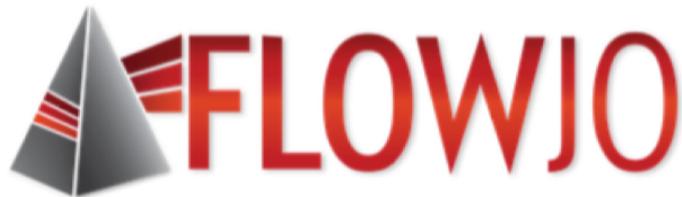


# Cytometry Data Analysis in FlowJo V10



Technical Resources & Product Innovation

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Josh Luthy

# Outline – Part I

## Intro to FlowJo

- Navigating the v10 Workspace
- Customizing Ribbons
- Creating and Editing Groups
- Graphs, Gating and Ancestry
- The Layout Editor
- Batching and Exporting Graphics
- The Table Editor and Exporting Statistics

# 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', and 'Configure'. Below the ribbon are two rows of icons: 'Navigate' (New Workspace, Add Samples..., Create Group..., Table Editor, Layout Editor, Preferences...) and 'Help' (Workspace Links, About, Contact FlowJo, Instrumentation). The main area is divided into two panes. The top pane shows a hierarchical tree of data groups with columns for 'Group', 'Size', and 'Role'. The bottom pane shows a detailed view of a sample, 'LD1\_NS+NS\_A01\_exp.fcs', with columns for 'Name', 'Statistic', and '#Cells'. The tree structure is as follows:

Group	Size	Role
{ } All Samples	33	Test
{ } Compensation	11	Compensation
{ } Immunophenotype Expt	21	Test
{ } Singlets		
{ } Live		
{ } Lymphocytes		
{ } Q1: CD3-, HLA-DR+		
{ } Q2: CD3+, HLA-DR+		
{ } Q3: CD3+, HLA-DR-		
{ } CD4		
Σ Count		
{ } CD8+		
Σ Count		
{ } IFN+		

Name	Statistic	#Cells
LD1_NS+NS_A01_exp.fcs		250342
{ } Singlets	91.9	230106
{ } Live	95.3	219268
{ } Lymphocytes	94.2	206540
{ } Live	100	206540
{ } Q1: CD3-, HLA-DR+	9.15	18891
{ } Q2: CD3+, HLA-DR+	0.080	165
{ } Q3: CD3+, HLA-DR-	79.5	164123
{ } CD4	76.7	125870
Σ Count	125870	
{ } CD8+	20.4	33466
Σ Count	33466	
{ } IFN+	0.48	162
Σ Median : Comp-PE-Cy7-A	1061	
{ } PERFORIN+	25.8	8640
Σ Median : Comp-PE-A (Per	1108	
{ } PERK+	1.33	444

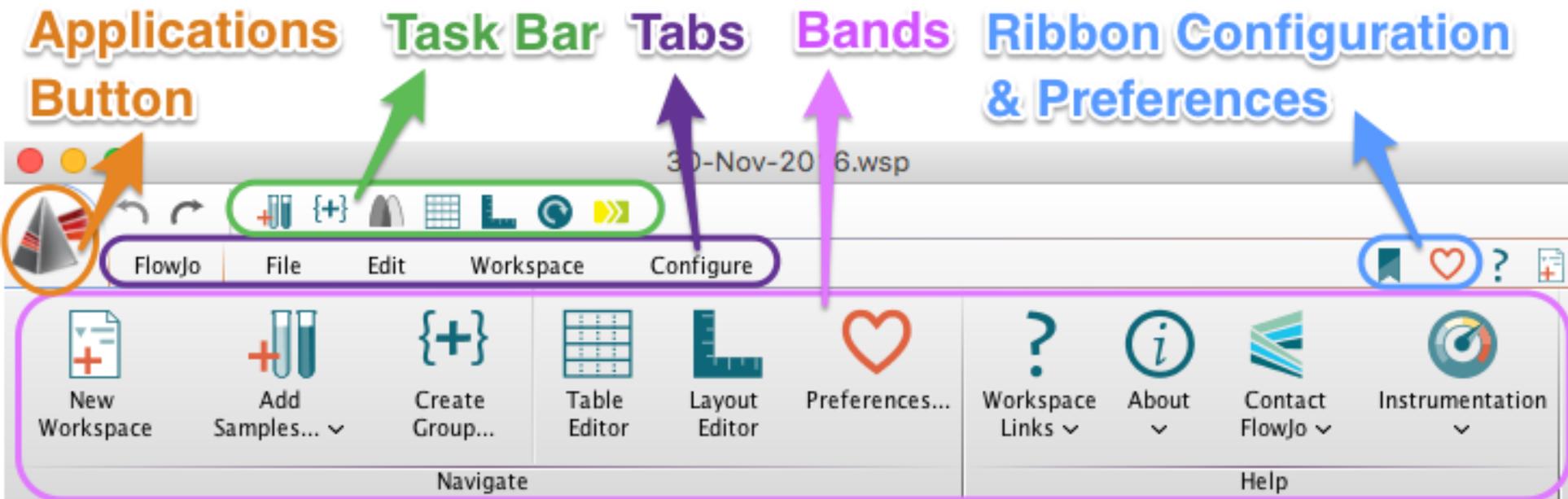
Ribbon  
Tabs and Bands

Groups and Group  
Analysis

Samples and  
sample analysis

# Ribbons, Tabs and Bands

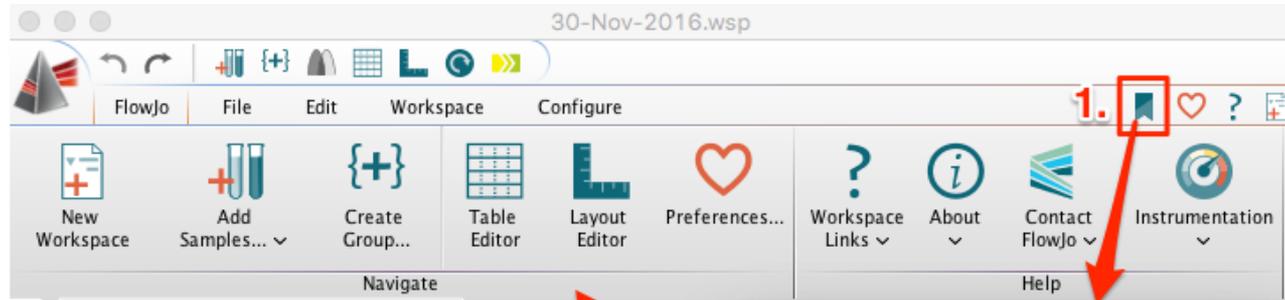
- Ribbon organization allows easy visual navigation of workspace functions.



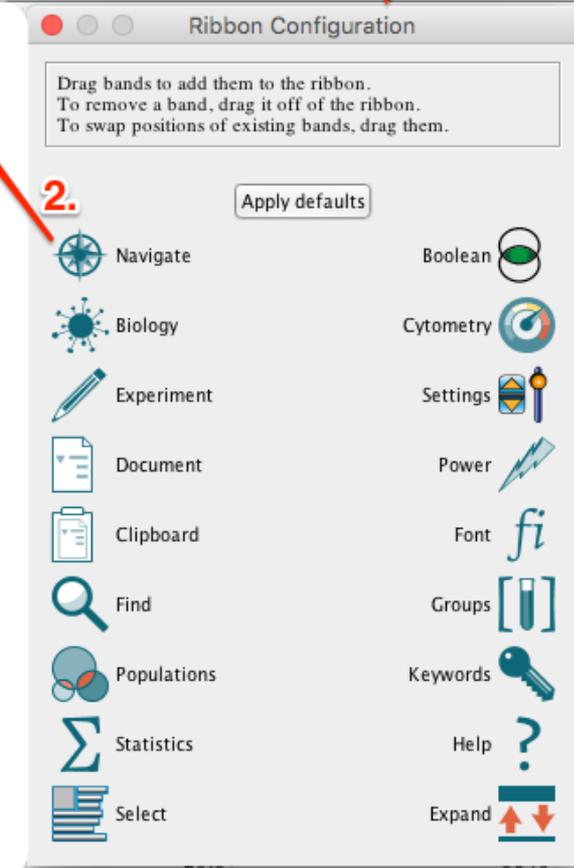
- Tabs group similar Bands together.
- Bands group similar Actions together.

# Customizing Ribbons

- Click on the Ribbon icon to configure



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



# Importing Data

Three Methods:  
1. Drag and drop into open workspace

2. Click the Add Samples button

3. From FlowJo Envoy

The screenshot shows the FlowJo software interface. The top menu bar includes 'FlowJo', 'File', 'Edit', 'Workspace', 'Tools', and 'Configure'. The toolbar contains several icons, with the 'Add Samples...' button (represented by a test tube icon) highlighted with a red box. Below the toolbar, there is a table with columns for 'Group', 'Size', and 'Role'. The table contains two rows: 'All' with Size 0 and Role 'Test', and 'Compensation' with Size 0 and Role 'Compensation'. On the right side, there is a sidebar with a 'Data' folder icon. A red arrow points from the 'Data' folder icon to the text 'Drag Samples Here' at the bottom of the image.

Group	Size	Role
All	0	Test
Compensation	0	Compensation

Drag Samples Here

# Group Pane

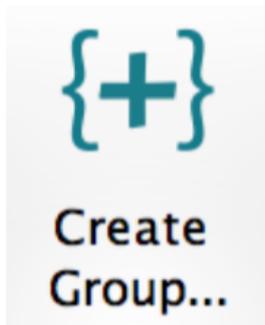
- The Group area lists all groups in the Workspace, # of samples in each group (Size), and the Role of that group (ex. Test, Compensation, Controls) .
- Groups act like folders to organize your samples, allows master gating and unique report generation.

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 (IFNg)		
IFNg+		
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.

A screenshot of a "Modify Group" dialog box. The title bar says "Modify Group". The dialog is divided into several sections:

- Appearance:** Name: "Master Gates", Color: a red square, Style: "Bold" (dropdown), Role: "None" (dropdown), Parameter Key: (empty text field).
- Sample Inclusion Criteria:** "Live group" (checked), "Synchronized" (unchecked). "Include samples that use the following staining:" (text) with a "Multiple" button. A text box contains: "Dead, HLA-DR, p-ERK1\_2, Blank, CD3, Perforin, CD38, IFNg, CD4, CD8". Below are two rows of filters: "\$FIL" (dropdown), "Contains" (dropdown), "FMO" (text field), "Choose..." (button); "Or" (dropdown), "\$FIL" (dropdown), "Contains" (dropdown), "LD" (text field), "Choose..." (button). "More Choices" and "Fewer Choices" buttons are below. "Show all keywords in menus" (checkbox) is also present.
- With reference to samples in another group:** "Only choose from" (radio), "Also include all" (radio selected). "samples in Group" (dropdown) shows "(No specified group)".
- Assignments:** Two rows, each with "Add Keyword:" (text field) and "With Value:" (text field).

