An Introduction to Fluorescence Resonance Energy Transfer (FRET) Technology and its Application in Bioscience

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Fluorescence Resonance Energy Transfer (FRET) is a physical phenomenon first described over 50 years ago, that is being used more and more in biomedical research and drug discovery today. FRET relies on the distance-dependent transfer of energy from a donor molecule to an acceptor molecule. Due to its sensitivity to distance, FRET has been used to investigate molecular interactions. FRET is the radiationless transmission of energy from a donor molecule to an acceptor molecule. The donor molecule is the dye or chromophore that initially absorbs the energy and the acceptor is the chromophore to which the energy is subsequently transferred. This resonance interaction occurs over greater than interatomic distances, without conversion to thermal energy, and without any molecular collision. The transfer of energy leads to a reduction in the donor's fluorescence intensity and excited state lifetime, and an increase in the acceptor's emission intensity. A pair of molecules that interact in such a manner that FRET occurs is often referred to as a donor/acceptor pair.

While there are many factors that influence FRET, the primary conditions that need to be met in order for FRET to occur are relatively few. The donor and acceptor molecules must be in close proximity to one another (typically 10-100 Å). The absorption or excitation spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor (Figure 1). The degree to which they overlap is referred to as the spectral overlap integral (J). The donor and acceptor transition dipole orientations must be approximately parallel. Assuming that the donor acceptor pairs are compatible the most critical element necessary for FRET to occur is close proximity of the pairs. Förster, demonstrated that the efficiency of the process (E) depends on the inverse sixth-distance between donor and acceptor (see Equation 1). [1]

$$\text{Eq 1.} \qquad E = R_o^6/(R_o^6 + r^6)$$

Where R_o is the Förster distance at which half the energy is transferred and r is the actual distance between donor and acceptor. The distance at which energy transfer is 50% efficient is referred to as the Förster radius (R_o). The magnitude of the R_o is dependent on the spectral properties of the donor and the acceptor. Förster distances ranging from 20 to 90 Å are most useful for studies of biological macromolecules. These distances are comparable to the diameters of many proteins, the thickness of biological membranes, and the distances between sites on multisubunit proteins. Any process that affects the energy transfer rate allows the process to be quantified. As a result, FRET is often referred to as a spectroscopic ruler. Note that the Förster distance (R_o) is dependant on a number of factors, including the fluorescence quantum yield of the donor in the absence of acceptor (fd), the refractive index of the solution (n), the dipole angular orientation of each molecule (k^2), and the spectral overlap integral of the donor and acceptor (J). See Equation 2.

$$\text{Eq 2.} \qquad R_o = 9.78 \times 10^3 (n^{-4} {}_* fd_* k^2 {}_* J)^{1/6} \text{ Å}$$
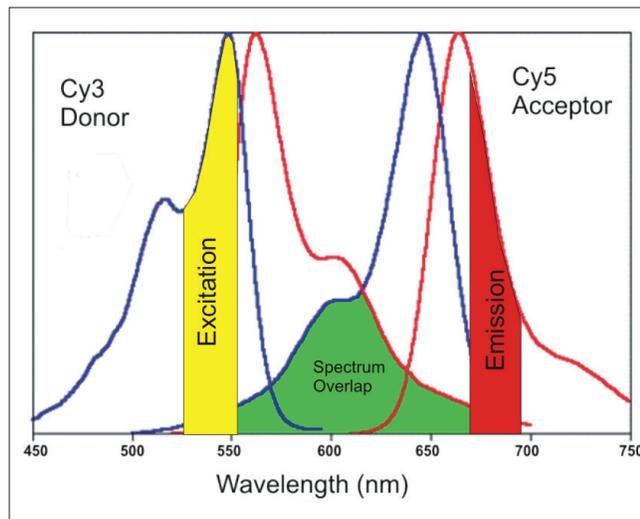
Figure 1. Schematic representation of the spectral overlap integral

As an example of the affect of distance on the efficiency of energy transfer one can use some arbitrary numbers for the Förster distance (Ro) and the actual distance (r). If Ro is arbitrarily set to 1 (Ro =1), and the distance between the donor and acceptor is also equal to 1, then r=Ro, and the equation for efficiency is $E = 1^6/(1^6 + 1^6)$, which is equal to 0.5 (i.e. 50%). This half maximal value is what the Förster distance is defined as being. If the distance is 10 x closer (e.g. r=0.1Ro), then $E = 1^6/(1^6 + 0.1^6) = 0.999999$, considerably more efficient. However, if the distance between the donor and the acceptor is 10 x further away (i.e. r=10Ro), then $E = 1^6/(1^6 + 10^6) = 0.0000001$. This extreme sensitivity for distance is what allows FRET to be used for proximity experiments.

