

Introduction to FlowJo v10

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The FlowJo v10 Workspace

An interface to organize your data and initiate actions

- Action Toolbar
- Groups and Group Analysis
- Samples and Sample Analysis

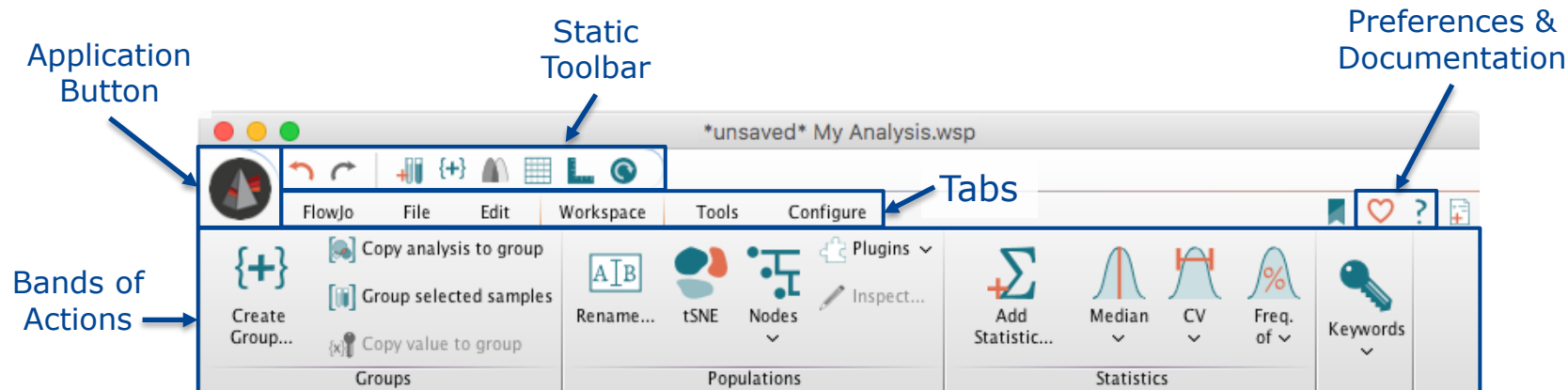
The screenshot displays the FlowJo v10 Workspace interface. The top menu bar includes 'FlowJo', 'File', 'Edit', 'Workspace', 'Tools', and 'Configure'. Below the menu bar is the Action Toolbar, which contains icons for 'New Workspace', 'Table Editor', 'Annotate Experiment...', 'Cell Cycle...', 'Proliferation Modeling...', 'Add Samples...', 'Layout Editor', 'Plate Editor', 'Kinetics...', 'Create Group...', 'Preferences...', 'Add Keyword', and 'Compare Populations...'. The main workspace is divided into three panes: 'Navigate', 'Experiment', and 'Biology'. The 'Navigate' pane shows a hierarchical tree of groups and samples. The 'Experiment' pane shows a table of statistics for the selected group. The 'Biology' pane shows a table of sample details.

Group	Size	Role
{ } All Samples	46	Test
{ } *STIM = NS+NS	5	Test
{ } *STIM = NS+PI	12	Test
{ } *STIM = PI+NS	5	Test
{ } *STIM = PI+PI	12	Test
{ } All Stain	20	Test
{ } Compensation	12	Compensation
{ } FMOs	14	Controls
{ } Master Gates	46	None
{ } Singlets		
{ } Lymphocytes		
{ } Live		

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
{ } Singlets	92.6	212691			
{ } Lymphocytes	93.3	198508			
{ } Live	97.9	194256			
{ } CD3+	79.8	155099			
{ } Q1: CD4-, CD8+	18.9	29349			
{ } Q2: CD4+, CD8+	0.64	994			
{ } Q3: CD4+, CD8-	78.4	121645			
{ } Q4: CD4-, CD8-	2.01	3123			
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
LD2_PI+PI_D02_exp.fcs		275465	LD2	PI+PI	D02

The Action Toolbar

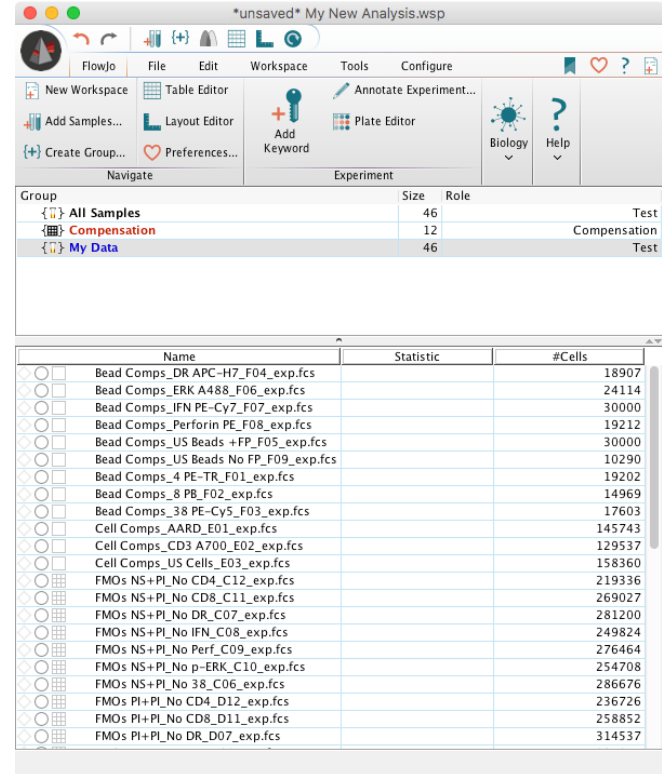
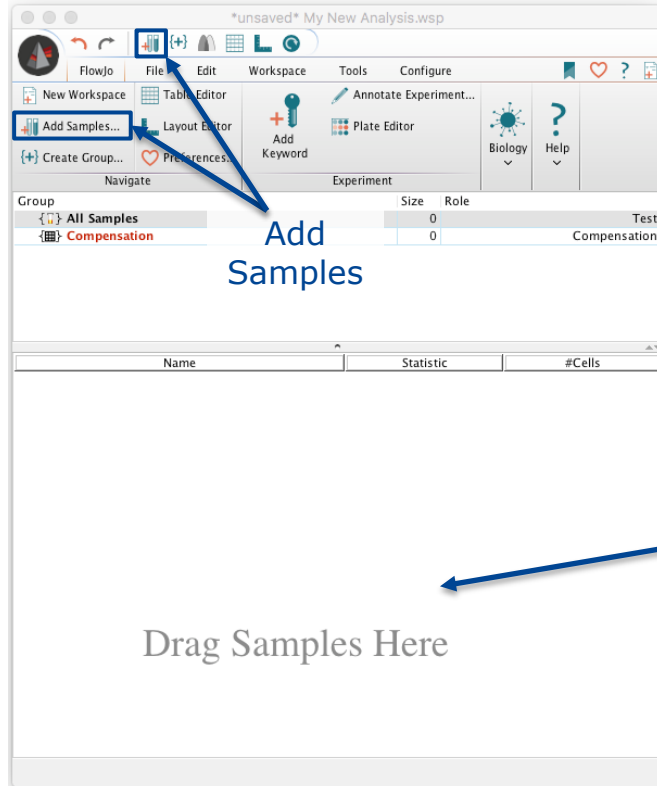
Allows visual navigation of workspace functions



- Tabs group similar Bands together
- Bands group similar Actions together
- Mouse-over an action button → tooltip + hotkey

Importing Data

Drag-and-drop files into Samples Pane, or click Add Samples



Group Pane

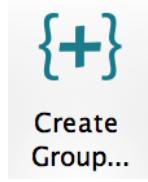
Groups act as folders to organize samples and initiate actions

Group	Size	Role
{ } All Samples	46	Test
{ } *STIM = NS+NS	5	Test
{ } *STIM = NS+PI	12	Test
{ } *STIM = PI+NS	5	Test
{ } *STIM = PI+PI	12	Test
{ } All Stain	20	Test
{ } Compensation	12	Compensation
{ } FMOs	14	Controls
▼ { } Master Gates	46	None
▼ { } Singlets		
▼ { } Lymphocytes		
▼ { } Live		

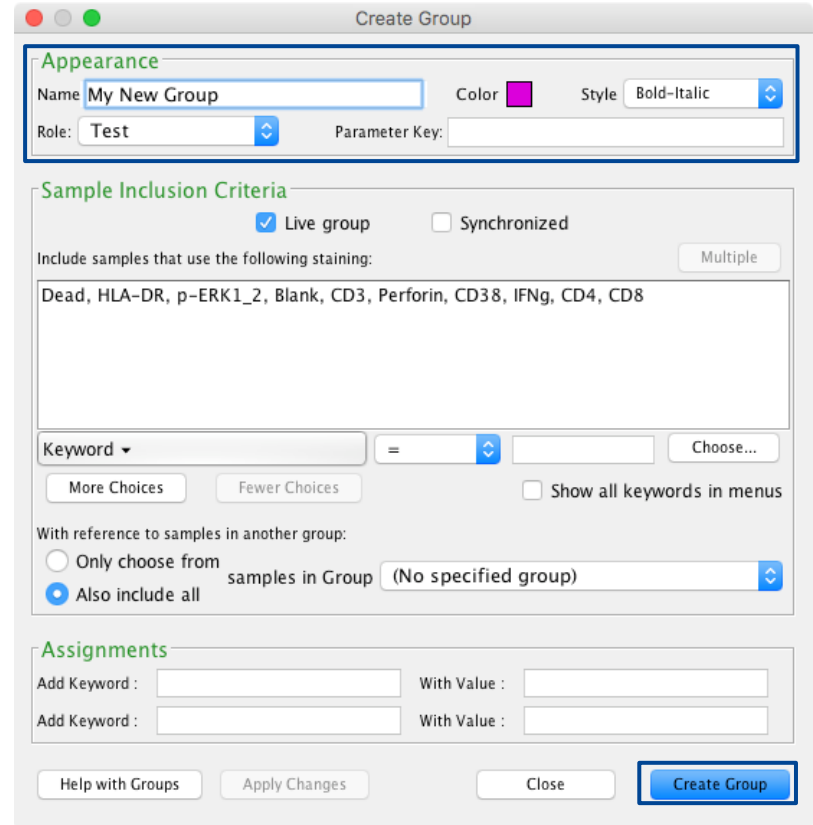
- The Group area lists all groups in the workspace, # of samples in each Group (Size) and the Role of that group
- Groups allow for master gating of multiple samples → Group-applied analysis gains the group color
- Groups can be used for batch reporting multiple samples

Creating and Editing Groups

Click the Create Group icon



- Type a Name
- Set options
- Click Create Group
- Drag-and-drop adds samples to the group
- Double click on an existing group to edit its properties



Create Group

Appearance

Name: Color: Style:

Role: Parameter Key:

Sample Inclusion Criteria

☒ Live group ☐ Synchronized

Include samples that use the following staining:

Keyword =

☐ 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

Specifies which samples are automatically included in a group

- A “Live group” automatically includes samples based on the user-defined sample inclusion criteria
- These criteria can include characters in the \$FIL field (File Name) or any other combination of keyword attributes

The screenshot shows the 'Modify Group' dialog box with the 'Sample Inclusion Criteria' section highlighted by a blue border. The 'Appearance' section at the top shows the group name as '*STIM = PI+NS', color as purple, and style as Bold. The 'Role' is set to 'Test'. The 'Sample Inclusion Criteria' section has 'Live group' checked and 'Synchronized' unchecked. Below this, it says 'Include samples that use the following staining:' followed by a list: 'Dead, HLA-DR, p-ERK1_2, Blank, CD3, Perforin, CD38, IFNg, CD4, CD8'. There is a 'Multiple' button to the right. Below the list, there is a dropdown menu showing '*STIM', followed by an equals sign, a dropdown showing 'PI+NS', and a 'Choose...' button. There are also 'More Choices' and 'Fewer Choices' buttons. A checkbox 'Show all keywords in menus' is unchecked. Below this, it says 'With reference to samples in another group:' followed by two radio buttons: 'Only choose from' (unchecked) and 'Also include all' (checked). To the right of the radio buttons is a dropdown menu showing '(No specified group)'. The 'Assignments' section at the bottom has two rows for 'Add Keyword' and 'With Value'. At the very bottom are buttons for 'Help with Groups', 'Apply Changes', 'Close', and 'Create Group'.

Samples Pane

Lists all samples within the selected group

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
▼ Singlets	92.6	212691			
▼ Lymphocytes	93.3	198508			
▼ Live	97.9	194256			
▼ CD3+	79.8	155099			
▶ Q1: CD4- , CD8+	18.9	29343			
▶ Q2: CD4+ , CD8+	0.64	993			
▶ Q3: CD4+ , CD8-	78.4	121644			
▶ Q4: CD4- , CD8-	2.01	3119			
▶ LD1_PI+NS_B01_exp.fcs		262774	LD1	PI+NS	B01
▶ LD1_PI+PI_D01_exp.fcs		244977	LD1	PI+PI	D01

- Displays sample-level gating analysis hierarchy
- Statistic and #Cells columns are displayed by default
- Additional Keyword attribute columns can be added

Keywords

Sample-level descriptive metadata

- Within the workspace, keywords can be:
 - Added
 - Displayed
- Keywords are used to:
 - Organize and sort samples
 - Create groups
 - Generate batch reports

The screenshot shows the FlowJo software interface. The 'Workspace' tab is selected in the top menu. The 'Keywords' icon is highlighted in the top right. A blue box highlights the 'Keywords' icon in the top right. A blue arrow points to the 'Keywords' icon with the text 'Add Keyword'. Another blue arrow points to the 'Name' column header with the text 'Right Click Column Header'. A yellow tooltip box is visible over the 'Edit Columns...' option in the context menu.

Group

Group	Size	Role
{ } All Samples	46	
{ } *STIM = NS+NS	5	
{ } *STIM = NS+PI	12	Test
{ } *STIM = PI+NS	5	Test
{ } *STIM = PI+PI	12	Test
{ } All Stain	20	Test
{ } Compensation	12	Compensation
{ } FM	14	Controls
{ } None	46	None

Right Click Column Header

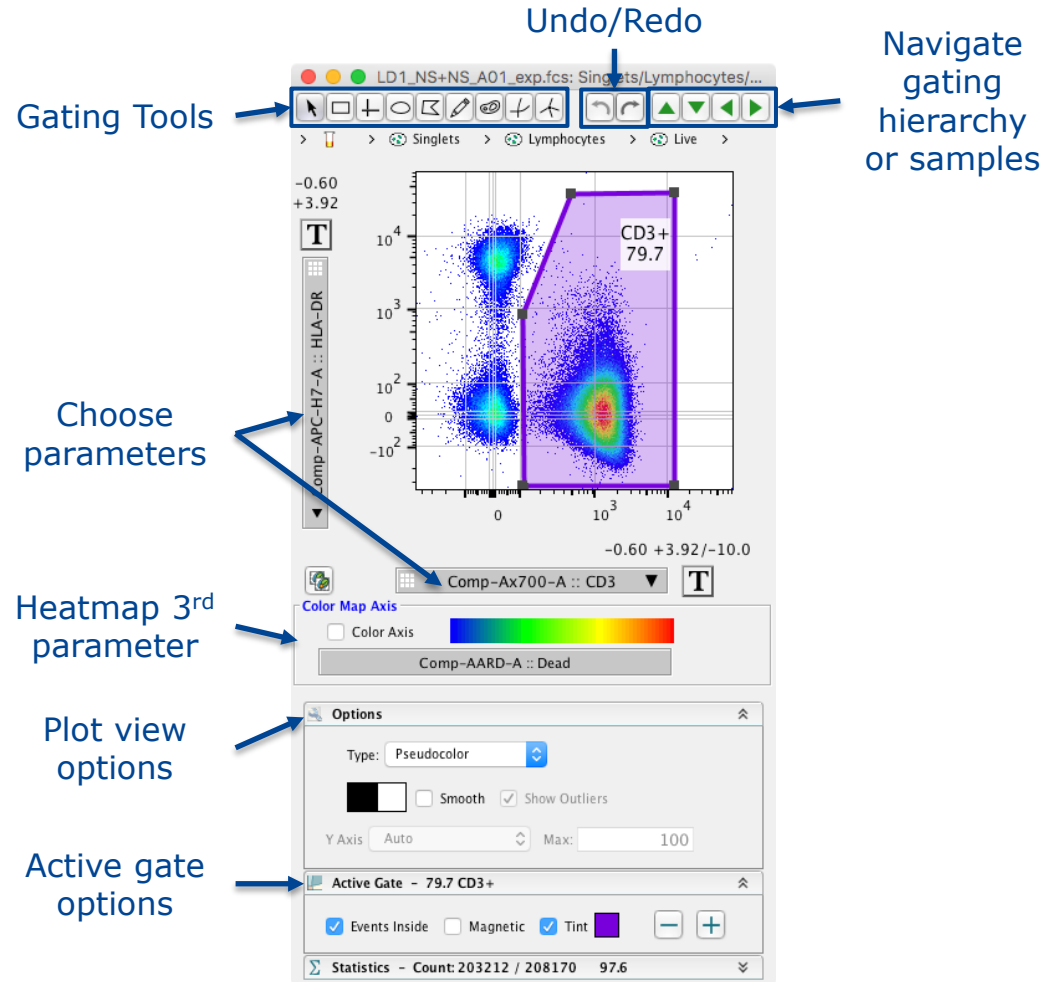
Open a dialog to choose the keyword columns shown in the workspace window.

Practice time



The Graph Window

- Facilitates data visualization and gating
- Click on an X or Y axis label to choose and view different measured parameters

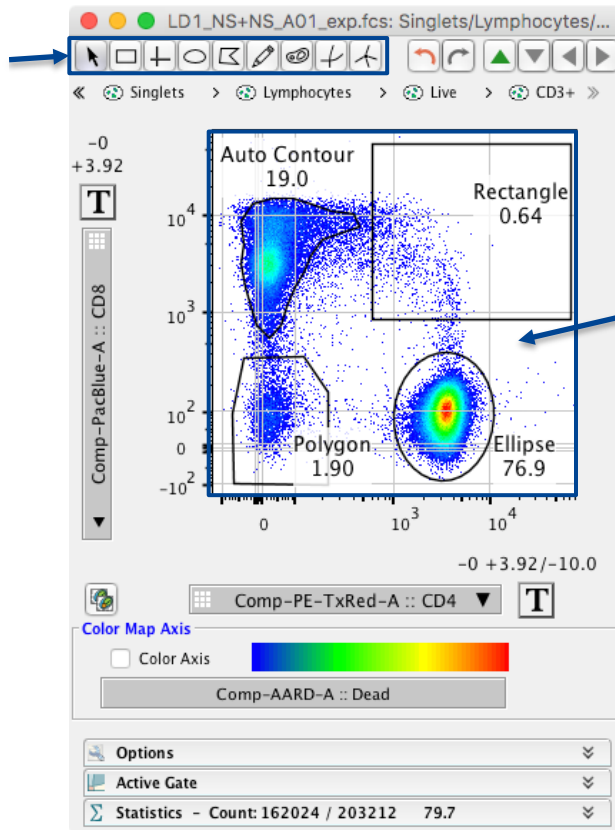


Gating Tools

Subset events into populations based on marker expression

- Draw a Gate →
Frequency of parent statistic
- Gates can always be modified or removed
- Double-click within a gate to focus on that population in a new graph window

Choose
a gating
tool



Draw a
gate

Gating Hierarchies

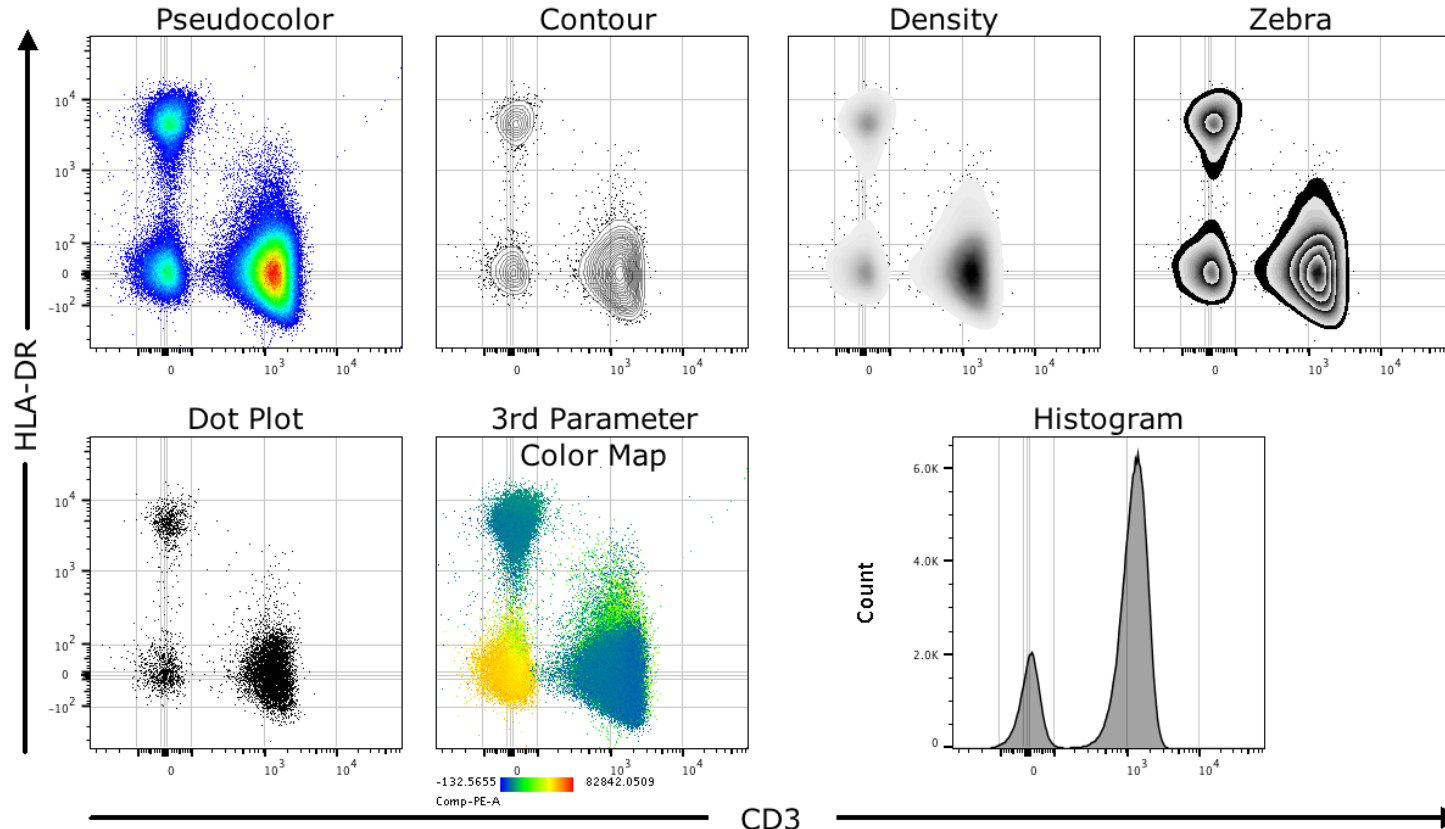
Display parent/child/sibling relationships between populations

- Viewed in the workspace samples pane
- Can be:
 - Collapsed or expanded
 - Drag-and-dropped to other gates, samples, groups, or areas of the program

Name	Statistic	#Cells
LD1_NS+NS_A01_exp.fcs		250342
LD1_NS+PI_C01_exp.fcs		229585
LD1_PI+NS_B01_exp.fcs		262774
▼ Singlets	95.3	250347
▼ Lymphocytes	89.6	224202
▼ Live	97.6	218763
▼ CD3+	82.9	181325
▼ Q1: CD4- , CD8+	24.0	43469
Σ Median : Comp-Ax488-A (p-ERK1_2)	407	
Σ Median : Comp-PE-A (Perforin)	62.0	
IFNg+	44.1	19160
Perf+	32.7	14221
pERK+	95.3	41446
IFNg-	55.9	24309
Perf-	67.3	29248
pERK-	4.65	2023
IFNg+Perf+pERK+	27.7	12056
IFNg+Perf+pERK-	1.47	637
IFNg+Perf-pERK+	14.6	6360
IFNg+Perf-pERK-	0.25	107
IFNg-Perf+pERK+	2.36	1028
IFNg-Perf+pERK-	1.15	500
IFNg-Perf-pERK+	50.6	22002
IFNg-Perf-pERK-	1.79	779
Q2: CD4+ , CD8+	0.56	1023
▼ Q3: CD4+ , CD8-	72.9	132235
Σ Median : Comp-Ax488-A (p-ERK1_2)	422	
Σ Median : Comp-PE-A (Perforin)	26.2	
Q4: CD4- , CD8-	2.54	4598

