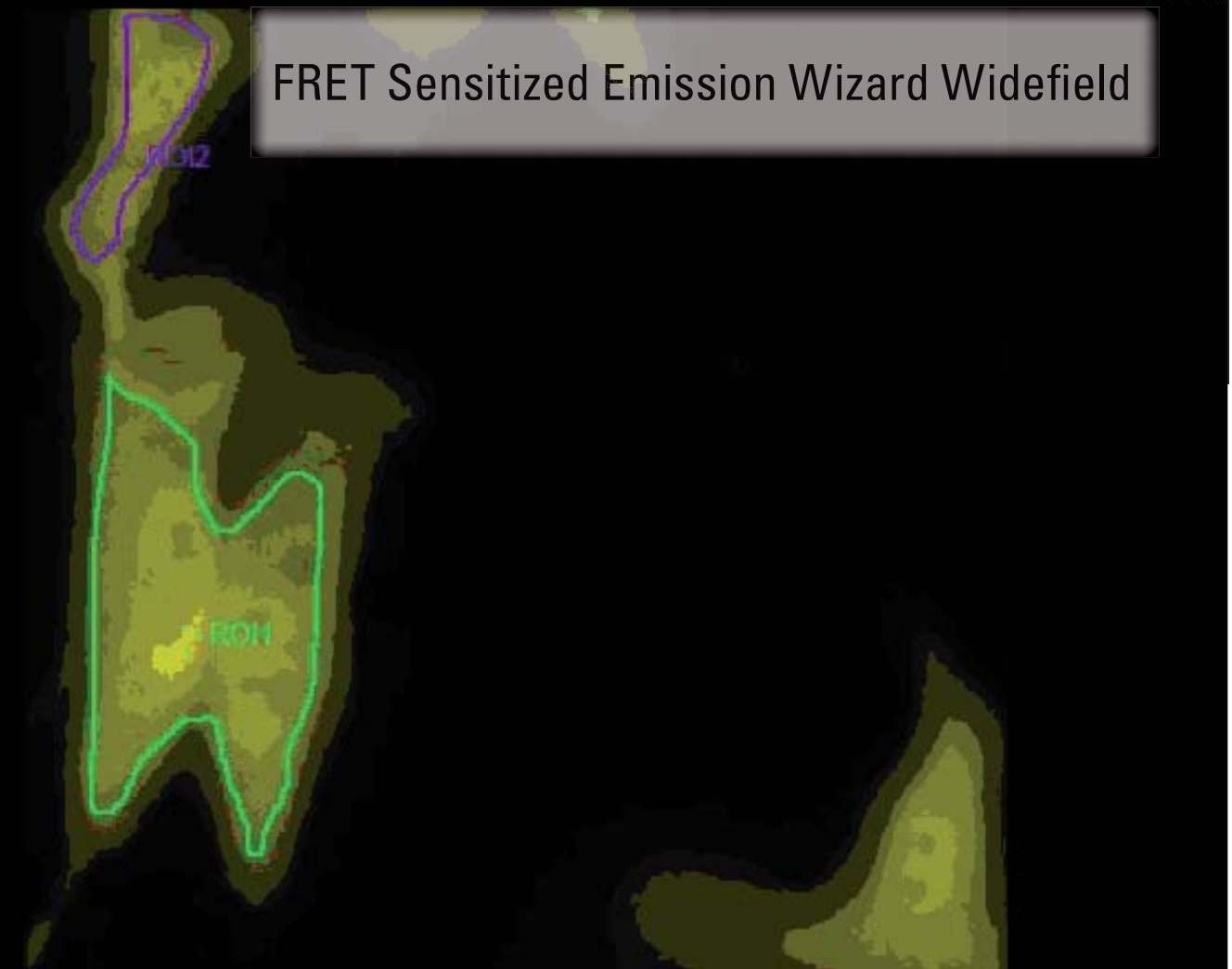
April
2009
No.04

WIDEFIELD APPLICATION LETTER

reSOLUTION

FRET Sensitized Emission Wizard Widefield



Leica
MICROSYSTEMS

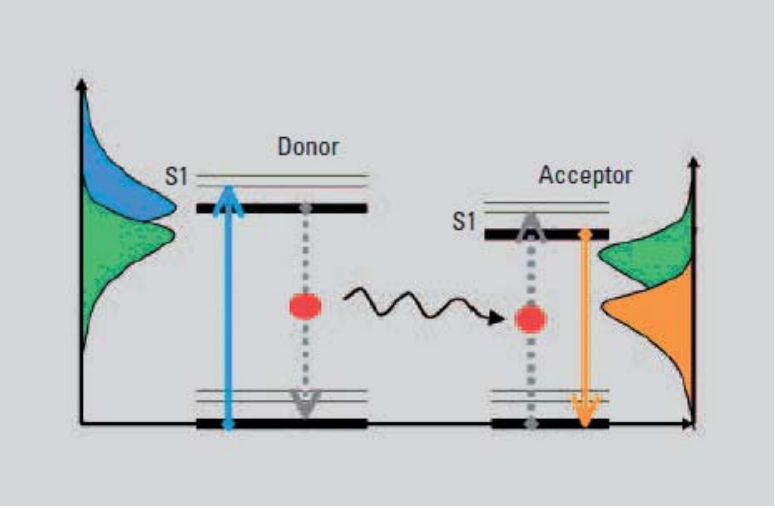
FRET SE with the Leica Advanced Widefield Systems AF7000, AF6500 and AF6000

FRET Sensitized Emission (FRET SE)

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:

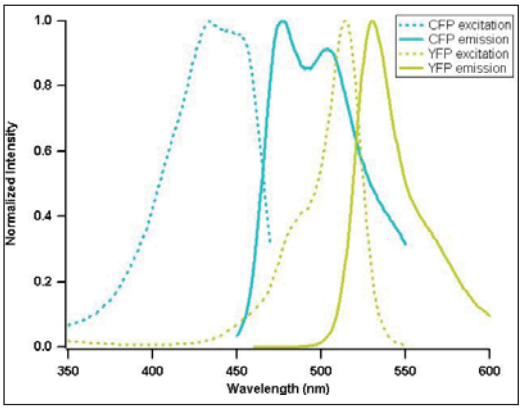
An excited fluorophore, called the donor, transfers its excited state energy to a light absorbing molecule which is called the acceptor. This transfer of energy is non-radiative. Sensitized Emission is one established method for the evaluation of FRET efficiencies. It can be applied to live cells as well as to fixed samples.



The Method:

FRET SE is a method mainly used to analyze protein – protein interactions or conformational changes of proteins. There are several prerequisites for FRET to occur:

- The Donor and Acceptor distance must be <10 nm
- Sufficient separation of excitation and emission spectra are necessary
- The Donor emission spectrum must overlap with the Acceptor absorption spectrum



Excitation and emission spectra of CFP/YFP FRET pair.
Courtesy: T. Zimmermann, EMBL

Due to the necessary overlap between Donor emission spectrum and Acceptor absorption spectrum each FRET measurement is done by sequential acquisition of three channels:

Donor channel: Donor excitation and Donor emission
FRET channel: Donor excitation, Acceptor emission
Acceptor channel: Acceptor excitation and Acceptor emission

Donor and Acceptor channel are used to eliminate crosstalk into the FRET channel. 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.

As this method is non-invasive, it is most frequently used for live cell experiments.

It is important that throughout the entire experiment and calibration routine, all measuring parameters such as camera exposure time, gain, excitation intensities must remain constant.

