[27] Protein Translocation Assays: Key Tools for Accessing New Biological Information with High-Throughput Microscopy

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Abstract

Redistribution technology is a cell-based assay technology that uses protein translocation as the primary readout for the activity of cellular signaling pathways and other intracellular events. Protein targets are labeled with the green fluorescent protein, and stably transfected cell lines are generated. The assays are read using a high-throughput, optical microscope-based instrument, several of which have become available commercially. Protein translocation assays can be formatted as agonist assays, in which compounds are tested for their ability to promote protein translocation, or as antagonist assays, in which compounds are tested for their ability to inhibit protein translocation caused by a known agonist. Protein translocation assays are high-content, high-throughput assays primarily used for profiling of lead series, primary screening of compound libraries, and as readouts for gene-silencing studies using siRNAs. This chapter describes two novel high-content Redistribution assay technologies: (1) The p53:hdm2 GRIP interaction assay, in which one high-content image feature is used for detection of primary hits, whereas a different feature is used to deselect compounds with unwanted mode of action, and (2) application of siRNAs to Redistribution assays, exemplified by knockdown of Akt isoforms in a FKHR translocation assay reporting on the PI3 kinase signaling pathway.

Pathway Screening Using BioImage Redistribution Technology

Imaging of cells by fluorescence microscopy has been an important tool in cell biology for more than a decade now, and recent years have seen rapidly growing interest in the application of high-throughput microscopy for studying cellular signaling pathways. It has been estimated that the number of high-content screens will increase by 50% over the coming years (Comley, 2005).

Cell-based protein translocation assays can be used to probe cellular signaling pathways that are otherwise difficult to study using traditional biochemical assays. Cellular proteins that translocate in response to external stimuli, such as transcription factors, receptors, kinases, and scaffolding

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proteins, can be tagged with fluorescent molecules and traced by fluorescence microscopy. BioImage Redistribution is a technology whereby a target protein is labeled with the green fluorescent protein (GFP) and expressed in a stable cell line. Redistribution assays allow for a ''pathway'' approach to screening in which an entire pathway is screened using a downstream target protein for fusion to GFP. When performing such a screen, the cell ''selects'' the actual target among the proteins in the pathway, which offers the possibility to discover entirely new classes of compounds with new modes of action, including protein translocation modulators and cell surface receptor modulators, as well as modulators of intracellular enzymes.

The ERF1 Redistribution assay is an elegant example illustrating the way in which a signaling pathway that has historically been difficult to study in high-throughput mode using biochemical methods can be probed with a protein translocation assay. ERF1 is a ubiquitously expressed membrane-distal reporter of the Ras–mitogen-activated protein kinase (MAPK) signaling pathway (Le Gallic *et al.*, 1999). The Ras-MAPK pathway is activated by a wide variety of receptors and is involved in growth and differentiation (Robinson and Cobb, 1997). The pathway is deregulated in various forms of cancer, and compounds inhibiting the pathway are already in clinical development (Hilger *et al.*, 2002). In short, activation of receptor tyrosine kinases, integrins, or ion channels leads to activation of Ras. Ras plays an essential role in the activation of Raf kinase, which directly phosphorylates and activates MEK1 and MEK2. MEK1/2 phosphorylates ERK1 and ERK2, which translocate to the nucleus, where they phosphorylate downstream targets, including ERF1. Unphosphorylated ERF1 is present in the nucleus, and the nuclear export of ERF requires phosphorylation by ERK1/2 (Le Gallic *et al.*, 1999). T24 cells, a human bladder cancer cell line, signal constitutively through the Ras-MAPK pathway due to a single mutation in HRas (Capon *et al.*, 1983). Thus, in T24 cells ERF1 is phosphorylated continuously by ERK1/2 and is exported from the nucleus (see Fig. 1A). If signaling through the Ras-MAPK pathway is prevented downstream of HRAS by the MEK inhibitor UO126 or by the Raf inhibitor BAY-43-9006, ERF1 is no longer phosphorylated by MEK1/2, and therefore accumulates in the nucleus (see Fig. 1B). In addition to inhibitors of the Ras-MAPK pathway, inhibition of the nuclear export machinery by leptomycin B also results in nuclear accumulation of ERF1. By measuring the translocation of the ERF1-GFP fusion from cytoplasm to nucleus in stably transfected T24 cells, compounds modulating the Ras/MAPK pathway downstream of HRAS and compounds modulating nuclear export can be identified. The translocation can be quantified by taking the logarithm of the ratio between fluorescence intensity in the nucleus and the cytoplasm (Log NucCyt Ratio). Figure 1C shows concentration–response curves of UO126, BAY-43-9006,

