LESSON PLAN

FCS Express High-Dimensional Data Reduction Tools Demonstration

Length: Approximately 75 minutes

Goal	To demonstrate high-dimensional data reduction tools in FCS Express™
Objectives	By the end of this module, participants will be able to: • Perform tSNE analysis and utilize tools to visualize tSNE plots in FCS Express. • Perform FlowSOM and utilize tools to visualize FlowSOM plots in FCS Express.
Materials	• Computer • Data files acquired on day 3 of training or any other sample data files
Prior to the Lesson	If you are not using the data from day 3 of training, identify a dataset to use for the demo.

Introduction

There are many different platforms that one can use to analyze mass cytometry data. We support FCS Express, a fully featured flow cytometry data analysis package that has been designed to work seamlessly with mass cytometry data. New Helios™ and CyTOF® XT instruments and Hyperion™ Imaging Systems will include a one-year license of FCS Express Flow software.

Performing high-dimensional data reduction is a quick and easy process with algorithms built directly into FCS Express. There is no need to rely on R or other outside software packages to perform dimensionality reduction. In the following primer you will learn how to set up, visualize, and prepare results for interpretation of the t-distributed stochastic neighbor embedding (tSNE) algorithms using the user interface in FCS Express. You will also be guided through preparation of data and charts using the FlowSOM (Self-Organizing Map) algorithm via FCS Express “pipeline” steps.

Demonstration

NOTE: A copy of FCS Express Flow is distributed with new Helios, CyTOF XT™ and Hyperion Imaging System instruments. You can claim your free copy of FCS Express via the license packet that was provided with the instrument. Additional licenses, support, and free training can be requested through De Novo Software™ any time at **support@denovosoftware.com**. Alternatively, you can download a one-month free trial from **https://denovosoftware.com/demo-overview/**.

NOTE: This is a brief overview. You can find additional online tutorials, short videos, and other resources at **denovosoftware.com/full-access/**.

Performing tSNE Using FCS Express

The steps to perform and interpret tSNE are explained in the section below. Briefly, the analysis workflow consists of merging/grouping all the data files to be examined in the experiment, selecting variables for the tSNE algorithm, running the algorithm, using plot visualizations to deconvolve/ungroup the individual data files, and using additional plots for visualization/interpretation. The following steps guide you through these processes in FCS Express.

Merge Data Files for Preparation of tSNE Run

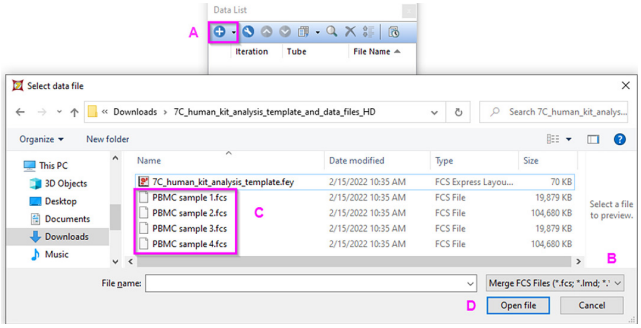
1 Open a new or existing FCS Express layout.

EXAMPLE: Use the **7C_human_kit_analysis_template.fey layout** and accompanying example data files within the zip folder. Alternatively, use your own layout and data files.

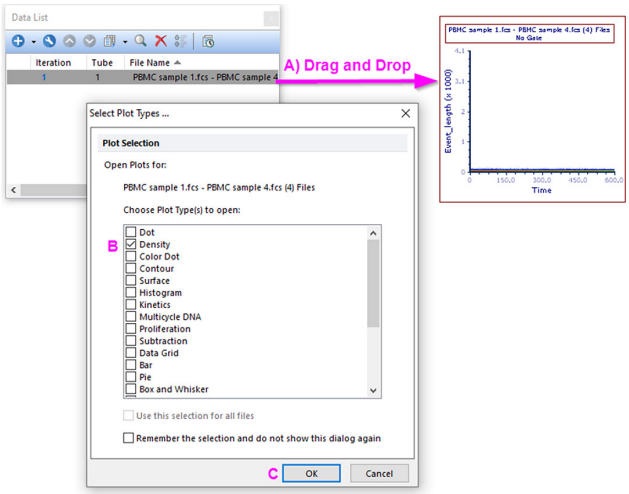
2 Select the **Data** tab ➔ **Data List**, and then click the blue plus (A) to **Select data file**.

3 Click **Options** (B) to **Merge FCS Files (*.fcs, *lmd, *.*)** (C), browse to select the data files to merge, and click **Open** (D).

The files selected are merged into one .fcs file in the Data List for the tSNE run.



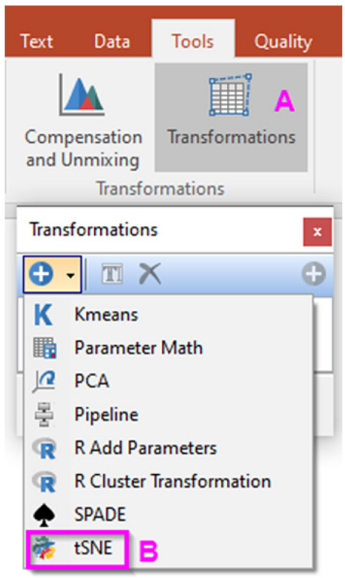
4 Insert a plot of the virtual merged data file by dragging and dropping it from the **Data List** to a blank spot or new page of the layout (A), choose **Density** from the Plot Selection dialog (B), and click **OK** to insert the plot (C).



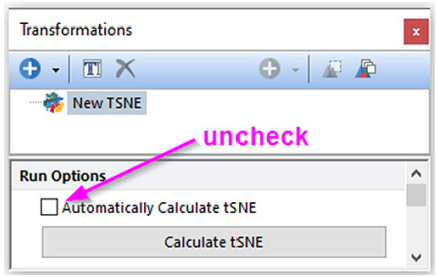
Choose Variables and Define the tSNE Settings

NOTE: The FCS Express manual contains additional details on all available options and variables for the **tSNE Run Options**.

1 Select the **Tools** tab, then select **Transformations** (A). Click the blue plus ⊕ and then choose **tSNE** (B) from the dropdown menu.

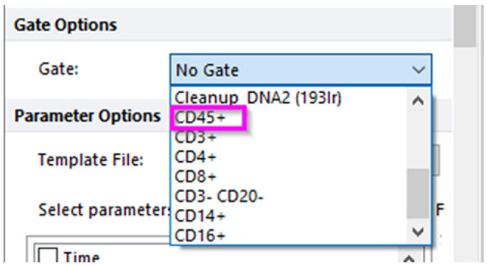


2 From **Run Options**, uncheck **Automatically Calculate tSNE**.

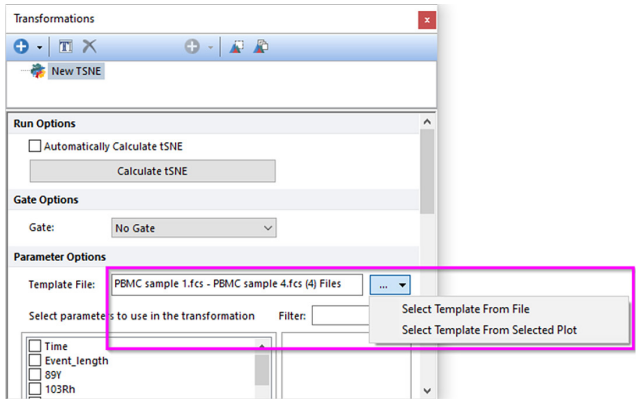


3 From **Gate** dropdown menu in the **Gate Options** section, choose a high-level gate that includes all your subsets of interest but excludes events such as debris, doublets, contaminating cells, etc. The tSNE transformation will be calculated only on these cells.

EXAMPLE: In the provided 7C_human_kit_analysis_template.fey with its example data files, choose CD45+. Your choice will depend on your experimental goal.



4 Check that the merged file (PBMC sample 1.fcs – PBMC sample 4.fcs (4) Files) is set as the template file for the tSNE calculation in the **Template File** field found in the **Parameter Options** section. If not, click on the plot inserted in Step 4 in the Merge Data Files for Preparation of tSNE Run section. Choose **Select Template From Selected Plot**.