At the bottom are buttons: "Help with Groups", "Apply Changes" (highlighted in blue), "Close", and "Create Group".

# 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] (button)
  - Dead, HLA-DR, p-ERK1\_2, Blank, CD3, Perforin, CD38, IFNg, CD4, CD8
  - Criteria rules (highlighted with a red box):
    - \$FIL (dropdown) Contains (dropdown) LD (text) Choose... (button)
    - And (dropdown) \$FIL (dropdown) Lacks (dropdown) FMO (text) Choose... (button)
    - And (dropdown) \*STIM (dropdown) = (dropdown) PI+PI (text) Choose... (button)
  - More Choices (button) Fewer Choices (button)  Show all keywords in menus
- With reference to samples in another group:**
  - Only choose from
  - Also include all samples in Group (No specified group) (dropdown)
- Assignments:** Add Keyword: [text] With Value: [text] (two rows)

Buttons at the bottom: 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)

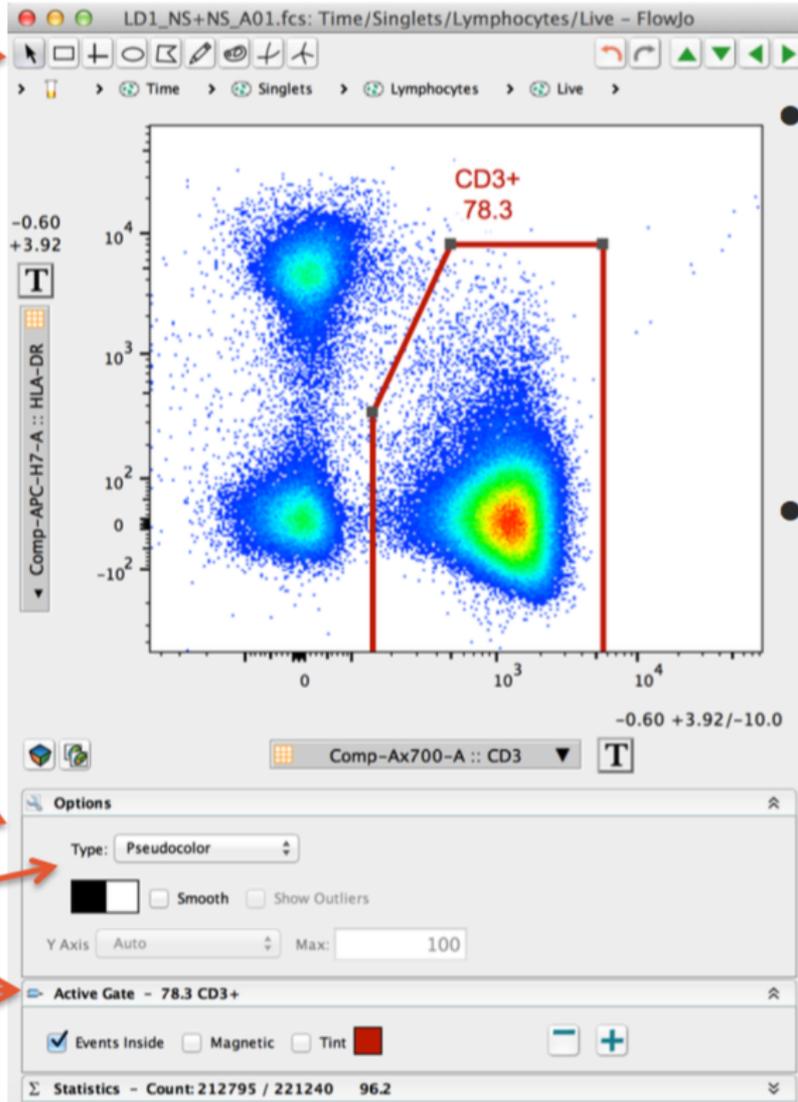
Name	Statistic	#Cells	*HIV Status	*PID	*STIM
LD1_NS+NS_A01.fcs		250342	Neg	LD1	NS+NS
LD1_NS+PI_C01.fcs		229585	Neg	LD1	NS+PI
LD1_PI+NS_B01.fcs		262774	Neg	LD1	PI+NS
Time	99.7	261964			
Singlets	96.2	252097			
Lymphocytes	93.7	236200			
Live	96.2	227167			
CD3+	81.4	184893			
Q1: CD4-, CD8+	24.0	44355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

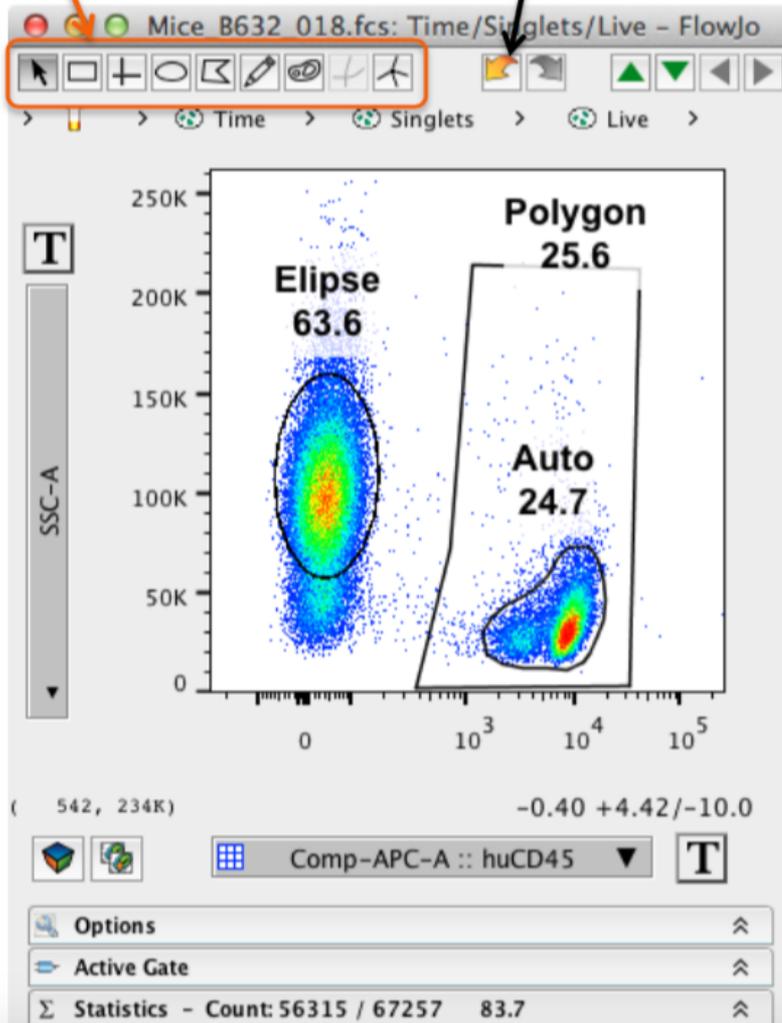
- Several different plot types are available to display flow data.
- Click on the Options Menu below the graph image and select Graph Type from the dropdown menu.

# Gating tools

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

Gating Tools

Undo!!

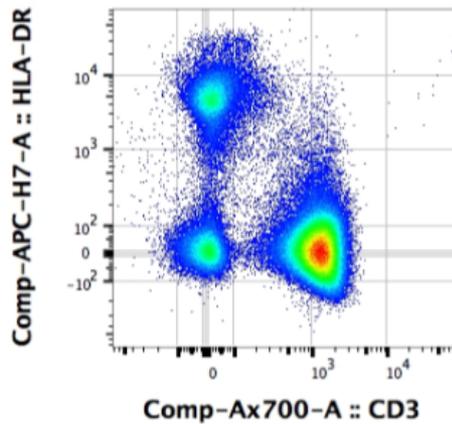


- Gates can always be modified or removed, so don't be shy.
- Explore the gating options and pick what works best for you.

