G protein-coupled receptor microarrays for drug discovery

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The dominance of G protein-coupled receptors (GPCRs) as a drug target class, coupled with the increased pace of target identification and expansion of compound libraries, presents a compelling need to develop technologies to screen multiple GPCRs simultaneously. To address this need, GPCR microarrays that require the co-immobilization of lipid molecules and the probe receptors of interest have been fabricated, using conventional robotic printing technologies. Assays to screen compounds for their pharmacological properties (binding affinity, relative potency and selectivity) using GPCR microarrays are discussed.

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▼ G-protein coupled receptors (GPCRs) represent the single most important class of drug targets – ~50% of current drugs target GPCRs, ~20% of the top 50 best-selling drugs target GPCRs: >US$23.5 billion in pharmaceutical sales per annum are ascribed to medications that address this target class [1,2]. GPCRs are associated with almost every major therapeutic category or disease class, including pain, asthma, inflammation, obesity, cancer, as well as cardiovascular, metabolic, gastrointestinal and CNS diseases [3]. The significance of GPCRs as a drug target lies in their physiological roles as cell-surface receptors responsible for transducing exogenous signals into intracellular response(s) [4,5]. There are 400–700 GPCRs in the human genome and ligands for ~200 of these have been discovered [3,6–8]. Although there is little conservation at the amino acid level among GPCR sequences, all of the receptors share a characteristic motif, consisting of seven distinct hydrophobic transmembrane regions (each is 20–30 amino acids in length), an extracellular N-terminus and an intracellular C-terminus (Fig. 1) [3–5].

Protein microarrays for drug candidate profiling

GPCRs are successfully used as drug targets because the binding of natural ligands to their paired GPCR(s) can be moderated by appropriate small molecule drugs [1,2]. Effective engineering of these drugs is essential, however, because aberrant binding to such a physiologically significant target class can lead to serious side effects. Structural data on GPCRs is limited and rational drug design is a challenge [3,9,10]; designing drugs that do not bind to non-targeted GPCRs is almost impossible. Currently, selectivity studies are conducted downstream in the drug discovery process; discarding compounds because of adverse binding at this stage makes the drug discovery process both expensive and time consuming. Given these considerations, and the strong possibility that so-called 'orphan' GPCRs – recently discovered as a result of the sequencing of the human genome [11,12] – could be valuable targets [6], there is a strong need for technologies that enable screening against multiple GPCRs simultaneously.

The value of the parallel analysis enabled by DNA microarrays [13] has inspired the development of protein arrays [14]. Beyond the use of protein abundance profiling as an analogue to gene expression profiling [15,16], protein arrays offer the possibility of highly parallel investigations of protein–small molecule and protein–protein interactions [17–20]. Because proteins are the primary candidates against which drugs are designed, the use of protein arrays for profiling candidate drug compounds is particularly attractive. Given the significance of GPCRs and other membrane-bound proteins (e.g. ion-channels), the lack of membrane-protein microarrays has been viewed as a major gap in protein microarray technology [14]. We recently fabricated GPCR microarrays using conventional robotic pin printing, and assays for screening compounds with these arrays [17–19].

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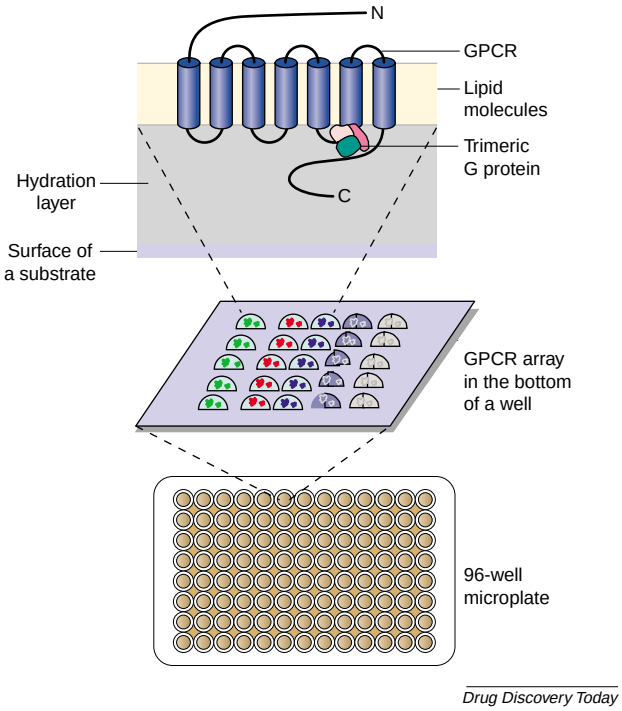

Figure 1. Schematic representation of a GPCR microarray in a 96-well microplate.

