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[4] The Ligand-Independent Translocation Assay: An Enabling Technology for Screening Orphan G Protein-Coupled Receptors by Arrestin Recruitment

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Abstract

Finding natural and/or synthetic ligands that activate orphan G protein-coupled receptors (oGPCRs) is a major focus in current drug discovery efforts. Transfluor® is a cell-based GPCR screening platform that utilizes an arrestin–green fluorescent protein conjugate (arrestin-GFP) to detect ligand interactions with GPCRs. The assay is ideally suited for oGPCRs because binding of arrestin-GFP to activated receptors is independent of the interacting G protein. Before embarking on a high-throughput screen, it is important to know that the target oGPCR can actually bind arrestin-GFP. This information was thought to be inaccessible, however, as arrestin-GFP recruitment is an agonist-driven process. This chapter describes an assay that enables GPCRs to be validated in Transfluor in the absence of ligand. This assay, termed the ligand-independent translocation (LITe) assay, utilizes a modified G protein-coupled receptor kinase to bypass the requirement of ligand for initiating arrestin-GFP translocation. Using the LITe assay, one can determine if an oGPCR binds arrestin-GFP and if the response is quantifiable by high-content screening instruments. In addition, the assay expedites the development and identification of oGPCR stable cell lines with the best Transfluor properties. In this way, the assay provides criteria for selecting the best oGPCRs to move forward for a Transfluor screening campaign. Moreover, the assay can be used for quality control purposes during the orphan receptor screen itself by providing positive translocation responses for calculation of *Z* prime values. In summary, the LITe assay is a powerful new technology that enables a faster and more reliable path forward in the deorphanization of GPCRs with Transfluor.

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Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of cell-surface receptors. They span the plasma membrane a characteristic seven times and function to transduce extracellular cues into intracellular signaling events that regulate numerous physiological processes. These receptors represent one of the most important targets for drug discovery as approximately 50% of all marketed drugs regulate their activity (Howard, 2001). The GPCR superfamily contains approximately 720 members, yet only a third of these have a known ligand and function (Wise *et al*., 2004). The remaining members are termed orphan receptors (oGPCRs) because their ligand and function are unknown. While many of the oGPCRs belong to the sensory class, approximately 160 represent potential therapeutic targets (Wise *et al*., 2004). Finding natural or surrogate ligands that activate these oGPCRs is essential for understanding their physiology and is a major focus in current drug discovery programs.

Upon binding ligand, GPCRs undergo conformational changes that promote their interaction with heterotrimeric guanine nucleotide-binding proteins (G proteins) composed of α, β, and γ subunits. This interaction triggers the exchange of guanosine 5′-diphosphate (GDP) for guanosine 5′-triphosphate (GTP) on the Gα subunit and the subsequent dissociation of the G protein from the receptor and the Gα subunit from the G$\beta\gamma$ subunits. The released G proteins regulate distinct effectors either positively or negatively, resulting in changes in the level of intracellular second-messenger signaling molecules. Ligand binding not only initiates intracellular signaling cascades, but also triggers mechanisms that limit the magnitude and duration of the signaling event. This process, termed desensitization, protects cells from being overstimulated and is mediated by two protein families, G protein-coupled receptor kinases (GRKs) and arrestins (Claing *et al*., 2002; Ferguson, 2001). The seven-member GRK family phosphorylates agonist-occupied receptors on serine and threonine residues located predominantly in the carboxyl-terminal tail to create high-affinity binding sites for arrestins. Arrestins comprise a family of four proteins that reside in the cytoplasm and translocate to and bind GRK-phosphorylated receptors at the plasma membrane. Binding of a single arrestin sterically prevents the receptor from activating additional G proteins, thereby terminating or ‘‘arresting’’ the receptor response. Arrestins subsequently target the desensitized receptor to clathrin-coated pits for endocytosis. Once internalized, GPCRs are either recycled back to the cell surface to respond again to agonist (resensitization) or degraded inside the cell (downregulation).

