Fanciful FRET

Steven S. Vogel,* Christopher Thaler, Srinagesh V. Koushik

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The validity of experiments based on Förster resonance energy transfer (FRET), an imaging technique widely used to measure protein-protein interactions in living cells, critically depends on the accurate and precise measurement of FRET efficiency. The use of FRET standards to determine FRET efficiency, and a consideration of such factors as how the abundance of FRET acceptors and the stoichiometry of donors and acceptors in a molecular complex can affect measured FRET efficiency, will enhance the usefulness with which FRET experiments can be interpreted.

Introduction

The number of PubMed-listed citations using Förster resonance energy transfer or FRET (also known as fluorescence resonance energy transfer) has increased 10-fold over the past 10 years (Fig. 1). This rapid growth in the application of FRET to biological problems may seem surprising considering that resonance energy transfer was first observed in the 1920s (*1*). What is the reason for the renewed interest in an obscure photo-physical phenomenon? In vivo validation of potential protein-protein interactions identified by pull-down assays (*2*) and yeast two-hybrid technology (*3*) is highly desirable. We suspect that the renewed interest in FRET results from the availability of user-friendly imaging instrumentation and from the development of spectral variants of green fluorescent protein (GFP), which can be used for in vivo FRET imaging (*4*, *5*) to substantiate claims of protein-protein interactions. Despite the great potential of FRET imaging for investigating protein-protein interactions in vivo, we contend that the popularity of FRET has risen in concert with a degradation in the validity of the interpretations of biological FRET experiments. This, we believe, is because many authors pay insufficient attention to the finer nuances of FRET theory. In our opinion, problems in FRET interpretation can be avoided by (i) using FRET standards to determine how accurately and precisely FRET efficiency is being measured and (ii) considering how changes in the stoichiometry and abundance of donors and acceptors can affect measured FRET efficiency and thus the interpretation of FRET experiments. Our goal in this review is to show how failure to address these issues can limit the usefulness of FRET analysis. Although most of these issues apply to FRET measurements using any fluorophore, we limit our discussion to FRET performed with spectral variants of GFP.

What Is FRET?

FRET is a physical phenomenon in which energy absorbed by a fluorophore is transferred to another molecule through a nonradiative pathway. For resonance energy transfer to occur, three specific conditions must be met. (i) The emission spectrum of the fluorophore, also called the donor, must overlap the acceptor molecule's absorbance spectrum (Fig. 2A). Although the acceptor can be (and often is) a fluorophore, this is not a requirement for FRET. (ii) The emission dipole of the donor and the absorption dipole of the acceptor must not be oriented perpendicular to each other (Fig. 2B). The interactions of most molecules are limited to the volume occupied by their electron cloud. For example, the single orbiting electron of a hydrogen atom occupies an electron cloud whose radius is ~0.1 nm. Some molecules can influence others at much greater distances by projecting electromagnetic fields well beyond the limits of their electron clouds. In asymmetrical molecules, these fields typically have a specific orientation. An electromagnetic field will exist in a molecule that has one region with a net positive charge and another region with a net negative charge. This configuration with two oppositely charged areas defines a dipole (two poles), whereas the magnitude and orientation of a dipole's electromagnetic field are described by a mathematical term, the dipole moment. In some molecules, as typified by chromophores, an induced oscillating dipole is formed when the molecule interacts with an external electromagnetic field, such as light. For FRET, excitation light induces an oscillating field in the donor. The donor's oscillating emission dipole can then potentially influence the absorption dipole of an acceptor to oscillate in synchrony (if their spectra overlap). The strength of

