

Using Bi-exponential Transformations
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What is Bi-exponential Transformation?

Flow cytometric immunofluorescence measurements are usually displayed on a 4-5 decade log scale to allow visualization of cells with a wide range of intensities on the same scale. However, after compensation is applied (by the user) to correct spectral overlap, cells often end up with fluorescence values of <0 . Thus, it is common that a large number of cells are displayed squished against the axis on a log scale, even when data is properly compensated. The poor spectral sensitivity of some detectors (especially in the red/far red channels) coupled with corrections for background light and electronic noise (automatically made by the instrument) can result in fluorescence values <0 even for uncompensated data. The cells become piled up in the first channel (against the axis) because the fluorescence parameters are displayed on a log scale where it is not possible to display "zero" or negative values. The spreading of a compensated population into negative data values is the result of statistical error in measurement that is inherent in the data collected on flow cytometers. Even though the measurement error is the same in uncompensated samples, the variation becomes obvious when a compensated population has a low mean and therefore appears in the low regions of the log scale. This is because log scales expand the view of data in the lower regions (first decade) and compress the view of data in the upper regions (fourth decade).

To better visualize these kinds of data files, the Diva and Flow-Jo software packages use "bi-exponential" (aka "Logicle") scaling, which incorporates linear scaling (with space for values below zero) for low values together with log scaling for high values. Note that the data is exactly the same, but FlowJo's display transform allows you to view the negative populations as nice symmetrical clusters instead of squished against the axis. Flow-Jo's display transformation process is based on a method developed by Dave Parks and Wayne Moore at Stanford University using a generalization of the hyperbolic sine function. The display functions approach true log for high data values and approach true linear around zero. This provides smooth, near-linear display of low and negative data values. The Diva software likely uses a similar method to compute bi-exponential transformations.

Bi-exponential Scaling (aka Transformation) has 2 important effects:

- Gets rid of the "squishing" of cells and allows proper visualization of cells with negative or dim fluorescence (ie, at axes origins), particularly important in the far-red channels and after compensation is applied.
- Ensures that the "visual" population center better correlates with the statistical center (median) (see Ref # 1 for more details).

- 1) **Bi-exponential Transformations of Analog (Calibur/Scan) FSC2.0 Files:**
 - a) You can do this ONLY if data files are compensated in Flow-Jo (ie., acquire comp controls and samples without hardware compensation, then use Flow-Jo to compensate).
 - b) In the Flow-Jo preferences menu "Workspace" window, under the "Compensation" heading, click "Allow custom transformations". This will allow you to change the transformations after you compensate your samples.

- Leave the default parameters as they can be changed later from the Platform->Compensation->Bi-exponential Transformation Menu
- c) **New:** In the latest FlowJo release (v8.8.6), you can choose to “Apply visualization (aka bi-exponential transformation) to all compensations” by checking this box in Workspace Preferences/Compensation. With this box checked, FlowJo will automatically transform when you apply compensation to a sample. If this box is NOT checked, the data will be displayed with standard log scaling UNLESS you choose Platform->Compensation->Bi-exponential Transformation Menu.
 - d) After the data has been compensated, open a datafile for a sample stained with all colors in the experiment and apply any live/dead gating, FSC/SSC gating etc. Select the fully gated population of a sample stained with all fluorochromes used in the experiment, and go to the “Platform” menu, choose “Compensate Sample->Bi-exponential Transformation”.
 - e) Try the default parameters first, but then change the default numbers as needed for:
 - i) Additional negative display space: increase from the default of 0 to 0.5 to 1 (or higher sometimes) if too many events are piled up on the axes.
 - ii) Number of positive decades: decrease from default of 4.5 to 4 or 3.5 to compress the low end of the scale if the neg/dim population appears “split”.

Bottom Line: By playing with these 2 display parameters to change the spreading of the low part of the scale, you can sometimes improve visualization. However, since the analog circuitry and the FSC 2.0 file format (even from a digital cytometry) do NOT record negative values, the data will still be truncated (piled-up) at zero), it may just not be as obvious.