Transfluor technology is a cell-based GPCR screening platform that utilizes an arrestin–green fluorescent protein conjugate (arrestin-GFP) to

detect ligand interactions with GPCRs (Oakley *et al.*, 2002). In the absence of agonist, the target receptor is inactive and arrestin-GFP is diffusely distributed in the cytoplasm. Upon activation of the receptor by agonist, arrestin-GFP translocates rapidly to the receptor at the plasma membrane and localizes with it in clathrin-coated pits. Two classes of GPCRs can be distinguished based on the stability of the receptor–arrestin complex (Oakley *et al.*, 2000, 2005). Class A or ''pit-forming'' receptors form transient complexes with arrestin-GFP that dissociate soon after the receptor is targeted to clathrin-coated pits. Class B or ''vesicle-forming'' receptors form stable complexes with arrestin-GFP that internalize as a unit into endocytic vesicles. The agonist-dependent redistribution of arrestin-GFP from a diffuse cytoplasmic pattern to a granular pattern at the plasma membrane (pits) or inside the cell (vesicles) provides a direct readout on the activation status of the target GPCR that is accurately quantitated by a variety of high-content screening instruments (Comley, 2005).

One of the unique features of Transfluor that makes it particularly well suited for screening oGPCRs is that the assay works for receptors independent of their interacting G protein (Oakley *et al.*, 2006). Published reports describe arrestin-GFP translocation to over 20 receptors that couple to $G\alpha_s$, over 20 receptors that couple to $G\alpha_i$, and over 30 receptors that couple to $G\alpha_q$. Therefore, screening an orphan GPCR with Transfluor, as opposed to traditional functional assays, requires no prior knowledge of the signaling pathway. Eliminating the need to determine the interacting G protein not only streamlines the drug discovery process but also may make it more productive. This is highlighted by reports describing GPCRs, such as the chemokine D6 receptor, that do not couple to classic G proteins but do bind arrestin-GFP (Galliera *et al.*, 2004). In fact, it has been suggested that the slow progress made using traditional assays in the deorphanization effort may indicate that many of the remaining oGPCRs signal through G protein-independent mechanisms (Wise *et al.*, 2004).

Before conducting a high-throughput screening campaign with Transfluor against a target orphan receptor, it is important to know that the receptor can bind arrestin-GFP and that the translocation response can be quantitated by the available automated image analysis system. However, without an agonist to activate the receptor (the case for all oGPCRs), how does one perform such assay validation? Faced with this issue, we developed an assay that allows evaluation of arrestin-GFP recruitment to receptors in the absence of ligand. This assay, termed the ligand-independent translocation (LITe) assay, utilizes a modified GRK to effectively take the place of ligand for initiating arrestin-GFP translocation to oGPCRs. This chapter describes the practice and utility of the LITe assay for oGPCRs to be screened with Transfluor.

Overview of the LITe Assay

Translocation of arrestin-GFP has been shown for many GPCRs to be dependent on both agonist occupancy and GRK phosphorylation. Several receptors, however, have been reported to recruit arrestin-GFP in the absence of ligand, including the 5-hydroxytryptamine 2C receptor (5HT2CR) and the human cytomegalovirus US28 receptor (Marion *et al.*, 2004; Miller *et al.*, 2003). Each of these receptors exhibits strong arrestin-GFP binding that is dependent on GRK-mediated phosphorylation but independent of agonist. GPCRs are thought to exist in an equilibrium between inactive and active conformations (Samama *et al.*, 1993). In the absence of agonist, most receptors spend the majority of their time in the inactive conformation. Occasionally, they isomerize into the active state and couple to G proteins to produce constitutive, or agonist-independent, activity. Binding of agonist markedly shifts the equilibrium by stabilizing the active conformation of the receptor. The unbound 5HT2C and US28 receptors are unique in that they spend the majority of their time in the active conformation, resulting in a high level of constitutive activity (Marion *et al.*, 2004; Miller *et al.*, 2003). These findings indicate that arrestin recruitment is not dependent on agonist binding per se, but on the adoption of the receptor active conformation. Without the stabilizing presence of the agonist, however, the active state for most GPCRs occurs too infrequently and is too short-lived for arrestin-GFP binding to be observed by fluorescence microscopy.