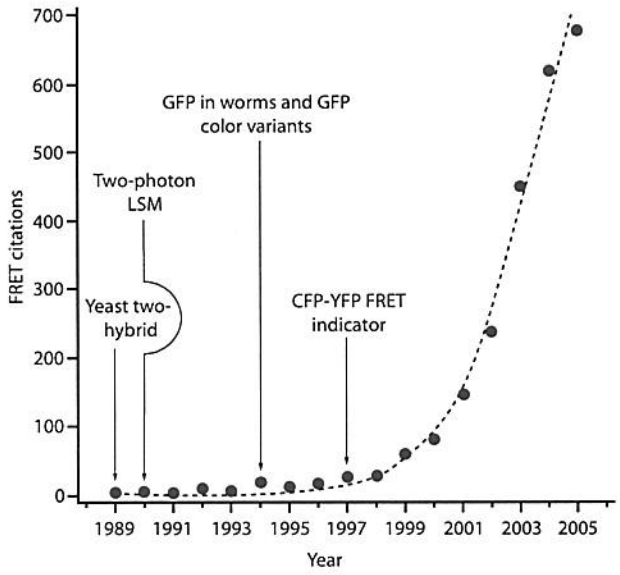
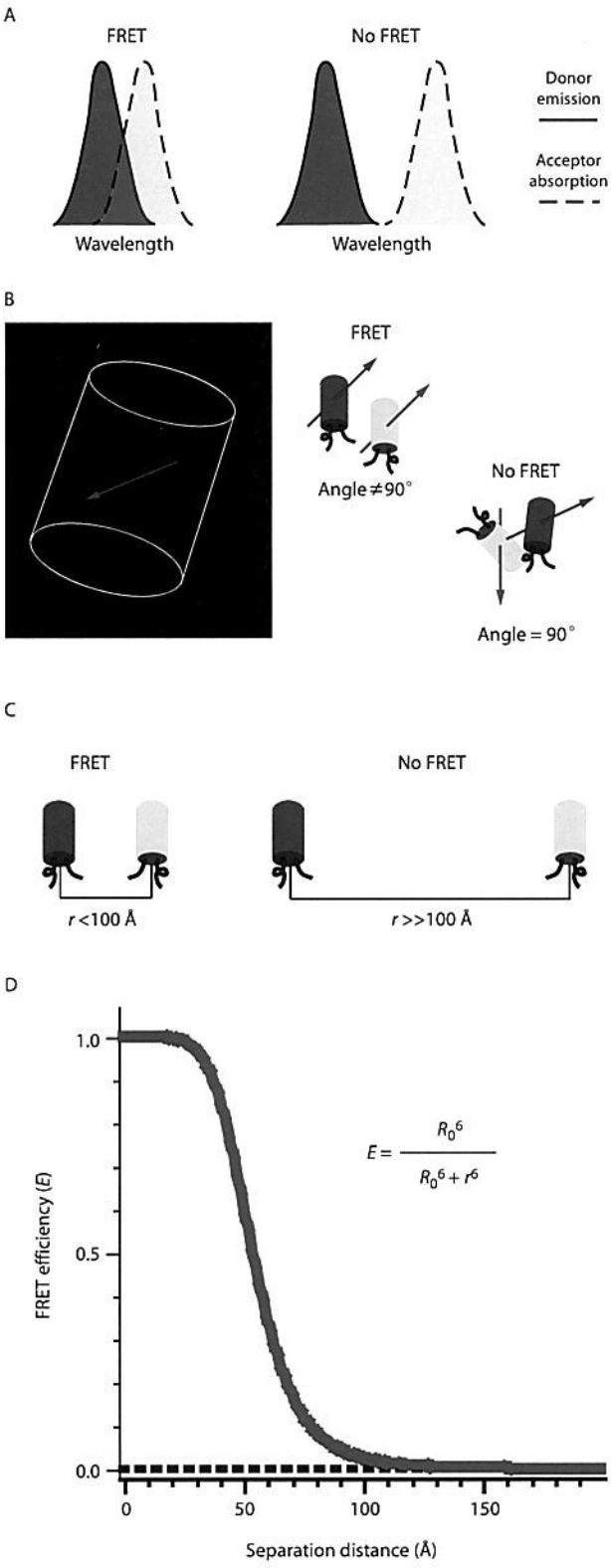


Fig. 1. The growth of PubMed (http://www.pubmedcentral.nih.gov/) citations containing the keyword "FRET" in the title or abstract over the past 16 years. Dates of important technological developments relevant to FRET are indicated with arrows.

National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Laboratory of Molecular Physiology, 5625 Fishers Lane, Bethesda, MD 20892, USA.

*Corresponding author. E-mail, stevevog@mail.nih.gov



such dipole-dipole coupling is a function of the electromagnetic field strength, as well as the relative orientation of the dipole moments. If the emission dipole of the donor is perpendicular to the absorption dipole of the acceptor, the oscillations will cancel each other out and there will be no net effect on the acceptor's dipole—in other words, no FRET. (iii) Donor and acceptor molecules must reside within 10 nm (100 Å) of each other (Fig. 2C). Förster showed that the efficiency of resonance energy transfer, or FRET efficiency (the fraction of the photon energy absorbed by a fluorescent molecule that is transferred to an acceptor through dipole-dipole interactions), has a sixth power dependence on the separation distance between the donor and the acceptor (*1*) (Fig. 2D). It is this strong dependence on separation distance that allows FRET imaging to be used to study molecular interactions on a 1- to 10-nm scale.

Why FRET?