GPCR microarray technology

The fabrication of GPCR microarrays presents fundamentally different issues from that of conventional DNA and protein microarrays [17–19]. Figure 1 is an idealized representation of a GPCR immobilized in a microarray. GPCRs require association with a lipid membrane to retain its correctly folded conformations and functions [21], therefore, GPCR microarrays need co-immobilization of the probe GPCR and the lipid membrane in which it is embedded. Furthermore, the lipid membrane needs to be offset from the surface to avoid the physical contact-induced misfolding or dysfunction of the extra-membrane domains of the receptor. An additional requirement is the need for the receptor–lipid complex to be stably associated with the surface throughout the assay. Covalent immobilization of the entire membrane is not desirable because lateral mobility is an intrinsic and physiologically important property of native membranes. For GPCRs, the GPCR–G protein complexes should be preserved after being arrayed onto a surface because the correct configuration of the receptor and the G protein itself are prerequisites for the binding of agonists to the receptor with physiological binding affinities [4].

GPCR contents

Cell membrane preparations containing GPCRs from a cell line overexpressing the receptor can be used directly, for fabrication of GPCR microarrays [17]. These GPCR membrane preparations were obtained from commercial vendors [Biosignal Packard (currently distributed by Amersham Biosciences, http://www1.amershambiosciences.com); PerkinElmer Life Sciences, http://lifesciences.perkinelmer.com/index.asp; Euroscreen, http:// www.euroscreen.be; Sigma Chemical, http://www.sigmaaldrich.com], and used with or without further modifications. [The cell line used, the concentration of the active receptor (B_{max} in pmol mg^{-1} of total protein) and the total protein concentration were specified by these vendors]. As expected, experiments suggested that the B_{max} value is the single most important factor influencing array performance; B_{max} values of >1 pmol mg^{-1} are required to get a reasonable signal:background ratio (data not shown). Homogeneity and the buffer composition of membrane preparations also have a significant role in printing quality and array performance. The homogeneity affects the packing density and uniformity of membrane fragments within a microspot; more homogeneous membrane fragments result in improved printing reproducibility. The buffer composition of the membrane preparation not only affects the functionality of the membrane proteins but also the wetting and de-wetting properties of the pin used for printing – an important factor in producing arrays of high quality.

Because of the direct use of unpurified membrane preparations from cells overexpressing the GPCRs of interest, endogenous receptors or other membrane-bound proteins in the preparations might cause problems with assay sensitivity and binding specificity. However, following examination of >16 different GPCRs, binding specificity and assay sensitivity were considered to be acceptable [17–19; and additional data not published]. Although there is no fundamental limit to the number of GPCRs in an array, practical limits on numbers are imposed by buffer compatibility, crosstalk between different ligands and a given receptor, and the availability of labelled ligands with high specificity and binding affinity.

Surface chemistry

Despite the discovery of supported lipid bilayers almost two decades ago [22], the interaction between membranes and surfaces is still poorly understood. It is believed to involve a delicate interplay of hydrophobic, electrostatic and surface hydration forces, that depends on the composition of the membrane and the functionality of the surface [23]. The nature of the surface determines the structure of lipid membranes supported on a substrate [18] and, thus, it was hypothesized that an ideal surface chemistry is one that provides supported membranes with the following characteristics: lateral fluidity, mechanical stability and correctly

folded conformation(s) of embedded proteins. Previously, lateral fluidity was the primary focus [23], however, all three characteristics were now considered significant for the practical realization of membrane microarray technology.

