FRET-based biosensors for protein kinases: illuminating the kinome

Jin Zhang*[a] and Michael D. Allen[b]

DOI: 10.1039/b706628g

Protein kinases are crucial components of intracellular signaling pathways which transmit signals by phosphorylation of downstream targets, altering their function. Efficient signal transduction requires precise kinase regulation within specific biological contexts, making tools that allow study of their dynamics *in situ* critical for understanding kinase function. Highlighted in this article is the design of genetically-encodable, FRET-based kinase biosensors with examples of their implementation to study kinase regulation in live biological contexts with high spatial and temporal resolution.

1. Introduction

Cellular function requires accurate translation of extracellular cues into functional responses *via* intracellular signaling cascades, many of which are critically regulated by protein kinases. Protein kinases enzymatically transfer the γ-phosphate of ATP to recipient amino acid side chains on protein targets to yield phosphoproteins with altered activities, interactions, localizations, or stability, thereby propagating the cellular signal. Dysregulation of protein kinases is involved in a variety of pathophysiologic states, including cancer, inflammatory and autoimmune disorders, and cardiac diseases. As a result, protein kinases have become one of the major therapeutic targets over the past 10 years.[1,2]

Many members in the kinome,[3] which includes 518 putative protein kinases in humans, 540 in mice, and 122 in yeast, have a plethora of downstream targets with diverse functional roles. In order to generate appropriate cell responses, precise spatial and temporal control of kinase activity must be achieved within a biological context. Understanding of the precise regulation of protein kinases holds the key to understanding the logic and language of signal transduction and developing effective therapeutic approaches targeting this class of enzymes.

However, investigation into regulation of kinases presents a challenge to traditional methods. For *in vitro* kinase assays, cells are lysed, and the phosphorylation of an exogenous substrate is monitored over time by radioactivity or phosphorylation-specific antibodies. The phosphorylation-specific antibodies can also be utilized to assess the degree of phosphorylation of an endogenous protein target within cell lysates by Western blot or in fixed cells with immunohistochemistry. All these assays can detect kinase activity, but some biological context is lost during cell fixation or lysis. Fortunately, the challenge of monitoring the dynamic changes in kinase activities in living systems has begun to yield to newly developed techniques[4–6]

[a]*Department of Pharmacology and Molecular Sciences, and The Solomon H. Snyder Department of Neuroscience and Department of Oncology, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Hunterian #307, Baltimore, MD, 21205, USA. E-mail: jzhang32@jhmi.edu; Fax: +1 410 955-3023; Tel: +1 410 502-0173*
[b]*Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, 21205, USA*

Jin Zhang

Dr Zhang received her BS degree in chemistry from Tsinghua University, and her PhD in chemistry from the University of Chicago. After finishing her postdoctoral training with Roger Tsien and Susan Taylor at the University of California, San Diego, she joined the Department of Pharmacology and Molecular Sciences at the Johns Hopkins University School of Medicine. She is currently an assistant professor of Pharmacology, Neuroscience and Oncology. Current research in her lab focuses on investigation of spatiotemporal regulation or dysregulation of protein kinases and second messengers in cell migration, energy metabolism and cancer development.

Michael D. Allen

Michael D. Allen received his Bachelors of Arts degrees in biology and chemistry from Transylvania University in Lexington, Kentucky. He is currently working towards his PhD in the Department of Pharmacology at the Johns Hopkins School of Medicine with Dr Jin Zhang. Current and future research interests include development of novel activity probes of signaling molecules for live cell imaging, in vivo imaging, and high throughput screening.

represented by a growing family of genetically-encodable FRET-based biosensors,[6] which can provide the spatial and temporal details of kinase regulation.

2. Designs of FRET-based kinase biosensors

Continuing efforts in developing new fluorescent proteins (FPs) have yielded a rainbow of variants with different fluorimetric properties.[7,8] Some pairs of fluorescent proteins in this rainbow can undergo fluorescence resonance energy transfer (FRET), the nonradiative transfer of energy from a donor to an acceptor fluorophore.[9] FRET is strongly dependent on interfluorophore distance and orientation. By incorporating a kinase-dependent molecular switch that undergoes a conformational reorganization upon phosphorylation between a FRET pair, one can produce a FRET-based kinase biosensor. Dynamic FRET changes can be read as changes in emission ratio of the two fluorophores, or alteration of fluorophore fluorescence lifetime.[10] Live cell imaging with these genetically-encoded kinase reporters permits the direct visualization of kinase activity in live cells.[11,12]

