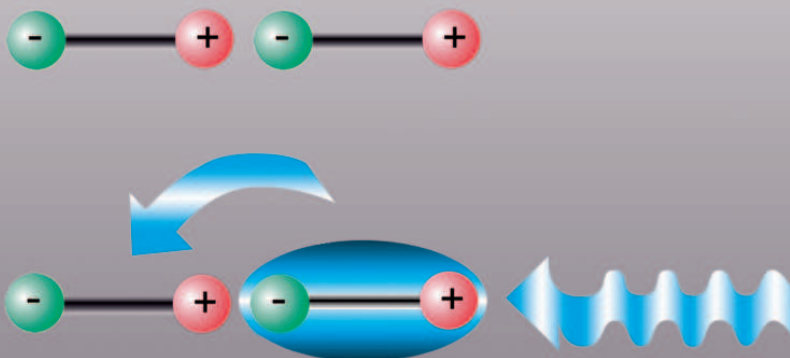
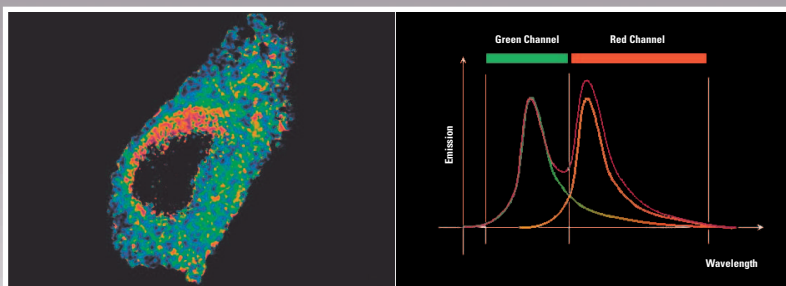
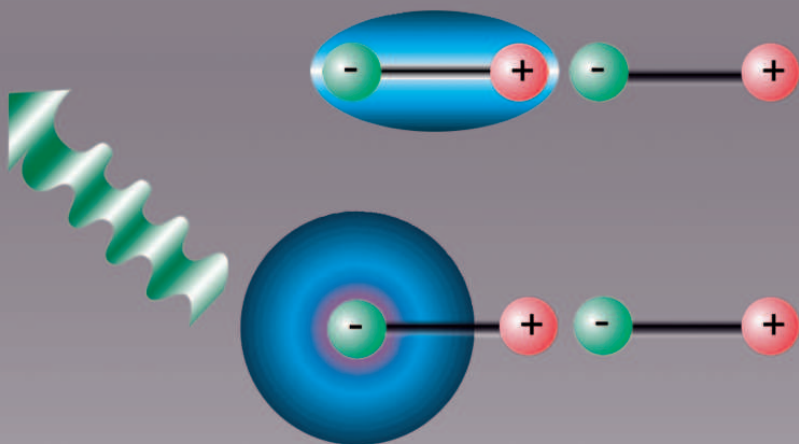


