

April
2009
No.04

WIDEFIELD APPLICATION LETTER



reSOLUTION



FRET Sensitized Emission Wizard Widefield

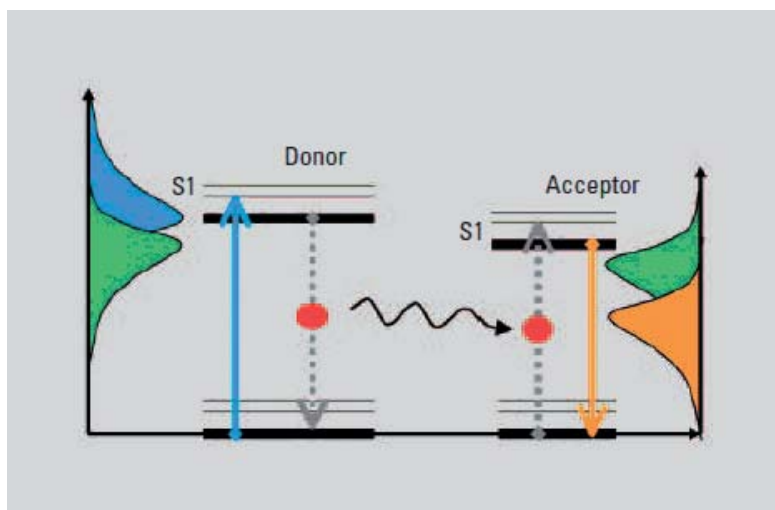
FRET SE with the Leica Advanced Widefield Systems AF7000, AF6500 and AF6000

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:

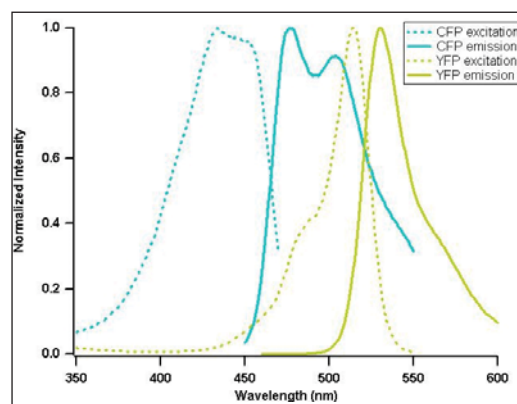
An excited fluorophore, called the donor, transfers its excited state energy to a light absorbing molecule which is called the acceptor. This transfer of energy is non-radiative. Sensitized Emission is one established method for the evaluation of FRET efficiencies. It can be applied to live cells as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

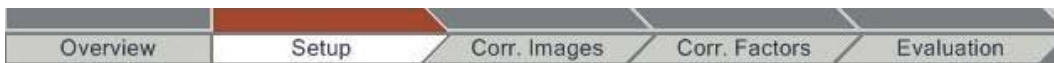


Note: Since the FRET Wizard requires two monitors this application also is greyed out if a second monitor is missing.

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

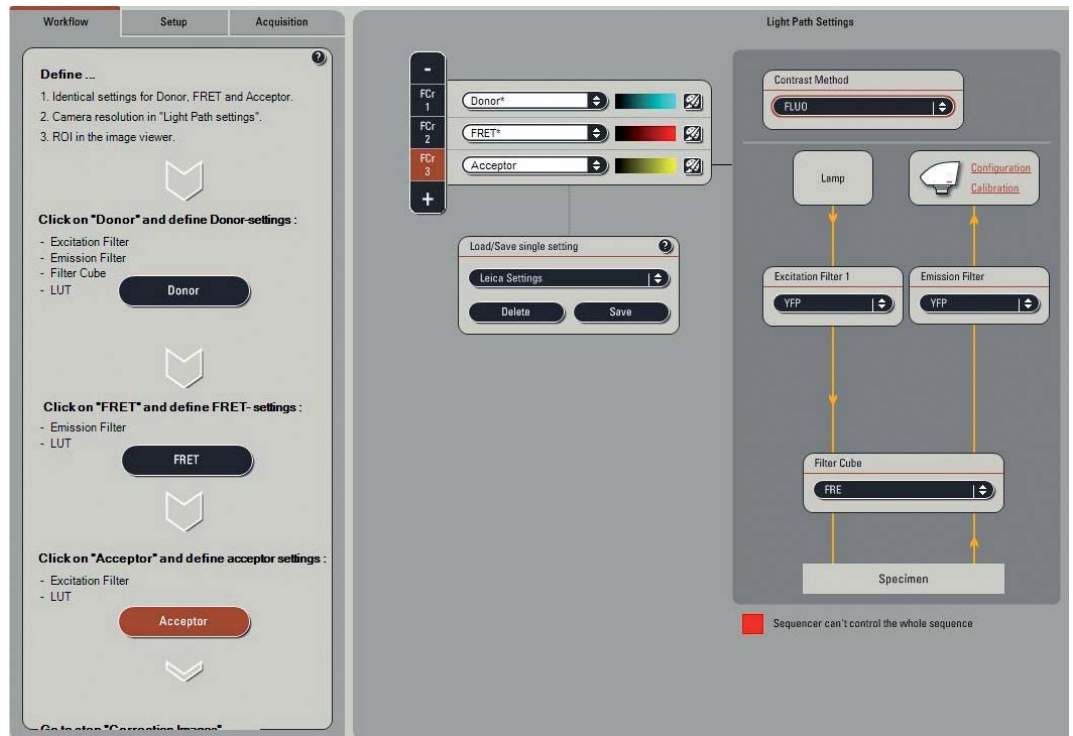
- Step 1: is dedicated to the imaging set-up.
- Step 2: executes control and FRET measurements.
- Step 3: guides through the calibration routine and calculates the calibration coefficients.
- Step 4: is dedicated to the execution of time-lapse experiments, evaluation of results and generation of experimental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

