

Open Review

Tools for GPCR drug discovery

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G-protein-coupled receptors (GPCRs) mediate many important physiological functions and are considered as one of the most successful therapeutic targets for a broad spectrum of diseases. The design and implementation of high-throughput GPCR assays that allow the cost-effective screening of large compound libraries to identify novel drug candidates are critical in early drug discovery. Early functional GPCR assays depend primarily on the measurement of G-protein-mediated 2nd messenger generation. Taking advantage of the continuously deepening understanding of GPCR signal transduction, many G-protein-independent pathways are utilized to detect the activity of GPCRs, and may provide additional information on functional selectivity of candidate compounds. With the combination of automated imaging systems and label-free detection systems, such assays are now suitable for high-throughput screening (HTS). In this review, we summarize the most widely used GPCR assays and recent advances in HTS technologies for GPCR drug discovery.

Keywords: G-protein-coupled receptors (GPCRs); high-throughput screening; high-content screening; functional assay; G-protein-dependent assay; G-protein-independent assay; label-free assay; functional selectivity

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Introduction

G-protein-coupled receptors (GPCRs), also known as 7 transmembrane receptors, are the largest family of cell surface receptors and account for approximately 4% of the protein-coding human genome^[1]. They are activated by a wide variety of stimulants, including light, odorant molecules, peptide and non-peptide neurotransmitters, hormones, growth factors and lipids, and control a wide variety of physiological processes including sensory transduction, cell-cell communication, neuronal transmission, and hormonal signaling.

After agonist binding, activated receptors catalyze the exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) on the α -subunit of heterotrimeric G proteins (composed of α -, β -, and γ -subunits), which in turn engages conformational changes that lead to the dissociation of $G\alpha$ from the dimeric $G\beta\gamma$ subunits^[2]. GPCRs coupled to $G\alpha_s$ and $G\alpha_{i/o}$ proteins activate or inhibit, respectively, adenylylate cyclase, the enzyme responsible for converting adenosine triphosphate (ATP) to 3',5'-cyclic adenosine monophosphate (cAMP). cAMP serves as a second messenger that activates protein kinase A (PKA) and other downstream effectors (previously reviewed^[3]). GPCRs coupled to $G\alpha_{q/10}$ alternatively

activate phospholipase C β (PLC β), which catalyzes the formation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃). IP₃ then binds and opens the endoplasmic IP₃-gated calcium channel, causing the release of calcium into the cytoplasm. GPCRs coupling to $G\alpha_{12/13}$ further activate the guanine nucleotide exchange factor RhoGEF, which in turn activates the small G protein RhoA.

In the presence of continuous agonist stimulation, GPCRs are phosphorylated by specific GPCR kinases (GRKs), and the recruitment of β -arrestins to the phosphorylated GPCRs eventually terminates G protein signaling and leads to a coordinated process of receptor desensitization, inactivation and internalization^[3]. The β -arrestins also facilitate the formation of multi-molecular complexes and provide a means for G protein-independent signaling of GPCRs, including those involving mitogen-activated protein (MAP) kinases, receptor and non-receptor tyrosine kinases, phosphatidylinositol 3-kinases (PI3K) and others^[4].

Given their importance in health and disease, together with their potential for therapeutic intervention via using small molecules as regulators, GPCRs represent the largest family of druggable targets. These receptors are the target of >50% of the current therapeutic agents on the market, including more than a quarter of the 100 top-selling drugs, with profits in the range of several billion US dollars each year^[5]. Therefore, GPCR assay development and GPCR ligand screening remain

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the major focus of drug discovery research worldwide. Historically, radioligand binding assays with receptor-containing membranes were used to identify compounds that target GPCRs. However, binding affinity data do not tell us whether the compound is an agonist or an antagonist, or more importantly, the overall potency of a compound under physiological conditions. Efforts have been made in the past few decades to develop signaling-dependent cell-based functional assays to provide more accurate and comprehensive data of the compounds targeting GPCRs.

An ideal assay for GPCR ligand screening should be simple, nonradioactive, robust, homogenous, and easily adapted to a microtiter plate format (96-, 384-, or 1536-well) for robotic automation. Since GPCR signaling consists of a series of spatial and temporal events, another important consideration is whether to measure a proximal or distal signaling step after GPCR stimulation. Measurement of events proximal to receptor activation will reduce the incidence of false positives^[6]; however, moving down the signal transduction cascade will enhance the signal-to-noise ratio due to signal amplification. Many GPCRs also activate multiple signaling pathways. Biased signaling, a phenomenon in which certain agonists display better efficacies in activating one pathway over others, is another critical issue to consider in functional screen development^[4, 7]. If a functional assay capturing only one signaling pathway is selected for screening compound libraries, potentially valuable compounds could be missed if the compound does display biased activity. Therefore, multiplexing of signaling pathways or assays representing an overall cellular response may be used to resolve such problems. In this review, we summarize the most widely used GPCR assays and recent advances in high-throughput screening (HTS) technology for GPCR drug discovery (Table 1).

Receptor binding assay

Receptor binding assay can be used to characterize in great detail the interaction between receptor and its ligands, such as the intrinsic affinity of ligands to the receptor, association/dissociation rates, and the density of receptor in tissues or cells^[8]. Receptor binding assay is a cell-free method theoretically suitable for any GPCR screening without involving downstream signaling from the receptor. This type of assay can also obtain agonists and antagonists in one experiment, but without distinguishing whether the candidate compound is an agonist, antagonist, or inverse agonist. However, the availability of labeled ligands greatly limits the application of this assay. It is practically useless for GPCR deorphanization.

The first radioligand binding assay was performed in 1970 by Lefkowitz *et al* using a radiolabeled hormone to determine the binding affinity for its receptor^[9]. Since then, ³H- or ¹²⁵I-labeled ligands have been widely used to characterize the affinity of a compound for a target GPCR, while non-labeled compounds can be characterized by their ability to displace the binding of a radiolabeled molecule to the target (orthosteric agonists/antagonists) or to modulate the affinity of a radiolabeled molecule for the target (some allosteric mod-

ulators)^[10]. The traditional radioligand binding assays require washing and filtration steps, which can only be scaled down to a 96-well format.

A homogenous scintillation proximity assay (SPA), which can be easily scaled down and automated for HTS applications, was developed more recently (reviewed in^[11]). In SPA, only the radiolabeled molecules binding to the GPCR immobilized on the surface of SPA beads can activate the scintillation beads, which produce photons detectable with a scintillation counter. SPA thus allows binding reactions to be tested without washing or filtration steps. Although radioligand binding gives a clear, unmistakable signal, radioligands are relatively expensive, problematic to dispose of, and some isotopes have inconveniently short half-lives. These drawbacks have led to the creation of highly sensitive nonradioactive alternatives.

