A guide to analyzing Attune CytPix Flow Cytometer data files in FCS Express 7 software

Introduction

The award-winning Invitrogen™ Attune™ CytPix™ Flow Cytometer combines acoustic focusing with a high-speed brightfield camera for simultaneous high-throughput flow cytometry and high-resolution brightfield imaging. Images of individual events and derived morphometric data can benefit an extensive, almost limitless range of flow cytometry applications. Invitrogen™ Attune™ Cytometric Software has automated image analysis capability to derive morphometric parameters (extended image-based parameters) using models pretrained on leukocytes and beads. These parameters may provide researchers a wealth of additional information for their assays beyond non–image-based cytometric methods.

This instruction guide is an introduction to the use of an additional software package, FCS Express™ software (De Novo Software, Pasadena, CA). The guide is intended to help users export data files from Attune Cytometric Software. Users can then open and adjust settings in FCS Express software to properly display and facilitate the analysis workflow when using Attune CytPix Flow Cytometer data files in either standard FCS file format or the ACS data file format that supports images and raw data. These instructions are being provided in part because we understand that users may want to take advantage of the workflow or analysis features provided by FCS Express software. We also highlight further analysis capabilities and features of FCS Express software to benefit Attune CytPix Flow Cytometer users. The pipeline feature allows users to access algorithms for high-dimensional data sets and provides innovative visualizations to help with data interpretation. This guide is not meant, however, to be a comprehensive resource for all features and functions available in FCS Express software. We suggest that you refer to the FCS Express software user guide or visit the De Novo Software website for help with the features not addressed here. These instructions will cover the needed steps for version 7 of FCS Express software; other versions may require modifications to these instructions, as menus and placement of settings may have changed.

User instructions

FCS Express software version 7.16.0047 or higher is required to open Attune CytPix Flow Cytometer ACS data files.

Dotmatics FCS Express

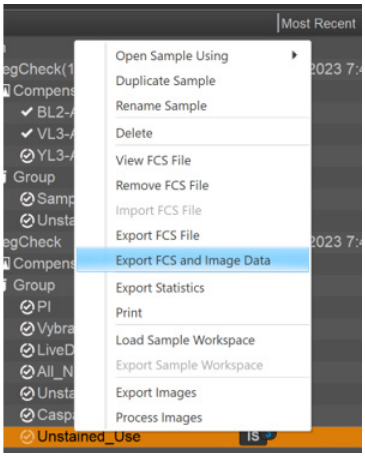
Note: Online tutorials and other helpful information can be accessed on the De Novo Software website, denovosoftware.com/full-access/, with a free De Novo Software account.

Exporting Attune CytPix Flow Cytometer data files from Attune Cytometric Software

Data files may be exported as standard FCS files (no images) or in the ACS file format (“FCS and Image Data” with images). Both are supported by FCS Express software.

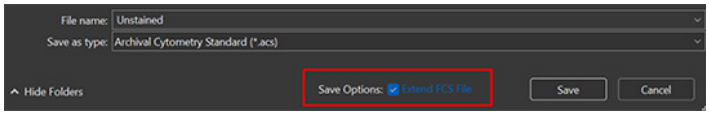
To export your data from Attune Cytometric Software to allow working with raw data and images in the ACS format:

1. Right-click on either the experiment (to export ACS files for all samples) or a single sample.
2. Choose the option **Export FCS and Image Data**.



Note: There are circles with a check mark next to the samples in the image. This icon indicates that the images have been processed in the software, which has generated image-based “extended parameters”. Users may choose to export data with or without the extended parameters in the next steps.

3. Check the **Extend FCS File** check box next to **Save Options**, and select **Save** at the bottom of the dialog.
4. Name the file, and then choose **Save**.



