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Single-cell Analysis of G-protein Signal Transduction*

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Terri Clister[‡], Sohum Mehta[‡], and Jin Zhang[‡§1]

From the [‡]Department of Pharmacology and Molecular Sciences and [§]The Solomon H. Snyder Department of Neuroscience and Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The growing use of fluorescent biosensors to directly probe the spatiotemporal dynamics of biochemical processes in living cells has revolutionized the study of intracellular signaling. In this review, we summarize recent developments in the use of biosensors to illuminate the molecular details of G-protein-coupled receptor (GPCR) signaling pathways, which have long served as the model for our understanding of signal transduction, while also offering our perspectives on the future of this exciting field. Specifically, we highlight several ways in which biosensor-based single-cell analyses are being used to unravel many of the enduring mysteries that surround these diverse signaling pathways.

All cells rely on signal transduction to communicate extracellular information to the intracellular machinery. In particular, G-protein signaling controls a multitude of diverse cellular functions, including responses to hormonal signals and environmental stimuli such as light and odor. It is estimated that ~1000 human genes encode G-protein-coupled receptors (GPCRs),[2] the initiators of G-protein signaling (1). Understanding G-protein signaling is therefore essential to unravel important signaling processes and to determine how the disruption of these processes can lead to disease. G-protein signaling begins with the activation of a GPCR by a corresponding ligand, which induces a conformational change in the receptor that transduces the external signal into the cell. This conformational change results in the recruitment and activation of heterotrimeric G-proteins, composed of Gα, Gβ, and Gγ subunits (G$\alpha\beta\gamma$). Specifically, the GPCR acts as a guanine nucleotide exchange factor (GEF) and converts the Gα subunit into its GTP-bound activated state. Once activated, Gα dissociates from the G$\beta\gamma$ dimer to activate various downstream effectors. Different Gα isoforms are known to associate with different GPCRs and/or effectors; thus, the specific downstream effects depend in part on the particular isoform that is activated. These effectors generate second messengers that both amplify the initial signal and modulate various downstream targets. G-protein signaling is regulated by the intrinsic GTPase activity of Gα, as well as by arrestin, which promotes GPCR internalization and mediates additional signaling pathways (2).

This classical view of G-protein signaling, revealed through years of painstaking biochemical study, has long served as a model for our understanding of intracellular signal transduction. However, recent studies are providing a more nuanced understanding of G-protein signaling through the use of single-cell analyses powered by fluorescent biosensors. These genetically encoded molecular tools offer a rapid and dynamic readout that enables the detection of biochemical activities within the native environment of a living cell and provide a unique platform for visualizing temporal and spatial information that traditional biochemical approaches often fail to capture. Such spatial and temporal information is crucial to our understanding of signaling dynamics and the complex interplay between different signaling cascades.

In this review, we first provide a brief summary of common biosensor design strategies. We then describe various studies that have used fluorescent biosensors and single-cell analyses to probe different aspects of G-protein signaling, specifically focusing on novel insights into GPCR activation, heterotrimeric G-protein dynamics, and second messenger production. Finally, we discuss some of our thoughts regarding the application of these techniques as well as the future of single-cell analyses of G-protein signaling.

Fluorescent Biosensors for Single-cell Analyses

Biosensors are engineered constructs that couple the detection of a biochemical event to an optical signal. Genetically encoded fluorescent biosensors in particular have greatly enhanced the study of biochemical processes in living cells (see Ref. 3). These modular tools are typically composed of a sensing unit that detects a specific biochemical activity and induces an observable change in the fluorescent signal from a reporting unit. For example, many proteins change their localization in response to the appearance or disappearance of binding partners in particular subcellular regions. Translocation-based biosensors, which combine a binding domain with a single fluorescent protein (FP), are thus able to report on the presence of specific molecules through the redistribution of fluorescence (Fig. 1*a*). The sensing unit can also comprise a molecular switch that modulates the fluorescent properties of the reporting unit. Generally, molecular switches are derived from proteins or protein fragments whose conformation changes in response to specific input signals. Inserting a molecular switch into a single FP results in a biosensor whose fluorescence intensity changes in response to an activity of interest (Fig. 1*b*). Alternatively, a molecular switch can be coupled to a pair of FPs that are capable of undergoing FRET (Fig. 1*c*), which involves the non-radiative transfer of excited state energy from a donor fluorophore to a nearby acceptor (4). These basic designs comprise a diverse

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[1] To whom correspondence should be addressed: Dept. of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 N Wolfe St., Baltimore, MD 21205. Tel.: 410-502-0173; Fax: 410-955-3023; E-mail: jzhang32@jhmi.edu.

[2] The abbreviations used are: GPCR, G-protein-coupled receptor; GEF, guanine nucleotide exchange factor; GRK, G-protein-coupled receptor kinase; α_2-AR, α_2-adrenergic receptor; β_2-AR, β_2-adrenergic receptor; PTHR, parathyroid hormone receptor; FP, fluorescent protein; FKBP, FK506-binding protein; AC, adenylyl cyclase; FlAsH, fluorescein arsenical hairpin binder; CID, chemically inducible dimerization.

