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Cellular Assays in HTS

Patricia Johnston

1. Introduction

Cell-based screens represent one of the most venerable approaches to lead generation, indeed, antimicrobial screens were the mainstay of drug discovery in the 1940s, 1950s, and 1960s ***(1)***. However, cellular assays also represent one of the most dynamic areas of innovation in high-throughput screening (HTS). The driver of this innovation is the demand for more leads from more complex targets. Genomics discovery efforts have generated many potential targets for HTS, but the biochemical nature of these novel targets may not be elucidated prior to their entry into the lead generation process. Therefore, these targets are frequently addressed in "gene to screen" paradigms that rely on cellular-screening approaches. Advances in cell-based screening approaches have also been driven by the need to address complex, multi-component target classes that may not be feasible in biochemical assays. Finally, the increasingly combative intellectual property playing field, and the need to accelerate the drug discovery process have driven innovations in cell-based screening methodologies.

The purpose of this chapter is to review cell-based screening methodologies currently in widespread use in the HTS laboratory. For the sake of brevity, I will focus only on eukaryotic systems, although there have been many recent advances in the screening of microbial systems both for the purposes of target validation ***(2)*** and lead generation ***(3,4)*** . Likewise, I will only discuss those methods that are compatible with HTS, in other words, cellular assays that support a minimal daily throughput of 5,000 samples.

From: *Methods in Molecular Biology, vol. 190: High Throughput Screening: Methods and Protocols*
Edited by: W. P. Janzen

2. Why Bother?

Cellular assay systems can be more complex and less well-defined than biochemical assays, and in some instances are more expensive and labor-intensive as well. So, why bother configuring a cell-based assay for HTS? The answers lie in the drug discovery strategy deployed for that target class and in the nature of the biological target. Drug discovery strategies focused on novel, potentially orphan targets require that screens be configured for targets for which the mechanism of action and relevance to disease have not been elucidated. In many such cases, the results of the HTS are expected to drive the validation of the target. Arguably, for these poorly characterized targets, a screen in which the target is expressed and regulated in a cellular environment ensures that the necessary substrates and cofactors of that target are available at physiologically appropriate concentrations. Thus, cellular assays negate the need to determine the appropriate cofactors, etc., prior to HTS. However, a cell-based approach may also be desirable for well-defined targets with clear disease relevance. For example, in cases where the molecular target is difficult or expensive to express and purify at the scale needed for HTS, a cell-based assay may represent the fastest, least expensive approach.

Complex targets which are very challenging to reconstitute in a biochemical assay represent another opportunity for cellular HTS applications. These would include targets that regulate a signaling pathway, e.g., the SOCS proteins, targets that are themselves regulated by poorly understood partners in a signaling pathway, or targets that require assembly of a regulatory complex. These targets are best screened in a cellular system in which all of the necessary components are pre-assembled.

In addition, cell-based screens may provide the means to identify compounds with a unique mechanism of action, for example, agonist or allosteric modulator of G-protein-coupled receptor (GPCR) targets. Finally, cellular HTS approaches may circumvent intellectual property restrictions on use of the cDNA for a target if a cell line is available that already expresses the gene of interest.

To summarize, though cell-based HTS may initially appear daunting, there are many targets for which a cell-based screen represents the fastest, cheapest path to lead generation. Furthermore, the complexity of cell-based assays may enable discovery of compounds with unique mechanisms of action. Certainly, cell-based screens ensure that intracellular targets are expressed in the appropriate physiological context and that active compounds have a favorable permeability profile.

3. Potential Cellular "Platforms"

The cellular platform or background chosen to approach a particular target has an enormous impact both on the development and the implementation of a HTS for that target. The choices are extensive, and include primary cell types and cell lines; both "native" and engineered. The choice is driven by the availability and behavior of the cells, as well as the amplitude and reproducibility of the signal attainable in that cellular system. Primary cells of human origin are the most physiologically relevant lead generation platform. Selected primary human cell types are commercially available and amenable to HTS ***(5)*** (Clonetics, Walkersville, MD), and various rodent primary cell types can be prepared at HTS scale. However, in general, primary cells cannot be obtained at the scale necessary for HTS. Thus, primary cell screens are typically positioned in the screening paradigm as low-throughput secondary assays.

