Fretting about FRET in Cell and Structural Biology

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Introduction

A sophisticated understanding of cellular processes and mechanisms requires knowledge of molecular mechanisms at a variety of scales in the living cellular milieu, often in real time. X-ray and electron-beam interrogations of biological systems have the capacity to provide information about the atomic scale for understanding the structure of proteins and nucleic acids in one or more physiologically relevant states of the molecules under investigation. This sheds light on biomolecular function in terms of catalysis, conformational change, and macromolecular assembly at extraordinarily high resolution. However, none of this information is in the context of a living cell.

Light and fluorescence emission microscopy in the visible region are the most popular means of making quantitative observations in live cells, because of the ability to observe dynamic processes in living cellular systems using these microscopies. The limited resolution of visible light (generally ~300 nm) used to make these observations, compared to the nanometer scale (1–10 nm) essential for understanding the molecular basis of many cellular processes, makes the molecular scale rather inaccessible. Consequently, these scales remain relatively uncharted in living cells.

Förster's resonance energy transfer (FRET) is one process involving the absorption of visible light whose measure is sensitive to the nanometer scale, making it most suitable to studying these scales in living cells. This review highlights important features of experiments that use FRET and on how to critically analyze FRET data.

The FRET Mechanism

In the FRET process, a donor fluorophore in its excited state nonradiatively transfers energy to an acceptor via a mechanism that may be understood as a dipole-induced dipole interaction (Förster, 1948). For FRET to occur, both a donor and acceptor fluorophore are required, wherein three important conditions have to be met. First, the emission spectrum of the donor fluorophore should overlap with the excitation spectrum of the acceptor molecule. Second, the physical orientation of the transition dipoles of donor and acceptor fluorophores should be favorable, and not orthogonal (perpendicular) to each other. And last and perhaps most relevant, the donor and acceptor fluorophores should be in close proximity to each other (1–10 nm) (see Figure 1A).

Given that the emission and the absorption spectra of the donor and acceptor molecules as well as the net orientation of their dipole moments do not change during the excited-state lifetime of the fluorophore, the efficiency of FRET, E, depends on the distance, r, separating them and is given by $E = (R_0)^6/[(R_0)^6 + (r)^6]$, where R_0 is Förster's radius. It typically ranges from 2 to 10 nm, providing a spectroscopic ruler at molecular dimensions (Stryer, 1978). R_0 is defined as that separation for which the energy transfer efficiency is 50% and is calculated using the expression

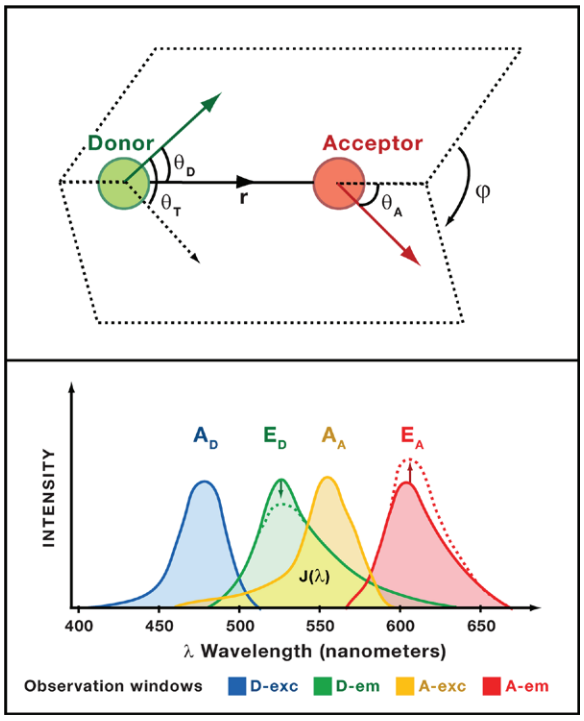

Figure 1. Schematic Depiction of the Fluorescence Resonance Energy Transfer Process

(A) Orientation of donor and acceptor transition dipoles. The relative angle between the two transition dipoles is responsible for depolarization of fluorescence upon energy transfer.

