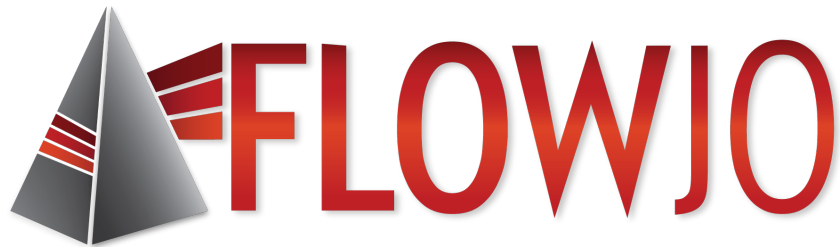


# Cytometry Data Analysis in FlowJo V10



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# The FlowJo v10 Workspace

- A graphical interface to organize your data.

The screenshot shows the FlowJo v10 Workspace interface. The top ribbon contains tabs for 'FlowJo', 'File', 'Edit', 'Workspace', 'Tools', and 'Configure'. Below the ribbon are several tool bands: 'Navigate' (New Workspace, Add Samples..., Create Group..., Table Editor, Layout Editor, Preferences...), 'Experiment' (Annotate Experiment..., Add Keyword, Plate Editor), 'Biology' (Cell Cycle..., Kinetics..., Compare Populations...), and 'Help' (About, Contact FlowJo, Instrumentation). The main workspace area displays a hierarchical tree of groups. The 'All Samples' group is expanded to show 'All Stain', 'Compensation', 'FMOs', and 'Master Gates'. The 'Master Gates' group is further expanded to show 'Time', 'Singlets', 'Lymphocytes', 'Live', 'CD3+', 'Q1: CD4-, CD8+', 'Geometric Mean : Comp-Ax488-A (p-ERK1\_2)', 'IFNg+', 'Freq. of Parent', 'Perf+', and 'pERK+'.

Ribbon  
Tabs and Bands

Groups and Group  
Analysis

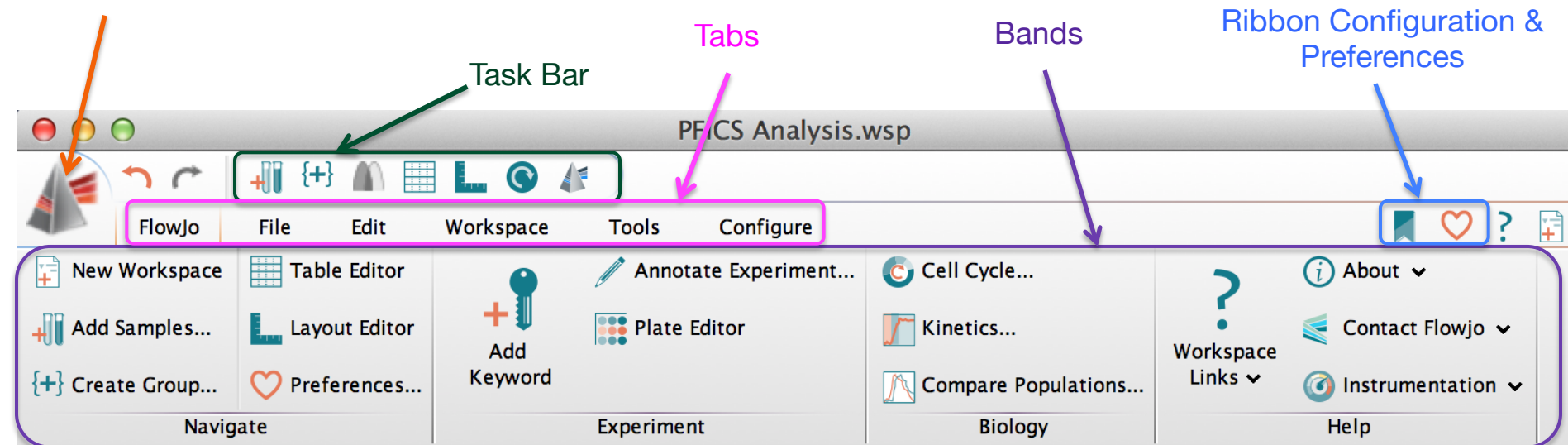
Name	Statistic	#Cells	*PID ▲	*STIM	Well ID:
LD1_NS+NS_A01_exp.fcs		250342	LD1	NS+NS	A01
LD1_NS+PI_C01_exp.fcs		229585	LD1	NS+PI	C01
LD1_PI+NS_B01_exp.fcs		262774	LD1	PI+NS	B01
LD1_PI+PI_D01_exp.fcs		244977	LD1	PI+PI	D01
LD2_NS+NS_A02_exp.fcs		330780	LD2	NS+NS	A02
LD2_NS+PI_C02_exp.fcs		286306	LD2	NS+PI	C02
LD2_PI+NS_B02_exp.fcs		279202	LD2	PI+NS	B02
Time	100.0	279199			
Singlets	96.3	268967			
Lymphocytes	91.3	245663			
Live	73.6	180798			
CD3+	81.7	147761			
Q1: CD4-, CD8+	25.1	37017			
Geometric Mean : Comp-Ax488-A (p-ERK1_2)	424				
IFNg+	64.1	23716			
Freq. of Parent	64.1				
Perf+	52.9	19580			
pERK+	93.2	34514			

Samples and  
sample analysis

# Ribbons, Tabs and Bands

- Ribbon organization allows easy visual navigation of workspace functions.

Application Button

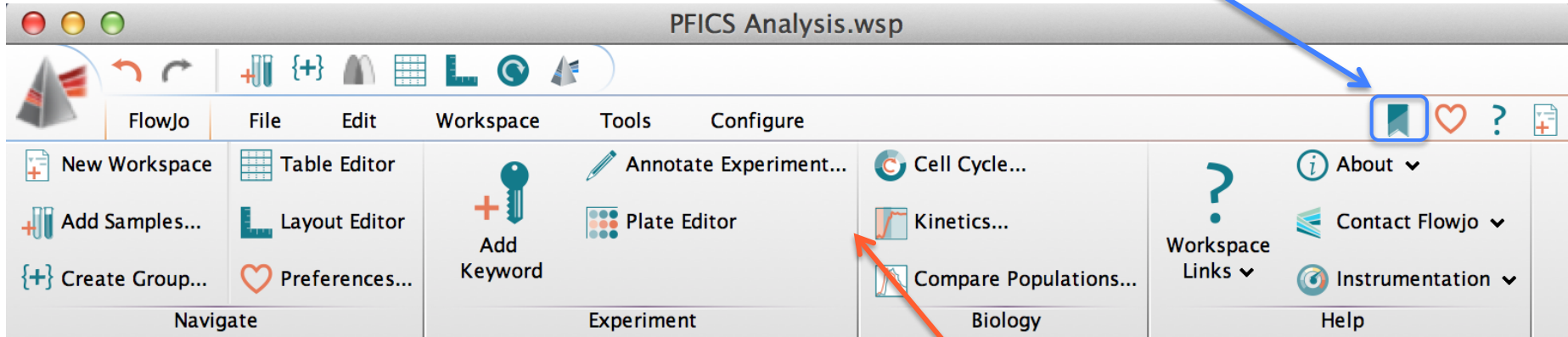


- Tabs group similar Bands together.
- Bands group similar Actions together.

# Customizing Ribbons

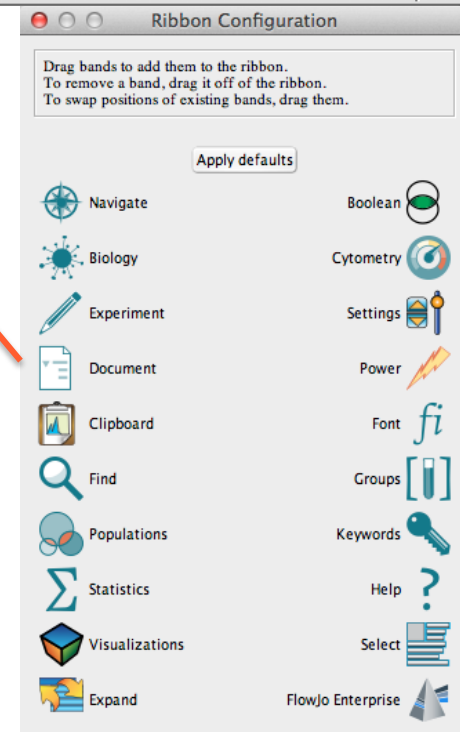
- Click on the Ribbon icon to configure

1.



- Drag the icon for any Band into the Ribbon → set of Actions added to your selected Tab.

2.

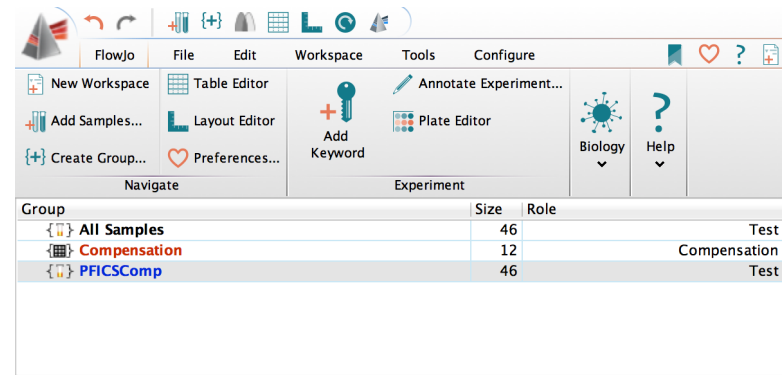
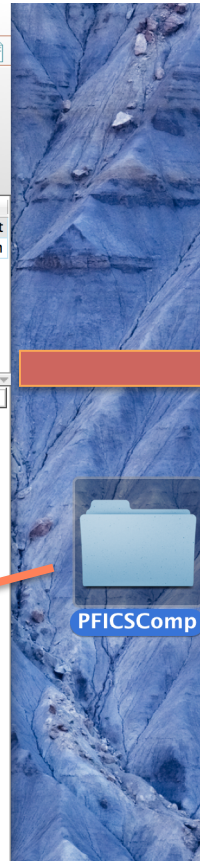
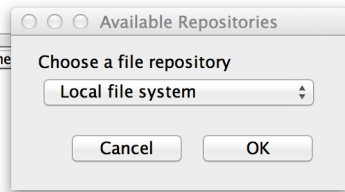
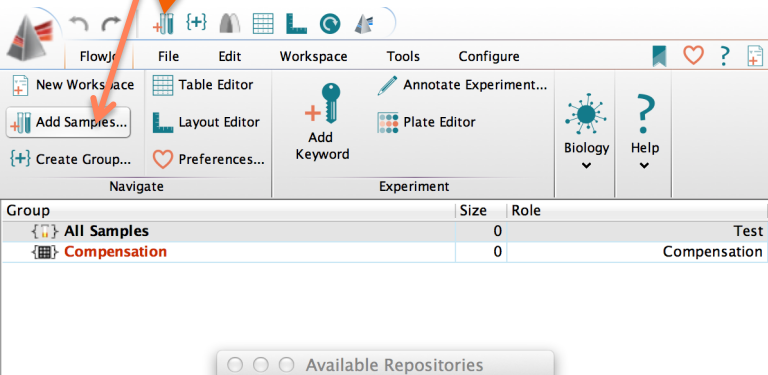
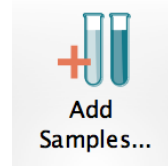




# Importing Data

Three possible methods:

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



	Name	Statistic	#Cells
<input type="checkbox"/>	Bead Comps_4 PE-TR_F01_exp.fcs		19202
<input type="checkbox"/>	Bead Comps_8 PB_F02_exp.fcs		14969
<input type="checkbox"/>	Bead Comps_38 PE-Cy5_F03_exp.fcs		17603
<input type="checkbox"/>	Bead Comps_DR APC-H7_F04_exp.fcs		18907
<input type="checkbox"/>	Bead Comps_US Beads +FP_F05_exp.fcs		30000
<input type="checkbox"/>	Bead Comps_ERK A488_F06_exp.fcs		24114
<input type="checkbox"/>	Bead Comps_IFN PE-Cy7_F07_exp.fcs		30000
<input type="checkbox"/>	Bead Comps_Perforin PE_F08_exp.fcs		19212
<input type="checkbox"/>	Bead Comps_US Beads No FP_F09_exp.fcs		10290
<input type="checkbox"/>	Cell Comps_AARD_E01_exp.fcs		145743
<input type="checkbox"/>	Cell Comps_CD3 A700_E02_exp.fcs		129537
<input type="checkbox"/>	Cell Comps_US Cells_E03_exp.fcs		158360
<input type="checkbox"/>	LD1_NS+NS_A01_exp.fcs		250342
<input type="checkbox"/>	LD1_PI+NS_B01_exp.fcs		262774
<input type="checkbox"/>	LD1_NS+PI_C01_exp.fcs		229585
<input type="checkbox"/>	LD1_PI+PI_D01_exp.fcs		244977
<input type="checkbox"/>	LD2_NS+NS_A02_exp.fcs		330780
<input type="checkbox"/>	LD2_PI+NS_B02_exp.fcs		279202
<input type="checkbox"/>	LD2_NS+PI_C02_exp.fcs		286306
<input type="checkbox"/>	LD2_PI+PI_D02_exp.fcs		275465
<input type="checkbox"/>	LD4_NS+NS_A03_exp.fcs		222740
<input type="checkbox"/>	LD4_PI+NS_B03_exp.fcs		224146

