29

High Content Translocation Assays for Pathway Profiling

Frosty Loechel, Sara Bjørn, Viggo Linde, Morten Præstegaard, and Len Pagliaro

Summary

This chapter describes the design and development of cell-based assays, in which quantitation of the intracellular translocation of a target protein—rather than binding or catalytic activity—provides the primary assay readout. These are inherently high content assays, and they provide feedback on cellular response at the systems level, rather than data on activities of individual, purified molecules. Multiple protein translocation assays can be used to profile cellular signaling pathways and they can play a key role in determination of mechanism of action for novel classes of compounds with therapeutic potential. This assay technology has developed from laboratory curiosity into main stream industrial research over the past decade, and its promise is beginning to be realized as data acquisition and analysis technology evolve to take advantage of the rich window into systems biology provided by translocation assays.

Key Words: Drug discovery; fluorescent proteins; high content screening; lead optimization; modes of action; multiple modes of action; off target effects; pathway profiling; pathway screening; protein translocation assays; redistribution assay.

1. Introduction

1.1. Cell Signaling and Protein Translocation

Cellular signaling pathways have long been studied, and their function dissected, with molecular and biochemical assays of various kinds. As powerful as these approaches have been, it is now clear that these nonsystems biological approaches give incomplete information about pathway biology. Because of this, assays performed at the cellular level have taken a predominant place in signaling studies, both in academia and in industry in recent years. Perhaps the most compelling reason to study pathways at the cellular level is the fact, now widely accepted, that all signal transduction occurs through transfer of mass within the cell *(1)*. Because of this, it is virtually impossible to assess the net effect of a signaling process without performing cell-based studies. A convergence of science and technology over the past decade has made this possible.

1.2. The Advent of Fluorescent Proteins

A milestone which enabled widespread development of cell-based protein translocation assays was the advent of the use of fluorescent proteins, and particularly the development of cell lines expressing fusion constructs between a target protein and a fluorescent protein. The first of these, *Aequoria victoria* green fluorescent protein (GFP) was initially purified and characterized by Shimomura and colleagues in 1962 *(2,3)*. The gene for GFP was cloned in 1992 *(4)* and expression of the fluorescent protein in other organisms was demonstrated in 1994 *(5)*. This pioneering work led to the rapid development of many variants of cell-based translocation assays, incorporating a

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

wide variety of parental cell lines, targets, and fluorescent proteins. The assays described in this chapter were all developed with *A. victoria* GFP, using an "enhanced" version of the protein conferring better folding, hence greater fluorescence intensity, at 37°C *(6)*; this approach can also be used with a variety of other fluorescent proteins that are now commercially available.

1.3. Development of High-Content Technology

In addition to the biological requirements for cell-based translocation assay systems, there was also a requirement for data acquisition and analysis capabilities beyond those found in the research laboratory of the mid1990s. This challenge has been met with the advent of integrated, image-based high content data acquisition and analysis systems, beginning with pioneering early models almost a decade ago *(7,8)* and progressing to more sophisticated models currently available (*see* **Subheading 2.2.1.**). During this early work, the term *high content* was coined *(8,9)* and it continues to describe complex assay systems, including cell-based protein translocation quantitation, well. This complex field is still undergoing rapid change and development, and it is dealt with in detail in **Subheading 2.** of this volume.

1.4. Pathway Profiling Enters Mainstream Drug Discovery

The concept of large scale industrial pathway profiling has long been intellectually attractive to basic scientists in the pharmaceutical and biotechnology industries. A number of academic groups have embraced this concept from a research perspective in recent years *(10–13)*, but industrial acceptance of the approach has been somewhat more reserved. One factor that has been missing for industry has been solid validation of the cost effectiveness of pathway profiling. This is because of several factors, including corporate confidentiality and the long cycle time for pharmaceutical development, resulting in a long wait for "real" market validation of the competitive advantages of integrating pathway profiling in pharmaceutical discovery and development. The promise of cell-based pathway profiling in industrial drug discovery is starting to be suggested by conference presentations, and evidence of its value is bound to appear in the literature over the next few years.

1.5. Examples of High Content Translocation Assays

Three examples of high content translocation assays are shown in **Fig. 1**, with a transcription factor, a glucose transporter, and a protein–protein interaction as the targets illustrated. Other similar examples have been described *(14–19)*.

The Forkhead (FKHR, FOXO1A) assay (**Fig. 1A**) monitors trafficking of the FKHR transcription factor between the cytoplasm and nucleus *(20)*. When the PI3K pathway is active, FKHR constantly becomes phosphorylated and is exported from the nucleus. When the pathway is inhibited, for example, by addition of the PI3K inhibitor wortmannin, FKHR accumulates in the nucleus. It is relatively simple to use an image analysis algorithm to quantify the translocation of FKHR between the nuclear and cytoplasmic compartments, as a measure of compound activity. Test compounds that bind to cells and fluoresce at the same wavelength as GFP give artifactual activity, and an internal fluorescence assay can easily be incorporated by mixing a small proportion of non-GFP expressing cells with the assay cell line, such that only 80% of the cells are green. If the algorithm is designed so that it reports the percentage of cells in the population that are green, then it is simple to deselect fluorescent compounds (those compounds that result in 100% of the cells in the population being green). Toxic test compounds are another source of artifact: when cells round up on the substrate, a substantial portion of the cytoplasm lies above (and perhaps below) the nucleus, making it appear as if the GFP-tagged target protein has

Fig. 1. *(Opposite page)* Three examples of high-content translocation assays used for pathway profiling. Images of a Forkhead nuclear translocation assay **(A)**, a GLUT4 membrane tranlocation assay **(B)**, and a p53-Hdm2 protein–protein interaction assay **(C)** are shown.

