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Research Report

GFP-based FRET analysis in live cells

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ARTICLE INFO

Article history:
Accepted 31 January 2006
Available online 10 March 2006

Keywords:
Fluorescence resonance energy transfer
Spectroscopy
microscopy
Protein–protein interaction
Green fluorescent protein
Spectra FRET

ABSTRACT

Fluorescence resonance energy transfer (FRET) is a widely utilized optical technique for measuring small distances of 1–10 nm in live cells. In recent years, its application has been greatly popularized by the discovery of green fluorescent protein (GFP) and many improved variants which make good donor–acceptor fluorophore pairs. GFP-based proteins are structurally stable, relatively inert, and can be reliably attached to points of interest. The combination of easy access to the GFP-based FRET technique and its obvious usefulness in many applications can lead to complacency. Potential problems such as light contaminants, e.g., bleed-through and cross-talk, and inconsistent donor and acceptor concentrations are easily overlooked and can lead to errors in FRET calculation and data interpretation. In this article, we outline possible pitfalls of GFP-based FRET and approaches that address these issues, including a "Spectra FRET" technique that can be easily applied to live cell studies.

1. Introduction

Fluorescence resonance energy transfer (FRET) is a technique for detecting inter- and intra-molecular distances of roughly 1–10 nm (Clegg, 1992; Selvin, 1995; Stryer, 1987). Two points of interest are labeled with different fluorophores–a donor fluorophore and an acceptor fluorophore–in which the acceptor absorption spectrum overlaps the emission spectrum of the donor. The excited donor fluorophore can dissipate the energy taken from a photon through several pathways, including normal fluorescent emission and non-radiative dipole–dipole coupling to a nearby acceptor, e.g., FRET. Energy transfer through the FRET mechanism decreases the donor fluorescence and increases the acceptor fluorescence. The efficiency of energy transfer is inversely proportional to the sixth power of the distance between the two fluorophores. Because of this strong distance dependency and the fact that FRET takes place in a range comparable to the size of many biological molecules, FRET has become an extremely useful reporter for molecular proximity in biological studies (Lakowicz, 1999; Miyawaki, 2003). Although FRET has a lower resolution than X-ray crystallography, it has the advantage of measuring in living biological systems under physiological conditions and, in many cases, in real time.

The development of multiple green fluorescent protein (GFP) mutants has greatly facilitated the application of FRET to studies in live cells (Heim and Tsien, 1996; Tsien, 1998). Multiple FRET pairs can be formed by mutant GFPs. The most widely used is the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) pair. The formation of a chromophore within these fluorescent proteins occurs automatically under physiological conditions, apparently due to an autocatalyzed biosynthesis of imidazolinone from residues Ser–Tyr–Gly (Barondeau et al., 2003; Cubitt et al., 1995). The same process occurs when the fluorescent protein is expressed as part of a fusion protein. Therefore, one can make tandem constructs between the fluorescent protein cDNA and the cDNA encoding the protein of interest. Transfection of cells with a mixture of tandem constructs containing the donor fluorophore cDNA and that containing the acceptor fluoro-

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doi:10.1016/j.brainres.2006.01.119

phore cDNA results in co-expression of fluorophore-labeled proteins in live cells. This routine molecular biological approach is not only easy to carry out but also highly specific. All the fluorescent proteins expressed in the cell are covalently attached to the protein to be studied. This ensures a low level of background fluorescence. A combination of the advance in fluorescence microscopy techniques and the development of GFP-based FRET pairs has resulted in fast adoption of the FRET approach to a wide spectrum of biological studies of protein trafficking, co-localizations, specific interactions, and conformational rearrangements (Miyawaki, 2003), including studies of proteins involved in auditory functions (see articles by R.M. Raphael and colleagues as well as by R. Hallworth and colleagues in this issue).

The popularity of FRET as a method for estimating distances in biological systems and its easy accessibility has lead to the development of a plethora of techniques for the measurement and quantification of FRET efficiency. FRET measurements may appear simple, but awareness of the potential pitfalls is crucial in order to avoid complications in the accuracy of fluorescence measurements and calculations down the line. This review outlines the major sources of light contaminations in FRET measurements and how they are resolved in several widely used FRET approaches. In addition, a recently developed "Spectra FRET" approach (Zheng, 2006) is presented as an example for measuring FRET signals in biological systems.