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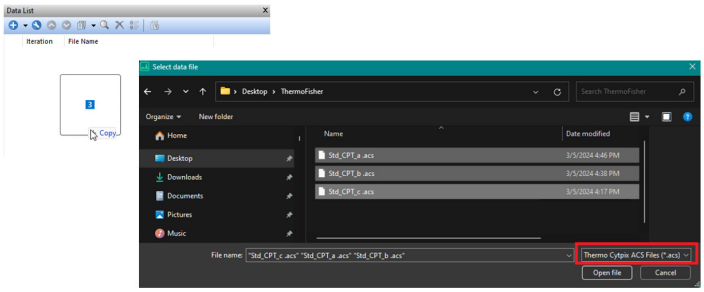
Opening Attune CytPix Flow Cytometer data files in FCS Express software

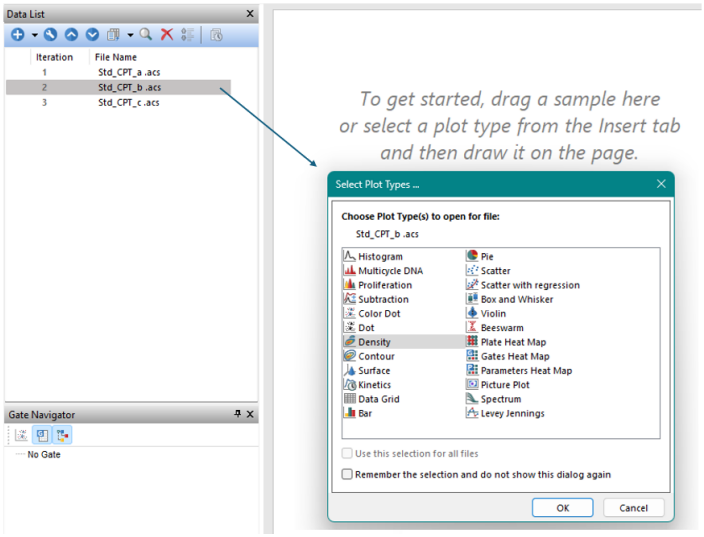
Attune CytPix Flow Cytometer ACS files are loaded into FCS Express software like any standard FCS files. Data files may be dragged and dropped onto the layout, dragged and dropped into the data list, or loaded via standard operating system dialogs. In the example below, we will add data files using the data list.

1. Select the desired Attune CytPix Flow Cytometer data files.
2. Drag the data files from the folder into the data list.

Note: Data files can also be added by clicking on the blue "**+**" icon in the Data List, navigating to the desired folder, choosing the file type from the drop-down menu, and selecting the desired files before clicking on **Open file**.

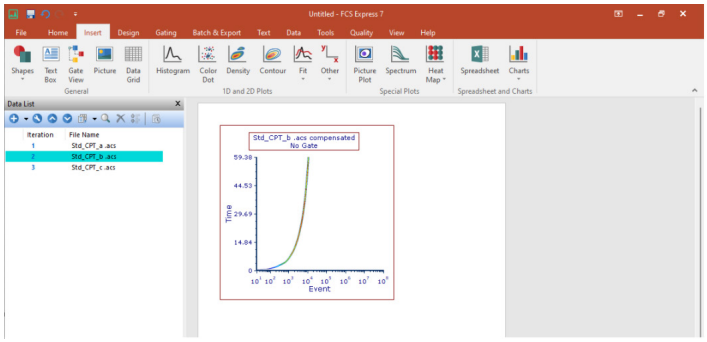


3. From the Data List, drag a data file onto the white space on the page.
4. Select one or more plot types.
5. Select **OK**.



Tip: You can select one or more plot types to generate in the workspace. You can also add plots by using the buttons for 1D and 2D Plots on the **Insert** tab.

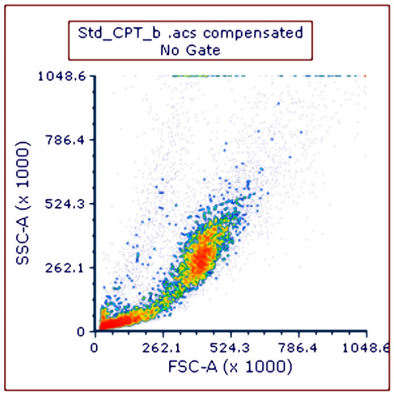
If, in the previous step, you selected plots to be generated, you will see a workspace that looks similar to the one shown below. Please see the section on **Working with images via data grids in FCS Express software for Attune CytPix Flow Cytometer data files** for information on utilizing data grids and accessing the image component of the ACS files.



