11.4 Widefield Multichannel Unmixing

Background

Widefield Multichannel Unmixing is a new function for the removal of crosstalk between fluorescent dyes in multichannel images with up to eight fluorescence channels using a traditional epifluorescence microscope. Crosstalk is a phenomenon which occurs, whenever dyes are excited by excitation light of more than one filter combination. This problem occurs for example, when dyes with broadly overlapping excitation spectra are used concurrently in one sample. Good examples are the spectral variants of fluorescent proteins, e.g. BFP, CFP, GFP and YFP. With traditional reflector filter sets it has been often difficult to achieve 100% signal separation for such dyes.

In essence this means, that a certain proportion of a signal in one channel is actually derived from another dye spilling over into the channel.

A parameter based separation is now available with the **Widefield Multichannel Unmixing** module. The procedure is separated into three steps:

- Measurement of the amount of crosstalk in 2 alternative procedures:
  - With appropriate reference samples
  - by automatic component extraction (ACE) from the sample to be unmixed
- Creation of the unmixing matrix file
- Unmixing of the image to be corrected by means of using the unmixing matrix file generated in step 2

NOTES:

- Non-fluorescent channels such as DIC or Phase contrast are automatically detected and disregarded by all unmixing functions. Such channels are automatically copied into the unmixed result images to facilitate merged views with fluorescent channels.

- The module Widefield Multichannel Unmixing is designed to work with images created using the AxioVision Multichannel Fluorescence module. For the creation of correct reference images the use of the AxioVision Multichannel module is required.

- The images used in these examples you will find on the AxioVision installation CD in the folder "Images".

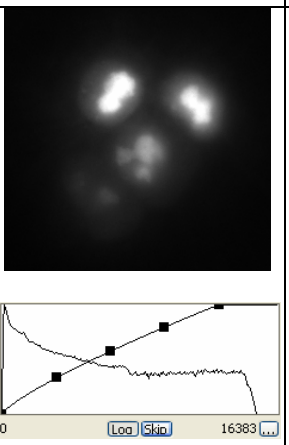
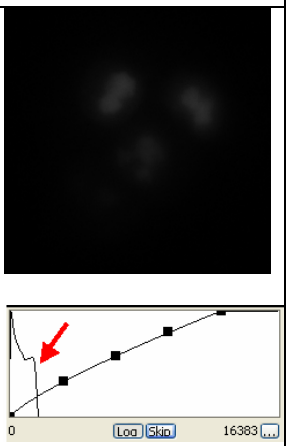
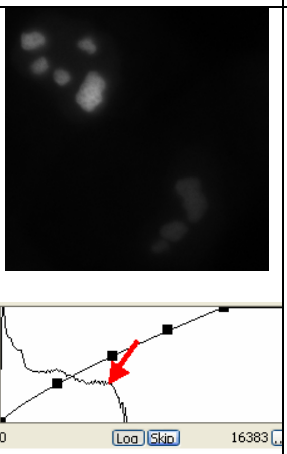
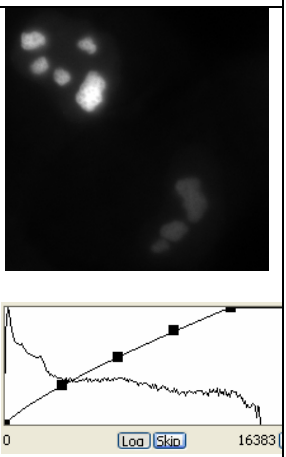
The Principle of unmixing

