

Two Decades of Genetically Encoded Biosensors Based on Förster Resonance Energy Transfer

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ABSTRACT. Two decades have passed since the development of the first calcium indicator based on the green fluorescent protein (GFP) and the principle of Förster resonance energy transfer (FRET). During this period, researchers have advanced many novel ideas for the improvement of such genetically encoded FRET biosensors, which have allowed them to expand their targets from small molecules to signaling proteins and physicochemical properties. Although the merits of “genetically encoded” FRET biosensors became clear once various cell lines were established and several transgenic organisms were generated, the road to these developments was not necessarily a smooth one. Moreover, even today the development of new FRET biosensors remains a very labor-intensive, trial-and-error process. Therefore, at this junction, it may be worthwhile to summarize the progress of the FRET biosensor and discuss the future direction of its development and application.

Key words: FRET, biosensor, fluorescent protein

Introduction

There is an increasing demand for visualization of pleiotropic intra- and extra-cellular properties at microscopic resolution. These may be biochemical parameters such as enzymatic activities and ion concentrations, physicochemical parameters such as membrane potential and tension, or cellular properties such as the cell cycle or cell lineage. To meet these demands, a number of indicators, herein called biosensors, have been developed over the past half century. The biosensors are comprised of such components as small organic fluorescent dyes, fluorescent proteins, or bioluminescent proteins. Because there are a number of excellent review papers on biosensors (Enterina *et al.*, 2015; Miyawaki and Niino, 2015; Sanford and Palmer, 2017; Ni *et al.*, 2018; Greenwald *et al.*, 2018), in this article we focus on genetically encoded intramolecular/unimolecular/single-chain biosensors based on the principle of Förster resonance energy transfer (FRET). In other words, the indicators that are the topic of this paper are comprised of two fluorescent proteins, are transduced within the cells by expression vectors, can be expressed stably in the cells and

organisms, and can be observed under a microscope without the need for exogenous dyes or chemicals. For simplicity, we will refer to these biosensors as FRET biosensors hereafter.

The current flourishing of fluorescence microscopy would not be possible without the discovery of green fluorescent protein (GFP) by the late Osamu Shimomura (Shimomura, 2005). One of the successful applications of GFP technology is the development of FRET biosensors such as the calcium indicator cameleon, being developed by Atsushi Miyawaki at the late Roger Tsien’s laboratory in 1997 (Miyawaki *et al.*, 1997). Following this report, the number of FRET biosensors and their applications have increased steadily, albeit not as fast as many developers might have expected (Supplementary Table 1). In this report, we will overview the progress of the FRET biosensors in the past two decades and discuss the future prospects.

Principle of FRET

FRET is a process by which a donor fluorophore in an excited state non-radiatively transfers its energy to a neighboring acceptor fluorophore (Förster, 1946; Jares-Erijman and Jovin, 2003). It should be emphasized that this is a non-

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radiative process and that “fluorescence resonance energy transfer” may be an inappropriate term. Because the principles and the pitfalls of FRET technology based on fluorescent proteins have already been described in previous review papers (Pietraszewska-Bogiel and Gadella, 2011; Vogel *et al.*, 2012; Lindenburg and Merks, 2014; Maryu *et al.*, 2018), here we will limit ourselves to briefly introducing the keywords referred to in this article.

The FRET efficiency, E , is represented by the following equations:

$$E = \frac{R_0^6}{R_0^6 + r^6}, \quad (1)$$

$$R_0^6 = c_0 \kappa^2 J n^{-4} Q_0, \quad (2)$$

$$J = \int \overline{f_D(\lambda)} \varepsilon_A(\lambda) \lambda^4 d\lambda, \quad (3)$$

where r is the distance between donor and acceptor (over the range of 1–10 nm), R_0 is the Förster distance, κ^2 is the parameter of the relative-orientation of the dipole moment of the donor emission and that of the acceptor absorption (range 0–4), n is the refractive index, Q_0 is the quantum yield of the donor in the absence of the acceptor, and J is the spectral overlap integrated over the wavelength λ with the normalized donor emission spectrum f_D and the acceptor molar extinction coefficient ε_A ; $c_0 = 8.8 \times 10^{-28}$ for R_0 in nm.

Among the parameters used in the equations, J , the spectral overlap between the donor and the acceptor, is the most critical for the initial choice of the donor and acceptor fluorescent proteins (Bajar *et al.*, 2016). For the design of the FRET biosensor, two other parameters—the distance, r , and the relative orientation between the donor and acceptor proteins, κ^2 —become the primary concern.

Mode of action

Basic structure

Most FRET biosensors contain at least one protein module, called the sensor domain. The sensor domain serves to translate the property of the observer’s interest into the appropriate conformational change of the peptide. The cellular event that induces the conformational change of the sensor domain can be a post-translational modification, such as phosphorylation or methylation, or a binding of ions or small molecules such as amino acids and sugars.

There are two main types of FRET biosensors. In the first type, both the donor and acceptor fluorescent proteins are fused directly to the sensor domain with minimum

linker peptides (Fig. 1A). When the sensor domain changes the conformation, the distance, r , and orientation factor, κ^2 , between the two fluorescent proteins will also change, resulting in the change in FRET efficiency. A typical example is the biosensor for c-Raf (Terai and Matsuda, 2005). Like many other protein kinases, c-Raf adopts a closed inactive conformation and open active conformation, the latter of which is induced by phosphorylation. Based on this knowledge, the biosensor for c-Raf was generated by fusing the donor and acceptor proteins to the C- and N-termini, respectively. As expected, the FRET efficiency was low when c-Raf is active and high when c-Raf was inactive (Fig. 1A). A large number of FRET biosensors for small molecules such as steroids, amino-acids, and sugars belong to this type (Supplementary Table 1).

The first type of FRET biosensor appears to be straightforward. However, it is not necessarily easy to find proper sites wherein the fused donor and acceptor fluorescent proteins could detect the conformational change of the sensor domain. In such cases, we should recall what the activity change means. An activity change in a protein inevitably results in a conformational change that can be detected by other entities, proteins in many cases; otherwise, the protein has no function. Thus, the second type of FRET biosensor uses an additional domain, the ligand domain, to recognize the conformational change of the sensor domain (Fig. 1B). A flexible linker is intercalated between the sensor domain and the ligand domain to facilitate the binding of the sensor domain to the ligand domain. There are many biosensors for protein kinases and small GTPases that belong to this type (Oldach and Zhang, 2014). Figure 1B shows the biosensor for Ras (Mochizuki *et al.*, 2001). Growth factor stimulation replaces the GDP on the Ras with GTP, inducing conformational change and binding to Raf. Taking this principle into consideration, in the Ras biosensor named Raichu, the Ras and Ras-binding domain of c-Raf are used as the sensor and ligand domains, respectively. Upon growth factor stimulation, the GTP-bound Ras binds to the Ras-binding domain of c-Raf, causing an increase in FRET (Fig. 1B).