(B) Overlap integral J(λ) between the donor emission (E_D) and acceptor absorption spectra (A_A). A_D and E_A are the donor absorption and acceptor emission spectra, respectively. Arrows depict the decrease in donor emission and increase in acceptor emission intensities upon energy transfer. Observation windows show excitation and emission wavelength bandwidths for a typical imaging experiment, indicating the potential for crosstalk between the different imaging channels. D, donor; A, acceptor; exc, excitation; em, emission.

Modified from Krishnan et al. (2001) with permission.

$R_0 = 8.79 \times 10^{-5} \times [n^{-4} \times Q \times \kappa^2 \times J(\lambda)]^{1/6}$, where R_0 is in Angstrom units, n is the refractive index of medium in the range of overlap, Q is the quantum yield of the donor in the absence of acceptor, and $J(\lambda)$ is the spectral overlap as shown in Figure 1B. κ^2 is the *orientation* factor, which depends on the relative orientation of the two dipoles (Figure 1A) and is defined by $\kappa^2 = [\text{Cos } \theta_T - 3 \text{ Cos } \theta_A \text{ Cos } \theta_D]$, where θ_T is the angle between donor (D) and acceptor (A) moments given by $\cos\theta_T = \sin\theta_A \sin\theta D \cos\phi + \cos\theta_D \cos\theta_A$, in which θ_D and θ_A are the angles between the separation vectors, R, and D and A, respectively, and ϕ is the azimuth between the planes (D,R) and (A,R). For freely rotating donor and acceptor fluorophores, a dynamic average value of κ^2 is taken to be 2/3.

Fortunately, distance changes during many biological processes such as protein-protein interactions, conformational changes during protein folding or ligand binding, or changes in the arrangement of molecular complexes occur over the length scales defined by R_0. FRET is exquisitely sensitive to the distance separating donor and acceptor molecules; it has a dependence on the sixth power of the distance separating the two fluorophores, and thereby small changes in distances (from the subnanometer to the nanometer) result in large changes in FRET efficiency, allowing the determination of distances separating the donor and acceptor molecules at this resolution.

Determining FRET

Every energy transfer event is associated with several characteristic changes in the fluorescence properties of both the donor and the acceptor. Design of a FRET assay depends on what property of the donor or acceptor is being monitored (the reader is referred to more detailed reviews for examples of different types of FRET measurements: Jares-Erijman and Jovin, 2003; Sekar and Periasamy, 2003). Rarely is simply the detection of FRET a sufficient yardstick to estimate molecular proximity. There are several quantitative features of this fluorescence phenomenon that affect FRET efficiency, and understanding these features is crucial for a critical examination of FRET data.

We briefly discuss the effects of FRET on the donor and acceptor, and then review different ways to measure FRET quantitatively. When FRET occurs, the donor fluorophore reduces its emission intensity, lifetime of the excited state, and net emission anisotropy. The reduction in lifetime makes it resistant to photobleaching. The reduction in donor intensity, and changes in fluorescence lifetime, emission anisotropy, and photobleaching rates, can be reliable measures of FRET. For an acceptor, FRET increases sensitized acceptor emission and decreases emission anisotropy; measuring donor-sensitized acceptor emission and its depolarization are quantitative indicators of FRET.

Two main types of determinations of FRET are possible. These are *steady-state* and *time-resolved* measurements; the method used strongly determines the instrumentation required and the information that may be obtained.

Steady-State Measurements

For measuring decrease in donor fluorescence due to FRET, images of donor intensity are acquired in the presence and absence of acceptor and FRET efficiency is calculated as $E = 1 - (F_{DA}/F_D)$, where F_{DA} is the donor fluorescence intensity in the presence of acceptor and F_D is the donor fluorescence intensity in the absence of acceptor. The intensity of the donor has to be corrected for the emission of the acceptor in the donor channel (termed crosstalk) and the amount of photobleaching of the donor. In addition, the concentrations of the donor in the presence and absence of the acceptors should be comparable for these corrections to be realized. Without these corrections, the efficiency calculated will be inaccurate. This is one of the simplest measures of FRET and is often used for FRET measurements where the concentrations of the donor and acceptor species are under the experimentalist's control, such as in cuvette-based methods.

