PRACTICAL MANUAL FOR
FLUORESCENCE
MICROSCOPY
TECHNIQUES

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chapter

Fluorescence Sensitized Emission FRET (SE-FRET)

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Abstract

Sensitised Emission (SE)-FRET is an fluorescence intensity based method that uses changes in fluorophore spectra to measure FRET. In the FRET scenario donor emission is decreased while acceptor emission is increased. CFP/YPF and GFP/mRFP pairs can be used to generate data with essential control experiments giving background corrections. When background data is subtracted from experimental data the degree of FRET (FRETc) can be determined.

We describe here a filter based setup to perform SE-FRET on a widefield microscope. SE-FRET is useful for measuring rapid changes protein-protein interaction in cells under live conditions where spatial information can also be followed – the classical example is the measurement of cellular Ca2+ fluxes. The concentration of CFP (GFP) and YFP (mRFP) in transfected cells needs to be controlled with similar levels of both fluorophores being optimal. The ideal SE-FRET probe is where a single protein contains both CFP/YFP or mRFP/GFP thereby bypassing the requirement for controlling individual protein concentrations.

1. Principle and Theory

Here we use the GFP/mRFP pair. When there is no protein-protein interaction between GFP/mRFP fusion protein pairs, FRET is not observed, and donor (GFP) excitation does not lead to excitation of the acceptor (mRFP). In contrast, when there is protein-protein interaction, FRET is observed, and
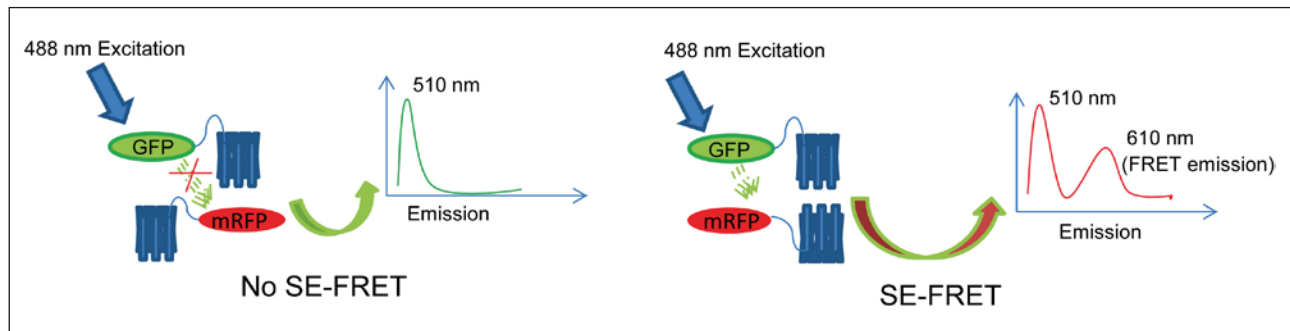

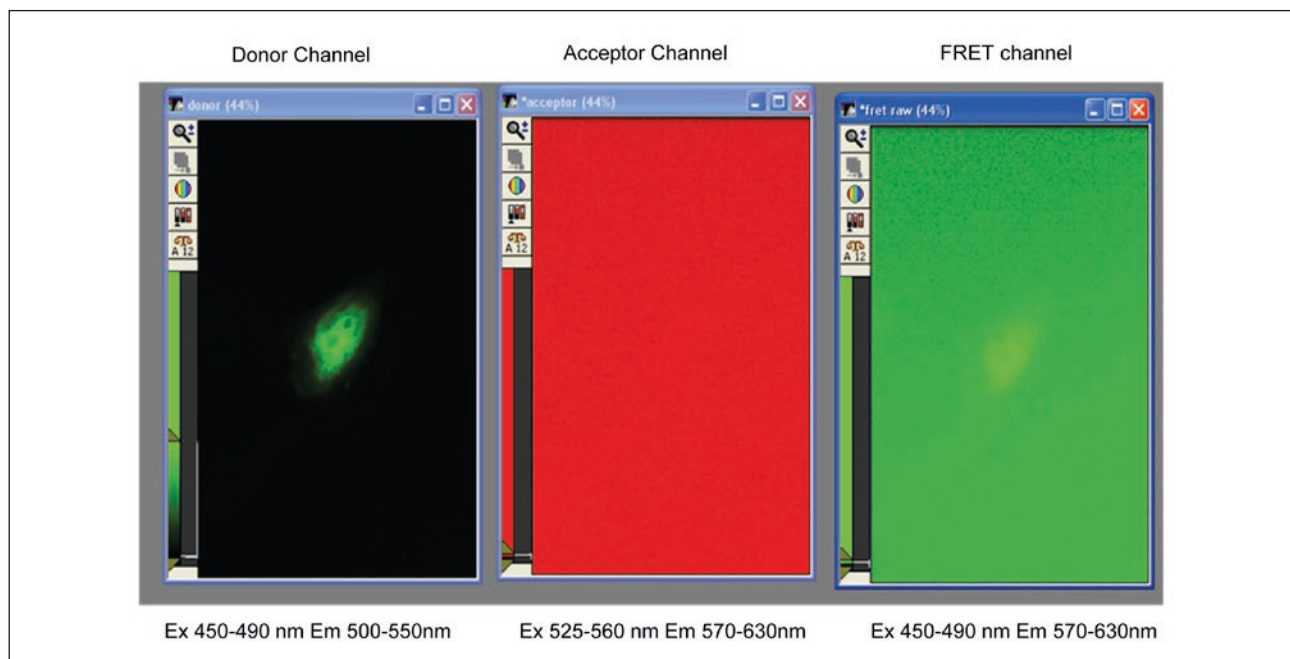**Figure 1** *Shown is the schematic representation of SE-FRET occurrence.*

Figure 2 *Shown is the image representation of donor channel emission and its cross-talk in FRET channel (Cross-talk coefficient B = 0.087) of donor alone (GFP) sample during donor excitation.*

the excitation of the donor leads to excitation of the acceptor. In the FRET scenario the donor emission is decreased and the acceptor emission is increased (Figure 1).

Theory of Sensitized Emission (SE)

SE-FRET is an flourecence intensity based method where in widefield implementation filters are used to separate donor/acceptor (GFP/mRFP signals). In SE-FRET the fluorescence emission of the acceptor (mRFP) that results from the radiatiolness energy transfer from an excited donor (GFP) is measured. Thus controls are critically important to correct data for; (i) Cross talk between donor to acceptor channels and (ii) Direct excitation of acceptor when donor is being excited (Figures 2 and 3). For controls, four samples are analysed. GFP (donor only), mRFP (acceptor only) and a pair of fusions which do not interact as a negative control. For the fourth sample, the positive control, we use of a tandem GFP-mRFP fusion protein.

A range of mathematical equations have be used to analyse SE-FRET data. We use here Youvan's method (equation 1; see Data analysis in section 5). Two coefficients, A and B, need to be determined. The coefficient B (Fd/Dd) is measured with donor (GFP) only (Figure 2) and represents the ratio of the signal obtained (Fd) in the FRET channel over the signal obtained in the donor (Dd) channel (Figure 2). The coefficient A (Fa/Aa) is measured with acceptor (mRFP) only (Figure 3) and is the ratio of the signal

obtained in the FRET channel (Fa) over the signal obtained in the acceptor channel (Aa; Figure 3). Both A and B coefficients are constant for any one set of experimental conditions.

2. Instrumentation

Here we use a dual view set-up on an Olympus widefield microscope with a 60x oil with appropriate GFP and mRFP excitation/emission filters and corresponding dichroic mirrors; a Xenon lamp for excitation, a CCD camera as detector and Metamorph software for image acquisition.

