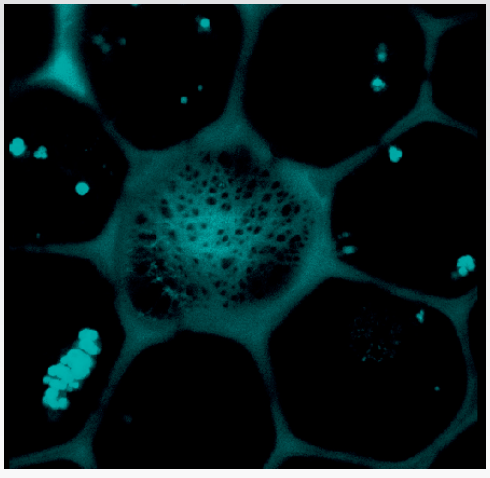
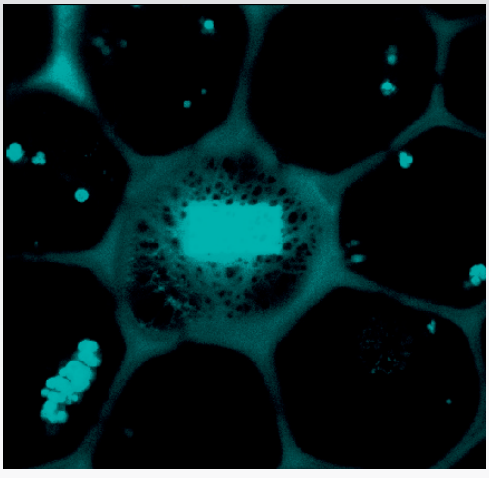
Dec.
2007
No. 28

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.

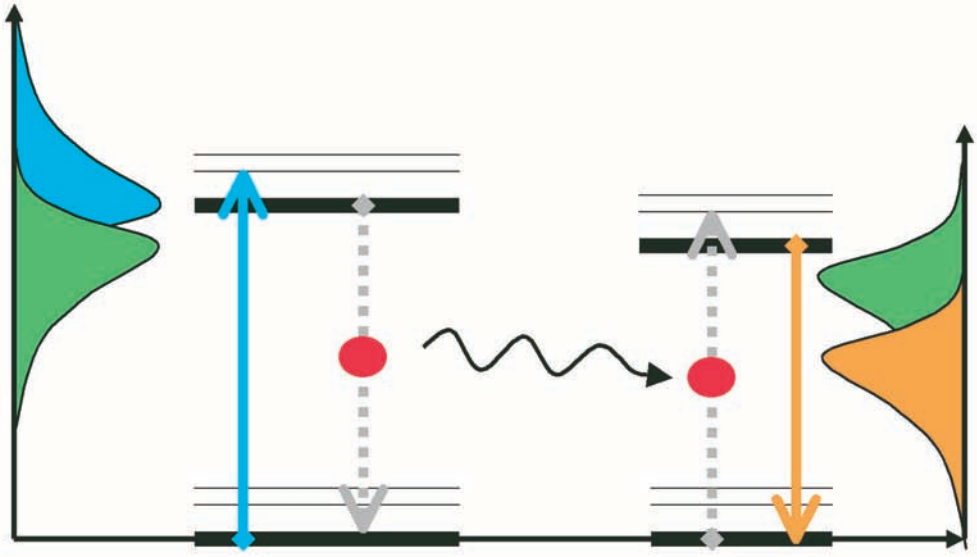
FRET Acceptor Photobleaching

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.