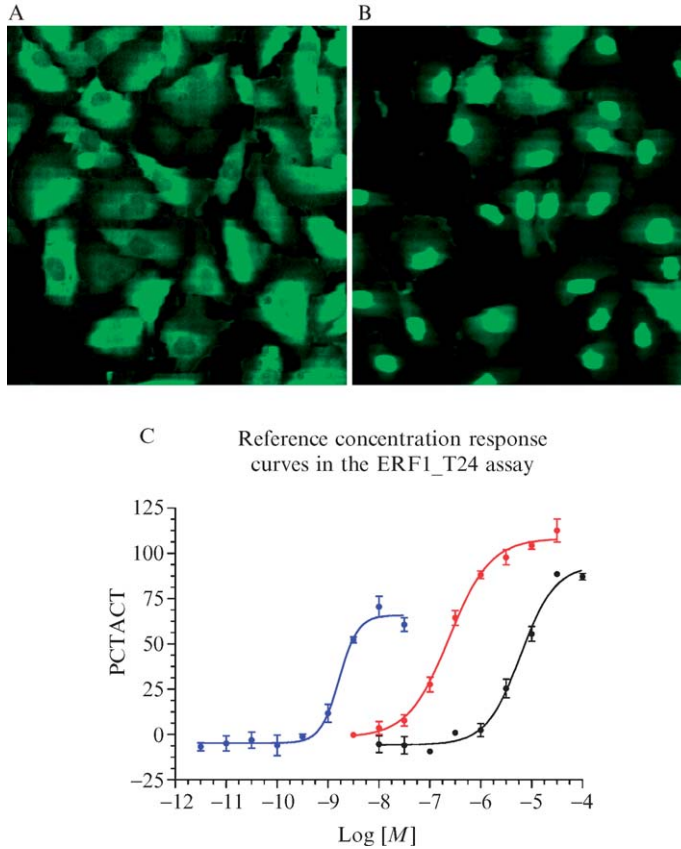

FIG. 1. (A and B) Images of T24 cells stably expressing GFP-ERF1. (A) Untreated cells. (B) Cells treated with 10 μM UO126 for 1 h at 37°. GFP-ERF1 accumulates in nuclei of cells treated with UO126, which can be measured as an increase in the image variable Log NucCyt ratio. (C) Concentration–response curve of UO126 (red), leptomycin B (blue), and BAY-43-9006 (black) in the ERF1 T24 Redistribution assay using Log NucCyt ratio as the response variable ($n = 3$). Scales are normalized to 100% activity for 10 μM UO126.

and leptomycin B when added to T24 cells expressing ERF1-GFP using Log NucCyt ratio as the primary response variable. The ERF1 Redistribution assay has been screened at BioImage using a 265,000 compound library, resulting in 1739 primary hits with an activity above 25%. Fluorescent compounds and toxic compounds causing cell rounding were removed by a second round of image analysis on the same set of images, and the chemical stability and drug-like structure of the hits were evaluated, leaving 718 compounds for retesting and secondary profiling. Two compounds were

eventually identified that exhibit activity in the ERF1 Redistribution assay and selectively inhibit the proliferation of Ras-MAPK pathway-dependent malignant melanoma cells (Grånäs *et al.*, 2006).

BioImage has developed a collection of commercially available protein translocation assays for probing diverse cellular signaling pathways and cellular functions. These include assays probing the MAKP/MEK/ERK pathway, the p39 MAPK pathway, the PI3 kinase pathway, the Wnt/β-catenin pathway, and the TGF-β pathway, as well as assays for GPCR internalization and nuclear hormone receptor translocation. In addition to the assays described earlier, we are developing novel assay types that expand the use of protein translocation using GFP. Two of these new techniques are presented in the following sections: (1) GRIP technology that allows for probing of specific protein–protein interactions outside of a signaling pathway context and (2) the use of siRNA to measure the effect of protein knockdown on cellular signaling pathways as measured in a Redistribution assay. These last two examples are detailed in this chapter.

p53-Hdm2 Protein–Protein Interaction Assay

The p53 tumor–suppressor plays a critical role in the prevention of human cancer. In the absence of cellular stress, the p53 protein is maintained at low steady-state levels and exerts very little effect on cell fate. In the presence of cellular stress, posttranslational modifications of p53 cause increased transcriptional activity and elevated protein levels, resulting in cellular changes such as cell cycle arrest, cellular senescence, and apoptosis (Gregorieff and Clevers, 2005). The antitumor activity of p53 is controlled by its negative regulator, Hdm2, through a feedback mechanism. Hdm2, which is overproduced in many tumors, binds p53 and inhibits its function by modulating its transcriptional activity and stability. Activation of p53 in tumor cells by inhibition of its physical interaction with Hdm2 is therefore a focus of cancer drug discovery.

The p53-Hdm2 GRIP assay is designed to measure the protein–protein interaction between p53 and Hdm2. GRIP is a protein–protein interaction discovery system that can be used to generate truly high-throughput-compatible cellular assays that allow screening for inhibitors of protein interactions. The technology is based on translocation of the human cAMP phosphodiesterase PDE4A4 (Terry *et al.*, 2003) to distinct cytoplasmic foci, where a bait protein (in this case Hdm2) is fused to the cAMP phosphodiesterase isoform PDE4A4B. PDE4A4 acts as an inducible anchor protein and the prey protein (in this case p53) is labeled with GFP. Figure 2 illustrates the configuration of the assay system: Treatment with the PDE4A4 Redistribution agonist RS25344 (Ashton *et al.*, 1994) leads to localization

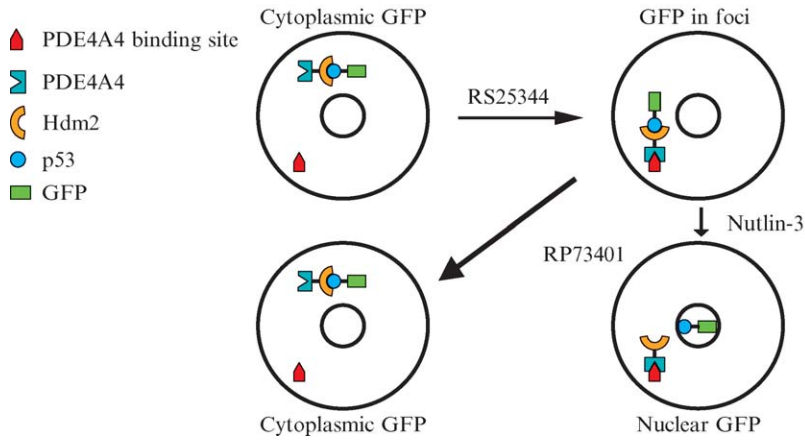

FIG. 2. Illustration of the p53–Hdm2 interaction assay. Fusion constructs of p53-GFP and PDE4A4-HDM2 are stably transfected into the same CHO parental cell line. Aggregation of PDE4A4 at punctate cytoplasmic foci in the presence of the inhibitor RS25344 provides a functional control for the assay. GFP fluorescence only enters the cell in the presence of compounds that disrupt the protein–protein interaction between p53 and HDM2.