Modes of action

In the design of the FRET biosensor, it is often assumed that the linkers connecting the fluorescent proteins with the sensor and ligand domains are flexible. If the linkers are flexible, the relative orientation between the donor and acceptor proteins changes in each energy transfer event evoked by the absorption of a single photon; therefore, in most probe designs the orientation factor, κ^2 , is set to 2/3, the average value assuming that the orientation can adopt any direction. Under this assumption, the FRET efficiency will correlate inversely with the distance between the donor and acceptor, resulting in a distance-dependent type biosensor as shown in the schema (Fig. 2A). One variant of this

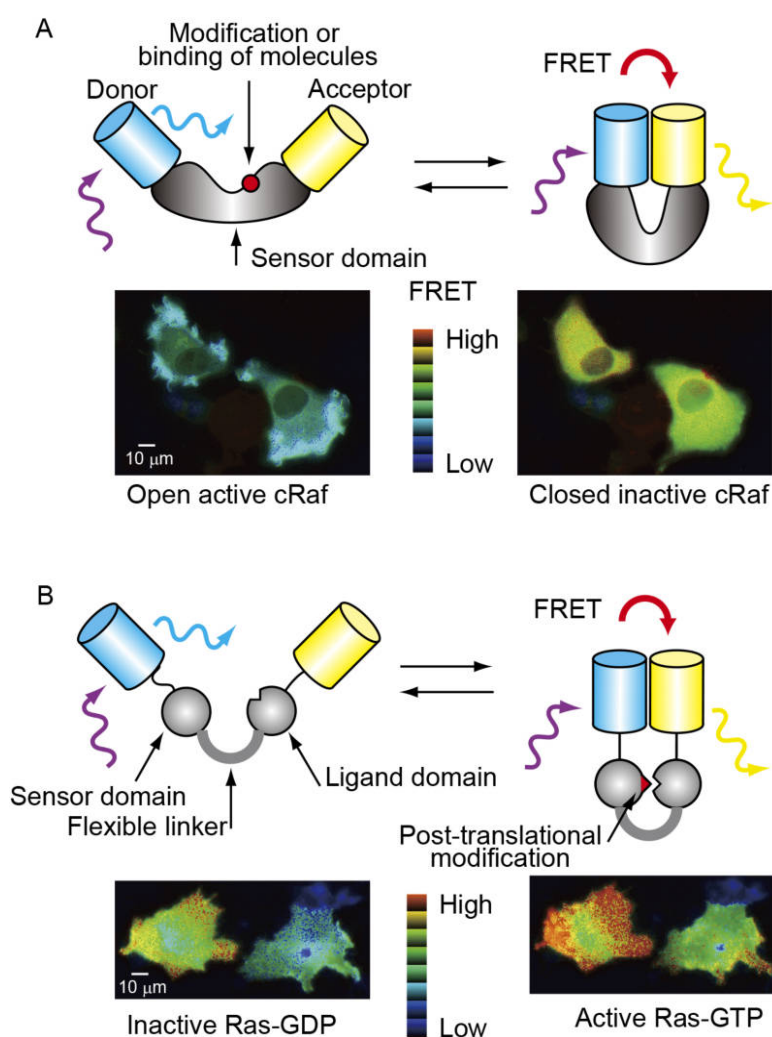


Fig. 1. Mode of action of the FRET biosensor. A. The simplest version of FRET biosensors consists of a sensor domain and the donor and acceptor fluorescent proteins. Post-translational modification or binding of other molecules changes the conformation so that the donor comes into close proximity to the acceptor and thereby increases the FRET efficiency. FRET images of the biosensor for cRaf are shown as an example. B. In the other version of FRET biosensors, the conformational change of the sensor domain is recognized by the ligand domain, which is ligated by a flexible linker. FRET images of the biosensor for Ras are shown as an example.

type of biosensor should be noted here. The second group of FRET biosensor—i.e., the biosensor following the calcium sensor—was to detect protease activity (Xu *et al.*, 1998). In this case, the linker that connects the donor and acceptor fluorescent proteins is the substrate peptide for caspase-3. Upon caspase-3 activation, the linker is cleaved to dissociate the donor protein from the acceptor fluorescent protein, culminating in the drop in FRET efficiency. Therefore, unlike many other FRET biosensors, the FRET biosensors for the proteases are irreversible (Fig. 2A).

The mode of action just described above is actually oversimplified. There are also FRET biosensors in which the binding of the sensor domain to the ligand domain decreases the FRET efficiency (Wang *et al.*, 2005). This implies

that the orientation of the fluorescent proteins is restricted and the orientation factor dominates the FRET efficiency over the distance between the donor and acceptor proteins, resulting in a so-called orientation-dependent biosensor (Fig. 2B). Noting the importance of the orientation of the donor and acceptor proteins, Nagai *et al.* developed a series of circular permutants (cp) to optimize the orientation of the donor and acceptor in order to maximize the FRET efficiency (Nagai *et al.*, 2004) (Fig. 2C).

A major factor limiting the dynamic range of the FRET biosensors is the basal FRET signal. As will be discussed later, almost all FRET biosensors exhibit significant FRET signals due to multimerization between the donor and acceptor fluorescent proteins, which is an intrinsic property

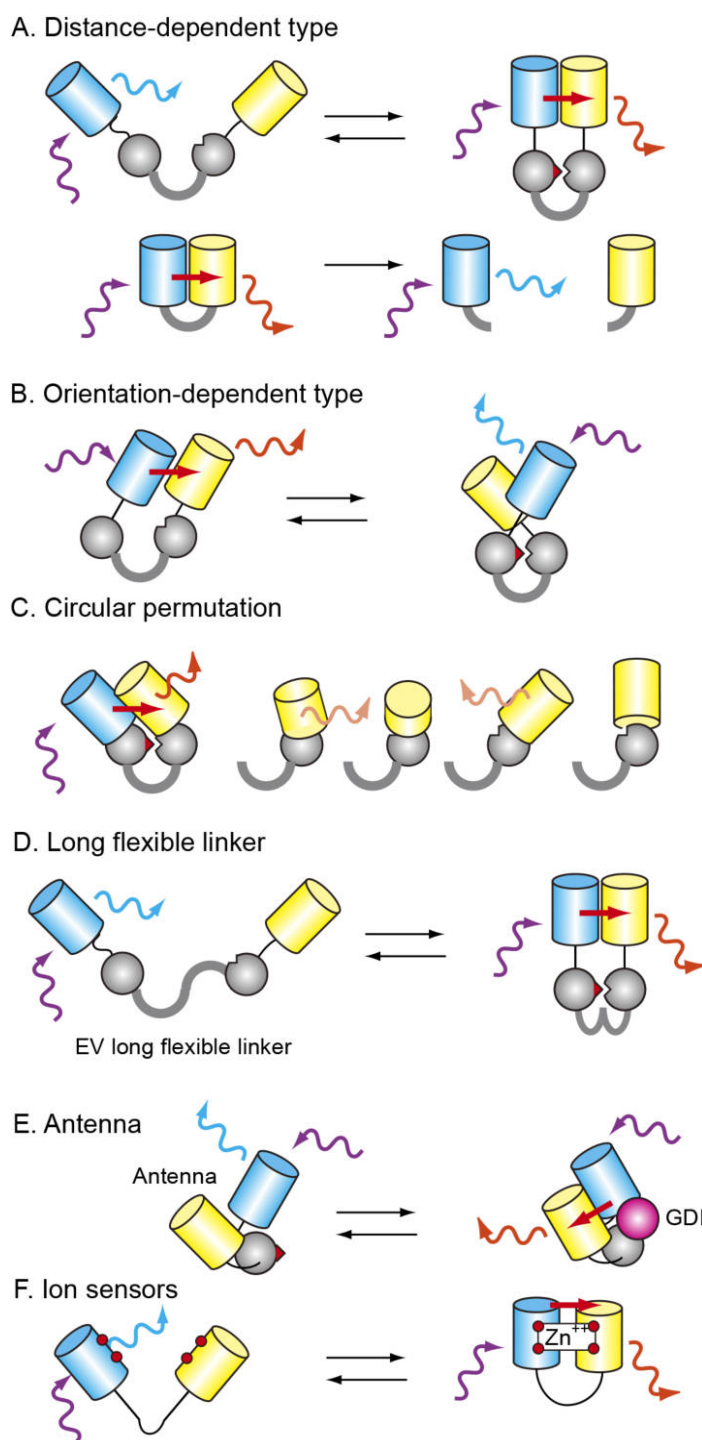


Fig. 2. Variation of FRET biosensors. A. The prototype design of the FRET biosensor. Here, the binding of the sensor and the ligand domain brings the donor into close proximity to cause FRET; this biosensor is thus called a distance-dependent type. A simple variant of this type are the FRET biosensors for proteases. B. In the orientation-dependent type, the basal FRET signal is reduced by the rotation of the fluorescent proteins. C. The circular permutants are often used to search for the best orientation of the fluorescent proteins. D. To minimize the basal FRET signal, flexible linkers of more than 100 amino acids are often used. E. The donor and acceptor proteins are fused with a short linker and fused to small GTPases just like an antenna. Binding of guanine nucleotide dissociation inhibitor (GDI) rotates the fluorescent proteins so that the FRET efficiency increases. F. The dimerization interface of the donor and acceptor proteins is modified to give the ions binding ability.

