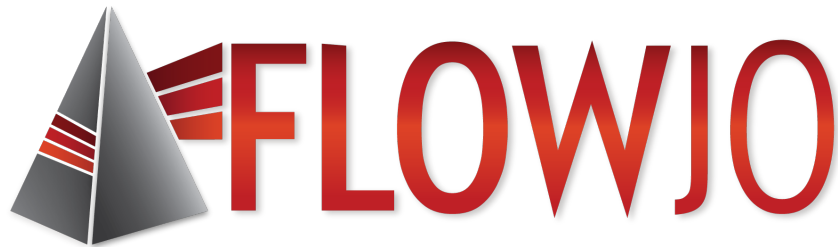


Cytometry Data Analysis in FlowJo V10



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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. The top ribbon contains tabs for FlowJo, File, Edit, Workspace, Tools, and Configure. The main workspace is divided into three panels: Group, Group Analysis, and Samples. The Group panel shows a hierarchical tree of groups, including 'All Samples', '20140711 Compensation', and '20140711 Rag1_BLT Act Baseline TQC'. The Group Analysis panel shows a detailed view of the selected group, including 'Time', 'Singlets', 'Live', and 'huCD45'. The Samples panel shows a list of samples with columns for Name, Statistic, #Cells, *PID, *Timepoint, *HIV Status, and *Sort.

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_J1873_021.fcs		57018	J1873	7_11_14	Neg	1
Mice_J1874_022.fcs		57954	J1874	7_11_14	Neg	1
Mice_J1875_023.fcs		68822	J1875	7_11_14	Neg	1
Mice_J1876_024.fcs		39520	J1876	7_11_14	Neg	1
Mice_J1877_025.fcs		63336	J1877	7_11_14	Neg	1
Mice_J1878_026.fcs		63752	J1878	7_11_14	Neg	1
Mice_J1879_027.fcs		75244	J1879	7_11_14	Neg	1
Mice_J1880_028.fcs		28002	J1880	7_11_14	Neg	1
Mice_J1881_029.fcs		25688	J1881	7_11_14	Neg	1
Mice_J1882_011.fcs		44850	J1882	7_11_14	Neg	1
Mice_J1883_012.fcs		46306	J1883	7_11_14	Neg	1
Mice_J1884_013.fcs		13702	J1884	7_11_14	Neg	1
Mice_J1885_014.fcs		48386	J1885	7_11_14	Neg	1

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.

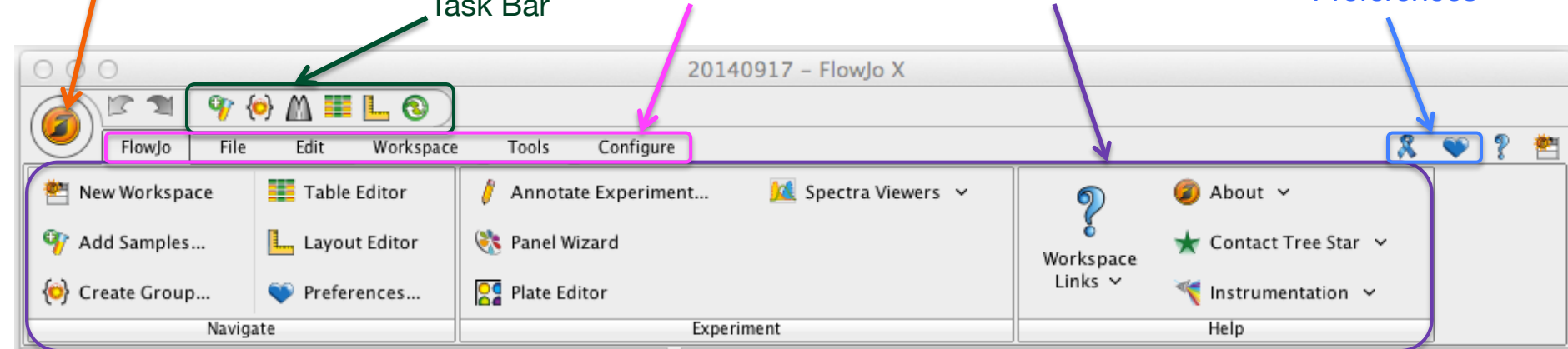
Application Button

Task Bar

Tabs

Bands

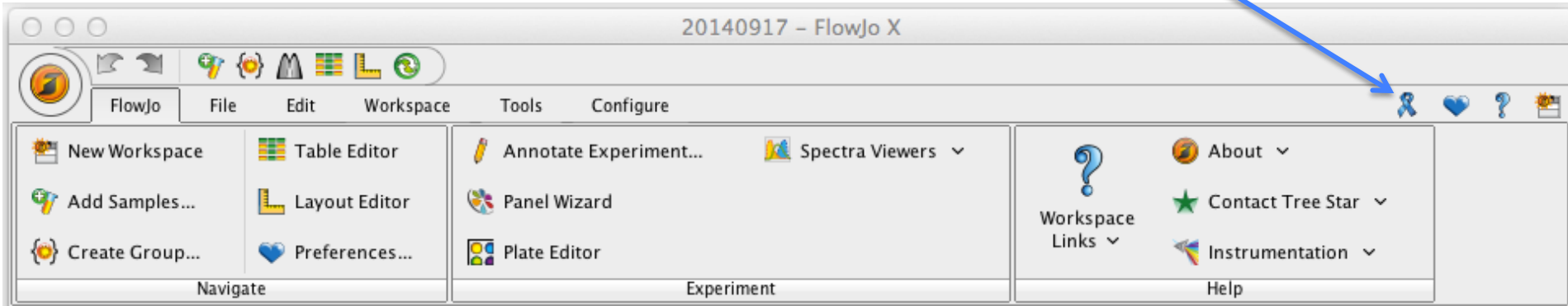
Ribbon Configuration & Preferences



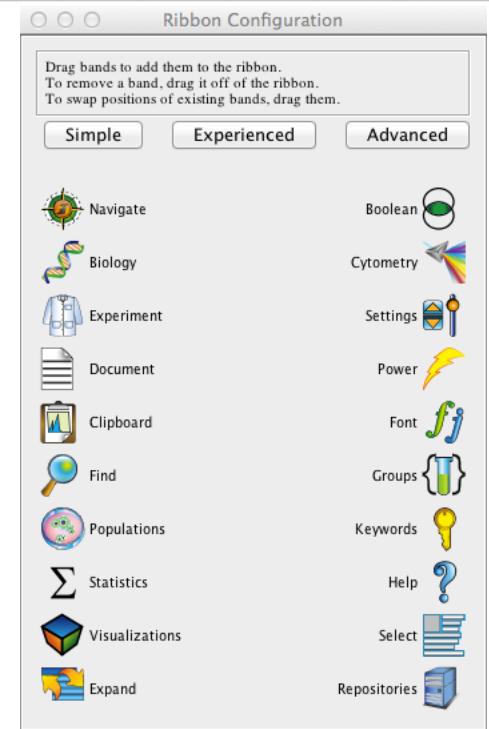
- Tabs group similar Bands together.
- Bands group similar Actions together.

Customizing Ribbons

- Click on the Ribbon icon to configure



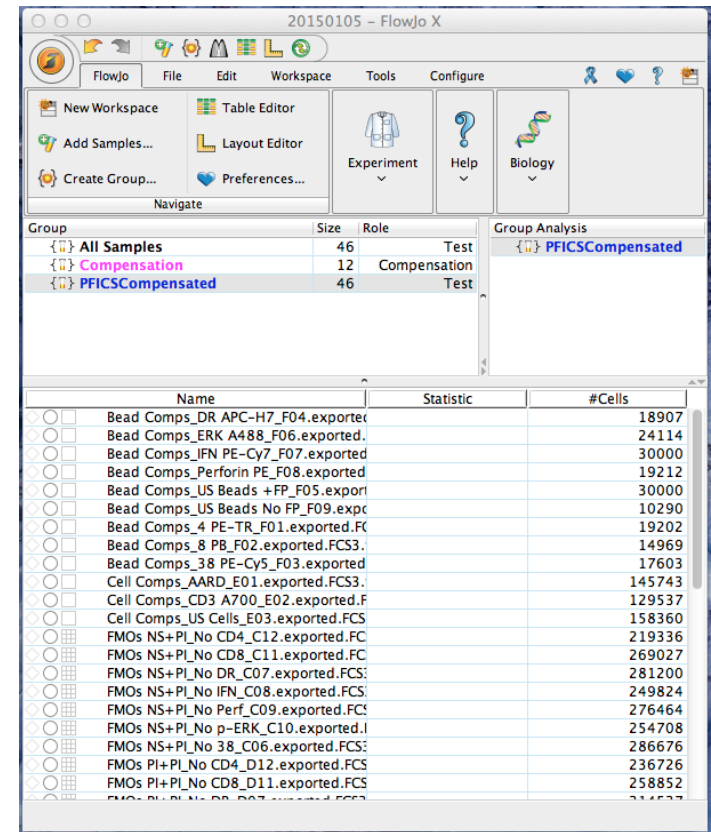
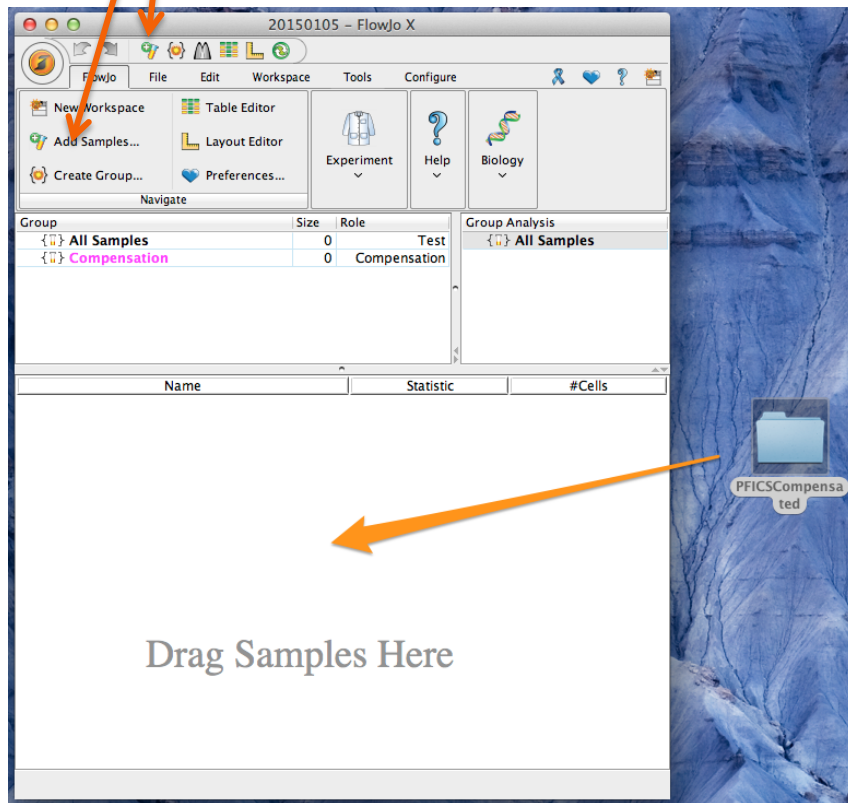
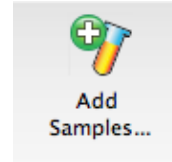
- Click on Simple, Experienced or Advanced to change the number of Tabs displayed.
- Drag the icon for any Band into the ribbon to add that set of Actions to your selected Tab.



