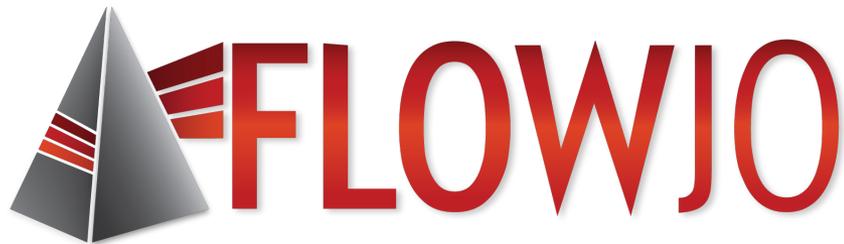


# Cytometry Data Analysis in FlowJo V10



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# The FlowJo v10 Workspace

- A graphical interface to organize your data.

PFICS Analysis.wsp

FlowJo File Edit Workspace Tools Configure

New Workspace Table Editor Add Samples... Layout Editor Create Group... Preferences...

Annotate Experiment... Add Keyword Plate Editor

Cell Cycle... Kinetics... Compare Populations...

Workspace Links About Contact FlowJo Instrumentation

| Group                                    | Size | Role         |
|--|------|--------------|
| All Samples                              | 46   | Test         |
| All Stain                                | 20   | Test         |
| Compensation                             | 12   | Compensation |
| FMOs                                     | 14   | Controls     |
| Master Gates                             | 34   | None         |
| Time                                     |      |              |
| Singlets                                 |      |              |
| Lymphocytes                              |      |              |
| Live                                     |      |              |
| CD3+                                     |      |              |
| Q1: CD4-, CD8+                           |      |              |
| Geometric Mean : Comp-Ax488-A (p-ERK1_2) |      |              |
| IFNg+                                    |      |              |
| Freq. of Parent                          |      |              |
| Perf+                                    |      |              |
| pERK+                                    |      |              |

Ribbon  
Tabs and Bands

Groups and Group  
Analysis

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

Samples and  
sample analysis

# Ribbons, Tabs and Bands

- Ribbon organization allows easy visual navigation of workspace functions.

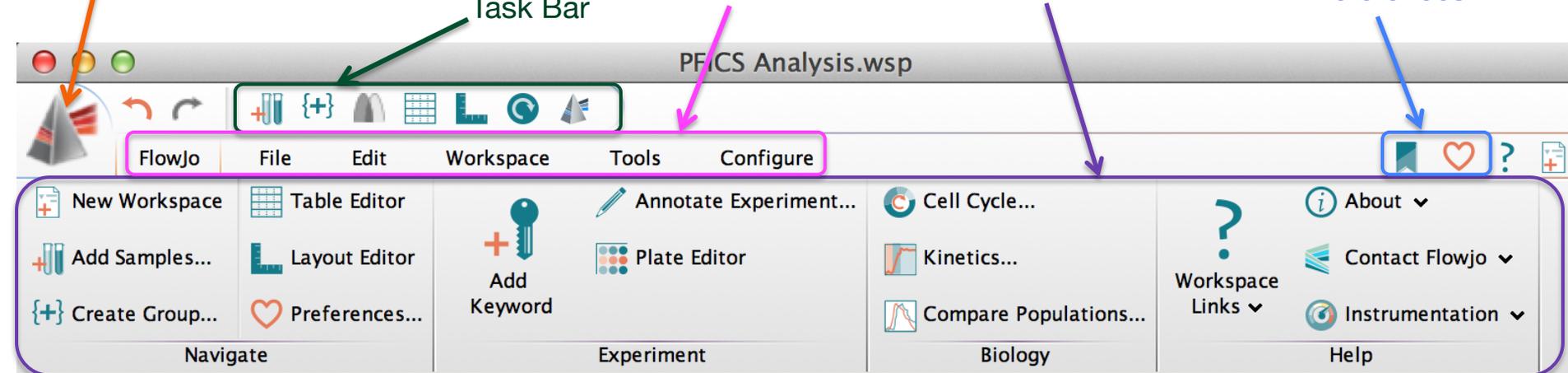
Application Button

Task Bar

Tabs

Bands

Ribbon Configuration & Preferences

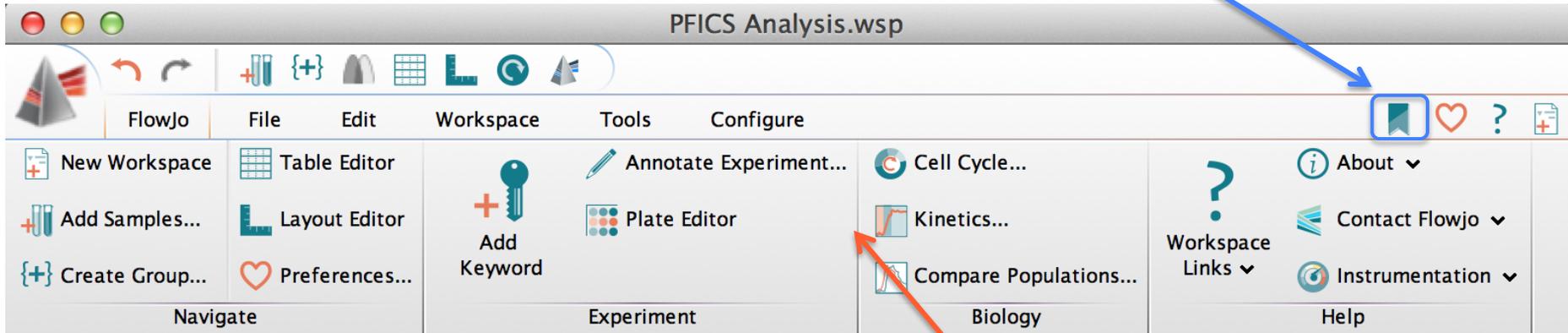


- Tabs group similar Bands together.
- Bands group similar Actions together.

# Customizing Ribbons

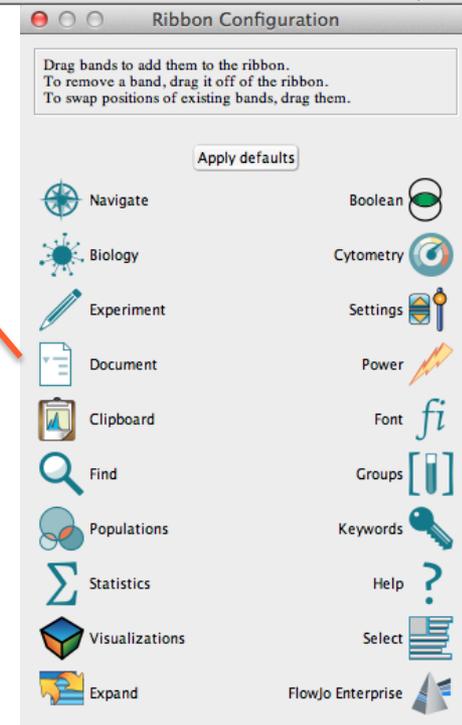
- Click on the Ribbon icon to configure

1.



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

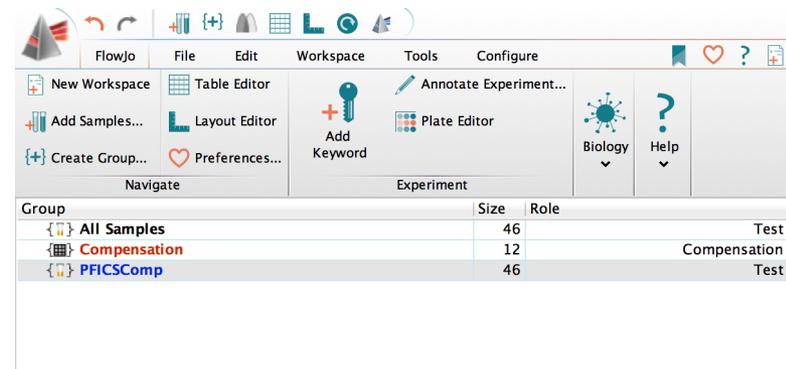
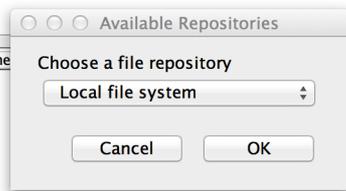
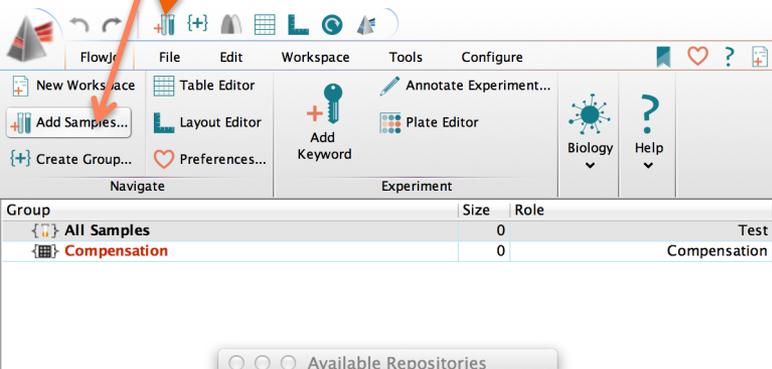
2.



