[3] Development of Assays for Nuclear Receptor Modulators Using Fluorescently Tagged Proteins

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Abstract

This chapter describes a method for designing cell-based assays to screen for nuclear receptor modulators. The basic strategy consists in following the movement of the receptors from the cytoplasm into the nucleus in response to ligand binding or analogous activating events. The receptors are tagged with green fluorescent protein for automated, fluorescent detection. In the case of constitutively nuclear receptors, they are engineered for cytoplasmic retention in the absence of an activating signal by fusing them to specific regions of the glucocorticoid receptor, which is found predominantly in the cytoplasm of cultured cells. The resulting chimeras respond to ligands or receptor modulators by translocating into the nucleus. This movement is monitored easily by automated fluorescent microscopy and serves as the basis for screening libraries. Finally, secondary assays built into the cell system can differentiate between modulators that stimulate, inhibit, or do not affect the transcriptional activity of the receptor under study. This approach has been validated for both the estrogen receptor and the retinoic acid receptor and should be applicable to any member of the superfamily, facilitating the identification of new ligands and selective receptor modulators.

Introduction

Nuclear Receptor Biology

Nuclear receptors are ligand-activated transcription factors that mediate a variety of important biological functions, including ion transport and salt balance, glucose and lipid metabolism, and the development and maintenance of reproductive organs. At the molecular level, a large number of human diseases result from the malfunctioning of these receptors. Examples include various forms of cancer caused, in part, by mutations in receptors such as the androgen, estrogen, or thyroid hormone receptors (Crescenzi *et al.*, 2003; Kato *et al.*, 2004; Shi *et al.*, 2002; Yamamoto *et al.*, 2001), congenital adrenal hypoplasia caused by DAX-1 mutations (Muscatelli *et al.*, 1994), mild early onset obesity caused by SHP mutations (Nishigori *et al.*, 2001), vitamin D-resistant rickets caused by mutations in the vitamin D receptor

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(Van Maldergem *et al*., 1996), familial glucocorticoid resistance caused by glucocorticoid receptor mutations (de Lange *et al*., 1997), and type I pseudohypoaldosteronism caused by mutations in the mineralocorticoid receptor (Tajima *et al*., 2000). Agonist or antagonist ligands can be utilized to modulate the transcriptional activity of these receptors and some have been used successfully in the clinic. Two challenges exist, however. First, finding selective ligands that have the desired effect in one target tissue (e.g., in the breast in the case of antiestrogens) without deregulating receptor signaling in other tissues (e.g., in the ovary or bone) has been generally difficult. Second, for a number of receptors, the ''orphan nuclear receptors,'' the endogenous ligands are still unknown. These orphan receptors have been the subject of much study and although ligands have been found relatively recently for several of them, the ligands of other orphans whose structures contain a ligand-binding pocket remain elusive. Given the clinical significance of the nuclear receptor superfamily and the therapeutic potential that modulators of these receptors do and can offer, we decided to develop cell-based assays to facilitate the identification of novel ligands and receptor modulators.

Rationale for the Development of Translocation Assays for Nuclear Receptor Ligand Discovery

Our goal was to develop assays for the discovery of nuclear receptor modulators that would offer maximal biological relevance and would detect a broad range of ligands (not just full agonists) through a signal that could be measured easily by automation and thus would be amenable to high-throughput applications. Conventional screens for receptor ligands usually use transcriptional activity as a readout. Transcriptional activity in response to ligand activation, however, can be cell line dependent, due to the coregulators that a cell type may express, and thus is not the best system for a broad screen. In the case of growth-promoting steroids, proliferation assays have also been used to screen for novel ligands. These assays, however, require several days to complete and are restricted in scope, as they may miss ligands that interact with the receptor and affect the transcription of a subset of genes without having an effect on proliferation (as could be the case for some selective modulators). Taking the aforementioned into consideration, we decided to exploit an early event in receptor activation as our assay readout: the cytoplasmic to nuclear translocation of the receptor that occurs upon ligand binding. This strategy can be used without the need for receptor manipulation in the case of receptors that are predominantly cytoplasmic in cell culture in the absence of hormone and undergo nuclear translocation only when bound by ligand,

