# Advanced flow cytometry data analysis with FlowJo™ v11 Software

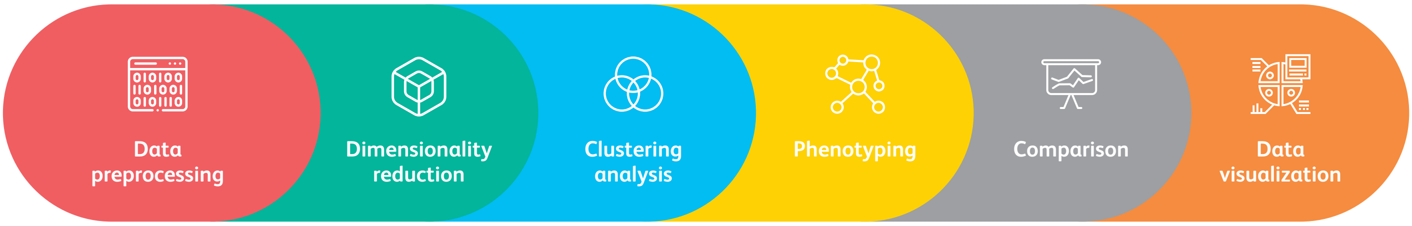
## A detailed overview of the full workflow to analyze high-dimensional flow cytometry data using advanced features in FlowJo™ v11 Software

Flow cytometry data often contains numerous parameters, making it challenging to visualize and interpret. Algorithmic tools can help improve and enhance the characterization of such complex data. FlowJo™ Software is a leading solution for single-cell flow cytometry analysis, offering a suite of machine learning tools that accelerate discovery, deepen insights, automate workflows and enable unbiased data exploration.

Let's see how you can investigate all the information available in your experiment in a few steps using tools that are built into FlowJo™ v11 Software.

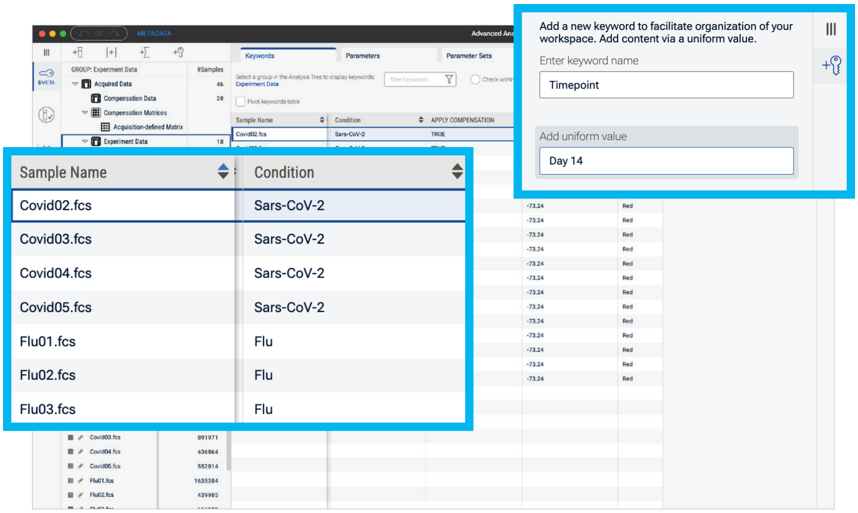
# FlowJo™ Software data analysis workflow

Drag-and-drop files into the sample pane or click **Add Samples**. FlowJo™ Software performs best if data is accessed from a local hard drive to avoid latency and disconnection issues.



## Step 1: Data preprocessing

The steps below prepare your files for high-dimensional analysis by adding categorical information to support comparisons, organizing parameters for clarity and removing technical artifacts from your data that could lead to misleading results.



Add a new keyword to facilitate organization of your workspace. Add content via a uniform value.

