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Mini-review and Review

Live-cell Imaging with Genetically Encoded Protein Kinase Activity Reporters

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ABSTRACT. **Protein kinases play pivotal roles in intracellular signal transduction, and dysregulation of kinases leads to pathological results such as malignant tumors. Kinase activity has hitherto been measured by biochemical methods such as *in vitro* phosphorylation assay and western blotting. However, these methods are less useful to explore spatial and temporal changes in kinase activity and its cell-to-cell variation. Recent advances in fluorescent proteins and live-cell imaging techniques enable us to visualize kinase activity in living cells with high spatial and temporal resolutions. Several genetically encoded kinase activity reporters, which are based on the modes of action of kinase activation and phosphorylation, are currently available. These reporters are classified into single-fluorophore kinase activity reporters and Förster (or fluorescence) resonance energy transfer (FRET)-based kinase activity reporters. Here, we introduce the principles of genetically encoded kinase activity reporters, and discuss the advantages and disadvantages of these reporters.**

Key words: kinase, FRET, phosphorylation, KTR

Introduction

Protein phosphorylation is a post-translational modification in which a specific enzyme, namely a protein kinase (hereinafter referred to simply as kinase), catalyzes the transfer of γ-phosphate of ATP to specific phosphoacceptor amino acids of target substrate proteins (Ubersax and Ferrell, 2007). More than 500 genes encoding protein kinases exist in the human genome, including genes for Ser/Thr kinases and Tyr kinases, and approximately 30% of proteins are estimated to be phosphorylated on at least one residue (Cohen, 2000; Ptacek *et al.*, 2005). Protein phosphorylation is involved in a wide range of cellular processes, such as proliferation, differentiation, survival, and cytoskeletal reorganization (Ubersax and Ferrell, 2007), and aberrant activation of kinases leads to pathological consequences, notably in malignant tumors (Frémin and Meloche, 2010). Indeed, small-molecule inhibitors targeting oncogenic kinases have become an important therapeutic paradigm for the treatment of cancer patients (Gross *et al.*, 2015; Zhang *et al.*, 2009).

Kinase and phosphorylation signaling have mostly been studied by means of population-level experiments such as western blotting. However, it is unclear whether individual cells behave in conformity with the averaged data. Live-cell imaging with fluorescent proteins (FPs) is a key technique to address this issue. Green fluorescent proteins (GFP) were discovered in the jellyfish *Aequorea victoria* in 1962 by Dr. Shimomura (Shimomura *et al.*, 1962). Over the past 50 years, the evolution of live-cell imaging with FPs has opened a door to the probing of a variety of biological processes. Currently, a broad range of fluorescent proteins beyond GFP are available from other marine organisms (Chudakov *et al.*, 2010). One advantage of FPs is that they are encoded by responsible genes, and thus researchers in

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the field of life science can easily introduce FPs and biosensors fused with FPs into cells and animals of interest by conventional gene delivery methods. Further, with the advent of newly developed fluorescence microscopes, it has become possible to apply FPs not only to label proteins, but also to more precisely visualize the subcellular localization of proteins beyond the optical diffraction limit (Huang *et al.*, 2009), to observe deeper tissues in living animals (Kamioka *et al.*, 2012), and to measure kinetic parameters (Aoki *et al.*, 2011; Sadaie *et al.*, 2014). Moreover, through the efforts of many groups, various genetically encoded fluorescent reporters based on the principle of Förster (or fluorescence) resonance energy transfer (FRET) have been developed, such as intracellular ions (Chen *et al.*, 2013; Miyawaki *et al.*, 1997), small GTPases (Aoki and Matsuda, 2009; Kiyokawa *et al.*, 2011; Mochizuki *et al.*, 2001), phospholipids (Nishioka *et al.*, 2008; Sato *et al.*, 2003), enzymatic reactions (Komatsu *et al.*, 2011; Ni *et al.*, 2006) and so on. Among them, kinase activation is one of the most intensively studied targets for FRET reporters.

Here, we focus on the applications of FPs to the monitoring of kinase activity in living cells; this review is primarily intended as a guide to the development and use of genetically encoded kinase activity reporters. Many excellent reviews provide details on the aspects of FP and FRET-based biosensors, and we encourage the interested reader to refer to them (Miyawaki, 2003; Muscheler *et al.*, 2007; Oldach and Zhang, 2014; Zhang *et al.*, 2002).

The mode of action of kinase activation and substrate phosphorylation

In order to develop a genetically encoded fluorescent reporter for kinase activity, we first must understand where and how the kinase is regulated and activated within a cell, and how the substrate behaves before and after phosphorylation. In many cases, the enzymatic activity of kinases is turned off when the kinases are at a steady state level, and turned on when they are externally and/or internally stimulated by growth factors, cytokines, or cell cycle progression (Fig. 1A). The kinase activation is often accompanied by a combination of several kinds of reactions. The simplest of these is the gene expression of the kinase itself, which serves as an activation mechanism *per se* (Fig. 1B). Translocation to different subcellular compartments such as the plasma membrane also triggers kinase activation (Fig. 1C). Under basal condition, kinases are often self-inactivated by steric constraints. For instance, a closed-inactive form of a kinase is incapable of binding to substrates, and thus the release of the auto-inhibition can be an essential process of kinase activation (Fig. 1D). Post-translational modification including phosphorylation is also a common mechanism as an activation switch for kinases (Fig. 1E). Finally, not only proteins but also secondary messengers such as calcium and cAMP act as regulatory factors that activate or inactivate kinases (Fig. 1F). Importantly, these events are not mutually exclusive, but rather complement each other to accomplish full activation of a kinase. Thereafter, the activated kinases then catalyze their substrates, generating phosphorylated proteins as a product. Some of the phosphorylated proteins are also known to be translocated to different subcellular spaces or degraded (Fig. 1G).

