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[5] High-Content Screening of Known G Protein-Coupled Receptors by Arrestin Translocation

By CHRISTINE C. HUDSON, ROBERT H. OAKLEY, MICHAEL D. SJAASTAD, and CARSON R. LOOMIS

Abstract

G protein-coupled receptors (GPCRs) have proven to be one of the most successful target classes for drug discovery. Accordingly, many assays are available to screen GPCRs, including radioactive-binding assays, second messenger signaling assays, and downstream reporter assays. One of

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the more novel approaches is the Transfluor technology, a cell-based assay that uses a detectable tag on a cytosolic protein, called arrestin, that is involved in the desensitization or inactivation of GPCRs. Monitoring the translocation of GFP-tagged arrestin from the cytosol to activated GPCRs at the plasma membrane measures the pharmacological effect of test compounds that bind the receptor target. Moreover, the Transfluor assay provides further, high-content information on the test compound itself and its effects on cell processes due to the fluorescent imaging of whole cells used in this screen. Screening known GPCRs with Transfluor against large compound libraries is best accomplished in cell lines stably expressing an optimum level of the target receptor. This chapter describes how to generate a clonal cell line stably expressing the known GPCR with suitable Transfluor properties. It then describes the steps involved in performing a Transfluor screen and discusses high content data resulting from the screen.

Introduction

G Protein-Coupled Receptors (GPCRs), G Protein-Coupled Receptor Kinases (GRKs), and Arrestins

G protein-coupled receptors comprise a large class of proteins that regulate many physiological functions such as sight, taste, smell, neurotransmission, cardiac output, and pain perception. In response to ligand binding, GPCRs activate heterotrimeric guanine nucleotide binding proteins (G proteins). Stimulation of the G protein results in its dissociation into an α subunit and a $\beta\gamma$ dimer that in concert initiates signaling cascades within the cell to produce a physiological response. Specifically, levels of the second messenger molecules—calcium and cyclic AMP—are altered, and transcription factors, such as NFAT and CREB, are activated to regulate gene expression. Agonist activation of GPCRs also begins the processes of receptor desensitization, internalization, and recycling (Ferguson, 2001). These processes are necessary to prevent overstimulation of the receptor in the presence of continuous agonist and are mediated by GRKs and arrestins.

G protein-coupled receptor kinases are a family of seven protein kinases responsible for phosphorylating GPCRs after agonist binding (Ferguson, 2001; Pitcher *et al.*, 1998). GRK1 and GRK7 are expressed only in the eye and phosphorylate rhodopsin. GRK2 and GRK3, also known as βARK1 and βARK2, are expressed ubiquitously and are recruited to activated GPCRs by the $\beta\gamma$ subunits of the G proteins. GRK4, GRK5, and GRK6 are also expressed ubiquitously, but unlike GRK2 and GRK3, they are anchored at the plasma membrane by either palmitoylation or electrostatic interactions. Upon agonist activation of a GPCR, there is a conformational change resulting in a more open receptor configuration.

If a GRK is in close proximity to a GPCR in its open conformation, the GRK is able to phosphorylate serine and threonine residues on the intracellular loops and C-terminal tail of the GPCR (Fig. 1, step 1).

Arrestins are a family of four cytoplasmic, scaffolding proteins responsible for turning off the G protein-dependent signaling of an activated GPCR (Ferguson, 2001). Arrestin1, or visual arrestin, and arrestin4,

