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Reporting from the Field: Genetically Encoded Fluorescent Reporters Uncover Signaling Dynamics in Living Biological Systems

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Abstract

Real-time visualization of a wide range of biochemical processes in living systems is being made possible through the development and application of genetically encoded fluorescent reporters. These versatile biosensors have proven themselves tailor-made to the study of signal transduction, and in this review, we discuss some of the unique insights that they continue to provide regarding the spatial organization and dynamic regulation of intracellular signaling networks. In addition, we explore the more recent push to expand the scope of biological phenomena that can be monitored using these reporters, while also considering the potential to integrate this highly adaptable technology with a number of emerging techniques that may significantly broaden our view of how networks of biochemical processes shape larger biological phenomena.

Keywords

signal transduction; imaging; biosensor; FRET; spatiotemporal dynamics

INTRODUCTION

All cells must sense environmental changes and make appropriate adjustments to their behavior. This remarkable ability requires the coordinated action of networks of signaling molecules whose properties are spatially and temporally regulated to achieve specific outcomes. It is therefore imperative to study the context-dependent interactions, reactions, and biochemical and biophysical changes that underlie dynamic signaling processes. Visualizing many of these events within their native biological contexts has become possible with the development of genetically encoded fluorescent reporters. As their name suggests, these reporters convert the detection of a specific biological parameter into an observable

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fluorescent signal. Because fluorescence occurs on the order of nanoseconds, it can capture events as they unfold in real time, be it over seconds, minutes, or hours. Being genetically encoded entails the introduction of a fluorescent reporter into living systems at the DNA level, followed by de novo synthesis, and subcellular targeting by the endogenous cellular machinery. Subcellular targeting makes it possible to control the specific loci at which the biological parameters are being detected. As a result of these properties, genetically encoded fluorescent reporters are proving extremely useful in elucidating the complex molecular mechanisms underlying dynamic signal transduction processes.

Several techniques predate the use of genetically encoded fluorescent reporters, and it is atop their shoulders that this technology was developed. At nearly 70 years old (1), immunofluorescence holds the distinction of being the first method to leverage the sensitivity of fluorescence for the detection and visualization of a host of specific intracellular components, including proteins, hormones, metabolites, messengers, and posttranslational modifications (2–5) and is largely responsible for establishing the field of molecular cell biology. Fluorescent analog cytochemistry (6, 7) and fluorescent chemical indicators (8) made it possible to directly observe the dynamics of specific processes in real time and in live cells, and considerably increased the level of detail with which intracellular phenomena could be investigated. Into this ground, the seed for genetically encoded fluorescent reporters was planted by the fortuitous discovery of *Aequorea victoria* green fluorescent protein (GFP). GFP attracted relatively little attention in the 30 years that passed between its initial discovery in 1962 and 1992 when the gene encoding GFP was cloned (reviewed in References 9 and 10). Heterologous expression in both bacteria and nematodes revealed GFP to be an intrinsically fluorescent protein (11, 12), a breakthrough that sparked a revolution in how intracellular processes could be observed. By using GFP as a genetically encodable fluorophore, it became possible to construct fluorescent reporters that are entirely encoded by DNA and that can be expressed by living cells and subsequently targeted to specific loci. A variety of fluorescent reporters have been constructed to enable the visualization of dynamic changes in the localization, activity, and interaction of signaling molecules with high spatial and temporal resolution.

In this review, we briefly discuss the basic principles and considerations that inform the design and construction of genetically encoded fluorescent reporters. We then explore some of the unique insights these biosensors provide into the spatial organization and temporal dynamics of intracellular signaling, followed by some of our thoughts regarding the future of genetically encoded fluorescent reporters and other tools for probing molecular processes in the native cellular context.

BUILDING A GENETICALLY ENCODED FLUORESCENT REPORTER

Signaling molecules are continually transforming—being born, turned over, relocalized, conformationally altered, and posttranslationally modified—and all the while interacting with one another. Genetically encoded fluorescent reporters can be placed within the native context of a living cell to directly report on these dynamic events in situ. Although reporters often vary in their specifics, the design principles involved in constructing a reporter that “speaks the language of the cell” remain largely universal.

In the most straightforward case, a fluorescent protein can be directly fused to a full-length cellular protein, reporting on its localization, synthesis, and turnover. Because a fluorescent protein typically does not encode any innate localization, distribution of the fluorescent signal can serve as a direct marker for localization of the target protein (Figure 1*a*). Furthermore, this design can be used to monitor protein synthesis or turnover because the accumulation or degradation of the reporter mirrors that of the target protein. Thus, in the context of a fluorescent reporter, full-length proteins serve as surrogates for their endogenous counterparts, providing information about expression, turnover, localization, and mobility.

Reporters can also be built that undergo a change in their fluorescent signal, allowing them to report on conformational changes in the protein of interest, as well as its interaction with other proteins. For example, the target protein and its interacting partner can be fused to two fluorescent proteins that can undergo fluorescence resonance energy transfer (FRET), such that their interaction promotes FRET. FRET is a photophysical phenomenon characterized by the nonradiative transfer of energy between two fluorophores—called the donor and acceptor—located in close proximity (<10 nm) to one another. Because energy transfer depends on distance and relative orientation between the two fluorophores, FRET can be exploited to report on protein-protein interactions and, in the case of a conformationally sensitive protein sandwiched between a FRET pair, conformational changes within the target protein.

Monitoring biochemical processes, such as the accumulation or degradation of a small molecule, or the activity of an enzyme, requires a “sensing element,” built from amino acid sequences of endogenous cellular proteins or de novo peptide sequences, to detect the target biochemical activity. A “reporting element,” consisting of one or more fluorescent proteins, or complementary fluorescent protein fragments in the case of bimolecular fluorescence complementation (13, 14), is then coupled to the sensing element, making fluorescence a proxy for biochemical status. Importantly, the sensing element often determines the specificity, sensitivity, and temporal resolution with which molecular events can be detected; the closely coupled reporting element should not in turn affect the function of the sensing element. For example, several protein domains change their localization in response to the production of intracellular messengers, and their translocation can be used to detect the presence of these molecules, such as the membrane translocation-based reporters for phosphoinositides (Figure 1*b*) (15–18). In other examples, the sensing elements behave like molecular switches and can undergo conformational changes in response to specific biochemical analytes, or posttranslational modifications (Figure 1*c*).

In the case of the widely used genetically encoded Ca^{2+} reporter cameleon, the molecular switch consists of calmodulin (CaM) and the M13 peptide, which binds to CaM in the presence of Ca^{2+} (19, 20). This molecular switch is sandwiched between a FRET pair, so that a change in free calcium concentrations is reported with a change in FRET. Because the ratio of donor and acceptor is fixed, changes in FRET can be conveniently monitored using changes in the acceptor-to-donor emission ratio. Ratiometric measurements largely eliminate artifacts that may arise through variations in sample thickness and reporter concentration. Similar designs that couple a molecular switch with a FRET pair have been

applied to build genetically encoded fluorescent reporters for accumulation of signaling molecules, such as cyclic AMP (cAMP) (21–23), or kinase activities (24, 25). In addition, molecular switches can also be inserted into single fluorescent proteins, such that conformational changes may change the fluorescence intensity (Figure 1c) or even shift the excitation or emission maxima of the fluorescent proteins, providing another ratiometric readout in the latter case.