such as the glucocorticoid receptor and the aryl hydrocarbon receptor. Receptors that are predominantly nuclear even when unliganded, such as the estrogen receptor, the vitamin D receptor, retinoic acid receptor (RAR), the peroxisome proliferator-activated receptors (PPARs), and others, first need to be engineered for cytoplasmic retention. A strategy for this engineering is presented later. Advantages of using the movement of the receptor from the cytoplasm to the nucleus as a readout in ligand screens are several. First, this event can be triggered by a wide variety of ligands (pure agonists, pure antagonists, selective modulators), giving the screen breath in its scope. Second, the movement of receptors into the nucleus can be monitored easily by fusing the receptor to green fluorescent protein (GFP) or another similar fluorescent protein. In addition, this biological process is less dependent on the cellular context and takes place in the order of minutes, giving a real-time live cell readout if desired.

GFP-Based Technology as a Powerful Tool for Automated Drug Discovery of Nuclear Receptor Modulators

With recent advances in fluorescent microscopy and the development of various forms of the GFP for labeling of fusion proteins *in vivo*, technologies for drug discovery have emerged that combine the strengths of automation with the power of *in vivo* or *in vitro* fluorescence detection. GFP is a particularly attractive reporter system for drug discovery because it can be measured in live cells, it does not require a cumbersome or lengthy assay for its detection but simply light excitation, it can be fused to most protein targets without altering their basic biological activity, its cellular location can be tracked, and its detection is automated easily. For these reasons, the cell-based assays described in this chapter are based on GFP technology. We and others have found that fusing GFP to members of the nuclear receptor superfamily or to chimeric receptors does not alter their function or their native cellular localization (Hager, 1999; Wan *et al.*, 2001). Because the nucleus can be stained with DNA dyes for easy detection and used as a reference, increases in GFP nuclear localization can be monitored effectively by automated microscopy.

Methods

Design of Translocating Nuclear Receptor Chimeras

Although all receptors shuttle within the cell, as mentioned earlier, some nuclear receptors are predominantly cytoplasmic in the absence of

hormonal stimulation. Such receptors can be fused to GFP and used directly to establish stable cell lines for high-content screening of nuclear receptor ligands/modulators. Many of the members of the superfamily, however, are found in the nucleus constitutively and require some engineering before they can be used in translocation-based drug screens. The general strategy for the design of translocating nuclear receptors consists in making chimeras between a constitutively nuclear receptor and one that translocates in response to hormone. The main idea is to combine domains such that the chimera remains in the cytoplasm when uninduced and shuttles into the nucleus when induced by ligands of the nuclear receptor of interest. Because its cellular location is very clearly cytoplasmic in uninduced culture cells, the glucocorticoid receptor can be used as a building block to construct chimeras between it and any constitutively nuclear receptor. Because it has been proven to work effectively, the principle that should be applied is to express the glucocorticoid receptor N terminus, DNA-binding domain, and partial ligand-binding domain sequences upstream of partial ligand-binding domain sequences from the nuclear receptor under study (illustrated in Fig. 1). A detailed protocol for the design of these chimeras is given next.

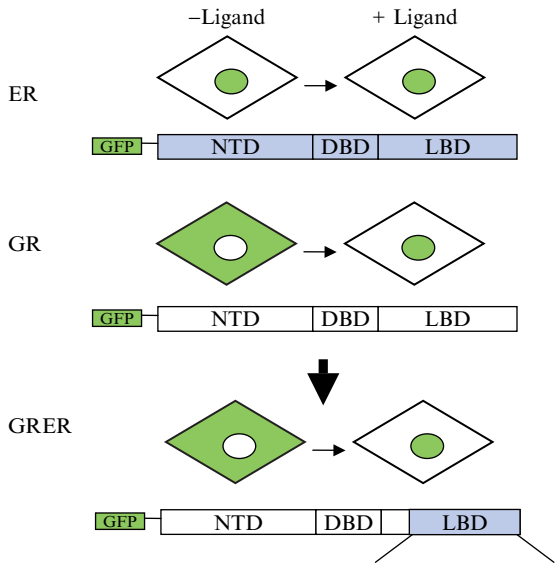

FIG. 1. Graphical representation of the general strategy used for creating nuclear receptors that shuttle between the cytoplasm and the nucleus in response to ligands/receptor modulators. The ligand-binding domain of any constitutively nuclear receptor could be used in place of the ER, which is the example shown here.