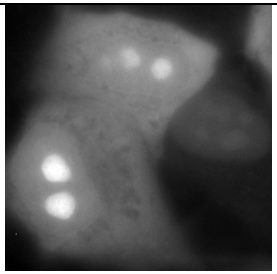
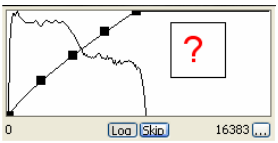
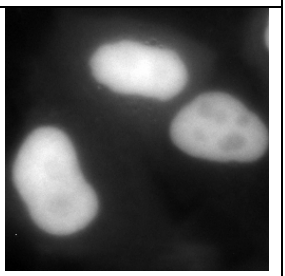
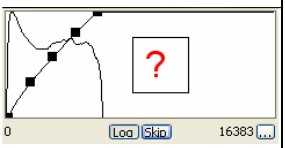
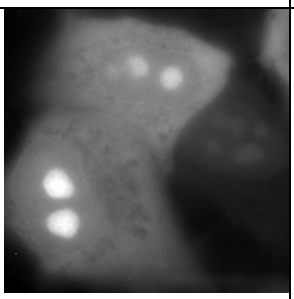
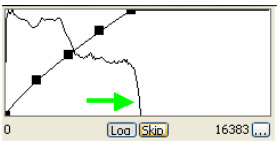
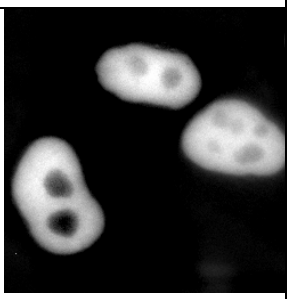
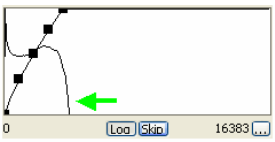
The following example tries to demonstrate the problem of cross talk as well as show the potential of unmixing such images. In this case, determination of cross talk is done using reference samples and not ACE.

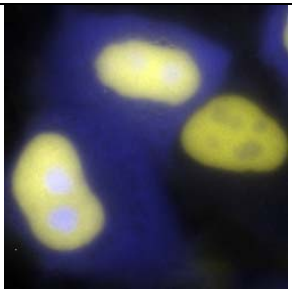
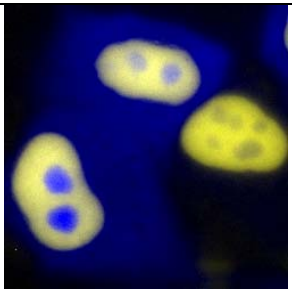
Reference sample A (CFP-Reference1.zvi): HeLa cells containing a virus proteins coupled to cyan fluorescent protein (CFP). This protein accumulates especially in the nucleolar regions of the cell nucleus.

Reference sample B (GFP-Reference1.zvi): HeLa cells containing a virus proteins coupled to green fluorescent protein (GFP). This protein accumulates in the nucleolar regions of the cell nucleus too.

Sample C: (RevCFP-H2-GFP_3.zvi): HeLa cells containing two proteins with different fluorescence: coupled to CFP is the same viral protein as in reference sample A (nucleoli); coupled to GFP is a histone protein, which stains the chromosomes and thus the entire nucleus a lesser degree of staining of the nucleoli.

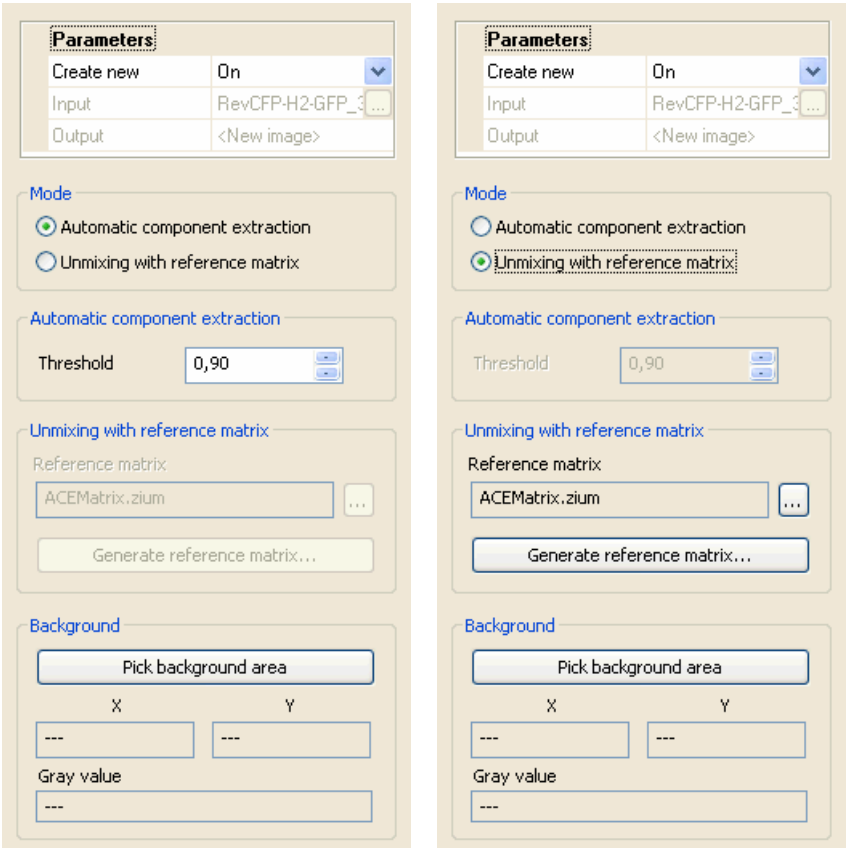
Sample	Channel 1(CFP)	Channel 2 (GFP)
A. Reference sample **CFP only**: A small degree of crosstalk is visible in channel 2 coming from channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		
B. Reference sample **GFP only**: A moderate degree of cross talk from channel 2 is visible in channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.		