The building blocks for fluorescent reporters are often cellular proteins or protein domains, and these endogenously derived components are likely to have endogenous partners. Interactions between an endogenous component and a reporter element can potentially buffer native cellular processes or interfere with biosensor function, should they occur. In the case of yellow cameleon, for instance, plasma membrane–targeting was shown to reduce the sensitivity of the reporter for Ca^{2+} (26). This was postulated to result from the formation of heterologous complexes with endogenous CaM located at the plasma membrane (27), though yellow cameleon was previously shown to be a poor binding partner for native CaM in vitro (19). One solution involved mutational optimization of the intramolecular CaM/M13 complex, thereby selecting against interference by native CaM (27). Another way to alleviate this problem is to identify alternative molecular switches that do not associate with CaM. Replacing the CaM/M13 pair from yellow cameleon with a molecular switch derived from the Ca^{2+} -binding protein troponin C (TnC) eliminated any ill effects stemming from endogenous CaM (26). This molecular switch is also fast, allowing the resulting reporters to react very quickly to Ca^{2+} dynamics (28). As alluded to above, in most cases, the kinetic properties of the molecular switch determine how rapidly a reporter can respond to changes in a given cellular parameter, thus controlling its temporal resolution.

INTRACELLULAR SIGNALING BECOMES VISIBLE

The advent of genetically encoded fluorescent reporters has acted as a catalyst for the study of complex, spatially compartmentalized, temporally dynamic signaling networks in their native biological context. Intracellular signaling networks represent the information-processing system by which cells decipher and respond to changes in their environment. For example, receptors on the cell surface detect extracellular cues and mediate the production of intracellular second messengers, which regulate the activity of signaling enzymes. However, being entirely drawn from this diverse yet limited set of components, signaling pathways are not always intrinsically specific. Cells instead link particular stimuli to appropriate responses by exerting control over the spatial arrangement of the signaling machinery and the dynamic properties of signaling processes. By monitoring the behavior of signaling molecules in living systems using genetically encoded fluorescent reporters, it is possible to directly visualize this spatiotemporal organization and understand the logic underlying intracellular signaling networks.

Spatial Organization of the Signaling Machinery

Signaling molecules are often segregated within the cell, and this nonuniform distribution of components helps to maintain specificity during intracellular signaling. Separation restricts the incidence of promiscuous biochemical reactions, while simultaneously affording cells the opportunity to assemble and facilitate specific signaling interactions. The cellular space

is therefore said to be compartmentalized into signaling domains (Figure 2). Genetically encoded fluorescent reporters bring a high degree of resolution to the study of signaling pathways and offer a detailed picture of compartmentalized signaling. Importantly, when using genetically encoded fluorescent reporters to visualize compartmentalized intracellular signals, subcellular targeting can direct these reporters to specific locations, in some cases below the resolution of conventional fluorescence microscopy, and enable the detection of specific pools of signaling activity. This section discusses the use of reporters, positioned in the midst of specific signaling domains, to probe the compartmentalization of signals by subcellular organelles, membrane microdomains, multi-protein complexes, and molecular gradients.

Compartmentalization of intracellular signals by membranes—Just as the plasma membrane divides the cell interior from the extracellular environment, and the endomembrane system divides the cell interior into functionally distinct organelles, so too does membrane compartmentation divide the signaling landscape (Figure 2a). Association with different subcellular compartments may be a general mechanism for modulating the activity of signaling proteins. For example, although the Ca^{2+} - and diacylglycerol (DAG)-dependent protein kinase, protein kinase C (PKC), is thought to signal predominantly from the plasma membrane, PKC is also known to translocate to other subcellular compartments in response to specific stimuli. In fact, targeted versions of a genetically encoded PKC activity reporter were able to detect PKC activity not only at the plasma membrane, but also on the Golgi and mitochondrial membranes, as well as within the cytosol and nucleus (29). Each of these subcellular compartments was further characterized by a unique PKC activity profile, determined by local phosphatase activity and second messenger production. In particular, Golgi-associated PKC remained active much longer than PKC at the plasma membrane (29). Similar behavior was recently reported for the DAG-dependent protein kinase, PKD (30), highlighting a possible role for the Golgi as a specialized signaling platform within the cell. These observations speak to the potential of a general approach using subcellularly targeted biosensors to examine how membrane compartmentation affects the activity of many other ubiquitous signaling enzymes.

Ca^{2+} signaling is also intricately linked to subcellular compartments. Although a ubiquitous intracellular messenger (31, 32), Ca^{2+} is potentially toxic and is excluded from the cytoplasm of all cells (33). Ca^{2+} signals consist of increases in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$), owing to influx across the plasma membrane or release from intracellular stores. The endoplasmic reticulum (ER) is the primary Ca^{2+} store, with the concentration of free Ca^{2+} in the ER ($[\text{Ca}^{2+}]_{er}$) being a critical determinant of cytosolic signaling. Whereas measurement of $[\text{Ca}^{2+}]_{er}$ dynamics can be challenging with previously available methods, such as small-molecule-based fluorescent Ca^{2+} indicators, $[\text{Ca}^{2+}]_{er}$ can be readily monitored using a genetically encoded Ca^{2+} reporter targeted into the ER lumen (34). Quantitative measurements are also possible because the Ca^{2+} -binding affinity of the reporter can be determined following both in vitro and in vivo calibrations. Thus, in HEK293 cells, ER-targeted expression of yellow cameleon revealed $[\text{Ca}^{2+}]_{er}$ to vary from approximately 500 μM in resting cells to 100 μM upon agonist-induced emptying of Ca^{2+} stores (34),

demonstrating how organelle-targeted biosensors can probe signaling domains in living cells.

It has also been suggested that mitochondria affect Ca^{2+} signaling by buffering cytosolic Ca^{2+} increases (35, 36). However, an enduring mystery surrounds the mechanism of Ca^{2+} uptake by mitochondria, whose single low-affinity Ca^{2+} uniporter is inconsistent with the rapid increases in mitochondrial Ca^{2+} concentrations observed upon cytosolic Ca^{2+} influx (37, 38). The existence of microdomains of high Ca^{2+} concentration near the mitochondrial surface, owing to close proximity with ER Ca^{2+} release channels, has been proposed as a mechanism to account for this discrepancy (39–42). Given their small size and transient nature, these microdomains are challenging to study, and direct evidence of their existence has not been forthcoming. Labeling of the mitochondrial and ER membranes with blue fluorescent protein (BFP) and GFP, respectively, revealed these organelles to be closely apposed in an interwoven network, providing indirect support for this hypothesis (41). Furthermore, studies using mitochondrially targeted aequorin, a bioluminescence-based Ca^{2+} reporter, suggested that ER Ca^{2+} release exposes the mitochondrial surface to a burst of Ca^{2+} exceeding the global $[\text{Ca}^{2+}]_c$ increase (41). Yet bioluminescence is typically too dim to provide enough spatial resolution to directly reveal Ca^{2+} microdomains. Instead, recent work with a genetically encoded fluorescent Ca^{2+} reporter, D1cpV, targeted to the outer mitochondrial membrane, finally offered direct visual evidence of ER/mitochondrial Ca^{2+} “hot spots.” The in vitro affinity of this reporter is ideal for monitoring large changes in Ca^{2+} concentration, and pixel-analyses of outer mitochondrial membrane D1cpV-expressing HeLa cells revealed Ca^{2+} microdomains of up to 20 μM covering approximately 10% of the mitochondrial surface during inositol (1,4,5)-trisphosphate (IP_3)-gated Ca^{2+} release (39).