Table 1 FRET-based kinase activity reporters (KAR) that utilize the modular design of a phosphoamino acid binding domain and kinase-specific substrate as the molecular switch

Reporter	Target	FRET pair	Signal change (%)[a]	Reference
AKAR	PKA	ECFP/cpVenus	40 ↑	13,20,28,47
Aktus	PKB	CFP/YFP	10 ↑	17
ATOMIC	ATM kinase	CFP/YFP	10 ↓	21
BKAR	PKB	ECFP/Citrine	10–25 ↓	18
CKAR	PKC	ECFP/Citrine	15–20 ↓	16
CrkII-based reporter	Abl	ECFP/Citrine	15–30 ↑	14
DKAR	PKD	CFP/YFP	10–20 ↓	22
EGFR reporter	EGFR	ECFP/Citrine	25–35 ↑	14
Erkus	Erk1	CFP/YFP	10 ↓	24
Phocus	IR	CFP/YFP	15–20 ↑	15
Picchu	Abl/EGFR	CFP/YFP	60 ↑	23
Src reporter	Src	ECFP/Citrine	30–35 ↓	14,19

[a] Best dynamic ranges are shown when different generations of reporters exist, with arrows representing the directions of FRET responses when plotted as changes in yellow over cyan emission ratio.

2.1 FRET-based kinase activity reporters

As one major class of kinase biosensors, kinase activity reporters serve as surrogate substrates for kinases and report the dynamics of phosphorylation by FRET changes. Such reporters have been developed for various serine/threonine and tyrosine kinases (Table 1).[13–24] These reporters utilize, as a molecular switch, a kinase substrate domain attached to a phosphoamino acid binding domain (PAABD). Upon phosphorylation of the substrate, the PAABD binds the phosphopeptide, altering the distance and/or orientation between donor and acceptor fluorophores, resulting in a detectable change in FRET (Fig. 1). Notably, such a molecular switch was also used to generate changes in fluorescence intensities in a series of single-fluorophore based kinase sensors for insulin receptor.[25]

For FRET-based kinase activity reporters, this modular design makes it possible to custom-design and engineer specific reporters for various kinases by selecting appropriate substrate domains and PAABDs. The substrate domain must be efficiently and specifically phosphorylated by the desired kinase, and once phosphorylated, readily recognized by the

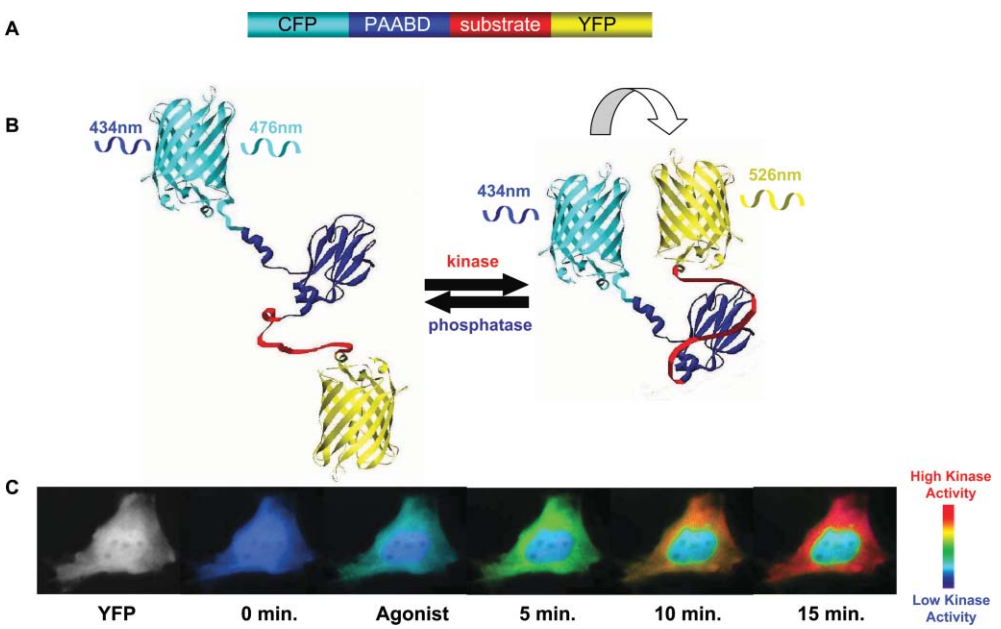

Fig. 1 A, General schematic of the functional domains of FRET-based kinase activity reporter (KAR). B, Structural representation of such sensors before and after phosphorylation by the target kinase, based on known structures of CFP, YFP and forkhead associated domain bound to a phosphopeptide. Shown is a phosphorylation-induced increase in FRET between CFP and YFP. C, A representative time course of emission ratio (yellow/cyan) change of A-Kinase Activity Reporter (AKAR), indicated by pseudocolor changes.