As information about the identity and primary sequence of every protein in the genome grows, so too will the importance of identifying biologically relevant protein-protein interactions. Various techniques performed on cell extracts, fixed cells, or simplified systems—such as immunolocalization, pull-down assays, and yeast two-hybrid analysis (*2*, *3*, *6*)—have been used to suggest functions for numerous gene products, as well as for identifying the proteins with which they potentially interact. Caveats associated with these experimental techniques, however, necessitate their validation with experiments conducted inside living cells. This requirement for credible, in vivo substantiation of these types of experiments, in conjunction with the ability to genetically tag and express proteins of interest in living cells and organisms with fluorescent proteins (FPs) (*7*) [a family of fluorescent proteins derived from jellyfish and corals (*8*)], has driven a renaissance in live-cell microscopy.

A typical experiment exploiting FP technology involves determining the interactions, if any, between two tagged proteins. If two proteins directly interact with each other, they should colocalize within the cell. Cells expressing putative interacting proteins tagged with unique spectral variants of green fluorescent protein (GFP), such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), can be used to test this expectation. If proteins do not colocalize, it is unlikely that they have a stable interaction (though the possibility of transient encounters cannot be eliminated). Conversely, regions of colocalization

Fig. 2. Requirements for FRET. (**A**) FRET can occur only if the emission spectrum of the donor (solid line) overlaps the absorption spectrum of the acceptor (dashed line). The overlapping region is shown in green. (**B**) The enhanced GFP chromophore is depicted in yellow within the protein's cylindrical structure. Its dipole moment (red arrow) has a specific orientation relative to the structure of the FP. If the emission dipole (red arrow) of a donor (blue cylinder) forms a 90° angle with the absorption dipole (green arrow) of the acceptor (yellow cylinder), FRET cannot occur. Dipole-dipole angles other than 90° will support FRET with a range of efficacies. (**C**) Resonance energy transfer is observed only when the distance separating the two fluorophores (r) is less than 1.8 × the Förster distance (R_0), typically less than 100 Å. (**D**) The dependence of FRET efficiency (E) on separation distance (r) is plotted for the Cerulean-Venus donor-acceptor pair. The Förster distance (R_0) for this donor-acceptor pair is 54 Å, and this calculation assumes that their dipoles are randomly oriented.

do not necessarily mean that the two proteins directly interact. The limits of resolution for conventional light microscopy (*9*) constrain the ability to interpret colocalization experiments.

The excitation volume of a two-photon microscope using a high–numerical aperture objective can be as small as 0.1 fl (*10*). The volume of a GFP molecule is ~30 × 10^{-9} fl (*11*). Thus, colocalization of two fluorophores in a single pixel can occur even if the tagged proteins represent only 2 out of potentially 3 million GFP-sized proteins that can occupy the excitation volume. From this perspective it becomes clear that the simple observation of colocalization should not, by itself, be considered strong supporting evidence for a direct molecular interaction. A statistically significant positive cross-correlation between spatial distributions of fluorophores, however, can suggest associations between proteins (*12*), but direct interactions should still not be implied.

How does the "FRET volume"—and the maximum number of GFP-sized molecules estimated to fit therein—compare to the excitation volume of light microscopy? The Förster distance (the separation distance for a specific donor-acceptor pair that yields a 50% FRET efficiency, assuming random orientation of dipoles) (*13*) dictates the volume in which FRET can occur. This volume can be thought of as being approximately equivalent to a sphere with a donor at its center and a radius equal to ~1.8 times the Förster distance. A typical Förster distance for FP FRET would be ~5 nm (*5*), yielding a FRET volume of 4 × 10^{-6} fl. Out of 100 GFP-sized molecules that can fit into this volume, only 1 needs to be an acceptor for FRET to occur; this is a substantial improvement in the likelihood that the association of the two proteins is meaningful compared with colocalization. However, although a positive FRET signal does indicate that donor and acceptor dipole moments interact with each other, it does not necessarily mean that these molecules bind to each other. It should also be clear from the requirements for FRET described above and shown in Fig. 2 that the absence of a positive FRET signal does not necessarily indicate that two molecules are not interacting with each other; their dipoles, for example, might be aligned perpendicular to each other. Despite these constraints, FRET imaging remains one of the only high-resolution cell biological methods for measuring protein-protein interactions in living cells.

How Is FRET Measured?

Numerous methods have been used for measuring FRET (*14*, *15*). Although it is not possible to discuss all of these methods in a short review, we will describe the theoretical basis used to measure FRET and highlight the limitations of some of these techniques. FRET methods can be divided into four fundamental categories: (i) methods that monitor changes in donor fluorescence; (ii) methods that examine changes in acceptor fluorescence; (iii) methods that simultaneously measure changes in both donor and acceptor fluorescence using spectral imaging; and (iv) methods that monitor changes in the orientation of the fluorophores (Fig. 3).