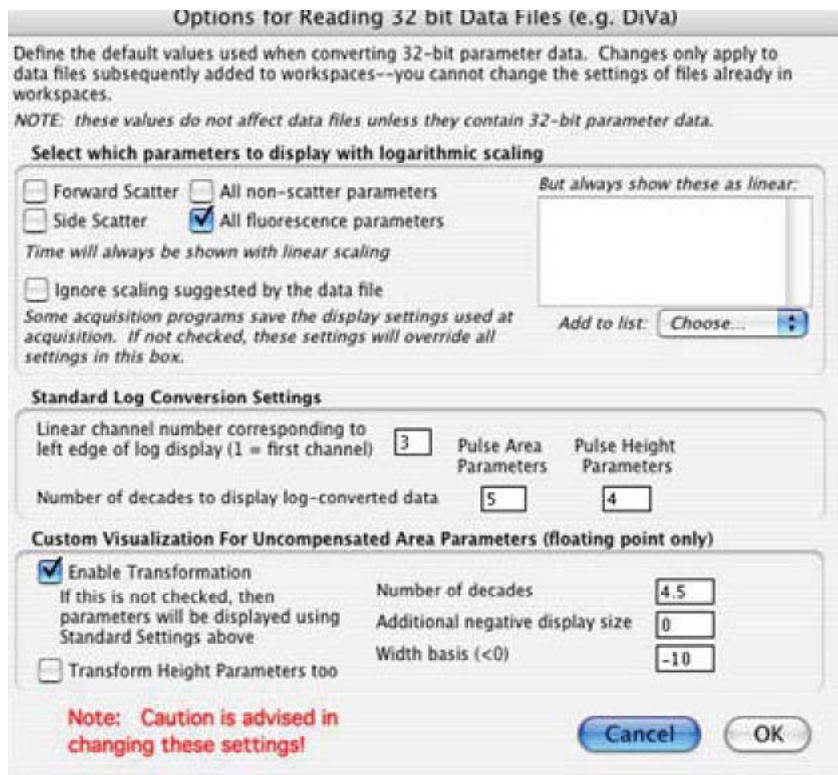
2) **Bi-exponential Transformations of Aria/LSR Digital Data:**

Overview: You can apply bi-exponential scaling to your uncompensated and/or compensated data. Note data must be exported from the BD FACSDiVaT software as FSC 3.0 files, which are saved in **linear, uncompensated** format. If the data was compensated during acquisition, this compensation matrix is included in the data file (in FlowJo, it is referred to as the **Acquisition matrix**). Therefore, the user must tell FlowJo in which format to display the data (log or linear.) The factory default FlowJo Preference is to show all fluorescence parameters in log and both side scatter and forward scatter in linear format.

It is recommended that you first apply a minor transformation to the uncompensated data when users export as directed below (Step 3) from the BD FACSDiVaT software, because this export generates data files that can have negative values for Area parameters. FlowJo uses a single automatic transformation for these parameters. Once this has been done to your satisfaction (ie., little to no pile-up of values on axes of all fluorescence parameters), compensate by applying the Acquisition compensation matrix, generating your own matrix or importing a matrix from another Workspace. When you then compensate these parameters (i.e., those that are already transformed), the compensated parameters by default will get the same minor transformation. This might be enough transformation--but often isn't. For this case (and whenever you compensate data), you can still choose to manually create a custom transformation for your data (Step 4 below).