PAABD. Incorporation of a PAABD, such as a 14-3-3 or forkhead associated domain as phosphoserine/threonine binding domains,[26] and Src-homolgy 2 (SH2) as phosphotyrosine binding domain,[27] converts substrate phosphorylation to a conformational change, which relies on intramolecular binding instead of structural changes of substrate domains. Thus a variety of substrate domains could be used in such a design without particular structural requirement.

An ideal kinase activity reporter should be specific, sensitive, and possibly reversible. The specificity of the reporter is largely determined by the amino acid sequence immediately surrounding the phosphorylation site of the substrate domain, although incorporating docking sites for certain kinases should enhance specificity. The sensitivity or response amplitude of kinase activity probes varies, determined by the difference in FRET efficiencies between phosphorylated and unphosphorylated reporter. In theory, kinase activity reporters are quite sensitive as one active kinase can phosphorylate multiple probes, amplifying response, thus allowing small pools of kinase to be detected. Reporter signal can be optimized through adjustment of fluorophore distance,[20] FRET partners,[28] and fluorophore orientation.[28] Additionally, reversibility of a reporter would facilitate continuous monitoring of the dynamic balance between kinase and phosphatase activities and may be achieved by utilizing PAABD with weaker binding affinity[20] and monomeric versions of FPs.[19,29] Detailed discussions are not provided here, as strategies for improving a kinase activity reporter have been previously reviewed.[6]

The unique advantages of these reporters are genetic encodability and targetability. These sensors can be generated as DNA constructs, and expressed in living cells, tissues and animals, circumventing usual problems of sensor delivery and allowing for real time tracking of activity with high temporal resolution. Furthermore, they can be tagged with subcellular targeting sequences and localized to distinct subcellular locations at which local pools of kinase activity can be studied, achieving high spatial resolution. In addition, in this unimolecular design, all modules are linked together and encoded by a single gene. The fixed donor to acceptor ratio allows simple measurements of emission ratios to be used, which has several advantages over single-wavelength monitoring, including minimizing sample-to-sample variations and providing quantitative measurement.[30]

2.2 Alternative FRET-based kinase biosensors

Kinase reporters have been constructed using alternative designs. For example, the kinase of interest itself can act as the molecular switch if its activation generates a conformational change. This conformational change can be translated to a change in FRET for tracking kinase activation, when the kinase is flanked by a FRET pair. This approach has been used to monitor the dynamics of Protein Kinase B (PKB)/Akt,[31,32] Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII),[33] mitogen activated protein (MAP) kinase, extracellular signal-regulated protein kinase 2 (ERK2)[34] and MAP kinase-activated protein kinase 2 (MK2)[35] in living cells. These reporters may be more specific than kinase activity probes as only the kinase to be studied is fluorescently tagged. However, a particular kinase may or may not undergo a dramatic change in conformation upon activation, limiting the generality of this design, and in some cases, dynamic range of the signal. Another alternative design is to sandwich a kinase substrate alone between a FRET pair. The conformational change in the substrate upon phosphorylation can be exploited as a kinase dependent switch to generate a change in FRET. Known examples in this class of reporters include those for monitoring cAMP-dependent protein kinase (PKA),[36] Protein Kinase C (PKC)[37] and ERK.[38] Capable of tracking dynamics in kinase activation or kinase mediated phosphorylation, these alternative kinase biosensors provide complementary high-resolution information of the spatiotemporal regulation of kinases.

3. Applications of FRET-based kinase biosensors

3.1 Visualization of dynamic and compartmentalized kinase activities in living cells

FRET-based kinase biosensors are powerful tools in elucidating the regulation of kinases in a cellular environment.[39–41] Highlighted below are a few representative examples.

