

Minireview

Get Ready to Sharpen Your Tools: A Short Guide to Heterotrimeric G Protein Activity Biosensors

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ABSTRACT

G protein-coupled receptors (GPCRs) are the largest class of transmembrane receptors encoded in the human genome, and they initiate cellular responses triggered by a plethora of extracellular stimuli ranging from neurotransmitters and hormones to photons. Upon stimulation, GPCRs activate heterotrimeric G proteins ($G\alpha\beta\gamma$) in the cytoplasm, which then convey signals to their effectors to elicit cellular responses. Given the broad biological and biomedical relevance of GPCRs and G proteins in physiology and disease, there is great interest in developing and optimizing approaches to measure their signaling activity with high accuracy and across experimental systems pertinent to their functions in cellular communication. This review provides a historical perspective on approaches to measure GPCR-G protein signaling, from quantification of second messengers and other indirect readouts of activity to biosensors that directly detect the activity of G proteins. The latter is the focus of a more detailed overview of the evolution of design principles for various optical biosensors of G protein activity with different experimental capabilities. We will highlight advantages and limitations

of biosensors that detect different G protein activation hallmarks, like dissociation of $G\alpha$ and $G\beta\gamma$ or nucleotide exchange on $G\alpha$, as well as their suitability to detect signaling mediated by endogenous versus exogenous signaling components or in physiologically relevant systems like primary cells. Overall, this review intends to provide an assessment of the state-of-the-art for biosensors that directly measure G protein activity to allow readers to make informed decisions on the selection and implementation of currently available tools.

SIGNIFICANCE STATEMENT

G protein activity biosensors have become essential and widespread tools to assess GPCR signaling and pharmacology. Yet, investigators face the challenge of choosing from a growing list of G protein activity biosensors. This review provides an overview of the features and capabilities of different optical biosensor designs for the direct detection of G protein activity in cells, with the aim of facilitating the rational selection of systems that align with the specific scientific questions and needs of investigators.

Introduction

G protein-coupled receptors (GPCRs) are a family of 7 transmembrane proteins that sense a wide range of extracellular stimuli and elicit specific cellular responses in eukaryotes (de Mendoza et al., 2014). For example, GPCRs in yeast sense pheromones to trigger mating (Dohlman, 2002; Dohlman and Slessareva, 2006; Alvaro and Thorner, 2016), while GPCRs in mammals respond to neurotransmitters, hormones, photons,

odors, or even mechanical stretch, among other stimuli (Weis and Kobilka, 2018; Wootten et al., 2018; Wingler and Lefkowitz, 2020). In mammals, GPCRs are involved in virtually every major physiological function, including sympathetic neurotransmission (Graham, 1990; Insel, 1996; Small et al., 2003), nociception and pain processing (Pan et al., 2008; Che, 2021; Uniyal et al., 2023), and glucose metabolism (Husted et al., 2017; Barella et al., 2021; Oliveira de Souza et al., 2021). GPCR signal transduction mechanisms have also fed our understanding of fundamental principles of cellular communication, like the existence of intracellular second messengers generated upon the action of extracellular “first messengers” like hormones (Sutherland, 1970; Hynes et al., 2013; Nair et al., 2019). From a biomedical perspective, dysregulation of GPCR pathways contributes to many diseases and disorders, including

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ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; EMTA, effector membrane translocation assay; FRET, fluorescence resonance energy transfer; GAP, GTPase-accelerating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine-nucleotide exchange factor; GPCR, G protein-coupled receptors; GRK3ct, C-terminal region of GPCR kinase 3; Nluc, nanoluciferase; ONE-GO, ONE vector G-protein optical; PKA, protein kinase A; PLC β , phospholipase C β ; RET, resonance energy transfer; RGS, regulators of G protein signaling; YFP, yellow fluorescent protein.

neuropsychiatric disorders (Santos et al., 2017; Sriram and Insel, 2018; Barman et al., 2021), neurodegeneration (Huang et al., 2017; Azam et al., 2020), cancer (Dorsam and Gutkind, 2007; Lappano and Maggiolini, 2011), and cardiovascular diseases (Wang et al., 2018). It is therefore not surprising that GPCRs have been and still are highly pursued pharmacological targets (Hauser et al., 2017; Sriram and Insel, 2018).

Despite the large number of GPCRs, the mechanisms by which they propagate signals inside the cell rely on a series of invariable initial steps. GPCRs initiate signaling primarily by activating heterotrimeric G proteins ($G\alpha\beta\gamma$) inside the cell, although some signaling cascades are also propagated via arrestins (Weis and Kobilka, 2018; Wingler and Lefkowitz, 2020; Maharana et al., 2022). GPCRs promote the exchange of GDP for GTP on the $G\alpha$ subunit of G protein heterotrimers, leading to the dissociation of GTP-loaded $G\alpha$ subunits and $G\beta\gamma$ dimers, which then act on their respective downstream effectors (Gilman, 1987; Knight et al., 2021) (Fig. 1A). Heterotrimeric G proteins are categorized into four families (G_s , $G_{i/o}$, $G_{q/11}$, $G_{12/13}$) based on the identity of the $G\alpha$ subunit (Neves et al., 2002) (Fig. 1B), which activate specific signaling cascades. For example, G_s stimulates adenylyl cyclase to elevate cellular cAMP, while G_i inhibits it (Sadana and Dessauer, 2009). In contrast, $G_{q/11}$ does not directly regulate adenylyl cyclase (Beazely and Watts, 2005; Sadana and Dessauer, 2009). Instead, $G_{q/11}$ activates phospholipase $C\beta$ (PLC β) isoforms, which promote the formation of inositol 1,4,5-trisphosphate and subsequent elevation of cytosolic Ca^{2+} (Hubbard and Hepler, 2006; Mizuno and Itoh, 2009; Lyon and Tesmer, 2013). In addition to PLC β isoforms, $G_{q/11}$ also activates a subfamily of Rho-GEFs (Chikumi et al., 2002; Lutz et al., 2007). Finally, G_{12} and G_{13} act on their own class of effectors, including a subfamily of Rho-GEFs different from those activated by $G_{q/11}$ proteins, which in turn regulate changes in the organization of the actin cytoskeleton and also alter gene expression (Suzuki et al., 2009; Aittaleb et al., 2010; Syrovatkina et al., 2016) (Fig. 1B).

An inflection point in the field of GPCR signaling, even before the identification of heterotrimeric G proteins or GPCRs,

was the development of an improved method to measure the second messenger cAMP (Gilman, 1970), which involves an interesting anecdote of prominent scientific characters. Reportedly (Gilman, 2012), Alfred Gilman secretly developed this method while working in the laboratory of Marshall Nirenberg, who far from recriminating the action rewarded it by communicating the work to the *Proceedings of National Academy of Sciences* listing Gilman as the sole author (Gilman, 1970). Having a rapid and reliable method to quantify cAMP enabled the identification of heterotrimeric G proteins as a guanine nucleotide-dependent protein component required for the transduction of signals from hormone receptors to adenylyl cyclase (Ross and Gilman, 1977a,b; Ross et al., 1977, 1978; Smigel et al., 1984), as well as subsequent discoveries leading to the reconstitution of this signal transduction mechanism with purified components (Caron and Lefkowitz, 1976; Caron et al., 1979; De Lean et al., 1980; Cerione et al., 1984; Samama et al., 1993; Weiss et al., 1996). This series of historical events resonates well with Sidney Brenner's quote "Progress in science depends on new techniques, new discoveries and new ideas, probably in that order" and exemplifies how technological developments have driven conceptual advances in the field of GPCR signaling.

Measuring GPCR Signaling via Indirect Readouts of G Protein Activity

In addition to measuring cAMP as the archetype of GPCR signaling readout, many other approaches have been developed to assess GPCR signaling by quantifying events downstream of specific types of G proteins (Fig. 2A). For example, GPCR-mediated $G_{q/11}$ activation has been monitored using different approaches to measure intracellular Ca^{2+} levels, including chemical probes (Tsien, 1980; Miyawaki et al., 1997) and genetically encoded calcium indicators (Mank and Griesbeck, 2008) (Fig. 2B). In contrast, activation of G proteins containing $G\alpha$ subunits of another family, $G_{12/13}$, is frequently monitored by using reporters of the gene regulatory function associated to activation of transcription factors

