FRET Acceptor PhotoBleaching (AB) in LASAF

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1- Generalities on FRET:

Fluorescence Resonance Energy Transfer (FRET) is a technique allowing study of proteins interactions, which are in proximities but beyond light microscopy resolution (classically, fluorescently labeled molecules over distances of 1-10 nm). Originally measured by fluorescence spectroscopy, FRET can also be measured by fluorescence microscopy. Since FRET occurs over distances similar to the size of proteins, it can be used to extend the resolution of the fluorescence microscope (typically >250 nm) to detect protein-protein interactions. Hence, it is an ideal approach to determine whether proteins that are co-localized at the level of light microscopy really interact with one another.

Method: FRET involves a nonradiative transfer of energy from an excited state donor fluorophore to a nearby acceptor. The energy transfer efficiency $FRET_{eff}$ is directly related to the distance r separating a given donor and acceptor pair by

$$FRET_{eff}=1/[1+(r/R_0)^6]$$

The resolution of FRET is thus defined by R_0 which is typically $< 10\text{-}70$ Å. R_0 depend of the extent of overlap between the donor emission and the acceptor spectra, the absorption coefficient for the acceptor, the quantum yield of the donor, and the relative orientation of the donor and acceptor. In any case, when distances separating donor and acceptor are superior of 2 R_0 , no FRET can occur. From this, it is possible to distinguish between 2 proteins being present in the same cytoplasmic compartment for instance, from those undergoing specific protein-protein interactions.

FRET Acceptor Bleaching involves measuring the donor "de-quenching" in the presence of an acceptor. This can be done by comparing donor fluorescence intensity in the same sample before and after destroying the acceptor by photobleaching. If FRET was initially present, a resultant increase in donor fluorescence will occur on photobleaching of the acceptor. The energy transfert efficiency can be quantified as:

$$FRET_{eff}= (D_{post}\text{-}D_{pre})/D_{post}$$

where D_{pos} is the fluorescence intensity of the Donor after acceptor photobleaching, and D_{pre} the fuorescence intensity of the Donor before acceptor photobleaching. The $FRET_{eff}$ is considered positive when $D_{post} > D_{pre}$.

Choice of donor-acceptor pair: The FRET AB requires that the acceptor is easily bleached under normal experimental conditions, and that the donor is relatively stable. Also, it requires that on photobleaching, the acceptor is no longer able to act as an efficient energy transfer acceptor by no longer absorbing an overlapping region with the donor. Few donor-acceptor pair follows these criteria: Cy3-Cy5, CFP-YFP, GFP-Cy3, and GFP-Cy3.5. Fluorescein-Rhodamine will FRET too. But this list is not exhaustive (please refer to our donor/acceptor list at the end of this application note).
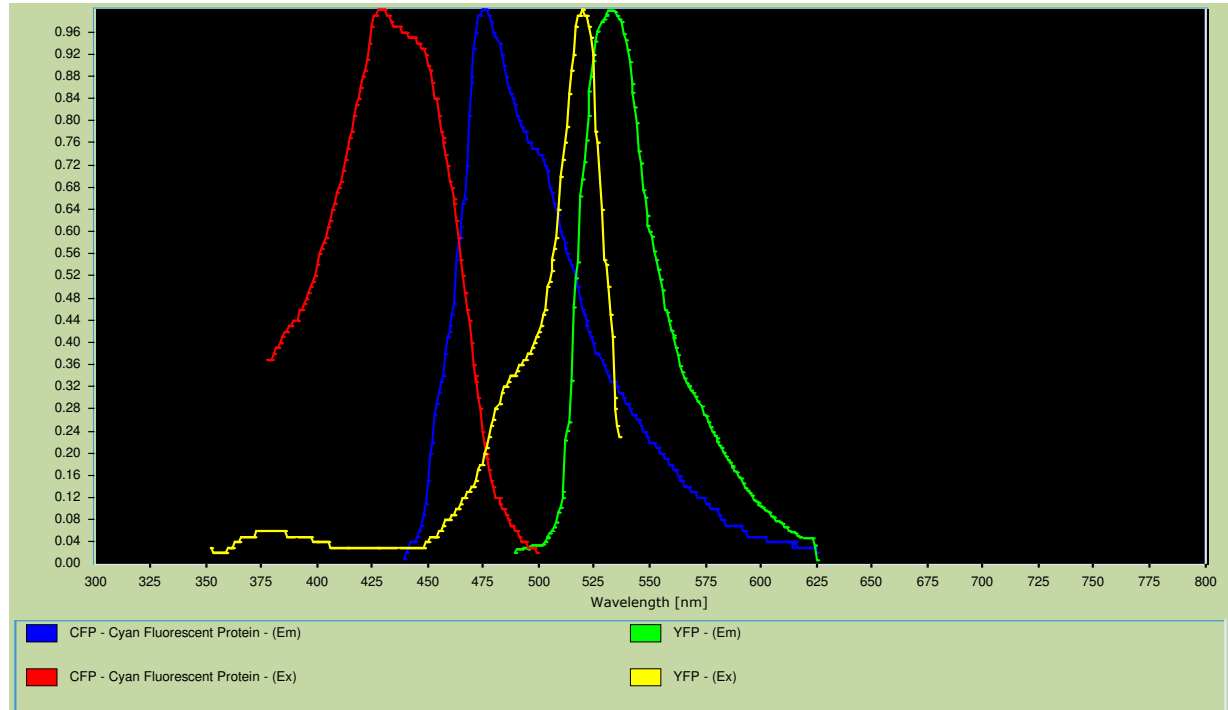
MICROSYSTEMS