The GRK2 isoform of the GRK family resides in the cytoplasm of cells and is recruited to agonist-activated receptors by liberated G$\beta\gamma$ subunits (Pitcher *et al.*, 1998). To develop a ligand-independent translocation assay, we altered GRK2 by adding a CAAX motif (where C is cysteine, A is a small aliphatic residue, and X is an uncharged amino acid) to its carboxyl terminus. This motif directs the attachment of a 20-carbon lipid group (geranylgeranyl) to GRK2 and results in the insertion of the kinase in the plasma membrane (Inglese *et al.*, 1992). The mutant GRK2 (referred to as GRK2m) is no longer dependent on agonist-mediated release of free G$\beta\gamma$ subunits for recruitment to the cell surface. By positioning the kinase constitutively at the membrane (via the mutation) and in the vicinity of the GPCR (via overexpression), GRK2m is poised to immediately phosphorylate receptors that transition into the active conformation before they isomerize back to the inactive state. As a result, even GPCRs with very short-lived active states can be efficiently phosphorylated and observed to recruit arrestin-GFP in the complete absence of agonist. We call the GRK2m-induced translocation of arrestin-GFP to unoccupied receptors the LITe assay.

A comparison of the classic agonist-induced translocation response and the LITe assay translocation response is shown in Fig. 1 for cells stably

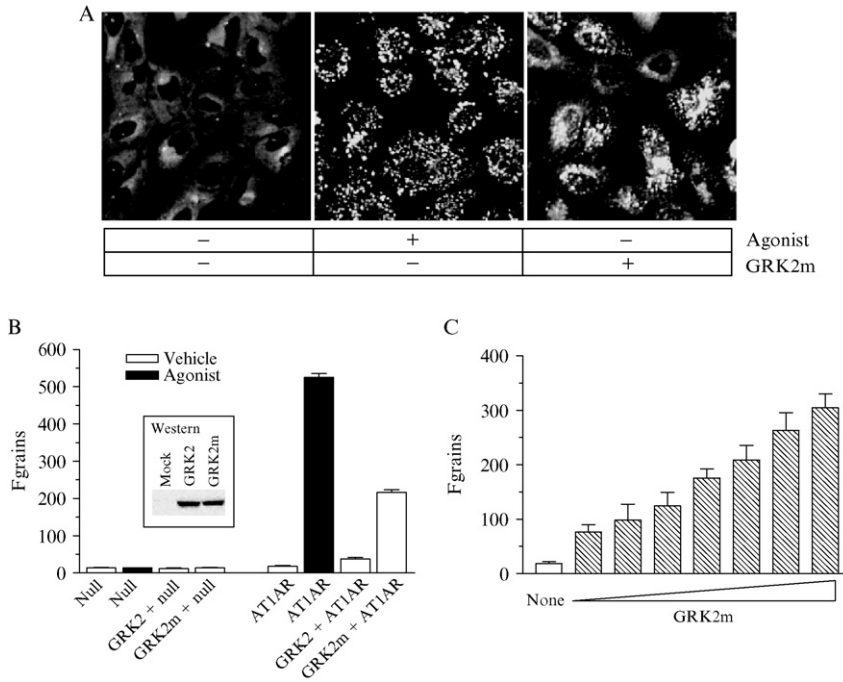

FIG. 1. Comparison of ligand-dependent and GRK2m-dependent translocation of arrestin-GFP to the AT1AR. Experiments were performed on U2OS cells stably expressing arrestin-GFP alone (null) or stably expressing both arrestin-GFP and the AT1AR (AT1AR). For the classic Transfluor assay, 100 n*M* angiotensin II (agonist) was added to cells for 30 min. For the LITe assay, GRK2m was transiently expressed using baculovirus, and the cells were analyzed the following day having never been exposed to the agonist. The INCell Analyzer 3000 was used for quantitation, and the reported parameter "Fgrains" is a measure of the fluorescent intensity of the spots resulting from localization of arrestin-GFP with the AT1AR in endocytic vesicles. (A) Confocal images showing distribution of arrestin-GFP in cells untreated (left image), treated with agonist (middle image), or transiently expressing GRK2m (right image). (B) Quantitation of arrestin-GFP translocation in cells untreated, treated with agonist, transiently expressing wild-type GRK2, or transiently expressing GRK2m. (Inset) Immunoblot of wild-type GRK2 and GRK2m. (C) Quantitation of arrestin-GFP translocation in cells transduced transiently with increasing amounts of GRK2m.