2. Potential problems in FRET experiments

In the following section, several common contaminating factors for FRET measurements will be discussed. These include (1) cross-talk, (2) bleed-through, (3) non-specific FRET, (4) mixture of fluorophore populations, and (5) variable expression levels of donor and acceptor. In addition, one must be aware that FRET measurements are usually based on specific assumptions. For example, re-absorption by the acceptor of the donor fluorescence emission (as opposed to the non-radiative energy transfer of FRET) is assumed to be negligible, which is often true at low fluorophore densities. For GFP-based FRET in biological experiments, the donor and acceptor fluorophores are tethered to biological molecules of interests. It is normally assumed that the absorption and emission properties of these fluorophores are unchanged in concatenated tandem constructs. The excitation and emission spectra should be checked if there is evidence suggesting otherwise. Furthermore, the pair of fluorophores can interact through ways other than FRET (Dexter, 1953), which may cause changes in the donor and acceptor fluorescence intensities in the same direction as the FRET mechanism. Failure to take these complications into consideration can lead to either miscalculation of the actual FRET efficiency or misinterpretation of the underlying biological process.

2.1. *Cross-talk*

Ideal FRET pairs are made of two fluorophores that have extensive overlap between the donor emission spectrum and the acceptor excitation spectrum, without overlapping excitation spectra or emission spectra. This is, however, rarely true in practice. GFP mutants, for example, have rather broad excitation and emission spectra that overlap significantly due to a relatively small Stoke's shift. As a result, a pair of GFP mutants may be excited (to different extents) by the same excitation light; their emissions may also be mixed extensively. One type of contaminating fluorescence signals in FRET experiments is caused by direct excitation of the acceptor by the donor excitation light. This is often called "cross-talk" (Fig. 1A). The extent of cross-talk is determined by the extinction coefficient of the acceptor fluorophore at the donor excitation wavelength as well as the relative excitation light intensities for the donor and the acceptor. Cross-talk can be determined experimentally by comparing the fluorescence intensity of an acceptor-only sample excited with the donor excitation light to that of the same sample excited with the acceptor excitation light. For the CFP–YFP pair measured under typical conditions, cross-talk may account for 15–25% of the YFP peak emission (Zheng et al., 2002).

2.2. *Bleed-through*

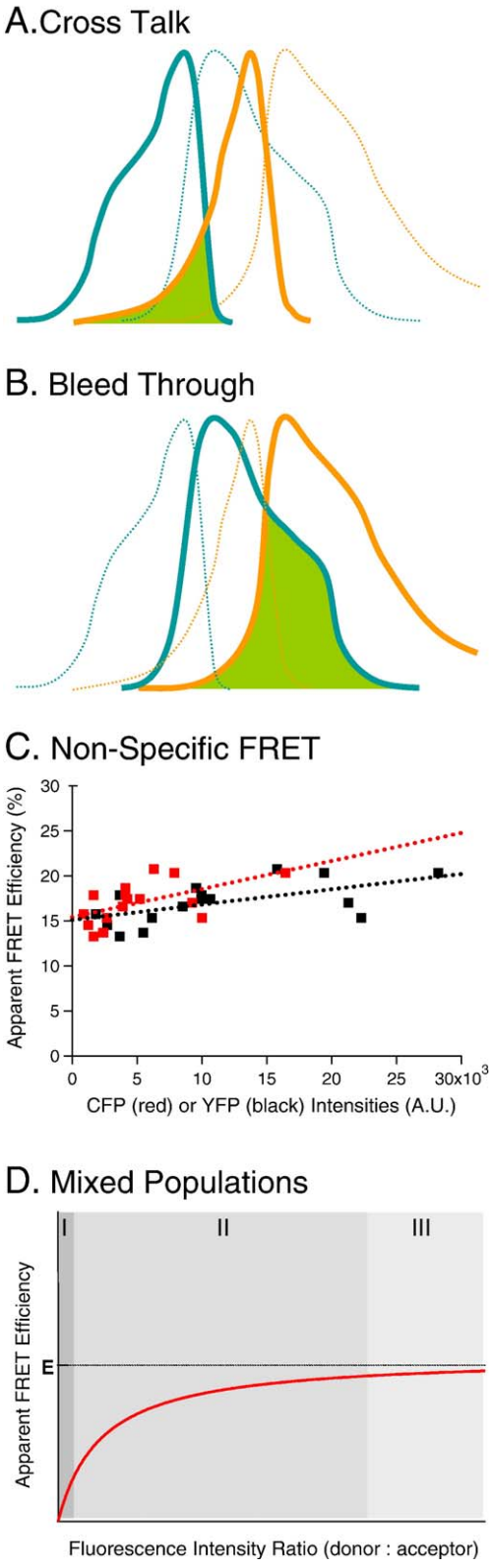
While cross-talk is caused by overlap in the excitation spectra, "bleed-through" is caused by overlap in the emission spectra. Bleed-through refers to the fluorescence emission from the donor fluorophore that is detected within the range of acceptor fluorescence (Fig. 1B). The extent of bleed-through is determined by the quantum yield of the donor fluorophore in the acceptor emission range. One can estimate the amount of bleed-through simply by examining the emission spectrum of the donor. The CFP emission at the YFP peak emission wavelength (530 nm), for example, can be as high as 50% of the CFP peak emission (measured at 480 nm).

