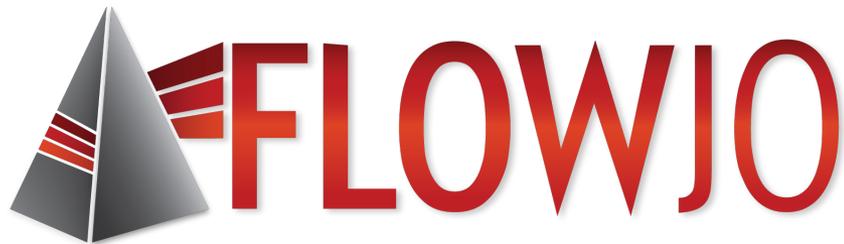


Cytometry Data Analysis in FlowJo V10



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Outline – Part I

- What is FlowJo?
- Navigating the V10 Workspace
- Customizing Ribbons
- Demo Data Background
- Creating and Editing Groups
- Graphs, Gating and Ancestry
- The Layout Editor
- Batching and Exporting Graphics
- The Table Editor



What is FlowJo?

- An integrated environment for viewing and analyzing flow cytometry data.
- Uniformly analyze whole experiments encompassing many related samples.
- Sophisticated tools allow generation of graphs and statistical reports, driving discovery of biological mechanisms.

The FlowJo v10 Workspace

- A graphical interface to organize your data.

The screenshot displays the FlowJo v10 workspace interface. At the top, there is a ribbon menu with tabs for 'FlowJo', 'File', 'Edit', 'Workspace', 'Tools', and 'Configure'. Below the ribbon, there are several toolbars and panels. The main workspace is divided into three main sections:

- Group Analysis:** A tree view showing the hierarchy of data groups. The selected group is '20140711 Rag1_BLT Act Baseline TQC', which is further divided into 'Time', 'Singlets', 'Live', and 'huCD45'. Under 'huCD45', there are four quadrants: 'Q1: CD4-, CD8+', 'Q2: CD4+, CD8+', 'Q3: CD4+, CD8-', and 'Q4: CD4-, CD8-'.
- Group Table:** A table showing the size and role of each group. The selected group has a size of 19 and a role of 'Test'.
- Sample Analysis Table:** A table listing individual samples with columns for Name, Statistic, #Cells, *PID, *Timepoint, *HIV Status, and *Sort. The table contains 29 rows of data, including samples like 'Mice_B629_015.fcs' and 'Mice_J1885_014.fcs'.

Ribbon
Tabs and Bands

Groups and
group analysis

Samples and
sample analysis

Ribbons, Tabs and Bands

- Ribbon organization allows easy visual navigation of workspace functions.

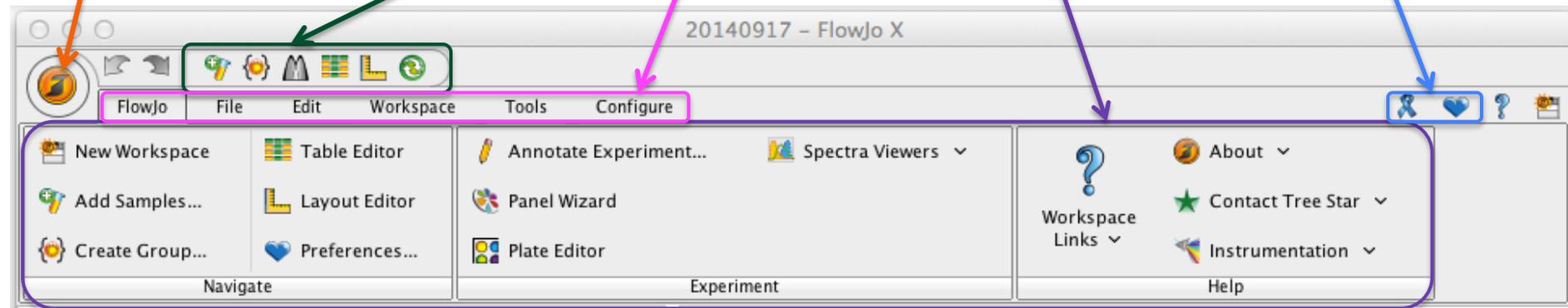
Application Button

Task Bar

Tabs

Bands

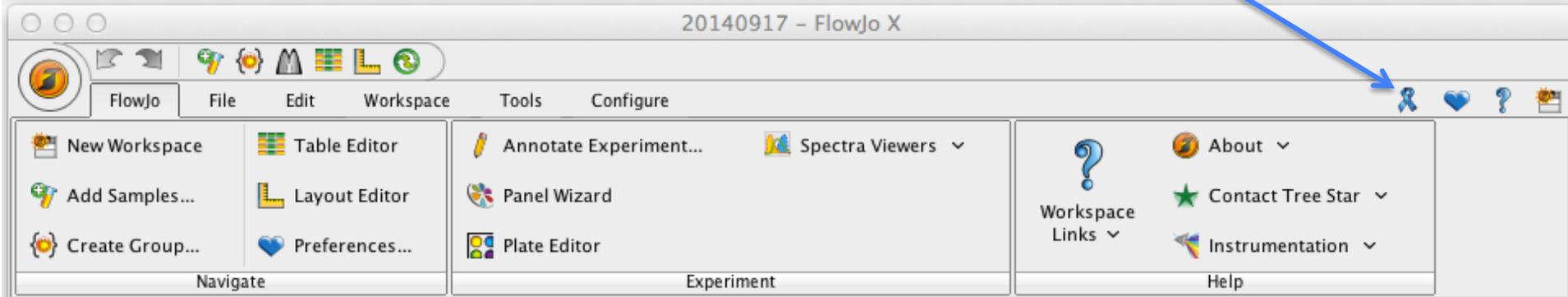
Ribbon Configuration & Preferences



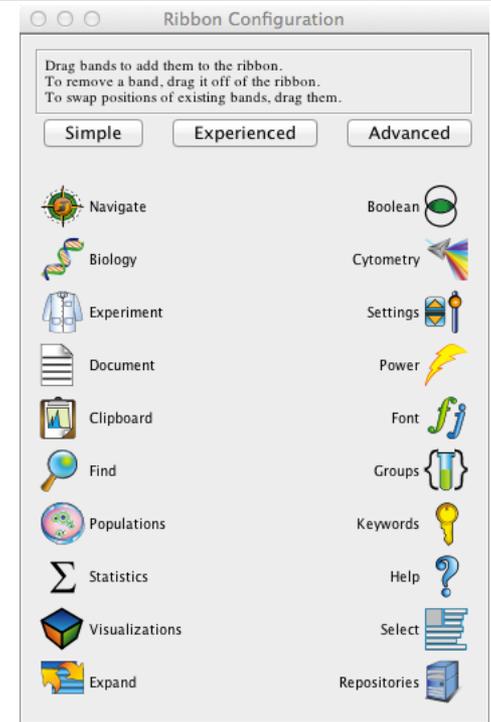
- Tabs group similar Bands together.
- Bands group similar Actions together.

