A guide to analyzing Attune NxT FCS data files using FlowJo software
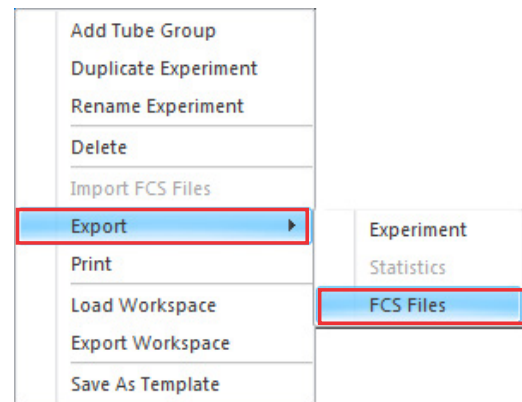
Background

Invitrogen™ Attune™ NxT Software on the Attune™ NxT Flow Cytometer records and outputs data in FCS 3.0 or 3.1 format. Importing these data into third-party flow cytometry analysis software requires setting the preferences of that software to properly display data. This instruction guide is intended to help users open and adjust certain settings in FlowJo™ analysis software (FlowJo, LLC; Ashland, OR) in order to properly display and facilitate the analysis workflow using Attune NxT Software FCS 3.1 data files. These instructions are not meant to be a comprehensive guide to all features and functions available in FlowJo software. We suggest you refer to the user guide or visit the FlowJo software website (**flowjo.com**) for information on the features not covered in this application note. These instructions will cover the required steps to analyze Attune NxT Software files using FlowJo software version 10.3 and 7.6.5; other versions may require modifications to these instructions, as menus and placement of settings may have changed.

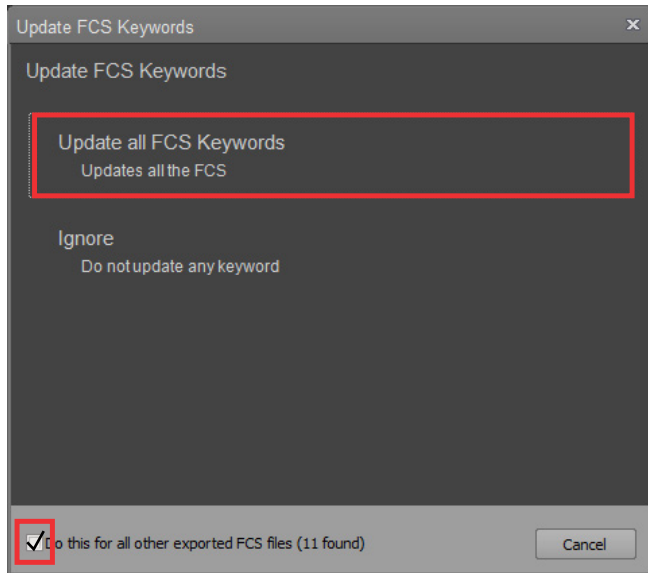## Exporting FCS files from Attune NxT Software

Export the Attune NxT Software data files to be analyzed in FlowJo software using these easy steps:

1. Right-click on the experiment to export FCS files from the experiment explorer.

2. Select **Export**, then **FCS Files**, from the drop-down menu.

3. In the **Select Folder** dialog box that opens, select the location to save the exported FCS files to (the default setting saves the FCS files as FCS 3.1).

   – **Note:** Older versions of FlowJo software require the Attune NxT FCS files to be exported as FCS 3.0. In this case, select **FCS 3.0** from the **Save As:** drop-down prior to exporting.

4. Click on **Select Folder** in the dialog box to export the FCS files.

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5. If any keyword (e.g., sample name, group name) or compensation has changed since the FCS files were recorded, a dialog box will be displayed allowing the updated sample information to be updated within the FCS file.

6. To update the keywords, check **Do this for all other exported FCS files** and click on **Update all FCS Keywords**.

Note: Selecting **Update all FCS Keywords** will modify the original FCS file. This modification is recorded in the FCS file using the $ORIGINALITY, $LAST_MODIFIER, and $LAST_MODIFIED keywords.