of fluorescent proteins. Because the probability of the binding between the donor and acceptor fluorescent proteins depends on the distance between them, a 116 amino acid-length flexible linker called the EV linker has been developed to reduce this basal signal and, in fact, this linker was shown to markedly increase the dynamic range of FRET biosensors (Komatsu *et al.*, 2011) (Fig. 2D). A similar 42 amino acid-length linker has also been developed by the Merckx laboratory (Vinkenborg *et al.*, 2009). More recently, Hodgson and his colleagues pursued a strategy that was the opposite of the approach adopted for the long flexible linkers: they proposed a novel type of FRET in which the donor and acceptor are directly linked to minimize the distance and fused to small GTPases just like an antenna (Hodgson *et al.*, 2016) (Fig. 2E). In this probe design, binding of the guanine nucleotide dissociation inhibitor (GDI) to the small GTPase twists the fluorescent proteins, increasing the FRET efficiency. Finally, we should note one last type of FRET biosensor having a very simple structure. Here, zinc-binding amino acid residues are introduced into the dimer interface of the donor and acceptor fluorescent proteins to form a zinc indicator (Fig. 2F) (Hessels *et al.*, 2015).

Fluorescent proteins for intramolecular FRET biosensors

CFP and YFP—the *de facto* standard

A number of fluorescent proteins have been developed and tested for their suitability to FRET biosensors. The merits and demerits of each FRET pair have been discussed previously (Bajar *et al.*, 2016; Mastop *et al.*, 2017; Martin *et al.*, 2018); therefore, in this report we will limit ourselves to a brief overview of the history. The first genetically encoded FRET biosensor for calcium, cameleon-1, was comprised of *Aequoria victoria* GFP and its color variant, blue fluorescent protein (BFP), as the acceptor and donor, respectively. However, to reduce autofluorescence and photodamage accompanied by the use of BFP, the BFP/GFP pair was soon replaced with the pair of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), both of which are also color variants of GFP (Miyawaki *et al.*, 1997). Since then, more than 90% of the FRET biosensors developed have used the CFP/YFP pair and offspring thereof (Supplementary Table 1). The reason why the fluorescent proteins derived from other organisms failed to surpass the CFP/YFP pair may be simply because many researchers kept improving CFPs and YFPs, and thus the speed of the improvement of this pair always surpassed that of the other fluorescent protein combinations. Alternatively, because the dimerization of the donor and acceptor is preferable for the high FRET efficiency, the susceptibility to dimerization of the CFP/YFP pair might have made it

invincible (Cranfill *et al.*, 2016).

cp mutants and YPet—the developer's choice for the acceptor

In terms of the general improvement of fluorescent proteins, the major goals have been resistance to low pH and photodamage, higher quantum efficiency and molar extinction co-efficient, and faster maturation periods. From these points of view, the first choices today will be Cerulean (Rizzo *et al.*, 2004) or Turquoise (Goedhart *et al.*, 2010) as donor, and citrine (Griesbeck *et al.*, 2001) or Venus (Nagai *et al.*, 2002) as acceptor. Meanwhile, there are also strategies for improving CFP and YFP for higher FRET efficiency. As already described, Nagai and Miyawaki took advantage of the β -can structure of YFP and developed a series of circular permutants of YFP, cpYFPs, which they hope to find the best orientation of the fluorophores (Nagai *et al.*, 2004) (Fig. 2C). On the other hand, Nguyen and Daugherty engineered a pair of CFP and YFP mutants, CyPet and YPet, for the best FRET efficiency (Nguyen and Daugherty, 2005). The CyPet/YPet pair exhibits several-fold higher FRET efficiency in comparison to the prototype CFP/YFP pair. Since their report, YPet has been used in many FRET biosensors, although CyPet is rather dim and has not gained much support.

Because both cpVenus and YPet have been used widely, it is worth discussing the mechanism underlying the high FRET efficiency by these two acceptor proteins. The original idea of the cpYFP mutant was to select the best orientation of the acceptor versus the donor (Fig. 2C). Thus one would expect that the cpYFPs selected for many FRET biosensors would vary depending on the 3D structure of the corresponding FRET biosensor. Strangely, however, among the several cp mutants, cp173 has been the most frequently chosen by far (Supplementary Table 1). This strongly suggests that the cp173 mutant must have some unknown advantage over the other cp mutants. In fact, it has been suggested that the cp173 mutant may efficiently dimerize with the donor in antiparallel configuration, resulting in high FRET efficiency (Kotera *et al.*, 2010). Meanwhile, the cause of the high FRET efficiency by the CyPet/YPet pair is itself controversial. The original developers negated the possibility that an increase in multimerization was the cause of the enhanced FRET (Nguyen and Daugherty, 2005). However, despite their ruling out this possible mechanism, later studies strongly suggested that the mutations acquired during the evolution of YPet conferred a dimerization property, and thereby enhanced the FRET efficiency between CFP and YPet (Vinkenborg *et al.*, 2007; Kotera *et al.*, 2010). In a related development, Lindenburg *et al.* showed that by introducing mutations at the dimerization interface of Cherry and Orange, which are originated from a *Discosoma* species, the FRET efficiency can be improved significantly (Lindenburg *et al.*, 2013).

Other FRET pairs—rising red stars

To use the FRET biosensor based on the CFP/YFP pair, cells are excited by blue light and monitored for cyan and yellow fluorescence. Therefore, researchers are forced to utilize the UV to blue range or orange to far-red range for the additional use of fluorescent probes (Fig. 3A). One idea is to use a fluorescent protein with a long Stokes shift. Ai *et al.* reported the development of a caspase sensor with mAmetrine, a blue light-excitable fluorescent protein with a long Stokes shift, as a donor and tdTomato, a red protein as the acceptor (Ai *et al.*, 2008). Similarly, Niino *et al.* used Sapphire as a donor and red fluorescent protein (RFP) as the acceptor (Niino *et al.*, 2009) (Fig. 3B). Both FRET biosensors were shown to be compatible with the biosensors comprising of the CFP/YFP pair. To use the yellow to red light region, mOrange and mCherry (Fig. 3C) were adopted as the donor and acceptor proteins, respectively, in biosensors for MT1-MMP (Ouyang *et al.*, 2010) and for Zn^{++} (Miranda *et al.*, 2012; Lindenburg *et al.*, 2013). More recently, the discoveries of fluorescent proteins in far-red to near-infrared range has enabled almost complete separation from the CFP/YFP pair. Zlobovskaya *et al.* reported the use of the mKate2 and iRFP pair (Zlobovskaya *et al.*, 2016), whereas Shcherbakova *et al.* reported miRFP720 and miRFP670 as the FRET pair (Shcherbakova *et al.*, 2018) (Fig. 3D). A potential problem with iRFP-derived fluorescent proteins is that the intracellular concentration of bilirubin, the extrinsic fluorophore for iRFP, may not be sufficient. This would become problematic particularly when these fluorescent proteins are expressed in mice.

