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Fluorescent protein FRET: the good, the bad and the ugly

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Dynamic protein interactions play a significant part in many cellular processes. A technique that shows considerable promise in elucidating such interactions is Förster resonance energy transfer (FRET). When combined with multiple, colored fluorescent proteins, FRET permits high spatial resolution assays of protein–protein interactions in living cells. Because FRET signals are usually small, however, their measurement requires careful interpretation and several control experiments. Nevertheless, the use of FRET in cell biological experiments has exploded over the past few years. Here we describe the physical basis of FRET and the fluorescent proteins appropriate for these experiments. We also review the approaches that can be used to measure FRET, with particular emphasis on the potential artifacts associated with each approach.

Why FRET?

Dynamic interactions between proteins are thought to have a key role in regulating most cellular signal transduction pathways. Although biochemical approaches to determining such interactions are common, weak or transient interactions might occur only within the natural cellular milieu of the proteins. Historically, colocalization by immunofluorescence microscopy in fixed cells has been a popular method for examining protein interactions *in situ*. Whereas most proteins are a few nanometers wide, however, the resolution of the fluorescence microscope is several hundred nanometers. By analogy, a typical fluorescence imaging experiment yields information equivalent to knowing that two students are present in a large lecture hall: merely localizing the two students to the same classroom yields no information about whether the students know each other or not.

Because many signaling pathways use the same cellular structures (e.g. clatherin-coated pits are used for internalizing many receptor complexes), such a crude measurement is suggestive at best and misleading at worst. The knowledge that two molecules are in fact adjacent, and not just in the same neighborhood, provides a much more reliable measure of their interaction. Electron microscopy provides the needed resolution but is limited by a lack of precise labeling strategies. Furthermore, these techniques are generally limited to use within fixed cells, which precludes dynamic measurements of live cells. Multicolor fluorescent protein (FP) imaging permits experiments in live cells, which are necessary for assays of transient interactions, but again this approach suffers from relatively poor spatial resolution.

A technique based on Förster resonance energy transfer (FRET) can overcome these limitations. FRET occurs between two appropriately chosen fluorophores only when the distance separating them is less than 10 nm [1–3]. Thus, FRET is well-suited to the study of protein interactions, which occur on a similar spatial scale. Over the past ten years, FRET approaches have gained popularity because of the ease of green fluorescent protein (GFP) targeting [4–6]. FRET between two differently colored fluorescent proteins (FP-FRET) has been widely used for two types of experiment.

First, integrated biosensors have been developed based on intramolecular FRET between FPs conjugated to opposing ends of an environmentally sensitive peptide or protein. For example, such sensors have been built for assays of intracellular Ca^{2+} [4,7], cAMP activity [8,9] and protease activity [10,11]. The availability of various biosensors is growing rapidly, and each assay is improving as successive generations of biosensors are developed. Although this strategy has seen some success, many biosensors report only a small change in FRET that barely exceeds the noise of many imaging systems.

Second, intermolecular FRET has been used to measure protein–protein interactions between two FP-labeled proteins. For example, FRET has been used to visualize many various protein interactions, such as oligomerization of receptors [12,13] and transcription factor interactions [14–16]. These procedures are more difficult to perform and to analyze than the biosensor work because of the added complication of variable stoichiometry between the two labeled proteins. Nevertheless, with appropriate experimental approaches and controls, such experiments are both feasible and informative. As the old adage goes, nothing that is truly worthwhile is ever easy!

In this review, we first describe the physical basis of FRET and the parameters that affect it. We then review the fluorescent proteins that are used in FRET-based experiments, in addition to the methods that are used to image FRET in cell biological applications. Particular emphasis will be given to the potential artifacts associated with each approach.

What is FRET, and what are the parameters that affect it?

FRET occurs between two fluorescent molecules that are sufficiently close. The energy flows from one molecule to

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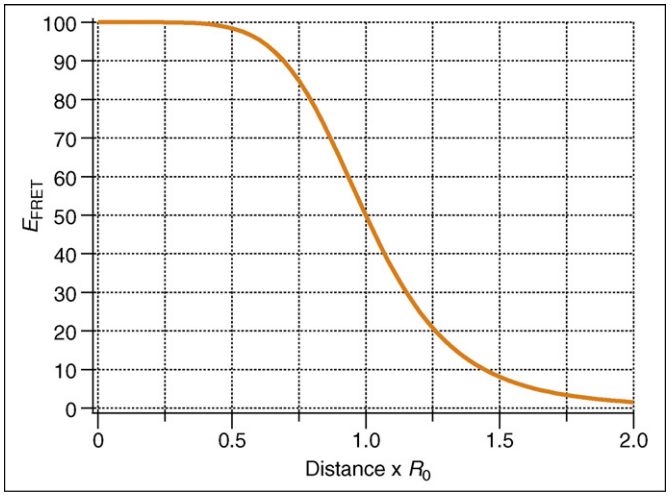

