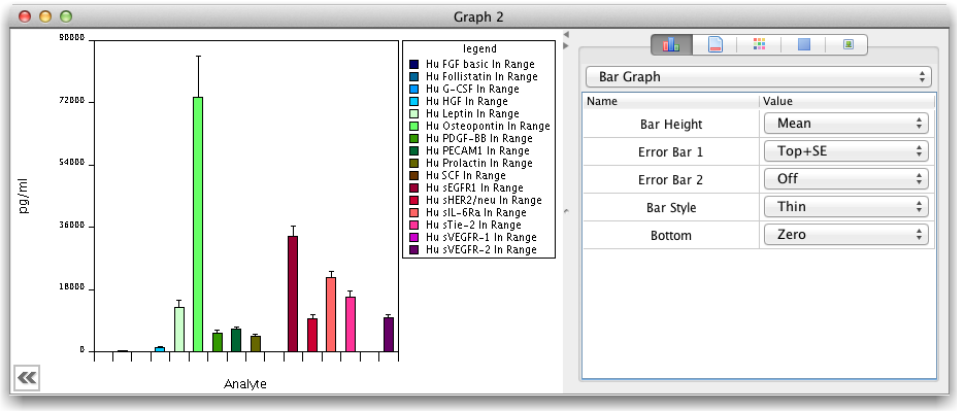


- Output Value: allows for the selection of the statistic, which determines the output value of each slice.
- Group Slice: turns the pie graph into a ringed pie graph.
- Draw Border: enables/disables the border of each ring.
- Labels: allows the Selection of ‘None’, ‘Percentage’, ‘Value’ or ‘Name’ as the labels for each slice.
- Label Color: allows the color of the labels to be changed
- Label font: allows the font of the labels to be changed.

Group Bar Graph

- A bar graph that shows individual values without statistical manipulation.
- Interleaved: if enabled, each group column is interleaved with the others.
- Draw Border: enables/disables the border around each column
- Border Color: allows the color of the column border to be changed.
- Gap: define the gap between columns.
- Group Gap: define the gap between groups.
- Gap Alignment: allows groups of columns to be justified in different parts on the x-axis.

Bar Graph

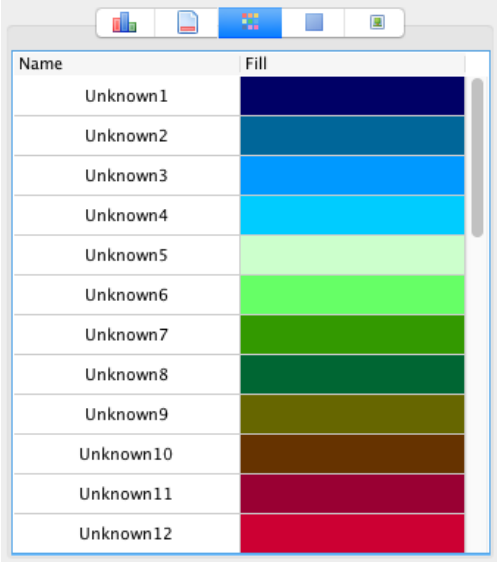


- Includes statistical manipulation on values within groups to provide each portion of the bar. This Graph type can also be used to create box and whisker plots.
- Bar Height: allows the height of each column to be defined based on a statistic for a given group
- Error Bar 1: allows the position of one of the error bars to be defined based on a statistic for a given group
- Error Bar 2: allows the position of the other error bars to be defined based on a different statistic for a given group
- Bar Style: allows for the manipulation of how the error bars are drawn
- Bottom: allows for the location of the 'bottom' of the bar to be set.
- Stacked Bar Graph: the stacked bar graph shows multiple bars either stacked or overlapped
- Superimposed: when enabled the bars are overlapped. Otherwise, they are stacked on top of each other.
- Gap: allows for the setting of a 'gap' between bars.
- Alignment: the alignment of the bars on the X axis.
- Dot/Line
- Draw Lines: if enabled, lines are drawn
- Draw Points: if enabled, points are drawn instead of lines.
- Regression Plot
- Histogram
- Column Scatter

Graph Axis Settings

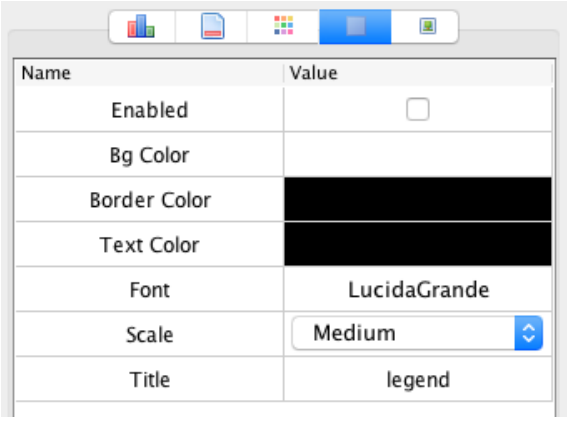
Various formatting options for the x and y axes are available in the Graph Axis Settings tab. These are divided into the categories of General, Labels, Scale, Major Tick Lines and Minor Tick Lines.