# Importing Data

Three possible methods:

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



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

Drag Samples Here

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

## Polyclonal PFICS Assay:

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



# PFICS Stim Conditions

- 2 Stims → 4 potential combinations

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

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

# Group Pane

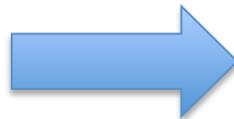
- The Group area lists all groups in the Workspace, # of samples in each group (Size), and the Role of that group (ex. Test, Compensation, Controls) .
- Groups act like folders to organize your samples, allows master gating and unique report generation.

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

Modify Group

**Appearance**

Name:  Color:  Style:

Role:  Parameter Key:

**Sample Inclusion Criteria**

Live group  Synchronized

Include samples that use the following staining:

Show all keywords in menus

With reference to samples in another group:

Only choose from

Also include all

**Assignments**

Add Keyword :  With Value :

Add Keyword :  With Value :

# Sample Inclusion Criteria

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

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

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

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

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)

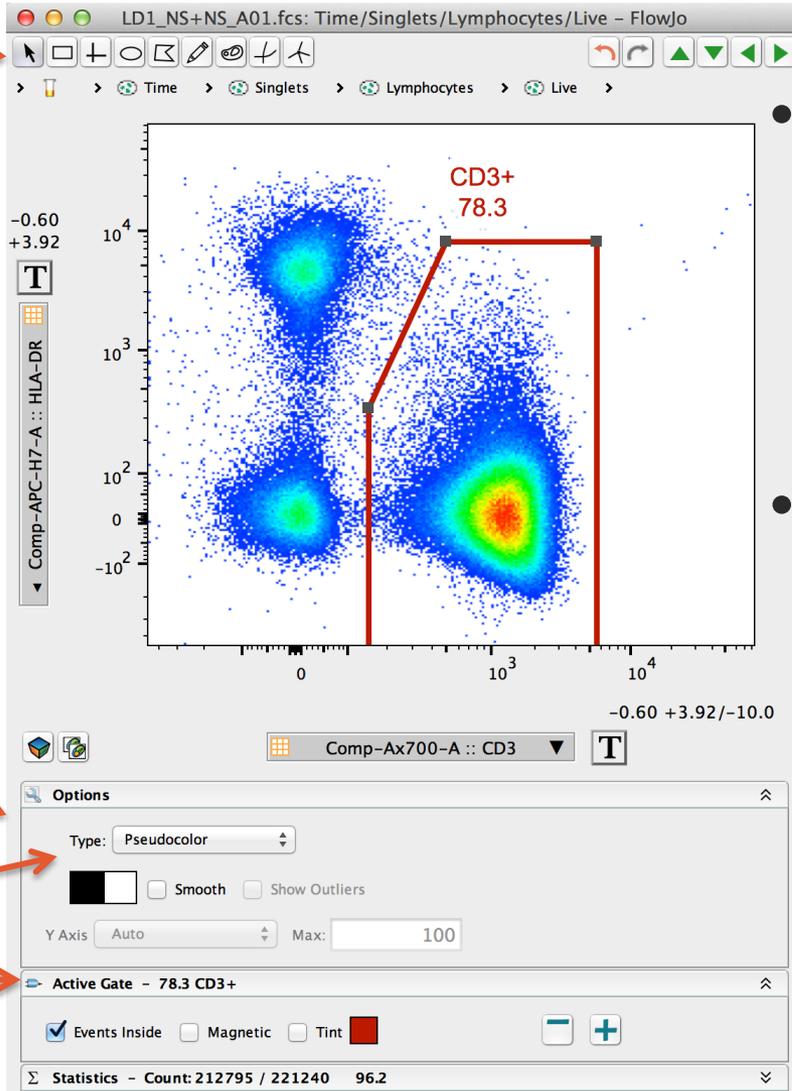
| Name              | Statistic | #Cells | *HIV Status | *PID | *STIM |
|-------------------|-----------|--------|-------------|------|-------|
| LD1_NS+NS_A01.fcs |           | 250342 | Neg         | LD1  | NS+NS |
| LD1_NS+PI_C01.fcs |           | 229585 | Neg         | LD1  | NS+PI |
| LD1_PI+NS_B01.fcs |           | 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

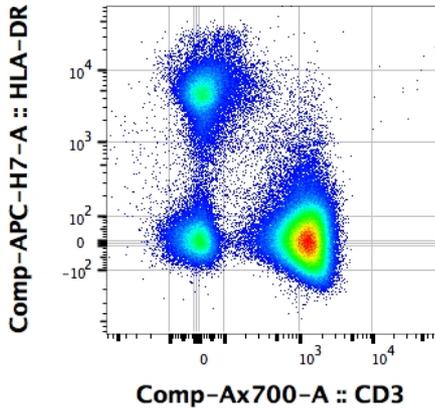
Active Gate Options

- 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.

