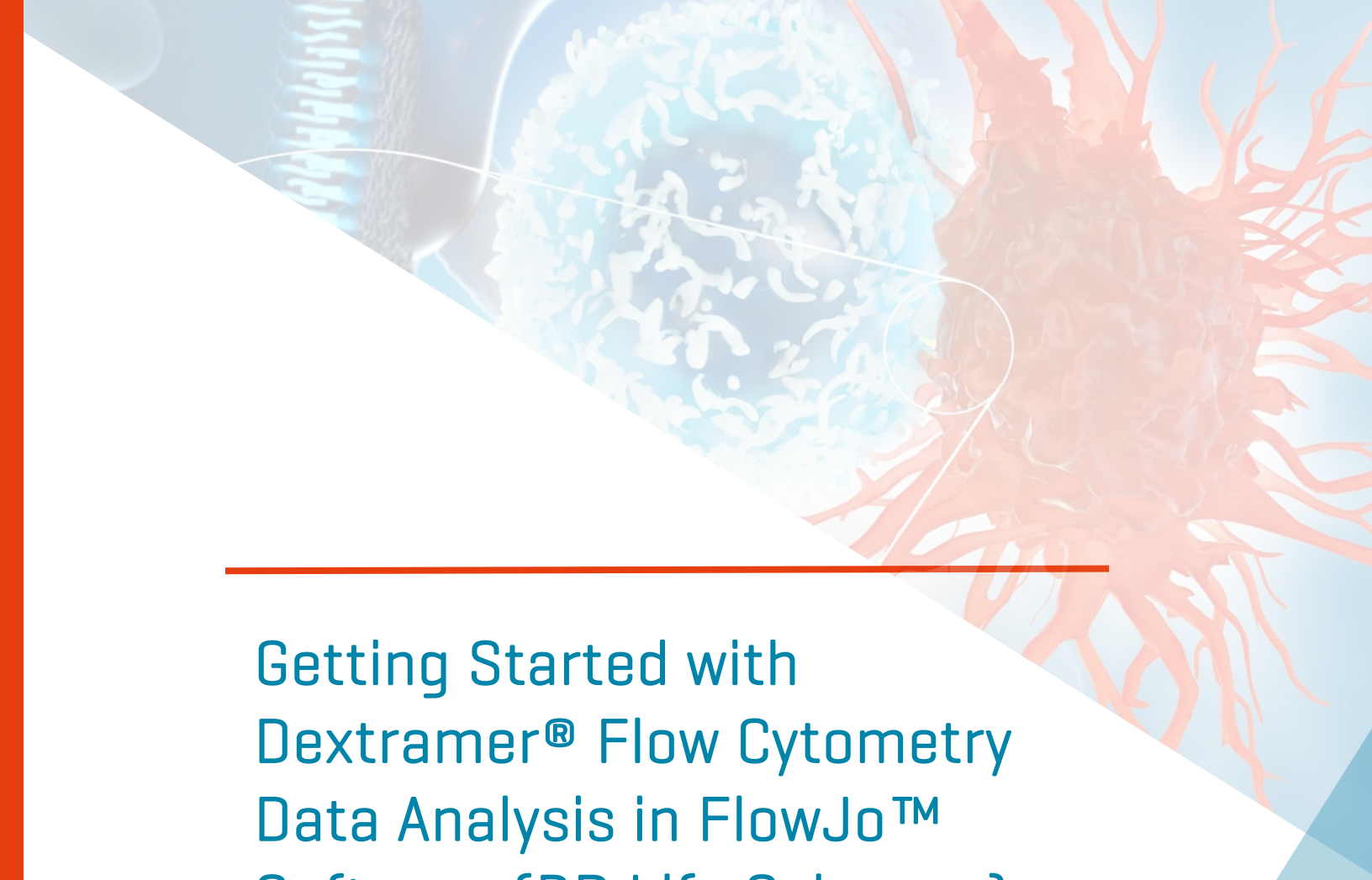

Getting Started with Dextramer® Flow Cytometry Data Analysis in FlowJo™ Software (BD Life Sciences)

After staining your cells with Dextramer® reagents, you will need to analyze your data to find your antigen-specific cells. This guide walks you through the basics of compensation, gating, and analyzing your Dextramer® stained samples in FlowJo™.

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2 Getting Started with Analyzing Dextramer® Flow Cytometry Data in FlowJo™ Software (BD Life Sciences)

2.1 Open a workspace in FlowJo™ Software

a. Open the FlowJo™ Software and log in using your credentials. This will open a new workspace. Alternatively, if another data set is already open in FlowJo™ Software
b. Select "New Workspace" in the upper left-hand corner (Fig. 1)

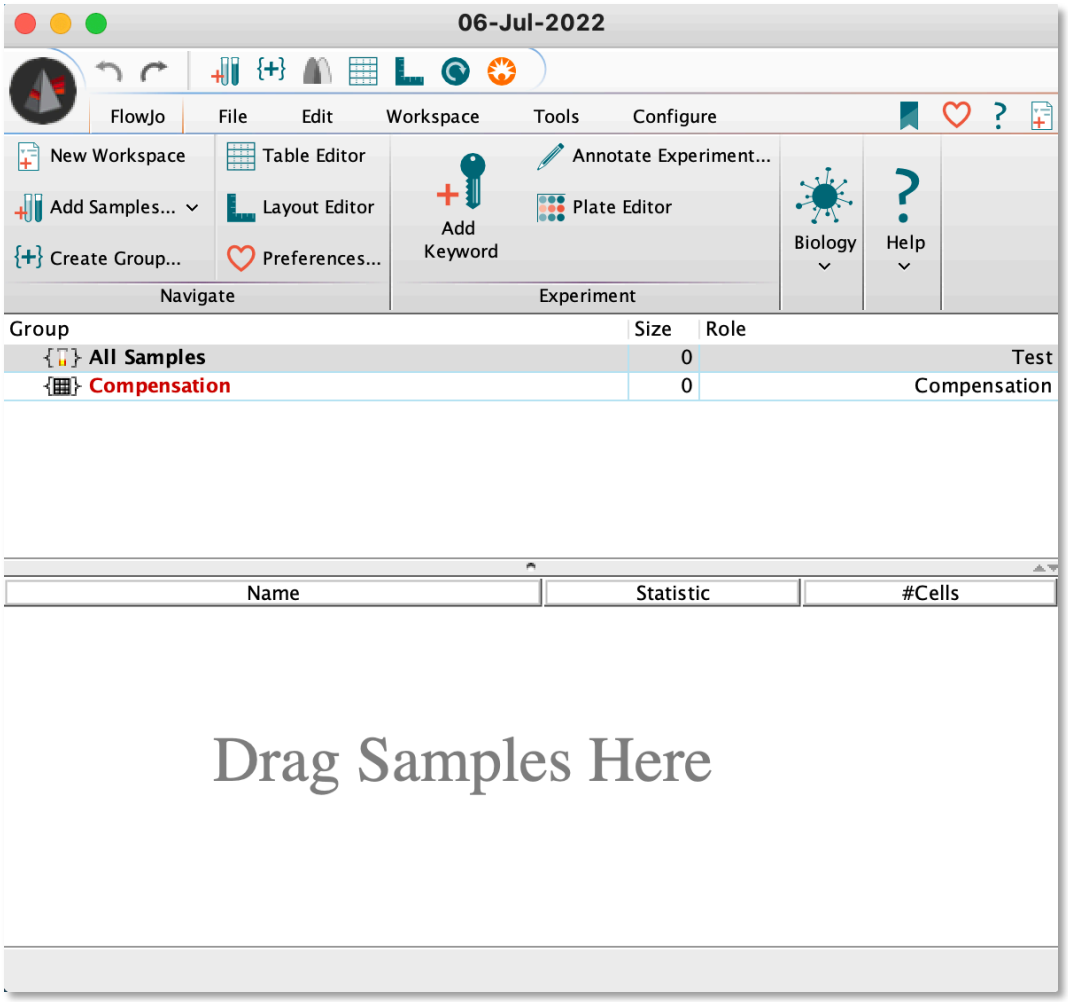

Fig. 2 A newly opened workspace.

2.2 Save the workspace in the desired format (Fig. 3)

a. Workspace (WSP) is the most common choice and will save a file containing:
    - The samples added to that workspace as FCS files
        - The pathway to the files will need to be referenced to easily re-open workspaces, so ideally, the file locations should not change

- Groups
- All analysis (gates, statistics, etc.)

b. Template (WSPT), useful for repeated analysis on samples with the same parameters, saves:
   - Group organization, gating strategy, graphical and tabular analysis set up

c. Archive (ACS), best if you often share data or collaborate with others on data analysis, saves:
   - FCS files and Workspace file as one file

d. Excel (XLS), best for plotting and analysis outside of FlowJo™, saves:
   - Exportable statistics.

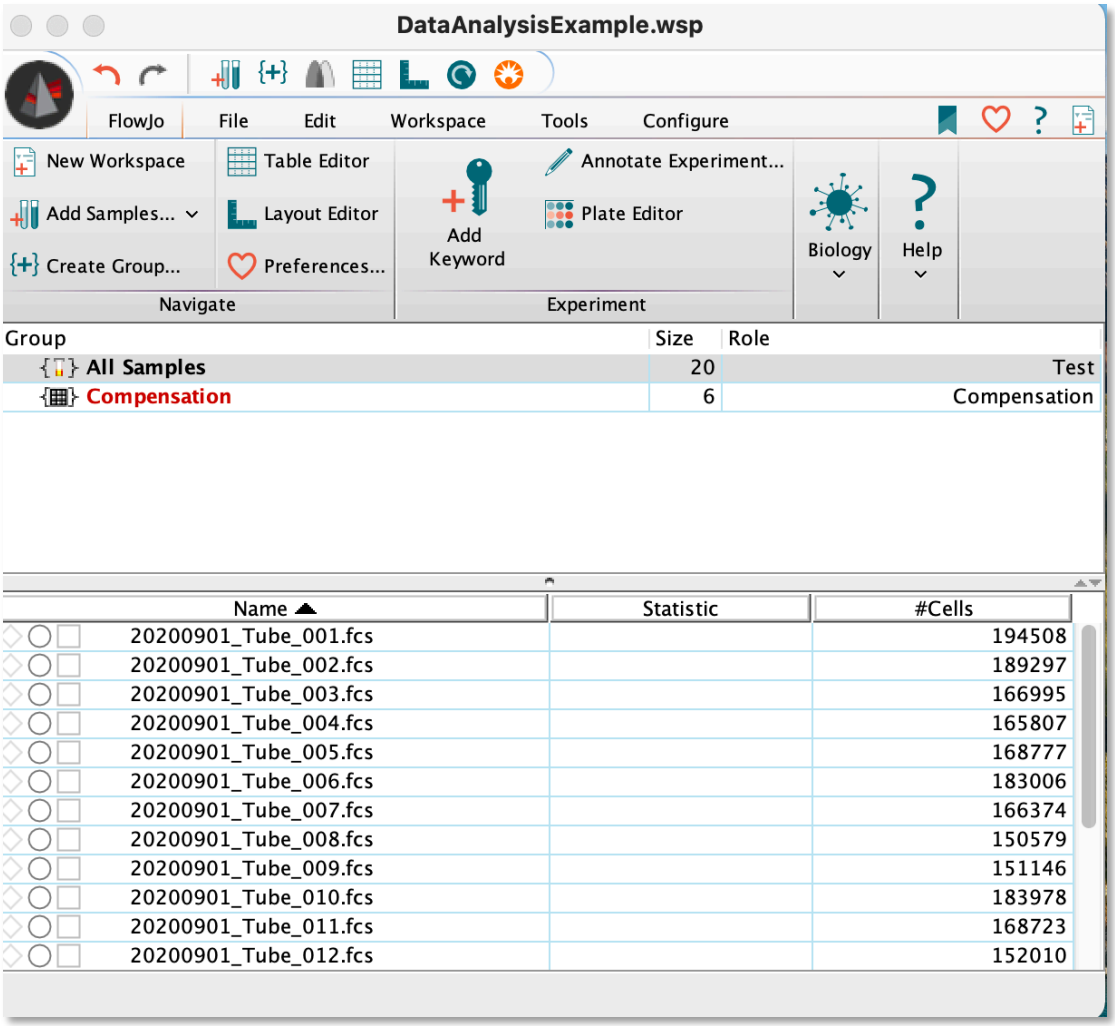

Fig. 4 A saved workspace with the samples added as FCS files.

2.3 Transfer FCS files

a. Move FCS files to a computer or external storage from the flow cytometer.

b. Open the folder containing the FCS files for analysis

c. Select the files and drag and drop into the workspace

2.4 Organize the workspace by groups

a. Organize the workspace by creating new groups as needed by selecting "Create Group" in the upper left-hand corner (Fig. 5).
   - Groups should be created based on similar sample properties or needing to analyze the data in the same way by applying the same gating strategy

b. Name the group, ideally to indicate the sample properties in this group

c. Add other parameters as needed:
   - keywords may enable FlowJo™ Software to auto-sort the right samples into this group.
   - For more details on keywords, see the Compensation group by right-clicking on the group and selecting edit group properties.

d. Alternatively, under the "All Samples" group in the main window, highlight the samples that you would like to transfer and drag and drop to the new group (Fig. 6).