Importing Data Into FlowJo

Three possible methods:

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

A screenshot of the FlowJo X software interface showing the "Add Samples" button and the "Drag Samples Here" area. The top menu bar includes "FlowJo", "File", "Edit", "Workspace", "Tools", and "Configure". Below the menu is a toolbar with icons for "New Workspace", "Add Samples...", "Create Group...", "Table Editor", "Layout Editor", "Preferences...", "Experiment", "Help", and "Biology". The main workspace is divided into two panes. The left pane, titled "Group", shows a list of groups: "All Samples" (Size 46, Role Test), "Compensation" (Size 12, Role Compensation), and "PFICSCompensated" (Size 46, Role Test). The right pane, titled "Group Analysis", shows "PFICSCompensated". At the bottom of the workspace is a large area labeled "Drag Samples Here". An orange arrow points from the "Add Samples..." button in the toolbar to the "Drag Samples Here" area. Another orange arrow points from the "Compensation" group in the left pane to the "Drag Samples Here" area. A blue folder icon labeled "PFICSCompensated" is shown being dragged into the "Drag Samples Here" area.

Name	Statistic	#Cells
Bead Comps_DR APC-H7_F04.exported		18907
Bead Comps_ERK A488_F06.exported		24114
Bead Comps_IFN PE-Cy7_F07.exported		30000
Bead Comps_Perforin PE-F08.exported		19212
Bead Comps_US Beads +FP_F05.exported		30000
Bead Comps_US Beads No FP_F09.exported		10290
Bead Comps_4 PE-TR_F01.exported.FCS3		19202
Bead Comps_8 PB_F02.exported.FCS3		14969
Bead Comps_38 PE-Cy5_F03.exported.FCS3		17603
Cell Comps_AARD_E01.exported.FCS3		145743
Cell Comps_CD3 A700_E02.exported.FCS3		129537
Cell Comps_US Cells_E03.exported.FCS3		158360
FMOs NS+PI_No CD4_C12.exported.FCS3		219336
FMOs NS+PI_No CD8_C11.exported.FCS3		269027
FMOs NS+PI_No DR_C07.exported.FCS3		281200
FMOs NS+PI_No IFN_C08.exported.FCS3		249824
FMOs NS+PI_No Perf_C09.exported.FCS3		276464
FMOs NS+PI_No p-ERK_C10.exported.FCS3		254708
FMOs NS+PI_No 38_C06.exported.FCS3		286676
FMOs PI+PI_No CD4_D12.exported.FCS3		236726
FMOs PI+PI_No CD8_D11.exported.FCS3		258852
FMOs PI+PI_No CD8_D12.exported.FCS3		214537

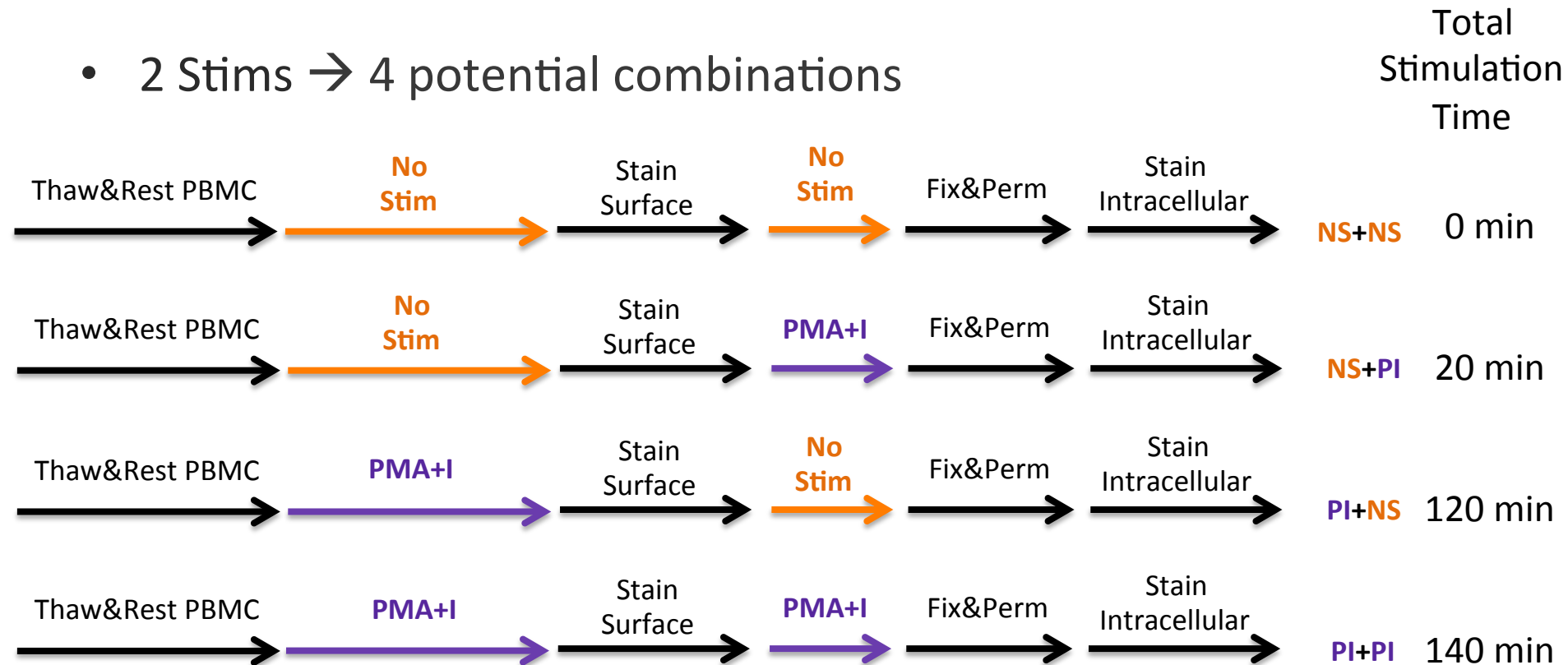
Today's Demo Data Set: Phospho-Flow + Intracellular Cytokine Staining (PFICS)

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



- 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

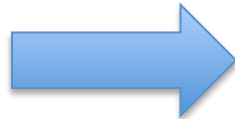
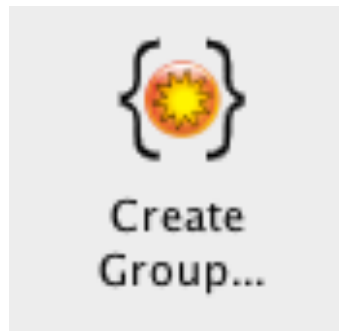
- The Group area lists all groups in the Workspace, # of samples in each group (Size), and the Role of that group (ex. Test, Compensation, Controls) .
- Groups act like folders to organize your sample files and allow unified master gating and analysis.

