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Illuminating the complexity of GPCR pathway selectivity – advances in biosensor development

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Abstract

It should come as no surprise that G protein-coupled receptors (GPCRs) continue to occupy the focus of drug discovery efforts. Their widespread expression and broad role in signal transduction underline their importance in human physiology. Despite more than 800 GPCRs sharing a common architecture, unique differences govern ligand specificity and pathway selectivity. From the relatively simplified view offered by classical radioligand binding assays and contractility responses in organ baths, the road from ligand binding to biological action has become more and more complex as we learn about the molecular mediators that underly GPCR activation and translate it to physiological outcomes. In particular, the development of biosensors has evolved over the years to dissect the capacity of a given receptor to activate individual pathways. Here, we discuss how recent biosensor development has reinforced the idea that biased signaling may become mainstream in drug discovery programs.

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Introduction

The binding of a drug or ligand to a GPCR will stabilize or shift the conformational equilibria in favor of specific conformations that will, in turn, recognize specific transducer proteins to facilitate protein–protein interactions [1]. These transducers will initially feed into different signaling cascades that may undergo crosstalk further downstream. Historically, drugs that bind to the orthosteric site of a given GPCR have been categorized as either being partial or full agonists, antagonists, or inverse agonists. In brief, partial agonists produce a response that is inferior to that of a full agonist, an antagonist occupies the binding site without efficacy, and an inverse agonist decreases the basal activity of a receptor (negative efficacy) [2]. While this terminology is still relevant today, our understanding of the complexity of GPCR signaling has forced us to revisit some of these concepts and invent new ones.

It is now well accepted that GPCRs can engage with multiple protein partners with different relative potencies/efficacies — a concept known as functional selectivity. As a consequence, the field has exploited both structural and functional data to develop compounds that favor the recruitment of one partner over another — otherwise known as ligand-biased signaling [3]. The distinction between partial agonism and ligand bias is muddled by an unclear framework that fails to emphasize the importance of assay sensitivity, signal amplification and intrinsic receptor bias. While the use of comparable assays can solve the issue of sensitivity and signal amplification, (the principal focus of this mini-review), the inherent differential coupling strength is an additional consideration that reflects the complexity of receptor pharmacology. Bidirectional allostery between ligand binding and effector coupling may also affect the population of receptors that are capable of binding a ligand. In the absence of intrinsic bias between two pathways that are equally coupled to a GPCR (i.e. G protein and β-arrestin), we would not observe a measurable difference when comparing a partial agonist. In contrast, a biased agonist would produce an unbalanced response favoring one pathway over another. Functional selectivity requires careful consideration in the context of promiscuous transducer coupling (i.e. G_s over G_i activation). If the intention is to compare two compounds in two pathways, the assays should be comparable and allow to measure both the potency (normally expressed as the EC_{50}) and the efficacy (corresponding to the maximal response). In other words, results should be interpreted with caution when comparing signal amplified assays (i.e. cAMP production, Ca^{2+} mobilization) with the response of a drug in an assay that measures direct protein–protein interactions with the effectors. In the case where the

coupling strength to one pathway is intrinsically greater than another, the ability of a partial agonist to engage a weakly coupled pathway could be missed. A prime example of such differential coupling strength is the more potent activation of G proteins over β-arrestin exhibited by many GPCRs. Similarly, the timeframe of the assay should also be taken into consideration. Endpoint assays that do not take into consideration the different kinetics of the biological process may lead to misinterpretation of the efficacy/potency of ligands.

While beyond the scope of this mini-review, it is equally important to consider both efficacy and potency when analyzing potentially biased ligands. If the goal is to understand the mechanism underlying functional selectivity and ligand bias, assay choice is especially important and should prioritize signal-to-noise with limited signal amplification. Above all, the ultimate consequence of these pharmacological concepts should be assessed by measuring functional outcomes.

