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# Genetically encoded fluorescent biosensors illuminate kinase signaling in cancer

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Protein kinase signaling networks stringently regulate cellular processes, such as proliferation, motility, and cell survival. These networks are also central to the evolution and progression of cancer. Accordingly, genetically encoded fluorescent biosensors capable of directly illuminating the spatiotemporal dynamics of kinase signaling in live cells are being increasingly used to investigate kinase signaling in cancer cells and tumor tissue sections. These biosensors enable visualization of biological processes and events directly *in situ*, preserving the native biological context and providing detailed insight into their localization and dynamics in cells. Herein, we first review common design strategies for kinase activity biosensors, including signaling targets, biosensor components, and fluorescent proteins involved. Subsequently, we discuss applications of biosensors to study the biology and management of cancer. These versatile molecular tools have been deployed to study oncogenic kinase signaling in living cells and image kinase activities in tumors or to decipher the mechanisms of anticancer drugs. We anticipate that the diversity and precision of genetically encoded biosensors will expand their use to further unravel the dysregulation of kinase signaling in cancer and the modes of actions of cancer-targeting drugs.

Kinases are of central importance in cellular signaling networks. In humans, 535 protein kinases have been identified, which can be further subclassified into seven major eukaryotic protein kinase families, as well as atypical and other kinases, based on primary sequence (1). In terms of residues that are targeted by phosphorylation, kinases mostly fall into two major groups: tyrosine kinases and serine/threonine kinases. Tyrosine kinases further comprise two classes, receptor tyrosine kinases (RTKs)<sup>2</sup> and nonreceptor tyrosine kinases (NRTKs), which play

important roles in cell–cell signaling and transmembrane signal transduction (2). Intracellular signal transduction invariably requires Ser/Thr kinase cascades and their downstream targets. The most recent report shows that nearly 300,000 phosphosites in humans have been experimentally identified and curated in PhosphoSitePlus ([www.phosphosite.org](http://www.phosphosite.org)),<sup>3</sup> (61) which represents over 65% of all posttranslational modification sites (3). This reflects the importance of Tyr/Ser/Thr kinases in controlling nearly every aspect of cellular life, such as the cell cycle, proliferation, differentiation, motility, and cell death or survival.

Under normal physiological conditions, kinase signaling must be precisely coordinated and integrated, and properly regulated differentiation signals are critical for preventing neoplasia, a type of abnormal and excessive tissue growth. In contrast, dysregulation of kinase signaling is frequently associated with oncogenesis. In tumor cells, it is common that key kinases are no longer adequately controlled, and excessive phosphorylation sustains signal transduction pathways in an activated state. Most tyrosine kinases remain stringently and negatively regulated in the absence of specific signals. When altered through gain-of-function mutation or chromosomal translocation, or overexpression and genomic amplification, they can become potent oncogenes by delivering continuous or enhanced signaling. For example, RTKs such as EGFR regulate cell growth and proliferation by signaling through intracellular MAP kinase cascades, and aberrant RTK activation is a frequent driver of malignancies, including breast and lung cancer (4). Meanwhile, direct mutational activation of MAP kinase cascades, such as the Ras-Raf-MEK-ERK MAP kinase signaling cascade, is arguably one of the most important oncogenic drivers of human cancers, such as in skin melanoma, thyroid, and colorectal cancer (5). NRTKs are also involved in integrating signal transduction through interactions with multiple receptors. Deregulation and overexpression of NRTKs such as Src have also been implicated in several human cancers, including melanoma, colon and breast carcinoma, and prostate cancer, with elevated Src activity promoting tumor growth as well as migratory and invasive potential (6, 7). In addition to canonical MAP kinase

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<sup>2</sup> The abbreviations used are: RTK, receptor tyrosine kinase; NRTK, nonreceptor tyrosine kinase; MAP, mitogen-activated protein; FP, fluorescent protein; PKA, protein kinase A; PKC, protein kinase C; PAABD, phospho-amino acid-binding domain; FLIM, fluorescence lifetime imaging microscopy; cpFP, circularly permuted fluorescent protein; NLS, nuclear localization signal; NES, nuclear export signal; ERK, extracellular signal-regulated

kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; *RLuc*, *Renilla* luciferase; LATS-BS, LATS biosensor; IMP, integrator of metastatic potential; EGF, epidermal growth factor; EGFR, EGF receptor; CML, chronic myelogenous leukemia; SH2, Src homology 2; VEGFR, vascular endothelial growth factor receptor; IFP, IR fluorescent protein; TEV, tobacco etch virus.

<sup>3</sup> Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.

pathways, dysregulation of special signaling modules such as the Hippo pathway is frequently observed in human cancers (8, 9). The Hippo pathway is a key regulator of organ size and tissue homeostasis by limiting cell growth. In the activated Hippo pathway, MST1/2 activates LATS1/2, which directly phosphorylates and inactivates the effectors YAP/TAZ. Mutations or gene fusions involving these kinases and regulators in the Hippo pathway can lead to persistent YAP/TAZ activation, which induces gene transcription and cancer initiation and development (10). Understanding the molecular mechanisms regulating kinase signaling is therefore essential to understanding their role in cancer. To this end, new and more powerful tools for interrogating kinases in fundamental research and clinical diagnosis, as well as drug discovery, will be extremely useful.