For more details, go to **thermofisher.com/ AttuneNxTSWUG**

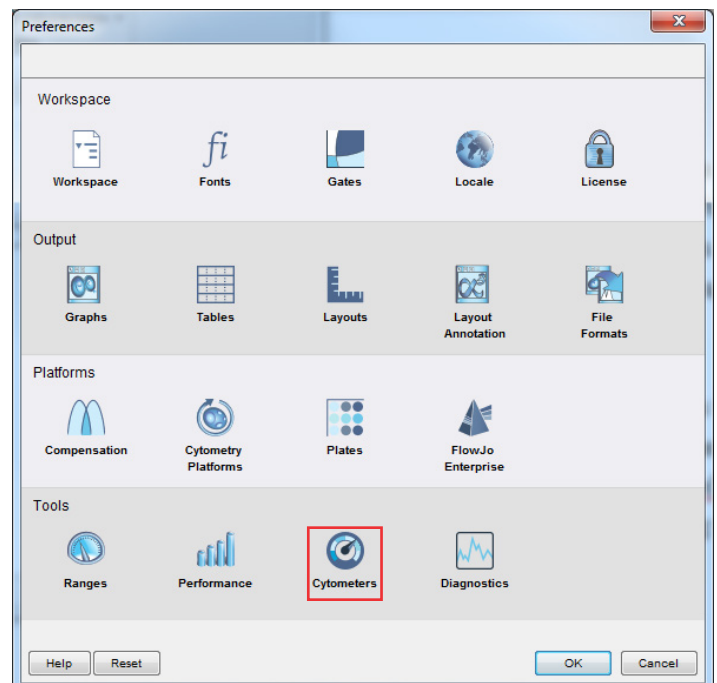## Analyzing Attune NxT FCS data files in FlowJo software v10.3

The default settings in FlowJo software should display Attune NxT FCS data correctly without modifying the individual software settings. The default values for the fluorescence parameters are set with the transform options enabled by a default width basis set at –1,000.
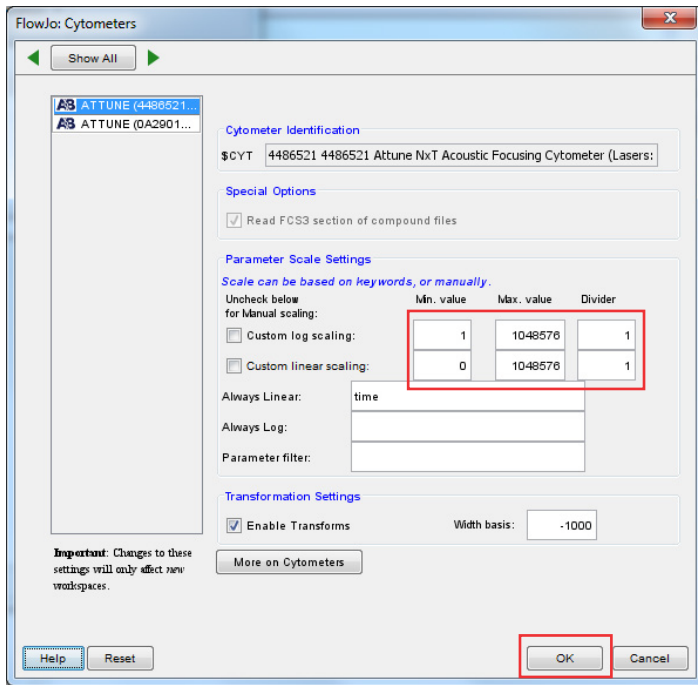
Note: The width basis may need to be adjusted, depending on the instrument settings used to acquire the Attune NxT data files.

Setting up FlowJo software v10.3 preferences
The preferences will need to be updated to display the event and width parameters correctly.

1. Open FlowJo software, and drag and drop the Attune NxT files to analyze in the software.

2. The FlowJo software should automatically recognize the FCS file as an Attune NxT file and set up the default transform preferences to correctly view the Attune NxT FCS file.

