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Critical Review

Dynamic Visualization of Signal Transduction in Living Cells: From Second Messengers to Kinases

Katie J. Herbst[1], Qiang Ni[1], and Jin Zhang[1,2]

[1]*Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, USA*

[2]*The Solomon H. Snyder Department of Neuroscience and Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA*

Summary

The study of signal transduction, or the highly regulated series of biochemical events which allow a cell to convert a given stimulus into a functional response, has seen a paradigm shift with a recent explosion in the number of genetically encoded FRET-based biosensors capable of detecting spatial and temporal regulation of various signaling events in living cells. The two classes of biosensors discussed, namely kinase activity and second messenger biosensors, utilize two fluorescent proteins (FP) suitable for FRET and convert a signaling event of interest into a conformational change in the biosensor that can be measured as a change in FRET between the two FPs. Individually, these biosensors have been used to elucidate many complex signal transduction mechanisms in various biological systems. However, it has become increasingly clear that it is often more desirable to study multiple signaling events simultaneously, allowing for precise correlation of the temporal profiles of multiple signaling molecules without the complication of cell to cell variability. With the design of spectrally distinct biosensors and new coimaging strategies, simultaneous imaging of multiple signaling events is not only possible, but has aided in mapping the intricate network of cellular signal transduction cascades. Furthermore, as aberrant signal transduction involving second messengers and kinases is implicated in numerous disease states, it is hopeful that these FRET-based biosensors and coimaging strategies can help to unravel the molecular links between altered signal transduction and certain disease states.

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Address correspondence to: Jin Zhang, 725 N. Wolfe Street, Hunterian 307, Baltimore, MD 21205, USA. Tel: (410) 502-0173. Fax: (410) 955-3023. E-mail: jzhang32@jhmi.edu

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INTRODUCTION

For cells to communicate information from their environment to both themselves and to other cells, they have developed a plethora of highly-regulated sequences of biochemical events which convert a given signal into a specific cellular response. At the heart of these ordered sequences of biochemical events, known as signal transduction pathways, are protein kinases, which constitute one of the largest gene families. All protein kinases carry out the same type of enzymatic reaction of transferring the γ-phosphate of ATP to hydroxyl groups on amino acid side chains of specific protein targets. Such phosphorylation of a target protein can alter the protein's function, activity, localization, and/or stability which are essential for proper signal propagation. Many kinases, diffusible serine/threonine kinases in particular, are often regulated by second messenger dynamics. Second messengers can be small molecules, lipids, or ions, and they frequently serve to amplify a physiological signal by regulating kinase activity. Well-known examples of second messengers include cyclic nucleotide monophosphates, diacylglycerol (DAG), phosphatidylinositol 3,4,5-triphosphate (PIP_3), and Ca^{++}. Changes in second messenger concentrations are exquisitely regulated both spatially and temporally under physiological conditions. In many cases, this regulation is also tightly coupled to spatiotemporal control of protein kinase activity.

For the purposes of achieving specific and efficient signal transduction, it is crucial that this spatiotemporal regulation of second messenger dynamics and protein kinase activity be properly regulated. If this regulation is not maintained, the cell and organism can suffer severe consequences. An incomplete list of pathophysiological states associated with aberrant kinase regulation includes cancer, neurodegenerative disorders, arthritis, autoimmune disorders, and diabetes. As such, protein kinases are emerging as a major group of therapeutic targets. In 1999, the first clinically useful protein kinase inhibitor, rapamycin, was approved for use as immunosuppressant (*1*). Since then the

number of FDA approved protein kinase inhibitors has increased steadily and there are currently around 30 candidate kinase inhibitors in clinical trials (*2*).