CONFOCAL APPLICATION LETTER

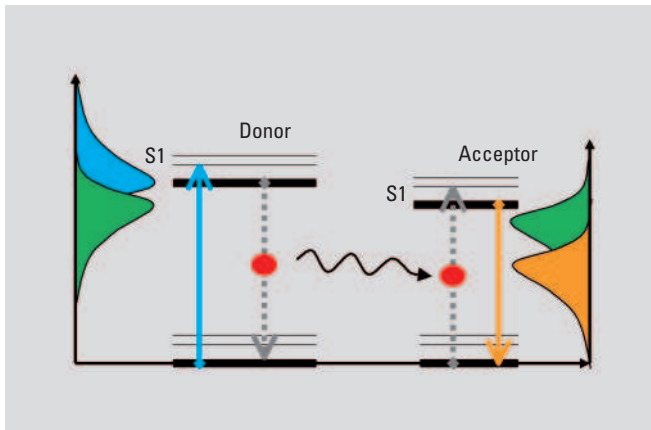


# reSOLUTION



LAS AF APPLICATION WIZARD  
FRET SENSITIZED EMISSION

# 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.

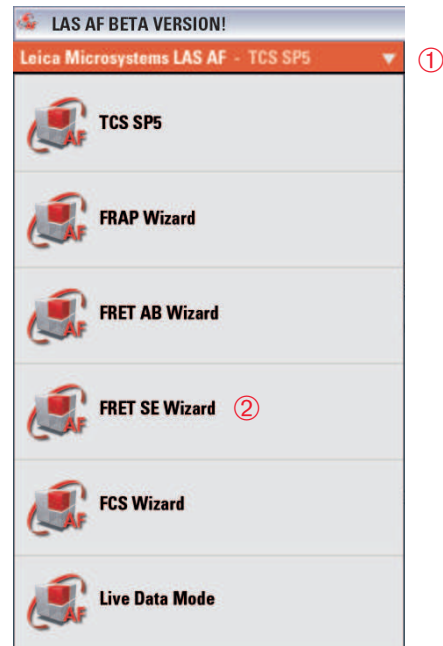
It is important to be aware that throughout the entire experiment and calibration routine, all measuring parameters such as gain, emission detection window, excitation intensities, zoom, format, scan speed, pinhole etc must remain constant.

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

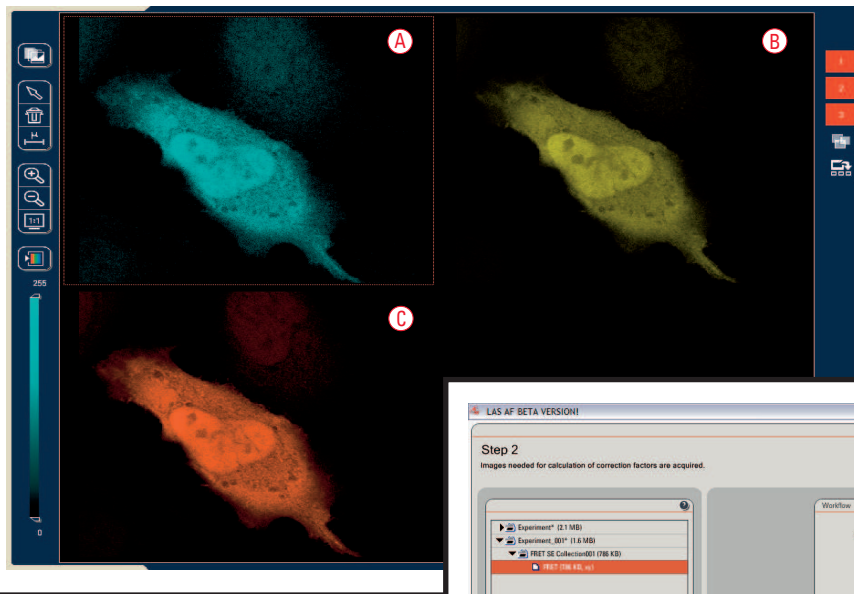
Within the LAS AF <sup>①</sup> menu bar you can find two wizards to perform FRET experiments: FRET AB (Acceptor Photo-Bleaching) and FRET SE (Sensitized Emission). This application note describes working with the FRET SE Wizard <sup>②</sup>.

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

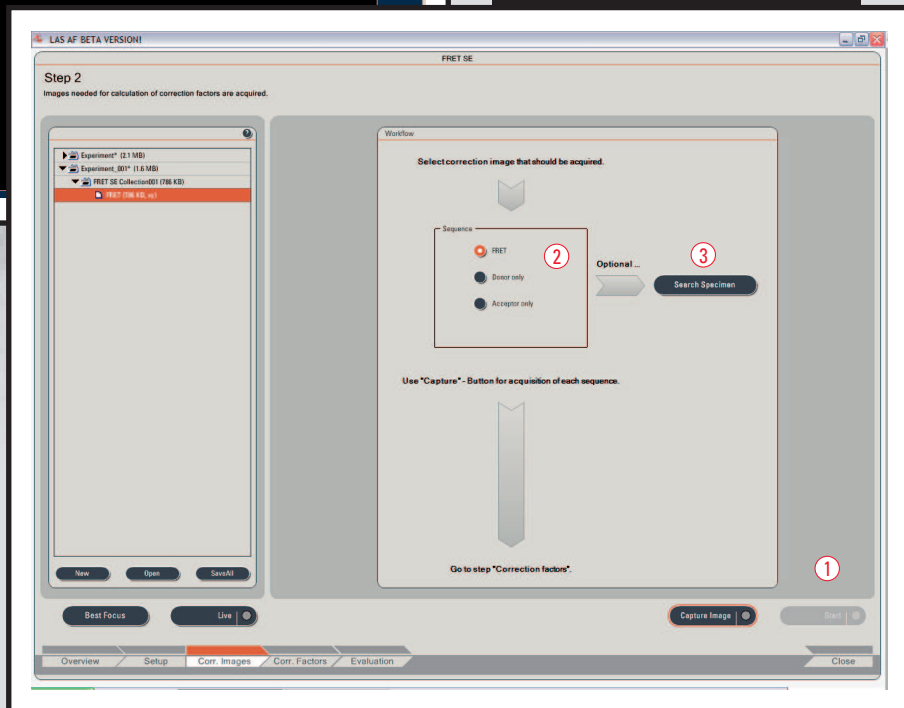
- Step 1: is dedicated to the imaging set-up.
- Step 2: executes control and FRET measurements.
- Step 3: guides through the calibration routine and calculates the calibration coefficients.
- Step 4: is dedicated to the execution of time-lapse experiments, evaluation of results and generation of experimental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