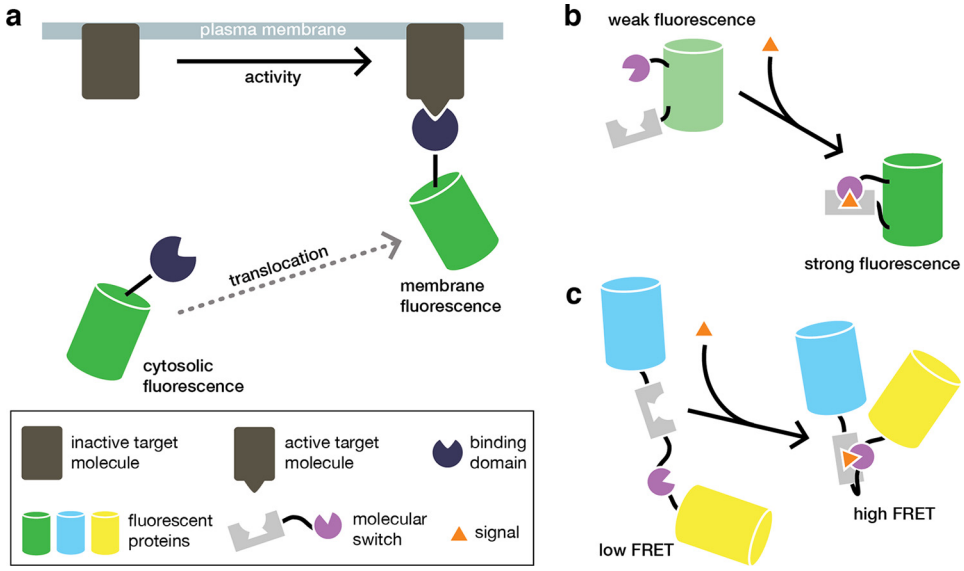

FIGURE 1. **Standard approaches for biosensor design.** *a*, translocation-based biosensors are generated by fusing a fluorescent protein to a protein or protein domain that specifically recognizes a molecule of interest. Here, GFP is fused to a binding domain that specifically recognizes the active conformation of a target protein located in the plasma membrane. The target protein becomes activated in response to an upstream signal, which induces the biosensor to translocate to the membrane and results in the redistribution of the fluorescent signal from the cytosol to the plasma membrane. *b*, in this example of an intensity-based biosensor, a molecular switch is inserted within a fluorescent protein such as GFP. The molecular switch consists of two protein fragments that associate in response to a specific input signal, which leads to a conformational change in the molecular switch and an increase in the fluorescence intensity of the biosensor. *c*, molecular switches are also used to generate FRET-based biosensors. Here, the molecular switch consists of two protein fragments fused in a single polypeptide that is sandwiched between two proteins capable of undergoing FRET. In response to the input signal, the molecular switch undergoes a conformational change that alters the distance and relative orientation of the fluorescent proteins, thereby resulting in a FRET change.

molecular toolkit for visualizing a myriad of signaling events in cells. Furthermore, because these biosensors can be directly expressed in and targeted throughout the cell, they are particularly powerful tools for monitoring biochemical processes at specific subcellular locations, which has proven invaluable for studying signaling dynamics.

Visualizing GPCR Signaling Behavior

GPCRs are a major family of plasma membrane receptors that are characterized by seven-transmembrane domains and classified according to their known structure and function. Understanding how these receptors transduce signals is essential to resolving the specificity and interplay of their downstream effects. In the classical model, ligand binding to a GPCR induces a conformational change in the receptor that activates Gα, which then dissociates from the receptor to activate effectors. Under prolonged ligand signaling, GPCRs are phosphorylated by G-protein-coupled receptor kinases (GRKs), leading to the recruitment of arrestin. GPCRs are then internalized via endocytosis, inactivated, and subsequently recycled back to the plasma membrane or targeted for degradation in the lysosome or proteasome. Biosensors have been applied in three general approaches to visualize GPCR signaling, mirroring the different steps in GPCR activation: 1) examining ligand-induced conformational changes; 2) monitoring G$\alpha\beta\gamma$ coupling; and 3) monitoring arrestin binding.