Many new binding assays are based on time-resolved fluorescence resonance energy transfer (TR-FRET) technology, such as DELFIA™ TRF from PerkinElmer, the LanthaScreen™ system from Invitrogen, and the Tag-lite™ system from Cisbio. Tag-lite™ is a newly developed homogeneous time-resolved fluorescence (HTRF) technology^[12] for assaying GPCR ligand binding in HTS format. A suicide enzyme (either SNAP- or CLIP-tag) is fused to the N-terminus of a GPCR without affecting its binding and activity, and a non-permeant substrate labeled with terbium cryptate fluorophore (Lumi4-Tb) is used to specifically and covalently label the receptors expressed on the cell surface. The ligands are labeled with red or green acceptors. The long fluorescence lifetime of the terbium cryptate allows a time-resolved measurement of FRET emission when all natural fluorescence disappears. The assay is carried out in a “mix and measure” format, which can be used not only for ligand binding studies but also for receptor activity analysis and GPCR dimerization assessment (as discussed below). The availability of Tagged-GPCR expressing cell lines and the fluorophore-labeled ligands are some of the limitations in this approach.

G-protein dependent functional assays

Ligand-binding assays are useful to identify new compounds that target GPCRs. Further analysis of the biological responses after compound binding will help complete the whole picture concerning the overall characteristics of the compound. Upon ligand binding, GPCRs change their conformation and activate coupled G proteins, which subsequently promote second messenger production via downstream effectors. The corresponding assays measuring either G protein activation or G protein-mediated events, including second messenger generation and reporter activation, are therefore defined as G-protein-dependent functional assays (Figure 1).

GTPγS binding assay

GTPγS binding assays directly measure the guanine nucleotide exchange of G proteins, an early event after GPCR activation, which is not subjected to amplification or regulation by other cellular processes^[6]. Typically, the accumulation of non-hydrolysable GTP analog, such as [³⁵S]-GTPγS, on the plasma

Table 1. Summary of GPCR screening technologies.

Assay classification	Commonly used assays	Commercial HTS Technology (Company)	Pros	Cons	Suitable GPCRs
Receptor binding assay	Radioligand binding assay	<ul style="list-style-type: none"> Filtration assay (PerkinElmer) SPA (GE Healthcare or PerkinElmer) 	High-throughput; less interference; obtain agonist and antagonist in one assay	Availability of radioligand; generation of radioactive waste; need secondary functional assay	any GPCR
G-protein dependent assays	Other tagged-ligand binding	<ul style="list-style-type: none"> DELFITM TRF (PerkinElmer); LanthaScreenTM system (Invitrogen); Tag-liteTM system (Cisbio) 	Non-radioactive; high-throughput; obtain agonist and antagonist in one assay	Availability of tagged-ligand; need secondary functional assay	any GPCR
	GTPγS binding	<ul style="list-style-type: none"> Filtration assay (PerkinElmer) SPA (GE Healthcare or PerkinElmer) DELFITM GTP Binding Assay (PerkinElmer) 	Functional cell-free assay; discrimination between full or partial agonists, neutral antagonists, inverse agonists, allosteric regulators	Relatively low signal to background window	Gα _{1/o}
CRE/MRE reporter assay	CRE/MRE reporter assay	<ul style="list-style-type: none"> SPA cAMP assay (GE Healthcare or PerkinElmer) HitHunterTM (DiscoverX); AlphaScreenTM (PerkinElmer); Fluorescence polarization-based cAMP kits (PerkinElmer, Molecular Devices and GE Healthcare); HTRFTM-based cAMP (Cisbio); cAMP GlosensorTM (Promega) 	Homogenous assay; high-throughput; large signal to background window	Need to know the coupling mechanism, not good for orphan GPCR	any GPCR
	cAMP assay	<ul style="list-style-type: none"> SPA cAMP assay (GE Healthcare or PerkinElmer) HitHunterTM (DiscoverX); AlphaScreenTM (PerkinElmer); Fluorescence polarization-based cAMP kits (PerkinElmer, Molecular Devices and GE Healthcare); HTRFTM-based cAMP (Cisbio); cAMP GlosensorTM (Promega) 	High-throughput; very sensitive	Need to know the coupling mechanism, not good for orphan GPCR	Gα _s , Gα _{1/o}
Ca ²⁺	Ca ²⁺	<ul style="list-style-type: none"> Fluo-3 or Fluo-4 (Invitrogen) and FLIPRTM automated real-time fluorescence plate readers (Molecular Device) 	High-throughput; functional assay for live cells; obtain agonist, antagonist and allosteric modulator in one assay	Fluorescent interference from compounds; not good for inverse agonist and slow binding agonist	Gα _q (with Gα _{15/16}); could be universal)
	IP _{1/3}	<ul style="list-style-type: none"> SPA IP₃ assay (PerkinElmer) AlphaScreenTM (PerkinElmer); HitHunterTM Fluorescence Polarization (DiscoverX); HTRF IP-OneTM (Cisbio) 	High-throughput; functional assay for live cells; good for slow binding ligands	Limited industrial validation	Gα _q (with Gα _{15/16}); could be universal)
G-protein independent assays	Receptor trafficking	<ul style="list-style-type: none"> Cellomics ArrayScanTM (Thermo Scientific); INCellTM Analyzer 3000 (GE Healthcare); OperaTM (Evotec Technologies); AcumenTM Explorer (TTP Lab Tech) 	Image based, high-content; functional assay for live cells; generic method for all GPCRs	Image based, relatively low throughput	any GPCR
	β-Arrestin recruitment assay	<ul style="list-style-type: none"> TransfluorTM (Molecular Device); BRET/FRET TangoTM (Invitrogen); PathHunterTM (DiscoverX); 	High-throughput; image or non-image based; functional assay for live cells; useful in biased signal detection; generic method for all GPCRs	Affinity for β-arrestin binding varies among GPCRs; less sensitive; need further pathway analysis	any GPCR
Label-free assay	Label-free assay	<ul style="list-style-type: none"> BINDTM RWG biosensors (SRU Biosystems) EpicTM RWG biosensors systems (Corning Inc); ECISTM (Applied Biophysics); xCELLigenceTM System (Roche and ACEA Biosciences) CelliKeyTM (MDS) 	Label-free functional assay in native live cells; summation of all cellular events	Possibly higher false positive and false negative rates; need special instrument and costly microplate; need further pathway analysis	any GPCR
	Receptor dimerization assay	<ul style="list-style-type: none"> BRET/FRET; PathHunterTM (DiscoverX); Tag-liteTM (Cisbio) 	GPCR heterodimers are considered new pharmacological targets	Very artificial system, can not assess GPCR dimerization in native state	any GPCR