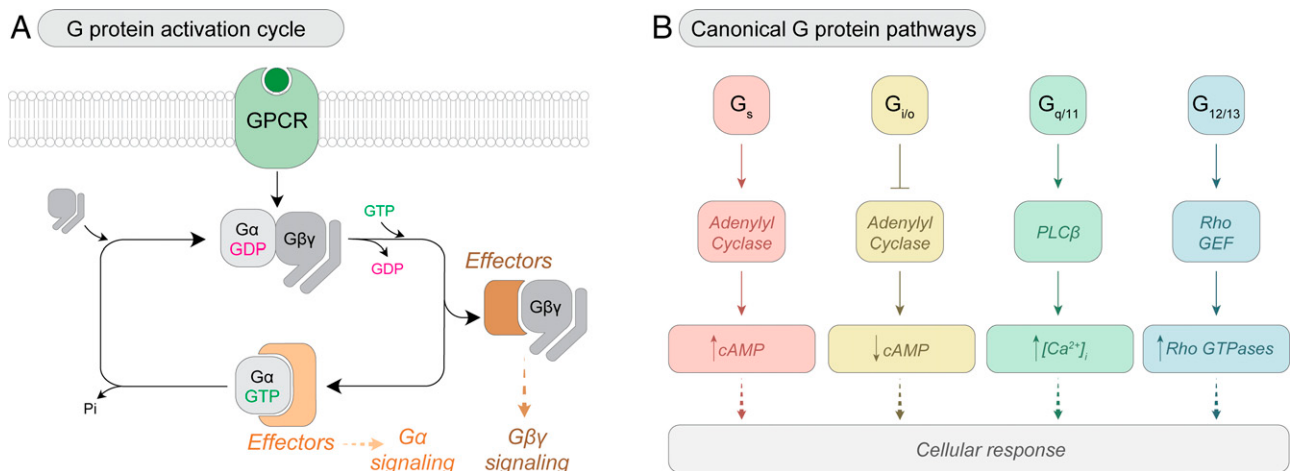


Fig. 1. G protein activation cycle and canonical signaling pathways. (A) GPCR-mediated activation of G proteins. Upon stimulation, GPCRs promote the exchange of GDP for GTP on the $G\alpha$ subunit of inactive $G\alpha\beta\gamma$ heterotrimer, triggering the dissociation of $G\beta\gamma$ from $G\alpha$ -GTP. Both $G\alpha$ -GTP and free $G\beta\gamma$ modulate their respective effectors to propagate signals to downstream cascades. Signaling is turned off once $G\alpha$ hydrolyzes GTP back to GDP, leading to reassociation with $G\beta\gamma$ into an inactive heterotrimeric complex. (B) G protein families and their canonical signaling pathways. G proteins are subcategorized into four families based on the identity of their $G\alpha$ subunits, which in turn have specific modulatory effects on different types of effectors.

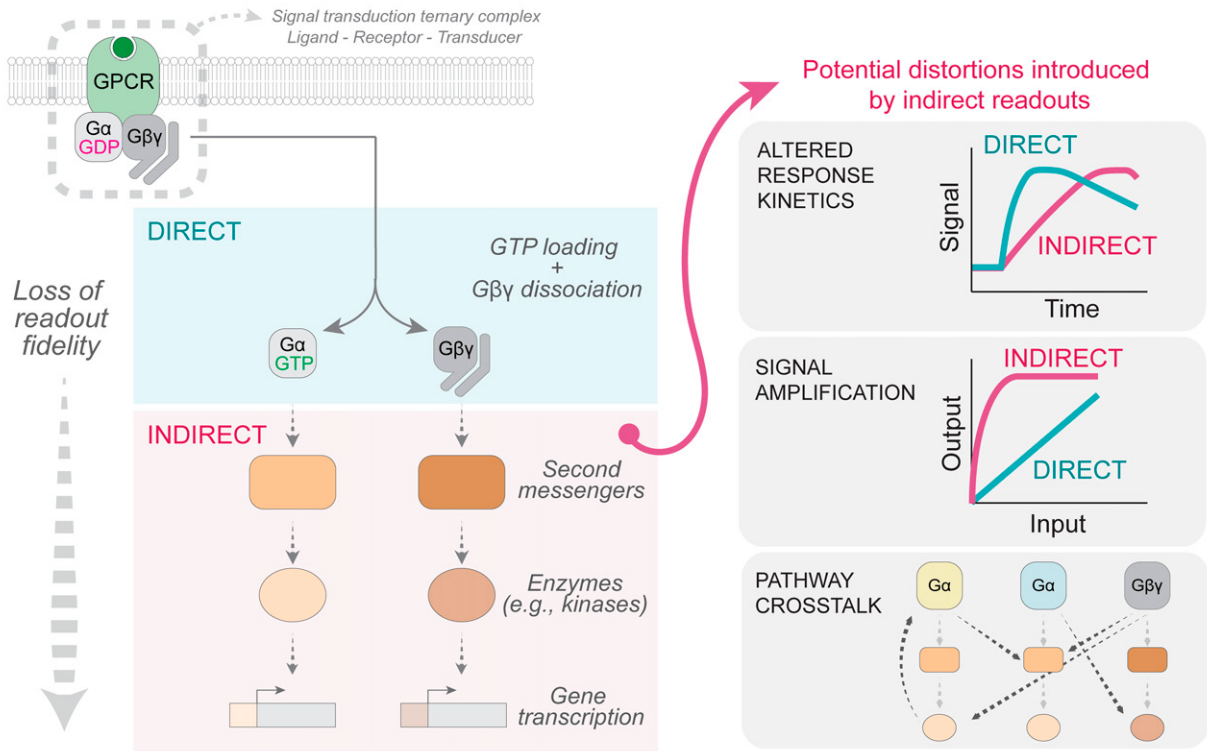
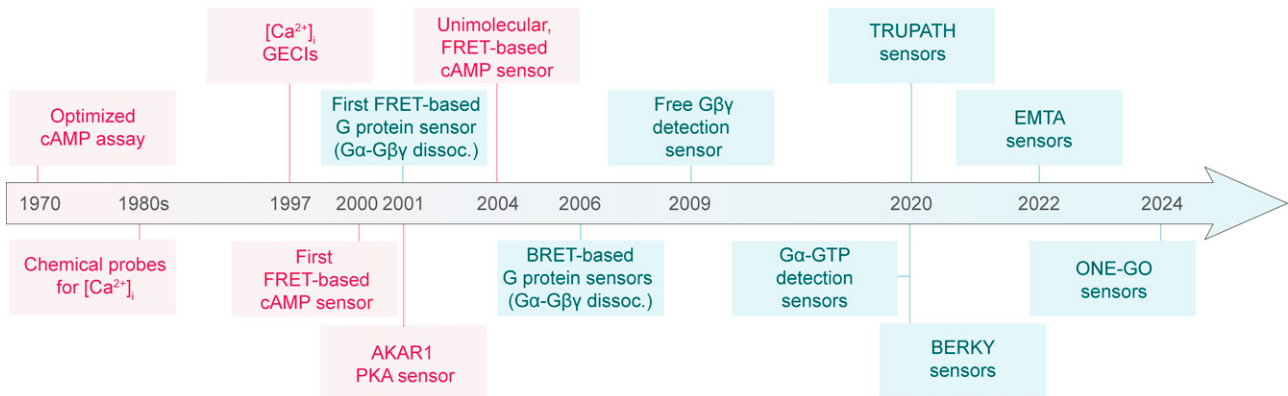
A Direct versus indirect readouts of GPCR-G protein signal transduction in cells**B** Timeline for the development of assays and sensor designs to monitor GPCR-G protein signal transduction

Fig. 2. Different approaches to assess GPCR-G protein signaling. (A) Left, schematic representation of GPCR-G protein signaling mechanisms and classification of different potential readouts as “DIRECT” (blue) or “INDIRECT” (red). Right, representation of potential distortions associated with indirect readouts, including changes in response kinetics, amplification events, or pathway crosstalk. (B) Timeline for the development of assays and sensor designs to monitor GPCR-G protein signal transduction.

like the serum-response factor (Treisman et al., 1998; Riento and Ridley, 2003; Siehler, 2009; Cheng et al., 2010; Watkins and Orlandi, 2021). Another general approach to assess GPCR signaling is the use of genetically encoded biosensors that measure the activity of kinases or levels of second messengers regulated by G proteins (Fig. 2, A and B). For example, there are several biosensors of cAMP (Zaccolo et al., 2000; Nikolaev et al., 2004) and protein kinase A (PKA) activity (Massengill et al., 2021; Zhang et al., 2001), a kinase controlled by cAMP (Sassone-Corsi, 2012), which therefore depend on G_s and/or G_i activity. Likewise, sensors for protein kinase C activity (Violin et al., 2003) are used to report $G_{q/11}$

signaling, given that this kinase depends on diacylglycerol and Ca^{2+} produced upon $PLC\beta$ activation (Hubbard and Hepler, 2006; Mizuno and Itoh, 2009). Assays like these that measure events indirectly linked to GPCR activation have been instrumental in providing key advances in the field (Fig. 2B), from discovery of GPCR ligands and their pharmacological properties (Costa and Herz, 1989; Lefkowitz et al., 1993; Bond et al., 1995; Yang et al., 2021) to the characterization of ligand binding pockets and the identification of G protein coupling sites (Ostrowski et al., 1992; Strader et al., 1994).

Despite the progress made using assays that measure indirect signaling events downstream of GPCR activation, these

approaches entail signal amplification and/or pathway crosstalk, which compromise the fidelity of the readout as a representation of the defining transduction event—i.e., activation of intracellular G proteins by GPCRs stimulated by extracellular ligands (or other cues) (Fig. 2A). For example, transcriptional changes occur at a much longer time scale than G protein activation, which is irrelevant when trying to understand G protein activation and/or deactivation kinetics. Synthesis of cAMP by adenylyl cyclases exemplifies well the problematic nature of signaling crosstalk. While adenylyl cyclases are typically regulated by $G_{\alpha s}$ and $G_{\alpha i}$, there is a plethora of other GPCR-mediated events that regulate them, including the formation of free $G\beta\gamma$, the elevation of Ca^{2+} , and the activation of protein kinase C or PKA, among others (Cooper, 2003; Halls and Cooper, 2011; Baldwin et al., 2019; Ostrom et al., 2022). Similarly, measuring Ca^{2+} as a readout of $G_{\alpha q}$ activation is also subject to distorting events, like cAMP-PKA dependent regulation of 1,4,5-trisphosphate-activated Ca^{2+} channels (Reiken et al., 2003; Wehrens et al., 2006) or the release of free $G\beta\gamma$, which modulates $PLC\beta$ isoforms (Chidiac and Ross, 1999; Dhyanani et al., 2020; Pfeil et al., 2020; Xiang et al., 2022). These confounding factors associated with a lack of readout fidelity are further compounded by the fact that many GPCRs couple promiscuously to different types of G proteins (Sandhu et al., 2019; Yang et al., 2021; Hauser et al., 2022). For example, the sphingosine-1-phosphate receptor 3 activates G proteins of the $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ families (Hauser et al., 2022; Siehler and Manning, 2002), leading to the simultaneous activation of multiple effector systems that undergo crosstalk with each other, making it very challenging to disentangle which G protein is responsible for which action of the receptor.