3. Methods

3.1 Materials Required

Any mammalian cell line of interest could be used. We chose here CHO-1 cells as they are easy to transfect and culture in 37°C CO_2 incubators. Fetal bovine serum (FBS), Antibiotics (penicillin and streptomycin),Trypsin, cDNA encoding GFP and mRFP acceptor and donor pairs, F-12K nutrient mixture [Kaighn's modification] media and transfection reagent (Turbofect). Glass bottom Matek dishes.

3.2 Sample Preparation

1. CHO-1 cells were grown in a 75 cm2 tissue culture flask up to 90% confluency in the complete growth media (1 x F-12 nutrient mixture [Kaighn's
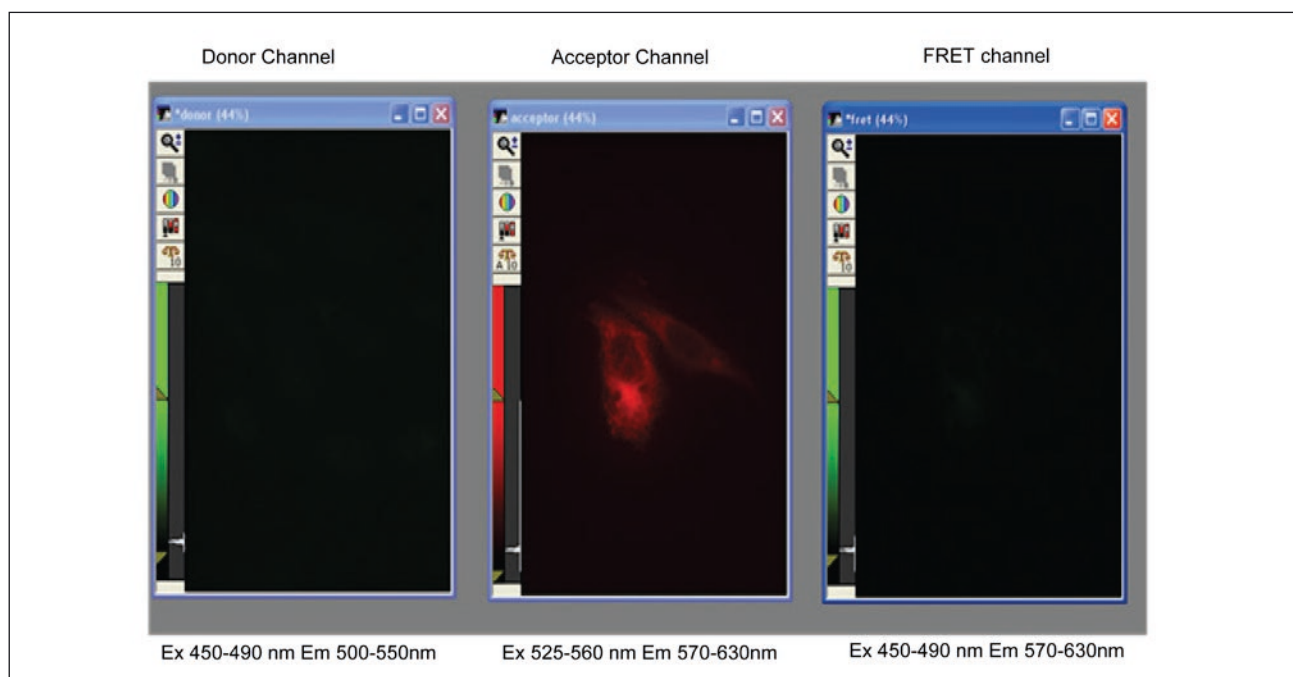

Donor Channel	Acceptor Channel	FRET channel
Ex 450-490 nm Em 500-550nm	Ex 525-560 nm Em 570-630nm	Ex 450-490 nm Em 570-630nm

Figure 3 *Shown is the image representation of acceptor channel emission and its cross-talk in FRET channel (cross-talk coefficient A = 0.043) of acceptor only (mRFP) sample during acceptor excitation.*
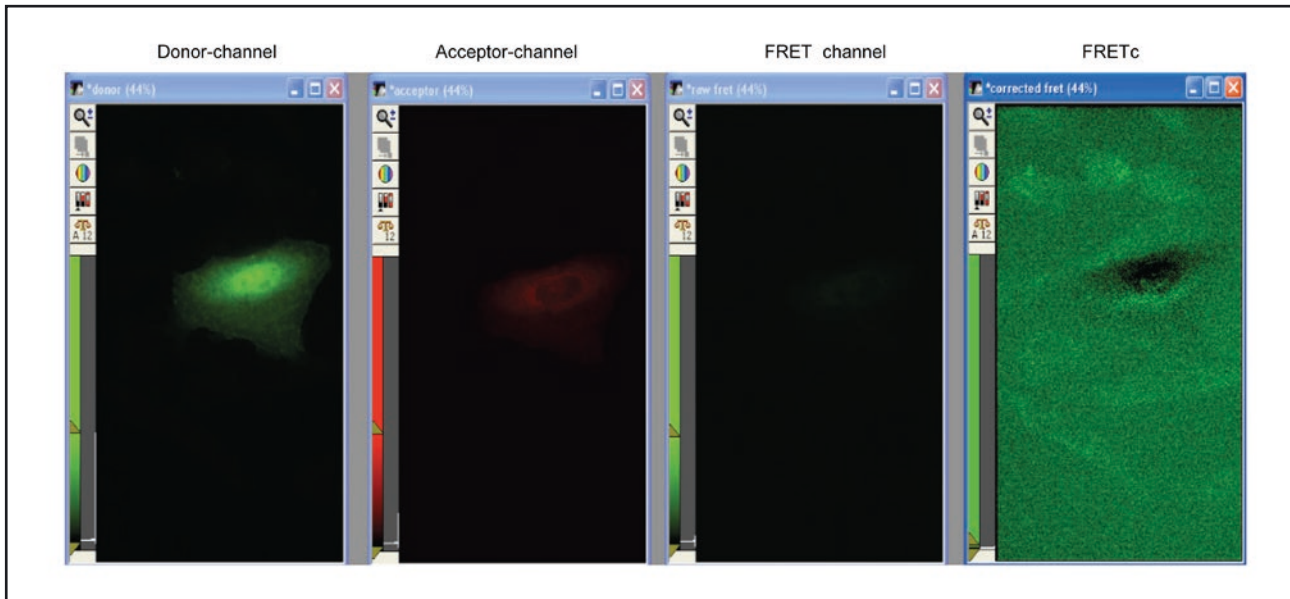
Figure 4 *A negative FRET control sample [GFP-NWASP and mRFP-Toca (non-interacting mutant)] expressed in CHO-1 cells upon 488 nm excitation during FRET measurement. FRETc image was obtained by performing the cross-talk correction on FRET channel image.*
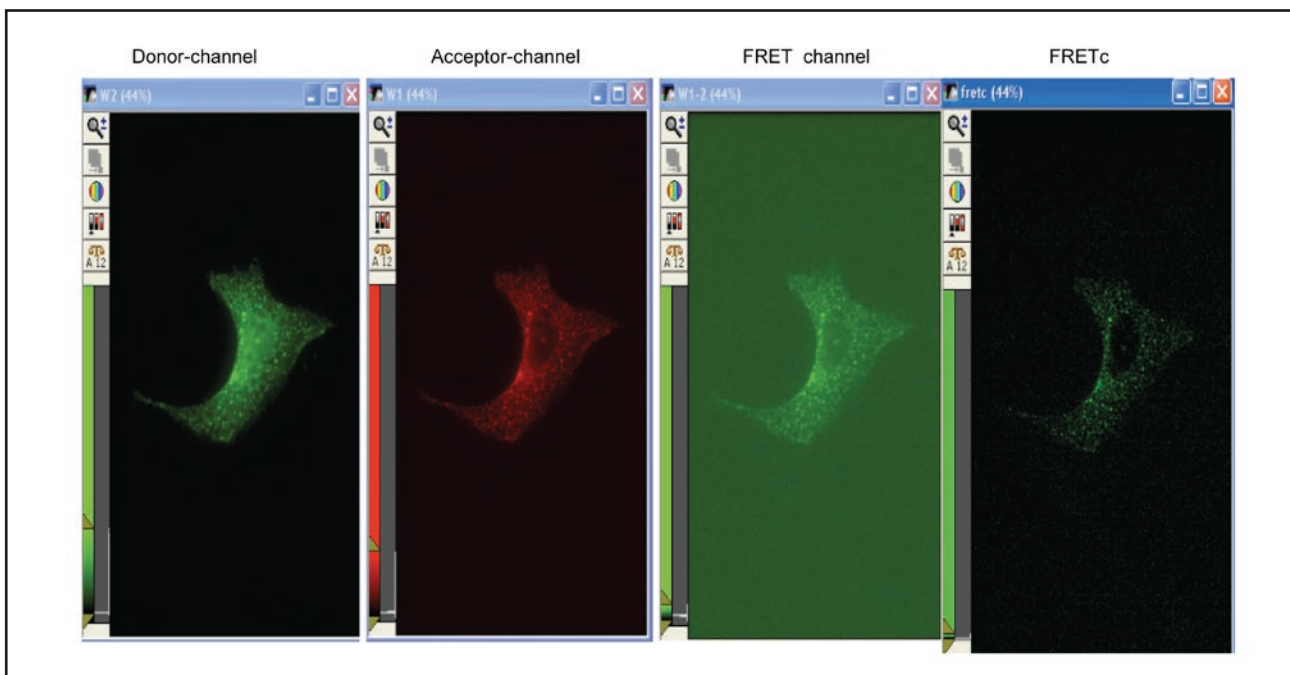