expressing arrestin-GFP and angiotensin II type 1A receptor (AT1AR). In the absence of agonist and GRK2m, arrestin-GFP is distributed evenly in the cytoplasm of the cells, indicating that the AT1AR, like most GPCRs, spends the majority of its time in the inactive conformation as no translocation is observed (Fig. 1A, left). Upon addition of a saturating concentration of agonist and stabilization of the receptor active state, arrestin-GFP

translocates to the AT1AR at the plasma membrane and internalizes with it into endocytic vesicles (Fig. 1A, middle). Quantitation of the response measures approximately 550 Fgrains and represents a 20-fold increase over basal levels (~25 Fgrains) (Fig. 1B).

For the LITe assay, transient transfection of GRK2m results in a dramatic translocation of arrestin-GFP into vesicles with the AT1AR in the complete absence of agonist (Fig. 1A, right). Quantitation of the LITe assay response measures approximately 250 Fgrains and represents a 10-fold increase over basal levels (Fig. 1B). Importantly, the GRK2m-induced translocation response is dependent on overexpression of the AT1AR, as no measurable redistribution of arrestin-GFP is detected upon expression of GRK2m in cells stably expressing arrestin-GFP alone (Fig. 1B, compare values for *GRK2m + null* and *GRK2m + AT1AR*). In addition, the LITe assay response is dependent on the membrane-localized form of GRK2. When expressed at similar levels, GRK2m induces a much greater translocation response than wild-type GRK2 (Fig. 1B, compare values for *GRK2 + AT1AR* and *GRK2m + AT1AR*). Finally, the magnitude of the ligand-independent translocation response is dependent on the amount of overexpressed GRK2m, as a stepwise increase in GRK2m levels produces a stepwise increase in the LITe assay response (Fig. 1C).

Utility of the LITe Assay

Early Validation of oGPCRs in Transfluor

The LITe assay is ideally suited for investigating the arrestin-binding properties of oGPCRs, as no ligand is available for these receptors. Knowing whether and to what extent an oGPCR can bind arrestin-GFP is of great value not only for selecting oGPCRs to move forward for a high-throughput screen but also for ensuring that Transfluor screens are conducted on receptor targets that work well in the assay. Although most agonist-activated receptors recruit arrestin-GFP, there are a few reported exceptions such as the β_3-adrenergic receptor (Cao *et al.*, 2000). In addition, arrestin-GFP translocation to some receptors is weak and difficult to quantitate. For these reasons, we recommend that all oGPCR targets be validated in Transfluor using the LITe assay prior to conducting a large screening campaign.

Early validation studies with the LITe assay are accomplished easily and quickly in transiently transfected cells. Although many cell types support Transfluor, we typically employ either HEK293 cells for their high transfection efficiencies or U2OS cells for their superior image quality (Oakley *et al.*, 2006). To perform the LITe assay, we transiently cotransfect two dishes of cells. The first dish receives the target oGPCR, arrestin-GFP, and empty

vector. The second dish receives the target oGPCR, arrestin-GFP, and GRK2m. Twenty-four hours after the transfection, cells are harvested and plated on 35-mm glass-bottom dishes. After an overnight incubation, we replace the culture medium with serum- and phenol-red free medium to reduce autofluorescence and then visualize the distribution of arrestin-GFP using a fluorescent microscope (63× objective and FITC filter set). We then compare the distribution of arrestin-GFP in cells expressing the target oGPCR in the absence and presence of GRK2m. In this way, the LITe assay on orphan receptors is similar to the classic agonist-induced translocation assay on known GPCRs, except that GRK2m takes the place of the agonist. A successful validation will show minimal arrestin-GFP recruitment in cells expressing the target oGPCR alone but a strong translocation response in cells coexpressing the target oGPCR and GRK2m.