In order to better understand these biological complexities, molecular tools or biosensors have been developed to quantify activity at different levels of signal transduction. In the context of GPCR pharmacology, these biosensors have largely integrated luminescence-based and/or fluorescence-based readouts. Here, we summarize the recent advances in biosensor development with a focus on bioluminescence resonance energy transfer (BRET)-based tools and how biased signaling has emerged as an important concept in drug discovery and development.

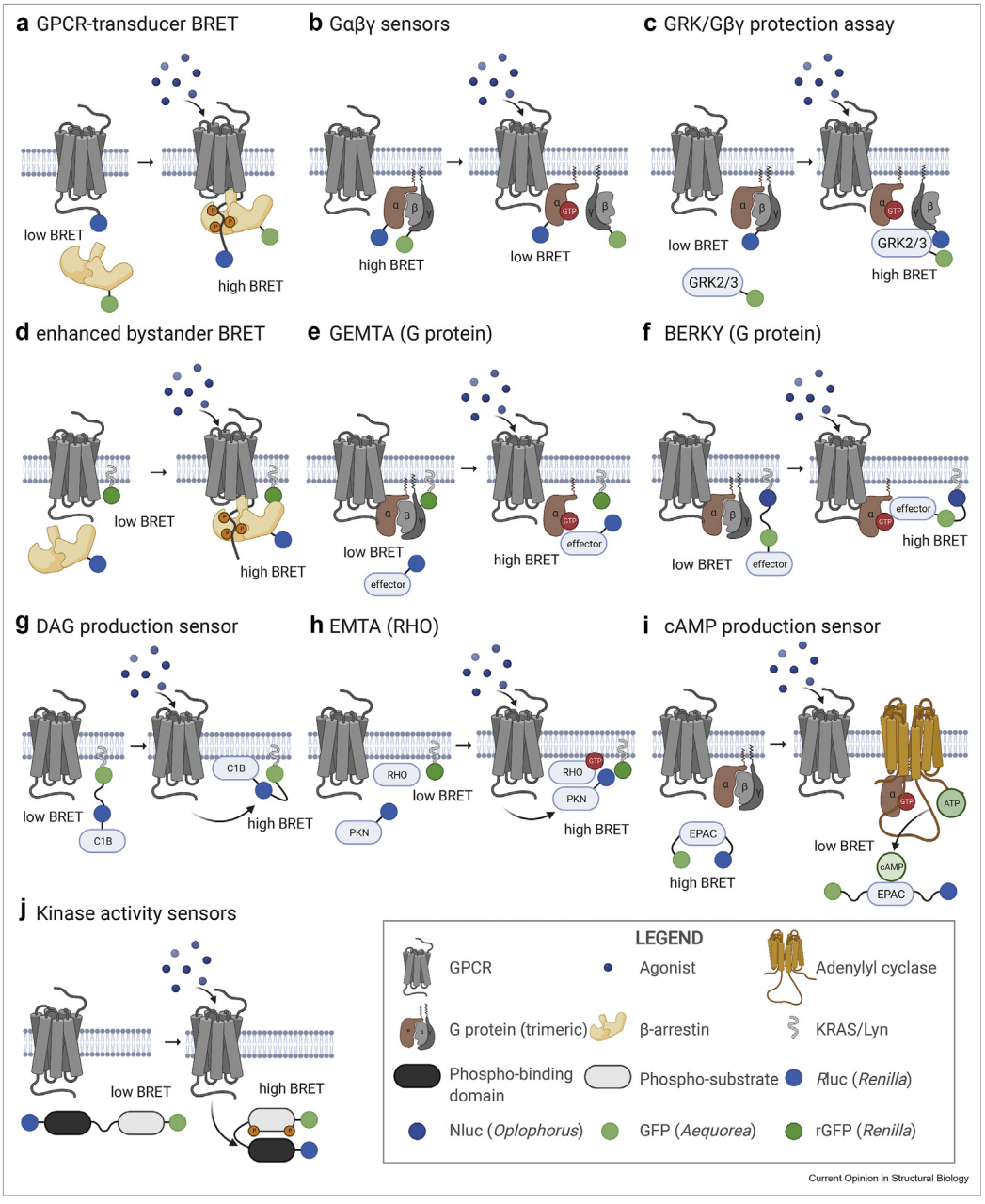
Measuring transducer coupling at the interface of GPCR signaling

