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Functional assays for screening GPCR targets

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G-protein-coupled receptors (GPCRs) are valuable molecular targets for drug discovery. An important aspect of the early drug discovery process is the design and implementation of high-throughput GPCR functional assays that allow the cost-effective screening of large compound libraries to identify novel drug candidates. Several functional assay kits based on fluorescence and/or chemiluminescence detection are commercially available for convenient screen development, each having advantages and disadvantages. In addition, new GPCR biosensors and high-content imaging technologies have recently been developed that hold promise for the development of functional GPCR screens in living cells.

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Introduction

In 2001, 50% of all newly launched drugs targeted G-protein-coupled receptors (GPCRs) and annual sales of these drugs was over $30 billion. In addition, it is estimated that 25% of the 100 top-selling drugs target GPCRs, demonstrating their value as 'drugable' discovery targets [1]. Endogenous ligands have been proposed for approximately 210 GPCRs; however, analyses of the human genome predict between 800 and 1000 GPCR genes. Excluding sensory GPCRs, this leaves a balance of over 100 'orphan' GPCRs for which endogenous ligands remain unidentified. Orphan GPCRs undoubtedly represent provisional targets for future drug discovery efforts as well as lucrative economic opportunities for the pharmaceutical industry [2]. The development of robust, reliable, and cost-effective functional screens for both known and orphan GPCRs is a major focus of the pharmaceutical industry. Furthermore, identification of endogenous or surrogate ligands for orphan GPCRs in functional assays can greatly facilitate target validation of these new targets. Several manufacturers have recently developed assay methodologies applicable to the development of new high-throughput functional GPCR screens and their utility will be discussed in this review.

GPCR activation and signaling

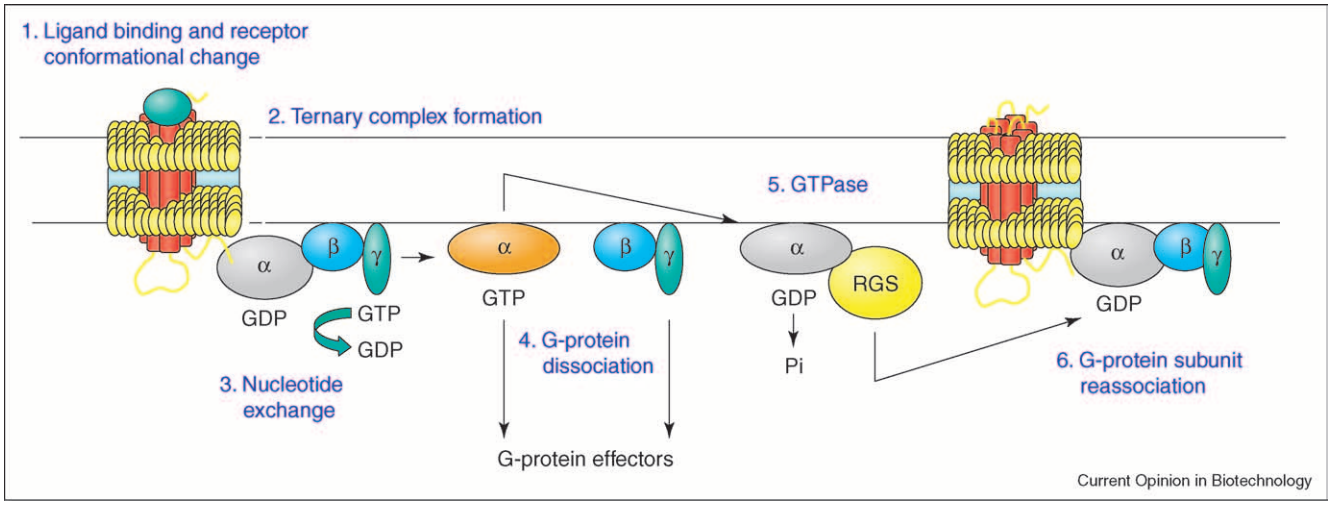
Ligand binding to GPCRs promotes conformational changes leading to G-protein coupling, the initiation of signal transduction pathways, and ultimately to cellular responses. G proteins are heterotrimeric containing α, β and γ subunits, and function by a cycle of subunit dissociation of α and βγ subunits superimposed by GPCR-mediated guanine nucleotide exchange (Figure 1).

G proteins are divided into four subfamilies (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) based on the structural and functional similarity of their α subunits [3]. Members of the different subfamilies act through a range of secondary messengers and signaling pathways (summarised in Figure 2). The βγ complexes, originally thought to primarily play roles in membrane attachment and in complex formation with inactive GDP-bound α subunits, also regulate several effector proteins [4•]. GPCR signaling can be regulated by desensitization and internalization, a process involving receptor phosphorylation, receptor complex formation with the cytoplasmic scaffold protein β-arrestin, recruitment of this resultant complex to clathrin-coated pits, endocytosis and, finally, either recycling to the membrane or lysosomal degradation [5].