FRET biosensors based on the intrinsic properties of fluorescent proteins

Generally speaking, the fluorescence of FRET biosensors should be robust to the surrounding milieu, but the fluorescence can be subject to changes in pH and ionic strength. There are groups of FRET biosensors that take advantage of this intrinsic property of fluorescent proteins. The pKa of YFP is approximately 6. This pH modulation of the YFP fluorophore has been used to construct pH sensor proteins (Hellwig *et al.*, 2004; Esposito *et al.*, 2008) (Fig. 4A). Recently, Morikawa *et al.* found that the fluorophore of YFP is sensitive to protein crowding (Morikawa *et al.*, 2016). They introduced a mutation to increase the sensitivity to the protein crowding and generated a protein-crowding sensor (Fig. 4B). Another intrinsic property of GFP-derived fluorescent proteins is the requirement of molecular oxygen for their autocatalytic chromophore synthesis. By using a hypoxia-tolerant flavin-binding fluorescent protein (FbFP) as the donor and YFP as the acceptor, Potzkei *et al.* invented an oxygen sensor (Potzkei *et al.*, 2012) (Fig. 4C). In this FRET biosensor design, FRET can-

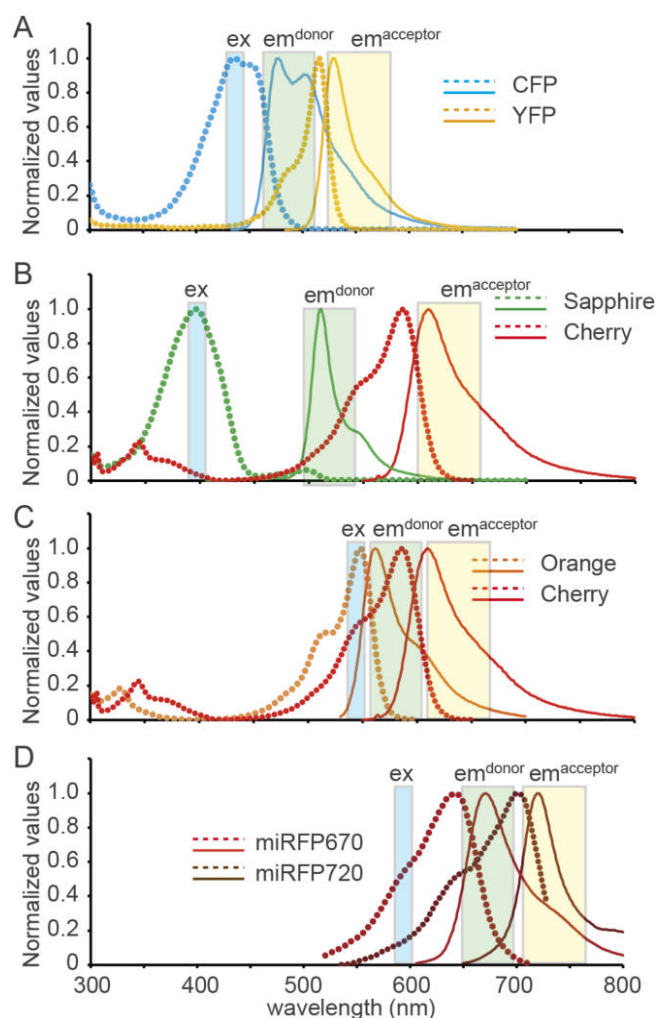


Fig. 3. Absorption and emission spectra of fluorescent proteins used for FRET. The blue, green, and yellow boxes indicate the wavelength range of filters used for the donor excitation (ex), donor emission (em^{donor}), and acceptor emission ($em^{acceptor}$), respectively.

not be observed under an anoxic condition because the fluorophore of YFP can be generated only in the presence of molecular oxygen. Of note, the maturation of YFP is an irreversible process; therefore, the probe cannot detect the decrease of oxygen. Lastly, for the development of a membrane-voltage sensor, bacterial rhodopsin was employed as the FRET acceptor, and was termed an electrochromic quencher (Gong *et al.*, 2014; Zou *et al.*, 2014). Upon depolarization of the membrane potential, the bacterial rhodopsin absorbs the excited energy of the fused fluorescent protein by a mechanism called electrochromic FRET (Fig. 4D), enabling the measurement of membrane potential by FRET.

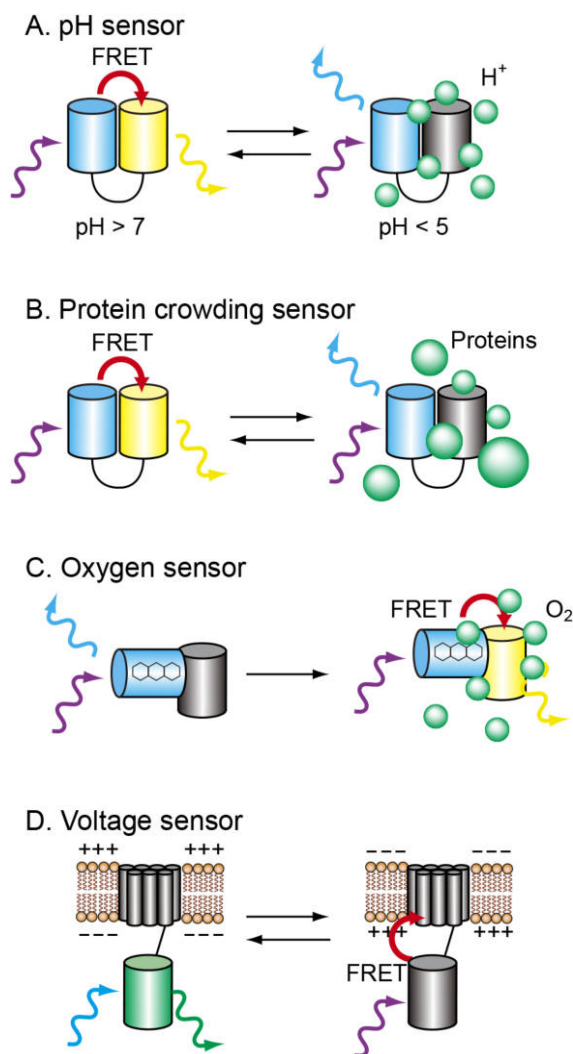


Fig. 4. FRET biosensors based on the intrinsic properties of fluorescent proteins. A. A pH sensor takes advantage of the pH-sensitive quenching of YFPs. B. A YFP mutant that is sensitive to protein crowding is used as the acceptor in the protein-crowding sensor. C. GFP derivatives require molecular oxygen for the maturation of fluorophore. In contrast, the donor fluorescent protein FbFP contains flavin as the fluorophore and does not require oxygen. D. Some voltage sensors use voltage-dependent rhodopsin as the acceptor.

Detection of FRET signals

Cells expressing the FRET biosensors can be analyzed after cell lysis or in intact cells by ratiometry with a microplate reader or a flow cytometer, or by fluorescence microscopy. Various methods for FRET microscopy and the unique features thereof have been discussed previously (Pietraszewska-Bogiel and Gadella, 2011); therefore, we will describe the current methods in brief using schematic diagrams.