Energy transfer between chromophores is based on well-established principles that have been integrated into engineered biosensors to measure anything from ligand binding and protein–protein interactions to conformational changes and from protein trafficking to second messenger production [4–6]. Proteins or protein fragments are engineered as fusion constructs with energy donor and acceptor pairs and expressed in living cells. The cells are subsequently exposed to a stimulus that results in a change in the energy transfer between donor-tagged and acceptor-tagged proteins. The energy transfer-based assay that has been used to generate the largest number of biosensors is known as BRET and is dependent on the proximity and orientation of luminescent donor and fluorescent acceptor molecules that allow for dipole–dipole coupling [5]. An extensive panel of BRET-based biosensors now exists to measure the activation of diverse pathways implicated in GPCR signaling (Figure 1a–j, Table 1).

Early efforts focused on the development of sensors that could detect interactions between GPCRs and their cognate partners – heterotrimeric G proteins [7]. Luciferase-tagged receptors were shown to form pre-coupled complexes with acceptor-tagged subunits of the heterotrimer that could be modulated by the addition of ligand. More than a decade later, crystallization efforts were ramping up and the Gα subunits were minimalized and engineered as mini G (mG) proteins to stabilize active GPCR conformations [8]. Soon thereafter, mG proteins were repurposed as BRET-based biosensors to profile receptor-G protein coupling [9]. Other well-known mediators of GPCR signaling, G protein-coupled receptor kinases (GRKs) and β-arrestin1/2, have also been subcloned as donor or acceptor fusion proteins or as conformational sensors to facilitate studies probing the downstream biases that ligands can confer through their interaction with the receptor [10–14] (Figure 1a). While important information could be inferred from these prior studies on G protein recruitment, they did not address one of the hallmarks of G protein activation–nucleotide exchange. First-generation sensors focused on the conformational rearrangement or heterotrimer dissociation associated with G protein activation [15]. To this end, luciferase was inserted into different sites within the Gα subunit to maintain functionality, and the Gβ or Gγ subunits were tagged with GFP producing a suite of sensors colloquially known as Gαβγ (pronounced Gaby) sensors allowing one to measure the activation of most Gα paralogues (Figure 1b, Table 1). In contrast to Gβγ, for which protein–protein interactions do not seem to be significantly affected by the addition of N-terminal tags, one cannot exclude that the insertion of large bioluminescent proteins in the Gα could have an impact on activity or protein–protein interactions. For this reason, an alternative approach was employed that relies on the sequestering of free Gβγ subunits by GRK2/3 following G protein activation through monitoring BRET between GRK2/3 and Gβγ [16–19] (Figure 1c). These approaches have been extended to nearly all G protein paralogues with progressive modifications to enhance the signal-to-noise ratio [20–29]. Although these biosensors indirectly demonstrate the catalytic potential of the G protein, definitive proof of nucleotide sensitive coupling has only been shown in the context of luciferase-tagged receptors with Venus-tagged Gβγ dimers in permeabilized cells exposed to apyrase or excess nucleotide [30].

The aforementioned sensors have been used in hundreds of studies related to structural biology and pharmacology, but they are inherently limited in their ability to study functional selectivity. Functional signaling complexes in native systems rely on unmodified protein stoichiometry. Supplementing a biological system with an excess of a given effector may facilitate protein–protein interaction and even activation, but whether these two signaling components would ever meet under endogenous circumstances is not clear.