Considerations for developing GPCR functional screens

There are several new paradigms regarding GPCR activation, signaling and regulation that should be considered when developing GPCR functional assays (Figure 3). The choice of a cell line for recombinant receptor expression can be an important consideration for several reasons. First, most GPCRs undergo post-translational modifications (e.g. palmitoylation and glycosylation) that can effect expression, pharmacology and function [6], and these should take place in the cell line of choice. Second, the third intracellular loop (IC3) and C-terminal tails of GPCRs are known to mediate association with several proteins called GIPs (G-protein interacting proteins), which can act in concert to influence GPCR expression, pharmacology, G-protein coupling and signaling [7•]. Thus, the presence of relevant GIPs in cells used to develop screens can influence the outcome. Third, GPCRs may undergo homodimerization and/or heterodimerization with other GPCRs [8]. Whereas homodimerization appears to be most important for regulating expression levels, heterodimerization can influence receptor expression and pharmacology, G-protein cou-

Figure 1



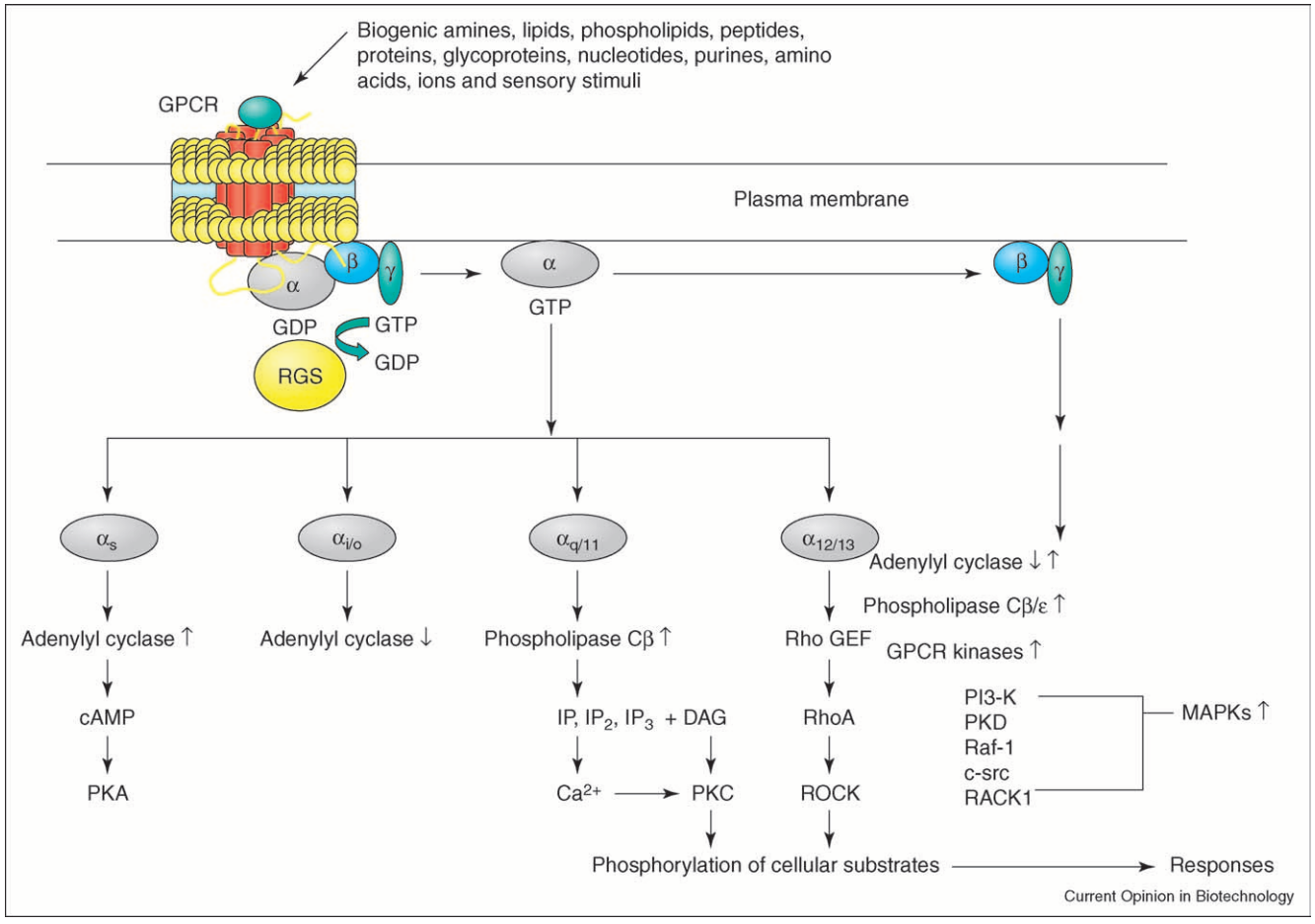
The G-protein activation/deactivation cycle. 1) The agonist–receptor interaction promotes a series of conformational changes favoring receptor coupling to G protein(s). 2) Formation of the agonist–receptor–G-protein ternary complex promotes a G-protein conformational change facilitating 3) the exchange of α-subunit-bound GDP for GTP. 4) The activated G protein then dissociates to form the GTP-bound α subunit and the βγ complex. The GTP-bound α subunit and βγ complex regulate the activity of specific intracellular effector proteins, leading to changes in the levels of secondary messengers (e.g. cAMP and calcium) and regulation of select signal transduction pathways. 5) The activity of the GTP-bound α subunit is terminated by hydrolysis of GTP to GDP by intrinsic GTPase activity of the α subunit. 6) The cycle is completed through the reassociation of the GDP-bound α subunit with the βγ complex. The kinetics of the G-protein activation/deactivation cycle are modulated by several accessory proteins including regulators of G-protein signaling (RGS) proteins [3].