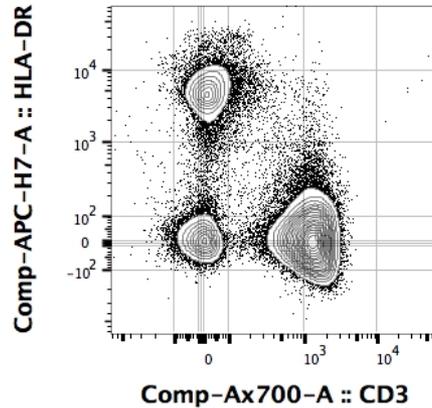
# Graph Display Options

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

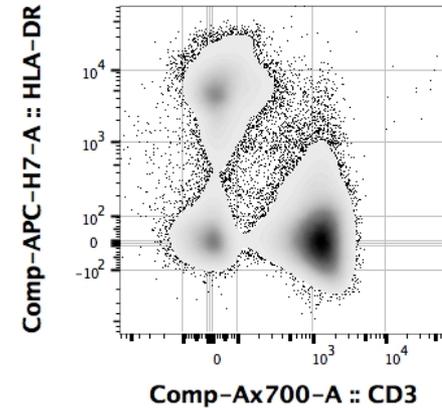
Pseudocolor



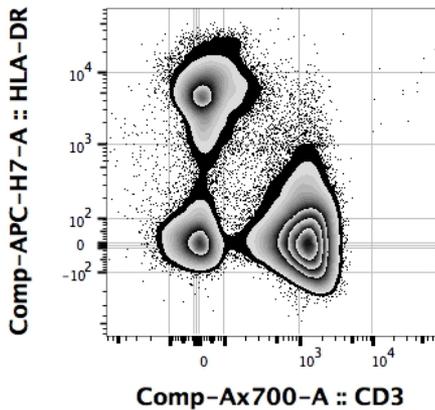
Contour



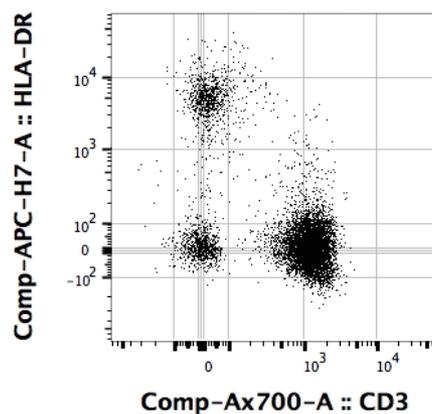
Density



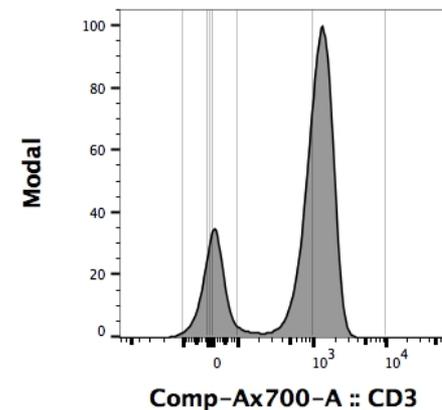
Zebra



Dot Plot



Histogram

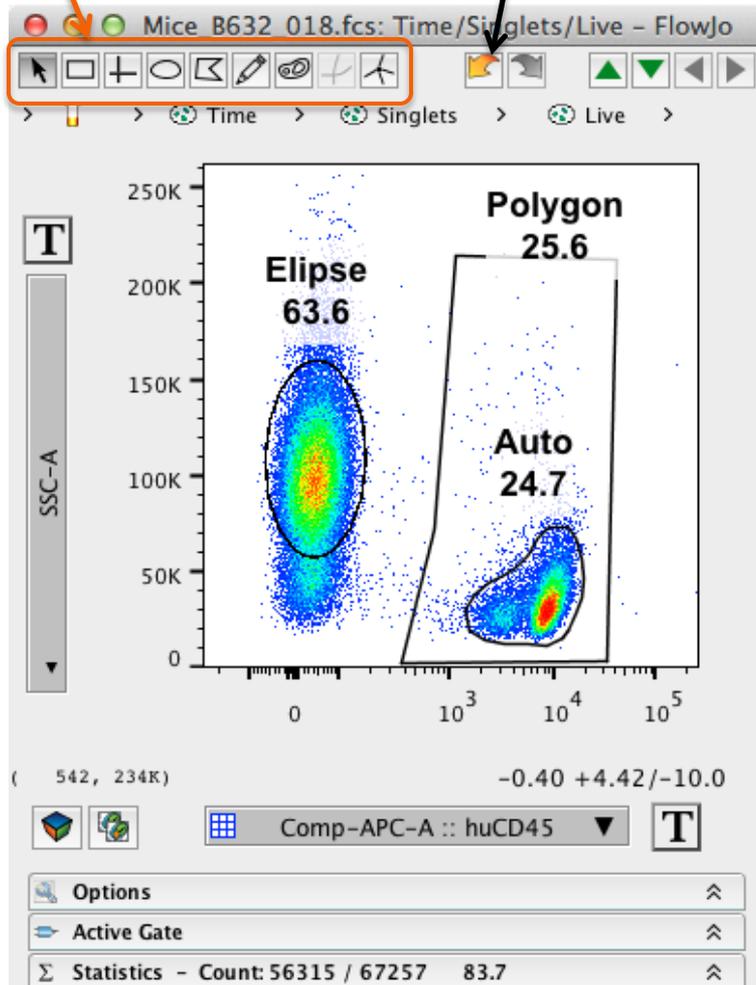


# Gating tools

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

Gating Tools

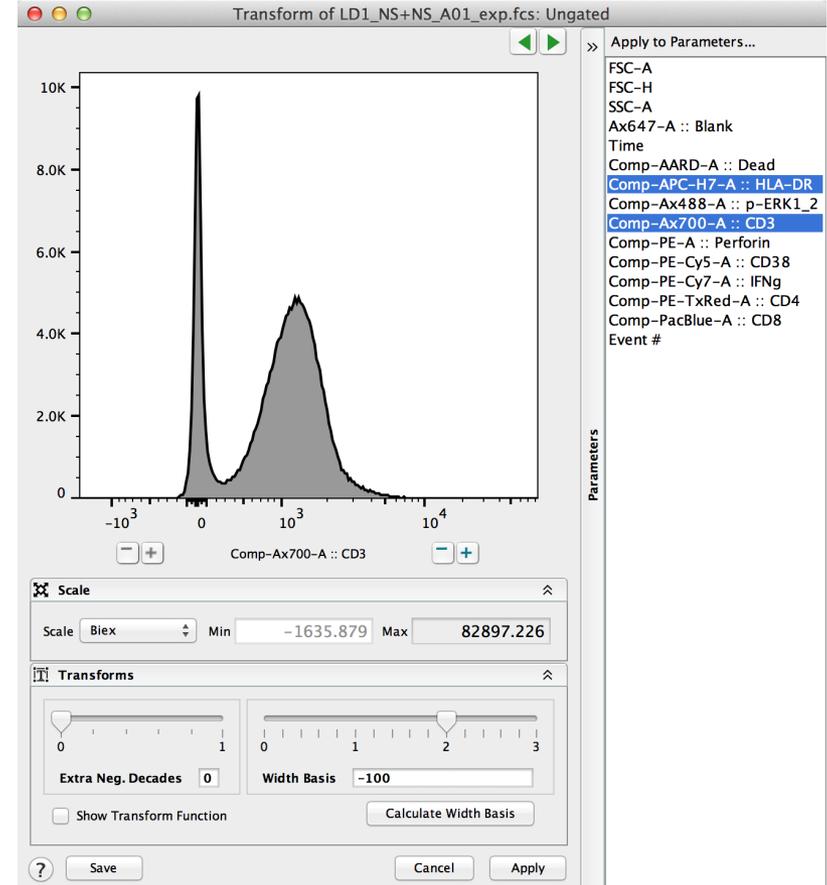
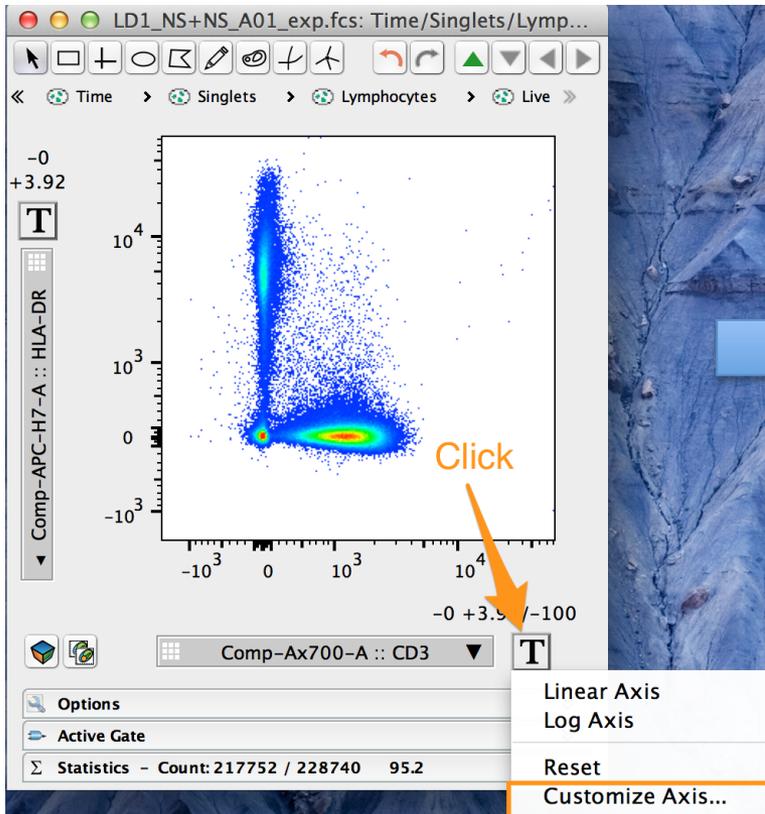
Undo!!



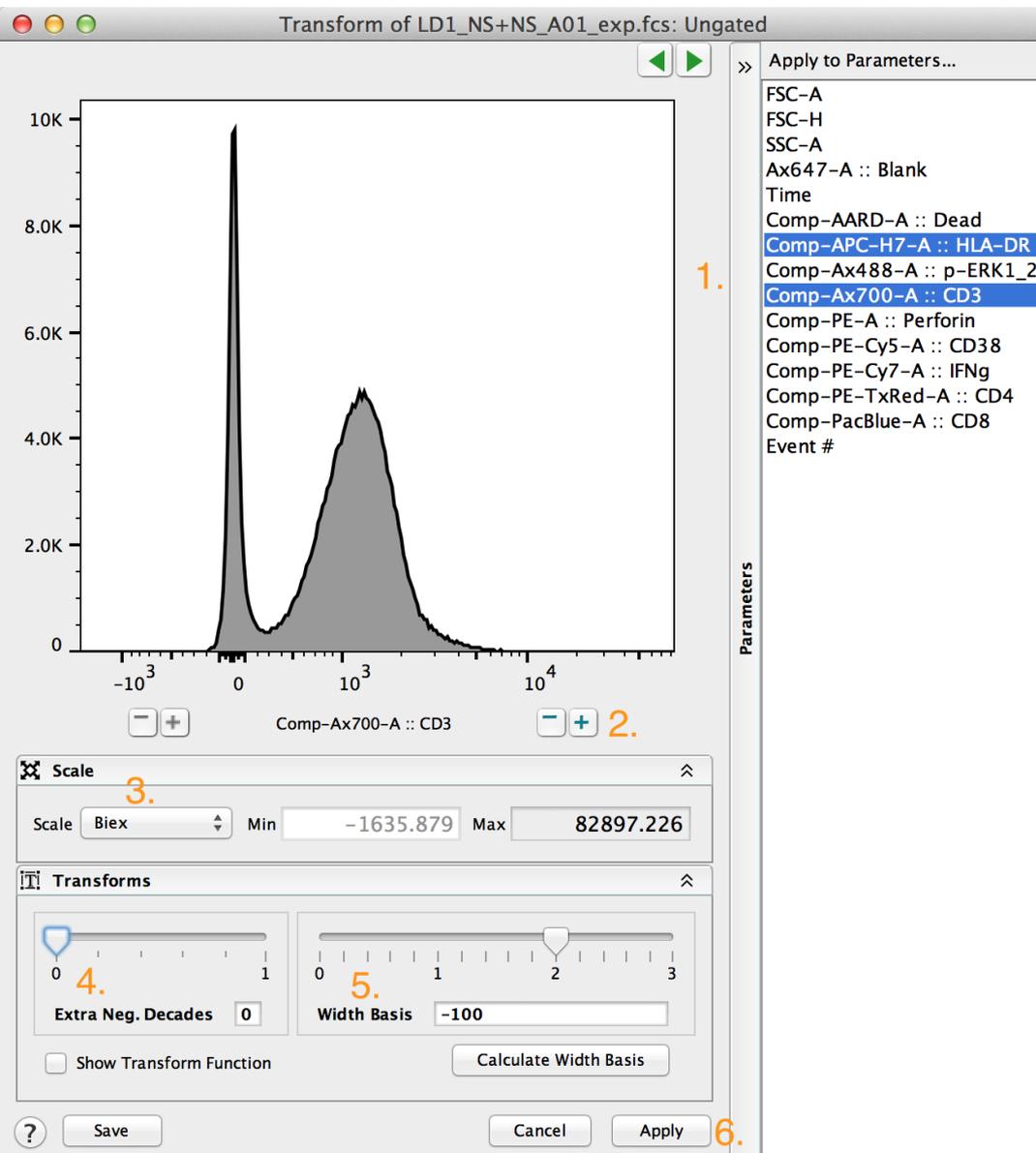
- Gates can always be modified or removed, so don't be shy.
- Explore the gating options and pick what works best for you.