Assessment of GPCR Signaling via Direct Measurement of G Protein Activity

Issues associated with using indirect readouts of GPCR signaling can be mitigated by the use of approaches that measure G protein activity *directly*. For the purpose of this review, we define “direct” measurements of G protein activity as those that determine either the dissociation of $G\alpha$ and $G\beta\gamma$ from preformed heterotrimers or nucleotide exchange leading to GTP loading on the $G\alpha$ subunit (Fig. 2A). Sensors that have the capacity to directly measure G protein activation have the advantage of representing the stimulus-GPCR-G protein signal transduction event more accurately, without being skewed or biased by amplification or interference from other pathways. Furthermore, biosensors that directly detect G protein activity allow insights into the kinetics of G protein activation and/or deactivation. In the following sections, we start by providing a brief introduction to the methodologies used to *directly* monitor G protein activity, followed by a case-by-case description of different classes of biosensors that will include 1) the principles underlying the biosensor design, 2) conceptual advances provided by their implementation, and 3) an assessment of their desirable features versus their limitations. However, we will not review other approaches that monitor receptor-proximal signaling events different from G protein activation. For example, we will not cover approaches used to detect physical coupling of G proteins to GPCRs (Galés et al., 2005; Audet et al., 2008; Laschet et al.,

2019), which does not always lead to G protein activation (Okashah et al., 2020; Jang et al., 2023), or to measure the recruitment of β -arrestins to receptors (Barak et al., 1997; Bertrand et al., 2002; Lee et al., 2016; Nuber et al., 2016), which does not always represent a signal propagation event. We direct the reader to other recent reviews that have covered approaches to measure GPCR-G protein coupling (Wright and Bouvier, 2021; Olsen and English, 2023) and β -arrestin signaling (Haider et al., 2019).

One general approach to measure GPCR signaling directly relies on imaging of fluorescent probes. For example, fluorescent proteins (e.g., GFP) can be fused to nanobodies that bind to GPCRs or G proteins. While nanobodies emerged in the GPCR field as tools for structural biology applications (Chun et al., 2012; Manglik et al., 2017), they have subsequently been repurposed for the development of biosensors (Irannejad et al., 2013; Stoeber et al., 2018; Che et al., 2020). Early examples of successful implementation of this type of biosensor can be found in the characterization of the prototypical GPCR-G protein axis composed of the β_2 adrenergic receptor and G_s (Irannejad et al., 2013; Manglik et al., 2017; Uchański et al., 2020). GFP-fused nanobody 80, which binds to the active conformation of β_2 adrenergic receptor or GFP-fused nanobody 37, which binds to the transient state, nucleotide-free conformation of receptor-bound G_s protein, have been used to track GPCR signaling with subcellular resolution (Irannejad et al., 2013). A powerful advantage of this type of approach is the high spatial resolution, but it is limited to select GPCRs and/or G proteins for which suitable nanobodies are available. This limitation has been mitigated for receptors by the development of “mini G proteins” as probes to broadly detect the active conformation of virtually any GPCR (Wan et al., 2018), yet they do not provide information on productive propagation of signaling to G proteins or the activation status of the latter. Another general limitation of using fluorescent probes is that the readout relies on fluorescence intensity measurements by imaging, and the changes in intensity display modest signal-to-noise ratios. This makes it technically challenging to make quantitative assessments of activity because fluorescence intensity is not only dependent on the response observed but also on the overall expression of the probe. This, together with the requirement for high-end imaging instrumentation, hinders the broad applicability of this type of approach.

In contrast to fluorescence intensity-based approaches, methods based on resonance energy transfer (RET) allow for ratio-metric measurements that correct for differences in expression of the probe and in many cases are feasible with instruments widely available in laboratories such as plate readers. RET is the process in which excess energy from a “donor” molecule is transferred to an “acceptor” chromophore via dipole-dipole interactions (Wu and Brand, 1994; Jones and Bradshaw, 2019). This only occurs when the donor/acceptor pair are in close proximity to each other (~ 10 Å) and in a permissive orientation (Wu and Brand, 1994; Jones and Bradshaw, 2019). Essentially, induction of a high energy state of the donor results in emission of light from the acceptor when RET occurs. Two major variants of RET-based approaches have been leveraged to develop assays based on the relative proximity of proteins to which donor and acceptor are fused. The first relies on a fluorescent donor, which gets excited by light; fluorescence resonance energy transfer (FRET) occurs when the acceptor is nearby (Wu and Brand,

1994; Jones and Bradshaw, 2019). The second RET variant uses a bioluminescent enzyme, instead of a fluorophore, as the donor. In this case, the energy that typically results in light emission upon substrate catalysis is transferred to a fluorescent acceptor via bioluminescence resonance energy transfer (BRET) (Wu and Brand, 1994; Jones and Bradshaw, 2019). Both FRET- and BRET-based systems have been leveraged to monitor protein-protein interactions that directly depend on the G protein activation status. The versatility and relative ease of use of RET approaches have enabled accurate and sensitive activity measurements, playing a pivotal role in uncovering how various natural ligands and drugs affect the GPCR-G protein transduction axis (Marullo and Bouvier, 2007; Lohse et al., 2012).

RET-Based Biosensors of G Protein Activity

The earliest example of a RET-based approach to directly measure G protein activity was based on measuring a loss of FRET upon dissociation of $G\alpha$ and $G\beta\gamma$ subunits fused to a FRET donor/acceptor pair (Janetopoulos et al., 2001). This general approach was adapted to BRET-based biosensors (dubbed “Gaby”) by replacing the fluorescent donor with a bioluminescent luciferase (Galés et al., 2006). The design principle of Gaby biosensors has been more recently leveraged to generate a suite of biosensors (dubbed TRUPATH) optimized by systematic assessment of the location of tags on the G protein subunits and the combinations of tagged G proteins subunits that lead to BRET responses with a large

dynamic range (Olsen et al., 2020). Yet another approach to measure $G\alpha/G\beta\gamma$ dissociation was developed by measuring RET between fluorescently tagged $G\beta\gamma$ and a luciferase-fused detector protein module that specifically binds to $G\beta\gamma$ only when it is not associated with $G\alpha$ (Hollins et al., 2009). In addition to approaches that measure $G\alpha/G\beta\gamma$ dissociation, other RET-based approaches rely on measuring the formation of $G\alpha$ -GTP, which is an even more direct readout of the nucleotide exchange factor activity of GPCRs (Syrovatkina et al., 2016; Weis and Kobilka, 2018). The first example of $G\alpha$ -GTP biosensors relied on the interaction between fluorescently tagged $G\alpha$ subunits and a luciferase-fused detector protein module that specifically binds to the GTP-bound form of the G protein (Maziarz et al., 2020), which was also converted into a unimolecular design [dubbed BERKY, for BRET sensor with ER/K linker and YFP (yellow fluorescent protein)] for the detection of endogenous, untagged $G\alpha$ -GTP (Maziarz et al., 2020). More recently, the multicomponent $G\alpha$ -GTP biosensors have been assembled into a single-vector design [one vector g protein optical biosensor (ONE-GO)] that improves dynamic range and applicability across experimental systems with endogenous GPCRs (Janicot et al., 2024). An alternative approach to measure $G\alpha$ -GTP, the effector membrane translocation assay (EMTA), relies on so-called bystander BRET. Essentially, a G protein effector fused to a BRET donor is recruited from the cytosol to the plasma membrane upon activation of membrane-bound G proteins, which in turn leads to BRET with an acceptor targeted to the plasma membrane due to the increased proximity and crowding effects on the