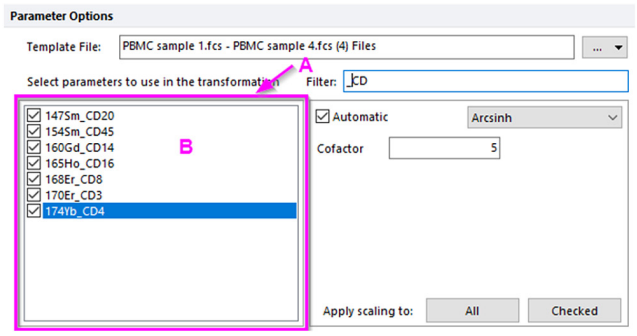
The **Template File** provides for the selection of raw data parameters associated with the file(s) for tSNE analysis.

EXAMPLE: If you are using the 7C_human_kit_analysis_template.fey layout and its associated example data files, the template file will be automatically filled with the data currently loaded in the layout.

5 From **Parameter Options**, check the box beside the parameters on which the tSNE transformation will be calculated from the list box at left. Enter **CD** into the **Filter** field (A) to narrow down the list, and choose the parameters of interest (B).

EXAMPLE: Select all the CD parameters (CD20, CD45, CD14, CD16, CD8, CD3, and CD4).

The chosen parameters for the algorithm are up to the user to define and depend on the experimental goals and desired results.

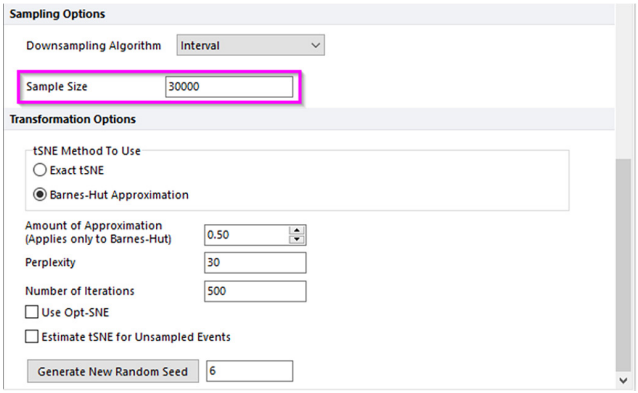


6 Enter the **Sample Size** in the **Sampling Options** section.

EXAMPLE: Enter 30000.

NOTE: If you wish to change additional downsampling and transformation options, you may do so in the **Sampling Options** section of the tSNE transformation dialog. See the FCS Express manual for more details on all of the **Sampling Option variables settings** and different **downsampling options**.

FCS Express has the ability to use **Opt-SNE** and the unique ability to upsample/**Estimate tSNE for Unsampled Events** for events that were not included in the tSNE run because of either gating or downsampling. Many of the default settings in this primer are chosen to allow for a faster tSNE run while you learn how to use the basics of the interface. See **tSNE performance in FCS Express** to get a better idea of how your data size and computer will impact tSNE run times.

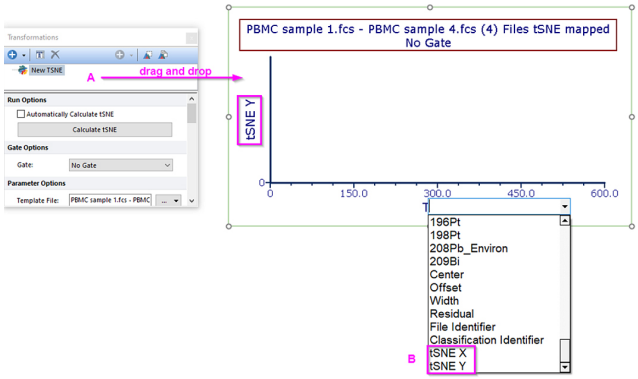


Run the tSNE Algorithm

1 Drag and drop the **New TSNE** transformation (A) from the upper pane of the Transformations window to the plot inserted in Step 4 of the Merge Data Files for Preparation of tSNE Run section. The plot title is subsequently appended with the text tSNE mapped.

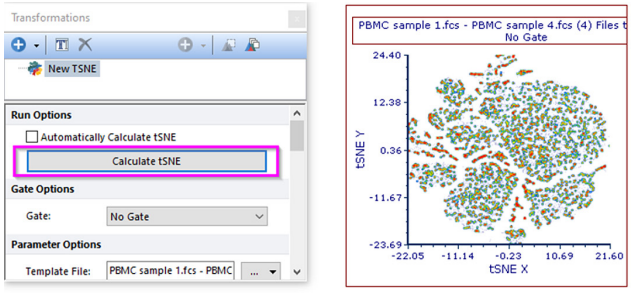
2 Change the plot X- and Y- axes to **tSNE X** and **tSNE Y** (B), respectively.

NOTE: The tSNE plot will not yet contain data because the tSNE transformation has not yet been calculated.



3 From **Run Options**, click the **Calculate tSNE button**. The plot to which the tSNE Transformation was applied will be populated when the calculation is complete.

EXAMPLE: The results from the example data files, calculated on a Sample Size setting of 30,000, are shown.

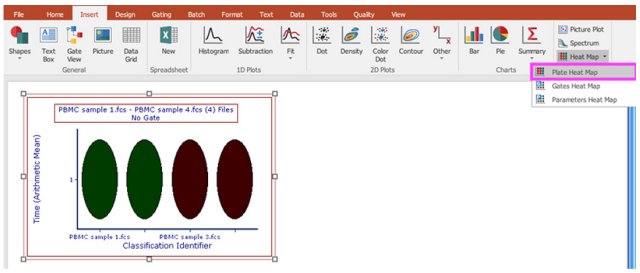


NOTE: While the algorithm is running, FCS Express will be unresponsive. When the tSNE calculation is complete, the tSNE X vs. tSNE Y plot will be populated automatically with data.

Deconvolution/Ungrouping Individual Data Files

After performing tSNE on a merged sample you will likely wish to view the results of each component file. You may also want to group component files from within the merged file for final analysis. In the next steps we will automatically create gates on each of the component files of the merged file to facilitate deconvolution. If you only wish to view the results on the merged file, proceed to Step 7.

1 On the **Insert** tab, select **Heat Map**. Then select **Plate Heat Map** from the dropdown menus and click once on an empty spot on the layout to create the **Plate Heat Map** plot.



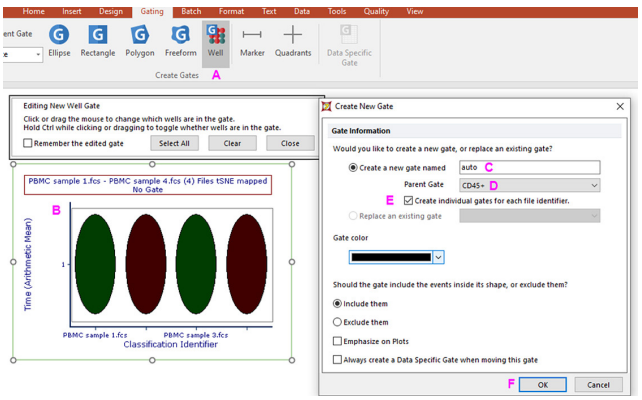
2 On the **Gating** tab, select **Well** (A). Then (B) click and drag your mouse cursor to encompass all the wells on your Plate Heat Map, which was created in the previous step.

3 Enter **auto** in the **Create a new gate named** field (C).

4 Select the gate on which the tSNE was calculated (D) (specified in Step 3 of the Choose Variables and Define the tSNE Settings; for the example choose CD45+) from the **Parent Gate** dropdown menu.