Starting the FRET Wizard:

Select the FRET SE Wizard in the pull down menu of the LAS AF main menu. The FRET SE Wizard is an optional module within LAS AF and is dongle protected. The application is greyed out if the FRET wizard is not licensed.

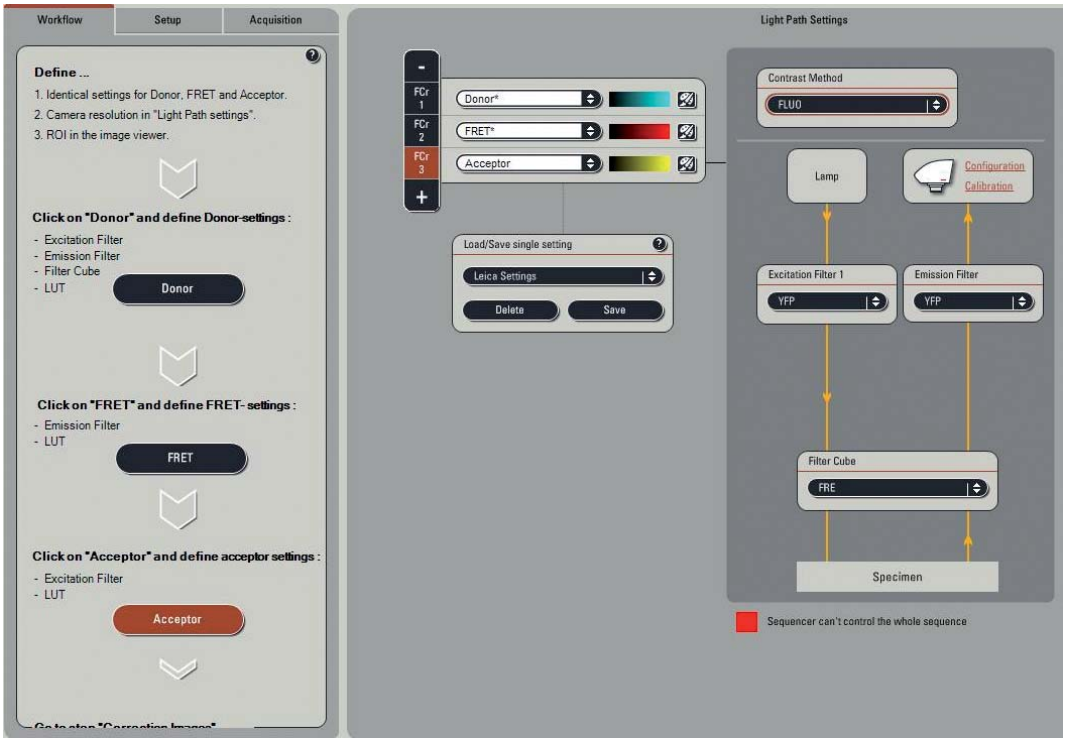
Note: Since the FRET Wizard requires two monitors this application also is greyed out if a second monitor is missing.

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: Setup

This step is for the setup of the acquisition channels and is the most difficult one. All three channels need to have identical settings for exposure time, gain and light intensity. These parameters need to be balanced to keep the signal in all channels below saturation whilst ensuring an image dynamic sufficient for FRET analysis.



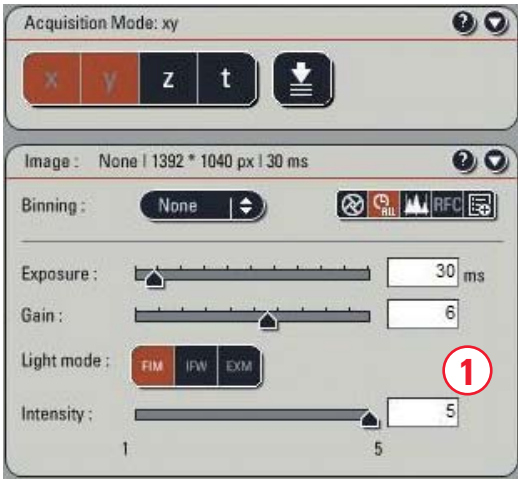
FRET Wizard Step 1: Setup of CFP/YFP FRET channels.
Donor channel: CFPex, CFPem
FRET channel: CFPex, YFPem
Acceptor channel: YFPex, YFPem

Workflow:

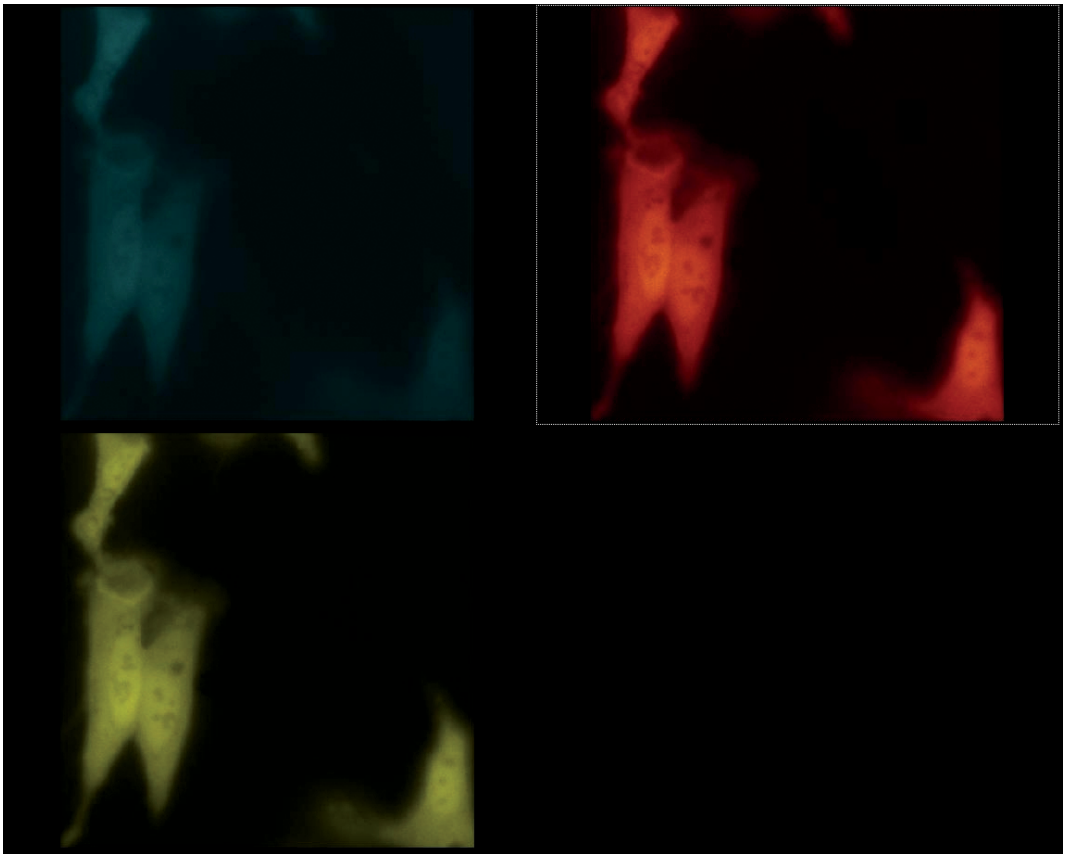
Start with the FRET sample. This sample shows signal in all three channels, enabling the user to balance the settings between the channels. Once the **acquisition parameters (1)** are defined, they will be used for both, the acquisition of the FRET experiment and the acquisition of the Donor only and Acceptor only controls in step 2. If the controls do not match the actual imaging conditions (saturation!) and therefore need to be readjusted, all previous measurements under the changed conditions will have to be repeated. All measurements performed under differing conditions should be discarded!

