Research Techniques Made Simple: Methodology and Applications of Förster Resonance Energy Transfer (FRET) Microscopy

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Classical biochemical techniques have contributed a great deal to our understanding of the mechanisms regulating fundamental biological processes. However, these approaches are typically end-point, population-based assays and are often insufficient in examining transient molecular events. Förster resonance energy transfer (FRET) microscopy is a powerful technique capable of investigating dynamic interactions between proteins and a plethora of biochemical signaling events based on the development of specific biosensors. This technique exploits the principle that when FRET occurs, energy from a donor fluorophore is transferred to an acceptor fluorophore only when certain conditions are met. These include dependence on both distance and fluorophore orientation. In this article, applications of FRET microscopy to protein interactions and modifications are discussed, and examples are given of the types of biosensors that can be developed. There are a number of methods to measure FRET. The most common modalities and specific advantages and shortcomings for each are reviewed. Finally, general considerations and guidelines for choosing a method are discussed.

Journal of Investigative Dermatology (2017) **137,** e185–e191; doi:10.1016/j.jid.2017.09.006

CME Activity Dates: 18 October 2017
Expiration Date: 17 October 2018
Estimated Time to Complete: 1 hour

Planning Committee/Speaker Disclosure: All authors, planning committee members, CME committee members and staff involved with this activity as content validation reviewers have no financial relationship(s) with commercial interests to disclose relative to the content of this CME activity.

Commercial Support Acknowledgment: This CME activity is supported by an educational grant from Lilly USA, LLC.

Description: This article, designed for dermatologists, residents, fellows, and related healthcare providers, seeks to reduce the growing divide between dermatology clinical practice and the basic science/current research methodologies on which many diagnostic and therapeutic advances are built.

Objectives: At the conclusion of this activity, learners should be better able to:
- Recognize the newest techniques in biomedical research.
- Describe how these techniques can be utilized and their limitations.
- Describe the potential impact of these techniques.

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INTRODUCTION

Macromolecular interactions and modifications are the foundation for every biological process and pathological condition occurring in our bodies, functioning in an extraordinarily complex and dynamic network of cellular signaling events. The advent of modern light-based microscopy has enabled researchers to observe molecules in their native habitat, in real-time, in living cells and tissues. However, the inherent applications of light-based microscopy to the study of protein interactions and modifications is somewhat limited. This can be overcome by combining conventional light-based microscopy with other techniques. Förster resonance energy transfer (FRET) is a process by which energy is transferred from one fluorophore (the donor) to a second

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Abbreviations: ER, endoplasmic reticulum; FLIM, fluorescence-lifetime imaging microscopy; FP, fluorescent protein; FRET, Förster resonance energy transfer

ADVANTAGES

- The ability to perform biochemical assays in real time in living cells
- A large number of FRET-based biosensors have already been created and characterized
- New biosensors can be generated to assess a protein of interest, although some expertise is necessary
- Some FRET measurement methods are relatively inexpensive and can be performed on standard wide field microscopes
- Potential applications in high-throughput screening

SHORTCOMINGS

- Data analysis can be complex, depending on sensors and methodology
- Some FRET measurement methods require expensive specialized equipment
- FRET efficiency can be quite low, and false positives and false negatives must be controlled for
- Some FRET-measurement methods are best suited as an endpoint assay
- Biosensors using FPs as donors and acceptors require careful characterization, because the bulky FP-tag can affect protein function

DEFINITIONS

- Fluorescence: The process by which a molecule that has absorbed light emits light at a longer wavelength than the absorbed light
- Nonradiative transfer: Transfer of energy without releasing a photon of light
- Anisotropy: Unequal physical properties along different axes
- Tension: Pulling force transmitted along an axis

fluorophore (the acceptor) in a nonradiative manner, rather than being emitted as a photon of light from the donor (fluorescence). This phenomenon is strongly dependent on the distance between the two fluorophores, occurs most efficiently when they are within 10 nm of each other (Figure 1), and decreases exponentially with increasing distance (Pietraszewska-Bogiel and Gadella, 2011). Therefore, FRET has historically been used to determine the close proximity of two molecules of interest.