Organelles also play host to specialized machinery responsible for carrying out their characteristic physiological functions. The ER, for one, oversees bulk folding of proteins in the secretory pathway. Protein folding in the ER requires a delicate homeostatic balance, which is actively maintained by the unfolded protein response (UPR) (43). UPR signaling is activated by unfolded proteins, an unusual stimulus that is difficult enough to observe using disruptive techniques, let alone in live cells. However, the ER maintains an oxidation state optimal for protein folding, and disruption of protein folding is likely to upset this balance. On the basis of this reasoning, Merksamer and colleagues (44) took advantage of an ER-targeted, redox-sensitive form of GFP to report on redox potential as a proxy for the accumulation of unfolded proteins in the ER of *Saccharomyces cerevisiae*. Together with a reporter for UPR-dependent transcriptional activation, it was possible to concurrently monitor both acute and downstream responses in populations of wild-type and UPR-deficient mutants treated with a variety of protein-unfolding stressors. This creative application of a rather simple biosensor design revealed functional links between the ER protein folding, modification, and quality control machineries.

Partitioning of signaling components into membrane microdomains—In

addition to compartmentalizing the cell interior, membranes are themselves compartmentalized. Lipid rafts are transient nanostructures, enriched in cholesterol and sphingolipids, that constitute ordered microenvironments within the plasma membrane (for review, see References 45 and 46). Many signaling proteins selectively partition into lipid

rafts, and rafts have garnered attention as signaling platforms because of their involvement in a number of intracellular signaling pathways (46).

Being only 10–200 nm in size, lipid rafts are difficult to study via light microscopy, hindering our understanding of raft-associated signaling events. However, advanced fluorescence microscopy techniques, such as fluorescence correlation spectroscopy (47, 48) and FRET, have permitted some study of the macromolecular organization of lipid rafts. In addition, genetically encoded fluorescent reporters targeted to membrane microdomains are being used to explore the regulation of signaling activity within these microdomains (Figure 2*b*). For example, when targeted to either lipid rafts or nonraft regions, a genetically encoded reporter for the GTPase Ras demonstrated that epidermal growth factor stimulation more rapidly activates Ras in lipid rafts, compared to the remainder of the plasma membrane (49). Conversely, a Src activity biosensor indicated that, although growth factor stimulation promotes coordinated Src activation in lipid rafts (50), Src is more rapidly and strongly activated in nonraft membrane regions (51). Using a similar approach, the serine/threonine kinase Akt was shown to be preferentially activated in rafts in response to growth factor stimulation (52). As we continue to probe membrane microdomains using various techniques including genetically encoded fluorescent reporters, a much more complete picture of the molecular organization and physiological function of raft-mediated signaling pathways will surely emerge.

Assembly of macromolecular complexes as signaling nanomachines—Scaffold proteins coordinate the assembly of signaling complexes, or “signalosomes,” and localize particular combinations of enzymes to specific cellular targets. Cells thereby generate multiple permutations of the signaling machinery, removing many of the constraints imposed by possessing only a finite complement of signaling proteins.

Targeting genetically encoded fluorescent reporters to specific multiprotein complexes therefore offers another strategy for teasing apart local signaling events. The cAMP-dependent protein kinase (PKA) undergoes subcellular localization by association with A-kinase anchoring proteins (AKAPs), which bind the regulatory subunits of PKA and tether the holoenzyme to specific targets (53, 54). Different PKA complexes assembled by distinct AKAPs may contain different PKA isoforms, which possess nonredundant signaling activity. Recently, genetically encoded cAMP reporters, targeted to different complexes associated with type I and type II PKA isoforms by using specific AKAP-binding sequences from the type I and type II regulatory subunits of PKA, revealed that distinct pools of cAMP arose from these signalosomes upon stimulation of the same cell surface receptors, allowing distinct stimuli to promote isoform-specific phosphorylation of cellular targets (55, 56). These studies offer a glimpse into the mechanisms that allow PKA isoforms to engage in nonredundant signaling. Using a similar approach to investigate various macromolecular signalosomes will likely illuminate the regulation and organization of isoform-specific signaling domains in this and other pathways.

An important function of multivalent scaffolds is to bind multiple signaling proteins, assembling them into a coordinated signaling machinery. Signaling dynamics captured in the local environment of these signalosomes could provide insights into signal coordination

and optimization by these multimeric signaling complexes, as suggested by a recent example using the prototypical scaffold protein AKAP79/150 (Figure 2c) (57). In response to muscarinic agonists, PKC phosphorylates the KCNQ2 subunit and inhibits potassium current through the M channel, an important step in regulating neuronal excitability. A genetically encoded C kinase activity reporter (CKAR) revealed that general PKC activity is not properly timed to control muscarinic receptor-induced channel downregulation (57). However, when CKAR was fused to AKAP79/150, local PKC activation was revealed to precisely match the kinetics of M current inhibition, demonstrating that the AKAP79/150-assembled signalosome synchronizes PKC activation with muscarinic stimulation to modulate current through the M channel (57). Future studies could dissect the molecular mechanisms for this temporal coordination.

Several scaffolding proteins have also been identified in mitogen-activated protein kinase (MAPK) cascades, and macromolecular complexes are important for MAPK signaling (58, 59). Use of a genetically encoded reporter for Ras activation recently highlighted the role of the scaffold Shoc2 in accelerating Ras-mediated activation of Raf in the signaling pathway consisting of Ras, Mek-Erk kinase, and extracellular signal-regulated kinase (Erk) (60). Genetically encoded reporters of MAPK activity, which have also recently been developed (61, 62), will undoubtedly fuel additional studies like those described above and provide a great deal of insight into the role of scaffolds in coordinating MAPK pathways.

Spatially restricted signals formed by diffusible gradients—Anchored enzymes give rise to spatial domains of diffusible signaling activity (Figure 2d). The imposition of spatial control by diffusible signals was first proposed by Turing (63) with regard to developmental biology and is now considered a fundamental organizing principle in intracellular signaling (64–66). Monitoring a diffusible signaling gradient is possible using genetically encoded fluorescent reporters, provided the biosensor is sensitive enough to respond to rapid changes in the local environment. The fast kinetics of reporters that detect rapid association-dissociation events are ideally suited for this. For example, a genetically encoded cAMP indicator expressed throughout the cytoplasm was able to detect a roughly linear gradient of cAMP originating at the leading edge of migrating cells (67). Another genetically encoded reporter, whose dissociation from β -importins acts as a marker for GTP-bound Ran, also enabled direct visualization of the RanGTP gradient, which extends outward from the chromosomes toward the spindle poles in both mitotic HeLa cells and extracts from *Xenopus* oocytes (68).

