Creating a Compensation Matrix

A compensation matrix is calculated using single color fluorescent control files that are collected on the ImageStream or FlowSight with all channels collected and in the absence of brightfield illumination or SSC. One file of between 500 and 1000 positively-stained events should be collected from a single color control sample for each fluorochrome in the experiment. These compensation control files are used to create a compensation matrix that is 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**.

Select the control files for compensation:

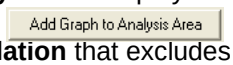
2. Click **Add Files** and control-select the appropriate –noBF compensation control files. There should be one for each fluorochrome used in the experiment. Click **Next** to load the files.

3. Verify the active channels used in the experiment and click **next**.

Validate the compensation matrix:

4. IDEAS will automatically identify the **single color positive cells** and assign them to the appropriate channel, create adjacent channel intensity dot plots, and auto-generate a compensation matrix. Matrix values with greater than 1% error are flagged red.

5. Adjust matrix values that are flagged if appropriate. To do this:

    a. Double-click the red value. The **Matrix Coefficient Intensity Plot** is displayed.
    b. If the green line does not fit the data points on the plot, click 

    c. Use a region tool  to select a new **positive population** that excludes any outliers.

6. Assign the **new population** to its channel using the appropriate drop down list.

7. A new matrix is calculated automatically and can be re-evaluated by double-clicking each cell in the matrix. Continue this process to obtain the lowest coefficient error value possible.

8. To view images with the applied matrix, select **Preview Images.** Double-click a representative image for each fluorochrome used in the experiment. IDEAS will display the compensated image.

9. If crosstalk images have black areas, the fluorochrome is **overcompensated,** and the corresponding matrix values should be manually reduced. If crosstalk images have bright areas, the matrix is **undercompensated,** and the corresponding matrix values should be manually increased. Repeat until crosstalk imagery is as close to background as possible and close the image gallery.

10. Click **Finish,** and save the compensation matrix.

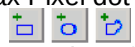
Copyright © 2007 Amnis

Quick Start Guide: Troubleshooting the Matrix

Troubleshooting Compensation

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 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. Open the poorly compensated data and use the tagging tool  to **create a training set** of poorly compensated events. They should include blanks and cover a range of intensities that follow the central tendency of all the populations in the intensity plots.
2. Save the training data by selecting tools, then **create data file from population**.
3. 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 IS100 systems or 0 to 4095 for ISX systems, on both axes.
   e. Create a combined population of non-saturated events.
   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.
4. **Open** the “training data set” (step 1) using the “compensation” template (step 2) and apply the current incorrect matrix.
5. Identify the matrix values that need adjusting using the **dot plots** and **images:**
   a. **Under-compensation** (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. **Over-compensation** (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.
6. Go to the **Compensation** dropdown, select **View Edit Matrix**.
7. **Manually** change the incorrect crosstalk matrix values identified in step5 by typing in new values. Start with changes of ~.1 or ~.05.
8. **Save** the new matrix.
9. **Open** the “training data set” (step 1) using the “compensation” template (step 2), and apply the new compensation matrix (step 8)
10. **Repeat** this process using smaller and smaller increments as you refine the matrix. Continue until the data is properly compensated.
11. Save the new matrix by selecting **OK**, apply a unique name and then click **Save.**
12. Once the matrix is corrected and saved it can be used for any applicable data files.

amnis

Copyright © 2007 Amnis