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REVIEW

Protein biosensors based on the principle of fluorescence resonance energy transfer for monitoring cellular dynamics

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Abstract Genetically-coded, fluorescence resonance energy transfer (FRET) biosensors are widely used to study molecular events from single cells to whole organisms. They are unique among biosensors because of their spontaneous fluorescence and targeting specificity to both organelles and tissues. In this review, we discuss the theoretical basis of FRET with a focus on key parameters responsible for designing FRET biosensors that have the highest sensitivity. Next, we discuss recent applications that are grouped into four common biosensor design patterns—intermolecular FRET, intramolecular FRET, FRET from substrate cleavage and FRET using multiple colour fluorescent proteins. Lastly, we discuss recent progress in creating fluorescent proteins suitable for FRET purposes. Together these advances in the development of FRET biosensors are beginning to unravel the interconnected and intricate signalling processes as they are occurring in living cells and organisms.

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Introduction

Since the discovery of fluorescent proteins (FPs) that are suitable for fluorescence resonance energy transfer (FRET) (Shaner et al. 2005), protein biosensors have rapidly become important tools for studying live cell molecular events. FRET was first described by Theodor Förster nearly sixty years ago as a non-radiative transfer of energy from a photo-excited donor to an acceptor fluorescent molecule located in close proximity (<100 Å) (Valeur 2002). The distance and orientation between the donor and acceptor governs the efficiency of the energy transfer. This efficiency can be determined by a fluorescence emission spectrum. In FRET biosensors, a biological event induces a conformational change in the biosensor, which in turn causes a detectable change in FRET efficiency as measured by the change of profile in the emission spectrum.

These FRET biosensors have many advantages over methods based on conjugating synthetic dyes: first, their fluorescence is acquired spontaneously; second, they can be constructed by simple genetic manipulations; third, they can be delivered into cells by transient transfection and subsequent expression; lastly, they can be targeted to organelles and tissues allowing imaging from single cells to whole organisms. A potential drawback of FRET biosensors is that FPs are relatively bulky (~50 Å) compared to synthetic dyes (~5 Å) and therefore may hinder protein activity. However, the activities of many proteins are not affected by fusion to FPs. A second potential drawback is that FPs gain fluorescence through the rate-limiting step of fluorophore maturation, which in some cases can be as long as 12 h, hindering the study of molecular events occurring during that period. To address this problem, many fast maturing FPs have been developed (Bevis and Glick 2002; Nagai et al. 2002; Pedelacq et al. 2006). A third drawback is that FPs are susceptible to photobleaching as is the case with any organic fluorescent dyes, which restricts their use in long term monitoring of cellular events. This problem can be solved by two-photon microscopy, which significantly lowers the donor FP photobleaching rate. This review will discuss recent applications of FRET biosensors.

The principle of FRET

The energy of an excited donor molecule is transferred to the acceptor by resonance coupling of the donor dipole and the acceptor dipole. The rate of this energy transfer, k_{FRET}, is described by the equations (Valeur 2002):

$$k_{\mathrm{FRET}} = \frac{1}{\tau_{\mathrm{D}}} \times \frac{9000 \ln(10) \kappa^2 Q_{\mathrm{D}} J}{128 \pi^5 n^4 N_{\mathrm{A}}} \times \frac{1}{R^6} = \frac{1}{\tau_{\mathrm{D}}} \left(\frac{R_0}{R} \right)^6 \quad (1)$$

$$R_0 = \left(\frac{9000 \ln(10) \kappa^2 Q_{\mathrm{D}} J}{128 \pi^5 n^4 N_{\mathrm{A}}} \right)^{\frac{1}{6}} \quad (2)$$