Lateral fluidity was examined using traditional fluorescence recovery after photobleaching (FRAP) experiments [23]. Tests for stable association with the surface and correct folding of the extramembrane domains were utilitarian in nature. The stability of supported membranes was examined by the ability of printed membrane microspots to resist desorption when drawn through the buffer–air interface. Owing to the importance of the extracellular N-terminus for ligand-binding to GPCRs, the ability of printed GPCR microspots to specifically bind to cognate ligands would provide information on whether the extramembrane domain was correctly folded and, simultaneously, serve as the litmus test for the technology.

A variety of surfaces have been investigated – some by design and some by screening. Of those tested, amine-presenting surfaces provide the best combination of those characteristics outlined previously: model lipid membranes are immobilized onto amine-presenting surfaces [e.g. γ-aminopropylsilane (GAPS)-derivatized surfaces] with rapid kinetics while maintaining the desired structures (preserved lateral fluidity and significant mechanical stability) [17; and additional data no shown]. Ligand binding to GPCR microarrays on these surfaces is specific; binding affinities are similar to those obtained using traditional methods [17–19]. Here, the focus will be on GPCR microarrays on GAPS-coated surfaces.

Printing technologies

Membrane microarrays can be fabricated in two, fundamentally different, ways. The first approach uses the direct immobilization of membranes onto micropatterned substrates consisting of membrane-binding and non-membrane-binding regions [24,25]. However, extending this approach to the fabrication of microspots of different compositions is challenging because of registration issues. The second approach uses direct printing of solutions of membranes or membrane proteins onto membrane-binding surfaces. Previously, printing of biomembranes was performed on substrates immersed in a buffer because of concerns about reduced membrane stability upon exposure to air [26–28].

The advent of DNA microarray technology has led to the development of several printing technologies that are capable of production-scale fabrication; the adoption of these methods with appropriate modifications would greatly facilitate the fabrication of membrane microarrays. It is known that lipid membranes are susceptible to environmental changes; lipid membranes supported on solid substrates are especially susceptible to desorption and/or deterioration in structure when withdrawn through an air–water interface [24,28]. As discussed previously, these issues have impeded the development of membrane microarrays, hence, the printing technologies referred to here generally involve printing and post-printing processes under ambient conditions.

Quill- and solid-pin printing have been examined as methods for fabricating GPCR microarrays; quill-pin (by Cartesian Technologies, http://www.cartesiantech.com) has been found to be more suitable for the large scale fabrication of GPCR microarrays [17,18]. Considerable optimization is required to print high-quality microarrays – currently, a single insertion of the pin into the GPCR sample can yield several hundred microspots leading to sample usage of ~0.5 nL or less per data point under optimal conditions. Other printing technologies, such as piezo type and ink-jet printers should be feasible but, as yet, have not been tested. It is important to understand the potential issues arising with each particular printing technology. For example, thermal ink-jet printing might adversely affect the integrity of GPCR complex, due to denaturation of the proteins or as a result of phase transitions of the membrane itself.

Labelled ligands

The work here has focused primarily on fluorescence detection because of the availability of fluorescence-based microarray scanners. However, the need for labelled ligands might limit the implementation of this technology in the drug discovery process. There are increasing numbers of fluorescently labelled ligands that are commercially available (see: Molecular Probes, http://www.probes.com; Phoenix Pharmaceuticals, http://phoenixpeptide.com; Amersham Biosciences; PerkinElmer; Sigma Chemical). Radioactivity-based detection should also be feasible, using high-resolution phosphorimagers (e.g. the Typhoon™ 9410, Amersham Biosciences), and could even provide superior data relative to fluorescence-based detection because of reduced problems with non-specific binding.

