ACS chemical biology

REVIEW

Design and Implementation of Cell-Based Assays To Model Human Disease

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ABSTRACT Cell-based assays, if appropriately designed, can be used to rapidly identify molecular mechanisms of human disease and develop novel therapeutics. In the last 20 years, many genes that cause or contribute to diverse disorders, including cancer and neurodegenerative disease, have been identified. With such genes in hand, scientists have created numerous model systems to dissect the molecular mechanisms of basic cellular and developmental biology. Meanwhile, techniques for high-throughput screening that use large chemical libraries have been developed, as have cDNA and RNA interference libraries that cover the entire human genome. By combining cell-based assays with chemical and genetic screens, we now have vastly improved our ability to dissect molecular mechanisms of disease and to identify therapeutic targets and therapeutic lead compounds. However, cell-based screening systems have yet to yield many fundamental insights into disease pathogenesis, and the development of therapeutic leads is frustratingly slow. This may be due to a failure of such assays to accurately reflect key aspects of pathogenesis. This Review attempts to guide the design of productive cellular models of human disease that may be used in high-throughput chemical and genetic screens. We emphasize two points: (i) model systems should use quantifiable molecular indicators of a pathogenic process, and (ii) small chemical libraries that include molecules with known biological activity and/or acceptable safety profiles are very useful.

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Received for review August 17, 2007 and accepted October 16, 2007.

Published online November 16, 2007

10.1021/cb700177u CCC: $37.00

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All experimental research on human disease depends on accurately replicating some aspect of pathogenesis in a controlled system, and no perfect models exist. Pharmaceutical companies are adept at using *in vitro* assays of protein function to identify novel inhibitors. If a specific therapeutic target is known, a biochemical assay can be readily developed, and this remains a desirable way to design and optimize new drugs. For example, the identification of Bcr-Abl as a therapeutic target for chronic myelogenous leukemia has allowed the discovery of effective therapies based on *in vitro* functional assays (*1*). However, many causative genes encode proteins of unknown function or without obvious catalytic sites amenable to targeting with *in vitro* assays. In addition, many proteins can only be evaluated in the context of an intact cell (*e.g.*, transmembrane proteins or proteins that function in a multicomponent complex), and thus in such cases cell-based assays are required for productive high-throughput screening (HTS).

In creating a disease model, a researcher must balance the ease of working with the system against the relevance to the disease process. Typically, "relevance" is determined empirically when predictions made by a simple system (*e.g.*, a cellular assay) are tested in a more complex system (*e.g.*, a transgenic mouse model). The balance between tractability and relevance becomes even more challenging when attempting to create a cellular model that is amenable to HTS. We argue that using a discrete and quantifiable molecular event that closely correlates with pathogenesis as the readout for cell-based HTS increases the likelihood of discovering compounds with activity in more stringent *in vivo* models. We contrast this strategy with that of using a cellular phenotype as an end point (*e.g.*, cell death in neurodegeneration models or cell proliferation in can-

cer models), which has a high potential for false positive and negative results.

For example, the identification of genes that cause neurodegenerative disease, such as superoxide dismutase (amyotrophic lateral sclerosis), amyloid precursor protein (Alzheimer's disease), polyglutamine proteins (Huntington's disease and others), and α-synuclein (Parkinson's disease), has led to a wealth of cellular and animal models ranging from yeast to mice, many of which have been adapted for HTS (*2*–*12*). It is possible to model neurodegeneration by culturing neuronal cells *ex vivo*, in which overexpression of toxic proteins causes cell death. This model appears to provide a simple, convenient readout, but the use of a phenotypic end point such as cell death is fraught with pitfalls. Neuronal cells certainly die in the course of neurodegeneration. However, tissue culture cells typically die within days of expression of a toxic protein, compared with degeneration, which generally takes years within the brain of an affected individual. Furthermore, ample evidence indicates that significant neuronal dysfunction occurs prior to detectable cell death (*13*–*15*). Myriad pathways influence cell death, and thus a compound can prevent cell death in this assay in many ways that do not relate to a disease-specific mechanism, such as the up-regulation of drug exporters or the down-regulation of apoptotic pathways. Thus, it is uncertain whether cell death models in tissue culture accurately represent crucial events in neurodegeneration. Not surprisingly, the ability of phenotypic cell models of neurodegeneration to predict success *in vivo* has been very low. The same holds true for cell proliferation models of cancer. As an alternative, we describe several assays that exploit molecular, rather than phenotypic, readouts.