where R is the distance between the donor and the acceptor. The Förster radius, R_0, is the distance between the donor and acceptor where the FRET efficiency is 50%. It is influenced by the following parameters: the quantum yield of the donor in the presence of the acceptor (Q_{D}); the refractive index (n) of the medium; Avogadro's number (N_{A}); the lifetime of the donor in the absence of acceptors (τ_{D}); the relative orientation factor (κ^2), which will be discussed in the following text; and lastly, the spectra overlap integral J, defined by

$$J = \int_0^\infty F_{\mathrm{D}}(\lambda) \varepsilon_{\mathrm{A}}(\lambda) \lambda^4 \, \mathrm{d}\lambda \quad (3)$$

where $F_{\mathrm{D}}(\lambda)$ is the donor emission profile and $\epsilon_{\mathrm{A}}(\lambda)$ is the acceptor molar extinction coefficient.

The FRET efficiency is defined by the equation:

$$E = \frac{k_{\mathrm{FRET}}}{k_{\mathrm{FRET}} + \tau_{\mathrm{D}}^{-1}} = \frac{R_0^6}{R_0^6 + R^6} \quad (4)$$

Equations 1–4 indicate that a higher donor quantum yield and larger overlap of donor-emission and acceptor-excitation spectra are key parameters to achieve greater energy transfer, which will result in a better FRET signal. Therefore, these are the important factors to consider when choosing FPs for FRET applications. Given a chosen FRET pair, the orientation factor (κ^2) and distance (R) determines the amount of energy that is actually transferred. The orientation factor characterizes the statistical average of the relative fluorophore orientation, which determines both how well the fluorophore dipoles are coupled and how efficiently energy is transferred. The orientation factor is 2/3 for free FRET pairs, but has a different value in biosensors where the movement of the FRET pairs is restricted. The second factor that affects the FRET signal is the distance (R) between the fluorophores. The most sensitive range of R is 0.7–1.4R_0, corresponding to 90–10% FRET efficiency (Fig. 1). R_0 is usually between 40 Å to 70 Å, hence, protein conformational change in this range is ideal for the largest dynamic range in FRET biosensors.

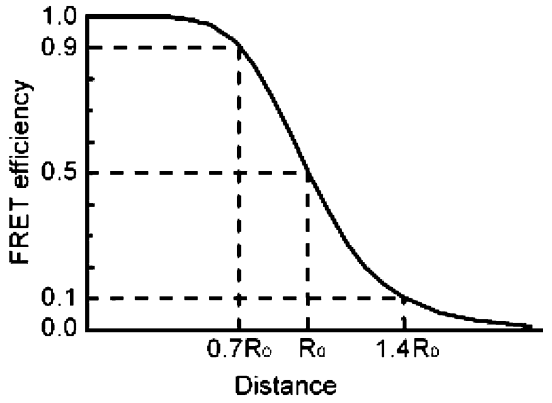

Fig. 1 FRET efficiency as a function of fluorophore distance. The FRET efficiency is 50% when the distance equals to the Forster radius (R_0). FRET efficiencies ranging from 10% to 90% correspond to distances from 1.4 R_0 to 0.7 R_0

Applications of FRET biosensors using FPs

FRET biosensors have been engineered to detect a broad range of molecular events such as protein-binding interactions, protein conformational changes, protein catalytic functions (for example, proteolysis, phosphorylation, dephosphorylation and GTPase activities), and concentration of biomolecules (for example, signalling molecules, cellular metabolites and nucleic acids). Table 1 provides examples of FRET biosensors based on their applications. They can be designed using four common patterns—intermolecular interactions, intramolecular interactions, proteolytic cleavage or multiple FPs (Fig. 2). Because the applications of FRET biosensors spans over a broader range, we will review the current advancement of FRET biosensors from the perspective of these four design patterns.

