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Identification of Orphan G Protein-Coupled Receptor Ligands Using FLIPR® Assays

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1. Orphan GPCRs

G protein-coupled receptors (GPCRs) make up the largest and most diverse family of transmembrane proteins and respond to a wide variety of stimuli including biogenic amines, peptides, bioactive lipids, hormones, and light (***1,2***). Agonist binding to these receptors activates intracellular signalling events mediated by G proteins, such as modulation of intracellular cyclic adenosine monophosphate (cAMP) levels or Ca^{2+} mobilization. To date, there are approx 250 characterized nonsensory GPCRs and a further 140 genes predicted to be GPCRs for which the endogenous or natural ligand is unknown—the "orphan" GPCRs (oGPCRs) (***3–5***). Historically, GPCRs, especially those in the aminergic receptor subfamily, have proved amenable to the design of synthetic agonists and antagonists of their activity. Of the top-selling prescription drugs in 2002, more than 33% act through GPCRs and provide greater than $25 billion in worldwide pharmaceutical sales. Therefore, considerable effort has been made to identify cognate ligands for oGPCRs and functionally characterize these receptors in order to elucidate their physiological and therapeutic relevance.

1.1. Promiscuous and Chimeric G Proteins

GPCRs exert their effects via activation of a variety of signaling pathways, mediated by the interaction of the receptor with its cognate G protein. There are four main families of G proteins whose functions are determined by their α

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subunit: $G\alpha_s$ activates adenylate cyclase, $G\alpha_i$ inhibits adenylate cyclase, $G\alpha_q$ activates phospholipase C, and $G\alpha_{12}$ has diverse signalling characteristics including modulation of Na^+/H^+ exchange and c-Jun N-terminal kinase (JNK) activation (***6***). For an oGPCR, not only is the ligand unknown, but also its G protein partner and the associated signaling cascade. One of the most successful high-throughput methods for oGPCR ligand screening is the measurement of changes in intracellular Ca^{2+} as a result of receptor-mediated phospholipase Cβ1 (PLC) activation (***7–11***) (**Table 1**). However, only a subset of naturally occurring G proteins signal through the PLC cascade; therefore, a mechanism is needed to channel a spectrum of downstream signaling pathways to a single measurable end point. To this end, the "promiscuous" G proteins e.g., $G\alpha_{15}$ or $G\alpha_{16}$, together with G protein chimeras, such as $G\alpha_{qi5}$ and $G\alpha_{qs5}$, are widely used. $G\alpha_{15}$ and $G\alpha_{16}$ are naturally occurring G proteins with the ability to couple to receptors which would normally signal via an alternative pathway (***12***). Using this characteristic, it is possible to "force" a receptor to respond to an agonist via PLC activation, thus considerably broadening the range of receptors that will give a measurable calcium mobilization response. Chimeric G proteins consist of $G\alpha_q$ in which the C-terminal five amino acids of this subunit are replaced by corresponding amino acids from the adenylate-cyclase linked G_i or G_s subunit to generate $G\alpha_{qi5}$ and $G\alpha_{qs5}$ respectively (***13***). Thus, these chimeras allow most G_i or G_s coupled receptors to signal via elevation of intracellular Ca^{2+}. However, it should be noted that although the majority of GPCR linked pathways can be manipulated in this way, this system is not universally applicable (***14***) and platforms utilizing cAMP response elements can be considered for putative G_i and G_s receptors that are unresponsive in a Ca^{2+} assay.

1.2. Fluorescent Calcium-Sensitive Dyes

Elevation of cytoplasmic Ca^{2+} resulting from receptor-coupled release from intracellular stores can be detected by using calcium-sensitive dyes such as Fluo-3 acetoxymethyl (AM) and Fluo-4 AM (Molecular Probes, www.Probes.com) which exhibit an increase in fluorescent intensity upon binding to Ca^{2+} (***15***). Incubation of the cells with the cell permeable indicator allows "loading" of the cytoplasm, and cleavage of the AM ester moiety by cytoplasmic esterases prevents the active dye from diffusing out of the cell. Because the AM form has a low aqueous solubility, a dispersion agent, e.g., Pluronic F-127, is used to facilitate cell loading. For some cell types, e.g., Chinese hamster ovary (CHO) cells, the inclusion of an anion exchange inhibitor, such as probenecid, is required for efficient cell loading (***16***). The Fluo-4 dye formulation requires the cells to be washed prior to processing to remove residual extracellular dye that can increase background signals. Recently, "no wash" dye formulations have