The ideal fluorescent ligands for GPCR microarray applications should have a high binding affinity (K_d) of several nanomolar, and a high specificity. For multiplexed binding assays using GPCR microarrays, a cocktail of fluorescently labelled ligands might be required. Alternatively, a single type of labelled ligand could be used as a probe for several different receptors, should it bind to these receptors with the desired affinity and specificity. For example, Bodipy-TMR (tetra- methylrhodamine)-CGP12177 binds to the β1 and β2 adrenergic receptors with similar affinity (K_d of 1–2 nM, data not shown). CGP12177 is an antagonist to β1

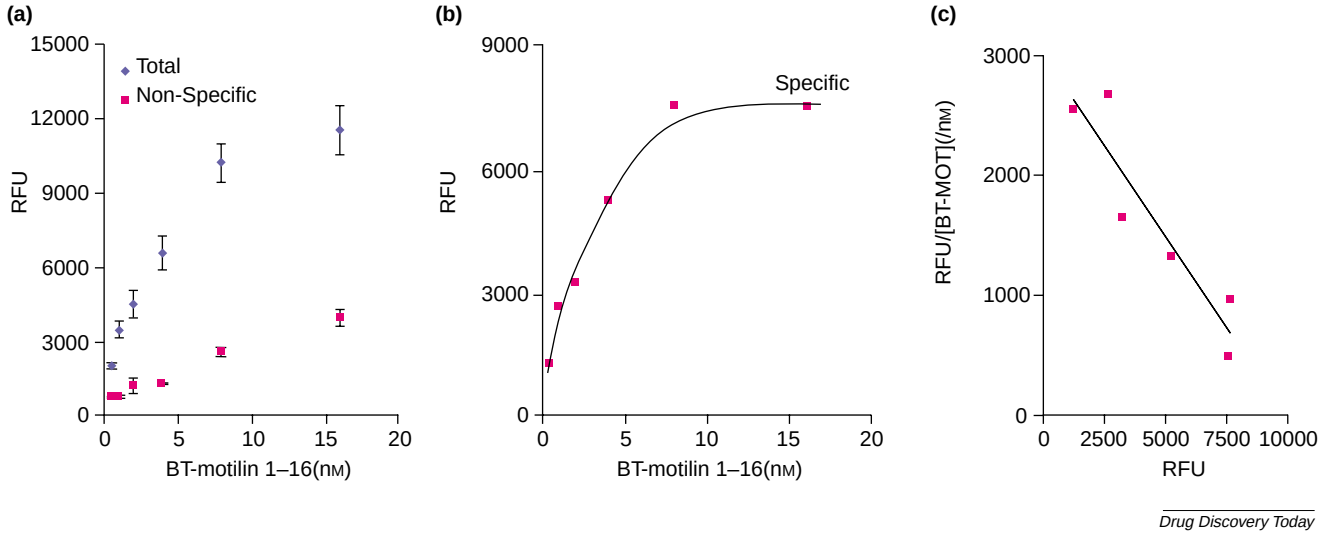

Figure 2. Saturation profile for the binding of fluorescently labelled ligands to GPCRs in the microarrays. **(a)** Plot of the fluorescence intensity [in relative fluorescence units (RFU)] of motilin receptor (MOTR) arrays as a function of the concentration of Bodipy-TMR-motilin 1–16 in the absence (equal to the total signal) and presence (representing the nonspecific signal) of excess unlabelled motilin 1–16. **(b)** Plot of the fluorescence intensity of the MOTR arrays due to the specific binding as a function of BT-motilin 1–16. **(c)** Scatchard plot for estimating the binding constant for BT-motilin 1–16.

and β2 receptors [29]. It is not essential that the labelled ligands give rise to similar intensity after binding to their respective receptors on the microarray. The effects of different compounds or compound concentrations on receptors can be measured in several ways, including absolute signal intensity, relative signal intensity or a ratiometric analysis with the use of two different dyes. Labelled ligands in the cocktail could be agonists and/or antagonists; the concentration of each ligand in the cocktail should be slightly greater (1–10-fold) than the K_d of the ligand to the paired receptor(s) to maximize total binding signal(s).

Bioassays for compound profiling

Several types of assays for compound profiling using GPCR microarrays have been described:

(i) Saturation assay to determine binding constants of labelled ligands to their receptors [17,18]. To do this, two subsets of microarrays are incubated individually with a buffered solution containing labelled ligand at different concentrations in the absence and presence of its unlabelled counterpart ligand in excess. The amount of specific binding is determined by subtracting the fluorescence signals of the first set of arrays from those of the second set of arrays, incubated with the labelled ligand (at the same concentration) and excess unlabelled ligand. Scatchard analysis was used to estimate the K_d [18]. To date, data has been obtained on more than 10 receptor–ligand pairs and the affinities estimated using microarrays are essentially the same as those obtained using other methods. An example – the binding of BT-motilin 1–16 to human motilin receptor microarrays – is shown in Figure 2.

