PRACTICAL MANUAL FOR FLUORESCENCE MICROSCOPY TECHNIQUES

Sohail Ahmed
Sudhaharan Thankiah
Radek Machán
Martin Hof
Andrew H. A. Clayton
Graham Wright
Jean-Baptiste Sibarita
Thomas Korte
Andreas Herrmann

chapter

Acceptor Photobleaching FRET (AP-FRET)

Thankiah Sudhaharan and Sohail Ahmed
Institute of Medical Biology, Singapore
Contact details of corresponding author: Thankiah Sudhaharan, sudhaharan.thankiah@imb.a-star.edu.sg

Index

Abstract

Although the concept of FRET has been known since the 1960s, its first implementation in the context of cell experiments was not until the 1990s. Bastiaens, Jovin, and colleagues pioneered the use of FRET to study membrane trafficking and cell signaling pathways[1]. Acceptor photobleaching (AP)-FRET was first implemented to track cholera toxin complex formation using the cyanine dye pairs Cy3 and Cy5[2]. Subsequently, when GFP and its variants became available, AP-FRET was used to follow 5HT receptor dimerization[3]. Initially, CFP and YFP were the fusion proteins of choice for FRET experiments as their spectral characteristics were well suited to FRET. However, GFP/mRFP or mCherry are preferred nowadays. AP-FRET is the least complicated method to perform FRET. It can utilize GFP/mRFP or GFP/mCherry pairs, like other FRET methods, as donor and acceptor, respectively. Moreover, AP-FRET can be performed on standard confocal microscopes, which are usually available in most institutions. In this chapter we describe in detail how to perform AP-FRET, define positive FRET, and design controls.

1. Principle and Theory

In simple terms, Förster Resonance Energy Transfer (FRET) is a non-radiative energy transfer process occurring due to interaction between the excited state of a donor and the ground state of an acceptor fluorophore. FRET occurs over distances of 10 nm or less (Figure 1). Hence FRET can be used as a molecular ruler working on the nanoscale. In biology FRET is widely used to measure biomolecular interactions such as protein-protein, protein-RNA, protein-ligand or protein-DNA. In this chapter, we focus on genetically encoded GFP and mRFP fluorescent proteins as tags to measure FRET between Cdc42 and CRIB fusion proteins by a method known as Acceptor Photobleaching (AP)-FRET. The fundamental aspect of the method lies in the comparison of donor fluorescence intensity (GFP) when in the presence or absence of an acceptor (mRFP or mCherry). If FRET is occurring between two fluorophores then it follows that eliminating the acceptor by means of photobleaching will increase donor fluorescence intensity. In contrast, in the absence of FRET, eliminating the acceptor should not affect the donor fluorescence intensity. Thus by measuring donor fluorescence intensity under these two different conditions should allow determining whether FRET is occurring or not. Another important advantage of AP-FRET is that it can be used to examine spatial aspects of protein-protein interactions in single cells under very precise conditions by choosing particular Regions Of Interest (ROIs). It should be noted that AP-FRET can only be performed on fixed samples, i.e. when fluorophore diffusion is prevented.

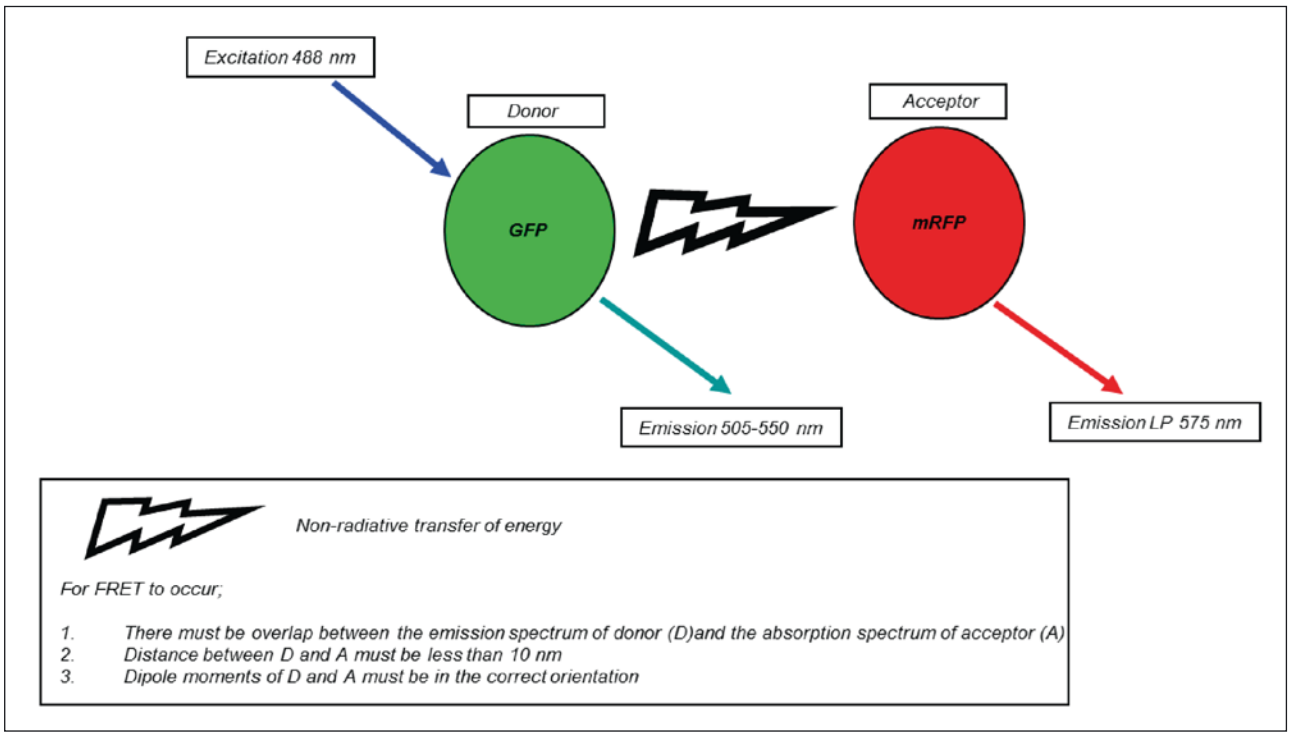


Figure 1 *Shown here is the pictorial representation describing FRET occurrence.*

2. Instrumentation

Either a confocal laser scanning microscope with lasers emitting at 488 and 561 nm for excitation with respective emission filter sets or a widefield microscope with appropriate filter sets and corresponding dichroic mirrors are necessary for the GFP and RFP pair. A 60 X water objective with high NA and either PMT detector (Confocal) or CCD camera (widefield) are also required.

3. Methods

A. Materials Required

We use CHO cells as a starting point for AP-FRET because this cell line expresses a wide range of GFP and mRFP fusion proteins well. In principle, any cell line of interest can be used for AP-FRET. Other required material include: CO_2 incubators (37 °C), Fetal Bovine Serum (FBS), antibiotics (penicillin and streptomycin), trypsin, plasmids with fluorescent tags (expressing the acceptor and donor fusion proteins), cell culture medium (F-12K nutrient mixture [Kaighn's modification] for CHO cells), transfection reagent (Turbofect), coverslips (diameter 18 mm) and hemacytometer. six well plate dishes, T75 flasks and 10 ml pipettes. para-formaldehyde, mounting media (without antifade) and microscopy cover slides.

B. Sample Preparation

CHO cells were grown in a 75 cm^2 tissue culture flask up to 90% confluency in the complete growth medium (1 x F-12K nutrient mixture [Kaighn's modification]) containing 10% fetal bovine serum qualified [FBS] and 1% antibiotics [penicillin and streptomycin]. Cells were detached from the flask, using 2 ml trypsin by incubating at 37 °C for 5 min and counted using a hemacytometer. For transfection, cells were seeded with a cell density of 1.5 x 10^5 cells in a 6-well tissue culture plate containing a 18 mm pre-washed and sterilized cover glass for 24 h. CHO cells were then transfected using Turbofect transient transfection reagent (other transfection reagents suited for the cell type can also be used) as per the following protocol. [Mix 3 µl of Turbofect with 1.5 µg of plasmid (1:2 (w/v), DNA/Turbofect) and 150 µl of serum free medium in a tube and let it stand for 25 min at RT]. Transfection mixes of respective plasmids were then transferred into different wells of a six well plate containing cells. The transfected cells in the six well plates were incubated at 37 °C in a CO_2 incubator for 24 h for protein expression to occur. Typically three transfections are used; (i) GFP-mRFP (tandem fusion) positive control. (ii) Co-expression of free GFP and mRFP (negative control). (iii) Co-expression of mRFP-Cdc42 and GFP-CRIB as experimental model. These positive and negative controls are essential to correlate to potential positive FRET scenarios. Other controls including point mutants or deletion constructs, fusion protein/GFP, or mRFP combinations should also be considered for rigorous analysis. 24 h after transfection, cells are washed 3 times with 1 x PBS and are fixed using 4% p-formaldehyde for 15 min and quickly washed with 50 mM Tris pH 8.0, 100 mM NaCl to rinse excess fixative and then washed again for 5 min. It is advisable to use the right fixative to keep the cell structures intact. The cover slip containing fixed cells are mounted on a microscopy slide using Hydromount, an aqueous non-fluorescing mounting media, and allowed to dry overnight.