The aim of detecting FRET signals is to quantify the

fraction of the On-state FRET biosensor (Fig. 5A). The sensitized FRET or intensity-based FRET method, which quantifies the ratio of the donor-derived versus the acceptor-derived fluorescence intensity, is the current standard in conventional fluorescence microscopy using FRET biosensors (Berney and Danuser, 2003) (Fig. 5B). Importantly, the simple ratio of fluorescence intensity of the donor channel versus the acceptor channel can be used for semi-quantitative measurement of the FRET efficiency. This is because the amount of spectral bleed-through from the donor and cross-excitation of the acceptor can be ignored in the intramolecular FRET biosensors (Aoki and Matsuda, 2009). If the microscope is equipped with a spectrometer, the fraction of the donor and the acceptor can be calculated by spectral unmixing (Zimmermann *et al.*, 2002; Komatsu *et al.*, 2018). Just to evidence the presence of FRET, the acceptor photobleaching method is conveniently applied, although this is an irreversible process (Bastiaens *et al.*, 1996) (Fig. 5C).

homo-FRET

The next two methods require special instrumentation. With polarized excitation light, the level of FRET efficiency can be estimated by measuring the ratio of parallel and vertical components of the emitted fluorescence (Clayton *et al.*, 2002) (Fig. 5D). Importantly, in this anisotropy measurement method, the same fluorescent protein can be used as the donor and acceptor, an arrangement known as homo-FRET. In principle, any fluorescent protein can be used for this method and it has been shown that many FRET biosensors can be instantaneously transformed to the homo-FRET biosensors (Ross *et al.*, 2018). Moreover, Kim *et al.* reported a microscope with photonic crystal analyzers that reliably detected fluorescence anisotropy with high precision (Kim *et al.*, 2017). With this novel technique, three homo-FRET biosensors were simultaneously monitored in the cells.

Fluorescence lifetime imaging microscopy (FLIM)

Lastly, the most robust system to quantify FRET is FLIM (Bastiaens and Squire, 1999) (Fig. 5E). This method requires only the lifetime of the donor fluorescence. Thus, by using non-radiative fluorescent proteins as the acceptor, multiplexed imaging can be achieved easily in FLIM (Murakoshi and Shibata, 2017). For guidance in the choice of FRET-detection method, Pietraszewska-Bogiel and Gadella published an excellent guideline (Pietraszewska-Bogiel and Gadella, 2011).

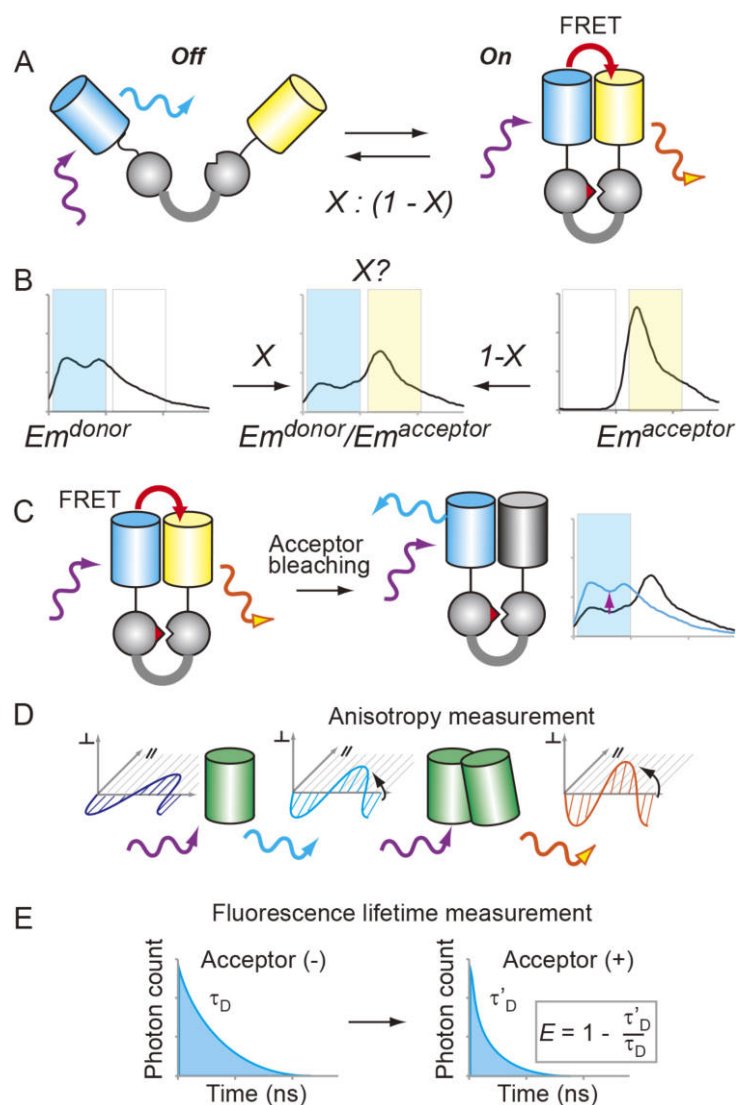


Fig. 5. Detection of FRET. A and B. FRET efficiency is estimated by measuring the contribution of the donor and the acceptor fluorescent proteins to the spectrum of the samples. The simplest method is to use the ratio of acceptor fluorescence intensity versus donor fluorescence intensity. The blue and yellow boxes indicate the filters used to acquire the photons for the donor and acceptor channels, respectively. If the spectra of the donor, acceptor, and sample are available, the contribution of the donor and acceptor can be determined by a method called spectral unmixing. C. A simple method to quantify the FRET efficiency is acceptor photobleaching. The increase in the donor fluorescence after photobleaching of the acceptor indicates FRET. D. By illuminating the donor with polarized emission light and measuring the fraction of parallel and perpendicular components of fluorescence, fluorescence from the donor and acceptor can be quantitatively analyzed. E. FRET causes a decrease in the fluorescence lifetime of the acceptor. Note that the FRET efficiency (E) can be measured accurately from the lifetimes in the presence and absence of the acceptor.

Evolution of FRET biosensors

Proteases

Next we overview how the application range of FRET biosensors has been steadily expanded (Fig. 6, Supplementary Table 1). The first genetically-encoded FRET biosensor was that of the Factor Xa protease (Mitra *et al.*, 1996). Because of its simple design (Fig. 2A, lower panel), the

strategy adopted in the Factor Xa reporter was adopted for other proteases, including caspases (Xu *et al.*, 1998), matrix metalloproteases (Yang *et al.*, 2007), granzyme B (Choi and Mitchison, 2013), and neutrophil elastase (Schulenburg *et al.*, 2016). In this class of FRET biosensors, the FRET signal becomes almost zero after the cleavage of the linker, which guarantees high signal-to-noise ratio. Therefore, FRET biosensors for proteases are routinely developed to demonstrate the proof of concept of new FRET pairs of flu-