Transformed cell lines of human origin are the most commonly used cell-based screening platform. These lines can be screened in their "native" state for targets naturally expressed in these cells, e.g., neuropeptide receptors expressed in the SK-N-MC neuroblastoma line ***(6)***. Many of these lines retain a highly differentiated phenotype and are an excellent platform on which to screen a complex physiological response, such as secretion, e.g., insulin secretion from insulinoma lines ***(7)***. Certain native cell lines serve as platforms in which to study the differentiation process itself. In these screens, a cocktail of stimuli is used to drive the cells down a differentiation path; an aspect of this differentiation process can be screened, or the terminally differentiated cells can be used to screen a particular physiological process. There are many cell lines amenable to differentiation studies; commonly used lines include C_2C_{12} ***(8)***, U937 ***(9)***, and L6 ***(10)*** cells.

Cell lines can be further engineered to express or over-express a target of interest. Some assay formats require co-expression of multiple proteins, or expression of "reporter" proteins. Expression can be transient or stable, and a number of expression systems employed, depending on the nature of the cell line and the target. Stable cell lines are most commonly generated by plamid transfection or retroviral infection. Plasmids can be introduced to the cell by internalization of DNA precipitates, electroporation, or DNA-liposome fusion (lipofection). Electroporation is the method of choice for suspension cultures, while lipofection is commonly employed for adherent cells. Clones can be isolated, or pooled cell populations can be utilized for HTS. Retroviral systems, in which the target DNA is introduced by viral infection are an attractive alternative to plasmid transfection, obviate the clonal selection step and can generate lines with high target expression levels.

Stable expression of the target is perhaps the easiest choice for HTS, but transient expression can be scaled up sufficiently to support a reasonable throughput. Transient transfection procedures generate a population of cells with generally high levels of target expression. Should isolation of a stable clone prove difficult, transient expression of a target may represent the fastest path to HTS. Batch lipofection is easily scaled-up and highly reproducible, but the reagents can be expensive for a full HTS campaign.

4. Common Cell-Based HTS Formats

Perhaps one of the oldest and most widely used eukaryotic HTS formats is the reporter assay *(7)*. Reporter assays can be designed to detect the transcriptional regulation of a particular gene, or they can be configured to detect activity of a signaling pathway. In reporter assays, the expression of a protein whose enzymatic activity is easily detected is linked to the biological activity of the target of interest. Transcriptional regulation reporter assays are configured by linking the promoter, or elements of the promoter, from the gene of interest to the coding region of the reporter gene. To detect activity of a signaling pathway, repeats of a particular response element are queued upstream of the reporter gene and regulate its expression in response to activation of the pathway. Potential reporter proteins include; chloramphenical acetyl transferase, firefly and renilla luciferase, secreted human growth hormone, β-galactosidase, secreted alkaline phosphatase, green fluorescent protein(s), (GFPs), and β-lactamase *(12)*. Of these, luciferase, β-galactosidase, and β-lactamase reporters are the most commonly used in HTS laboratories.

There are both advantages and disadvantages to reporter screens. Widespread implementation of this assay format has driven the development of multiple instrumentation platforms and a diversity of reagent kits compatible with them. The cost of reagents for reporter screens, though not low, is acceptable, and many of the commercially available reagents provide advantages such as greater signal amplitude or stability. Most importantly, reporter screens are highly amenable to miniaturization *(13)*.

Reporter screens have their limitations as well. Reporter gene transcription triggered by receptor activation generates an indirect signal removed both spatially and temporally from the target. One could argue that this indirect and amplified signal might obscure subtle modulation of the receptor. For example, reporters designed to detect Gs-coupled receptor activity are often configured to amplify the signal via multiple copies of the cAMP response element. Such amplification can result in unusual dose-dependent pharmacology *(14)*. Reporter assays designed to detect the activity of receptors that signal via calcium mobilization typically utilize the NFAT-response element. However,

NFAT-mediated gene expression is dependent on both the amplitude and duration of the calcium flux ***(15)***, and therefore NFAT-driven reporter assays cannot be used to detect rapid calcium-mediated responses. For these reasons it may be desirable to design screens that detect signaling events more proximal to the receptor, such as receptor phosphorylation, G-protein recruitment, cAMP generation, or calcium mobilization.

HTS of cellular calcium mobilization was greatly facilitated by the introduction of the FLIPR (Molecular Devices, Sunnyvale, CA), a fluorescent-imaging system that collects information about the calcium flux in each well of a microplate (both 96- and 384-well) simultaneously ***(16)***. Intracellular calcium concentrations are measured by preloading the cells with a calcium-sensitive fluorescent dye, such as Fluo3 ***(17)***. FLIPR reads at subsecond intervals, which enables capture of the response kinetics. This feature, along with the liquid-handling capabilities of the device, facilitates identification of subtle effects on receptor activity, and successive liquid additions enable discrimination of agonist, allosteric modulation, and antagonist activity within the same plate of cells.