One of the most widely used applications of FRET in the life sciences is FRET microscopy using genetically encodable biosensors containing fluorescent proteins (FPs) as the donor

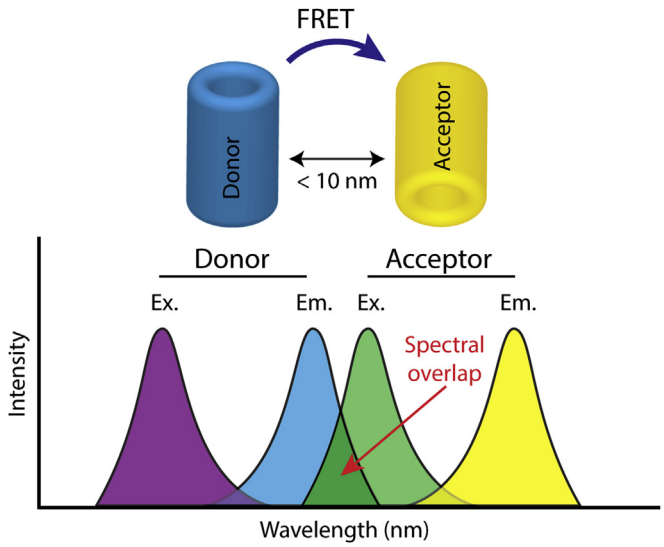

Figure 1. Requirements for FRET. The donor molecule emission (Em.) and acceptor molecule excitation (Ex.) spectra must overlap. FRET efficiency is highest when the donor and acceptor molecules are within 10 nm of each other and their dipoles are at a parallel orientation. FRET, Förster resonance energy transfer.

and acceptor fluorophores (Day and Davidson, 2012). For this reason, FRET has sometimes been dubbed fluorescence resonance energy transfer. Often, cyan and yellow FPs are used, because there is sufficient spectral overlap between the emission of cyan FP and the excitation of yellow FP for FRET to occur (Figure 1), and these FPs have a high quantum yield (i.e., emission efficiency). Other suitable pairs of FPs include green and red pairs, which offer advantages for live cell imaging such as low phototoxicity and less photobleaching. In addition to using two FPs as donor and acceptor molecules, other fluorophores including quantum dots, lanthanides, SNAP-tags, and fluorescein arsenical hairpin binder (i.e., FLAsH)-based tags, among others, can be used on their own or in combination with FPs to generate FRET biosensors (Emami-Nemini et al., 2013; Rajendran et al., 2014; Stanisavljevic et al., 2015). For the sake of simplicity, we will focus on sensors using FPs in our examples.

Unlike conventional biochemical approaches, FRET-based biosensors facilitate the examination of protein interactions and signaling events in their normal cellular environment, in many cases in living cells. Because FRET is performed in intact samples, it provides a distinct advantage for studying skin biology, because its use enables investigators to examine functions in specific layers of the epidermis, for instance in three-dimensional epidermal equivalent culture models. The use of FRET probes allows the investigator to retain spatial information that can be lost when using traditional biochemistry techniques. Here, we describe common applications of FRET biosensors (Figure 2) and the methods used to measure FRET.

APPLICATIONS

Protein-Protein Interactions

Because FRET is highly dependent on the distance between the two FPs, it is often used to observe the interactions