4. Data Acquisition

Any type of fluorescence microscope offering the necessary settings and controls can be used to perform the experiment. Although the Zeiss LSM

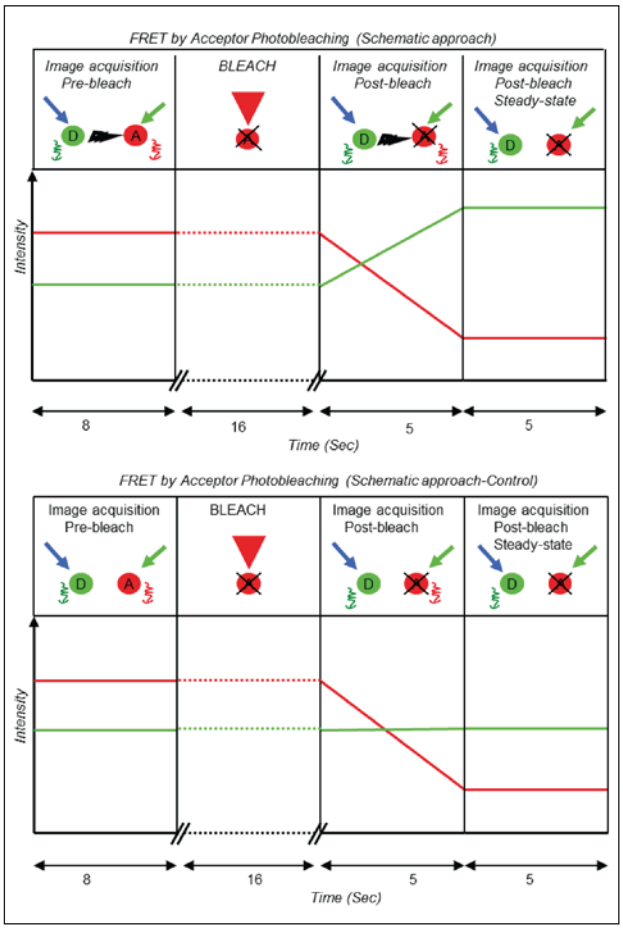


Figure 2 *Schematic representation is describing actual FRET and non-FRET situation expected from a typical FRET experiment.*

510 confocal microscope is described in detail here, confocal microscopes from other manufacturers are suited as well. The software control sequences are given in the Appendix. If another type of confocal microscope is used, the equivalent software control windows have to be identified while the actual steps remain the same.

The AP-FRET experiment is running as a time sequence over a period of 35 s. Cells with similar modest levels of GFP/mRFP expression are chosen. Cells with high over expression should be avoided.

Dual channel recording (GFP and mRFP) is initiated pre-bleach and fluorescence intensity is measured for 8 s. Acceptor bleaching is then initiated for 16 s, followed by dual channel imaging for further 10 s. The bleaching time was selected in such a way that the rate of decrease in fluorescence intensity was approx. 66 u/s (see data analysis section b). Under the experimental conditions, the rate of change in fluorescence intensity post-bleaching can be followed accurately (Figure 2).

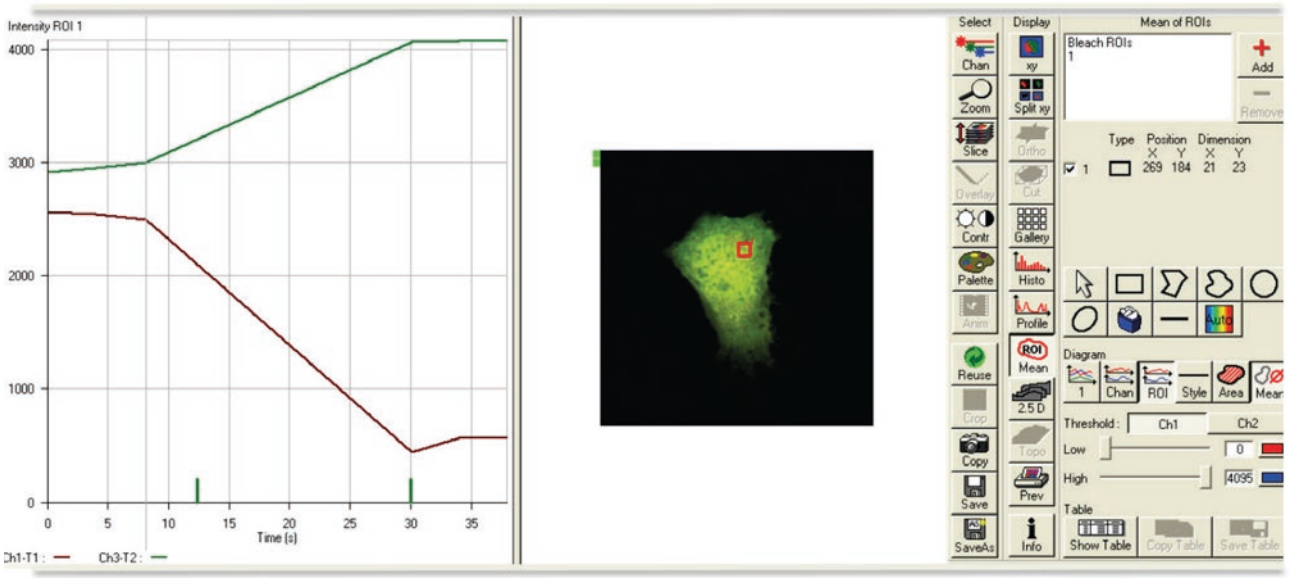


Figure 3 *A positive FRET control (GFP-mRFP) tandem fusion sample expressed in CHO-1 cells is undergoing FRET measurement. The sample was prepared as described in the method by co fusing GFP and mRFP and fixing after gene expression.*

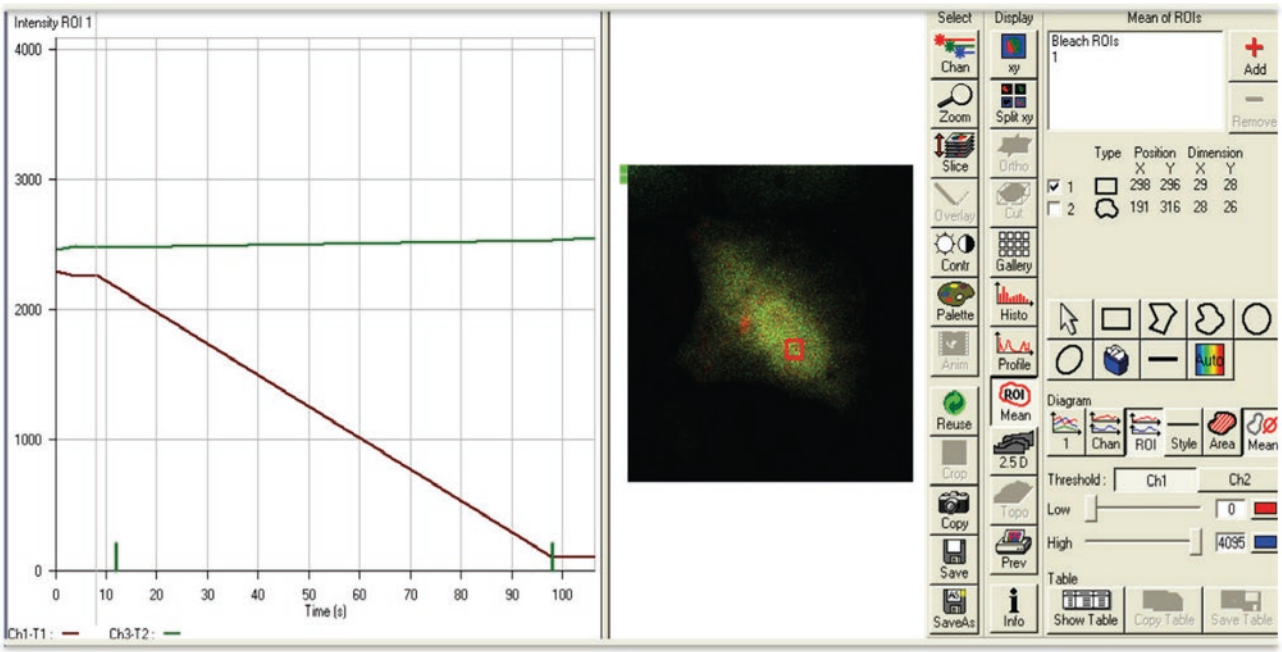


Figure 4 *A negative FRET control (free GFP and mRFP) sample expressed in CHO-1 cells is undergoing FRET measurement. The sample was prepared as described in the method by co-expressing free GFP and mRFP and fixing after gene expression.*

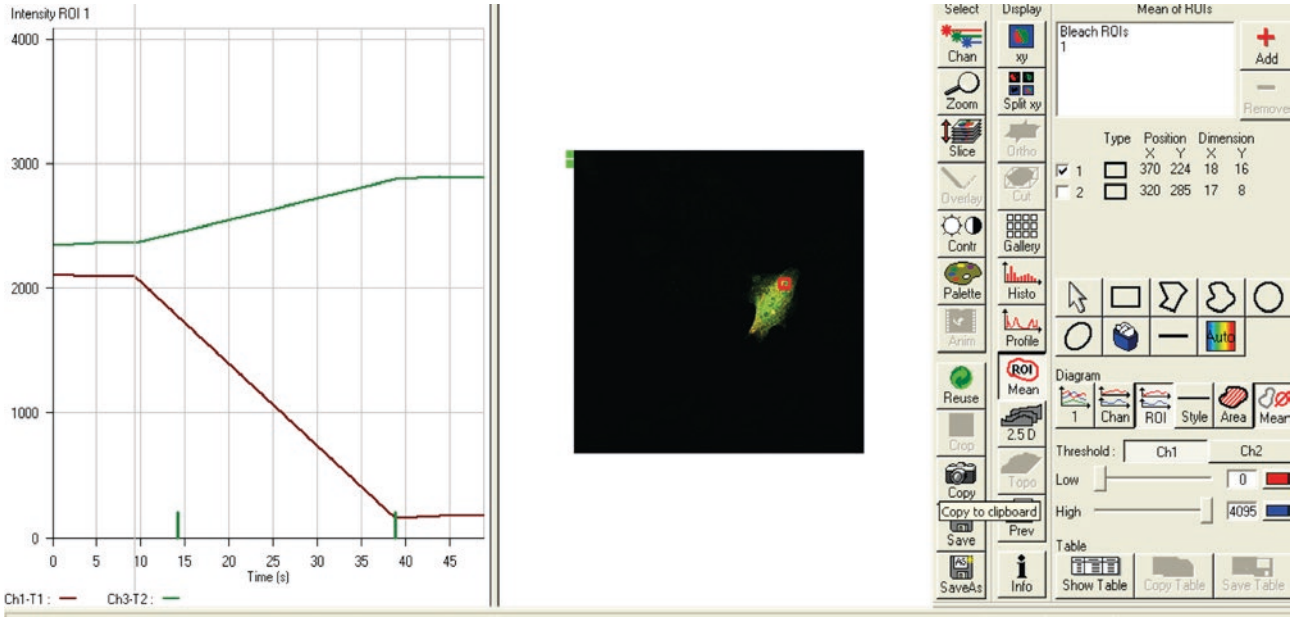


Figure 5 *Experimental FRET sample co-expressing GFP-Cdc42 and mRFP-CRIB in CHO-1 cells. The CRIB domain is a Cdc42 binding domain of N-WASP protein that binds Cdc42 with high affinity and is fused to mRFP.*

In the control FRET negative scenario, there is little change in GFP intensity post-bleaching. In contrast, the positive FRET scenario exhibits significant change in GFP fluorescence intensity post-bleaching. The following four steps are used in order to collect the images and data.

