Creating a Compensation Matrix

A compensation matrix is calculated using single color fluorescent control files that are collected on the ImageStream in the absence of brightfield illumination. One file of between 500 and 1000 positive events should be collected for each fluorochrome in the experiment. 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** from the Compensation menu.
2. Click 

and navigate to the single color no brightfield compensation control files.
3. Highlight each fluorescent control file using Ctrl-select and click **Open**. A list of each compensation control file to be loaded appears in the directory.
4. When all control files have been added to the directory, click 

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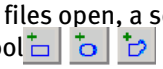
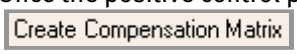
5. A prompt to **combine the control files** comes up. Select No to save on disk space or Yes to combine each control sample into a single duplicate data file.
6. After the files open, a scatter plot of channel 1 **area vs. aspect ratio** is displayed. Using a region tool  identify the single cells to use as your compensation population.
7. Select the newly created population from the **compensation population** drop down list, or use the 'All' population if few outliers are present.
8. IDEAS will automatically identify the **single color positive cells** for each channel and display adjacent channel scatter plots with these events displayed in their respective colors.
9. Under positive populations is a list of each color channel. From the drop down list next to the channel names, select the appropriate single color **positive population** and do this for each fluorochrome used in the experiment. For example, if FITC was used, in channel 3 (green) select the 

population.
10. Once the positive control populations are assigned to their corresponding channels, click . 

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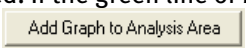
11. To view the matrix, click on the **Compensation Matrix** tab in the upper right hand corner.
12. **Validate the matrix** by right-clicking each cell of the matrix.
13. The **Matrix Coefficient Intensity Plot** is displayed. If the green line of best fit does not exactly overlay the data points on the plot, click 

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14. Using a region too  select a new **positive population** that excludes the outliers.
15. Assign the **new population** to its channel using the appropriate drop down list.
16. Click 

again to re-calculate the matrix using the new populations.
17. Click 

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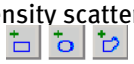
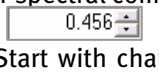
Quick Start Guide: Troubleshooting the Matrix

Troubleshooting the Compensation Matrix

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. Create and save a **Compensation Template** with these critical parameters (update the template for each IDEAS version upgrade);
   a. Set the image gallery display properties  for each channel from 0 to 100.
   b. Create adjacent channel Peak Intensity scatter plots  .
   c. On each plot, use the region tool  to create a population with no saturation. You will have three regions (R1, R2, R3).
   d. In the region manager, extend the regions from 0 to 1022 counts on both axes.
   e. Create a combined population of the intersection (R1 and R2 and R3).
   f. Create adjacent channel Intensity dot plots  using the non-saturated events.
   g. Save this template as an .ast file and use this template throughout the process.
2. **Open the poorly compensated data** using the original matrix and the Compensation Template.
3. Using the tagging tool  to **create a training set** hand-select mis-compensated events (ignoring outliers) that cover a range of intensities and includes unlabeled cells.
4. **Save the training set** as a separate.rif by choosing "Create Data File from Population" from the Tools menu, and select the training set. Check the save .rif box and click OK.
5. **Open the training set** .rif using the original compensation matrix and the Compensation Template.
6. Draw regions that identify the **unlabeled and single color positive** populations.
7. Identify the matrix values that need adjusting by inspecting the **scatter 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. **Open** the training set .rif.
9. **Browse** for the incorrect matrix and press open.
10. Click on **Advanced**, and under spectral compensation click the folder button  .
11. When the matrix is displayed  **manually change the incorrect crosstalk matrix values** identified in step 7. Start with changes of ~.1 or ~.05 and use smaller and smaller increments as you refine the matrix.
12. Click **Save**, append *manual* to the matrix name, then click OK. Click OK to close the advanced window.
13. Leave the .cif name the same and IDEAS will **overwrite the old .cif**. Press OK twice to navigate through the warnings.
14. When prompted for an analysis template, use the **Compensation Template**.
15. If the data still appears to be mis-compensated**, repeat steps 8-15**.

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