Most of the kinase reporters exploit, to a greater or lesser degree, the aforementioned changes in the kinase itself or its substrates in the process of kinase activation and inactivation. The kinase activity reporters can be classified into two categories: the single-fluorophore kinase activity reporters and the FRET-based kinase activity reporters (Miyawaki and Niino, 2015). We will introduce examples and discuss the pros and cons of these two types of reporters.

Single-fluorophore kinase activity reporters

A single-fluorophore kinase activity reporter is defined as a biosensor that harbors only a single FP. An advantage of the single-fluorophore kinase activity reporters is that they are often easier to develop than the FRET-based kinase activity reporters. In addition, single-fluorophore kinase activity reporters enable multiplex imaging with differently colored FPs, while FRET-based reporters are often associated with the difficulty in multiplexed imaging. There are also some drawbacks to the use of single-fluorophore kinase activity reporters. First, these reporters are more susceptible to the focus drift effect and object motion than ratiometric FRET-based imaging. Second, it is difficult to monitor subcellular kinase activation by most of the single-fluorophore kinase activity reporters.

Table I lists the most commonly used single-fluorophore kinase activity reporters.

Localization- or translocation-based kinase activity reporters

In extreme cases, the translocation of proteins upon activation has been employed to probe kinase activity with a single-fluorophore. Upon activation, some kinases move to different subcellular compartments, because the redistribution of kinases is required for their activation and/or substrate phosphorylation. In either case, we can trace kinase activation by tagging the kinases with an FP and observing their subcellular distribution. For instance, Akt, also known as protein kinase B, and CRaf are localized at the cytosol in the basal state, and translocated to the plasma membrane by binding to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) or to GTP-loaded active Ras, respectively (Haugh *et al.*, 2000; Rocks *et al.*, 2006) (Fig. 2A, left). Therefore, membrane recruitment of Akt and CRaf fused with FP functions as a marker of their activation, respectively. Of note, Akt

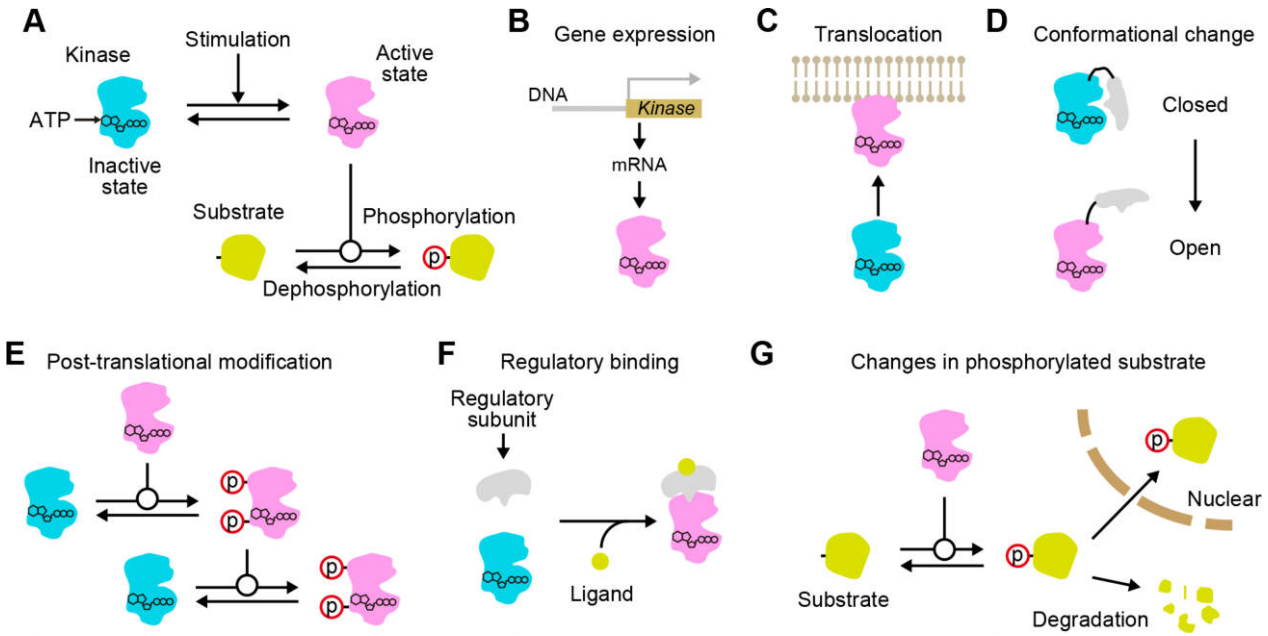

Fig. 1. Mechanisms of kinase activation. (A) Inactive kinase (left) is activated (right) by stimulation, leading to the phosphorylation of substrates. (B) Gene expression increases the amount of constitutively active kinase protein. (C) Subcellular translocation from the cytoplasm to plasma membrane induces kinase activation. (D) Closed-inactive kinase is activated upon stimulation by releasing intramolecular auto-inhibition and exposing the substrate-binding site. (E) Post-translational modification such as phosphorylation increases kinase activity. (F) Dissociation or association of the regulatory protein through ligand binding regulates kinase activation. (G) The phosphorylated substrate undergoes subcellular translocation such as nuclear import or degradation.

and CRaf carrying FP can also be interpreted as a reporter of PIP_3 and Ras activity, respectively. In another example, the nuclear import of ERK MAP kinase has been widely used as a marker of ERK activation (Lidke *et al.*, 2010; Raisner *et al.*, 2005). MEK binds to and anchors ERK at the cytoplasm in the resting state. Upon stimulation, ERK is phosphorylated by MEK and is subsequently dissociated from MEK, followed by the nuclear translocation (Fig. 2A, right). In both cases, the expression level of reporters is of critical importance in order to accurately quantify kinase activation; the expression level must be comparable with the number of the binding partners, namely Ras, PIP_3, and MEK. Therefore, co-expression of binding proteins might help to increase the dynamic range of reporter translocation in response to stimulation (Fujioka *et al.*, 2006; Terai and Matsuda, 2005). To prevent unnecessary signal transduction by the reporters themselves, kinase-dead mutants or truncated mutants containing the responsible domain for the translocation would work better than full-length kinases fused with FPs.