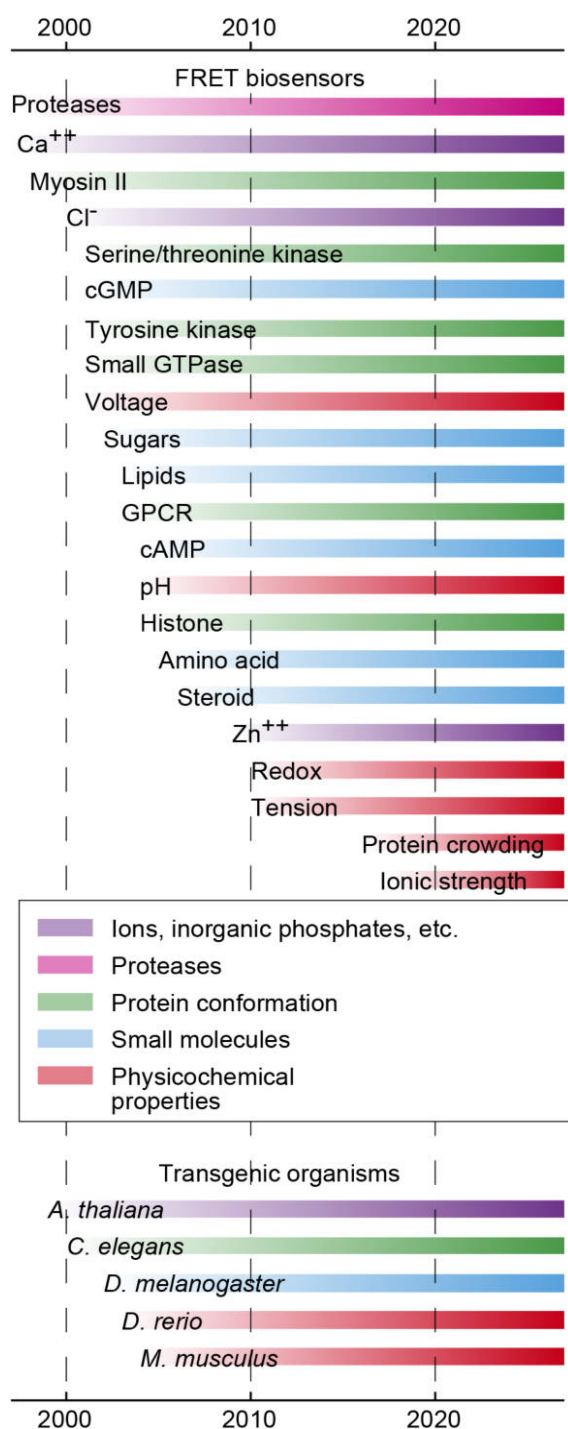


Fig. 6. Evolution of FRET biosensors and transgenic multicellular organisms. The upper panel shows the year of the development of each class of FRET biosensor. The lower panel shows the years of the development of transgenic organisms expressing the calcium sensor cameleon.

orescent proteins (Supplementary Table 1). In contrast to the other FRET biosensors, the response of the FRET bio-

sensors against proteases is irreversible, meaning that time derivative of FRET signals represents the protease activity at each time point. By this reason, the FRET biosensors for proteases *in vivo* may not as sensitive as can be anticipated by the wide dynamic range *in vitro*.

Ions, inorganic phosphates, and heavy metals

The successful development of the FRET biosensors for calcium in 1997 (Miyawaki *et al.*, 1997; Persechini *et al.*, 1997) encouraged researchers to develop FRET biosensors for other ions and inorganic molecules, including Cl⁻, Mg²⁺, K⁺, Zn²⁺, Cu⁺, and inorganic phosphate. Most of the FRET biosensors for ions adopt a simple design that consists of an ion-binding motif sandwiched by the donor and acceptor fluorescent proteins as exemplified in Fig. 1. The ion-binding motives should be carefully chosen by the high specificity and appropriate affinity, which matches the sub-cellular concentrations of the ions of interest. For example, the first two FRET biosensors for calcium, which used either calmodulin or calmodulin-binding domain from smooth muscle myosin light chain kinase (Miyawaki *et al.*, 1997; Osibow *et al.*, 2006), suffered from low efficiency of transgenic mouse production or failed to monitor calcium concentration of ER. To resolve these problems, a calcium binding domain of troponin C or a kringle domain from apolipoprotein were employed in the other calcium sensors (Heim and Griesbeck, 2004; Osibow *et al.*, 2006). A unique ion sensor is the FRET biosensor for chloride (Kuner and Augustine, 2000). The chloride biosensor Clomeleon took advantage of the sensitivity of YFP, but not CFP, to chloride ions. Another variant that utilize the intrinsic property of the fluorescent protein is the zinc biosensor eZinCh, which chelates a zinc ion between the donor and acceptor proteins to induce dimerization (Fig. 2F). Genetically encoded fluorescent sensors for Zn²⁺ and Cu⁺ have been extensively reviewed recently (Hao *et al.*, 2018).

Protein conformation

Soon after the development of the FRET biosensors for calcium ions and proteases, those of Myosin II (Suzuki *et al.*, 1998) was reported, heralding the most flourishing class of FRET biosensors for three key enzymes of signal transduction, small GTPases (Mochizuki *et al.*, 2001), tyrosine kinases (Kurokawa *et al.*, 2001; Ting *et al.*, 2001), and serine/threonine kinases (Zhang *et al.*, 2001). It may be worth describing the evolution of the FRET biosensors for small GTPases to figure out what have been modified for the better biosensors. In the first FRET biosensor for Ras, Raichu-Ras, the pair of Ras and Ras-binding domain of Raf was sandwiched by the FRET pair of fluorescent protein, CFP and YFP (Fig. 7A). The binding of Ras-GTP to Raf causes FRET. Soon after the report of Raichu-Ras, a FRET biosensor with a simple structure was reported for the monitoring

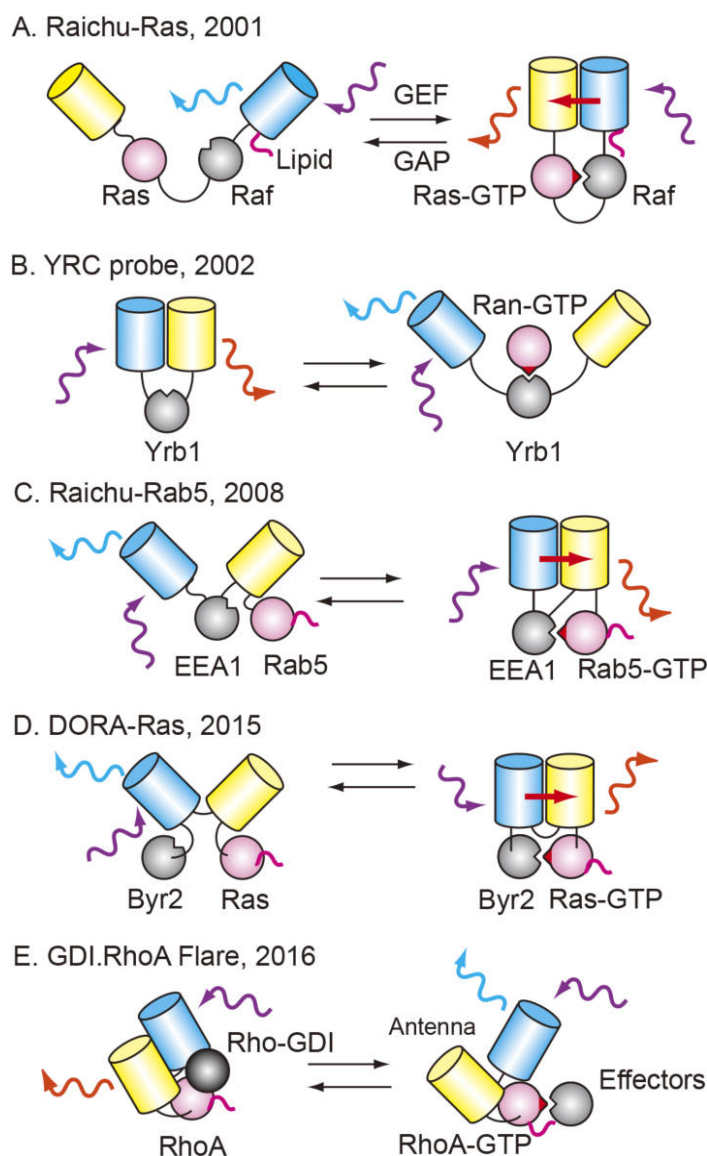


Fig. 7. Evolution of FRET biosensors for small GTPases. A. Raichu-Ras, the archetype FRET biosensor for small GTPases. B. YRC probe for Ran. C. Raichu-Rab5. D. DORA-Ras. E. GDI.RhoA Flare. The biosensors generally are comprised of the acceptor fluorescent protein YFP (yellow), small GTPases (magenta), linker, GTPase-binding domain (grey), and the donor fluorescent protein CFP (cyan). The red curves indicate C-terminal lipid moiety. Note that YRC (B) does not contain GTPase and that GDI.RhoA Flare does not contain the GTPase-binding domain.

of Ran, a nuclear small GTPase (Fig. 7B). In this FRET biosensor design, Yrb1, a Ran-binding protein was sandwiched by the FRET pair. The endogenous Ran-GTP binds to Yrb1 and decreases the basal FRET signal. Similar FRET biosensors were also developed for Rho-family GTPases (Itoh *et al.*, 2002; Yoshizaki *et al.*, 2003). A problem of the earlier FRET biosensors was related to the subcellular localization of the FRET biosensors. Most small GTPases are localized at membrane by the lipid modification at either C- or N-terminus. In the first generation biosensors for Ras, the membrane targeting signal is chopped

out of the GTPases and fused to the C-terminus of the fluorescent protein. This modification may not recruit the FRET biosensors to the physiological subcellular locations. To overcome this problem, following generations of FRET biosensors carry small GTPases at the C-terminus (Fig. 7C, D) (Kitano *et al.*, 2008). Notably, in the latest generations of the FRET biosensor, in which the dynamic ranges have been improved, the donor and acceptor fluorescent proteins are tandemly placed (Ng *et al.*, 2015; Hodgson *et al.*, 2016), so that they seem to operate in the orientation-dependent manner (Fig. 2B, Fig. 7E).

