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REVIEW

Small-Molecule Screening: Advances in Microarraying and Cell-Imaging Technologies

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ABSTRACT Cell-permeable small molecules can be used to modulate protein function selectively, rapidly, reversibly, and conditionally with temporal and quantitative control in biological systems. The identification of these chemical probes can require the screening of large numbers of small molecules. With the advent of new technologies, small-molecule high-throughput screening is widely available. This Review focuses on the emerging technologies of microarray screening platforms and high-content screening formats.

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Small molecules have been exploited in biological experiments throughout human history on an *ad hoc* basis. However, only recently have they been viewed as general tools to systematically explore protein function in biological systems. Perhaps the most significant challenge in this pursuit is the discovery of a small molecule that can selectively modulate either a given protein function or a specific biological phenotype (physiological effect), an approach termed "chemical genetics" (*1–5*). The identification process can be achieved by the systematic screening of large numbers of small molecules, known as high-throughput screening (HTS). Traditionally, HTS has been the realm of the pharmaceutical industry; however, in the last decade, new robotic and analysis technologies have made HTS available to all life science researchers.

Broadly speaking, three types of assays are available for quantifying small-molecule interactions with biological systems (Figure 1): protein-, cell-, and organism-based assays.

Protein-Based Assays. There are a wide range of techniques that can be used to detect protein-ligand interactions, some examples include: radioligand assays (*6*); affinity selection chromatography (*7*) coupled with NMR (*8*) or mass spectroscopy (*9*); isothermal calorimetry (*10*); surface plasmon resonance (*11*); and a variety of fluorescent techniques such as fluorescence polarization (*12*), fluorescence correlation (*13*), and FRET (*14*). Cell-free, bead-based assays for detection of small-molecule–protein binding include scintillation proximity (*15*), AlphaScreen (*16*), and flow cytometry assays (*17*).

Cell-Based Assays. Cellular experiments can be broadly classified as enzyme-linked immunosorbent and similar assays (*18*), expression reporter gene assays that monitor cell responses at the transcription/translation level (*19*), second messenger assays that

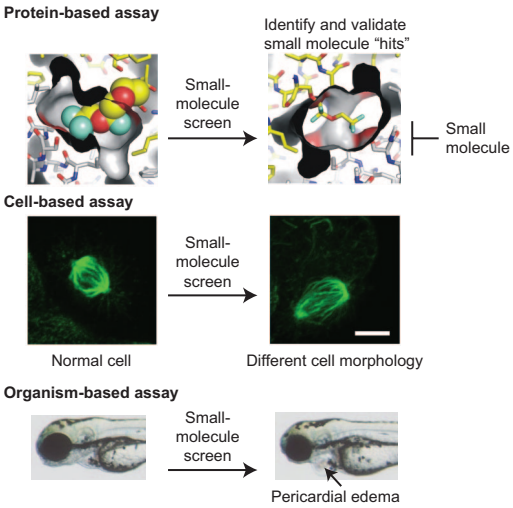

Figure 1. Small molecules can be used in assays with proteins, cells, or organisms.

monitor signal transduction following activation of cell surface receptors (*20*), and imaging assays that monitor overall response of cells to external stimuli (*21*). For reporter gene assays, colorimetric (*22*) and chemi- and bioluminescence (*23*) readouts are used, although fluorescent methods are the most common detection method. In second messenger and imaging assays, fluorescent techniques are primarily used. Several fluorescent methods (*24*) for cell-based assays have been developed, including fluorescence correlation (*13*), time-resolved fluorescence, FRET (*14*), and flow cytometry assays (*17*).

Organism-Based Assays. In organism-based assays, imaging techniques are generally used that monitor phenotypic changes *via* fluorescent and visible light microscopy.

