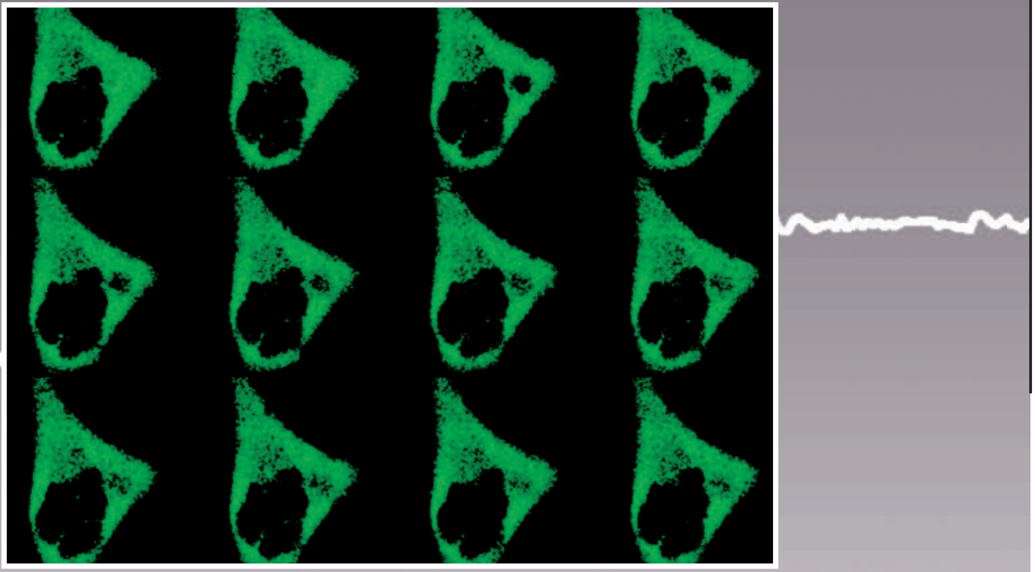
Oct. 2006
No. 21

CONFOCAL APPLICATION LETTER

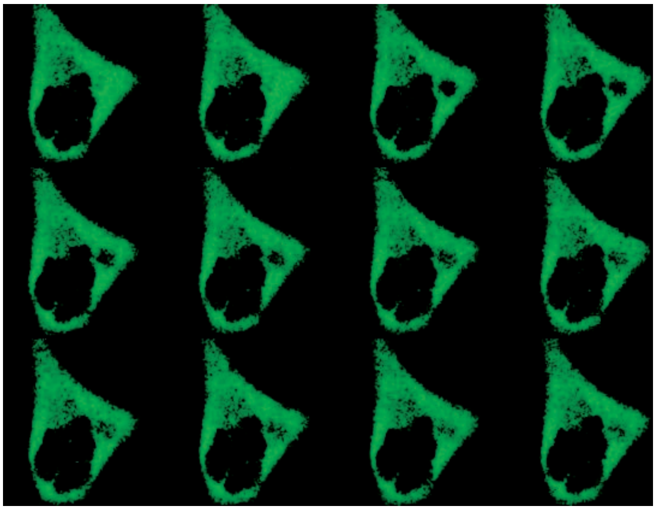
reSOLUTION



LAS AF APPLICATION WIZARD
FRAP WITH TCS SP5

MICROSYSTEMS

FRAP with TCS SP5 (LAS AF 1.5.1)



FRAP with Tubulin-GFP transformed HeLa cells.

Fluorescence recovery after photobleaching (FRAP) has been considered the most widely applied method for observing translational diffusion processes of macromolecules. The resulting information can be used to determine kinetic properties like the diffusion coefficient, mobile fraction and transport rate of the fluorescently labeled molecules. FRAP employs irradiation of a fluorophore in a living sample with a short laser pulse. Modern laser scanning microscopes like the Leica TCS SP5 have the advantage of using a high intensity laser pulse for bleaching and a low intensity laser pulse for image recording. With the LAS-AF application wizard you can choose between different ways to carry out a FRAP experiment. You are able to adapt timing parameters for various experiments, e.g. moderate, fast or multi-step kinetics.

For very fast kinetics the FlyMode (pp. 9) is the best method. In this mode, reading out the signal during the x-fly back of the scanner provides a time resolution between lines instead of between frames for the FRAP experiment.

Depending on the necessary bleaching power you may choose ROI bleach or ROI with Zoom In bleach combined with one or multiple bleach steps. Free y-format will reduce scanning time during bleaching if multiple bleach intervals are needed.

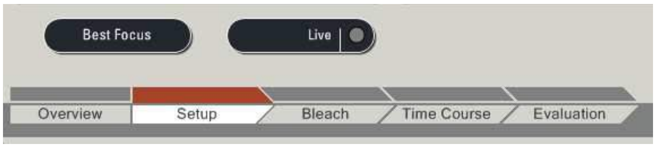
FRAP – step by step

Choose the FRAP-Wizard

First the FRAP routine without FlyMode is described. At the bottom of the wizard interface the working steps are displayed as buttons.

Step 1: Setup – Setting parameters for pre- and postbleach imaging

Click on the **Setup** button to adjust hardware parameters for pre- and postbleach imaging.



Acquisition speed

For freely diffusing molecules 1400 Hz line frequency scan speed with bidirectional scan should be used. In combination with an image format of 256 x 256 pixels one can record one image every 118 ms.

Excitation light

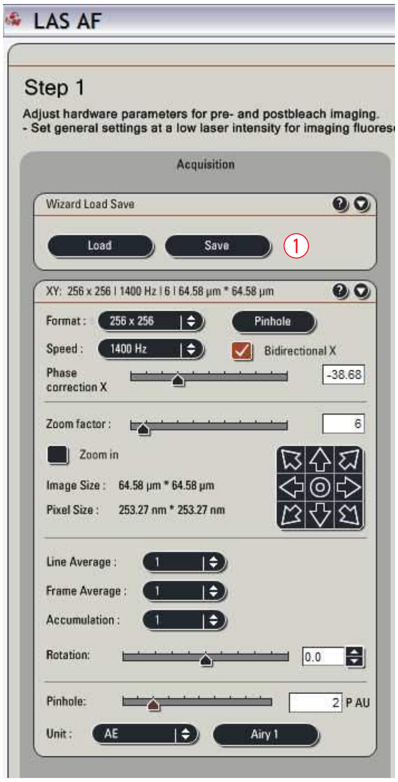
To allow highest dynamic range between monitoring and bleaching adjust Argon laser power to 100% with the configuration/laser menu. For imaging set the AOTF values at low percentage.

Pinhole size

You may set the pinhole sizes to 2 airy units if you work with thin cell layers. You will collect more information about kinetics in the depth and improve the signal-to-noise ratio.

Note:

Set the intensity below saturation and slightly above zero as setting to zero can interfere with data analysis. An appropriate lookup table (glow over/glow under) can help to adjust gain/offset. Make sure to use the same gain settings for all experiments. For reproducibility it is recommended to save ① the settings that include all settings from the **Setup, Bleach** and **Time Course** Tabs.



Step 1

Adjust hardware parameters for pre- and postbleach imaging.
- Set general settings at a low laser intensity for imaging fluores

XY: 256 x 256 | 1400 Hz | 6 | 64.58 µm * 64.58 µm

Format : 256 x 256

Speed : 1400 Hz

☑ Bidirectional X

Phase correction X -38.68

Zoom factor : 6

Image Size : 64.58 µm * 64.58 µm

Pixel Size : 253.27 nm * 253.27 nm

Line Average : 1

Frame Average : 1

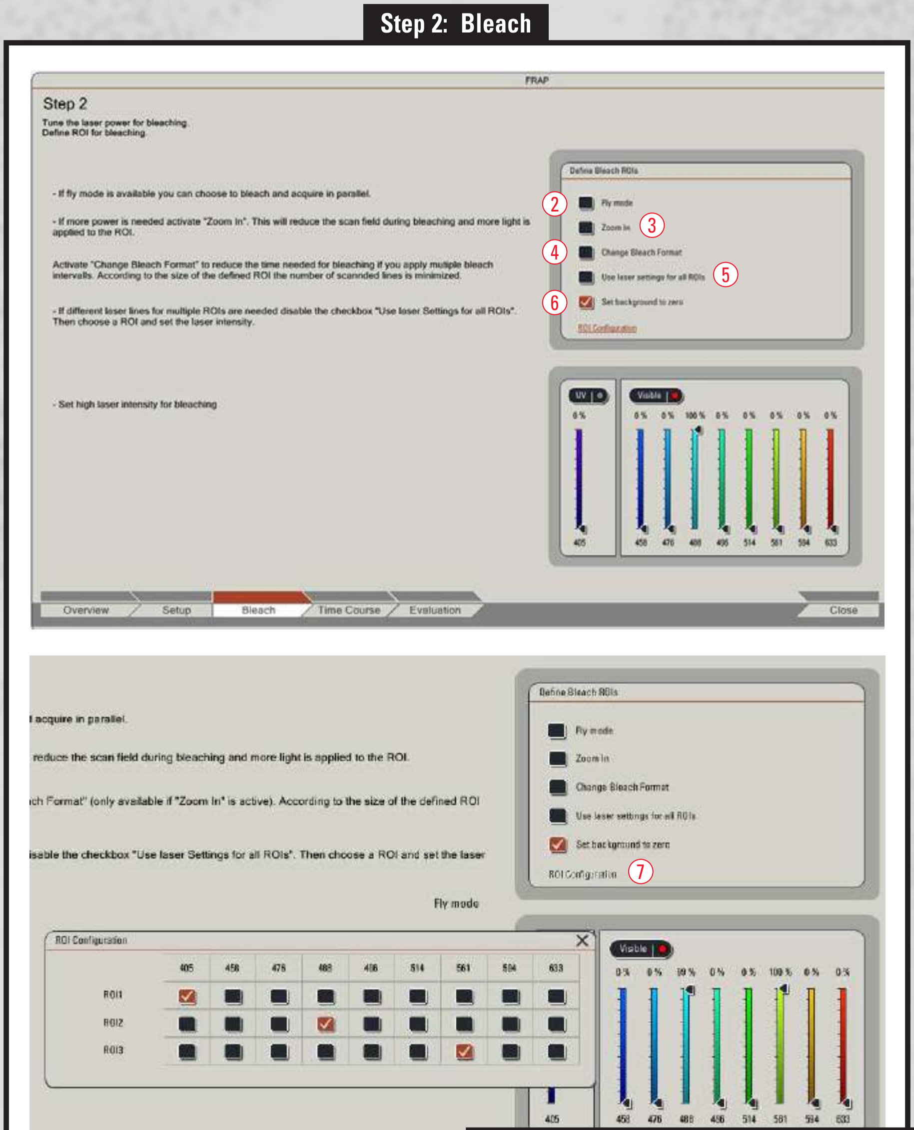
Accumulation : 1

Rotation: 0.0

Pinhole: 2 P AU

Unit : AE

Step 2: Bleach



FRAP

Step 2

Tune the laser power for bleaching.
Define ROI for bleaching.

- If fly mode is available you can choose to bleach and acquire in parallel.
- If more power is needed activate "Zoom In". This will reduce the scan field during bleaching and more light is applied to the ROI.

Activate "Change Bleach Format" to reduce the time needed for bleaching if you apply mutiple bleach intervalls. According to the size of the defined ROI the number of scannded lines is minimized.

- If different laser lines for multiple ROIs are needed disable the checkbox "Use laser Settings for all ROIs". Then choose a ROI and set the laser intensity.

- Set high laser intensity for bleaching

Define Bleach ROIs

☐ Fly mode
☐ Zoom In
☐ Change Bleach Format
☐ Use laser settings for all ROIs
☑ Set background to zero

ROI Configuration

UV 405	Visible 458	476	488	496	514	561	594	633
0 %	0 %	0 %	100 %	0 %	0 %	0 %	0 %	0 %

acquire in parallel.

reduce the scan field during bleaching and more light is applied to the ROI.

ch Format" (only available if "Zoom In" is active). According to the size of the defined ROI

isable the checkbox "Use laser Settings for all ROIs". Then choose a ROI and set the laser

Fly mode

Define Bleach ROIs

☐ Fly mode
☐ Zoom In
☐ Change Bleach Format
☐ Use laser settings for all ROIs
☑ Set background to zero

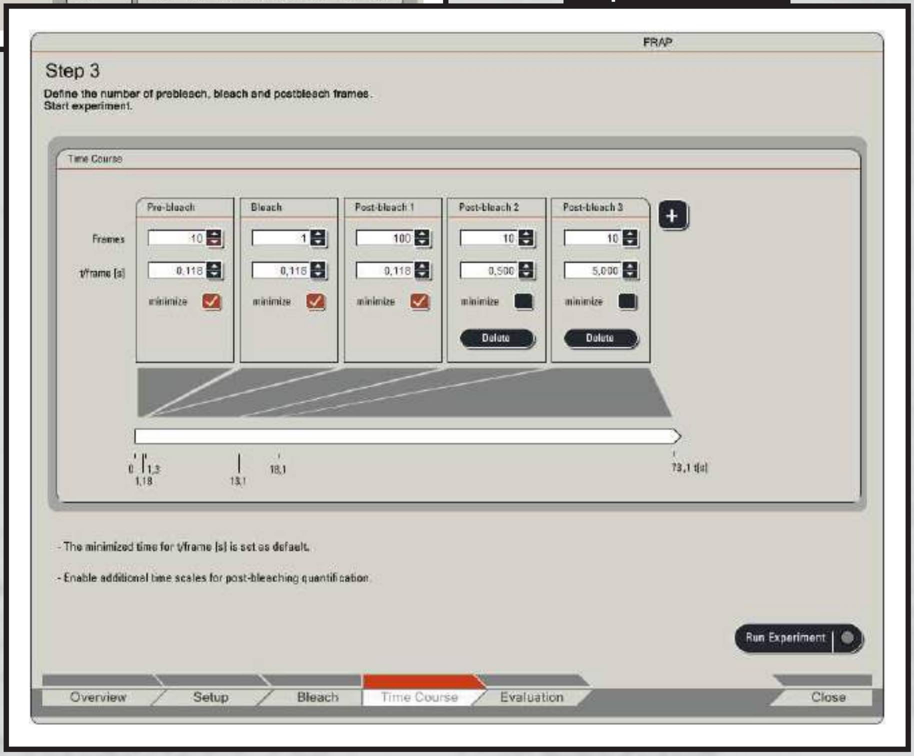
ROI Configuration

ROI Configuration

	405	458	476	488	496	514	561	594	633
ROI1	☑	☐	☐	☐	☐	☐	☐	☐	☐
ROI2	☐	☐	☐	☑	☐	☐	☐	☐	☐
ROI3	☐	☐	☐	☐	☐	☐	☑	☐	☐

405	Visible 458	476	488	496	514	561	594	633
	0 %	0 %	99 %	0 %	0 %	100 %	0 %	0 %

Step 3: Time Course



FRAP

Step 3

Define the number of prebleach, bleach and postbleach frames.
Start experiment.

Time Course

	Pre-bleach	Bleach	Post-bleach 1	Post-bleach 2	Post-bleach 3
Frames	10	1	100	10	10
t/frame [s]	0,118	0,118	0,118	0,500	5,000
minimize	☑	☑	☑	☐	☐

- The minimized time for t/frame [s] is set as default.
- Enable additional time scales for post-bleaching quantification.

Step 2: Bleach – Define parameters for bleaching

Click on the **Bleach** button to set the parameters for bleaching.

You can choose any of the following options:

FlyMode ②

Allows faster time resolution for the whole FRAP series

Zoom In ③

For most bleaching applications we recommend the **Zoom In** option. This reduces the scan field during bleaching and more light is applied to the ROI.

Set background to zero ⑥

This option is recommended when **Zoom In** is active. Thus the background outside the exposed ROI is not bleached

Change Bleach Format ④

According to the size of the defined ROIs the number of scanned lines is reduced during bleaching (strip scan). You may use this option to speed up the bleaching when multiple bleach intervals, e.g. 10 or more, are needed. This option can be combined with **Zoom In**.

Use laser settings for all ROIs ⑤

When several ROIs should be exposed with the same laser lines one can choose this option.

Now draw the ROI for bleaching and define the AOTF value(s) to tune the laser power for bleaching.

Bleaching with several laser lines and several ROIs

Click on **ROI Configuration** ⑦ in the Define **Bleach ROIs** box when individual laser lines should be active for several ROIs.

Photo-activation

You can use the FRAP wizard for photo-activation as well. Open the UV-shutter and use the 405 laser line instead of the 488 laser line.

Preconditions for effective bleaching in resonant scanning mode

If very fast scan modes are needed (e.g. measurement of diffusion in aqueous media) you may scan bidirectional in 512 x 128 format which results in a very short time per frame, e.g. 18 ms. Here it is recommended to apply multiple bleach frames, e.g. three or four to apply sufficient light for bleaching.

Furthermore the resonant scanner needs some time for settling if the zoom value is changed. Therefore the zoom option that is applied only for bleaching is not available in step two for the resonant scanning mode. However, one can define a higher zoom value for the complete FRAP series in step one.

Step 3: Time Course – Define number of prebleach, bleach and postbleach intervals

Next, choose **Time Course** to define number of prebleach, bleach and postbleach intervals.

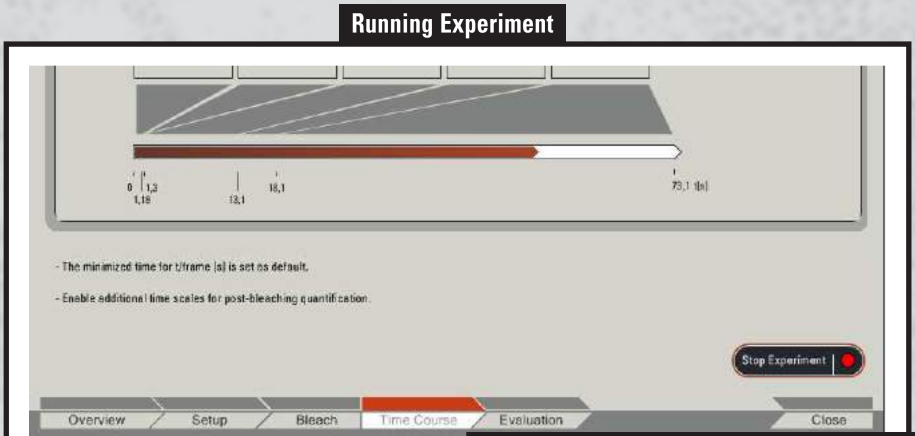
A typical experiment with 1400 Hz scan speed (bidirectional scan) and 256 x 256 format can be defined as follows:

Prebleach	10 frames	minimized time frame →	118 ms each interval
Bleach	1 frame	minimized time frame →	118 ms each interval
Postbleach 1	100 frames	minimized time frame →	118 ms each interval
Postbleach 2	10 frames	→	1 sec each interval
Postbleach 3	10 frames	→	5 sec each interval

Acquisition speed

The acquisition speed should be adjusted to resolve the dynamic range of the recovery with good temporal resolution. Thus ideally acquire at least 10 data points during the time required for half of the recovery.

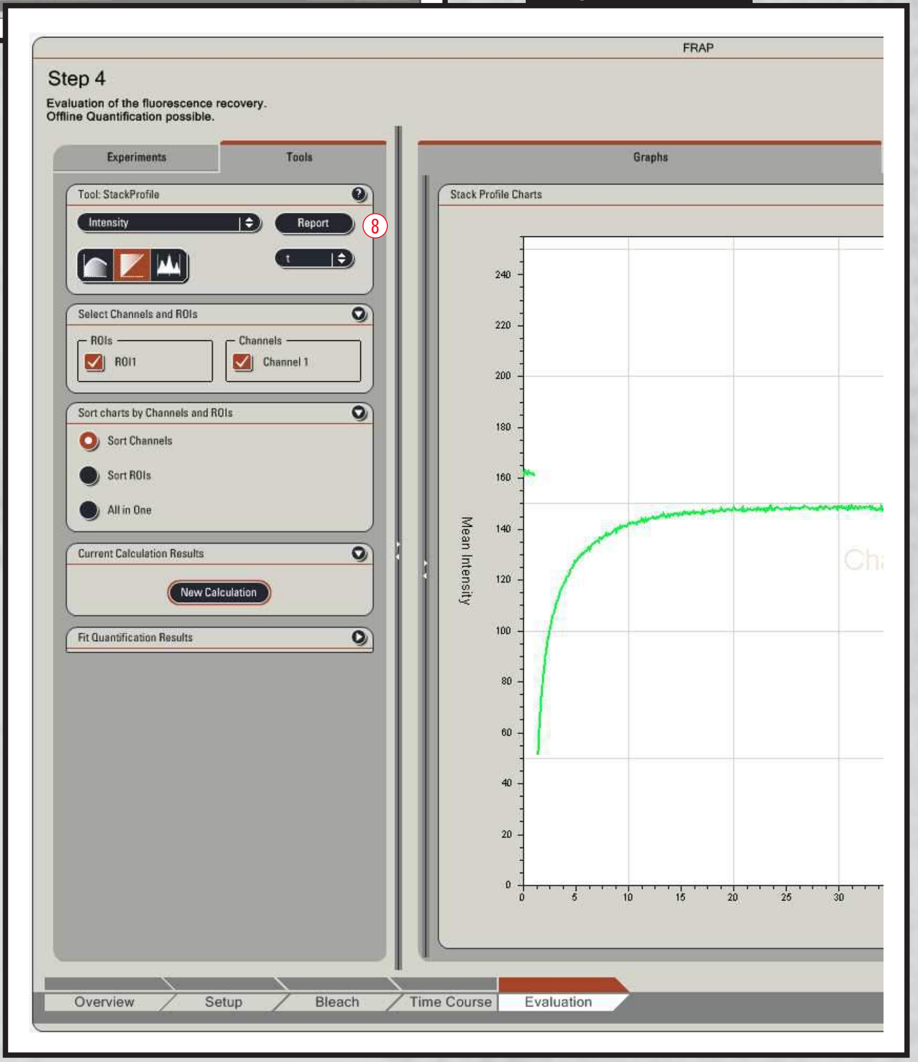
Running Experiment



- The minimized time for t/frame [s] is set as default.