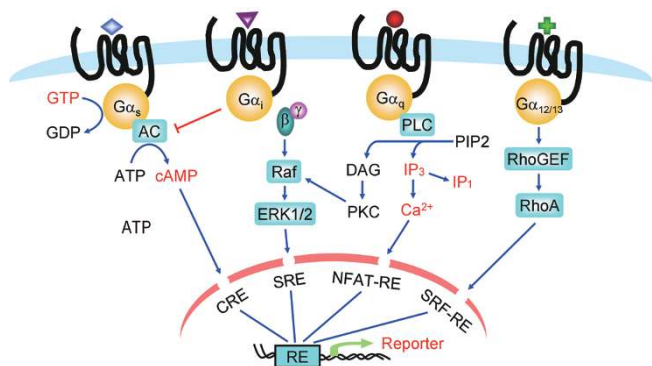


Figure 1. Receptor binding and G-protein-dependent assays. Schematic representation of receptor binding and major pathways activated by different G proteins. Red indicates the detection points of commonly used HTS assays: GTP γ S binding, cAMP detection, IP $_3$ /IP $_1$ detection, Ca $^{2+}$ flux and reporter expression.

membrane prepared from cells expressing GPCRs of interest is measured after agonist stimulation. Unlike receptor binding assays, the GTP γ S binding assay allows discrimination between full or partial agonists, neutral antagonists, inverse agonists, and allosteric regulators^[13]. In reality, this assay is experimentally more feasible for receptors coupled to G $\alpha_{i/o}$, which is the most abundant G protein in many cells and has a faster GDP-GTP exchange rate than other G proteins^[6]. Nevertheless, [35 S]-GTP γ S binding assays can also be used with GPCRs that couple to the G α_s and G α_q families of G proteins, especially in artificial expression systems, or using receptor-G α chimeras, or by immunoprecipitation of [35 S]-GTP γ S-labeled G α (reviewed in^[13]). A problem with this assay is that it requires a filtration step through glass fiber to separate free and bound [35 S]-GTP γ S, which limits assay throughput. With the development of SPA technology, the filtration step can be omitted and GTP γ S binding assays can be adopted for high-throughput screening^[14, 15].

With the increased desire to move assays to a non-radioactive format, a GTP binding assay based on time-resolved fluorescence (TRF) technology utilizing a non-radioactive, non-hydrolysable, europium-labeled GTP analog, GTP-Eu, has been developed for GPCR screening (DELFIATM GTP binding assay from PerkinElmer)^[16]. However, this assay still requires filtration and washing steps. The DELFIATM GTP binding assay has been validated on several GPCRs, including α_2 -adrenergic^[17], neuropeptide FF2 receptor^[18], dopamine D3 receptor^[19] and muscarinic receptors^[20], and the results are comparable with those obtained using traditional [35 S]-GTP γ S binding assays^[21].

cAMP assay

Assays measuring cellular levels of cAMP are dependent on the activity of adenylyl cyclase, which is regulated by GPCRs coupled to G α_s or G $\alpha_{i/o}$ protein. G α_s positively stimulates the activity of adenylyl cyclase, resulting in increased cellular cAMP. In contrast, activation of G α_i leads to a negative

regulation of adenylyl cyclase and a decrease in cAMP levels. Screening G α_s -coupled receptors is generally straightforward, whereas screening G $\alpha_{i/o}$ -coupled receptors, especially for G $\alpha_{i/o}$ -coupled receptor antagonists, could be extremely difficult to achieve with high precision using cAMP detection methods. This difficulty arises because of the requirement to pre-stimulate adenylyl cyclase with forskolin, which should be titrated during assay optimization, to inhibit the response with agonist and then measure reversal of the agonist effect with antagonists. In addition, to counteract the natural degradation of cAMP to AMP by phosphodiesterase (PDE) enzymes, an inhibitor of PDE (eg, IBMX) might be required in the system during assay optimization. cAMP levels are typically measured using a competition assay in which cellular cAMP competes with an introduced, labeled form of cAMP for binding to an anti-cAMP antibody.

Radiometric assays, such as the SPA cAMP assay from GE Healthcare and the FlashPlateTM cAMP assay from PerkinElmer using 125 I-labeled cAMP, are widely used. More recently, these assays have been replaced with fluorescence- or luminescence-based homogenous assays to avoid the use of radioactivity. There are several newer radio-free approaches for cAMP detection. One such assay is based on Enzyme Fragment Complementation (HitHunterTM) and was introduced by DiscoverX (<http://www.discoverx.com>). Cellular cAMP competes with cAMP labeled with a small peptide fragment of β -galactosidase for binding to an anti-cAMP antibody. The resulting free labeled-cAMP complements with the enzyme fragment, producing active β -galactosidase, which is detected with fluorescent or luminescent substrates^[22, 23]. AlphaScreenTM from PerkinElmer is a sensitive bead-based proximity chemiluminescent assay. Cellular cAMP competes with a biotinylated cAMP probe recognized by a streptavidin donor and anti-cAMP antibody-conjugated acceptor beads. Release of the biotinylated cAMP from the antibody results in the dissociation of the streptavidin donor from its acceptor, which can be measured as a decrease in the chemiluminescent signal (<http://www.perkinelmer.com>). In addition, fluorescence polarization (FP)-based cAMP kits are available from PerkinElmer, Molecular Devices and GE Healthcare. When exposed to polarized light, the emission from an antibody-bound fluorescent-labeled cAMP is also polarized due to restricted molecular rotation. When the labeled cAMP is replaced on the antibody by cellular cAMP, the emission becomes more depolarized because it can rotate freely in solution. With the availability of red-shifted fluorophores, the signal-to-noise ratios have been greatly improved^[24]. Furthermore, HTRF-based cAMP detection is available from Cisbio. With this method, novel donor (cAMP antibody labeled with europium cryptate) and acceptor (cAMP labeled with a modified allophycocyanin dye) pairs are designed to increase the stability of the signal and make this assay highly sensitive and reproducible for cAMP measurement^[12]. Finally, the cAMP GlosensorTM assay is a luciferase biosensor-based assay from Promega^[25]. Upon cAMP binding, the conformational change in the biosensor leads to the activation of luciferase and increased light output.

This type of assay can be used to measure GPCR function in a non-lytic live-cell format, enabling facile kinetic measurements of cAMP accumulation or turnover in living cells. The assay also offers a broad dynamic range, showing up to 500-fold changes in light output. Extreme sensitivity allows the detection of $G\alpha_i$ -coupled receptor activation or inverse agonist activity in the absence of artificial stimulation by compounds such as forskolin (<http://www.promegea.com/glosensor>). A direct comparison of AlphaScreenTM, HTRF, HitHunterTM, and FP cAMP assays suggests that there are advantages and disadvantages in each method^[26]. The AlphaScreenTM and HTRF assays are recommended for cells expressing low levels of GPCRs because of their higher sensitivities.