FRET using intermolecular interactions

Binding interactions between proteins are essential for signal transduction and catalytic activation. For instance, many protein pathways are activated by the association of membrane receptors, which activate enzymes that propagates the signals. To study these binding interactions, FRET biosensors are created by fusing the donor and acceptor FP separately to the interacting proteins of interest. When the intermolecular interaction of these separate fusion proteins occur, the donor and acceptor are consequently brought closer together to create an intermolecular FRET signal corresponding to the location and time of the interaction (see Fig. 2A). Biosensors of this class have proven to be as invaluable tools to study the association mechanisms of membrane receptors as well as the cytoplasmic proteins. Recently, an inflammatory cytokine receptor subunit, IL-17RA, was shown to multimerize and preassemble in the plasma membrane of HEK293 cells. When HEK293 cells were coexpressed with IL-17RA fused to CFP and YFP, the plasma membrane of transfected HEK293 cells displayed a strong FRET signal indicating the association of CFP and YFP tagged IL-17RA (Kramer et al. 2006). Using a similar approach, a biosensor was constructed to study the association of G-protein coupled receptors (GPCRs) with G-proteins. Gα was tagged with CFP and G$\beta\gamma$ with YFP (Azpiazu and Gautam 2004). Before activation by GPCRs, Gα-CFP and G$\beta\gamma$-YFP subunits bind together, producing a high FRET signal. After activation, Gα-CFP and G$\beta\gamma$-YFP separate, resulting in the loss of FRET. Studies using this biosensor suggested the mechanism behind the specificity of G-protein signalling pathways was the stochastic collision between GPCRs and G-proteins. In another example, the direct interaction of transcription factors Erg and Jun was demonstrated by fusing them to YFP and CFP respectively (Camuzeaux et al. 2005).

In addition to studying protein associations, intermolecular FRET has also been applied to probe the effects of small molecules in signal transduction pathways. Here, the biosensor mechanism usually relies on the small-molecule-dependent binding of two separate domains that bring a FRET pair into close proximity and consequently, increasing the FRET signal. This mechanism has been used to construct biosensors for cAMP where YFP is fused to protein kinase A (PKA) and CFP to its cAMP-dependent binding substrate (Lissandron et al. 2005; Zaccolo et al. 2005). Using this biosensor, it was discovered that cAMP is generated by cells in discrete functional compartments inside cardiac myocytes (Zaccolo et al. 2002, 2005; Zaccolo and Pozzan 2002). We have seen from the examples described above

Table 1 List of protein biosensors

Application	Examples	Biosensor FRET mechanism	Sensory domain(s)	FRET pair	Source
Protein binding interaction	Multimerization of IL-17RA	Inter	IL-17RA with itself	CFP YFP	Kramer et al. (2006)
	GPCR subunit association	Inter	Gα with G$\beta\gamma$	CFP YFP	Azpiazu and Gautam (2004)
	Transcriptional factor Erg and Jun interaction	Inter	Erg with Jun	CFP YFP	Camuzeaux et al. (2005)
Protein conformational change	Sensing membrane potential	Intra S	Potassium channel voltage sensing domain	ECFP EYFP	Sakai et al. (2001)
GTPase	Activation and signalling of rac and cdc42	Intra M Or Intra S	Cdc42 or rac with GTPase binding domains	CFP YFPECFPEYFP	Itoh et al. (2002), Seth et al. (2003)
Protease activity	Caspases	Cleavage	Caspase proteolytic substrate	CFP YFP Cerulean Venus	Chiang and Truong (2005), Jones et al. (2000), Nagai and Miyawaki (2004), Onuki et al. (2002), Xu et al. (1998)
	Calpain	Cleavage	Calpain proteolytic substrate	ECFP EYFP	Stockholm et al. (2005)
	Factor Xa	Cleavage	Factor Xa proteolytic substrate	BFP5 RSGFP4	Mitra et al. (1996)
Kinase/phosphotase activity	MLCK and MLCP	Intra S	RMLC (regulatory myosin light chain)	ECFP Citrine	Yamada et al. (2005)
	Kinetics and potencies of 12 known PKC ligands	Intra S	PKCδ	ECFP EYFP	Braun et al. (2005)
	Detection of PKC activities	Intra S	Truncated pleckstrin containing PH and DEP domains	ECFP EYFP	Schleifenbaum et al. (2004)
	Phosphorylation by insulin receptor	Intra M	Phosphorylation recognition domain and its binding substrate	CFP YFP	Sato et al. (2002), Sato and Umezawa (2004)
	Activities of EGFR, Src and Ab1	Intra M	SH2 with phosphorylation substrates for EGFR, Src and Ab1	CFP YFP	Ting et al. (2001)
	Activation of Src	Intra M	SH2 with phosphorylation substrates for Src	CFP YFP	Wang et al. (2005)
Metabolic molecules	Glucose	Intra S	Glucose binding protein	ECFP EYFP	Fehr et al. (2004), Fehr et al. (2003), Ye and Schultz (2003)
	Maltose	Intra S	Periplasmic binding proteins	ECFP EYFP	Fehr et al. (2002)
	Glutamine	Intra S	Glutamate/aspartate binding protein ybeJ	ECFP Venus	Okumoto et al. (2005)