Figure 1



Monitoring biased signaling using biosensors at different levels of signal transduction. Schematic representation of the principal biosensors used at the interface of the agonist-bound GPCR (**a to d**), effector engagement of active signaling species (**e and f**), and finally signal amplified events (**g to j**). **(a)** GPCR-transducer BRET requires that both the receptor and the transducer be fused to either a donor or an acceptor. β-arrestin is shown as an example of a transducer, but other examples are listed in Table 1. **(b)** Heterotrimeric G protein dissociation/reassociation BRET sensors now exist for all G protein families and almost all paralogues. Different donor and acceptor pairs and insertion points exist, but the biological concept is the same. **(c)** G protein activation can also be assessed indirectly by monitoring the recruitment of G protein-coupled receptor kinase 2/3 (GRK2/3) to free Gβγ. **(d)** Bystander BRET and enhanced bystander BRET (ebBRET) monitor ligand-dependent protein trafficking by using markers of organelles fused to an acceptor protein. **(e)** G protein effector membrane translocation assay (GEMTA) sensors are built on ebBRET and employ effectors of the different G protein families to measure recruitment to the plasma membrane. **(f)** BRET biosensors with ER/K linker and YFP (BERKY) sensors similarly use effectors of the different G protein families and a marker of the plasma membrane, but they are constructed as flexible unimolecular proteins. **(g)** Diacylglycerol (DAG) production can be measured using a unimolecular sensor composed of the cysteine-rich zinc finger (C1B) DAG-binding domain of protein kinase C (PKC) tethered to the plasma membrane that folds upon itself to produce BRET. **(h)** Different active species can be detected using effector membrane translocation assay (EMTA), such as the small GTPase Ras homolog family member (Rho), which upon activation will bind to protein kinase N (PKN) at the plasma membrane. **(i)** cAMP production can be measured using the release of the autoinhibitory conformation of exchange proteins directly activated by cAMP (EPAC) after binding of cAMP, which results in a decrease in BRET. **(j)** The kinase activity of different GPCR-regulated kinases such as PKC and ERK1/2 can be measured using unimolecular sensors composed of a phospho-substrate and a phospho-binding domain connected by a flexible linker that, once

One such example is the glucagon receptor (GCGR), for which structural and functional data exist to support its interaction with G_s and G_i [31]. While the G_s data is strongly supported by *in vivo* and studies in native systems, the ability of GCGR to activate G_i under physiological conditions is still an open question. There are also examples of non-canonical transducer interactions, such as the unproductive coupling of V_2R-G_{12} or the inverse coupling of 5-HT_7 receptors that may lead to changes in BRET signal using these tools but do not necessarily equate to activation [32,33]. Careful characterization of the functionality of these complexes requires orthogonal assays to ascertain pathway activation.

Pros and cons to signal amplified readouts

Such limitations often motivate the choice to move toward signal amplified readouts such as Ca^{2+} mobilization, the production of diacylglycerol and cAMP, the activation of Ras homolog family member (Rho) proteins, or the phosphorylation of ERK1/2 where native stoichiometry and structure can be maintained. Binding domains from proteins that are sensitive to the mobilization/production of second messengers or active-state proteins have been modified or used as building blocks to make biosensors that measure protein kinase C (PKC) activity, the production of cAMP [exchange proteins directly activated by cAMP (EPAC)] or diacylglycerol (C1B), Rho activation [protein kinase N (PKN)] and ERK1/2 phosphorylation [34–37] (Figure 1g–j).

While signal amplified readouts can be advantageous from the perspective of not modifying the transducer and often producing robust signals, it becomes a double-edged sword scenario when dissecting the contribution of different effectors to the response. In the case of a receptor that is coupled to both G_s and $G_{i/o}$ where G_s would stimulate the production of cAMP and G_i would inhibit this, the use of the EPAC sensor would provide an indication of either the preference for one family over another or the relative abundance of one family within the cell over another. Likewise, sensors of Rho activation like PKN will not distinguish activity from a receptor that is coupled to both $G_{q/11}$ and $G_{12/13}$ that both can activate the Rho pathway. There are also potential cases of crosstalk in signal amplified readouts where it becomes more difficult to distinguish the relative efficacy of drugs. A prime example of this comes from the use of the EPAC sensor in a system expressing calcium-sensitive adenylyl cyclase for GPCRs that are coupled to both G_q and G_s. In the context of drug discovery, assays that measure upstream events at the interface of the receptor–transducer complex provide higher certainty that the compound is acting through the receptor of interest and not through another protein that indirectly has an effect on the signaling readout. Ultimately, to have physiological relevance, ligand bias should result in a differential signaling outcome, which should be detectable using sensors monitoring downstream signals.