Over the past 2 decades, the study of protein kinase signaling has been greatly enhanced by the design and application of various genetically encoded fluorescent biosensors capable of effectively monitoring kinase activities in living cells (11). The rapid and spatially precise optical readout of fluorescent biosensors makes it possible to visualize biological analytes and events directly *in situ*, preserving the native biological context and providing more detailed spatiotemporal information than can be obtained *in vitro*. For instance, the rapid dynamics of RTK activity can be easily monitored in live cells using biosensors but are difficult to capture using biochemical approaches, such as Western blotting. Furthermore, biosensors can be used to observe variations in kinase activity at the single-cell or even subcellular level. Whereas biosensors have already been widely used to study key cancer-associated kinases in normal cells and tissues, they are now increasingly being applied in cancer models. In this review, we first provide a brief summary of common biosensor design strategies. We then describe various studies that have used fluorescent biosensors to probe different types of kinase activities, especially in cancers.

### Common biosensor design strategies

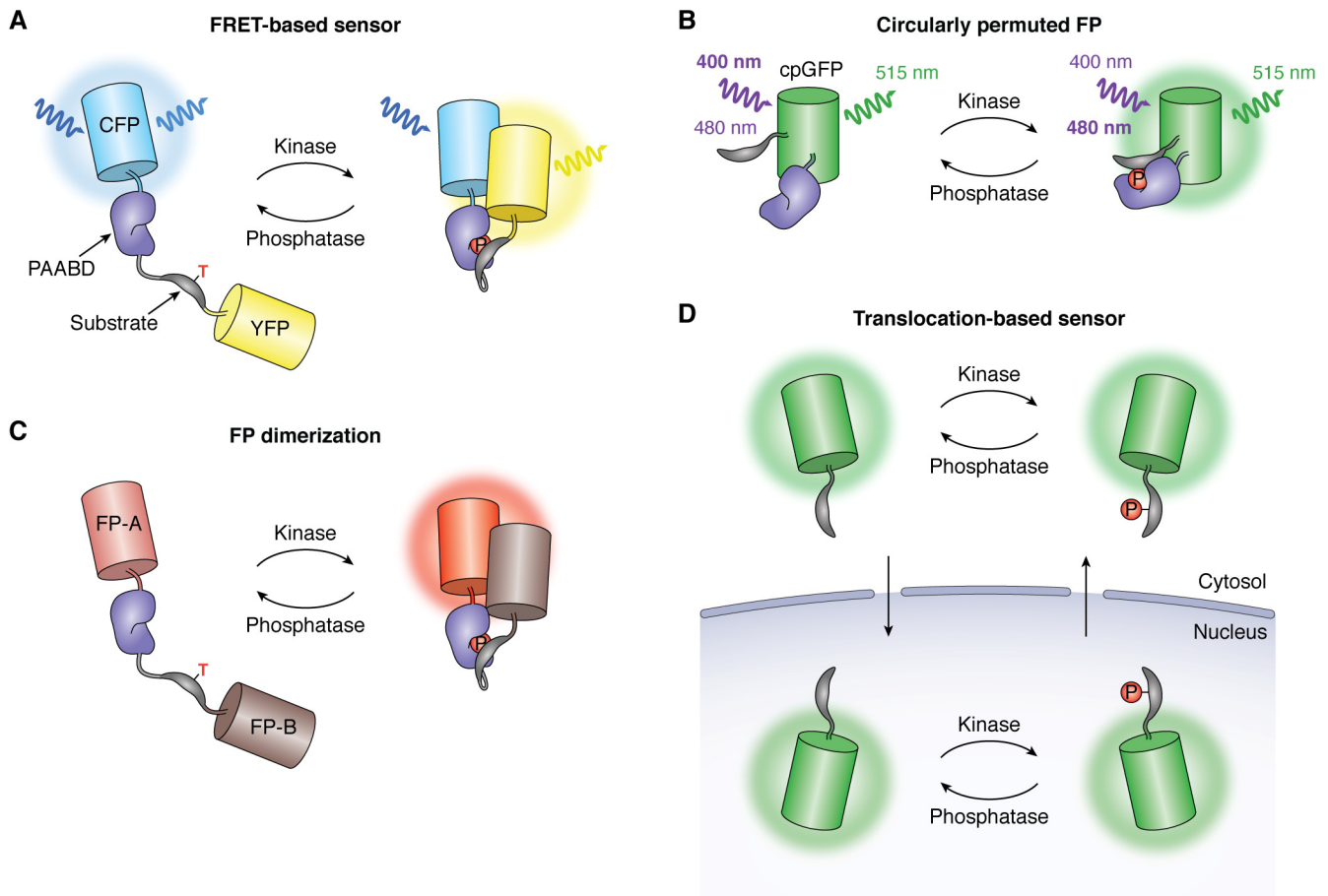
Genetically encoded fluorescent biosensors for kinase activity typically include two essential components: a sensing unit to detect changes in the signaling event and a reporting unit to translate the state of the sensing unit into a quantifiable readout. The sensing unit generally comes from a whole or central part of a substrate protein that is sensitive to the kinase of interest and functions as a “molecular switch” to control the behavior of the reporting unit. The reporting unit typically consists of one or more fluorescent protein (FP) variants, although recombinant luciferases can also be used depending on the requirements of the assay, yielding biosensors that variously change their photophysical properties or localization in response to phosphorylation. More details of biosensor design, including signaling targets, sensing components, and FPs, were recently reported by Greenwald *et al.* (12) and can also be found in our Fluorescent Biosensor Database (biosensordb.ucsd.edu).<sup>3</sup> Although the development and optimization of such reporters commonly require intensive efforts, more researchers can adapt these tools using an established pipeline for kinase activity reporter development (11). Furthermore, based on feedback from applications, more and more sensitive and effective tools

will continue to be generated using various approaches, including screening-based methods (13), to further expand our understanding of physiological and pathological processes.