Table 1 continued

Application	Examples	Biosensor FRET mechanism	Sensory domain(s)	FRET pair	Source
Signalling molecules	cAMP	Inter	PKA with cAMP-dependent binding substrate	CFP YFP	Zaccolo et al. (2002, 2005), Zaccolo and Pozzan (2002)
	IP_3	Intra S	InsP3 receptors	CFP YFP	Remus et al. (2006), Tanimura et al. (2004)
	cGMP	Intra S	GKI and PDE	CFP YFP	Nikolaev et al. (2006)
	Estrogen receptor ligand	Intra S	Estrogen receptor ligand binding domain	CFP YFP	De et al. (2005)
	Ca^{2+} in ER	Intra S	apoK1-er	CFP YFP	Osibow et al. (2006)
	Ca^{2+}	Intra M	CaM M13	CFP YFP BFP GFP	Miyawaki et al. (1997)
Other molecule	Specific RNA sequence	Intra S	HIV-1 Rev protein	ECFP EYFP	Endoh et al. (2005)

Note: Intra S: Intramolecular single domain; Intra M: Intramolecular multiple domain interaction; Inter: Intermolecular interactions; Cleavage: Biosensor cleavage

that intermolecular FRET biosensors provide direct evidence of protein binding events, which is ideal for studying protein associations in vivo. The available intermolecular biosensors to this date cover only a small fraction of the total protein binding interactions, hence, we expect to see a broader the range of protein binding interactions being detected with new biosensors in the near future. Currently, one of the limitations of intermolecular FRET biosensors is that using one FP pair can detect only one pair of protein association. The solution to the problem is to use multiple FRET pair as will be discussed below.

FRET using intramolecular interactions

The changes of protein conformation are usually resulted from biochemical stimulation, including either environmental changes such as the concentration of small molecules and membrane voltage or modifications performed by other proteins such as kinases and phosphatases. By monitoring these protein conformation changes with FRET, we can indirectly detect their inducing molecular events (see Fig. 2B). Indeed, sensory proteins with relatively large conformation changes such as IP_3-receptor, cGMP-dependent protein kinase I, estrogen receptor ligand binding domain, HIV-1 Rev protein, glucose-binding protein, periplasmic binding protein and glutamine-binding protein ybeJ exhibit sufficient conformational changes to be used to detect concentration changes of IP_3 (Remus et al. 2006; Tanimura et al. 2004), cGMP (Nikolaev et al. 2006), estrogen receptor ligand (De et al. 2005), specific RNA sequence (Endoh et al. 2005), glucose (Fehr et al. 2003, 2004; Ye and Schultz 2003), maltose (Fehr et al. 2002) and glutamate (Okumoto et al. 2005), respectively. These small molecules are important in cellular signalling and metabolic pathways and hence, these biosensors will help to study their dynamics in the cell. For example, a glucose sensor capable of sensing glucose concentration within the physiological range was employed to study the dynamics of glucose metabolism in COS-7 cells (Fehr et al. 2003). The study showed the rapid glucose consumption in COS-7 cells by probing glucose concentration in the cytosol while changing the