Obviously, cell-based screens designed to detect calcium mobilization are limited to those receptors that signal through this pathway. Co-expression of a promiscuous (Gα15, Gα16), or chimeric G protein can "switch" the signal of GPCRs so that they couple to $InsP_3$ generation and calcium release ***(18)***. These G-protein switching strategies have made calcium-mobilization screening approaches applicable to many more receptors. However, screens designed to detect receptor activity against a backdrop of stable, high-level promiscuous G protein expression are often susceptible to artifacts: false-positives derived presumably from other cell-surface receptors hi-jacking the G protein.

Although the FLIPR has facilitated advances in calcium-mobilization screens, these assays remain difficult to configure, relatively slow, and fraught with potential artifacts. The optics of the device limit fluorescence detection to the bottom of the well. This reduces background fluorescence, but also requires that the cells be firmly adhered. If the cells detach or move during liquid addition, the signal is compromised. Likewise, the best signal is obtained from densely seeded wells, which can generate large cell-culture requirements for implementation in the high-throughput laboratory.

Other signaling events proximal to the receptor can be screened including: receptor phosphorylation, dimerization, or internalization; cAMP-generation; or G protein recruitment. Of these approaches, cAMP-detection assays are the most amenable to HTS. These screens are designed to detect cAMP levels present in the cell 30–60 min after receptor activation. The cells are lysed and the cAMP level quantified, typically by an immuno-assay. Numerous kits are

marketed for cAMP quantitation on multiple-assay platforms, including radioactive (Amersham Pharmacia Biotech, Uppsala Sweden, or NEN Life Sciences Inc, Boston MA), luminescent (Tropix, or Biosignal, Montreal Canada), and fluorescent-assay systems (LJL Biosystems, Sunnyvale CA). The advantages of cAMP generation screens are related to the assay format and the nature of the target. Some of these assay systems are homogenous and amenable to miniaturization, others are compatible only with 96-well plates. All of the assays are fairly expensive. The most significant disadvantage of this approach is apparent in screens of receptors linked to Gi. These screens are frequently limited by a small dynamic range of signal.

Reporter, cAMP, or calcium-mobilization screens are ideally suited for the identification of agonist activities, but antagonist identification does not require a cellular assay. Typically, antagonist screens are configured as binding assays utilizing isolated receptors or membrane preparations. However, cell-based approaches to antagonist identification can be attractive alternatives to biochemical binding assays. Cell-based ligand-binding assays can be configured with scintillant-impregnated plates (Cytostar T plates, Amersham Life Sciences, Arlington Heights, IL; Flashplates, NEN Life Science Products Inc, Boston, MA). Cellular monolayers are established in the wells and radiolabeled ligand is added to the media. Binding to the surface receptor excites the scintillant via a proximity effect, and the radiolabel in solution is silent. This approach removes the need to prepare membranes, thereby reducing the cell-culture requirements for HTS, and reduces the number of wash steps required with membrane-binding assays. Furthermore, the short compound-incubation periods obviate the cytotoxicity complications present in other assay formats such as reporter screens designed to detect antagonist activity.

Cell-based proximity assays have also proven useful to screening transporters. Cell-based screens for transporter targets, such as amino acid or neurotransmitter carriers, are preferred when ligand binding to the transporter is complicated by either high nonspecific binding by sticky ligands, or low expression of the transporter. Additionally, cell-based approaches to transporter targets enable identification of allosteric modulators that might be missed in a binding assay.

Proliferation assays are another common cell-based screening format in which scintillant-impregnated plates can be used. Proliferation assays can be configured to address a wide diversity of targets that either stimulate or inhibit cell growth: including cell-surface growth factor or cytokine receptors, intracellular enzymes, or multidrug-resistance pumps. Direct quantitation of ^{14}C-thymidine incorporation into DNA provides one of the most sensitive assays for cellular proliferation. Likewise, assays designed to quantify protein

synthesis by detection of ^{35}S-methioine incorporation are one of the most sensitive methods to detect cytotoxic compounds. The scintillant-impregnated plates described above enable both of these assays. However, the radioactive nature of these screens can make them a less attractive choice for HTS than fluorescent or colorimetric approaches utilizing tetrazolium dyes such as WST1, MTT, or XTT (Boehringer Mannheim Biochemicals).