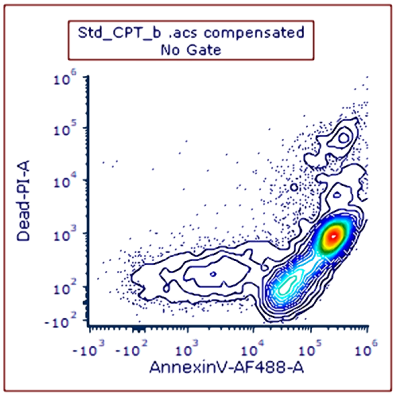
Working with 2D plots and parameters in FCS Express software

In most cases, no additional adjustments to plots or scaling are needed to visualize Attune CytPix Flow Cytometer data in FCS Express software. To adjust and change the parameters on a plot:

1. Choose each axis title and select the parameter you would like to display. In this example, FSC-A is displayed on the x-axis and SSC-A on the y-axis.

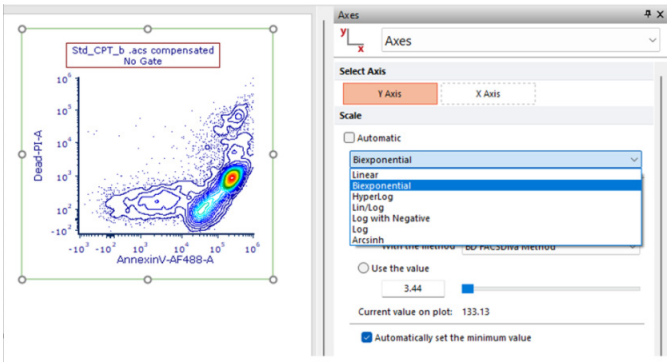


2. Add any additional plot to the layout via the **Insert** tab using your preferred plot type (e.g., density, color dot).

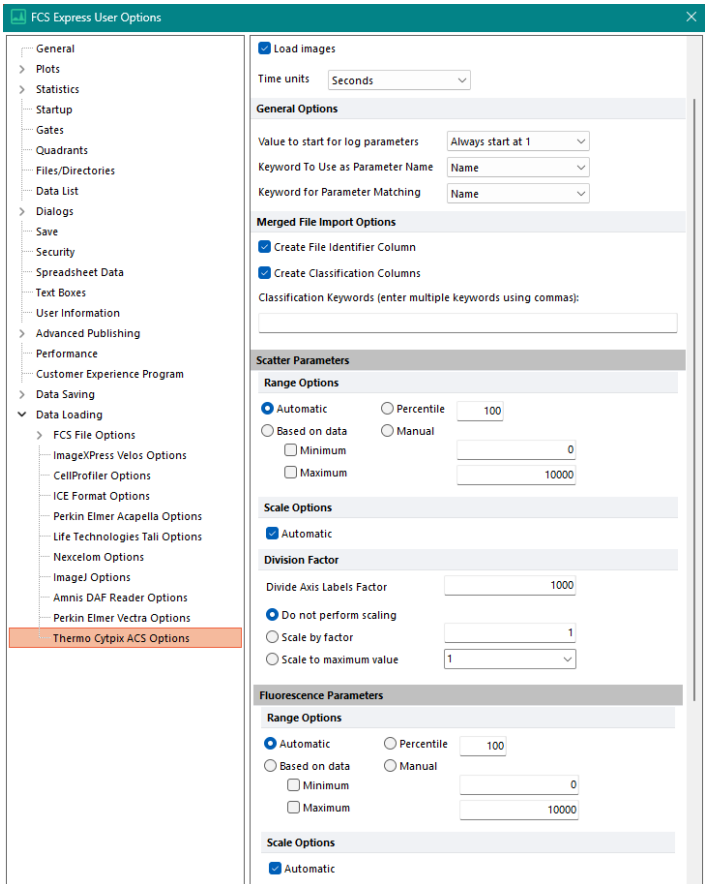


Note: If you would like to change the axis scaling, there are several options to choose from. To change the axis scaling, follow the optional steps below:

3. Select the plot, right-click, and select **Format** to bring up the formatting window.
4. Choose the drop-down menu at the top of the formatting window and select **Axes**.
5. Adjust scalings as desired.

