
Rainbow Bead Standardization

UWCCC Flow Cytometry Laboratory

<https://cancer.wisc.edu/research/resources/flow/>

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Why Standardize My Flow Assay?

Assay standardization in flow cytometry improves reproducibility and reduces cytometer set-up time. For assays evaluating protein expression levels using MFI (median fluorescence intensity), this standardization is critical for reducing user set-up variation among replicates. For all flow assays, standardization expedites cytometer set-up and data analysis.

How Does Rainbow Bead Standardization Work?

The observed fluorescence intensity on a flow cytometer is dependent on many non-biological parameters including laser power, alignment, temperature, and optical efficiency. When setting up an experiment, we adjust the sensitivity of the detectors to optimize instrument performance for the run, but these voltages may not be optimal for the next week or even the next day and adjustments must be made each time the assay is run. Rainbow beads (Spherotech, Cat. RFP-30-5A Rainbow Fluorescent Particles Mid-Range) are hard-dyed fluorescent particles that emit as a single peak in each instrument channel. The core can provide these beads at our reduced cost. Acquiring these beads under optimized instrument settings generates a set of target values that reflect instrument sensitivity. For future runs, rainbow beads are acquired first and the voltages are adjusted until the bead peaks hit the target values ($\pm 10\%$, closer is better). At this point, set-up is complete and collection of compensation and test samples can proceed. This method of standardization is very robust and able to accommodate both daily variations and larger changes in performance such as would be seen after cytometer maintenance and alignment.

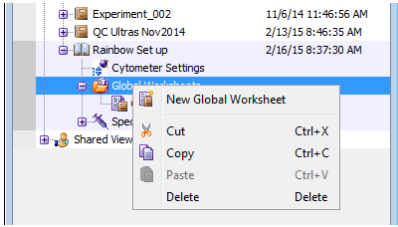
Do I Still Have to Compensate?

Yes! Standardization tells you where to set voltages (instrument sensitivity) using multicolor beads, but compensation uses single stained controls to account for fluorescence spillover. Compensation is still critical for obtaining good multicolor flow cytometry data. For the most accurate compensation, there are three basic rules that must be followed:

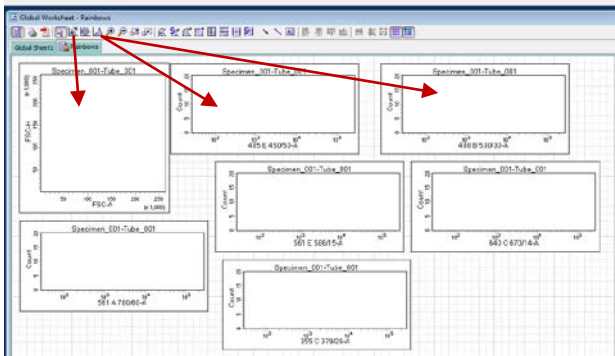
1. The compensation control must **be as least as bright** as the stained sample.
2. The negative and positive populations must have **matched autofluorescence**: cells to cells, beads to beads, etc.
3. The fluorochrome used for the control must **exactly match the fluorochrome** used in the experiment. FITC cannot be used instead of GFP, tandem dyes must be from the same vial as the experimental stain.

How Do I Standardize in Diva?

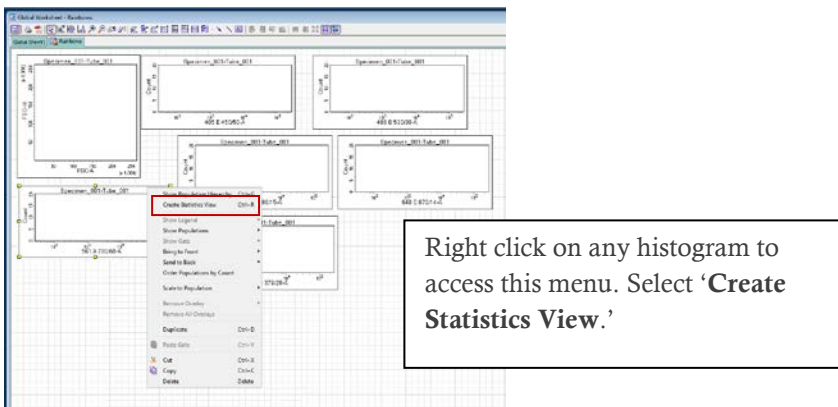
1. Set optimized voltages manually using a fully stained sample and checking the individual comps (do not record, calculate, or apply comp yet).
2. Create a new Global Worksheet entitled 'Rainbows.'