Figure 2 shows the results of the LITe assay performed on four different oGPCRs. In the absence of GRK2m, no translocation response is observed for oGPCR1, oGPCR2, or oGPCR3 (Fig. 2, top). In each case, arrestin-GFP is distributed evenly in the cytoplasm of the cells. A weak vesicle response, however, is observed for oGPCR4, as small spots can be seen in the cytoplasm of the cells (Fig. 2, top). This suggests that oGPCR4 either possesses a significant degree of constitutive activity or that a natural ligand for this receptor is present in the medium. Upon coexpression of GRK2m, a profound redistribution of arrestin-GFP into vesicles is observed for each

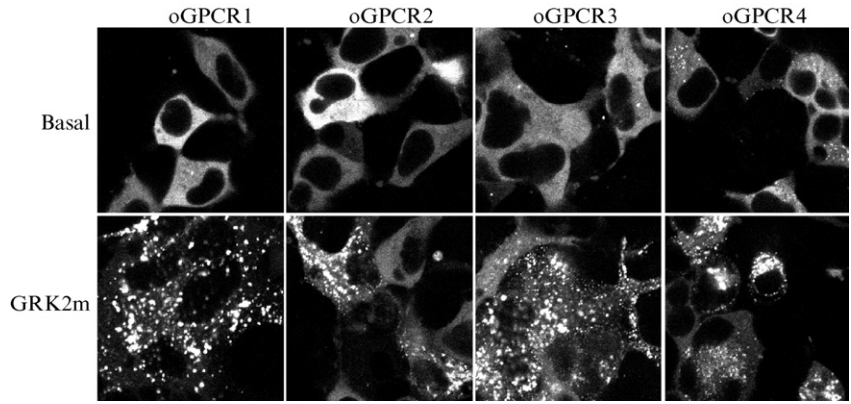

FIG. 2. LITe assay response for four oGPCRs expressed transiently in HEK293 cells. HEK293 cells were transfected transiently by calcium phosphate precipitation with arrestin-GFP, an oGPCR (oGPCR1, oGPCR2, oGPCR3, or oGPCR4), and either empty vector or GRK2m. Thirty-six hours later, the distribution of arrestin-GFP was visualized by fluorescence microscopy. (Top) Confocal images of the distribution of arrestin-GFP in cells expressing the oGPCR alone (basal). (Bottom) Confocal images of the distribution of arrestin-GFP in cells expressing both oGPCR and GRK2m.

of the orphan GPCRs (Fig. 2, bottom). These results in the LITe assay indicate that all four orphans strongly bind arrestin-GFP upon isomerization into the active conformation and suggest that each receptor will be a good candidate for a Transfluor screening campaign.

A negative or weak response in the LITe assay does not automatically disqualify an oGPCR as a target for a Transfluor screen. GPCRs can be genetically modified in their carboxyl-terminal tail to improve their response in Transfluor (Oakley *et al.*, 2006). The molecular motif mediating the stable binding of arrestin-GFP to vesicle-forming GPCRs, such as the AT1AR, has been identified and shown to be transferable (Oakley *et al.*, 1999, 2001). This motif is a cluster of serine/threonine residues positioned properly within the receptor carboxyl-terminal tail that serves as a primary site of GRK phosphorylation. Adding this motif to a receptor can convert a weak pit response into a strong vesicle response without significantly impacting receptor pharmacology. We have successfully employed this genetic alteration on many known and orphan receptors to better enable their detection in Transfluor.

Validation of oGPCR Stable Cell Lines for Transfluor Screening Campaigns

After validating an orphan receptor in Transfluor using the LITe assay in transiently transfected cells, we develop a stable cell line for each target receptor to be screened against a large compound library. Transfluor screening campaigns can be conducted on cells transiently or stably expressing the oGPCR of interest. The transient approach is achieved quickly but can result in more variability in the translocation response due to heterogeneity in receptor expression levels. Stables require a longer time investment but can result in individual clones that express a uniform level of receptor and give a more uniform translocation response. We utilize both approaches but favor stable expression of the target oGPCR for large screens that stretch over days and weeks. We have found the LITe assay to be instrumental for the rapid identification of the oGPCR stable clone with the best properties for a Transfluor screen.