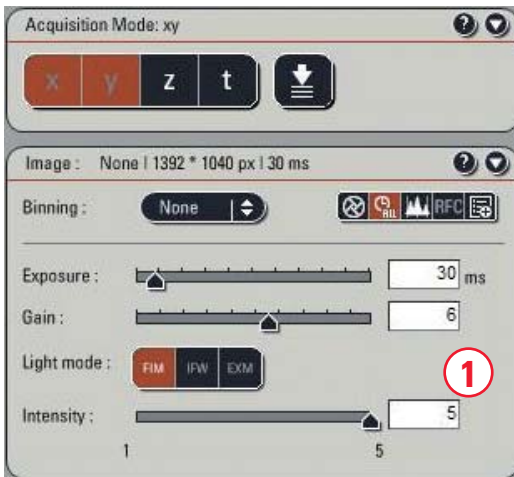
Workflow:

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!

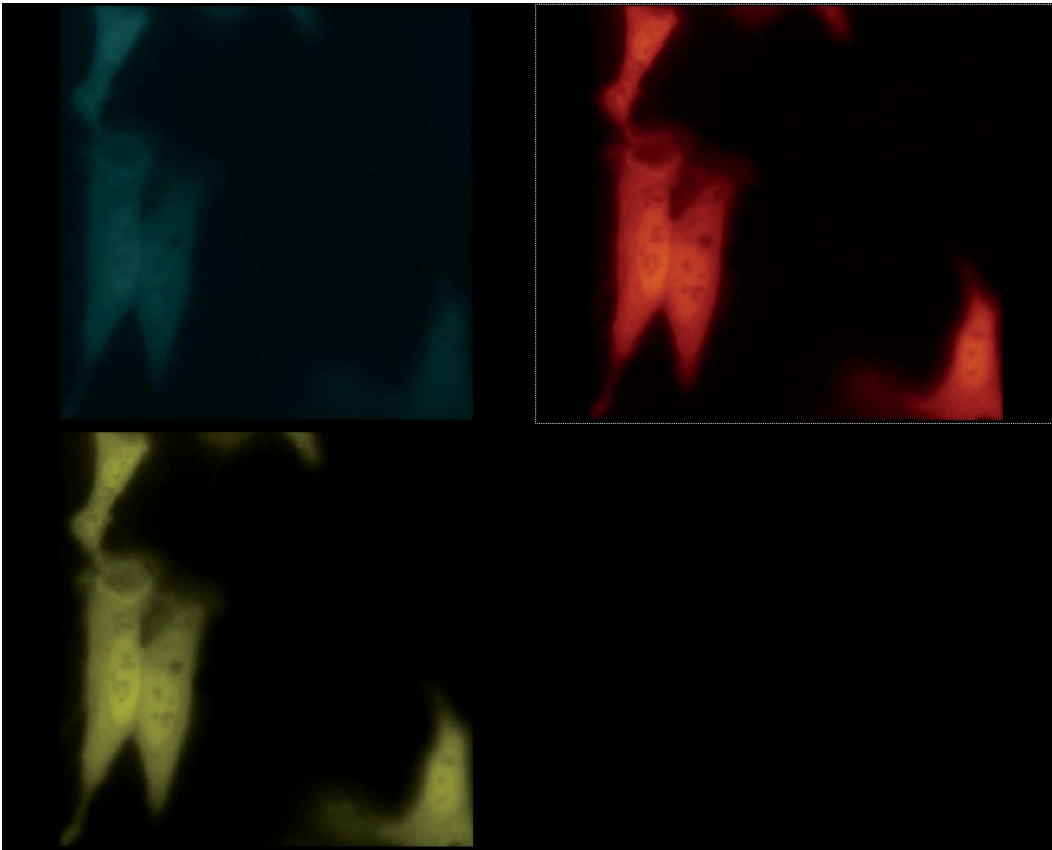
Note: Press the Quick Look-Up Table button to check for images saturation. Only image areas showing no saturation can be used for quantification.



