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CONFOCAL APPLICATION LETTER

reSOLUTION

Dye Separation

Leica
MICROSYSTEMS

Introduction

Today a wide variety of fluorescent dyes and fluorescent proteins are available for multicolor fluorescence microscopy. Recorded signals from these fluorescent molecules provide complex information about multilabeled samples, often necessitating quantification or localization/colocalization analysis.

If significant overlap of the excitation or emission spectra of multiple fluorophores occurs it becomes difficult to distinguish between the different signals. Consider a combination of the four fluorophores Alexa 488, Alexa 546, Alexa 568 and TOTO-3 (see Fig. 1). It is difficult to separate emission signals from these dyes due to their strong spectral overlap, which results in signals from multiple dyes in each channel. This phenomenon is termed crosstalk, or bleed-through. Interpreting multicolor images can be challenging in this case because they arise from a mixture of signals from multiple dyes.

Crosstalk

There are different options to avoid and/or remove crosstalk of fluorophores for multi-labeled samples.

For example, when using simultaneous scan mode there are acquisition strategies to minimize crosstalk. One way is to optimize the detection range to avoid crosstalk. Reducing the excitation light for each respective fluorophore will also reduce the emission intensity, which in turn reduces the degree of crosstalk. But, if the degree of overlap is too strong (Alexa 546/Alexa 568 or Dapi/FITC) it is better to choose the sequential scan mode.

However, sequential scan may not be the best choice when speed is important (i.e. for live cell imaging). Simultaneous detection of all dyes may be necessary; sequential scan mode may be too slow. In addition, samples that are stained with multiple fluorophores that are excited by the

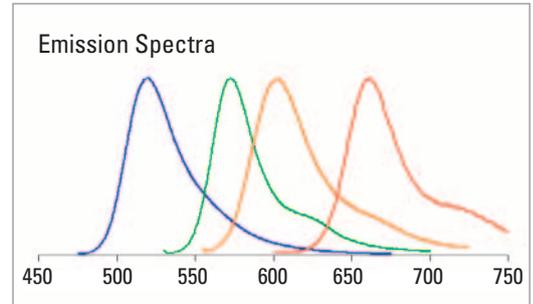
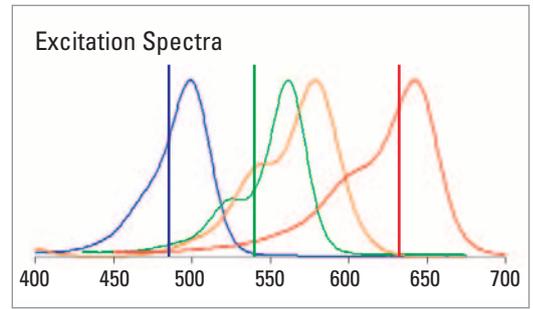


Fig.1: Excitation and emission spectra of Alexa 488, Alexa 546, Alexa 568 and TOTO-3.

same laser line (see example Fig. 1) will exhibit crosstalk despite using sequential scan. In these cases a mathematical restoration of dyes into separate channels may be necessary. This will be discussed in the following.

Consider a FITC/TRITC double-labeled sample.

See in Fig. 2a the emission spectrum of only one dye. The total emission light collected from FITC will be distributed in both channels. Here the green channel collects about $\frac{3}{4}$ of the entire green signal while $\frac{1}{4}$ of the signal spills over into the red channel.

For the red channel a similar situation exists (Fig. 2b). The total light collected from TRITC will be distributed in both channels. Here we estimate $\frac{4}{5}$ of the red signal is seen in the red channel and $\frac{1}{5}$ of the signal goes into the green channel.

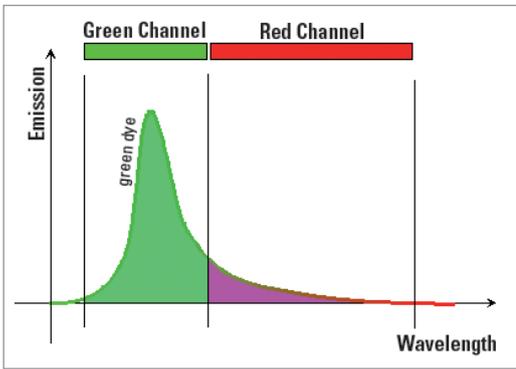


Fig. 2a: Emission spectrum of the green channel
 We estimate here:
 $\frac{3}{4}$ of all FITC emission goes into the green channel
 $\frac{1}{4}$ of all FITC emission goes into the red channel

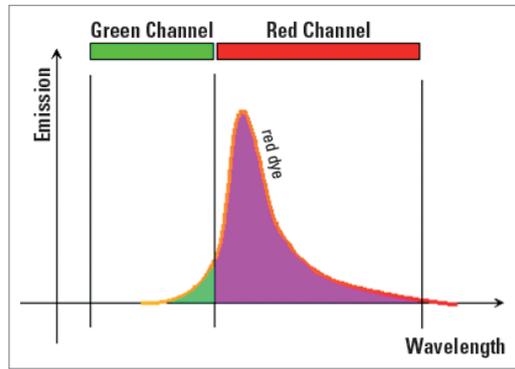


Fig. 2b: Emission spectrum of the red channel
 We estimate here:
 $\frac{1}{5}$ of all TRITC emission goes into the green channel
 $\frac{4}{5}$ of all TRITC emission goes into the red channel

In a double-stained sample (Fig. 2c), signals from both dyes will be present. In our example you will record $\frac{3}{4}$ FITC + $\frac{1}{5}$ TRITC in the green channel and $\frac{1}{4}$ FITC + $\frac{4}{5}$ TRITC in the red channel.

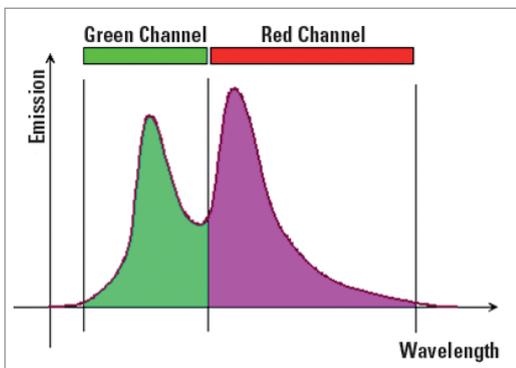


Fig. 2c: Emission signals of a double-labeled sample. The black curve represents the sum of the signals of both fluorophores.

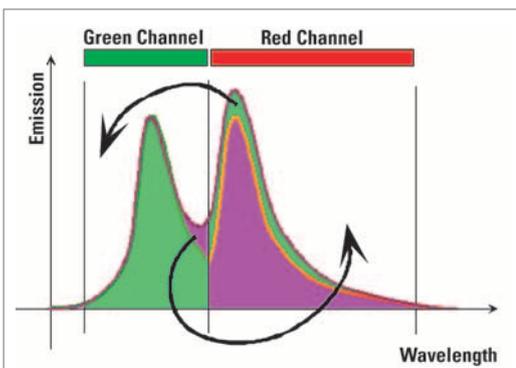
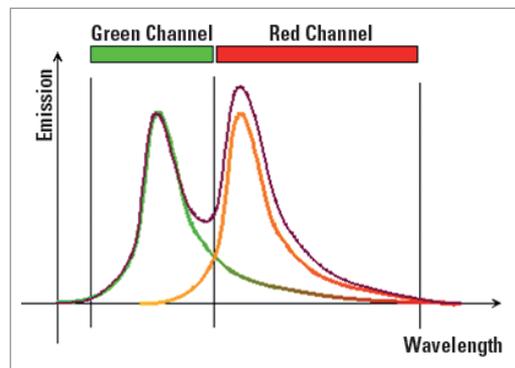


Fig. 2d: Removing crosstalk:
 $\frac{1}{4}$ of all FITC emission has to be removed from red channel
 $\frac{1}{5}$ of all TRITC emission has to be removed from green channel

The goal is now to separate the signals, so that each channel contains only the signal from one dye. This means that $\frac{1}{5}$ of the TRITC signal has to be removed from the green channel, and $\frac{1}{4}$ of the FITC signal has to be removed and redistributed

from the red channel. The resulting image will be free of crosstalk (Fig. 2d).

Expressed in mathematical terms you will have two equations with two unknowns:

$$\text{Green channel: } G = \frac{3}{4}\text{FITC} + \frac{1}{5}\text{TRITC}$$

$$\text{Red channel: } R = \frac{1}{4}\text{FITC} + \frac{4}{5}\text{TRITC}$$

There are different options to find these coefficients to solve this mathematical problem:

- you may use reference measurements
→ **Channel + Spectral Dye Separation** tool

- you may estimate the coefficients and subtract crosstalk manually
→ **Manual Dye Separation** tool

- you may use computation through statistical analysis (Intensity Correlation)
→ **Automatic Dye Separation** tool

1. Dye Separation: Background

Dye Separation Based on Linear Unmixing

The Linear Unmixing method was initially developed for processing multiband satellite images. In general the algorithm is based on the following assumption: the total emission signal S of every channel λ is expressed as a linear combination of the contributing dyes $FluoX$. A_x represents the amount of contribution by a specific fluorophore.