PKA microcompartmentalization. A-Kinase Activity Reporter 1 (AKAR1) was designed to track PKA activity in living cells.[13] When tethered to PKA holoenzyme *via* a sequence from A-Kinase anchoring protein (AKAP), phosphorylation of the reporter is accelerated, compared to the untethered reporter. Thus, the catalytic subunit of PKA, despite its diffusibility, when released from PKA holoenzyme, would preferentially phosphorylate a local pool of substrates. This example provided direct illustration of functional effects of compartmentalization of a kinase with its substrate.

Taking this approach further, Dodge-Kafka *et al.* modified AKAR2, the second generation of AKAR, through introduction of docking sites for PKA holoenzyme and negative regulator phosphodiesterase (PDE) derived from AKAPs.[42] The reversible AKAR2 was generated by replacing a high-affinity pair in AKAR1, 14-3-3 and a serine-containing substrate, with a lower-affinity pair, forkhead associated domain 1 (FHA1) and a threonine containing substrate.[20] This reversibility makes it possible to follow transient responses. As shown in this study,[42] a transient response due to recruitment of PDE to PKA-AKAR became more sustained with co-expression of constitutively active MAP-Kinase Kinase (MEK). It was then discovered that PKA, PDE, and ERK as well as additional cAMP-dependent regulators of ERK are all local residents of a signaling complex mediated by mAKAP. Thus, the FRET-based PKA sensor was instrumental in elucidating the functions of mAKAP as a cAMP signaling module that integrates signals from both cAMP and ERK pathways to bi-directionally regulate cAMP signaling. Notably, these biosensors can be targeted to signaling complexes or used as scaffolds of artificial signaling complexes that mimic those found in nature for elucidating spatial regulation of kinases *via* submicroscopic localization and redistribution.

Hyperinsulinaemia effects in adipocytes

Chronic hyperinsulinaemic conditions are typical of type 2 diabetes. The effects of such conditions on cAMP/PKA signaling were examined using the AKAR2 biosensor in adipocytes.[20] It was shown that chronically high insulin levels delay PKA activation in response to β-adrenergic stimulation, despite overproduction of cAMP under the same conditions. Furthermore, disruption of PKA scaffolding mimics the interference of insulin with β-adrenergic receptor signaling. This observation led to the finding that β-adrenergic receptor and PKA are compartmentalized in adipocytes and chronically high insulin levels may disrupt the close apposition of β-adrenergic receptors and PKA, identifying a new mechanism for crosstalk between heterologous signal transduction pathways.

Long-range activation of Src in mechanosensing. Src, a non-receptor tyrosine kinase, plays an important role in regulating many cellular processes including transduction of mechanic signals *via* regulation of integrin-cytoskeleton interaction. A FRET-based Src activity reporter was used to study the spatial and temporal dynamics of Src activity during mechanotransduction.[19] Wang *et al.* showed that when a local mechanical stimulus was applied to cells expressing this reporter, a wave of Src activity occurred from the site of stimulation to distal parts of the cell periphery in the opposite direction to the applied force. It was further shown that this force-induced, directional and long-range activation of Src depends on both actin polymers and microtubules. In this example, spatial difference in Src activity was revealed and quantified using the FRET-based kinase biosensor, providing direct evidence for the critical role of Src kinase in mechanotransduction.

Oscillatory kinase activity. FRET-based kinase biosensors can provide temporal information of dynamically regulated kinases. Oscillations in concentrations of second messengers, such as calcium, have been observed, and carefully examined in living cells in order to decode the specific regulatory information encoded in such oscillations for controlling cellular processes, such as gene expression.[43] Yet a general method with sufficient temporal resolution is still needed to test how protein kinases, many of which are activated by second messengers to mediate downstream cellular processes, may participate in this process. FRET-based kinase biosensors, with millisecond to second resolution,[44] are suitable for examining oscillations in kinase activities. Indeed oscillatory activity of Protein Kinase C was visualized by a FRET-based C-kinase activity reporter (CKAR).[16] Targeting of this reporter to the plasma membrane, where PKC is activated, revealed oscillatory phosphorylation in HeLa cells in response to histamine. Such oscillatory kinase activity may emerge as a general theme of kinase regulation as PKA activity oscillation was recently captured in retinal explants.[29]

3.2 Visualization of kinase regulation deeper in tissues

While analysis of kinase dynamics in living cells provides valuable information of kinase regulation within cellular context, the ability to image kinase activity and regulation in intact tissues puts us one step closer to the goal of understanding kinase regulation and function *in vivo*.