The oGPCR stables are made in U2OS cells because of their superior adherence properties and image quality (Oakley *et al.*, 2006). We begin by transfecting U2OS cells stably expressing arrestin-GFP with the target oGPCR. Antibiotics are applied in order to select for the oGPCR-expressing cells, and the surviving cells are dilution plated to achieve individual clones. To quickly find the best oGPCR clone for an ensuing Transfluor screen, we utilize the LITe assay. Cells from each stable clone are plated into two wells of a 96- or 384-well plate. One well is left untreated for assessment of the basal translocation response, and the other well is transiently transfected with GRK2m. This can be accomplished with traditional lipid-based transfection

procedures or by transducing the cells with baculovirus containing GRK2m. The advantage of the baculovirus gene delivery approach is that infection efficiencies greater than 95% can be achieved in U2OS cells. After an overnight incubation, the plates are analyzed on a high-content screening instrument. We then compare, qualitatively and quantitatively, the distribution of arrestin-GFP in the absence and presence of GRK2m for each clone.

To be used in a Transfluor high-throughput screen, the orphan receptor stable clone should meet two criteria. First, in the absence of GRK2m, little to no arrestin-GFP translocation should be observed in the cells. The reason for this requirement is that compounds that weakly activate the oGPCR will be better detected in a ''clean'' rather than ''noisy'' background. The top part of Fig. 3 shows images of appropriate basal responses for three different oGPCRs stably expressed in U2OS cells. We strongly recommend a visual inspection of the basal response to take advantage of the high-content value of Transfluor. Viewing the image not only confirms the absence of translocation for a clone receiving a low quantitative score, but also provides information on the overall health of the cells. Receptor clones with a large number of cells displaying vacuolated, rounded-up, and/or overly elongated morphology are discarded as these features predict poor growth and viability.

The second requirement of an oGPCR stable clone is that GRK2m expression induces a large, uniform translocation response that can be

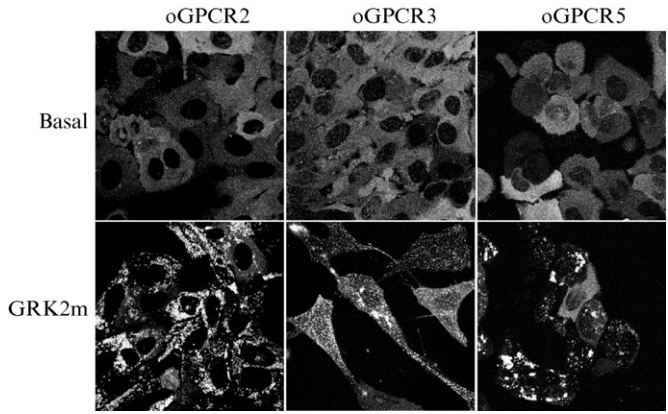

FIG. 3. LITe assay response for three oGPCRs stably expressed in U2OS cells. U2OS cells stably expressing arrestin-GFP and oGPCR2, oGPCR3, or oGPCR5 were plated in 35-mm glass-bottom dishes. For the LITe assay, the stable cell lines were either untreated (basal) or exposed to baculovirus containing GRK2m for 18 h. The distribution of arrestin-GFP was then visualized by fluorescence microscopy. (Top) Confocal images of the distribution of arrestin-GFP in each oGPCR stable in absence of GRK2m (basal). (Bottom) Confocal images of the distribution of arrestin-GFP in each oGPCR stable in the presence of GRK2m.

quantitated easily by the high-content screening instrument of choice. Clones in which the LITe assay response is heterogeneous across the population of cells or poorly quantitated are discarded. The lower part of Fig. 3 shows images of the LITe assay responses for the three different oGPCRs stably expressed in U2OS cells. In each case, almost all the cytoplasmic arrestin-GFP has redistributed into pits and/or vesicles, indicating that the translocation response is very strong. Additionally, both the magnitude and the phenotype of the response are uniform across most of the cells. Quantitation of the LITe assay response for each oGPCR stable measures approximately 300 Fgrains and represents a five- to eightfold increase over basal levels (Fig. 4). Thus, these oGPCR stables are excellent choices for a Transfluor screen, as the combination of a low basal response and a large GRK2m-induced response ensures that each cell line possesses a large signal window for detecting active compounds.

