



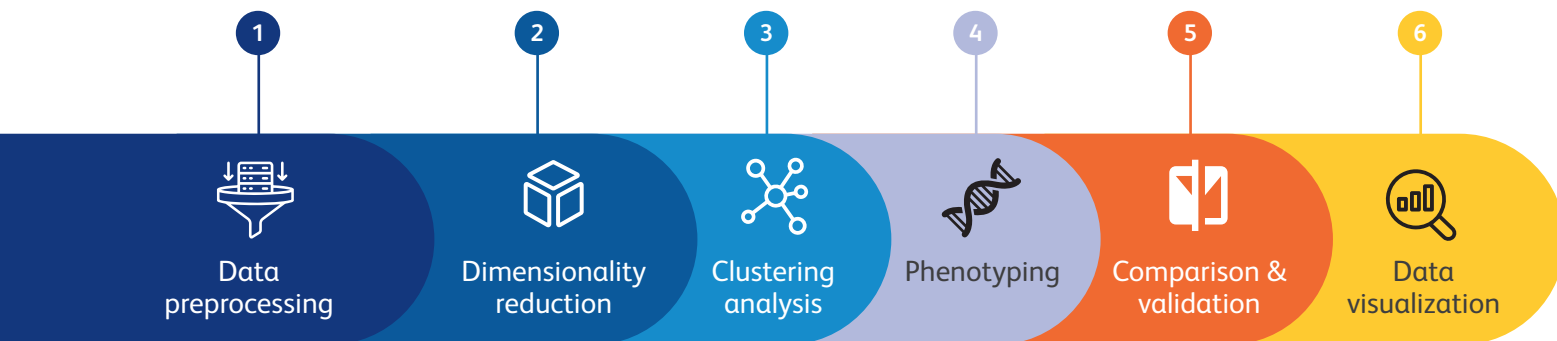
Advanced flow cytometry data analysis with FlowJo™ Software v10

A detailed overview of the full workflow to analyze high-dimensional flow cytometry data using advanced FlowJo™ Software features.

Flow cytometry data often contains information across a large number of parameters, making it challenging to visualize and interpret. With more parameters and more complex data in an experiment, algorithmic tools can enhance and improve the characterization of cytometric populations. FlowJo™ Software is the leading platform for single-cell flow cytometry analysis and provides a number of machine learning tools to either accelerate the discovery process, add depth, automate or provide an unbiased approach.

Let's see how you can investigate all the information available in your experiment in a few steps using tools or plugins that are built in FlowJo™ Software.