### FRET-based biosensors

The most commonly used biosensors for measuring kinase activity dynamics in single cells are based on FRET. FRET-based biosensors are engineered by sandwiching the sensing unit between two FPs that act as a FRET pair and utilize the phosphorylation-induced conformational change in the sensing unit to modulate nonradiative energy transfer between the FPs (Fig. 1A). At the turn of the millennium, Zhang *et al.* (14) developed a first-generation protein kinase A activity reporter (AKAR1), in which a consensus phosphorylation sequence for protein kinase A (PKA; LRRASLP) was tethered by a flexible linker to the phospho-amino acid-binding domain (PAABD) 14-3-3 $\tau$ , which is capable of specifically recognizing and binding the phosphorylated peptide. Importantly, phosphorylation of the substrate is determined by the balance between kinase and phosphatase activity, with PKA-mediated phosphorylation expected to increase the intramolecular binding between 14-3-3 $\tau$  and the substrate, whereas dephosphorylation by cellular phosphatases should reverse this effect. Because kinase activity typically changes against a backdrop of relatively constant phosphatase activity, the resulting conformationally induced FRET changes between a flanking CFP and YFP pair should allow this design to faithfully report net increases and decreases in kinase activity. However, the high affinity of the 14-3-3 $\tau$  domain was found to protect AKAR1 from dephosphorylation, yielding a largely irreversible FRET response, and all subsequent variants (*e.g.* AKAR2 (15), AKAR3 (16), and AKAR4 (17)) have instead utilized the lower-affinity forkhead-associated 1 (FHA1) domain, in conjunction with a modified PKA substrate (LRRATLVD), to more accurately capture PKA activity dynamics. Since then, a diverse family of FRET-based sensors have been developed based on the same general design for monitoring the activity of a multitude of different kinases, including but not limited to the insulin receptor (18), Abl (19), protein kinase C (PKC) (20), Src (21), Akt (22), and p38 (23). However, not all efforts to develop kinase biosensors based on this chimeric molecular switch will be successful, in which case an intrinsic conformational change in a substrate protein may alternatively be used as the sensing unit. For instance, Zhou *et al.* (24) took advantage of the native phosphorylation-induced conformational change in full-length 4EBP1 when designing the sensing unit for their mTOR complex 1 activity reporter (TORCAR). Furthermore, this design has also been adapted to yield a specific phosphatase activity biosensor for calcineurin, in which case acutely regulated phosphatase activity drives the changes in FRET (25).

FRET increases the occurrence of donor-sensitized emission by the acceptor while quenching direct donor fluorescence. Responses from FRET-based biosensors can therefore be measured using the acceptor/donor emission ratio, with the fixed 1:1 ratio of the donor and acceptor FPs enabling these sensors to provide a quantitative readout. However, FRET also results in a shortening of the fluorescence lifetime ( $\tau$ ) of the donor FP, which can be measured via fluorescence lifetime



**Figure 1. Genetically encoded biosensors for visualizing kinase activity in living cells.** *A*, FRET-based biosensors utilize a kinase-inducible molecular switch to modulate the relative distance and orientation of a pair of FPs in response to changes in biosensor phosphorylation, thereby coupling endogenous kinase activity to biosensor FRET efficiency. As shown here, the molecular switch is often constructed by fusing a substrate peptide for a specific kinase to a PAABD, such that recognition of the phosphorylated substrate by the PAABD drives a conformational change in the biosensor. *B*, single-fluorophore kinase biosensors have recently been generated in which the substrate peptide and PAABD are inserted within the sequence of a cpFP, whereby the recognition of the phosphorylated substrate by the PAABD distorts the barrel of the cpFP, thus altering its photophysical behavior. In the example shown here, phosphorylation of the sensor leads to a shift in the maximum excitation wavelength of cpGFP from 400 to 480 nm, producing an excitation-ratiometric response. *C*, an alternative strategy for constructing single-fluorophore biosensors retains the same overall configuration of FRET-based sensors but replaces the FRET FP pair with a pair of dimerization-dependent FPs. This design also relies on the molecular switch to modulate the proximity of the dimerization-dependent FPs, as bringing FP-A and B into close proximity results in enhanced fluorescence emission by FP-A and has been used to develop red-fluorescent kinase sensors. *D*, instead of relying on a conformational change, translocation-based kinase biosensors use phosphorylation to control the behavior of nuclear localization and export signals that are incorporated within the substrate sequence. At rest, the dephosphorylated biosensor accumulates in the nucleus, whereas elevations in kinase activity drive the translocation of the biosensor into the cytosol, whereby the ratio of nuclear-to-cytoplasmic fluorescence serves as a dynamic readout of kinase activity.