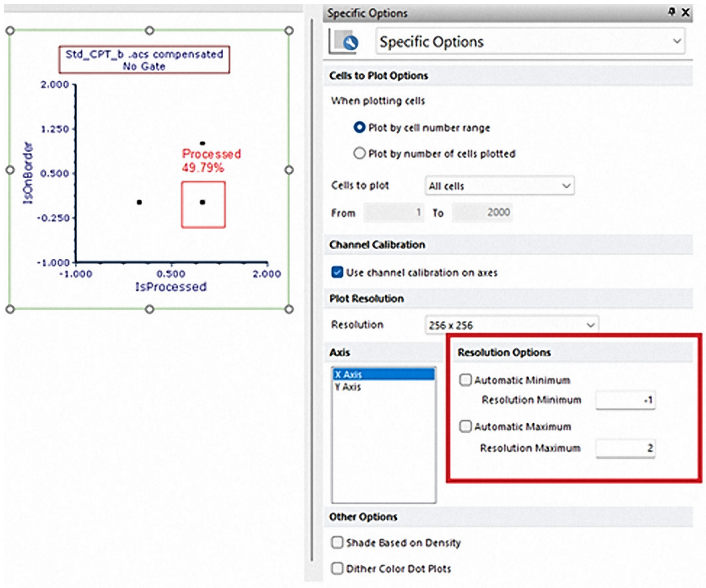


Adjusting the scaling may be useful when working with the **Extended Parameters**. Additionally, there are **user options specific to the Attune CytPix Flow Cytometer** accessible via the **File** tab → **Options** → **Data Loading** → **Thermo Cytpix ACS Options**. These options will allow you to set preferences for how you would like to visualize and scale data sets.

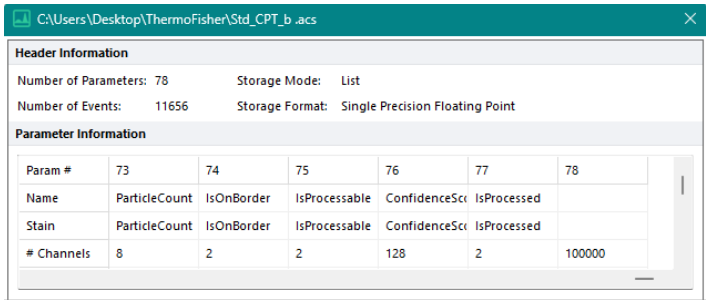


Tip: Setting the Scatter and Fluorescence Parameters **Range Options** to use **Based on data** with both **Minimum** and **Maximum** checked, and linear scaling, may be useful for display of extended parameters on plots. The scale formula for manually scaled plot parameters will be retained and applied automatically to all plots in the layout when selected.

In addition to adjusting scaling, a user can modify the range of data displayed on a plot by using **Resolution Options** from the **Format** → **Specific Options** section. This will optimize the display of data within a desired range.



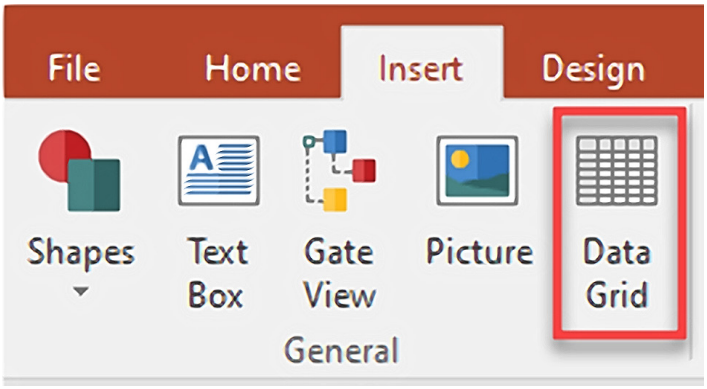
Tip: To determine the best range to set for **Resolution Minimum** and **Resolution Maximum**, the user may observe the range collected by the Attune CytPix Flow Cytometer by selecting a plot, clicking on the **Data** tab, and selecting **View Header** under **Parameter Information. # Channels** will indicate the maximum of the range.



