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Methods 40 (2006) 279–286
METHODS
www.elsevier.com/locate/ymeth

# Analyzing protein kinase dynamics in living cells with FRET reporters

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Accepted 27 June 2006

**Abstract**

Genetically encoded reporters based on fluorescence resonance energy transfer (FRET) are being developed for analyzing spatiotemporal dynamics of kinase activities in living cells, as the activities of this class of enzymes are often dynamically regulated and spatially compartmentalized within specific signaling context. Here we describe a general modular design and engineering strategies for the development of activity reporters for kinases of interest, using A-kinase activity reporter (AKAR) as an illustrative example. Discussed here are basic structure of such reporters, design considerations, reporter gene construction, cellular and in vitro characterization. Strategies for improving specificity, dynamic range or sensitivity, reversibility and integrity of the reporter as well as basic methods for live-cell time-lapse imaging using these reporters are summarized. Discussion of using this approach in the study of MAPK cascades is also provided. These FRET-based kinase activity reporters, along with analogous probes based on alternative designs, provide real-time tracking of kinase dynamics with subcellular resolution, which should complement other methods and offer great opportunities to delineate the molecular mechanisms underlying the complex regulation of kinases.


*Keywords:* FRET; Kinase; Live-cell imaging; Fluorescent protein; Compartmentalization

## 1. Introduction

Protein kinases are enzymes that catalyze the transfer of phosphate from ATP to specific amino acid residues on protein substrates. By doing so, they modulate the activities of multiple protein substrates and regulate various cellular processes ranging from cell growth, proliferation, division, to apoptosis. Many kinases are known to have broad specificity and can phosphorylate and regulate a diversified list of substrates. Adding to the complexity, protein kinases themselves are subject to multiple levels of regulation including post-translational modifications as well as protein–ligand and protein–protein interactions. It is increasingly apparent that dysregulation of protein kinases and abnormal phosphorylation is often a cause or consequence of disease and consequently protein kinases have been suggested as a major class of drug targets [1]. Thus various members of the protein kinase family [2], along with their substrates and upstream regulators, are crucial and versatile components of the signal transduction networks, which are responsible for converting changes in intra- and extracellular environment to appropriate biological responses.

Within the complex signal transduction networks, protein kinases are often the key nodes connected to multiple upstream signals and various downstream substrates. In many cases, specific and optimized modulation in signaling events requires spatial compartmentalization of kinases activities. In the case of cAMP-dependent protein kinase (PKA), given its ubiquitous presence in mammalian cells and its widespread involvement in many parallel signaling cascades, A-kinase anchor proteins (AKAPs) [3] are often required to provide a molecular framework to orient PKA towards selected substrates. Disruption of PKA anchoring

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doi:10.1016/j.ymeth.2006.06.013

by peptides that antagonize PKA–AKAP interactions often disables cAMP-dependent signaling [4,5], emphasizing the essential role of PKA anchoring in signal transduction. In the case of mitogen activated protein kinases (MAPKs), scaffold proteins have been proposed to interact with MAPK pathway components that consist of MAPK and upstream kinases (i.e. MAPKK and MAPKKK) to create functional signaling modules and enhance the efficiency and specificity in the phosphorylation relay that eventually lead to differential activation of MAPK [6–8]. For example, in hepatocyte growth factor (HGF) stimulated epithelial cells, it has been shown that the scaffold protein paxillin facilitates formation of the Raf-MEK-ERK complex at the focal adhesions of the cells and its phosphorylation by the activated ERK in turn leads to its interaction with focal adhesion kinase (FAK) and subsequent activation of phosphoinositide 3-kinase (PI3K), which could be responsible for the migratory and tubulogenic responses [9]. Likewise, compartmentalization of other kinases and their substrates has been suggested to be a key determinant in the specificity of other signaling pathways, although the molecular basis and cellular consequences of such compartmentalization are less well understood [10,11].

In order to better understand protein kinases within their signaling context, with spatial compartmentalization and multiple levels of regulation maintained, methods are needed to analyze protein kinase dynamics in living cells. Ideally high spatial and temporal resolution should be achieved to allow following the activity of a particular kinase in different compartments and signaling microdomains and tracking its activity changes continuously. Furthermore, such information of protein kinase dynamics should be obtained from single cells because some important aspects of signal transduction such as transient and oscillatory activities can be averaged out during studies of a population of cells.