3. To check and update the FlowJo software preferences, open the preferences by clicking the **Preferences** button (♡).

4. In the Preferences window, select **Cytometers** in the Tools group.
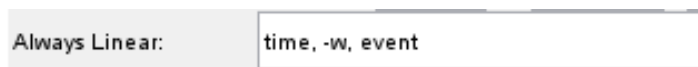
Analyzing Attune NxT data files

The Attune NxT data files can be analyzed in FlowJo software as described here. If the Attune NxT FCS file contains an embedded spillover matrix ($SPILLOVER), the fluorescence parameters will automatically be compensated. For instructions on defining a compensation matrix using compensation controls, refer to the user documentation for FlowJo software.

1. Open FlowJo software, and drag and drop the Attune NxT data files.

2. Double-click on a file to open the plot window.

5. Select the **Attune NxT** cytometer from the list that opens up (the cytometers list is populated when an FCS file with a unique $CYT is opened for the first time).
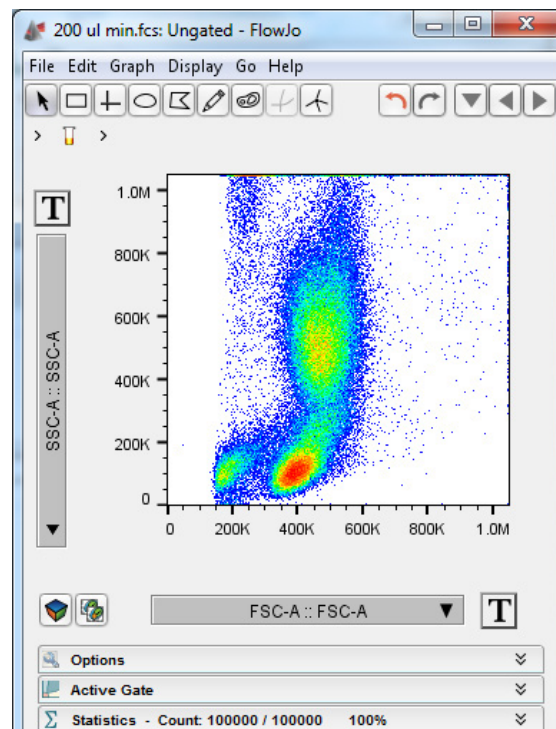

6. Add "time," "-w", and "event" to the list of parameters that are **Always Linear.**
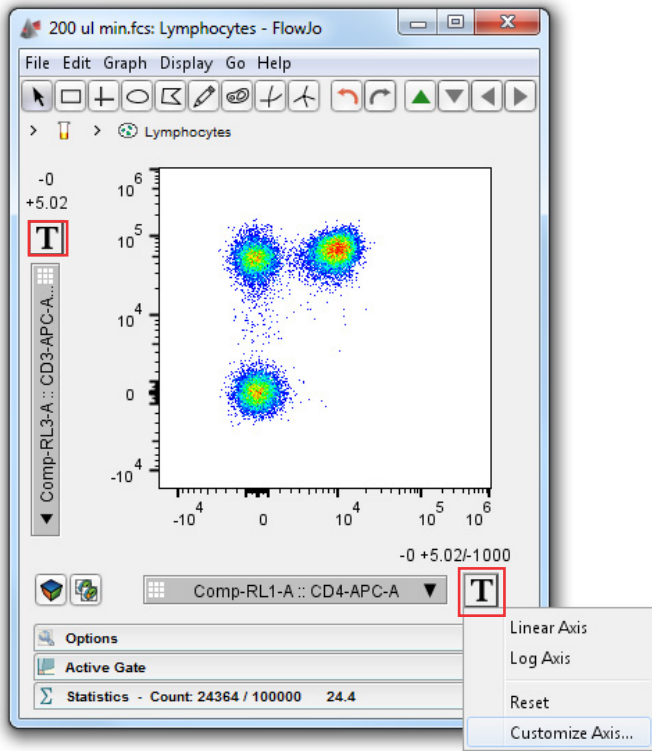

7. By default, the scaling is set to custom for both log and linear scaling at 1,048,576 (2^{20}). This scale may not be appropriate for the time, event, and width parameters. These parameters will require scales to be set manually on a case-by-case basis.