# Transforming Data

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

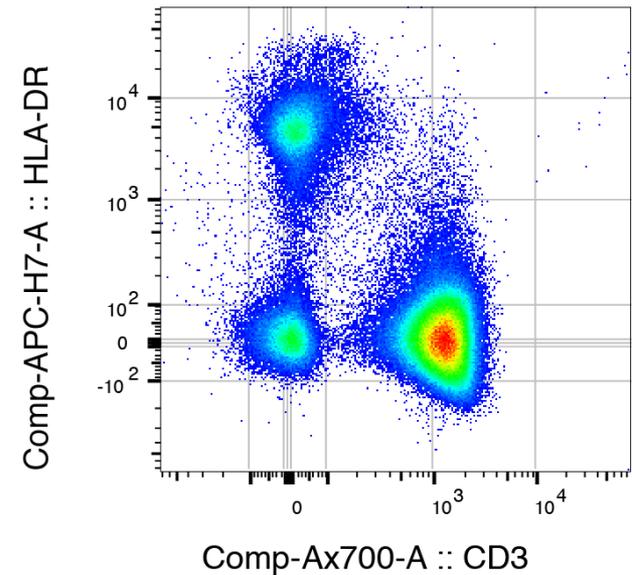
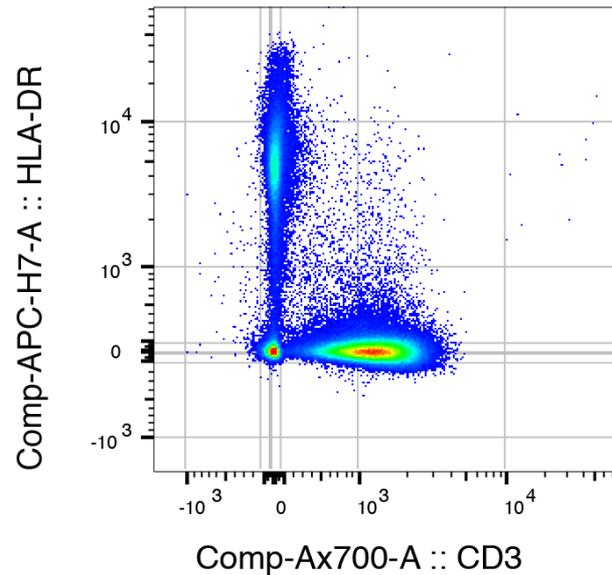
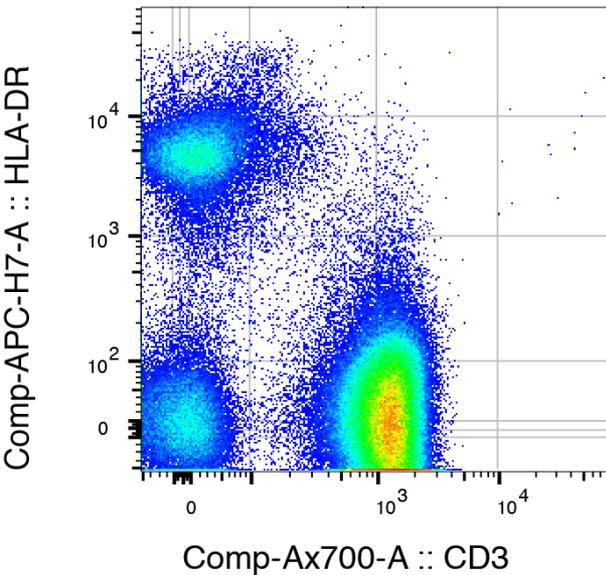