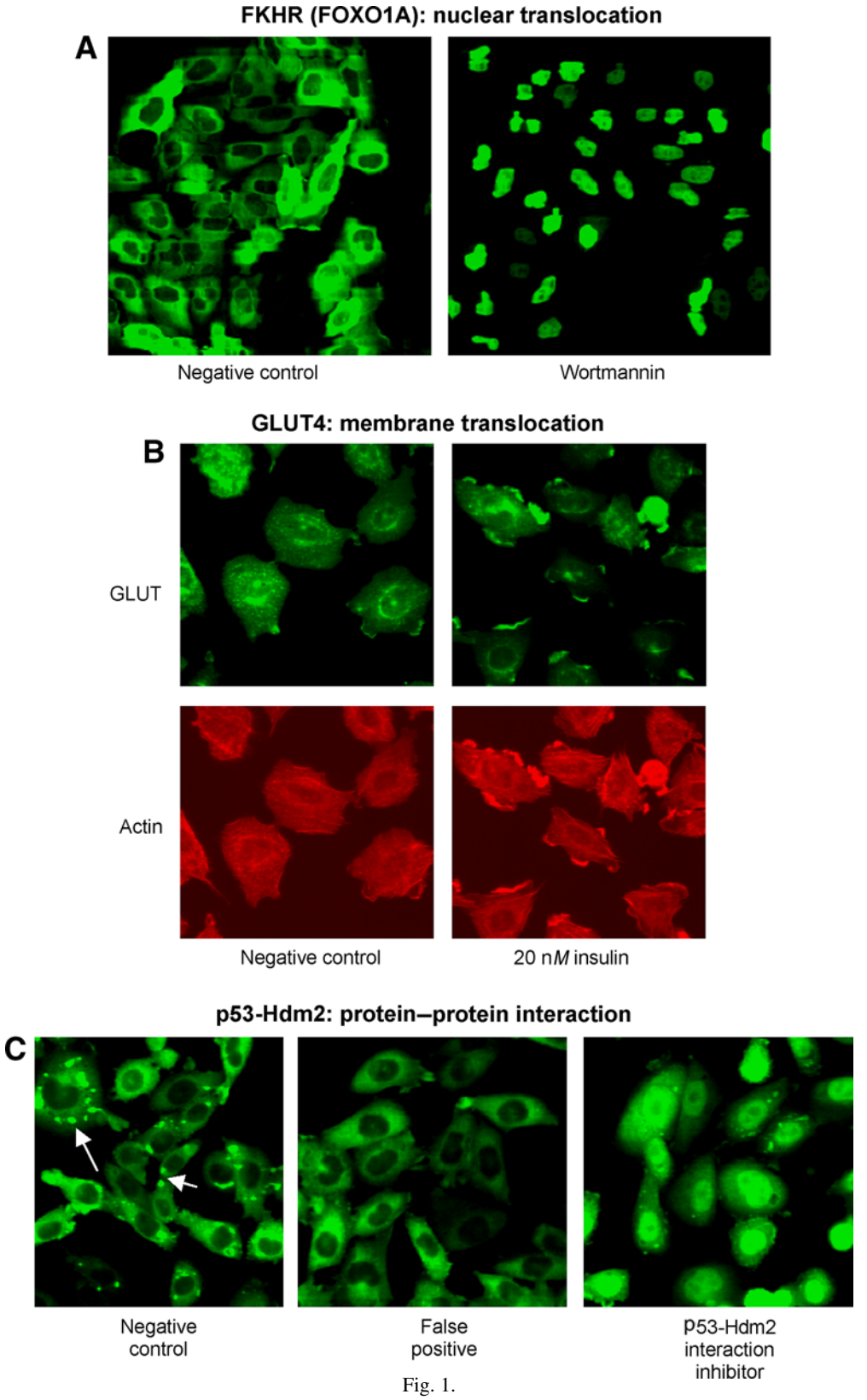
FKHR (FOXO1A): nuclear translocation
A
Negative control
Wortmannin
GLUT4: membrane translocation
B
GLUT
Actin
Negative control
20 n*M* insulin
p53-Hdm2: protein–protein interaction
C
Negative control
False positive
p53-Hdm2 interaction inhibitor

Fig. 1.

translocated to the nucleus. Algorithms can be designed to measure and report the cytoplasmic area for each cell, allowing such toxic compounds to be deselected.

The GLUT4 assay (**Fig. 1B**) measures translocation of the glucose transporter GLUT4 from intracellular vesicles to actin structures located near the plasma membrane, in response to insulin stimulation. Staining of the fixed cells with fluorescent phalloidin allows detection of the reorganized actin cytoskeleton, and gives the possibility of high content analysis of colocalization of GLUT4 with these actin structures.

The p53-Hdm2 assay illustrated in **Fig. 1C** is a high content screening assay based on a "bait and prey" technology, termed GRIP, for measuring protein–protein interactions (*see* **ref. *21*** for an overview of this approach). The bait protein, in this case Hdm2, is fused to an inducible anchor, the phosphodiesterase PDE4A4, and is reversibly localized in compact cytoplasmic foci. The prey protein, GFP-p53, binds to the prey protein and is, therefore, localized to the same foci. Compounds that disrupt the binding between p53 and Hdm2 result in dissociation of GFP-p53 from the foci and translocation of fluorescence to the nucleus. False-positive test compounds that interfere with the anchor cause dissociation of the p53-Hdm2 complex from the foci, but the GFP-p53 remains in the cytoplasm, providing an internal deselection parameter.

2. Implementation of High Content Translocation Assays for Pathway Profiling

We describe how to design a cell-based translocation assay, generate an assay cell line, and optimize the assay; validation of the assay is an essential part of assay design and development. Finally, we give an example of how translocation assays can be used for pathway screening and hit deconvolution.

