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An Introduction to FRET, with an Emphasis on the Optics Involved.

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Fluorescent Resonant Energy Transfer (FRET) is a process that involves two fluorochromes (molecules that have the potential to fluoresce) with overlapping emission and excitation spectra. FRET is useful in determining intra-molecular and intermolecular reactions both spatially and temporally. It can be used to follow and record the interactions of proteins, enzymes, DNA, and RNA well below the 200 nanometer (10-9 meter) resolution limit of the optical microscope at its highest magnification and with the highest numerical aperture (NA) optics. In fact, FRET only allows resolution of interactions between 1 and 5nm, which is its beauty as well as its curse. Several excellent references discussing FRET in detail are listed in the bibliography. This application note is intended as a basic introduction only, with special emphasis on the optics required for the protocol.

If one fluorochrome has an emission spectrum that overlaps significantly with the absorption spectrum of a second fluorochrome, and if the two fluorochromes are physically very close to each other (generally between 1 and 6nm (the forster distance)), then energy from the first (donor) molecule may be transferred to the second (acceptor) molecule. This is a non-radiative phenomenon (no photons are emitted from the donor) in which energy from the donor (shorter wavelength emission) fluorochrome is transferred to the acceptor (longer wavelength emission) fluorochrome. When this happens, the donor fluorochrome's fluorescence is quenched (reduced in total light output). The acceptor's emission is triggered by energy absorbance or transfer from the donor and results in a wavelength appropriate emission from the acceptor. As noted above, this transfer is exquisitely distance sensitive, and as accidental FRET is an extremely rare event (effectively never happening) it is a reliable indicator of molecular interaction.

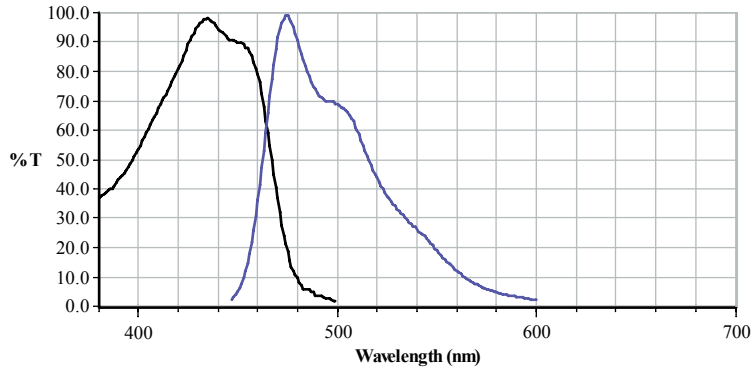
In theory, this is a fairly simple optical arrangement: an excitation filter and dichroic mirror to match the wavelength of the donor molecule, and an emission filter to match the fluorescence emitted by the acceptor. The microscopy would involve exciting only the donor, and imaging or recording only the emission of the acceptor. The presence of an emission from the acceptor, without its being directly illuminated or excited, should indicate FRET.

However, as a technique it is not simple due to several confounding issues including; overlapping absorption and emission wavelengths, the potential for motion, noise inherent in wide-field microscopy, and autofluorescence within the cells being imaged.

Overlapping Spectral Wavelengths

The emission and absorption characteristics of two different fluorochromes are not necessarily distinct. Take, for example, one of the most commonly used FRET pairs used in biological investigations, the Cyan and Yellow Fluorescent Proteins (CFP, YFP), which are two mutants of Green Fluorescent Protein (GFP). The Cyan Fluorescent Protein (CFP) has an absorption maximum of 435nm, with emission at 480nm (Figure 1).

Figure 1

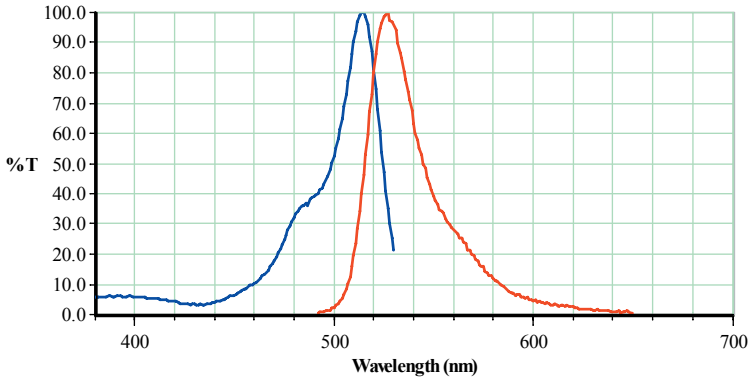


Cyan Fluorescent Protein (CFP)

black graph = absorption
blue graph = emission

The Yellow Fluorescent Protein (YFP), has a maximum absorption of 505nm with emission at