Protocol for the Design of Translocating Chimeras

1. Obtain (or construct using standard subcloning procedures) a glucocorticoid receptor (GR) mammalian expression vector tagged with GFP at the N terminus and containing an antibiotic marker under a bacterial promoter. The rat GR works well, yet other species such as the mouse or human GR should give similar results. Note: there should be a short polypeptide between GFP and GR to isolate the GR structure from the GFP structure, which is quite rigid (Hager, 1999).

2. Analyze the restriction enzyme sites in the construct to identify unique sites (or double cutters if necessary) that cut the GR in the sequence corresponding to the loop between helices 1 and 3 of the ligand-binding domain (LBD) (by convention GR does not have a helix 2). Additionally, identify a restriction enzyme that cuts at the very end of the GR expression sequence or just into the vector sequence. The idea is to remove a fragment corresponding to the C terminus of the LBD of the GR, maintaining only sequences that encode helix 1 and a region of the loop between helices 1 and 3. It may be necessary to insert a polylinker with unique restriction enzyme sites or to create a unique site by silent point mutagenesis using polymerase chain reaction in the desired location (Mackem *et al.*, 2001). Although addition of a polylinker will introduce extra sequence, it may facilitate future chimera construction. We have found that the net addition of three amino acids (from polylinker sequences) to the loop between helices 1 and 3 does not disturb the overall receptor structure or its function (see Fig. 2 for an example). Note: when choosing the final digestion strategy, keep in mind the subsequent steps in chimera construction to ensure subcloning compatibility.

3. Digest 1–2 μg of the expression vector with the single cutter restriction enzyme (or do a partial digestion if not using a single cutter) to obtain a linear fragment lacking the desired region of the GR LBD as defined in step 2. Gel purify the fragment. This is the ''vector fragment.''

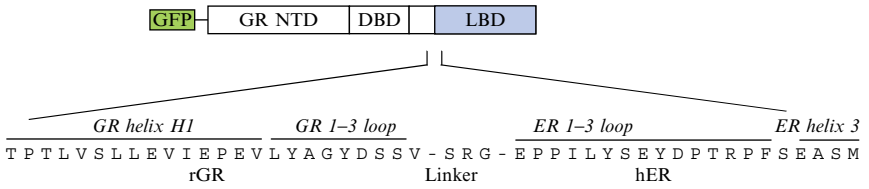

FIG. 2. Detailed view of the amino acid sequence at the fusion site between glucocorticoid and estrogen receptor chimeras showing the addition of three residues introduced by the presence of a linker used for cloning purposes. A small duplication of the loop between helices 1 and 3 results from this chimera construction based on the homology comparison of the receptor sequences (see Fig. 3).

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rGR    438 KLCLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRY 497
hER α  183 RYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRL 242

       498 RKCLQAGMN----LEARKTKKKIKGIQQ----------ATAGVSQDTSENPNKTIV---- 539
       243 RKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLWPSPLMIKRSK 302

       540 --PAALPQLTPTLVSLLEVIEPEVLYAGYDSSVPDSAWRIMTTLNMLGGRQVIAAVKWAK 597
       303 KNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAK 362

       598 AILGLRNLHLDDQMTLLQYSWMFLMAFALGWRSYRQSSGNLLCFAPDLIIN-EQRMSLPC 656
       363 RVPGFVDLTLHDQVHLLECAWLEILMIGLVWRS-MEHPGKLL-FAPNLLLDRNQGKCVEG 420

       657 MYDQCKHMLFVSSELQRLQVSYEEYLCMKTLLLLSS----VPKEGLKSQE-------LFD 705
       421 MVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLD 480

       706 EIRMTYIKELGKAIVKREGNSSQNWQRFYQLTKLLDSMHEV 746
       481 KITDTLIHLMAKAGLTLQ----QQHQRLAQLLLILSHIRHM 517
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FIG. 3. Amino acid sequence homology between rGR and hER α proteins shows how the receptors align in their secondary structure. Residues corresponding to helix 1 are underlined for reference.

