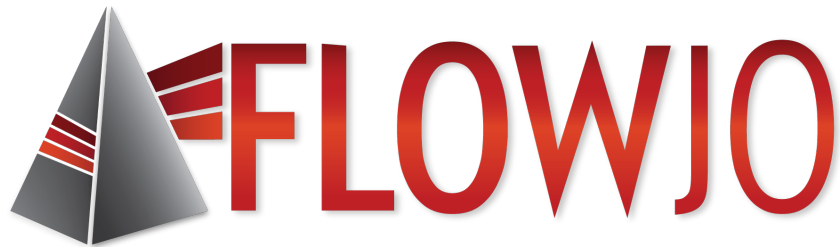


# Cytometry Data Analysis in FlowJo V10



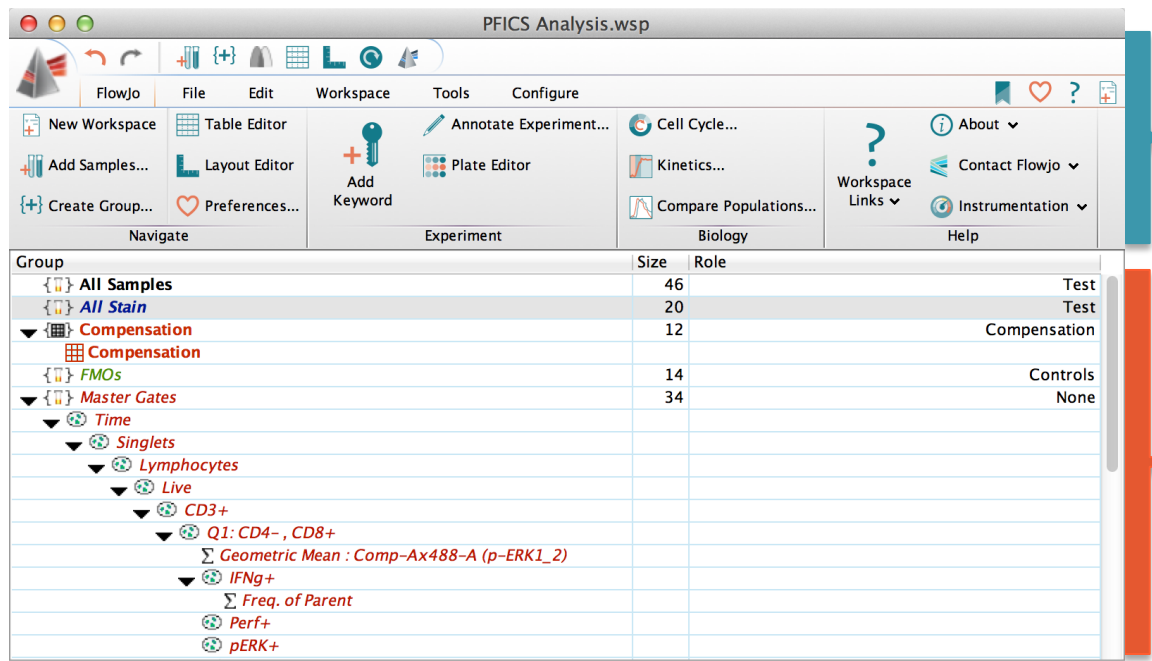
Timothy Quinn Crawford, PhD  
Application Scientist  
FlowJo, LLC  
[timc@flowjo.com](mailto:timc@flowjo.com)

# Outline

- Navigating the V10 Workspace
- Demo Data background
- Creating and editing Groups
- Keyword attributes and their uses
- The Graph Window, gating and ancestry
- The Layout Editor – exporting graphics
- The Table Editor – exporting statistics
- Workspace Templates
- Compensation

# The FlowJo v10 Workspace

- A graphical interface to organize your data.



Ribbon  
Tabs and Bands

The screenshot shows the "Groups and Group Analysis" table in the FlowJo v10 Workspace. The table has columns for "Name", "Statistic", "#Cells", "\*PID", "\*STIM", and "Well ID". The data is organized into a hierarchical tree structure. A red arrow points from the text "Groups and Group Analysis" to the table.

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			

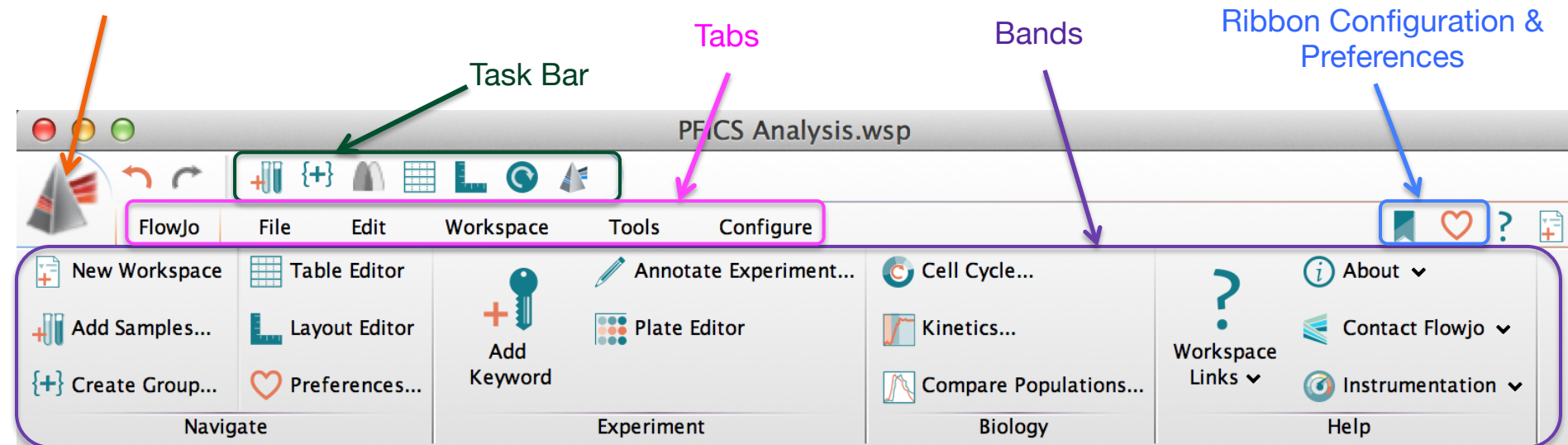
Groups and Group  
Analysis

Samples and  
sample analysis

# Ribbons, Tabs and Bands

- Ribbon organization allows easy visual navigation of workspace functions.

Application Button

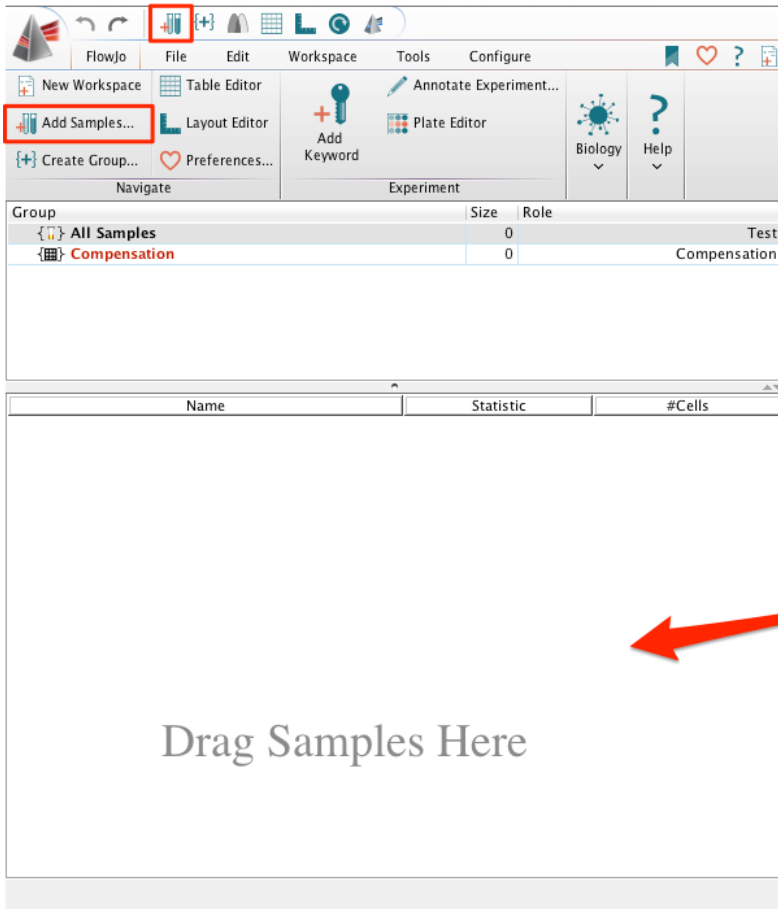
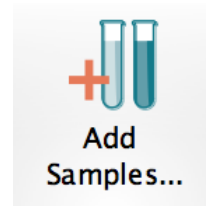


- Tabs group similar Bands together.
- Bands group similar Actions together.

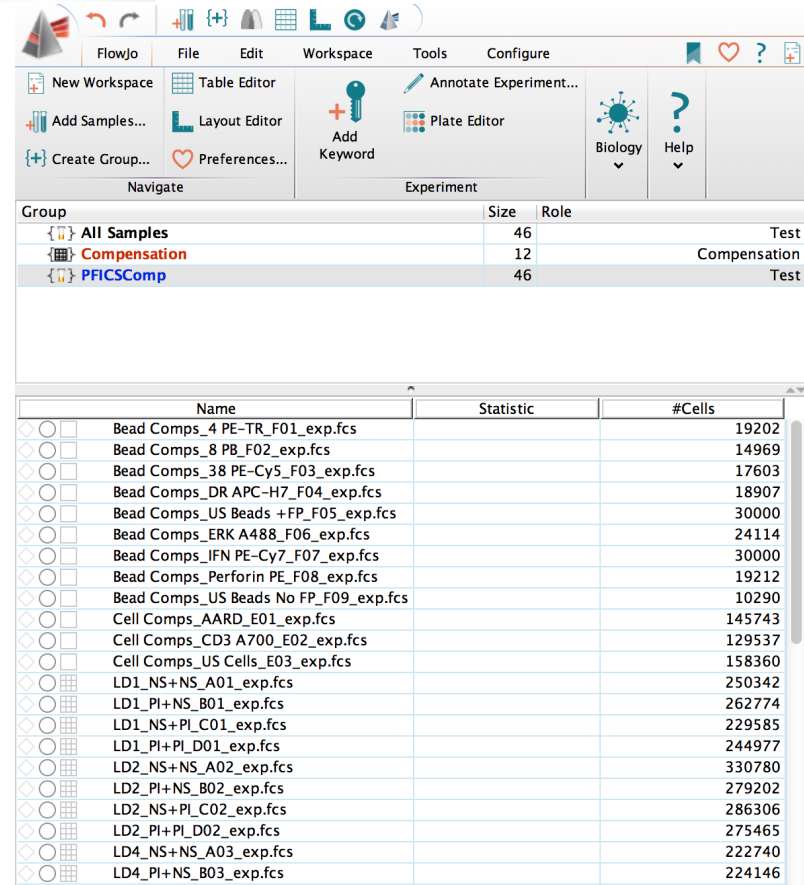


# Importing Data

Drag and drop into Samples Pane or click the Add Samples button



Drag Samples Here



Group	Size	Role
{ } All Samples	0	Test
{ } Compensation	0	Compensation

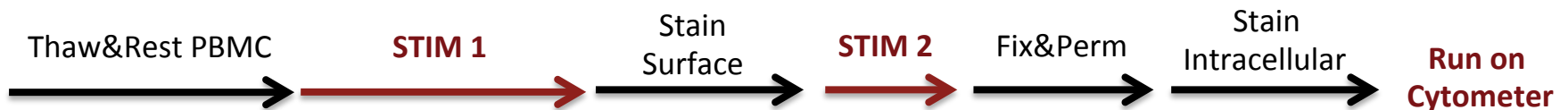
Name	Statistic	#Cells
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_4 PE-TR_F01_exp.fcs		19202
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_8 PB_F02_exp.fcs		14969
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_38 PE-Cy5_F03_exp.fcs		17603
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_DR APC-H7_F04_exp.fcs		18907
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_US Beads +FP_F05_exp.fcs		30000
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_ERK A488_F06_exp.fcs		24114
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_IFN PE-Cy7_F07_exp.fcs		30000
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_Perforin PE_F08_exp.fcs		19212
<input type="checkbox"/> <input type="checkbox"/> Bead Comps_US Beads No FP_F09_exp.fcs		10290
<input type="checkbox"/> <input type="checkbox"/> Cell Comps_AAARD_E01_exp.fcs		145743
<input type="checkbox"/> <input type="checkbox"/> Cell Comps_CD3 A700_E02_exp.fcs		129537
<input type="checkbox"/> <input type="checkbox"/> Cell Comps_US Cells_E03_exp.fcs		158360
<input type="checkbox"/> <input type="checkbox"/> LD1_NS+NS_A01_exp.fcs		250342
<input type="checkbox"/> <input type="checkbox"/> LD1_PI+NS_B01_exp.fcs		262774
<input type="checkbox"/> <input type="checkbox"/> LD1_NS+PI_C01_exp.fcs		229585
<input type="checkbox"/> <input type="checkbox"/> LD1_PI+PI_D01_exp.fcs		244977
<input type="checkbox"/> <input type="checkbox"/> LD2_NS+NS_A02_exp.fcs		330780
<input type="checkbox"/> <input type="checkbox"/> LD2_PI+NS_B02_exp.fcs		279202
<input type="checkbox"/> <input type="checkbox"/> LD2_NS+PI_C02_exp.fcs		286306
<input type="checkbox"/> <input type="checkbox"/> LD2_PI+PI_D02_exp.fcs		275465
<input type="checkbox"/> <input type="checkbox"/> LD4_NS+NS_A03_exp.fcs		222740
<input type="checkbox"/> <input type="checkbox"/> LD4_PI+NS_B03_exp.fcs		224146



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

## Polyclonal PFICS Assay:

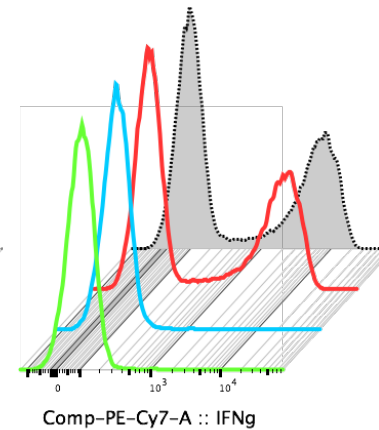
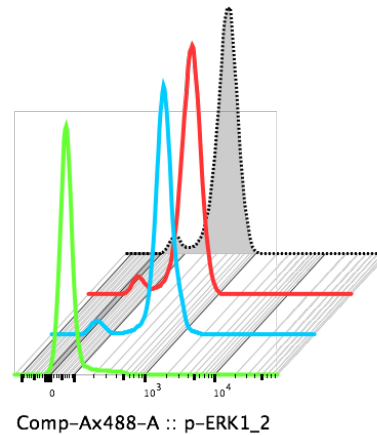
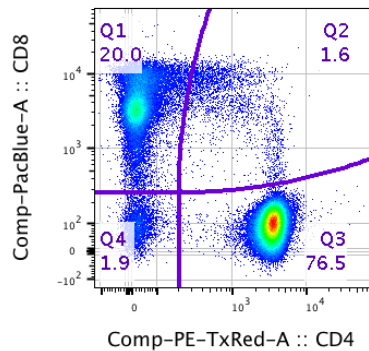
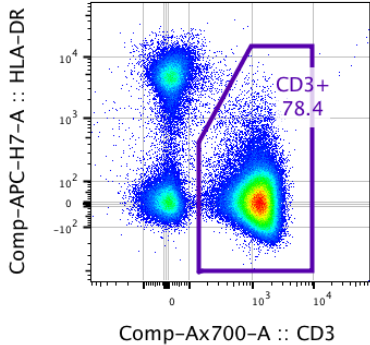
- Thaw and rest cryopreserved human PBMC overnight
- Stimulate with PMA+Ionomycin (PI) for 2 hours or rest (NS) while blocking protein secretion → signaling and cytokines
- Stain for viability (AARD) and surface antigens (CD3, CD4, CD8, CD38 and HLA-DR)
- Stimulate PI for 20 minutes or NS rest
- Fix,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



# PFICS Stim Conditions

- 2 Stimulations → 4 potential \*STIM combinations/conditions

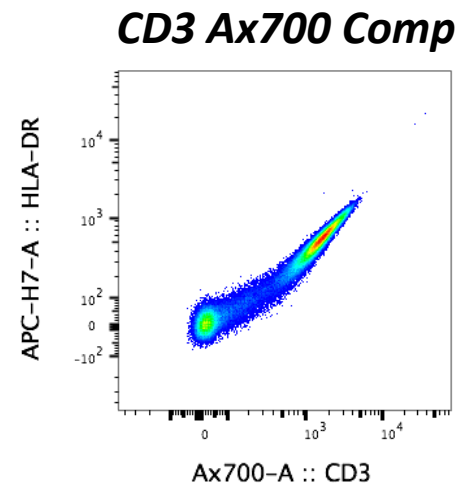
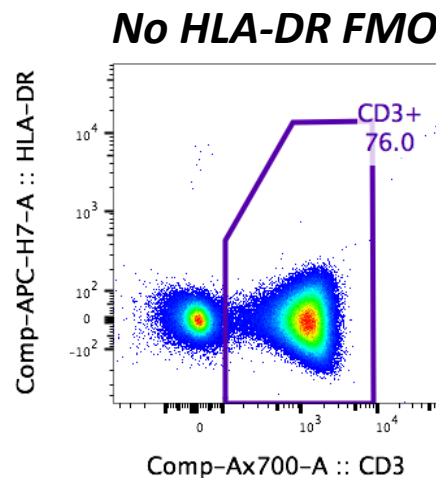
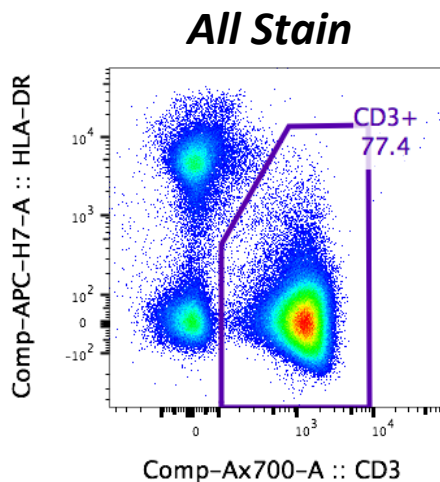
*STIM Condition	Total *STIM Time	phospho-ERK Response	IFN- $\gamma$ Response
NS+NS	0 min	-	-
NS+PI	20 min	++++	-
PI+NS	120 min	+++	+++
PI+PI	140 min	+++	+++



Sample Name	
LD1_NS+NS_A01_exp.fcs	
LD1_NS+PI_C01_exp.fcs	
LD1_PI+NS_B01_exp.fcs	
LD1_PI+PI_D01_exp.fcs	

# PFICS Samples

- 46 Total Samples
  - 20 experimental *All Stain* samples – Stained with all reagents in the panel → Real Experiment
  - *14 Fluorescence Minus One (FMO)* controls – Leave one reagent out of panel → Gating Control
  - *12 Compensation* controls – Stained with single reagent to isolate the fluorochrome emission spectrum and determine spillover into detectors.