For all types of assays, the trend is toward developing increased throughput. To achieve this, highly miniaturized assay formats such as microarrays and microfluidic systems (*25*) are increasingly prominent, and a greater emphasis is being placed on highly sensitive detection methods. Confocal fluorescence microscopy, which allows sample analysis with high spatial and temporal resolution, and related techniques have improved sensitivity both in cell-free and cell-imaging assays (*26*). This review will not attempt to be comprehensive but instead will concentrate on the use of microarrays and cell-imaging techniques in small-molecule screens. These two important emerging technologies are rapidly expanding areas of research that provide a flexible, high-throughput means of assessing small-molecule interactions with biological systems.

Microarrays in Small-Molecule Screens. Microarray technologies based on surface-bound small molecules (*27*) and proteins (*28*) have successfully identified novel small-molecule–protein interactions. Small-molecule and protein microarray screening involves the high-density immobilization of small molecules or purified recombinant proteins, respectively, as spatially discrete spots onto a modified glass or gel surface followed by incubation with putative binding partners. The mobile binding partner is usually labeled with a fluorescent tag, such as an organic dye (*29*), which allows for small-molecule–protein complex detection with fluorescence microscopy; however, label-free techniques, such as surface plasmon resonance (*30*), have also been used (Figure 2).

In protein microarrays, the proteins are attached to the support by covalent bond formation between free amines on the surface of the protein and epoxy- or aldehyde-modified slides. In this approach, an array of randomly oriented proteins is formed. This may cause specific binding domains to bind to or be concealed by the solid support, and this will give inconsistent results. An alternative immobilization procedure involves an affinity interaction between a His6-tagged protein and a nickel-coated surface (*31*). As the tagged protein is

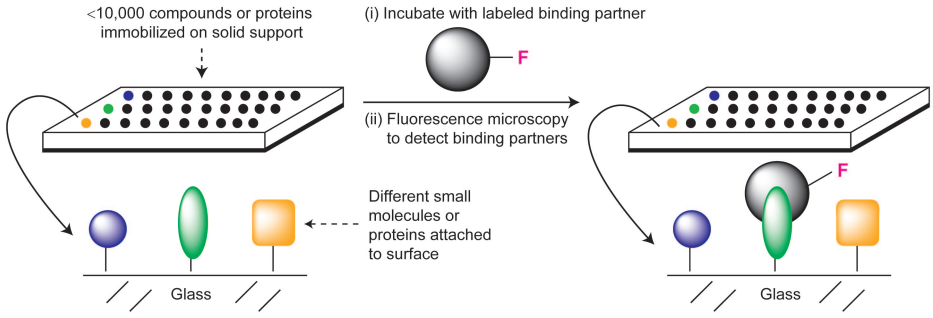

Figure 2. Small-molecule and protein microarrays: different small molecules or proteins are attached to a glass slide (usually 25 × 75 mm) and probed with a fluorescently labeled binding partner. Fluorescence microscopy is used to detect small-molecule–protein interactions.

formed as a fusion, the orientation of the proteins is more uniform and may lead to more reliable results.

Protein microarrays are used to detect direct binding interactions between small molecules and proteins and to probe biological systems. In an example of a direct binding assay, a yeast proteome-wide array was probed with two compounds known to influence the target of the rapamycin pathway. Several protein interaction partners for each molecule were determined in this way (*32*). In another example, a protein microarray was used to demonstrate the cyclic AMP-dependent interaction between the *Escherichia coli* proteins phosphodiesterase and the Per-Arnt-Sim (PAS) domain of the enzyme (*33*).

Some problems associated with arraying proteins onto a surface include denaturation and dehydration of the proteins and inaccessible immobilization conformations. Encapsulation of proteins in a sol–gel minimizes these difficulties because the gel mimics an aqueous environment and allows free rotation of the proteins. This methodology was used to obtain quantitative data for the inhibition of protein kinase A with the known inhibitors H7 and H89 (*34*).