To dissect common signal transduction mechanisms and therapeutically target protein kinases, it is necessary to understand the spatiotemporal regulation of these enzymes in their native cellular environment where the entire signaling network can remain intact. Conventional methods of studying protein kinases, however, provide static snapshots of signal transduction cascades and are inefficient at capturing both the spatial and temporal dynamics of kinases in living cells. These issues have been circumvented with the recent development of fluorescence resonance energy transfer (FRET)-based kinase activity biosensors, which, by use of fluorescent proteins (FP), have proven to be an effective way to monitor both spatial and temporal kinase regulation in living cells (*3*). There also exist a series of FRET-based biosensors that can detect second messenger dynamics in living cells (*4*). Both FRET-based kinase activity biosensors and FRET-based second messenger biosensors have been utilized to visualize the real-time cellular responses to perturbations of a given biological system, and they have become powerful tools for monitoring signaling dynamics in living cells (*5, 6*).

Although individually, both FRET-based kinase activity biosensors and FRET-based second messenger biosensors have provided nondestructive methods to study real-time signaling events in living cells, these probes can provide much more information when they are imaged concurrently (*7*). For example, this makes it possible to establish the dynamic interactions between these signaling molecules directly in their native context. Specifically, when a second messenger biosensor is coimaged with a kinase activity biosensor, the precise kinetic correlation between second messenger dynamics and kinase activity can be obtained, thus providing insights as to how spatiotemporally regulated second messenger dynamics drive the spatiotemporal regulation of kinase activity. Coimaging of different biosensors can be implemented by a variety of means, including physically separating the biosensors into distinct subcellular locations via unique amino acid targeting sequences, or by using spectrally distinct fluorophores (*7*, *8*). Regardless of the coimaging approach taken, coimaging of FRET-based biosensors is expected to make significant contributions toward the goal of generating detailed dynamic signaling maps which depict the complexity of signal transduction in time and space in a given living system.

FRET-BASED BIOSENSORS FOR TRACKING SECOND MESSENGER AND KINASE DYNAMICS

Two fluorophores in close proximity with appropriate relative orientation can undergo FRET, or the nonradiative transfer of energy from an excited donor fluorophore to an acceptor fluorophore (*5*). All FRET-based biosensors exploit this biophysical phenomenon by translating a specific biochemical event, for example, protein phosphorylation or change in second messenger concentration, into a conformational change in the biosensor which alters the distance and/or orientation between the two fluorophores and thus the ability for the two fluorophores to undergo FRET. Importantly, whether FRET increases or decreases between the basal and stimulated states of the biosensor is biosensor-dependent (*9*).

Modular Design

Among various designs of FRET-based kinase biosensors that have been reported (*3*), the most common modular design is that of the kinase activity reporter (KAR): a kinase inducible molecular switch consisting of a kinase-specific substrate region and a phosphoamino acid binding domain (PAABD), flanked by a pair of FPs suitable for FRET (*5*) (Fig. 1A). Numerous reporters with this design exist for both serine/threonine and tyrosine kinases (Table 1). Such a modular design allows one to change only the substrate portion of the reporter to generate new biosensors that are specific for different kinases. For example, B-kinase activity reporter (BKAR) and D-kinase activity reporter (DKAR) were engineered by replacing the substrate domain in C-kinase activity reporter (CKAR) with consensus substrates for PKB and PKD, respectively (*15, 18*).

Currently, there are FRET-based second messenger biosensors to detect cellular fluctuations in Ca^{++} (*27, 28*), PIP_3 (*29, 30*), 3′,5′-cyclic adenosine monophosphate (cAMP) (*31–33*), 3′,5′-cyclic guanosine monophosphate (cGMP) (*34*), nitric oxide (*35*), and more. Many of these biosensors rely on a ligand-induced conformational change in the binding protein in order to alter the distance and/or orientation between the two FPs that flank the second messenger binding domain (Fig. 1B). If, however, there is not a large enough ligand binding-induced conformational change in the binding protein to induce a FRET change, one must come up with other strategies to induce such a change. One such strategy, which has been implemented for a PIP_3 biosensor, indicator for phosphoinositides based on Akt (InPAkt), is to incorporate a pseudoligand to the biosensor that binds to the binding domain in the absence of natural ligand binding but is displaced upon natural ligand binding, thereby generating a conformational change (*29*). In principle, this modular design can be extended to other ligands using corresponding ligand-binding domains and engineered pseudoligands.