Customizing Ribbons

- Click on the Ribbon icon to configure



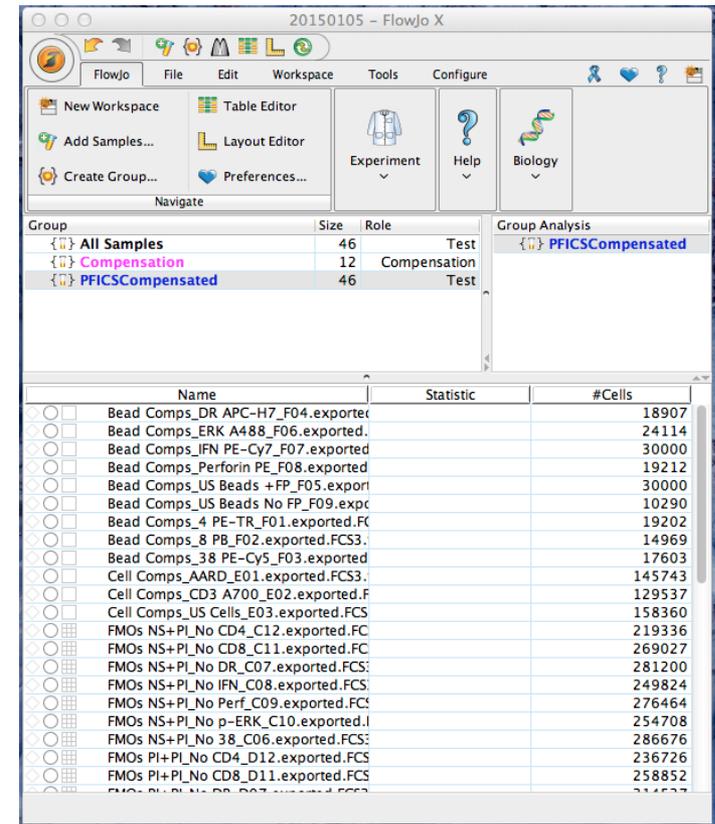
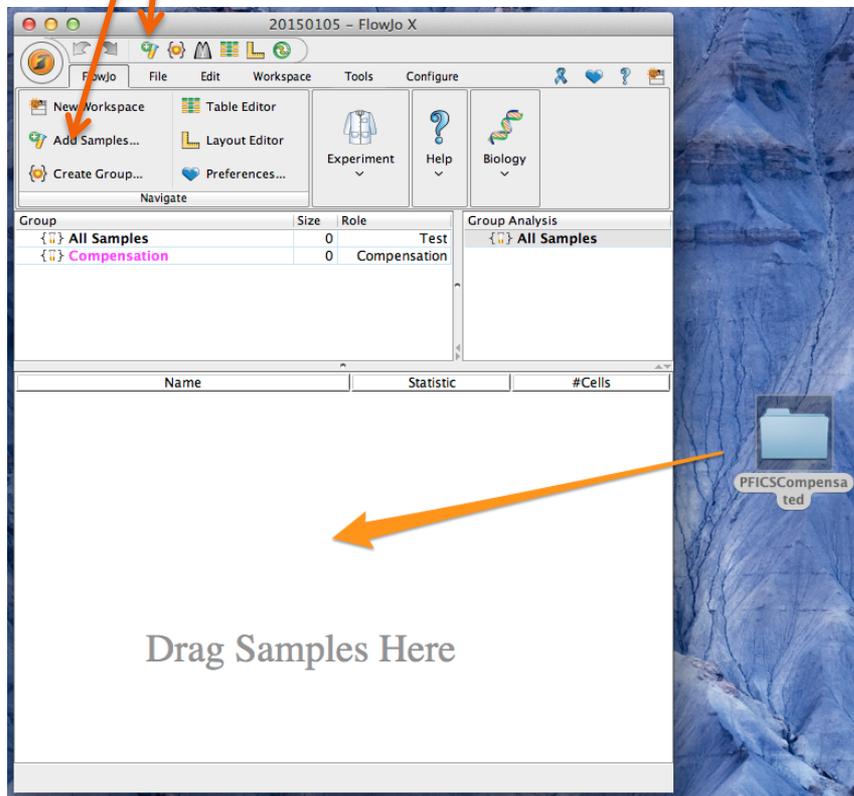
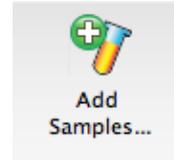
- Click on Simple, Experienced or Advanced to change the number of Tabs displayed.
- Drag the icon for any Band into the ribbon to add that set of Actions to your selected Tab.



Importing Data Into FlowJo

Three possible methods:

1. Drag and drop into samples pane
2. Click Add Samples button
3. Press  ;



Today's Demo Data Set: Phospho-Flow + Intracellular Cytokine Staining (PFICS)

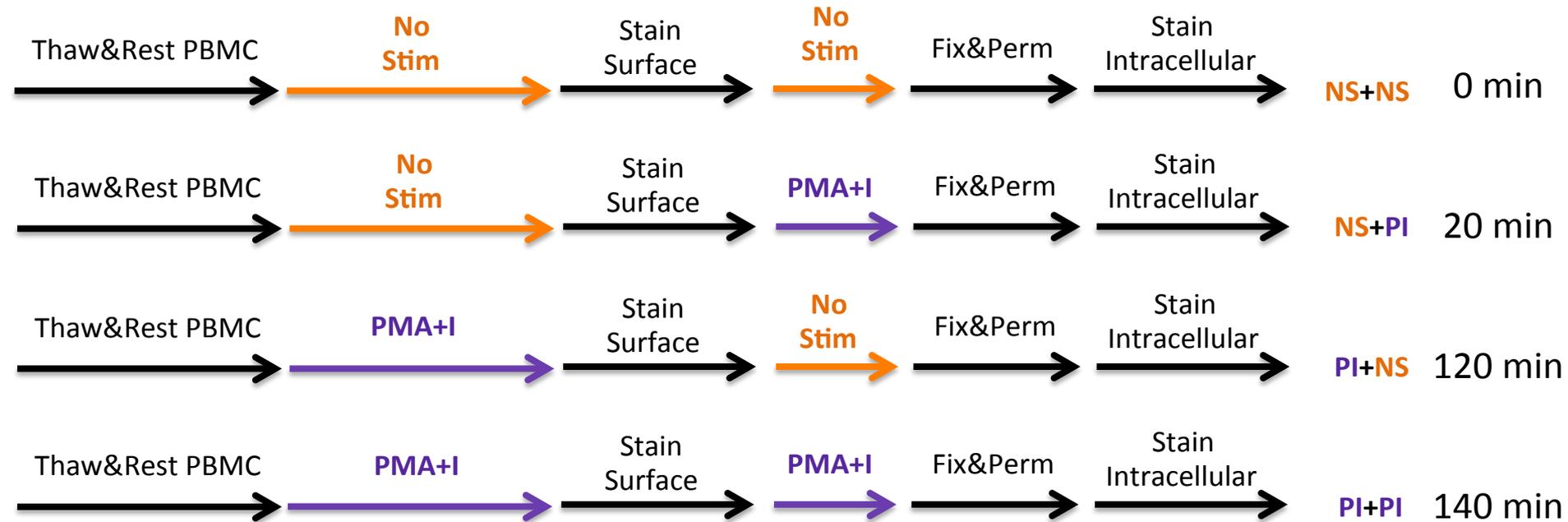
Polyclonal PFICS Assay:

- Thaw and rest cryopreserved human PBMC overnight
- No Stim (NS) or stimulate with PMA+Ionomycin (PMA+I or PI) for 2 hours
- Stain for viability (AARD) and surface antigens (CD3, CD4, CD8, CD38 and HLA-DR)
- No Stim or stimulate PMA+I for 20 minutes
- Fix, permeabilize and stain for intracellular antigens (phospho-ERK1/2, IFN- γ and Perforin)

PFICS Stim Conditions

- 2 Stims → 4 potential combinations

Total
Stimulation
Time



- 5 donors X 4 conditions = 20 experimental samples
- 1 donor with 7 FMOs X 2 stim conditions = 14 FMO controls
- 12 Compensation Controls

Groups and Group Analysis

- The Group area lists all groups in the Workspace, # of samples in each group (Size), and the Role of that group (ex. Test, Compensation, Controls) .
- Groups act like folders to organize your sample files and allow unified master gating and analysis.

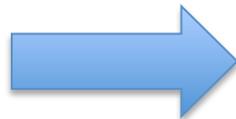
Group	Size	Role
{ } All Samples	46	Test
{ } Compensation	12	Compensation
{ } Export	20	Test
{ } FMOs	14	Controls
▼ { } PFICSCompensated	34	Test
Time		
Single Cells		
Lymphocytes		
Live		
CD3+		
Q1: CD4- , CD8+		
Σ Median : p-ERK1_2 (Comp-Ax488-A)		
INFg+		
Perf+		
pERK+		
Q2: CD4+ , CD8+		
Q3: CD4+ , CD8-		
Q4: CD4- , CD8-		

Group Analysis
▼ { } PFICSCompensated
Time
Single Cells
Lymphocytes
Live
CD3+
Q1: CD4- , CD8+
Σ Median : p-ERK1_2 (Comp-Ax488-A)
INFg+
Perf+
pERK+
Q2: CD4+ , CD8+
Q3: CD4+ , CD8-
Q4: CD4- , CD8-

- Group Analysis displays all analysis within a group.

Creating and Editing Groups

- To create a new group type \mathbb{H} G, or click the Create Group Icon located in either the task bar at the top of the workspace, or within the Navigate band.



- Double click on an existing group to edit its properties.

A screenshot of a software dialog box titled "Create Group". The dialog is organized into several sections:

- Appearance:** Includes a "Name" text field, a "Color" dropdown menu (currently set to a blue square), a "Style" dropdown menu (currently set to "Plain"), a "Role" dropdown menu (currently set to "None"), and a "Parameter Key" text field.
- Sample Inclusion Criteria:** Features a "Live group" checkbox (checked) and a "Synchronized" checkbox (unchecked). Below this is a text area labeled "Include samples that use the following staining:" containing the text "CCR5, HLA-DR, CD38, CD4, huCD45, CD3, CD8, Dead". To the right of this text area is a "Multiple" button. Below the text area is a "Keyword" dropdown menu, an "=" operator dropdown, and a "Choose..." button. There are also "More Choices" and "Fewer Choices" buttons, and a "Show all keywords in menus" checkbox.
- With reference to samples in another group:** Contains two radio buttons: "Only choose from" (unchecked) and "Also include all" (checked). To the right of "Also include all" is a dropdown menu labeled "samples in Group" with the value "(No specified group)".
- Assignments:** Contains two rows, each with an "Add Keyword:" text field and a "With Value:" text field.

At the bottom of the dialog are four buttons: "Help with Groups", "Apply Changes", "Close", and "Create Group".

