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[14] Adenoviral Sensors for High-Content Cellular Analysis

By JONATHAN M. KENDALL, RAY ISMAIL, and NICK THOMAS

Abstract

To maximize the potential of high-content cellular analysis for investigating complex cellular signaling pathways and processes, we have generated a library of adenoviral encoded cellular sensors based on protein translocation and reporter gene activation that enable a diverse set of assays to be applied to lead compound profiling in drug discovery and development. Adenoviral vector transduction is an efficient and technically simple system for expression of cellular sensors in diverse cell types, including primary cells. Adenoviral vector-mediated transient expression of cellular sensors, either as fluorescent protein fusions or live cell gene reporters, allows rapid assay development for profiling the activities of

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candidate drugs across multiple cellular systems selected for biological and physiological relevance to the target disease state.

Introduction

There is an emerging realization in drug discovery that pursuing industrialized high-throughput screening (HTS) of massive compound libraries, while generating huge quantities of data, has not significantly improved productivity or generated better drugs. These deficiencies are driving new efforts to improve the drug development process by integrating data from a range of disciplines (Bleicher *et al.*, 2003) into a more unified and knowledge-based process (Bajorath, 2002). This trend is accompanied by a shift in emphasis away from solely the number of data points that can be generated towards a focus on the quality of data obtained (Walters *et al.*, 2003). Recent developments in high-throughput microscopy (Ramm and Thomas, 2003; Mitchison, 2005; Price *et al.*, 2002) allow high-definition intracellular analysis at very high rates, with high-end systems imaging as many as 30,000 wells per day, with 500 cells analyzed per well. Coupled with cellular sensors of appropriate sensitivity and sophistication (Taylor *et al.*, 2001) and matching image analysis software, these high-content screening (HCS) and high-content analysis (HCA) platforms provide the potential to generate detailed and informative functional data at levels of throughput that were previously obtainable only with instrumentation yielding low-complexity data. Ongoing developments in hardware and software are continually improving the power and statistical robustness of cellular imaging, allowing HCA to play an increasingly significant role in decision making toward the early identification of viable and safe drug compounds.

A critical requirement for high-content analysis is the availability of specific and sensitive sensors for key cellular pathways and processes; it is these sensors that are at the heart of producing data-rich cellular images. Many sensors for cellular analysis use genetically encoded elements (Guerrero and Isacoff, 2001; Tsien, 2005; Wouters *et al.*, 2001; Zhang *et al.*, 2002) either as the complete sensor, for example, a green fluorescent protein (GFP)–fusion protein, or as part of a sensor system, for example, a reporter gene generating a fluorescent product.

Cell-based assays used for drug discovery have traditionally used stable cell lines to express genetically encoded sensors (Lundholt *et al.*, 2005; Pagliaro and Praestegaard, 2001). While these engineered cells are ideally suited to primary screens requiring large numbers of cells, the timescales and resources required to generate stable cells generally restrict sensor-expressing cell lines to standard cell types such as HEK293 (Hamdan *et al.*, 2005), CHO (Oosterom *et al.*, 2005), and U2OS (Oakley *et al.*, 2002).

One of the major reasons for the limited diversity of stable cell lines are the restraints imposed by plasmid-based transfection procedures. Chemical transfection procedures using cationic lipid complexes (Dass, 2004), polymers (Dennig, 2002), or peptides (Fischer *et al*., 2001) often need extensive optimization (Colosimo *et al*., 2000) and generally yield only low transfection efficiencies in nontransformed and primary cells (Kiefer *et al*., 2004; Welter *et al*., 2004; Young *et al*., 2004). While alternative physical transfection procedures, including novel methods based on electroporation (Distler *et al*., 2005), are available, these have not been used extensively to generate sensor expressing cell lines.

To overcome the limitations of using stably engineered cells and hence to enable a more diverse set of assays to be applied in lead compound profiling, we have generated a library of adenoviral encoded cellular sensors based on protein translocation and reporter gene activation.

Design and Construction of Adenoviral Sensors for Cellular Assays

Adenoviral vectors have a number of distinct advantages over chemical transfection methods and other viral vectors as a universally applicable cell delivery vehicle for the rapid development of transient cellular assays in different cell types, including primary cells (Table I). Adenoviral vectors are compatible with the transduction of a large range of cell types, including terminally differentiated cells, and have high transduction efficiency, allowing sensor expression to be achieved at a low multiplicity of infection (MOI), minimizing perturbation of cellular processes. Sensor expression from adenoviral vectors does not require integration into the host cell genome, allowing sensor expression without possible modification of chromatin structure or activity arising from random DNA sequence insertion.

Recombinant adenoviral vectors for expression of cellular sensors are engineered (Fig. 1) by replacing the E1 gene of the viral genome (Bett *et al*., 1993; Hitt *et al*., 1997) with cDNA encoding the gene of interest fused to a fluorescent protein or with a reporter gene construct. Removal of the E1 gene renders the adenoviral vectors replication deficient; however, they can be propagated by infection of cells such as HEK293 that provide the E1 function by complementation. To further increase the capacity of the vectors for insertion of foreign DNA, the vector system has a further deletion in the nonessential E3 region of the genome (Bett *et al*., 1994).

