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].

[1] Neher & Neher, J. Microsc. 213, 1 (2004), p. 46-62
[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.
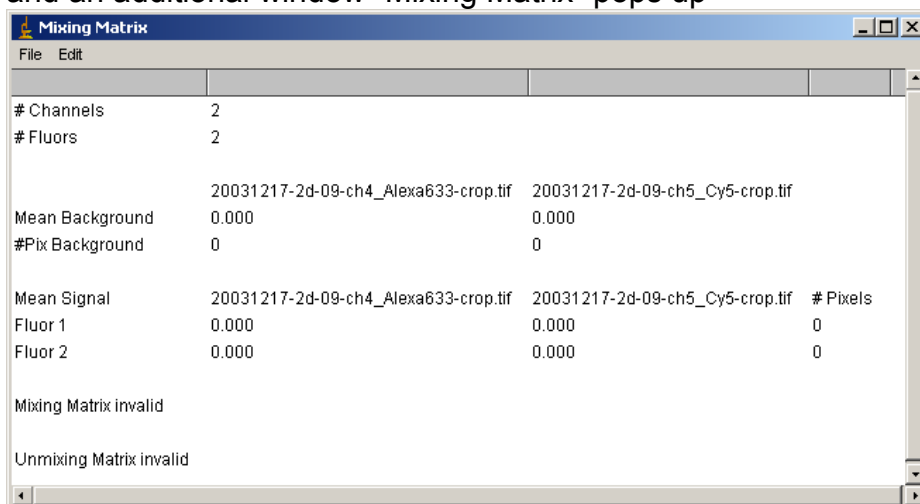

b) Open the plugin "Measure Mixing Matrix". Select all color channels, type in the number of fluorochromes used in this experiment, and press "Start

Measurements". The plugin window now looks like this
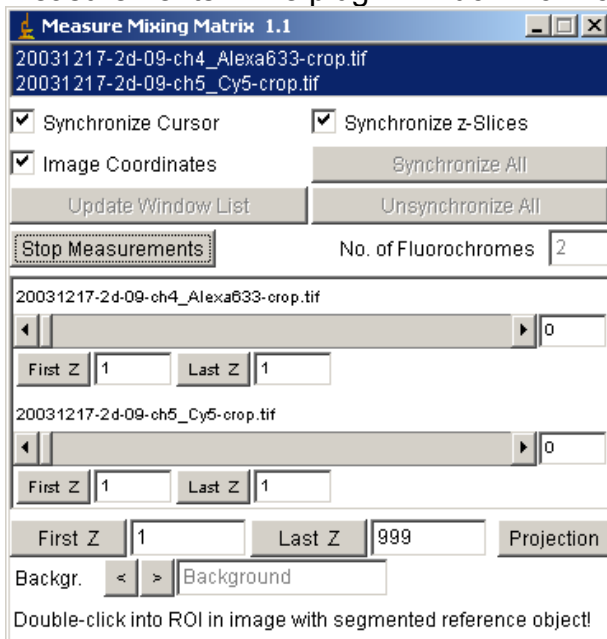
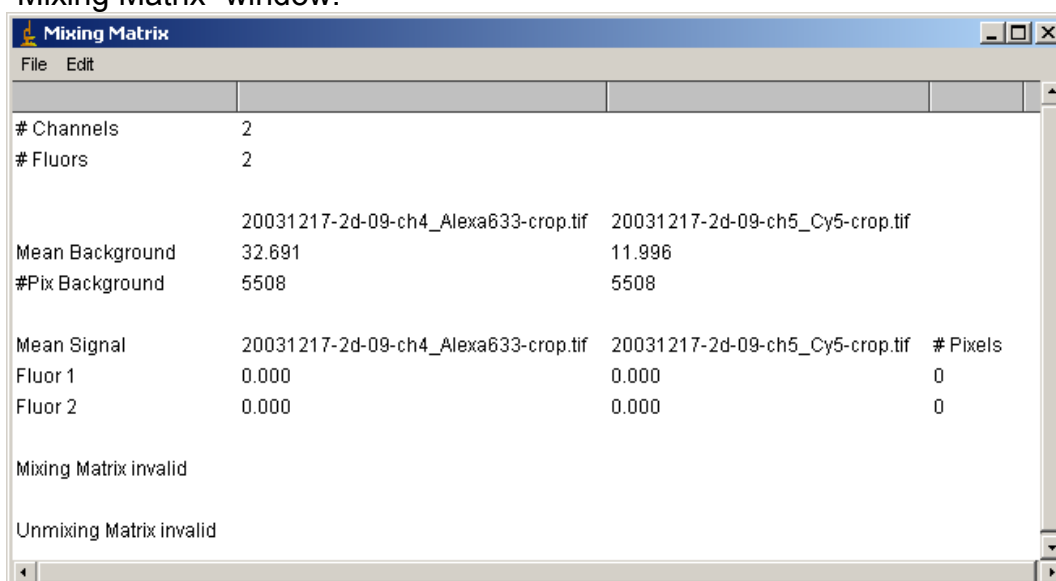and an additional window "Mixing Matrix" pops up