IP₃/IP₁ and Ca²⁺ assays

Stimulation of $G\alpha_q$ or $G\alpha_i$ coupled-GPCRs activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol biphosphate (PIP₂) to form two second messengers, inositol 1,4,5-triphosphate (IP₃) and DAG. While DAG activates protein kinase C (PKC), IP₃ activates the IP₃ receptor on the endoplasmic reticulum (ER) resulting in an efflux of Ca²⁺ from the ER to the cytoplasm and an elevation of intracellular Ca²⁺. IP₃ is very rapidly hydrolyzed to IP₂, then to IP₁ and finally to inositol by a series of enzymatic reactions^[27]. The radioactive IP₃ assay measures ³H-inositol incorporation and is a traditional assay for the assessment of PLC activity, but it is not suitable for the screening of large compound collections because it requires a cell wash step and generates radioactive waste. An SPA platform has been developed to achieve higher throughput and homogenous assays in measuring IP accumulation.

There are a few non-radiometric technologies used for the measurement of IP₃, including AlphaScreenTM (PerkinElmer) and HitHunterTM Fluorescence Polarization (DiscoverX) assay. Recently, a homogeneous, non-radioactive TRF assay for measuring IP accumulation, IP-One HTRFTM, was released by Cisbio (<http://www.htrf.com>). The basis for the assay is a reduction in energy transfer between acceptor IP₁ and a europium-conjugated IP₁ antibody as cellular IP₁ accumulates and replaces the acceptor IP₁ in binding the IP₁ antibody. Compared to earlier kits using IP₃-binding proteins to specifically measure IP₃, the IP-One assay takes advantage of the fact that LiCl inhibits the degradation of IP₁, the final step in the inositol phosphate cascade, allowing it to accumulate in the cell and to be measured as a substitute for IP₃. This assay also does not require a kinetic readout. Data reported by Cisbio show that the assay can be used with endogenously or heterologously expressed GPCRs in either adherent or suspension cells to quantitate the activity of agonists, antagonists, and inverse agonists. Additionally, the efficacy data from the IP-One assay correlate well with those from calcium assays and traditional IP₃ assays (<http://www.htrf.com>). This assay can be adopted for ultra high-throughput screening in the 1536-well plate format and has been tested with cell lines expressing M₁ acetylcholine, FFAR1, vasopressin V1b, and neuropeptide S receptors^[28].

As previously mentioned, intracellular Ca²⁺ is another second messenger for GPCR signaling. GPCRs that naturally couple to $G\alpha_q$ produce a ligand-dependent increase in intracellular Ca²⁺. However, $G\alpha_i$ /*or* $G\alpha_s$, or $G\alpha_{12}$ -coupled GPCRs can also be “switched” to induce an increase in intracellular Ca²⁺ either by the expression of a chimeric G-protein ($G\alpha_{q15}$ or $G\alpha_{q05}$) or a promiscuous G-protein ($G\alpha_{16}$ or $G\alpha_{15}$) (reviewed in^[29]). The Ca²⁺ assay is very popular in GPCR screening owing to the availability of cell-permeable Ca²⁺-sensitive fluorescent dyes (such as Fluo-3 and Fluo-4) and automated real-time fluorescence plate readers, such as FLIPRTM from Molecular Device. Molecular Device also offers fluorescent dye kits, which contain proprietary quenching molecules that allow cellular loading of dye without the need of subsequent cell washing to remove excess dye^[30]. The integrated pipetting capabilities of the FLIPRTM allow ultra high-throughput screening in 384- or 1536-well format with the ability to detect agonists, antagonists, and allosteric modulators all in one assay. The use of fluorescent dyes can also be replaced by the use of Ca²⁺-sensitive biosensors. Recombinant expression of the jellyfish photoprotein aequorin, which provides an intense luminescent signal in response to elevated intracellular Ca²⁺ in the presence of a coelenterazine derivative, has also been developed for functional screens of GPCRs^[31]. With the overexpression of promiscuous G-proteins, the Ca²⁺ assay does not require the prior knowledge of G protein coupling conditions and signaling pathways of the receptor, so it is widely used to de-orphan GPCRs^[32,33].

Although Ca²⁺ assays are robust and easily amenable to HTS, there are some important shortcomings. They cannot be used to screen for inverse agonists because increases in basal Ca²⁺ are not observed in cells expressing constitutively active $G\alpha_q$ -coupled receptors. In addition, calcium flux occurs rapidly and transiently and is not suitable to detect slow binding agonists. In such conditions, an IP₁ accumulation assay will be more useful. Furthermore, false positive signals obtained from fluorescent and nuisance compounds are a problem, and the sensitivity is often insufficient to allow the use of primary cells.

Reporter assay

GPCR activation is well known to alter gene transcription via responsive elements for second messengers including the cAMP response element (CRE), the nuclear factor of activated T-cells response element (NFAT-RE), the serum response element (SRE) and the serum response factor response element (SRF-RE, a mutant form of SRE), all of which are located within the gene promoter regions (reviewed in^[34]). Therefore, cell-based reporter assays provide another popular and cost-effective HTS platform for GPCR screening. Reporter gene constructs usually contain second messenger responsive elements upstream of a minimal promoter, which in turn regulate the expression of a selected reporter protein. Commonly used reporters are enzyme proteins with activities linked to a variety of colorimetric, fluorescent or luminescent readouts, such as luciferase, alkaline phosphatase, β -galactosidase,

β -lactamase and a variety of fluorescent proteins. Among them, luciferase is the reporter of choice, especially in high-throughput screening due to its sensitivity, broad dynamic range, lack of endogenous activity and low interference coming from the compounds^[35].

The advantages of reporter gene assays include the wide linearity and sensitivity of the technique and a large signal-to-background ratio, making them suitable for the detection of weak GPCR agonists or allosteric modulators. Reporter gene assays are also easy to set up and can be scaled down to extremely low assay volumes in 1536- or 3456-well formats. Despite these advantages, some concerns have been raised, such as the requirement for long incubation periods, difficulty in antagonist detection due to reporter accumulation and the higher potential for false positives because the signal event is distal from receptor activation. Concerns about the long incubation time and accumulation of reporter have been addressed through the use of destabilized reporters (available from Promega). The higher false positive rate due to the distal signaling event could be partially resolved with the co-expression of a constitutively expressed internal control^[35], so compounds nonspecifically affecting gene transcription could be ruled out. Chen *et al* demonstrated that by combining pathway-specific reporter assays, all four major G protein subfamilies and downstream pathways could be studied in one luciferase reporter assay format. This combination could help establish receptor-G protein profiles for specific receptors and aid drug screening for pathway-specific GPCR modulators^[34].

Generic G-protein independent functional assays

Receptor internalization assay

The concept of the GPCR internalization assay is based on the common phenomenon of GPCR desensitization, which has been demonstrated for numerous GPCRs (reviewed in^[36]). In the desensitization process, GRKs phosphorylate agonist-activated GPCRs on specific serine and threonine residues, and cytosolic β -arrestins are recruited to the cell membrane by GRK-phosphorylated GPCRs. β -Arrestins uncouple GPCRs from their cognate G proteins and target the receptors to clathrin-coated pits for endocytosis. With the development of image-based, high-content screening (HCS) systems, the internalization of GPCRs is now a quantifiable process.