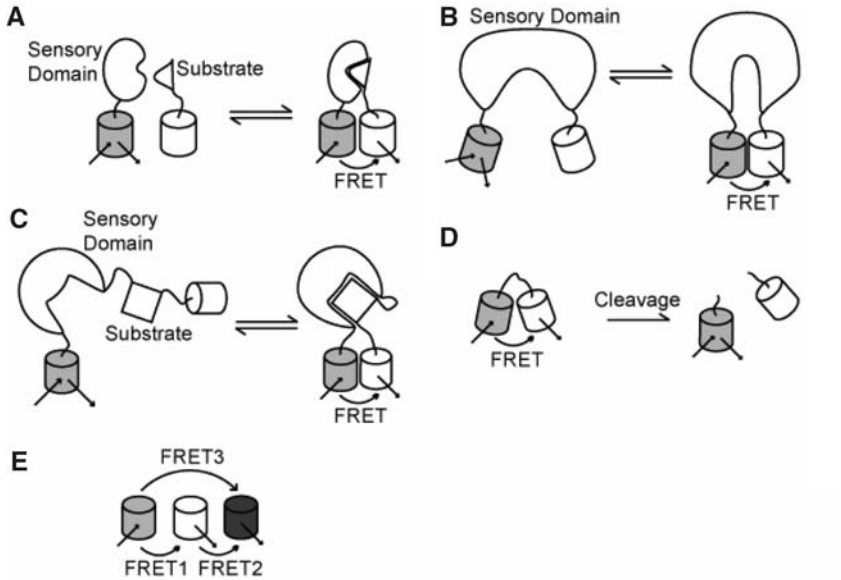

Fig. 2 Schematics showing common FRET biosensor constructs. White cylinders are YFPs, grey cylinders are CFPs and black cylinders are RFPs. Arrows pointing into the FPs are excitation light and those pointing outwards from the FPs are their emission light. (**A**) Intermolecular FRET biosensor uses the binding between separate sensory domain and substrate to create FRET. Intramolecular FRET biosensors using (**B**) a sensory domain alone, and (**C**) the binding between a sensory domain to its substrate. (**D**) Cleavage biosensors detect the substrate cleavage between FRET pairs. (**E**) FRET using three FPs creates three FRET signals

extracellular glucose concentration or inhibiting glucose transport by cytochalasin B.

In the above examples, target molecules are detected by directly binding to the biosensors. Other intramolecular FRET biosensors are constructed such that instead of binding, the sensory domain is modified by enzymes and is thus able to detect molecular events associated with the activities of the modifying enzyme. For example, a biosensor able to detect phosphorylation of regulatory myosin light chain (RMLC) was constructed utilizing the conformational change of RMLC upon phosphorylation and dephosphorylation by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), respectively (Yamada et al. 2005). In another example, a Ca^{2+} biosensor without the parasitic Ca^{2+}-buffering properties was created using the apoK1-er domain, which undergoes a reversible conformational change in a Ca^{2+}-dependent reaction with calreticulin and a protein disulfide isomerase (Osibow et al. 2006). Because of this unique Ca^{2+}-dependent reaction, the biosensor is sensitive to Ca^{2+} concentration levels in the physiological range of the endoplasmic reticulum (10–700 μM). Proteins may undergo conformational changes without chemically reacting with other molecules. For instance, the voltage-sensing domain of a potassium channel protein changes conformation when triggered with changes in membrane potential. A membrane potential biosensor was cleverly constructed utilizing the rotational conformation change of this protein that causes changes in the orientation factor rather than separation between the FRET pair (Sakai et al. 2001).