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.
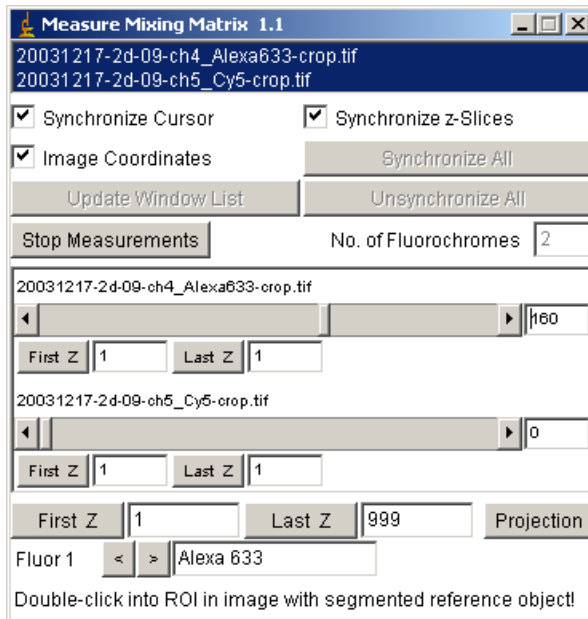
You can see the entries for mean background and number of pixels in the "Mixing Matrix" window.

Mixing Matrix			
File Edit			
# Channels	2		
# Fluors	2		
	20031217-2d-09-ch4_Alexa633-crop.tif	20031217-2d-09-ch5_Cy5-crop.tif	
Mean Background	32.691	11.996	
#Pix Background	5508	5508	
Mean Signal	20031217-2d-09-ch4_Alexa633-crop.tif	20031217-2d-09-ch5_Cy5-crop.tif	# Pixels
Fluor 1	0.000	0.000	0
Fluor 2	0.000	0.000	0
Mixing Matrix invalid			
Unmixing Matrix invalid			

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.
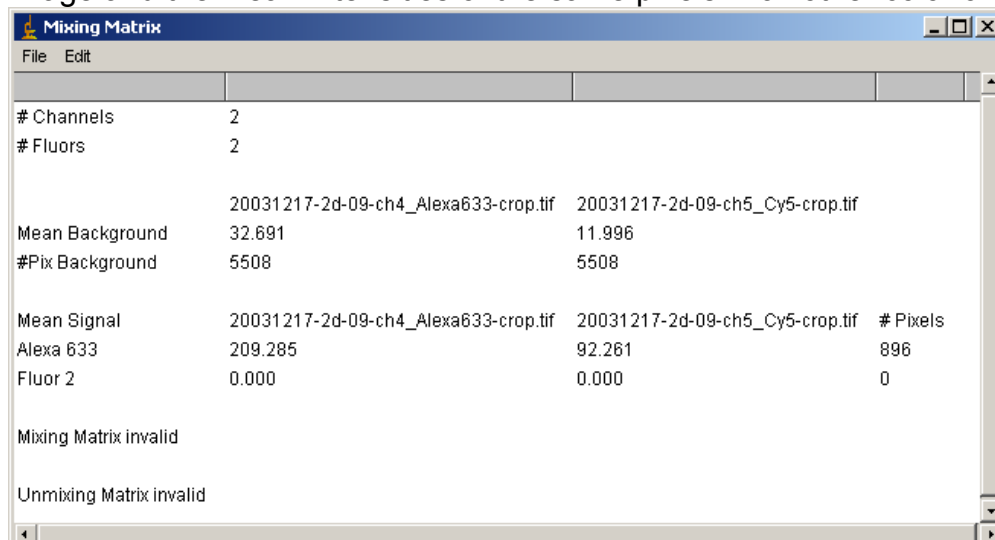
The ROI mostly contains pixels of the reference object, but also background pixels or pixels of other objects. To segment the reference object, adjust the threshold slider in the plugin window for a channel, where the object is best visible
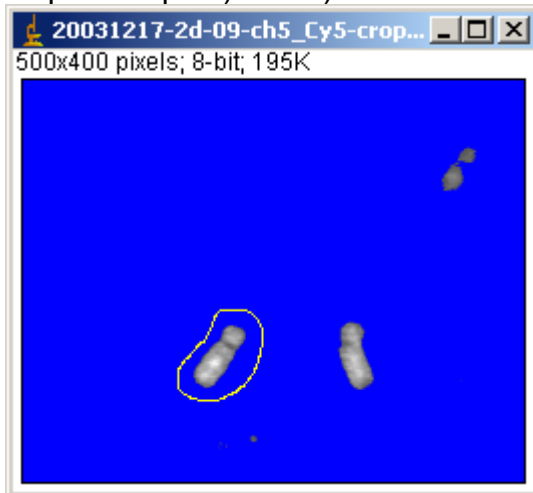
Measure Mixing Matrix 1.1

