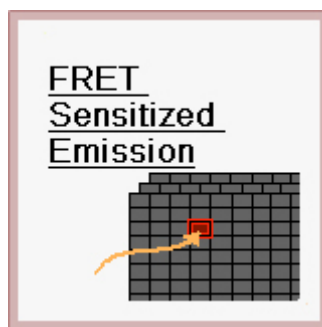


## FRET Sensitized Emission

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### Function

The FRET Sensitized Emission wizard is used for measuring the FRET efficiency. For this purpose, the fluorescence emission of the acceptor that results from the radiation-free energy transfer of an excited donor molecule is measured. However, the accuracy of the measurements can be affected by optical crosstalk, excitation of the acceptor through the excitation wavelength of the donor or impact of the background on the signal. The use of this method under the guidance of the wizard allows for correcting these phenomena and thereby contribute to optimizing the image and the measurement of the FRET share.

Three different types of cells (references) are required for the FRET experiment:

- ▶ Cells containing only the donor dye.
- ▶ Cells containing only the acceptor dye.
- ▶ FRET cells containing donor as well as acceptor dyes and in which the FRET occurs.

The first two samples provide the reference values that are required for correcting the optical crosstalk and the possible excitation of the acceptor through the wavelength of the donor. The third sample is the actual FRET sample on which the measurement is performed.

The FRET Sensitized Emission wizard is divided into three steps:

- ▶ In Step 1, the recording settings for donor, acceptor and FRET sample are defined or default values are loaded and adjusted.
- ▶ In Step 2, three channel images are sequentially recorded "line by line" and the ROIs are plotted that are used for correcting the optical crosstalk and the background effect as reference:
  - Channel A:** Excitation with excitation wavelength of donor and detection with parameter settings of donor (donor channel)
  - Channel B:** Excitation with excitation wavelength of donor and detection with parameter settings of acceptor (FRET channel)
  - Channel C:** Excitation with excitation wavelength of acceptor and detection with parameter settings of acceptor (acceptor channel)
- ▶ In Step 3, the FRET experiment and the analysis of the FRET efficiency are performed. In addition, several options are available to further optimize the resulting image and to save the measurement results.

### Procedure

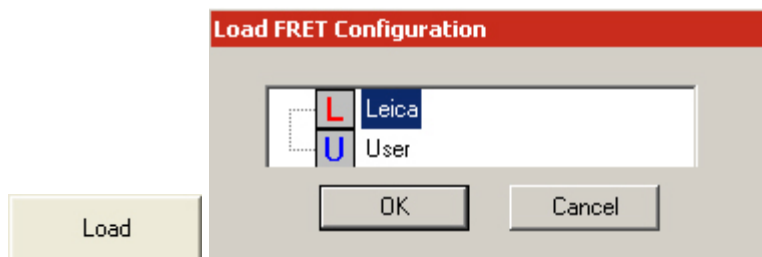
#### Step 1

**Note**

The complete experiment can be skipped if you are only interested in viewing the measurement results of saved data records. For this purpose, click the button:

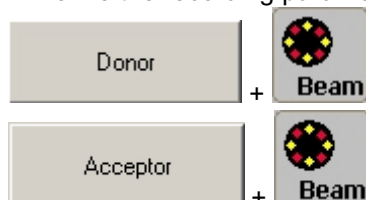


- 1 ▶ Load the predefined settings for recording the donor and acceptor.



Or:

- ▶ Define the recording parameters for the donor and acceptor.

**Note:**

One channel and one excitation wavelength are used to record images of the donor and acceptor.

- 2 ▶ The integration rate of image recordings to improve signal/noise ratio.

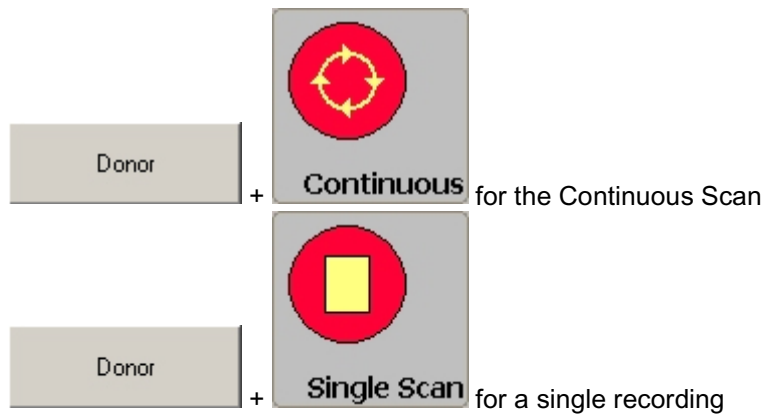


- 3 ▶ If necessary, zoom in on and center a section of the specimen.

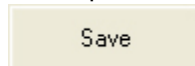


- 4 To determine the desired integration share for the image quality of donor and acceptor:

- ▶ Record an image of the donor (or acceptor and FRET sample)



- 5 ▶ If required, save the settings made for future FRET experiments.

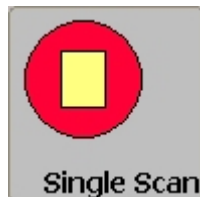


- 6 ▶ Proceed to the next step.



## Step 2

- 1 ▶ Start the recording.



The result will be three sequentially recorded channel images.

**Channel A:** Excitation with excitation wavelength of donor and detection with parameter settings of donor (donor channel)

**Channel B:** Excitation with excitation wavelength of donor and detection with parameter settings of acceptor (FRET channel)

**Channel C:** Excitation with excitation wavelength of acceptor and detection with parameter settings of acceptor (acceptor channel)

In this case, the *Continuous* button is used only for image optimization.