Moving toward native stoichiometry and bias – detecting GTP-loaded Gα by BRET

Next-generation BRET-based biosensors are built on those from previous generations by combining protein trafficking with enhanced bystander BRET (ebBRET) to monitor pathway activation (Figure 1d). The ebBRET assay relies on the natural coevolution of luciferase and GFP from *Renilla reniformis* for improved energy transfer and dynamic range [6,38]. Markers of different compartments are tagged with rGFP, and cytosolic effectors or effectors that have been engineered to localize to the cytosol are tagged with *R*lucII to monitor trafficking that is dependent on activation. The G protein effector membrane translocation assay (GEMTA) has been successfully applied to 3 out of the 4 families of G proteins – $G_{i/o}$ (Rap1Gap-*R*lucII), $G_{q/11}$ (p63RhoGEF-*R*lucII), and $G_{12/13}$ (PDZRhoGEF-*R*lucII or p115RhoGEF-*R*lucII) [39] (Figure 1e). These biosensors recognize the GTP-bound form of the Gα subunit providing a direct measure of the consequence of G protein activation – the step immediately downstream of pathway activation. For G_s, the search for an effector that can be transformed into an equivalent biosensor has proven difficult. One likely explanation is the intrinsic ability of G_s to leave the membrane upon activation [40,41]. This movement was exploited to create a biosensor that monitors G_s leaving the membrane by monitoring BRET between G_s-*R*lucII and rGFP anchored to the plasma membrane through the polybasic sequence with the prenylated CAAX box of the GTPase first identified in Kirsten Rat Sarcoma virus (KRAS) [39,41]. While this particular sensor is a direct measure of the consequence of G_s activation, the G_s is modified (*R*lucII insertion) and requires heterologous expression. Moreover, it does not provide an indication of the duration of G protein activity contrary to the sensors available for the other G protein families. The ebBRET-based sensors of GRK2 and β-arrestin1/2 recruitment have also been engineered to monitor receptor engagement of other transducers [6,33].

Simultaneous efforts by others took a different approach in developing unimolecular biosensors called

phosphorylated, will result in the binding of the two, thus bringing the acceptor and donor pair closer together. For the sake of simplicity, the donor and acceptor variants have been grouped as either *R*luc (*Renilla* luciferase) or Nluc (*Oplophorus* nanoluciferase) and GFP (*Aequorea*) or rGFP (*Renilla*), respectively. Created with BioRender.com.

Table 1

Nonexhaustive summary of BRET-based biosensors that measure responses for numerous signaling pathways. Signaling pathways are grouped according to the primary transducer that forms an active signaling complex with the agonist-bound receptor. For the sake of simplicity, the donor and acceptor variants have been grouped as either *R*luc (*Renilla*) or Nluc (*Oplophorus*), and GFP (*Aequorea*) or rGFP (*Renilla*), respectively, and the insertion sites have not been included.