In addition to cross-talk and bleed-through, a frequently encountered source of light contamination is the external light. This "background" light is usually at a constant level under identical experimental conditions and can be easily quantified and subtracted. For example, one can measure the intensity of a "blank region" as a way to estimate the background contamination.

2.3. *Non-specific FRET*

Intense fluorescence signal is often advantageous in FRET experiments. To achieve high fluorescence intensity, one can either increase the power of the excitation light or increase the number of fluorophores from which fluorescence emission is recorded. As the increase in excitation power has an upper limit and is always penalized by increased photobleaching, there is a practical limitation in using higher levels of excitation light. Enhancing the fluorescence signal by expressing higher densities of fluorophore-tagged molecules may introduce another problem: as the fluorophore density increases, the average distance between unassociated donor and acceptor fluorophores drops. As a result, the probability of finding a donor and an acceptor within the FRET distance increases. Existence of this non-specific FRET can be easily detected when the measured FRET values are plotted against the fluorescence intensity of either the donor or the acceptor (here, the fluorescence intensity is used to represent roughly

the fluorophore density). One such plot is given in Fig. 1C. The detectable dependence of FRET on the fluorescence intensity may suggest the occurrence of non-specific FRET. (However, transfer of the donor fluorescence to the acceptor fluorescence may also occur through a non-FRET, re-absorption mechanism, especially when the fluorophore density is high).



2.4. *Mixture of fluorophore populations*

The simple case scenario, in which all donor fluorophores and acceptor fluorophores form one-to-one pairs, rarely exists in biological experiments. More likely, fluorophores exist in several other forms, for example, uncoupled fluorophores and pairs of like fluorophores. Fluorescence emission from these fluorophores contributes to the total fluorescence intensity and reduces the apparent FRET efficiency. To illustrate the issues, one can consider a simple case in which the biological sample contains the donor–acceptor pair as well as the free donor and the free acceptor at concentrations of DA, *D*, and A, respectively. Without FRET, the donor and acceptor fluorescence intensities, F_D and F_A, respectively, would be directly proportional to the fluorophore concentrations:

$$F_D = (D + DA) \cdot S_D \quad (1)$$

$$F_A = (A + DA) \cdot S_A \quad (2)$$

in which S_D and S_A are constants that reflect the properties of the recording system and the fluorophore, such as the transfer function of the fluorescence detector, excitation light intensity, the fluorophore extinction coefficient, and the quantum yield. FRET causes a decrease in the donor intensity and an increase in the acceptor intensity. These changes in the

Fig. 1 – Potential problems in FRET experiments. A. Cross-talk. Excitation (solid curves) and emission (dotted curves) spectra of the donor (blue) and the acceptor (yellow) fluorophores are superimposed. The shaded area represents the overlap between the donor and acceptor excitation spectra. Excitation light in this range will cause cross-talk excitation of the acceptor fluorophore. B. Bleed-through. Excitation (dotted curves) and emission (solid curves) spectra are color-coded as in A. The shaded area represents donor bleed-through emissions in the acceptor range. C. Dependence of the apparent FRET efficiency on the fluorescence intensity. Concatenated CFP–YFP dimers with a 37-amino acid linker were expressed in HEK293 cells. FRET was quantified using the Spectra FRET approach, and plotted as a function of the CFP (red symbols) or the YFP (black symbols) intensity. In order to observe the intensity dependence, cells with expression levels expended over a 30-fold range were used. Dotted lines represent linear fits of the data. The positive slopes suggest that non-specific FRET between neighboring molecules may have occurred. Note that the two fitting lines intersect with the y axis at the same point (about 15% FRET efficiency), as expected from a non-specific FRET mechanism. D. The apparent FRET efficiency depends on the donor-to-acceptor fluorescence ratio. The distribution of the apparent FRET efficiency is constructed using Equation 6 with an intrinsic FRET efficiency E, which is indicated by the dotted line. Only at high donor-to-acceptor ratios (Region III), the apparent FRET efficiency approaches the true FRET efficiency.

fluorescence intensity are determined by the FRET efficiency, E, and the concentration of donor–acceptor pairs:

$$F_D^{FRET} = DA \cdot E \cdot S_D \quad (3)$$

$$F_A^{FRET} = DA \cdot E \cdot \frac{\varepsilon_D}{\varepsilon_A} \cdot S_A \quad (4)$$

in which ε_D and ε_A are molar extinction coefficients for the donor and acceptor, respectively, at the donor excitation wavelength.

