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

Acta Pharmacologica Sinica (2012) 33: 372–384; doi: 10.1038/aps.2011.173; published online 23 Jan 2012

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 guanidine diphosphate (GDP) for guanidine 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 Ga from the dimeric Gβγ subunits[2]. GPCRs coupled to Gaq and Gas/ai proteins activate or inhibit, respectively, adenylyl 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 Gai/ir alternatively activate phospholipase Cβ (PLCβ), 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 Gas/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[1]. 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...
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; 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. 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. 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. Since then, $^3$H- or $^{125}$I-labeled ligands have been widely used to characterize the affinity of a compound for a target GPCR, while nonlabeled 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 modulators). 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. Typically, the accumulation of non-hydrolysable GTP analog, such as $[^{35}]$-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 | • 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 |
|                      | Other tagged-ligand binding | • DELFIATM 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 |
| G-protein dependent assays | GTPγS binding | • Filtration assay (PerkinElmer) | Functional cell-free assay; discrimination between full or partial agonists, neutral antagonists, inverse agonists, allosteric regulators | Relatively low signal to background window | Gαi/o |
| CRE/MRE reporter assay | cAMP assay | • SPA cAMP assay (GE Healthcare or PerkinElmer) • HitHunterTM (DiscoveRx); • AlphaScreenTM (PerkinElmer); • Fluorescence polarization-based cAMP kits (PerkinElmer, Molecular Devices and GE Healthcare); • HTRF™-based cAMP (Cisbio); • cAMP GlosensorTM (Promega) | 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 | Gαi (with Goi5,i6 could be universal) |
|                      | Ca2+ | • Fluo-3 or Fluo-4 (Invitrogen) and FLIPR™ 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 | Gαi (with Goi5,i6 could be universal) |
| IP3 | • SPA IP3 assay (PerkinElmer) • AlphaScreenTM (PerkinElmer); • HitHunter™ Fluorescence Polarization (DiscoveRx); • HTRF IP-One™ (Cisbio) | High-throughput; functional assay for live cells; good for slow binding ligands | Limited industrial validation | Gαi (with Goi5,i6 could be universal) |
| G-protein independent assays | Receptor trafficking | • Cellomics ArrayScan™ (Thermo Scientific); • INCell Analyzer 3000 (GE Healthcare); • Opera™ (Evotec Technologies); • Acumen™ 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 | • TransFluo™ (Molecular Device); • BRET/FRET • Tango™ (Invitrogen); • PathHunter™ (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-arrrestin binding varies among GPCRs; less sensitive; need further pathway analysis | any GPCR |
| Label-free assay | BIND™ RWG biosensors (SRU Biosystems) • Epic™ RWG biosensors systems (Corning Inc); • ECIS™ (Applied Biophysics); • xCELLigence™ System (Roche and ACEA Biosciences); • CellKey™ (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 | BRET/FRET; • PathHunter™ (DiscoveRx); • Tag-lite™ (Cisbio) | GPCR heterodimers are considered new pharmacological targets | Very artificial system, can not assess GPCR dimerization in native state | any GPCR |
membrane prepared from cells expressing GPCRs of interest is measured after agonist stimulation. Unlike receptor binding assays, the GTPγS-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 Gα<sub>q</sub>, which is the most abundant G protein in many cells and has a faster GDP-GTP exchange rate than other G proteins [6]. Nevertheless, [35S]-GTPγS binding assays can also be used with GPCRs that couple to the Gα and Gα<sub>i/o</sub> families of G proteins, especially in artificial expression systems, or using receptor-Gα<sub>i/o</sub> chimeras, or by immunoprecipitation of [35S]-GTPγS-labeled Gα (reviewed in [13]). A problem with this assay is that it requires a filtration step through glass fiber to separate free and bound [35S]-GTPγS, which limits assay throughput. With the development of SPA technology, the filtration step can be omitted and GTPγS 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 α2-adrenergic [17], neuropeptide FF2 receptor [18], dopamine D3 receptor [19] and muscarinic receptors [20], and the results are comparable with those obtained using traditional [35S]-GTPγS binding assays [21].