Sample	Channel 1(CFP)	Channel 2 (GFP)
C. 2-channel fluorescence sample (CFP and GFP): Before Unmixing: How much signal from channel 2 is visible in channel 1 (cross talk) and vice versa? **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.	 	 
D. 2-channel fluorescence sample (CFP and GFP): After Unmixing: A net percentage of pixel intensity values from channel 2 are added to the signal in channel 1. **Image histogram:** x-axis: pixel gray values. y-axis: relative number of pixels in logarithmic scale; identical settings of the display curve.	 	 

	CFP&GFP-sample original pseudocolor mode	CFP&GFP-sample unmixed pseudocolor mode
E. 2-channel fluorescence sample (CFP and GFP): Comparison "before" and "after" unmixing: shows a markedly improved signal separation (shown at identical display settings.		

The Widefield Multichannel Unmixing function

The **Widefield Multichannel Unmixing** function is the central function for unmixing samples:



Using the function dialog, you can select whether the unmixing should take place via "automatic component extraction" (left-hand image) or using a reference matrix (right-hand image). If you choose unmixing using a reference matrix, you will need a reference matrix file containing the corresponding information for the unmixing.

If such a file does not yet exist, you can start a wizard via the 

button, which will lead you through the process of generating a reference matrix file.

Working with the basic functions

The basic functions in the **Widefield Multichannel Unmixing ⇒ Basic functions** menu are the functions for unmixing that will already be familiar to you from the previous version of AxioVision. These functions are still available, for compatibility reasons and for use in Commander scripts. The following two examples describe how you can use these functions for unmixing.

Unmixing with reference samples

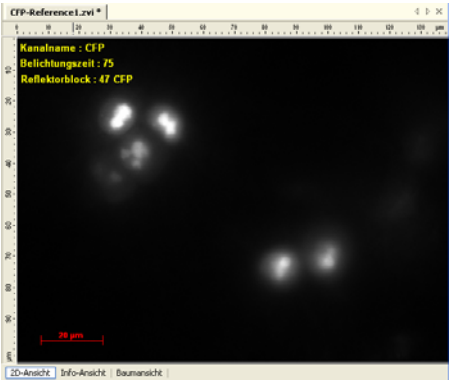
This example shows you how to measure cross talk using reference samples and use this information to unmix a 2 channel fluorescence image.

The same images are used as in example 1.

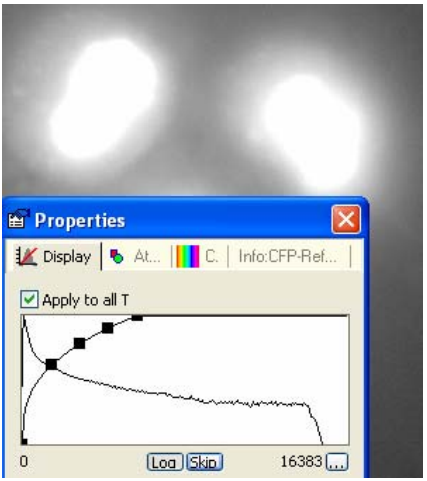
- Open the first reference image "CFP-Reference1.zvi". It is a 3 channel image. The first channel contains transmitted light information (DIC). The CFP signal is visible strongest in channel 2 (Zeiss filter set #47). Change into b/w mode (

) and channel 

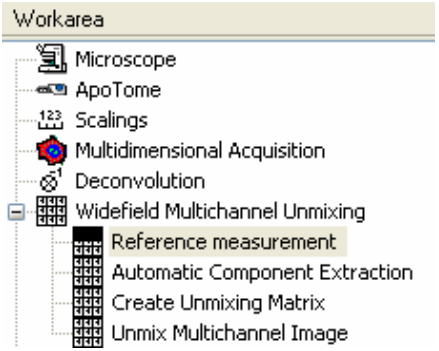
.