Samples and Sample Analysis

- Displays the sample list and associated analysis of the currently selected group.
- Statistic and #Cells columns are displayed by default. Additional information can be displayed as columns. (Workspace Tab → Add Keywords or Configure Tab → Edit Columns)

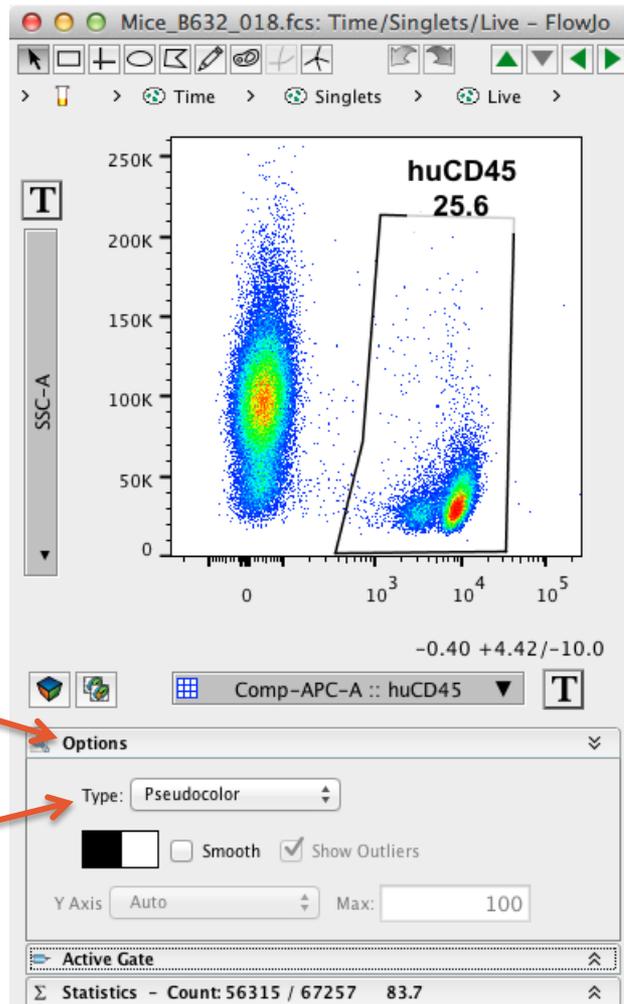
Name	Statistic	#Cells	*PID	*Timepoint ▲	*HIV Status	*Sort
▶ Mice_B629_015.fcs		79508	B629	7_11_14	Neg	1
▶ Mice_B630_016.fcs		181870	B630	7_11_14	Neg	1
▶ Mice_B631_017.fcs		130338	B631	7_11_14	Neg	1
▶ Mice_B632_018.fcs		69680	B632	7_11_14	Neg	1
▶ Mice_B633_019.fcs		59878	B633	7_11_14	Neg	1
▶ Mice_J1872_020.fcs		41158	J1872	7_11_14	Neg	1
▶ Mice_I1873_021.fcs		57018	I1873	7_11_14	Neg	1

SOP

- Double click on a sample to open a Graph Window and add gates.

Graphs

- The Graph Window facilitates data visualization and gating.



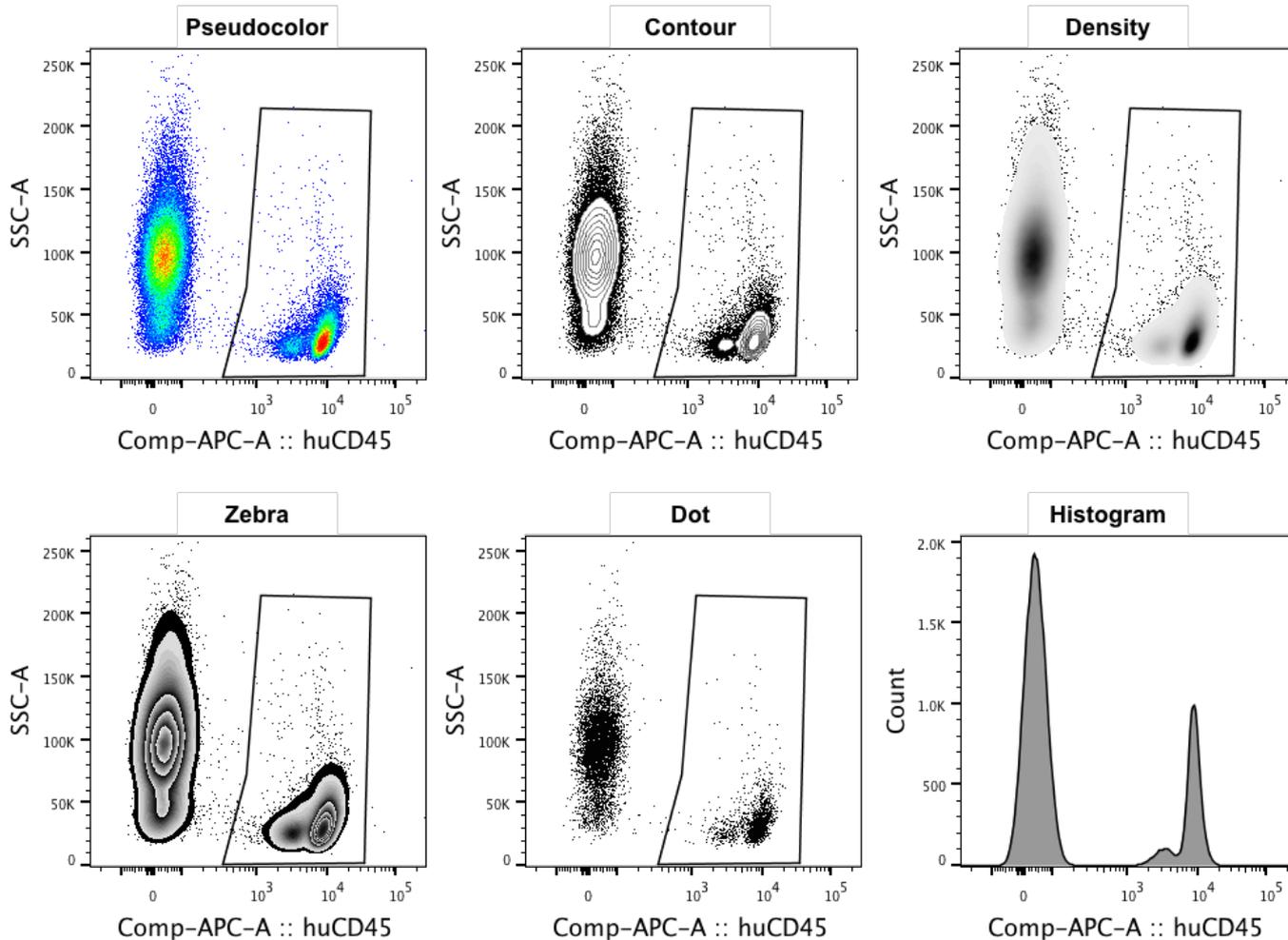
- Several different kind of plots are available to display flow data.
- Click on the Options Menu below the graph image and select graph Type from the dropdown menu.

Options

Graph Type

Graph Display Options

- Try them all and pick what pleases you, or best represents your data.

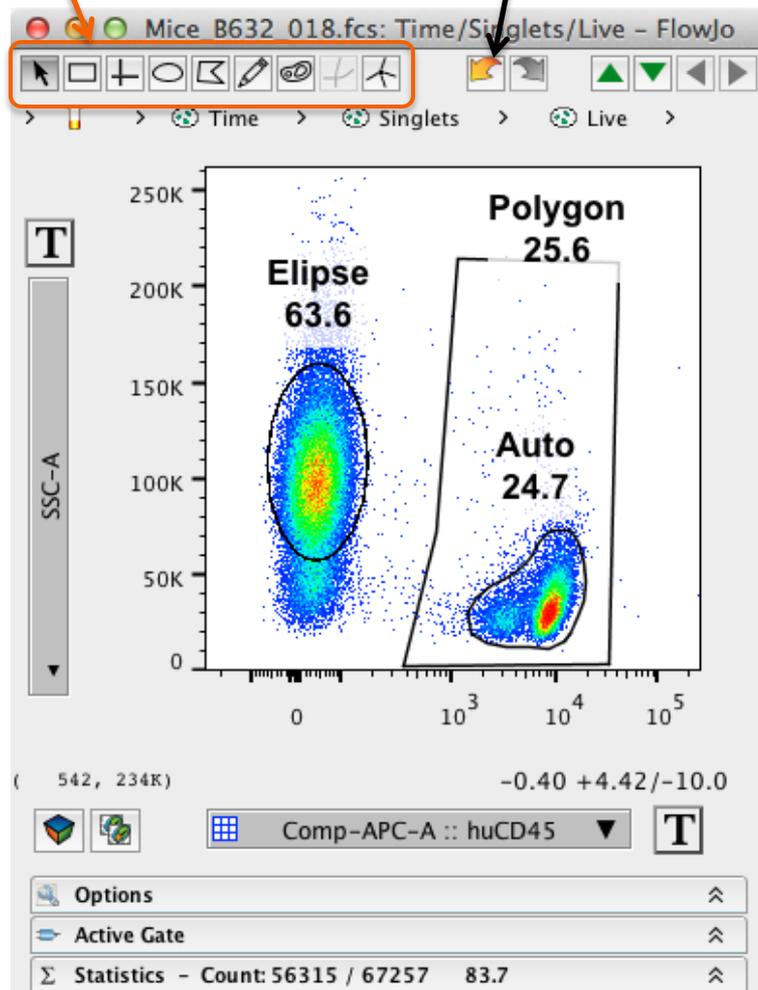


Gating tools

- Are located at the top of a Graph Window.