cAMP assay

Assays measuring cellular levels of cAMP are dependent on the activity of adenylate cyclase, which is regulated by GPCRs coupled to Gα<sub>q</sub>, or Gα<sub>q, i/o</sub> protein. Gα<sub>i/o</sub> positively stimulates the activity of adenylate cyclase, resulting in increased cellular cAMP. In contrast, activation of Gα<sub>q</sub> leads to a negative regulation of adenylate cyclase and a decrease in cAMP levels. Screening Gα<sub>q</sub>-coupled receptors is generally straightforward, whereas screening Gα<sub>q, i/o</sub>-coupled receptors, especially for Gα<sub>q, i/o</sub>-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 adenylate 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 (e.g., 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 FlashPlate<sup>TM</sup> cAMP assay from PerkinElmer using [125I]-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 (HitHunter<sup>TM</sup>) 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]. AlphaScreen<sup>TM</sup> 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 [23]. 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 [22]. Finally, the cAMP Glosensor<sup>TM</sup> 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 $G_{\alpha_q}$-coupled receptor activation or inverse agonist activity in the absence of artificial stimulation by compounds such as forskolin (http://www.promega.com/glosensor). A direct comparison of AlphaScreen™, HTRF, HitHunter™, and FP cAMP assays suggests that there are advantages and disadvantages in each method[30]. The AlphaScreen™ and HTRF assays are recommended for cells expressing low levels of GPCRs because of their higher sensitivities.

**IP$_3$/IP$_1$ and Ca$^{2+}$ assays**

Stimulation of $G_{\alpha_i}$ or $G_{\alpha_q}$-coupled GPCRs activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol bisphosphate (PIP$_2$) to form two second messengers, inositol 1,4,5-triphosphate (IP$_3$) and DAG. While DAG activates protein kinase C (PKC), IP$_3$ activates the IP$_3$ receptor on the endoplasmic reticulum (ER) resulting in an efflux of Ca$^{2+}$ from the ER to the cytoplasm and an elevation of intracellular Ca$^{2+}$. IP$_3$ is very rapidly hydrolyzed to IP$_2$, then to IP$_1$ and finally to inositol by a series of enzymatic reactions[27]. The radioactive IP$_3$ assay measures $^{3}$H-inositol incorporation and is a standard 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 IP$_3$, including AlphaScreen™ (PerkinElmer) and HitHunter™ Fluorescence Polarization (DiscoverX) assay. Recently, a homogeneous, non-radioactive TRF assay for measuring IP accumulation, IP-One HTRF™, was released by Cisbio (http://www.htrf.com). The basis for the assay is a reduction in energy transfer between acceptor IP$_3$ and a europium-conjugated IP$_3$ antibody as cellular IP$_3$ accumulates and replaces the acceptor IP$_3$ antibody. Compared to earlier kits using IP$_3$-binding proteins to specifically measure IP$_3$, the IP-One assay takes advantage of the fact that LiCl inhibits the degradation of IP$_3$, the final step in the inositol phosphate cascade, allowing it to accumulate in the cell and to be measured as a substitute for IP$_3$. 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 IP$_3$ 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 $M_4$ acetylcholine, FFAR1, vasopressin V1b, and neuropeptide S receptors[28].