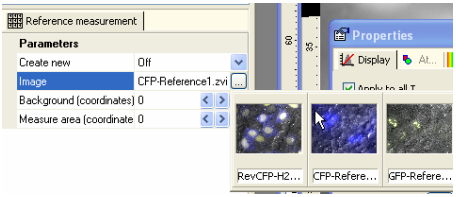
- Open the properties dialog (menu **View ⇒ Properties**) and adjust the display line in a way to enhance the gray value visibility (e.g. **Best Fit** and **Gamma value** ~ 0.5). Now it is easier to distinguish true image background from signal derived from the fluorescent dye.



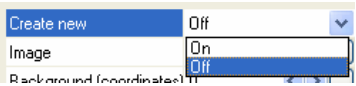
- In the workarea select Widefield Multichannel Unmixing and then **Reference measurement**.



- The image "CFP-Reference1.zvi" is selected automatically as **Input** image because it the active image in the foreground.

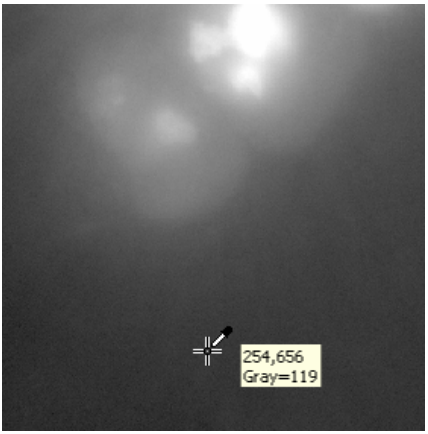


- If it is not selected, switch the **Create new** function to **Off**



and select the image from the pop up gallery.

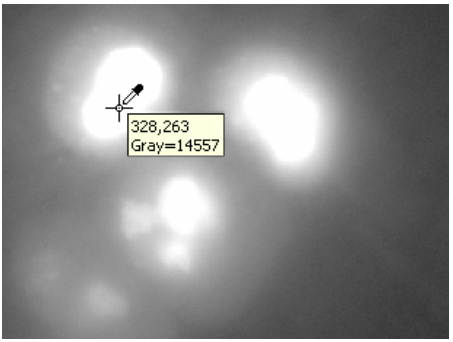
- In the edit field Background (coordinates) click on the ... button.



- Select a suitable region in the image for background correction (ideally without any signal coming from the dye). The pixel coordinates (x/y) as well as the gray value of the selected pixel are shown.

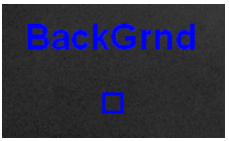
- Click at an suitable position once in the image. The x coordinate is shown in the input field.

- Click the ... button in the input field Measure area (coordinates).



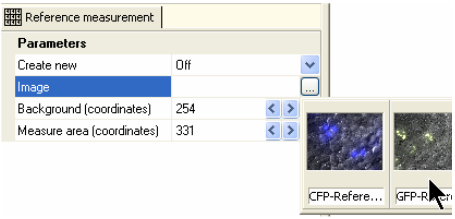
- Search for a suitable region in the image for measurement. Ideally search for a region with an intensity as high as possible.

- Click the Start button to carry out measurement.



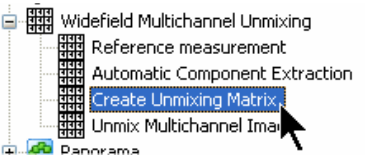
- The regions taken for measurement are shown in the image. Save the image, because it's used later for generation of the unmixing matrix.

- Open the second reference image **GFP-Reference1.zvi**. The third channel contains the GFP fluorescence (fluorescence filter set # 44).

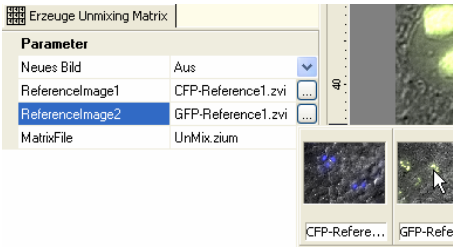


- Repeat the steps 2-7 also for this image.

