Dynamic Imaging Using Fluorescence Resonance Energy Transfer

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Recently, fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) have been the buzzwords for monitoring protein-protein interactions in living cells. These methodologies provide higher temporal (< 1 ns) and spatial resolution (1–10 nm) than near field microscopy (50 nm). FRET microscopy is an excellent method for imaging the structure and localization of proteins under physiological conditions, since other methods, including X-ray diffraction and electron microscopy, require the specimens to be fixed.

WHAT IS FRET?

FRET is a quantum mechanical process in which radiationless transfer of energy from a donor to an acceptor molecule occurs (Figure 1). For FRET to occur between a donor and an acceptor, the following four conditions must be fulfilled. 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 between 1 and 10 nm. Third, the donor emission dipole moment, the acceptor absorption dipole moment, and their separation vectors must be in a favorable mutual orientation. Finally, the emission of the donor should have a high quantum yield (7).

The main application of FRET is to provide spatial information between two proteins at a higher resolution than can be achieved using conventional microscopy. The technique has been called a “spectroscopic ruler”, based on Förster’s basic rate equation for a donor and acceptor pair at a distance “ r ” from each other (4,10). Many contemporary biological questions can be addressed using FRET. These include the extent of co-localization of proteins, clustering of membrane trafficking proteins, dimerization of proteins, transcription mechanisms, transduction pathways, molecular motor motions, and protein folding (9,11).

INSTRUMENTATION

Digitized video FRET (DVFRET) imaging provides 2-D spatial distribution of steady-state protein-protein interactions (8). An advantage of DVFRET is that it allows the use of any excitation wavelength using interference filters for various fluorophore pairs. The disadvantage of DVFRET is that out-of-focus information is present in the FRET signal of the protein under investigation. In confocal FRET (C-FRET) and two-photon excitation FRET (2p-FRET) microscopy, the out-of-focus information is not imaged so that only the FRET signal at the se-

lected focal plane is collected (9).

In a DVFRET system, any fluorophore pair combination can be used with the appropriate filter sets, but, in the case of confocal systems, the available laser lines on any confocal imaging system limit the specific fluorophore pairs that can be used. Theoretically, in a multiphoton system, any fluorophore combination could be used if the Ti:sapphire laser is tunable from 700 and 1000 nm. However, it is reasonably difficult to tune lasers for both donor and acceptor excitation wavelengths without a laser expert. Consider the example of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) as FRET pairs. The two-photon excitation wavelength for CFP is around 820 nm and that for YFP is around 940 nm. Hence, the laser has to be tuned about 120 nm.

There are additional advantages of the multiple-photon approach. The excitation is in one single focal plane, unlike confocal microscopy where the excitation occurs throughout the path of the beam. The depth of focus is increased as compared with confocal imaging, and deep tissue imaging is a practical option. In addition, since the excitation is in the infrared region, significantly less damage occurs to living cells than in confocal imaging.

EXPERIMENTAL METHODOLOGY

To process FRET data using most software packages, a set of images should be collected. These images are collected from three different preparations of the same specimen. These are single-labeled with the donor fluorophore, single-labeled with the acceptor fluorophore, and double-labeled with both donor and acceptor fluorophores. All three specimens are imaged using both donor and acceptor excitation wavelengths, and should the occurrence of FRET need to be verified, a negative control could be included.

In any imaging experiment, whether it is DVFRET, C-FRET, or 2p-FRET, one has to optimize the fluorophore concentration of donor and acceptor molecules. In general, the acceptor concentration should be greater than that of the donor. This is required to increase the probability of dipole moment orientation of donor and acceptor molecules. It is important to perform a negative control to verify the occurrence of FRET.

IMAGE LIST FOR A GENERALIZED FRET EXPERIMENT

Single-Label Donor Slide

Select a well-expressed cell by using the bright field and arc lamp illumination. Adjust the gain and the black level of the donor and acceptor channel and acquire an image. The acceptor channel image is the spectral bleed-through from the donor emission signal using donor excitation wavelength.

Single-Label Acceptor Slide

The acceptor channel image is the acceptor bleed-through when excited by the donor excitation wavelength.

Double-Labeled Donor and Acceptor Slide

The gain and the black level adjustment for each channel should be kept constant for all other image acquisitions (acceptor alone and D+A). Therefore, it is very important to spend time in setting up the appropriate gain and black levels for image acquisition. FRET signal is obtained along with both donor and acceptor bleed-through signals in the acceptor channel with the donor excitation wavelength.

BLEED-THROUGH CORRECTION

Donor and acceptor spectral bleed-through is an important issue in contaminating the FRET signal in the acceptor channel. Acceptor bleed-through is the direct excitation by donor wavelength and its emission in the acceptor channel. There have been a number of attempts to remove the spectral bleed-through signal from the acquired images (5,12,13). It is also equally important to correct for fluorophore expression level variation (3). For example, Figure 2 demonstrates the requirement of correction algorithm to obtain true FRET signal. To extract the FRET signal, one could use the ratio imaging of donor intensity to acceptor intensity (A/D), and this gives a qualitative measure of FRET (8).