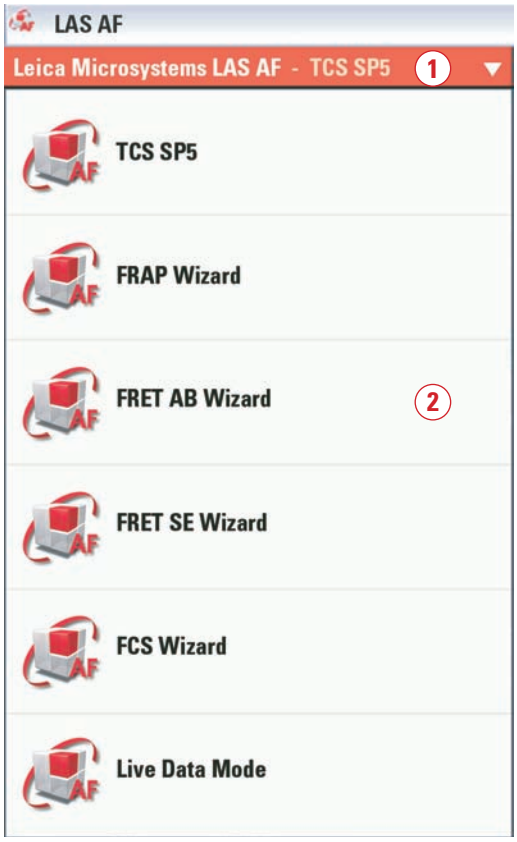
In the event of FRET an excited fluorophore transfers its energy to another light absorbing molecule.

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.



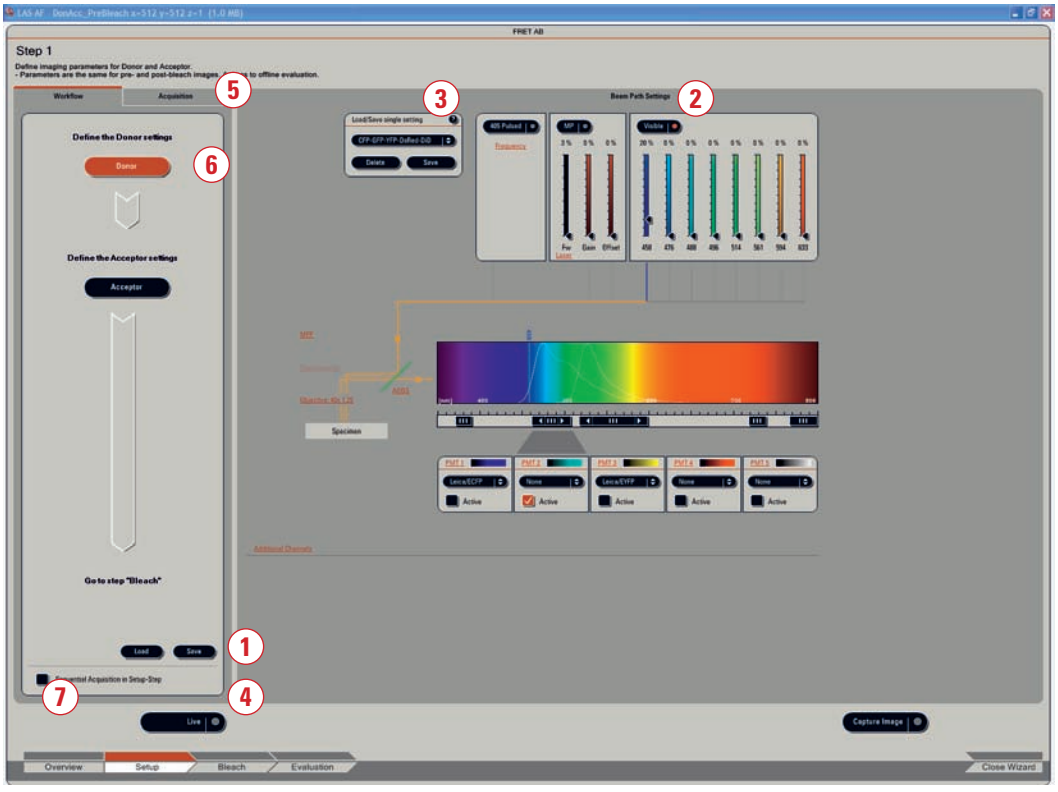
FRET Acceptor Photobleaching – Step by Step

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%.