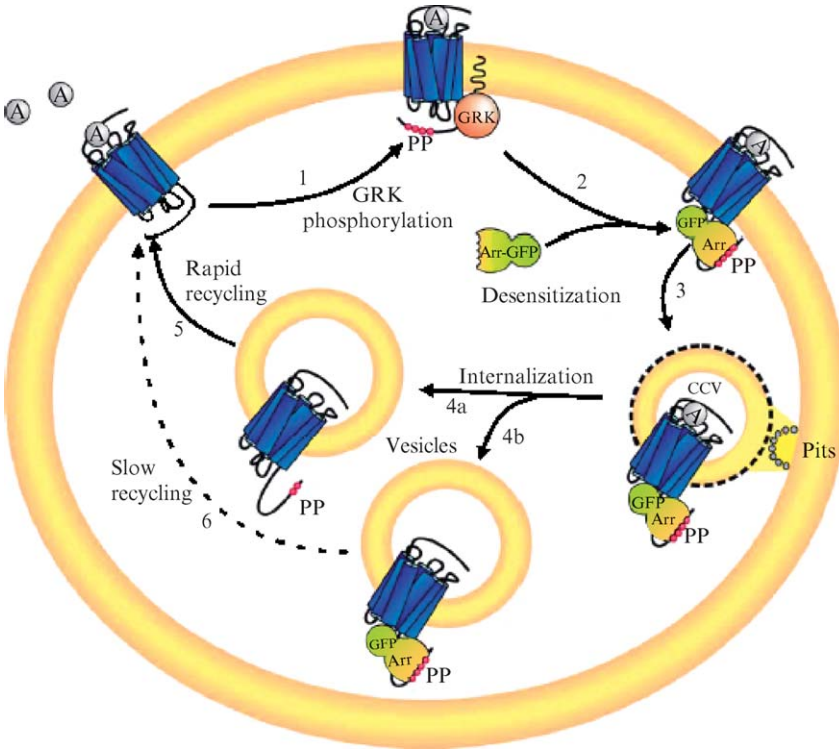


FIG. 1. Arrestin translocation to agonist-activated GPCRs and desensitization. Step 1: Upon agonist binding (A), GPCRs activate G proteins (not pictured) and change conformation to expose a site in the C-terminal tail for phosphorylation (PP) by GRK kinases. Step 2: Arrestin or a fusion protein of arrestin and GFP (Arr-GFP) binds the agonist-occupied, GRK-phosphorylated GPCR and occludes the receptor from binding and activating its G protein in a process termed desensitization. Step 3: Arrestin then targets the desensitized GPCR to clathrin-coated pits, wherein in step 4 the GPCR is internalized in clathrin-coated vesicles and delivered to endosomes. Step 4a: For some GPCRs, arrestin dissociates at or near the plasma membrane and is excluded from receptor-containing vesicles. Step 4b: In contrast, arrestin remains associated with other receptors and traffics with them into endocytic vesicles. Step 5: GPCRs that dissociate from β-arrestin at or near the plasma membrane are recycled rapidly, whereas in step 6, GPCRs that remain associated with arrestin are recycled slowly.

or cone arrestin, are only expressed in the eye. Arrestin2, or β-arrestin1, and arrestin3, or ß-arrestin2, are expressed ubiquitously. Arrestins translocate and bind to agonist-activated GPCRs that have been phosphorylated by GRKs (Fig. 1, step 2). Arrestins bind to the GPCR at intracellular sites that prevent the G protein α subunit from associating with the GPCR. Consequently, G protein-dependent signaling is terminated. Arrestin binding also targets the GPCR for endocytosis in clathrin-coated pits by acting as a scaffold linking the GPCR to internalization proteins such as clathrin and AP-2 (Fig. 1, step 3) (Goodman *et al*., 1996; Laporte *et al*., 2000). Some GPCRs bind arrestin weakly and dissociate from the arrestin once in clathrin-coated pits at the plasma membrane. These GPCRs are called class A GPCRs and recycle more quickly back to the cell surface to be available for agonist stimulation (Fig. 1, steps 4a and 5). Other GPCRs bind arrestin with high affinity and internalize with arrestin into endosomal vesicles. These GPCRs are called class B GPCRs and recycle more slowly (Fig. 1, steps 4b and 6) (Oakley *et al*., 2000, 2005).

ArrestinGFP Translocation Assay

Because almost all GPCRs associate with arrestin upon agonist binding, one can measure GPCR activation, or inactivation, by monitoring the translocation of a fluorescently labeled arrestin from the cytosol to an activated GPCR at the plasma membrane (Barak *et al*., 1997; Oakley *et al*., 2002, 2006). In Transfluor technology, a green fluorescent protein (GFP) is fused to the carboxy terminus of arrestin, forming an arrestinGFP fusion protein. Both arrestinGFP and the target GPCR are overexpressed in the same cell. In the absence of an agonist, the arrestinGFP is distributed uniformly throughout the cytoplasm and is excluded from the nucleus. This can be visualized in live cells using a fluorescent microscope with the

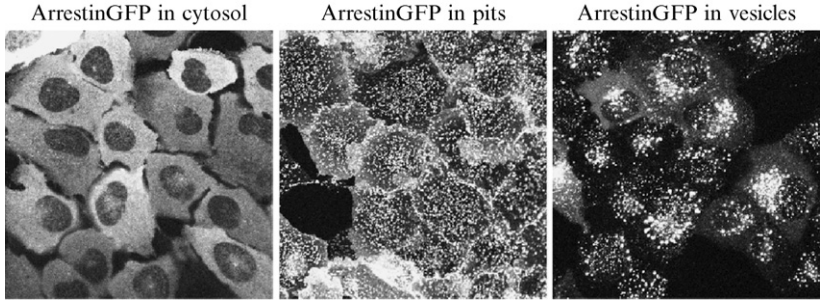


FIG. 2. Confocal microscopy of arrestinGFP translocation in cells. Images of live U2OS cells stably expressing a known GPCR and arrestinGFP were captured in real time before (left) and after 8 (middle) or 44 (right) min of stimulation with agonist.