This method uses spectral signatures (emission spectra) as references. In the case of multi-fluorescence images, even combined and mixed emission signals can be clearly separated into the dyes that contribute to the total signal.

In other words, the system calculates the distribution coefficients of all the dyes in the different channels.

$$S(\lambda) = A_1 x Fluo1(\lambda) + A_2 x Fluo2(\lambda) + A_3 x Fluo3(\lambda)...$$

1.1 Channel Dye Separation

For correct unmixing it is necessary to find regions with pure dyes in the sample as references. The best way to do this is to use controls that contain only one of the dyes. This approach reduces the risk of taking spectra with slight contributions of other fluorophores as references. However, multi-labeled samples may also be used if there are areas within the specimen that clearly contain single dye regions without colocalization. The distribution coefficients will be measured, and the sample can be analyzed. If you need to separate n different dyes, it is sufficient to collect n different channels; no 'spectrum' must be recorded.

1.2 Spectral Dye Separation

This method is preferred for lambda stacks. Mathematically it is the same equation used for the **Channel Dye Separation**. Here, the set of coefficients for a dye is the 'spectrum'.

This method requires the appropriate reference spectra, which can be measured with a Lambda scan or which can be taken from the literature. The spectra can be stored in a spectra database.

General information:

Autofluorescence

Autofluorescence of cells may be a significant problem in fluorescence microscopy. By means of the **Channel** and **Spectral Dye Separation** tool you can treat autofluorescence as another fluorophore (unstained sample as reference) and thus remove it from the specimen-specific signal.

In the same way background may be removed, assuming the background of the sample is homogeneous.

Separation of non-balanced fluorophores

Sometimes the emissions of different fluorophores are not well balanced in intensities. In this case a weak signal may be partially overlaid by the crosstalk coming from a strong signal. With the Dye Separation tool it is possible to separate both fluorophores.

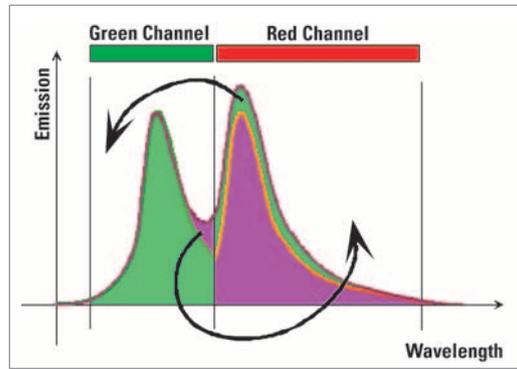
Separation of fluorophores excited with one laser line

Even if your sample is stained with two dyes excited by the same laser line, the **Dye Separation** tool can successfully separate the two fluorophores. For example, a sample containing both Alexa 488 and GFP requires 488 nm light for excitation of both fluorophores. As mentioned above, a sequential scan will not eliminate crosstalk in this example. However, even though the emission spectra extremely overlap, it is still possible to separate the signals using reference samples.

Note: The **Spectral Dye Separation** tool cannot be used in the special case where the fluorophores are both excited by a single laser line AND their intensities are significantly different.

1.3 Manual Dye Separation

In the **Manual** tool the distribution coefficients are not calculated by the system but are estimated by the user, i.e. no references are needed. Let's explain it using the example described above.



To get crosstalk-free images we estimate that $\frac{1}{5}$ of the TRITC signal has to be removed from the green channel and $\frac{1}{4}$ FITC signal has to be removed from the red channel. The user only needs to apply the estimated numbers to a matrix.

Matrix		
	Ch1	Ch2
Dye1	1	0,25
Dye2	0,2	1

Note: If your system is equipped with the Colocalization Analysis tool (license is required), you can use the cytofluorogram to get the coefficients (see page 22).

Dye Separation based on Intensity Correlation

The *Leica Automatic Dye Separation (Weak & Strong)* software analyses the correlation of the grey values of the pixels in different channels. A scatter plot known as a cytofluorogram represents such correlations and is used for example in colocalization analysis.

The cytofluorogram (Fig. 3) shows grey values of channels one and two on the x- and y-axis, respectively. Each pixel in the scatter plot represents an intensity pair (green-red) of the original detection channels. Crosstalk of the green dye into the red channel is defined by the angle of the data cloud with the x-axis (0° defining 0% crosstalk). In the same manner, crosstalk of the red dye into the green channel is defined by the angle of the data cloud with the y-axis.

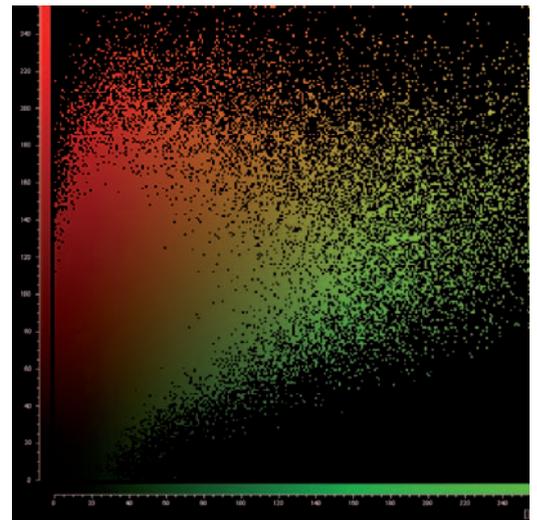


Fig. 3: Cytofluorogram shows the intensity relationships between two channels.

1.4 Automatic Dye Separation: Weak and Strong

The **Automatic Dye Separation** tool uses a mathematical procedure, called cluster analysis, for classifying objects into homogenous groups. In our case the objects being classified are the grey values of the pixels, which are acquired in different detection channels.

After identifying clusters of homogenous image data, the best-fit line for the clouds in the cyto-fluogram is determined. Crosstalk correction is achieved by 'moving' the fitted clouds to the axes (Fig. 4a–4d). The advantage of this method is that no spectral information is needed – the main distributions are found by fitting.

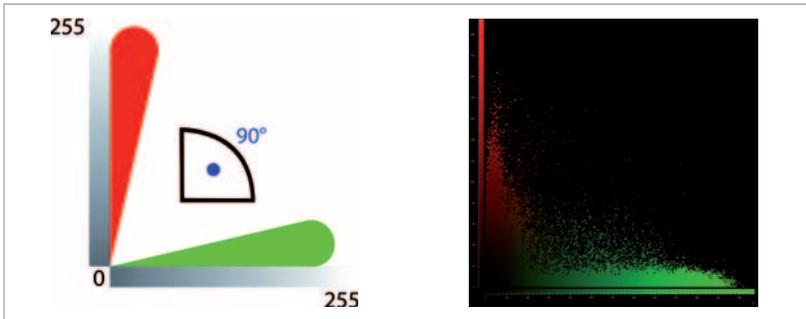


Fig. 4a: Ideal separation of fluorescent signals, without crosstalk; each channel is related to its own dye.

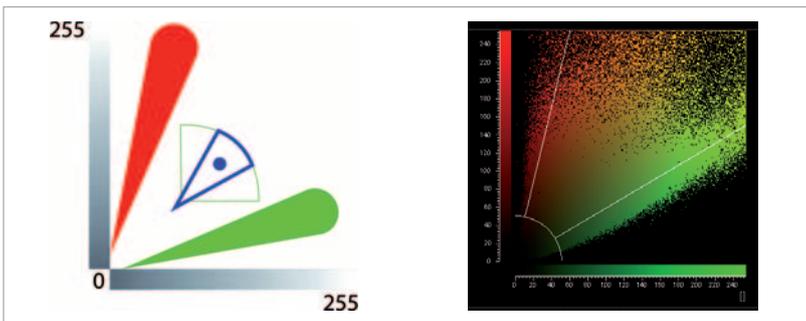


Fig. 4b: Fluorescent signals if crosstalk occurs: the clouds are tilted towards the diagonal.