Signals from enzyme activity reporters can be blurred out by diffusion, making it difficult to use them in resolving activity gradients. By contrast, tethering these biosensors to larger structures can limit their mobility and improve spatial resolution. In this manner, a plasma membrane-targeted PKA activity reporter could be used to visualize a PKA signaling gradient in migrating cells that matched the aforementioned cAMP gradient (67). Additionally, expression of a chromosomally targeted activity reporter for aurora B kinase detected a gradient of activity that was centered on the spindle midzone in HeLa cells (69). This aurora B activity gradient was further overlaid by a gradient of aurora B activation, also centered around the midzone, which was controlled by the interaction of aurora B with

spindle microtubules. Consequently, the gradient of aurora B–dependent phosphorylation was hypothesized to mark the position of the spindle midzone (69).

In pituitary cells, fusion of genetically encoded fluorescent reporters to the Ca^{2+} -activated transmembrane adenylyl cyclase 8 revealed the occurrence of both cAMP and Ca^{2+} microdomains in the vicinity of this enzyme (70, 71), underscoring the potential of genetically encoded fluorescent reporters to probe even miniscule signaling gradients. Targeting an aurora B activity reporter to either the centromere or the kinetochore likewise revealed the formation of a nanoscale gradient of aurora B activity when chromosome biorientation physically pulled kinetochore-bound substrates away from centromere-associated aurora B (72), establishing a molecular basis for tension sensing by mitotic chromosomes.

Dynamics of Signaling Pathways

Signal transduction networks operate like complex computational systems, wherein regulatory interactions between signaling molecules form interlinked circuits that control the dynamics of signal flow. Capturing signaling dynamics in real time with genetically encoded fluorescent reporters represents an important advance in untangling the wiring of signaling circuits. Reporters of cAMP accumulation and PKA activity recently brought to light a PDE-dependent negative feedback circuit governing a dose-dependent switch from transient to sustained signaling during β -adrenergic receptor (β AR) stimulation in cardiomyocytes (73). Subnanomolar doses of isoproterenol, a β AR agonist, produced transient bursts of cAMP/PKA signaling, which became sustained upon higher levels of stimulation. Negative feedback from β AR-associated phosphodiesterase 4 (PDE4) was shown to attenuate signaling at low doses, whereas stronger stimulation overcame this threshold by dissociating PDE4 from receptors and enabling persistent cAMP/PKA activity (73).

Bistability, which is the ability of a system to occupy either of two stable states, allows a signaling pathway to convert graded stimuli into switch-like, all-or-none responses (74, 75). MAPKs, such as c-jun N-terminal kinase (JNK), are known to be ultrasensitive and also to exhibit bistability (76). Although this behavior is readily observed using lysates prepared from individual *Xenopus* oocytes, there have been few demonstrations in mammalian systems because the same experimental approach would require assaying populations of cells. However, by communicating the responses from individual cells, genetically encoded fluorescent reporters can probe signaling dynamics that are lost at the population level, as was recently shown with a JNK activity reporter (JNKAR) (61). When expressed in HeLa cells, JNKAR activation was clearly seen to possess ultrasensitivity in response to anisomycin stimulation. In addition, intermediate amounts of stimulation yielded two discrete cell populations with either very high or very low JNKAR activity, revealing a bistable system (61).

Genetically encoded fluorescent reporters can also be used to monitor oscillatory signaling dynamics, such as the Ca^{2+} -dependent PKC activity oscillations observed using CKAR in HeLa cells treated with histamine (77). The Ca^{2+} oscillator has been a topic of extensive research (78), and fluorescent reporters have begun to reveal additional oscillatory signals that are involved in regulating various important cellular processes. For example, patterning

of the retinal ganglion cell (RGC) axonal network involves waves of spontaneous electrical activity that spread across the RGC layer. Though cAMP/PKA signaling had previously been implicated in establishment and refinement of RGC axonal projections, the link between retinal waves and the cAMP/PKA signaling cascade was not understood. Using genetically encoded fluorescent reporters, cAMP and PKA activities were shown to oscillate in response to depolarization-induced Ca^{2+} influx caused by spontaneous electrical activity in RGCs (79). Importantly, single-cell assays are best suited for characterizing such oscillatory behaviors because they can capture activity dynamics, which are often lost when observing asynchronous cell populations. Such oscillations can have important functional roles. For instance, Ca^{2+} , cAMP, and PKA activity were recently shown to undergo coordinated oscillations and form a tightly integrated signaling circuit in pancreatic β -cells. These oscillatory patterns of activation allow PKA to exercise effective spatially localized signaling control, or switch to being a global regulator that can translocate into the nucleus and control gene expression (80). This type of oscillatory signaling circuit may be a more widespread mechanism for integrating diverse inputs to control functional outcomes than currently appreciated. The development and application of genetically encoded biosensors will therefore be critical to investigating various cellular functions that involve oscillatory controls and frequency modulation.

Given the complexity of intracellular signaling dynamics, more quantitative approaches, rather than simplistic wiring diagrams (81), are needed to dissect the spatiotemporal regulation of signaling networks. A recent study combined observations of a genetically encoded reporter for nuclear factor κB (NF- κB) localization with mathematical simulations to study the dynamics of information processing in immune signaling (82). Tumor necrosis factor α (TNF α)-treated cells were shown to produce stereotypical oscillations in NF- κB trafficking, characterized by both digital and analog signaling dynamics. High-throughput imaging revealed the stochastic, rather than deterministic, nature of this pathway, as not all cells in a population responded to TNF α treatment (82). Using a stochastic mathematical model, the authors were then able to recapitulate many of the behaviors of this system, including the dynamic control of gene expression by the interaction of digital and analog NF- κB signaling dynamics. The power of mathematical modeling, coupled with high-throughput, quantitative analyses of single-cell behaviors, will facilitate the development of a more holistic view of intracellular signaling networks.

Integrated Cellular Responses

Signaling networks are the engines of cellular decision making. Within these networks, pathways frequently interact, and pathway cross regulation mediates cellular responses by shifting dynamic equilibria toward particular outcomes. Probing network interactions is no small feat, but the wide range of genetically encoded fluorescent reporters enables the use of multiple biosensors to monitor different, cross regulatory pathways. In neutrophils, uniform exposure to the chemoattractant fMet-Leu-Phe induces spontaneous polarization, and reporters for phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and the GTPase RhoA revealed that mutually negative interactions between these pathways are essential for the self-organization of cell polarity (83). A genetically encoded PKA biosensor also revealed a PKA activity gradient in migrating cells (67). Gradient formation was shown to depend on

the tethering of PKA to integrins at the leading edge, which function as AKAPs, and abolishing these interactions disrupted cell polarity. In addition, localized PKA was required for the accumulation of PIP₃ at the leading edge, visualized using a PIP₃ indicator, suggesting that concerted interactions between PKA and PIP₃ signaling activity help promote and maintain polarity during cell migration (67).

Robust biological outcomes arise via the integration of spatial signaling domains, dynamic circuits, and cross regulatory interconnections. This is elegantly illustrated by the recent use of genetically encoded reporters for cAMP and cyclic GMP (cGMP) to study the differentiation of cultured hippocampal neurons (84). cAMP promotes axon formation, whereas cGMP promotes the formation of dendrites. These messengers are mutually antagonistic, and local fluctuations likely drive symmetry breaking. Reciprocal regulation was observed to generate local double-negative feedback in which cAMP both inhibited the synthesis and stimulated the degradation of cGMP, and vice versa (84). A localized cAMP domain in the developing axon was also seen to promote long-range suppression of cAMP accumulation in the rest of the cell. Conversely, although cGMP accumulation was locally suppressed in the axon, global cGMP levels, and thus dendrite formation elsewhere in the cell, were unaffected (84). Thus, the authors were able to observe in rich detail how these cells combine local and long-range negative feedback between reciprocal pathways to form restricted signaling domains, ensuring the development of several dendrites and only one axon.