pling and signal transduction. The primary question regarding heterodimerization is whether a heterodimer pair is the appropriate therapeutic target and if it should be considered in assay development. It is very likely that the complement of GPCR interacting proteins and signaling molecules present in cell lines typically used to express recombinant GPCRs for screen development could be very different from those present in normal and/or disease-relevant tissues and cells. In addition, recombinant expressed GPCRs might display ligand-independent signaling (constitutive activity) and this activity may need to be manipulated during assay optimization; constitutive activity might need to be reduced for optimization of agonist screens or increased for optimization of inverse agonist screens. Furthermore, if identification of partial agonists is desired, the constitutive activity of the GPCR should be reduced so they can be differentiated from full agonists. Often, GPCR constitutive activity can be titrated by modulating the levels of receptor expression, but some GPCRs are constitutively active even when expressed at lower densities. If disease-relevant cells endogenously expressing the receptor of interest can be used for screen development, these complicating factors may be less of an issue.

Several GPCRs clearly activate multiple G proteins [9,10••]. Although multiplicity of coupling in some cases can be an artifact of receptor overexpression in recombinant expression systems, it has also been clearly established for some endogenously expressed GPCRs. More importantly from a drug discovery perspective, the potential for agonist trafficking, a phenomenon in which certain agonists display different efficacies in activating one pathway over another, may be a critical issue to consider in functional screen development for agonist or antagonist screens [11]. If a single functional assay capturing only one signaling pathway is selected for screening compound libraries, potentially valuable compounds may be missed if the compound does display functional selectivity [12]. Furthermore, G-protein-independent signalling, such as activation of MAP kinase (mitogen-activated protein kinase) pathways, can also add to the multiplicity of potential signaling for a given GPCR [12]. If the therapeutically relevant signaling pathway is not known for a GPCR that displays multiplicity in G-protein coupling, it may be necessary to develop and implement multiple screens in a screening campaign.

An ideal GPCR screen should be simple, nonradioactive, robust (i.e. high signal-to-noise ratio), homogenous, contain minimal reagent additions, and be amenable to a microtiter plate format (96-, 384- or 1536-well) to facilitate robotic automation. Another consideration is whether to measure a proximal or distal signaling step. Measurement of events proximal to GPCR activation will reduce the incidence of false positives [13]; however, signal-to-noise ratios can be enhanced moving down the signal transduction cascade owing to signal amplifica-

Figure 2



GPCR signaling pathways. Members of the $G\alpha_s$ subfamily primarily act to stimulate adenylyl cyclase to produce the secondary messenger, cAMP. Increases in cellular cAMP lead to activation of protein kinase A (PKA) and the phosphorylation of specific cellular substrates leading to cellular responses. The $G\alpha_{i/o}$ G-protein subfamily includes $G\alpha_i$, $G\alpha_o$ and $G\alpha_z$, all of which act primarily to inhibit adenylyl cyclase. The $G\alpha_{q/11}$ subfamily members all act to stimulate phospholipase Cβ, an enzyme promoting the hydrolysis of membrane-associated phosphatidylinositol species (PI, PIP and PIP_2) to form inositol phosphates (IP, IP_2 and IP_3) and diacylglycerol (DAG), an activator of protein kinase C (PKC). These ultimately lead to increases in levels of intracellular calcium and activation of PKC, as well as other calcium-dependent proteins. The main effector system activated by the $G\alpha_{12/13}$ subfamily is the guanine nucleotide exchange factor RhoGEF, which in turn activates the small G protein RhoA. The βγ complexes, originally thought to primarily play roles in membrane attachment and in complex formation with inactive GDP-bound α subunits, also regulate several effector proteins [4•].