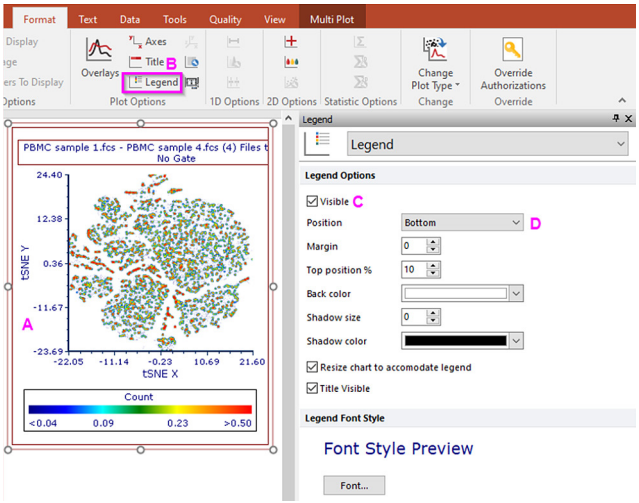
5 Select the **Create individual gates for each file indentifier** checkbox (E).

6 Choose black from the **Gate color** dropdown menu and click **OK** (F).

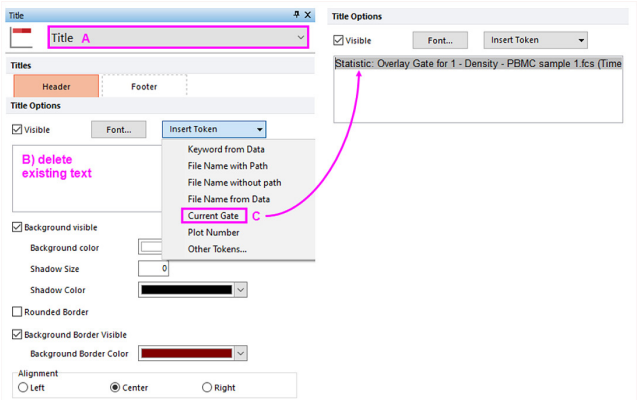


7 To visualize the results of the tSNE calculation on many parameters at once:

a Click the tSNE plot to select it (A), click the **Legend** on the **Format** tab (B), check the **Visible** box (C), and choose **Bottom** from the **Position** dropdown menu (D).

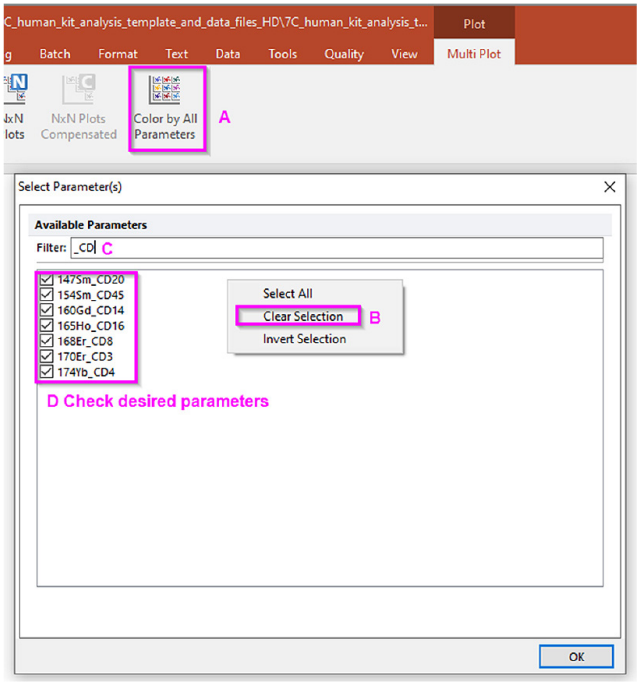


b Choose **Title** from the dropdown menu (A), delete existing text or tokens in the **Title Options** text field (B). Then choose **Current Gate** from the Insert Token menu (C). The gate applied to the plot will now appear in the plot title.



c Click the **Plot** tab family, then select **Multi Plot** tab and click the Color by All Parameters button (A). Right-click in the window to **Clear Selection** (B), enter CD in the Filter: field to narrow down the list (C), and check the CD parameters that are of interest regarding the tSNE analysis (D). Then click **OK**.

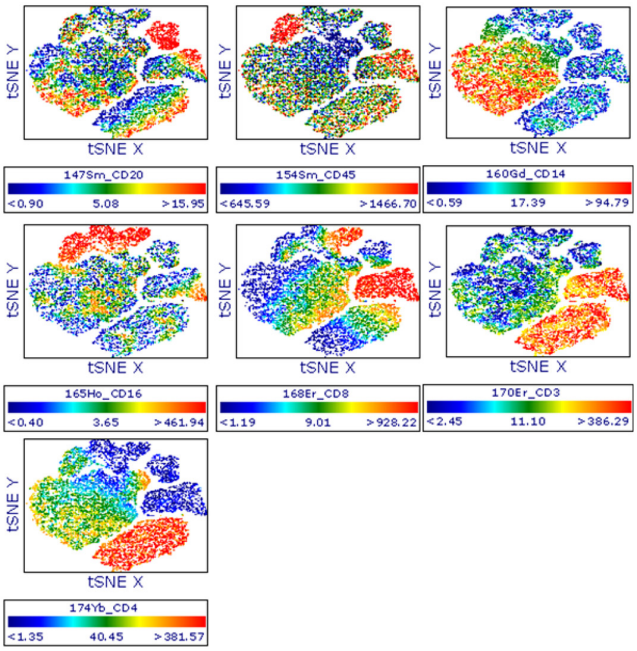
EXAMPLE: Choose all the CD parameters (CD20, CD45, CD14, CD16, CD8, CD3, and CD4).



d Drag your mouse cursor around the group of plots created in the previous step to select them and resize, if desired. Repeat Step 7a to add a Legend.

The Multi Plot array will now show descriptive Legends, where each plot displays the intensity of expression of the indicated parameter.

NOTE: You may **reduce plot resolution via Specific Options** if the plot colors are faint due to having only few events to visualize. For example, if you completed Steps 1–7 and/or tSNE was calculated on a small sample size (i.e., extensively downsampled).



8 To view the results from the individual component files, select the tSNE multiplot array/group and its parent plot by clicking on the layout page (outside of any plots, so no objects are selected), and press Cmd[mac]-/Ctrl[PC]-**A** to select all. Then from the **Gating** tab, select **Current Gate** from the dropdown menu. Choose the automatically created gate that corresponds to the original component data file of interest.

The multiplot array/group and its parent plot are regated to show only the events corresponding to the specified original data file. Repeat as needed for the other component files.

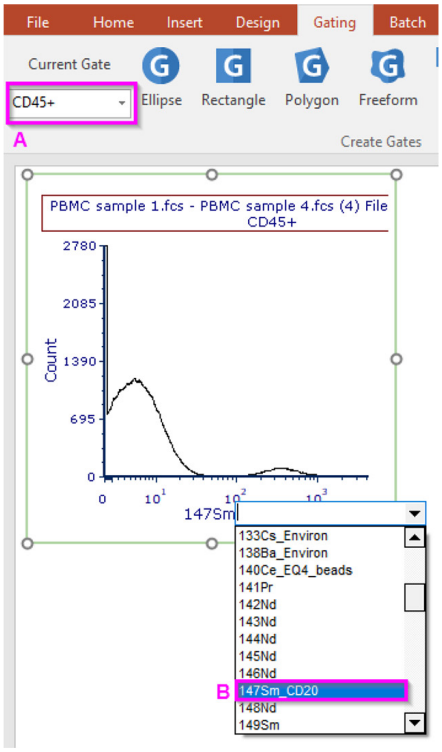
9 (Optional) To view the results from the individual component files on separate layout pages, **select**, **copy**, and **paste** the multiplot array/group and its parent plot to a **new page** in the layout. Then from the **Gating** tab, select **Current Gate** from the dropdown menu, and choose the automatically created gate that corresponds to the original component data file of interest.

10 To evaluate expression across the tSNE continents via **Histogram Parameter Overlays**:

a On the **Insert** tab, select **Histogram** button. Then click once on a blank spot of the layout (or a new page) to insert the plot.