## Step 1: Setting of experimental conditions



## Step 2: Acquire image sequences of controls for calibration



### 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 (A) + (B) consists of donor (light blue) and FRET (Yellow). The excitation wavelength is selectively chosen for donor excitation. The second sequence (C) contains acceptor fluorescence (orange). The excitation light is selective for acceptor excitation.

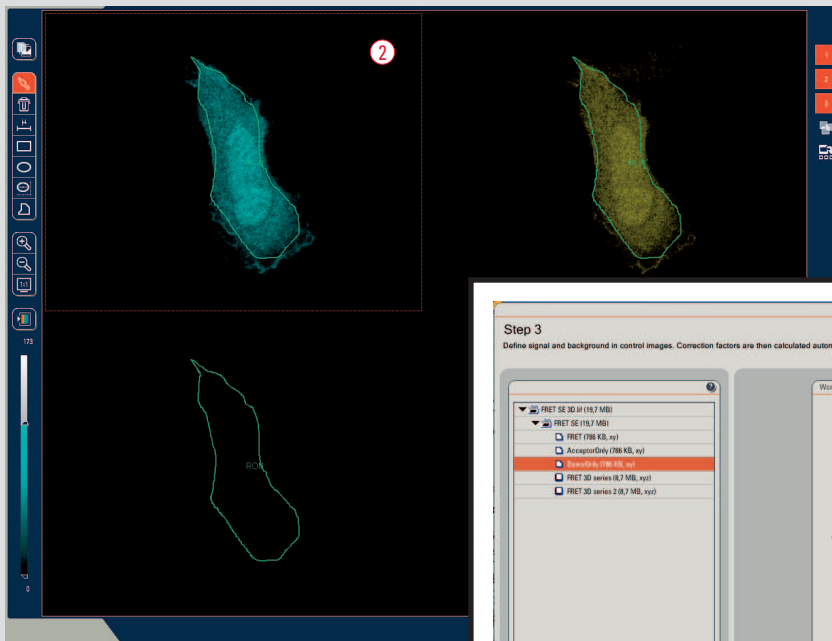
5. Slowly increase the excitation light of the acceptor (in our example 514 nm) until the signal of the acceptor (orange) is just below saturation. Do not change the PMT gain or offset as this will also change the imaging conditions for the FRET (yellow) signal detection!
6. Define the number of averages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

