

Principal Papers in FRET Technology: The Use of Fluorescence Resonance Energy Transfer (FRET) in the Study of Molecular Dynamics in the Living Cells

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1. Basic Concept of FRET

FRET is nominally the non-radiative transfer of energy from a donor molecule to the acceptor molecule, in which an excited donor fluorophore transfers energy to a lower-energy acceptor fluorophore via a short-range (10nm) dipole-dipole interaction. Binding interactions between donor-labeled and acceptor-labeled proteins can bring fluorophores within the appropriate distance for FRET to occur. Application of FRET to microscopy has become an important tool for live-cell detection of molecular interactions between fluorescently labeled molecules. Yet few FRET studies quantify the stoichiometry of molecular interactions.

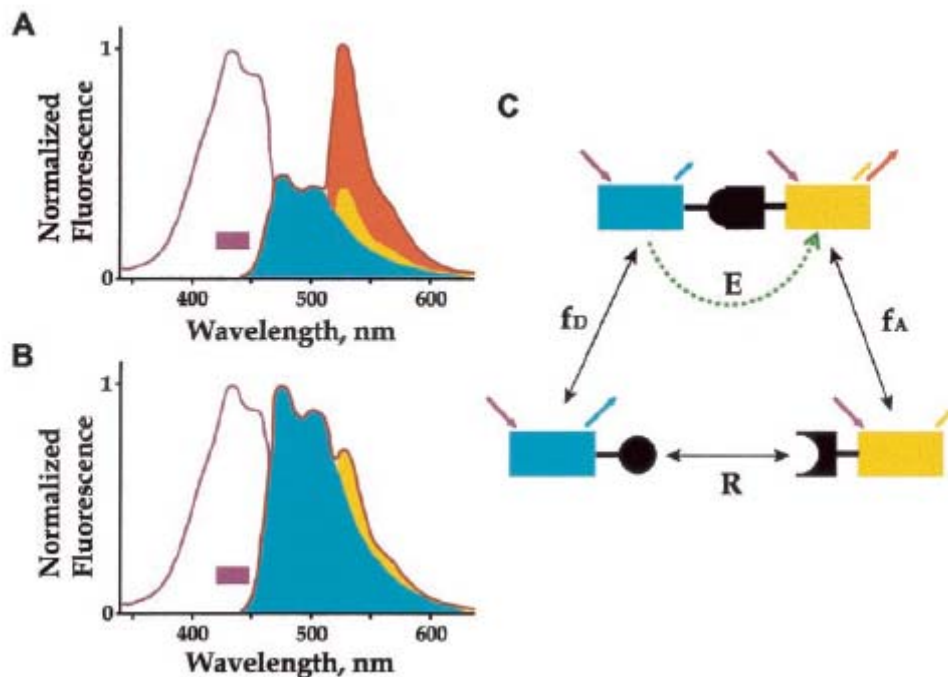


FIG.1. Dipoles of two interacting fluorophores

What is the meaning of the word fluorescence? Fluorescence is a particular method of de-excitation of an excited molecule; there are, however, several such methods of de-excitation. Some of those methods of de-excitation involve emission of light others do not. And other pathways combine non-radiative transitions with radiative emission. Contrast fluorescence with: vibration relaxation; phosphorescence; and internal conversion. Vibration relaxation is merely a transition from one excited vibration level within an excited electronic state to a lower energy vibration level. Such a transition is a consequence of the fact that the vibration states are not exactly harmonic, in other words a harmonic in vibration potentials permit otherwise orthogonal states to exchange energy. Next let us consider phosphorescence. Phosphorescence is similar to fluorescence in that both are types of luminescence. Fluorescence is light emitted from a singlet excited state and phosphorescence is light emitted from a triplet state. The basic idea concerns the number of ways that total spin of an atom proton plus electron—can be achieved. There are three states with $s = 1$: $|1\ 1_i\rangle$, $|1\ 0_i\rangle$, and $|1\ -1_i\rangle$, whereas there is only one way to achieve the orthogonal state $s = 0$: $|0\ 0_i\rangle$. $\hbar^2 s(s + 1)$ is the Eigen value of the S^2 .) Since triplet states that are derived from a singlet state in the de-excitation process are of lower energy phosphorescence is of longer wavelength. The longer lifetime of phosphorescence is beyond the scope of the present treatment.