The most direct methods for measuring FRET efficiencies are based on monitoring changes in donor fluorescence (either lifetime or intensity) in the presence and absence of acceptor. The two most common variants of this approach are FLIM-FRET (a method that measures FRET by monitoring changes in a donor's fluorescent lifetime, that is, how rapidly a population of fluorophores emits light after a short excitation pulse), based on fluorescent lifetime imaging (FLIM) (*16*–*18*); and acceptor bleaching (an approach that measures the intensity of donor fluorescence before and after photobleaching acceptors) (*19*–*21*). The fluorescent lifetime of a donor should get shorter if FRET is occurring; thus, by comparing the lifetime of the donor in the presence and absence of acceptors, the FRET efficiency can be measured. Similarly, measuring the fluorescence intensity of a donor before and after photobleaching acceptors is equivalent to measuring the intensity of the donor in the presence and absence of acceptors. Bleaching the acceptors should produce an increase in the donor's fluorescent emission if FRET had been occurring.

FLIM-FRET analysis typically uses curve-fitting algorithms to estimate the donor's fluorescent decay constant (a variable that parameterizes the kinetics of a fluorophore's decay) in the presence and absence of acceptors. A ratio of these time constants is then used to calculate FRET efficiency. Assumptions involved in curve fitting, such as the appropriateness of specific decay models for a particular sample, or even the specific method used for fitting must be considered to attain reasonable results with this method. Furthermore, FLIM analysis typically requires detecting large numbers of emitted photons for accurate results, and thus prolonged exposure to excitation irradiation. This, in turn, can result in bleaching, photodamage, and a degradation of the time resolution of the method. FLIM measurements also require specialized instrumentation.

An advantage of acceptor bleaching is that it can be performed on normal wide-field microscopes. For accurate FRET measurements, this technique requires complete bleaching of the acceptor (which can take several minutes) without bleaching the donor. Furthermore, when implemented with FP derivatives, the accuracy of these FRET measurements may be compromised by some of the peculiar photo-physics reported with FPs (*22*). For instance, the emission intensity of some FPs changes either stably (*23*), transiently (*24*), or reversibly (*25*) upon exposure to specific wavelengths of light. Other FPs display alterations in the wavelengths of their emissions (*26*). Clearly, photobleaching controls with isolated donors and acceptors must be included to rule out the possibility of such changes in response to high-intensity light.

The most popular methods used for measuring FRET involve monitoring acceptor emission as a result of donor excitation (*27*). The main advantage of this approach is that, like acceptor bleaching, it can be performed on simple wide-field fluorescent microscopes that are widely used in cell biology laboratories. This technique is commonly referred to as the three-cube method because it involves acquiring three different images using three fluorescent filter sets. First, a FRET image is generated by using a filter set that excites the donor but measures emission from the acceptor. Images obtained with filter sets that measure emission from donors or acceptors when directly excited are then used to correct the "FRET image." These corrections are required because the emission of the donor can bleed through into the FRET image and because excitation wavelengths that are used to excite donors can excite acceptors in some cases. The intensity observed in the corrected FRET image not only encodes information about the amount of FRET in the sample, but is also a function of the amount of sample present, the excitation intensity, the excitation wavelength, and the instrumentation used (filters, objectives, detectors, and the like). Thus, the FRET signal measured is not typically comparable to that obtained in experiments conducted on other microscopes. In contrast, a measured FRET efficiency for

a particular sample should be the same regardless of what microscope or method is used to obtain it. Thus, the challenge of this method lies in transforming the intensity information encoded in the corrected FRET image into units of FRET efficiency. Recently, variants of this method calibrated using either FLIM-FRET (*28*) or acceptor-bleaching (*29*, *30*) have been implemented to measure actual FRET efficiencies. An additional advantage of the three-cube approach is its speed of data acquisition (typically requiring a few seconds as compared to minutes for most of the other FRET imaging approaches). Thus, these FRET methods have a distinct advantage for applications requiring time-lapse or three-dimensional FRET imaging in living cells.