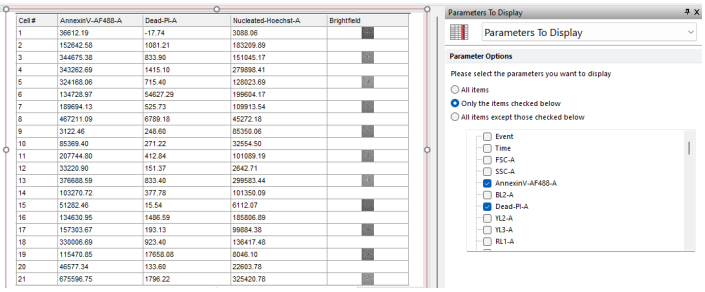
Working with images via data grids in FCS Express software for Attune CytPix Flow Cytometer data files

Data grids allow users to visualize the images obtained during acquisition. Data grids can have gates applied and create new gates. Multiple data grids can be used to visualize differences in images between gated populations or data files.

1. Select **Insert** tab → **Data Grid**.



2. Click on an area in the layout to insert the **Data Grid.** The data grid will show all parameters by default, with the Brightfield image parameter shown in the last column. In this example, the image has been modified to display desired parameters.

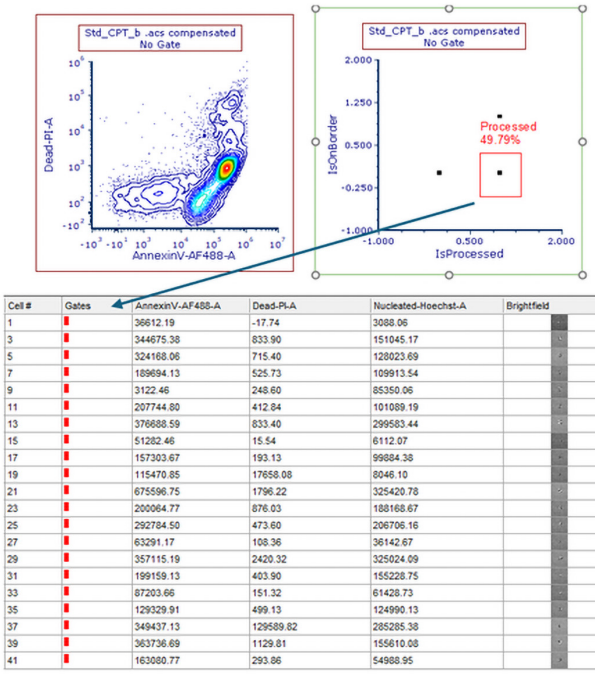


To format the data grid to show specific parameters or only images:

3. Select **Data Grid** → right-click → **Format**.
4. Select **Parameters to Display** from the drop-down menu. Using **Only the items checked below**, select the parameters to display in your data grid. Images are contained in the Brightfield parameter, usually last in the list.

Gating data grids and visualizing images

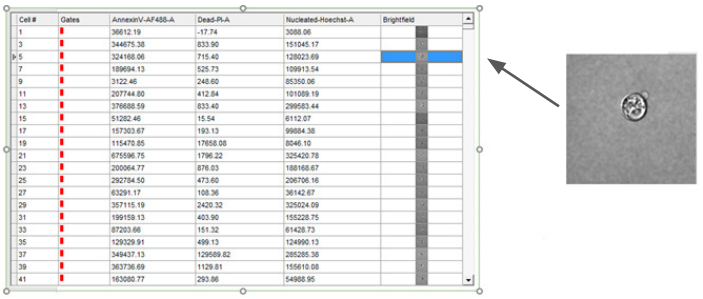
Gates may be applied to a data grid by dragging a gate onto the **Data Grid** or by selecting the **Data Grid** and modifying the **Current Gate** using the drop-down menu on the **Gating** tab. Data sets from the Attune CytPix Flow Cytometer may not include images of every event acquired. In those cases, users may create a gate on the "IsProcessed" parameter in order to visualize only events with associated images. An "IsProcessed" parameter value of "1" indicates an event for which an image exists. Once gates are created they may be dragged and dropped onto a **Data Grid** as shown below, to visualize the events and images associated with the event. Additionally, users may use the **Format → Gates To Display** feature to show a colored icon associated with the gate within which an event falls.