Figure 5 *Shown is the CHO-1 cell expressing positive control (GFP-mRFP) tandem fusion image in donor, acceptor and FRET channel respectively obtained using 488 nm excitation. FRETc image was obtained by performing the cross-talk correction on FRET channel image.*

modification] media) containing 10% FBS and 1% antibiotics [penicillin and streptomycin].

2. Cells were detached from the flask, using 2 ml trypsin and incubating at 37°C for 5 min. Cells were counted using a hemacytometer.

3. For transfection, cells were seeded with a cell density of 1.5 x 104 cells in a tissue culture Matek glass bottom dish and left for 24 hours.

4. CHO-1 cells were transfected using Turbofect transient transfection reagent 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 media in a tube and allow it to stand for 25 min at room temperature. Transfection mixes of different plasmids were transferred into different wells of a 6 well plate containing

cells. The transfected cells in 6 well plates were incubated in 37°C for 24 hours to allow protein expression to occur. Typically 5 transfections were carried out in parallel:
a) GFP-mRFP tandem fusion (positive control)
b) Experimental sample (coexpression of mRFP-Toca + GFP-NWASP). Protein pairs that are known to interact be independent methods.
c) Coexpression of mRFP-Toca (mutant) + GFP-NWASP (negative control)
d) GFP alone expressing sample and
e) mRFP alone expressing sample.

5. On the day of fluorescence measurements, cells were harvested and rinsed 2 times with PBS containing 2.0 ml of Hank's Balanced salt media (Gibco).

4. Data Acquisition

Switch on microscope Olympus IX81 live cell system and accessories and allow microscope to warm up (Screen shot prints are given in Appendix). Briefly, the following four steps were used to collect images.

(i) Switch-on the microscope. Switch on the microscope with dual view setup and start Metamorph software. Create a new folder to save image data in order to carry out the experiment.
(ii) Select an objective and focus the microscope. Select 60X oil objective and focusing the sample open software window. To focus the sample use white light then look for cells having fluorescence signal using Xenon lamp.
(iii) Configure the software for acquisition and calculating crosstalk coefficients A and B
(iv) Capture FRET images for data analysis.

5. Data Analysis

After determining the coefficients A and B, equation 1 is used to determine FRET and is called FRETc for corrected data.

$$Fc = Ff - \left[\frac{Fd}{Dd} \cdot Df \right] - \left[\frac{Fa}{Aa} \cdot Af \right] \quad (1)$$

This data analysis protocol has been automated in the Metamorph FRET module.

6. Data Verification

It is important to use negative and positive controls in the experiments to eliminate possible artifacts.

For negative control we use GFP and mRFP fusion protein pairs that we know do no interact. For the positive control we use a tandem GFP-mRFP fusion that will give maximum FRET.

To validate the data obtained with the widefield protocol the samples can be analysed using spectral imaging on a confocal microscope. In the present case an FV1000 Olympus microscope was used. The data was collected in the lambda scan mode by exciting the FRET (tandem fusion GFP-mRFP) sample with donor excitation (GFP, 488 laser line). The emission was scanned through by adjusting the filter range in the lambda mode from 475-675 nm. Basically the emission scan covers the donor emission (490-550nm) along with FRET emission (575-675 nm) using single donor excitation. Figure 6 shows a typical example of spectral imaging data of FRET sample obtained in the region of interest (ROI) in lambda scan mode. The GFP-mRFP expressing cell used for spectral imaging before and after acceptor photobleaching is shown in figure 6 (a). The spectral data in the ROI of the cell which is subjected to pre and post acceptor photobleach (using 559 nm laser line) to abolish FRET in the ROI is shown in figure 6 (b and c, respectively). From the data it can be observed that the FRET emission peak seen in the ROI of GFP-mRFP sample is lost upon photobleaching the acceptor, simultaneously the donor (GFP) intensity increased due to de-quenching effect of acceptor bleaching (Figure 6b and c).