of PDE4A4 into compact foci, and through the interaction between p53 and Hdm2, the GFP tag (on p53) is recruited to PDE4A4-binding sites, resulting in GFP-labeled foci. The PDE4A4 inhibitor RP73401 antagonizes the effect of RS25344, causing focus dispersal, thereby serving as a universal reference compound for the assay system (Alvarez *et al.*, 1995).

Nutlin-3 is a small molecule p53–Hdm2 interaction inhibitor, developed by a combination of screening approaches and rational drug design (Vassilev *et al.*, 2004), which serves as a reference compound in the assay. Nutlin-3 causes loss of GFP fluorescence from the PDE4A4 foci (Fig. 2) by dissociating the interaction between PDE4A4-Hdm2[1–124] and GFP-p53 [1–312]. This can be measured through image analysis software that first identifies spots and subsequently calculates the primary response. In this assay, the primary response (quantified by the Fgrains parameter) is the average in each image of a dimensionless number defined for each spot as the ratio between the sum of spot pixel values divided by the sum of pixel values in a region surrounding the spot. The Fgrains variable was found to produce Z factors values around 0.5 to 0.8 for the p53-Hdm2 assay and was superior to other spot features, such as number of spots per cell, total area of spots per cell, and average intensity of spots.

Whereas Nutlin-3 specifically dissociates the p53–Hdm2 interaction, other compounds, such as RP73401, dissociate the PDE4A4 spots (PDE4A4 dislocators) without breaking the p53–Hdm2 interaction. Both types of

compounds, that is, p53–Hdm2 interactors and PDE4A4 dislocators, resulted in spot dispersal, and by only using the Fgrains variable, we were unable to distinguish between the two modes of action. However, it is well known that Hdm2 is a negative regulator of p53 and that treatment with Nutlin-3 results in nuclear accumulation of the p53 transcription factor (Vassilev *et al.*, 2004). This suggested that monitoring GFP-p53[1–312] translocation to the nucleus might be useful for discriminating between p53–Hdm2 interactors and PDE4A4 dislocators. This turned out to be true. Using the logarithm of the ratio between fluorescence intensity in the nucleus and the cytoplasm (Log NucCyt Ratio) to quantify GFP-p53 [1–312] translocation resulted in a significant response when cells were treated with Nutlin-3, whereas treatment with RP73401 showed no response. Figure 3A illustrates the translocation events described earlier, and Fig. 3B and C show concentration–response curves of Nutlin-3 and RP73401 for the output variables Log NucCyt Ratio and Fgrains on normalized scales. The EC_{50} value for Nutlin-3 was $\sim$1.5 μM when Fgrains or Log NucCyt ratio was used as an output variable, whereas the EC_{50} for RP73401 was $\sim$20 nM when using Fgrains. Thus, by using multiple readouts from one assay and one set of images, it was possible to accelerate the compound deselection process during a screen. In addition, we eliminated the need for running a PDE4A4 counterscreening assay, thereby saving time and resources.

Protocol for Generation of p53-Hdm2_CHO Assay Cell Line

CHOhIR cells are stably cotransfected with GFP-p53[1–312] and PDE4A4-Hdm2[1–124] plasmids using the FuGene6 reagent and maintained with 0.5 mg/ml geneticin/G418 and 1 mg/ml zeocin in Ham's F-12 nutrient mixture containing glutamax-1 supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL penicillin, 100 μg/mL streptomycin, Stable transfectants are FACS sorted and cloned to obtain a homogeneous population of cells. p53-Hdm2_CHO cells are grown in a humidified incubator at 37° and 5% CO_2 and are passaged twice weekly by resuspension with 0.25% trypsin/0.53 mM EDTA solution for 3 min at room temperature.

Assay Protocol

1. The day before the assay is performed, seed p53-Hdm2_CHO cells in 96-well Packard ViewPlates at a density of 7000 cells/well in 100 μl Nut. mix.F-12 (HAM) medium containing 1 μM RS25344, 10% by volume FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.5 mg/ml geneticin, and 1 mg/ml zeocin. Incubate for 24 h at 37°, 5% CO_2 with lid.

