Visualization of growth signal transduction cascades in living cells with genetically encoded probes based on Förster resonance energy transfer

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Fluorescence probes based on the principle of Förster resonance energy transfer (FRET) have shed new light on our understanding of signal transduction cascades. Among them, unimolecular FRET probes containing fluorescence proteins are rapidly increasing in number because these genetically encoded probes can be easily loaded into living cells and allow simple acquisition of FRET images. We have developed probes for small GTPases, tyrosine kinases, serine–threonine kinases and phosphoinositides. Images obtained with these probes have revealed that membrane protrusions such as nascent lamellipodia or neurites provide an active signalling platform in the growth factor-stimulated cells.

Keywords: Förster resonance energy transfer; fluorescence resonance energy transfer; fluorescence protein; signal transduction

1. PROBES BASED ON THE PRINCIPLE OF FRET

In this post-genome project era, the characterization of protein networks has become a major focus of scientific interest. To build a model of a signalling network, it is essential to obtain the spatio-temporal activities of each signalling molecule. Accordingly, a number of research groups have been working extensively to develop probes for the visualization of protein activities and second messengers in living cells. Many of these probes developed to date have been based on the principle of Förster (or fluorescence) resonance energy transfer (FRET). The successful use of green fluorescent protein (GFP) in the development of genetically encoded FRET probes has greatly widened their application to routine cell biology research (Bastiaens & Squire 1999; Pollok & Heim 1999; Hailey *et al.* 2002; Zhang *et al.* 2002; Jares-Erijman & Jovin 2003; Miyawaki 2003; Sekar & Periasamy 2003). Here, we provide an overview of the GFP-based unimolecular FRET probes developed primarily in our laboratory and discuss the future directions of FRET imaging.

2. GENETICALLY ENCODED FRET PROBES

FRET is a process by which radiationless transfer of energy occurs from a donor fluorophore to an acceptor placed in close proximity to the donor (Tsien & Miyawaki 1998; Pollok & Heim 1999; Jares-Erijman & Jovin 2003). There are innumerable applications of this principle and here we will focus on the probes wherein both the donor and the acceptor are fluorescence proteins and incorporated into a single probe. Hereafter,

we will refer to these genetically encoded unimolecular FRET-based probes as simply FRET probes. The advantages of these FRET probes are that they can be easily loaded into the cells, and they also allow a simple acquisition of FRET images that can be unambiguously evaluated (Kurokawa *et al.* 2004). A variety of probes have been developed for the monitoring of signalling proteins and second messengers, as summarized in tables 1 and 2, respectively. Originally, GFP and a blue-emitting mutant of GFP (BFP) were used as the FRET pair, but most recent probes employ a cyan-emitting mutant (CFP) as the donor and a yellow-emitting mutant of GFP (YFP) as the acceptor, because the latter FRET pair generally yields stronger FRET signals. Several improved versions of CFP and YFP mutants are now available and some are optimized specifically for FRET (Nagai *et al.* 2004; Nguyen & Daugherty 2005).

3. PROBES FOR PROTEIN KINASES

A large number of the FRET probes aim at the monitoring of protein kinase activities (table 1). There are two approaches to achieve this goal. The first group of probes, called substrate-type FRET probes hereafter, is designed to monitor the phosphorylation level of the specific substrate of each kinase. A prototype of this group is Picchu, which is designed to monitor the tyrosine phosphorylation of the CrkII adaptor protein (Kurokawa *et al.* 2001). The CrkII adaptor protein includes SH2 and SH3 domains (Matsuda *et al.* 1992). The SH2 domain binds to phosphotyrosine-containing peptides and mediates intermolecular or intramolecular interaction (Matsuda *et al.* 1991). The activation of tyrosine kinases including Abl and epidermal growth factor (EGF)