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.

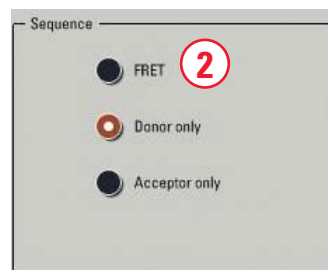
2. Filter cube FRET set 11532630 for use with AF6000 and AF6500. This set consists of three filter cubes for the Donor channel, the FRET channel and the Acceptor channel. Since all cubes contain an emission filter there is no need for an additional CFP or YFP cube for eyepiece view.

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.



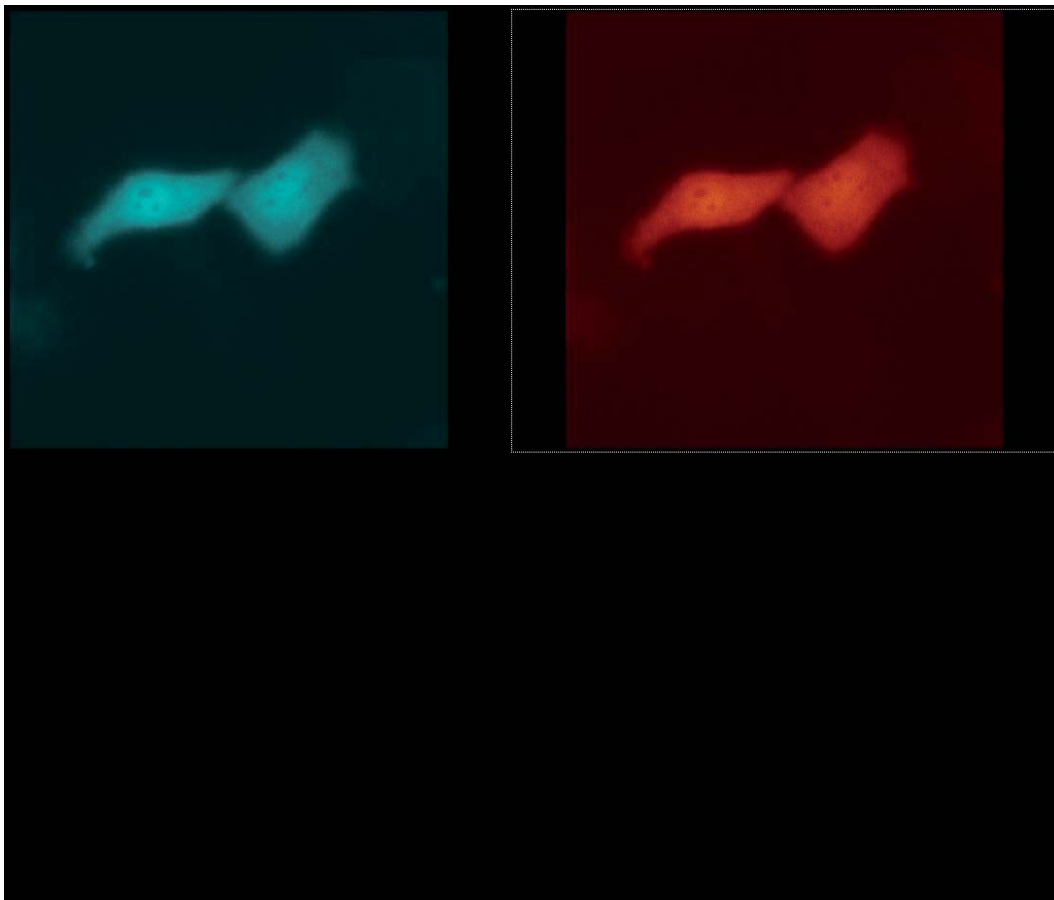
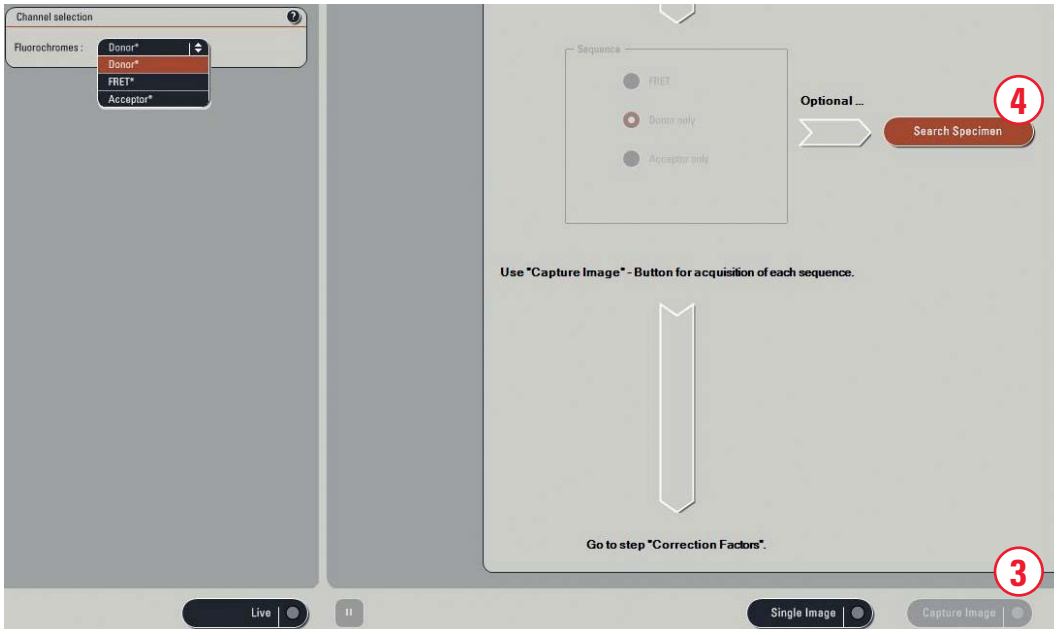
Workflow:

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)**.

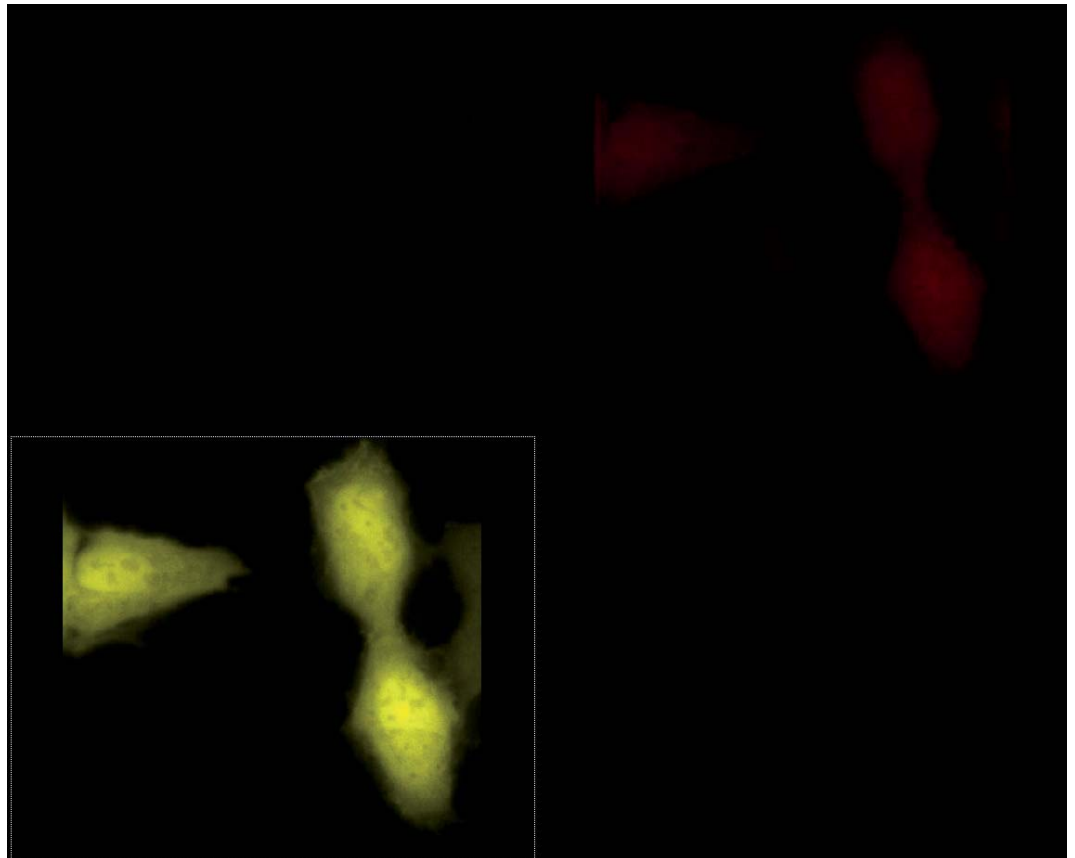
In all other cases the acquisition starts with the Donor only sample. The image dynamic of all three channels can be checked by pressing **Search Specimen (4)**. This will activate a pull down menu for channel selection and will deactivate the **Capture image (3)** button. Once all three channels show 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 dishes 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.