The first FRET biosensor for protein kinases, cAMP-responsive tracer (ART), was reported in 2000 (Nagai *et al.*, 2000). In ART, Kemptide, a well-characterized synthetic substrate peptide of PKA was sandwiched by the donor and acceptor fluorescent proteins. The basal FRET signal was reduced upon PKA phosphorylation of the biosensor. Soon after the initial success of the development, drastic improvement of the dynamic range of the PKA FRET biosensor was achieved by the use phosphate-binding domain of 14-3-3 (Zhang *et al.*, 2001). Almost same time, FRET biosensors for tyrosine kinases were also reported from two research groups (Kurokawa *et al.*, 2001; Ting *et al.*, 2001). Both groups used SH2 domain for the detection of phosphotyrosine of the substrate domain in the FRET biosensors. Thereafter, a large number of FRET biosensors for protein kinases have been reported to cover all seven classes of protein kinases, with essentially the same backbones (Oldach and Zhang, 2014; Maryu *et al.*, 2018).

Small molecules

In 2000, the targets of FRET biosensors were expanded to cGMP (Sato *et al.*, 2000).

The cGMP biosensor was the first-in-class FRET biosensor to target small molecules such as nucleotides, sugars (Fehr *et al.*, 2002), lipids (Sato *et al.*, 2003; Violin *et al.*, 2003), amino acids (Okumoto *et al.*, 2005), and steroids (De *et al.*, 2005), which were all developed within several years of the first report. The number and the varieties of FRET biosensors of this class are increasing steadily (Supplementary Table 1). However, it should be noted that careful controls are required for quantitative measurements of these small molecules due to the effect of pH and ionic strength (Moussa *et al.*, 2014).

Physicochemical properties

The biosensor for membrane potential (Sakai *et al.*, 2001) was the first-in-class of the FRET biosensors for assessing the physicochemical properties of cells, including pH (Hellwig *et al.*, 2004), redox (Yano *et al.*, 2010), tension (Grashoff *et al.*, 2010), protein crowding (Morikawa *et al.*, 2016), oxygen concentration (Patzke *et al.*, 2012), and ionic strength (Liu *et al.*, 2017). Because of the increasing demand from neuroscientists, a huge number of voltage sensors have already been developed (Lin and Schnitzer, 2016). Meanwhile, the FRET biosensor for tension is attracting particular interest in the field of mechanobiology (Ma and Salaita, 2018).

Cellular heterogeneity as revealed by FRET biosensors

The benefit of genetically encoded biosensors is exempli-

fied when biosensors are expressed in the cells of interest, but the stable expression of FRET biosensors in cells may be more difficult than expected. For example, it has often been reported that the biosensor-expression is considerably heterogeneous in cell clones established with a linearized plasmid DNA (Maares *et al.*, 2018). This heterogeneity may be caused by gene silencing or by homologous recombination between the coding DNAs of donor and acceptor fluorophore, when both are derived from *Aequoria victoria* GFP (Komatsubara *et al.*, 2015). The level of gene silencing may be promoter-dependent and the frequency of homologous recombination may vary by the percentage of homology, the length of insertion between the two fluorescent proteins, and cell lines. These uncertainties may be the reason why this inconvenient truth is not necessarily shared among researchers. Here it will suffice to say that this potential pitfall during the establishment of cell lines can be overcome either by the use of transposon-mediated gene transfer or reduction of the homology between the donor and the acceptor (Aoki *et al.*, 2012; Komatsubara *et al.*, 2015).

When the FRET biosensors are introduced into the cell lines by the aforementioned methods, the expression level is stable during the passage and homogenous among the cells (Aoki *et al.*, 2012; Komatsubara *et al.*, 2015). This homogenous and stable expression of the FRET biosensor has allowed researchers to shed light on the heterogeneity of the activity of protein kinases such as ERK (Fig. 8). Albeck *et al.* found that cells could exhibit stochastic ERK activation in the presence of a low concentration of epidermal growth factor (EGF) (Albeck *et al.*, 2013). We also observed similar stochastic ERK activation and found that in some cell types this ERK activation can be propagated to the neighboring cells in a manner dependent on EGF-family proteins and EGF receptors (Aoki *et al.*, 2013). Similar fluctuation of protein kinase activity was also found in AMPK (Hung *et al.*, 2017). It should be emphasized that timelapse imaging is a powerful approach to untangle the mechanism behind the heterogeneity of enzymatic activities. Looking at a video, we can easily tell whether the heterogeneity reflects an intrinsic fluctuation (Albeck *et al.*, 2013), activation/suppression mediated by the adjacent cells (Aoki *et al.*, 2013), or memory before cell divisions (Yukinaga *et al.*, 2014; Yang *et al.*, 2017).

Intravital imaging with FRET biosensors

One of the most exciting applications of the FRET biosensor is intravital imaging, which fully capitalizes on the advantages of the “genetically encoded” probe. Within seven years after the initial report, cameleon was already expressed in five major experimental multicellular organisms, *A. thaliana* (Allen *et al.*, 1999), *C. elegans* (Kerr *et al.*, 2000), *D. melanogaster* (Fiala *et al.*, 2002; Reiff *et al.*,

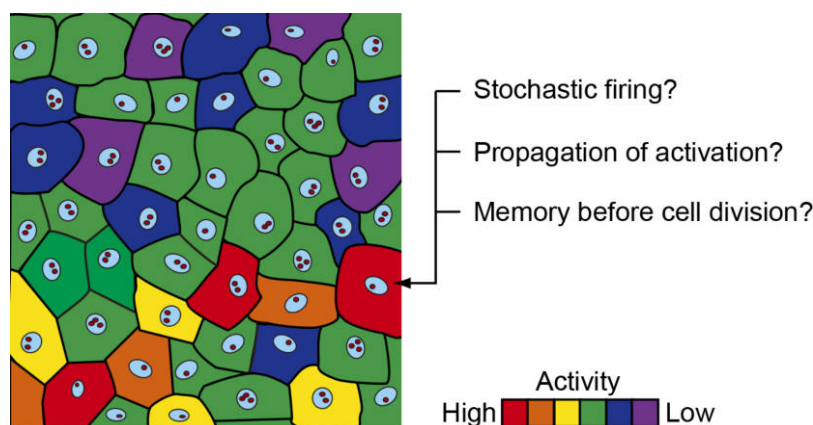


Fig. 8. The snapshot of molecular activity. A snapshot of the activity of signaling molecules such as ERK MAP kinase sometimes exhibits remarkable heterogeneity, which may be caused by stochastic fluctuation, propagation of activation waves, or a memory before cell divisions. Timelapse imaging for longer than the cell cycle period is the best way to infer the mechanism.