Many substrates phosphorylated by protein kinases are also subjected to changes in subcellular distribution. The transcription factors known as Forkhead box Os (FOXOs) are phosphorylated by Akt, and then translocated from nucleus to cytoplasm (Burgering, 2003). Intriguingly, Akt phosphorylates serine/threonine amino acid residues located at or near the nuclear localization signal (NLS), resulting in their nuclear export and cytoplasmic retention. Based on this idea, GFP-fused FOXOs are used as Akt activity reporters (Katsura *et al.*, 2015; Maryu *et al.*, 2016). In a similar way, p38 activity has been investigated by quantifying the subcellular distribution of MK2/MAPKAPK2, which is a substrate of p38 MAP kinase (Almholt *et al.*, 2004a); the phosphorylation of MK2 by p38 induces the translocation from nucleus to cytoplasm.

Degradation-based kinase activity reporters

Protein stability and degradation can be altered by phosphorylation, providing us with other principles for kinase activity reporters (Fig. 2B). Brugge and her colleagues reported FIRE (Fra-1-based integrative reporter of ERK), which consists of YFP fused to the PEST domain of Fra-1 (Albeck *et al.*, 2013). Because phosphorylation of Fra-1 by ERK (or downstream kinase RSK) suppresses degradation of the Fra-1 PEST domain (Casalino *et al.*, 2003; Vial and Marshall, 2003), the fluorescence intensity of FIRE increases upon ERK activation (Fig. 2B). Fusion of the phosphorylation-regulated degradation domains to a fluorescent protein, such as in the FIRE reporter, paves the way to the generation of single-fluorophore kinase activity reporters. It is noteworthy that the dynamics of FIRE inten-

Table I. Single fluorophore-based kinase activity reporters

Target kinase	Name of probe	Reference
Localization-based reporter (substrate, if the substrate is fused with FP)		
Akt	GFP-AktPH	(Haugh *et al.*, 2000)
Akt (FoxO1, 3)	FoxO1-Clover	(Gross and Rotwein, 2016)
BTK	BTK-PH-GFP	(Varnai, 2005)
CDK2	DHB	(Spencer *et al.*, 2013)
DGK	GFP-DGK	(Shirai *et al.*, 2000)
ERK	GFP-ERK2, GFP-ERK5	(Carter *et al.*, 2009; Rubinfeld *et al.*, 1999)
p38	EGFP-p38	(Gong *et al.*, 2010)
p38 (MAPKAPK2/MK2)	EGFP-MK2	(Gong *et al.*, 2010)
JAK (STAT)	STAT-GFP	(Samsonov *et al.*, 2013)
JNK	GFP-JNK3	(McDonald *et al.*, 2000)
JNK (PDK-1)	PDX-1-GFP	(Kawamori *et al.*, 2003)
Lats (YAP)	GFP-YAP	(Bao *et al.*, 2011)
MEKK2	MEKK2-GFP	(Schaefer *et al.*, 1999)
PKA	PKAcat-GFP	(Almholt *et al.*, 2004b)
PKC	γ-PKC-GFP	(Sakai *et al.*, 1997)
PKC (KRas, MARCKS)	GFP-KRas, MARCKS-GFP	(Bivona *et al.*, 2006; Ohmori *et al.*, 2000)
PKD (HDAK5)	GFP-HDAK5	(Vega *et al.*, 2004)
Raf	Raf-GFP	(Knight *et al.*, 2006; Rocks *et al.*, 2006)
TGFbR (Smad)	EGFP-Smad	(Schmierer and Hill, 2005)
Degradation-based reporter (substrate)		
ERK (FosL)	FIRE	(Albeck *et al.*, 2013)
IKK (IκB)	IκBα-EGFP	(Nelson *et al.*, 2002)
Susceptible cpFP-based reporter (substrate)		
Src (IRS-1)	cyan-, green-, yellow-sinphos	(Kawai *et al.*, 2004)
Kinase translocation reporter (KTR) system		
JNK	JNK-KTR	(Regot *et al.*, 2014)
ERK	ERK-KTR	(Regot *et al.*, 2014)
p38	p38-KTR	(Regot *et al.*, 2014)
PKA	PKA-KTR	(Regot *et al.*, 2014)
Akt	Akt-FoxO3A-KTR	(Maryu *et al.*, 2016)

sity exhibit a much slower time scale (~ 12 hours) than the fluctuations of ERK activity (~ 30 min) (Albeck *et al.*, 2013; Aoki *et al.*, 2013). This could be attributable to the mechanism of this reporter, which is based on protein degradation and synthesis.

Inflammatory signaling is also investigated by the degradation of inhibitors of κB (IκBs) and nuclear localization of transcription factor NF-κB (Nelson *et al.*, 2002). Under an unstimulated condition, NF-κB is sequestered in the cytoplasm by binding to IκBs, which masks the NLS of NF-κB. Upon stimulation, IκB kinase (IKK) phosphorylates IκBs, leading to their ubiquitination and degradation. Consequently, the NF-κB is released from the IκB to enter the nucleus, and initiates its transcription. Therefore, the degradation of IκBs indeed reports IKK activity.