- Enable additional time scales for post-bleaching quantification.

Step 4: Evaluation



Step 4

Evaluation of the fluorescence recovery.
Offline Quantification possible.

Duration of the FRAP experiment

Initial experiments should be conducted until no noticeable further increase in fluorescence intensity is detected.

If you want your ROIs & Time Course included in your saved settings you can do this in Step1. Proceed again to **Time Course** and click **Run** to start the experiment. The experiment runs automatically and leads to the evaluation step.

Note:

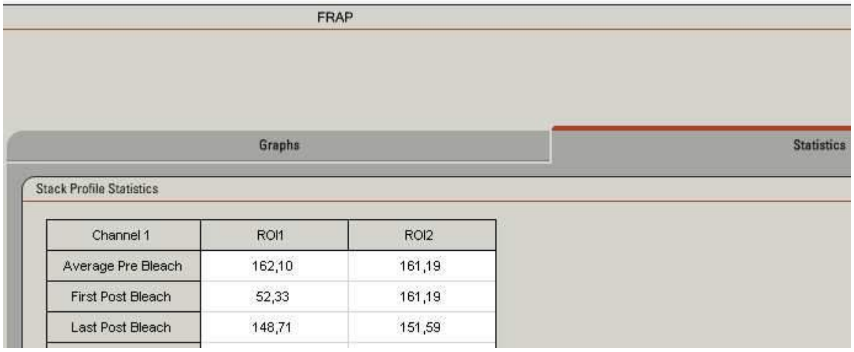
If you use fluorescent proteins usage of postbleach 2 & 3 may lead to intensity changes during transition between the different time scales. Altering the imaging frequency during the experiment can alter the fraction of fluorescent protein driven into dark states (see Weber et al., 1999).

The experiment can be stopped while it is already running e.g. during postbleach. The user will then be guided to the evaluation step. This is particularly useful if the total time for full recovery is not known, since it allows to end the experiment during postbleach as soon as full recovery (i.e. no more increase in intensity) is reached. There is no need to wait until the predicted number of frames has been acquired.

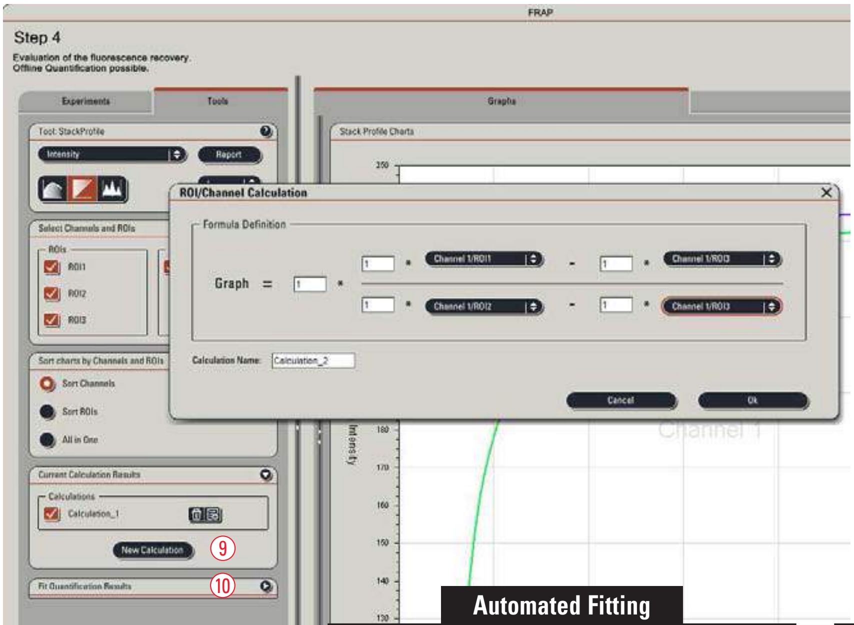
Step 4: Evaluation

Now the recovery chart is displayed. The chart shows all intensity values averaged over the ROIs for all frames. This chart can be exported to Excel via right mouse click. The **Report** button ⑧ generates a data sheet in xml format. To compare different experiments it is essential to use bleaching regions with the same size, relative position in the cell and scanning parameters. You can save the experiment procedure in the **Setup** step (p. 3).

Step 4: Evaluation



Channel 1	ROI1	ROI2
Average Pre Bleach	162,10	161,19
First Post Bleach	52,33	161,19
Last Post Bleach	148,71	151,59



Step 4

Evaluation of the fluorescence recovery.
Offline Quantification possible.

☑ ROI1
☑ ROI2
☑ ROI3

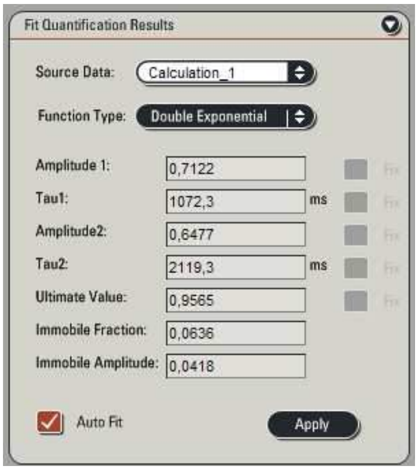
● Sort Channels
○ Sort ROIs
○ All in One

☑ Calculation_1

Graph = 1 * (1 * Channel 1/ROI1 - 1 * Channel 1/ROI3) / (1 * Channel 1/ROI2 - 1 * Channel 1/ROI3)

Calculation Name: Calculation_2

Automated Fitting



Source Data: Calculation_1

Function Type: Double Exponential

Amplitude 1: 0,7122

Tau1: 1072,3 ms

Amplitude2: 0,6477

Tau2: 2119,3 ms

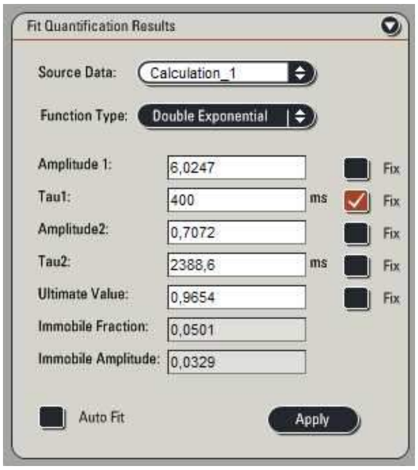
Ultimate Value: 0,9565

Immobile Fraction: 0,0636

Immobile Amplitude: 0,0418

☑ Auto Fit

User Defined Fitting



Source Data: Calculation_1

Function Type: Double Exponential

Amplitude 1: 6,0247 ☐ Fix

Tau1: 400 ms ☑ Fix

Amplitude2: 0,7072 ☐ Fix

Tau2: 2388,6 ms ☐ Fix

Ultimate Value: 0,9654 ☐ Fix

Immobile Fraction: 0,0501

Immobile Amplitude: 0,0329

☐ Auto Fit

You can display the relevant FRAP data such as last prebleach or first postbleach value in the statistics tab.

Usually the chart has to be corrected for background bleaching which is caused during imaging. Define a second ROI, e.g. enclosing the whole cell, and click on **New Calculation** ⑨ on the **Current Calculation Results** box. This opens a window where you can enter the desired formula using the values of all ROIs as well as arbitrary constants which can be used to subtract offset values. The result appears in a second chart at the bottom.

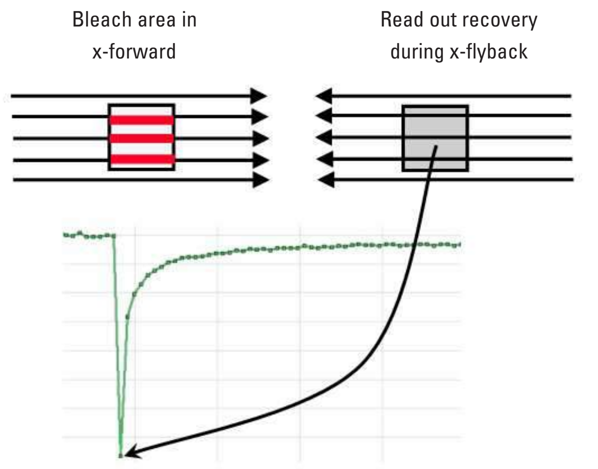
Fitting of FRAP data

Quantities like the relaxation time (τ) or amount of immobile fraction can be determined by single or double exponential fitting: After correction the calculated data is ready to be processed by LAS AF according to Levenberg Marquardt (see Press et al., 1993, pp 681-687). Click on Fit Quantification Results ⑩ and choose which ROI should be applied with which channel or which calculation should be used as source data, e.g. Calculation_1. Apply either an automated fit (see figure "Automated Fitting") or a user defined fit using fixed values for certain parameters e.g. for tau1 (see figure "User Defined Fitting"). The resulting fit is then overlaid with the calculated graph.

FRAP – FlyMode:

You may reduce the time resolution down to 0.35 ms since the measurement of recovery is already done between lines instead of between frames. This means the measurement of recovery starts closest possible to the zero time (t_0) of the postbleach intensity.

The FlyMode combines both, the bleach scan and first image scan after bleaching. Bleaching is performed during the forward motion using ROI scan features together with high laser power. During fly back, the laser intensity is set to imaging values (AOTF switching works within microseconds). Thus, the first image is acquired simultaneously with the bleaching frame. And consequently, the delay time between bleaching and data acquisition is less than half the time needed to scan a single line.

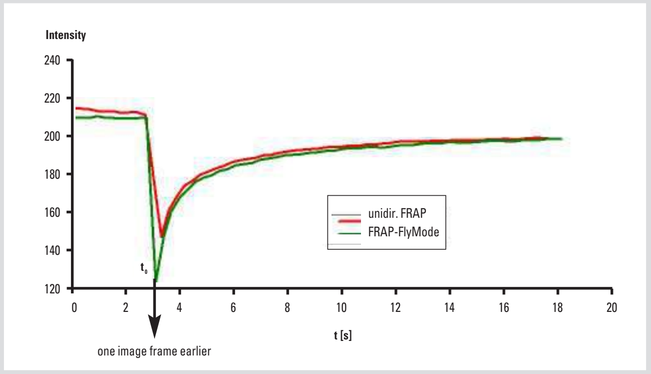


Free diffusion of FITC in glycerol, 1400 Hz scan speed and 256x256 format. With FRAP-FlyMode readout starts one frame earlier compared to unidirectional FRAP.

FlyMode operation is fully automated; just activate bidirectional scan in step 1 (setup), activate FlyMode in step 2 and proceed as already shown above.

Forward and fly back scan are displayed as separate channels during the experiment. The viewer shows on the left side the forward scan and on the right side the fly back scan.

Note:
Zoom In and **Change Format** for bleaching is not available in the FlyMode.



With the FlyMode you can catch the closest point of recovery (t_0) because it can already be acquired within the bleach frame.

Suggested background reading:

- Axelrod, D., D.E. Koppel, J. Schlessinger, E. Elson, W.W. Webb. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys. J. 16: 1055-1069 (1976)
- Braeckmans, K., L. Peeters, N.N. Sanders, S.C. De Smedt, J. Demeester. Three-Dimensional Fluorescence Recovery after Photobleaching with the Confocal Scanning Laser Microscope. Biophys. J. 85: 2240-2252 (2003)
- Beaudouin, J., D. Gerlich, N. Daigle, R. Eils, J. Ellenberg. Nuclear Envelope Breakdown Proceeds by Microtubule-Induced Tearing of the Lamina. Cell 108, 2002: 83-96
- Ellenberg, J., E.D. Siggia, J.E. Moreira, C.F. Smith, J.F. Presley, H.J. Worman, J. Lippincott-Schwartz. (1997) Nuclear membrane dynamics and reassembly in living cells: Targetting of an inner nuclear membrane protein in interphase and mitosis. J. Cell. Biol. 138: 1193-1206 (1997)
- Lippincott-Schwartz, J., E. Snapp, A. Kenworthy. Studying protein dynamics in living cells. Nature 2: 444-456 (2001)
- Lippincott-Schwartz, J., N. Altan-Bonnet, G.H. Patterson. (2003) Photobleaching and photoactivation:following protein dynamics in living cells. Nature Cell Biology Suppl: 7-14 (2003)
- Phair, R.D., T. Misteli. High mobility of proteins in the mammalian cell nucleus. Nature 404 (6778): 604-609 (2000)
- Phair, R.D., T. Misteli. Kinetic modelling approaches to in vivo imaging. Nat Rev Mol Cell Biol. 2: 898-907 (2001)
- Misteli, T., A. Gunjan, R. Hock, M. Bustink, D.T. Brown. Dynamic binding of histone H1 to chromatin in living cells. Nature 408: 877-880 (2000)
- Press, W.H., B.P. Flannery, S.A. Teukolsky, W.T. Vetterling. Numerical Recipes in C: The Art of Scientific Computing, 2nd edition, Cambridge University Press (1993)
- Rabut, G., J. Ellenberg. Photobleaching techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, iFRAP, and FLIP, in Live Cell Imaging: A Laboratory Manual. Goldman, R.D. and Spector, D.L. (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York: 101-126 (2005)
- Schröder, J., T.N. Bui. Analyse der Proteindynamik mit einem nicht-fluoreszierenden Protein. Biospektrum 12 (5): 515-517 (2006)
- Siggia, E.D., J. Lippincott-Schwartz, S. Bekiranov. Diffusion in inhomogenous media: Theory and simulations applied to a whole cell photobleach recovery. Biophys. J. 79: 1761-1770 (2000)
- Snapp, E.L., N. Altan, J. Lippincott-Schwartz. Measuring protein mobility by photobleaching GFP chimeras in living cells. Curr. Prot. Cell. Biol., chapter 21.1 (2003)
- Weber, W., V. Helms, J.A. McCammon, P.W. Langhoff. Shedding light on the dark and weakly fluorescent states of green fluorescent proteins. Proc Natl Acad Sci USA, 96 (11): 6177-6182 (1999)

Internet resources:

http://www.embl.de/eamnet/html/molecular_dynamics_2005.html

http://www.dkfz.de/tbi/projects/imagingAndFunctionalScreening/diffusionalDynamicsNucleus.jsp

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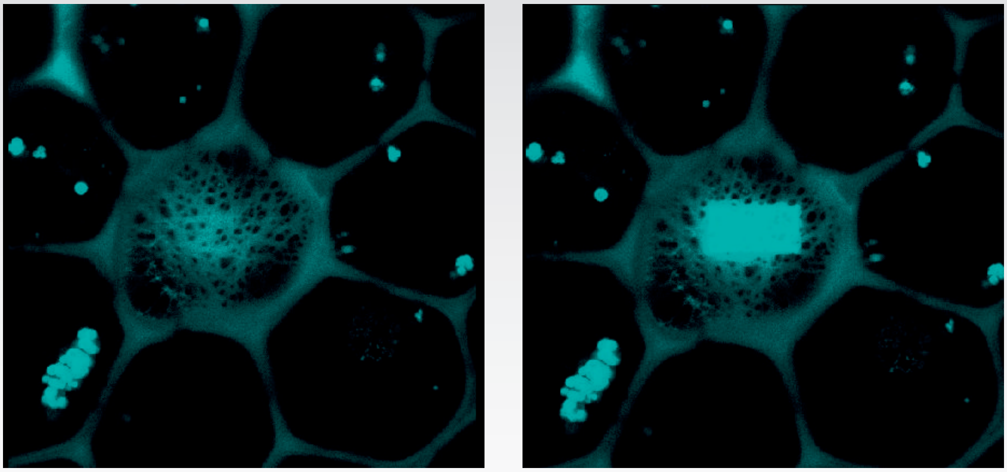
No. 28

CONFOCAL APPLICATION LETTER

reSOLUTION

FRET Acceptor Photobleaching
LAS AF Application Wizard



Leica
MICROSYSTEMS

FRET with Leica TCS SP5
LAS AF Version 1.7.0

Introduction
Fluorescence Resonance Energy Transfer (FRET) is a technique which allows insight into interactions between proteins or molecules in proximities beyond light microscopic resolution. The principle: an excited fluorophore, called donor, transfers its excited state energy to a light absorbing molecule which is called acceptor. This transfer of energy is non-radiative. Acceptor Photobleaching is one established method for the evaluation of FRET efficiencies. It is usually applied to fixed samples, as any relocation of donor molecules during measurement will lead to false intensity correlations.

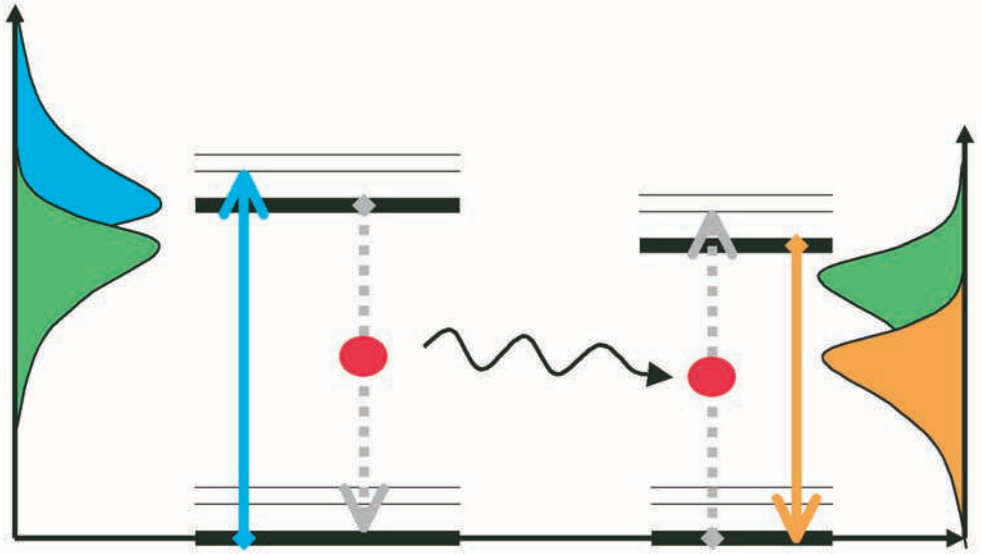
FRET Acceptor Photobleaching

The method:
In the event of FRET the donor encounters a quenching of fluorescence due to its energy transfer to the acceptor. The donor fluorescence will be unquenched after photobleaching of the acceptor. The difference of fluorescence intensity of the donor before and after photobleaching gives a direct indication to the FRET efficiency and can be quantified as follows:

$$FRET_{eff} = (D_{post} - D_{pre}) / D_{post}$$

where D_{post} is the fluorescence intensity of the donor after photobleaching and D_{pre} is the fluorescence intensity of the donor before photobleaching.

In the event of FRET an excited fluorophore transfers its energy to another light absorbing molecule.

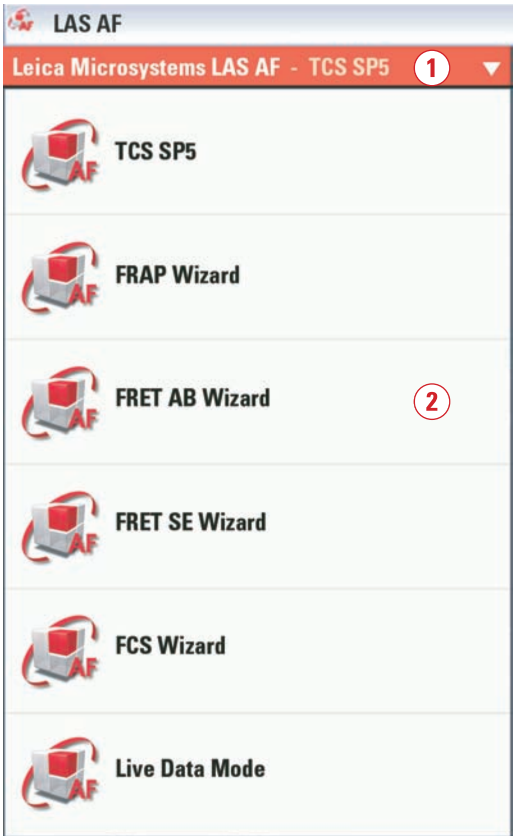


FRET wizards in Leica Application Suite Advanced Fluorescence (LAS AF)

In the LAS AF **(1)** menu bar you can find two wizards to perform FRET experiments: FRET AB (Acceptor Photobleaching) and FRET SE (Sensitized Emission). This application letter describes working with the FRET AB Wizard **(2)**.

The wizard consists of 3 steps and an overview of the experimental workflow.

Step 1 is dedicated to the imaging set-up. Step 2 allows the definition of bleaching conditions. The experiment is executed in between Step 2 and 3. Step 3 allows the evaluation of results and the generation of experimental reports.