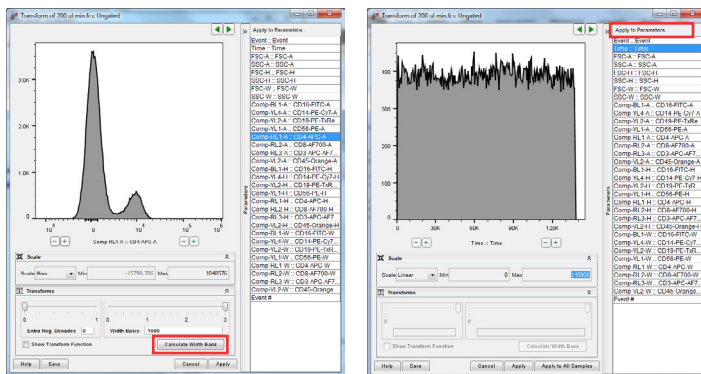8. Click **OK** to save the changes.

Note: Changes made to the cytometer preferences will only apply to newly created workspaces in FlowJo software.

3. Select the desired parameters and add required gates.

4. If the scale or range for a selected parameter needs to be adjusted, click the **T** button adjacent to the parameter name. From the drop-down menu, select **Customize Axis…**.

5. The transform options dialog box will be displayed, showing the transformation options and range for the selected parameter.
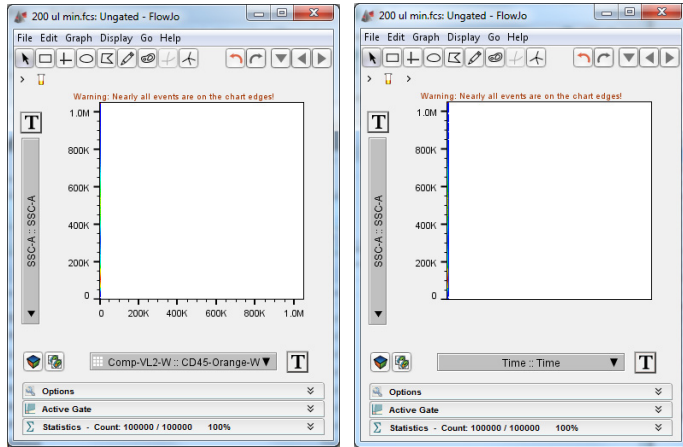

6. The upper or lower limits can be adjusted as needed to scale the displayed data.

7. Adjustments to the W**idth Basis** value used by the biexponential transform may be required if the instrument settings were not correctly set for a parameter. The W**idth Basis** slider can be used to adjust the data as required. The goal is to set this value so that the entirety of the dimmest/negative population is visible without losing the ability to resolve the positive populations (i.e.6, dim population begins to merge with the negative population).

8. The transform options dialog box can also be used to update the default ranges and scales for each parameter as part of the cytometer setting preferences. To apply the specified range and/or scale settings to a set of parameters, select the parameters in the **Apply to parameters…** window. Updating the defaults will only apply to newly created workspaces in FlowJo software.

Note: Data points that are saturated on the instrument may appear as a line at the top or right sides of the plot even when the range is extended, as they represent the maximum value the instrument is capable of producing. Data points that are outside the set min/max for the selected parameter will also appear as a line at the left, top, right, or bottom of a plot. FlowJo software v10.3 will display a warning stating that a percentage of the events are on the chart edges.

Note: The time, event, and width parameters will require their ranges to be manually set using the transform options dialog box. FlowJo software does not automatically adjust the plot to the data for these parameters. As a starting place, width parameters should be set with a minimum of 0 and a maximum of 128. Time and event need to be set based on the upper time and/or event data value within the FCS file.