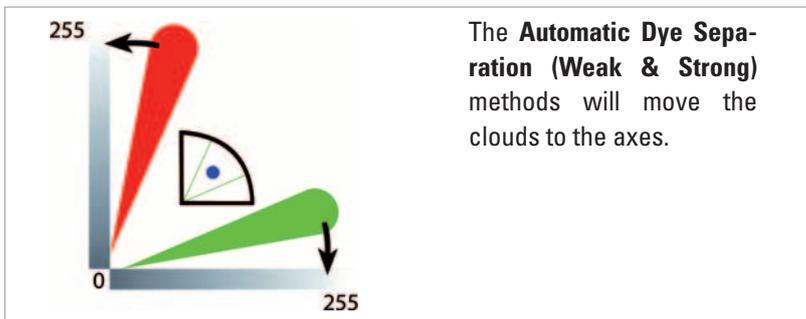


Fig. 4c: Correction of crosstalk

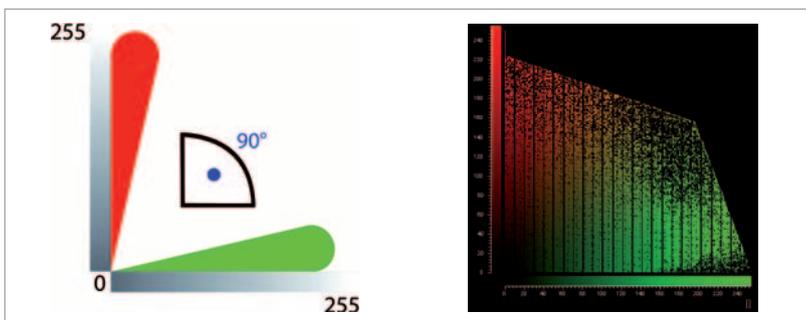
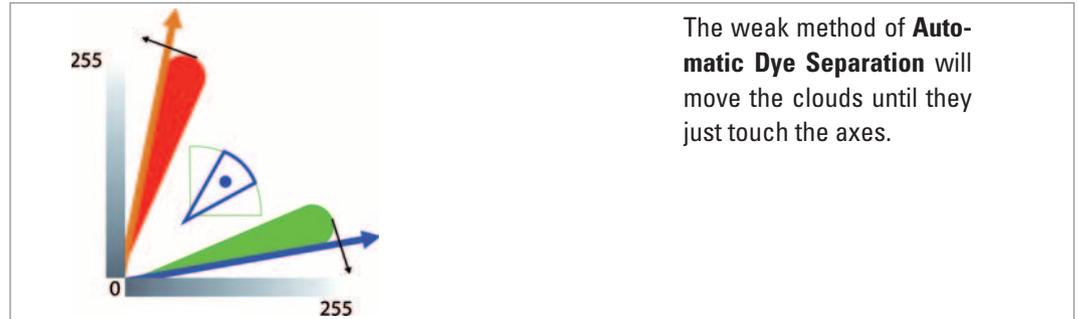


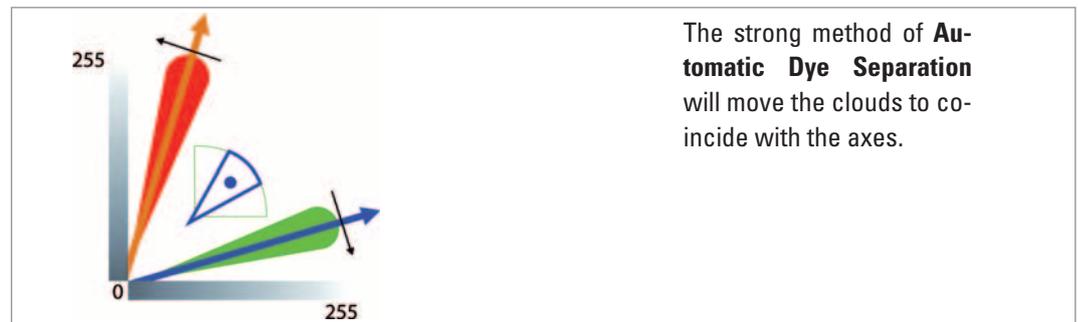
Fig. 4d: After processing: Ideal separation of fluorescent signals, each channel is related to its own dye.

What is the difference between Weak and Strong Dye Separation?

Weak:



Strong:



2. Dye Separation: Choosing the Right Tool

Channel Dye Separation

- When pure dyes are present in the sample or references are available
- For separation of two fluorophores with strong emission overlap, excited with the same excitation line
- For separation of autofluorescence

Spectral Dye Separation

- When reference spectra are available
- For lambda-series
- For separation of autofluorescence

Manual Dye Separation

- When no reference spectra are available
- When **Automatic Dye Separation** (see below) have failed

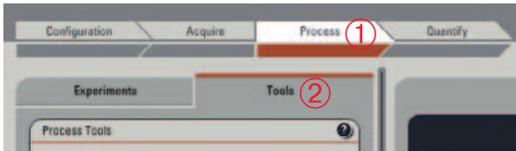
Automatic Dye Separation: Weak & Strong

- When no reference spectra are available; precondition: good signal to noise ratio
- **Weak** method: weak background and noise reduction
- **Strong** method: strong background and noise reduction

In order to get reasonable results with any of the **Dye Separation** tools, it is important to have images with good signal to noise ratios.

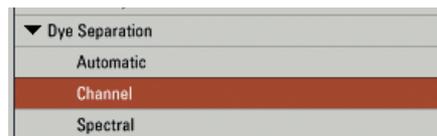
3. Dye Separation in LAS AF

You can find the **Dye Separation** tool under **Process** ① and the tab **Tools** ②.



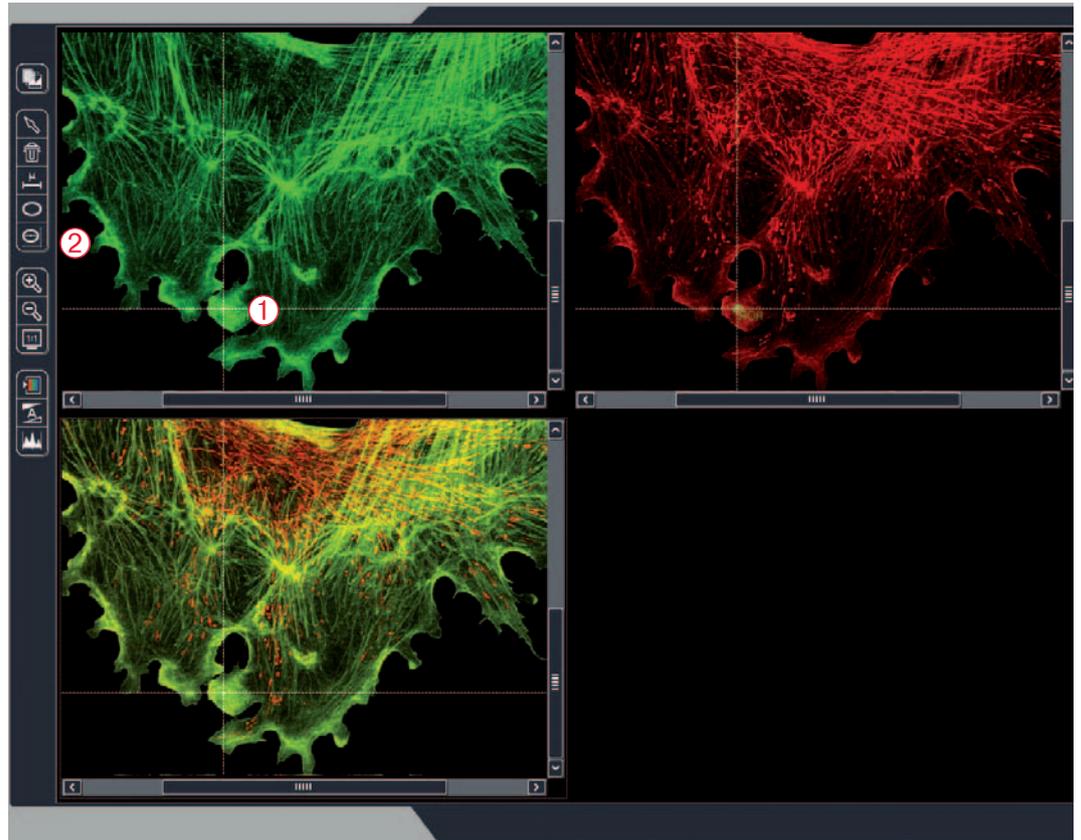
3.1 Channel Dye Separation: Step by Step

Select the **Channel Dye Separation** tool. To determine the distribution coefficients of the fluorophores (i.e. degree of crosstalk) you need to define reference regions within your image or series. You may use multi-labeled samples with pure dye regions (see paragraph A). Reference regions may also be defined on separately acquired single-dye control images (see paragraph B). Note that you have to keep detection parameters identical for the reference images.



A. When pure dye in a multi-labeled sample is present

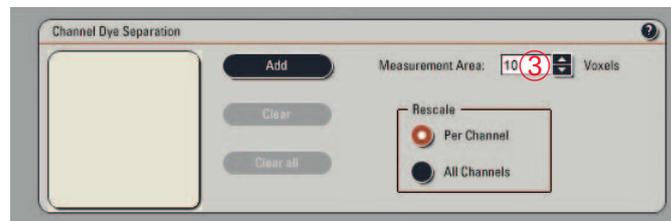
1. Place the crosshair in the viewer **①** to a position that clearly contains a single dye.
You can also draw a ROI manually after activating the ROI function in the viewer **②**.