imaging microscopy (FLIM). Thus, FLIM represents an alternative approach to monitoring FRET-based biosensors. Notably, because FLIM is less sensitive to the loss of donor emission intensity caused by scattering in tissues, FLIM-FRET can be effectively applied *in vivo*. For example, Weitsman *et al.* (26) generated a FLIM-FRET-compatible version of the EGFR activity biosensor phosphorylation indicator of CrkII chimeric unit (Picchu) (27) by replacing YFP and CFP with mRFP1 and EGFP, respectively, and used the resulting Picchu-FLIM sensor to investigate EGFR activity in tumor xenografts. Another benefit of FLIM-FRET is that it does not require fluorescence emission by the acceptor, which can potentially alleviate some challenges of ratiometric imaging, such as spectral bleed-through. To this end, Chen *et al.* (28) recently modified AKAR3 to generate FLIM-AKAR, which they imaged using 2-photon FLIM to monitor PKA activity in brain tissue. This sensor contains monomeric EGFP as a bright donor and cpsREACH, which displays a

high absorption coefficient but low quantum yield, as a “dark” acceptor, which provides a better signal/noise ratio and less bleed-through from the acceptor into the donor channel. Similar strategies have recently been applied to AKAR3EV and AKAR4 to yield the FLIM-FRET-based sensors AKARet (29) and AKAR5 (30), respectively.

### Single-fluorophore biosensors

Along with FRET-based biosensors, single-fluorophore biosensors mainly based on circularly permuted fluorescent proteins (cpFPs) have emerged as another popular biosensor design strategy. Circular permutation involves rearranging the linear sequence of a typical FP to relocate the N and C termini to be within the rigid  $\beta$ -barrel so that the conformational change of the embedded molecular switch can more easily alter the conformation and fluorescence of the cpFP. Single-fluorophore biosensors based on cpFPs have advantages of a smaller spectral



footprint. However, although numerous single-fluorophore sensors have been developed to monitor different cellular analytes, such as calcium (31, 32), cAMP (33), and metabolic products (34), the application of this sensor design to visualize kinase activity has been largely unexplored. To this end, Mehta *et al.* (35) recently engineered a series of single-fluorophore biosensors in which cpGFP is sandwiched between a kinase substrate peptide and PAABD, giving rise to a novel suite of excitation ratiometric kinase activity reporters (ExRai-KARs), wherein the maximum excitation peak of cpGFP undergoes a phosphorylation-dependent shift from  $\sim 400$  to  $\sim 488$  nm, with  $\sim 515$ -nm (green) emission, in response to elevated kinase activity (Fig. 1B). The resulting sensors showed a substantial improvement compared with best-in-class FRET-based reporters, such as an almost 2-fold higher dynamic range and signal/noise ratio in the case of ExRai-AKAR. With its high sensitivity and robustness to submaximal kinase stimulation, this design may thus represent a promising new strategy for developing high-performance kinase activity sensors.

Continuing with this same substrate-cpFP-PAABD configuration, Mehta *et al.* (35) were also able to develop single-color KARs based on cp-T-sapphire, which exhibits single excitation and emission maxima at  $\sim 400$  and  $\sim 513$  nm, respectively, and cpBFP, which displays single excitation and emission peaks at  $\sim 385$  and  $\sim 450$  nm, respectively. By utilizing an alternative design based on FP dimerization (36), they were further able to develop red fluorescent single-color KARs (Fig. 1C). Given that complex cell behaviors are regulated by highly integrated kinase signaling networks, these single-color kinase sensors are highly desirable tools for simultaneously examining multiple kinase activities, as well as the cross-regulation between kinases, in single cells. Indeed, through the use of their single-color KARs, Mehta *et al.* (35) were able to successfully perform 4–6-parameter multiplexed imaging to simultaneously monitor changes in kinase activities, as well as cAMP and  $\text{Ca}^{2+}$  elevations, in single cells.

### Translocation-based biosensors

In contrast to the above biosensors, translocation-based biosensors are entirely different in design, which depends on relocating the sensor to a predetermined subcellular site rather than directly altering FP photophysics. The basic design principle for kinase translocation reporters stems from the endogenous sequences found in kinase substrates that contain a nuclear localization signal (NLS) and/or nuclear export signal (NES) neighboring the phosphorylation sites. Phosphorylation and dephosphorylation of these sites alter the recognition of these NLS and NES motifs, which results in translocation in and out of the nucleus (Fig. 1D). For instance, Spencer *et al.* (37) initially developed a biosensor of CDK2 activity by fusing Venus (YFP) to the C-terminal domain of human DNA helicase B (HDHB), which exits the nucleus in response to CDK2-dependent phosphorylation. Gross *et al.* (38) similarly generated an Akt translocation sensor by fusing full-length FoxO1 to the green FP Clover, and Maryu *et al.* (39) divided the central region of FoxO3a, containing the Akt phosphorylation site and NLS and NES sequences, to generate their own Akt kinase sensor. In contrast to these endogenous sequences, Regot *et al.* (40)