Recently, a photon-efficient FRET method has been developed that monitors changes in the intensity of donors and acceptors simultaneously using spectral imaging. Spectral imaging microscopy measures a complete emission spectra for each pixel (*31*). Encoded in the emission spectrum is information about the abundance of donors and acceptors, as well as about the FRET efficiency. Theoretical approaches for measuring FRET using spectral imaging have been described (*32*) and implemented (*33*) using two-photon microscopy (*34*) (a form of microscopy in which two coincident infrared photons, each with only half of the energy required to excite a particular fluorophore, are used to excite a fluorophore). An advantage of using spectral imaging to measure FRET is that in addition to yielding FRET efficiency, it also measures the abundance of donors and acceptors. Because two-photon excitation is usually implemented with tunable lasers, wavelengths that efficiently excite both donors and acceptors can be selected. This is important because the judicious selection of excitation wavelengths for specific samples can maximize the signal-to-noise ratio of both donor and acceptor signals (*33*). This FRET method requires specialized equipment for spectral imaging, two-photon excitation, and purified samples of the donor and acceptor fluorophores to provide reference spectra.

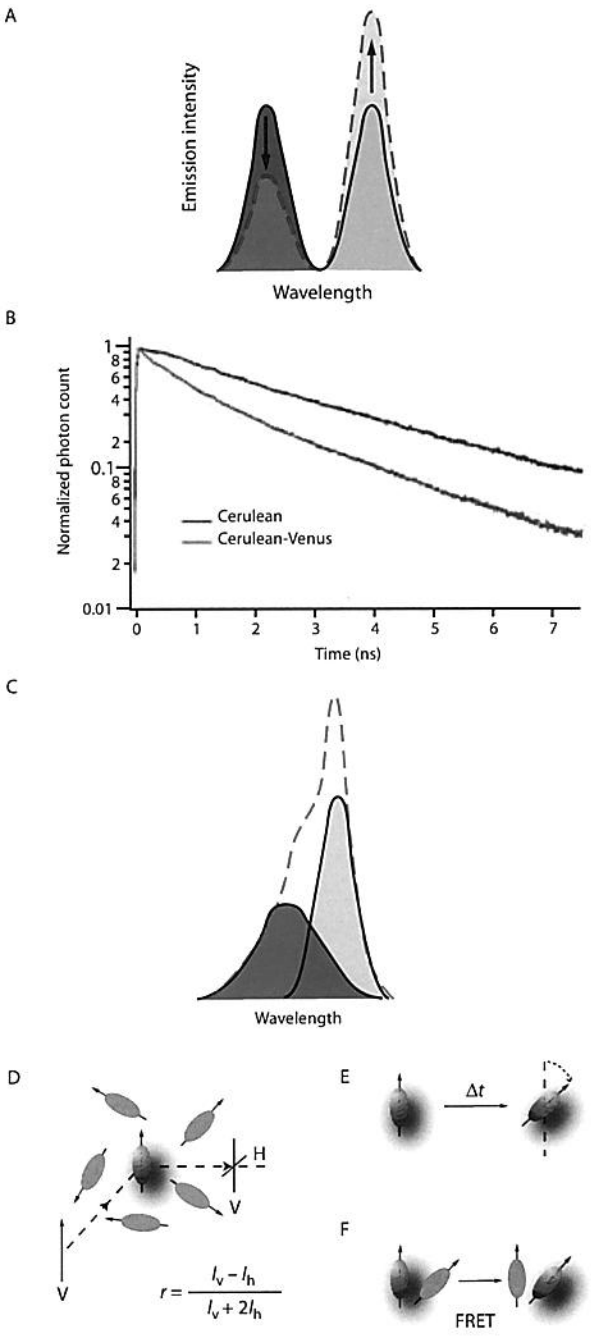


Fig. 3. Measuring FRET. (**A**) When FRET occurs between two fluorophores, the intensity of the donor's fluorescent emission (blue) will decrease, and the intensity of the acceptor's emission (yellow) will increase [see transition from solid black curve (no FRET) to dashed red curve (FRET)]. These changes form the basis of FRET measurements obtained by monitoring either donor or acceptor intensity. (**B**) Changes in a donor's fluorescent lifetime decay can also be used to measure FRET. The blue decay curve was obtained from cells transfected with Cerulean. The red decay curve was obtained from cells transfected with a construct in which Cerulean is closely attached to Venus (*33*). The area between these curves represents the energy that was transferred by FRET from donor (Cerulean) to acceptor (Venus) in the Cerulean-Venus construct. (**C**) The complex emission spectrum observed (red dashed line) in cells that are expressing two fluorophores is the linear sum of the emission spectra from donors (blue) and acceptors (yellow). Excitation wavelength–dependent changes in the shape of the complex emission spectra can be used to measure FRET (*33*). (**D**) Fluorescence anisotropy can be used to measure FRET. When a randomly oriented population of fluorophores is excited with linearly polarized light, molecules whose absorption dipole is oriented parallel to the polarization axis are preferentially excited. The resulting anisotropy (r), a measure of the degree of orientation, can be determined by measuring the emission intensity through vertically (I_v) and horizontally (I_h) oriented polarizers. The anisotropy signal will decrease (called depolarization) if the fluorophore can change its orientation in the time interval between when it is excited and when it emits a photon. (**E**) Depolarization can also occur when the fluorophore transfers its excitation energy to a neighboring molecule with a different orientation. (**F**) Because energy transfer can occur much more rapidly than molecular rotation, particularly for FPs, depolarization due to FRET can be distinguished from depolarization due to motion.