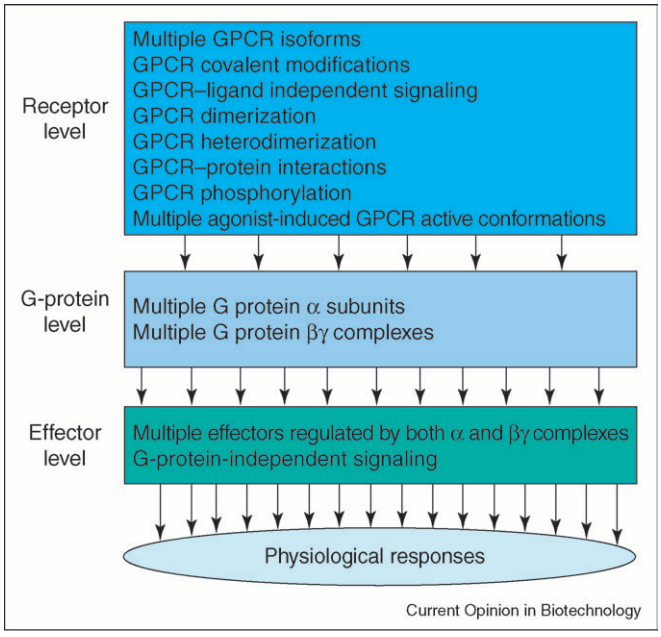
tion. Recently, several companies have introduced assay kits that are suitable for functional screening of GPCRs. Common functional assays currently used for GPCR drug discovery are listed in Table 1.

Widely used functional assay platforms for GPCRs

Guanine nucleotide binding assays

GPCR-mediated guanine nucleotide exchange is monitored by measuring [^{35}S]GTPγS binding to plasma membranes prepared from cells expressing GPCRs of interest. This platform is attractive because guanine nucleotide exchange is a proximal event to receptor activation and is not subject to amplification or regulation by other cellular processes [13]. Unfortunately, this assay is generally limited to $G_{i/o}$-coupled receptors, because $G_{i/o}$ is the most abundant G protein in most cells and has a faster GDP–GTP exchange rate than other G proteins [13]. The assay also requires a filtration step to separate free and bound [^{35}S]GTPγS, which limits assay throughput. The high assay background observed for G_s- and G_q-coupled receptors can be reduced by immunoprecipitation of the desired GTPγS-bound α subunit and filtration can be avoided using SPA^{TM} beads (scintillation proximity assay, GE Healthcare; http://www.gehealthcare.com) [14,15]. Perkin Elmer (http://www.perkin-elmer.com) has recently released a non-radioactive time-resolved fluorescence (TRF) GTP-binding assay using lanthanide chelate technology (europium-labeled GTP), but this still requires a filtration and washing step. The TRF

Figure 3



New paradigms regarding GPCR activation, signaling and regulation that should be considered in developing functional assays.

GTP-binding assay has been validated using membranes expressing recombinant GPCRs including α_2-adrenergic, motilin, serotonin 5-HT5a, neurotensin, muscarinic M_1, and neuropeptide FF_2 receptors and the results are comparable with those obtained in traditional [^{35}S]GTPγS binding assays [16•,17].

cAMP assays

Several homogenous assay platforms for cAMP measurement in whole cells or for adenylyl cyclase activity in membranes are commercially available and have been recently reviewed [18••,19••]. Screening G_s-coupled receptors is generally straightforward, whereas screening $G_{i/o}$-coupled receptors in cAMP assays can be considerably more difficult. To maximize the inhibition signal, it is often necessary to stimulate adenylyl cyclase with forskolin (a direct activator of adenylate cyclase) and this should be titrated during optimization of the assay. Radiometric GE Healthcare SPA™ and Perkin Elmer FlashPlate™ cAMP assays have been widely used, but have been replaced by fluorescence or luminescence-based homogenous assays to avoid the use of radioactivity. For example, Cisbio International (http://www.htrf-assays.com) has developed a sensitive high-throughput homogenous cAMP assay (HTRF) based on time-resolved fluorescence resonance energy transfer technology. This assay has been validated for both G_s- (β_2-adrenergic, histamine H_2, melanocortin MC_4, CGRP and dopamine D_1) and $G_{i/o}$-coupled (histamine H_3) receptors. cAMP assay kits based on fluorescence polarization are commercially available from Perkin Elmer, Molecular Devices (http://www.moleculardevices.com), and GE Healthcare and have been successfully miniaturized. They generally have low signal-to-noise ratios that can be improved using new red-shifted fluorophores [19••]. Perkin Elmer also offers the AlphaScreen™ cAMP assay, a sensitive bead-based chemiluminescent assay requiring laser activation and a special endpoint reader. DiscoveRx (http://www.discoverx.com) offers a homogenous high-throughput cAMP assay kit called HitHunter™ based on a patented enzyme (β-galactosidase) complementation technology using either fluorescent or luminescent substrates [20–22]. Gabriel *et al.* [18••] have reported a direct comparison of the AlphaScreen™, HTRF, HitHunter™ and FP cAMP assay platforms and suggest that there are advantages and disadvantages for each method. One of their recommendations is to use AlphaScreen™ or HTRF for cells expressing low levels of GPCRs owing to higher sensitivities. For cells expressing higher GPCR densities, they suggest that FP and HitHunter™ are sufficient platforms.

Inositol phosphate accumulation assays

Inositol phosphate (IP) accumulation assays have been used to develop functional screens for G_q-coupled GPCRs, but they are radioactive and non-homogenous. SPA™ technology has been applied to develop higher throughput homogenous assays for IP accumulation. One approach utilizes metal ions immobilized on the SPA™ bead that bind [^{3}H]IP via their phosphate groups [23]. Another utilizes yttrium silicate immobilized on SPA™

Table 1

Common functional assays for screening GPCRs.