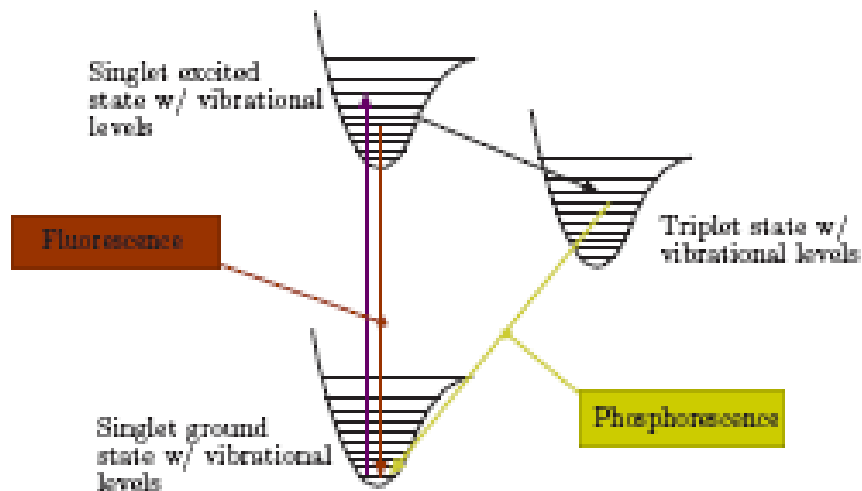


FIG. 2: .The three methods of deexcitation explained quantum mechanically.

Finally the de-excitation process can be accomplished by internal conversion. This process involves energy loss to the solvent through collisions and anharmonicities in vibrational states. Having explained the first of the words in the acronym FRET, we can now concentrate on the second word—resonance. The best and most commonly used metaphor is that of coupled pendula diagrammed below. If two pendula have a spring connecting their rods then when one pendulum is set

swinging (the donor molecule absorbs a photon) the other pendulum of couple will begin swinging (the acceptor molecule emits a photon).

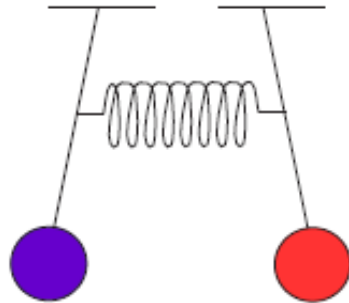


FIG. 3. The classical metaphor for resonance—two coupled pendula

The baffling point about FRET is that for the non-radiative transfer of energy to occur there must be an overlap between the emission profile (the function of intensity versus wavelength) of the donor and the absorption profile of the acceptor. Yet these are precisely the conditions for radiative transfer, also. The point is that the two processes—radiative and non-radiative transfer—have very different dependences on distance.

2. Qualitative Description of FRET

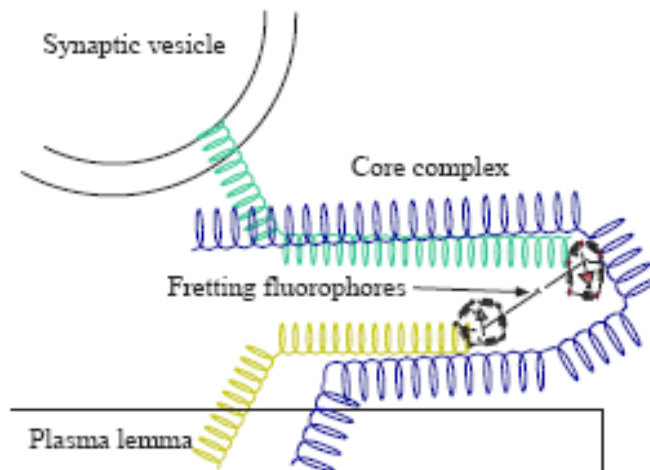


FIG. 4: FRET and the core-complex. Part of an experiment to prove that the core complex is intimately involved in some instances of synaptic vesicle fusion.