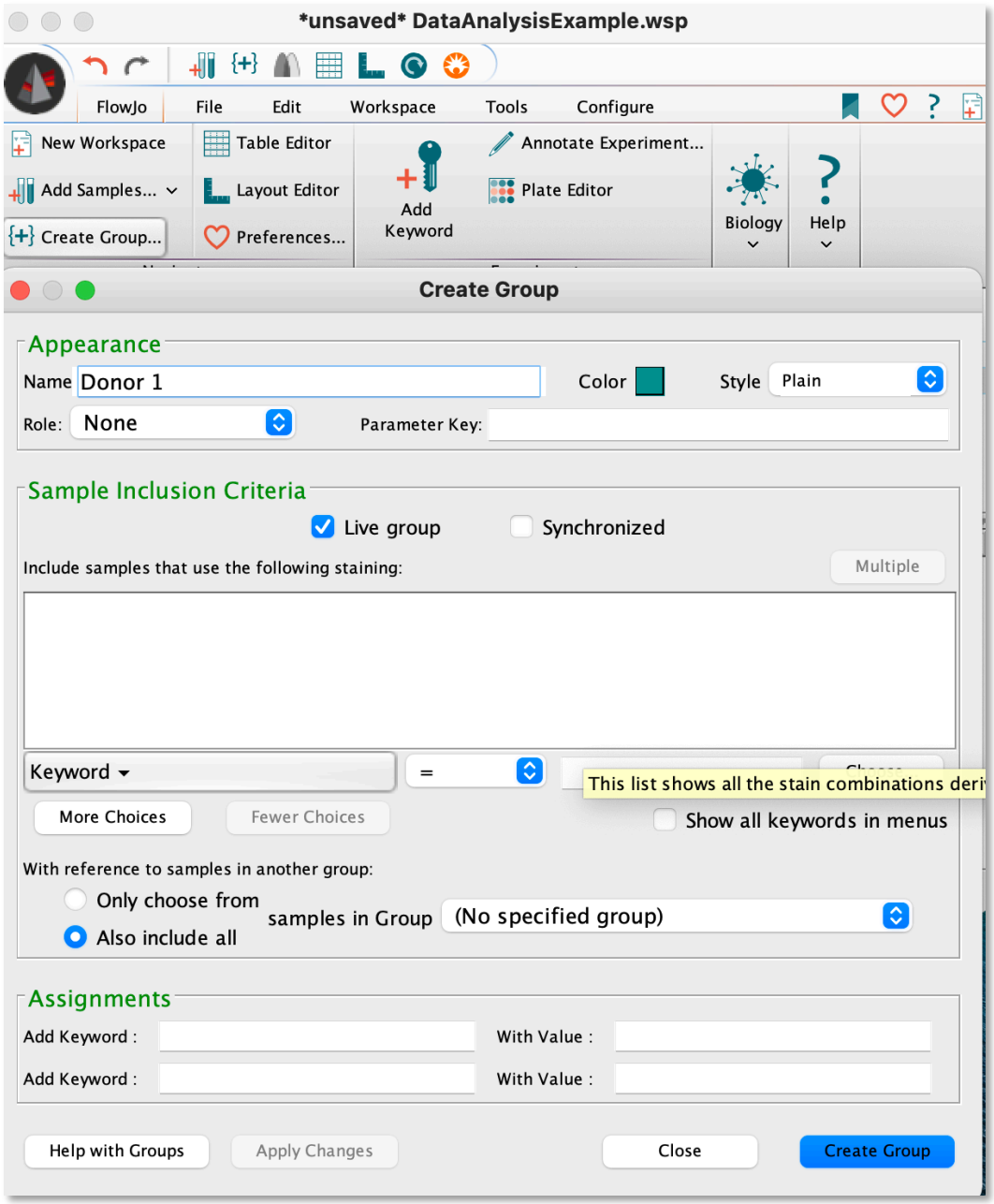

Fig. 7 Creating groups.

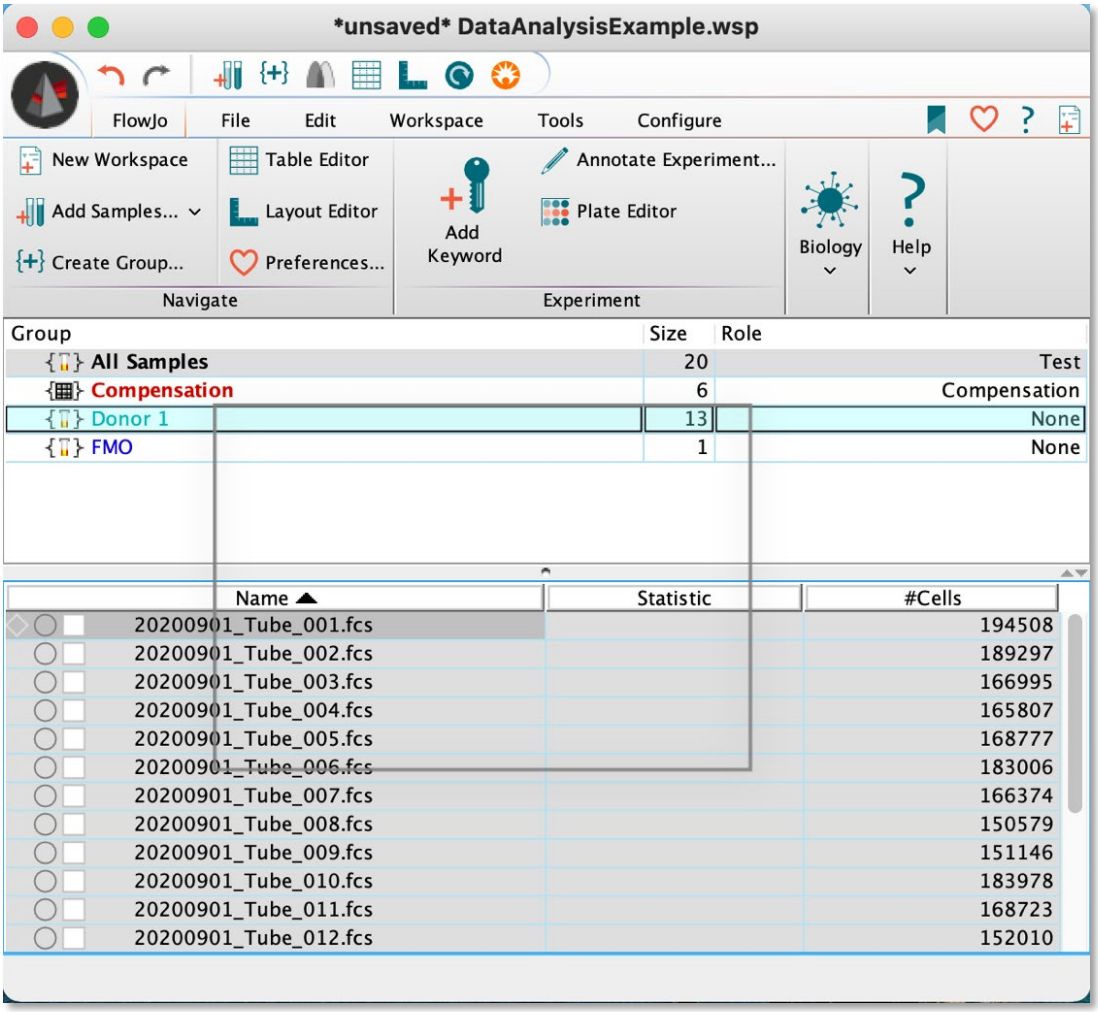

Fig. 8 Organizing samples into groups.

3 Checking Sample Quality

Start by performing a fast quality check on the data set to ensure that there have been no instrumental issues during acquisition (Fig. 9)

3.1 Check sample quality

a. Select the "All Samples" group, then "Tools" in the upper taskbar, and finally, "Check Sample Quality"
   - When you do this, FlowJo™ Software automatically plots the median light intensity values for each laser line over time which demonstrates if voltages were changed, if a clog occurred, or if other issues occurred with the laser line during acquisition

b. Use the "Inspect" tool under the Workspace tab or double-click the circle to the left of the sample to see the resulting plots and sample information
   - These plots provide information on potential errors during acquisition, including the severity and duration of these errors, communicated by a color output.
   - Circle icons left of the samples will indicate sample quality according to color; blue = good quality, green = ok, violet = irregular, and red= problematic sample.

Based on this information, you may choose to exclude samples from the analysis.

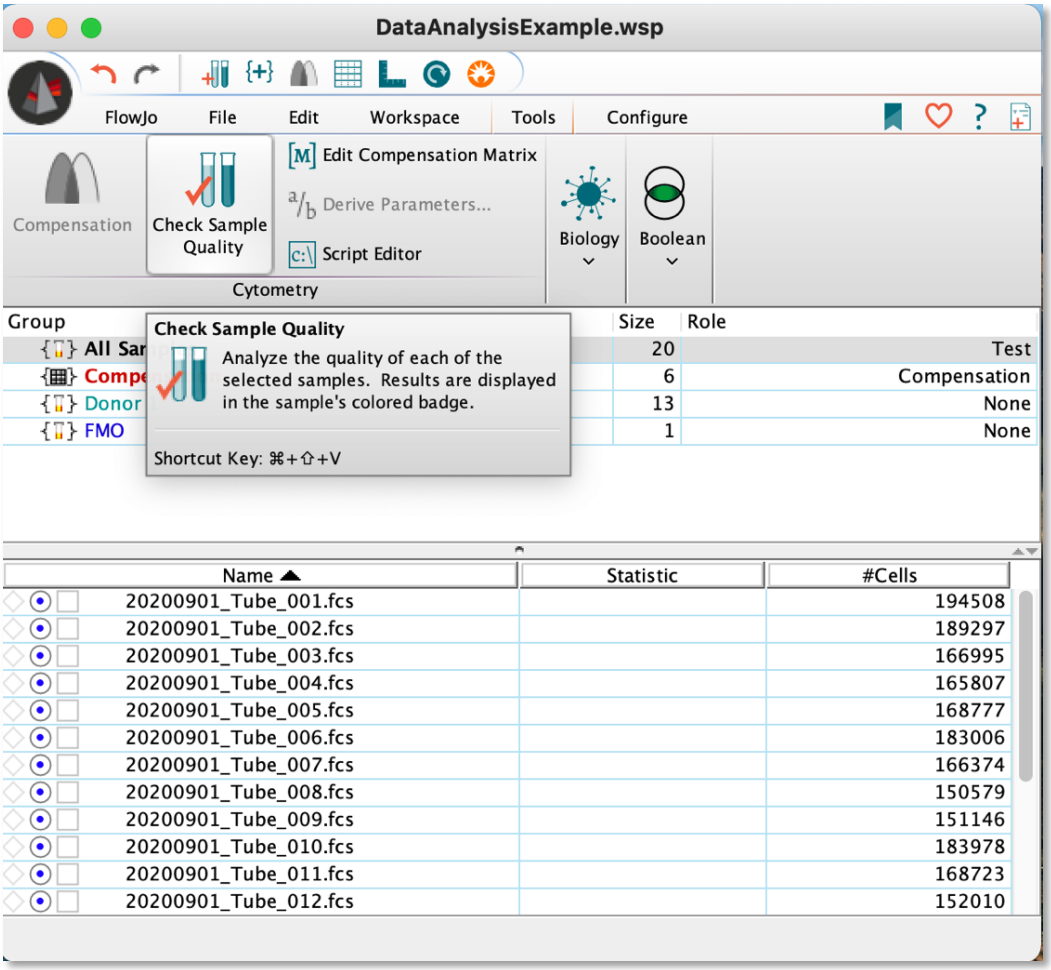

Fig. 10 Initiating data quality check. Once selected, sample quality check will be performed automatically.

3.2 Viewing laser lines for a sample as a preliminary quality check

a. To view the plots associated with a sample, double click on the circle to the left of the sample (Fig. 11)
   - Trends for used detectors should fluctuate along the linear mid-line indicating steady laser lines and acquisition.

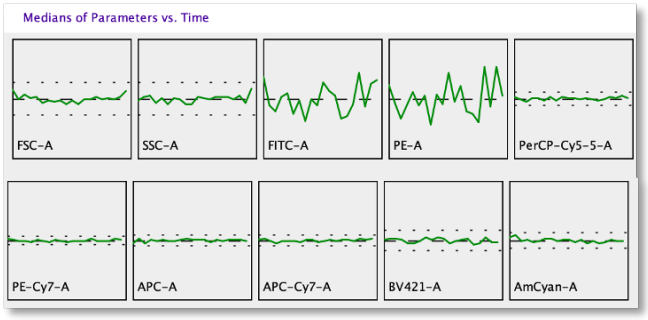

Fig. 12 Example of steady laser lines.