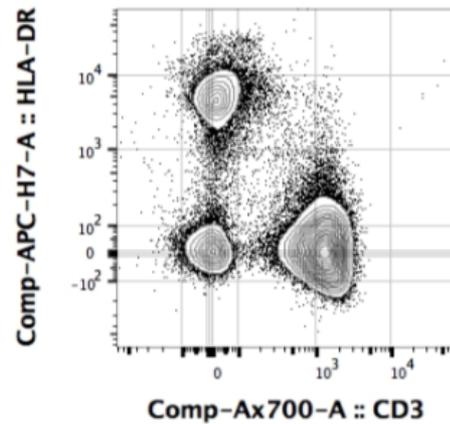
# Graph Display Options

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

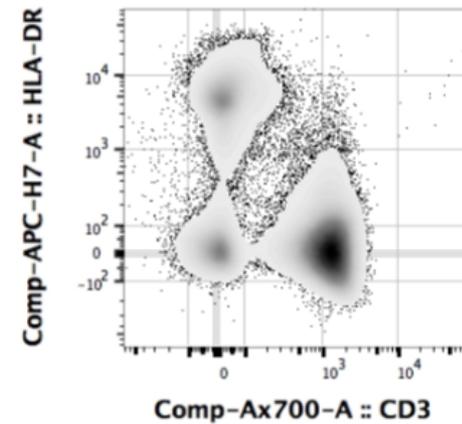
Pseudocolor



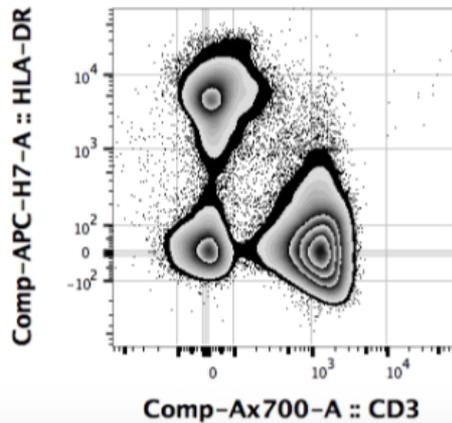
Contour



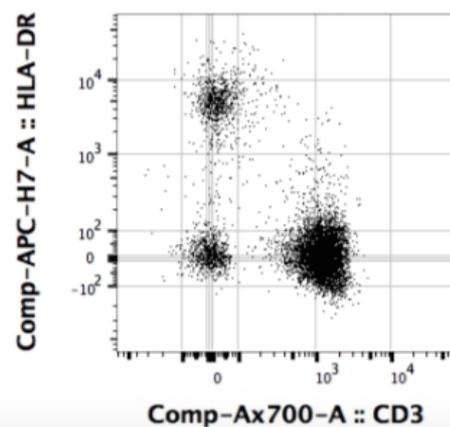
Density



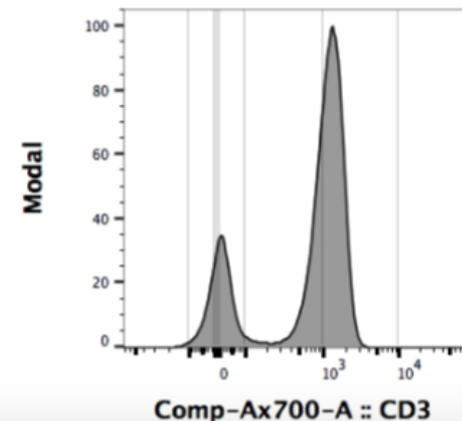
Zebra



Dot Plot

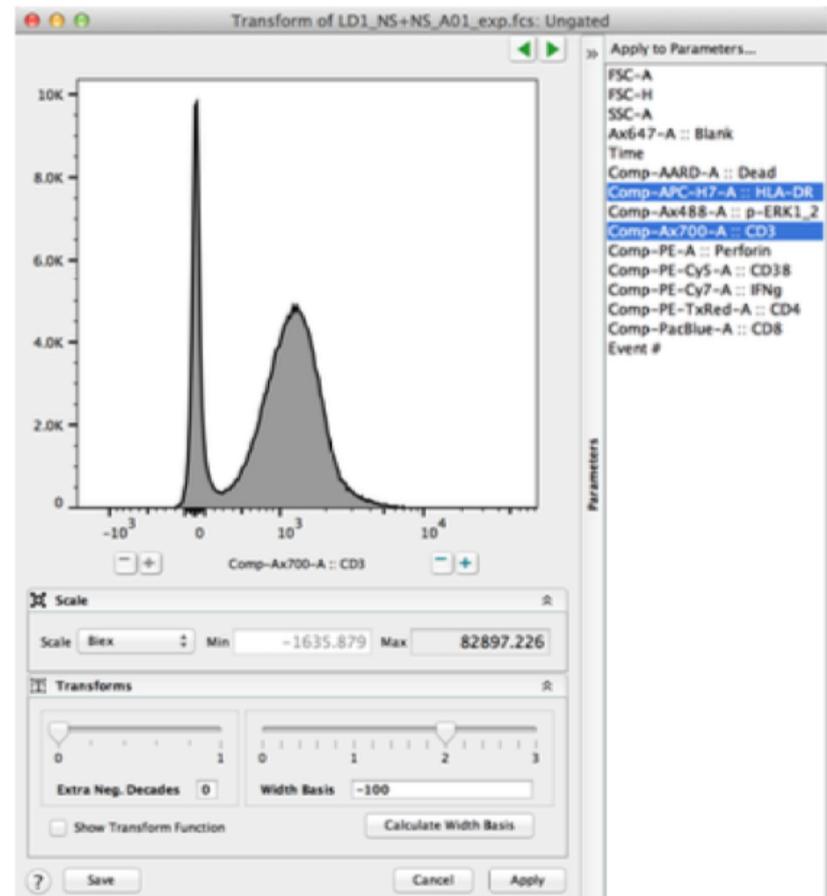
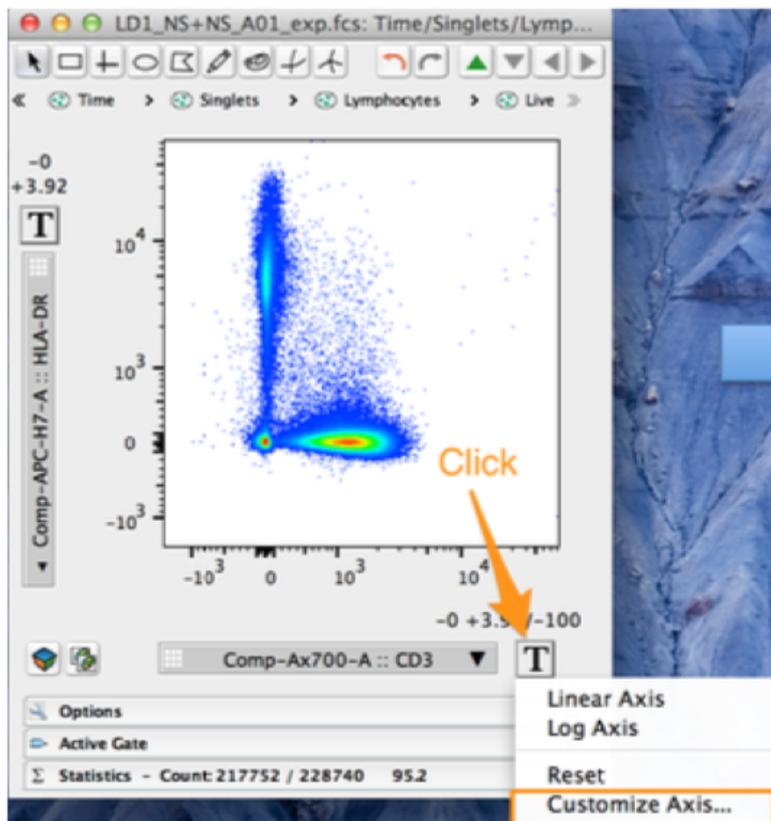