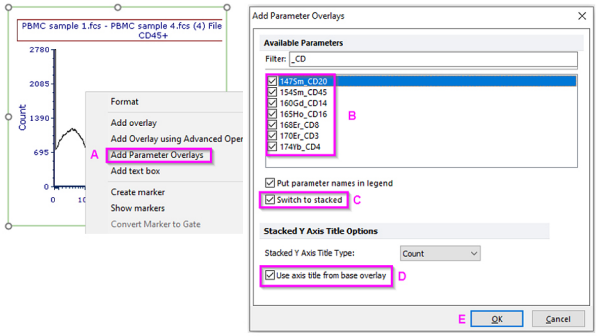
b On the **Gating** tab, select a gate of interest from the **Current Gate** dropdown menu (A). Then change the parameter X-axis parameter to a parameter of interest (B).

EXAMPLE: Choose the gate on which the tSNE was calculated, CD45+. Then select 147Sm_CD20.



c Right-click on the plot and choose **Add Parameter Overlays** from the pop-up menu (A). Then select additional parameters of interest (B). Check the **Switch to stacked** (C) and **Use axis title from base overlay** (D) boxes. Click **OK** (E).

EXAMPLE: Select all the CD parameters including CD20, CD45, CD14, CD16, CD8, CD3, and CD4 from the **Available Parameters** list.

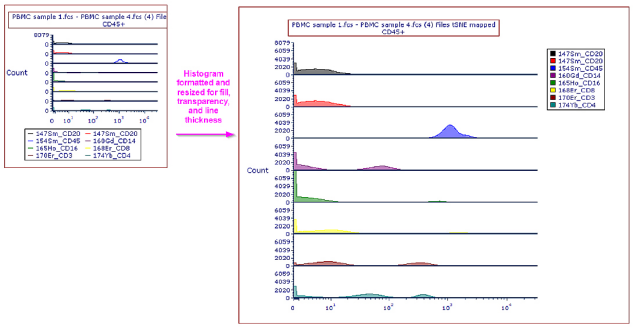


d Repeat Step 7a to add a Legend on the right side of the plot.

e Expand the width and height of the plot to better visualize the data.

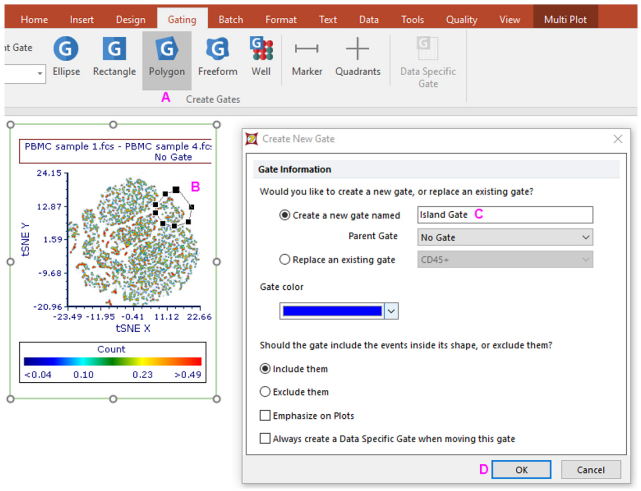
f (Optional) To access the histogram **overlay** property formatting options, select **Overlays** on the **Format** tab to adjust fill, transparency, line thickness, smoothing, and other formatting options.

EXAMPLE: For the data used in this primer we recommend a smoothing of 10 and using transparency fill set to 50.



11 Create a gate on the tSNE plot and apply it to the histogram parameter overlay plot to interrogate expression among the tSNE islands.

a On the **Gating** tab, select **Polygon** (A) and draw a gate on one of the tSNE islands within the parent plot shown in Step 7, which shows the virtual merged data file (B). Then enter **Island Gate** (C) and click **OK** (D).



b Click on the histogram parameter overlays plots shown in Step 10f to select it. On the **Gating** tab, select **Island Gate** from the **Current Gate** dropdown menu.

The plot has now been regated to show only events within the island gate. You may resize or reposition the island gate. Notice the change in the histogram parameter overlay plot to evaluate expression of different populations or islands within the tSNE plot.

Performing FlowSOM Using FCS Express

FlowSOM is a transformation that employs Self-Organizing Map clustering and Minimal Spanning Trees. These steps are followed by a Consensus Clustering step in which metaclusters are identified. Briefly, the steps in a FlowSOM analysis are to merge the data files from your experiment, choose variables for the FlowSOM algorithm, run the algorithm, use plot visualizations to deconvolve/ungroup the individual data files, and use additional plots to examine the results at the metacluster level. The following procedure guides you through this workflow in FCS Express.

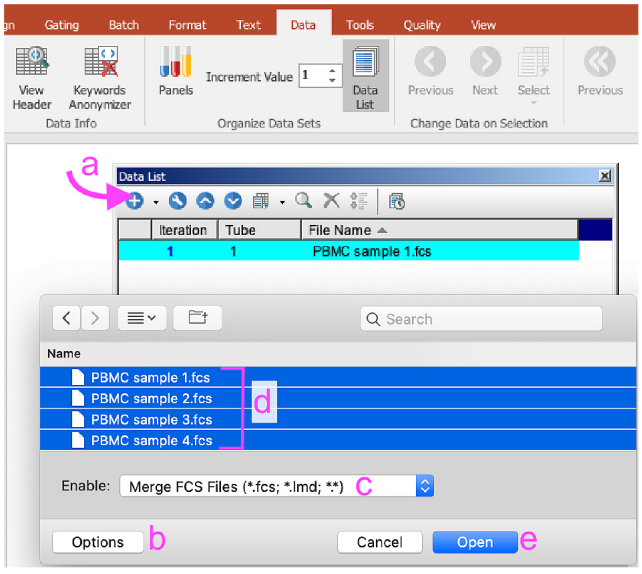
Merging Data Files for Preparation of FlowSOM

NOTE: If you have already completed the previous section (Performing tSNE Using FCS Express), proceed to Step 3.

1 Open a new or existing FCS Express layout.

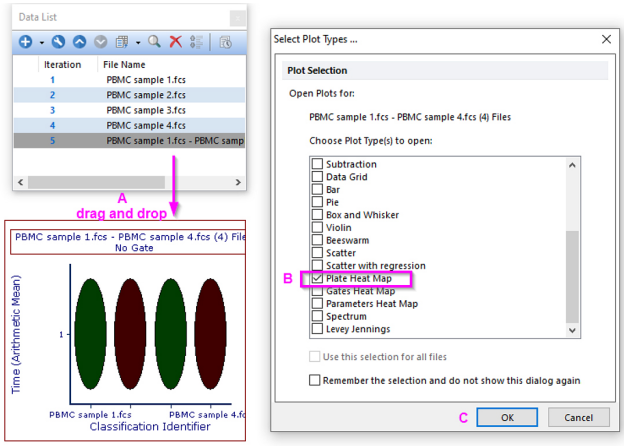
EXAMPLE: Use the **7C_human_kit_analysis_template.fey layout** and accompanying example data files within the zip file. However, you may use any layout and data files.

2 To create a merged data file select **Data List** from the **Data** tab. Then click the blue plus ⊕ to Select data file (a). Select **Options** (b) to **Merge FCS Files (*.fcs, *lmd, *.*)** (c). Browse to select data files to merge (d). Click **Open** (e).



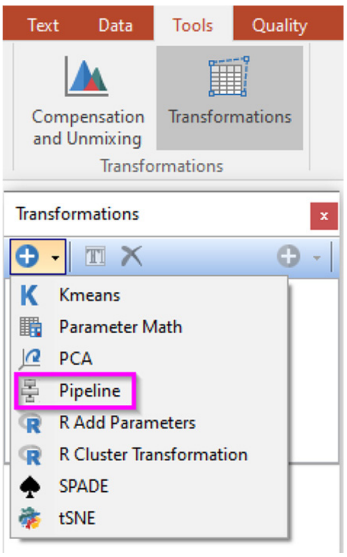
The files selected will be merged into one .fcs file in the Data List for the FlowSOM run.

3 Drag and drop a data file from the Data List to a blank spot on the layout or to a **new page** (A) to insert a plot of the virtual merged data file. Choose **Plate Heat Map** (B). Click **OK** (C).