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

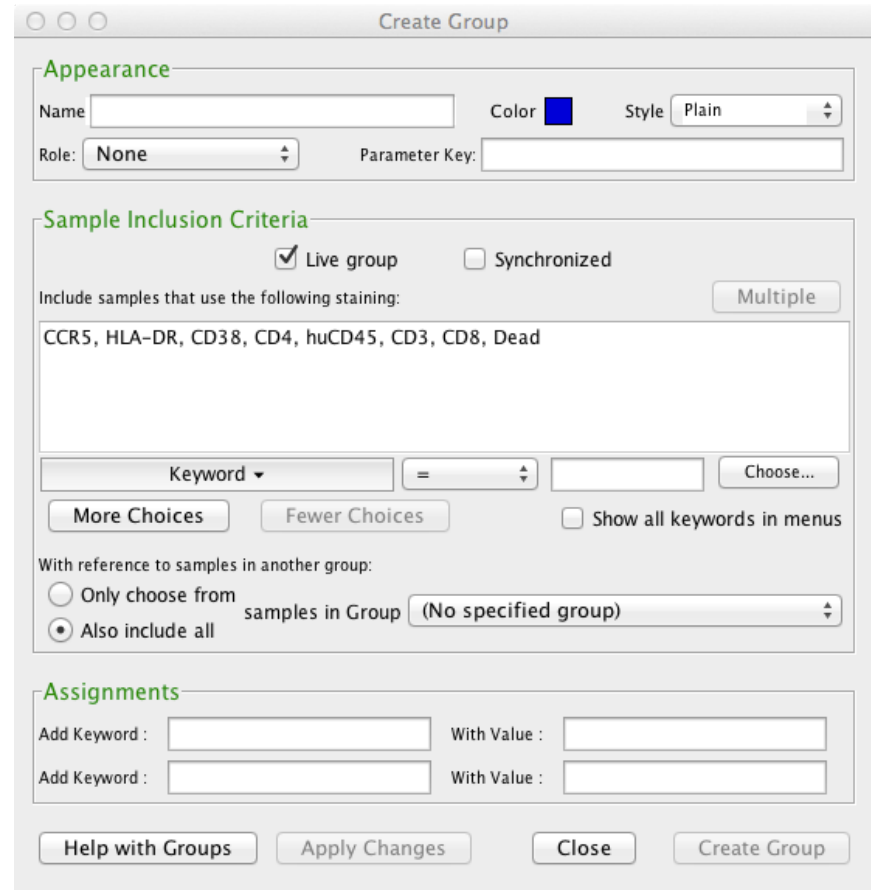
- Group Analysis displays all analysis within a group.

Creating and Editing Groups

- To create a new group type  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 the 'Create Group' dialog box. The dialog has a title bar with three window control buttons and the text 'Create Group'. It is divided into several sections: 'Appearance' with fields for Name, Color (a blue square), Style (a dropdown menu showing 'Plain'), Role (a dropdown menu showing 'None'), and Parameter Key; 'Sample Inclusion Criteria' with checkboxes for 'Live group' (checked) and 'Synchronized', a text area for 'Include samples that use the following staining:' containing 'CCR5, HLA-DR, CD38, CD4, huCD45, CD3, CD8, Dead', and a 'Multiple' button; a section for keyword assignment with a 'Keyword' dropdown, an equals sign, a text field, and a 'Choose...' button; and 'Assignments' with two rows of 'Add Keyword' and 'With Value' fields. At the bottom are buttons for '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)

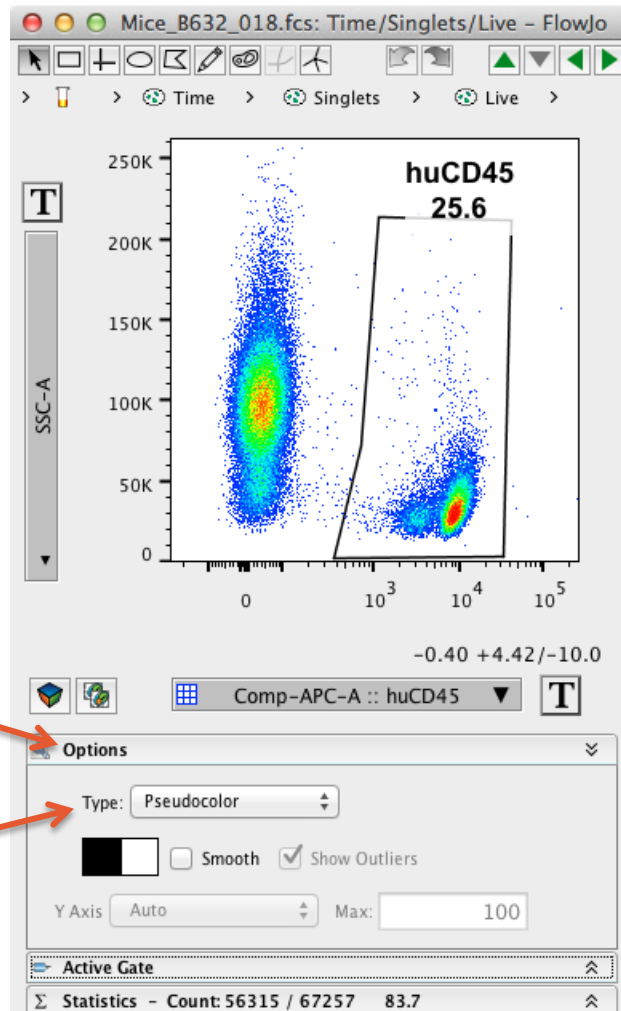
Name		Statistic	#Cells	*PID	*Timepoint ▲	*HIV Status	*Sort
◊ ○ ▢ ▶	Mice_B629_015.fcs		79508	B629	7_11_14	Neg	1
◊ ○ ▢ ▶	Mice_B630_016.fcs		181870	B630	7_11_14	Neg	1
◊ ○ ▢ ▶	Mice_B631_017.fcs		130338	B631	7_11_14	Neg	1
◊ ○ ▢ ▶	Mice_B632_018.fcs		69680	B632	7_11_14	Neg	1
◊ ○ ▢ ▶	Mice_B633_019.fcs		59878	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

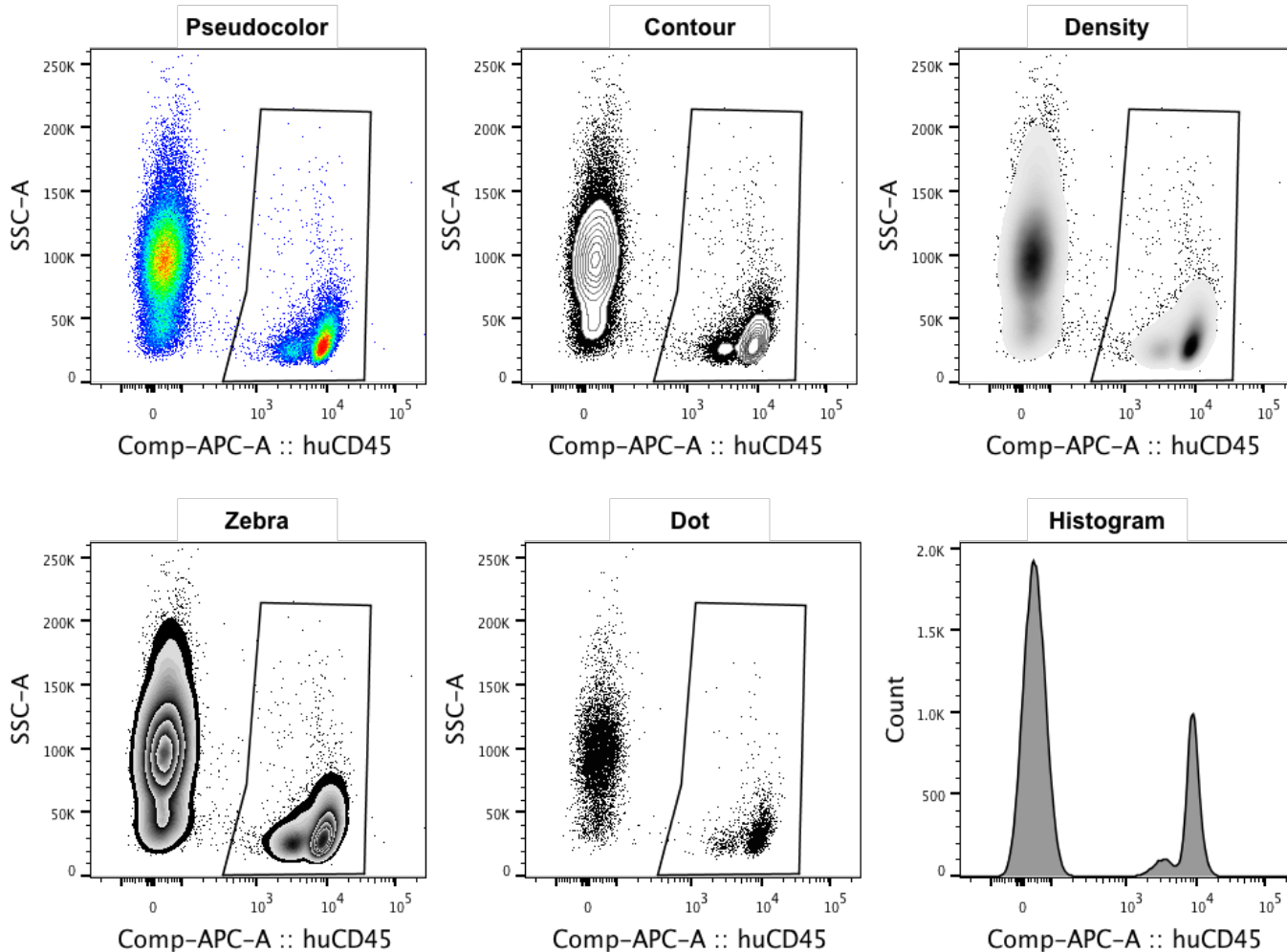
- The Graph Window facilitates data visualization and gating.



- 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.