(i) Start the microscope, software, and laser. Switch on the microscope and open the software window. Create a new folder to save image data in order to carry out the experiment.

(ii) Select an objective and focus the microscope. Select C-Apochromat 63 x 1.2 W objective by using Vis and Micro buttons in the expert mode. Click on Vis and Micro buttons in the software to focus on the sample using white light. Look for cells featuring fluorescence tags by using the mercury lamp and focus on them. Excite the GFP/mRFP fusion proteins of with the 488 and 561 nm laser lines as excitation source, while selecting [405/488/561] as dichoric mirror and [490,565] as secondary dichoric mirrors.

(iii) Configure laser scanning and detection for confocal image acquisition. Monitor the emission by selecting GFP (BP 505-550) and mRFP (LP 575) emission filters to record the fluorescence intensity. Select ROI and photobleach by using 70% of the power of the 561 nm laser and select appropriate iterations so that at least 95% of the fluorescence intensity is bleached.

(iv) Configure the bleaching and time lapse settings for acceptor photobleaching. Perform bleaching by running three pre-scan images and three post-bleach scans.

Figure 3 shows an experiment with GFP-mRFP tandem fusion protein expression in CHO-1 cells. The tandem GFP-mRFP protein is generated from a cDNA construct where GFP encoding DNA is linked to mRFP encoding DNA directly. This tandem fusion protein is predicted to give maximum FRET and serves as positive control. For the negative control, we use free GFP and mRFP protein expression in CHO-1 cells (Figure 4). The experiment uses the small GTPase of the Rho family, Cdc42, as a GFP fusion protein. The CRIB domain is a Cdc42 binding domain that binds Cdc42 with high affinity and is fused to mRFP. An AP-FRET experiment using GFP-Cdc42 and mRFP-CRIB is shown in Figure 5.

5. Data Analysis

A. FRET Efficiency

Background intensity data should be obtained by marking three ROIs outside the cell area for both GFP and mRFP channels and computing the average value. The respective background average value should then be subtracted from the GFP and mRFP fluorescence intensity values for each time frame. To calculate the FRET efficiency in percentage, E (%), the background subtracted values of GFP pre-bleaching and GFP post-bleaching should be used. These values can be obtained by using the “show table” function on the software and then by “exporting” the data as Excel files. The E (%) can be obtained using the following equation:

$$E(\%) = \frac{I_{GFP(post-bleach)} - I_{GFP(pre-bleach)}}{I_{GFP(post-bleach)}} x\,100$$

B. Pearson Product Moment Correlation Coefficient – "r"

A very important parameter that can be extracted from the AP-FRET experiments is the Pearson product moment correlation coefficient "r", a dimensionless index that ranges from -1.0 to 1.0. "r" can be determined by comparing rates of change in GFP fluorescence intensity with those in mRFP post bleaching. Thus a time series is performed on bleaching mRFP whereby GFP and mRFP intensities are recorded. An "r" value of -1.0 indicates a perfect fit with the linear relation and suggests that the increase in one parameter correlated with the decrease in the other parameter. "r" is calculated using the following equation;

$$r = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}$$

Where x and y are the sample means average (array1, GFP intensity) and average (array2, mRFP intensity), respectively.

6. Data Verification

The AP-FRET experiment generates two data types, (i) E (% FRET effiiciency) and (ii) "r" (Pearson product moment correlation coefficient). The E and "r" values for the two controls (tandem GFP-mRFP fusion and free GFP/mRFP) can be used to define positive FRET. Positive FRET is defined as having a FRET efficiency value of > 3% and cross-correlation "r" values of between -0.7 to -1.0. Data from a minimum of about 10-12 cells should be collected for the calculation of "r".

7. Applications and Limitations

By choosing different ROIs, it is possible to gain spatial and conformational information about protein-protein interactions. In our own work on the actin cytoskeleton and cell morphology, we have used AP-FRET to examine protein-protein interactions. In particular, we have examined the protein-protein interactions involved in Cdc42 and Rif induced filopodia. Small GTPases of the Rho family are well known to reorganize the actin cytoskeleton downstream of Ras and growth factor receptors. These signaling pathways involve recruitment of protein complexes to sites in the plasma membrane to remodel actin-membrane structures. Filopodia are small actin rich protrusions that are dynamic with a turnover every 1-2 min. Cdc42 is known to bind

Table 1 Typical FRET data table complete with necessary controls showing FRET efficiency and correlation coefficient with standard deviation.

Protein	% FRET E (±SD)	r (±SD)
Controls		
GFP-mRFP (tandem fusion)	28.64 ± 3.69	-0.99 ± 0.01
Cyto-mRFP / GFP	1.91 ± 1.49	-0.17 ± 0.63
mRFP -IRSP53 + Cytp-GFP	2.12 ± 1.49	-0.16 ± 0.55
GFP-N-WASP + Cyto mRFP	2.73 ± 1.90	-0.63 ± 0.42
Experimental		
mRFP-Cdc42V12 + GFP-CRIB (domain)	18.40 ± 3.56	-0.99 ± 0.01
mRFP-Cdc42N17 + GFP-CRIB (domain)	2.34 ± 2.26	-0.09 ± 0.75
mRFP-N-WASP + GFP-Cdc42V12	10.17 ± 2.42	-0.97 ± 0.02
mRFP-N-WASP + GFP-Cdc42N17	2.42 ± 1.66	-0.47 ± 0.49
mRFP-IRSp53 + GFP-Cdc42V12	9.79 ± 3.47	-0.94 ± 0.06
mRFP-IRSp53 + GFP-Cdc42N17	2.69 ± 2.68	-0.1 ± 0.69

IRSp53, Toca1, and N-WASP. In turn, IRSp53 interacts with F-actin and actin modulators. The latter group includes Mena, Eps8, mDia1/2, and Dynamin. An example of such an AP-FRET analysis is shown in Table 1. Controls are critical in this analysis for the definition of a positive FRET. The first control is using the free protein pairs, GFP/mRFP versus a tandem fusion GFP-mRFP protein. This control defines minimum and maximum FRET, respectively. Second control experiment consists in using the experimental proteins with free GFP/mRFP as pairs. In this case FRET should not be observable. A last control uses point mutants (N17 in the case of Cdc42) in which protein-protein interactions are prevented. The data in Table 1, which compares controls with experiments, clearly show that Cdc42V12 interacts with CRIB, N-WASP and IRSp53 in vivo, but Cdc42N17 does not. We have used AP-FRET to examine spatial interactions of Cdc42, Rif, and IRSp53 with target proteins[14]. More specifically, we have examined if these proteins interact with filopodia. Interestingly, we have been able to show by AP-FRET that IRSp53 interacts with Mena, Eps8, mDia1, and Dynamin but not with mDia2 in filopodia[17].

Limitations of AP-FRET include:
(i) The need for samples to be fixed.
(ii) The expression levels of donor and acceptor have to be carefully selected to aim for a 1:1 ratio.
(iii) Several control measurements are needed to determine non-specific FRET.

8. Conclusions

AP-FRET is the most straightforward and intuitive method for measuring FRET. On one hand, if two fusion proteins, such as GFP-Cdc42 and mRFP-CRIB, are not interacting with each other, then there is no reason why bleaching of mRFP-CRIB should increase the fluorescence intensity of GFP. On the other hand, if the two fusion proteins are interacting and their dipole moments are in the correct orientation, while being 10 nm or less apart, then FRET should occur. In the positive FRET case, bleaching of mRFP-CRIB should affect the fluorescence of GFP-Cdc42 (a de-quenching process). Further, the rate of decrease in mRFP-CRIB fluorescence intensity should correlate with the rate of increase of GFP-Cdc42 intensity. By the use of two simple equations, the values for E and “r” can be determined and positive FRET identified.

