Fluorescence resonance energy transfer microscopy: a mini review

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Abstract. Fluorescence resonance energy transfer (FRET) microscopy is a better method than the x-ray diffraction, nuclear magnetic resonance, or electron microscopy for studying the structure and localization of proteins under physiological conditions. In this paper, we describe four different light microscopy techniques to visualize the interactions of the transcription factor CAATT/enhancer binding protein alpha (C/EBPα) in living pituitary cells. In wide-field, confocal, and two-photon microscopy the FRET image provides two-dimensional spatial distribution of steady-state protein–protein interactions. The two-photon imaging technique provides a better FRET signal (less bleedthrough and photobleaching) compared to the other two techniques. This information, although valuable, falls short of revealing transient interactions of proteins in real time. The fluorescence lifetime methods allow us to monitor FRET signals at the moment of the protein interactions at a resolution on the order of subnanoseconds, providing high temporal, as well as spatial resolution. This paper will provide a brief review of the above-mentioned FRET techniques. © *2001 Society of Photo-Optical Instrumentation Engineers.*
[DOI: 10.1117/1.1383063]

1 Introduction

The physics and chemistry of fluorescence resonance energy transfer (FRET) have been well studied theoretically and experimentally for many years,[1–10] but only with recent technical advances has it become feasible to apply FRET to biomedical research.[11–24] The measured energy transfer signal in the spectrofluorometer configuration does not provide two-dimensional distribution of protein associations. The technology advancements in light microscopy imaging and development of different fluorophores have generated a lot of interest among biologists to obtain spatial and temporal distribution of protein associations in living cells.[9–12,18–22]

FRET is a quantum mechanical process in which direct radiationless transfer of energy from a donor (D) to an acceptor (A) molecule occurs. For FRET to occur between a donor and acceptor, the following four conditions must be fulfilled.[1–3] First, the donor emission spectrum must significantly overlap the absorption spectrum of the acceptor. Second, the distance between the donor and acceptor fluorophores must fall within the range of $\sim 1 - \sim 10$ nm. Third, the donor emission dipole moment, the acceptor absorption dipole moment, and their separation vectors must be in favorable mutual orientation. Finally, the donor should have a high quantum yield.

The main application of FRET is as a "spectroscopic ruler," based on Förster's basic rate equation for a donor and acceptor pair at a distance r from each other.[1,2] The Förster distance is the distance between the donor and the acceptor at

which half the excitation energy of the donor is transferred to the acceptor while the other half is dissipated by all other processes, including light emission. The efficiency drops if the distance between D and A molecules changes from the Förster distance. Finally, it is important to understand the physics of FRET before implementing FRET techniques for biological applications.[3] In this mini review we will discuss briefly four FRET microscopy techniques used for visualizing protein associations in a single living cell.

2 Fluorophore Pairs for FRET Imaging

To localize the proteins or to quantitate the distance between the proteins one would require two fluorophore molecules to attach to the proteins under investigation. Conventional (FITC and Cy3) or different mutant forms of green fluorescent proteins (GFPs) could be used as fluorophore pairs.[13,14,24] There are a number of conventional and GFP flurophores used for FRET including FITC–Rhodamine, Alexa488–Cy3, Cy3–Cy5, BFP–GFP, BFP–Red Fluorescent Protein (RFP), BFP–YFP, Cyan Fluorescent Protein (CFP)–YFP, and CFP–RFP. Many more can be found in the literature with their respective spectra and filter combinations.[22] It is important to note that one should have a good expression (or labeling) level of proteins with the selected fluorophore. It is also important to use better optics, a high quantum efficiency charge coupled device camera (for real time $2p$-FRET), or photomultiplier tubes to acquire the FRET images.

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It is important to carefully select filter combinations that reduce the spectral bleedthrough background to improve the signal-to-noise ratio for the FRET signals. These filters are commercially available from either Chroma Technology Corporation (www.chroma.com) or Omega Optical, Inc. (www.omegafilters.com). For the studies described here, the same dichroic mirror was used to acquire both the donor and acceptor images. This is important because there can be small differences in the mechanical position of the dichroic mirror from one filter cube to another, and the use of different cubes can introduce artifacts in the processed FRET images.