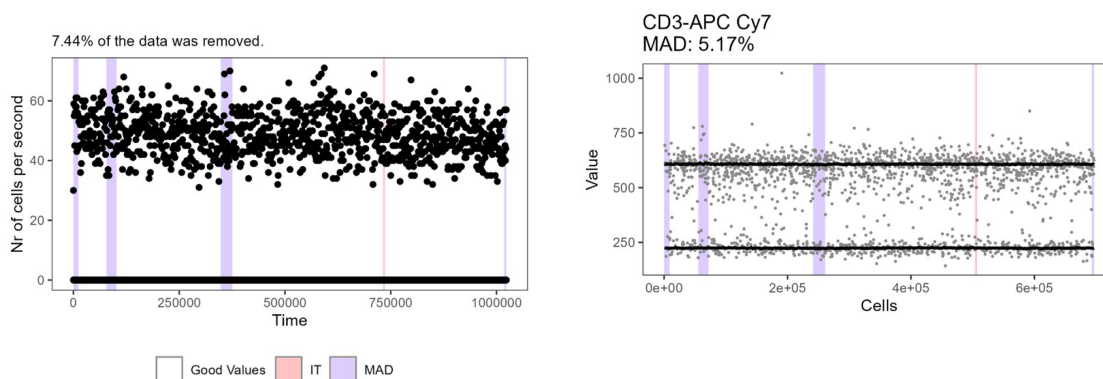




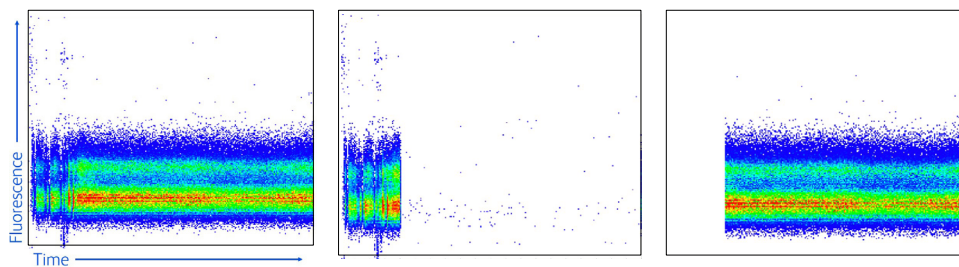
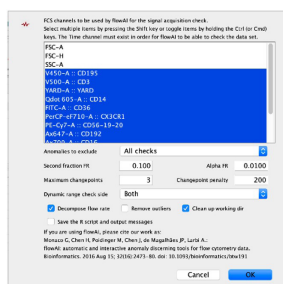
Step 1: Data preprocessing

Quality control of data is an important step in your analysis workflow, especially when many parameters are measured and manually inspecting every combination of parameters would be tedious. The processes outlined below help remove technical artifacts from your data that could lead to false discoveries and prep your files for easier interpretation.

- **Data clean up:** Technical issues at the cytometer can introduce questionable events into your files, lowering the quality of the data. Algorithms such as **FlowClean**, **FlowAI** and **PeacoQC**, all enabled as FlowJo™ Software plugins, automatically identify and remove “bad events” in your FCS files by tracking data consistency over time. FlowAI or FlowClean are best run on uncompensated data.



Abnormal events removed using PeacoQCv

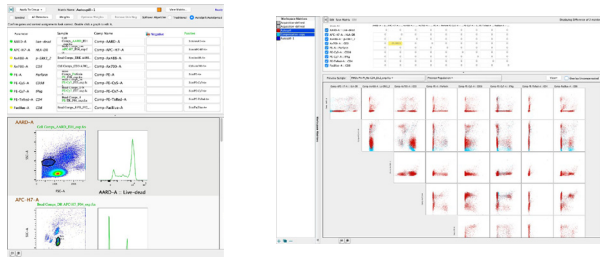


All Events

“Bad” events

“Good” events

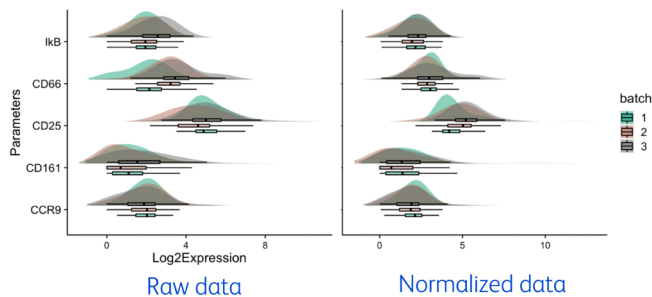
- **Compensation:** Compensate and transform raw data into a format suitable for analysis.



Example with AutoSpill/AutoSpread

- For either a traditional or spectral process to correct for spillover, FlowJo™ Software has several options to ameliorate high-parameter compensation. **AutoSpill** for example uses robust linear regression to calculate spillover coefficients for automatic gating thereby eliminating many steps in the compensation workflow. For more information, check our [AutoSpill/AutoSpread flyer](#).

- **Normalize:** Normalization can be used to remove variance between batches due to differences in the cytometer, the staining protocol or operator inconsistencies. **CytoNorm**, **CyCombine** and **MNN** (Mutual Nearest Neighbors) minimize batch effects by modeling and correcting for technical variability between samples.