As previously mentioned, intracellular Ca$^{2+}$ is another second messenger for GPCR signaling. GPCRs that naturally couple to $G_{\alpha_q}$ produce a ligand-dependent increase in intracellular Ca$^{2+}$. However, $G_{\alpha_{11}}$, $G_{\alpha_{15}}$, or $G_{\alpha_5}$-coupled GPCRs can also be “switched” to induce an increase in intracellular Ca$^{2+}$ either by the expression of a chimeric G-protein (G$_{\alpha_j}$ or G$_{\alpha_o}$) or a promiscuous G-protein (G$_{\alpha_{15}}$ or G$_{\alpha_5}$) (reviewed in[30]). The Ca$^{2+}$ assay is very popular in GPCR screening owing to the availability of cell-permeable Ca$^{2+}$-sensitive fluorescent dyes (such as Fluo-3 and Fluo-4) and automated real-time fluorescence plate readers, such as FLIPR™ 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 FLIPR™ 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 Ca$^{2+}$-sensitive biosensors. Recombinant expression of the jellyfish photoprotein aequorin, which provides an intense luminescent signal in response to elevated intracellular Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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 IP$_3$ 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, $\beta$-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 GPCRs 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\(^{[56]}\)). 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 (e.g., measurement of intracellular \(\text{Ca}^{2+}\) concentration for \(G_{\alpha_{i}}\)-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\(^{[57]}\)). 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\(^{\text{TM}}\) Series from Thermo Scientific (http://www. thermo.com.cn/HCS), while others are equipped with confocal optics, such as the INcell\(^{\text{TM}}\) Analyzer 3000 from GE Healthcare and Opera\(^{\text{TM}}\) from Evotec Technologies. More recently, the development of a laser-scanning fluorescence microplate cytometer, for example, the Acumen\(^{\text{TM}}\) Explorer from TTP Lab Tech, offers even higher throughput in these multiplexing assays.

In contrast to the aforementioned \(\text{Ca}^{2+}\), 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\(^{[39]}\). 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\(^{\text{TM}}\) system or the granularity analysis module of the INCell\(^{\text{TM}}\) 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,
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”[144]. 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 Gαi-coupledGPCRs, which traditionally suffer from a small assay window in second messenger detection systems, and orphan GPCRs.

The first commercialized β-arrestin recruitment assay, Transflour™, was originally licensed by Norak Biosciences in 1999 from Duke University Medical Center and is now available from Molecular Devices. The Transflour™ 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™[46-47]. Taking advantage of these HCS instruments, the Transflour™ 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 vise 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
are brought into proximity, which results in the activation of the enzyme, cleavage of the substrate and generation of a chemiluminescent signal. Similar to Tango™ GPCR assays, PathHunter™ 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 PathHunter™ 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
either angle- or wavelength-shift. 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 BIND<sup>TM</sup> (SRU Biosystems) and Epic<sup>TM</sup> (Corning Inc) systems. Although initially designed for in vitro molecular binding assays, both systems were successful in studying cell morphological changes and GPCR signaling<sup>61, 62</sup>.

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<sup>63</sup>. 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.<sup>41</sup> Since then, electrical-based detections have been applied to study a wide variety of cellular events, including cell adhesion and spreading.<sup>65</sup>, cell morphological changes<sup>66</sup>, and cell death.<sup>67</sup> 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) xCELLigence<sup>TM</sup> 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<sup>68, 69</sup>. A more high-throughput system, CellKey<sup>TM</sup>, was developed by MDS Analytical Technologies, which is designed to detect acute cellular responses in 96- and 384-well formats.

Based on both the CellKey<sup>TM</sup> and Epic<sup>TM</sup> systems, scientists observed distinct response profiles depending on the G-protein pathway activated<sup>62, 70, 71</sup>. Several studies compared the Epic<sup>TM</sup> system and traditional Ca<sup>2+</sup> or cAMP assays using CHO cells expressing the muscarinic M2, M3 or dopamine D3 receptors. Most of the results from the Epic<sup>TM</sup> were consistent with data from cAMP or Ca<sup>2+</sup> readouts with few exceptions, and the Epic<sup>TM</sup> was found to detect weak activity that was not observed with the label-based assays<sup>72, 73</sup>. Many studies with GPCR ligand sets also demonstrated similar rank-order potency values between CellKey<sup>TM</sup> impedance and traditional Ca<sup>2+</sup> or cAMP assays<sup>74-76</sup>.

