Spectral Unmixing for ImageJ – Documentation

Purpose:
Spectral unmixing is an algorithm, which is applied to fluorescence images to correct for spectral bleedthrough between different color channels. E.g. the emission spectra of the fluorochromes CFP and YFP partially overlap, and CFP signals also appear in the YFP fluorescence channel. This overlap can be corrected by spectral unmixing.

Algorithm:
The algorithm applied is a simple matrix algorithm as used e.g. in ¹ and ². It measures the spectral bleedthrough between color channels from reference images. The relative intensity of each fluorochrome in all color channels is stored in a matrix here called the “mixing matrix”. The inverse of this matrix is stored and is used to correct the bleedthrough in images of “real” experiments. These images have to be recorded under the same imaging conditions as the reference images. The above example for CFP and YFP is an example of two fluorochromes and two color channels, but the algorithm can be used for any number of fluorochromes and any number of color channels. But there have to be at least as many color channels as fluorochromes.

Installation:
Have the plugin Sync_Windows_1.3 or higher installed. It is available from the ImageJ website. Put Spectral_Unmixing_1.2.jar and Jama-1.0.1.jar into the ImageJ plugins-folder or a subfolder and restart ImageJ. A submenu “SpectralUnmixing” of the Plugins menu appears, which contains the entries “Display Mixing Matrix”, “Measure Mixing Matrix” and “Unmix”. Jama-1.0.1.jar is the .jar-file of the Java Matrix Package ³.

² Zimmermann et al., FEBS Letters 531 (2992) p. 245-249
³ http://math.nist.gov/javanumerics/jama/
Usage:

Preparation of reference samples
Reference samples ideally are prepared from the same biological material and under the same staining and embedding conditions as the samples for the “real” experiments. Additionally, each fluorochrome has to have a well-known position in the sample, where it does not colocalize to other fluorochromes. There are generally two ways to achieve this:

1. There are well-separated structures in the sample, and each fluorochrome labels only one of these structures. E.g. a metaphase chromosome spread, where each fluorochrome marks a different chromosome. In this case one needs only one reference sample for all fluorochromes. Of this reference sample one has to record a set of images containing all color channels.

2. One prepares one reference sample for each fluorochrome, in which the sample is only stained with this fluorochrome. In this case one has to take one set of images of each reference sample, each set containing all color channels.

Measuring the unmixing matrix
The method to measure the unmixing matrix is a bit different in each of the two cases. For an example the matrix is measured for a metaphase spread with FISH-stained chromosomes #5 and X (Courtesy of Marion Cremer, LMU München). The X-chromosome is stained with Alexa 633 and chromosome #5 is stained with Cy5.

Case 1: One reference sample for all fluorochromes.

a) Open the images of all color channels in ImageJ. Make sure, that you open the images in an order, which is convenient for you, e.g. first the blue fluorescence channels, then the green, then the red fluorescence channels. For unmixing, the channels have to be in the same order. You can check the order in the “Window” menu of ImageJ.

b) Open the plugin “Measure Mixing Matrix”. Select all color channels, type in the number of fluorochromes used in this experiment, and press “Start
Measurements”. The plugin window now looks like this and an additional window “Mixing Matrix” pops up.

c) To measure the background in all images, select a ROI, which has only background signal in all images. It is sufficient to do this in one image. The ROI is drawn synchronously in all images. Then double-click into the ROI.
You can see the entries for mean background and number of pixels in the “Mixing Matrix” window.

```
<table>
<thead>
<tr>
<th>Channels</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluors</td>
<td>2</td>
</tr>
<tr>
<td>Mean Background</td>
<td>32.691</td>
</tr>
<tr>
<td>#Pix Background</td>
<td>5558</td>
</tr>
<tr>
<td>Mean Signal</td>
<td>20031217-2d-09-ch4_Alexa633-crop.tif</td>
</tr>
<tr>
<td>Fluor 1</td>
<td>0.000</td>
</tr>
<tr>
<td>Fluor 2</td>
<td>0.000</td>
</tr>
</tbody>
</table>

d) To measure the relative contributions of fluorochrome 1 (here Alexa 633) to the fluorescence channels, first click onto the right arrow of the lower control in the plugin window. The text next to it changes from “Background” to Fluor 1. You can give the fluorochrome a more intuitive name in the adjacent text box. Then draw a ROI around the respective reference object.
The ROI mostly contains pixels of the reference object, but also background pixels or pixels of other objects. To segment the reference object, adjust the threshold slider in the plugin window for a channel, where the object is best visible.

The respective image now looks like this:

To perform the actual measurement, double-click into the ROI in the image, where you segmented the reference object. The plugin measures the mean intensities of the pixels within the ROI, which are above the threshold in this image and the mean intensities of the same pixels in all other color channels.
f) Repeat steps d) and e) for the other fluorochromes.

![Image of spectral unmixing](image-url)

g) After the last fluorochrome has been measured, the mixing matrix and the unmixing matrix are displayed.

![Image of mixing matrix](image-url)

h) Save the data to disk by using the “File->Save As...” command in the File menu of the Mixing Matrix window.

**Case 2: One reference sample for each fluorochrome.**

a) Load all color channels for the reference object for the first fluorochrome, start “Measure Mixing Matrix”, measure the background and the intensities of the first fluorochrome as shown above.

b) To measure the intensities of the second fluorochrome in all color channels, press “Stop Measurements” in the plugin window.

c) Close all images (color channels) for the first fluorochrome.
d) Open all color channels with the reference object for the second fluorochrome. Be careful to do this in the same order as you opened the images for the first chromosome. Otherwise your color channels will be mingled in the matrix.

e) Press “Update Window List” in the plugin window and then select all the color channels of the reference object for fluorochrome 2.

f) Press “Start Measurements” and measure the intensities like shown above. The values are added to the measurements.

g) Repeat steps b) to f) for the other fluorochromes. After the last measurement you again get the mixing matrix and the unmixing matrix.

h) Save the data to disk.

You can display a saved mixing/unmixing matrix with the plugin “Display Mixing Matrix”.

Doing the actual unmixing

a) Open the all color channels of the measurement, which you want to unmix.

b) Open the plugin “Unmix”.

c) Load the file containing the unmixing matrix. The number of fluorochromes and channels is displayed for checking.

d) Press “Unmix”.

e) A window “Select Channels” pops up.

Open images are displayed in the window in the order, in which they were opened. You can select different images or a different order with the dropdown-lists for each channel.


The unmixed images are of the same image type (8-bit, 16-bit or 32-bit) as the image of channel 1. If “32-bit float output” is selected, the unmixed images are of type 32-bit. The Unmix Channels plugin can be used in macros. It also works with the function “Record Macro”, so it is easy to create a macro, which unmixes large amounts of data.