BIOSENSOR	DESCRIPTION	REFERENCE
G_s		
GPCR-*R*luc8 + GFP-mGs	mG sensor – measures the active conformation of the GPCR capable of engaging G_s	[9]
GPCR-GFP + $G\alpha_s$-*R*luc	GPCR-$G\alpha_s$ interaction assay	[25]
$G\alpha_s$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[7,15,20,26,27]
$G\alpha_s$-*R*luc + GFP-KRas	Bystander BRET sensor – measures $G\alpha_s$ leaving the plasma membrane	[41]
$G\alpha_s$-67-*R*luc + rGFP-CAAX	ebBRET sensor – measures $G\alpha_s$ leaving the plasma membrane	[39]
*R*luc-ERKsubstrate-GFP	REV sensor – measures phosphorylation of ERK1/2	[35]
*R*luc-EPAC-GFP	EPAC sensor – detects the production of cAMP stimulated by G_s	[36,37]
$G_{i/o}$		
GPCR-*R*luc + GFP-mGsi	mG sensor – measures the active conformation of the GPCR capable of engaging $G_{i/o}$	[9]
$G\alpha_{i1}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[15,20,27]
$G\alpha_{i2}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[15,20,27]
$G\alpha_{i3}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,23,27]
$G\alpha_{oA}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,23,24,27]
$G\alpha_{oB}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,23,24,27]
$G\alpha_z$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,22]
$G\alpha_{gust}$-117-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20]
*R*luc-EPAC-GFP	EPAC sensor – detects the production of cAMP inhibited by $G_{i/o}$	[36,37]
Rap1Gap-*R*luc + $G_{i/o}$ + rGFP-CAAX	GEMTA sensor – measures activation of $G_{i/o}$ at the plasma membrane	[39]
Lyn11-Nluc-ER/K-GFP-KB-1753	BERKY sensor – measures activation of $G_{i/o}$ at the plasma membrane	[42]
*R*luc-ERKsubstrate-GFP	REV sensor – measures phosphorylation of ERK1/2	[35]
$G_{q/11}$		
GPCR-*R*luc + GFP-mGsq	mG sensor – measures the active conformation of the GPCR capable of engaging $G_{q/11}$	[9]
$G\alpha_q$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,21,27]
$G\alpha_{11}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,27,28]
$G\alpha_{15}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20]
GFP-CAAX-*R*luc-C1B	DAG biosensor – measures the production of DAG at the plasma membrane	[34]
GFP-FHA1-FHA2-TLKI-TLKD-*R*luc-C1B	PKC biosensor – measures the activation of PKC	[34]
p63RhoGEF-*R*luc + $G_{q/11}$ + rGFP-CAAX	GEMTA sensor – measures activation of $G_{q/11}$ at the plasma membrane	[39]
Lyn11-Nluc-ER/K-GFP-GRK2^{RH}	BERKY sensor – that measures activation of $G_{q/11}$ at the plasma membrane	[42]
PKN-RBD-*R*luc + rGFP-CAAX	Rho GTPase activation at the plasma membrane	[34]
*R*luc-ERKsubstrate-GFP	REV sensor – measures phosphorylation of ERK1/2	[35]
$G_{12/13}$		
GPCR-*R*luc + GFP-mG12	mG sensor – measures the active conformation of the GPCR capable of engaging $G_{12/13}$	[9]
$G\alpha_{12}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,27,28]
$G\alpha_{13}$-*R*luc + Gβ + GFP-Gγ	'Gαβγ' sensor – measures the dissociation/rearrangement of Gα and the Gβγ dimer	[20,27,28]
PKN-RBD-*R*luc + rGFP-CAAX	Rho GTPase activation at the plasma membrane	[34]

Table 1 (*continued*)

