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Multiplexed imaging of signaling networks by genetically encoded, fluorescent protein-based biosensors

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Abstract

Signal transduction processes are a necessary component for multicellular life, and their dysregulation is the basis for a host of syndromes and diseases. Thus, it is imperative that we discover the complex details of how signal transduction processes result in specific cellular outcomes. One of the primary mechanisms of regulation over signaling pathways is through spatiotemporal dynamics; However, traditional methods are limited in their ability to reveal such details. To overcome these limitations, researchers have created a host of genetically encodable, fluorescent protein-based biosensors to study these dynamic processes in real time in living cells. Due to the complexities and interconnectedness of signaling pathways, it is thus desirable to use multiple biosensors in individual cells to better elucidate the relationships between signaling pathways. However, multiplexed imaging with such biosensors has been historically difficult. However, recent developments in designs and multiplexing strategies has led to vast improvements in our capabilities. In this review, we provide perspectives on the recently developed biosensor designs and multiplexing strategies that are available for multiplexed imaging of signal transduction pathways.

Keywords

biosensor; signal transduction; multiplexed imaging; fluorescent protein; signaling

Introduction

For life to function, cells need to be able to sense and respond to their environment. Furthermore, multicellular life requires communication between cells, tissues, and organs. These critical sensing and communication abilities occur through complex, tightly controlled signal transduction circuits that enable individual cells, tissues, and organs to respond appropriately to stimuli and meet the needs of the organism. When these pathways become dysregulated, illnesses such as cancer, diabetes, cardiovascular disease, and neurodegenerative disorders may develop[1–3]. This fact underscores the critical need to be able to understand these complex circuits in order to prevent and treat disease and improve human health.

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Traditional methods to study these pathways, such as immunohistochemical and *in vitro* assays, have provided significant insight into signal transduction mechanisms, but they are vastly limited in providing quantitative spatiotemporal and reflect the population average, which may not reflect the heterogenous responses of individual cells[4].

Since the advent of fluorescent protein (FP) utilization to study biological processes[5,6], there has been an explosion of new FPs and the creation of genetically encodable, FP-based biosensors: engineered proteins that alter their fluorescence properties in response to a given trigger, which we have recently reviewed in detail[7]. A major advantage of using biosensors to study signal transduction networks is that these tools can illuminate signaling processes in real time, vastly increasing our ability to determine the strict spatiotemporal dynamics of specific signaling pathways. Furthermore, biosensor imaging allows researchers to study these signaling events in the context of single living cells, resolving previously unseen details such as spatiotemporal dynamics or cell heterogeneity.

Thus far, individual biosensors are often used in isolation. While this approach has led to a richer and deeper understanding of signal transduction networks, signaling pathways do not operate in seclusion. When these FP-based biosensors are used to study isolated processes, researchers are unable to discern the interrelationship between two or more pathways, such as whether they are synergistic, antagonistic, upstream, or downstream from one another. Thus, there is much to be gained by using multiple biosensors to simultaneously examine multiple pathways in the same cell, as this will yield a richer understanding of these complex signal transduction circuits.

Multiplexed imaging is an advantageous approach to study these important signaling networks, but it is not without its own caveats. The challenge of multiplexed imaging with biosensors arises from two important facts: the visible spectrum is a finite resource, and FPs have relatively large spectral footprints (Figure 1). These two facts mean that attempting to multiplex with multiple FPs can easily lead to overlapping spectra, masking our ability to simultaneously detect multiple signaling events. However, the unique advantages of genetically encodable biosensors have led researchers to continually develop creative methods to bypass the issue of spectral overlap and reduce the spectral footprints of current imaging techniques. Here, we first provide a general overview of common biosensor designs, followed by a discussion of several methods that have been developed to enable multiplexed imaging of fluorescent biosensors.

Overview of Biosensor Design

Extensive efforts have been made to develop FP-based biosensors in order to visualize signal transduction activities in real time in living cells. These FP-based biosensors all share a similar overall design, in which one or more FPs serve as a “reporting unit” in conjunction with a “sensing unit” derived from endogenous protein sequences that detects a specific signaling event (Figure 2), such as second messenger binding or phosphorylation by a specific kinase. Coupling of the sensing and reporting units allows the detection of a signaling event to induce a change in the fluorescence readout from the biosensor, and how this coupling is implemented defines the nature of the fluorescence change.

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Modulating FRET between a pair of FPs.

Förster resonance energy transfer (FRET) is a photophysical phenomenon where an excited "donor" FP nonradiatively transfers its excited-state energy to a nearby "acceptor" FP via dipole-dipole interactions (see Greenwald et al.[7], Newman et al.[8], and Cardullo et al.[9] for a more detailed overview). The efficiency of this nonradiative energy transfer is strictly dependent on distance: only when the two fluorophores are in close proximity (<10 nm) will FRET occur[10]. Thus, FRET-based biosensors can be generated by fusing a pair of FPs capable of undergoing FRET to a molecular switch that changes its conformation in response to a particular signaling event (Figure 2A). Changes in the conformation of the switch modulate the proximity of the FPs, thereby altering FRET. Experimentally, this can be monitored by measuring the ratio of donor-sensitized acceptor fluorescence (i.e., acceptor emission following donor excitation) to donor-direct fluorescence, providing a quantitative, dynamic readout of biochemical activity[10]. FRET-based biosensors have been used extensively to measure a variety of different biochemical processes, from the dynamics of second messengers such as Ca^{2+} and cAMP to enzymatic activities such as kinase/phosphatase dynamics[7]. For example, the Protein Kinase A kinase activity reporter (AKAR) utilizes cyan and yellow FPs on the N-terminal and C-terminal ends of the biosensor, with a PKA-specific substrate sequence and a phospho-amino acid binding domain (PAABD) sandwiched in between[11]. Upon increases in PKA activity, PKA will phosphorylate the substrate sequence, leading to interactions with the PAABD. This phosphorylation-induced intramolecular interaction causes the two FPs to come into close proximity, which increases FRET (Figure 2A). This modular design has proven extremely versatile and has been extended to measure a variety of different enzyme activities beyond kinases[7].

Modulating ddFP proximity.