In conclusion ➔ Precondition for FRET:
- Overlap of Donor emission and Acceptor absorption spectra.
- Sufficient separation of excitation and emission spectra.
- Donor and Acceptor distance typically ranges from 2 to 10 nm.

(Example of excitation and emission spectra for CFP and YFP fluorophores)

Examples of applications for FRET:
- Conformational changes of macromolecules (proteins).
- Association/Dissociation of macromolecules (protein subunits, DNA oligomers).
- Changes in concentration of small metabolites (camp) or ions (calcium signals).
- Membrane potential changes.

Limitations of FRET AB:

- When bleaching the acceptor, the donor may also be bleached.
- Specimen can moves during the bleaching procedure, which limits the application mostly to fixed or immobile samples.
- Acceptor can be difficult to bleach.

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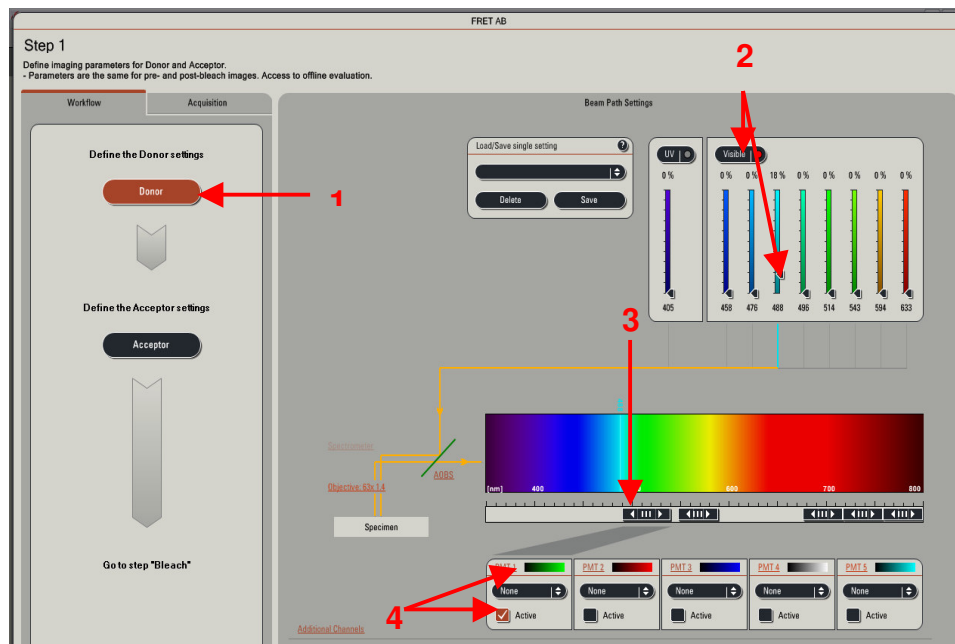Vol. 4   September 2006

2- FRET AB using LASAF wizard:

A – Activate the FRET AB wizard: Click on the upper left corner on the **Leica Microsystems LASAF** drop-down window to access the applications. Choose FRET AB Wizard as shown on the picture.
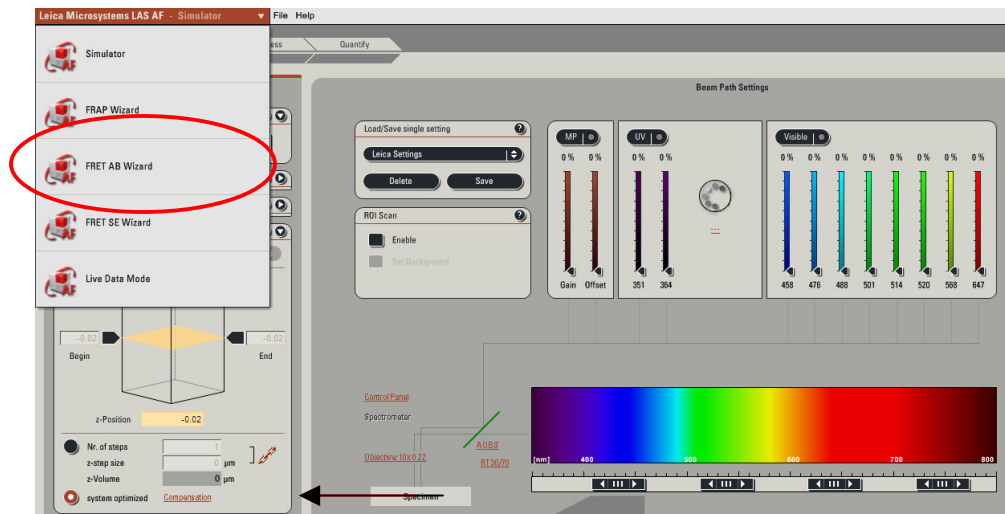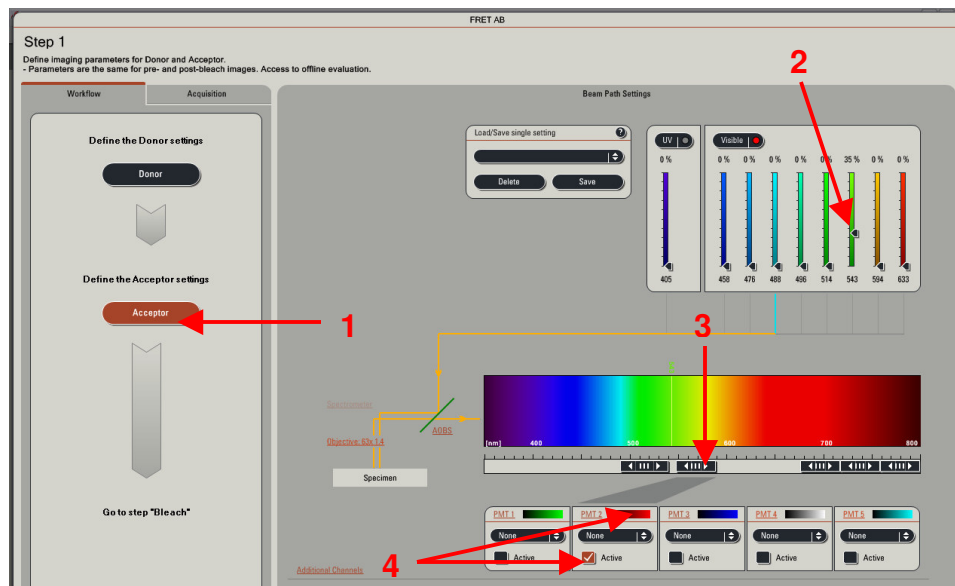

B – Step 1.1 ➔ Donor Setup:

1- Activate the Donor.
2- Activate the laser line corresponding to your Donor excitation (488 Argon laser in this example).
3- Position your PMT in accordance with your Donor emission.
4- Activate your PMT and change the color if needed by clicking on the color line.