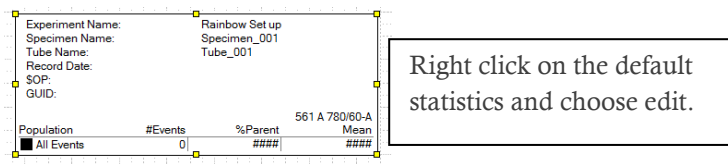
3. In the 'Rainbows' worksheet, create a FSC/SSC dot plot with a P1 polygon gate to define beads, and a histogram plot for each fluorescent channel in the experiment.



4. Make a statistics layout to show the median fluorescent intensity for each histogram.



5. Edit the default statistics view so that it shows the desired information.



Preparing Rainbow Beads

Dilute one drop of well-mixed rainbow beads in 300 μ L of flow buffer.

Rainbow Initial Set-up Quick Reference

1. Optimize cytometer voltages using your samples.
2. Collect the rainbow beads without any compensation and record median fluorescence intensity for each channel.
3. Collect and apply compensation.
4. Acquire your samples.

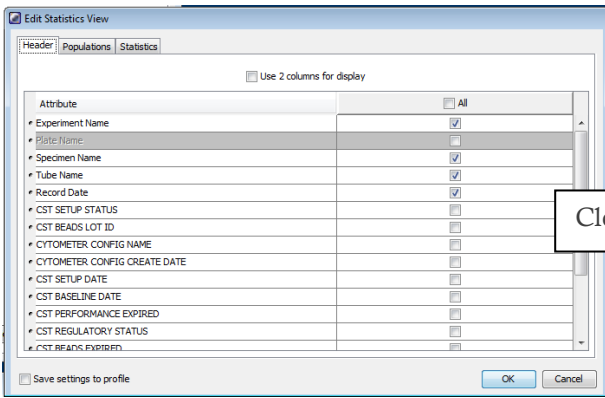
Set-up Already Standardized Assay

1. Acquire rainbow beads and adjust detector voltages to hit MFI target values determined as above.
2. Proceed with compensation.
3. Acquire samples.

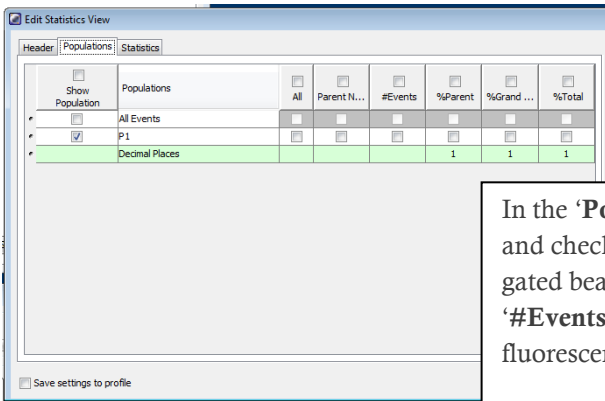
How Do I Change Bead Lots?

Changing bead lots is simple as long as you have beads from both lots.

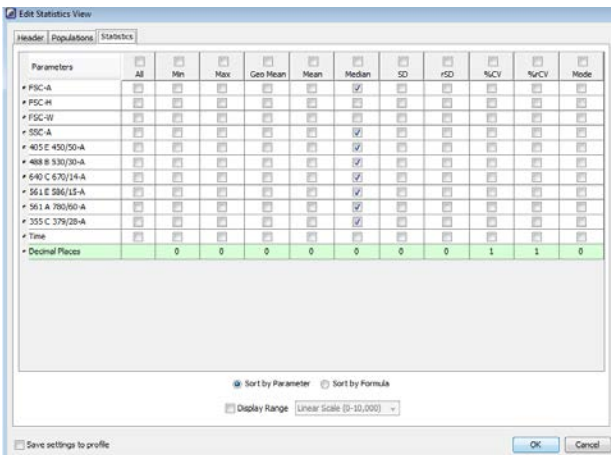
1. Run old bead lot and adjust voltages to hit target values.
2. Acquire new bead lot at those settings and note new target values.