Graph Color Settings



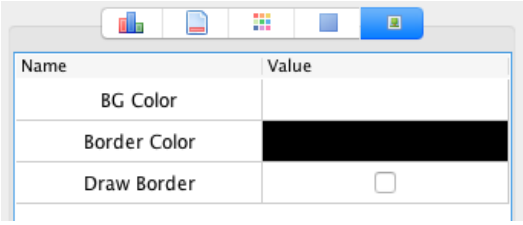
Click on the Fill color associated with a particular sample to choose a new color.

Graph Legend Settings



Format various elements related to the graph legend.

Graph Border and Background

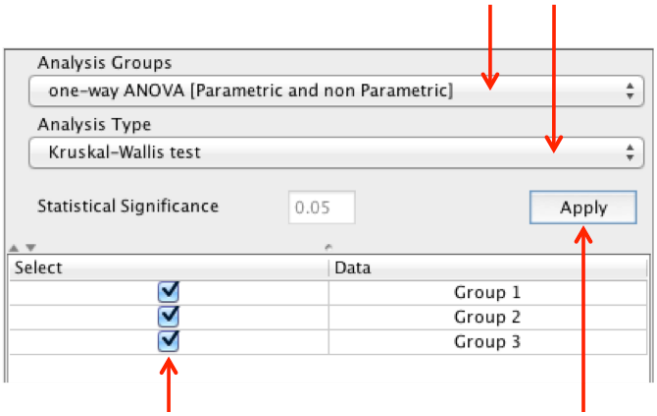


Choose a color for the border and graph background. Choose to display or hide the border.

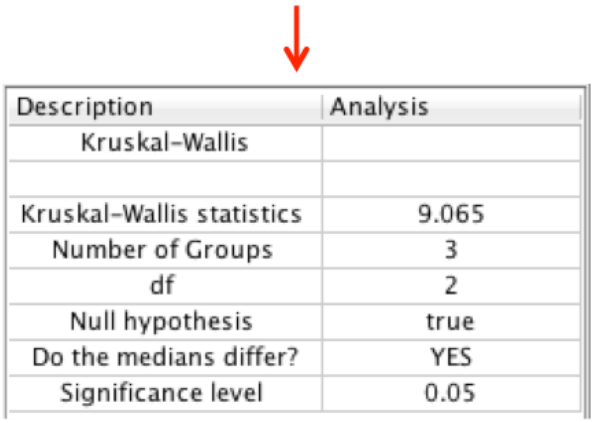
Statistical Analysis

To perform a statistical analysis on data contained in a graph, in the Statistics section click ‘Analysis’ associated with the particular graph in the Bead Data window. This displays the available statistical tests and the list of samples to compare in the ‘Analysis’ window.

To perform a statistical test, choose an Analysis Group along with an Analysis Type from the drop-down menus.



Select the groups that you wish to compare and click ‘Apply’. The result will be displayed in the Description and Analysis columns adjacent.



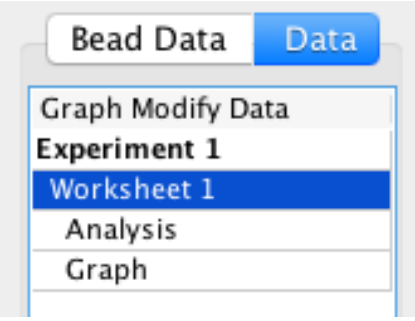
Description	Analysis
Kruskal-Wallis	
Kruskal-Wallis statistics	9.065
Number of Groups	3
df	2
Null hypothesis	true
Do the medians differ?	YES
Significance level	0.05

The options with the Analysis Group are:
- t tests (Parametric and non-Parametric)
- One-way ANOVA (Parametric and non-Parametric)