Table 1
Examples and Year of Publication of Orphan Receptor–Ligand Pairings That Used Ca^{2+} Flux As the Assay Readout

Ligand	Receptor	Year	Reference
Cortistatin	MrgX2	2003	***7***
Sphingosine 1-phosphate	GPR3/6/12	2002	***8***
BAM22 and related fragments	SNSR3/4	2002	***9***
KISS-1	GPR54	2001	***10***
Melanin concentrating hormone	MCH2	2001	***11***

*These citations are illustrative and not comprehensive as in some cases several groups identified the same receptor–ligand pair.

been developed and are commercially available, e.g., fluorometric imaging plate reader (FLIPR®) Calcium 3 assay reagent (Molecular Devices). The main advantages these provide over the protocols in which wash steps are required is an increase in throughput capability and a reduction in the stress put on the cells, especially for fragile cells or those that are weakly adherent. We have found both Fluo-4 and Calcium 3 reagents to have excellent signal sensitivity. However, when using Calcium 3 reagent we have found high background signals with lipid ligands and greater variability between cell types than when using Fluo-4.

1.3. Fluorometric Imaging Plate Reader

The protocols detailed here describe the use of the FLIPR 96-well microplate system for the measurement of intracellular calcium levels. The FLIPR hardware contains optic, liquid-handling, and temperature-control systems together with data collection and analysis software. The FLIPR comprises a 96-well pipettor that simultaneously adds compounds to a microplate containing the cell type to be tested. The cell monolayer is then excited with an argon laser, and the resulting fluorescence change in response to compound treatment is detected by a charge-coupled device (CCD) camera in real time (*see* www.moleculardevices.com for more information). When monitoring an agonist treatment using Fluo-4 or Calcium 3 assay reagent, a typical assay can be run in approx 4 min per plate.

1.4. Selection of Ligand Library

The success rate of ligand identification for an orphan receptor will depend on a number of factors including ligand library selection and the concentrations used. We commonly use a 10 μ*M* concentration for small molecule ligands and bioactive lipids, and a 1 μ*M* concentration for peptides. The choice

of compound library will depend on whether a natural or a synthetic ligand is required. A selection of commercially available GPCR ligand libraries (96-well format) is listed in the "Materials" section (**Subheading 2.3.**). An alternative source of ligands is the use of high-performance liquid chromatography (HPLC) fractions prepared from tissue extracts (***17***).

2. Materials

2.1. Transient Transfection of HEK293 (Human Embryonic Kidney) Cells

1. Microbiological Safety Cabinet (class II).
2. Microscope.
3. CO_2 incubator set at 37°C with humidified 5% CO_2/95% air e.g., Hereaus.
4. Hemocytometer (Sigma, www.sigmaaldrich.com).
5. Rechargeable pipetman.
6. 225-cm^3 Flasks, vented cap (Costar, www1.fishersci.com).
7. HEK293 cells (Human embryonic kidney) (Invitrogen, www.invitrogen.com). Note: alternative cell lines such as CHO or COS-7 can also be used.
8. Dulbecco's modified Eagle medium (DMEM) + 10% fetal calf serum, 2 m*M* L-glutamine, 25 m*M* HEPES, 1X MEM nonessential amino acids.
9. Serum free minimal media, e.g., OptiMEM (Invitogen).
10. Cationic lipid transfection agent, e.g., Lipofectamine Plus (Invitrogen).
11. Mammalian expression constructs for $G\alpha_{15}$ (Genbank: AF493904, plasmid available from Molecular Devices) and $G\alpha_{qi5}$ (plasmid available from Molecular Devices).

2.2. FLIPR Assay

1. 1X Trypsin 0.25 mg/mL (Invitrogen).
2. Phosphate-buffered saline (PBS) without calcium or magnesium.
3. 96-well black, clear-bottomed microplate, sterile (Costar).
4. Probenecid (Sigma).
5. Type B 96-well black FLIPR tips (Molecular Devices).
6. FLIPR (Fluorometric Imaging Plate Reader) (Molecular Devices).
7. Sterile reservoirs (Costar).
8. 1 *M* sodium hydroxide.