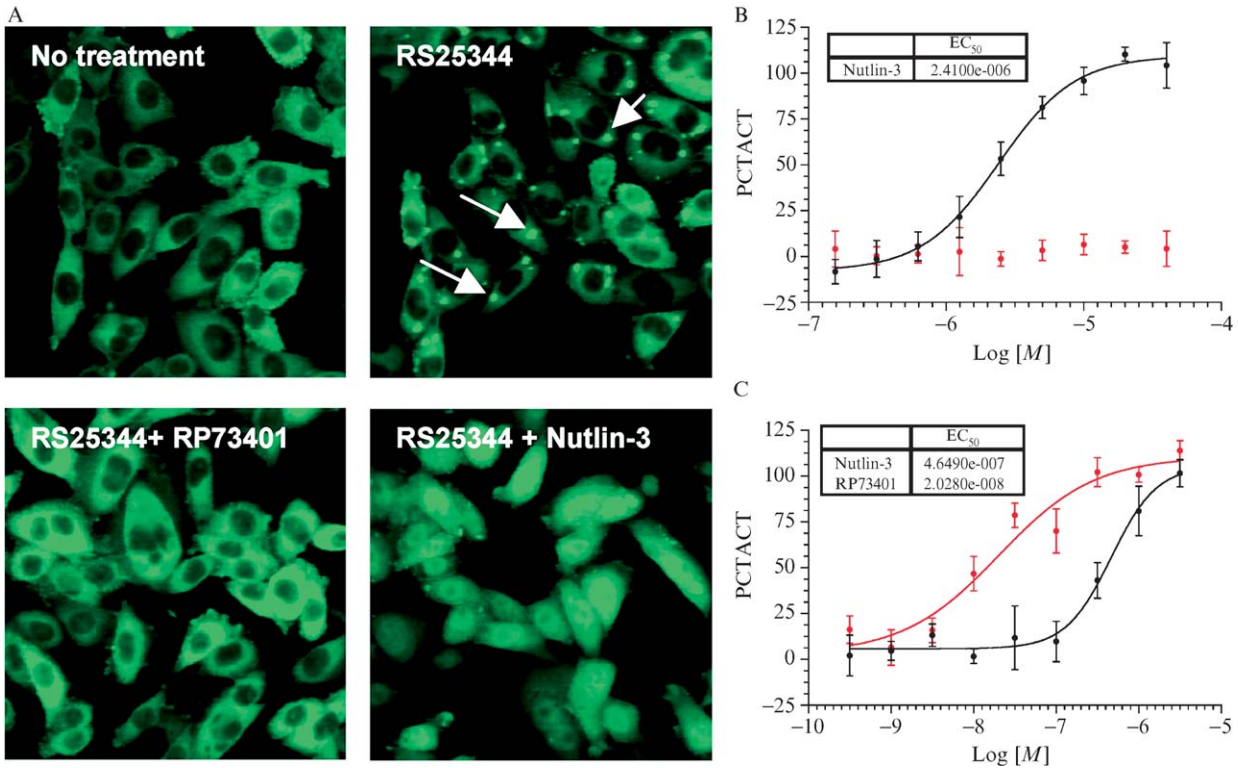

FIG. 3. (A) Images illustrating that RS25344-induced focus formation is dispersed by both Nutlin-3 and RP73401; only Nutlin-3 results in nuclear fluorescence. (B and C) Concentration–response curves of Nutlin-3 (black) and RP73401 (red) for the output variables Log NucCyt ratio (B) and Fgrains (C) on normalized scales ($n = 5$).

2. The next day inspect cells under a microscope to ensure that they look healthy and uncontaminated and that there is no ''edge effect,'' that is, uneven distribution of cells in the edge wells.

3. Prepare assay buffer: Ham's Nutrient Mixture F12 medium containing 10% by volume FBS, 10 m*M* HEPES, and 100 IU/mL penicillin, 100 μg/mL streptomycin.

4. Prepare 400× controls: Negative control: 100% dimethyl sulfoxide (DMSO). Positive control: 4 m*M* Nutlin-3 in DMSO. Final concentrations in assay is 10 μM Nutlin-3/0.25% DMSO.

5. Pipette 1 μl of the controls in the wells of a 96-well polystyrene compound plate; the negative control is placed in wells [A1;D1] and [E12; H12] and the positive control in wells [E1;H1] and [A12;D12].

6. Add 199 μl assay buffer to the compound plate and mix on a shaker table.

7. Transfer 100 μl from compound plate to cell plate.

8. Incubate cell plate with lid for 2 h in a CO_2 incubator.

9. Gently decant assay buffer.

10. Add fix solution (4% formaldehyde in phosphate buffer [pH = 7.0], 150 μL/well).

11. Fix cells for 10 min at room temperature.

12. Wash cells four times with PBS (300 μl/well).

13. Decant PBS and stain cell nuclei by adding 100 μl 1 μM DRAQ5 (Biostatus) in PBS containing 1 m*M* $MgSO_4$.

14. Image and analyze assay plates on a suitable imaging system by measuring Fgrains and Log NucCyt ratio.

Use of High-Content Assays in RNAi Studies

High-content imaging assays have the potential for being the preferred technology format for RNAi studies (for a review about RNAi, see Mittal, 2004). Imaging assays have the advantage, compared to alternative technologies, such as various forms of reporter and ELISA assays, that cells are visualized directly. In addition to the primary functional readout, for example, protein translocation, high-content imaging assays enable the user to acquire and evaluate secondary data from the cells. This is a very valuable feature considering that RNAi studies are quite prone to both false-negative and false-positive results. For instance, cell morphology data can be used to estimate toxicity arising from procedural disturbance derived from siRNA transfection or shRNA expression. In general, transfection efficiency, knockdown efficiency, and transfection-related toxicity are common problems in siRNA studies, but are relatively straightforward to control when using high-content imaging assays. For instance, fluorescence-tagged siRNAs have been

widely used to estimate transfection efficiency by simple fluorescence microscopy of transfected cells. Although useful, this method has a tendency to overestimate the percentage of cells having sufficient knockdown to generate a functional effect. When applying siRNAs to GFP-based translocation assays, we have successfully used a GFP-specific siRNA as a control for transfection efficiency as well as an indirect measure of knockdown level (see Fig. 5). In this case, the level of GFP fusion protein expression following transfection of GFP-specific siRNA is assumed to be representative for the general transfection efficiency and indicative for general knockdown effects. For target discovery purposes, a good control would be to employ a control siRNA that is related directly to the primary functional assay readout, such as H-Ras siRNA in the ERF Redistribution assay mentioned earlier.