1. The high energy (low wavelength) dipole begins oscillating after absorption of and appropriate photon in addition to the fact that the donor is emitting a longer wavelength photon.
2. The low energy (longer wavelength) dipole feels the oscillations in the electric field and at the resonant frequency absorbs the energy.
3. Now the acceptor is excited and emits in the longest wavelength photon of the FRET event.
4. One of the key qualitative points is that the FRETing fluorophores have to be within, say, 50 Ås or each other. This necessity of close proximity for the donor and acceptor is responsible for the fantastic utility of FRET in neural biology. FRETing fluorophores on proteins prove that the two proteins are close enough for biologically relevant interactions (Fig. 4).

3. The Elusive Connection between Experiment and Theory in FRET

Efficiency E is defined as the fraction of energy (in photons) absorbed by the donor that was subsequently transferred to the acceptor. We will show the following; efficiency

$$\text{efficiency} = E = \frac{R_0^6}{R_0^6 + R^6} \quad (1)$$

where:

1. R_0 : Forster distance,
2. R : the distance between the centers of the fluorophore dipole moments. More relevant to experimental proof of the existence of FRET is the following expression;

$$E = 1 - \frac{F_{DA}}{F_D}, \quad (2)$$

where:

1. F_{DA} : fluorescence intensity of the donor in the presence of the acceptor,
2. F_D : fluorescence intensity of the donor when the acceptor is far away. Equation (2) makes sense because, when the acceptor is close to the donor F_{DA} should be low and the efficiency should be close to one. When the acceptor is far away then $F_{DA} = F_D$ and efficiency equals 0.

4. Application of FRET Techniques in Optical Microscopy

Microscope configurational parameters for fluorescence resonance energy transfer investigations vary with the requirements of the fluorophores, specimen, and imaging mode(s), but virtually any upright or inverted microscope can be retrofitted for FRET microscopy. In general, the microscope should be equipped with a high-resolution (12-bit) cooled and intensified CCD camera system coupled to quality

interference filters having low levels of crosstalk (minimum blocking level) and bandpass regions corresponding closely to the fluorophore spectra. The detector sensitivity determines how narrow the filter bandpass can be and still enable data acquisition to proceed at acceptable speeds with a minimum of spectral bleed-through noise. In most cases, a single dichromatic mirror coupled to excitation and emission filter wheels or sliders should be used to acquire images in order to minimize or eliminate image shifts.

Widefield fluorescence microscopy suffers from fluorophore emission originating above and below the focal plane to yield images with significant out-of-focus signal that reduces contrast and leads to image degradation. This problem is compounded in FRET microscopy because of the inherently low signal levels produced as a result of resonance energy transfer. Digital deconvolution techniques can be coupled to optical sectioning in order to reduce or eliminate signals away from the focal plane, but the process is computationally intensive and may not be fast enough for many dynamic FRET imaging experiments. Laser scanning confocal techniques can be applied to FRET microscopy to produce a significant improvement in lateral resolution, while enabling the collection of serial optical sections at intervals approaching real time. The major drawback of confocal microscopy is the limitation of excitation wavelengths to the standard laser lines available for a particular system, which restricts the choice of fluorophore donor and acceptor pairs in resonance energy transfer experiments. Multiphoton excitation can also be employed in combination with FRET techniques and is less damaging to cells due to the longer excitation wavelengths involved. In addition, autofluorescence artifacts and photobleaching of the specimen are less likely to occur within the restricted excitation volume characteristic of multiphoton excitation.

A typical microscope configuration capable of observing living cells in culture with several fluorescence resonance energy transfer imaging motifs is presented in Figure 7. The inverted tissue culture microscope is equipped with a standard tungsten-halogen lamphouse on the pillar in order to examine and record the cells using standard brightfield, phase contrast, or differential interference contrast (**DIC**) illumination. Note that the latter two contrast enhancing techniques can be employed in combination with fluorescence to reveal the spatial location of fluorophores within the cellular architecture. A standard Peltier-cooled CCD camera system is attached to the microscope trinocular head for widefield fluorescence and brightfield image capture.

Resonance energy transfer experiments are conducted with the multispectral microscope illustrated in Figure 5 using either widefield illumination (arc discharge lamp) or a real-time scanning confocal attachment equipped with a high-speed Nipkow disk system. The argon-krypton laser beam is first filtered through an acousto-optic tunable wavelength device to select specific excitation wavelengths before passing to the confocal scan head. Images are collected using two high-resolution Gen III-intensified cooled CCD cameras reading separate channels and spooled to a host computer. Scanning the specimen in the lateral (**x** and **y**) and axial

(z) planes enables collection of optical sections for three-dimensional image reconstruction. A variety of image processing software programs are compatible with the illustrated microscope configuration.