Note: Press the Quick Look-Up Table button to check for images saturation. Only image areas showing no saturation can be used for quantification.

Note: EM CCD cameras usually have a higher dynamic range and a higher bit depth than conventional cameras. The higher the bit depth the easier it is to balance the acquisition parameters between Donor, FRET and Acceptor channel.



FRET Wizard acquisition interface: The Wizard ensures that exposure time, camera gain and intensity are the same for all acquisition channels. Changing one of these parameters for one channel will automatically apply the change to all other channels.



FRET Wizard image viewer. All three channels show enough signal for FRET quantification. No part of the image is saturated.

Note: In case the acquisition parameters do not match the Donor only or Acceptor only controls, try first to find cells within the controls matching the acquisition parameters before going back to step 1 and start the set-up all over again.

Hardware for FRET acquisition

For FRET acquisition three FRET sets can be used:

1. External filter wheel CFP/YFP FRET set 11522073 for use with AF7000. This set allows high-speed FRET acquisition using the Leica external filter wheels for FRET excitation and FRET emission. The FRET set comes already with a matching filter cube. The cube is only equipped with a dichroic mirror but does not contain an excitation filter nor emission filter. For eyepiece view it is therefore strongly recommended to add a CFP cube 11513892 and a YFP cube 11513893.

The external filter wheel FRET set requires the control of two filter wheels and therefore only can be used with an AF7000.

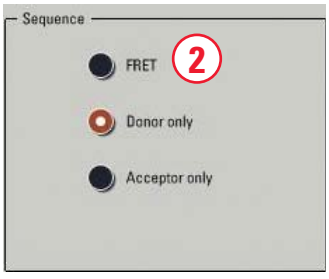
Note: External filter wheel solutions for other FRET pairs are available or can be offered on request.

2. Filter cube FRET set 11532630 for use with AF6000 and AF6500. This set consists of three filter cubes for the Donor channel, the FRET channel and the Acceptor channel. Since all cubes contain an emission filter there is no need for an additional CFP or YFP cube for eyepiece view.

3. External filter wheel FRET set 11888363 for TIRF. This set includes a FRET emission filter wheel and a matching filter cube. For excitation the TIRF AOTF is used. Since the cube does not contain an emission filter, it is strongly recommended to add a CFP cube and a YFP cube for eyepiece view.

Step 2: Corr. Images

In this step the Donor only control image and the Acceptor only control image are taken. If no FRET time series is required, this step can also be used to acquire the images of the FRET sample. This can be the case if fixed FRET samples are used. FRET time-lapse experiments are done in Step4.



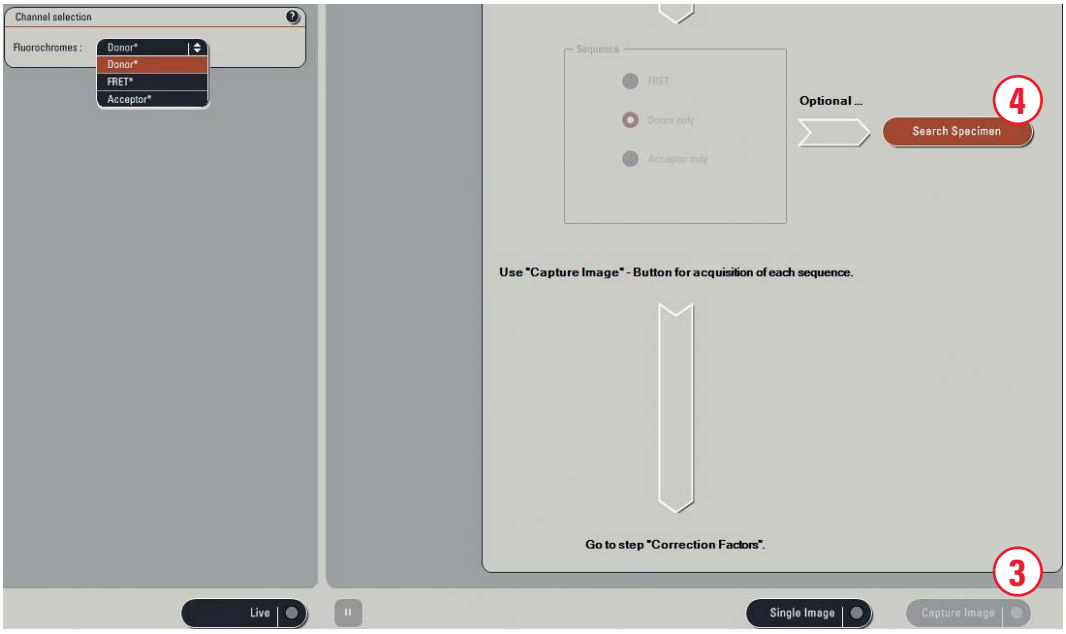
Workflow:

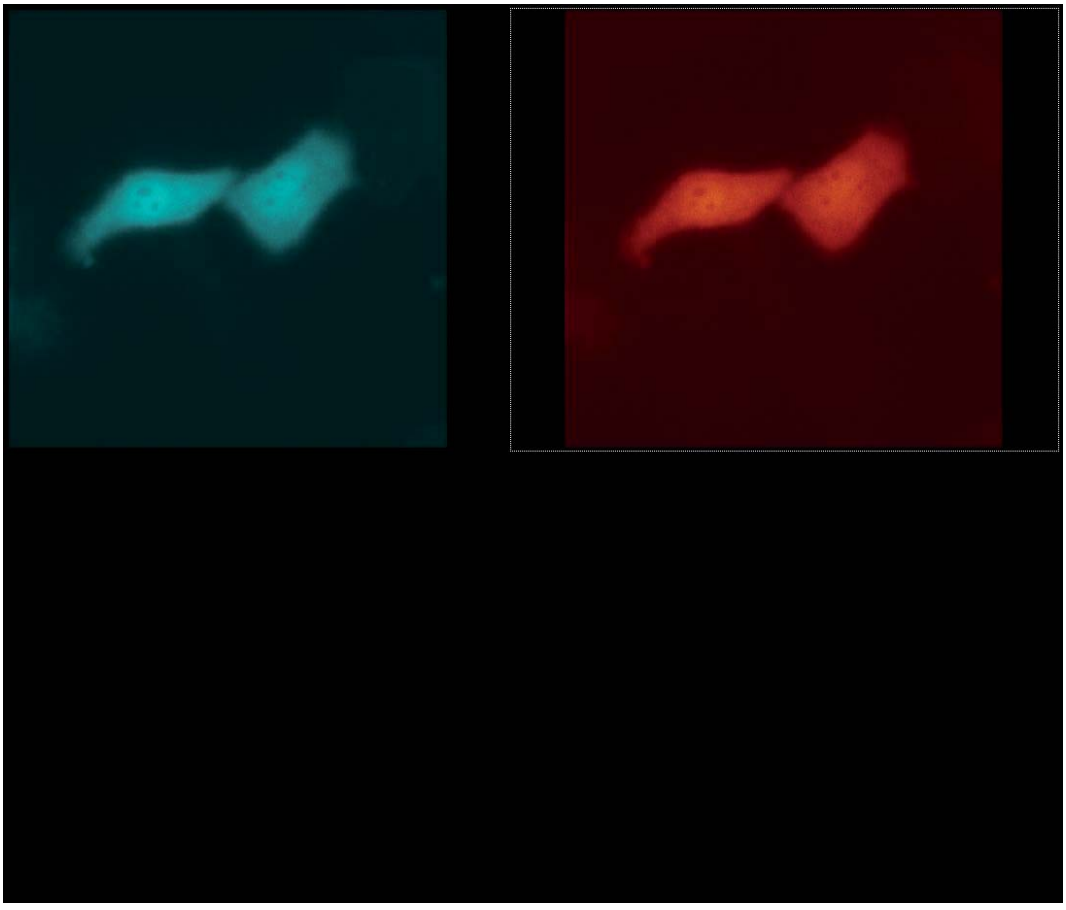
If you want to acquire one FRET time point only, you can start step 2 with the acquisition of the FRET images by activating the FRET radio button **(2)** and pressing **Capture Image (3)**.