7. Limitations

SE-FRET is an indirect method to measure FRET. In SE-FRET images and data need to be corrected and analysed before the presence or absence of FRET can be determined. Critically important in the SE-FRET method described here is to look for cells that have similar levels of donor and acceptor protein concentration. The SE-FRET experiments can be optimised by insuring the expression levels of donor and acceptor are similar to endogenous levels. Also one should check that protein localization of the GFP/mRFP fusion proteins is similar to that found for the endogenous protein. If protein concentration of the fusion protein pairs are different then protein concentration normalisation can be carried, However, this approach is not preferred. Normalisation can be achieved by determining A and B coefficients with protein concentrations of donor and acceptor as for the experimental condition. In SE-FRET specific controls should be considered in advance and are essential for assessing the presence or absence of protein-protein interactions. With regard to controls, point mutants of fusion proteins that do not change
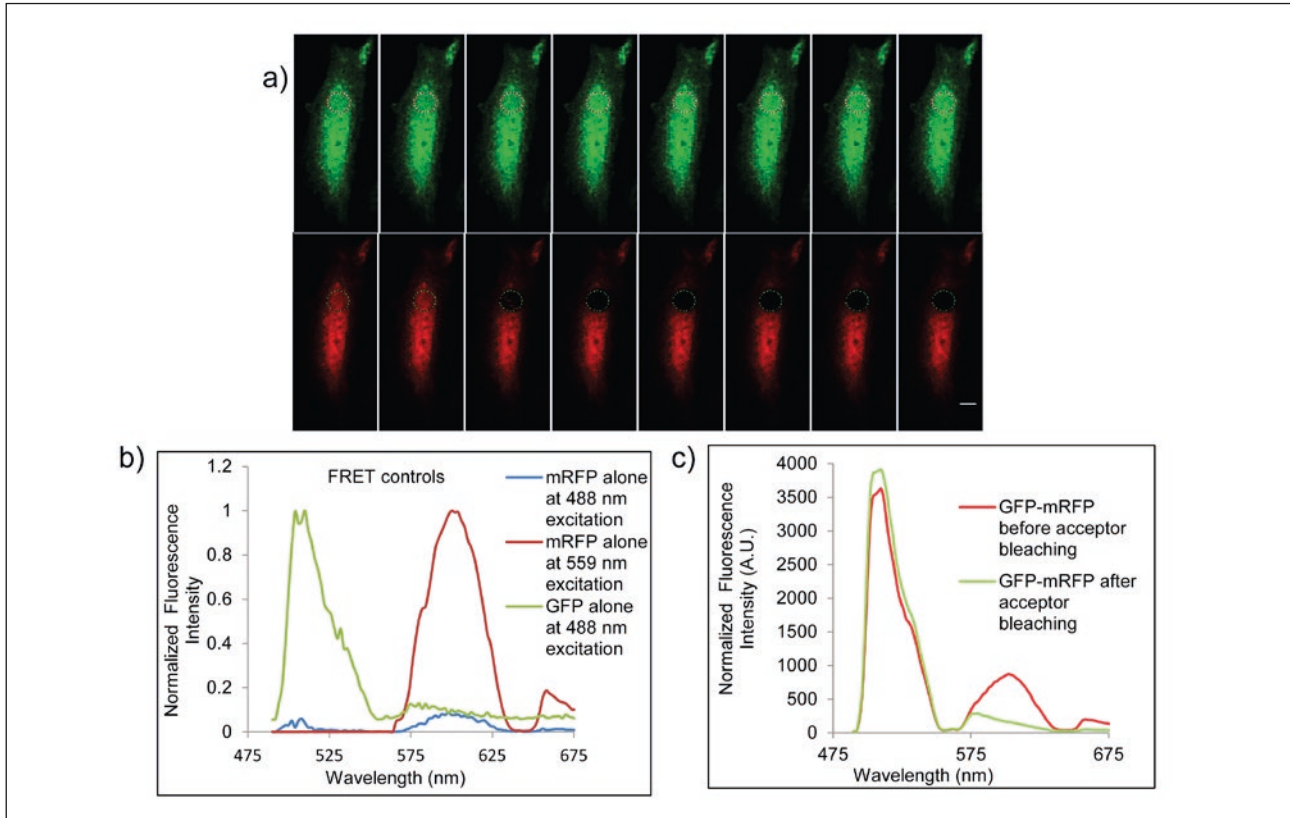
Figure 6 Shown is the spectral imaging (SE-FRET) mode data (a) positive control sample (GFP-mRFP) tandem fusion expressed in CHO-1 cells image in donor and acceptor channel respectively upon 488 nm excitation. The ROI was photo bleached using 561 nm laser and used for spectral image FRET measurement. (b) Spectral image measurement data from control cells. (c) Spectral image measurement data of (a) before and after acceptor bleaching.

the overall size or structure but affect protein-protein interaction, are powerful negative controls.

8. Conclusion

Controls and validation in SE-FRET are key for obtaining accurate data. The SE-FRET allows event occurring in the msec range to be followed with good spatial resolution (see range of uses for SE-FRET in Table 1). Thus SE-FRET is the FRET method of choice for monitoring rapid events in live cells.

Table 1 Table describing the publication list of SE-FRET method applications used in cells with various fluorescent proteins FRET pairs.

| Application	FRET-PAIR	References
Reviews on Sensitized emission FRET		(Padilla-Parra and Tramier, 2012)[1] (Periasamy et al, 2008)[2] (Miyawaki, 2011)[3] (Jares-Erijman and Jovin, 2003)[4]
Selected Recent Applications		
Observing distinct micro domains	mCFP-mCit	(Abankwa and Vogel, 2007)[5]
FRET efficiency measurement in live cells	CFP-YFP	(Chen et al, 2006)[6]
Homo- and heterodimerization of two human peroxisomal ABC transporters	CFP-YFP	(Hillebrand et al, 2007)[7]
Structure and localization of functional neurokinin-1 receptors	CY3-CY5	(Meyer et al, 2006)[8]
Homodimerization and internalization of galanin type 1 receptor	CFP-YFP	(Wirz et al, 2005)[9]
Role of calmodulin in MAPK signaling	CFP-YFP	(Moreto et al, 2009)[10]
Serotonin transporter oligomerization documented in RN46A cells and neurons	GFP-mCherry	(Fjorback a et al, 2009)[11]
Dynamic but not constitutive association of calmodulin with rat TRPV6 channels	CFP-YFP	(Derler et al, 2006)[12]
AKAP79-mediated targeting of the cyclic AMP-dependent protein kinase	CFP-YFP	(Gardner et al, 2006)[13]
Identification of plasma membrane macro- and microdomains	CFP-YFP	(Kobrinsky et al, 2005)[14]
Investigation of the dimerization of proteins from the epidermal growth factor receptor family	CFP-YFP	(Liu et al, 2007)[15]

References and Further Reading

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[11] Fjorback, A. W., Pla, P., Muller, H. K., Wiborg, O., Saudou, F., and Nyengaard, J. R., 2009, Serotonin transporter oligomerization documented in RN46A cells and neurons by sensitized acceptor emission FRET and fluorescence lifetime imaging microscopy: Biochem Biophys Res Commun, 380, 724-8.