In general the donor and acceptor moieties are different, in which case FRET can be detected by the appearance of fluorescence of the acceptor or by quenching of donor fluorescence. The donor probe is always a fluorescent molecule. Note that luminescent molecules behave as a fluorescent molecule in regards to their emission. With appropriate excitation, its electrons jump from the ground state (So) to a higher vibrational level. Very rapidly (within picoseconds) these electrons decay to the lowest vibrational levels (S1) and eventually decay (within nanoseconds) back to the So state and a photon of light is emitted. When conditions for FRET to occur are

met, decay as donor fluorescence and energy transfer to the acceptor will compete for the decay of excitation energy. With resonance energy transfer, the photon is NOT emitted, but rather the energy is transferred to the acceptor molecule, whose electrons in turn become excited as described for the donor molecule. The subsequent return to the ground state emits a photon (Figure 2).
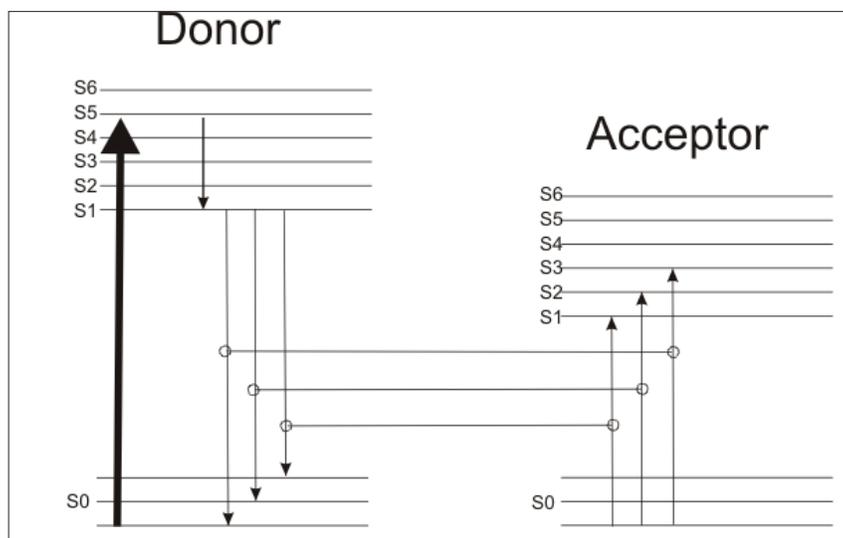

Figure 2. Schematic depicting electron vibrational energy states that occur during FRET.

Measurement

The detection and quantitation of FRET can certainly be accomplished in a number of different ways. Because FRET can result in both a decrease in fluorescence of the donor molecule as well as an increase in fluorescence of the acceptor, a ratio metric determination of the two signals can be made. The advantage of this method is that a measure of interaction can be made that is independent of the absolute concentration of the sensor. Because not all acceptor moieties are fluorescent, they can be used as a means to quench fluorescence. In these instances, those interactions that result in a fluorescent donor molecule coming in close proximity to such a molecule would result in a loss of signal. Inversely, reactions that remove the proximity of a fluorescent donor and a quencher would result in an increase in fluorescence. One such example would be protease assays. These assays usually involve a fluorescent moiety on one terminus and a quenching molecule on the other terminus separated by a peptide containing the protease cleavage sequence.