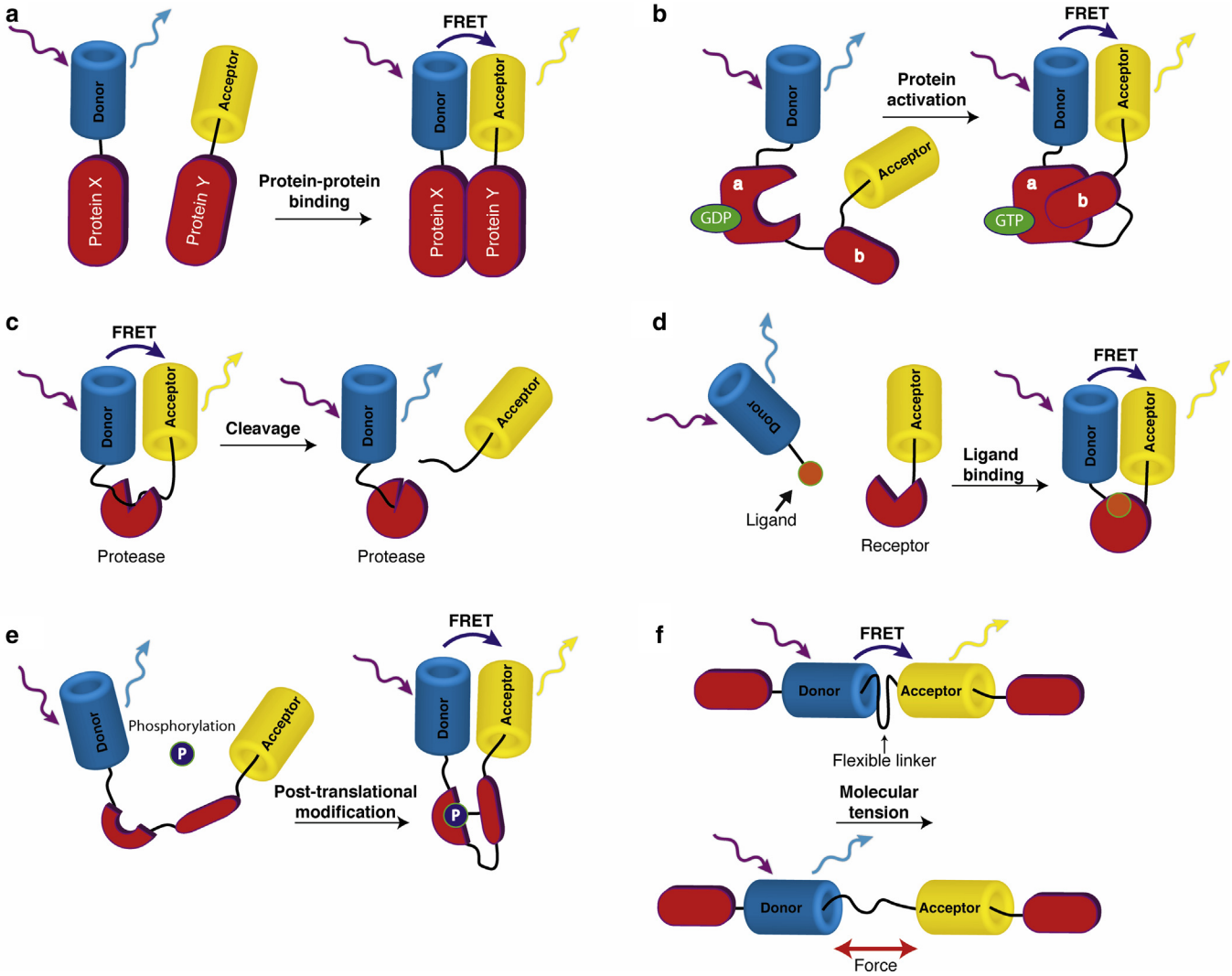

Figure 2. Common applications of FRET sensors. (**a**) FRET can be used to study protein-protein interactions. Here, two proteins of interest (X and Y) are tagged with a donor and acceptor, and FRET occurs upon interaction between the two proteins. (**b**) In this biosensor, an inactive GTPase (**a**), bound to GDP, does not interact with a downstream effector protein that is part of the same fusion protein (**b**). Once activated, the GTP-bound GTPase interacts with the effector, and FRET occurs. (**c**) A biosensor can be designed to examine the activity of a protease. Here, protease-mediated cleavage inhibits FRET. (**d**) A ligand tagged with a donor will FRET with a receptor tagged to an acceptor upon ligand binding. Biosensors can be designed to observe conformational changes in a protein of interest. (**e**) For example, posttranslational modifications such as phosphorylation induce conformational changes that cause the biosensors to FRET. (**f**) Biosensors can be designed to assess tension within a protein of interest by incorporating a FRET module with a flexible linker that loses the ability to FRET when force is applied to the molecule. FRET, Förster resonance energy transfer; GDP, guanosine diphosphate; GTP, guanosine triphosphate.