Another biosensor design strategy takes advantage of the native and obligatory dimerization of RFPs. These single-color biosensors make use of two variants of the same FP that must dimerize in order to emit fluorescence. Alford et al. generated the first ddFP through rigorous molecular evolution to create an RFP pair where one FP has an intact chromophore but is quenched in the monomeric state[12] (Figure 2B), while the second FP does not contain an intact chromophore but relieves the quenched state of the first monomer upon dimerization. With this clever FP pairing, Alford and colleagues designed biosensors for Ca^{2+} and caspases. The Ca^{2+} biosensor is a split version of the cameleon FRET sensor, in which one ddFP is linked to the N-terminal end of calmodulin and the other ddFP is linked to the M13 peptide, which will interact with calmodulin after Ca^{2+} binding, thereby bringing the two ddFPs together to increase fluorescence. The caspase sensor, however, is designed such that upon caspase activation, a linker between the two ddFPs is cleaved, leading them to dissociate and decrease fluorescence.

This biosensor design has recently been utilized to develop additional signaling biosensors. For example, Ding et al.[13] and Mehta et al. [14] used ddFPs to create new FRET-inspired kinase sensors with a much smaller spectral footprint. Specifically, they created ERK biosensors by replacing the FPs in a traditional FRET-based biosensor with a ddFP pair, so that upon phosphorylation of the biosensor the ddFPs will come together (Figure 2B).

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Recently, Kim et al.[15] also created new GTPase biosensors with ddFPs, in which a small GTPase of interest is tagged with one of the ddFPs and an effector sequence is tagged with the other ddFP; the effector sequence will only bind to the GTPase in the GTP-bound state. Thus, the two ddFPs only dimerize and increase in fluorescence when GTP is bound and the GTPase is active.

Modulating the fluorescence of a single FP.

Conceptually, single -FP -based biosensors are similar to FRET based biosensors in that upon activation of the switch, there is a change in the fluorescence properties of the FP. However, these biosensors have a unique design that relies on the switch's ability to modify FP fluorescence. For example, by directly inserting the switch domains within the FP, changes in the switch conformation upon detection of a signaling event will change the fluorescence readout (Figure 2C). While single-fluorophore biosensors with an incorporated molecular switch are extremely advantageous for multiplexed imaging, the engineering of these biosensors can be more complex. Nevertheless, the unique advantage of only utilizing a single FP per biosensor and the large dynamic range observed for many of these single-fluorophore biosensors has spurred greater efforts to expand their development.

One strategy for incorporating a switch into a single FP is to insert the sequence for the switch domain directly within the sequence of the FP, as was done for the cAMP biosensor Flamindo[16,17]. In this design, the cAMP binding protein EPAC is inserted into a YFP variant, Citrine[18], such that under conditions of low cAMP, the EPAC domain is compact, allowing the beta-barrel of the FP to fully close, making the biosensor fluorescent. However, when cAMP binds to EPAC, the domain undergoes a conformational shift that perturbs the beta barrel structure, leading to a decrease in fluorescence.

Another approach to couple a switch to a FP is to insert a circularly permutated FP (cpFP) into a bipartite molecular switch. Circular permutation rearranges the linear sequence of a polypeptide to shift the positions of the N-and C-termini while maintaining the native structure of the folded peptide[19]. Thus, the termini of the FP are now within the beta-barrel itself, making it possible to insert a switch and allow modulation of the FP beta barrel, thereby controlling fluorescence. This was done to develop GCaMP, one of the earliest single-FP biosensors for Ca^{2+}[20]. Nikai et al. fused calmodulin at the C-terminus of cpEGFP, with the M13 peptide connected to the N- terminus. Ca^{2+} binding to the calmodulin domain induces interactions between calmodulin and the M13 peptide, which results in the closing of the beta barrel of cpEGFP and an increase in fluorescence (Figure 2C).

Inducing FP translocation.

The simplest biosensors are those where a protein or a functional domain is fused to a single FP. Although this design does not involve conformational changes in the sensing domain or changes in the biophysical behavior of the FP, translocation-based biosensors have proven valuable in signal transduction studies. A couple of key examples include the tagging of kinases, such as ERK, to monitor kinase translocation to and from the nucleus[21], or connecting the PH-domain from Akt to an FP to monitor phosphatidylinositol (3,4,5)-

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triphosphate (PIP3) accumulation at the plasma membrane[22]. In this design, the FP translocates to the site at which the signaling activity is occurring. A more recent approach, however, has been to create translocation reporters in which the FP is relocalized to a predetermined location in response to signaling activity. For example, Regot et al exploited short sequences containing an NLS and an NES in conjunction with a kinase-specific substrate sequence, designed in such a way that phosphorylation disrupts the NLS and activates the NES, causing the sensor to translocate from the nucleus to the cytoplasm (Figure 2D)[23]. Regot et al also highlighted the generalizability of this approach by developing kinase translocation reporters (KTRs) for ERK, p38, JNK, and PKA.

While translocation-based biosensors yield excellent temporal resolution, spatial resolution is ultimately limited as a consequence of the design. These sensors rely on translocation from one region of the cell to another as their readout, and thus they cannot be modified with additional localization sequences to assay changes in kinase activity in specific subcellular regions such as the Golgi apparatus, mitochondria, or plasma membrane.

Multiplexing Strategies

Imaging multiple FRET-based biosensors

While FRET-based biosensors have been indispensable in signal transduction research, the presence of two distinct FPs in a FRET biosensor consumes a large portion of the available spectral space, limiting their utility for multiplexed imaging. In order to utilize these well-established biosensors for multiplexed imaging, researchers have developed clever techniques to bypass this hurdle, such as developing and using orthogonal FRET pairs, taking advantage of fluorescence lifetime imaging, or developing biosensors based on FRET between identical FPs, known as homo-FRET.

Orthogonal FRET pairs.—Commonly utilized FRET pairs are green-red (GR) and cyan-yellow (CY). However, GFP and YFP have considerable spectral overlap, eliminating the possibility of imaging these two common FRET biosensors together (Figure 1A). We previously reported a technique to obtain orthogonal FRET pairs that significantly reduces spectral cost[24]. In this method, two different biosensors have the same FRET acceptor (e.g., RFP) but spectrally distinct donors. By sequentially acquiring each donor-acceptor pair (e.g..,. excite CFP and immediately read red and cyan fluorescence, then excite YFP and read yellow and red fluorescence,) one can independently monitor changes of the two FRET sensors. This way, the spectral footprint is reduced to three FPs. The ability of both CFP and YFP to transfer energy to RFP allowed us to create a YR-based cAMP biosensor and a CR-based PKA biosensor; we were then able to demonstrate that cAMP concentrations and PKA activity can be uncoupled, in that PKA activity remains sustained despite a transient cAMP increase. One of the advantages to this technique is that it doesn’t require special filters that wouldn’t already be found in a common epifluorescent microscope set up to detect GFP, CFP, YFP, and RFP. However, this technique is limited on the total number of processes that can be monitored simultaneously, since the excitation/emission regions from three FP preclude the visible light portion of the spectrum from being used for other biosensors.