Histogram

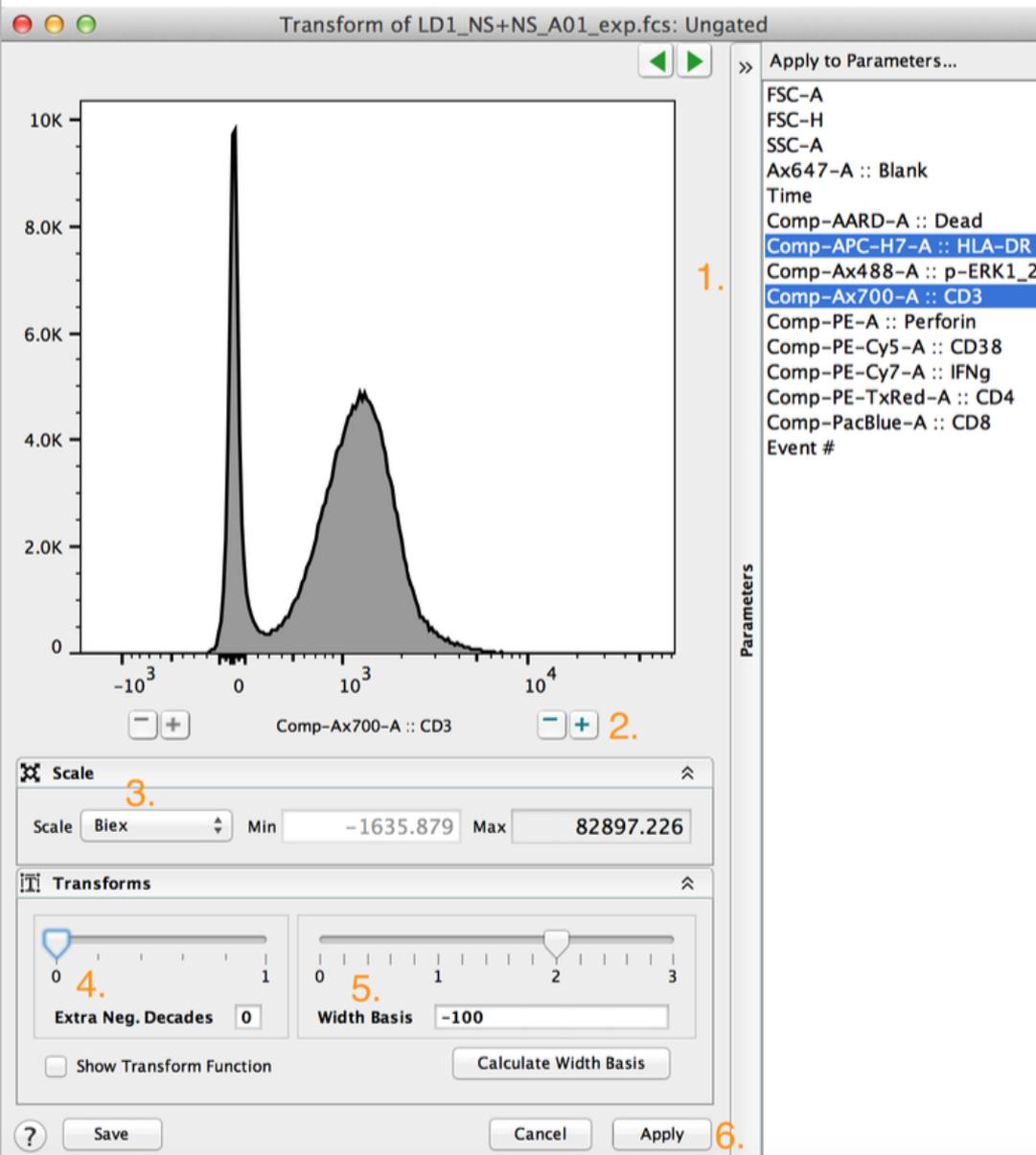


# Transforming Data

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

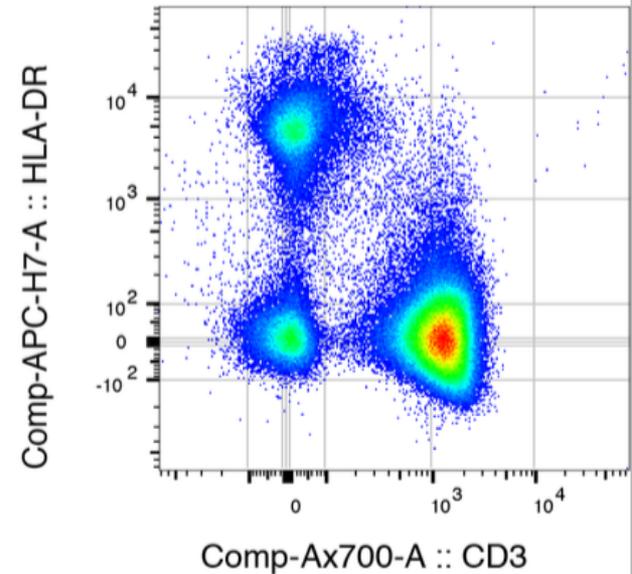
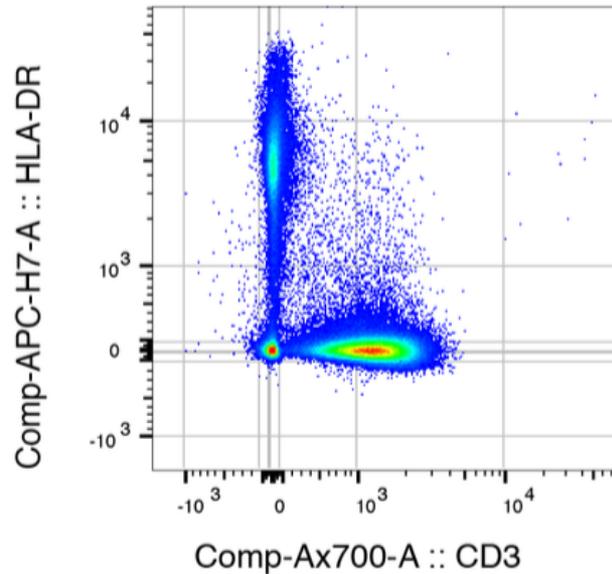
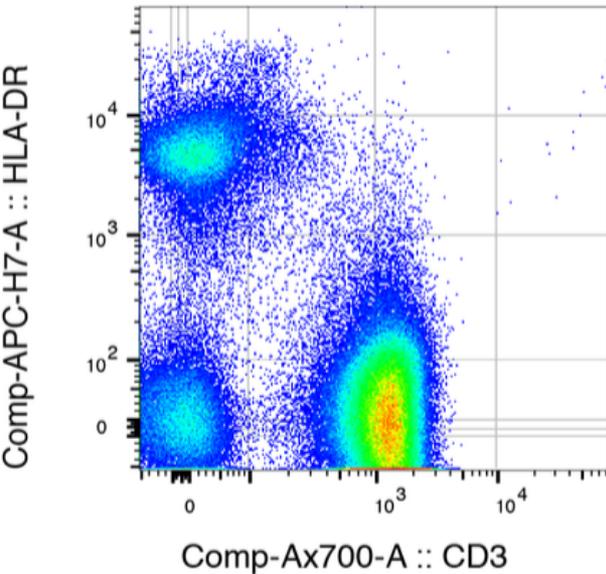


# Transform Options



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.

# Adding Statistics

- Add a statistic to any gated population selected within a sample gating hierarchy.

The screenshot shows the FlowJo software interface with the following elements:

- Workspace:** The top menu bar includes 'Workspace', 'Tools', and 'Configure'. The 'Add Statistic...' button is highlighted with a red box and labeled '2. Select Add Statistic'.
- Statistics Panel:** A panel on the right contains icons for 'Median', 'CV', 'Freq. of', and 'Keywords'. The 'Median' icon is highlighted with a red box and labeled '3. Choose Statistic'.
- Add Statistic Dialog:** A dialog box titled 'Add Statistic...' is open, showing a list of statistics. 'Median' is selected and highlighted with a green box, labeled '4. Choose Parameter'. The 'Parameter' field is set to 'IFNg+'. The 'Add' button is highlighted with a red box and labeled '5. Click Add'.
- Population Selection:** In the main workspace, the population 'Q1: CD4+, CD8+' is selected and highlighted with a red box and labeled '1. Select Population'.
- Data Table:** A table at the bottom shows the results of the analysis, with columns for 'Name', 'Statistic', and 'Count'. The 'Median' value for the selected population is 463536.

Name	Statistic	Count
LD1_NS+NS_A01_exp.fcs		46
↳ All Samples		46
↳ All Stain		20
↳ Compensation		12
↳ FMOs		14
↳ Master Gates		34
LD1_PI+NS_B01_exp.fcs		463536
LD2_PI+NS_B02_exp.fcs		407999
LD4_PI+NS_B03_exp.fcs		229585
LD12_PI+NS_B04_exp.fcs		286306
LD14_PI+NS_B05_exp.fcs		199393
LD1_NS+PI_C01_exp.fcs		347208
LD2_NS+PI_C02_exp.fcs		343027
LD4_NS+PI_C03_exp.fcs		286676
LD12_NS+PI_C04_exp.fcs		281200
LD14_NS+PI_C05_exp.fcs		249824
FMOs NS+PI_No 38_C06_exp.fcs (Control)		276464
FMOs NS+PI_No DR_C07_exp.fcs (Control)		254708
FMOs NS+PI_No IFN_C08_exp.fcs (Control)		269027
FMOs NS+PI_No Perf_C09_exp.fcs (Control)		219336
FMOs NS+PI_No p-ERK_C10_exp.fcs (Control)		244977
FMOs NS+PI_No CD8_C11_exp.fcs (Control)		
FMOs NS+PI_No CD4_C12_exp.fcs (Control)		
LD1_PI+PI_D01_exp.fcs		

- Statistic nodes can be group-applied just like a gate.

# 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**

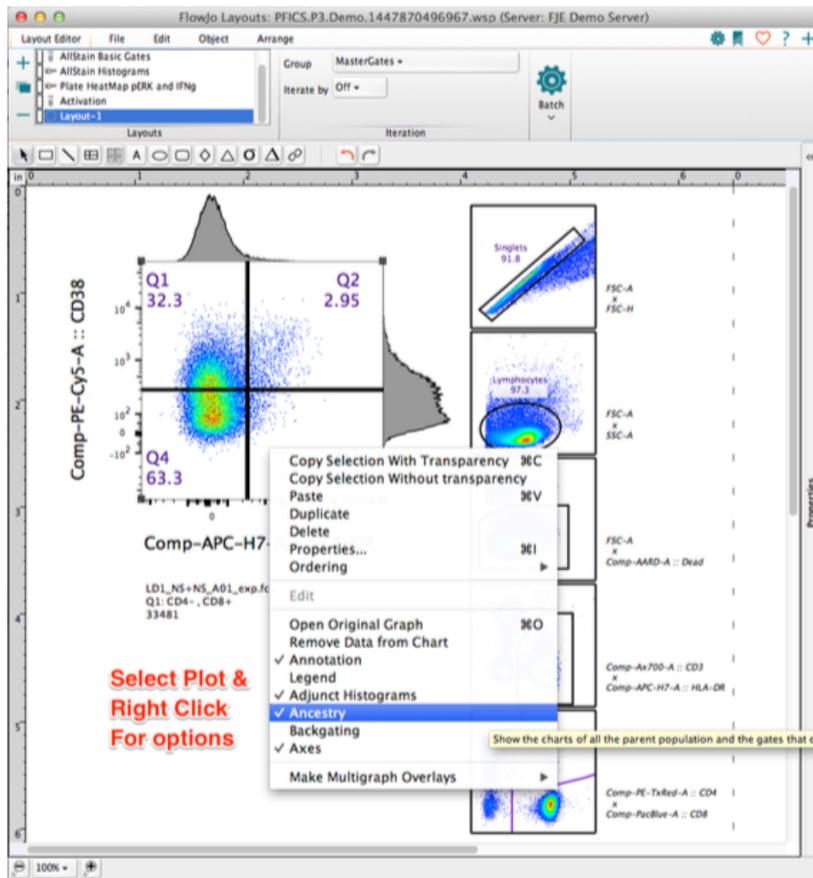
**Batch Report Format**

**Drag & Drop**

The screenshot displays the FlowJo software interface. On the left, a hierarchical tree shows the population structure under 'LD1\_NS+NS\_A01\_exp.fcs', including 'Singlets', 'Lymphocytes', 'Live', 'CD3+', and 'Q1: CD4-, CD8+'. On the right, a grid of plots is visible. The top row contains three plots: 'CD8+ T Cells' (p-ERK1\_2), 'pERK+', and 'IFNg+', each with a percentage value (94.3, 44.3, and 33.6 respectively). The bottom row contains five plots: 'Singlets' (95.8), 'Lymphocytes' (94.8), 'Live' (95.9), 'CD3+' (81.0), and 'Q1' (23.9). The interface includes a menu bar (File, Edit, Object, Arrange), a toolbar, and a 'Properties' panel on the far right.

# Within Layout Editor

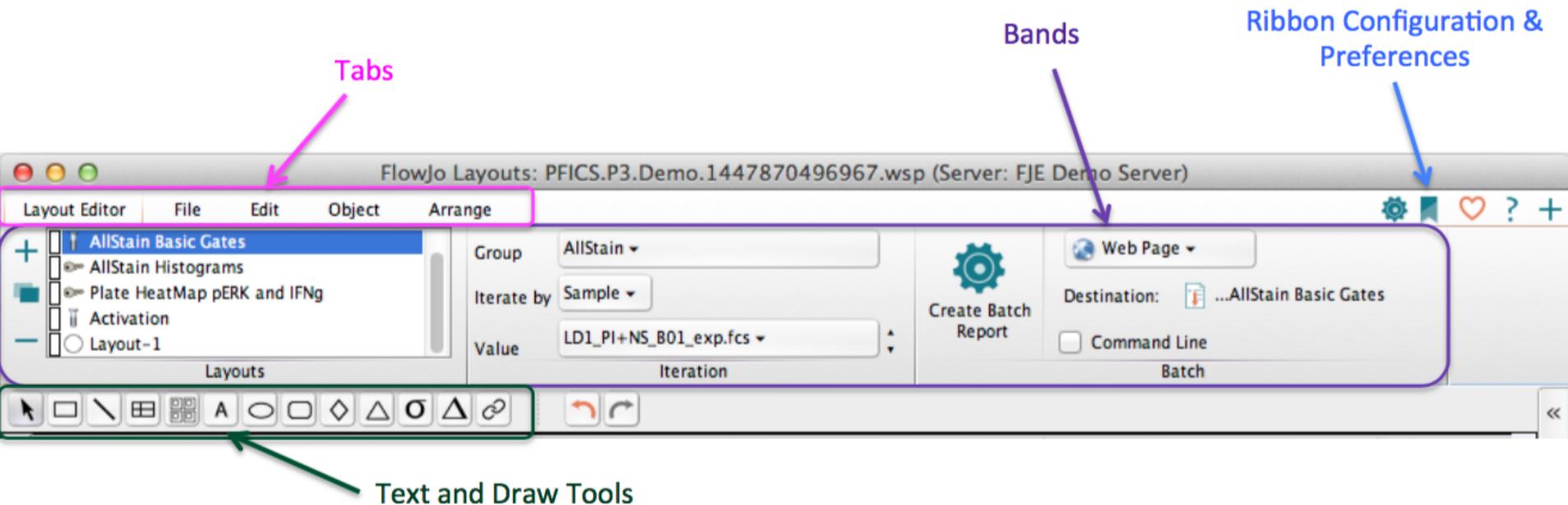
- Graphs can be organized and re-formatted.
- Statistics, keywords, text and even shapes or objects can be added to illustrate your analysis.