between two proteins of interest. In the case of intermolecular FRET, one protein contains the donor FP and the other the acceptor FP. When the two proteins come close enough together (i.e., through binding), then FRET occurs between the FPs (Figure 2a). When performing intermolecular FRET analysis, variation in the expression levels of the two FP-containing proteins must be taken into account, complicating data analysis. However, when properly controlled, this method enables researchers to visualize where in a cell two proteins are interacting. For example, De Filippo et al. (2017) coexpressed the melanogenic enzyme tyrosinase tagged with a donor FP and a trafficking mutant version of G-protein–coupled receptor 143 tagged with an acceptor FP and determined that, unlike wild-type G-protein–coupled receptor 143, the trafficking mutant interacts with tyrosinase at the plasma membrane (Figure 3).

Many of the newer FRET biosensors are intramolecular probes containing both the acceptor and donor within the same construct. Intramolecular probes can still be used to examine protein-protein binding, if designed properly. For example, intramolecular biosensors have been generated that measure Rho family GTPase activation by including both the GTPase and a downstream effector protein domain in the same sensor (Nakamura et al., 2006). In this case, when bound to guanosine diphosphate, the inactive GTPase and effector do not interact, producing no FRET. When bound to guanosine triphosphate, the active GTPase binds to the effector, and FRET occurs (Figure 2b).

Enzymatic cleavage

FRET biosensors can also be generated to examine the enzymatic activity of a protein of interest (Zauner et al.,

