High-content assays for ligand regulation of G-protein-coupled receptors

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High-content assays rely on the imaging of cellular events. They can be used to monitor the activation of G-protein-coupled receptors (or other receptors), their internalization into the cell, or alterations in their amount. In addition, multiplexed assays can provide further information about the characteristics of the receptor. Recent improvements in throughput using high-content screening platforms means that such assays are now an integral element of functional analysis in the drug discovery process.

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▼ G-protein-coupled receptors (GPCRs) are the most amenable set of targets for small-molecule medicines in the pharmaceutical industry. Despite the considerable number of clinically effective medicines that function by binding to GPCRs, the absolute number of GPCRs that are targets for current medicines represents only a small subset of the total encoded by the human genome. The human genome sequencing programme identified a large number of prospective GPCRs [1,2], and recent analysis has predicted a total of 367 non-chemosensory GPCRs in humans [3]. The endogenous ligands for a significant number of these GPCRs are still unknown. De-orphanization of ligand–GPCR pairings is thus a major focus of many companies who hope that many of these GPCRs might be identified and subsequently validated as potential therapeutic targets [4–7]. Recently, owing to the desire to identify ligands that interact with orphan GPCRs, emphasis has shifted from ligand-binding assays to functional assays [8] because it is clearly impossible to perform binding assays in the absence of a ligand. Many functional assays have been based either on elevation of intracellular Ca^{2+} [7,9] or on the promotion of transcription to enable synthesis and detection of reporter gene constructs [10].

Assays based on agonist-mediated elevation of intracellular Ca^{2+} have been particularly effective in de-orphanization of GPCRs [7]. However, several other assays are available that rely on cell imaging and the ability of either the GPCR itself, or a second protein that interacts with it, to alter in cellular location, amount, or spectral properties in a ligand-dependent manner. Such approaches are generically termed high-content assays [11] as several pieces of information about the behaviour of the GPCR can be gathered in parallel and because they offer a means to multiplex assay endpoints. Previously, such high-content assays were limited by a relatively slow throughput and cumbersome data analysis systems. However, significant improvements in both the instrument platforms available and the algorithms used to analyse the cellular location and trafficking of proteins has encouraged their widespread use. Several assays are now available that are well-suited to high-content screening.

Assays based on protein translocation

Variants of the autofluorescent green protein from the jellyfish *Aequoria victoria* have been developed that have both altered emission spectra and enhanced fluorescence properties, and this has revolutionized many aspects of cell biology in recent years [12,13]. In addition, it opened up possibilities for the development of ligand-screening assays for GPCRs based on cell imaging. That the vast majority of GPCRs internalize from the cell surface into acidic endosomes in response to agonist challenge has been well-established for many years [14,15]. However, direct visualization of this process following expression of a form of the β_2-adrenoceptor, in which a green fluorescent

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protein (GFP) was appended to the C-terminal tail of the GPCR [16], initiated interest in this process as a direct screening strategy. Importantly, attachment of such polypeptides to the C-terminus of GPCRs does not generally disrupt ligand binding, and – at least in the types of heterologous cell expression systems favoured for ligand screening – does not appear to significantly alter GPCR internalization and recycling [17,18]. A significant number of GPCRs have now been tagged in this way. Although ligand-induced-internalization of GPCRs is generally viewed as an indication of agonism, this is not necessarily the case. Internalization of the rat cholecystokinin-A receptor by a ligand devoid of efficacy in second messenger generation assays has been reported [19]. However, the extent of internalization for the majority of GPCRs is efficacy-dependent, with partial agonists generally less effective than full agonists. The opioid receptors have been particularly well studied in this regard. It is generally considered that the relatively poor ability of morphine to cause internalization of the μ opioid receptor reflects the partial agonist nature of this compound.

Limitations in monitoring GPCR internalization

High-content screening platforms are essentially pseudo-confocal imaging systems developed to analyse multiple cells in microtitre plate wells simultaneously. Such platforms rely on the availability of specific algorithms that measure the appearance and intensity of intracellular fluorescent patches or aggregates. Potential limitations on the quality of data that might be obtained using this approach arise from two issues. First, when expressed in heterologous cell systems, a significant fraction of many GPCRs is incompletely processed and not delivered effectively to the plasma membrane [20]. This is the case in cell lines that either stably or transiently express the GPCR construct of interest. In addition to GFP variants being autofluorescent, the endoplasmic reticulum or Golgi organelles in which they are trapped are often close to the recycling endosomal pools that contain GPCRs internalized in response to ligand challenge; signal-to-background ratio can therefore be limited. Furthermore, the steady state between plasma-membrane-localized GPCR and internalized GPCR reflects the balance of internalization and recycling. In certain cases, the fraction of GFP-tagged GPCR that becomes internalized can be relatively minor. Despite these concerns, high-content screens have been successfully applied to ligand-induced internalization of a range of GPCRs [21], and data with acceptable reproducibility have been obtained (Fig. 1).