Graph Display Options

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

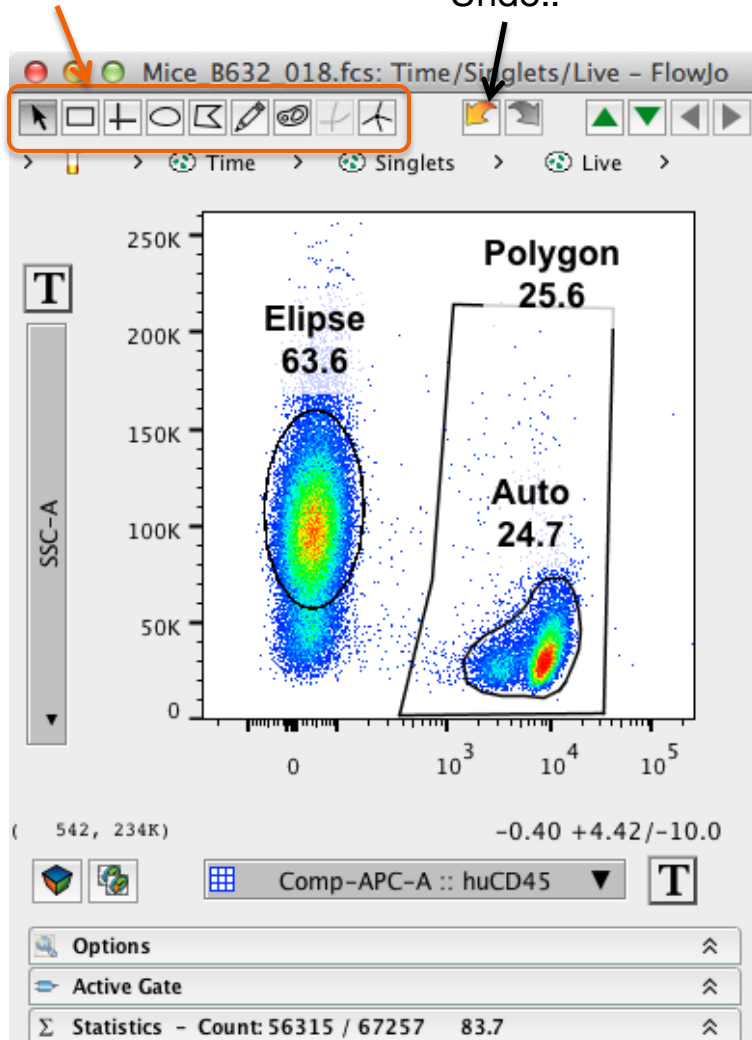


Gating tools

- Are located at the top of a Graph Window.

Gating Tools

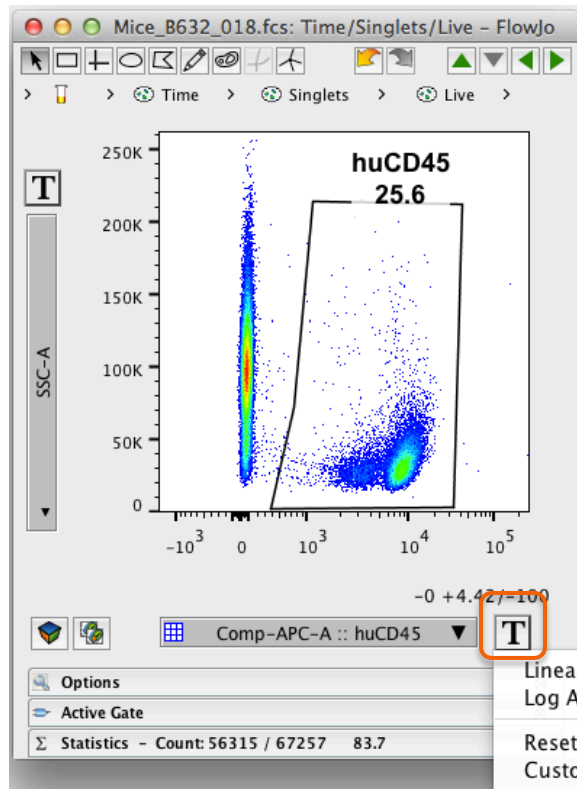
Undo!!



- 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

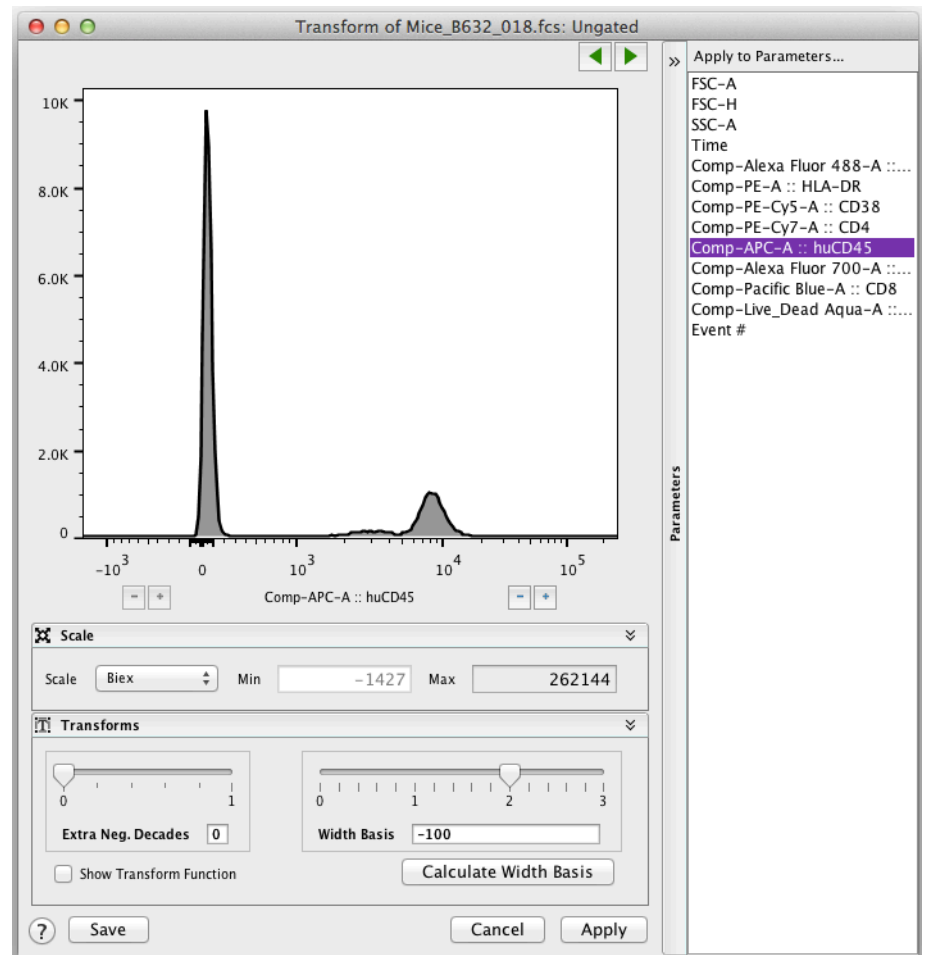
- Your data may initially look ‘squished’.
- Click the Transformation icon to change the visual display.



Custom Transform

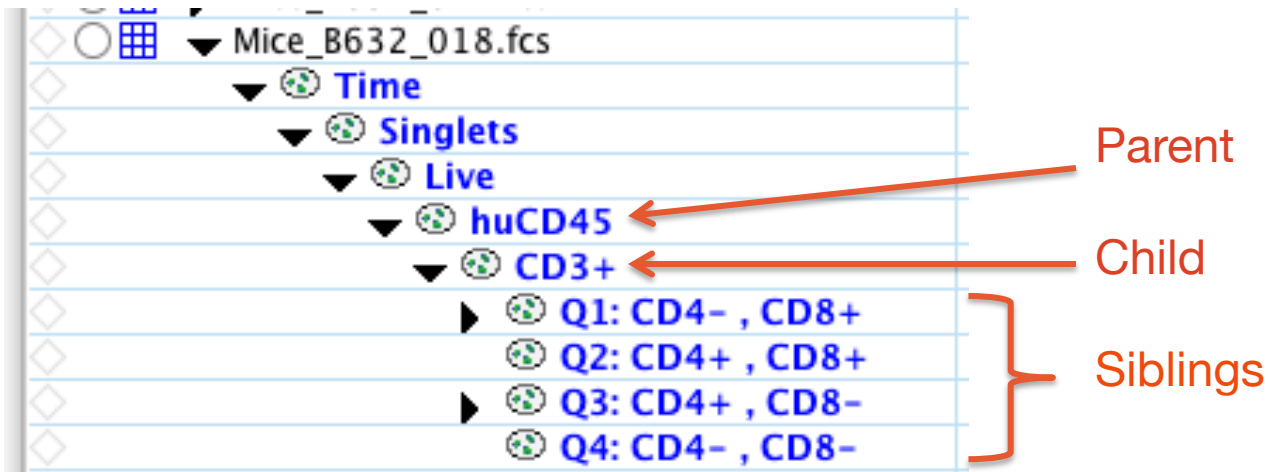
Linear Axis
Log Axis

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.