**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:**  
Ch1 (A): 0 Ch2 (B): 0 Ch3 (C): 0

**ROI Intensity:**  
Ch1 (A): 65 Ch2 (B): 7 Ch3 (C): 0

Accept

Calculate factors

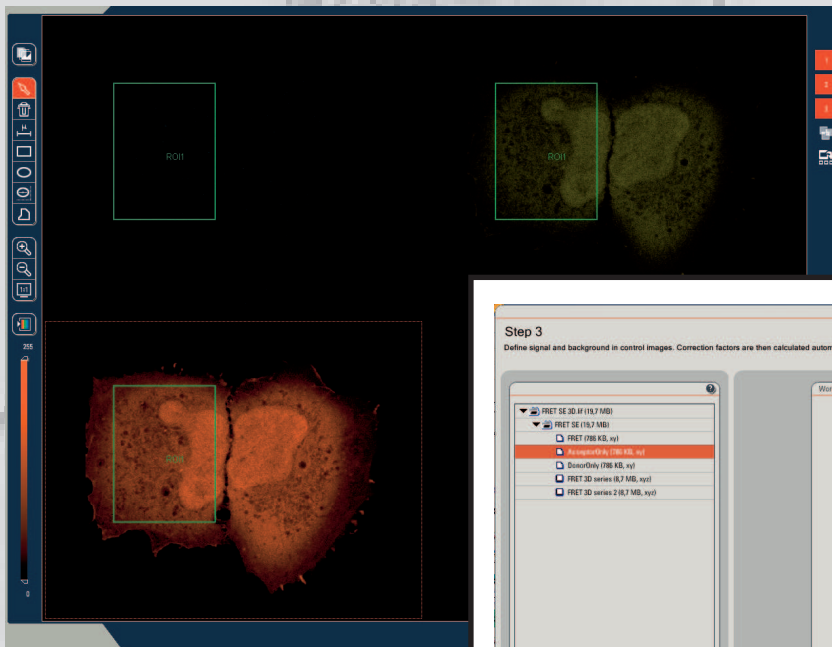
**Correction factors - FRET SE**

$\alpha$ : 0  $\beta$ : 0  $\gamma$ : 0  $\delta$ : 0

Load Save

Live Capture Image Start

Overview Setup Corr. Images **Corr. Factors** Evaluation Close



### Step 3: Calibration

**Step 3**  
Define signal and background in control images. Correction factors are then calculated automatically.

**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:**  
Ch1 (A): 1 Ch2 (B): 21 Ch3 (C): 21

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

Accept

Calculate factors 4

**Correction factors - FRET SE**

$\alpha$ : 0  $\beta$ : 0  $\gamma$ : 0  $\delta$ : 0

Load Save

Live Capture Image Start

Overview Setup Corr. Images **Corr. Factors** Evaluation Close

## 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)
<b>Specimen: FRET</b>	Signal (Donor)	Signal (FRET)	Signal (Acceptor)
<b>Specimen: Donor only</b>	Signal (Donor)	Signal < Channel 1 (x-talk)	No Signal
<b>Specimen: Acceptor only</b>	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.

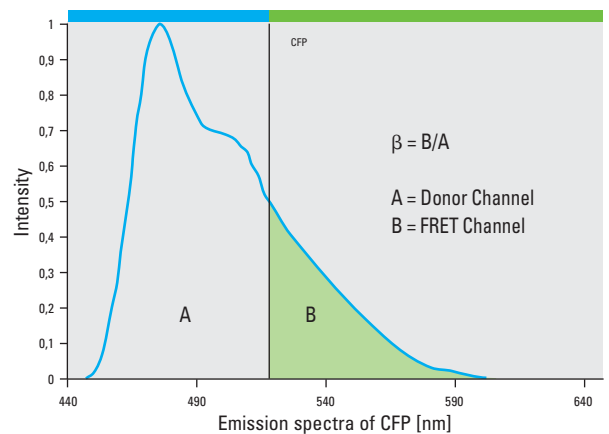
6. Continue to the next step **Evaluation** to perform live cell measurements after calibration and to retrieve FRET efficiencies.



## Calibration Factors:

1. Donor only reference generates correction factor  
Calibration Factors:

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



2. The Acceptor only reference generates correction factors  $\alpha$ ,  $\gamma$ ,  $\delta$ .

$\alpha$  corrects for acceptor cross-excitation cross-talk:

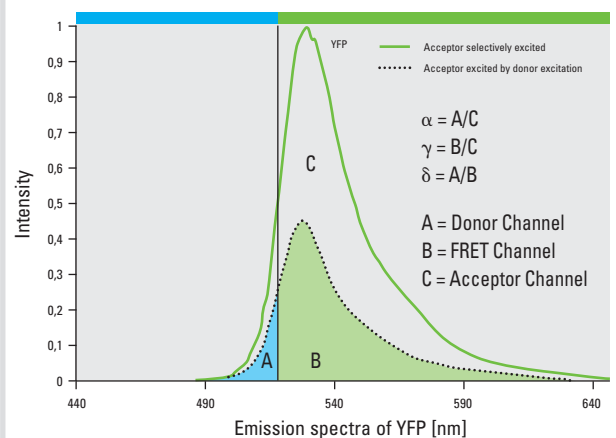
$$\alpha = A/C$$

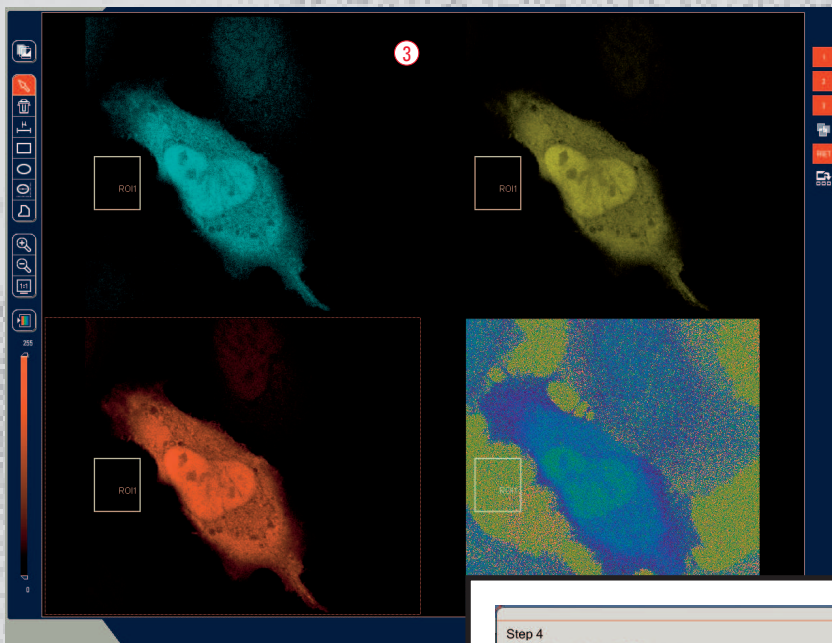
$\gamma$  corrects for acceptor cross-excitation:

$$\gamma = B/C$$

$\delta$  corrects for FRET cross-talk:

$$\delta = A/B$$





**Step 4: Calculation of FRET efficiencies and live cell measurements**

**Step 4**  
 Define time lapses and acquire the FRET-sequence.  
 Evaluation of FRET-Efficiency is automatically done. The table of results and FRET-Efficiency images are shown. Optionally background subtraction of FRET-data can be performed.

**Experiments** | **Tools**

- FRET SE 30.11 (18.7 MB)
- FRET SE (18.7 MB)
  - FRET (766 KB, .myf)
  - AcceptOnly (766 KB, .xy)
  - DonorOnly (766 KB, .xy)
  - FRET 3D series (8.7 MB, .myf)
  - FRET 3D series 2 (8.7 MB, .myf)

**Workflow**

FRET-efficiency calculated according to Method 1

**Optional ...**

Change method: Method 1 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): 3 Ch3 (C): 3 2

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

Accept

Define Time Lapses / Z-Stack

Acquisition Mode

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

Live | Capture Image | Run Experiment | Close

---

**Step 4**  
 Define time lapses and acquire the FRET-sequence.  
 Evaluation of FRET-Efficiency is automatically done. The table of results and FRET-Efficiency images are shown. Optionally background subtraction of FRET-data can be performed.

**Experiments** | **Tools** | **Acquisition**

Acquisition Mode: .myf

Z-Stack:

Z-Offset: Set Points

z-Position: -0.02

Nr. of steps: 2  
 z-step size: 0.01 µm  
 z-Volume: 0 µm

System optimized

Time interval: 0 h 0 m 2 s 31 ms

Duration: 1 h 0 m 0 s 0 ms

Stacks: 1

Reset | Accept

**Workflow**

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): 3 Ch3 (C): 3

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

Accept

Define Time Lapses / Z-Stack

Acquisition Mode 4

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

Live | Capture Image | Run Experiment 5 | Close



**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  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  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:**