When conducting siRNA studies using high-content imaging assays, it is quite evident that a cell population transfected with siRNA is not a uniform population, but rather a distribution of cells ranging from full knockdown to no effect. This is obviously also true for reporter-type assays in which, however, the cell population cannot be evaluated as individual cells, but only as a whole. This is a very common problem in siRNA studies. Transfection of siRNA may result in close to 100% knockdown in a fraction of transfected cells, but if only 50% of the cells are efficiently transfected, the final assay activity profile will be hidden in background noise. This prompts the question: Is there a way to use high-content imaging assays to only include cells with a certain level of knockdown or transfection efficiency in the image analysis? For large siRNA studies, such as target discovery screens, one suggestion is to express an unstable fluorescent protein in the assay cell line that does not interfere with the functional assay readout, for example, express a red or blue unstable fluorescent protein in a translocation assay cell line using a green fluorescent protein as the primary readout. If siRNA directed against the unstable fluorescent protein is included as a fraction in all siRNA transfections, the intensity of this fluorophore represents transfection efficiency and can be used to set a cutoff for cells that should be included in the image analysis.

Translocation assays based on expression of fluorescent fusion proteins can be performed on live cells, creating an opportunity to do multiple measurements in time. This can be beneficial when effects of siRNAs against proteins with divergent or unknown half-life are studied. The time from transfection to effective siRNA-mediated knockdown is normally 2 to 3 days, but can easily vary from overnight to several days and should, in principle, be optimized for each siRNA. Considering the often long response time to siRNA-mediated knockdown, the assay itself should preferably be reasonably short to obtain a reproducible readout. High-content imaging assays, including translocation assays, have the advantage

that most assay time lines range from minutes to 1 to 2 h, whereas reporter assays usually are performed overnight.

By far the broadest application of high-content imaging assays is currently small academic research studies of cell signaling pathways where siRNAs are used to link protein activities to biological functions. However, several academic laboratories and commercial institutions have commenced doing focused target discovery using RNAi technologies. Large libraries of siRNAs or retroviral vectors expressing shRNA have been screened to identify new members of signaling pathways and new targets for drug discovery (Berns *et al.*, 2004; Brummelkamp *et al.*, 2004). For the reasons mentioned earlier, high-content imaging assays are good candidate assays for such broad RNAi screens.

Use of siRNA-Mediated Knockdown to Validate Akt Isoform Dependency of a FKHR Redistribution Assay

The FKHR Redistribution assay is an example of a high-content imaging assay that has been biologically validated with Western blots, reference compound testing, and siRNA studies. The mammalian transcription factors FKHR, FKHRL1, and AFX function as key regulators of insulin signaling, cell cycle progression, and apoptosis downstream of phosphoinositide 3-kinase (PI3K). In growing cells, Forkheads are kept inactive through Akt-mediated phosphorylation of three conserved threonines. Phosphorylation of these threonines causes binding to 14–3-3 proteins in the nucleus followed by nuclear export and cytoplasmic retention. Nuclear export of Forkhead proteins is dependent on the classical NES/Crm1 pathway. When PI3K/Akt signaling is inhibited, FKHR translocates to the nucleus, where it activates target genes involved in growth arrest and apoptosis (Burgering and Kops, 2002).

We tested a range of reference compounds inhibiting PI3K, Akt, and nuclear export to validate that the FKHR Redistribution assay detects inhibition of individual components of the PI3K pathway upstream of FKHR. As shown in Fig. 4A, all of these reference compounds have expected activities in the FKHR Redistribution assay. Furthermore, a PI3K inhibitor (wortmannin) and an Akt inhibitor (compound 13 in Barnett *et al.*, 2005) inhibit phosphorylation of Akt and FKHR at relevant serine residues (Fig. 4B).

The FKHR Redistribution assay has been further characterized by transfection of Akt isoform-selective siRNAs (see later for a general protocol for siRNA transfection). Figure 5A shows that transfection of a GFP-specific siRNA prevents expression of FKHR-GFP, although some residual expression is detectable in a fraction of cells, indicating that the