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[15] Liu, P., Sudhaharan, T., Koh, R. M., Hwang, L. C., Ahmed, S., Maruyama, I. N., and Wohland, T., 2007, Investigation of the dimerization of proteins from the epidermal growth factor receptor family by single wavelength fluorescence cross-correlation spectroscopy: Biophys J, 93, 684-98.
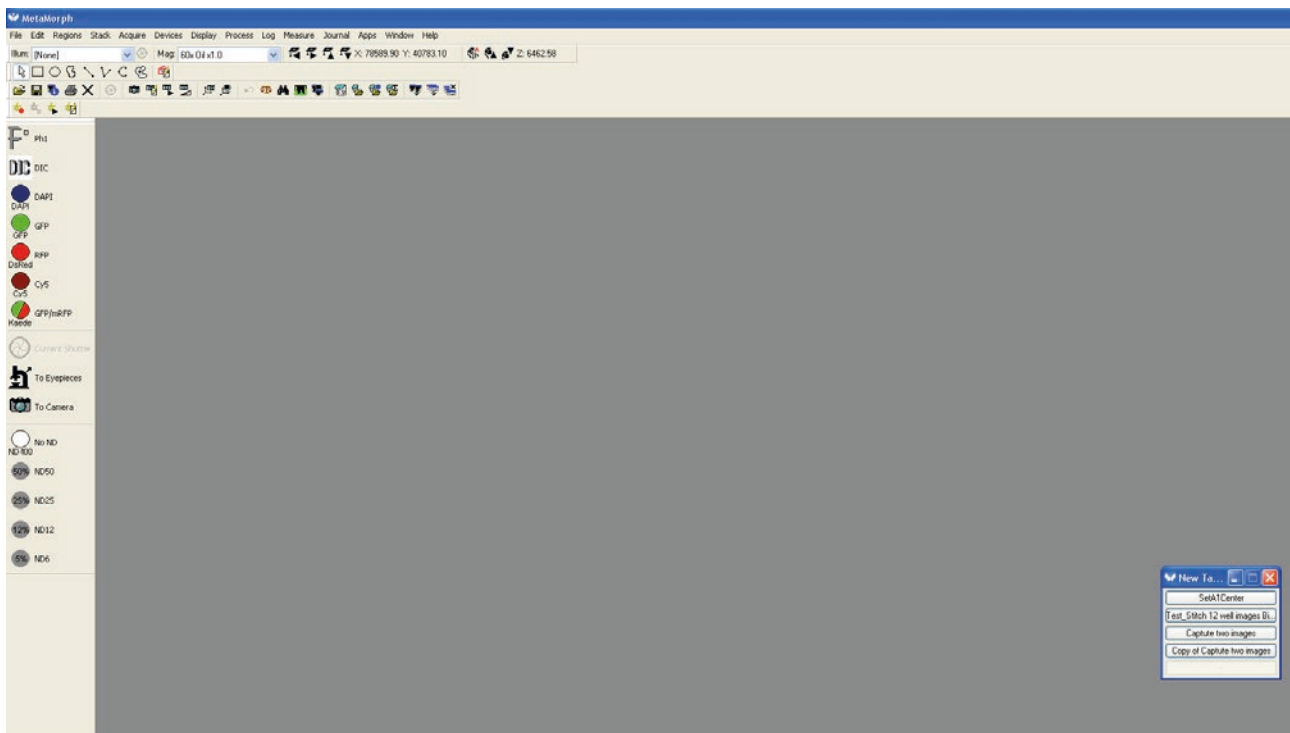
Appendix
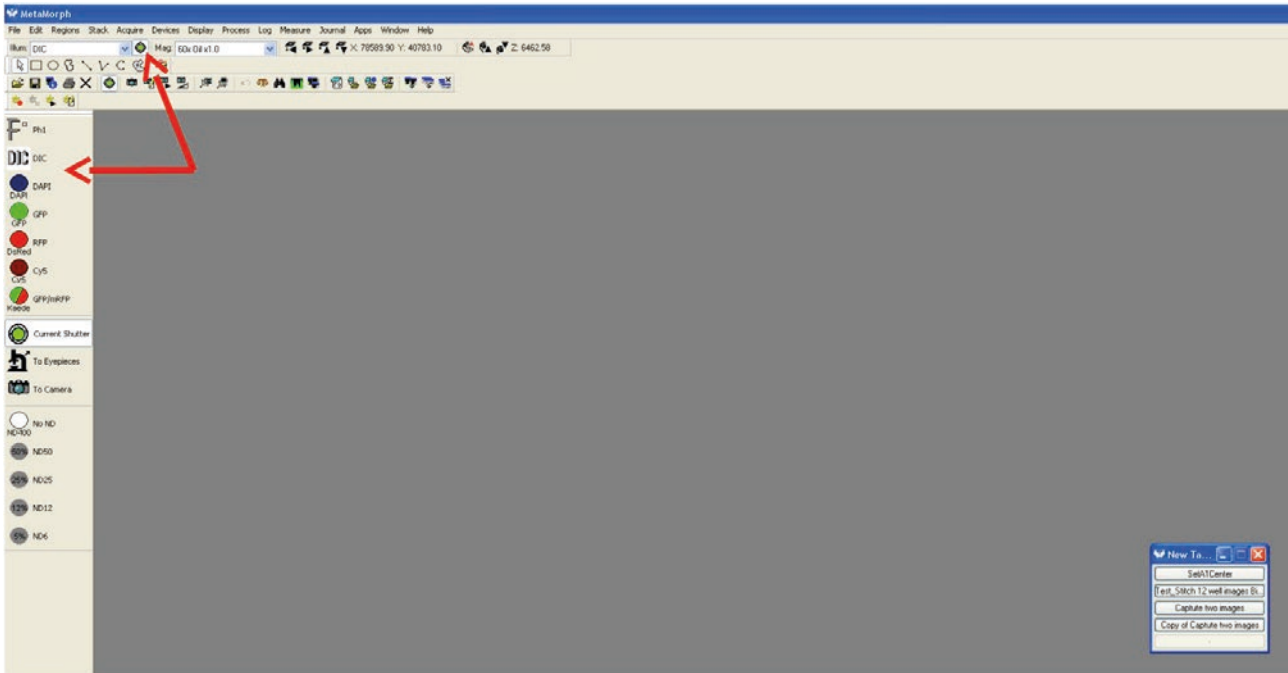
Image Acquisition Using Metamorph

→ Click on Metamorph software
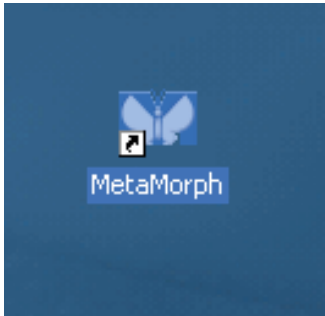
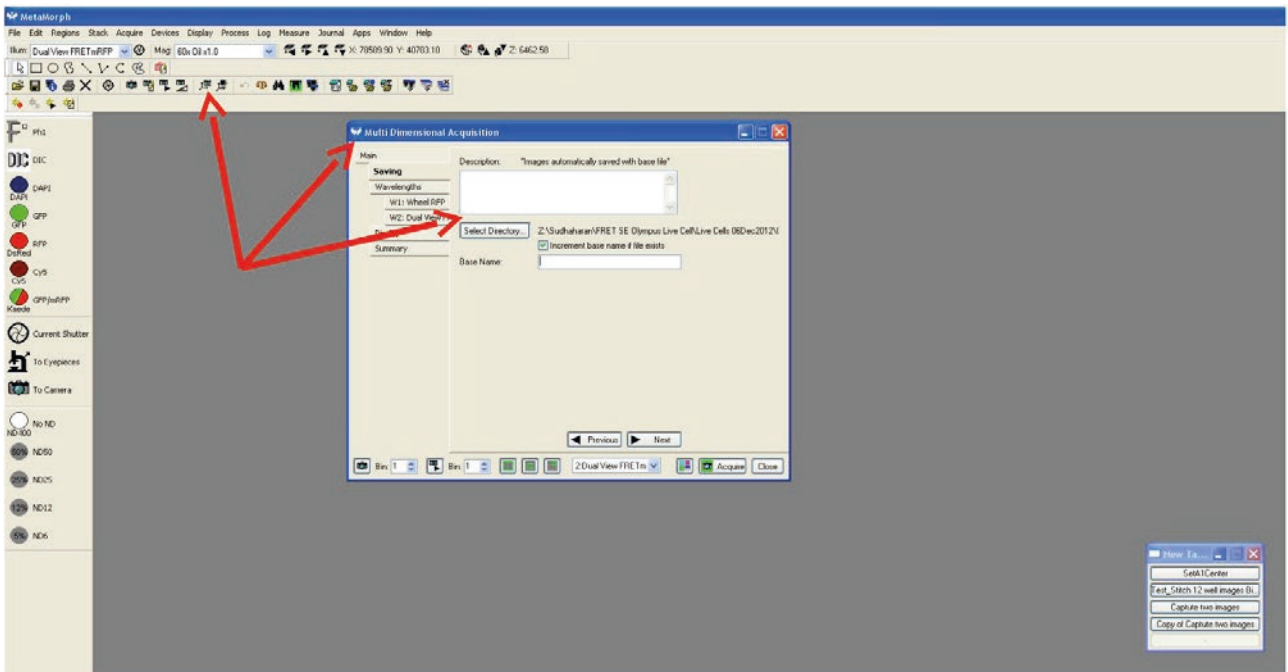→ Metamorph opens with the following window