Figure 1. FRET efficiency versus distance. The FRET efficiency (E_{FRET}) varies with the sixth power of distance between donor and acceptor. As a result, there is a steep fall in E_{FRET} with increasing distance. The Förster radius (R_0) is the distance at which 50% FRET occurs. Owing to the strong distance dependence, FRET is usually detected only when the two fluorophores are closer than $1.5R_0$.

the other by non-radiative transfer. In other words, the first molecule does not emit a photon that is then absorbed by the second molecule. Rather, the energy is coupled through the fluorescent dipoles, which radiate energy in the same manner as a radio antenna. The theory of 'resonance energy transfer' was developed by Förster and, in honor of his contribution, the effect has been named after him [17]. The Förster theory shows that FRET efficiency (E_{FRET}) varies as the sixth power of the distance between the two molecules (r):

$$E_{FRET} = 1/[1 + (r/R_0)^6] \qquad \text{(Equation I)}$$

where R_0 is the characteristic distance where the FRET efficiency is 50%, which can be calculated for any pair of fluorescent molecules (see later). The FRET efficiency is shown graphically in Figure 1. Because of the $1/r^6$ dependence, the curve has a sharp fall off. For distances less than R_0 the FRET efficiency is close to maximal, whereas for distances greater than R_0 the efficiency is close to zero. For distances close to R_0 FRET can be used as a molecular ruler, and indeed FRET has been adapted for such purposes in structural biology by using precision spectroscopic approaches [18]. For most cell biological applications, however, the signal-to-noise ratios available limit FRET experiments to a more binary readout (i.e. a measurement will distinguish between 'high-FRET' and 'low-FRET', or simply between 'FRET and no-FRET').

As mentioned earlier, R_0 can be calculated for any pair of fluorescent molecules. The value of R_0 in an aqueous solution is determined by a fairly simple equation with well-known input parameters [1,3]:

$$R_0 = [2.8 \times 10^{17} \cdot \kappa^2 \cdot Q_D \cdot \varepsilon_A \cdot J(\lambda)]^{1/6} \text{ nm} \qquad \text{(Equation II)}$$

where κ^2 represents the angle between the two fluorophore dipoles, Q_D is the donor quantum yield, ε_A is the maximal acceptor extinction coefficient ($Mol^{-1}\ cm^{-1}$), and $J(\lambda)$ is the spectral overlap integral between the normalized donor fluorescence, $F_D(\lambda)$, and the acceptor excitation spectra, $E_A(\lambda)$:

$$J(\lambda) = \int F_D(\lambda) \cdot E_A(\lambda) \cdot \lambda^4 d\lambda \qquad \text{(Equation III)}$$

Although the mathematics might look complicated, most of the parameters are constants that are easily found in the literature. The two terms that need further explanation are κ^2 and $J(\lambda)$. κ^2 simply says that the FRET coupling depends on the angle between the two fluorophores in much the same way as the position of a radio antenna can affect its reception. If the donor and acceptor are aligned parallel to each other, the FRET efficiency will be higher than if they are perpendicular. This degree of alignment defines κ^2. Although κ^2 can vary between 0 and 4, it is usually assumed to be 2/3, which is the average value integrated over all possible angles. For almost any realistic situation κ^2 is close to 2/3, and there is usually nothing that one can do to adjust this value (although some researchers have attached FPs rigidly to their proteins of interest, which could lead to marked effects [19,20]). The overlap integral, $J(\lambda)$, is the region of overlap of the two spectra (Figure 2). The other parameters that can affect FRET are the quantum yield of the donor and the extinction coefficient of the acceptor. Thus, to maximize the FRET signal, one must choose the highest quantum yield