During the last decade significant progress has been made in our understanding of the structure and function of protein kinases and a variety of new methods have been developed for the investigation of this class of enzymes. One approach for monitoring kinase dynamics with high spatial and temporal resolution is based on fluorescence resonance energy transfer (FRET), which is particularly useful for analyzing kinase dynamics within cellular context. FRET is a radiationless energy transfer process by which the transfer of excited state energy from an excited donor fluorophore to an acceptor fluorophore occurs [12]. FRET efficiency is largely determined by the spectral overlap between the donor emission and the acceptor absorption, quantum yield of the donor, distance between the donor and acceptor, and the relative orientation of the donor and acceptor. Because FRET only occurs when the donor and acceptor are in molecular proximity (i.e. <10 nm apart) and the efficiency of this process is dependent on the inverse sixth power of the distance between the donor and acceptor, it can provide a sensitive measure of molecular events such as protein conformational changes and protein–protein interactions. The limitation, however, is that the donor and acceptor must be maintained in close proximity, as FRET is effective only when they are 1–10 nm apart. On the other hand, fluorescent proteins (FPs) offer the enormous benefit of genetically encodable fluorescence labeling. Newer generation of FPs not only have overcome many issues of early FPs in terms of expression, folding, maturation, photostability, oligomerization, and environmental sensitivity, but more importantly now offer several color variants that are suitable for FRET applications [13,14]. The marriage between FRET and FPs has yielded FP-based FRET reporters that enable visualization of complex cellular processes such as second messenger dynamics, enzyme activation/activities and protein–protein interactions in living cells [15,16]. In this review we mainly focus on the development of FRET reporters for monitoring the dynamics of PKA and MAPK, yet this approach can be readily applied to other members of the protein kinase family.

## 2. Methods

Several designs have been devised to generate FP-based FRET reporters for analyzing protein kinase dynamics. In this section we will focus on a general modular design of kinase activity reporters (Fig. 1), which is used in many current kinase reporters [17–24], followed by discussion of two alternative designs.

### 2.1. Basic structure of a kinase activity reporter

Our design of a kinase activity reporter consists of four basic modules that are linked in sequence, namely a FRET donor (e.g. CFP), a substrate domain, a phosphoaminoacid binding domain, and a FRET acceptor (e.g. YFP), with an optional targeting motif (Fig. 1). In this general design, the kinase of interest phosphorylates the substrate domain, whereupon the concatenated phosphoaminoacid binding domain intramolecularly binds the phosphorylated substrate to generate a conformational change, which is relayed to the FRET pair flanking the tandem fusion protein, producing a FRET change. Dephosphorylation of the substrate domain by phosphatases disrupts the intramolecular complex and reverses the FRET change. This unimolecular design, in which all modules are encoded by a single gene, offers several advantages including fixed donor to acceptor ratio for simple FRET measurement, easy targeting, and a defined mechanism based on intramolecular interaction therefore less interference with endogenous interacting partners.

### 2.2. Design considerations

#### 2.2.1. Substrate domain

The first step in designing a kinase activity reporter is to identify a specific phosphorylation substrate domain for

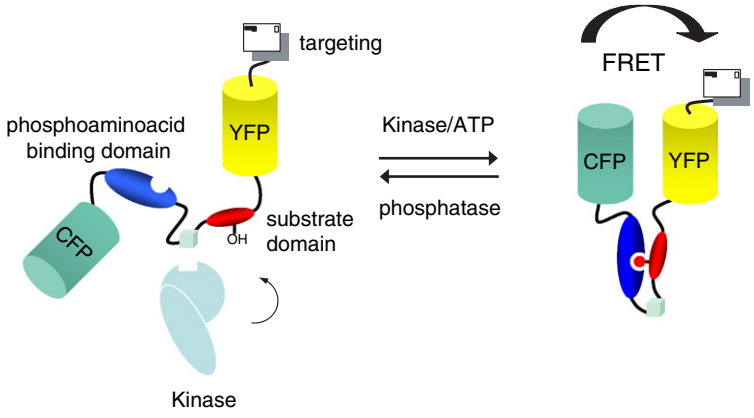


Fig. 1. Basic structure of a kinase activity reporter. A kinase activity reporter consists of four basic modules that are linked in sequence, namely a FRET donor (e.g. CFP), a phosphoaminoacid binding domain, a substrate domain, and a FRET acceptor (e.g. YFP). Additional elements include defined docking sites for recruiting the kinase of interest and localization sequences for targeting the reporter to different subcellular compartments or protein complexes.