Graph Window Display Options

Customize your data visualization

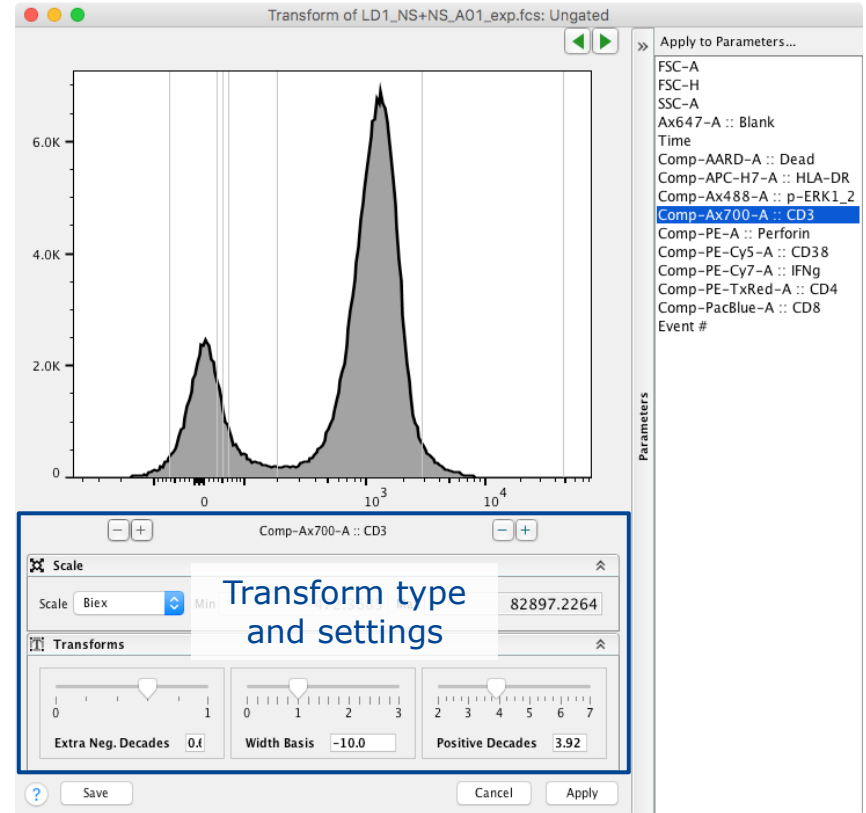
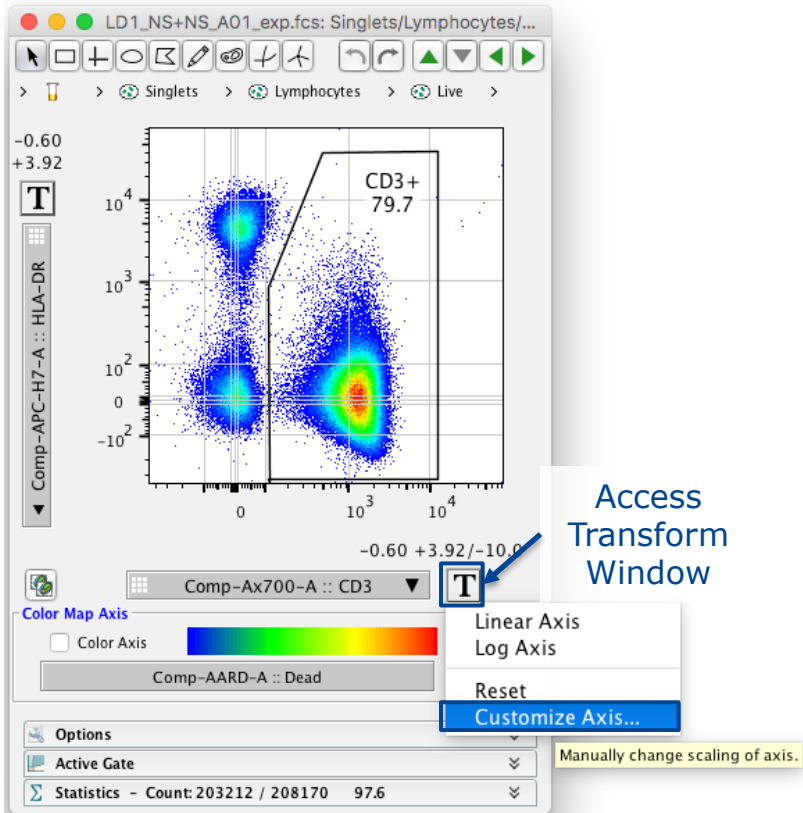


Practice time



Transforming Data

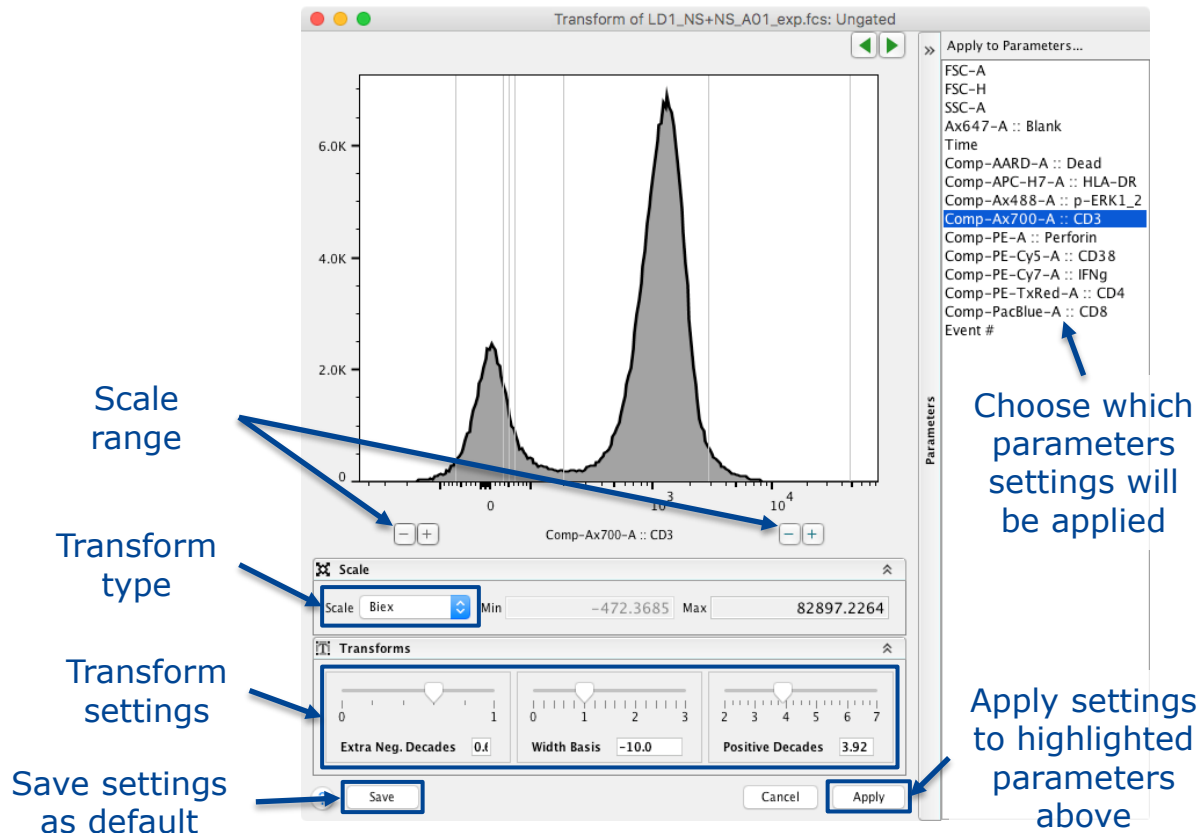
Click the Transformation [T] button → Customize Axis...



Transforming Data

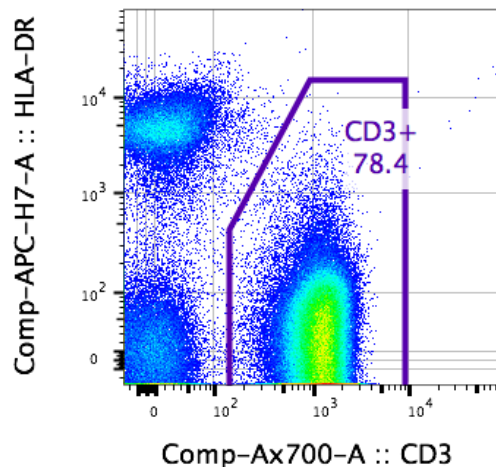
Only affects visual display & scaling of data, not the raw values

- +/- Buttons adjust range
- Sliders adjust transform
 - Extra Neg Decades
 - Width Basis
 - Positive Decades
- Apply button → applies selected settings to chosen parameters

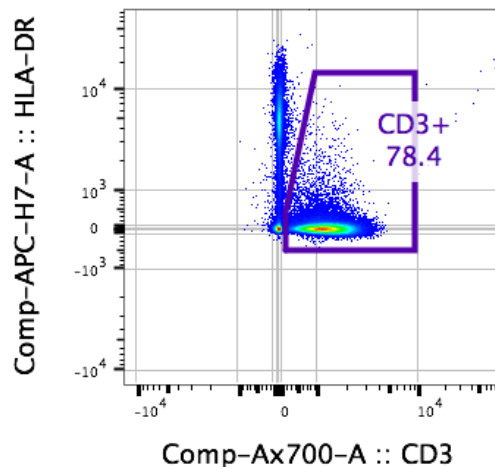


Transformation

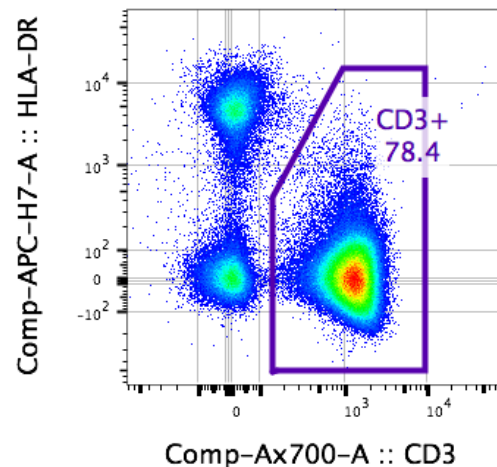
Extra Neg Decades = 0
Width Basis = -1
Extra Pos Decades = 3.92



Extra Neg Decades = 1
Width Basis = -200
Extra Pos Decades = 3.92



Extra Neg Decades = 0.6
Width Basis = -10
Extra Pos Decades = 3.92



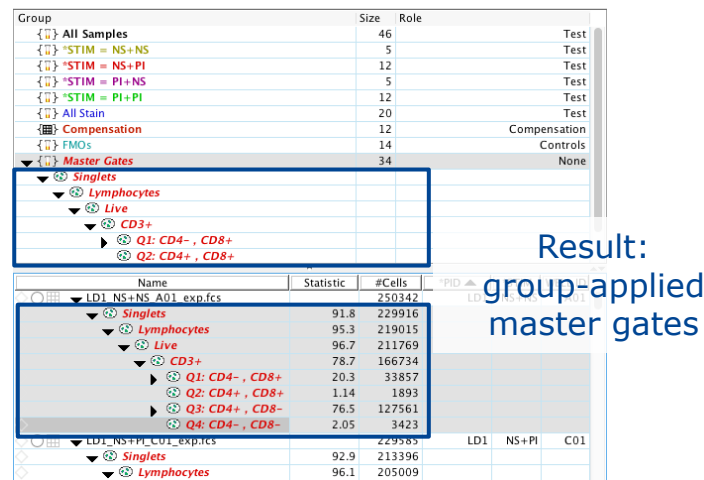
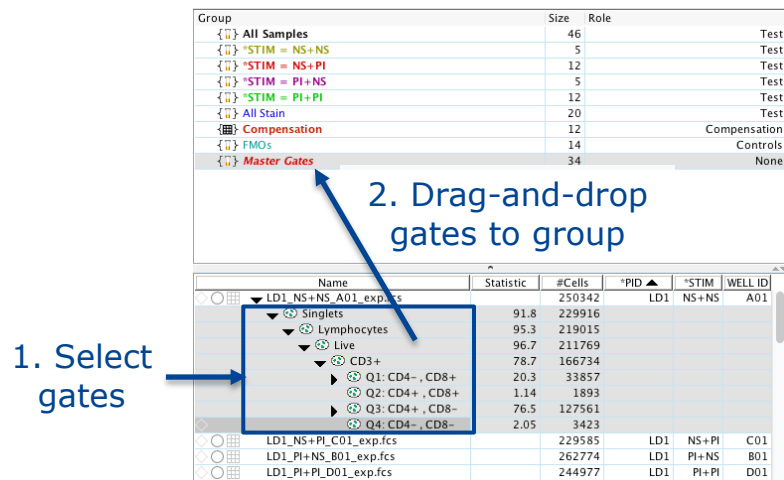
- Gets rid of the “squishing” of cells
- Ensures the visual population center better correlates with the statistical center (median)
- Makes high resolution compensated digital cytometry data more appealing to the eye

Practice time



Group Application of Gates

Allows for master gating of multiple samples

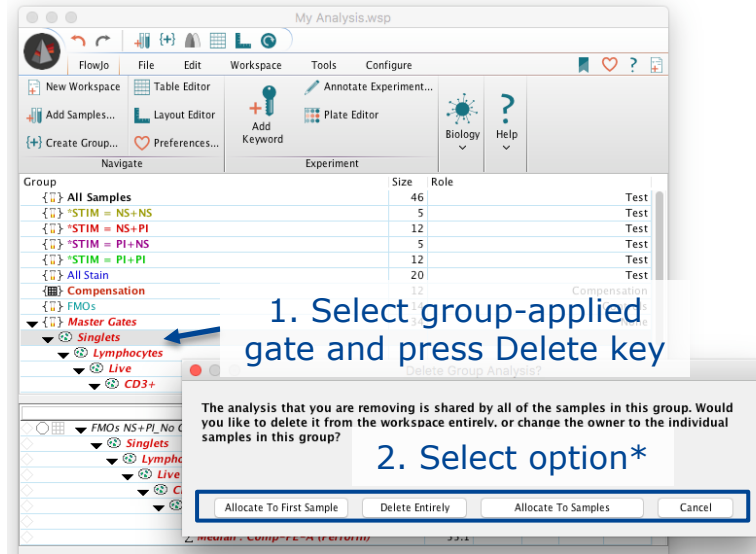


- Select gray gates on a single sample and:
 - drag-and-drop gates from a single sample to a group, or
 - right click on gates and choose "Copy analysis to group"
- Group-applied gates turn the group color to denote that they are identical

Group-Applied Gates

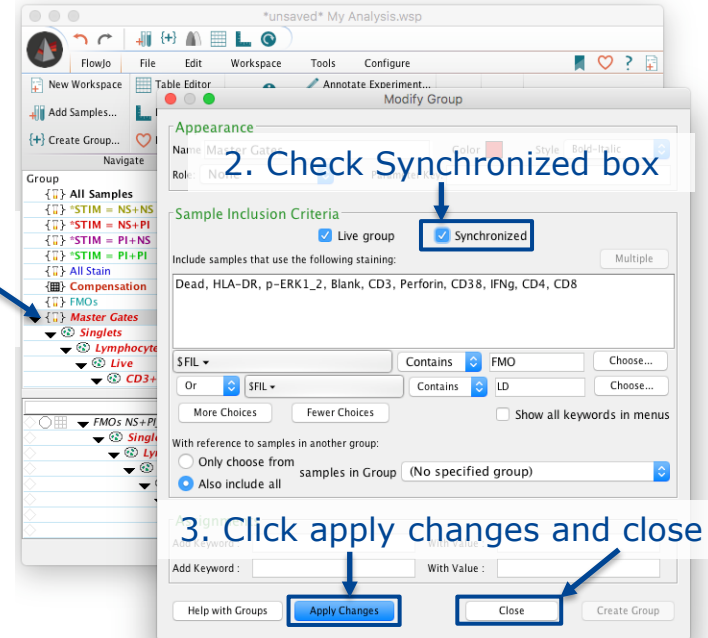
- Can be
 - Modified on a single sample
 - Removed or deleted from the group gating tree (A)
 - Synchronized through the group properties menu (B)

(A)



(B)

1. Double click on group



*Delete Entirely → gates removed completely

Allocate → removes group gating, deeps gates on sample(s)

Additional Statistics

To enumerate properties of gated populations

- Add a statistic to any gated population within a sample gating hierarchy
- Statistic Nodes can be group-applied just like a gate
- Example statistics:
 - Count
 - Median
 - Standard Deviation (SD)

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

- 1. Select Population(s):** An arrow points to the 'CD3+' population in the 'Groups' panel on the left.
- 2. Click Add Statistic:** An arrow points to the 'Add Statistic...' button in the 'Statistics' panel at the top.
- 3. Choose statistic:** A dropdown menu is open, showing various statistics. 'Median' is selected.
- 4. Choose parameter(s):** A sub-menu is open for the 'Median' statistic, showing various parameters. 'Q1: CD4- , CD8+' is selected.
- 5. Click Add:** An arrow points to the 'Add' button at the bottom right of the 'Add Statistic...' dialog.

The 'Add Statistic...' dialog box contains the following text:

Choose a statistic and any applicable parameters below.
Press the Add button to apply them to your analysis

Population: Q1: CD4- , CD8+
Parameter: Time
Comp-AARD-A :: Dead
Comp-APC-H7-A :: HLA-DR
Comp-Ax488-A :: p-ERK1_2
Comp-Ax700-A :: CD3
Comp-PF-A :: Perforin
Comp-PE-Cy5
Comp-PE-Cy7-A :: IFNγ
Comp-Facilin-A :: CD6
Event #
Percentile:
Freq. of: [Choose...]

Buttons: Close, Add

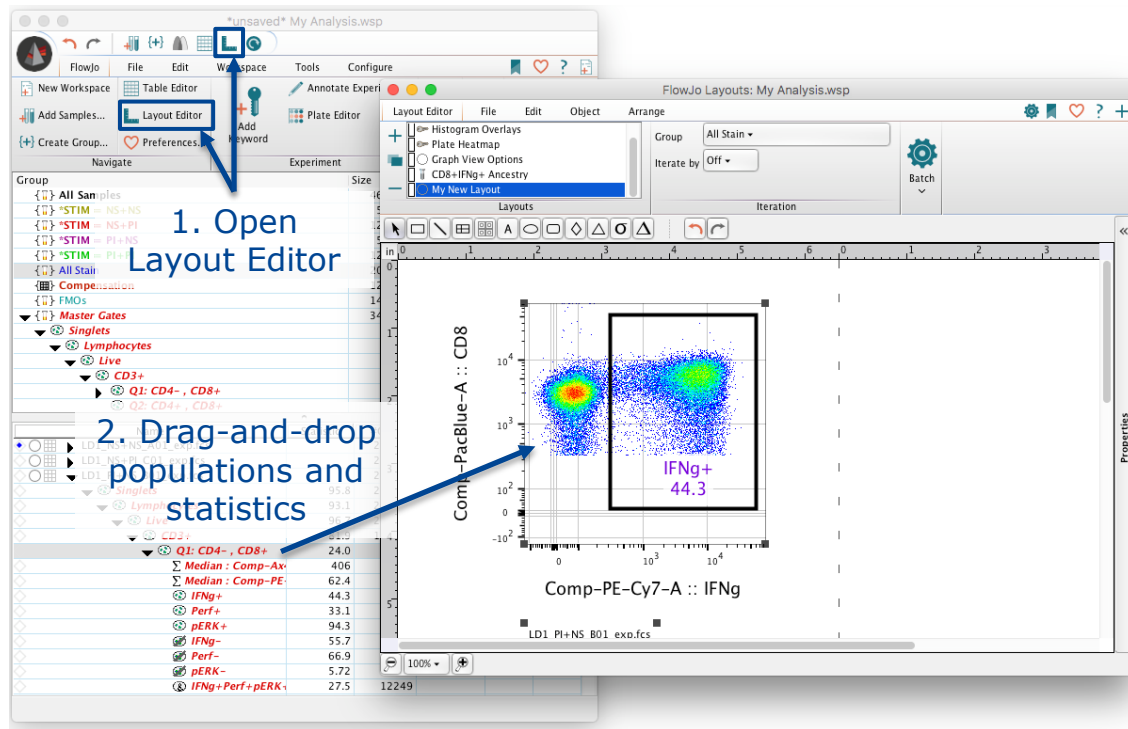
Practice time



The Layout Editor

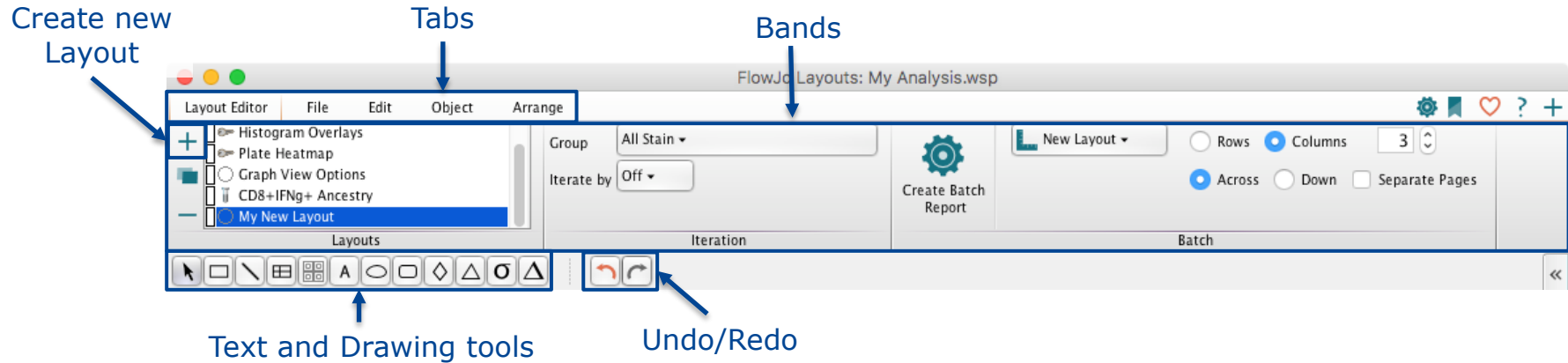
A tool for creating graphical reports

- To create a layout:
 - Click on the Layout Editor icon in the workspace ribbon
 - Drag-and-drop populations from a sample gating hierarchy in the workspace to the Layout Editor window