In small-molecule microarray fabrication, compounds are synthesized and immobilized onto a chemically modified surface. Methods for *in situ* synthesis of microarrayed compounds include photolithographic (*35*) and maskless photodirected syntheses (*36*). However, the most widely used approach involves synthesis of compound libraries followed by printing onto glass slides. These libraries, generated by parallel or "split–mix" synthesis, must include a suitable functional group for chemoselective attachment to the glass surface. Several mild coupling reactions are available, including formation of silyl ethers (*37*) and amides (*38*), Michael additions (*39*), and Diels–Alder reactions (*40*). Alternative immobilization protocols, such as nonselective photoinduced cross-linking (*41*) and noncovalent affinity interactions (*42*), have also been used. This approach is used both to detect binding interactions between small molecules and proteins and in enzymatic functional assays.

Direct binding assays that use small-molecule microarrays have identified small-molecule ligands for many purified proteins, including the yeast transcriptional corepressor Ure2p (*43*), calmodulin (*44*), and the quorum sensing protein CarR from *Erwinia carotovora* (*38*). Although purified proteins are usually used in binding assays on microarrays, this technology has been extended to the use of proteins from crude cell lysates. For example, Bradner *et al.* (*45*) incubated small-molecule microarrays with cellular lysates containing overexpressed epitope-labeled proteins from mammalian cells. Detection of small-molecule–protein interactions was achieved by treatment of the microarrays with fluorescently labeled antibodies that interact with the epitope tag (*45*).

Various approaches are available for enzymatic functional assays with small-molecule microarrays. One example entails a surface-bound reporter group that translates enzymatic activity into fluorescent readouts. Yao and coworkers (*46, 47*) utilized coumarin–enzyme substrate conjugates immobilized on glass slides in a screen of hydrolytic enzymes. Hydrolysis of the nonfluorescent coumarin–substrate complexes results in the fluorescent coumarin being unmasked and indicates substrate-dependent enzyme profiles. Another approach to enzymatic functional assays, demonstrated by Gosalia *et al.* (*48*) in a screen for caspase inhibitors, entails arraying small molecules held within glycerol droplets onto glass slides. Sequential aerosol deposition of the caspase enzymes and a fluorogenic coumarin-containing enzyme substrate allowed monitoring of enzyme–substrate interactions without the need for surface linkage of the small molecule to the slides.

Some of the limitations associated with immobilization of ligands are addressed in dry chemical microarrays or microarrayed compound screening (*49*). For these microarrays, up to 10,000 compounds are printed in DMSO onto polystyrene sheets that are the size of a conventional 96-well plate and are then dried (Figure 3). An agarose gel embedded with the target biomolecule is applied onto the sheet to introduce the compounds into the assay. This approach has identified inhibitors of HIV integrase (*50*) and caspase (*51*). The visualization

KEYWORDS

Genotype: Heritable genetic information specific to each individual.

Phenotype: The observable characteristics of a cell or organism. A phenotype is created through interaction of the genotype with the environment.

Assay or screen: Used to assess the effects of a substance, such as a small molecule, on a protein, cell, or organism.

Chemical genetics: The use of small molecules to perturb protein and signaling pathways of biological systems.

Cell imaging: The use of visible light and fluorescent microscopy techniques to assess phenotypic changes in fixed and living cell populations.

High-content screening: Cell- or organism-based assays that use automated imaging techniques to detect multiple phenotypic responses.

Small molecule: Organic molecule with a molecular weight typically <500 Da.

High-throughput screening: Process by which large numbers of small molecules are screened for biological activity rapidly and in parallel.

Small-molecule microarray: Array of small molecules immobilized as spatially discrete spots onto a modified glass or gel surface.