2.1. Assay Design

2.1.1. Cell Lines

The performance of cell-based assays is a combination of ease of use, reproducibility, and cell-line biology. The very best cell-based assays are derived from cells having the ideal biology for the assay and straightforward handling in terms of culture and automation; however, ease of use and biology often do not go hand in hand, and in such cases it might be necessary to make a compromise on the choice of cell line. Assay biology will of course vary from assay-to-assay, whereas conditions of cell handling by and large are known variables. For high content imaging assays we find that several parameters concerning the choice of cell line are important for the final assay quality (*see* **Note 1**):

1. Cell line morphology. The ideal cell line has flat morphology in a uniform monolayer, strong attachment to assay plates, and minimal tendency to form cell aggregates.
2. Culture conditions. Media, doubling time, and transfectability are important factors. Many primary cells require specialized media supplements or feeder layers, grow very slowly, are difficult to transfect by standard transfection agents, and senesce after relatively few doublings, whereas the opposite is true for many standard cell lines. This might not be a key issue for small experimental studies, but for high throughput assays in routine use, a rule of thumb is that simple culture conditions result in robust and reproducible assays.
3. Source of cells. Many laboratories, both in academia and industry, culture cell lines that have been around for years without knowing the original source of the cell lines. Some of these cell lines will have drifted significantly from the equivalent cell lines from a validated vendor. Therefore, only use cells or cell lines with known history and employ cell banking to maintain the cell passage number as close as possible to the source.
4. Assay alignment. Considering these cell culture guidelines along with the assay biology can be crucial for the final assay development process. Specialized biology often requires specialized cells, and in such cases it might be necessary to do significant optimization of cell handling to obtain acceptable assay quality. On the other hand, standard biology or ubiquitous signaling pathways typically can be measured in standard cell lines, and in such cases there is normally no reason to complicate assay

development by choosing an exotic cell line. However, a few additional considerations may be important for the final choice of cell line. First, the species of the cell line might be important for either biological or technical reasons. If an assay is intended for use with siRNAs, a human cell line will be required because most siRNA collections are directed against human mRNAs. Second, cell alignment with other assays is an important but often overlooked issue. If an assay is used for determining structure-activity relationships for compound series along with other cell based assays, for example, functional assays, it might be desirable to use the same parental cell line for all assays to reduce inter-assay variability, i.e., the structure-activity relationships will be based on real biological activity without interference from secondary effects coming from the use of different cell lines. Thoughtful planning before initiating cell line development, both in terms of operational and biological issues, is always a good investment.

2.1.2. Selection of Targets

If studies in the literature show convincing translocation of the protein of interest on stimulation or inhibition of the signal pathway, then there is a good chance that a robust translocation assay can be developed using a GFP fusion to the target protein. The images published in such articles (in which protein localization is visualized either by immunofluorescence or GFP-tagging) allow a reasonably good evaluation of how easy it will be to quantify the target protein's translocation in the final assay. Marginal translocation is unlikely to result in a high-quality assay.

In cases in which the target itself does not undergo translocation but exerts its cellular activity in static location (or submicroscopic movement), a good strategy is to select a pathway component downstream of the target that will translocate in response to activation or deactivation of the target. This indirect strategy opens up for assaying substances that have activity in the pathway upstream of the protein chosen for translocation, and can thus be used for identification of compound classes that inhibit the pathway by a variety of mechanisms. Deconvolution of compound mode of action should be performed subsequently. When using this strategy it is crucial to investigate the cellular activity of the pathway (*see* **Subheading 2.3.3.**).

2.1.3. Choice of Fluorescent Protein

The objective is to design and construct a genetic fusion between a fluorescent protein and the target protein, which can be expressed in the selected cell line, and in which the fusion protein will display the same translocation behavior as the target protein in response to a given stimulus. A number of fluorescent proteins with various properties are commercially available today. A very important consideration is that the fluorescent properties of the protein should be compatible with the detection equipment, i.e., it can be efficiently excited by the light source of the platform, and the emission wavelength can be detected. When the fluorescent protein is to be used as a marker of target protein translocation, it is important that the fluorescent protein does not itself harbor any signals that direct it to a cellular compartment or cause oligomerization. One should also choose a fluorescent protein that is as fluorescent as possible under the conditions tested, because the fewer molecules that needs to be expressed to do the job, the less intrusive it is on cellular functions.

In mammalian cells, EGFP is a good choice. It has been optimized to express well, and it has a long and excellent track record as a translocation marker. Its fluorescent properties are compatible with the most commonly used imaging platforms.

2.1.4. Choice of Expression Vector

The most important elements to consider in the expression vector are the promoter and selective marker. Both should be considered in relation to the host cell line that is to be transfected. The promoter should be one that expresses well in the host cell, also after many passages if the objective is to generate a stable cell line. Viral promoters such as pCMV and pSV40 can be used in most cell lines. These are considered to be strong promoters and will give rise to highly fluorescent cells. On the other hand, overexpression of the fusion protein might be deleterious to the

normal cellular biology to be assayed, in which case a weaker promoter should be used; alternatively, cell clones should be selected for study not based on the maximal level of fluorescence but rather by their biology. The selective marker should be one that is effective in the host cell line, also during prolonged propagation. If the cell line already contains another DNA construct with a selective marker one must use a different selection for the fusion construct.

2.1.5. Choice of Orientation of the Fusion Protein

Some fluorescent proteins, for example, those based on *Aequorea* GFP such as EGFP, are amenable to fusion at both the N-terminus and the C-terminus, but one should consider whether there is a preferable orientation based on the fusion partner biology. For example, if either terminus of the protein of interest is known to harbor a localization signal or a phosphorylation site, it should probably not be chosen as a point of fusion to the fluorescent protein. Similarly, if the three-dimensional structure of the protein shows that one of the termini is buried, or if a terminal domain is known to interact with other proteins, it should probably be avoided as a point of fusion. If no such information is available, both orientations should be tested.

The length and composition of the linker between the two fusion partners might be of importance for proper function, but this parameter may be difficult to optimize for each construct. As a rule of thumb, a length of 10–20 amino acid residues is frequently used in the linker region, and ideally it should not contain any cryptic biologically active sites such as phosphorylation sites or protease recognition sites (*see* **Fig. 2**).

2.1.6. Stable vs Transient Transfection

Before going into the details of cell line development, it might be helpful to discuss differences between high content translocation assays based on stable cell lines expressing fluorescent probes vs assays based on transient transfection (*see* **Note 2**). Many researchers tend to think that transient assays are faster and easier than assays based on stable cell lines. This holds true for small-scale studies, or research experiments, in which an analysis can be based on few cells and the requirement for reproducibility is low. One advantage of transient assays is that the choice of cell line is not confined to single cell lines, but rather restricted by cell line transfection efficiencies. Often, viral transduction rather lipid-based transfection is required in order to obtain data of reasonable quality.