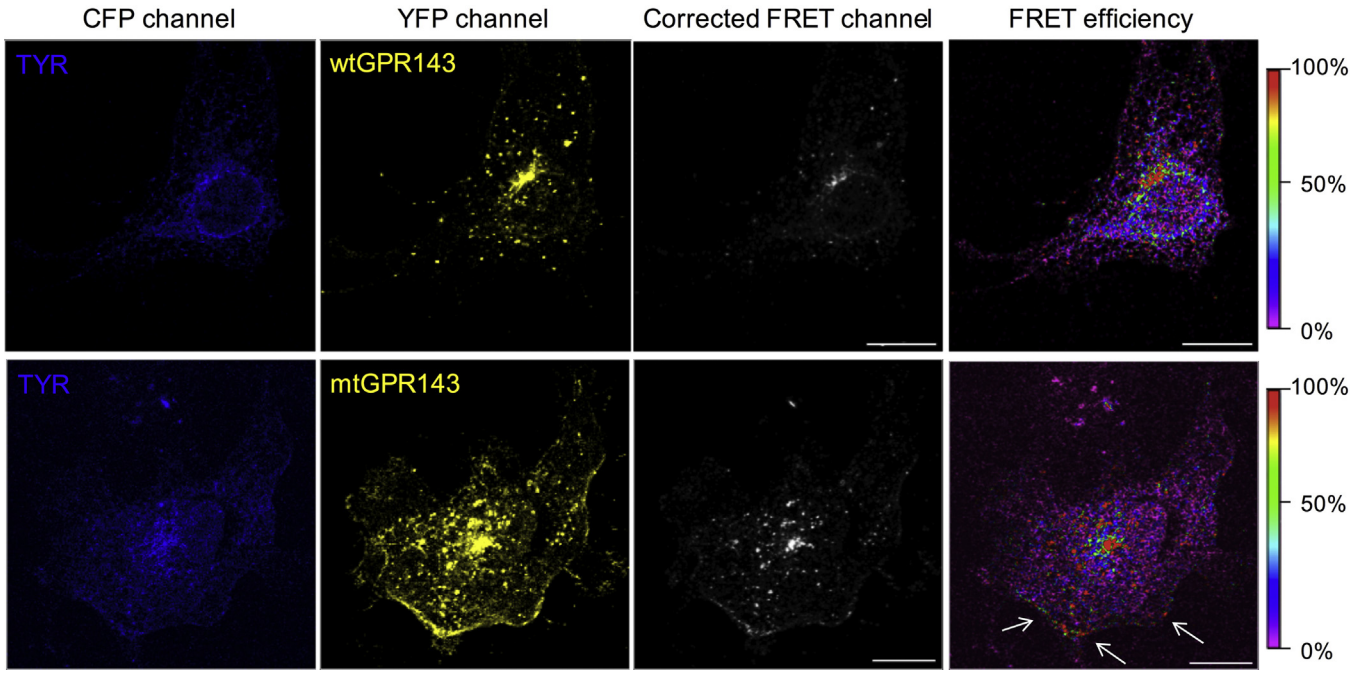

Figure 3. FRET of G-protein-coupled receptor 143-EYFP and tyrosinase-CFP in COS7 cells. In this example, the authors use sensitized emission FRET to observe where in COS7 cells G-protein–coupled receptor 143 (GPR143) tagged with YFP (the acceptor) is interacting with the melanogenic enzyme tyrosinase (TYR) tagged with CFP (the donor). A color scale of the localized FRET efficiency is shown to the right. Both wild-type (wt) and a trafficking deficient mutant (mt) GPR143 interact with TYR in vesicles in the perinuclear and peripheral regions of the cell, but only the trafficking deficient mutant is found to interact with TYR at the plasma membrane. This example highlights the ability of FRET microscopy to differentiate localized changes in protein-protein interactions. White arrows indicate the plasma membrane regions where the FRET signal is localized. Scale bar = 20 μm. Reprinted with permission from De Filippo et al., 2017. CFP, cyan fluorescent protein; FRET, Förster resonance energy transfer; YFP, yellow fluorescent protein.

2011). For example, a biosensor can be designed to include a peptide containing the cleavage sequence of a protease of interest. In this case, at steady state the acceptor and donor FPs are within close enough proximity that efficient FRET occurs (Figure 2c). When the protease of interest becomes active, it cleaves the peptide sequence within the sensor, and the donor and acceptor FPs move apart. This is measurable by both a decrease in the FRET signal and an increase in the donor signal, which results from loss of donor quenching. This method was used by Li et al. (2014) to show that kynurenine increases matrix metalloproteinase activity using a commercially available matrix metalloproteinase assay fluorimetric kit.

Conformational changes

In addition to a dependence on distance, FP orientation is crucial for FRET, because FRET occurs most efficiently when the dipole of the acceptor and donor FPs are parallel to each other. These properties make FRET a powerful technique for examining conformational changes within a protein of interest. A multitude of stimuli induce conformational changes in protein structure, leading to alterations in cell signaling. For example, ligand binding to a receptor often induces conformational changes that recruit downstream signaling partners. Therefore, biosensors sensitive to ligand binding can be designed to either decrease or increase FRET upon association with ligand, allowing real-time examination of receptor activation. Experiments can be designed to detect FRET between a ligand and a receptor by coupling each to a donor or acceptor (intermolecular FRET), and FRET occurs upon ligand binding (Figure 2d). Alternatively, if the donor and acceptor are within the receptor (intramolecular FRET), ligand binding can induce a conformational change that causes FRET to occur. These approaches have been heavily used in characterizing G-protein–coupled receptors and their ligands (Emami-Nemini et al., 2013).