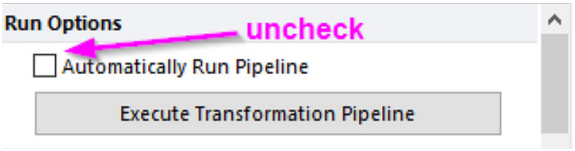


Choosing Variables and Defining the FlowSOM Settings

1 Click **Transformations** on the **Tools** tab. Click the blue plus and choose **Pipeline**.

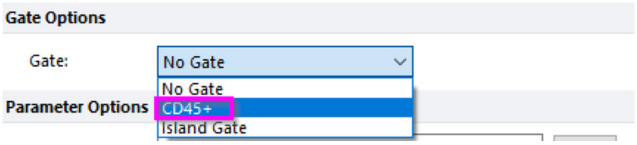


2 Uncheck **Automatically Run Pipeline** from Run Options.



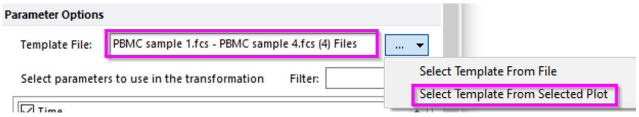
In the **Gate Options** section, choose a high-level gate that includes all your subsets of interest but excludes events such as debris, doubtlets, and contaminating cells from the **Gate** dropdown menu. The Transformation Pipeline is calculated only on these cells. The selection depends on your experimental goal.

EXAMPLE: Choose CD45+.



Check that the merged file is now set in the **Template File** field in the **Parameter Options** section for the tSNE calculation. If not, click on the plot inserted in Step 3 of the Merging Data Files for Preparation of FlowSOM section, and choose **Select Template From Selected Plot**.

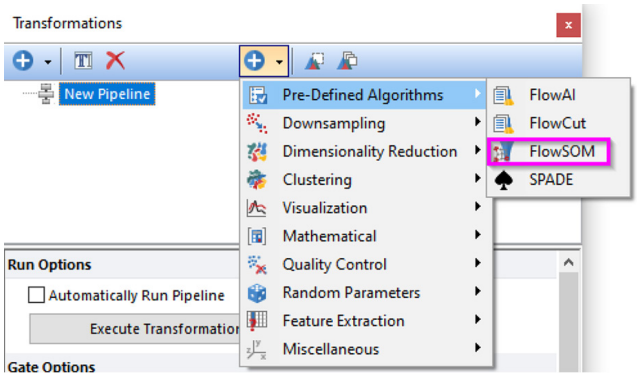
EXAMPLE: PBMC sample 1.fcs – PBMC sample 4.fcs (4) Files is set as the Template File.



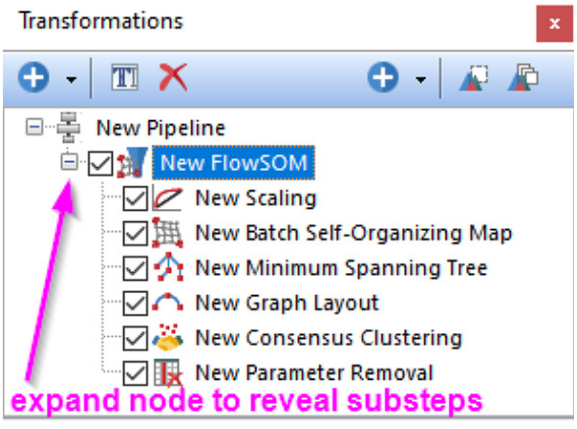
The **Template File** provides the raw data parameters associated with the file(s) selected for the FlowSOM analysis.

EXAMPLE: For the 7C_human_kit_analysis_template.fey layout and the associated example data files, the template file will automatically contain the data currently loaded in the layout.

3 Click the blue plus at the top of the **Transformations** window on the right. Select **Pre-Defined Algorithms** and then FlowSOM.



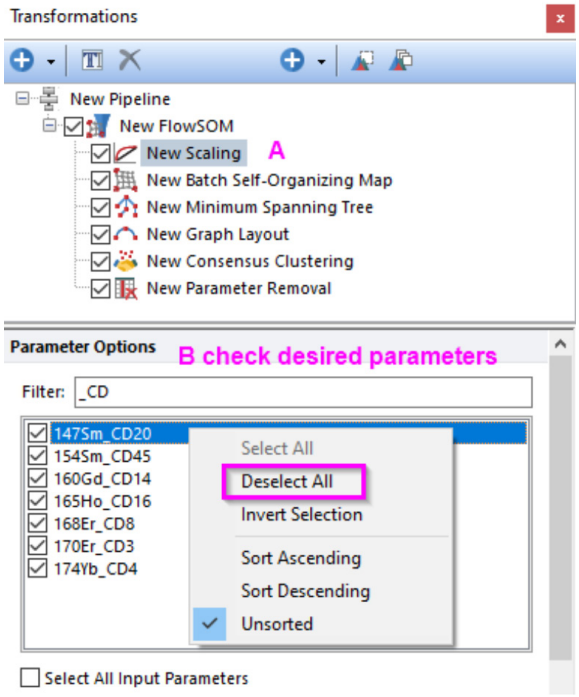
4 Click to expand the New FlowSOM node to reveal its six substeps.



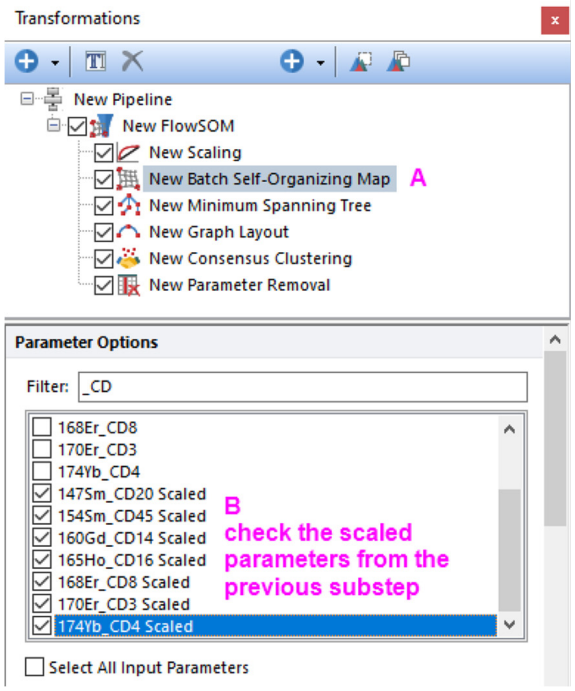
5 Click on **New Scaling** (A). Select the parameters under Parameter Options (B) that the Self-Organizing Map will use to calculate.

NOTE: Right-click within the box to **Deselect All** first and enter **CD** in the **Filter**: field to narrow down the list.

EXAMPLE: Select all the "CD" parameters (CD20, CD45, CD14, CD16, CD8, CD3, and CD4). The chosen parameters depend on the experimental goals.



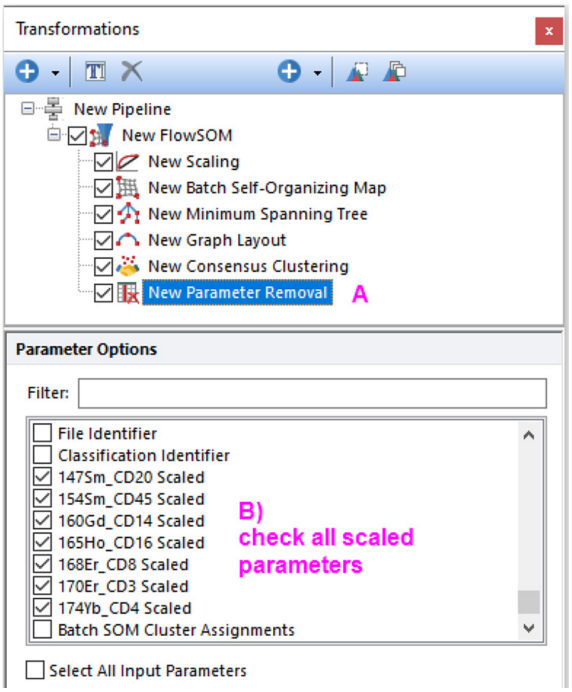
6 Select **New Batch Self-Organizing Map** (A). Choose the **Parameter Options** (B) defined in the previous substep, if they are not selected.