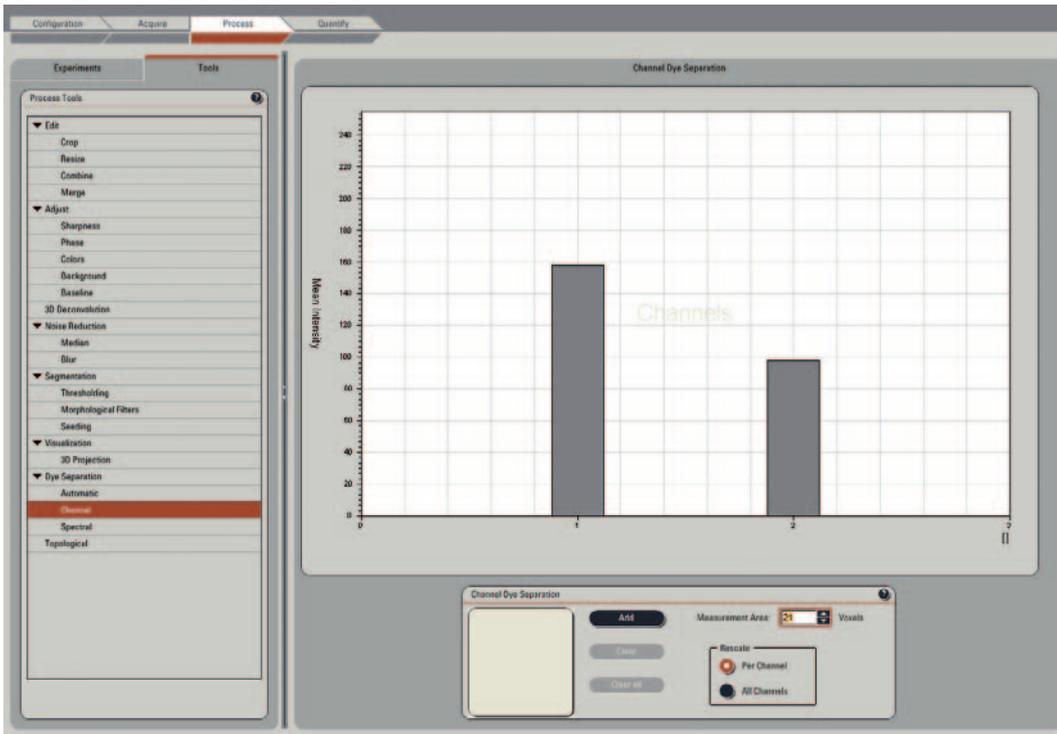


Bovine pulmonary artery endothelial cells (BPAEC); green: BODIPY FL phalloidin, F-actin; red: MitoTracker Red CMXRos, mitochondria.

Note: If there is no crosshair visible in the viewer you can activate it by clicking on the **Crosshair** button .

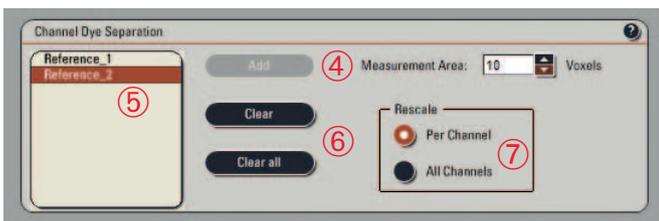
2. In the field **Measurement Area** **③** you may adjust the size of the reference region (in voxels).





The histogram visualizes the color distribution inside of the reference region.

3. Click **Add** ④ to define the chosen position as a reference region to determine the distribution coefficients of this fluorescent dye.
Every reference region you define is added to the list box ⑤.
Clear and **Clear all** ⑥ delete a marked reference or all references, respectively.



4. Repeat steps 1-3 for all dyes used in your image.
5. Choose a method of rescaling ⑦ for the resulting images or series.

There are two options for rescaling:

Per Channel: All channels are rescaled separately to spread the dynamic range of the images over the entire range of bit depth (e.g. 8 bit from 0 to 255). This operation results in brighter images, but these images cannot be further quantified.

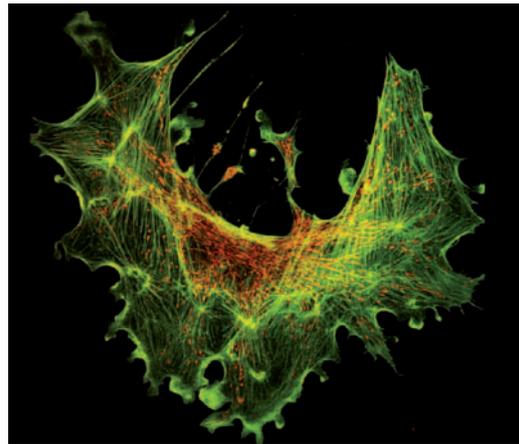
All Channels: All channels are rescaled together using the same factor, thereby maintaining the proportion. In this case only one channel gets the maximum bit depth.

6. Click **Apply** ⑧ to perform the image processing.

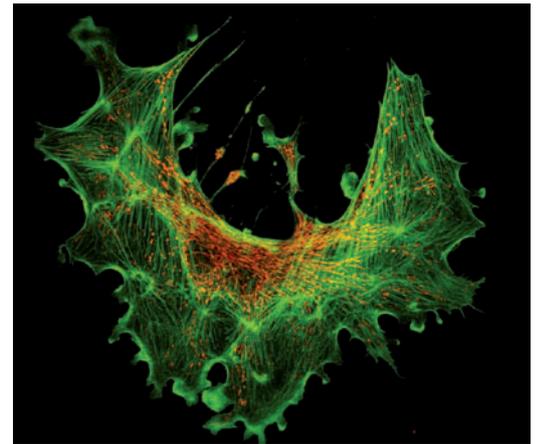


To preview the changes, press the **Preview** button. The **Reset** function allows you to go back to the default settings.

Original image with crosstalk



Resulting image without crosstalk



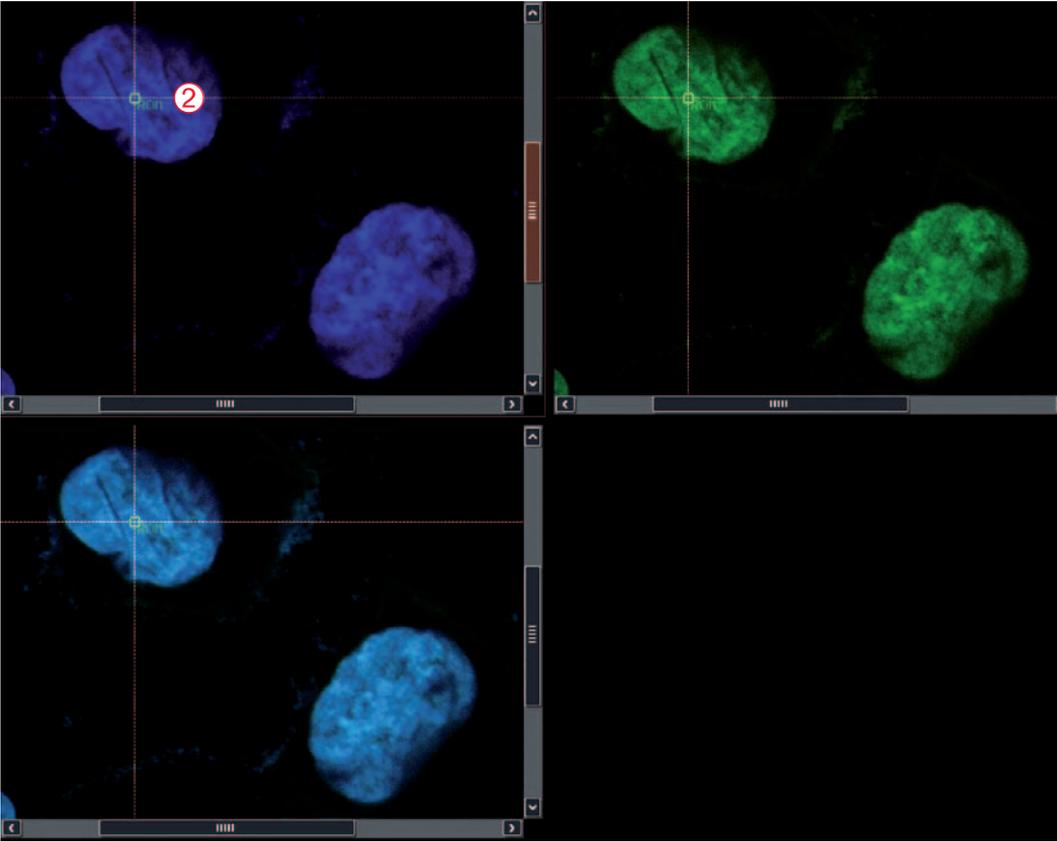
B. Using reference images

If you want to use control images of single labeled specimens as reference samples, you must capture all of the images using the same detection parameters that were chosen for the multi-labeled sample.