Drag Samples Here

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

## Polyclonal PFICS Assay:

- Thaw and rest cryopreserved human PBMC overnight
- Stimulate with PMA+Ionomycin (PI) for 2 hours or rest (NS) while blocking protein secretion → signaling and cytokines
- Stain for viability (AARD) and surface antigens (CD3, CD4, CD8, CD38 and HLA-DR)
- Stimulate PI for 20 minutes or NS rest
- Fix, perm and stain for intracellular antigens (phospho-ERK1/2, IFN- $\gamma$  and Perforin)



# PFICS Stim Conditions

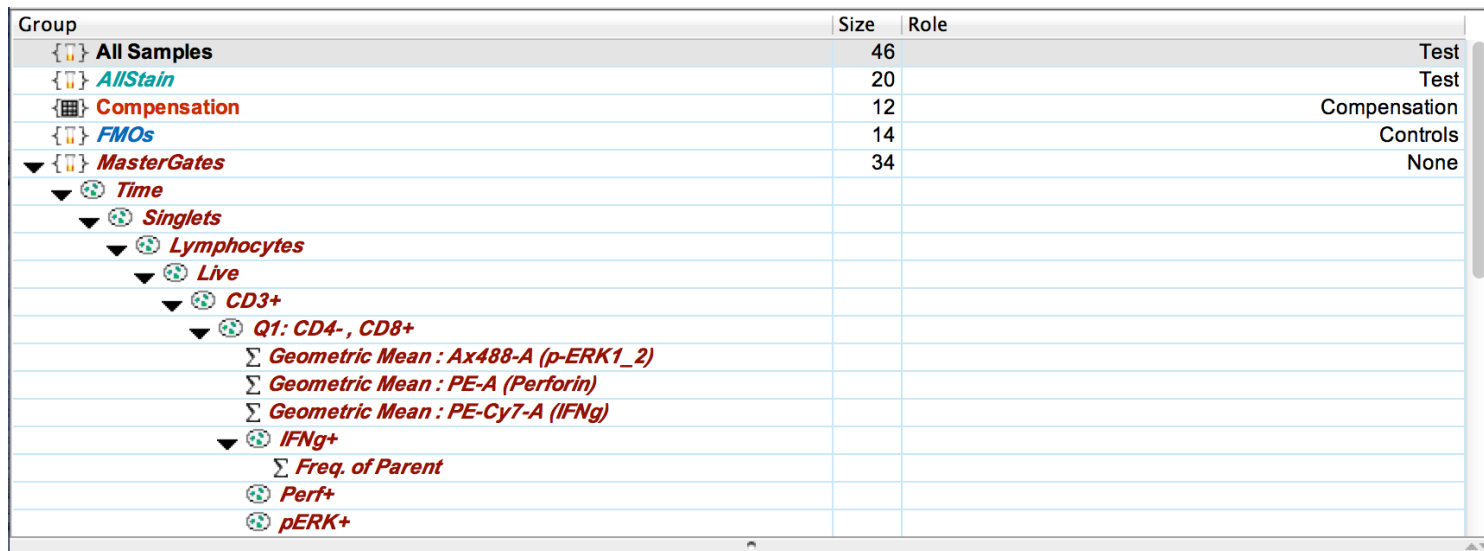
- 2 Stims → 4 potential combinations

Condition	Total Stim Time	phospho-ERK Response	IFN- $\gamma$ Response
NS+NS	0 min	-	-
NS+PI	20 min	++++	-
PI+NS	120 min	+++	+++
PI+PI	140 min	+++	+++

- 5 donors X 4 stim conditions = 20 experimental All Stain samples
- 1 donor with Fluorescence Minus One (FMO) controls  
7 x 2 stim conditions = 14 FMOs
- 12 Compensation Controls

# Group Pane

- 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 samples, allows master gating and unique report generation.

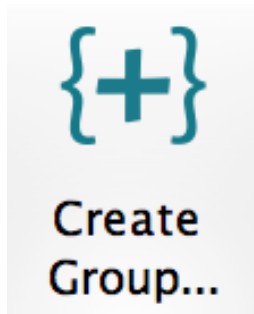


Group	Size	Role
{ } All Samples	46	Test
{ } AllStain	20	Test
{ } Compensation	12	Compensation
{ } FMOs	14	Controls
▼ { } MasterGates	34	None
{ } Time		
▼ { } Singlets		
▼ { } Lymphocytes		
▼ { } Live		
▼ { } CD3+		
▼ { } Q1: CD4-, CD8+		
Σ Geometric Mean : Ax488-A (p-ERK1_2)		
Σ Geometric Mean : PE-A (Perforin)		
Σ Geometric Mean : PE-Cy7-A (IFNγ)		
▼ { } IFNγ+		
Σ Freq. of Parent		
{ } Perf+		
{ } pERK+		

- Group owned analysis gains the group color.

# 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.

Modify Group

**Appearance**

Name:  Color:  Style:

Role:  Parameter Key:

**Sample Inclusion Criteria**

Live group  Synchronized

Include samples that use the following staining:

Show all keywords in menus

With reference to samples in another group:

Only choose from

Also include all

**Assignments**

Add Keyword :  With Value :

Add Keyword :  With Value :

# Sample Inclusion Criteria

- Live groups automatically include samples based on user-defined inclusion criteria.

- Criteria could include the staining panel, a keyword, characters in the file name, or any combination of these features.

The screenshot shows a 'Modify Group' dialog box with the following sections:

- Appearance:** Name: PI+PI, Color: [teal square], Style: Bold-Italic, Role: Test, Parameter Key: [empty].
- Sample Inclusion Criteria:**  Live group,  Synchronized. Include samples that use the following staining: [Multiple] Dead, HLA-DR, p-ERK1\_2, Blank, CD3, Perforin, CD38, IFNg, CD4, CD8. Rules: \$FIL Contains LD, And \$FIL Lacks FMO, And \*STIM = PI+PI. Buttons: More Choices, Fewer Choices, Show all keywords in menus.
- With reference to samples in another group:**  Only choose from,  Also include all. samples in Group: (No specified group).
- Assignments:** Add Keyword: [empty] With Value: [empty], Add Keyword: [empty] With Value: [empty].
- Buttons:** Help with Groups, Apply Changes, Close, 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)

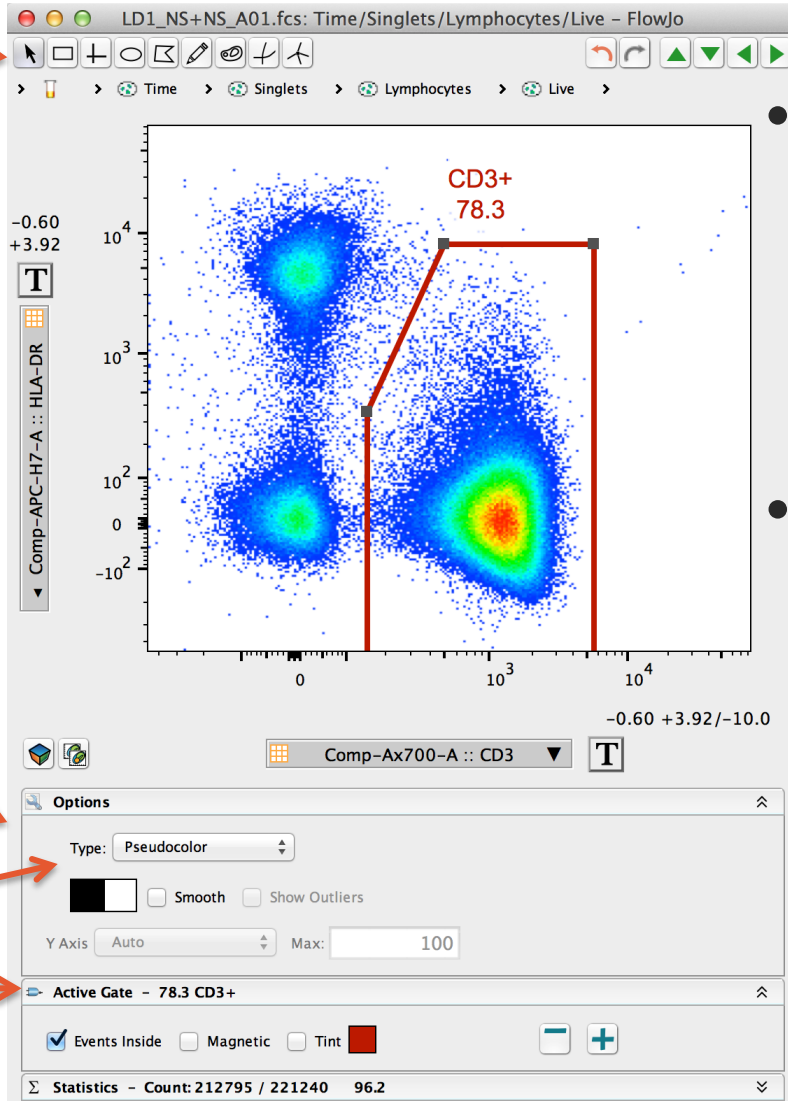
Name	Statistic	#Cells	*HIV Status	*PID	*STIM
LD1_NS+NS_A01.fcs		250342	Neg	LD1	NS+NS
LD1_NS+PI_C01.fcs		229585	Neg	LD1	NS+PI
LD1_PI+NS_B01.fcs		262774	Neg	LD1	PI+NS
Time	99.7	261964			
Singlets	96.2	252097			
Lymphocytes	93.7	236200			
Live	96.2	227167			
CD3+	81.4	184893			
Q1: CD4-, CD8+	24.0	44355			
Q2: CD4+, CD8+	1.13	2090			
Q3: CD4+, CD8-	72.7	134352			
Q4: CD4-, CD8-	2.22	4096			
LD1_PI+PI_D01.fcs		244977	Neg	LD1	PI+PI

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

# The Graph Window

- Facilitates data visualization and gating.

Gating Tools



Plot View Options

Graph Type

Active Gate Options

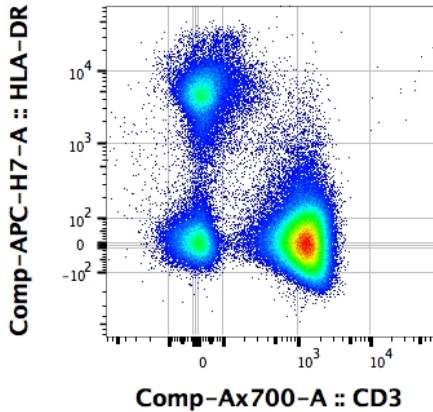
- Several different plot types are available to display flow data.
- Click on the Options Menu below the graph image and select Graph Type from the dropdown menu.