appropriate filters for GFP excitation and emission (Fig. 2, left). Within seconds of agonist addition, the arrestinGFP translocates from the cytoplasm to the activated GPCRs and forms clathrin-coated pits at the plasma membrane. These pits resemble small fluorescent dots distributed evenly over the cell surface (Fig. 2, center). If a class A GPCR is stimulated, the pits will remain visible as long as the agonist is present. If a class B GPCR is stimulated, the arrestinGFP will internalize with the GPCRs from the pits to larger endocytic vesicles within several minutes. These vesicles resemble larger and brighter fluorescent dots and typically concentrate in the perinuclear region (Fig. 2, right). The magnitudes of these fluorescent pit and vesicle translocation responses of class A and B receptors can be quantitated on a variety of automated image analysis systems to distinguish different pharmacological profiles of GPCR ligands.

Stable Expression of a Known GPCR for the ArrestinGFP Translocation Assay

Transient Validation

In the development of a cell line stably expressing a known GPCR for the arrestinGFP translocation assay, the target GPCR is first validated in the Transfluor assay in transiently transfected cells. Several cell types have been used for the arrestin translocation assay, but U2OS osteosarcoma cells are recommended for screening campaigns because they adhere strongly to glass and plasticware and their flattened morphology yields high-quality images. We transfect U2OS cells stably expressing arrestinGFP with the target receptor. This is best accomplished using FuGENE6 on cells at 50% confluence. The following day, seed the cells into a 35-mm glass-bottom dish. After an overnight incubation, remove the growth medium and replace with 1 ml of serum and phenol red free-MEM with 10 m*M* HEPES. Incubate cells at 37° for 30 min to 1 h. During this incubation, examine the cells on a fluorescence microscope with a 20× or higher objective and the appropriate filter to visualize GFP. In the absence of agonist, neither pits nor vesicles should be present in the cells. If pits or vesicles are found, take careful note of their abundance and distribution. Stimulate cells with the agonist specific for the transfected GPCR. Within 2 to 45 min of stimulation, look for cells that have pits or vesicles.

Evaluation of Response

Prior to agonist addition, the arrestinGFP should be distributed uniformly throughout the cytoplasm of the cells and excluded from the nucleus (Fig. 2, left). After agonist addition, the arrestinGFP should be

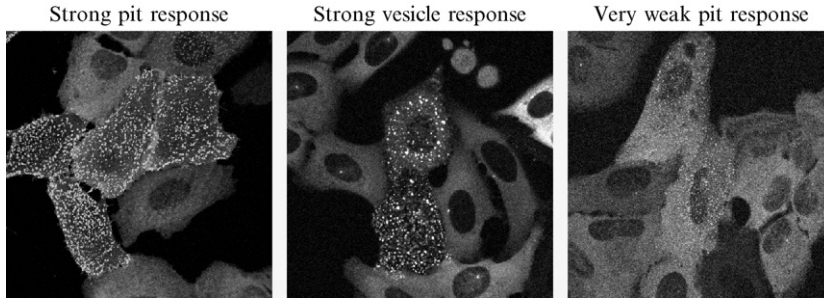


FIG. 3. ArrestinGFP translocation to transiently expressed GPCRs. U2OS cells stably expressing arrestinGFP were transiently transfected with three different GPCRs. Agonists were added, and confocal images of the translocation of arrestinGFP in the live cells were collected.

completely cleared from the cytoplasm and redistributed to clathrin-coated pits or endocytic vesicles (Fig. 3, left and middle). If the transfected receptor is a class A GPCR, arrestinGFP will only complex with agonist-occupied receptors in clathrin-coated pits at the plasma membrane. Pits are usually 0.5–2 μm in size (Fig. 3, left) and remain present on the surface of cells for at least 1 h in the continued presence of agonist. If the transfected receptor is a class B GPCR, arrestinGFP will traffic with the receptor from the clathrin-coated pits into endocytic vesicles. Vesicles are 3 to 5 μm in size (Fig. 3, middle) and remain present inside the cell for several hours even after agonist removal.

Occasionally, transient transfection of a GPCR yields either no arrestin GFP translocation in response to agonist or only a very weak translocation to tiny pits with the majority of the arrestinGFP remaining in the cytoplasm (Fig. 3, right). In these cases, the target receptor can be modified to improve or optimize the arrestinGFP translocation. The molecular motif that allows class B GPCRs to bind tightly to arrestinGFP and traffic together to endocytic vesicles has been identified and found to be transferable (Oakley *et al.*, 1999). Modifying a weak or nonresponding GPCR with this motif usually results in receptors that produce a robust Transfluor response that can be quantitated easily.