4. Obtain an expression vector or cDNA for the nuclear receptor of interest. Run a pairwise comparison of the amino acid sequences for the GR and this receptor, marking the various helices to get an idea of how the two LBDs align (an example of the comparison between rGR and hER is shown in Fig. 3). This can be done using Blast or other public software. Analyze the DNA sequence of the receptor under study for restriction enzyme sites to obtain the region of the LBD equivalent to the deleted GR. Design the final construct to have as close to a full LBD as may be feasible, minimizing areas of deletion or duplication. We have found that duplicating a small region of the loop between helices 1 and 3 is preferable to having a net deletion in this area (Martinez *et al.*, 2005).

5. Once the best digestion strategy has been found, digest 3 to 4 μg of the receptor of interest and gel isolate the main LBD fragment. This is the "insert."

6. Analyze the compatibility of the vector and insert fragments for ligation. Blunt end fragments if necessary using standard procedures to fill in or digest overhangs. Ensure that no coding sequences are deleted in this process.

7. Perform conventional ligation reactions and use the ligation products to transform competent bacteria. Plate the bacteria on agar plates with the selection antibiotic and grow colonies overnight. Pick colonies and prepare plasmid DNA. Digest plasmid DNA with restriction enzymes that can predict the presence and orientation of the insert. Sequence candidate constructs to ensure the correct fusion point and the presence of wild-type sequences. Prepare batches of plasmid DNA for storage and for use in subsequent steps.

8. Use the plasmid DNA obtained in step 7 to transiently transfect adherent mammalian cells. For example, transfect NIH 3T3 cells using the Lipofectamine 2000 reagent following the manufacturer's protocol. Briefly, plate cells on cover glass chambers (such as on the Nunc Lab Tek II series) and allow them to attach to the plate at least overnight. Transfect the cells (at about 60 to 70% confluency) the next day and incubate them for 24 h. View the cells growing on cover glass chambers in an inverted fluorescent microscope to detect the expression and localization of the GFP-tagged chimera. The chimera should be found mainly in the cytoplasm. Next, test its ability to translocate into the nucleus by adding a cognate ligand for the receptor whose LBD is present in the construct. Treat a separate well with glucocorticoids (i.e., dexamethasone) as a negative control (dexamethasone should not bind to or translocate the chimera unless the LBD is from a receptor that cross-reacts with glucocorticoids such as potentially the mineralocorticoid receptor). Incubate for 1 to 3 h and monitor translocation by fluorescence microscopy. It is important to evaluate the phenotype of the chimeric receptor in more than one cell type to establish if cytoplasmic retention is a generalized phenomenon.

Note: for specific examples of how this complete design strategy can be applied, please see the published studies by Mackem *et al.* (2001) and Martinez *et al.* (2005).

This protocol should yield cytoplasmic chimeric receptors that respond to ligands by translocating into the nucleus. If the chimera is not efficiently retained in the cytoplasm or if it does not translocate in response to ligand, the fusion site may need to be optimized. The studies described in the references mentioned earlier should serve as guides for specific design strategies. From our experience, however, the optimal fusion site for the chimeras is between helix 1 and helix 3 of the LBD.

Construction of Mammalian Cell Lines Stably Expressing GFP-Tagged Chimeric Receptors

It has been reported that nuclear receptors can behave differently when they are stably expressed versus transiently expressed in cells (Smith *et al.*, 1993). This is thought to be in part due to processing differences and to differential association with heat shock proteins and immunophilins (Botos *et al.*, 2004). Because these associations can influence cytoplasmic/nuclear retention of the receptors, it is advisable to establish stable expression of the chimeras to ensure their cytoplasmic localization and their response to ligands by nuclear translocation. Regardless, stable cell lines are necessary for drug screening, as they avoid the need for expensive and repeated transient transfections and they offer a more homogeneous expression.