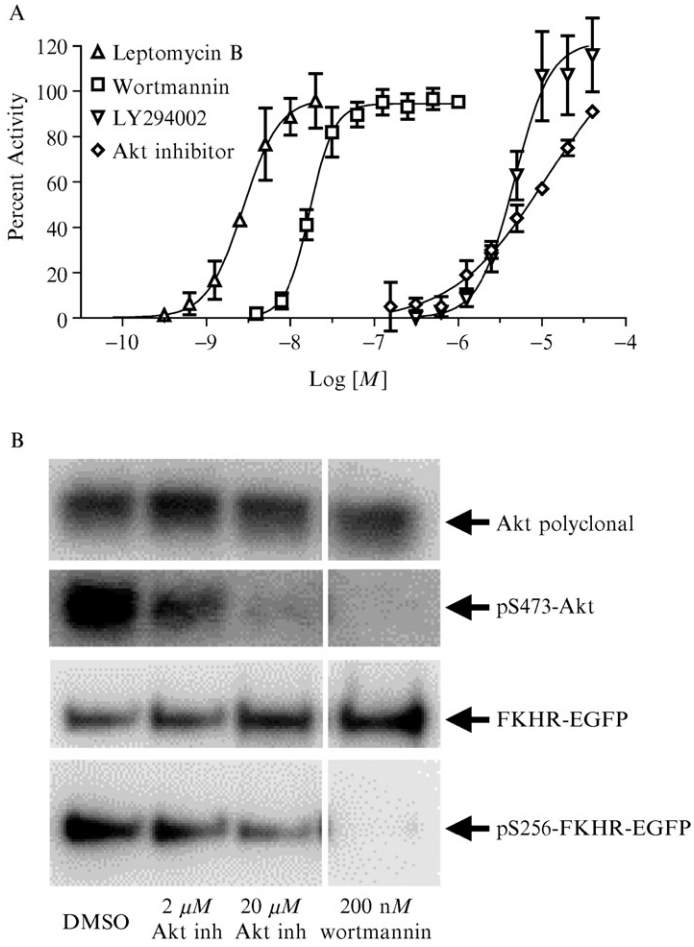

FIG. 4. Validation of FKHR Redistribution assay by reference compound testing and Western blotting. (A) Concentration–response curves of PI3K inhibitors (wortmannin and LY294002), a reference Akt inhibitor [compound 13 in Barnett *et al.* (2005)], and the nuclear export inhibitor leptomycin B. Protein translocation is detected as the logarithm of the ratio between nuclear and cytoplasmic fluorescence (Log NucCyt ratio) in FKHR-GFP expressing U2OS cells and normalized to the activity of 150 n*M* wortmannin (100% activity). (B) Western blots on cell lysates from the FKHR Redistribution assay cell line. Activation of Akt by phosphorylation of Ser473 and deactivation of FKHR by phosphorylation of Ser256 can be inhibited by wortmannin (PI3K inhibitor) and an Akt inhibitor [compound 13 in Barnett *et al.* (2005)].

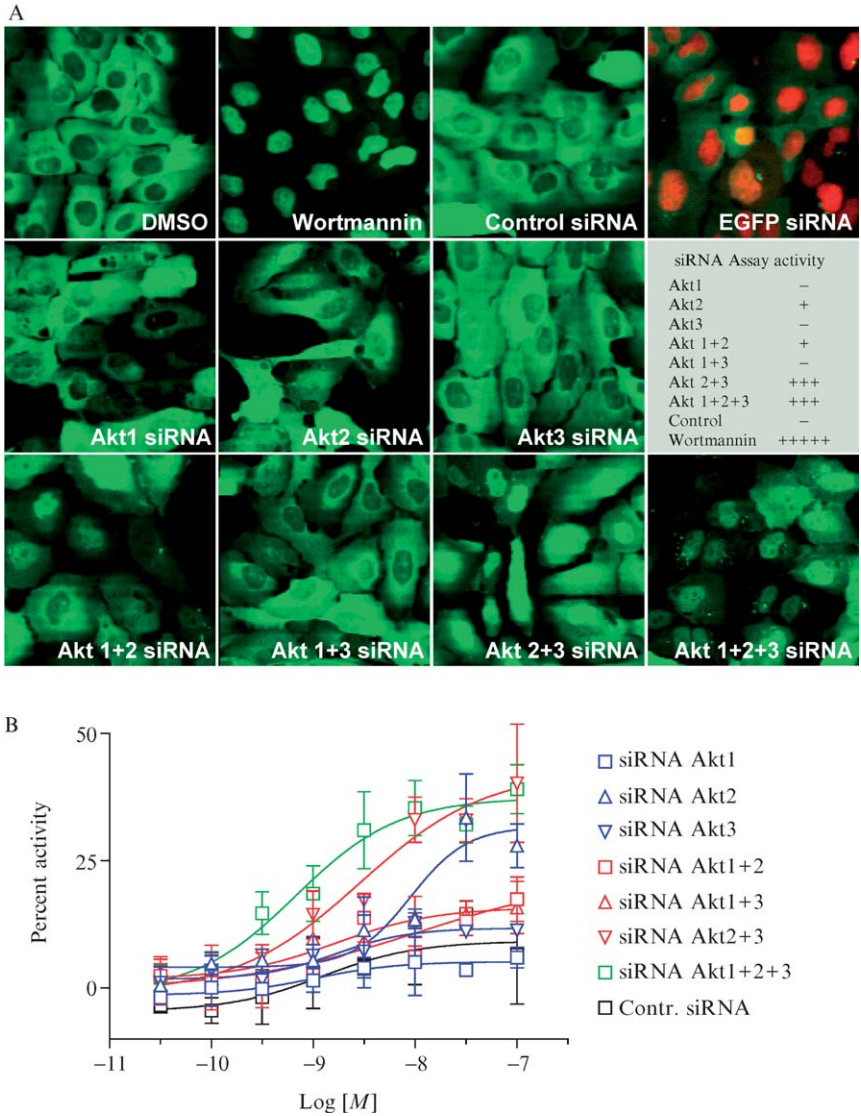

FIG. 5. Activity of Akt isoform-specific siRNAs in FKHR-GFP Redistribution assay. (A) Representative images showing effects of Akt siRNA transfection. The FKHR-GFP assay is performed 72 h posttransfection of Akt isoform-selective siRNAs (20 n*M* per siRNA). DRAQ5-stained red nuclei in cells transfected with EGFP siRNA show that cells are present in the image, despite knockdown of FKHR-GFP expression. Selective knockdown of Akt isoforms has been verified by TaqMan PCR (done in collaboration with ALTANA Pharma AG). (B) Concentration–response curves derived from analysis of images acquired from Akt siRNA transfections performed in triplicate. Concentrations are plotted as total siRNA concentrations. Percentage activity is calculated relative to the activity of 150 n*M* wortmannin, which is defined as 100% activity.