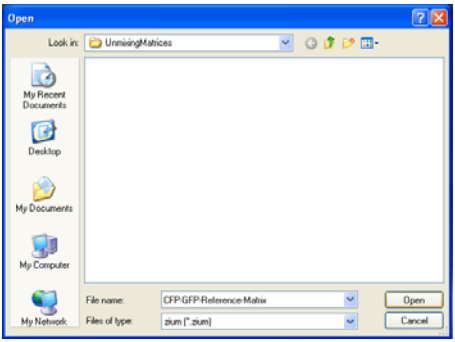
- In the workarea select the function **Create Unmixing Matrix** from the **Widefield Multichannel Unmixing** function group.



- 10. Select the input images accordingly: CFP-Reference1 as **ReferenceImage1**, GFP-Reference1.zvi as **ReferenceImage2**. Please take care to enter channels and reference images in the same order. If you use images with more than two channels, the function is extended accordingly.

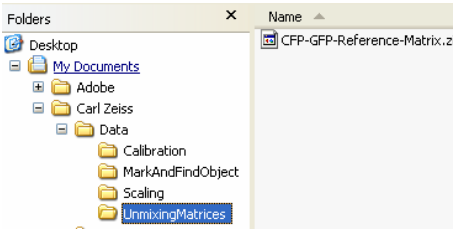


- 11. To define a name for the unmixing matrix file, click the ... button in the input field MatrixFile. In the following dialog you can enter a name for the matrix file.

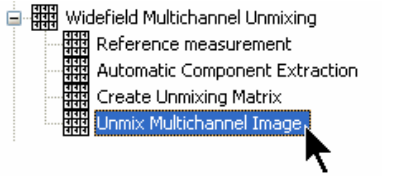


- Click the Open button to accept the filename.

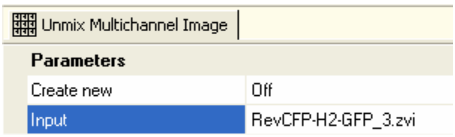
- Click the Start button to generate the unmixing matrix file. The file is saved – like other AxioVision files – in the user folder.



- In the workarea select the function **Unmix Multichannel Image** from the **Widefield Multichannel Unmixing** function group.



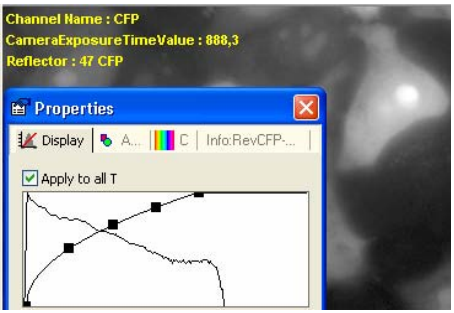
- Open the image to unmix and select it as **Input** image. In this example, the image "RevCFP-H2-GFP_3.zvi" is used.



- Switch to the black and white view (

) and activate channel 2 (

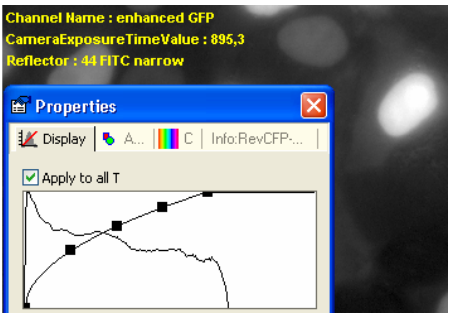
).



- Right click in the image and select **Properties**. Set the display characteristic line in such a way, that the gray values are displayed extremely amplified. So you can easily detect, where the background is not caused by the sample.

- Switch to channel 3 

and repeat the setting of the display characteristic line like in previous step.



- Switch back to the color view and switch off channel 1 

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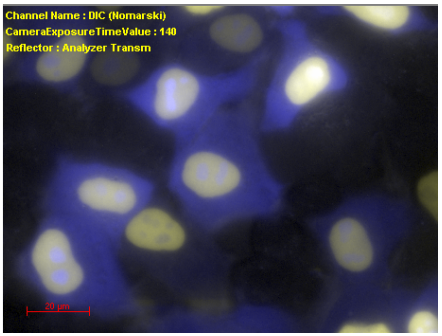
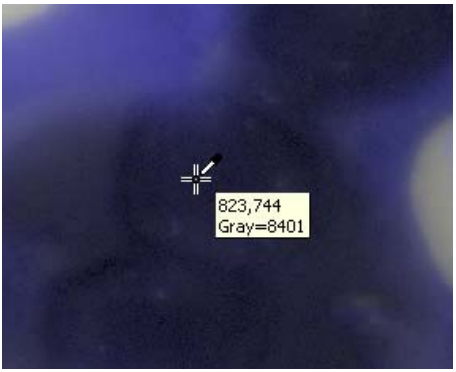
- Now you can separate the two fluorescence colors clearly from the background signal.