To date, most high-throughput, cell-based screens have been whole-well cell assays that quantify one molecular event or provide a single readout of a complex biological process (*eg*, measurement of intracellular Ca^{2+} concentration for $\text{G}\alpha_q$ -coupled GPCR activation) – they have been one-dimensional. HCS is a relatively new technology, introduced approximately 10 years ago, that combines high-resolution fluorescence microscopy with automated image analysis (reviewed in^[37]). This technology offers multi-dimensional or multi-parametric readouts by monitoring various biomolecules labeled with different fluorophores. The multi-parametric data generated by HCS can also provide temporal and spatial information for biomolecules, thus enabling a more sophisticated understanding of responses in the cell after stimulation. There are

a few HCS systems currently available; some are based on automated epi-fluorescent microscopes, such as the Cellomics ArrayScan™ Series from Thermo Scientific (<http://www.thermo.com.cn/HCS>), while others are equipped with confocal optics, such as the INCell™ Analyzer 3000 from GE Healthcare and Opera™ from Evotec Technologies. More recently, the development of a laser-scanning fluorescence microplate cytometer, for example, the Acumen™ Explorer from TTP Lab Tech, offers even higher throughput in these multiplexing assays.

In contrast to the aforementioned Ca^{2+} , cAMP and reporter assays, the internalization assay is independent of the associated G protein subclass or individual GPCR intracellular signaling pathway. Thus, there is no need to have prior knowledge of the signaling pathways of a GPCR before using this assay. The internalization assay is particularly useful for de-orphaning GPCRs, while the imaging-based GPCR internalization assays also offer the general advantage of the HCS format.

There are several ways to monitor the internalization process of GPCRs. For GPCRs with known ligands, fluorophore-labeled specific ligands can be used to detect the internalization of the receptors. Fluorescent ligands have been used since the mid-1970s. When coupled to the growing exploitation of imaging-based HCS analysis, it is clear that fluorescent molecules offer a safer, more powerful and more versatile alternative to traditional radioligand binding assays^[38]. Another way to visualize the internalization of a GPCR is to co-internalize a specific antibody, directed either against an extracellular domain of the receptor or against an N-terminal epitope tag^[39]. The primary antibody is co-internalized with the receptor upon agonist stimulation and then detected with a fluorophore-labeled secondary antibody. Considering the cost of antibodies and the tedious procedure of immunofluorescent staining, this method is commonly used for GPCR signaling studies but not large-scale drug screening. GPCRs tagged with fluorescent proteins (GFP or RFP) are the most common setup for HCS of receptor internalization (Figure 2) and are widely used for receptor deorphanization. When internalized, these tagged-GPCRs form grain-like objects within the cells. The Spot detector bio-application in the ArrayScan™ system or the granularity analysis module of the INCell™ Analyzer can identify, analyze and quantify these grain-like structures^[40, 41]. With the EGFP-tagged GPR120 internalization assay, Hirasawa and colleagues de-orphaned this receptor and found that unsaturated long-chain free fatty acids activate GPR120 and promote GLP-1 secretion^[42].

β -arrestin recruitment assay

β -arrestins are cytosolic proteins that bind to ligand-activated GPCRs, uncouple the receptors from G proteins and target the receptors to clathrin-coated endocytic vesicles. β -arrestin recruitment is a ubiquitous mechanism of negative regulation of GPCR signaling that has been demonstrated for almost all GPCRs^[43]. More recently, β -arrestins have themselves been shown to act as signaling scaffolds for numerous pathways,

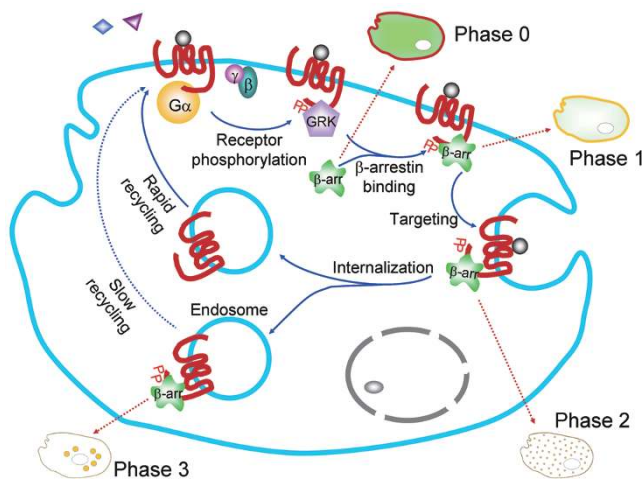


Figure 2. TransfluoR™ and receptor internalization assays. Schematic representation of TransfluoR™ β-arrestin recruitment assay multiplexed with a receptor internalization assay. The receptor is tagged at the C-terminus with RFP, and β-arrestin is tagged with GFP. In the resting state, the receptor is located on the cell membrane and β-arrestin is localized in the cytoplasm (Phase 0). A few seconds to several minutes after stimulation, the receptor is phosphorylated by GRK and β-arrestin is recruited to the cell membrane (Phase 1). β-arrestin binding leads to the internalization of the receptor-β-arrestin complex, initially forming internalization pits (Phase 2). For fast-recycling GPCRs, the receptor dissociates with β-arrestin and returns to the plasma membrane. For slow-recycling GPCRs, the receptor-β-arrestin complex traffics towards the endosome and forms large vesicles (Phase 3).

such as c-Src, ERK 1/2, and Akt, in a G protein-independent manner^[4]. G protein and β-arrestin pathways were found to be distinct and could be pharmacologically modulated independently with “biased ligands”^[44]. Therefore, β-arrestin-based assays are more interesting, potentially providing new insights into the functional selectivity (biased agonism) of GPCR signaling. These insights may help eliminate undesirable side effects by activating certain pathways but not others. Therefore, β-arrestin recruitment assays provide novel, universal, and G-protein independent ways for GPCR screening and drug discovery. Such assays are particularly useful for the screening of Gα_i-coupled GPCRs, which traditionally suffer from a small assay window in second messenger detection systems, and orphan GPCRs.

The first commercialized β-arrestin recruitment assay, TransfluoR™, was originally licensed by Norak Biosciences in 1999 from Duke University Medical Center and is now available from Molecular Devices. The TransfluoR™ assay is performed using GFP-tagged β-arrestin. The redistribution of diffuse β-arrestin-GFP from the cytoplasm to agonist-occupied receptor-containing pits or vesicles can be monitored quantitatively with a high content imaging system, such as the INCell™ Analyzer, ArrayScan™, Acumen™ or ImageXpress™^[45–47]. Taking advantage of these HCS instruments, the TransfluoR™ assay provides robust high-throughput screening for compounds targeting GPCRs^[48, 49]. The benefits of this assay include the

following: (1) no fluorescent dyes or secondary substrates are required; (2) the cell imaging assay allows parallel detection of putative compound liabilities, such as cytotoxicity; (3) visualization of β-arrestin localization (to the membrane or to the vesicles) and (4) multiplexing with the receptor internalization assay (Figure 2) and providing additional information regarding drug effect with respect to ligand-induced trafficking. Like any other imaging-based assays, to obtain high quality images for further software analysis, the cell type used should be grown in a monolayer, have good adherence properties and have a large cytoplasm-to-nucleus ratio. Before conducting a HTS screening for orphan GPCRs, a ligand independent translocation assay (LITE™), which utilizes a constitutively active GRK2 to phosphorylate the receptor and initiate GPCR-β-arrestin interaction, is required to verify that the receptor can indeed recruit β-arrestin-GFP after stimulation^[50].