# Group Pane

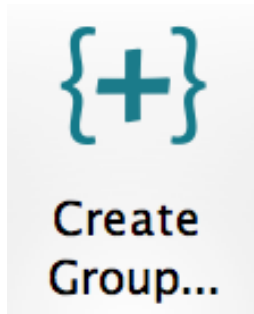
- The Group area lists all groups in the Workspace, # of samples in each group (Size), and the Role of that group (ex. Test, Compensation, Controls) .
- Groups act like folders to organize your samples, allows master gating and unique report generation.

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

- 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 "Appearance" section shows "Name: All Stain", "Color: Blue", "Style: Bold-Italic", "Role: Test", and "Parameter Key:". The "Sample Inclusion Criteria" section has "Live group" checked and "Synchronized" unchecked. It lists "Dead, HLA-DR, p-ERK1\_2, Blank, CD3, Perforin, CD38, IFNg, CD4, CD8". The "\$FIL" dropdown is set to "LD". The "With reference to samples in another group:" section has "Also include all" selected and "samples in Group" set to "(No specified group)". The "Assignments" section has two empty "Add Keyword" and "With Value" fields. Buttons at the bottom include "Help with Groups", "Apply Changes", "Close", and "Create Group".

# Sample Inclusion Criteria

- **Live groups** automatically include samples based on user-defined **Sample Inclusion Criteria**.
- Sample Inclusion Criteria could include the staining panel, characters in the \$FIL (file name), a user defined Keyword attribute, or a combination of features.

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

- Appearance:**
  - Name: PI+PI
  - Color: Blue
  - 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
  - \$FIL Contains LD
  - And \$FIL Lacks FMO
  - And \*STIM = PI+PI
  - 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 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 Keyword attributes can be displayed as 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.



# Add New Keywords

- Keywords attach descriptive metadata to samples
- Examples:
  - FCS file standard required keywords (ex. \$FIL, \$PnS)
  - User defined descriptive Keywords (ex. Patient ID, Timepoint)
- Workspace Tab → Keywords Band → **Add Keyword** allows user to define new Keywords and add metadata

The screenshot shows the FlowJo software interface. The 'Workspace' tab is selected in the top menu. In the 'Keywords' band, the 'Add Keyword' button (represented by a key icon with a plus sign) is highlighted with a red box. Below the main interface, a table displays sample data with columns for Name, Statistic, PacBlue-A reagent, Patient ID, and Timepoint.

Name	Statistic	PacBlue-A reagent	Patient ID	Timepoint	
LD1_NS+NS_A01_exp.fcs			CD8	LD1	Day 0
LD1_NS+PI_C01_exp.fcs			CD8	LD1	Day 0
LD1_PI+NS_B01_exp.fcs			CD8	LD1	Day 0
LD1_PI+PI_D01_exp.fcs			CD8	LD1	Day 0
LD2_NS+NS_A02_exp.fcs			CD8	LD2	Day 0
LD2_NS+PI_C02_exp.fcs			CD8	LD2	Day 0
LD2_PI+NS_B02_exp.fcs			CD8	LD2	Day 0
LD2_PI+PI_D02_exp.fcs			CD8	LD2	Day 0

# Display Existing Keywords

- Configure Tab → Settings Band → **Edit Columns** allows user to display Keywords as columns in the workspace samples pane

The screenshot shows the FlowJo software interface. The 'Configure' tab is selected in the top menu bar. In the 'Settings' band, the 'Edit Columns...' button is highlighted with a red box. Below the settings, a table lists sample groups:

Group	Size	Role
All Samples	46	Test
AllStain	20	Test
Compensation	12	Compensation
Compensation		
	14	Controls

The 'Edit Columns' dialog box is open, showing a list of 'All Column Values' on the left and 'Columns To Display' on the right. The '\*SAMPLEID' column is selected in the left list. A red arrow points from the text 'Double Click to Add' to the '\*SAMPLEID' entry. The dialog also includes buttons for 'Add Column', 'Remove Column', 'Remove All', 'Save', 'Default', and 'Use as workspace default' (checked). The 'Columns To Display' list includes: Name, Statistic, WELL ID, \*PID, \*STIM, HIV Status, and \*SAMPLEID.

	WELL ID	*PID	*STIM	*HIV Status
	A01	LD1	NS+NS	Neg
	C01	LD1	NS+PI	Neg
	B01	LD1	PI+NS	Neg
	D01	LD1	PI+PI	Neg
	A02	LD2	NS+NS	Neg
	C02	LD2	NS+PI	Neg
	B02	LD2	PI+NS	Neg
	D02	LD2	PI+PI	Neg

Double Click to Add

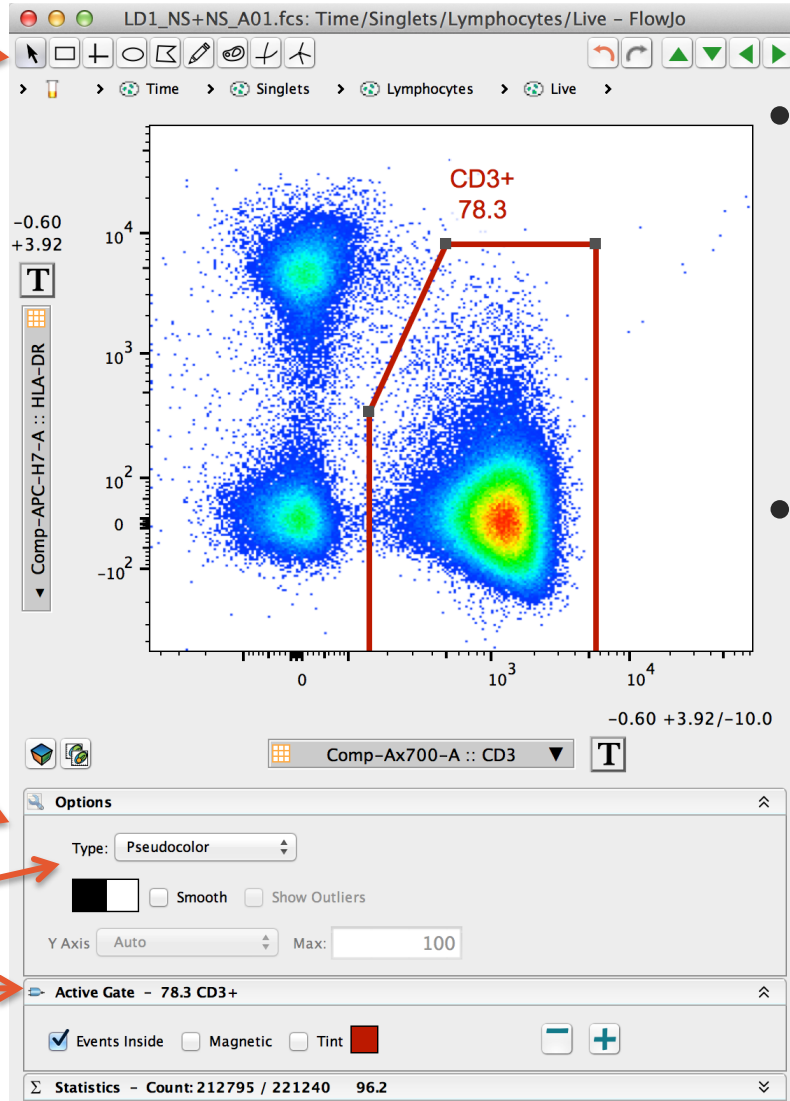
Click

OK

# The Graph Window

- Facilitates data visualization and gating.

Gating Tools



Plot View Options

Graph Type

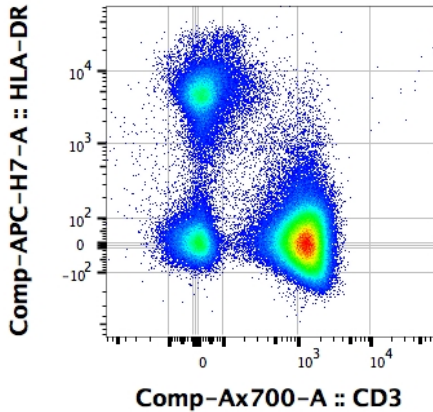
Active Gate Options

- 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.

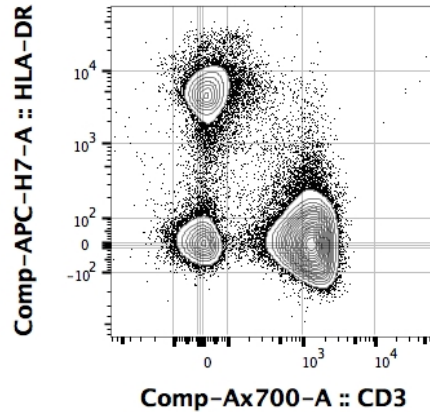
# Graph Display Options

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

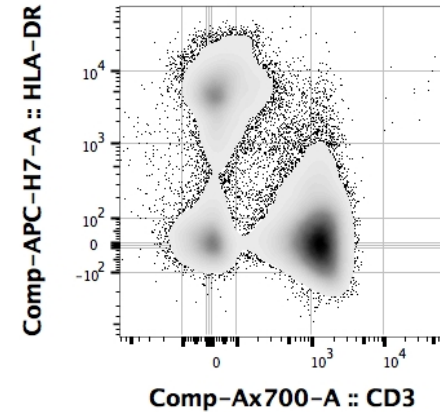
Pseudocolor



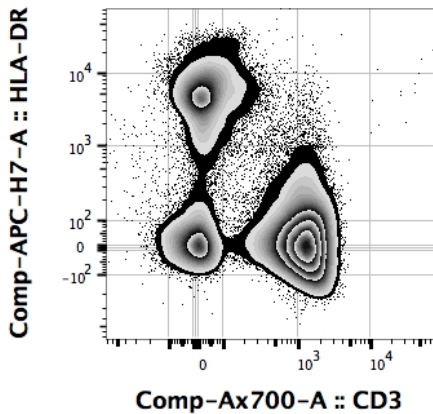
Contour



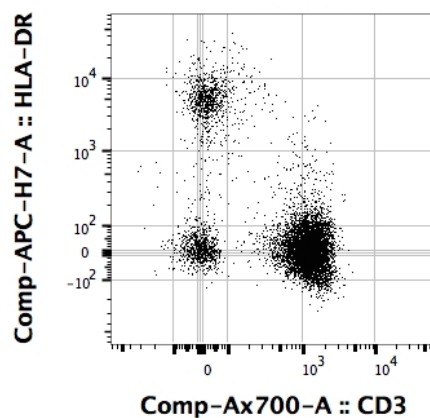
Density



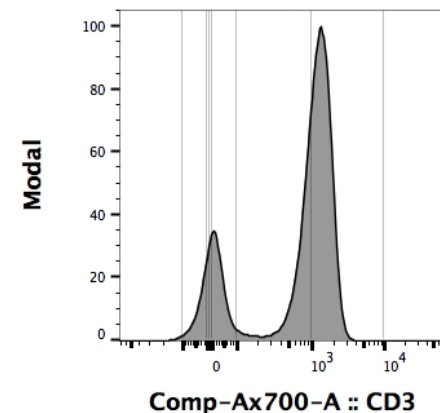
Zebra



Dot Plot



Histogram

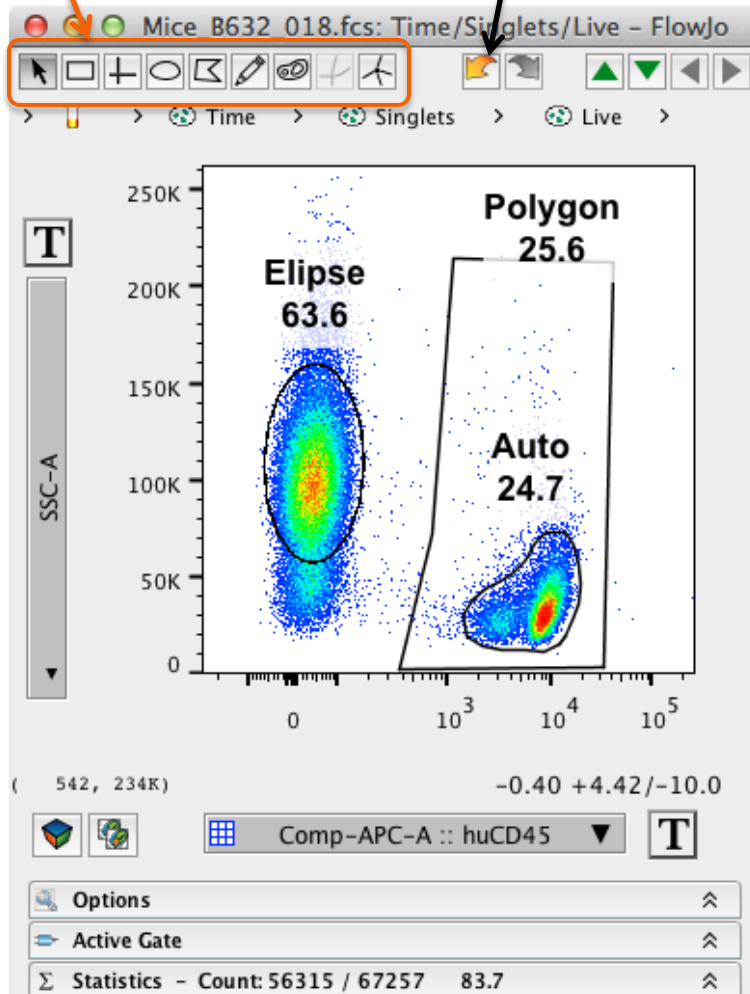


# Gating tools

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

Gating Tools

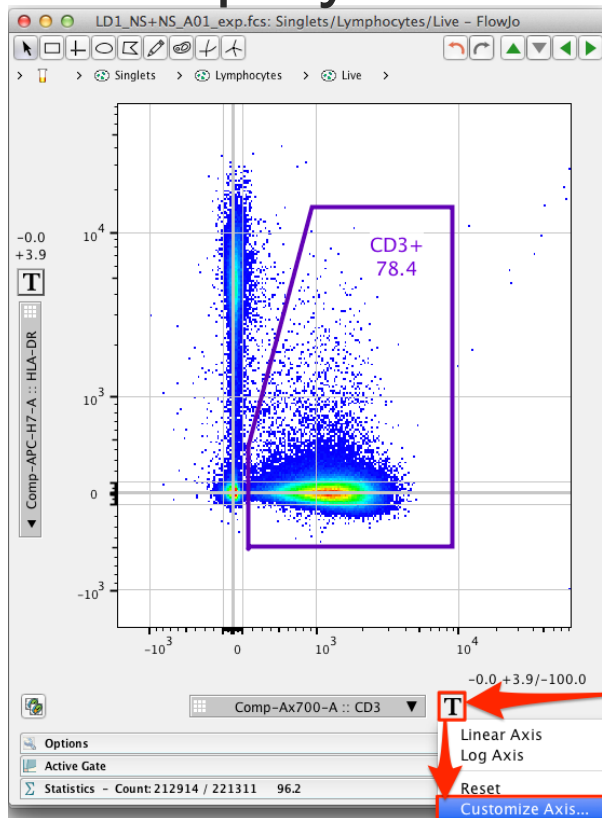
Undo!!



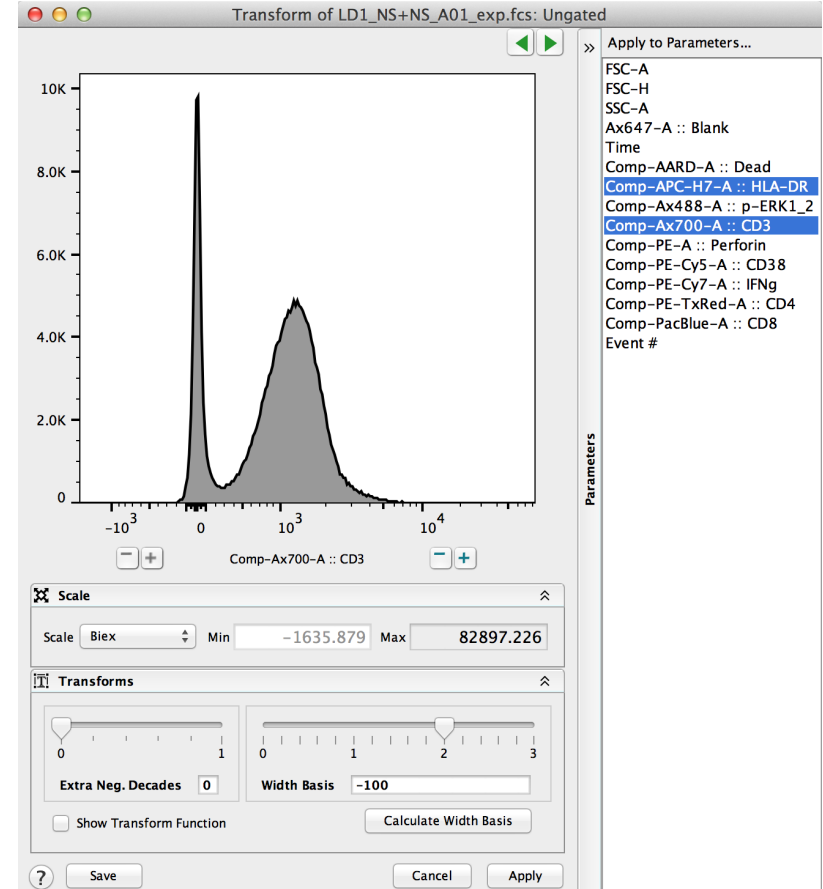
- Gates can always be modified or removed, so don't be shy.
- Explore the gating options and pick what works best for you.