In contrast to what was described above, not all proteins undergo significant conformational changes to induce observable FRET differences. In these cases, a sensory domain is fused with its binding substrate and sandwiched between a FRET pair. When the sensory domain is stimulated by the molecular event of interest, it binds to the substrate protein inducing a large overall conformational change that changes the FRET signal (see Fig. 2C). This substrate-binding mechanism has been demonstrated in early studies on Ca^{2+} biosensors using calmodulin and its

binding peptide (Miyawaki et al. 1997). The binding of calmodulin to the binding peptide is reversible depending on the Ca^{2+} concentration. When Ca^{2+} concentration rises to 1 μM, calmodulin binds to the peptide to bring CFP and YFP closer, increasing FRET; when Ca^{2+} concentration drops below 0.1 μM, calmodulin dissociates from the peptide, decreasing FRET. Further improved variants of Ca^{2+} biosensors have been created to monitor cellular Ca^{2+} dynamics and signalling (Mank et al. 2006; Miyawaki et al. 1999; Truong et al. 2001). For example, by targeting mitochondria with a yellow cameleon Ca^{2+} biosensor (Miyawaki et al. 1997), researchers have shown that mitochondria in mouse skeletal muscle cells take up Ca^{2+} during neuron stimulated contraction and release Ca^{2+} during relaxation. Furthermore, the Ca^{2+} dynamics in the mitochondria is several milliseconds behind that of the cytosol (Rudolf et al. 2004). The cameleon biosensors (Miyawaki et al. 1997, 1999) have also been used in transgenic flies (Diegelmann et al. 2002; Fiala and Spall 2003; Fiala et al. 2002; Mank et al. 2006), mice (Hara et al. 2004; Nyqvist et al. 2005; Tsujino et al. 2005), zebrafish (Higashijima et al. 2003) and nematodes (Kerr et al. 2000) to detect cellular activities under external stimulations. For instance, researchers were able to visualize Ca^{2+} concentration changes in olfactory projection neurons of *Drosophila* brain when stimulated by odorant, providing a system to model olfaction (Fiala et al. 2002). Biosensors using a similar substrate-binding mechanism have also been created to detect protein activities such as kinases (Braun et al. 2005; Sato et al. 2002; Sato and Umezawa 2004; Schleifenbaum et al. 2004; Ting et al. 2001; Wang et al. 2005) and GTPases (Itoh et al. 2002; Seth et al. 2003).

Presently, the major application of intramolecular biosensors is to detect enzyme activities and the concentration of small molecules as we have seen in the examples discussed above. In the coming years, we expect to see biosensors for most of the important signalling and metabolic molecules. On the technical side, it is easier to characterize the FRET signal from intramolecular biosensors because it is less sensitive to the relative concentration of the biosensor than intermolecular biosensors. Hence, this type of biosensors is preferred in quantitative molecular cell biology studies. However, one of the challenges is to improve the dynamic range of intracellular biosensors in order to achieve higher signal-to-noise ratio. At the moment, intramolecular biosensors having the largest dynamic range is constructed using interacting sensory domains, which could be further optimized by circularly permutated FPs (cpFPs) as will be discussed below.