One example focused on the role of retinal waves in temporal regulation of PKA activity.[29] In the early stages of retinal development, retinal ganglion cells (RGCs) spontaneously send a wave of action potentials across the ganglion cell layer. These retinal waves play an important role in the development of vision. Because PKA has been implicated in playing a major role in retinal development, its spatiotemporal dynamics were investigated in rat retinal explants. Using AKAR2.2, a variant of AKAR2 with improved kinetics due to the addition of antidimerization mutations to both members of the FRET pair, spontaneous oscillations in PKA activity were visualized, which are temporally correlated with spontaneous depolarizations associated with retinal waves, supporting retinal wave control of these PKA activity dynamics. This work establishes a connection between spontaneous neural activity with temporal oscillations in kinase activity during retinal development through the use of a FRET-based, tissue imaging approach.

Visualization of kinase activity at different subcellular regions was also achieved using tissue imaging by Gervasi *et al.* in their study of PKA dynamics in neurons of mouse brain slices.[45] AKAR genes were delivered by Sindbis virus transduction of individual cortical brain slices. Distinct kinetics of PKA activation were observed in response to stimulation of the Gs-coupled, 5-HT7 receptor at different locations within cells: rapid at the plasma membrane, slow in the cytosol, and even slower in the nucleus. Thus kinase biosensor imaging in morphologically intact mammalian tissue revealed the potential physiological relevance of PKA signal integration at the subcellular level.

3.3 Kinetic modeling using quantitative imaging data

Kinases, as key regulatory nodes, function within signaling networks. Integrating experimental measurements and mechanistic computational models may facilitate quantitative understanding of the regulation and function of kinases at the systems level. For this purpose, quantitative measurements are needed for feeding into mechanistic models as well as for verifying predictions generated by the models. In this context, these FRET-based kinase biosensors can provide quantitative information about the activation and inactivation kinetics of kinases and spatial distribution of their activities in living cells. In a recent example, live-cell imaging using AKAR provided quantitative data, which was used in systems modeling of PKA-mediated phosphorylation in neonatal cardiac myocytes in response to G-protein coupled receptor stimuli and UV photolysis of "caged" cAMP.[46] It was shown that cAMP accumulation is rate-limiting in PKA-mediated phosphorylation downstream of the β-adrenergic receptor. Localized release of cAMP *via* uncaging triggered gradients of PKA-mediated phosphorylation, enhanced by phosphodiesterase activity and PKA-mediated buffering of cAMP. These results demonstrate that combining live-cell FRET imaging and mechanistic computational models can further

our understanding of spatiotemporal signaling.

3.4 High-throughput screening with FRET-based kinase reporters for drug discovery

Development of a high-throughput compatible assay platform for FRET-based kinase biosensors should open up new applications for these probes, such as high throughput screening of kinase activators and inhibitors and for activity profiling of kinases in diseases. One such assay has been developed recently by testing the ratiometric readout of AKAR3, currently the most sensitive AKAR variant with optimized fluorophore orientation, in a high-throughput plate reader format (Fig. 2).[47] This assay format, combined with a genetically targetable kinase activity reporter, allowed simple, rapid, and convenient high-throughput reading of dynamic kinase activities with high spatiotemporal resolution. For instance, AKAR3 was tagged with a nuclear export signal (NES) and localized predominately in the cytosol so that cytosolic PKA activity was recorded without contamination from nuclear activity, which has slower kinetics due to diffusion of the PKA catalytic subunit from cytosol to nucleus. In this case, sensor targeting led to improved maximum signal as well as increased spatial and temporal resolution. This technique could be used to reveal how individual signaling microdomains, such as kinase-containing signaling complexes, are affected by drugs or other perturbations with high-throughput. Furthermore, as a proof of principle, a pilot screen using 160 compounds from the Johns Hopkins Clinical Compound Library[48] identified known activators and inhibitors of PKA signaling. It is expected that further improvement of these biosensors and detection methods will lead to powerful live-cell high-throughput assays for a variety of kinase targets with the potential of identifying compounds with novel modes of action, for example, by acting on a subpopulation of the target kinase.