Adenoviral vector entry into the cell (transduction) involves a number of interactions between proteins on the capsid coat of the vector and target cell surface (Vellinga *et al*., 2005). The process starts with absorption of the virus onto target cells through interaction with the coxsackie and adenovirus receptor (CAR) on the cell surface, and entry of the adenoviral vector

TABLE I
COMPARISON OF CHEMICAL AND VIRAL GENE DELIVERY METHODS AND VECTORS

	Chemical	Adenoviral	MMLV (retroviral)	Lentiviral (retroviral)	Baculoviral	Adenoviral advantages
Tropism	Broad	Broad	Broad	Broad	Restricted	Can be used to transduce many different cell types
Transduction efficiency	Low	High	Low	Low	Low	Effective for cell types that are difficult to transfect Low multiplicity of infection required for transduction
Terminally differentiated cells	Variable	Yes	No	Yes	Yes	Enables sensor expression and analysis in physiologically relevant cell types
Expression	Transient/ stable	Transient	Stable	Stable	Transient/ stable	No adverse effects from integration of gene into host cell chromosome

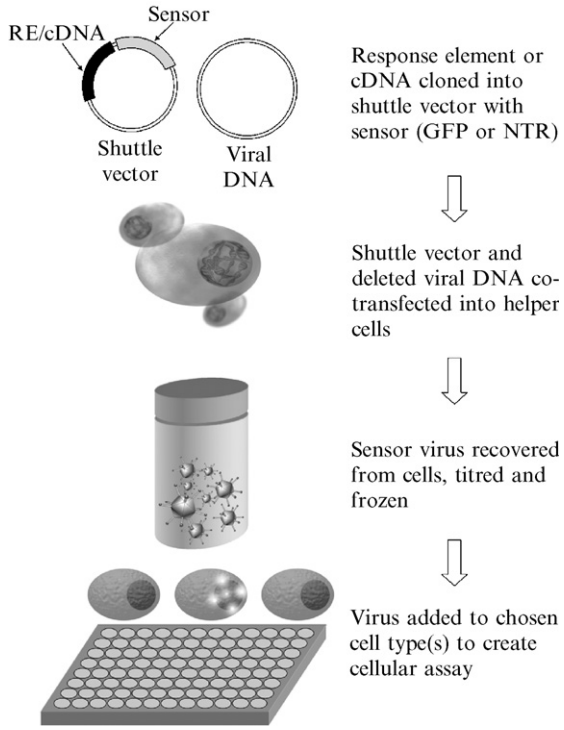

FIG. 1. Engineering adenoviral sensors and assays. A shuttle vector containing a response element (RE) linked to nitroreductase (NTR) for a reporter gene assay or a cDNA sequence linked to GFP for a translocation assay is complemented with modified viral DNA sequences in cotransfection of helper cells in tissue culture. Recombination of sensor and viral DNA, supplemented by additional viral proteins encoded in the helper cells, allows production of replication-deficient adenovirus particles. The sensor encoding adenovirus is recovered from the cell growth media, purified, titered to a defined level of infectivity, and frozen. The addition of adenovirus to assay host cells results in sensor expression without viral replication.

follows via interaction of the capsid penton base with cell surface integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Viral internalization occurs through clathrin-mediated endocytosis followed by pH-dependent release from endosomes into the cytoplasm. Subsequent transport to the nucleus allows DNA replication and transcription to proceed from an epichromosomal location, resulting in sensor expression.

Transduction by adenoviral vectors is a quick and simple process and does not require any specialized equipment or additional reagents. The adenoviral vector is simply added to cells in culture medium and cells are cultured to allow sufficient expression of the encoded sensor to permit

TABLE II
COMPARISON OF EFFICIENCY OF ADENOVIRAL AND CHEMICAL METHODS FOR GFP SENSOR DELIVERY

	% transduction/transfection with GFP-glucocorticoid receptor		
	Adenoviral	Fugene 6	Lipofectamine
HeLa	83 ± 11	55 ± 8	51 ± 8
U2OS	81 ± 12	24 ± 11	14 ± 8
HepG2	29 ± 12	10 ± 8	5 ± 4
SW1353	32 ± 32	9 ± 11	7 ± 3

detection and analyses. Expression can be detected within 12 h of transduction, and the majority of GFP translocations and reporter genes can be analyzed 24 h after transduction of target cells.

Adenoviral vectors can efficiently transduce a broad range of cell types and provide higher efficiency delivery than chemical transfection (Table II). They are particularly attractive for studies in primary cells and nondividing cells associated with differentiated tissues such as brain and heart that are refractory to chemical methods. Endogenous levels of CAR and integrins vary between cell types and determine the efficiency of a particular cell line to transduction. However, the amount of virus added to cells can be optimized readily to achieve a desired level of expression, and transduction efficiency is very reproducible, allowing routine use with large quantities of cells, providing an ideal assay development tool.

Adenoviral vectors have been used previously as research tools to transduce cells with GFP translocation sensors (Kajimoto *et al*., 2001; Kang and Walker, 2005) and reporter gene assays (Hartig *et al*., 2002; McPhaul *et al*., 1993). However, despite the wide use of adenovirus for target expression (Darrow *et al*., 2003; Ogorelkova *et al*., 2004) for drug screening applications, these vectors have not seen significant use for delivery of sensors for high-content analysis on automated imaging platforms. To increase the potential of HCA in drug activity profiling, we have assembled a library of adenoviral sensors based on cDNA-GFP fusion proteins and a complementary live cell reporter gene assay (Thomas, 2002) based on the bacterial enzyme nitroreductase and a quenched cyanine dye, CytoCy5S.