the kinase of interest. This may be achieved by choosing a known substrate or designing a substrate based on consensus sequences. Such consensus substrate sequences can be determined either from peptide library screening data [25,26] or based on known substrates. For instance, a consensus sequence for substrates of ERK1 has been identified as PL(S/T)P using the latter approach [27]. When designing AKAR1 [17], a PKA activity reporter, a known PKA specific substrate kemptide (LRRASLG), was selected as a candidate substrate.

Once identified, this substrate sequence may need to be further modified in such a way that the phosphorylated substrate also fits a consensus binding sequence of the phosphoaminoacid binding domain. In the case of AKAR1 [17], a modified kemptide (LRRASLP) was then used because the phosphoaminoacid binding domain used in this reporter, 14-3-3, has a consensus binding sequence R(R/K)(F/R/S/Y)pS(W/Y/F/L)P, where pS denotes phosphoserine.

In addition to the amino acid sequence immediately surrounding the phosphorylation site of the substrate domain, many kinases including MAPKs also rely on docking sites to interact with specific substrates [28]. Therefore for these kinases, incorporation of docking sites may be necessary in order to achieve high specificity.

### 2.2.2. Phosphoaminoacid binding domain

The phosphoaminoacid binding domain in this reporter design provides a conformational switch by converting phosphorylation of the substrate domain to a change in FRET. Incorporation of such a module generalizes the design so that a variety of substrate domains can be used, not just those that drastically change their conformation upon phosphorylation [29–31].

Phosphoaminoacid binding domains are modular protein domains that recognize phosphoaminoacid consensus sequences. Known modular protein domains include phosphoserine/threonine binding domains [32] such as 14-3-3, forkhead associated (FHA) domain, and some WW domains, as well as phosphotyrosine binding domains [33] such as Src-homology 2 (SH2) and phosphotyrosine binding (PTB) domain. Ideally the consensus sequence of the chosen binding domain should be compatible with the phosphorylation substrate sequence. In AKAR1, 14-3-3 was used because its consensus sequence encompasses a consensus sequence for PKA phosphorylation.

### 2.2.3. FRET pair

Aside from the general requirements for the successful use of FPs in an imaging experiment [13], additional considerations for a FRET pair include sufficient spectral overlap between the donor emission and the acceptor excitation, sufficient spectral separation of both the excitation and emission between the donor and acceptor. At present, CFP/YFP is still the FRET pair of choice in terms of FRET efficiency, although recent advances in development of FPs have yielded a variety of FP color variants with improved properties and new color pairs are becoming available [13,34]. Among CFP/YFP FRET pairs, currently ECFP and Cerulean [35] are commonly used CFP variants while Citrine [36] and Venus [37] are commonly used YFP variants. It is also important to note that monomeric FPs [38] should be used in these reporters [20] in order to minimize interference due to dimerization/oligomerization of the FPs. In fact, a simple replacement of the weakly dimeric FP with its monomeric version can lead to improvement of both reversibility and dynamic range of the reporter [23].

### 2.2.4. Linker regions

Linker regions between individual modules can also play important roles, especially the linker between the substrate domain and the phosphoaminoacid binding domain. When structural information of the intramolecular complex formed between the binding domain and the phosphorylated substrate is available, an optimal linker sequence may be rationally designed [15]. However in most cases, linkers of varied length and composition need to be tested in a trial-and-error manner in order to determine an optimal

linker sequence. For instance, three linkers of different length, i.e. 7 amino acid (aa), 14 aa and 19 aa, were incorporated between the substrate sequence and FHA1 domain during the development of AKAR2 [24]. It was found that the construct containing the 14 aa linker provided a greater response and faster kinetics compared to the other two constructs. As a general starting point, flexible linkers like GGSGG may be used.

#### 2.2.5. Targeting motif

Such kinase activity reporters can be diffusible throughout the cell or specifically targeted to various subcellular compartments, such as plasma membrane, nucleus, endoplasmic reticulum (ER), and mitochondria [39,40], by incorporating targeting motifs in the reporter gene (Fig. 1). This is not only useful for improving the signal to noise ratio in detecting localized kinase activity [20] but also can be effective in achieving simultaneous imaging of multiple signaling events in different subcellular compartments in a single cell [40,41].