Note: CytoNorm is run before data concatenation whereas CyCombine and MNN are run after concatenation

- **Doublet and debris removal:** Continue with manual removal of irrelevant events, such as debris, doublets and marginal events. Apply appropriate gating strategies to define specific cell populations of interest.
- **Annotation:** Meta-data, information about the data, can be added to your FCS files either at the time of collection using the acquisition software or post-acquisition in FlowJo™ Software. Keywords should describe experimental set up (timepoint, condition, tissue, etc.). Use the **Plate Editor** and **Keywords** band functions to add meta-data in FlowJo™ Software. Additionally, numeric keywords can be included as parameters and gated on in the Graph window.
- Capture metadata in the context of the experiment and store/share instrument configuration for panel design in **BD® Research Cloud**.

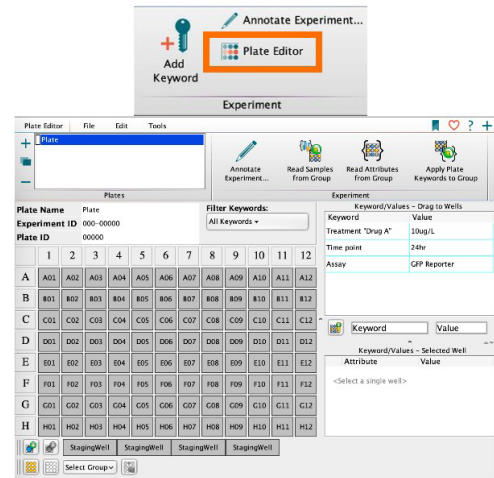
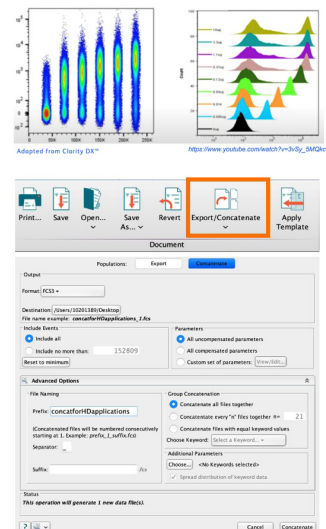


Plate editor

Well ID	Doxycycline (ug/ml)	Cohort	Sex	Age (mos)	Transgene	Panel
A01	0.5	C57black6	F	8	WT	B
A02	1.0	Balbc	F	18	IKKBS34A	A
A03	2.5	Balbc	M	11	IKKGLZ	A
A04	2.5	Balbc	M	13	IKKBS34A	A
A05	10.0	C57black6	M	17	IKKBS34A	B
A06	25.0	C57black6	F	7	IKKGLZ	A
A07	10.0	C57black6	F	6	IKKAS37E	B
A08	10.0	Balbc	F	18	IKKBS36E	B
A09	25.0	C57black6	F	9	IKKGLZ	A
A10	0.0	C57black6	M	8	IKKBS34A	A
A11	50.0	Balbc	M	8	IKKGLZ	B
A12	0.0	Balbc	F	6	IKKBS34A	B
B01	0.5	C57black6	M	13	IKKBS34A	A
B02	1.0	Balbc	F	7	IKKGLZ	B
B03	2.5	C57black6	F	17	IKKAS37E	B
B04	2.5	Balbc	F	6	IKKBS36E	A
B05	10.0	Balbc	F	14	IKKGLZ	B
B06	25.0	C57black6	M	16	IKKAS37E	B
B07	10.0	Balbc	M	11	IKKBS36E	A
B08	10.0	C57black6	M	15	IKKGLZ	A
B09	25.0	Balbc	F	13	IKKBS34A	A
B10	0.0	C57black6	M	8	IKKGLZ	B
B11	50.0	C57black6	F	14	IKKBS34A	A
B12	0.0	C57black6	F	14	IKKBS34A	B

Keyword band

- **Concatenation:** Merging multiple samples into a single concatenated file allows them to be computationally analyzed together and directly compared. Samples can be separated later by gating on keyword-based parameters included in the merged file.