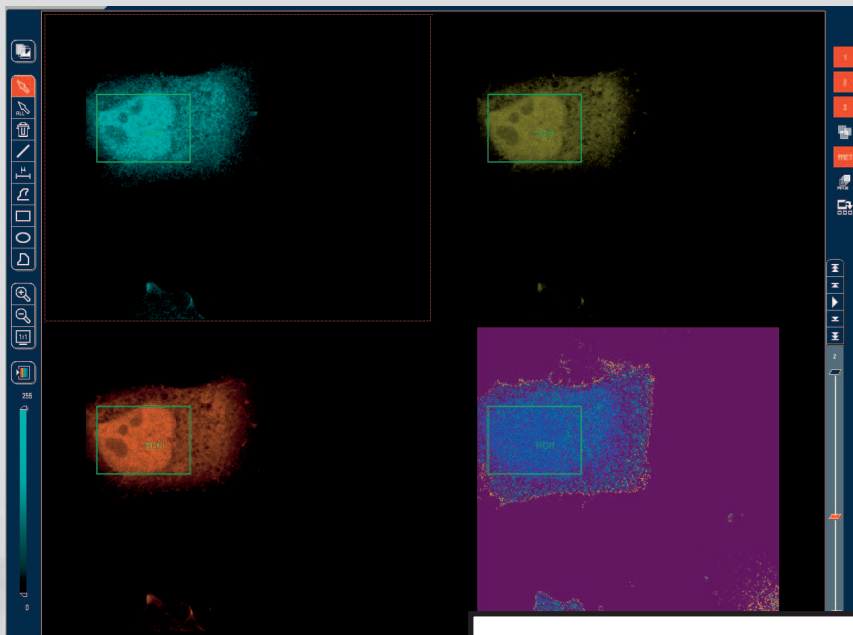
$$E_A(i) = \frac{B - A \times \beta - C \times (\gamma - \alpha \times \beta)}{C \times (1 - \beta \times \delta)}$$

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.

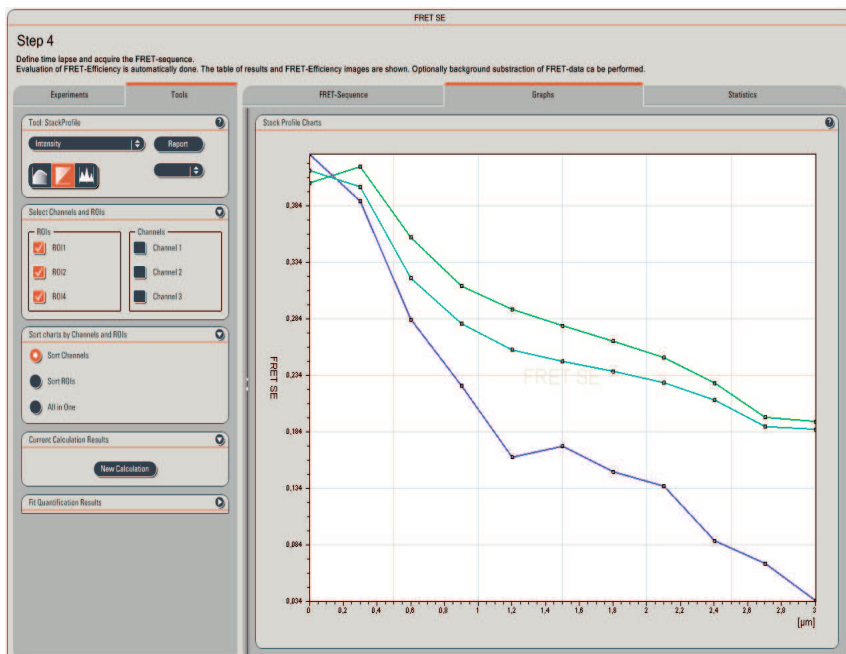
**Method 3:**

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

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



**Step 4: Calculation of FRET efficiencies and live cell measurements**



**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 subtraction of FRET-data can be performed.

Experiments Tools

Tool: StackProfile  
 Intensity Report

Select Channels and ROIs  
 ROIs:  ROI1  ROI2  ROI4  
 Channels:  Channel 1  Channel 2  Channel 3

Sort charts by Channels and ROIs  
 Sort Channels  
 Sort ROIs  
 All in One

Current Calculation Results  
 New Calculation

Stack Profile Statistics

	ROI1	ROI2	ROI4
FRET	0,292630556191256	0,161722117293816	0,256663229507594
Channel1	139,68	48,40	84,28
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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