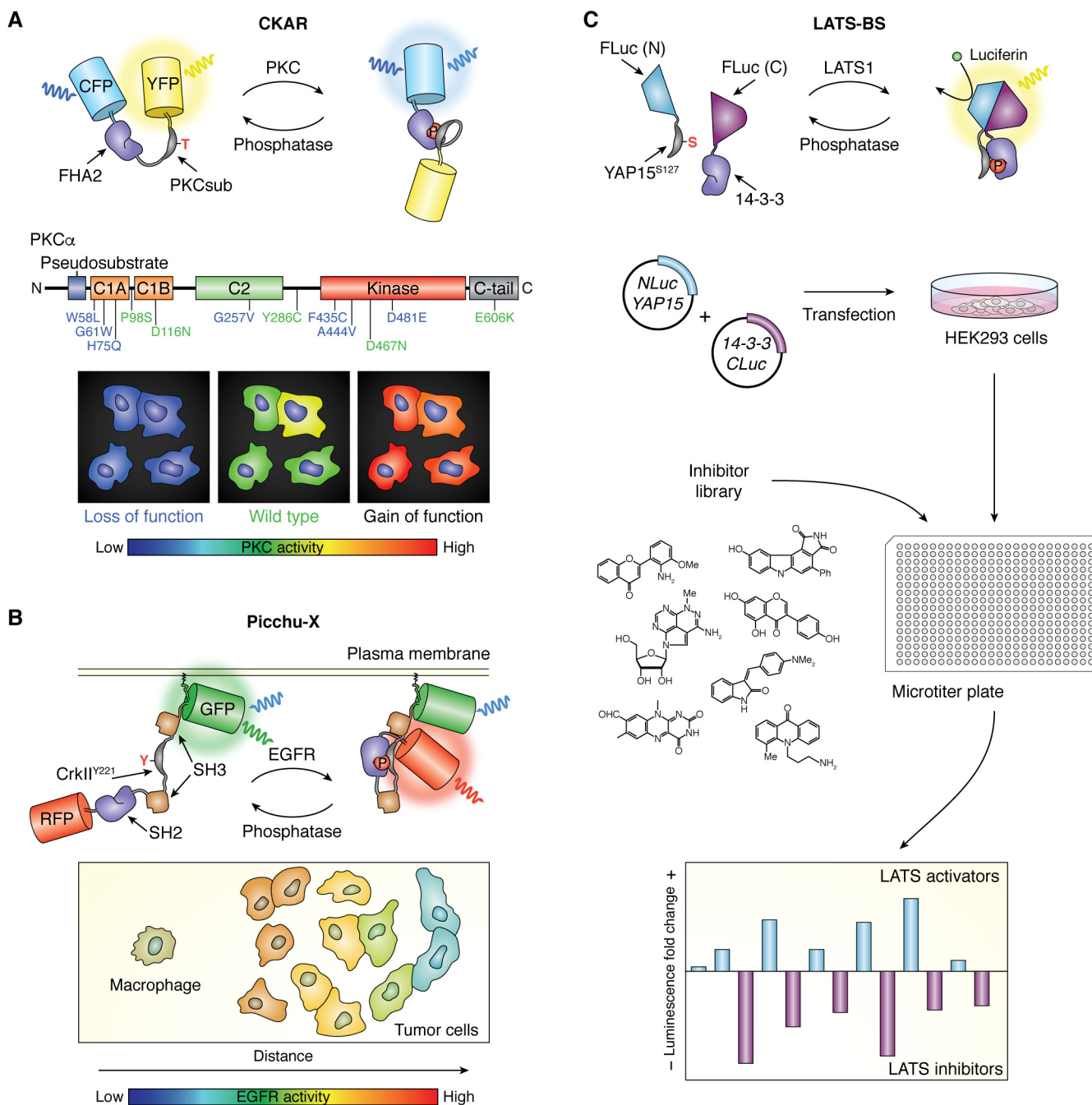
have demonstrated the generalizability of this approach by engineering a family of kinase translocation reporters based on a minimal translocation domain that contains a kinase-specific substrate peptide fused in tandem to a bipartite NLS and an NES, such that phosphorylation inhibits the NLS and activates the NES. This design, which relies on cellular machinery such as nuclear import to function, has been applied to PKA, c-Jun N-terminal kinase, p38, and ERK activity.

### Bioluminescence-based biosensors

An alternative biosensing strategy is to develop bioluminescence-based biosensors that incorporate luminescent proteins as the reporting unit. Luciferases are a well-known group of luminescent proteins that emit light as a byproduct of the catalytic oxidation of a luciferin substrate. Because these enzymes do not rely on external illumination to produce a signal, bioluminescence-based readouts are not affected by the autofluorescence, photobleaching, or phototoxicity that can occur during fluorescence imaging and thus have been considered a potential way to enhance biosensor sensitivity and versatility. A typical strategy for designing luminescent sensors is to utilize the reversible fragment complementation of a split luciferase as a means of dynamically controlling light output. For example, Herbst *et al.* (41) developed a luminescent PKA activity reporter (lumAKAR) based on a biomolecular design in which the kinase-inducible molecular switch from FRET-based AKAR is divided into two fragments (substrate peptide and PAABD) that are fused to complementary N- and C-terminal portions of *Renilla* luciferase (RLuc). Elevations in PKA activity cause this bimolecular switch to form a complex, which reconstitutes catalytically active RLuc. This generalizable design was also applied to monitor the dynamics of other kinases, such as PKC (41). RLuc fragment complementation was also used by Stefan *et al.* (42) to investigate dynamic protein kinase complexes, wherein the regulatory and catalytic subunits of PKA were fused to complementary N- and C-terminal RLuc fragments. Azad *et al.* also recently developed a bioluminescence-based LATS biosensor (LATS-BS) consisting of complementary N- and C-terminal luciferase fragments fused to a 15-amino acid YAP peptide (YAP15), which contains the Ser-127 LATS phosphorylation site, and a 14-3-3 $\tau$  domain (43) (Fig. 2C).