When assays are conducted routinely and the requirement for assay quality increases, it becomes complex and tedious to run assays based on transient transfections. First, there can be a considerable expenditure of time and transfection reagent used for each experiment. Second, the quality of a high content imaging assay will depend on the uniformity of a cell population in terms of expression level and biological response, both of which are very problematic to achieve with transient assays. The biological response often varies substantially among cells in a nonclonal population, from cells without any detectable response to cells with clear biological activity. There is no way around this other than development of clonal stable cell lines. Third, as assays based on transient transfection imply complex operational steps, it is complicated to maintain assay reproducibility and robustness, especially when assay protocols are transferred between laboratories or end users. Fourth, if viral transduction is necessary to obtain an acceptable percentage of expressing cells in transient assays, biosafety is an important concern, in particular when working with human oncogenes. The requirements for biological containment will be high for all steps involving living cultures and that might be operationally unachievable.

For these reasons, we prefer to develop high-content imaging assays as stable clonal cell lines expressing the protein of interest fused to a fluorescent protein, usually GFP. Such cell lines have uniform expression of the GFP-fusion protein and the biological response is normally aligned within the cell population in terms of timing and the level of response. Image analysis can be done in a robust and reproducible manner with high demands on assay quality. As a consequence these assay cell lines can be transferred between laboratories with very short start-up time for new users.

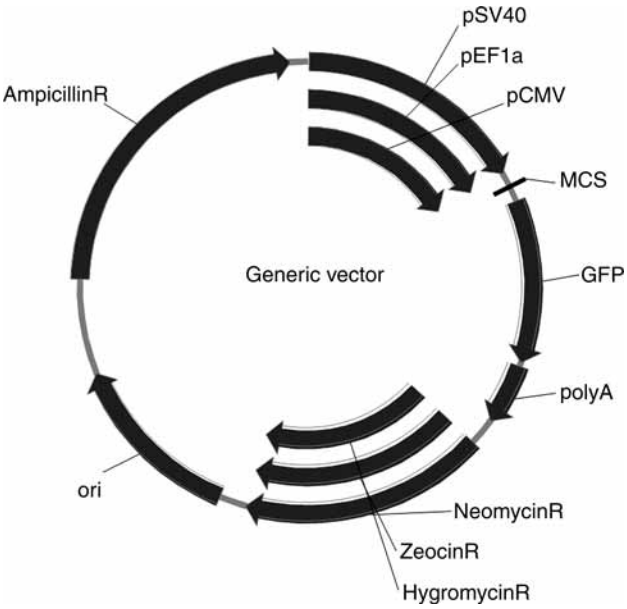

Fig. 2. A generic fusion construct vector map. A typical construct used to create fusions to GFP at the C-terminal of the protein of interest; the multiple cloning site is before GFP. To create fusions of the opposite orientation the MCS would be behind GFP. The fusion protein is expressed from a promoter, for example a viral promoter pCMV or pSV40 or a cellular promoter pEF1a. The plasmid is selected with for example g418, zeocin or hygromycin. In addition there are functions for propagation and selection in *Escherchia coli*.

2.2. Assay Development

2.2.1. Transfection

The first step in the development of stable cell lines for high content translocation assays is transfection of the expression plasmid into suitable host cell lines. Based on knowledge in the literature, complexity of the assay biology, and operational issues, we normally select three to eight candidate host cell lines that are developed in parallel as stable cell lines expressing the GFP-fusion protein of interest. The reason for developing several cell lines in parallel is that the attrition rate for high content assay cell line development is quite high—more than 50%. Many cell lines fail because of unworkable assay biology and some because of problems with protein expression. Among standard human cell lines that work well technically are U2OS, HeLa, and RKO, all of which can be easily transfected using standard transfection reagents such as FuGENE6 or Lipofectamine 2000.

2.2.2. Selection of Stable Cell Line

After transfection, cells are exposed to the relevant antibiotic selection agent, for which a resistance gene is carried on the expression plasmid. Several good selection agents are available including Geneticin (G418), blasticidin, and Zeocin. A resistant population of cells with stable integration of the expression plasmid will grow up after 2–4 wk of culture in selection medium. This cell population varies from cells with no expression of the GFP-fusion protein to cells showing high expression. A good estimate of the biological response of a cell population requires a high fraction of fluorescent cells. This fraction can be optimized by passing the cell population through a fluorescence-activated cell sorter that can be adjusted to collect cells having a defined level of fluorescence. Having a "green" fraction of the stable cell population, the biological activity profile of the cells can be assayed.

2.2.3. Testing the Stably Transfected Cell Line

A protocol for the initial translocation test should be prepared, based on current knowledge of the target protein's translocation behavior. The best available reference compounds (either agonist or antagonist) should be used. The compounds should be tested in concentration response, because cell lines can vary considerably in their sensitivity, and the potency of the same compound purchased from two different suppliers can also vary. Important assay parameters to consider include serum concentration and incubation time. If in doubt, test several different combinations of conditions and time.

Keep in mind that the cell line will be heterogeneous. Some of the cells will not contain detectable levels of the GFP-tagged target protein, and there can be a considerable range of expression levels. It is commonly observed that of the GFP-expressing cells, only a subset show the expected translocation behavior. In some cases it is possible to develop an assay on the multiclonal stably transfected cell line, but in general it is worth taking the time and effort to clone the cell line, in order to maximize assay quality and robustness.

2.2.4. Testing Clones

A simple procedure for obtaining a clonal cell line is to perform dilution cloning. The stably transfected cells are seeded in 96-well plates at a density of 0.5–1 cell per well. After 2–3 wk growth, the plates can be replicated, giving two plates for test of translocation and one mother plate for propagation. A clone should be as homogeneous as possible, both with respect to the expression level of the GFP-tagged target protein and the translocation response. Do not assume that the brightest cells will make the best assay cell line; overexpression can in some cases give aberrant subcellular localization or cellular toxicity. Cellular morphology and growth rate are also factors that can be used when selecting clones. Evaluating the translocation response can in some cases be difficult, because of the fact that the clones by definition are not tested using an optimized protocol. It is sometimes advantageous to perform a limited amount of optimization on the stably transfected nonclonal cell line, in order to develop a protocol that is sufficiently good to allow selection of the best clones.