In all other cases the acquisition starts with the Donor only sample. The image dynamic of all three channels can be checked by pressing **Search Specimen (4)**. This will activate a pull down menu for channel selection and will deactivate the **Capture image (3)** button. Once all three channels show enough image dynamic and no saturation, exit **search specimen** and press **Capture image**. Follow the same procedure for the Acceptor only sample. Typically, FRET, Donor only and Acceptor only sample are on different petri dished or slides.

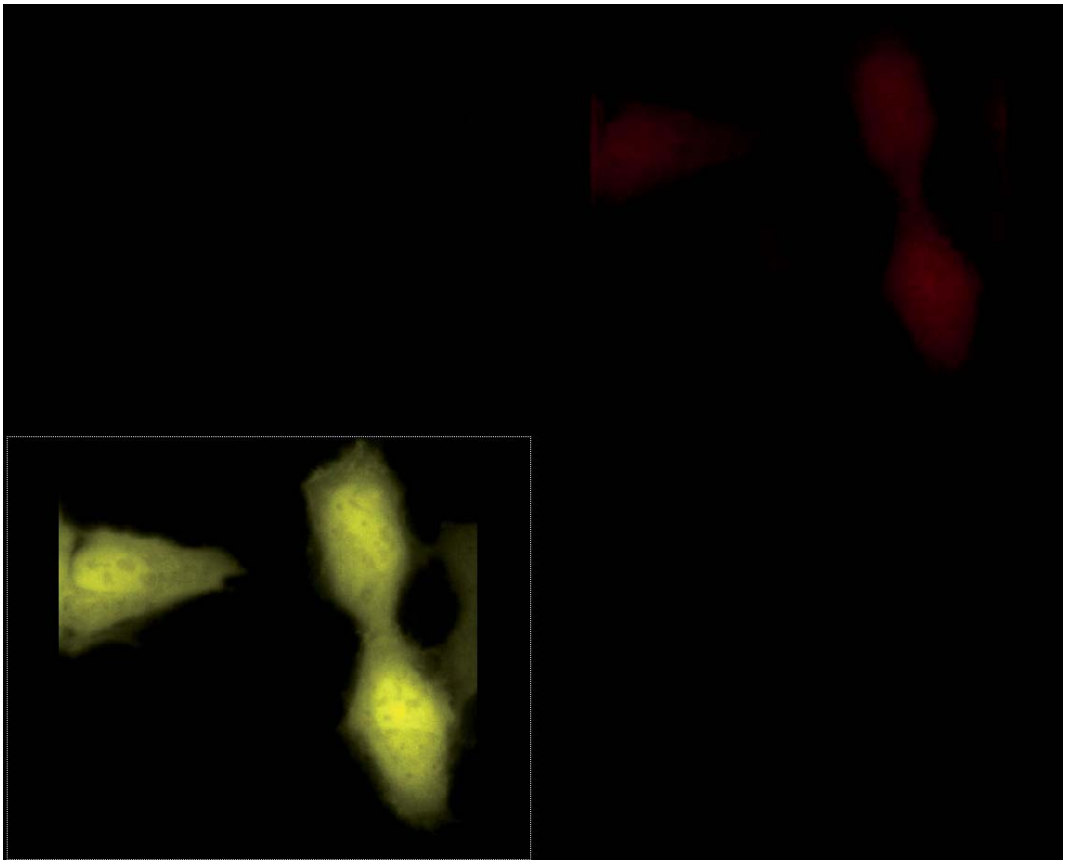
For eyepiece view it might be necessary to select a filter cube other than specified in the channel setup step 1.

Attention! Changing filter cubes on the microscope will automatically change the filter cube in the active acquisition channel of the FRET Wizard. It is therefore mandatory to swivel in the correct filter cube before starting acquisition.





Typical example of a Donor only sample. Channel 1 shows the Donor only image, channel 2 its cross talk into the FRET channel. Channel 3 is the Acceptor channel and should not show an image. Image information in channel 3 might derive from auto fluorescence of the sample, fluorescencing cell medium or straylight. In all these cases the FRET experiment should be stopped and counter measures taken.



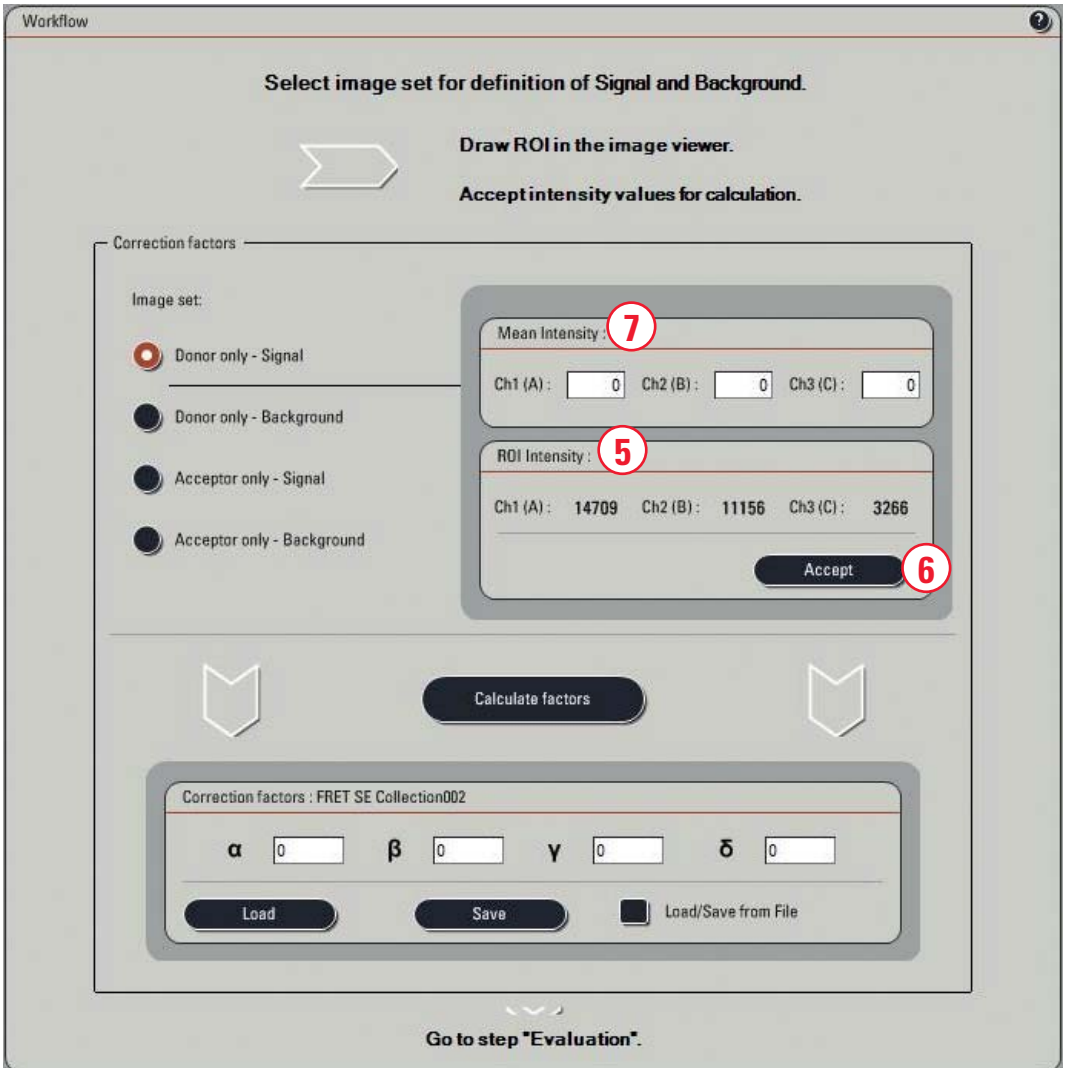
Typical example of an Acceptor only sample. Channel 3 shows the Acceptor only image, channel 2 its cross talk into the FRET channel. Channel 1 is the Donor channel and and should not show an image. Image information in channel 1 might derive from auto fluorescence of the sample, fluorescencing cell medium or straylight. In all these cases the FRET experiment should be stopped and counter measures taken.