# Transform Options



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

# Effects of Transformation



## Effects:

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

# Boolean Combination Gates

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

**2. Select Create Combination Gates**

**3. Abbreviate names and click**

**1. Highlight single marker gates**

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

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

# The Layout Editor

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

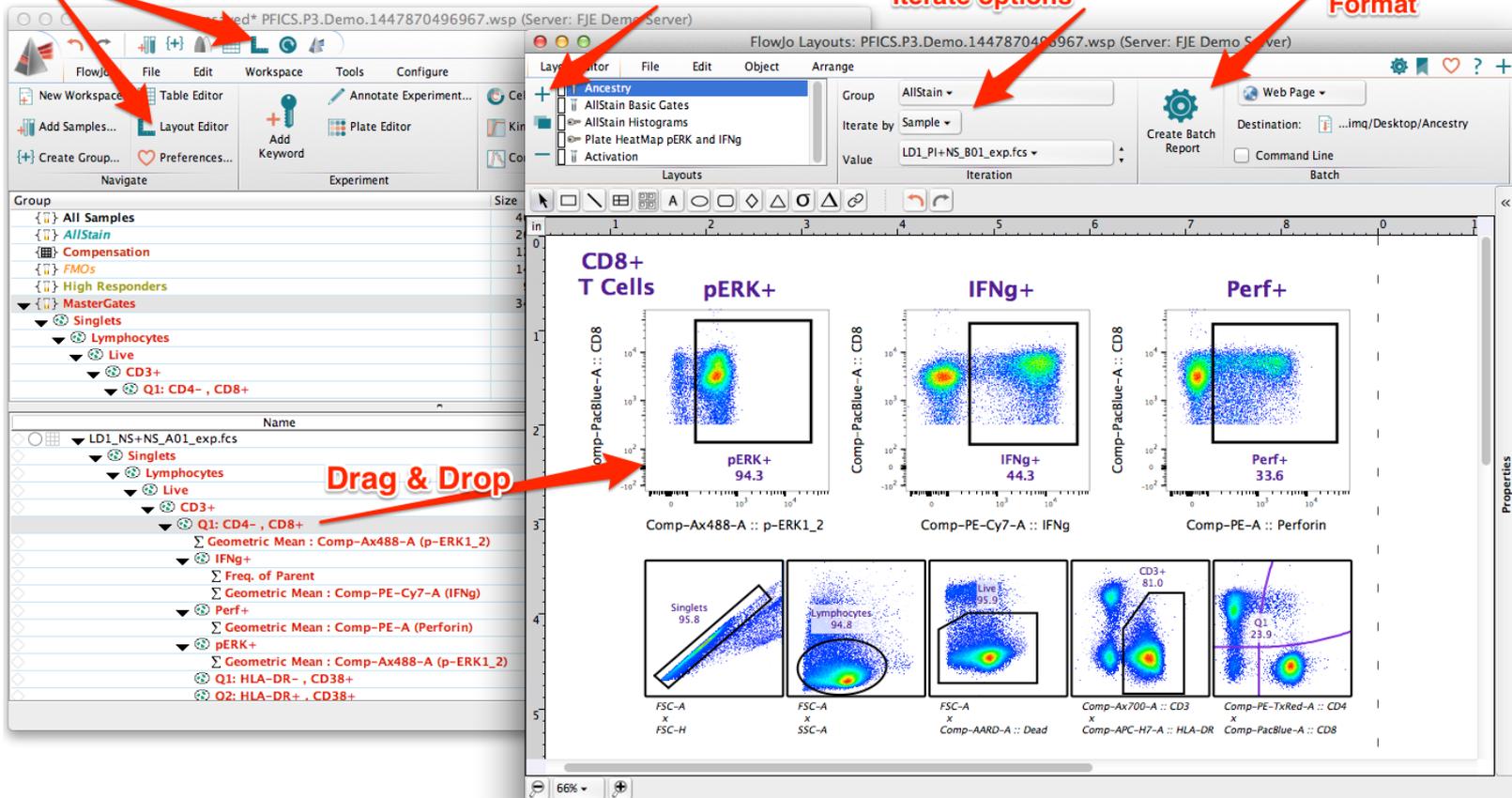


Layout Editor

Create Layouts

Specify Group and Iterate options

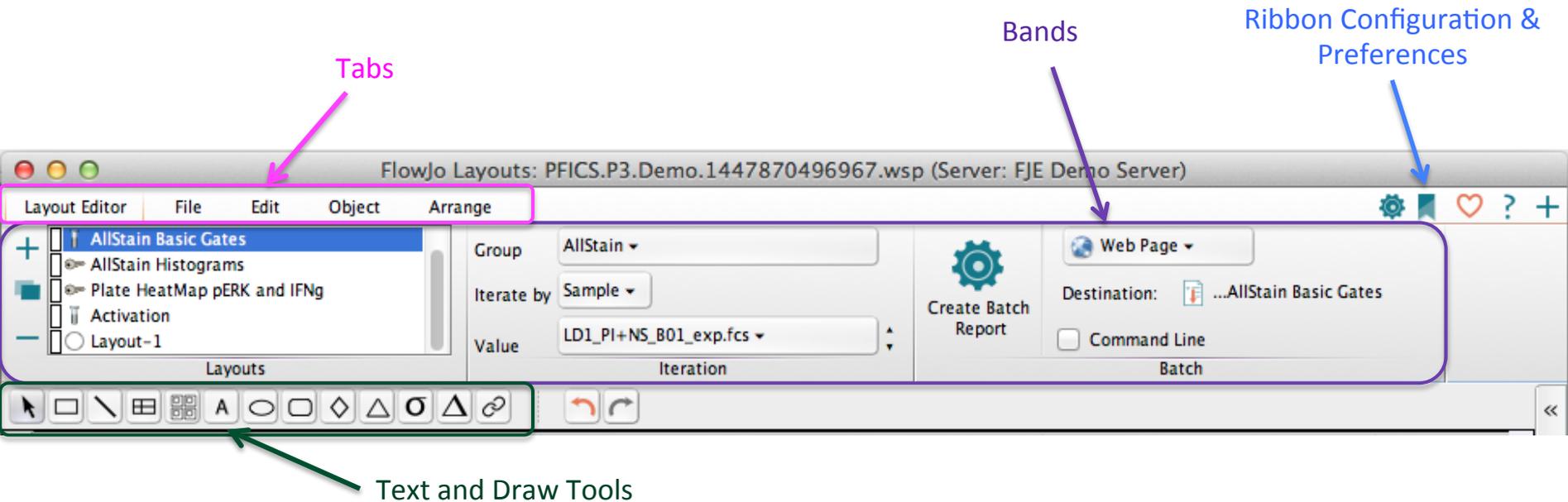
Batch Report Format



Drag & Drop

# Working in Layout Editor

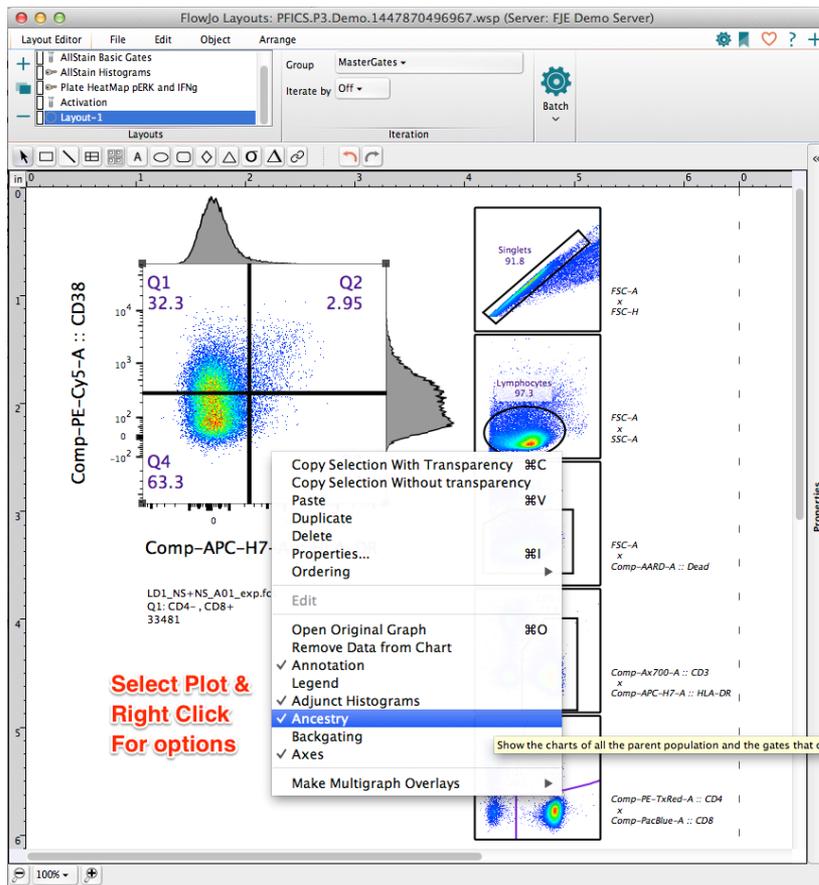
- Similar to the Workspace. Layout Editor has its own customizable Ribbon with Tabs and Bands to organize actions.



- 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.



Select Plot &  
Right Click  
For options

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

# Working in Layout Editor

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

**Tabs**

**Axis Parameters**

**Graph Type and Options**

**Annotate Tab**

**Gate Annotation options**

**Axis Label options**

Graph Definition

LD1\_NS+NS\_A01\_exp.fcs

Specify Annotate Fonts Legend

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

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

Type: Pseudocolor

Contour Levels: 5%

Smoothing

Show Outliers

Use Large Dots

Show Grid

Y Axis: Auto Max: 1000

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

Apply Cancel OK

Graph Definition

LD1\_NS+NS\_A01\_exp.fcs

Specify Annotate Fonts Legend

Annotation

Show Gates  Show Frequencies

Axes  Show Population Names

Adjunct Histograms

Ancestry  Backgating

Horizontal

X Axis

Hide Ticks  Hide Numbers  Hide Label

Label:

Y Axis

Hide Ticks  Hide Numbers  Hide Label

Label:

Apply Cancel OK

# Batch Analysis of Layout Editor Graphics

**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' dropdown menus, 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' options, 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 Location to write report
- Click Create Batch Report

The screenshot shows the FlowJo software interface with the 'Create Batch Report' dialog box open. The dialog box has several fields and a button:

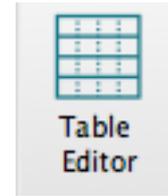
- Group:** AllStain
- Iterate by:** Sample
- Value:** LD1\_PI+NS\_B01\_exp.fcs
- Report Type:** Web Page
- Report Location:** Destination: ...AllStain Basic Gates
- Batch:**  Command Line
- Button:** Create Batch Report

Red arrows point from the following labels to the corresponding fields in the dialog box:

- Group** points to the 'Group' dropdown.
- Iterate by** points to the 'Iterate by' dropdown.
- Report Type** points to the 'Report Type' dropdown.
- Report Location** points to the 'Destination' field.
- Batch!** points to the 'Create Batch Report' button.

# The Table Editor

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



Open Table Editor

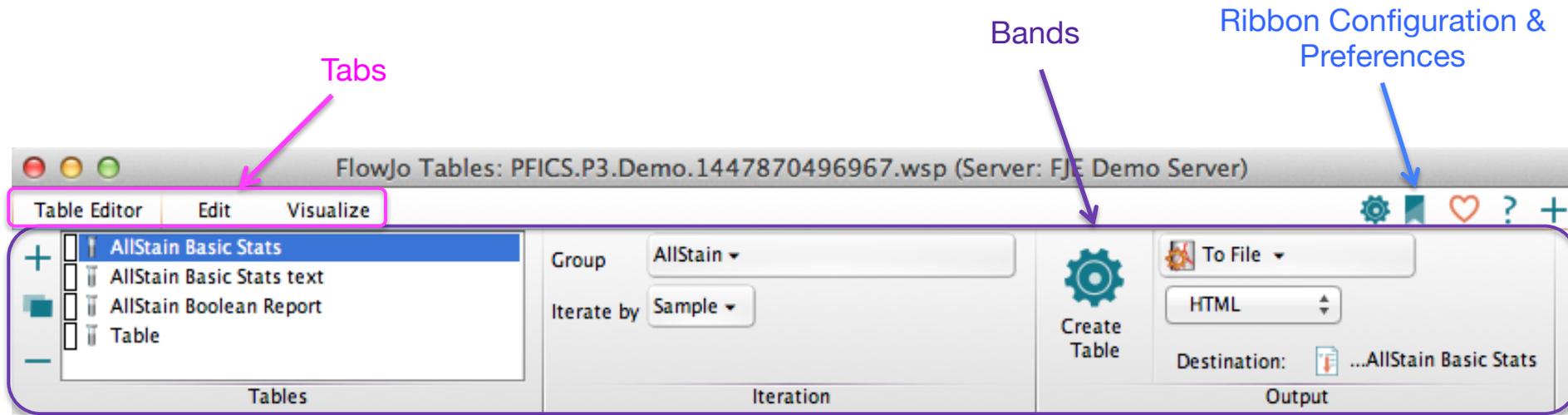
FlowJo Tables: PFICS.P3.Demo.1447870496967.wsp (Server: FJE Demo Server)