Molecular Indicators in Disease Models. The value of a cell-based assay is greatly enhanced if the end point is specific to the disease mechanism. Most basic cellular phenotypes that can be readily measured (*e.g.*, proliferation, death, and process extension) are under the control of multiple regulatory pathways. When such readouts are used in screening assays to identify genetic or chemical modifiers, there is consequently a great chance that a nonspecific effect will account for the result. Conversely, when a highly specific molecular event (*e.g.*, protein folding) is monitored within a cell, the odds are much lower that nonspecific processes could account for the result. Here, we provide examples of molecular events monitored in cell-based assays that have been or could be adapted to HTS.

Protein Aggregation in Neurodegeneration. Misfolding and aggregation of pathogenic peptides within the cell is a central feature of many neurodegenerative diseases. Polyglutamine diseases are a family of at least nine dominantly inherited neurodegenerative disorders (*16*). Each derives from an expanded glutamine tract that destabilizes a target protein. This leads to misfolding, aggregation, and toxicity in central nervous system neurons. Various relatively inaccurate methods have been used to quantify intracellular aggregation: direct counting of large cellular inclusions by microscopy; filter trap assays, in which cell lysates are passed through a membrane with small pore size to detect large aggregates; and Western blots to detect high-mobility complexes. Such approaches either are not amenable to HTS or are unable to detect subtle changes in aggregation.

Fluorescence resonance energy transfer (FRET) is a powerful method for detecting protein interactions and can be exploited in cell-based assays for HTS (Figure 1, panel a). FRET involves the direct transfer of energy from a donor to an acceptor molecule, which is detected by spectroscopy. The green fluorescent protein derivatives cyan (CFP) and yellow (YFP) fluorescent proteins are useful FRET donor/acceptor pairs in cell-based assays. The fusion of aggregation-prone proteins to CFP and YFP allows quantitative detection of FRET based on protein interactions (Figure 1, panel b). Cells expressing these fusion proteins are cultured in a microtiter format, and the FRET signal is quantitatively measured by using a micrometer-based fluorescence plate reader (*17*). The utility of this system was demonstrated by screening the National Institute of Neurological Disorders and Stroke (NINDS) compound collection (www.msdiscovery.com) (*18*, *19*), which is composed of U.S. Food and Drug Administration (FDA)-approved drugs and natural products. Eleven compounds were identified that inhibited intracellular polyglutamine protein aggregation (*20*). Six of these compounds diminished neurodegeneration in a *Drosophila* model of neurodegeneration created by overexpression of the pathogenic peptide that causes Huntington's disease (*20*), providing a striking degree of corroboration *in vivo*. The quantitative nature of the FRET-based assay also facilitated genetic studies to determine molecular mechanisms that regulate protein aggregation (*17*).