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Table 1. Genetically encoded FRET probes for kinases, methylases and GTPases. (Abbreviations: EGFR, epidermal growth factor receptor; SH2, *src* homology 2 domain; PTB, protein tyrosine-binding domain; KID, kinase-inducible domain; PH, pleckstrin domain; DEP, dishevelled-Egl10-pleckstrin domain; RBD, Ras-family binding domain; PTHR, parathyroid hormone receptor; α_2A AR, α_2A androgen receptor. Variants of YFP and CFP such as citrine, venus and enhanced YFP are not distinguished in this table.)

subject	structure	name	references
<i>phosphorylation of tyrosine kinase substrates</i>			
EGFR	CFP-SH2 (Shc)-substrate (synthetic)-YFP	EGFR-indicator	Ting <i>et al.</i> (2001)
EGFR	EGFR-CFP-PTB (Shc)-YFP	FLAME	Offterdinger <i>et al.</i> (2004)
EGFR	α -helix-YFP-CrkII-CFP	Picchu-Z	Itoh <i>et al.</i> (2005)
insulin receptor	CFP-substrate (IRS1)-SH2 (p85)-YFP	Phocus	Sato <i>et al.</i> (2002)
Abl, EGFR	YFP-CrkII-CFP	Picchu	Kurokawa <i>et al.</i> (2001)
Abl, EGFR	CFP-CrkII-YFP	Abl indicator	Ting <i>et al.</i> (2001)
Src	TM (Cbp)-YFP-substrate (Cbp)-SH2 (Csk)-CFP	chimera	Matsuoka <i>et al.</i> (2003)
Src	CFP-SH2 (Src)-substrate (synthetic)-YFP	Src indicator	Ting <i>et al.</i> (2001), Wang <i>et al.</i> (2005)
<i>serine-threonine kinase substrate phosphorylation</i>			
PKA	GFP-KID (PKA)-BFP		Nagai <i>et al.</i> (2000)
PKA	CFP-[14-3-3]-substrate (synthetic)-YFP	AKAR	Zhang <i>et al.</i> (2001)
PKA	RGFP-substrate (kemptide)-BGFP	ART	Nagai <i>et al.</i> (2000)
PKA, PKC	YFP-PH and DEP (pleckstrin)-GFP	KCP, KCAP	Brumbaugh <i>et al.</i> (2006)
PKC	CFP-FHA2 (Rad53P)-substrate(synthetic)-YFP	CKAR	Violin <i>et al.</i> (2003)
Akt/PKB	CFP-[14-3-3]-substrate-YFP	Aktus	Sasaki <i>et al.</i> (2003)
MLCK, Rho kinase	CFP-myosin light chain-YFP	CRCit	Yamada <i>et al.</i> (2005)
<i>conformational change of serine-threonine kinases</i>			
Akt/PKB	GFP-Akt-YFP		Calleja <i>et al.</i> (2003)
PKC	CFP-PKC δ -YFP	CY-PKC δ	Braun <i>et al.</i> (2005)
Akt/PKB	PH (Akt)-YFP-Akt-CFP	Akind	Yoshizaki <i>et al.</i> (2007)
c-Raf, B-Raf	YFP-Raf-CFP	Prin	Terai & Matsuda (2005, 2006)
ERK	YFP-ERK-CFP	Miu2	Fujioka <i>et al.</i> (2006)
<i>histon methylation</i>			
histon	CFP-chromodomain-histon-YFP	K9, K27 reporter	Schleifenbaum <i>et al.</i> (2004)
<i>small GTPases</i>			
Ras, Rap1, Ral, R-Ras	YFP-Ras-RBD-CFP	Raichu	Mochizuki <i>et al.</i> (2001), Takaya <i>et al.</i> (2004, 2007)
Rho, Rac, Cdc42, TC10	YFP-RBD-Rho-CFP	Raichu	Itoh <i>et al.</i> (2002), Yoshizaki <i>et al.</i> (2003), Kawase <i>et al.</i> (2006)
Rho, Rac, Cdc42	YFP-RBD-CFP	Raichu-CRIB	Itoh <i>et al.</i> (2002), Yoshizaki <i>et al.</i> (2003)
Rho	RBD-CFP-YFP-RhoA	RhoA biosensor	Pertz <i>et al.</i> (2006)
Cdc42	Cdc42-YFP-GBD-ACV-CFP	GEF sensor	Seth <i>et al.</i> (2003)
N-WASP/Cdc42	CFP-N-WASP-YFP	N-WASP-BS	Lorenz <i>et al.</i> (2004)
<i>GPCR</i>			
PTHR, α_2A AR	PTHR-CFP-PTHR-YFP	PTHR-cam, α_2A AR-cam	Villardaga <i>et al.</i> (2003)