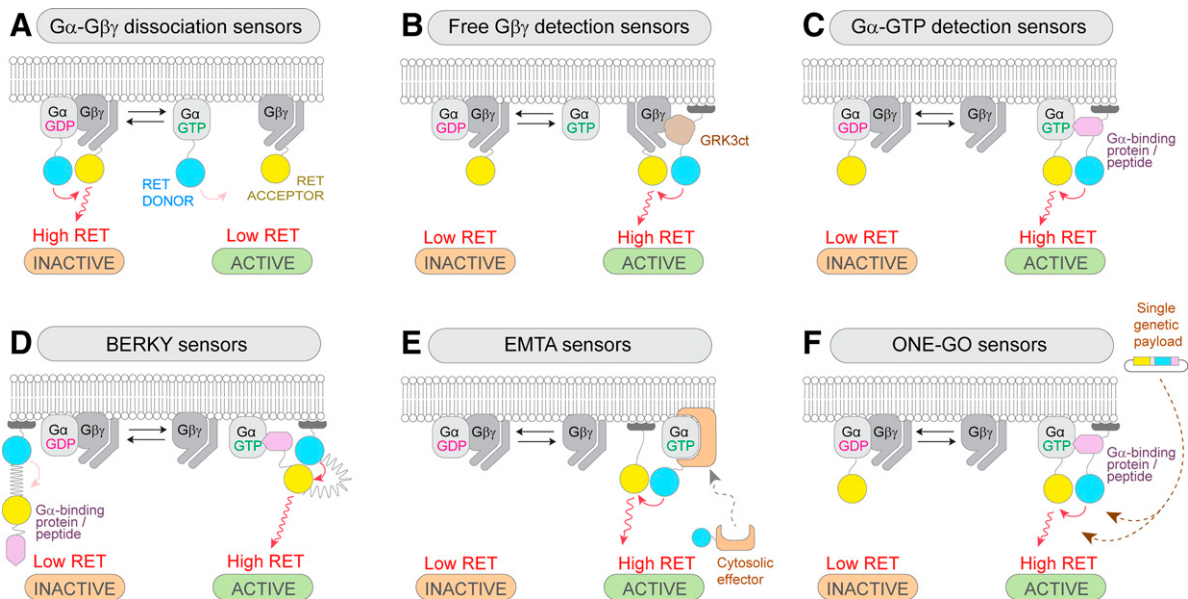


Fig. 3. Design of RET-based sensors to directly monitor G protein activity in cells. (A) $G\alpha$ - $G\beta\gamma$ dissociation sensors. In the inactive state, a $G\alpha$ fused to a RET donor (blue) interacts with $G\beta\gamma$ fused to a RET acceptor (yellow), leading to high RET. After G protein activation, the $G\alpha$ and $G\beta\gamma$ subunits dissociate (or rearrange), leading to a decrease in RET. (B) Free $G\beta\gamma$ detection sensors. In the inactive state, $G\beta\gamma$ fused to a RET acceptor (yellow) is associated with $G\alpha$ fused to a RET donor (blue) and attached to the plasma membrane. After G protein activation, $G\beta\gamma$ is released, allowing it to interact with GRK3ct fused to a RET donor (blue), leading to an increase in RET. (C) $G\alpha$ -GTP detection sensors. Upon G protein activation, a $G\alpha$ fused to a BRET acceptor (yellow) binds to detector modules fused to a BRET donor (blue) and attached to the plasma membrane that specifically bind to $G\alpha$ -GTP species, leading to high BRET. (D) BERKY sensors. As in (C), this biosensor design relies on binding of active G proteins to specific detector modules, but BRET increases arise from intramolecular interactions between a BRET donor (blue) and a BRET acceptor (yellow) within the same biosensor construct, which is attached to the plasma membrane. (E) EMTA sensors. Upon G protein activation, an effector molecule fused to a BRET donor (blue) is recruited from the cytosol to membranes via binding to $G\alpha$ subunits, which leads to increased BRET with a bystander membrane-anchored BRET acceptor (yellow). (F) ONE-GO sensors. Biosensor designs analogous to those in (C) are delivered to cells as a single genetic payload, enabling robust activity measurements across diverse cell types.

two-dimensional plane of the membrane rather than by direct high-affinity binding between the components fused to the BRET pair (Avet et al., 2022). Figure 3 summarizes the different RET-based biosensor designs that are detailed here, including the discussion of their respective strengths and limitations.

Biosensors that Detect the Dissociation of $G\alpha$ and $G\beta\gamma$

FRET-Based Sensors of $G\alpha$ - $G\beta\gamma$ Dissociation. The seminal generation of FRET-based biosensors that detect $G\alpha$ - $G\beta\gamma$ dissociation (Janetopoulos et al., 2001) represented a major advance in directly monitoring GPCR signaling responses. By fusing a CFP (FRET donor) to the $G\alpha 2$ subunit and a YFP (FRET acceptor) to the $G\beta$ subunit (Fig. 3A), the authors quantified loss of FRET as a measure of receptor-mediated activation in *Dictyostelium discoideum* (Janetopoulos et al., 2001). This biosensor design was later mimicked in mammalian cells by tagging $G\alpha i1$ with YFP and $G\beta 1$ with CFP (Bünemann et al., 2003) and then further developed to include additional members of the $G_{i/o}$ family (Frank et al., 2005) and $G_{\alpha s}$ (Hein et al., 2006). This FRET-based system was later optimized for the $G_{i/o}$ family, $G_{\alpha q}$, and $G_{\alpha 13}$ by using a more photostable CFP variant (mTurquoise2), which was sensitive enough to detect endogenous GPCR activity in some cases (Adjobo-Hermans et al., 2011; van Unen et al., 2016; Mastop et al., 2018). An example of conceptual advance made possible thanks to this biosensor was the assessment of whether $G\alpha$ and $G\beta\gamma$ dissociate and/or rearrange after activation. By measuring FRET interactions between $G\alpha$ subunits of the $G_{i/o}$ family, and $G\beta 1\gamma 2$ tagged with CFP at varying positions, studies suggested that G proteins undergo a molecular rearrangement in which the αA - αB loop of $G\alpha i$ moved closer to the N-terminus of $G\beta 1$ while distancing from the C-terminus of $G\gamma 2$ (Bünemann et al., 2003; Frank et al., 2005), which went against the prevalent model at the time that the $G\alpha$ and $G\beta\gamma$ subunits fully dissociated. Another example of the utility of this type of biosensor was to identify which combinations of different $G\alpha$ and $G\gamma$ proteins ($G\alpha i1$, 2, or 3 with either $G\gamma 1$, 2, or 4) led to heterotrimers that were more efficiently activated by GPCRs (Gibson and Gilman, 2006), which revealed that not only $G\alpha$ but also the $G\gamma$ subunits contribute to productive GPCR coupling.

A major advantage of FRET-based biosensors is their high spatial and temporal resolution, which allows for single-cell imaging and investigating signaling events that occur within subcellular compartments. However, FRET-based biosensors tend to display suboptimal signal-to-noise ratios, which compromises the dynamic range of the responses observed and their reproducibility. Moreover, both instrumentation and data processing required for FRET-based approaches pose a limitation for their wide adoption—i.e., there is not only a need for sophisticated microscopy, but the data obtained requires relatively complex corrections to achieve reliable results that are not confounded by photobleaching, spurious excitation of fluorophores, and/or signal spillover across different channels.

BRET-Based Sensors of $G\alpha$ - $G\beta\gamma$ Dissociation. Based on some of the limitations listed previously for FRET-based approaches, it is easy to envision the motivation for the subsequent development of BRET-based biosensors. In BRET, the fluorescent donor of FRET is replaced by a luminescent enzyme, thereby bypassing the need for light excitation and many of the associated complications, such as photobleaching,

or the requirement of cumbersome corrections for light spill-over across channels. Moreover, background luminescence tends to be negligible, which provides improved signal-to-noise ratios compared with FRET. Lastly, instrumentation to carry out BRET measurements, like plate readers equipped for luminescence, is widely available in many laboratories. An early example of BRET biosensors to detect GPCR responses consisted of a direct adaptation of earlier FRET sensors to detect $G\alpha$ - $G\beta\gamma$ dissociation (Fig. 3A). These BRET-based sensors, dubbed “Gaby” for analogy to $G\alpha\beta\gamma$, relied on a $G\alpha$ subunit fused to *Renilla reniformis* luciferase as donor and $G\beta\gamma$ fused to a GFP (Galés et al., 2006). While the design of this biosensor was initially implemented specifically for $G\alpha i1$ (Galés et al., 2006), it was subsequently extended to 10 different $G\alpha$ subunits, including members of all four G protein families (Saulière et al., 2012), or more thoroughly optimized for $G_{\alpha s}$ and $G_{\alpha olf}$ by testing multiple positions for the insertion of the BRET components (Yano et al., 2017). More recently, the Gaby biosensor design has been optimized to maximize the dynamic range of the responses detected by systematically testing combinations of $G\alpha$ subunits tagged with a luciferase at various positions of their sequence with different $G\beta\gamma$ subunits tagged with an acceptor fluorescent protein (Olsen et al., 2020). This open-source biosensor platform, called TRUPATH, was optimized for 14 different $G\alpha$ subunits, including the first probes of this class for $G\alpha 15$ and $G_{\alpha gust}$, and in most cases had an improved dynamic range compared with first-generation Gaby biosensors. Another iteration of the Gaby design to detect $G\alpha$ - $G\beta\gamma$ dissociation, named G-CASE (Schihada et al., 2021), for G protein tricistronic activity sensors, consisted of assembling all components in a single plasmid and replacing the original donor fused to $G\alpha$ (i.e., *Renilla* luciferase) with a newer generation luciferase, namely nanoluciferase (Nluc), of smaller size but increased brightness and signal stability (Hall et al., 2012; Machleidt et al., 2015).