# Transforming Data

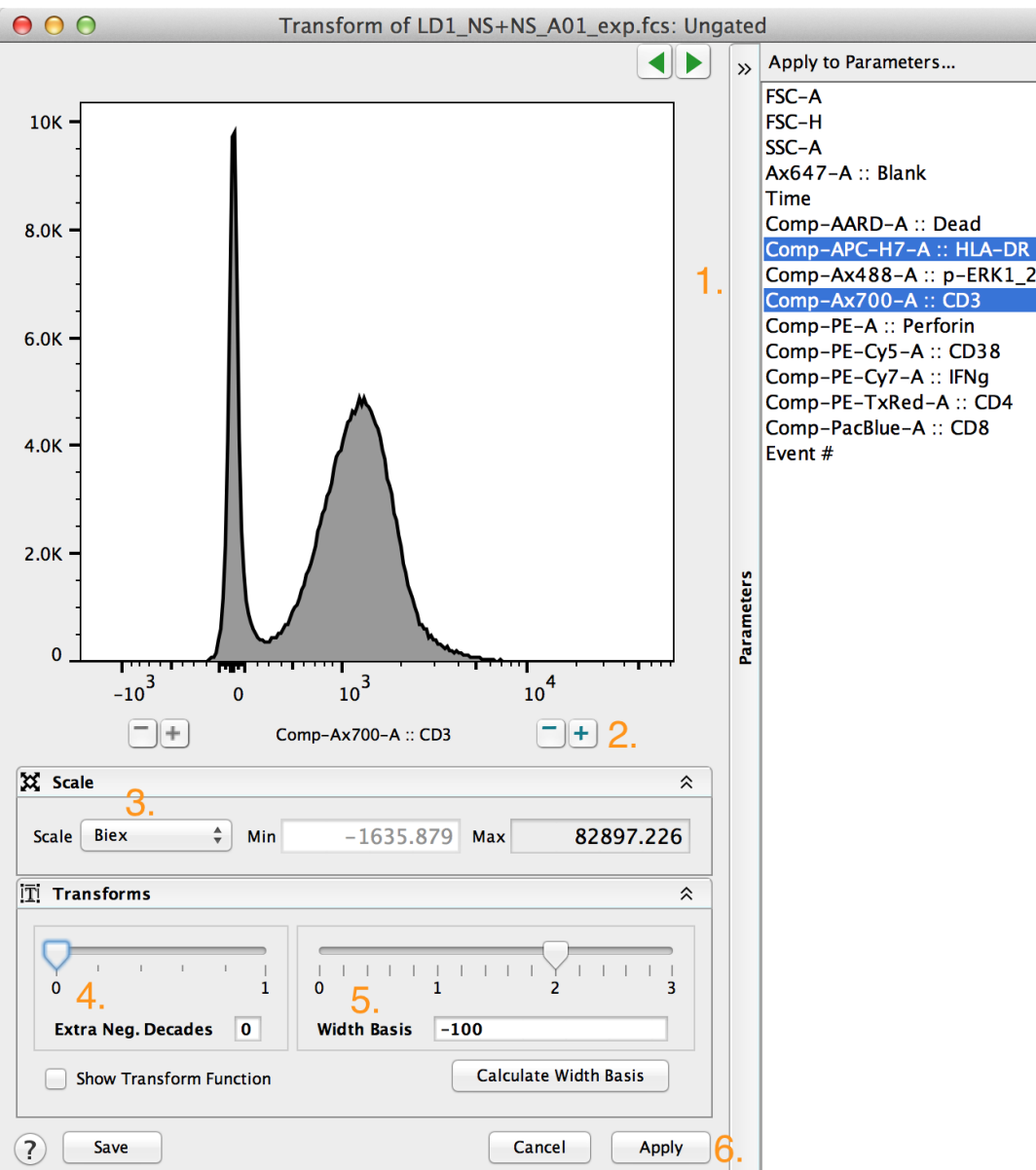
- Your data may initially look ‘squished’.
- Click the Transformation [ T ] button and Select Customize Axis... to change the visual display.



Manually change scaling of axis.



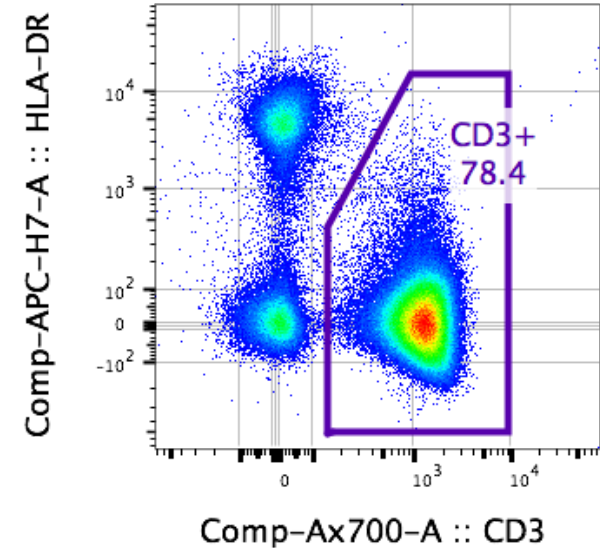
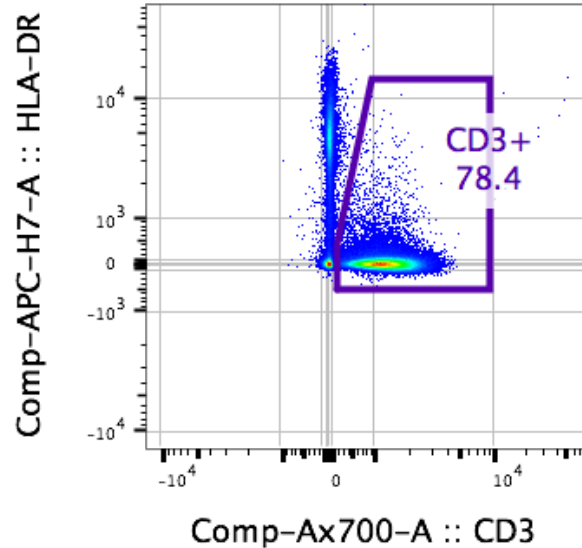
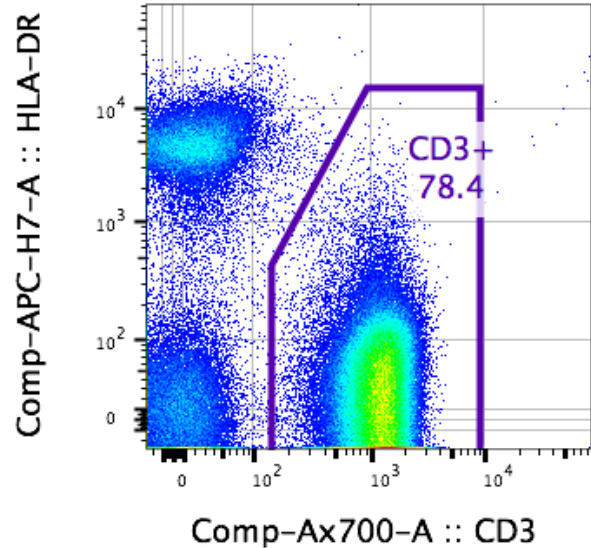
# Transform Options



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. Applies the transformation settings to all selected parameters



# Effects of Transformation



1. Gets rid of the “squishing” of cells.
2. Ensures the visual population center better correlates with the statistical center (median).
3. Makes 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**

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		
Q1: CD4-, CD8+		82
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	

# Adding Statistics

- Add a statistic to any gated population selected within a sample gating hierarchy.
- Statistic nodes can be group-applied just like a gate.

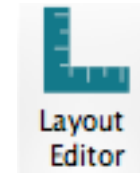
The screenshot shows the FlowJo software interface with the following components and annotations:

- Tools Menu:** The 'Tools' menu is open, and the 'Add Statistic...' option is highlighted with a red box and labeled '2. Select Add Statistic...'.
- Sample Gating Hierarchy:** A tree view on the left shows a sample 'LD1\_NS+NS\_A01\_exp.fcs' with a gated population 'Q1: CD4-, CD8+' selected. This step is labeled '1. Select Population'.
- Populations Table:** A table at the top right lists various populations and their counts. The 'Q1: CD4-, CD8+' population is highlighted.
- Add Statistic... Dialog:** A dialog box is open, showing a list of statistics. 'Geometric Mean' is selected, labeled '3. Choose Statistic'. Below it, 'Comp-PE-A :: Perforin' is selected, labeled '4. Choose Parameter'.
- Dialog Buttons:** The 'Add' button at the bottom right of the dialog is highlighted with a red box and labeled '5. Click Add'.

Group	Count	Label
All Samples	46	Test
AllStain	20	Test
Compensation	12	Compensation
FMOs	14	Controls
MasterGates	34	None
PFICSComp	46	Test
PI+PI	5	Test

# The Layout Editor

- A tool for creating graphical reports.
- Click on the Layout Editor icon.
- Drag populations from a sample to Layout Editor.



Layout Editor

Create Layouts

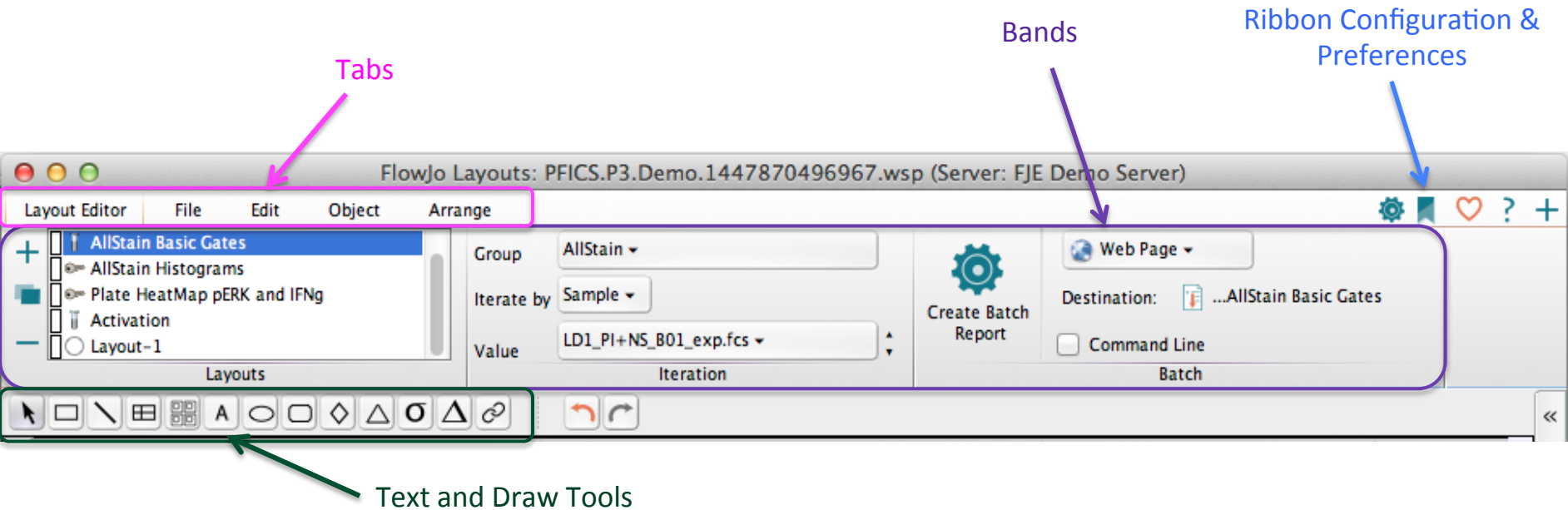
Specify Group and Iterate options

Batch Report Format

The screenshot displays the FlowJo software interface. The 'Layout Editor' window is open, showing a list of populations on the left and a grid of plots on the right. Red arrows point to various features: 'Layout Editor' points to the window title; 'Create Layouts' points to the 'Layouts' list; 'Specify Group and Iterate options' points to the 'Group' and 'Iterate by' dropdowns; 'Batch Report Format' points to the 'Create Batch Report' button and 'Destination' field. A 'Drag & Drop' arrow points from a population in the left pane to a plot in the grid. The plot grid shows several plots, including 'CD8+ T Cells', 'pERK+', 'IFNγ+', and 'Perf+', each with a corresponding histogram below it. The histogram for 'CD8+ T Cells' shows a population of 94.3. The histogram for 'IFNγ+' shows a population of 44.3. The histogram for 'Perf+' shows a population of 33.6. The histogram for 'CD8+ T Cells' also shows a population of 94.3. The histogram for 'IFNγ+' shows a population of 44.3. The histogram for 'Perf+' shows a population of 33.6. The histogram for 'CD8+ T Cells' also shows a population of 94.3. The histogram for 'IFNγ+' shows a population of 44.3. The histogram for 'Perf+' shows a population of 33.6.

# Working in Layout Editor

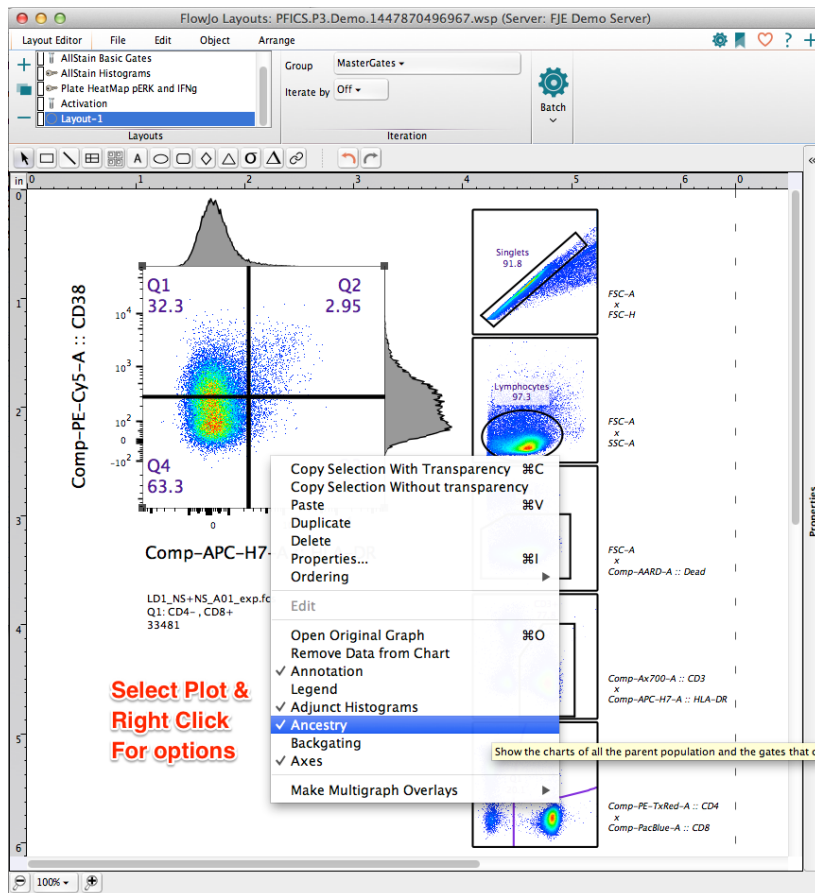
- Similar to the Workspace. Layout Editor has its own customizable Ribbon with Tabs and Bands to organize actions.



- 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

The image displays two instances of the 'Graph Definition' dialog box in a layout editor. The left instance is the 'Specify' tab, and the right instance is the 'Annotate' tab. Red arrows point to various elements in the dialog boxes, highlighting key features.

**Specify Tab (Left):**

- Tabs:** A red box highlights the 'Specify', 'Annotate', 'Fonts', and 'Legend' tabs.
- Axis Parameters:** Red arrows point to the 'X Axis' (Comp-APC-H7-A :: HLA-DR) and 'Y Axis' (Comp-PE-Cy5-A :: CD38) dropdown menus.
- Graph Type and Options:** Red arrows point to the 'Type' (Pseudocolor), 'Contour Levels' (5%), and checkboxes for 'Smoothing', 'Show Outliers', 'Use Large Dots', and 'Show Grid'.
- Scale:** Red arrows point to the 'Scale' section, showing 'Width: 100%', 'Height: 100%', and 'Lock Shape'.

**Annotate Tab (Right):**

- Gate Annotation options:** Red arrows point to checkboxes for 'Annotation', 'Show Gates', 'Axes', 'Adjunct Histograms', 'Ancestry', 'Show Frequencies', and 'Show Population Names'.
- Axis Label options:** Red arrows point to checkboxes for 'Hide Ticks', 'Hide Numbers', and 'Hide Label' for both X and Y axes, along with 'Label' input fields.

**Background Graphs:**

- Top left: A histogram and a scatter plot. The scatter plot has a gate labeled 'Q1 32.3' and another gate labeled 'Q4 63.3'. The axes are 'Comp-PE-Cy5-A :: CD38' (y-axis) and 'Comp-APC-H7-A :: HLA-DR' (x-axis).
- Bottom left: A scatter plot with a gate labeled 'Q1 20.1'. The axes are 'Comp-PE-TxRed-A :: CD4' (y-axis) and 'Comp-PacBlue-A :: CD8' (x-axis).



# Batch Analysis of Layout Editor Graphics

**Specify Group & Iteration options**      **Report Type & Location**

The screenshot shows the FlowJo software interface. At the top, there are two red arrows pointing to the 'Group' and 'Iterate by' fields, labeled 'Specify Group & Iteration options'. Another red arrow points to the 'Create Batch Report' button, labeled 'Then Click Create Batch Report'. A fourth red arrow points to the 'Web Page' and 'Destination' fields, labeled 'Report Type & Location'. The main area displays several flow cytometry plots, including a large one on the left with quadrants Q1 (24.2%), Q2 (1.97E-3%), Q3 (5.90E-3%), and Q4 (75.7%), and a vertical stack of smaller plots on the right showing various cell populations like Singlets (95.4%), Lymphocytes (93.9%), Live (94.2%), CD3+ (80.0%), and Q1 (23.9%).

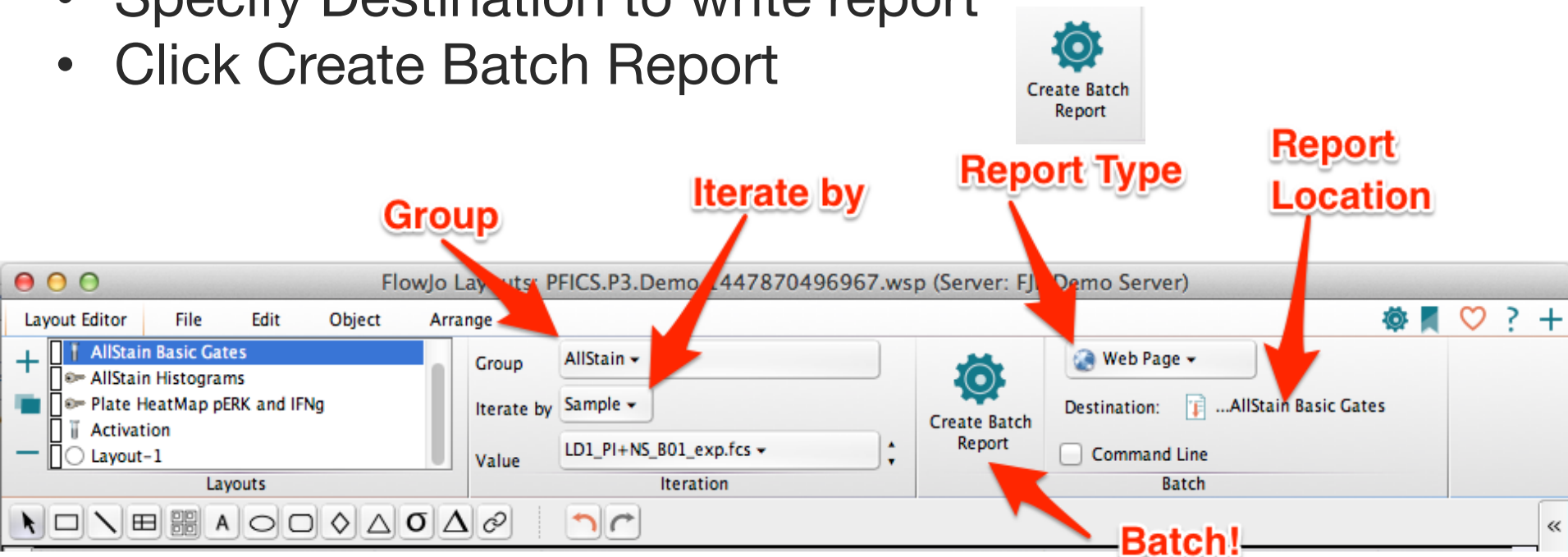
**Then Click Create Batch Report**

- 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 Destination to write report
- 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.