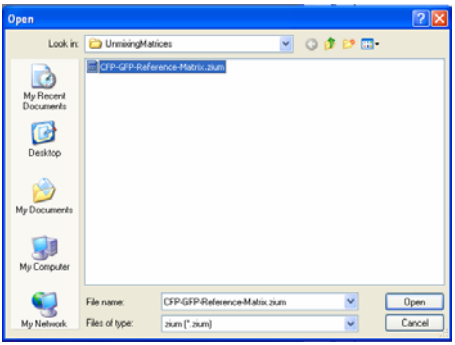
- Click the … button in the input field Background (coordinates). In the image search for a suitable region for the background measurement (preferably without a signal caused by the fluorescence dye).



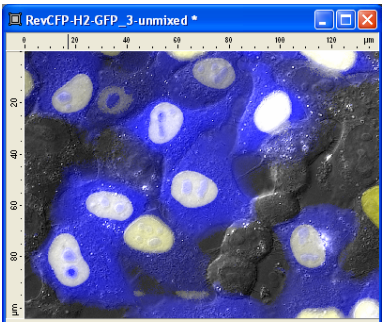
- The pixel coordinates (x/y) as well as the gray value of the selected pixel are shown. Click at an suitable position once in the image. The x coordinate is shown in the input field.

- Enter a name for the **Output** image.

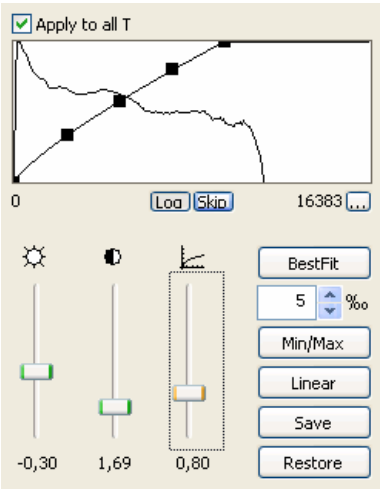
- Click the … button in the input field MatrixFile to select the unmixing matrix file. Click the Open button to load the file. The dialog is closed.



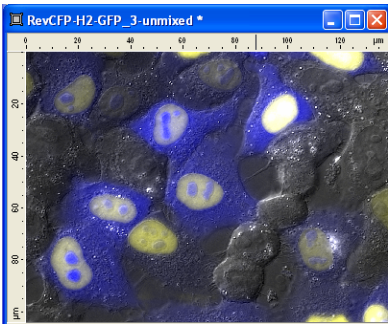
- Click the Start button to start the **unmixing**. The result image shows a clear separation of the fluorescence channels 2 (CFP) and 3 (GFP). Channel 1 without fluorescence (DIC) is copied to the output images unchanged.



- To optimize the display, you should set the display characteristic line for channel 2 and 3 accordingly:



- Save the output image.

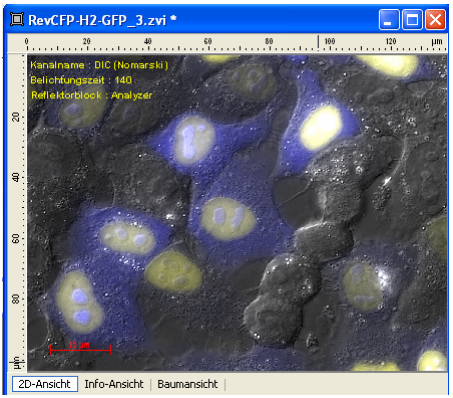


Unmixing of a multichannel image using ACE

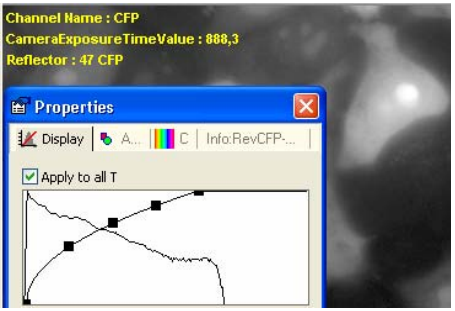
This example shows you how to measure cross talk directly an image for unmixing with Automatic Component Extraction (ACE) and how to use it for unmixing of samples.