(ii) Competitive binding assay to examine the relative potency of compounds against the binding of a pre-selected labelled ligand to receptor(s) [17,18]. For this purpose, multiple sub-arrays are treated individually with a given compound at varying concentrations in the presence of a labelled ligand or a cocktail of labelled ligands at a fixed concentration. The fluorescence intensity of each receptor microspot is examined as a function of the compound concentration, and an IC_{50} value is subsequently extracted. The relative potencies of two different ligands (ICI118551 and xamoterol) against the binding of Bodipy-TMR-CGP12177 have been examined using human β1-adrenergic receptor microarrays (Fig. 3); results show that the relative potencies obtained using β1 microarrays are comparable to those obtained using homogeneous assays.

The tolerance of GPCR microarrays to DMSO (dimethyl sulfoxide) has been examined; DMSO concentrations of up to 5% in the assay solution have no obvious effect on ligand binding to the receptors in the microarrays (data not shown).

Bioassays for compound selectivity screening

The selectivity of a potential drug compound for a targeted GPCR over other GPCRs is an extremely important factor in drug development. GPCRs can be selectively arrayed –

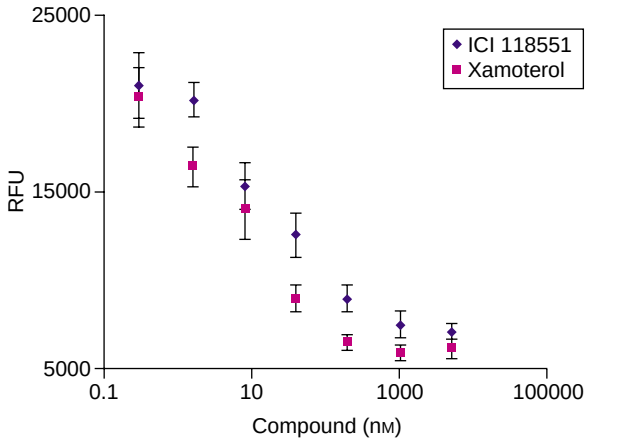

Figure 3. The concentration-dependent inhibition of Bodipy-TMR-CGP12177 (BT-CGP) binding by ICI118551 and xamoterol to microarrays of the β1-adrenergic receptor (β1) [shown in relative fluorescence units (RFU)]. The relative differences in affinity are consistent with the known relative potencies of these inhibitors.

functionally, physiologically, or family-based; and the 'themed' GPCR arrays can be used for compound selectivity screening:

Screening compounds against GPCRs from different families

A microarray consisting of the β1-adrenergic receptor, neurotensin receptor subtype 1 (NTR1) and dopamine-receptor subtype 1 (D1) was used to demonstrate this assay [17]. The results show that fluorescently labelled neurotensin (BT-NT) specifically binds to NTR1 in the microarrays; only excess neurotensin, (not CGP12177 nor SCH23390) inhibits the binding of BT-NT to the NTR1 [17] – neurotensin is a natural agonist for NTR1. These results demonstrate that the immobilized GPCRs retain their native specificity in the array.

Screening compounds against GPCRs within a family

Microarrays consisting of three members from the adrenergic receptor subfamily (β1, β2, and α_{2A}) have been used for compound selectivity screening [17,18]; the results show that fluorescently labelled CGP12177 specifically binds to the β1 and β2 arrays. Furthermore, ICI118,551, a drug compound targeted towards adrenergic receptors, was found to have significantly higher affinity for β2 (K_i value of 0.6 nM) relative to β1 (K_i value of 25 nM) [17,18], consistent with that reported in the literature [30]. The ability to measure small changes in fluorescence (~twofold) confirms the overall robustness of these assays and because the affinity of a ligand depends on whether the GPCR is complexed to a G-protein, the data indirectly indicate that the GPCR-G protein complex is preserved in the microspots.