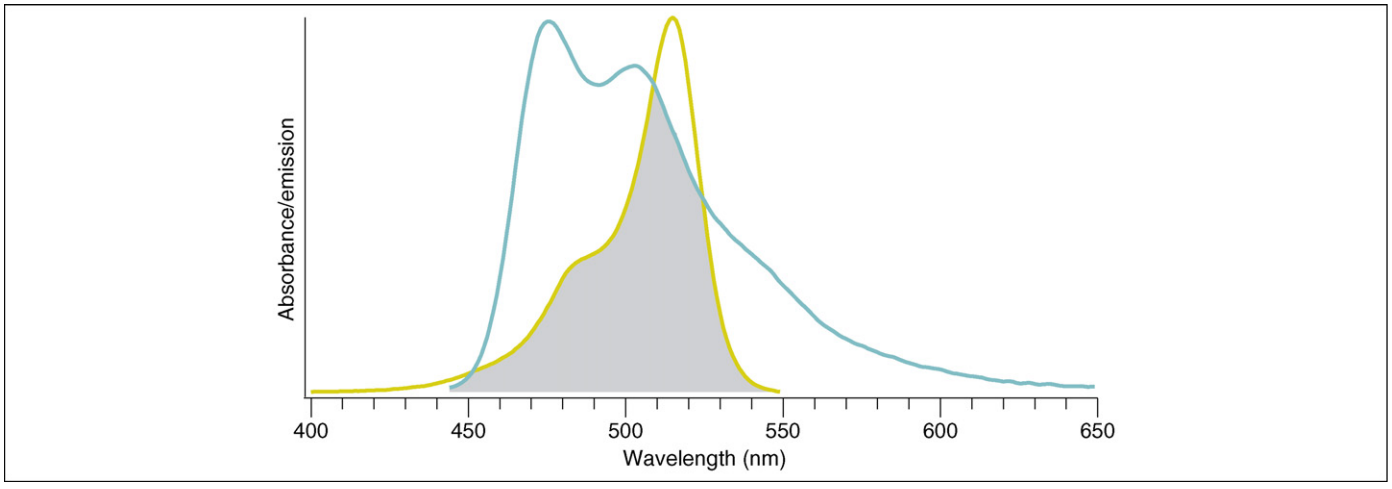

Figure 2. Spectral overlap between CFP and YFP. The spectral overlap between donor and acceptor is determined as the overlap between the emission spectrum of the donor (CFP, blue line) and the absorbance spectrum of the acceptor (YFP, yellow line). The spectral overlap is indicated by the grey area.

donor, the highest absorbing acceptor, and fluorophores with significant overlap in their spectra. This theory has been repeatedly verified by experiment, and there are no other ways to maximize FRET for non-aligned probes.

Each of the parameters described affects the R_0 calculation only by the sixth power. Thus, a doubling of the donor quantum yield results in only a 12.5% change in R_0. Because almost all fluorophores used in FRET imaging experiments have high quantum efficiencies ($\geq$50%) and strong absorption ($\geq$50 000 $cm^{-1}\,M^{-1}$), the range of possible R_0 values is limited to between 4 and 6 nm [21,22], and most FRET pairs have $R_0 \approx 5$ nm. Because FRET efficiency is steeply dependent on the distance separating the FRET pair and the relative orientation of the fluorophores, FRET can be used to detect changes in protein–protein interactions that arise from changes in the affinity between the two proteins or changes in the conformation of their binding. It is worth repeating that, for cell biological applications in which FRET is being imaged, experiments generally differentiate only between two states, and other information is needed to aide in the molecular interpretation of the observed FRET changes.

Real world issues affecting FRET measurements

In practice, many issues complicate FRET measurements and can lead to misleading or even meaningless results [23]. A principal issue is that the donor and acceptor fluorophores might be of different brightness. In theory this should not be a problem but, because most instruments can measure only a limited dynamic range, it might be that one of the fluorophore channels is saturated or that the dimmer fluorophore image is more affected by systematic noise. Thus, in our experience it is best to use a donor and acceptor that are of comparable brightness. Another factor that can limit FRET detectability is a donor:acceptor stoichiometry that is outside the range of 10:1 to 1:10 [24]. For FRET measurements of protein–protein interactions, where one partner might be in excess, this factor can be a serious limitation. The main problem is then to measure a small amount of FRET in a background of fluorescent labels that are not undergoing FRET. There is really nothing that can be done to improve this situation, and therefore many possible protein–protein interaction experiments that fall into this category are simply unsuitable for examination by FRET. For biosensors built with one donor and one acceptor, however, the stoichiometry is guaranteed to be 1:1; thus, this issue never arises and the amount of 'cross-talk' is constant.

The next two issues arise from cross-talk, or bleed-through, between the two fluorophore colors. First, the acceptor can be excited directly with light that is chosen to excite the donor. Second, fluorescence from the donor can similarly leak into the detection channel for the acceptor fluorescence. Because these two sources of cross-talk come from the photophysics of organic fluorophores and will be present for any FRET pair, they must be addressed when FRET is measured. Choosing fluorophores that are spectrally separated reduces the cross-talk but also reduces the overlap integral, $J(\lambda)$, which in practice usually decreases the detectability of the FRET signal more than it helps to eliminate the cross-talk problem.