The first approach depends on conformational changes in the ligand-bound GPCR. Several biosensors have been developed to monitor GPCR activation and explore the functional effects of GPCR conformational dynamics. Vilardaga *et al.* (5) developed a pair of GPCR biosensors, based on the α_2-adrenergic receptor (α_2-AR) and the parathyroid hormone receptor (PTHR), which were used to determine the unique kinetics of each receptor (~40 ms and ~1 s, respectively). These biosensors were generated by inserting CFP into the third intracellular loop of each receptor, with YFP fused to the C terminus, such that ligand-induced conformational changes in the receptor cause a change in FRET (Fig. 2*a*). Unfortunately, the intramolecular FP adds bulk that can adversely interfere with downstream signaling. This same group created a modified sensor to mitigate this issue by using FlAsH (fluorescein arsenical hairpin binder), a small-molecule dye that only fluoresces when bound to a specific peptide sequence. Hoffman *et al.* (6) replaced the FP in the intercellular loop of α_2-AR with the binding motif for FlAsH and fused CFP to the C terminus of the receptor to make an improved GPCR biosensor. The reduced bulk improved the biosensor response 5-fold without changing the observed kinetics or inhibiting downstream signaling. These early GPCR biosensors set the stage for further studies of GPCR signaling.

Multiple experimental studies have demonstrated that GPCRs adopt distinct conformations in response to different ligands, leading to the hypothesis that different downstream signaling pathways are coupled to specific GPCR conformations (7–13). Recently, Malik *et al.* (14) created a series of biosensors that contain the GPCR-binding domain of different Gα subunits to study ligand-specific conformational changes and subsequent differences in downstream effects. These biosensors contain full-length β_2-AR followed by YFP, a flexible linker, CFP, and a C-terminal fragment from a particular Gα subunit (14) (Fig. 2*b*). Gα proteins have been shown to bind a cytosolic groove on activated GPCRs, and the C terminus of Gα is important for transducing signals between GPCRs and G$\alpha\beta\gamma$. By creating multiple biosensors, each containing a