Applications of GPCR microarrays in drug discovery

A wide range of technologies are available to screen compounds against GPCRs. The increased pace of target identification [11,12] and the increasing size of compound libraries [9,10] continues to drive the development of these technologies, which can now be classified as either cell-based or GPCR-membrane-based [31]. The cell-based assays use intact cells that express or overexpress a GPCR of interest; these assays offer the advantage that the functional activation of GPCRs by candidate compounds can be monitored – readout is mainly based on the generation of secondary messengers [e.g. Ca^{2+}, cAMP, Ins(1,4,5)P_3 and so on]. Cell-based assays, including reporter gene assays, β-arrestin and GPCR-GFP translocation assays (i.e. receptor internalization and endosome formation) have been described [31]. GPCR-membrane-based assays use membrane preparations obtained from a cell-line stably overexpressing the receptor. Compound binding is monitored through competition assays, using a fluorescent or radioactive ligand as a probe [4]. Non-cell-based assays to monitor the activation of GPCRs are mostly limited to monitoring GTP-GDP exchange at the GPCR-associated Gα protein using GTP analogues (^{35}S -GTPγS or Eu-GTP) [4,32].

Despite the interest and the overwhelming number of current and future GPCR targets, few methods have been described for the simultaneous study of multiple GPCRs. Recently, two research groups have suggested that arrays of transiently transfected cell clusters or GPCR-transfected cells on barcoded substrates, could be used for multiplexed compound screening [33,34]. (GPCR microarrays are naturally suited to the simultaneous analysis of multiple GPCRs.) Figure 4 suggests how GPCR microarrays can contribute to the various stages of drug discovery – they can be used at all stages of drug discovery before preclinical testing, although their greatest relevance is in the lead identification and validation stages (Fig. 4a). To date, most studies have involved spatially segregated arrays on slides, although the feasibility of fabricating and using GPCR microarrays in a microplate format has also been examined. An ideal product would be GPCR microarrays at the bottom of 96- or 384-well plates (Fig. 4b), the choice of content depending on the stage of drug discovery and individual customer needs, as discussed in the following section.

Target validation

One of the fundamental applications of protein arrays is target validation. By profiling the differential expression of proteins using antibody arrays and correlating the changes to a disease phenotype [14–16], several putative targets (and biomarkers) to a particular disease can be identified. GPCR microarrays (and microarrays of other receptors or

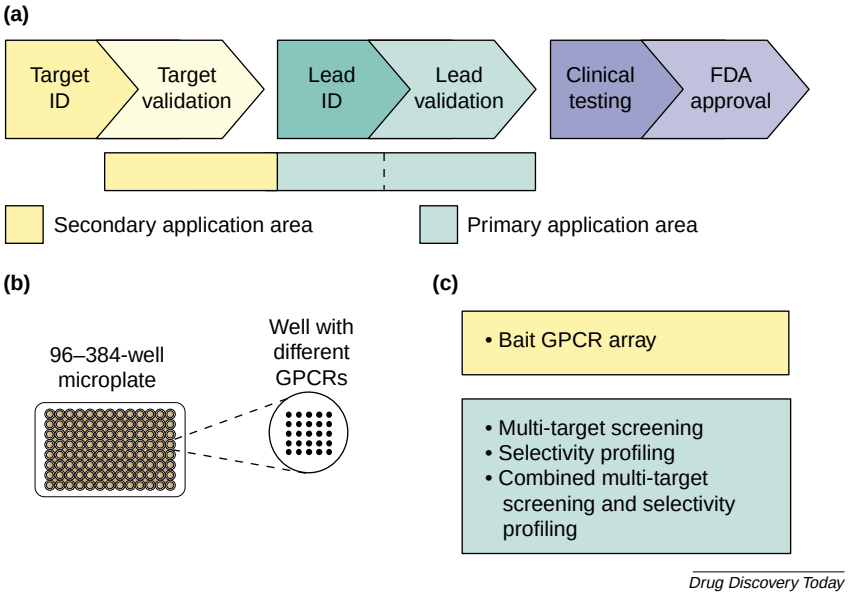

Figure 4. The use of GPCR microarrays in drug discovery. **(a)** Drug discovery processes and the targeted applications of GPCR microarray technologies. **(b)** Potential product concept: microarrays of multiple GPCRs in 384-well microplate. **(c)** GPCR microarrays can also be used in target identification and are well suited for applications in lead identification and optimization.

membrane-bound molecules) could also serve an analogous function as bait molecules to identify differentially expressed proteins (other 'bait' molecules include peptides and aptamers).