Table Editor

Open Table Editor

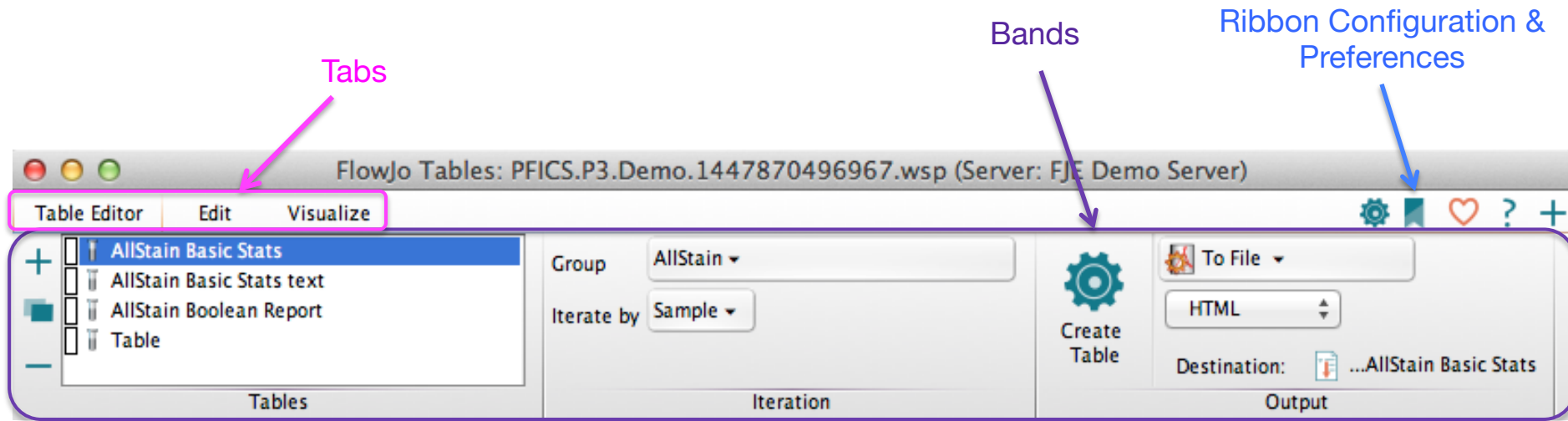
FlowJo Tables: PFICS.P3.Demo.1447870496967.wsp (Server: FJE Demo Server)

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.

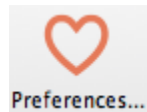
# Table Editor Visualize Tools

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

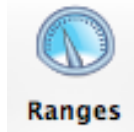
- Highlight row(s), then select the visualization.

- Expected Ranges can be set within Preferences

→ Ranges



Preferences...



Ranges

The screenshot shows the FlowJo Tables software interface. The 'Visualize' tab is selected, and the 'Heat Map' tool is highlighted with a red arrow and the text '2. Apply visualization tool'. The table below shows various cell populations and their parameters.

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

# Workspace Templates

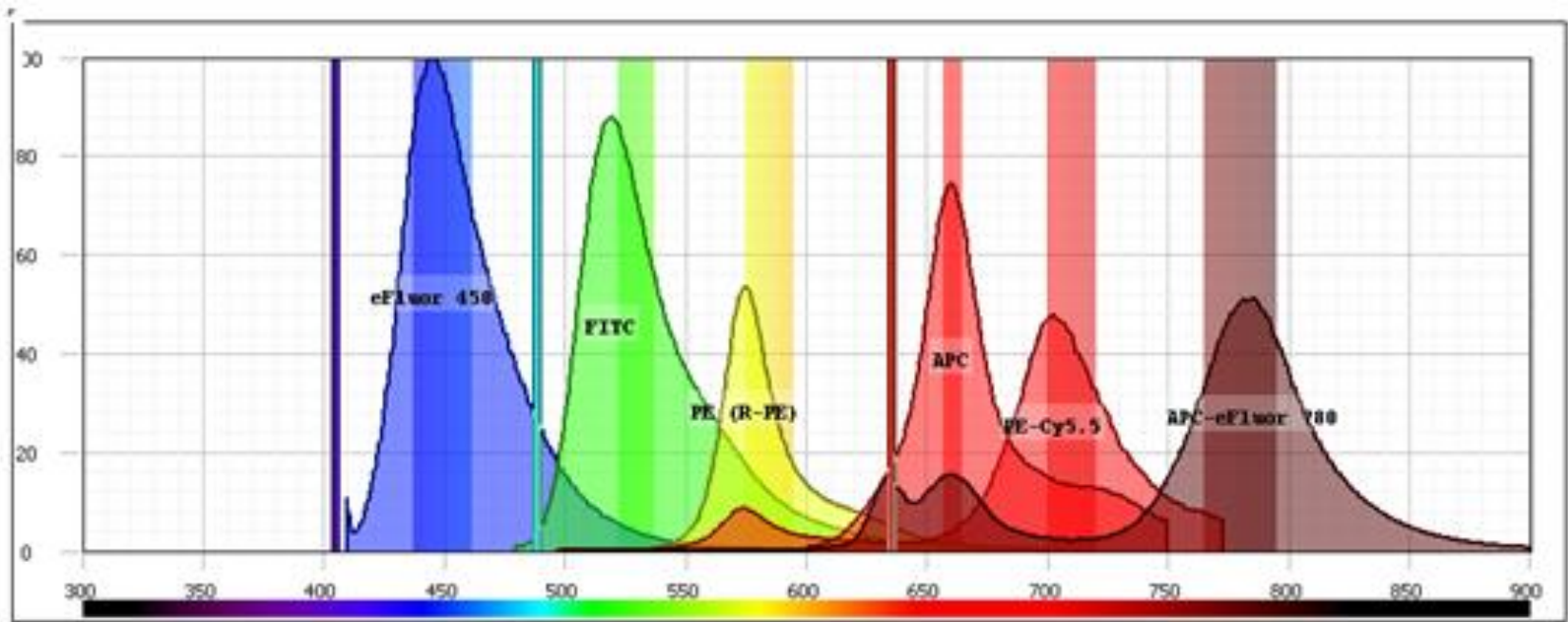
- Allows saving all analysis reports in your workspace without data.
- Streamlines repetitive analysis of multiple runs using the same staining panel(s).
- File Tab → Document Band → Save As... → **Export as a Template (WSPT)**

The screenshot shows the FlowJo software interface with the title bar '\*unsaved\* PFICS Analysis.wsp'. The 'File' menu is open, and the 'Export as Template (WSPT)' option is highlighted with a red box. The interface includes a toolbar with icons for Open..., Print..., Save, Save As..., Revert, Export/Concatenate, Apply Template, Find, and FCS Scan. The 'Save As...' dropdown menu is visible, showing options: Save as Workspace (WSP), Save as Archive (ACS), Export as Template (WSPT), and Export to Excel (XLS). The 'Export as Template (WSPT)' option is highlighted with a red box. Below the menu, a table displays the workspace structure and data.

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

# 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.

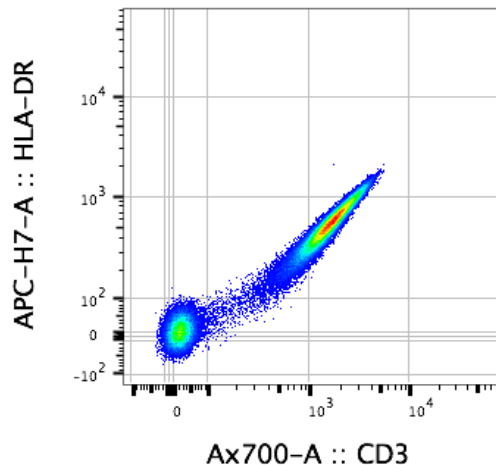


# PFICS Compensation Controls

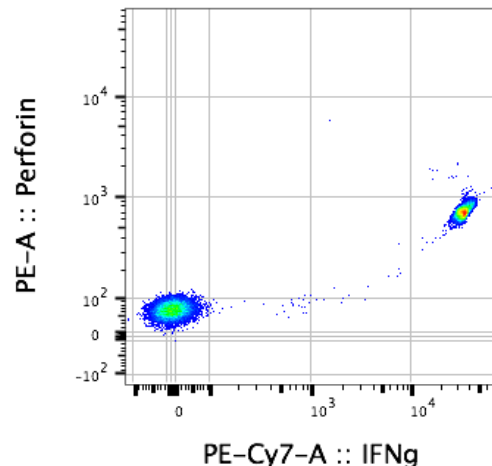
- **PBMC Cells**

1. Unstained Cells
2. AARD
3. CD3 Alexa700

**CD3 Ax700  
Cell Comp**



**IFNg PE-Cy7  
Bead Comp**



- **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. *IFNg PE-Cy7*
9. *Perforin PE*

Tim's Additional Rule (#4)

-Treat your comps like you treat your cells.



# Compensation I

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



in the task bar.

2. Click the Compensation Tool

Group

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

Name

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

1. Highlight Compensation Group

The wizard auto gates samples

Group

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

Name

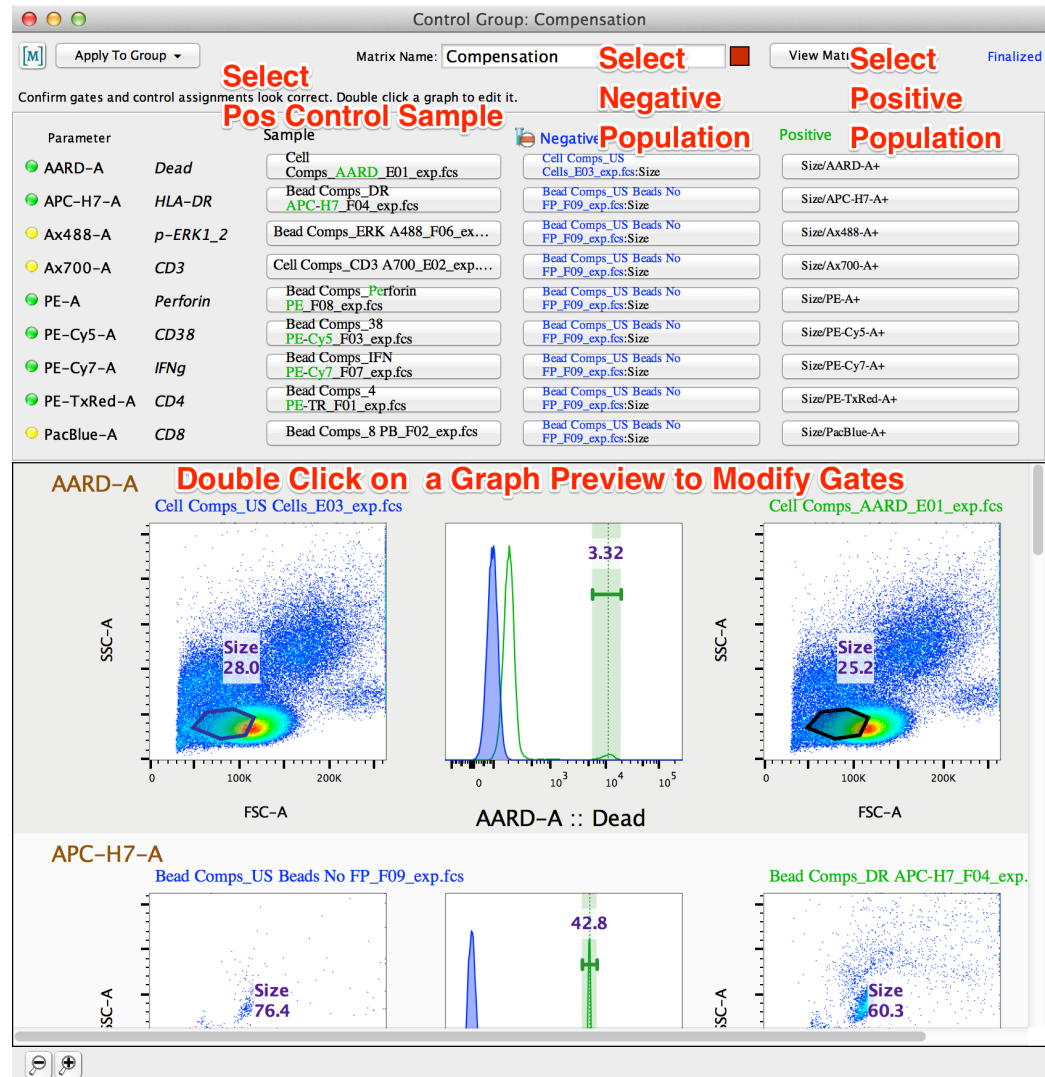
Name	Statistic	#Cells
Bead Comps_DR APC-H7_F04_exp.fcs (Control)		18907
Size	60.3	11396
APC-H7-A+	42.8	4873
Bead Comps_ERK A488_F06_exp.fcs (Control)		24114
Size	66.8	16113
Ax488-A+	47.1	7593
Bead Comps_IFN PE-Cy7_F07_exp.fcs (Control)		30000
Size	70.4	21132
PE-Cy7-A+	52.5	11095
Bead Comps_Perforin PE_F08_exp.fcs (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 II

- Define positive and negative sample populations.

For each Parameter

- Choose from the dropdown lists for each parameter, or drag and drop to populate fields.
- Double click graph preview to modify gates.



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 Dead	Cell Comps <input type="text" value="AARD_E01_exp.fcs"/>	<input type="text" value="Cell Comps_US Cells_E03_exp.fcs:Size"/>	<input type="text" value="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>  
 ←  
 ←

**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 III

- Preview and apply the calculated matrix

**Select Color**      **Name Matrix**      **Edit Matrix**      **Save a copy of the Matrix**

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

**Applied Matrix Badge is Color Coded**

**Add a Matrix from file**

**Preview Matrix effect on a sample**

Workspace Matrices

Compensation

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

Preview Sample: LD1\_NS+NS\_A01\_exp.fcs      Preview Population:      View       Overlay Uncompensated

APC-H7-A :: HLA-DR      Ax488-A :: p-ERK1\_2      Ax700-A :: CD3      PE-A :: Perforin      PE-Cy5-A :: CD38      PE-Cy7-A :: IFNg

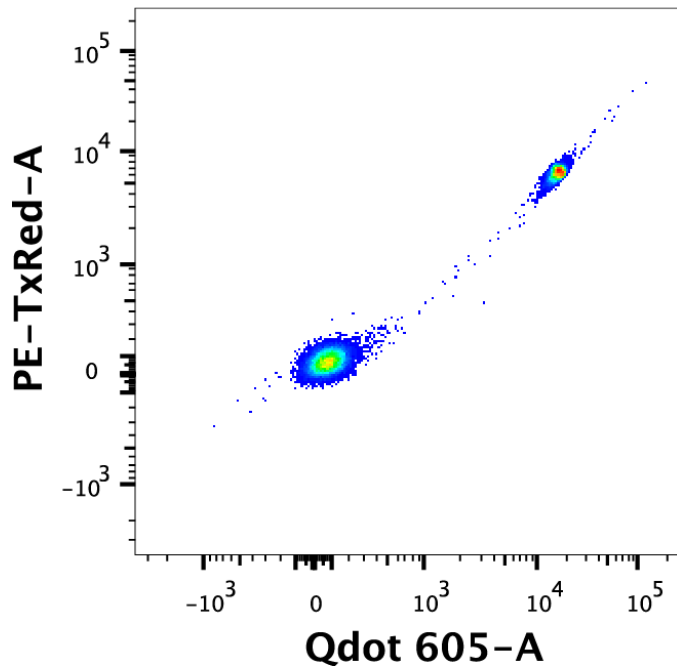
AARD-A

APC-H7-A

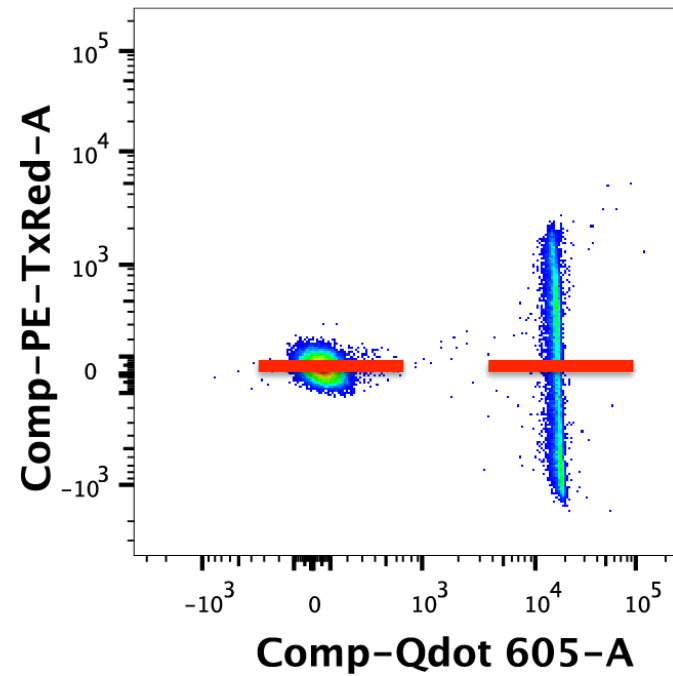
# Effect of Compensation



## Uncompensated



## Compensated



# Outline – Part II

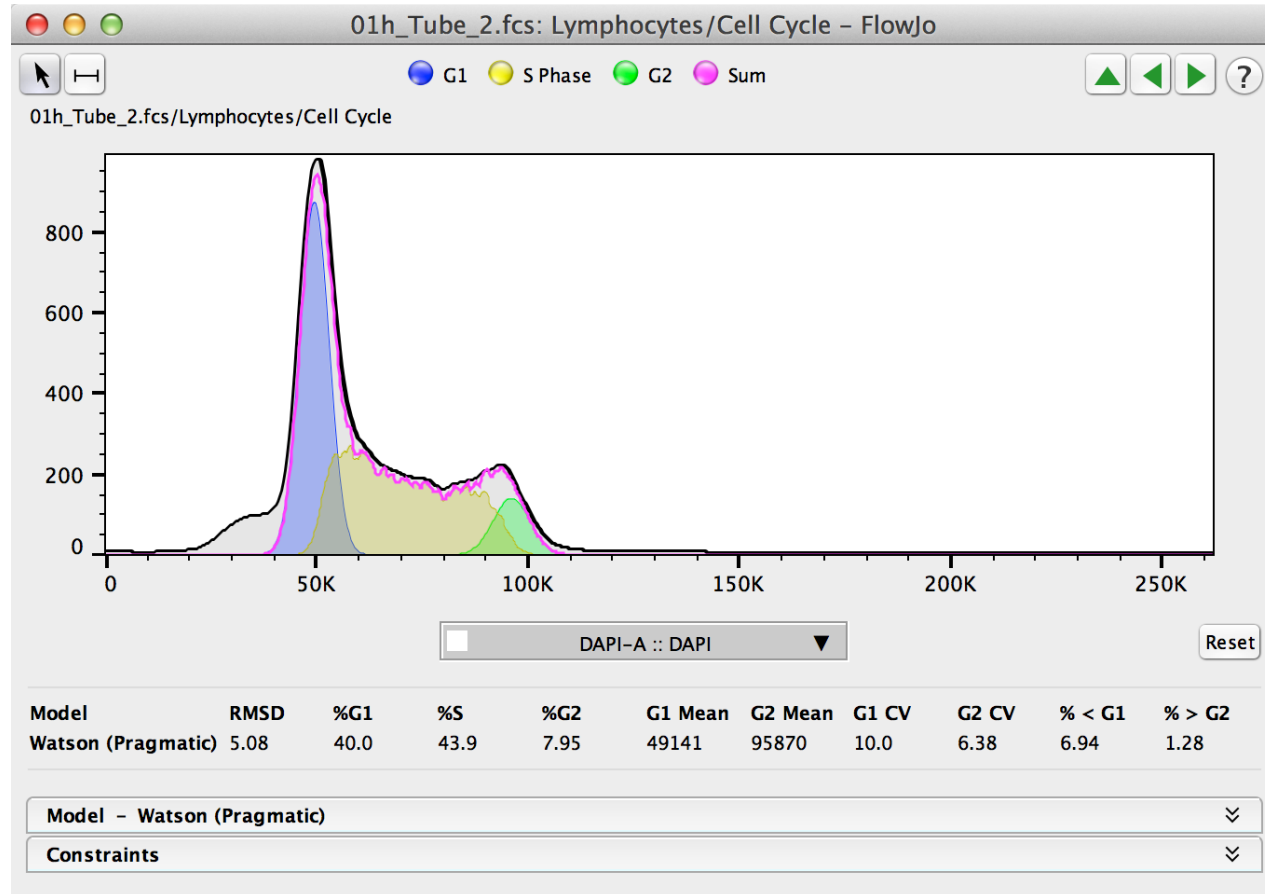
## Advanced Tools and Platforms

- Cell Cycle Analysis
- Plate Tools
- Export/Concatenate
- Plugins
- Additional Resources
- Q&A

# Cell Cycle Analysis

- The Cell Cycle platform allows 1D modeling of cell cycle phases based on DNA content.