Gating Tools

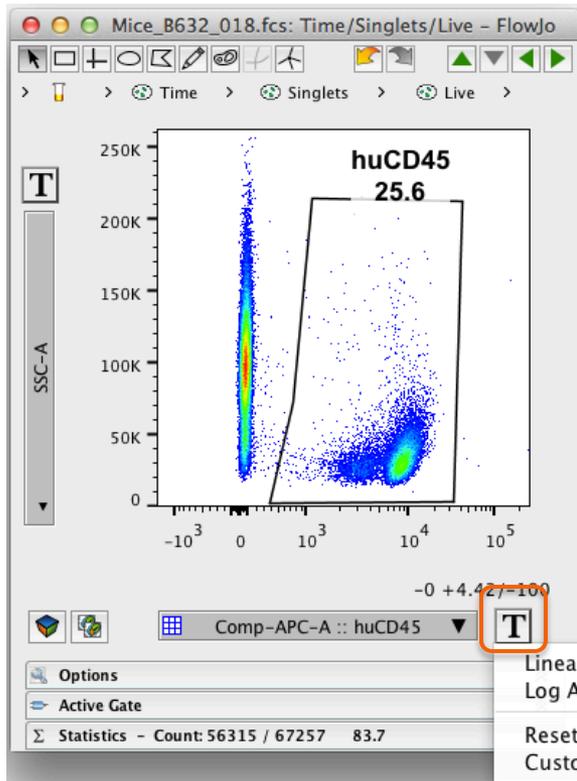
Undo!!



- Gates can always be modified or removed, so don't be shy.
- Explore the gating options and pick what works best for you.

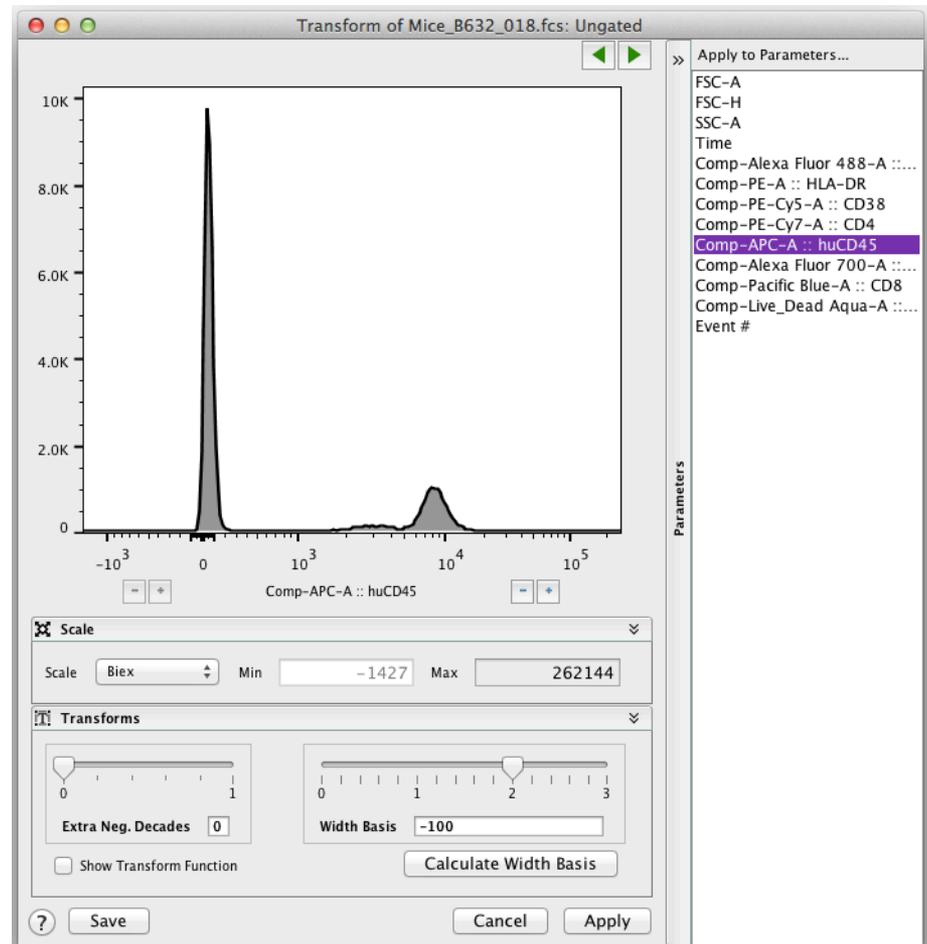
Transforming Data

- Your data may initially look ‘squished’.
- Click the Transformation icon to change the visual display.



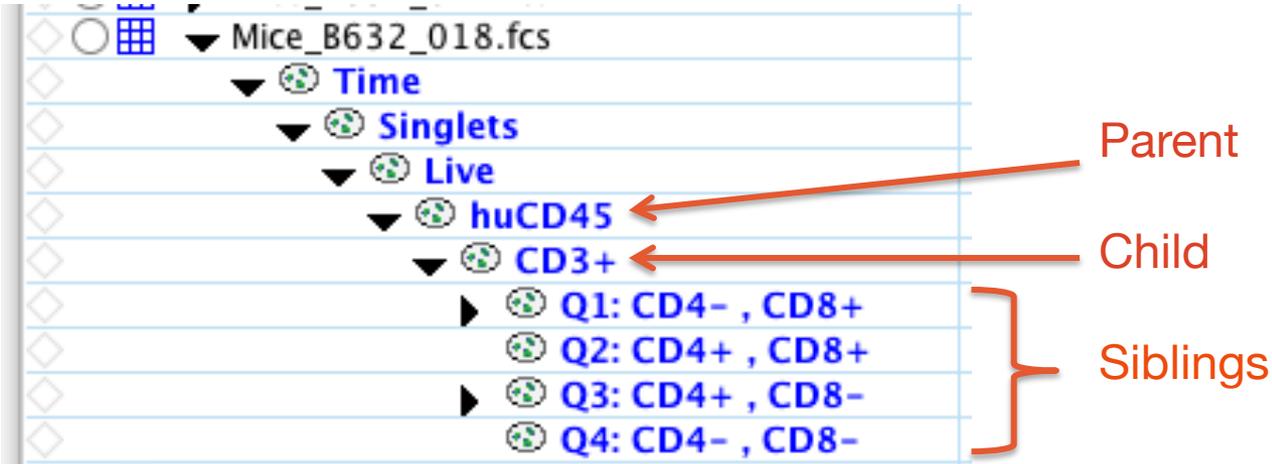
Custom Transform

Linear Axis
Log Axis
Reset
Customize Axis...



Gating Hierarchy

- When you create a gate on a sample, FlowJo shows you this gate (subset population) as a genealogical tree.
- The subset population is a child of the parent sample.



The Layout Editor

- A tool for creating graphical reports.
- Type  L, or click on the Layout Editor icon.
- Drag populations from a sample to Layout Editor.



Layout Editor

The screenshot shows the FlowJo software interface. The main window is titled "20140917 Tabs_Group" and "FlowJo Layouts---20140920". The interface includes a menu bar (File, Edit, Object, Arrange), a toolbar, and a workspace. The workspace is divided into several panels:

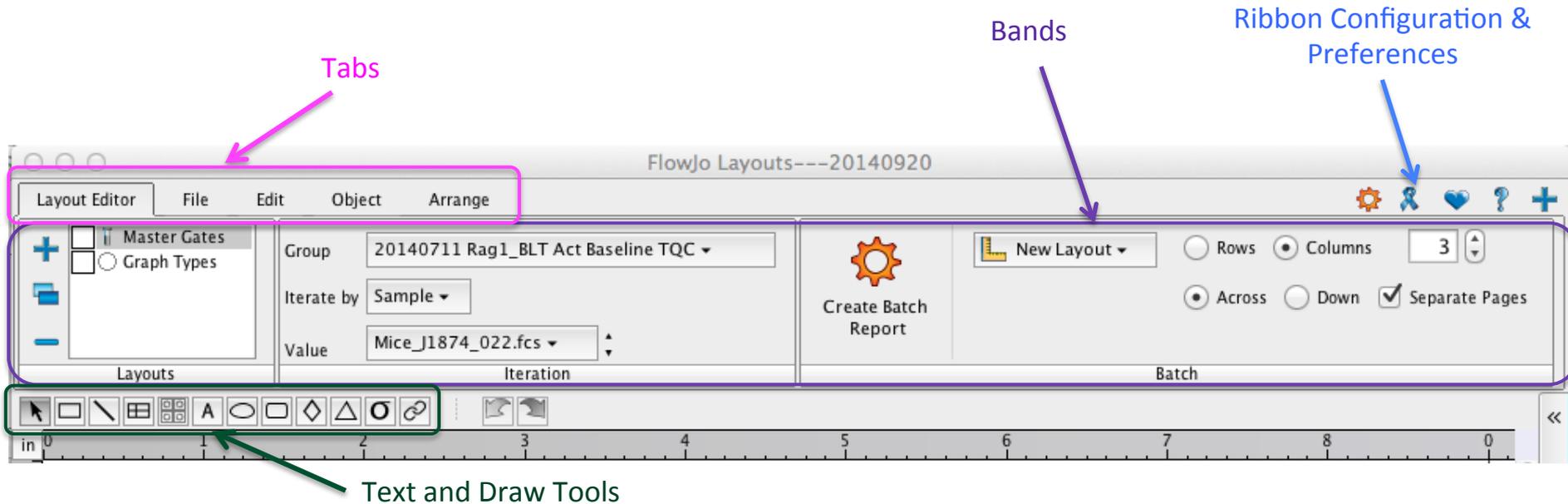
- Left Panel:** A tree view showing the sample structure. The selected population is "Mice_B632_018.fcs" > "Time" > "Singlets" > "Live" > "huCD45" > "CD3+".
- Right Panel:** A table showing the statistics for the selected population.
- Main Plot Area:** Two flow cytometry plots. The left plot shows "CD3+" (81.7%) and the right plot shows a 2x2 quadrant plot for "CD3+" (Q1: 34.4%, Q2: 2.16%, Q3: 62.3%, Q4: 1.09%).