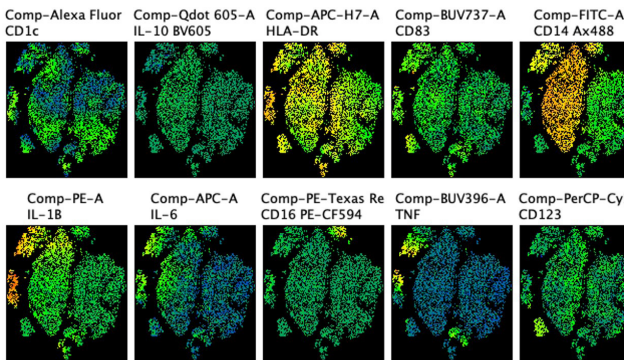
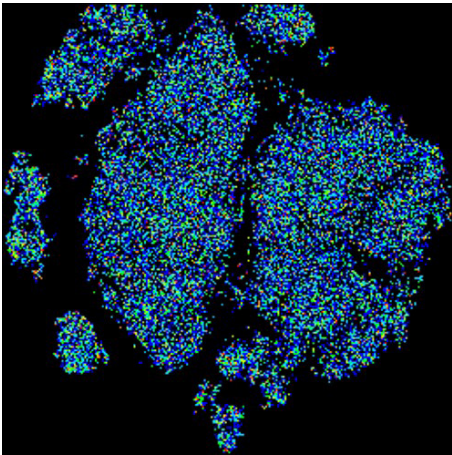


Concatenation options in FlowJo™ Software

Step 2: Dimensionality reduction

Dimensionality reduction techniques, such as Uniform Manifold Approximation and Projection (**UMAP**) and t-distributed stochastic neighbor embedding (**t-SNE**), can help simplify data while preserving their essential characteristics. These methods allow effective visualization of complex datasets and aid in identifying patterns within the data. Reduce the dimensionality of the data for easy visualization with:

- **t-SNE:** Generates new parameters optimized in such a way that observations/data points that are close to one another in high-dimensional space are plotted close together in the reduced data space created via the t-SNE parameters.
- **UMAP:** Provides a similar approach while attempting to preserve more of the overall structure of the high-dimensional data by factoring the distance between data points in high-dimensional space into low-dimensional mapping.

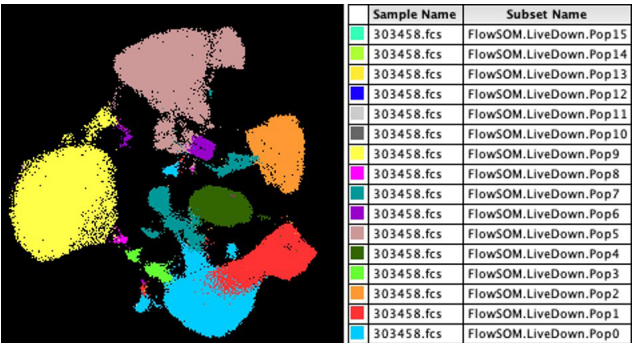


Heat map antigen expression - orientation

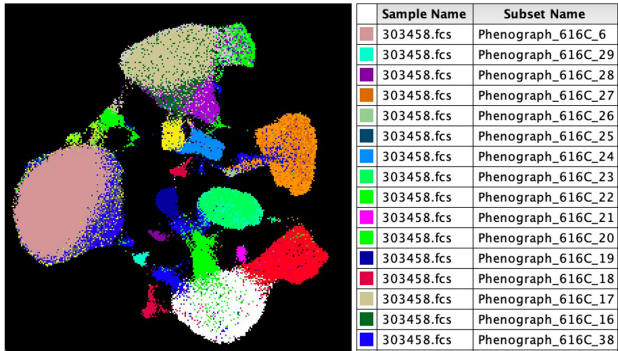
Step 3: Clustering analysis

Clustering allows you to identify populations in your samples without manual gating by grouping cells into distinct clusters based on the similarity of their features. Common clustering algorithms include self-organizing maps (SOM), partitioning algorithms and density-based clustering. This can be applied as a complete gating analysis or as a means of identifying subsets within manually gated high-level populations.