1. Select the first single-dye control image in the experiment tab ①.

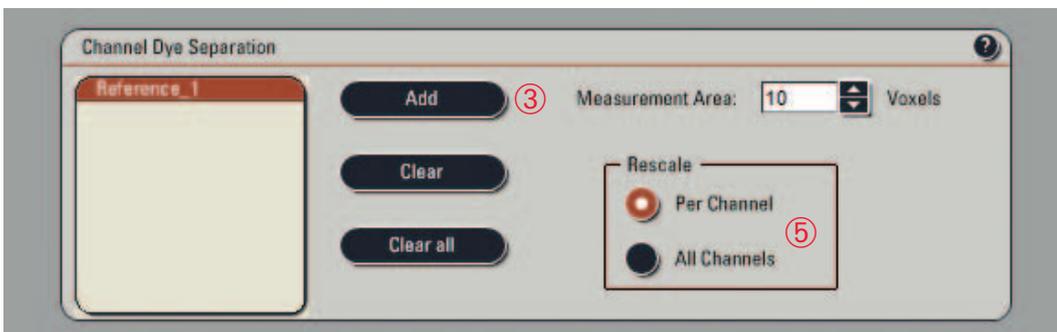


- Place the crosshair in the viewer **②** to an appropriate position.
You can also draw a ROI manually.



HeLa cells, reference 1: single-labeled cells imaged using the same conditions as the double-labeled sample; cyan (1. channel): Dapi, nucleus; green (2. channel): crosstalk of Dapi.

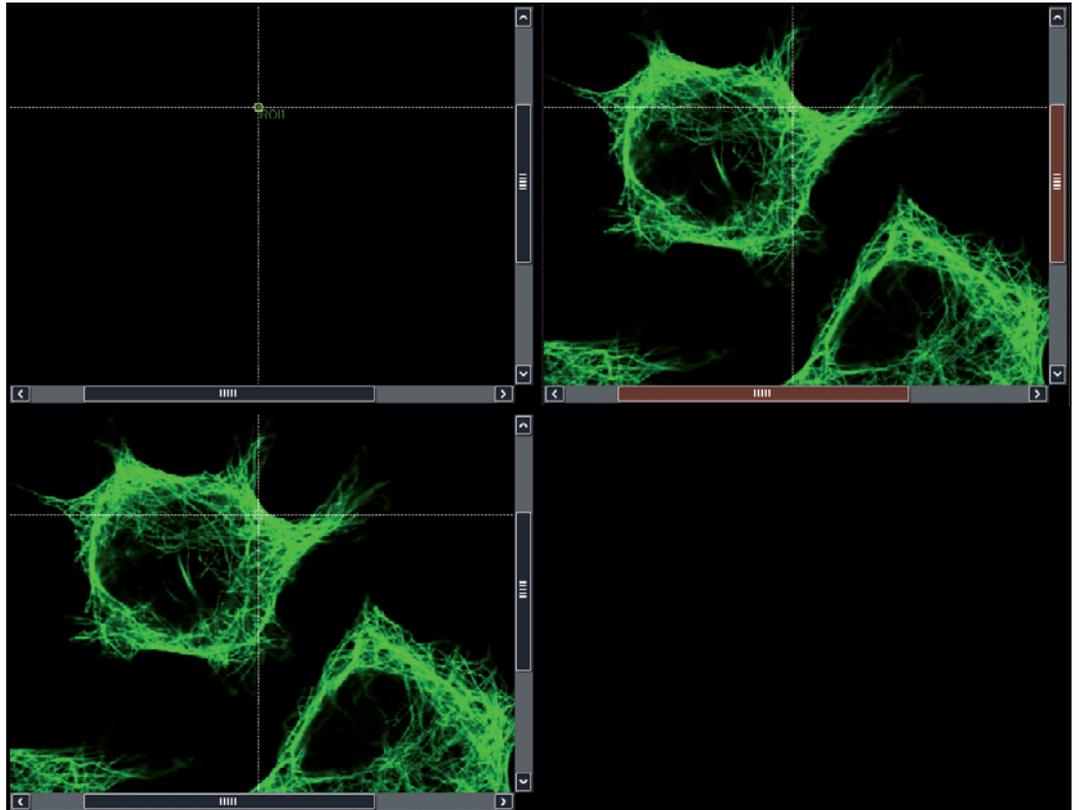
- Click **Add** **③** to define this position.



4. Select the second single-dye control image in the experiment tab ④.



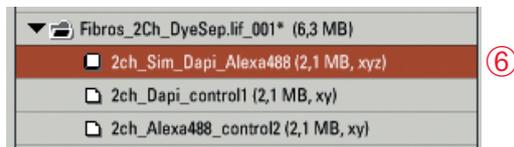
5. Place the crosshair in the viewer and again click **Add** ③ to define the second coefficient.



6. Repeat this process for all of the dyes used.

7. Choose a method of rescaling ⑤ for the resulting images (see page 12).

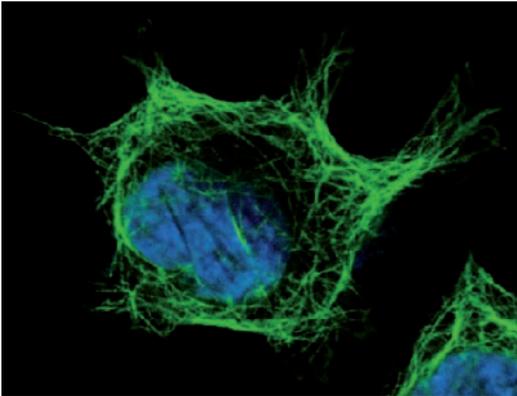
8. Select the images to be unmixed ⑥.



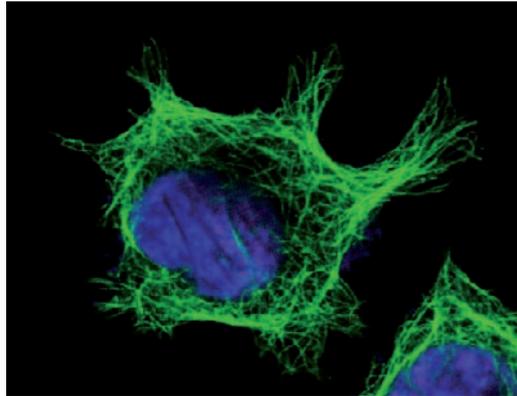
9. Click **Apply**.

Separation of two fluorophores

Original image with crosstalk



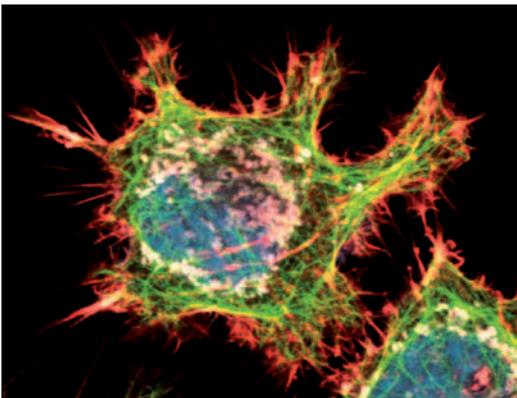
Resulting image without crosstalk



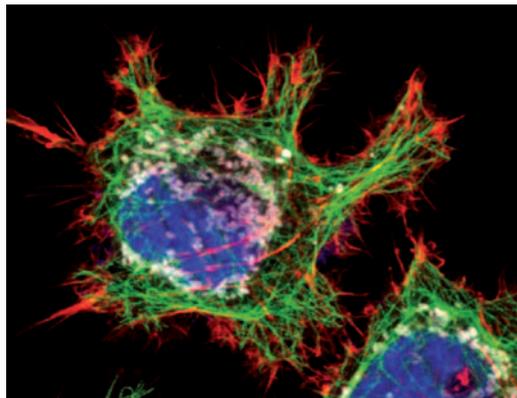
HeLa cells (fibroblasts); blue: Dapi, nucleus, green: Alexa 488, tubulin.

Separation of four fluorophores

Original image with crosstalk



Resulting image without crosstalk

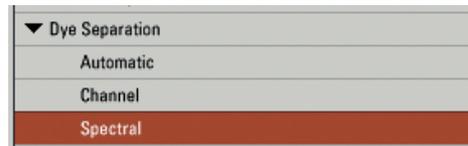


HeLa cells (fibroblasts); blue: Dapi, nucleus; green: Alexa 488, tubulin; red: TRITC phalloidin, actin; grey: Mito Tracker Red CMXRos, mitochondria.

3.2 Spectral Dye Separation: Step by Step

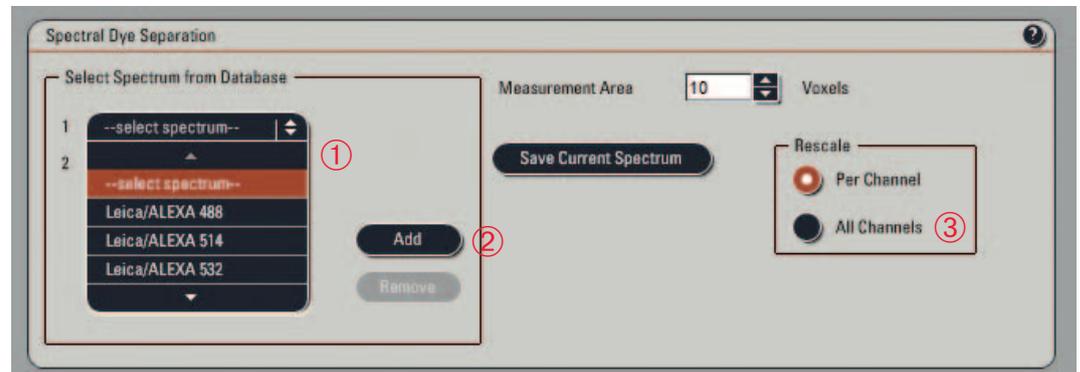
Select the **Spectral Dye Separation** tool.