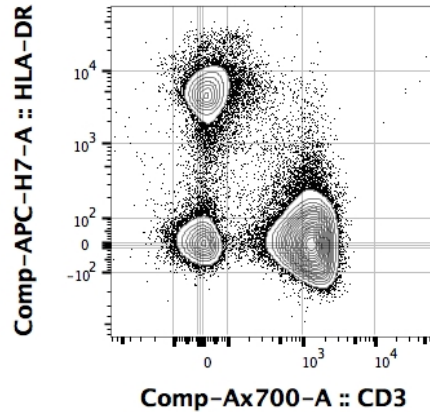
# Graph Display Options

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

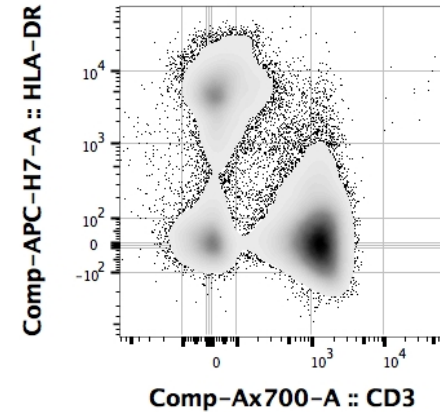
Pseudocolor



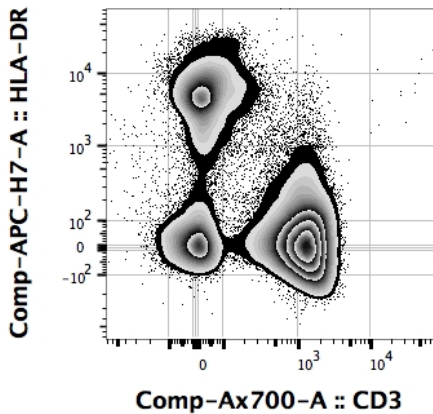
Contour



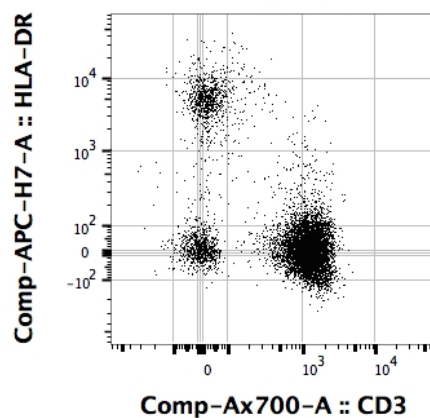
Density



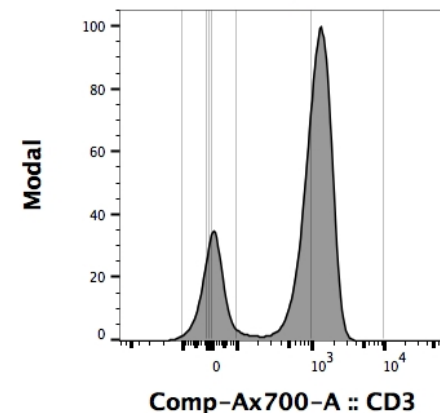
Zebra



Dot Plot



Histogram

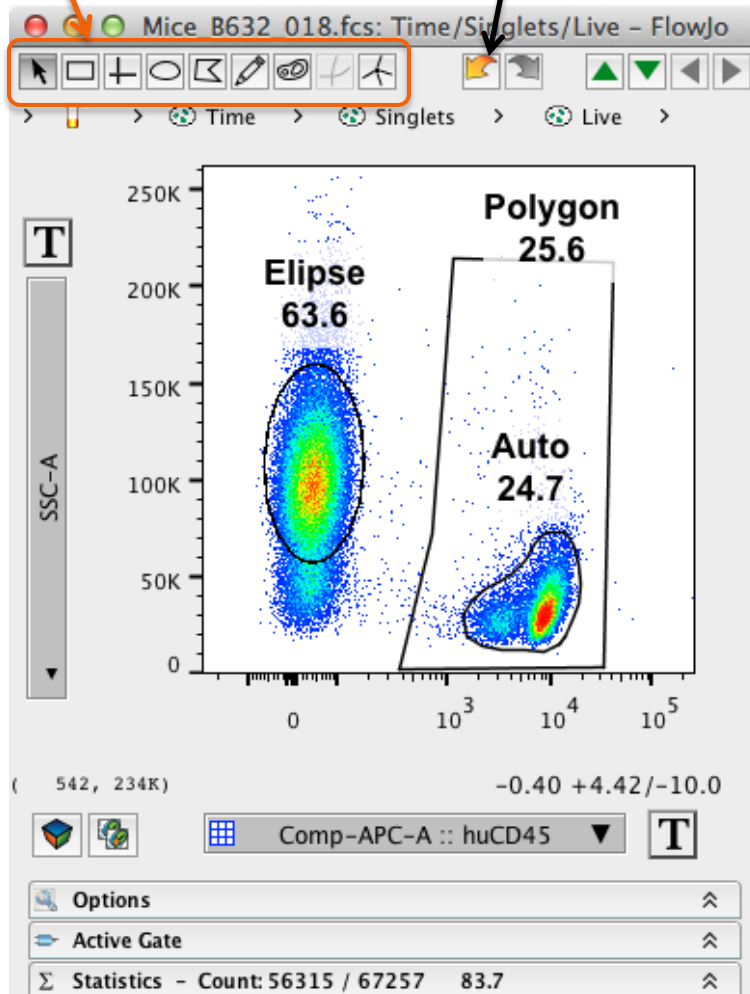


# Gating tools

- Are located at the top left in a Graph Window.

Gating Tools

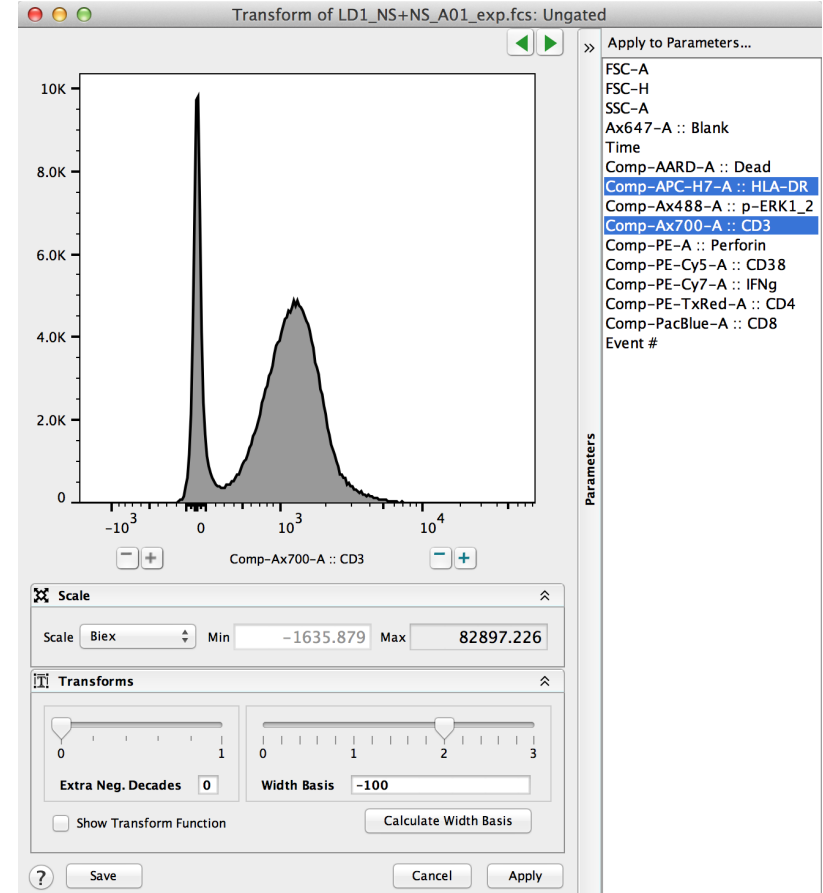
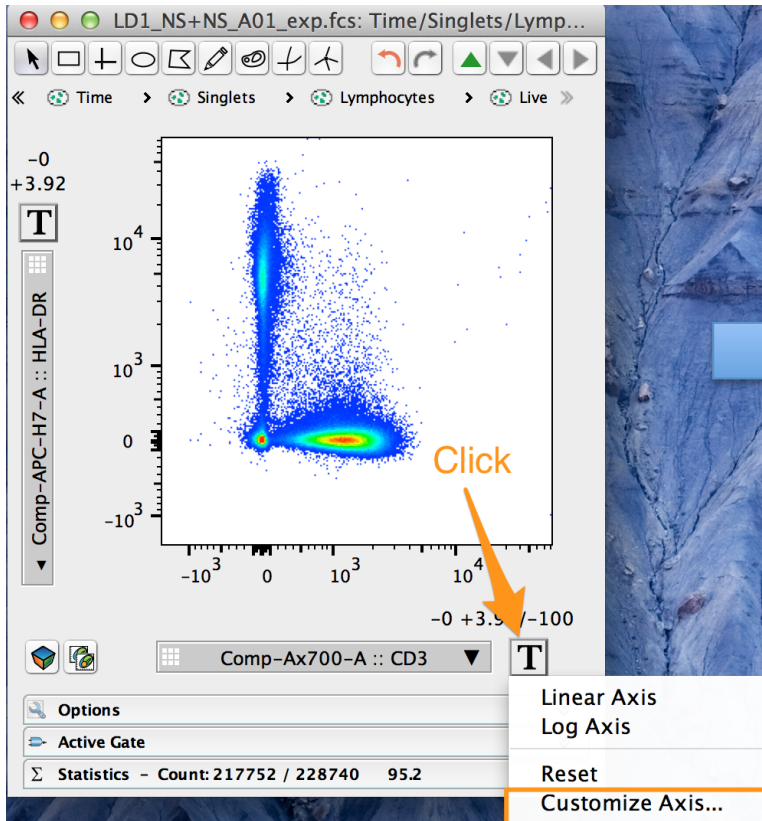
Undo!!



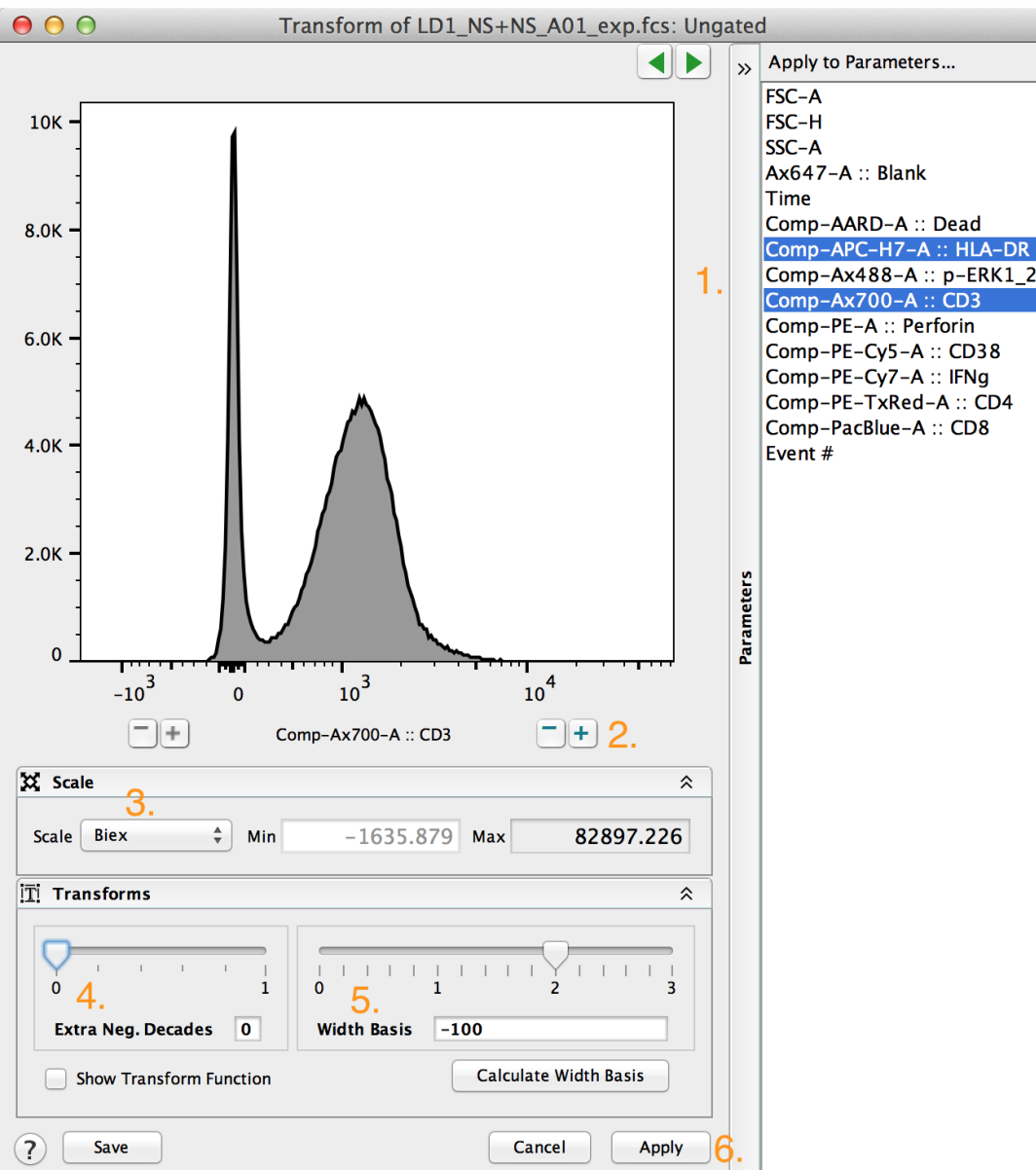
- Gates can always be modified or removed, so don't be shy.
- Explore the gating options and pick what works best for you.

# Transforming Data

- Your data may initially look ‘squished’.
- Click the Transformation [ T ] button and Select Customize Axis... to change the visual display.