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It is also possible to choose alternate FRET pairs that minimize spectral overlap between FPs, allowing imaging of two completely orthogonal FRET biosensors in the same cell. For example, the use of CY-FRET biosensors with Orange-Red (OR) FRET biosensors has worked for multiplexed imaging[25]. However, the spectral differentiation between these pairs is still narrow (Figure 1B). So, while this approach is possible, it is still not ideal for multiplexed imaging.

Researchers have thus worked to create orthogonal FRET pairs that exhibit even greater spectral separation. For example, Shcherbakova et al recently developed a highly red-shifted FRET-pair consisting of the near-infrared (nIR) FPs miRFP670 and miRFP720, thereby allowing them to construct a biosensor that shows excellent spectral separation from the standard CY FRET pair[26] (Figure 1C). Using this new FRET pair, Shcherbakova and colleagues created a nIR-FRET-based Rac1 GTPase biosensor that they were able to pair with a CY-FRET-based biosensor for the GTPase RhoA, enabling them to detect antagonism between these two GTPases in migrating cells. Furthermore, they were able to pair their nIR Rac1 biosensor with a CY-biosensor for Rac1-GDI (G-nucleotide dissociation inhibitor) binding and uncovered that Rac1 activation at the leading edge directly followed binding and release of Rac1 by GDIs. Finally, they demonstrated the generalizability of this nIR FRET pair by developing nIR biosensors for JNK and PKA kinase activities.

Fluorescence-lifetime imaging microscopy (FLIM).—All fluorophores exhibit a characteristic lifetime that is dependent on the availability of both radiative and nonradiative paths for the decay of excited-state energy[27,28]. When two FPs are undergoing FRET, the donor depletes its excited state more rapidly through non-radiative energy transfer to the acceptor, thereby shortening the donor fluorescence lifetime. Thus, a powerful method by which FRET-based biosensors can be imaged is by fluorescence-lifetime imaging microscopy (FLIM), which involves directly measuring the donor fluorescence lifetime instead of the intensity of sensitized emission by the acceptor (Figure 3). By eliminating the need to monitor acceptor emission, FLIM offers a major advantage over intensity-based FRET measurements[29] and allows the possibility to use non-emitting, or “dark”, acceptor FPs thereby increasing the spectral space available for multiplexing[30–32]. This was recently illustrated by Demeautis et al.[33], who constructed a FLIM-FRET ERK activity reporter (EKAR) by pairing mTFP with the dark acceptor ShadowG[30], as well as an AKAR variant containing the long-Stokes-shift FP LSSmOrange[34] paired with mKate2 [35]. Each donor exhibits a characteristic fluorescence decay time in response to pulsed 440 nm excitation. Upon changes in ERK and PKA activity come alterations in FRET between the donor and acceptor FPs, resulting in detectable changes in the fluorescence lifetime of each acceptor. Using this approach, the authors were able to simultaneously measure ERK and PKA activity in response to EGF in HeLa cells. By using dark acceptors in conjunction with donors that are mutually excited by the same laser, Demeautis and colleagues were able to simplify microscope setup. However, this simplification does not detract from the drawback of using FLIM since it requires specialized instrumentation to detect fluorescence decay[36].

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FRET between identical fluorophores (homo-FRET).—A key requirement for FRET is overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor (Figure 4A). While this is commonly implemented using FPs with distinct spectra (hetero-FRET), FRET is also possible between a spectrally identical donor and acceptor (homo-FRET) because individual FPs often exhibit sufficiently overlapping excitation/emission spectra (Figure 4B). Thus, using homo-FRET based biosensors would free up much of the visible spectrum because each biosensor contains FPs in only one color. However, since the donor and acceptor are spectrally identical, they can't be distinguished via emission intensity or lifetime. Thus, homo-FRET sensors must be imaged using a different fluorescence property: polarization.

Given their size, FPs rotate very little during the lifetime of the excited state[22]. Thus, when excited using plane-polarized light, an FP will emit light that is polarized in the same direction as the incident illumination. During FRET, however, the acceptor FP is unlikely to be in the same orientation as the donor, resulting in a loss of polarization of the emitted light. Thus, homo-FRET can be measured by monitoring the polarization of the fluorescence emission via fluorescence anisotropy imaging, whereby an increase in FRET corresponds to a decrease in anisotropy (Figure 4C). This approach was previously explored by Warren et al., who used the PH domain of Akt fused to the red FP mCherry to monitor the accumulation of 3'-phosphoinositides at the plasma membrane[22]. In this case, homo-FRET occurs as a result of molecular crowding among the mCherry-containing sensor molecules at the membrane, providing a quantitative readout of phosphoinositide accumulation in addition to biosensor translocation. The authors were then able to perform multiplexed imaging by simultaneously measuring changes in cytosolic calcium concentrations using the CY-FRET based calcium sensor TN-L15 [37].

More recently, we reported an expansion of homo-FRET-based biosensor imaging by developing a suite of biosensors for monitoring kinase and second messenger dynamics [38]. We developed a family of homo-FRET biosensors, termed fluorescence anisotropy reporters (FLAREs) for PKA, ERK, PKC, myosin light chain kinase, Ca^{2+}, and cAMP. Using these homo-FRET biosensors, we were able to demonstrate multiplexed imaging under several different cellular conditions and stimuli. Of particular interest, we were able to detect synchronized oscillations of Ca^{2+} and cAMP in the MIN6 pancreatic β-cell line, and we were also able to simultaneously detect Ca^{2+} and PKA activities *in vivo* in skeletal muscle of mice. Additionally, we demonstrated three-parameter imaging of PKA activity, ERK activity, and Ca^{2+} release in HEK293 cells. Thus, major advantages of this technique include the ability to measure up to three parameters simultaneously, compatibility with *in vivo* imaging, and the availability of several available colors with common laser or filter sets; however, the microscope would need to be equipped with the ability to measure polarized light.