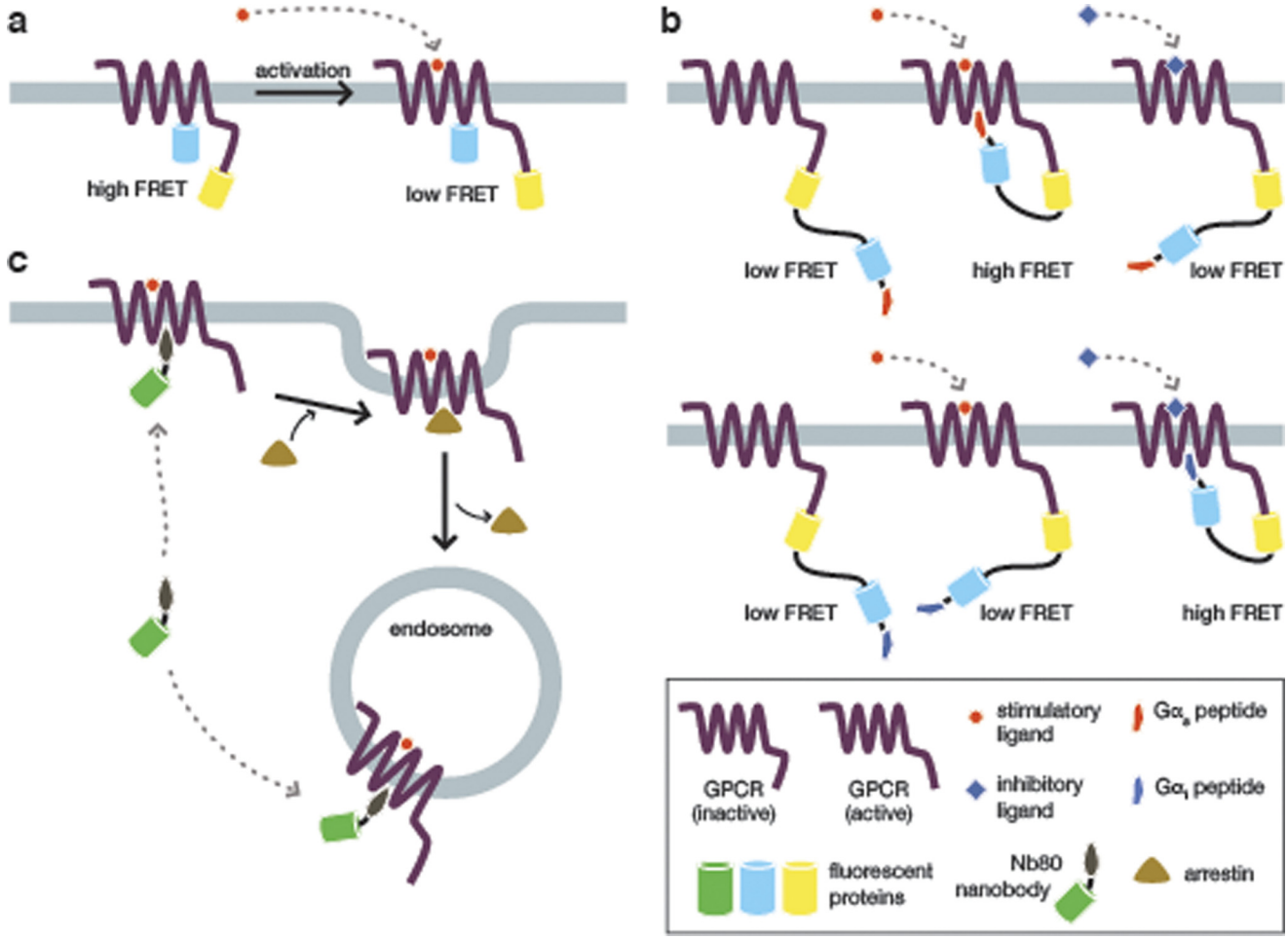

FIGURE 2. **GPCR activation dynamics revealed using biosensors.** *a*, a FRET-based biosensor for measuring GPCR activation dynamics. Vilardaga *et al.* (5) generated a pair of biosensors based on the α_2-AR and the PTHR, in which CFP was inserted within the third intracellular loop and YFP was fused to the C terminus of the full-length receptor. In both biosensors, receptor activation leads to a conformational change that decreases the FRET between the two fluorescent proteins. When expressed in cells, these biosensors made it possible to directly visualize the ligand-induced conformational dynamics of these two receptors with millisecond precision. *b*, a family of biosensors illuminates ligand-specific conformational changes and G-protein coupling. In a recent study, Malik *et al.* (14) generated a panel of biosensors consisting of the β_2-AR fused to YFP, a flexible linker, and CFP. Each sensor also contained a fragment from a particular Gα protein at the far C terminus. In cells expressing the biosensor variant containing a Gα_s fragment (*upper panel*), only a corresponding "stimulatory" agonist was able to activate the biosensor and elicit a FRET response. Conversely, only an "inhibitory" agonist was able to elicit a FRET change in cells expressing the Gα_i-fused biosensor (*lower panel*), thus demonstrating that different conformational changes induced by specific ligands can link a single receptor to diverse downstream pathways. *c*, detecting endogenous GPCR activation in endosomes. Most GPCR biosensors report on the behavior of exogenously expressed GPCR constructs. Thus, to investigate endogenous GPCR dynamics, Irannejad *et al.* (18) fused GFP to a nanobody that specifically binds the active conformation of the β_2-AR. This probe decorates the plasma membrane in stimulated cells but is displaced following the binding of arrestin during GPCR internalization. However, this translocation-based biosensor was subsequently observed to label the resulting endosomes, indicating that the β_2-AR is still active in these compartments.

unique Gα subunit, it is possible to probe the link between a specific Gα and GPCR activation induced by different ligands. For example, the authors found that treating β_2-AR with one of two inhibitory ligands enhanced the binding of Gα_i over Gα_s. These results confirm previous studies that established Gα_i as being responsible for inhibitory GPCR signaling and Gα_s as being responsible for stimulatory GPCR signaling. Furthermore, structural studies have shown that a highly conserved (E/D)RY motif in β_2-AR forms an "ionic lock" with neighboring residues when cells are treated with an inhibitory ligand, and mutating certain residues in this motif has been shown to cause constitutive GPCR signaling (15–17). Using the aforementioned biosensors, the authors found that mutating either Glu/Asp or Arg increased the association of Gα_s to β_2-AR; however, as with previous studies, only the Glu/Asp mutations induced the increased cAMP response caused by Gα_s signaling. Using these modified FRET-based GPCR biosensors, this group was able to directly visualize the links between specific ligand-induced conformational changes and the downstream effects controlled by specific Gα proteins.

Classically, endocytosis is thought to result in the inactivation and recycling of GPCRs; however, the question of whether GPCRs are inactive in endosomes has recently been debated. To examine this question directly and determine whether GPCRs could remain active in endosomes, Irannejad *et al.* (18) developed a translocation-based biosensor that specifically binds the β_2-AR in its active conformation to probe endogenous GPCR activation (Fig. 2*c*). This biosensor comprises GFP fused to a nanobody, Nb80, that specifically binds activated β_2-AR after isoprenaline treatment. The nanobody is competed off by arrestin binding, which leads to receptor endocytosis. This group observed that GFP-tagged Nb80 translocated to the

plasma membrane upon β_2-AR activation and then again to endosomes once the receptor was internalized, revealing that at least a subset of endogenous β_2-AR is active in endosomes. The biosensor used in this study binds endogenous β_2-AR, thereby circumventing concerns regarding artifacts due to exogenous GPCR biosensor expression and providing valuable insights into endosomal GPCR activity.