Figure 2

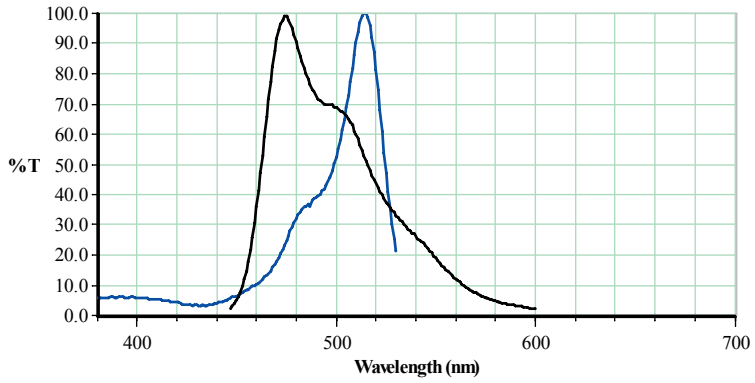


Yellow Fluorescent Protein (YFP)

blue graph = absorption
red graph = emission

530nm (Figure 2).
The emission curve (at least at the maxima) for the cyan protein almost completely overlaps with the absorption curve for the yellow protein, which is one of the prerequisites for FRET (Figure 3), and would seem to make the two molecules an excellent FRET pair.

Figure 3



CFP emission, YFP Absorption

black graph = cyan emission
blue graph = yellow absorption

Intuitively, FRET should be possible by exciting the CFP with light of approximately 436nm (a large spike in the mercury emission spectra), and recording or imaging any emission of about 530nm. Unfortunately, all the fluorescent proteins including cyan and yellow have coincident emission tails, such that there is considerable overlap (minimum of about 25%) between the emissions of the cyan fluorescent protein and the yellow fluorescent protein, therefore the emission from cyan may be read/recorded in the yellow channel. This emission may then be misinterpreted as FRET when; in fact, it is simply spectral bleedthrough of the extended emission from the donor protein. Furthermore, while the excitation maxima of cyan and yellow fluorescent proteins are spectrally quite distinct (435nm and 505nm) respectively, the absorption spectrum of YFP is broad and has some overlap with the absorption spectrum of CFP. Thus it becomes possible that YFP can be directly excited by 436nm light. This raises the question whether the YFP emission is caused by FRET or by direct excitation of 436nm light. Any and all possible FRET pairs must be tested to determine if the excitation of the donor will directly excite the acceptor.

As described below, ratio methods (dividing CFP emission by YFP emission) will resolve many of these issues. However, it is still critical to correct for the emission overlap between cyan and yellow fluorescent proteins.

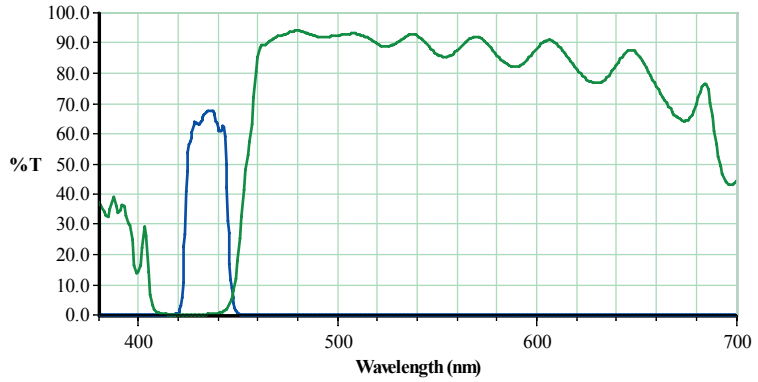
Obviously, this problem would not occur if there were no free cyan molecules. Unfortunately this will never occur when intermolecular FRET is being measured for stoichiometric reasons, and is extremely unlikely to occur when measuring intramolecular FRET events. Thus the cyan will be excited at 436nm and emit with a 480nm maxima but there will be a concomitant tail out to 520-550nm that will show up in the yellow emission spectra. Thus, it is necessary to subtract the donor emission and divide the two emissions in order to determine if a FRET reaction has occurred.

Subtraction of this cyan emission signal from the total FRET-plus-cyan signal seems simple and straightforward, except for the fact that this contamination by the cyan emission tail can, in some situations, be a very large proportion of the FRET signal.