Susceptible circular permutated-GFP (cpGFP)-based kinase activity reporter

The fluorescence of FPs is usually robust and unaffected by the external change, because the β-barrel fold of FPs reduces the accessibility of outside solvent. Interestingly, however, the chromophore of circular permutated GFP at residues 144-145 (cp145-GFP) is more sensitive to neighboring events than normal GFPs (Baird *et al.*, 1999; Miyawaki and Niino, 2015). Based on this principle, single fluorophore-based genetically encoded calcium indicators (GECIs) have been developed by many laboratories (Lin and Schnitzer, 2016; Nagai *et al.*, 2001; Nakai *et al.*, 2001; Pérez Koldenkova and Nagai, 2013). This approach is potentially applicable to the development of kinase activity reporters. Indeed, the pioneering work of Umezawa and coworkers has demonstrated proof-of-concept for the design of kinase activity reporters with cp145-FPs (Kawai *et al.*, 2004). In their tyrosine kinase reporters, which are known as "sinphoses", the cp145-FPs are sandwiched between the substrate domain and phosphorylation recognition domain together with a localization signal domain (Fig. 2C). It is anticipated that, upon tyrosine kinase activation, the phosphorylation of the substrate domain brings it into close proximity to the phosphorylation recognition domain, leading to the change in fluorescence of cp145-FPs. Inter-

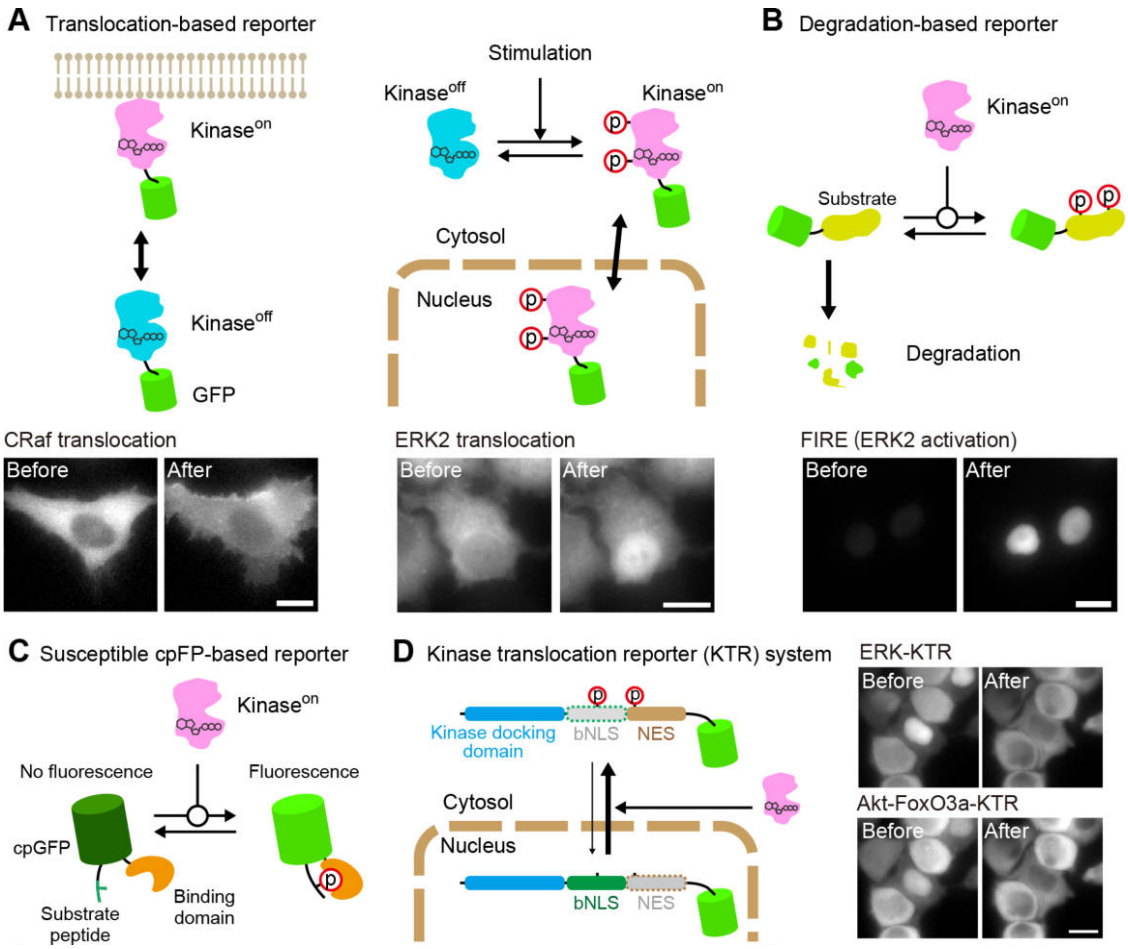

Fig. 2. Single-fluorophore kinase activity reporters. (A) Translocation-based kinase activity reporter. Kinase activation often takes place in a specific subcellular compartment such as the plasma membrane (upper left) and nucleus (upper right), and this allows us to illuminate kinase activation by the translocation of kinase fused with FP. CRaf proteins fused with FP are recruited to the plasma membrane upon EGF stimulation (lower left). Nuclear accumulation of ERK2 proteins fused with FP is evident after ERK activation (lower right). Scale bar, 15 μm. (B) Degradation-based kinase activity reporter. An activated kinase phosphorylates a substrate (yellow), leading to increased protein stability (upper). The abundance of FIRE, a degradation-based ERK activation reporter, is increased by ERK activation induced by EGF (lower). Scale bar, 15 μm. (C) Susceptible cpFP-based reporter. cpFP is fused with the phosphorylated peptide and its binding domain (orange). Once the peptide (green) is phosphorylated by activated kinase, the intramolecular binding between the peptide and binding domain undergoes a change in the chromophore of cpFP, increasing or decreasing the fluorescence. (D) KTR system. Shown here is the basic design of the KTR (left). The unphosphorylated form of KTR is retained in the nucleus by the function of bipartite NLS (bNLS). Upon phosphorylation, the bNLS and NES are inactivated and activated, respectively, and therefore the phosphorylated KTR is exported to the cytosol. EGF-induced Akt and ERK activation are manifested as nuclear export of Akt-FoxO3a-KTR (upper right) and ERK-KTR (lower right), respectively. Scale bar, 15 μm.

estingly, cp145-EGFP shows an increase in green fluorescence, whereas cp145-ECFP exhibits a decrease in cyan fluorescence (Kawai *et al.*, 2004). Although the development and improvement of susceptible cpGFP-based reporters requires time-consuming and labor-intensive steps, this approach has great potential for overcoming the limitations of the dynamic range and signal-to-noise ratio of kinase activity reporters.