Fig. 5 : The multispectral microscope illustrated

Based upon the fundamental principles of the phenomenon, a number of important practical points should be considered when fluorescence resonance energy transfer measurements are conducted with an optical microscope:

- The concentrations of donor and acceptor fluorophores must be closely controlled. The statistically highest probability of achieving fluorescence resonance energy transfer occurs when a number of acceptor molecules surround a single donor molecule.
- Photobleaching must be eliminated because the artifact can alter the donor-to-acceptor molecular ratio, and therefore, the measured value of the resonance energy transfer process.
- The donor fluorescence emission spectrum and the acceptor absorption spectrum should have a substantial overlap region.
- There should be minimal direct excitation of the acceptor in the wavelength region utilized to excite the donor. A common source of error in steady state FRET microscopy measurements is the detection of donor emission with acceptor filter sets.
- The emission wavelengths of both the donor and acceptor must coincide with the maximum sensitivity range of the detector.
- The donor absorption and emission spectra should have a minimal overlap in order to reduce the possibility of donor-to-donor self-transfer.

- The donor molecule must be fluorescent and exhibit sufficiently long lifetime in order for resonance energy transfer to occur.
- The donor should exhibit low polarization anisotropy to minimize uncertainties in the value of the orientation (κ -squared) factor. This requirement is satisfied by donors whose emission results from several overlapping excitation transitions.
- When using antibody labeling techniques, reagents conjugated with donor and acceptor fluorochromes should not be altered in their biological activity. Any reduction in activity will seriously affect the validity of resulting resonance energy transfer measurements.
- Because fluorescence resonance energy transfer requires the donor and acceptor molecules to have the appropriate dipole alignment and be positioned within 10 nanometers of each other, the tertiary structure of the reagents to which the molecules are attached must be considered. For example, when donor-acceptor molecules can be attached to different structural locations (such as the carboxy or amino terminus) on a protein, it is possible that FRET will not be observed even though the proteins do interact, because the donor and acceptor molecules are located on opposite ends of the interacting molecules.
- Living cells labeled with green fluorescent protein mutants for FRET investigations should be analyzed using traditional immunohistochemical techniques to verify that the tagged protein adopts the same intracellular habitat and properties as the native counterpart.

In order for the fluorescence resonance energy transfer phenomenon to provide meaningful data as a tool in optical microscopy, both specimen preparation and imaging parameters must be optimized. The selection of appropriate donor and acceptor probes and the manner in which they are employed as molecular labels is a major challenge. In addition, once a labeling strategy that permits energy transfer has been elucidated, a wide spectrum of techniques may be used to perform the measurement itself. A majority of quantitative fluorescence microscopy investigations are conducted by measuring the intensity of fluorescence emission. Fluorescence intensity-based detection of FRET is typically achieved by monitoring changes in the relative amounts of emission intensity at the two wavelengths corresponding to the donor and acceptor chromophores. When conditions are appropriate for fluorescence resonance energy transfer to occur, an increase in acceptor emission (**I(A)**) is accompanied by a concomitant decrease in donor emission (**I(D)**) intensity.

Although a change in the relative emission intensity of either donor or acceptor can be taken as indicative of resonance energy transfer, the customary approach is to utilize the ratio of the two values, **I(A)/I(D)**, as a measure of FRET. The value of the ratio depends upon the average distance between donor-acceptor pairs and is insensitive to differences in the path length and volume accessed by the exciting light beam. Any specimen condition that induces a change in the relative distance between the molecular pairs produces a change in the ratio of donor and acceptor emission. Consequently, FRET can be observed in the microscope by preferential excitation of a donor fluorophore and detection of the increased emission of an interacting acceptor fluorophore, accompanied by a reduction in donor fluorescence produced by quenching due to energy transfer. Measurement of FRET employing the intensity-monitoring approach is termed **steady state** fluorescence resonance energy transfer imaging.