Group	Size	Role
{ } All Samples	29	Test
{ } 20140711 Compensation	10	Compensation
{ } 20140711 Rag1_BLT Act Baselin	19	Test

Name	Statistic
▼ Mice_B632_018.fcs	
▼ Time	99.1
▼ Singlets	97.4
▼ Live	83.7
huCD45	25.6
▼ CD3+	81.7
Q1: CD4-, CD8+	34.4
Q2: CD4+, CD8+	2.16
Q3: CD4+, CD8-	62.3
Q4: CD4-, CD8-	1.09

Working in Layout Editor

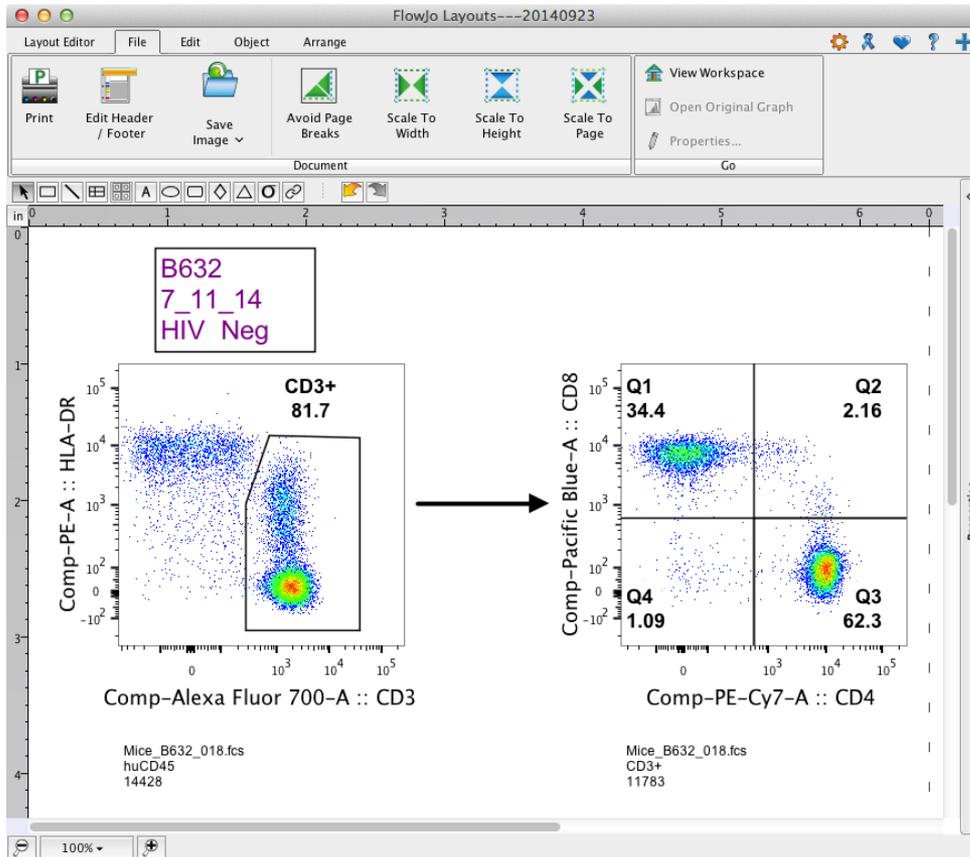
- Similar to the Workspace, the Layout Editor has its own customizable Ribbon with Tabs and Bands to organize actions.



- Try clicking on the different tabs to see what types of actions are available.

Within Layout Editor

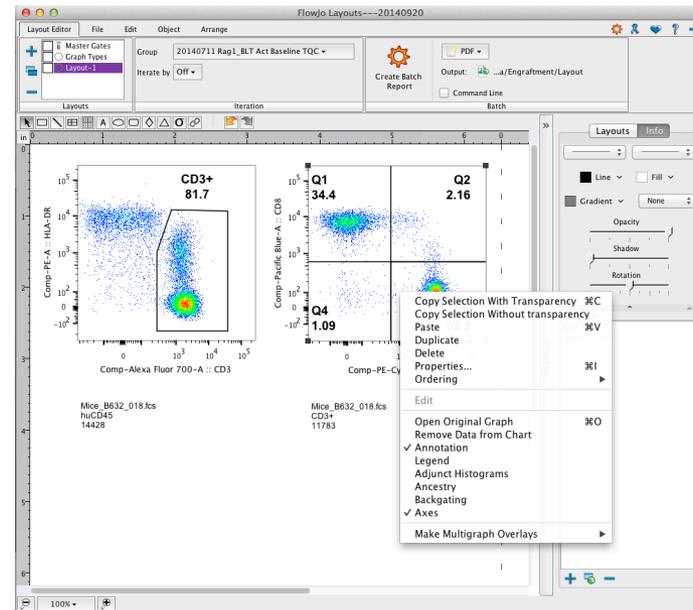
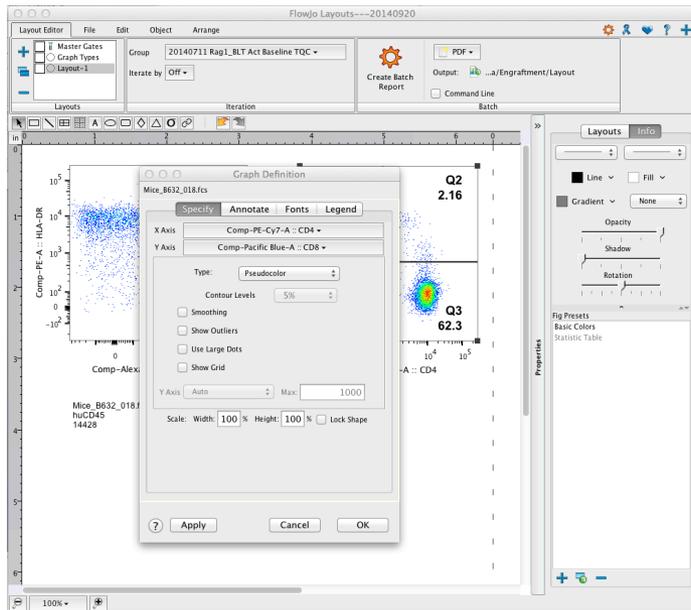
- Graphs can be organized and re-formatted.
- Statistics, keywords, text and even shapes or objects can be added to illustrate your analysis.



- We encourage you to explore the tools and display features available to improve the visualization of your data.

Working in Layout Editor

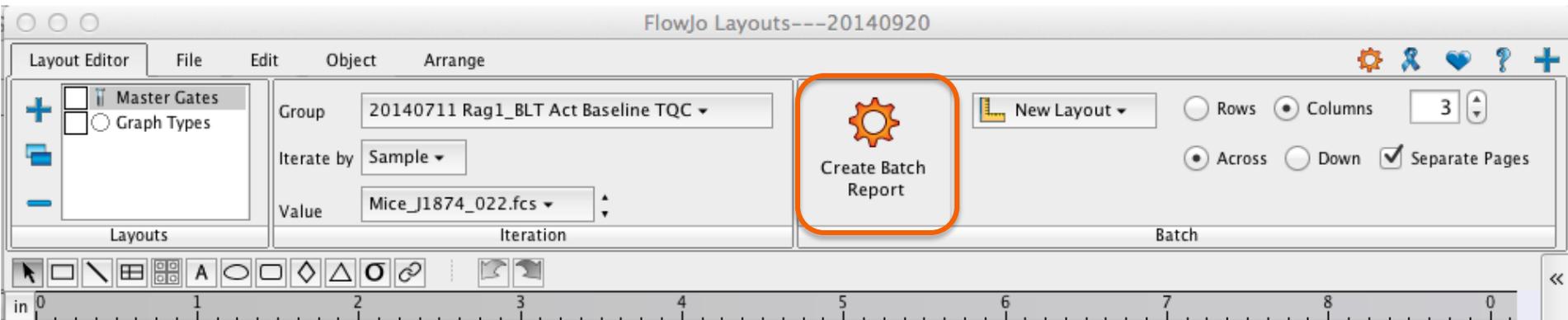
- Click once on a graph or object to select it.
- Double Click a graph to change its properties.