The first conceptual advance provided by the implementation of Gaby biosensors was reported in the same publication in which they were initially described (Galés et al., 2006). More specifically, by using various Gaby sensor designs with different locations of the donor within the structure of $G\alpha i1$, the authors proposed a model in which G protein activation leads to relative movements between $G\alpha i1$ and $G\beta\gamma$ subunits instead of a complete dissociation (Galés et al., 2006), which was in agreement with prior findings using analogous FRET-based sensors (Bünemann et al., 2003). Another advance made possible by leveraging Gaby biosensors was facilitating the elucidation of the activation profile of novel GPCR ligands across different G protein subtypes, as was done for the angiotensin II analog [1 Sar 4 Ile 8 Ile]-angiotensin II (Saulière et al., 2012) or for synthetically derived pepducins, some of which activate G_s at the $\beta 2$ -adrenergic receptor without triggering arrestin-mediated pathways (Carr et al., 2014). The TRUPATH platform has similarly enabled studies into novel ligands and how they activate different G protein subtypes, as was done at the M_2 receptor (Jiang et al., 2022), or into mechanisms of G protein activation, like how amino acids conserved across $G\alpha$ subunits affect $G\beta\gamma$ release (Knight et al., 2021). Additionally, the ease of use of the TRUPATH platform renders it compatible with higher-throughput experiments (DiBerto et al., 2022), which can be leveraged to address productive GPCR/G protein coupling at a larger scale than previously possible (Olsen et al., 2020). As for the G-CASE design, the improvement in

reproducibility along with a careful procedure for data analysis allows for the more reliable determination of GPCR constitutive activity (Schihada et al., 2021).

Gaby, TRUPATH, and G-CASE biosensors all provide clear advantages in terms of ease of implementation and systematic profiling of activation of different G proteins. However, all these biosensor designs also share limitations. A major drawback in using this sensor design is the need to express both exogenous $G\alpha$ and $G\beta\gamma$ in significant excess to lead to productive BRET pairs over nonproductive pairing with endogenous, untagged G protein subunits. This overexpression affects the stoichiometry of signaling components, promotes G protein pairs that would not necessarily exist in endogenous systems, and can influence the normal behavior of G protein-mediated signaling pathways. For example, it has been reported that the subtype of $G\gamma$ subunit present in a heterotrimer plays a critical role in the spatiotemporal regulation of G proteins (Masuho et al., 2021), suggesting that the overexpression of exogenous $G\gamma$ subunits required with these biosensor designs will affect the properties of G protein responses detected. Moreover, the requirement to introduce multiple exogenous components makes Gaby and TRUPATH sensors difficult to implement in systems that are not easily transfected. In this regard, a recent report with an optimized version of this type of biosensor design for G proteins of the $G_{i/o}$ family has succeeded in measuring responses in primary neurons (Xu et al., 2024), leading to observations that highlight some of the limitations mentioned. For example, they found that responses in primary cells differed from those observed in cell lines with overexpressed receptors and that even when studying endogenous receptors in neurons the responses were skewed depending on the type of exogenous, tagged $G\gamma$ subunit required to assemble the biosensor system in cells (Xu et al., 2024). The TRUPATH biosensor design presents some additional limitations arising from the focus on increasing dynamic range. For example, to achieve larger responses, a particular subclass of $G\gamma$ subunits (consisting of $G\gamma_1$, $G\gamma_9$, and $G\gamma_{11}$) with low membrane affinity and fast translocation kinetics (Masuho et al., 2021; Kankanamge et al., 2022) became the preferred choice in 9 out of the 14 TRUPATH biosensors. While the features of this class of $G\gamma$ subunits likely contribute to the improved dynamic range because $G\alpha$ and $G\beta\gamma$ will not only dissociate from each other but also become spatially separated in different cellular compartments, the kinetics of activation and the ability to reform heterotrimers after activation are probably markedly different from those expected for many other common combinations on G protein subunits found naturally in specific cell types (Masuho et al., 2021; Xu et al., 2024). Finally, the insertion of bulky tags, like luciferases or fluorescent proteins, on $G\alpha$ subunits can sometimes modify how they handle nucleotides and thereby alter their activity (Gibson and Gilman, 2006). For example, almost all TRUPATH sensors for the $G_{i/o}$ family have a luciferase inserted in the αA - αB helix, a location where similar insertions lead to acceleration of nucleotide exchange rates on $G\alpha_{i1}$ (Gibson and Gilman, 2006).

RET-Based Sensors of “Free” $G\beta\gamma$. Instead of measuring G protein heterotrimer dissociation by tagging the $G\alpha$ and $G\beta\gamma$ subunits with RET donor/acceptor pairs, as with Gaby or TRUPATH sensors, another approach was developed by leveraging a separate “detector module” for $G\beta\gamma$ dissociated from $G\alpha$ subunits (Hollins et al., 2009) (Fig. 3B). More

specifically, the C-terminal region of GPCR kinase 3 (GRK3ct), was used as a detector module based on prior evidence demonstrating that it can bind to $G\beta\gamma$ dimers only when they are “free” (Lodowski et al., 2003). Thus, when fused to a RET donor (a luciferase or CFP), it would transfer energy to a YFP acceptor fused to $G\beta\gamma$ only when the G protein dimer is dissociated from $G\alpha$ (Hollins et al., 2009). The design of the BRET version of this biosensor was subsequently optimized (Masuho et al., 2015a) by replacing the original donor (*Renilla* luciferase) fused to GRK3ct with Nluc to increase the sensitivity and reproducibility of activation readouts (Masuho et al., 2015a).

The immediate conceptual advance provided by implementing this biosensor design, which accompanied its initial description (Hollins et al., 2009), was about addressing conflicting information on whether $G\beta\gamma$ subunits fully dissociated from the $G\alpha$ subunit (Akgoz et al., 2004; Nobles et al., 2005; Galés et al., 2005, 2006; Digby et al., 2006, 2008; Lambert, 2008). More specifically, it was demonstrated using this biosensor that at least some $G\beta\gamma$ subunits dissociated enough from $G\alpha$ upon activation to allow for the binding of an effector (e.g., GRK3) (Hollins et al., 2009). This biosensor also allowed delving deeper into the mechanisms by which regulators of G protein signaling (RGS) proteins affect G protein activity. While acceleration of G protein deactivation by RGS proteins is easily explained by their GTPase-accelerating protein (GAP) activity (Ross and Wilkie, 2000; Neubig and Siderovski, 2002), early evidence showed that they also accelerated activation rates (Doupnik et al., 1997; Saitoh et al., 1997), a seemingly paradoxical phenomenon for a GAP. Lambert and colleagues set out to test two models proposed to explain this by leveraging the GRK3ct free $G\beta\gamma$ sensor. One model proposed that acceleration of activation required a “physical scaffold” function of RGS proteins, where, in addition to the GAP activity, other domains within the protein could bridge G proteins and GPCRs together, thereby accelerating new cycles of activation (Sierra et al., 2000; Neitzel and Hepler, 2006). The other model proposed a “kinetic scaffold” in which the GAP activity of RGS proteins was sufficient to ensure rapid conversion of $G\alpha$ -GTP into $G\alpha$ -GDP to prevent GPCR substrate depletion at high rates of nucleotide exchange, thereby allowing for the net outcome of improved activation rates (Ross and Wilkie, 2000; Zhong et al., 2003; Smith et al., 2009). By means of precise kinetic measurements of free $G\beta\gamma$ using a combination of RGS-insensitive and fast GTPase mutants in the presence or absence of RGS proteins, it was concluded that the GAP function alone of RGS proteins is sufficient to explain both the increase in deactivation rate and the increase in the onset of G protein activity (Lambert et al., 2010). In addition to the findings described here, the second-generation biosensor that leveraged the brighter BRET donor Nluc led to better systematic profiling of various aspects of G protein regulation, like determining the G protein coupling preferences of GPCRs (Masuho et al., 2015b, 2023), the G protein specificity of RGS proteins (Masuho et al., 2020), and the impact of different combinations of $G\beta$ and $G\gamma$ subtypes on G protein signaling kinetics and efficacy (Masuho et al., 2021). Another area in which this same system has provided novel insight is in dissecting the functional consequences of disease-associated mutations in either G proteins (Fuchs et al., 2013; Marivin et al., 2016; Lohmann et al., 2017; Muntean et al., 2021) or their regulators (DiGiacomo et al., 2020; Masuho et al., 2020). Additionally, this optimized BRET sensor has been leveraged to elucidate noncanonical mechanisms of G protein regulation, including

the dissection of what G protein active species propagates signaling initiated by a class of nonreceptor activators characterized by containing a $G\alpha$ -binding-and-activating motif (Parag-Sharma et al., 2016; Maziarz et al., 2018; Garcia-Marcos, 2021, 2024) or defining how the neuronal protein called GINIP biases GPCR-mediated neuromodulator responses to favor $G\beta\gamma$ signaling over $G\alpha$ -GTP signaling (Park et al., 2023; Luebbers et al., 2024).

A benefit of the biosensor design based on using a separate detector module for free $G\beta\gamma$ subunits is that the $G\alpha$ subunit does not need to be tagged, which mitigates concerns about the potential deleterious or unexpected effects that this may have on G protein activity (Galés et al., 2006; Gibson and Gilman, 2006; Yano et al., 2017). Additionally, GRK3ct by itself does not interact with GPCRs or preformed G protein heterotrimers (Hollins et al., 2009), a desirable feature for a detector module as its expression will not directly modulate the intrinsic enzymatic reactions that govern G protein activity. However, like Gaby sensors, a limitation of this system is the requirement to express exogenous $G\alpha$, $G\beta$, and $G\gamma$, which can impact the properties of the G protein responses measured. Moreover, the need for the GRK3ct detector module adds one more genetic component to be introduced into cells, further contributing to the difficulty of implementing this type of biosensor in cell that are not easily transfectable.