receptor induces the phosphorylation of CrkII on tyrosine 221, which residue in turn binds to the SH2 domain intramolecularly (Rosen *et al.* 1995). This phosphorylation-induced conformational change was detected by Picchu as shown in figure 1a (Kurokawa *et al.* 2001). In Cos7 cells expressing Picchu, stimulation with EGF induces a rapid and robust increase in the level of FRET, which strongly correlates with the level of phosphotyrosine-containing proteins within the cells. A flaw of this type of probe is that the diffusion of the probe is too fast to delineate the subcellular localization of tyrosine kinase activity. To overcome this flaw, the probe was fused to the membrane-targeting signal of K-Ras protein, generating the Picchu-X probe. A variation of this approach is to fix the Picchu probe to the molecule of interest by the use

of a pair of α -helix peptides that form a stable dimer (figure 1b; Itoh *et al.* 2005). By restricting the localization of the Picchu probe to the C terminus of the EGF receptor, we observed that the kinase activity of EGF receptor is retained during endocytosis. A direct fusion of the substrate-type probe to the EGF receptor has also been shown to be successful (Offterdinger *et al.* 2004).

The activity of serine-threonine kinases is also detected by the substrate-type FRET probes. In the simplest form, the substrate peptide is sandwiched by YFP and CFP (Nagai *et al.* 2000; Yamada *et al.* 2005; Brumbaugh *et al.* 2006). The conformational changes of the substrate peptides induced by phosphorylation of the substrate lead to the changes in FRET efficiency. To improve the gain of the FRET signal, other probes use

Table 2. Genetically encoded FRET probes for low-molecular weight substances. (Abbreviations: C1, C1 domain; CRD, cysteine-rich domain. Variants of YFP and CFP such as citrine, venus and enhanced YFP are not distinguished in this table.)

subject	structure	name	references
<i>lipids</i>			
PIP ₃	CFP-PH (GRP)-YFP-membrane anchor	Fllip	Sato <i>et al.</i> (2003)
PI(4,5)P ₂	CFP-PH (PLC δ)-YFP	CYPHR	Violin <i>et al.</i> (2003)
PI(3,4)P ₂	CFP-PH (TAPP1)-YFP-membrane anchor	Pippi-PI(3,4)P ₂	Yoshizaki <i>et al.</i> (2007)
IP ₃	CFP-IP ₃ receptor-YFP	LIBRA, fretino, FIRE	Tanimura <i>et al.</i> (2004), Sato <i>et al.</i> (2005) and Remus <i>et al.</i> (2006)
Diacylglycerol	CFP-C1 (PKC β)-YFP	DAGR	Violin <i>et al.</i> (2003)
Diacylglycerol	CFP-CRD (PKC)-YFP-membrane anchor	Daglas	Sato <i>et al.</i> (2006)
<i>ions</i>			
calcium	CFP-calmodulin-M13-YFP	cameleon	Miyawaki <i>et al.</i> (1997)
calcium	CFP-kringle domain (apoK1)-YFP	apoK1-er	Osibow <i>et al.</i> (2006)
chloride ion	CFP-linker-YFP	clomeleon	Kuner & Augustine (2000)
calmodulin	BGFP-MLCK-RGFP	FIP-CA, CB	Romoser <i>et al.</i> (1997)
<i>hormones, cyclic nucleotides, and amino acids</i>			
androgen	CFP-androgen binding domain-coactivator-YFP	ficaro	Awais <i>et al.</i> (2006)
oestrogen/ androgen	CFP-oestrogen or androgen receptor-YFP	CEY, CAY	De <i>et al.</i> (2005)
glutamate	CFP-glutamate-binding domain-YFP	FLIPE	Okumoto <i>et al.</i> (2005)
cGMP	CFP-GKI-YFP	cygnet	Honda <i>et al.</i> (2001)
cGMP	YFP-phosphodiesterase-CFP	pGES-DE2	Nikolaev <i>et al.</i> (2006)
cAMP/Epac	CFP-Epac-YFP	CFP-Epac-YFP, ICUE, Epac2-camps	DiPilato <i>et al.</i> (2004), Nikolaev <i>et al.</i> (2004) and Ponsioen <i>et al.</i> (2004)
sugar	CFP-sugar-binding domain-YFP	nanosensors	Fehr <i>et al.</i> (2005)
<i>miscellaneous</i>			
K ⁺ channel	Kv potassium channel-CFP-YFP	VSFP1	Sakai <i>et al.</i> (2001)
HIV Rev	YFP-Rev peptide-CFP	YRFnC-11ad	Endoh <i>et al.</i> (2005)