Fluorescence polarization anisotropy measurements can be used to measure FRET (*35–39*) (Fig. 3D). FRET methods based on anisotropy compare the orientation of the molecules excited (by linearly polarized light) with the orientation of the molecules that emit fluorescence (in response to excitation). In the absence of FRET, the molecule excited is the molecule emitting fluorescence, and thus its orientation will be highly correlated, particularly for large, slow-moving fluorophores such as FPs. If FRET occurs, the molecule excited may be different from the molecule emitting, and thus the correlation between their orientations will decrease substantially. Anisotropy has been used to monitor FRET in samples where donors and acceptors are different fluorophores (*40*), and it is the only method capable of measuring FRET between a single fluorophore (called homo-FRET) (*41*, *42*). Homo-FRET has been used to monitor dimerization reactions (*37*) and has great potential for following subunit interactions of homo-oligomers (*36*). The theoretical basis for interpreting and quantifying FRET efficiency measurements using anisotropy warrants further study.

Considering the many methods used for measuring FRET, each with its own specific experimental nuances and underlying assumptions, leads us to conclude that the possibility of errors in the accuracy of FRET measurements is real.

The Accuracy of FRET Measurements

In many studies, the accuracy (how close a measured value is to the actual value) of FRET measurements is not known. When, for example, a 10% FRET efficiency is measured, should we consider if the actual transfer efficiency is 5% or 15%? Such errors could affect our interpretations of a FRET experiment and limit the usefulness of FRET for studying protein-protein interactions.

Biologists use FRET primarily to determine the separation distance between two fluorophores or to measure a structural change as a result of a biological perturbation. Because of the sixth power dependence of FRET efficiency on fluorophore separation distance (Fig. 2D), even small errors in the accuracy of FRET measurements can have large effects on both the absolute separation distances estimated from such experiments and on the relative changes. Hence, the accuracy required for any particular FRET experiment must be evaluated on a case-by-case basis with this nonlinear dependence in mind. Furthermore, FRET efficiencies measured in cells are often an ensemble measurement arising from both specific protein-protein interactions and from random associations (see below). Unraveling the origins of a FRET signal under these conditions involves statistical comparisons between the experiment and controls. In this situation, knowledge of the accuracy as well as the precision (how well repeated measurements of a quantity agree) of a FRET measurement is essential.

Why has it not been possible to determine the accuracy of FRET measurements? The reason is primarily because FRET standards (molecules with known FRET efficiencies) have not been defined or adopted by the scientific community. Without standards, cross-comparisons between FRET methods are not possible. Studies using multiple methods for measuring FRET efficiency are seldom conducted and, when attempted (*43*), frequently fail to yield the same results, indicating the possibility of experimental or conceptual errors. However, with FRET standards, separate laboratories using any method for measuring FRET efficiency would be able to properly calibrate their system to yield accurate experimental results.

Further confounding our ability to determine the accuracy of FRET measurements is the common practice of using indices instead of FRET efficiencies as the experimental result (*43*). FRET indices vary from method to method. In its simplest form, a FRET signal is generated at a wavelength that primarily excites the donor. The fluorescence emission is then detected through either an acceptor-specific filter or a donor-specific filter, and these values are divided to form a FRET index. With more complex indices, corrections for spectral bleed-through and direct excitation of acceptor are applied to form corrected indices. Finally, in the most complex index schemes, corrected FRET indices are normalized either to the donor signal or to the acceptor signal. Unlike FRET efficiency, which should be the same for a particular sample regardless of the method or microscope used to measure it, FRET indices, even for the same samples, can vary widely. We contend that their use frustrates comparison of results with those obtained from other laboratories using other methods. Because indices often have a nonlinear dependence on the abundance of donors or acceptors (*30*, *43*), the use of these indices can exaggerate or diminish the perception of the underlying FRET efficiency. In our opinion, their use should be avoided whenever possible. For example, consider a simple case in which the use of an index might seem justified: a FRET-based calcium indicator composed of a single donor and acceptor covalently attached to each other through a calcium-binding moiety. Calibration curves are used to equate changes in the FRET signal to changes in the concentrations of free calcium. There is no obvious reason to know the actual change in FRET efficiency to observe a change in the free calcium concentration with such an indicator. Yet, we would argue that knowledge of the value and magnitude of the change in FRET efficiency used to detect changes in the concentration of free calcium could alter our confidence in such measurements. A change in calcium concentration based on a 10% FRET efficiency would be much more compelling than one based on a 1% change. Similarly, measurements based on changes in FRET efficiency obtained with donor-acceptor separation distances comparable to the Förster distance (as compared to measurements made when the donor-acceptor separation distances are either much greater or smaller than the Förster distance) would be more compelling because in this regime, small changes in distance yield large changes in FRET efficiency. Thus, even for this simple case we conclude that, if a FRET measurement can be reported as a FRET efficiency, it should be.