The same images are used as in example 1.

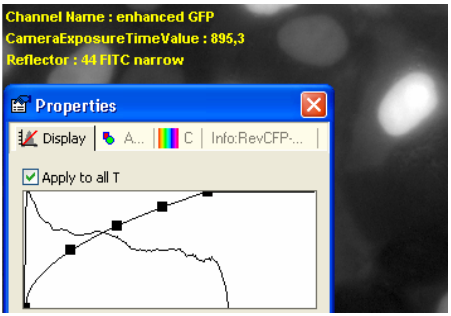
- 1. Open the image to unmix. In this example the image "RevCFP-H2-GFP_3.zvi" is used.



- Switch to the black and white view (Off) and activate channel 2 (2). Right click in the image and select **Properties**. Set the display characteristic line in such a way, that the gray values are displayed extremely amplified. So you can easily detect, where the background is not caused by the sample.

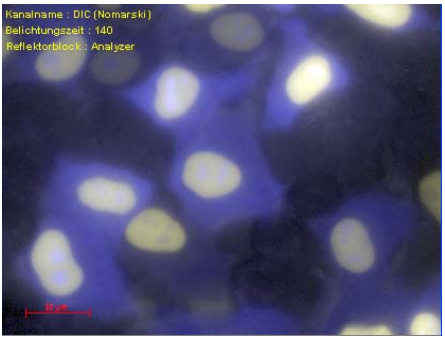


- Switch to channel 3  and repeat the setting of the display characteristic line like in previous step.



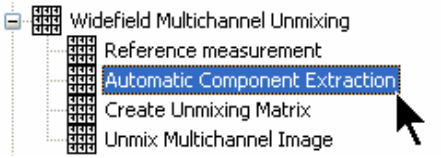
- Switch back to the color view and switch off channel 1

.

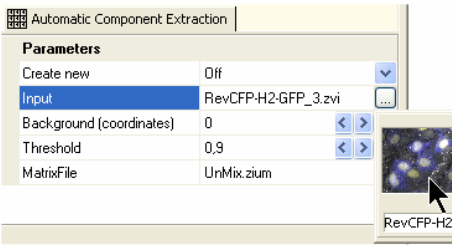


- Now you can separate the two fluorescence colors clearly from the background signal.

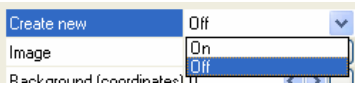
- In the workarea select the function **Automatic Component Extraction** from the **Widefield Multichannel Unmixing** function group.



- The reference image "RevCFP-H2-GFP_3.zvi" is selected automatically as Input image because it the active image in the foreground.



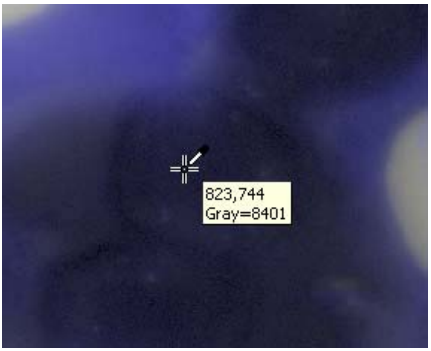
- If it is not selected, switch the **Create new** function to Off



and select the image from the pop up gallery.

- Click the  button in the input field 

. In the image search for a suitable region for the background measurement (preferably without a signal caused by the fluorescence dye). The pixel coordinates (x/y) as well as the gray value of the selected pixel are shown.

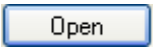


- Click at an suitable position once in the image. The x coordinate is shown in the input field.

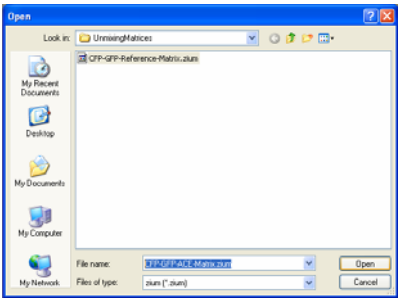
- The predefined threshold in the input field **Threshold** can be used unchanged 

. For further information about this parameter, please read in the online help.