7 (Optional) If you wish to change other options within any of the FlowSOM substeps, you may do so. Details on the settings are available in the FCS Express manual **here**.

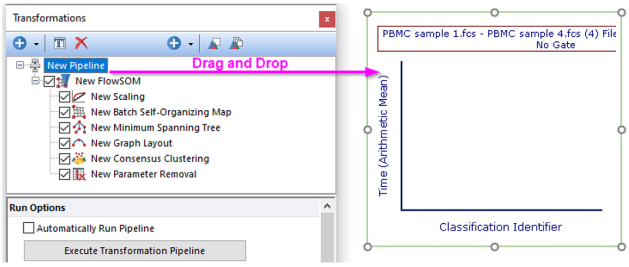
8 (Optional) Add a **downsampling step** before the New Scaling substep in the Pipeline.

9 Select **New Parameter Removal** (A). Then select the scaled parameters (defined in the previous substep), if they are not selected (B). This step prevents duplicate copies of these parameters from appearing in your plot axes.

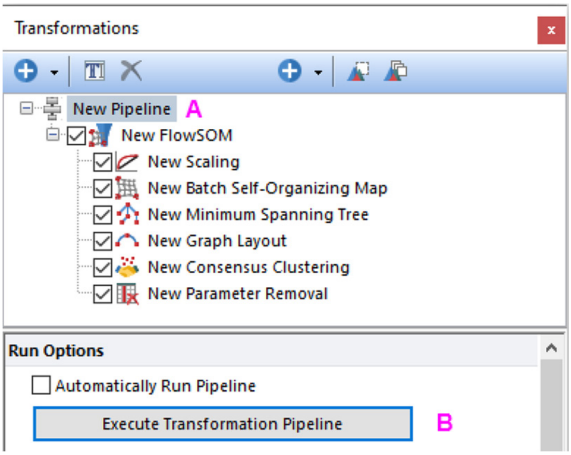


Running the FlowSOM Algorithm

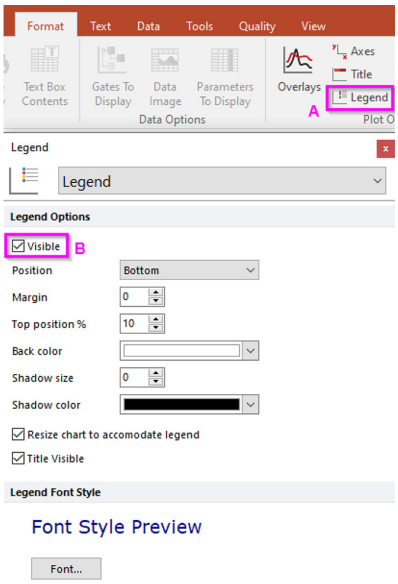
1 Drag and drop the **New Pipeline** transformation from the upper pane of the **Transformations** window to the Plate Heat Map plot inserted in Step 3 in the Merge Data Files for Preparation of tSNE Run section (note that plot title changes).



2 Select **New Pipeline** (A) in the upper pane of the Transformations window. Click **Execute Transformation Pipeline** (B) under **Run Options**.

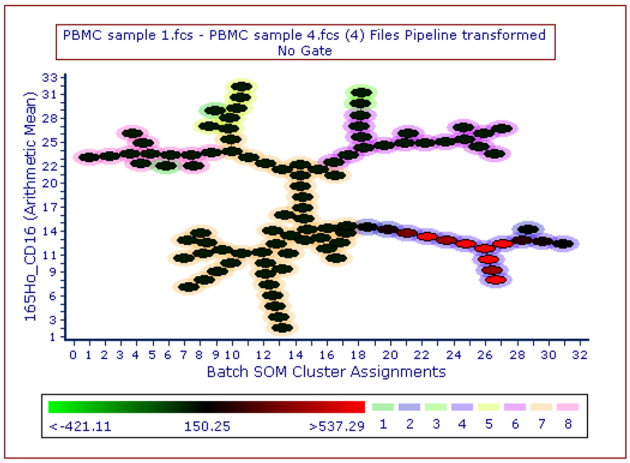


3 On the **Format** tab, select **Legend** (A) and check the **Visible** box (B).



4 Click on the X- and Y-axes to change them to Batch SOM Cluster Assignments and any parameter of your choice (e.g., CD16), respectively. Enter CD16 or any other text after clicking on the Y-axis to filter the list.

The red-green gradient in the legend displays the mean expression level of the Y-parameter for each node (the internal color of the cluster). The discrete color swatches 1–8 on the right side of the legend denote the metaclusters (the peripheral color of the cluster).

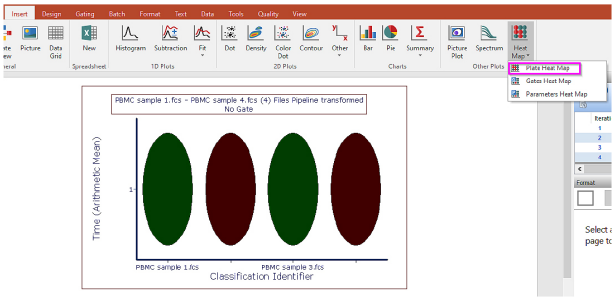


Deconvolving/Ungrouping Individual Data Files

You can view the FlowSOM results of each component file after performing FlowSOM on a merged sample. You can also group component files from within the merged file for final analysis. In the next steps we automatically create gates on each component file of the merged files to facilitate deconvolution. If you only wish to view the results on the merged file, proceed to the next section, Interrogating Metacluster Phenotypes.

1 On the **Insert** tab, select **Heat Map**, then **Plate Heat Map**. Click once on an empty spot on the layout to create the Plate Heat Map plot.

NOTE: The plot title should contain Pipeline transformed. If it does not, click on the FlowSOM plot from the previous step prior to performing this step.

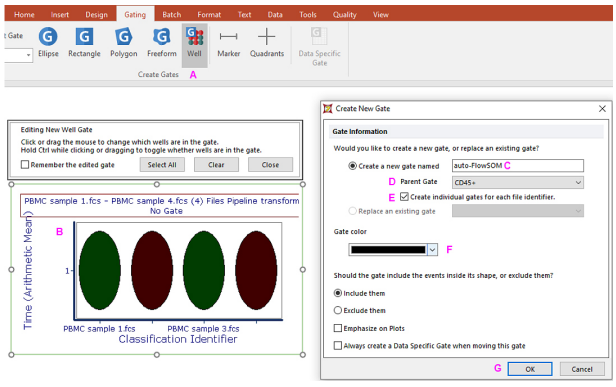


2 On the **Gating** tab, select **Well** (A) and then click and drag your mouse to encompass all the wells on the Plate Heat Map that was created Step 3 in the Merge Data Files for Preparation of tSNE Run section (B). Enter auto-FlowSOM in the **Create a new gate named** field (C).

3 Select the gate on which the FlowSOM was calculated (D). This was the gate specified in Step 3 of the Choosing Variables and Defining the FlowSOM Settings section.

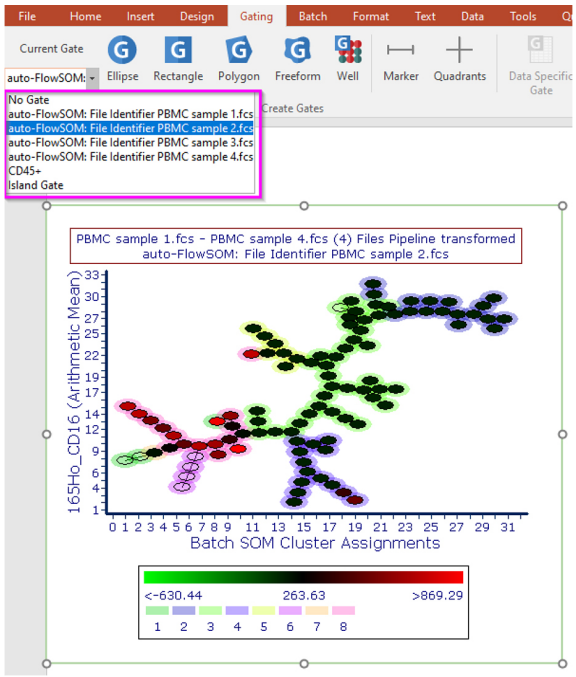
EXAMPLE: Choose CD45+ from the Parent Gate dropdown menu (E).