Lead identification and lead validation

Although membrane microarrays could prove to be useful in target validation, the primary application of GPCR microarrays is in the area of lead identification and validation. Lead validation ('secondary screening') will be discussed first because the applicability of GPCR microarrays to biochemical pharmacology studies (potency, selectivity) is clear from the experiments described previously. A key question, however, is whether the binding assays can be conducted in a multiplexed manner; that is, whether the assays are feasible if the sample 'target' solution contains a cocktail of labelled ligands and one (or more) candidate drug compound(s). Experiments to demonstrate the feasibility of estimating K_d values for labelled ligands (crosstalk issues might limit the number of receptor–ligand pairs) simultaneously with IC_{50} values for compounds (unpublished results) have been successful. The use of label-independent methods of detection will obviate the need for competition assays and, therefore, the need to use cocktails of labelled ligands [35].

The use of GPCR microarrays for identifying 'hits' during primary screening is equally powerful. Two scenarios are discussed here: primary screening against GPCRs with known ligands and screening against orphan GPCRs.

GPCRs with known ligands The technology described previously can be adopted readily once robustness and feasibility in a 384-well format is demonstrated. The use of arrays containing both GPCR targets and other GPCRs that would subsequently be the subject of selectivity studies is envisioned. The ability to integrate aspects of hit identification and selectivity screening into one assay is attractive because it can streamline the process of drug discovery significantly and can lead to substantial cost savings.

Orphan receptors Cell-based assays [for example, the measurement of Ca^{2+} flux using a fluorometric imaging plate reader system (FLIPR®, by Molecular Devices, http://www.moleculardevices.com) dominate primary screening [31]; the lack of known ligands precludes the use of competition assays, and a random screen of available labelled ligands would be ineffective by comparison with monitoring GPCR activation using cell-based assays. Nevertheless, cell-based assays are cumbersome, difficult to reproduce and not suited to screening against multiple GPCRs.

Figure 1 suggests that the GPCR–G protein complex is maintained in the microarray, yielding the interesting possibility of using GPCR microarrays in the study compound agonism. Preliminary results suggest that it is possible to monitor the activation of arrayed GPCRs using GTPγS analogues, although further experimental work is required to confirm feasibility (data not shown). The ability to conduct functional assays using GPCR microarrays opens the door for de-orphanizing 'orphan' receptors – an exciting possibility that would change our perception of the potential of protein arrays.

Concluding remarks

GPCR microarrays comprise a powerful drug discovery technology. It offers multiplexing, requires minimal sample quantities, offers archivability of precious receptors, is amenable to a variety of platforms, including HTS and can be used broadly across several stages of drug discovery. The monitoring of agonism (or antagonism) using GPCR microarays is also a very exciting possibility. Most of the infrastructure and several key components needed to

implement this GPCR microarray technology in drug discovery are commerically available. For example, the newest generation of Cartesian printers provide the ability to print large numbers of GPCR microarrays in a microplate format, and several scanners that enable the detection of microarrays in a microplate are available [e.g. the LS 400 fluorescence detection scanner from Tecan (http://www.tecan.com) and the Typhoon™ 9410 for radioactive detection]. GPCR microarrays can be stored for extended periods of time under dry conditions at 4°C – an additional practical advantage that should see GPCR microarrays emerge as one of the dominant technologies for screening against this most important class of targets.

GPCRs constitute one class of key membrane-bound molecules; however, the scope of membrane microarray technology extends well beyond GPCRs. The fabrication of microarrays containing GPCRs and the epidermal growth factor (EGF)-receptor [35] have been demonstrated, as has multiplexed compound screening using these arrays (unpublished results). The use of microarrays of lipids derivatized with carbohydrates (gangliosides) for screening of toxins and inhibitors has been published recently [36].

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