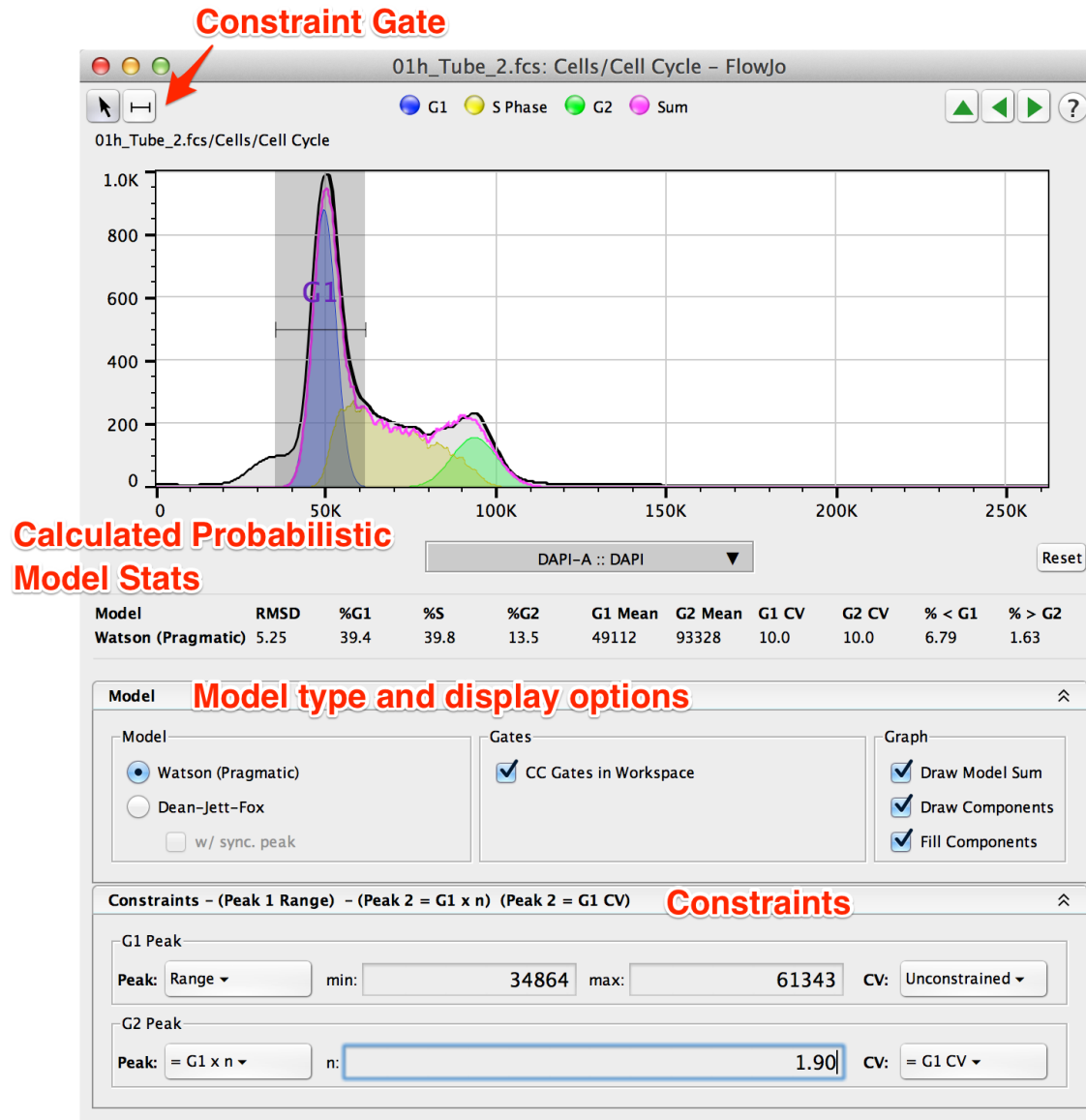
- V10 has 1D Watson pragmatic (polynomial S-phase fit) and Dean-Jett-Fox (Gaussian fit) models.





# Cell Cycle Analysis Workflow

- Initiate CC modeling from the Biology Band
- Select model type
- Set constraints on G1/G2 peaks and CVs.
- Set model individually for each sample, or group-apply a model to all samples in a group.





# Cell Cycle Analysis Reporting

**FlowJo Tables: Cell Cycle.wsp**

Column	Population	Statistic	Parameter	Name
1	Cells/Cell Cycle	RMSD		
2	Cells/Cell Cycle	%G1		
3	Cells/Cell Cycle	%S		
4	Cells/Cell Cycle	%G2		
5	Cells/Cell Cycle	G1 Mean		

**FlowJo Layouts: Cell Cycle.wsp**

Layout Editor | File | Edit | Object | Arrange

Group: Experiment 1  
Iterate by: Sample  
Value: 01\_h\_Control\_No\_Edu.fcs

Layouts: [Icons]

Count

DAPI-A :: DAPI

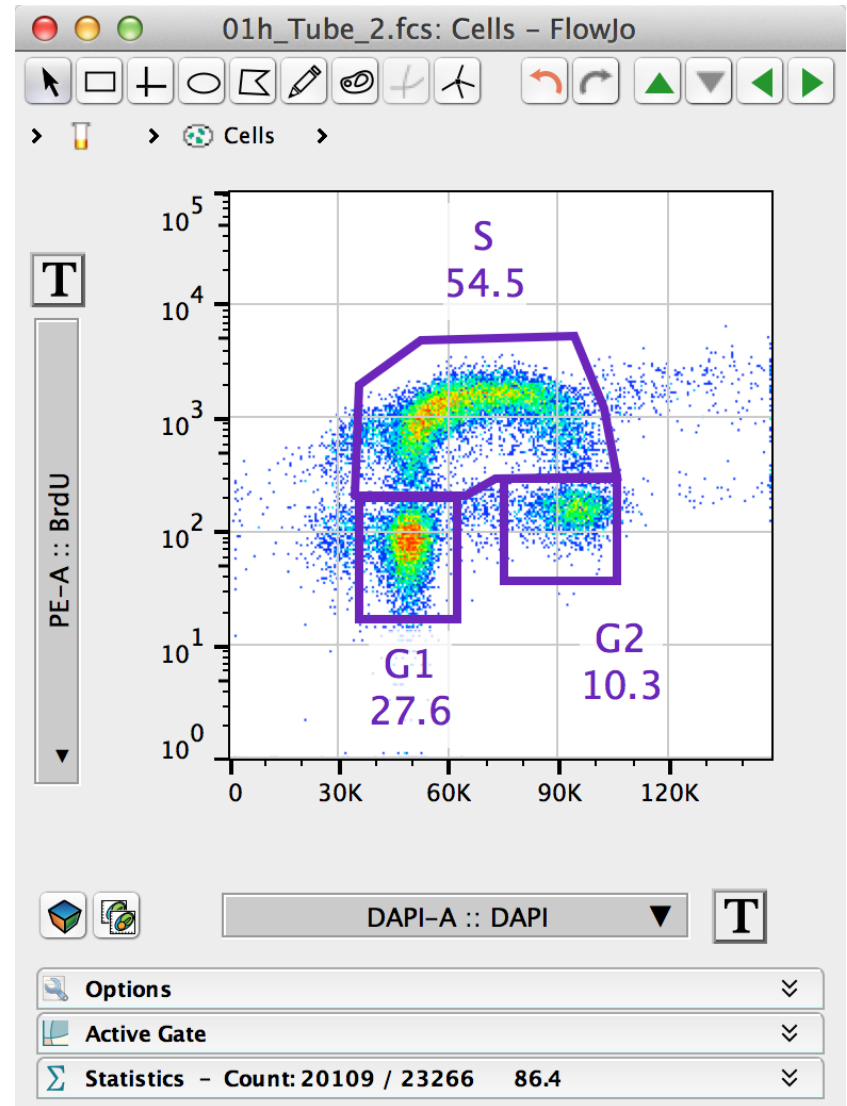
**Statistics:**

- RMSD : 3.80
- %G1 : 35.8
- %S : 40.2
- %G2 : 17.7
- G1 Mean : 45046
- G2 Mean : 85595
- G1 CV : 9.31
- G2 CV : 9.30
- % less G1 : 4.99
- % greater G2 : 3.03

**Drag and Drop Cell Cycle Node to TE or LE**

# Cell Cycle Analysis in 2D

- Note that if using an S-phase specific marker in your panel, you can also use standard 2D gating to define G1, S, and G2.
- This may produce results that are more accurate than the 1D probabilistic models.



# 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.

The screenshot shows the Plate Editor interface with the following components:

- Menu Bar:** Plate Editor, File, Edit, Tools
- Plates Panel:** 20120116 PFICS T...
- Experiment Panel:** Annotate Experiment..., Read Samples from Group, Read Attributes from Group, Apply Plate Keywords to Group
- Metadata:** Plate Name: 20120116 PFICS TQC; Experiment ID: 000-00000; Plate ID: cd9353c6-d77c-4005-9772-0659...
- Filter Keywords:** All Keywords
- Plate Grid:** 8 rows (A-H) x 12 columns (1-12). Well A01 is selected.
- Keyword/Values - Drag to Wells:**

Keyword	Value
Time point	24hr
Treatment "Drug A"	10ug/L
Assay	GFP Reporter
- Keyword/Values - Selected Well:**

Attribute	Value
<del>SP23N</del>	Comp-PE-Cy7-A
<del>SP23S</del>	IFNg
<del>SPILLOVER</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

# Plate Statistics Heatmap

**1. Open the Layout Editor window**

**3. Select the Group with samples you wish to view heat mapped statistics for**

Group	Size	Role
All Samples	46	Test
AllStain	20	Test
Compensation	12	Compensation
Compensation	14	Controls
MasterGates		None
Singlets		
Lymphocytes		
Live		
CD3+		
Q1: CD4-, CD8+		
Geometric Mean: Comp-Ax488-A (p-ERK1_2)		

Name	Statistic	#Cells	*PID	*STIM	WELL ID
LD1_NS+NS_A01_exp.fcs		250342	LD1	NS+NS	A01
Singlets	91.8	229939			
Lymphocytes	97.3	223640			
Live	95.9	214524			
CD3+	77.6	166566			
Q1: CD4-, CD8+	20.8	33378			
Geometric Mean: Comp-Ax488-A (p-ERK1_2)	74.2				
IFNg+	1.04	347			
Freq. of Parent	30.1	10 <sup>255</sup>			
Perf+	7.7	115			
pERK+					
Q1: HLA-DR-, CD38+	32.3	10781			
Q2: HLA-DR+, CD38+	2.83	946			
Q3: HLA-DR+, CD38-	1.52	509			
Q4: HLA-DR-, CD38-	63.3	21142			
IFNg-	99.0	33031			
Perf-	69.9	23325			
pERK-	95.3	31819			
IFNg+Perf+pERK+	0.13	44			
IFNg+Perf+pERK-	0.49	162			
IFNg+Perf-pERK+	0.015	5			
IFNg+Perf-pERK-	0.41	136			
IFNg-Perf+pERK+	2.94	980			
IFNg-Perf+pERK-	26.6	8867			
IFNg-Perf-pERK+	1.59	530			
IFNg-Perf-pERK-	67.9	22654			
Q2: CD4+, CD8+	1.44	2394			
Q3: CD4+, CD8-	76.6	127512			
Q4: CD4-, CD8-	1.99	3315			
LD1_NS+PI_C01_exp.fcs		229585	LD1	NS+PI	C01
Singlets	92.9	213388			
Lymphocytes	97.8	208665			
Live	96.3	200963			
CD3+	78.1	156978			
Q1: CD4-, CD8+	18.8	29572			
Geometric Mean: Comp-Ax488-A (p-ERK1_2)	505				

**4. Drag and drop a Statistic node onto the Plate tool box**

**2. Click the Plate Tool Button, then click on the open layout window.**

**(Drag Plate Statistics Here)**

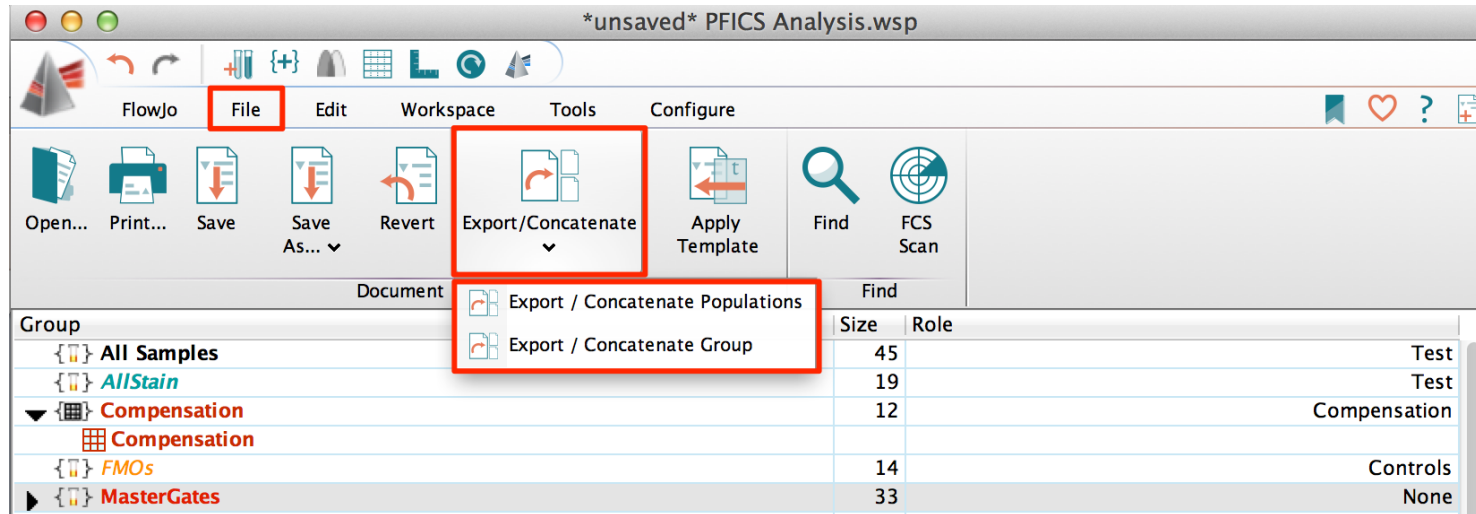
**Data will populate the Plate tool box**  
**Sample position is based on WELL ID**

	1	2	3	4	5
A	Blue	Blue	Blue	Blue	Blue
B	Grey	Grey	Grey	Grey	Grey
C	Yellow	Yellow	Yellow	Yellow	Yellow
D	Grey	Grey	Grey	Grey	Grey

Sample Group: AllStain  
 Statistic: Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Geometric Mean: Comp-Ax488  
 :: heatmap range = 67.4 - 599  
 :: displayed as Heatmap

# Export/Concatenate

- Initiation from the Workspace



- Two options:
  - Export/Concatenate Populations → select gated populations on sample gating hierarchy
  - Export/Concatenate Group → select group or group owned gate in the groups pane

# Concatenate Options

- **Output**

- Format: select file format (FCS3 or CSV)
- Destination: specify save location
- File name example: displays example of naming scheme as specified in Advanced Options → File Naming

- **Include Events**

- Include all events is the only option when concatenating

- **Parameters**

- Suggestion: [Always leave Choose All uncompensated parameters selected.](#)  
If the sample is compensated, compensated parameters will be written to the concatenated file.

- **File naming**

- Prefix: specify a common prefix
- Suffix: specify a suffix

- **Status**

- Number of files that will be generated

Populations: Export or Concatenate

Populations:

Output

Format: FCS3

Destination: /Users/timq/Desktop/PFICS.Example  
File name example: concat\_1\_LD1.fcs

Include Events

Include all  
 Include no more than: 199577

Parameters

All uncompensated parameters  
 All compensated parameters  
 Custom set of parameters:

Advanced Options

File Naming

Prefix: concat

(Concatenated files will be numbered consecutively starting at 1. Example: prefix\_1\_suffix.fcs)

Separator: \_

Suffix: LD1.fcs

Group Concatenation

Concatenate all files together  
 Concatenate every "n" files together n= 4  
 Concatenate files with equal keyword values  
Choose Keyword:

Additional Parameters

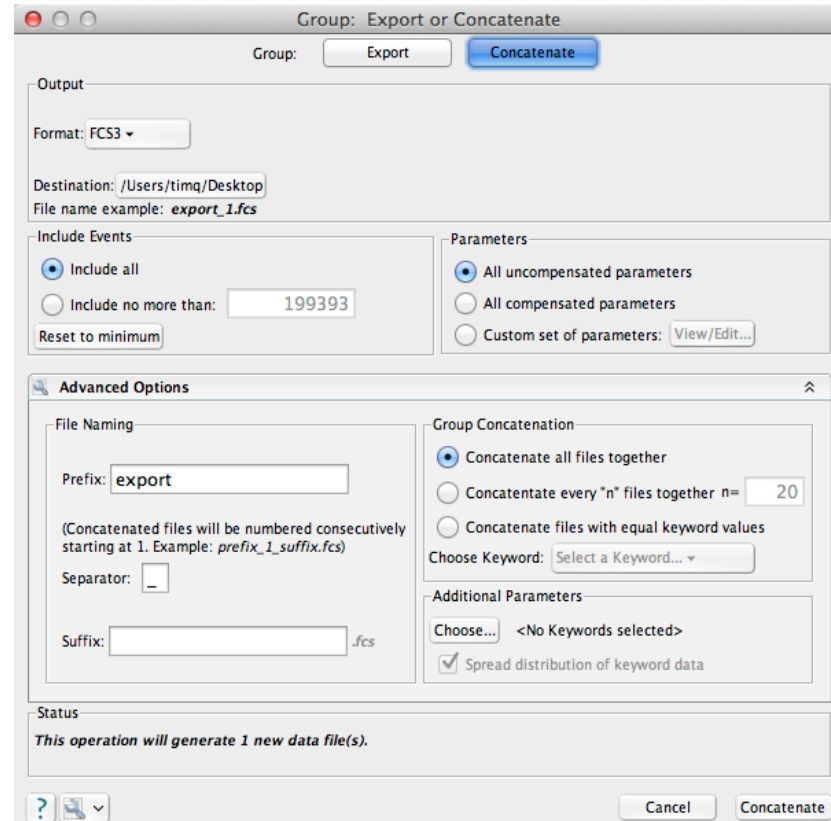
\*Condition  
 Spread distribution of keyword data

Status

This operation will generate 1 new data file(s).