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<sup>76-79</sup>, endogenous receptor profiling in cell lines commonly used in drug discovery<sup>62, 74, 77</sup>, systemic cell biology studies of GPCR signaling<sup>77, 79</sup>, 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.<sup>70</sup> 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<sup>80</sup>, 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.<sup>81</sup> 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.<sup>82, 83</sup> Therefore, compounds that specifically target GPCR heterodimers or affect receptor dimerization may have the potential to achieve specific therapeutic effects.<sup>84</sup> 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 PathHunter<sup>TM</sup> GPCR dimerization system (DiscoveRx) and Tag-lite<sup>TM</sup> 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<sup>85, 86</sup>). 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-lite<sup>TM</sup> is a method combining TR-FRET with SNAP-tag<sup>TM</sup> technology (Cisbio), enabling quantitative analysis of protein-protein interactions.
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 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 GPCP 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 β2 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 (β2 adrenergic receptor and Gαs) 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.

Acknowledgements

The authors thank Chang-sheng DU for discussion and critical reading of this manuscript. This work was supported by grants from the Ministry of Science and Technology of China (2009ZX09302-001 and 2008DFB30150), the National Natural Science Foundation of China (31071227 and 90713047), and the Shanghai Commission of Science and Technology (09DZ2260100).

References

1 Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 2003; 63: 1256–72.

2 Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: conserved structure and molecular mechanism. Nature 1991; 349: 117–27.

3 Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. Nat Rev Mol Cell Biol 2002; 3: 639–50.

4 Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arenergic. Science 2005; 308: 512–7.

5 Klabunde T, Hessler G. Drug design strategies for targeting G-protein-coupled receptors. ChemBiochem 2002; 3: 928–44.

6 Milligan G. Principles: extending the utility of [35S]GTP gamma S binding assays. Trends Pharmacol Sci 2003; 24: 87–90.

7 Kenakin T. Efficacy at G-protein-coupled receptors. Nat Rev Drug Discov 2002; 1: 103–10.

8 Bylund DB, Toews ML. Radioisogand binding methods: practical guide and tips. Am J Physiol 1993; 265: L421–9.

9 Lefkowitz RJ, Roth J, Pastan I. Radioreceptor assay of adrenocorticotropic hormone: new approach to assay of polypeptide hormones in plasma. Science 1970; 170: 633–5.

10 Trinkle C, Weyand O, Schroter A, Mohr K. Using a radiolalloster to test predictions of the cooperativity model for gallamine binding to the allosteric site of muscarinic acetylcholine M(2) receptors. Mol Pharmacol 1999; 56: 962–5.

11 Glickman JF, Schmid A, Ferrand S. Scintillation proximity assays in high-throughput screening. Assay Drug Dev Technol 2008; 6: 433–55.

12 Degorce F, Card A, Soh S, Trinquet E, Knapik GP, Xie B. HTRF: A technology tailored for drug discovery — a review of theoretical aspects and recent applications. Curr Chem Genomics 2009; 3: 22–32.

13 Harrison C, Traynor JR. The [35S]GTPgamma S binding assay: approaches and applications in pharmacology. Life Sci 2003; 74: 489–508.

14 Ferrer M, Kolodin GD, Zuck P, Peltier R, Berry K, Mandala SM, et al. A fully automated [35S]GTPgammaS scintillation proximity assay for the high-throughput screening of Gi-linked G protein-coupled receptors. Assay Drug Dev Technol 2003; 1: 261–73.

15 Johnson EN, Shi X, Cassaday J, Ferrer M, Strulovici B, Kunapuli P. A 1536-well [35S]GTPgammaS scintillation proximity binding assay for ultra-high-throughput screening of an orphan galphai-coupled GPCR. Assay Drug Dev Technol 2008; 6: 327–37.

16 Labrecque J, Wong RS, Fricker SP. A time-resolved fluorescent lanthanide (Eu)-GTP binding assay for chemokine receptors as targets in drug discovery. Methods Mol Biol 2009; 552: 153–69.

17 Frang H, Mukkala VM, Syssto R, Oliikka P, Hurskainen P, Scheinin M, et al. Nonradioactive GTP binding assay to monitor activation of g-protein-coupled receptors. Assay Drug Dev Technol 2003; 1: 275–80.

18 Engstrom M, Narvanes A, Savola JM, Wurster S. Assessing activation of the human neuropeptide FF2 receptor with a non-radioactive GTP binding assay. Peptides 2004; 25: 2099–104.

