





## Compensation

IDEAS® uses a wizard to automatically calculate a compensation matrix for your experiment. During data acquisition a file of between 500 and 1000 positively-stained events should be collected from a single color control sample for each fluorochrome in the experiment. Compensation control files should be collected with **all channels active and brightfield and SSC turned off**. Each compensation file is then loaded into IDEAS and used to measure the spectral crosstalk of each fluorochrome into each channel, and is applied to the experiment data when the file is opened or the experiment is batch processed.

### *To create a compensation matrix:*

1. Select **New Matrix** when opening a .rif, or from the IDEAS toolbar select **Compensation** then **Create New Matrix**.
2. Click **Add Files** and control-select the appropriate –noBF control files. One for each fluorochrome in the experiment. Click **Next** to load the files.
3. Verify the active channels used in the experiment and click **next**.
4. IDEAS will automatically identify the **single color positive cells**, create adjacent channel intensity dot plots, and auto-generate a compensation matrix. Matrix values with greater than 1% error are flagged red. If all values are black click Finish.



### *Validate the compensation matrix:*

5. If some values are flagged red, optimize the matrix. To do this:
  - a. Double-click the red value in the matrix. 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 an image and IDEAS will display the compensated version of the image in the gallery window.
  - a. If crosstalk images have black areas, the fluorochrome is **overcompensated**, and the corresponding matrix values should be manually reduced.
  - b. 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.
9. Click **Finish**, and save the compensation matrix

# Troubleshooting Compensation

Sometimes an applied matrix produces poorly compensated data. This can happen for a number of reasons including saturation, autofluorescence, or using the wrong population. Ideas lets you manually adjust the matrix using this method.

## To troubleshoot and repair a compensation matrix:

1. Open the poorly compensated (comp) data and use the tagging tool  to **create a training set** of poorly compensated events. Include blanks and cover a range of intensities.
2. Save the training data by selecting tools, then **create data file from population**.
3. Create and save a **Comp Template** or use the template in 9 color comp training data folder;
  - a. Set the image gallery display properties  for each channel from 0 to 150.
  - b. Create adjacent channel Raw Max Pixel dot plots and gate out saturated events.
  - c. Create adjacent channel Intensity dot plots using the non-saturated events.
  - d. Save this template as an .ast file and use for future compensation troubleshooting.
4. **Open** the “training data set” with the “compensation” template 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**: Single color positive population curves into the crosstalk channel.
    - ii. **Images**: the crosstalk channel contains an apparent fluorescent mirror-image.
  - b. **Over-compensation** (crosstalk coefficient is too high):
    - i. **Plots**: Single color positive population curves off the plot.
    - ii. **Images**: the crosstalk channel contains dark spots matching the bright spots in peak channel.
6. Go to the **Compensation** dropdown, select **View Edit Matrix**.
7. **Manually** change the incorrect matrix values from step 5. Start with changes of  $\sim .1$  or  $\sim .05$ .
8. **Save** the new matrix.
9. **Open** the “training data set” (step 1) using the “compensation” template (step 3), 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 file.