Genetic Encodability

The most distinguishing characteristic of FRET-based biosensors that makes them powerful tools for studying signaling events in living systems is that they are genetically encoded. In other words, FRET-based biosensors are delivered to living cells, tissues, or whole organisms as DNA plasmids, and they rely on the organism to provide all of the necessary machinery to make a chimeric protein that is the functional biosensor. Consequently, delivery of the biosensors is nondestructive so that signaling dy-

Table 1
FRET-based KARs using a kinase-inducible molecular switch (phosphoamino acid binding domain and kinase-specific substrate) sandwiched between a pair of FPs suitable for FRET

Sensor	Target	FRET pair	Dynamic range[a]	Notes	2nd Messenger regulator
AKAR (*10*)	PKA	ECFP/cpVenusE172	30–40% ↑	3rd Generation	cAMP
AktAR (*11*)	Akt/PKB	Cerulean/cpVE172	25–35% ↑	Raft and nonraft targeted	PIP_3
Aktus (*12*)	Akt/PKB	CFP/YFP	10% ↑	Golgi and mitochondria targeted	PIP_3
ATOMIC (*13*)	ATM kinase	CFP/YFP	10% ↓	Histone targeted	
Aurora B Kinase Sensor (*14*)	Aurora B Kinase	CFP/YFP	50% ↑	Targeted to centromeres, chromatin, and cytosol	Ca^{++}(indirect)
BKAR (*15*)	Akt/PKB	ECFP/Citrine	10–25% ↓	Reversible	PIP_3
CKAR (*16*)	PKC	ECFP/Citrine	15–20% ↓	PM targeted is most sensitive	Ca^{++}, DAG
Crk II-based reporter (*17*)	Abl kinase	ECFP/Citrine	15–30% ↑	Observe increased Abl activity in membrane ruffles	
DKAR (*18*)	PKD	CFP/YFP	10–20% ↓	Ca^{++} plays a role in PKD activation	Ca^{++}
EGFR-reporter (*17*)	EGFR	ECFP/Citrine	25–35% ↑	Observe propagation of signal from membrane	
EKAR (*19*)	Erk	eGFP/mRFP1	10–20% ↑	Contains 72-glycine linker	
Erkus (*20*)	Erk1	CFP/YFP	10% ↓	Uses ERK-docking domain	
Phocus-2pp (*21*)	IR	CFP/YFP	15–20% ↑	Reversible	
Picchu (*22*)	Abl/EGFR	CFP/YFP	60% ↑	PM targeted and diffusible	
ROZA (*23*)	ZAP-70	CFP/YFP	15% ↓	Lipid raft targeted	
Src-reporter (*24*)	Src	ECFP/Citrine	30–35% ↓	More sensitive version with ECFP/YPet (*25*)	
Srcus (*26*)	c-Src	CFP/YFP	10–15% ↓	Can be targeted to EGFR	

[a]Dynamic range represents percentage change in emission ratios between the basal and stimulated states with an upward arrow indicating an increase in yellow to cyan emission ratio.

namics can be visualized in living systems (*36*). And for this same reason, they can be directed to subcellular regions via specific amino acid targeting sequences that are added to the biosensor. Targeting of biosensors to specific locations is important for studying the spatial regulation of signaling dynamics, and, as will be discussed in later sections, represents one proven strategy for the coimaging of FRET-based biosensors (*29, 31*).

One great advantage for targeting biosensors to subcellular regions of interest is the ability to investigate the functional roles of various signaling microdomains that are believed to exist in a cellular context but are difficult to study due to their dynamic nature and small sizes. This has recently been illustrated with an Akt Activity Reporter (AktAR) (*11*) in an effort to directly measure Akt activity within lipid rafts in real time, as well as distinguish Akt signaling between raft and nonraft regions of the plasma membrane. By independently targeting AktAR to either lipid raft or nonraft regions of the plasma membrane, Gao et al. observed differential Akt signaling in the distinct plasma membrane microdomains. Specifically, the study shows that although Akt can be activated via PDGF independently in nonraft regions, albeit not as robustly as in lipid rafts, IGF-stimulated Akt activity in nonraft regions is dependent on the integrity of lipid rafts.