Our understanding of network behavior in intracellular signaling will benefit from the ability to simultaneously follow multiple signaling pathways within the same cell. Many strategies are being pursued to enable multiparameter imaging of cellular processes (85, 86). The main challenge is to maximize spectral separation of the biosensors being coimaged, while maintaining a large dynamic range for detection of the signals. Coimaging a genetically encoded, FRET-based biosensor with the fluorescent Ca²⁺ indicator fura-2 is a widely used approach (87), despite some spectral overlap between fura-2 and the popular FRET pair consisting of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). This was recently used to explore cross talk in the regulation of vascular smooth muscle tone, revealing the temporal coordination of Ca²⁺ and cAMP signaling in greater detail (88).

Given the variety of fluorescent protein color variants (89–91), simultaneously imaging multiple genetically encoded fluorescent reporters is also possible (85). However, FRET-based reporters, while being highly versatile in terms of the biological parameters they can detect, are also very challenging to coimage. Some FRET pairs have been developed for coimaging (80, 86, 92–95), but most reporters built for this purpose are still encumbered by difficult spectral separation or diminished dynamic range. Alternatively, some researchers have taken more creative approaches, such as in the development of a biosensor for cGMP using BFP as the donor fluorescent protein and a nonfluorescent YFP variant as a “dark” FRET acceptor (96). This reporter was imaged together with a standard CFP/YFP-FRET cAMP biosensor and the red-shifted Ca²⁺ indicator fura-red, allowing simultaneous tracking of cAMP, cGMP, and Ca²⁺ signaling dynamics. Another strategy combines two FRET-based reporters into a single polypeptide, wherein spectrally distinct donor fluorescent proteins from each biosensor can undergo FRET using a shared acceptor. Such a single-

chain, dual-specificity biosensor was recently employed to simultaneously monitor localized oscillations in PKA activity and cAMP accumulation in pancreatic β -cells (80). As these and other techniques continue to mature and enter the mainstream, widespread application of multiparameter imaging should greatly help unravel the wiring of signaling networks.

LIGHTING THE WAY FORWARD

Though genetically encoded fluorescent reporters have come a long way since their inception, in many ways, we have yet to fully realize the extent to which these biosensors might allow researchers to unravel the intricacies of intracellular signaling. There are ample opportunities to push this technology further and integrate it with other techniques to gain access to greater insights and a more complete understanding of the molecular basis of signal processing and transduction.

Growing the Brand: Expanded Reporter Capacity

One of the most foreseeable future developments of genetically encoded fluorescent reporters is the rapidly expanding list of signaling events that can be captured by these reporters. Moreover, as the design of biosensors continues to progress, genetically encoded fluorescent reporters will become increasingly apt for the study of a growing assortment of biological processes. For example, biosensors have largely been confined to studying a relatively narrow range of signaling processes, such as those occurring within single cells and generally unfolding over the course of several minutes. Yet, biological phenomena vary greatly in both their temporal and spatial scales. Developmental processes, for instance, occur gradually over several hours or days, whereas some enzymatic processes are fleeting. In addition, whereas some processes require the participation of just a few molecules, others only take shape across tissues or entire organisms. What is becoming clear is that all of these processes are potentially open to investigations driven by genetically encoded fluorescent reporters.

Temporal scale—Several studies have already utilized biosensors to monitor the signaling activity dynamics associated with long-term biological processes. A genetically encoded fluorescent reporter was recently used to monitor activation of the GTPase Cdc42 during embryogenesis in *Drosophila melanogaster* (97). Using this reporter, investigators showed that Cdc42 remains inactive throughout the embryo until about 15 h into development; at this point, it becomes activated in several tissues at the onset of organogenesis. In addition, this reporter was combined with genetic studies to examine the importance of Cdc42 activation during the development of the anterior corner cell motor neuron (97).

A novel genetically encoded fluorescent reporter for cyclin B/Cdk1 activity, the major catalytic force promoting entry into M phase, was also recently developed and used to elucidate the coordination of events leading up to mitosis (98). This reporter was modeled on the prototypical kinase activity biosensor design (99), and was used to show that activation of cyclin B/Cdk1 coincides with initiation of prophase, gradually increasing to its maximum activity over a 30-min period. Use of this reporter also revealed that activation of the anaphase-promoting complex/cyclosome, an early event in prophase, requires much lower levels of cyclin B/Cdk1 activity than are needed to promote nuclear envelope

breakdown, which occurs much later, demonstrating how multiple activity thresholds can be set by a single enzyme to trigger sequential processes (98). Both of the aforementioned studies relied on biosensors with ratiometric readouts, which are ideal for monitoring signaling processes over long periods of time because reporter expression levels, as well as sample size and shape, are liable to vary greatly over the course of an experiment.

Although the above examples used FRET-based reporters, biosensors based on other designs can also be used for long-term imaging. For example, fragments derived from the E3 ubiquitin ligase substrates Cdt1 and Germinin were fused to red and green fluorescent proteins, respectively, to generate a dual-color fluorescent ubiquitin-based cell cycle indicator (Fucci) (100). These proteins are reciprocally degraded at the onset of the S and G₁ phases of the cell cycle, respectively, providing an indication of the G₁-to-S transition (100). A similar dual-color indicator system has also been developed based on the translocation of two different biosensors, allowing tracking of all four cell cycle phases (101). In addition, the Fucci system was recently adapted for use in zebrafish, wherein it was used to explore the coordination of cell cycle progression with tissue differentiation (102).

Although developmental processes often last for hours, the pace of some discrete signaling events is more rapid, on the order of seconds or less. For example, cell surface receptors must catalyze the rapid transformation of extracellular cues into intracellular actions, and the activation of G protein-coupled receptors (GPCRs), which involves rearrangement of the transmembrane helical domains, has been shown to elicit downstream signaling events within seconds of ligand binding (103–105). Furthermore, ligand-induced conformational changes within a GPCR are inferred to occur on the order of milliseconds (103, 106). In fact, genetically encoded reporters of GPCR activation are able to reveal that conformational rearrangements in the parathyroid hormone receptor occur within only 40 ms of hormone binding, whereas studies of the α 2A adrenergic receptor indicated slower activation kinetics of about 1 s (107, 108). More recently, allosteric agonists were found to reverse ligand-stimulated activation of the M2 muscarinic receptor with kinetics (80–200 ms) that were much faster than antagonist-induced reversal (>400 ms) (109).