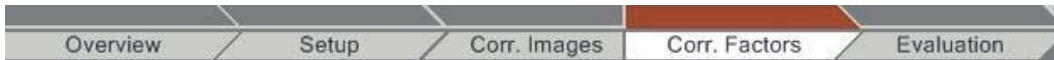


Typical example of a Donor only sample. Channel 1 shows the Donor only image, channel 2 its cross talk into the FRET channel. Channel 3 is the Acceptor channel and should not show an image. Image information in channel 3 might derive from auto fluorescence of the sample, fluorescing cell medium or straylight. 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 should not show an image. Image information in channel 1 might derive from auto fluorescence of the sample, fluorescing 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).

Workflow

Select image set for definition of Signal and Background.

Draw ROI in the image viewer.
Accept intensity values for calculation.

Correction factors

Image set:

- Donor only - Signal
- Donor only - Background
- Acceptor only - Signal
- Acceptor only - Background

Mean Intensity: **7**

Ch1 (A): Ch2 (B): Ch3 (C):

ROI Intensity: **5**

Ch1 (A): 14709 Ch2 (B): 11156 Ch3 (C): 3266

Accept **6**

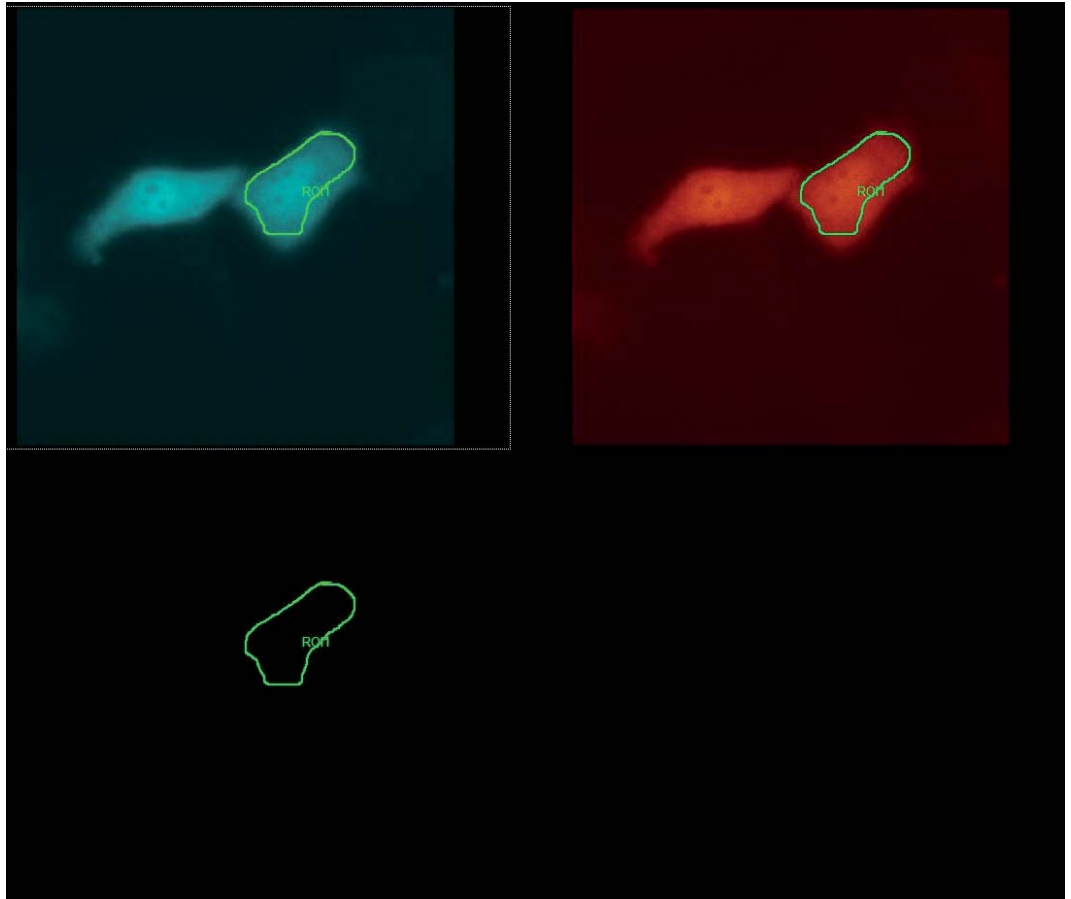
Calculate factors

Correction factors : FRET SE Collection002

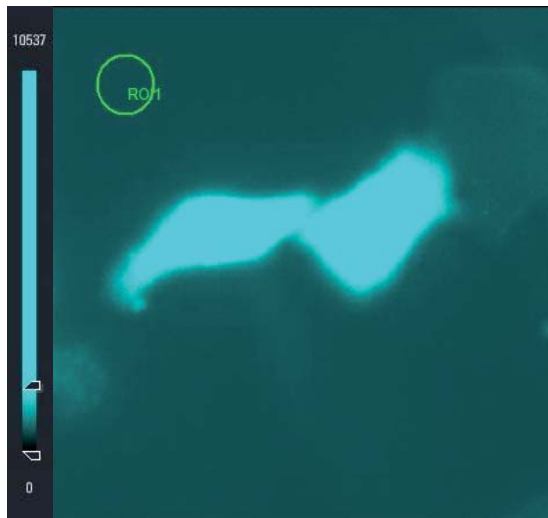
α β γ δ

Load **Save** Load/Save from File

Go to step "Evaluation".



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.

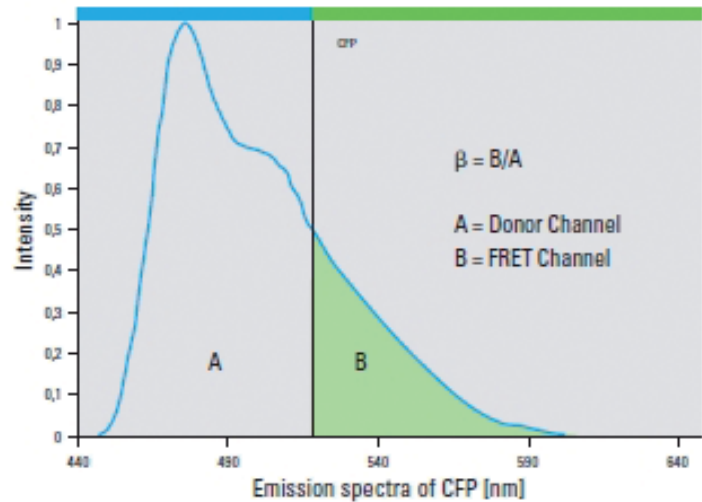
The correction factors are calculated as follows:

Calibration Factors:

1. Donor only reference generates correction factor

Calibration Factors:

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



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

α corrects for acceptor cross-excitation cross-talk:

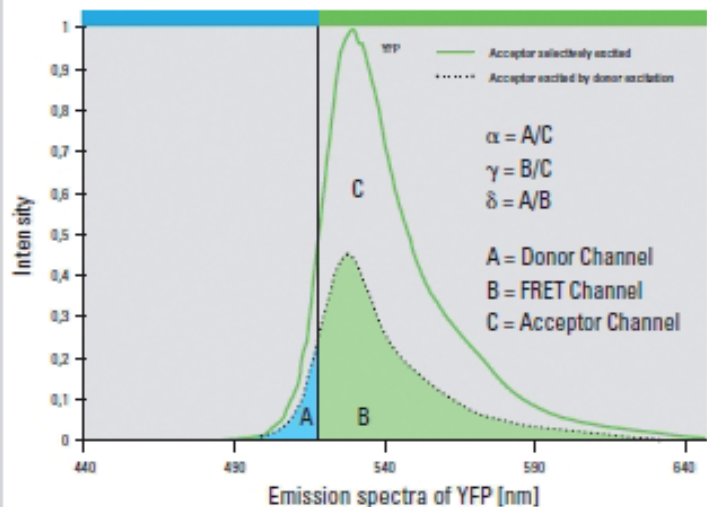
$$\alpha = A/C$$

γ corrects for acceptor cross-excitation:

$$\gamma = B/C$$

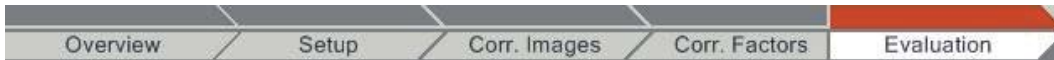
δ corrects for FRET cross-talk:

$$\delta = A/B$$



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 stoichiometry (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.