Before establishing stable cell lines, the investigator should consider whether to use a constitutive or an inducible system for expression of the chimeras. While constitutive expression is more economic and simple and does not require activation steps, some cells may not tolerate high receptor levels and may silence receptor expression or lose the inserted construct after a few passages. Constitutive systems are less problematic in this regard, yet they require extra steps during the establishment of the cell line and during experimental procedures to induce receptor expression. Our experience has been that fibroblasts and nonendocrine cells usually tolerate receptor expression well, while some cells of endocrine origin (such as C127) tend to be less tolerant of exogenous receptors.

Note: see section entitled ''Automated Measurements of Nuclear Translocation'' on secondary assays before establishing stable cell lines.

If the end user decides to establish stable cell lines with constitutive expression of the chimera, standard protocols can be used, such as the one outlined by Martinez *et al.* (2006). If inducible systems are preferred, we recommend following the procedures outlined by Walker *et al.* (1999). In either case, established cell clones should be analyzed for GFP-chimera expression (see later), and clones that are homogeneous and show good expression over several passages should be chosen for further studies.

Preliminary Evaluation of a Cell-Based Assay for Nuclear Receptor Ligands Using Fluorescence Microscopy

Before proceeding onto drug screens, the stably expressed chimeric receptor should be evaluated for its translocation efficiency in response to known ligands. This is usually done manually before miniaturization for automated procedures. It is important to establish the response of the chimera to a broad set of ligands and to measure the dose responsiveness of the translocation event, as both of these parameters constitute the foundation of successful high-content drug screens. Although the endogenous ligands of a number of orphan receptors have not been identified, synthetic ligands exist for several of them. These are available commercially through Calbiochem, Cayman, Tocris, and other providers.

Simple Protocol for Evaluation of Known Ligands

1. Plate 50,000 to 200,000 cells per well of a two-well cover glass chamber (Nunc Lab Tek II series) and allow cells to attach and grow for a day.
2. Treat cells with vehicle or with individual known ligands (full and partial agonists and antagonists) for 1 to 5 h.
3. Visualize cells as described earlier for transiently transfected cells. If the GFP expression is not very strong or if the medium gives off

autofluorescence, it may be necessary to visualize the cells in phosphate-buffered saline (PBS) or Hank's balanced salt solution (HBSS). When imaging is prolonged for longer than 10 min, we recommend adding serum to the buffers. The cells can be observed and imaged live or can be observed first, then fixed, and imaged after fixation. Fixation protocols (we use 3.5% paraformaldehyde in PBS for 10 min at room temperature) should not significantly alter the distribution of the chimeric receptor. It is good practice, however, to always visualize the cells before fixing them to ensure this. Although the fixation state is optional, it can give added flexibility, as these cells can be imaged up to 48 h after fixation if stored properly. Store the fixed cells at 4° in PBS containing 0.1% paraformaldehyde and protected from the light. Some signal loss may be experienced after 48 h of storage.

Note: It is expected that all cognate ligands of the receptor under study will cause the receptor to translocate into the nucleus, although some antagonists may require high concentrations, that is, micromolar, and/or may not result in complete translocation of the chimera. If any known ligand does not induce translocation, it would be advantageous to evaluate whether it can inhibit translocation in response to other ligands. If this is the case, other such translocation inhibitors could be screened for by cotreating the cells with an agonist that induces translocation together with drugs or molecules from compound libraries and monitoring for cytoplasmic retention.

Protocol for Manual Evaluation of Dose Responsiveness and EC_{50} Calculations

To determine if the assay is feasible for measuring the potency and efficacy of novel ligands/modulators, the dose responsiveness of the translocation process can be measured and EC_{50} values calculated. Although several imaging systems are equipped with cytoplasmic to nuclear translocation algorithms available in their software packages (discussed later), it is advisable to go through these measurements manually at least once to ensure proper understanding of the calculations and correct setup of the analysis journals.