Because fluorescence emission occurs within nanoseconds, it does not impinge on the temporal scale of the signaling events that can be detected with biosensors. Rather, as discussed above (see “Building a genetically encoded fluorescent reporter”), the ability to monitor fast processes using genetically encoded fluorescent reporters is determined by the properties of molecular switches whose kinetics need to be similarly fast, if not faster. For example, a genetically encoded cAMP biosensor recently implicated rapid cAMP degradation in the shaping of signaling microdomains, although the rate of degradation was likely underestimated owing to relatively slow cAMP dissociation from the reporter (110). Similarly, in many genetically encoded Ca²⁺ reporters, the molecular switch responds too slowly to resolve very rapid Ca²⁺ fluctuations. Attempting to address this, Mank and colleagues (28) have developed an enhanced version of their previous TnC-based Ca²⁺ reporter (see above), exhibiting increased dynamic range and faster response kinetics. The off rate of the reporter was found to take the form of a double-exponential decay, with major and minor time constants of 142 ms and 867 ms, respectively, allowing visualization of faster dynamics than can be observed using prior genetically encoded Ca²⁺ reporters (28). In

addition, a small-molecule-based Ca^{2+} indicator, calcium-green FAsH, has been shown to exhibit response kinetics that are faster still, with off rates exceeding $2,000 \text{ s}^{-1}$ (111). This indicator can be targeted to specific subcellular locations via tight binding between its arsenic moiety and a short peptide sequence containing pairs of closely spaced thiols, representing an example of genetically targetable fluorescence (reviewed in References 112 and 113). Continued improvements along these lines will move the use of genetically encodable/targetable fluorescent reporters inexorably toward the study of a wider range of biological processes.

Biological contexts—Living tissues maintain native biological contexts both intra- and intercellularly, and genetically encoded fluorescent reporters are already being used to probe signaling processes in such settings. Recently, a widely used Ca^{2+} reporter, GCaMP-2, was used for Ca^{2+} imaging in olfactory bulb slices to elucidate the role of external tufted cells in controlling the intrinsic electrical rhythms of olfactory bulb glomerular networks (114). In another study, living pituitary slices from mice ubiquitously expressing a cAMP reporter were used in tissue imaging to analyze the effects of somatostatin receptor activation on pituitary cAMP signaling and also to probe the role of specific somatostatin receptor isoforms in mediating the effects of therapeutic somatostatin analogs (115). Inducible gene-expression systems can also be used, which reduces the potential for adverse effects stemming from whole-body reporter expression. For example, transgenic mice expressing a tetracycline-inducible cAMP reporter were used to observe cAMP dynamics in pancreatic β -cells (116). Glucose stimulation was observed to produce synchronous cAMP responses across entire β -cell islets, illustrating the potential to visualize tissue-level behaviors.

Genetically encoded fluorescent reporters can also report on signaling activities in the context of intact organisms. For example, transgenic *Drosophila* expressing the cAMP biosensor, PKA-GFP, enabled visualization of PKA activity in the brains of adult fruit flies (117). However, in vivo imaging, especially in living animals, is complicated by the fact that most fluorescent proteins are excited and emit light at visible wavelengths. Unfortunately, visible light does not penetrate deeply into tissues, as it is readily absorbed and scattered (118, 119). One possible solution is to use two-photon imaging (120, 121) on surgically exposed tissues. Two-photon imaging offers better tissue penetration and reduced photodamage compared to short-wavelength illumination. This technique was recently used with a genetically encoded caspase activity reporter to monitor the dynamics of cytotoxic T-cell activity in mice receiving subcutaneously injected tumors (122).

Light of approximately 650 to 900 nm is best suited for deep-tissue imaging (118, 119), and molecular engineering efforts are also being directed toward shifting fluorescent proteins to longer wavelengths to accommodate this. Mutagenesis of a red fluorescent protein found in the sea anemone *Entacmaea quadricolor* gave rise to the far-red fluorescent protein Katushka, and its monomeric derivative mKate (123). Both of these proteins exhibit excitation and emission maxima at 588 and 635 nm, respectively, and produce much brighter signals compared to other red and far-red fluorescent proteins. Additional engineering of Katushka has led to the development of a pair of near-infrared fluorescent proteins with emission peaks of 650 and 670 nm (124), and an infrared fluorescent protein has also recently been developed on the basis of a bacterial phytochrome, which excites and

emits at 684 and 708 nm, respectively (125). Continued efforts in engineering far-red/infrared fluorescent proteins or genetically targetable fluorophores, and importantly, their evolution and incorporation into the design of biosensors, should facilitate real-time measurement of various signaling activities in deep tissues of living animals.

Spatial scale—When one zooms in to focus on local contexts within a cell, be it during living-cell, tissue, or whole-organism imaging, the spatial resolution is ultimately limited by the diffraction of visible light (~250 nm), which occurs on roughly the same scale as most intracellular structures. As discussed above in the section “Partitioning of signaling components into membrane microdomains,” one way to achieve specific detection of signaling activities within these local contexts is to genetically target reporters to specific microdomains, assuming a targeting motif is available. The need to examine the impact of local biological contexts on signaling events will drive the widespread use of the genetic targeting approach to send a variety of different fluorescent reporters to signaling microdomains, such as specific membrane compartments and molecular signalosomes.

Several techniques have been invented that seek to achieve higher spatial resolution than conventional fluorescence microscopy (reviewed in Reference 126). For example, stimulated emission depletion increases resolution by narrowing the point spread function of the excitation beam with an overlaid depletion laser, which suppresses peripheral emission from out of focus fluorophores. Stimulated emission depletion has already been used for live-cell imaging with fluorescent proteins (127, 128) and targeted fluorophores (129). Another family of super-resolution techniques, known as PALM (130) and STORM (131), utilize fluorophores that can undergo photoconversion either between a nonfluorescent and a fluorescent form (photoactivation) or between two different emission wavelengths (photoswitching). Sparse illumination of the sample results in photoconversion of a subset of fluorophore-labeled molecules, which are imaged and then bleached. Iteration of this process allows the construction of super-resolution images, based on the localization of individual fluorescent molecules. In addition to tracking the localization of specific signaling molecules in super resolution, marriage between super-resolution imaging and other types of genetically encoded fluorescent reporters, for example those involving activity-dependent molecular switches, stands as an exciting path to consider to visualize various signaling processes with even finer spatial resolution than has been possible.

Integrating Targeted Perturbations

Given that signaling pathways are highly dynamic and intimately interconnected, dissecting causal relationships between specific players within these networks requires both reporters for monitoring intracellular signaling events with high spatial and temporal resolution and tools for perturbing these processes with matching resolution. Typically, the activation of pathways is achieved by stimulating cells with hormones or pharmacological agonists. Yet, this top-down approach does not permit examination of individual steps and branches within a signaling pathway. Expressing constitutively active or inactive signaling proteins is one way around this, but this approach is marred by inflexibility in terms of when and where the specific processes can be targeted. Selective perturbation of discrete signaling activities within live cells would therefore be an important addition to our molecular toolkit. Two

basic approaches are available for achieving such targeted perturbations (Figure 3). In the first, an approach based on small-molecule-induced dimer formation is used to drive specific signaling activities at selected times and places in the cell. The other uses light to discretely perturb signaling molecules.