Motion

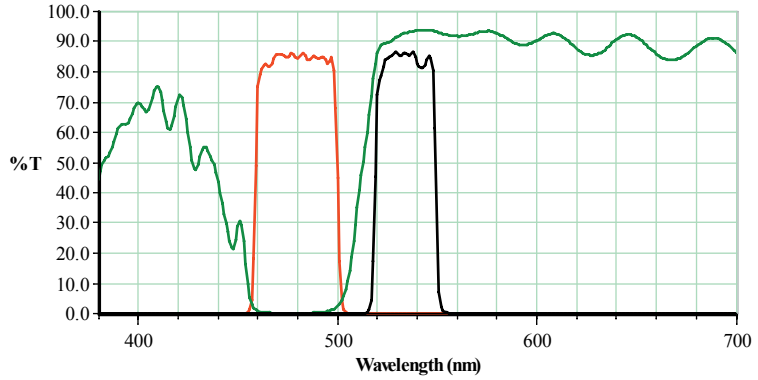
To collect FRET data two images are needed, one of which defines the CFP emission intensity, the other the YFP emission intensity. A ratio of the two images is then calculated. Therefore, there must be no movement during the acquisition of the two images. Obviously, the best way to avoid motion is to acquire both images simultaneously, using a dual detection system. Several devices, including those from Optical Insights, Hamamatsu, and PTI, are currently

Figure 4



Typically mounted into microscope

blue graph = d436/20x cyan exciter filter
green graph = 455dclp, dichroic mirror



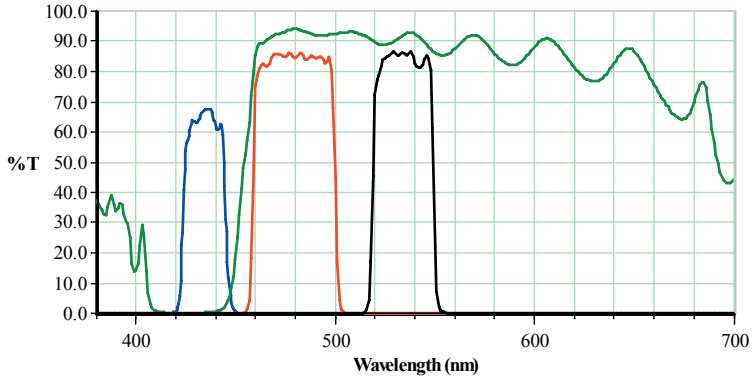
Emission beamsplitter set, mounted in the splitter units

red graph = d480/40m, cyan emission filter
green = 510dclp, dichroic
black = d535/30m, yellow emission filter

available. The optical configuration for these devices would appear similar to Figure 4.

However, simultaneous acquisition is not always practical. Use of a fairly rapid emission filter wheel is the next best alternative. The investigator acquires the image or data from the excited donor (CFP), and then moves the wheel as quickly as possible, to acquire the image from the

Figure 5



CFP excitation/beamsplitter, with CFP and YFP single emitters

blue graph = d436/20x, cyan exciter
green = 455dclp, cyan dichroic
red = d480/40m, cyan emitter
black = d535/30m, yellow emitter
(please note that there are several possible YFP emitters…)

acceptor emission (YFP). See Figure 5 for the optics involved with this configuration. In addition, the acquisition time of the camera or detector must be rapid enough to minimize any movement. This means that there must be enough photons present at the detector to form an image, and that the camera must be sensitive enough to collect the photons.

It is important to keep in mind that much of this work is done on live cells in aqueous solution, a fact that presents other issues involving motion, such as the motion arising from the use of perfusion pumps. It is imperative that the microscope and all attachments be kept very stable. Vibration isolation tables are recommended and quite common for these applications.