C – Step 1.2 ➜ Acceptor Setup:

1- Activate the Acceptor.
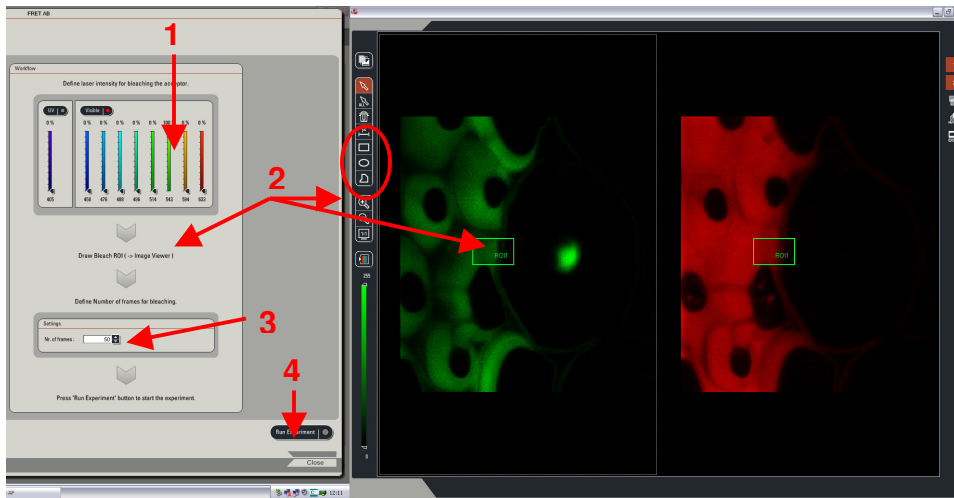2- Activate the laser line corresponding to your Acceptor excitation (543 nm laser in this example).
3- Position your PMT in accordance with your Acceptor emission.
4- Activate your PMT and change the color if needed by clicking on the color line.

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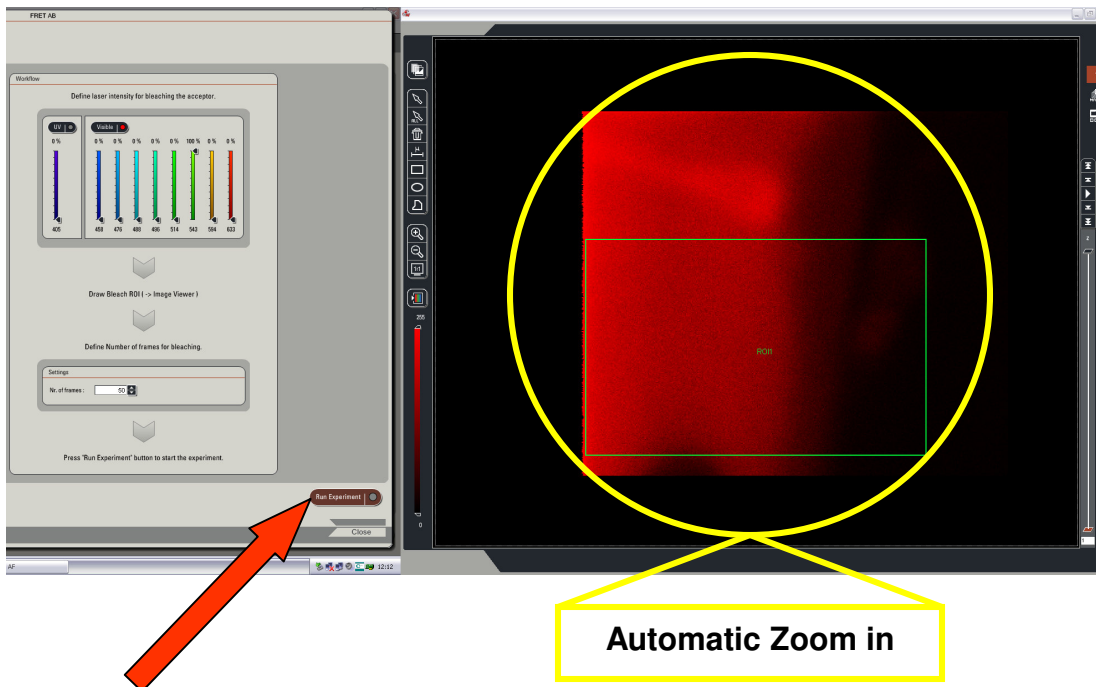# Confocal Application Notes
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D – Step 2.1 ➔ Bleaching Setup:

1- Position your Acceptor laser line at 100% for the bleaching setup (or less if your sample is very sensitive to bleaching).
2- Draw a Region of Interest (**ROI**) inside your image, corresponding to the bleaching area and the region where the FRET efficiency will be calculated. You can choose your ROI shape by clicking on the left image side (rectangular, round, or free drawing ROI shape).
3- Enter the number of Frame that the bleaching will be applied in side the ROI.
4- Press "**Run Experiment**" to start the experiment.