Interpreting FRET Measurements

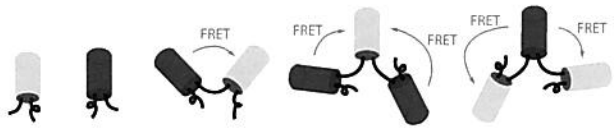
Assuming accurate FRET efficiency measurements, the evaluation of a FRET experiment must consider the precision of the experimental determination. The degree of precision required in a measurement is dictated by the biological states that the researcher is trying to differentiate. These states might have an intrinsic variance with biological significance. For example, the distance separating a donor and acceptor might not be discrete but rather may comprise a distribution of separation distances in a population of molecules. Such behavior is typically observed when a donor is attached to an acceptor by a flexible tether. The precision measured in a FRET experiment will be a convolution of the instrumental error of the measurement and the underlying biological variability. Thus, the instrumental error of the method used to measure FRET can limit the ability to observe biological variability. The simplest method for measuring FRET

efficiency involves calculating the ratio of the fluorescent emission from the donor in the presence and absence of acceptor (*41*). The emission can be measured by counting the individual photons detected. In this situation, counting statistics will limit the best instrumental precision possible (*44*); the more photons counted, the better the precision that can be attained. Data-acquisition systems constrain the number of discrete events that can be counted in an experiment. Thus, digitization resolution can limit precision. With 8-bit digitization, a sample with a 5% FRET efficiency should have a variance of at least 10% due to counting statistics. With 12-bit digitization, this variance can theoretically be reduced to ~2%, and with 16-bit digitization it falls below 1%. Thus, although less than 1% instrumental error can be achieved with current technology, the 12-bit resolution of modern confocal microscopes, and of typical high-quality charge-coupled device cameras, limits the precision of FRET measurement to at best 2%. Although extensive signal averaging might increase this precision, biological variance superimposed on this instrumental error can further degrade one's ability to differentiate FRET efficiency differences. In our laboratory, the precision of FRET efficiency measurements depends on the method used and ranges from 3 to 9%.

Because of the nonlinear sixth power dependence of FRET efficiency on separation distance, the precision of a FRET measurement will have different meanings, in terms of molecular structure, at different absolute FRET efficiency values. For example, a FRET efficiency of 10 ± 5% for a donor-acceptor pair with a Förster distance of 5.4 nm predicts a separation distance of 7.8 nm, but with a range between 6.8 and 8.3 nm (assuming random orientation of dipoles). In contrast, a FRET efficiency of 50 ± 5% (the same precision as in our previous measurement) predicts a separation distance of 5.4 nm but with the range limited to ± 0.2 nm.

Even assuming high accuracy and precision, it remains important to realize that other factors influence the interpretation of measured FRET efficiencies. Most important is knowledge of the local concentration of acceptors. Donors targeted to the cytoplasm in the presence of high acceptor concentrations (4 mM, assuming a Förster distance of 5 nm) will produce FRET signals as a result of random acceptor distributions (*41*). In essence, at these high acceptor concentrations, a randomly placed donor will have a high probability of always being within 10 nm of at least one acceptor. If these donors and acceptors are confined to subcellular regions, such as in membranes, as few as 100 acceptors per μm^2 can result in "random FRET" (*45*). In our laboratory, we find that almost any pair of integral membrane proteins labeled with a donor and an acceptor will generate approximately a 5% random FRET efficiency when coexpressed in the same membrane under normal imaging conditions (*46*). Determining what fraction of a FRET signal arises from specific protein-protein interactions as compared to that arising from random interaction due to overcrowding requires knowledge of the local abundance of acceptors in the sample. Random FRET between membrane-targeted donors and acceptors has important ramifications when considering what constitutes a valid negative control for FRET experiments. Clearly, it is not legitimate to use membrane-bound donors coexpressed with cytosolic acceptors as a negative control for a positive FRET signal observed when membrane-resident donors and acceptors are expressed together.