- Right click the graph for even more options.
- Hold down shift and click on multiple graphs to select and edit their properties simultaneously.

Batch Analysis of Layout Editor Graphics

- Batch operations perform repetitive analysis on multiple samples, applying the layout to an entire set of samples.
- Within the Layout Editor Tab, Look for the Create Batch Report icon.



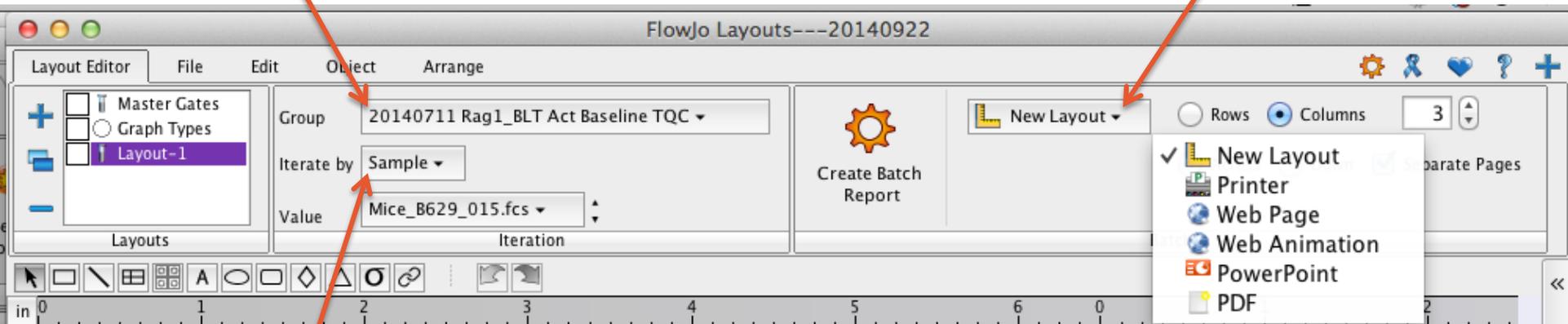
Batch Analysis of Layout Editor Graphics

- Specify the group you wish to batch, and how to iterate the batch process (ex. by sample or keyword), then specify where you want the batch output to go. Finally, click on



Batch Output

Group



Iteration Criteria

The Table Editor

- A tool for creating statistical reports.
- Type \boxplus T, or click on the Table Editor icon.
- Drag populations from sample to Table Editor.



Table Editor

FlowJo Tables:20140922

Table Editor Edit Visualize

Export 1
Export 2

Group: 20140711 Rag1_BLT Act Baseline TQC

Iterate by: Sample

To Clipboard
To Printer
Batch To Current Layout

Batch to Figure
To File: Excel

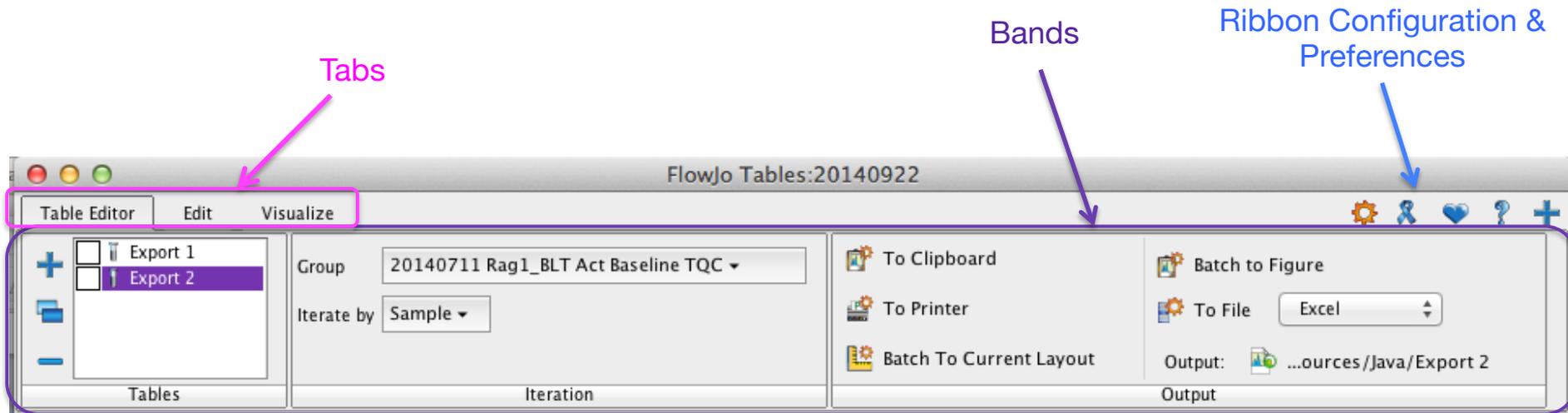
Output: ...ources/Java/Export 2

Col...	Population	Statistic	Parameter	Name
1	Σ Time/Singlets/Live/huCD45	Freq. of Parent		
2	Σ Time/Singlets/Live/huCD45/CD3+	Freq. of Parent		
3	Σ Time/Singlets/Live/huCD45/CD3+/Q1: CD4-, CD8+	Freq. of Parent		
4	Σ Time/Singlets/Live/huCD45/CD3+/Q3: CD4+, CD8-	Freq. of Parent		
5	*Sample ID			
6	*Timepoint			

Name	Statistic
Mice_B632_018.fcs	
Time	99.1
Singlets	87.4
Live	83.7
huCD45	25.8
CD3+	81.7
Q1: CD4-, CD8+	34.4
Q2: CD4+, CD8+	2.16
Q3: CD4+, CD8-	62.3
Q4: CD4-, CD8-	1.09

Within Table Editor

- Again, the Table Editor has its own customizable Ribbon with Tabs and Bands to organize actions.



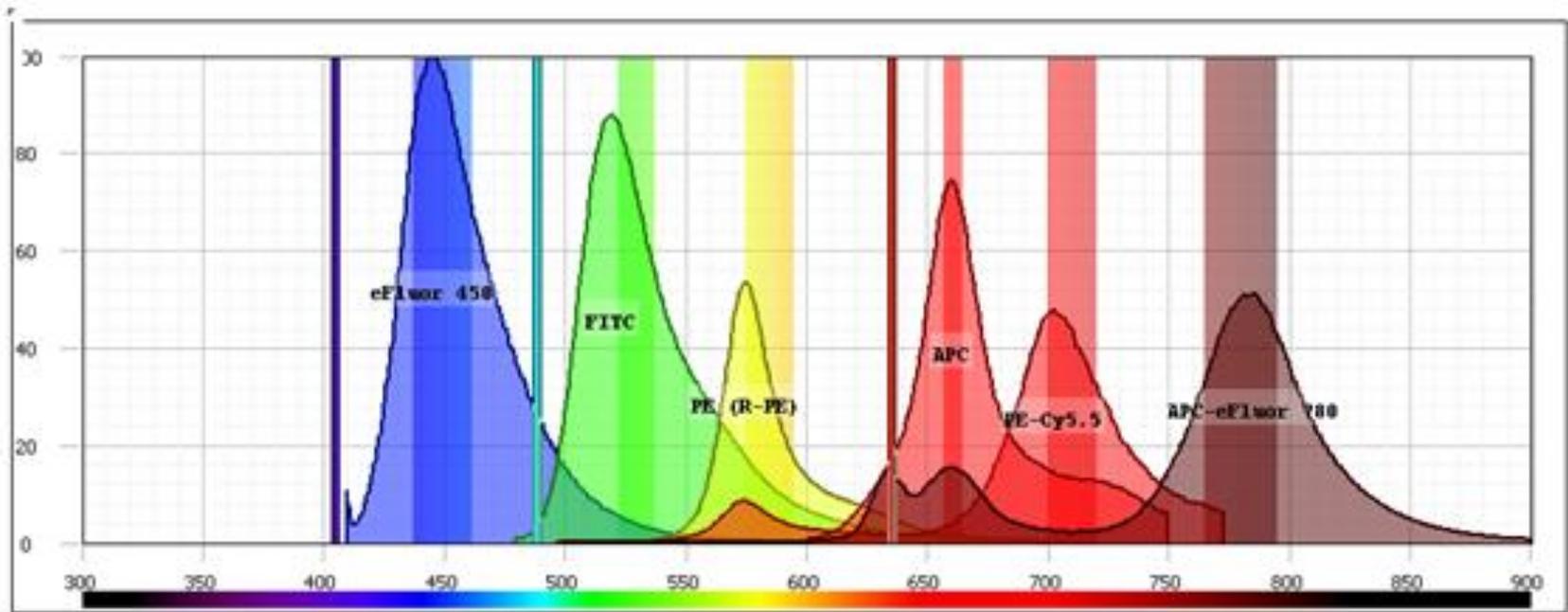
- Specify the group you wish to batch, and how to iterate the batch process, then in the Output band, specify where you want the batch output to go.

Outline – Part II

- Compensation
- Templates
- Command Line FlowJo

Compensation

- Compensation corrects for spillover between fluorochrome emission spectra.