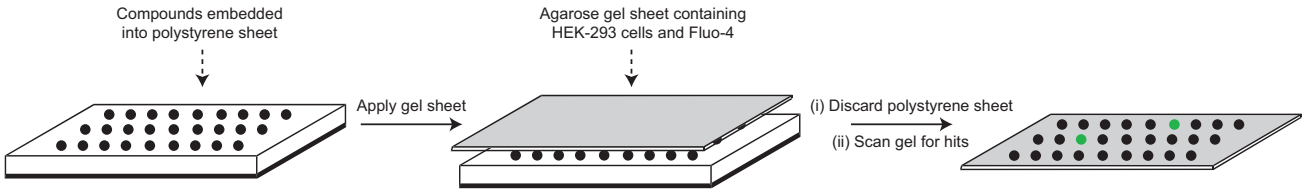

Figure 3. Microarrayed compound screening. An agarose gel layer containing engineered HEK-293 cells and calcium ion indicator Fluo-4 is applied to a sheet of polystyrene embedded with small molecules. The small molecules diffuse from the polystyrene into the gel. After incubation, fluorescence microscopy is used to detect the hits.

methods for this type of microarray are often specific to the assay. For example, in the screen for HIV integrase inhibitors, a radiometric detection method was used, whereas in the caspase inhibitor assay, time-resolved fluorescence was applied. One interesting application of this technology is the extension to a cell-based screening format for the identification of G-protein receptor agonists (*52*). Human embryonic kidney (HEK)-293 cells, expressing a human dopamine receptor and a chimeric G protein, and the fluorescent calcium ion indicator Fluo-4 were incorporated into the agarose sheet and then placed onto the microarrayed compounds, which diffused into the gel. Agonistic binding of the compounds with the dopamine receptor increased calcium ion levels. Binding of the calcium ions to Fluo-4 resulted in enhanced fluorescence (Figure 3).

The first small-molecule microarray suitable for cell-based screens was developed by Bailey *et al*. (*53*). Cytotoxic small molecules, contained within a biodegradable polymer to facilitate sustained release of compound into the cell layer, were arrayed on glass slides. A monolayer of mammalian cells was cultured over the slide, and this allowed the arrayed small molecules to diffuse into proximal cells. After incubation, both visible and fluorescent microscopy were used to analyze the slides. This technology is highly promising despite some technical limitations due to cross-contamination. An alternative format is a cell-based array in which cells are printed onto the slide. This technology has been investigated primarily in cellular immune responses and cancer profiling (*54*) and has not yet been transferred to small-molecule applications.

High-Content Screening in Small-Molecule Screens. High-content screening can be described as cell- or organism-based assays that use automated imaging techniques such as fluorescence microscopy to detect multiple phenotypic responses (*21, 55*). This is especially useful in the assessment of phenotypic changes, such as cell migration (*56*), that are difficult to quantify by other means.

Fluorescent microscopy of cells is dependent upon labeling of intracellular structures. In endpoint experiments, this can be done by fixing cells prior to staining of cellular the apparatus, such as the nuclei or Golgi apparatus; however, for real-time analysis of cellular mechanisms, alternative labeling procedures are required. Chemical, antibody, or endogenous methods can be used to label cellular proteins. One chemical method of protein labeling involves covalent attachment of the dye to the protein of interest before microinjection into a cell. Genetically fusing a receptor protein to the target protein is an alternative labeling method. Growing cells that express the modified protein are treated with a small-molecule–antibody–fluorophore that enters the cells and binds to the receptor, thereby labeling the desired protein (*57*). Quantum dot nanoparticles have also been used to label proteins. These are stable and less toxic than organic dyes and have very narrow excitation bands, which facilitate multiplexing, so they may be ideal labels for single-cell imaging (*58*). Endogenous labeling is achieved by genetically encoding a variant of GFP (*59, 60*) as a fusion to the protein of interest.