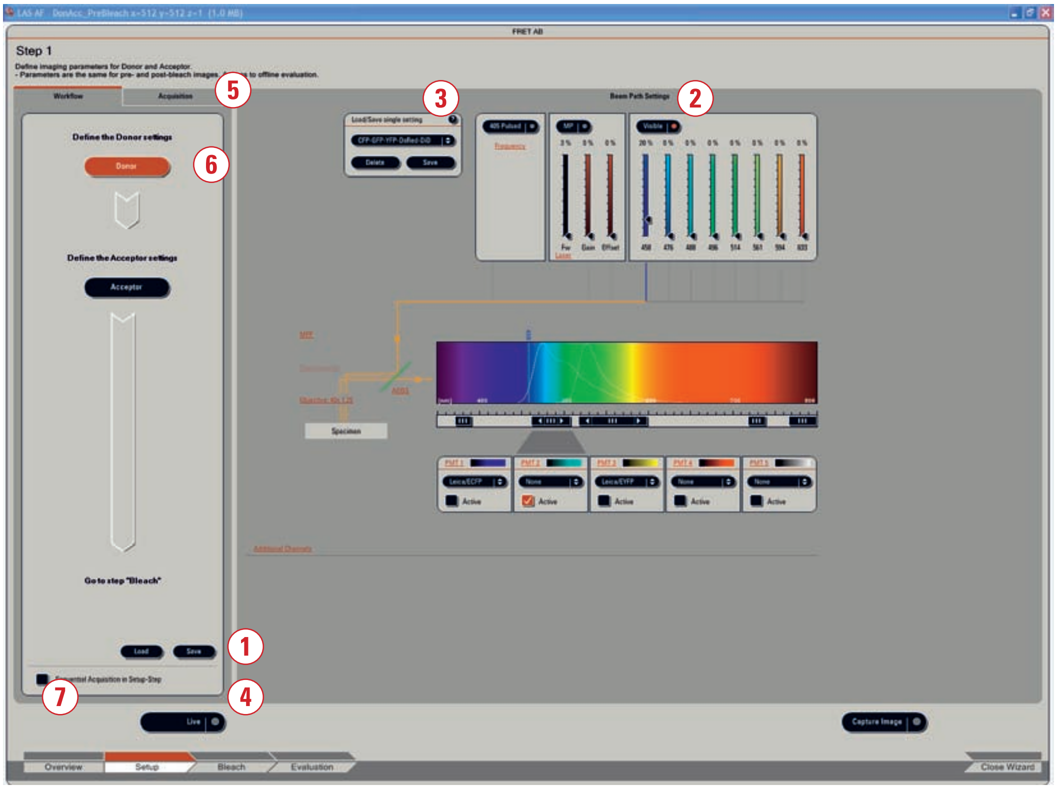
FRET Acceptor Photobleaching – Step by Step

Step 1: Setting of experimental conditions
Define the imaging conditions for donor and acceptor fluorescence by following the workflow. You may start your experiment with previously saved imaging conditions. Use the **Load** and **Save** options **(1)**. If you want to establish imaging conditions yourself you must start out with the **Beam Path Settings** **(2)**.

1. Begin by simultaneous excitation and detection of the donor and acceptor. You can load existing settings from the IPS list **(3)**, or set up the imaging conditions manually (e.g.: Donor = CFP excitation 458 nm; emission 462- 510 nm; Acceptor = YFP excitation 514 nm; emission 518-580 nm). This enables you to assess the fluorescence intensities, PMT gain, and laser dose for each label. It also allows to judge where

you may find donor and acceptor fluorescence and where they coincide. Do all adjustments by using the **Live** button (4). Check for appropriate imaging resolution. You may change the zoom factor via control panel or with the tools under **Acquisition** (5).

2. Now follow the workflow and change the imaging conditions to donor detection only (6). by switching off the acceptor detection channel and setting the laser light of the acceptor down to 0%.



LAS AF DonAcc_PreBleach x=512 y=512 z=1 (1.0 MB)

Step 1

Define imaging parameters for Donor and Acceptor.
- Parameters are the same for pre- and post-bleach images. [illegible] to offline evaluation.

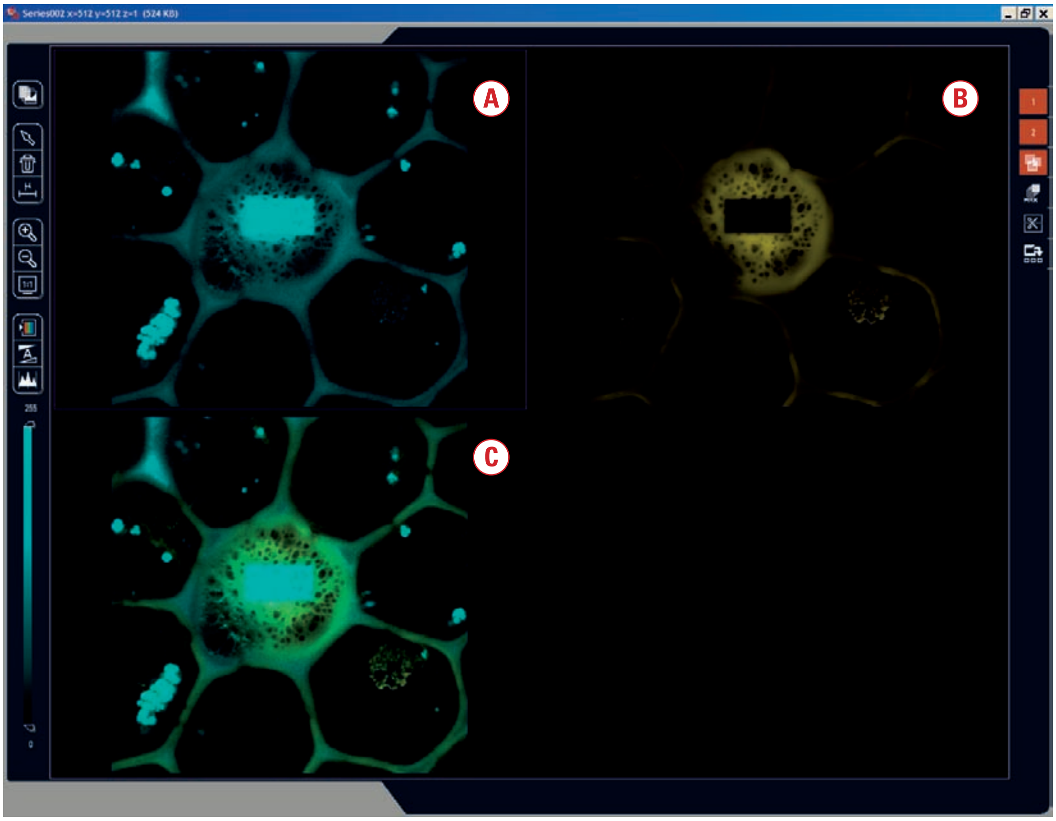
3. Start **Live Scan** (4) for image optimization.

4. Continue by defining the acceptor imaging set-up. Turn the donor excitation light down to 0%, turn off donor detection channel, turn on acceptor detection channel instead and then set the excitation laser line of the acceptor for adequate excitation light.

5. Start **Live Scan** (4) for image optimization.

6. If you would like to see both donor and acceptor acquired together, you need to activate the sequential imaging option via checkbox (7). Image acquisition of donor and acceptor is done in a line by line sequential scan mode.

7. The images visible in the viewer are generated by means of line by line sequential scan. Images **(A)** + **(B)** show donor (blue) and acceptor (yellow). The image bottom left shows the overlay of both fluorescence signals **(C)**. To display an overlay image activate this button. The overlay image will help you to choose the appropriate region for acceptor photobleaching.



8. Define the number of averages for best image quality with the tools under **Acquisition** **(5)**.

9. Proceed to the next step **Bleach** to define bleaching conditions.

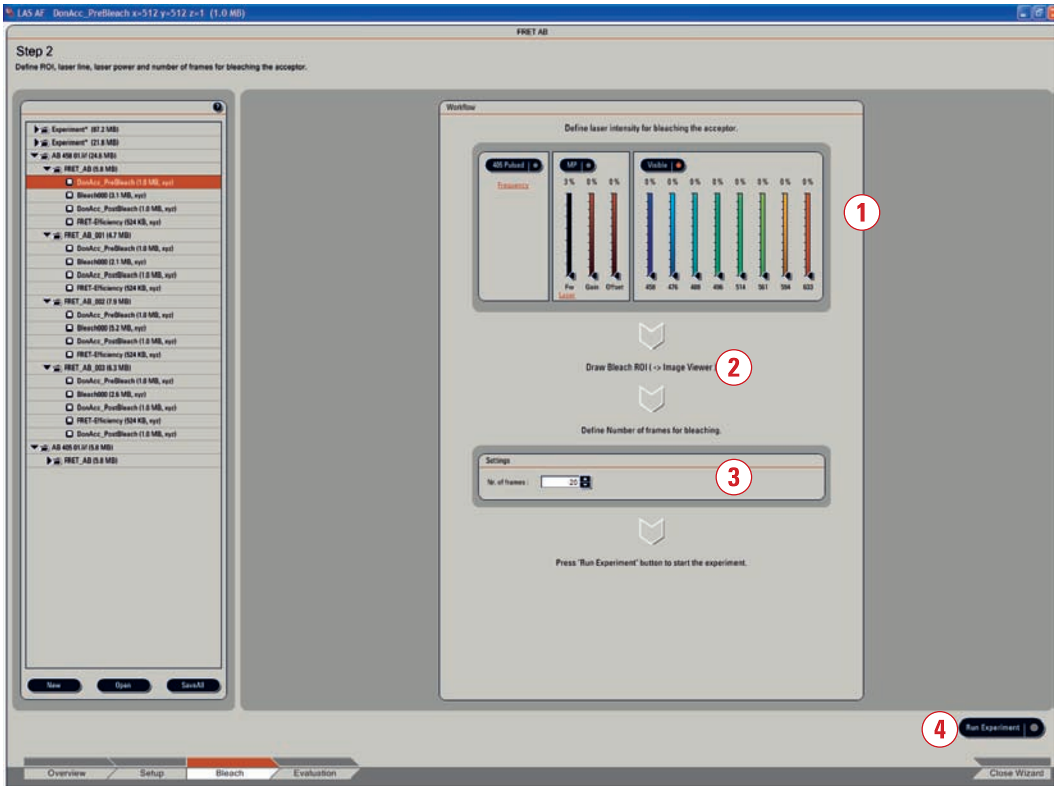
Step 2: Define Acceptor Photobleaching Conditions

1. Begin with the choice of laser line and intensity for acceptor photobleaching **(1)**.

2. Draw a region of interest (ROI) **(2)** around the area you wish to bleach.

3. Select the number of bleaching iterations **(3)**. If you have defined averaging under **Acquisition** in step 1, this will apply to the bleaching as well. So please consider the bleach duration accordingly.

4. You may now run the bleaching experiment **(4)**. The measurement will start by imaging the donor and acceptor before bleaching, followed by the bleaching of the acceptor and finally imaging the donor and acceptor after bleaching. The imaging conditions before and after bleaching are identical. The bleaching progress may be followed by the progress bar or bleaching progress in the image and may be stopped if necessary **(4)**.



LAS AF DonAcc_PreBleach x=512 y=512 z=1 (1.0 MB)

Step 2

Define ROI, laser line, laser power and number of frames for bleaching the acceptor.

Define laser intensity for bleaching the acceptor.

Draw Bleach ROI (-> Image Viewer)

Define Number of frames for bleaching.

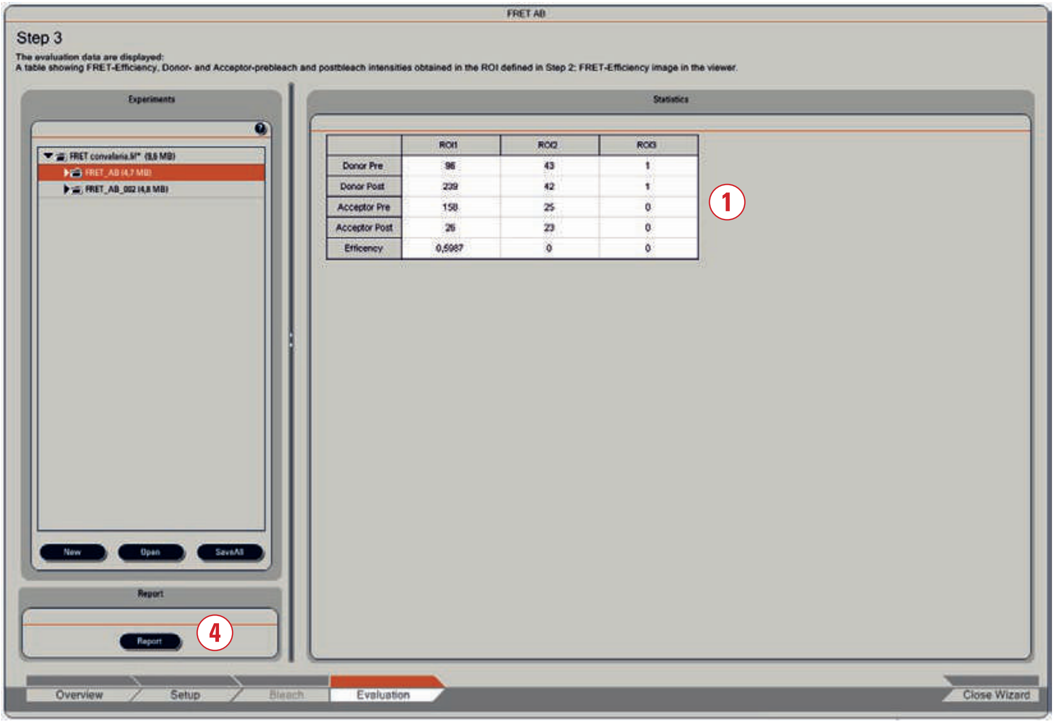
Nr. of frames : 20

Press 'Run Experiment' button to start the experiment.

Step 3: Evaluation

1. The image shown now in the viewer is the FRET efficiency image. Mean values of FRET efficiency within the bleached region (ROI1) are displayed in the user interface **(1)**.

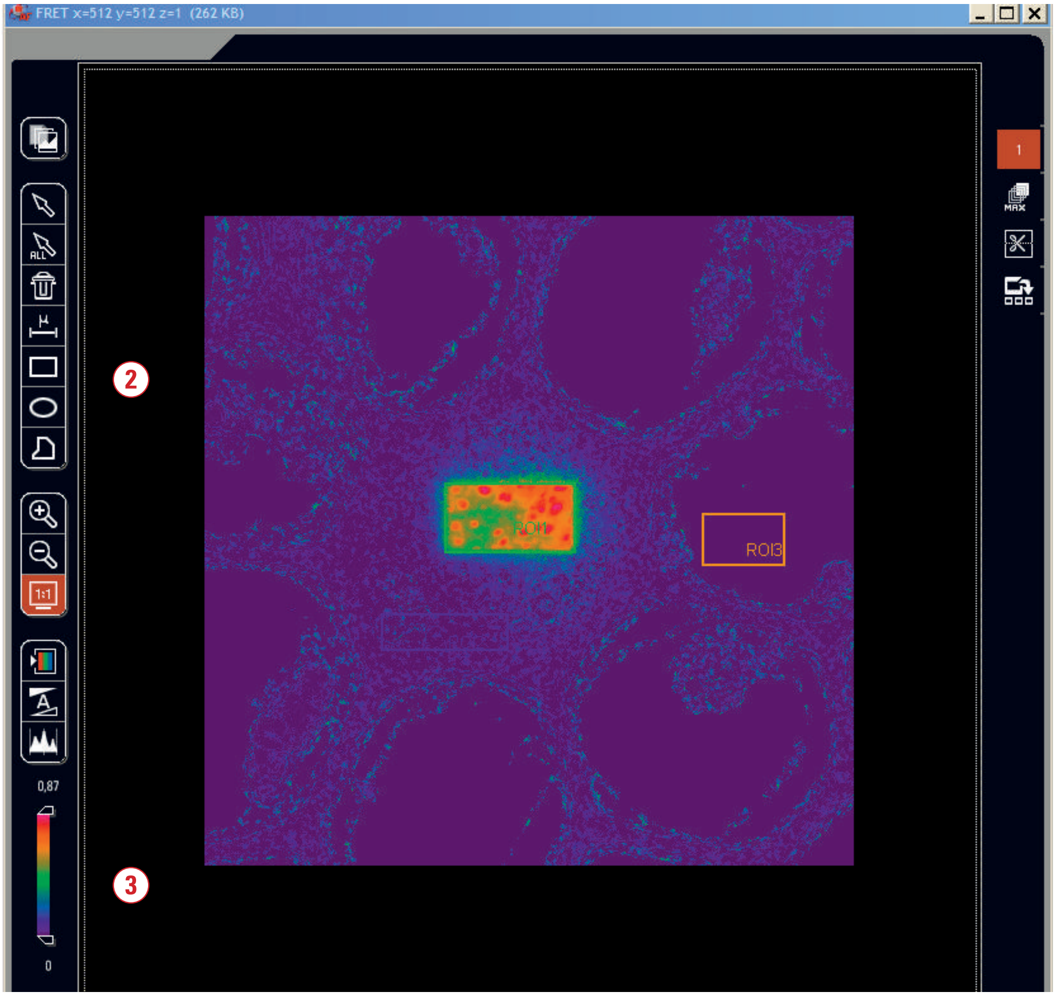
2. You may choose additional regions of interest for better interpretation of results **(2)**.

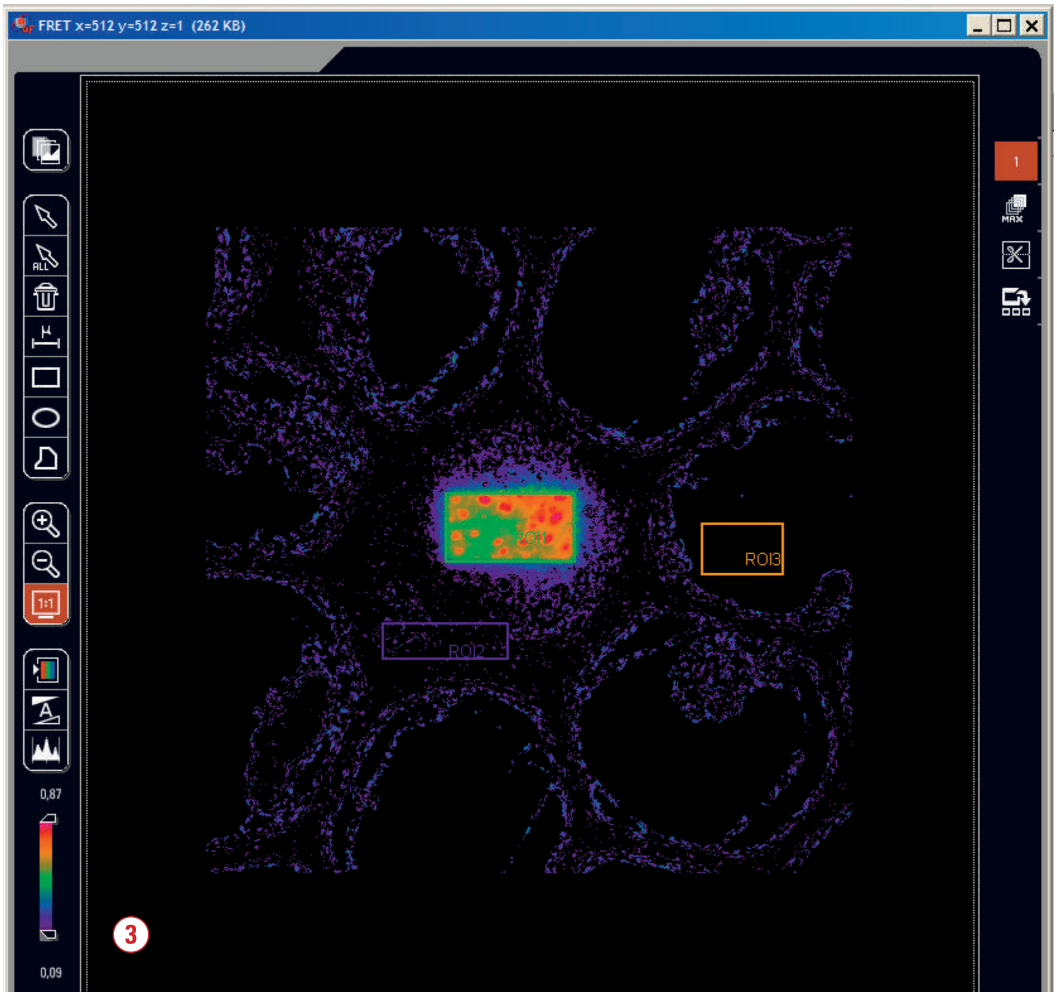


Step 3

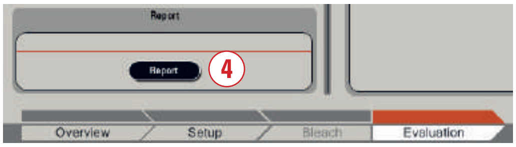
The evaluation data are displayed:
A table showing FRET-Efficiency, Donor- and Acceptor-prebleach and postbleach intensities obtained in the ROI defined in Step 2: FRET-Efficiency image in the viewer.

	ROI1	ROI2	ROI3
Donor Pre	96	43	1
Donor Post	239	42	1
Acceptor Pre	158	25	0
Acceptor Post	26	23	0
Efficency	0,5987	0	0





3. FRET efficiencies may also be estimated via the intensity display in the viewer **(3)**. The displayed LUT may be changed for better visualization or understanding by clicking on intensity wedge. Color coding for high or low values may be altered by moving the wedge ends up and down. This may, for example, set the purple background to black.



4. Saving data: Images are saved via Save As by right mouse click on the experiment in the experiment tree. Regions of interest (ROIs) and images that have undergone changes (e.g. FRET efficiency images with LUT changes or added annotations) are saved by a right mouse click on the viewer. The experimental data is saved by creating a report **(4)**.