Quality Control for Transfluor Screening Campaigns

The actual Transfluor screen on the chosen oGPCR stable clone must be robust and reproducible such that active compounds can be reliably distinguished from the background noise. The most widely used parameter for evaluating the quality of a high-throughput screening assay is the *Z* prime value (Zhang *et al.*, 1999). The *Z* prime value is a statistical value used to determine assay robustness and reproducibility and is derived from the dynamic range and variability of positive and negative controls. A *Z* prime value of 0.5 or more indicates that data are of excellent quality. As the dynamic range becomes greater and the variability becomes smaller, the *Z* prime value approaches a perfect score of 1.0. The natural ligand provides the positive control for calculating *Z* prime values in screens for activators of known GPCRs. With no such ligand available for oGPCRs, *Z* prime values cannot be determined on the actual target receptor and an important quality control is lost.

We, however, have found that the LITe assay response can be used as a positive control for calculating *Z* prime values and evaluating the integrity of the oGPCR Transfluor screen. This is shown in Fig. 5 for a small screen of approximately 6000 compounds. U2OS cells stably expressing oGPCR3 are plated in 384-well plates. Wells 373–384 (column 24) are exposed to GRK2m-containing baculovirus and serve as the positive control. Wells 361–372 (column 23) are left untreated and serve as the negative control. After an overnight incubation, compounds are added to wells 1–360 (columns 1–22) and vehicle to wells 361–384. The plates are then analyzed on a high-content screening instrument. Figure 5A plots the basal and GRK2m-induced translocation responses for each of the 384-well plates. The basal

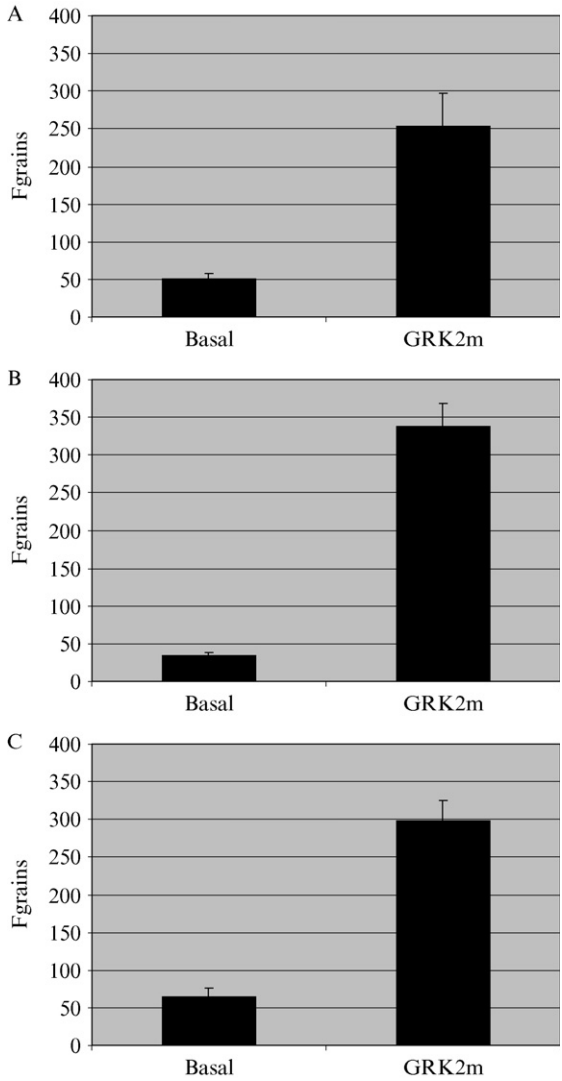

FIG. 4. Quantitation of the LITe assay response for three oGPCRs stably expressed in U2OS cells. U2OS cells stably expressing arrestin-GFP and oGPCR2 (A), oGPCR3 (B), or oGPCR5 (C) were plated in 384-well plates. For the LITe assay, wells were either untreated (basal) or exposed to baculovirus containing GRK2m for 18 h. The plates were then analyzed for arrestin-GFP translocation using the INCell Analyzer 3000.