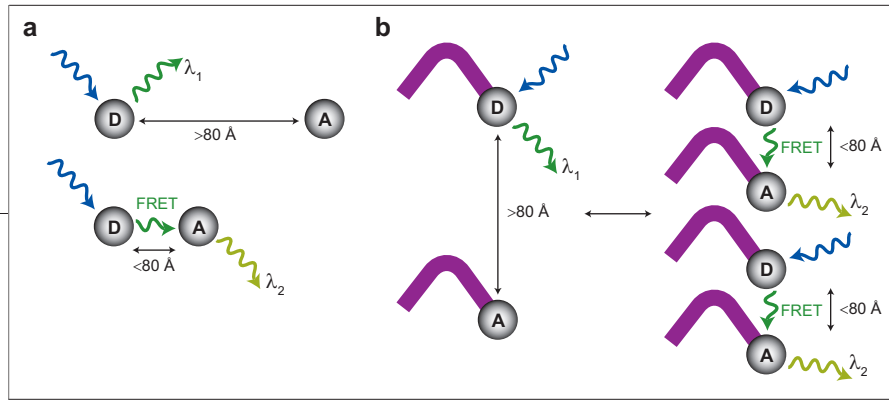

Figure 1. Use of FRET to detect protein aggregation in cells. a) FRET involves direct energy transmission between a donor and an acceptor molecule. When donor/acceptor distance exceeds ~80 Å, no FRET occurs, and donor excitation produces an emission of only λ_1. The proximity of the donor/acceptor pair results in FRET upon donor excitation, and donor excitation produces a new emission of λ_2. b) When aggregation-prone proteins associated with neurodegenerative diseases are tagged with FRET donor/acceptor pairs (*e.g.*, CFP/YFP), it is possible to measure protein self-association quantitatively in an intact cell based on the amount of FRET.

Specific Modulation of Gene Expression. Specific modulators of gene expression have important therapeutic potential. For example, a large body of evidence suggests that improving glutamate clearance might ameliorate amyotrophic lateral sclerosis. The glutamate transporter, GLT-1, which removes glutamate from the synaptic cleft, is thus a potential therapeutic target. A spinal cord slice culture was used to screen the NINDS compound collection for molecules that increase GLT-1 expression. β-Lactam antibiotics were identified as leads, and the treatment of mice with the antibiotics increased GLT-1 expression *in vivo*, although the effect on disease progression in a transgenic mouse model was small (*21*). It remains to be seen whether modulation of GLT-1 expression will have any benefit in patients.

In a second example, a cell-based assay was used to screen >800,000 compounds to identify molecules that would cause selective read-through of a nonsense stop codon (*22*). The investigators identified a compound with activity in a mouse model of muscular dystrophy. This compound corrected the deficit induced by an aberrant stop codon in the dystrophin gene. These two approaches demonstrate the feasibility of appropriately constrained cell-based assays to identify compounds with high probabilities of activity *in vivo*.

KEYWORDS
Cell-based assay: A system that utilizes a living cell as a means to measure a physiological or pathophysiological process.
High-throughput screening: The utilization of an enzymatic, biochemical, or a cell-based assay to evaluate hundreds or thousands of small molecules or genes in parallel.
Disease modeling: The representation of a particular aspect of a disease using an *in vitro*, cell-based, or animal system.
Neurodegenerative disease: A neurological disease that derives from progressive, inexorable loss of neuronal function and cell death.

Conformational Change in Nuclear Receptors. Intracellular nuclear receptors (NRs) are important therapeutic targets in human disease. For example, the estrogen receptor (ER) is strongly implicated in breast cancer, and the androgen receptor (AR) in prostate cancer (*23, 24*). Antagonists of ER (tamoxifen) and AR (bicalutamide) have potent, but short-lived, utility in the treatment of metastatic disease, so improved inhibitors are needed. NR function has been studied for decades through the use of reporter genes, whereby a hormone responsive promoter is linked to an easily measured transcriptional product (*e.g.*, luciferase) (*25*). This interposes multiple steps between the initial binding of the ligand and the measured activity of the NR: hormone-induced conformation change, nuclear translocation, recruitment of transcriptional assembly proteins, and translation of mRNA, among many other steps (Figure 2, panel a). Inhibition of any of these steps can produce nonspecific responses if a cell-based screen is used to detect antagonists.

A conformation-based assay of NR activation utilizes a discrete molecular event, which reduces detection of nonspecific responses (*26*). In this system, an NR, such as AR, is tagged at the amino and carboxy termini with CFP and YFP peptides. In the absence of hormone, the termini of the receptor remain separated in space, and no FRET occurs. After ligand binding, the receptor undergoes a conformational change that brings the amino and carboxy termini into proximity, thus producing a FRET signal (Figure 2, panel b). By transfecting this AR conformational reporter in cells, a physiological model of AR activity was established that is very amenable to HTS. The conformational assay reports a very early event in receptor signaling that occurs within several minutes of ligand binding (*26*).