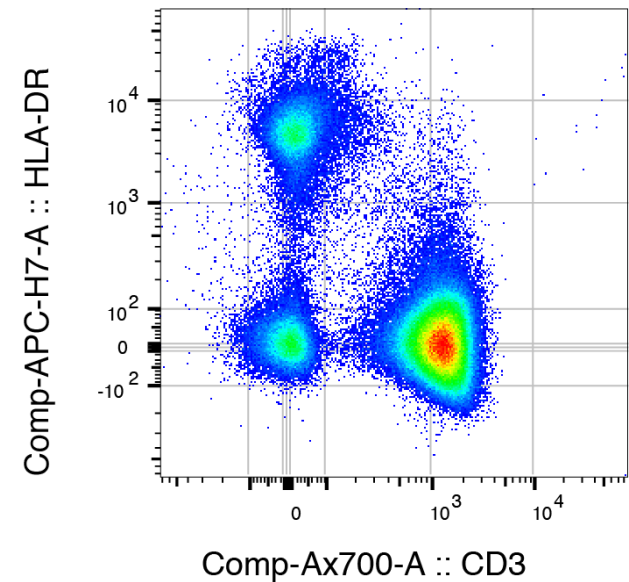
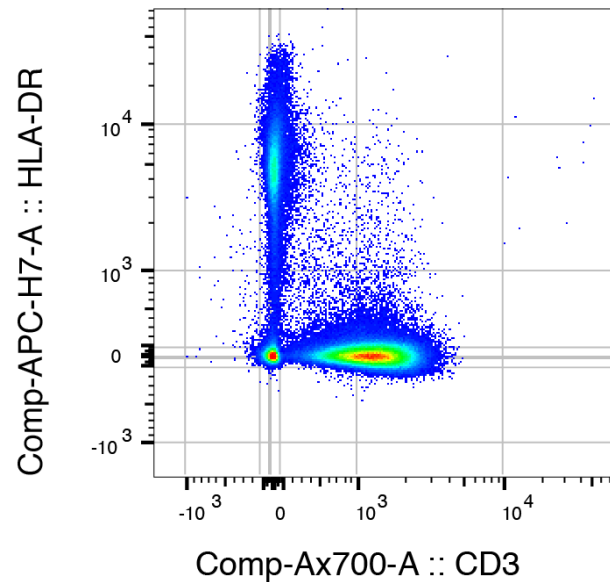
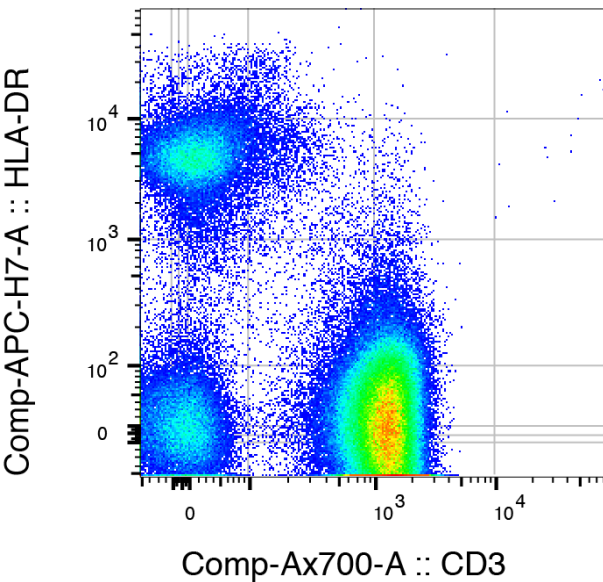


# Transform Options



1. Select parameter(s)
2. Add or remove extra Pos. decades/range on top end
3. Select scale (Biex displays linear around zero and log further out)
4. Add or remove extra Neg. decades/range on bottom end
5. Width basis scales how much visual display is given to linear vs. log range of the Biex scale
6. Click the Apply button at bottom right to apply the transformation settings to selected parameters

# Effects of Transformation



## Effects:

1. Gets rid of the “squishing” of cells.
2. Ensures the visual population center better correlates with the statistical center (median).
3. Make high resolution compensated digital cytometry data more appealing to the eye.

# Boolean Combination Gates

- Calculate all possible combinations based on single marker gates ( $\# \text{combinations} = 2^{\# \text{gates}}$ ).

**2. Select Create Combination Gates**

**3. Abbreviate names and click**


**1. Highlight single marker gates**

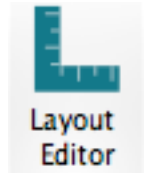
Group	Size	Role
{ } FMOs	0	Controls
{ } High Responders	4	Test
{ } MasterGates	8	None
{ } Singlets		
{ } Lymphocytes		
{ } Live		
{ } CD3+		
{ } Q1: CD4-, CD8+		
Σ Geometric Mean : Comp-Ax488-A (p-ERK1_2)		
{ } IFNg+		
Σ Freq. of Parent		
Σ Geometric Mean : Comp-PE-Cy7-A (IFNg)		
{ } Perf+		
Σ Geometric Mean : Comp-PE-A (Perforin)		
{ } pERK+		
Σ Geometric Mean : Comp-Ax488-A (p-ERK1_2)		
{ } Q1: HLA-DR-, CD38+		

Name	Size	Role
{ } Q1: CD4-, CD8+		
Σ Geometric Mean : Comp-Ax488-A (p-ERK1_2)	74.8	
{ } IFNg+	1.02	342
Σ Freq. of Parent	1.02	
Σ Geometric Mean : Comp-PE-Cy7-A (IFNg)	635	
{ } Perf+	30.1	10055
Σ Geometric Mean : Comp-PE-A (Perforin)	814	
{ } pERK+	4.70	1568
Σ Geometric Mean : Comp-Ax488-A (p-ERK1_2)	775	



# 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.

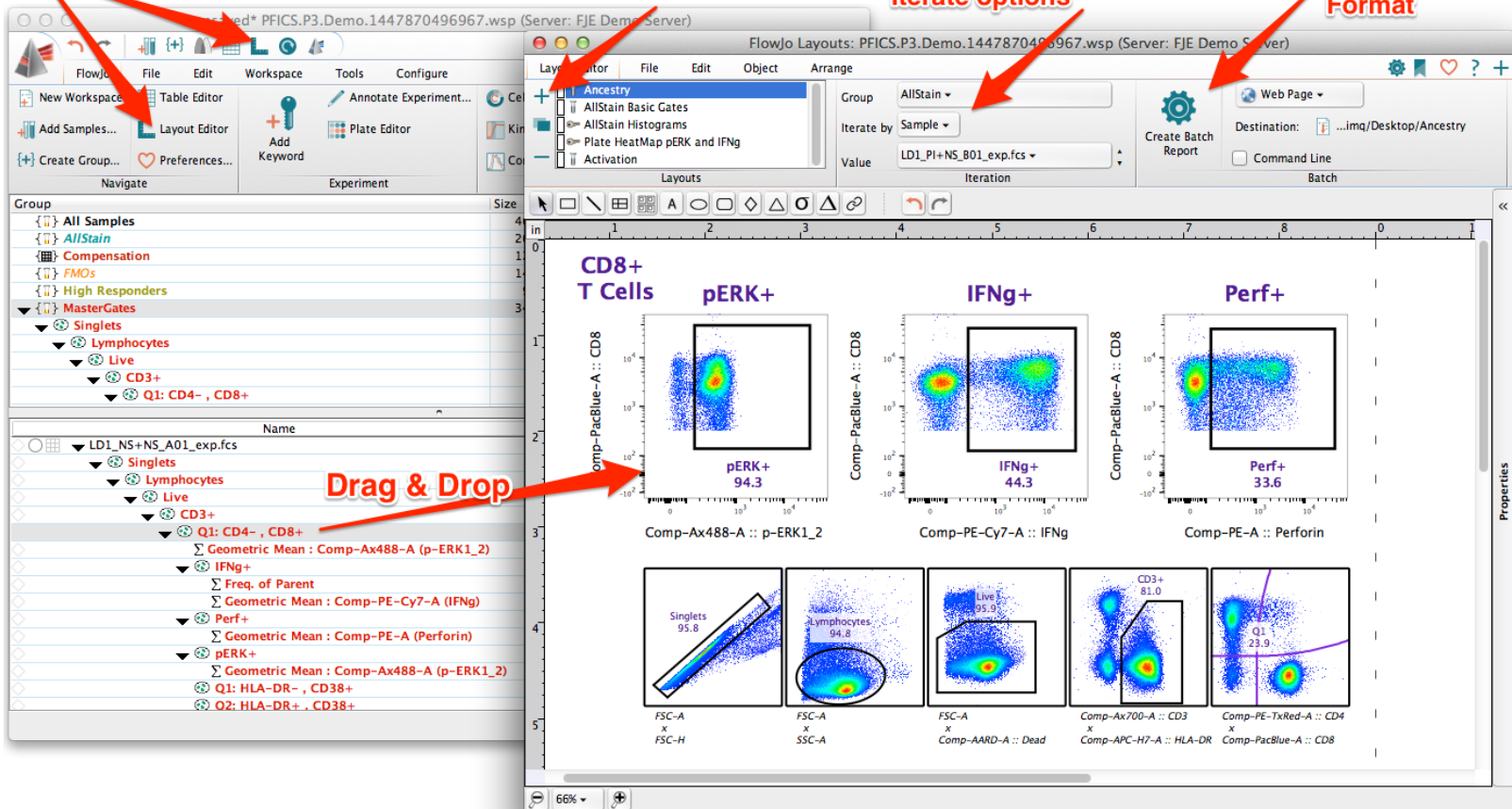


Layout Editor

Create Layouts

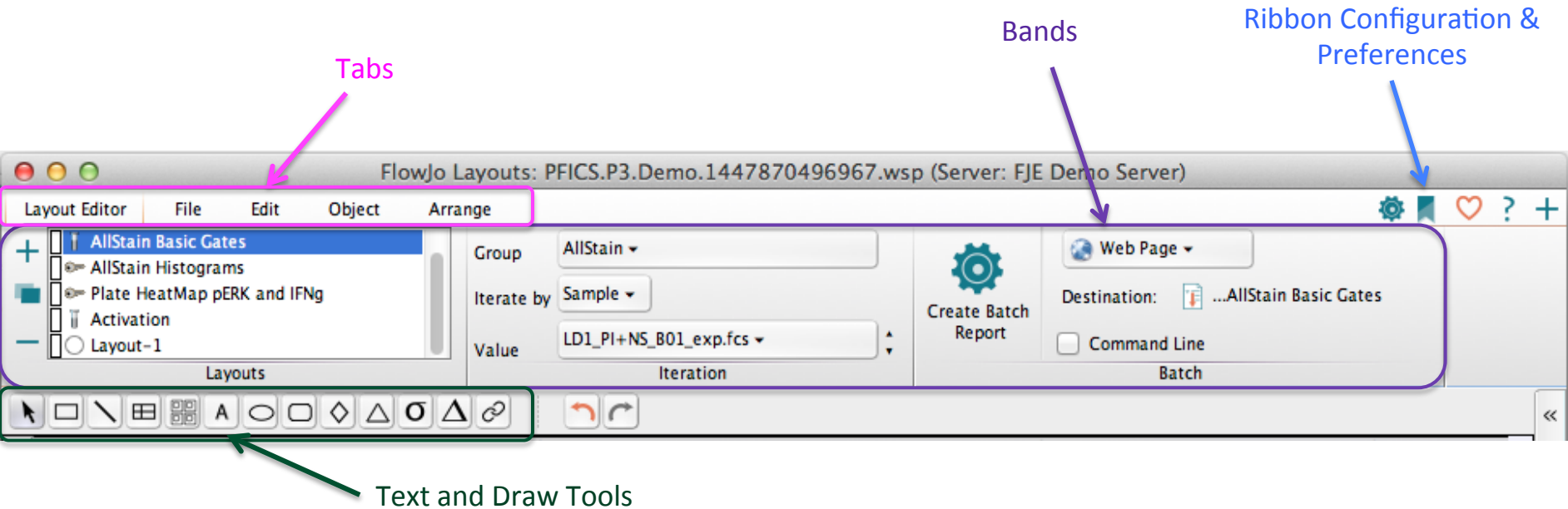
Specify Group and Iterate options

Batch Report Format



# Working in Layout Editor

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

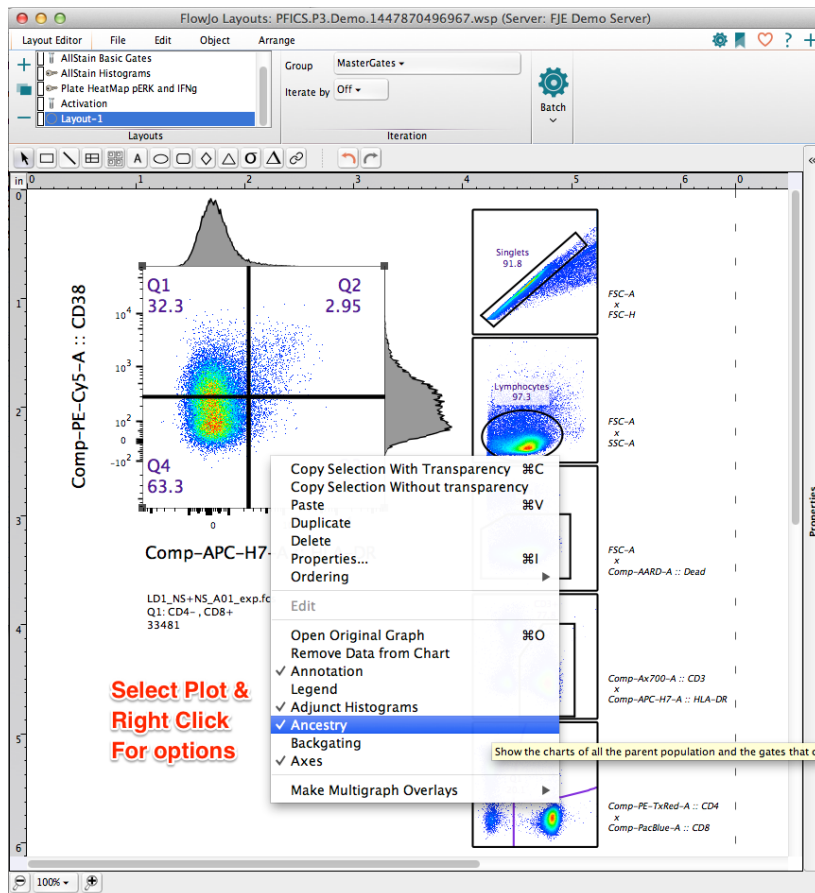


- Try clicking on the different tabs to see what types of actions are available.