3.3 Checking acquisition flow over time

a. Manually go through each sample to observe the flow consistency over the acquisition time

b. Select the "All Samples" group. Change the X axis to time and the Y axis to SSC

c. Click through to ensure steady and constant acquisition flow (Fig. 13).

Other trends in the acquisition flow could indicate a partial clog, an aspirated sample, or loss of fluorescence and should be gated out during analysis.

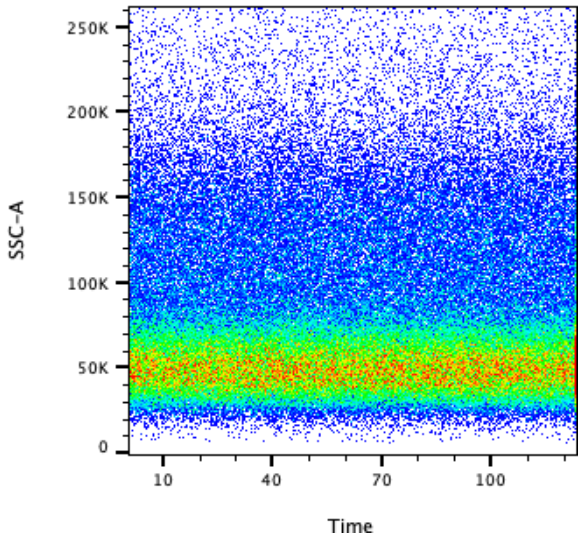

Fig. 14 Example of a steady flow profile.

4 Compensation

Compensation calculates and corrects the spectral overlap between fluorophores on the reagents used in the panel. Technically, it is a linear algebraic correction to un-mix signals received in a single photodetector from multiple dyes (Fig. 15).

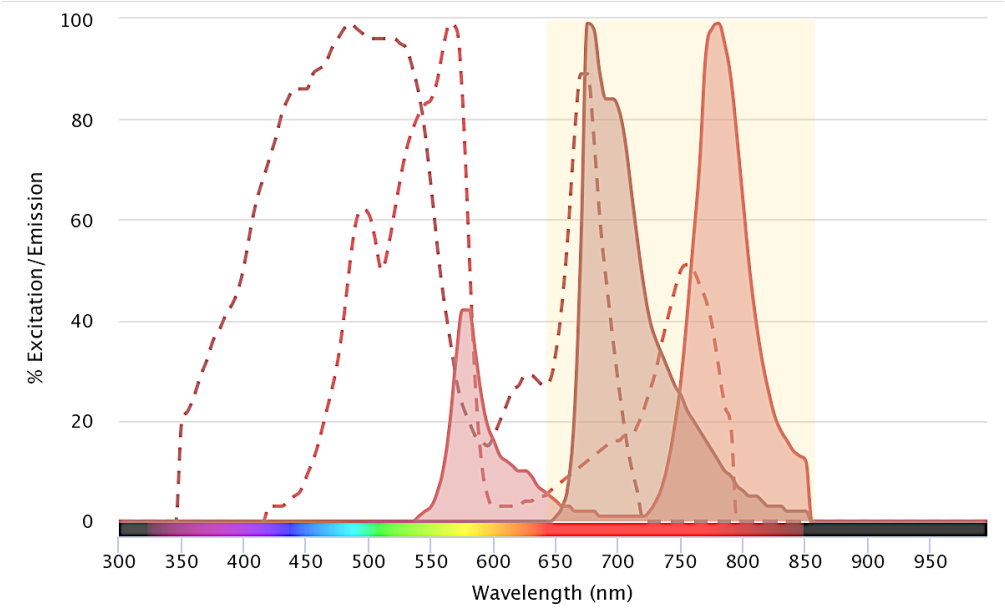

Fig. 16 Example of spectral overlap between two fluorophores (PE-Cy7 and PerCP-Cy5.5 have significant spectral overlap as highlighted in yellow). Spectral excitation/emission profiles from fluorofinder.com.

For samples acquired by flow cytometry, performing compensation on single-stained controls is required before analyzing experimental samples. Compensation can be done using:

- The acquisition software on the instrument (if FCS3 compatible). If needed, the compensation can be re-calculated in FlowJo™ Software later
- FlowJo™ Software or other similar software.

In all cases, the calculated compensation coefficients should **not** be manipulated manually.

4.1 Naming Conventions

If including "compensation" or "comp" in the title of the compensation controls during acquisition, FlowJo™ Software will automatically direct samples to the "Compensation" group, when FCS files are dropped into your workspace.

4.2 Rules for Compensation

- Compensation will require both unstained and single-stained controls in your experiment
- To the extent possible, use the same reagent lot as used in the full panel

- Compensation controls should show equal or brighter staining than samples.
  - Generally, it is recommended to stain single-stained controls at the same titrations as within the full panel
- Optimally, acquire 5,000-10,000 events/sample if using compensation beads
  - If working with cell-based compensation controls, the number of cells acquired depends on the cell type that you are working with.

4.3 Adding Compensation to your samples in FlowJo™ Software

a. Select the "Compensation" group. If the suggested naming practices were followed, the compensation control files will already be here. Alternatively, drag and drop the files into the "Compensation" group (Fig. 17).

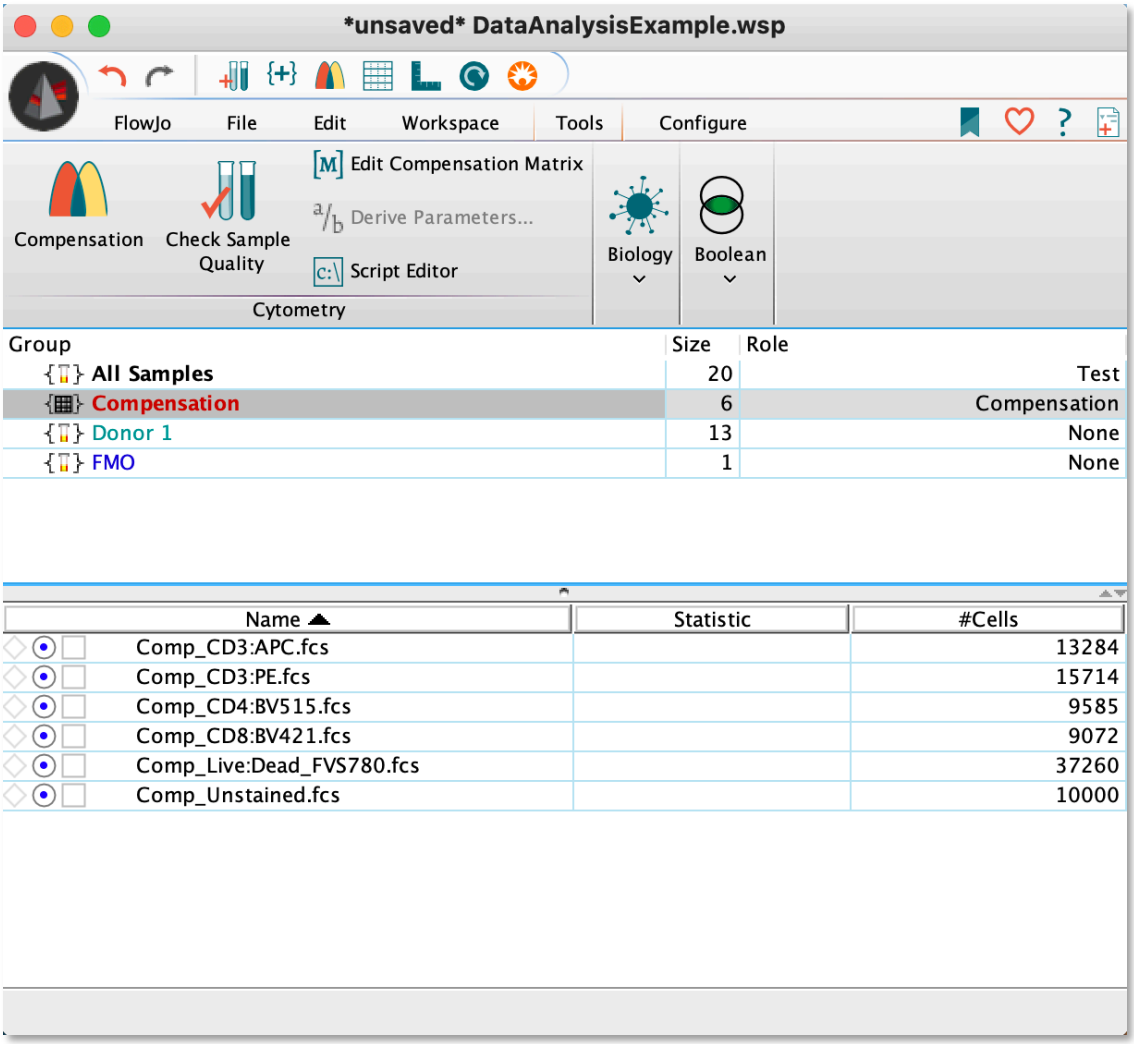

Fig. 18 Example of the compensation control files with the correct naming conventions in the "Compensation" group.

4.4 Calculating the compensation matrix

a. Select the "Compensation" group then select the compensation icon from the top task bar. When selected, a notification will appear at the bottom of the screen informing of the ongoing compensation matrix calculation. This will take a few moments dependent on the number of controls.

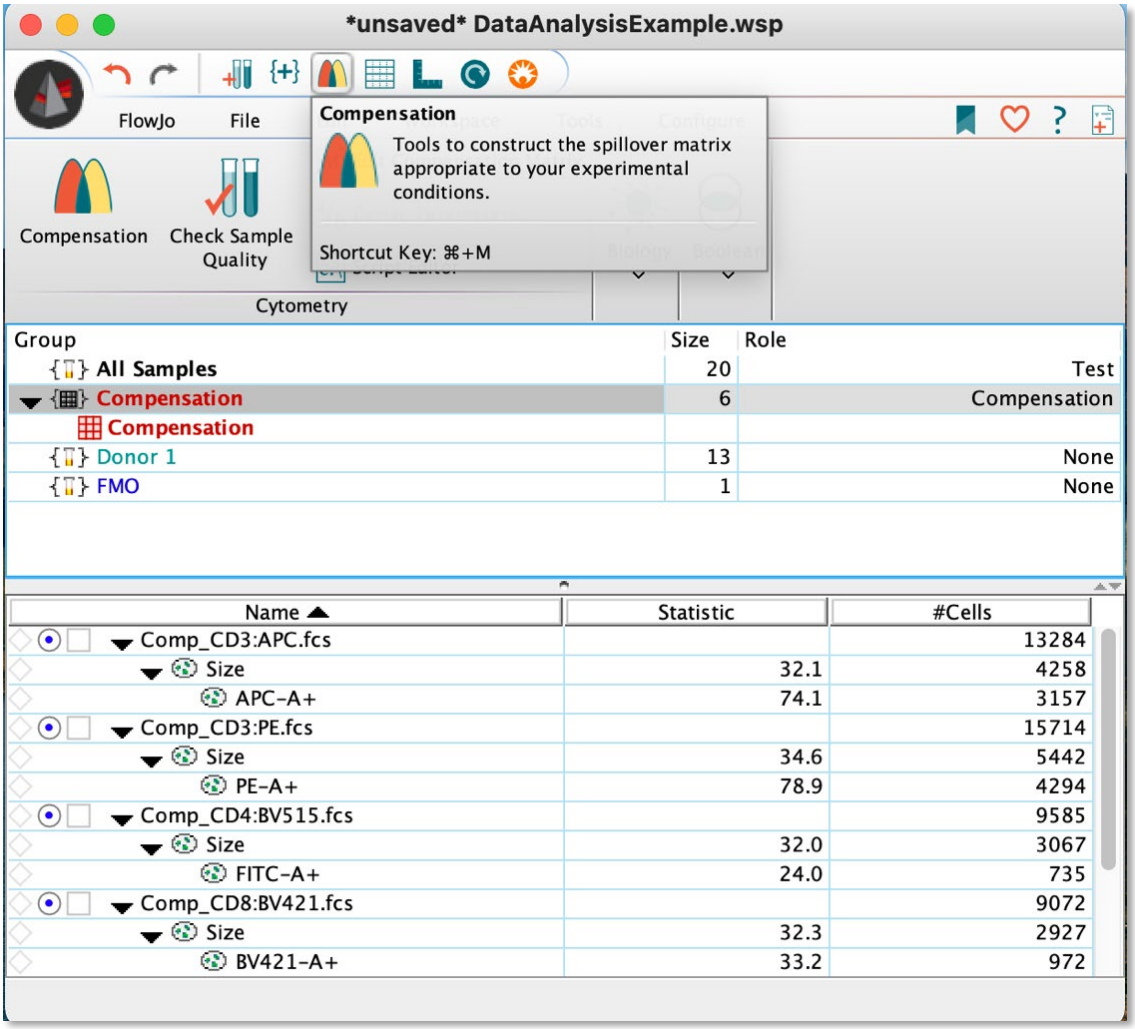

Fig. 19 Initiating compensation. Once selected, compensation will be calculated automatically.