Another study which illustrates the value of genetically targeting FRET-based biosensors to specific subcellular locations is by Wang, et al. in which they investigated the role that Src kinase plays in response to mechanical stimuli (*24*). Using a KAR for Src kinase and human umbilical vein endothelial cells (HUVEC) as a model system, the authors monitored the response of the Src kinase biosensor to mechanical forces. To detect Src activity in the lamellipodia, the areas on the outskirts of the cell that are important for transduction of mechanical force-induced signal, the authors targeted the Src reporter to the plasma membrane via an N-terminal plasma membrane targeting sequence. By applying a local mechanical force to a single HUVEC, the authors observed an immediate activation of Src that propagated to the edges of the cell in the opposite direction of the applied force. Furthermore, because of the membrane-targeted Src KAR, they were also able to observe protrusions of lamellipodia at the edges of the cell.

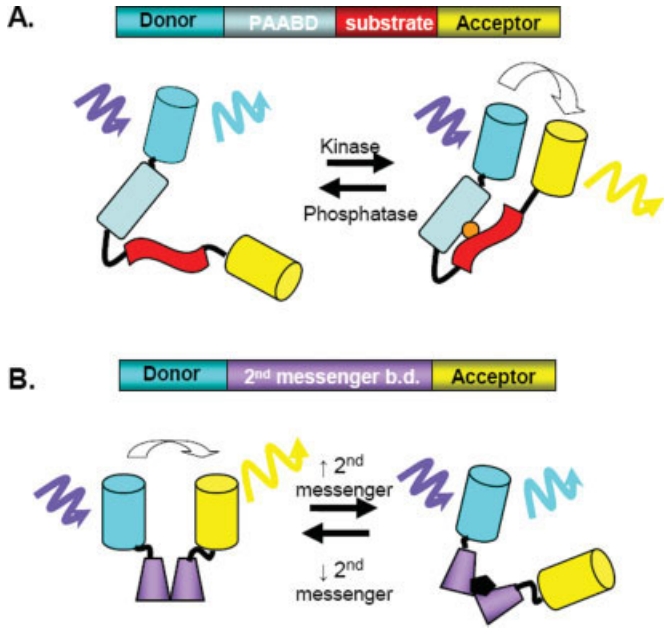

Figure 1. Modular design of genetically encoded FRET-based biosensors. (A) General design of a KAR that increases in FRET in response to endogenous kinase activation. PAABD, phosphoamino acid binding domain; substrate, kinase specific substrate; orange circle, phosphorylated amino acid. (B) General design of a FRET-based second messenger biosensor that decreases in FRET upon second messenger accumulation.

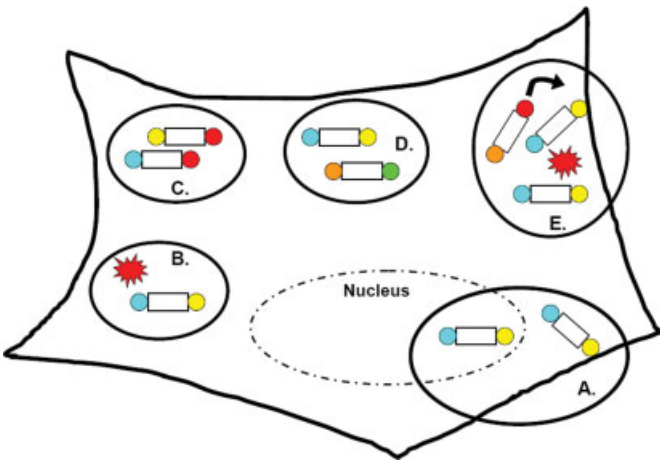

Figure 2. Coimaging strategies that can be used to monitor multiple signaling events simultaneously. (A) FRET-based biosensors that utilize identical FRET pairs can be coimaged if they are genetically targeted to distinct cellular locales such as the plasma membrane and nucleus. (B) A FRET-based biosensor can be coimaged with a spectrally distinct single fluorophore dye without the need for spatial separation. (C) Two FRET-based biosensors can be coimaged in the same cellular location if they utilize spectrally distinct energy donors and the same acceptor; CR and YR are a suitable example. (D) Two spectrally distinct FRET-based biosensors, such as mTFP/Citrine and mAmetrine/tdTomato, can be coimaged without the need for physical separation. (E) Using a combination of coimaging strategies allows for simultaneous imaging of several signaling events.