1. Plate and treat cells as described in the previous section. Fix the cells as described earlier and stain nuclei with DAPI. Image on both the GFP and the DAPI channels at least 20 cells per concentration of ligand whose contours are clearly delineated (we recommend using a 40× objective and imaging areas with multiple cells in them). Superimpose or combine the GFP and the DAPI images and save these dual-channel files without overriding the original separate files.

2. Using any standard imaging software (such as MetaVue), open the combined files and define the area of the nucleus by the DAPI signal. Use

the contour of the cell to define the full area of each individual cell (shown in Fig. 4). Choose an area outside the cell to measure the intensity of the background in the GFP channel. Measure and record the average background intensity per pixel, the integrated intensity of the GFP signal over the full cell area, and the integrated intensity of the GFP signal over the nucleus. Multiply the background value per pixel times the area of the cell or the nucleus. Subtract this value from the corresponding integrated intensities of the whole cell or the nucleus. Repeat these calculations for about 20 cells per ligand concentration. Express the fluorescence in the nucleus as a percentage of the total cell fluorescence and average the percentage nuclear fluorescence for each concentration.

3. Plot out the average percentage nuclear fluorescence against drug concentration. Using graphing software, define a semilog scale by choosing

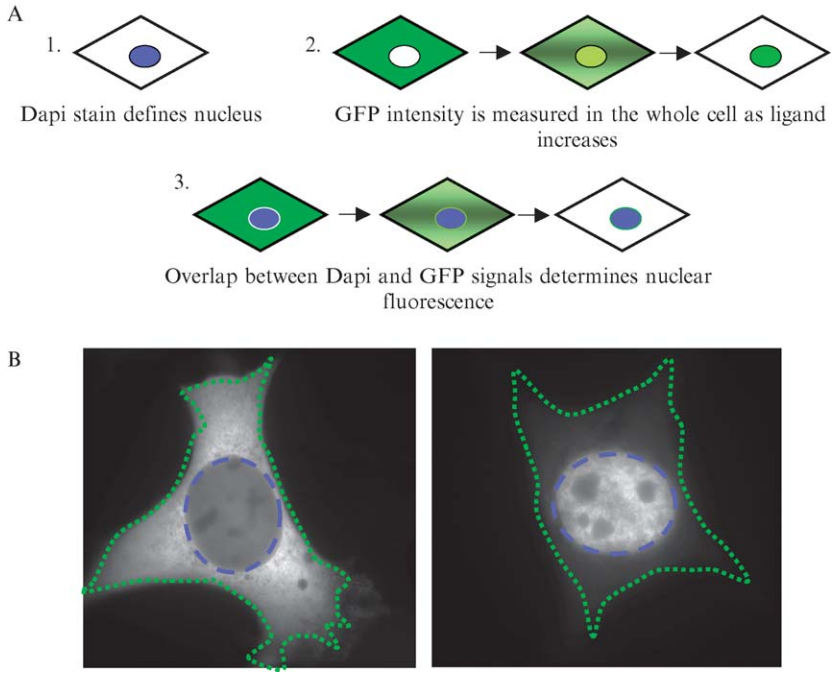

FIG. 4. Measurements of receptor nuclear translocation. (A) Schematic of the procedure for defining the nucleus and the area of the whole cell showing increased overlap in the nuclear dye and the GFP signal as ligand concentration increases. (B) A cell with predominantly cytoplasmic fluorescence (left) and one with predominantly nuclear fluorescence (right) are shown with the areas where intensity measures should be taken and used for calculations highlighted. Blue line delineates the nucleus; the green line delineates the whole cell.

logarithmic units for the *x* axis. Fit the curve to standard formulas for EC_{50} calculations assuming a receptor–agonist interaction (Zhang and Danielsen, 1995). From this calculation, obtain the maximal value and the EC_{50} for the translocation process. The maximum corresponds to ligand efficacy; the EC_{50} defines ligand potency.