4.5 Auto-compensation by FlowJo™ Software

a. When completed, the compensation parameter window will automatically open. This is the first attempt at compensating the data by the FlowJo™ Software (Fig. 20).

The resulting compensation must be quality checked and adjusted. The color of the circle left of the parameter also provides insights into needed adjustment. Appropriately adjusted samples will have green or yellow indicators.

Fig. 21 Example of the automatically generated compensation parameter window before "Choosing Parameters".

4.6 Adjusting the Compensation

a. First, edit the parameters to include only the used detectors.

b. Select the "Parameter" button in the left column and "Choose Parameters".

c. Hold down the command or control key and select the relevant detectors.

d. "Choose Selected Parameters". This will update the parameters as well as the compensation control plots below (Fig. 12 and 13).

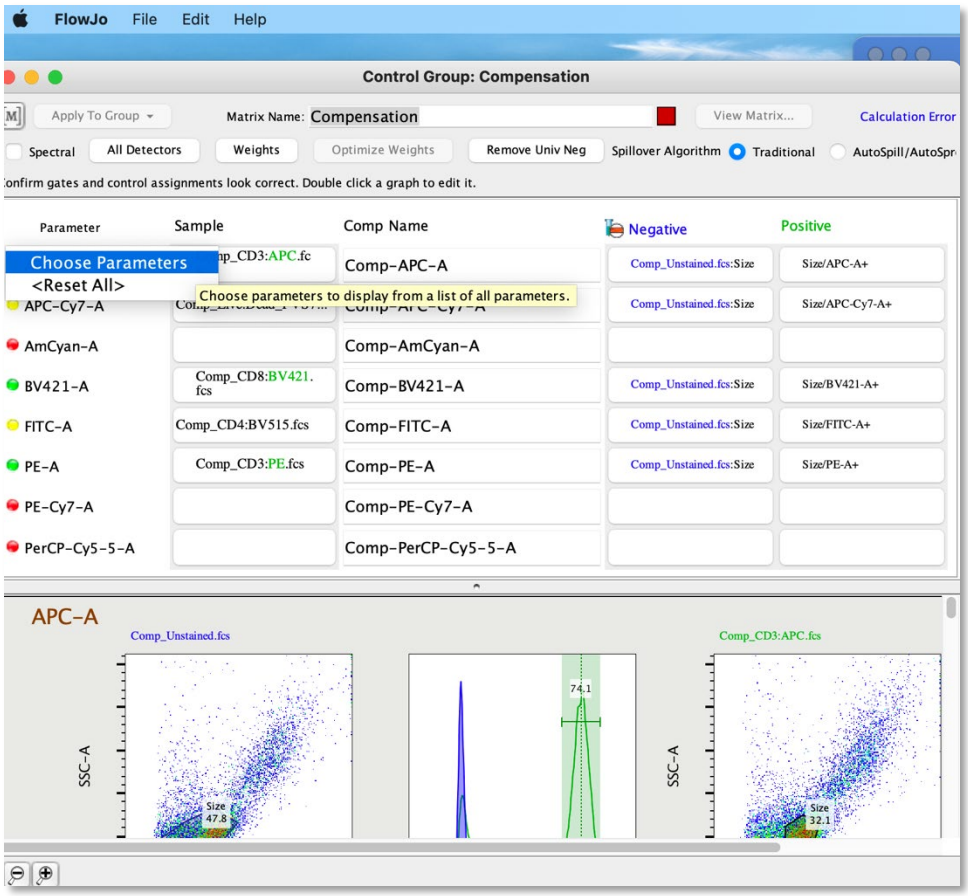

Fig. 22 Using the "Choose Parameters" drop down menu to modify included parameters, part 1.

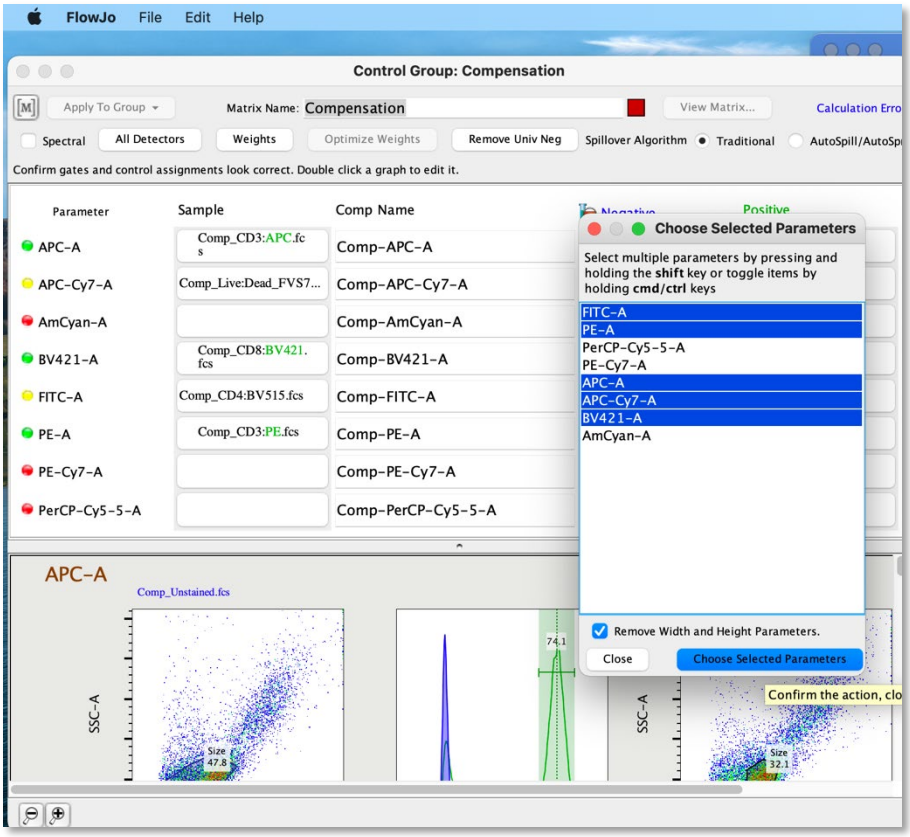

Fig. 23 Using the "Choose Parameters" drop down menu to modify included parameters, part 2.

4.7 Check the compensation of each sample

a. Confirm that the appropriate negative control sample was selected (shown in blue, unstained cells or beads) and

b. Confirm that the appropriate positive control sample was selected (shown in green, single-stained cell or bead sample)
   - If the correct files were not auto selected, click on the file name and a drop-down menu will appear, select the correct file (Fig. 24).

4.8 Adjust the gating of compensation controls

The FlowJo™ Software will set a preliminary gate for the cell population being analyzed. Often this needs to be corrected (Fig. 25).

a. For each single-stained sample, adjust the population as needed. The goal is to select populations that are as bright or brighter than the experimental samples and provide a clear negative (shown in blue) and positive (shown in green) signal with matched autofluorescence, not to select the cells of interest (Fig. 26).

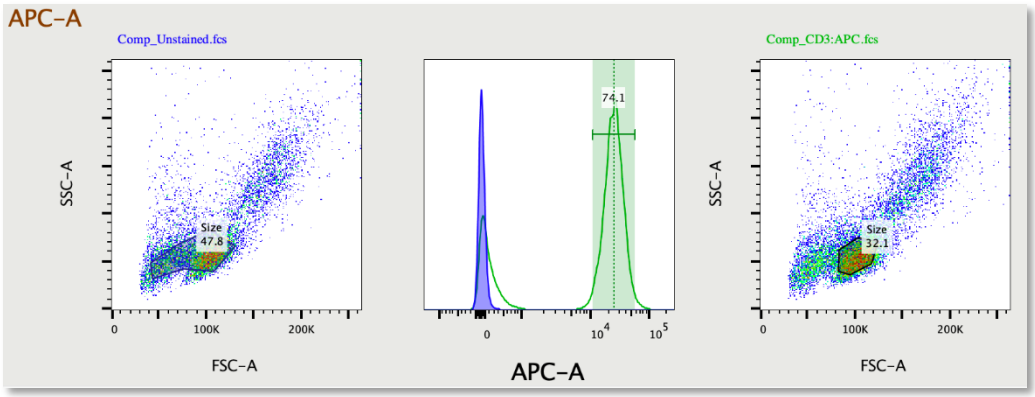

Fig. 27 Example of automated population selection by FlowJo™ Software.

4.9 Adjust gating

a. To adjust, double click on the scatter plots and adjust the gates to include relevant cells based on the stain being investigated. Then, double click the histogram plot and adjust the green gate to cover the peak representing positive staining (Fig. 28).

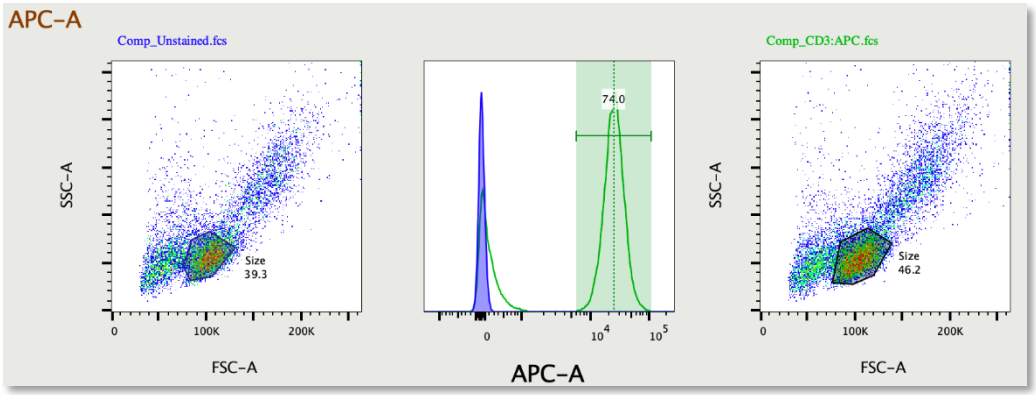

Fig. 29 Example of adjusted gates for negative and positive staining.

4.10 Transformation of data

Transformation of the data may be required as part of this process to better visualize which cells to select.

a. To do this, double click on any plot and select the "T" button on either axis, "Customize Axis", and adjust the population using the +/- tools. Once one population is transformed, the rest will be transformed the same way (Fig. 30).

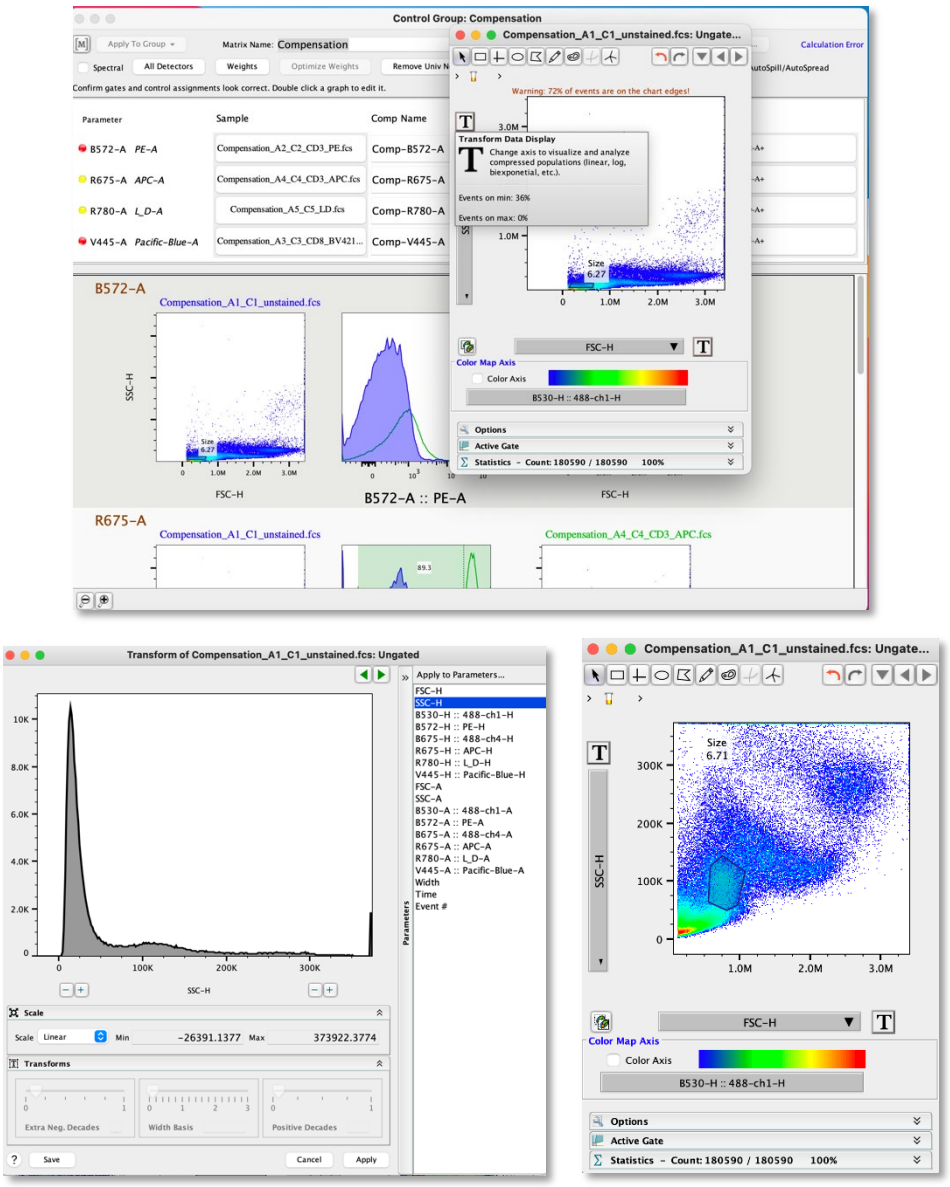

Fig. 31 Example of transforming the data for better visualization of cell populations.