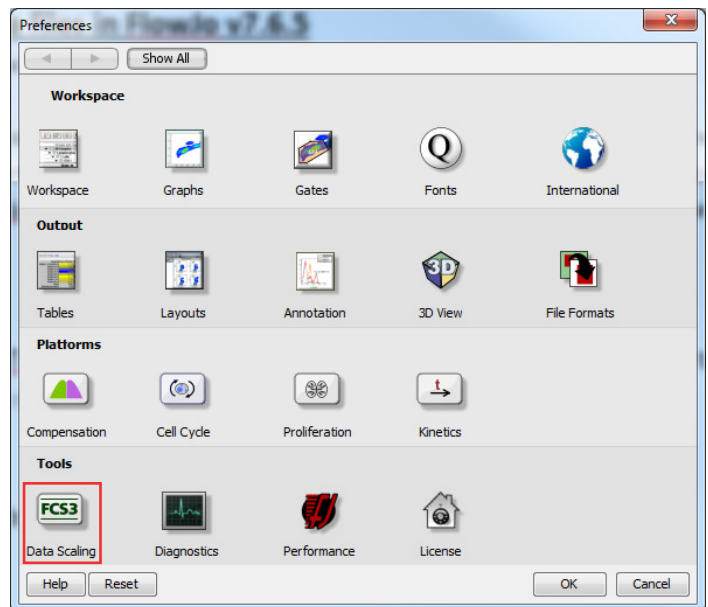## Analyzing Attune NxT FCS data files in FlowJo software v7.6.5

Note: Attune NxT data files should be exported as FCS 3.0 when performing analysis using FlowJo software 7.6.5.

The default settings in FlowJo software 7.6.5 will display uncompensated Attune NxT FCS 3.0 data files correctly without requiring modification of individual software settings. When analyzing compensated data files, adjustments to the biexponential transform settings are required even if linear and log plots are scaling properly.
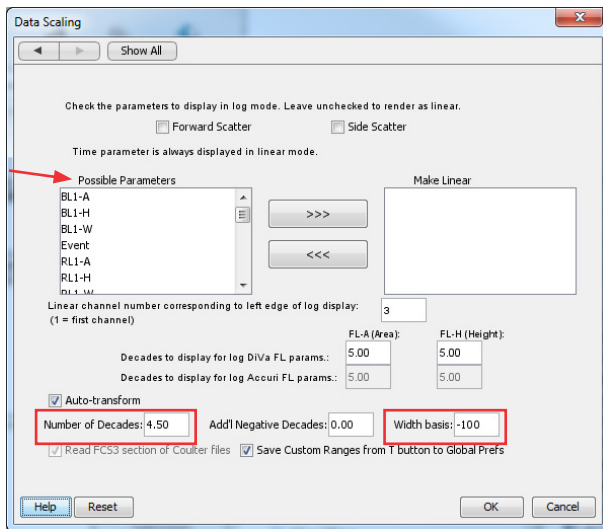
Setting up FlowJo software v7.6.5 preferences

To facilitate the analysis of Attune NxT data files using FlowJo software v7.6.5, the default preferences can be set to minimize the adjustment of individual software settings each time an Attune NxT FCS file is analyzed.

1. Open FlowJo software, and drag and drop the Attune NxT data files to analyze in the software.

2. Open the FlowJo software preferences by clicking on **Edit**, then **Preferences**.

3. Select **Data Scaling** in the lower left of the Preferences window. This will open the **Data Scaling** options.
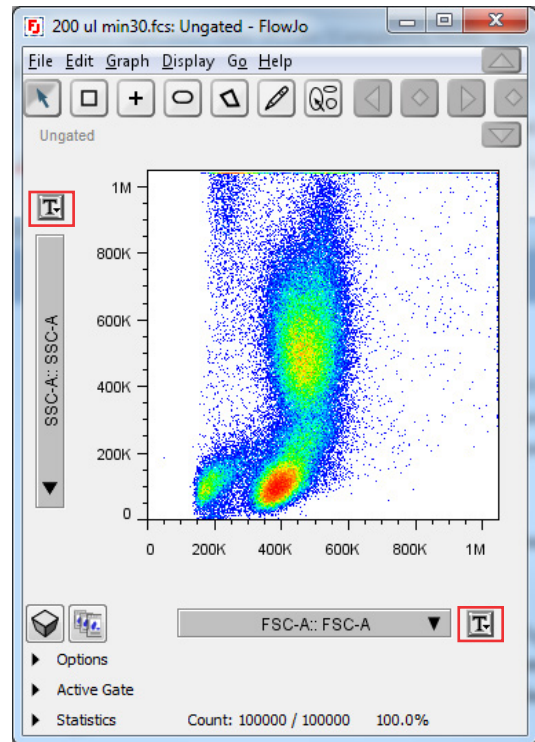

4. Select the Attune NxT parameters from the **Possible Parameters** list. The parameters should be set to linear (event, and all width parameters).