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

# Working in Layout Editor

- 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.

# Working in Layout Editor

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

**Tabs**

**Specify** **Annotate** **Fonts** **Legend**

**Axis Parameters**

**Graph Type and Options**

**Annotate Tab**

**Gate Annotation options**

**Axis Label options**

**Specify** **Annotate** **Fonts** **Legend**

Annotation

Show Gates

Axes

Adjunct Histograms

Ancestry

Show Frequencies

Show Population Names

Backgating

Horizontal

X Axis

Hide Ticks

Hide Numbers

Hide Label

Label:

Y Axis

Hide Ticks

Hide Numbers

Hide Label

Label:

LD1\_NS+NS\_A01\_exp.fcs

LD1\_NS+NS\_A01\_exp.fcs

Q1: CD4-, CD8+ 33481

Q1 32.3

Q4 63.3

Comp-PE-Cy5-A :: CD38

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

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

Max: 1000

Apply Cancel OK

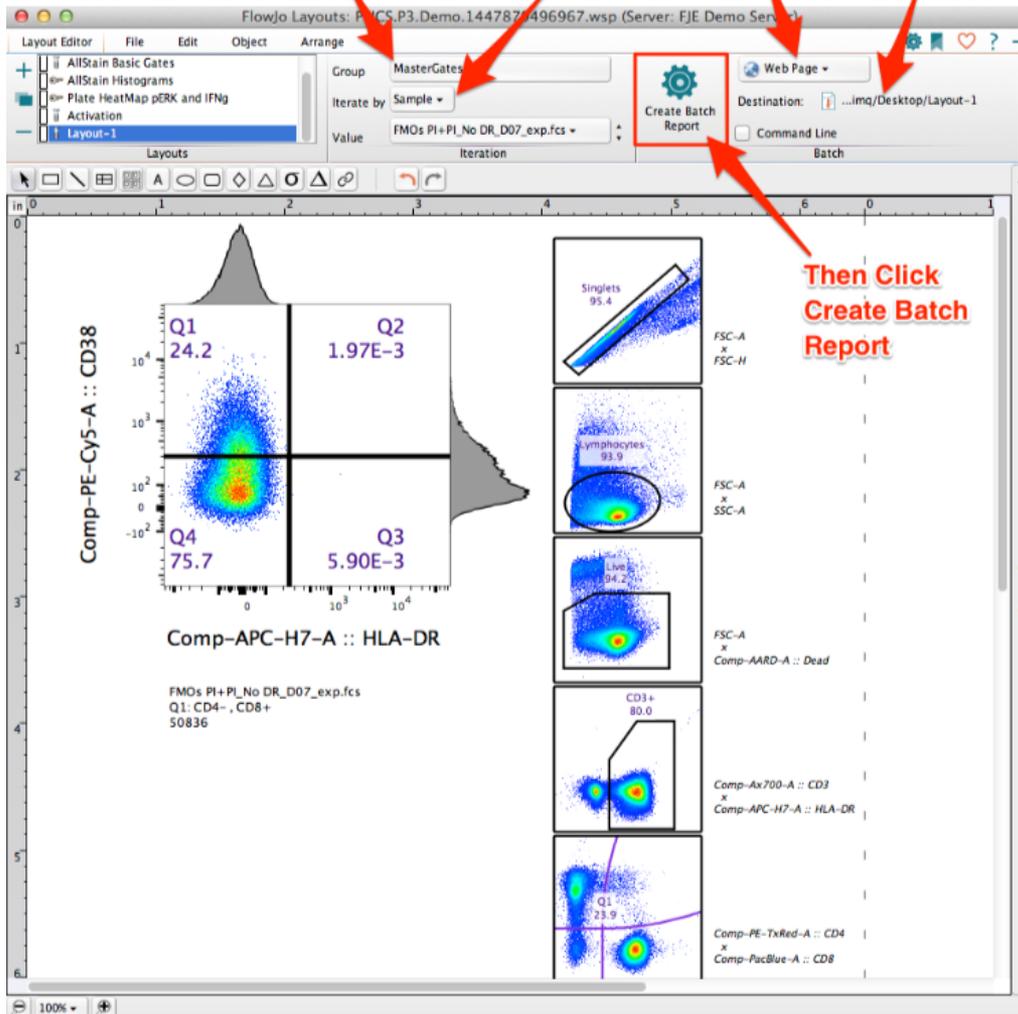
Apply Cancel OK

Comp-PE-TxRed-A :: CD4

Comp-PacBlue-A :: CD8

# Batch Analysis of Layout Editor Graphics

Specify Group & Iteration options      Report Type & Location

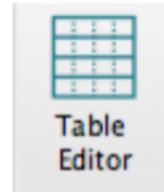


- Batch operations perform repetitive analysis on multiple samples, applying the layout to an entire set of samples.
- Specify Group, Iterate by, Report type and Location, then Click Create Batch Report .



# The Table Editor

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



Open Table Editor

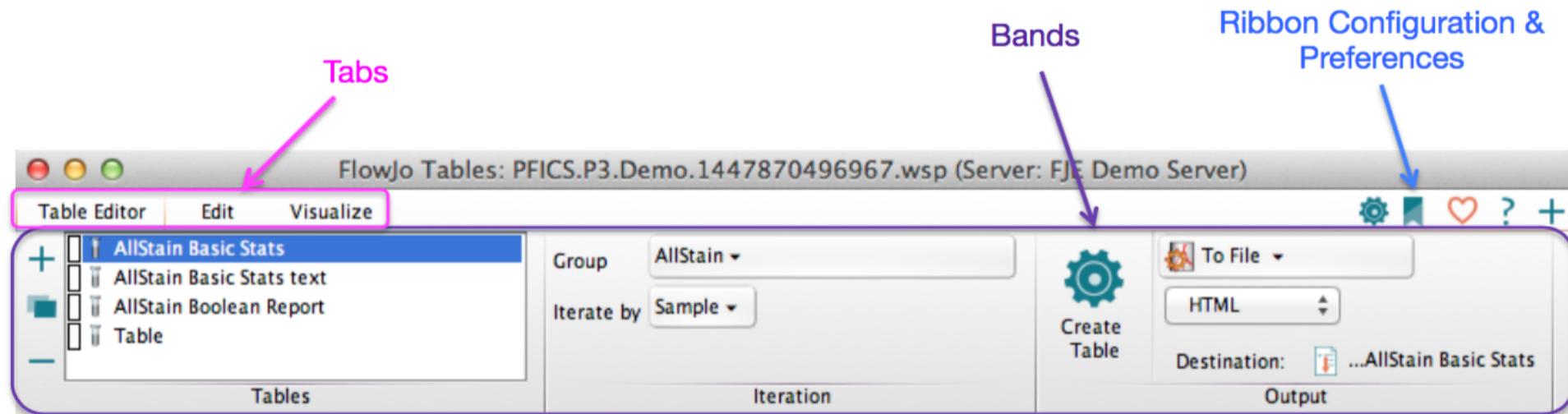
FlowJo Tables: PFICS.P3.Demo.1447870496967.wsp (Server: FJE Demo Server)

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.

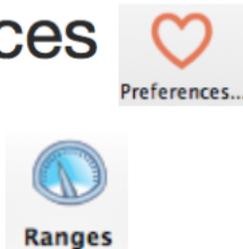
# Table Editor Visualize Tools

- 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.

Ancestry Subset Statistic For	*PID	*STIM	pERK GMF	% IFNg+	% Perf+	CD4/CD8 Ratio	HLA-DR+,I	% 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_NS...	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_PL...	LD12	PI+NS	327	45.3	40.8	1.94	1.50	84.8	4632	408
LD12_PL...	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_PL...	LD14	PI+NS	366	17.7	18.2	1.66	1.21	94.8	3708	650
LD14_PL...	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

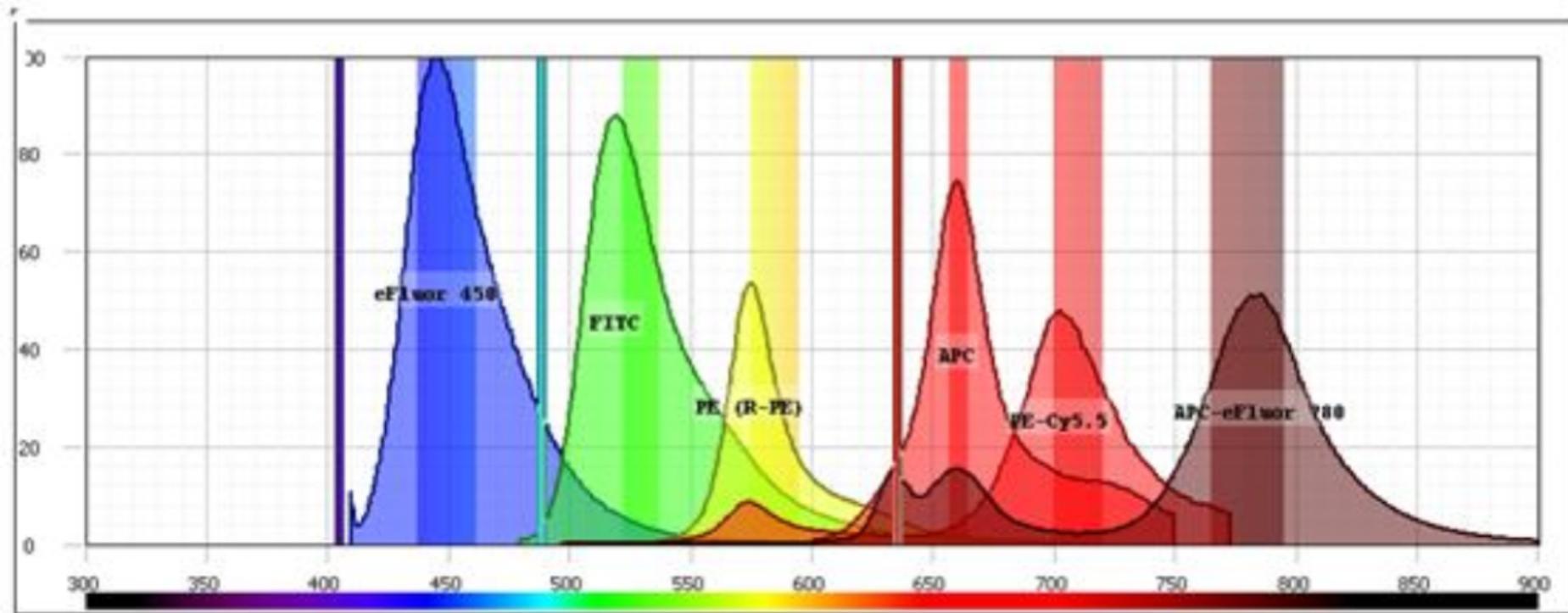
# Outline – Part II

## Advanced Tools and Platforms

- Compensation
- Export/Concatenate
- Cell Cycle Analysis
- Plugins
- Templates
- Additional Resources

# 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.

# Compensation

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



2. Click the Compensation Tool

1. Highlight Compensation Group

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

The wizard auto gates samples

Group	Size	Role
{ } All Samples	12	Test
{ } Compensation	12	Compensation
{ } PFICS Compensation Controls	12	Test

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

# Compensation

- Then fills in the suggested positive and negative populations.
- Choose alternate populations from dropdown list for each parameter.
- Double click on preview graphs to modify auto-gates.