4 Check the **Create individual gates for each file identifier** checkbox (E) and choose black from the **Gate color** dropdown menu (F). Click **OK** (G).



5 Click on the FlowSOM plot to select it. On the **Gating** tab, select **Current Gate** dropdown menu and then choose the first automatically created gate. This gate corresponds to the first original component data file in the virtual merged file.

The plot is now regated to show only the events corresponding to the original data file. Repeat for the other component files (optional).

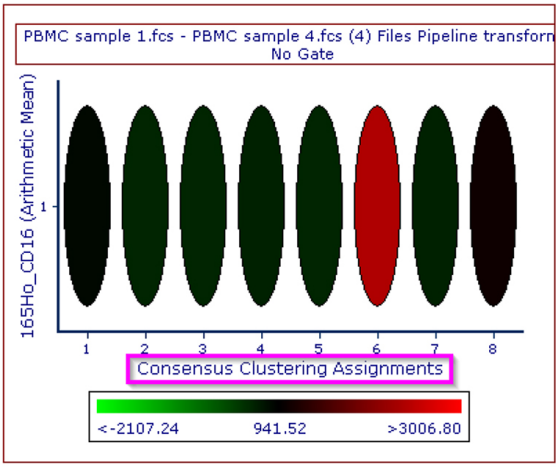


6 (Optional) To view the results from the individual component files in separate plots side by side within the layout, **select**, **copy**, and **paste** the plot as many times as you have component files. Then, on the **Gating** tab, select the **Current Gate** dropdown menu and choose the automatically created gate that corresponds to the original component data file of interest. Repeat this on each new plot as needed for the other component files.

Interrogating Metacluster Phenotypes

1 Press Cmd[mac]-/Ctrl[PC]-**D** on your keyboard to duplicate the plot, and move it below or next to the existing plot.

2 Click on the X-axis to change it to **Consensus Clustering Assignments**.



3 On the **Gating** tab, select **Well** (A) and then click and drag your mouse cursor to encompass all the wells on your Plate Heat Map created in the previous step (B). Enter Metacluster in the **Create a new gate named** field (C).

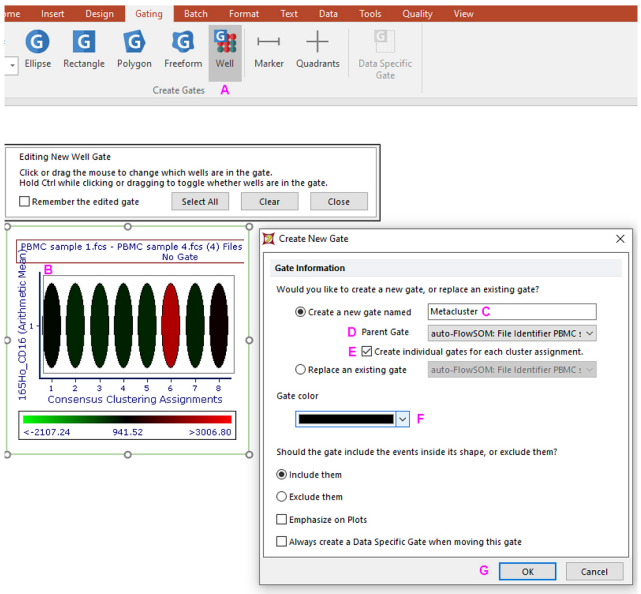
4 From the **Parent Gate** dropdown menu (D) do one of the following:

a If you only want to interrogate metaclusters at the level of the merged file, then select the gate on which FlowSOM was calculated (CD45 in the example).

b If you want to interrogate metaclusters at the level of the original component data files, select a gate with the prefix AutoFlowSOM that were created in Steps 1–4 in the Deconvolving/ Ungrouping Individual Data Files section. These gates correspond to one of your original component files.

EXAMPLE: Choose the AutoFlowSOM-Automatic Classification Gate PBMC sample 1.fcs gate.

5 Check the **Create individual gates for each file identifier** checkbox (E). Then choose black from the **Gate color** dropdown menu (F). Click **OK** (G).

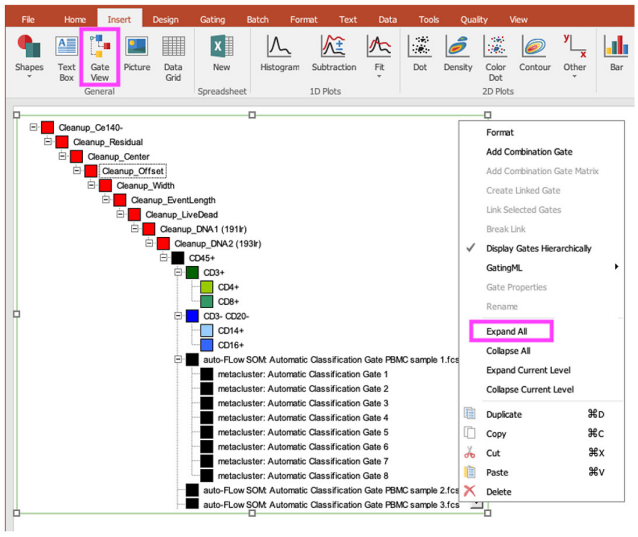


NOTE: If you choose to create the metacluster gates as children of an AutoFlowSOM gate that corresponds to one of the original component files, and would like to interrogate these metaclusters within the other component files, you may **use the Clipboard to duplicate these gates and copy them as children of the other component file gates**. This will enable the phenotypic metacluster analysis for each of the component files.

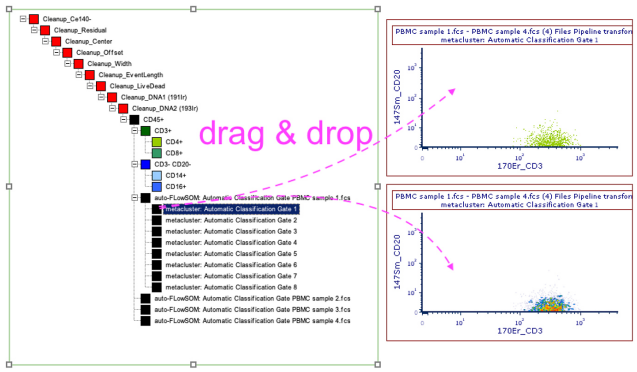
6 Click **Density** and/or **Color Dot** on the **Insert** tab, then click once on a blank spot of the layout to insert a new plot.

7 Change the parameters of this new plot to any of your choice (e.g., CD3 vs. CD20).

8 Click **Gate View** on the **Insert** tab, and click once on your layout to insert the object. Right-click within the object and select **Expand All** to reveal the new gates within your existing hierarchy.



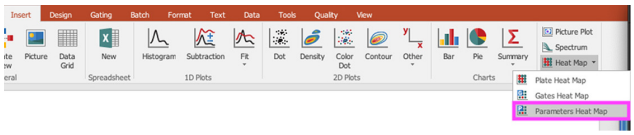
9 Drag and drop the first metacluster gate using its black color block square to the Density and/or Color Dot plot created in Step 6 to regate the plot(s). Repeat this step with the other metacluster gates to interrogate the other metaclusters. Alternatively, duplicate the existing plot(s) as needed, regating each time on a different metacluster gate. This allows you to view the results from multiple metaclusters simultaneously.



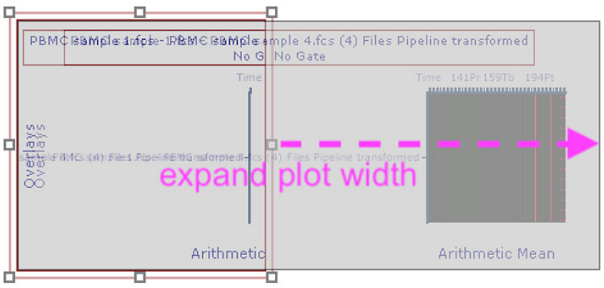
We will now evaluate expression across the metaclusters using a Parameters Heat Map.