Lastly, FRET signals can be reduced if the two fluorophores are not aligned (i.e. $\kappa^2 \approx 0$) or if they are simply not within a distance close to R_0. If two labeled proteins interact, but the fluorescent labels are on opposite sides of the complex, then there might not be a FRET signal, even though the proteins of interest are bound. In the experience of most laboratories, these kind of false negatives are common, especially with FP-based FRET. Often, several labeling strategies are needed before a sufficient FRET signal is detected [25]. Each of these issues can be mitigated by an informed choice of the fluorophore pair to be used.

Fluorescent proteins and FRET

Genetic labeling with fluorescent proteins has revolutionized live-cell imaging experiments and led to increased interest in FRET techniques. Mutations of GFP [26,27] and the discovery of coral-derived proteins [28,29] have led to a broad palette of different colored proteins [29]. As we describe here, however, many of the problems generally associated with FRET are particularly acute for FP-FRET. First, because the excitation and emission spectra of many FPs are broad, there can be significant cross-talk. Second, the large size of FPs (4.2-nm long with a 2.4-nm diameter barrel [30,31]; Figure 3) occupies much of the useful FRET distance [21], resulting in practical maximal FRET efficiencies of ~40%. Third, for assays of protein–protein interactions, it is important to ensure that the FPs used interact minimally on their own; therefore, mutations that eliminate FP dimerization should be included in all FRET experiments [32]. The naturally occurring weak dimerization of some FPs, however, can be used to increase the FRET signal in a biosensor. Lastly, FPs come in a wide range of brightness. For example, one popular donor, enhanced cyan fluorescent protein (ECFP), has fivefold less brightness than its yellow acceptor partner, EYFP [33].

There are several potential FRET pairs among the currently available FPs. The first pair developed was blue FP (BFP) coupled with GFP [34], but the poor photophysical properties of BFP made this pair impractical, although recently optimized BFPs might make this pair useful for some applications [35,36]. The first effective pair comprised CFP as the donor and YFP as the acceptor [4]. Other pairs include GFP or YFP as the donor coupled with orange or red derivatives such as mKO [37] or mCherry [38], and an orange donor coupled with a red acceptor. The orange and red coral-derived proteins have long excitation tails, however, causing direct acceptor excitation to be a key limitation to their use.

Thus, CFP and YFP remain the 'best' FRET pair, despite the significant cross-talk in both the excitation and emission spectra of these two FPs (Figure 4). Some limitations of this pair have been mitigated through the creation of new FPs with superior excitation coefficients and quantum yields, such as the optimized CFPs mCerulean [39] and SCFP3A [40], and the optimized YFPs mCitrine [29], SYFP2 [40] and mVenus [41]. Cross-talk in CFP-to-YFP FRET is a particular problem when using a 458-nm laser on a confocal microscope because this light is not optimal for CFP excitation and elicits considerable

