Quick Start Guide: Compensation 4.0

Creating a Compensation Matrix 4.0

A compensation matrix is calculated using single color fluorescent control files that are collected on the ImageStream in the absence of brightfield illumination and SSC. One file of between 500 and 1000 positive events should be collected for each fluorochrome in the experiment, using the instrument configuration that is used for collecting the data. Once the matrix is created it can then be applied to the experiment data when batch processing or opening a raw image file.

To create a compensation matrix:

1. Select **New Matrix** when opening a .rif, or from the IDEAS toolbar select **Compensation** then **Create New Matrix**.

Step 1: Select the channels and control files for compensation:

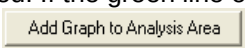
2. "Check" each active channel that corresponds to each fluorochrome used in the experiment.

3. Select **Add Files**, and navigate to the –noBF compensation control files, and add each control file. After pressing **Next** IDEAS will open those files.

Step 2: Validate the compensation matrix:

4. IDEAS will automatically identify the **single color positive cells** for each channel.
5. Adjacent channel dot plots are displayed in their respective colors and populations are assigned to channels automatically. The compensation matrix is calculated and displayed.

6. **Validate the matrix** by right-clicking each cell of the matrix.

7. The **Matrix Coefficient Intensity Plot** is displayed. If the green line of best fit does not exactly overlay the data points on the plot, click 

8. Using a region tool  select a new **positive population** that excludes the outliers.

9. Assign the **new population** to its channel using the appropriate drop down list ie. Change 2_Positive to R2 as up date for the FITC channel.

10. The new values are calculated automatically and can be evaluated by right clicking each cell in the matrix. Continue this process to obtain the lowest coefficient error value possibly, typically to the thousands place or better.

11. Click **Finnish,** and save the compensation matrix.

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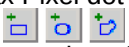
Quick Start Guide: Troubleshooting the Matrix

Troubleshooting Compensation 4.0

Sometimes an applied matrix produces poorly compensated data. This can happen for a number of reasons: 1) miscalculation of the compensation matrix by inclusion of inappropriate events (such as doublets, saturated pixel events, or artifacts), 2) controls used for matrix calculation differ significantly from the experimental samples (different cell type, different probe), or 3) cells exhibit substantial autofluorescence. This protocol describes a method for manually generating and validating a compensation matrix for difficult samples.

To troubleshoot and repair a compensation matrix:

1. With the poorly compensated data open, use the tagging tool  to **create a training set** of poorly compensated events. They should cover a range of intensities and include some unlabeled cells.
2. Save the training set as the Compensation population. These are the events you will use to manually adjust the matrix.
3. In IDEAS go to the **Compensation** dropdown and select **View/Edit Matrix.**
4. Check **Select a population from current file**. Select the training set created in step 1.
5. Press **Preview,** and IDEAS will open the training set.
6. Create and save a **Compensation Template** with these critical parameters;
   a. Set the image gallery display properties  for each channel from 0 to 100.
   b. Create adjacent channel Raw Max Pixel dot plots  .
   c. On each plot, use the region tool  to create a population with no saturation.
   d. In the region manager, extend the regions from 0 to 1022 for Gen1 systems or 0 to 4095for ISX systems, on both axes.
   e. Create a combined population of the intersection (R1 and R2 and R…depending on the number of active channels). The goal here is to eliminate saturated events from the compensation calculation.
   f. Create adjacent channel Intensity dot plots  using the non-saturated events.
   g. Save this template as an .ast file and use this template for future compensation troubleshooting.
7. Identify the matrix values that need adjusting using the **dot plots** and **images:**
   a. **Undercompensation** (crosstalk coefficient is too low):
      i. **Plots:** Intensity mean for the single color positive population is higher than the unlabeled population in the crosstalk channel or the intensity in the crosstalk channel trends diagonally upwards.
      ii. **Images:** the crosstalk channel contains an apparent fluorescent mirror-image.
   b. **Overcompensation** (crosstalk coefficient is too high):
      i. **Plots:** Intensity mean for the single color positive population is lower than the unlabeled population in the crosstalk channel or the intensity in the crosstalk channel trends diagonally downwards.
      ii. **Images:** the crosstalk channel contains dark spots corresponding to the bright spots in the fluorescent channel of interest.
8. Go to the **Compensation** dropdown, select **View Edit Matrix**.
9. **Manually** change the incorrect crosstalk matrix values identified in step7 by typing in new values. Start with changes of ~.1 or ~.05.
10. Check **Select a population from current file**, and choose the population without saturated pixels.
11. Press **Preview**, and IDEAS will apply the new compensation values to the training set.
12. **Repeat** steps 8 through 11 using smaller and smaller increments as you refine the matrix. Continue until the data is properly compensated.
13. Save the new matrix by selecting **OK**, apply a unique name and then hit **Save.**
14. Once the matrix is corrected and saved it can be used for any applicable data files.

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