An extremely popular method called donor dequenching after acceptor photobleaching is a modification of this method, and involves monitoring the increase in donor fluorescence after photobleaching the acceptor. Donor intensity is recorded in the presence of acceptor, following which the acceptor is completely bleached. The donor intensity is recorded again and the FRET efficiency is calculated as $E = 1 - (F_{DA}/F_{DABI})$, where F_{DA} is the donor fluorescence intensity in the presence of acceptor and F_{DABI} is the donor fluorescence intensity after the acceptor has been bleached. The donor and acceptor pairs chosen for such an assay should be such that the acceptor can be easily photobleached and the donor should be resistant to photobleaching by the wavelength chosen for bleaching the acceptor. Corrections for donor photobleaching are extremely important here, compared to the previous method, because of the long photobleaching times usually used to bleach out the acceptor completely. Also, the amount of crosstalk in the donor channel has to be estimated. The advantage of this method is that FRET efficiency can be estimated in an imaging mode on a per-pixel basis (Bastiaens et al., 1996), providing optically resolved spatial information about nanometer-scale proximity. This method unfortunately cannot be used for experiments where rapid reorganization of donor and acceptor occurs during photobleaching times required to bleach acceptors.

Measuring rates of photobleaching of the donor is another way of measuring FRET and is easily implemented on a simple wide-field imaging system (Jares-Erijman and Jovin, 2003). In this case, the rate of donor photobleaching in the presence and absence of the acceptor is compared and the FRET efficiency is calculated as $E = (\tau_{DA}/\tau_D) - 1$, where τ_D is the rate of photobleaching in the absence of acceptor and τ_{DA} is the rate in its presence.

Perhaps the most widely used method to observe FRET is to estimate the extent of sensitized emission of the acceptor. These measurements are, however, con-

taminated by crosstalk from many sources (i.e., donor emission in the acceptor channel and donor excitation by the acceptor excitation wavelength) and appropriate corrections have to be made. This method is not quantitative unless a whole host of factors are known about the photophysics of the donor and acceptor system; however, it is the method of choice for most single-molecule FRET measurements where single-molecule fluorescence energy transfer is being monitored at the donor and acceptor emission wavelengths simultaneously.

A very important consideration in the above FRET measurements, especially detection of sensitized emission, is to monitor fluorophore intensities in detection channels for the donor and acceptor, individually, before proceeding with measurements that have both donor and acceptor present. This helps estimate the amount of crosstalk and allows estimation of appropriate correction factors for crosstalk (Nagy et al., 1998).

Energy transfer can also take place between like fluorophore species by exactly the same Förster's mechanism. This is known as homo-FRET or concentration-dependent depolarization (Weber, 1954). Homo-FRET may be measured by determining the anisotropy of fluorescence emission, a measure of how polarized the fluorescence emission is. When fluorophores are excited by polarized light, fluorescence emission is polarized unless the fluorophore rotates during the excited-state lifetime or transfers its energy to another molecule that is oriented differently from the direction of excitation polarization. A decrease in anisotropy when the rotational dynamics of the fluorophore is unaltered is indicative of FRET between like fluorophores; increasing FRET consequently results in decreased anisotropy. This is a useful method to monitor aggregation states of large molecules where the fluorophore emission is not completely depolarized due to rotational motion. FRET efficiency is calculated by the expression $E = 1 - (r/r_0)$, where r and r_0 represent anisotropy under conditions conducive to FRET or not, respectively. Steady-state anisotropy measurements are simple to implement under a normal epifluorescent microscope (Varma and Mayor, 2005).