5. Set the **Number of Decades** to 4.50 and set the **Width basis** to –100.

6. Click **OK** to save the changes.

Note: Changes made to the cytometer preferences will only apply to newly created workspaces in FlowJo software.

Analyzing Attune NxT data files

Attune NxT data files can be analyzed in FlowJo software as described here. If the Attune NxT FCS file contains an embedded spillover matrix ($SPILLOVER), the fluorescence parameters will automatically be compensated. For instructions on defining a compensation matrix using compensation controls, refer to the user documentation for FlowJo software.
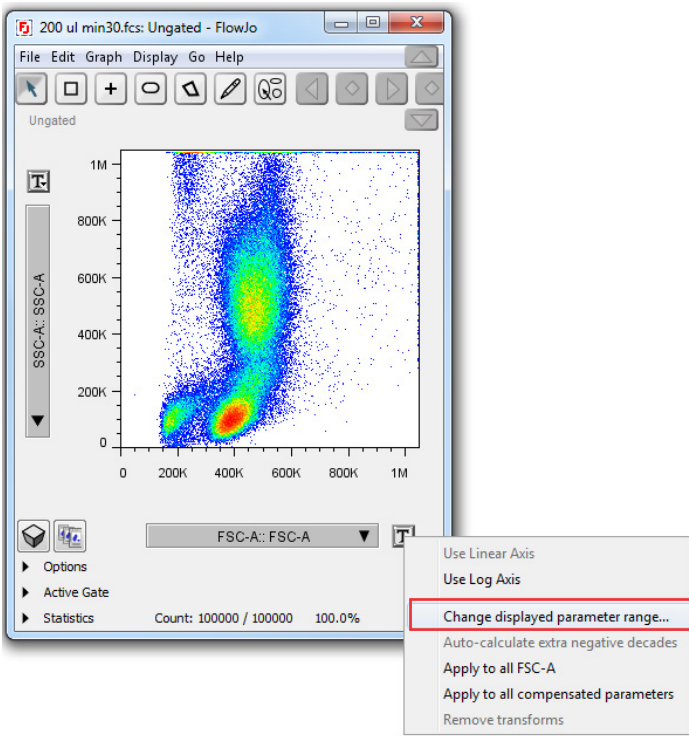
1. Open FlowJo software, and drag and drop the Attune NxT data files to analyze in the software.

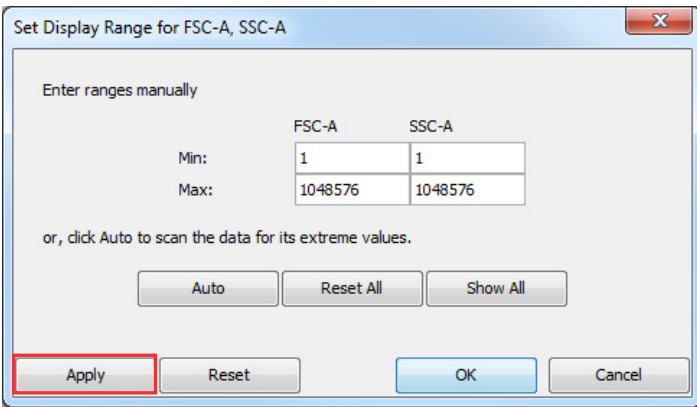2. Double-click on a file to open the plot window.

3. Set the desired parameters for each axis and add required gates.

4. If the scale or range for a selected parameter needs to be adjusted, click the **T** button adjacent to the parameter name.

Transform settings—uncompensated data

1. For uncompensated parameters, the transform options allow the parameter scale to be set to either linear or log, and allow adjustment of the range.

Note: Data points that are saturated on the instrument may appear as a line at the top or right sides of the plot even when the range is extended as they represent the maximum value the instrument is capable of producing. Data points that are outside the set min/max for the selected parameter will also appear as a line at the left, top, right, or bottom of a plot. FlowJo software v7.6.5 will display a warning stating that a percentage of the events are on the chart edges.