References

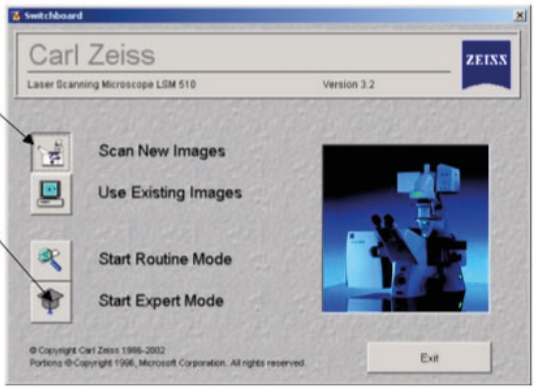
[1] Microscopic imaging tracks the intracellular processing of a signal transduction protein: fluorescent labeled protein kinase C βI. Bastiaens, P.I.H. and Jovin T.M. Proc.Natl.Acad.Sci.USA, 1996,93, 8407-8412.
[2] Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. Bastiaens, P.I.H., Majoul I.V.,Verveer P.J.,Soling H-D and Jovin T.M.The EMBO Journal 1996, 15. 4246-4253.
[3] Serotonin 5-HT2C receptor homodimer biogenesis in the endoplasmic reticulum: real-time visualization with confocal fluorescence resonance energy transfer. Herrick-Davis K, Weaver BA, Grinde E, and Mazurkiewicz, JE, J. Biol Chem, 2006, 281, 27109-16.
[4] Development of probes for cellular functions using fluorescent proteins and fluorescence energy transfer. Miyawaki A. Annu. Rev. Biochem, 2011, 80, 31.1-31.17.
[5] Visualization of molecular activities inside living cells with fluorescent labels. Bunt G. and Wouters FG. International Review of Cytology, 2004, 237, 205-277.
[6] FRET imaging. Jares-Erijman EA and Jovin TM. Nature Biotechnology, 2003, 21, 1387-1395.
[7] Fluorescence resonance energy transfer microscopy of localized protein interactions in the living cell nucleus. Day RN, Periasamy A. and Schaufele F. Methods, 2001, 25, 4-18.
[8] Fluorescenceresonance energy transfer (FRET) measurement by gradual acceptor photobleaching: Van Munster E.B, Kremers GJ, Adjobo-Hermans M J, and Gadella TW, Jr. J Microsc., 2005, 218, 253-62.
[9] APPL proteins FRET at the BAR: direct observation of APPL1 and APPL2 BAR domain-mediated interactions on cell membranes using FRET microscopy. Chial, H. J., Lenart, P., and Chen, Y. Q., PLoS One, 2010, 5, e12471.
[10] Fluorescence resonance energy transfer (FRET) analysis demonstrates dimer/oligomer formation of the human breast cancer resistance protein (BCRP/ABCG2) in intact cells. Ni Z, Mark ME, Cai X, and Mao Q. Int J Biochem Mol Biol, 2010, 1, 1-11.
[11] FRET with multiply labeled HERG K(+) channels as a reporter of the in vivo coarse architecture of the cytoplasmic domains. Miranda P, Manso DG, Barros F, Carretero L, Hughes TE, Alonso-Ron C, Dominguez P, and de la Pena P. Biochim Biophys Acta, 2008, 1783, 1681-99.
[12] Determination of in vivo dissociation constant, KD, of Cdc42-effector complexes in live mammalian cells using single wavelength fluorescence cross-correlation spectroscopy. Sudhaharan, T, Liu, P, Foo, YH, Bu, W, Lim, KB, Wohland, T, and Ahmed, S., J Biol Chem, 2009, 284, 13602-9.
[13] The Toca-1-N-WASP complex links filopodial formation to endocytosis. Bu W, Chou AM, Lim KB, Sudhaharan T and Ahmed S. J Biol Chem, 2009, 284, 11622-36.
[14] The Cdc42 effector IRSp53 generates filopodia by coupling membrane protrusion with actin dynamics. J Lim KB, Bu W, Goh WI, Koh E, Ong SH, Pawson T, Sudhaharan T, and Ahmed S. J. Biol Chem, 2008, 283, 20454-72.
[15] Cdc42 interaction with N-WASP and Toca-1 regulates membrane tubulation, vesicle formation and vesicle motility: implications for endocytosis Bu W, Lim KB, Yu YH, Chou AM, Sudhaharan T, and Ahmed S. PLoS One, 2010, 5, e12153.
[16] Rho GTPase Cdc42 is a direct interacting partner of Adenomatous Polyposis Coli protein and can alter its cellular localization. Sudhaharan T, Goh WI, Sem KP, Lim KB, B, W, and Ahmed S. PLoS One, 2010, 6, e16603.
[17] Rif-mDia1interaction is involved in filopodium formation independent of Cdc42 and Rac effectors. Goh WI, Sudhaharan T, Lim, KB, Sem, KP, Lau, CL, and Ahmed, S., J Biol Chem, 2011, 286, 13681-94.

Applications	FRET Pairs	References
Reviews on FRET		(Miyawaki, 2011)[4] (Bunt and Wouters,2004)[5] (Jares-Erijman and Jovin, 2003)[6] (Day et al, 2001)[7]
Selected Recent Applications		
Gradual acceptor photobleaching method.	CFP and YFP	(Van Munster et al, 2005)[8]
APPL1 and APPL2 BAR domain-mediated interactions on cell membranes.	CFP and YFP	(Chial et al, 2011)[9]
Dimer/oligomer formation of the human breast cancer resistance protein (BCRP/ABCG2) in intact cells.	CFP and YFP	(Ni et al, 2011)[10]
HERG K (+) channels as a reporter of the in vivo coarse architecture of the cytoplasmic domains.	CFP and YFP	(Miranda et al, 2008)[11]
Determination of in vivo dissociation constant, Kd, of Cdc42-effector complexes in live mammalian cells.	GFP and mRFP	(Sudhaharan et al, 2009)[12]
The Toca-1-N-WASP complex links filopodial formation to endocytosis.	GFP and mRFP	(Bu et al, 2009)[13]
The Cdc42 effector IRSp53 generates filopodia by coupling membrane protrusion with actin dynamics.	GFP and mRFP	(Lim et al, 2008)[14]
Cdc42 interaction with N-WASP and Toca-1 regulates membrane tubulation, vesicle formation and vesicle motility.	GFP and mRFP	(Bu et al, 2010)[15]
Rho GTPase Cdc42 is a direct interacting partner of Adenomatous Polyposis Coli protein and can alter its cellular localization.	GFP and mRFP	(Sudhaharan et al, 2011)[16]
Rif-mDia1 interaction is involved in filopodium formation	YFP and mRFP	(Goh and others, 2011)[17]

Appendix

Starting the LSM 510 Software

- Double click the LSM 510 icon
- Select "Scan New Images"
- Select "Start Expert Mode"



Turning on the Lasers

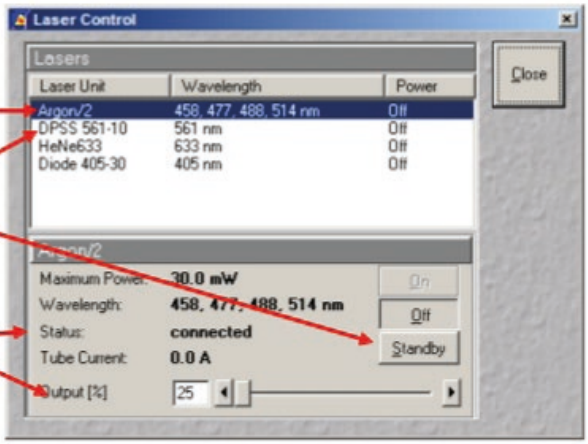
1) Select Acquire

2) Select Laser

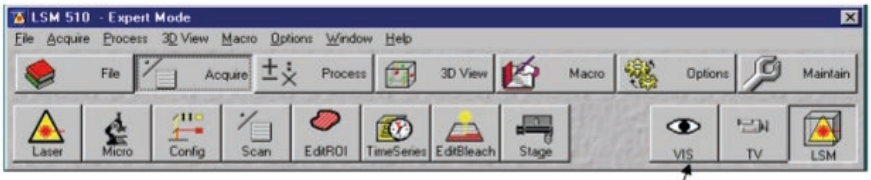
3) Click Standby for Argon Laser and wait for it to warm up. Once the status says "*READY*" switch it ON.

4) Set Output(%) so that tube current is in between 5.5A to 6.5A

5) Click on DPSS 561 Laser and switch it ON.



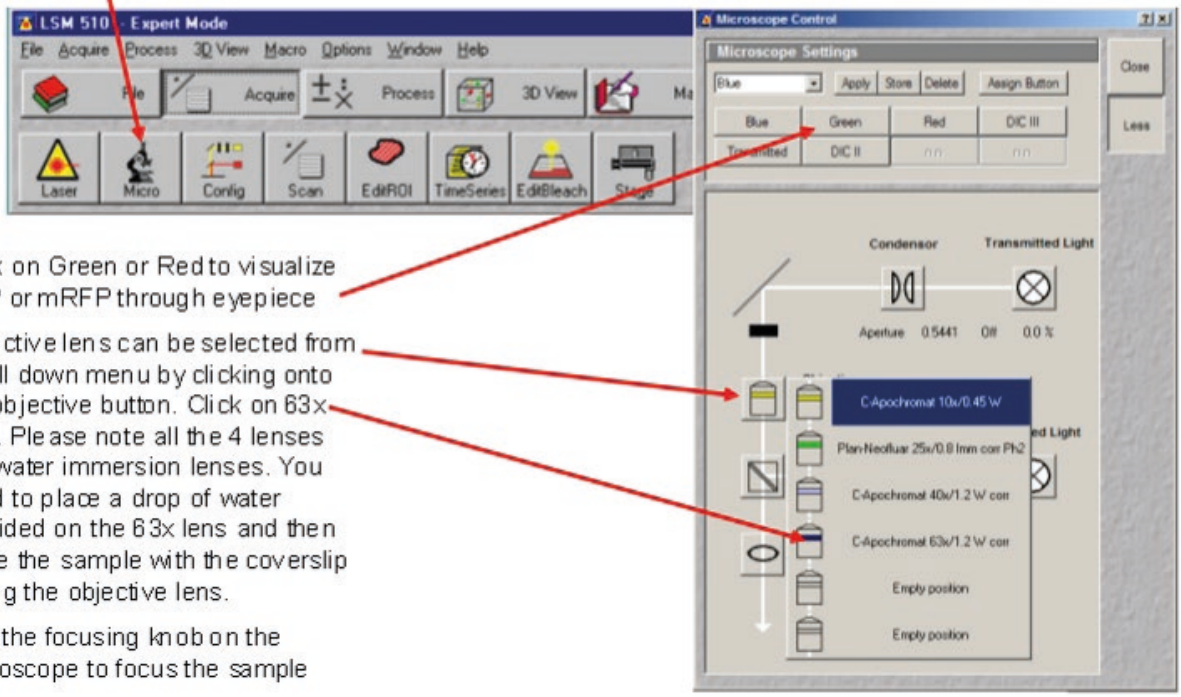
Change Between Direct Observation and Laser Scanning



To view the sample through the eyepiece, click on "VIS". Toggle between VIS and LSM button in main menu to automatically switch between direct observation and laser scanning

Selecting an Objective and Focusing the Sample

1) Select "Micro" (Main menu *Acquire*)



2) Click on Green or Red to visualize GFP or mRFP through eyepiece

3) Objective lens can be selected from a pull down menu by clicking onto the objective button. Click on 63x lens. Please note all the 4 lenses are water immersion lenses. You need to place a drop of water provided on the 63x lens and then place the sample with the coverslip facing the objective lens.