Time-Resolved Methods

Fluorescence Lifetime Measurements. Time-resolved measurements can be used to estimate the decrease in donor lifetime expected as a consequence of FRET. The efficiency of FRET is calculated as $E = 1 - (\tau/\tau_d)$, where τ and τ_d are the fluorophore lifetime in the presence or absence of the acceptor, respectively. Time-resolved measurements yield additional information about the FRET phenomenon. If, for example, a FRET efficiency of 50% is calculated in a steady-state measurement, it could mean that either all fluorophores undergo FRET with 50% efficiency or half of them undergo FRET with 100% efficiency. The problem could be resolved by measuring lifetime of the donor fluorophore. In the first instance, a single lifetime is expected that is shorter than the lifetime observed in the absence of the acceptor. In the second instance, however, the fluorescence decay would no longer be a single exponential; two lifetimes would be observed with corresponding amplitudes, one that resembles the lifetime of the donor in the absence of the acceptor and another that is much shorter. The extent of change in lifetime is related to the efficiency of FRET, whereas the amplitude of the fast-decay component attributable to FRET is related to the fraction of donor species undergoing FRET.

Fluorescence Anisotropy Decay Measurements. Measuring decay of emission anisotropy in the time domain often allows the FRET process to be simply distinguished from any other process of anisotropy decay such as segmental motions and rotation of fluorophores, due to a separation of timescales. The rate of decay of anisotropy because of FRET is directly related to FRET efficiency and provides information about the distance between fluorophores, independent of the fraction of fluorophores undergoing FRET (Gautier et al., 2001).

In general, time-resolved measurements require expensive instrumentation, usually a pulsed laser or a frequency-modulated light source for excitation, a time-correlated photon-counting device or devices capable of monitoring changes in fluorescence decay at the nanosecond timescale, and elaborate software for acquiring data as well as data-fitting modules for calculating the lifetimes and rotational correlation time for time-resolved fluorescence and anisotropy decays (Bastiaens and Squire, 1999; Dong et al., 2003).

What Information Can FRET Reveal?

FRET has been used to study a variety of nanometer- and subnanometer-scale proximity-related questions regarding biomolecules in solution or inside cells. Some examples of the recent uses of FRET are detailed below.

Conformational Changes at the Subnanometer Scale during Protein Dynamics

Understanding conformational dynamics at the nanometer and subnanometer scale during changes induced upon ligand binding, mechanical stresses, and nucleotide hydrolysis provides a window into the mechanism of action of these complex machines. Correct positioning of fluorescent tags on a molecule in action can provide this information (see, for example, Figure 2). Determining molecular motions of a myosin motor while it undergoes its ATPase cycle (Shih et al., 2000) or structural transitions in single molecules of RasGTPase as it undergoes interactions with effectors (Arai et al., 2006) has revealed new insights into the functioning of nature's machines. The ability to incorporate nonnatural fluorescent amino acids paves the way for a new era for the use of FRET in studying protein dynamics in a test tube and in vivo. For example,