Suggested readings:

- Gadella, T. W. J., Van der Krogt, G. N. M. & Bisseling, T.: GFP-based FRET Microscopy in Living Plant Cells. Trends Plant Sci. 4 (7): 287-291, 1999
- Lippincott-Schwartz, J., Snapp, E. & Kenworthy, A. K.: Studying protein dynamics in living cells. Molecular Cell Biology, 2, 444-456, 2001
- Wouters, F.S., Verveer, P.J. & Bastiaens, P.I.H.: Imaging biochemistry inside cells. Trends Cell Biol., 11(5): 203-11, 2001

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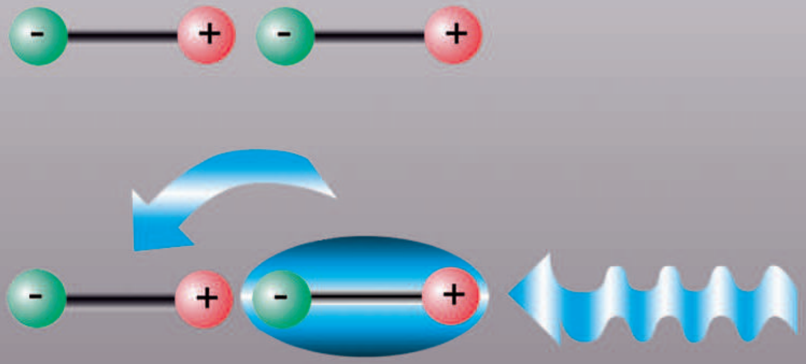
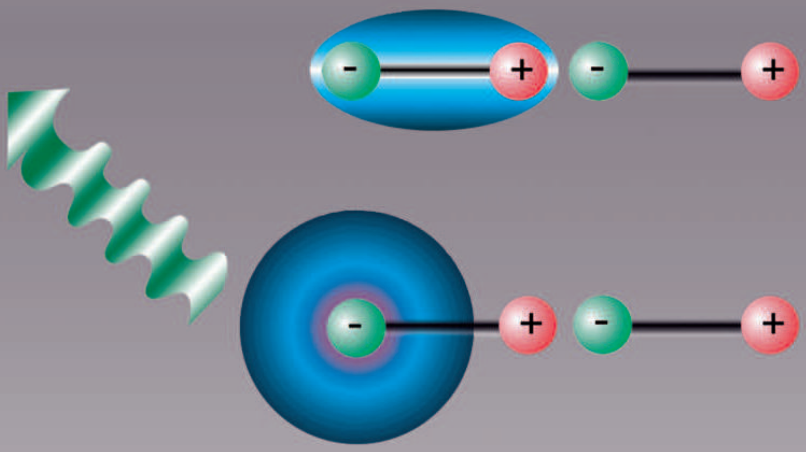
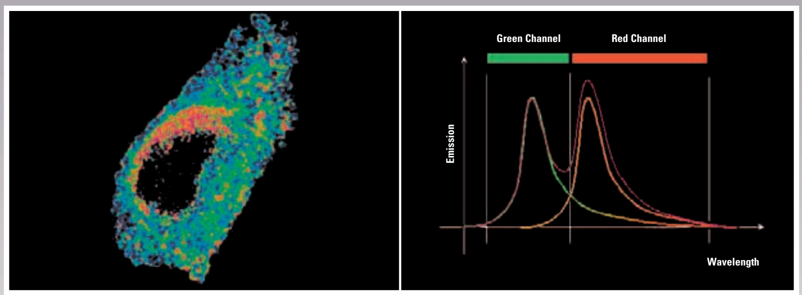
2006
No.20

CONFOCAL APPLICATION LETTER



reSOLUTION

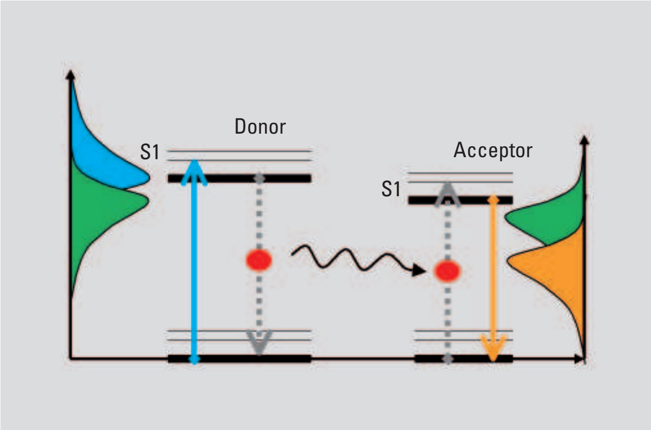




LAS AF APPLICATION WIZARD
FRET SENSITIZED EMISSION

Leica
MICROSYSTEMS

FRET with Leica TCS SP5 (LAS AF)



FRET Sensitized Emission

Fluorescence Resonance Energy Transfer (FRET) is a technique, which allows insight into the interactions between proteins or molecules in proximities beyond light microscopic resolution.

The Principle:
An excited fluorophore, called the donor, transfers its excited state energy to a light absorbing molecule which is called the acceptor. This transfer of energy is non-radiative.

Sensitized Emission is one established method for the evaluation of FRET efficiencies. It can be applied to live cells as well as to fixed samples.

The Method:
As this method is non-invasive, it is most frequently used for live cell experiments. Because crosstalk is an important issue, controls and complex correction calculations are inherent to the method. Measurements are executed by detection of the fluorescent signals of the donor and FRET as well as acceptor in a line by line sequential scan acquisition.

Proper excitation conditions play a major role. In the first sequence, the donor must be excited to generate donor fluorescence and sensitized emission of the acceptor. In the second sequence, the selective excitation of the acceptor will create acceptor fluorescence.

FRET sample preparations must therefore include references of donor in the absence of the acceptor (donor only control) and acceptor in the absence of the donor (acceptor only control).

Ideally, all references are included in the same preparation. The donor and acceptor references are used to obtain calibration coefficients to correct for excitation and emission cross talk.

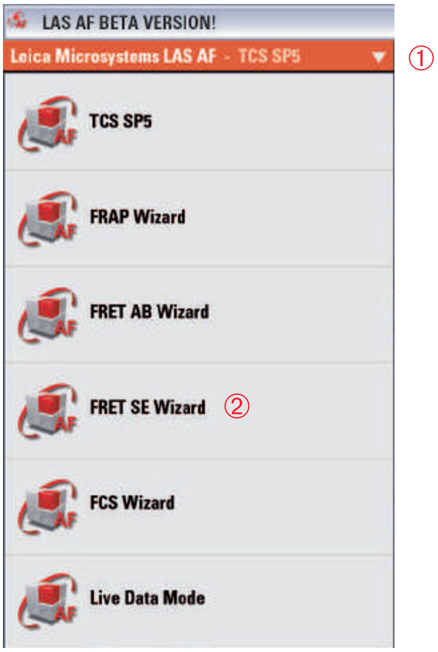
It is important to be aware that throughout the entire experiment and calibration routine, all measuring parameters such as gain, emission detection window, excitation intensities, zoom, format, scan speed, pinhole etc must remain constant.

FRET Wizards in Leica Application Suite Advanced Fluorescence (LAS AF)

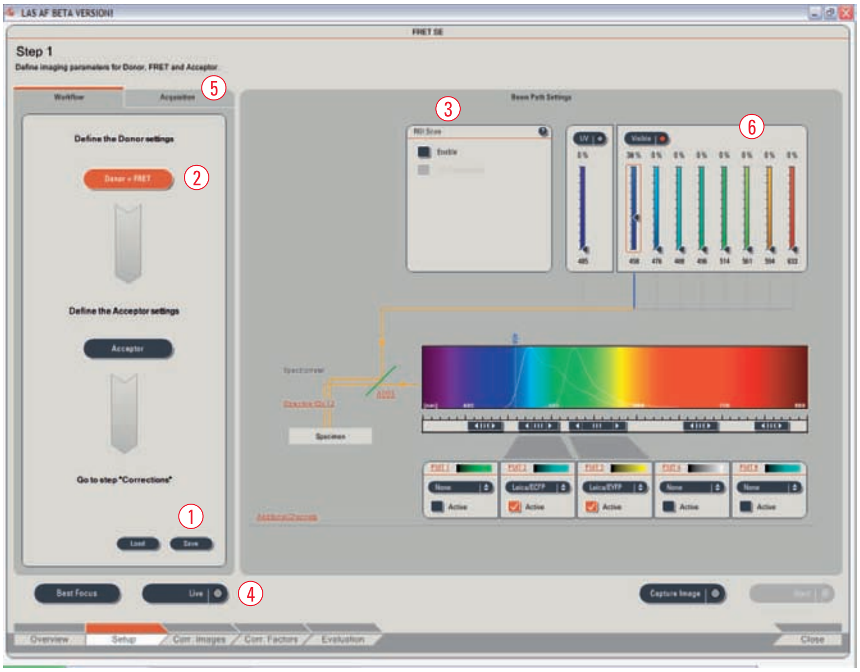
Within the LAS AF ① menu bar you can find two wizards to perform FRET experiments: FRET AB (Acceptor Photo-Bleaching) and FRET SE (Sensitized Emission). This application note describes working with the FRET SE Wizard ②.

The wizard consists of 4 steps and in addition, an overview of the experimental workflow.

- Step 1: is dedicated to the imaging set-up.
- Step 2: executes control and FRET measurements.
- Step 3: guides through the calibration routine and calculates the calibration coefficients.
- Step 4: is dedicated to the execution of time-lapse experiments, evaluation of results and generation of experimental reports.



Step 1: Setting of experimental conditions



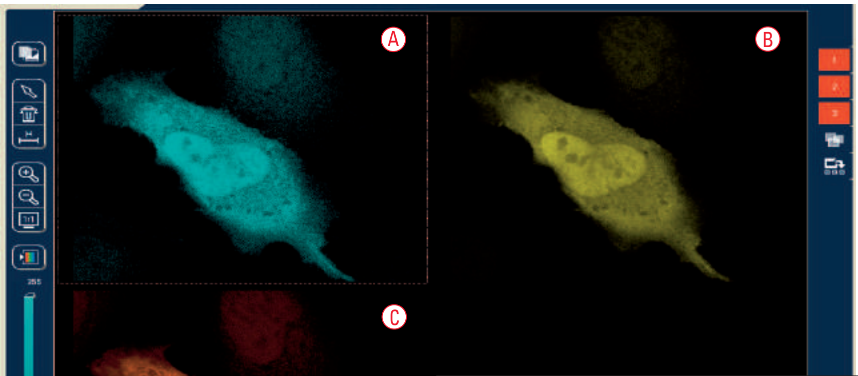
Step 1

Define imaging parameters for Donor, FRET and Acceptor

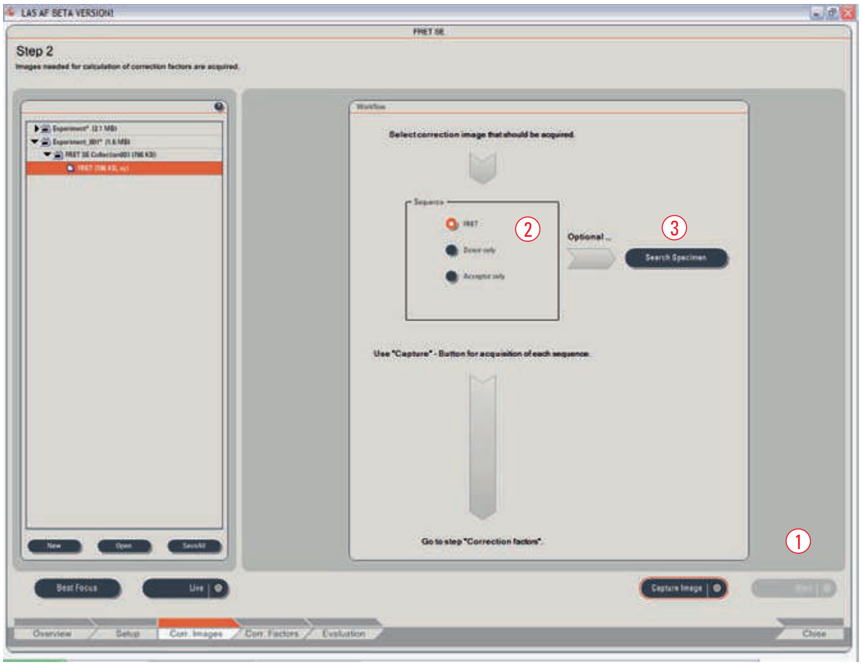
Define the Donor settings

Define the Acceptor settings

Go to step "Corrections"



Step 2: Acquire image sequences of controls for calibration



Step 2

Images needed for calculation of correction factors are acquired.

Select correction image that should be acquired.

Use "Capture" - Button for acquisition of each sequence.

Go to step "Correction factors".

Note:

It is vital to keep all measurements under identical conditions. If your controls do not match the actual imaging conditions (saturation!) and if you have therefore to readjust, all previous measurements under the changed conditions will have to be repeated. All measurements performed under differing conditions should be discarded!

FRET Sensitized Emission – Step by Step

Step 1: Setting of experimental conditions

Begin the imaging set-up with the FRET sample with all fluorescence information (donor, FRET and acceptor) in the same specimen. Define imaging conditions for donor, FRET and acceptor fluorescence by following the workflow. You may start your experiment with previously saved imaging conditions. Use the **Load** and **Save** options ①. If you want to establish imaging conditions yourself you must start out in the **Beam Path Settings** ③.

1. Image acquisition of donor, FRET and acceptor will automatically be done in a line by line sequential scan mode, after the definition of donor, FRET and acceptor. Start defining the donor and the FRET detection (**Donor + FRET**) ② via the **Beam Path Settings** window ③. Begin by simultaneous excitation and detection of the donor and acceptor (e.g.: Donor = CFP excitation 458nm; emission 462-510nm; Acceptor = YFP excitation 514nm; emission 518-580nm). This enables you to assess the fluorescence intensities, PMT gain, and laser dose for each label. Make all adjustments by using the **Live** button ④. Check for appropriate imaging resolution. You may change the zoom factor via the control panel or with the tools under **Acquisition** ⑤.

2. Reduce now the laser light of the acceptor down to 0% ⑥. Re-adjust the acceptor PMT to be slightly below saturation. You are now exciting only selectively for the donor and have properly defined your conditions for the donor and FRET imaging.

3. Continue by defining the acceptor set-up ② via the **Beam Path Settings** window ③. Turn the donor excitation light down to 0%. Choose a new LUT for the directly excited acceptor signal (e.g.: light blue = donor; yellow = FRET; orange = acceptor) for better discrimination. Start **Live Scan** ④.

4. The images you see in the viewer are generated by means of a line by line sequential scan. The first sequence Ⓐ + Ⓑ consists of donor (light blue) and FRET (Yellow). The excitation wavelength is selectively chosen for donor excitation. The second sequence Ⓒ contains acceptor fluorescence (orange). The excitation light is selective for acceptor excitation.

5. Slowly increase the excitation light of the acceptor (in our example 514 nm) until the signal of the acceptor (orange) is just below saturation. Do not change the PMT gain or offset as this will also change the imaging conditions for the FRET (yellow) signal detection!

6. Define the number of averages for best imaging conditions. The desired acquisition mode may be chosen with the tools under **Acquisition** ⑤.

7. Proceed to the next step **Corr. Images** to acquire control images of the specimen for calibration.

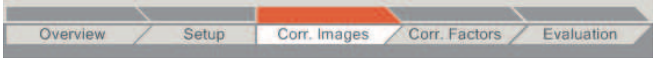
Step 2: Acquire image sequences of controls for calibration

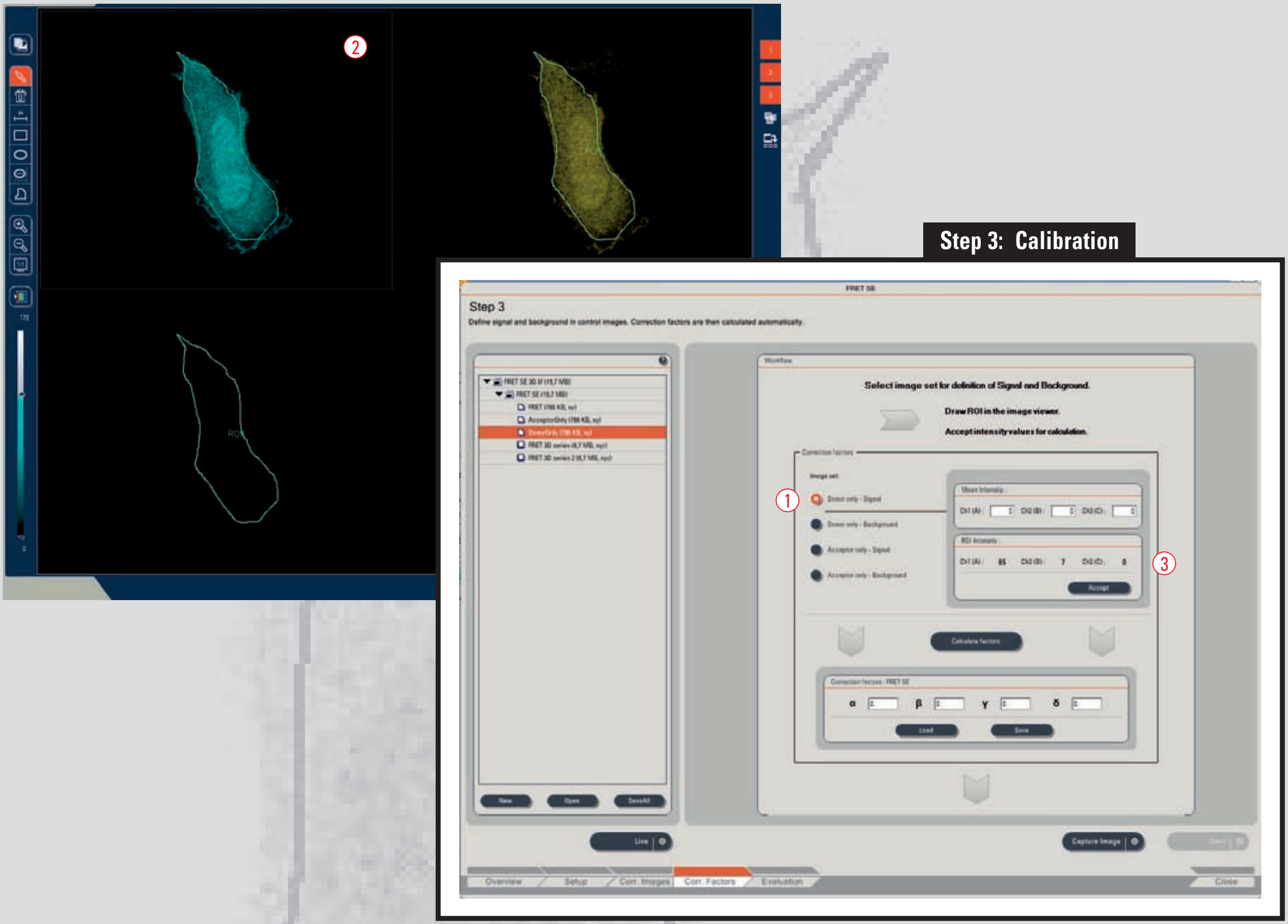
1. In step 2 you may first take an image set of your FRET sample since it is already under the scope and nicely set-up. Acquire the sequence via **Capture Image** ①. The image set will be automatically named 'FRET'.

2. Continue with the measurements of the controls (**Donor only** and **Acceptor only**) ②. To keep track of the specimen choose the correct radio button for the according specimen.

3. If controls are on separate slides you will now change specimen. For a better field of vision you may want to go back to zoom 1 to be able to find adequate cells to properly match the intensities and to avoid saturated regions. Use the function button **Search Specimen** ③. You now have access to all the acquisition tools needed. When you exit **Search Specimen** you will automatically return to the zoom factor and resolution conditions of all previous measurements in the experiment.