### 2.3. Reporter gene construction

Once the FRET pair, phosphoaminoacid binding domain, and substrate sequence are determined, a cDNA construct containing all these modules can be generated using standard molecular cloning techniques. Mutant constructs that serve as proper controls should also be generated. These include mutation of the phosphorylation site in the substrate domain to a non-phosphorylable amino acid and mutations of residues in the phosphoaminoacid binding domain that are critical for its binding to the substrate. In the case of AKAR1 [17], two mutants, S475A (with mutation of serine to alanine in the modified kemptide) and K280E (with mutation of one critical residue at the active site of 14-3-3 binding domain) were generated.

### 2.4. Cellular characterization

Cellular characterization is essential for the applications of such kinase activity reporters in living cells. To identify a promising candidate reporter, the following experiments should be carried out. First, transfection efficiency and expression patterns should be examined in several different cell lines. In the case of AKAR1 [17], both the cyan and yellow fluorescence were uniformly distributed in the cytosolic compartment but excluded from the nucleus probably due to the large size of the reporter, when the construct was transfected into HeLa cells. A similar expression pattern was also observed in CHO, COS-7, and HEK293 cells.

Second, FRET response should be observed in cell lines expressing the kinase of interest upon activation by known stimuli and this response should be abolished by treatment with kinase specific inhibitors. In addition, stimulation of other kinases should not lead to the same changes. For instance, in HeLa cells transfected with AKAR1, an emission ratio increase of 25–50% was detected upon addition of forskolin (Fsk) to stimulate adenylyl cyclase and activate PKA. This response can be blocked by a relatively specific PKA inhibitor H-89 as well as PKA specific inhibitor PKI.

### 2.5. In vitro characterization

In vitro characterization of the reporter is often necessary in order to confirm and complement the cellular results. The reporter can be tested in vitro by purifying the chimeric protein from bacteria and measuring the FRET change upon phosphorylation with the appropriate kinase and ATP. This FRET change should be abolished by removal of either the kinase or ATP from the reaction. Different kinases can be used to evaluate the specificity of the reporter.

For in vitro characterization of AKAR1 [17], chimeric protein of AKAR1 was expressed as N-terminal $His_6$ tag fusions in *Escherichia coli* and purified by nickel chelate chromatography. Purified AKAR1 chimeric protein was treated with the catalytic subunit of PKA in the corresponding reaction buffer at 25 °C. The fluorescence spectra were measured with an excitation wavelength of 434 nm before and after addition of 1 mM ATP. This treatment resulted in a yellow over cyan emission ratio change of 30% in 5–10 min. Omission of either PKA or ATP abolished the FRET change, indicating that the response requires PKA phosphorylation. In the same way, AKAR1 was also tested against calmodulin-dependent kinase II, PKCβII, PKCδ, and PKG as representative serine/threonine kinases whose consensus sequences have some overlap with the sequence of modified kemptide. None of these kinases gave significant FRET changes in 1 h, therefore the FRET response of AKAR1 is relatively PKA specific.

### 2.6. Validation with control constructs

Given positive and consistent results from both cellular and in vitro characterization, further validation with control constructs should be carried out. It is expected that mutation of the phosphorylation site in the substrate domain and mutations of critical residues in the phosphoaminoacid binding domain should abolish or diminish the FRET response respectively. Indeed, unlike AKAR1, mutant S475A did not show FRET response, while mutant K280E gave a much smaller response both in living cells and in vitro [17]. These results support the designed mechanism for the FRET response of the reporter.

### 2.7. Improvement

Once a kinase activity reporter is successfully developed, further improvement may be necessary to meet the specific requirements of different applications. Here we discuss several proven strategies for the improvement of important properties of the reporter.

#### 2.7.1. Specificity

One of the key challenges in designing a kinase activity reporter is achieving high specificity. Oriented peptide

library approach [25,26] has been an effective strategy for determining an optimal substrate sequence for the kinase of interest. However, this approach has the limitation that the predicted optimal sequences are based on in vitro data, which does not always predict their specificity within the cellular context. An alternative to the oriented peptide library approach is to design a sequence based on endogenous substrate sequences of the kinase [23]. The challenge here, however, is that the exact endogenous substrate sequences of many kinases and the crucial elements encoding their specificity are yet to be determined. To further complicate this issue, it is well known that many kinases have overlapping substrate specificities. When this is indeed the case, higher specificity may be achieved by targeting the reporter to the proximity of the kinase of interest via incorporation of a docking site or tethering to anchoring or adaptor proteins [17,42].