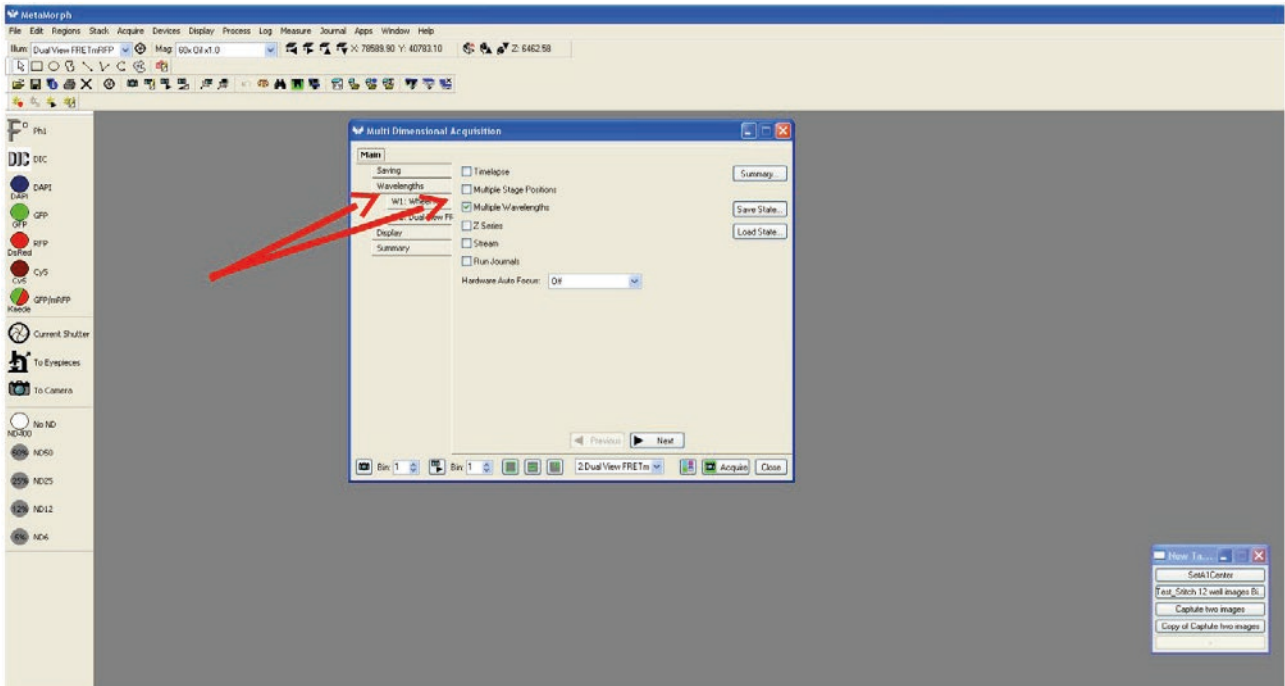→ Click on DIC and shutter to open to view the cells through eyepiece

→ Click on Multidimensional button
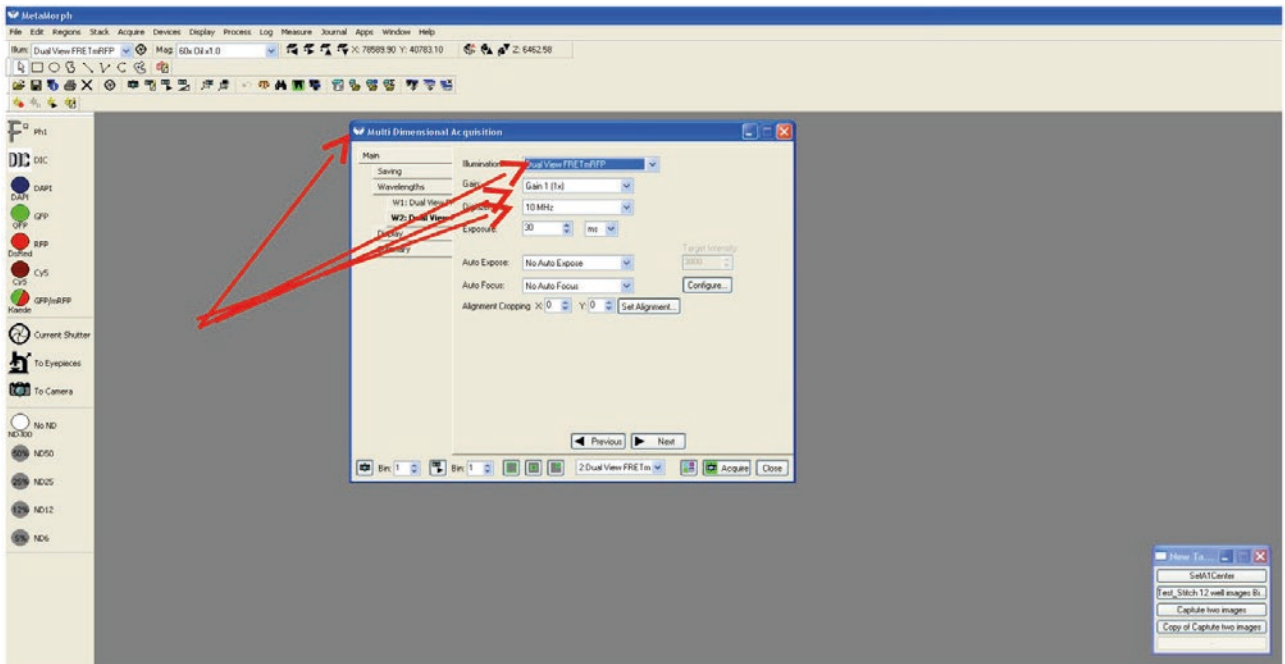→ The window opens up a new page
→ In the new window create a folder to save images by clicking select directory

→ Click on wavelength and multiple wavelengths to select different channels

→ Now select the required wavelengths using illumination button
→ Keep the Gain at 1 and Digitizer at 10 MHz
→ Adjust the exposure time to the desired levels