Working in the Layout Editor

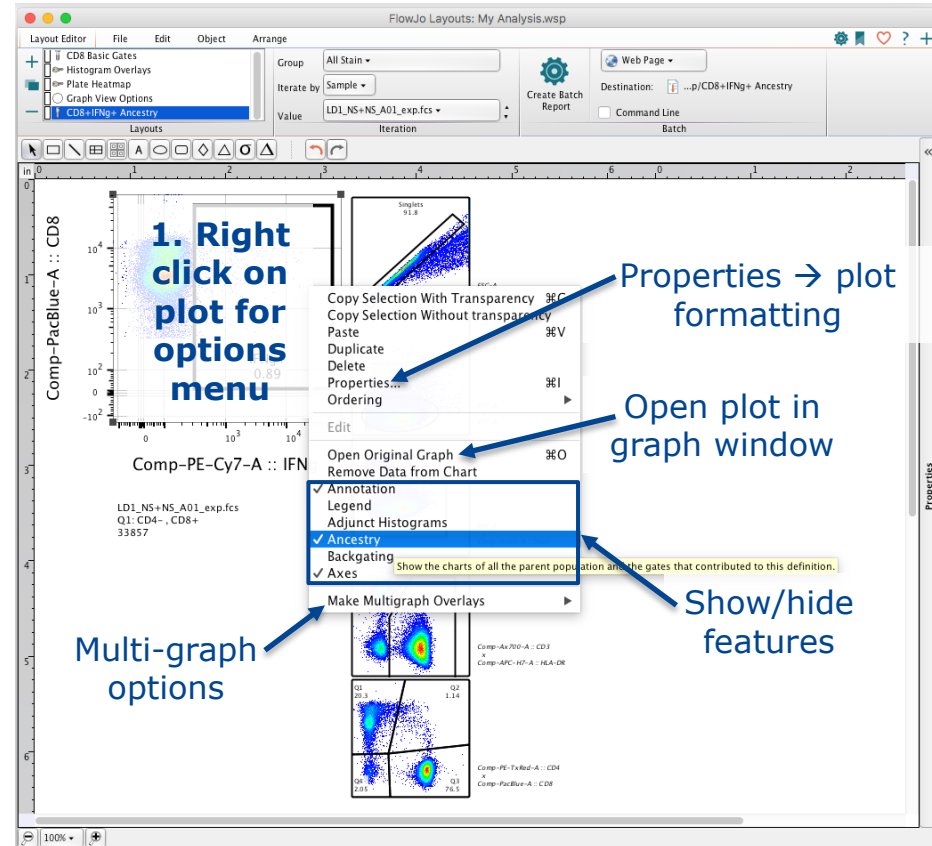
- Similar to the Workspace, the Layout Editor has its own action taskbar with tabs and bands to organize actions



- Try clicking on the different tabs to see what types of actions are available
- Click the + button to create new layout reports

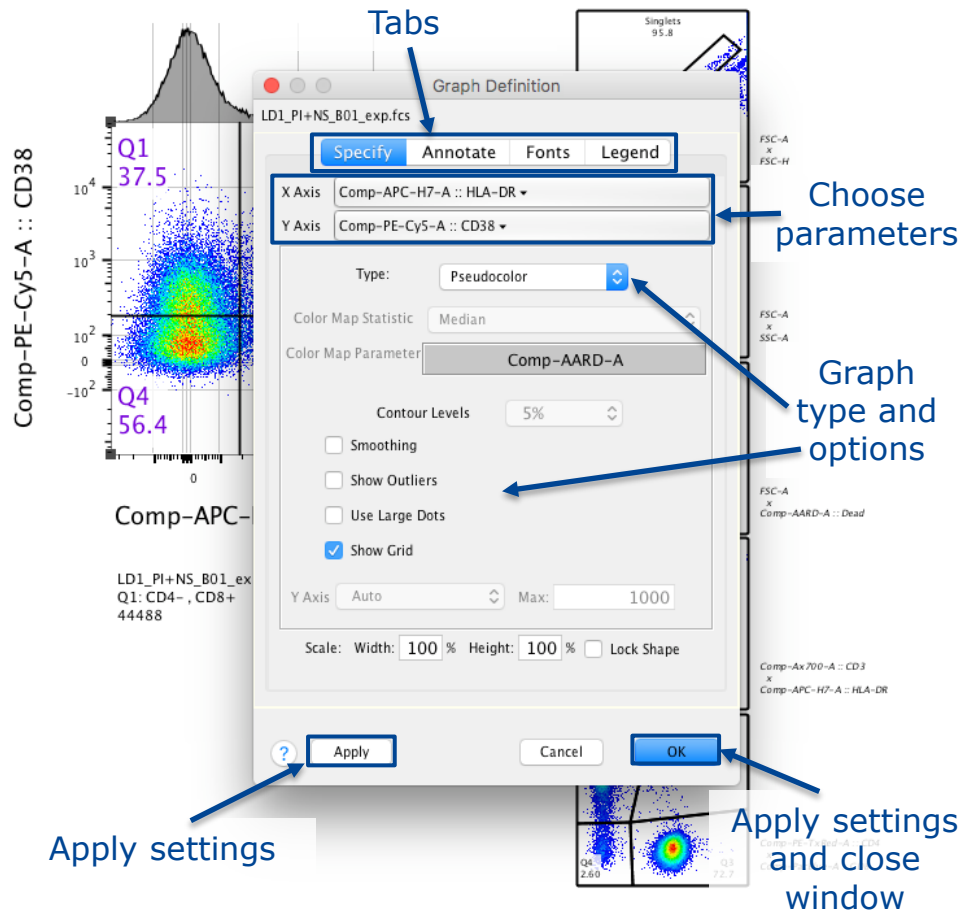
Within the Layout Editor

- Graphs can be organized and reformatted
- Statistics, keywords, text and shapes or objects can be added to illustrate your analysis
- Right click on a graph plot for a list of options including Ancestry, Backgating and Properties/Formatting
- Right click on plot axis label → Parameter selection (also available w/in graph properties)



Formatting Graph Plots in the Layout Editor

- Right click on plot and select Properties → Graph Definition window
- 4 tabs with formatting options for:
 - Specify graph style
 - Annotation
 - Fonts
 - Legend



Batch Analysis of Layout Graphics

Applies the layout across multiple samples in a group

- To batch a layout:
 - Specify a group
 - Sample
 - Panel
 - Keyword
 - Set batch options
 - Click "Create Batch Report"

Select group & iterate by option Set batch options

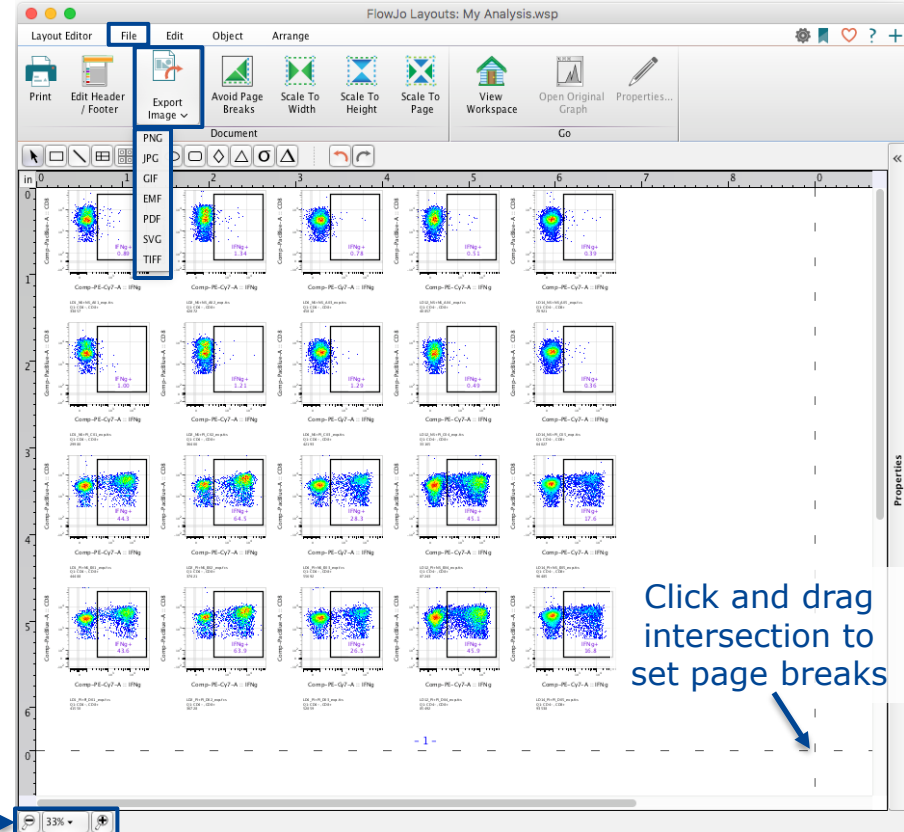
Then click "Create Batch Report"

Default Result: A new batch layout will be created, applying the current layout to multiple samples within the specified group

Export Image Options

Are available under the File tab

- Options include:
 - PNG
 - JPG
 - GIF
 - EMF
 - PDF
 - SVG
 - TIFF

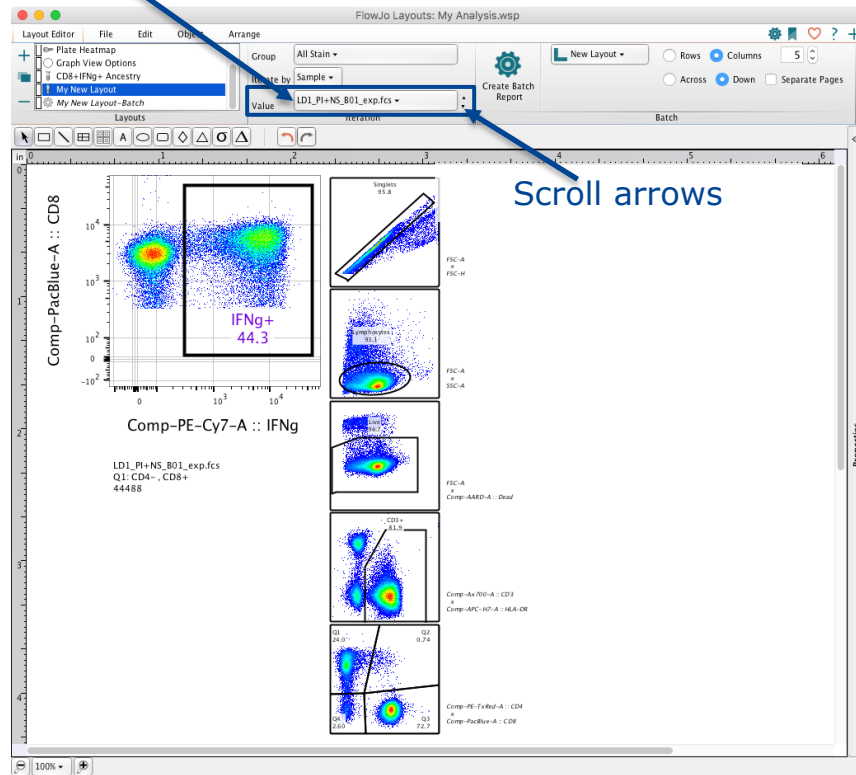


Iteration

Enables scrolling through samples in a group

- With iteration by set to Sample:
 - Click on the Value menu to select and view a specific sample in the specified group
 - Click the up/down arrows next to the value menu to scroll through the samples in a group one by one

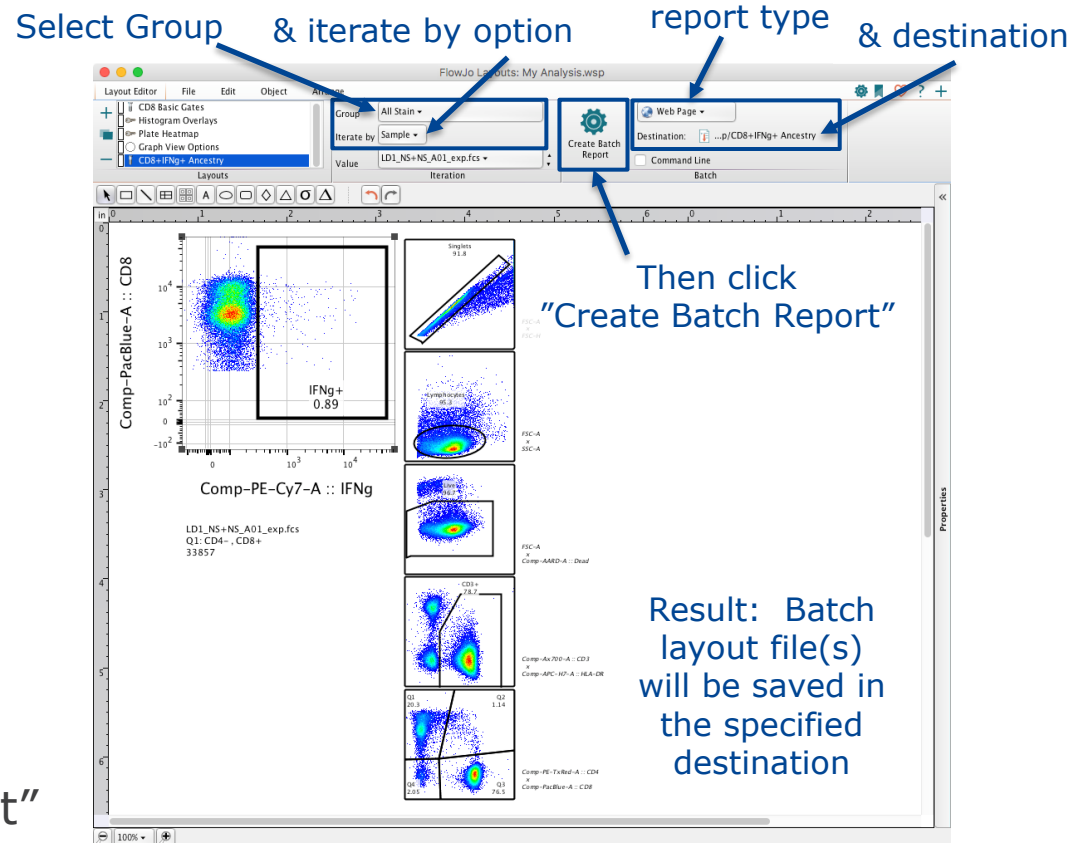
Value menu



Batch Options

Enable reports to be exported directly out of FlowJo

- To save batch report to disc:
 - Specify a group
 - Choose iterate by option
 - Choose report type
 - Printer
 - Web Page
 - PPT
 - PDF
 - Choose destination (location to save)
 - Click “Create Batch Report”



Practice time



The Table Editor

A tool for creating statistical reports

- To create a Table:
 - Click on the Table Editor icon in the workspace ribbon
 - Drag-and-drop Populations and Statistics to the Table Editor window
 - Click "Create Table"

1. Open Table Editor

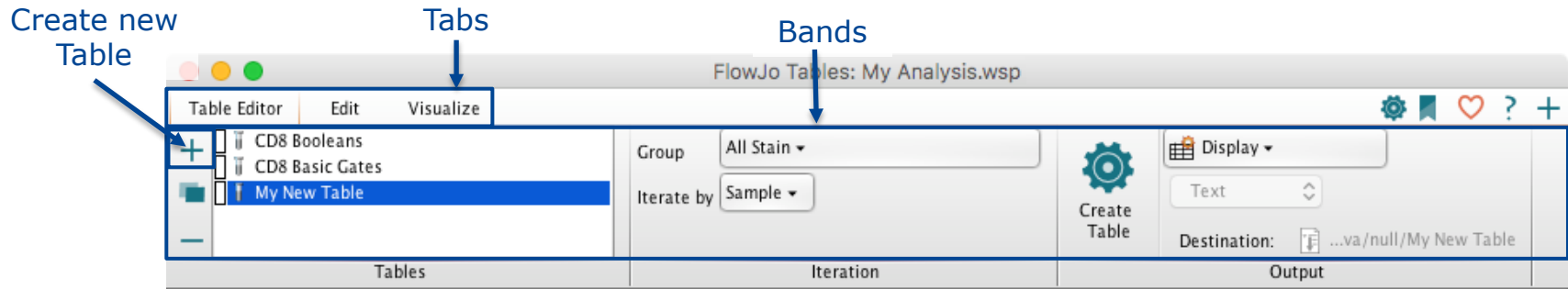
2. Drag-and-drop populations and statistics

3. Click "Create Table"

Column	Population	Statistic	Parameter
1	Singlets/Lymphocytes/Live	Freq. of Par...	
2	Singlets/Lymphocytes/Live/CD3+	Freq. of Par...	
3	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Freq. of Par...	
4	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Median	Comp-Ax488-A :: p-ERK...
5	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Median	Comp-PE-A :: Perforin
6	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/IFNg+	Freq. of Par...	
7	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Perf+	Freq. of Par...	
8	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/pERK+	Freq. of Par...	
9	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q2: HLA-DR-, CD38+	Freq. of Par...	
10	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q2: HLA-DR+, CD38+	Freq. of Par...	
11	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q3: HLA-DR+, CD38-	Freq. of Par...	
12	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q4: HLA-DR-, CD38-	Freq. of Par...	

Within the Table Editor

- Again, the Table Editor has its own taskbar ribbon with tabs and bands to organize actions



- Click the + button to create new tables
- Enumerating and displaying table statistics is similar to batching layouts
 - Specify the group you wish to report, then click "Create Table"
 - Tables can also be sent to a layout report, or saved in various standard file formats (click the Display menu for options)

Table Editor Visualize Tools

Add visual formatting to your displayed

- Options include:
 - Heat Map
 - Standard Deviation
 - Expected Range
- To add a visualization
 - Highlight rows(s)
 - Select a visualization option to apply
 - Click "Create Table"

FlowJo Tables: My Analysis.wsp

Table Editor Edit Visualize

Heat Map Standard Deviation Expected Range

Time Series Correlation

Plots

1. Highlight rows

2. Select visualization to apply

Column	Population	Statistic	Parameter
1 Σ	Singlets/Lymphocytes/Live	Freq. of Parent	
2 Σ	Singlets/Lymphocytes/Live/CD3+	Freq. of Parent	
3 Σ	Formula		
4 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4+, CD8-	Freq. of Parent	
5 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Freq. of Parent	
6 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Median	Comp-Ax488-A :: p-ERK1_2
7 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+	Median	Comp-PE-A :: Perforin
8 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/IFNg+	Freq. of Parent	
9 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Perf+	Freq. of Parent	
10 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/pERK+	Freq. of Parent	
11 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q1: HLA-DR-, CD38+	Freq. of Parent	
12 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q2: HLA-DR+, CD38+	Freq. of Parent	
13 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q3: HLA-DR+, CD38-	Freq. of Parent	
14 Σ	Singlets/Lymphocytes/Live/CD3+/Q1: CD4-, CD8+/Q4: HLA-DR-, CD38-	Freq. of Parent	

Table Editor Output

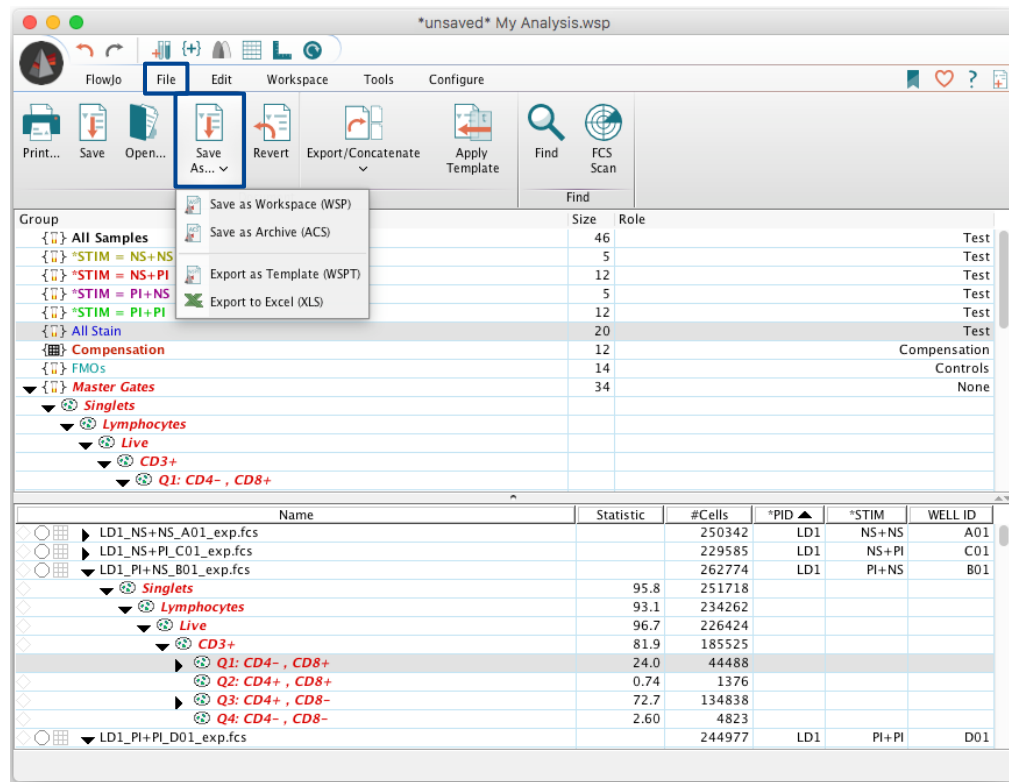
- Visual formatting is maintained if table is created for Display, saved as HTML or sent to a layout
- Values in a displayed table can be copied and pasted outside of FlowJo
- To save a table in text, CSV, Excel or SQL format, choose that option using the Display button next to "Create Table"

	*PID	*STIM	Q1: CD4- , CD8+ p-ERK1_2 Median	pERK+ Freq. of Parent	IFNg+ Freq. of Parent	Perf+ Freq. of Parent	IFNg+Perf+pERK+ Freq. of Parent	CD4/CD8 Ratio
LD1_NS...	LD1	NS+NS	68.2	4.62	0.89	30.2	0.13	▲ 3.77
LD1_NS...	LD1	NS+PI	550	95.0	1.00	30.0	0.42	▲ 4.09
LD1_PI+	LD1	PI+NS	406	94.3	44.3	33.1	27.5	▲ 3.03
LD1_PI+	LD1	PI+PI	401	94.4	43.6	32.3	26.6	▲ 3.05
LD2_NS...	LD2	NS+NS	75.5	0.33	1.34	55.5	0.033	2.79
LD2_NS...	LD2	NS+PI	590	92.3	1.21	52.8	0.59	▲ 3.02
LD2_PI+	LD2	PI+NS	472	93.5	64.5	51.4	47.0	▲ 2.87
LD2_PI+	LD2	PI+PI	454	93.5	63.9	50.7	46.4	▲ 2.91
LD4_NS...	LD4	NS+NS	77.8	7.58	0.78	20.9	0.044	1.51
LD4_NS...	LD4	NS+PI	641	97.2	1.29	23.5	0.30	1.51
LD4_PI+	LD4	PI+NS	489	96.8	28.3	23.6	19.8	▼ 1.20
LD4_PI+	LD4	PI+PI	484	96.3	26.5	22.4	18.3	▼ 1.22
LD12_N...	LD12	NS+NS	62.0	3.86	0.51	36.7	0.056	▲ 3.62
LD12_N...	LD12	NS+PI	481	89.6	0.49	34.7	0.26	▲ 4.26
LD12_PL...	LD12	PI+NS	390	84.6	45.1	39.7	21.1	1.93
LD12_PL...	LD12	PI+PI	381	83.5	45.9	40.2	21.3	1.93
LD14_N...	LD14	NS+NS	67.4	3.86	0.39	14.2	0.054	2.10
LD14_N...	LD14	NS+PI	530	95.5	0.36	13.7	0.15	2.29
LD14_PL...	LD14	PI+NS	396	94.6	17.6	18.1	12.7	1.65
LD14_PL...	LD14	PI+PI	384	93.0	16.8	18.1	11.5	1.66
Mean			370	70.7	20.2	32.1	12.7	2.52
SD			191	39.7	23.1	13.1	15.4	0.95

Workspace Templates

Save all analysis without referencing data

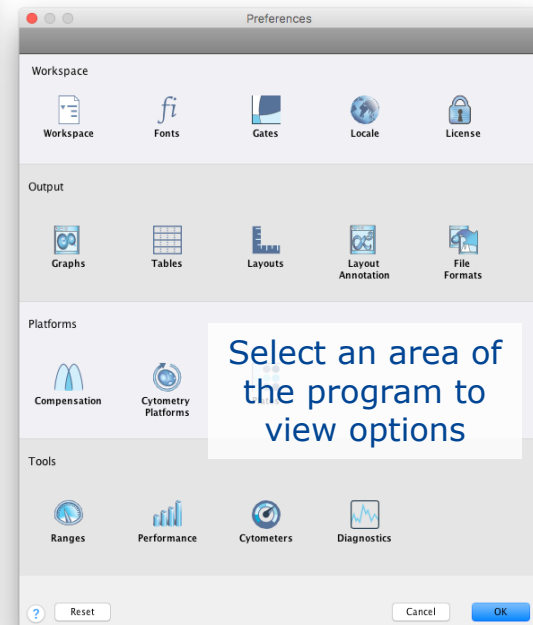
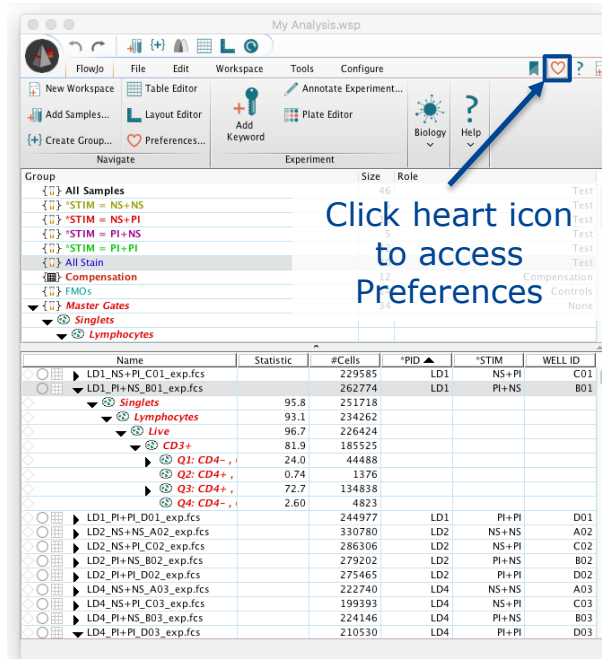
- Save as options are located within the File tab
 - WSP (Normal Save)
 - ACS (Zip file, incl. data)
 - WSPT (Template)
- Templates streamline repetitive analysis of multiple runs using the same staining panel(s)



Preferences

To specify your own personalized defaults

- Click the heart icon at the top left corner of a window to access Preferences
- Allows each user to modify default functionality and appearance



Thank You!