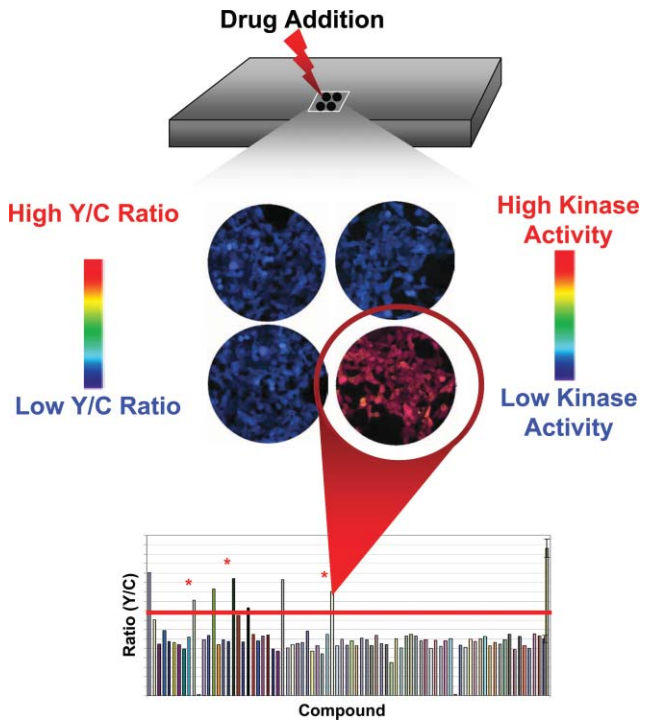

Fig. 2 Schematic representation of the application of kinase biosensors in live-cell, high throughput screens for novel pathway modulators. Individual compounds are added to each well of the microtiter plates, which contain living mammalian cells expressing a kinase biosensor. Cyan and yellow fluorescence intensities are read by a fluorescence plate reader and emission ratios are calculated to detect which wells contain compounds that may activate or inhibit the kinase. High yellow/cyan (Y/C) emission ratios (red) represent potential agonists.

3.5 Sensor engineering for future applications

Existing and new kinase biosensors will continue to be improved and modified for specific applications. To improve the assay sensitivity and allow capturing of subtle changes of kinase activity, new phosphoamino acid binding domains may be identified or engineered to be used in kinase biosensors. Further improvement of current FP and FRET pairs would also be of tremendous help. On the other hand, isoform specific kinase biosensors will be useful in elucidating isoform dependent regulations in different cell and tissue types.

Furthermore, to achieve a better understanding of the dynamic signaling networks, it is essential to establish correlation between regulations of kinases and other signaling molecules in the networks. To this end, kinase biosensors with orthogonal fluorimetric properties may be utilized, along with other compatible techniques, such as patch clamp, for simultaneous monitoring of multiple signaling molecules within the same or different signaling pathways.

4. Future considerations and concluding remarks

The list of kinases tracked using FRET-based biosensors will continue to expand to meet the demands of the scientists whose favorite kinases have yet to be illuminated. Alongside the study of additional mammalian kinases, the kinases of other organisms should also be examined with this approach. Kinase biosensors can be used to gain knowledge about plant, fungal, and protozoan signaling, as well as that of bacteria and viruses, leading to identification of new drug targets and new opportunities for therapy, and possibly even elucidation of the evolutionary paths taken by different species for evolving kinase regulatory networks.

The realization of *in vivo* tracking of kinase activities using these FRET-based kinase biosensors is on the horizon. Currently, *in vivo* FRET imaging primarily focuses on surgically-exposed regions of interest on larger organisms using multi-photon microscopy.[49,50] Fibred fluorescence microscopy, which uses a small-diameter fibre-optic probe to

provide real-time images, offered a promising alternative, with high-resolution for deep region imaging.[51] Probes constructed of near infrared fluorophores or with incorporation of bioluminescent moieties, as well as improved *in vivo* imaging technologies should allow the imaging of whole organisms, establishing tracking of multi-tissue/system kinase dynamics simultaneously, therefore providing a global picture of kinase function.

In conclusion, combining the unique features of genetic encodability, targetability, high sensitivity, and specificity, FRET-based kinase biosensors can provide real-time tracking of kinase signaling in native environments ranging from single cells to whole organisms with high spatial resolution. Their applications will undoubtedly continue to expand our knowledge of kinome dynamics in multiple organisms and hopefully lead to the discovery of novel and useful pharmacological agents and therapeutics.

Acknowledgements

The work of the authors is supported by the National Institutes of Health (DK073368 and CA122673), a Scientist Development Award from the American Heart Association, a Young Clinical Scientist Award from Flight Attendant Medical Institute, 3 M, and the W.M. Keck Center (to J. Z.). We would like to thank Qiang Ni for helpful discussions and other lab members for critical reading of the manuscript.

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