#### 2.7.2. Dynamic range/sensitivity

The limited dynamic range of FP-based FRET reporters is often suspected to be insufficient to detect subtle yet biologically significant signals. However, this apparent limit should not be an intrinsic problem of the method and significant improvement in the dynamic range of such reporters has been achieved [43].

Because FRET is sensitive to the distance and/or orientation between the donor and acceptor, several effective strategies along this line have been developed. First, circularly permuted FPs (cpFPs) can be used to replace the original FP [43,44]. Since the difference between a cpFP and FP is only their C- and N-terminal locations, this replacement is likely to generate a different orientation between the FRET pair. Next, linker variations between individual domains and alternative phosphoaminoacid binding domains can be explored. These changes are expected to alter the distance and/or orientation between the FRET pair and therefore FRET efficiency. While this remains a trial-and-error process, it has been demonstrated that computational modeling can help to provide some rational guidelines [45].

Additionally, a better FRET pair can lead to further improvement of dynamic range of the reporter. The efforts in engineering new FPs have been mostly focused on improvement of the properties of individual FPs [35–37]. However, this may or may not help to improve FRET efficiency between a pair of FPs. Recently, an approach using molecular evolution based on fluorescence activated cell sorting (FACS) was applied to optimize a CFP/YFP pair for FRET [46]. The resulting pair, CyPet/YPet, achieved more than sixfold dynamic range improvement over the parental pair. While promising, the mammalian expression of this new pair still needs further engineering before it can supersede the current CFP/YFP pair [13].

#### 2.7.3. Reversibility

The FRET response of AKAR1 was largely irreversible in living cells [17]. It was hypothesized that tight binding of the phosphorylated substrate by the phosphoaminoacid binding domain (i.e. 14-3-3) can prevent the phosphorylated substrate from being dephosphorylated by phosphatases and lead to apparent irreversibility of the reporter. In order to continuously monitor the dynamic balance between the kinase and phosphatase activities, however, it is desirable that the kinase activity reporter be reversible. Therefore to achieve better reversibility, the phosphoaminoacid binding domain of choice should have a moderate binding affinity, e.g. submicromolar, toward the phosphorylated substrate [24]. Consistent with this hypothesis, the replacement of the 14-3-3 with FHA1, a modular phosphothreonine binding domain with a weaker binding affinity than that of 14-3-3, led to the generation of AKAR2, a reversible AKAR reporter [24]. Additionally, use of monomeric FPs as FRET donor and acceptor should also help to improve reversibility.

#### 2.7.4. Integrity

A unimolecular kinase activity reporter has a defined response mechanism of FRET change. As such it is important that the reporter remain intact in the cellular context because proteolysis would render the reporter unresponsive. One indication of proteolyzed reporter is imperfect colocalization of fluorescent signals from the donor and acceptor. Because proteolysis mostly occurs at the linker regions, modifications of the linker composition can be carried out to improve its resistance to proteolysis.

### 2.8. Application—differential subcellular dynamics of PKA activity

FRET-based kinase activity reporters can provide valuable information about compartmentalization and dynamic regulation of kinases activities. For instance, the reversible PKA reporter AKAR2 [24] has been used in live-cell time-lapse experiments to monitor the differential PKA activities within the cytosolic and nuclear compartments (Fig. 2).

One typical imaging setup we use consists of a Zeiss Axiovert 200 M microscope with a 40×/1.3 NA oil-immersion objective lens, and a cooled CCD camera MicroMAX BFT512 (Roper Scientific) controlled by METAFLOUR software (Universal Imaging). Dual-emission ratio imaging for CFP/YFP uses a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters (475DF40 for CFP and 535DF25 for YFP) altered by a Lambda 10-2 filter changer (Sutter Instruments).