You may unmix by choosing reference spectra from a spectral database (see paragraph A) or you may add your own measured dye spectra (see paragraph B).



A. Using reference spectra from a database

1. Select the corresponding fluorophore from the database ①.
If your image contains multiple fluorophores click **Add** ② to choose additional spectra.



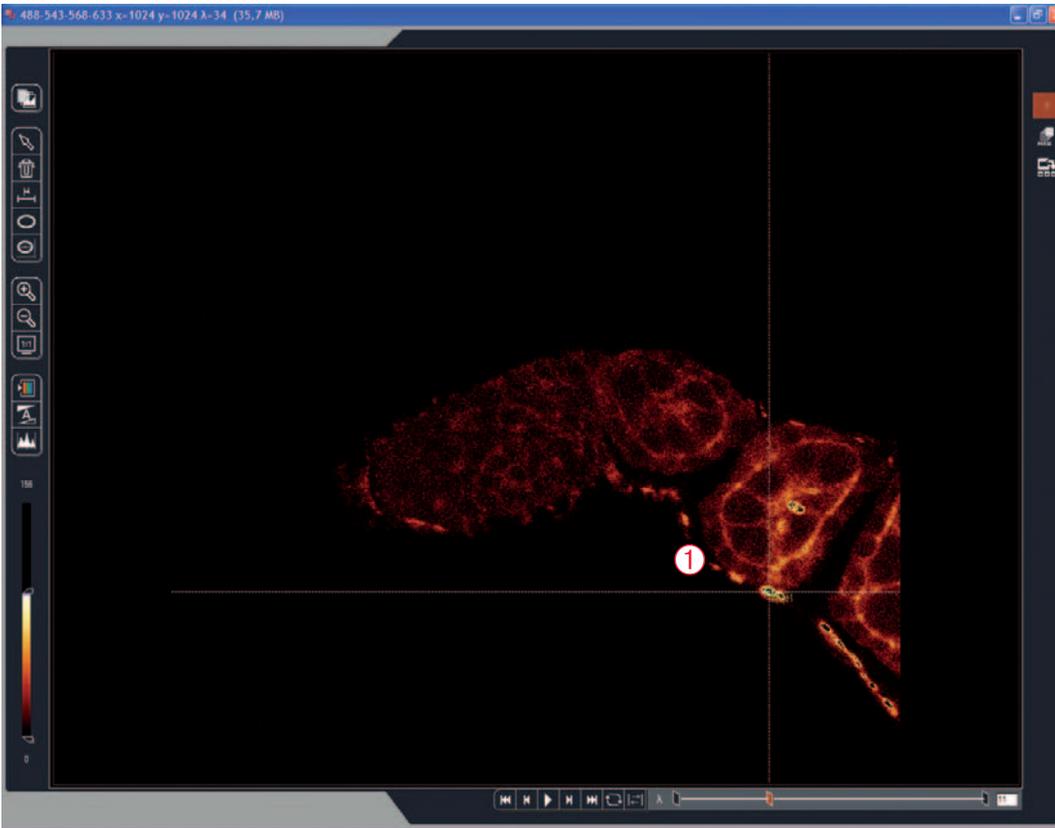
2. Choose a method of rescaling ③ (see **Channel Dye Separation**, page 12).

3. Click **Apply**.

B. Using measured reference spectra

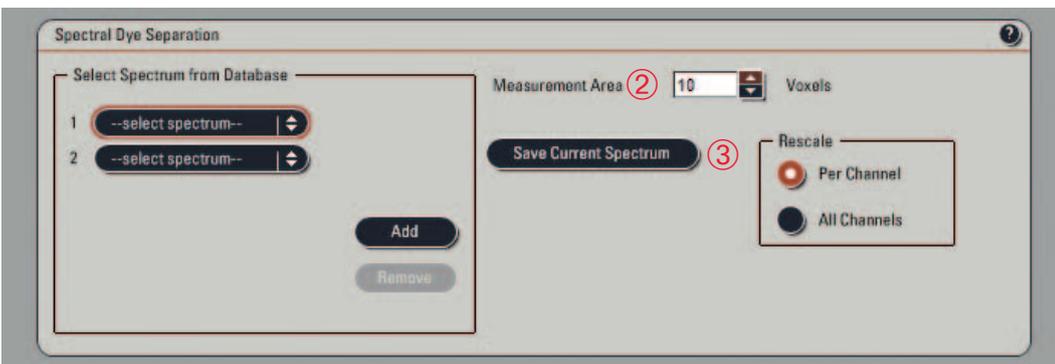
1. Place the crosshair ① in a region of your choice. You can also draw a ROI manually.

Note: If there is no crosshair visible in the viewer you may activate it by clicking on the Crosshair button .