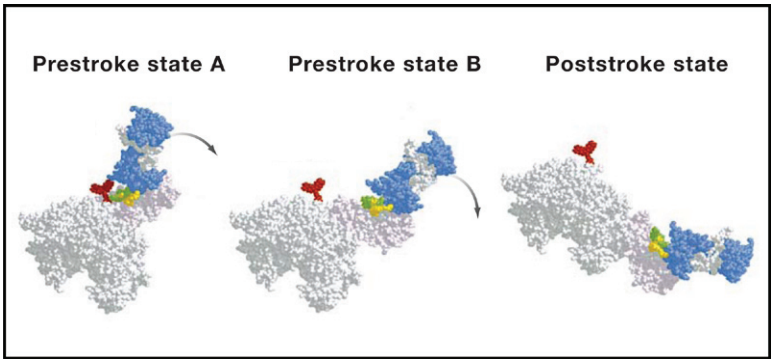

Figure 2. Location of Donor and Acceptor Fluorophores on Myosin Heavy and Regulatory Light Chains for FRET Studies
The heavy chain (HC) is colored white, the essential light chain (EL) is colored light violet, and the regulatory light chain (RLC) is colored blue. Tetramethylrhodamine-5-maleimide (the acceptor dye) attached to HC-Cys250 is colored red, and Oregon green 488 maleimide (the donor dye) is colored green or yellow according to its attachment to the RLC at either RLC-Cys114 or RLC-Cys116 (residues 113 and 115 in the chicken skeletal sequence), respectively. The distance between the donor and acceptor dyes decreases dramatically going from the poststroke to the prestroke states, leading to a large increase in FRET efficiency. Modified from Shih et al. (2000) with permission.

a recent study utilizing fluorescent nonnatural amino acids incorporated at different positions in calmodulin in a cell-free *Escherichia coli* translation system revealed structural transitions induced in calmodulin upon calcium binding (Kajihara et al., 2006).

Protein-Protein Interactions In Vivo

FRET has been extensively used to study protein-protein interactions in solution. However, the ability to combine imaging and FRET has paved the way for understanding the spatial and temporal interactions of proteins and other tagged molecules inside a living cell. The spatial and temporal interactions of transcription factors with histones and DNA architecture within the mammalian cell nucleus are beginning to be mapped using FRET methods (Cremazy et al., 2005), in the hope that understanding the complex and dynamic organization of these components inside the cellular nucleus might help elucidate mechanisms of gene expression. These studies, although in their infancy, indicate the power of FRET to uncover information vital to our understanding of dynamic changes in vivo.

Activation State of Signaling Cascades in Cells

FRET has been a very powerful way of monitoring the activation of signaling cascades as reviewed by Meyer and Teruel (2003) and Miyawaki (2003). For example, activation of RhoGTPases in live cells can be imaged using FRET biosensors. In this system, the GTPase binding domain of an effector protein is tagged with donor and acceptor fluorophores so that they undergo intramolecular FRET; binding of the RhoGTPase of interest relieves this FRET. Thus, depending upon the activation status of the GTPase, the binding domain will either be attached to the GTPase or not, resulting in changes in donor fluorescence with respect to the acceptor fluorescence; the ratio of donor to acceptor fluorescence will provide information on the spatial distribution of the activation state of the signaling GTPase. Similar methodology may be extended to other signaling molecules and modules, providing a real-time in vivo sensor of activation of key signaling nodes with the use of GFP-based FRET sensors (see Figure 3).

Structure of Macromolecular Complexes in Living Cells

Generally, the FRET process provides information on nanometer-scale proximity between donor-acceptor pairs (at least while using small organic fluorophores including those associated with fluorescent proteins); however, FRET may also be deployed to obtain information greater than the scale dictated by Förster's radii of fluorophores. This involves monitoring the change in FRET efficiency of an ensemble of fluorophores by changing donor and acceptor concentrations and having a rigorous theoretical model to explain how these changes could affect FRET efficiency. Two recent examples of this approach are illustrated in studies on nanometer-scale structures of lipid-anchored proteins and dynamics of a protein-translocating channel (Sharma et al., 2004; Snapp et al., 2004).

Interpreting FRET Data from Experiments

Most scientific research papers reporting FRET data will indicate how the FRET experiments were done. As indicated above, there are several caveats with each type of FRET measurement. Some obvious aspects of the experimental system must be detailed so that readers may be made aware of the concentrations of the species involved, potential angular orientations of the fluorophores employed, crosstalk factors inherent in the imaging system, and appropriate controls for positive and negative FRET signals.

Donor and Acceptor Fluorophore Concentration

While monitoring FRET between two species in membranes, knowledge of the concentration of donors and acceptors is crucial to distinguishing between nonspecific FRET arising from a high density of fluorophores and specific FRET due to molecular interactions. In membranes or in a two-dimensional surface, relatively low concentrations of fluorophores will inevitably contribute to a small fraction of membrane molecules that approach Förster's radius to generate (false or trivial) FRET signals; at 12,000/μm^2, the average separation is ~10 nm, close to the R_0 of some fluorophores. Under these conditions, significant FRET could occur without any specific interaction between donor and acceptor species with typical fluorophores. Thus, elaborate controls to monitor donor and acceptor concentrations as well as membrane localization are important to ensure detectability of a specific donor-acceptor interaction.