Finally, cell-based assays can be designed to measure changes in protein expression. These screens can be configured to quantify protein expression on the cell surface, within the cell, or secreted into the medium via immunocytochemical techniques ***(5,19)***. The signal is determined by the modification of the secondary antibodies; fluorescent and colorimetric formats are available and compatible with multiple instrumentation platforms. Implementation of these approaches is of course dependent on the availability of an antibody specific for the protein(s) of interest. These screens are straightforward to develop and typically inexpensive, but are hindered by the need for multiple wash steps. Changes in protein expression can also be monitored by detection of mRNA expression. However, assays to quantify changes in mRNA expression have not been implemented at a high-throughput scale ***(20,21)***.

5. Implementation Challenges

Cell-based screens generate unique challenges for HTS implementation. These challenges arise during the development and validation of cell-based screens and continue through implementation in HTS during which the cell culture must be scaled to support daily production of hundreds of microplates for a campaign of weeks or months duration. Implementation challenges tied to the nature of cell culture at scale are beyond the scope of this review. Key challenges tied to cell-based screen development, regardless of the assay format, include the growth and adherence properties of the cells, solvent tolerance, cytotoxicity complications, and the stability of the cellular phenotype.

Growth and adherence issues are at the heart of many assay reproducibility problems. In screens dependent on long incubation periods (24–48 h), variations in cell growth across the plate can generate significant signal drift that usually manifests as edge effects. This differential growth is typically due to temperature variations or media evaporation, and can be controlled by careful regulation of the humidity and temperature of the incubator chambers, and by ensuring that the microplates are distributed evenly in the incubators. Poor or inconsistent cellular adherence can also produce significant variation in the signal of certain assay formats. Screen formats particularly prone to cellular adherence artifacts include calcium mobilization assays utilizing FLIPR, cellular enzyme-linked immunosorbent assays (ELISAs), or other assays in which

the cell monolayers must be washed, and any assay requiring removal and transfer of incubation media. Poor adherence can be improved by coating the surface of the microplate with a charged substrate such as poly-lysine or with extracellular matrix components such as fibronectin or collagen. Coated plates are commercially available, or plates can be prepared in-house at reasonable expense.

Solvent and compound intolerances as evidenced by cytotoxicity are more difficult challenges to address. In general, solvents such as dimethyl sulfoxide (DMSO) interfere with cell-based assays at concentrations of 1% or less. Higher seeding densities or serum concentration can mitigate these effects slightly, but a low solvent tolerance may be inherent to a cellular platform. Likewise, cytotoxicity complications apparent in assays that require long incubation periods are inevitable, and in many cases can only be addressed by changing the assay configuration or by a secondary assay triage protocol.

6. Future of Cell-Based Screening

The two drivers of innovation in cell-based screening methodologies are the need to miniaturize and the desire to capture temporal and spatial data on target activity: "high-content" screens. Miniaturization refers both to efforts focused on scaling to smaller well volumes; 96, 384, 1536, etc., but also to different screening platforms such as microfluidics that minimize reagent needs. The challenges associated with smaller well volumes are amplified versions of those apparent with any microplate-based cell assay, i.e., cell growth, adherence, solvent tolerance, etc. One unique challenge to lower volume screening is cell seeding, particularly for adherent cells. Smaller well volumes can increase bubbling or impede even liquid distribution across the bottom of the well, both of which can result in uneven cell attachment. This challenge is tied directly to the state of the art in liquid handling instrumentation, and will evolve as these instruments evolve. Cell-based screening on chips, however, is rich with unique implementation challenges and opportunities. Chip technologies such as those available from Caliper Technologies (Caliper Technologies Corp., Mt. View CA), require fewer cells and have the potential for rapid ligand challenge and read times. These properties generate opportunities for screening of primary cells and may enable screening of transient events such as channel activity.

High content screening, such as that enabled by Cellomics technology (Pittsburg PA), though not compatible with high throughput, represents a trend in cell-based screening towards capture of richer data on more complex aspects of target activity ***(22)***. These technologies enable direct visualization, in real-time if desired, of target movement in the cell, and represent unique methods to study receptor activity or transcriptional regulation.

7. Conclusion

Cell-based high throughput screens are a powerful tool in the lead generation process. Cellular assays though potentially challenging can represent the fastest path to lead identification for novel, poorly characterized targets. A cellular approach provides insight into the permeability profile of the active compounds and enables identification of compounds with unique mechanisms of action. Recent advances in the instrumentation applicable to cellular screening have enabled closer examination of the intricacies of intracellular signaling events. These advances suggest that cellular approaches will prove as fruitful a source of new leads in the twenty-first century as the antimicrobial screens were for the last.

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