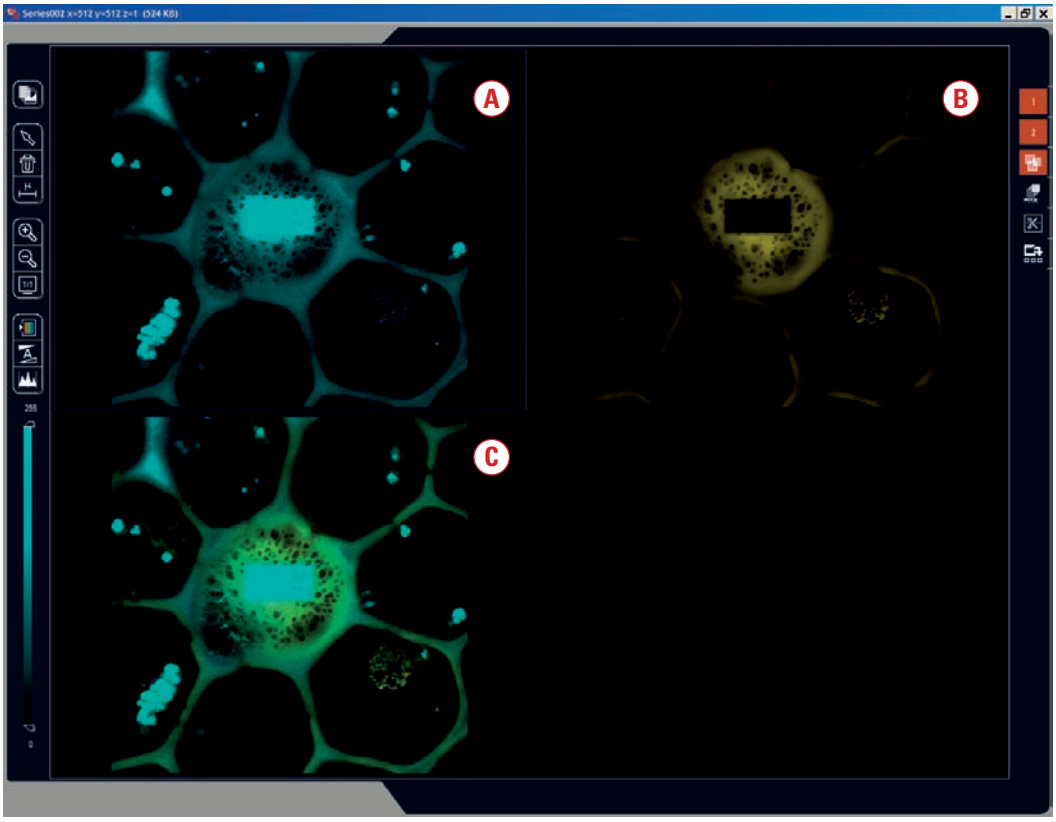
3. Start **Live Scan (4)** for image optimization.

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.