Downstream signaling often involves posttranslational modifications of signaling components, the most common of which is phosphorylation. The addition of a phosphate group can have multiple effects on protein structure through alterations in hydrophobicity, electrostatic attractive and repulsive forces, and protein-protein interactions. For example, phosphorylation of common recognition domains can alter interactions between domains of the same protein or between binding partners, and FRET biosensors can be designed to examine these alterations (Figure 2e). Finally, environmental changes such as pH and ionic concentration can induce conformational rearrangements that can be examined with FRET. For example, Celli et al. (2016) used the cameleon-based endoplasmic reticulum (ER)-targeted calcium sensor D1ER to show that ER calcium is released after barrier perturbation in human epidermal equivalent cultures (Figure 4).

Molecular tension sensors

Mechanotransduction, the conversion of mechanical stimuli into biochemical responses, is emerging as a critical regulator of a number of cellular behaviors, including proliferation, differentiation, and migration, as well as pathological conditions such as hypertension, atherosclerosis, myopathies, and

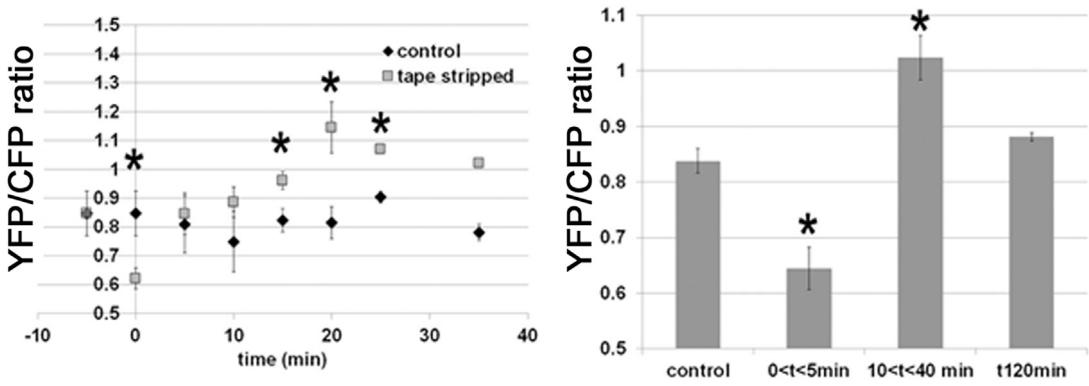

Figure 4. Calcium fluxes in reconstructed human epidermal equivalent cultures. Here, the authors use the endoplasmic reticulum (ER)-targeted, FRET-based calcium sensor D1ER to examine stress-induced ER calcium release in reconstructed human epidermal equivalents. In this case, the presence of calcium induces FRET to occur between CFP (the donor) and YFP (the acceptor). A form of sensitized emission, the ratio of YFP/CFP fluorescence is reported by the authors as an indicator of FRET. As calcium increases, the relative ratio of YFP/CFP increases. The authors used this method to that show ER calcium is released in response to barrier perturbation (tape stripping), as evidenced by the decreased YFP/CFP ratio at early timepoints. ER calcium is then replenished (increased YFP/CFP ratio) over the course of minutes and eventually returns back to homeostatic levels. Data are presented as mean ± standard error of the mean. Asterisks indicate statistically significant deviations from unperturbed stratum granulosum calcium levels, $P < 0.02$. Modified with permission from Celli et al., 2016. CFP, cyan fluorescent protein; FRET, Förster resonance energy transfer; min, minute; YFP, yellow fluorescent protein.

cancer progression. Mechanotransduction is mediated by a variety of cellular components, and the cytoskeleton with its associated adhesive organelles and motor proteins is a major contributor. At the molecular level, mechanotransduction is often facilitated via protein conformational alterations that are the result of tension within the amino acid backbone of a protein. Recently developed FRET-based force sensors are capable of measuring tension within mechanosensitive adhesive organelles. These include components of focal adhesion, which link cells to their extracellular environments, and adherens junctions, which couple cell-cell interfaces. A FRET module containing both the donor and acceptor FPs joined by a calibratable, flexible linker region is genetically inserted into the backbone of the protein of interest. Under conditions of low tension, the donor and acceptor FPs are in close enough proximity for efficient FRET to occur. As tension is applied to the molecule, the FPs move apart, and FRET decreases (Figure 2f). For example, Borghi et al. (2012) developed a tension sensor for the epithelial cell-cell adhesion molecule E-cadherin and showed that it is under constitutive tension generated by actomyosin, which is increased at cell-cell contacts upon stretching. There are a number of caveats to these sensors, because intermolecular FRET must be controlled for, and there is an assumption that the protein acts as a linear spring, which has not been confirmed in all cases.