Monitoring interaction between soluble molecules by FRET does not suffer from such a large "false" positive problem; significant FRET is detectable only

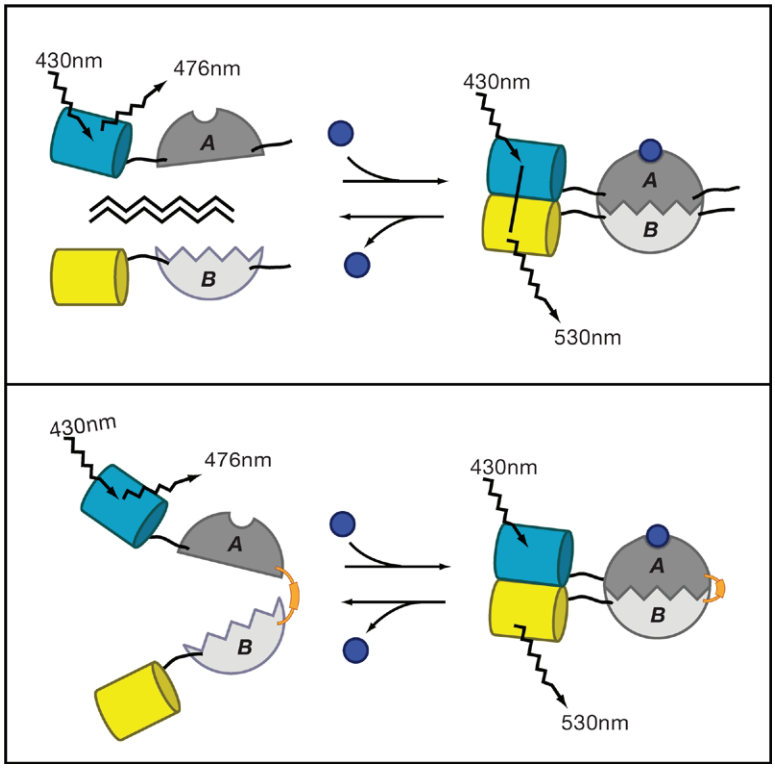

Figure 3. Design of GFP-Based FRET Sensors

(A) The basic principle of bimolecular fluorescent indicators. CFP and YFP (or any other suitable donor and acceptor pair) are fused to domains A and B, respectively, which interact with one another depending upon the ligand binding or modification of domain A. There are many variations in the design; CFP and YFP can be swapped, or signal-dependent dissociation of the two domains can be used. Incoming and outgoing saw-toothed arrows indicate excitation and emission at the stated wavelengths, respectively.

(B) The basic design of unimolecular fluorescent indicators.

Modified from Miyawaki (2003) with permission.

at 250,000 molecules/μm^3 or at ~3–5 mM concentrations of donor and acceptor molecules without any specific interaction between the two tagged species. However, proper controls for concentration are necessary, especially if the proteins of interest are overexpressed. If a specific protein complex is formed, the stoichiometry of donors and acceptors will dictate how the FRET signal could vary with changing donor and acceptor concentrations. In a nonspecific FRET process, increasing acceptor concentration while keeping the donor concentration fixed generally increases FRET efficiency as the probability of transferring to any acceptor increases. If there is a specific complex of defined stoichiometry, this increase will conform to a specific trend; theoretical modeling of this trend is necessary to understand the structure of this complex.

Coexisting Levels of Unlabeled Donor and Acceptor Species

If experiments are conducted in vivo in the presence of competing endogenous components, titration of the donor and acceptor may be necessary to give optimum results. False negative results could be attributed to the dilution effect of unlabeled, endogenous species.