SIMULTANEOUS TRACKING OF SECOND MESSENGER AND KINASE ACTIVITY

Although genetically encoded FRET-based biosensors have been valuable tools for mapping individual signaling events, coimaging of two or more biosensors, or multiparameter imaging, offers the advantage of mapping the spatiotemporal relationship between multiple signaling events in the same cell. This is particularly desirable for elucidating the precise kinetic correlations between second messenger dynamics and kinase activity. For example, using biosensors for cAMP and PKA expressed individually in retinal neurons, Dunn et al. observed that retinal waves drive oscillations in both cAMP concentration and PKA activity (*37*). However, because the cAMP and PKA oscillations were observed in separate experiments, the precise correlation between the cAMP oscillations and the PKA oscillations was not determined. The remainder of this section will discuss the challenges associated with multiparameter fluorescence imaging and some of the strategies that can be implemented to circumvent these challenges.

Challenges Associated with Coimaging

The most confining limitation of multiparameter fluorescence imaging is the limited number of spectrally distinct fluorophores in the visible range that can be utilized in fluorescence-based biosensors (*7*). For the specific case of genetically encoded FRET-based biosensors, this limitation is even more restricting for two reasons: first because each biosensor requires two FPs, and second because most FPs have relatively broad excitation and emission spectra, although this can be partly alleviated with appropriate excitation and emission filters. Despite this, coimaging two FRET pairs that utilize four spectrally distinct FPs has been demonstrated recently (*38*). As a result, researchers can now apply a number of coimaging techniques, and in some cases a combination of such techniques, to coimage two or more signaling events in the same cell (*39*). The coimaging strategy implemented generally depends on the biological questions being asked. Different strategies for multiparameter fluorescence imaging will be discussed in the following subsections.

Coimaging of Signaling Activities via Physical Separation of the Biosensors

One strategy to coimage FRET-based biosensors is to physically separate the two probes within the same cell via distinct subcellular targeting sequences (Fig. 2A). Because the two bio-

sensors will be in different regions of the cell, this strategy can be used with biosensors that are spectrally similar. This coimaging strategy is useful if the two signaling events of interest occur in distinct subcellular locations, or when asking questions about how a signaling event at one cellular location affects signaling elsewhere in the cell. For instance, DiPilato et al. were interested in understanding the precise temporal correlation between cAMP accumulation at the plasma membrane and PKA activity in the nucleus (*31*). To study this, they targeted the FRET-based Indicator of cAMP Using Epac (ICUE) to the plasma membrane and coexpressed this biosensor with nuclear localized A kinase activity reporter (AKAR). Both biosensors utilize CFP/YFP (CY) FRET pairs, but they can be coimaged because they are physically separated in the cell. Upon activation of β2-adrenergic receptors, an immediate accumulation of cAMP at the membrane was observed. In response to the same treatment, however, nuclear targeted AKAR did not respond until 5–10 min after a sustained accumulation of cAMP at the membrane. On the other hand, using a similar coimaging approach, cAMP accumulation in the nucleus was shown to be almost as rapid as at the membrane. This data suggests that a relatively slow translocation of the catalytic subunit into the nucleus after the activation of the cytosolic PKA holoenzyme provides the temporal control of receptor-mediated nuclear PKA signaling.

Coimaging via physical separation of biosensors was also applied to simultaneously track PIP_3 production at the plasma membrane and Akt (or PKB) activity in the nucleus. To do this, a plasma membrane targeted InPAkt was coimaged with a nuclear-targeted BKAR (*29*). In this case, a rapid yet transient increase of PIP_3 at the membrane was observed, and this response was followed by a delayed and gradual BKAR response in the nucleus. This data suggests that Akt can remain active as it translocates from the membrane to the nucleus, even as PIP_3 accumulation at the plasma membrane diminishes.