Biosensors that Detect $G\alpha$ -GTP Formation

BRET-Based Sensors of $G\alpha$ -GTP with Membrane-Bound Detector Modules. While $G\beta\gamma$ dissociation is closely related to GPCR activation, an even more direct approach to record receptor function would be to measure the event that defines their catalytic activity as guanine-nucleotide exchange factors (GEFs), i.e., the exchange of GDP for GTP on $G\alpha$. In addition to being a more direct readout of GPCR activity, measuring $G\alpha$ -GTP would enable the characterization of regulatory proteins that affect $G\alpha$ independently of $G\beta\gamma$ such as guanine nucleotide dissociation inhibitor (GDIs) (Willard et al., 2004) or some nonreceptor GEFs (Tall et al., 2003; Garcia-Marcos et al., 2009; Garcia-Marcos, 2024).

Inspired by the GRK3ct free $G\beta\gamma$ biosensor design (Hollins et al., 2009), the first technology able to measure levels of $G\alpha$ -GTP relied on Nluc-fused detector modules that specifically interact with YFP-tagged $G\alpha$ -GTP proteins (Fig. 3C) (Maziarz et al., 2020). Careful consideration was placed into the choice of detector modules to have the following three features: 1) *specificity* toward GTP-bound $G\alpha$, but not $G\alpha$ -GDP, of one particular G protein family; (2) high *affinity* but reversible binding to G proteins to allow tracking of both activation and deactivation kinetics; and (3) no direct effect on the enzymatic activity of the target $G\alpha$ protein to measure activity with high *fidelity*. Detector modules with these features have been identified and implemented in biosensors for all G protein families, including $G_{i/o}$, G_s , $G_{q/11}$, and $G_{12/13}$ (Maziarz et al., 2020; Janicot et al., 2024).

One advance enabled by this biosensor design has been the elucidation of mechanisms of G protein regulation that differentially affect $G\alpha$ and $G\beta\gamma$. For example, the GDI activity of GoLoco motifs was shown to promote free $G\beta\gamma$ release while dampening $G\alpha$ -GTP formation in cells (Maziarz et al., 2020), a feature suggested by some observations in vitro that had remained untestable in cells due to the lack of methods to directly measure $G\alpha$ -GTP (Ghosh et al., 2003; Webb et al., 2005).

Similarly, by using parallel measurements of $G\alpha$ -GTP and free $G\beta\gamma$, it was shown that different nonreceptor GEFs have varying abilities to generate either $G\alpha$ -GTP or free $G\beta\gamma$ and differ in how they interplay with GPCRs to regulate G proteins (Garcia-Marcos, 2021). This biosensor design provided mechanistic insights into how pro-oncogenic features endowed by somatic mutations found in either $G\alpha_q$ (Maziarz et al., 2020) or $G\alpha_s$ (Janicot et al., 2024) rely on alterations in their nucleotide loading status to become hyperactive. For example, it was found the R247Q mutation in $G\alpha_q$ renders it insensitive to the GAP activity of RGS proteins (Maziarz et al., 2020) and that the R201C mutation in $G\alpha_s$ only leads to partial occupancy with GTP in cells and that this mutant protein can be further activated by GPCRs (Janicot et al., 2024). By leveraging the ability to monitor the catalytic GEF activity of GPCRs directly, this biosensor design was also leveraged to map similarities and differences between the structural determinants of G proteins required to couple to receptors. More specifically, it was found that G proteins containing $G\alpha_i$ subunits rely largely on their C-terminus to couple to their cognate GPCRs, whereas those containing $G\alpha_s$ rely on their $\alpha N/\beta 1$ hinge region, in addition to the C-terminus, to achieve specific coupling to their cognate GPCRs (Janicot et al., 2024). Another advance enabled by this biosensor design relates to the ability to customize it to dissect G protein activation within distinct subcellular compartments, which underlies the idea of location-bias—i.e., that different signaling outputs can be encoded by the cellular location at which G protein activation by GPCRs happens. To address this type of question, Eiger and colleagues modified the detector module in the original design by replacing the sequence targeting it to the plasma membrane by one to deliver it specifically to endosomes, which revealed differences in activation of $G\alpha_i$ by the receptor CXCR3 at the plasma membrane or endosomes depending on the chemokine used to stimulate cells (Eiger et al., 2022).

Some advantages of this biosensor design over previously described $G\alpha$ - $G\beta\gamma$ dissociation sensors arise from the fact that it relies on endogenously expressed $G\beta\gamma$ (Maziarz et al., 2020; Janicot et al., 2024), which has two direct implications. One is that all the spatiotemporal and coupling specificity aspects of G protein regulation dictated by $G\beta\gamma$ are left intact. The second implication is that exogenous $G\alpha$ can be expressed at low levels because there is no need to saturate endogenous $G\beta\gamma$ to obtain measurable signals, as the BRET pair is formed with a separate detector module component. This is in contrast with the requirements of designs like Gaby or TRUPATH, for which endogenous G proteins have to be outcompeted by the exogenous G protein subunits tagged with the BRET donor/acceptor pair. Nevertheless, the need for an exogenous, tagged $G\alpha$, even at low levels, still poses some minor concerns about the fidelity of the responses observed. Moreover, the need for at least two genetic components, $G\alpha$ and detector module, limits the implementation of this biosensor design to transfectable cells.

Unimolecular BRET-Based Sensors of $G\alpha$ -GTP (BRET Sensor with ER/K Linker and YFP). As part of the same study that developed the $G\alpha$ -GTP sensors described earlier, another biosensor design was envisioned to measure the activity of endogenous G proteins, which had not been possible to date (Maziarz et al., 2020). The design of the biosensor (BERKY) consisted of repurposing $G\alpha$ -GTP or free $G\beta\gamma$ detector modules as components of a unimolecular construct with

a BRET donor (Nluc) and BRET acceptor (YFP) connected by a bistable ER/K α -helix (Sivaramakrishnan and Spudich, 2011). The construct was anchored to the plasma membrane on the opposing terminus of the detector module, such that binding of the latter to active G proteins at the plasma membrane favors the bent conformation of the ER/K linker. Upon bending of the linker, the BRET donor and acceptor become closer to each other, leading to an increase in BRET (Fig. 3D) (Maziarz et al., 2020). The modular design of BERKY constructs allowed the development of sensors for different G α subtypes (i.e., G α_i , G α_q , and G α_{13}) by keeping the same BERKY “core” and simply swapping the detector modules (Maziarz et al., 2020).

The main advance provided by this biosensor design was the ability to characterize for the first time the properties of G protein activation not only directly but also under endogenous conditions. For example, it was found that the kinetics of endogenous G α -GTP formation and generation of G $\beta\gamma$ occurred at the same rate but that the rate of activation was distorted (i.e., slowed down) upon expression of exogenous G proteins (Maziarz et al., 2020). Moreover, based on its compact design as a single genetic component, it was possible to implement BERKY biosensors to study endogenous GPCR signaling in difficult-to-transfect cell types (Maziarz et al., 2020). This included recordings of G protein activation and deactivation with subsecond resolution in primary neurons, becoming the first example of G protein activity measurements in which all signaling components are expressed with native stoichiometry and in a well-differentiated cell type instead of a cell line. Since then, BERKY biosensors have been leveraged to carefully dissect mechanisms by which GPCR signaling is fine-tuned in neurons (Park et al., 2023) or in cancer cells (Zhao et al., 2023) while preserving endogenous levels of expression of all the signaling components involved.

Adding to the intrinsic strength of measuring signaling with high fidelity by preserving endogenous expression conditions, BERKY biosensors were shown to have no impact on signaling events downstream of G proteins (Maziarz et al., 2020). Thus, not only do BERKY biosensors not affect directly the early signaling events that they detect at the plasma membrane (i.e., G protein activity), but they also mitigate concerns that may arise from interference due to frequent feedback mechanisms triggered by downstream signaling events. While the unimolecular design of BERKY biosensors makes their implementation with endogenous GPCRs feasible even in difficult-to-transfect cells, a potential weakness is that the dynamic range of the responses detected in this context is small. Endogenous responses are small by definition compared with what is observed in systems that rely on exogenous expression of signaling components, and the maximal amplitude of BRET differences detectable by BERKY sensors is also constrained by the spatial separation of different modules in this unimolecular design and the affinity of the detector modules for G proteins. Finally, another limitation of BERKY biosensors developed to date is the lack of one that detects G α_s -GTP, which leaves out representation for one of the four G protein families.