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

Drag Populations & Statistics

# Within Table Editor

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



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

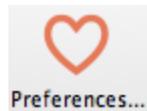
# Table Editor Visualize Tools

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

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

- Expected Ranges can be set within Preferences

→ Ranges



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

Table Editor Edit **Visualize**

Heat Map **2. Apply visualization tool**

Standard Deviation

Expected Range NK Cells

Time Series Correlation 3D Plot

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

# Table Editor Output

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

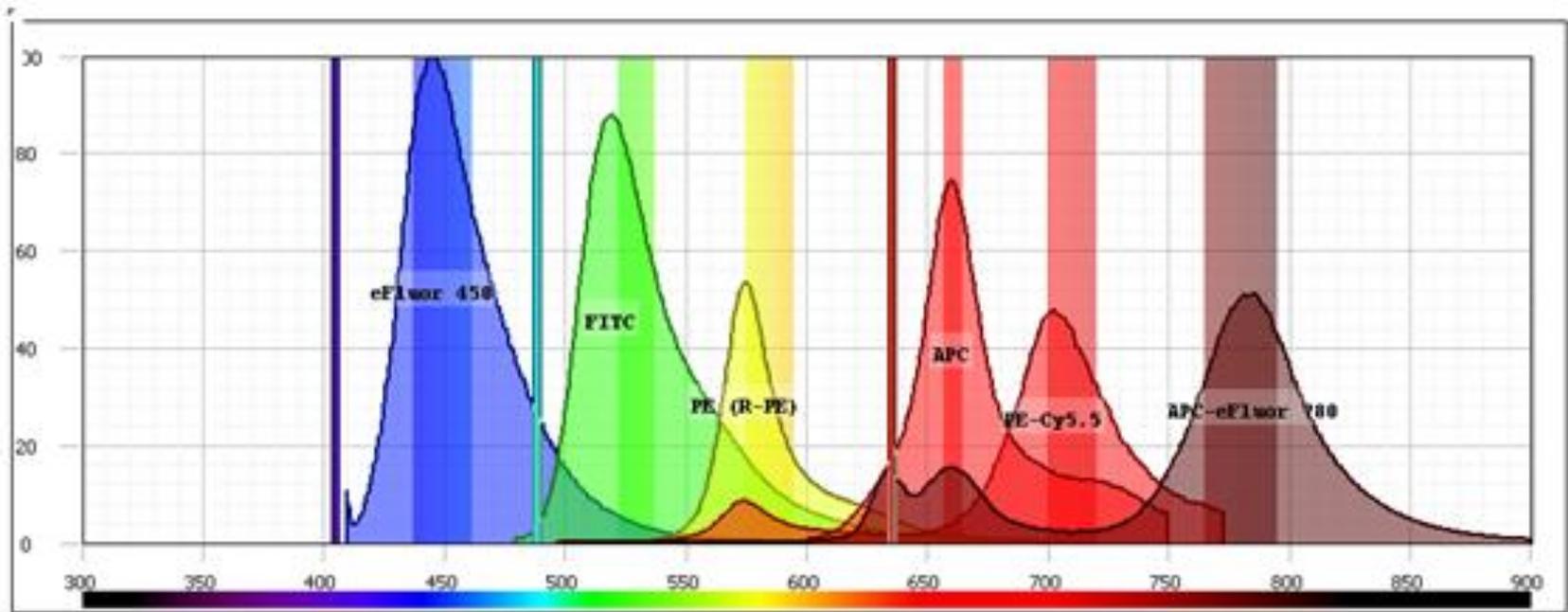
- Other file types (ex. Text, CSV, Excel) produce statistics tables lacking visualization formatting.

Table - AllStain Basic Stats

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

# Compensation

- Compensation corrects for spillover between fluorochrome emission spectra.



- Compensation is essential for multicolor panels

# Three Rules of Compensation

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

# PFICS Compensation Controls

- PBMC Cells

1. Unstained Cells
2. AARD
3. CD3 Alexa700

- Compensation Beads

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

# Compensation

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



in the task bar.

2. Click the Compensation Tool

1. Highlight Compensation Group

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

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

The wizard auto gates samples

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

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

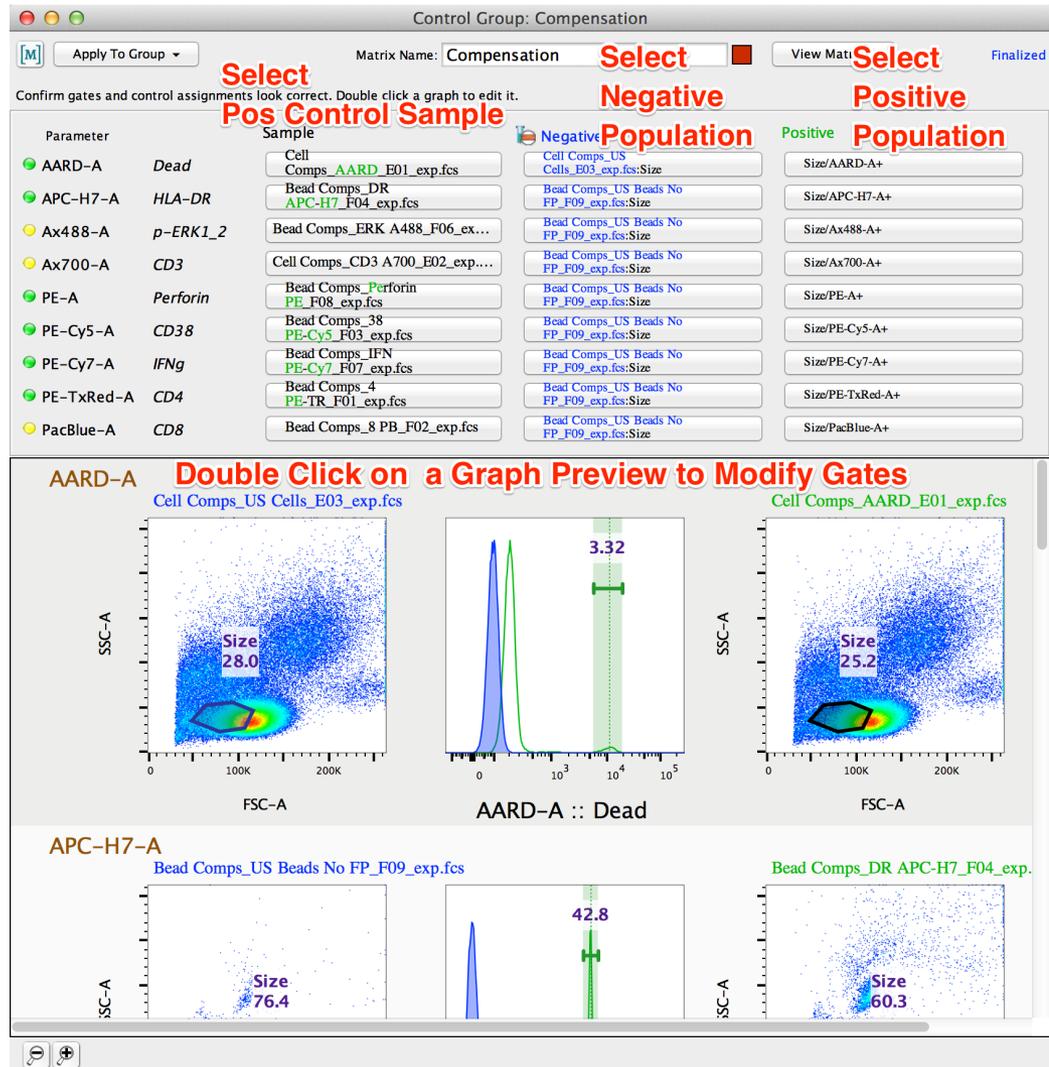
# Compensation

- Then fills in the positive and negative.

- Choose from the dropdown lists for each parameter.

- Double click preview graphs to modify gates.

For each Parameter



Control Group: Compensation

Apply To Group ▾ Matrix Name:   Finalized

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

| Parameter   | Sample  | Negative   | Positive                           |
|---|---|--|------------------------------------|
| <input checked="" type="radio"/> AARD-A <i>Dead</i> | Cell<br>Comps <input type="text" value="AARD_E01_exp.fcs"/> | <input type="radio"/> Cell Comps_US<br>Cells <input type="text" value="E03_exp.fcs:Size"/> | <input type="radio"/> 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\_AARD\_E01\_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  
 Cell Comps\_US Cells\_E03\_exp.fcs :: Size  
 Size  
 Size/AARD-A+

<Clear>

**Use Positive drop down list**

to Choose Positive population

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

Select Color

Name Matrix

Edit Matrix

Save a copy of the Matrix

The screenshot displays the 'Workspace Matrices' panel with a matrix named 'Compensation' selected. The matrix is a 10x10 table with values ranging from 0 to 39.6125. Below the matrix is a 'Preview Sample' section showing a grid of 6x2 flow cytometry plots for various markers and populations. The interface includes a menu bar with 'Edit' and 'Save Matrix' options, and a toolbar with icons for 'Cytometry', 'Biology', 'Boolean', and 'Visualizations'.

| 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 :: ... |
|----------------|-----------------|-----------------|----------------|------------------|-----------------|------------------|-------------------|------------------|
| 100            | 0.0351          | 0.3746          | 0.0685         | 0.0382           | 0.1447          | 0.0399           | 0.064             | 24.1599          |
| 0              | 100             | 0               | 3.2511         | 0.0169           | 0.8078          | 39.6125          | 0.056             | 0                |
| 1.8492         | 0               | 100             | 0              | 0.0119           | 0               | 0                | 0                 | 0                |
| 0.1713         | 34.835          | 0.1071          | 100            | 0                | 1.0301          | 10.2007          | 0                 | 0.0443           |
| 0              | 0.0125          | 0.3404          | 0.0375         | 100              | 14.4881         | 1.3119           | 37.6694           | 0                |
| 0              | 3.0045          | 0.0253          | 7.7547         | 1.6106           | 100             | 12.018           | 0.7082            | 0                |
| 0              | 5.7598          | 0.0603          | 0.3117         | 1.8877           | 0.368           | 100              | 0.8245            | 0                |
| 0              | 0.0291          | 0.1118          | 0.0572         | 23.9323          | 52.786          | 6.0459           | 100               | 0                |
| 16.9144        | 0               | 0.0597          | 0              | 0.0076           | 0               | 0                | 0.0063            | 100              |