We screened the NINDS compound collection against the AR conformation assay and a traditional luciferase reporter transcription assay. The two assays were equally sensitive to known antagonists, but the conformation-based assay was much more specific (unpublished data). Using the mean value of the nonantagonist treated cells as a control, we observed that the conformation-based assay identified the top 5% of hits at approximately three standard deviations from the mean, whereas the transcription-based assay required seven standard deviations from the mean to reduce the hit list to a similar number of compounds. This suggests that the transcriptional assay is much more prone to nonspecific effects. In fact, very few of the hits identified by the transcription screen were also effective in

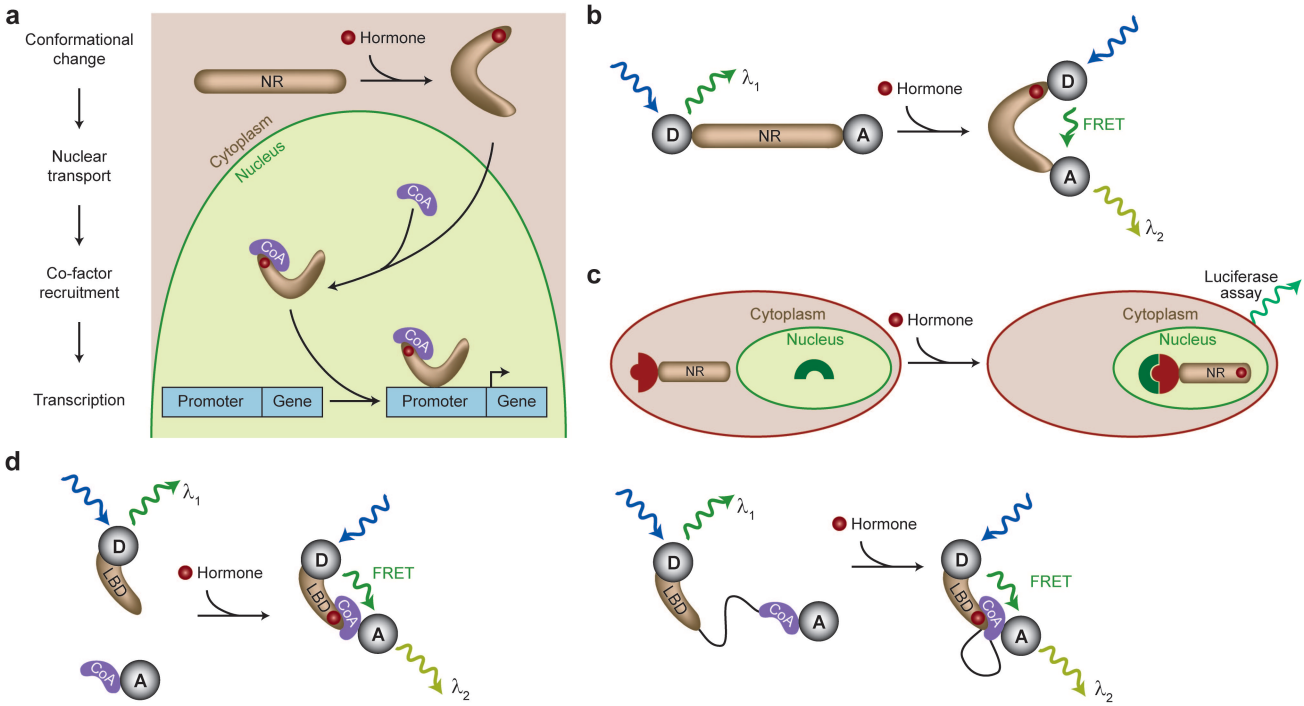

Figure 2. Use of FRET to monitor NR function. a) NRs undergo multiple steps of processing after ligand activation, which can produce nonspecific hits during a screen. b) The amino and carboxy termini of an NR are tagged with a FRET donor (D) and acceptor (A). Conformational change induced by hormone binding reduces the intramolecular distance and increases the FRET signal. c) The amino terminus of an NR is tagged with one-half of a luciferase enzyme. The second half is tagged with a nuclear localization sequence and is constitutively nuclear. Nuclear translocation of the NR allows reconstitution of the luciferase activity. d) The LBD of an NR is tagged with a FRET donor, and a coactivator protein (CoA) is tagged with a FRET acceptor. Hormone binding induces intermolecular FRET. Alternatively, a single fusion protein has a FRET donor fused to the LBD, fused in turn to a coactivator peptide motif, and then fused to a FRET acceptor. Hormone binding induces intramolecular FRET.

secondary assays of antiandrogen activity, and those that were effective were also identified by the conformation screen (unpublished data). Many NRs, including ER, could be adapted to a conformational assay. This method has been used to characterize compounds that induce subtle conformational changes of intracellular ER-α and -β (*27*), illustrating how specific molecular readouts can also be used to better characterize regulators of protein function in secondary analyses.

Nuclear Localization. Several assays that model other steps along the NR activation pathway are also amenable to HTS. Upon ligand binding, AR concentrates in the nucleus, where it forms dimers prior to activating gene expression (*26*). A highly quantitative cell-based screen was developed to detect antagonists of AR nuclear translocation by using a genetically encoded bioluminescent indicator (*28*). AR was fused to the amino-terminal half of Renilla luciferase, while the carboxy-terminus of Renilla luciferase contained a potent nuclear localization signal, making it constitutively