# 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.



- Right Click on a graph plot for Ancestry and Backgating options
- Right click and select Properties for additional graph formatting

# Working in Layout Editor

- Double Click a graph to change its properties/formatting with 4 tabs of Graph Definition options

**Tabs**

**Axis Parameters**

**Graph Type and Options**

**Annotate Tab**

**Gate Annotation options**

**Axis Label options**

Graph Definition

LD1\_NS+NS\_A01\_exp.fcs

Specify Annotate Fonts Legend

X Axis: Comp-APC-H7-A :: HLA-DR

Y Axis: Comp-PE-Cy5-A :: CD38

Type: Pseudocolor

Contour Levels: 5%

Smoothing

Show Outliers

Use Large Dots

Show Grid

Y Axis: Auto Max: 1000

Scale: Width: 100% Height: 100%  Lock Shape

Apply Cancel OK

Graph Definition

LD1\_NS+NS\_A01\_exp.fcs

Specify Annotate Fonts Legend

Annotation

Show Gates  Show Frequencies

Axes  Show Population Names

Adjunct Histograms

Ancestry  Backgating

Horizontal

X Axis

Hide Ticks  Hide Numbers  Hide Label

Label:

Y Axis

Hide Ticks  Hide Numbers  Hide Label

Label:

Apply Cancel OK

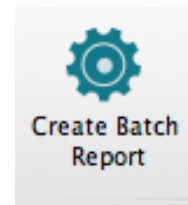
# Batch Analysis of Layout Editor Graphics

The screenshot displays the FlowJo software interface. At the top, the title bar reads "FlowJo Layouts: F:\CS.P3.Demo.144787\496967.wsp (Server: FJE Demo Service)". The main window is divided into several sections:

- Layout Editor:** Contains a tree view on the left with folders like "AllStain Basic Gates", "AllStain Histograms", "Plate HeatMap PERK and IFNg", "Activation", and "Layout-1".
- Group & Iteration options:** A section with a "Group" dropdown set to "MasterGates", an "Iterate by" dropdown set to "Sample", and a "Value" dropdown set to "FMOs PI+PI\_No DR\_D07\_exp.fcs".
- Report Type & Location:** A section with a "Create Batch Report" button (highlighted with a red box and arrow), a "Web Page" dropdown, a "Destination" field set to "...img/Desktop/Layout-1", and a "Command Line" checkbox.
- Main Plot Area:** A large multi-panel plot. The top-left panel is a histogram of "Comp-PE-Cy5-A :: CD38" with a gate labeled "Q1 24.2". Below it is a 2x2 quadrant plot of "Comp-PE-Cy5-A :: CD38" vs "Comp-APC-H7-A :: HLA-DR" with gates labeled "Q2 1.97E-3", "Q3 5.90E-3", and "Q4 75.7". To the right are several smaller plots: "Singlets 95.4", "Lymphocytes 93.9", "Live 94.2", "CD3+ 80.0", and "Q1 23.9". Each plot has its own axis labels and gate percentages.

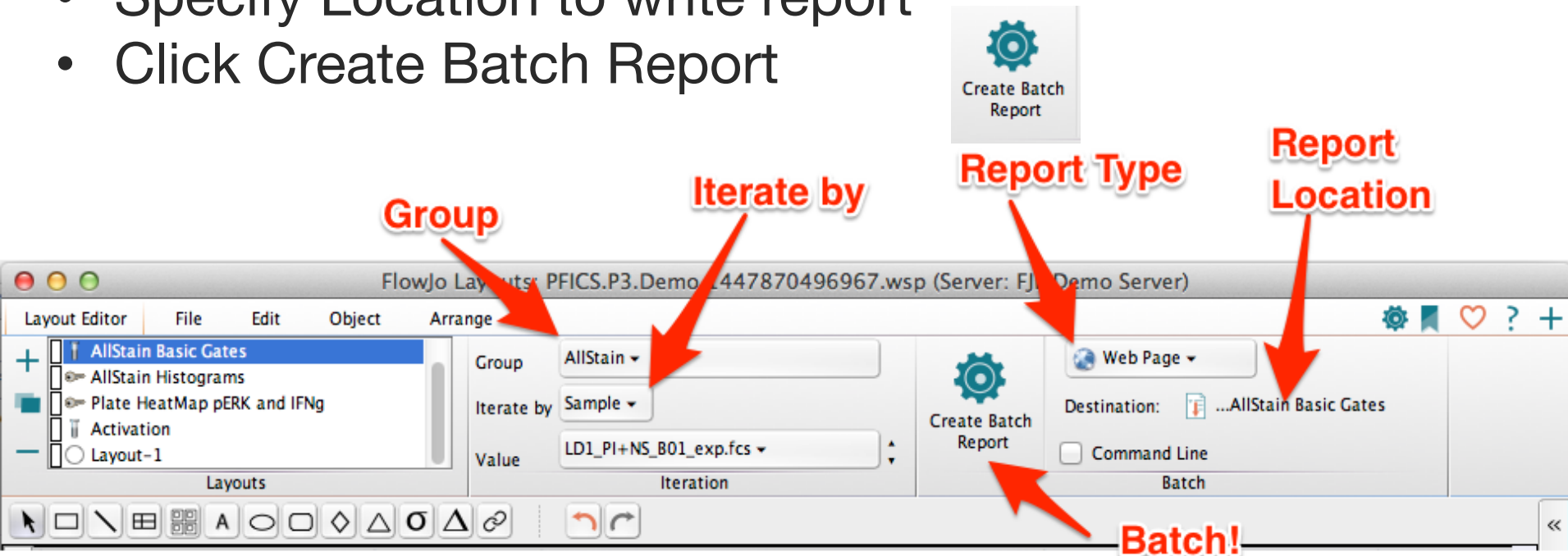
Red arrows point from the text labels to the corresponding UI elements: "Specify Group & Iteration options" points to the Group and Iterate by dropdowns; "Report Type & Location" points to the Create Batch Report button and the Destination field; "Then Click Create Batch Report" points to the Create Batch Report button.

- Batch operations perform repetitive analysis on multiple samples, applying the layout to an entire set of samples.
- Specify Group, Iterate by, Report type and Location, then Click Create Batch Report .



# Batch Report Layouts

- Specify Group
- Choose Iterate by option
  - Sample
  - Panel
  - Keyword
    - Iterate By (must be Same for all samples displayed in layout)
    - Discriminator (must be Different for all samples displayed in layout)
- Specify type of Report
- Specify Location to write report
- Click Create Batch Report



# The Table Editor

- A tool for creating statistical reports.
- Type  $\boxplus$  T, or click on the Table Editor icon.
- Drag Populations & Statistics to Table Editor.



Table Editor

Open Table Editor

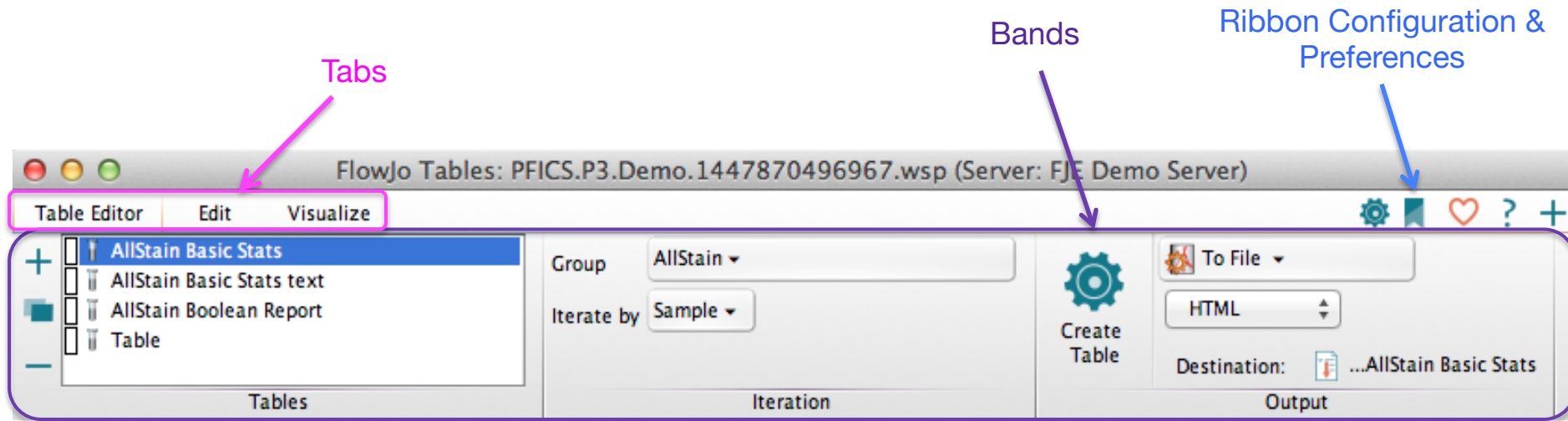
FlowJo Tables: PFICS.P3.Demo.1447870496967.wsp (Server: FJE Demo Server)

Col...	Population	Statistic	Parameter	Name
1	*PID			
2	*STIM			
3	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Geometric Mean	Comp-Ax488-A	pERK GMF
4	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/IFNg+	Freq. of Parent		% IFNg+
5	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Perf+	Freq. of Parent		% Perf+
6	Formula			CD4/CD8...
7	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q2: HLA-DR+, CD38+	Freq. of Parent		HLA-DR+, ...
8	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/pERK+	Freq. of Parent		% pERK+
9	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/IFNg+	Geometric Mean	Comp-PE-Cy7-A	IFNg GMF
10	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Perf+	Geometric Mean	Comp-PE-A	Perf GMF
11	Singlets/Lymphocytes/Live	Freq. of Parent		Viability
12	Singlets/Lymphocytes/Live/CD3+	Freq. of Parent		% CD3+
13	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Freq. of Parent		% CD8+
14	Singlets/Lymphocytes/Live/CD3+/Q3: CD4+, CD8-	Freq. of Parent		% CD4+

Drag Populations & Statistics

# 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.

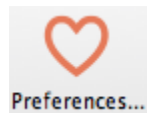
# Table Editor Visualize Tools

- Table formatting/visualization options such as heat mapping are contained within the Visualize Tab.

- Highlight row(s), then select the visualization.

- Expected Ranges can be set within Preferences

→ Ranges



FlowJo Tables: PFICS.P3.Demo.1447870496967.wsp (Server: FJE Demo Server)

Table Editor Edit **Visualize**

Heat Map **2. Apply visualization tool**

Standard Deviation

Expected Range NK Cells

Time Series Correlation 3D Plot

C...	Population	Statistic	Parameter	Name
1	*PID			
2	*STIM			
3	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+	Geometric Mean	Comp-Ax488-A	pERK GMF
4	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+ /IFNg+ <b>1. Highlight Rows</b>	Freq. of Parent		% IFNg+
5	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+ /Perf+	Freq. of Parent		% Perf+
6	Formula			CD4/CD8 R...
7	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+/Q2: HLA-DR+ , CD38+	Freq. of Parent		HLA-DR+ ,C...
8	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+ /pERK+	Freq. of Parent		% pERK+
9	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+ /IFNg+	Geometric Mean	Comp-PE-Cy7-A	IFNg GMF
10	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+ /Perf+	Geometric Mean	Comp-PE-A	Perf GMF
11	Singlets/Lymphocytes/Live	Freq. of Parent		Viability
12	Singlets/Lymphocytes/Live/CD3+	Freq. of Parent		% CD3+
13	Singlets/Lymphocytes/Live/CD3+/Q1: CD4- , CD8+	Freq. of Parent		% CD8+
14	Singlets/Lymphocytes/Live/CD3+/Q3: CD4+ , CD8-	Freq. of Parent		% CD4+



# Table Editor Output

- Formatting/visualization options are maintained when a table is batched to either Display or HTML formats.