FRET detecting proteolytic cleavage

Proteases belong to a major class of enzymes essential to many cellular processes such as the initiation and propagation of the apoptosis pathway. Because proteases usually cleave their substrate irreversibly, the detection of protease activity inevitably requires the cleavage of the biosensor (see Fig. 2D). A frequent target for this class of FRET biosensors are caspases due to their importance in the apoptosis pathway and high peptidal substrate specificity (Chiang and Truong 2005; Jones et al. 2000; Nagai and Miyawaki 2004; Onuki et al. 2002; Xu et al. 1998). The caspase biosensors is constructed by fusing a FRET pair to the *N*- and *C*-terminal of their peptide substrates, which is usually four residues with the sequence specific to the caspase of interest. When caspase cleaves the substrate in the biosensor, the FRET signal dramatically decreases as the FP pair is separated. By monitoring the FRET signal, the dynamics of caspase-8 activation in single cell was studied showing that caspase-8 activation occurs earlier than caspase-3 during apoptosis, which suggested along with other evidences that caspase-3 activation is dependent on caspase-8 (Luo et al. 2003). Recently, by using both caspase-3 and Ca^{2+} biosensors in a cell co-culture, the two molecular events were imaged simultaneously showing that the activation of caspase-3 during apoptosis is accompanied by a cytosolic Ca^{2+} concentration rise and fall (Chiang and Truong 2005). Since the discovery of caspases in the early 90s, caspase biosensors have played critical roles in the discovery of the biochemical properties of caspases and their biological roles inside cells. Beyond caspases, biosensors for other proteases, such as

calpain (Stockholm et al. 2005) and Factor Xa protease (Mitra et al. 1996), were also made based on the same principle to image in vivo protease activities.

FRET using multiple FPs

As was mentioned in the previous sections, FRET experiments using one pair of FPs provide information on a single molecular event, however in many cases signalling and metabolic pathways are composed of multiple simultaneous molecular events. To image two molecular events, FRET biosensors using multiple FPs have been used. As each FRET pair occupies a large portion of the visible spectrum, it is difficult to introduce a second distinct FRET pair. To solve the problem, a triplet of CFP-YFP-mRFP was used to measure three distinct FRET signals—CFP to YFP, CFP to mRFP, YFP to mRFP (see Fig. 2E) (Galperin et al. 2004; He et al. 2005). As a proof-of-concept experiment to simultaneously monitor the three FRET events, the three-domain complex of Rab5 and EEA.1sh was detected and the interaction between EGFR with Grb2 and Cbl was confirmed (Galperin et al. 2004). In a similar experiment, the trimerization of TRAF2 was confirmed in living cells (He et al. 2005). Currently, the only triplet FPs for multiple FRET is CFP-YFP-RFP. Since the CFP-YFP FRET is very high and the YFP-RFP FRET is barely detectable, only a handful of studies were conducted using multiple FRET to this date. We expect multiple FRET to become more popular as more RFP mutants are created with better properties as FRET acceptors.

Fluorescent proteins for FRET

One of the challenges in using and designing FRET biosensors is the signal-to-noise ratio (SNR). One way to achieve high SNR is by choosing the right FPs or circularly permutated FPs (cpFPs) for FRET. Since the discovery of green fluorescent protein (GFP) in *Aequorea Victoria* (jellyfish), many FP variants have been designed or discovered. These FPs can be classified into five groups according to their peak emission wavelengths: blue, cyan, green, yellow, and orange-red. Based on colour, there are three types of common FRET pairs: blue donor to green acceptor, cyan to yellow/orange and green to orange/red. Optimal FRET pairs have high spectra overlap, acceptor quantum yield and donor molar extinction coefficient (Table 2). Recently, FP pairs such as CyPET and YPET have been created specifically for FRET (Nguyen and Daugherty 2005). In this case, random mutagenesis and gene shuffling were used to create variants of CFP and YFP. These variants were then fused together and screened for improved FRET signals. The resulting CyPET and YPET exhibited a 20-fold ratiometric FRET change when used in caspase-3 biosensors comparing to only threefold using the parental CFP-YFP pair. In addition to designing FRET pairs with better spectral properties, most of the FPs used in FRET are engineered to be monomeric or weakly dimeric to avoid the oligomerization of FPs, which in biosensors may cause interactions that result in parasitic FRET signals. Furthermore, oligomerization may constrain the kinetics of the biosensor or even cause aggregation, making strongly oligomerizing FPs such as DsRed (Clontech) and MiCy (Karasawa et al. 2004) relatively difficult to use. Recently, cpFPs were used to optimize the FRET dynamic range of biosensors (Mank et al. 2006; Nagai et al. 2004; Zapata-Hommer and Griesbeck 2003). Since the circular permutation of a FP creates new N- and C-termini for protein fusion, it has a different FRET orientation factor when spliced into biosensors. Particular cpFPs will have a large gain in dynamic range as a result of favourable changes in both the distance and orientation factors for FRET. The YC3.60 calcium biosensor was created by replacing the FRET acceptor Venus with its circular permutation variant at Asp-173. The variant showed a dynamic range of 600% in comparison to the 120% of the original YC2.12 and YC3.12 (Nagai et al. 2004). The improvement of dynamic range by circularly permutated FPs is case-dependent because certain FP circular permutations that benefit one biosensor may not improve other biosensors. Hence, circular permutation of FP must be carried out on a case-by-case basis to optimize biosensor dynamic range.