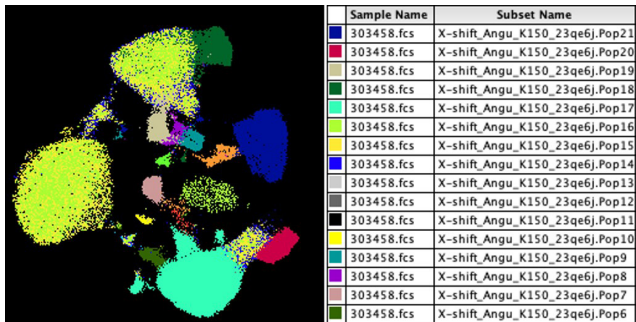
- **FlowSOM:** Clusters data by first morphing a grid into a self-organized map of the data by moving grid nodes to where there are more cells, then using k-means clustering to partition the nodes into a user defined number of clusters.
- **Phenograph:** Clusters data by using the density of neighboring cells to determine cutoffs, automatically determining the best number of clusters.
- **XShift:** Clusters data similarly to Phenograph but includes a subsampling step that can make it much faster.



FlowSOM



Phenograph

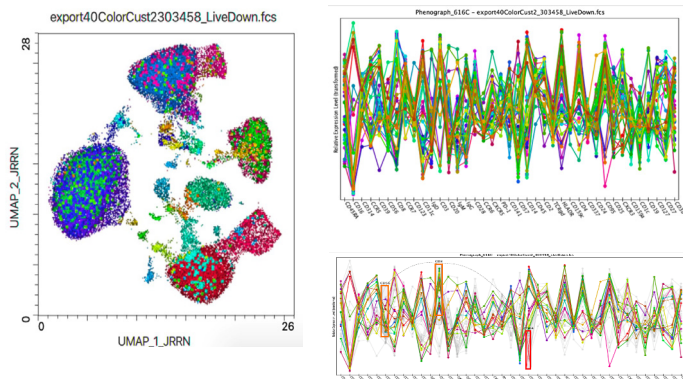


XShift

Step 4: Phenotyping

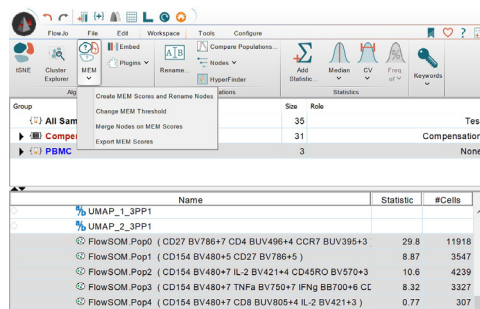
Mapping clusters to a phenotype is essential to completing an analysis. There are multiple tools in FlowJo™ Software to help you interpret clusters and drive biological insights.

- **ClusterExplorer:** FlowJo™ Software includes an interactive tool that can be used to interrogate populations of interest after performing clustering and dimensionality reduction. It displays clusters projected on a t-SNE or UMAP plot, heatmaps, bar charts and line plots to visualize trends between clusters, plus a host of controls for delving deeper into the data. For more details, [see our ClusterExplorer flyer](#).



ClusterExplorer allows the user to profile clusters through an interactive set of views that show the data as dot plots, heat maps and line plots.

- **Marker Enrichment Modeling (MEM):** MEM is a phenotyping tool available in FlowJo™ Software as of version 10.9. MEM assigns quantitative names to clusters based on marker expression compared to a user-defined reference and allows cluster refinement.