Alternatively, several non-imaging-based β-arrestin recruitment assays, such as Bioluminescence Resonance Energy Transfer (BRET), PathHunter™ technology (DiscoverX) and the Tango™ assay (Invitrogen), are available. The BRET assay was one of the earliest approaches utilized for assessing GPCR-β-arrestin interactions and can be scaled for HTS^[51, 52]. The receptor of interest is tagged at the C-terminus with a fluorescent protein tag (such as eGFP2, GFP10 or YFP) and the β-arrestin is tagged with a *Renilla* luciferase (RLuc) or vice versa. Upon β-arrestin recruitment, the two tags come into close proximity and the light emitted from the RLuc reaction excites the GFP, which then emits a detectable signal at a higher wavelength (Figure 3A). BRET is calculated as the ratio of the two emissions (GFP/RLuc). It was reported that increased BRET sensitivity can be achieved by using RLuc8/YPet and RLuc8/RGFP as donor/acceptor couples^[53].

Invitrogen’s Tango™ GPCR Assay System is a platform based on a protease-activated reporter gene (Figure 3B). β-arrestin is fused to a TEV protease, while GPCR is extended at its C-terminus with a protease cleavage site followed by the transcription factor Gal-VP16^[54]. Upon GPCR activation, protease-tagged arrestin is recruited to the receptor and the Gal-VP16 that is fused to the receptor is cleaved and enters the nucleus to regulate the transcription of a β-lactamase reporter gene. The β-lactamase catalyzes the cleavage of a modified substrate tagged with two fluorophores, and the change in FRET signal between these two fluorophores can be monitored. Tango™ GPCR assays have been validated for a diverse array of GPCRs, including receptors related to each of the major G protein pathways and activated by a variety of ligand types^[55, 56].

The PathHunter™ assay developed by DiscoverX utilizes enzyme fragment complementation of β-galactosidase and subsequent enzymatic activity to measure receptor-β-arrestin interactions (Figure 3C). In this assay, β-arrestin is fused to an N-terminal deletion mutant of β-galactosidase that is catalytically inactive, and GPCR is tagged at the C-terminus with a small (4 kDa) fragment derived from the deleted N-terminal sequence of β-galactosidase (ProLink™). Upon GPCR-β-arrestin interaction, the two parts of β-galactosidase

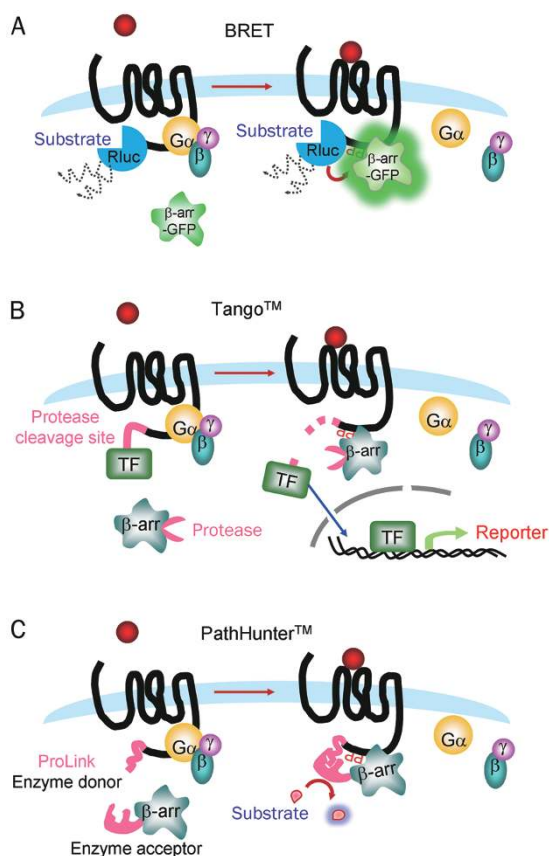


Figure 3. Non-imaging-based β -arrestin recruitment assays. (A) BRET assay. The GPCR is tagged with a RLuc, and β -arrestin is tagged with GFP, or vice versa. Upon β -arrestin recruitment, the two tags come into close proximity and the light emitted from the RLuc reaction excites the GFP, which then emits a detectable signal at a higher wavelength. (B) TangoTM assay. β -arrestin is fused to a protease, while GPCR is extended at its C-terminus with a protease cleavage site followed by a transcription factor (TF). Upon β -arrestin recruitment, the TF fused to the receptor is cleaved and enters the nucleus to regulate the transcription of a reporter gene. (C) PathHunterTM assay. β -arrestin is fused to a deletion mutant of β -galactosidase that is catalytically inactive, and GPCR is tagged with a small fragment derived from the deleted sequence of the enzyme (ProLinkTM). Upon GPCR- β -arrestin interaction, the two parts of β -galactosidase are brought into close proximity, which results in cleavage of the substrate and generation of a chemiluminescent signal.

are brought into proximity, which results in the activation of the enzyme, cleavage of the substrate and generation of a chemiluminescent signal. Similar to TangoTM GPCR assays, PathHunterTM has also been validated for a diverse array of GPCRs and is widely accepted in industrial and academic drug-screening laboratories^[57, 58]. Both assays also have commercially available, assay-ready cell lines for a large number of GPCRs. There are several advantages in the PathHunterTM assay, including a homogeneous assay with enzyme-amplified robust signal. The chemiluminescent signal is also low in background and resistant to interference from fluorescent compounds. One disadvantage of this platform is that the time window for measurement is limited, so the assay only

captures a snapshot of β -arrestin-receptor binding during the period of substrate incubation. However, this limitation can also be viewed as an advantage when using instruments capable of real-time detection to study the kinetics of GPCR- β -arrestin interaction.

Label-free whole cell assays

Label-free technologies, which emerged within the past few years, have the potential to substantially change some aspects of whole-cell assays, including GPCR screening (reviewed in^[59]). Many assays widely employed for GPCR ligand discovery tend to provide reductionist views of cell signaling. These assays are extremely robust and have been successfully implemented for measuring one particular functional activity of GPCRs, but often fail to account for the summation of events associated with the activation of one or more receptors. The emergence of label-free technologies presents a different strategy in measuring signal transduction, with integrated or cumulative responses rather than the resolution of individual events. Label-free, whole cell assays generally employ a biosensor that converts the summation of ligand-induced changes in living cells to optical, electrical, calorimetric, acoustic, magnetic or other quantifiable signals. They can detect changes in cellular features including adhesion, proliferation, migration, and cell death.