4. Proceed from Corr. Images to the next step Corr. Factors to generate calibration factors.





Step 3: Calibration

Step 3

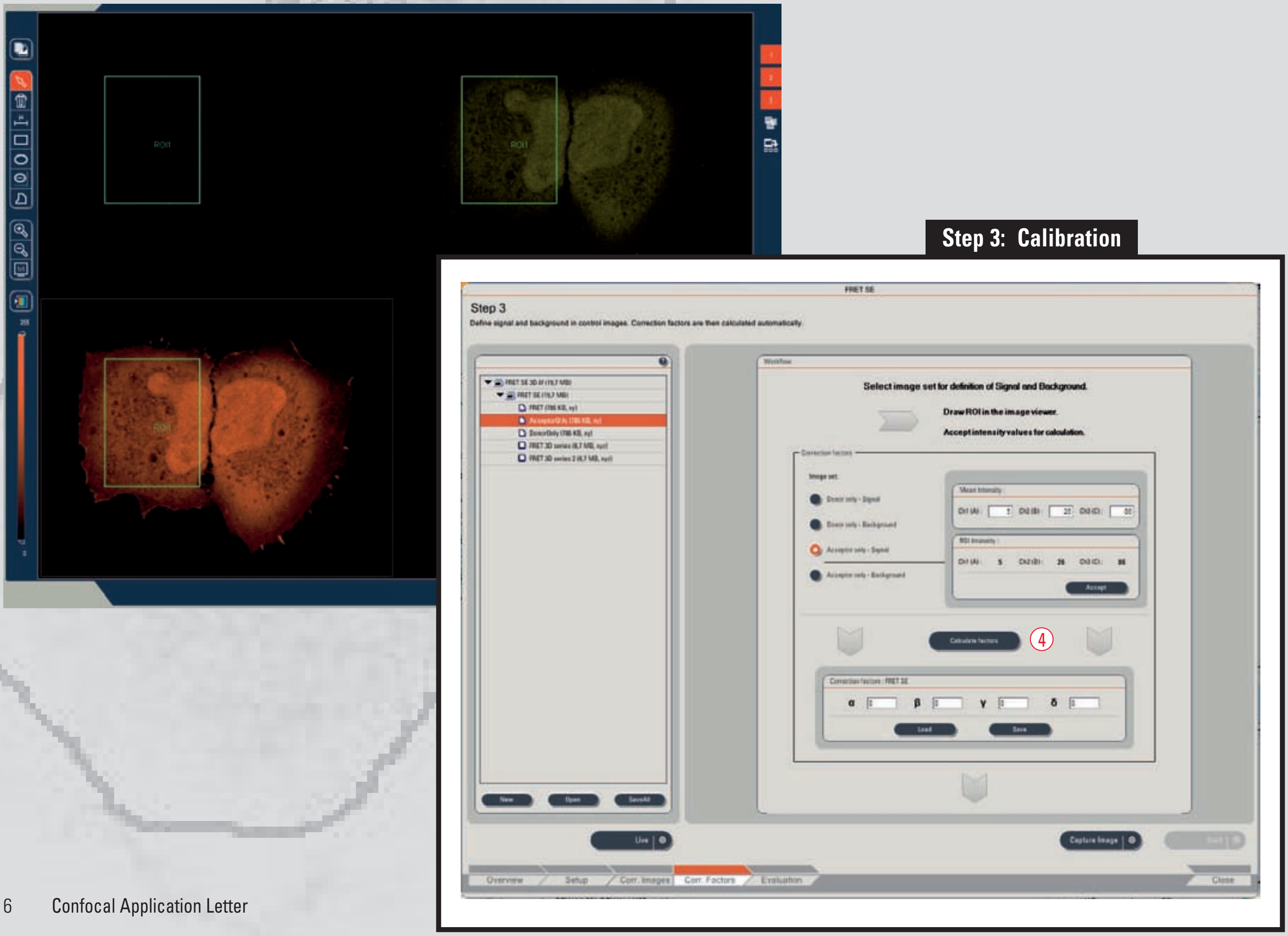
Define signal and background in control images. Correction factors are then calculated automatically.

Select image set for definition of Signal and Background.

Draw ROI in the image viewer.

Accept intensity values for calculation.

ROI Intensity: Ch1 (A): 85 Ch2 (B): 7 Ch3 (C): 0



Step 3: Calibration

Step 3

Define signal and background in control images. Correction factors are then calculated automatically.

Select image set for definition of Signal and Background.

Draw ROI in the image viewer.

Accept intensity values for calculation.

ROI Intensity: Ch1 (A): 5 Ch2 (B): 26 Ch3 (C): 86

Step 3: Calibration

1. Ensure you have the appropriate image set active in the experiment overview to match the indication marked by the radio button in the interface ①.

2. Draw a region of interest (ROI) in the image where only donor signal is found ②. The mean intensities within the ROI are shown in the workflow interface ③. Press **Accept** to use values for calculation of calibration coefficients.

3. Background subtraction is optional. It is only necessary if the images show background above 0. If this is the case, draw a ROI in the image where only background signal is found and press **Accept to** use these values.

4. Continue likewise for acceptor only sequence.

   For ease of interpretation intensity distribution check the table showing the intensity distribution for measured specimen (e.g. CFP-YFP):

	Channel 1 (A)	**Channel 2 (B)**	**Channel 3 (C)**
Specimen: FRET	Signal (Donor)	Signal (FRET)	Signal (Acceptor)
Specimen: Donor only	Signal (Donor)	Signal < Channel 1 (x-talk)	No Signal
Specimen: Acceptor only	Very little to no Signal (x-excited x-talk Acceptor)	Signal < Channel 3 (x-Excitation)	Signal (Acceptor)

5. Press **Calculate Factors** to generate correction factors ④.

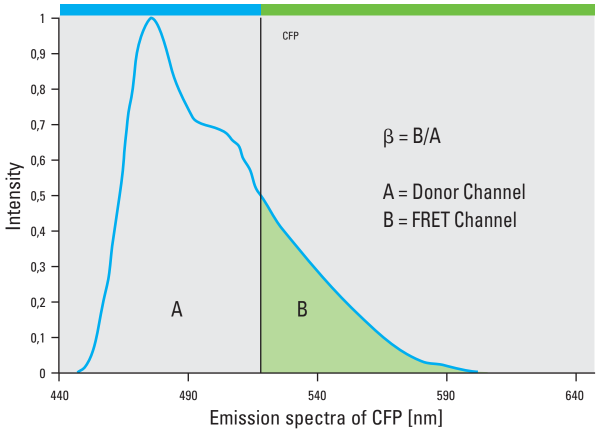
All calculated calibration factors will be applied to the existing FRET sequences (fixed sample analysis) or any time-lapse experiment to follow. Precondition: all measurements remain under unchanged imaging conditions.

6. Continue to the next step **Evaluation** to perform live cell measurements after calibration and to retrieve FRET efficiencies.

Calibration Factors:

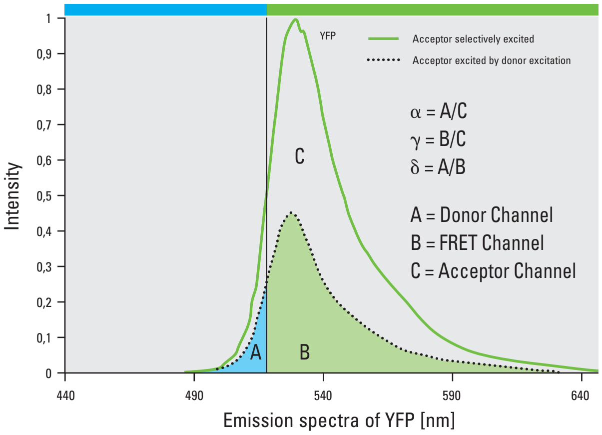
1. Donor only reference generates correction factor Calibration Factors:

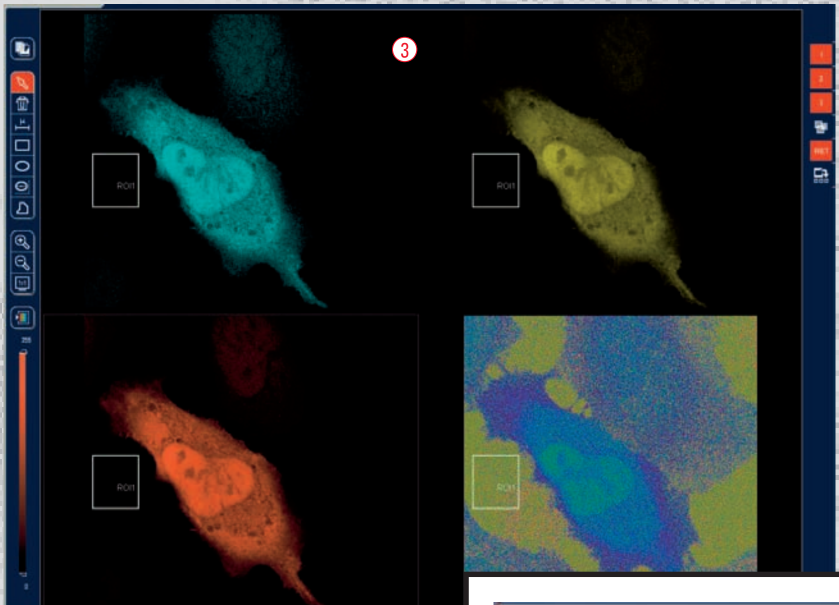
β corrects for donor cross-talk: $\beta = B/A$



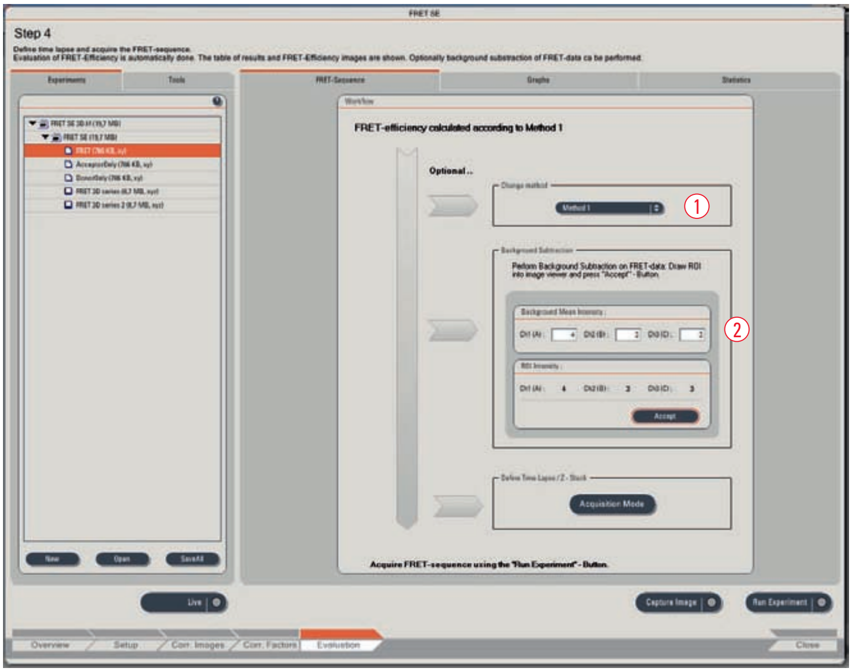
2. The Acceptor only reference generates correction factors α, γ, δ.

α corrects for acceptor cross-excitation cross-talk:
$\alpha = A/C$
γ corrects for acceptor cross-excitation:
$\gamma = B/C$
δ corrects for FRET cross-talk:
$\delta = A/B$





Step 4: Calculation of FRET efficiencies and live cell measurements



FRET SE

Step 4

Define time lapse and acquire the FRET-sequence.
Evaluation of FRET-Efficiency is automatically done. The table of results and FRET-Efficiency images are shown. Optionally background substraction of FRET-data ca be performed.

FRET-efficiency calculated according to Method 1

Optional ..

Change method: Method 1

Background Subtraction

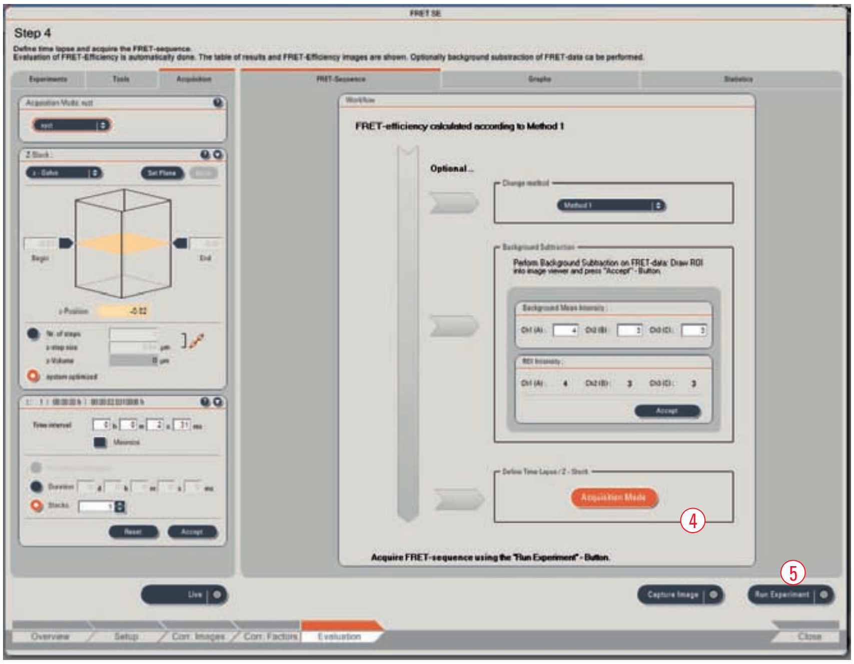
Perform Background Subtraction on FRET-data: Draw ROI into image viewer and press "Accept" - Button.

Background Mean Intensity: Ch1 (A): 4 Ch2 (B): 2 Ch3 (D): 2

ROI Intensity: Ch1 (A): 4 Ch2 (B): 2 Ch3 (D): 3

Define Time Lapse / Z - Stack

Acquire FRET-sequence using the "Run Experiment" - Button.



FRET SE

Step 4

Define time lapse and acquire the FRET-sequence.
Evaluation of FRET-Efficiency is automatically done. The table of results and FRET-Efficiency images are shown. Optionally background substraction of FRET-data ca be performed.

FRET-efficiency calculated according to Method 1

Optional ..

Change method: Method 1

Background Subtraction

Perform Background Subtraction on FRET-data: Draw ROI into image viewer and press "Accept" - Button.

Background Mean Intensity: Ch1 (A): 4 Ch2 (B): 2 Ch3 (D): 2

ROI Intensity: Ch1 (A): 4 Ch2 (B): 3 Ch3 (D): 3

Define Time Lapse / Z - Stack

Acquire FRET-sequence using the "Run Experiment" - Button.

Step 4: Calculation of FRET efficiencies and live cell measurements

Before running a time-lapse experiment you may choose the calculation method to calculate and represent the apparent FRET efficiencies ①. You may also change the method after running the experiment. Method 1 is automatically applied.

If your FRET data shows a background above 0 you may run a background subtraction ②. Draw a ROI in the image viewer where only background signal is found ③. Press **Accept** to include the background subtraction into calculations. You may also fill in a value by yourself to match background variations more precisely.

For time-lapse experiments choose imaging conditions under **Acquisition** in the **Acquisition Mode** ④ and start your series with **Run Experiment** ⑤.

Calculation of FRET efficiency:

EA is the apparent FRET efficiency. A, B, C correspond to the intensities of the 3 signals (donor, FRET, acceptor) and α, β, γ and δ are the calibration factors generated by acceptor only and donor only references.

Method 1:

$$E_A(i) = \frac{B - A \times \beta - C \times \gamma}{C}$$

Ref. Wouters et al., TRENDS in Cell Biology, Vol 11, No.5, May 2001: 203-211

Method 2:

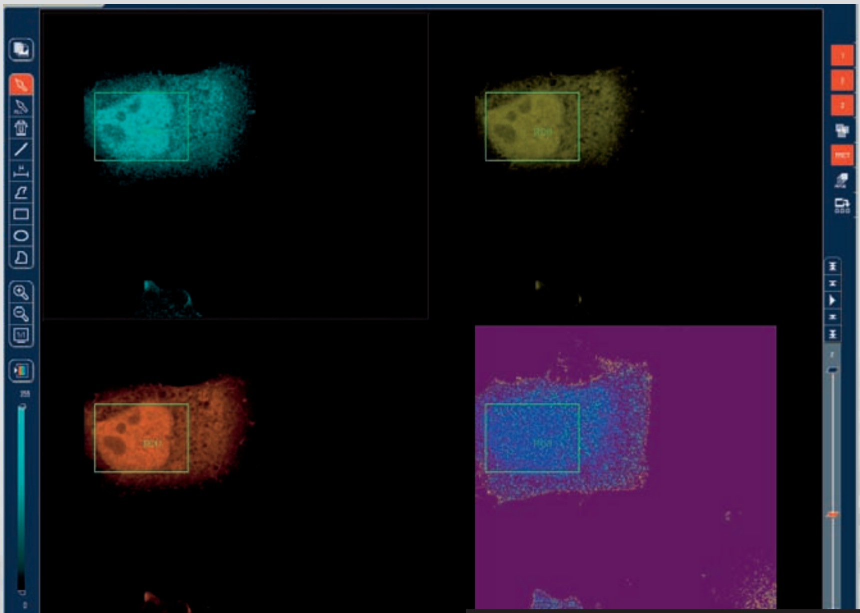
$$E_A(i) = \frac{B - A \times \beta - C \times (\gamma - \alpha \times \beta)}{C \times (1 - \beta \times \delta)}$$

Ref. Van Rheenen, J., M. Langeslag, K. Jalink: Correcting Confocal Acquisition to Optimize Imaging of Fluorescence Resonance Energy Transfer by Sensitized Emission. Biophysical Journal, Vol. 86, April 2004: 1-13.

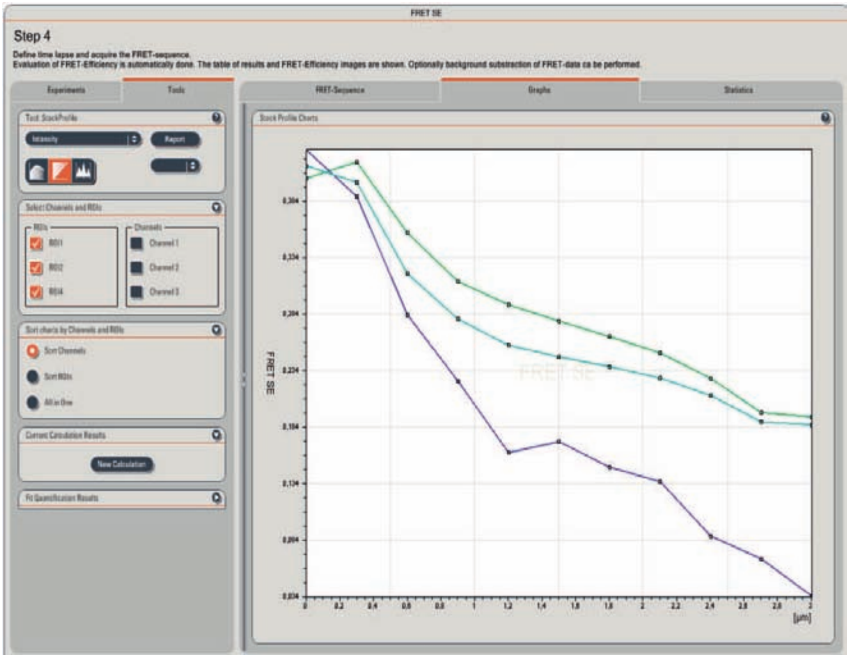
Method 3:

$$E_A(i) = \frac{B}{A}$$

The Ratiometric Calculation is used in samples with a fixed stochiometry (1:1) of donor and acceptor (e.g. Cameleons).



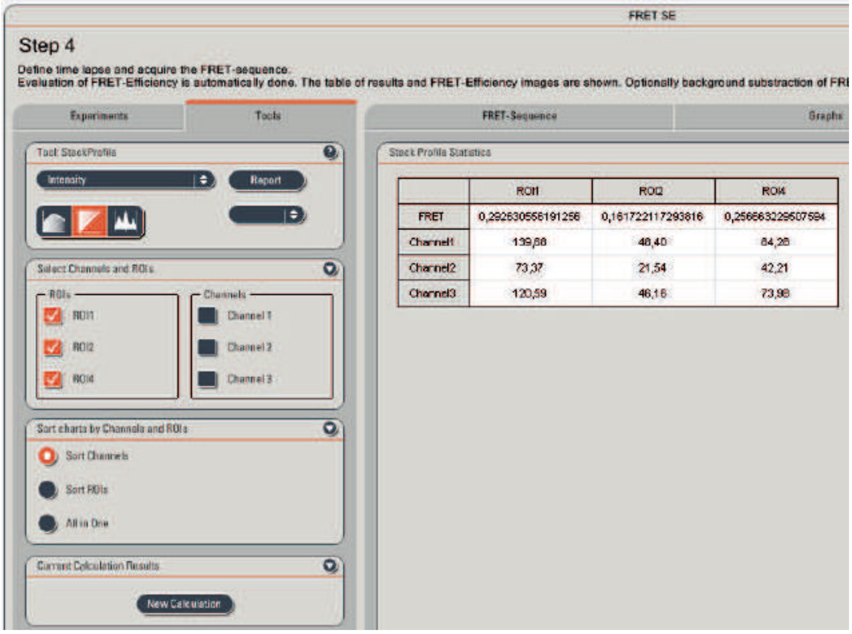
Step 4: Calculation of FRET efficiencies and live cell measurements



FRET SE

Step 4

Define time lapse and acquire the FRET-sequence.
Evaluation of FRET-Efficiency is automatically done. The table of results and FRET-Efficiency images are shown. Optionally background substraction of FRET-data ca be performed.



FRET SE

Step 4

Define time lapse and acquire the FRET-sequence.
Evaluation of FRET-Efficiency is automatically done. The table of results and FRET-Efficiency images are shown. Optionally background substraction of FRI

	ROI1	ROI2	ROI4
FRET	0,292630556191256	0,161722117293816	0,256663229507594
Channel1	139,88	48,40	84,26
Channel2	73,37	21,54	42,21
Channel3	120,59	46,16	73,98

To see FRET and intensity values displayed in a graph during the experiment change to the tab sheet **Graph**. Draw one or multiple ROIs in the image viewer to see intensities displayed in the graph.

For apparent FRET efficiencies change to tab sheet **Statistics**.

Single FRET images (e.g. fixed specimen) can be analyzed too, but will show results only under **Statistics** and not in the graphical display. Change to tab sheet **Statistics** and use ROI functionality to choose the appropriate regions of interest in the image.