Bystander BRET-Based Sensors of G α -GTP (EMTA). Another approach developed after the two G α -GTP biosensors systems described previously to measure the activity of untagged, and in some cases endogenous, G proteins is the biosensor design EMTA (Avet et al., 2022). In general, this

assay also relies on the use of G α -GTP detector modules, but in this case the measurements are based on the principle of bystander BRET. Instead of relying on the high-affinity binding between the proteins fused to the donor/acceptor pair, bystander BRET leverages the increase of random collisions due to crowding effects, typically occurring upon coincident localization of the donor-acceptor pair on the two-dimensional plane of the surface of a biological membrane (Kuravi et al., 2010). This principle was implemented in the EMTA platform by using donor-fused detector modules that are recruited from the cytosol to the plasma membrane due to binding to active G proteins, which in turn leads to an increase of BRET with a “bystander” acceptor (YFP) constitutively attached to the plasma membrane (Fig. 3E) (Avet et al., 2022). While this design was successfully implemented to establish biosensors for 11 G protein subtypes covering the G $\alpha_{q/11}$, G $\alpha_{i/o}$ and G $\alpha_{12/13}$ families, the lack of a suitable detector module precluded it for G α_s (Avet et al., 2022). For the latter, the authors mimicked a previously described design (Martin and Lambert, 2016) relying on loss of bystander BRET signal between donor-tagged G α_s and membrane-bound acceptors, which leverages the G α_s translocation as an indirect readout of G protein activity (Avet et al., 2022). Of note, a detector module for G α_s -GTP has been recently identified (Janicot et al., 2024) and shown to report GPCR responses when implemented in a bystander BRET assay format equivalent to that of the EMTA, suggesting that the latter platform could now be harmonized in the same format for representative members of all G protein families.

Because EMTA biosensors function with untagged G proteins, they are in principle compatible with experiments using either exogenous or endogenous G proteins. By leveraging the large dynamic range of detection with exogenous G proteins and exogenous GPCRs in HEK293 cells, the EMTA platform enabled large-scale profiling of the coupling preferences of 100 receptors with 12 different G proteins, which revealed some differences with analogous datasets established previously through other approaches (Avet et al., 2022). For example, compared with a previous coupling dataset based on the use of G protein chimeras and a downstream signaling activity readout (Inoue et al., 2019), or to a dataset from the IUPHAR “Guide to Pharmacology” based on curated information from the literature (Harding et al., 2023), the EMTA platform reported a higher percentage of GPCRs that could activate the G $\alpha_{12/13}$ family (Avet et al., 2022). The EMTA biosensor design also allowed for interrogation of G protein activity with subcellular resolution, as demonstrated by showing G $\alpha_{q/11}$ activation at early endosomes through targeting of the bystander acceptor to this particular location (Wright et al., 2021). As for the suitability of EMTA biosensor to report the activity of endogenous G proteins and/or endogenous GPCR, the evidence is more limited. While the authors provided two examples of responses detected in cell lines for an endogenous receptor with endogenous G proteins, most other cases of endogenous GPCR responses relied on the expression of exogenous G proteins (Avet et al., 2022).

While EMTA biosensors allow for direct and robust measurements of untagged GPCRs and G proteins, a potential concern is interference with the signaling mechanism under investigation. It is possible that the expression of detector modules at the levels required for this assay could interfere with G protein signaling, which has not been directly addressed

to date. In some cases, like with the sensors for $G_{i/o}$ family proteins, the detector module used (Rap1GAP) could bind to inactive G proteins even better than to active G proteins and block their activation, since Rap1GAP has been shown to be a GDI for $G_{\alpha i}$ (Webb et al., 2005; Willard et al., 2007). The EMTA also has limitations in the context of investigating G protein activation kinetics, because shuttling of the detector modules between subcellular compartments will make the rates of BRET responses different from the actual kinetics of G protein activation. Finally, the broad applicability of this system across cell types that are difficult to transfect remains to be established, given that there is a minimum requirement of expressing simultaneously two genetic components (detector module and bystander acceptor).

Single-Vector BRET-Based Sensors of $G\alpha$ -GTP (ONE-GO).

While some of the $G\alpha$ -GTP biosensors described here allow measurement of GPCR activity with high fidelity and minimal to no interference with signaling, two general limitations still apply to them: the lack of broad applicability in physiologically relevant systems that are hard to transfect (e.g., primary cells) and the difficulty of implementation for non-experts. To address these limitations, the components of the biosensors described in earlier (i.e., using membrane-anchored detector modules) were repurposed to generate a new design, the ONE-GO biosensor (Fig. 3F) (Janicot et al., 2024). The ONE-GO biosensor design relies on the expression of YFP-tagged $G\alpha$ along with the specific detector modules, both introduced into cells as one genetic payload and expressed in optimal proportions to achieve acceptor-to-donor ratios conducive to a larger dynamic range of the responses detected. To achieve this, the sequence encoding the $G\alpha$ -YFP component was positioned immediately downstream of the promoter, whereas the detector module fused to Nluc was placed after a low efficiency internal ribosome entry sites sequence (Bochkov and Palmenberg, 2006; van Unen et al., 2016), which leads to high acceptor-to-donor expression ratios. This biosensor design did not require the expression of exogenous $G\beta$ and $G\gamma$ subunits, relying instead on the assembly of functional G protein heterotrimers formed by binding of endogenous $G\beta\gamma$ to the exogenous $G\alpha$ -YFP expressed in low amounts. The ONE-GO genetic constructs were assembled into a lentiviral vector backbone and kept under the size limit for packaging to make them compatible with transduction into cells that are hard to transfect (e.g., primary cells). In total, 10 ONE-GO biosensors were developed for different $G\alpha$ subtypes covering all four G protein families (Janicot et al., 2024).

The advances allowed by ONE-GO biosensors include enabling the interrogation of endogenous GPCR activity in cell lines and in a broad range of primary cell cultures like neurons, fibroblasts, or endothelial cells and across multiple assay formats, as for the parallel interrogation of hundreds of experimental conditions at once (Janicot et al., 2024). The versatility and ease of use were leveraged to interrogate context-dependent GPCR signaling, a feature known to influence response outcomes (Kenakin, 2018, 2019; Marti-Solano, 2023) but that had not been addressed by directly measuring G protein activity. A conclusion from these efforts was that context dependence is a prevalent feature of GPCR signaling even at the earliest event of the signal transduction mechanism (i.e., nucleotide exchange on G proteins elicited by the action of the receptor). This contrasts with the prevailing view that context dependence, also referred to as *system bias*, is determined by

how downstream signaling cascades are wired in a particular cellular context and that receptor proximal events are largely insensitive to it (Kenakin, 2018, 2019). For example, it was shown that the selectivity of the protease-activated receptor 1 for different G proteins depends on the cell type in which it is expressed. Additionally, receptors presumed to couple to G proteins of the same class (G_i and G_o) only do so for some GPCRs but not others in primary neurons (Janicot et al., 2024). Moreover, the G protein coupling selectivity of protease-activated receptor 1 depended not only on cell type but also on cell state, as demonstrated in experiments using a cellular model of cardiac fibrosis showing that quiescent fibroblasts and transformed myofibroblasts display different profiles of G protein activation (Janicot et al., 2024). In addition to enabling the interrogation of endogenous GPCR activity in many different cell types, the simplicity of delivering ONE-GO biosensors as a single genetic payload with preoptimized BRET acceptor-to-donor expression ratios also facilitates their implementation in a wide variety of assay formats, especially those requiring the interrogation of many conditions in parallel. For example, this allowed the functional profiling of clinically used antipsychotics across dozens of different GPCRs and of multiple naturally occurring genetic variants of several target GPCRs (Janicot et al., 2024).

ONE-GO biosensors combine robust performance in recombinant systems, similar to other systems like EMTA or TRUPATH, with their applicability with endogenous GPCRs, including in physiologically relevant systems like primary cells. These features stem from the single-plasmid, compact design, which permits convenient deployment without cumbersome optimization of expression conditions or troubleshooting to achieve adequate working conditions because the biosensor performance is largely preestablished by the design of the genetic construct. However, ONE-GO sensors do require the expression of low amounts of exogenous G proteins. While this does not have detectable effects on downstream signaling (Janicot et al., 2024), it could be a concern in situations in which recording activity with the highest possible fidelity is required.

Conclusions and Perspectives

This review provides an overview on the progress made through the development of biosensors to directly detect G protein activity and the different features associated with each particular design (summarized in Fig. 4). A key conclusion of this review is that every biosensor design has strengths and weaknesses and that choosing to use a particular one should be tailored to the specific scientific questions and needs of investigators. An important general consideration when using biosensors is that, by definition, any tool used to measure a particular biological phenomenon (in this case, GPCR-mediated signaling) is bound to affect the process under investigation to some degree. What is key to assess and consider is to what degree the assay distorts the phenomenon under investigation and whether this is acceptable for the specific scientific question that one intends to answer. For example, if the priority is to investigate signaling of endogenous GPCRs and G proteins with the highest possible fidelity, BERKY biosensors would be an adequate choice given that effects on signaling events downstream of G protein activation are undetectable. However, the small amplitude of the responses detected with these biosensors would represent an unwarranted challenge if studying

Biosensor Design	Activation event measured	G protein subunit tagged	Endogenous GPCR compatibility	Endogenous G α ?	Endogenous G $\beta\gamma$?	Applicability in primary cells	Targetable to sub-cellular compartments
G α -G $\beta\gamma$ dissociation <i>FRET</i>	G α -G $\beta\gamma$ dissociation	G α + G $\beta\gamma$	Low	No	No	Low	No
G α -G $\beta\gamma$ dissociation <i>BRET</i>	G α -G $\beta\gamma$ dissociation	G α + G $\beta\gamma$	Low	No	No	Low	No
Free G $\beta\gamma$ “detector” <i>BRET / FRET</i>	Release of free G $\beta\gamma$	G $\beta\gamma$	Low	No	No	Low	No
G α -GTP “detectors” <i>BRET</i>	Formation of G α -GTP	G α	Low	No	Yes	Low	Yes [#]
BERKY <i>BRET</i>	Formation of G α -GTP	None	High	Yes	Yes	High	Yes
EMTA <i>BRET</i>	Formation of G α -GTP	None	High	Yes	Yes	Low	Yes [#]
ONE-GO <i>BRET</i>	Formation of G α -GTP	G α	High	No	Yes	High	Yes