Different techniques can be used to correct the bleed-through signals and give a more accurate estimation of FRET. Acceptor photo-bleaching is a prominent method wherein the acceptor is bleached completely in a double-labeled cell and the increase in the donor signals is observed. Based on post-bleach and pre-bleach intensities of the donor, the efficiency of FRET is calculated (6). The main problem with this method is that it is not suitable for live cells where prolonged irradiation of the double-labeled cells can cause damage. Moreover, in the process of photo-bleaching, both the acceptor and donor molecules may also be bleached to some extent when the donor excitation spectra overlap the acceptor excitation spectra (as in the case of BFP and dsRED). In addition, if the acceptor is more photostable, then it requires a long time to bleach and the focal plane of interest may shift and cell viability may be compromised. There are other methods based on different mathematical calculations (3,5,12,13) whereby the process itself does not involve any damage to the cells, but there are issues in the way that a specific method corrects for bleed-through.

All of the methods described above are based on intensity; the efficiency of FRET is determined by the intensity observed in the FRET channel. However, it can be difficult to analyze quantitatively the FRET signal because of bleed-through problems.

LIFETIME FRET IMAGING

An alternative technique for localizing and quantitating the FRET signal is lifetime FRET imaging. Here the need for any bleed-through correction is eliminated. For FRET applications using the lifetime imaging technique, only the change in the lifetime of the donor molecules in the presence and ab-

Figure 1. FRET. Illustration of Jablonski's energy level diagram to demonstrate the occurrence of FRET (7). S0 and S1 represent the ground and first singlet states, while the T1 is a triplet electronic state. The closely spaced states within each electronic level represent the vibrational energy level. Emission from S1 to S0 directly is the fluorescence signal, and emission from T1 to S0 is the phosphorescence signal.

Figure 2. 2p-FRET imaging. Two-photon PFRET data analysis (3) was implemented for CFP- and YFP-C/EBP α protein expressed in GHFT1-5 cells. The donor excitation wavelength was 820 nm, and the acceptor excitation wavelength was 940 nm. The emission filter for donor and acceptor was 485/30 and 535/50 nm, respectively. The energy transfer efficiency before and after correction is 37.1% and 27.6%, respectively. These data were acquired using the Radiance2100™ and the Coherent™ ti:sapphire laser system (Bio-Rad Laboratories, Hercules, CA, USA).

Figure 3. Lifetime FRET imaging. BFP-dsRED-C/EBPα protein dimerization in pituitary GHFT1-5 living cells. Time-resolved donor [BFP-C/EBPα] images were acquired in the presence (AD_0 , AD_1 , AD_2 , and AD_3) and absence (D_0 and D_1) of the acceptor (dsRED-C/EBPα). The single exponential decay of the donor alone lifetime (τ_D ; red color) and double exponential decay of the donor in the presence of the acceptor (τ_{DA1} and τ_{DA2} ; red color) were processed. In the absence of acceptor, there is no second component lifetime. However, in the presence of acceptor, 2-D distributions of change in the lifetimes of donor ($\tau_D = 3.7$ ns, $\tau_{DA1} = 2.1$ ns and $\tau_{DA2} = 0.78$ ns at position “c”), clearly indicate the occurrence of protein dimerization. Moreover, the second component represents the distance distribution between donor and acceptor molecules. These images were acquired using a high-speed gating camera (LaVision, Germany) and a high-speed ti:sapphire laser coupled to a Nikon TE300 epifluorescent microscope. The excitation wavelength is 365 nm (doubled 730 nm), and the emission for donor and acceptor is 460/50 and 610/60 nm, respectively (2).

sence of acceptor molecules is measured. Such measurements would be advantageous for understanding environmental factors for FRET measurements in a heterogeneous sample.

This aspect is of particular importance because of the very definition of lifetime: the average time for which the molecule spends in the excited state before returning to the ground state and not the number of molecules that go to the excited state. More precisely, it is the time taken for the initial intensity to decay to $1/e$ (where e is natural logarithm number 2.718282) and is independent of the initial intensity. Therefore, the change in either expression level or concentration of cells does not affect the lifetime. Moreover, since FRET is a quantum mechanical process that takes place in the excited state, lifetime imaging is an appropriate tool to study FRET quantitatively (2). Figure 3 shows a set of images for estimating the dimerization of C/EBP α proteins in GHFT1-5 cells (1).

The z-axis lifetime distribution of donor in the presence of acceptor is more challenging to determine. We are currently working on implementing z-axis optical sectioning of the lifetime images. The lifetime FRET images not only estimate the distance between donor and acceptor molecules more precisely but also allow the localization of more pairs of protein-protein interactions. This is difficult to implement in the intensity-based FRET images because of multiple spectral bleed-through signals. Another way one could follow the donor lifetime 3-D distributions (or 4-D) in the presence and absence of acceptor is by implementing the FRET measurements using both confocal and two-photon microscopy.

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