Instead of selecting each cell population individually, the same gate may be applied to all samples in the "Compensation" group, if relevant. To do this, in the main window, click and drag the size gate to the "Compensation" group or right click on the gate in the main window and select "apply to group".

Once completed for each parameter, all the Parameters selected at the beginning of the compensation process should now have a yellow or green icon, not red.

4.11 Viewing the calculated compensation matrix

a. Within the compensation window, in the upper right-hand corner select "View Matrix". The matrix will be calculated based on the adjustments

b. Select "Preview Sample", then select a single-stained control from the dropdown menu. Check for linear-oriented populations without smears or diagonal staining. On the right-hand side, check the "overlay uncompensated data" to show how the compensation has impacted the data. Check each single-stained and unstained control (Fig. 32 - Fig. 33).

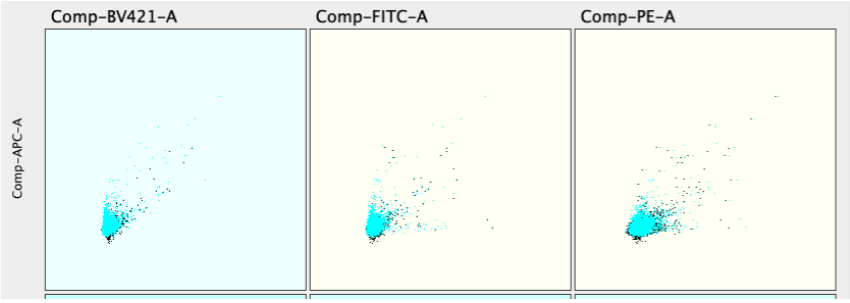

Fig. 34 Example of expected unstained control of adjusted data (black) and uncompensated data (blue).

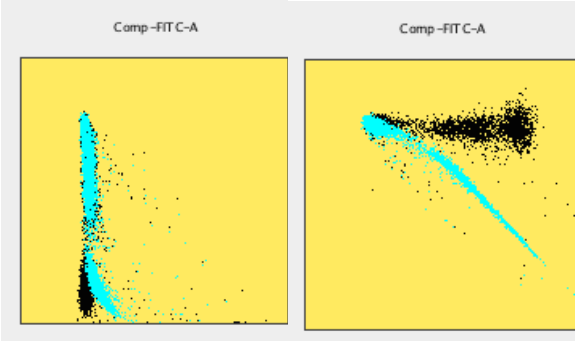

Fig. 35 Example of proper compensation adjustment of adjusted data (black) and uncompensated data (blue).

c. If compensation is not adequate (Fig. 36), close the compensation matrix window, adjust compensation, and view the new matrix by repeating the previous compensation steps as described in section 4.6-4.11.

d. When satisfied, select the "Apply to Group" button in the upper left-hand corner and select which group(s) to apply the compensation matrix to.

e. Alternatively, click and drag the [M] matrix symbol in the upper left-hand corner to the group in the main window. Once the matrix has

been applied to a group, a matrix icon will now appear to the left of the sample in the main window (Fig. 37).

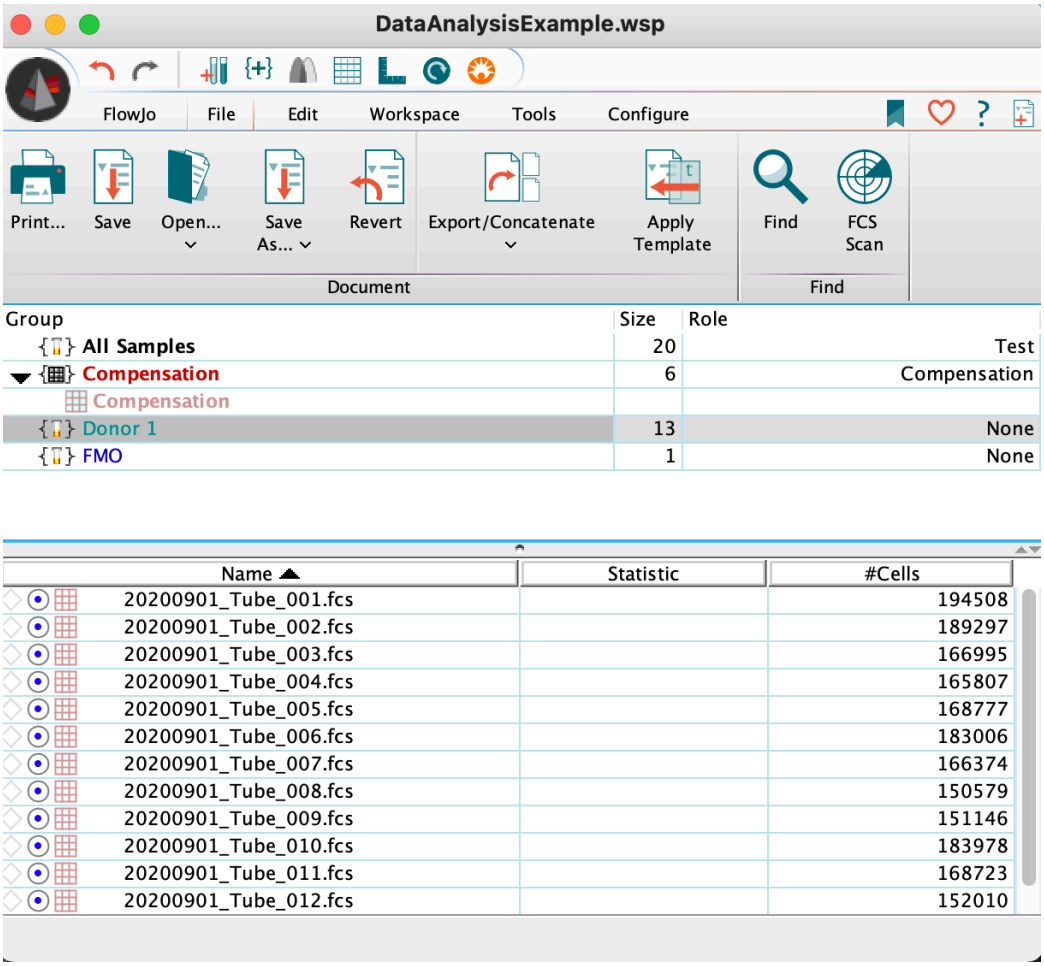

Fig. 38 Example of the compensation matrix applied to a group of samples.

5 Setting Gates and Statistics for Experimental Samples

a. To adjust the visual presentation of the data, use the Width Basis slider tool function within the Transform tool with fluorescent parameters on the x and y axis changing one fluorochrome at a time.

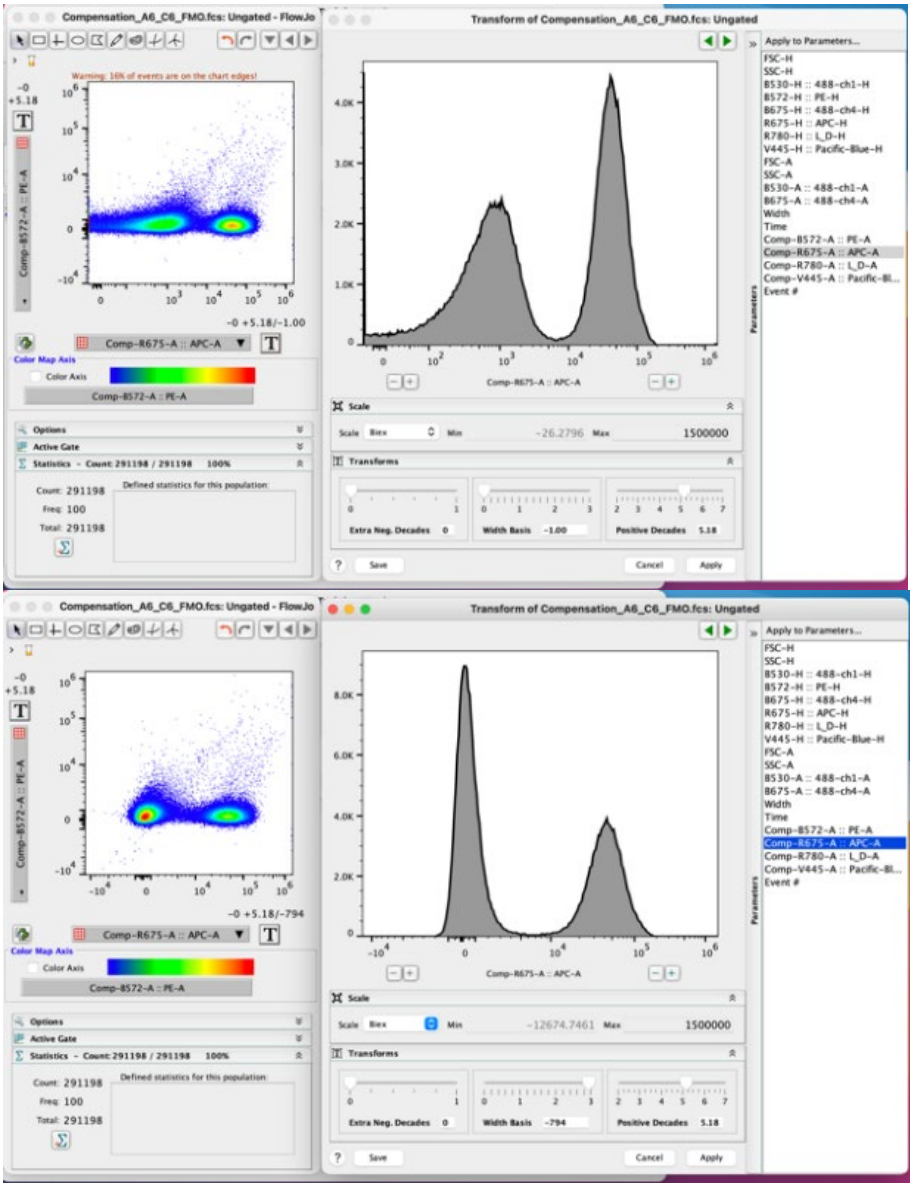

Fig. 39 Example of using the Width Basis tool to change visual presentation of the data (adjustment from 0 to 3).

5.1 Determine the appropriate gate for the MHC Dextramer® reagents

a. Open the corresponding Negative Control Dextramer® sample with a conjugated fluorochrome matching the experimental Dextramer® reagent by double clicking on the sample name in the main window. Use the Negative Control Dextramer® to determine the appropriate gate for the MHC Dextramer® reagent(s) used in your experiment.

b. Set gates based on the gating strategy for the experimental samples (Fig. 40). Different gate drawing tools are available in the upper task bar within the plot window. Double click on the new gated population to open a new plot where the next iterative gate can be set.

    a. Gate on steady flow (SSC vs. Time).
    b. Gate out debris.
    c. Gate on lymphocytes if comfortable identifying them based on the scattering pattern.
    d. Gate on singlets (cells along the diagonal on the FSC H vs. FSC A plot). If desired, a second round can be performed (cells along diagonal on the SSC H vs. SSC A plot). Forward scatter provides information about cell size while side scatter provides information on intracellular granularity.
    e. Gate on live, CD3+ cells.
    f. Optional dump gate for removal of other cells (ex. CD19 for monocytes, CD4+ T cells, etc.).
    g. Finally, plot CD8+ vs. Dextramer® and set the Dextramer® positive gate.