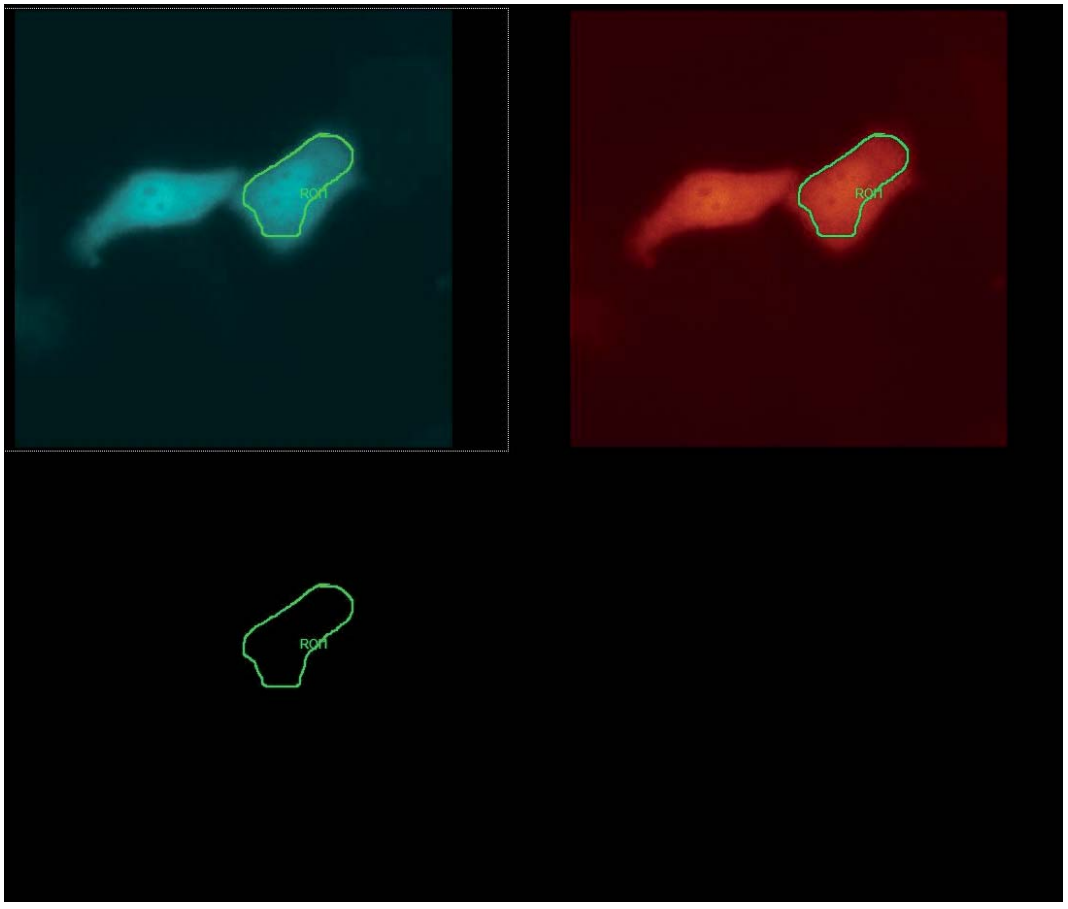
Step 3: Corr. Factors

In this step the cross talk between Donor, FRET and Acceptor channel is identified.

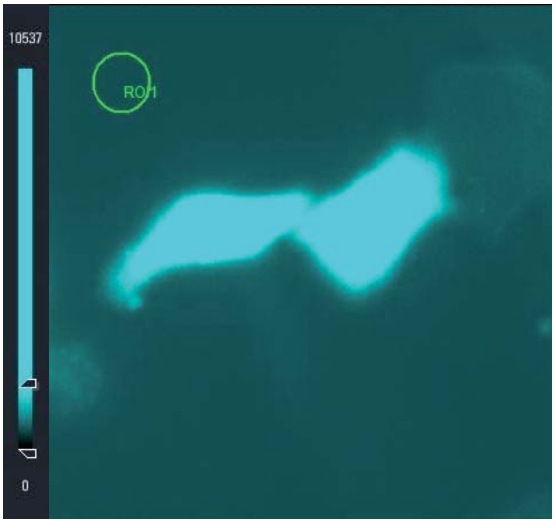
Workflow:

Start with the Donor only signal ROI. The mean intensity of the ROI in all channels is displayed in the ROI intensity table **(5)**. Once **Accept (6)** is pressed, the values will be taken over for cross talk calculation and will be displayed in the mean intensity table **(7)**.





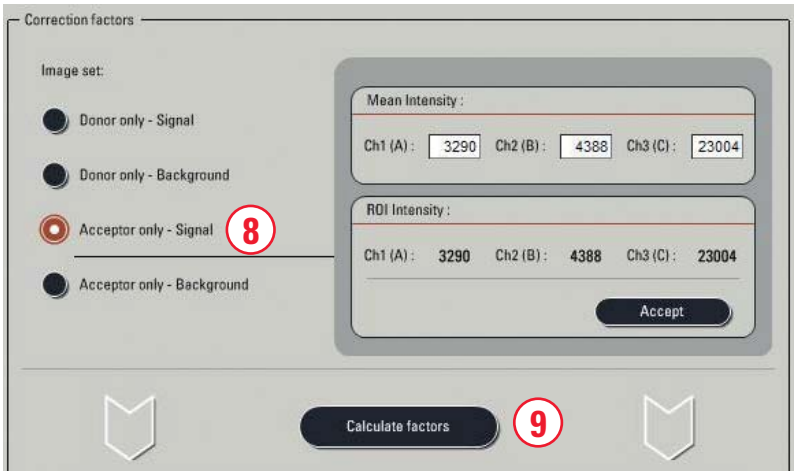
Donor only signal ROI in Donor, FRET and Acceptor channel



Scaling of the Donor only image reveals background image information on the upper right corner and the lower left corner of the image. These areas need to be avoided when drawing the background ROI.