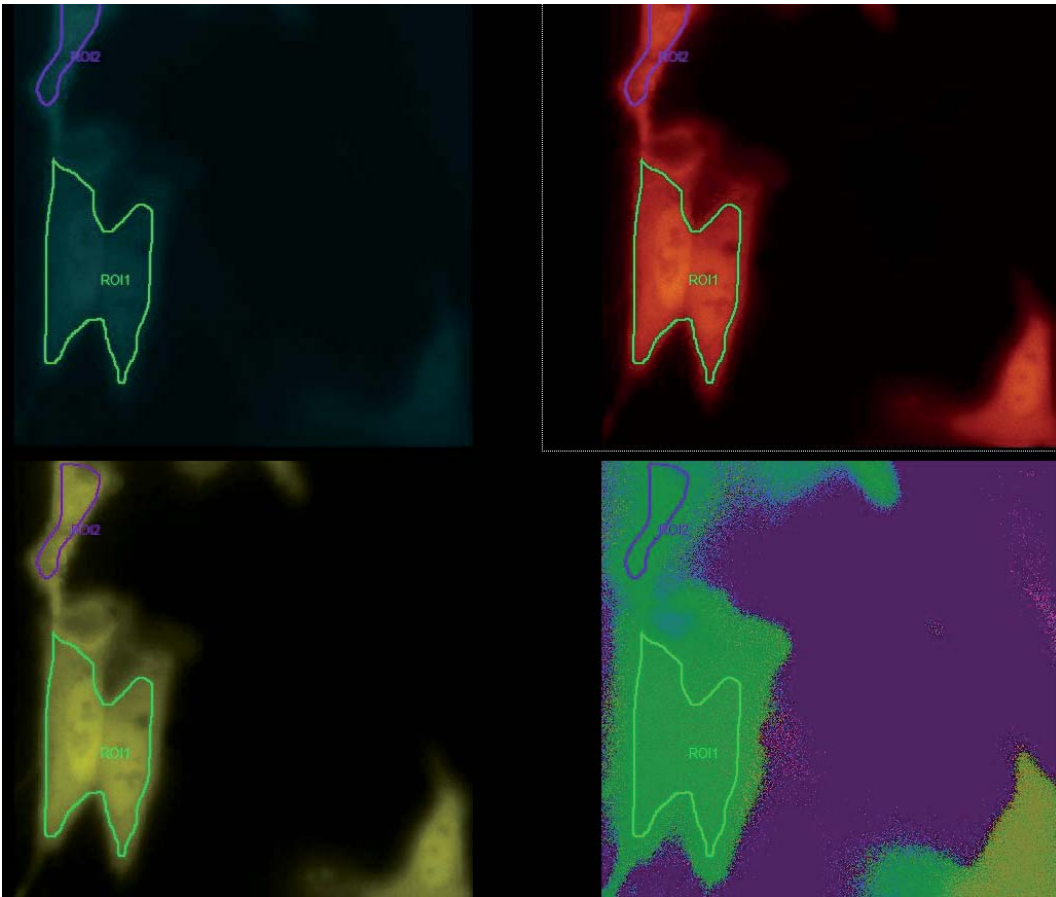
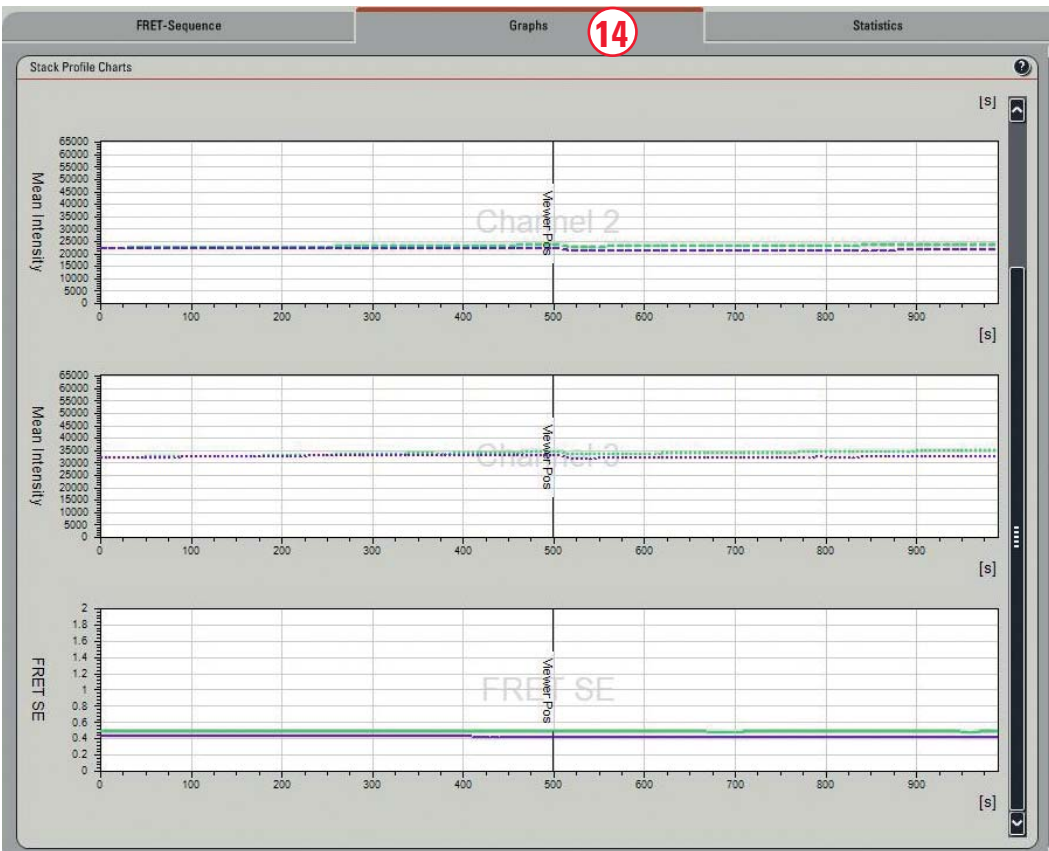