20031217-2d-09-ch4_Alexa633-crop.tif
20031217-2d-09-ch5_Cy5-crop.tif

☑ Synchronize Cursor   ☑ Synchronize z-Slices

☑ Image Coordinates   Synchronize All

Update Window List   Unsynchronize All

Stop Measurements   No. of Fluorochromes 2

20031217-2d-09-ch4_Alexa633-crop.tif

◄   ► 160

First Z 1   Last Z 1

20031217-2d-09-ch5_Cy5-crop.tif

◄   ► 0

First Z 1   Last Z 1

First Z 1   Last Z 999   Projection

Fluor 1   <   >   Alexa 633

Double-click into ROI in image with segmented reference object!

The respective image now looks like this:

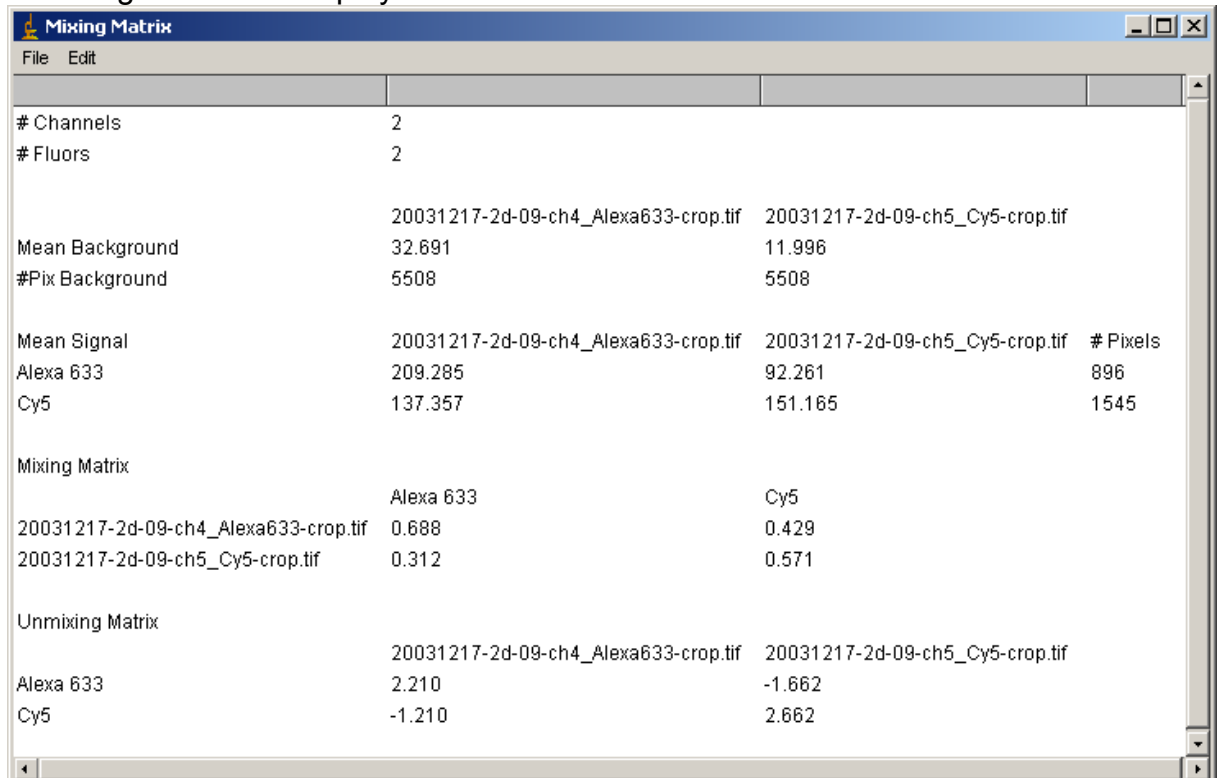**20031217-2d-09-ch4_Alexa63...**
500x400 pixels; 8-bit; 195K