Cell #	Gates	AnnexinV-AF488-A	Dead-PI-A	Nucleated-Hoechst-A	Brightfield
1		36612.19	-17.74	3088.06	
3		344675.38	833.90	151045.17	
5		324168.06	715.40	128023.69	
7		189694.13	525.73	109913.54	
9		3122.46	248.60	85350.06	
11		207744.80	412.84	101089.19	
13		376688.59	833.40	299583.44	
15		51282.46	15.54	6112.07	
17		157303.67	193.13	99884.38	
19		115470.85	17658.08	8046.10	
21		675596.75	1796.22	325420.78	
23		200064.77	876.03	188168.67	
25		292784.50	473.60	206706.16	
27		63291.17	108.36	36142.67	
29		357115.19	2420.32	325024.09	
31		199159.13	403.90	155228.75	
33		87203.66	151.32	61428.73	
35		129329.91	499.13	124990.13	
37		349437.13	129589.82	285285.38	
39		363736.69	1129.81	155610.08	
41		163080.77	293.86	54988.95	

Note: The **Data Grid** at bottom right of the graph was selected (see blue arrow), **Processed** gate was chosen to display from the **Gates To Display** dialog, and the Processed gate was applied to limit visualization to only events with images.

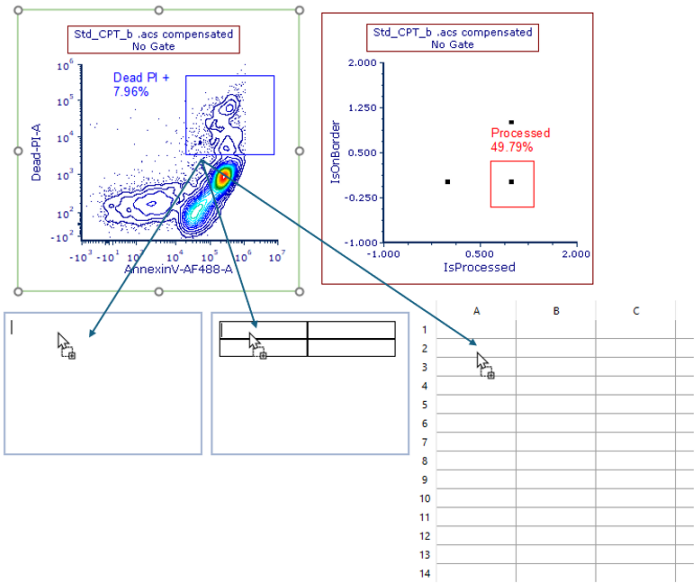
Images may also be inserted into the layout by dragging and dropping from the **Data Grid** onto a blank space in the layout. This is an effective tool to display representative images of a given population in your reports.

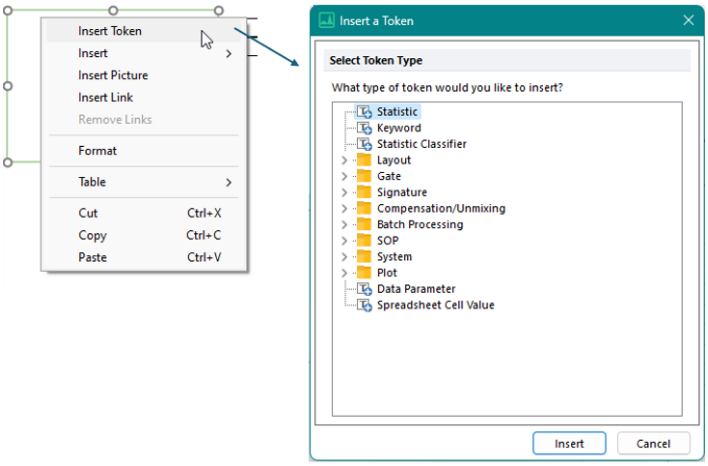


Working with statistics

Statistics can easily be accessed from any plot via a right click on the plot and selecting **Statistics → Gate Statistics**.

Additionally, statistics can be introduced into text boxes, tables, and spreadsheets by dragging a plot onto the object or by right-clicking in an object and using the **Insert a Token** dialog as shown. A token is live updating text that displays information such as statistics, FCS file keywords, layout properties, system information, and more.