Enter keyword name: Timepoint

Add uniform value: Day 14

| Sample Name | Condition |
|---|---|
| Covid02.fcs | Sars-CoV-2 |
| Covid03.fcs | Sars-CoV-2 |
| Covid04.fcs | Sars-CoV-2 |
| Covid05.fcs | Sars-CoV-2 |
| Flu01.fcs | Flu |
| Flu02.fcs | Flu |
| Flu03.fcs | Flu |

### Annotation

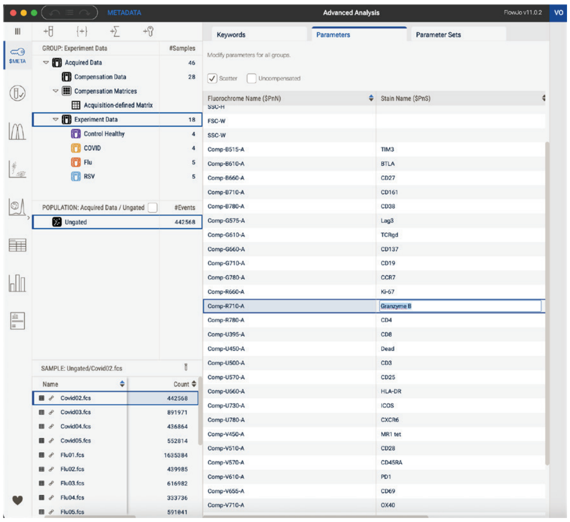
Metadata, information about the data, can be reviewed and added to your FCS files either at the time of collection using the acquisition software, or post-acquisition in the **Keywords** tab of the **Metadata Manager.** Keywords can describe experimental set up (timepoint, condition, tissue, etc.) and be used to categorize samples later in **Tables** or **Charts** or facilitate smart batching in **Reports**.

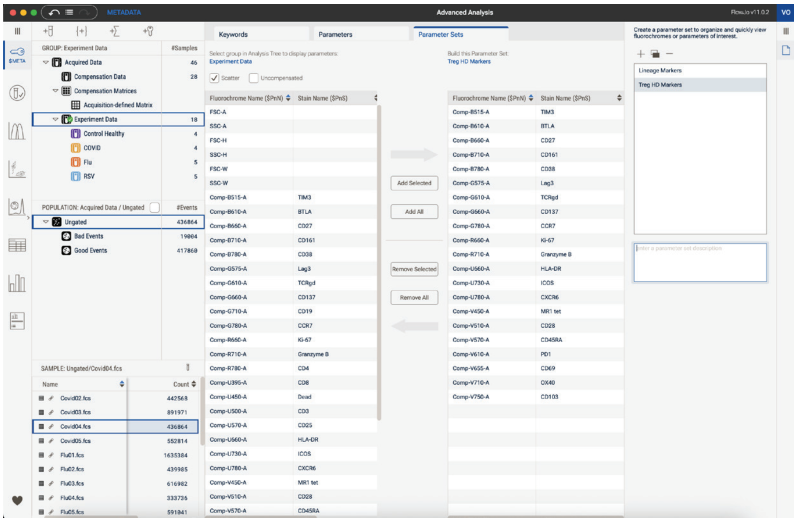
*Note:* Virtually Concatenated Populations (VCP) eliminates the need for keyword-based parameters in operations like clustering or dimensionality reduction, but they are still needed for organizing **Tables**, **Charts** or performing advanced batch operations in **Reports**.

### Parameter management

Edit **Fluorochrome** and **Stain** names in the **Parameters** tab to correct typos or align parameter labels across data sets. Group desired parameters into **Parameter Sets** for quick access.