Questions?

Compensation in FlowJo v10



Previewing Compensation

Double click on the square matrix badge next to a sample name

My Analysis.wsp

FlowJo File Edit Workspace Tools Configure

New Workspace Table Editor Add Samples... Layout Editor Create Group... Preferences... Add Keyword Annotate Experiment... Plate Editor Cell Cycle... Kinetics... Proliferation Modeling... Compare Populations...

Group All Samples 46 Size Role

- {+} *STIM = NS+NS 5
- {+} *STIM = NS+PI 12
- {+} *STIM = PI+NS 5
- {+} *STIM = PI+PI 12
- {+} All Stain 20
- ▼ Compensation 12
- FMOs 14
- Master Gates 34
- Singles
- Lymphocytes
- Live

Name	Statistic	#Cells	*PID
Bead Comps_DR APC-H7_F04_exp.fcs		18907	
Bead Comps_ERK A488_F06_exp.fcs		24114	
Bead Comps_IFN PE-Cy7_F07_exp.fcs		9000	
Bead Comps_Perforin PE_F08_exp.fcs		19212	
Bead Comps_US Beads +FP_F09_exp.fcs		30000	
Bead Comps_US Beads No FP_F09_exp.fcs		10290	
Bead Comps_4 PE-TR_F01_exp.fcs		19202	
Bead Comps_8 PE_F03_exp.fcs		14969	
Bead Comps_38 PE-Cy5_F03_exp.fcs		17603	
Cell Comps_AARD_F01_exp.fcs		145743	LD1
Cell Comps_CD3 A700_F02_exp.fcs		129537	LD1
Cell Comps_CD3 A700_F02_exp.fcs		158360	LD1
FMOs NS+PI_No DR_C07_exp.fcs		219336	LD1
FMOs NS+PI_No DR_C07_exp.fcs		269027	LD1
FMOs NS+PI_No DR_C07_exp.fcs		281200	LD1
FMOs NS+PI_No IFN_CD8_exp.fcs		249824	LD1
FMOs NS+PI_No rert_L02_exp.fcs (Contr		276464	LD1
FMOs NS+PI_No p-ERK_C10_exp.fcs (Con		254708	LD1
FMOs NS+PI_No 38_C06_exp.fcs (Control		286676	LD1

Double click a square badge

Workspace Matrices

Acquisition-defined

Trade Comp Spectral Comp AutoSpill Comp AutoSpill Comp Autofluor sub

Displaying matrix 'Acquisition-defined'

Show All

AARD-A : D... APC-H7-A : A488-A : A700-A : PE-A : Perforin PE-Cy5-A : PE-Cy7-A : L PE-TxRed-A : PacBlue-A :

100	0.0354	0.3566	0.0766	0.0439	0.1591	0.0406	0.0881	24.0643
0	100	0	3.2542	0.0121	0.8053	39.4941	0.056	0
1.8552	0	100	0	0.0085	0	0	0	0
-0.1121	35.058	0.0982	100	-0.1126	1.2014	10.612	-0.1002	0
0	0.0134	0.3399	0.0379	100	14.5027	1.3134	37.6909	0
0	2.984	0.0256	7.712	1.6022	100	11.9274	0.7023	0
0	5.7673	0.0598	0.3117	1.8872	0.3682	100	0.8245	0
0	0.0289	0.1116	0.0566	23.9197	52.7587	6.0519	100	0
16.8535	0	0.0597	0	0.0055	0.0075	0	0.0063	100

Edit Matrix SSM

Preview Sample: Bead Comps_DR APC-H7_F04_exp.fcs Preview Population View Overlay Uncompensated

Workspace Matrices

Comp-APC-H7-A Comp-A488-A Comp-A700-A Comp-PE-A Comp-PE-Cy5-A Comp-PE-Cy7-A Comp-PE-TxRed-A

1) Select Compensation group

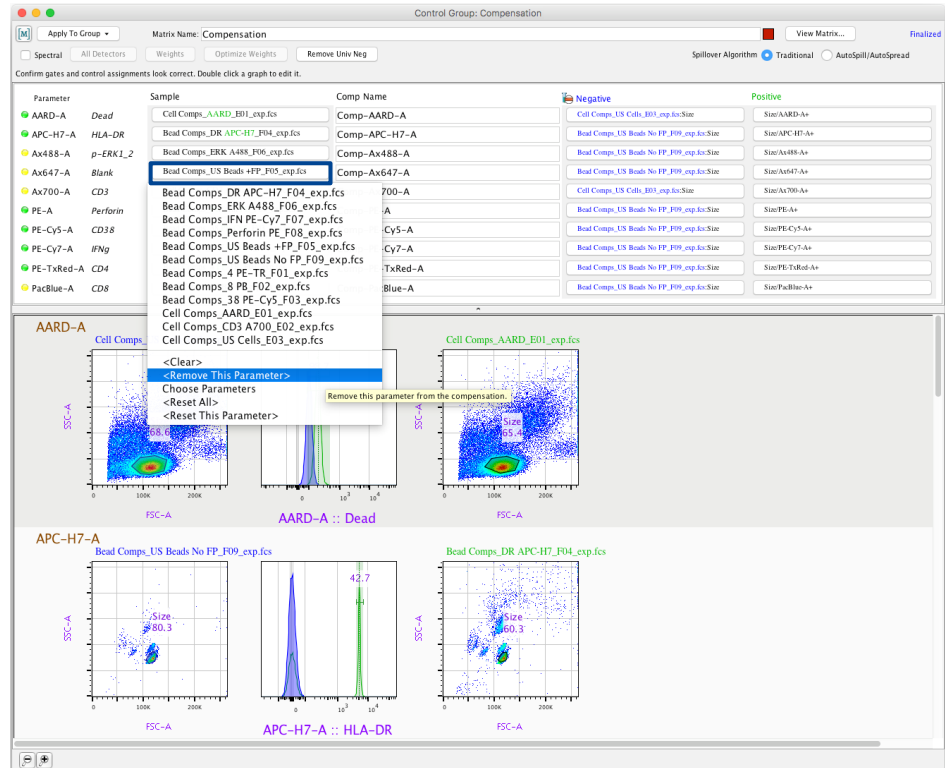
2) Select Compensation action

RESULT: Compensation matrix setup window will open

Choose or Remove Parameters

Click on a Sample column field → drop-down options, including:

- Single color control selection
- Remove Parameter
- Choose Parameters
- Reset All or single parameter



Default is Traditional Compensation

Options for utilizing Spectral unmixing math and AutoSpill robust linear regression can be selected in the header

Spectral

Traditional

AutoSpill

Control Group: Compensation

Matrix Name: My New Compensation

Apply To Group ▾

☒ Spectral ☐ All Detectors ☐ Weights ☐ Optimize Weights ☐ Remove Univ Neg

Spillover Algorithm: ☒ Spectral ☐ Traditional ☐ AutoSpill/AutoSpread

View Matrix... Finalized

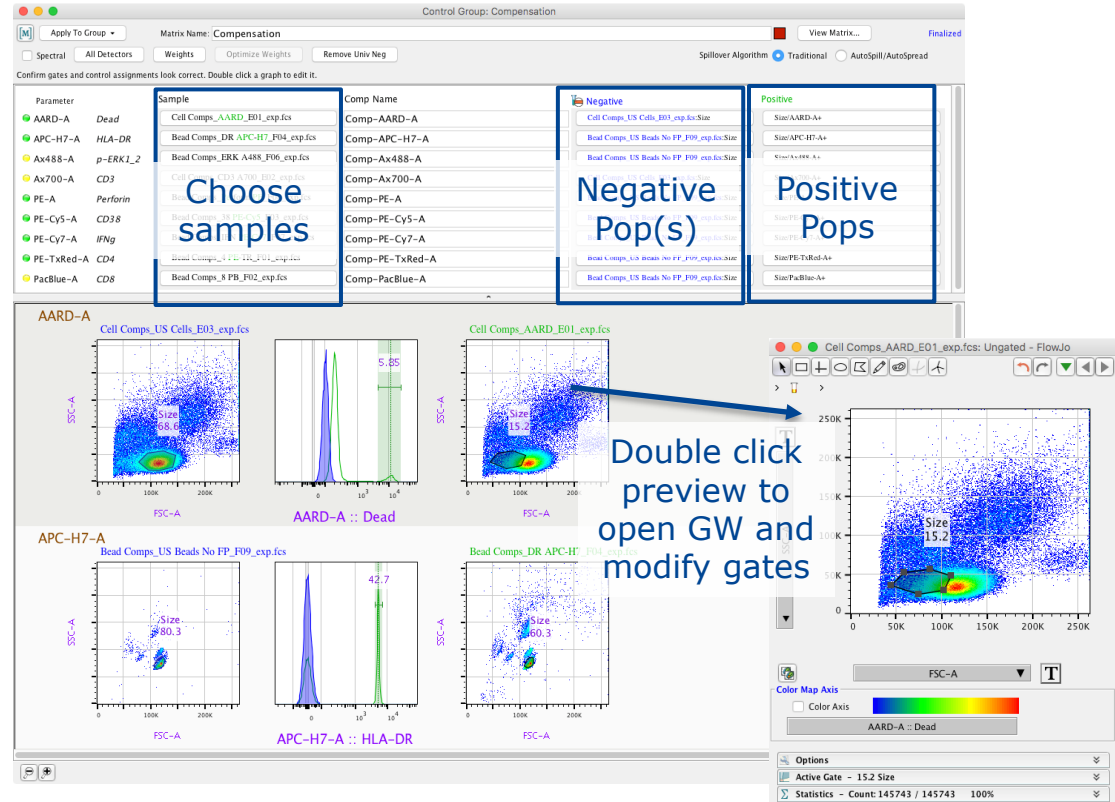
Confirm gates and control assignments look correct. Double click a graph to edit it.

Parameter	Sample	Comp Name	Negative	Positive
AARD-A Dead	Cell Comps_AARD_E01_exp.fcs	Comp-AARD-A	Cell Comps_US Cells_E03_exp.fcs:Size	Size/AARD-A+
APC-H7-A HLA-DR	Bead Comps_DR APC-H7_F04_exp.fcs	Comp-APC-H7-A	Bead Comps_US Beads No FP_F09_exp.fcs:Size	Size/APC-H7-A+
Ax488-A p-ERK1_2	Bead Comps_ERK A488_F06_exp.fcs	Comp-Ax488-A	Bead Comps_US Beads No FP_F09_exp.fcs:Size	Size/Ax488-A+
Ax700-A CD3	Cell Comps_CD3 A700_E02_exp.fcs	Comp-Ax700-A	Cell Comps_US Cells_E03_exp.fcs:Size	Size/Ax700-A+
PE-A Perforin	Bead Comps_Perforin PE_F08_exp.fcs	Comp-PE-A	Bead Comps_US Beads No FP_F09_exp.fcs:Size	Size/PE-A+
PE-Cy5-A CD38	Bead Comps_38 PE-Cy5_F03_exp.fcs	Comp-PE-Cy5-A	Bead Comps_US Beads No FP_F09_exp.fcs:Size	Size/PE-Cy5-A+
PE-Cy7-A IFNγ	Bead Comps_IFN PE-Cy7_F07_exp.fcs	Comp-PE-Cy7-A	Bead Comps_US Beads No FP_F09_exp.fcs:Size	Size/PE-Cy7-A+
PE-TxRed-A CD4	Bead Comps_4 PE-TR_F01_exp.fcs	Comp-PE-TxRed-A	Bead Comps_US Beads No FP_F09_exp.fcs:Size	Size/PE-TxRed-A+
PacBlue-A CD8	Bead Comps_8 PB_F02_exp.fcs	Comp-PacBlue-A	Bead Comps_US Beads No FP_F09_exp.fcs:Size	Size/PacBlue-A+

Review and Adjust Gates

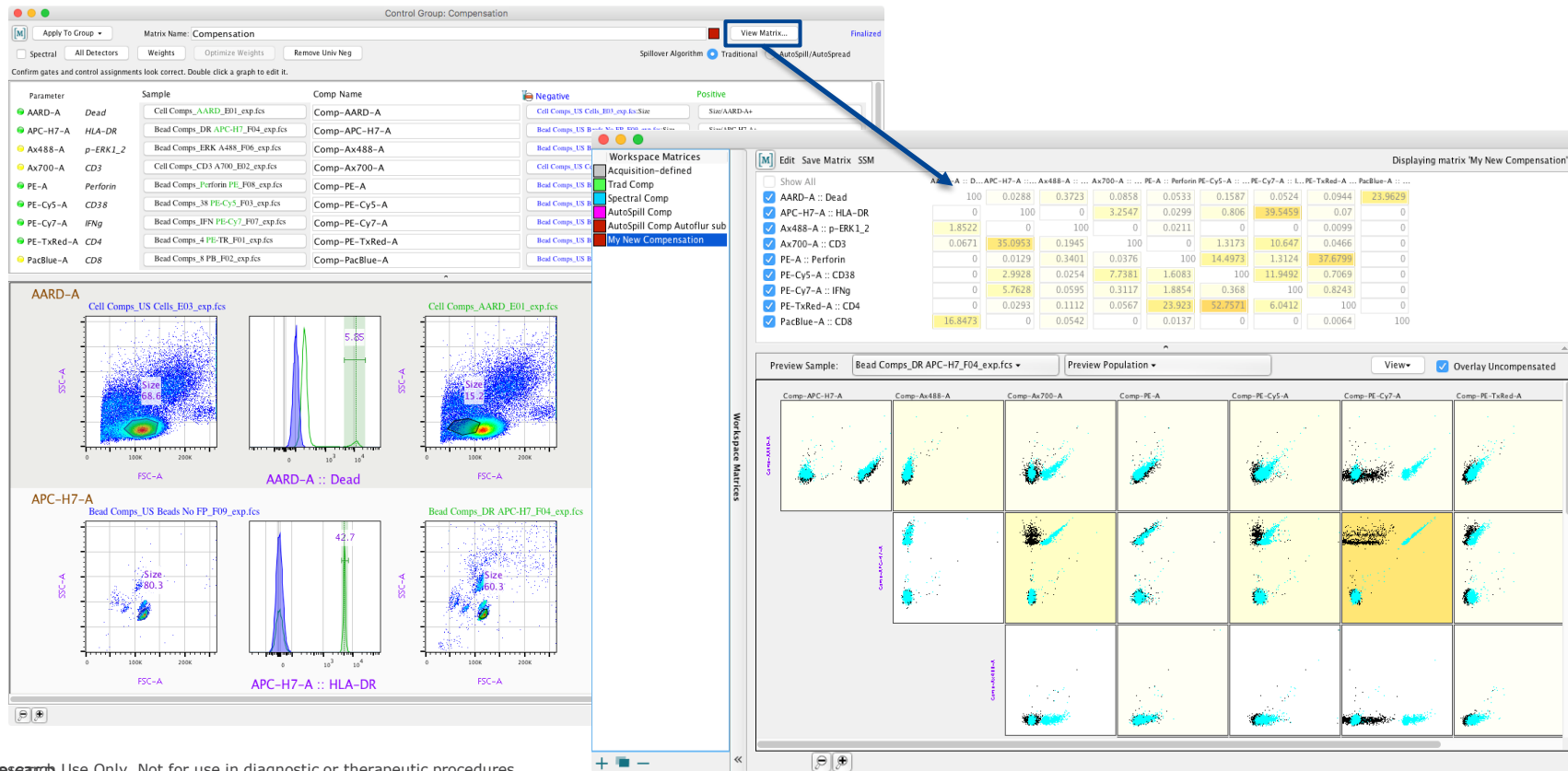
Parameters used and auto-gating for populations

- Define positive and negative
- Choose from dropdowns lists or drag-and drop populations from workspace gating tree
- Double click graph plot preview to modify gates



View Matrix

Once choices are finalized, click to view and apply



Apply Compensation

Select matrix, then drag-and-drop

Drag and drop M to a group or sample
→ applies selected matrix

Selected Matrix

Badge turns the color of applied matrix

Displaying matrix 'My New Compensation'

	AARD-A :: D...	APC-H7-A :: ...	Ax488-A :: ...	Ax700-A :: ...	PE-A :: Perforin	PE-Cy5-A :: ...	PE-Cy7-A :: L...	PE-TxRed-A :: ...	PacBlue-A :: ...
AARD-A :: D...	100	0.0288	0.3723	0.0858	0.0533	0.1587	0.0524	0.0944	23.9629
APC-H7-A :: ...	0	100	0	3.2547	0.0299	0.806	39.5459	0.07	0
Ax488-A :: p-ERK1,2	1.8522	0	100	0	0.0211	0	0	0.0099	0
Ax700-A :: CD3	0.0671	35.0953	0.1945	100	0	1.3173	10.647	0.9466	0
PE-A :: Perforin	0	0.0129	0.3401	0.0376	100	14.4973	1.3124	37.6799	0
PE-Cy5-A :: CD38	0	2.9928	0.0254	7.7381	1.6083	100	11.9492	0.7069	0
PE-Cy7-A :: IFNγ	0	5.7628	0.0595	0.3117	1.8854	0.368	100	0.8243	0
PE-TxRed-A :: CD4	0	0.0293	0.1112	0.0567	23.923	52.7571	6.0412	100	0
PacBlue-A :: CD8	16.8473	0	0.0542	0.0137	0	0	0	0.0064	100

Preview Sample: Bead Comps_DR APC-H7_F04_exp.fcs

Preview Population: View Overlay Uncompensated

Workspace Matrices

Comp-APC-H7-A

Comp-Ax488-A

Comp-Ax700-A

Comp-PE-A

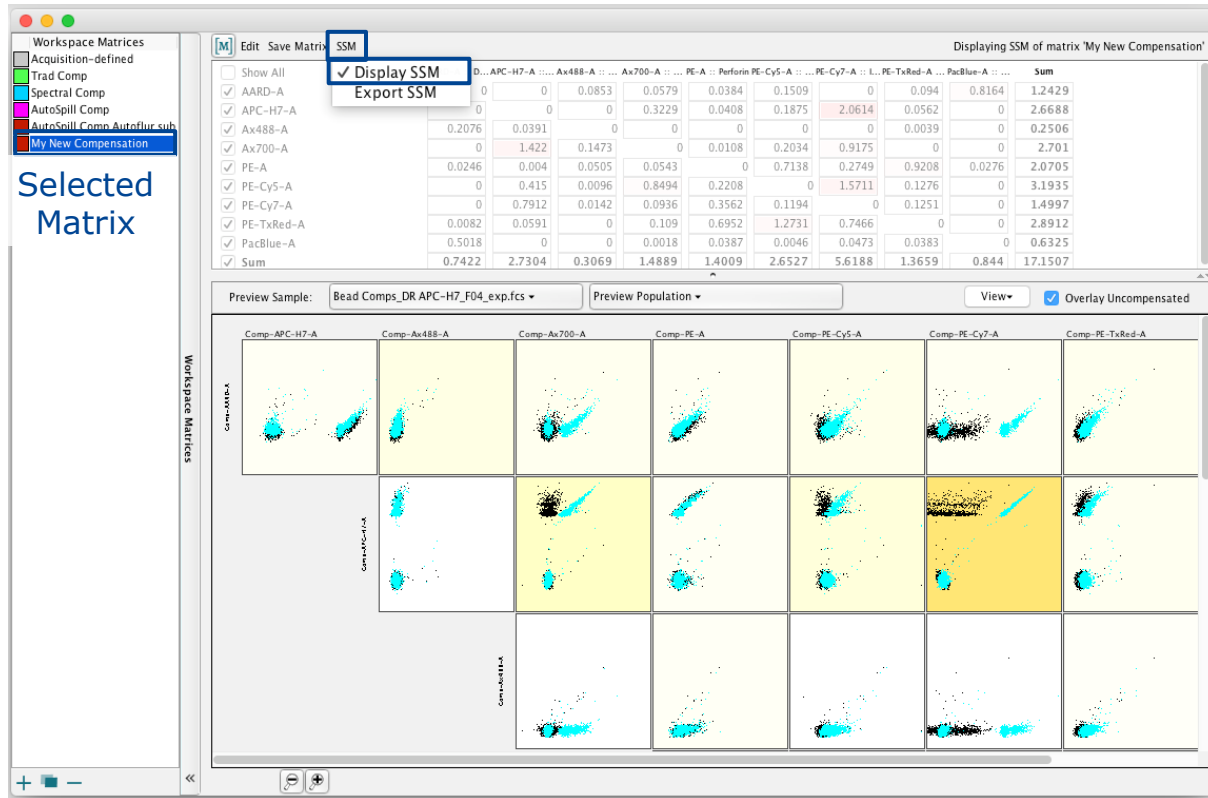
Comp-PE-Cy5-A

Comp-PE-Cy7-A

Comp-PE-TxRed-A

Accessing the Spillover Spreading Matrix

Select matrix, then click SSM to Display or Export



Using a Spillover Spreading Matrix

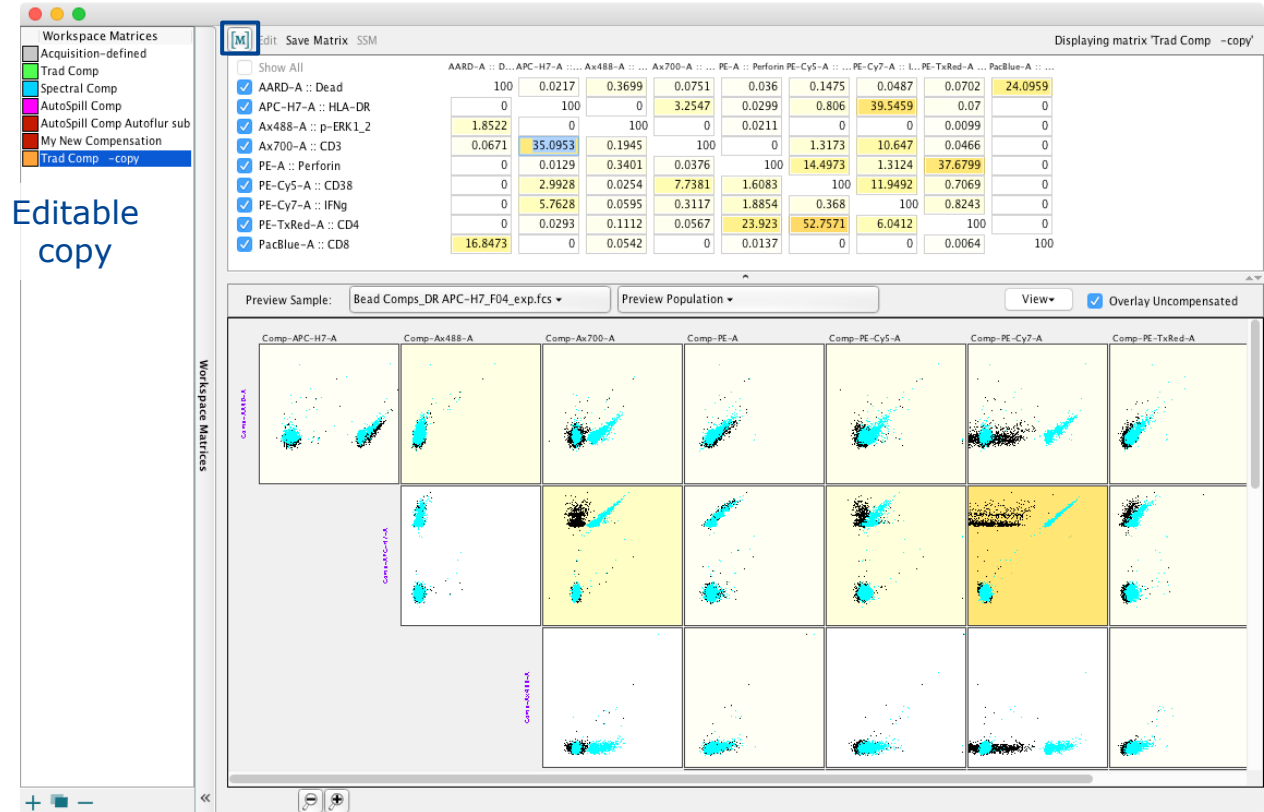
Large numbers with red shading → primary detector's fluorochrome (columns) will spread in secondary detector's channel (Rows)

→ Loss of sensitivity for low expressing populations in that secondary channel *if* that primary fluor antigen is expressed on the same population.