Table 2 Spectral properties of several FRET pairs

Proteins	Donor/ Acceptor	Ex (Steinmeyer et al. 2005)	Em (Steinmeyer et al. 2005)	Extinction coefficient (EC)	Quantum yield (QY)	Relative brightness (ECxQY)	pK_a	R_0	Oligomerization	Source
Blue-Green										
EBFP (Blue)	D	380	440	29,000	0.31	9.0	N/A		Weakly dimeric	Clontech
EGFP (Green)	A	484	507	56,000	0.60	33.6	N/A	4.5 nm	Weakly dimeric	Clontech
Cyan-Yellow/Orange										
Cerulean (Cyan)	D	433	475	43,000	0.62	26.7	4.7		Weakly dimeric	Rizzo et al. (2004)
Venus (Yellow)	A	515	528	92,200	0.57	52.5	6.0	5.3 nm	Weakly dimeric	Nagai et al. (2002)
mCitrine (Yellow)	A	516	529	77,000	0.76	58.5	5.7	5.2 nm	Monomeric	Griesbeck et al. (2001)
CyPet (Cyan)	D	435	477	35,000	0.51	17.9	5.0		Weakly dimeric	Nguyen and Daugherty (2005)
YPet (Yellow)	A	517	530	104,000	0.77	80.1	5.6	N/A	Weakly dimeric	Nguyen and Daugherty (2005)
MiCy (Green-Cyan)	D	472	495	27,250	0.90	24.5	6.6		Dimeric	Karasawa et al. (2004)
mKO (Orange)	A	548	559	51,600	0.60	31.0	5.0	5.4 nm	Monomeric	Karasawa et al. (2004)
Green-Orange/Red										
T-Sapphire (Green)	D	399	511	44,000	0.60	26.4	4.9		Weakly dimeric	Zapata-Hommer and Griesbeck (2003)
mOrange (Orange)	A	548	562	71,000	0.69	49.0	<6.5	5.7 nm	Monomeric	Shaner et al. (2004)
TDimer2 (Red)	A	552	579	120,000	0.68	81.6	4.8	6.3 nm	Functionally monomeric	Yang et al. (2005)

Conclusion and perspectives

FRET biosensors are valuable tools in monitoring molecular events in living cells. Here, we have reviewed the concepts and applications of FRET biosensors. They are widely used for their unique advantages in cellular imaging, including spontaneous fluorescence, simple genetic manipulations, ease of delivery inside cells, and flexibility in targeting to organelles and tissues. One of the challenges in FRET biosensor design is the availability of good FRET pairs. Consequently, many recent studies on FPs are focused on creating variants that are especially designed for FRET applications (Karasawa et al. 2004; Nguyen and Daugherty 2005; Rizzo et al. 2004). Another challenge in biosensor design is ensuring that the sensory domain traverses conformations with distance and orientation factors favourable for the FRET signals. Looking into the future, we anticipate FRET biosensors will allow us to simultaneously image the many coordinated and complex molecular events of signalling and metabolic pathways within transgenic animals.

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