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Optical approaches for single cell and subcellular analysis of GPCR-G protein signaling

Dinesh Kankanamge¹, Kasun Ratnayake¹, Kanishka Senarath¹, Mithila Tennakoon, Elise Harmon, and Ajith Karunarathne*

Department of Chemistry and Biochemistry, The University of Toledo, 2801 W. Bancroft, Toledo, OH 43606, USA

Abstract

G protein-coupled receptors (GPCRs), G proteins and their signaling associates, are major signal transducers that control a majority of cellular signaling and regulate key biological functions including immune, neurological, cardiovascular, and metabolic processes. These pathways occupy over one-third of drugs on the market, however, the current understanding of their function is limited and primarily derived from cell-destructive approaches providing an ensemble of static, multi-cell information about the status and composition of molecules. Spatiotemporal behavior of molecules-involved is crucial to understanding in vivo cell behaviors both in health and disease, and the advent of genetically encoded fluorescence proteins and small fluorophore-based biosensors has facilitated the mapping of dynamic signaling in cells with subcellular acuity. Since we and others have developed optogenetic methods to regulate GPCR-G protein signaling in single cells and subcellular regions using dedicated wavelengths, the desire to develop and adopt optogenetically-amenable assays to measure signaling has motivated us to take a broader look at the available optical tools and approaches compatible with measuring single-cell and subcellular GPCR-G protein signaling. Here we review such key optical approaches enabling the examination of GPCR, G protein, secondary messenger, and downstream molecules such as kinase and lipid signaling in living cells. The methods reviewed employ both fluorescence and bioluminescence detection. We not only further elaborate the underlying principles of these sensors, but we also discuss the experimental criteria and limitations to be considered during their use in single-cell and subcellular signal-mapping.

Keywords

GPCR; G protein; Signal Transduction; Fluorescence; FRET; BRET; Real-Time Assay; Imaging; Single Cell Analysis; Microscopy; Translocation

*Corresponding Author, Ajith.karunarathne@utoledo.edu.

¹Dinesh Kankanamge, Kasun Ratnayake, and Kanishka Senarath contributed equally to this work.

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INTRODUCTION

GTP binding protein (G protein) coupled receptors (GPCRs) represent the largest family of cell surface receptors found in all eukaryotic genomes and control the majority of cell signaling. Since assay-development is leveraged on chemical modifications and behavior of molecules involved in subcellular spaces, driven by atomic level interactions, we first review key events in GPCR-G protein signal transduction. All GPCRs share a common structure with seven transmembrane (TM) domains, an extracellular amino terminus (NT), and an intracellular carboxyl terminus (CT). Transmembrane regions are connected through three extracellular and three intracellular cytosolic loops. Heterotrimeric G proteins, that interact with GPCRs, consisting of three subunits; $G\alpha$, $G\beta$, and $G\gamma$, interact with the cytosolic-face and the core created by a counter-clockwise barrel structure of seven TM helices of the receptors [1,2]. Activation of GPCRs by ligands promotes GDP to GTP exchange on the $G\alpha$ subunit, which results in a conformational change in $G\alpha$ that leads to the dissociation of heterotrimer to $G\alpha$ GTP and $G\beta\gamma$ (Fig. 1) [3–5]. Generated $G\alpha$ and $G\beta\gamma$ initiate downstream signaling networks by interacting with effector molecules such as adenylyl cyclase (AC), phosphoinositide 3-kinase (PI3K), phospholipase C (PLC) and ion channels [6,7]. Additionally, the resultant free $G\beta\gamma$ subunits translocate from the plasma membrane (PM) to internal membranes (IMs) in a $G\gamma$ dependent manner [8] and has been shown to modulate $G\beta\gamma$ -effector activation at the PM [7].

GPCRs are activated by a diverse array of ligands such as hormones, lipids, neurotransmitters, glycoproteins and cytokines [2]. Light sensitive GPCRs in the vision system, called opsins, also possess the same 7-TM structure, bind to the inverse agonist retinal, and are activated upon exposure to light [9]. Activated GPCRs and G proteins regulate a large cohort of physiological functions, including sense (vision, taste and smell), cellular secretion, neurotransmission, metabolism, cell growth, differentiation, immune and inflammatory responses. Nearly one-third of all drugs on the market target GPCRs because of aberrant behaviors in GPCR-G protein pathways in diseases including immune and nervous system disorders, cardiovascular diseases, cancer, and diabetes. The limitation is that these drugs only cover ~10% of GPCRs found in the human genome [10,11]. Therefore, identification of molecules to expand the control to the rest of the GPCRs is paramount to human health.

Several GPCR-classifications based on their sequence and functional similarities are currently being used [12,13]. In the commonly used A-F classification, Class-A GPCRs or rhodopsin-like GPCRs represent ~80% of all GPCRs. In addition to the 7-TM structure, Class-A GPCRs possess a palmitoylated cysteine at the Ct [12]. Class-B, or secretin family has nearly 70 GPCRs and contains Nt region of over 100 residues that is stabilized by disulfide bonds. Among Class-B receptors, Vasoactive intestinal peptide, Calcitonin, Corticotropin releasing hormone, Glucagon, Parathyroid hormone, and adhesion subfamily GPCRs are included. Class-C encapsulates gamma-aminobutyric acid (GABA), metabotropic glutamate, calcium-sensing, and taste receptors [13]. Receptors in this family contain a large Nt region (~ 600 residues) connected to the TM1 through a loop containing cysteine. Classes D-F represent fungal pheromone receptors, cyclic adenosine monophosphate (cAMP) receptors, and frizzled/smoothed receptors [12].

GPCRs can also be classified based on the type of heterotrimer G α subunit they interact. Stimulatory (G α s)-pathway activates ACs thus catalyzing the formation of the second messenger cyclic AMP (cAMP). Generated cAMP then activates cAMP stimulated protein kinase A (PKA), which phosphorylates target proteins in the cell. These interactions are attenuated by phosphodiesterases (PDEs), which break down cAMP and dampen the signal [14]. Activation of the inhibitory G protein (G α i/o)-pathway inhibits ACs, decreases cAMP, and deactivates PKA to induce opposite effects of those elicited by G α s [15,16]. G α q-pathway stimulates PLC, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂), a PM-phospholipid and generates two second messengers: inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ diffuses through the cytosol, binds IP₃ receptors in the endoplasmic reticulum (ER), and releases stored Ca²⁺ to increase the Ca²⁺ level in the cell. DAG, which stays on the PM, stimulates protein kinase C (PKC) activation, and thereby phosphorylating target proteins [17]. G α 12/13-pathway signals by activating Rho family GTPases [18,19].

In addition to signaling triggered by G α GTP, the liberated G $\beta\gamma$ subunits also regulate crucial processes required for cell survival and homeostasis [20–22]. G $\beta\gamma$ has been shown to increase the activity of mitogen-activated protein kinase (MAPK) signaling pathways through PI3K/Akt and PLC β , as well as through insulin-like growth factor receptor 1 (IGFR1) and c-Src tyrosine kinase [22,23]. MAPK pathways promote survival, differentiation, proliferation and higher resistance to apoptosis [24,25]. An upstream regulator of matrix metalloproteinases (MMPs), signal transducer and activator of transcription 3 (STAT3) and STAT1 are also controlled by G $\beta\gamma$ [26]. MMPs are activated by extracellular signal-regulated (ERK) and c-Jun N-terminal kinase (JNK)-MAPK pathways and facilitate cell proliferation through the degrading extracellular matrix. CXCR7 inhibition reduces MMP-9 and MMP-2 activities in breast cancer cells [27], while it enhances MMP-12 expression by CXCR4 activation-induced ERK activation [28]. Additionally, G $\beta\gamma$ promotes prostate cancer cell migration by activating ERK1/2 upon Gi-coupled GABAB receptor activation [29].

GPCR - G protein signaling can be detected using the activities of many molecules in the pathway including the GPCR (Fig.1). Measurement of signaling at various depths in the signaling pathway requires detection of; (i) conformational changes in the receptor-domains upon ligand binding, (ii) interactions of the GPCR with G protein heterotrimer, G α and G $\beta\gamma$ subunits and downstream proteins that recognize the activated GPCR, (iii) GPCR activation-induced changes in protein interactions such as G $\alpha\beta\gamma$ heterotrimer dissociation and G $\beta\gamma$ translocation, (iv) GPCR activation induced generation of second messengers such as cAMP and Ca²⁺ as well as activation of a variety of downstream signaling such as protein phosphorylation and cell-behavior changes. We uncovered the spectral selectivity of several non-rhodopsin opsin GPCRs expressing cells can be exposed to designated wavelengths without activating opsins. This motivated us to characterize signaling probes to interrogate opsin-induced subcellular GPCR-G protein activation in single cells [9,30]. Since available methods lacked the ability to directly capture the dynamics of heterotrimer dissociation either due to lack of sensitivity or these methods only measured downstream signaling of activated G proteins, we developed G $\beta\gamma$ translocation-based G γ 9 assay [31]. We begin our commentary by reviewing G γ 9 translocation assay and supporting its potential using live

cell imaging data. We then discuss the other optical methods that have already been employed as well as approaches that can be adopted for single-cell and subcellular signaling detection.

1. Real-time detection of single-cell and subcellular GPCR-G protein activation using the $G\gamma 9$ assay

Heterotrimeric G-proteins are localized to the PM while being in transient interactions with GPCRs. $G\alpha$ subunits are palmitoylated and myristoylated at their N-terminal (Nt) cysteine [32], whereas the C-terminus (Ct) of $G\gamma$ in $G\beta\gamma$ dimer is prenylated either with a farnesyl or a geranylgeranyl lipid anchor [33]. These lipids supports the localization of heterotrimeric G proteins at the PM. Additionally, positively charged amino acids and hydrophobic aromatic amino acids at the Ct of $G\gamma$ has also been a crucial factor for PM localization of heterotrimers [8]. The inactive $G\alpha(\text{GDP})$ has high affinity to interact with $G\beta\gamma$ and form the heterotrimer. Upon GPCR activation by external stimuli, GDP in $G\alpha(\text{GDP})$ exchanges to a GTP, inducing heterotrimer-dissociation and generating activated G proteins, $G\alpha(\text{GTP})$ and $G\beta\gamma$ [34]. Using live cell imaging, it has been shown that the generated free $G\beta\gamma$ reversibly translocate from the PM to the internal membranes (IMs) [35,36]. We believe that the property differences between the PM and IMs as well as the higher surface area of IMs compared to the PM supports translocation. Using a fluorescent protein attached to either $G\beta$ or $G\gamma$ subunit, $G\beta\gamma$ translocation from the PM to IMs was observed.

This translocation of $G\beta\gamma$ from the PM to IMs depends on the type of $G\gamma$ [35,37]. There are 12 different G protein gamma subunits which exhibit distinct translocation rates upon GPCR activation [38,36]. $G\gamma 9$, 11 and 1 showed faster translocation compared to the rest while $G\gamma 9$ exhibited the fastest translocation half time ($T_{1/2} < 10$ s) for most GPCRs [38,7]. Recently, an assay based on $G\beta\gamma 9$ translocation was developed to monitor GPCR activation in living cells [31] (Fig. 2A, B). For example, activation and deactivation of endogenous $\alpha 2$ -adrenergic receptor ($\alpha 2$ -AR) in HeLa cells was demonstrated using $G\gamma 9$ translocation assay in the presence of agonist (norepinephrine, 100 μM) and antagonist (yohimbine, 100 μM) (Fig. 2). Not only was this assay used to measure activation and deactivation of both G_s - and G_i/o -coupled GPCR, but it was also able to detect activation of subcellular G protein through the localized optical activation of opsins [31,9,39]. Investigating various signaling events inside a cell and in organelles is crucial for understanding cellular signaling mechanisms involve in variety of physiological processes. Though chemical ligands were useful to decipher cellular signaling mechanisms, limitations such as ligand diffusion, lack of methods to deliver ligands to specific location of interest, low temporal or spatial control of the signaling activation have been limitations. However, the use of light and light activatable proteins allowed precisely control signaling in subcellular regions with higher temporal acuity.

Light activatable GPCRs; also called opsins, have been widely used as optogenetic tools for subcellular G-protein-GPCR activation detection [9,31,6,17]. For example, in single cells expressing blue opsin, diffraction limited 445 nm blue light was able to activate opsins and G-proteins only in blue light exposed region [31]. Similarly, temporal activation of blue

opsin was also shown where removal of optical stimuli resulted in deactivation of blue opsin which was observed by the recovery of the PM-bound G γ 9 (Fig. 2C). When using opsins as optogenetic tool for subcellular G-protein activation, the spectral overlapping properties of opsins and reporter fluorescent protein tethered to G-protein should be selected such that imaging wavelength should not activate the opsin. For example, blue opsin has a maximum absorption wavelength of 414 nm and its absorption spectrum spans around approximately 380 nm - 490 nm. Therefore, when conducting subcellular experiments using blue opsin, G-proteins tagged with fluorescent proteins with excitation wavelength above 500 nm, i.e. YFP-515 nm, mCherry-594 nm [39]. Blue light absorbing opsins such as blue opsin and melanopsin have been used to conduct subcellular G-protein activation in imaging experiments [31,9]. However, when the absorption spectrum of opsin even has a minor overlap with the excitation light or emission range of the fluorescence protein in the sensor, we have observed that imaging of fluorescence proteins can activates opsins. However, such opsin activations due to imaging can be minimized by reducing the intensity of the excitation light, exposure time and frequency of imaging. Use of efficient optics, including single photon sensitive camera allow the optimization of above factors. Similar considerations are applied to other fluorescence-based biosensors described below when used in combination with optogenetic signaling activation.

Over ensemble averages from cell destructive methods, investigation of signaling events inside a cell and in selected organelles is crucial since the resultant information can pinpoint aberrant mechanisms. The above data (Fig. 2) show that optogenetic methods surpass traditional methods with limitations such as ligand diffusion, inability to deliver ligands to specific location of interest and low temporal or spatial control of the signaling activation. Since, G protein heterotrimer activation is controlled by the activated GPCRs; the extent of G γ translocation can be quantified to identify the relative efficiency of the receptor activation. The aforementioned G γ 9 assay has been used to measure the activation of GPCRs and G-proteins in living cells in a variety of investigations [6,7,17,8,40–43].

2. Single molecule detection of GPCR activation

Determining the dynamics of GPCR dimerization is important for interpreting complex signaling pathways and GPCR induced biased signaling. A technique for imaging the equilibrium between monomer-dimer formation of N-formyl peptide receptor (FPR) at single molecule level was developed [44,45]. Here, Alexa Fluor 594 dye has been conjugated with ϵ -amine of lysine residue in N-formyl peptide (FP). Addition of this dye-peptide conjugate to CHO cells expressing a non-active mutant of FPR (D71A) has demonstrated an equilibrium between monomer and dimer ratio of FPR. Fluorescence imaging conducted using total internal reflection fluorescence (TIRF) microscopy at 37 °C resulted in the appearance of fluorescence spots corresponding to dye-conjugated GPCRs, and 2D Gaussian distribution analysis was conducted to resolve single vs multiple spots for identification of corresponding monomer-dimer equilibrium.

Recently, a single molecule TIRF imaging method was developed to identify activation and conformational dynamics of metabotropic glutamate receptor 3 (mGluR3) using mGluR3 fused with tetramethylrhodamine-labelled HaloTag [46]. These experiments were conducted

in HEK293 cells to monitor changes in diffusion and distribution of mGluR3 upon ligand addition in real-time to assess GPCR oligomerization. The Bayesian-hidden Markov model was used to analyze the trajectories of mGluR3 diffusion. In addition, a dual color TIRF imaging of mGluR3-Cy3 and G protein G α i/o tethered with SNAP-Cell 647-SiR ligand was used to identify receptor-G protein coupling dynamics [47]. D2-dopamine-like receptor (D_{2L}R) activation was visualized using TIRF microscopy-based single molecule imaging [48]. The D_{2L}R were fluorescently labelled using SNAP tags or fluorescent ligands to monitor the formation of homodimers and monomer-dimer equilibrium of D₂R in living CHO cells. This method can also be applied for detecting spatial and temporal organization of GPCRs in living cells at the single molecule level. Here D_{2L}R fusion protein with SNAP tag at Nt was synthesized and covalently conjugated to a fluorescent ligand, Alexa 546-benzyl guanine. An overview of single molecule detection of GPCR activation by TIRF imaging is depicted in Fig. 3.

A single-molecule fluorescence spectroscopic method to investigate signaling activation of beta2-adrenergic receptors β 2-ARs) has been developed [49]. Conjugated Cy3 and Cys265 dyes were located at helix6 of β 2-AR, near the cytoplasmic end. The dye conjugated β 2-ARs were immobilized in phospholipid nanodiscs and connected to a quartz surface for detection by TIRF microscopy. β 2-ARs were activated by agonist formoterol and by inverse agonist ICI-118,551 (ICI) to monitor the active states and receptor conformational changes through agonist binding. Cy3 labelled β 2-ARs were imaged and fluorescence intensity, along with trajectories of Cy3, were quantified to identify active and inactive state populations of β 2-ARs. The assignments of Cy3 fluorescence for active and inactive states were based on the corresponding active and inactive conformation of helix VI of the β 2-AR crystal structure. Results showed that upon addition of agonist, the equilibrium shifted towards active state population, while the antagonist shifted the equilibrium towards inactive state population of β 2-AR. Overall, single molecule imaging of GPCRs can be used *in vitro* and *in vivo* to decipher receptor activation-inactivation dynamics as well as GPCR oligomerization patterns. In the future, single molecule methods can be used in combination with subcellular optogenetics. For instance, since TIRF microscopy can illuminate bottom PM to activate a limited number of opsins in a diffraction limited region as well as image labels on the opsin to capture its conformational changes using distinct wavelengths (Fig. 3).