Location and Orientation of Fluorescent Probes

It is important to remember that the FRET scale is very close to the size of a 30 kDa globular protein, which is an ~3 nm sphere. Therefore, inappropriate locations of fluorophores on interacting partners can lead to misleading negative results. For example, monitoring FRET between two proteins by using donor and acceptor fluorophores could potentially give false negative results if the fluorescent tags on the protein are located at distances too large for FRET. Thus, where possible, the location of the donor and acceptor fluorophores must be carefully chosen. The orientation of the fluorophores is another important aspect, as transfer efficiency depends strongly on the orientation of the fluorophores (see Figure 1); attention should be paid to the orientation of donor and acceptor probes with respect to each other and changes that could occur during any alteration in conformation. Wherever possible, small and freely rotating fluorophores should be chosen. Orientation and distance are intimately coupled in the determination of FRET; changes in either parameter can dramatically affect FRET readouts. Often, changes in FRET efficiency have been falsely attributed to a change in distance between the molecules, whereas in fact the orientation between donor and acceptor probes has been altered (Jares-Erijman and Jovin, 2003).

Choice of Fluorophore

The choice of fluorophore is an extremely important aspect, as this explicitly determines the scale of the measurement. There are a host of FRET pairs available that are either genetically encoded or may be attached to proteins or nucleic acids (Shaner et al., 2005; Zhang et al., 2002). Besides matching donor and acceptor excitation and emission spectra, there could be artifactual aggregation of donor and acceptor species, as noted in studies using certain GFP isoforms (Zacharias et al., 2002). At high concentrations achieved in membranes, this may give rise to false indications of protein-protein proximity. Another consideration is that the R_0 of any pair of fluorophores chosen should be very close to the expected distance scale in the experiment, as small-distance mismatches where the R_0 is much smaller than the distance to be sampled may lead to the lack of detectable FRET or false negatives.

Quantitative Analysis and Comparison with Theoretical Models

Many researchers ignore the quantitative aspect of FRET and look for black and white answers, and often they do not indicate how the experimental accuracy of FRET efficiency in their experiments was determined. These are also important considerations when critically evaluating FRET data. Constructing a theoretical model with available distance estimates may provide a check on the potential location and expected efficiency of FRET. Deviations from these expectations in the FRET experiment may help to refine the existing theoretical model.

Conclusions

With the ability to sense changes in the subnanometer to nanometer scale, in cuvette-based and imaging-based systems, coupled with the advent of a variety of intrinsically fluorescent proteins (Shaner et al., 2005), FRET has truly become a noninvasive technique allowing the study of nanometer spatial and temporal scales in a variety of cellular systems. Protein-protein interactions determined using immunoprecipitation and yeast two-hybrid-type experiments are all amenable to FRET-based analysis. Specifically transient, weak interactions in a fraction of molecules can easily be inferred using a FRET assay. FRET is also very useful in studying conformational changes over the subnanometer to nanometer scale in live cells, and this has allowed the development of activity sensors for a vast array of signaling systems (Meyer and Teruel, 2003; Miyawaki, 2003). Utilizing FRET techniques combined with theoretical modeling, where FRET efficiency may be modulated by structural and fluorescence changes, has opened the way to studying structures at the nanoscale in larger-scale protein assemblies in vivo (Rao and Mayor, 2005).

FRET is a very useful high-contrast technique because specific molecules of interest may be labeled with nonperturbing fluorophores or by using intrinsic fluorophores such as tryptophans in some proteins. The fast dynamics of the FRET process, usually picoseconds, allows measurement of transient dynamic interactions not accessible to most other spectroscopic techniques also capable of measuring dynamic conformational changes and aggregation states of proteins in solution such as NMR. Although three-dimensional structure at the Angstrom scale made accessible by X-ray crystallography and cryo-electron microscopy tomography has revealed innumerable protein structures, the structural changes leading to function remain largely unclear. FRET nicely fills this niche, taking advantage of this structural knowledge and locating this information in a living system in real time.

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