Translocation of GPCR-interacting proteins

An equally generic assay derives from the interaction of β-arrestins with GPCRs that have become phosphorylated in response to agonists. This interaction is central to the internalization of GPCRs via clathrin-coated pits owing to the ability of β-arrestins to interact with clathrin adapter proteins [22]. Although specific details of the processes remain unclear, agonist-occupation and the associated phosphorylation of GPCRs can result in massive and rapid translocation of GFP-tagged forms of β-arrestins from the cytosol of transfected cells to the plasma membrane [23]. Thus, GPCRs can essentially be resolved into two categories: (1) high-affinity interactions, where β-arrestins co-internalize with the GPCR, and (2) low-affinity interactions, where β-arrestins separate from the GPCR at an early stage in GPCR internalization [24]. High-affinity interactions appear to be defined by groups or patches of aliphatic hydroxy amino acids that presumably are sites of phosphorylation. In terms of high-content screens, high-affinity interactions of GPCRs with β-arrestin GFP isoforms are easier to analyse and quantitate because they result in redistribution of the GFP into punctate aggregates that are easily identified and scored. By contrast, translocation of any protein from cytosol to plasma membrane is difficult to monitor and quantitate owing to limitations associated with defining the plasma membrane at the edge of cells, and developing effective algorithms.

Both β-arrestin-1 and β-arrestin-2 (also known as arrestin-2 and arrestin-3, respectively) have been used in such assays. There have been reports of certain GPCRs showing significant selectivity for β-arrestin-1 versus β-arrestin-2, but such reports are few. However, it appears that, overall, β-arrestin-2 generally displays equivalent-or-higher affinity for agonist-occupied GPCRs than β-arrestin-1; as such, different forms of β-arrestin-2 tagged with fluorescent proteins have been widely used in screening programmes. The key hydroxy amino acids that appear to define high-affinity β-arrestin interactions are frequently located in the C-terminal tail of GPCRs. Thus, high-content screening applications can use replacement of the wild-type C-terminal tail of a GPCR with one known to interact effectively with β-arrestins. Clearly, consideration must be given to whether this might alter the pharmacology of a GPCR, but, in general, this region is not central to ligand binding. Although we have only tested a relatively small number of GPCRs, the C-terminal tails of the rat thyrotropin-releasing hormone receptor-1 and the human orexin-1 receptor are among our 'gold standards' in this regard. Norak Biosciences (http://www.norakbioscience.com/) hold key patents on the use of β-arrestins in such translocation assays [25]; others have therefore modified the means of detection of such interactions. For example, agonist regulation of GPCR–β-arrestin interactions appears to be well-suited to bioluminescence resonance energy transfer techniques [26,27].

However, as this technique is not suitable for single-cell or cell imaging assays, it cannot be considered as a high-content approach.

Several other proteins translocate in the cell in response to GPCR activation and might thus be suitable for high-content screens. Significant attention has been given to the pleckstrin homology (PH) domain of phospholipase-C-δ1 as a monitor of the inositol phospholipids–inositol 1,4,5-trisphosphate signalling pathway [28,29]. In contrast to the systems discussed earlier, one drawback of this and other potential systems is that it is essentially a kinetic assay rather than an endpoint assay because translocation is transient even in the continued presence of an agonist. Thus, correct timing of the assay is essential and might alter from cell-type to cell-type and for each individual GPCR screened.