2.2.1. Fluo-4 AM Protocol

1. Fluo-4 AM (Molecular Probes).
2. Pluronic F-127 (Molecular Probes).
3. Dimethyl sulfoxide (DMSO).
4. 1X Hank's balanced salt solution (HBSS).
5. Bovine serum albumin (BSA).

2.2.2. Calcium 3 Assay Reagent (No Wash)

1. FLIPR Calcium 3 assay reagent (Molecular Devices).
2. 1X HBSS.
3. 1 *M* HEPES.

2.3. GPCR Ligands

1. RBI Library of Pharmacologically Active Compounds (LOPAC): 640 small molecule ligands (agonists + antagonists) for known GPCRs (Sigma).
2. Prestwick chemical library: 880 pharmacologically active compounds (Prestwick Chemical, Inc.; www.prestwickchemical.com).
3. Prestwick peptide library: 240 known peptide ligands (Prestwick Chemical, Inc.).
4. Biomol lipid library: 203 bioactive lipids (Biomol; www.Biomol.com).
5. Biomol Orphan Ligand Library: 84 compounds with defined or putative biological activity whose protein-binding partners are unknown (Biomol).

3. Methods

The methods in the Hsubsequent sections use HEK293 cells and the Lipofectamine Plus transfection reagent. Alternative standard cell lines, e.g., CHO, COS-7, and alternative cationic lipids, can also be used.

Note: all cell handling to be carried out in a Microbiological Safety Cabinet Class II.

3.1. Transient Transfection of HEK293 Cells

1. Cells grown to 60–80% confluency in 225cm^2 vented flask.
2. Prewarm OptiMEM to 37°C.
3. Prepare solution A: 15 µg oGPCR plasmid DNA, 5 µg $G\alpha_{15}$ plasmid, 5 µg Gq_{i5} plasmid, 90 µL Plus reagent, 2.25 mL OptiMEM.
4. Incubate Solution A at room temperature for at least 15 min.
5. Meanwhile, prepare Solution B: 45 µL Lipofectamine (cationic lipid), 2.25 mL OptiMEM.
6. Combine solutions A and B. Mix gently. Incubate at room temperature for 15 min.
7. Add 15 mL OptiMEM.
8. Remove cells from incubator. Wash in 15 mL OptiMEM and then aspirate off the liquid.
9. Add the DNA/lipofectamine/OptiMEM mix.
10. Incubate at 37°C for 4–5 h.
11. Aspirate the transfection mix and add 40 mL full growth media.
12. Return to CO_2 incubator set at 37°C with humidified 5% CO_2/95% air.
13. Prepare mock-transfected cells alongside receptor transfected cells in order to determine responses resulting from the cellular background (*see* **Note 1**).

3.2. FLIPR Assay

3.2.1. Cell Preparation

1. Twenty-four hours posttransfection, wash cells in 10 mL prewarmed PBS and then aspirate off the liquid.
2. Add 2 mL trypsin. Incubate for 1–2 min at 37°C.
3. Gently tap flask to detach the cells. Add 4 mL growth media + serum. Count cells using a hemocytometer.
4. Seed cells into black, clear-bottomed 96-well plates at a density of 5×10^4 cells per well in a 100 µL volume *(see* **Notes 2–4**).
5. Culture cells for a further 24 h.

3.2.2. Compound Preparation

1. Compounds diluted to working concentration in round-bottomed, 96-well plates (*see* **Note 5**).
2. Compound working stocks are made up at 4X concentration for Fluo-4 assay and 5X concentration for the FLIPR Calcium 3 assay, to account for the dilution that occurs on addition to the cell plate; e.g., in the Fluo-4 assay, for a screening concentration of 10 µ*M*, compound addition plate contains 40 µ*M* stock.
3. Small molecule ligands and bioactive lipids are screened at a final assay concentration of 10 µ*M* and peptides at 1µ*M*.
4. Peptides are diluted to the appropriate concentration in HBSS containing 0.1% BSA. All other compounds are diluted in HBSS where possible (*see* **Note 6**).