Most of the label-free instruments available for GPCR screening use either an optical or electrical biosensor to detect cellular changes after stimulation. The optical biosensors are also called resonant waveguide grating (RWG) biosensors, which utilize grating structures embedded in the bottom of microtiter plates (Figure 4B). When illuminated with broadband or monochromatic light, these grating surfaces reflect a narrow band of light that is characteristic of the refractive index near the grating surface. These instruments can detect

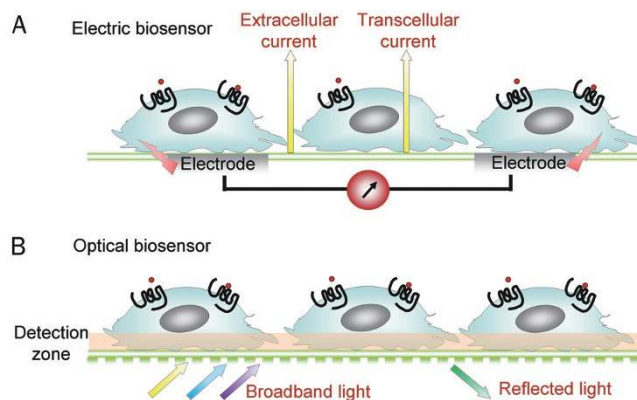


Figure 4. Principles of the biosensors for label-free, whole-cell detection. (A) Schematic representation of an electrical biosensor. Cells are cultured on the surface of arrayed gold microelectrodes. A low AC voltage at variable frequencies is applied to the cell layer and both the extracellular current and transcellular current are measured. (B) Schematic representation of an optical biosensor. Cells are cultured on the surface of the biosensor with an embedded grating structure. Only the mass redistribution within the bottom portion of cells is directly measured.

either angle- or wavelength-shift^[60]. The refractive index is influenced by the physical properties of the cell layer in contact with the grating surface. When a GPCR is activated and subsequent signal transduction changes the biomolecular concentration within an approximately 200 nm range of the contact surface, the disturbance in local refractive index can be detected as a shift in resonant angle or wavelength. The current optical-based instruments include BINDTM (SRU Biosystems) and EpicTM (Corning Inc) systems. Although initially designed for *in vitro* molecular binding assays, both systems were successful in studying cell morphological changes and GPCR signaling^[61, 62].

Electrical biosensors, also known as impedance-based biosensors, mainly consist of a substrate, an electrode and a cell layer in close contact with the electrode (Figure 4A). Giaever and Keese of GE first reported the use of impedance to measure cellular processes^[63]. In their early studies, fibroblasts cultured on thin-film gold electrodes were found to impede the flow of a very weak alternating current. The impedance change could be monitored in real-time, and the fluctuation of impedance was dependent on ATP concentration and actin polymerization, and was thus linked to cellular motion^[64]. Since then, electrical-based detections have been applied to study a wide variety of cellular events, including cell adhesion and spreading^[65], cell morphological changes^[66], and cell death^[67]. It is now generally accepted that the impedance value is the sum of cellular events, including the relative density of cells over the electrode surface and the relative adherence of these cells.

Applied Biophysics launched ECISTM, the first commercially available instrument for electrical-based whole cell detection with high-throughput capability (up to 96-well detection), in 1995. The more recently available (2008) xCELLigenceTM System from Roche Applied Science and ACEA Biosciences is also built to fit into cell culture incubators and to measure long-term cellular responses that occur over hours to days. Both systems have been used to detect GPCR activity for hours^[68, 69]. A more high-throughput system, CellKeyTM, was developed by MDS Analytical Technologies, which is designed to detect acute cellular responses in 96- and 384-well formats.

Based on both the CellKeyTM and EpicTM systems, scientists observed distinct response profiles depending on the G-protein pathway activated^[62, 70, 71]. Several studies compared the EpicTM system and traditional Ca²⁺ or cAMP assays using CHO cells expressing the muscarinic M2, M3 or dopamine D3 receptors. Most of the results from the EpicTM were consistent with data from cAMP or Ca²⁺ readouts with few exceptions, and the EpicTM was found to detect weak activity that was not observed with the label-based assays^[72, 73]. Many studies with GPCR ligand sets also demonstrated similar rank-order potency values between CellKeyTM impedance and traditional Ca²⁺ or cAMP assays^[74-76].

The sensitivity, precision and high-throughput of some label-free instruments warrant their use in HTS, although the cost of consumables might limit broader application. These systems have also been used for ligand selectivity studies^[76-78],

endogenous receptor profiling in cell lines commonly used in drug discovery^[62, 74, 77], systemic cell biology studies of GPCR signaling^[77, 79], and many other aspects of GPCR research. Like any other functional assay, label-free, whole cell assays are also prone to false positives. Another unique problem associated with label-free systems is the possibility that signaling through multiple pathways with opposing effects may cause a lack of overall response, resulting in a false negative outcome^[70]. In combination with traditional assays, biosensor-based label-free methods have a promising future and should further strengthen the role of GPCRs in drug discovery and development.

Receptor dimerization assay

Many, but perhaps not all^[80], GPCRs interact with each other at the plasma membrane to form dimers, oligomers or even higher-order complexes. The dimerization of GPCRs can be observed early after biosynthesis and profoundly impacts receptor pharmacology and signaling (reviewed in^[81]). For class C GPCRs, heterodimerization is obligatory for receptor function. Many class A GPCRs, however, can function as homodimers when individually expressed in cells, and heterodimerization between class A GPCRs may lead to distinct and unique signaling properties even when stimulated with the same ligand, a phenomenon termed "heteromer-directed signaling specificity." Such phenomena are believed to be involved in the physiological roles of GPCRs and in disease-specific dysregulation of a receptor effect^[82, 83]. Therefore, compounds that specifically target GPCR heterodimers or affect receptor dimerization may have the potential to achieve specific therapeutic effects^[84]. As a result, there is considerable interest in designing assays to assess the effect of compounds on GPCR dimerization. Various technologies have been established to monitor receptor dimerization, including resonance energy transfer approaches (FRET or BRET) and the recently developed PathHunterTM GPCR dimerization system (DiscoverRx) and Tag-liteTM GPCR dimer assay (Cisbio).

In commonly used FRET or BRET-based approaches, donor and acceptor molecules are genetically fused to the C-terminus of GPCRs, which are overexpressed in the cells (Figure 5A). Resonance energy transfer occurs when donor and acceptor molecules are brought into close proximity as a consequence of GPCR dimerization (reviewed in^[85, 86]). However, one limitation of such traditional FRET and BRET assays is that in the overexpression system, resonance energy transfer can also occur within the intracellular compartments such that it is difficult to demonstrate a specific signal resulting only from a direct interaction of proteins at the cell surface. In addition, low signal-to-noise ratio resulting from the intrinsic fluorescence background of a cell and the overlap between the emission spectra of FRET donors and acceptors is another limitation of the commonly used resonance energy transfer techniques.