Suggested background reading:

- Gadella T.W.J., G.N.M. Van der Krogt, T. Bisseling. GFP-based FRET Microscopy in Living Plant Cells. Trends Plant Sci. 4(7) 287-291 (1999)
- Gordon et al.: Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy. Biophysical Journal, Vol. 74 2702-2713 (1998)
- Lippincott-Schwartz, J., E. Snapp and A.K. Kenworthy. Studying protein dynamics in living cells. Molecular Cell Biology, 2 444-456 (2001)
- Van Rheenen, J., M. Langeslag, K. Jalink: Correcting Confocal Acquisition to Optimize Imaging of Fluorescence Resonance Energy Transfer by Sensitized Emission. Biophysical Journal, Vol. 86 1-13 (2004)
- Wouters F.S., P.J. Verveer & P.I.H. Bastiaens. Imaging biochemistry inside cells. Trends Cell Biol., Vol. 5: 203-211 (2001)
- Zal T., R.J. Gascoigne: Photobleaching-Corrected FRET Efficiency Imaging of Live Cells. Biophysical Journal, Vol. 86 3923-3939 (2004)
- Zhang, J., R.E. Campbell, A.Y. Ting, R.Y. Tsien: Creating new fluorescent probes for cell biology. Nature, Vol. 3 (2002)

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Singapore		Tel. +65 6779 7823	Fax +65 6773 0628
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Sweden:	Sollentuna	Tel. +46 8 625 45 45	Fax +46 8 625 45 10
Switzerland:	Glattbrugg	Tel. +41 1 809 34 34	Fax +41 1 809 34 44
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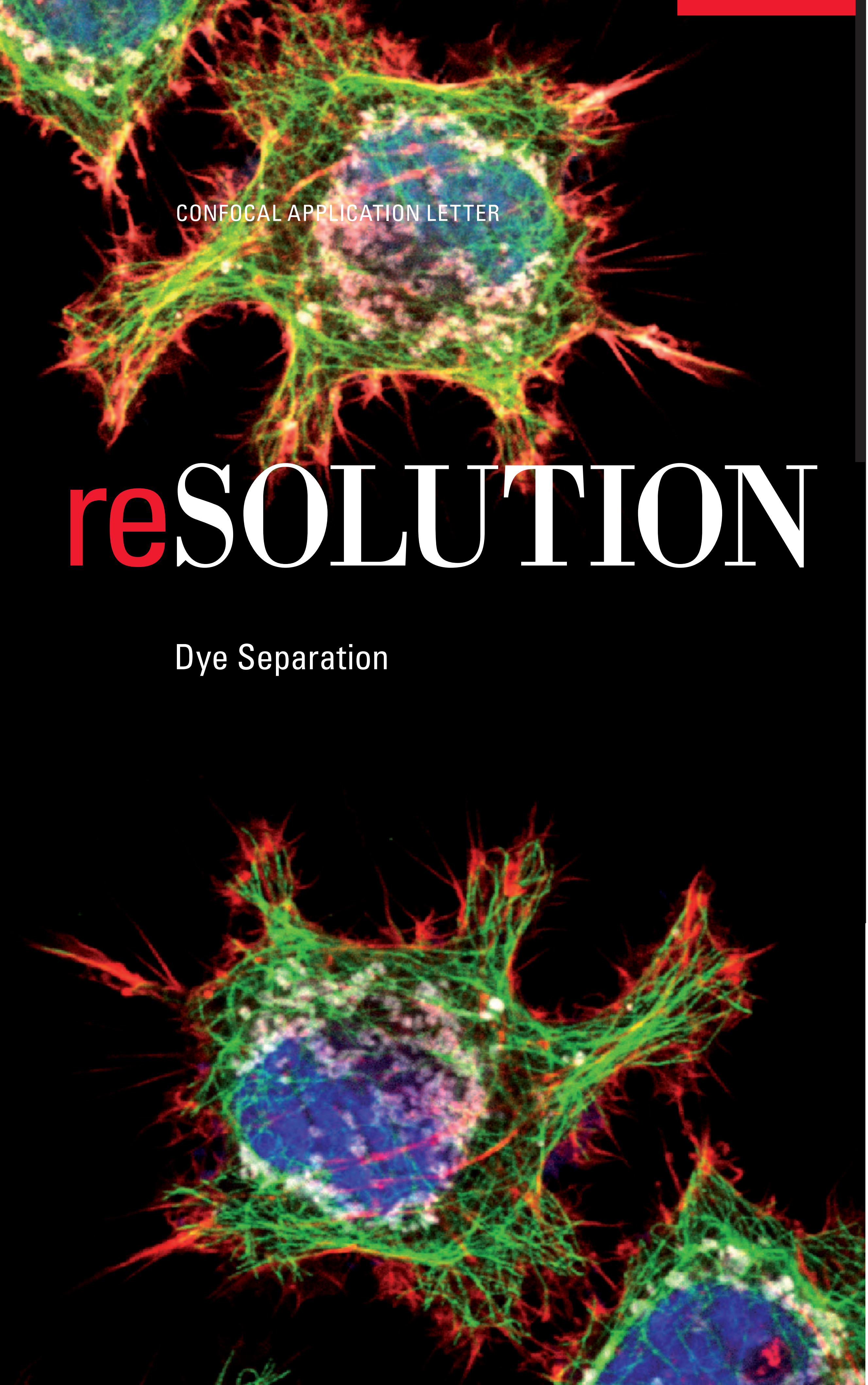
CONFOCAL APPLICATION LETTER

reSOLUTION

Dye Separation



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.

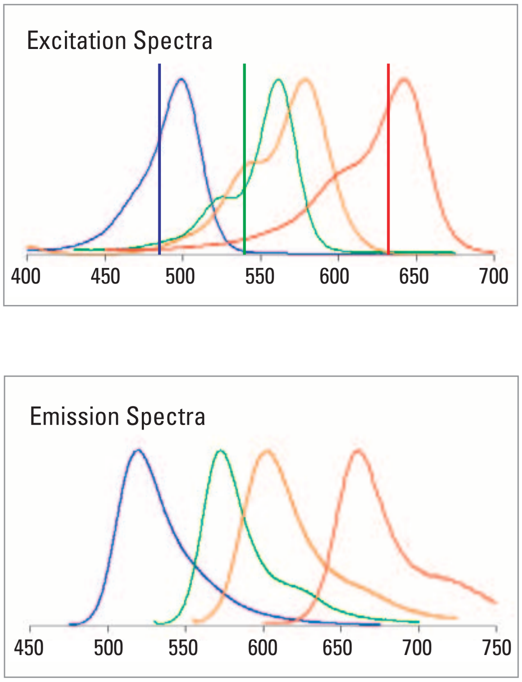


Fig.1: Excitation and emission spectra of Alexa 488, Alexa 546, Alexa 568 and TOTO-3.

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 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 $3/4$ of the entire green signal while $1/4$ 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 $4/5$ of the red signal is seen in the red channel and $1/5$ of the signal goes into the green channel.

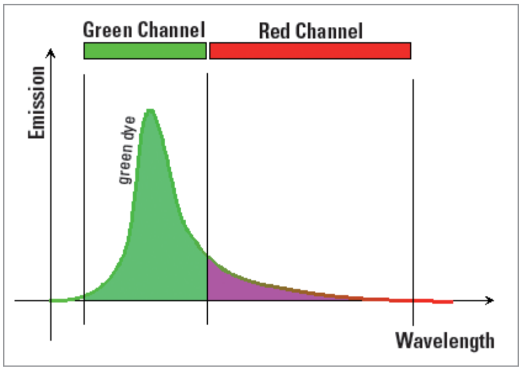


Fig. 2a: Emission spectrum of the green channel
We estimate here:
3/4 of all FITC emission goes into the green channel
1/4 of all FITC emission goes into the red channel

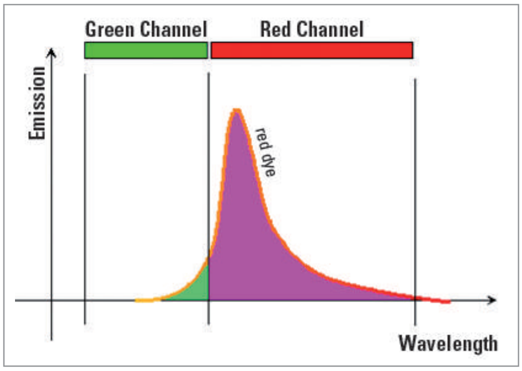


Fig. 2b: Emission spectrum of the red channel
We estimate here:
1/5 of all TRITC emission goes into the green channel
4/5 of all TRITC emission goes into the red channel

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

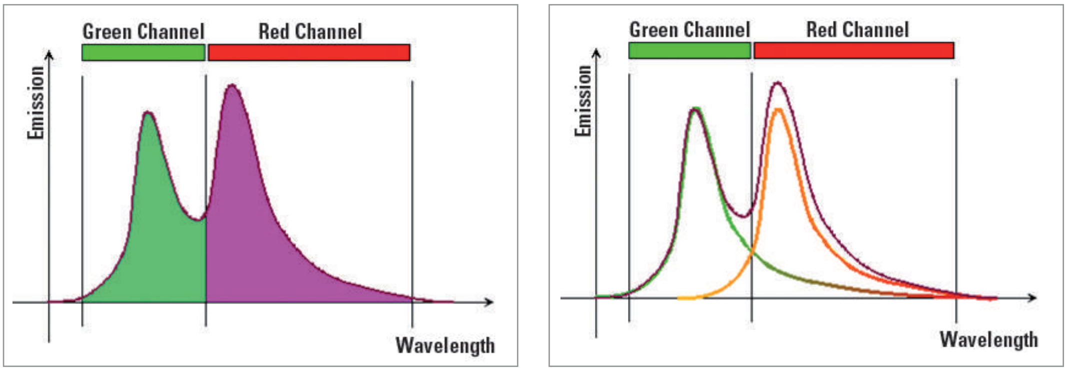


Fig. 2c: Emission signals of a double-labeled sample. The black curve represents the sum of the signals of both fluorophores.

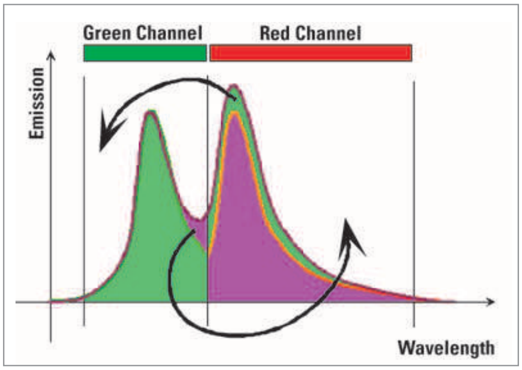


Fig. 2d: Removing crosstalk:
1/4 of all FITC emission has to be removed from red channel
1/5 of all TRITC emission has to be removed from green channel

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:

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

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 homogenous.

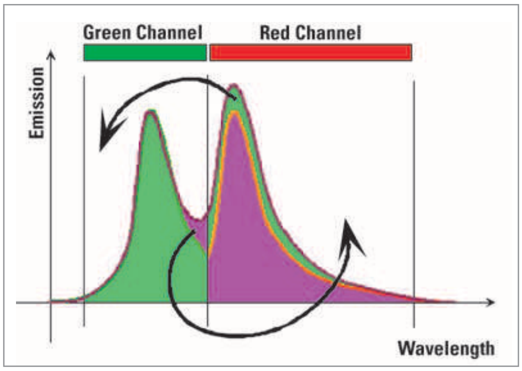
Separation of non-balanced fluorophores
Sometimes the emissions of different fluorophores are not well balanced in intensities. In this case a weak signal may be partially overlaid by the crosstalk coming from a strong signal. With the Dye Separation tool it is possible to separate both fluorophores.

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.

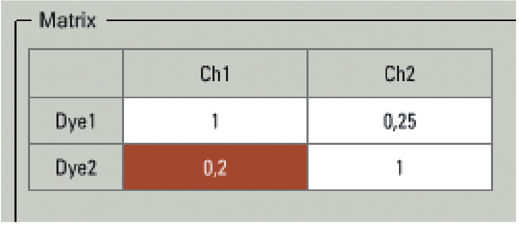
Note: The **Spectral Dye Separation** tool cannot be used in the special case where the fluorophores are both excited by a single laser line AND their intensities are significantly different.

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 1/5 of the TRITC signal has to be removed from the green channel and 1/4 FITC signal has to be removed from the red channel. The user only needs to apply the estimated numbers to a 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.

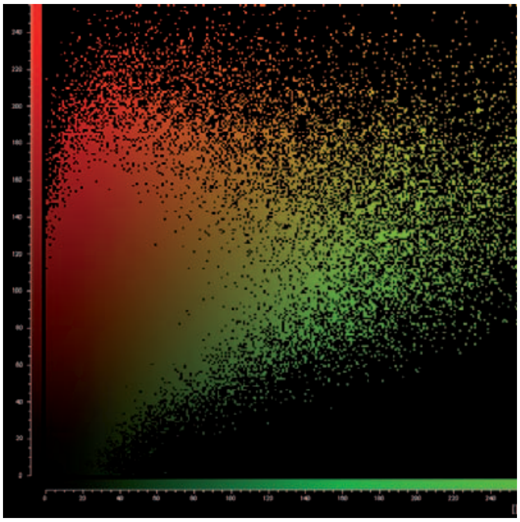


Fig. 3: Cytofluorogram shows the intensity relationships between two channels.

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 cytofluogram 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.

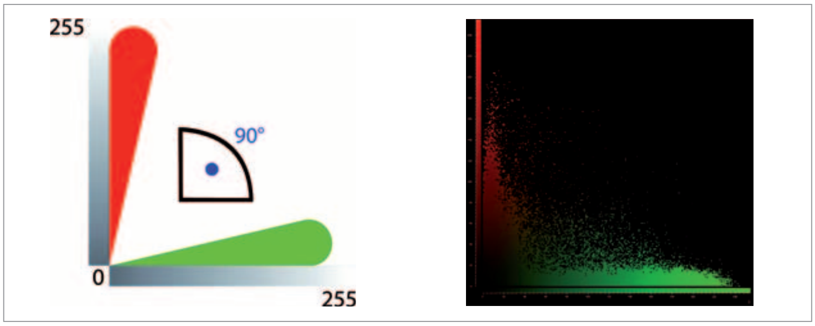


Fig. 4a: Ideal separation of fluorescent signals, without crosstalk; each channel is related to its own dye.

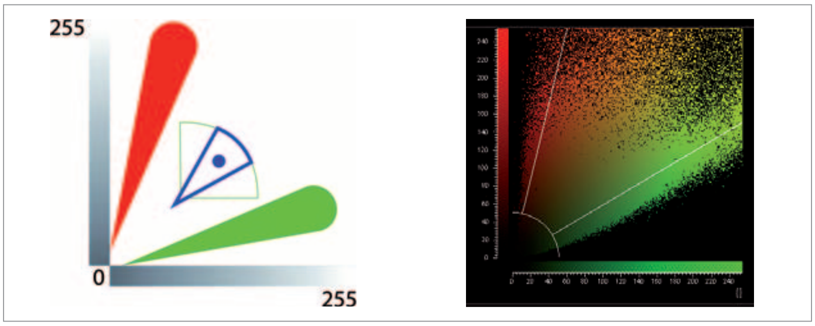
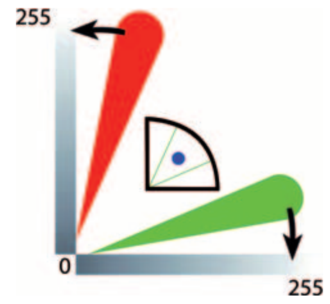


Fig. 4b: Fluorescent signals if crosstalk occurs: the clouds are tilted towards the diagonal.



The **Automatic Dye Separation (Weak & Strong)** methods will move the clouds to the axes.

Fig. 4c: Correction of crosstalk

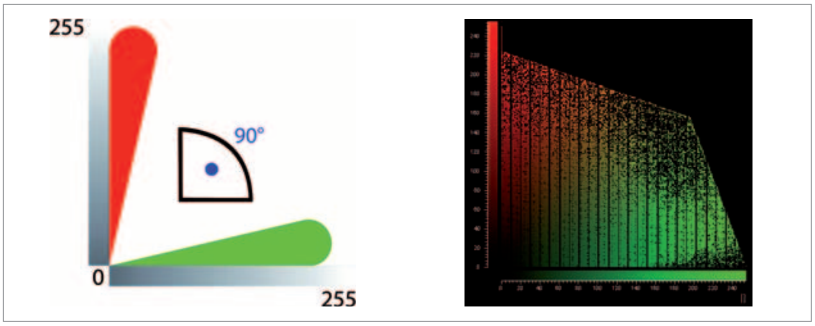
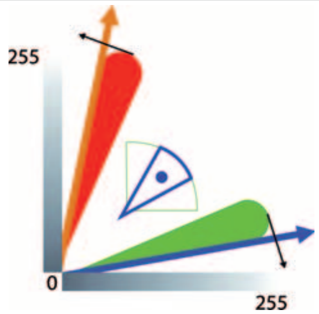


Fig. 4d: After processing: Ideal separation of fluorescent signals, each channel is related to its own dye.

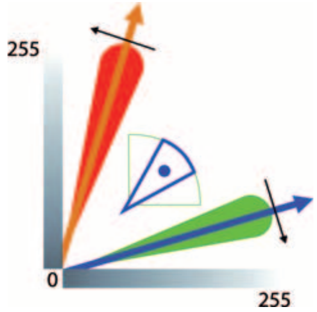
What is the difference between Weak and Strong Dye Separation?

Weak:



The weak method of **Automatic Dye Separation** will move the clouds until they just touch the axes.

Strong:



The strong method of **Automatic Dye Separation** will move the clouds to coincide with the axes.

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

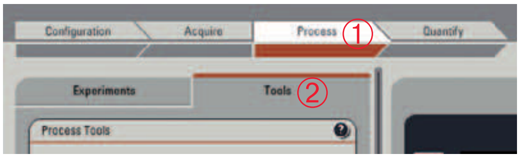
Automatic Dye Separation: Weak & Strong

- 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.

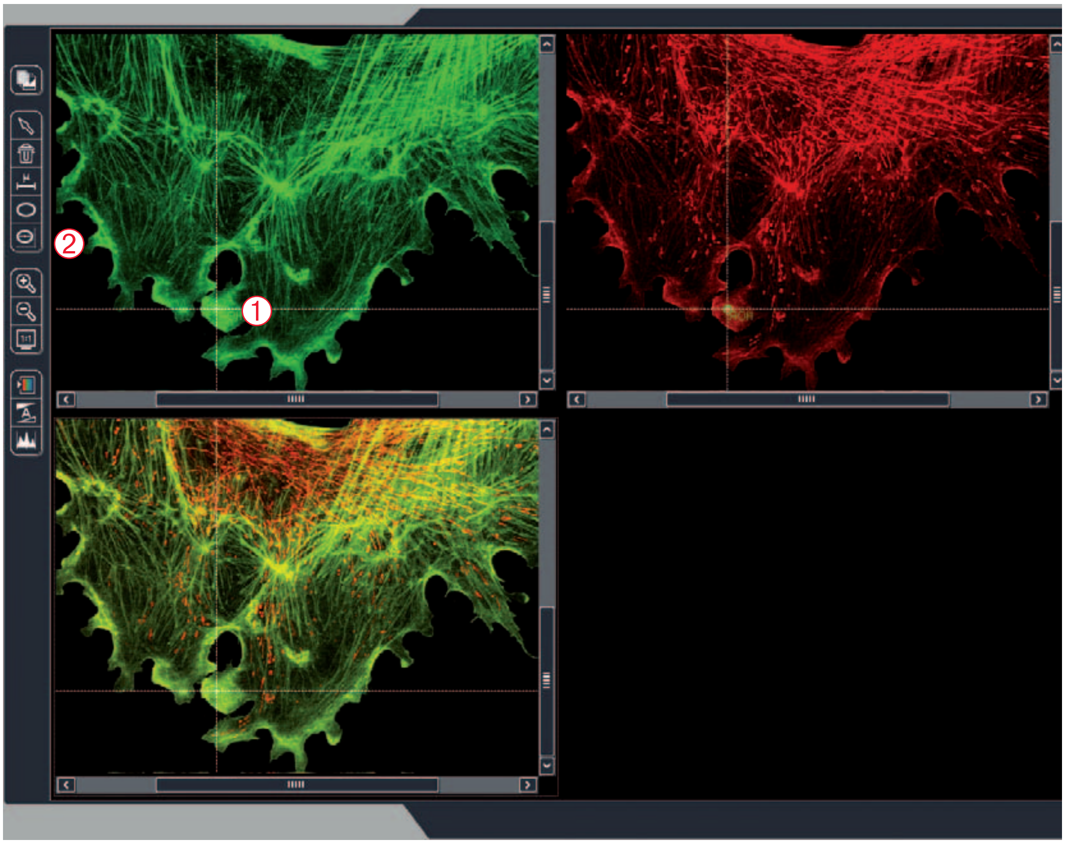


3.1 Channel Dye Separation: Step by Step

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

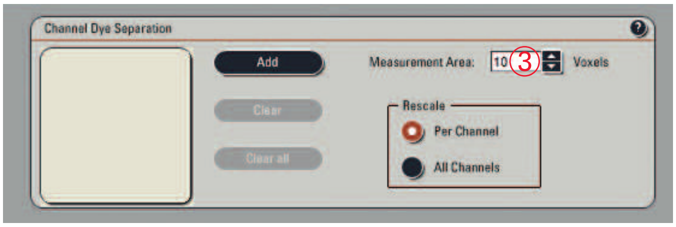
1. Place the crosshair in the viewer ① to a position that clearly contains a single dye.
You can also draw a ROI manually after activating the ROI function in the viewer ②.