Kinase translocation reporter (KTR) system

Recently, Covert and his colleagues reported a rationally designed kinase reporter system, KTR (kinase translocation reporter) (Regot *et al.*, 2014). The KTR system is comprised of a kinase docking site, bipartite NLS (bNLS), and NES, and phosphorylation sites are located within the bNLS and NES (Fig. 2D). The bNLS sequences are defined as $KRX_{10\text{-}12}K(K/R)(K/R)$, and acidic residues (D and E) should be enriched in the central linker region. The authors optimized the bNLS sequence to be negatively regulated by

phosphorylation. Introducing negatively charged amino acids or phosphorylation within the NES sequence increases its export activity. Therefore, the phosphorylation of KTR by kinases induces the translocation from nucleus to cytoplasm. The kinase specificity is achieved by adding a kinase docking site and optimizing the phosphorylation site. For MAP kinases, the addition of a specific docking site (e.g., FQFP for ERK) is sufficient to change the kinase specificity of KTR, and in this way, the authors could develop JNK-, ERK-, and p38-KTRs. For AGC kinases such as PKA, the specificity of KTR should be achieved by optimizing the phosphorylation sequence, though there is limited flexibility for the sequence space. Finally, the authors showed the great potential of the KTR system for multiplexed imaging in living cells. Based on this idea, we have developed an Akt activation reporter, Akt-FoxO3a-KTR, and demonstrated multiplexed imaging of Akt and ERK activity in proliferating cells (Maryu *et al.*, 2016). The KTR system opens the possibility of developing a single-fluorophore activity reporter for kinases that do not show any characteristic changes in subcellular distribution described above.

FRET-based kinase activity reporters

FRET is a non-radiative process in which the excitation energy of a donor fluorophore is transferred to an acceptor fluorophore (Förster, 1946). In principle, FRET efficiency denoted as E is described as follows:

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

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

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

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 donor absorption and acceptor transition moments (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; $c_0 = 8.8 * 10^{-28}$ for R_0 in nm (Jares-Erijman and Jovin, 2003; Padilla-Parra and Tramier, 2012; Ueda *et al.*, 2013). The orientation factor, κ^2, can be approximated as 2/3, when the donor and acceptor fluorophores are randomly oriented. However, as mentioned later, this approximation is available only when highly mobile, small molecules are used for donor and acceptor fluorophores, and is not applied to the case where FPs are used. Based on these equations, FRET efficiency is inversely proportional to the sixth power of distance between donor and acceptor fluorophores, and therefore FRET is very sensitive to the distance. This is the reason why FRET is known as a spectroscopic ruler (Stryer, 1978).

In practical terms, for the development of a FRET biosensor, the distance and relative orientation between donor and acceptor FPs are the main factors that influence the FRET efficiency once a pair of donor and acceptor fluorophores is fixed. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are preferably used for the sensitized FRET, because YFP allows the easy acceptor photobleaching, which provides a simple and practical measure of FRET (Kiyokawa *et al.*, 2011; Miyawaki, 2003). Meanwhile, the pair of GFP and red fluorescent protein (RFP) is applied to fluorescence lifetime microscopy (FLIM)-FRET, because of the high photostability and single component of the fluorescence lifetime in GFP.

The sensitized-FRET and FLIM-FRET methods are two major approaches for visualizing FRET signals in living cells (Miyawaki, 2011; Padilla-Parra and Tramier, 2012; Yasuda, 2006). Under the FRET condition, the donor fluorescence and lifetime are decreased, while the acceptor emits fluorescence through energy transfer. The sensitized-FRET method utilizes these reciprocal changes in the donor and acceptor fluorescence for evaluating to what extent FRET takes place. Therefore, the spectral overlaps, such as bleed-through of the donor in the acceptor detection channel and cross-excitation of the acceptor excited by donor excitation light, should be carefully corrected (Aoki and Matsuda, 2009; Fujioka *et al.*, 2006). Meanwhile, the FLIM-FRET method measures the fluorescence decay of a donor fluorophore on the order of nano-seconds, and calculates its lifetime. To accomplish this, the FLIM-FRET method requires special equipment, e.g., a pulsed-laser and a high-sensitive detector (Day and Davidson, 2012; Sun *et al.*, 2013). The sensitized-FRET method is technically easier but less quantitative than the FLIM-FRET method (van Munster and Gadella, 2005; Yasuda, 2006). The FRET-based kinase activity reporters are listed in Table II.

Intermolecular, or bimolecular, FRET biosensors

FP-based FRET biosensors are roughly classified into two categories: intermolecular (or bimolecular) FRET biosensors and intramolecular (or unimolecular) FRET biosensors (Aoki *et al.*, 2008; Oldach and Zhang, 2014). In the former case, a polypeptide fused with a donor FP is expressed along with a separate polypeptide attached to an acceptor FP. The increase in FRET signals is observed upon binding between the donor and acceptor FP-fused proteins (Fig. 3A). Under the dissociation condition of the intermolecular FRET biosensor, the distance between donor and acceptor