transfection is successful, but knockdown is not 100% effective. Transfection of a nontargeting control siRNA has no effect on FKHR-GFP localization (compared to DMSO-treated cells). This is also the case for transfection of Akt1- and Akt3-specific siRNAs, whereas transfection of Akt2 siRNA leads to slight redistribution of FKHR from the cytoplasm to the nucleus. When the Akt1-, Akt2-, and Akt3-specific siRNAs are mixed before transfection either as mixtures of two siRNAs or as a mix of all three siRNAs, only the combination of Akt2 and Akt3 siRNA and the combination of all the Akt siRNAs give rise to significant translocation of FKHR-GFP to the nucleus. This indicates that Akt2 and Akt3 are important for FKHR regulation, at least in the assay cell line, whereas Akt1 is not. All shown siRNA knockdowns have been validated by quantitative polymerase chain reaction (PCR) on RNA purified from parallel transfections (data not shown). Figure 5B shows concentration–response curves of siRNA transfections in the FKHR assay using the Log NucCyt ratio as a variable on a normalized scale (100% activity is treatment with 150 n*M* wortmannin for 1 h). These data suggest that transfection of Akt siRNAs is not as effective in terms of FKHR redistribution as treatment with reference compounds such as wortmannin. For example, transfection of a mix of all three Akt siRNAs results in 40% activity, as compared to the 100% activity achieved with 150 n*M* wortmannin. This is probably due to the fact that reference compounds (inhibition of enzymatic activity) act differently than siRNAs (knockdown of expression). Knockdown mediated by siRNA is rarely 100% effective, and the remaining amount of protein may be sufficient to partly rescue protein function, whereas inhibition of enzymatic activity directly blocks protein function for the total pool of the relevant enzyme. We conclude that translocation of FKHR-GFP is regulated by Akt2 and Akt3 but not Akt1 in the FKHR Redistribution assay cell line presented here.

Protocol: Test of Functional Effects of siRNAs in Redistribution or Translocation Assays

General Protocol for siRNA Transfection in 96-Well Plates

Reagents: siRNA in 20 μM stock solutions, Lipofectamine 2000 (Invitrogen), Opti-MEM medium (Invitrogen)

1. The day before transfection plate cells in 96-well Packard ViewPlates at a density of 1500 to 2000 cells/well in 100 μl/well growth medium without penicillin–streptomycin. Incubate for 24 h at 37°, 5% CO_2 with lid.

2. At the day of transfection dilute at 5× concentration of final siRNA concentration in 15 μl/well Opti-MEM. The optimal final siRNA

concentration for transfection of standard cell lines such as HeLa and U2OS cells is around 10 n*M* and a good range for concentration response studies is 0.1 to 100 n*M*. A few siRNAs can be mixed, but the total concentration should not exceed 50 to 100 n*M* depending on the cell type. Stocks of siRNA should be handled with RNase-free filter tips as other types of RNA.

3. Dilute Lipofectamine 2000 reagent in Opti-MEM medium. Use 0.15 μl/well lipofectamine 2000 diluted in 10 μl/well Opti-MEM. Mix by pipetting and incubate for 5 to 10 min at room temperature.
4. Mix siRNA and Lipofectamine 2000 dilutions (15 + 10 = 25 μl/well).
5. Incubate at room temperature for 20 min.
6. Add 25 μl/well siRNA and Lipofectamine 2000 transfection mix to cell plates.
7. Incubate plates 24 h.
8. Change medium on plates to fresh growth medium.
9. Incubate for 48 h.
10. Run Redistribution or translocation assay according to relevant assay protocol.
11. Image and analyze assay plates on a suitable imaging system.

Assay-Specific Cell-to-Cell Heterogeneity Plays a Role in Assay Quality

Development of Redistribution assays comprises transfection and selection of high-performing clonal cell lines, followed by thorough optimization of assay conditions, image acquisition, and image analysis. Details of clone generation and assay condition optimization have been reviewed elsewhere (Loechel *et al*., 2006). Here, we describe an often overlooked factor in the development of high-content assays: the number of cells to analyze in order to achieve the best possible *Z* factor.

Most traditional biochemical assays performed on cells in microtiter plates, such as reporter assays and ELISA assays, result in a single assay response per well, with no opportunity to assess the heterogeneity of the cell population in individual wells or to remove the contribution of nonresponders in order to lower assay noise. In contrast, high-content imaging assays allow assessment of the variation between cells in the same well, thus enabling assay developers to sort cells based on primary response or secondary image features, as well as to adjust the number of analyzed cells to achieve the best possible *Z* factor (Zhang *et al*., 1999). The standard deviation (SD) of So and Smax used for computing the *Z* factor is calculated as the SD between the average response in the So wells and Smax wells, respectively, the so-called interwell variation. However, even though the