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

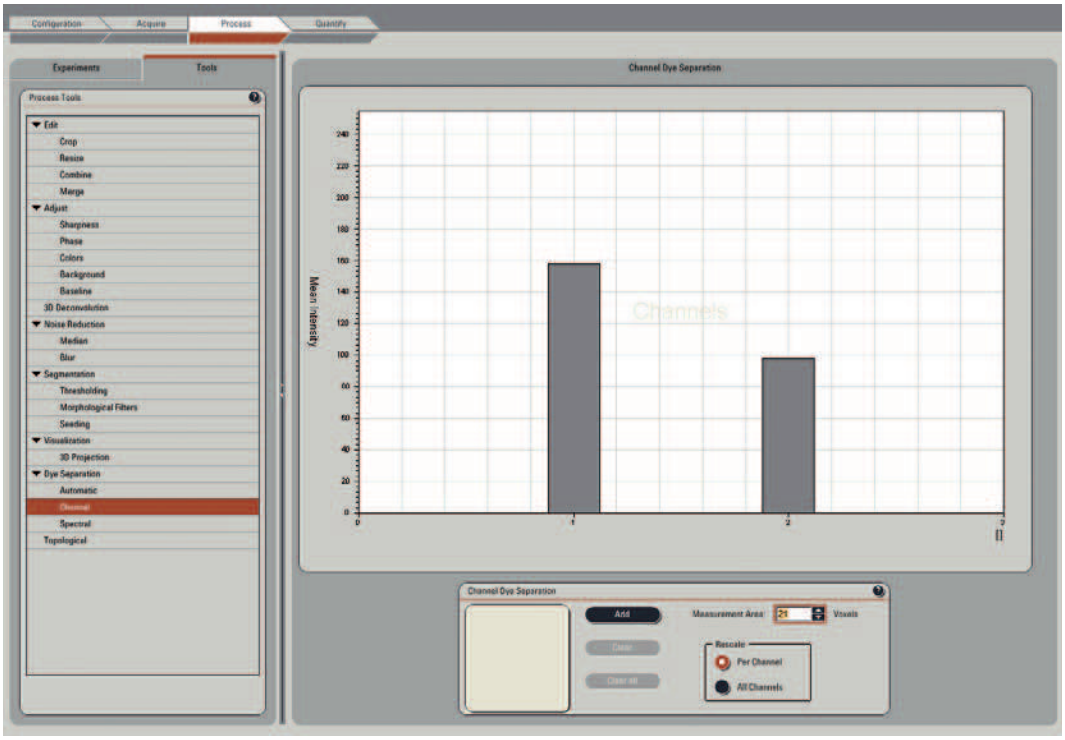
Note: If there is no crosshair visible in the viewer you can activate it by clicking on the **Crosshair** button ⊖.

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



Measurement Area: 10 Voxels

Rescale
● Per Channel
○ All Channels



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.

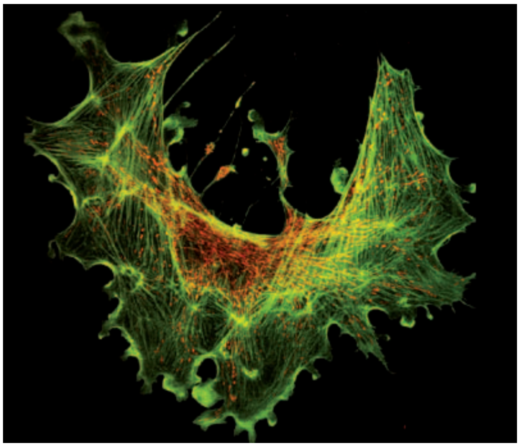
There are two options for rescaling:
Per Channel: All channels are rescaled separately to spread the dynamic range of the images over the entire range of bit depth (e.g. 8 bit from 0 to 255). This operation results in brighter images, but these images cannot be further quantified.

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.

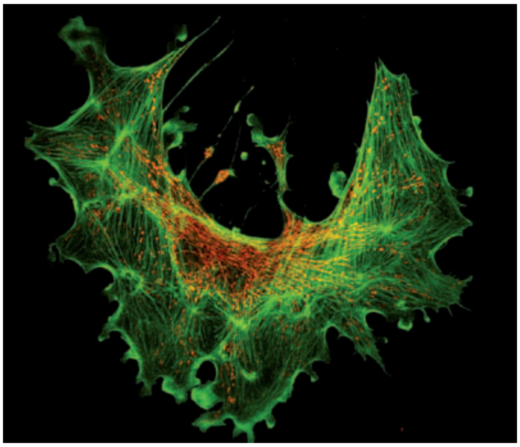
6. Click **Apply** ⑧ to perform the image processing.

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

Original image with crosstalk



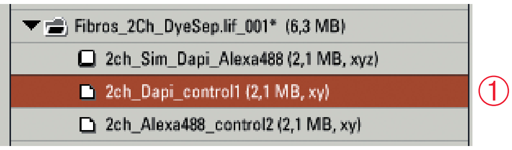
Resulting image without crosstalk



B. Using reference images

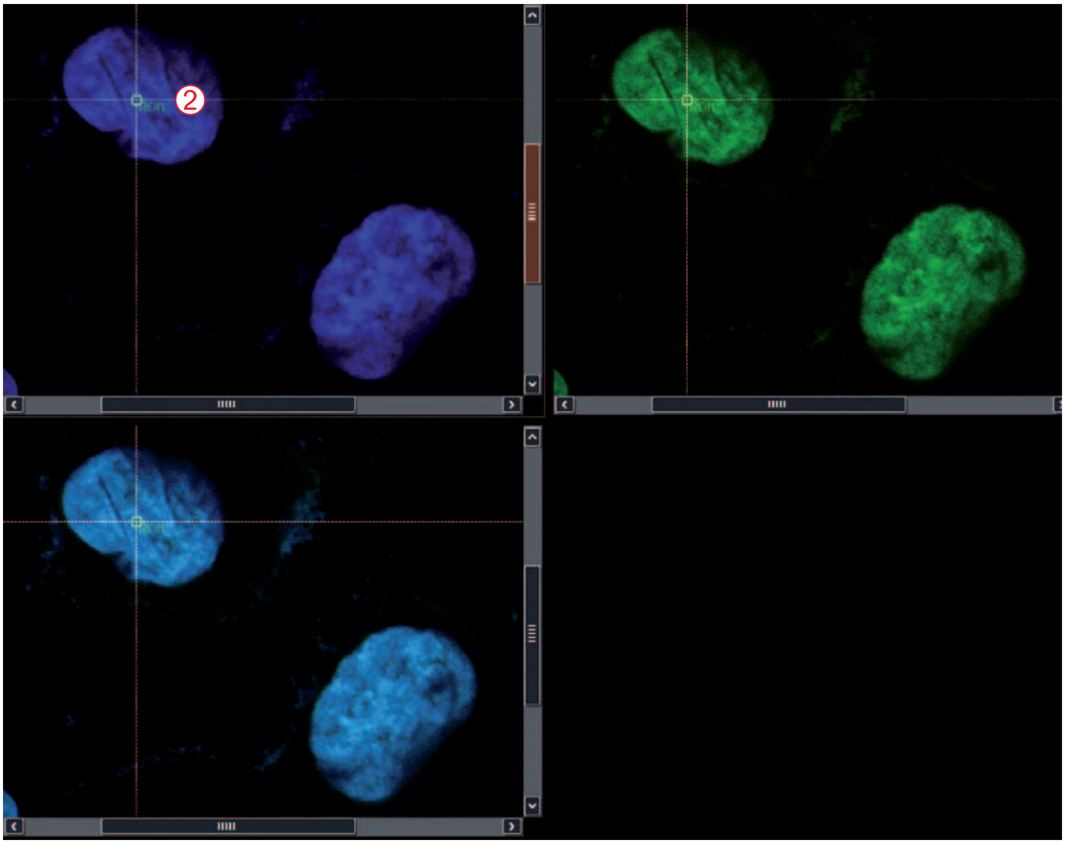
If you want to use control images of single labeled specimens as reference samples, you must capture all of the images using the same detection parameters that were chosen for the multi-labeled sample.

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



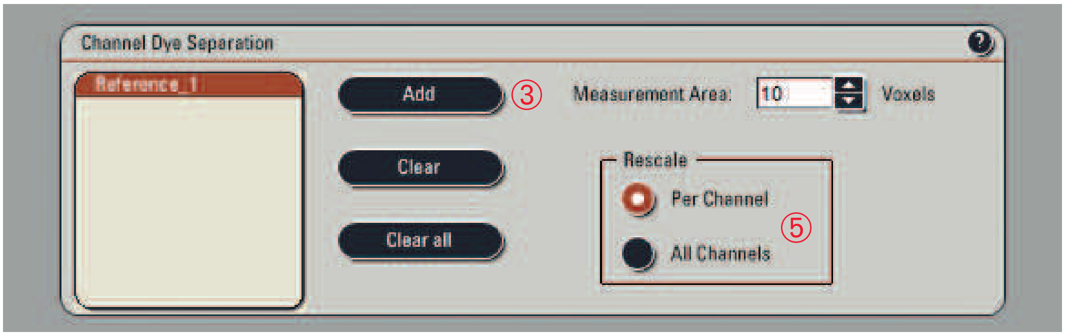
Fibros_2Ch_DyeSep.lif_001* (6,3 MB)
2ch_Sim_Dapi_Alexa488 (2,1 MB, xyz)
2ch_Dapi_control1 (2,1 MB, xy)
2ch_Alexa488_control2 (2,1 MB, xy)

2. 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.

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


Channel Dye Separation

Reference_1

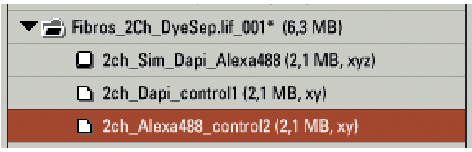
Measurement Area: 10 Voxels

Rescale

● Per Channel

○ All Channels

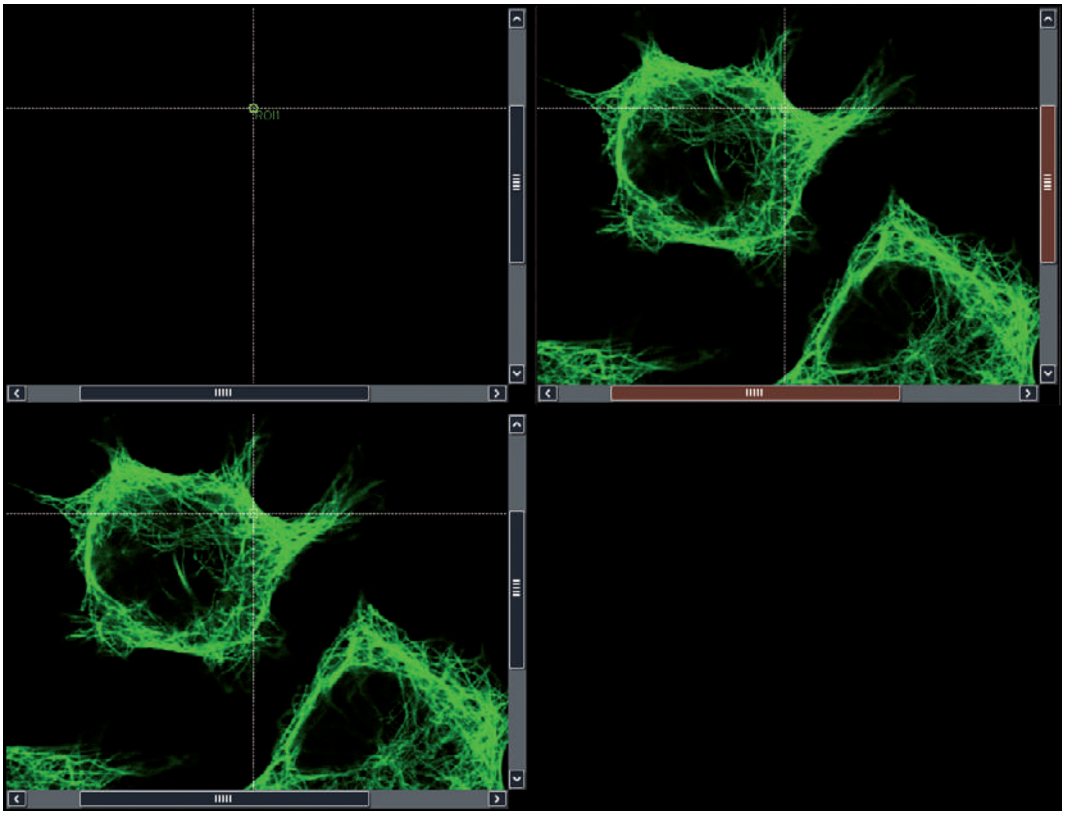
4. Select the second single-dye control image in the experiment tab ④.



Fibros_2Ch_DyeSep.lif_001* (6,3 MB)
2ch_Sim_Dapi_Alexa488 (2,1 MB, xyz)
2ch_Dapi_control1 (2,1 MB, xy)
2ch_Alexa488_control2 (2,1 MB, xy)

④

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

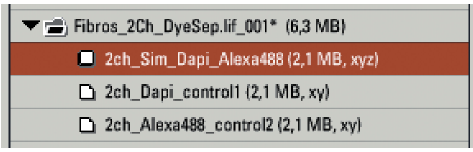


HeLa cells, reference 2: single-labeled cells imaged using the same conditions as the double-labeled sample; no signal recorded in the 1. channel; green (2. channel): Alexa 488, tubulin.

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.

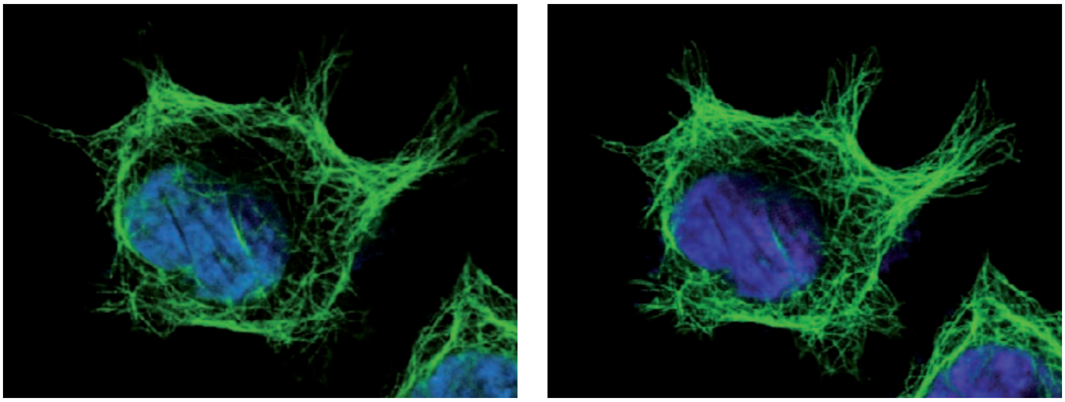


Fibros_2Ch_DyeSep.lif_001* (6,3 MB)
2ch_Sim_Dapi_Alexa488 (2,1 MB, xyz)
2ch_Dapi_control1 (2,1 MB, xy)
2ch_Alexa488_control2 (2,1 MB, xy)

⑥

9. Click **Apply**.

Separation of two fluorophores

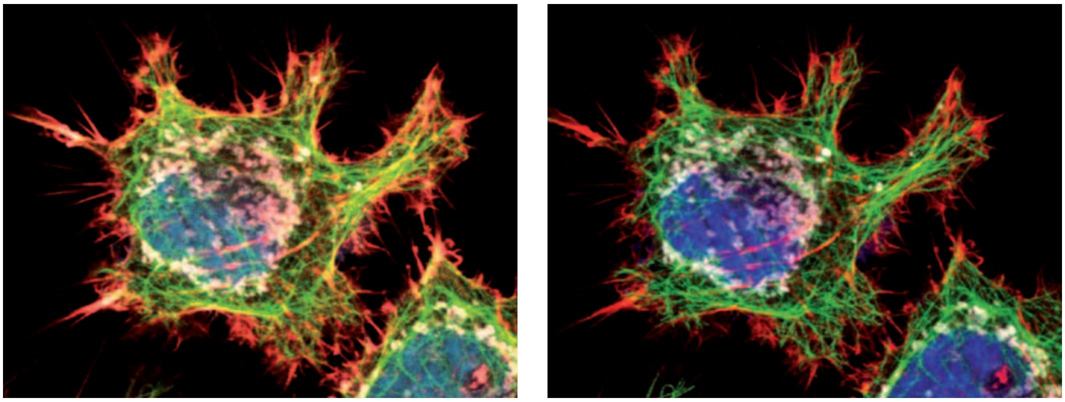


Original image with crosstalk

Resulting image without crosstalk

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

Separation of four fluorophores



Original image with crosstalk

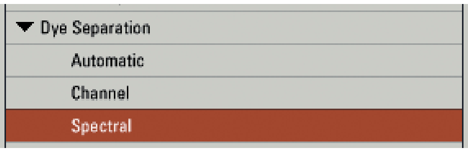
Resulting image without crosstalk

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

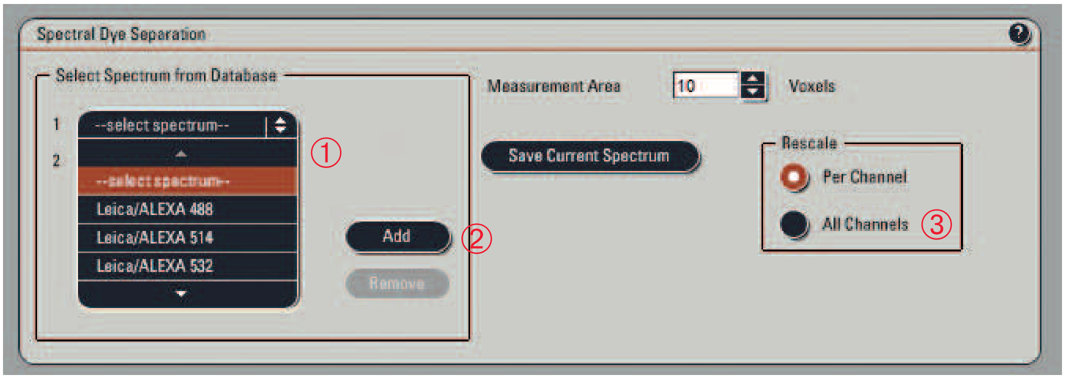
Select the **Spectral Dye Separation** tool.

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.



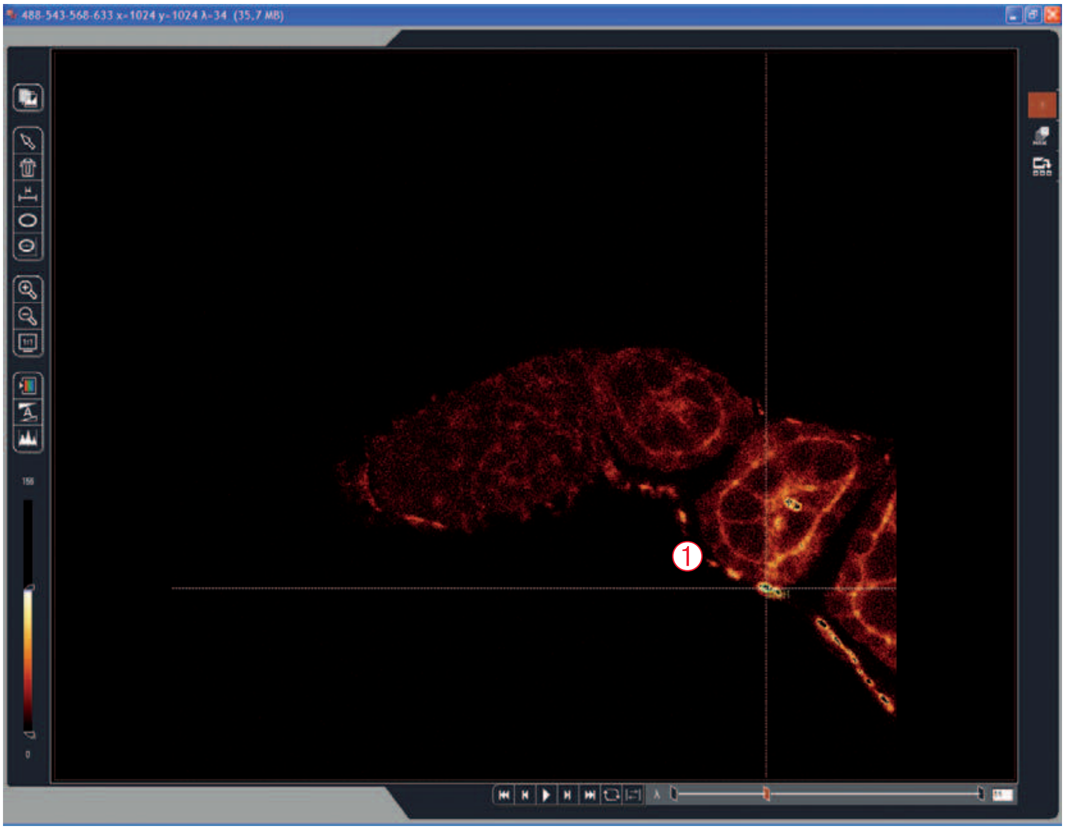
2. Choose a method of rescaling ③ (see **Channel Dye Separation**, page 12).