10 On the **Insert** tab, select **Heat Map** and then Parameters Heat Map. Click once on an empty spot on the layout to create the **Parameters Heat Map** plot.

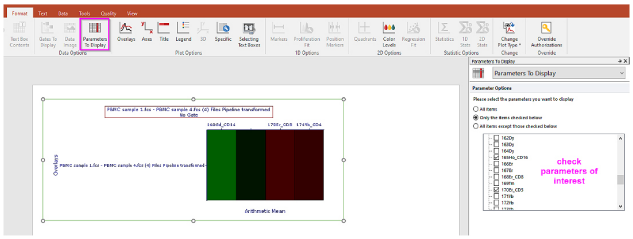
NOTE: The plot title will be amended with Pipeline transformed. If not, click on the FlowSOM plot from a previous step and try this step again.



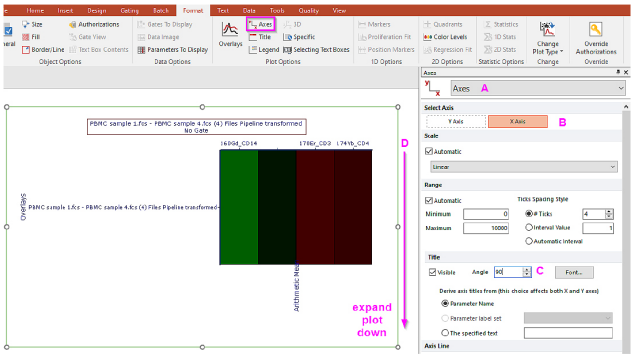
11 Expand the plot width of the **Parameters Heat Map**.



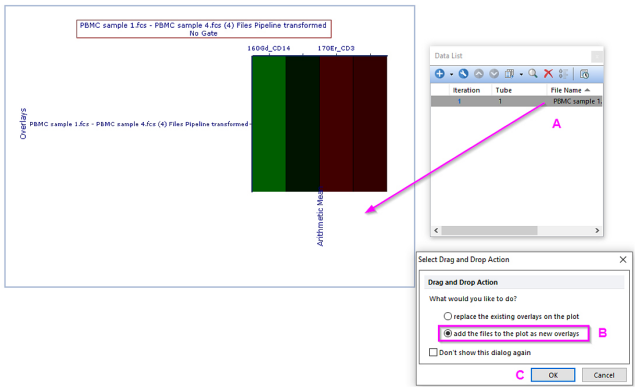
12 Click **Parameters To Display** on the **Format** tab. Select the desired parameters under Parameter Options in the window.



13 Choose **Axes** in the toolbar (A), then select **X Axis** (B), scroll down, and enter 90 in **Angle** (C). Expand the plot height to see the vertically oriented X-axis labels clearly (D).



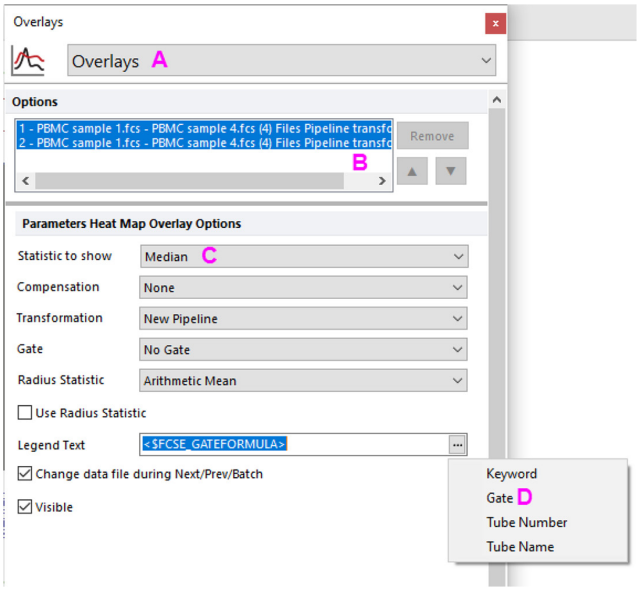
14 Drag and drop the merged filename from the Data List to the Parameters Heat Map (A). Click **add the files to the plot as new overlays** (B), then click **OK** (C).



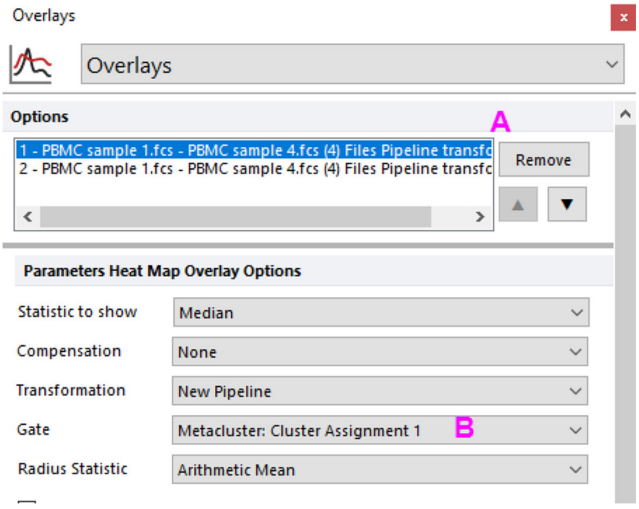
15 Repeat the preceding step for each metacluster.

NOTE: You may need to expand the height of the plot to see all of the metaclusters as horizontal Y-axis labels.

16 Choose **Overlays** in the toolbar on the **Format** tab (A). Click in the list at the top and select all overlays in the list at the top (via Cmd[mac]-/Ctrl[PC]-**A**) (B). Then select **Median** from the **Statistic to show** dropdown menu (C) and **Gate** for the **Legend Text** (D).

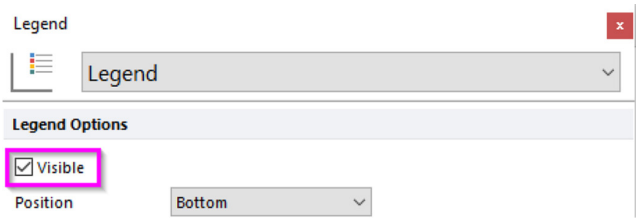


17 Select the first overlay in the list at the top (A). Then select the first metacluster **Metacluster: Cluster Assignment 1** from the **Gate** options (B).



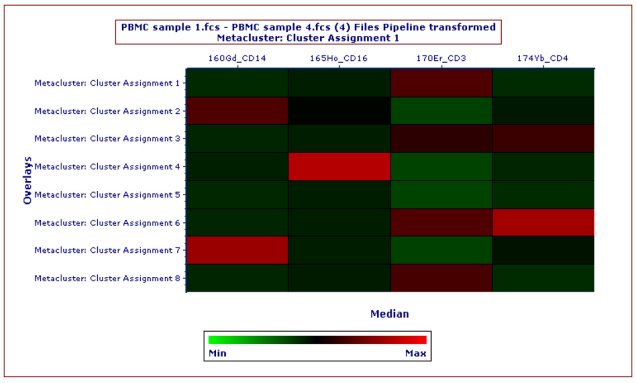
18 Repeat the preceding step for each overlay, so that overlay #2 is gated on the Metacluster: Cluster Assignment 2, #3 on Metacluster: Cluster Assignment 3, etc.

19 Choose **Legend** in the dropdown menu and check the **Visible** box.



The plot will now resemble those shown below.

20 To adjust the logic by which colors are assigned (i.e., rows vs. columns options, pictured below), color levels (palettes), color scales, radius visibility for displaying additional statistics, and other options, see **Specific Options**, **Color Levels**, and **Overlays formatting options** on the **Format** tab.



Summary

Now that you know the basics of high-dimensional analysis in FCS Express, you can perform tSNE and FlowSOM analysis on mass cytometry data. For additional practice, there are additional tutorials on the De Novo Software website. For a more in-depth training on FCS Express, contact your Standard BioTools™ field applications specialist who will connect you to your De Novo Software FAS.

Learn more: **fluidigm.com/about/contactus/tech-support**

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