3. Detecting GPCR-G protein activation at single-cell resolution using translocation-based sensors

3.1 Cyclic adenosine 3',5'-monophosphate (cAMP)

Upon Gs-coupled GPCR activation, G α sGTP activates ACs and catalyzes the cyclization of adenosine triphosphate (ATP), generating cAMP [50,51]. The resultant cAMP regulates many crucial physiological responses including metabolism, insulin secretion, calcium homeostasis, muscle contraction, cell adhesion, and gene transcription [52–54]. Epac is cAMP-guanine exchange factor (cAMP-GEF) and a cAMP-regulated GEF for Ras family members Rap1 and Rap2, resulting in their activation [52,53]. Epac contains a regulatory region with one (Epac1) or two (Epac2) cAMP-binding domains, a Dishevelled, Egl-10, Pleckstrin (DEP) domain, and a catalytic region for GEF activity [53,54]. Both Epac1 and

Epac 2 domains have been used as unimolecular cAMP sensors [55,56]. It has been shown that upon forskolin-induced adenylate cyclase activation and cAMP production induce translocation of Epac1 to the plasma membrane (PM) [53]. During isoproterenol induced β -AR activation and cAMP generation, rapid Epac1 translocation has been reported in A431 cells as well [53]. Similarly Epac1 responses were observed in OVCAR-3, ACHN, RCC10, MDCK, N1E-115, HeLa, Rat-1, GE11, and H1299 cell lines upon activation of ACs [53]. Epac probes, especially the latest including pH sensitive Ci/Ce Epac2-camps [57], Epac1-based ICUE3 [58] were able to detect cAMP fluctuations/dynamics pancreatic β -cell line-MIN6 [57] and HEK-293 cells [58] with an improved spatial and temporal resolution [55].

A two-component cAMP sensor was engineered using CFP tagged regulatory subunit of cAMP-dependent protein kinase (PKA) targeted to the PM using a prenylation motif $\text{RI}\beta$ -CFP-CaaX) and YFP tagged catalytic subunit (Ca) of PKA (PKA-Ca-YFP) [59]. Due to the high affinity of PKA-Ca-YFP for $\text{RI}\beta$ -CFP-CaaX, in the absence of cAMP, both constructs are located at the PM. Upon G α s-coupled GPCR activation, cAMP interacts with the regulatory subunit $\text{RI}\beta$ -CFP-CaaX), releasing the PKA-Ca-YFP. This is indicated by translocation of YFP to the cytosol. Therefore, cAMP generation can be monitored either by measuring the extent of PKA-Ca-YFP translocation FRET loss between CFP and YFP. Thus, this sensor can be used both as a translocation-based as well as a FRET-based sensor to measure the cAMP level in living cells. By replacing, the YFP on PKA-Ca-YFP with a red fluorescence protein such as mCherry will allow the use of this sensor to measure subcellular cAMP production induced by blue and green light sensitive optogenetic signaling triggers such as blue opsin and photoactivatable ACs [60,61].

3.2 Diacylglycerol (DAG)

Two distinct signaling molecules downstream of activated GPCRs, G α qGTP and G $\beta\gamma$, activates phospholipase C (PLC) which triggers the hydrolysis of PIP_2 at the PM, generating DAG and IP_3 [62]. Once DAG is generated at the PM, proteins containing cysteine-rich small globular DAG-binding domains (C1) are recruited to the PM to interact with DAG [62]. Meanwhile, IP_3 diffuses to the cellular interior and binds to IP_3 receptors on the endoplasmic reticulum (ER), which releases Ca^{2+} , and thus inducing a positive feedback on PLC activation and enhancing DAG generation at the Golgi [62,63]. Protein kinases PKC and PKD, as well as Rac-GAPs chimerins, are the two main classes of immediate downstream proteins that bind to DAG, and thus their affinity for DAG has been utilized in sensor development to detect GPCR activation induced DAG generation [62]. One of the first-generation DAG sensors contained a green fluorescent protein (GFP) fused to the C1 domain of PKC γ , and its translocation to membranes upon GPCR activation served as a readout for monitoring DAG production (Fig. 4) [62]. The regulatory domains of conventional PKC isoforms (PKC α , β I, β II, and γ) contain a cysteine rich C1 domain, which consists of tandem ~50 residue long protein domains (C1A and C1B), including a consensus sequence of six-cysteine and two-histidine residues that coordinate two Zn^{2+} [64]. GFP tagged first C1-domain of PKC γ is used as the DAG sensor. This fusion construct has been used in the rat basophilic leukemia 2H3 (RBL) model cell line to monitor the activation of platelet activation factor (PAF) receptor, which stimulate PLC β , and induces a transient translocation of Cys-domains from the cytosol to the PM. Fluorescence

confocal microscopy is used to monitor the translocation of the GFP-C1 construct/sensor using 488 nm excitation and 515 nm emission [65]. Another unimolecular fluorescence sensor for DAG has been reported in which a circularly permuted GFP (cpGFP) from G-GECO1 is placed between a pseudo substrate domain and the C1 domain of PKC isoform, PKC δ [66]. Since the binding DAG to the C1 domain separates the pseudo substrate from the enzyme C1, circularly permuted FP was positioned in portions of the PKC δ that could undergo a large conformational changes following DAG binding [66]. Functionality of this assay has been shown in HEK-293 cells [66]. Since this assay involves protein translocation similar to G γ 9 assay, it can be used to measure single cell and subcellular GPCRs and G protein signaling in live cells, in real time.

3.3 PIP2

Detection of PIP2 in living cells was reported in 1998 using a GFP tethered pleckstrin homology domain (PH) of PLC δ 1 which has a higher binding affinity to the phosphoinositol headgroup of PIP2 [67,68]. Since this GFP-PH biosensor selectively detects the concentrations of PIP2 on the PM, it has been widely used primarily to monitor activation of Gq-coupled GPCRs [69–71,17,72,42,9]. Additionally, it was also used for detecting G $\beta\gamma$ mediated PLC β activation after Gi/o pathway activation as well [6]. Once PIP2 is hydrolyzed, IP3 along with PHPLC δ 1 sensor translocate to cytosol. The overview of the sensor and a schematic of how PIP2 being measured is shown in (Fig. 5). This reporter has a higher affinity towards IP3 compared to PIP2, which limits its efficacy to estimate PIP2 levels in cells [73,74]. However, a high affinity fluorescence reporter for PIP2 has been designed using a PIP2 binding domain called ‘tubby’, found in a membrane bound transcription factor [75,76]. To achieve a better reporter translocation kinetics and an increased affinity towards PIP2 over IP3, a point mutation of R332H on tubby domain was introduced. Additionally, together with melanopsin (a Gq and Gi/o coupled opsin) [17], this PIP2 sensor has been used to detect single cell and subcellular G protein activation [9].

3.4 PKC

Phosphokinase C (PKC) is involved in the pathophysiology of cancer. PKC senses the formation of secondary messengers such as diacylglycerol (DAG) and calcium upon GPCR activation. The real-time detection of PKC signaling is achieved using either translocation-based or FRET-based techniques [77–79]. PKC has kinase and regulatory domains, including C2, C1a and C1b regulatory subdomains. C2 domain has higher Ca²⁺ binding affinity, while C1b prefers DAG. In the translocation-based PKC sensor, binding of C1b domain to DAG was utilized in the presence of calcium to recruit PKC to the PM upon receptor activation. PKC γ -GFP and PKC β II-GFP fusion constructs have been generated and expressed in cells to visualize the activation of GPCRs [80]. The activation of downstream secondary messengers, such as DAG and calcium, is essential for PKC β II-GFP sensor translocation from cytosol to the PM upon GPCR activation. However, only activation of Gq-coupled GPCRs can be monitored, and, as reported, PKC β II-GFP sensor translocation was not observed upon activation of Gs and Gi coupled receptors, such as β 2-AR and D2R, respectively [78]. Receptor desensitization dynamics and signaling cross-talk between different Gq-coupled GPCRs, such as angiotensin 1A (AT1AR) and endothelin (ETAR) receptors, expressed in the same cellular system have been monitored using this sensor [78].

PKC γ -GFP translocation was monitored upon activation of NMDA and mGluR1 receptors in living cells as well [80].

3.5 Sensors for detecting active-GPCR conformations.

3.5.1 Mini G proteins—Since G proteins are basically localized to the PM, their binding to GPCR upon agonist stimulation does not change their subcellular localization as was observed during β -arrestin recruitment [81,82]. One of the major challenges to detect GPCR and G protein interactions during receptor activation is the limited life time of the receptor-G protein complex formation. To overcome these limitations, mini G proteins (mG) were generated [83,84] as the engineered version of the GTPase domain of the G α subunits with a truncated Nt, which also lack lipid modifications that target them to the PM. Similar to a corresponding heterotrimer, mGs recognize ligand binding-induced structural changes in GPCRs [83]. The family of mG is now extended to all classes of G α subunits, including G α_i , o, s, q, and 13 [84,81]. It has been found that fluorescently tagged mG proteins generally localized in the cytoplasm and nucleus of mammalian cells. Insertion of a Nt nuclear export signal restricted their localization to cytosol, improved recognition of activated GPCR. The rapid translocation of cytosolic mG to the PM upon agonist induced GPCR activation and reverse back to the cytosol upon receptor-inactivation, thus can be used to assay conformational status GPCRs [81]. These mG proteins can also be utilized as FRET as well as BRET sensors to detect GPCRs activation-deactivation cycles.

3.5.2 Nanobodies (Nbs)—Conformation-specific single-domain cameloid antibody fragments were used to obtain an active-state crystal structure of receptors [85,86]. Compared to heterotrimeric antibodies, nanobodies consist of monomeric single domain with a compact prolate shape which gain the full antigen binding capacity and access to cavities or clefts on biological molecules [87,88]. Agonist activated GPCRs alone were not easy to crystallize due to the dynamic nature of receptors. For instance, spectroscopic studies revealed that a large fraction of agonist-bound receptors primarily stay in their inactive state [88]. Therefore, strategies have been developed using nanobodies to determine the structure of the agonist-bound receptor in its active conformation. Nanobody 80 (Nb80) was identified after screening a library of single chain nanobodies against agonist-bound β 2-AR in its active conformation (Fig. 6) [88]. Nb80 also showed G protein like properties and binding studies showed that β 2-AR-Nb80 strongly stabilized the active conformation of the β 2-AR compared to that in the β 2-AR-G α_s [89]. Fluorescently tagged Nb80 has been used to interrogate the location-biased activation of β -ARs in living cells [90]. Nb80-GFP was cytosolic, and upon ligand addition, Nb80-GFP rapidly recruited to the active-state β 2-AR on the PM [90]. This probe is also employed to examine the activation of β 1-AR located in IMs using a cell permeable β 1-AR agonist, dobutamine. Upon addition of dobutamine in the presence of an inhibitor for β 1-AR on the PM, Nb80 recruitment to IM-localized β 1-AR was observed [90]. Recent work using Nb80 showed that statins attenuate internalization of activated β 2-AR, indicating the broader utility of statins in treating cardiovascular diseases [91]. Similar nanobodies were used to identify the active conformation of M2 muscarinic (Nb9–8) and μ -opioid receptors (Nb39, 33) [88]. Therefore, Nbs can be used as a powerful tool to detect active-state GPCRs at the subcellular locations in single cells.

4. Fluorescence and Bioluminescence Resonance Energy Transfer (FRET- and BRET)- based detection of GPCR conformational changes

Resonance energy transfer is a phenomenon commonly utilized to measure the interaction between two associating molecules with the help of donor and acceptor proteins. In order to transfer energy between two molecules, the emission spectra of the donor and the absorption spectra of the acceptor should adequately overlap. Additionally, the donor and acceptor molecules should be in close proximity (10–100 Å) and possess appropriate dipole orientations [92,93]. FRET uses non-radiative energy transfer from a donor fluorophore to an acceptor fluorophore to detect the proximity between two proteins, (Fig. 7) [94,95]. It can also be used to map dynamic proximity and conformational changes between proteins. FRET efficiency is determined by three major criteria: (i) distance between the donor and the acceptor, (ii) spectral overlap of the donor emission spectrum and the acceptor absorption spectrum, and (iii) the relative orientation of the donor emission dipole and the acceptor absorption dipole. The typical distance for efficient FRET between donor and acceptor ranges from 1–10 nm. FRET efficiency is highest when both dipoles are in line (parallel) and lowest when they are perpendicular. FRET is also affected by environmental conditions such as solvent type, degree of hydration, ionic strength, pH and temperature that can affect dipole interactions [96].

FRET efficiency (E) and the intermolecular distances (R) are described by the following equations [96].

$$E = 1 - (I_{da}/I_d)$$

$$R = \left[\frac{R_0^6}{E} - R_0^6 \right]^{\frac{1}{6}}$$

Where, I_{da} : signal of the donor with presence of the acceptor I_d : signal of the donor without the acceptor R_0 : Förster distance (the distance at which a 50% probability of FRET transfer versus donor fluorescence)

CFP and YFP are the well-established and commonly used donor and acceptor pair in most FRET-based cell biology experiments. In a FRET-based sensor, the two interacting proteins are fused to CFP and YFP fluorophores [97]. When the donor CFP is excited at its proper excitation wavelength (440 nm), the emission occurs at 480 nm. A state of electronic polarity and an accompanying dipole moment are established for the electron resonance of an excited fluorophore. The energy of excitation dipole is non-radiatively transferred to the acceptor fluorophore with an absorption dipole of similar resonance frequency (in parallel transition dipole orientation with the donor) to the donor, and when the acceptor comes close proximity to the donor (Fig. 7). Changes in this ratio are directly related to the measurement of interactions between two molecules or two subunits of the same molecule [94].

In an experimental FRET setting, the measurements of acceptor signal in the presence (F_{AD}) and absence (F_A) of the donor are acquired, and the correction factors are calculated prior to determination of the FRET ratio (FR). Correction factor calculations require individually-labeled donor and acceptor, to eliminate signals from non-FRET events such as bleed-through (spill-over) and cross-excitation of donor and acceptor fluorophores [98]. In these calculations, non-FRET donor fluorescence in acceptor emission wavelength (R_{D1}) and bleed through of directly excited donor in acceptor emission signal (R_{D2}) will be eliminated respectively from acceptor signal in the presence of the donor and acceptor emission signal due to direct excitation (R_{A1}). In the experimental settings, the following information is acquired cells expressing either donor CFP(C) or acceptor YFP (Y) tagged proteins. For cells expressing only CFP, CC (CFP excitation-CFP emission), CY and YY will be captured to calculate R_{D1} and R_{D2} and for cells expressing only YFP, YY and CY images will be captured to calculate R_{A1} .

4.1 FRET-based detection of conformational changes in GPCRs

GPCR activation is usually measured by monitoring downstream events such as protein phosphorylation. However, these approaches fail to provide dynamic changes associated with receptor activation. During the last two decades, several optical techniques have been developed to monitor receptor activation by observing GPCRs in intact cells, which provided insight into the kinetic and spatial properties of GPCR activation and G protein mediated signaling [99]. Since it was believed that GPCR activation involves a conformational change of the cytoplasmic regions of transmembrane helices III and VI [100–102], a series of receptor mutants were generated by inserting CFP and YFP at various positions of the third intracellular loop and the Ct of receptors (Parathyroid hormone receptor-PTHr and $\alpha 2$ -AR) to monitor those movements [100,103,104]. HEK 293 cells expressing a mutant PTHr with CFP in the third cytosolic loop and a YFP at the Ct showed a strong emission at 535 nm - the characteristic emission wavelength of YFP, when CFP was excited with 436 nm light [100]. This indicates FRET between CFP and YFP and was confirmed by acceptor (YFP) photobleaching FRET changes. In control experiments, PTHr only with CFP inserted in the intracellular loop III showed corresponding CFP emission at 480 nm with 436 nm light excitation, and a PTHr with YFP at the Ct alone did not show a noticeable emission at 535 nm. When PTHr was activated with PTH, a symmetrical increase in CFP emission and decrease in YFP emission was observed. This resulting rapid decrease in the bleed through-corrected emission intensity ratio (F_{535}/F_{480}) indicated that the change was due to a decrease in FRET. Similar intramolecular FRET results were observed with $\alpha 2$ -AR in Chinese hamster ovary (CHO) and neuronal PC12 cells as well [100]. Since $\alpha 2$ -AR is a class I GPCR and PTHr is a class II GPCR, this common FRET loss with both receptors activation suggested that the conformational change in transmembrane helices III and VI is similar in both receptor classes [100].

Agonist induced conformational changes have been observed with $\beta 2$ -ARs as well (Fig. 8). A mutated $\beta 2$ -AR with a CFP molecule in the intracellular loop III and YFP at the Ct showed a reduced FRET ratio (F_{YFP}/F_{CFP}) upon $\beta 2$ -AR activation suggesting a conformational change in the receptor [105]. The separated donor and acceptor labels upon activation were proximally located at the receptor inactive state. Many similar kinds of

FRET sensors have been reported to detect and analyze GPCR conformational changes, including A_{2A}-adenosine, β_1 -adrenergic, and bradykinin receptors [106,97,107,108]. Additionally, since donor - acceptor pairs in these assays are stationary, they can capture GPCRs activation in spatially confined regions as well.

Although the CFP/YFP is a well-established FRET pair, it has an intrinsic drawback due to the larger size of CFP and YFP, compared to the protein/s of interest which could alter their functional integrity. To overcome this, a FRET acceptor with a small, membrane-permeant fluorescein derivative with two arsen-(III) substituents (fluorescein arsenical hairpin binder; FAsH) targeted to a short tetracysteine sequence genetically encoded to the protein of interest has been developed [109]. This CFP/FAsH-tetracysteine FRET pair has been shown to provide greater agonist-induced FRET signals for α_2 -ARs, with similar kinetics and downstream signaling indicating that receptor functions are not altered by the FRET pair[109].