Employing these sensors alone and in combination (Fig. 2) provides the potential to place reporter gene assays at the terminus of multiple signaling pathways to determine the route of action and specificity of candidate drugs and to deploy GFP translocation sensors at key intervention points on the same pathways to increase the resolution and precision of activity profiling data.

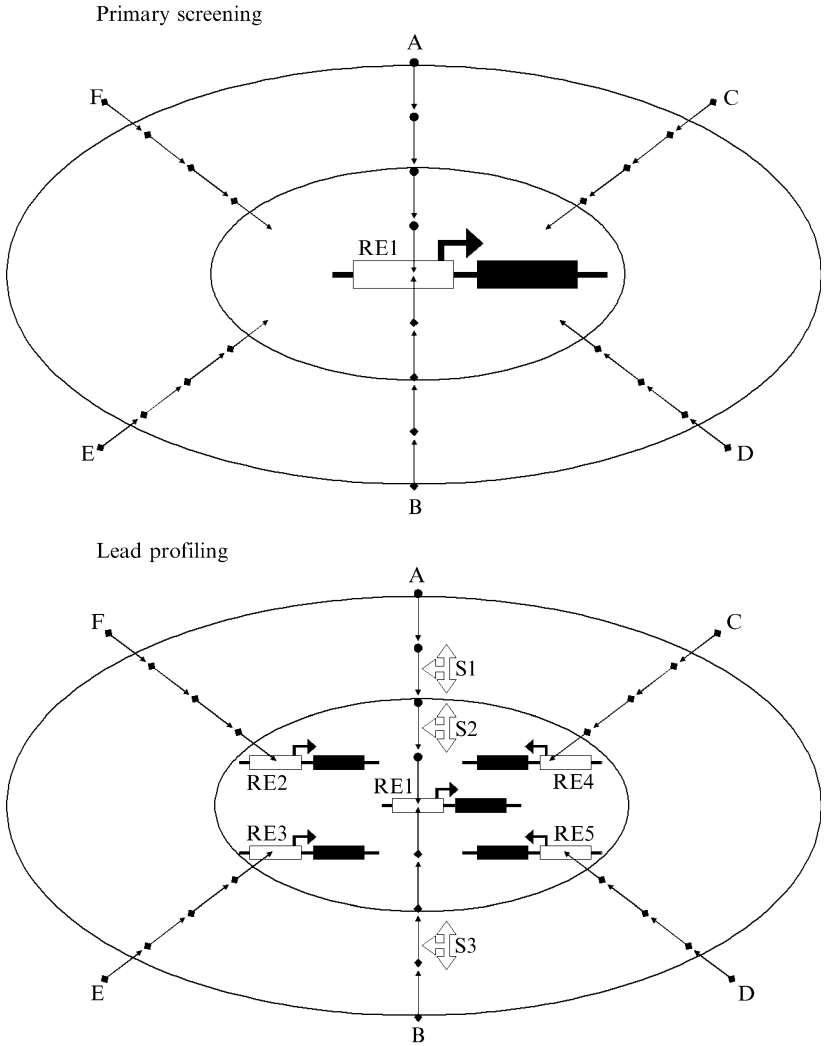

FIG. 2. Application of adenoviral sensors to lead profiling in drug development. A typical primary screening scenario for an assay using a stable reporter gene cell line is illustrated. In the assay, compounds may interact with a number of cell-signaling pathways (A–F). Action of a compound on a target pathway A, linked to the reporter gene via a response element (RE1), will generate an assay "hit." Other compounds may also induce or repress reporter gene activity through alternative pathways (B) impinging on the same response element, producing a false-positive hit. In addition, compounds acting on the target pathway (A) may also interact with other pathways (C, D, E, and F) not connected to the reporter gene. Applying adenoviral sensors to lead profiling allows the activity of compounds arising from primary screening to be characterized in detail. Using a panel of sensors transiently expressed in one or more cell

Validation of Adenoviral Sensors

To validate the performance of adenovirus-encoded sensors, we have compared assay data obtained using transient viral expression with data from the same sensors expressed in transient plasmid-based expression assays and in stable cell lines.

Analysis of the translocation of a rapidly responding translocation sensor, an EGFP fusion to the pleckstrin homology (PH) domain of the delta-1 isoform of phospholipase C, showed comparable assay data (Fig. 3) from a stable CHO cell line and CHO cells transduced with the same fusion protein using adenoviral delivery. Analysis of dose–response curves obtained from image analysis yielded EC_{50} values for ATP stimulation of 6.3 μM for the stably expressed sensor and 13.2 μM for the virally encoded sensor.

Comparison of a reporter gene assay carried out using plasmid transfection and adenoviral transduction showed significantly improved assay statistics using the adenoviral vector (Fig. 4C). Signal to noise (assay range/root sum SD squared) was 7.3 for plasmid assays and 55.3 for adenoviral assays at 100 μM forskolin. Adenoviral transduction allowed nitroreductase (NTR) reporter gene assays to be run in either live or fixed cell format using both HTS and HCS platforms (Fig. 4D) with increased precision, yielding forskolin EC_{50} values (95% confidence limits) of 0.8–4.5 μM for plasmid assays and 0.4–0.8 μM for adenoviral assays.