D – Step 2.2 ➔ Experiment running: Bleaching Step

Automatic Zoom in

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Once activated the experiment will run automatically in sequence:
1- First, images of the Donor and Acceptor prebleaching will be taken.
2- The bleaching will begin by a zoom in of the ROI. Bleaching of the ROI will be done as determined by the number of frame chosen.
3- Images of the Donor and Acceptor postbleaching will be taken.

E – Step 3 ➔ Results

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

- Experiment* (8.4 MB)
  - Image000 (524 KB, xy)
  - FRET_AB (3.9 MB)
    - DonAcc_PreBleach (524 KB, xyz)
    - Bleach000 (2.6 MB, xyz)
    - DonAcc_PostBleach (524 KB, xyz)
    - FRET-Efficiency (262 KB, xyz)

	ROI1	ROI2	ROI3	ROI4
Donor Pre	36	36	72	0
Donor Post	73	90	151	0
Acceptor Pre	86	153	141	2
Acceptor Post	23	39	27	0
Efficency	0.498	0.5923	0.519	0.1

$$\text{Efficiency} = \frac{\text{Donor}_{\text{post-bleach}} - \text{Donor}_{\text{pre-bleach}}}{\text{Donor}_{\text{post-bleach}}}$$
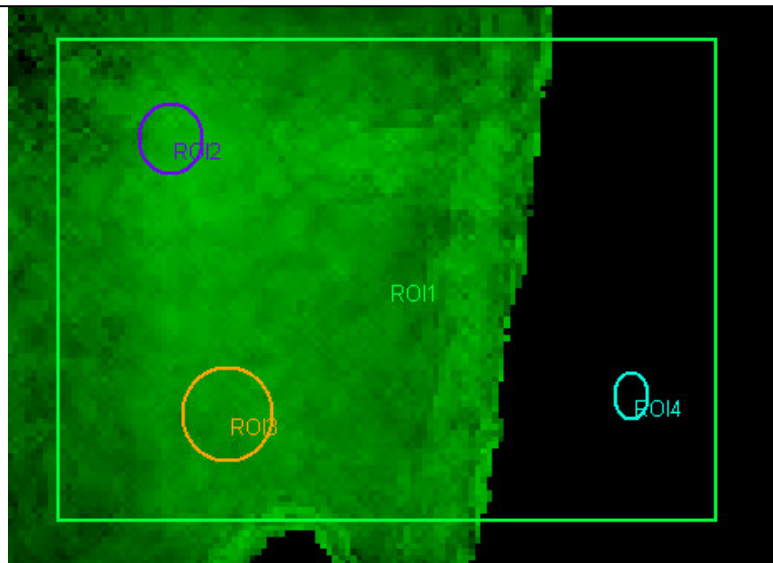
In this example, the ROI1 values are corresponding to the total ROI bleached. Sub-ROIs (ROI2 and 3) can be drawn inside the bleached area in order to measure more discrete FRET events. The ROI4 was drawn outside the cell in order to measure the background. If the background values were high, these values would need to be subtracted from the donor pre- and postbleaching in order to compensate for the background noise.

3 - Donor-Acceptor pair:

For you information, we have listed here the Donor-Acceptor pair, which will FRET when doing a FRET AB.
Remember that FRET will occur when R_0 is <10-70 Å.

	R_0 (Å)					
Donor	**Acceptor**					
	Alexa Fluor 488	**Alexa Fluor 546**	**Alexa Fluor 555**	**Alexa Fluor 568**	**Alexa Fluor 594**	**Alexa Fluor 647**
Alexa Fluor 350	50					
Alexa Fluor 488	NA	64	70	62	60	56
Alexa Fluor 546		NA		70	71	74
Alexa Fluor 555			NA		47	51
Alexa Fluor 568				NA		82
Alexa Fluor 594					NA	85
Alexa Fluor 647						NA

Donor	Acceptor	R_0 (Å)
Fluorescein	**Tetramethylrhodamin**	55
Cy3	**Cy5**	>50
CFP	**YFP**	50
BFP	**GFP**	40
CFP	**GFP**	48
GFP	**YFP**	57

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