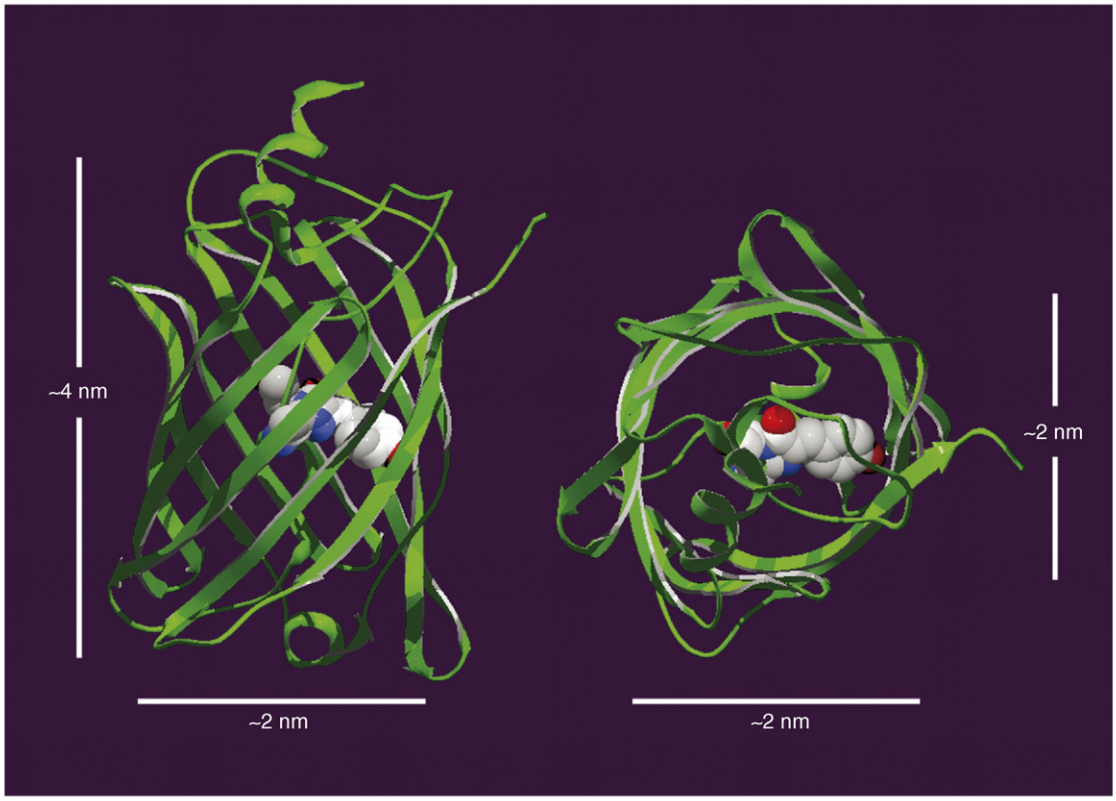

Figure 3. Crystal structure of GFP. The protein is cylindrical, with a diameter of ~2.4 nm and a length of 4.2 nm. The cylinder consists of 11 β strands with a single α helix running along its axis. The chromophore is located in the α helix at the center of the protein. All fluorescent proteins have a similar structure.

direct excitation of YFP. Another newly created teal FP (mTFP1) [42] is excited more efficiently at 458 nm than is CFP, but its use with YFP is limited by even greater spectral overlap of the fluorescence. An additional CFP–YFP pair, called CyPET and YPET, has been coevolved to maximize FRET in a specific construct [43]; however, the enhancement of FRET in this pair is due to intrinsic dimerization between the two FPs [44]. As predicted from the Förster theory, this pair is not as efficient as the other optimized pairs when the dimerization is removed.

Because there are numerous methods for imaging and measuring FP-FRET, useful comparisons of FP-FRET partners and methods have been carried out by many groups using conjoined FRET constructs [24,33,45]. These quantitative 'apples-to-apples' comparisons have shown that the most efficient pair is mCerulean to mVenus, although substitution of SCFP3A for mCerulean, or SYFP2 or YPET for mVenus, produces statistically similar results.

Methods of imaging FRET for cell biology applications

Because each of the FP-FRET pairs has a distinct pathology that complicates its use, it is important to understand the method with which FRET is being detected. It is recommended that researchers use as many different measurement methods as feasible when first beginning to establish the FRET methodology for a given experiment. Once the system and the possible results are well understood, then the simplest approaches can be used for ongoing procedures. The list of methods that have been developed to image FRET is extensive [46]. All of these strategies for measuring FRET can be applied to FP-FRET experiments but, on the basis of practical considerations, five general approaches have proved particularly useful.

First, two-channel imaging with an algorithm that corrects for excitation and emission crosstalk (first described in Ref. [47], also see [48,49]); second, acceptor photobleaching (also called donor dequenching) [50–52]; third, fluorescence lifetime imaging (FLIM) [53–55]; fourth, spectral imaging [56,57]; and fifth, fluorescence polarization imaging [58,59]. Each of these approaches has strengths and weaknesses. On the one hand, for example, two-channel imaging is the simplest method, but it requires the most complicated set of controls. On the other hand, FLIM can yield an unambiguous measurement of FRET efficiency, but instrumentation to measure the nanosecond lifetimes is expensive and not yet widely available to cell biology laboratories.

Sensitized emission

Sensitized emission, or two-color ratio imaging with controls, is the simplest method of imaging FRET. The donor is excited by a specific wavelength of light, and the signal is collected by using emission filters chosen for the donor fluorescence and the acceptor fluorescence. If there were no cross-talk between the excitation and fluorescence of the two fluorophores, then sensitized emission would be a perfect method. Because cross-talk between FPs is a significant problem, however, extensive control experiments are needed to establish the presence of FRET; thus, it is difficult to quantify FRET with this approach [60]. Most investigators have access to a fluorescence microscope that can be adapted easily to the two-channel imaging required for this