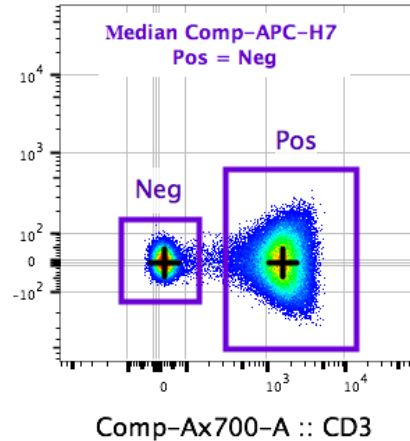
Show All	B515-A	C... B610-A	... B660-A	... B710-A	... B780-A	... C575-A	... C610-A	... P... C660-A	... T... C710-A	... F... C780-A	... B... B670-A	... B... B730-A	... C... B780-A	... C... U310-A	... C... U450-A	... L... U500-A	... C... U570-A	... C... U660-A	... C... U740-A	... C... U785-A	... C... V450-A	... L... V510-A	... C... V570-A	... C... V605-A	... L... V655-A	... KL... V710-A	... C... V750-A	... T... V785-A	Sum		
✓ B515-A	0	0.3386	0.3656	0.2974	0.1239	0.621	0.4031	0.0041	0.2543	0	0.1968	0.0021	0	0.1381	0.0467	0.336	0.3264	0.1082	0.0023	0	0	0.3933	0.2711	0.2993	0.1361	0	0	0	4.6644		
✓ B610-A	0.1127	0	2.6497	1.7932	0.8328	1.1919		0.92	1.0927	0.7799	2.3488	0.5584	0.0615	0.2085	0.0195	0	0.5252	1.2206	0.3809	0.2716	0.2722	0	0.3333	2.9096	1.7473	0.8158	0.9363	1.0448	44.1706		
✓ B660-A	0.1364	0.5315	0	2.3386	1.3725	0.5631	0.4942	3.2433	2.3834	1.1011		2.0263	0.2674	0.3172	0	0	0.1873	2.0194	0.5647	0.4282	0.6809	0.0722	0.5765	0.7269	8.8741	1.31	1.5452	1.921	47.7461		
✓ B710-A	0.1354	0.5441	1.0453	0	2.2489	1.9492	0.5709	0.2963	4.6855	1.6941	2.118	3.2236	0.4014	0.4654	0.0065	0.0511	0.1883	0.4156	1.0155	0.6654	0.7323	0.0938	0.5453	0.3853	1.3669	5.7264	2.737	3.1052	36.4127		
✓ B780-A	0.2341	0.6986	0.5431	0.7403	0	0.8329	0.6307	0.1459	0.3853	8.5401	0.2949	0.3526	0.4928	1.566	0.0459	0	0.2651	0.1327	0.4942	2.1341	0.9449	0.0057	0.6124	0.4714	0.36	0.377	2.4492	0.1191	39.9286		
✓ C575-A	0.0424	1.9982	0.8639	0.6754	0	0	2.9765	0.3844	0.5238	0.342	0.457	0.1293	0.0367	0.2014	0	0	2.7368	0.1877	0.1293	0.0986	0.1759	0	1.6275	1.5294	0.5182	0.2372	0.2317	0.2867	16.6903		
✓ C610-A	0	0.347	1.0752	1.0007	0.48	0.5437	0	0.6176	1.2095	0.8448	0.7095	0.3916	0.0942	0.1008	0	0	0.1334	0.3651	0.2312	0.1763	0	0	0.0052	0.8931	0.6319	0.3944	0.4322	0.5483	11.2257		
✓ C660-A	0.238	1.0988	2.1611	7.4463	2.391	2.2169	1.0328	0	3.8736	3.0857	0	1.3753	0.2239	0.3759	0.0053	0	0.2182	1.9735	0.7133	0.5421	0.84	0.0058	0.6127	1.0494	2.9001	2.1461	1.3706	0.2064	81.0784		
✓ C710-A	0.2323	0.6409	3.4267	2.6679	2.2866	4.034	1.0822	0.3501	0	3.1982	0	3.7772	0.4419	0.4325	0.0281	0	0.4711	0.2663	1.2556	0.6027	1.0011	0	0.7166	0.3659	0.7419	8.1753	2.4534	2.1129	68.9194		
✓ C780-A	0.1681	0.5254	0.3026	0.371	0.9366	0.5497	0.1097	0.3004	0	0.2124	0.1826	0.2638	0.679	0	0	0	0.1168	0	0.2428	1.0268	0.4652	0	0.2974	0.1936	0.0943	0.0834	0.8785	8.4637	28.7071		
✓ B670-A	0	0.0715	1.0139	0.7532	0.4003	0.2028	0	0.7894	0.8355	0.7507	0	1.6043	0.3281	0	0	0	0	0.1893	0.1496	0.1257	0	0	0.0994	0	0.3975	0.2077	0.1932	0.3082	8.4203		
✓ B730-A	0	0.1368	0.2564	1.4657	0.6963	0.5515	0.1605	0	1.8614	1.1939	0.1382	0	0.4558	0.2842	0.0843	0	0.128	0	0.7259	0.5524	0	0	0.1326	0	0.136	0.883	1.1473	1.3907	12.3809		
✓ B780-A	0	0.2077	0	0.4514	3.0128	0.4101	0.2436	0	0.4743	6.352	0.21	0.7009	0	1.1959	0.1289	0	0	0.3985	1.9665	0.2052	0	0.2013	0.3174	0	0.1392	0.2938	0	5.4068	23.7013		
✓ U390-A	0	0.1216	0	0	0	0.4568	0.1426	0	0	0	0	0	0	0	0	0	0.4907	0.2984	0.2899	0	0.002	0.1043	0.2781	0	0.1676	0.1306	0	0	0.1305	0	
✓ U450-A	0.1743	0.2722	0	0.1743	0	1.9562	0.3811	0.1386	0	0	0	0	0	0	0	0	3.0982	0.1685	0.9	1.9053	0	0.16785	0.3189	0.2847	0.2392	0.3414	2.0067	0.6367	0.618	0.2763	15.542
✓ U500-A	0.5774	0.422	0	0.2431	0	3.1989	0.7145	0	0.2549	0.0119	0.3459	0	0	0	0	0	1.3282	0.6859	0	1.6785	0.3189	0.2847	0.2392	0.3414	2.0067	0.6367	0.618	0.2763	0	0.2098	0.0146
✓ U570-A	0.0665	3.7848	1.3291	0.6307	0.2852	2.6041	5.8792	0.7111	0.6288	0.4377	0.7776	0.2031	0	0	0	0	0.5972	0.1268	0.0711	0	0.4764	0.3182	0.2725	0.5088	0	2.7078	2.4427	0.5675	0.1576	0.1342	51.9846
✓ U660-A	0	0.1707	2.9006	1.2139	0.6039	0.4625	0.306	2.3327	1.4807	0.1038	8.2951	1.8182	0.325	0.9696	0.134	0.113	0.1055	0	0.16182	1.3161	0.0777	0.0063	0.1344	0.2498	2.5206	0.6624	0.7575	0.889	30.4962		
✓ U740-A	0	0.2222	0.4975	2.8962	2.4377	0.552	0.2029	0	0.2127	1.4977	0	5.3914	0.6845	1.7906	0.169	0.0985	0.1624	0.1376	0	2.9492	0.1723	0	0.1178	0.0048	0.1208	0.8175	2.2198	2.6786	2.7037		
✓ U785-A	0	0.1545	0	0.3911	0.757	0.3086	0	0	0.2901	1.1045	0.2235	0.2231	0.7381	3.1007	0.3551	0.2563	0.2959	0	0.4701	0	0	0	0.3055	0.2381	0	0	0.6004	2.5838	12.3964		
✓ V450-A	0.0446	0.0598	0	0	0	0.4282	0.1316	0	0.0538	0	0.0028	0	0	0.10718	1.0495	0.3322	0.209	0.0465	0.034	0	0	0.6973	0.3662	0.2574	0.1036	0.0513	0.0629	0.1	5.1025		
✓ V510-A	0.7941	0.2297	0	0.1547	0.0793	1.6991	0.7555	0.087	0.1627	0	0.203	0.1256	0	1.7821	0.10184	2.1514	0.7977	0.1951	0.1277	0.0752	0.9707	0	1.4708	1.2915	0.5662	0.2906	0.3788	0.3997	15.8066		
✓ V570-A	0	0.1696	0.7577	0.6661	0.3129	7.6938	2.7641	0.3708	0.6984	0.5536	0.6443	0.3454	0	0.3164	0.1472	0	3.3054	0.5143	0.3617	0.2696	0.7871	0.0108	0	2.4184	1.5168	0.7472	0.985	1.1078	28.3642		
✓ V605-A	0	0.6501	0.9981	0.8946	0.4701	2.035	4.712	0.6073	0.9499	0.7616	2.0555	0.7046	0.0731	0.3739	0.0795	0.0627	0.9419	1.4062	0.5725	0.4948	0.4507	0.0812	0.4823	0	2.3078	1.2075	1.5683	1.7195	26.6607		
✓ V655-A	0	0.0852	0.9766	0.7422	0.3716	0.2956	0.6482	0.7012	0.7338	0.611	2.5225	1.205	0.1616	0.4517	0.0979	0	0.1138	2.5522	0.8206	0.6425	0.5817	0.1265	0.1449	0.7955	0	1.9323	2.2523	2.2755	21.8419		
✓ V710-A	0	0.1485	0.5393	3.0619	1.0766	0.5284	0.1851	0.0896	1.6216	0.9927	0.2695	4.7297	0.4512	1.2071	0.1098	0	0.0846	0.2322	2.6149	1.7435	0.7764	0.1344	0.201	0.1698	0.4375	0	5.6643	8.6018	33.6714		
✓ V750-A	0	0.1209	0	0.9053	1.4112	0.192	0	0.0029	0.4393	1.1097	0.0047	1.5818	0.35	1.419	0.0604	0	0.0911	0	2.0175	2.0226	0.4913	0.1015	0.1343	0.1044	0.1377	0.9992	0	8.4272	22.124		
✓ V785-A	0	0.0542	0	0.2421	0.8591	0	0.1295	0	0.2055	1.4545	0.0037	0.428	0.3196	1.9129	0.062	0	0.1037	0.0614	0.6316	2.6335	0.5631	0.0813	0.1321	0.1187	0.1355	0.3181	0.3046	0	13.9497		
✓ Sum	2.9563	14.7051	41.153	59.2293	35.0533	62.5467	39.2397	11.902	26.6119	37.6502	52.2474	31.0801	6.1706	25.3843	4.9514	5.676	14.589	12.9983	16.2825	21.4049	15.6332	4.6098	13.3453	25.2163	33.5904	27.6594	33.7732	69.9257	745.5853		

Editing Compensation

Click Edit → Copy where you can change values



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

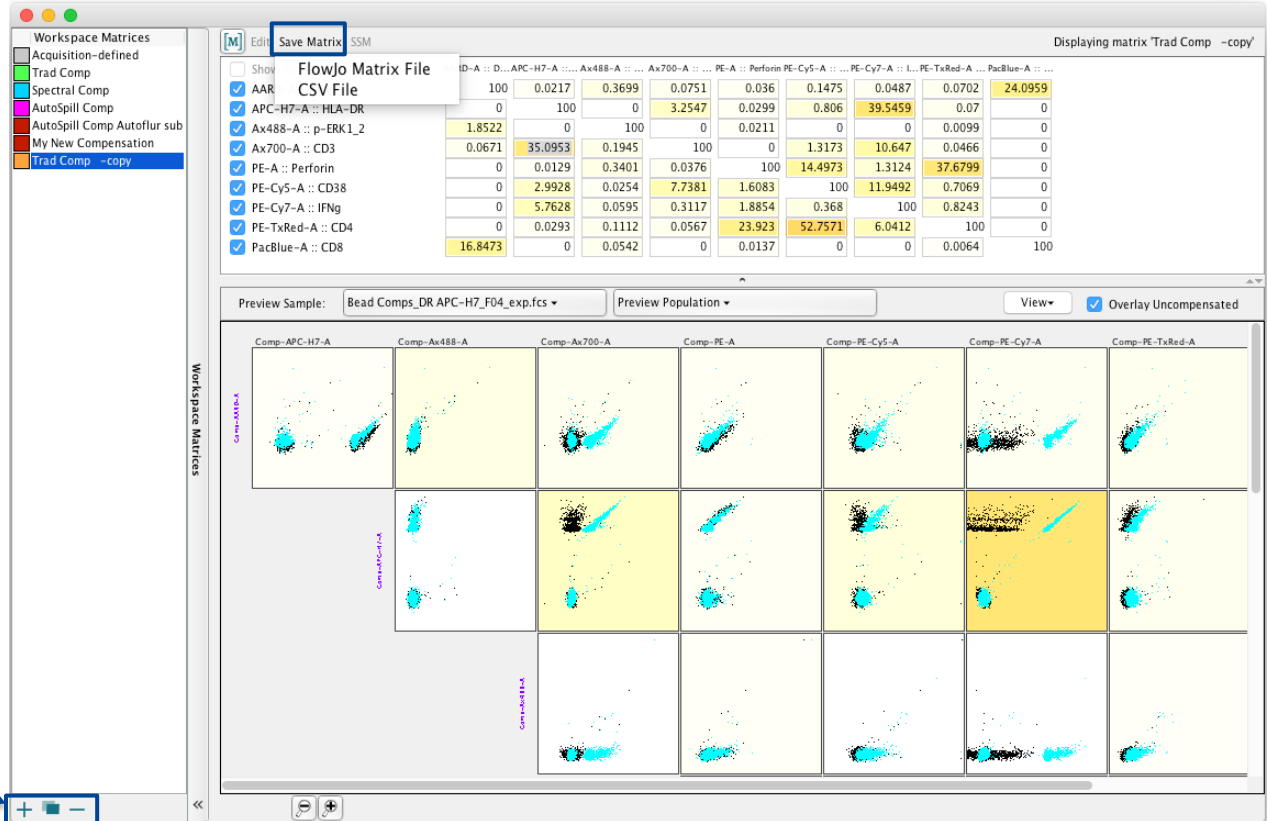


Saving Compensation

Click Save Matrix

- CSV → use outside of FlowJo
- FlowJo Matrix (.mtx) → import back into FlowJo workspace

Add, duplicate or delete a matrix



Plugin setup

Plugins

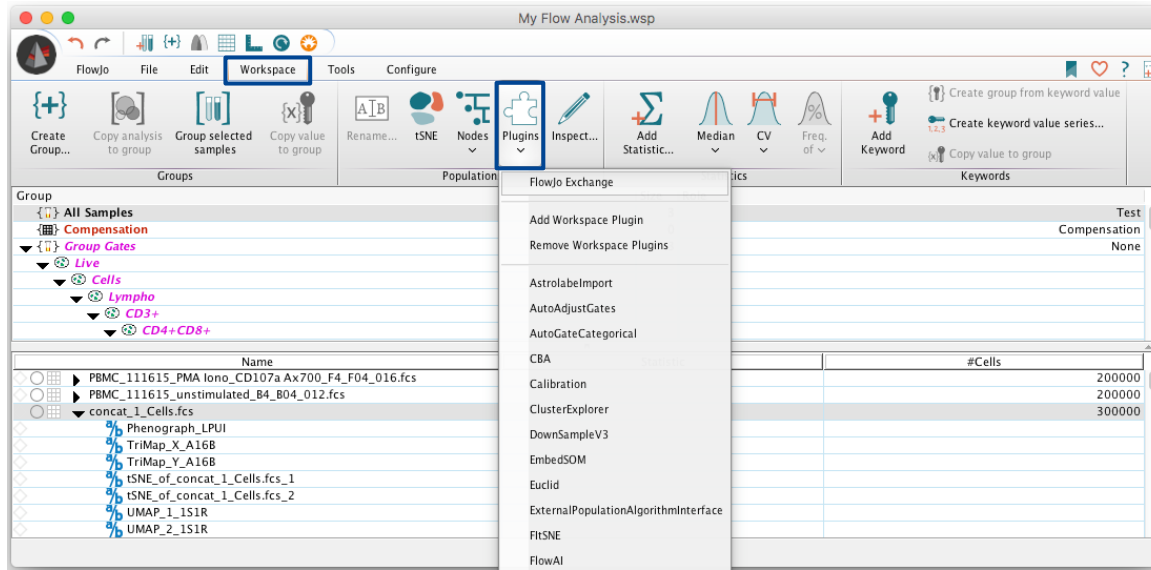
Apps that run some external algorithm or function

- Can be used to:
 - Cleanup samples
 - Embed data in fewer dimensions (dimensionality reduction)
 - Classify events into populations based on similarity (clustering)
 - Visualize population relationships (comparisons)
- Must be downloaded and installed prior to use

The Plugin Menu

Is located in the Workspace Tab → Populations Band

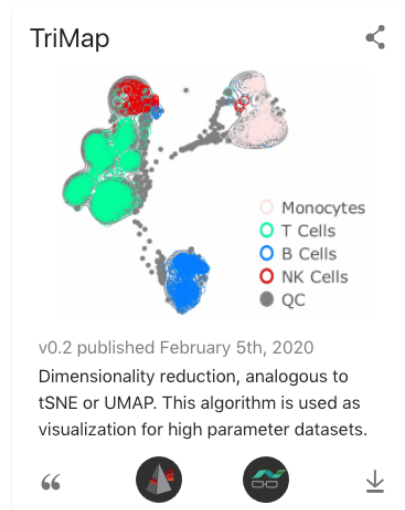
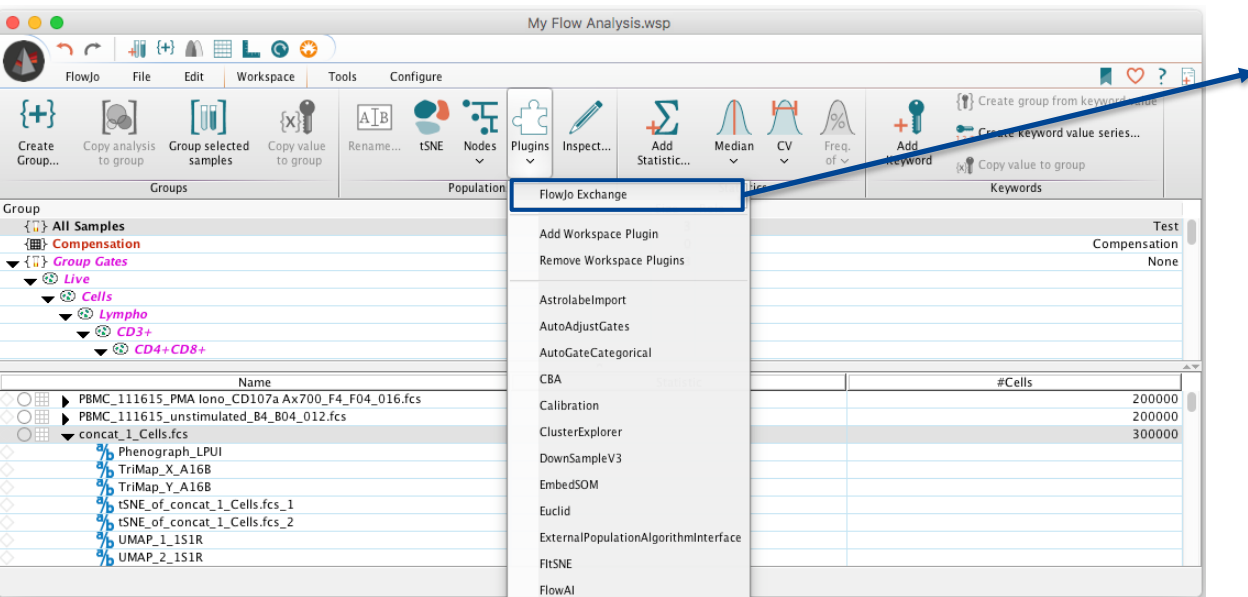
- Once plugins are downloaded and installed, they will be:
 - listed in the drop-down Plugins menu
 - used like an action button to initiate a plugin process



Download Plugins

From the FlowJo Exchange

- 1st plugin menu item is a link to the Exchange

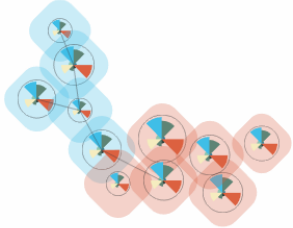


The FlowJo Exchange

Lists all available plugins with download links

- Click on a link to download the plugin and associated documentation

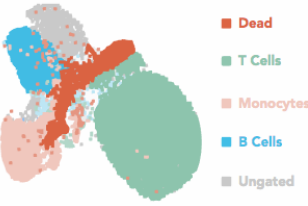
FlowSOM



v2.9 published November 21st, 2020
Cluster using Self-Organizing Maps

“   ⬇

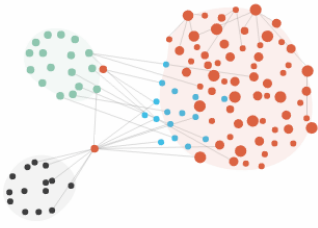
UMAP



v3.1 published March 25th, 2020
A dimensionality reduction technique similar to t-SNE

“   ⬇

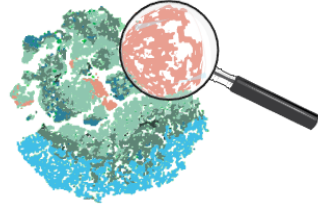
Phenograph



v3.0 published September 26th, 2020
Delineate clusters by unsupervised nearest-neighbors grouping of biological parameters.

“   ⬇

ClusterExplorer



v1.5.7 published December 8th, 2020
ClusterExplorer illustrates a profile of relative intensity values across parameters in flow cytometry data.