19 Leopoldo M, Lacivita E, Colabufo NA, Contino M, Berardi F, Perrone R. First structure-activity relationship study on dopamine D3 receptor agents with N-(4-(4-arylpiperazin-1-yl)butyl)arycarboxamide structure. J Med Chem 2005; 48: 7919–22.

20 Zhang HY, Watson ML, Gallagher M, Nicolle MM. Muscarinic receptor-mediated GTP-Eu binding in the hippocampus and prefrontal cortex is correlated with spatial memory impairment in aged rats. Neurobiol Aging 2007; 28: 619–26.

21 Koval A, Kopein D, Puruvanov V, Katananae VL. Europium-labeled GTP as a general nonradioactive substitute for [35S]GTPgamma S in high-throughput G protein studies. Anal Biochem 2010; 397: 202–7.

22 Eglen RM, Singh R. Beta galactosidase enzyme fragment complementation as a novel technology for high throughput screening. Comb Chem High Throughput Screen 2003; 6: 381–7.

23 Weber M, Ferrer M, Zheng W, Inglese J, Strulovici B, Kunapuli P. A 1536-well cAMP assay for Gs- and Gi-coupled receptors using enzyme fragmentation complementation. Assay Drug Dev Technol 2004; 2: 39–49.

24 Williams C. cAMP detection methods in HTS: selecting the best from the rest. Nat Rev Drug Discov 2004; 3: 125–35.

25 Wgdal SS, Anderson JL, Vildigiris G, Shultz J, Wood KV, Fan F. A novel bioluminescent protease assay using engineered firefly luciferase. Curr Chem Genomics 2008; 2: 16–28.

26 Gabriel D, Vernier M, Pfeifer MJ, Dasen B, Tenaillon L, Bouhelar R. High throughput screening technologies for direct cyclic AMP measurement. Assay Drug Dev Technol 2003; 1: 291–303.

27 Berridge MJ. Inositol trisphosphate and calcium signalling. Nature 1993; 361: 315–25.

28 Liu K, Titus S, Southall N, Zhu P, Inglese J, Austin CP. Assay Drug Dev Technol 2008; 6: 433–55.

29 Emrey R, Ranki NB. Screening G protein-coupled receptors: measurement of intracellular calcium using the fluorometric imaging plate reader. Methods Mol Biol 2009; 565: 145–58.

30 Zhang Y, Kowal D, Kramer A, Dunlop J. Evaluation of FLIPR calcium 3 assay kit — a new no-wash fluorescence calcium indicator reagent. J...
31 Eglen RM, Reisine T. Photoproteins: important new tools in drug discovery. Assay Drug Dev Technol 2003; 8: 571–7.

32 Robas NM, Fidock MD. Identification of orphan G protein-coupled receptor ligands using FLIPR assays. Methods Mol Biol 2005; 306: 17–26.

33 Hansen KB, Brauner-Osborne H. FLIPR assays of intracellular calcium in GPCR drug discovery. Methods Mol Biol 2009; 552: 269–78.

34 Cheng Z, Garvin D, Paguio A, Stecha P, Wood K, Fan F. Luciferase reporter assay system for deciphering GPCR pathways. Curr Chem Genomics 2010; 4: 84–91.

35 Fan F, Wood KV. Bioluminescent assays for high-throughput screening. Assay Drug Dev Technol 2007; 5: 127–36.

36 Salahpour A, Barak LS. Visualizing receptor endocytosis and trafficking. Methods Mol Biol 2011; 756: 311–23.

37 Zanella F, Lorenz JB, Link W. High content screening: seeing is believing. Trends Biotechnol 2010; 28: 237–45.

38 Middleton RJ, Kellam B. Fluorophore-tagged GPCR ligands. Curr Opin Chem Biol 2005; 9: 517–25.

39 Hislop JN, von Zastrow M. Analysis of GPCR localization and trafficking. Methods Mol Biol 2011; 746: 425–40.

40 Taylor HD, Haskins JR, Giuliano KA. High content screening: a powerful approach to systems cell biology and drug discovery. Totowa, New Jersey Humana Press Inc; 2007.