- Other file types (ex. Text, CSV, Excel) produce statistics tables lacking visualization formatting.

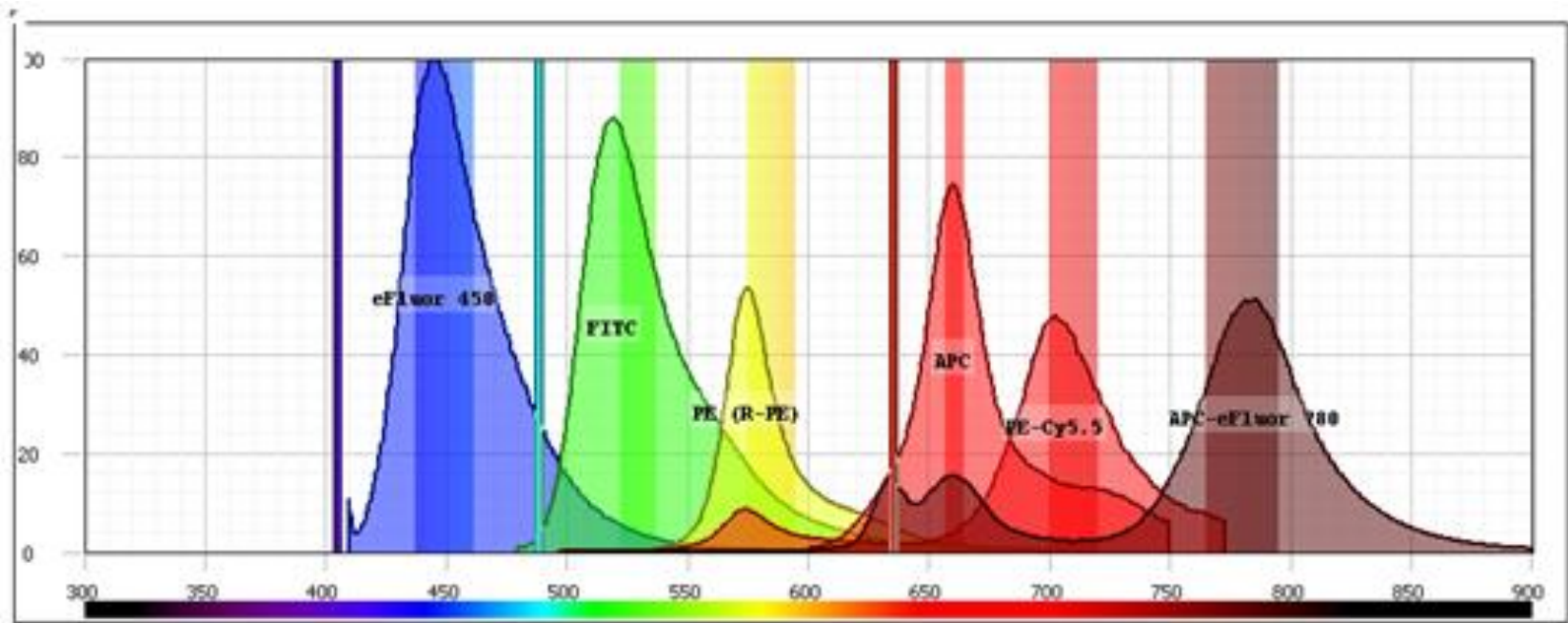
Table - AllStain Basic Stats

Ancestry Subset Statistic For	*PID	*STIM	pERK GMF	% IFNg+	% Perf+	CD4/CD8 Ratio	HLA-DR+,	% pERK+	IFNg GMF	Perf GMF
LD1_NS...	LD1	NS+NS	74.1	1.09	30.2	▲ 3.81	2.95	4.70	642	812
LD1_NS...	LD1	NS+PI	503	0.96	30.0	▲ 4.13	2.72	94.9	504	809
LD1_PI+...	LD1	PI+NS	375	44.3	33.6	▲ 3.04	2.26	94.3	4917	807
LD1_PI+...	LD1	PI+PI	373	43.8	32.7	▲ 3.06	1.94	94.5	4907	816
LD2_NS...	LD2	NS+NS	75.6	1.83	55.9	2.80	2.07	0.45	509	818
LD2_NS...	LD2	NS+PI	496	1.91	53.4	▲ 3.01	1.87	91.0	425	752
LD2_PI+...	LD2	PI+NS	420	64.0	52.1	▲ 2.86	1.27	92.6	5894	739
LD2_PI+...	LD2	PI+PI	407	63.7	51.4	▲ 2.91	1.46	92.7	5768	734
LD4_NS...	LD4	NS+NS	86.6	1.05	21.1	1.52	2.71	8.08	494	740
LD4_NS...	LD4	NS+PI	596	1.74	23.6	1.52	2.80	97.1	403	775
LD4_PI+...	LD4	PI+NS	456	28.2	23.8	▼ 1.21	1.74	96.8	5298	577
LD4_PI+...	LD4	PI+PI	449	26.5	22.6	▼ 1.22	1.48	96.4	5035	566
LD12_N...	LD12	NS+NS	67.5	0.74	37.5	▲ 3.64	2.93	4.14	755	440
LD12_N...	LD12	NS+PI	414	0.50	35.3	▲ 4.28	3.19	89.3	683	444
LD12_PI...	LD12	PI+NS	327	45.3	40.8	1.94	1.50	84.8	4632	408
LD12_PI...	LD12	PI+PI	319	46.1	41.4	1.94	1.64	83.7	4793	403
LD14_N...	LD14	NS+NS	72.4	0.50	14.3	2.11	1.90	4.11	689	811
LD14_N...	LD14	NS+PI	483	0.45	13.8	2.30	2.19	95.5	595	829
LD14_PI...	LD14	PI+NS	366	17.7	18.2	1.66	1.21	94.8	3708	650
LD14_PI...	LD14	PI+PI	351	17.0	18.3	1.67	1.10	93.2	3565	644
Mean			336	20.4	32.5	2.53	2.05	70.7	2711	679
SD			167	23.0	13.4	0.96	0.65	39.5	2259	152



# 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

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



in the task bar.

2. Click the Compensation Tool

1. Highlight Compensation Group

Group	Size	Role
{ } All Samples	12	Test
{ } <b>Compensation</b>	12	Compensation
{ } PFICS Compensation Controls	12	Test

Name	Statistic	#Cells
Bead Comps_DR APC-H7_F04_exp.fcs (Control)		18907
Bead Comps_ERK A488_F06_exp.fcs (Control)		24114
Bead Comps_IFN PE-Cy7_F07_exp.fcs (Control)		30000
Bead Comps_Perforin PE_F08_exp.fcs (Control)		19212
Bead Comps_US Beads +FP_F05_exp.fcs (Control)		30000
Bead Comps_US Beads No FP_F09_exp.fcs (Control)		10290
Bead Comps_4 PE-TR_F01_exp.fcs (Control)		19202
Bead Comps_8 PB_F02_exp.fcs (Control)		14969
Bead Comps_38 PE-Cy5_F03_exp.fcs (Control)		17603
Cell Comps_AARD_E01_exp.fcs (Control)		145743
Cell Comps_CD3 A700_E02_exp.fcs (Control)		129537
Cell Comps_US Cells_E03_exp.fcs (Control)		158360

The wizard auto gates samples

Group	Size	Role
{ } All Samples	12	Test
{ } <b>Compensation</b>	12	Compensation
{ } PFICS Compensation Controls	12	Test

Name	Statistic	#Cells
Bead Comps_DR APC-H7_F04_exp.fcs (Control)		18907
Size	60.3	11396
APC-H7-A+	42.8	4873
Bead Comps_ERK A488_F06_exp.fcs (Control)		24114
Size	66.8	16113
Ax488-A+	47.1	7593
Bead Comps_IFN PE-Cy7_F07_exp.fcs (Control)		30000
Size	70.4	21132
PE-Cy7-A+	52.5	11095
Bead Comps_Perforin PE_F08_exp.fcs (Control)		19212
Size	71.0	13645
PE-A+	55.4	7559
Bead Comps_US Beads +FP_F05_exp.fcs (Control)		30000
Size	70.7	21206
Ax647-A+	100.0	21197
Bead Comps_US Beads No FP_F09_exp.fcs (Control)		10290
Size	76.4	7859
Bead Comps_4 PE-TR_F01_exp.fcs (Control)		19202
Size	66.1	12699
PE-TxRed-A+	48.9	6205
Bead Comps_8 PB_F02_exp.fcs (Control)		14969
Size	66.7	9988

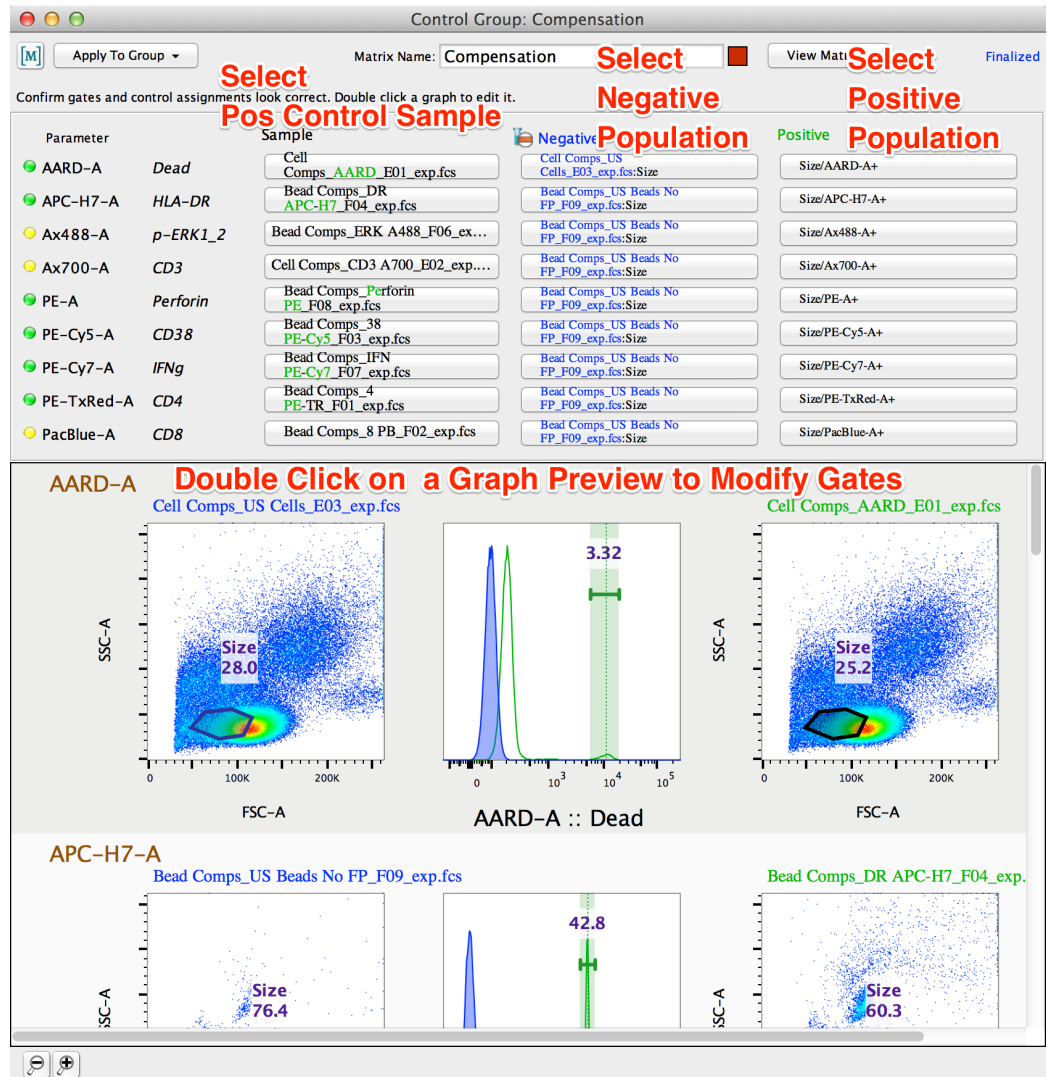
# Compensation

- Then fills in the positive and negative.

- Choose from the dropdown lists for each parameter.

- Double click preview graphs to modify gates.