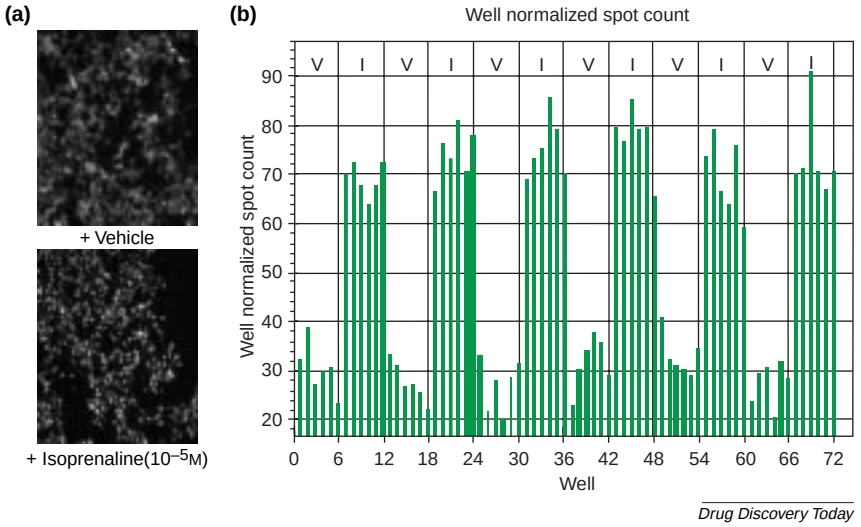

Figure 1. High-content analysis of agonist-stimulated internalization of a β_2-adrenoceptor–green fluorescent protein (GFP) fusion protein. HEK293 cells stably expressing a β_2-adrenoceptor–GFP fusion protein [34] were seeded into wells of a microtitre plate. Individual wells were treated either with vehicle or 10^{-5}M isoprenaline for 30 mins. **(a)** Cells were subsequently imaged using a Cellomics Arrayscan II (http://www.cellomics.com) and **(b)** the well-normalized spot count measured.

Assays based on altered protein concentration

All GPCRs display some level of constitutive activity. That is, they are able to transduce signals in the absence of an agonist ligand. The extent of constitutive activity varies significantly between different GPCRs and can often be increased substantially by judicious mutagenesis [30]. Many of these mutations also result in destabilization of the GPCR such that it denatures more easily and displays a reduced half-life in cells [31–34]. Importantly, binding of ligands to such mutationally modified GPCRs rescues the destabilized phenotype [35]. In the absence of any specific adaptive processes, the steady-state level of any protein in a cell represents a balance between its rate of synthesis and degradation. Therefore, in this situation the effect of the ligand is to cause upregulation of the protein [36].

Introduction of mutations into a GPCR might alter its function or detailed pharmacology. However, the pragmatic view, particularly when screening for small-molecule ligands for orphan GPCRs, is that hits from such screens can subsequently be characterized against the wild-type receptor. Attachment of a reporter protein such as GFP to the C-terminal tail of an appropriately mutated GPCR thus provides a high-content assay for ligand-induced protein stabilization [37,38]. More quantitative assays can be developed by attachment of easy-to-assay reporter proteins such as *Renilla luciferase* [39] or attachment of a G protein [40] to the C-terminal tail of the destabilized GPCR. Indeed, following co-expression of a constitutively active and destabilized mutant of the β_2-adrenoceptor tagged at the C-terminus with *Renilla luciferase*, and an equivalent mutant of the α_{1b}-adrenoceptor C-terminally tagged with GFP, parallel measurement of fluorescence and luminescence enabled detection of compounds selective between these two GPCRs [39]. Such upregulation is both ligand-concentration dependent and time-dependent.

At least conceptually, it might be anticipated that the observed EC_{50} for ligand-induced upregulation should be highly correlated with the K_i to bind the mutated GPCR [37]. In several examples, good correlations of this type have been observed [39]. However, this is not always the case, with the EC_{50} being significantly higher [38]. The basis for these discrepancies has not been systematically explored; however, as the period of exposure to ligands in such assays is usually in the region of 24h, issues such as ligand stability need to be explored. Furthermore, studies where EC_{50} is significantly higher than the K_i will probably result in a lack of detection of compounds with low affinity. However, as there is no requirement for previous knowledge of ligands for the receptor, this type of assay might be useful for the identification of antagonist ligands for orphan GPCRs in small-molecule chemical libraries.

Assays based on altered spectral properties

The ideal assay for a high-content screen for GPCR activation would involve either a GPCR or an associated protein