4.2 BRET-based detection of GPCR conformational changes

Detection of GPCR activation-induced generation of second messengers is the most common way of measuring the GPCR activation. Over the last decade, several luminescence-based quantitative methods were developed due to their high throughput adaptability and minimum background signals. These methods are also advantageous as they eliminate downstream reporter expression as well as extensive image analysis [110]. The use of bioluminescent luciferases in BRET assays provides a means of illuminating GPCRs and G proteins in order to obtain the mechanics and dynamics of the GPCR pathway signaling. In BRET sensors, the donor fluorescent protein is replaced by a luciferase enzyme. Luciferases generate light (440–480 nm) by catalyzing the oxidation of a specific organic substrate (eg: Coelenterazine, furimazine) in the presence of ATP. The generated luminescence excites the acceptor fluorescent protein [111,112]. Typically, Renilla luciferase (RLuc) and GFP are used as the donor and acceptor molecules in BRET. RLuc oxidizes its substrate, coelenterazine in to coelenteramide emitting light at ~470 nm, which is subsequently excite the nearby GFP for its excitation [113]. Excited GFP then emits green light at 510 nm. In BRET-based measurements, the emission intensity ratio of the luciferase and the fluorescence protein is calculated [114]. YFP or GFP are the most common BRET acceptors employed to probe receptor conformational rearrangements associated with GPCR activation.

Nanoluc (NLuc) has proven to be ~150 times more luminescent than RLuc when used in BRET assays [115]. NLuc is synthesized from *Olophorus* luciferase which works well due to its inherent luminescence and the fact that it contains a small catalytic subunit structurally similar to iLBPs or homodimers [115]. Since NLuc is significantly smaller in size than RLuc, it is a good candidate for tagging proteins. Its robustness to withstand environmental influences such as pH changes also makes NLuc more preferable over other luciferases. Despite the small size of NLuc, it provides a very bright and sustained luminescence with a higher physical stability that provides a means for intramolecular BRET assays. As a genetic reporter, NLuc helps to quantify cellular processes by optimizing light intensity using furimazine as the substrate. Furimazine was designed as an improved luminogenic substrate

for NLuc [115]. When comparing inter- and intracellular BRET, intracellular BRET models produced substantially increased net BRET observations and demonstrated a clear trend from repression to potentiation as cell density increased [116]. BRET provides the ability to identify orphan receptors and transducers coupled to a given receptor, while also monitoring the effects of mutations that promote signaling-biases to further explore the specific residues and receptor-domains involved in ligand-specific biased signaling [117]. Conformational BRET analysis also better informs the drug discovery process by providing more information about receptor conformations [117,118]. Nevertheless, the main impetus of using NLuc as a BRET donor is to improve sensitivity of assays that detect drug actions on GPCRs. The donor NLuc is inserted in the third cytosolic loop (between positions 251 and 252 in $\beta 2$ -AR), and the acceptors, either YFP or GFP are attached to the C-terminus of the receptor (at 369 position) [117]. Since these positions are the ones which detect the rotation of transmembrane helix 5 and the outward movement of transmembrane helix 6, which results in intracellular loop 3, shifting away from the C-terminus of the receptor, a loss of BRET ratio (YFP (527 nm) /NLuc luminescence (460 nm)) can be observed with receptor activation, as a result of the distance increase between YFP and NLuc [119,117].

5. Intermolecular FRET and BRET detection of GPCR activation

GPCR-G protein activation takes place in sub-second time scale to initiate downstream signaling [120,121]. Optical techniques, such as FRET and BRET, have developed to visualize GPCR activation and its downstream signaling machinery, from ligand-receptor binding to G protein dependent and independent signaling [122]. Table 1 summarizes various FRET- and BRET-based sensors that can be utilized to inspect intermolecular interactions between receptor-G protein and G protein-G protein. Upon GPCR activation, $G\alpha$ bound GDP exchanges with a GTP, resulting in the dissociation of the heterotrimer into $G\alpha$ GTP and $G\beta\gamma$ dimer [123–125]. Therefore, appropriate FRET or BRET probes can be designed to examine intermolecular receptor-G proteins and G protein-G protein interactions, thus measuring GPCR and G protein activation in real-time [126,127,122].

5.1 GPCR - G protein interactions

Interactions between GPCR and $G\alpha$ subunit have been characterized in detail for rhodopsin and $G\alpha$ transducin (Gt). Structural data suggest that both $G\alpha$ and $G\beta\gamma$ in the heterotrimer involve interaction with the GPCR. Gt heterotrimer activation by rhodopsin further showed that the primary G protein coupling site in the receptor is at the intracellular loop 3 which exhibits interactions with C-terminus of $G\alpha$ during receptor activation [128–130]. This was recently demonstrated using the crystal structure of agonist bound $\beta 2$ -AR [131]. Additional regions of $G\alpha$ subunit were also reported to interact with GPCRs [132,133,126], while $G\beta\gamma$ subunits interact with GPCRs to stabilize the receptor- $G\alpha$ interaction interface [134–136]. The flexible helical domain of $G\alpha$ exhibits major displacements and re-orientation during receptor activation, which has been proven using BRET probing within $G\alpha$ subunit [121]. Initially, FRET between receptor and G protein have been shown by purified, reconstituted, fluorescently labelled $\beta 1$ -AR and G protein subunits [137,138]. Later, similar studies were performed using genetically encoded fluorescence proteins (FRET) and luciferase (BRET) fusions. For these studies, GPCRs were fluorescently labeled at the C-terminus, where the

labeled receptors were functionally intact [122], and G proteins were labelled at various sites. G β and G γ were most frequently labelled at their Nt due to minimal functional impairment to the heterotrimer and G protein function [139,140,120], but G γ can also be labeled at its C-terminus [140] which removes the CaaX motif of the G γ subunits and disrupts their localization on the PM. The C-terminally tagged G γ were able to maintain interactions with G α , however, the resultant G $\beta\gamma$ was unable to control G $\beta\gamma$ -effectors activities [120]. To study the receptor-G protein pre-coupled model, basal BRET was measured by BRET between B₂ bradykinin receptor-G α_q , protease activated receptor-1-G α_i and δ -opioid receptors-G α_i [141–143]. These studies revealed that receptor and G proteins are located nearby or pre-coupled before the activation. To monitor realtime GPCR-G protein interactions and activation, BRET was measured between β 2-AR Ct (with the energy donor Rluc) and G α_s , where the energy acceptor (GFP10, a blue shifted GFP variant) was inserted between the linker 1 region and the GTPase domain of G α_s [144].

FRET studies were conducted using YFP tagged β 1-AR at the C-terminus of Adenosine_{2A} receptor (A_{2A} R) and N-terminally CFP tagged G γ_2 (Fig. 9). CFP was then excited 436 nm, and FRET change (acceptor/YFP emission at 535 nm) was expected when receptor-G proteins were assembled in close proximity in living cells [145]. Similarly, FRET between α 2-AR and G proteins has been demonstrated by tagging α 2-AR at Ct with YFP and G γ_2 at Nt with CFP and transiently expressed these constructs in HEK293T cells. Upon stimulation with 100 μ M norepinephrine, CFP fluorescence decreased and YFP fluorescence increased. Resultant FRET increase can be measured by calculating the ratio of F_{YFP} over F_{CFP} [146].

5.2 G protein - G protein interactions

FRET between G α and G $\beta\gamma$ was first measured by inserting donor and acceptor fluorophores on G α (Fluorescein 5-thiocyanate) and G β (Eosin 5-thiocyanate) as early as 1994 by Richard Neubig and coworkers [147]. This study revealed the mechanism and kinetics of G protein association [147]. To measure FRET between G α and G β , CFP was inserted in the helical domain of G α_2 and Nt of G β , while retaining functional interactions between G proteins [148]. To visualize the FRET-change between G α and G $\beta\gamma$, conventional fluorescence microscopic techniques were used with the cells expressing G α_2 -CFP and β -YFP. Fluorescence was measured by exciting the cells using blue light (420–460 nm) and observing the emission in the yellow range (525 to 540 nm). With the saturation dose of ligand, FRET signal at the PM decreased rapidly due to heterotrimer dissociation [148]. GPCR activation induced inter-subunit rearrangement that was detected by the decreased FRET between G α_i -YFP with either CFP tagged G γ_2 or G β_1 . Single-cell FRET determination exhibited a robust FRET between G α_i -YFP and G γ_2 -CFP in the absence of agonist confirming formation of heterotrimers. However, addition of noradrenaline to activate α 2-AR in HEK cells demonstrated 30–35% of decreased FRET due to subunit dissociation [140]. C-terminal tagging of G γ_2 exhibited cytosolic localization due to lack of prenylation and still showed a detectable FRET indicating functional interaction of G $\beta\gamma$ dimer with G α_i -YFP [140]. Selectivity in G α_i and G β subunits dissociation during α 2-AR activation has been explored by tagging G α_i subfamilies with YFP variant citrine at the α b- α c loop after Ala-121 of G α_{i1} and Ala-114 of G α_{i2} and G α_{i3} [149]. Similarly, CFP was inserted in the Nt of G $\beta_{1,2}$ and 4. To measure G protein activation dynamics during GPCR

activation, a FRET assay was developed and various combinations of $G\alpha_i$ and $G\beta$ were used to gain the subunit selectivity [149]. With the evolution of FRET techniques, one of the major obstacle was the enhancement of photophysical properties of the fluorophores, to make them capable of quantitative long term imaging without any fluorescence artifacts [150]. It was previously explained that an enhanced versions of fluorescence proteins increase the sensitivity of the FRET measurements [151,152], but the donor and acceptor expression ratio also influence the FRET measurements when FRET donor and acceptor were introduced to cells using separate plasmids [150]. A series of $G\alpha_i$ -based FRET sensors were used to investigate G protein activation directly with high spatial and temporal resolution [150]. These new generation $G\alpha_i$ -based FRET sensors consist of bright fluorescence protein variants with high quantum yields, stability and the improved ability to transfect the cells with single plasmid encoding both the acceptor and donor. In order to achieve sensitive and robust FRET measurements, $G\alpha_i$ subunits were fused with the most photostable monomeric cyano fluorescence protein, mTurquoise2 (mTq2) [150]. As the acceptor, circularly permuted Venus (cpVenus) has been fused to Nt of $G\gamma_2$ [150]. These single plasmids encoding $G\alpha_i$ -based FRET biosensors established their suitability for single cell live cell microscopy and measurement of fast kinetics associated with G protein activation, and the validity of $G\alpha_i$ FRET sensors in single cells FRET analysis demonstrated several different G_i -coupled receptors, including the LPA_2 , M_3 and BK_2 receptors [150].

FRET-based assays to examine the G_{α_s} activation-deactivation kinetics in living cells were developed [145] to measure the signaling of the G_s pathway signaling in living cells, including GPCR and G protein activities and second messenger (cAMP) levels. These events have been difficult to map in real-time in living cells. To examine the FRET between G_{α_s} and $G\beta\gamma$, YFP was inserted into G_{α_s} as previously described [153] and CFP was tethered to the Nt of $G\gamma_2$. HEK293T cells transfected with A_{2A} receptor, G_{α_s} -YFP, β_1 , and CFP- γ_2 showed that the fluorescence was initially localized to the PM. Upon stimulation, CFP fluorescence increased with a reduction in YFP fluorescence, resulting in a decrease in YFP-to-CFP fluorescence ratio, indicating G protein activation and subunit dissociation [145]. FRET experiments in β_1 -AR, analyzed using monoexponential FRET decay curves, exhibited similar results, indicating that β_1 -AR-driven G_{α_s} activation is similar to A_{2A} R-driven G_{α_s} activation [145]. Studying G_{α_q} -activation is important because involvement of G_{α_q} signaling is reported in many pathophysiological functions, including cancer and heart muscle regulation [154–156]. Recent development of FRET based techniques to examine GPCR-G protein activation have led to the generation of a G_{α_q} -based FRET sensor where G_{α_q} was tagged with mTq in its α -helical domain and $G\gamma_2$ was tagged with YFP [157,158]. After validating the functionality of the G_{α_q} -mTq, basal FRET experiments were conducted in the presence of YFP- $G\gamma_2$. A truncated version of mTq-consisting G_{α_q} sensor (G_{α_q} -mTq 6) exhibited higher basal FRET compared to full length mTq tagged G_{α_q} sensor. HeLa cells expressing G_{α_q} -mTq 6 and YFP- $G\gamma_2$ exhibited a decrease in FRET signal upon activation of histamine H1 receptor, thus enabling determination of Gq signaling in real-time in single cells [159]. Similarly, YFP tagged $G\beta_1$ has been used to examine G protein separation during muscarinic M1 activation [160]. To measure the activity of $G_{\alpha_{12/13}}$ pathway, a FRET sensor was generated with human $G_{\alpha_{13}}$ [161]. Based on the sequence homology between $G\alpha_i$, G_{α_q} with $G_{\alpha_{13}}$, a truncated mTq2 (after removal of last

nine amino acid of mTq2) was inserted after Q144 of human $G\alpha_{13}$ and was found to be nonfunctional. However, later a fluorescently tagged and functional $G\alpha_{13}$ variant was created, and FRET was determined by co-transfecting mTurquoise2- $G\alpha_{13}$ untagged-G β and 173cpVenus- $G\gamma$ on separate plasmids. Upon activation of LPA2-receptor, a change in the FRET ratio was observed, indicating $G\alpha_{13}$ pathway activation [161].

5.3 GPCR - β -arrestin interactions

Agonist-dependent activation of GPCRs also induces rapid phosphorylation and desensitization of the receptor [162]. Phosphorylated GPCRs exhibit increased affinity for cytosolic interacting proteins, such as β -arrestin [162]. To determine GPCR signaling activation by measuring β -arrestin recruitment to the receptor, FRET- and BRET-based methods have been developed [163,164]. FRET between β 2-AR-mCFP and β -arrestin2-mYFP upon stimulation of β 2-AR was observed (Fig. 10), where the stimulated β 2-AR recruited cytosolic β -arrestin2 [164]. PTHR-YFP activation and deactivation was also measured using β -arrestin-CFP recruitment-based FRET [100]. These results confirmed that FRET between receptor and β -arrestin2 can be used to assess agonist-induced receptor conformations upon activation.

The BRET-based assay to examine GPCR and β -arrestin interactions was developed using C-terminally luciferase-fused β 2-AR and β -arrestin-YFP, which showed agonist dependent increased BRET signal [165]. Thyrotropin releasing hormone receptor and gonadotropin-releasing hormone receptor interactions with β -arrestin were also examined using renilla luciferase tagged receptor and β -arrestin-YFP [166]. A robust BRET increase observed made this assay suitable for high-throughput screening, and thus validated this method for assaying more GPCRs, including chemokine, opioids, dopamine, and prostaglandin receptors [167–171].

5.4 GPCR -G protein-coupled receptor kinase (GRK) interactions

GRKs facilitate desensitization of GPCRs by phosphorylating them. Although GRK directly interacts with GPCRs, only few FRET and BRET studies available for GPCR GRKs interactions. Oxitocin receptor (OTR) C-terminally fused to luciferase and GRK2-YFP exhibited a short-lived BRET signal [172]. This indicated that GRK2 transiently interacts with the activated receptor and subsequently dissociates, once the receptor is phosphorylated. These results were further confirmed using co-immunoprecipitation and GRK2 translocation assays [122,172]. Luciferase tagged glucagon-like peptide receptor and GRK2-GFP interactions were similarly observed (Fig. 11) [173]. In a similar study, neurokinin receptor (NKR) interactions with GRK2 and GRK5 were determined using BRET [174]. It was further confirmed that, unlike GRK2, GRK5 can associate with the receptor before activation, [174].

6. FRET and BRET-based detection of GPCR-induced effector activities

6.1 Adenylyl Cyclase - cAMP sensors

6.1.1 Epac-based cAMP probes—The activation of Gs-coupled GPCRs, like β 1-AR, promote the Gas-GTP mediated activation of ACs. ACs produces cAMP using ATP as the

substrate. The resultant cAMP activates effector proteins, including cAMP-dependent protein kinase A, two isoforms of exchange proteins activated by cAMP (Epac1 and Epac2) and cyclic nucleotide gated ion channels [175]. These cAMP-triggered signaling governs a cohort of diverse cellular and physiological processes such as gene expression, cell division and migration, exocytosis and insulin secretion, and cardiac contractility [176–179]. Monitoring cAMP and its effector proteins interactions is widely used to measure Gs-coupled GPCR activation. Additionally, since Gi-coupled GPCRs inhibit ACs, the reduction of cAMP production induced by Gs-coupled GPCRs or forskolin is used to measure Gi-pathway functions [180]. Utilizing the FRET, fluorescent cAMP indicators are developed exploiting cAMP binding domains in Epac or PKA [56].

The cAMP-Epac biosensors can measure spatiotemporal changes of cellular cAMP concentrations. Epac backbone contains either the full-length Epac1 protein or a single-domain containing only the cAMP binding module of Epac1 or Epac2 [56,181]. FRET changes between donor CFP and acceptor YFP are observed upon binding of cAMP to the Epac inserted between CFP and YFP [182]. Therefore, ratiometric measurements of fluorescence intensities between donor and acceptor are measured for quantitative determination of cellular cAMP level [181,56]. Epac1-cAMP biosensor was developed by fusing CFP and YFP to Nt and Ct of the protein respectively [56]. The fusion Epac2-cAMP protein was developed by inserting cAMP binding domain B of Epac2 between YFP and CFP. Here, YFP is tethered to H4 and CFP is on $\alpha 6$ helices of the domain B. Enhanced cyano fluorescence protein (eCFP) and enhanced yellow fluorescence protein (eYFP) are the most common donor-acceptor pair in most FRET sensors [183]. However, the higher sensitivity of eYFP to pH and other ion concentration changes has been a problem [184].

6.1.2 PKA-based cAMP probe—Binding of cAMP is required for PKA activation. This PKA-based cAMP probe is designed by attaching fluorescence proteins CFP and YFP to regulatory (R) and catalytic (C) subunits of PKA respectively [185]. In reduced cellular cAMP levels, the holotetrameric conformation of PKA exists as its inactive CFP-R₂C₂-YFP form exhibiting the highest FRET (Fig 12). The binding of cAMP to R₂ domain releases the active C-domain, inducing a conformational change on the sensor. This causes the two fluorescence proteins to diverge, terminating the FRET [186]. However, the tetrameric form of PKA requires a cooperative binding of cAMP to four interconnected cAMP binding sites with varying binding affinities [187]. This makes the activation mechanism of PKA multifaceted and delayed. As a solution for this, modified FRET biosensors for cAMP were developed containing single cAMP binding domains to eliminate catalytic properties and cooperative binding [56].