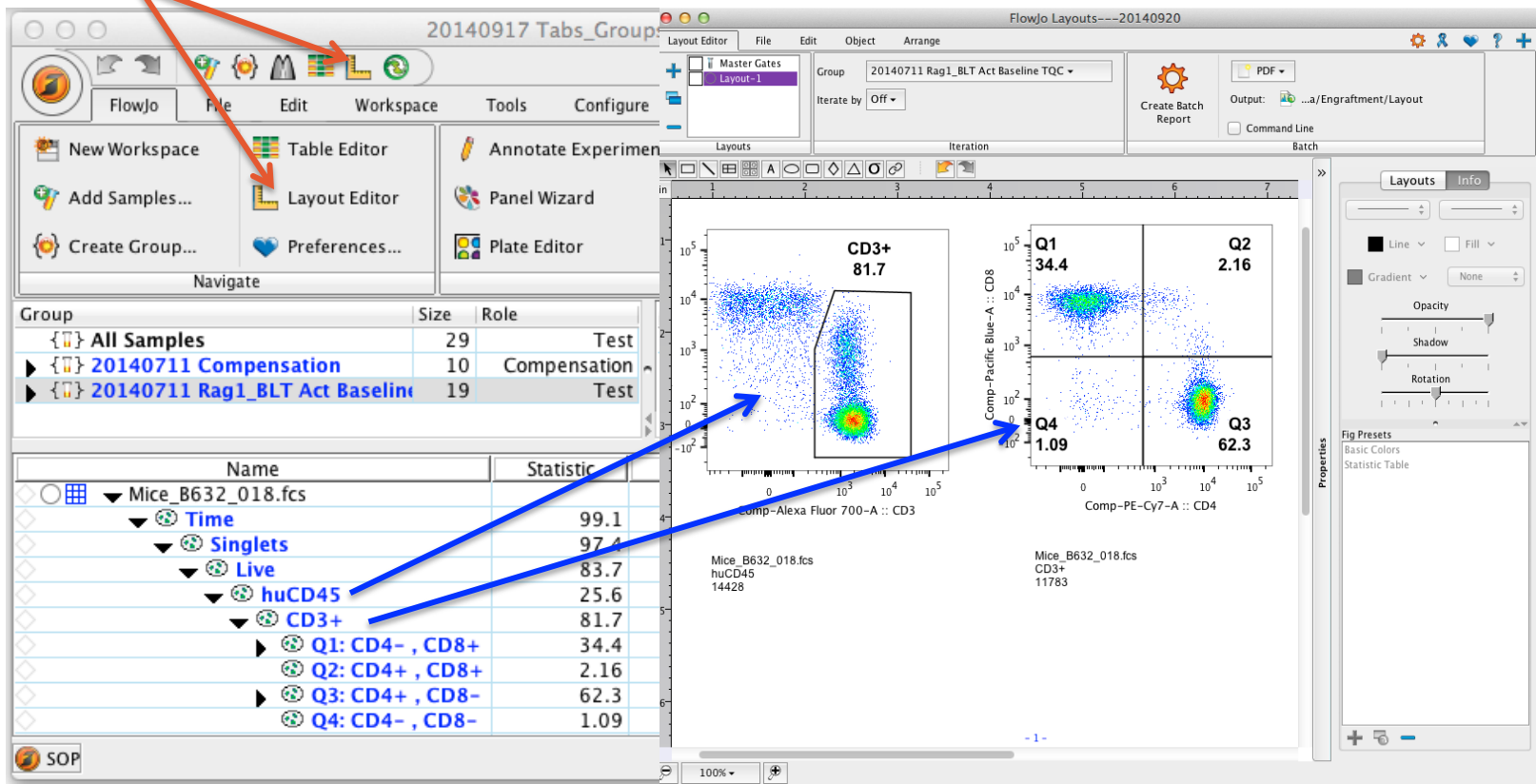
The Layout Editor

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



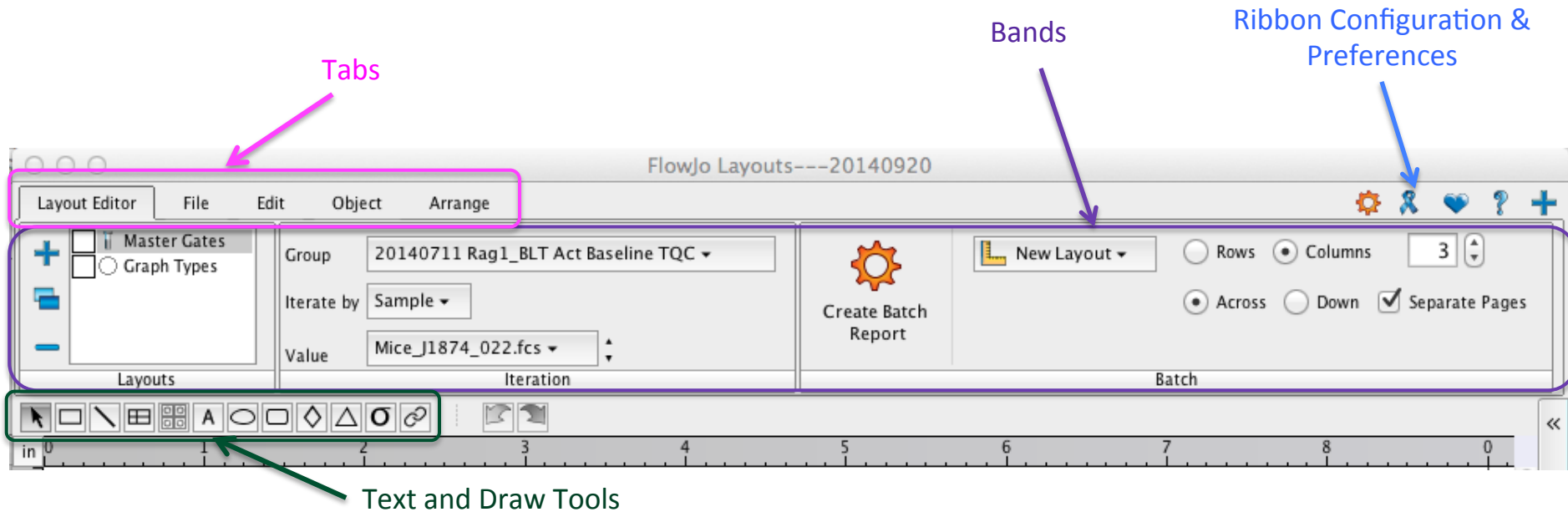
Layout
Editor

Layout Editor



Working in Layout Editor

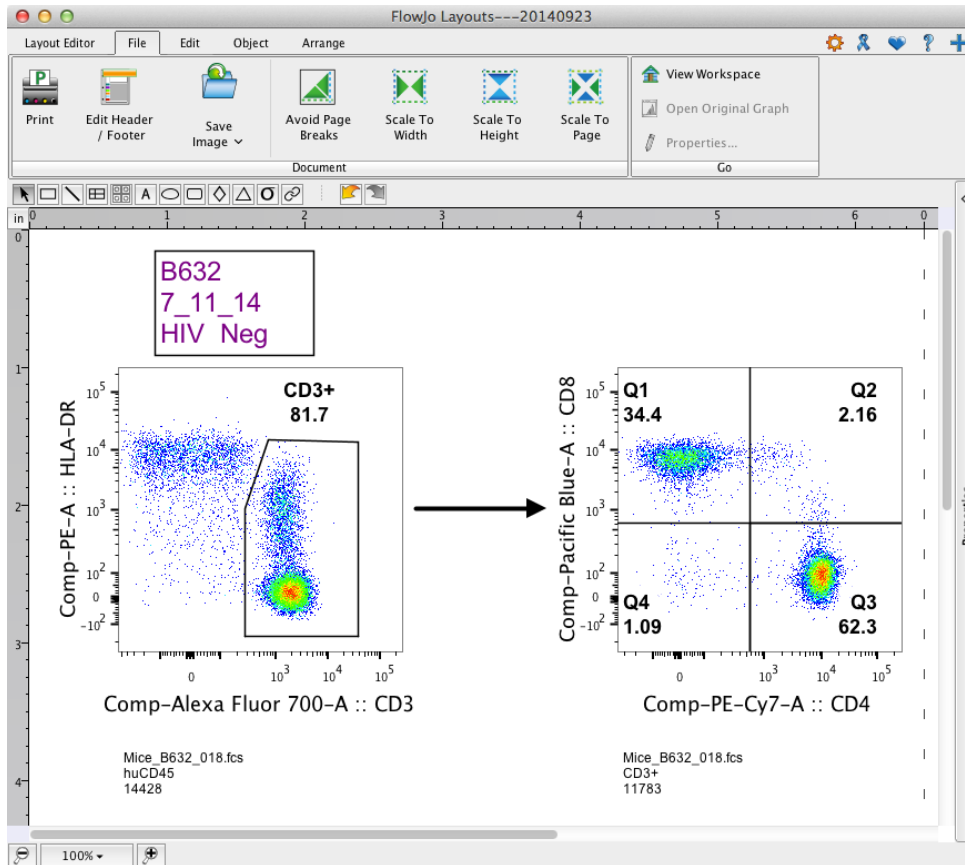
- Similar to the Workspace, the Layout Editor has its own customizable Ribbon with Tabs and Bands to organize actions.