41 Haasen D, Schnapp A, Valler MJ, Heilker R. G protein-coupled receptor internalization assays in the high-content screening format. Methods Enzymol 2006; 414: 121–39.

42 Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, et al. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med 2005; 11: 90–4.

43 Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 2002; 115: 455–65.

44 Violin JD, Lefkowitz RJ. Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci 2007; 28: 416–22.

45 Bowen WP, Wylie PG. Application of laser-scanning fluorescence microscopy to high content screening. Assay Drug Dev Technol 2006; 4: 209–21.

46 Eggingel C, Brand L, Ullmann D, Jager S. Highly sensitive fluorescence detection technology currently available for HTS. Drug Discov Today 2003; 8: 632–41.

47 Haasen D, Wolff M, Valler MJ, Heilker R. Comparison of G-protein coupled receptor desensitization-related beta-arrestin redistribution using confocal and non-confocal imaging. Comb Chem High Throughput Screen 2006; 9: 37–47.

48 Oakley RH, Hudson CC, Cruickshank RD, Meyers DM, Payne RE Jr, Rhem SM, et al. The cellular distribution of fluorescently labeled arrestins provides a robust, sensitive, and universal assay for screening G protein-coupled receptors. Assay Drug Dev Technol 2002; 1: 21–30.

49 Garippa RJ, Hoffman AF, Gradi G, Kirsch A. High-throughput confocal microscopy for beta-arrestin-green fluorescent protein translocation G protein-coupled receptor assays using the Evotec Omega. Methods Enzymol 2006; 414: 99–120.

50 Oakley RH, Hudson CC, Sjaastad MD, Loomis CR. The ligand-independent translocation assay: an enabling technology for screening orphan G protein-coupled receptors by arrestin recruitment. Methods Enzymol 2006; 414: 50–63.

51 Bertrand L, Parent S, Caron M, Legault M, Joly E, Angers S, et al. The BRET2/arrestin assay in stable recombinant cells: a platform to screen for compounds that interact with G protein-coupled receptors (GPCRs). J Recept Signal Transduct Res 2002; 22: 533–41.

52 Hamdan FF, Audet M, Garneau P, Pelletier J, Bouvier M. High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer-based beta-arrestin2 recruitment assay. J Biol Screen 2005; 10: 463–75.

53 Kamal M, Marquez M, Vauthier V, Leloir A, Froguel P, Jockers R, et al. Improved donor/acceptor BRET couples for monitoring beta-arrestin recruitment to G protein-coupled receptors. Biotechnol J 2009; 4: 1337–44.

54 Barnea G, Strapps W, Herrada G, Berman Y, Ong J, Kloss B, et al. The genetic design of signaling cascades to record receptor activation. Proc Natl Acad Sci U S A 2008; 105: 64–9.

55 Doucette C, Vedvik K, Koepnick E, Bergsma A, Thomson B, Turek-Etienne TC. Kappa opioid receptor screen with the Tango beta-arrestin recruitment technology and characterization of hits with second-messenger assays. J Biol Screen 2009; 14: 381–94.

56 Hanson BJ, Wetter J, Bercher MR, Kopp L, Fuerstenau-Sharp M, Vedvik KL, et al. A homogeneous fluorescent live-cell assay for measuring 7-transmembrane receptor activity and agonist functional selectivity through beta-arrestin recruitment. J Biol Screen 2009; 14: 798–810.

57 Yin H, Chu A, Li W, Wang B, Shelton F, Otero F, et al. Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. J Biol Chem 2009; 284: 12328–38.

58 Zhao X, Jones A, Olson KR, Peng K, Wehrman T, Park A, et al. A homogeneous enzyme fragment complementation-based beta-arrestin translocation assay for high-throughput screening of G-protein-coupled receptors. J Biol Screen 2008; 13: 737–47.

59 Scott CW, Peters MF. Label-free whole-cell assays: expanding the scope of GPCR screening. Drug Discov Today 2010; 15: 704–16.