e) 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.

Mixing Matrix

File Edit

# Channels	2		
# Fluors	2		
	20031217-2d-09-ch4_Alexa633-crop.tif	20031217-2d-09-ch5_Cy5-crop.tif	
Mean Background	32.691	11.996	
#Pix Background	5508	5508	
Mean Signal	20031217-2d-09-ch4_Alexa633-crop.tif	20031217-2d-09-ch5_Cy5-crop.tif	# Pixels
Alexa 633	209.285	92.261	896
Fluor 2	0.000	0.000	0
Mixing Matrix invalid			
Unmixing Matrix invalid			

f) Repeat steps d) and e) for the other fluorochromes.

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

Mixing Matrix

File   Edit

# Channels	2		
# Fluors	2		
	20031217-2d-09-ch4_Alexa633-crop.tif	20031217-2d-09-ch5_Cy5-crop.tif	
Mean Background	32.691	11.996	
#Pix Background	5508	5508	
Mean Signal	20031217-2d-09-ch4_Alexa633-crop.tif	20031217-2d-09-ch5_Cy5-crop.tif	# Pixels
Alexa 633	209.285	92.261	896
Cy5	137.357	151.165	1545
Mixing Matrix			
	Alexa 633	Cy5	
20031217-2d-09-ch4_Alexa633-crop.tif	0.688	0.429	
20031217-2d-09-ch5_Cy5-crop.tif	0.312	0.571	
Unmixing Matrix			
	20031217-2d-09-ch4_Alexa633-crop.tif	20031217-2d-09-ch5_Cy5-crop.tif	
Alexa 633	2.210	-1.662	
Cy5	-1.210	2.662	

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".
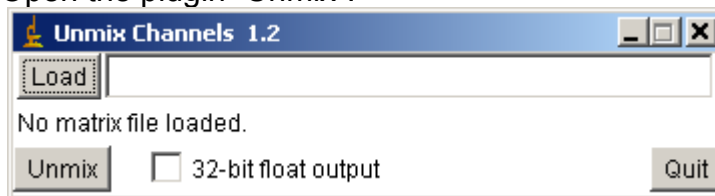
c) Load the file containing the unmixing matrix. The number of fluorochromes
and channels is displayed for checking.
d) Press "Unmix".
e) A window "Select Channels" pops up.
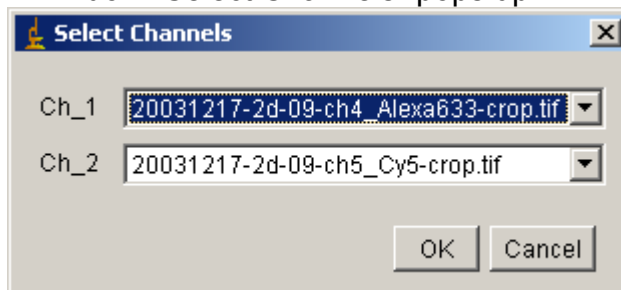
Open images are displayed in the window in the order, in which they were
opened. You can select different images or a different order with the
dropdown-lists for each channel.
f) Press "OK". The unmixed images appear.

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.