3.2.3. Preparation of Fluo-4 Loading Dye and Loading the Cells (48 h Posttransfection)

Note: keep loading dye protected from direct light (*see* **Note 7**).

1. Prepare 100X probenecid stock (250 m*M*) by dissolving 0.71 g probenecid in 5 mL of 1 *M* sodium hydroxide. Make up to 10 mL with PBS. Prepare fresh on the day of the assay.
2. To make up loading dye solution for one assay plate, thaw one vial (50 µg) Fluo-4 and resuspend in 20 µL DMSO.
3. Add 20 µL 20% pluronic F-127 and mix.
4. Add dye/pluronic mixture to 11 mL serum free growth medium.
5. Add 1.1 mL 100X probenecid stock.
6. Remove growth medium from the cells and replace with 100 µL warm (37°C) loading dye solution (*see* **Note 8**).
7. Incubate for 1 h at 37°C in 5% CO_2/95% air.
8. Wash cells three times with 150 µL of wash buffer per well (HBSS/2.5 m*M* probenecid, pH 7.4) (*see* **Note 9**). When using peptide ligands include 0.1% BSA.
9. Incubate at room temperature for 15 min prior to processing within the FLIPR.

Table 2
FLIPR® Program Settings

Laser setting	0.6 W
Exposure length	0.4 s
Addition volume	50 µL
Pipettor height	180 µL (Fluo-4) or 230 µL (Calcium 3)
Addition speed	35 µL/s
Addition start	After sample 10 (after 10 s)
Read time (total = 2 min)	60 samples every 1 s followed by 20 samples taken every 3 s

3.2.4. Preparation of Calcium 3 Assay Reagent ("No Wash") and Loading of Cells

1. Prepare 100X probenecid stock (250 mM) by dissolving 0.71 g probenecid in 5 mL 1 *M* sodium hydroxide. Make up to 10 mL with PBS. Prepare fresh on the day of the assay.
2. To prepare the reagent buffer, pipet 10 mL of 10X Hanks Balanced Salt Solution, 2 mL of 1 *M* HEPES buffer solution, and 1 mL of 100X probenecid (final in-well concentration of 2.5 m*M*), into 86 mL cell culture treated water.
3. Thaw one vial FLIPR Calcium 3 assay reagent and equilibrate to room temperature.
4. Dissolve contents of one vial completely in 10 mL of reagent buffer and then add to the remaining buffer. Adjust pH to 7.4 and adjust volume to 100 mL with water.
5. Remove cell plates from incubator.
6. Add an equal volume (100 µL) of assay reagent to each well. Growth medium does not have to be removed. (*see* **Note 8**).
7. Incubate cell plates for 1 h at 37°C/5% CO_2 and then equilibrate to room temperature for 10 min prior to reading on the FLIPR.

3.2.5. Reading the Assay Plates on the FLIPR

Program the FLIPR to take readings as detailed in **Table 2** (**Notes 10–11**).

3.2.6. Data Analysis

Results are displayed as graphs in 96-well format. An agonist-dependent, receptor-mediated response is characterized by a distinctive calcium signal which returns to the baseline level (**Fig. 1A**). Care should be taken to identify non-receptor-mediated signals (**Fig. 1B**) such as those produced by calcium ionophores or compounds that disrupt the lipid bilayer. A numerical value representing the response can be obtained by exporting the statistics of each curve. The statistics most commonly used are Max-Min and Sum (area under the curve).