“  ⬇

Installing Plugins

Requires a few initial steps

For each new Plugin you want to use:

- Step 1 – Download the Plugin package and unzip
- Step 2 – Place Plugin.jar in your Plugins folder

If this is your first time installing a Plugin

- Step 3 – Setup/Configure Preferences

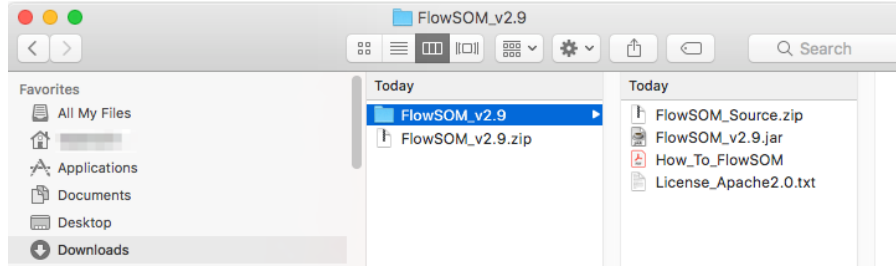
If the plugin requires R

- Step 4 – Install R (if not already installed)
- Step 5 – Run the Plugin package installation script within R

Steps 1 & 2

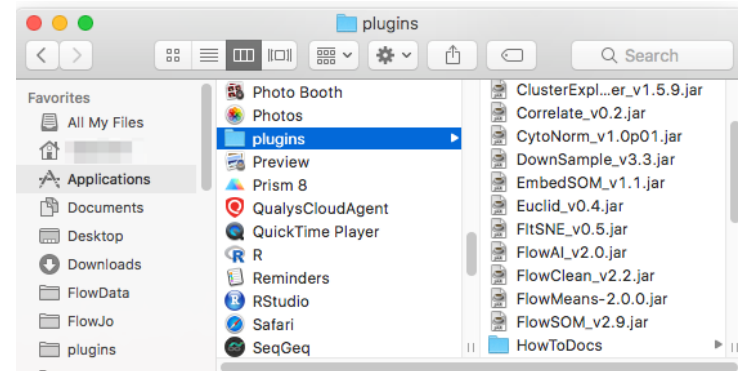
Apply to all plugins

1. Download plugin package and unzip



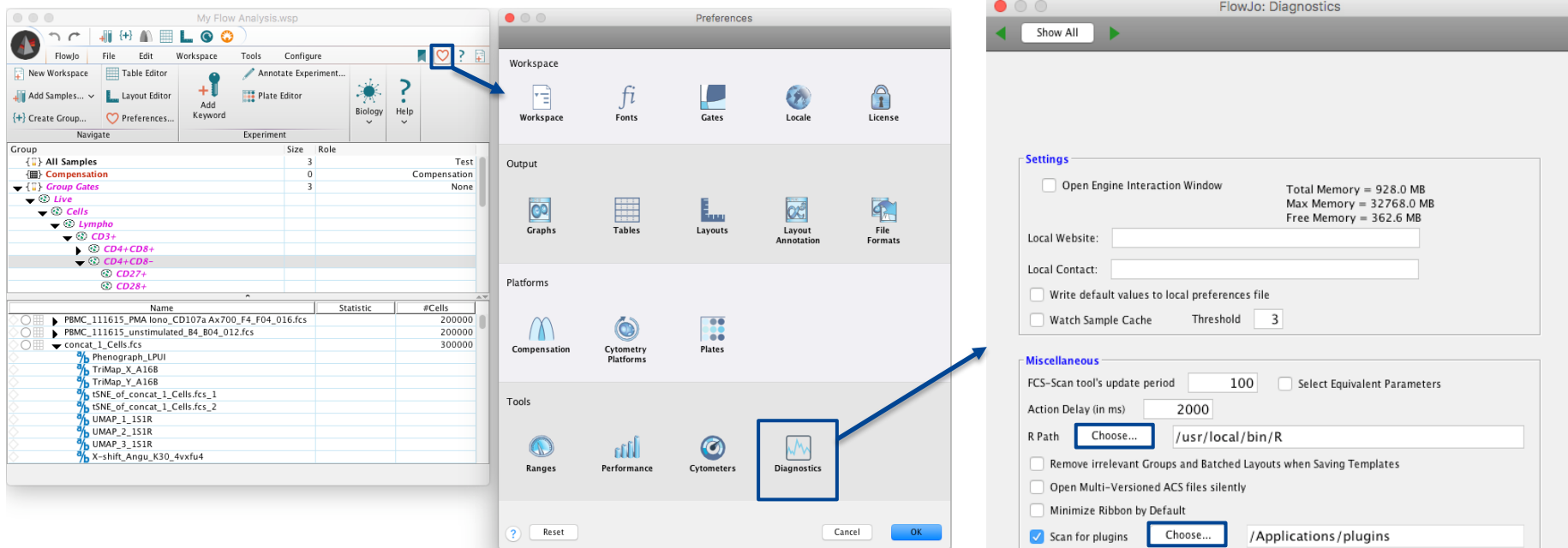
2. Put the Plugin.jar file in your plugins folder

- Restart FlowJo → the plugin name will display in your Plugins action menu



Step 3 – Configure Diagnostics Preferences

Tells FlowJo the location of your plugins and R installation



The first screenshot shows the FlowJo software interface with the 'Configure' button highlighted in the top ribbon. The second screenshot shows the 'Preferences' dialog box with the 'Diagnostics' icon highlighted. The third screenshot shows the 'FlowJo: Diagnostics' settings window with the 'R Path' and 'Scan for plugins' options highlighted.

- Choose the location your plugins folder and R installation
- Restart FlowJo → applies the change

Step 4 – Install R

Required for certain plugins to run

- Navigate to <https://cran.r-project.org/>
- Download and Install the R base package for your operating system

Download and Install R

Precompiled binary distributions of the base system and contributed packages, **Windows and Mac** users most likely want one of these versions of R:

- [Download R for Linux](#)
- [Download R for \(Mac\) OS X](#)
- [Download R for Windows](#)
- In addition to the base R package, also download and install:
 - X-Quartz – If using a Mac. Link: <https://www.xquartz.org/>
 - R-Tools – If using a PC

[base](#)

Binaries for base distribution. This is what you want to **install R for the first time**.

[contrib](#)

Binaries of contributed CRAN packages (for R $\geq$ 2.13.x; managed by Uwe Ligges). There is also information on [third party software](#) available for CRAN Windows services and corresponding environment and make variables.

[old contrib](#)

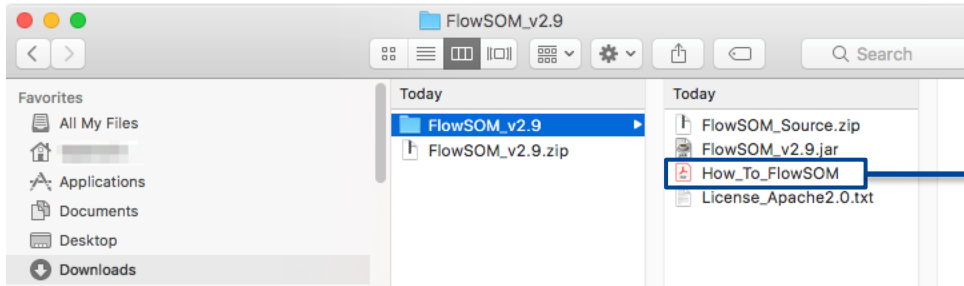
Binaries of contributed CRAN packages for outdated versions of R (for R $<$ 2.13.x; managed by Uwe Ligges).

[Rtools](#)

Tools to build R and R packages. This is what you want to build your own packages on Windows, or to build R itself.

Step 5 – Installing R Packages

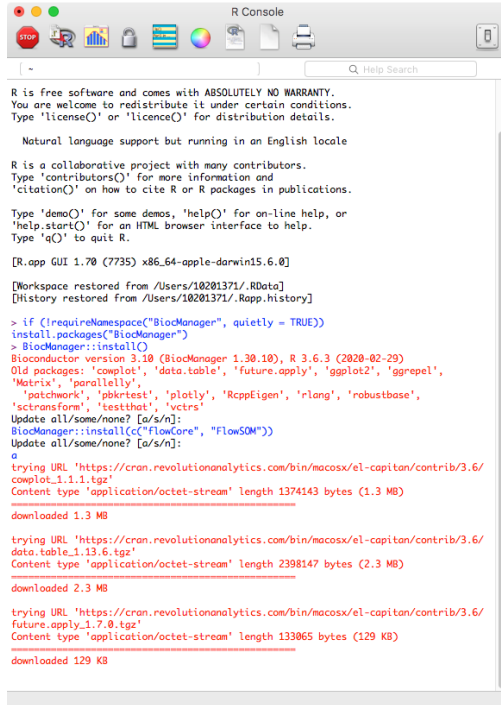
- For each Plugin that utilizes the R environment, the algorithm package installation script must first be run within R.
- Each Plugin downloaded from the FlowJo Exchange site will come with a “How To” document, which specifies the packaging scripts for any R dependencies.



```
if (!requireNamespace("BiocManager", quietly = TRUE))  
  install.packages("BiocManager")  
BiocManager::install()  
BiocManager::install(c("flowCore", "FlowSOM"))  
install.packages("pheatmap")
```

Step 5 – Installing R packages

- Open R and copy/paste, or type the script into the R console



```
R Console

R is free software and comes with ABSOLUTELY NO WARRANTY.
You are welcome to redistribute it under certain conditions.
Type 'license()' or 'licence()' for distribution details.

Natural language support but running in an English locale

R is a collaborative project with many contributors.
Type 'contributors()' for more information and
'citation()' on how to cite R or R packages in publications.

Type 'demo()' for some demos, 'help()' for on-line help, or
'help.start()' for an HTML browser interface to help.
Type 'q()' to quit R.

[R.app GUI 1.70 (7735) x86_64-apple-darwin15.6.0]

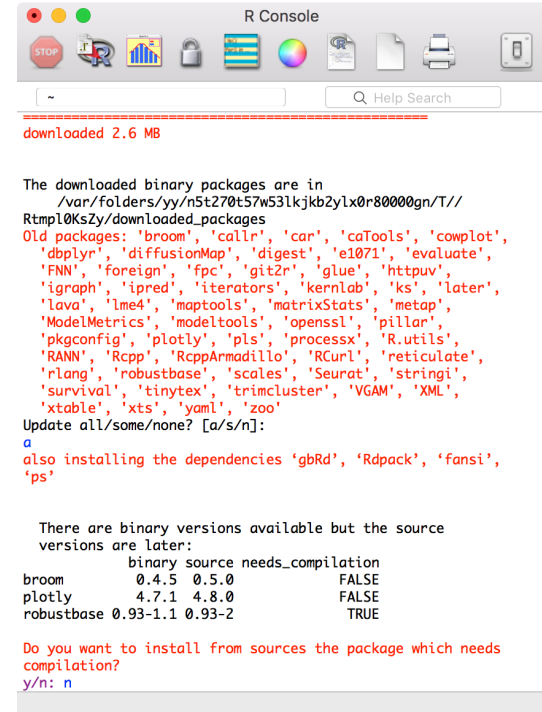
[Workspace restored from /Users/10201371/.RData]
[History restored from /Users/10201371/.Rapp.history]

> if (!requireNamespace("BiocManager", quietly = TRUE))
+ install.packages("BiocManager")
> BiocManager::install()
Bioconductor version 3.10 (BiocManager 1.30.10), R 3.6.3 (2020-02-29)
Old packages: 'cowplot', 'data.table', 'future.apply', 'ggplot2', 'Matrix',
'parallelly',
'patchwork', 'pkrtest', 'plotly', 'RcppEigen', 'rlang', 'robustbase',
'sctransform', 'testthat', 'vctrs'
Update all/some/none? [a/s/n]:
BiocManager::install(c("FlowCore", "FlowSOM"))
Update all/some/none? [a/s/n]:
a
trying URL 'https://cran.revolutionanalytics.com/bin/macosx/el-capitan/contrib/3.6/
cowplot_1.1.1.tgz'
Content type 'application/octet-stream' length 1374143 bytes (1.3 MB)
downloaded 1.3 MB

trying URL 'https://cran.revolutionanalytics.com/bin/macosx/el-capitan/contrib/3.6/
data.table_1.13.6.tgz'
Content type 'application/octet-stream' length 2398147 bytes (2.3 MB)
downloaded 2.3 MB

trying URL 'https://cran.revolutionanalytics.com/bin/macosx/el-capitan/contrib/3.6/
future.apply_1.7.0.tgz'
Content type 'application/octet-stream' length 133065 bytes (129 KB)
downloaded 129 KB
```

- You will notice the package components being downloaded
- If prompted to update a previously installed package, select all (a), unless the component requires compiling, then select no (n)



```
R Console

downloaded 2.6 MB

The downloaded binary packages are in
/var/folders/yy/n5t270t57w53lkjkb2ylx0r80000gn/T//
Rtmp10KsZy/downloaded_packages
Old packages: 'broom', 'callr', 'can', 'caTools', 'cowplot',
'dbplyr', 'diffusionMap', 'digest', 'e1071', 'evaluate',
'FNN', 'foreign', 'fpc', 'git2r', 'glue', 'httpuv',
'igraph', 'ipred', 'iterators', 'kernelab', 'ks', 'later',
'lava', 'lme4', 'mapproj', 'matrixStats', 'metap',
'ModelMetrics', 'modeltools', 'openssl', 'pillar',
'pkgconfig', 'plotly', 'pls', 'processx', 'R.utils',
'RANN', 'Rcpp', 'RcppArmadillo', 'RCurl', 'reticulate',
'rimg', 'robustbase', 'scales', 'Seurat', 'stringi',
'survival', 'tinytex', 'trimcluster', 'VGAM', 'XML',
'xtable', 'xts', 'yaml', 'zoo'
Update all/some/none? [a/s/n]:
a
also installing the dependencies 'gbRd', 'Rdpack', 'fansi',
'ps'

There are binary versions available but the source
versions are later:
      binary source needs_compilation
broom    0.4.5  0.5.0                FALSE
plotly   4.7.1  4.8.0                FALSE
robustbase 0.93-1.1 0.93-2                TRUE

Do you want to install from sources the package which needs
compilation?
y/n: n
```

Available Plugins

Can be classified into several groups

Pre-processing	Dimensionality Reduction	Clustering	Interpretation +Dig Deeper
Downsample	tSNE	X-Shift	ClusterExplorer
<i>CytoNorm</i>	UMAP	<i>FlowSOM</i>	HyperFinder
<i>FlowAI</i>	TriMAP	Phenograph	<i>Euclid</i>
<i>FlowClean</i>	<i>EmbedSOM</i>	<i>flowMeans</i>	
IndexSort			

Bold-Italic → Requires R



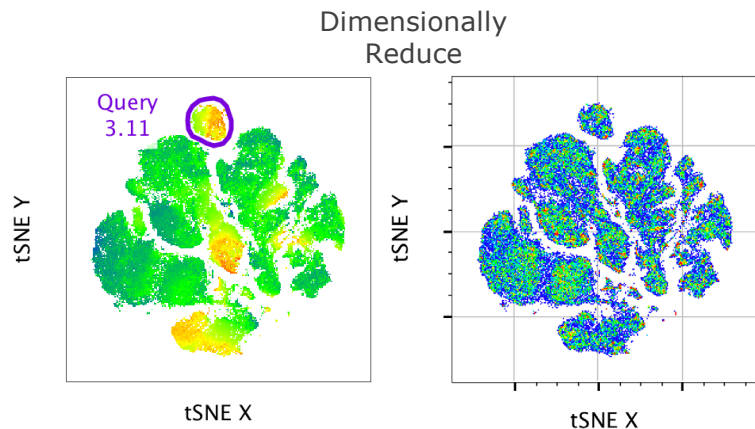
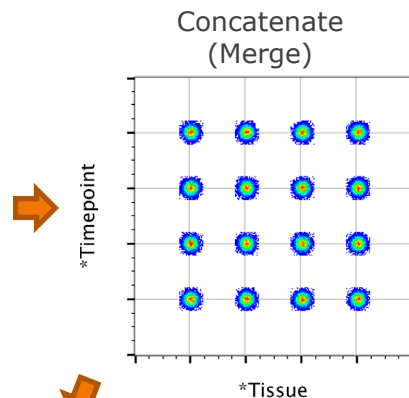
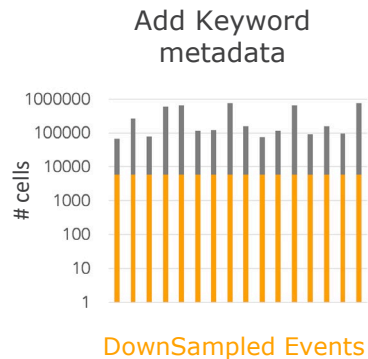
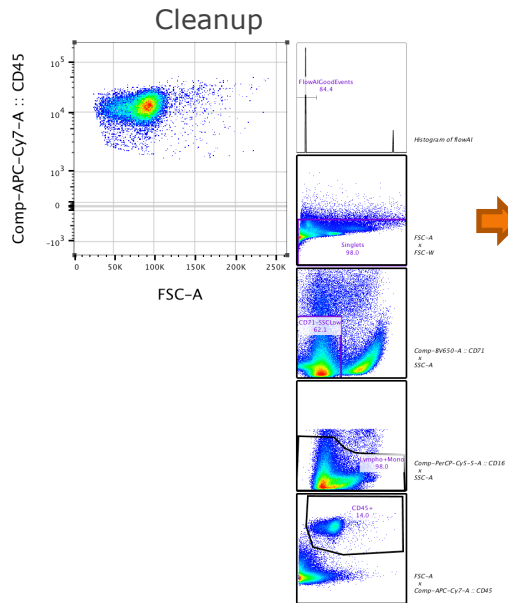
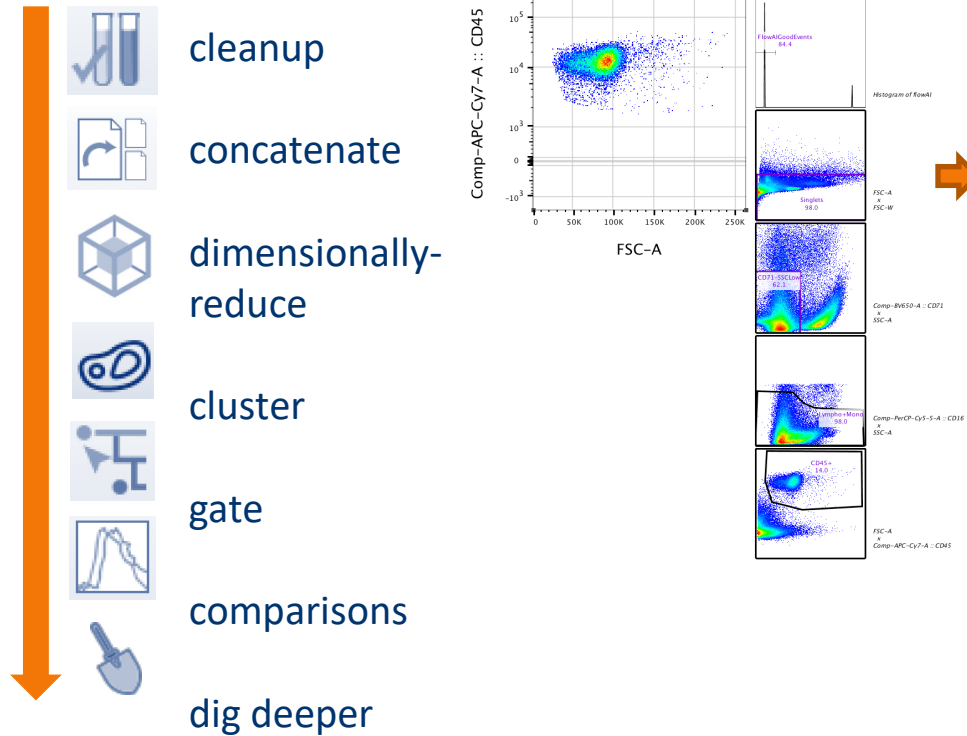
Visualization	Utility
<i>ViolinBox</i>	CBA
<i>MiST</i>	StainIndex
<i>Sunburst</i>	<i>iCellR</i>
Correlate	PluginWizard

Why Pugins?

- Plugins Enable:
 - Quick access to algorithmic processes
 - Point and click interface for non-programmers
- Allowing for:
 - Data clean up
 - Population discovery
 - Exploration
 - Visualization
 - Computational Sorting

Employing Plugins

As part of a discovery workflow



Employing Plugins



cleanup



concatenate



dimensionally-
reduce



cluster



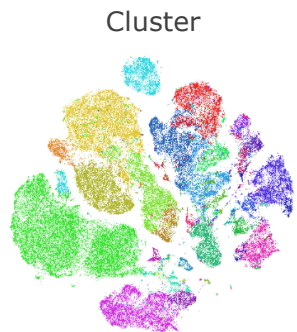
gate



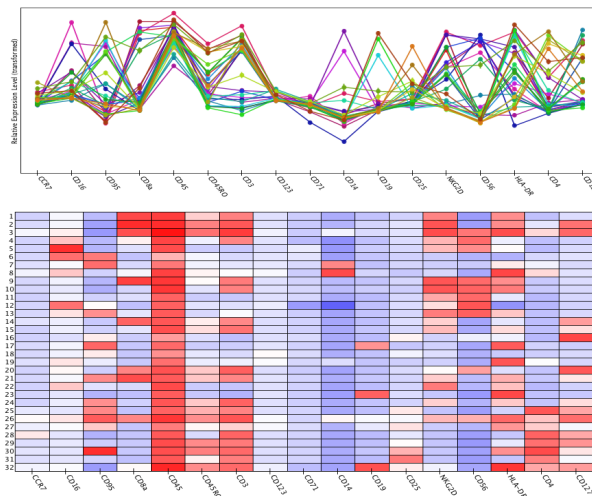
comparisons



dig deeper



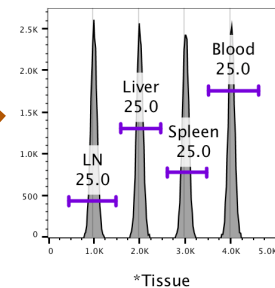
Explore



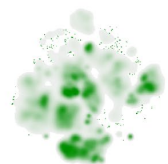
Identify
Differentially Expressed
Populations



Gate



Compare Conditions



blood



spleen



liver



lymph node

Additional Resources

To empower your discovery

- Download Plugins:

<https://www.flowjo.com/exchange/>

- Searchable Documentation

<https://docs.flowjo.com/flowjo>

- FlowJo University

<https://www.flowjo.com/learn/flowjo-university/flowjo>

- Technical Support

flowjo@bd.com

Dimensionality Reduction and Clustering

Analysis Workflow

for Discovery



cleanup



concatenate



dimensionally-
reduce



cluster



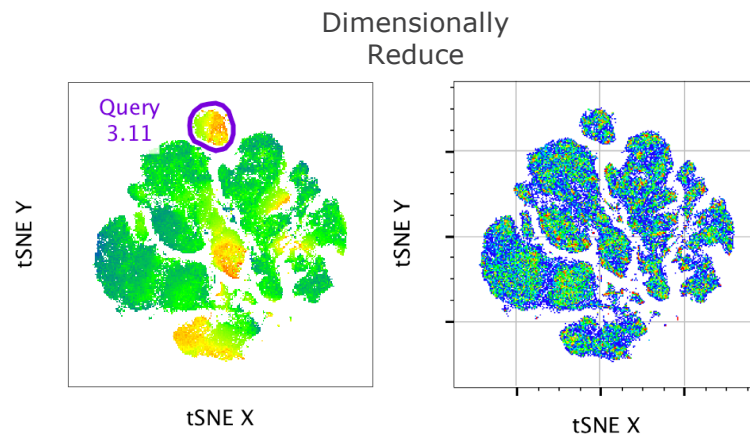
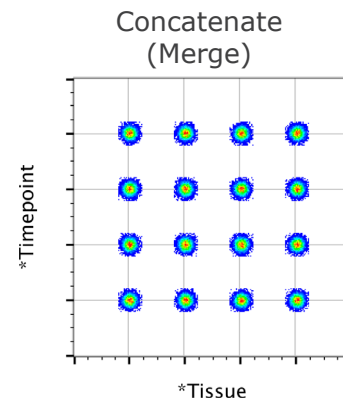
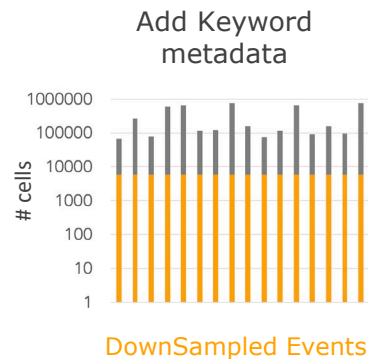
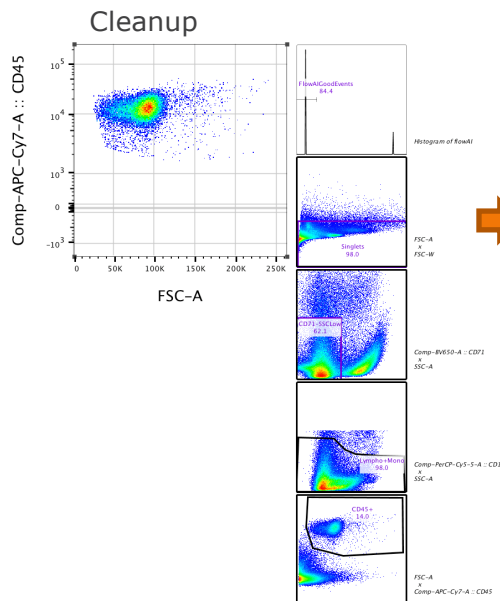
gate



comparisons



dig deeper



Analysis Workflow

for Discovery



cleanup



concatenate



dimensionally-
reduce



cluster



gate

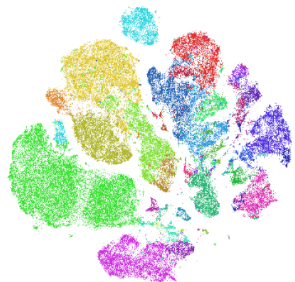


comparisons

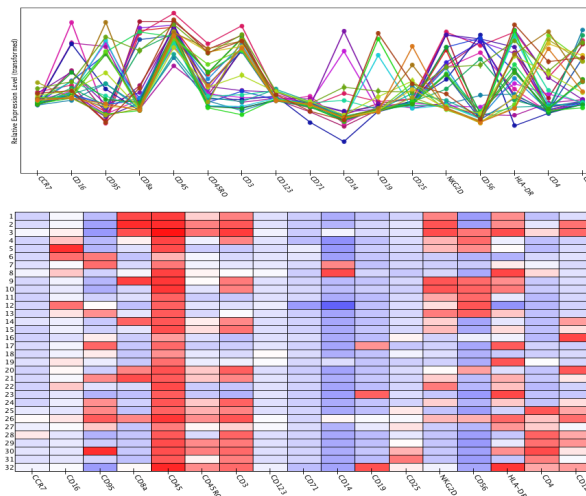


dig deeper

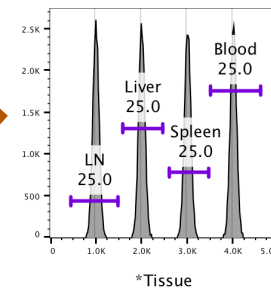
Cluster



Explore



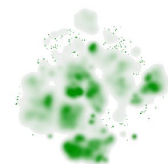
Gate



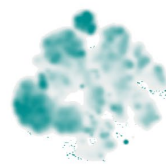
Identify
Differentially Expressed
Populations



Compare Conditions



blood



spleen



liver



lymph node

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IndexSort			

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<i>MiST</i>	StainIndex
<i>Sunburst</i>	<i>iCellR</i>
Correlate	PluginWizard

Dimensionality Reduction

creates new derived parameters



cleanup



concatenate



dimensionally-reduce



cluster



gate

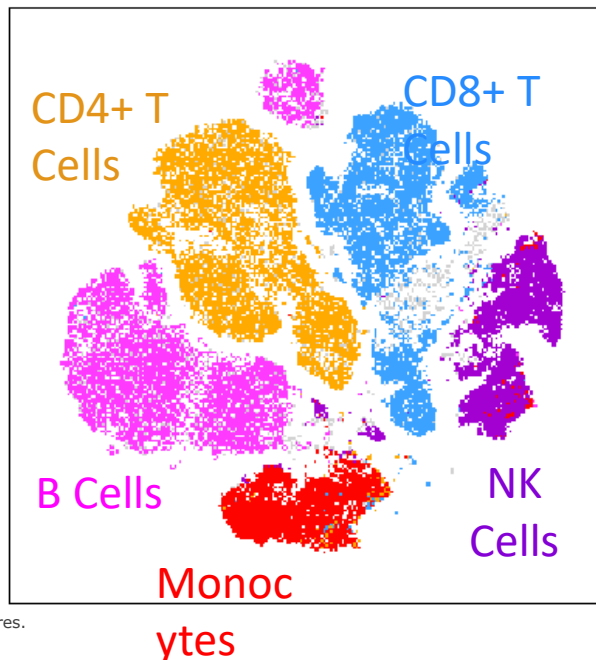


comparisons



dig deeper

- Goal: low-dimensional representation of a high-dimensional dataset that preserves the overall structure of the data as much as possible

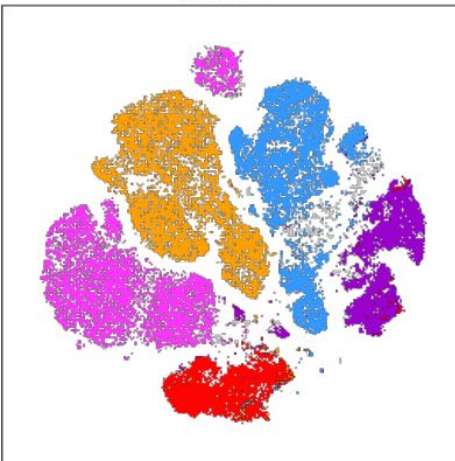
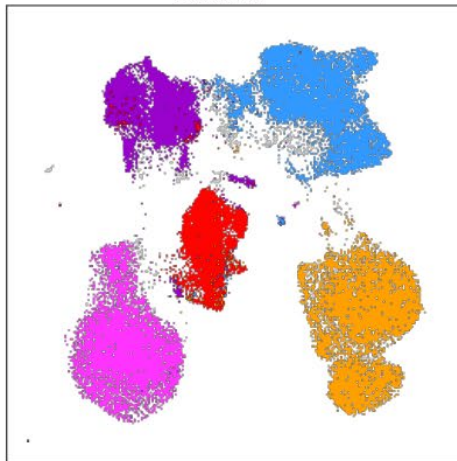
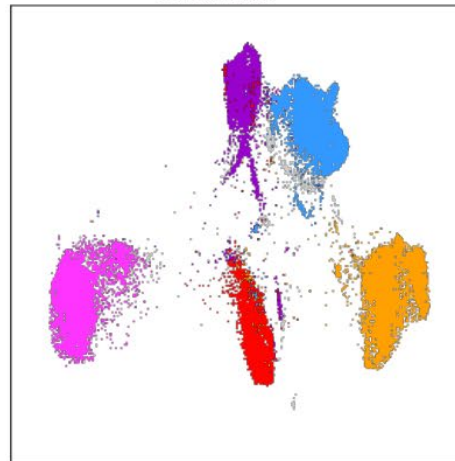
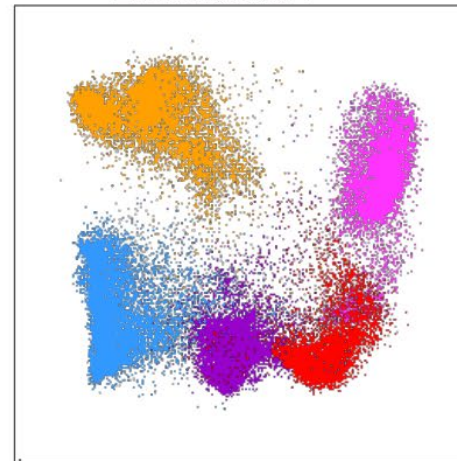


- Events with a similar multi-dimensional expression pattern group together within the dimensionally reduced data space

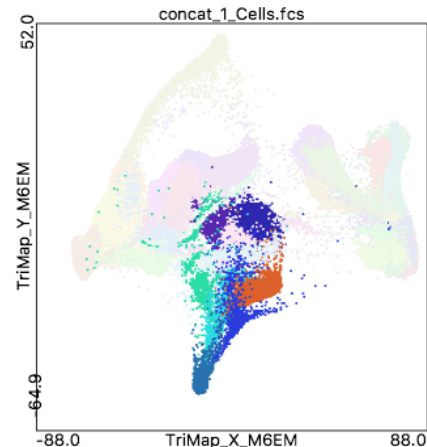
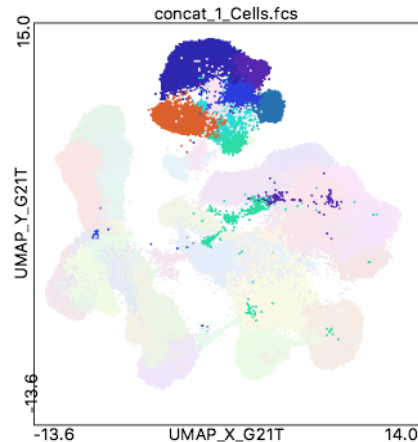
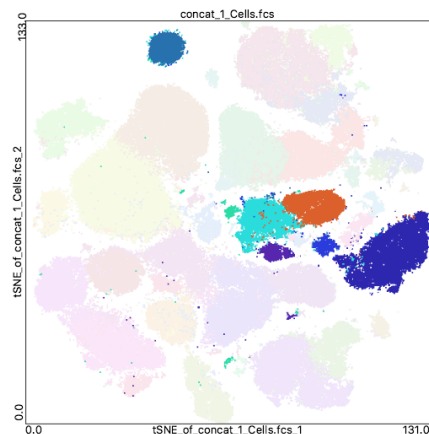


Dimensionality Reduction Options

- t-SNE = t-Distributed Stochastic Neighbor Embedding
- UMAP = Uniform Manifold Approximation and Projection
- TriMAP = Triplet Manifold Approximation and Projection
- EmbedSOM = 2D visualization of FlowSOM clustering output

tSNE**UMAP****TriMAP****EmbedSOM****■ Monocytes****■ B Cells****■ CD4+ T Cells****■ CD8+ T Cells****■ NK Cells**

-
- Relative Expression Level (transformed)
- CD8 BUV805
- CI 12
CI 14
CI 15
CI 18
CI 21
CI 23
CI 39
- Genes on x-axis: CcmB, TIC, IL-5, B6B30, IL-13, B6B60, IFN γ , B6B700, CD133, B6B700, CD238, FE, Perform, A4394, TCR α , CD, FE, CQ5, RevD3, FE, CQ5, IL-22, FE, CQ7, IL-21, A4647, CD107a, A4700, or A, CD3, APC-H7, CCR4, BUI195, Live, Dead, IV, Blue, CD4, BUI496, CD25, BUI496, CD39, BUI563, CD95, BUI662, CD95, BUI737, CD8, BUI805, IL-2, A4621, CD155, BUI480, CD45RO, BUI570, IL-17A, BUI605, A467, A4650, CD69, BUI711, TNF α , BUI750, CD27, BUI786



BD RESTRICTED

t-SNE

the GUI details

Run Name: tSNE_of_concat_1_Cells.fcs

Population: concat_1_Cells.fcs

Comp-B515-A :: GzmB FITC
Comp-B610-A :: IL-8 BB630
Comp-B660-A :: IL-13 BB660
Comp-B710-A :: IFNg BB700
Comp-B780-A :: CD137 BB790
Comp-G575-A :: CD28 PE
Comp-G610-A :: Perforin Ax594
Comp-G660-A :: TCR GD PE-Cy5
Comp-G710-A :: FoxP3 PE-Cy55
Comp-G780-A :: IL-22 PE-Cy7
Comp-R670-A :: IL-21 Ax647
Comp-R730-A :: CD107a Ax700 or APC-R700
Comp-R780-A :: CD3 APC-H7
Comp-U390-A :: CCR7 BUV395
Comp-U450-A :: Live Dead UV Blue
Comp-U500-A :: CD4 BUV496
Comp-U570-A :: CD25 BUV563
Comp-U660-A :: CD39 BUV661
Comp-U740-A :: CD95 BUV737
Comp-U785-A :: CD8 BUV805

Select All Compensated Select All Uncompensated

Learning Configuration: ☒ Auto (opt-SNE) ☐ Manual
opt-SNE will end early exaggeration when Kullback-Leibler Divergence (KLD) drops off and it will stop iterating when KLD rate of change slows to <0.2%. The learning rate below has also been suggested based on the opt-SNE approach.
(See Belkina, et al. <https://doi.org/10.1038/s41467-019-13055-y>)

iterations: 1000 perplexity: 30 learning rate (eta): 21000
KNN algorithm: Exact (vantage point tree)
gradient algorithm: Barnes-Hut

? Close Run



FlowJo™ v10

Select Parameters – Only highlighted parameters will be considered

Learning Configuration – Defaults to Opt-SNE

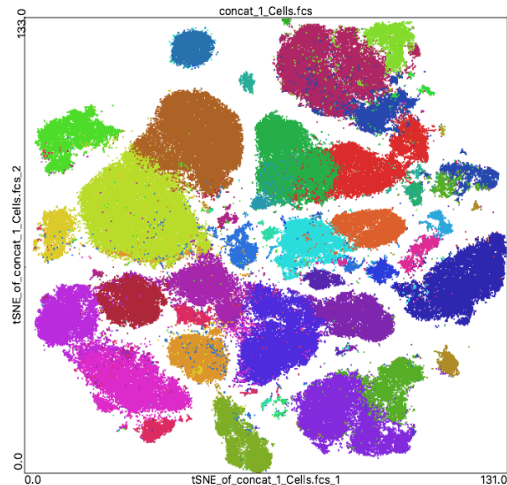
Iterations – Maximum number of iterations

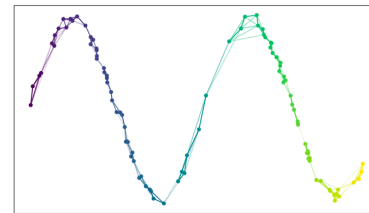
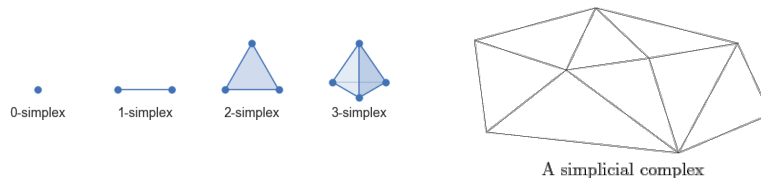
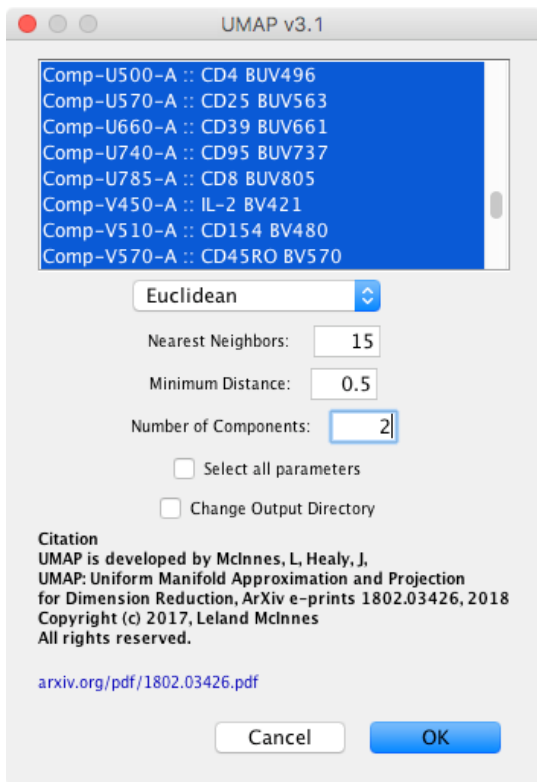
Perplexity – Related to the number of nearest neighbors that is used in learning algorithms. May be viewed as a knob that sets the number of effective nearest neighbors. & Learning Rate

Learning Rate (eta) – How fast you get to a solution. Small → gradual changes per iteration, slower. Large → larger changes, faster.

KNN – Exact (vantage point tree) vs Annoy (Random Forest Projection)

Gradient Algorithm – Barnes-Hut vs Fit-SNE



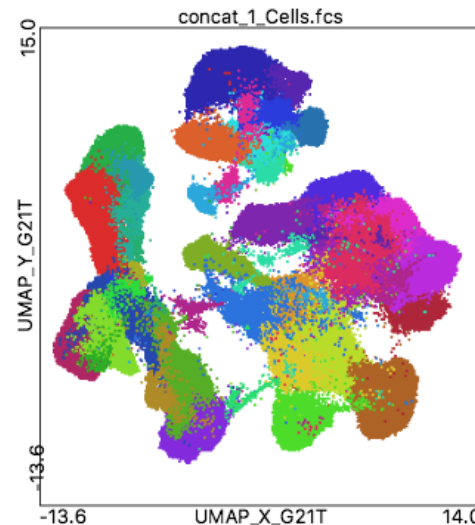


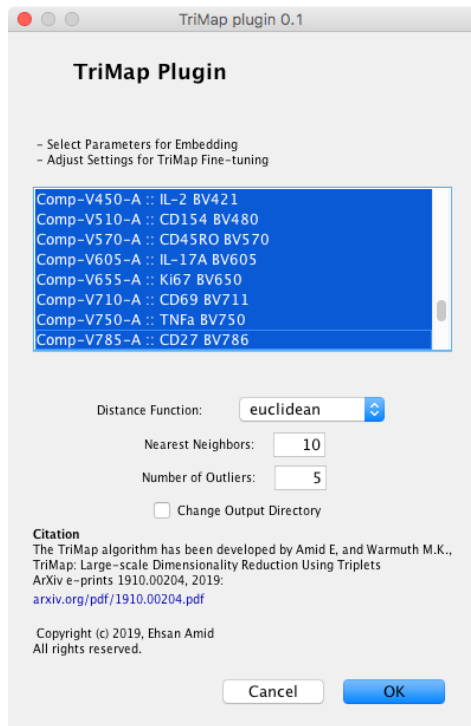
• How does it work?

- Neighbor graph style approach that uses simplices to build a manifold approximation of the data structure, making an effort to preserve the topological global structure of that data.

• How well does it work?

- Equally meaningful representations compared with t-SNE
- Better representation of multi-branched continuous trajectories (ex. hematopoietic development, lineage relationships)
- Faster than Barnes-Hut tSNE
- Scales better with high #s of parameters
- Improved Global Structure representation
- Can produce multiple components



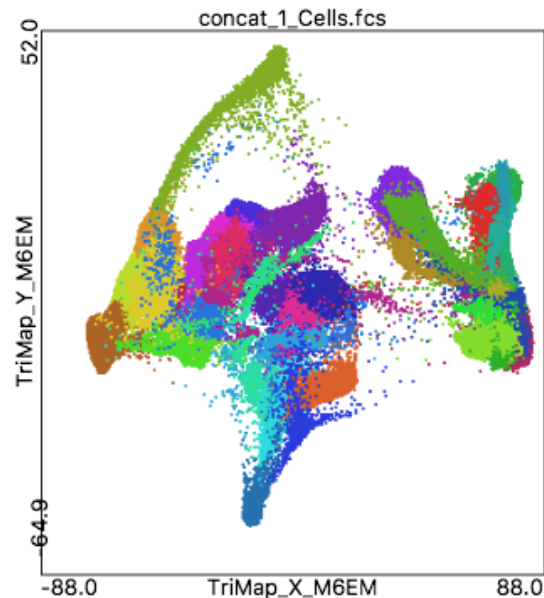


• How does it work?

- Semi-supervised metric learning
- Initialized with low dimensional PCA embedding, and this embedding is then modified using a set of selected triplets from the high-dimensional representation

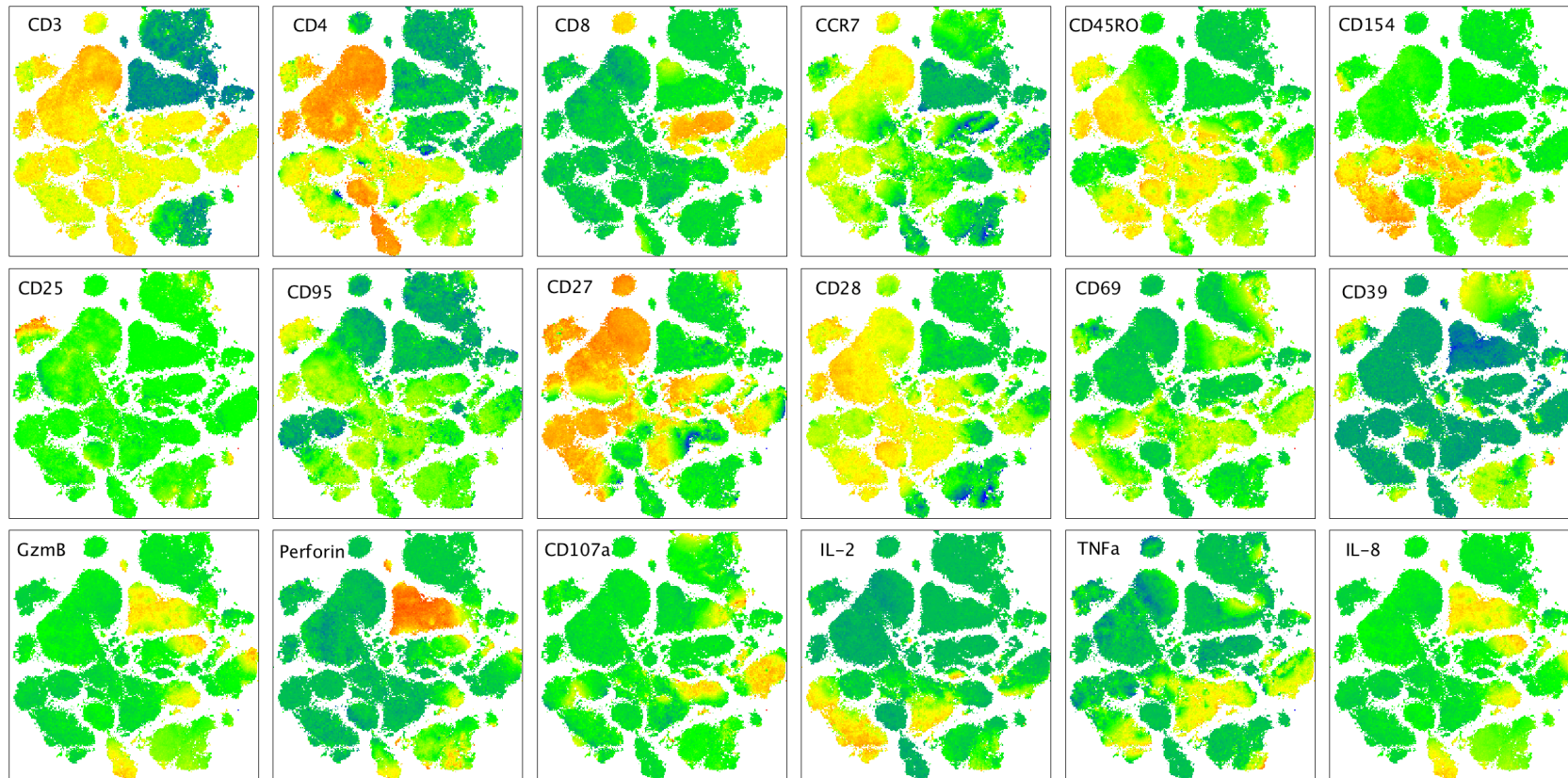
• How well does it work?