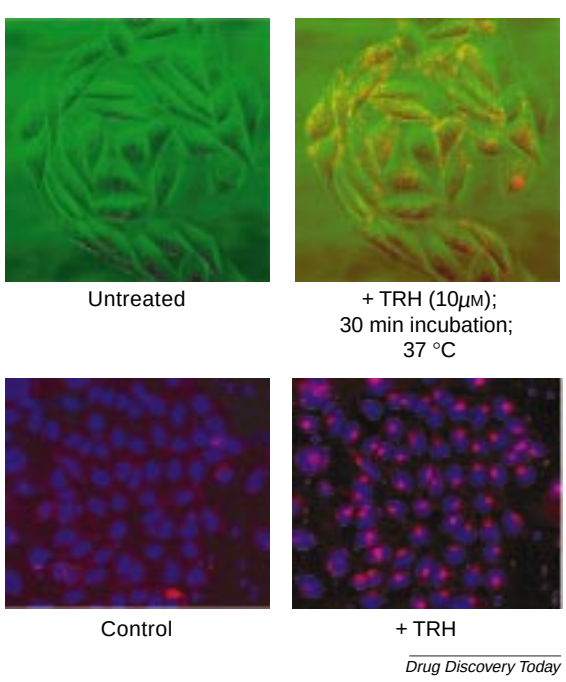

Figure 2. High content CypHer-5 assays for the activation of a VSV-tagged form of the thyrotropin releasing-hormone (TRH) receptor-1. **(a)** HEK293 cells stably expressing an N-terminally VSV-tagged form of the rat thyrotropin releasing hormone receptor-1 were untreated or exposed to TRH (10 μM) for 30 min at 37°C. Patches of cells were then visualized to detect fluorescence of CypHer-5 (red). **(b)** An N-terminally VSV-tagged form of the rat TRH receptor-1 was expressed stably in CHO cells that were pre-incubated with anti-VSV antibody labelled with CypHer-5 and Hoechst nuclear stain (blue). Cells were then treated with TRH (10 μM) for 0 (control) or 30 min (+ TRH) at 37°C and imaged on an Amersham Biosciences INCell analyser 3000. Red colour represents protonated CypHer-5 and reflects the internalized GPCR. Figure 2b was provided by Elaine Adie, Amersham Biosciences (http://www.amershambiosciences.com).

whose spectral properties change in response to activation. Potentially, assays based on resonance energy transfer techniques can offer this. Indeed, as GPCRs appear to exist and potentially function as dimeric or oligomeric species [41,42], there was considerable interest in several reports indicating that agonist ligands could induce or inhibit GPCR dimerization [43].

In either fluorescence or bioluminescence resonance energy transfer studies, the effectiveness of energy transfer is crucially related to the distance between the partner proteins [44]. Ligands that alter the interactions between GPCR monomers would thus be expected to alter the energy transfer signal. This has indeed been observed in several studies [45–47]. However, although the details remain contentious, it is clear that, in many cases, addition of GPCR ligands to cells expressing GPCRs tagged with resonance energy transfer-competent molecules does not result in alterations in energy transfer [48–50]. These studies indicate that many GPCRs do not alter their state of oligomerization in response to ligands. This lack of generality means that such a methodology does not offer a practical general approach to GPCR ligand screening.

A more promising approach relates to the dissociation of G proteins into separate α- and β/γ-subunits stimulated by GPCR activation. FRET-competent variant G protein α- and β-subunits from *Dictyostelium discoideum* have been produced by fusion to cyan- and yellow-fluorescent proteins. Addition of the chemoattractant cAMP as agonist for the GPCR resulted in a reduced FRET signal [51,52]. Perhaps more surprisingly, G-protein activation (as monitored by FRET) reached a dose-dependent steady-state level; the G protein heterotrimer would normally be expected to rapidly dissociate and reassociate upon during continuous stimulation. Although the authors suggest that construction of similar energy-transfer pairs of mammalian G proteins should enable direct *in situ* mechanistic studies and their application in ligand-screening programmes, such a task is not trivial. Although G proteins from different species are highly homologous, and many groups have generated functional forms of mammalian G proteins that incorporate autofluorescent proteins within the sequence [53–55], progress towards an equivalent FRET-based assay has been slow. However, progress is being made.

Multiplexed assays

Although based on an alteration of fluorescence intensity at the same wavelength – rather than a shift in the spectrum – the recent development of pH-sensitive cyanine dyes as a means of monitoring GPCR activation is currently one of the most promising novel approaches [56,57]. As discussed earlier, the vast majority of GPCRs internalize into acidic endosomal vesicles in response to agonist occupancy. CypHer-5 is a pH-sensitive cyanine dye derivative that, when excited with red laser light, fluoresces only when protonated. As the pKa of this dye is close to 6.1, significant fluorescence is only detected in an acidic environment. In intact-cell, high-content assays, GPCRs modified with an appropriate epitope tag in the extracellular N-terminal region can bind epitope tag antibodies labelled with CypHer-5. However, these antibody–GPCR complexes are not observed if the cell culture medium is in the region of pH 7.4 because the dye displays minimal fluorescence. The dye only fluoresces when the GPCR is activated by a ligand that results in internalization of both the GPCR and the CypHer-5-labelled antibody bound to it. This provides a red signal that can be easily monitored using either a conventional confocal microscope (Fig. 2a) or a high-content screening platform