nuclear. With nuclear translocation of AR, the reconstitution of these split fragments produces quantifiable bioluminescence in the presence of the appropriate substrate (Figure 2, panel c). This assay was used to screen for compounds that inhibit nuclear translocation of AR, a discrete step in the AR activation pathway. The assay yielded important leads and was even adapted to an *in vivo* assay in mice (*28*). This work exemplifies how another important molecular event (nuclear localization) can be adapted to a quantitative cell-based model for HTS.

Cofactor Interactions. To activate gene expression, many NRs recruit cofactors that utilize LXXLL motifs to bind to NR ligand binding domains (LBDs) (*29*). No single NR–cofactor interaction is known to be absolutely critical in the progression of disease; however, it

is possible that inhibitors of these interactions could be useful therapeutics. Two reports describe the use of FRET reporters to identify novel inhibitors of NR–coactivator interactions. In the first, a FRET acceptor (YFP) is fused to the LBD of either the peroxisome proliferator activated receptor-γ or ER-α. A FRET donor is fused to NR coactivators CREB-binding protein or steroid receptor coactivator-1 (SRC-1) (Figure 2, panel d) (*30*). Known agonists of the two NRs increased NR association with the coactivators, while antagonists inhibited the ligand-induced cofactor association. In the second, YFP was fused to the ER-α LBD, attached by a flexible linker sequence to the LXXLL motif of SRC-1 fused to CFP (Figure 2, panel d) (*31*). This intracellular reporter also demonstrated the ligand-dependent association of ER with a coactivator and was used to determine the activity of various compounds as agonists and antagonists of the LXXLL-LBD association. The FRET assays of NR–coactivator association replicated the findings of other assays, such as immunoprecipitation and yeast two hybrid. However, unlike these other techniques, the FRET assay may be more likely to identify leads with *in vivo* activity because it uses an intracellular, quantifiable molecular event and it is readily adaptable to HTS.

HTS: Start with Small Libraries. The choice of a library is critical to the success of a screening project. A comprehensive discussion of library design is beyond the scope of this Review (for an excellent review of ideal compound composition, see ref (*32*)), but it is important to consider the size and cost of the library, how to determine the mechanisms of action of hits, and how to translate them into viable drugs. Although some hits from large synthetic chemical libraries have been translated into important therapies, discovering new lead compounds *via* HTS by using these libraries has not been widely successful, and many companies are now developing smaller, focused libraries in hopes of greater success rates (*33, 34*).