# Concatenating Groups

- Highlight a group or group owned population. The group should contain all the samples you wish to export.
- Choose Export/Concatenate Group and click the Concatenate button at the top of the UI
- **Group Concatenation**
  - Concatenate all files together
  - Concatenate every “n” files together
  - Concatenate files with equal keyword values
- **Additional Parameters**
  - Creates new derived parameter(s) from selected Keyword attribute(s) → Order files or group together based on common feature (example: Timepoint or Sick vs Healthy)





# 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 software's main window with the 'File' menu open. The 'Export/Concatenate' option is selected, and a dropdown menu is visible with 'Export / Concatenate Populations' highlighted. Below the menu, a table displays the population statistics for various samples.

Name	Statistic	#Cells	*STIM	*Condition
LD1_NS+NS_A01_exp.fcs		250342	NS+NS	1
Singlets	92.2	230744		
Lymphocytes	95.9	221311		
Live	96.2	212865		
LD1_NS+PI_C01_exp.fcs		229585	NS+PI	2
Singlets	93.2	213887		
Lymphocytes	96.7	206750		
Live	96.5	199577		
LD1_PI+NS_B01_exp.fcs		262774	PI+NS	3
Singlets	96.1	252600		
Lymphocytes	93.6	236368		
Live	96.2	227312		
LD1_PI+PI_D01_exp.fcs		244977	PI+PI	4
Singlets	96.1	235439		

The screenshot shows the 'Populations: Export or Concatenate' dialog box. The 'Concatenate' button is selected. The 'Advanced Options' section is expanded, showing the following settings:

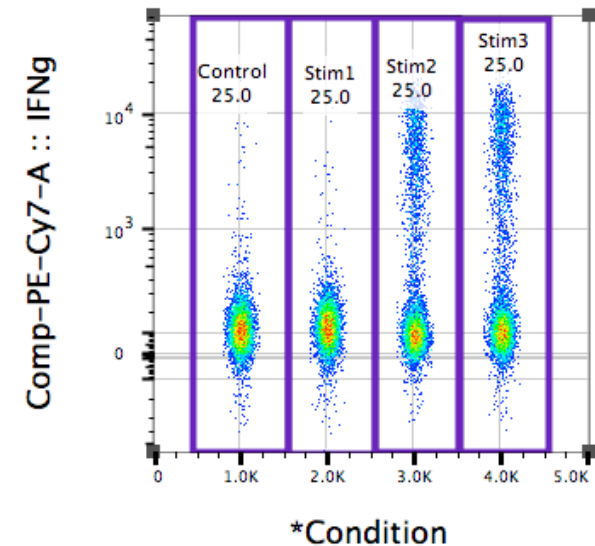
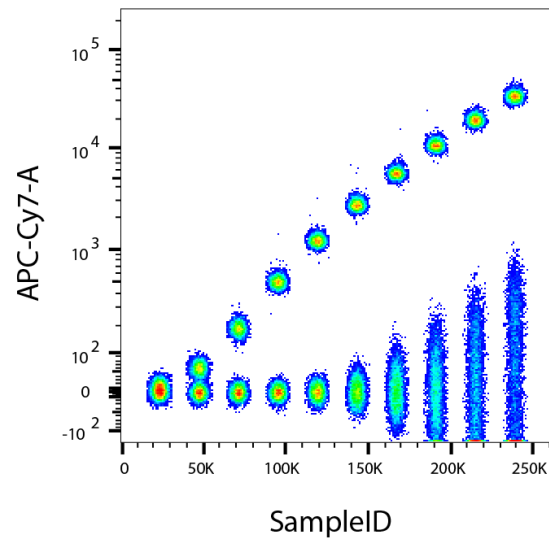
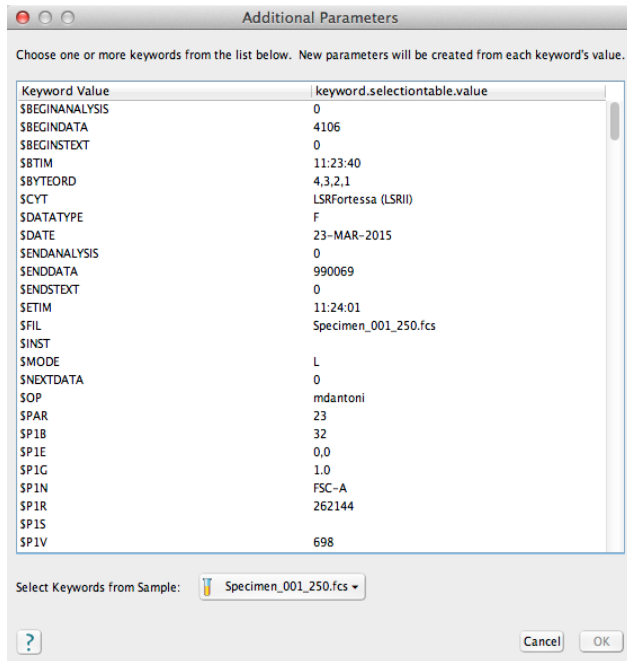
- File Naming:** Prefix: `concat`, Separator: `_`, Suffix: `LD1_Live`.fcs
- Group Concatenation:**  Concatenate files with equal keyword values. Choose Keyword: `*PID`
- Additional Parameters:**  Spread distribution of keyword data

Status: This operation will generate 1 new data file(s).



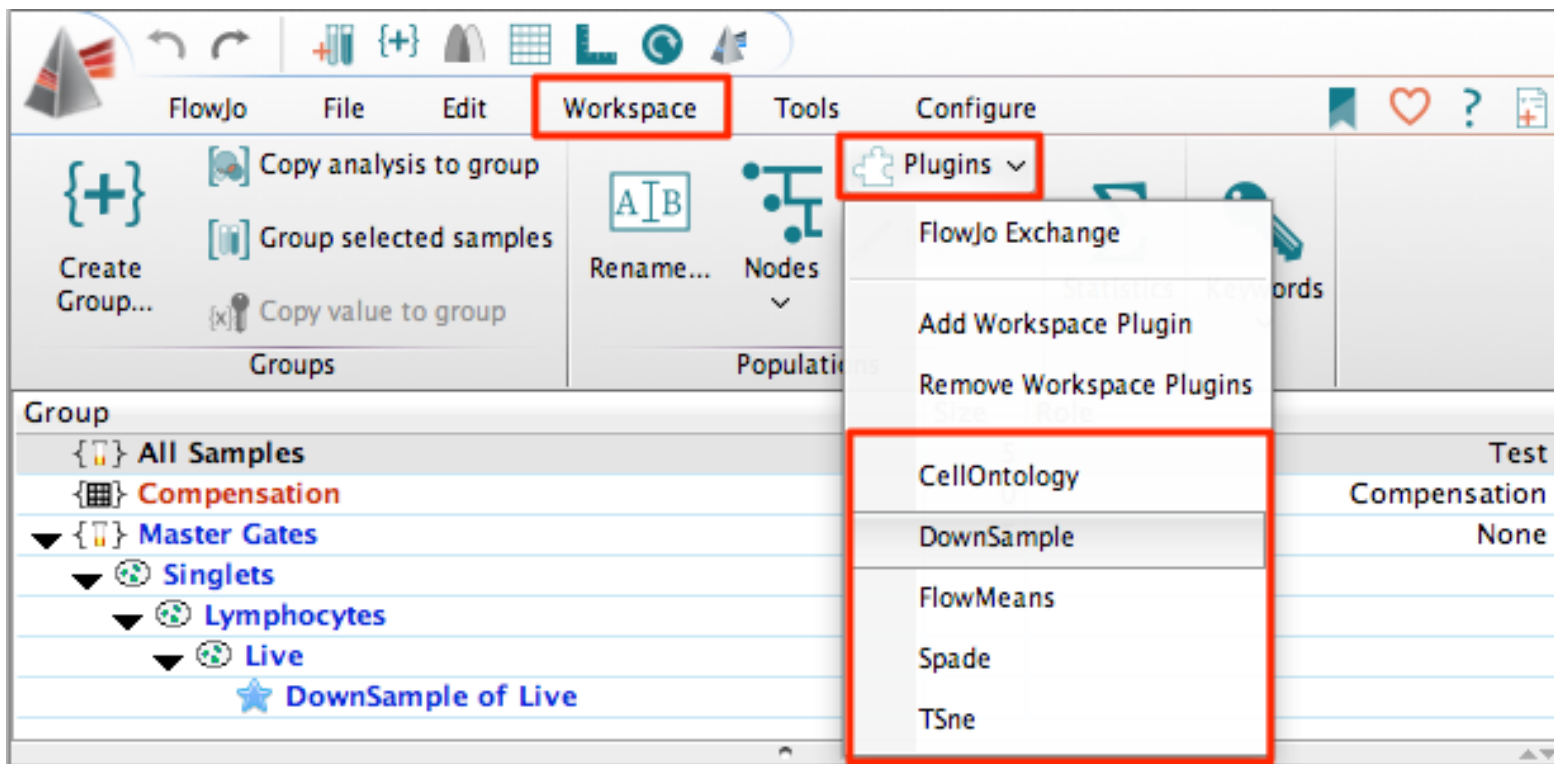
# Additional Parameters

- 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.



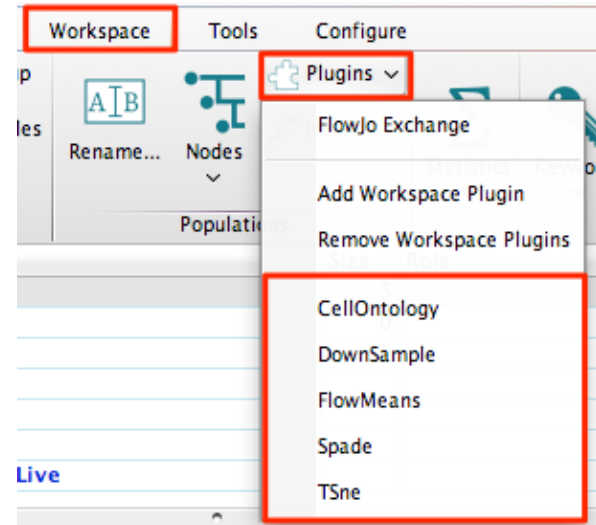
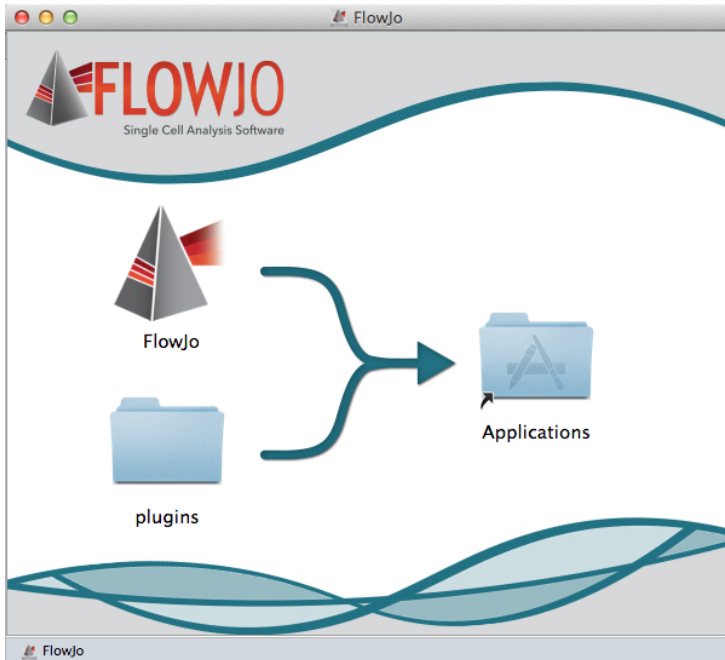
# Plugins

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



# Installing Plugins

- 5 plugins are included with the FlowJo v10.2 release
- Download the installation package for your OS and follow the instructions.
- Open FlowJo and look under the Workspace tab → Populations band → Plugins menu.



# Currently Available Plugins

- ***DownSample*** - Create a child gate containing a limited number of events, selected randomly from the parent population
- ***tSNE*** – Dimensionally reduce high parameter data into 2D
- ***SPADE*\*** - Automated clustering with minimal spanning tree
- ***FlowMeans*\*** - Automated clustering with K-means
- ***CellOntology*\*** - Query the GO Database to identify unknown populations.

\*Requires the R Statistical Computing Environment

Available at: <https://cran.r-project.org/>

# When you run a Plugin

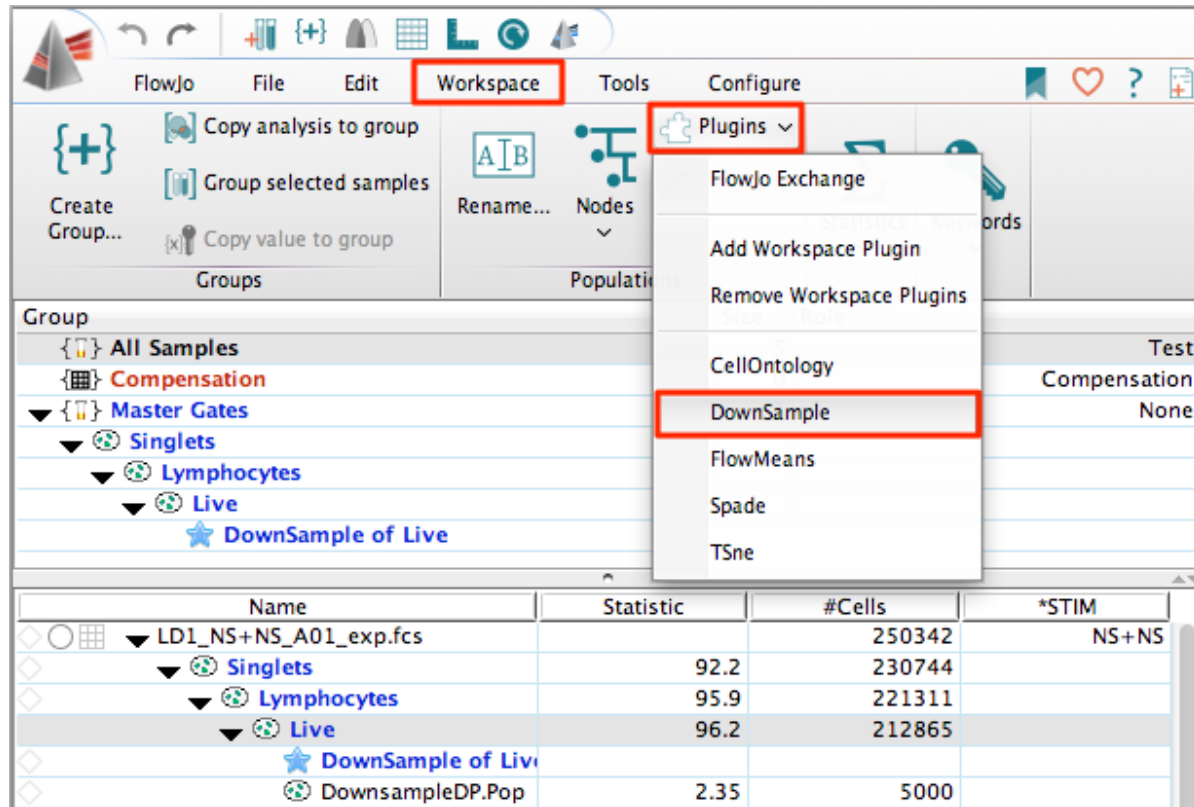
- You must save the FlowJo Workspace first
  - If not, prompted to save
- Many plugins take a gated population from FlowJo, uses it to run some sort of operation or algorithm calculation producing associated derivative files, and returns results to FlowJo.
- The derivatives files are saved in a folder, created the first time a plugin is run in a Workspace.
- The folder is named the same as the Workspace and saved in the same location as the Workspace.
- All subsequent plugins run from that Workspace will be saved to that same derivatives folder.

# DownSample

- Selects a limited number of data points/events from a sample or gated population
  - Events are evenly distributed across parent sample or gated population → random
  - Creates a gate containing selected events
  - Purposes:
    - Reduce number of events for algorithm calculation
    - Normalize cell number to compare distribution of populations across samples

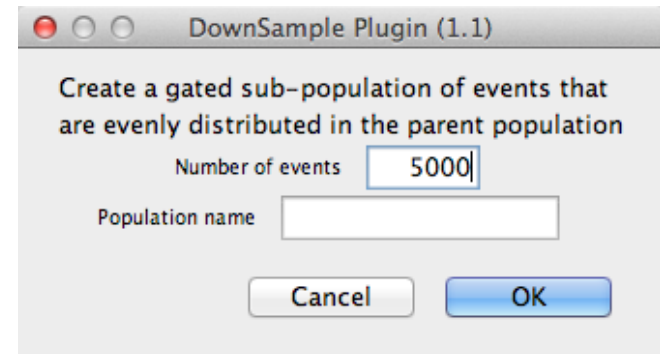
# DownSample

- Initiating DownSample from the Workspace



The screenshot shows the FlowJo software interface. The 'Workspace' menu is highlighted in red. The 'Plugins' dropdown menu is open, and 'DownSample' is highlighted in red. The 'DownSample of Live' population is selected in the workspace. The table below shows the statistics for the populations.