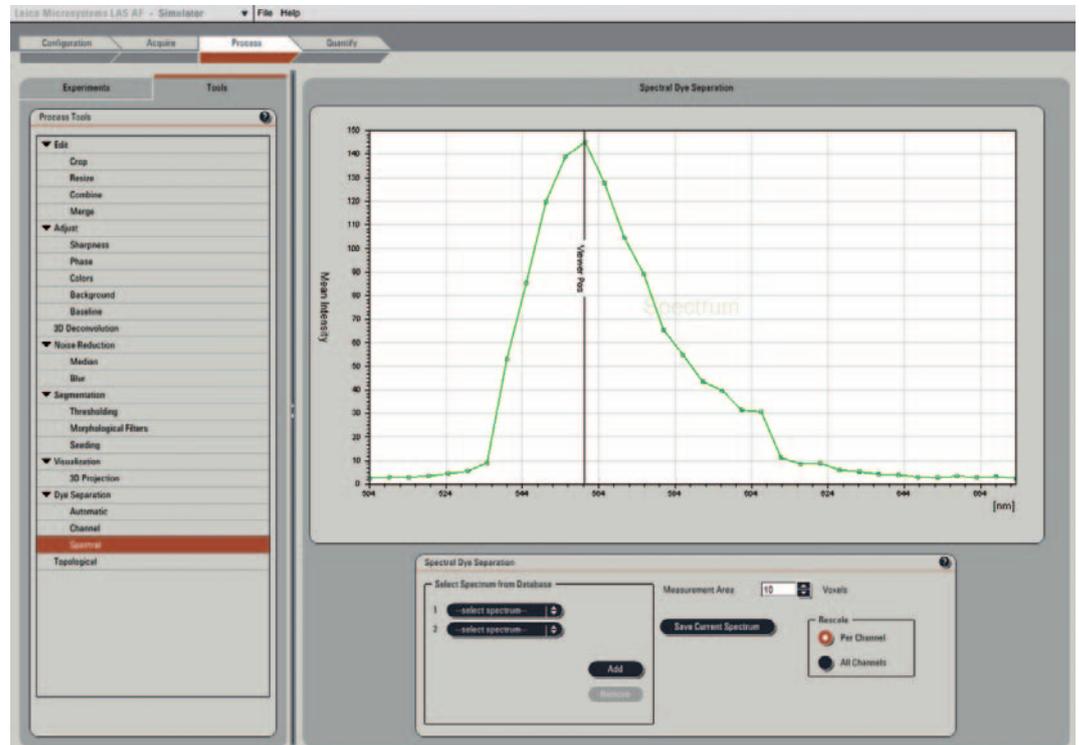


2. In the field **Measurement Area** ② you may alter the size of the reference region (in voxels).

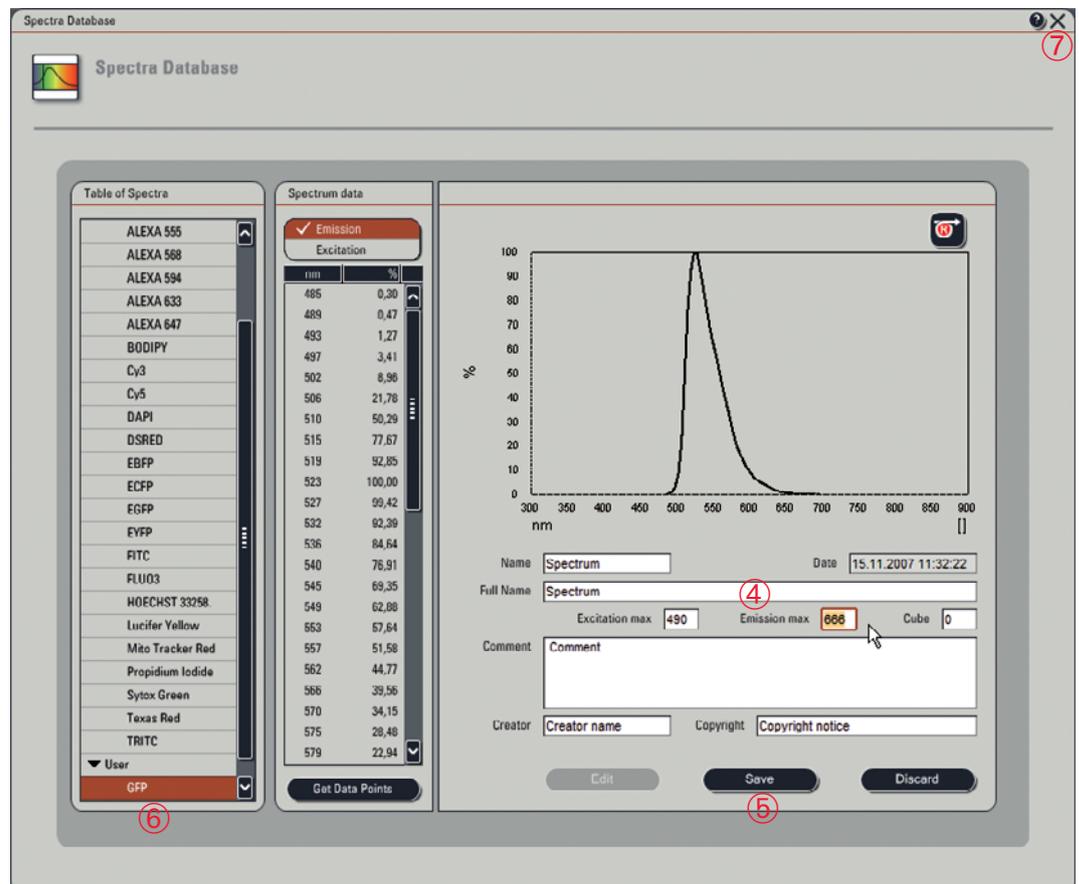
3. Click **Save Current Spectrum** ③ to add the actual spectrum to the **Spectra Database**.



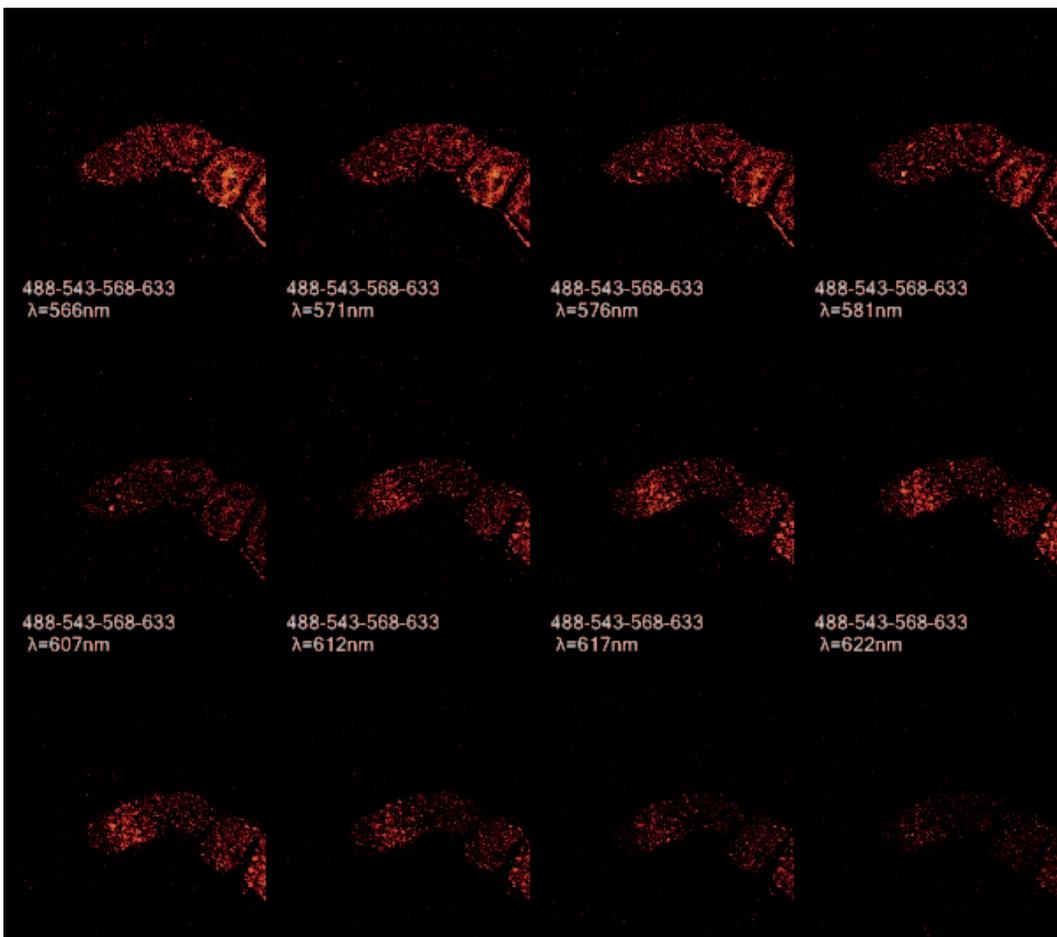
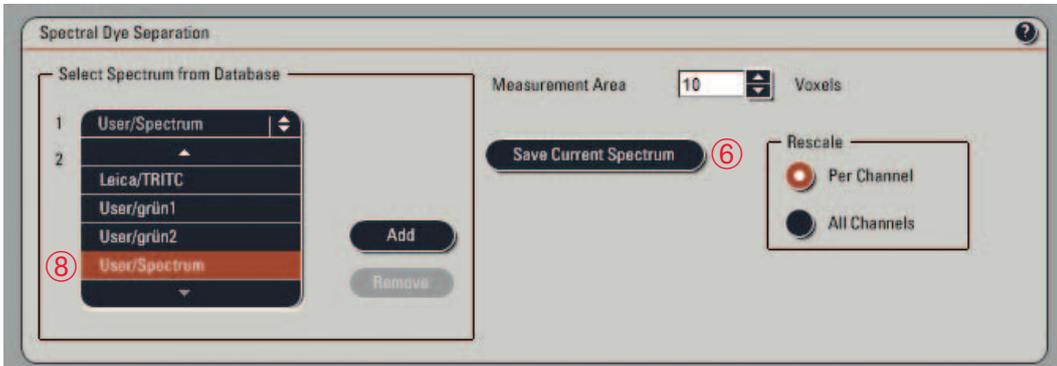
A dialog box will open automatically.



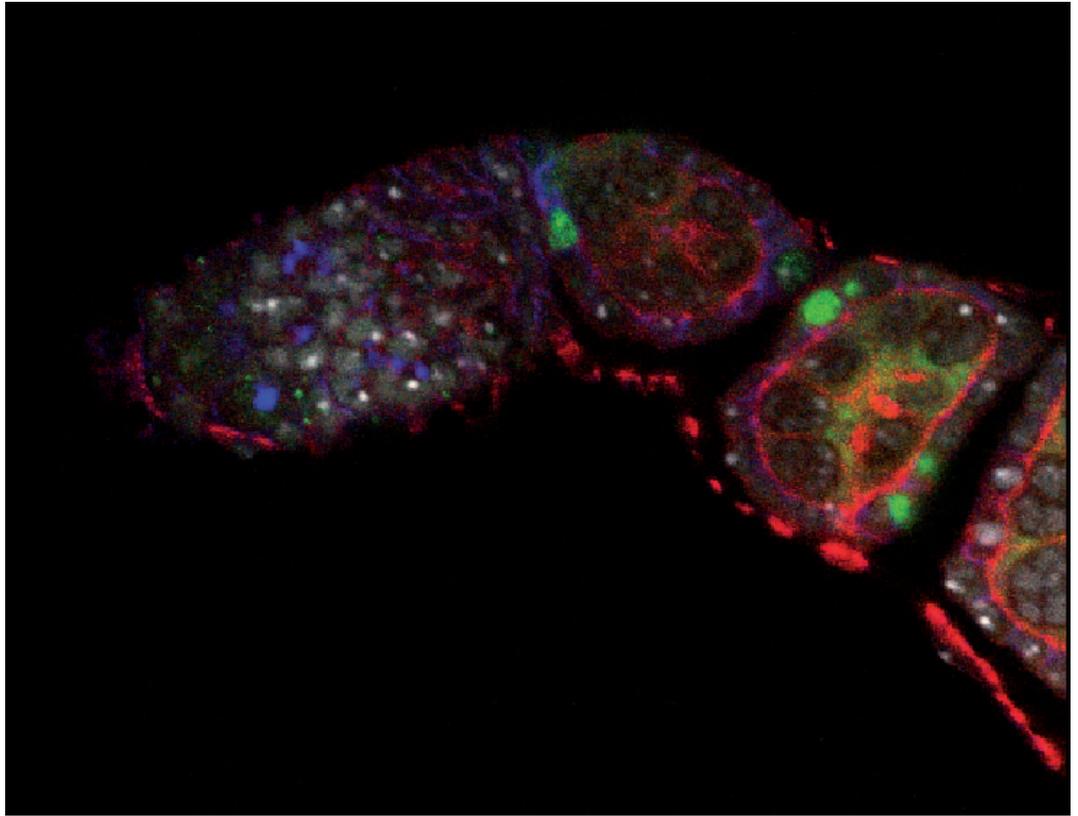
The measured emission spectrum of the fluorophore is displayed and can be saved in the spectra database.