- Meaningful representations
- Faster than tSNE
- Scales well (comparable to UMAP)
- Preserves *Global Structure*



3rd Parameter Heat Map

orient and identify



Query Gate

and explore



cleanup



concatenate



dimensionally-
reduce



cluster



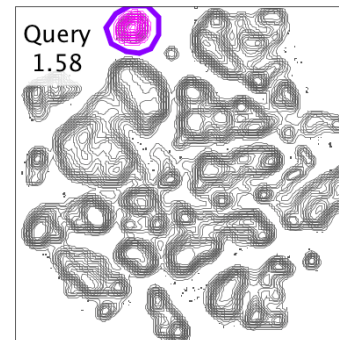
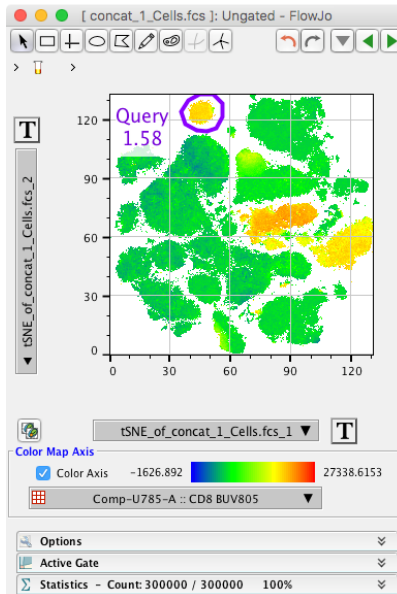
gate



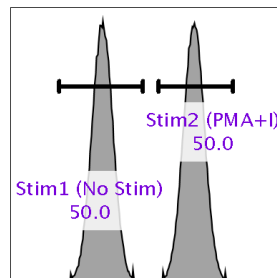
comparisons



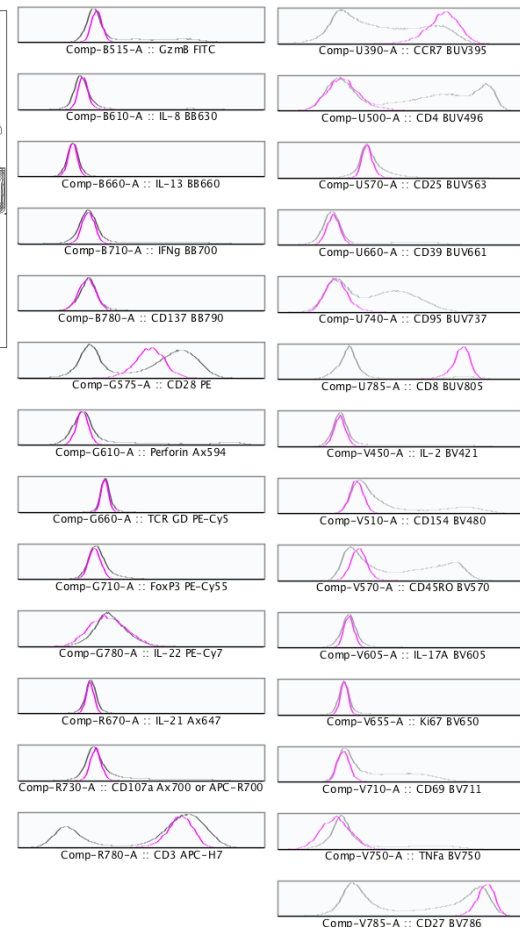
dig deeper



Sample Name	Subset Name	Count
concat_1_Cells.fcs	Query	4748
concat_1_Cells.fcs	Ungated	300000



Name	Statistic
Query	1.58
Stim1 (No Stim)	98.3
Stim2 (PMA+I)	1.66



Clustering

creates populations



cleanup



concatenate



dimensionally-
reduce



cluster



gate

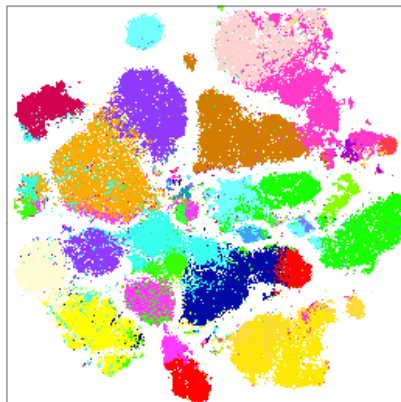


comparisons



dig deeper

tSNE Y

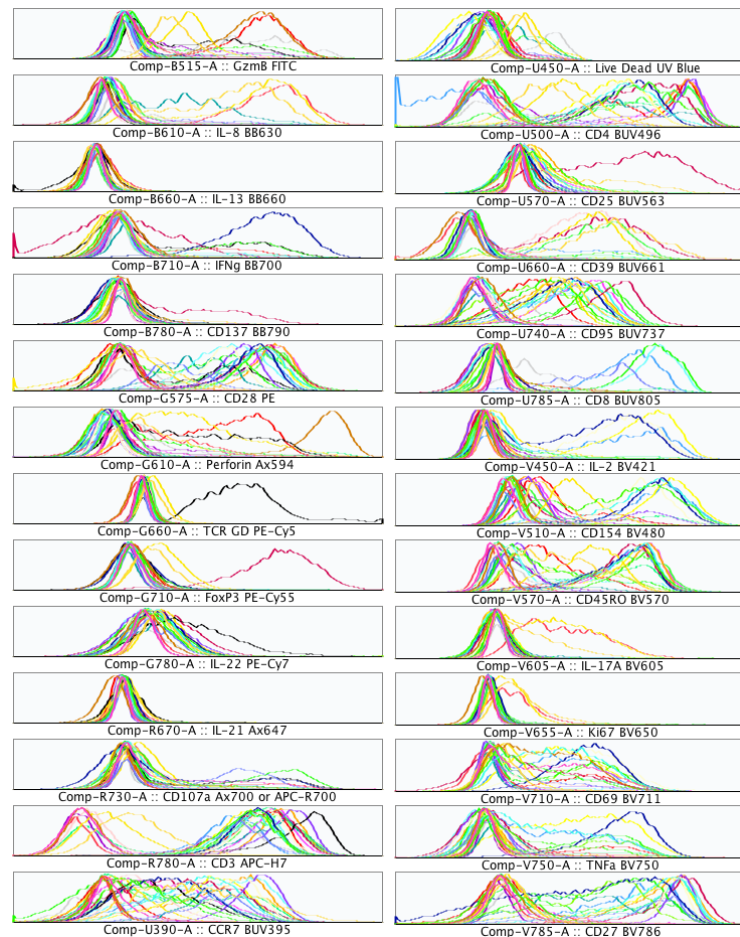


tSNE X

- Overlay cluster populations in the LE
- Right click and choose Make Multigraph Overlays → Histograms



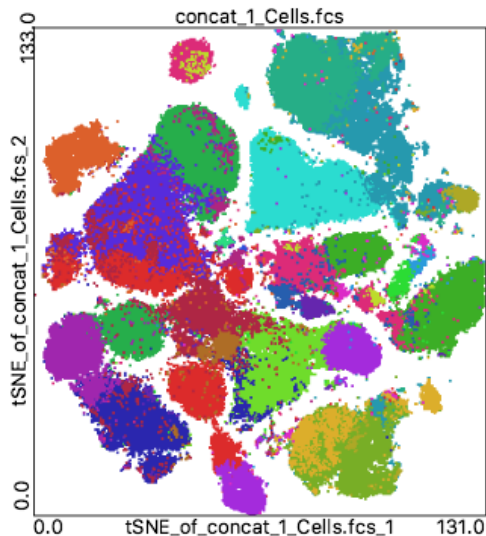
FlowJo™ v10



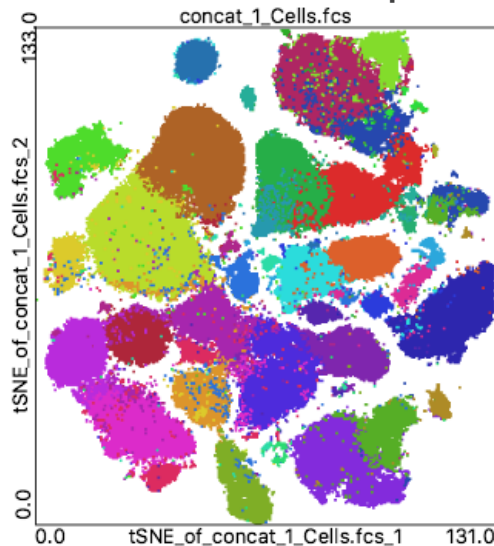
Clustering Options

- X-Shift – Fast KNN Density Estimation
- PhenoGraph – Nearest Neighbors Graph → Communities
- FlowSOM – Self Organizing Map

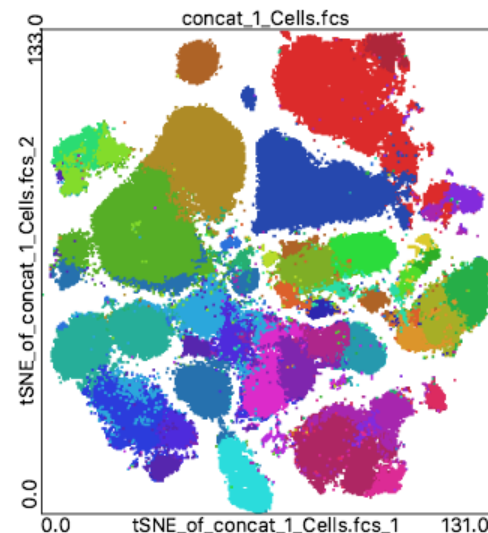
X-Shift



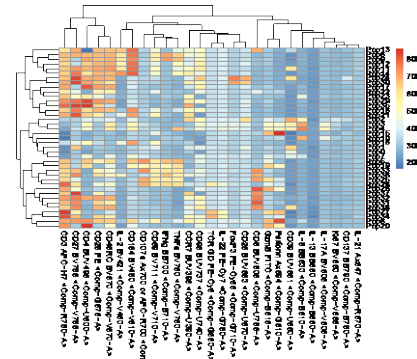
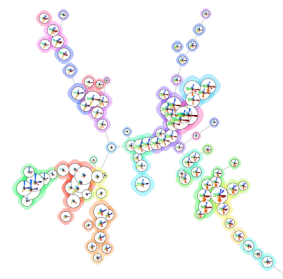
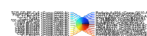
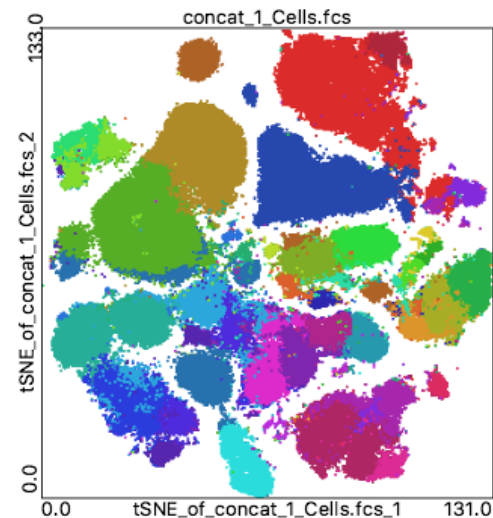
PhenoGraph



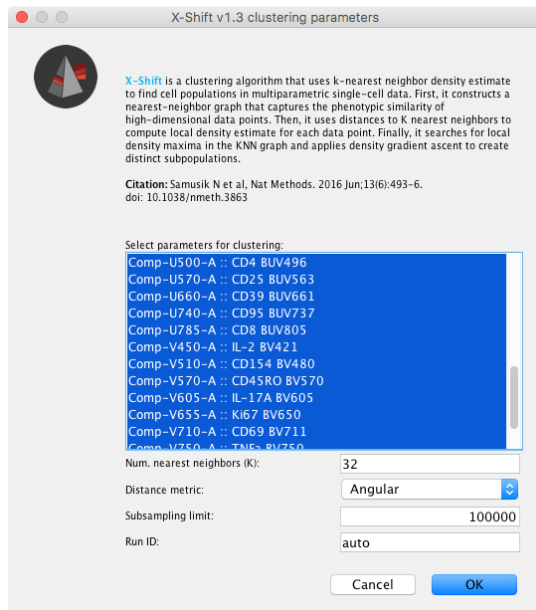
FlowSOM



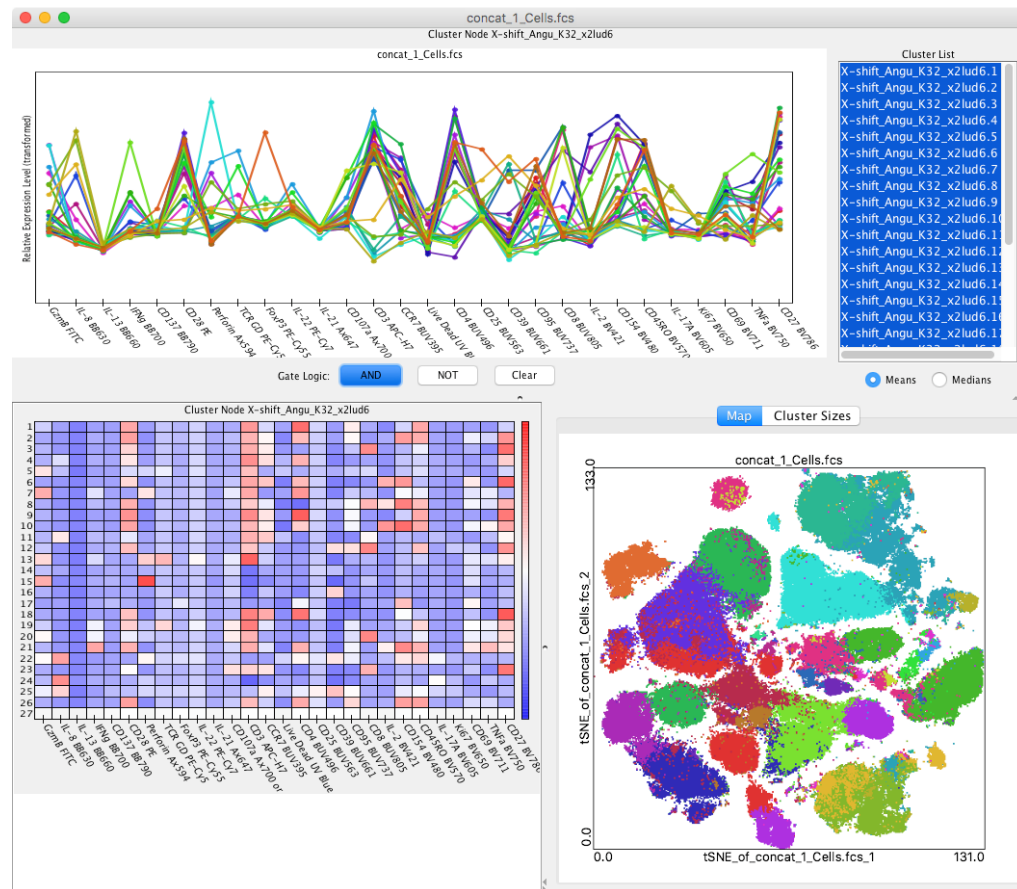
Heatmap showing the correlation of 39 variables. The variables are listed on both the x and y axes. The color scale ranges from blue (low correlation) to red (high correlation). The diagonal is dark red, indicating a correlation of 1.0. The heatmap shows various patterns of positive and negative correlations between the variables.



- Analyzes using a self-organizing map and K nearest neighbors to generate cluster populations



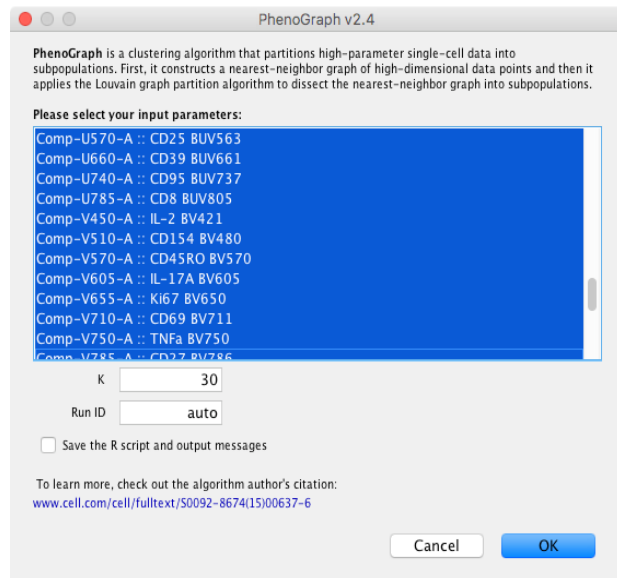
- K-nearest neighbor density estimation



PhenoGraph



FlowJo™ v10



- Constructs a nearest neighbor graph and partitions the graph into communities



ClusterExplorer

gating and comparison of cluster populations



cleanup



concatenate



dimensionally-reduce



cluster



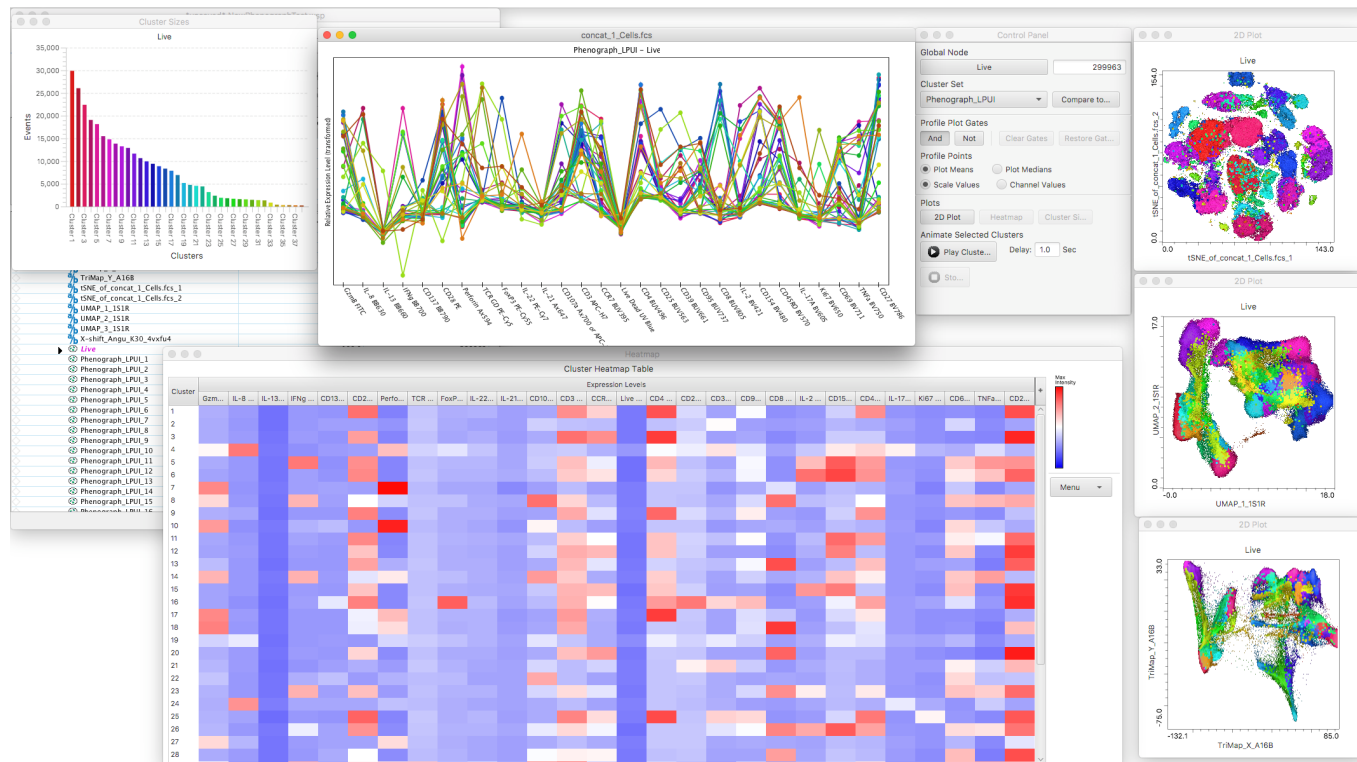
gate



comparisons



dig deeper



Now What?

- You've found one or more interesting populations by high parameter clustering
- You would like to isolate this population for additional study, but don't have a hierarchical gating tree...

Question becomes: How do you sort a population identified with a clustering algorithm?



HyperFinder

generate an optimized gating strategy for any population



cleanup



concatenate



dimensionally-reduce



cluster



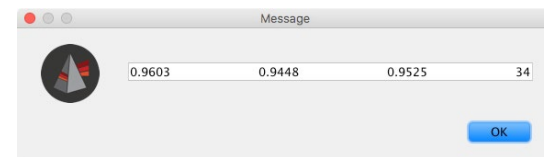
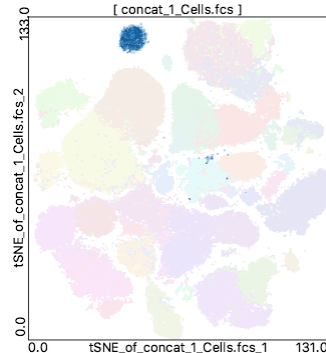
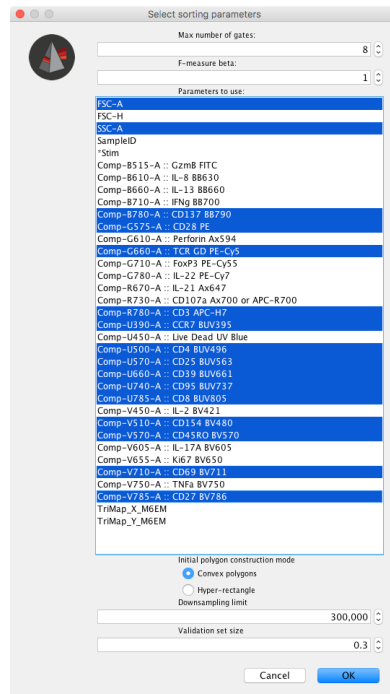
gate



comparisons



dig deeper



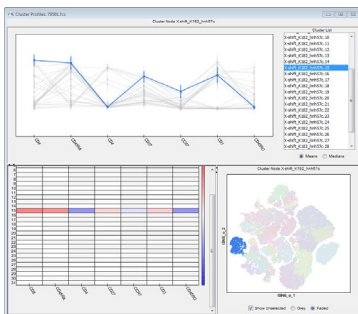
Computational Sorting Workflow

1) Clustering

FlowJo plugins
X-shift
PhenoGraph
FlowSOM

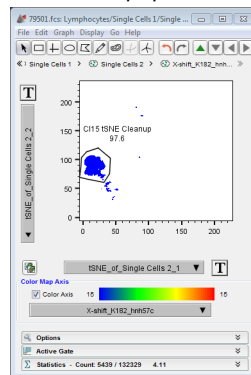
Finding the cluster of interest

ClusterExplorer FlowJo plug-in

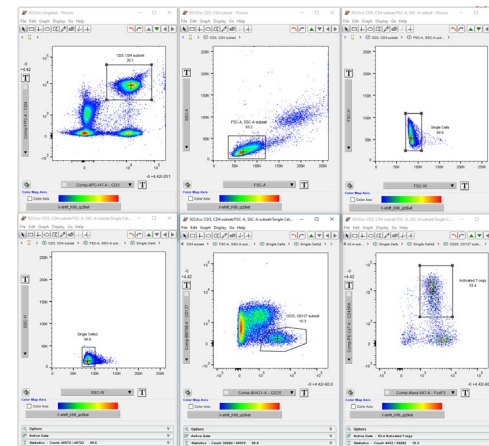


t-SNE Cleanup

Removing the outliers by
t-SNE gating on the
clustered population



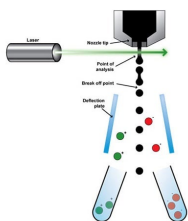
2) Conventional gating



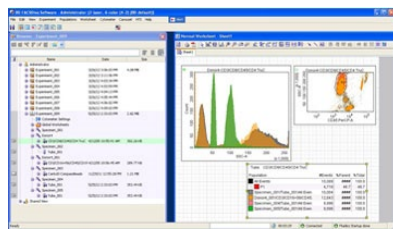
Use cases:

- 1) Shortens the gating strategy
- 2) Try and capture the same population while leaving some markers out

Sorting

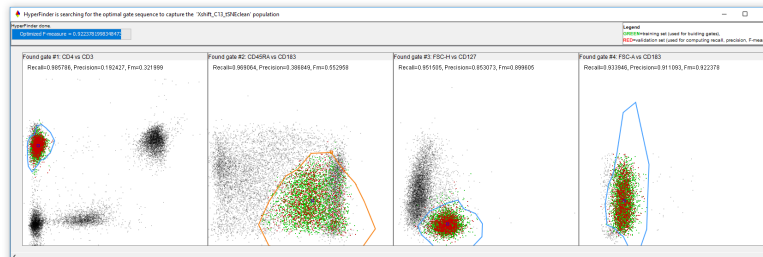


BD FACSDiva™



BD FACSDiva™ Export function
available in FlowJo v10.6+

HyperFinder



10-15 min
on 100K
dataset

Thank You!

Questions?

Thank You!

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