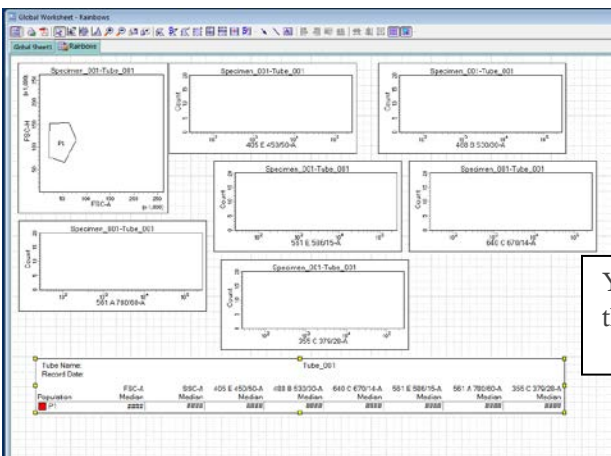
Clean up the header information



In the 'Populations' tab, uncheck 'All Events' and check 'P1' so that only statistics from the gated bead region are displayed. Uncheck '#Events' etc. to make more space for fluorescence values.



In the 'Statistics' tab, uncheck 'Mean' and check 'Median' for all the parameters of interest. If the scatter of your cells does not drastically differ from the scatter of the rainbow beads, you may be able to set target values for FSC and SSC.

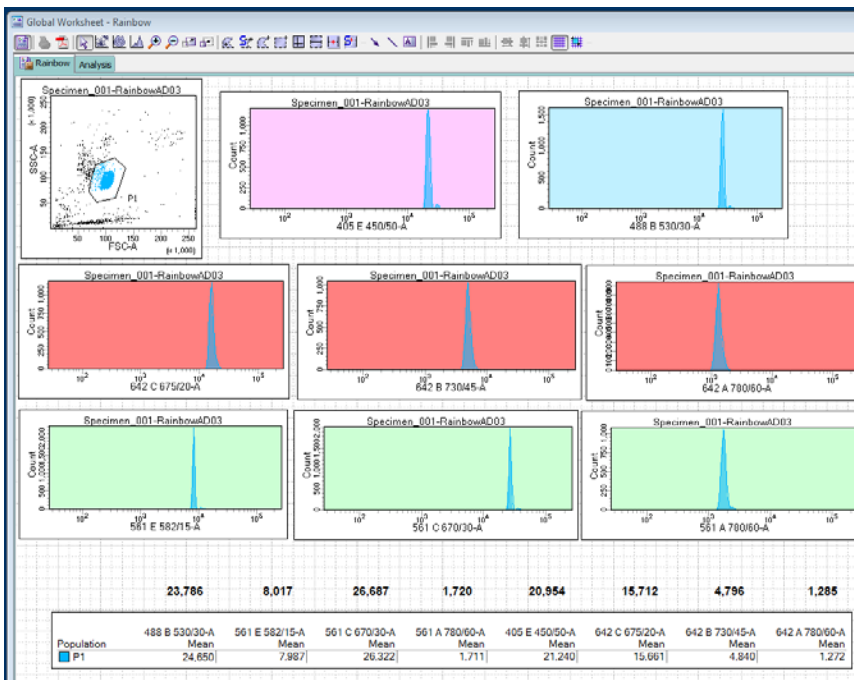


You should now have a layout something like this.

6. Acquire ~10,000 rainbow bead events.
7. The median values from the statistics window are your target values. For convenience, you can type them in text boxes and put them next to the appropriate channel in the statistics window. This way, if you save a template your target values will be listed right next to where the stats show up (see image below).
8. Proceed with compensation and sample acquisition.

Running Your Assay After Standardization

1. Open the Rainbows worksheet.
2. Acquire beads and adjust voltages until the median values match your target values ($\pm 10\%$). **Make sure Diva is not applying compensation to the tube!**
 - a. Compensation will affect target values. Check for compensation in the 'Compensation' tab of the 'Cytometer' window. If comp values are present, select all and clear compensation.
3. Proceed with compensation and sample acquisition.



Target values from initial standardization typed into text boxes.

Voltages were adjusted to hit the target values to within 10%.