60 Fang Y. Label-free cell-based assays with optical biosensors in drug discovery. Assay Drug Dev Technol 2006; 4: 583–95.

61 Fang Y, Ferrie AM, Li G. Probing cytoskeleton modulation by optical biosensors. FEBS Lett 2005; 579: 4175–80.

62 Fang Y, Li G, Ferrie AM. Non-invasive optical biosensor for assaying endogenous G protein-coupled receptors in adherent cells. J Pharmacol Toxicol Methods 2007; 55: 314–22.

63 Giaever I, Keese CR. Monitoring fibroblast behavior in tissue culture with an applied electric field. Proc Natl Acad Sci U S A 1984; 81: 3761–4.

64 Giaever I, Keese CR. Micromotion of mammalian cells measured electrically. Proc Natl Acad Sci U S A 1991; 88: 7896–900.

65 Tiruppathi C, Malik AB, Del Vecchio PJ, Keese CR, Giaever I. Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. Proc Natl Acad Sci U S A 1992; 89: 7919–23.

66 Giaever I, Keese CR. A morphological biosensor for mammalian cells. Nature 1993; 366: 591–2.

67 Zhu J, Wang X, Xu X, Abassi YA. Dynamic and label-free monitoring of natural killer cell cytotoxic activity using electronic cell sensor arrays. J Immunol Methods 2006; 309: 25–33.

68 Yu N, Atienza JM, Bernard J, Blanc S, Zhu J, Wang X, et al. Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. Anal Chem 2006; 78: 35–43.

69 McLaughlin JN, Shen L, Holinstat M, Brooks JD, Dibenedito E, Hamm HE. Functional selectivity of G protein signaling by agonist peptides and thombin for the protease-activated receptor-1. J Biol Chem 2005; 280: 25048–59.