phosphoserine/phosphothreonine-binding modules such as 14-3-3 and FHA2, which amplify the phosphorylation-induced conformational change of the probes (Zhang *et al.* 2001; Sasaki *et al.* 2003; Violin *et al.* 2003). These substrate-type probes for serine–threonine kinases suffer the same flaw as do the substrate-type probes for tyrosine kinases. They can be used to monitor the kinase activity averaged over the entire cell area, but in most cases they lack the spatial information at the subcellular level unless the probe is fixed to the organelle or visualized with a highly sensitive CCD camera.

The second group of probes for kinases, called kinase-type FRET probes hereafter, is designed to monitor directly the conformational change of kinases. Protein kinases generally include a catalytic and a regulatory domain, the latter of which associates with the former to hold the enzyme in ‘a closed inactive state’. Upon stimulation, the regulatory domain dissociates from the catalytic domain to drive the enzyme into ‘an open active state’. Such a conformational change has been successfully monitored as a change in the FRET efficiency in the probes for PKC, Akt, Raf and ERK (Calleja *et al.* 2003; Braun *et al.* 2005; Terai & Matsuda 2005; Fujioka *et al.* 2006; Yoshizaki *et al.* 2007). The three-dimensional structural data of both the inactive and the active forms are necessary for the rational design of FRET probes; however, such structural data are

still not available for most protein kinases. This is the primary reason that most of the FRET probes reported in the data share a plain structure in common; i.e. the whole protein is sandwiched by CFP and YFP. As easily expected, this simple strategy does not work in many kinases. Fortunately, the N and C termini of GFP are located in close proximity to each other; therefore, as an alternative strategy, GFP could be inserted into the linker region that connects the domains of protein kinases without perturbing their three-dimensional structure. For example, we have developed a FRET probe for Akt wherein CFP is inserted within the regulatory domain of Akt (figure 1c; Yoshizaki *et al.* 2007).

An advantage of the kinase-type FRET probes is that they can monitor both the activity and the localization of kinases. An example showing the relationship between the structure and the localization of the kinase is the probe for ERK (Fujioka *et al.* 2006). ERK adopts a closed inactive conformation when it is bound to MEK in the cytoplasm. Growth factor-induced MEK activation results in the phosphorylation of ERK, which triggers the dissociation of the MEK–ERK complex. Upon release from MEK, ERK adopts the open active conformation and enters into the nucleus. This sequence of phosphorylation, nuclear import and dephosphorylation has been visualized using the FRET probe (Fujioka *et al.* 2006; figure 1d).

Figure 1. FRET probes for protein kinases. (a) Structure and mode of action of Picchu, a probe for tyrosine phosphorylation of the CrkII adaptor protein. The SH2 domain of CrkII binds intramolecularly to its Tyr²²¹, when it is phosphorylated. (b) Non-covalent binding of the Picchu probe to EGF receptor. Lower panels show the FRET image of a Cos cell expressing Picchu or Picchu-Z before and after stimulation with EGF. (c) Akind, a probe for Akt. CFP is inserted between the PH domain and the catalytic domain. Akt recruitment to the plasma membrane via the PH domain is followed by phosphorylation and conformational change. (d) Miu2, a probe for ERK. ERK liberated from MEK adopts an open active conformation. Lower panels show the FRET image of cells expressing Akind, Prin-c-Raf and Miu2 before and after stimulation with growth factor.