Name	Statistic	#Cells	*STIM
LD1_NS+NS_A01_exp.fcs		250342	NS+NS
Singlets	92.2	230744	
Lymphocytes	95.9	221311	
Live	96.2	212865	
DownSample of Live			
DownsampleDP.Pop	2.35	5000	



The dialog box titled 'DownSample Plugin (1.1)' contains the following text and controls:

Create a gated sub-population of events that are evenly distributed in the parent population

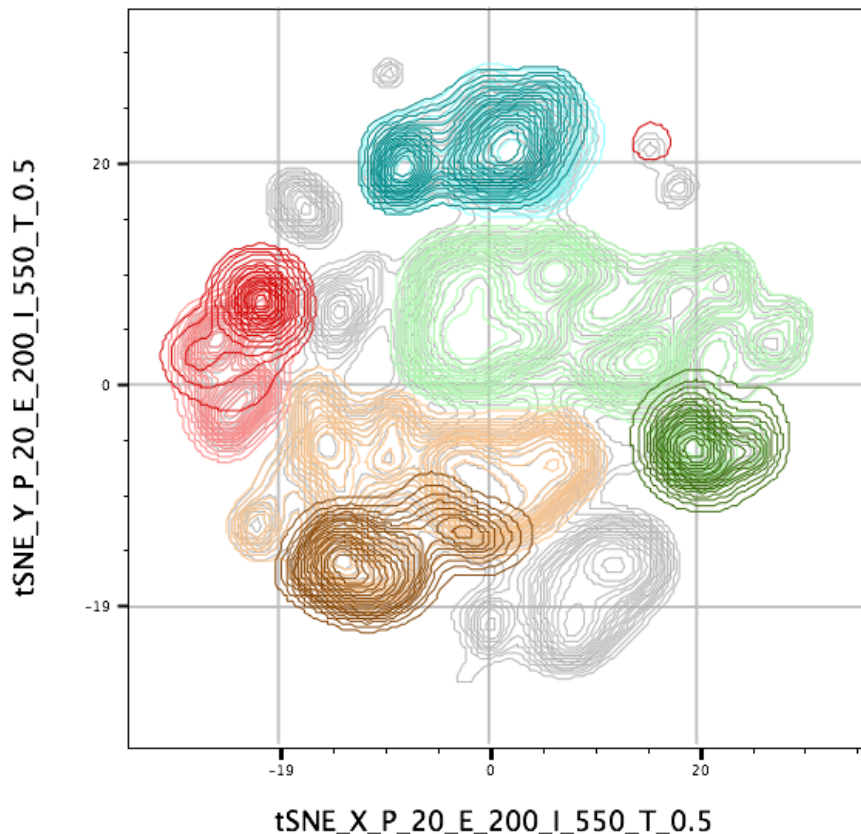
Number of events

Population name

Cancel

# tSNE

- T-Distributed Stochastic Neighbor Embedding (tSNE)
  - An algorithm for performing dimensionality reduction
  - Allows visualization of complex multi-dimensional data in fewer dimensions while still maintaining the structure of the data



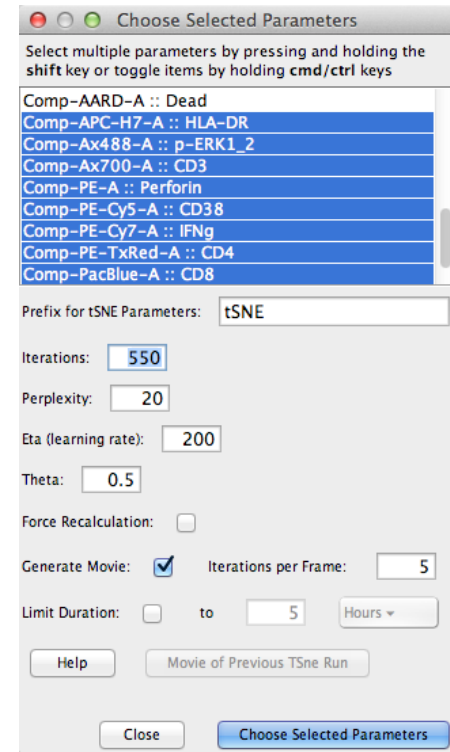
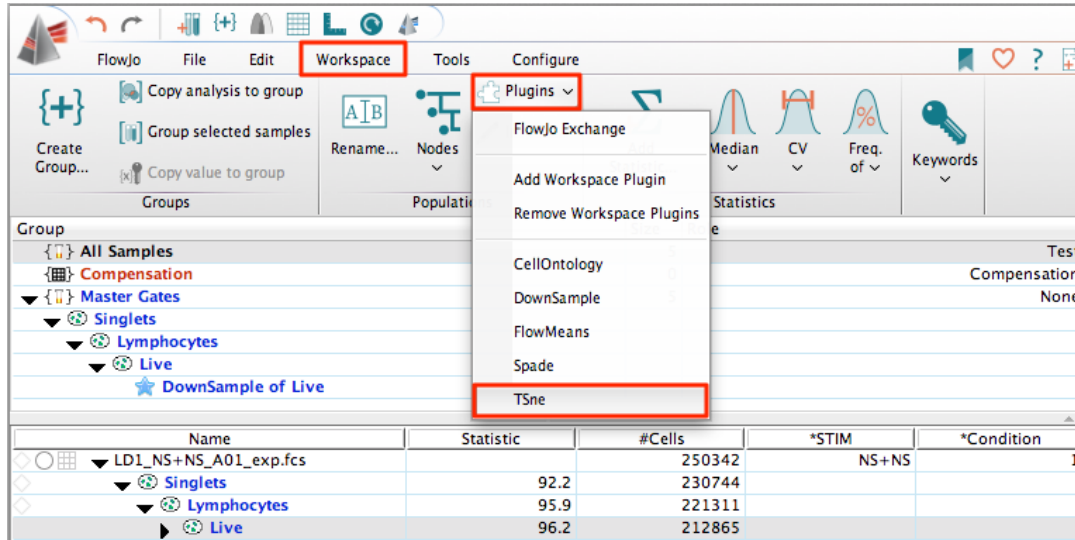
	Full Gating Path
■	Lymphocytes/DownsampleDP.Pop/Q3: CD3+ , CD19-/Q3: CD4+ , CD8-/CD127-CD25+
■	Lymphocytes/DownsampleDP.Pop/Q3: CD3+ , CD19-/Q3: CD4+ , CD8-
■	Lymphocytes/DownsampleDP.Pop/Q3: CD3+ , CD19-/Q1: CD4- , CD8+/CD127-CD27+
■	Lymphocytes/DownsampleDP.Pop/Q3: CD3+ , CD19-/Q1: CD4- , CD8+
■	Lymphocytes/DownsampleDP.Pop/Q1: CD3- , CD19+/IgD+
■	Lymphocytes/DownsampleDP.Pop/Q1: CD3- , CD19+
■	Lymphocytes/DownsampleDP.Pop/Q4: CD3- , CD19-/CD56+/CD197+
■	Lymphocytes/DownsampleDP.Pop/Q4: CD3- , CD19-/CD56+
■	Lymphocytes/DownsampleDP.Pop

Maaten and Hinton (2008). "Visualizing data using t-SNE." Journal of Machine Learning Research, 9: 2579–2605.



# tSNE

- Initiating tSNE from the Workspace



- Iterations** – Maximum number of iterations the algorithm will run.
- Perplexity** – Perplexity is related to the number of nearest neighbors that is used in learning algorithms. In tSNE, the perplexity may be viewed as a knob that sets the number of effective nearest neighbors. The most appropriate value depends on the density of your data. Generally a larger / denser dataset requires a larger perplexity.

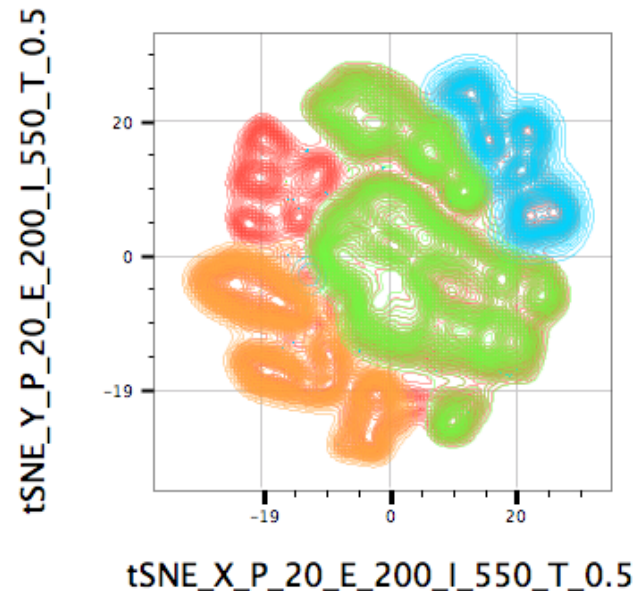
# tSNE

- Creates two new derived parameters from user selection, optimized in such a way that observations/data points which were close to one another in the raw high dimensional data are close in the reduced data space.

The screenshot shows the FlowJo software interface. The 'Settings' panel is visible, with the 'Hide Derived Parameters' button highlighted. Below the settings, a table lists various parameters and their statistics. The following table is extracted from the screenshot:

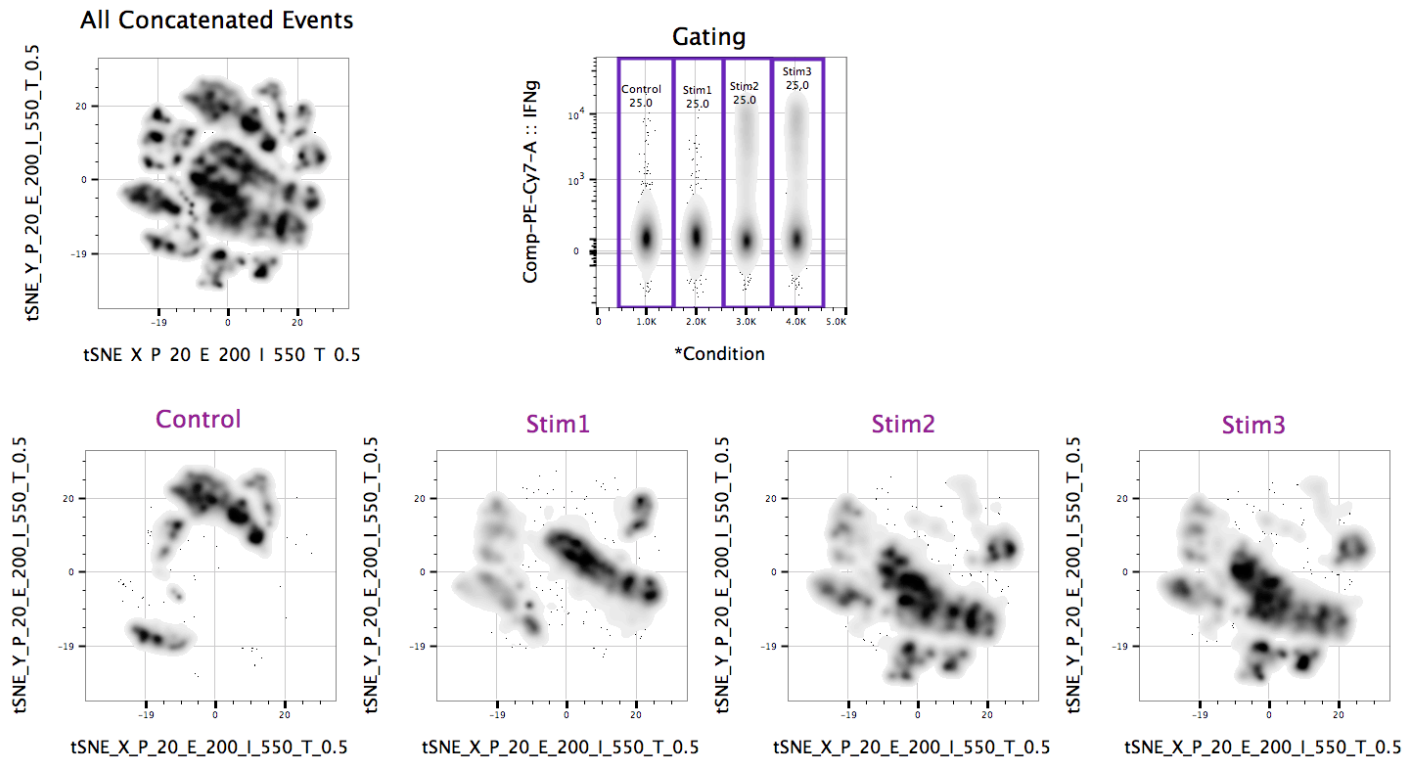
Name	Statistic	#Cells	*STIM	*Condition
LD1_NS+NS_A01_exp.fcs		250342	NS+NS	1
LD1_NS+PI_C01_exp.fcs		229585	NS+PI	2
LD1_PI+NS_B01_exp.fcs		262774	PI+NS	3
LD1_PI+PI_D01_exp.fcs		244977	PI+PI	4
concat_1_LD1.fcs		20000		*Condition
Spade.2151145				
% Singlets	100	20000		
% Lymphocytes	100	20000		
% Live	100	20000		
% tSNE_X_P_20_E_200_I_550_T_0.5				
% tSNE_Y_P_20_E_200_I_550_T_0.5				

	Sample Name	Subset Name	Count
■	concat_1_LD1.fcs	Q3: CD4+ , CD8-	12057
■	concat_1_LD1.fcs	Q1: CD4- , CD8+	3504
■	concat_1_LD1.fcs	CD3-HLA-DR-	2033
■	concat_1_LD1.fcs	Live	20000



# tSNE

- Practical Considerations
  - Cleaning up the data
  - DownSample
  - Parameter Selection
  - Workflow

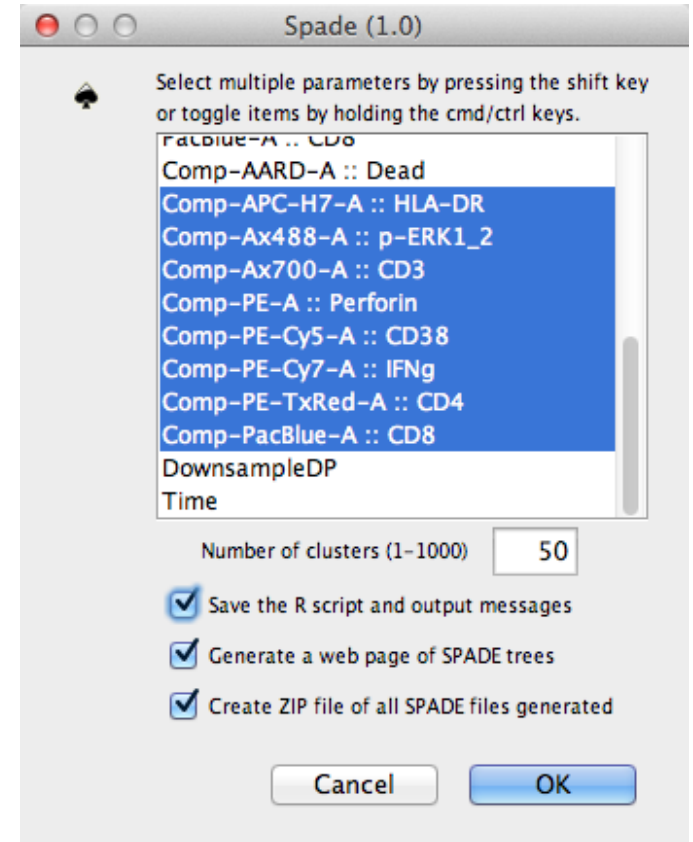
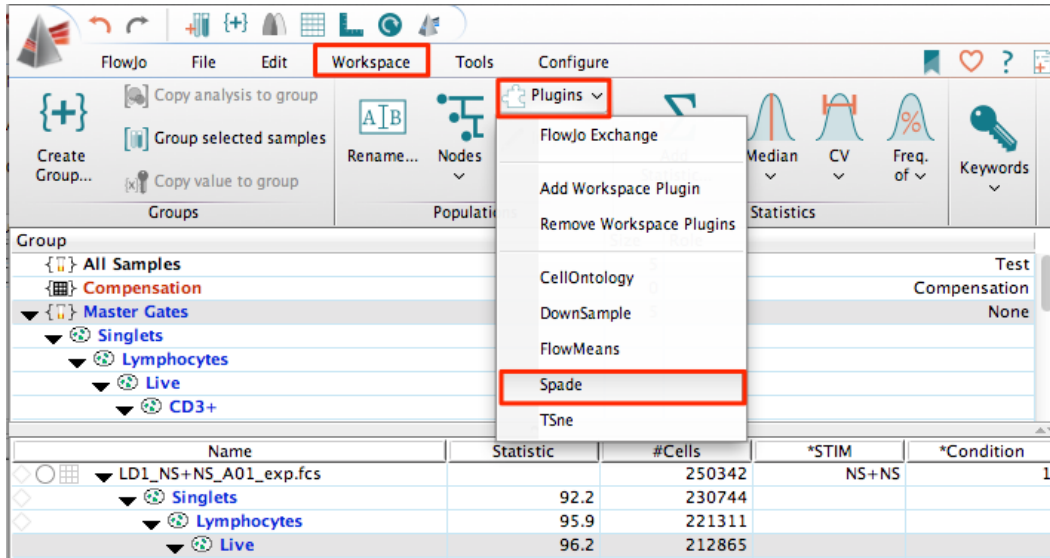


# SPADE

- Spanning tree Progression of Density normalized Events (SPADE) is an algorithmic visualization tool for high dimensional flow and mass cytometry data.
- Requires R and the R package SPADE
  - `install.packages("devtools")`
  - `library(devtools)`
  - `devtools::install_github("nolanlab/Rclusterpp")`
  - `source("http://bioconductor.org/biocLite.R")`
  - `devtools::install_github("nolanlab/spade")`
- Produces
  - Gated cluster populations as children of the parent reference population in the FlowJo workspace
  - Zip file containing a network GML file, PDFs of the graphs, tables, and FCS files with the "cluster" column appended.

# SPADE

- Initiation from the Workspace



- Enter the desired Number of Clusters and click OK

# SPADE

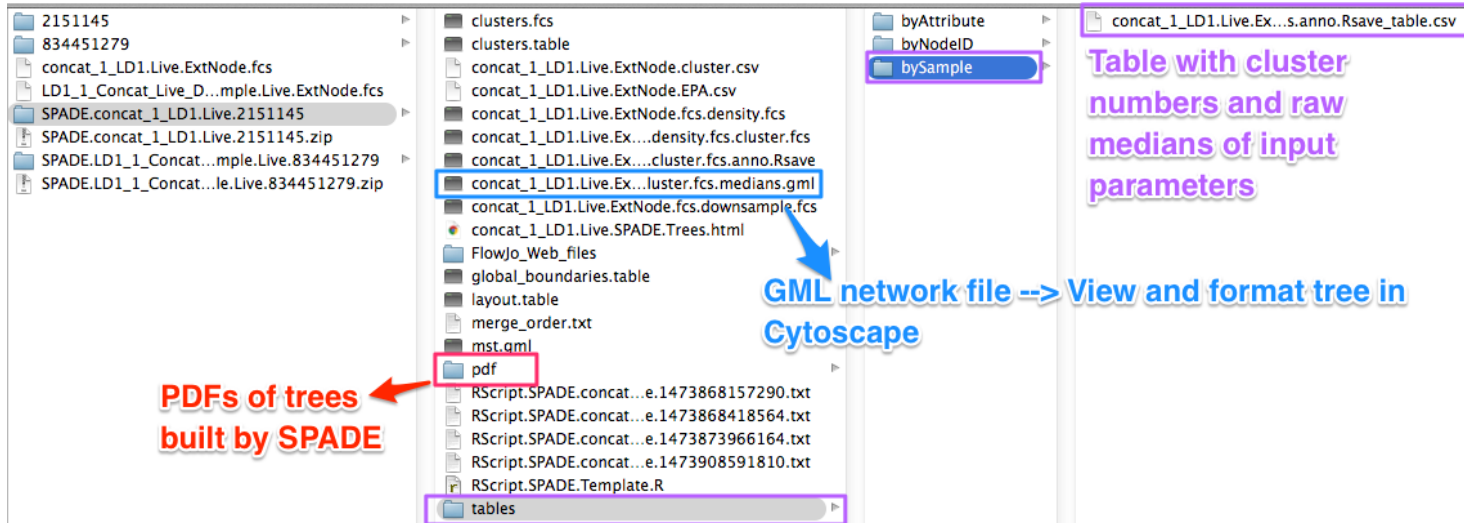
- Populations in the workspace represent events within clusters of the spade tree
- Double Click on the Spade of 'Population' node, then select Get Zip File

The screenshot displays the SPADE software interface. On the left, a tree view shows a hierarchy of nodes. The 'Spade of Live' node is selected, and a red arrow points to it with the text 'Double Click'. The tree view includes columns for 'Name', 'Statistic', and '#Cells'. The right side of the image shows a dialog box titled 'Spade (1.0)'. The dialog box contains a list of parameters to be selected, a 'Number of clusters (1-1000)' field set to 50, and three checked options: 'Save the R script and output messages', 'Generate a web page of SPADE trees', and 'Create ZIP file of all SPADE files generated'. The 'Get Zip File' button is highlighted with a red box.