BIOSENSOR	DESCRIPTION	REFERENCE
Lyn11-Nluc-ER/K-GFP-Rhotekin RBD	BERKY sensor – measures Rho GTPase activation at the plasma membrane	[42]
PDZRhoGEF-*R*luc + rGFP-CAAX	GEMTA sensor – measures activation of $G_{12/13}$ at the plasma membrane	[39]
p115RhoGEF-*R*luc + rGFP-CAAX	GEMTA sensor – measures activation of $G_{12/13}$ at the plasma membrane	[39]
Lyn11-Nluc-ER/K-GFP-PRG^{RH}	BERKY sensor – measures activation of $G_{12/13}$ at the plasma membrane	[42]
Gβγ		
GRK2-GFP + Gα + Gβ + $G\gamma_5$-*R*luc	GRK/Gβγ protection assay	[18]
masGRK3-*R*luc/Nluc + Gα + Gβγ-GFP	GRK/Gβγ protection assay	[16,17,19]
Lyn11-Nluc-ER/K-GFP-GRK3ct	BERKY sensor – measures the release of Gβγ at the plasma membrane	[42]
GRK2/3		
GRK2-*R*luc + rGFP-CAAX	ebBRET GRK2 recruitment assay	[33]
GPCR-GFP + *R*luc-GRK2	GPCR-GRK2 interaction assay	[11]
GPCR-*R*luc + GRK2-GFP	GPCR-GRK2 interaction assay	[14,33]
GPCR-*R*luc + GRK3-GFP	GPCR-GRK2 interaction assay	[14]
β-arrestin1/2		
GPCR-*R*luc + GFP-β-arrestin2	GPCR-β-arrestin interaction assay	[14]
GPCR-GFP + β-arrestin2-*R*luc	GPCR-β-arrestin interaction assay	[14]
GPCR-*R*luc + GFP-β-arrestin1	GPCR-β-arrestin interaction assay	[14]
β-arrestin1-*R*luc + GPCR-GFP	GPCR-β-arrestin interaction assay	[14]
β-arrestin1-*R*luc + rGFP-CAAX	ebBRET β-arrestin recruitment assay	[39]
β-arrestin2-*R*luc + rGFP-CAAX	ebBRET β-arrestin recruitment assay	[6,39]
*R*luc-β-arrestin2-GFP	Double brilliance β-arrestin conformation sensor	[12]

'BRET biosensor with ER/K linker and YFP' (BERKY) that detect the formation of Gα-GTP or free Gβγ. To this end, peptides or protein fragments were selected as follows: KB-1753 for $G_{i/o}$, RGS domain of GRK2 for $G_{q/11}$, RH domain of PDZRhoGEF for $G_{12/13}$, the C-terminal region of GRK3 for Gβγ and Rhotekin RBD for Rho-GTP [42] (Figure 1f). Like the GEMTA sensors, BERKY sensors contain a membrane localization motif from Lck/Yes novel tyrosine kinase (Lyn) to measure G protein activation at the plasma membrane, but are based on NanoBRET and employ donor and acceptor pairs from different species [Nluc (*Oplophorus gracilirostris*) and YFP (*Aequorea victoria*), respectively]. BERKY sensors have the advantage that they are a single genetic component, which facilitates their implementation; however, the stoichiometry is fixed, which limits the signal-to-noise ratio, and the different origins of the donor and acceptor pair have inferior energy transfer compared to luciferase and GFP from *R. reniformis*.

Concluding remarks and future perspectives

Structural biology continues to have an enormous influence on the field of GPCR pharmacology. Diverse ligands coupled with genetic variants have provided a basis for the study of bias. One could argue that structural biology has, in a sense, driven the development of biosensors toward a platform that can better unravel the unique factors that drive functional selectivity in relevant models. While great strides have been made to profile transducer coupling for many GPCRs, perhaps the next frontier in biased signaling will be to integrate two emerging concepts known as location and kinetic bias [43,44] – adding another level of complexity and yet another hurdle to overcome for biosensor development. Moving biosensors to *in vivo* settings also represents an area of active research that will undoubtedly generate powerful tools to assess the physiological relevance of functional selectivity and biased signaling [45,46]. Finally, even if we may never be able to bring different biosensors on par with one another to compare pathway selectivity, a basic understanding of the advantages and limitations of the tools used will increase the likelihood of obtaining meaningful data from multiplexing.

Conflict of interest statement

M.B. is the president of the scientific advisory board for Domain Therapeutics. M.B. has filed patent applications related to some of the biosensors described in this work, and the technology has been licensed to Domain Therapeutics. S.C.W. declares no competing interests.

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** of outstanding interest

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