Noise and Registration

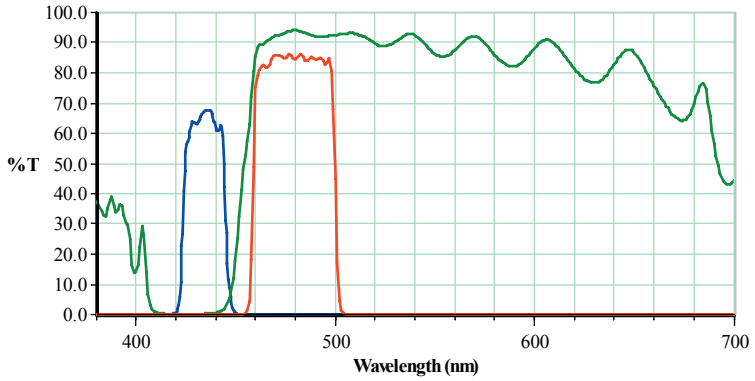
Signals involved in FRET are so small that they can easily become lost in the noise (background brightness) of the microscope. One logical solution is to determine the ratio between the emissions of the donor and acceptor molecules, instead of trying to measure only the FRET emission. The donor emission should decrease at the same time that the acceptor emission

increases. This division process makes it possible to isolate much of the noise, thus enhancing the true FRET emission. While division of two images is considerably more difficult than addition or subtraction, most morphometry software packages now include a module that allows for ratiometric analysis. It must be kept in mind that division of two images requires that the two be in pixel registration. Please note that some very early papers claimed to detect FRET signals by excitation of the donor and image/calculation of emission at the acceptor only, without ratiometry. This approach is now considered naïve.

Mercury light sources are notoriously noisy. Many experts consider them unacceptable for ratio imaging, yet they remain the most common light source in current use. Xenon light sources are considered more stable and less noisy. However xenon doesn't offer the advantage of energy spikes that are present in the mercury source. Furthermore, even a properly aligned and cleaned microscope is likely to provide several percentage points of noise before the image reaches the camera or detector, and there is the added noise of the detector itself to consider. If the measured ratio for a common FRET relationship were around 12%, it would be easy to lose much of that in the noise of the microscope, making it unlikely that those statistics would be reliable.

Some researchers do not have emission filter wheels or dual detectors and cannot employ either of the ratio techniques listed above. Some try to use two separate cubes to measure FRET. One cube would be a typical donor (e.g. CFP) cube: 436/20x, 455dclp, and 480/40m (Figure 6). The other cube would have the same exciter and dichroic, with the acceptor (e.g. YFP) emission, for example, 436/20x, 455dclp, and 535/30m (Figure 7).

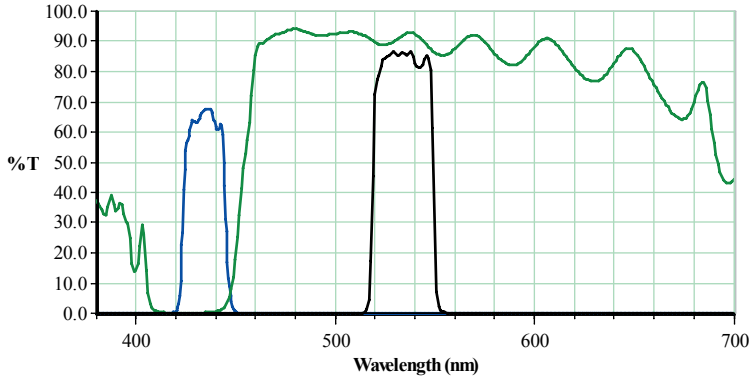
Figure 6



CFP set, Chroma 31044v2

blue = d436/20x, excitation filter
green = 455dclp, dichroic mirror
red = d480/40m, emission filter

Figure 7



CFP excitation and beamsplitter, with YFP emission filter

blue graph = d436/20x, CFP excitation filter
green = 455dclp, CFP dichroic
black = d535/30m, YFP/FRET emission filter
(please note that there are several possible YFP emission options…)

This approach presents several problems. One is the constant threat of motion, as the two cubes must be moved into and out of position. This threat can be lessened, but not eliminated, by using a turret system. Another potential problem is the time required to move the cubes, during which the cells may also move. Furthermore, the microscope and cubes must be nearly perfectly machined and aligned; else the two images will not be in alignment at the camera or detector. In this case, the image from one cube may go to a slightly different location on the detector, due to beam deflection and wedge (deviation from perfect parallelism) associated with either the mirror or the emission filter, or with machining of the parts within the microscope. Many software packages now provide an algorithm that will re-align two images, but it is still an extra step and not something that happens automatically.