With a few GPCRs, arrestinGFP translocation is observed before the agonist is added to the cells. This basal translocation response may result from receptors having a high degree of constitutive activity or be caused by agonist already present in the culture media, especially in fetal bovine serum (FBS). While generally not problematic, basal activity may raise background levels in untreated cells and cause difficulty in developing or growing a cell line containing a GPCR that is under constant stimulation by its agonist. For this condition, culturing cells in reduced amounts of FBS or

in a mixture with dialyzed FBS or charcoal/dextran-treated–FBS (stripped serum) is recommended to reduce undesirable levels of basal activity and improve cell growth.

Development of Stable Cell Lines for Known Receptors

Transfection and Drug Selection. After validating a GPCR in Transfluor using transiently transfected cells, we then develop a stable cell line for the receptor. Stable, clonal cell lines are advantageous for screening, because with optimal and uniform levels of receptor expression, there is less variability during screening and ideal screening statistics can be achieved. We begin by transfecting U2OS cells stably expressing arrestinGFP with the target receptor or mock DNA. The next day, the cells are split into a 35-mm glass-bottom dish for evaluating transfection efficiency and into a 15-cm dish for drug selection. If the transfection efficiency is acceptable, we add the appropriate selection antibiotic to the 15-cm dish and to the dish of cells that was transfected with mock DNA. This dish serves as a control to monitor the time needed to completely kill all nontransfected cells.

Evaluating Antibiotic-Resistant Cells (Parent Mix). The surviving antibiotic-resistant cells are known as the parent mix. Before continuing forward with stable development, we evaluate arrestinGFP translocation to the known receptor in the parent mix for two purposes. First is to ensure that the translocation response observed in transiently transfected cells is preserved in cells stably expressing receptors. Second is to evaluate the percentage of cells demonstrating the translocation response. Both results are obtained by seeding one 35-mm glass-bottom dish with 250,000 to 300,000 parent mix cells. After an overnight incubation at 37°, we assess the arrestinGFP translocation response in the absence and presence of a saturating concentration of the target receptor ligand. The magnitude of the agonist-induced response should be preserved, and the percentage of stimulated cells showing the response should have increased markedly as compared to that obtained after the initial transfection.

Clone Selection and Expansion. If the assessment of percentage responding cells in the parent mix is acceptable, then clonal cell populations are obtained by dilution plating in 15-cm gridded dishes with very low cell densities. In approximately 1.5 weeks, colonies on the 15-cm gridded dishes should have grown to an optimal size for picking, ~100 cells per colony. We isolate selected colonies using cloning cylinders and transfer each clone into 1 well of a 24-well plate. If the percentage of positive responding cells in the parent mix equaled 30 to 50%, then ~40 colonies are picked for screening. If the percentage of positive responding cells is less than 30%, then 70 to 100 colonies are picked. As clones grow to confluence in the 24-well plate, feed

as necessary. When cells are 70 to 100% confluent in the wells, aspirate media and wash with phosphate-buffered saline (PBS). Add 100 μl of trypsin to the well and incubate at 37° until the cells round up. Then add 1 ml of media. Using a pipette, resuspend the cells and transfer 200 μl to duplicate wells of a 96-well, glass-bottom, black plate and seed the remaining cells in a six-well plate with 2 ml of media.

Identification of Positive Clones. Clones are initially screened in a 96-well glass-bottom plate after an overnight incubation. Replace seed media with 100 μl of phenol red-free MEM and 10 mM HEPES and incubate at 37° for 30 min to 1 h. Next, carefully add 25 μl of a 5× maximal dose of agonist to one of the pair of wells and 25 μl of dilution buffer to the other well. Incubate at 37° for 10 to 20 min. Examine the live cells on the fluorescence microscope and look for clones that meet the criteria described later. Identify three to eight of the best clones to further expand and characterize.