4. PROBES FOR SMALL GTPASES

(a) **Design of the FRET probe for small GTPases**
Small GTPases, also called low-molecular weight GTPases or Ras superfamily GTPases, are molecular switches that adopt either a GDP-bound inactive or a GTP-bound active state. These molecular switches are integrated into virtually all intracellular signal transduction pathways and regulate cell growth, differentiation, migration, apoptosis, etc. (Bos 1997; Takai *et al.* 2001). Thus, visualization of the on and off states of the small GTPases will enable us to see live images of the activities of each pathway. In contrast to protein kinases, the conformational change of many small GTPases is limited to their effector regions, which

cannot be readily detected by the simple fusion of fluorescent proteins. Two methods, both of which use the specific binding of the activated GTPases to their effector proteins, have been developed to observe such conformational changes of GTPases. The probe designated the effector type has a simpler structure, consisting of an effector domain sandwiched by CFP and YFP (figure 2a; Itoh *et al.* 2002; Yoshizaki *et al.* 2003; Lorenz *et al.* 2004). The binding of the activated GTPases to the effector in the probes increases the distance between CFP and YFP. These probes possess two serious flaws. First, the amount of FRET signal is limited by the number of the activated GTPases. The number of small GTPase molecules is typically between

Figure 2. FRET probes for small GTPases. (a) Structure of effector-type probes. The binding of the cognate GTPase to the effector dissociates the association of CFP and YFP. (b) Archetypal structure of Raichu-type unimolecular FRET probes. (c) Localization of active Ras, RalA and Rap1 in Cos cells as visualized by Raichu probes. (d) Localized activity of Rho-family GTPases in HeLa cells. The white arrows indicate the direction of migration.

10^4 and 10^6 per cell. GFP has to be expressed at least at 10^5 molecules per cell to yield a specific fluorescence signal over the background autofluorescence when observed with the fluorescence microscope imaging system used routinely in many laboratories. This means that the FRET signal obtained by the effector-type probe barely exceeds the sensitivity of the imaging system at best. Second, the expression of the effector-binding domains has been shown to inhibit competitively the authentic signalling cascade. The more successful approach to designing probes for small GTPases is to incorporate both the GTPase and the effector into a single molecule (Mochizuki *et al.* 2001; Itoh *et al.* 2002; Yoshizaki *et al.* 2003; Takaya *et al.* 2004, 2007; Kawase *et al.* 2006). A schematic presentation of the archetypal probe of this group, Raichu, is shown in figure 2b.

(b) FRET probes for Ras-family GTPases

Several important findings have been discovered by the use of these FRET probes. It has been visually demonstrated that the expression level of the EGF receptor determines whether or not signals generated by the local application of EGF can be propagated laterally to the entire plasma membrane. Sawano *et al.*

applied EGF locally to single cells expressing the Raichu-Ras probe and found that Ras is activated only at the site of EGF application when the expression level of EGF receptor is low but that Ras is activated on the entire plasma membrane when the expression level of EGF receptor is high (Sawano *et al.* 2002). The latter fact explains why many cancer cells overexpress the EGF receptor. A difference in the subcellular localization of the active Ras-family GTPases has also been revealed by the use of FRET probes. In contrast to the classical Ras proteins, which are activated primarily at the plasma membrane, Rap1 was found to be activated around the perinuclear endomembrane compartments (Mochizuki *et al.* 2001). Rap1 functions similarly to or antagonistic to the classical Ras proteins depending on the cell context (for review, see Bos 1997). The reason for this ambivalent action of Rap1 remains elusive, but the difference in the subcellular localization of activation may provide a clue to this enigma.