Autofluorescence

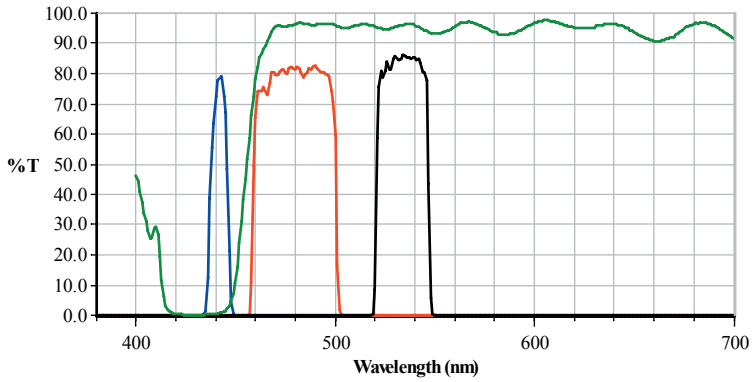
As with all fluorescence techniques, the endogenous fluorescence in a cell/tissue (the autofluorescence) can rear its ugly head. Some samples will have little autofluorescence, while others such as liver or brain slices may have a large autofluorescence signal. In fact, virtually every sample will have some level of autofluorescence. This should always be evaluated before the experiment by using the optics necessary for all the FRET applications to determine what the cells look like, without any external fluorescence added. There are a surprising number of researchers that go through all the steps necessary to do fluorescence work without ever imaging (checking) their preparations to determine the background levels of light emission. This is a particular problem with any cell that is highly pigmented.

Usually the autofluorescence can be subtracted from the signal of interest, since this unwanted emission tends to be broader and less specific in both intensity and wavelength. Occasionally special steps have to be taken, such as moving the bandpass of the emission filter in order to maximize the wanted signal and minimize the autofluorescence. Unfortunately, there are no optics that can determine what is 'wanted' and what is considered 'noise'. This has to be determined by the researcher with a variety of control experiments.

Mitigating Techniques

Using a laser scanning confocal microscope for FRET can minimize most of the above problems. The diffraction limited spot, coupled with the PMT (photomultiplier tube) detection of only that one spot in time, greatly reduces the overall noise in the system and makes it much easier to isolate the FRET relationship. Confocal microscopes also commonly have multiple detectors so that the two images may be acquired simultaneously, as shown with filters in (Figure 8). See the reference list for more details concerning confocal applications.

Figure 8



442nm laser set for CFP/YFP FRET emission ratio

blue graph = z442/10x, laser clean-up filter
green = z442rdc, dichroic
red = hq480/40m, cyan emission filter
black = hq535/30m, yellow/fret emission filter
(please note that there are several possible YFP/FRET emitters…)

In either type of microscopy, it is a great advantage if the FRET relationship can be broken (or established) on demand using a chemical, electrical, or mechanical challenge to the cell/tissue. This allows for a built-in control of FRET versus non-FRET using the same field of view in the microscope. This technique is much better than relying on the average intensity of single probe samples (donor-only cells and acceptor-only cells) for the multiple images to be used in the ratio pairs. This technique is very paradigm/experiment specific, however, and is not always possible.

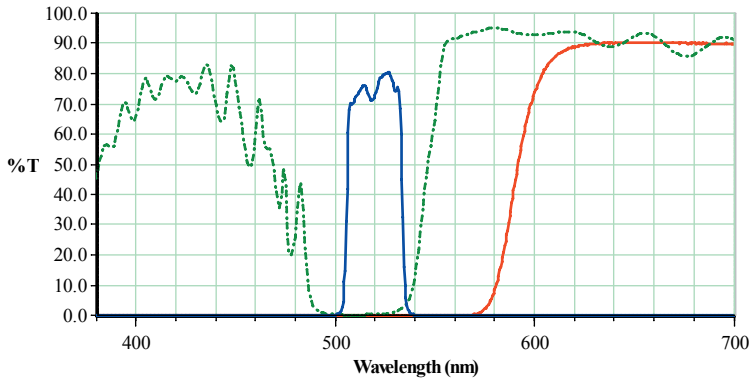
Acceptor Bleaching

Acceptor bleaching is a new method that offers great promise for wide-field and confocal FRET microscopy. In this protocol, a ratio is determined for the emission of the donor using images from before and after bleaching the acceptor.

Since FRET is a non-radiative energy transfer process, the absence (or destruction) of the acceptor should show as a slightly increased emission from the donor.

Again, using CFP/YFP as our example, you would acquire an image of the cyan emission (d480/40m) using cyan excitation (d436/20x) just as if you were imaging cyan protein alone (set 31044, Figure 6). Then a special cube/filter set would be used to completely (95%+) photobleach (destroy) the YFP (Figure 9a, 9b, and 9c show three different designs for widefield, while 9c is a laser set). The CFP filters would be used again to acquire the cyan emission. If there had been a FRET relationship between the two fluorochromes, the cyan emission will have increased slightly after bleaching the yellow protein.