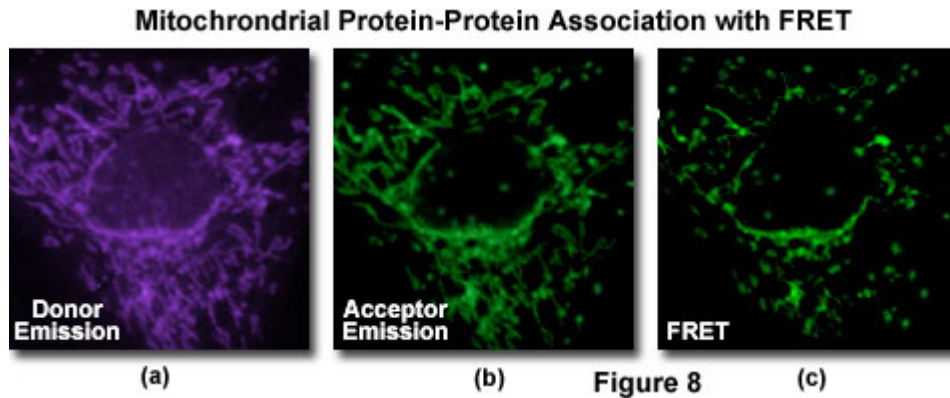


Fig. 6: A typical investigation of intracellular protein-protein association in a living cell culture

Appropriate donor and acceptor probes are selected on the basis of their absorption and emission spectral characteristics. For maximum resonance energy transfer, the donor emission spectrum should substantially overlap the absorption spectrum of the acceptor. In addition, there should be minimal direct excitation of the acceptor fluorophore at the excitation maximum of the donor, and there should not be significant emission overlap between the donor and acceptor in the wavelength region at which acceptor emission occurs. In practice, it can be difficult to identify donor-acceptor pairs that satisfy these requirements. The situation is often complicated by the fact that the commercially available fluorescence filter sets are not completely effective in passing only the desired wavelengths, and a small percentage of light outside the design passband may be transmitted. Unless very well characterized and controlled expression systems are used, the precise concentration of the donor and acceptor fluorophores may be difficult to determine. Additional corrections may be also be required for autofluorescence, photobleaching, and background fluorescence.

A typical investigation of intracellular protein-protein association in a living cell culture is illustrated in Figure 6 for events associated with apoptosis, a physiological process of cellular death resulting from an intricate cascade of sequential interactions. Gene products directly involved in the chain of events can be labeled by fusion to appropriate members of the fluorescent protein family (in this case, BFP and GFP) for co-expression in the same cell in order to probe specific associations by FRET. The proteins involved with apoptosis interact within the mitochondria and display a gradual decrease in binding as programmed cell death proceeds. Thus, an image of donor emission contains only fluorescence from the BFP-labeled proteins, while the corresponding acceptor emission profile (Figure 9(b)) illustrates signals due to proteins labeled with GFP (and some contribution from donor emission). A FRET filter, as described below, reveals fluorescence derived from resonance energy transfer between the two proteins

Among the factors that may potentially affect the accuracy of fluorescence resonance energy transfer measurements in general, several are highly specific to

the optical microscope. A primary target in microscopy investigations is to obtain high resolution images, and this requires particular attention to the quality and performance of optical filters employed to spectrally discriminate among the absorption and emission wavelengths of the donor and acceptor. In order to maximize the signal-to-noise ratio (without deleteriously affecting the specimen or the process being investigated), it is necessary to carefully balance the intensity and time of exposure to excitation light with the concentration of donor and acceptor fluorophores and the detector efficiency. If the concentration of donor-acceptor fluorophores is excessive, self-quenching can occur, affecting the accuracy of FRET measurements. Photobleaching is a problem with all fluorophores, and can affect the donor-acceptor ratio, altering fluorescence measurements. Excess illumination intensity can also damage specimens, particularly those containing living cells or tissues.

5. Donor photobleaching fluorescence resonance energy transfer (pbFRET),

A technique known which exploits the photobleaching process to measure FRET, is often applied in the study of fixed specimens. Based on pixel-by-pixel analysis, the method has been applied to measure proximity relationships between cell surface proteins labeled with fluorophore-conjugated monoclonal antibodies. Photobleaching FRET is founded on the theory that a fluorophore is sensitive to photodamage only when it is elevated to an excited state. Statistically, only a small proportion of molecules are in an excited state at any one time, and therefore, fluorophores with longer fluorescence lifetimes have a higher probability of suffering photodamage and exhibit a higher rate of photobleaching.