Ideal clones contain 100% of the cells responding to agonist by completely clearing the cytosol of arrestinGFP and redistributing it to pits

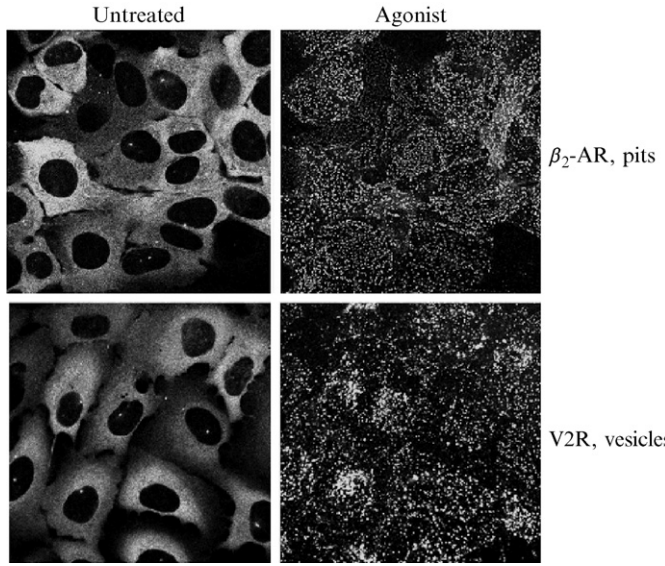


FIG. 4. Clonal populations of Transfluor cell lines expressing known GPCRs. (Top) Confocal images of a clonal population of U2OS cells stably expressing the β_2-adrenergic receptor and arrestinGFP in the absence (untreated) or presence of isoproterenol (agonist). The β_2-adrenergic receptor is a class A receptor and produces a pit response. (Bottom) Confocal images of a clonal population of U2OS cells stably expressing the vasopressin 2 receptor and arrestinGFP in the absence (untreated) or presence of arginine vasopressin (agonist). The vasopressin 2 receptor is a class B receptor and produces a vesicle response. Modified from Oakley *et al.* (2002) with permission of Mary Ann Liebert.

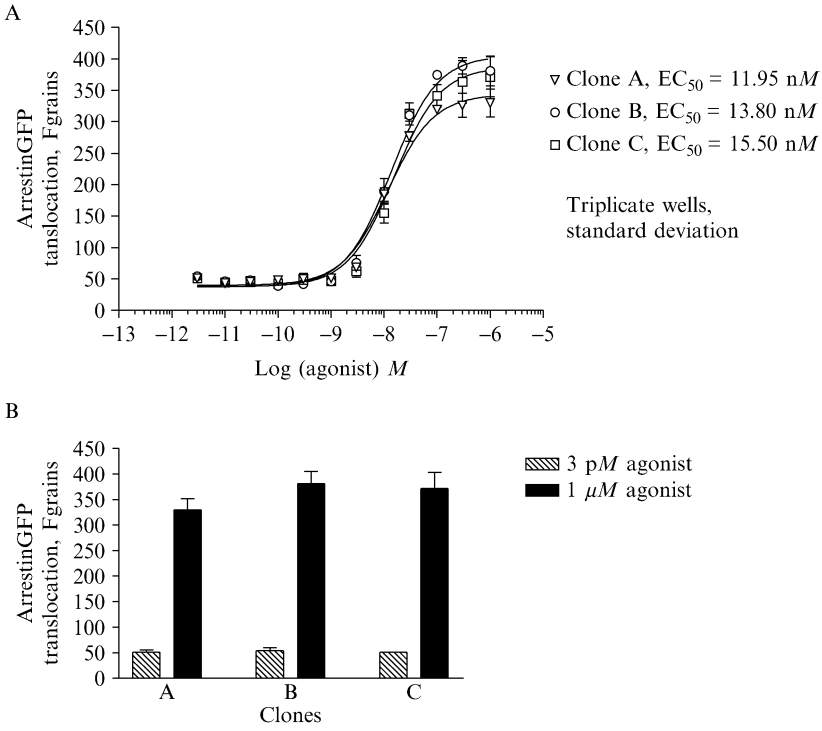
or vesicles (Fig. 4, right). The arrestinGFP levels in untreated cells should be uniform and consistent within a clone and void of multiple localized concentrations or aggregates of the arrestinGFP (Fig. 4, left). Very little to no basal arrestinGFP translocation should occur in the absence of agonist. These clones should consist of healthy cells that have a regular, epithelial morphology. Avoid clones with cells that are elongated, rounded, unusually large, or have a high presence of vacuoles.

Choosing the Best Clone for the Screen

Seed the clones that were selected by visual assessment in triplicate rows or columns of a 96- or 384-well plate. Incubate at 37° overnight. The next day replace seed media with serum/phenol red free MEM with 10 m*M* HEPES (100 μl for a 96-well plate and 20 μl for a 384-well plate), and incubate at 37° for 30 min to 1 hr. Then to the triplicate rows or columns, add 25 μl for a 96-well plate or 10 μl for a 384-well plate of twelve 1:3 dilutions of a 5 or 3× stock of agonist, respectively, to generate a 12-point concentration–response curve. Incubate at 37° for 30 min. Fix the cells with 4% formaldehyde in PBS and an appropriate nuclear stain such as Hoechst or DRAQ5. Fix for 45 min at ambient temperature. Acquire images and quantitate the arrestinGFP translocation response for each of the clones on an image analysis instrument. Quantitate EC_{50} concentrations for the 12 point curve, and signal to background, signal to noise, and *Z* prime values for the minimum and maximum agonist concentration values for each clone (Zhang *et al*., 1999).