There are two general ways to experimentally estimate E. FRET can be measured by the fractional decrease in the donor intensity or the fractional increase in the acceptor intensity. In the first case, the apparent FRET efficiency, E^{app}, of the system mentioned above would be:

$$E^{app} = \frac{F_D^{FRET}}{F_D} = \frac{DA}{D + DA} \cdot E \quad (5)$$

An example of this approach is the quantification of donor de-quenching after acceptor photobleaching (Miyawaki and Tsien, 2000). Alternatively, FRET can be measured using enhanced acceptor emission, in which the apparent FRET efficiency will be:

$$E^{app} = \frac{F_A^{FRET}}{F_A} \cdot \frac{\varepsilon_A}{\varepsilon_D} = \frac{DA}{A + DA} \cdot E \quad (6)$$

There are several variants of this general approach, including the "netFRET" method (Gordon et al., 1998) and the "three-cube" method (Erickson et al., 2001).

Eqs. (5) and (6) demonstrate a general problem for FRET quantification in biological systems. Regardless of whether FRET is quantified by the decrease in the donor intensity or by the increase in the acceptor intensity, if all the fluorophores are not correctly paired, the observed FRET efficiency will always be lower than the true efficiency, E. This is because free or unpaired fluorophores contribute to the total fluorescence intensity but not to the FRET signal. At equal amount of D and DA, for example, the apparent FRET efficiency according to Eq. (5) will be only one half of the true efficiency, $E^{app} = 0.5E$.

2.5. *Variable fluorophores expression levels*

The problem mentioned above is exacerbated when the expression levels of the donor and the acceptor fluorophore vary from one sample to another. Variable expressions, unfortunately, are quite common in biological experiments. The relationship between the apparent FRET efficiency and the concentrations of the donor and acceptor fluorophores is illustrated in Fig. 1D. In the schematic illustration, the apparent FRET efficiency is calculated from the enhanced acceptor emission according to Eq. (6) and is plotted against the fluorescence intensity ratio between the donor and acceptor. The figure can be divided into three regions. In region I, there are substantial amounts of uncoupled acceptor molecules that do not contribute to FRET (DA << A). As a result, the apparent FRET efficiency is much lower than the true efficiency. Region III, on the other hand, is ideal in that most of the acceptor fluorophores should be coupled to a donor (DA >> A) and the apparent efficiency approaches the true value. However, in order for the apparent efficiency to be close to the true efficiency, the donor-to-acceptor ratio needs to be quite high. This often translates into low acceptor intensities, which are hard to quantify accurately and more prone to contaminations from autofluorescence and background light. An additional problem for region III is that FRET is quantified as the change of the low acceptor intensity in the presence of the high donor intensity. The calculated FRET efficiency values thus tend to be inaccurate. Region II is where the donor intensity and the acceptor intensity are comparable and easy to measure. This region, like region I, is where the apparent efficiency depends strongly on the donor-to-acceptor ratio. In the "spectra FRET" method described later, accurate estimation of the intrinsic FRET efficiency is achieved by including data from all three regions.

It has been shown that the expression level of many proteins in *Xenopus laevis* oocytes can be well controlled by the amount of RNA injected, yielding a consistent donor-to-acceptor ratio among oocytes (Sigel and Minier, 2005; Zheng and Zagotta, 2004). Expression in cell lines by gene transfection, on the other hand, does not produce consistent fluorophore ratios. Even experiments involving "twin constructs", in which both the donor and the acceptor are encoded within the same plasmid, still yielded variable donor-to-acceptor expression ratios (see Fig. 2I). Thus, any FRET measurement using transfected cells must take into account the variable donor-to-acceptor ratio.

3. FRET quantification techniques

As discussed above, FRET may be measured by either the decrease of the donor fluorescent emission or the increase of the acceptor fluorescent emission. Many techniques have been developed for quantifying FRET that are based on measuring changes in the fluorescence intensity. While our discussion here is limited to only these intensity-based techniques, FRET can be quantified by many other attributes. Excellent discussions of various FRET quantification methods can be found in many reviews (Clegg, 1992; Selvin, 1995). A few widely used FRET approaches, based on (1) donor de-quenching, (2) enhanced acceptor emissions, and (3) comparison of donor–acceptor emission ratios, are outlined below. Emphasis is given to how each approach deals with the various potential contamination factors discussed above.