Experimental evidence supporting this concept has demonstrated that the photobleaching time of a fluorophore varies inversely with its excited-state lifetime. The occurrence of resonance energy transfer reduces the fluorescence lifetime of the donor molecule, effectively protecting it against photobleaching. Calculations of pbFRET are based on the decreased rate of donor photobleaching relative to that measured for the donor in the absence of resonance energy transfer. The measurement of photobleaching in FRET studies requires a relatively long timeframe, and therefore is most applicable to fixed cell specimens in which temporal data is not important and the effect on cell function from photobleaching is not an issue. In some respects, the donor photobleaching technique is less complicated than sensitized emission measurement, although fitting of time constants to photobleaching curves involving multiple components presents some additional difficulties.

6. Acceptor Photo-bleaching

The energy transfer efficiency can also be determined by acceptor photobleaching techniques, in which the change in donor emission quenching is measured by comparing the value before and after selectively photobleaching the acceptor molecule. Analysis of the change in donor fluorescence intensity in the

same specimen regions, before and after removal of the acceptor, has the advantage of requiring only a single specimen preparation, and directly relates the energy transfer efficiency to both donor and acceptor fluorescence.

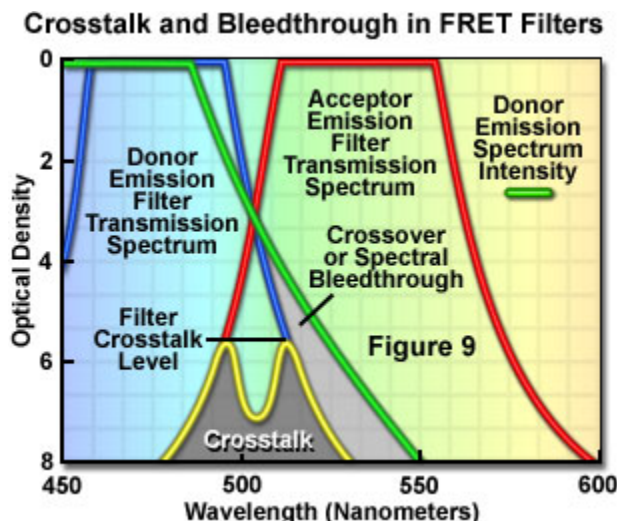


Figure 7: schematic illustrations of crossover (spectral bleed-through) and filter crosstalk

Accurate measurement of fluorescence resonance energy transfer in the microscope requires compensation for all of the potential error sources. A straightforward technique to correct for detection of donor fluorescence with the acceptor emission filter and acceptor fluorescence with the donor emission filter (due to crossover or spectral bleed-through) has been developed. The method also corrects for the dependence of FRET on the concentrations of the donor and acceptor fluorophores. The measurement strategy, which requires a minimum of spectral information, utilizes a combination of three filter sets and can be readily implemented. The donor, FRET, and acceptor filter sets are designed to isolate and maximize three specific signals: donor fluorescence, acceptor fluorescence attributable to FRET, and the directly excited acceptor fluorescence, respectively. In practice, three different specimens, containing just donor, just acceptor, and both donor and acceptor are examined with each of the three filter sets, and the resulting data manipulated arithmetically to correct for crossover and for uncontrolled variations in donor-acceptor concentrations.

Presented in Figure 7 are schematic illustrations of crossover (spectral bleed-through) and filter crosstalk, two significant problems that must be overcome in order to achieve quantitative results in fluorescence resonance energy transfer experiments. Crossover or bleed-through is manifested by an overlap of the donor fluorescence emission spectrum with the bandpass region of the acceptor emission interference filter in Figure 9, resulting in donor emission signal (unwanted wavelengths) being transmitted through the emission filter. In contrast, filter crosstalk describes the minimum attenuation (blocking) level over a specific range of two filters placed together in series, and is of concern when matching excitation and emission filters for fluorescence sets. Dichromatic mirrors are often included in

crosstalk evaluation of fluorescence filter combinations. Although two emission filters are rarely placed in the light path at the same time, the spectra are drawn together in Figure 9 to simultaneously illustrate both concepts. Note that the two filter spectra (blue and red curves) represent light transmittance by the interference filters, whereas the donor emission curve (green) is a plot of intensity versus wavelength.