4) Use the focusing knob on the microscope to focus the sample

The Right Way to Scan the Sample

SINGLE TRACK

Use for **single**, double and triple labelling

Simultaneous scanning only

ADVANTAGES

Faster image acquisition

DISADVANTAGES

Cross talk between channels

MULTI TRACK

Use for double or triple labelling

Sequential scanning, line by line or frame by frame

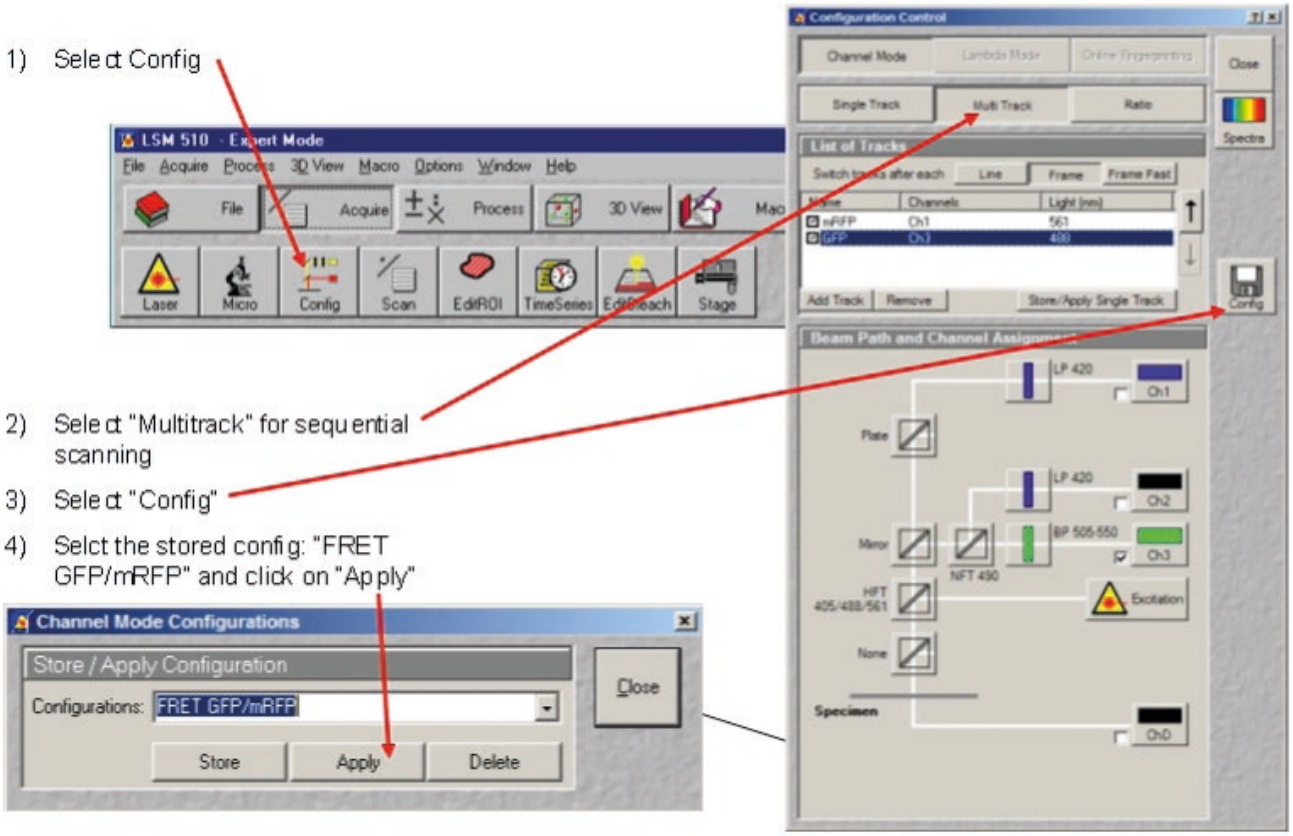
ADVANTAGES

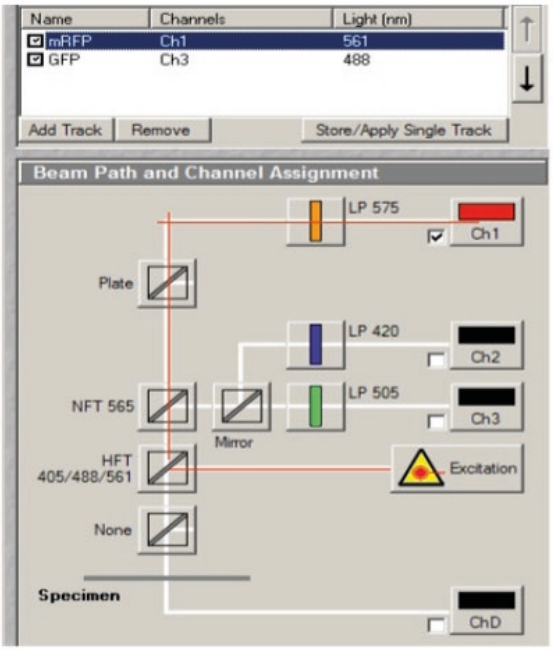
When one track is active, only one detector and one laser is switched on. This dramatically reduces crosstalk.

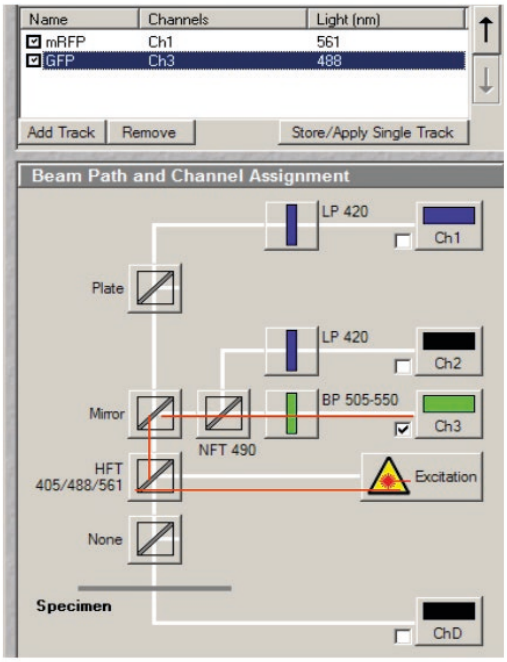
DISADVANTAGES

Slower image acquisition

Apply a Stored Configuration







Set the Parameters for Scanning

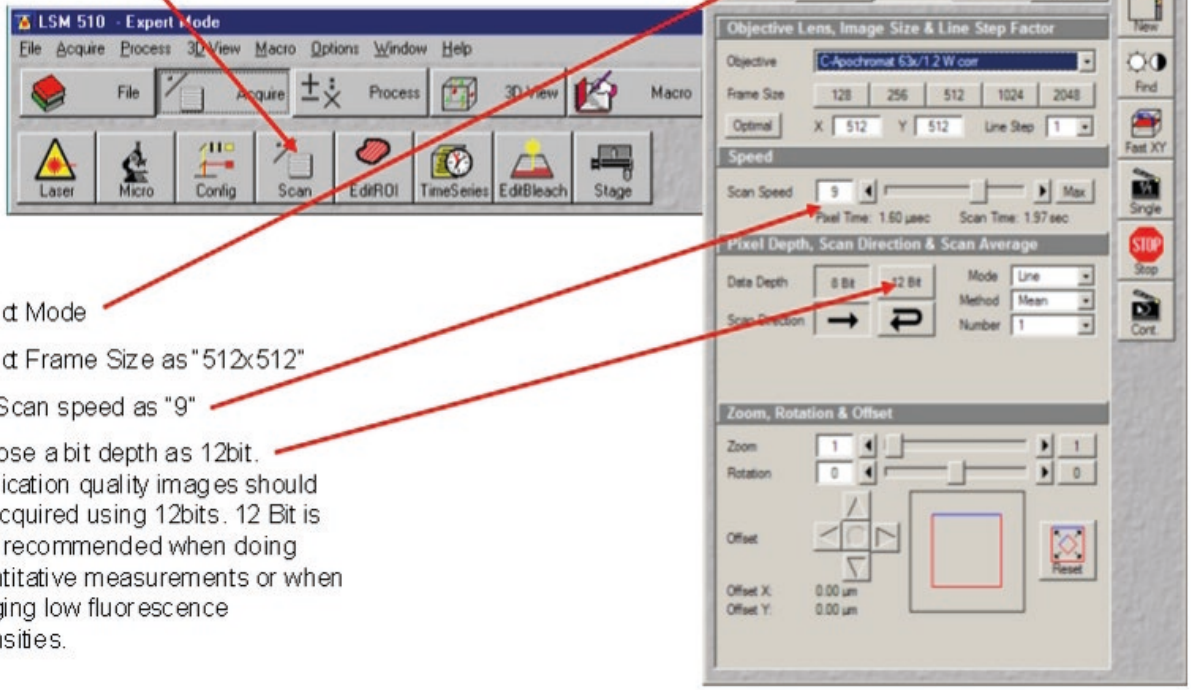
1) Select Scan

2) Select Mode

3) Select Frame Size as "512x512"

4) Set Scan speed as "9"

5) Choose a bit depth as 12bit. Publication quality images should be acquired using 12bits. 12 Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.