Application of Adenoviral Sensors to High-Content Analysis

Transient expression of adenoviral sensors allows rapid assay development following a number of key steps. Preliminary literature research should be carried out to determine the biological requirements for the assay and to gather information that is relevant to the drug target and pathway of interest to aid in the choice of one or more sensors. At this stage one or more appropriate cell lines should be selected, taking into account endogenous expression of any key components in the signaling

types, the interactions of compounds with a range of signaling pathways may be profiled. An adenoviral nitroreductase (NTR) reporter gene sensor using the same response element used in the primary screen (RE1) may be used to confirm on-target (pathway A) and off-target (pathway B) activity detected in the primary screen. The target pathway may be additionally monitored using adenoviral GFP fusion protein translocation sensors (S1 and S2) upstream of the reporter gene to determine the site of action of the candidate drug at higher resolution. Similarly, further sensors (S3) may be deployed on pathways known to interact with the readout used in primary screening to gain further information on compounds showing off-target activity. Finally, a battery of adenovirus-encoded NTR reporter gene assays using a wide range of response elements (RE2-RE5) may be employed to monitor further off-target activity of hit compounds against other cellular signaling pathways.

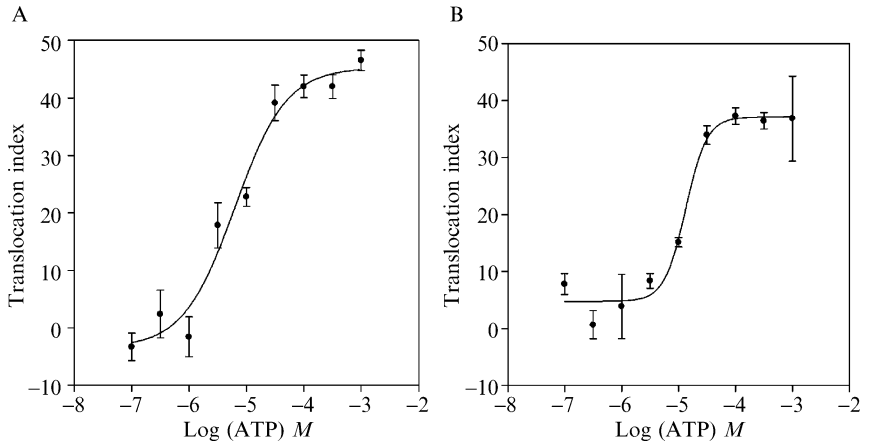

FIG. 3. GFP translocation sensor under stable and transient expression conditions. (A) CHO cells stably expressing a EGFP-phospholipase C delta-1 PH domain (PLCD1-PH) fusion were treated with a range of ATP concentrations to induce translocation of the fusion protein from the plasma membrane to the cytoplasm and the redistribution of the protein quantified by automated image analysis. (B) The same fusion protein was transiently expressed in CHO cells using an adenoviral vector and ATP-induced translocation measured under identical conditions.

pathway(s) under investigation. Any available information on response dynamics, suitable agonists and antagonists for positive controls, and cell culture conditions should be evaluated to design a prototype assay protocol. Once this basic protocol is established, optimization of viral transduction can proceed. At this stage experiments will be required to optimize the MOI (the amount of virus that should be added to each cell). During this period of assay development the aim is to establish the efficiency of transduction, to confirm that no toxic effects occur, and to determine that sensor response to a chosen stimulus can be detected. Once the optimum MOI has been established, further assay development activities addressing cell seeding density, incubation time, replicate numbers, and other assay design factors can be finalized to maximize the assay performance for reproducibility, signal to noise, and other metrics. The protocols and procedures described next are typical of those used in our laboratory for expression and analysis of adenoviral-encoded sensors.

EGFP-Glucocorticoid Receptor (GR) Translocation Sensor

The glucocorticoid receptor, a member of the steroid receptor superfamily, resides predominantly in the cytoplasm and accumulates in the