GPCR activity can also be monitored via the dissociation of G$\alpha\beta\gamma$ or by the binding of arrestin. One question that has remained unclear in the field is whether G$\alpha\beta\gamma$ associates with GPCRs prior to receptor activation or whether they are only recruited to the receptor after its conformational change. Nobles *et al.* (19) used a bimolecular FRET sensor to study the interaction between G$\alpha\beta\gamma$ (Gα_o or Gα_s, Gβ_1, and Gγ_2) and multiple receptors (α_2-AR, muscarinic acetylcholine receptor M_4, A_1 adenosine receptor, and D2S dopamine receptor). Each component was tagged with either CFP or YFP, and the FRET responses were monitored between different combinations of G-protein and receptor. The authors used these biosensors to demonstrate that specific G-proteins tend to "precouple" with specific receptors. For example, α_2-AR precouples with Gα_o but not with Gα_s, whereas the known Gα_s receptor prostacyclin precouples with Gα_s but not with Gα_o. This precoupling model conflicts with other studies that instead suggest a diffusion-controlled model for the interaction between GPCRs and heterotrimeric G-proteins. These studies used FRET biosensors for PTHR and α_{2A}-AR but did not observe any precoupling (12, 20). The reason for these discrepancies is unclear, and further studies are thus needed to resolve the nature of G-protein-receptor coupling.

The binding of arrestin to GPCRs is another indicator of GPCR activity, specifically long-term GPCR activity leading to endocytosis. Arrestin is recruited to phosphorylated GPCRs and is necessary for receptor endocytosis via clathrin-coated pits. Violin *et al.* (21) developed a bimolecular FRET reporter of arrestin binding to study the specificity of GRKs, which are the enzymes responsible for phosphorylating GPCRs and hence recruiting arrestin. The authors fused CFP to the β_2-AR and YFP to β-arrestin and found that the recruitment of β-arrestin to β_2-AR can act as an indicator of endogenous and exogenous GRK activity. This study also revealed a high degree of redundancy in GRK specificity, with the amount of GRK activity being proportional to the kinetics of the arrestin-receptor interaction, leading the authors to conclude that the regulation of GRK, and subsequent GRK regulation of GPCRs, is a mechanism to control the length of GPCR activation. Krasel *et al.* (22, 23) also used FP-fused proteins to study the interaction between β_2-AR and β-arrestin. These FRET studies, which use the same biosensor design described above, reveal the kinetics of β-arrestin binding to the receptor, as well as the reliance of this interaction on GRK activity. The authors also found that the C terminus of the receptor aids in β-arrestin binding and subsequent receptor internalization, which they suggest may occur through the recruitment of other proteins to aid in internalization.

A more recent study used multiple FRET biosensors to study interactions between PTHR and both arrestin and G$\alpha\beta\gamma$ (24). For some GPCRs, such as the β_2-AR, G$\alpha\beta\gamma$ and arrestin are generally thought to bind sequentially. However, studies have suggested that G$\alpha\beta\gamma$ and arrestin can bind PTHR together. Previous work by Feinstein and colleagues (12, 25) showed that, unlike β_2-AR, PTHR induced prolonged cAMP signaling. PTHR-arrestin complexes were specifically associated with this prolonged cAMP signaling, a finding that calls into question the classical model of G-protein signaling and recycling. To understand how PTHR signaling and β_2-AR signaling differ with regard to prolonged cAMP signaling, Wehbi and colleagues (27) used multiple FRET biosensors, which were described previously (reviewed in Ref. 26) to study the interactions between PTHR, arrestin, and G$\alpha\beta\gamma$ by tagging PTHR, Gα_s, G$\beta\gamma$, and arrestin with CFP or YFP and monitoring FRET between pairs of tagged proteins. Using this approach, arrestin and G$\beta\gamma$ were found to associate with each other and simultaneously bind the PTHR. This arrangement prolonged cAMP production (as previously observed) (12, 25) through continued Gα_s activation from within endosomes, thereby revealing the mechanism underlying prolonged cAMP signaling in the PTHR pathway. Prolonged cAMP signaling, based on receptor internalization via arrestin, was also shown to be the main downstream signaling difference between two agonists of the V2 receptor (V2R) (27).

Monitoring the Dynamics of Heterotrimeric G-proteins

Following GPCR activation, G$\alpha\beta\gamma$ carries the signal to various downstream effectors. Classically, G$\alpha\beta\gamma$ dissociates from the GPCR and disassembles after the GPCR activates Gα by exchanging its bound GDP for GTP. There are multiple isoforms of the Gα subunit, each of which is involved in specific downstream signaling (28). For instance, Gα_s induces adenylyl cyclase (AC) to produce cAMP, whereas Gα_i inhibits AC activity. Biosensors that focus on G$\alpha\beta\gamma$ are thus important tools for studying the diversity and specificity of G-protein signaling. FRET biosensors can be used to monitor G$\alpha\beta\gamma$ activity through the disassembly of the subunits or through the association of a G-protein subunit with an effector, which forms the next step of the G-protein signaling pathway.