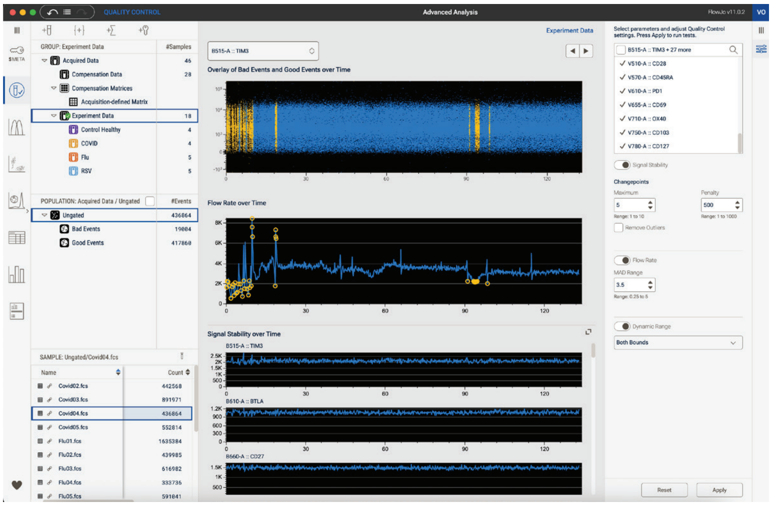
If your algorithmic workflow focuses on a subset of many parameters, simply selecting your desired set each time parameters are required can significantly streamline the process and save a lot of time.





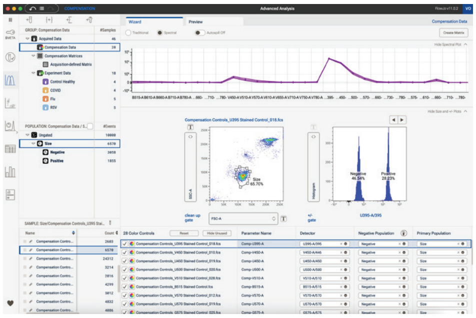
## Data clean up

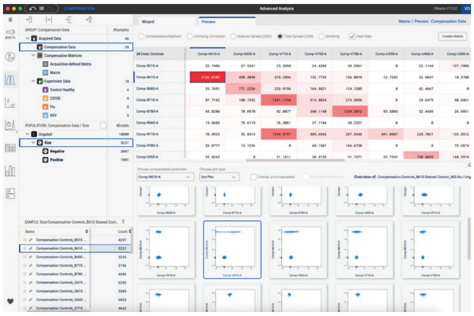
Quality control (QC) of data is an important step in your analysis workflow, especially when many parameters are measured and manually inspecting every combination of parameters would be tedious. Issues at the cytometer can introduce technical outliers into your files, lowering the quality of the data. Use the onboard QC check in FlowJo™ v11 Software, modeled after FlowAI, to automatically inspect your files and remove time-based abnormalities. The algorithm checks for irregularities in flow rate, signal stability and dynamic range. Review the visual report to see which “bad events” events were removed, then continue your downstream analysis on the “good events” subpopulation which is automatically added to your gating hierarchy.

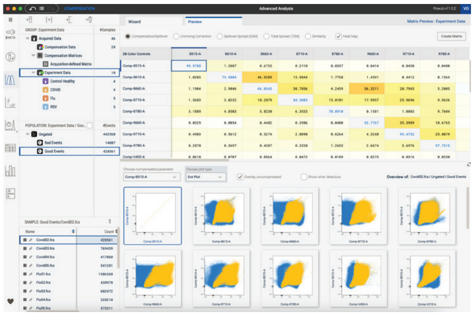


## Compensation or Unmixing

Correct for spillover using the **Compensation Wizard**. The Wizard will assign controls to specific parameters, auto-gate the data and provide both 2D plots and spectral signatures for you to ensure accuracy.







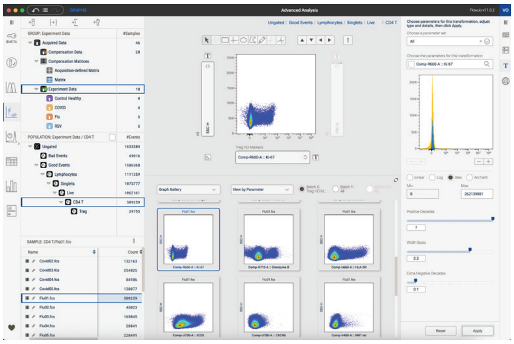
Use the **Preview** tab to review your matrix and the associated quality metrics such as spillover spread and cosine similarity.

To improve high-parameter compensation or unmixing, **AutoSpill** is a built-in feature that uses robust linear regression to calculate spillover coefficients. It automates gating and eliminates a key step in the compensation workflow, while iteratively refining results for optimal accuracy.