Assay (company)	Biological measurement	Kit reagents	Basis	Endpoint	Advantages	Disadvantages
[^{35}S]GTPγS binding	Membrane-based GPCR-mediated guanine nucleotide exchange	[^{35}S]GTPγS	Irreversible [^{35}S]GTPγS binding to receptor-activated G proteins	Radiometric	Proximal to receptor activation	Radioactive, non-homogenous, requires a filtration step
Eu-GTP™ binding (Perkin Elmer)	Membrane-based GPCR-mediated guanine nucleotide exchange	Europium-GTP	Binding of europium-labeled GTP to receptor-activated G proteins	Time-resolved fluorescence	Proximal to receptor activation, nonradioactive	Non-homogenous, requires a filtration step
SPA™ (GE Healthcare)	Cell- or membrane-based, cAMP accumulation	Assay buffer, SPA™ beads conjugated with a cAMP MAb, [^{125}I]camp	ELISA based-competition of cAMP with [^{125}I]cAMP for binding to MAb conjugated to SPA™ beads, loss of signal due to reduced proximity of [^{125}I]cAMP and the SPA™ bead	Radiometric	Sensitive, homogenous, amenable to automation	Radioactive, relatively expensive
FlashPlate™ (Perkin Elmer)	Cell- or membrane-based, cAMP accumulation	Buffer, FlashPlate™ with cAMP MAb attached, [^{125}I]camp	ELISA based-competition of cAMP with [^{125}I]cAMP for binding to cAMP MAb conjugated to scintillant-coated wells, loss of signal due to reduced proximity of [^{125}I]cAMP and MAb in wells	Radiometric	Homogenous, amenable to automation	Radioactive, relatively expensive
AlphaScreen™ (Perkin Elmer)	Cell-based cAMP accumulation	cAMP MAb conjugated acceptor bead, streptavidin-coated donor beads with chemi-luminescence compound, biotinyl-cAMP	cAMP competes with biotinyl-cAMP binding to high-affinity streptavidin-coated donor beads, loss of signal due to reduced proximity of acceptor-donor bead	Luminescence	High sensitivity, homogenous, amenable to automation, cost effective, broad linear range of detection	Temperature- and light-sensitive, color quenching, special endpoint detector required
Fluorescence polarization (Perkin Elmer, Molecular Devices, GE Healthcare)	Cell- or membrane-based cAMP accumulation	cAMP MAb, fluorescent-labled camp	cAMP competes with Fluor-cAMP binding to cAMP MAb, loss of signal due to decrease in rotation and polarization	Fluorescence polarization	Homogenous, amenable to miniaturization and automation	Lower signal-to-noise (may be improved with red-shifted dyes)
HTRF cAMP (Cisbio)	Cell-based, cAMP accumulation	cAMP MAb conjugated with eurocryptate, acceptor molecule labeled camp	cAMP competes with acceptor-labeled cAMP binding to europium-conjugated cAMP MAb, loss of signal due to reduced europium-acceptor molecule proximity	Time-resolved fluorescence	Broad linear range, high signal-to-noise, homogenous, amenable to automation	
HitHunter™ (DiscoveRx)	Cell-based, cAMP accumulation	cAMP MAb, ED-cAMP conjugated peptide, acceptor protein, lysis buffer	cAMP competes with ED-cAMP for complementation of β-Gal activity with binding of acceptor peptide, loss of signal as enzyme complementation is reduced	Fluorescence or luminescence	Low compound interference, high sensitivity, homogenous, amenable to automation	Relatively expensive
IP Accumulation	Cell-based IP accumulation	None	Filtration to separate [^{3}H]inositol and [^{3}H]IPs	Radiometric	Sensitive, can be used for constitutively active G_q-coupled GPCRs	Low throughput, some automation possible

Table 1 Continued

Assay (company)	Biological measurement	Kit reagents	Basis	Endpoint	Advantages	Disadvantages
IP_1^{TM} (Cisbio)	Cell-based IP_1 accumulation	Europium-conjugated IP_1 MAb, acceptor-labeled IP_1	Loss of signal as IP_1 competes for binding of acceptor-labeled IP_1 binding to europium-MAb	Time-resolved fluorescence	Sensitive, homogenous, amenable to automation, can be used for constitutively active G_q-coupled GPCRs	Limited industrial validation
FLIPR™ (Molecular Devices)	Cell-based, increases in intracellular calcium	Calcium sensitive dye; Calcium-3	Increased fluorescence as intracellular dye binds calcium	Fluorescence	Sensitive, homogenous, amenable to automation	Cannot be used for inverse agonist screens, fluorescence quenching
AequoScreen™ (EuroScreen)	Cell-based, increases in intracellular calcium	Cells lines expressing select GPCRs along with promiscuous or chimeric G proteins and a mitochondrially targeted version of apoaequorin	Calcium-sensitive aequorin generates a luminescent signal when a coelenterazine derivative is added	Luminescence	Sensitive, homogenous, amenable to automation	Cannot be used for inverse agonist screens
Reporter gene	Cell-based, increases in reporter gene expression due to increases in second messengers	Several promotor plasmids and reporters are commercially available	GPCR changes in secondary messengers alter expression of a selected reporter gene	Fluorescence, luminescence, absorbance	Cost effective, sensitive, homogenous, amplification of signal	Long incubations and high false-positive hit rate, distal to receptor activation
Melanophore (Arena Pharmaceuticals)	Cell-based, changes in pigment dispersion	None	Melanosomes aggregate with inhibition of PKA, disperse with activation of PKA or PKC	Absorbance	Sensitive, homogenous, no cell lysis, amenable to automation	Time-consuming to produce stable cell lines expressing GPCRs