Janetopoulos *et al.* (29) created the first FRET biosensor for G$\alpha\beta\gamma$ signaling based on the classical understanding of the dissociation of Gα from the G$\beta\gamma$ dimer by fusing YFP and CFP to Gβ and Gα_s, respectively. This group observed a transient, decreasing FRET response in *Dictyostelium discoideum* upon GPCR activation, corresponding to the dissociation of the heterotrimeric components. Bünemann *et al.* (30) then modified this design to study the dissociation of the Gα_i family of inhibitory G-proteins by fusing YFP to Gα_i, and by fusing CFP to three different regions of the G$\beta\gamma$ dimer (Fig. 3*a*). Interestingly, two of the three resulting biosensors produced increasing FRET responses, which is contrary to the expected response if the heterotrimer subunits were dissociating from each other. This implies a continued interaction between the tagged subunits upon GPCR activation and questions the classical view of G$\alpha\beta\gamma$ disassembly upon the activation of G-protein signaling, at least for Gα_i. These results suggest that, unlike the dissociation observed with Gα_s, Gα_i proteins undergo a conformational rearrangement but do not fully dissociate. These conclusions are supported by fluorescence recovery after photobleaching (FRAP) experiments that show the same dissociation or contin-

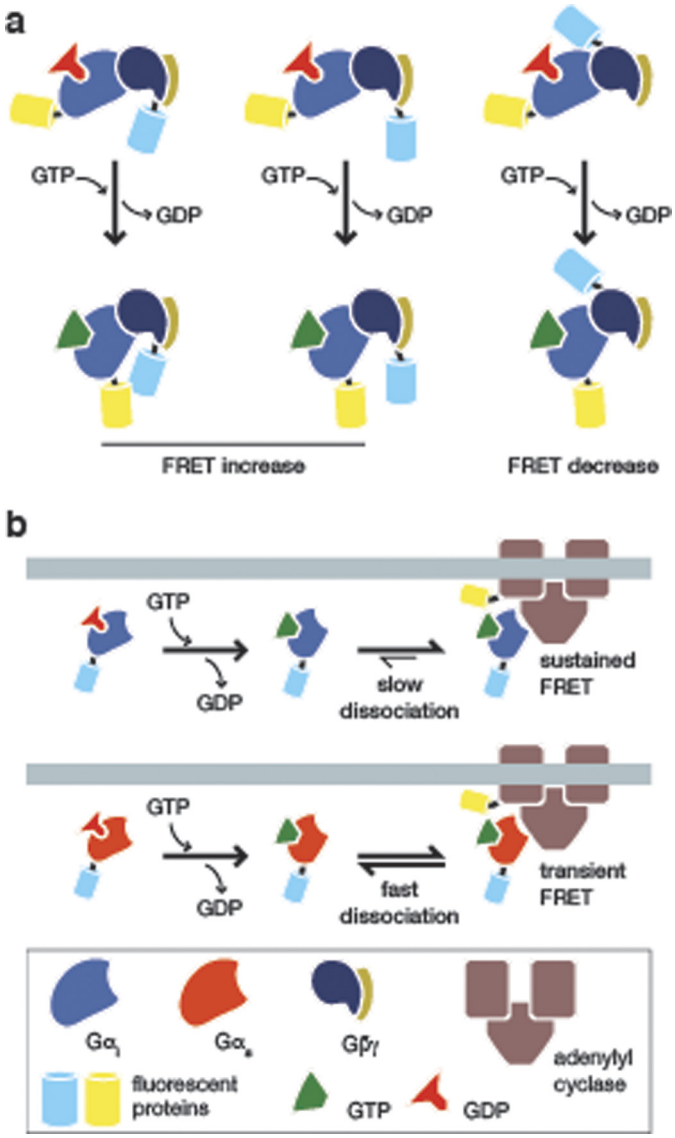

FIGURE 3. **Visualizing heterotrimeric G-protein signaling.** *a*, conformational rearrangement upon activation of the G_i heterotrimer. To test the dissociation of G_i upon activation by a GPCR, Bünemann *et al.* (30) generated a series of biosensors by fusing YFP to $G\alpha_i$ and CFP to three different positions on the $G\beta\gamma$ dimer. Although one of these biosensors produced a FRET decrease upon GPCR stimulation (*right panel*), the other two both produced increasing FRET responses (*left* and *middle panels*), suggesting that G_i proteins undergo a conformational rearrangement upon activation and do not fully dissociate, as in the case of the G_s heterotrimer. *b*, monitoring $G\alpha_i$ interaction with adenylyl cyclase. Milde *et al.* (35) recently examined the interaction between $G\alpha_i$ and adenylyl cyclase by fusing CFP to $G\alpha_{i1}$ and YFP to AC5. The activation of $G\alpha_{i1}$ by GPCR signaling results in its interaction with AC5, leading to a FRET increase (*upper panel*). However, when compared with $G\alpha_s$ (*lower panel*), $G\alpha_{i1}$ was observed to dissociate very slowly from AC5, which the authors suggested may be a mechanism for the self-regulation of AC5 activity in cells. For clarity, $G\beta\gamma$ is not shown.

ued association of different $G\alpha$ subunits after GPCR activation (31).