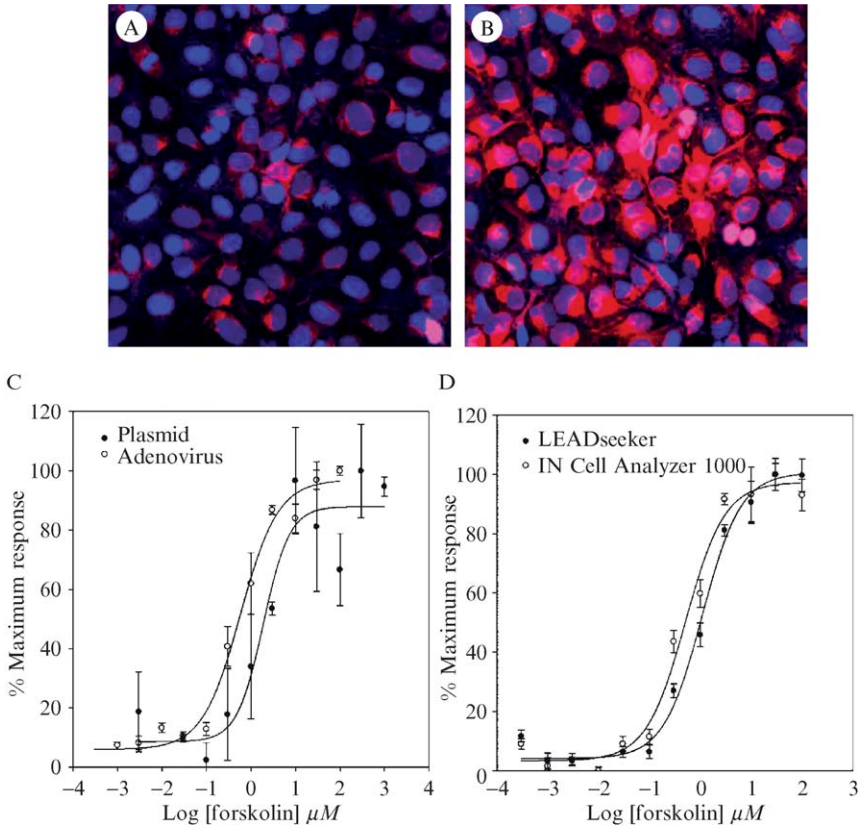

FIG. 4. Nitroreductase (NTR) reporter gene assay. A CRE-NTR construct containing four repeats of the cAMP response element (CRE) upstream of a minimal promoter was used to measure transcriptional activity of cAMP-binding protein (CREB) in transient assays in response to forskolin stimulation. Cells transduced with adenoviral vector encoding the CRE-NTR construct were loaded with CytoCy5S NTR substrate and imaged on IN Cell Analyzer 1000 in the absence (A) and presence (B) of forskolin. (C) Comparison of transient live cell assays performed using plasmid transfection and adenoviral delivery of the CRE-NTR construct showing improved reproducibility with the adenoviral vector. (D) Fixed cell CRE-NTR assay analyzed using macroimaging (LEADseeker) and automated microscopy (IN Cell Analyzer 1000).

nucleus when glucocorticoids such as cortisol bind to the receptor (Kumar and Thompson, 2005). A consequence of the nuclear translocation is the binding of GR to specific DNA elements in the promoter regions of target genes and concomitant regulation of gene transcription rates. The absolute response following gene transcription will depend on the cellular context.

A fusion protein of GR and EGFP provides a sensor to examine the distribution of the receptor within the nucleus and cytoplasm in response to agonists or antagonists of the receptor. The EGFP–GR chimera functions normally in cytoplasmic/nuclear translocation and gene activation and responds to pharmacologically relevant stimuli (Walker *et al.*, 1999). The adenoviral vector containing the gene encoding EGFP fused to the GR enables expression of the transgene in cultured cells, allowing the intracellular translocation of the fusion protein to be monitored on automated epifluorescent microscopes in the presence of an appropriate agonist such as dexamethasone.

Reagent Preparation

Prepare the following reagents. Culture medium; Dulbecco's Modified Eagle's Medium (DMEM), with 10% fetal calf serum (FCS), 2 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Culture medium + charcoal-stripped FCS (CS-FCS); DMEM with 2 m*M* L-glutamine and 10% CS-FCS. Agonist; dexamethasone, prepare 50 μM stock in ethanol and store in suitable aliquots at –20°. Phosphate-buffered saline. Fixative; formalin 10%, dilute to 5% in phosphate-buffered saline (PBS). Hoechst nuclear dye; prepare 10 μM stocks in PBS, store at –20°, and protect from direct light. Prepare assay test compounds by diluting in culture medium + CS-FCS to concentrations twice that of the desired final assay concentration.

Assay Procedure

1. The day before starting the assay, detach logarithmically growing HeLa cells by treatment with trypsin. Adjust the cell count to 2.5×10^4 ml with culture medium to a final volume of 20 ml (i.e., 5×10^5 cells in 20 ml culture medium). Thaw the EGFP-GR adenoviral vector (GE Healthcare) by placing the tube on ice. Add 50 μl (2.5×10^7 ifu) adenovirus to the cell suspension and mix by inverting the tube gently. At this cell density, this procedure will deliver the optimum 50 MOI to the HeLa cells.
2. Dispense 200 μl of the cell suspension per well of a 96-well imaging quality plate. Incubate the cells for 24 h at 37°, 5% CO_2 in a tissue culture incubator.
3. Warm all assay reagents to 37°. Decant the culture medium from the cells and add 100 μl of medium containing CS-FCS. Incubate the cells for 1 h at 37°, 5% CO_2.
4. Add 100 μl of the prepared two-fold dilution stocks of the test compounds.
5. Incubate the plates at 37°, 5% CO_2 for 30 min.

6. Decant the assay medium from the cells. Add 100 μl fixative to each well and incubate for 15 min at room temperature.
7. Decant the fixative and wash the cells in each well with 200 μl PBS.
8. Decant the PBS, add 100 μl of 2.5 μM Hoechst to each well and incubate for 15 min to stain nuclei.
9. Image the cells using automated microscopy (e.g., IN Cell Analyzer 1000 or IN Cell Analyzer 3000, GE Healthcare).

The fixed cell protocol just described provides a convenient assay procedure that does not require complex scheduling where large numbers of test compounds are to be evaluated; however, preliminary experiments may be required to determine the optimal time for fixation following stimulus. Alternatively, the procedure may be carried out using live cell imaging to follow translocation in a dynamic assay. For live cell assays it is advisable to stagger the timing of addition of test compounds to coincide with image acquisition times to ensure that each well is imaged at the same relative time following stimulus. For live cell assays, the Hoechst nuclear stain (5 μM) should be added to the test compounds prior to addition to the cells.