Imaging multiple single-fluorophore biosensors

Using single-fluorophore and single-color strategies maximizes the possibilities for simultaneous imaging of multiple signaling activities in individual cells due to the relatively minimal amount of the electromagnetic spectrum occupied by each biosensor, making it

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much more feasible to perform multi-parameter imaging than when using FRET-based biosensors. However, one caveat with many of the single fluorophore biosensors is that they are intensiometric in contrast to ratiometric, and intensity-based sensors are more prone to imaging artifacts[39]. Despite this caveat, recently reported single-FP biosensors are proving to be an invaluable resource to probe the workings of signal transduction through multiplexed imaging.

A recent example of two-parameter imaging with single-color biosensors is the ddFP-based GTPase biosensors created by Kim et al. In their report, they successfully used a red H-Ras biosensor with a green Rac1 sensor in randomly migrating cells [15]. In this study, Kim and colleagues identified that both GTPases were active at the leading edge of cells, but that the Ras and Rac1 biosensors had slightly different spatial localizations. In another example of two-parameter imaging with single fluorophore biosensors, Harada and colleagues were able to perform successful *in vivo* multiplexed imaging of Ca^{2+} and cAMP dynamics in cerebral cortical astrocytes by simultaneously imaging G-GECO with their red-fluorescent cAMP indicator Pink Flamindo, a red version of the single-fluorophore cAMP biosensor described above [40].

There have been a handful of reports where researchers used single-fluorophore biosensors for three-parameter imaging. For example, Zhao et al. utilized directed evolution combined with bacterial screening to develop additional Ca^{2+} indicators in a variety of colors, including green (G-GECO), blue (B-GECO), and red (R-GECO) [41]. This effort enabled them to simultaneously image local Ca^{2+} dynamics in the cytoplasm, nucleus, and mitochondria in single HeLa cells, revealing compartment-to-compartment variations in Ca^{2+} signaling in individual cells. Furthermore, they demonstrated that these new Ca^{2+} sensors can be used in conjunction with other biosensors, such as the FRET-based Ateam1.03 biosensor[42] to simultaneously monitor Ca^{2+} and ATP dynamics.

Among single-fluorophore sensors, the KTR biosensor design is also suited for three-way multiplex imaging. For example, Regot et al in 2014 successfully measured the temporal dynamics of JNK, p38, and ERK in 3T3 cells treated with anisomycin, an inhibitor of eukaryotic protein synthesis [23]. In these experiments, Regot and colleagues discovered that p38 inhibition led to an increase in JNK and ERK activities and that p38 activation by anisomycin altered ERK activity fluctuations rather than inducing an overall decrease in ERK activity. In a more recent example, Maryu et al. used an mKusabira Orange [43]-tagged ERK KTR in conjunction with their own eGFP-tagged Akt KTR and an mCherry-tagged fluorescent cell-cycle indicator [44]. Although they relied on computationally heavy linear unmixing with these sensors, Maryu and colleagues discovered a strong correlation between the cell cycle and ERK and Akt cooperativity in response to EGF.

We recently reported another example of three-parameter imaging employing novel single-fluorophore biosensors containing a cpFP and a kinase-dependent molecular switch [14]. Upon inserting a PKA substrate and PAABD into cpEGFP, we found that the modified EGFP chromophore appeared to exhibit both the neutral and anionic states observed in wtGFP [6]. Interestingly, phosphorylation of the substrate domain, leading to interactions between the substrate and PAABD, shifts the chromophore ionic state such that there

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is a change from 400 nm to 488 nm excitation peaks. Thus, we were able to observe a greater than twofold increase in kinase activity by taking the ratio of 488/400-excited fluoresce intensity in response to stimuli. We demonstrated the generalizability of this cpEGFP-based PKA biosensor design, termed ExRai-AKAR, by also creating biosensors for monitoring PKC and Akt activities. Notably, we were able to utilize the ExRai-AKAR in three-parameter imaging in primary neurons in combination with a ddFP-based ERK sensor (RAB-EKAR-EV) and blue Ca^{2+} sensor (BCaMP)[45].

In the same report, we also generated teal and blue intensiometric biosensors for monitoring PKA and PKC activity based on cp-T-Sapphire and cpBFP. Combining these probes with a previously developed red Ca^{2+} indicator, RCaMP[45], and the yellow cAMP indicator Flamindo2 [17] then enabled us to successfully perform four-parameter multiplexed imaging, in which we simultaneously monitored PKA, cAMP, PKC, and Ca^{2+} dynamics in the cytoplasm of single HeLa cells.

Maximizing the possibilities by combining designs and approaches

Above, we have described several techniques and designs for studying signal transduction processes through multiplexed imaging of biosensors. However, each technique or design need not be utilized in isolation. For example, Ohta et al. combined two single-fluorophore biosensors with a FRET-based ATP biosensor in MIN6 cells[46]. Specifically, they used the B-GECO1[41] Ca^{2+} sensor and the ATeam 1.03 ATP sensor[42] together with a new cAMP biosensor that they developed, known as R-FlincA. In this study, they observed extreme cellular heterogeneity in response to glucose, an observation that wouldn't have been possible with traditional population-level studies.

In another example, Piljic et al. used subcellular targeting of biosensors to perform three-parameter imaging of FRET-based probes[47]. Specifically, they used a plasma membrane localized CY FRET-based biosensor for PKC, a cytosolic-localized CY-FRET sensor for CaMKII, and an OR FRET-based translocating biosensor for Annexin A4. In order to verify the ability to differentiate between the two CY probes, Piljic and colleagues pretreated cells with a PKC inhibitor prior to ionomycin treatment and observed that ionomycin no longer induced a FRET change near the plasma membrane. Thus, to measure the three parameters, they selected regions of interest in the cytoplasm for CaMKII, at the membrane for PKC, and around both cytosol and membrane for monitoring the Annexin A4 FRET-based biosensor. To push the limits of multiplexed imaging, Piljic and colleagues then incorporated a Ca^{2+} indicator dye, Fura Red. Although the dye had significant bleed through with the mCherry in the Annexin A4 probe, the authors were still able to measure Annexin A4 accumulation at the membrane by tracking mOrange translocation from the cytosol to the membrane, thereby achieving four-parameter imaging with three FRET-based probes and a synthetic dye.

Using the cpFP based kinase biosensors we recently developed, we were also able to use the subcellular targeting approach to simultaneously image six different activities (Figure 5)[14]. Specifically, we monitored plasma membrane and nuclear PKA and ERK activities using targeted variants of sapphireAKAR and RAB-EKAR-EV, along with the cytosolic accumulation of cAMP via Flamindo 2[17] and Ca^{2+} via RCaMP[45].