3 Microscopic Cellular Assays

FRET efficiency increases with an increase in the percentage of donor and acceptor overlap spectrum and correspondingly the bleedthrough signal increases in the FRET channel. The efficiency of FRET could also be improved by optimizing the concentration of the fluorophore used for protein labeling. One should reduce or optimize the fluorophore concentration to reduce the self-interactions, such as donor–donor or acceptor–acceptor interactions. For example, the FRET signal should be measured by keeping the donor at an optimal concentration level by varying the concentration of the acceptor. The FRET signal increases with the increase in acceptor concentration. By keeping the selected acceptor concentration one could repeat to find the donor optimal concentration. These optimal concentrations could be used for labeling the proteins for FRET imaging. It is also important to note that the orientation of the donor and acceptor dipole moments play key roles for FRET to occur. So, to increase the probability for FRET to occur one should consider having a higher concentration of the acceptor than the donor. Sometimes this may not be a strict rule for various proteins under investigation. If FRET occurs the acceptor signal increases (sensitized signal) and the donor signal decreases. After removing the background and autofluorescence signal from the donor and acceptor, the ratio of A/D would provide the resultant FRET image.

4 Problems Associated with FRET Images

Fluorescence microscopy is an important tool for two- or three-dimensional visualization of protein–protein interactions in a living specimen. This is a noninvasive technique that allows us to look inside cells and tissues with more detail by using fluorophore-labeling techniques. Labeling these proteins with different fluorophore molecules could allow monitoring of the protein structure and composition. Each fluorophore molecule used for FRET imaging has a characteristic absorption and emission spectrum that should be considered for characterizing the FRET signal. Moreover, there are other problems encountered in FRET microscopy imaging such as autofluorescence, detector, and optical noise, photobleaching, and spectral bleedthrough signal. It is important to implement the correction in order to quantitate the FRET signal or in calculating the distance between the two proteins.[22,25]

The first condition for energy transfer dictates that the efficiency of FRET will improve with increased overlap of the donor fluorophore emission spectra with the absorption spectra for the acceptor fluorophore (see Figure 1). The problem we encounter, however, is that if the spectral overlap of donor and acceptor fluorophores is increased, there is increased
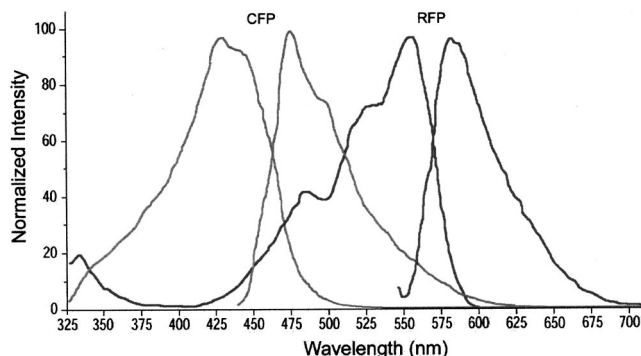

Fig. 1 Illustration of excitation and emission spectral overlap of donor (Cyan Fluorescent Protein, CFP) and acceptor (Red Fluorescent Protein, RFP or DsRed).

spectral bleedthrough signal detected in the acceptor channel. The spectral bleedthrough results from a combination of both the acceptor fluorophore excited by the donor excitation light, plus the donor emission detected in the acceptor channel. The result is a high and variable background signal from which a weak FRET-based acceptor emission must be extracted. In addition to removing the spectral bleedthrough, it is important to implement correction for the concentration or expression level of the fluorophore labeled to the proteins. This would improve the accuracy in the calculation of the distance between the proteins involved in energy transfer.