3. Click **Apply**.

B. Using measured reference spectra

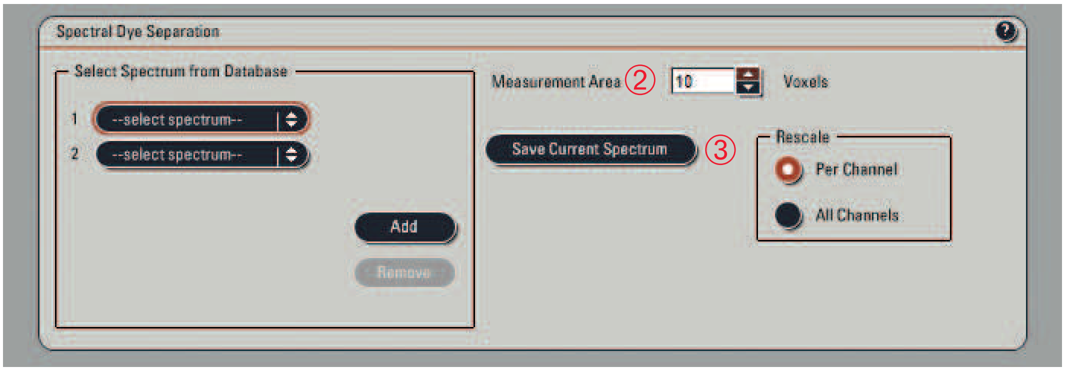
1. Place the crosshair ① in a region of your choice. You can also draw a ROI manually.

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).

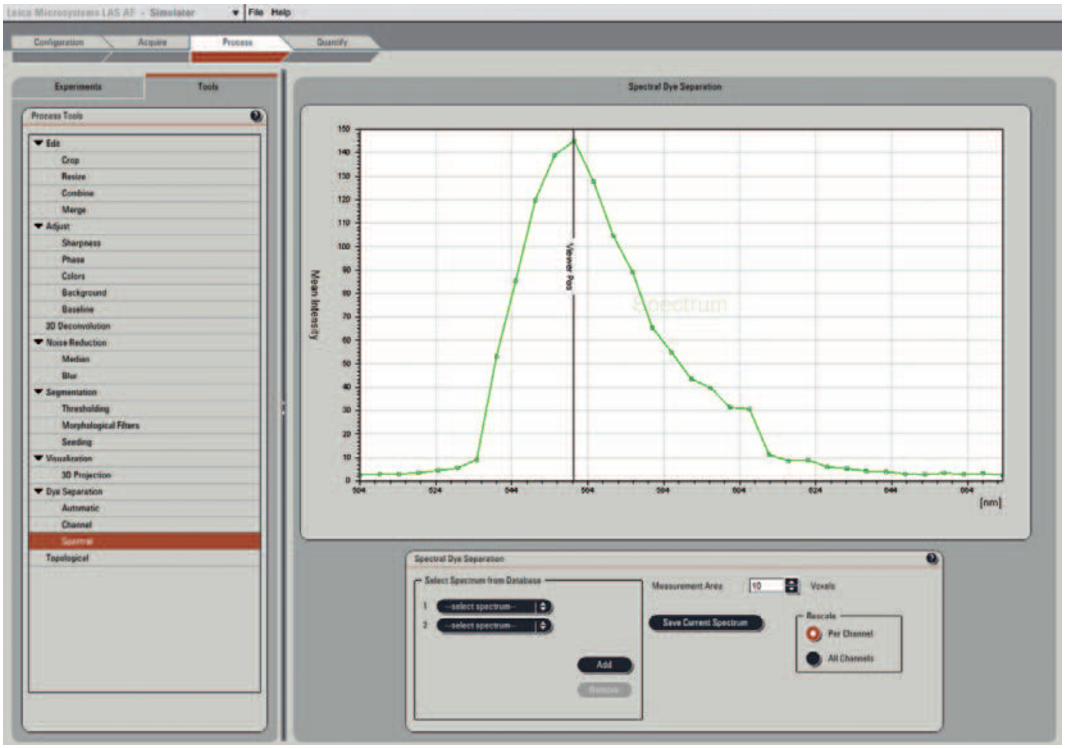
3. Click **Save Current Spectrum** ③ to add the actual spectrum to the **Spectra Database**.



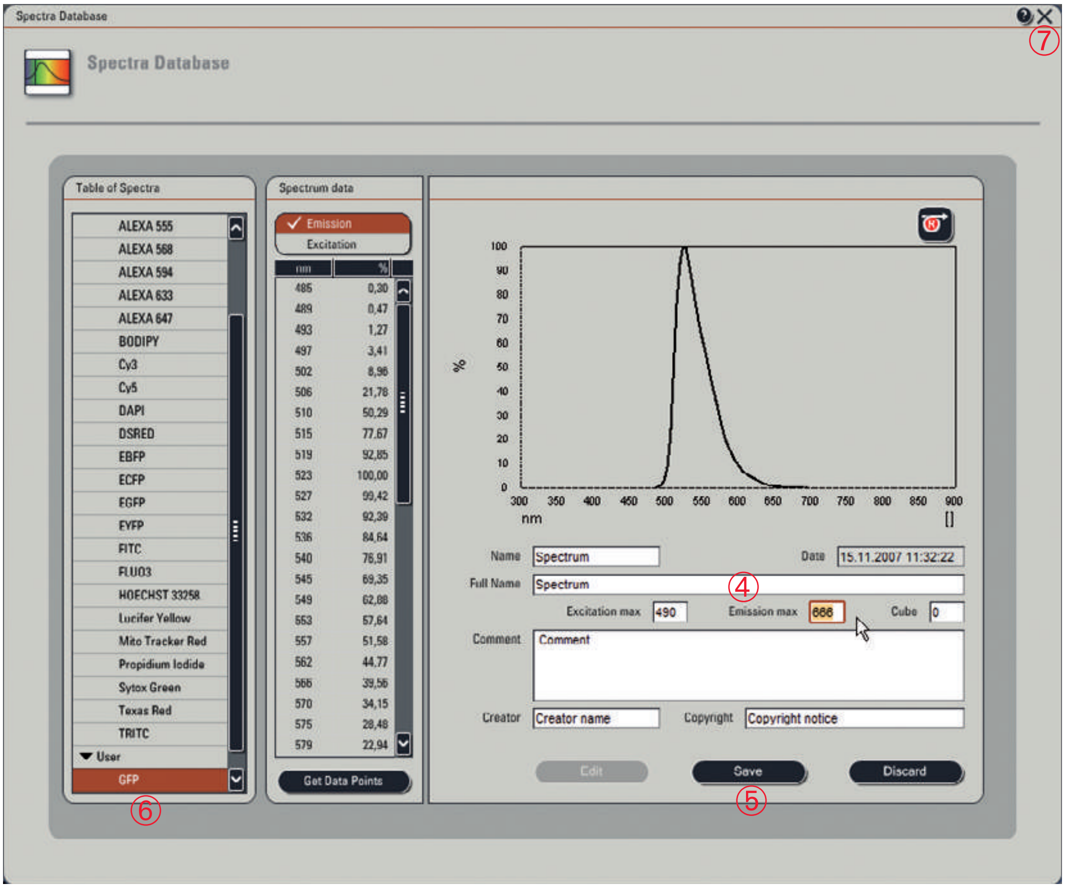
Measurement Area ② 10 Voxels

Rescale
● Per Channel
○ All Channels

A dialog box will open automatically.



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

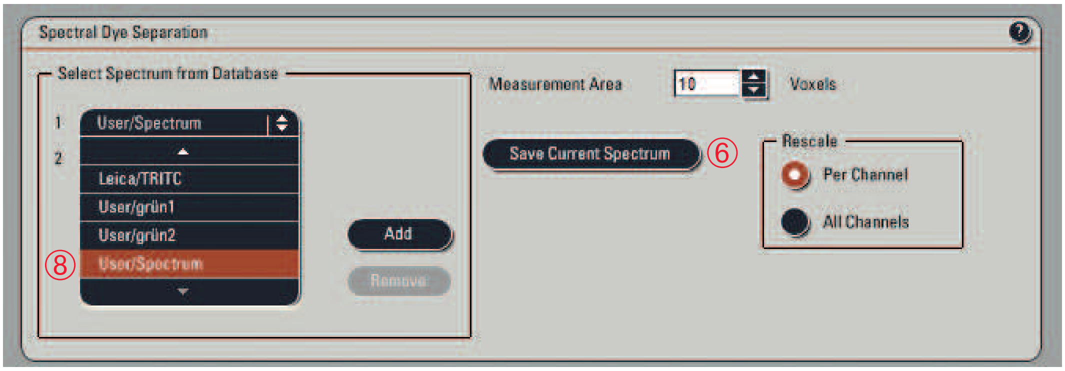


nm	%
485	0,30
489	0,47
493	1,27
497	3,41
502	8,96
506	21,78
510	50,29
515	77.67
519	92,85
523	100,00
527	99,42
532	92,39
536	84,64
540	76,91
545	69,35
549	62,88
553	57,64
557	51,58
562	44.77
566	39,56
570	34,15
575	28,48
579	22,94

Name: Spectrum
Date: 15.11.2007 11:32:22
Full Name: Spectrum
Excitation max: 490
Emission max: 666
Cube: 0
Comment: Comment
Creator: Creator name
Copyright: Copyright notice

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.


Spectral Dye Separation

Select Spectrum from Database

1 User/Spectrum

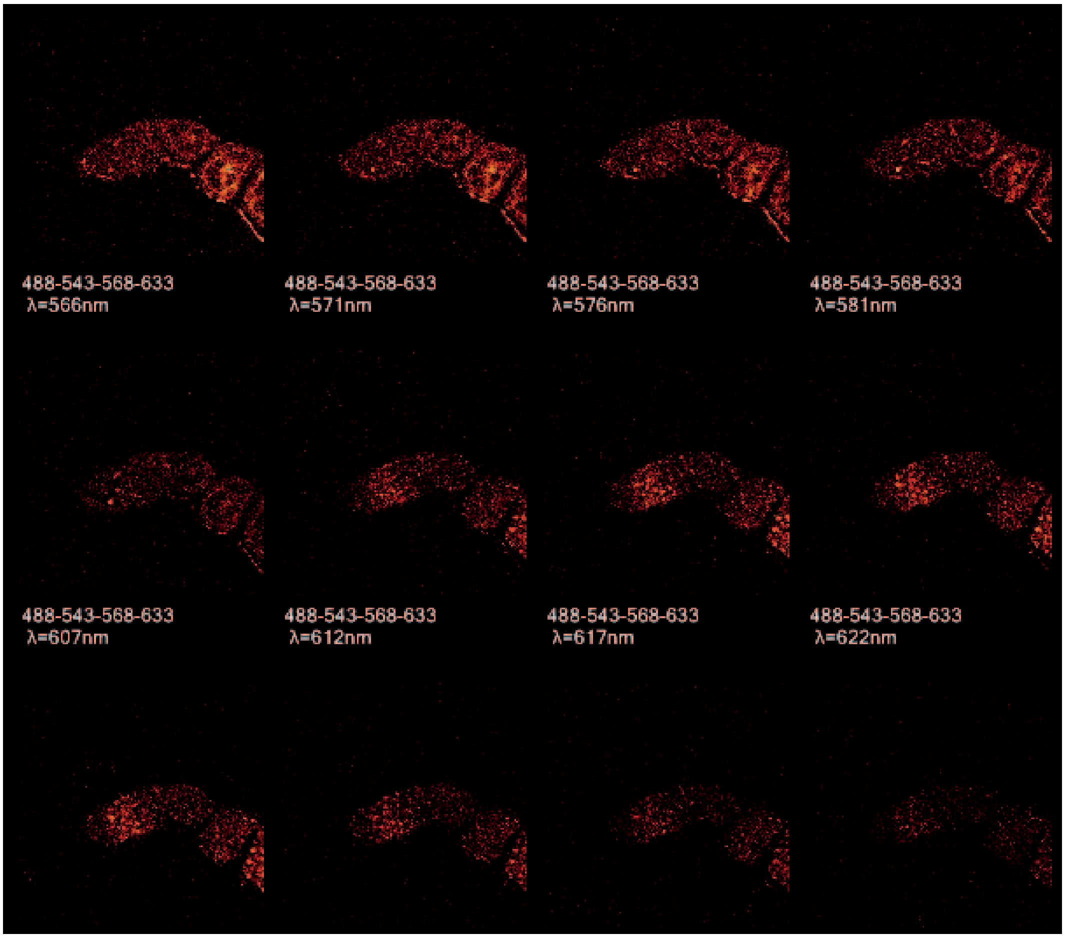
2

Measurement Area 10 Voxels

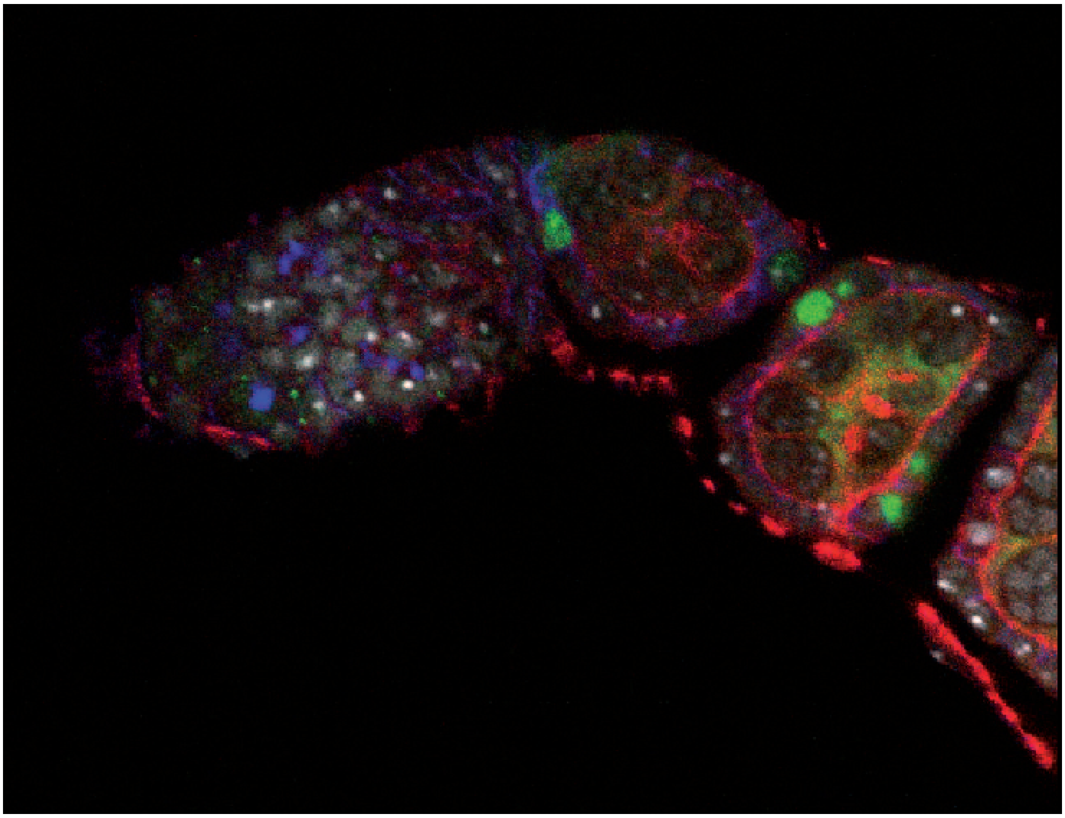
Rescale

● Per Channel

○ All Channels



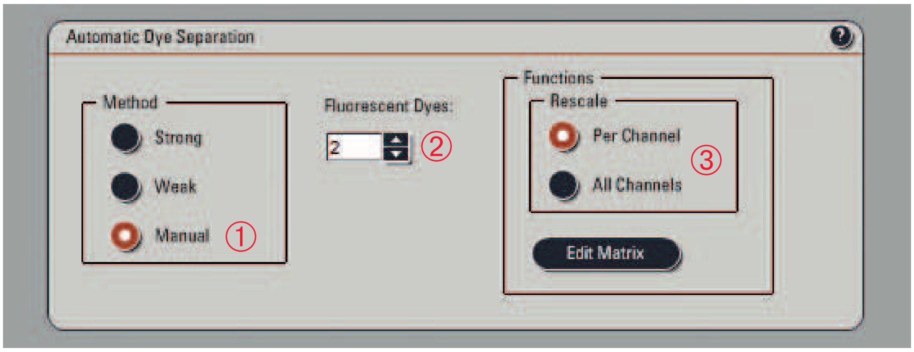
Part of the image gallery of a lambda series of *Drosophila melanogaster* stained with Alexa 488, Alexa 546, Alexa 568 and TOTO-3.



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.



Method
○ Strong
○ Weak
● Manual ①

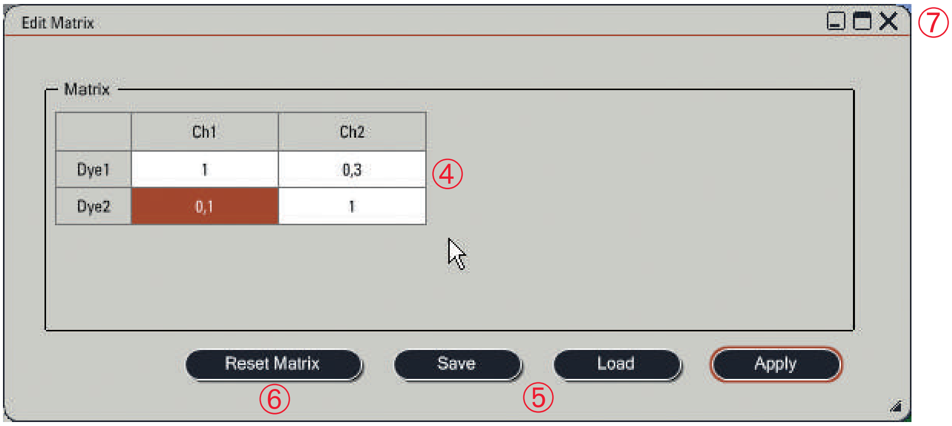
Fluorescent Dyes: 2 ②

Rescale
● Per Channel ③
○ All Channels

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).



Matrix

	Ch1	Ch2
Dye1	1	0,3
Dye2	0,1	1

④

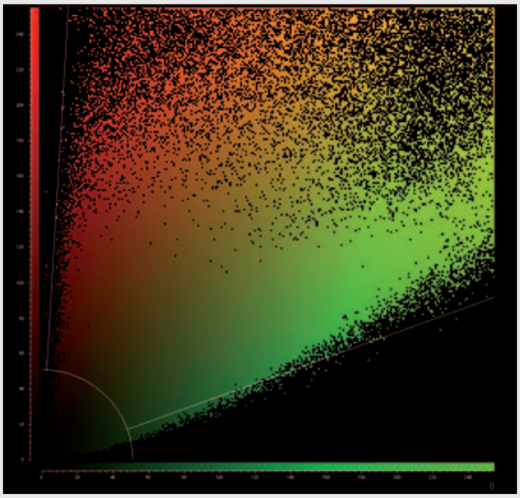
3. Type the desired coefficients into the matrix fields ④. In this example we correct $^{1}/_{3}$ cross-talk from the green dye in the red channel and $^{1}/_{10}$ cross-talk from the red dye in the green channel.

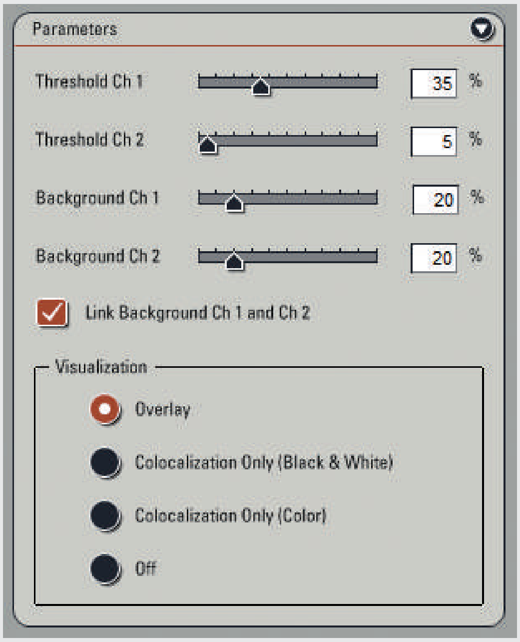
The matrix can be saved and reloaded ⑤ for reproducibility. Keep in mind that in order to use the same contribution coefficients in the matrix, identical recordings using the same parameters must be taken.

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.





Parameters

Threshold Ch 1 35 %

Threshold Ch 2 5 %

Background Ch 1 20 %

Background Ch 2 20 %

☑ Link Background Ch 1 and Ch 2

Visualization

● Overlay

○ Colocalization Only (Black & White)

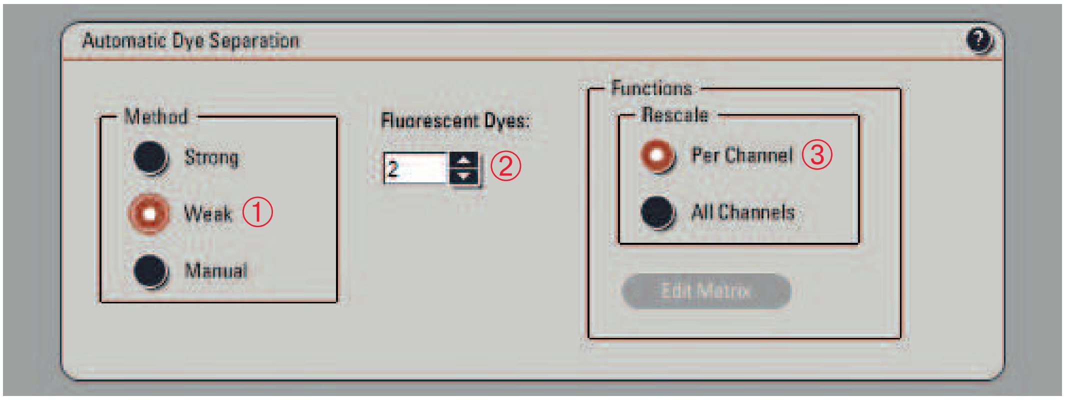
○ Colocalization Only (Color)

○ 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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