Abbreviations: β-Gal, β-galactosidase; ED-cAMP, enzyme fragment donor–cAMP conjugate; Eu-GTP, europium-labeled GTP; IP, inositol phosphate; MAb, monoclonal antibody; PKA, protein kinase A; PKC, protein kinase C; SPA, scintillation proximity assay; TRF, time-resolved fluorescence.

beads that bind IPs but not inositol [24]. Recently, a homogeneous TRF assay for measuring IP accumulation, called IP-One, has been released by Cisbio. The basis for the assay is a reduction in energy transfer between acceptor IP_1 and a europium-conjugated IP_1 antibody as IP_1 accumulates. Previous kits using IP_3-binding proteins to specifically measure IP_3 have been difficult to use because of a short incubation period and limited signal-to-noise ratios, owing to the rapid conversion of IP_3 to IP_2 and IP_1. The IP-One platform has been validated for cells expressing the recombinant muscarinic M_1, vasopressin V_{1A}, oxytocin, histamine H_2, $P2Y_1$, chemokine CCR5, and metabotropic mGluR1 and mGluR5 receptors.

Intracellular calcium assays

Functional assays designed to measure intracellular calcium ($[Ca^{2+}]_i$) are popular owing to the availability of calcium-sensitive fluorescent dyes and automated real-time charge-coupled device (CCD)-based fluorescence plate readers, such as FLIPR® (Molecular Devices) [25]. Activation of GPCRs can lead to increases in $[Ca^{2+}]_i$ through different mechanisms, including IP_3 release of intracellular stores from the endoplasmic reticulum, entry of calcium across the plasma membrane via calcium permeable channels, and by mechanisms that export or re-sequester calcium after receptor activation. Thus, active test compounds should be evaluated as to whether they act directly via GPCR activation [22]. Two recent advances have further increased the capacity of the FLIPR® platform. First, a new fluorescent dye kit called Calcium-3 (Molecular Devices) has been developed that allows cellular loading of dye without the need for subsequent cell washing. A comparison of 5-HT_{2C} and mGluR5 receptor agonist EC_{50} values obtained using Fluo4 dye or the Calcium-3 kit gave similar results [26]. The components of the Calcium-3 kit are proprietary making it difficult to evaluate their potential pharmacological properties [27]. Second, the FLIPR® instrument can now be configured with 1536-well pipetting heads to increase throughput and reduce reagent volumes. Although the FLIPR® method works well for both agonist and antagonist screening, it cannot be used to screen for inverse agonists because increases in basal $[Ca^{2+}]_i$ are not observed in cells expressing constitutively

active G_q-coupled receptors. This platform has been widely used for the deorphanization of GPCRs [28,29••].

A variant of the FLIPR® assay that utilizes the recombinant expressed jellyfish photoprotein, aequorin, has also been developed for functional screens of GPCRs. Aequorin is a calcium-sensitive reporter protein that generates a luminescent signal when a coelenterazine derivative is added. Euroscreen (http://www.euroscreen.be) offers engineered cell lines, called AequoScreen™ [30], in which different GPCRs and promiscuous or chimeric G proteins together with a mitochondrially targeted version of apoaequorin are all expressed, to conveniently develop high-throughput $[Ca^{2+}]_i$ assays. One disadvantage of this platform is that a lengthy preincubation period (4–18 h) is required before a test compound can be evaluated. Another variant of the $[Ca^{2+}]_i$ assay has been recently developed for G_s- and $G_{i/o}$-coupled GPCRs. In this assay, increases in intracellular cAMP concentration regulate the opening of recombinant expressed modified cyclic nucleotide-gated (CNG) calcium channels, resulting in increases in $[Ca^{2+}]_i$ which can be measured using FLIPR® [31]. Assay-ready cells stably expressing a variety of recombinant human G_s- and G_i-coupled receptors and the CNG are available from BD Biosciences (ACT*One* cell lines; http://www.bdbiosciences.com).