6.1.3 HCN2-camps sensor—A FRET-based approach was employed to monitor the spatiotemporal distribution of cAMP in adult cardiomyocytes, primarily to determine the differential regulation of cardiomyocyte contraction by $\beta 1$ - and $\beta 2$ -ARs [188]. The single cAMP binding domain of the hyperpolarization activated cyclic nucleotide-gated potassium channel 2 (HCN2) is sandwiched between YFP and CFP. Upon cAMP binding-induced conformational changes in the channel were observed by monitoring the FRET between CFP and YFP.

6.1.4 CAMYEL sensor—Even though there are many commercial kits available to detect cAMP, BRET-based cAMP measurements are popular since they are simple, rapid, cost effective and versatile. CAMYEL cAMP sensor was constructed using YFP-Epac-RLuc arrangement and is capable of rapid quantification of cAMP generation in living cells [189]. CAMYEL exhibited reduction in BRET signal indicating Gs-pathway activation in RAW264.7 cells [189]. This sensor also exhibited Gi-pathway activation induced cAMP reduction in CHO cells [189].

6.2 PIP2 sensors

Since there are several limitations in the PH domain of PLC δ translocation-based PIP2 sensor, a PIP2 FRET sensor has been designed to reliably and quantitatively detect PIP2 dynamics in living cells and to improve the high-throughput adaptability of the assay [190]. This sensor measures changes in overall fluorescence intensity of multiples cells. Here, the acceptor and the donor, for instance CFP and YFP are individually tethered to PH-PLC δ 1 and expressed in equimolar quantities in cells. Both sensors are co-localized to the PM and exhibited FRET. Once PIP2 is hydrolyzed, since the sensor leave the PM and the FRET signal is lost (Fig. 13). Different cell types such as NIH3t3, HEK293, A431, RBL and COS-7 expressing this FRET based PIP2 sensor have been captured GPCR activation and PIP2 hydrolysis. This PIP2 FRET sensor can be used to measure single cell signaling with optogenetics signaling activation. However, excitation light for CFP as well as light emitted by YFP are likely to activate some optogenetic constructs. This will results data lacking a baseline to understand signaling when the GPCRs are inactive. Alternatively, FRET acceptor-donor pairs with red shifted excitation emission profiles as well as the less commonly used phytochrome based optogenetic tools, are worthy of exploring [191].

6.3 DAG

Intermolecular and intramolecular FRET sensors to measure GPCR activation-induced DAG generation were developed. In CYPHR intermolecular sensor, PH domain of PLC δ 1 is fused to the FRET pair, CFP and YFP (CFP-PH δ -YFP). The PH domain of PLC δ 1, binds to PIP $_2$ in the PM, and translocates to the cytosol upon PIP $_2$ hydrolysis [192]. Generation of DAG and Ca $^{2+}$ reduce the intermolecular FRET. DAG dynamics at subcellular membranes have been measured genetically encoded DAG sensor based on intramolecular FRET [193]. Here, a cysteine-rich domain (CRD) from protein kinase C β is used as a specific DAG-binding domain between CFP and YFP through α -helical linkers consisting of repeated EAAAR sequences, and a single di-glycine motif is introduced within one of the rigid linkers as a hinge [193]. Upon DAG generation, CRD binds to DAG which induces a flip-flop-type conformational change of the sensor, resulting in an increased FRET between CFP and YFP. This sensor named as 'Daglas' is used to detect localized DAG concentrations and dynamics upon targeting the sensor to specific subcellular localizations [194,193,195].

6.4 Protein kinase B (AKT)

To overcome drawbacks in early FRET biosensors for Akt, Eevee-iAkt was designed with enhanced sensitivity and specificity. Eevee-iAkt was employed to measure Akt activity in different subcellular locations including the PM, cytosol and mitochondria [196]. This

intramolecular sensor includes phosphopeptide binding domain (PBD), a linker, and the substrate sequence inserted between CFP and YFP [197]. Because of the phosphorylation of the substrate, the FRET is increased due to the binding of PBD to the phosphorylated substrate, leading to a conformational change in the sensor. The modified Eevee-iAkt sensor consists of an optimized fluorescent protein pair, YPet and ECFP and a long flexible EV linker to optimize the Eevee backbone [196]. In order to monitor the differential activity of Akt in various subcellular locations, the Ct of the construct was tagged with desired localization sequences such as nuclear export sequence (NES), nuclear localization signal (NLS) and signal sequence for prenylation (CaaX) [198].

6.5 Calcium

The activity of Ca^{2+} as a second messenger governs numerous essential cellular activities such as development, differentiation and apoptosis [199]. Cameleon is a FRET-based fluorescence indicator for Ca^{2+} , designed using GFP and a calcium binding protein in the cytosol, calmodulin (CaM). Sequentially connecting CFP-CaM-CaM binding peptide of myosin light chain kinase (MLCKp)-YFP, the conventional yellow cameleon (YC) sensor was constructed [200]. Ca^{2+} binding to CaM module of cameleon conformationally changes cameleon while causing the attached MLCKp to fold. This increases the FRET as the distance between the donor and acceptor decreases [104,200]. The change in acceptor and donor fluorescence ratios indicate Ca^{2+} availability in different cellular compartments including cytoplasm, nucleus and ER [201,200]. Nevertheless, the limited dynamic range is a drawback in traditional cameleons which makes it challenging to detect minor changes in Ca^{2+} concentrations. The first generation YC6.1 consists of the serial fusion of CFP, the Nt fragment of CaM (NCaM), Gly-Gly, CKKp, Gly-Gly, the C-terminal fragment of CaM (C-CaM) and YFP [202]. YC6.1 with a modified structure of CaM in complex with CaM binding peptide derived from CaM-dependent kinase kinase (CKKp) shows an improved sensitivity [203]. YC6.1 is developed by fusing the CaM binding peptide at the linker region between the Nt and Ct domains of CaM [204]. Here, the distance between the N- and C-termini of CaM in the CKKp bound structure is considerably shorter than 40 Å. Since this sensor can be modified to measure Ca^{2+} in different cellular compartments, it is suited to measure Ca^{2+} in cytosol as well as internal compartments.

6.6 PKA

AKAR is a ratiometric FRET sensor generated to determine PKA activity in cells [205]. This intramolecular FRET sensor consists of CFP (donor), phosphoamino binding domain, PKA-specific phosphorylatable peptide sequence and YFP (acceptor) [206]. When PKA is inactive, basal FRET between two fluorophores is small. Active PKA phosphorylates the substrate region and increases its specificity for FHA1, bringing two fluorophores closer, resulting in FRET increase (Fig. 14) [207]. Since then, a series of AKAR sensors have been generated using flexible linkers and improved variants of fluorescence proteins, such as enhanced CFP, CyPet, YPet, Venus and circularly permuted Venus [207]. Subcellular PKC activity was monitored using AKAR sensor targeted to various localization. By inserting a PH domain to interact with phosphoinositides on the PM, AKAR3 sensor was generated to monitor the PKA activation at the PM [207]. To measure the PKA activity only in the

cytoplasm, AKAR3-NES was used, and mitochondrial PKA activation was measured using DAKAP-AKAR3.

6.7 PKC

PKC is activated by DAG generated as a result of GαqGTP induced PLCβ activation and subsequent hydrolysis of PIP2. DAG generation and IP3 mediated Ca²⁺ response regulates the PKC enzyme activity. C kinase activity reporter (CKAR) was constructed analogous to AKAR [206]. AKAR is a unimolecular FRET reporter consisting of CFP, PKC-substrate sequence responsible for PKC mediated phosphorylation, FHA2 (phosphothreonine-binding domain), and YFP. Phosphorylation of the substrate sequence by PKC facilitates the intramolecular trapping of phosphorylated substrate sequence and FHA2, creating conformational changes required for the FRET change. HeLa cells expressing this sensor were treated with histamine instead of PDBu, a direct activator of PKC exhibited significant and reproducible change in FRET, while it also captured physiological activation of PKC by Gq-coupled GPCRs [208]. Several variants of the CKAR reporter were generated to examine highly dynamic PKC activation in living cells and subcellular specificities of CKAR phosphorylation and dephosphorylation [208]. The next generation of FRET-based PKC activity reporters with flexible linkers (EV linkers) exhibited enhanced detection expanded the understanding of spatiotemporal regulation of PKC activity [205].

7. Luminescence-based detection of GPCR-G protein signaling

7.1 Protein translocation-based detection of GPCR-G protein activation

During GPCR-G protein signal transduction, many proteins have been identified to translocate between various cellular compartments including cytosol, nucleus and the PM [209–211]. For instance, GPCR activation induces cytosol to PM translocation of SH2 domain containing proteins [209], arrestins [210], and PKC family members [211]. During quantification of these subcellular changes, new probes have been developed, with a calcium (Ca²⁺)-sensitive, luminescent photo-protein, aequorin, fused to a protein of interest, either (i) aequorin-β arrestin (βarr2-AEQ), or (ii) aequorin-PKCβ (PKCβ-AEQ) [211]. Upon GPCR activation, these aequorin-fused signaling proteins initially localized in the cytosol, translocate to the PM due to the larger concentration of Ca²⁺ at the inner leaflet of the PM compared to the cytosol [212,213]. In order for aequorin to be luminescent, it requires a hydrophobic prosthetic group, coelenterazine, which converts the apo-aequorin to active aequorin [214]. After Ca²⁺ binds to the high affinity sites in aequorin, it oxidizes coelenterazine into coelenteramide with the production of CO₂ and emission of blue light measurable at ~470 nm. Since the aequorin activity is proportional to the Ca²⁺ concentration, the measured light emission provides reliable quantification of the Ca²⁺ concentration near the PM [215].

PathHunter assay uses an enzyme fragment complementation approach comprised of two inactive enzyme fragments incorporated by GPCR conformational changes to generate active enzyme, to monitor GPCR activation [110,216,217]. The probe generation includes fusion of the Ct of the GPCR to a mutated Nt α fragment peptide derived from β-galactosidase, termed ProLink. The Ct of β-arrestin is tethered to an Nt deletion mutant of β-

galactosidase, termed the enzyme acceptor (β -arrestin-EA). Upon GPCR activation, GRK2 mediated phosphorylation of the C_t of the GPCR, β -arrestin-EA translocate to the PM and bind to the phosphorylated receptor. This drives the complementation of inactive β -galactosidase fragments resulting in active β -galactosidase enzyme to catalyze the substrate hydrolysis and generate chemiluminescence, thus enabling the detection of GPCR activation by quantifying β -galactosidase activity in the presence of β -galactosidase luminogenic substrates (Fig. 15) [110].

In LinkLight / β -arrestin assay, the C_t of a GPCR is fused to Tobacco Etch Virus protease (TEV) with a flexible peptide sequence (G4S)₃, and β -arrestin is linked to an inactive permutated luciferase (pLuc) [218]. This pLuc is constructed through the insertion of a TEV protease cleavage sequence (ENLYFQR) as a linker connecting the C luciferase fragment (CLuc) (amino acids 234 to 550) in front of N luciferase fragment (NLuc) (amino acids 2 to 233) [218]. Receptor-activation-induced β -arrestin recruitment causes the cleavage of pLuc by GPCR-fused TEV protease, and the subsequent refolding of luciferase results in the active enzyme detectable by chemiluminescence generation in the presence of exogenous luciferase substrates (Fig. 16) [218].

Tango GPCR Assay comprises of two fusion protein constructs: a transcription factor tethered to the C_t of GPCR through a protease cleavage site and a protease-tagged β -arrestin [219]. Upon receptor activation and β -arrestin recruitment, the protease cleaves and releases the GPCR-fused transcription factor, which then enters the nucleus and promotes the activation of reporter genes [219]. Usually these reporter genes are either β -lactamase or luciferase, which generate luminescence in the presence of their substrates [219–221].

Gq-GPCR activation induced signaling requires precise measurement and quantification of effector response. Recently, a split luciferase-based sensor was designed to measure Gq-coupled GPCR activation in living cells [222]. Here, a catalytically inactive domains of split luciferase CBRC (small C-terminal fragment) and CBRN (complementary Nt domain fragment) were tethered to G α q and PLC β 3 respectively [223–225]. Upon Gq-GPCR activation, α q and PLC β 3 become proximal and thus generates the active luciferase, catalyzing the conversion of luciferin to oxyluciferin, producing bioluminescence (Fig. 17). In this study, fusion proteins of G α q and PLC β 3 with luciferase fragments were designed and tested to identify constructs giving best signal to noise ratio for the bioluminescence signal. Gq-coupled GPCRs such as human histamine 1 receptor (hH₁R), muscarinic acetylcholine receptors 1, 3, 5 (hM_{1,3,5}Rs) and neurotensin receptor (hNTS₁R) were activated by their cognate ligands and signaling was measured using this split luciferase sensor [222]. The efficacies of different ligands on same family of Gq-GPCRs were also tested. This sensor is a potential tool for the identification of orphan-Gq-coupled GPCRs in variety of cells using bioluminescence imaging [222]. These luminescence-based methods are likely to be developed in to sensors to measure single cell and subcellular GPCR-G protein signaling, however may require single photon sensitive photon detectors.

7.2 Measurement of GPCR-governed gene expression

Reporter genes are synthesized in response to activation of a specific signaling pathway and are monitored by measuring their enzymatic activities mainly using color, fluorescence or

luminescence generation [226]. Reporter-gene systems have been widely used to study gene expression regulation, including alkaline phosphatase, β -galactosidase, β -glucuronidase, β -lactamase, GFP and luciferase [227–229]. Among them, luciferase is the most widely used reporter because of its wide dynamic range, sensitivity, easy quantitation, and lack of endogenous activity which results in minimal background producing a high signal-to-noise ratio [230]. GPCR activation results in G protein activation followed by the heterotrimer dissociation into $G\alpha$ and $G\beta\gamma$ subunits, which then initiates downstream pathways leading to gene transcription induced by various response elements (REs), including cAMP response element (CRE), nuclear factor of activated T-cells response element (NFAT-RE), serum response element (SRE) and serum response factor response element (SRF-RE) [226,231].

These assays usually employ plasmids/reporter vectors expressing destabilized luciferase gene under the control of response elements, such as CRE-, NFAT-RE-, SRE-, SRFRE- (i.e., RE-promoter-luciferase), or they employ stable cell lines with RE-promoter-luciferase integrated into the chromosome. These plasmids are used to measure major GPCR signaling events: cAMP generation (Fig. 18) [231], Ca^{2+} mobilization, ERK/MAPK activity, and small G protein activation (i. e., RhoA) [226]. If the endogenous expression of the receptor is low, expression of the GPCR is also needed to obtain a better signal. After receptor activation, the resulting luminescence is measured by Glo-luciferase assay. An increased induction response with both SRE- and SRF-RE reporters has been developed with an addition of protein degradation sequences (hPEST and hCL) to the C-terminus of luciferase [226].

8. Conclusions and future perspectives

Compared to cell destructive methods, real-time examination of signaling in living cells, especially with single cell as well as subcellular resolution, provides physiologically pertinent and pharmacologically more relevant information. Approaches described here only summarize a fraction of probes so far developed (Table 1, 2), since our emphasis was only to illustrate approaches that have been used or have the potential to be used as subcellular detectors of GPCR-G protein signaling. In future, the development of new imaging approaches as well as fluorophores, luminogenic enzymes and substrates with brighter emission and appropriate spectral properties will allow optical probing of GPCR and G protein signaling in living animals and correlate cellular-subcellular signaling with physiological responses as well as animal behaviors.