Some Examples

Genetically encoded fluorescent dyes, such as Green Fluorescent Protein (GFP) and related molecules blue, cyan, yellow and red have provided the ability to perform FRET in vitro, particularly in living cells [2]. These proteins form FRET pairs with each other as well as with conventional dyes. They can be attached to other proteins genetically or covalently yet still retain their fluorescent capability. These dyes have the utility of being genetic elements that can be linked with other genes to form chimeric proteins. These chimeric proteins contain a GFP (or related fluorescent protein element) and a putative binding domain. With different chimeric proteins (one donor and one acceptor) protein-protein interactions can be investigated. Only when the donor/acceptor pairs interacted through protein-protein interactions would FRET result. (Figure 3)
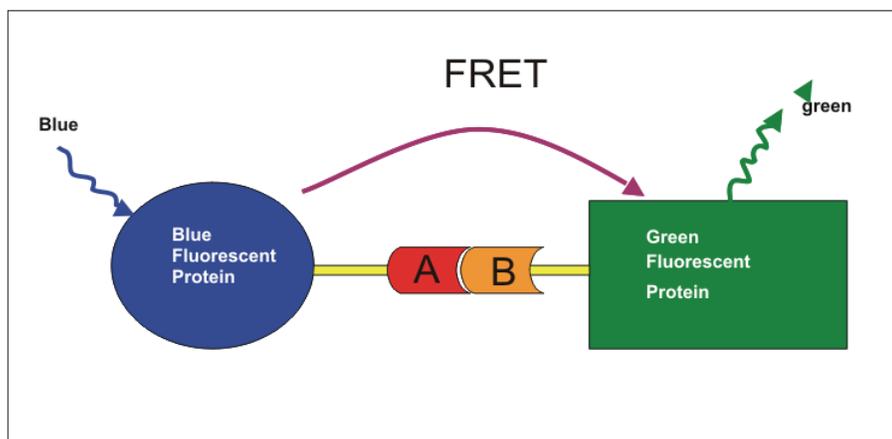

Figure 3. Schematic representation of the interaction of two different fluorescent protein chimeras. Protein-protein interactions between proteins labeled A and B bring Blue fluorescent protein and Green fluorescent proteins in close enough proximity to allow for FRET to occur. In this example, excitation of blue fluorescent protein results in the emission of fluorescence by Green fluorescent protein.

The organic cyanine dyes Cy3, Cy5, Cy5.5 and Cy7, which emit in the red range (>550 nm), offer a number of advantages. Their emission range is such that background fluorescence is often reduced. Additionally large distances (>100 Å) can be measured as a result of the high extinction coefficients and good quantum yields. Even donor-acceptor pairs with separated emission spectra (i.e. low overlap integral) result in acceptable Förster distances. For example, Cy3, which emits maximally at 570 nm and Cy5, which emits at 670 nm, have a Förster distance >50 Å. Large separation between pairs allows the measurement of acceptor emission as a result of FRET without interference from donor emission. In addition these molecules can be linked directly to specific locations in synthetically produced nucleic acids, allowing FRET to be used to assess nucleic acid annealing.
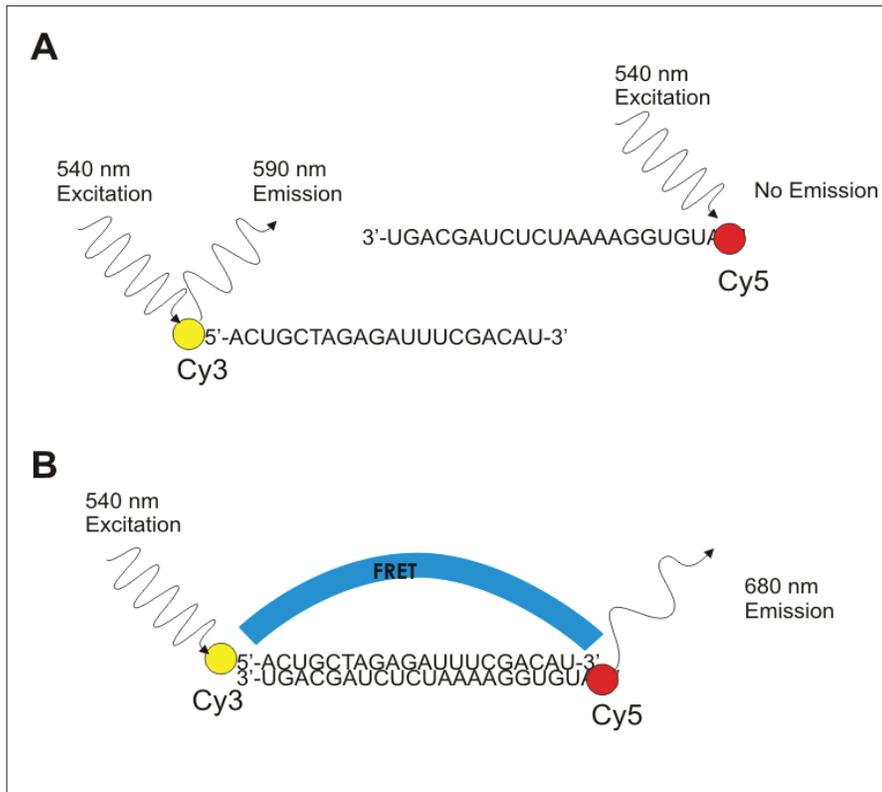
Figure 4. Schematic representation of FRET occurring between Cy3 and Cy5 fluorescent moieties when labeled oligonucleotides are annealed.