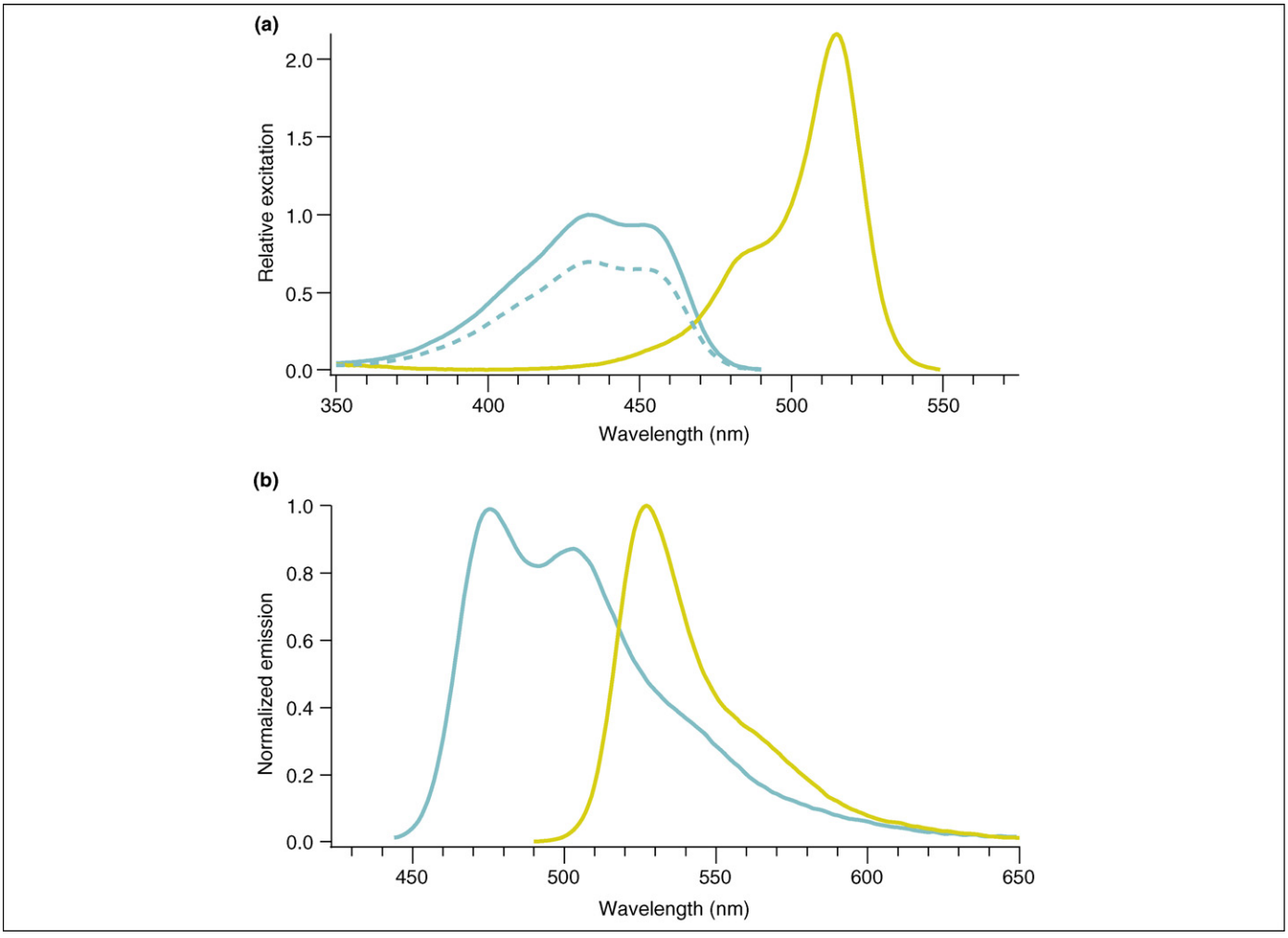

Figure 4. Overlap in the excitation and emission spectra of CFP and YFP. These two proteins have considerable overlap in both excitation **(a)** and emission **(b)** spectra. Direct excitation of the acceptor (YFP, yellow line) can be significant depending on the wavelength used for excitation of the donor (CFP, blue lines) owing to the higher extinction coefficient of YFP as compared with CFP. This overlap is especially problematic when enhanced CFP (dotted blue line) is used as the donor and can be partially overcome by using CFP variants with higher extinction coefficients such as mCerulean (unbroken blue line) or SCFP3A. The broad fluorescence emission spectrum of CFP shows considerable intensity in the region of YFP emission.

method, but the necessary control experiments require considerable image processing to subtract the cross-talk components, which greatly increases the noise and uncertainty in the measurement.

Several corrective approaches have been developed for sensitized emission FRET imaging [47,48,61,62]. The concept is to use different filter combinations with samples that contain only the donor, only the acceptor, or the putative FRET sample with both the donor and acceptor. This permits the researcher to determine the amount of expected cross-talk in both excitation and emission and to subtract it from the FRET measurement. In theory this approach is fine, but the need for image processing increases all of the noise that is initially in the images. Thus, if the FRET signal is small, then it might not be possible to measure it by this approach. A quantitative comparison has detailed the strengths and weaknesses of many of these approaches [63].