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References

1. Hanlon CD, Andrew DJ (2015) Outside-in signaling--a brief review of GPCR signaling with a focus on the Drosophila GPCR family. *J Cell Sci* 128 (19):3533–3542. doi:10.1242/jcs.175158 [PubMed: 26345366]
2. Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3 (9):639–650. doi:10.1038/nrm908 [PubMed: 12209124]

3. Jastrzebska B (2013) GPCR: G protein complexes--the fundamental signaling assembly. *Amino Acids* 45 (6):1303–1314. doi:10.1007/s00726-013-1593-y [PubMed: 24052187]
4. Hamm HE (1998) The many faces of G protein signaling. *J Biol Chem* 273 (2):669–672. doi: 10.1074/jbc.273.2.669 [PubMed: 9422713]
5. Johnston CA, Siderovski DP (2007) Receptor-mediated activation of heterotrimeric G-proteins: current structural insights. *Mol Pharmacol* 72 (2):219–230. doi:10.1124/mol.107.034348 [PubMed: 17430994]
6. Siripurapu P, Kankanamge D, Ratnayake K, Senarath K, Karunarathne A (2017) Two independent but synchronized Gbetagamma subunit-controlled pathways are essential for trailing-edge retraction during macrophage migration. *J Biol Chem* 292 (42):17482–17495. doi:10.1074/jbc.M117.787838 [PubMed: 28864771]
7. Senarath K, Payton JL, Kankanamge D, Siripurapu P, Tennakoon M, Karunarathne A (2018) Ggamma identity dictates efficacy of Gbetagamma signaling and macrophage migration. *J Biol Chem* 293 (8):2974–2989. doi:10.1074/jbc.RA117.000872 [PubMed: 29317505]
8. O'Neill PR, Karunarathne WK, Kalyanaraman V, Silvius JR, Gautam N (2012) G-protein signaling leverages subunit-dependent membrane affinity to differentially control betagamma translocation to intracellular membranes. *Proc Natl Acad Sci U S A* 109 (51):E3568–3577. doi:10.1073/pnas.1205345109. [PubMed: 23213235]
9. Karunarathne WK, Giri L, Kalyanaraman V, Gautam N (2013) Optically triggering spatiotemporally confined GPCR activity in a cell and programming neurite initiation and extension. *Proc Natl Acad Sci U S A* 110 (17):E1565–1574. doi:10.1073/pnas.1220697110 [PubMed: 23479634]
10. Fang Y, Kenakin T, Liu C (2015) Editorial: Orphan GPCRs As Emerging Drug Targets. *Front Pharmacol* 6 (295):295. doi:10.3389/fphar.2015.00295 [PubMed: 26696893]
11. Hauser AS, Attwood MM, Rask-Andersen M, Schioth HB, Gloriam DE (2017) Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* 16 (12):829–842. doi: 10.1038/nrd.2017.178 [PubMed: 29075003]
12. Hu GM, Mai TL, Chen CM (2017) Visualizing the GPCR Network: Classification and Evolution. *Sci Rep* 7 (1):15495. doi:10.1038/s41598-017-15707-9 [PubMed: 29138525]
13. Alexander SP, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Davies JA, Collaborators C (2017) THE CONCISE GUIDE TO PHARMACOLOGY 2017/18: G protein-coupled receptors. *Br J Pharmacol* 174 Suppl 1 (Suppl Suppl 1):S17–S129. doi:10.1111/bph.13878 [PubMed: 29055040]
14. Godinho RO, Duarte T, Pacini ES (2015) New perspectives in signaling mediated by receptors coupled to stimulatory G protein: the emerging significance of cAMP e flux and extracellular cAMP-adenosine pathway. *Front Pharmacol* 6:58. doi:10.3389/fphar.2015.00058 [PubMed: 25859216]
15. Kang M, Chung KY, Walker JW (2007) G-Protein Coupled Receptor Signaling in Myocardium: Not for the Faint of Heart. *Physiology* 22 (3):174–184 [PubMed: 17557938]
16. Kamp TJ, Hell JW (2000) Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. *Circ Res* 87 (12):1095–1102 [PubMed: 11110765]
17. Kankanamge D, Ratnayake K, Samaradivakara S, Karunarathne A (2018) Melanopsin (Opn4) utilizes Galphai and Gbetagamma as major signal transducers. *J Cell Sci* 131 (11). doi:10.1242/jcs.212910
18. Siehler S (2009) Regulation of RhoGEF proteins by G12/13-coupled receptors. *Br J Pharmacol* 158 (1):41–49. doi:10.1111/j.1476-5381.2009.00121.x [PubMed: 19226283]
19. Pfreimer M, Vatter P, Langer T, Wieland T, Gierschik P, Moepps B (2012) LARG links histamine-H1-receptor-activated Gq to Rho-GTPase-dependent signaling pathways. *Cell Signal* 24 (3):652–663. doi:10.1016/j.cellsig.2011.10.014 [PubMed: 22100544]
20. Khan SM, Sleno R, Gora S, Zylbergold P, Laverdure JP, Labbe JC, Miller GJ, Hebert TE (2013) The expanding roles of Gbetagamma subunits in G protein-coupled receptor signaling and drug action. *Pharmacol Rev* 65 (2):545–577. doi:10.1124/pr.111.005603 [PubMed: 23406670]
21. Khalil BD, Hsueh C, Cao Y, Abi Saab WF, Wang Y, Condeelis JS, Bresnick AR, Backer JM (2016) GPCR Signaling Mediates Tumor Metastasis via PI3Kbeta. *Cancer Res* 76 (10):2944–2953. doi: 10.1158/0008-5472.CAN-15-1675 [PubMed: 27013201]

22. Gulati S, Jin H, Masuho I, Orban T, Cai Y, Pardon E, Martemyanov KA, Kiser PD, Stewart PL, Ford CP, Steyaert J, Palczewski K (2018) Targeting G protein-coupled receptor signaling at the G protein level with a selective nanobody inhibitor. *Nat Commun* 9 (1):1996. doi:10.1038/s41467-018-04432-0 [PubMed: 29777099]
23. Perrault R, Wright B, Storie B, Hatherell A, Zahradka P (2011) Tyrosine kinase-independent activation of extracellular-regulated kinase (ERK) 1/2 by the insulin-like growth factor-1 receptor. *Cell Signal* 23 (4):739–746. doi:10.1016/j.cellsig.2010.12.008 [PubMed: 21215800]
24. Wang J, Xia Y (2012) Assessing developmental roles of MKK4 and MKK7 in vitro. *Commun Integr Biol* 5 (4):319–324. doi:10.4161/cib.20216 [PubMed: 23060952]
25. Urnukhsaikhan E, Cho H, Mishig-Ochir T, Seo YK, Park JK (2016) Pulsed electromagnetic fields promote survival and neuronal differentiation of human BM-MSCs. *Life Sci* 151:130–138. doi:10.1016/j.lfs.2016.02.066 [PubMed: 26898125]
26. Yuen JW, Poon LS, Chan AS, Yu FW, Lo RK, Wong YH (2010) Activation of STAT3 by specific Galpha subunits and multiple Gbetagamma dimers. *Int J Biochem Cell Biol* 42 (6):1052–1059. doi:10.1016/j.biocel.2010.03.017 [PubMed: 20348012]
27. Wani N, Nasser MW, Ahirwar DK, Zhao H, Miao Z, Shilo K, Ganju RK (2014) C-X-C motif chemokine 12/C-X-C chemokine receptor type 7 signaling regulates breast cancer growth and metastasis by modulating the tumor microenvironment. *Breast Cancer Res* 16 (3):R54. doi:10.1186/bcr3665 [PubMed: 24886617]
28. Tan CT, Chu CY, Lu YC, Chang CC, Lin BR, Wu HH, Liu HL, Cha ST, Prakash E, Ko JY, Kuo ML (2008) CXCL12/CXCR4 promotes laryngeal and hypopharyngeal squamous cell carcinoma metastasis through MMP-13-dependent invasion via the ERK1/2/AP-1 pathway. *Carcinogenesis* 29 (8):1519–1527. doi:10.1093/carcin/bgn108 [PubMed: 18487224]
29. Xia S, He C, Zhu Y, Wang S, Li H, Zhang Z, Jiang X, Liu J (2017) GABABR-Induced EGFR Transactivation Promotes Migration of Human Prostate Cancer Cells. *Mol Pharmacol* 92(3):265–277. doi:10.1124/mol.116.107854 [PubMed: 28424220]
30. Karunarathne WK, Giri L, Patel AK, Venkatesh KV, Gautam N (2013) Optical control demonstrates switch-like PIP3 dynamics underlying the initiation of immune cell migration. *Proc Natl Acad Sci U S A* 110 (17):E1575–1583. doi:10.1073/pnas.1220755110 [PubMed: 23569254]
31. Senarath K, Ratnayake K, Siripurapu P, Payton JL, Karunarathne A (2016) Reversible G Protein betagamma9 Distribution-Based Assay Reveals Molecular Underpinnings in Subcellular, Single-Cell, and Multicellular GPCR and G Protein Activity. *Anal Chem* 88 (23):11450–11459. doi:10.1021/acs.analchem.6b02512 [PubMed: 27778511]
32. Linder ME, Middleton P, Hepler JR, Taussig R, Gilman AG, Mumby SM (1993) Lipid modifications of G proteins: alpha subunits are palmitoylated. *Proc Natl Acad Sci U S A* 90 (8):3675–3679 [PubMed: 8475115]
33. Mumby SM, Casey PJ, Gilman AG, Gutowski S, Sternweis PC (1990) G protein gamma subunits contain a 20-carbon isoprenoid. *Proc Natl Acad Sci U S A* 87 (15):5873–5877. doi:10.1073/pnas.87.15.5873 [PubMed: 2116011]
34. Gilman AG (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* 56:615–649. doi:10.1146/annurev.bi.56.070187.003151 [PubMed: 3113327]
35. Akgoz M, Kalyanaraman V, Gautam N (2004) Receptor-mediated reversible translocation of the G protein betagamma complex from the plasma membrane to the Golgi complex. *J Biol Chem* 279 (49):51541–51544. doi:10.1074/jbc.M410639200 [PubMed: 15448129]
36. Saini DK, Kalyanaraman V, Chisari M, Gautam N (2007) A family of G protein betagamma subunits translocate reversibly from the plasma membrane to endomembranes on receptor activation. *J Biol Chem* 282 (33):24099–24108. doi:10.1074/jbc.M701191200 [PubMed: 17581822]
37. Saini DK, Karunarathne WK, AnGaswamy N, Saini D, Cho JH, Kalyanaraman V, Gautam N (2010) Regulation of Golgi structure and secretion by receptor-induced G protein betagamma complex translocation. *Proc Natl Acad Sci U S A* 107 (25):11417–11422. doi:10.1073/pnas.1003042107 [PubMed: 20534534]

38. Ajith Karunarathne WK, O'Neill PR, Martinez-Espinosa PL, Kalyanaraman V, Gautam N (2012) All G protein betagamma complexes are capable of translocation on receptor activation. *Biochem Biophys Res Commun* 421 (3):605–611. doi:10.1016/j.bbrc.2012.04.054 [PubMed: 22538369]
39. Ratnayake K, Kankanamge D, Senarath K, Siripurapu P, Weis N, Tennakoon M, Payton JL, Karunarathne A (2017) Chapter 1 - Measurement of GPCR-G protein activity in living cells In: Shukla AK (ed) *Methods in Cell Biology*, vol 142 Academic Press, pp 1–25 [PubMed: 28964328]
40. Giri L, Patel AK, Karunarathne WK, Kalyanaraman V, Venkatesh KV, Gautam N (2014) A G-protein subunit translocation embedded network motif underlies GPCR regulation of calcium oscillations. *Biophys J* 107 (1):242–254. doi:10.1016/j.bpj.2014.05.020 [PubMed: 24988358]
41. O'Neill PR, Gautam N (2014) Subcellular optogenetic inhibition of G proteins generates signaling gradients and cell migration. *Mol Biol Cell* 25 (15):2305–2314. doi:10.1091/mbc.E14-04-0870 [PubMed: 24920824]
42. Ratnayake K, Payton JL, Lakmal OH, Karunarathne A (2018) Blue light excited retinal intercepts cellular signaling. *Sci Rep* 8 (1):10207. doi:10.1038/s41598-018-28254-8 [PubMed: 29976989]
43. Samaradivakara S, Kankanamge D, Senarath K, Ratnayake K, Karunarathne A (2018) G protein gamma (Ggamma) subtype dependent targeting of GRK2 to M3 receptor by Gbetagamma. *Biochem Biophys Res Commun* 503 (1):165–170. doi:10.1016/j.bbrc.2018.05.204 [PubMed: 29864421]
44. Kasai RS, Kusumi A (2014) Single-molecule imaging revealed dynamic GPCR dimerization. *Curr Opin Cell Biol* 27:78–86. doi:10.1016/j.ceb.2013.11.008 [PubMed: 24480089]
45. Kasai RS, Suzuki KG, Prossnitz ER, Koyama-Honda I, Nakada C, Fujiwara TK, Kusumi A (2011) Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. *J Cell Biol* 192 (3):463–480. doi:10.1083/jcb.201009128 [PubMed: 21300851]
46. Yanagawa M, Hiroshima M, ToGashi Y, Abe M, Yamashita T, Shichida Y, Murata M, Ueda M, Sako Y (2018) Single-molecule diffusion-based estimation of ligand effects on G protein-coupled receptors. *Sci Signal* 11 (548). doi:10.1126/scisignal.aao1917
47. Yanagawa M, Hiroshima M, ToGashi Y, Abe M, Yamashita T, Shichida Y, Murata M, Ueda M, Sako Y (2018) Single-molecule diffusion-based estimation of ligand effects on G protein-coupled receptors. *Sci Signal* 11 (548). doi:10.1126/scisignal.aao1917
48. Tabor A, Weisenburger S, Banerjee A, Purkayastha N, Kaendl JM, Hubner H, Wei LX, Gromer TW, Kornhuber J, Tschammer N, Birdsall NJM, Mashanov GI, Sandoghdar V, Gmeiner P (2016) Visualization and ligand-induced modulation of dopamine receptor dimerization at the single molecule level. *Sci Rep* 6:33233. doi:ARTN3323310.1038/srep33233 [PubMed: 27615810]
49. Lamichhane R, Liu JJ, Pljevaljcic G, White KL, van der Schans E, Katritch V, Stevens RC, Wuthrich K, Millar DP (2015) Single-molecule view of basal activity and activation mechanisms of the G protein-coupled receptor beta2AR. *Proc Natl Acad Sci U S A* 112 (46):14254–14259. doi:10.1073/pnas.1519626112 [PubMed: 26578769]
50. Serezani CH, Ballinger MN, Aronoff DM, Peters-Golden M (2008) Cyclic AMP: master regulator of innate immune cell function. *Am J Respir Cell Mol Biol* 39 (2):127–132. doi:10.1165/rcmb.2008-0091TR [PubMed: 18323530]
51. Kamenetsky M, Middelhaufe S, Bank EM, Levin LR, Buck J, Steegborn C (2006) Molecular details of cAMP generation in mammalian cells: a tale of two systems. *J Mol Biol* 362 (4):623–639. doi:10.1016/j.jmb.2006.07.045 [PubMed: 16934836]
52. Schmidt M, Dekker FJ, Maarsingh H (2013) Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. *Pharmacol Rev* 65 (2):670–709. doi:10.1124/pr.110.003707 [PubMed: 23447132]
53. Ponsioen B, Gloerich M, Ritsma L, Rehmann H, Bos JL, Jalink K (2009) Direct spatial control of Epac1 by cyclic AMP. *Mol Cell Biol* 29 (10):2521–2531. doi:10.1128/MCB.01630-08 [PubMed: 19273589]
54. de Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL (2000) Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. *J Biol Chem* 275 (27):20829–20836. doi:10.1074/jbc.M001113200 [PubMed: 10777494]
55. Patel N, Gold MG (2015) The genetically encoded tool set for investigating cAMP: more than the sum of its parts. *Front Pharmacol* 6:164. doi:10.3389/fphar.2015.00164 [PubMed: 26300778]

56. Nikolaev VO, Bunemann M, Hein L, Hannawacker A, Lohse MJ (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. *J Biol Chem* 279 (36):37215–37218. doi:10.1074/jbc.C400302200 [PubMed: 15231839]
57. Everett KL, Cooper DM (2013) An improved targeted cAMP sensor to study the regulation of adenylyl cyclase 8 by Ca²⁺ entry through voltage-gated channels. *PLoS One* 8 (9):e75942. doi: 10.1371/journal.pone.0075942 [PubMed: 24086669]
58. DiPilato LM, Zhang J (2009) The role of membrane microdomains in shaping beta2-adrenergic receptor-mediated cAMP dynamics. *Mol Biosyst* 5 (8):832–837. doi:10.1039/b823243a [PubMed: 19603118]
59. Dyachok O, Isakov Y, Sagetorp J, Tengholm A (2006) Oscillations of cyclic AMP in hormone-stimulated insulin-secreting beta-cells. *Nature* 439 (7074):349–352. doi:10.1038/nature04410 [PubMed: 16421574]
60. Lindner R, Hartmann E, Tarnawski M, Winkler A, Frey D, Reinstein J, Meinhart A, Schlichting I (2017) Photoactivation Mechanism of a Bacterial Light-Regulated Adenylyl Cyclase. *J Mol Biol* 429 (9):1336–1351. doi:10.1016/j.jmb.2017.03.020 [PubMed: 28336405]
61. Efetova M, Schwarzel M (2015) Photoactivatable Adenylyl Cyclases (PACs) as a Tool to Study cAMP Signaling In Vivo: An Overview In: Zaccolo M (ed) *cAMP Signaling: Methods and Protocols*. Springer New York, New York, NY, pp 131–135. doi:10.1007/978-1-4939-2537-7_10
62. Antal CE, Newton AC (2013) Spatiotemporal dynamics of phosphorylation in lipid second messenger signaling. *Mol Cell Proteomics* 12 (12):3498–3508. doi:10.1074/mcp.R113.029819 [PubMed: 23788531]
63. Kunkel MT, Newton AC (2010) Calcium transduces plasma membrane receptor signals to produce diacylglycerol at Golgi membranes. *J Biol Chem* 285 (30):22748–22752. doi:10.1074/jbc.C110.123133 [PubMed: 20519514]
64. Steinberg SF (2008) Structural basis of protein kinase C isoform function. *Physiol Rev* 88(4): 1341–1378. doi:10.1152/physrev.00034.2007 [PubMed: 18923184]
65. Oancea E, Teruel MN, Quest AFG, Meyer T (1998) Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells. *Journal of Cell Biology* 140 (3):485–498. doi:DOI 10.1083/jcb.140.3.485 [PubMed: 9456311]
66. Tewson P, Westenberg M, Zhao Y, Campbell RE, Quinn AM, Hughes TE (2012) Simultaneous detection of Ca²⁺ and diacylglycerol signaling in living cells. *PLoS One* 7(8):e42791. doi: 10.1371/journal.pone.0042791 [PubMed: 22912738]
67. Stauffer TP, Ahn S, Meyer T (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P₂ concentration monitored in living cells. *Curr Biol* 8 (6):343–346 [PubMed: 9512420]
68. Varnai P, Balla T (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[³H]inositol-labeled phosphoinositide pools. *J Cell Biol* 143 (2):501–510 [PubMed: 9786958]
69. Billups D, Billups B, Challiss RA, Nahorski SR (2006) Modulation of Gq-protein-coupled inositol trisphosphate and Ca²⁺ signaling by the membrane potential. *J Neurosci* 26 (39):9983–9995. doi: 10.1523/JNEUROSCI.2773-06.2006 [PubMed: 17005862]
70. Hao JJ, Liu Y, Kruhlak M, Debell KE, Rellahan BL, Shaw S (2009) Phospholipase C-mediated hydrolysis of PIP₂ releases ERM proteins from lymphocyte membrane. *J Cell Biol* 184(3):451–462. doi:10.1083/jcb.200807047 [PubMed: 19204146]
71. Horowitz LF, Hirdes W, Suh BC, Hilgemann DW, Mackie K, Hille B (2005) Phospholipase C in living cells: activation, inhibition, Ca²⁺ requirement, and regulation of M current. *J Gen Physiol* 126 (3):243–262. doi:10.1085/jgp.200509309 [PubMed: 16129772]
72. Raucher D, Stauffer T, Chen W, Shen K, Guo S, York JD, Sheetz MP, Meyer T (2000) Phosphatidylinositol 4,5-Bisphosphate Functions as a Second Messenger that Regulates Cytoskeleton-Plasma Membrane Adhesion. *Cell* 100 (2):221–228. doi:10.1016/s0092-8674(00)81560-3 [PubMed: 10660045]