5 Different FRET Microscopic Techniques

5.1 Digitized Video FRET (DVFRET) Microscopy

Any fluorescence microscopy (inverted or upright) can be converted to a DVFRET microscopy. There are number of papers listed in the literature for various protein studies using the DVFRET system.[4,9,12,13,16–20] The important aspects of the FRET imaging system are using good sensitivity detectors, filters, and objective lenses. High sensitivity detectors would help to reduce the data acquisition time and one could use a narrow band pass filter for excitation and emission to reduce the spectral bleedthrough noise. It is advisable to use a single dichroic to acquire the D and A images using the donor excitation wavelength in the double-labeled cells. This could be achieved by using excitation and emission filter wheels in the microscope system. This kind of option would help to reduce any spatial shift of D and A images, since the FRET image is obtained by pixel-by-pixel ratioing (A/D) of D and A images. But some of the signal in the FRET image results from the spectral bleedthrough due to the overlap spectral property of D and A fluorophores. To obtain the optimal FRET signal, it is important to correct for the bleedthrough (or crosstalk) signal in the acceptor channel. There are a couple of papers that attempted to correct the bleedthrough signal.[16,25,26] Most of the correction schemes were implemented by collecting a series of images of donor alone, acceptor alone, and double labeled cells, at both donor and acceptor excitation wavelengths.

5.2 Confocal FRET (CFRET) Microscopy

A major limitation to the DVFRET microscopy technique is that the emission signals originating from above and below
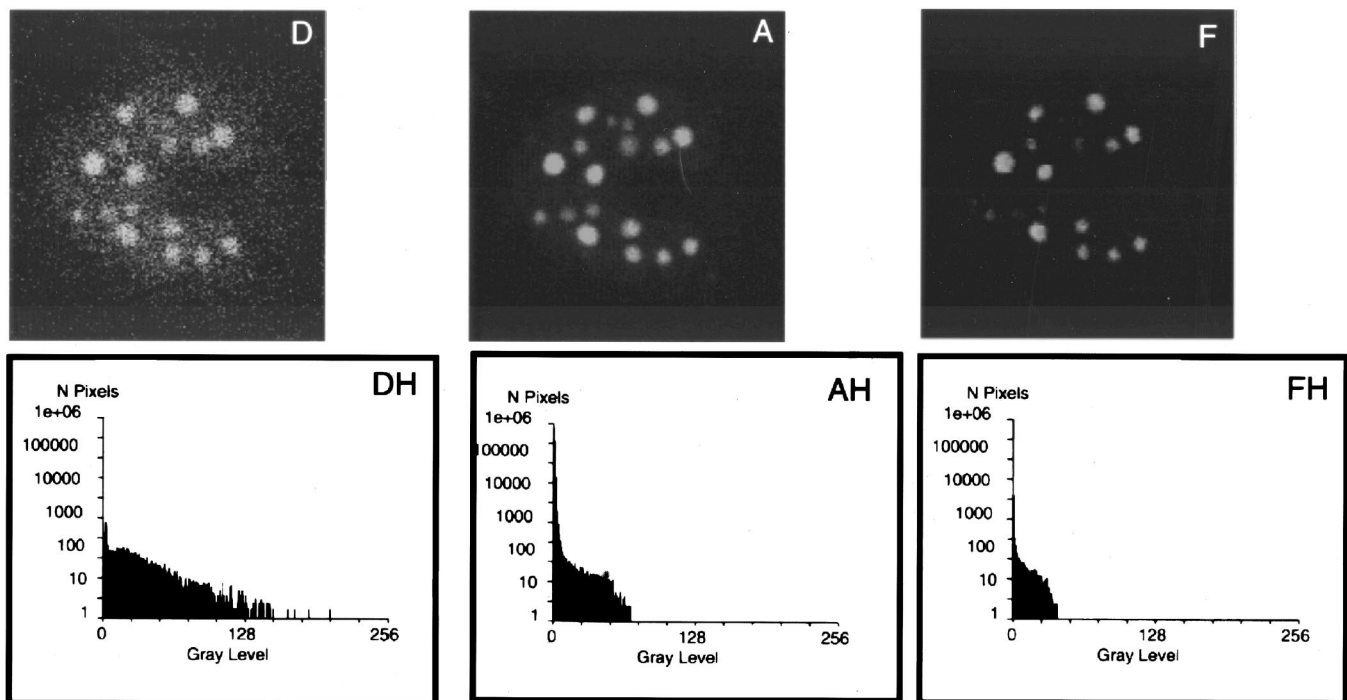
Fig. 2 The C-FRET systems were used for CFP–RFP pair to visualize the C/EBPα proteins in a single cell using Nikon PCM2000 laser scanning confocal microscopy. The excitation wavelength used to excite the donor molecule (CFP–C/EBPα) was 457 nm from an argon ion laser. A HeNe green laser line (543 nm) was used to acquire the acceptor (RFP–C/EBPα) image. Using the excitation wavelength 457 nm both the donor and acceptor images were acquired from the cells expressed both the proteins (CFP–RFP–C/EBPα). The energy transfer signals were processed from these images using the software by removing the entire noise and bleedthrough signals as shown in (F) (CFPem-480/30; RFPem-610/60). The respective histograms below the figures clearly demonstrate the noise signal in the acceptor channel (AH) and the processed true FRET signals (FH).