3) **Bi-exponential Transformation of Uncompensated Diva Data**

- a) **First**, export the Diva data files as “FCS 3.0” files (see Note below for more info about this data format). To apply Bi-exponential scaling to your data, there are 2 Flow-Jo Workspace Preferences that need to be changed (see below). These Flow-Jo Preferences **MUST** be changed from the default **before** importing Diva files into a Flow-Jo Workspace! Note that you can save different Preference configurations for future use.
- b) In the Flow-Jo preferences menu “Workspace” window, under “Compensation” heading, click “Allow custom transformations” – this will allow you to change the transformations after you compensate your samples (same as Step 1b above). Leave the default parameters as they can be changed later from the Compensation_>Bi-exponential Transformation Menu.
- c) Under the Heading “Reading Diva and other 32 bit Data files” (bottom right) click “Define Conversions for 32-bit data files”. At the bottom of the next window, you’ll see “Custom Visualization for Uncompensated Area Parameters (floating point only): check “Enable Transformations” – this will apply minor transformation to the Uncompensated data when it is imported.



Now you need to optimize the transformation settings to find those that allow the best scaling of your uncompensated data. There are 3 settings to be tweaked:

- a) “Number of decades”
 - i) decrease if the “negative” population is splitting in two or looks wide, (recommend starting at 3.5 or 4.0)
 - ii) increase if “negative” population is too compressed
- b) “Additional negative display size” (recommend leaving at “0”)

- c) “Width basis” is a negative number representing the total number of channels compressed to a linear scale (centered around the “0” value”). Usually this needs to be increased from the default of “-10” to accommodate the large spread in the far red channels.
 - i) increase the number if you see a distribution of events where population looks split
 - ii) decrease if neg/dims are spread over too much of the scale

The most important setting to optimize at this point is the Width Basis (WB), which determines how much of the low end of the scale is linear, as well as the steepness of the transition from linear to log. I find for most datasets, you need to change the default setting of -10 to around -50 to -100 to make sure there is enough linear region to accommodate the spreading induced by compensation. Check it out by importing 1 or 2 data files (from fully stained samples) into the Workspace. If the scale looks reasonable for the uncompensated data, bring in your compensation controls and define and apply a compensation matrix – you’ll need to check the scaling on all parameters (or at least check far red (PE-Cy7 or APC-Cy7) vs FITC channels – you’ll typically see more values below zero and thus “squishing”) with PE-Cy7/APC-Cy7, but if you only optimize this channel the FITC negs will seem too compressed – sometimes you have to find the right compromise.

Important: you should be optimizing the scaling by looking at a fully gated sample – dead cells and debris are usually at the lowest end of the scale, and if you optimize to accommodate these events (which will ultimately be gated out anyway), you will end up with too much unoccupied space at the low end of the scale after the dead cells/debris are gated out!

If you still see too much squishing you need to increase the WB (eg, change from -50 to -75). If the data seems to compressed at the low end of the scale you need to decrease the WB (eg., change from -50 to -25). To change the WB, change the Preferences for Diva 32-bit data in the Flow-Jo Preferences menu **FIRST**, then create a New Workspace and import the same data files you have been working with. Note that once you have defined your compensation matrix, you can import it into the New Workspace (original Workspace has to be open) so you don’t have to define a new matrix every time you try this! You can also drag gates into the new workspace from the old workspace so you don’t have to draw new ones. With this step don’t worry about perfection – the most important this is to optimize the width basis. The other settings can be tweaked by applying a Custom Compensation as described below. In a given Workspace, you can find out what the WB was set at for that file by choosing Workspace/Workspace Preferences from the Top menu bar.

2) Custom Transformation of Compensated Data:

- a) Select a fully-stained sample (that has fluorescence in all channels), gated to remove dead or unwanted cells (i.e., lymphocyte gate), and then choose "Define Transformation..." from the compensation menu. If you don’t have

a fully stained sample you can concatenate files (see Flow-Jo manual). A custom transformation is computed based on the distribution of the data, which results in a better visualization.