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These examples demonstrate that through the clever use of multiple methods and sensor designs, we can push the limits of what is possible to utilize multiplexed imaging of biosensors to examine cell signaling pathways.

Conclusion and Perspectives

Our ability to perform multiplexed biosensor imaging to study signal transduction has increased dramatically over the last decade. This explosion in our capabilities has led to several exciting discoveries, ranging from revelations about crosstalk between PKA and ERK [33] to realizations concerning the extent of cellular heterogeneity[44]. While we have come a long way from multiplexed *in vitro* kinase assays from cell lysates[48], constantly pushing on the boundaries of our capabilities will lead to new applications and new discoveries.

Biosensors in nearly every cell-compatible hue of the electromagnetic spectrum have been created, but there is only a limited amount of space on the spectrum and in a cell, creating an apparent limit on how many processes could be imaged simultaneously. However, Querard and colleagues recently reported the use of photoswitchable FPs to reversibly change the colors of the FPs in a cyclic manner, allowing the detection of fluorescence emission from a particular label even if there are spectrally-interfering FPs in the cell; they were able to successfully demonstrate the specific detection of four different variants of GFP in the same cell[49]. While Querard et al demonstrated multiplexed imaging with this technique, they did not use this system with biosensors, nor did they study signal transduction pathways specifically. However, this technique could lead to greater improvements in multiplexed imaging and thus our knowledge and understanding of signal transduction.

An additional promising route to increasing the potential for multiplexing is in using bioluminescence resonance energy transfer (BRET) biosensors. Recently Ruigrok et al reported the use of three different BRET-based biosensors that had different acceptor FPs but identical luciferase isoforms to detect the calmodulin-dependent activation of three different transient receptor potential (TRP) channels[50]. While Ruigrok and colleagues did not express all three versions of their biosensor in individual cells, they were able to mix three different Hek-293 cell populations that had each been individually transfected with one of the biosensors and then detect individual biosensor activation in the mixed populations. Additionally, Suzuki et al. recently demonstrated 5-way imaging of subcellularly targeted BRET constructs based on variants of nano-lantern[51]. These studies open the door to being able to also use BRET biosensors in a way to open up the spectral space available for multiplexed imaging.

Another area of interest is to use multiplexed imaging *in vivo*. While several biosensors have been shown to work *in vivo*, such as pink Flamindo in mouse brains[40], ERK KTR in *C. elegans* development[52], or EKAR in mouse skin[53], actual multiplexing *in vivo* is a new avenue to be explored that will yield a great harvest of biological knowledge.

Finally, another technology to explore in conjunction with multiplexed imaging is high-throughput imaging. One thing we have learned from single-cell analysis is that there is

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greater cell-to-cell variability among genetically identical populations than we could have imagined previously[54]. For example, Ryu and colleagues, using a microfluidic chip to enable high-throughput imaging of PC-12 cells in response to various concentrations and pulses of either EGF or NGF, discovered that while EGF stimulated proliferation in the vast majority of cells, a few cells responded by differentiating and forming neurites[55]. This cellular heterogeneity underscores the importance of assaying a larger number of cells than is typically possible through traditional low-throughput imaging methods. Performing multiplexed imaging of signal transduction pathways in a high throughput manner will serve to greatly enhance our understanding of cell biology and signal transduction crosstalk.

In conclusion, we have discussed several of the key techniques and overall concepts behind multiplexed imaging of signaling pathways. There are many more examples and biosensors available, which we recently thoroughly reviewed [7]. We encourage those studying signal transduction pathways to utilize these amazing tools in their research and consider what they could learn through multiplexed imaging.

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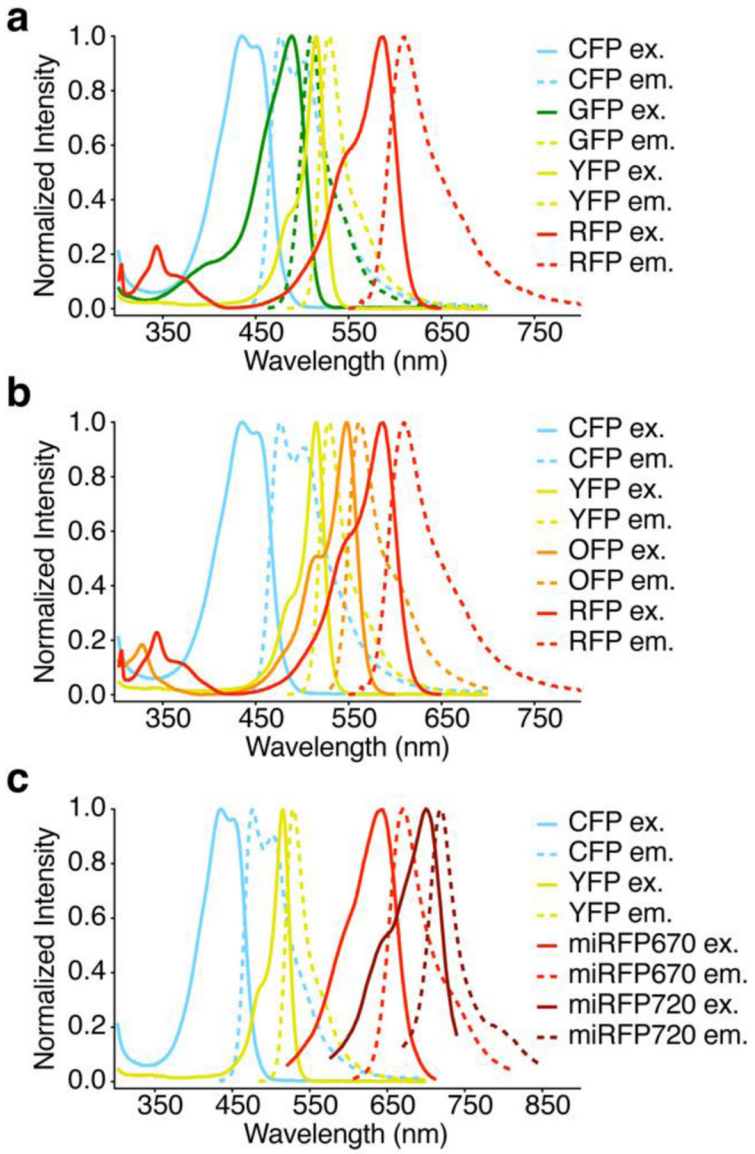

Figure 1. Spectral overlap between fluorescent proteins.