2.2.5. Optimization

Assay optimization can be a complicated process, because of the fact that there is a large number of parameters to test, generally far too many to allow simultaneous testing in all possible combinations. A good strategy is to focus on two or three parameters at the beginning of the optimization process. Once the best conditions are established for these, move on to test other parameters. Return occasionally to the previously tested parameters if necessary, and retest them in the evolving assay protocol. This is an iterative process that can be repeated as desired until satisfactory assay quality is achieved.

When testing assay parameters, choices must constantly be made as to which conditions should be used in the final assay protocol. These choices can be made based on: (1) maximizing translocation response, evaluated both by visual examination of the images and by the output from the image analysis algorithm; (2) maximizing a key assay parameter such as Z'; (3) minimizing plate-to-plate and day-to-day variability. Important parameters to test include:

1. Serum concentration. Serum contains a myriad of growth factors that activate signaling pathways and affect the translocation of target proteins, often in unpredictable ways. Low serum concentration or serum-free conditions are preferable, because they allow better control of signal pathway activation through addition of defined agonists/antagonists and enhanced sensitivity for serum-binding test compounds, but a balance must be struck with cell viability and morphology. Some cell lines require high serum concentrations, particularly for extended incubations.
2. Assay time. Translocation events can occur on a time-scale of seconds, minutes, or even hours. Optimization is required for each target protein. Very long assay times (e.g., 18 h) can be problematic, because test compounds are often cytotoxic and cause cell rounding, making it difficult or impossible to measure translocation.

3. Cell density. Cell viability and signal pathway activation are sometimes improved when using confluent cells. On the other hand, certain image analysis algorithms work best with well-separated cells. A compromise is sometimes necessary.
4. Temperature. Temperature can be a consideration with assay times of less than an hour. Compound addition is generally performed at room temperature, and the cell plate is then returned to the 37° CO_2 incubator. Thus, the cells are exposed to temperature variations that can affect translocation, and these temperature effects can vary from plate-to-plate or from well-to-well. Strategies for avoiding these effects include the use of prewarmed assay buffer for compound addition and minimizing the amount of time that the cell plate is outside of the incubator *(22)*.
5. Plate coating. Good cell adhesion is crucial for translocation assays. Poorly adherent cells can be washed off the plate during compound addition or plate wash. In addition, if the cells round up during the assay because of insufficient adhesion to the substrate, it can be difficult to accurately quantify translocation with image analysis software. Choice of cell line is important (*see* **Subheading 2.1.1.**). Cell adhesion can often be improved by coating the plates with reagents such as fibronectin, collagen, or polylysine.
6. Fixation. Translocation assays can be performed either on live cells or on fixed cells. Live cell assays are sometimes useful as a prelude to assay optimization, but it is necessary to use a fixed cell format in order to obtain high throughput. A 5–10 min incubation with 4% formalin buffer is an easy and effective fixation method.
7. Nuclear staining. Image analysis algorithms typically require a nuclear marker in order to measure translocation. Hoechst 33258 is a very effective nuclear stain (added to the fixed cells as a 1 μ*M* solution in PBS). DRAQ5 is another useful nuclear stain that has the advantage that it also stains RNA, though to a lesser extent than DNA. This allows it to be used as both a nuclear marker (intense staining) and a cytoplasmic marker (weaker staining), by using two different thresholds in the image analysis algorithm. DRAQ5 is used at a concentration of 0.3–1 μ*M*, depending on cell density. For some cell lines, DRAQ5 might not give sufficient contrast between the nucleus and cytoplasm, necessitating the use of Hoechst 33258.
8. Cell line stability. Multiple vials of the assay cell line should be frozen. A good assay cell line can be continuously cultured for 1–2 mo with no reduction in assay quality. A cell line that is unstable with respect to translocation response or GFP expression levels is of little value.
9. Image based instrument platforms. A number of image-based instrument platforms for high content analysis are currently available, and the approach described in this chapter is platform-independent. The data in this chapter were acquired with the GE Healthcare (Piscataway, NJ) IN Cell 3000; however, these assays are currently running on a wide range of instruments, including the Cellomics ArrayScan, the Evotec Opera, the CompuCyte ICyte, the Molecular Devices Discovery 1, the BD Biosciences Atto Pathfinder HT, and others.
10. Image-analysis algorithms. Manufacturers of the major imaging platforms provide standard algorithms with the instruments. Alternatively, it is possible for users with programming expertise to generate custom algorithms using programs such as MATLAB.
11. Assay optimization example: FKHR (FOXO1A) assay. The U2OS cell line was selected for this assay, because it is a human cell line with good morphology and a functional PI3K signal pathway. It was transfected with a plasmid expressing FKHR-GFP and a stable clone was selected, giving the assay cell line U2OS PS1564 GS cl. 1A. The assay was optimized as a 1 h assay at 37°C in the presence of 0.7% serum, with the PI3K inhibitor wortmannin as the positive control compound. After fixation, the cells were stained with DRAQ5 and read on the INCell 3000 Analyzer, allowing calculation of test compound activity with respect to nuclear accumulation of FKHR, as well as calculation of cell rounding (toxicity) and fluorescence. The assay cell line is stable up to passage 25 (three to four cell doublings per passage).

2.3. Assay Validation

Validation of the assay is crucial before it is released for use. The most important factors to validate are target identity, regulation of target activity, and presence of an intact and functional signaling pathway (**Fig. 3**).