Additional factors, which can potentially introduce significant errors, also require correction when steady state FRET measurement techniques are employed. Furthermore, careful control of the donor and acceptor fluorophore concentrations is desirable. Fluorophore concentration determinations can be partially avoided through the application of **time-resolved** fluorescence measurements, which provide a method of obtaining average lifetimes without a precise knowledge of donor concentrations. The technique enables quantitative determination of donor-acceptor separation distances, and is based on measurements of the donor lifetime in the presence and absence of the acceptor. Measuring fluorescence intensity decay as a function of time elucidates the emission dynamics of the excited-state molecule, and consequently, more detailed information about the nature of the donor-acceptor interaction may be obtained. Graphical plots of intensity decay illustrate time-averaged details of the fluorescence decay process (see Figure 10(a)), which are unresolved when employing steady state techniques. Measurements indicating the same value for average lifetime, when recorded as steady state intensity normalized to absorption, may correspond to significantly different decay curve shapes in time-resolved data plots, indicating differences in the intermolecular processes involved.

The fluorescence lifetime (τ) of a fluorophore is the characteristic time that a molecule exists in the excited state prior to returning to the ground state. Representing fluorescence decay in a simplified single exponential form following a brief pulse of excitation light, the fluorescence intensity as a function of time (t) is given by the equation:

$$I(t) = I_0 \exp(-t/\tau)$$

where $I(0)$ is the initial fluorescence emission intensity immediately after the excitation light pulse, and $I(t)$ is the fluorescence intensity measured at time t . The fluorescence lifetime (τ) is defined as the time required for the intensity to decay to $1/e$ of its initial value (approximately 37 percent of $I(0)$; Figure 10(a)), and is the reciprocal of the rate constant for fluorescence decay from the excited state to the ground state.

The primary overall advantage of time-resolved versus steady state FRET measurements is that donor-acceptor separation distances can be mapped with greater quantitative accuracy. This results in part because fluorescent lifetimes do not depend upon local intensity or concentration, and are largely unaffected by photobleaching of the fluorophores. Fluorescence lifetimes are, however, highly sensitive to fluorophore environment, and even molecules with similar spectra may

display distinct lifetimes under different environmental conditions. Because scattering does not affect fluorophore lifetimes, measurements of lifetime variation can provide information that is specifically related to local molecular processes.

The time-domain technique for measuring fluorescence lifetime relies essentially on single-photon counting and requires a detection system with sufficient temporal resolution to collect nearly 100 percent of the photons generated by each excitation pulse. Although phase-resolved techniques are relatively less demanding to perform, they are not generally as sensitive as the photon-counting approach. When phase modulation is employed to resolve complicated multifluorophore lifetimes, long exposure times to damaging excitation illumination can prove to be excessive for some specimens, and also may not provide sufficient temporal resolution for live-cell processes. The preferred technique depends upon both the information required from the investigation and the type of specimen being studied.