Despite these difficulties, sensitized emission measurements can be useful for dynamic experiments in which FRET changes are large, because acquisition of the two images is simultaneous. This situation is especially true for biosensors, where the FRET change is large and the stoichiometry of the donor and acceptor is known to be 1:1. In the experiment shown in Figure 5, for example, a protease biosensor has been engineered to have a high FRET efficiency but, when the peptide linker is cleaved, the FRET drops to zero, thereby creating a large FRET change that demonstrates specific protease activity at a given time and space within the live cell (e.g. [10,64]).

Acceptor photobleaching

Acceptor photobleaching, or donor dequenching, is also simple but is limited to a single measurement. The concept is that the donor fluorescence is quenched owing to FRET because some of the donor fluorescence energy is used to make acceptor fluorescence. Photobleaching the acceptor fluorophore releases this quenching and increases the donor fluorescence. If FRET is present, the donor fluorescence must increase when the acceptor is removed. For these experiments, it is important to ensure that acceptor photobleaching does not degrade the donor, and that the acceptor is photobleached down to ~10% of its initial value. Both of these constraints are easy to meet with the use of a laser scanning confocal microscope.

This method has the advantage that it is straightforward, quantitative and performed on a single sample. The FRET

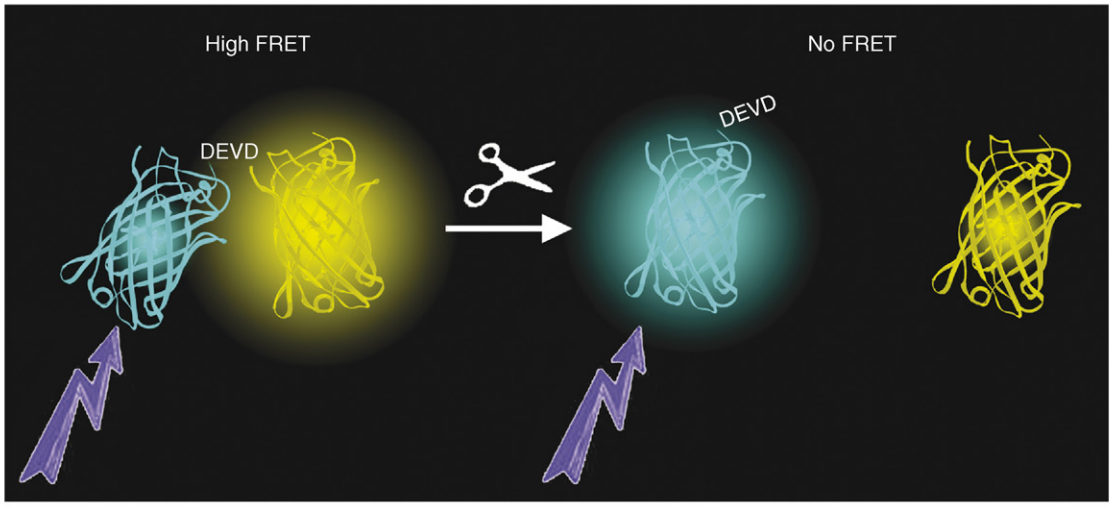

Figure 5. Biosensor for caspase-3 protease activity. FRET-based biosensors for protease activity can be constructed by directly fusing the donor and acceptor FPs through a protease sensitive linker with the sequence Asp-Glu-Val-Asp (DEVD). In the absence of protease activity, the biosensor will show a high FRET efficiency, owing to the short distance between donor and acceptor. Activation of Caspase3 (represented by scissors) results in cleavage of the linker, which enables donor and acceptor to diffuse away from each other, thereby abolishing FRET.

efficiency can be calculated by subtracting the donor intensity in the presence of the acceptor from its intensity after photobleaching of the acceptor, and by normalizing this value to the intensity after bleaching. Of course, the main disadvantage is that this method is destructive; as a result, it can be used only once, and is thus poorly suited for dynamic measurements. This approach can also be somewhat slow, depending on the time it takes to photobleach the acceptor (usually ~1 min with laser excitation; longer if a lamp is used). Nevertheless, it is almost always worthwhile to perform an acceptor photobleaching measurement at the end of the experiment, regardless of what other methods are being used.

Fluorescence lifetime imaging microscopy

FLIM is the most rigorous method for measuring FRET, and it is also less prone to cross-talk artifacts because it looks only at the donor fluorescence [53,65]. All fluorescent molecules show an exponential decay in their fluorescence on the nanosecond timescale, and the rate of this exponential decay is sensitive to environmental changes that quench the fluorescence. Thus, the basic concept of FLIM is related to that of acceptor photobleaching. The donor fluorescence is quenched by FRET, and the amount of quenching can be determined by measuring the shortening of the fluorescence decay time of the donor in the presence of FRET. In this manner, FLIM gives an unambiguous value of the FRET efficiency. FLIM-FRET measurements are not as sensitive to direct acceptor excitation artifacts as other methods, and they can also be used with acceptors that are not fluorescent [66]. Both of these advantages serve to expand the number of useful FP-FRET pairs.