For each Parameter



Control Group: Compensation

Apply To Group ▾ Matrix Name:   Finalized

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

Parameter	Sample	Negative	Positive
<input checked="" type="radio"/> AARD-A <i>Dead</i>	Cell Comps <b>AARD_E01_exp.fcs</b>	Cell Comps_US Cells_E03_exp.fcs:Size	Size/AARD-A+

**Use Sample drop down list**

to select Pos Control Sample and

**Choose or Remove Parameters**

Bead Comps\_DR APC-H7\_F04\_exp.fcs :: Size  
 Bead Comps\_ERK A488\_F06\_exp.fcs :: Size  
 Bead Comps\_IFN PE-Cy7\_F07\_exp.fcs :: Size  
 Bead Comps\_Perforin PE\_F08\_exp.fcs :: Size  
 Bead Comps\_US Beads +FP\_F05\_exp.fcs :: Size  
 Bead Comps\_US Beads No FP\_F09\_exp.fcs :: Size  
 Bead Comps\_4 PE-TR\_F01\_exp.fcs :: Size  
 Bead Comps\_8 PB\_F02\_exp.fcs :: Size  
 Bead Comps\_38 PE-Cy5\_F03\_exp.fcs :: Size  
  
 Cell Comps\_CD3 A700\_E02\_exp.fcs :: Size  
 Cell Comps\_US Cells\_E03\_exp.fcs :: Size

<Clear>  
 <Remove This Parameter> ←  
 Choose Parameters ←  
 <Reset All>  
 <Reset This Parameter>

**Use Negative drop down list**

to Select Negative Sample or Population

Bead Comps\_DR APC-H7\_F04\_exp.fcs :: Size  
 Bead Comps\_ERK A488\_F06\_exp.fcs :: Size  
 Bead Comps\_IFN PE-Cy7\_F07\_exp.fcs :: Size  
 Bead Comps\_Perforin PE\_F08\_exp.fcs :: Size  
 Bead Comps\_US Beads +FP\_F05\_exp.fcs :: Size  
 Bead Comps\_US Beads No FP\_F09\_exp.fcs :: Size  
 Bead Comps\_4 PE-TR\_F01\_exp.fcs :: Size  
 Bead Comps\_8 PB\_F02\_exp.fcs :: Size  
 Bead Comps\_38 PE-Cy5\_F03\_exp.fcs :: Size  
 Cell Comps\_AARD\_E01\_exp.fcs :: Size  
  
 Size  
 Size/AARD-A+

<Clear>

**Use Positive drop down list**

to Choose Positive population

Size  
  
 <Clear>

- Note that you can always create your own gates on a sample and then choose those from the drop down menus.
- When set up is complete, select View Matrix (top right) to Modify, Apply, Save or Preview the matrix you've created.



# Compensation

Select Color

Name Matrix

Edit Matrix

Save a copy of the Matrix

The screenshot displays the 'Workspace Matrices' panel with a matrix named 'Compensation' selected. The matrix is a 10x10 table with values ranging from 0 to 39.6125. Below the matrix is a 'Preview Sample' section showing a grid of 10 flow cytometry plots for various markers: APC-H7-A :: HLA-DR, Ax488-A :: p-ERK1\_2, Ax700-A :: CD3, PE-A :: Perforin, PE-Cy5-A :: CD38, and PE-Cy7-A :: IFNg. The plots show the effect of the compensation matrix on the sample data.

**Applied Matrix Badge is Color Coded**

**Add a Matrix from file**

**Apply Matrix with Drag and Drop onto Group or Sample**

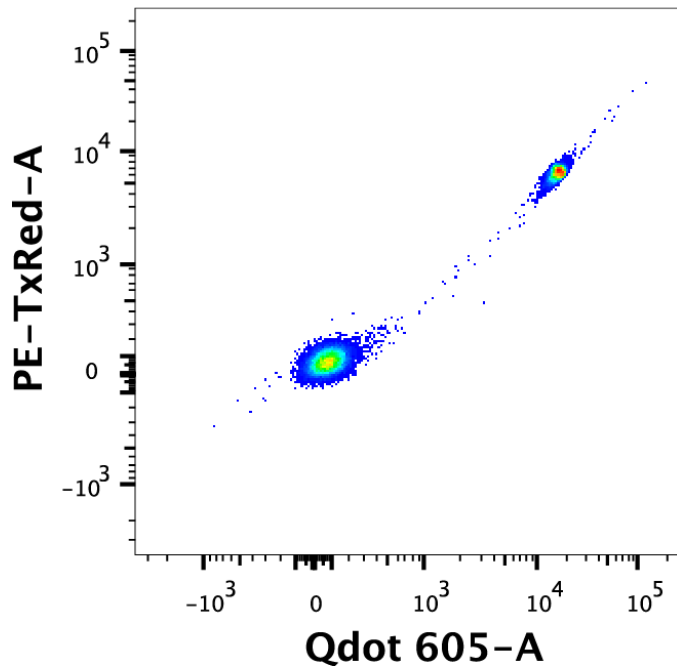
**Preview Matrix affect on a sample**

AARD-A :: Dead	APC-H7-A :: HLA-DR	Ax488-A :: p-ERK1_2	Ax700-A :: CD3	PE-A :: Perforin	PE-Cy5-A :: CD38	PE-Cy7-A :: IFNg	PE-TxRed-A :: CD4	PacBlue-A :: CD8
100	0.0351	0.3746	0.0685	0.0382	0.1447	0.0399	0.064	24.1599
0	100	0	3.2511	0.0169	0.8078	39.6125	0.056	0
1.8492	0	100	0	0.0119	0	0	0	0
0.1713	34.835	0.1071	100	0	1.0301	10.2007	0	0.0443
0	0.0125	0.3404	0.0375	100	14.4881	1.3119	37.6694	0
0	3.0045	0.0253	7.7547	1.6106	100	12.018	0.7082	0
0	5.7598	0.0603	0.3117	1.8877	0.368	100	0.8245	0
0	0.0291	0.1118	0.0572	23.9323	52.786	6.0459	100	0
16.9144	0	0.0597	0	0.0076	0	0	0.0063	100

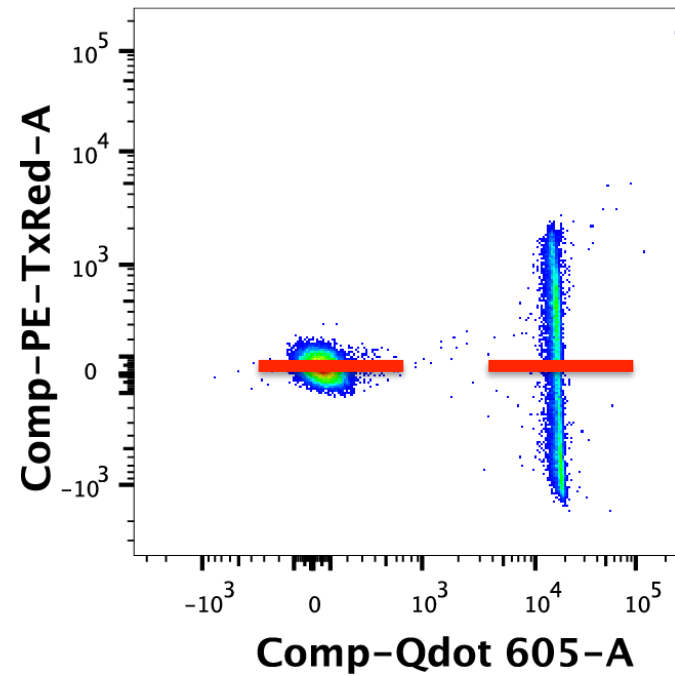
# Effect of Compensation



## Uncompensated



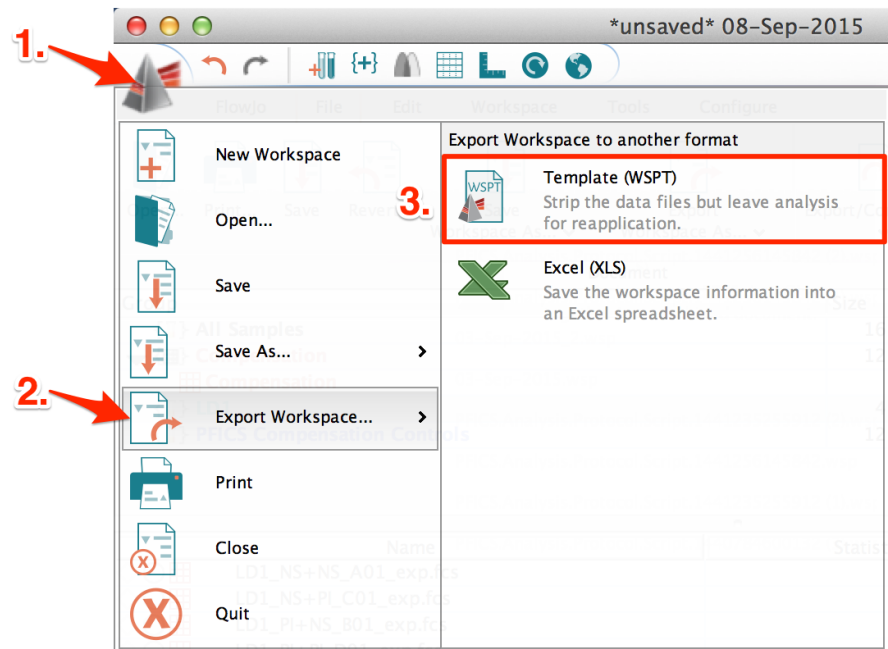
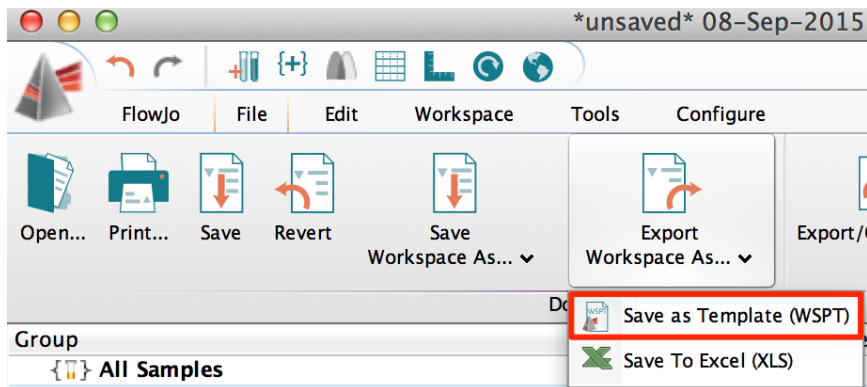
## Compensated





# Workspace Templates

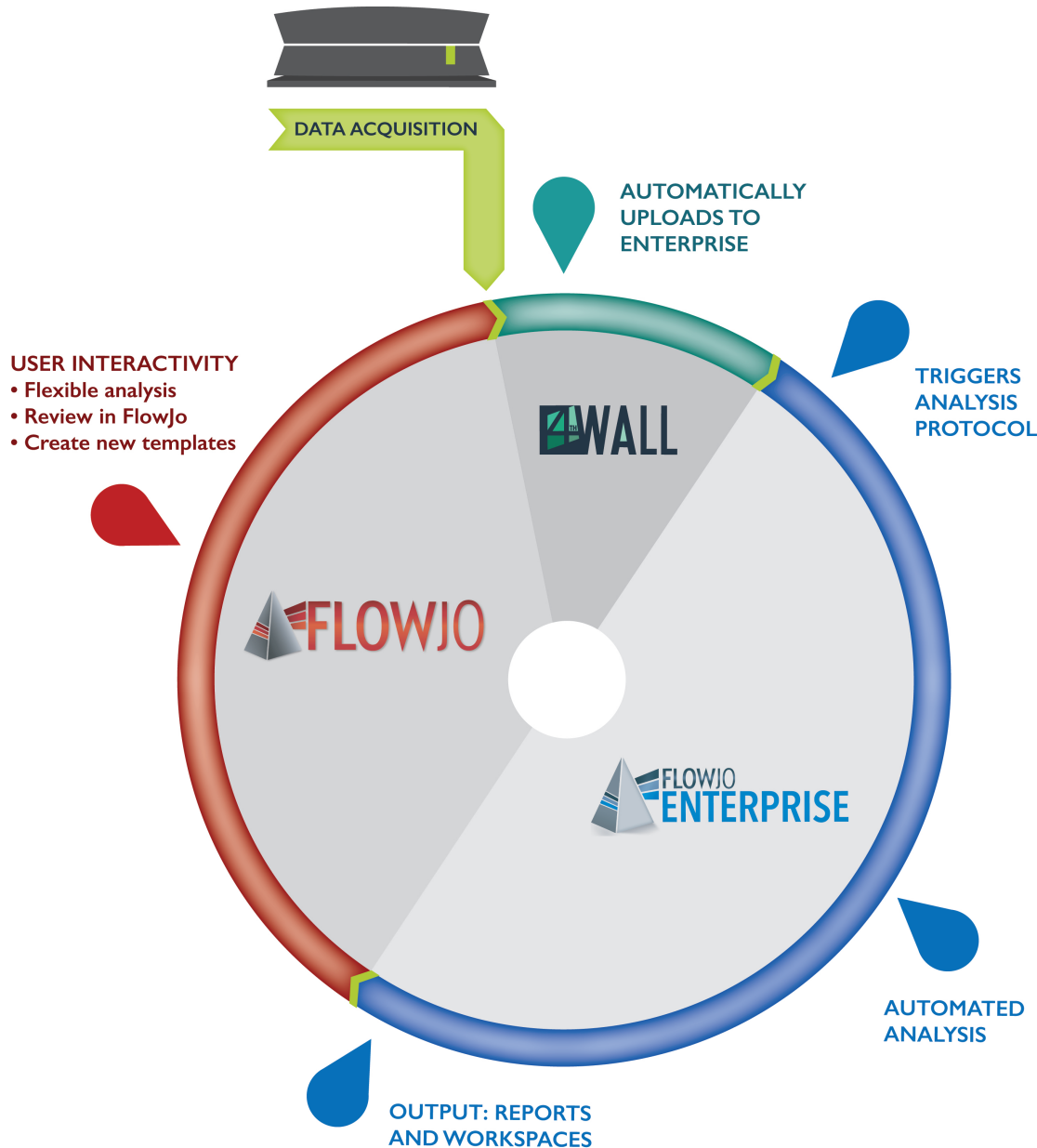
- Allows saving all analysis reports in your workspace without data.
- Streamlines repetitive analysis of multiple runs using the same staining panel(s).
- File Tab → Document Band → Export Workspace As... Save as a Template



# FlowJo Enterprise:

- is a server-based version of FlowJo v10, designed to assist with data archiving, analysis, and report generation for high dimension, high throughput flow or mass cytometry data.
- can handle data upload directly from the cytometer, store it on a secure server, and provide computational power and automated analysis features for scientists.
- is an optional add-on component of the FlowJo Licensing Server (FLS) institutional site license.
- is offered as 1 of 4 tiered packages, with each tier introducing additional features and levels of service.