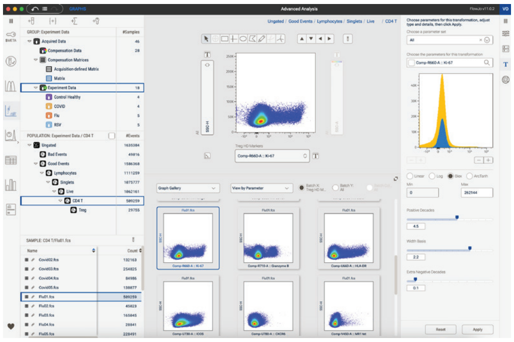
New to FlowJo™ v11 Software is the **Correction Matrix**, a tool used to correct unmixing matrices by applying a direct parameter-parameter adjustment, greatly improving on the parameter-detector adjustment necessary to adjust spectral matrices currently.  For more information, see our Correction Matrix documentation. https://flowjo.com/docs/flowjo11/compensation-and-unmixing-2/correction-matrix)

## Transforms

Expand or compress data on a graph to enhance the visual separation of cell populations; a crucial step in advanced workflows. Dimensionality reduction and clustering tools rely on this transformed data to form islands or identify distinct cell clusters.



*Plots before Transform*

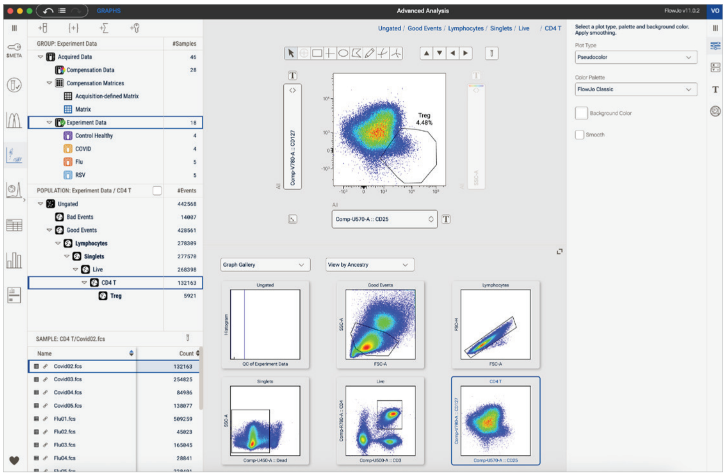


*Plots after Transform*

The algorithm interprets the data as it appears to you. Use the ‘**T**’ button or the **Transforms** property to select a scale, adjust axis ranges and tailor the visualization space for parameters that will be used in downstream algorithms.

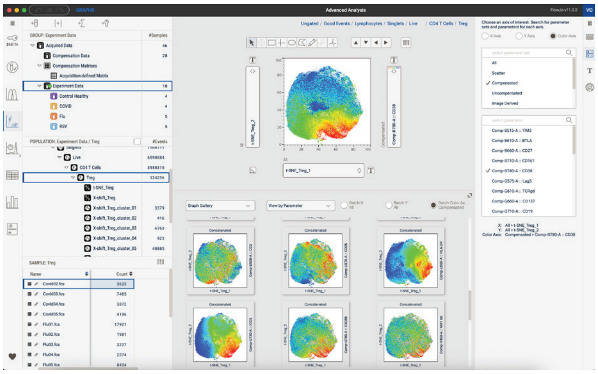
## Doublet and debris removal

Continue with manual removal of irrelevant events such as debris, doublets and dead cells. Apply appropriate gating strategies to define specific cell populations of interest to be used as inputs for dimensionality reduction and clustering.