In the example depicted in Figure 4, two complementary RNA oligonucleotides are labeled with Cy3 and Cy5 respectively. When these labeled molecules are not annealed (Figure 4A), excitation of an RNA oligonucleotide labeled with Cy3 with light at 540 nm results only in the emission of light by Cy3 at 590 nm, while the complementary RNA-oligo labeled with Cy5 does not emit any light at 590 nm or its true emission wavelength of 680 nm. However, when the two oligonucleotides are allowed to anneal, the close proximity of the molecules allows for FRET transfer to occur. This results in the emission of light at 680 nm when the annealed molecule is excited with 540 nm light. Note that not all of the emission of Cy3 at 590 nm is lost, but a significant portion is.
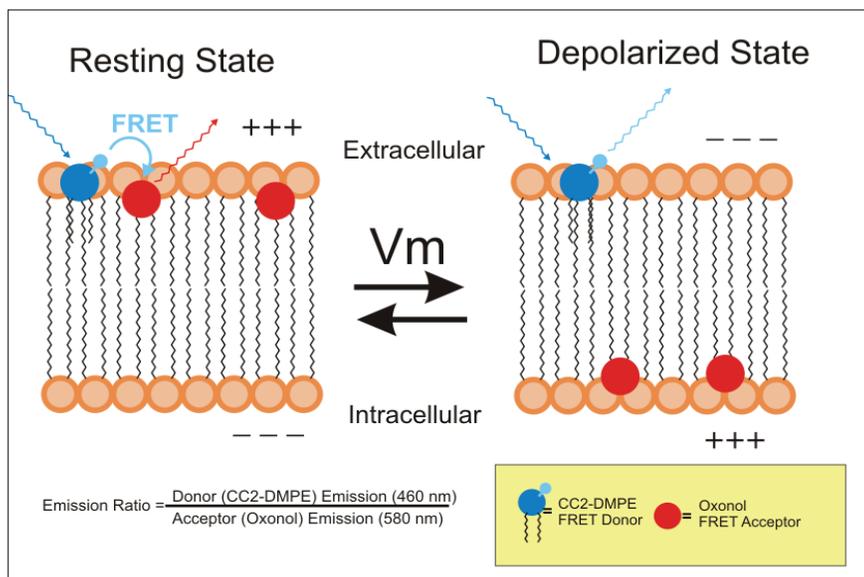

Figure 5. Schematic Diagram of FRET activity utilized by VSPs.