Reporter gene assays

Cell-based reporter gene assays provide another popular and cost-effective high-throughput functional homogenous assay platform for screening GPCR targets. Advantages of this platform include miniaturization to a 1536-well format and full robotic automation. Reporter assays are based on the ability of GPCR-mediated secondary messengers such as cAMP (CRE responsive element) or calcium (AP1 or NFAT response elements) to activate or inhibit a responsive element placed upstream of a minimal promoter, which in turn regulates the expression of a selected reporter protein. Commonly used reporters include β-galactosidase, luciferase, green fluorescent protein (GFP) and β-lactamase [19••,32–34]. Disadvantages of this platform include a requirement for long incubation intervals and the fact that the signaling event measured is distal from receptor activation, which can result in a high number of false-positives. Bresnick *et al.* [35•] reported the development of a universal reporter assay suitable for screening GPCRs that has been used to determine G-protein coupling of orphan GPCRs. This approach utilized both NFAT–β-lactamase and CRE–β-lactamase promoter–reporter systems as well as chimeric G proteins.

The development of universal GPCR functional screens

Attempts have been made to design universal GPCR functional screens that use a single common assay endpoint. Promiscuous G proteins such as $G_{\alpha 15/16}$, which allow coupling of G_s and $G_{i/o}$-coupled GPCRs to stimulate phospholipase Cβ (PLCβ), can be used to force coupling of these receptors to increases in $[Ca^{2+}]_i$. Several G_s- and $G_{i/o}$-coupled receptors couple to $G_{\alpha 16}$ and this coupling can be improved by engineering a G-protein chimera in which the backbone of $G_{\alpha 16}$ is combined with the C-terminal tail of the G protein $G_{\alpha z}$ (another member of the $G_{\alpha i}$ G-protein subfamily) [36,37]. Alternatively, several G-protein chimeras including $G_{q/s}$, $G_{q/i}$, $G_{q/o}$, $G_{q/z}$ have been engineered by replacing the last five to nine C-terminal amino acids of the G protein that the receptor typically couples to with the backbone of G_q [38–41]. In theory, these chimeras should allow screening of all G-protein coupled receptors in the FLIPR® intracellular calcium assay, thus facilitating the design of a universal functional assay. Molecular Devices offers expression vectors or cells expressing $G_{q/s}$, $G_{q/i}$, $G_{q/o}$, and $G_{q/z}$ (LiveWare®) for convenient assay development. The introduction of an N-terminal myristoylation sequence into $G_{q/i}$ has also been reported to increase its overall coupling efficiency [38]. Kostenis has recently reviewed the use of promiscuous G proteins and G-protein chimeras in GPCR functional screen development and reports that certain arrangements are superior and that a generic universal FLIPR® assay using one engineered G protein may not be optimal [41]. A popular method for screening compounds to identify endogenous or surrogate ligands for orphan GPCRs is to use the FLIPR® assay in combination with promiscuous G proteins or G-protein chimeras [28,29••,42,43]. Chimeric G proteins have also been used to extend the range of insect-cell-based functional assays for human GPCRs [44]. An important point to remember concerning the use of either promiscuous G proteins or G-protein chimeras is that although they might couple efficiently to the GPCR of interest, the pharmacology of the receptor may be altered [40,41].

Arena Pharmaceuticals (http://www.arenapharm.com) utilizes the Melanophore screening platform as a high-throughput, homogenous, functional screen for G_s-, $G_{i/o}$- and G_q-coupled GPCRs. This assay is based on the ability of transiently or stably expressed GPCRs to alter the distribution of melanin-containing pigment granules known as melanosomes in *Xenopus* skin cells called melanophores. Specifically, transfected melanophores will either aggregate or disperse melanosomes depending on whether the GPCR is $G_{i/o}$- or $G_{s/q}$-coupled, respectively. This allows for a simple readout based on light transmission through cell monolayers. The assay is simple, low cost (less than five cents/well), does not require cell lysis before reading, has been fully automated, and is adapted to 384- and 1536-well microtiter plate formats. Furthermore, this platform has allowed us to screen for surrogate ligands and inverse agonists for orphan GPCRs, because we are able to promote constitutive activation of GPCRs, by simple overexpression [45–47].