Chemically inducible dimers—Oligomerization controls the activity of many proteins, and exogenously triggered dimerization is an effective means of selectively activating discrete biochemical pathways (132). Using binding domains for the immunosuppressive compound rapamycin, it is possible to generate chimeric proteins that will specifically associate upon treatment with this drug (Figure 3a). Chemical dimerization systems are easily introduced into live cells because the protein components can be genetically encoded, and rapamycin can readily permeate cellular membranes. With this approach, constitutively active and dominant negative forms of Rac were induced to translocate to the plasma membrane, where they were used to rapidly and selectively perturb local signaling activity, and similarly induced localization of a Rac-specific guanine nucleotide exchange factor allowed selective activation of endogenous Rac (133). This chemical dimerization strategy revealed Rac-dependent and -independent branches of phosphoinositide 3-kinase signaling in neutrophil polarization (134). In a recent twist on this technique, a rapamycin-binding domain was introduced into the catalytic domain of the focal adhesion kinase. The resulting fusion protein showed activity only in the presence of rapamycin, demonstrating a novel strategy for engineering direct allosteric regulation of enzymes (135).

Like genetically encoded fluorescent reporters, inducible dimerization systems can be targeted to a variety of subcellular compartments (133, 136). Along these lines, induced dimerization was recently employed to further explore the formation of ER-mitochondrial Ca^{2+} microdomains (137). Targeted expression of the dimerization domains allowed selective induction of ER-mitochondrial junctions upon brief rapamycin treatment. Attaching ratio-metric pericam, a genetically encoded Ca^{2+} reporter, to one of the domains also enabled direct monitoring of Ca^{2+} transients within these junctions. Notably, these transients were dependent on the spacing between the ER and mitochondrial membranes, which could be controlled by linkers introduced into the dimerization domains (137). This opens up the possibility of selectively perturbing signaling processes at specific cellular sites while directly monitoring the outcome of these site-directed perturbations by coexpressing similarly targeted biosensors.

Optically inducible switches—Light alternatively offers a noninvasive approach to targeted perturbations, in contrast to the more passive role it plays in observing these processes. Because light can be precisely manipulated with both high spatial and temporal resolution, it engenders the ability to specify both the timing and location of these perturbations. Several methods have been developed over the years to enable optical control of signaling processes (138). A classic technique uses cell-permeable second messenger analogs, whose biological activities are masked by the presence of a photo-labile protecting group. This method has been successfully applied to a number of intracellular messengers, including Ca^{2+} , nitric oxide (NO), cyclic nucleotides, and IP_3 (reviewed in Reference 139), and a membrane-permeant, photoactivatable phosphatidylinositol 3-phosphate analog was

also recently developed (140). Given their widespread use, it should come as no surprise that these molecules have already been used with genetically encoded fluorescent reporters. For example, uncaging of a cAMP analog was used to transiently activate PKA (141) and also to generate cAMP gradients in neonatal cardiac myocytes (142), both of which could be observed using genetically encoded reporters.

Several photoactivatable proteins are also available, extending optical control to channel activity, enzymatic activity, and protein-protein interactions. For example, a number of light-sensitive ion channels, related to bacterial rhodopsin proteins, have been discovered (Figure 3*b*) (143–146). The single-celled flagellate, *Euglena gracilis*, was also found to possess a photoactivatable adenylyl cyclase (147). Researchers have additionally engineered some of their own photoswitchable proteins, including a light-inducible ionotropic glutamate receptor (148, 149). A photoactivatable Rac variant has also been generated (Figure 3*c*) (150) and used to monitor the effects of localized Rac activation in both *Drosophila* and *Xenopus* transgenic animals (151, 152). Furthermore, a light-activated, reversible protein-protein interaction system was also developed, offering a noninvasive alternative to chemically induced dimerization (153). Although exogenous cofactors are needed in some cases to assemble the optically inducible switches, these systems are at least genetically targetable and are in principle more amenable to some in vivo applications where noninvasive approaches for selectively triggering cellular processes are highly desirable.

Genetically encoded fluorescent reporters and genetically targetable perturbations represent the ability to acutely perturb and continuously monitor signaling processes (154) within living biological systems. Systematic dissection of the interrelationship between signaling components using these technologies, combined with quantitative modeling, should lead to a more comprehensive understanding of dynamic signaling networks.

Tapping into Native Biochemistry

Like many fundamental biological processes, signal transduction pathways use chemistry as the basis for their molecular logic. Traditionally, studying the chemistry of biological molecules involved quantitative analyses of their properties, behaviors, and activities in test tubes. Using this in vitro biochemistry approach, scientists have learned a great deal about the actions and properties of specific biomolecules, enabling the elucidation and mapping of the biochemical pathways that contribute to cellular functions. However, it is becoming clear that biological context plays a critical role in governing the behaviors of biomolecules, keenly influencing their functional outcomes. With the help of the new molecular tools discussed here, in particular genetically encoded fluorescent reporters, it is possible that biochemical studies can instead be performed directly in living biological systems (155–158).

Such native biochemistry studies make it possible to reveal behaviors of biomolecules that only occur in their native biological contexts, thereby enabling the elucidation of complex regulation. For example, de novo purine biosynthesis involves the actions of six enzymes that carry out ten consecutive chemical steps. Although in vitro studies have provided little evidence for interactions between these enzymes, cellular imaging using fluorescent protein-

tagged enzymes revealed that a dynamic multienzyme complex, or “purinosome,” exists in cells to carry out purine biosynthesis in a highly coordinated fashion (155).

Furthermore, native biochemistry can help characterize the influence of specific cellular properties on the actions of various biomolecules while also enumerating the parameters of the relevant biochemical reactions. A recent study combined biosensor imaging, immunohistochemistry, and mathematical modeling to demonstrate that certain cellular geometries, in particular dendritic diameter and surface-to-volume ratio, favor local accumulation of specific molecules near the plasma membrane in the establishment of PKA and MAPK microdomains (157). In another example, using a novel approach termed enzyme substrate imaging, measurement of the Michaelis-Menten kinetics of protein tyrosine phosphatase 1B (PTP1B) revealed an enzyme substrate gradient and demonstrated the spatial regulation of PTP1B activity (158).

Importantly, native biochemical studies can also analyze biochemical parameters of molecules in contexts wherein their functional cellular partners are preserved, thereby linking biochemical changes to functional effects. In a recent example, a FRET-based reporter of CaM was used to determine the concentration of free, Ca^{2+} -unbound CaM, or apoCaM, in live cells containing functioning Ca^{2+} channels, and electrophysiology was used to characterize channel regulation. This quantitative analysis allowed the authors to discover a novel mechanism of channel regulation, whereby the distal carboxyl tail of a channel can retune channel affinity for apoCaM so that natural variations in CaM concentration affect the strength of Ca^{2+} -dependent feedback modulation (156).

Together, the insights gained through native biochemistry can enrich and advance our knowledge about various biomolecules involved in cellular processes. With regard to understanding signal transduction processes, native biochemistry provides a platform naturally suited to quantitative characterization of the biochemical properties, behaviors, and activities of various signaling molecules in the native contexts of living biosystems, which should lead to a more comprehensive understanding of signaling networks.

CONCLUDING REMARKS

Dawn is only just breaking for genetically encoded fluorescent reporters. Tremendous insights have been obtained into the spatiotemporal organization that informs the logic of intra-cellular signaling, yet there is a vast potential for these molecular tools to probe and uncover the spatial and temporal details of biological processes on a variety of scales. Although we have focused on molecular processes underlying intracellular signaling events, the applications of these genetically encoded fluorescent reporters are much broader (159), given the flexibility in adapting this technology for visualizing a variety of biochemical and biophysical processes (160). This broad applicability, combined with the capability of capturing molecular events in their native biological contexts and suitability for quantitative analyses, renders this reporter technology at the forefront of several emerging technologies suited for studying various phenomena in their native biological contexts. Together, these technologies promise to unravel the molecular logic of signal transduction and, in a broader context, various other biological phenomena.