70 Peters MF, Vaillancourt F, Heroux M, Vaiqette M, Scott CW. Comparing label-free biosensors for pharmacological screening with cell-
based functional assays. Assay Drug Dev Technol 2010; 8: 219–27.
71 Leung G, Tang HR, McGuinness R, Verdonk E, Michelotti JM, Liu VF. Cellular dielectric spectroscopy: a label-free technology for drug discovery. J Lab Autom 2005; 10: 258–69.
72 Dodgson K, Gedge L, Murray DC, Coldwell M. A 100K well screen for a muscarinic receptor using the Epic label-free system — a reflection on the benefits of the label-free approach to screening seven-transmembrane receptors. J Recept Signal Transduct Res 2009; 29: 163–72.
73 Tran E, Ye F. Duplexed label-free G protein-coupled receptor assays for high-throughput screening. J Biomol Screen 2008; 13: 975–85.
74 Ciamborone GJ, Liu VF, Lin DC, McGuinness RP, Leung GK, Pitchford S. Cellular dielectric spectroscopy: a powerful new approach to label-free cellular analysis. J Biomol Screen 2004; 9: 467–80.
75 Peters MF, Knappenberger KS, Wilkins D, Sygowski LA, Lazor LA, Liu J, et al. Evaluation of cellular dielectric spectroscopy, a whole-cell, label-free technology for drug discovery on Gi-coupled GPCRs. J Biomol Screen 2007; 12: 312–9.
76 McGuinness RP, Proctor JM, Gallant DL, van Staden CJ, Ly JT, McGuinness RP, Proctor JM, Gallant DL, van Staden CJ, Ly JT, et al. Enhanced selectivity screening of GPCR ligands using a label-free cell based assay technology. Comb Chem High Throughput Screen 2009; 12: 812–23.
77 Verdonk E, Johnson K, McGuinness R, Leung G, Chen YW, Tang HR, et al. Cellular dielectric spectroscopy: a label-free comprehensive platform for functional evaluation of endogenous receptors. Assay Drug Dev Technol 2006; 4: 609–19.
78 Lee PH, Gao A, van Staden C, Ly J, Salon J, Xu A, et al. Evaluation of dynamic mass redistribution technology for pharmacological studies of recombinant and endogenously expressed G protein-coupled receptors. Assay Drug Dev Technol 2008; 6: 83–94.
79 Fang Y, Li GG, Peng J. Optical biosensor provides insights for bradykinin B(2) receptor signaling in A431 cells. FEBS Lett 2005; 579: 6365–74.
80 Meyer BH, Segura JM, Martinez KL, Hovius R, George N, Johnsson K, et al. FRET imaging reveals that functional neurokinin-1 receptors are monomeric and reside in membrane microdomains of live cells. Proc Natl Acad Sci U S A 2006; 103: 2138–43.
81 Rozenfeld R, Devi LA. Exploring a role for heteromerization in GPCR signaling specificity. Biochem J 2011; 433: 11–8.
82 Rozenfeld R, Gupta A, Gagnidze K, Lim MP, Gomes I, Lee-Ramos D, et al. AT1R-CBR heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II. EMBO J 2011; 30: 2350–63.
83 Gassmann M, Shaban H, Vigot R, Sansig G, Hailer C, Barbieri S, et al. Redistribution of GABAB(1) protein and atypical GABAB responses in GABAB(2)-deficient mice. J Neurosci 2004; 24: 6086–97.
84 Rozenfeld R, Devi LA. Receptor heteromerization and drug discovery. Trends Pharmacol Sci 2010; 31: 124–30.
85 Achour L, Kamal M, Jockers R, Marullo S. Using quantitative BRET to assess G protein-coupled receptor homo- and heterodimerization. Methods Mol Biol 2011; 756: 183–200.
86 Cottet M, Albizu L, Comps-Agran L, Trinquet E, Pin JP, Mouilliac B, et al. Time resolved FRET strategy with fluorescent ligands to analyze receptor interactions in native tissues: application to GPCR oligomerization. Methods Mol Biol 2011; 746: 373–87.
87 Comps-Agran L, Maurel D, Rondard P, Pin JP, Trinquet E, Prezeau L. Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to G protein-coupled receptor oligomerization. Methods Mol Biol 2011; 756: 201–14.
88 Maurel D, Comps-Agran L, Brock C, Rives ML, Bourrier E, Ayoub MA, et al. Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. Nat Methods 2008; 5: 561–7.
89 Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, et al. Crystal structure of rhodopsin: A G protein-coupled receptor. Science 2000; 289: 739–45.
90 Hanson MA, Stevens RC. Discovery of new GPCR biology: one receptor structure at a time. Structure 2009; 17: 8–14.
91 Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, et al. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. Nature 2007; 450: 383–7.
92 Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, et al. The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 2008; 322: 1211–7.
93 Wu B, Chien EY, Mol CD, Fentali G, Liu W, Katrich V, et al. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science 2010; 330: 1066–71.
94 Chien EY, Liu W, Zhao Q, Katrich V, Han GW, Hanson MA, et al. Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. Science 2010; 330: 1091–5.
95 Shimamura T, Shiroishi M, Weyand S, Tsujimoto H, Kobilka TS, et al. Structure of the human histamine H1 receptor complex with doxepin. Nature 2011; 475: 65–70.
96 Rasmussen SG, Devree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the beta(2) adrenergic receptor-Gs protein complex. Nature 2011; 477: 549–55.
97 Sela I, Golan G, Strajbl M, Rivenzon-Segal D, Bar-Haim S, Bloch I, et al. G protein coupled receptors — in silico drug discovery and design. Curr Top Med Chem 2010; 10: 638–56.
98 Senderowitz H, Marantz Y. G Protein-Coupled Receptors: target-based in silico screening. Curr Pharm Des 2009; 15: 4049–68.
99 Gruber CW, Muttenthaler M, Freissmuth M. Ligand-based peptide design and combinatorial peptide libraries to target G protein-coupled receptors. Curr Pharm Des 2010; 16: 3071–88.
100 Trophsa A, Wang SX. QSAR modeling of GPCR ligands: methodologies and examples of applications. Ernst Schering Found Symp Proc 2006; (2): 49–73.