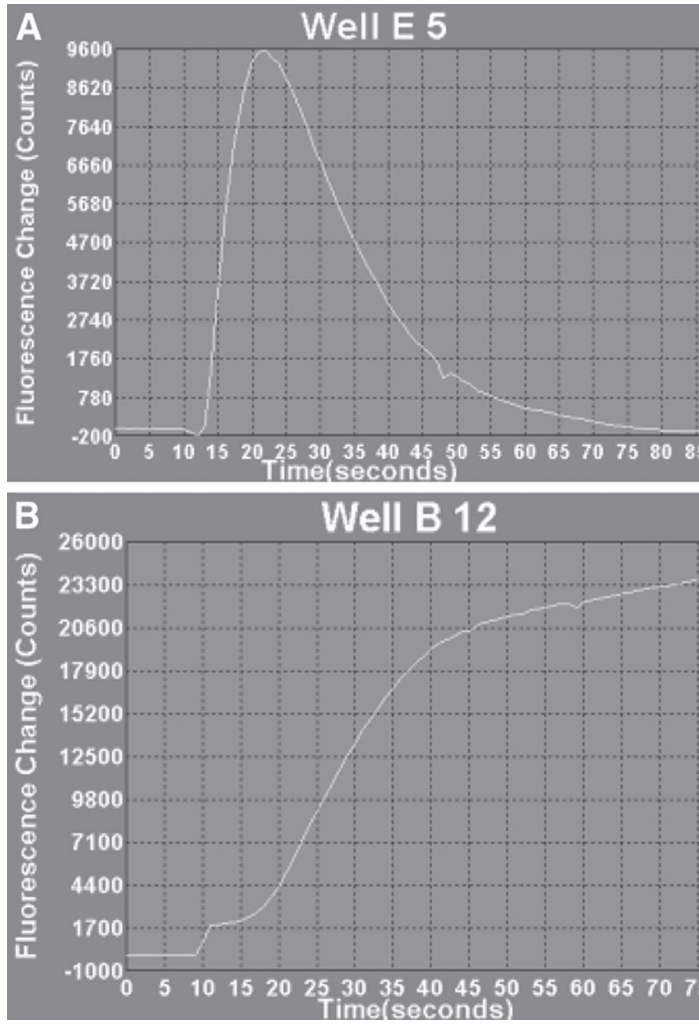

Fig. 1. (**A**) Example of a receptor-mediated fluorescence/calcium response characterized by a sharp peak within seconds of agonist addition (compound added at 10 s), followed by a return to baseline. (**B**) Example of a non-receptor-mediated fluorescence/calcium response characterized by slow onset and not returning to baseline.

Subtraction of the mock Ca^{2+} signal from the oGPCR transfected signals will identify any responses caused by receptors endogenous to the cell line used.

4. Notes

1. The transfection efficiency of the cell line and protocol can be checked by transfecting with a β-galactosidase reporter to estimate the percentage of cells that

have taken up the expression plasmid (β-galactosidase assay system, Invitrogen). Alternatively, a parallel transfection of a tagged receptor can be used.
2. Fragile or weakly adherent cells (e.g., HEK293) are best seeded on poly D-lysine coated plates, especially when using a protocol that includes wash steps.
3. To reduce well-to-well and plate-to-plate variability, a Multidrop dispenser (ThermoQuest) can be used for addition of cells to the assay plate.
4. On the day of the assay, the cells should be 90–100% confluent in the microplate. The 96-well FLIPR reads fluorescence across the middle of the well. Therefore, any spaces in the monolayer in this region will result in signal reduction.
5. When using round- or V-bottomed plates for compound preparation, at least 10 µL dead volume is required for the FLIPR liquid handling system (50 µL for flat-bottomed plates).
6. When testing compounds which require DMSO for solubilization, check the DMSO tolerance of your assay—most cell based screens are sensitive to DMSO >1%.
7. Do not expose the loading dye to direct light. When loading the cells, turn off the light in the tissue culture cabinet. When equilibrating the plates to room temperature, keep them covered.
8. When testing multiple plates, stagger the dye loading step at 5-min intervals (if carrying out a single compound addition) so that each plate is incubated for approx 1 h in loading dye. Shorter incubation times can affect sensitivity because of insufficient dye loading. Longer incubation times can increase background levels and affect cell viability. Signal stability can be tested by using different load times and incubation temperatures (room temperature vs 37°C).
9. Prior to carrying out an experiment, test the wash protocol on your cell type of choice and then check the monolayer under the microscope to ensure cells are not being dislodged.
10. The pipettor height should be above the volume of the loaded cells, but below the total volume once the compound is added. This prevents loss of compound through splashes on the sides of the well.
11. Prior to placing the cell plates in the FLIPR, wipe the bottom with an antistatic cloth to remove dust or fingerprints that may affect the signal.

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