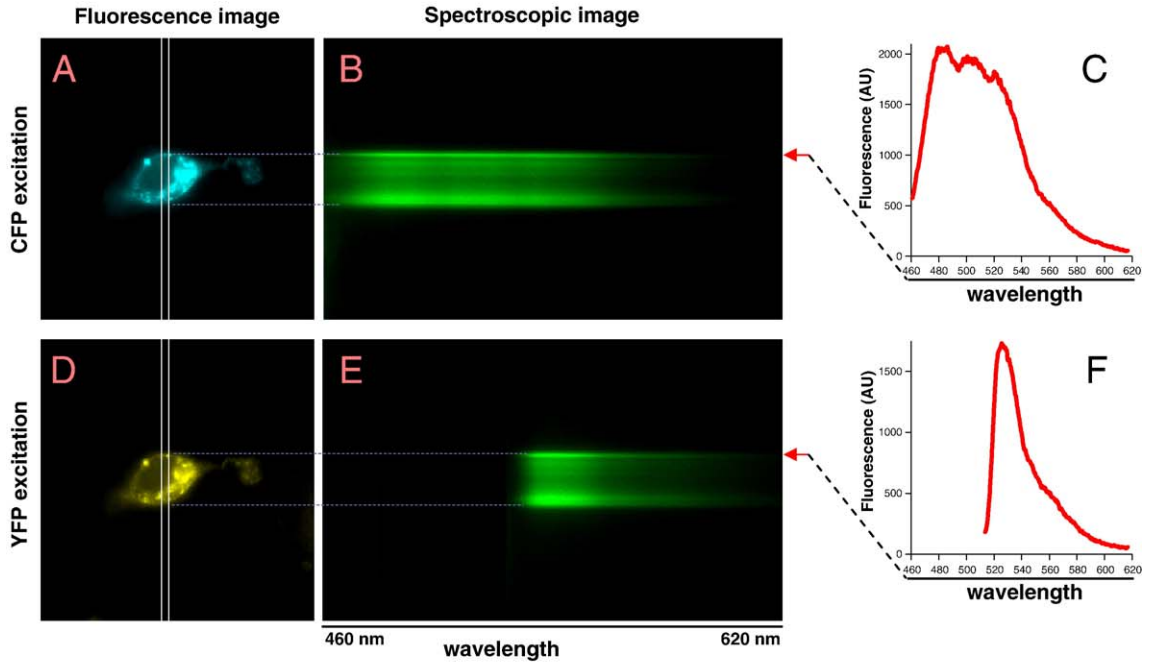
3.1. *Donor de-quenching*

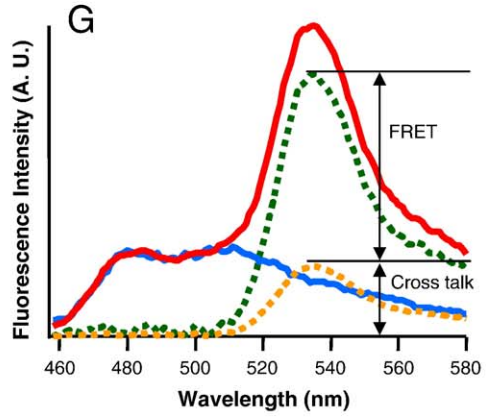
Donor de-quenching is a technique in which an increase in the fluorescence intensity of the donor is measured after the acceptor is destroyed by photobleaching. The energy transfer that was occurring between the donor and the acceptor can no longer occur in the absence of the acceptor, so FRET can be measured as an increase in the donor emission:

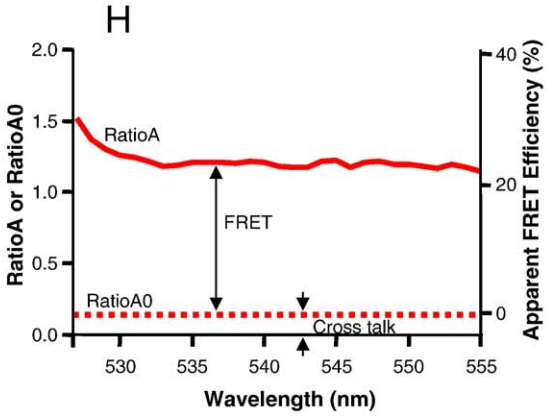
$$E = 1 - \frac{F_D}{F'_D} \quad (7)$$

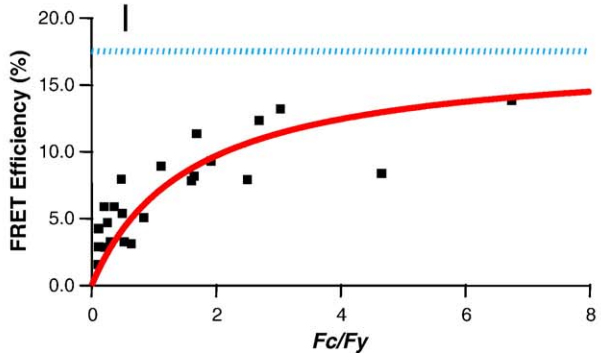
in which F_D and F'_D are the donor intensity before and after photobleaching of the acceptor, respectively. Unlike the photobleaching process intrinsic to photoexcitation, an

increase in fluorescence is generally hard to achieve unless FRET is present, making donor de-quenching an attractive approach to identify positive FRET signals (Miyawaki and Tsien, 2000). Nonetheless, measurements can only be taken once from each sample since the photobleaching process is irreversible. In addition, incomplete acceptor photobleaching can lead to underestimation of FRET (Berney and Danuser, 2003). As donor de-quenching measures changes in donor fluorescence, it is less affected by either cross-talk (the excitation light used to bleach the acceptor usually does not bleach the donor efficiently) or bleed-through (donor emission can be measured at a wavelength range away from the acceptor emission). Mixtures of fluorophore populations will cause underestimation of the FRET efficiency (Tron et al., 1984). To better observe FRET, cells with low CFP-to-YFP ratios should be used. Under such conditions, most of the donor (CFP) is coupled to an acceptor (YFP). Ideally, the relationship between the apparent FRET efficiency and the expression level









of the fluorophores should be used to determine the true FRET efficiency.

3.2. *Acceptor enhanced emission*

Another technique for calculating FRET is to measure the enhancement of acceptor emission in the presence of FRET. This is also known as sensitized emissions. Instead of measuring the increase in fluorescence of the donor, as with donor de-quenching, an increase in fluorescence of the acceptor is measured when the donor fluorophore transfers energy to the acceptor fluorophore during the occurrence of FRET:

$$E = \frac{\varepsilon_A}{\varepsilon_D}\left(\frac{F'_A}{F_A} - 1\right) \quad (8)$$

in which F_A and F'_A are the acceptor intensity in the absence and in the presence of the donor, respectively. There are several different methods designed to measure FRET in this way, such as "netFRET" (Gordon et al., 1998), "three-cube FRET" (Erickson et al., 2001), as well as the "Spectra FRET" method discussed below. Common to these methods, light contaminations due to cross-talk and bleed-through are estimated separately from acceptor-only and donor-only samples using the same equipment setting for FRET measurements. These contaminating fluorescent emissions are subsequently subtracted from the total fluorescent signal measured from the donor-plus-acceptor samples. In netFRET (Gordon et al., 1998) and its modified form (Xia and Liu, 2001), FRET is assumed to arise from the bimolecular interaction with a certain equilibrium constant in the form $D + A \leftrightarrow DA$. Algorithms are developed to correct for variable protein expression levels with a ratio that compares the concentration of donor–acceptor molecules (donor-tagged molecules associated with acceptor-tagged molecules) to the concentration of free donors and free acceptors. A general approach is applied in the spectra FRET method, for which no specific form of interaction is assumed. The mixture of fluorophore populations and variable donor-to-acceptor ratios is dealt with by analyzing the dependence of the apparent FRET to the donor-to-acceptor ratio (see below).

3.3. *Donor–acceptor emission peak ratio*

A technique for rapid detection of FRET changes is to compare donor–acceptor emission peak ratios with and without FRET (Miyawaki and Tsien, 2000). Since FRET is the transfer of energy from a donor to an acceptor, the fluorescence intensity of the donor will decrease and the fluorescence intensity of the acceptor will increase. The ratio between the acceptor intensity over the donor intensity before FRET will be smaller than the ratio after FRET. As FRET changes the numerator and the denominator in opposite directions, the ratio measures both the decrease in the donor emission and the increase in the acceptor emission at the same time and hence is very sensitive to changes in FRET.

$$\text{FRET\_index} = \frac{F_A}{F_D} \quad (9)$$

The measurement of fluorescence ratio is not as complicated as the techniques previously described and can be quickly determined at the peak emission of each fluorophore. It is thus a good method for measuring changes in distance. As it is intended to be a quick test for FRET changes, contaminations such as cross-talk and bleed-through are normally not corrected. The FRET signal measured from the donor-to-acceptor ratio is also affected by mixed fluorophore populations and variable expression levels, which should be considered in choosing experimental conditions.