MEASUREMENT METHODS

Acceptor photobleaching

One of the most straightforward methods of detecting FRET involves acceptor depletion. During FRET, energy is transferred from the donor to the acceptor, essentially quenching (i.e., decreasing) the light emitted by the donor. Therefore, if the acceptor is selectively depleted through photodestruction, the emission of the donor FP will increase accordingly, assuming the process of photobleaching also abolishes acceptor absorption. Images of the donor emission are taken before and after photobleaching the acceptor, and the increase in intensity of donor FP emission is used as an indicator of FRET. Acceptor photobleaching can be easily implemented on a wide variety of microscope systems, with the condition that photobleaching of the acceptor does not bleach the donor FP. However, this method has some limitations. It is relatively slow, is innately destructive, and is affected by sample movement during acquisition. Therefore, it is most suitable for fixed sample applications. Nevertheless, this method is a good first step because it can quickly provide useful information about differences between experimental samples and verify that FRET is occurring before optimizing other FRET methods.

Sensitized emission

Ratio-based FRET measurements are founded on the principle that during FRET the acceptor fluorescence emission is responsive, or is sensitized, to the donor emission. As FRET is increased, the emission for the donor FP decreases, resulting in an increased FRET/donor ratio. This method requires the acquisition of a number of sample and control images. In the simplest variation, in the case of an intramolecular FRET sensor, images of the donor (donor wavelength excitation and emission) and FRET (donor wavelength excitation and acceptor wavelength emission) signals are acquired, processed, and presented as a FRET/donor ratio image. For intermolecular FRET, the acquisition and processing is much more complicated, because varying levels of donor and acceptor concentration and localization require correcting for direct excitation of acceptor and bleed through of donor emission into the FRET signal. Therefore, calculation of a corrected FRET image requires the use of multiple controls and correction factors (Broussard et al., 2013). However, there are a number of advantages of this method, including ease of implementation on both wide field and confocal microscopes. In addition, this method is suitable for live cell imaging of dynamic events, because image acquisition can be quite fast depending on microscope setup.

Fluorescence-lifetime imaging microscopy (FLIM)-FRET

Fluorescence occurs when an excited fluorophore spontaneously emits a photon, dropping back to the ground state. The frequency at which this occurs, or fluorescence lifetime, is unique for each fluorophore and can be affected by local environmental factors that

Table 1. General guidelines for choosing a FRET method

Sample Type: Fixed	Sample Type: Live Cell — Slow Dynamics	Sample Type: Live Cell — Fast Dynamics
Acceptor photobleaching	Acceptor photobleaching	Sensitized emission
Polarized anisotropy	FLIM-FRET	Polarized anisotropy

Abbreviations: FLIM, fluorescence-lifetime imaging microscopy; FRET, Förster resonance energy transfer.

quench fluorescence. In the case of FRET, the presence of an acceptor quenches (i.e., decreases) the fluorescence of the donor through nonradiative transfer of energy, resulting in a decrease in fluorescence lifetime of the donor. FLIM can be used to measure fluorescence lifetime, typically by use of either time- or frequency-domain methods. Both of these methods enable the calculation of FRET efficiency using different instrumentation. Major advantages of FLIM-FRET include that it is not dependent on protein concentration, is less affected by photobleaching, and requires imaging of only the donor FP. However, fluorescence lifetime can be affected by other environmental factors such as pH, the presence of ions, and the refractive index of the medium, necessitating carefully controlled experiments. FLIM imaging is relatively slow and expensive; specialized instrumentation for either time- or frequency-domain measurements is also required but can be incorporated into existing wide field or confocal microscope setups. Finally, many fluorophores exhibit multi-exponential decay curves in living cells, requiring complex data analysis during quantitation.