73. Hirose K (1999) Spatiotemporal Dynamics of Inositol 1,4,5-Trisphosphate That Underlies Complex Ca^{2+} Mobilization Patterns. *Science* 284 (5419):1527–1530. doi:10.1126/science.284.5419.1527 [PubMed: 10348740]
74. Nash MS, Young KW, Willars GB, Challiss RA, Nahorski SR (2001) Single-cell imaging of graded $\text{Ins}(1,4,5)\text{P}_3$ production following G-protein-coupled-receptor activation. *Biochem J* 356 (Pt 1): 137–142. doi:10.1042/bj3560137 [PubMed: 11336645]
75. Santagata S, Boggon TJ, Baird CL, Gomez CA, Zhao J, Shan WS, Myszkowski DG, Shapiro L (2001) G-protein signaling through tubby proteins. *Science* 292 (5524):2041–2050. doi:10.1126/science.1061233 [PubMed: 11375483]
76. Quinn KV, Behe P, Tinker A (2008) Monitoring changes in membrane phosphatidylinositol 4,5-bisphosphate in living cells using a domain from the transcription factor tubby. *J Physiol* 586 (12): 2855–2871. doi:10.1113/jphysiol.2008.153791 [PubMed: 18420701]
77. Gallegos LL, Newton AC (2011) Genetically encoded fluorescent reporters to visualize protein kinase C activation in live cells. *Methods Mol Biol* 756:295–310. doi: 10.1007/978-1-61779-160-4_17 [PubMed: 21870234]
78. Feng X, Zhang J, Barak LS, Meyer T, Caron MG, Hannun YA (1998) Visualization of Dynamic Trafficking of a Protein Kinase C βII /Green Fluorescent Protein Conjugate Reveals Differences in G Protein-coupled Receptor Activation and Desensitization. *J Biol Chem* 273 (17):10755–10762. doi:10.1074/jbc.273.17.10755 [PubMed: 9553141]
79. Gallegos LL, Kunkel MT, Newton AC (2006) Targeting protein kinase C activity reporter to discrete intracellular regions reveals spatiotemporal differences in agonist-dependent signaling. *J Biol Chem* 281 (41):30947–30956. doi:10.1074/jbc.M603741200 [PubMed: 16901905]
80. Sakai N, Sasaki K, Ikegaki N, Shirai Y, Ono Y, Saito N (1997) Direct visualization of the translocation of the gamma-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein. *J Cell Biol* 139 (6):1465–1476 [PubMed: 9396752]
81. Wan Q, Okashah N, Inoue A, Nehme R, Carpenter B, Tate CG, Lambert NA (2018) Mini G protein probes for active G protein-coupled receptors (GPCRs) in live cells. *J Biol Chem* 293 (19): 7466–7473. doi:10.1074/jbc.RA118.001975 [PubMed: 29523687]
82. Barak LS, Ferguson SS, Zhang J, Caron MG (1997) A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem* 272 (44):27497–27500. doi:10.1074/jbc.272.44.27497 [PubMed: 9346876]
83. Carpenter B, Tate CG (2016) Engineering a minimal G protein to facilitate crystallisation of G protein-coupled receptors in their active conformation. *Protein Eng Des Sel* 29 (12):583–594. doi: 10.1093/protein/gzw049 [PubMed: 27672048]
84. Nehme R, Carpenter B, Singhal A, Strege A, Edwards PC, White CF, Du H, Grisshammer R, Tate CG (2017) Mini-G proteins: Novel tools for studying GPCRs in their active conformation. *PLoS One* 12 (4):e0175642. doi:10.1371/journal.pone.0175642 [PubMed: 28426733]
85. Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK (2011) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* 469 (7329):175–180. doi:10.1038/nature09648 [PubMed: 21228869]
86. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R (1993) Naturally occurring antibodies devoid of light chains. *Nature* 363 (6428):446–448. doi:10.1038/363446a0 [PubMed: 8502296]
87. Lauwereys M, Arbabi Ghahroudi M, Desmyter A, Kinne J, Holzer W, De Genst E, Wyns L, Muyldermans S (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J* 17 (13):3512–3520. doi:10.1093/emboj/17.13.3512 [PubMed: 9649422]
88. Manglik A, Kobilka BK, Steyaert J (2017) Nanobodies to Study G Protein-Coupled Receptor Structure and Function. *Annu Rev Pharmacol Toxicol* 57:19–37. doi:10.1146/annurev-pharmtox-010716-104710 [PubMed: 27959623]
89. Rasmussen SGF, Choi H-J, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK (2011) Structure of a nanobody-stabilized active state of the $\beta(2)$ adrenoceptor. *Nature* 469 (7329):175–180. doi:10.1038/nature09648 [PubMed: 21228869]

90. Irannejad R, Pessino V, Mika D, Huang B, Wedegaertner PB, Conti M, von Zastrow M (2017) Functional selectivity of GPCR-directed drug action through location bias. *Nat Chem Biol* 13 (7): 799–806. doi:10.1038/nchembio.2389 [PubMed: 28553949]
91. Tennakoon M, Kankanamge D, Senarath K, Fasih Z, Karunarathne A (2019) Statins Perturb Gbetagamma Signaling and Cell Behavior in a Ggamma Subtype Dependent Manner. *Mol Pharmacol* 95 (4):361–375. doi:10.1124/mol.118.114710 [PubMed: 30765461]
92. Sekar RB, Periasamy A (2003) Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J Cell Biol* 160 (5):629–633. doi:10.1083/jcb.200210140 [PubMed: 12615908]
93. Tsien RY, Bacskaï BJ, Adams SR (1993) FRET for studying intracellular signalling. *Trends Cell Biol* 3 (7):242–245 [PubMed: 14731760]
94. Masi A, Cicchi R, Carloni A, Pavone FS, Arcangeli A (2010) Optical methods in the study of protein-protein interactions. *Adv Exp Med Biol* 674:33–42 [PubMed: 20549938]
95. Schaferling M, Nagl S (2011) Förster resonance energy transfer methods for quantification of protein-protein interactions on microarrays. *Methods Mol Biol* 723:303–320. doi:10.1007/978-1-61779-043-0_19 [PubMed: 21370073]
96. Telser A (2002) FUNDAMENTALS OF LIGHT MICROSCOPY AND ELECTRONIC IMAGING. *Shock* 17 (5):442
97. Hoffmann C, Gaietta G, Bunemann M, Adams SR, Oberdorff-Maass S, Behr B, Vilardaga JP, Tsien RY, Ellisman MH, Lohse MJ (2005) A FLAHS-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat Methods* 2 (3):171–176. doi:10.1038/nmeth742 [PubMed: 15782185]
98. McCann JJ, Choi UB, Zheng L, Weninger K, Bowen ME (2010) Optimizing methods to recover absolute FRET efficiency from immobilized single molecules. *Biophys J* 99 (3):961–970. doi:10.1016/j.bpj.2010.04.063 [PubMed: 20682275]
99. Lohse MJ, Nikolaev VO, Hein P, Hoffmann C, Vilardaga JP, Bunemann M (2008) Optical techniques to analyze real-time activation and signaling of G-protein-coupled receptors. *Trends Pharmacol Sci* 29 (3):159–165. doi:10.1016/j.tips.2007.12.002 [PubMed: 18262662]
100. Vilardaga J-P, Bunemann M, Krasel C, Castro M, Lohse MJ (2003) Measurement of the millisecond activation switch of G protein-coupled receptors in living cells. *Nat Biotechnol* 21:807. doi:10.1038/nbt838 [PubMed: 12808462]
101. Sheikh SP, Vilardaga J-P, Baranski TJ, Lichtarge O, Iiri T, Meng EC, Nissenson RA, Bourne HR (1999) Similar Structures and Shared Switch Mechanisms of the β 2-Adrenoceptor and the Parathyroid Hormone Receptor. *J Biol Chem* 274 (24):17033–17041. doi:10.1074/jbc.274.24.17033 [PubMed: 10358054]
102. Vilardaga JP, Frank M, Krasel C, Dees C, Nissenson RA, Lohse MJ (2001) Differential conformational requirements for activation of G proteins and the regulatory proteins arrestin and G protein-coupled receptor kinase in the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein. *J Biol Chem* 276 (36):33435–33443. doi:10.1074/jbc.M011495200 [PubMed: 11387315]
103. Tsien RY (1998) The green fluorescent protein. *Annu Rev Biochem* 67:509–544. doi:10.1146/annurev.biochem.67.1.509 [PubMed: 9759496]
104. Miyawaki A, Tsien RY (2000) Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol* 327:472–500 [PubMed: 11045004]
105. Reiner S, Ambrosio M, Hoffmann C, Lohse MJ (2010) Differential signaling of the endogenous agonists at the beta2-adrenergic receptor. *J Biol Chem* 285 (46):36188–36198. doi:10.1074/jbc.M110.175604 [PubMed: 20837485]
106. Lohse MJ, Bunemann M, Hoffmann C, Vilardaga JP, Nikolaev VO (2007) Monitoring receptor signaling by intramolecular FRET. *Curr Opin Pharmacol* 7 (5):547–553. doi:10.1016/j.coph.2007.08.007 [PubMed: 17919975]
107. Chachisvilis M, Zhang YL, Frangos JA (2006) G protein-coupled receptors sense fluid shear stress in endothelial cells. *Proc Natl Acad Sci U S A* 103 (42): 15463–15468. doi:10.1073/pnas.0607224103 [PubMed: 17030791]

108. Rochais F, Vilardaga JP, Nikolaev VO, Bunemann M, Lohse MJ, Engelhardt S (2007) Real-time optical recording of beta1-adrenergic receptor activation reveals supersensitivity of the Arg389 variant to carvedilol. *J Clin Invest* 117 (1):229–235. doi:10.1172/JCI30012 [PubMed: 17200720]
109. Hoffmann C, Gaietta G, Bunemann M, Adams SR, Oberdorff-Maass S, Behr B, Vilardaga J-P, Tsien RY, Ellisman MH, Lohse MJ (2005) A FAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat Methods* 2:171. doi:10.1038/nmeth742 [PubMed: 15782185]
110. Zhao X, Jones A, Olson KR, Peng K, Wehrman T, Park A, Mallari R, Nebalasca D, Young SW, Xiao SH (2008) A homogeneous enzyme fragment complementation-based beta-arrestin translocation assay for high-throughput screening of G-protein-coupled receptors. *J Biomol Screen* 13 (8):737–747. doi:10.1177/1087057108321531
111. den Hamer A, Dierickx P, Arts R, de Vries J, Brunsveld L, Merckx M (2017) Bright Bioluminescent BRET Sensor Proteins for Measuring Intracellular Caspase Activity. *ACS Sens* 2 (6):729–734. doi:10.1021/acssensors.7b00239 [PubMed: 28670623]
112. Zhao H, Doyle TC, Wong RJ, Cao Y, Stevenson DK, Piwnicka-Worms D, Contag CH (2004) Characterization of coelenterazine analogs for measurements of Renilla luciferase activity in live cells and living animals. *Mol Imaging* 3 (1):43–54. doi:10.1162/153535004773861714 [PubMed: 15142411]
113. Woo J, von Arnim AG (2008) Mutational optimization of the coelenterazine-dependent luciferase from Renilla. *Plant Methods* 4 (1):23. doi:10.1186/1746-4811-4-23 [PubMed: 18826616]
114. Dacres H, Michie M, Trowell SC (2012) Comparison of enhanced bioluminescence energy transfer donors for protease biosensors. *Anal Biochem* 424 (2):206–210. doi:10.1016/j.ab.2012.02.028 [PubMed: 22387387]
115. Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, Otto P, Zimmerman K, Vidugiris G, Machleidt T, Robers MB, Benink HA, Eggers CT, Slater MR, Meisenheimer PL, Klaubert DH, Fan F, Encell LP, Wood KV (2012) Engineered luciferase reporter from a deepsea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem Biol* 7 (11): 1848–1857. doi: 10.1021/cb3002478 [PubMed: 22894855]
116. Sleno R, Petrin D, Devost D, Goupil E, Zhang A, Hebert TE (2016) Designing BRET-based conformational biosensors for G protein-coupled receptors. *Methods* 92:11–18. doi:10.1016/j.ymeth.2015.05.003 [PubMed: 25962643]
117. Picard LP, Schonegge AM, Lohse MJ, Bouvier M (2018) Bioluminescence resonance energy transfer-based biosensors allow monitoring of ligand- and transducer-mediated GPCR conformational changes. *Commun Biol* 1 (1):106. doi:10.1038/s42003-018-0101-z [PubMed: 30271986]
118. Devost D, Sleno R, Petrin D, Zhang A, Shinjo Y, Okde R, Aoki J, Inoue A, Hebert TE (2017) Conformational Profiling of the AT1 Angiotensin II Receptor Reflects Biased Agonism, G Protein Coupling, and Cellular Context. *J Biol Chem* 292 (13):5443–5456. doi:10.1074/jbc.M116.763854 [PubMed: 28213525]
119. Yoshida T, Kakizuka A, Imamura H (2016) BTeam, a Novel BRET-based Biosensor for the Accurate Quantification of ATP Concentration within Living Cells. *Sci Rep* 6:39618. doi: 10.1038/srep39618 [PubMed: 28000761]
120. Gales C, Rebois RV, Hogue M, Trieu P, Breit A, Hebert TE, Bouvier M (2005) Real-time monitoring of receptor and G-protein interactions in living cells. *Nat Methods* 2 (3):177–184. doi:10.1038/nmeth743 [PubMed: 15782186]
121. Gales C, Van Durm JJJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nat Struct Mol Biol* 13:778. doi:10.1038/nsmb1134 [PubMed: 16906158]
122. Lohse MJ, Nuber S, Hoffmann C (2012) Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. *Pharmacol Rev* 64 (2): 299–336. doi:10.1124/pr.110.004309 [PubMed: 22407612]
123. Wettschureck N, Offermanns S (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev* 85 (4):1159–1204. doi:10.1152/physrev.00003.2005 [PubMed: 16183910]

124. Frank M, Thumer L, Lohse MJ, Bunemann M (2005) G Protein activation without subunit dissociation depends on a G α (i)-specific region. *J Biol Chem* 280 (26):24584–24590. doi: 10.1074/jbc.M414630200 [PubMed: 15866880]
125. Levitzki A, Klein S (2002) G-protein subunit dissociation is not an integral part of G-protein action. *ChemBiochem* 3 (9):815–818. doi: 10.1002/1439-7633(20020902)3:9<815::AIDCBIC815>3.0.CO;2-E [PubMed: 12210980]
126. Oldham WM, Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 9 (1):60–71. doi:10.1038/nrm2299 [PubMed: 18043707]
127. Birnbaumer L (2007) The discovery of signal transduction by G proteins: a personal account and an overview of the initial findings and contributions that led to our present understanding. *Biochim Biophys Acta* 1768 (4):756–771. doi:10.1016/j.bbame.2006.09.027 [PubMed: 17141178]
128. Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* 455 (7212): 497–502. doi:10.1038/nature07330 [PubMed: 18818650]
129. Choe HW, Kim YJ, Park JH, Morizumi T, Pai EF, Krauss N, Hofmann KP, Scheerer P, Ernst OP (2011) Crystal structure of metarhodopsin II. *Nature* 471 (7340):651–655. doi:10.1038/nature09789 [PubMed: 21389988]
130. Choe HW, Park JH, Kim YJ, Ernst OP (2011) Transmembrane signaling by GPCRs: insight from rhodopsin and opsin structures. *Neuropharmacology* 60 (1):52–57. doi:10.1016/j.neuropharm.2010.07.018 [PubMed: 20708633]
131. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK, Kobilka BK (2011) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* 477 (7366):549–555. doi:10.1038/nature10361 [PubMed: 21772288]
132. Cai K, Itoh Y, Khorana HG (2001) Mapping of contact sites in complex formation between transducin and light-activated rhodopsin by covalent crosslinking: use of a photoactivatable reagent. *Proc Natl Acad Sci U S A* 98 (9):4877–4882. doi:10.1073/pnas.051632898 [PubMed: 11320237]
133. Bae H, Cabrera-Vera TM, Depree KM, Graber SG, Hamm HE (1999) Two Amino Acids within the α 4 Helix of GailMediate Coupling with 5-Hydroxytryptamine1BReceptors. *J Biol Chem* 274 (21):14963–14971. doi:10.1074/jbc.274.21.14963
134. Taylor JM, Jacob-Mosier GG, Lawton RG, Remmers AE, Neubig RR (1994) Binding of an alpha 2 adrenergic receptor third intracellular loop peptide to G beta and the amino terminus of G alpha. *J Biol Chem* 269 (44):27618–27624 [PubMed: 7961678]
135. Azpiazu I, Cruzblanca H, Li P, Linder M, Zhuo M, Gautam N (1999) A G protein gamma subunit-specific peptide inhibits muscarinic receptor signaling. *J Biol Chem* 274 (50):35305–35308. doi:10.1074/jbc.274.50.35305 [PubMed: 10585394]
136. Taylor JM, Jacob-Mosier GG, Lawton RG, VanDort M, Neubig RR (1996) Receptor and membrane interaction sites on Gbeta. A receptor-derived peptide binds to the carboxylterminus. *J Biol Chem* 271 (7):3336–3339. doi:10.1074/jbc.271.7.3336 [PubMed: 8631928]
137. Heithier H, Frohlich M, Dees C, Baumann M, Haring M, Gierschik P, Schiltz E, Vaz WL, Hekman M, Helmreich EJ (1992) Subunit interactions of GTP-binding proteins. *Eur J Biochem* 204 (3): 1169–1181 [PubMed: 1312936]
138. Koestler M, Heithier H, Baumann M, Dees C, Hekman M, Vaz W (1989) Association-dissociation of purified subunits of GTP-binding proteins measured by fluorescence energy transfer. *J Protein Chem* 8 (3):406
139. Ruiz-Velasco V, Ikeda SR (2001) Functional expression and FRET analysis of green fluorescent proteins fused to G-protein subunits in rat sympathetic neurons. *J Physiol* 537 (Pt 3):679–692 [PubMed: 11744747]
140. Bunemann M, Frank M, Lohse MJ (2003) Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc Natl Acad Sci U S A* 100 (26):16077–16082. doi: 10.1073/pnas.2536719100 [PubMed: 14673086]