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Glossary

Fluorescence resonance energy transfer (FRET)	a through-space transfer of excited-state energy from a donor fluorophore to an acceptor fluorophore
Bimolecular fluorescence complementation	the interaction between two proteins fused to complementary fragments of a “split” fluorescent protein regenerates the functional fluorescent protein
Aequorin	a Ca^{2+} -dependent bioluminescent protein originally discovered in the jellyfish <i>Aequorea victoria</i>

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SUMMARY POINTS

1. Genetically encoded fluorescent reporters are a diverse family of molecular tools capable of visualizing dynamic signaling events in real-time and in live biosystems, including cells, tissues, and organisms.
2. The design of genetically encoded fluorescent reporters is highly modular and adaptable to a multitude of biochemical and biophysical processes.
3. Subcellular targeting allows genetically encoded fluorescent reporters to probe the mechanisms of compartmentalized signaling.
4. Measuring the dynamics of signaling processes can be achieved at the single-cell level using genetically encoded fluorescent reporters and, when combined with mathematical modeling, can help dissect the wiring of the signaling networks.
5. Parallel application of multiple fluorescent reporters to observe interrelated signaling pathways can be used to unravel the network behaviors involved in cellular decision making.

FUTURE ISSUES

1. Given the complex interrelationship between signaling molecules within networks, the ability to perform multiparameter imaging is desirable. Reporters with better signals and increased spectral separation are needed for improved coimaging to simultaneously track multiple cellular parameters within individual cells.
2. The development of molecular switches with faster response kinetics will help increase the temporal resolution of biosensors and aid the detection of fast signaling processes.
3. In vivo imaging studies will benefit from improvements in the engineering of far-red and infrared fluorophores.
4. Further advances in super-resolution microscopy and the marriage between super-resolution imaging and different types of genetically encoded fluorescent reporters should allow visualization of various signaling processes with finer spatial resolution than has been possible.
5. Further integration of genetically encoded fluorescent reporters with methods for genetically targeted biochemical perturbations will significantly increase the ability of native biochemistry to unravel the molecular basis of biological phenomena.

Figure 1.

Major classes of genetically encoded fluorescent reporters. (a) Reporters for visualizing the native localization of endogenous proteins. Direct fusion to a fluorescent protein enables observation of the subcellular distribution of numerous proteins. Shown are epifluorescence images of MIN6 cells expressing a fluorescent protein-tagged form of the type II regulatory subunit of protein kinase A (PKA) (*left*) and HEK293 cells expressing the β 1 adrenergic receptor tagged with a fluorescent protein (*right*), along with respective schematic illustrations of green fluorescent protein (GFP) fused to either a cytosolic (*left*) or a transmembrane protein (*right*). (b) Reporters based on effector-guided translocation. Fusion of a fluorescent protein to a specific effector domain can be used to monitor second messenger dynamics. As illustrated here, conversion of phosphatidylinositol (4,5)-bispophosphate (PIP₂) into inositol (1,4,5)-trisphosphate (IP₃) and diacylglycerol (DAG) causes dissociation of a particular fluorescent protein-tagged pleckstrin homology (PH) domain from the plasma membrane. Confocal fluorescence images show reporter distribution before (*left*) and after (*right*) phospholipase C (PLC) activation in Cos-7 cells. (c) Reporters based on kinase/phosphatase activity. Fusion of a fluorescent protein to a substrate can be used to monitor kinase/phosphatase activity. As illustrated here, activation of a kinase (ATP → Kinase → Phosphatase → P_i) causes the substrate to bind to a fluorescent protein (GFP), leading to a change in fluorescence intensity. Confocal fluorescence images show reporter distribution before (*left*) and after (*right*) kinase/phosphatase activation in Cos-7 cells.

(c) Reporters in which a molecular switch is used to detect a biochemical event. Molecular switches can be designed to respond to a variety of parameters. Fluorescence resonance energy transfer (FRET)-based reporters utilize molecular switches sandwiched between donor and acceptor fluorescent proteins, such as CFP and YFP (*left*). In a kinase activity reporter, phosphorylation (*red circle*) mediates conformational changes in the kinase activity-dependent molecular switch, modulating FRET between the fluorescent protein pair. Alternatively, a molecular switch based on a pair of interacting proteins, such as calmodulin (CaM) and the M13 peptide, can be inserted within a fluorescent protein (*right*). Ca^{2+} binding causes a conformational rearrangement that increases fluorescence intensity. Images in panel c depict HeLa cells expressing the PKA activity reporter AKAR, along with ratiometric images showing the FRET response of AKAR upon forskolin treatment (*left, bottom*), and HEK293 cells expressing the Ca^{2+} indicator GCaMP responding to thapsigargin treatment (*right, bottom*). Images colored to reflect increasing FRET (*left*) or increasing fluorescence intensity (*right*).

Figure 2.

Studying compartmentalized signaling with subcellularly targeted biosensors. (a) Local signaling within and around subcellular organelles. By incorporating a particular targeting motif into the primary sequence of a genetically encoded reporter, it is possible to detect specific signaling activities at subcellular loci including, but not limited to (i) the cytosol, (ii) the plasma membrane, (iii) the nucleus, (iv) the Golgi apparatus, (v) the endoplasmic reticulum, and (vi) the mitochondria. (b) Signaling events within membrane microdomains. Reporters can be further localized to membrane microdomains using specific targeting

motifs and can distinguish between raft- and nonraft-associated signaling activities. (c) Coordinated macromolecular signalosomes. Instead of relying on subcellular targeting motifs, reporters can be targeted to macromolecular complexes, for example, via direct fusion to scaffolding proteins, allowing for visualization of biochemical events specific to these complexes. (d) Diffusible biochemical gradients. Biosensors can also be used to observe gradients of signaling activity. Given that it can rapidly respond to changes in its immediate environment, the response of a reporter will vary depending on its position within the gradient. AC, adenylyl cyclase; AKAP, A-kinase anchoring protein; Akt, a serine-threonine protein kinase; PKC, protein kinase C.

Figure 3.

Three examples of targeted perturbations. (a) In chemically inducible dimer formation, rapamycin drives the association of an FKBP-rapamycin-binding domain (FRB domain) with the 12-kDa FK506-binding protein (FKBP12). This can be used, for example, to direct the activity of a given enzyme toward substrates in a location-specific manner. (b) Channelrhodopsins are relatives of the bacterial rhodopsins. These ion channels are inactive in the dark and become activated upon the light-induced isomerization of an associated retinol moiety. (c) Photoactivatable Rac consists of the light-oxygen-voltage (LOV) domain and the Jα helical extension from the *Avena sativa* phototropin 1 protein fused to the N terminus of a constitutively active Rac isoform. In the absence of illumination, a complex between the LOV and Jα domains prevents Rac from interacting with its effector proteins. A reversible, light-induced conformational change disrupts the LOV-Jα complex, permitting the expression of Rac activity with a high degree of temporal and spatial control.