By exploiting the genetic targetabilty of FRET-based biosensors, these studies illustrate the ease with which two FRET-based biosensors that utilize spectrally similar FPs can be coimaged when they are physically separated. Because many current FRET-based biosensors utilize CFP and YFP as the FRET pair, this strategy can be implemented easily with existing biosensors.

Coimaging FRET-Based Biosensors via Spectrally Distinct Fluorophores

To fulfill the spectral requirement of FRET, the emission spectrum of the energy donor must sufficiently overlap the excitation spectrum of the energy acceptor. However, to avoid spectral bleed-through in filter-based fluorescence microscopy, the fluorescence properties of the two fluorophores must be sufficiently distinct. Unfortunately, because the emission spectra of most FPs frequently span up to 100 nm, it is difficult to find two combinations of FPs for coimaging that can both function as FRET pairs and avoid spectral bleed-through (*7*). There are a number of ways around this problem such as: coimaging a FRET biosensor with a spectrally distinct single-fluorophore biosensor (*16*) (Fig. 2B), coimaging two FRET pairs that use distinct donors and a common acceptor (*40*) (Fig. 2C), and utilizing FPs that have unique excitation and emission properties that make them suitable as FRET partners yet distinguishable from the other FPs in a filter-based setup (*38, 39*) (Fig. 2D).

Coimaging a FRET-Based KAR with a Spectrally Distinct Single-Fluorophore Second Messenger Biosensor. Using a plasma membrane targeted CKAR (MyrPalm-CKAR), Violin, et. al. observed oscillations in histamine-induced PKC activity, and hypothesized that these oscillations correlated with the well characterized cytoplasmic histamine-induced Ca^{++} oscillations in HeLa cells (*16*). To study this, they coimaged MyrPalm-CKAR, a CY biosensor, with the Ca^{++} indicator Fura red, a fluorescent dye that is excited near 410 nm, emits near 610 nm, and increases in fluorescence upon Ca^{++} binding. By coimaging these two biosensors, the authors observed that regardless of the characteristics of the Ca^{++} oscillations in the cytoplasm, MyrPalm-CKAR displayed oscillations that were coupled to the Ca^{++} oscillation pattern, with a 10–15 sec delay.

Kunkel et al. used a similar coimaging strategy by imaging cytosolic BKAR, a CY biosensor, with a spectrally distinct single fluorophore biosensor, an RFP-tagged PH domain of Akt, which translocates from the cytoplasm to the plasma membrane upon PIP_3 binding to serve as a readout for PIP_3 production (*15*). By concurrently imaging these two biosensors, it was shown that PIP_3 production at the plasma membrane precedes Akt activity in the cytosol, revealing a rapid succession of events from PIP_3 production, to Akt activation, to phosphorylation of substrates by active Akt.

In practice, there are a few caveats associated with this particular coimaging strategy. First, single fluorophore imaging is more susceptible to sample variation, and it is therefore important to keep expression levels of the biosensor similar between samples (*8*). Second, in the specific case of coimaging Fura red with a CY biosensor, care should be taken to limit the amount of Fura red loaded because the Fura red emission profile overlaps that of CY FRET (*16, 39*). When these considerations are properly accounted for, coimaging of a FRET biosensor and a single-fluorophore biosensor can be a powerful coimaging technique.

Coimaging Dual FRET-Based Biosensors. As aforementioned, coimaging two FRET-based biosensors is limited by the number of spectrally distinct FPs that can be used as functional FRET pairs. One strategy to overcome this limitation is to coimage two biosensors that utilize spectrally distinct donors with a common acceptor. Because it has been recently shown that CFP can be an energy donor for RFP variants, and because YFP is an established donor for RFP, it is possible to generate functional CFP/RFP (CR) and YFP/RFP (YR) FRET biosensors. Further, these probes will be suitable for coimaging because CFP and YFP have sufficiently distinct excitation properties and there