141. Philip F, Sengupta P, Scarlata S (2007) Signaling through a G Protein-coupled receptor and its corresponding G protein follows a stoichiometrically limited model. *J Biol Chem* 282 (26): 19203–19216. doi:10.1074/jbc.M701558200 [PubMed: 17420253]
142. Ayoub MA, Maurel D, Binet V, Fink M, Prezeau L, Ansanay H, Pin JP (2007) Real-time analysis of agonist-induced activation of protease-activated receptor 1/Galphi1 protein complex measured by bioluminescence resonance energy transfer in living cells. *Mol Pharmacol* 71 (5): 1329–1340. doi:10.1124/mol.106.030304 [PubMed: 17267663]
143. Audet N, Gales C, Archer-Lahlou E, Vallieres M, Schiller PW, Bouvier M, Pineyro G (2008) Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing delta-opioid receptors and heterotrimeric G proteins. *J Biol Chem* 283 (22):15078–15088. doi:10.1074/jbc.M707941200 [PubMed: 18381293]
144. Yu JZ, Rasenick MM (2002) Real-time visualization of a fluorescent G(alpha)(s): dissociation of the activated G protein from plasma membrane. *Mol Pharmacol* 61 (2):352–359 [PubMed: 11809860]
145. Hein P, Rochais F, Hoffmann C, Dorsch S, Nikolaev VO, Engelhardt S, Berlot CH, Lohse MJ, Bunemann M (2006) Gs activation is time-limiting in initiating receptor-mediated signaling. *J Biol Chem* 281 (44):33345–33351. doi:10.1074/jbc.M606713200 [PubMed: 16963443]
146. Hein P, Frank M, Hoffmann C, Lohse MJ, Bunemann M (2005) Dynamics of receptor/G protein coupling in living cells. *EMBO J* 24 (23):4106–4114. doi:10.1038/sj.emboj.7600870 [PubMed: 16292347]
147. Neubig RR, Connolly MP, Remmers AE (1994) Rapid kinetics of G protein subunit association: a rate-limiting conformational change? *FEBS Lett* 355 (3):251–253 [PubMed: 7527348]
148. Janetopoulos C, Jin T, Devreotes P (2001) Receptor-mediated activation of heterotrimeric G-proteins in living cells. *Science* 291 (5512):2408–2411. doi:10.1126/science.1055835 [PubMed: 11264536]
149. Gibson SK, Gilman AG (2006) Galpha and Gbeta subunits both define selectivity of G protein activation by alpha2-adrenergic receptors. *Proc Natl Acad Sci U S A* 103 (1):212–217. doi: 10.1073/pnas.0509763102 [PubMed: 16371464]
150. van Unen J, Stumpf AD, Schmid B, Reinhard NR, Hordijk PL, Hoffmann C, Gadella TW Jr., Goedhart J (2016) A New Generation of FRET Sensors for Robust Measurement of Galphi1, Galphi2 and Galphi3 Activation Kinetics in Single Cells. *PLoS One* 11(1):e0146789. doi: 10.1371/journal.pone.0146789 [PubMed: 26799488]
151. Klarenbeek JB, Goedhart J, Hink MA, Gadella TW, Jalink K (2011) A mTurquoise-based cAMP sensor for both FLIM and ratiometric read-out has improved dynamic range. *PLoS One* 6(4):e19170. doi:10.1371/journal.pone.0019170 [PubMed: 21559477]
152. Sparta B, Pargett M, Minguet M, Distor K, Bell G, Albeck JG (2015) Receptor Level Mechanisms Are Required for Epidermal Growth Factor (EGF)-stimulated Extracellular Signal-regulated Kinase (ERK) Activity Pulses. *J Biol Chem* 290 (41):24784–24792. doi:10.1074/jbc.M115.662247 [PubMed: 26304118]
153. Hynes TR, Mervine SM, Yost EA, Sabo JL, Berlot CH (2004) Live cell imaging of Gs and the beta2-adrenergic receptor demonstrates that both alphas and beta1gamma7 internalize upon stimulation and exhibit similar trafficking patterns that differ from that of the beta2-adrenergic receptor. *J Biol Chem* 279 (42):44101–44112. doi:10.1074/jbc.M405151200 [PubMed: 15297467]
154. Akhter SA, Luttrell LM, Rockman HA, Iaccarino G, Lefkowitz RJ, Koch WJ (1998) Targeting the receptor-Gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science* 280 (5363):574–577 [PubMed: 9554846]
155. Wetschreck N, Rutten H, Zywiets A, Gehring D, Wilkie TM, Chen J, Chien KR, Offermanns S (2001) Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galphi1 in cardiomyocytes. *Nat Med* 7 (11):1236–1240. doi:10.1038/nm1101-1236 [PubMed: 11689889]
156. Dorn GW 2nd (2005) Physiologic growth and pathologic genes in cardiac development and cardiomyopathy. *Trends Cardiovasc Med* 15 (5):185–189. doi:10.1016/j.tcm.2005.05.009 [PubMed: 16165015]

157. Wedegaertner PB, Chu DH, Wilson PT, Levis MJ, Bourne HR (1993) Palmitoylation is required for signaling functions and membrane attachment of Gq alpha and Gs alpha. *J Biol Chem* 268 (33):25001–25008
158. Wilson PT, Bourne HR (1995) Fatty acylation of alpha z. Effects of palmitoylation and myristoylation on alpha z signaling. *J Biol Chem* 270 (16):9667–9675 [PubMed: 7536745]
159. Adjobo-Hermans MJ, Goedhart J, van Weeren L, Nijmeijer S, Manders EM, Offermanns S, Gadella TW Jr. (2011) Real-time visualization of heterotrimeric G protein Gq activation in living cells. *BMC Biol* 9:32. doi:10.1186/1741-7007-9-32 [PubMed: 21619590]
160. Jensen JB, Lyssand JS, Hague C, Hille B (2009) Fluorescence changes reveal kinetic steps of muscarinic receptor-mediated modulation of phosphoinositides and Kv7.2/7.3 K+ channels. *J Gen Physiol* 133 (4):347–359. doi:10.1085/jgp.200810075 [PubMed: 19332618]
161. Mastop M, Reinhard NR, Zuconelli CR, Terwey F, Gadella TWJ Jr., van Unen J, Adjobo-Hermans MJW, Goedhart J (2018) A FRET-based biosensor for measuring Galpha13 activation in single cells. *PLoS One* 13 (3):e0193705. doi:10.1371/journal.pone.0193705 [PubMed: 29505611]
162. Shenoy SK, Lefkowitz RJ (2003) Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *J Biol Chem* 278 (16): 14498–14506. doi:10.1074/jbc.M209626200 [PubMed: 12574160]
163. Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, Pineyro G (2003) Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci U S A* 100 (20):11406–11411. doi:10.1073/pnas.1936664100 [PubMed: 13679574]
164. Violin JD, Ren XR, Lefkowitz RJ (2006) G-protein-coupled receptor kinase specificity for beta-arrestin recruitment to the beta2-adrenergic receptor revealed by fluorescence resonance energy transfer. *J Biol Chem* 281 (29):20577–20588. doi:10.1074/jbc.M513605200 [PubMed: 16687412]
165. Angers S, Salahpour A, Joly E, Hilaiet S, Chelsky D, Dennis M, Bouvier M (2000) Detection of 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci U S A* 97 (7): 3684–3689. doi:10.1073/pnas.97.7.3684 [PubMed: 10725388]
166. Kroeger KM, Hanyaloglu AC, Seeber RM, Miles LE, Eidne KA (2001) Constitutive and agonist-dependent homo-oligomerization of the thyrotropin-releasing hormone receptor. Detection in living cells using bioluminescence resonance energy transfer. *J Biol Chem* 276 (16):12736–12743. doi:10.1074/jbc.M011311200 [PubMed: 11278883]
167. Hamdan FF, Audet M, Garneau P, Pelletier J, Bouvier M (2005) High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer 1-based beta-arrestin2 recruitment assay. *J Biomol Screen* 10 (5):463–475. doi: 10.1177/1087057105275344 [PubMed: 16093556]
168. Qiu Y, Loh HH, Law PY (2007) Phosphorylation of the delta-opioid receptor regulates its beta-arrestins selectivity and subsequent receptor internalization and adenylyl cyclase desensitization. *J Biol Chem* 282 (31):22315–22323. doi:10.1074/jbc.M611258200 [PubMed: 17565992]
169. Coulon V, Audet M, Homburger V, Bockaert J, Fagni L, Bouvier M, Perroy J (2008) Subcellular imaging of dynamic protein interactions by bioluminescence resonance energytransfer. *Biophys J* 94 (3):1001–1009. doi:10.1529/biophysj.107.117275 [PubMed: 17921204]
170. Klewe IV, Nielsen SM, Tarpo L, Urizar E, Dipace C, Javitch JA, Gether U, Egebjerg J, Christensen KV (2008) Recruitment of beta-arrestin2 to the dopamine D2 receptor: insights into anti-psychotic and anti-parkinsonian drug receptor signaling. *Neuropharmacology* 54 (8):1215–1222. doi:10.1016/j.neuropharm.2008.03.015 [PubMed: 18455202]
171. Masri B, Salahpour A, Didriksen M, Ghisi V, Beaulieu JM, Gainetdinov RR, Caron MG (2008) Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc Natl Acad Sci U S A* 105 (36):13656–13661. doi: 10.1073/pnas.0803522105 [PubMed: 18768802]
172. Hasbi A, Devost D, Laporte SA, Zingg HH (2004) Real-time detection of interactions between the human oxytocin receptor and G protein-coupled receptor kinase-2. *Mol Endocrinol* 18 (5):1277–1286. doi:10.1210/me.2003-0440 [PubMed: 14976224]

173. Jorgensen R, Kubale V, Vrecl M, Schwartz TW, Elling CE (2007) Oxyntomodulin differentially affects glucagon-like peptide-1 receptor beta-arrestin recruitment and signaling through Galpha(s). *J Pharmacol Exp Ther* 322 (1):148–154. doi:10.1124/jpet.107.120006 [PubMed: 17395766]
174. Jorgensen R, Holliday ND, Hansen JL, Vrecl M, Heding A, Schwartz TW, Elling CE (2008) Characterization of G-protein coupled receptor kinase interaction with the neurokinin-1 receptor using bioluminescence resonance energy transfer. *Mol Pharmacol* 73 (2):349–358 [PubMed: 17986524]
175. Dremier S, Kopperud R, Doskeland SO, Dumont JE, Maenhaut C (2003) Search for new cyclic AMP-binding proteins. *FEBS Lett* 546 (1):103–107 [PubMed: 12829244]
176. Lee KA (1991) Transcriptional regulation by cAMP. *Curr Opin Cell Biol* 3 (6):953–959 [PubMed: 1667580]
177. Prasad KN, Cole WC, Yan XD, Nahreini P, Kumar B, Hanson A, Prasad JE (2003) Defects in cAMP-pathway may initiate carcinogenesis in dividing nerve cells: a review. *Apoptosis* 8 (6): 579–586. doi:10.1023/A:1026179324295 [PubMed: 14574064]
178. Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S (2000) cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nat Cell Biol* 2 (11):805–811. doi:10.1038/35041046 [PubMed: 11056535]
179. Zagotta WN, Olivier NB, Black KD, Young EC, Olson R, Gouaux E (2003) Structural basis for modulation and agonist specificity of HCN pacemaker channels. *Nature* 425 (6954):200–205. doi:10.1038/nature01922 [PubMed: 12968185]
180. Storch U, Straub J, Erdogmus S, Gudermann T, Mederos YSM (2017) Dynamic monitoring of Gi/o-protein-mediated decreases of intracellular cAMP by FRET-based Epac sensors. *Pflugers Arch* 469 (5–6):725–737. doi:10.1007/s00424-017-1975-1 [PubMed: 28386636]
181. DiPilato LM, Cheng X, Zhang J (2004) Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc Natl Acad Sci U S A* 101 (47):16513–16518. doi:10.1073/pnas.0405973101 [PubMed: 15545605]
182. Ponimaskin EG, Heine M, Zeug A, Voyno-Yasenetskaya T, Salonikidis PS (2007) Monitoring Receptor-Mediated Changes of Intracellular cAMP Level by Using Ion Channels and Fluorescent Proteins as Biosensors In: Chattopadhyay A (ed) *Serotonin Receptors in Neurobiology*. Frontiers in Neuroscience. CRC Press/Taylor & Francis/Taylor & Francis Group, LLC., Boca Raton (FL),
183. Patterson G, Day RN, Piston D (2001) Fluorescent protein spectra. *J Cell Sci* 114 (Pt 5):837–838 [PubMed: 11181166]
184. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol* 20 (1):87–90. doi:10.1038/nbt0102-87 [PubMed: 11753368]
185. Zaccolo M, De Giorgi F, Cho CY, Feng L, Knapp T, Negulescu PA, Taylor SS, Tsien RY, Pozzan T (2000) A genetically encoded, fluorescent indicator for cyclic AMP in living cells. *Cell Biol* 2 (1):25–29. doi:10.1038/71345
186. Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* 295 (5560):1711–1715. doi:10.1126/science.1069982 [PubMed: 11872839]
187. Diller TC, Madhusudan, Xuong NH, Taylor SS (2001) Molecular basis for regulatory subunit diversity in cAMP-dependent protein kinase: crystal structure of the type II beta regulatory subunit. *Structure* 9 (1):73–82 [PubMed: 11342137]
188. Nikolaev VO, Bunemann M, Schmitteckert E, Lohse MJ, Engelhardt S (2006) Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching beta1-adrenergic but locally confined beta2-adrenergic receptor-mediated signaling. *Circ Res* 99 (10):1084–1091. doi: 10.1161/01.RES.0000250046.69918.d5 [PubMed: 17038640]
189. Jiang LI, Collins J, Davis R, Lin KM, DeCamp D, Roach T, Hsueh R, Rebres RA, Ross EM, Taussig R, Fraser I, Sternweis PC (2007) Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J Biol Chem* 282 (14):10576–10584. doi:10.1074/jbc.M609695200 [PubMed: 17283075]

190. van der Wal J, Habets R, Varnai P, Balla T, Jalink K (2001) Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. *J Biol Chem* 276 (18):15337–15344. doi:10.1074/jbc.M007194200 [PubMed: 11152673]
191. Chernov KG, Redchuk TA, Omelina ES, Verkhusha VV (2017) Near-Infrared Fluorescent Proteins, Biosensors, and Optogenetic Tools Engineered from Phytochromes. *Chem Rev* 117(9): 6423–6446. doi:10.1021/acs.chemrev.6b00700 [PubMed: 28401765]
192. Violin JD, Zhang J, Tsien RY, Newton AC (2003) A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J Cell Biol* 161 (5):899–909. doi: 10.1083/jcb.200302125 [PubMed: 12782683]
193. Sato M, Ueda Y, Umezawa Y (2006) Imaging diacylglycerol dynamics at organelle membranes. *Nat Methods* 3 (10):797–799. doi:10.1038/nmeth930 [PubMed: 16990811]
194. Ferraz-Nogueira JP, Diez-Guerra FJ, Llopis J (2014) Visualization of phosphatidic acid fluctuations in the plasma membrane of living cells. *PLoS One* 9 (7):e102526. doi:10.1371/journal.pone.0102526 [PubMed: 25025521]
195. Ueda Y, Ogiso H, Sato M, Umezawa Y, Okazaki T, Kobayashi T (2015) Asymmetrical diacylglycerol dynamics on the cytosolic and luminal sides of a single endomembrane in living cells. *Sci Rep* 5:12960. doi:10.1038/srep12960 [PubMed: 26264517]
196. Komatsu N, Aoki K, Yamada M, Yukinaga H, Fujita Y, Kamioka Y, Matsuda M (2011) Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol Biol Cell* 22 (23):4647–4656. doi:10.1091/mbc.E11-01-0072 [PubMed: 21976697]
197. Ni Q, Titov DV, Zhang J (2006) Analyzing protein kinase dynamics in living cells with FRET reporters. *Methods* 40 (3):279–286. doi:10.1016/j.ymeth.2006.06.013 [PubMed: 16908183]
198. Miura H, Matsuda M, Aoki K (2014) Development of a FRET biosensor with high specificity for Akt. *Cell Struct Funct* 39 (1):9–20 [PubMed: 24212374]
199. Clapham DE (2007) Calcium signaling. *Cell* 131 (6):1047–1058. doi:10.1016/j.cell.2007.11.028 [PubMed: 18083096]
200. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997) Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature* 388 (6645):882–887. doi:10.1038/42264 [PubMed: 9278050]
201. Miyawaki A, Griesbeck O, Heim R, Tsien RY (1999) Dynamic and quantitative Ca²⁺ measurements using improved cameleons. *Proc Natl Acad Sci U S A* 96 (5):2135–2140 [PubMed: 10051607]
202. Truong K, Sawano A, Mizuno H, Hama H, Tong KI, Mal TK, Miyawaki A, Ikura M (2001) FRET-based in vivo Ca²⁺ imaging by a new calmodulin-GFP fusion molecule. *Nat Struct Biol* 8 (12):1069–1073. doi:10.1038/nsb728 [PubMed: 11702071]
203. Osawa M, Tokumitsu H, Swindells MB, Kurihara H, Orita M, Shibamura T, Furuya T, Ikura M (1999) A novel target recognition revealed by calmodulin in complex with Ca²⁺-calmodulin-dependent kinase kinase. *Nat Struct Biol* 6 (9):819–824. doi:10.1038/12271 [PubMed: 10467092]
204. Porumb T, Yau P, Harvey TS, Ikura M (1994) A calmodulin-target peptide hybrid molecule with unique calcium-binding properties. *Protein Eng* 7 (1):109–115 [PubMed: 8140087]
205. Komatsu N, Aoki K, Yamada M, Yukinaga H, Fujita Y, Kamioka Y, Matsuda M (2011) Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol Biol Cell* 22 (23):4647–4656. doi:10.1091/mbc.E11-01-0072 [PubMed: 21976697]
206. Zhang J, Ma Y, Taylor SS, Tsien RY (2001) Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc Natl Acad Sci U S A* 98 (26):14997–15002. doi:10.1073/pnas.211566798 [PubMed: 11752448]
207. Allen MD, Zhang J (2006) Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem Biophys Res Commun* 348 (2):716–721. doi:10.1016/j.bbrc.2006.07.136 [PubMed: 16895723]
208. Violin JD, Zhang J, Tsien RY, Newton AC (2003) A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J Cell Biol* 161 (5):899–909. doi: 10.1083/jcb.200302125 [PubMed: 12782683]