Table 1. TRANSGENIC MOUSE LINES EXPRESSING FRET BIOSENSORS

Target	Method	Tissue distribution	Reference
Ca ⁺⁺	Conventional*	Langerhans' islands	(Hara <i>et al.</i> , 2004)
MLCK	Conventional	smooth muscle cells	(Isotani <i>et al.</i> , 2004)
Ca ⁺⁺	Conventional	neuron	(Tsujino <i>et al.</i> , 2005)
cAMP	Conventional	cardiomyocyte	(Nikolaev <i>et al.</i> , 2006)
Ca ⁺⁺	Conventional	neuron	(Heim <i>et al.</i> , 2007)
Caspase 3	ROSA26 knock-in	Cre-dependent	(Tomura <i>et al.</i> , 2009)
cAMP	Conventional	ubiquitous	(Calebiro <i>et al.</i> , 2009)
Ca ⁺⁺	Conventional	glia	(Atkin <i>et al.</i> , 2009)
Caspase 3	Conventional/insulator	ubiquitous	(Yamaguchi <i>et al.</i> , 2011)
PKA, ERK	Tol2-mediated	ubiquitous	(Kamioka <i>et al.</i> , 2012)
Rac1	ROSA26 knock-in	Cre-dependent	(Johnsson <i>et al.</i> , 2014)
PKA, ERK	Tol2-mediated	Cre-dependent	(Goto <i>et al.</i> , 2015)
AMPK	Tol2-mediated	ubiquitous	(Konagaya <i>et al.</i> , 2017)
RhoA	ROSA26 knock-in	Cre-dependent	(Nobis <i>et al.</i> , 2017)
Akt	ROSA26 knock-in	Cre-dependent	(Warren <i>et al.</i> , 2018)

* The conventional method denotes the injection of linearized DNA into oocytes.

2002), *D. rerio* (Higashijima *et al.*, 2003), and *M. musculus* (Hara *et al.*, 2004) (Fig. 6). However, these organisms were not necessarily widely used thereafter, because the expression level was often insufficient for observation of the cells of interest. In *A. thaliana* the transgene was found to suffer from gene silencing (Deuschle *et al.*, 2006). Similarly, the expression of FRET biosensors was reported to be a difficult task in mice (Hara *et al.*, 2004; Tsujino *et al.*, 2005; Calebiro *et al.*, 2009). The difficulties may have arisen from the homologous recombination, toxicity, or gene silencing. Although the reasons for the failure were not clearly identified, several approaches have been shown to alleviate the problem. First, Hara *et al.* reported that a transgenic mouse line expressing cameleon could be generated by using a tissue-specific promoter (Hara *et al.*, 2004). Second, expression cassettes have been inserted into the ROSA26 locus to evade gene silencing (Tomura *et al.*,

2009; Johnsson *et al.*, 2014). In a third approach, Yamaguchi *et al.* used an insulator sequence to prevent the expression cassette from gene silencing (Yamaguchi *et al.*, 2011). Finally, we found that Tol2 transposase-mediated gene transfer could produce transgenic mice expressing a FRET biosensor with very high efficiency (Kamioka *et al.*, 2012). These advances notwithstanding, the number of mouse lines expressing FRET biosensors is still small (Table I). This may be partly because the instrumentation required for the intravital imaging of mice is not necessarily accessible to many researchers and partly because the merit of this technology is not widely appreciated (Hirata and Kiyokawa, 2016). Therefore, it may be worth referring to recent discoveries that were achieved only by the use of transgenic mice expressing the FRET biosensor, or FRET mice, as they are often known.

In a study using cultured epithelial cells, we noticed that

ERK activation could be propagated to neighboring cells in an EGFR-dependent manner (Aoki *et al.*, 2013). The direction of the propagation was almost random. We next examined whether similar propagation of ERK activation could be observed in mouse epithelial cells (Hiratsuka *et al.*, 2015). We found that the ERK activation propagation rarely occurs in normal mice, and when it does, it is initiated from a few cells and propagated to the neighboring cells in a firework-like burst of activity. We named this phenomenon the spatial propagation of radial ERK activity distribution, or SPREAD for short. The average diameter of SPREAD is approximately 0.1 mm and the duration is one half hour. SPREAD appears to promote G2/M exit to G1. Interestingly, when the skin was wounded, the ERK activation was always propagated from the wound edge. The ERK activation wave could reach a few millimeters from the wound edge. Importantly, we recently found that the epithelial cells move collectively against the ERK activation wave (Aoki *et al.*, 2017). The role of growth factors in cell migration has been reported in developing fruit flies, but this is the first report to show that the repetitive waves of activation of a growth factor signaling pathway can regulate collective cell migration.

Future perspectives

Here, we have limited ourselves to a discussion of only genetically encoded intramolecular FRET biosensors, the future of which may partly depend on the advent of other imaging techniques (Miyawaki and Niino, 2015; Sanford and Palmer, 2017; Ni *et al.*, 2018; Greenwald *et al.*, 2018). The most formidable rival for the intramolecular FRET biosensors is the single fluorescent protein (SP)-based biosensor. In the field of neuroscience, neuronal activity is measured by calcium influx and membrane voltage, and both these phenomena can be measured by either FRET biosensors or SP-based biosensors. In fact, various derivatives of the SP-based calcium sensor GCaMP are dominating FRET biosensors as the preferred choice for calcium imaging, particularly in intravital setup (Nakai *et al.*, 2001; Grienberger and Konnerth, 2012). Similarly, SP-based sensors are more frequently used for the measurement of neuronal voltage than the FRET-based sensors (Lin and Schnitzer, 2016). A common feature to these two types of indicators is the time scale required for recording, which is sometimes on the order of milliseconds. Quantification of FRET requires images of the donor and acceptor, which may take a few seconds for ordinary microscopy setup, rendering the FRET biosensor less popular among neuroscientists. Furthermore, both calcium and voltage are digital signals, so that the frequency of the signals is more important than their strength. A major virtue of FRET biosensors over SP-based sensors stems from the ratiometry of the former, which is robust and suitable for the measure-

ment of strength. However, the SP-based sensors can also be transformed to ratiometric sensors by the addition of suitable fluorescent protein for the calibration (Nagai *et al.*, 2001; Zhao *et al.*, 2011). Therefore, it is not a critical difference whether the probe is SP-based or FRET-based. We should choose the better ones every time depending on the properties to be observed.

Higher sensitivity and a lower signal-to-noise ratio are always suitable targets for improvement. These could be achieved not only by improved FRET biosensors but also by better image-acquisition devices. Over the last two decades, an increase in the sensitivity of detectors, such as the CMOS camera and photon detectors, has markedly improved the quality of images acquired by the FRET biosensors. Meanwhile, even though FLIM has been regarded as an ideal method for the quantification of FRET signals, the earlier instruments did not necessarily yield better results than did the widefield or confocal microscopes in terms of image quality and time resolution. FLIM microscopes with higher sensitivity are awaited to expand the applications of FRET biosensors.

Another potential application of the intramolecular FRET biosensors is drug screening using cell lines expressing a FRET biosensor. Conventional fluorescent microplate readers do not have enough sensitivity to quantify the FRET signal of the cell lines. To circumvent this problem, FRET biosensors fused to bioluminescent proteins, i.e., FRET-BRET hybrid biosensors, have recently been developed, and are expected to become a promising new application of FRET biosensors (Aper *et al.*, 2016; Komatsu *et al.*, 2018), because these hybrid type biosensors can take advantage of the bioluminescence-based screening methods.

Trends in cell biology are often reflected in registered keywords. We have moved from population to single cell analysis, from mean to heterogeneity, and from a reductionist approach to systems analysis. It is hoped that another important shift is forthcoming: from western blotting with activity-specific antibodies to FRET imaging for cellular function.

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