### Applications of biosensors in cancer

From their introduction nearly 20 years ago, genetically encoded kinase biosensors have provided numerous insights into the regulation of kinase signaling in physiological processes. In light of this success, the use of biosensors to study kinase signaling in cancer has attracted increasing interest in recent years. From cultured cancer cell lines to tumor tissue, biosensors provide direct *in situ* measurements of the spatio-temporal dynamics of kinase activity and play an increasingly crucial role in diverse studies, ranging from the molecular dissection of kinase signaling pathways in cancer to monitoring the effects of cancer drugs on specific kinase pathways and targets for improved cancer therapy and drug discovery.



**Figure 2. Using genetically encoded kinase biosensors to study cancer.** *A*, studying cancer-associated kinases at the single-cell level. PKC has long been considered to be an oncogene due to the ability of phorbol esters, which potently activate PKC, to promote tumorigenesis. However, when Antal *et al.* (44) probed the role of PKC in cancer by examining the effect of various cancer-associated mutations on PKC activity using the FRET-based PKC activity reporter CKAR, most mutations were found to either have no effect on PKC activity or be loss-of-function mutations. For example, among 12 PKC $\alpha$  mutations tested, seven exhibited reduced activity compared with WT PKC $\alpha$ , whereas the remaining five showed no difference; no gain-of-function mutants were observed. Their results strongly suggest that PKC is in fact a tumor suppressor and highlight the importance of using biosensors to dissect cancer-associated signaling in the native context of living cells. *B*, illuminating heterogenous signaling in tumor tissues. Tumors are highly complex and intricately organized tissues. Interactions among cells within different parts of the tumor can form diverse microenvironments associated with unique signaling dynamics, and genetically encoded biosensors can be powerful tools for elucidating the mechanisms of this heterogeneity. For example, by using the FRET-based EGFR biosensor Picchu-X to visualize EGFR signaling in tumor xenografts, Weitsman *et al.* (27) were able to observe substantial heterogeneity in EGFR activation within the tumor. Because they were imaging biosensor responses in intact tissue, they were further able to correlate the responses to the proximity of different tumor structures and cell types, revealing that proximity to infiltrating macrophages was strongly associated with elevated EGFR activity in tumor cells. *C*, biosensors are increasingly being used in high-content studies to dissect signaling pathways and identify drug targets. Azad *et al.* (43) recently applied this approach using their newly developed bioluminescent LATS1 sensor to identify upstream regulators of the Hippo pathway. By culturing biosensor-transfected HEK293 cells in microtiter plates and treating them with a large panel of kinase inhibitors, they were able to identify both activators and inhibitors of LATS1 kinase, thus providing new details of Hippo pathway regulation.

### Dissecting oncogenic kinase signaling in living cells

Cell function and behavior are dynamically regulated by kinase signaling pathways, which often play a key role in oncogenesis. The use of genetically encoded biosensors to rapidly and sensitively probe changes in kinase activities at the single-cell level can thus provide novel and valuable insights into the mechanisms of aberrant kinase signaling in cancer biology.

PKC has long been regarded as an oncogenic kinase based on the ability of various PKC isozymes to act as high-affinity intracellular receptors for tumor-promoting phorbol esters. However, a 2015 study in which Antal *et al.* (44) used the genetically encoded PKC activity sensor CKAR to systematically characterize 46 cancer-associated mutations known to alter PKC signaling has helped to largely overturn this long-standing dogma. By co-expressing CKAR in COS7 cells along with different PKC variants bearing mutations in the regulatory or kinase domains and monitoring the FRET ratio change in response to pharmacological stimulation of PKC, the authors found ~61% of PKC mutations to be loss-of-function, with most either reducing or abolishing PKC activity, whereas none were activating (Fig. 2A). Their analysis of cancer-associated mutations from diverse cancers and throughout the PKC family revealed that PKC isozymes are generally inactivated in cancer, supporting a tumor-suppressive function for this kinase. Further supporting the role of PKC as a tumor suppressor, correcting a loss-of-function PKC $\beta$  mutation via CRISPR-mediated genome editing in a patient-derived colon cancer cell line suppressed anchorage-independent growth and reduced tumor growth in a xenograft model. Based on these results, it is not difficult to see why over 30 years of clinical trials using PKC inhibitors to treat cancer would not only have failed, but in some instances also worsened patient outcomes. Thus, we can only hope that biosensor-based studies will play an even greater role in guiding clinical studies in the future.

Generally, biosensor development and optimization are focused on ensuring the specificity of a biosensor for a single kinase. Nevertheless, Midde *et al.* (45) recently presented an intriguing counterexample to this strategy, in which a substrate protein shared by multiple kinases was applied to develop a biosensor to indicate metastatic potential. Specifically, the authors based their biosensor design on the Tyr-phosphorylated protein CCDC88A (GIV/Girdin), which they had revealed through phosphoproteomics analyses to be a key metastasis-specific phosphoevent across a variety of solid tumors. Furthermore, because GIV integrates prometastatic signals from multiple oncogenic receptors, these multimodular biosensors were named integrators of metastatic potential (IMPs). Using these IMP sensors, the authors were successfully able to capture the heterogeneity of metastatic potential within primary lung and breast tumor cells at steady state, sensitively detect the few cells with the highest metastatic potential, and even track the metastatic potential of tumor cells during metastatic progression and the development of drug resistance. These findings indicate that IMPs are effective tools for measuring the diversity and plasticity of metastatic potential of tumor cells in a sensitive and unbiased way.