GPCR biosensors

There are several recent examples of 'GPCR biosensors' that have been invaluable in helping to characterize GPCR activation and signaling in living cells. Such systems might also provide alternative functional assay platforms for GPCR drug discovery in the near future. For example, bioluminescence resonance energy transfer or BRET has been used to study the interaction of GPCRs fused to Renilla luciferase (Rlu) with the cytoplasmic scaffold protein β-arrestin fused to GFP [48]. BRET is a novel platform which allows real-time evaluation of protein–protein interactions in living cells. In this example, BRET is based on the transfer of energy between recombinant expressed GPCR–Rlu and β-arrestin–GFP when they are in close proximity after the addition of the luciferase substrate coelentcrazine. Vrecl *et al.* [49••] have reported the development of a screening platform using BRET and mutant β-arrestin 2 proteins that are either phosphorylation-independent in their interaction with GPCR or which lack the sites important for their interaction with clathrin-coated pits. These mutants appear to have a significantly greater residence time in clathrin-coated pits when associated with class A receptors, resulting in a sufficient signal-to-noise ratio for a microtiter plate-based assay. Charest *et al.* [50] have recently developed a BRET-based biosensor in which β-arrestin is sandwiched between Renilla luciferase and yellow fluorescent protein (YFP). When β-arrestin interacts with and activates a GPCR, energy transfer is increased. The increase in energy transfer is proposed to result from a conformational change in the GPCR, which brings the N- and C-terminal ends of β-arrestin together. The authors suggest that this could be a useful biosensor for the large-scale screening of GPCRs. Measurement of real-time interactions of the human oxytocin receptor and G-protein-coupled receptor kinase GRK2 have also been reported using BRET [51] and this technology has also been used to study the interaction of GPCRs and G proteins in living cells. This method can capture rapid ligand-induced increases in bioluminescence resulting from the interaction of the receptor and G protein, as well as a slower decrease in luminescence that represents receptor desensitization and may have application in the identification of ligands for orphan GPCRs [52]. Hamdan *et al.* [53•] have recently described a BRET screening assay for measuring GPCR and β-arrestin interactions in which a GPCR is fused to enhanced yellow fluorescent protein (eYFP) and β-arrestin is fused to Rlu. This assay was used to screen a library of small molecules for antagonists of the chemokine receptor CCR5 in a 96-well format. We anticipate that BRET assays for evaluating GPCR interactions with G proteins or β-arrestins will become more popular once signal-to-noise ratios can be optimized.

Fluorescence resonance energy transfer or FRET, which is similar to BRET, has also been used to evaluate GPCR and protein interactions in real time in living cells [54–56] (see also Update). For example, β_2-adrenergic receptor C-terminally fused to the donor cyan fluorescent protein (CFP) and β-arrestin fused to the acceptor yellow fluorescent protein (YFP) will undergo energy transfer if they are closer than 100 Å [56]. This system has been used to show that the initial kinetics of β-arrestin–YFP is limited by the kinetics of GRK-2-mediated phosphorylation of the receptor [56]. Furthermore, repeated stimulation of the receptor leads to accumulation of GRK-phosphorylated receptor that can bind β-arrestin very rapidly. The first use of FRET to evaluate the kinetics of GPCR-mediated G-protein activation in living cells (*Dictyostelium discoideum*) utilized recombinant expressed fluorescent protein fusions of α (fused to CFP) and βγ subunits (fused to YFP) [57,58]. Azpiazu and Gautam [59] recently reported the use of CFP-tagged Gα subunits and YFP-tagged β subunits in a FRET study to evaluate muscarinic receptor activation of a G protein; activation of the receptor reduced FRET. This system was used to demonstrate that sensor molecules are sensitive to the sequence of activation and receptor numbers, and showed that receptors and G proteins function as mutually exclusive multimolecular complexes. Using a different approach, Hoffman *et al.* [60••] introduced both CFP and a small membrane-permeable fluorescein derivative, called FlAsH, into a short tetracysteine sequence of the human adenosine A_{2A} receptor. Using this system they were able to monitor receptor activation in live cells by FRET. The utility of these new live cell based approaches for the development of generic high-throughput functional screening of GPCRs remains to be established, but we feel that they hold substantial promise.

High-content imaging

High-content imaging is a new technology platform that generally allows measurement of a number of assay variables at one time. It is an assay technology that has continued to mature in recent years. High-content assays for GPCRs generally rely on the measurement of protein translocation or receptor internalization and trafficking [61–63]. These assays were developed very early on in the high-content field and have consequently achieved a significant level of validation and acceptance in the industry. Future developments are likely to involve the application of the technology to target validation following hit identification in screening campaigns.

Assay throughput continues to be an issue that limits the use of high-content imaging for larger primary screening campaigns; however, throughputs of around 100 plates per day are now claimed by some vendors. High-content imaging can therefore be considered as a primary screening tool for carefully selected targets, such as orphan GPCRs with poorly defined G-protein signaling properties and 7-TM proteins which are more distantly related to traditional GPCRs. For example, Borchert *et al.* [64]

recently reported the development of a high-content assay for activators of the Wnt-Frizzled pathway based on the translocation of β-catenin. The assay was used to screen a library of 51 000 compounds and produced a reasonable 0.6% hit rate.

Conclusions

There are several options for developing high-throughput GPCR functional assays with a number of assay kits commercially available. The emphasis for new assays has shifted from radiometric to fluorescence- or chemiluminescence-based endpoint detection together with the use of homogenous microtiter-plate-based platforms. Several new paradigms concerning GPCR expression, signaling and regulation need to be considered when developing functional GPCR screens and the use of therapeutically relevant cells expressing physiological densities of GPCRs is advised for GPCR screen development. Developments in FRET and BRET have enabled the development of live cell GPCR biosensors that may be configured into high-throughput formats in the near future. In addition, advances in automated high-content imaging will ultimately lead to development of new primary screening technologies.

Update

A recent review by Milligan and Bouvier [65] describes in depth the application of FRET and BRET to study GPCR–protein interactions such as GPCR dimerization. The benefits and limitations of these methodologies are comprehensively covered.

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