Channel Settings – Adjust the Pinhole

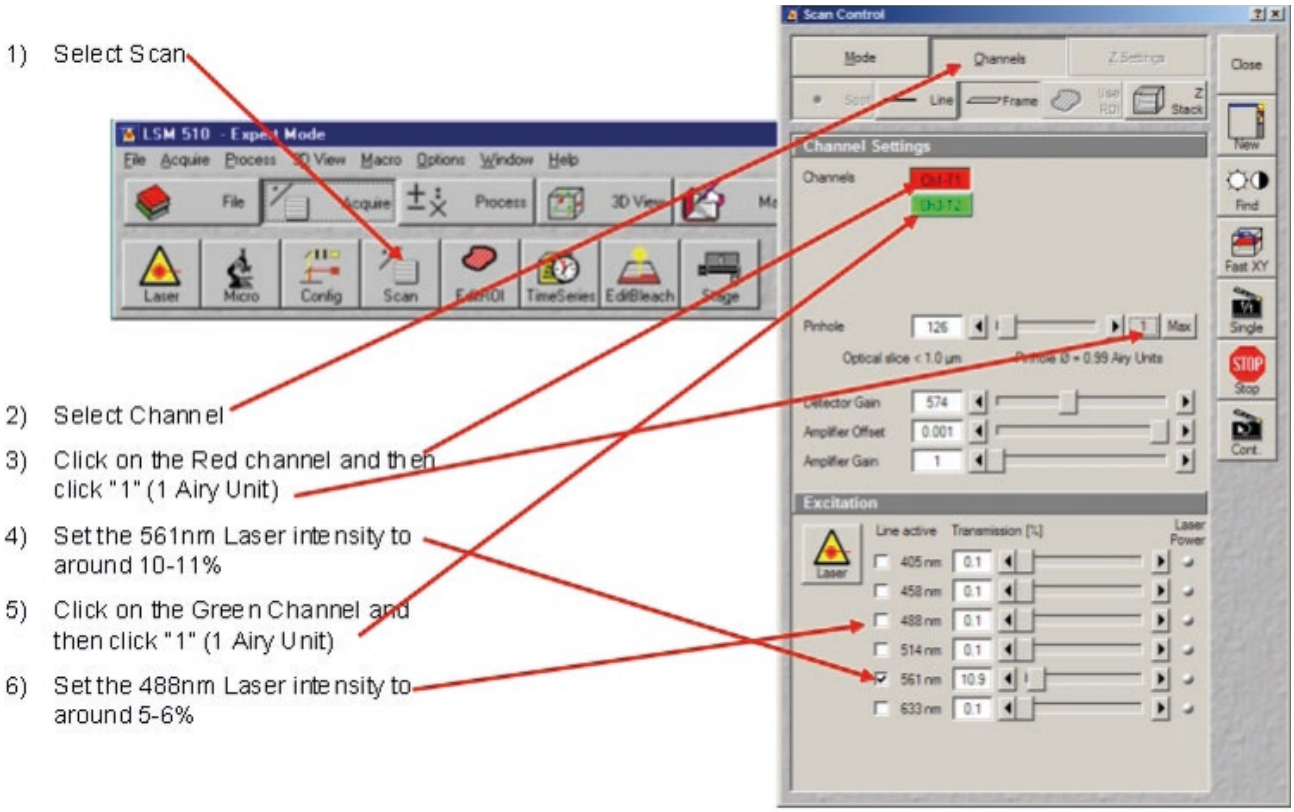


Image Acquisition – Fast Scanning

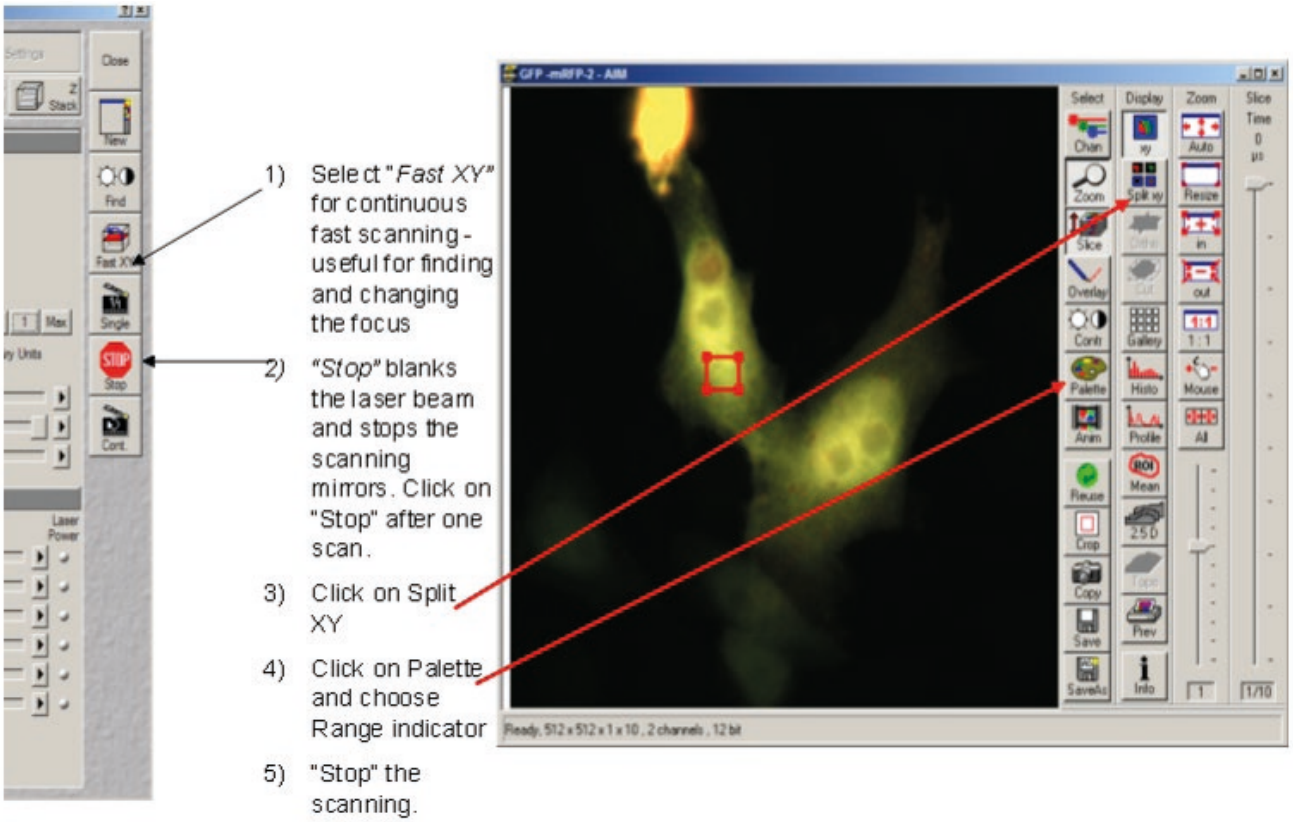
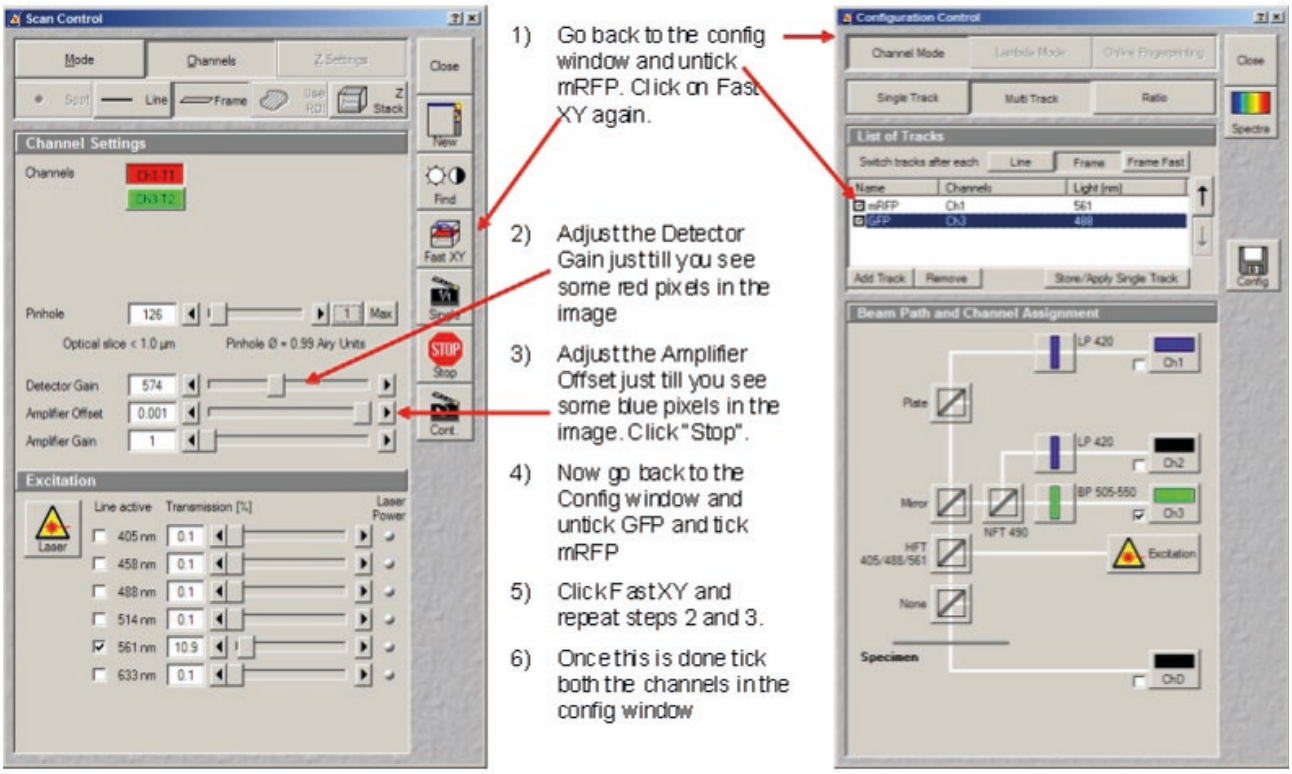
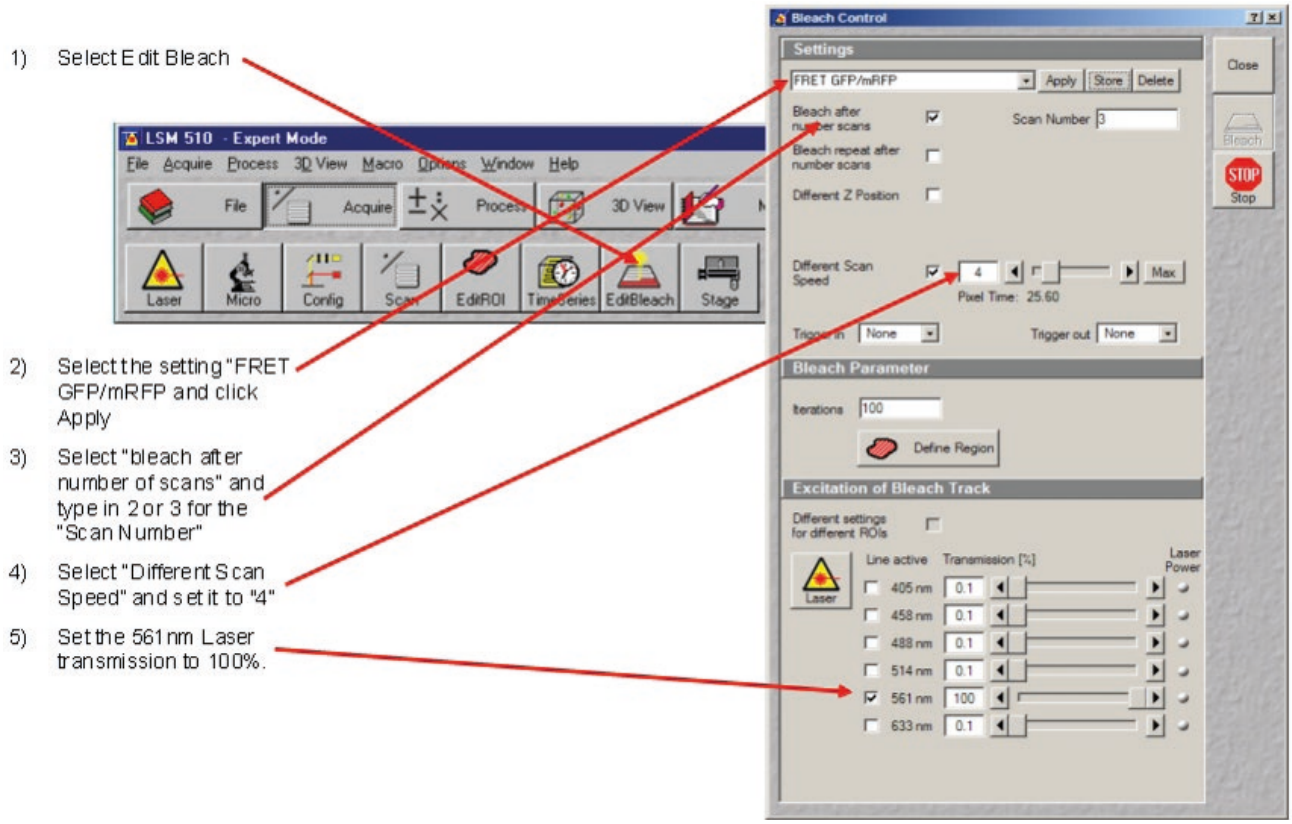