Using another $G\alpha\beta\gamma$ dissociation biosensor, Kataria *et al.* (32) recently studied the specific role of the protein resistant to inhibitors of cholinesterase 8 (Ric8), a molecular chaperone with putative non-receptor GEF activity, in *D. discoideum* chemotaxis (see Ref. 33 for a discussion of Ric8 regulation of heterotrimeric G-proteins). The chemoattractant cAMP is known to activate $G\alpha\beta\gamma$ and the monomeric G-protein Ras, which are necessary for coordinating chemotaxis. In this study, Ric8 was found to be necessary for chemotaxis when there is a shallow cAMP gradient. Using a bimolecular sensor composed of $G\alpha_2$-CFP and $G\beta$-YFP, the authors studied the dissociation of the heterotrimeric subunits, and hence the activation of G-protein signaling, in response to cAMP agonist stimulation in wild-type and *ric8*-null cells. In cells lacking Ric8, the total FRET change was less than half that observed in wild-type cells, and the response was slower. These results, together with biochemical data, indicated that Ric8 acts as a GEF to reactivate inactive $G\alpha_2$-containing G-proteins and sustain G-protein signaling without continued receptor signaling. This sustained $G\alpha$ signaling results in downstream effects on Ras that lead to concentrated Ras activity at the leading edge of a migrating cell.

Heterotrimeric G-proteins are the main link between GPCRs and downstream effects, with different $G\alpha$ subunit isoforms able to activate different effectors. Thus, a more complete picture of the interactions between $G\alpha\beta\gamma$ isoforms and various effectors will improve our understanding of how different $G\alpha$ subunits shape the diversity of G-protein signaling. To this end, a number of biosensors have been developed to probe the interactions between $G\alpha\beta\gamma$ components and downstream effectors. For example, Sadana *et al.* (34) previously used a FRET probe to study the dynamics of the interaction between $G\alpha_s$ and adenylyl cyclase 5 (AC5). However, it was unclear how the dynamics of $G\alpha_i$ differed from those of $G\alpha_s$. Recently, Milde *et al.* (35) reported the development of a FRET biosensor to monitor the association between $G\alpha_i$ and AC5 (Fig. 3*b*). This bimolecular sensor contains YFP fused to the N terminus of AC5 and CFP attached to $G\alpha_{i1}$. Upon activation of $G\alpha_{i1}$ by the α_{2a}-AR, the authors observed rapid interaction between $G\alpha_{i1}$ and AC5. Surprisingly, this response was followed by the slow dissociation of $G\alpha_{i1}$ from AC5, which differed from the dynamics of the $G\alpha_s$-AC5 interaction and was not altered by increasing the intrinsic GTPase activity of $G\alpha_{i1}$. The authors concluded that the slow deactivation kinetics of AC5-bound $G\alpha_{i1}$ can delay the reassembly of the $G\alpha\beta\gamma$ complex and hence may be a mechanism for AC5 self-regulation.

Tracking Second Messenger Dynamics

Second messengers are small molecules whose intracellular levels are tightly regulated by the cell to amplify and propagate signals to diverse downstream effectors. In the context of G-protein signaling, these can include cAMP, inositol 1,4,5-trisphosphate, diacylglycerol, and Ca^{2+}, and fully understanding G-protein signaling dynamics requires knowing how these different second messenger pools are generated. For example, Chakir *et al.* (36) used biosensors to study cAMP production in response to β_2-AR stimulation in canine heart cells with synchronous or dyssynchronous heart failure and to investigate the mechanism of cardiac resynchronization therapy. Using a FRET biosensor that contains a known cAMP-binding protein sandwiched between CFP and YFP, this group found that cardiac resynchronization therapy improves stimulatory G-protein signaling in both synchronous and dyssynchronous heart failure models. Specifically, cells from treated canines produced more cAMP because the inhibitory $G\alpha_i$ subunit was inhibited by regulators of G-protein signaling (RGS2 and RGS3). Because

$G\alpha_i$ is inhibited, β_2-AR signaling is biased to the $G\alpha_s$ pathway, which activates AC and increases cAMP production, thereby restoring normal physiological conditions.

Similarly, Verma *et al.* (37) used a Ca^{2+} biosensor to measure the effect of various agonists and GRKs on D1-D2 heteromer dopamine receptor signaling. The homo-oligomers of the dopamine receptors have been well studied, but the dynamics of the D1-D2 hetero-oligomer are not well characterized. A FRET-based Ca^{2+} probe, composed of the Ca^{2+}-binding protein calmodulin fused to the calmodulin-binding peptide M13 and flanked by CFP and YFP, was used in this study to measure intracellular Ca^{2+} levels in response to a D1-specific agonist, a D1-D2-specific activating agonist, and GRK knockdown. This group found that the binding of a D1 agonist, even without full activation, is sufficient to induce an increase in Ca^{2+} that can be attenuated by GRK activity.