Polarized anisotropy

Another method of detecting FRET uses polarized light to examine the anisotropic characteristics (i.e., directional variance) of donor emission. When a field of fluorophores is excited with polarized light, only a subset of fluorophores are excited, those whose absorption vectors are oriented parallel to light polarization. Because of the size of FPs and the timescale at which measurements are taken, there is minimal FP rotation, and emitted light remains anisotropic, polarized in the direction of excitation. If a donor FP transfers energy to an acceptor molecule (FRET), then the emitted photon is polarized based on the orientation of the acceptor FP, resulting in a decrease in anisotropy. Samples are excited with polarized light using either an inherently polarized laser or a polarization filter placed in the excitation light path. A variable polarization filter in the emission path is then used to collect light parallel and perpendicular to the excitation orientation. These images are used to calculate the anisotropy, and decreased values of anisotropy indicate a greater amount of FRET. This imaging modality is quite rapid and well suited for high-content screening and live cell imaging. However, it is less sensitive than other methods to FRET efficiency and is often used for qualitative assessment, especially in the case of high FRET versus low FRET conditions.

CHOOSING A METHOD

Because each of the methods used for FRET measurement has its own set of pros and cons, there is no overall best method. Moreover, many FRET-based biosensors have their own eccentricities. Some general guidelines to choosing an appropriate method include the following (see also Table 1). For fixed samples, acceptor photobleaching and FLIM-FRET would be appropriate, because there are no dynamic or time considerations. In the case of live cell imaging of very dynamic events, sensitized emission and polarized anisotropy have the advantage of rapid acquisition. Sensitized emission can often provide more information than polarized anisotropy; however, for intermolecular FRET it requires a multitude of additional controls. FLIM-FRET methods provide an advantage for intermolecular FRET measurements, because they are not dependent on the stoichiometry of the donor and acceptor FPs. Therefore, FLIM-FRET would be preferred over other methods as long as an appropriate acquisition timescale

MULTIPLE CHOICE QUESTIONS

1. Which of the following applications can be assessed using FRET microscopy?
   A. Protein-protein interactions
   B. Tension within a molecule of interest
   C. Ligand binding to a receptor
   D. All of the above

2. What physical parameters affect FRET efficiency?
   A. The orientation of the donor and acceptor molecules
   B. The distance between the donor and acceptor molecules
   C. Both A and B
   D. Neither A nor B

3. Which method of measuring FRET is best used first?
   A. Sensitized emission
   B. Acceptor photobleaching
   C. FLIM-FRET
   D. Polarization anisotropy

4. To obtain a corrected intermolecular FRET image using sensitized emission, one must correct for which of the following?
   A. Bleed-through of donor emission into the FRET signal
   B. Direct excitation of acceptor by donor excitation
   C. Both A and B
   D. Neither A nor B

5. Measuring FRET using acceptor photobleaching is based on the principle of which of the following?
   A. Dequenching of the acceptor molecule
   B. Quenching of the acceptor molecule
   C. Dequenching of the donor molecule
   D. Quenching of the donor molecule

can be achieved. Overall, the best practice is to use and compare all methods available to determine what works best for a biosensor under any given experimental conditions.

CONCLUSION

FRET-based microscopic assays leverage a simple physical phenomenon, the transfer of energy from a donor to acceptor molecule, to address complex biological questions that many other techniques cannot achieve. These include the ability to visualize localized, dynamic protein-protein interactions and biochemical signaling events in real time and in living cells. The potential applications of FRET microscopy to both basic and clinically relevant research questions are mostly limited to the availability of biosensors and accessibility to an appropriate method for visualizing FRET. As these are improved and become more accessible, FRET-based microscopic studies have remarkable potential to contribute both to our understanding of the fundamentals of life and to the development of high-throughput screening assays for drug discovery.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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