2.3.1. Target Identity

The most straightforward method is to perform Western blotting on a cell extract using a specific antibody against the target. Obtaining a specific band at the expected location on the blot

(relative to a molecular weight marker) is good evidence that the target identity is correct. Include a cell extract of the parental cell line as a negative control. It is sometimes not possible to obtain antibodies of sufficiently high specificity for Western blotting. Instead an siRNA approach using siRNAs directed against the fusion protein can be applied. Target expression level can be monitored by Western blotting against GFP, or alternatively by fluorescence imaging of the siRNA treated cells. This latter approach provides the advantage of detecting potential cytotoxic effects of the siRNA protocol that could compromise the result. A significant down-regulation, without cytotoxic effects, with one or more of the siRNAs is a safe indicator that the identity of the fusion is correct. It is important to include appropriate negative and positive control siRNAs.

2.3.2. Regulation of Target Activity

After confirming the identity of the GFP fusion protein in the cell line, it should be confirmed that its activity is regulated properly by the relevant signaling pathway, if it is feasible to do so. This is relatively straightforward to test in cases in which the target translocates in a phosphorylation-dependent manner following stimulation of the cells, because activity can be monitored using phospho-specific antibodies. This can be done with Western blotting on cell lysates, which has the advantage that in many cell lines there is endogenous target protein, which will get modified simultaneously with the GFP fusion protein. The regulation of the endogenous protein can then be used as an internal reference. When performing such Western blots it is important to use a sensitive procedure to allow identification of even low levels of endogenous protein. Immunofluorescence with phospho-specific antibodies is another approach that provides information about subcellular localization and phosphorylation level of the target, though it cannot distinguish between the endogenous target protein and the GFP fusion protein. The fluorescence microscopy images can be analyzed using the same technique as in the translocation assay and can, thus, be used in a more quantitative manner.

2.3.3. Presence of an Intact and Functional Signaling Pathway

When generating a clonal assay cell line it is important to verify that the intracellular pathway of interest is functional and properly regulated. Validating all of the components of a pathway would in many cases be a long and difficult process, so instead a limited number of key components of the pathway are selected for analysis. When possible, proteins for which phospho-specific antibodies are available should be selected, which makes it simple to assess the state of activation using Western blotting or ELISA. If information about intracellular localization simultaneously with phosphorylation level is required, immunofluorescence will be a better choice (remember to use secondary antibodies conjugated with a fluorophore whose fluorescence spectrum does not overlap with that of GFP). Once the components are selected, a plan for which samples to prepare can be made. Translocation assays are as a standard run in 96-well plates. However, in order to prepare samples for Western blotting larger cell quantities are required, and cell extracts are prepared from cultures in six-well plates. When treating cells in a different format it is important to reproduce the assay conditions as closely as possible, because parameters such as media volume and cell density can influence the biology of the pathway.

In order to modulate the pathway, select a set of reference compounds that either activate or inhibit specific pathway components. These compounds should be as specific as possible and be well described in literature (*see* **Note 3**). The expected activity profile of the compounds is later used as a reference when the actual data have been generated. If there are discrepancies they should be investigated, details rechecked and reconsidered, and if necessary the experiments are repeated until unequivocal results are obtained. It is crucial at this point in assay development to be confident that the assay is giving the desired read-out before continuing with compound testing.

Discrepancies occasionally occur and there can be several explanations for this. Two of the major causes are that the reference compounds are less specific than described in the literature

and have off-target effects, or that the signaling pathway in the assay cell line is not as described in the literature (*see* **Note 4**). Less than optimal specificity of reference compounds can sometimes be dealt with by using a panel of moderately specific compounds, rather than relying on a single reference compound. It is also possible to obtain specific modulation of the pathway using a siRNA approach; however, issues with off-target effects and toxicity must be addressed in the experimental design. A much more severe problem is a signaling pathway that does not respond as expected, because this indicates that the pathway biology is aberrant. This could be a clonality issue, and testing of other clones might lead to identification of a fully functional cell line. Overexpression of the GFP fusion protein could perturb the pathway either because of increased activity at its level in the pathway or a dominant negative effect. In either case the assay cannot be properly validated and should be redesigned, either by choosing a different cell line, reducing the expression level of the fusion protein, or modifying the fusion protein construct (e.g., by eliminating catalytic activity with a well-chosen mutation).

2.4. Pathway Screening

Figure 4 shows an example of how translocation assays can be used for pathway screening. The phosphoinositide-3-kinase (PI3K) pathway is activated when a ligand binds to a growth factor receptor (GFR) on the cell surface. PI3K generates PIP_3 in the plasma membrane, leading to translocation of Akt and other pH domain-containing proteins from the cytoplasm to the plasma membrane. Akt phosphorylates FKHR, resulting in export of the FKHR transcription factor from the nucleus to the cytoplasm. Both Akt and FKHR translocate, and we have developed high content translocation assays for these two targets. When the FKHR translocation assay is used to screen a library of compounds, hit compounds are obtained that inhibit the pathway at the level of FKHR translocation, as well as at all levels upstream of FKHR.

The mechanism of action of the hit compounds can be deconvolved using a panel of translocation assays and traditional assays. The Akt translocation assay ***(20)*** can be used to distinguish between compounds that inhibit PI3K, GFR, or Akt itself (positive in the Akt assay) from compounds that work downstream of Akt (negative in the Akt assay). The mechanism of action of upstream hits can be further defined using kinase inhibition assays (receptor tyrosine kinase inhibition assays or PI3K inhibition assays). Compounds that specifically inhibit nuclear export of FKHR can be distinguished from general nuclear export (Crm1) inhibitors by using the Rev translocation assay. Crm1 inhibitors will be positive in both the FKHR and Rev translocation assays and negative in the Akt translocation assay. Specific FKHR translocation inhibitors will be negative in the Rev translocation assay.

A validated functional assay is an important part of pathway screening. In the case of the PI3K pathway, which many cancer cells are dependent on for proliferation, a good functional assay is a matched pair of cancer cell lines, one of, which is dependent on the PI3K pathway for cell growth and one of which is not. Hit compounds should give clear differential killing of the two cancer cell lines. Hence the best-hit compounds from a pathway screen will give convincing activity in the downstream functional assay, with mechanism of action elucidated by the upstream assay panel.