Assay Analysis

Typical image acquisition and analysis procedures for the EGFP-GR sensor using IN Cell Analyzer 1000 and IN Cell Analyzer 3000 are outlined. Image acquisition and procedures on other platforms will vary, but follow the same basic procedure. For image acquisition on a lamp-based imager (e.g., IN Cell Analyzer 1000), use a Q505 long pass dichroic with filters selected for excitation at 360 and 475 nm and emission at 535 nm for both channels. A 10$\times$ objective provides sufficient resolution for the assay, but a 20$\times$ objective may be employed if preferred. For image acquisition on the line scanning confocal IN Cell Analyzer 3000, EGFP is imaged using 488-nm laser line excitation with emission monitored through the 535-45-nm filter and the Hoechst nuclear stain imaged using 363-nm laser line excitation and a 450-65-nm filter emission filter.

Image analysis is performed using nuclear trafficking analysis software to quantitatively measure the fluorescence intensity in the cytoplasm and the nucleus of each cell. The software provides single cell and population averaged data for the nuclear/cytoplasmic (Nuc/Cyt) ratio of sampled nuclear and cytoplasmic EGFP-GR intensities as a measure of intracellular sensor distribution. Typical data for the EGFP-GR sensor expressed in HeLa cells and imaged on IN Cell Analyzer 1000 are shown in Fig. 5.

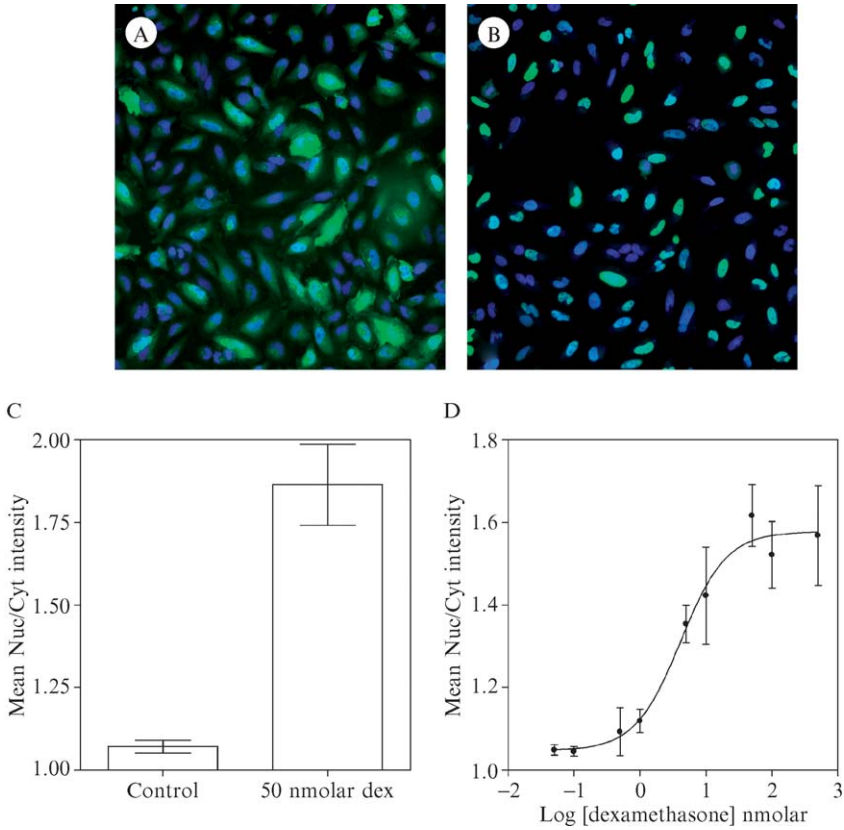

FIG. 5. Adenoviral EGFP-glucocorticoid receptor (EGFP-GR) sensor. HeLa cells expressing the EGFP-GR were imaged on IN Cell Analyzer 1000 after a 30-min incubation in the absence (A) or presence (B) of dexamethasone. Automated image analysis (C) was used to determine the nuclear/cytoplasmic (Nuc/Cyt) distribution of the sensor in 30 replicate control and dexamethasone-treated wells yielding an assay Z factor (Zhang *et al.*, 1999) value of 0.47. Exposure of transduced cells to a range of dexamethasone concentrations (D) yielded an EC_{50} of 4.1 n*M*.

Nuclear Factor of Activated T Cells (NFAT) Nitroreductase Reporter Gene Sensor

Nuclear factor of activated T cell transcription factors (Macian, 2005) are a family of at least four (NFAT1–4) structurally and functionally related proteins expressed in the immune system and in muscle, cardiac, and neuronal cells. In T cells, ligand binding at antigen receptors results in cell activation, leading to cytokine production, which plays a major role in

modulating the immune system. A key feature of this type of regulated transcription in T cells is the Ca^{2+} calmodulin-dependent activation of NFAT transcription factors via activation of the phosphatase calcineurin. Activated calcineurin dephosphorylates NFAT proteins, inducing a conformational change that unmasks a nuclear localization sequence inducing NFAT proteins to translocate to the nucleus where they bind to NFAT response elements and modulate gene expression. In the nucleus, NFATs may form synergistic complexes with other transcription factors, including AP-1 and NF-κB, to alter gene transcription. Expression and quantitation of a NTR reporter gene, cloned downstream of a basal promoter and NFAT response elements, provide a measure of the level of activity along the NFAT pathway in application areas such as differentiation, apoptosis, and immunosupression.