Because the cost of a large library is high, and the success rate is low, we suggest that academic laboratories begin by screening small collections of biologically active molecules in cell-based assays. Ideally, compounds should be cell-permeant, nontoxic, and nonmutagenic and have good bioavailability. These characteristics will make them more effective in live cells and improve their likelihood of activity *in vivo*. We also recommend that the collection include natural products and drugs already known to be safe and/or effective in humans.

Screening an established chemical space offers many advantages. These compounds are generally known to be biologically active, which increases their chance of modifying the molecular event of interest. Also, a compound that influences a discrete molecular event within a cell need not directly bind the protein target. Protein behavior (*e.g.*, aggregation) is often subject to the influence of cell signaling pathways (*17, 35*), which can create opportunities for therapeutic modulation as well as determination of important pathogenic mechanisms.

Putative mechanisms of action of many natural products and known drugs have often been previously described. This can provide an instant wealth of information about a confirmed hit and greatly aids further pharmacological and genetic evaluation of molecular mechanisms. A lead compound can thus become a "biological probe" in the identification of novel regulatory pathways and new therapeutic targets. In addition, libraries of FDA-approved drugs and natural products are composed of compounds safe for human use, and this expedites their translation from discovery to the clinic (*36*). Natural products often have very good pharmacokinetic profiles, and nearly half of currently approved drugs mimic them or have been inspired by them (*37*). For these reasons, we suggest that using a library that includes natural products and compounds of known function, with *in vivo* tolerability, greatly enhances the chance of finding a useful lead.

Screening known compounds presents potential disadvantages, including the perceived lack of novelty and the difficulties of creating novel intellectual property (IP). However, assay development, chemical and genetic screening, and subsequent characterization of pathways that regulate key pathological mechanisms are vital aspects of translational research. The creation of novel IP from leads is essential for successful translational research and is certainly more challenging when one is working with previously described compounds. It is always vital to determine a structure–activity relation-

KEYWORDS
Nuclear hormone receptors: Proteins that sense the presence of specific hormones and transduce their activity by direct binding to particular promoter elements and regulation of gene expression. Before they bind hormone, these receptors may reside in the cytoplasm or nucleus, but they are localized to the nucleus after ligand activation.
Library choice: The selection of collections of small molecules for use in high-throughput screens.
Molecular readouts: The use of specific protein conformational change, protein–protein interactions, or changes in subcellular localization as the basis of a cellular assay to identify chemical or genetic modifiers.

ship (SAR) for any promising lead. This can help validate its putative mechanism and may also reveal a more efficacious compound with a novel structure and that is more amenable for IP protection. The ZINC database can help identify commercially available compounds for SARs (*38*). Finally, it remains possible to protect an off-patent compound for a new clinical indication, especially if novel formulations are created and new mechanisms of action are defined. Many small companies have now been started based on rescreening known compounds to develop drugs for novel indications. This may ultimately constitute a highly productive use of the "therapeutic chemical space". Ultimately, the advantages of rescreening previously characterized compounds should not be discounted.

Conclusion. Mammalian cell based HTS has not yet lived up to its promise of drug and target discovery. We argue that cell-based disease models that rely on a discrete molecular event will improve the chances of finding valuable leads. For academic research, cellular assays based on validated disease mechanisms increase the odds of identifying factors or signaling pathways that modulate a key event. Such assays may be exploited for complementary DNA or RNA interference library screens and for subsequent molecular studies of pathogenesis. These screens are not limited to mechanisms relevant only to neurodegeneration or cancer but can be extended to any molecular event that defines disease progression. The design of specific intracellular molecular end points, and the careful choice of libraries, will facilitate informative investigations that make the best use of precious resources for an academic laboratory or small enterprise.

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