**For Each Parameter:**

**Select Single Stained Samples Negative Positive**

**Negative Populations Positive Populations**

Parameter	Sample	Negative	Positive	
APC-H7-A	HLA-DR	Bead Comps_DR APC-H7_F04_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/APC-H7-A+
Ax488-A	p-ERK1_2	Bead Comps_ERK A488_F06_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/Ax488-A+
Ax700-A	CD3	Cell Comps_CD3 A700_E02_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/Ax700-A+
PE-A	Perforin	Bead Comps_Perforin PE_F08_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/PE-A+
PE-Cy5-A	CD38	Bead Comps_38 PE-Cy5_F03_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/PE-Cy5-A+
PE-Cy7-A	IFNg	Bead Comps_IFN PE-Cy7_F07_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/PE-Cy7-A+
PE-TxRed-A	CD4	Bead Comps_4 PE-TR_F01_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/PE-TxRed-A+
PacBlue-A	CD8	Bead Comps_8 PB_F02_exp.fcs	Cell Comps_US Cells_E03_exp.fcs:Size	Size/PacBlue-A+

**APC-H7-A**

Cell Comps\_US Cells\_E03\_exp.fcs

Size 59.1

42.7

Bead Comps\_DR APC-H7\_F04\_exp.fcs

Size 73.5

**Double Click on Preview Graph to Adjust Gating**

**Ax488-A**

Cell Comps\_US Cells\_E03\_exp.fcs

Size 59.1

47.4

Bead Comps\_ERK A488\_F06\_exp.fcs

Size 79.2

## Control Group: Compensation

Matrix Name:

Finalized

ts look correct. Double click a graph to edit it.

Sample

 Negative

Positive

**Use Sample drop down list**

**Use Negative drop down list**

**Use Positive drop down list**

**to select Pos Control Sample and**

**to Select Negative Sample  
or Population**

**to Choose Positive population**

**Choose or Remove Parameters**

Bead Comps\_DR APC-H7\_F04\_exp.fcs  
Bead Comps\_ERK A488\_F06\_exp.fcs  
Bead Comps\_IFN PE-Cy7\_F07\_exp.fcs  
Bead Comps\_Perforin PE\_F08\_exp.fcs  
Bead Comps\_US Beads +FP\_F05\_exp.fcs  
Bead Comps\_US Beads No FP\_F09\_exp.fcs  
Bead Comps\_4 PE-TR\_F01\_exp.fcs  
Bead Comps\_8 PB\_F02\_exp.fcs  
Bead Comps\_38 PE-Cy5\_F03\_exp.fcs  
**Cell Comps\_AARD\_E01\_exp.fcs**  
Cell Comps\_CD3 A700\_E02\_exp.fcs  
Cell Comps\_US Cells\_E03\_exp.fcs

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

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  
**Cell Comps\_US Cells\_E03\_exp.fcs :: Size**  
Size  
Size/AARD-A+

<Clear>

Size  
**Size/AARD-A+**  
<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

**Set Color**   **Name Matrix**   **Edit Matrix**   **Save a Copy**   **Calculate SSM**

The screenshot displays the FlowJo software interface for compensation. The top menu bar includes 'FlowJo', 'File', 'Edit', 'Workspace', and 'Tools'. The 'Edit' menu is open, showing options: 'Edit Compensation Matrix', 'Derive Parameters...', and 'Script Editor'. The 'Workspace Matrices' panel shows 'Acquisition-defined' and 'Compensation'. The 'Edit Matrix' window is active, displaying a matrix for 'Compensation' with various parameters and a list of selected markers: APC-H7-A :: HLA-DR, Ax488-A :: p-ERK1\_2, PE-Cy5-A :: CD38, and PE-TxRed-A :: CD4. A red box highlights the 'M' icon in the top left of the matrix window. A large red arrow points from this icon to the text 'Apply the Matrix to a File or Group by Dragging and Dropping the "M" Icon'. The matrix data is as follows:

APC-H7-A	Ax488-A	Ax700-A	PE-A	Perforin	PE-Cy5-A	PE-Cy7-A	PE-TxRed-A	PacBlue-A
100	-0.9682	2.9091	0.7394	0.0255	39.5223	0.1274	1.7935	
-0.1452	100	-0.2591	0.5269	-0.5628	-0.1996	0.0545	1.2597	
35.0401	0.1715	100	-0.1713	1.2007	10.5874	0	0	
0	0.1292	0	100	14.284	1.252	37.5816	0.3947	
3.0078	0	7.7513	1.6658	100	12.0697	0.7171	0.1195	
5.7255	0	0.2542	1.9835	0.2482	100	0.8283	0.2602	
0	0	0	24.1284	52.6343	6.0498	100	0.389	
0	-0.3995	-0.1735	0.3292	-0.3642	-0.1292	0.0117	100	

The bottom panel shows a 'Preview Population' window with a grid of flow cytometry plots for various markers: Ax488-A :: p-ERK1\_2, Ax700-A :: CD3, PE-A :: Perforin, PE-Cy5-A :: CD38, PE-Cy7-A :: IFNg, and PE-Tx. A red arrow points from the 'M' icon to the text 'Preview Matrix on a Sample'.

# Spillover Spreading Matrix

Workspace Matrices  
 Acquisition-defined  
 Compensation

[M] Edit Save Matrix **SSM** Displaying matrix 'Compensation'

SSM  
 Select a matrix that was created with FlowJo's compensation wizard and click to display or export the SSM

Ax700-A :: ...	PE-A :: ...	Perforin PE-Cy5-A :: ...	PE-Cy7-A :: ...	PE-TxRed-A :: ...	PacBlue-A :: ...
2.9091	0.7394	0.0255	39.5223	0.1274	1.7935
-0.2591	0.5269	-0.5628	-0.1996	0.0545	1.2597

[M] Edit Save Matrix SSM Displaying SSM of matrix 'Compensation'

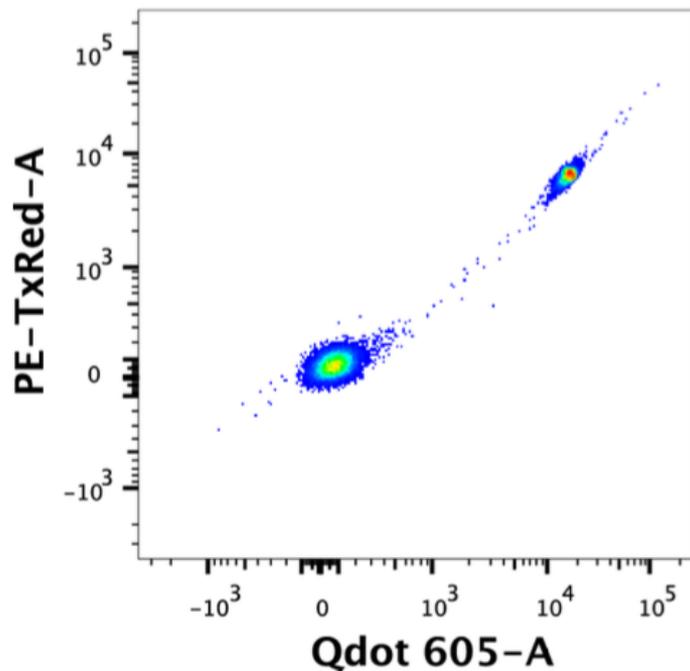
Show All  
 APC-H7-A :: HLA-DR  
 Ax488-A :: p-ERK1\_2  
 Ax700-A :: CD3  
 PE-A :: Perforin  
 PE-Cy5-A :: CD38  
 PE-Cy7-A :: IFNg  
 PE-TxRed-A :: CD4  
 PacBlue-A :: CD8

APC-H7-A :: ...	Ax488-A :: ...	Ax700-A :: ...	PE-A :: ...	Perforin PE-Cy5-A :: ...	PE-Cy7-A :: ...	PE-TxRed-A :: ...	PacBlue-A :: ...
0	0	0.3044	0.094	0.0041	2.1057	0	0.1985
0	0	0	0.0793	0	0	0	0.117
1.4624	0.1909	0	0	0	0.9375	0	0
0	0	0.0381	0	0.726	0.2686	0.9951	0.0933
0.4192	0	0.9256	0.2223	0	1.5937	0.1248	0.0638
0.781	0	0.0844	0.374	0.0831	0	0.1276	0.0538
0.053	0	0.1025	0.7051	1.2957	0.7624	0	0.0651
0	0	0	0.0638	0	0	0	0