Reagent Preparation

Prepare the following reagents. Culture medium; DMEM, with 10% FCS, 2 m*M* L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Agonists; phorbol myristate acetate (PMA) and ionomycin, prepare stock solutions in ethanol and store in suitable aliquots at –20°. PBS. Fixative; formalin 10%, dilute to 2% in PBS. Hoechst nuclear dye; prepare 25 μM stocks in PBS, store at –20°, and protect from direct light. Prepare assay test compounds by diluting in culture medium + CS-FCS to concentrations twice that of the desired final assay concentration. CytoCy5S solution; reconstitute with DMSO to a concentration of 1 to 5 m*M*. Dilute in serum-free media to 10 μM immediately before use.

Assay Procedure

1. The day before starting the assay prepare a suspension of trypsinized U2OS cells in culture medium. Thaw the NFAT-NTR adenoviral vector (GE Healthcare) by placing the tube on ice. Add 6 μl of adenovirus/5 $\times$ 10^5 cells and mix by inverting the tube gently to transduce cells at the optimal MOI of 6.

2. Seed cells into a 96-well assay plate at 15,000 cells per well and incubate for 24 h at 37°, 5% CO_2, 95% humidity.

3. Prior to starting the assay, prepare solutions of test compounds at an appropriate concentration in serum-free culture medium. Warm all assay reagents to 37°.

4. After 24 h in culture remove medium from cells and add 90 μl of test compound solution per well. Use 0.1 μg/ml PMA and/or 2 μM ionomycin

as positive controls. Add 90 μl of serum-free culture medium to negative control wells.

5. Incubate cells for 10 min at 37° and then add 10 μl of 10 μM CytoCy5S (GE Healthcare) nitroreductase substrate to each well. Incubate cells for a further 12 to 16 h at 37°, 5% CO_2, 95% humidity.

6. Measure CytoCy5S fluorescence intensity (excitation maximum 647 nm, emission maximum 667 nm) by imaging. For live cell macroimaging (e.g., LEADseeker, GE Healthcare), image the plate without further processing. For live cell imaging by automated microscopy, stain cell nuclei by the addition of 10 μl of 25 μM Hoechst for 30 min.

7. For imaging of fixed cells, decant the assay medium, add 100 μl fixative to each well, and incubate for 30 min at room temperature.

8. Decant the fixative and wash the cells in each well with 200 μl PBS. Decant the PBS and add 100 μl of 2.5 μM Hoechst to each well to stain nuclei.

Assay Analysis

The typical image acquisition and analysis procedures for NTR reporter gene assays using IN Cell Analyzer 1000 are outlined. Image acquisition and procedures on other platforms will vary, but will follow the same basic procedure.

1. Acquire images from each well using the 10× objective and a single tile located at the center of the well to capture data from 200 to 300 cells/assay. Assays at lower cell density may require more images. Acquisition times are typically 200 to 500 ms for the Hoechst nuclear stain (ex. 360/40 nm, em. 535/50 nm) and 100 to 400 ms for CytoCy5S (ex. 620/60 nm, em. 700/75 nm).

2. Measure CytoCy5S fluorescence in cells using automated Object Intensity image analysis to determine the intensity of the red fluorescent NTR product in cells segmented using the Hoechst nuclear marker.

Data from application of the NFAT-NTR sensor in a multiplexed live cell assay in conjunction with a stably expressed GFP fusion protein are shown in Fig. 6. Transduction of the adenoviral NTR sensor into a cell line reporting cell cycle position via imaging of EGFP intensity and distribution allowed the cell cycle distribution of ionomycin-stimulated gene expression mediated through NFAT to be correlated with cell cycle position on an individual cell basis. Data from this study also demonstrate that delivery of a reporter gene sensor using an adenoviral vector at an optimal MOI does not perturb the cell cycle (Fig. 6D) nor diminish the expression or alter the behavior of a coexpressed GFP fusion protein.