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

Within Layout Editor

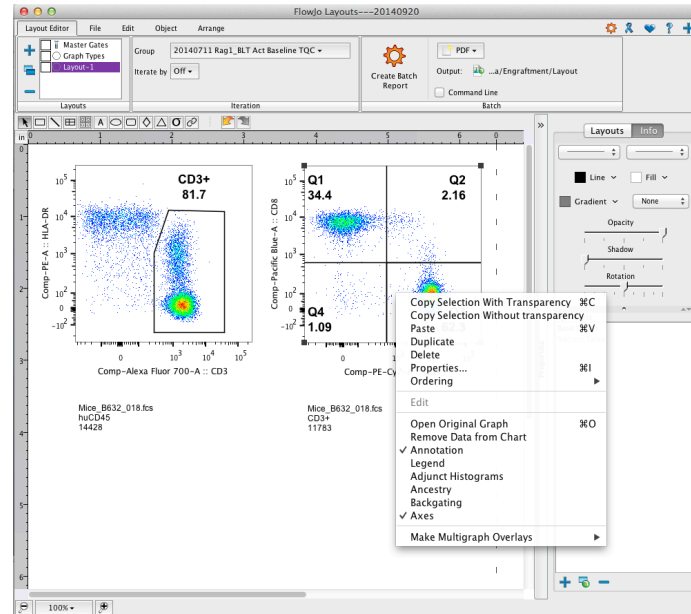
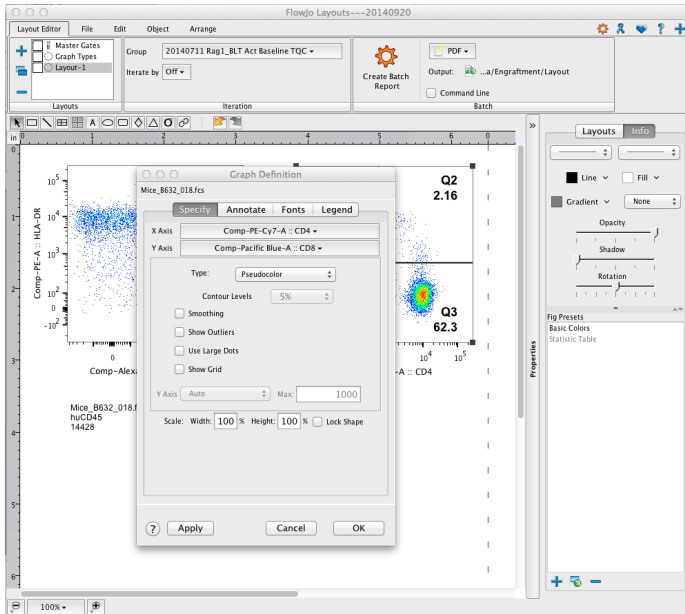
- Graphs can be organized and re-formatted.
- Statistics, keywords, text and even shapes or objects can be added to illustrate your analysis.



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

Working in Layout Editor

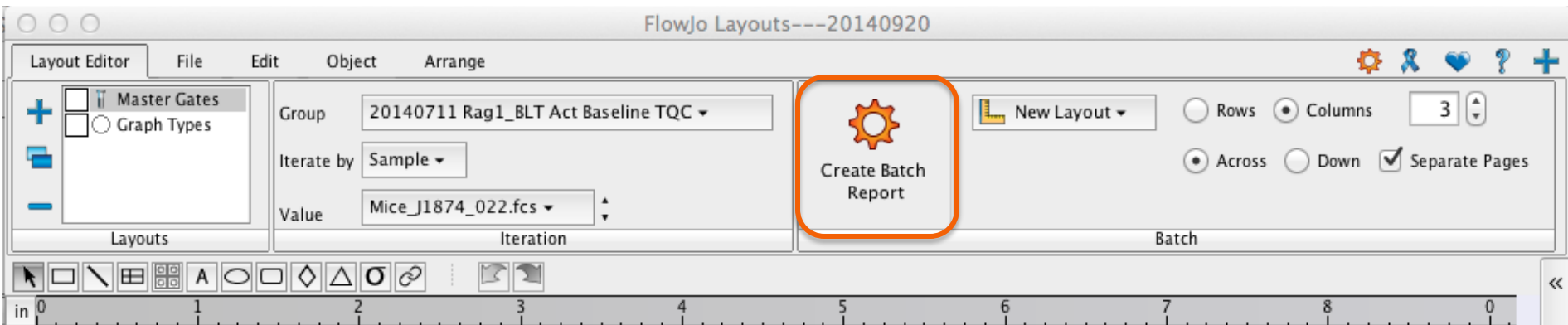
- Click once on a graph or object to select it.
- Double Click a graph to change its properties.



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

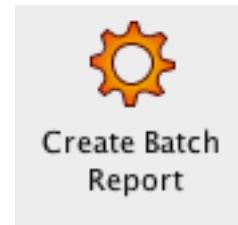
Batch Analysis of Layout Editor Graphics

- 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.



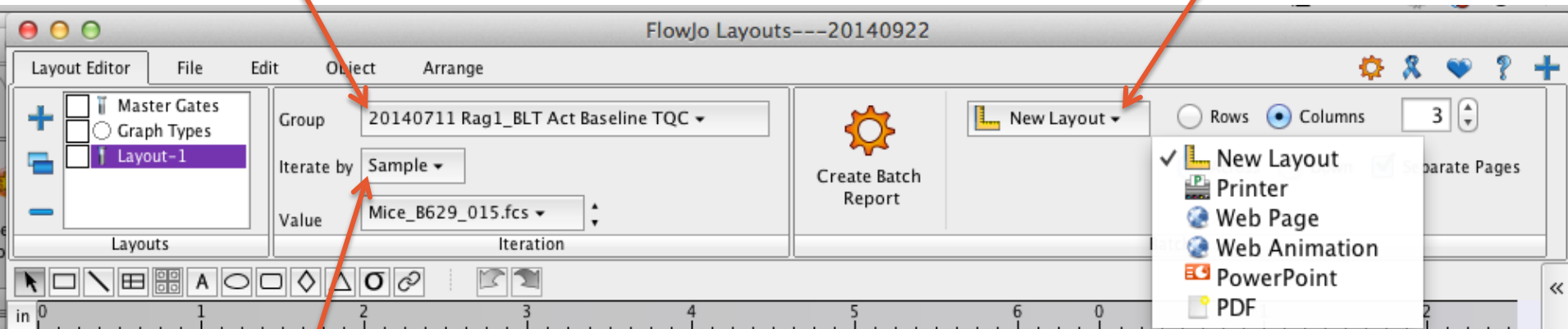
Batch Analysis of Layout Editor Graphics

- 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

The screenshot shows the FlowJo software interface with the Table Editor window open. The Table Editor window displays a list of populations and their statistics. The populations are listed in a table with columns for Population, Statistic, Parameter, and Name. The populations are:

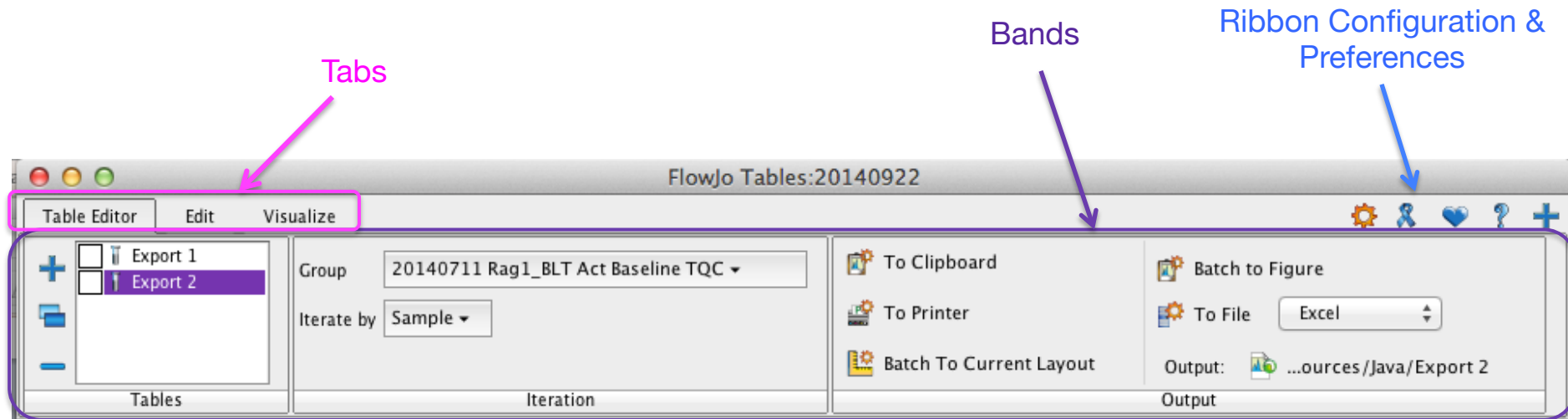
Population	Statistic	Parameter	Name
Time/Singlets/Live/huCD45	Freq. of Parent		
Time/Singlets/Live/huCD45/CD3+	Freq. of Parent		
Time/Singlets/Live/huCD45/CD3+/Q1: CD4-, CD8+	Freq. of Parent		
Time/Singlets/Live/huCD45/CD3+/Q3: CD4+, CD8-	Freq. of Parent		
*Sample ID			
*Timepoint			

The populations are listed in a table with columns for Population, Statistic, Parameter, and Name. The populations are:

Population	Statistic	Parameter	Name
Time/Singlets/Live/huCD45	Freq. of Parent		
Time/Singlets/Live/huCD45/CD3+	Freq. of Parent		
Time/Singlets/Live/huCD45/CD3+/Q1: CD4-, CD8+	Freq. of Parent		
Time/Singlets/Live/huCD45/CD3+/Q3: CD4+, CD8-	Freq. of Parent		
*Sample ID			
*Timepoint			