HeLa cells are plated onto sterilized glass coverslips in 35-mm glass-bottom dishes (MatTek) and grown to 50–70% confluency in DMEM supplemented with 10% FBS at 37 °C in 5% $CO_2$. Cells are transfected with a mammalian expression vector (pcDNA3) containing AKAR2 and cultured at 37 °C. Typically cells are imaged 18–36 h post-transfection although fluorescent reporters can be observed in just 4–6 h. In preparation for imaging, cells are washed with Hanks' balanced salt solution (HBSS) buffer (pH 7.4), and maintained in the dark at room temperature.

Cells expressing intermediate levels of fluorescent reporters and showing normal morphology are selected for imaging. The first panel of Fig. 2 shows such a cell expressing AKAR2 with fluorescence uniformly distributed throughout the cell. Three sets of images are acquired, including Cyan direct (exc 420, em 475), FRET (exc 420, em 535) and Yellow direct (exc 495, em 535). Grayscale images are collected with no saturated pixels by controlling the excitation intensity with neutral density filtration and by adjusting the exposure time. For a typical time-lapse experiment with AKAR, exposure time is 100–1000 ms and images are acquired every 10–60 s.

For data processing, fluorescent images are background corrected by subtracting autofluorescence intensities of untransfected cells (or background with no cells) from the emission intensities of cells expressing fluorescent reporters. The intensity within selected cell regions of interest (ROI) is measured in both Cyan direct (exc 420, em 475), and FRET (exc 420, em 535) channels. The ratios of emissions are then calculated at different time points. Since yellow fluorescent protein is more photobleachable, the intensity within ROI is also measured in Yellow direct channel (exc 495, em 535) to monitor its photobleaching. In any case, prolonged illumination should be avoided to minimize photobleaching during the experiment. Pseudocolor images can also be generated using the METAFLOUR software to indicate the emission ratio of yellow over cyan.

As shown in Fig. 2, when Fsk was added to the cells, an emission ratio increase in the cytosol was detectable within 1 min and reached a maximum within 3–4 min. On the other hand, the emission ratio in the nucleus stayed low until 5–10 min after Fsk stimulation, consistent with previous observation using nuclear targeted AKAR1 [17]. The slow response in the nucleus is consistent with slow diffusional translocation of the catalytic subunit of PKA into the nucleus after its dissociation from the regulatory subunits in the cytoplasm upon cAMP elevation [47,48]. Removal of the stimulant and addition of H-89, a PKA inhibitor, resulted in a decrease in emission ratio first in the cytosol followed by propagation into the nucleus. The emission ratio returned to the basal level over 30–50 min period.

### 2.9. Application of FRET system to MAPK

While there is no published example of MAPK reporters based on the above discussed design yet, two alternative designs of FRET-based reporters have been applied to MAPK. One example is a set of FRET-based probes for extracellular signal-regulated protein kinase (ERK) based on Ets1 and Elk1 transcription factors [30]. These reporters contain substrate sequences from either transcription factor, which are known to be phosphorylated by ERK, sandwiched between CFP and YFP (Fig. 3A). For one construct, residues 1–138 from Ets1 containing two phosphorylation sites and a docking domain for ERK was used. Different sequences from Elk1, containing two phosphorylation sites and a FXFP motif that binds ERK, were used for several other constructs. In vitro studies showed that these probes can generate a FRET decrease in the presence of active ERK, and are unaffected by c-jun N-terminal kinase (JNK) or p38 MAPK. Different signal amplitudes of FRET decrease were observed for different constructs with

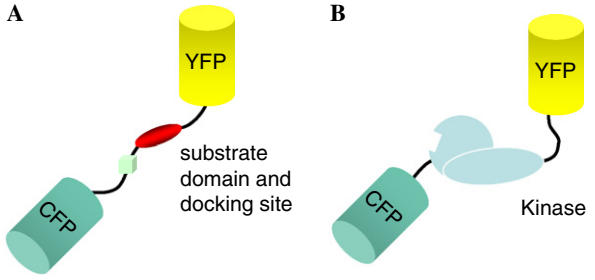


Fig. 3. Application of FRET system to MAPK. (A) An alternative design consisting of a substrate domain sandwiched between a FRET donor and a FRET acceptor. (B) An alternative design with the kinase of interest sandwiched between a FRET donor and a FRET acceptor.