Repeat the whole procedure for Acceptor only signal ROI and Acceptor background ROI. Pressing the Acceptor only radio button **(8)** will automatically show the Acceptor only images in the image viewer.

The correction factors are calculated by pressing **Calculate factors (9)**. Please make sure the intensities of all ROIs are taken over in the mean intensity table. Otherwise the calculation of the correction factors will be wrong.



ROI intensities are stored in the mean intensity table by pressing Accept

Depending on the FRET formula not all correction factors might be required for FRET calculation.

All numbers in the correction factors table can manually be overwritten.

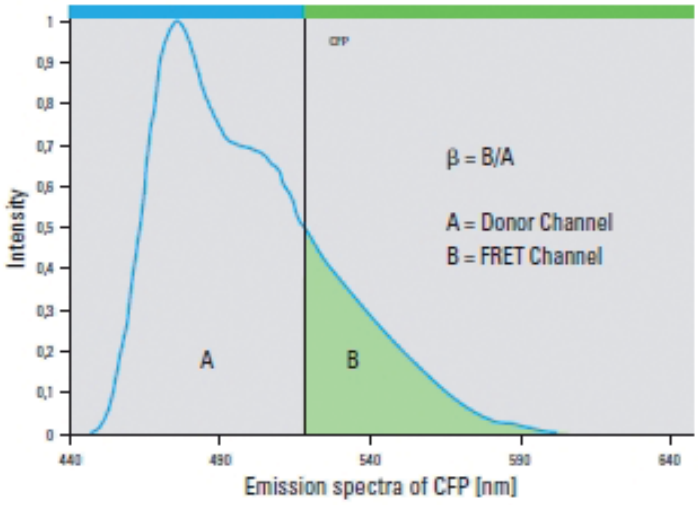
Correction factors table showing cross talk correction factors α, β, γ and δ. In our example the correction factor β indicates a 67% crosstalk of the Donor channel into the FRET channel. The Donor channel is considered to be 100%. Correction factor β and γ usually show the highest crosstalk values whereas α and δ are rather small.

The correction factors are calculated as follows:

Calibration Factors:

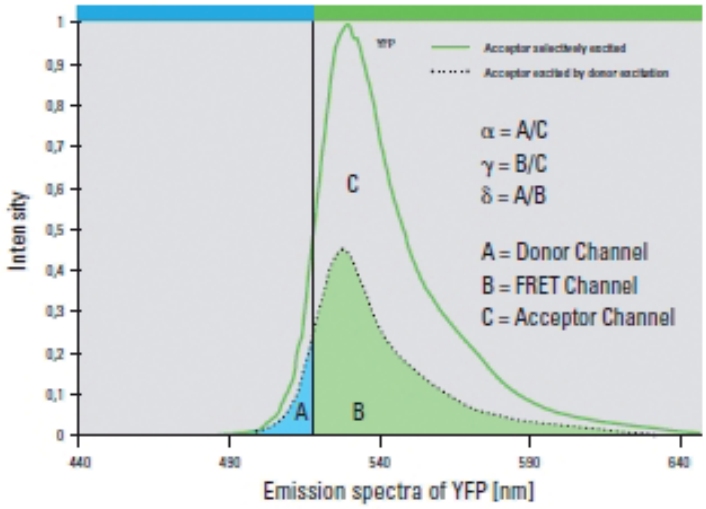
1. Donor only reference generates correction factor Calibration Factors:

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



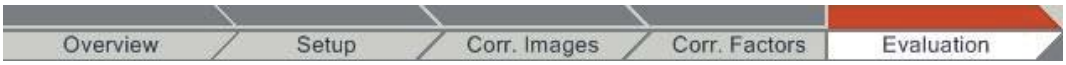
2. The Acceptor only reference generates correction factors α, γ, δ.

α corrects for acceptor cross-excitation cross-talk:
α = A/C
γ corrects for acceptor cross-excitation:
γ = B/C
δ corrects for FRET cross-talk:
δ = A/B



A: Signal of Donor channel
B: Signal of FRET channel
C: Signal of Acceptor channel

Step 4: Evaluation

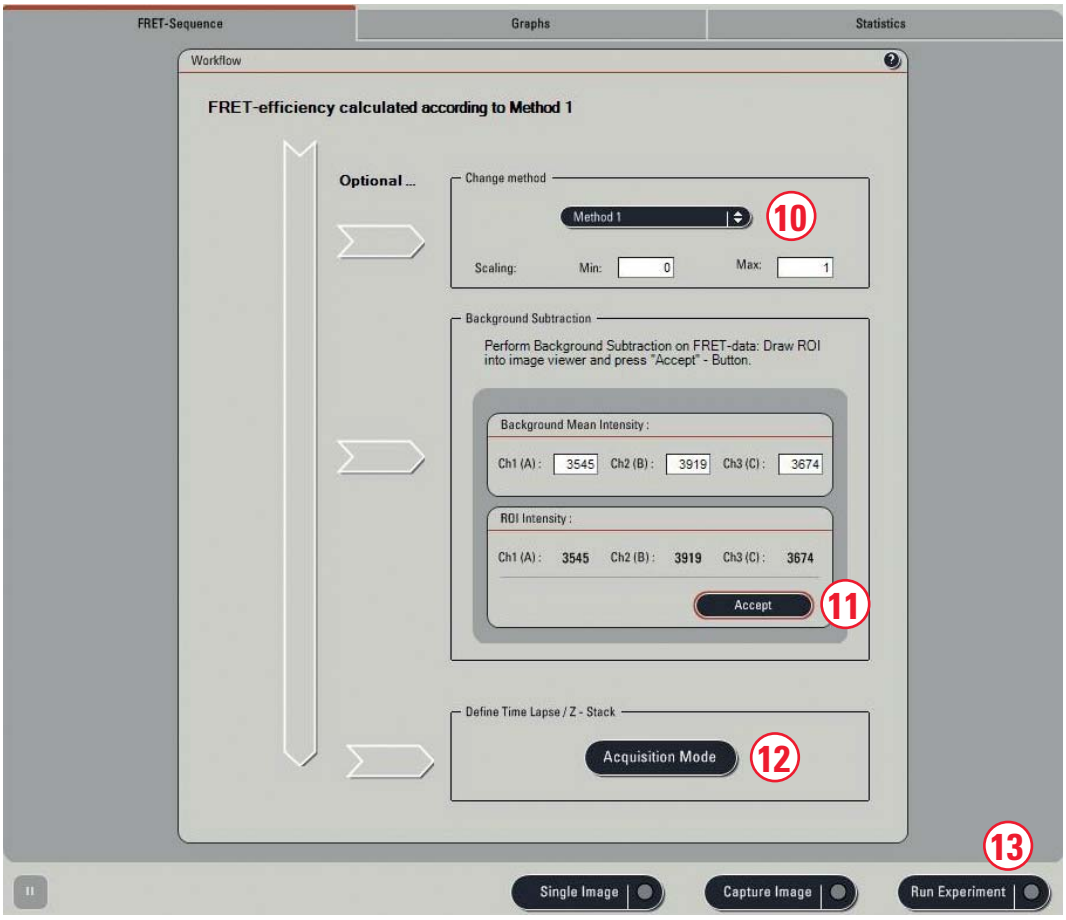


Use this step to acquire FRET time-lapse experiments and to analyze FRET data.

Workflow:

Before running a time-lapse experiment you may choose the **calculation method** (10) to calculate and represent the apparent FRET efficiencies. You may also change the method after running the experiment. Method 1 is automatically applied. For background subtraction draw a ROI in the image viewer where only background signal is found. Press **Accept** (11) 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** (12) and start your series with **Run Experiment** (13).