4. Spectra FRET: a case study

"Spectra FRET" is a spectroscopy-based approach to accurately quantify FRET efficiency in live cells. The approach is based on the same framework outlined by Clegg (1992). It measures FRET as the enhanced acceptor emission. The method takes advantage of the easy accessibility of two types of equipment, the spectrograph and the CCD camera, both of which are increasingly popular in biomedical research and can be easily set up for epifluorescence microscopy. The recording and data analysis procedures are relatively simple and can be automated with compatible equipment configuration.

The recording system for Spectra FRET is built around a regular epifluorescence microscope (Zheng, 2006). The basic requirement for the microscope is a shutter-controlled excitation light source, two filter cubes (for donor and acceptor

Fig. 2 – Spectra FRET. A single HEK 293 cell expressing CFP- and YFP-tagged CLC_0 chloride channel subunits was observed with CFP (A) or YFP (D) excitation. Spectroscopic images from the region under the slit (indicated by rectangles) were recorded with each excitation (B and E). In the spectroscopic images, the y axis represents the position of the cell, the x axis represents the wavelength. The fluorescence intensity values along a horizontal line constitute the emission spectrum; those measured from the upper membrane region (red arrows) are shown in C and F. Notice several bright strips in B and E between the membrane signals which represent fluorescence from intracellular sources. G. Removal of the bleed-through and cross-talk contaminations. A CFY emission spectrum (blue curve) is normalized to the CFP peak of an emission spectrum from a cell expressing CFP–YFP tandem dimers (red curve). The extracted YFP spectrum (green curve, which represents the difference between the red curve and the blue curve) contains both the FRET emission and the cross-talk emission (yellow curve). H. The difference between RatioA (solid line) and RatioA0 (dotted line) indicates the existence of the FRET signal. Note that RatioA0 corresponds to the zero FRET efficiency level (right axis). I. An example of the Spectra FRET measurement. Each symbol represents the measurement from a single HEK293 cell transfected with plasmids containing cDNAs encoding both CFP- and YFP-tagged CLC_0 subunits in a modified pIRES2-EGFP vector (Clonetech). The red curve represents a fit of the data set to a model for the dependence of the apparent FRET efficiency on the fluorescence intensity ratio. The dotted blue line represents the expected FRET efficiency.

excitation, respectively), and a single output port to which the spectrograph is attached. The spectrograph should have an input slit that has adjustable width and can be easily moved in and out of the light path. The grating inside the spectrograph is normally installed on a rotational turret, allowing easy selection of the recording wavelength range. The spectrograph is attached to the output port of the microscope via a compatible mounting mechanism. The CCD camera is in turn attached to the output port of the spectrograph. Because the spectral resolution is largely determined by the spectrograph, an HQ quality CCD camera should be sufficient for this type of measurements.

To record FRET, a fluorescent cell is brought into the field-of-view of the camera. This is done with the input slit of the spectrograph moved out of the light path, and the grating set at a small angle at which it is equivalent to a mirror projecting the cell image to the camera (Figs. 2A and D). The slit is then moved into the light path to cover the region of the cell from which fluorescence signals are to be measured. The grating is rotated to the desired angle for light of a selected wavelength range to be projected to the camera. Two spectroscopic images are taken using the donor and the acceptor excitation filter cube, respectively (Figs. 2B and E). A fluorescence emission spectrum is constructed from each image using the fluorescence intensity values along a horizontal line whose position corresponds to the part of the cell to be measured (Figs. 2C and 2F). In the example shown in Fig. 2, the bright signal from the cell membrane (red arrows) is quantified by a horizontal line along the bright strip. An advantage of the Spectra FRET method is that fluorescence signals from different parts of the cell can be conveniently identified and selected. For Spectra FRET, the same procedure outlined above is carried out with control cells expressing only donor fluorophores or only acceptor fluorophores, as well as experimental cells expressing donor and acceptor fluorophores.