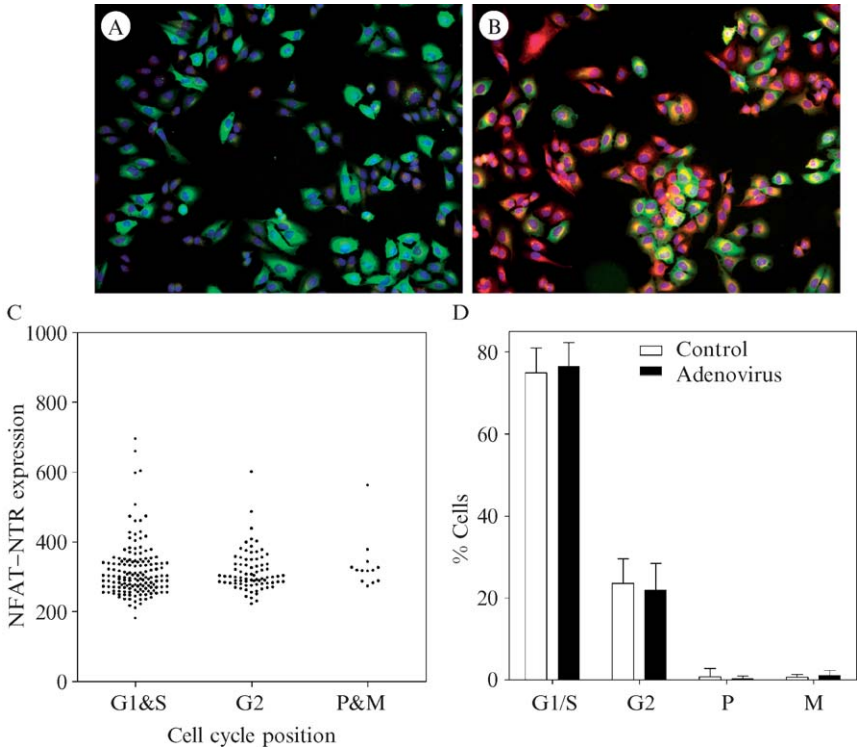

FIG. 6. NFAT-NTR reporter gene sensor in multiplexed assay. U2OS cells stably expressing a cyclin B1-EGFP fusion protein under the control of the cyclin B1 promoter, which dynamically reports cell cycle position (Thomas *et al*., 2005), were transduced with adenovirus encoding an NFAT-NTR reporter gene construct. NTR expression was imaged in live cells on IN Cell Analyzer 1000 in the absence (A) and presence (B) of 1 μM ionomycin. Automated image analysis was used to determine the cell cycle position and ionomycin-induced NFAT reporter gene expression for each cell (C) and to compare cell cycle distribution in cells in the presence and absence of adenovirus (D).

Conclusions and Future Perspectives

The development of cell-based assays for secondary screening of compounds identified as hits in high-throughput primary screens has traditionally involved using stable cell lines. This approach can severely limit the depth and breadth of interrogation of biological activity of candidate drugs through limitation of the scope and relevance of biological model systems that can be engineered in standard laboratory cell lines. Adenoviral vector transduction provides an efficient and technically simple system

for transient expression of assay sensors in diverse backgrounds from transformed to primary cells, providing the potential to profile the biological activity of compounds across a very diverse interrogation matrix.

Biological pathways are often viewed simplistically as a linear chain of cause-and-effect relationships, with the ERK 1 signaling pathway (Cobb *et al*., 1994) being a typical example. Signal transduction starts at the EGF receptor (EGFR) at the cytoplasmic membrane. Binding of EGF causes receptor activation, transduction of signal to STAT1/3 in the cytoplasm, and internalization of the EGFR. Once activated, STAT1/3 translocate from the cytoplasm to the nucleus where the activated transcription factors promotes gene expression from STAT responsive control elements.

Viewed as an isolated element, this signaling pathway has three levels and at least one intervention point on each level at which virally encoded assays can be engineered to report signal transduction: receptor internalization, STAT translocation, and STAT-induced gene expression. EGFR internalization may be detected by viral expression of EGFR tagged to bind a pH responsive fluorescent dye that increases its fluorescence when the receptor is internalized into acidic vesicles (Adie *et al.*, 2003). STAT translocation may be measured by viral expression of a EGFP–STAT fusion protein, and STAT-induced gene expression may be measured by viral expression of a NTR reporter gene under control of an STAT response element. This basic strategy of inserting sensors at key points in signaling pathways to flag the passage of signal transduction events can be repeated and expanded for many pathways, targeted to distinguish routing of signaling in divergent, convergent, and interacting pathways, and the scope of interrogation diversified by expression of the same sensing networks in a variety of host cells reflecting different genotypes and phenotypes.

In the future we envisage data from multiple sensors expressed in parallel in diverse cellular backgrounds providing a high-definition view of drug activity and specificity, with each adenoviral sensor providing the equivalent of a pixel in an image. When viewed individually they may yield only a small amount of information; as a whole they provide the full picture.

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[15] Cell-Based Assays Using Primary Endothelial Cells to Study Multiple Steps in Inflammation

By THOMAS MAYER, BERND JAGLA, MICHAEL R. WYLER, PETER D. KELLY, NATHALIE AULNER, MATTHEW BEARD, GEOFFREY BARGER, UDO TÖBBEN, DEBORAH H. SMITH, LARS BRANDÉN, and JAMES E. ROTHMAN

Abstract

Cell-based assays are powerful tools for drug discovery and provide insight into complex signal transduction pathways in higher eukaryotic cells. Information gleaned from assays that monitor a cellular phenotype can be used to elucidate the details of a single pathway and to establish patterns of cross talk between pathways. By selecting the appropriate cell model, cell-based assays can be used to understand the function of a specific cell type in a complex disease process such as inflammation. We have used human umbilical vein endothelial cells to establish three cell-based, phenotypic assays that query different stages of a major signaling pathway activated in inflammation. One assay analyzes the tumor necrosis factor α (TNFα)-induced translocation of the transcription factor NF-κB from the cytoplasm into the nucleus 20 min after stimulation with TNFα. Two more assays monitor the expression of E-selectin and VCAM-1, 4 and 24 h after stimulation with TNFα. Indirect immunofluorescence and high-throughput automated microscopy were used to analyze cells. Imaging was performed with the IN Cell Analyzer 3000. All assays proved to be highly robust. Z' values between 0.7 and 0.8 make each of the three assays well suited for use in high-throughput screening for drug or probe discovery.

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