Excitation and emission spectra of various fluorescent proteins (FPs) that span the visible spectrum. While numerous FPs have been developed with distinct excitation and emission peaks, and thus characteristic “colors”, all FPs have broad (>100 nm) spectral footprints that pose a challenge to multiplexing experiments. (a) CFP and YFP are a commonly used FRET pair, as are GFP and RFP. However, considerable overlap between the CFP, GFP, and YFP spectra preclude the use of these FRET pairs for multiplexed imaging. (b) OFP and RFP have been used as an orthogonal FRET pair with CFP and YFP. Nevertheless, spectral contamination is likely between OFP and RFP, as well as between OFP and YFP, due to their substantial overlap, which can diminish biosensor signals. (c) The novel FRET pair of mIRFP670 and miRFP720 shows much better spectral separation from CFP and YFP and may therefore enable more robust multiplexing of FRET-based sensors.

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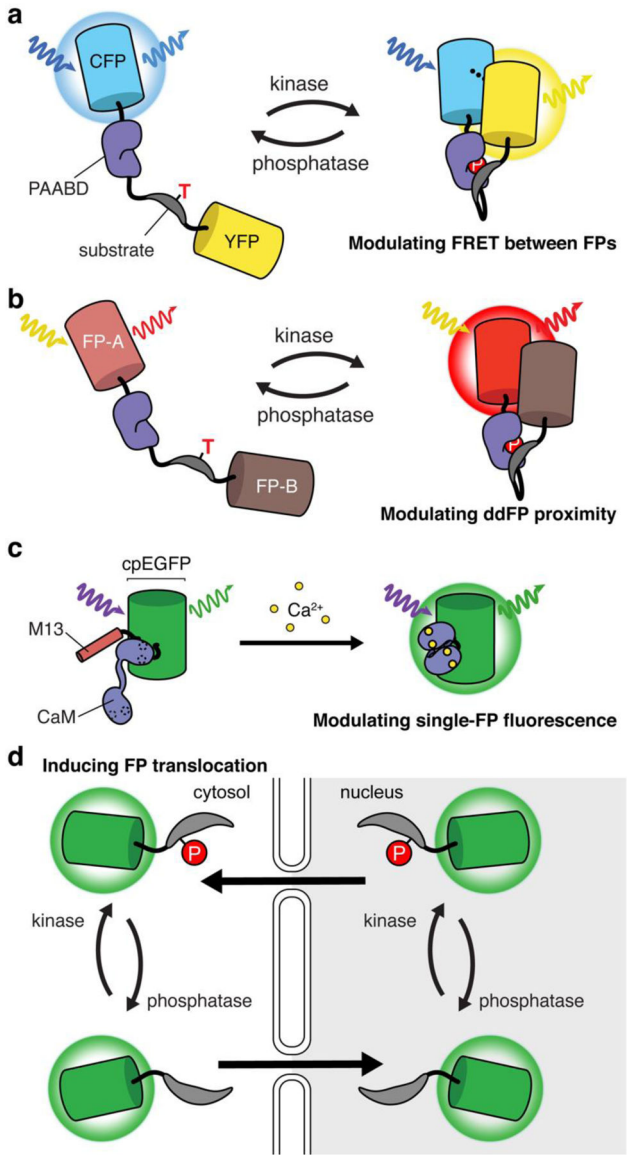

Figure 2. Common designs for genetically encoded fluorescent biosensors.

(a) Biosensors designed to modulate FRET between a pair of FPs often incorporate a conformationally dynamic molecular switch to control the relative distance and orientation of the attached FPs in response to a biochemical activity of interest. For example, FRET-based kinase activity reporters generally utilize a molecular switch where a specific kinase substrate sequence is tethered to a phosphoamino acid binding domain (PAABD). In the presence of kinase activity, the PAABD will bind the phosphorylated substrate and induce a conformational change in the biosensor that alters FRET. (b) A similar strategy can be used to modulate the proximity, and thus fluorescence intensity, of a ddFP pair. (c) Another way to modulate FP fluorescence intensity is to directly insert the molecular switch components into a single FP. In the Ca^{2+} sensor GCaMP, for example, the M13 peptide and calmodulin (CaM) are attached to the internal N- and C-termini, respectively, of circularly permuted EGFP (cpEGF). In the absence of Ca^{2+}, the switch components

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adopt and open conformation that disrupts the GFP β-barrel and decreases fluorescence, while the binding of Ca^{2+} to CaM causes the switch to close, restoring the GFP barrel and increasing fluorescence intensity. (d) Inducing the translocation of an FP is the oldest and simplest biosensor design strategy, and depends only on the intrinsic localization behavior of the attached sensing unit protein. Recently, this approach was used to develop a family of kinase activity reporters by fusing an FP to a substrate kinase peptide containing nuclear localization and export sequences, so that phosphorylation of the substrate controls the nuclear-cytoplasmic shuttling of the biosensor.