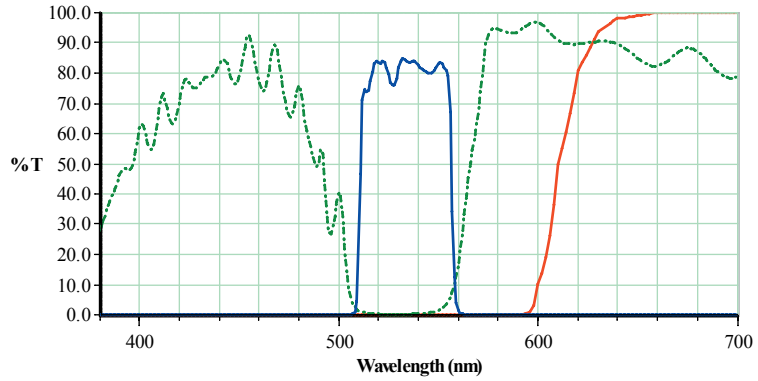
Figure 9a



Yellow Fluorescent Protein (YFP) bleaching set

blue graph = d520/30x, YFP bleaching exciter
green = 545dclp, dichroic
red = og590lp, emission blocking glass for safety

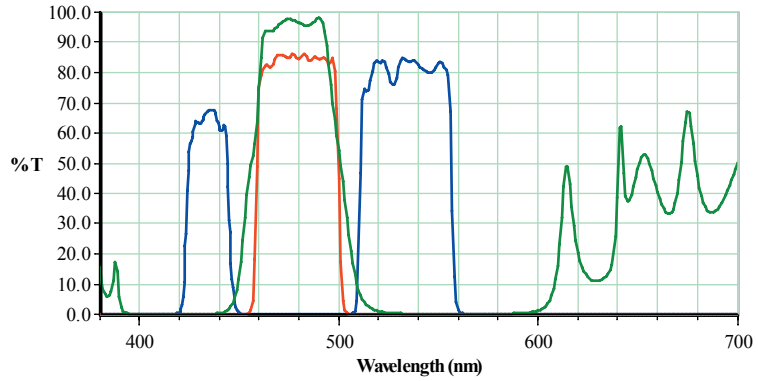
Figure 9b



YFP bleaching set, v2

blue graph = d535/50x, YFP bleaching excitation
green = 565dclp, dichroic
red = rg610lp, emission blocking glass for safety

Figure 9c



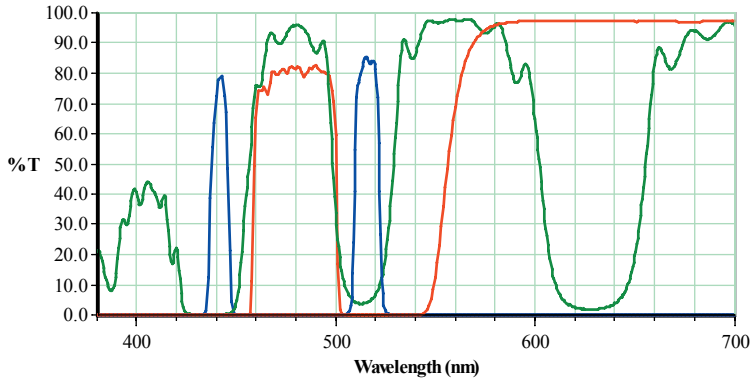
YFP bleaching set, v3
(for use with an excitation filter wheel, without changing the dichroic)

blue graph = d436/20x, cyan exciter
green = custom bandpass dichroic (…dmbp5.tfd)
red = d480/40m, cyan emission filter
blue = d535/50x, yellow bleaching filter
(note: care must be taken in using this set without proper blocking optics in the eyepieces)

This technique provides a built-in control, since the same field of view can be ratioed before and after bleaching. There is no longer a need to 'break' the FRET bond, nor is there an issue with the cyan emission bleeding into the yellow emission channel. Also eliminated is the necessity of using average cyan and yellow intensities from cells with single transfections.