Conclusions and Future Perspectives

Single-cell analyses using fluorescent biosensors are clearly a powerful method to study signaling dynamics in pathways such as G-protein signaling. The genetic encodability and modular nature of biosensors provide a convenient way to create sensors for different processes by fusing FPs to appropriate sensing units. However, biosensors are not without faults. FPs can disrupt the natural functions of the sensing unit due to their added bulk, whereas the sensing unit itself can perturb the natural signaling system. For example, a biosensor that contains a catalytically inactive protein as the sensing unit may buffer the natural ligand pool and affect native signaling (38). However, these effects can be reduced by using enhanced biosensors, thus lowering the required biosensor concentrations, as well as by performing the proper controls. The drawbacks of biosensors are also balanced by their principle advantage in single-cell analysis: the ability to directly observe living cells in real time and collect both temporal and spatial information on biochemical activities.

Single-cell analyses also pose an interesting dilemma, in that they can reveal dynamics that are rare or hidden at the population level but may also reveal a wide range of behaviors across a population, making it difficult to tease out the relevant dynamics. This natural heterogeneity can hinder our understanding of signaling dynamics, but it can also contain functional information. Studies have presented different functional models for how noise in a population is created and how it can affect function (39, 40). For instance, stochastic differences may contribute to the ability of a subpopulation of cells to exceed a threshold and differentiate, which is an important event in many processes, including development and immune cell differentiation. Single-cell analyses are something of a mixed blessing in this respect as they can provide valuable information that is lost in population experiments, but the natural heterogeneity of a signal may hinder the understanding of its function unless the role of the heterogeneity can also be determined.

In addition to monitoring signaling dynamics, it is also possible to perturb specific biochemical processes at the single-cell level by using optogenetics or chemically inducible dimerization (CID) to directly manipulate cellular processes with spatiotemporal precision. Combining biosensor-based single-cell analyses with these novel tools offers new opportunities to expand our understanding of signaling dynamics in general and G-protein signaling in particular. Masseck *et al.* (41) provide an excellent overview of current optogenetic methods and tools to study G-protein signaling, particularly at the GPCR level. Optogenetics encompasses techniques in which light is used to control the activity of specific proteins. Opsins, a class of GPCRs, are ideal for optogenetics because these light-sensing receptors can be experimentally activated by light. Optogenetics is therefore readily applicable to the study of G-protein signaling.

A recent study selected opsins based on their ability to be activated by specific wavelengths of light that do not overlap with those necessary for fluorescent biosensor use (42). The authors found three distinct opsins that meet this criterion and enable the specific activation of GPCRs coupled to $G\alpha_s$, $G\alpha_q$, or $G\alpha_i$ while simultaneously using FRET biosensors to measure downstream effects. The three opsins developed are: a blue variant of rhodopsin (termed bOpsin) to activate $G\alpha_{i/o}$ signaling; melanopsin to activate $G\alpha_q$ signaling; and a fusion construct between a jellyfish GPCR domain that binds $G\alpha_s$ and the fluorophore component of bOpsin (termed CrBlue). When these opsins were activated by an optical input, fluorescently tagged heterotrimeric G-protein subunits translocated into the cytosol, indicating successful activation of the receptor and G-protein signal transduction. Beyond combining the optical activation of G-protein signaling with the imaging of downstream activity, the authors demonstrated that the light-activated receptors could be activated at distinct cellular locations while activity was measured throughout the cell. These studies present a powerful tool to activate distinct GPCRs at specific, confined locations in the membrane, which the authors used to direct the growth of neurites. When the optical input was directed at the edge of a neurite and steadily moved away, lamellipodia were observed to expand in the direction of the optical input, concomitant with the retraction of a distal neurite. The ability to locally activate GPCRs opens new possibilities for ways to control and study G-protein signaling dynamics.

CID is another method of directing protein activity that utilizes the induced dimerization or association of specific protein domains. For example, the FK506-binding protein (FKBP) and FKBP12-rapamycin-binding domain (FRB) proteins dimerize in the presence of rapamycin, which is a useful tool for directing the association or translocation of proteins (43). To study the specificity of heterotrimeric G-protein signaling, Putyrsky *et al.* (44) used this system to tether different components of the $G\alpha\beta\gamma$ heterotrimer to the plasma membrane. They then observed the downstream effect by measuring intracellular cAMP and Ca^{2+}. FKBP was fused to $G\alpha_q$ or $G\alpha_s$ subunits or to $G\gamma$ (remained associated with $G\beta$), and FRB was targeted to the membrane. Upon rapamycin treatment, the G-protein subunits translocated to the plasma membrane and induced downstream signaling independent of receptor activation. This study demonstrates a new technology for selectively studying G-protein signaling diversity controlled by distinct heterotrimeric G-proteins, as well as wide-ranging applications for studying other aspects of G-protein signaling.

Optogenetics and CID are effective methods to directly control different aspects of protein behavior that, together with

fluorescent biosensors, provide a promising new way to probe the dynamics of G-protein signaling. Future studies utilizing these molecular tools to control, perturb, and analyze G-protein signaling at the single-cell level will enable far more comprehensive and detailed studies of the spatiotemporal dynamics of G-protein signaling, including GPCR activation, heterotrimeric G-protein activity, and downstream functions.

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