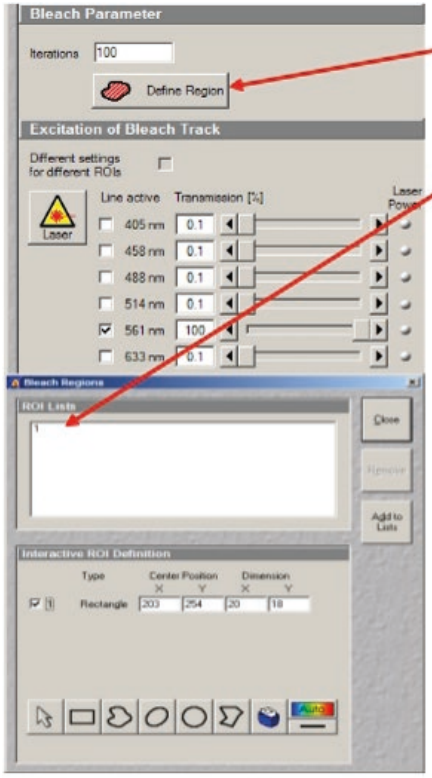
Image Acquisition – Adjusting Gain and Offset



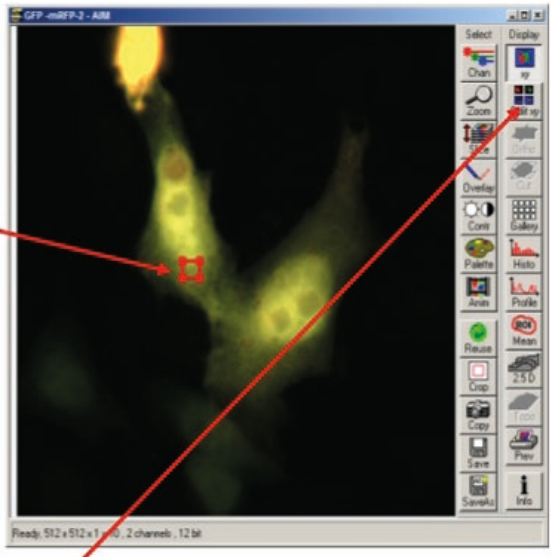
Setting up Bleaching Parameters (Acceptor Photobleaching)



Define the ROI for Bleaching



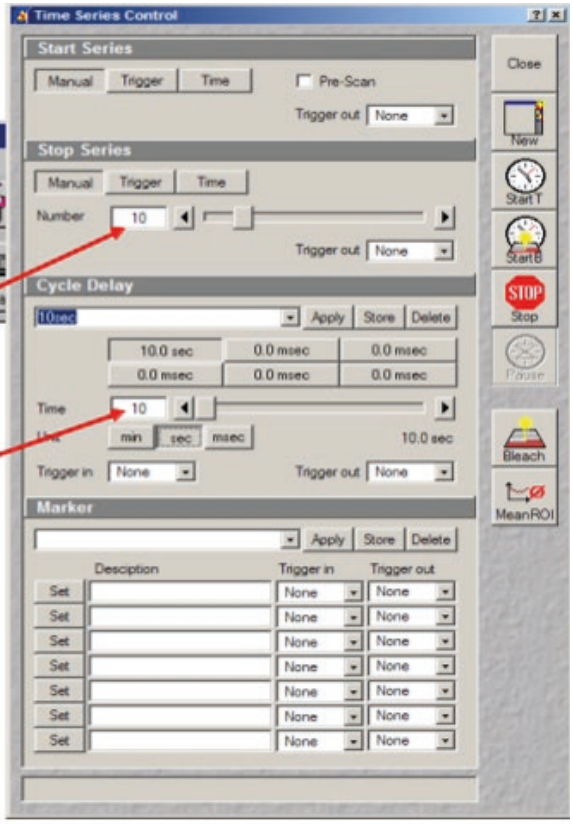
1) Click on "Define Region" in the "Edit Bleach" window
2) Click on "1" in the ROI lists.
3) On the image already scanned with the Fast XY function place the ROI at the desired location
4) You may resize the ROI as desired.
5) Make sure the ROI is not too big as bleaching might take much longer.
6) You may click on Split XY to view the 2 channels separately



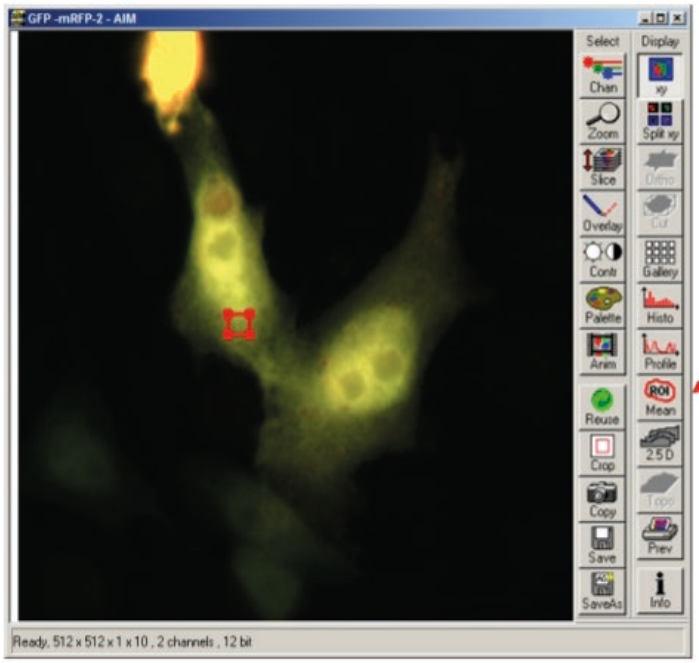
Set the Time Series to Perform Experiment

1) Select Time Series

2) We will take a total of 10 images per slide. Hence type in 10 at the Stop series slot.
3) We shall continuously capture images and thus set the cycle delay to 0.
4) Click on "Start B". This will start the time lapse and the bleaching process. Since we set 3 scans before bleaching, we will have 3 images before bleaching and 7 after bleaching.