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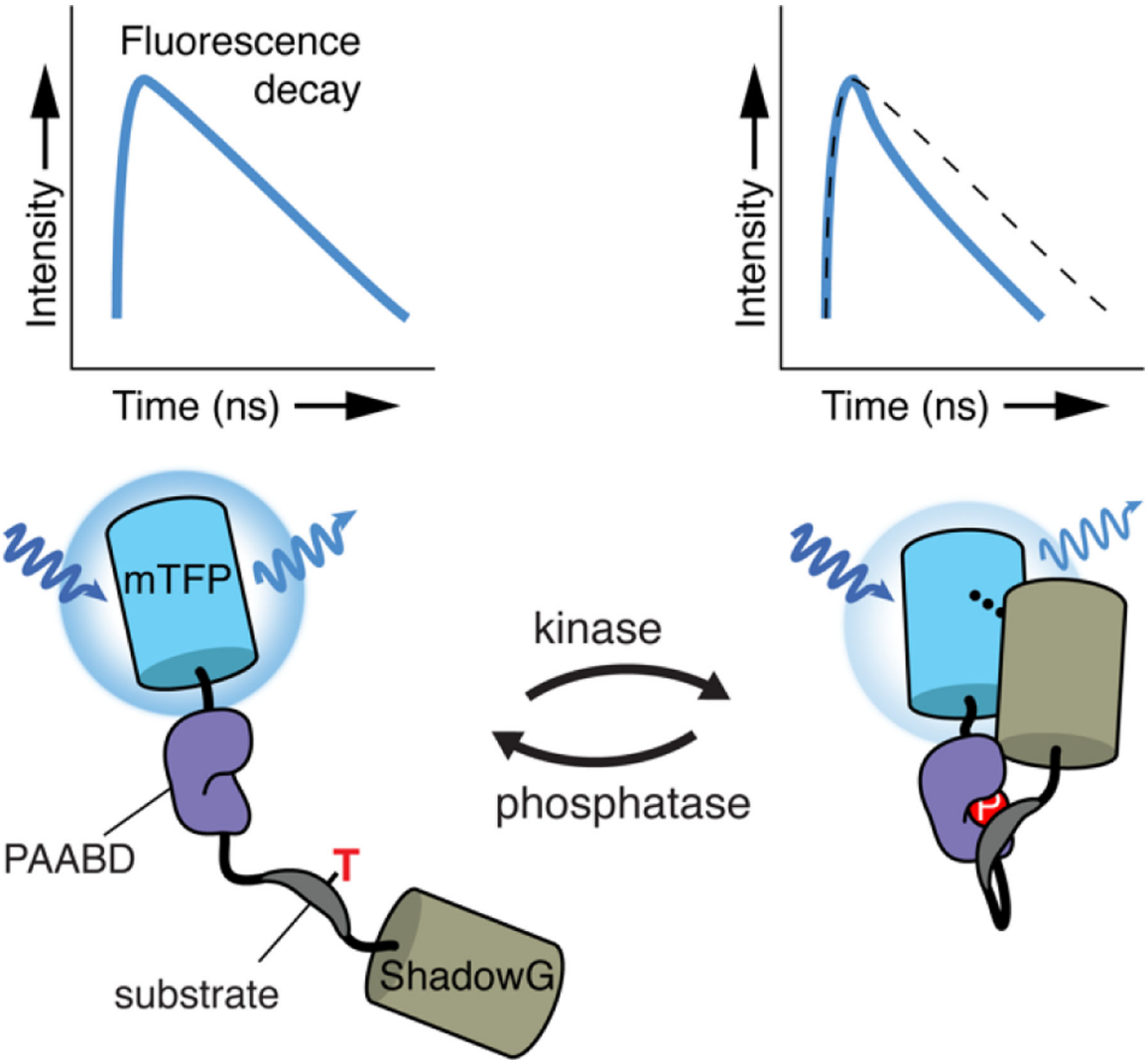

Figure 3. Fluorescence lifetime imaging of FRET-based biosensors.

Fluorescence lifetime imaging microscopy (FLIM) is a powerful technique for monitoring FRET-based biosensors and is increasingly being used for multiplexing. FLIM involves measuring the fluorescence decay curve of the FRET donor over very fast (e.g., nanosecond) timescales. Energy transfer to the acceptor will decrease the fluorescence lifetime of the donor FP (e.g., mTFP), and thus change the slope of the fluorescence decay curve. Because FLIM only measures donor fluorescence, the acceptor can be a so-called “dark” FP that does not emit fluorescence (e.g., ShadowG). This frees up additional spectral space for greater multiplexed imaging.

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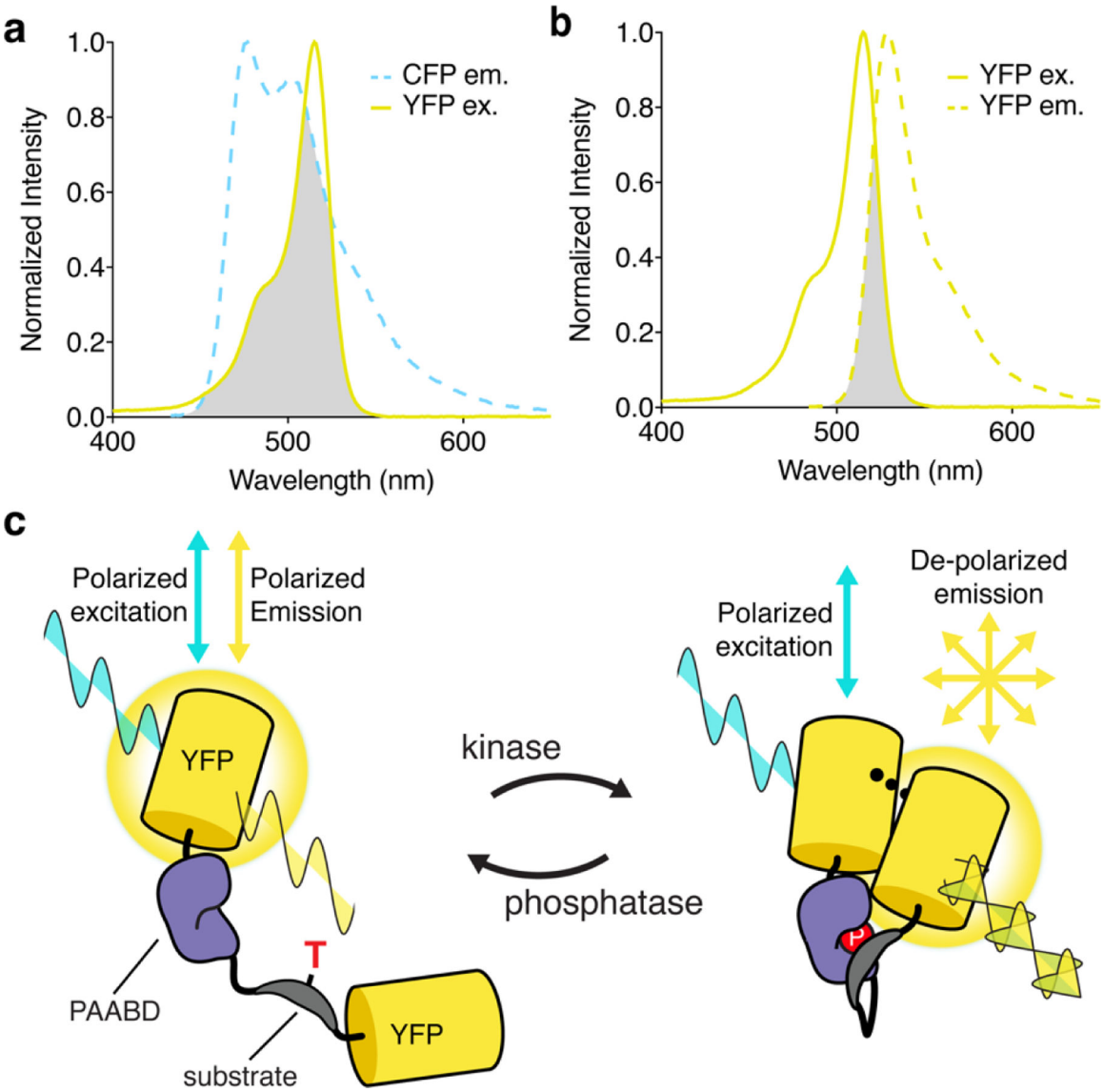

Figure 4. Single-color FRET sensors based on homo-FRET and anisotropy imaging.