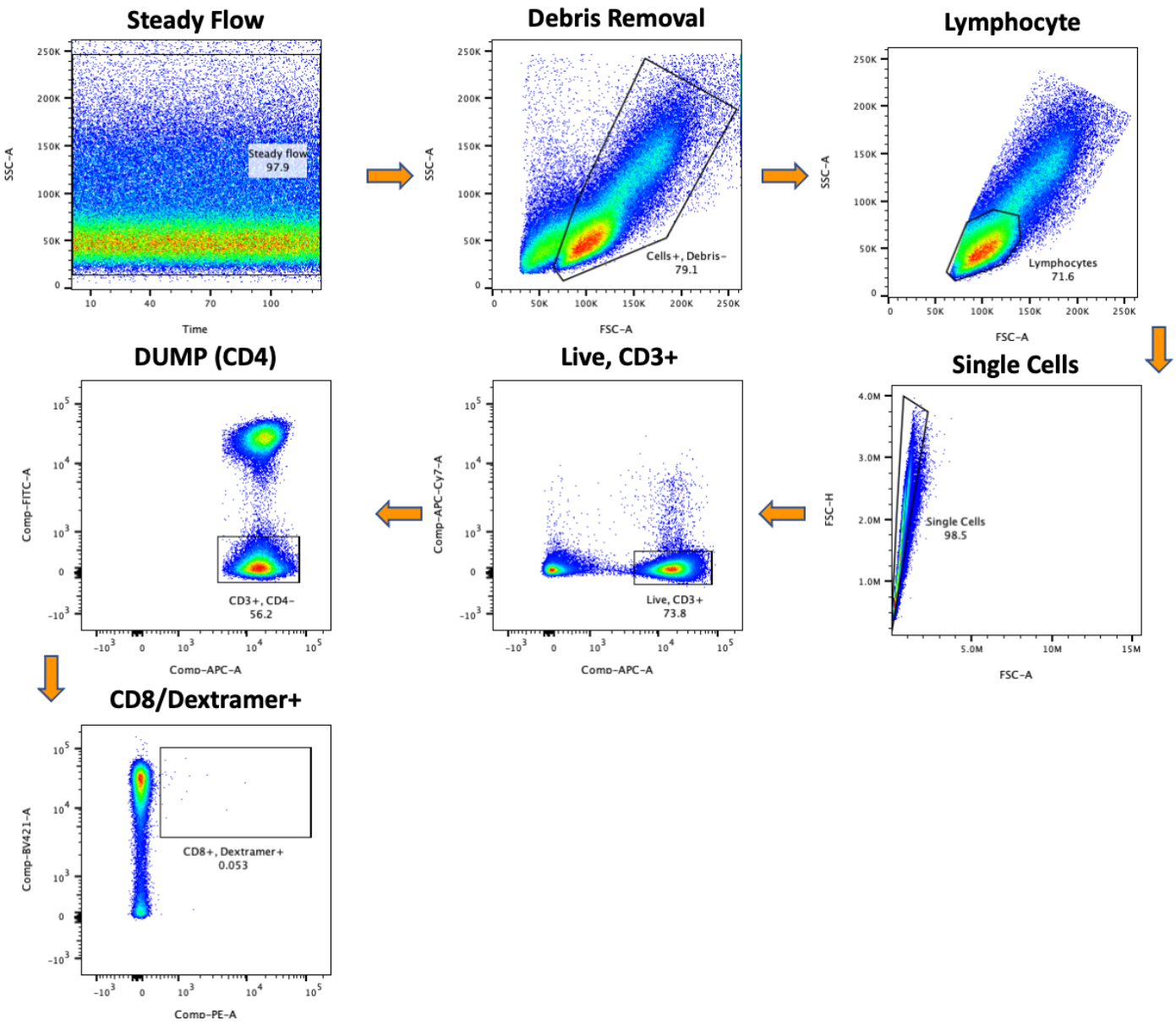

Fig. 41 Example gating strategy for detection of antigen-specific CD8+ T cells stained with MHC I Dextramer® reagents.

5.2 Add Statistical values to samples

Once gates are set, statistical values may also be added. For example, if interested in the magnitude of staining of a particular molecule, add the MFI statistic to the sample.

a. To do this, go to the gate value, right click, and select "add statistic". Then select "Median" or "Geometric Mean" from the left menu and the fluorescence parameter from the right menu and select "Add". The main window should now update to include the MFI (Fig. 42).

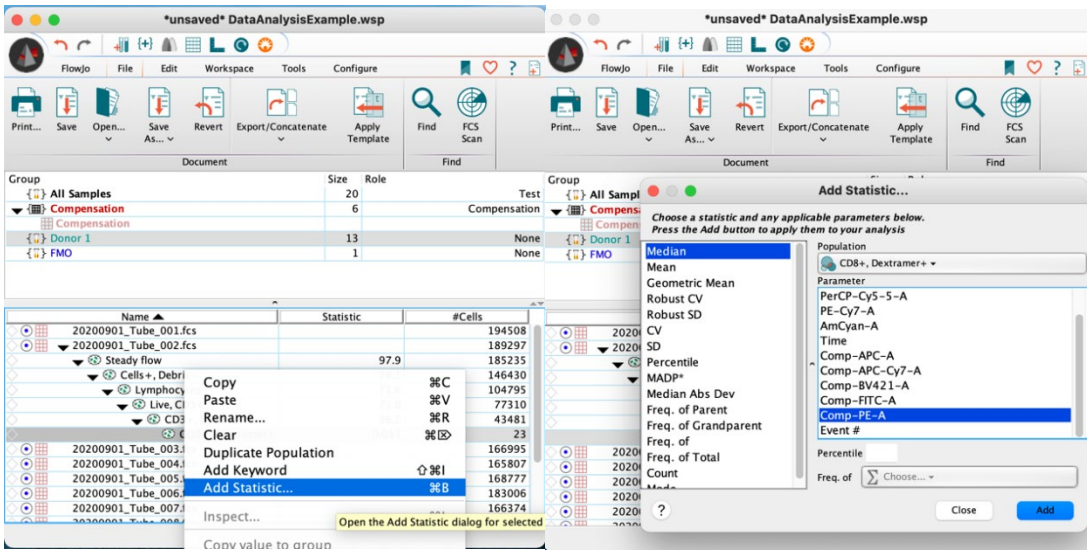

Fig. 43 Example of how to add statistics to a group or sample.

5.3 Copy analysis to group

a. Copy the gating strategy and statistics to the experimental sample(s) by highlighting the gates and dragging and dropping to a sample or group or alternatively by right clicking and selecting “Copy analysis to group”.

6 Analyzing and Styling Experimental Samples

Once the gating strategy has been applied, check each sample that the analysis strategy was applied to and ensure that the gates were applied appropriately, adjusting as needed. Once the gates are adjusted to satisfaction, the results can be visualized by individual selection one-by-one or alternatively in groups, using the "Layout Editor" tool to quickly generate graphical reports, or the "Table Editor" tool in the upper task bar to generate tabular reports.

6.1 Layout Editor Tool

a. Select the Layout Editor tool from the main workspace task bar to open a new window. Select the dotted line and adjust the space as needed (Fig. 44).

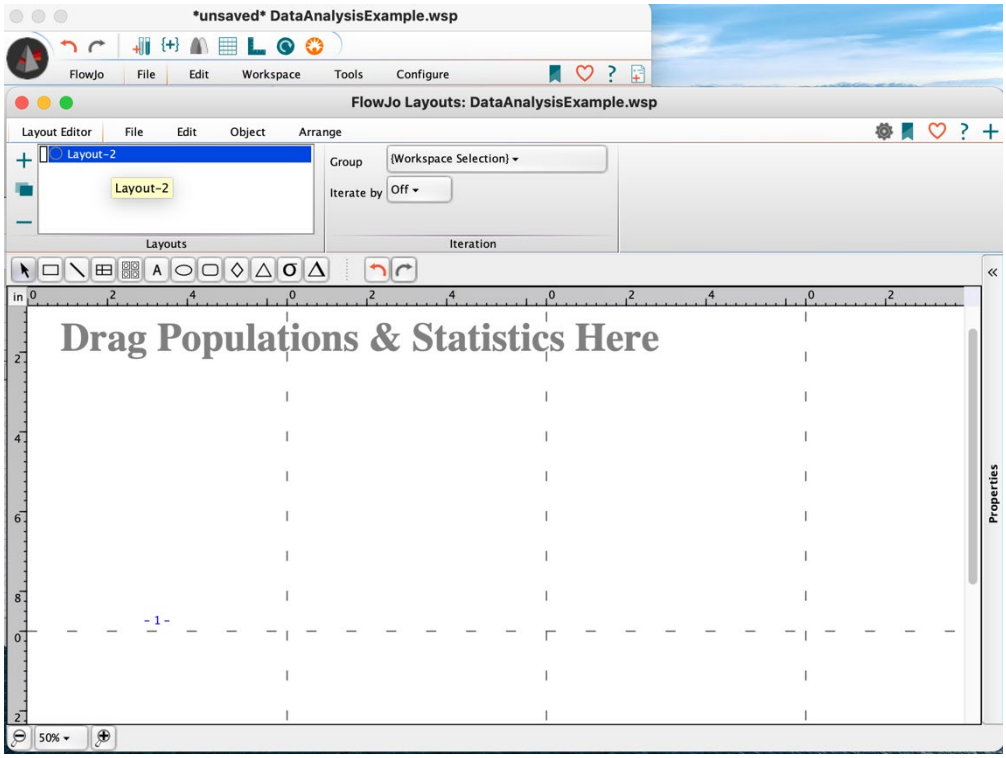

Fig. 45 The Layout Editor Tool window.

6.2 Add results to the Layout Editor tool

a. In the main window, select desired results and drag and drop them into the layout editor to visualize them simultaneously

b. To edit multiple graphs in the layout editor at the same time, in the same way, select them all. Alternatively, create a layout for the analysis of one sample and then use the "Batch Report" function to repeat the same analysis with the same organization style for all other similar samples.

c. For a structured style, drag the sample(s) into the "Grid" tool under Layouts (to maintain the axis, you must drag and drop it into the Layout editor first and then into the placed grid) (Fig. 46).

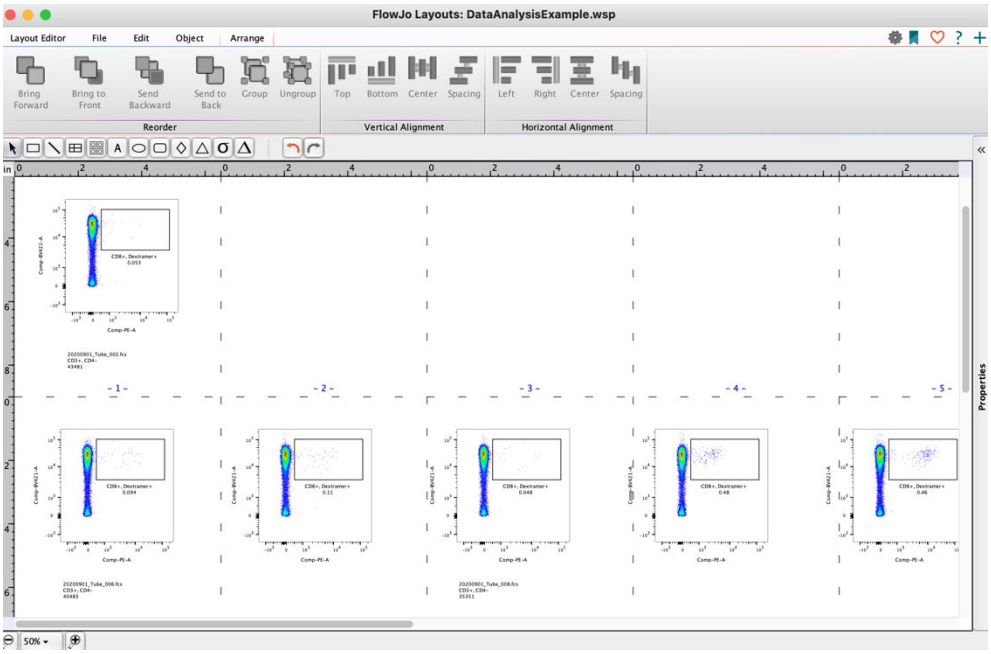

Fig. 47 Example of Layout Editor with flow plots

6.3 Quick overview of the gating strategy

a. If desired, quickly view the gating strategy by right clicking a plot and selecting "Ancestry".

6.4 Back gating

a. Confirm where the cells in the final plotted population originated from in the various gates by right clicking and selecting "Back gating". Back gating can also assist with identifying strategies for reducing background by optimizing gates (Fig. 48).