the focal plane contribute to out-of-focus signal that reduces the contrast and seriously degrades the image. Digital deconvolution microscopy in the DVFRET system would help to localize the proteins at different optical sections, but this requires all intensive computational process to remove the out-of-focus information from the optical sectioned FRET images.[12,15] Moreover, optical sectioning in the DVFRET system may be good for the dimerization and not for the dynamic FRET imaging.

Laser scanning confocal FRET (CFRET) microscopy can overcome this limitation owing to its capability of rejecting signals from outside the focal plane and acquiring the signal in real time. This capability provides a significant improvement in lateral resolution and allows the use of serial optical sectioning of the living specimen.[27,28] A disadvantage of this technique is that the wavelengths available for excitation of different fluorophore pairs are limited to standard laser lines. If a confocal microscope is equipped with ultraviolet (UV) laser and UV optics, it is possible to use the BFP fluorophore. As mentioned above, however, this fluorophore is sensitive to photobleaching, and this is exacerbated by the intense laser source. Standard laser lines do allow CFRET to be used for many fluorophore combinations including CFP–RFP, GFP–rhodamine or Cy3, FITC, or Alexa488–Cy3,[22] or Rhodamine and Cy3–Cy5.[20]

5.2.1 *Image Acquisition and Processing*

The cDNA sequence for transcription factor C/EBPα was fused in frame with the sequence encoding either the CFP or RFP color variants. For transfection, pituitary GHFT1-5 cells were harvested and transfected with 3–10 mg of purified expression plasmid encoding the CFP or RFP fusion proteins by electroporation.[18] For imaging, a cover slip with a monolayer of transfected cells was placed in a specially designed chamber for the microscope stage.[29] Cells expressing both the CFP and RFP tagged C/EBPα protein were identified by 440 and 535 nm excitation using an arc lamp source coupled to the Nikon TE200 epifluorescence microscope, respectively. A 457 nm filter was placed in the argon–ion laser filter slot and then the laser was driven at high current to obtain a few microwatts of power at the specimen plane to excite the donor molecule (CFP) to obtain donor and acceptor (RFP) images. Then, the processing software was used to remove the spectral bleedthrough and other noises to obtain true FRET signal as shown in Figure 2. The respective histograms clearly demonstrate the noise involved in the acceptor channel [Figure 2(AH)] and the gray level considerably reduced in the true FRET signal histogram [Figure 2(FH)].