There are, however, has several limitations to FLIM that prevent it from being the dominant approach for FRET imaging. First, measurements of nanosecond lifetimes are complicated; thus, the instrumentation is expensive to obtain and to maintain, and is not yet widely available. In addition, FLIM is usually a slower imaging method, potentially requiring several minutes for each image, which limits its applicability in many FRET experiments. This might change in the future as more user-friendly commercial systems that are faster and more efficient continue to be developed. Another complication is that the lifetimes of FPs in live cells are often multi-exponential, which requires more comprehensive data to be acquired for the analysis of FRET and furthers slows FLIM-FRET measurements. Lastly, other environmental factors, such as autofluorescence background or a change in pH, can also shorten the measured fluorescence lifetime; thus, care must be taken in interpreting FLIM-FRET data in living cells.

Spectral imaging

Spectral imaging is a variation on the sensitized emission method, whereby, instead of acquiring data through two channels, the complete emission spectrum containing both the donor and acceptor fluorescence is collected on excitation of the donor. This method is the typical approach used for cuvette spectroscopy experiments, but is a more recent improvement for imaging systems. The concept is that collection of the whole fluorescence spectrum enables overlapping fluorescent spectra to be separated by using not just the peak of the fluorescence emission but also the distinct shapes of the spectral tails [24,33]. By collecting the spectrum from both the donor and the acceptor, it is possible to determine the amount of donor fluorescence and acceptor fluorescence.

This method requires specialized spectral imaging equipment, but such systems are readily available on many confocal microscopes and can be added onto a conventional fluorescence microscope at modest cost. Previous determination of the amount of cross-talk due to direct excitation of the acceptor, or use of two excitation wavelengths, permits determination of the amount of FRET [24]. The principal drawback of this approach is the reduced signal-to-noise ratio associated with acquiring the complete spectrum rather collecting it through two

channels with a filter-based system. As more commercial systems are being developed and installed, however, the use of spectral imaging for FRET is increasing; thus, it is expected that spectral imaging will become one of the main methods for performing FRET imaging experiments.

Polarization anisotropy imaging

The last method is based on measurements of fluorescence polarization [58], which offers particular advantages for high-contrast discrimination of FP-FRET [59,67,68]. This concept is based on the fact that polarized light excitation selects a population of fluorescent molecules aligned parallel to the excitation polarization. Immediately after excitation, most of the fluorescence will be polarized parallel to the excitation; thus, the fluorescence will be anisotropic in terms of polarization. This anisotropy will disappear if the molecules rotate during the nanosecond fluorescent lifetime but, because FPs are large and rotate slowly, their fluorescence does not depolarize much. If there is FRET between two FPs that are slightly misaligned, then the fluorescence will come out at a different angle, which will look the same as a rotation of the FP.

The main strength of this approach is the ease of measuring fluorescence polarization parallel and perpendicular to the excitation with high signal-to-noise. Because these data can be acquired rapidly and minimal image processing is needed, this approach is well suited for applications in high-content screening. Any direct excitation of the acceptor must be avoided, however, because it can decrease the donor signal and reduce the signal-to-noise ratio of the measurement. In addition, although the polarization FRET technique is superb in discriminating between the presence and absence of FRET, it is not a good approach for differentiating between strong and weak FRET. Furthermore, polarization can be degraded in high numerical aperture lenses, so polarized FRET experiments should be limited to imaging with lenses with a numerical aperture of $\leq$1.0.

Concluding remarks and future perspectives

Although FP-based FRET experiments offer tremendous potential to reveal molecular dynamics in living cellular systems, as yet there is not a perfect FRET pair. The optimized versions of CFP and YFP still provide the most effective pair for general use. Likewise, there is no perfect method to measure FRET, although the approaches described here all have strengths that can be leveraged depending on the particular experimental situation under investigation. As more optimized FPs become available – in particular, bright red FPs that might be appropriate as acceptors for GFP or YFP donor FPs – FP-FRET should become even more useful for protein–protein interaction studies in live cells. So far, the broad absorption spectra of the red FPs, in addition to the lower quantum yields of the monomeric versions, make them difficult to use for FRET. The rapid pace of improvements in FPs, however, lends optimism that such proteins will be available in the future and will help to revolutionize further this new approach to elucidating intracellular biochemical mechanisms.

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