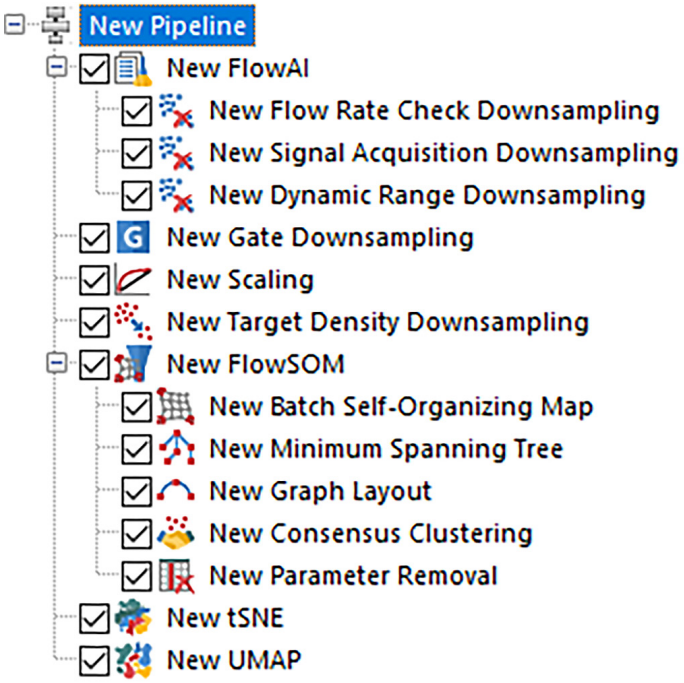


Working with high-dimensional analysis tools in FCS Express software

FCS Express software offers high-dimensional analysis tools via Pipelines. Pipelines enable researchers to use the flexible and intuitive interface of FCS Express software to perform advanced data analysis and processing steps, without the need for external applications such as R or Python. Within FCS Express software you have access to many of the most common flow cytometry algorithms and processing steps that are also applicable to your Attune CytPix Flow Cytometer data sets. Pipelines increase the computational flexibility and granularity of running algorithms and data transformations while giving users the unique ability to customize the data processing workflow. The steps below will assist you in accessing and implementing pipelines, but we suggest viewing the extensive resources on the De Novo Software website to understand the tools offered and how they should be implemented practically.

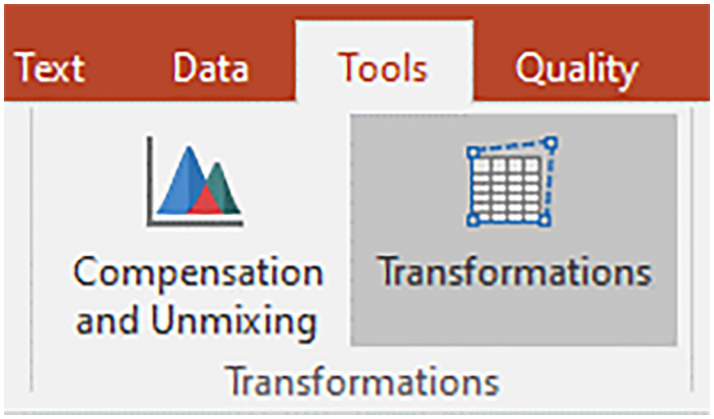
Please see:

1. Pipelines overview
2. Learn more about available Pipeline Steps and their operation

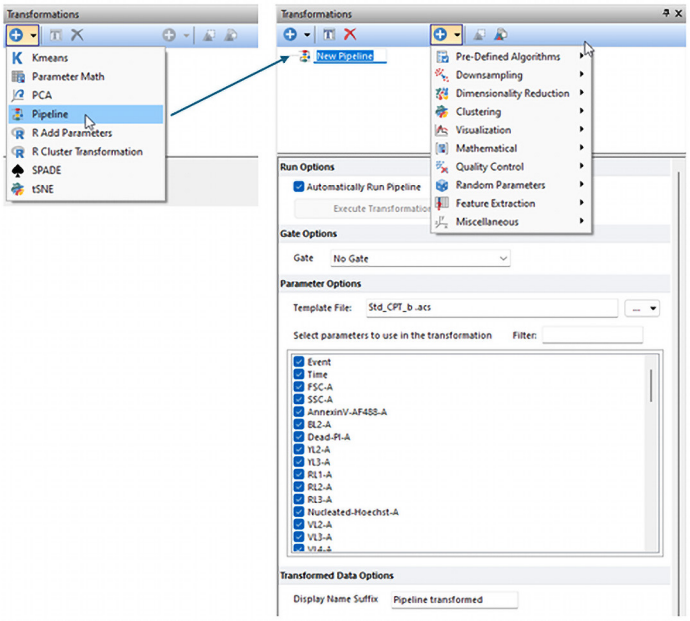


To access high-dimensional analysis tools using a pipeline:

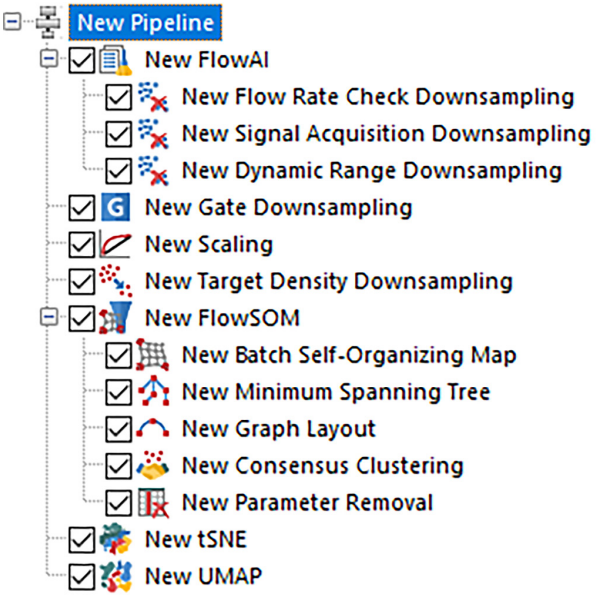
1. Select the **Tools** tab → **Transformations**.



2. Select the blue "+" icon at the far left and select **Pipeline**.
3. Select the pipeline created.
4. Select the blue "+" icon at the right to select a pipeline step to insert. Add additional steps as desired.

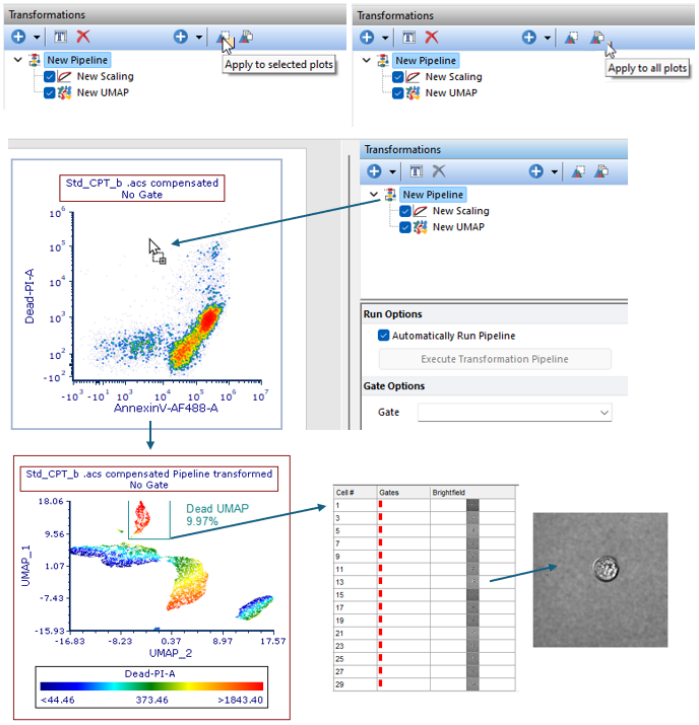


Users may select as many pipeline steps as needed to achieve the desired results. Actions within the pipeline can be checked or unchecked.



To run a pipeline on a data set, choose the method below that best suits your needs. Once a pipeline is applied, the series of steps will begin running. If running a step results in new parameters being created (i.e., UMAP, tSNE), the new parameters will be accessible on any plot the pipeline is applied to after the run is complete.

- Select specific plots for the pipeline: choose **Apply to selected plots** or select **Apply to all plots.**
- Drag and drop the pipeline root step onto a plot.



Gates created on the pipeline-transformed plot may be dragged onto a **Data Grid** to visualize events and images associated with the population or cluster of interest. Additionally, representative images may be dragged onto the layout for further reporting.

Note: When high-dimensional analysis is performed, it is often necessary to merge multiple files into one file before running a pipeline, and files are then deconvolved via gates to compare file-to-file variability and clustering. To learn more about the process of merging ACS files for use in high-dimensional data reduction pipeline steps, please see **How can image ACS files from the Attune CytPix be merged for high dimensional analysis?**

Learn more at **thermofisher.com/cytpix**