- Compensation is essential for multicolor panels

Three Rules of Compensation

- First, there must be a single stained control for every parameter in the experiment!
- In Addition, there are three *rules* for ‘good’ compensation controls.
 1. Controls need to be at least as bright or brighter than any sample the compensation will be applied to.
 2. Background fluorescence should be the same for the positive and negative control.
 3. Compensation controls **MUST** match the exact experimental fluorochrome.

PFICS Compensation Controls

- PBMC Cells

1. Unstained Cells
2. AARD
3. CD3 Alexa700

- Compensation Beads

1. Unstained Beads with Fix and Perm
2. CD4 PE-TexasRed
3. CD8 Pacific Blue
4. CD38 PE-Cy5
5. HLA-DR APC-H7
6. Unstained Beads without Fix and Perm
7. p-ERK1/2 Alexa 488
8. IFN-g PE-Cy7
9. Perforin PE

Compensation Wizard

- Select a Compensation Group in the groups window, then click  in the task bar.



20140917 Tabs_Groups_Samples.wsp - FlowJo X

Control Group: Compensation

Apply To Group [M] Matrix Name: Compensation View Matrix... Finalized

Confirm gates and control assignments look correct. Double click a graph to edit it.

Parameter	Sample	Negative	Positive
Alexa Fluor 488-A	CCR5	Bead Comps_Unstained Beads_010.fcs:Size	Size/Alexa Fluor 488-A+
PE-A	HLA-DR	Bead Comps_Unstained Beads_010.fcs:Size	Size/PE-A+
PE-Cy5-A	CD38	Bead Comps_Unstained Beads_010.fcs:Size	Size/PE-Cy5-A+
PE-Cy7-A	CD4	Bead Comps_Unstained Beads_010.fcs:Size	Size/PE-Cy7-A+
APC-A	huCD45	Bead Comps_Unstained Beads_010.fcs:Size	Size/APC-A+
Alexa Fluor 700-A	CD3	Cell Comps_Unstained Cells_001.fcs:Size	Size/Alexa Fluor 700-A+
Pacific Blue-A	CD8	Bead Comps_Unstained Beads_010.fcs:Size	Size/Pacific Blue-A+
Live_Death Aqua-A	Dead	Cell Comps_Unstained Cells_001.fcs:Size	Size/Live_Death Aqua-A+

Name	Statistic	#Cells	*PID	*T
Bead Comps_CCR5 FITC_009.fcs		24728		7
Bead Comps_Unstained Beads_010.fcs		24549		7
Cell Comps_AARD Comp_002.fcs		1368...		7
Bead Comps_CD8 PacBlue_005.fcs		25459		7
Bead Comps_HLA-DR PE_006.fcs		24782		7
Bead Comps_CD4 PE-Cy7_004.fcs		25451		7
Cell Comps_Unstained Cells_001.fcs		98670		7
Bead Comps_CD45 APC_008.fcs		25369		7
Cell Comps_CD3 Alexa700 Comp_003.fcs		1355...		7
Bead Comps_CD38 PE-Cy5_007.fcs		26148		7

Alexa Fluor 488-A
Bead Comps_Unstained Beads_010.fcs

SSC-A vs FSC-A plot showing a gate labeled **Size 83.3**.

Alexa Fluor 488-A :: CCR5

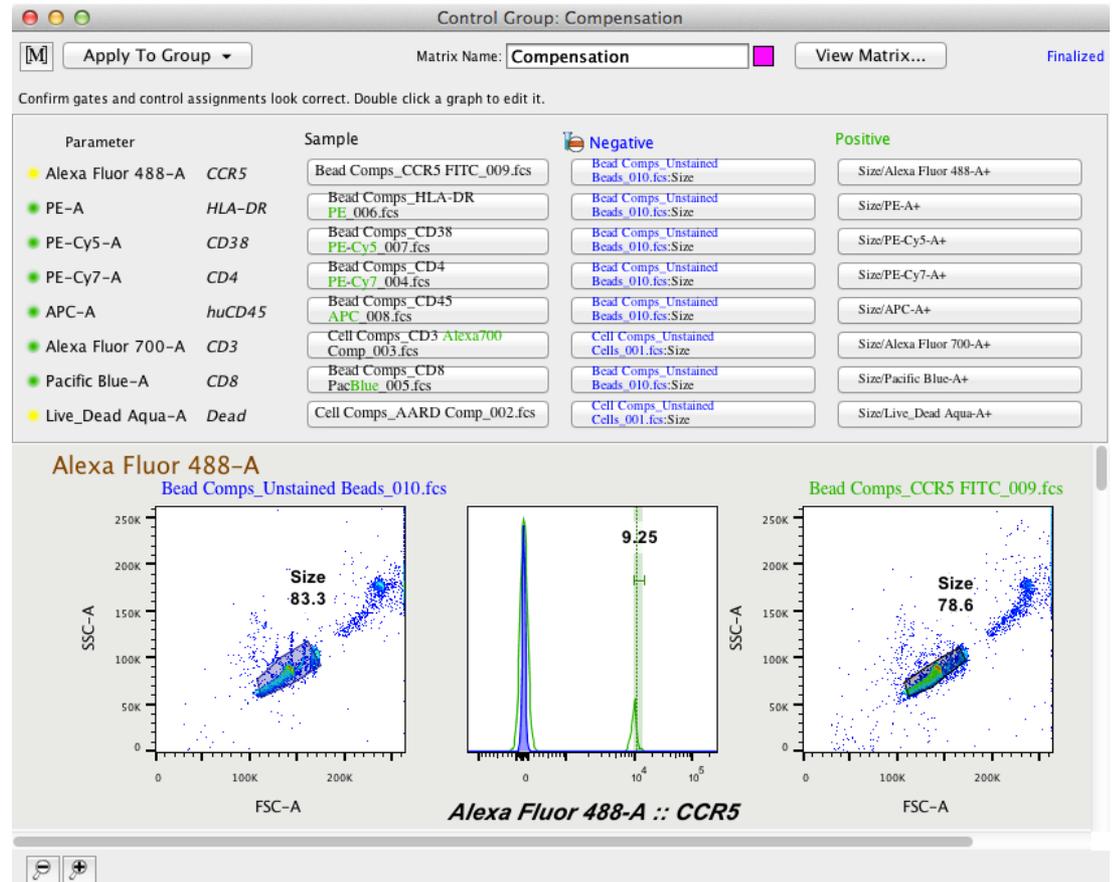
9.25

SSC-A vs FSC-A plot showing a gate labeled **Size 78.6**.

SOP

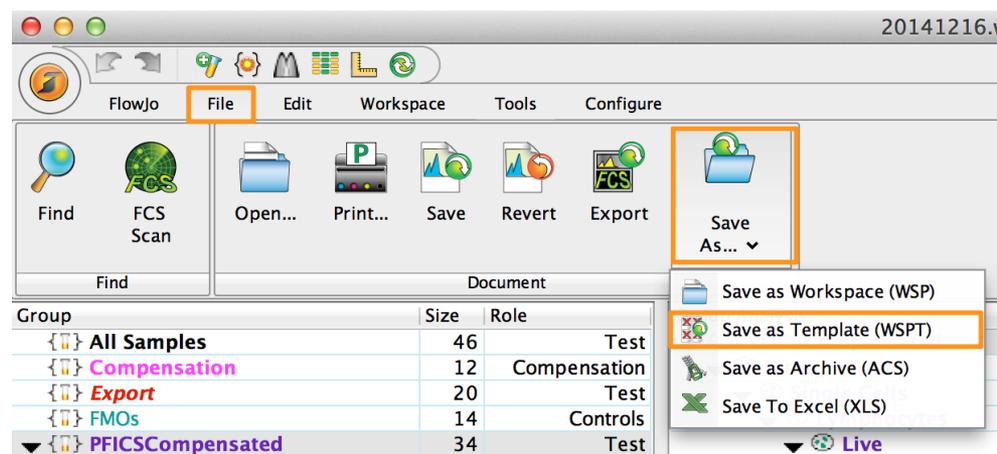
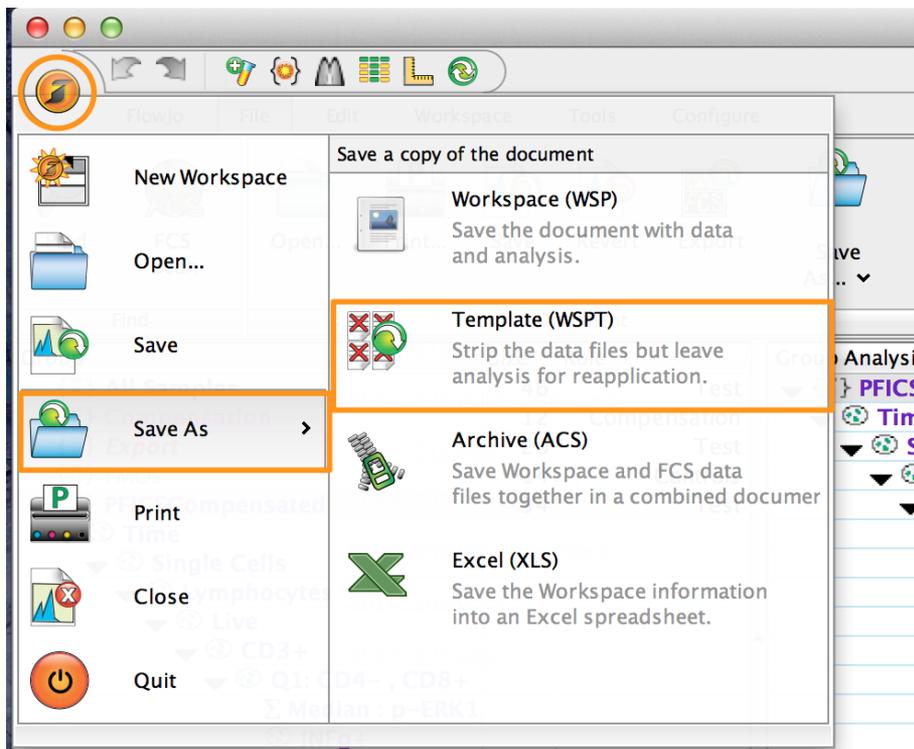
Compensation Wizard

- The wizard auto gates your compensation samples and fills in the positive and negative.
- To select a different sample, or gate choose from the dropdown lists.
- Double click on a graph in the wizard to modify the gate



Workspace Templates

- Allow saving all analysis reports in your workspace without data
- Streamlines repetitive analysis of multiple runs using the same staining panel(s).