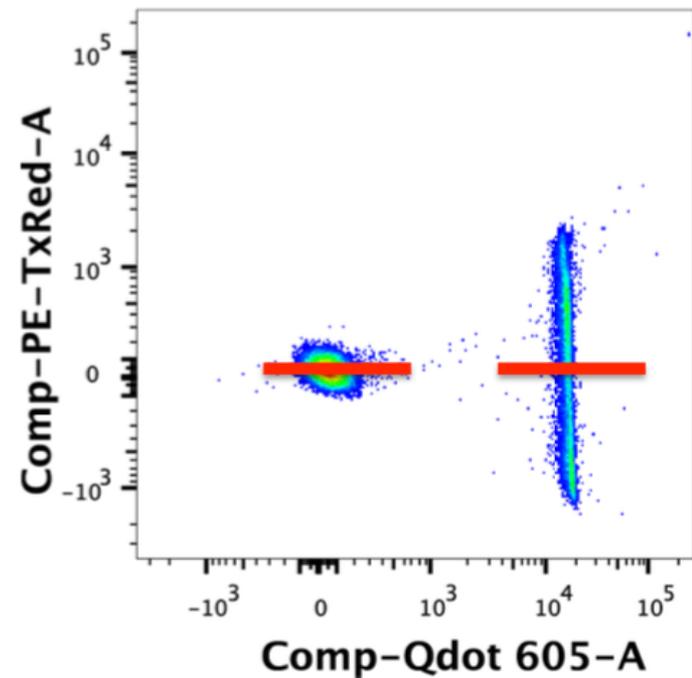
# Effect of Compensation



## Uncompensated

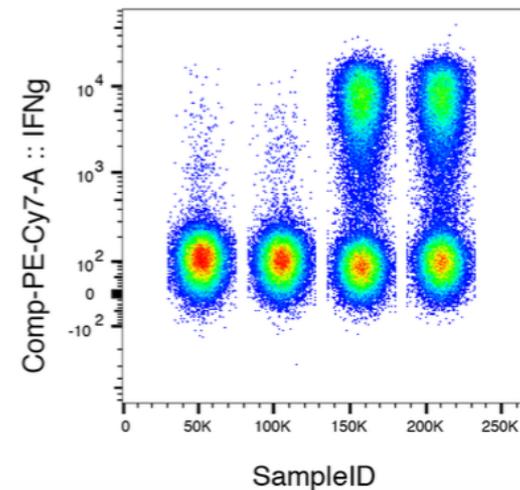
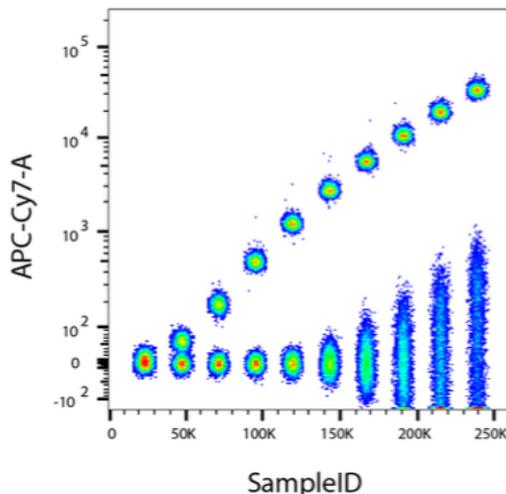


## Compensated



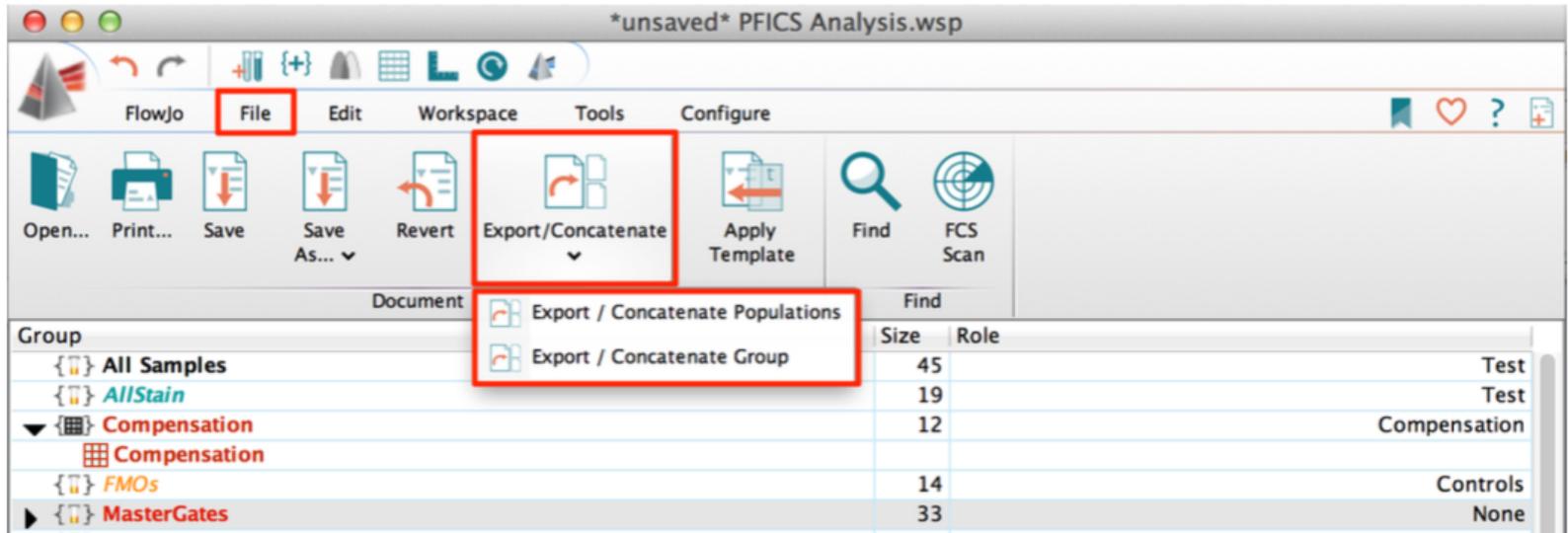
# Export and Concatenate

- Add and embed keyword metadata
- Merge data from multiple files
- Identify specific populations
- Isolate events for further computational analysis
- Titrate reagents for optimal staining and stimulation conditions
- Visualize responses



# Export or Concatenate Data

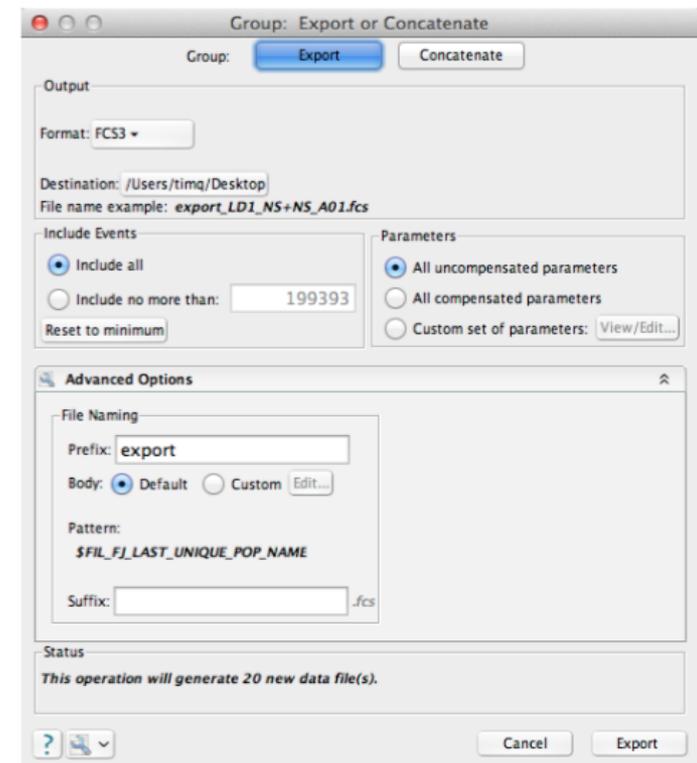
- The Data Export/Concatenate... action button is located by default in the Document band within the workspace File tab.



- Choose from two options in the drop down menu:
  - 1) Export/Concatenate Populations
    - subset of events defined by gating hierarchy/phenotype
  - 2) Export/Concatenate Group
    - all or a subset of events independent of phenotype

# Export Options

- **Output panel**
  - Format – selects file format (FCS3 or CSV)
  - Destination – specifies directory where output files will be saved
  - File name example – displays example of naming scheme as specified in Advanced Options → File Naming
- **Include Events panel** – Include all events or down-sample randomly with Include no more than #
- **Parameters panel** – Choose All uncompensated parameters (default selection) is recommended. If the sample is compensated, compensated parameters will also be written to the file.
- **Advanced Options File naming panel**
  - Prefix – specifies a common prefix to add
  - Body – specifies the keywords to create a unique name for each file
  - Pattern – displays keyword pattern for body naming scheme
  - Suffix – specifies a suffix to add
- **Status panel**
  - Tells how many files will be produced



# Concatenating Populations

- Highlight the equivalent population nodes within the gating tree of samples you wish to merge.
- Choose Export/Concatenate Populations.

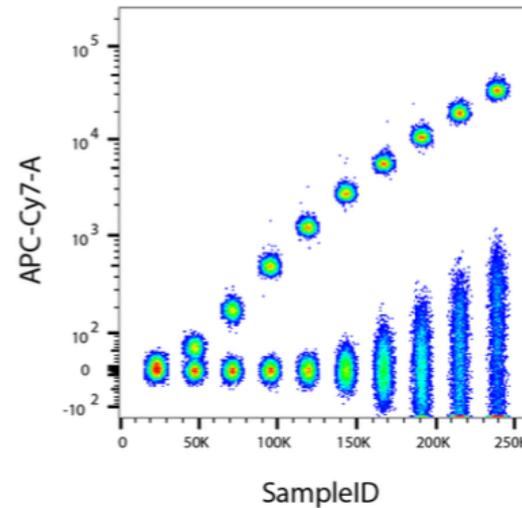
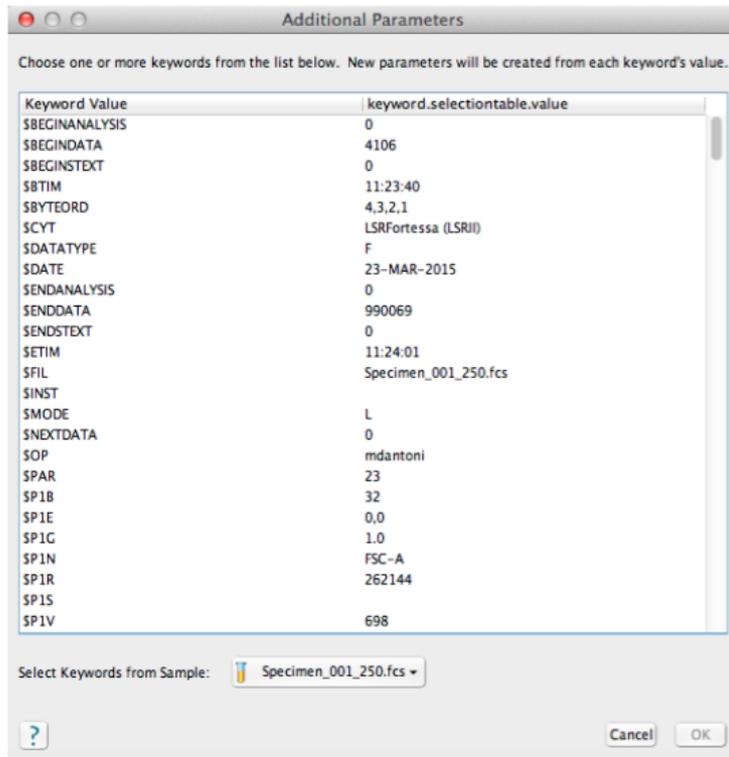
The screenshot shows the FlowJo software interface. The top menu bar includes FlowJo, File, Edit, Workspace, Tools, and Configure. Below the menu is a toolbar with icons for Open, Print, Save, Revert, Save As, Export Workspace, Data Export / Concatenate, Find, and FCS Scan. The main workspace displays a gating tree under the 'Group' section, with 'All Samples' expanded to show 'Rainbow Bead Voltage Titration' and 'Beads'. A 'Data Export / Concatenate...' dialog box is open, showing a table of data.