(c) FRET probes for Rho-family GTPases

Rho-family GTPases regulate many aspects of cell function involving the reorganization of actin cytoskeleton, such as cell migration and cytokinesis

(Etienne-Manneville & Hall 2002). Typical phenotypes of active RhoA, Rac1 and Cdc42 are the induction of stress fibre, lamellipodia and filopodia, respectively, implying that these Rho-family GTPases are the master regulators of the cytoskeleton. In migrating cells, the stress fibre is prominent in the uropod in the rear side and the lamellipodia and filopodia are hallmarks of the leading edge at the front. Thus, it is not surprising that FRET probes have revealed high activities of Rac and Cdc42 at the leading edge of migrating cells (figure 2c; Itoh *et al.* 2002). However, an unexpected finding was made using the FRET probe for RhoA. The prominent stress fibre at the rear side of the cells and the antagonistic action of RhoA on the Rac activity had suggested high RhoA activity at the rear and low activity at the front of the migrating cells. What was observed by the FRET probe was that RhoA activity is high not only at the rear side but also at the front end (figure 2d; Kurokawa & Matsuda 2005). Thus, the same GTPase may function well at the different subcellular compartments and transmit signals to distinct effectors. The images obtained with FRET probes are a versatile tool for revealing such distinct roles of each signalling pathway. It should also be noted that the results obtained by the fluorescence protein-based probes for Rho-family GTPases are essentially the same as those obtained by the FRET probes with chemical fluorophores (Pertz *et al.* 2006).

5. FRET PROBES FOR LIPIDS, IONS AND NUCLEOTIDES

Taking advantage of their different binding specificities to phosphoinositides, the pleckstrin homology (PH) domains tagged to GFP are used to monitor the change in the concentration of phosphoinositides (Hirose *et al.* 1999). However, this method is based on the plasma membrane translocation of the GFP-PH domain; therefore, it is vulnerable to artefacts that affect the cell shape, particularly the thickness of the cytoplasm. Umezawa and his colleagues applied the FRET technology to visualize the level of phosphoinositides by using the PH domain (Sato *et al.* 2003). The FRET probe for PIP₃ consists of CFP, the PH domain of GRP, the hinge region, YFP, and the membrane anchor from the amino-terminus (figure 3a). In the absence of PIP₃ on the plasma membrane, the hinge region is flexible, resulting in a large distance between CFP and YFP. In the presence of a high volume of PIP₃ on the plasma membrane, the GRP PH domain binds tightly to the plasma membrane via PIP₃, resulting in the loss of flexibility of the hinge region. Under this condition, CFP is fixed in close proximity to YFP, increasing the FRET efficiency. An attractive characteristic of this probe is that it can be easily modified into a probe for other phosphoinositides and diacylglycerol by replacing the PH domain having affinity to these lipids. We have shown that the Akt activation is prominent at nascent lamellipodia and correlates more to the level of PI(3,4)P₂ than to that of PIP₃ (figure 3b; Yoshizaki *et al.* 2007). This observation strongly suggests that PIP₃-5-phosphatase plays a positive role in the regulation of Akt activity.

Figure 3. FRET probes for phosphoinositides. (a) Structure of Flip-type probes for phosphoinositides. The binding of the lipid-binding domain to the inner surface of the cell membrane fixes the probe to the membrane, resulting in a decrease in the distance between CFP and YFP. (b) Localization of (i) PIP₃ (Pippi-PI(3,4,5)P₃) and (ii) PI(3,4)P₂ (Pippi-PI(3,4)P₂) in Cos cells before and after EGF stimulation.

FRET probes for ions and cyclic nucleotides have also been developed successfully as summarized in table 2. These probes compete with the chemical probes, which are often easier to use and brighter than GFP. However, in the upcoming era of *in vivo* imaging, genetically encoded FRET probes will be widely employed because they can be readily integrated into the genome and expressed constitutively.