Table II. FRET-based kinase activity reporters

Target	Name of probe	Reference
Intermolecular FRET		
Akt, PDK1	Akt-Venus, CFP-PDK1;	(Yoshizaki *et al.*, 2006)
AMPK	bimABKAR, bimABKAR-Kras	(Depry *et al.*, 2015)
CRaf MEK	CFP-c-Raf-pm MEK-YFP	(Terai and Matsuda, 2005)
ERK	bimEKAR	(Depry *et al.*, 2015)
ERK MEK	mVenus-MEK1 mCFP-ERK2;CFP-ERK MEK-YFP	(Aoki *et al.*, 2011; Fujioka *et al.*, 2006)
JNK	bimJNKAR	(Depry *et al.*, 2015)
Intramolecular FRET: (1) kinase type		
Akt	Akind, ReAktion	(Ananthanarayanan *et al.*, 2007; Yoshizaki *et al.*, 2006)
Aurora A kinase	AURKA biosensor	(Bertolin *et al.*, 2016)
B-Raf	Prin-BRaf	(Terai and Matsuda, 2006)
C-Raf	Prin-CRaf	(Terai and Matsuda, 2005)
CAMKII	Camuiα, Camk2a(314)-F40	(Piljić *et al.*, 2011; Takao *et al.*, 2005)
DAPK	DAPK1(334)-F40	(Piljić *et al.*, 2011)
EGFR	FLAME	(Offterdinger *et al.*, 2004)
ERK	Miu2	(Fujioka *et al.*, 2006)
FAK	FERM sensor, SPASM sensor	(Papusheva *et al.*, 2009; Ritt *et al.*, 2013)
MELK	YFP-xMELK K/R-CFP	(Le Page *et al.*, 2011)
MK2	GMB	(Neininger *et al.*, 2001)
MLCK	MLCK-FIP	(Chew *et al.*, 2002)
PAK1	Pakabi	(Parrini *et al.*, 2009)
PDK1	Lyn-PARE	(Gao *et al.*, 2011)
PKC	CY-PKCδ	(Braun *et al.*, 2005)
Intramolecular FRET: (2) substrate type		
Abl	Abl indicator, Pickles	(Mizutani *et al.*, 2010; Ting *et al.*, 2001)
Akt	Aktus, BKAR, AktAR, Eevee-Akt, Eevee-iAkt	(Gao and Zhang, 2008; Komatsu *et al.*, 2011; Kunkel *et al.*, 2005; Miura *et al.*, 2014; Sasaki *et al.*, 2003)
AMPK	AMPKAR, ABKAR, T2AMPKAR, AMPKAR-EV	(Chennell *et al.*, 2016; Konagaya *et al.*, 2017; Sample *et al.*, 2015; Tsou *et al.*, 2011)
ATM kinase	Atomic	(Johnson *et al.*, 2007)
Aurora B kinase	Aurora B sensor, Aurora B kinase sensor	(Chu *et al.*, 2011; Fuller *et al.*, 2008)
Cdk1	Cyclin B1-Cdk1 biosensor	(Gavet and Pines, 2010)
ERK	Erkus, EKAR, EKAREV, ERKy, EKAR2G1, EKAR-TVV	(Fritz *et al.*, 2013; Harvey *et al.*, 2008; Komatsu *et al.*, 2011; Sato *et al.*, 2007; Tomida *et al.*, 2012; Vandame *et al.*, 2014)
EGFR	Picchu, EGFR indicator, Picchu-Z, PicchuEV, FLAME	(Itoh *et al.*, 2005; Komatsu *et al.*, 2011; Kurokawa *et al.*, 2001; Ting *et al.*, 2001)
FAK	FAK biosensor	(Seong *et al.*, 2011)
Insulin receptor	Phocus	(Sato *et al.*, 2002)
JNK	JNKAR1, JNKAR1-EV	(Fosbrink *et al.*, 2010; Komatsu *et al.*, 2011)
MARK	MARK-AR1	(Timm *et al.*, 2011)
MLCK, Rho kinase	CRCit	(Yamada *et al.*, 2005)
mTORC1	TORCAR	(Zhou *et al.*, 2015)
p38	PerKy-p38	(Tomida *et al.*, 2015)
PKA	AKAR1, AKAR2, AKAR3, AKAR3EV	(Allen and Zhang, 2006; Komatsu *et al.*, 2011; Zhang *et al.*, 2001, 2005)
PKC	CKAR, KCP-1, Eevee-PKC	(Komatsu *et al.*, 2011; Schleifenbaum *et al.*, 2004; Violin *et al.*, 2003)
PKD	DKAR	(Kunkel *et al.*, 2007)
PLK1	PLK1 FRET-probe	(Macůrek *et al.*, 2008)
ROCK/Rho kinase	Eevee-ROCK	(Li *et al.*, 2017)
RSK	Eevee-RSK	(Komatsu *et al.*, 2011)
S6K	Eevee-S6K	(Komatsu *et al.*, 2011)
SAP3K	SAP3K reporter	(Tomida *et al.*, 2009)
Src	Src indicator, Src reporter, Srcus, Src biosensor, BG-Src biosensor	(Hitosugi *et al.*, 2007; Ouyang *et al.*, 2008; Su *et al.*, 2013; Ting *et al.*, 2001; Wang *et al.*, 2005)
SYK	SYK biosensor	(Xiang *et al.*, 2011)
TAK1	Eevee-TAK1	(Takaoka *et al.*, 2016)
ZAP70	ROZA, ROZA-XL	(Cadra *et al.*, 2015; Randriamampita *et al.*, 2008)