Image viewer displaying FRET image and FRET channels during a running experiment. ROIs can be added, changed, or deleted during image acquisition. The mean intensities and the apparent FRET efficiency of the ROIs will be updated in the statistics table and in the experiment graph during image acquisition.



FRET Graph of two ROIs displayed 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.

The screenshot shows the 'Statistics' tab of the FRET-Sequence software. On the left, there are acquisition controls including 'Acquisition Mode: xyt', 'Channel selection', 'Fluorochromes: Donor', and 'Time Interval' settings. The main area displays 'Stack Profile Statistics' with two tables. The first table shows FRET values for ROI1 and ROI2 across three channels. The second table provides detailed statistics for Channel 1, including Mean Value, Pixel Count, Pixel Sum, Length, Frame Count, Variance, Standard Deviation, Average Deviation, Max Amplitude, Max Position, Min Amplitude, Min Position, and Center Of Mass Pos. A third table shows similar statistics for Channel 2. At the bottom, there are buttons for 'Live', 'Single Image', 'Capture Image', and 'Run Experiment'.

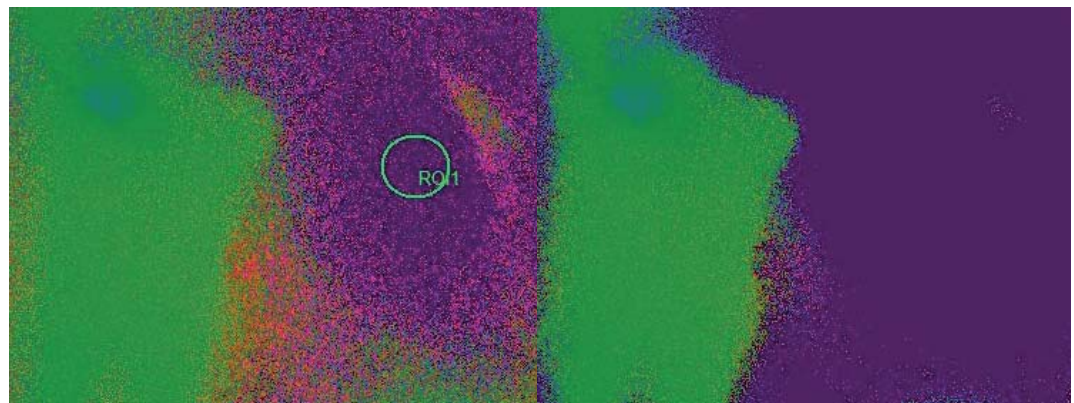
	ROI1	ROI2
FRET	0.476224000649346	0.415622472259601
Channel1	8477.88	10193.8
Channel2	23188.43	21931.98
Channel3	34121.28	32881.13

Channel 1	ROI1	ROI2
Mean Value	8397.76	10077.96
Pixel Count	1836400	348300
Pixel Sum	15,39 10E9	3,51 10E9
Length	989.99 s	989.99 s
Frame Count	100	100
Variance	11922.87	76108.22
Standard Deviation	109.19	276.88
Average Deviation	106.36	266.3
Max Amplitude	8607.15	10696.29
Max Position	0.00 s	0.00 s
Min Amplitude	8243.31	8723.84
Min Position	960.00 s	969.99 s
Center Of Mass Pos.	491.66 s	487.48 s

Channel 2	ROI1	ROI2
Mean Value	22776.46	21674.26
Pixel Count	1836400	348300
Pixel Sum	11.8 10E9	7.64 10E9

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

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