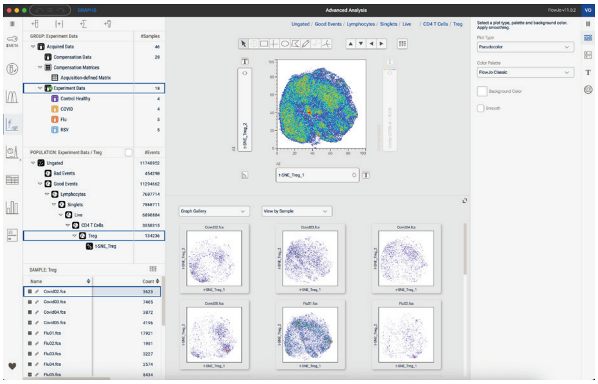
# Step 2: Dimensionality reduction

Dimensionality reduction techniques 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, built into FlowJo™ v11 Software.



## t-SNE

Generates new parameters that are optimized in such a way that data points that are close to one another in high-dimensional space are plotted close together in the low-dimensional data space created via the t-SNE parameters.



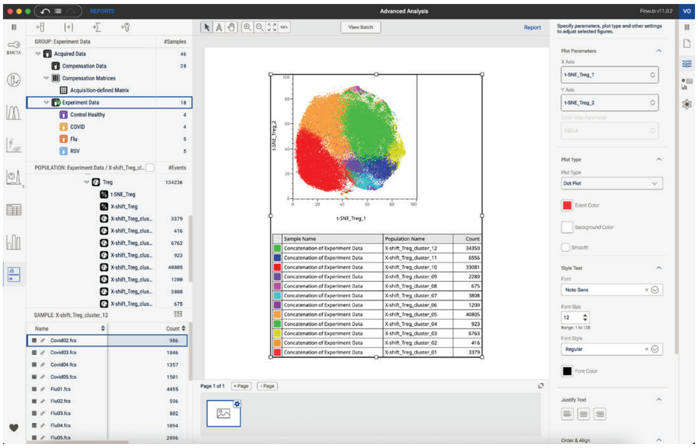
## Virtual concatenation

Merging multiple samples allows them to be computationally analyzed together and directly compared. Rather than create a new concatenated file, FlowJo™ v11 Software creates Virtually Concatenated Populations (VCP) with the click of a toggle, which allows clustering or dimensionality reduction to be run on an entire experiment, while preserving the ability to view the results on the individual samples.

# 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.

Automate population identification in FlowJo™ v11 Software using **X-Shift**, a fast and high-performing clustering algorithm designed for the discovery of rare cell populations.

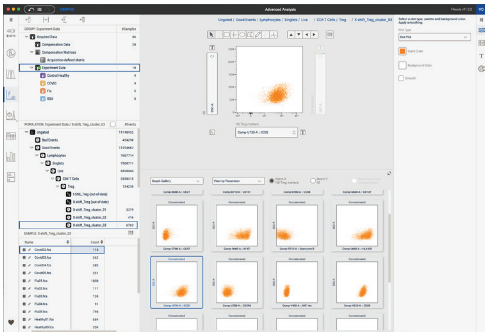


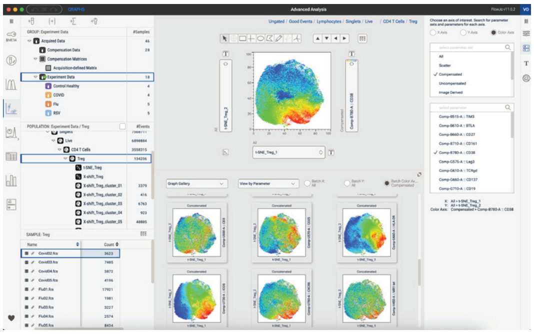
| | Sample Name | Population Name | Count |
|---|---|---|---|
| 🟩 | Concatenation of Experiment Data | X-shift_Treg_cluster_12 | 34350 |
| 🟦 | Concatenation of Experiment Data | X-shift_Treg_cluster_11 | 6556 |
| 🟥 | Concatenation of Experiment Data | X-shift_Treg_cluster_10 | 33081 |
| 🟪 | Concatenation of Experiment Data | X-shift_Treg_cluster_09 | 2280 |
| 🟪 | Concatenation of Experiment Data | X-shift_Treg_cluster_08 | 675 |
| 🟦 | Concatenation of Experiment Data | X-shift_Treg_cluster_07 | 3808 |
| 🟪 | Concatenation of Experiment Data | X-shift_Treg_cluster_06 | 1200 |
| 🟧 | Concatenation of Experiment Data | X-shift_Treg_cluster_05 | 40805 |
| 🟩 | Concatenation of Experiment Data | X-shift_Treg_cluster_04 | 923 |
| 🟨 | Concatenation of Experiment Data | X-shift_Treg_cluster_03 | 6763 |
| 🟧 | Concatenation of Experiment Data | X-shift_Treg_cluster_02 | 416 |
| 🟥 | Concatenation of Experiment Data | X-shift_Treg_cluster_01 | 3379 |