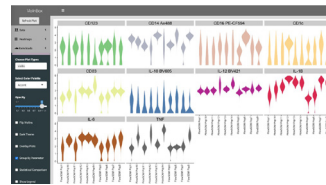
Creating MEM scores

- **Tracking Responders Expanding (T-REX):** T-REX is a workflow plugin that creates dimensionality reduction parameters, identifies the areas of difference between two user-specified conditions, clusters the data and then identifies the clusters that match the region of difference.

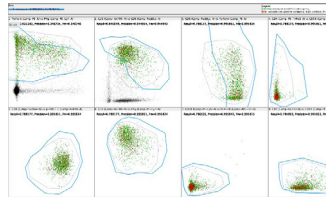
Step 5: Comparison and validation

Comparing distributions of data is an important goal in many applications. Numerous experiments are designed to test whether two populations are statistically significantly different.

- **ViolinBox:** This tool allows users to generate violin or box and whisker plots for multiple parameters to compare populations

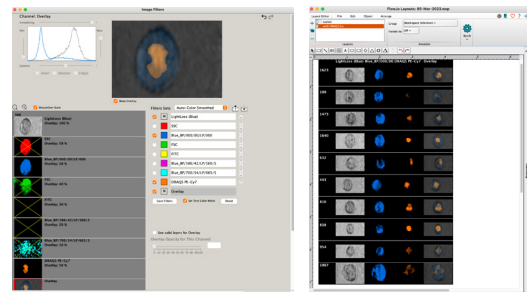


- **HyperFinder:** This tool creates manual gates that recapitulate interesting populations identified via algorithmic analysis. This gating strategy can be exported for use on a BD FACSAria™ or BD FACSDiscover™ S8 Cell Sorter and used to sort complex populations and validate discoveries made in computational analysis.



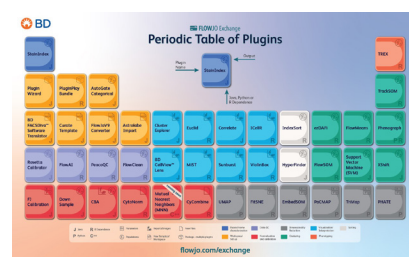
Step 6: Data visualization

Visualizing flow cytometry data is essential for interpreting results and communicating findings effectively. Many of the tools described thus far include graphical outputs to assist in this. There are many other options available on the FlowJo Exchange visualization tab. Additionally, the BD CellView™ Lens Plugin allows you to visualize images of cells collected on the BD FACSDiscover™ S8 Cell Sorter.



BD CellView™ Lens Plugin in FlowJo™ Software

- Embracing advanced data and then identifying analysis techniques will undoubtedly help extract meaningful insights and enhance your research outcomes to contribute to scientific discoveries. This workflow serves as a general guideline, and specific analysis strategies may vary depending on the research objectives and data characteristics. The tools used here are either built into FlowJo™ Software or available as plugins that can be downloaded at: <https://www.flowjo.com/exchange>. A handy periodic table of plugins is available at <https://info.flowjo.com/table-plugin>.



Resources

- Introduction to FlowJo flyer: https://f.hubspotusercontent20.net/hubfs/2566672/Marketing/FlowJo_Introduction.pdf
- Recorded Webinars <https://www.flowjo.com/learn/webinars/recorded>
- FlowJo University <https://www.flowjo.com/learn/flowjo-university/flowjo>
- Documentation <https://docs.flowjo.com/flowjo>
- FlowJo newsletter <https://www.flowjo.com/newsletter>
- FlowJo Exchange <https://www.flowjo.com/exchange>
- Try FlowJo™ Software for free <https://www.flowjo.com/solutions/flowjo/free-trial>
- FlowJo Support <https://www.flowjo.com/contac>

If you have any further questions or require assistance with your flow cytometry data analysis, our team of experts is always here to support you. Happy analyzing!



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