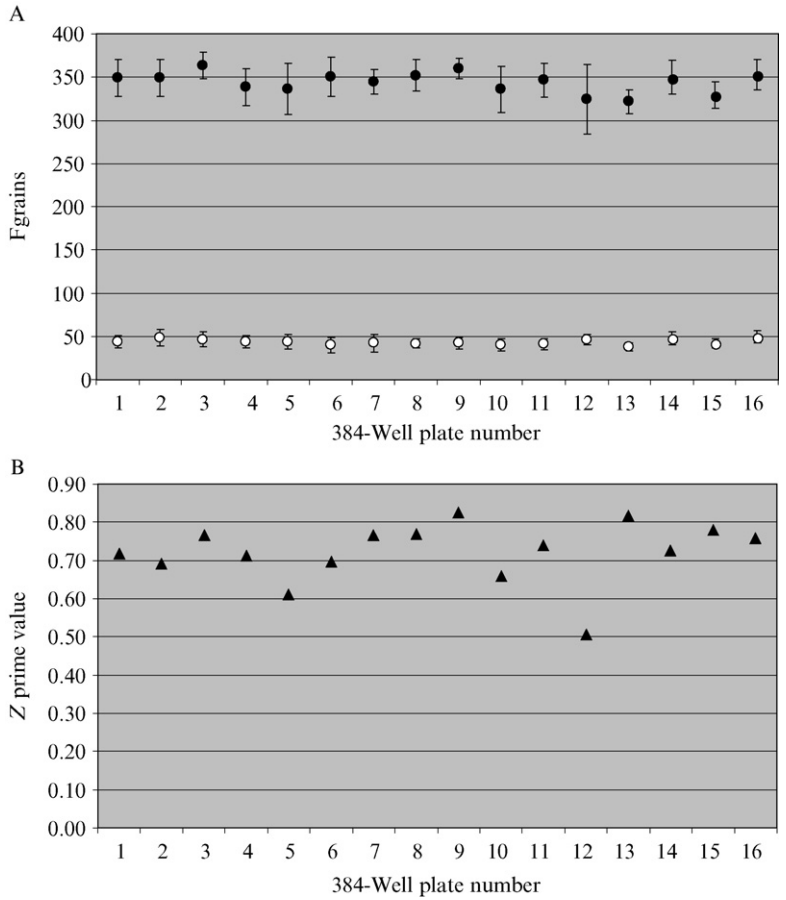

FIG. 5. LITe assay response provides quality control for oGPCR Transfluor screening campaigns. U2OS cells stably expressing arrestin-GFP and oGPCR3 were plated in 384-well plates for a Transfluor agonist screen. For the LITe assay, wells in column 23 of each plate were left untreated (basal) and wells in column 24 of each plate were exposed to baculovirus containing GRK2m. The following day compounds were added to remaining wells (columns 1–22) and plates were analyzed on the INCell Analyzer 3000 for arrestin-GFP translocation. (A) Quantitation of arrestin-GFP translocation in the absence (○) and presence of GRK2m (●) across 16 consecutive plates. (B) Calculated Z' prime values for each of the plates shown in A using the basal and GRK2m-induced responses for the negative and positive controls, respectively.

response (negative control) averaged 44 Fgrains, whereas the GRK2m-induced response (positive control) averaged 344 Fgrains, representing an eightfold signal to background ratio. The calculated Z prime values from each 384-well plate are shown in Fig. 5B and range from 0.51 to 0.82 with

an overall average of 0.72, indicative of a high-quality screen. We have found similar high *Z* prime values for other oGPCRs and in large screening campaigns of greater than 100,000 compounds.

Conclusion

The LITe assay employs a modified GRK2 that constitutively resides at the plasma membrane to bypass the requirement of an agonist for inducing arrestin-GFP translocation to GPCRs. The assay has proven to be of great value for validating oGPCRs in Transfluor, for identifying the oGPCR stable clones with the best Transfluor properties, and for serving as a quality control during the Transfluor screen. In this way, the LITe assay ensures that the orphan receptor screening campaigns performed with Transfluor have the highest probability of success.

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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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