## 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. Examples are **View by Parameter** or **Heatmap Color Axes** in the **Graph Gallery**.

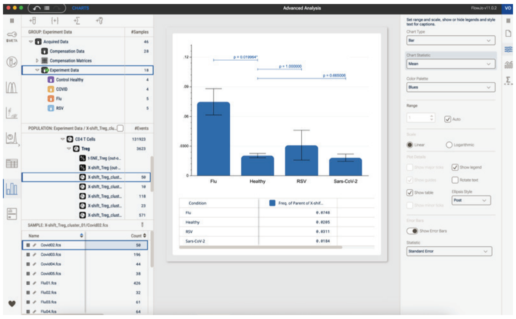
Additional tools such as **Cluster Explorer** and **Marker Enrichment Modeling** (MEM) are available in FlowJo™ v10 Software, to which FlowJo™ v11 Software analyses can be exported. These tools will come to FlowJo™ v11 Software soon and are free to use with your FlowJo Portal license.

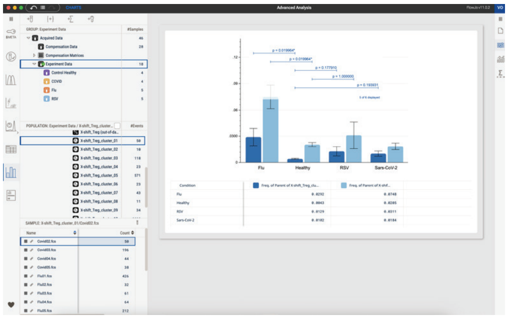




## Step 5: Comparison

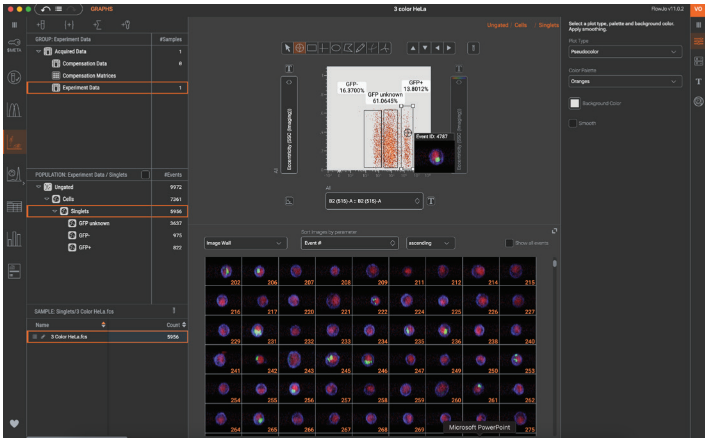
Comparing distributions of data is an important goal in many applications. Numerous experiments are designed to test whether two populations are statistically significantly different. Create a list of statistics in **Tables** to visualize them in **Charts** and perform tests for statistical significance.