Name	Statistic	#Cells	*PMT Voltage
Specimen_001_250.fcs		10717	250
Beads	82.2	8811	
Specimen_001_300.fcs		10528	300
Beads	84.7	8916	
Specimen_001_350.fcs		10479	350
Beads	84.6	8866	
Specimen_001_400.fcs		10502	400
Beads	85.0	8923	
Specimen_001_450.fcs		10497	450
Beads	85.1	8935	
Specimen_001_500.fcs		10497	500
Beads	85.1	8934	
Specimen_001_550.fcs		10468	550
Beads	84.6	8861	
Specimen_001_600.fcs		10506	600
Beads	84.6	8886	
Specimen_001_650.fcs		10489	650
Beads	85.4	8958	
Specimen_001_700.fcs		10500	700
Beads	84.8	8900	

The screenshot shows the 'Populations: Export or Concatenate' dialog box. The 'Populations' section has 'Export' and 'Concatenate' buttons, with 'Concatenate' selected. The 'Output' section shows 'Format: FCS3', 'Destination: /Users/timq/Desktop/ExportDemo', and 'File name example: export\_1.fcs'. The 'Include Events' section has 'Include all' selected, with a 'Reset to minimum' button and a value of 8811. The 'Parameters' section has 'All uncompensated parameters' selected. The 'Advanced Options' section includes 'File Naming' (Prefix: export, Separator: \_, Suffix: .fcs) and 'Group Concatenation' (Concatenate all files together selected, n=10). The 'Additional Parameters' section has 'Spread distribution of keyword data' checked. The 'Status' section indicates 'This operation will generate 1 new data file(s)'. The dialog has 'Cancel' and 'Concatenate' buttons at the bottom.

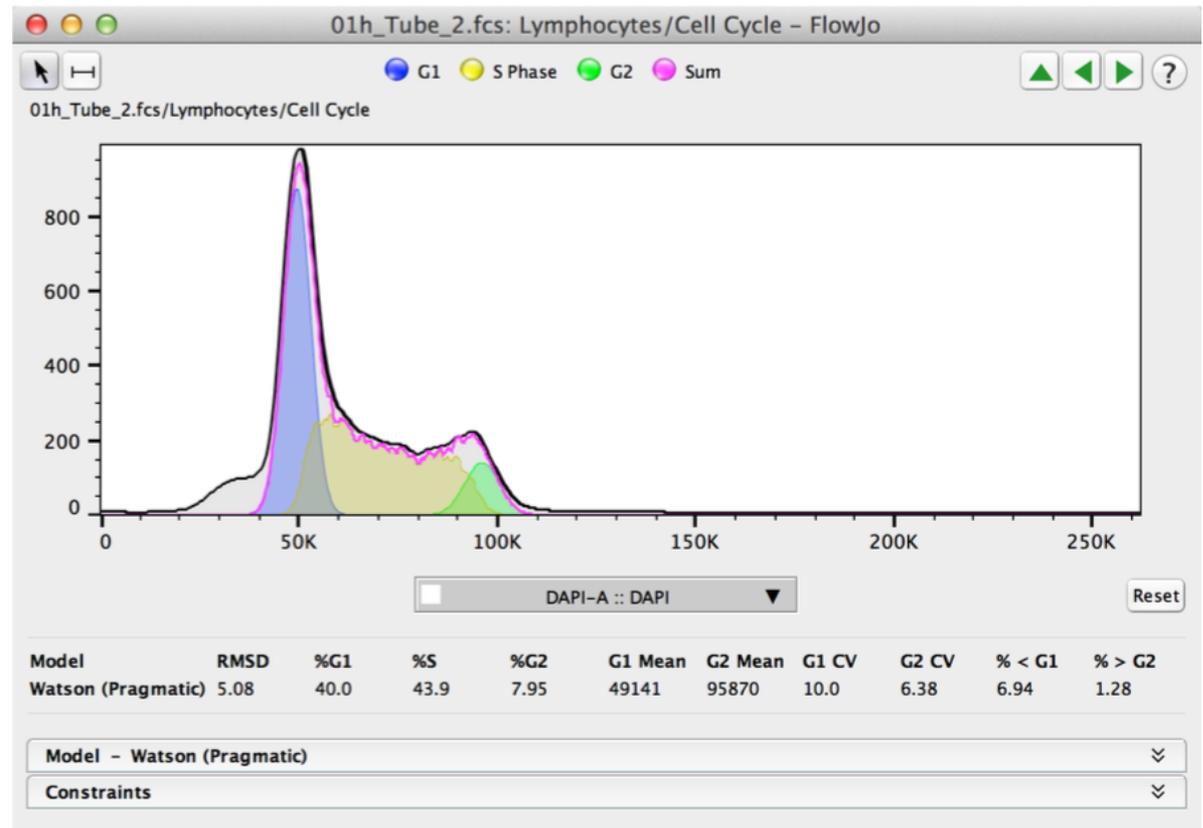
# Additional Parameters

- You can select one or more keywords to create new parameters in the concatenated output file.
- Note that you will always get a new parameter called Sample ID in the concatenated file. Selecting Sample ID allows you to see the different samples that were merged.



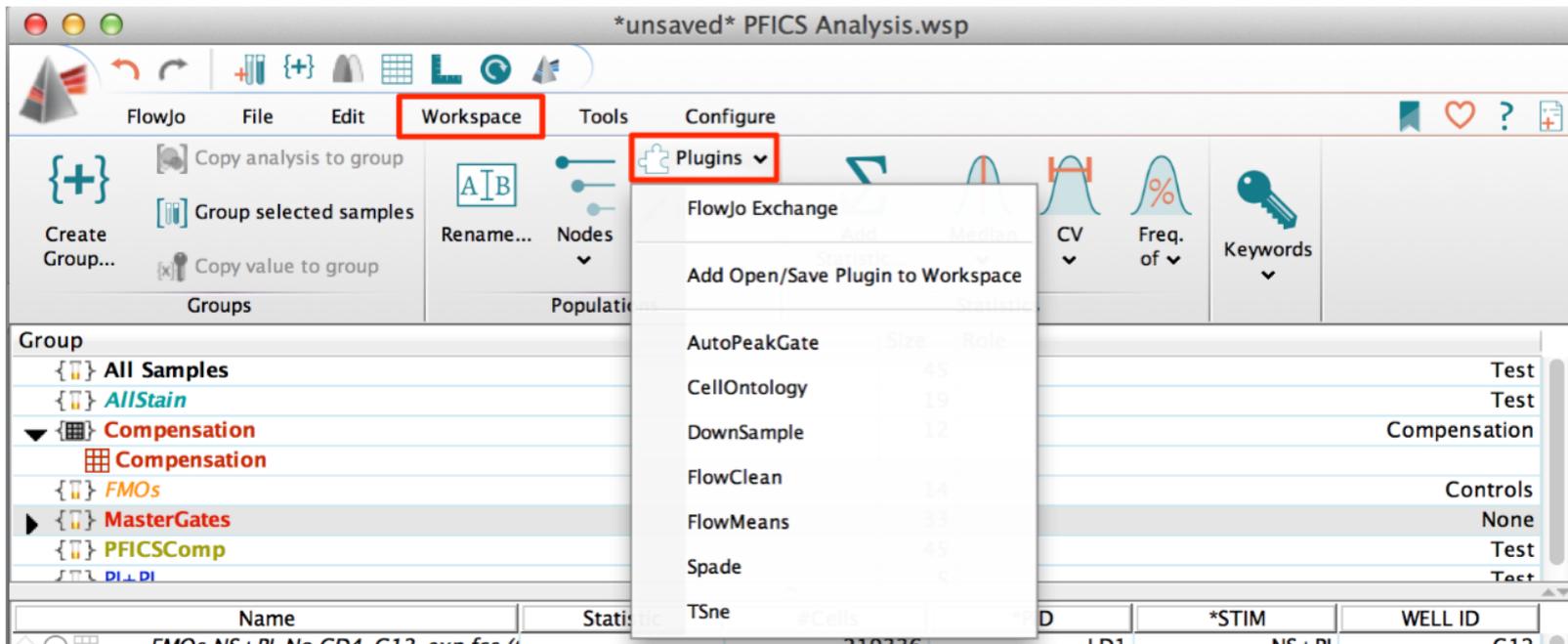
# Cell Cycle Analysis

- 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.



# Plugins

- Java programs that extend the functionality of FlowJo.
- Access from the Plugins menu  
Workspace→Populations band→Plugins menu



- Download the latest Plugins from The FlowJo Exchange: [exchange.flowjo.com](https://exchange.flowjo.com)

# 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

The screenshot shows the PFICS Analysis software interface. The title bar indicates the file is '\*unsaved\* PFICS Analysis.wsp'. The 'File' menu is open, and the 'Export as Template (WSP)' option is highlighted with a red box. The 'Save as Archive (ACS)' option is also highlighted with a red box. The 'Export to Excel (XLS)' option is also visible. The 'Find' search bar is visible below the menu. The main workspace area shows a table with columns for 'Group', 'Size', and 'Role'. The table contains the following data:

Group	Size	Role
{ } All Samples	45	Test
{ } AllStain	19	Test
▼ { } Compensation	12	Compensation
{ } Compensation		
{ } FMOs	14	Controls
▶ { } MasterGates	33	None

# Additional Training Resources

- Recorded webinars are available here: [www.flowjo.com/learn/webinars](http://www.flowjo.com/learn/webinars)
- Guided instructional courses: [www.flowjo.com/learn/flowjo-university](http://www.flowjo.com/learn/flowjo-university)
- The Daily Dongle blog has great tips and tricks: [flowjo.typepad.com/](http://flowjo.typepad.com/)



## Questions?

- FlowJo is here to help!
- Contact [flowjo@bd.com](mailto:flowjo@bd.com) with any questions or concerns regarding your analyses questions.
- If you have specific questions for me, feel free to reach out: [joshua.luthy@bd.com](mailto:joshua.luthy@bd.com)