Name	Statistic	#Cells
concat_1_LD1.fcs		20000
Spade.2151145		
Singlets	100	20000
Lymphocytes	100	20000
Live	100	20000
Spade of Live		
TSne of Live		
CD3+		16050
CD3-HLA-DR+	9.22	1844
CD3-HLA-DR-	10.2	2033
Control	25.0	5000
CD3-HLA-DR-	11.1	557
Unknown Pop1	0.50	25
DownsampleDP	100	20000
Spade.Live.Pop1	1.10	221
Spade.Live.Pop2	0.27	55
Spade.Live.Pop3	0.92	183
Spade.Live.Pop4	0.46	92
Spade.Live.Pop5	0.84	169
Spade.Live.Pop6	0.26	52
Spade.Live.Pop7	1.10	220
Spade.Live.Pop8	1.32	264
Spade.Live.Pop9	0.43	87
Spade.Live.Pop10	0.76	153
Spade.Live.Pop11	0.95	189
Spade.Live.Pop12	0.48	95
Spade.Live.Pop13	0.30	60
Spade.Live.Pop14	0.54	109
Spade.Live.Pop15	3.13	626

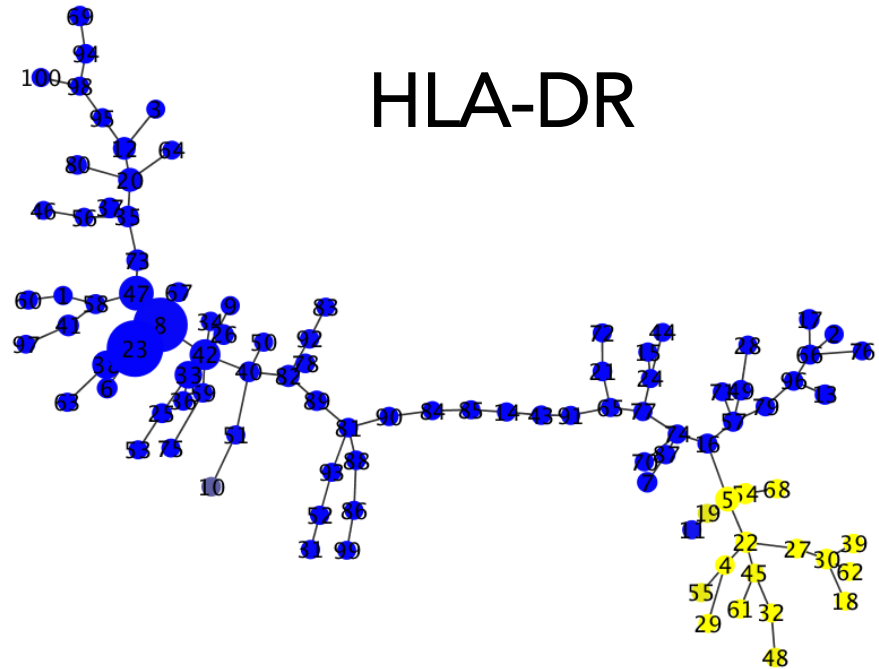
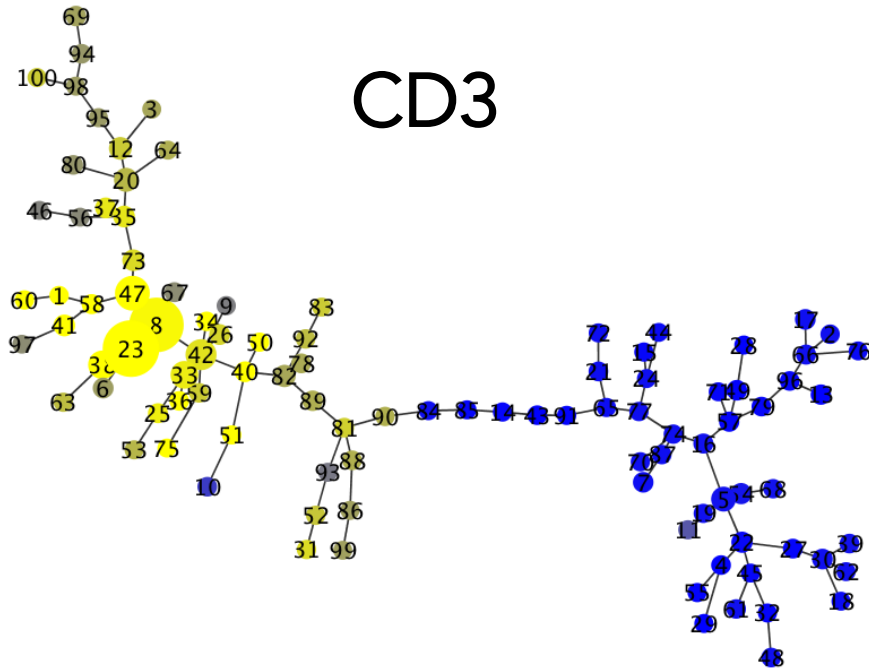
# Within the SPADE .zip

- GML file – Tree network can be visualized using Cytoscape.
- pdf folder - contains PDFs of all the trees built by SPADE
- Tables → By Sample → .csv – spreadsheet with raw medians for every parameter used in the SPADE calculation



# SPADE

- GML file can be visualized in Cytoscape.

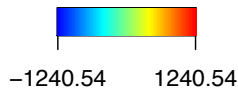
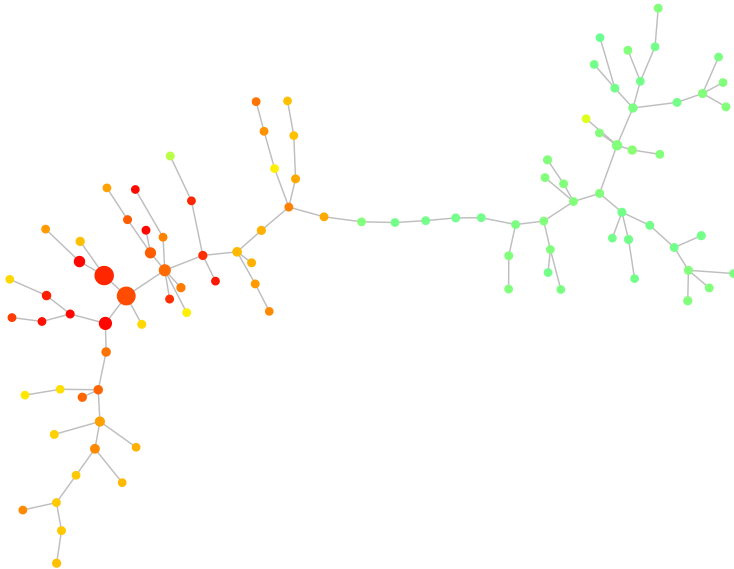




# SPADE

- PDFs aren't as useful, but the By Sample CSV table may be helpful for calling expression.

LD1\_1\_Concat\_Live\_Downsample.Live.ExtNode  
raw\_mediansFJComp.Ax700.A  
(Used for tree-building)

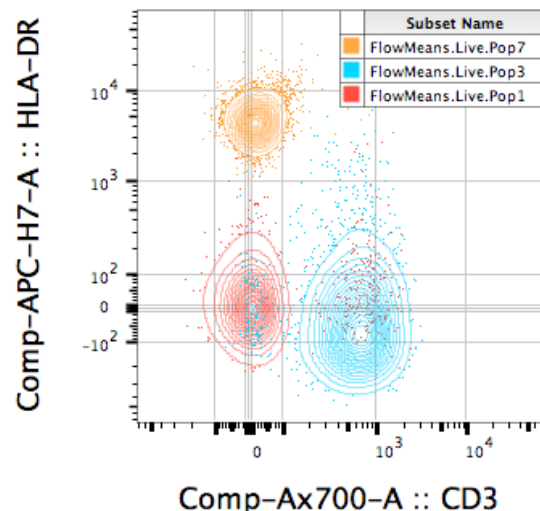
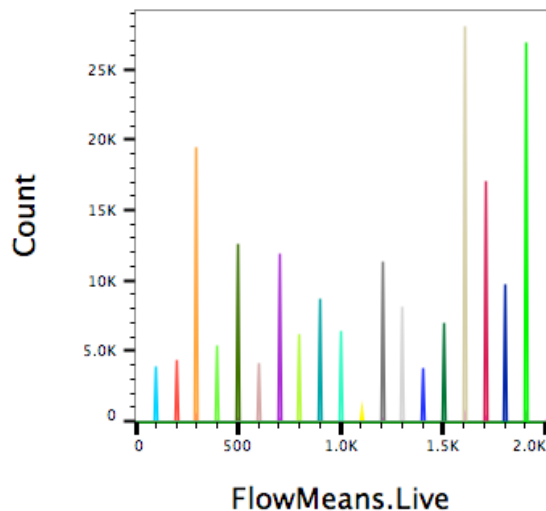


Range: 0.02 to 0.98 pctile

ID	count	CD150	FcgR	IL7R
		raw_mediansFJComp.PE.A_clust	raw_mediansFJComp.PerCP.Cy5.5.A_clust	raw_mediansFJComp.PE.Cy7.A_clust
1	117	-91.29728699		-187.1949005
2	75	-114.8877792		1425.162476
3	607	131.198349		942.6101685
4	144	45.06650162		231.4508133
5	176	540.3177338		638.2731018
6	2213	-133.9013367		3068.497314
7	9016	413.6738434		1986.239563
8	126	671.3719482		1045.242371
9	1157	559.7160034		1440.166504
10	111	2026.342407		847.7639771
11	120	2658.671143		-195.1321869
12	405	1883.511353		752.9630127
13	94	391.3848114		5319.604004
14	118	482.8102112		531.129425
15	253	-140.9054565		2870.712402
16	54	-137.2172623		450.6687164
17	66	322.586792		1074.81543
18	632	343.6929016		3988.859375
19	358	1934.900391		-187.7943344
20	84	87.44278717		820.6205444

# FlowMeans

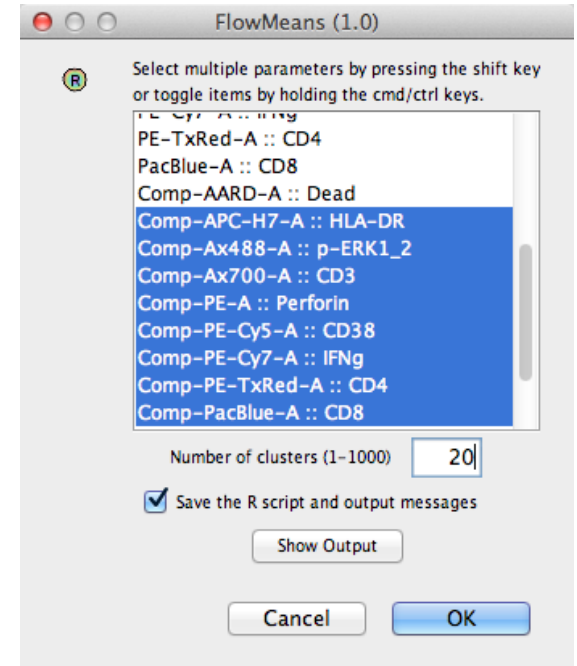
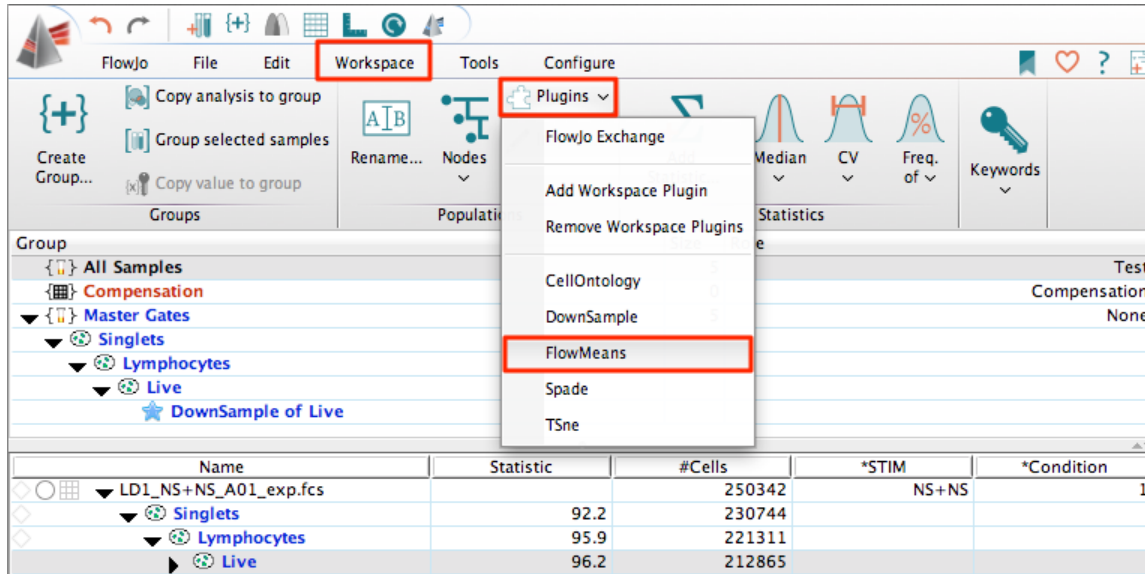
- FlowMeans is a method for automated identification of cell populations based on K-means clustering.
- Requires R and the R package flowMeans
  - `source("https://bioconductor.org/biocLite.R")`
  - `biocLite("flowMeans")`
- Produces gated cluster populations as children of the parent reference population.



Aghaeepour, N. et. al. (2011) Rapid cell population identification in flow cytometry data. *Cytometry A*, DOI: 10.1002/cyto.a.21007

# FlowMeans

- Initiating flowMeans from the Workspace



- Enter the desired Number of Clusters and click OK

# Additional Plugin Resources

## The FlowJo Exchange

<http://exchange.flowjo.com/>

- Future plugin releases
- Featured plugins
- Updates
- Developer documentation
- Scripts

## Documentation

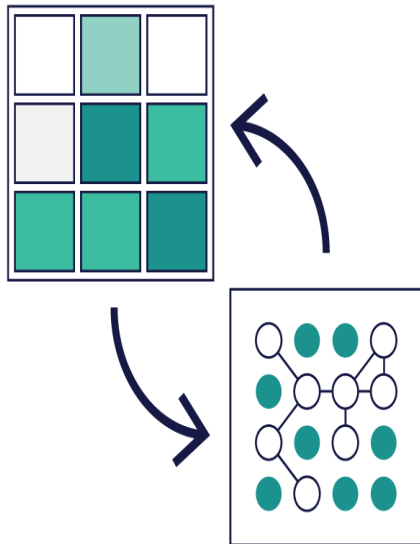
<http://docs.flowjo.com>

- Search for Plugins → pages describing plugin setup and functionality

# Additional Training Resources

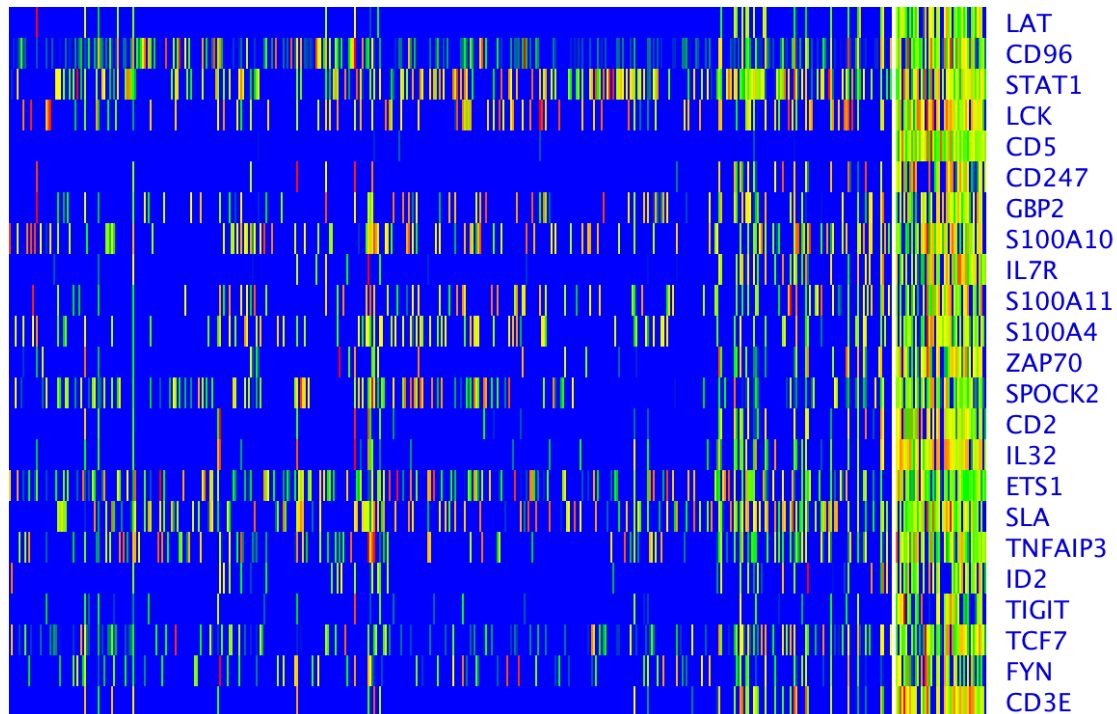
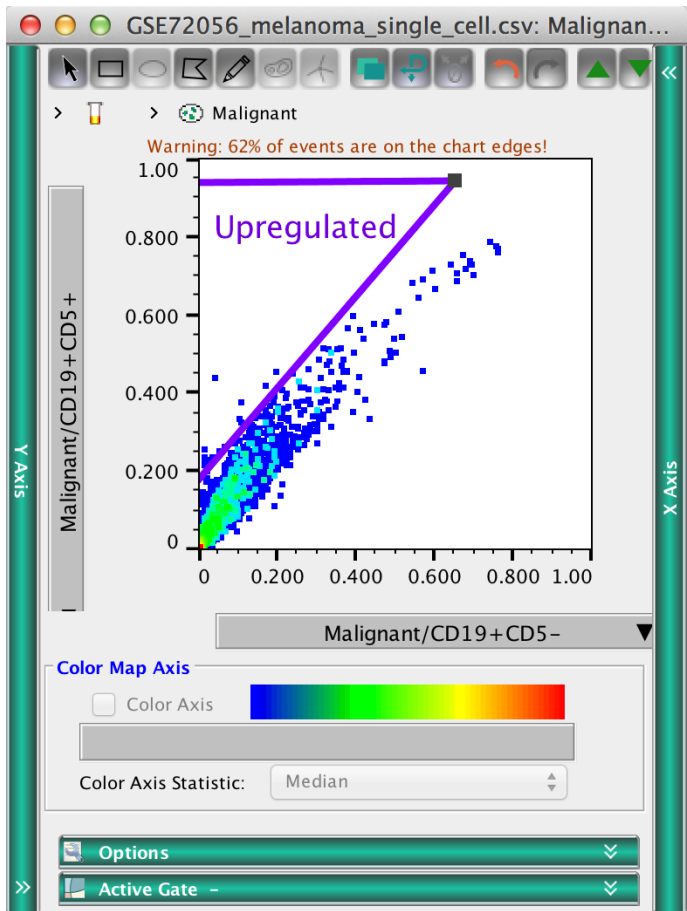
- Webinars on basic and advanced features of FlowJo, held on the 1<sup>st</sup> and 3<sup>rd</sup> Thursday of each month.
- Webinar Schedule can be found at <http://www.flowjo.com/webinars/>
- Technical Documentation for V10 can be found at <http://docs.flowjo.com/>
- The Daily Dongle provides tips,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

- Single cell gene expression analysis software.
  - Gate on individual genes, gene sets or synthetic parameters to define populations.
  - Pivot the graph to compare populations, define differentially expressed genes and identify new novel populations.
  - Visualize differences.



CD19+CD5-

CD19+CD5+



[www.flowjo.com/solutions/seqgeo](http://www.flowjo.com/solutions/seqgeo)



## 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.

# Thank You!