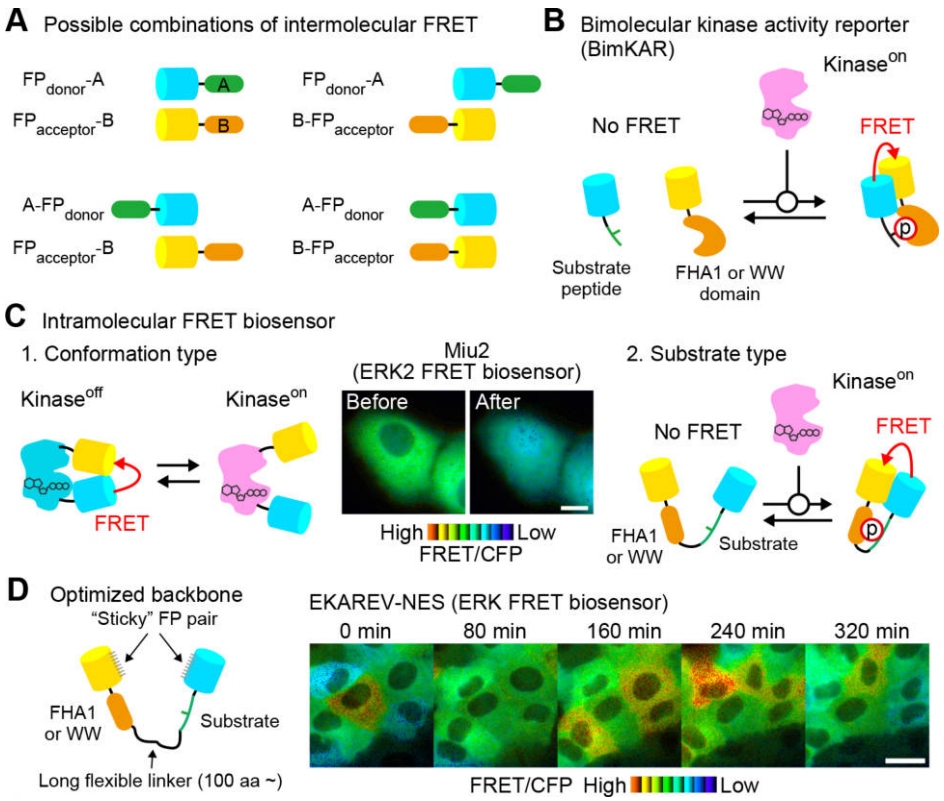

Fig. 3. FRET-based kinase activity reporters. (A) Schematic representation of possible combinations of intermolecular FRET. Donor (cyan FP) and acceptor (yellow FP) are fused with A and B domains, respectively, at the N- or C-terminus of each FP. (B) Bimolecular kinase activity reporter (BimKAR). An activated kinase phosphorylates a substrate peptide fused with FP. Thereafter, a phospho-peptide binding domain (orange) with FP interacts with the phosphorylated peptide (green), bringing the donor FP in close proximity to the acceptor FP. (C) Intramolecular FRET biosensors can be roughly divided into two types. The first one is the conformation type, which monitors the conformation change in a kinase upon activation (left). An example of a conformation-type FRET biosensor, Miu2 (ERK2 FRET biosensor), is depicted before and after EGF treatment, showing the induction of open conformation and nuclear accumulation (middle). The second one is the substrate type, which serves as a substrate, indicating FRET signals in a phosphorylation-dependent manner (right). Scale bar, 15 μm. (D) Optimized backbone for the intramolecular FRET biosensor, called the Eevee system, which includes a long flexible linker and sticky FP pair (left). Stochastic ERK activation is observed in proliferating cells with an Eevee biosensor, the EKAREV-nuclear export sequence (NES) (ERK FRET biosensor), which is a substrate-type intramolecular FRET biosensor. Scale bar, 20 μm.

fluorophores can be quite a bit greater than the Förster distance, which is a few nm. For this reason, the relative distance between donor and acceptor FPs is mainly attributed to the increase in FRET in the intermolecular FRET. The advantage of this intermolecular FRET system is the simplicity of development of the biosensor in comparison to the intramolecular FRET biosensor. The possible configurations of FPs and binding domains (for example, A and B) are limited to 4 types: FP_{donor}-A and $FP_{acceptor}$-B, FP_{donor}-A and B-$FP_{acceptor}$, A-FP_{donor} and $FP_{acceptor}$-B, and A-FP_{donor} and B-$FP_{acceptor}$ (Fig. 3A). The combination of FPs showing the highest response should be selected as an intermolecular FRET biosensor.

The bimolecular kinase activity reporter (bimKAR) is a good example of an intermolecular FRET biosensor for monitoring kinase activity (Depry *et al.*, 2015; Herbst *et al.*, 2011). The bimKAR is comprised of two polypeptides, i.e., the FP_{donor}-FHA1 domain or FP_{donor}-WW domain and the substrate peptide-$FP_{acceptor}$ (Fig. 3B). The FHA1 domain and WW domain are phosphorylated peptide-binding domains that recognize the pT-X-X-D motif and pS/pT-P motif, respectively. The CMGC kinases (cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases) phosphorylate the pS/pT-P motif, and therefore the WW domain is suitable for the phosphorylated peptide-binding domain. In other kinases, the phosphorylated peptide motif should be optimized to pT-X-X-D for recognition of the FHA1 domain. The target kinase, which is activated upon stimulation, phosphorylates the substrate peptide-$FP_{acceptor}$, followed by the binding of the phosphorylated substrate-$FP_{acceptor}$ to FP_{donor}-WW or FP_{donor}-FHA1. Thus, the FRET signal is increased upon kinase activation. The expression levels of donor and acceptor fluorophores usu-

ally differ from each other within individual cells, and therefore careful spectral corrections such as bleed-through and cross-excitation are necessary to obtain accurate FRET signals. This image correction process usually reduces the signal-to-noise ratio by the propagation of error. This issue may be partly overcome by using a self-cleaving 2A peptide to express almost similar amounts of donor and acceptor fluorophores. Because the donor-to-acceptor molar ratio is fixed, the bleed-through and cross-excitation are cancelled out by simple ratiometry (Szymczak *et al.*, 2004). In addition, the binding domain for the intermolecular FRET biosensor often acts as a competitor binding to phosphorylated endogenous proteins, resulting in a possible perturbation of intracellular signaling. For these reasons, less effort has been devoted to developing intermolecular FRET biosensors than intramolecular FRET biosensors.