Fig. 4. Comparisons of the features of direct G protein activity biosensors. The table in this figure summarizes the characteristics of the RET biosensors highlighted in this review. [#] indicates demonstrated use of targetability feature, whereas others indicated as “Yes” are only theoretically possible.

both GPCRs and G proteins at endogenous levels is not required for the specific question. In some cases, a reasonable compromise could be to use ONE-GO biosensors to study endogenous GPCRs even in primary cells, with the compromise that some exogenous, tagged G protein needs to be expressed. Systems like EMTA or the free G $\beta\gamma$ sensor based on the GRK3ct detector module would mitigate tagging of the G α subunit, although they might be challenging to implement in cells that are difficult to transfect. Several biosensors have proven to be suitable for investigating the rapid kinetics of G protein activation and deactivation, including those that detect free G $\beta\gamma$ or G α -GTP based on membrane-anchored detector modules, BERKY, or ONE-GO biosensors. In some cases, like when investigating some non-GPCR regulators of G proteins, it might be critical to detect either G α -G $\beta\gamma$ dissociation or G α -GTP formation to understand the mechanisms at play, which would then determine which biosensor to implement. If the priority is to detect large responses, and working in highly transfectable cells is acceptable for the intended goal, the TRUPATH design would be a good choice given that it was specifically developed for this purpose, although systems like EMTA or ONE-GO also display large responses and are suitable for scaling up conditions for high-throughput applications in cell lines. This series of examples does not intend to be exhaustive or definitive but tries to illustrate the importance of taking into consideration the relationship between the question under investigation and the features of the biosensor system. We hope that this review provides other investigators with a reference to make informed decisions in this regard.

A number of challenges associated with existing biosensors still remain to be addressed. As summarized previously, there is a wealth of biosensor options to measure G protein activity

directly using recombinant systems in transfectable cell lines, and there has been significant progress in developing new approaches to investigate the activity of endogenous GPCRs and G proteins in different cell types. For the latter, there is still room for improvement. For example, while biosensors relying on recombinant protein expression have been systematically optimized to improve the dynamic range of detection and reproducibility (Masuho et al., 2015a; Olsen et al., 2020), biosensors suitable for endogenous G proteins (e.g., BERKY) perform less robustly (Maziarz et al., 2020). This is not surprising given that endogenous responses are bound to be smaller than those obtained in overexpression systems, yet it represents a limitation to overcome to facilitate their wide adoption. Another important aspect of any biosensor platform is to have a wide or comprehensive coverage of G protein subtypes to capture different signaling modes by GPCRs. While G_s activation can be detected with various biosensor platforms using tagged G protein subunits, there are no biosensors suitable to measure the activity of either endogenous G α s or endogenous G α olf, even for the two platforms in which this is theoretically possible, BERKY and EMTA (Maziarz et al., 2020; Avet et al., 2022). This is also true for some G α subunits of the G_{i/o} family involved in sensory perception like transducins (G α t1, G α t2) or gustducin (G α gust). Both the limitation of dynamic range and G protein type coverage could be overcome by identifying new proteins or peptides as detector modules. For example, one would expect that higher affinity detector modules might improve the sensitivity of biosensors or enable new G protein specificities. Regarding the latter, a recently described G α s-binding peptide (Janicot et al., 2024) holds the promise of enabling biosensors for endogenous G_s activity. Other avenues for improving the dynamic range of detection could include

exploring alternative donor-acceptor pairs or their relative orientation (Fritz et al., 2013). As a final note related to the relationship between the dynamic range of detection of biosensors and their ability to report the activity of endogenous signaling proteins, the recently described ONE-GO biosensor platform represents an interesting compromise and illuminates an avenue that could be capitalized to improve other systems. ONE-GO biosensors are broadly applicable with endogenous GPCRs in many different cell types, and, even though they rely on exogenous expression of tagged G proteins, their potential interference is largely mitigated by the compact single-plasmid design that enables low expression levels and adequate BRET donor-to-acceptor ratios. Thus, it is conceivable that mimicking a similar design to existing biosensor platforms that are currently implemented exclusively in cell lines overexpressing GPCRs might enable their implementation in other cell types with endogenous GPCRs. A proof of principle for the idea has already been provided with the free $G\beta\gamma$ sensors that relies on GRK3ct as a detector module (Janicot et al., 2024). An orthogonal strategy to directly measure endogenous G protein activity would be to leverage gene-editing approaches to tag endogenous signaling components (White et al., 2020; W. Jang, preprint, DOI:<https://doi.org/10.1101/2024.03.05.583500>) for biosensor applications. This could be achieved by the editing of endogenous G proteins or by combining endogenous G protein modification with the expression of other components (e.g., detector modules) required to assemble the biosensor design. Although this approach would require generating gene-edited versions of each particular cell type of interest, which might or might not be feasible depending on the particular case, an attractive idea would be to generate whole genetically modified organisms (e.g., mice) for broader applicability.

There are two potentially overlapping broad areas in which the future developments of G protein activity biosensors could have a transformative effect. One area pertains to the improvement of imaging-based tools to gain spatial resolution in recording, whereas the other area relates to gaining the ability to record G protein activity in intact tissues or even in whole animals. The transformative potential of improving imaging-based tools arises, at least in part, from the ongoing interest in dissecting the relationship between the subcellular localization of active G proteins and signaling outcomes (Eichel and von Zastrow, 2018; Stoeber et al., 2018; Tsvetanova et al., 2021; Kumar and Puthenveedu, 2022; Radoux-Mergault et al., 2023; Klauer et al., 2024). While it is becoming increasingly evident that GPCRs and G proteins operate as functional signaling units in subcellular compartments like endosomes or the Golgi apparatus, among others, it has been challenging to date to directly monitor in real time these signaling events *in situ*. Moreover, gaining spatial resolution would represent an advance not only in this emerging area of organellar signaling but also in more classical, yet equally inaccessible, aspects of compartmentalized signaling. For example, distinguishing G protein activation events in soma, dendrites, or synapses of neurons could propel significant advances in neurobiology. Even gaining the ability to monitor endogenous G protein activity without subcellular resolution but simply with single-cell resolution would also enable the answering of questions of relevance in systems with cellular heterogeneity, like cocultures, organoids, or tissue sections. The information summarized in this review makes evident that much of the progress done in developing G protein activity biosensors has been achieved through BRET-based technologies,

which are not well suited for imaging, or at least for imaging with sufficient resolution, but allow for high signal-to-noise ratios in populations of cells. Thus, it is likely that progress in this area will require envisioning biosensors based on other principles of signal detection. The first approach that comes to mind is FRET, as it might be possible to repurpose many of the BRET biosensor designs' components. While some FRET biosensors of G protein activity have been reported, there is still much room for improvement and optimization based on advances in fluorophore brightness, chemogenetic, or circularly permuted RET pairs. Other promising approaches are fluorescence lifetime imaging (Chen et al., 2014; Levitt et al., 2020; Vu and Arai, 2023; Tilden et al., 2024), 2-photon polarization microscopy (Lazar et al., 2011; Yellen and Mongeon, 2015; Bondar and Lazar, 2017), and other biosensor designs that rely on conformational changes of fluorescent proteins that result in brightness intensity changes (Mehta et al., 2018; Kostyuk et al., 2019), all of which might enable single wavelength measurements, thereby overcoming many of the data processing and corrections required for more traditional sensitized-emission FRET approaches used so far.

As for the second area for potentially transformative progress, the development of biosensors compatible with measurements in intact tissues or organisms would enable the investigation of GPCR-G protein signaling in a context that accounts for the complex intercellular relationships and mechanical properties *in vivo* that cannot be properly recapitulated by cultured cells. For example, one could envision leveraging biosensors in brain slices to dissect GPCR signaling in neurons or even specific types of neurons. This would not only better preserve the identity of the cell type under study but would also account for the potential influence of other cell types, like glia, in shaping the signaling responses under study. Similarly, certain disease-associated phenomena occur at the tissue level rather than cell autonomously, so their influence on GPCR signaling cannot be properly assessed with isolated cells. For example, almost any form of tissue fibrosis leads to changes in the mechanical properties of tissues. Since mechanosensitivity is emerging as a regulatory mechanism of many GPCRs (Scholz et al., 2015; Leiphart et al., 2019; Lin et al., 2022; Wilde et al., 2022), including but not limited to adhesion GPCRs, capturing the behavior of GPCR signaling under these environmental changes would be highly desirable. For the same reasons, approaches that allow the monitoring of GPCR signaling in intact tissues would also be advantageous to test new drugs under development and gain insights into the relationship between their direct effects at the molecular level and the desired therapeutic effects at the organismal level. The development of this next generation of biosensor designs compatible with measurements in intact tissues will most likely rely on imaging-based approaches (see previous paragraph) and/or efficient means to express the constructs of interest in defined cell types within specific tissues. The latter could be achieved through viral delivery approaches with suitable cell-specific promoters, or even through knocking in the biosensor constructs in the genome of a model organism like a mouse as conditional alleles, which could leverage the wide range of genetic tools already available to conditionally control gene expression through recombination.

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Data Availability

This review article contains no datasets generated or analyzed during the present study.

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