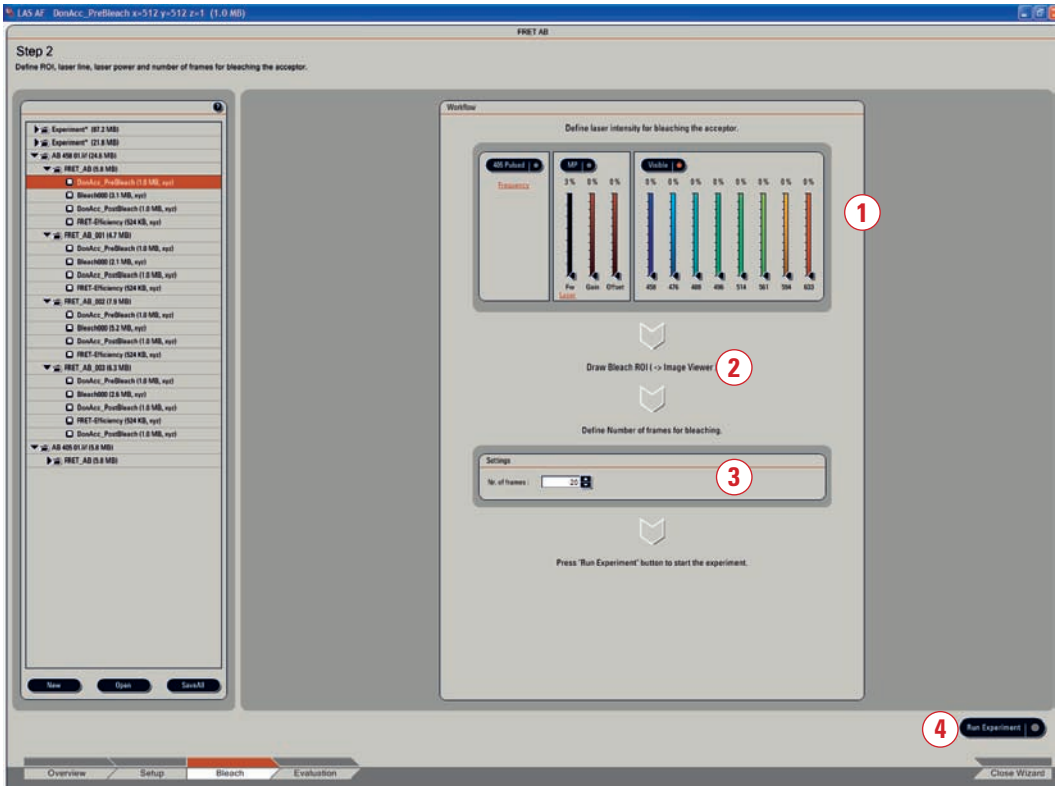
Step 2: Define Acceptor Photobleaching Conditions

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.

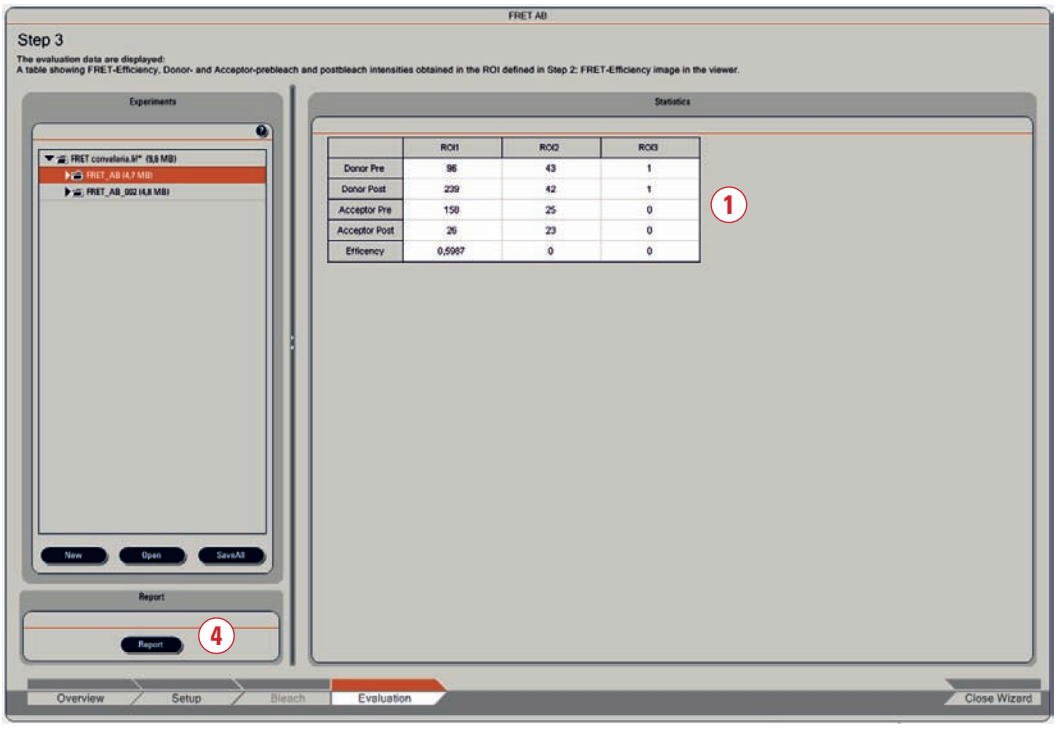
4. You may now run the bleaching experiment **(4)**. The measurement will start by imaging the donor and acceptor before bleaching, followed by the bleaching of the acceptor and finally imaging the donor and acceptor after bleaching. The imaging conditions before and after bleaching are identical. The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