One difficulty with this procedure is the need to assure that the yellow (acceptor) fluorescence is completely bleached (destroyed) without affecting the fluorescence of the (cyan) donor pro-

Figure 9d



YFP bleaching set for 442/514nm laser systems, designed to leave the mirror in position and move the clean-up filters and the emitters

blue graph = z442/10x, helium-cadmium clean-up filter, CFP excitation
green = z442/514rpc, polychroic to reflect 442nm and 514nm laser line
red = hq480/40m, CFP emission filter
blue = z514/10x, argon clean-up filter, to bleach YFP
red = og550lp, absorption glass emitter for safety only

tein. This must be carefully checked with adequate controls. Issues may also arise with regard to completely bleaching the yellow (acceptor) molecule in a time frame that is suitable for the particular application, and to minimize motion artifacts.

Another potential problem is that the resultant ratio will be very small, and therefore its accuracy is difficult to ascertain. Even so, this technique offers great hope for wide field and confocal FRET applications, with its built in control mechanism.

Summary

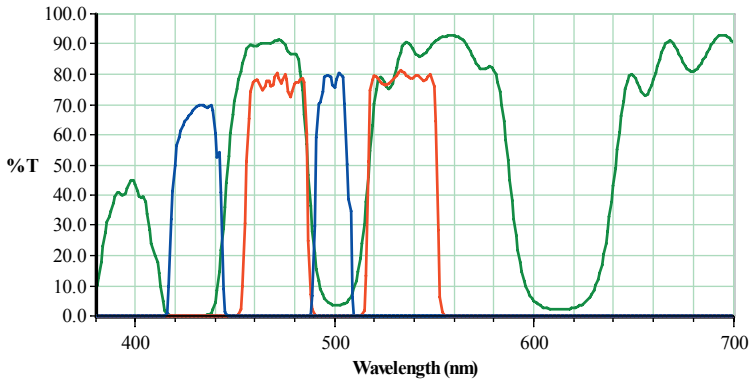
FRET is a difficult technique, however when done properly it can be extremely valuable as it allows measures of protein-protein interaction in living cells. As the technique works at the nanometer level, and if fluorescent proteins are being used as the reporters, care should be taken to define where the fluorescent protein gene sequence is inserted within the protein (generally carboxyl or amino terminus) to maximize the chance of interaction. Furthermore, to avoid most of the issues described above and to ensure that there actually is a relationship, initial FRET measurements should be performed in a fluorimeter in free solution. If the fluorimeter results are positive, the next set of experiments should occur with a confocal if at all possible. However, despite its associated problems, FRET has been shown to work quite successfully in many cases. The molecular constructs and manipulations are actually the hardest part. The optical arrangement of filters and mirror is fairly straightforward. The exciter filter should match the absorption maximum of the donor molecule, with the mirror's reflection band coinciding with the exciter filter. The transmission band of the mirror and the emission filters should match the emission maximum of the acceptor molecule, and donor emission for ratio analysis. A common mistake is to use the dichroic mirror from the acceptor set instead

of the dichroic for the donor. For our CFP/YFP example, the optics would include: 440/20x, 455dclp, 480/40m, and 535/30m (Figure 5).

As with all applications however, there is more than one approach and multiple techniques are available. This can sometimes depend a great deal on the hardware available to the researcher. We have shown cases above using dual detectors simultaneously, sequential detection with emission filter wheels, and single acquisitions by moving single cube designs.

There also exists the possibility of using both excitation and emission filter wheels for special applications. This equipment makes it possible to leave a polychroic (dual dichroic) in place in the microscope while sequentially selecting single excitation filters and single emission filters. Even though FRET, in absolute terms, only requires the excitation of the donor, many researchers want to determine the presence of the acceptor. See our dual set (Figure 10) for single band exciter, single band emitter set.

Figure 10



CFP/YFP dual mirror, with single exciters and emitters.
Chroma set 86002v2

blue graph = s430/25x, cyan exciter
blue = s500/20x, yellow exciter
green = dual dichroic, polychoic
red = s470/30m, cyan emission
red = s535/30m, yellow emission

Please contact your nearest expert for more advanced techniques and/or applications. When all is said and done, probably the best way to do FRET is with FLIM, but that is another story…

Some Additional Reading

Bastiaens, P.I.H., and A. Squire. 1999. Fluorescence lifetime imaging microscopy: spatial resolution of biochemical processes in the cell. Trends Cell Biol. 9, 48-52.

Chalfie, Martin, and Steven Kain. 1998. GFP: Green Fluorescent Protein, Preparation,

Applications and Protocols. Wiley-Liss, NY, USA.
Chamberlain, C.E., V.S. Kraynov, and K.M. Hahn. 2000. Imaging spatiotemporal dynamics of Rac activation in vivo with FLAIR. Methods Enzymol. 325, 389-400.