209. Pawson T, Nash P (2000) Protein-protein interactions define specificity in signal transduction. *Genes Dev* 14 (9):1027–1047 [PubMed: 10809663]
210. Shenoy SK, Lefkowitz RJ (2003) Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochem J* 375 (Pt 3):503–515. doi: 10.1042/BJ20031076 [PubMed: 12959637]
211. Giorgi C, Romagnoli A, Agnoletto C, Bergamelli L, Sorrentino G, Brini M, Pozzan T, Meldolesi J, Pinton P, Rizzuto R (2011) Translocation of signalling proteins to the plasma membrane revealed by a new bioluminescent procedure. *BMC Cell Biol* 12:27. doi: 10.1186/1471-2121-12-27 [PubMed: 21658234]
212. Giorgi C, Romagnoli A, Agnoletto C, Bergamelli L, Sorrentino G, Brini M, Pozzan T, Meldolesi J, Pinton P, Rizzuto R (2011) Translocation of signalling proteins to the plasma membrane revealed by a new bioluminescent procedure. *BMC Cell Biology* 12 (1):27. doi: 10.1186/1471-2121-12-27 [PubMed: 21658234]
213. Marsault R, Murgia M, Pozzan T, Rizzuto R (1997) Domains of high Ca^{2+} beneath the plasma membrane of living A7r5 cells. *EMBO J* 16 (7):1575–1581. doi:10.1093/emboj/16.7.1575 [PubMed: 9130702]
214. Yu Z, Taylor JL, He Y, Ni J (2015) Enlightenment on the aequorin-based platform for screening Arabidopsis stress sensory channels related to calcium signaling. *Plant Signal Behav* 10 (11):e1057366. doi:10.1080/15592324.2015.1057366 [PubMed: 26336841]
215. Bakayan A, Domingo B, Vaquero CF, Peyrieras N, Llopis J (2017) Fluorescent Protein-photoprotein Fusions and Their Applications in Calcium Imaging. *Photochem Photobiol* 93 (2): 448–465. doi:10.1111/php.12682 [PubMed: 27925224]
216. von Degenfeld G, Wehrman TS, Hammer MM, Blau HM (2007) A universal technology for monitoring G-protein-coupled receptor activation in vitro and noninvasively in live animals. *FASEB J* 21 (14):3819–3826. doi:10.1096/fj.07-9597com [PubMed: 17942828]
217. Yan YX, Boldt-Houle DM, Tillotson BP, Gee MA, D'Eon BJ, Chang XJ, Olesen CE, Palmer MA (2002) Cell-based high-throughput screening assay system for monitoring G protein-coupled receptor activation using beta-galactosidase enzyme complementation technology. *J Biomol Screen* 7 (5):451–459. doi:10.1177/108705702237677 [PubMed: 14599361]
218. Eishingdrelo H, Cai J, Weissensee P, Sharma P, Tocci MJ, Wright PS (2011) A cell-based protein-protein interaction method using a permuted luciferase reporter. *Curr Chem Genomics* 5:122–128. doi:10.2174/1875397301105010122 [PubMed: 22207892]
219. Barnea G, Strapps W, Herrada G, Berman Y, Ong J, Kloss B, Axel R, Lee KJ (2008) The genetic design of signaling cascades to record receptor activation. *Proc Natl Acad Sci U S A* 105 (1):64–69. doi:10.1073/pnas.0710487105 [PubMed: 18165312]
220. Yan Y, Xu TH, Harikumar KG, Miller LJ, Melcher K, Xu HE (2017) Detection of Membrane Protein Interactions by Cell-based Tango Assays. *Bio Protoc* 7 (22):e2903. doi:10.21769/BioProtoc.2903
221. Kroeze WK, Sassano MF, Huang XP, Lansu K, McCorvy JD, Giguere PM, Sciaky N, RothBL (2015) PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nat Struct Mol Biol* 22 (5):362–U328. doi:10.1038/nsmb.3014 [PubMed: 25895059]
222. Littmann T, Ozawa T, Hoffmann C, Buschauer A, Bernhardt G (2018) A split luciferase-based probe for quantitative proximal determination of Galphaq signalling in live cells. *Sci Rep* 8(1): 17179. doi:10.1038/s41598-018-35615-w [PubMed: 30464299]
223. Chen H, Zou Y, Shang Y, Lin H, Wang Y, Cai R, Tang X, Zhou JM (2008) Firefly luciferase complementation imaging assay for protein-protein interactions in plants. *Plant Physiol* 146(2): 368–376. doi:10.1104/pp.107.111740 [PubMed: 18065554]
224. Luker KE, Smith MC, Luker GD, Gammon ST, Piwnica-Worms H, Piwnica-Worms D (2004) Kinetics of regulated protein-protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. *Proc Natl Acad Sci U S A* 101 (33):12288–12293. doi:10.1073/pnas.0404041101 [PubMed: 15284440]
225. Misawa N, Kafi AK, Hattori M, Miura K, Masuda K, Ozawa T (2010) Rapid and high-sensitivity cell-based assays of protein-protein interactions using split click beetle luciferase

complementation: an approach to the study of G-protein-coupled receptors. *Anal Chem* 82 (6): 2552–2560. doi:10.1021/ac100104q [PubMed: 20180537]

226. Cheng Z, Garvin D, Paguio A, Stecha P, Wood K, Fan F (2010) Luciferase Reporter Assay System for Deciphering GPCR Pathways. *Curr Chem Genomics* 4:84–91. doi: 10.2174/1875397301004010084 [PubMed: 21331312]
227. Herschman HR (2004) Noninvasive Imaging of Reporter Gene Expression in Living Subjects In: *Advances in Cancer Research*, vol 92 Academic Press, pp 29–80 [PubMed: 15530556]
228. Jiang T, Xing B, Rao J (2008) Recent developments of biological reporter technology for detecting gene expression. *Biotechnol Genet Eng Rev* 25 (1):41–75. doi:10.5661/bger-25-41 [PubMed: 21412349]
229. Basu C, Kausch A, M Chandlee J (2004) Use of β -glucuronidase reporter gene for gene expression analysis in turfgrasses, vol 320. doi:10.1016/j.bbrc.2004.05.128
230. Paguio A, Stecha P, Wood KV, Fan F (2010) Improved dual-luciferase reporter assays for nuclear receptors. *Curr Chem Genomics* 4:43–49. doi:10.2174/1875397301004010043 [PubMed: 21687560]
231. Fan F, Wood KV (2007) Bioluminescent assays for high-throughput screening. *Assay Drug Dev Technol* 5 (1):127–136. doi:10.1089/adt.2006.053 [PubMed: 17355205]

Fig. 1:

Major steps in GPCR - G protein activation process. These molecular events are exploited to develop optical strategies to detect and quantify the resultant signaling. Image shows a homology model of a GPCR with its 7TM structure. Upon its activation by a ligand, the GPCR undergoes conformational changes (curved arrow). This results in heterotrimer ($G\alpha\beta\gamma$) dissociation, generating $G\gamma$ (purple structure - PDB ID: 3AH8) and $G\beta\gamma$ (yellow and blue structures - PDB ID: 3AH8). A variety of proteins (grey and blue) recognize the activated GPCR (grey arrow) as well as $G\alpha\text{GTP}$ (purple arrow) and $G\beta\gamma$ (blue arrows). Additionally, $G\beta\gamma$ reversibly-translocate to internal membranes (IMs) black arrows.

Fig. 2: Measurement of GPCR-G protein activation using G γ 9 assay.

A. Schematic representation of G $\beta\gamma$ translocation and G γ 9 assay. Red cylinder shows the fluorescence tag on G γ 9. **B.** A HeLa cell expressing GFP- γ 9 shows primarily a PM distribution (left). GFP- γ 9 translocate to IMs upon activation of endogenous α 2-AR with 100 μ M norepinephrine (Nor-epi). Addition of yohimbine (α 2-AR inhibitor) results in reverse translocation of G $\beta\gamma$ back to the PM. White arrow-IMs and Yellow arrow-PM. Plot shows GFP fluorescence dynamics on the PM (black curve) and IMs (red curve), indicating α 2-AR activation and subsequent deactivation. **C.** *Upper row:* A HeLa cell expressing blue opsin-GFP (not shown) and mCherry- γ 9 shows primarily a PM distribution (left) before opsins activation. Blue opsin was reconstituted with 11-cis retinal (50 μ M) prior imaging. Upon optical activation (OA) of blue opsin in a spatially confined region (blue boxed region) using 445 nm light, mCherry- γ 9 translocated from the PM (yellow arrow) to IMs (blue arrow) only in the light-exposed area (middle). Upon OA termination, mCherry- γ 9 translocated back to the PM (right image). *Bottom row shows* enlarged images of the optically activated subcellular region of the cells. Note the initially PM-bound G γ 9 translocation on OA and reverse upon OA termination. Plot shows the mCherry fluorescence in IMs of blue light exposed (red) and unexposed (black) areas of the cell, indicating localized signaling activation by blue opsin and signaling detection using G γ 9 assay. Scale bar –5 μ m.

Fig. 3:

Overview of single molecule detection of GPCR activation. The dye can be tethered to a location of interest in the GPCR. Orange color-excitation beams and the evanescent field at the interface, red color-TIRF emission.

Fig. 4: DAG sensor translocation.

The DAG sensor containing GFP fused to the C1 domain from PKC γ , translocate to the PM to interact with generated DAG, serving as a readout for monitoring DAG production.

Fig. 5 :
Overview of translocation-based PIP2 sensor, engineered using the PH domain of PLC δ 1.

Fig. 6 :
Interaction of Nb80 (purple) with activated $\beta 2$ -AR (gray), Mimicking GPCR-heterotrimer interaction. PDB ID: 3P0G

Fig. 7 :
Jablonski diagram showing Fluorescence (Förster) resonance energy transfer (FRET). A donor chromophore, in its electronic excited state may transfer energy to an acceptor chromophore through nonradiative dipole-dipole coupling.

Fig. 8: GPCR activation induced receptor conformational change.

Major conformational changes in transmembrane regions and intracellular loops of β_2 -AR (PDB ID: 3P0G) during activation shows a 9.5 Å (5.7 Å to 15.2 Å) distance change between Arg131 (red) and Leu272 (blue). Indicated is also the transmembrane (TM) helix 6 displacement, and the distance between Asn322 (yellow) and Cys327 (orange) reduces in 2.0 Å (6.3 Å to 4.3 Å) representing TM helix 7 conformational change relative to the inactive β_2 -AR structure (PDB ID: 2RH1).

Fig. 9 :
Overview of FRET between the tagged Ct of the receptor and the G protein γ subunit.

Fig. 10:
Overview of FRET detection of GPCR activation using receptor-β-arrestin interactions.

Fig. 11:
Overview of BRET detection of GPCR activation using receptor-GRK interactions.

Fig. 12: Schematic representation of the PKA-based cAMP probe.

YFP and CFP are tagged to the C and R subunits of PKA respectively. In low cAMP levels, GFP-tagged holotetrameric form of PKA exhibits maximum FRET as the two fluorescence proteins are close to each other. Increase in cAMP results in a FRET reduction as PKA changes its conformation. Binding of cAMP to the R subunit of PKA induces dissociation of C and R subunits, resulting in FRET reduction.

Fig. 13:

Overview of the FRET based PIP2 sensor. Left and right panels show sensor orientations before and after PIP2 hydrolysis.

Fig.14: Schematic representation of AKAR sensor for PKA.
Phosphorylated substrate binding region (blue circle) increases its affinity for phosphoamino binding domain (FHA1, orange), bringing donor (CFP) and acceptor (YFP) together, increasing the FRET.

Fig. 15: Principle of PathHunter β-arrestin assay.

Activation of β-galactosidase (ProLink) fused-GPCR results in β-arrestin recruitment to the phosphorylated Ct of the GPCR. This interaction generates functional β-galactosidase enzyme, which hydrolyzes the substrate resulting in chemiluminescence.

Fig.16: Principle of LinkLight assay.

β-arrestin fused with permutated luciferase (pLuc) fragments; CLuc and NLuc, linked via TEV protease cleavage sequence (ENLYFQR) interacts with the Tobacco Etch Virus protease (TEV)-fused phosphorylated C-terminus of GPCR upon ligand-induced receptor activation. The distance changes between the TEV-tagged GPCR and the pLuc leads to proteolytic cleavage of the pLuc, and the cleaved luciferase fragments refold, reconstructing the active luciferase through fragment complementation. This reconstituted luciferase-generated luminescence in the presence of a chemical substrate indicates activation-induced GPCR phosphorylation.

Fig. 17:
Detection of Gq-PCR activation using the split luciferase sensor. Here, PLC β -G α_q interaction is exploited to generate luminescence.

Fig. 18: Principle of GPCR activation induced reporter gene expression.

With GPCR activation, generated Gαs-GTP activates AC, inducing cAMP production. Downstream of cAMP, generation of cAMP response element-binding protein (CREB) transcription factor, its translocation to the nucleus and interactions with the cAMP response elements (CRE) increase the transcription of the reporter gene luciferase. The resultant luminescent signal signaling not only indicates transcription, but also captures GPCR-G protein signaling process.

Table 1:

FRET and BRET based biosensors

Biosensor Name	Target	Number of molecules	Physical type	Pubmed ID	Addgene plasmid #
Gai3 v1	Gai	Bimolecular or other	FRET	16371464	
Gai-Gβ1	Gai	Bimolecular or other	FRET	14673086	
Gai-Gγ2	Gai	Unimolecular	FRET	14673086	
Gai2 Sensor v2	Gai	Bimolecular or other	FRET	26799488	#69624
Gai1 Sensor v2	Gai	Bimolecular or other	FRET	26799488	#69623
RLucII-117-Gas + GFP10-GY1	Gas	Bimolecular or other	BRET	27499021	
Gas Sensor	Gas	Bimolecular or other	FRET	16963443	
Gaq Sensor (v2)	Gaq	Bimolecular or other	FRET	21619590	
Ga13 Sensor (v2)	Ga13	Unimolecular	FRET	29505611	#112933
HCN2-camps	cAMP	Unimolecular	FRET	17038640	
Epac1-camps	cAMP	Unimolecular	FRET	15231839	
Epac2-camps	cAMP	Unimolecular	FRET	15231839	
mICNBD-FRET	cAMP	Unimolecular	FRET	27003291	
CAMYEL	cAMP	Unimolecular	BRET	17283075	
BFP-PMCA-GFP	Ca ²⁺	Unimolecular	FRET	17901055	
AKAR4	PKA	Unimolecular	FRET		#61619
CKAR	PKC	Unimolecular	FRET	12782683	#14860

Table 2:

Optical techniques to study GPCR-G protein pathway signaling

Principle / design	Sensor / assay	Effectors assayed	Resolution and throughput
	Fluorescently tagged G β and G γ s [7,38]	GPCR and G proteins	Single cell and high throughput
	Mini G proteins[84,83]	GPCR	Single cell and high throughput
	Nanobodies [90]	GPCR	Single cell
	PKA-Ca[59]	Adenylyl cyclases	Single cell and high throughput
	C1 domains of PKC[78,77,79]	PLC β and DAG	Single cell
Protein translocation	PH domain of PLC δ [68,17]	PLC β and PIP2	Single cell
TIRF	AlexaFluor, Cy3, SNAP dyes tethered to GPCR[46,48,49]	GPCR	Single molecule
FRET and BRET	GPCR-G protein and G protein-G protein[145,146,35,150]	GPCR	Single cell and high throughput
	GPCR- β -arrestin [164,165,167]	GPCR- β -arrestin	Single cell and high throughput
	GPCR-GRK [172,174]	GPCR- β -arrestin	Single cell and high throughput
	Epac [56,181]	Adenylyl cyclases-cAMP	Single cell
	HCN2-camps [188]	Adenylyl cyclases-cAMP	Single cell
	CAMYEL [189]	Adenylyl cyclases-cAMP	High throughput
	Eevee-iAkt [196–198]	PKB	Single cell
	Cameleon [104,200]	Ca ²⁺	Single cell and subcellular
	AKAR[206,207]	PKA	Single cell and subcellular
	CKAR [77]	PKC	Single cell
Luminescence	PathHunter assay[110,216,217]	GPCR- β -arrestin	High throughput
	LinkLight assay [218]	GPCR- β -arrestin	High throughput
	Tango GPCR Assay[219–221]	GPCR-PLC β 3	High throughput
	Reporter gene expression[231,226]	Response elements (CRE, SRE, NFAT-RE)	High throughput