How is FRET quantified and corrected for various contaminations using the spectroscopic data? First, the contribution from donor bleed-through is removed from the donor–acceptor emission spectrum (Fig. 2G, red curve) by subtracting from it a donor spectrum that has been normalized to the peak fluorescence in the donor emission region (Fig. 2G, blue curve). This results in an extracted acceptor spectrum that is free of donor contamination (Fig. 2G, green dotted curve). Next, the contribution from cross-talk is removed from this extracted spectrum. The amount of cross-talk can be estimated from the two spectra collected from acceptor-only cells using the donor and the acceptor excitation light, respectively. The ratio between these two spectra, which is termed RatioA0, represents the efficiency of cross-talk that is specific to the donor–acceptor pair and the recording system. Multiplying RatioA0 to the total acceptor emission from a donor–acceptor sample in respond to the acceptor excitation light (not shown) yields the amount of the cross-talk signal (Fig. 2G, yellow dotted curve). The difference between the extracted acceptor spectrum and the cross-talk is the FRET signal. In Spectra FRET, the ratio between the extracted acceptor spectrum and the total acceptor emission spectrum with the acceptor excitation is calculated, which is termed RatioA, and compared to RatioA0 (Fig. 2H). The apparent FRET efficiency from an individual cell, E^{app}, can be calculated as

$$E^{app} = \left(\frac{\text{RatioA}}{\text{RatioA0}} - 1\right)\frac{\varepsilon_A}{\varepsilon_D} \quad (10)$$

As both bleed-through and cross-talk are subtracted from the whole wavelength range, the method is more tolerant to low signal-to-noise ratios and more reliable. In addition, any contamination signal (e.g., autofluorescence and background light) can be easily identified as they usually have distinct spectral properties from those of the fluorophores.

As one may expect, errors in FRET estimation due to mixed fluorophore populations and variable expression levels cannot be corrected from measurement of individual cells. In the Spectra FRET method, expression levels of the donor and the acceptor are quantified as the fluorescence intensities at the peak of the respective emission spectrum. The ratio between the donor peak intensity (Fc) and the acceptor peak intensity (Fy) is calculated as Fc/Fy. The apparent FRET efficiency measured from each cell is plotted against the Fc/Fy value of the same cell (Fig. 2I). (Note that Fc/Fy is not the ratio of the fluorophore densities per se. FRET causes a decrease in the donor intensity and an increase in the acceptor intensity; how much the fluorescence intensities change depends on the FRET efficiency which, in turn, depends on the fluorophore densities!) As expected from Eq. (6) and Fig. 1D, at low Fc/Fy, the apparent FRET efficiency is also low. But as Fc/Fy increases, so does the apparent FRET efficiency. At high Fc/Fy values, the apparent FRET efficiency eventually plateaus off at the actual FRET efficiency. While the shape of the distribution depends on the biological system from which FRET is measured, the plateau of the distribution should represent the true FRET efficiency regardless of the system. Cells with high Fc/Fy are needed to gain an accurate estimate of the true FRET efficiency.

The Spectra FRET technique has been recently applied to both membrane proteins and cytosolic proteins expressed in cultured cells. A step-by-step procedure can be found in a recent article (Zheng, 2006). The use of a spectrograph has many advantages, such as accurate subtraction of bleed-through and cross-talk light contaminants, a built-in control for system linearity (both RatioA and RatioA0 are expected to be wavelength-independent, as shown in Fig. 2H), easy separation of the fluorescence signal from the cell membrane versus those from cytosolic structures. As with other FRET quantification methods based on enhanced acceptor emission, measurements taken in spectra FRET are non-destructive.

5. Conclusions

GFP-based FRET provides a highly sensitive reporter for intermolecular distances in live cells, which is not easily matched by other techniques. The popularity of this optical technique already prompts development of real-time assays of protein–protein interactions in live cells with astonishing sensitivity and reliability. It is almost guaranteed that new ways of utilizing FRET will be developed. Research into new fluorescent molecules continues to yield improved donor–acceptor pairs. Improvements in the optical technology,

including the manufacture of new light sources and filters, enhancements of spectroscopic equipment for biological studies, and development of more sensitive light detectors, etc., continue to open up new possibilities for better FRET techniques. It is likely that some of the current problems associated with FRET measurement may be attenuated or even eliminated in the near future, allowing faster and more sensitive detection of dynamic biological processes in live cells. Until then, we must fully understand the potential technical issues affecting FRET measurements and make appropriate corrections. Used properly, FRET can serve as an extremely useful "molecular ruler".

Acknowledgments

We are grateful to Dr. Edward Lachica (Olympus USA) for support of the Spectra FRET imaging system, Dr. Yuechueng Liu (University of Oklahoma) and Dr. Tsung-Yu Chen (UC Davis) for the generous gifts of CFP–YFP fusion constructs and fluorescent CLC channel constructs, respectively, and Tom Hill (UC Davis) for critical reading of the manuscript.

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