Note: from our experience, full agonists exhibit similar efficacies, as expected, while antagonists vary in their efficacy. A typical range for percentage nuclear fluorescence is between 20% in the absence of ligand and 75 to 80% at the highest concentrations of ligand.

Automated Measurements of Nuclear Translocation

The power of automation can be applied to measure translocation as a function of drug concentration. Among the imaging systems currently available with software for automated calculations of cytoplasmic to nuclear shuttling are the Discovery-1 from Universal Imaging, the IC 100 originally designed by Q3DM, the INCell analyzer from General Electrics, and the ArrayScan from Cellomics. The standard algorithms that can be purchased with these imaging systems allow for various degrees of end-user manipulation. When ready to begin drug screens, the investigator should test the various systems to see which one best fits the specific cell-based assay. We have had good results using both imaging and analysis tools of the IC 100 from Q3DM/Beckman, as well as the Discovery-1 system from Universal Imaging. We have not tested other systems directly.

When setting up automated imaging and data analysis, several parameters should be optimized. The choice of multiwell plates is an important component of both image quality and assay cost. Glass bottom, optic plastic and regular plastic plates can be tested unless the manufacturer of the imaging system clearly recommends a particular type of plate. Cell density and cell growth conditions for miniaturization will also need to be optimized. For the actual imaging, the best exposure time for each channel, the number of regions imaged per well, and the range for focus draft between channels will need to be determined. We have found it helpful to always focus on the DAPI channel without refocusing on the GFP channel. This ensures good focus even when the GFP signals are weak or when dust particles interfere with fluorescence detection. Once the parameters have been optimized, screens can proceed. Analyzed data should be generated from each run for the unbiased identification of hits, that is, of compounds, small molecules or other biologically active agents that cause translocation of the chimeric receptor into the nucleus (or that inhibit agonist-induced translocation). These hits should be retested for confirmation and then evaluated in secondary assays.

Built-in Secondary Assays

A cell-based screening protocol is not complete until hits are evaluated in secondary assays that test the ability of the hit to modulate a particular biological event in a manner that is distinct from the screening assay. Because the screening here is based on translocation of the receptor from the cytoplasm to the nucleus, an appropriate secondary assay would measure a downstream event in receptor action. We recommend building such an assay into the original stable cell line system for maximal relevance and ease of measurement. A good complement to the fluorescent-based primary translocation assay is a luciferase-based assay for transcriptional activity. When establishing stable cell lines as described earlier, a reporter construct can be introduced into the cells together with the expression vector for the chimeric receptor and a selection marker. Because the chimera contains the DNA-binding domain of the GR, the reporter gene (such as luciferase) should be driven by a promoter containing glucocorticoid response elements, that is, MMTV, 5xGRE, or tyrosine aminotransferase. Luciferase activity in response to the hits from the screen can be measured in a 96-well format in high- or low-throughput fashion, depending on the number of hits obtained. Luciferase assays should also be performed with individual hits in the presence of a known agonist to measure the potential antagonist activity of the hits. Alternative secondary assays amenable to automation include whole cell or *in vitro*-binding competition studies or measuring certain phenotypes specific to the activation of the receptor under study (such as apoptosis of a particular cell type by a ligand or morphological changes induced by ligand-activated differentiation).

Perspectives and Future Applications

The movement of proteins between compartments in the cell in response to biological signals can be measured using fluorescence technology and offers multiple opportunities for drug discovery applications. In the case of nuclear receptors, translocation from the cytoplasm to the nucleus triggered by ligand binding can be measured automatically by tagging the receptor fluorescently. This movement can be mimicked in nontranslocating nuclear receptors by designing glucocorticoid receptor chimeras as described in this chapter. To date, several orphan receptors await the identification of their cognate ligands, as do the over 200 nuclear receptors expressed in *Caenorhabditis elegans*. Conventional approaches to find these ligands have not yet succeeded. The strategy presented here could aid in catalyzing this discovery. In addition, modulators of many other proteins whose cellular localization is altered or regulated by signaling cascades could be targeted for fluorescence cell-based drug screens.

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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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