The GPCR dimerization assay with Tag-liteTM is a method combining TR-FRET with SNAP-tagTM technology (Cisbio), enabling quantitative analysis of protein-protein interactions

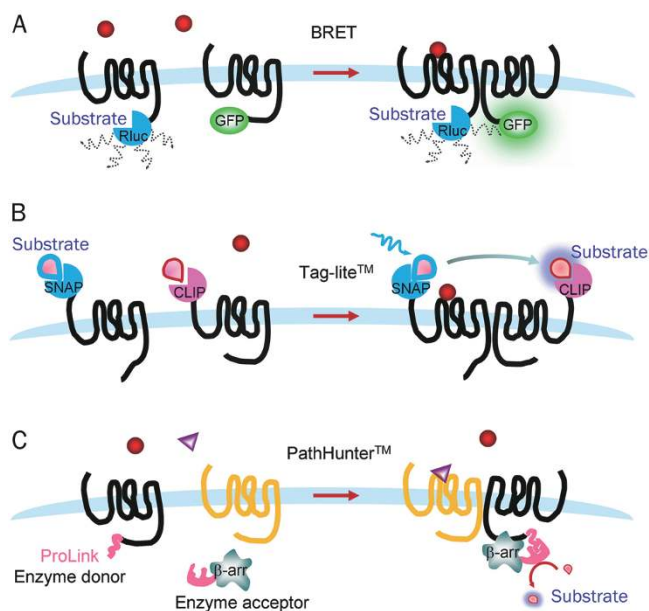


Figure 5. Receptor dimerization assays. (A) BRET dimerization assay. GPCRs are tagged with a RLuc donor and a GFP acceptor. Upon receptor dimerization, the two tags come into close proximity and energy transfer occurs. (B) Tag-lite™ dimerization assay. GPCRs are tagged with either a SNAP- or CLIP-tag at the N-terminus, which can be subsequently labeled with their corresponding cell-impermeable substrates carrying appropriate TR-FRET-compatible fluorophores. (C) PathHunter™ heterodimerization assay. β -arrestin is fused to the larger portion of a β -galactosidase enzyme acceptor, and the ProLink™ tag is attached to one of the GPCR targets. A second untagged GPCR can be introduced into the cells, and the transactivation effects of the untagged GPCR on the ProLink™-tagged GPCR can be measured by the recruitment of β -arrestin to the tagged-GPCR using PathHunter detection reagents.

at the surface of living cells in a 96- or 384-well format. In this assay, GPCRs are tagged with either a SNAP- or CLIP-tag at the N-terminus, which can be subsequently labeled with their corresponding cell-impermeable substrates carrying appropriate TR-FRET-compatible fluorophores, typically using terbium cryptate as a donor and a green or red fluorescent molecule as an acceptor (Figure 5B). Several possible dimer combinations exist in this assay: 1/4 of the dimers contain both receptors labeled with the donor, 1/4 of the dimers contain both receptors labeled with the acceptors, and 1/2 of the dimers contain one receptor labeled with the donor and one receptor labeled with the acceptor. Only the last fraction will emit the FRET signal^[87]. This assay was validated with well-known GPCR dimers and the oligomeric assembly of both class A and class C GPCRs was confirmed^[88].

The PathHunter™ GPCR dimerization system from DiscoverX is another platform for GPCR heterodimerization analysis. Cell lines utilized in the previously described PathHunter™ β -arrestin recruitment assay can be used as the starting material. In these cell lines, β -arrestin is fused to the larger portion of the β -galactosidase enzyme acceptor, and the smaller 42-amino acid ProLink™ tag is attached

to one of the GPCR targets. A second untagged GPCR can be introduced into the cells and the transactivation effects of the untagged GPCR on the ProLink™-tagged GPCR can be measured by the recruitment of β -arrestin to the tagged-GPCR using PathHunter detection reagents (Figure 5C). The transactivation strength can be estimated as a ratio between the cellular response to the agonist of the untagged GPCR and the response to the agonist of ProLink™-GPCR. The assay can be used to investigate the interaction between GPCR pairs, as well as screen for compounds that modulate GPCR activity through enhancing or disrupting GPCR heterodimerization in a 384-well format (http://www.discoverx.com/documents/DRx_poster_Heterodimer_DOT09_REV1.pdf).

In silico drug discovery

GPCR drug discovery usually relies on HTS for hit identification. However, with the development and maturation of computational methods for drug discovery, HTS may be complemented by *in silico* screening. This combination of approaches will reduce both time and cost through reducing the number of compounds to be experimentally tested and increase the probability of identifying novel lead compounds. Both ligand-based and structure-based methods for *in silico* screening have been successfully applied to GPCR drug discovery.

Structure-based screening requires reliable 3D structures for the target protein, which poses a challenge for GPCRs because of the heterogeneity of this receptor family and the lack of crystallographic data. Until 2008, the only available GPCR structure was that of bovine rhodopsin^[89]. In the last few years, a number of different technological developments^[90] have resulted in the structures of several new GPCRs, including β 2 adrenergic receptor^[91], A2a adenosine receptor^[92], CXCR4 chemokine receptor^[93], dopamine D3 receptor^[94] and histamine H1 receptor^[95]. Additionally, the first structure of a signal transduction complex (β 2 adrenergic receptor and G_{α_s}) has also been reported^[96]. Nevertheless, the relatively limited 3D information for GPCRs is an obstacle for structure-based drug discovery. In the absence of crystal structures, both homology modeling and *ab initio* techniques have been applied to model the 3D structures of GPCRs and used for *in silico* screening (reviewed in^[97, 98]).

When a crystal structure of the targeted protein is not available and reliable modeling is not feasible, a problem common to many GPCRs, ligand-based drug discovery methods remain the major computational approach for the analysis of the growing data sets for GPCR ligands. Ligand-based screening is highly efficient and can be productive with sufficient information on known ligands. Incorporating ligand information with homology modeling has also been applied with good results (reviewed in^[97, 99, 100]).

Conclusion

Choosing the right primary HTS assay for GPCRs, one of the most important protein families for therapeutic targeting, is critical in early drug discovery. With the availability of all the aforementioned assays, a few important things must also be

considered. First, the choice of cell line for GPCR expression might affect assay development because such cell line might lack correct post-translational modification and dimerization of GPCRs, and the expression of important signaling molecules. Second, the level of GPCR expression also needs to be carefully monitored because significant overexpression might lead to ligand-independent signaling and shifts in G protein coupling. Third, the functional selectivity (biased signaling) of ligands might complicate the screening process. Assays based on one signaling pathway might miss potentially valuable compounds acting on other pathways. Therefore, multiplexing of assays or a summed readout should always be considered. With the advancement of high-content imaging and label-free, whole cell technologies, new GPCR functional assays might provide more comprehensive and physiologically relevant information on lead compounds and might improve the success rate in drug discovery.

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