# FlowJo Enterprise Components



Email:  
[enterprise@flowjo.com](mailto:enterprise@flowjo.com)  
for information

# The Plate Editor

- Viewer to add keywords in a plate format
- Located in the visualizations Band within the Tools Tab
- Add new keyword/value pairs to the right. Drag and drop on selected wells.

Plate Editor

Plate Editor File Edit Tools

20120116 PFICS T...

Annotate Experiment... Read Samples from Group Read Attributes from Group Apply Plate Keywords to Group

Plates Experiment

Plate Name 20120116 PFICS TQC Filter Keywords: All Keywords v

Experiment ID 000-00000

Plate ID cd9353c6-d77c-4005-9772-0659...

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01 <sub>34</sub>	A02 <sub>34</sub>	A03 <sub>34</sub>	A04 <sub>34</sub>	A05 <sub>34</sub>	A06	A07	A08	A09	A10	A11	A12
B	B01 <sub>34</sub>	B02 <sub>34</sub>	B03 <sub>34</sub>	B04 <sub>34</sub>	B05 <sub>34</sub>	B06	B07	B08	B09	B10	B11	B12
C	C01 <sub>34</sub>	C02 <sub>34</sub>	C03 <sub>34</sub>	C04 <sub>34</sub>	C05 <sub>34</sub>	C06 <sub>34</sub>	C07 <sub>34</sub>	C08 <sub>34</sub>	C09 <sub>34</sub>	C10 <sub>34</sub>	C11 <sub>34</sub>	C12 <sub>34</sub>
D	D01 <sub>34</sub>	D02 <sub>34</sub>	D03 <sub>34</sub>	D04 <sub>34</sub>	D05 <sub>34</sub>	D06 <sub>34</sub>	D07 <sub>34</sub>	D08 <sub>34</sub>	D09 <sub>34</sub>	D10 <sub>34</sub>	D11 <sub>34</sub>	D12 <sub>34</sub>
E	E01 <sub>15</sub>	E02 <sub>15</sub>	E03 <sub>15</sub>	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01 <sub>9</sub>	F02 <sub>9</sub>	F03 <sub>9</sub>	F04 <sub>9</sub>	F05 <sub>9</sub>	F06 <sub>9</sub>	F07 <sub>9</sub>	F08 <sub>9</sub>	F09 <sub>9</sub>	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Keyword/Values - Drag to Wells

Keyword	Value
Time point	24hr
Treatment "Drug A"	10ug/L
Assay	GFP Reporter

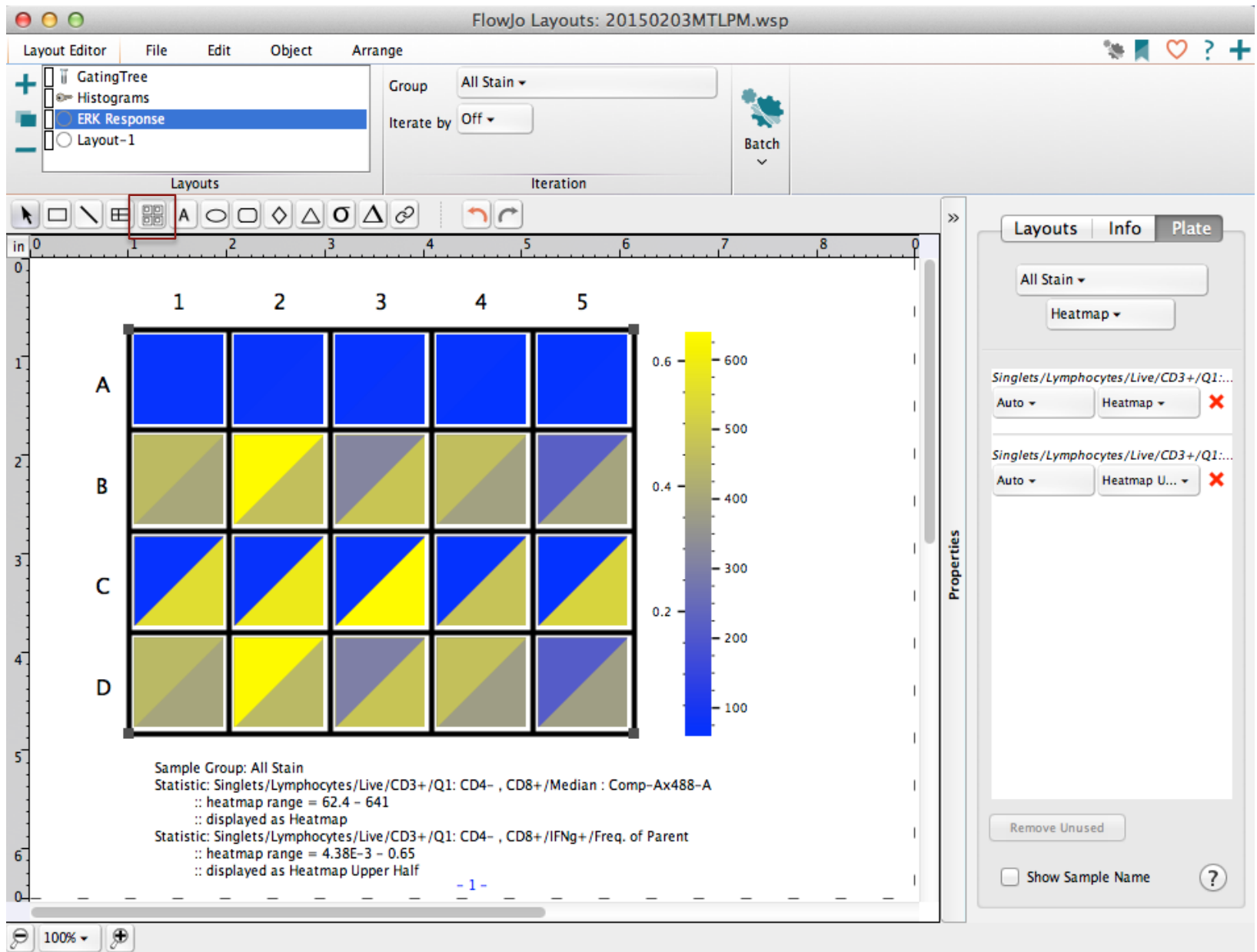
Keyword Value

Keyword/Values - Selected Well

Attribute	Value
<del>SP23N</del>	Comp-PE-Cy7-A
<del>SP23S</del>	IFNg
<del>SSPILOVER</del>	9,Ax700-A,Pac...
<del>SSRC</del>	LD1
<del>*Condition</del>	1
<del>*DDATE</del>	01_14_13
<del>*HIV Status</del>	Neg
<del>*PID</del>	LD1
<del>*SAMPLEID</del>	LD1
<del>*STIM</del>	NS+NS
<del>*TDATE</del>	01_16_13
<del>EXPORT TIME</del>	18-JAN-2013-...
<del>GUID</del>	df526c9f-60fc...
<del>TUBE NAME</del>	NS+NS
<del>WELL ID</del>	A01
<del>STOT</del>	250342

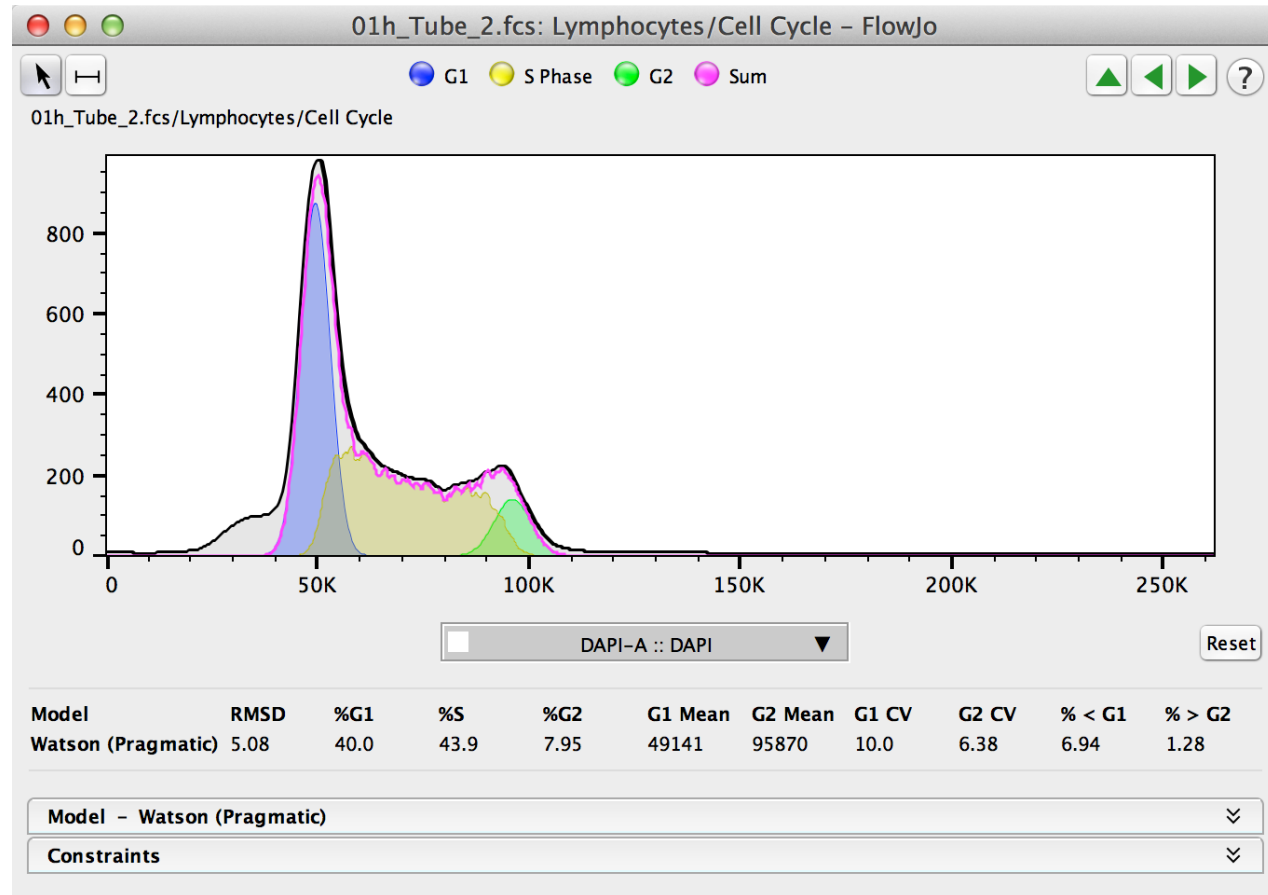
Select Group v

# Plate Visualizations



# Cell Cycle Analysis

- The Cell Cycle platform allows 1D modeling of cell cycle phases based on DNA content
- V10.1 has 1D Watson and Dean-Jett-Fox models.



# Additional Training Resources

- Webinars on basic and advanced features of FlowJo, held on the 1<sup>st</sup> and 3<sup>rd</sup> 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](mailto:techsupport@flowjo.com) for general questions and support.
- Contact [timc@flowjo.com](mailto:timc@flowjo.com) for science questions, additional training resources and information on FlowJo Enterprise.

# Thank You!