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).

Note: Method 3 is a simple ratiometric FRET measurement. The FRET ratio can also be done in the Ca^{++} imaging tool within quantify.

To see FRET and intensity values displayed in a graph during the experiment change to the tab sheet **Graph** **(14)**. Draw one or multiple ROIs in the image viewer to see intensities displayed in the graph.

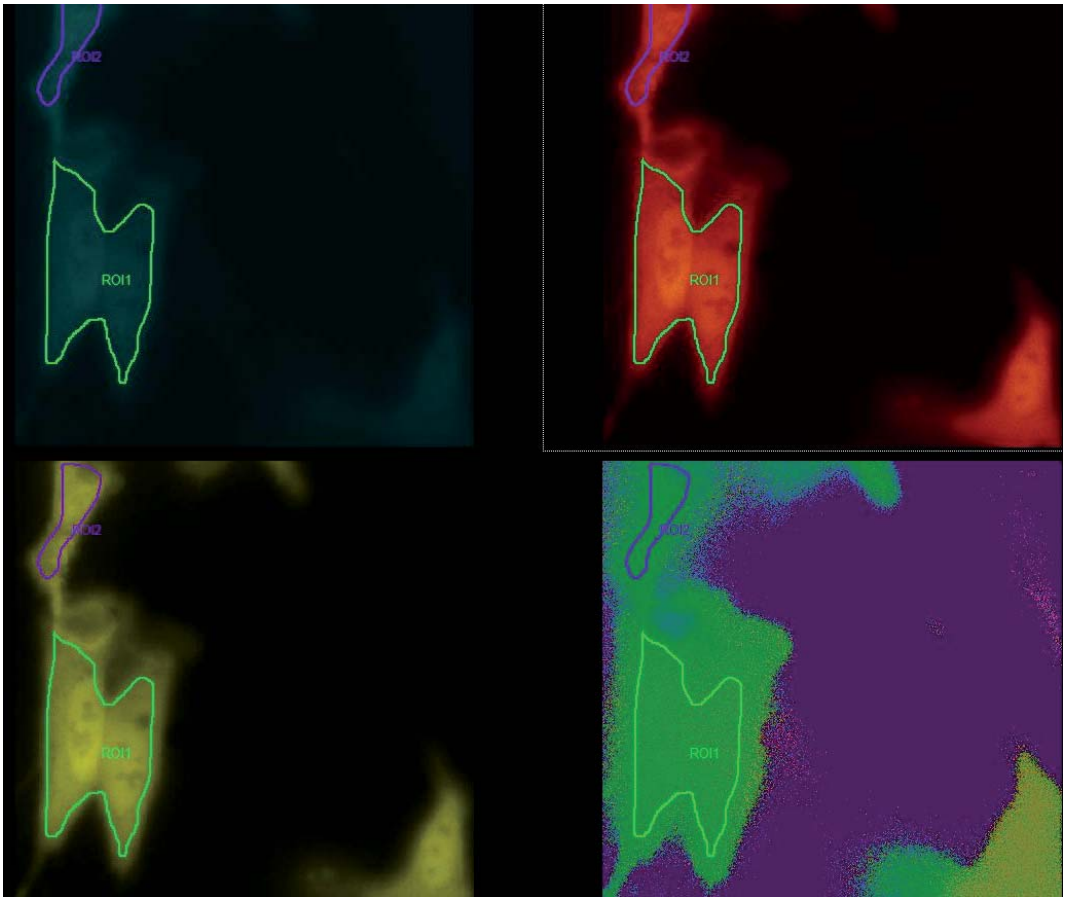
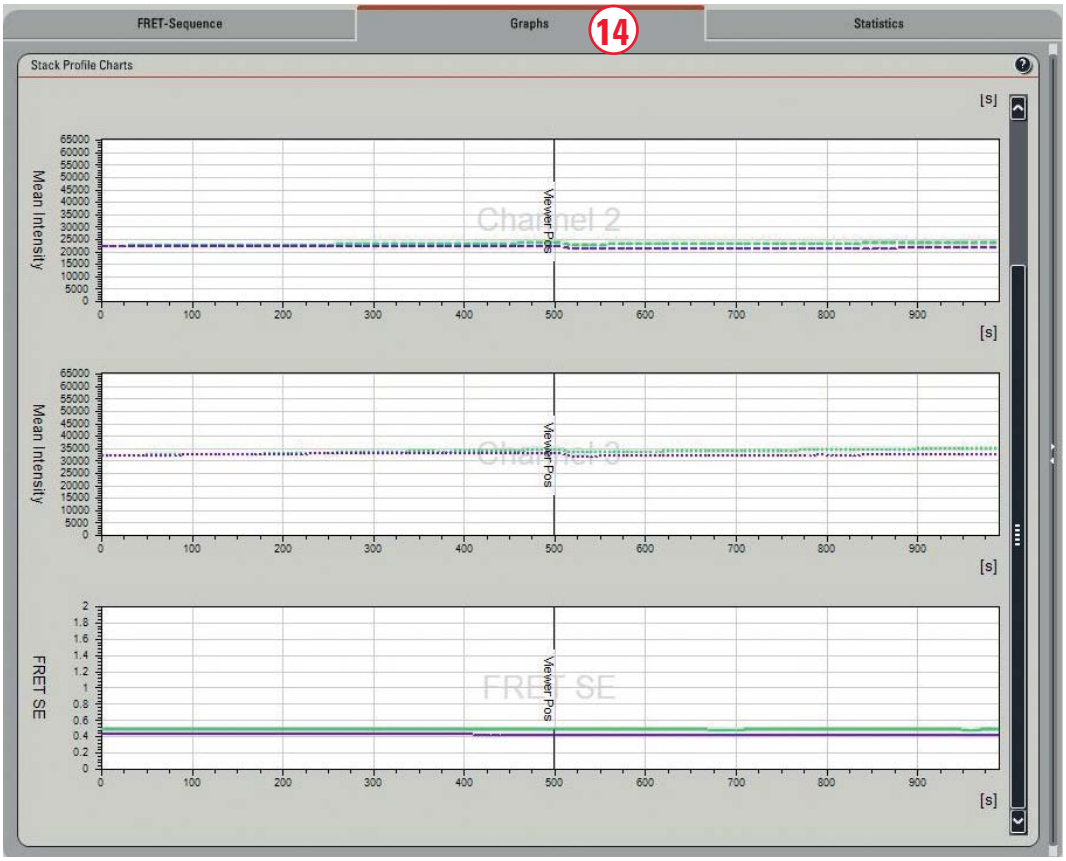
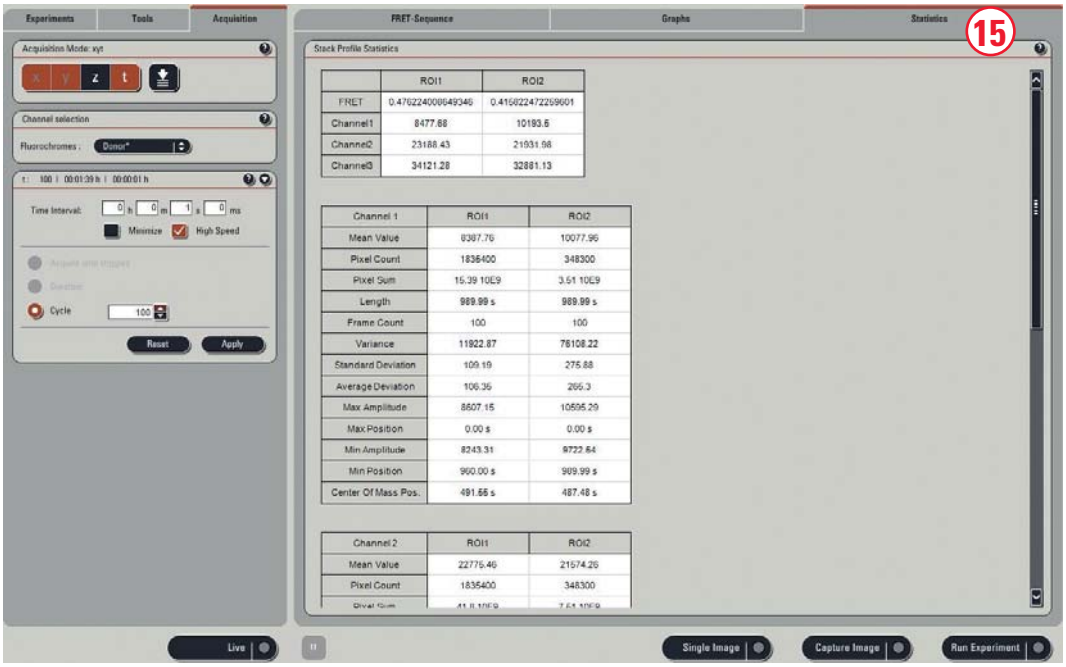