For t tests, the options with the Analysis Type are:
- Unpaired t tests with Welch-Satterthwaite approximation
- Unpaired t tests
- Unpaired t test Equal Variance
- Paired t tests
- Mann-Whitney

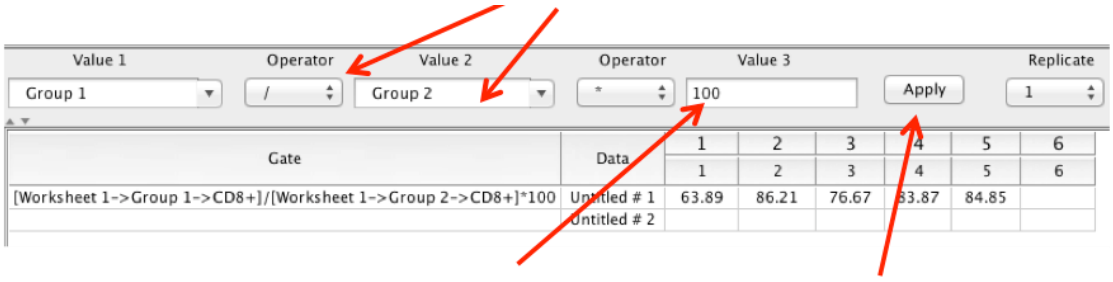
For **One-way ANOVA,** the options with the **Analysis Type** are:
• One-way analysis of Variance
• Kruskal-Wallis test

Another option for graphing data is in the Graph Modify Data Window. This can be accessed by selecting a Worksheet in the Data window (next to the tab for the Bead Data window.



This is where calculations involving group data (as displayed in the Graph Data Worksheets) can be performed. For example, proportions of one group (or individual samples) can be compared to another, with the result automatically converted into a new graph.

For example, the percentage that Group 1 is of Group 2 can be calculated as follows:

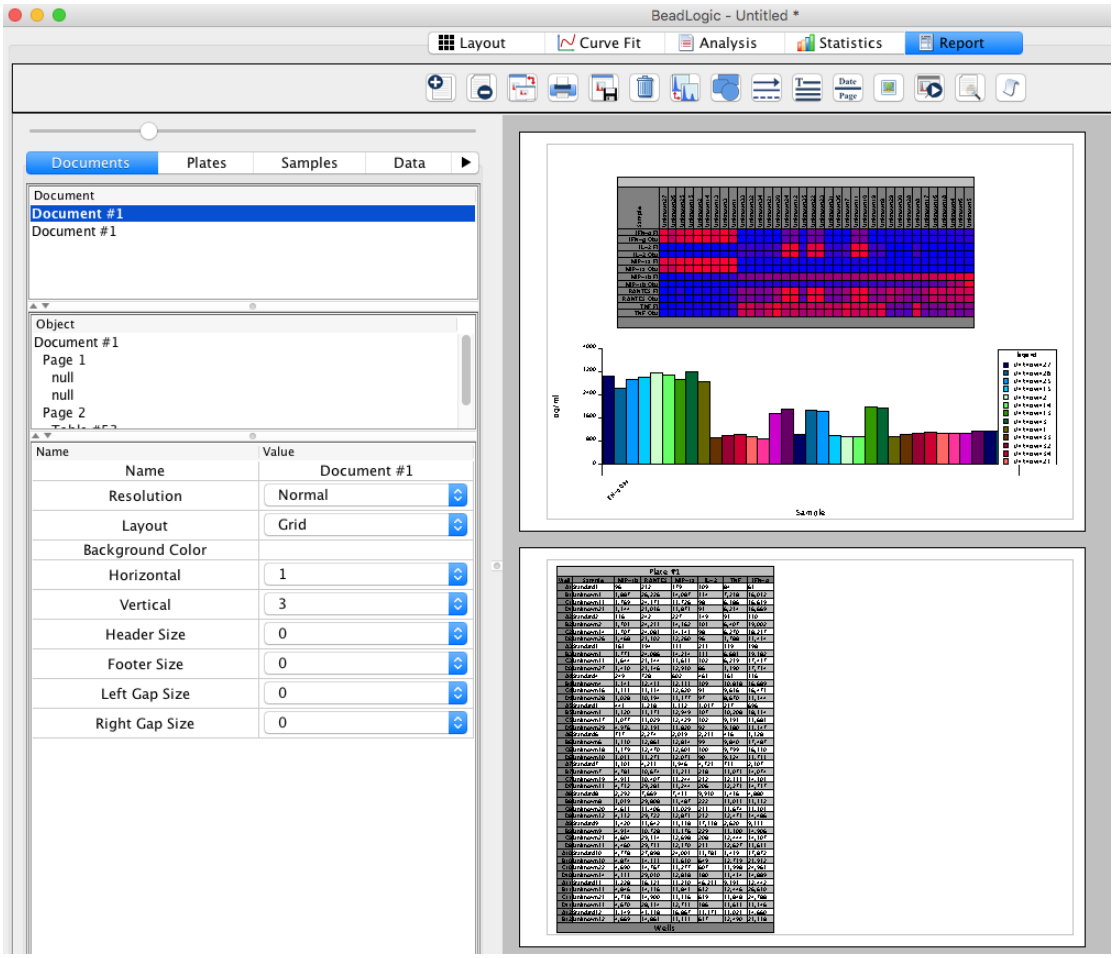


Enter a number in the Value 3 field and click ‘Apply’.

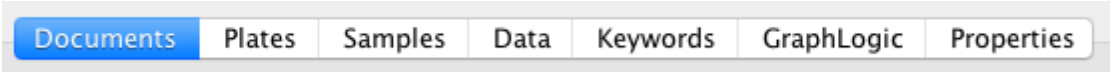
By default, the first graph in the Graph Data window is titled ‘Graph 1’ and the first Graph in the Graph Modify Data window is ‘Graph 2’. This is why when adding a new graph to the Graph Data window it appears as ‘Graph 3’. Names of all graphs, analyses, worksheets and experiments can be renamed by clicking on the title when it is selected.

BeadLogic - Report

Reports can be generated, containing any of the elements created as part of the data analysis.

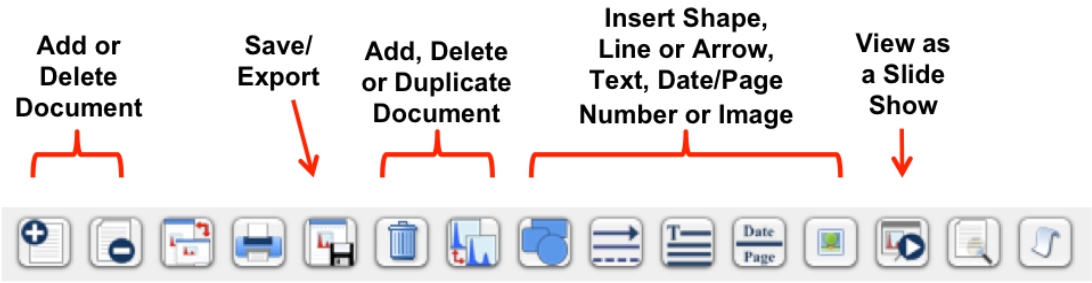


These elements along with various formatting options can be selected from the panels below the tabs to the left of the page layout. These tabs are:



To add an element, click-and-drag it from the panel directly onto the page.

Reports can be annotated and edited using the elements in the toolbar at the top of the screen. These include:



Layout and library templates: To create a layout template, click the template button in the toolbar and press ‘Save’. This allows the user to save a basic report template including headers and footers. Data is then added to the report manually. To save a Library Template, create a report including all data (graphs, tables, heatmaps, etc.). Then, click Library at the bottom of the screen and click ‘Save’. Enter the library name and description and click ‘Ok’. To load a Library Template, select ‘Library’, choose a library and click ‘Apply’. This will load the library template and repopulate all the data-associated elements with the results from the new analysis.

New or Delete a Document - Documents can be organized in the **List of Documents** tab above the file inspector.

Page Setup - Change the page layout between portrait (**Screen**) and landscape (**Paper**).

Print and **Save as PDF**.

Add a plot, delete item and duplicate plot - Highlight the appropriate population or document, either in the samples list or on the page layout, to add, delete or duplicate it.

Add stats table – Highlight the populations in the Navigator that have had statistics applied to them. Then, click the button and drag it onto the page.

Add shapes, arrows/lines and text – click on the appropriate button and drag it to the page layout. These can be resized once they are on the page.

Date and Page Numbers – click to add the date or page numbers to the document.

Add Image – insert an image from a file

Slide Show – Once in slide show mode, progress the slides forward by clicking the mouse. Right click to provide more navigation options.

Display Preview Page – Click on any page in the preview to jump straight to that page.

Click through the tabs above the File Inspector to customize and manage your document.

A default report showing all regression curves can be generated by right-clicking on an analyte in the Analytes panel in the Curve Fit tab and selecting 'Send all to report' → 'Default Analyte Report'.