Within Table Editor

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



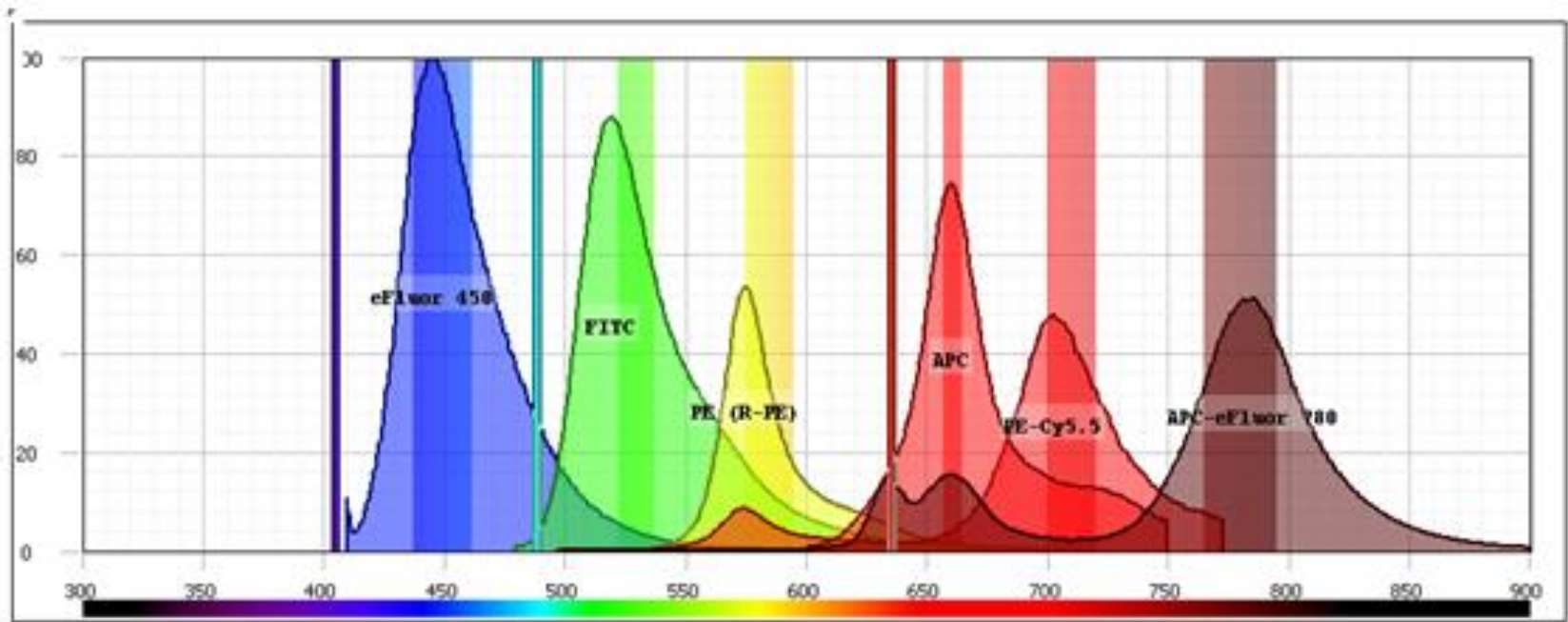
- Specify the group you wish to batch, and how to iterate the batch process, then in the Output band, specify where you want the batch output to go.

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 ▾ Matrix Name: **Compensation** View Matrix... Finalized

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

Parameter	Sample
Alexa Fluor 488-A CCR5	Bead Comps_CCR5 FITC_009.fcs
PE-A HLA-DR	Bead Comps_HLA-DR PE_006.fcs
PE-Cy5-A CD38	Bead Comps_CD38 PE-Cy5_007.fcs
PE-Cy7-A CD4	Bead Comps_CD4 PE-Cy7_004.fcs
APC-A huCD45	Bead Comps_CD45 APC_008.fcs
Alexa Fluor 700-A CD3	Cell Comps_CD3 Alexa700 Comp_003.fcs
Pacific Blue-A CD8	Bead Comps_CD8 PacBlue_005.fcs
Live_Death Aqua-A Dead	Cell Comps_AARD Comp_002.fcs

Negative

Bead Comps_Unstained Beads_010.fcs:Size
Bead Comps_Unstained Beads_010.fcs:Size
Bead Comps_Unstained Beads_010.fcs:Size
Bead Comps_Unstained Beads_010.fcs:Size
Bead Comps_Unstained Beads_010.fcs:Size
Cell Comps_Unstained Cells_001.fcs:Size
Bead Comps_Unstained Beads_010.fcs:Size
Cell Comps_Unstained Cells_001.fcs:Size

Positive

Size/Alexa Fluor 488-A+
Size/PE-A+
Size/PE-Cy5-A+
Size/PE-Cy7-A+
Size/APC-A+
Size/Alexa Fluor 700-A+
Size/Pacific Blue-A+
Size/Live_Death Aqua-A+

Group Analysis

Group	Size	Role
All Samples	29	Test
20140711 Compensation	10	Compensation
20140711 Rag1_BLT Act Baseli	19	Test