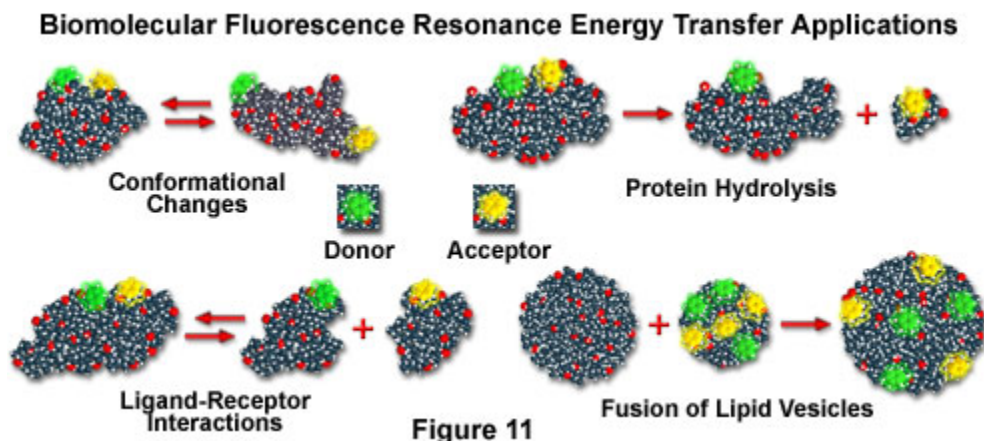
Fluorescence lifetime measurements have proven to be a sensitive indicator of FRET, and have particular advantages in live-cell studies because of the independence of lifetime measurements upon factors such as concentration and light path length, which are difficult to control in living specimens. A primary advantage of performing FRET studies by fluorescence lifetime measurement lies in the fact that it is possible to distinguish energy transfer even between donor-acceptor pairs with similar emission spectra. When fluorescence lifetimes are measured directly (in contrast to the use of steady state values), a determination of FRET is possible without the photodestruction of the donor or acceptor fluorophores. Because FRET reduces the fluorescence lifetime of the donor molecule through energy transfer to the acceptor, a direct comparison of the donor lifetime in the presence of the acceptor (**(DA)**) to that in the absence of the acceptor (**(D)**), enables the calculation of a FRET efficiency value (**(E(T))**) for each image pixel.

Depending upon the technique, fluorescence lifetime measurements require the specimen to be exposed to either high-frequency repetitive pulses of excitation light, or to continuous sinusoidally modulated light. In studies with living cells, the effect of high-intensity illumination must always be evaluated. Regardless of the method, the reference lifetime of the donor without acceptor must be determined under experimental conditions identical to those of the donor-acceptor measurement. One means of accomplishing this with a single specimen is to measure the donor-only lifetime after photobleaching destruction of the acceptor following the energy transfer experiment.

7. Conclusions

In biological investigations, the most common applications of fluorescence resonance energy transfer are the measurement of distances between two sites on a macromolecule (usually a protein or nucleic acid) or the examination of *in vivo* interaction between biomolecular entities. Proteins can be labeled with synthetic fluorochromes or immunofluorescent fluorophores to serve as the donor and

acceptor, but advances in fluorescent protein genetics now enable researchers to label specific target proteins with a variety of biological fluorophores having differing spectral characteristics. In many cases, the amino acid tryptophan is used as an intrinsic donor fluorophore, which can be coupled to any number of extrinsic probes serving as an acceptor.



If macromolecules are labeled with a single donor and acceptor, and the distance between the two fluorochromes is not altered during the donor excited state lifetime, then the distance between the probes can be determined from the efficiency of energy transfer through steady state measurements, as discussed above. In cases where the distance between the donor and acceptor fluctuates around a distribution curve, such as protein assemblies, membranes, single-stranded nucleic acids, or unfolded proteins (see the scenarios presented in Figure 11), FRET can still be employed to study the phenomena, but time-resolved lifetime measurements are preferred. Several biological applications that fall into both cases are illustrated in Figure 11, including conformational changes, dissociation or hydrolysis, fusion of membrane-like lipid vesicles, and ligand-receptor interactions.

Although various methods are available for the measurement of fluorescence resonance energy transfer in the optical microscope, none are completely without disadvantages. Some techniques require more elaborate and expensive instrumentation, while others are based on assumptions that must be carefully validated. Certain approaches are appropriate for fixed specimens, but cannot be applied to living cell systems, while other methods must incorporate significant corrective calculations or data analysis algorithms. It is certain, however, that FRET analysis shows great promise for further development in the utility and scope of biological applications. Dramatic improvements in instrumentation have occurred in recent years, particularly with respect to time-resolved techniques.

Fluorescence lifetime measurements that were only accomplished with extreme difficulty in the past are now aided by mature picosecond and nanosecond technologies. Advances in fluorescent probe development have produced smaller and more stable molecules with new mechanisms of attachment to biological targets. Fluorophores have also been developed with a wide range of intrinsic

excited state lifetimes, and a significant effort is being placed on development of a greater diversity in genetic variations of fluorescent proteins. Entirely new classes of fluorescent materials, many of which are smaller than previous fluorophores and allow evaluation of molecular interactions at lower separation distances, promise to improve the versatility of labeling and lead to new applications of the FRET technique.

Contributing Authors and References

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