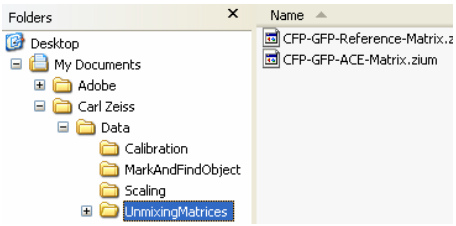
- To define a name for the unmixing matrix file, click the  button in the input field 

. In the following dialog you can enter a name for the matrix file. Click the 

button to accept the filename.



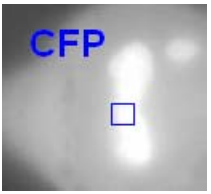
- Click the Start button to generate the unmixing matrix file. The file is saved – like other AxioVision files – in the user folder.



- The measurement regions defined by ACE are drawn to the image using the channel color.

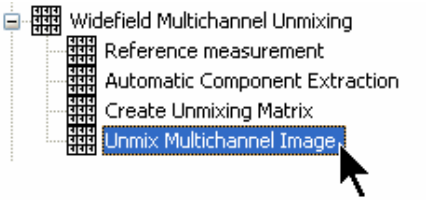
- Switch to the black and white view (Off) and activate the accordant channel.

Channel 2:

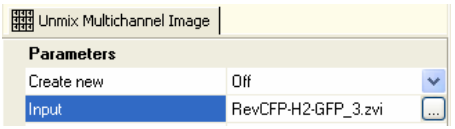


Channel 3:

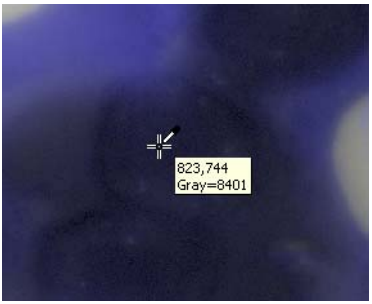
- In the workarea select the function **Unmix Multichannel Image** from the **Widefield Multichannel Unmixing** function group.



- Open the image to unmix and select it as **Input** image. In this example, the image "RevCFP-H2-GFP_3.zvi" is used.

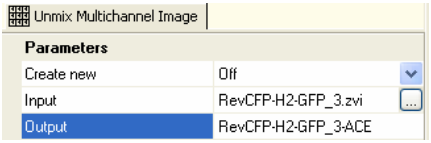


- Now repeat the background measurement:

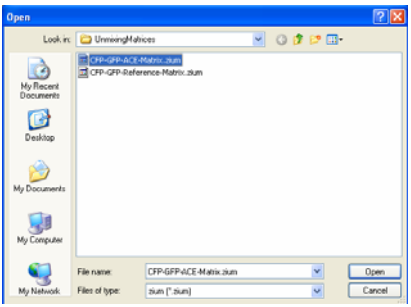


- Click the [...] button in the input field Hintergrund (Koordinaten) and then click in a background region (see also step 7).

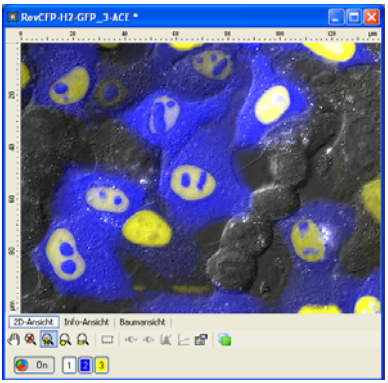
- Enter a name for the **Output** image.



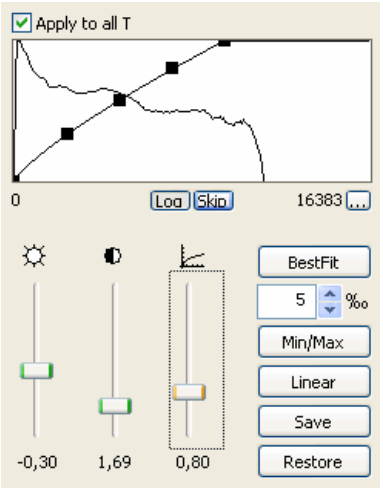
- Click the [...] button in the input field MatrixFile to select the **unmixing matrix file**. Click the Open button to load the file. The dialog is closed.



- Click the Start button to start the unmixing. The result image shows a clear separation of the fluorescence channels 2 (CFP) and 3 (GFP). Channel 1 without fluorescence (DIC) is copied to the output images unchanged.



- To optimize the display, you should set the display characteristic line for channel 2 and 3 accordingly:



- Save the output image.