### Note

*The recorded images feature a digital resolution of 12 bit. For this reason, the dynamic range covers 4,096 different intensity values. However, the amount of data to be saved increases.*

### a) Donor only

- ▶ In the *View* window, change to the donor channel.



- 2 ▶ In the *Donor only* field, click on the *Signal* button.

Donor only		ROI	3002	198	62
Signal	Apply	A:	0	B: 0	C: 0
Background	Apply	A:	0	B: 0	C: 0

- ▶ Plot an ROI in the image where only donor dye (signal) is found.



### Note

The capital letters *A*, *B* and *C* represent the three channels.

The numbers above the three channels represent the mean intensity value of the ROI in the respective channel. If several ROIs were plotted, they represent the overall mean value of all ROIs in the respective channels. These are reference values that are used for the subsequent calibration and measurement. To accept the values for your measurements, click on the *Apply* button.

ROI	3002	198	62
Apply	A: 3002	B: 198	C: 62

- 3 ▶ In the *Donor only* field, click on the *Background* button.

Donor only		ROI	39	52	53
Signal	Apply	A:	3002	B: 198	C: 62
Background	Apply	A:	0	B: 0	C: 0

- ▶ Plot an ROI in the image where no signal is found (background).



### Note

The capital letters *A*, *B* and *C* represent the three channels.

The numbers above the three channels represent the mean intensity value of the ROI in the respective channel. If several ROIs were plotted, they represent the overall mean value of all ROIs in the respective channels. These numbers can be used to determine the amount of background effect in the different

channels. These are reference values that are used for the subsequent calibration and measurement. To accept the values for your measurements, click on the Apply button.

## b) Acceptor only

- 1 ▶ In the View window, change to the acceptor channel.



- 2 ▶ In the Acceptor only field, click on the Signal button.

Acceptor only		ROI	53	81	1304
Signal	Apply	A:	0	B:	0
Background	Apply	A:	0	B:	0

- ▶ Plot an ROI in the image where only acceptor dye is found.



### Note

The capital letters A, B and C represent the three channels.

The numbers above the three channels represent the mean intensity value of the ROI in the respective channel. If several ROIs were plotted, they represent the overall mean value of all ROIs in the respective channels. These are reference values that are used for the subsequent calibration and measurement. To accept the values for your measurements, click on the Apply button.

ROI	53	81	1304			
Apply	A:	53	B:	81	C:	1304

- 3 ▶ In the Acceptor only field, click on the Background button.

Acceptor only		ROI	31	48	49
Signal	Apply	A:	53	B:	81
Background	Apply	A:	0	B:	0

- ▶ Plot an ROI in the image where no signal is found (background).

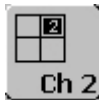
**Note**

The capital letters A, B and C represent the three channels.

The numbers above the three channels represent the mean intensity value of the ROI in the respective channel. If several ROIs were plotted, they represent the overall mean value of all ROIs in the respective channels. These numbers can be used to determine the amount of background effect in the different channels. These are reference values that are used for the subsequent calibration and measurement. To accept the values for your measurements, click on the Apply button.

**c) FRET Sample**

- 1 ▶ In the View window, change to the FRET channel.



- 2 ▶ In the FRET Sample field, click on the Background button.

FRET Sample		ROI	35	51	53
Background	Apply	A:	<input type="text" value="0"/>	B:	<input type="text" value="0"/>
		C:	<input type="text" value="0"/>		

The numbers above the three channels represent the mean intensity value of the ROI in the respective channel. If several ROIs were plotted, they represent the overall mean value of all ROIs in the respective channels. These values are used for the FRET efficiency measurement. To accept the values for your measurements, click on the Apply button.

- 3 ▶ Proceed to the next step.

**Step 3**

- 1 ▶ Adjust the necessary time parameters for live specimen:



& see [Recording time image series](#)

**Note**

If no settings are performed, the default value is 1 frame.

- 2 ▶ Start the FRET experiment.



- 3 The values in the *Calibration Coefficients* field represent the constants that the software calculated from the calibration measurement in Step 2. They describe the crosstalk between the channels.

$a$  = share of acceptor signal in the donor channel (channel A).  
Correction factor from the acceptor sample = donor emission (donor excitation) / acceptor emission (acceptor excitation)


$a$  = share of donor signal in the FRET channel (channel B).  
Correction factor from the donor sample = acceptor emission (donor excitation) / donor emission (donor excitation)

$c$  = share of acceptor signal in the FRET channel (channel B).  
Correction factor from the acceptor sample = acceptor emission (donor excitation) / acceptor emission (acceptor excitation)

**Note**

*If you activate the Manual Correction check box, you can manually correct the values calculated by the software.*

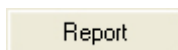
- 4 Finally, the FRET effectiveness is automatically calculated by the Leica Confocal software for the complete frame or the plotted ROIs.

The calculated values are displayed in the *Calibrated Results* field. To learn about the meaning of the different formula components, click on the question mark in the corner (  ).

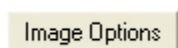
Several options are also available in this step to further optimize the resulting image and to save the measurement results.



This function creates an analysis graph that represents the time-based behavior of the median intensity during the data recording.



You can save the complete FRET experiment in xml format. The file contains the image recording of donor, acceptor and FRET, the number of recorded frames and their analysis data, the measurement parameters and the hardware parameters defined for the experiment.



*Visualization factors*

The display of the FRET signal and the FRET effectiveness can be intensified

here.

#### *Thresholds*

A threshold can be defined for each channel to perform a noise correction.

#### *Filter*

If the Filter source (Blur) check box is selected, a blur filter is applied. This filter type filters the high frequencies out of the image. That is, the distinctive transitions from low to high intensity values are reduced. This filter can be used to remove noise from an image.

#### *Apply-keys*

The Apply buttons are used to accept the optimized images in the experiment.