will be minimal contamination of the two FRET channels. For example, FRET-based biosensors for caspase activities using these FRET pairs have been constructed and imaged simultaneously in cells with minimal signal contamination (*40*). This serves as a proof of principle that a CR and a YR FRET-based biosensor can be used for coimaging of two distinct signaling events in the same cell. However, for FRET-based second messenger and kinase biosensors, an important challenge is to develop CR and YR FRET-based biosensors that have sufficient dynamic range to track often subtle but physiologically relevant changes of signaling activities. In this regard, a CR AKAR and YR ICUE have been developed with emission ratio changes up to 50 and 60%, respectively. Furthermore, coimaging of these two biosensors has been achieved to track cAMP dynamics and PKA activity concurrently in single living cells (Allen M.D. et al., unpublished). Because this CR/YR coimaging strategy has already proven successful, it could immediately impact studies such as the one by Dunn et al. (*37*) where, instead of looking at cAMP and PKA dynamics independently, the two events could be simultaneously monitored and the spatiotemporal relationship of cAMP and PKA, elucidated.

As an alternative to coimaging FRET pairs with a shared acceptor FP, recent efforts to engineer new FPs with distinct spectral properties have made it possible to coimage two FRET biosensors that utilize four different FPs. For example, Ai et al. engineered a GFP mutant, mAmetrine, with a long Stokes-shift, or the shift between fluorophore absorption and emission maxima, that was used to obtain two distinct coimageable FRET pairs (*38*). Specifically, mAmetrine was found to be a suitable FRET donor for the orange fluorescent protein (OFP), tdTomato, and this FRET pair was coimaged with another FRET pair using the monomeric teal FP, mTFP1, as the energy donor for the acceptor YFP mCitrine. They incorporated these two spectrally distinct FRET pairs into biosensors of caspase activity and demonstrated that indeed these two pairs can be coimaged in HeLa cells to detect the onset of apoptosis. However, despite using carefully chosen filter sets, there still exists some degree of cross-excitation between the donors; most noticeably, the filter set used to excite mAmetrine also results in 14% excitation of mTFP1. As a result, simple correction factors have been derived to obtain corrected mAmetrine and mCitrine intensities based on the fact that both mAmetrine and mCitrine use the same emission filter set. It is worth to point out, however, that this corrected mCitrine intensity also includes contribution from the cross-excitation of mCitrine using the mTFP1 excitation filter set. In principle, this value is different from the value that should be used to calculate the corrected mAmetrine intensity although in practice the difference is most likely small and therefore inconsequential. For additional information regarding the degree of spectral overlap between these and other coimageable FRET pairs, see the recent review by Carlson and Campbell (8).

Coimaging more than Two Biosensors via a Combination of Coimaging Strategies. mKO, an OFP variant, was originally introduced as a FRET acceptor for the CFP, MiCy (*41*). Recently, however, it was shown that mKO can also be used as a FRET donor for mCherry. Utilizing this information, Piljic and Schultz hypothesized that such an orange/red (OR) FRET biosensor could be coimaged with a standard CY biosensor (*39*). To demonstrate this, they generated an OR biosensor (ORNEX4) that detects Ca^{++}-dependent annexin A4 association with the plasma membrane. In this study, they demonstrated that OR- and CY- FRET biosensors can be coimaged in the same subcellular location with sufficient signal separation. Moreover, by combining this coimaging technique with physical separation of two CY-biosensors, one at the plasma membrane and one in the cytosol, and by adding Fura red to the equation, they were able to image four signaling events simultaneously in a single cell. Although the biosensors used in this study were not used for simultaneous tracking of second messenger and kinase dynamics, in principle, these FPs can certainly be incorporated into current second messenger biosensors and KARs to test them for such applications (Fig. 2E).

CONCLUDING REMARKS

The development of FRET-based KARs and second messenger biosensors in the last decade has led to a paradigm shift in the study of signal transduction, specifically, by enabling the study of endogenous signaling dynamics in living systems. Further, via the engineering of new FPs with unique spectral properties and the development of new dual-imaging strategies, these biosensors can be used concurrently as a method to monitor the precise relationships between multiple signaling events. Using these biosensors to dissect discrepancies between signaling dynamics in normal and various disease states could provide further understanding of the underlying molecular mechanisms and ultimately lead to new therapeutic agents that target protein kinases.

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