Clegg, R.M. 1992. Fluorescence resonance energy transfer and nucleic acids. Methods Enzymol. 211: 353-389.

Gordon, G.W., G. Berry, X.H. Liang, B. Levine, and B. Herman. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. Biophys. J. 74, 2702-2713.

Haugland, R.P. 1996. Handbook of Fluorescent Probes and Research Chemicals, 6th Ed. Molecular Probes, Eugene, OR, USA.

Herman, B. 1998. Fluorescence Microscopy, 2nd Edition. Springer-Verlag, NY, USA.

Herman, B., and S.M. Fernandez. 1982. Dynamics and topographical distribution of surface glycoproteins during myoblast fusion: a resonance energy transfer study. Biochemistry. 21: 3275-3283.

Heim, R., and R.Y. Tsien. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol., Feb. 1;6(2): 178-82.

Inoue, S. 1986. Video Microscopy. Plenum Press, NY, USA.

Karpova, T.S., C.T. Baumann, L. He, X. Wu, A. Grammer, P. Lipsky, G.L. Hager, and J.G. McNally. 2003. Fluorescence resonance energy transfer from cyan to yellow fluorescent protein detected by acceptor photobleaching using confocal microscopy and a single laser. Journal of Microscopy, Vol 209, Issue 1, page 56.

Kenworthy, A.K., N. Petranova, M. Edidin. 2000. High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. Mol. Biol. Cell. 11, 1645-1655.

Lakowicz, J.R. 1999. Principles of fluorescence spectroscopy, 2nd Edition. Kluwer Academic – Plenum, NY, USA.

Mason, W.T., Editor. 1999. Fluorescent and Luminescent Probes for Biological Activity. Academic Press, CA, USA.

Matayoshi, E.D., G.T. Wang, G.A. Krafft, and J. Erickson. 1990. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. Science. 147: 954-958.

Murphy, Douglas B. 2001. Fundamentals of Light Microscopy and Electronic Imaging. Wiley-Liss, USA.

Pawley, James B., Editor. 1998. Handbook of Biological Confocal Microscopy. Plenum Press, NY, USA.

Reichman, J. 2000. Handbook of Optical Filters for Fluorescence Microscopy. HB1.1.
Sekar, R.B., and A. Periasamy. 2003. Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. J. Cell Biol. 160, 629-633.

Slavik, Jan, Editor. 1995. Fluorescence Microscopy and Fluorescent Probes. Plenum Press, NY, USA.

Stanley, C. Michael. 2003. Filters and mirrors for applications in fluorescence microscopy. Methods in Enzymology, Vol 360, page 394. Elsvier Science, USA.

Stanley, P.E., and L.J. Kricka, Editors. 2002. Bioluminescence and Chemiluminescence, Progress and Current Applications. World Scientific Publications, London, UK.

Sullivan, Kevin F., and Steve A. Kay, Editors. 1999. Green Fluorescent Proteins. Academic Press, London, UK.

Szollosi, J., S. Damjanovich, S.A. Mulhern, and L. Tron. 1987. Fluorescence energy transfer and membrane potential measurements monitor dynamic properties of cell membranes: a critical review. Prog. Biophys. Molec. Biol. 49: 65-87.

Tron, L., J. Szollosi, S. Damjanovich, S.H. Helliwell, D.J. Arndt-Jovin, and T. M. Jovin. 1984. Flow cytometric measurements of fluorescence resonance energy transfer on cell surfaces. Quantitative evaluation of the transfer efficiency on a cell-by-cell basis. Biophys. J. 45: 939-946.

Uster, P.S., and R.E. Pagano. 1986. Resonance energy transfer microscopy: observations of membrane-bound fluorescent probes in model membranes and in living cells. J. Cell Biol. 103: 1221-1234.

Wallrabe, Horst, Michael Stanley, Ammasi Periasamy and Margarida Barroso. 2003. Journal of Biomedical Optics, Vol 8, Issue 3, pg 339. SPIE, IBOS.

Wolf, D.E., A.P. Winiski, A.E. Ting, K.M. Bocian, and R.E. Pagano. 1992. Determination of the transbilayer distribution of fluorescent lipid analogues by nonradiative fluorescence energy transfer. Biochemistry, 31: 2865-2873.

Wu, P., and L. Brand. Resonance energy transfer: methods and applications. Anal. Biochem. 218, 1-13.

Xia, Z., and Y. Liu. 2001. Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscope. Biophys. J. 81, 2385-2402.

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