Group Analysis

Name	Statistic	#Cells	*PID
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

Size 83.3

SSC-A

FSC-A

Alexa Fluor 488-A :: CCR5

9.25

SSC-A

FSC-A

Bead Comps_CCR5 FITC_009.fcs

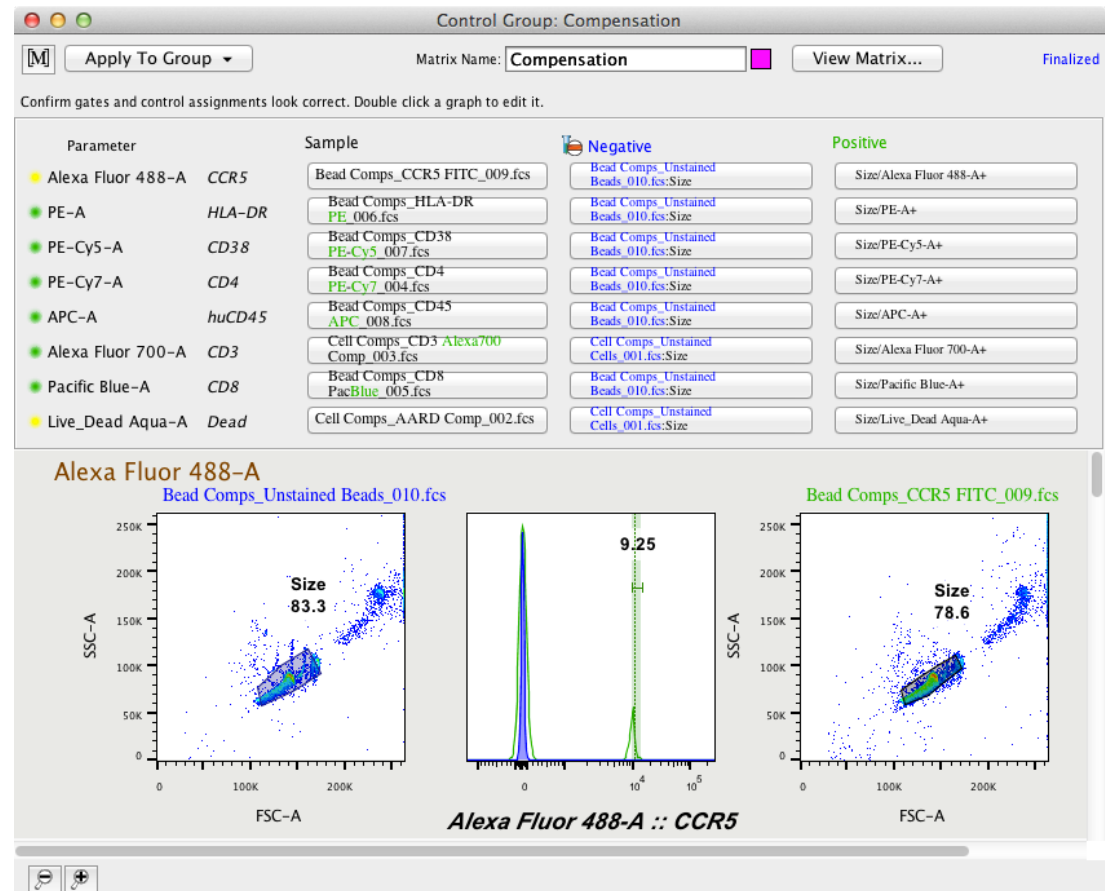
Size 78.6

SSC-A

FSC-A

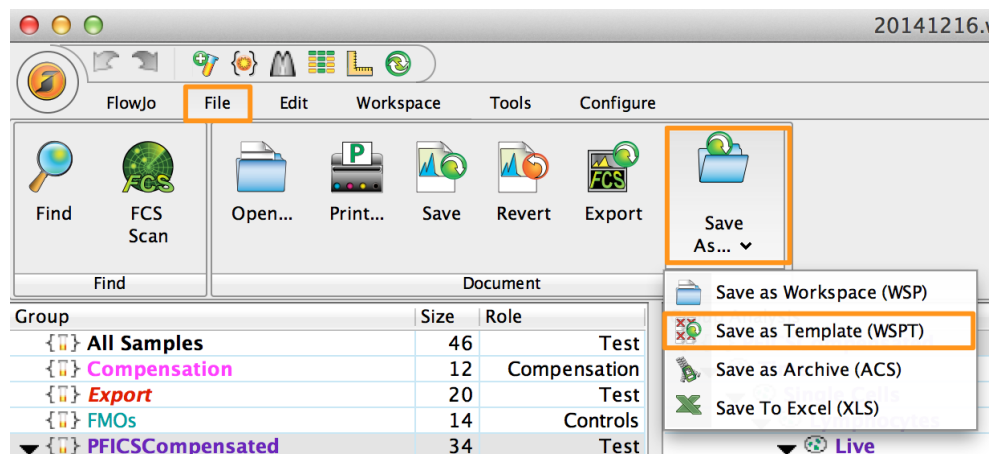
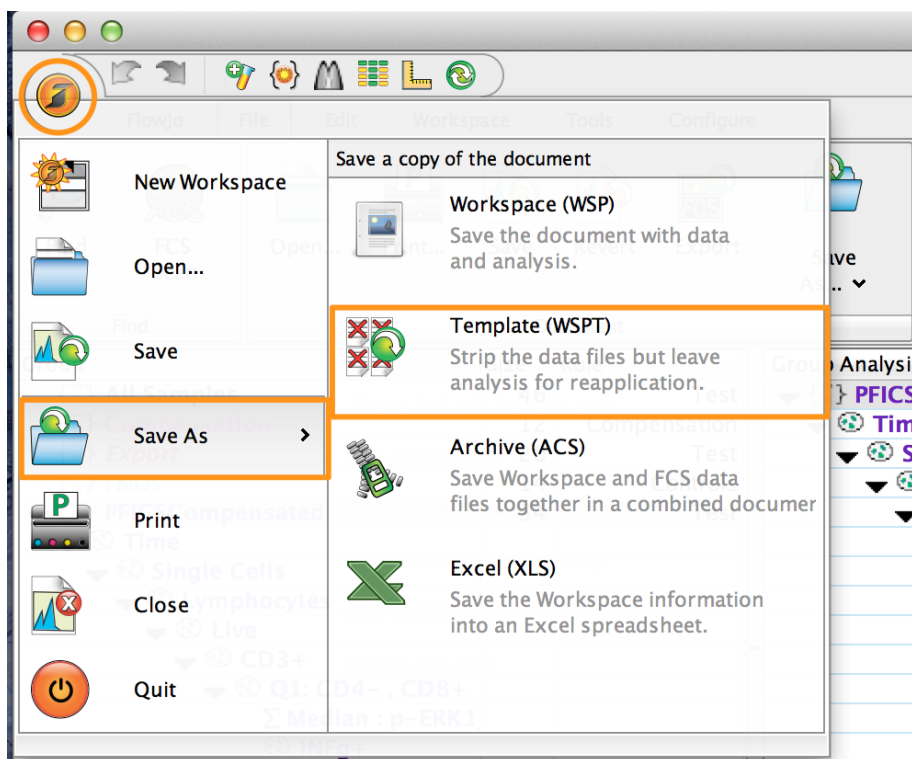
Compensation Wizard

- 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).

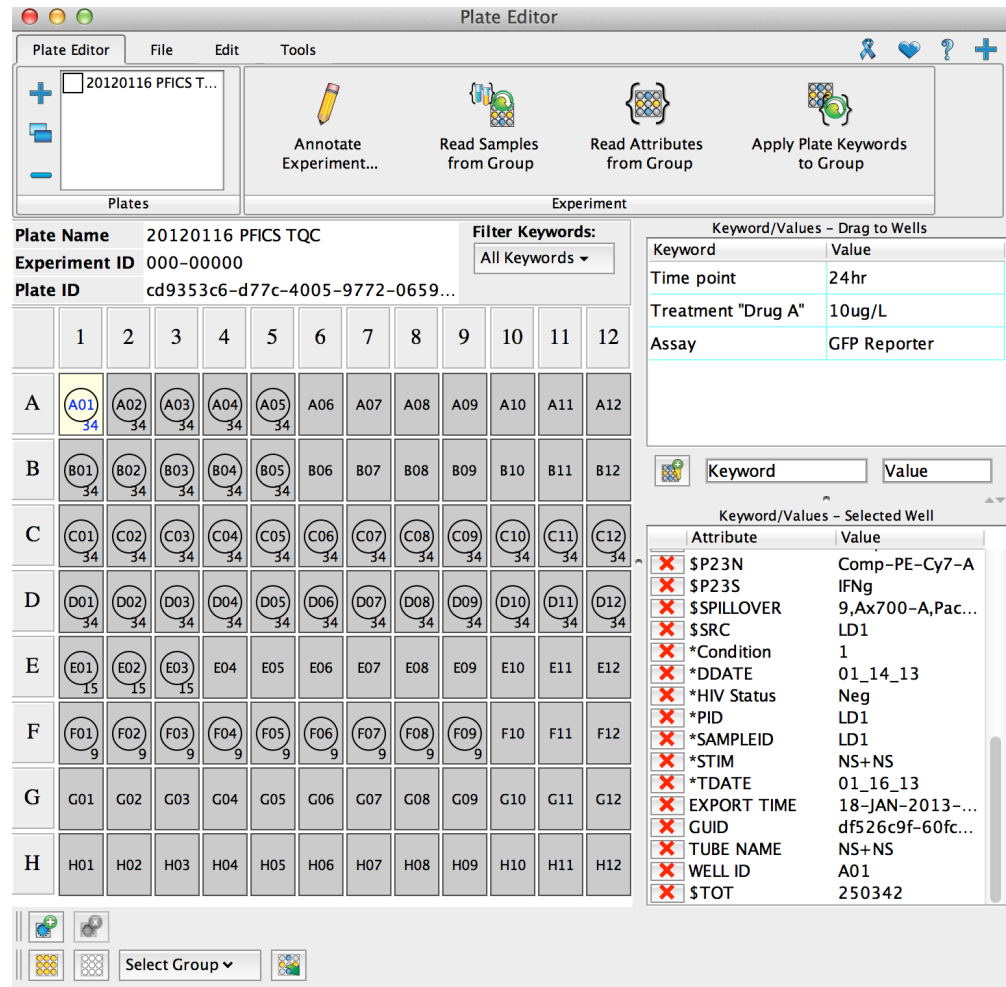


Command Line FlowJo (Ninja Skills)

- 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.

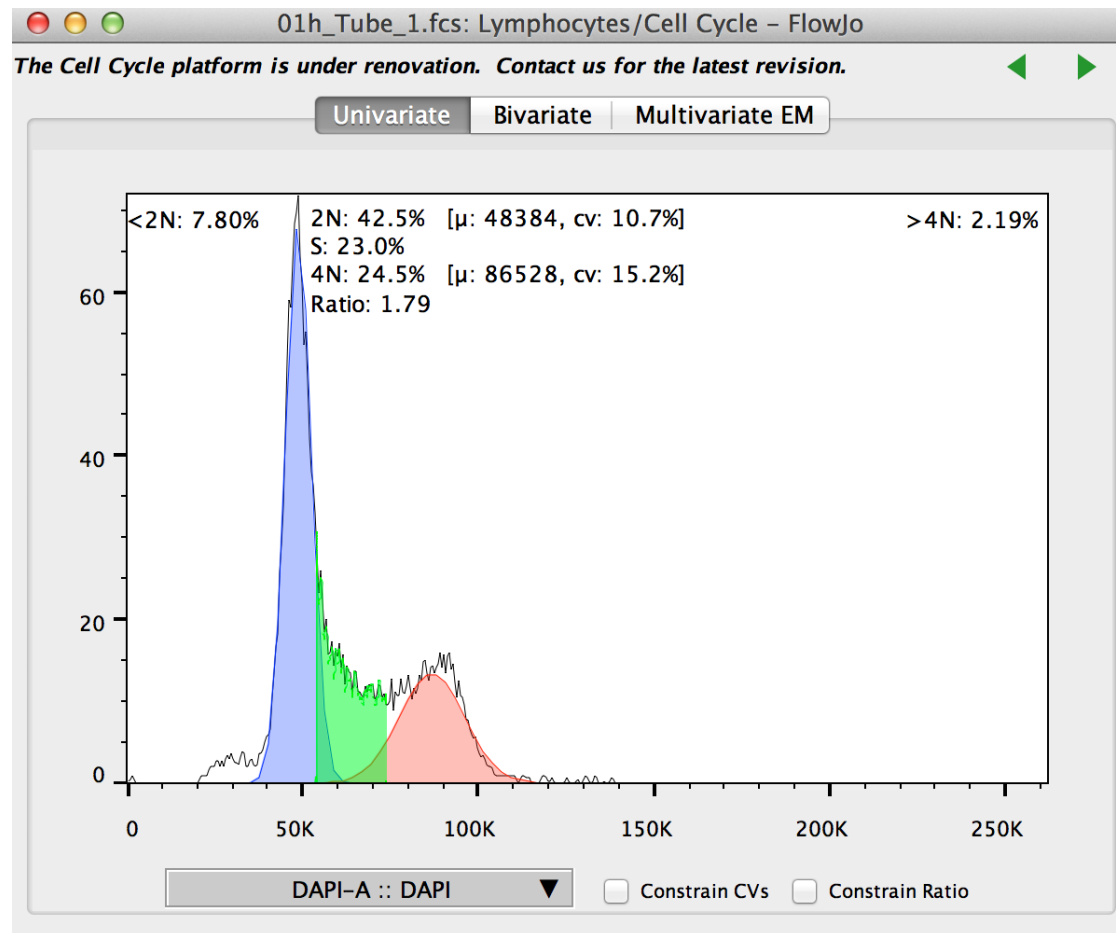
The Plate Editor

- 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!