Image viewer displaying FRET image and FRET channels during a running experiment. ROIs can be added, changed, or deleted during image acquisition. The mean intensities and the apparent FRET efficiency of the ROIs will be updated in the statistics table and in the experiment graph during image acquisition.

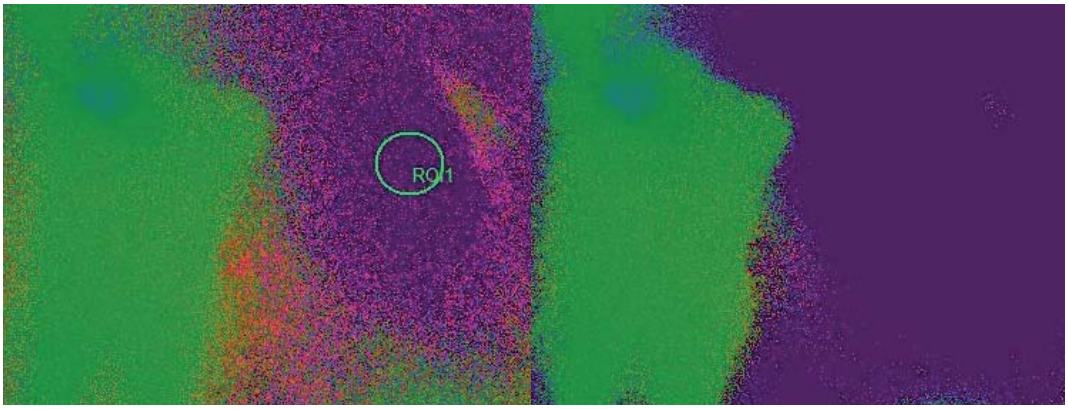


FRET Graph of two ROIs dispayed during a running experiment.

For apparent FRET efficiencies change to tab sheet **Statistics** **(15)**. 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.



Note: Depending on the background ROI the Apparent FRET values outside the FRET cells can become extremely high. This is an effect of background noise divided by background noise. This can be avoided by drawing FRET ROIs only in regions where FRET is expected.



Impact of ROI selection on FRET intensities. In the upper right corner of the left image a high apparent FRET efficiency is suggested. Apparently there can not be FRET since no cells are located in this area. Choosing a different background ROI in the right image makes the FRET intensities in the upper right corner disappear. Please note that in both images the apparent FRET efficiency within the live cells is not changing.

Impressum
Authors:
Markus Schechter and Gabriele Burger,
Leica Microsystems

“With the user, for the user”
Leica Microsystems

Leica Microsystems operates globally in four divisions, where we rank with the market leaders.

• Life Science Division

The Leica Microsystems Life Science Division supports the imaging needs of the scientific community with advanced innovation and technical expertise for the visualization, measurement, and analysis of microstructures. Our strong focus on understanding scientific applications puts Leica Microsystems’ customers at the leading edge of science.

• Industry Division

The Leica Microsystems Industry Division’s focus is to support customers’ pursuit of the highest quality end result. Leica Microsystems provide the best and most innovative imaging systems to see, measure, and analyze the microstructures in routine and research industrial applications, materials science, quality control, forensic science investigation, and educational applications.

• Biosystems Division

The Leica Microsystems Biosystems Division brings histopathology labs and researchers the highest-quality, most comprehensive product range. From patient to pathologist, the range includes the ideal product for each histology step and high-productivity workflow solutions for the entire lab. With complete histology systems featuring innovative automation and Novocastra™ reagents, Leica Microsystems creates better patient care through rapid turnaround, diagnostic confidence, and close customer collaboration.

• Surgical Division

The Leica Microsystems Surgical Division’s focus is to partner with and support surgeons and their care of patients with the highest-quality, most innovative surgical microscope technology today and into the future.

The statement by Ernst Leitz in 1907, “with the user, for the user,” describes the fruitful collaboration with end users and driving force of innovation at Leica Microsystems. We have developed five brand values to live up to this tradition: Pioneering, High-end Quality, Team Spirit, Dedication to Science, and Continuous Improvement. For us, living up to these values means: **Living up to Life**.

Active worldwide

Australia:	North Ryde	Tel. +61 2 8870 3500	Fax +61 2 9878 1055
Austria:	Vienna	Tel. +43 1 486 80 50 0	Fax +43 1 486 80 50 30
Belgium:	Groot Bijgaarden	Tel. +32 2 790 98 50	Fax +32 2 790 98 68
Canada:	Richmond Hill/Ontario	Tel. +1 905 762 2000	Fax +1 905 762 8937
Denmark:	Herlev	Tel. +45 4454 0101	Fax +45 4454 0111
France:	Rueil-Malmaison	Tel. +33 1 47 32 85 85	Fax +33 1 47 32 85 86
Germany:	Wetzlar	Tel. +49 64 41 29 40 00	Fax +49 64 41 29 41 55
Italy:	Milan	Tel. +39 02 574 861	Fax +39 02 574 03392
Japan:	Tokyo	Tel. +81 3 5421 2800	Fax +81 3 5421 2896
Korea:	Seoul	Tel. +82 2 514 65 43	Fax +82 2 514 65 48
Netherlands:	Rijswijk	Tel. +31 70 4132 100	Fax +31 70 4132 109
People’s Rep. of China:	Hong Kong	Tel. +852 2564 6699	Fax +852 2564 4163
Portugal:	Lisbon	Tel. +351 21 388 9112	Fax +351 21 385 4668
Singapore		Tel. +65 6779 7823	Fax +65 6773 0628
Spain:	Barcelona	Tel. +34 93 494 95 30	Fax +34 93 494 95 32
Sweden:	Kista	Tel. +46 8 625 45 45	Fax +46 8 625 45 10
Switzerland:	Heerbrugg	Tel. +41 71 726 34 34	Fax +41 71 726 34 44
United Kingdom:	Milton Keynes	Tel. +44 1908 246 246	Fax +44 1908 609 992
USA:	Bannockburn/Illinois	Tel. +1 847 405 0123	Fax +1 847 405 0164

and representatives in more than 100 countries

www.leica-microsystems.com

Leica
MICROSYSTEMS