Compare EC_{50} values that were collected for each clone from the imaging instrument to known EC_{50} values in the literature determined from signaling assays or to K_d values from binding experiments performed with the same agonist. Choose clones with Transfluor EC_{50} values similar to those found in the literature with other assays. Repeat concentration–response curves to ensure consistent results and run multiple agonists, if available, to check for expected pharmacology. Accordingly, if an antagonist screen is the goal, run concentration inhibition curves with known antagonists. Check IC_{50} values from the Transfluor assay with those reported in the literature for signaling assays or with K_i values from binding assays. Also consider how quickly the cells of a clone double and the kind of screening statistics each clone yields. Pick the clone that has a short doubling time and gives high signal to noise and *Z* prime values in the Transfluor assay. This ensures that the clone grows well, has low background and noise, and has a wide assay window for hits. Once the clone is decided upon, expand the cells and freeze down a large quantity of low passage cells for this clone to be able to expand from in the future.

Figure 5 shows quantitative data for three clonal cell lines of the same target GPCR. Concentration–response curves and the EC_{50} values of the clones (Fig. 5A) were in close proximity to each other and within a half-log of the literature K_d value for the agonist. Statistical analysis of the three clones (Fig. 5B) showed that all had excellent Z prime values and would provide an adequate assay window to identify hits. Clones differed only slightly in the average quantitation of their arrestinGFP translocation maxima and their basal noise. Because all three clones grew similarly in culture, clone B was chosen for the screen based on having the highest signal:noise and Z prime values.



Clone	Ave min	SD min	N min	Ave max	SD max	N max	S:B	S:N	Z prime
A	51.33	4.04	3.00	329.67	22.23	3.00	6.42	12.32	0.72
B	54.33	5.03	3.00	381.00	23.64	3.00	7.01	13.51	0.74
C	50.67	0.58	3.00	371.33	31.94	3.00	7.33	10.04	0.70

FIG. 5. Quantitative data from the INCell 3000, generation 1, of positive, clonal Transfluor cell lines. (A) Concentration–response curves with EC50 values of three clonal populations of cells stably expressing a known GPCR and arrestinGFP. (B) Statistical analysis of the Transfluor response in the three clonal cell populations.

Screening Known Receptors with Transfluor Technology

Meeting the Demand of High-Throughput Screening with Transfluor

High-throughput screening assays need to be predictive of the pharmacology of a test compound on its target, simple and easy to perform, robust, and automatable. The arrestinGFP translocation assay has been shown to meet all of these requirements. First, it has been used successfully to determine the pharmacology of known agonists and antagonists for several GPCRs (Ghosh *et al.*, 2005; Oakley *et al.*, 2006). Next, the Transfluor assay is performed easily because it involves a few, basic steps (Fig. 6). In contrast to other high-content screening assays, which require primary and secondary antibody incubations and washes after cell fixation, no wash steps are necessary with Transfluor. In addition, all steps in the assay are automated easily by liquid-handling robotics such as MultiDrops and MiniTraks, and detection of assay results has been validated on multiple, automated fluorescent imaging platforms (Oakley *et al.*, 2006). Furthermore, the assay is very reproducible and gives excellent screening statistics, including *Z* prime values above 0.5, ensuring an optimal screening assay window.

Preparation Steps for Screening

Scale-up of Cells. The stable clone of cells expressing the target GPCR must be expanded to supply the cells needed for the actual screen. The number of compounds to be screened, the number of plates used in the screen, the number of cells seeded per well, the amount of excess void

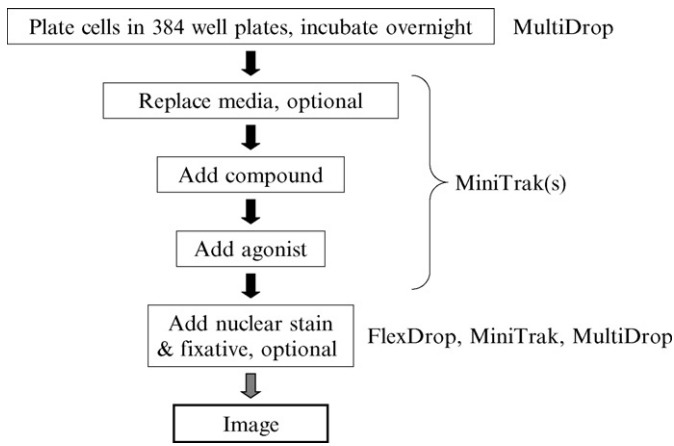


FIG. 6. Transfluor screening protocol steps with associated liquid-handling robotics.