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

**Applied Matrix Badge is Color Coded**

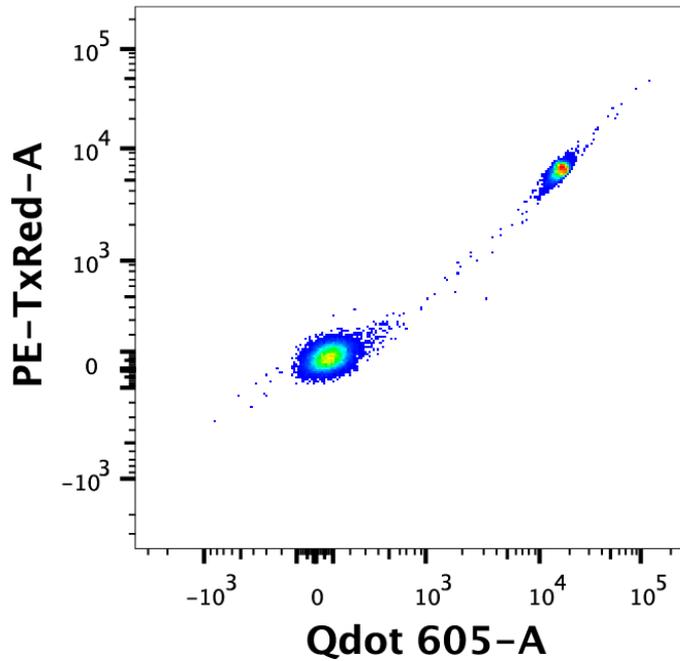
**Add a Matrix from file**

**Preview Matrix effect on a sample**

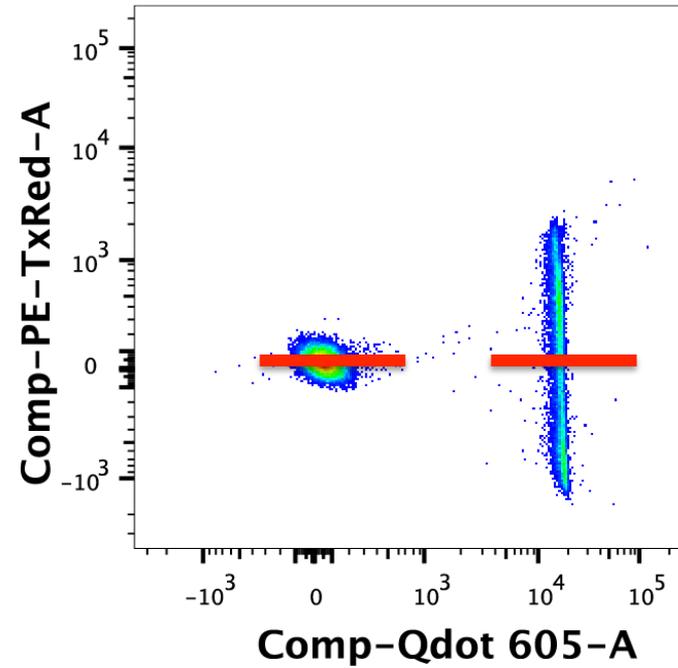
# Effect of Compensation



## Uncompensated

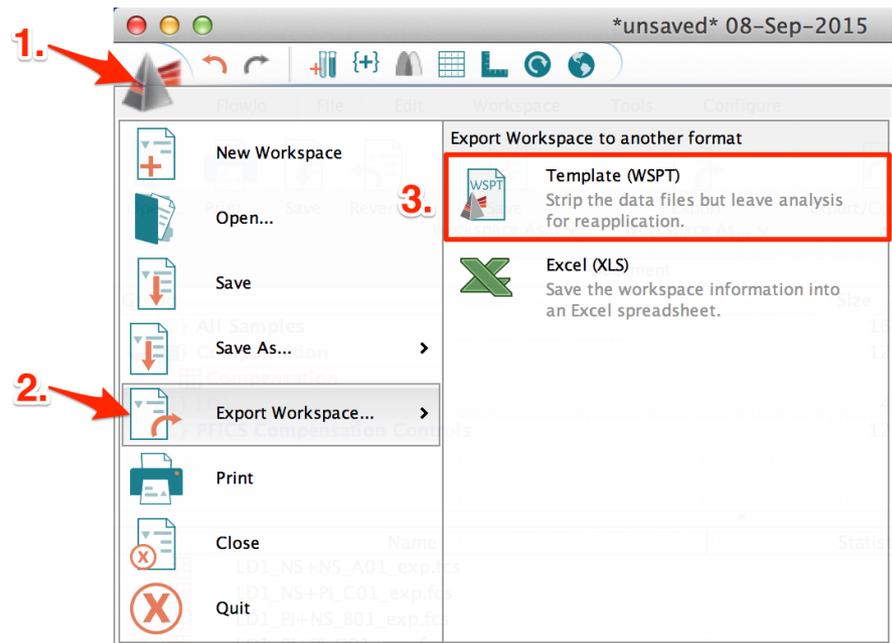
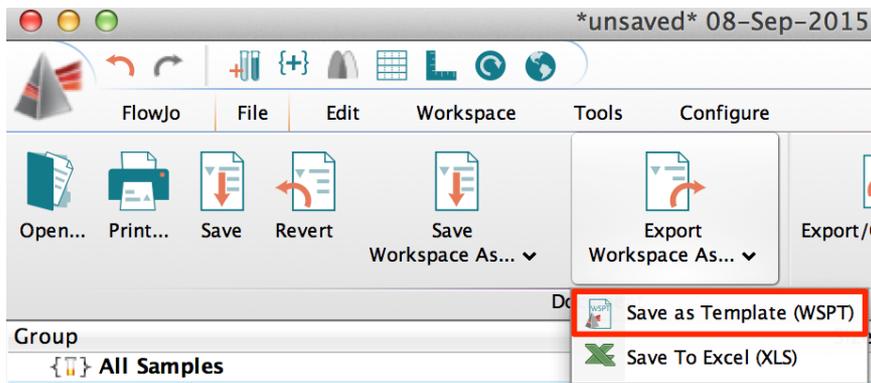


## Compensated



# Workspace Templates

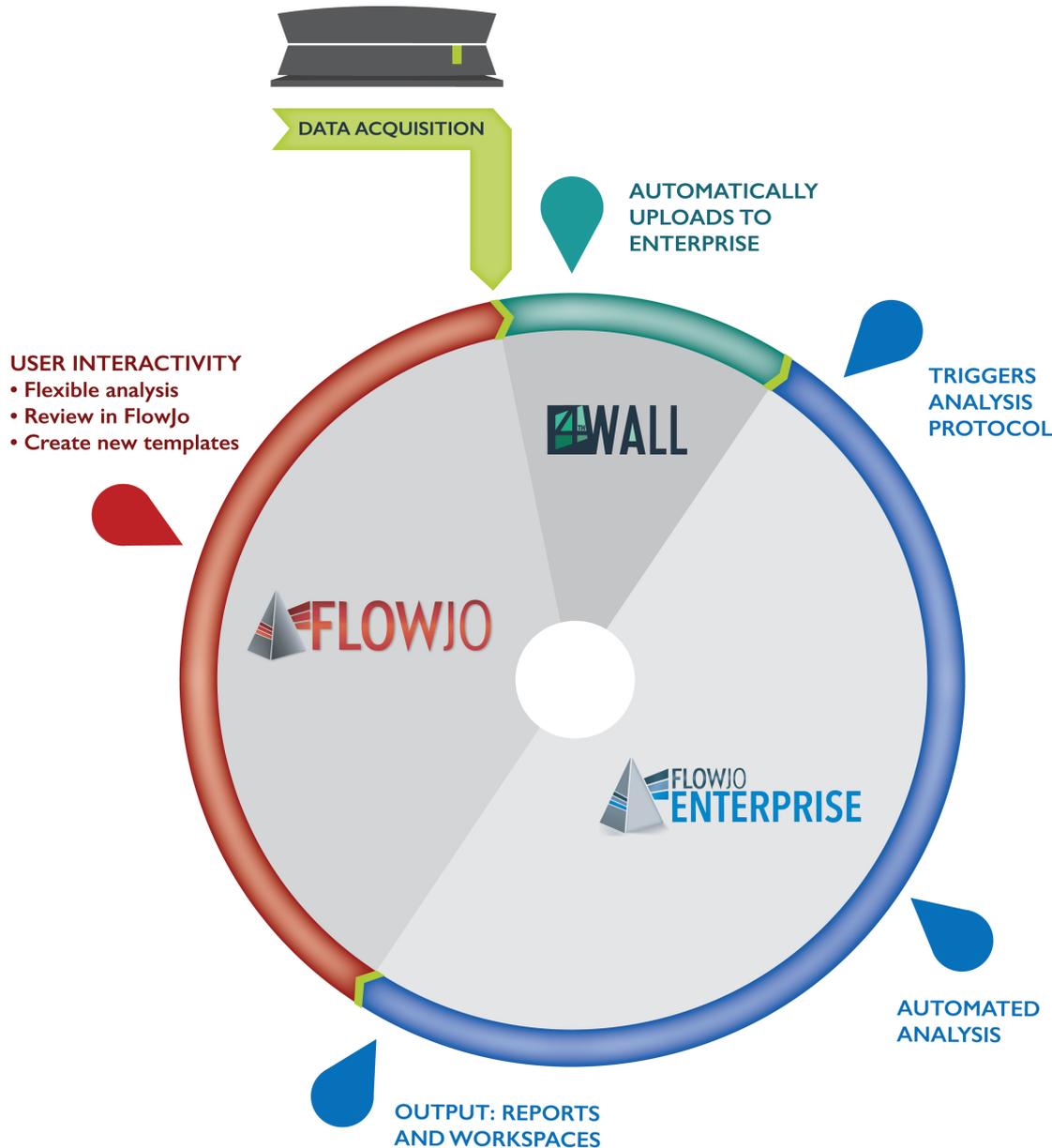
- Allows saving all analysis reports in your workspace without data.
- Streamlines repetitive analysis of multiple runs using the same staining panel(s).
- File Tab → Document Band → Export Workspace As... Save as a Template