3. Notes

1. A cell-based translocation assay is much more than just a cell line ***(23)***. Generating a cell line for a translocation assay that stably expresses the protein of interest is a fairly straightforward task, given sufficient time and basic molecular biology and cell culture skills. However, an established cell line is only the starting point for generating a reproducible and robust translocation assay. A broad range of parameters can influence the cellular activity of the pathway and thereby biological relevance of substances tested in the assay. For example, cell density influences activity of certain pathways and can thereby affect the translocation of the target. Serum present in culture medium activates GFRs affecting multiple pathways and cell cycle. Another parameter is the dynamics of the translocation event. In some

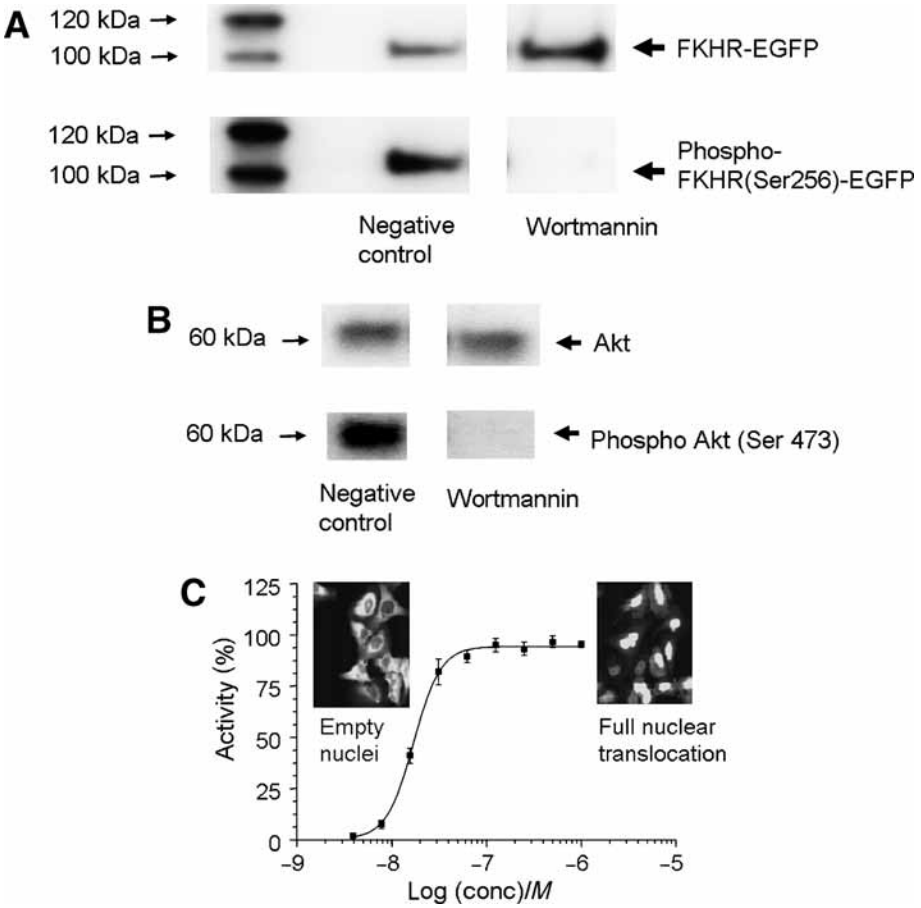

Fig. 3. Validation of FKHR-GFP translocation assay. Target regulation validated by phospho-FKHR specific antibody. In the negative control, FKHR-GFP is phosphorylated; wortmannin treatment leads to significant decrease in phosphorylation **(A)**. Proper regulation of the pathway in the FKHR-GFP assay cell line validated using phospho-Akt specific antibody. Akt is phosphorylated and active in the negative control; wortmannin treatment eliminates Akt phosphorylation **(B)**. In the negative control, FKHR-GFP is inactive and distributed in cytoplasm. Increasing concentrations of wortmannin leads to Akt inhibition, FKHR activation, and translocation of FKHR to the nucleus **(Insets C)**.

cases it is a transient event requiring investigation of the optimal time-point for measurement. Many other parameters need investigation. The relationship between the parameters is impossible to predict and generation of a translocation assay from a cell line therefore includes a long optimization phase.

2. Translocation studies published in the literature are most often performed using transient transfections, because this is a quick and relatively easy way to generate the small number of images needed for a scientific article. For some targets, generation of a stable cell line is the major stumbling block in assay development. This problem can generally be resolved either by testing multiple cell lines, by reducing target protein expression levels (e.g., by using an inducible promoter), or by selective mutations in the target protein (e.g., elimination of kinase activity by mutation of a key amino acid in the catalytic site).
3. Good reference compounds are important for development of a good translocation assay. They are used during assay optimization, assay validation, and as controls in the final assay formatting. Unfortunately, perfect reference compounds are seldom if ever available, so one must make do with what has been described in the literature and is commercially available. Reference compounds are rarely completely specific, so it is important not to use too high a concentration for activation/inhibition of the relevant signaling pathway component, in order to avoid side effects (inhibition of off-pathway enzymes or cellular

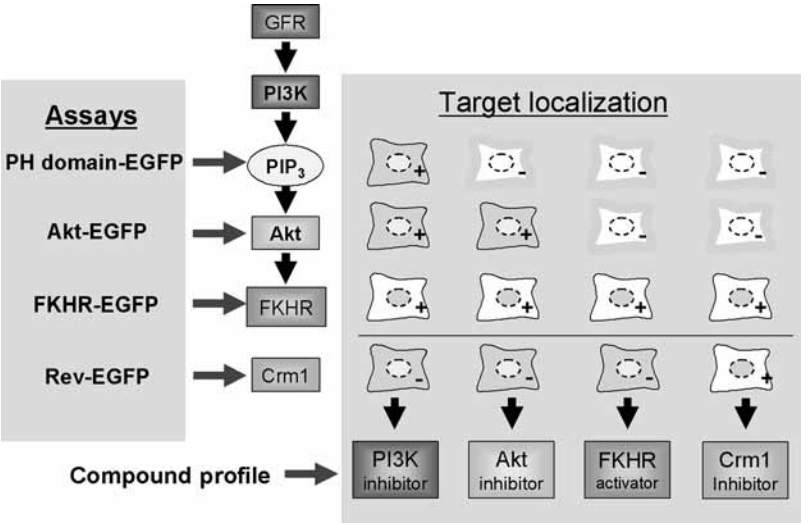

Fig. 4. Scheme for hit deconvolution/pathway screening. Four translocation assays targeting different levels of the PI3-kinase pathway are used for pathway screening and hit deconvolution by creating a family of different compound profiles. The target localization matrix generated by a series of assays at various levels in a signaling pathway enables rapid segmentation of the pathway space in which compounds are acting; subsequent chemo-proteomic analysis can lead to rapid mechanism of action identification.