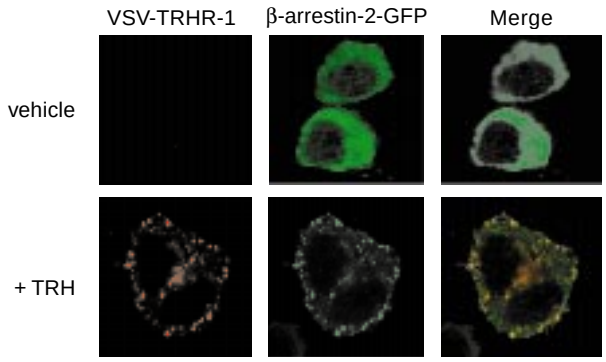

Figure 3. Multiplexing CypHer-5 and β-arrestin-2-GFP assays demonstrates high affinity interactions between the thyrotropin releasing-hormone (TRH) receptor-1 and β-arrestin-2. HEK293 cells were transfected to transiently express both an N-terminally vesicular stomatitis virus (VSV)-tagged form of the rat TRH receptor-1 and β-arrestin-2–GFP. CypHer-5-labelled anti-VSV antibody was added and samples incubated with vehicle or TRH (10 μM) for 30 min at 37°C. Cells were then visualized using a Zeiss confocal microscope (http://www.zeiss.com). In the absence of agonist, β-arrestin-2–GFP is evenly distributed in the cytosol but the GPCR is not detected because at the pH of the medium CypHer-5 does not display significant fluorescence. After agonist treatment both the GPCR and β-arrestin-2–GFP translocate. The red colour in the image of the GPCR indicates that the CypHer-5 associated with the GPCR is in an acidic environment. The yellow colour in the merged picture indicates that the GPCR and β-arrestin-2 are co-localized and were internalized as a complex.

(Fig. 2b) such as the IN Cell analyser 3000 (Amersham Biosciences; http://www.amershambiosciences.com). This approach has been used successfully to monitor agonist-mediated internalization of GPCRs that couple predominantly to each of the G_s-, G_i-, and G_q–G_{11}-mediated signalling pathways [57], and provides both a good signal-to-noise ratio and reproducible pharmacology, regardless of whether assays are run in agonist or antagonist detection formats. The only previous knowledge required to initiate a GPCR screen is that the labelled antibody is able to bind the epitope-tagged GPCR.

A clear advantage over simply monitoring the cellular redistribution of GPCRs tagged at the C-terminus with an autofluorescent protein is that, whilst a significant fraction of the GPCR might be trapped inside the cell (and not delivered to the plasma membrane), this is not accessible to the CypHer-labelled antibody and is thus not detected in the assay. Another significant advantage of the CypHer-5 dye is that it fluoresces at the red-end of the spectrum. This means that it can be used in parallel with other translocation assays that use GFP-tagged proteins. For example, following agonist stimulation of cells co-expressing a form of the rat thyrotropin releasing hormone receptor-1 tagged at the N-terminus with the vesicular stomatitis virus (VSV)-epitope and β-arrestin-2 GFP, the appearance of yellow intracellular vesicles indicates a high-affinity interaction between the two polypeptides owing to their co-internalization (Fig. 3). By contrast, introduction of β-arrestin-2 GFP and agonist stimulation of cells expressing a form of the β_2-adrenoceptor modified at the N-terminus with the VSV-epitope tag results in cells with red intracellular vesicles and a green corona at the plasma membrane. This indicates that, although the β-arrestin-2 GFP interacts with the β_2-adrenoceptor, it does so with relatively low affinity, and the two proteins are rapidly separated from one another.

A further useful but surprising feature of CypHer-5 is that, although cell fixation is expected to dissipate proton gradients, the dye becomes fluorescent when formaldehyde is used as the fixative. It is therefore possible to reuse plates of fixed cells for subsequent further analysis. This basic approach is potentially suitable for monitoring the internalization of other cell-surface receptors [58]. As the transactivation of growth factor receptors, (i.e. the receptor for epidermal growth factor) by GPCRs is a common process [59,60], it might be possible to develop generic screens for GPCR activation by monitoring internalization of a CypHer-5-labelled growth factor receptor. This would then act as a surrogate marker for GPCR activation.

Concluding remarks

High-content screening assays have become of increasing importance in the drug discovery process. This reflects both improvements in speed and analysis of samples, and the capacity to multiplex assays. Several assay formats are now available and this is expected to increase markedly in the future.

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