volume of cell seeding solution, and the number of screening days all factor into the total number of cells necessary to complete the screen. To achieve this expansion of cells, low passage number cells are expanded into high surface area cell culture vessels such as roller bottles or cell factories. Cells in the high surface area vessels are fed as necessary until the cells are near confluence, at which time they can be harvested by trypsin or nonenzymatic dissociation reagents and seeded into either more high surface area vessels for further expansion or directly into the assay plates.

Optimization and Validation Experiments. Several important optimization and validation experiments need to be performed on the receptor stable prior to the screen. For example, the appropriate seeding density of U2OS cells expressing the GPCR target and arrestinGFP has to be obtained to ensure the highest-quality data and screening statistics; 5000 cells per well can be used as a starting density for a 384-well plate. In addition, incubation times and temperatures need to be optimized in regards to the arrestinGFP translocation response and constraints with automation. Furthermore, screening conditions for control agonists and/or antagonists need to be determined that address their cost, their final dimethyl sulfoxide concentrations in the screen, their possibility to nonspecifically bind to plasticware used in liquid handling, their stability over time and temperatures, and the concentration(s) to be used in the screen. Finally, a multiplate quality control test run should be done with all the optimized conditions to verify that appropriate screening statistics are achievable. If possible, a test run should be included with a subset of compounds from the larger library to mimic screening day conditions and confirm that all aspects of the screen, including the cellular response in the assay, liquid-handling robotics, and image acquisition and analysis, are all functioning correctly.

Screening a Known GPCR in the ArrestinGFP Translocation Assay

Liquid-Handling Assay Protocol. An outline of a typical Transfluor screening protocol is shown in Fig. 6. Liquid-handling robots are used to seed 384-well plates with an appropriate number of cells per well. The plates are incubated overnight at 37°/5% CO_2. The next day the media in the cell assay plates is removed and replaced with assay buffer (serum and phenol red-free MEM with 10 m*M* HEPES). Test compounds are then transferred from compound library plates to the cell assay plates. For agonist screens, plates are incubated for 40 min at room temperature. The assay is then stopped by addition of a solution of formaldehyde and nuclear stain. For antagonist screens, after incubating the cells with compounds for 40 min, an appropriate concentration of agonist is added to each

well for an additional incubation. The assay is then stopped as described earlier with the formaldehyde/nuclear stain addition.

Imaging Plates. After fixation, the assay plates can be sealed and imaged with the fixative still on them. Proper excitation and emission filters are selected to excite and detect the GFP fluorophore, and the fluorophore is used to stain nuclei of the cells. Neutral density filters, exposure times, camera gain, binning of pixel data, and the order of detecting fluorophores must be optimized to yield an unsaturated image that is highly resolved in the shortest period of time. The image size from a well also needs to be optimized for speed of acquisition and to ensure an adequate number of cells for the quantitation. In addition, a z axis offset of the objective may be set to help visualize the arrestinGFP translocation in the cells. This is especially useful for visualizing the pit response that is best seen at the surface where the cells sit on the plate bottom.

Data Analysis

Algorithms used by imaging platforms usually identify the number of cells present in the image(s) of a well by counting the number of fluorescently labeled nuclei. The algorithms then quantitate the number of fluorescent spots per well or per cell, the area and size of the spots, and/or the intensity of the spots. These data are used to quantitate the appearance of a pit or vesicle response if an agonist screen was performed or the absence of a pit or vesicle response in the case of an antagonist screen. Raw data can be used directly or it can be transformed further with software to calculate percentage inhibition or percentage response in relation to control wells. Mean and standard deviations are calculated from positive and negative controls on each plate and are then compared across all plates of the screen. Signal to background, signal to noise, and Z prime values are calculated from the positive and negative controls on each plate and averaged for the entire screen to determine the validity and accuracy of the screen. Responses from wells that meet a specified threshold are flagged as a hit by the software and can be reviewed visually.