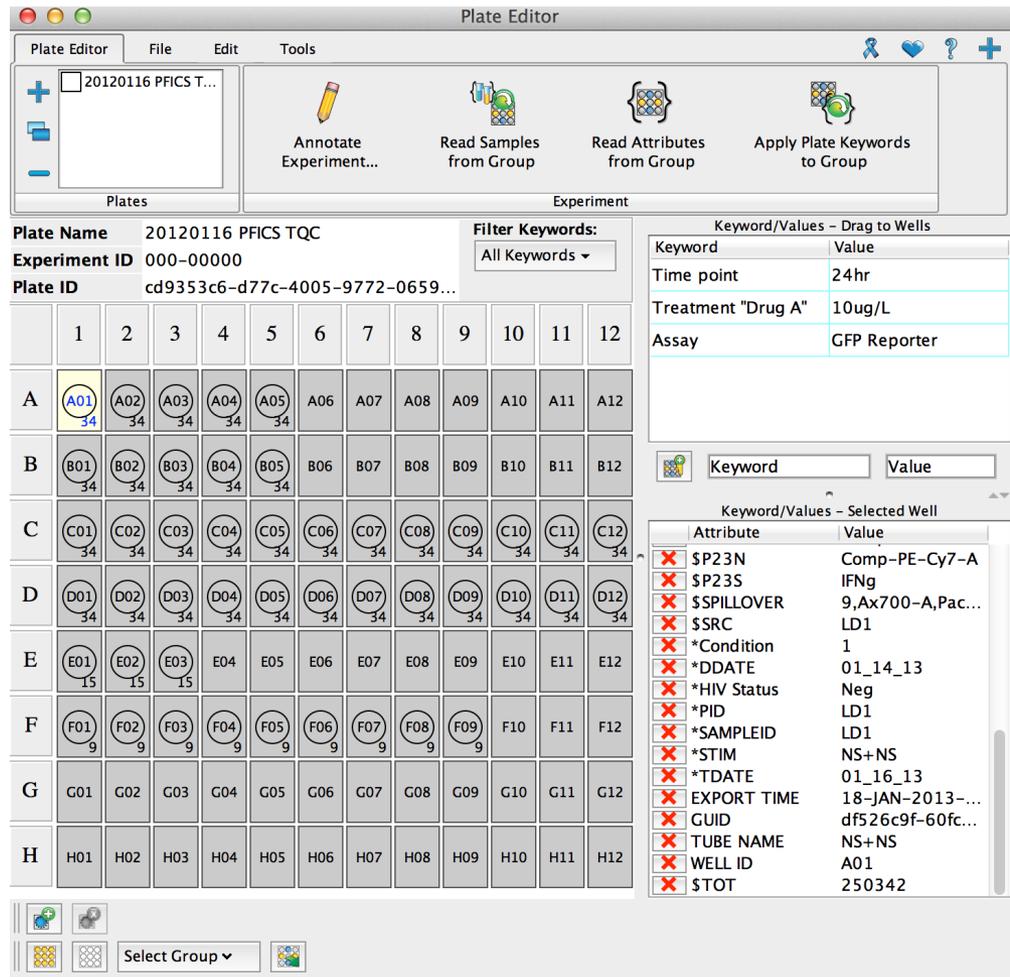


Command Line FlowJo (Ninja Skills)

- FlowJo can be run “headless” through command line interface, applying templates and generating automated reports.
- **FlowJo Enterprise** is a server based version of FlowJo 10 that spawns command line tasks through a web browser interface.
- Designed to assist with data management, analysis and report generation for high dimensional, high throughput flow or mass cytometry.

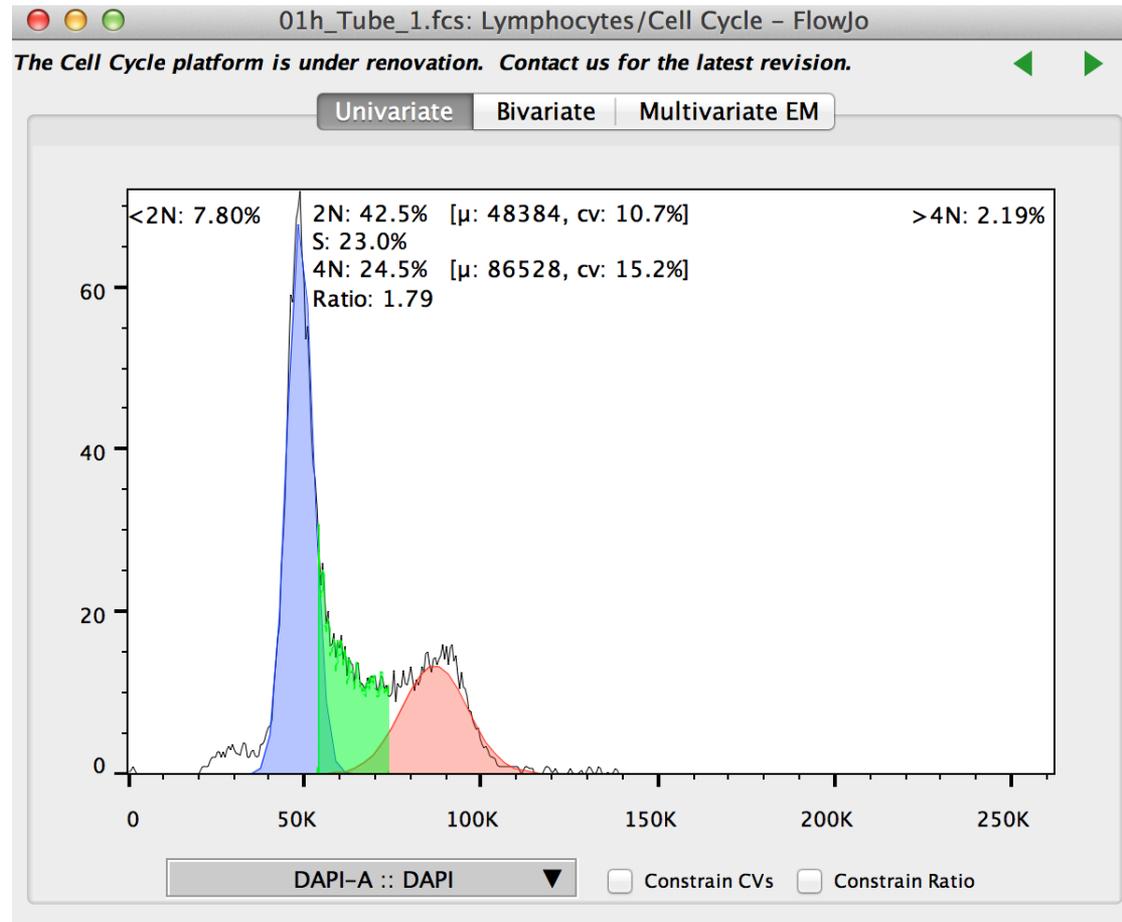
The Plate Editor

- Viewer to add keywords in a plate format
- Can export layout for use in FACS Diva acquisition software.
- Located in the visualizations Band within the Tools Tab
- Add new keywords/Value pairs to the right. Drag and drop on selected wells.



Cell Cycle Analysis

- The Cell Cycle platform allows 1D modeling of cell cycle phases based on DNA content
- Currently under development, the upcoming point release of v10.0.8 will have 1D Watson and Dean-Jett-Fox models, similar to FlowJo v9 for Mac



Additional Training Resources

- Webinars on basic and advanced features of FlowJo, held on the 1st and 3rd Thursday of each month.
- Webinar Schedule can be found at <http://www.flowjo.com/webinars/>
- Technical Documentation for V10 can be found at <http://docs.flowjo.com/>
- The Daily Dongle provides tips, tricks and answers to common questions.
<http://flowjo.typepad.com/>



Questions?

- FlowJo is here to help with all your cytometry analysis needs.
- Contact techsupport@flowjo.com for general questions and support.
- Contact timc@flowjo.com for science questions, additional training resources and information on FlowJo Enterprise.

Thank You!