→ Click on live to open a new window for selecting right cells for imaging
→ Also select the required ND filter to reduce bleaching while imaging
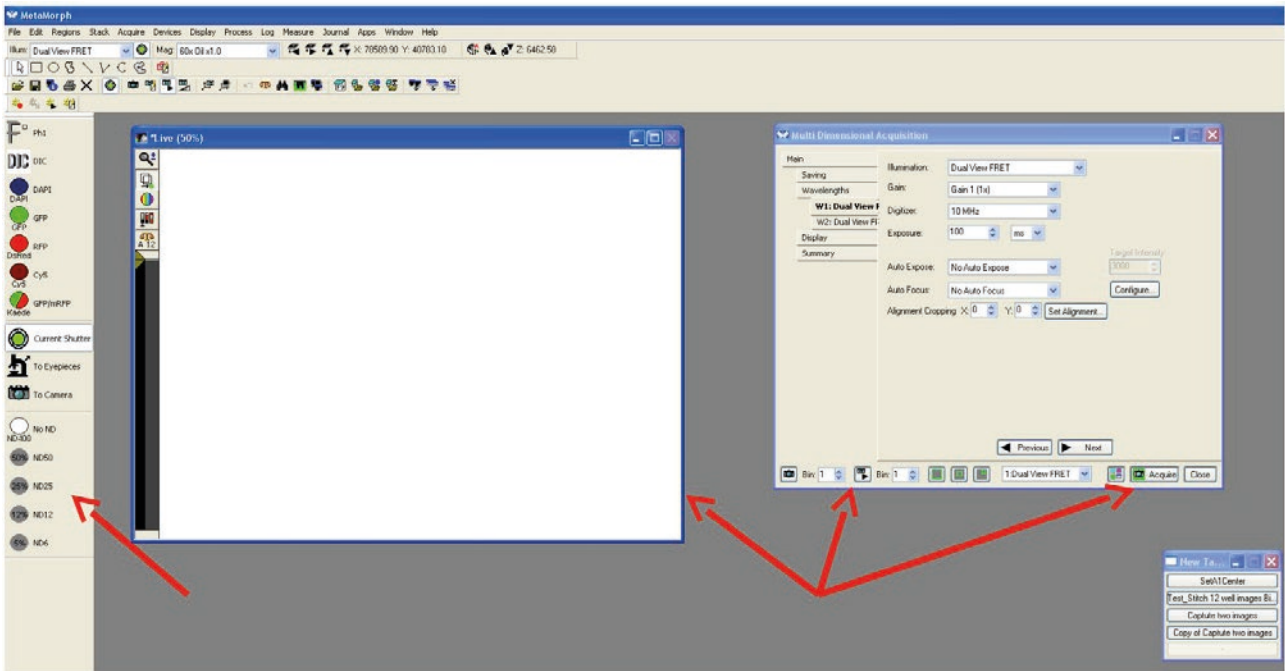
→ The following window shows two channel image collected for crosstalk correction of GFP
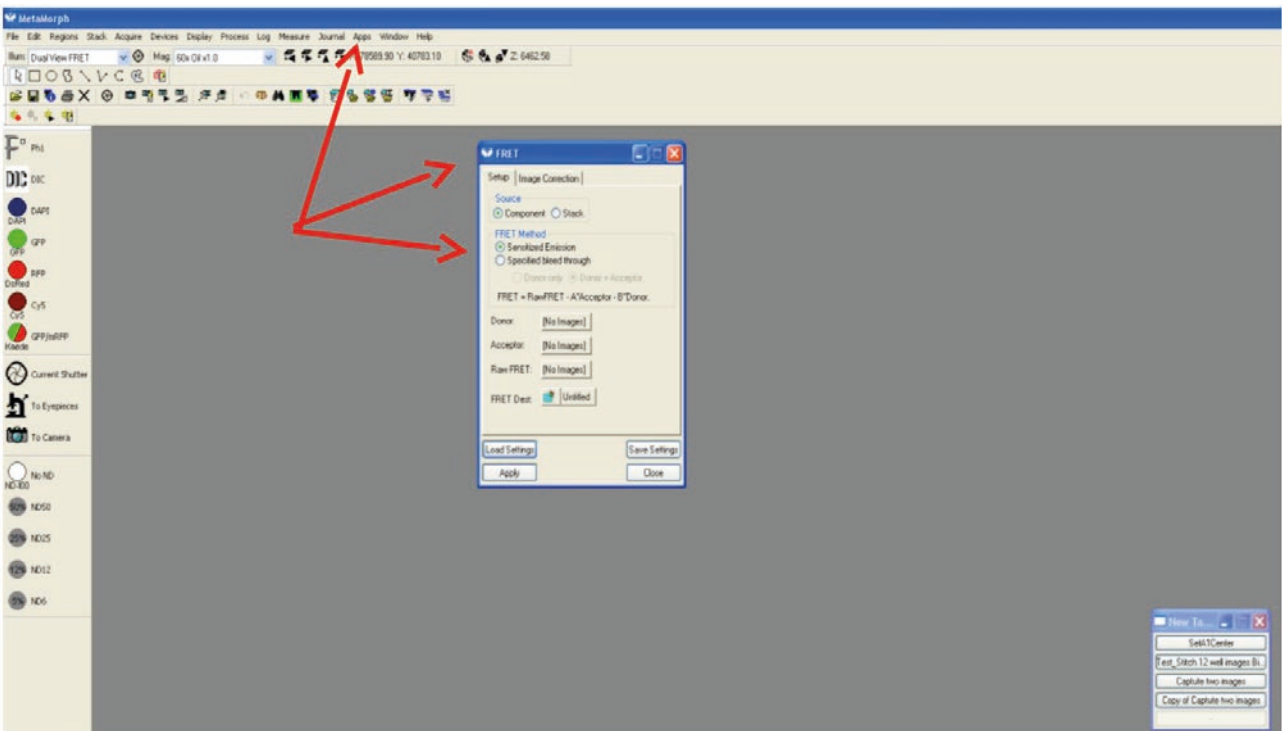→ Repeat the same for RFP correction factor using RFP wavelength
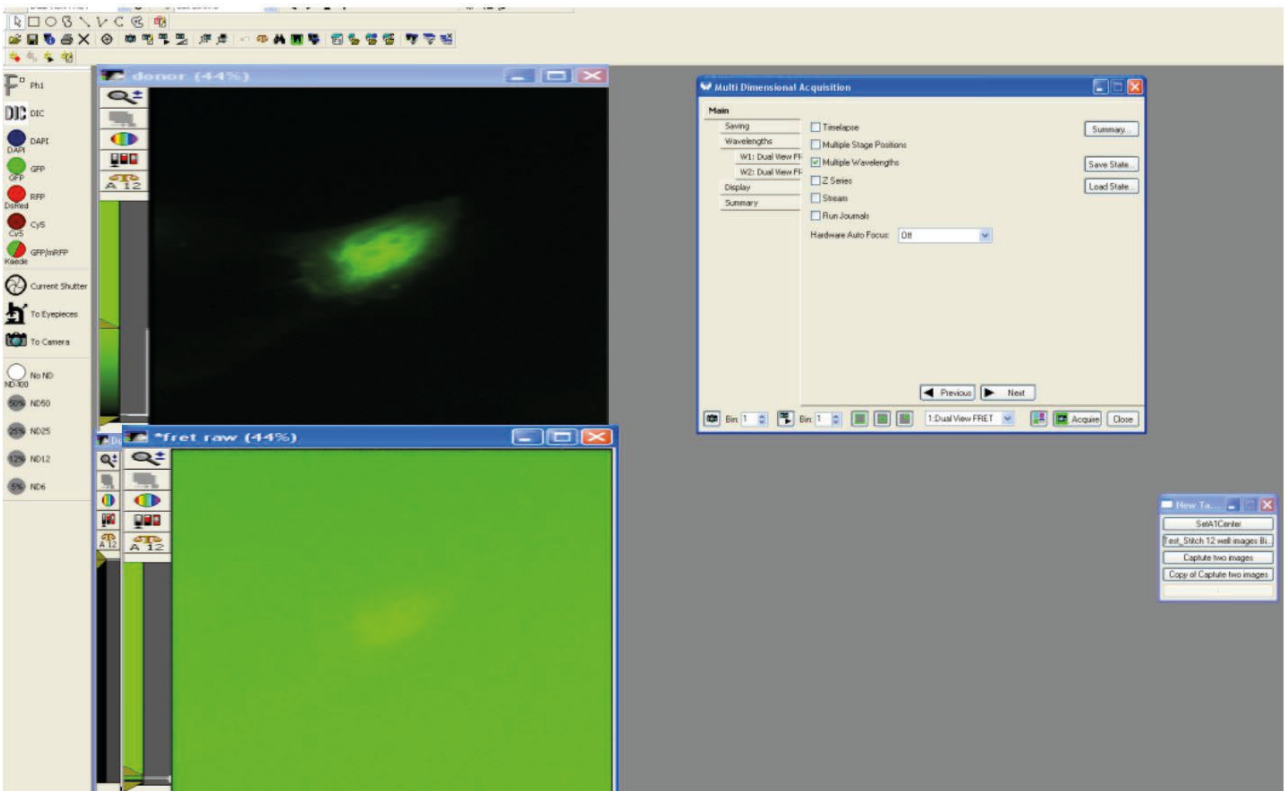
→ Finally select acquire to get three channel image for experimental sample to perform corrected FRET
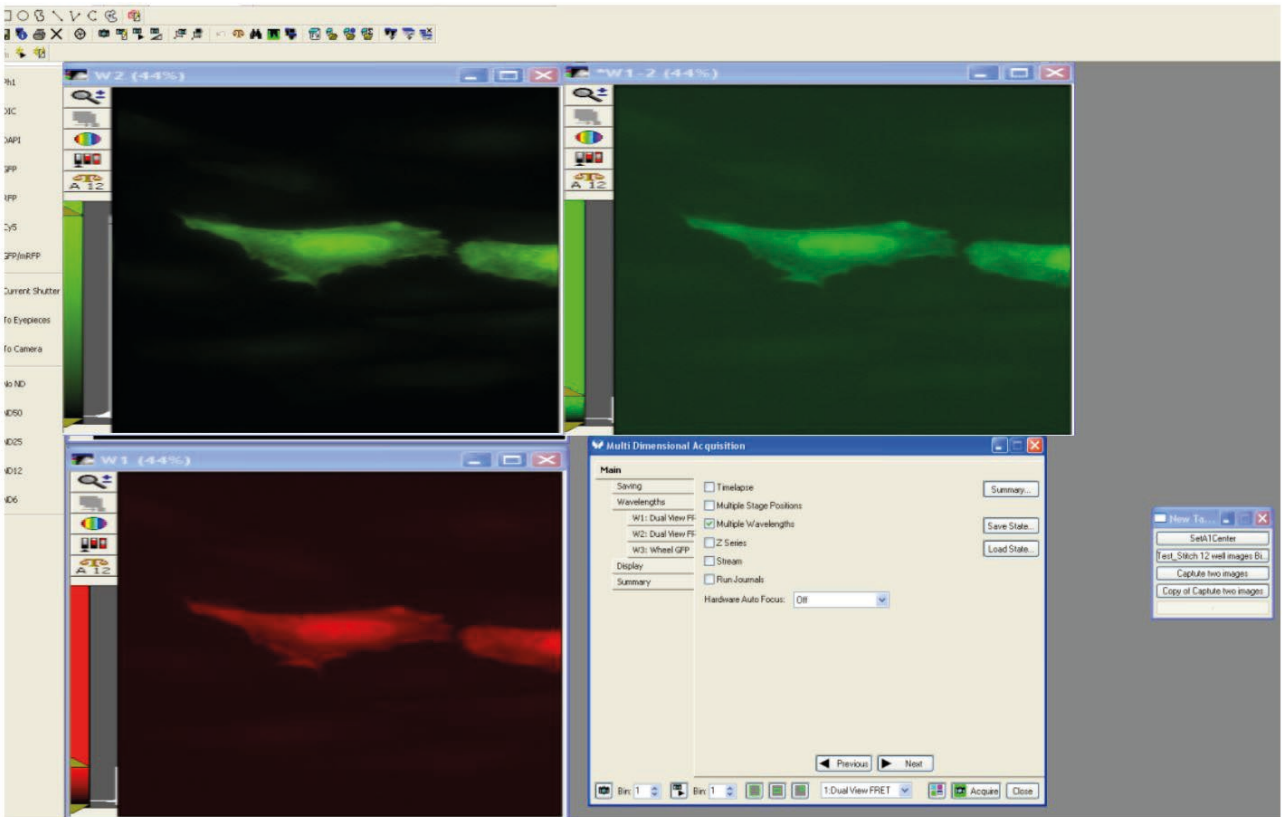
→ The collected images could be used for obtain FRETc
→ Click on Apps and select FRET from Metamorph software and select sensitized emission method