### Imaging kinase activities in tumor tissues

Tumors are complex tissues that engage in multifaceted interactions with the surrounding host environment, which ultimately shape the course of disease progression. Applying biosensors at the tissue level is therefore an extremely exciting and powerful approach to studying the role of kinase-mediated signaling in the occurrence and development of tumors.

Kinase activity can be influenced by changes in both intracellular signaling networks and the external environment. Particularly within the complex tumor microenvironment, the regulation of kinase signaling can be quite intricate, and kinase activities are often heterogeneous. Intratumoral heterogeneity is considered a major mechanism underlying treatment failure using molecularly targeted therapies. For example, EGFR is overexpressed or aberrantly activated in most common solid tumors, including non-small-cell lung cancer and cancers of the breast, prostate, and colon, yet despite the development of numerous EGFR-targeted drugs with specific mechanisms of action in recent years, clinical testing has revealed relatively poor response rates. Importantly, the failure of cells to respond to drugs could be linked to differences in the external microenvironment and not to EGFR expression. Therefore, Weitsman *et al.* (26) utilized their FLIM-FRET-based EGFR biosensor Picchu-FLIM, which was delivered into a murine model of basal-like breast cancer *in vivo* via liposomes, to examine the intratumoral heterogeneity of EGFR activity. This work revealed substantial heterogeneity in both EGFR activity and EGFR inhibitor efficacy in a murine breast cancer model. Furthermore, the proximity of macrophages to tumor cells was positively associated with tumor cell EGFR activity and was found to at least partially account for the observed intratumoral heterogeneity (Fig. 2B). This result was consistent with the demonstration that tumor cells and macrophages interacted through a paracrine loop involving the macrophage growth factor CSF-1 released from tumor cells and the reciprocal release of EGF by macrophages (46). This association was also found to persist in the presence of an EGFR inhibitor. The same effect of macrophage infiltration on EGFR activation was also seen in colorectal cancer xenografts, but not in non-small-cell lung cancer xenografts expressing a constitutively activated EGFR mutant that could not be affected by macrophages (26).

Vascular permeability, or the extent to which a blood vessel wall allows the flow of small molecules or whole cells in and out of the vessel, is markedly increased in tumor tissues, and this “hyperpermeability” is associated with angiogenesis and metastasis. In microvascular endothelial cells, the cAMP/PKA pathway mediates endothelial barrier function by suppressing vascular permeability. Yamauchi *et al.* (47) utilized the PKA activity reporter AKAR3EV, which is an improved AKAR3 containing a flexible long Eevee linker (48), to understand the contribution of PKA to vascular hyperpermeability in tumor tissue. Through a combination of *in vivo* two-photon excitation microscopy and AKAR3EV-expressing transgenic mice, the level of PKA activity was revealed to be significantly lower in intratumoral endothelial cells compared with subcutaneous endothelial cells. Meanwhile, a modified Miles assay, in which higher extravasation of Evans blue dye was observed in tumor



tissue, confirmed the hyperpermeability of the tumor vessels. PKA activation with a cAMP analogue further alleviated tumor vascular hyperpermeability, suggesting that low PKA activity in endothelial cells may be responsible for vascular hyperpermeability in tumor tissues.

### Screening for kinase inhibitors and evaluating drug effects

Because they offer a direct, real-time readout of kinase activity, kinase biosensors can potentially be used to monitor drug-targeting efficacy as well as to understand the spatiotemporal changes of kinase activity in response to inhibitors. For example, imatinib was developed to treat chronic myelogenous leukemia (CML), which is mediated by the Bcr-Abl gene fusion product, yet acquired resistance to imatinib has prompted the search for novel tyrosine kinase inhibitors directed against Bcr-Abl mutants. As such, there is an emerging need for *in vivo* tools that can rapidly and sensitively monitor Bcr-Abl activity and evaluate drug resistance as well as guide clinical therapies. With this in mind, Tunceroglu *et al.* (49) utilized Picchu (26) to profile inhibitor efficacy and thus monitor drug resistance in CML cells. However, because Picchu can also respond to both Abl and EGFR activity, Mizutani *et al.* (50) instead developed a more specific and sensitive FRET-based biosensor based on CrkL, which is the most characteristic Bcr-Abl substrate. In the resulting phosphorylation indicator of CrkL *en* substrate (Pickles), CrkL is fused to an SH2 domain and sandwiched between Venus and ECFP, so that intramolecular binding of the SH2 domain to Tyr-207-phosphorylated CrkL increases FRET. Using this sensor, they assessed the effect of imatinib on Bcr-Abl activity in CML and evaluated the influence of second-generation drugs on Bcr-Abl mutants, finding that the T315I mutation conferred resistance to both nilotinib and dasatinib, whereas the G250E mutation showed dose-dependent resistance to nilotinib. By applying this sensor in patient cells, the authors were also able to monitor disease status during imatinib therapy.