Harvesting High Content Data

In addition to numerical data that are collected for measuring the arrestinGFP translocation, other information is also collected that can yield more high content information, such as the number of cells per well, the fluorescence intensity of the GFP or nuclei stain channels, overall cell morphology, and size/area of nuclei (Ghosh *et al.*, 2005; Oakley *et al.*, 2006). This additional, high content information can be harvested and used to automatically filter wells that have been flagged as hits into groups with

similar characteristics. For example, by filtering the hits that have decreased number of cells in the well or smaller nuclei, one can separate out the wells that might have toxicity issues. Similarly, by filtering the hits that exceed a certain fluorescence intensity of the GFP channel and/or the nuclei stain channel, one can separate out the wells that have fluorescent compounds that could cause a false flagging of a well as a hit.

Figure 7 shows images of flagged wells that were automatically scored to rule out false hits quickly and prioritize the hits to further pursue. Figure 7A and B are active hits from an agonist and antagonist screen, respectively. These images are within numerical threshold limits for the fluorescence of their nuclei stain and the GFP signal. The images of these active hits are clear of artifacts, precipitates, and excess fluorescence. Cells appear to be in good health with no signs of starvation or toxicity. Figure 7C is an image of a false-positive fluorescent well in an antagonist screen. The fluorescence intensities in the GFP and nuclear stain channels are saturating and preclude the discernment of any other cellular details, such as the presence of pits or vesicles. Figure 7D is an image of a toxic, false-positive well for an antagonist screen. There are fewer cells per well and the cells are severely rounded. The size of the nuclei has shrunk and few, healthy cells are present that could show a Transfluor response. Figure 7E is an image of a

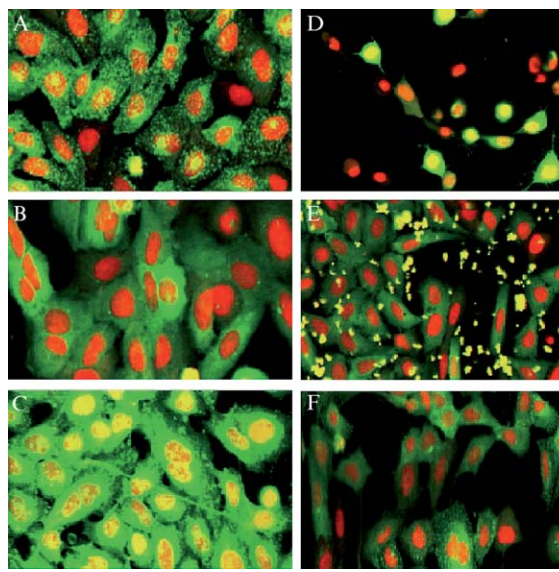


FIG. 7. Transfluor screening hits. (A) Active hit for agonist screen. (B) Active hit for antagonist screen. (C) Fluorescent false positive. (D) Toxic false positive. (E) Precipitate. (F) Miscellaneous; image is partially out of focus. Images from INCell 3000 generation 1.

well with a precipitate that appears as an excess of fluorescent spots throughout the well in either or both the GFP and nuclear stain channels. Precipitates can mask the arrestinGFP translocation response, cause toxicity to the cells, and appear as false-positive hits in an agonist screen. Figure 7F is an example of a flagged well that can be scored as miscellaneous. It is an image of an out-of-focus well. The quantitation for this well is inaccurate, as only a few cells in the well are in focus for measuring the arrestin GFP translocation response.

Conclusion

By exploiting the nearly universal process of GPCR desensitization to measure GPCR activation by arrestinGFP translocation in whole cells, Transfluor provides high content information on the test compound itself and its effects on cell processes. The Transfluor assay utilizes a cell line expressing a fluorescently tagged arrestin that resides in the cytosol of unstimulated cells. Upon transfection of a target GPCR and stimulation with a known agonist, the fluorescently tagged arrestin molecules translocate from the cytosol to the receptors at the plasma membrane, forming fluorescently labeled pits or endocytic vesicles. The redistribution of the fluorescence within whole cells can be measured by high-throughput, imaging platforms. Optimum screening conditions with the Transfluor assay are obtained with the generation and selection of a clonal cell line permanently expressing the target GPCR and arrestinGFP. ArrestinGFP translocation results are then collected easily using a simple Transfluor screening protocol. Imaging instruments used in the screening of known GPCRs for arrestinGFP translocation can make use of multiple data parameters, algorithms, and assays run in parallel to acquire high-content information on the test compounds, cells, and signaling events within the cells. This information can be used to automatically score the images of flagged wells, reduce the number of images to inspect visually, eliminate false-positives quickly, and prioritize hits for further pursuit. With the advent of running a second messenger GPCR assay in parallel with Transfluor (Turpin *et al.*, 2005), a new chapter opens in high-content screening of GPCRs in which researchers are able to measure activation of a GPCR by both signaling and desensitization assays being performed in one well.

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