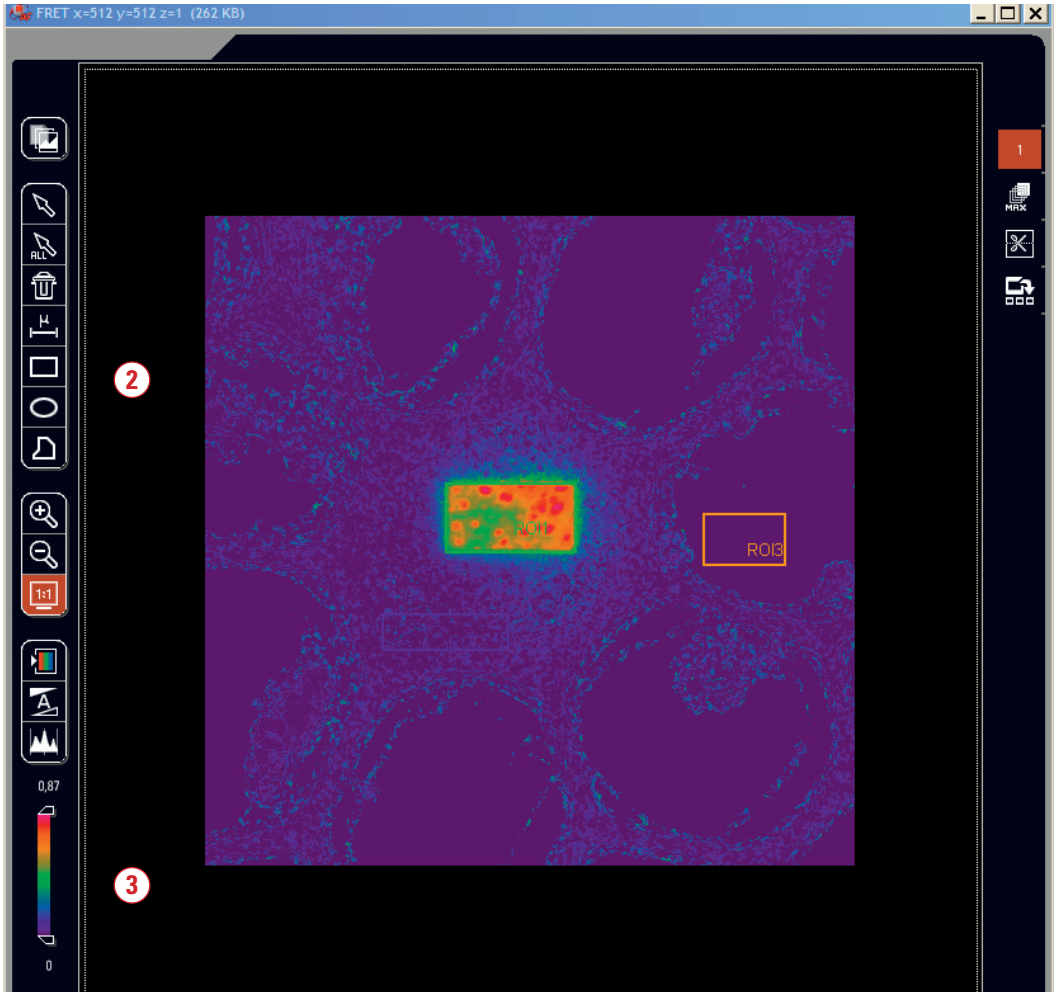


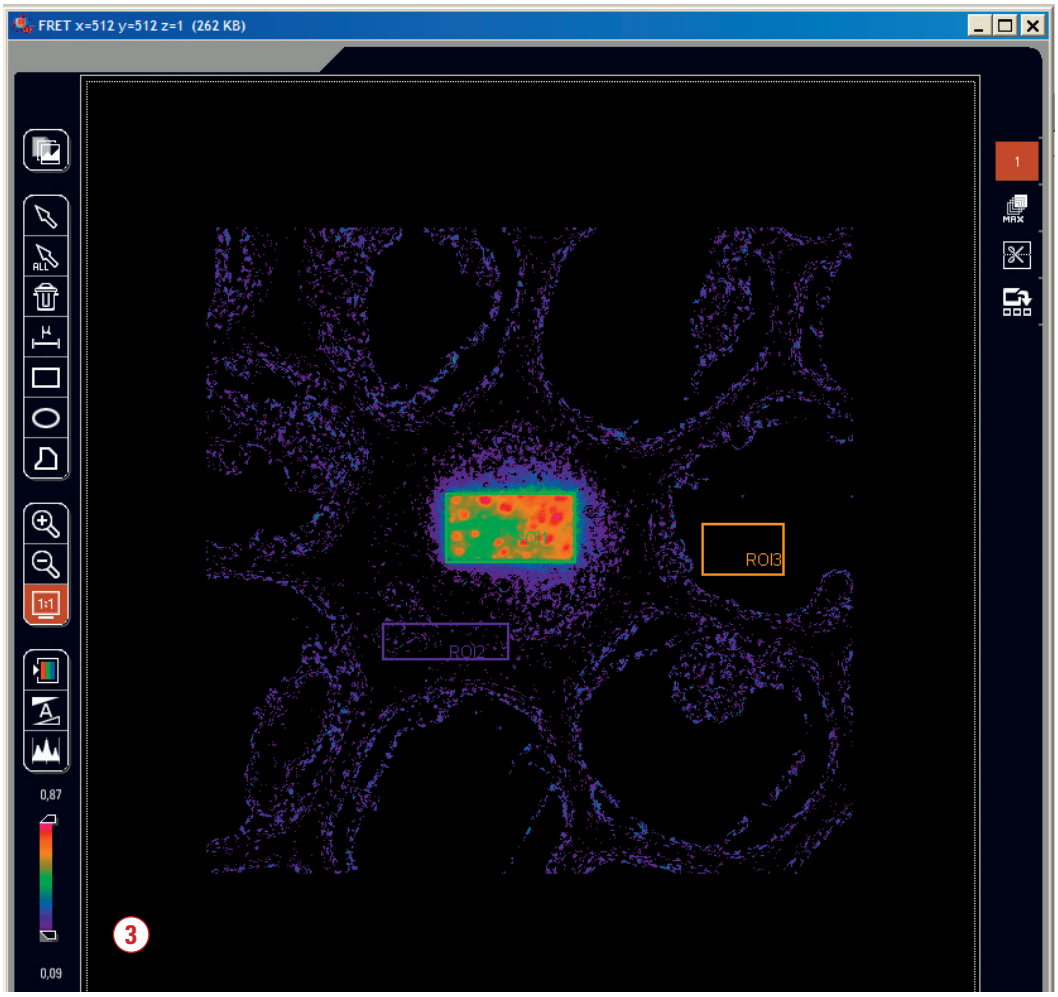
Step 3: Evaluation

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)**.

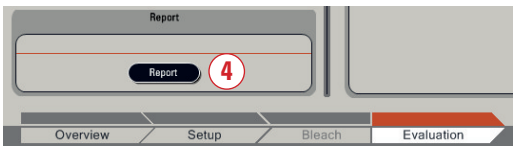
2. You may choose additional regions of interest for better interpretation of results **(2)**.


FRET AB
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.
Experiments
Statistics
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
1
New
Open
SaveAll
Report
Report
4
Overview
Setup
Bleach
Evaluation
Close Wizard


FRET x=512 y=512 z=1 (262 KB)
2
3
ROI1
ROI3
0,87
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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