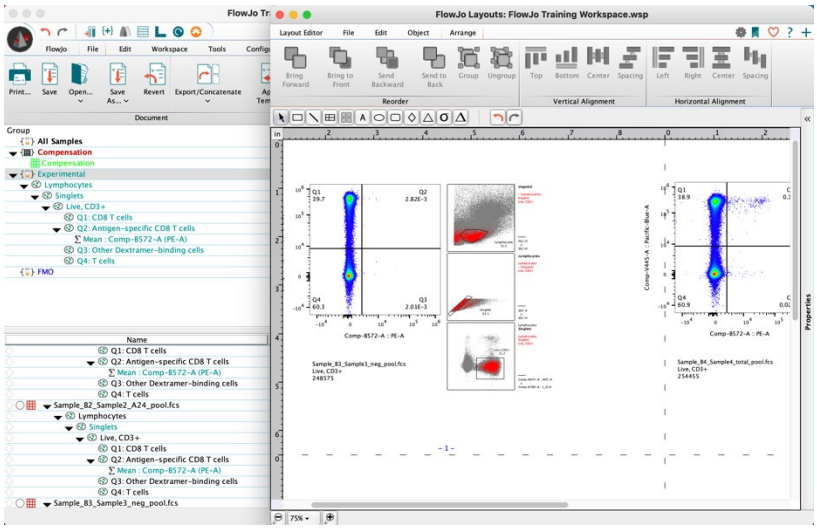

Fig. 49 Example of back gating.

6.5 Change plot properties

a. Change multiple plot properties by double clicking on the plot. This opens a new window with multiple tabs.

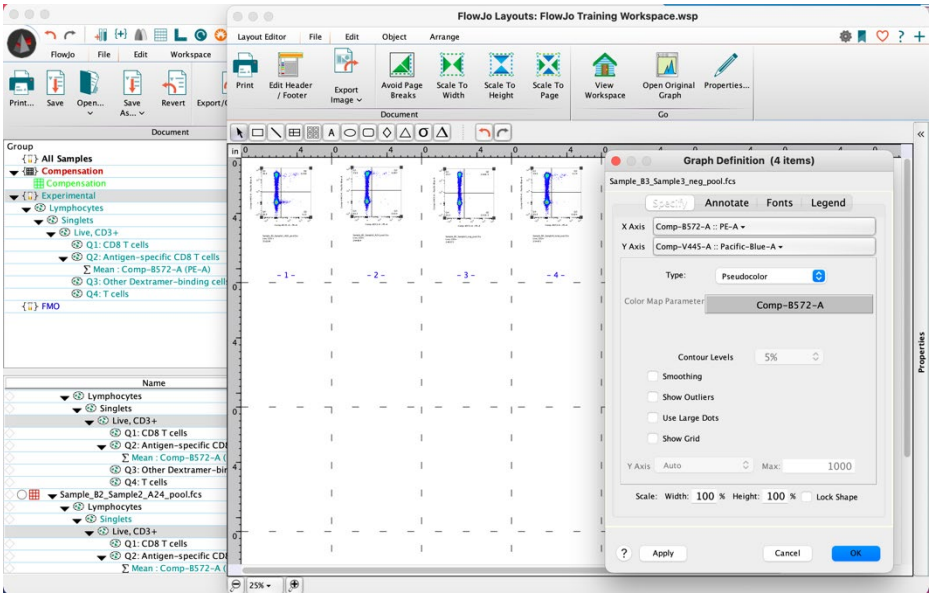

Fig. 50 Changing plot properties in the Layout Editor.

- **Specify tab**: Change the plot type and data visualization parameters. In the event of changing the Y axis to histogram, change the scale to Modal. To separate the populations for better visualization, right click on the plot, scroll down to "Histograms" and select the degree of offset preferred. To change the sample order or color, highlight the sample in the Legend
- **Annotate tab**: Change the axis parameters (tick marks, and labels) and what text is shown within the plot (gate positions, frequencies, population names)

- **Fonts tab**: Change the font, size and color of the text shown in plots
- **Legend tab**: Change what information appears in the legend.

Additionally, the object tab at the top of the layout editor allows you to change other style parameters of the plots, such as line style and thickness, background colors of the plots, etc.

6.6 Create overlays

a. Create overlays of data by highlighting the group in the main window and dragging and dropping onto the population inside of the layout editor. Alternatively, highlight the group in the main window, right click, "Select Equivalent nodes", and drag and drop on to the population in the layout editor (Fig. 51).

6.7 Remove layer in overlays

a. To de-select any populations being represented in the layout editor, select the legend, highlight sample to remove, right click, and "Remove Layer".

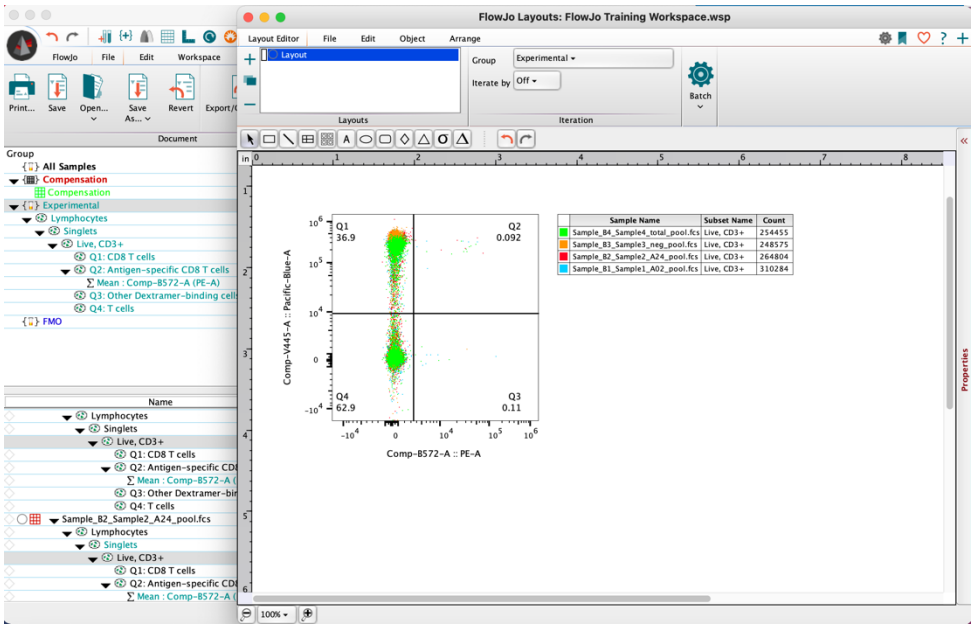

Fig. 52 Example of making overlays in Layout Editor.

6.8 Change population color

a. Change colors of the populations by clicking on the colored square next to the sample name and choosing a new color. To change the sample order, select the sample in the legend and drag to the desired position (Fig. 53).

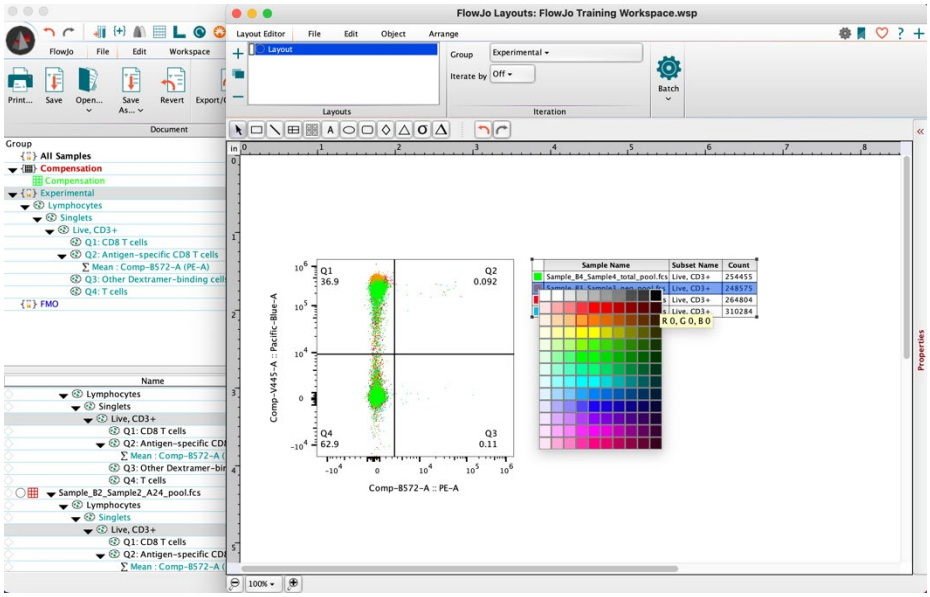

Fig. 54 Example of how to change color of an overlay in Layout Editor.

6.9 Repeat analysis for every sample

a. To perform the same analysis for every sample with the same layout, select “Create Batch Report”. Under the “Layout Editor” tab in the Layout Editor task bar, change to iterate by sample. Then select the Batch cog, change the organization parameters, and select “Create Batch Report”.

6.10 Iterating samples by panel

a. If an overlay was created, change the iteration to "Panel". Iterating by panel is like iterating by sample but iterates by N samples to combine them

b. To properly iterate by panel, the sample order within the group must be in the correct order, and the number, N, must be changed to the number of overlayed samples.

6.11 Iterating samples by keywords

If the data in a group is not in the order, you would like to batch; a keyword can be added to assign an order.

a. To do this, go to the main workspace and select "Add Keyword". Then add a keyword such as "Order" and numerical values for the order you would like the samples to be overlayed.

6.12 Iterating samples by a set of keywords

Adding an additional "set" keyword enables grouping samples together.

a. To do this, iterate by keyword, choosing to iterate by set and discriminate by order.

6.13 Include a control within each overlayed plot that does not change

a. To do this first drag the control population onto the overlay
b. Right click and select "Set as Control" within the legend. The text within the legend will now change to italics and the control will be included in each iteration during the batch analysis
c. Any analysis done can be exported and saved as a photo in various formats or as a PDF.

For more information on making and exporting plots, see Graphical Reports in FlowJo™ Software or more at FlowJo™ University.

6.14 Table Editor Tool

Selecting the Table Editor tool from the main workspace task bar will open a new space to organize numerical data (Fig. 55).

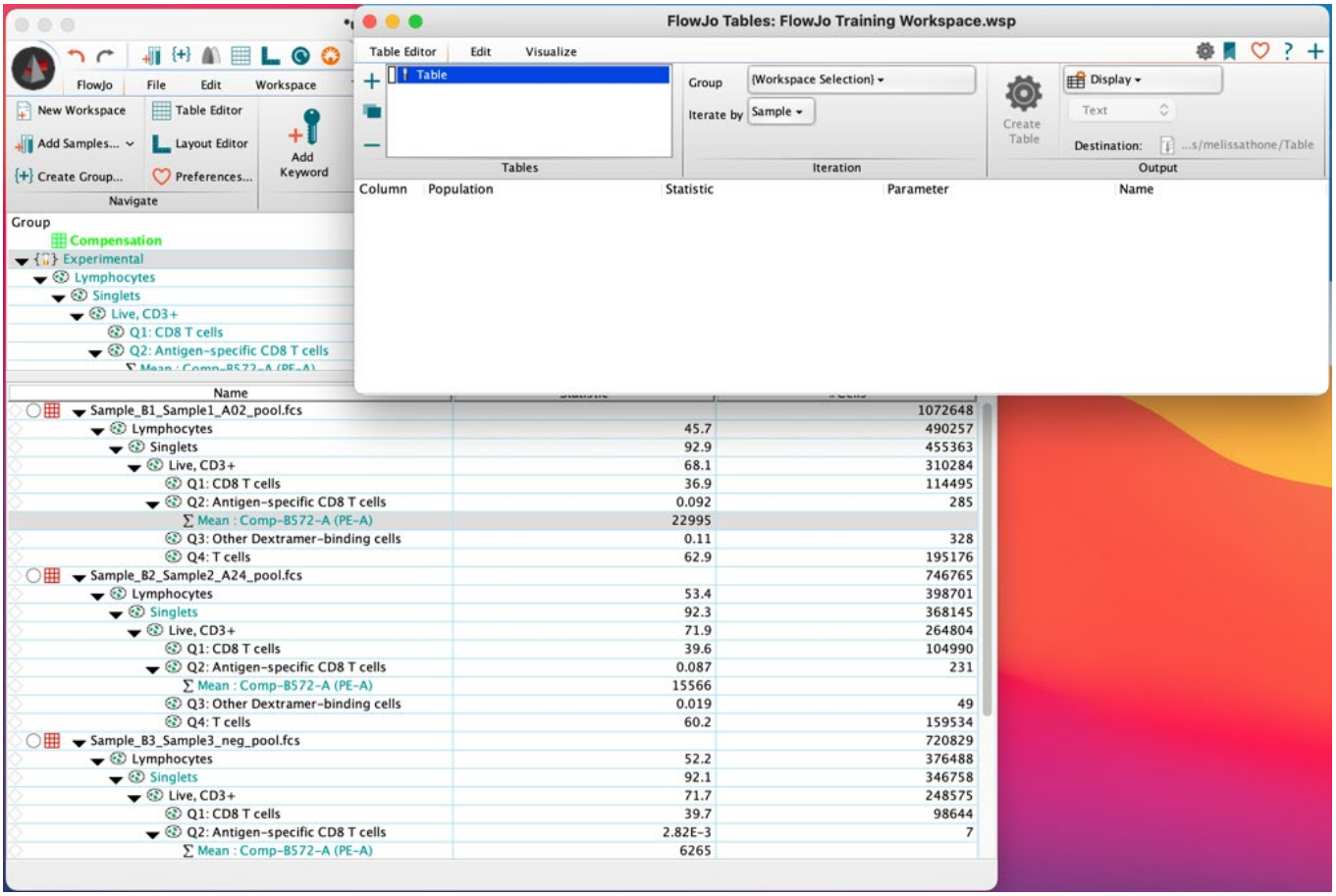

Fig. 56 The Table Editor tool window.