4. Fill in the fields ④ accordingly. By clicking on **Save** ⑤ the spectrum is saved under **User** in the **Table of spectra** ⑥.
5. Press **X** ⑦ to go back to the **Spectral Dye Separation** dialog. The saved spectrum is now available in the list under **User** ⑧. You may continue as described under paragraph A.



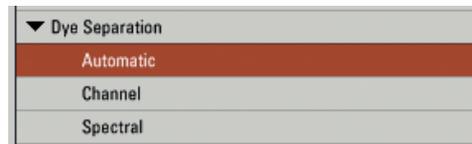
Part of the image gallery of a lambda series of *Drosophila melanogaster* stained with Alexa 488, Alexa 546, Alexa 568 and TOTO-3.

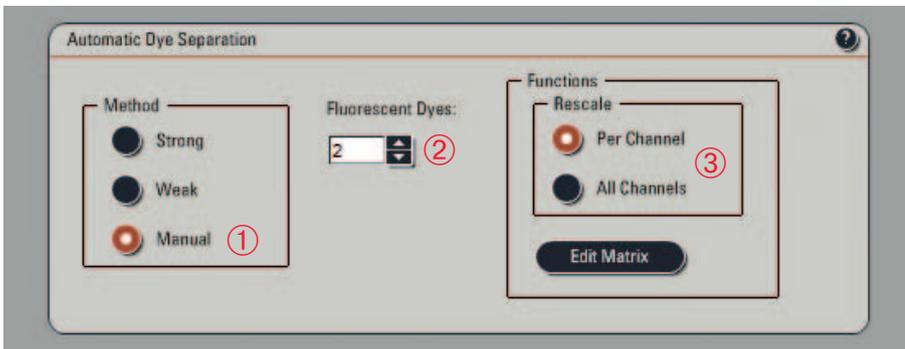


Fluorescence signals are separated after processing the spectral data with the Spectral Dye Separation tool.
Courtesy of Dr. Ralf Pflanz, MPI Biophysical Chemistry, Göttingen

3.3 Manual Dye Separation

Clicking on **Automatic** opens a dialog box, where you can choose the settings for either the **Manual Dye Separation** or the **Automatic Dye Separation** methods. Choosing **Manual** opens a new dialog window.

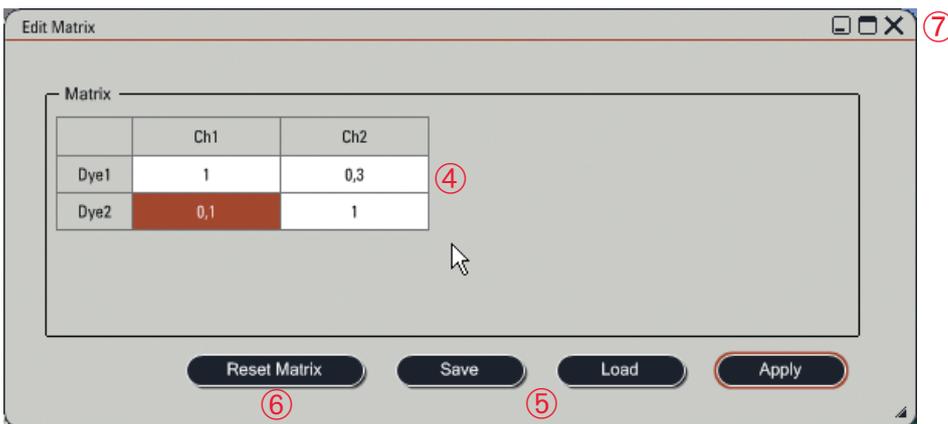




1. Select the **Manual** ① method in the **Automatic Dye Separation** window. A new dialog box opens that will allow you to unmix the crosstalk of one channel from the other manually.

Note: The field **Fluorescent Dyes** ② reflects the number of dyes used during image acquisition. LAS AF recognizes this and automatically displays the number of channels.

2. Choose a method of rescaling ③ the resulting images or series (see **Channel Dye Separation**, page 12).



3. Type the desired coefficients into the matrix fields ④. In this example we correct $\frac{1}{3}$ cross-talk from the green dye in the red channel and $\frac{1}{10}$ cross-talk from the red dye in the green channel.

The matrix can be saved and reloaded ⑤ for reproducibility. Keep in mind that in order to use the same contribution coefficients in the matrix, identical recordings using the same parameters must be taken.

The **Reset Matrix** ⑥ button allows you to go back to the default settings.

4. Close the **Edit Matrix** dialog **7** (see page 21) and click **Apply** **8**.



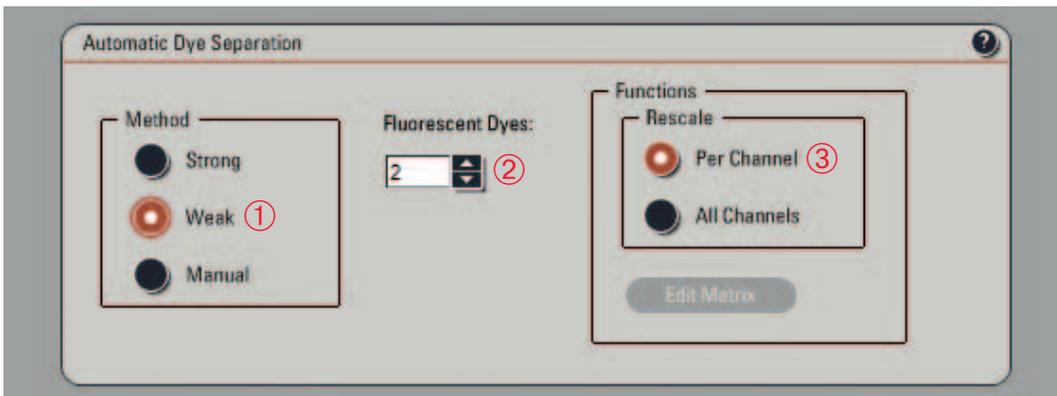
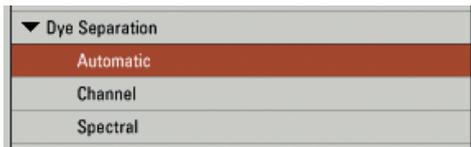
Note: 1. The **Edit Matrix** button is active only if you have selected the **Manual** option under **Method**.
2. Lambda scans cannot be processed with the **Automatic Dye Separation** tool.

A screenshot of a software interface. On the left is a cytofluorogram showing a dense cloud of points with a color gradient from red to green. On the right is a 'Parameters' control panel. It contains four sliders: 'Threshold Ch 1' at 35%, 'Threshold Ch 2' at 5%, 'Background Ch 1' at 20%, and 'Background Ch 2' at 20%. There is a checked checkbox for 'Link Background Ch 1 and Ch 2'. Below these is a 'Visualization' section with four radio buttons: 'Overlay' (selected), 'Colocalization Only (Black & White)', 'Colocalization Only (Color)', and 'Off'.

If the Colocalization tool is available on your system, you can use the cytofluorogram to determine the coefficients. By defining the threshold for both channels you can obtain a best-fit line for the clouds. In this example the coefficients are 0.35 for channel 1 and 0.05 for channel 2 (see explanation under **Automatic Dye Separation: Weak and Strong**, page 6ff)

3.4 Automatic Dye Separation: Weak and Strong

Choose the Automatic Dye Separation tool.



1. Select between method **Weak** or **Strong** ①.

Note: The field **Fluorescent Dyes** ② reflects the number of dyes used during image acquisition. LAS AF recognizes this and automatically displays the number of channels.

2. Choose a method of rescaling ③ the resulting images or series (see **Channel Dye Separation**, page 12).



3. **Apply** ④ transfers the unmixed data file to **Experiments**.

To preview the changes, press the **Preview** ⑤ button. The **Reset** ⑥ function allows you to go back to the default settings.

References:

F. Olschewski, Living Colors, Excellent Solutions for Live Cell Imaging, G.I.T. Imaging & Microscopy 2/2002

T. Zimmermann, Spectral Imaging and Linear Unmixing in Light Microscopy, Adv Biochem Eng Biotechnol. 2005;95:245-65

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