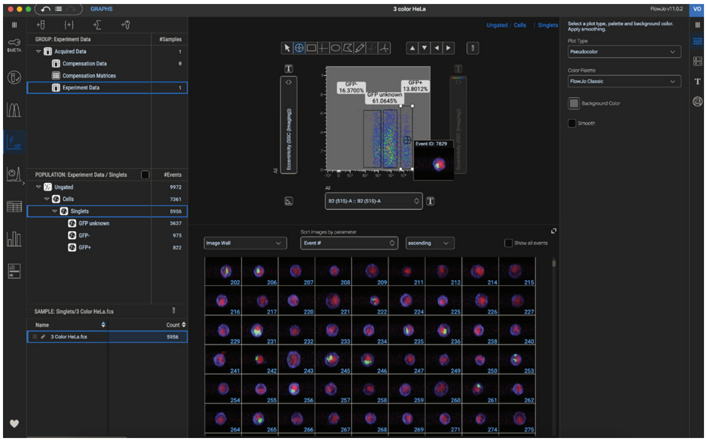




## Step 6: Data visualization

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. Additionally, the integrated **Image Wall** allows you to visualize images of cells collected on the BD FACSDiscover™ S8 Cell Sorter and BD FACSDiscover™ A8 Cell Analyzer.





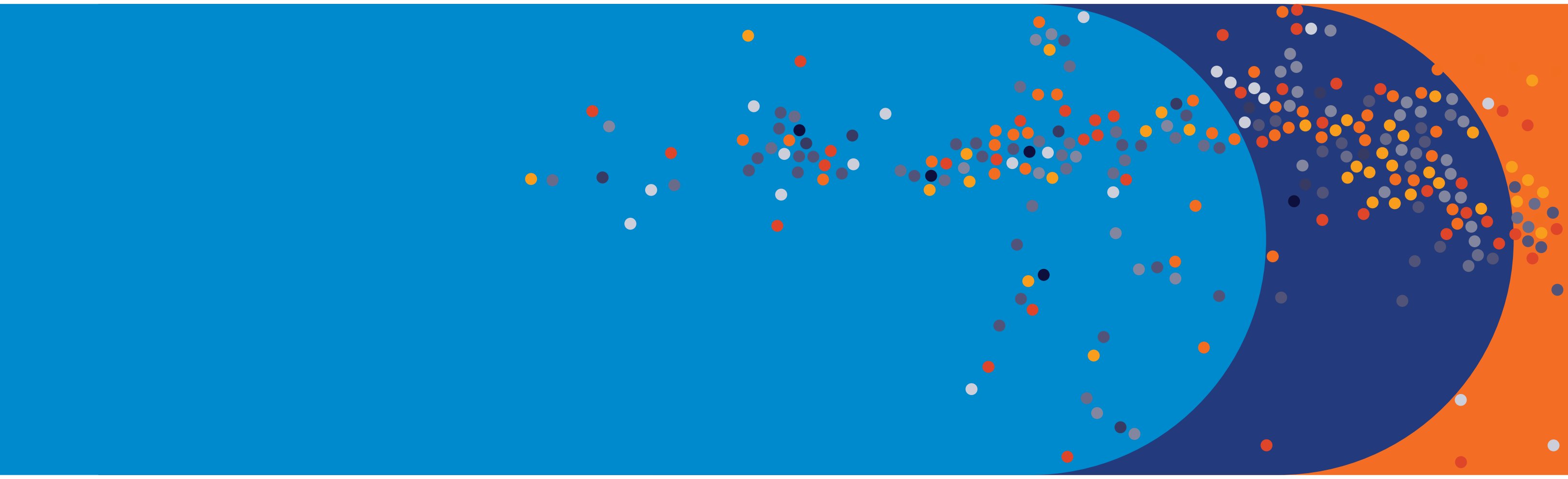
Embracing advanced data analysis techniques will help you extract meaningful insights to drive scientific discoveries.

This data analysis workflow described here serves as a general guideline, and specific analysis strategies may vary depending on research objectives and data characteristics.

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!

# Resources

- Webinars: https://flowjo.com/learn/webinars
- FlowJo University: https://www.flowjo.com/learn/flowjo-university/flowjo-v11
- Documentation: https://www.flowjo.com/docs/flowjo11
- Try FlowJo™ Software for free: https://flowjo.com/flowjo/free-trial
- FlowJo newsletter: https://www.flowjo.com/newsletter
- FlowJo Support: https://www.flowjo.com/contact
- Introduction to FlowJo™ Software brochures: https://flowjo.com/learn/marketing-materials



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