5.3 Two-Photon FRET (TFRET) Microscopy

As mentioned above, the advantage of CFRET over DVFRET lies in the ability to reject the out-of-focus signal that originates from outside the focal plane. A significant improvement over CFRET could be achieved by eliminating the out-of-focus signal altogether by limiting excitation to only the fluorophore at the focal plane. This is precisely what two-photon excitation microscopy does. Two-photon absorption was theoretically predicted by Goppert-Mayer[30] in 1931, and it was

experimentally observed for the first time in 1961 by using a ruby laser as the light source.[31] Denk[32] and others[24] experimentally demonstrated the original idea of two-photon fluorescence scanning microscopy in biological samples. The rate of two-photon excitation is proportional to the square of the instantaneous intensity. With extremely high instantaneous intensity, two photons of light (at approximately twice the wavelength normally required to excite a fluorophore) can simultaneously occupy the fluorophore absorption cross section. The energies of these photons are combined to excite the fluorophore. Because two-photon excitation occurs only in the focal volume, the detected emission signal is exclusively infocus light. Further, since two-photon excitation uses longer wavelength light, it is less damaging to living cells, thus limiting problems associated with fluorophore photobleaching and photodamage, as well as intrinsic fluorescence of cellular components.[33,24]

Cells expressing the fluorophore tagged protein should be identified by respective excitation wavelengths using an arc lamp light source coupled to the microscope. For $2p$ excitation, the Ti:sapphire laser wavelength should be tuned from 900 to 700 nm, in order to see no (or minimum) signal from the acceptor alone labeled proteins. The excitation wavelength should be selected in such a way that no (or minimum) acceptor signal should be visualized in the FRET channel. For example, for the BFP and RFP pair, we found that no RFP signal was seen at 740 nm.[21,22] The BFP tagged protein molecule was excited with an infrared wavelength from the Ti: sapphire laser, which caused a considerable amount of signal at 740 nm. 740 nm was used as an excitation wavelength to acquire the donor and acceptor images. Before using the BFP–RFP combination, we used the BFP alone to monitor the bleedthrough signal in the RFP channel (FRET or acceptor channel). The acquired image should have backgrounds subtracted and bleedthrough corrected to obtain a $2p$-FRET image.

5.4 Lifetime Imaging FRET (LFRET) Microscopy

Each of the fluorescence microscopy techniques described above use intensity measurements to reveal fluorophore concentration and distribution in the cell. Recent advances in camera sensitivities and resolutions have improved the capability of these techniques to detect dynamic cellular events. Unfortunately, even with these improvements in technology, currently available fluorescence microscopic techniques do not have high-speed (<second) time resolution to fully characterize the organization and dynamics of complex cellular structures. In contrast, the time-resolved fluorescence microscopic technique allows the measurement of dynamic events at very high temporal resolution. Moreover, the spectral bleedthrough or crosstalk is a problem to recognize whether one is observing the sensitized emission or the bleedthrough signals. Also, the strength of the signal depends on the excitation intensity and the fluorophore concentration. In contrast, lifetime measurements are independent of excitation intensity or fluorophore concentration. To date, most measurements of fluorescence lifetimes have been performed in solution or in cells in suspension.[3] Fluorescence lifetime imaging microscopy was developed to overcome this limitation to allow fluorescence lifetime measurements to be acquired from living cells in culture.[34–40]

The combination of lifetime and FRET (LFRET) will provide high spatial (nanometer) and temporal (nanoseconds) resolution when compared to steady state FRET imaging. Importantly, spectral bleedthrough is not an issue in LFRET imaging because only the donor fluorophore lifetime is measured. The presence of acceptor molecules within the local environment of the donor that permit energy transfer will influence the fluorescence lifetime of the donor. By measuring the donor lifetime in the presence and the absence of acceptor one can accurately calculate the distance between the donor- and acceptor-labeled proteins.

6 Conclusion

In this paper we described four FRET microscopic techniques (DVFRET, CFRET, TFRET, and LFRET) to localize the proteins in a single living cell. As an example the CFRET results show that FRET imaging of GFP-fusion proteins can furnish a wealth of information on the physical interactions between protein partners. As explained it is important to remove the spectral bleedthrough and other noises to obtain the true sensitized emission. Moreover, the LFRET would have been an alternative technique to reduce the noise in calculating the distance between donor and acceptor molecules.

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