Src up-regulation and activation governed by the tumor microenvironment may affect drug targeting in pancreatic cancer. To probe the spatiotemporal regulation of Src activity in pancreatic cancer and determine the activity changes in response to the anti-invasive Src inhibitor dasatinib, Nobis *et al.* (21) constructed a FLIM-FRET-based version of a previously described Src biosensor to visualize Src activity in pancreatic cancer (51). In both 3D organotypic cultures and live pancreatic tumors, a spatially distinct gradient of Src activity was observed within invading tumor cells, which was greatly enhanced at the invasive border relative to the tumor cortex. The authors observed that upon treatment with dasatinib, cells at the invasive borders switched from a predominantly active to an inactive cell state, which correlated with impaired metastatic capacity *in vivo*. They also found that the extent to which Src activity responded to dasatinib treatment was correlated by the distance of tumor cells from the host vasculature, further illustrating how biosensors can be used to uncover drug penetrance *in vivo* and thereby map areas of poor drug-targeting efficiency within specific tumor microenvironments.

Kinase biosensors can also be applied to directly screen for more effective inhibitors. For example, Azad *et al.* (43) devel-

oped a bioluminescence-based LATS biosensor (LATS-BS, described above) to monitor the activity of the Hippo core component LATS kinase, which they used to perform a screen for upstream kinases capable of modulating LATS kinase activity. By applying a library of 80 small-molecule kinase inhibitors to treat HEK293A cells expressing this sensor, the authors identified six kinase inhibitors that activated the biosensor, in addition to six inhibitors that reduced the biosensor signal (Fig. 2C). Of these “activating” inhibitors, the vascular endothelial growth factor receptor (VEGFR) inhibitor SU4312 showed the most dramatic effect on LATS. Further verification using multiple VEGFR inhibitors suggested that VEGFR signaling was indeed a regulator of the Hippo pathway. Specifically, activated VEGFR was found to inhibit LATS and activate the downstream Hippo effectors YAP and TAZ, which participates in regulating blood vessel formation in both physiological and pathological settings.

### Conclusions and perspectives

Genetically encoded kinase activity biosensors have emerged as powerful tools to illuminate kinase signaling networks in both physiological and pathological settings. The commonly used biosensor designs described above are all capable of rapidly and sensitively visualizing dynamic changes in kinase activity, thereby providing important new insights into the dysregulation of kinase signaling in cancer as well as into the molecular mechanisms of cancer-targeting drugs.

The use of biosensors to perform tissue-level imaging of kinase signaling in whole tumors represents an extremely exciting new area of cancer research. However, *in vivo* fluorescence imaging generally suffers from problems such as phototoxicity and tissue penetrability of visible light. The development of biosensors incorporating various recently engineered IR fluorescent proteins (IFPs) (52), which are capable of excitation and emission at wavelengths from 650 to 900 nm (*e.g.* the near-IR window (53)), has thus opened a window onto *in vivo* activity imaging. For example, Tchekanda *et al.* (54) have developed an IFP-based reporter for detecting the spatiotemporal dynamics of protein–protein interactions, wherein a protein fragment complementation assay based on IFP1.4 was used to monitor  $\beta_2$ -adrenergic receptor signaling in U2OS cells. Continued engineering efforts have also produced improved IFPs with much higher brightness and quantum yield and have even been used to carry out *in vivo* tumor imaging in intact mouse brains (55, 56).

Even with these advances, whole-tumor imaging of kinase signaling, such as efforts to carefully delineate relationships between kinase activity and the tumor microenvironment, represents a tremendous technical challenge, which is made even more daunting by the real-time, dynamic nature of biosensor imaging. Alternatively, biosensors that are capable of “memorizing” differences in kinase activities across a heterogeneous and complex tumor could enable retrospective examination of chronic signaling differences over the course of tumor development and evolution. In neuroscience, the concept of such an activity “integrator” has recently been realized for the study of neuronal activity and neuromodulator signaling in the brain (57–59). Many of these snapshot reporters share a similar



design in which co-application of an external (e.g. light) and internal (e.g.  $\text{Ca}^{2+}$ ) stimulus are required to trigger an AND-gate that drives the tobacco etch virus (TEV) protease-mediated cleavage and release of a transcription factor to induce the expression of an FP reporter gene. Thus, any cell that experiences the internal stimulus (e.g.  $\text{Ca}^{2+}$  elevation) during the recording period, which is defined by the external stimulus (e.g. illumination), will be permanently marked with fluorescence. In a more recent example, Wintgens *et al.* (60) applied a split TEV protease system to monitor the recruitment of adapter proteins to ligand-activated RTKs, including the epidermal growth factor receptor (ERBB), the insulin receptor (INSR), and the hepatocyte growth factor receptor (HGFR) families. Binding of the activated RTK and adaptor reconstitutes the split TEV, causing cleavage of a TEV substrate sequence between the RTK and the Gal-VP16 transcription factor, which then migrates into the nucleus to initiate luciferase expression. Using this integrator, the entire history of RTK activation during ligand treatment is recorded and converted into a luminescent signal. In addition, these RTK-split-TEV recruitment assays were validated in dose-dependent inhibition assays using ERBB family-selective antagonists. Although lacking the AND-gate design of neuronal activity integrators, this work hints at the potential for developing kinase activity integrators to assist with whole-tumor recording of kinase activities in cancer research.

Together, IFP-based biosensors and “snapshot” integrators are effective new tools that promise to deepen our understanding of kinase signaling in cancer by enabling the study of complex specimens. We expect that far more comprehensive and detailed future studies, including investigations of kinase activation, heterogeneity, and responses to drug treatment, will be made possible using these tools.

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