Transform settings—compensated data

To properly visualize compensated data, a special transformation, called biexponential, is applied to the compensated data. This setting can be adjusted globally as described above in the section titled **Setting up FlowJo software preferences**. Adjustment of the individual parameters is described here. These settings are only recommended starting points and may need to be optimized for each data file.

1. For compensated parameters, the transform value can be adjusted by selecting **Change transform values…** in the transform options drop-down box.

1. Select the desired transformation (linear vs. log) from the transform drop-down box.

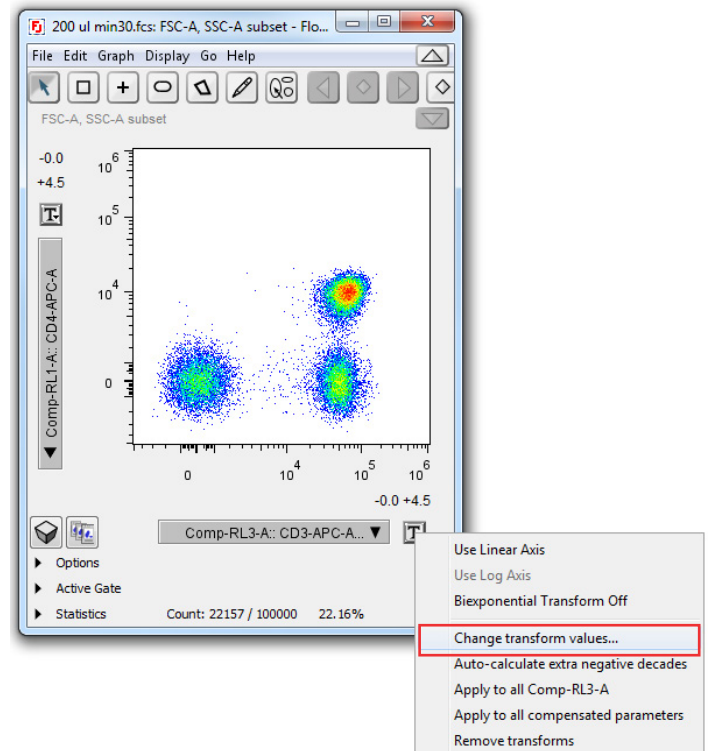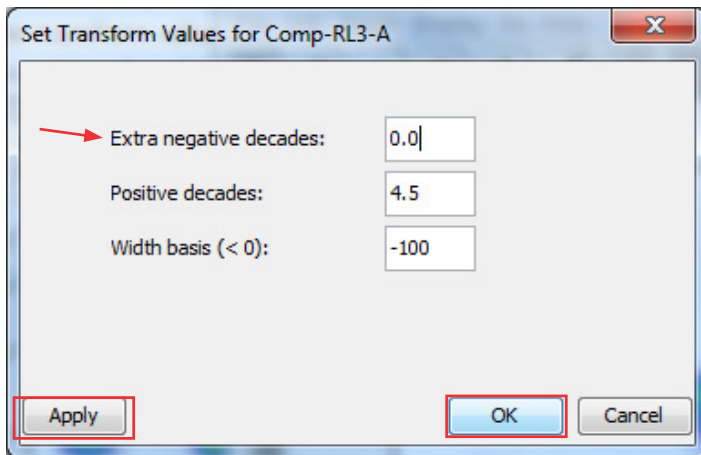2. To adjust the axis range, select **Change displayed parameter range…**.

3. The default range set by the FCS data file will be displayed. The upper or lower limits can be adjusted as needed to scale the displayed data. Values can be previewed by clicking **Apply**. When settings are optimized, click **OK**.

2. This will open the **Set Transform Values** dialog box. These options are the same as those in the global preferences except they are only applied to the selected parameter. Set the **Extra negative decades** starting at 0.0 and increase as necessary to bring data on scale. Then set the **Width basis** to –100 and increase as necessary in order to compress negative populations that may appear too diffuse. Ensure that the **Positive decades** is set to 4.5 decades.

3. Click **Apply** to see the value applied to the data, then click **OK** when finished.

4. Repeat steps 1 through 3 for each compensated fluorescence parameter that requires modification.

These instructions are based on our understanding of the default display of the data in the respective software.

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