# FlowJo Enterprise:

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

# FlowJo Enterprise Components



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

# The Plate Editor

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

Plate Editor

Plate Editor File Edit Tools

20120116 PFICS T...

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

Plates Experiment

Plate Name 20120116 PFICS TQC Filter Keywords: All Keywords v

Experiment ID 000-00000

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

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

Keyword/Values - Drag to Wells

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

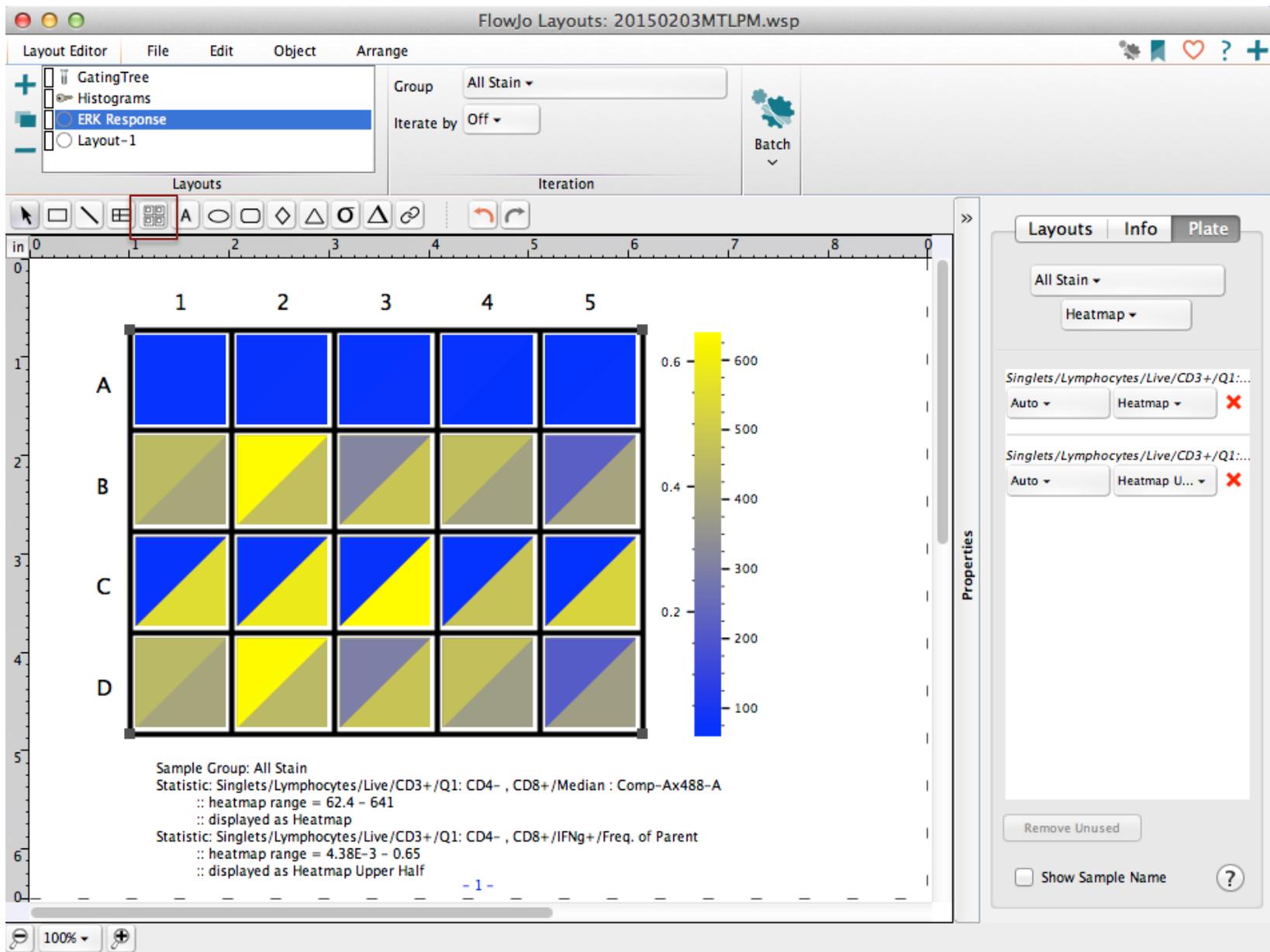
Keyword Value

Keyword/Values - Selected Well

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

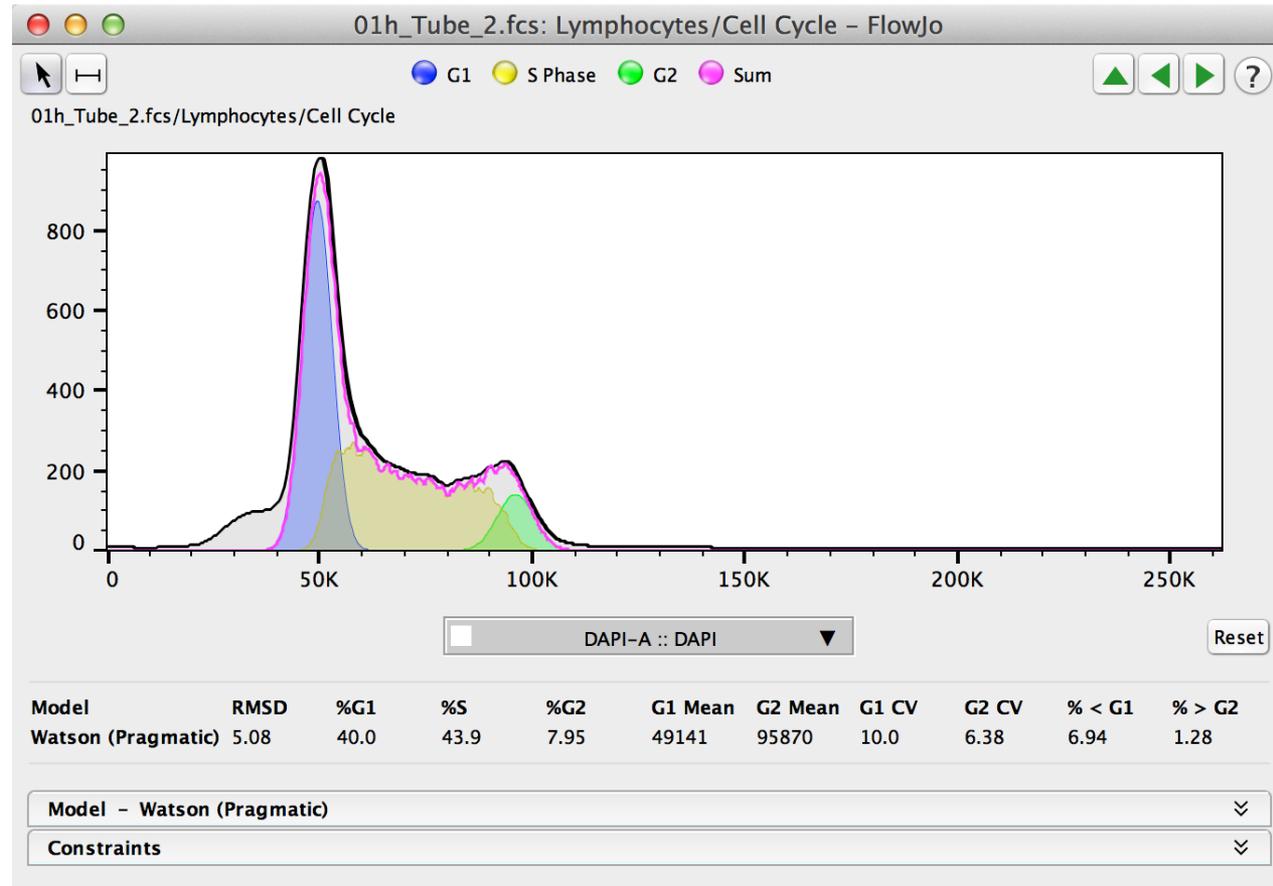
Select Group v

# Plate Visualizations



# Cell Cycle Analysis

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



# Additional Training Resources

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



## Questions?

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

# Thank You!