Intramolecular, or unimolecular, FRET biosensors

Intramolecular (or unimolecular) FRET biosensors bear both donor and acceptor FPs in a single polypeptide, and therefore FRET occurs primarily within the biosensor (Fig. 3C). Unlike in intermolecular FRET biosensors, the relative orientation between donor and acceptor FPs plays an important role in the FRET efficiency. Intramolecular FRET biosensors for monitoring kinase activity typically have one of two designs, namely, the conformation-type or substrate-type design (Fig. 3C). The former type detects conformational changes in a kinase or its substrate upon activation by FRET (Fig. 3C, left). For example, Miu2, a FRET biosensor for conformational change in ERK2, demonstrates an open and active conformation after EGF stimulation, concomitant with the nuclear translocation (Fujioka *et al.*, 2006) (Fig. 3C, middle). Meanwhile, in the latter type, the FRET biosensor includes not only a substrate peptide that is phosphorylated by the target kinase, but also a phosphorylation recognition domain such as a WW domain or FHA1 domain (Fig. 3C, right). Intramolecular FRET biosensors have clear advantages—that is, they permit easier introduction of the expression vector, they do not require spectral corrections because the donor-to-acceptor molar ratio is always 1:1 (Sabouri-Ghomi *et al.*, 2008), and they exhibit empirically higher sensitivity and gain as compared with the intermolecular FRET biosensors. Therefore, the intramolecular FRET biosensors have been widely utilized in cell biology.

The serious caveat is the difficulty associated with the development of intramolecular FRET biosensors (Aoki *et al.*, 2012). For instance, it must be decided which FPs, sensor and ligand domains should be included, how the order of these domains should be configured, and how long the optimal linker between the domains should be. Most of these difficulties are derived from the orientation-dependent features of the intramolecular FRET biosensor (Jares-Erijman and Jovin, 2003). The precise structures of the sensor and ligand domains are rarely available. In practical terms, the addition or removal of only one amino acid of linker length has a major effect on the FRET efficiencies (Horikawa *et al.*, 2010). It is almost impossible to predict the effect of the orientation-dependent factor of intramolecular FRET biosensors, and therefore the development of an intramolecular FRET biosensor requires labor-intensive, trial-and-error characterizations.

To circumvent the difficulties inherent in the development of intramolecular FRET biosensors, a number of FP variants that are optimized for FRET applications have been generated by several research groups (Bajar *et al.*, 2016; Karasawa *et al.*, 2004; Lam *et al.*, 2012; Nguyen and Daugherty, 2005; Rizzo *et al.*, 2004). In addition, we have reported a rationally designed optimal backbone for intramolecular FRET biosensors (Komatsu *et al.*, 2011). This backbone is named the extension for enhanced visualization by evading extra FRET (Eevee) system. This Eevee system has two characteristic features: a long flexible linker, called an EV linker, and a "sticky" FP pair (Fig. 3D, left). The long flexible linker renders the biosensor completely "distance-dependent", thereby precluding the "orientation-dependent" effect on FRET. In addition, the long flexible linker lowers basal FRET levels. The "sticky" FP pair—for example YPet and ECFP—enhances the gain of the FRET signal induced by stimulation (Kotera *et al.*, 2010). Applying the Eevee system to the previously reported kinase activity reporters has improved them several-fold. By using the highly sensitive ERK FRET biosensor, EKAREV, stochastic ERK activations were visualized in proliferating cultured cells (Albeck *et al.*, 2013; Aoki *et al.*, 2013) (Figure 3D, right). Further, the use of the Eevee system enabled us to develop new biosensors for S6K and RSK without many optimization steps. Thus, the Eevee system reduces the laborious steps required for prototyping biosensors. Despite advances in the rational backbone for FRET biosensors, much work is still needed for the development and characterization of a FRET biosensor for protein kinases, because many peptide sequences that are phosphorylated by a given kinase are not expected to be phosphorylated when they are included in a FRET biosensor.

Future perspectives

The development of genetically encoded kinase activity reporters is still challenging. However, by employing a broad range of techniques, the development of these reporters has become feasible. For instance, Pertz and his colleagues reported versatile tool kits for the development of intramolecular FRET biosensors (Fritz *et al.*, 2013). Their techniques allowed efficient and comprehensive characterization of intramolecular FRET biosensors. Further, the FLIM-FRET with a non-fluorescent protein enables multi-

plexed imaging of molecular activity (Laviv *et al.*, 2016). While one of the remaining difficulties is to find the peptide sequences to be phosphorylated by a target kinase, a recent advance in phosphoproteomics, kinase-interacting substrate screening (KISS) (Amano *et al.*, 2015), facilitated the development of a novel ROCK FRET biosensor (Li *et al.*, 2017).

Finally, we will consider the future perspectives related to the imaging of kinase activity. First, the manipulation of kinase activity in cells or animals is becoming an indispensable technique for the evaluation of kinase functions *in vitro* and *in vivo*. Chemically induced dimerization has been reported to modulate kinase activity by small chemical compounds (Aoki *et al.*, 2011; Derose *et al.*, 2013). More recently, an optogenetic approach has become an indispensable tool for controlling kinase activity in space and time by light (Schlipalius *et al.*, 2012; Wilson *et al.*, 2017), clearly dissecting the causal relationship between kinase activation and its physiological function (Aoki *et al.*, 2013, 2017; Wilson *et al.*, 2017). Second, the *in vivo* imaging of kinase activity sheds new light on the role of kinase activation in a more physiological and pathological context (Hiratsuka *et al.*, 2015; Kamioka *et al.*, 2012; Mizuno *et al.*, 2014; Tomida *et al.*, 2012). Further progress will require a deeper understanding of the mechanisms of kinase activation, a development of improved FPs for live-cell imaging, and continued efforts to combine these different technologies.

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