→ Feed in the correction factor coefficient values by selecting image correction button in FRET window
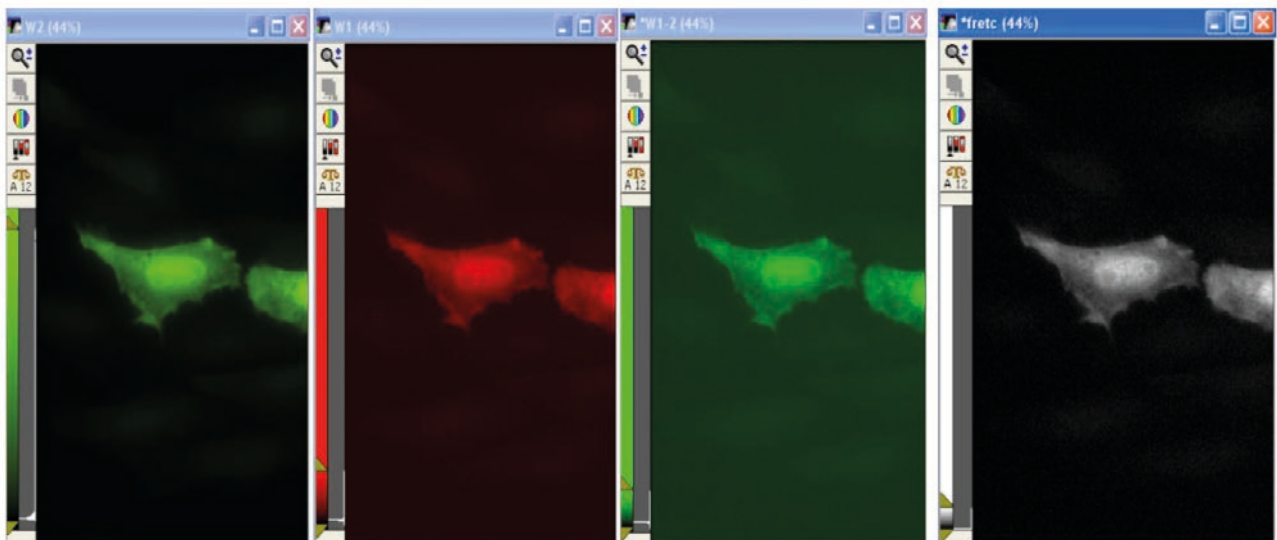→ Draw a ROI outside the image and select regions in background subtraction and click apply to get FRETc
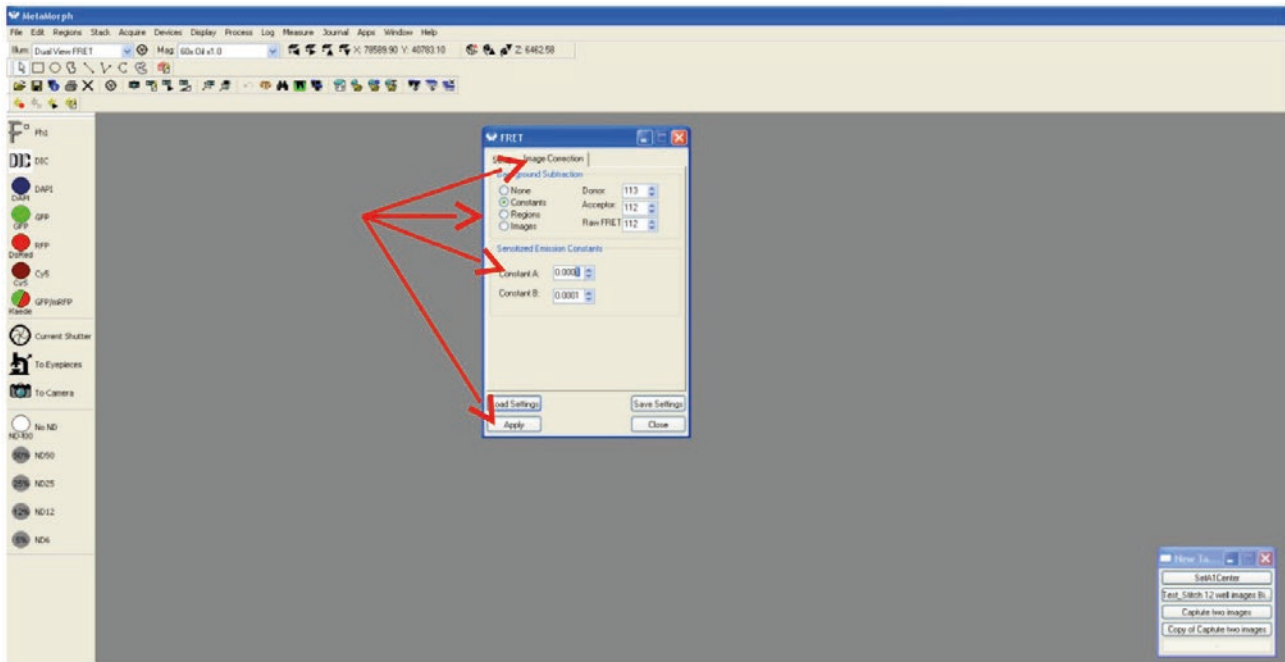
| Donor-channel | Acceptor-channel | FRET channel | FRETc |

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Material can be downloaded from www.picoquant.com

www.picoquant.com