6. LIMITATIONS AND PROSPECTS

(a) Sensitivity

As described above, the number of FRET probes is increasing rapidly; however, many technical problems with these probes remain to be solved. First, the sensitivity of the FRET probe is not as high as expected. This is primarily because the gain of FRET probes does not generally exceed 50% (Kurokawa *et al.* 2004). Here, the gain means the difference in the FRET ratio, i.e. the lowest versus the highest values of (YFP fluorescence)/(CFP fluorescence) of the probe excited at 440 nm. The gain may be improved remarkably by the use of circularly permuted fluorescent proteins, which changes the direction of the dipole moment of the fluorescence proteins (Nagai *et al.* 2004). For the application of permutants, however, the linker regions connecting the fluorescence proteins to the probe must be rigid. At the moment, information on the rigidity of the linker region of each probe is extremely limited, and it is unlikely that the use of a permuted fluorescence protein will improve the gain of the probe. Thus, the development of fluorescent proteins optimized for FRET, like CYpet, is eagerly awaited (Nguyen & Daugherty 2005).

(b) Impedance of the authentic signalling pathways

FRET probes never deduce signals without interfering with the internal signalling cascades. In our typical FRET imaging set-up, a single cell expresses approximately 10^6 probes per cell. Because the numbers of each signalling molecule typically range from 10^3 to 10^6 molecules per cell, the probe outnumbers the intrinsic signalling molecules under most experimental conditions. Thus, the upstream signal reaching the molecule of interest is inevitably either attenuated or enhanced by the expression of the FRET probes. This issue is particularly important when the positive or negative feedback loop plays a pivotal role in the field under investigation.

(c) Simultaneous monitoring of two FRET probes in a single cell

It is often necessary to visualize two signalling activities simultaneously. For this, two FRET pairs whose excitation and emission do not impede each other should be chosen. YFP and a red fluorescent protein as a second FRET pair have been successfully used for the monitoring of protein–protein interaction (Galperin et al. 2004). However, the number of unimolecular FRET probes that include red fluorescence proteins as acceptors is extremely limited at the moment, and these probes must be further improved before two FRET probes can be used simultaneously in a single cell.

(d) Stable expression of FRET probes

A merit of genetically encoded fluorescent proteins is their stable expression within the cells; however, in most FRET applications reported to date, the probes have been expressed transiently. In our experience, the genetically encoded FRET probes meet two challenges for stable expression. First, the DNA of CFP and YFP frequently recombine during the integration to the genome. This problem is particularly serious when retroviruses are used as the vector. Second, the fluorescence of probes often decays during the passage of the cells. This problem may be ascribable to the oxidative stress caused by GFP. Thus, the future FRET probes should contain fluorescent proteins whose genomes share little homology with each other and which are less toxic to the cells than the GFP used currently.

(e) In vivo imaging

The application of FRET probes in living animals creates greater challenges than the use of FRET probes in tissue culture cells. The first and largest is the low transmittance of visible light in the tissues, which renders the fluorescence imaging applicable only to small and transparent model animals or embryos. Many research groups and companies are trying to develop fluorescence proteins in the infrared wavelength range, which will widen the application of FRET probes *in vivo*. Another approach to alleviate the low transmittance of visible light is the use of two-photon excitation microscopy (Fan et al. 1999), in which near infrared light is used for the excitation to decrease the absorption by the tissues and reduce the background autofluorescence. The second problem to be solved is

the difference in the transmittance of the fluorescence between the donor and the acceptor. Since the FRET level is estimated by the ratio of the fluorescence from the donor to that from the acceptor, this difference in transmittance makes an estimation of the FRET efficiency extremely difficult. This problem may be overcome by fluorescence lifetime microscopy (Bastiaens & Squire 1999). In this method, the lifetime of the donor fluorescence is detected to measure the FRET efficiency, negating the necessity to measure the fluorescence from the acceptor.

7. CONCLUSIONS

Genetically encoded FRET probes now cover a wide area of signalling pathways. Although there remain numerous problems that must be overcome in the future, the desire of biologists to view the action of molecules in living cells will keep driving the developers to increase the number of probes, to polish up the current probes and to seek new applications in living animals.

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