6.15 Add results to the Table Editor tool

a. Drag and drop populations or statistical values into the Table Editor window. Similar to the batch function described in the Layout Editor section, you can also iterate by sample (Fig. 57).

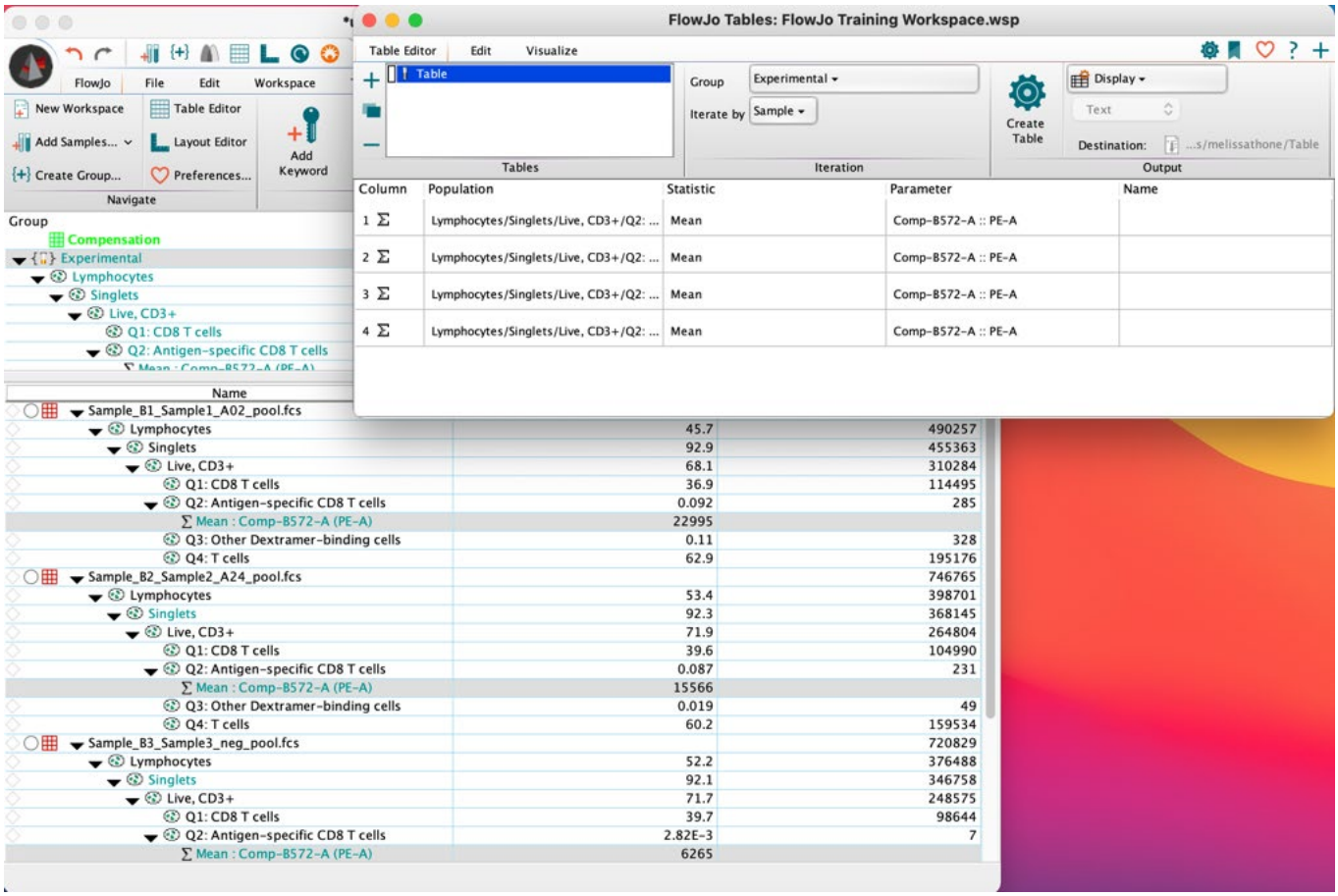

Fig. 58 Example of dragged and dropped populations in the Table Editor window.

6.16 Make a Heatmap

a. To make a heatmap of the values, select the values of interest, and under the "Visualize" tab select heatmap. This should add heatmap signals next to the values in the table. Returning to the "Table Editor" tab and pressing "create table" generates the heat map (Fig. 59).

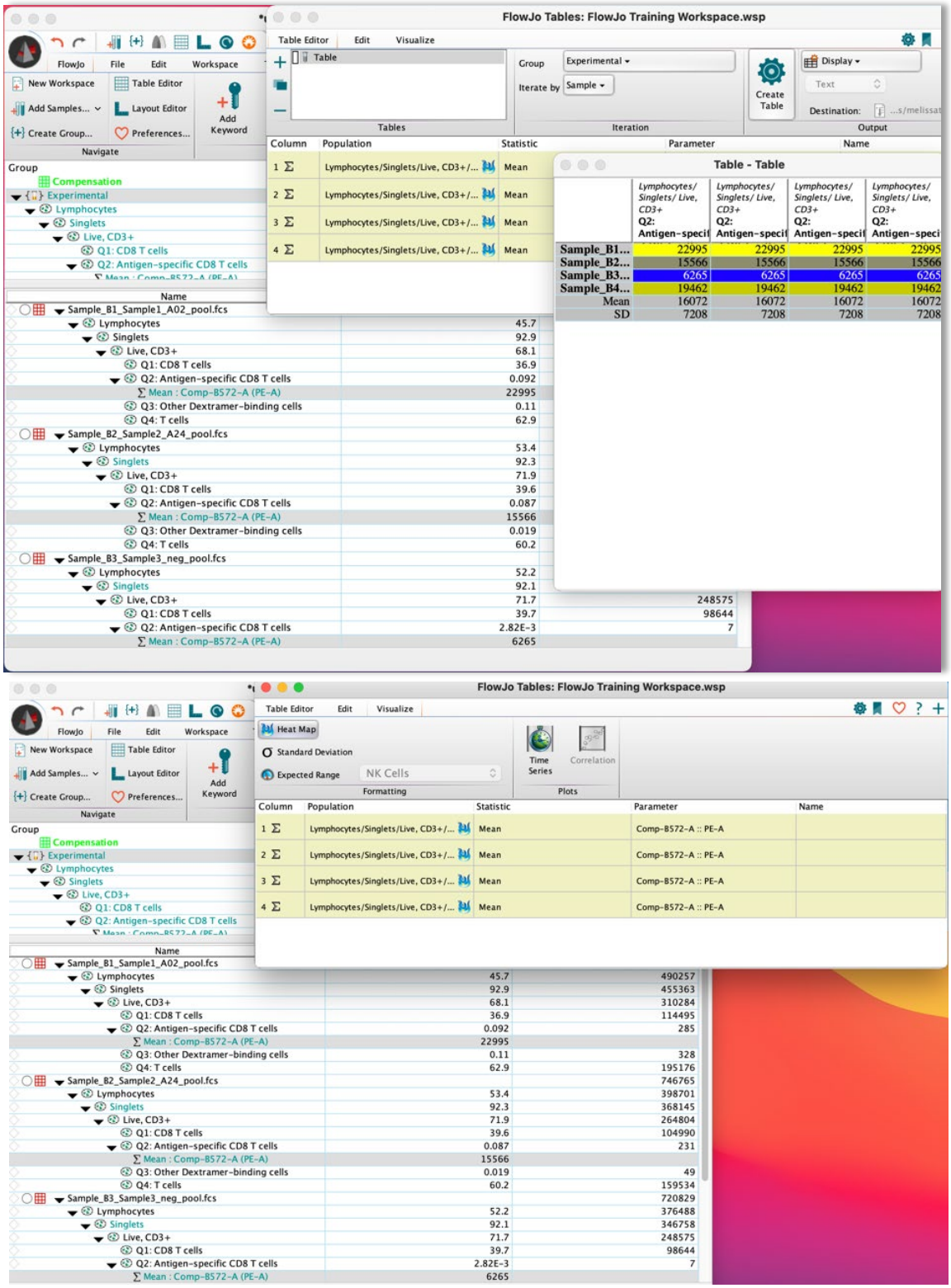

Fig. 60 Using the Table Editor Tool to create heat maps.

6.17 Export as CSV file

a. This can be saved and exported as a CSV file to do further analysis in Excel or GraphPad Prism. Alternatively, export to the current layout so that the graphical and statistical data can be visualized simultaneously (Fig. 61)

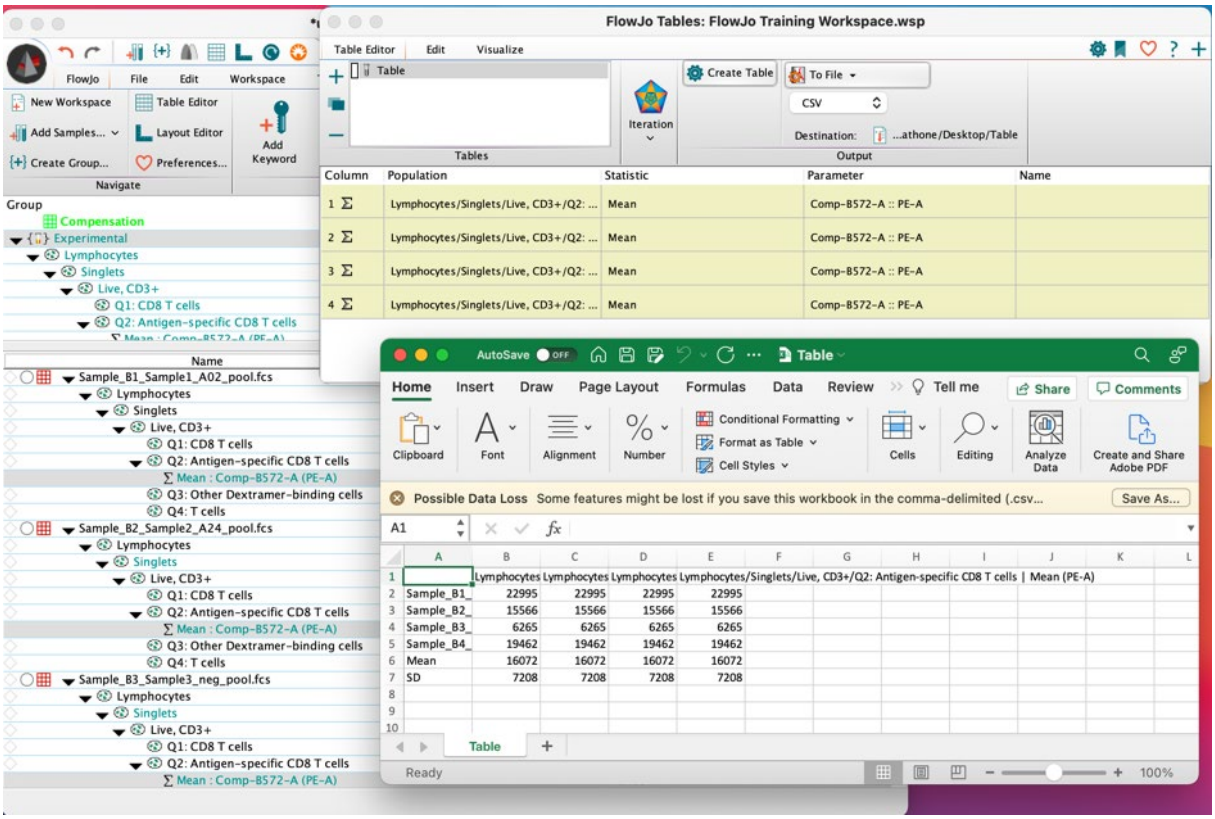

Fig. 62 Saving and exporting Table Editor data as a CSV file.

b. For more information on making and exporting Tables, see Tabular Reports in FlowJo™ Software or more at FlowJo™ University.

7 Creating a Template

Templates are useful for repeated analysis on similar samples with the same panel design.

7.1 Setting up a template

a. The parameter names must remain the same for a template to work in the future. Parameter names are sometimes the detector name alone or a combination of the detector name and the stain name
b. The panel design must not change, and the same flow cytometer should be used to acquire the data
c. Once you have finished analyzing your data, applied the gates to your samples, and properly set up the iterations in the layout and table editor tools, you can save them as a template to be applied in the future.

For more information, please see Templates in FlowJo™ Software.