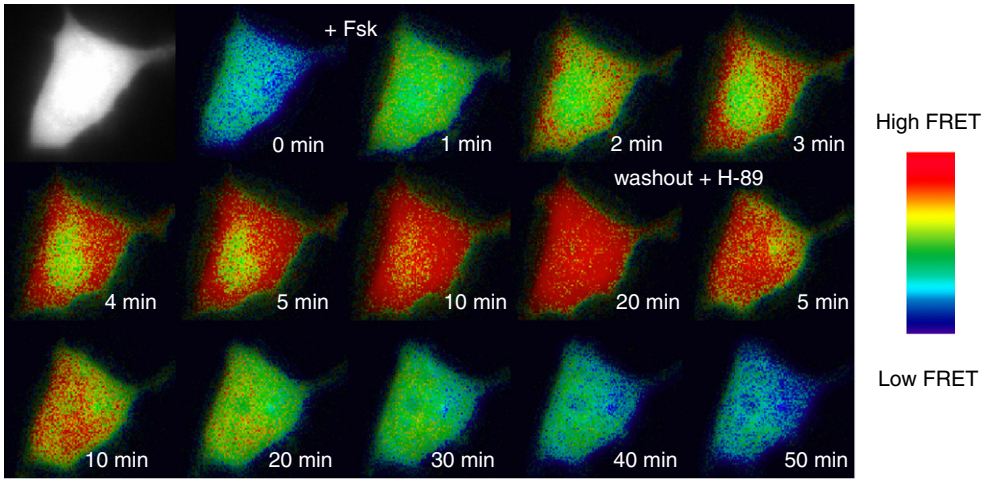


Fig. 2. Pseudocolor images of a live HeLa cell expressing AKAR2 in response to forskolin (Fsk). Yellow to cyan emission ratio increases due to phosphorylation-induced changes in FRET, which can be reversed by the removal of Fsk and addition of a PKA inhibitor, H-89.

shorter substrate sequences corresponding to bigger FRET changes. Although in vitro data were promising, similar results were not obtained when these probes were used in living cells, suggesting additional improvement is necessary for them to be useful in cellular applications.

Yet another approach was taken to study intracellular activation of ERK using FRET (Fig. 3B). In this case, ERK2 itself was sandwiched between CFP and YFP to detect global conformational change of ERK2 [49], which may alter the distance and/or orientation between N- and C-termini that were shown in close proximity in the crystal structure. It was shown that binding of MAP/ERK kinase (MEK) to the probe could increase FRET while phosphorylation of Thr188 and Tyr190 would trigger the dissociation of MEK from the probe, leading to a decrease in FRET. This probe was used to visualize activation of ERK by MEK in HeLa cells. Such design provides an effective means of analyzing kinase dynamics when kinase activation leads to a conformational change within the kinase itself, and may provide complementary information to that obtained from kinase activity reporters.

## 3. Discussion and concluding remarks

FP-based FRET reporters provide a means to directly visualize cellular signaling events in real time and space. Here we reviewed the general design of a class of such reporters that could enable dynamic visualization of compartmentalized kinase activities in live cells, tissues, and whole organisms. In these unimolecular reporters, FRET can be assessed by the ratio of acceptor to donor emissions upon donor excitation, providing a straightforward ratiometric measurement. Such ratiometric measurement can cancel out some sample-to-sample variations, such as variations in light source and cell thickness [50]. Even so, proper controls (Sections 2.3, 2.4 and 2.6) are always needed to ensure emission ratio changes are due to phosphorylation events instead of other factors such as intracellular pH changes, which may differentially affect the spectral properties of the donor and acceptor. In addition, these genetically encoded reporters can be easily targeted to different cellular compartments by introduction of targeting motifs in its structure. When necessary, localizations below the resolution of conventional fluorescence microscopy can be achieved [17]. Last but not least, it is important to realize that overexpression of any reporters may disturb the cellular systems under investigation. In depth discussion about potential perturbations caused by fluorescent reporters of different designs can be found in reference [15].

It is expected that these kinase activity reporters will be further utilized in various applications to gain valuable information about the spatial and temporal dynamic of kinase signaling events, as well as the dynamic interplay and crosstalk between different signaling events and pathways. Furthermore, this methodology should be generally applicable to many kinase family members. It is hopeful that kinome-wide tracking of cellular activities may be eventually achieved, adding time and space dimensions and dynamic information to the current map of signal transduction networks.

## Acknowledgments

This work was supported by NIH grant DK073368, the Young Clinical Scientist Award Program of Flight Attendant Medical Institute, 3M, and a Scientist Development Award from the American Heart Association to J.Z.

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