The stoichiometry of donors and acceptors in a molecular complex can also dramatically alter FRET efficiency (*41*). This can best be understood by considering the measured FRET efficiencies of some FP constructs (Fig. 4), and an even deeper appreciation of the complexities of interpreting FRET measurements can be obtained by conducting thought experiments involving defined mixtures of these constructs. For example, a CV construct that contains one Cerulean (*47*) (a CFP variant) covalently attached to one Venus (*48*) (a YFP variant) has a FRET efficiency of 45% (Fig. 4) (*33*). This construct has a Venus:Cerulean ratio (V/C) of 1. A closely related construct containing two Cerulean molecules attached to one Venus has almost the same FRET efficiency (41%) but has a V/C value of 0.5, whereas a near-identical construct containing two molecules of Venus attached to one Cerulean has a V/C ratio of 2 and a FRET efficiency of 70%. These counterintuitive findings arise because FRET efficiency is based on the donor's environment (not the acceptor's). A Cerulean molecule alone has a FRET efficiency of 0% because it has no acceptors. A FRET efficiency for Venus alone cannot be defined because in this situation it is acting as an acceptor, and donors are not present. The FRET efficiency is a function of how many acceptors are near the donor and how close they are. Consider a more complex scenario where more than one construct can be present in a sample. Our first example is a cell expressing equal amounts of Cerulean and Venus (the first thought experiment in Fig. 4). If the concentration of Venus is low (<<4 mM), we can assume that random FRET will not occur. Because the



Thought experiments

Mixture	1:1 mix of C and V	1:1 mix of V and CV	1:1 mix of C and CV	1:1 mix of V and CVC	1:1 mix of C and VCV
Estimated FRET efficiency	$E = 0\%$	$E = 45\%$	$E = 22.5\%$	$E = 41\%$	$E = 35\%$
Estimated V/C ratio	V/C = 1	V/C = 2	V/C = 0.5	V/C = 1	V/C = 1

Fig. 4. Cartoon of Venus (*48*), Cerulean (*47*), and three molecular constructs that transfer energy by FRET from Cerulean (blue cylinder) to Venus (*48*) (yellow cylinder). FRET efficiency (*E*) of Cerulean alone is defined as 0%, and for the three remaining constructs it was measured by spectral imaging. The details of their construction and methods of FRET efficiency measurements are described in (*33*). Red arrows indicate the primary direction of resonance energy transfer in these constructs. The table shows the predicted FRET efficiency (*E*) and ensemble Venus:Cerulean ratio (V/C) for five different 1:1 mixtures of the constructs depicted above. Note that samples with the same V/C values can have different FRET efficiencies.

monomeric forms of Cerulean and Venus used in this thought experiment should not bind to each other (*49*), we expect an ensemble FRET efficiency of 0% and a V/C ratio of 1. This situation, however, would have the same V/C ratio as one in which a cell expresses the CV construct alone, but in that situation the FRET efficiency was 45%. A cell expressing equal amounts of Venus and CV construct should have the same ensemble FRET efficiency as a cell expressing the CV construct alone, but its V/C ratio will be 2 instead of 1. Similarly, a cell expressing only the VCV construct will also have a V/C ratio of 2, but its FRET efficiency was 70%. Clearly, interpretation of FRET signals requires knowledge of the stoichiometry of donors and acceptors in the specific protein complexes. Although the relative abundance of donors and acceptors can be determined in living cells with some FRET methods (*28*, *33*), these ensemble measurements do not necessarily translate into the donor-acceptor stoichiometry of a specific complex of interest. Single-molecule FRET analysis, however, can potentially determine these values (*50*–*52*).

Despite the many limitations involved in interpreting FRET measurements outlined above, FRET remains one of the best methods for studying protein-protein interactions in living cells. FRET can also be used to validate structural details revealed by x-ray crystallography and electron microscopy, but in a living cell. The challenges of using FRET imaging to study protein-protein interactions lie in devising ways to overcome these limitations. An appreciation of the precision of FRET methods, as well as an understanding of how the dynamics of acceptor abundance, random FRET, and donor-acceptor stoichiometry affect FRET efficiencies, can substantially reduce the number of erroneous interpretations of FRET experiments that have been observed in recent studies. In conclusion, the development, characterization, free dissemination, and ultimate scholarly acceptance of specific FRET standards will go a long way toward eliminating errors in the accuracy of FRET measurements.

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