Voltage Sensor Probes (VSPs) are a Fluorescence Resonance Energy Transfer (FRET)-based assay technology used for high-throughput ion channel drug discovery. The FRET donor is a membrane-bound, coumarin-phospholipid (CC2-DMPE), which binds only to the exterior of the cell membrane. The FRET acceptor is a mobile, negatively charged, hydrophobic oxonol [either $DiSBAC_2(3)$ or $DiSBAC_4(3)$], which will bind to either side of the plasma membrane in response to changes in membrane potential (Figure 5). It is the distance dependence that is utilized with VSPs. Only when the acceptor $DISBAC_2(3)$ is located on the exterior of the cell membrane is FRET capable of taking place. Resting cells have a relatively negative potential, so the two probes associate with the exterior of the cell membrane, resulting in efficient FRET. Exciting the CC2-DMPE donor probe (at ~400 nm) generates a strong red fluorescence signal (at ~590 nm) from the oxonol acceptor probe. When the membrane potential becomes more positive, as occurs with cell depolarization, the oxonol probe rapidly translocates (on a subsecond time scale) to the other face of the membrane (Figure 5). Thus, each oxonol probe "senses" and responds to voltage changes in the cell. This translocation separates the FRET pair, so exciting the CC2-DMPE donor probe now generates a strong blue fluorescence signal (at ~460 nm) from the CC2-DMPE probe. Depolarization of the cell, which causes $DISBAC_2(3)$ to relocate to the inner side of the membrane, would be expected to result in a decrease in FRET activity.

Lanthanide compounds, such as europium and terbium, have been effectively used as donors in FRET reactions. These compounds provide very good signal/noise ratios as a result of their long fluorescent half-life and spectral characteristics. The emission of these compounds has a sharply spiked profile with a large Stoke's shift from excitation. In addition, the lengthy fluorescent half-life allows for measurement to begin after the cessation of excitatory light. The delay between excitation and measurement (msec range) allows for background fluorescence from the organic acceptor molecule, with a half-life in the nanosecond range, to dissipate. Thus only donor-acceptor emission is measured when the appropriate delay is enabled.

The donor molecule need not always involve a fluorescent compound. Luminescent molecules emit photons in a fashion very similar to fluorescence. The primary difference is that the electron excitation is not the result of photon absorption, but rather from the release of chemical energy contained within the molecule. When the excited electrons return to their ground energy can be released as a photon of light or transferred via RET to an acceptor molecule if the conditions are correct. While more limited in the number of molecules that can be utilized, this technology has the advantage that there is no external excitation of the acceptor molecule.

Issues

When designing FRET experiments there are a number of issues that need to be considered. The most obvious issue is the matter of close proximity. Depending on the assay design, close proximity will either be established or removed during the assay, resulting in a change in signal that can be measured. Appropriate donor/acceptor pairs need to be selected. The pairs need to have enough spectral overlap for efficient energy transfer to take place, yet have enough of a difference in spectrums as to be distinguishable from one another. The choice of filters for fluorescent wavelength selection is also critical to the success or failure of experimental detection of FRET. The excitation filter for the donor has to be able to selectively excite the donor molecule, while minimizing direct excitation of the acceptor molecule. Contaminating direct excitation of the acceptor molecule can be accounted for using appropriate controls, but large amounts will make interpretation of the data more difficult. As shown in figure 1, the filters used for Cy3 excitation, minimized excitation of Cy5, while providing sufficient excitation signal for Cy3.

Another important concern in regards to detection of FRET involves analyte concentration. Only those molecules that interact with one another will result in FRET. If large amounts of donor and acceptor molecules are present, but do not interact, the amount of FRET taking place would be quite low. In this example, while the donor and the acceptor molecules may be very easy to detect separately the actual amount of FRET activity may not be sufficient to detect. In regards to FRET the actual "analyte" being measured are donor/acceptor pairs rather than the individual components. Additionally, both the donor and acceptor molecules need to be in sufficient concentration in order for FRET to take place. Most binding events are a dynamic process that reaches a steady state. If one of the components of the reaction is in short supply then the total amount bound will be naturally low. For example, transient transfections with two different genetic elements that result in one of the elements not being efficiently translated into protein will result in poor levels of FRET. Cellular localization can play a role as well. If one molecule is located in the cytoplasm while the other molecule has a nuclear location, there will be no interaction with each other despite sufficient quantities of each.

FRET Applications

- Structure and conformation of proteins [3]
- Spatial distribution and assembly of proteins [4]
- Receptor/ligand interactions [5]
- Immunoassays [6]
- Structure and conformation of nucleic acids [7]
- Real-time PCR assays and SNP detection [8,9]
- Nucleic acid hybridization [10]
- Distribution and transport of lipids [11]
- Membrane fusion assays [12]
- Membrane potential sensing [13, 14]
- Fluorogenic protease assays [15]
- Indicators for cyclic AMP [16].

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