toxicity). If a gold standard reference compound for a particular target is not available, using a panel of moderately specific compounds can often be useful in assay validation. Finally, RNAi reagents can sometimes be used as a substitute for reference compounds.

4. When performing pathway screening on a large compound library, it is not unusual to find off-pathway hits. The off-pathway mechanism is revealed by lack of activity in the upstream assays used for deconvolution of mechanism of action, or by a mismatch in potency in the screening assay and the downstream functional assay. Occasionally, off-pathway hits are found that are very potent in the functional assay. It can be worthwhile pursuing such hits: a compound with an unexpected mechanism of action that gives high potency in the functional assay can turn out to be extremely valuable as a drug candidate. The possibility of finding such compounds is one of the side benefits of performing a well-designed pathway screen.

References

1. Westwick, J. K. (2004) Drug discovery in the cellular context. *Eur. Pharm. Rev.* **1,** 79–84.
2. Shimomura, O., Johnson, F. H., and Saiga, Y. (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol.* **59,** 223–239.
3. Shimomura, O. (2005) The discovery of aequorin and green fluorescent protein. *J. Microsc.* **217,** 1–15.
4. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992) Primary structure of the Aequorea victoria green-fluorescent protein. *Gene* **111,** 229–233.
5. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.
6. Yang, T. -T., Cheng, L., and Kain, S. R. (1996) Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucl. Acids Res.* **24,** 4592, 4593.
7. Kamentsky, L. A., Gershman, R. J., Kamentsky, L. D., Pomeroy, B. M., and Weissman, M. L. (1996) CompuCyte corporation. Pathfinder system: computerizing the microscope to improve cytology quality assurance. *Acta Cytol.* **40,** 31–36.
8. Giuliano, K. A. and Taylor, D. L. (1998) Fluorescent-protein biosensors: new tools for drug discovery. *Trends Biotechnol.* **16,** 135–140.

9. Ghosh, R. N., Chen, Y. T., DeBiasio, R., et al. (2000) Cell-based, high-content screen for receptor internalization, recycling and intracellular trafficking. *Biotechniques* **29,** 170–175.
10. Bailey, S. N., Wu, R. Z., and Sabatini, D. M. (2002) Applications of transfected cell microarrays in high-throughput drug discovery. *Drug Discov. Today* **7,** S113–S118.
11. Clemons, P. A. (2004) Complex phenotypic assays in high-throughput screening. *Curr. Opin. Chem. Biol.* **8,** 334–338.
12. Perlman, Z. E., Mitchison, T. J., and Mayer, T. U. (2005) High content screening and profiling of drug activity in an automated centrosome-duplication assay. *Chembiochemistry* **6,** 218.
13. O'Rourke, N. A., Meyer, T., and Chandy, G. (2005) Protein localization studies in the age of 'Omics.' *Curr. Opin. Chem. Biol.* **9,** 82–87.
14. Terry, R., Cheung, Y. F., Praestegaard, M., et al. (2003) Occupancy of the catalytic site of the PDE4A4 cyclic AMP phosphodiesterase by rolipram triggers the dynamic redistribution of this specific isoform in living cells through a cyclic AMP independent process. *Cell Signal* **15,** 955–971.
15. Almholt, D. L. C., Loechel, F., Nielsen, S. J., et al. (2004) Nuclear export inhibitors and kinase inhibitors identified using a MAPKAP kinase 2 Redistribution® screen. *Assay Drug Dev. Technol.* **2,** 7–20.
16. Almholt, K., Tullin, S., Skyggebjerg, O., Scudder, K., Thastrup, O., and Terry, R. (2004) Changes in intracellular cAMP reported by a Redistribution assay using a cAMP-dependent protein kinase-green fluorescent protein chimera. *Cell Signal* **16,** 907–920.
17. Pedersen, H. C. and Pagliaro, L. (2004) Redistribution approach for cellular analysis. *Genet. Eng. News* **24,** 36–38.
18. Grånäs, C., Lundholt, B. K., Heydorn, A., et al. (2005) High content screening for G protein coupled receptor targets using cell-based protein translocation assays. *Comb. Chem. High-Throughput Screen* **8,** 301–309.
19. Grånäs, C., Lundholt, B. K., Loechel, F., et al. (2006) Identification of RAS-mitogen activated protein kinase signaling pathway modulators in an ERF1 Redistribution® screen. *J. Biomol. Screen* **11,** in press.
20. Lundholt, B. K., Linde, V., Loechel, F., et al. (2005) Identification of Akt pathway inhibitors using redistribution screening on the FLIPR and the IN Cell 3000 analyzer. *J. Biomol. Screen* **10,** 20–29.
21. Pagliaro, L., Felding, J., Audouze, K., et al. (2004) Emerging classes of protein-protein interaction inhibitors and new tools for their development. *Curr. Opin. Chem. Biol*. **8,** 442–449.
22. Lundholt, B., Scudder, K., and Pagliaro, L. (2003) A simple technique for reducing edge effect in cell-based assays. *J. Biomol. Screen* **8,** 566–570.
23. Pagliaro, L. and Pra estegaard, M. (2001) Transfected cell lines as tools for high-throughput screening: a call for standards. *J. Biomol Screen* **6,** 133–136.