- b) The custom transformation will be applied to all samples that are compensated the same way in the workspace; hence it only need be computed once for each unique compensation matrix (and when computed for other matrices, requires a sample compensated with that matrix as an input to the customization). Note that for Custom Compensation, the software determines optimal settings individually for each parameter, based on the distribution of values.
- c) remember to **verify all gates again after transformation** as transformation may “reveal” events which are now below/outside your gate!
- d) The default parameters generally will work well for 18-bit acquisition where the lymphocytes are pinned to a top value of about 100 (10^2).
- e) User-Specified Parameters in the transformation definition:
 - (i) "Additional negative display size" - how much visual space to devote to events that have values less than zero. Increase from 0 to 0.5 or 1 if negs are too squished on the axes.
 - (ii) "Positive decades of display" - how many decades of dynamic range is shown for events greater than zero. By default, this is 4.5--even if you export 5.5 decade data, use 4.5 or lower, otherwise too much visual space is devoted to the lowest (meaningless) decade – the negs will be spread over too much of the scale.
 - (iii) If the "negative" population is splitting in two or is too wide, then decrease the positive decades. Positive decades should be roughly the number of decades from the top of your autofluorescent lymphocytes to the top of the scale plus 1. So, if you put lymphocytes topping out at 10^2 (3.5 decades from the top), this value is typically 4.5.

NOTE: If not enough linearization was applied to uncompensated data during import into FlowJo, you can improve visualization somewhat by changing amount of negative and positive display space, but dim/negs will still look squished and odd. If this happens, increase width basis and –re-import into new Workspace.

Final Notes: You can often achieve similar visualization by changing the scaling parameters in different ways (eg., leaving Width basis at -10 or decreasing total decades will both compress the negs into one subset). But remember that compensation induces much more spread in the data, and if the linearization is not sufficient, you will often end-up with weird looking squished populations. Generally, you'll get better visualization of dim/neg subsets by starting with a higher width basis. Suggested steps:

- Try a few different width basis settings in the Diva prefs – test out by importing 1 or 2 files for fully stained sample.
- select the one that gives best visualization of dim/negs in far red channels (ie, negs not split, but not taking up too much space either)
- check to see if this setting is OK for FITC negs (if they are too compressed, try again with a lower width basis)
- Once the WB looks reasonable, import all data files, compute compensation matrix
- If needed, fine-tune scaling with a custom transformation (show more neg space, decrease or increase spreading of dim subsets by increasing or decreasing positive decades (respectively

- The final optimal scaling boils down to personal taste & which subsets you want to emphasize visually

Note from Treestar on Diva Data File Types:

Note that exporting Diva data as "experiment" and "FCS 3.0" are identical with respect to contents of the data. However, the keywords and/or file names may be different. According to Treestar, "FCS 3.0" is a more intelligible version because it contains everything, whereas the "experiment" format relies on some data in the BD database (which FlowJo can't access.) **Use "Experiment if you want to save the instrument configuration settings (PMT voltages, etc). However, Flow-Jo can't interpret certain Diva parameters such as the Area Scaling Factor (ASF), which can result in FSC-A looking weird. Therefore from a Flow-Jo perspective it is best to save as FCS 3.0.**

Diva file naming convention - FlowJo can sometimes "lose the link" to your FCS files if their names are longer than 16 characters. Also, do NOT use dashes, symbols (eg.,+ or -) or other non-alphanumeric characters in your file name. This happens only in the context of moving the workspace from one computer to another, or from one web-server to another. Its best to use shorter file names when exporting DiVa files to FlowJo - your analyses will be more robust over time.

Useful FlowJo Manual Web pages:

(<http://www.flowjo.com/v8/html/displaytransformprocedure.html>) procedure
(<http://www.flowjo.com/v8/html/displaytransformdigital.html>) digital data display
(<http://www.flowjo.com/v8/html/displaytransform.html>) overview

Sources:

This Guide was compiled using information from the Flow-Jo User guide, the Purdue flow cytometry blog, selected references (below) and experience gained from my endless trials. Please let me know if you find mistakes or ambiguities.

Cindy

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1. Herzenberg, L.A., Tung, J., Moore, W.A., Herzenberg, L.A. & Parks, D.R. Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* **7**, 681-685 (2006).

2. Parks, D.R., Roederer, M. & Moore, W.A. A new "Logicle" display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. *Cytometry A* **69**, 541-551 (2006).