(a) The considerable overlap between the CFP emission spectrum and YFP excitation spectrum allows for efficient energy transfer. (b) The emission and excitation spectra of individual FPs, such as YFP, also exhibit some overlap, which enables homo-FRET. (c) The response from a homo-FRET-based biosensor can be measured by monitoring fluorescence polarization. In the absence of FRET, polarized excitation will result in identically polarized fluorescence emission by a subset of biosensors that are aligned with the plane of illumination, as FPs rotate slowly compared with the lifetime of the excited state. However, given the fact that a FRET acceptor is unlikely to be in the exact same orientation of the donor FP, an increase in FRET will result in a decrease in polarized fluorescence emission. This principle has enabled the development of single-color FRET-based biosensors, including a suite of fluorescence anisotropy reporters (FLAREs) for monitoring various signaling activities. Shown here is a FLARE kinase activity reporter.

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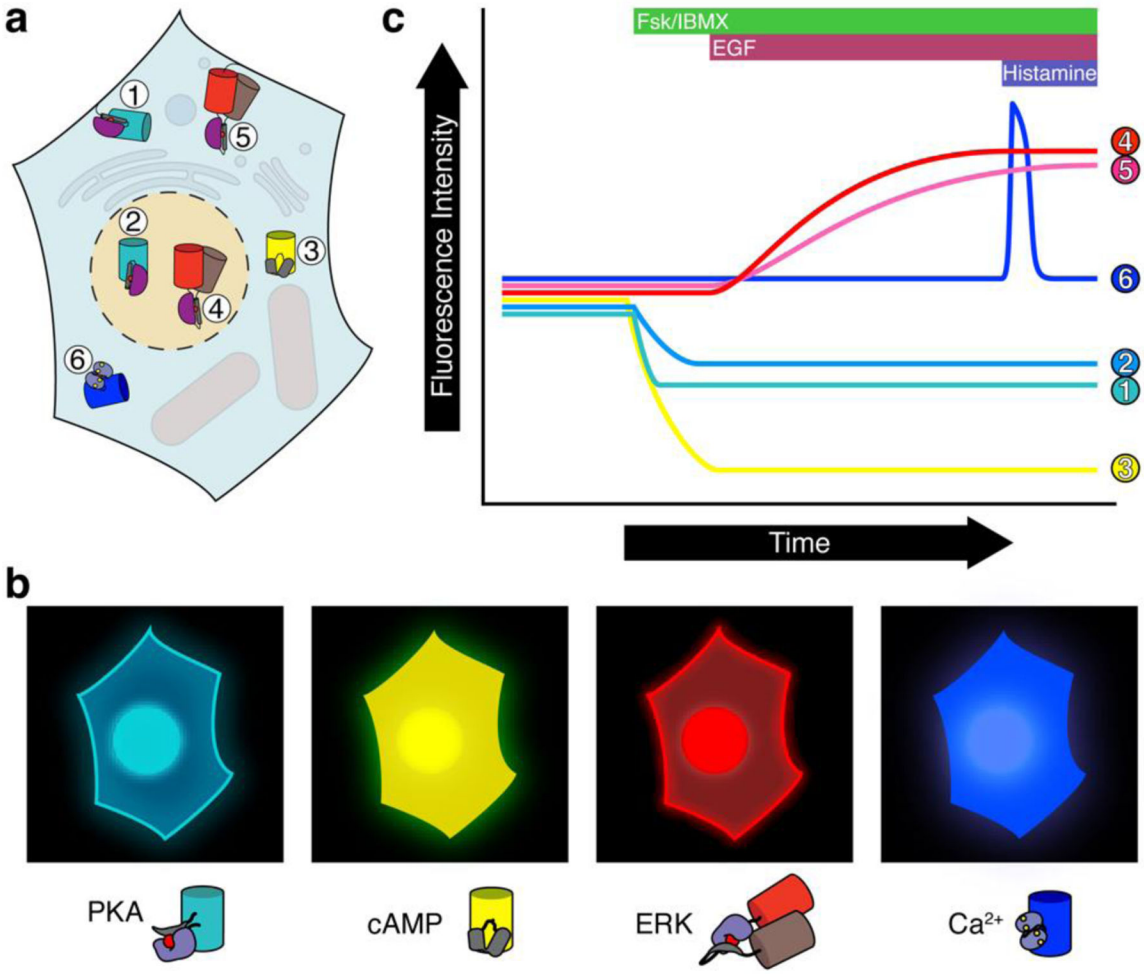

Figure 5. Combining strategies to maximize multiplexing.

(a) Single-FP biosensors greatly reduce the amount of spectral space required to image individual signaling activities, meaning more biosensors can be imaged simultaneously. However, the fluorescence signals from biosensors can also be physically separated by targeting these probes to distinct subcellular compartments. (b) By combining these complementary strategies, we are able to simultaneously detect both spatially and spectrally distinct biosensor signals, permitting even greater multiplexed imaging within the same amount of spectral space (i.e., with the number of biosensor colors). (c) We recently applied this approach to multiplexed imaging using single-color PKA (sapphireAKAR; teal), cAMP (Flamindo2; yellow), ERK (RAB-EKAR-EV; red), and Ca^{2+} (B-GECO; blue) sensors to simultaneously measure (1) plasma mebrane and (2) nuclear PKA activity, (3) cytosolic cAMP accumulation, (4) nuclear and (5) plasma membrane ERK activity, and (6) cytosolic Ca^{2+} dynamics in single cells. Sequential induction of cAMP/PKA, ERK, and Ca^{2+} signaling yields distinct responses from each biosensor and subcellular compartment.