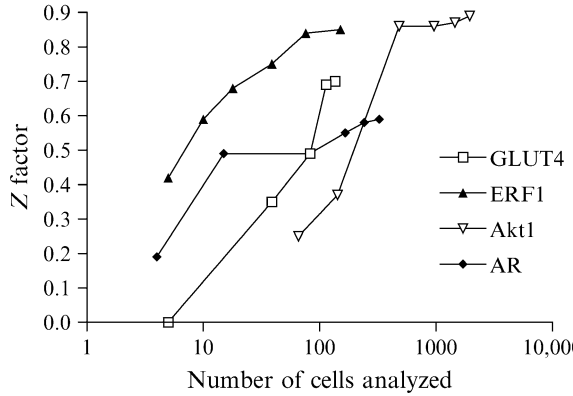

FIG. 6. Assay quality (Z factor) as a function of the number of cells analyzed. Images were acquired on an IN Cell Analyzer 3000 (GE Healthcare). The number of cells in an image was reduced by changing the scan width and scan length, thereby reducing the field of view. The number of cells analyzed was increased by acquiring multiple images from different positions of the same well. The GLUT4 assay and the Akt1 assay are membrane translocation assays, i.e., their primary response is the translocation of GLUT4 or Akt1 from the cytoplasm to the membrane as measured by the area of fluorescent spots in the cell membrane. The ERF1 assay and the androgen receptor (AR) assay are nuclear translocation assays, whose primary output is the translocation of ERF1 or AR from cytoplasm to nucleus as measured by the variable Log NucCyt.

intrawell variation does not appear directly in the Z factor, it has a direct influence on the precision of the average response from each well. In other words, the more variation between individual cells in the same well (intrawell variation), the more cells need to be quantified per well to achieve the same assay Z factor. We find that the intrawell variation in Redistribution assays depends strongly on the identity of the assay. This is illustrated in Fig. 6, which shows the relationship between number of analyzed cells and Z factor of various Redistribution assays. The ERF1 Redistribution assay (described earlier) and the androgen receptor (AR) Redistribution assay are assays whose primary output is the translocation of ERF1 or AR from cytoplasm to nucleus as measured by the variable Log NucCyt. Both assays yield acceptable Z factors, even when relatively few cells per well are analyzed. The ERF1 assay gave a Z factor of 0.42 when only an average of 5 cells per well was analyzed, whereas the AR assay had a Z factor of 0.49 when an average of 15 cells per well was analyzed. Both assays showed rapidly increasing Z factors as the number of analyzed cells increases up to around 100 to 150 cells. The GLUT4 assay and the Akt1 assay are both membrane translocation assays, that is, their primary output is the translocation of GLUT4 or Akt1 from the cytoplasm to the membrane

as measured by the area of fluorescent spots in the cell membrane. It is our experience that membrane translocation assays generally have higher intrawell variation compared to nuclear translocation assays, as exemplified by the GLUT4 and Akt1 assays. The GLUT4 assay needed around 40 cells, and the Akt1 assay needed around 140 cells to achieve Z factors above 0.3.

In practice, the number of cells analyzed in high-content assays is often determined by the field of view of the microscope and lenses used for image acquisition. Thus, optimization of the number of cells needed for analysis is sometimes deemphasized as an important factor for overall assay quality because of technical limitations. Certainly, it is always best to optimize assay conditions, such as serum concentration, incubation time, and temperature, in order to develop robust assays that require a fairly low number of analyzed cells to achieve good assay quality. However, if a large variation between cells is caused by the endogenous biology of the cells, such as the cells being in different growth phases, having different shapes, and so on, the only way to achieve a satisfactory Z factor can be to increase the number of analyzed cells. Most high-content platforms include the possibility of acquiring multiple images from different positions of the same well, thereby increasing the number of analyzed cells. Because of the increase in image acquisition time per well, this is often undesirable when developing high-content assays for screening purposes, but it can be the only way to achieve a good Z factor for translocation assays characterized by high cell-to-cell variation.

Future Developments

The ability to assess biological responses at the cellular level using optical microscopy as a primary tool has a long and productive legacy, dating as far back as early work by pioneers such as Leevenhoek and Koch (Wilson, 1995). Recent decades have seen dramatic improvements in our abilities to monitor and manipulate biological systems with tools, including fluorescent analog cytochemistry (Wang, 1989), novel fluorescent dyes (Haugland, 2005), and fluorescent proteins (van Roessel and Brand, 2002). As valuable as these advances have been from a research perspective, their integration into industrialized settings, such as drug discovery, has been limited by the lack of tools for rapidly acquiring and analyzing the enormous quantity of image information that must be processed to evaluate biological responses at the cellular level. These aspects of the field have changed, and continue to change, rapidly, with the introduction of instruments designed for high-speed, optical microscope-based acquisition of cellular images from multiwell plates. A number of such instruments are currently available commercially and are reviewed elsewhere in this

volume. These instruments have quickly eased the bottleneck for data acquisition that presented challenges to the field for a number of years and have instead moved the bottleneck to data analysis. Work in this field is progressing rapidly, and the coming generation of instrument platforms promises to provide both the hardware and the software support necessary to realize the full potential of protein translocation assays.

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[28] High-Content Screening of Functional Genomic Libraries

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Abstract

Recent advances in functional genomics have enabled genome-wide genetic studies in mammalian cells. These include the establishment of high-throughput transfection and viral propagation methodologies, the production of large-scale cDNA and siRNA libraries, and the development of sensitive assay detection processes and instrumentation. The latter has been significantly facilitated by the implementation of automated microscopy and quantitative image analysis, collectively referred to as high-content screening (HCS), toward cell-based functional genomics application. This technology can be applied to whole genome analysis of discrete molecular and phenotypic events at the level of individual cells and promises to

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