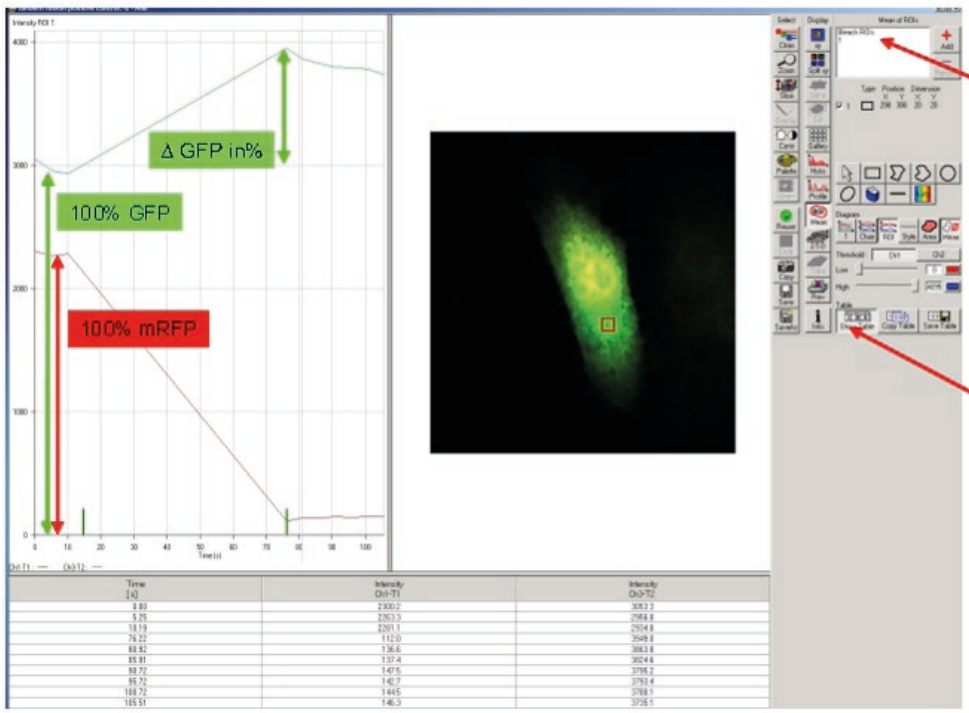


FRET Result – Mean ROI



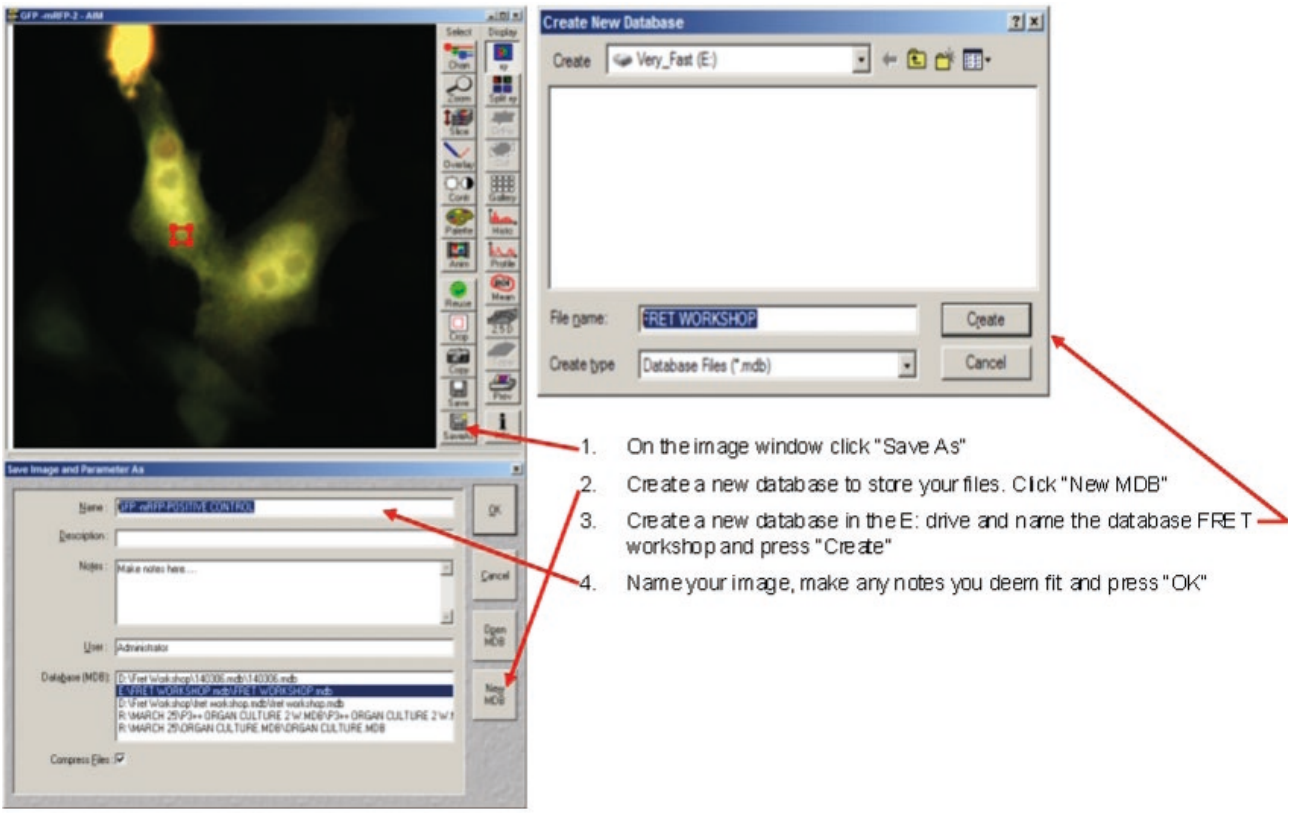
1) As the images are being captured, you might want to see how the average intensity of your ROI changes before bleaching and especially after the bleaching of the Acceptor (mRFP) changes. Click on "Mean ROI" in the image window.

FRET Result – Mean ROI

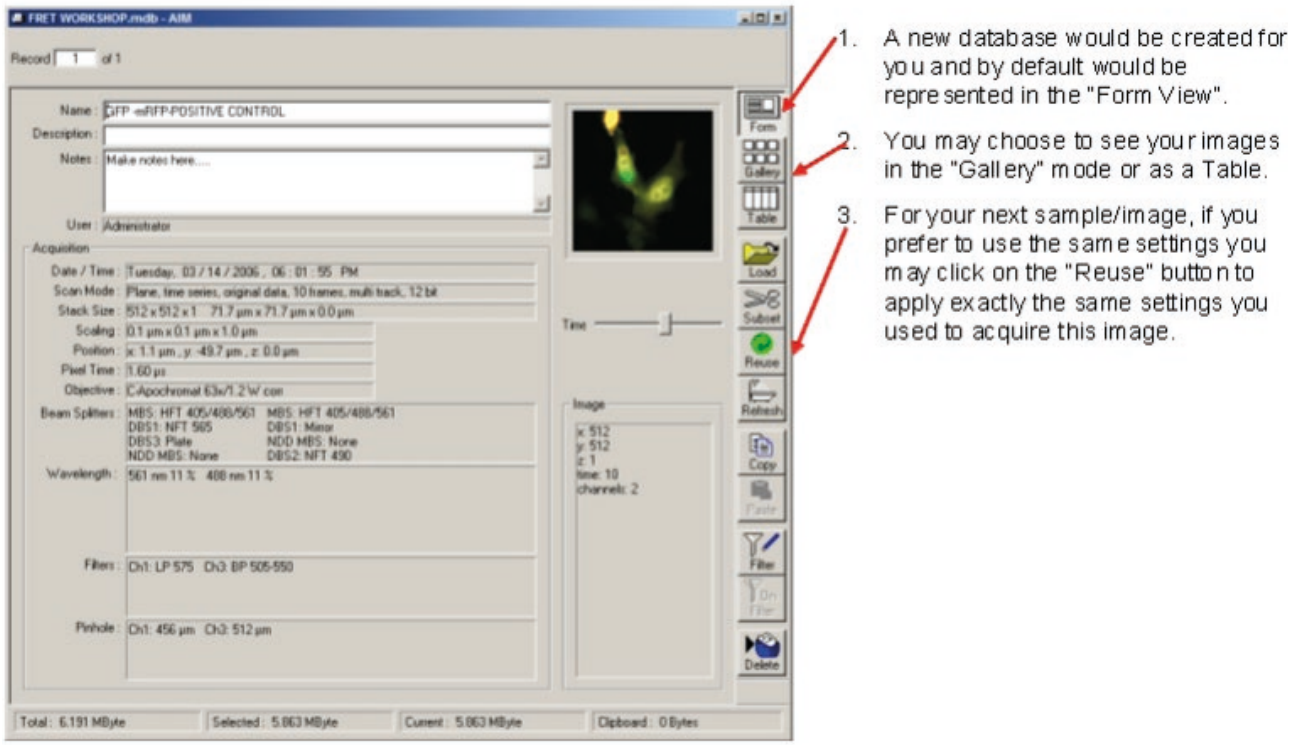


1) Click on Bleach ROI to see the ROI you had selected for bleaching.
2) The bleaching might take a while.
3) At the point bleaching starts note the increase in the intensity of Donor (GFP)
4) Click on "Show Table" to see the average intensity of your ROI in numbers.

Saving Data – Database



Saving Data – Database – Form View



PicoQuant GmbH
Rudower Chaussee 29
12489 Berlin
Germany

Material can be downloaded from www.picoquant.com

www.picoquant.com