Confocal and laser scanning microscopes, with their flexible resolution capabilities, are typically used for detection of fluorescence emission because background fluorescence from unbound fluorophores and dead cells is minimized (*61*). Low-resolution microscopy can analyze a cell population, showing characteristics such as cell proliferation, whereas high-resolution analysis of a single cell allows visualization of cellular structures and intracellular translocations (*62*). Several fluorescence techniques are available for cell imaging. Fluorescence lifetime imaging, which monitors localized changes in the fluorescent lifetime of probes, is used for

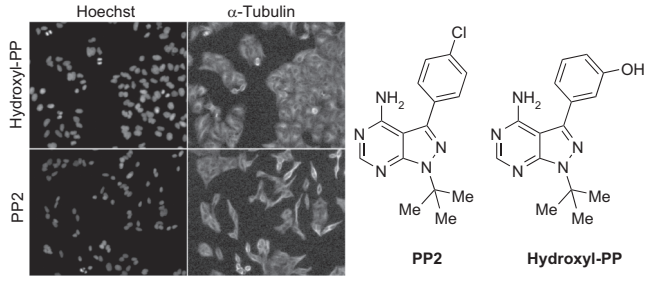

Figure 4. Microscope images of A549 cells stained with Hoechst dye or α-tubulin antibody after treatment with either hydroxyl-PP or PP2. The PP2-treated cells are more elongated and have a more condensed nuclear structure compared to the hydroxyl-PP-treated cells (*65*).

tracking dynamic changes in cells. For example, fluorescence lifetime imaging microscopy was used to map binding of the anticancer drug topotecan in nuclear structures of breast tumor cells (*63*). Fluorescence microscopy can be combined with other techniques such as Raman spectroscopy. When Raman and fluorescent imaging are integrated into one microscope, Raman visualization of the small molecule and fluorescence imaging of the protein are possible (*64*).

Cell imaging could prove vital in the discovery of small molecules with novel modes of action. One interesting example is a cell-morphology-based screen for small-molecule inhibitors of novel cellular targets in human cancer cell lines. A collection of small molecules, including known protein kinase inhibitors, was screened against four different cell lines. Following staining of DNA and microtubules, microscopy was used to assess characteristics of both the cell population and individual cells. Computational analysis was then employed to assign a distinct morphological signature to each small molecule. In this screen, hydroxyl-PP (a hydroxylated analogue of the kinase inhibitor PP2; Figure 4) was shown to have a different signature and a different mode of action than PP2 despite the close structural relationship (*65*). Other examples of small-molecule screens that utilize cell imaging include the discovery of inhibitors of cytokinesis in *Drosophila* cells (*66*) and identification of Akt pathway inhibitors (*67*).

Imaging technology has been applied to organism-based screens on *Danio rerio* (zebrafish) embryos and *Caenorhabditis elegans* (nematode worms). Zebrafish and *C. elegans* make good models for human disease as there is a high level of convergence in the proteomes of these organisms with the human proteome (*68*). The higher level of complexity of these organisms compared with cultured cells allows more information to be obtained from phenotypic screens. Both zebrafish and *C. elegans* are optically transparent, a trait that makes imaging an ideal readout with either classic morphology or fluorescent proteins expressed in cells or organs. Furthermore, the short life cycle and small size of zebrafish embryos and *C. elegans* make them suitable for HTS. In a recent example, Kwok *et al.* (*69*) screened >14,000 compounds for the induction of defects in wild-type *C. elegans*. Microscopy was used to identify 308 compounds that induced visible phenotypic changes, such as slow growth, lethality, and morphological defects (*69*). More extensive phenotypic screens have been conducted on both wild-type and mutant zebrafish embryos. For example, treatment of wild-type zebrafish embryos with a diverse small-molecule library identified a compound that delayed embryo development and led to abnormal brain, heart, and jaw morphology (*70*). In other studies, small molecules have been shown to suppress mutations that cause a cardiovascular defect (*71*) and mitotic arrest (*72*).

Perspectives and Challenges. Microarray technology that uses small molecules is primarily employed in the evaluation of protein–ligand binding interactions, although methodologies for enzymatic functional assays and cellular assays have also been demonstrated. The development of highly miniaturized homogeneous functional assays in which both binding partners are mobile remains a challenge in this field because of the difficulties associated with cross-contamination of reaction sites. Similarly, improved detection methods, such as highly sensitive imaging techniques, that allow for real-time analysis of the assay are vital. Cell-imaging technology has been extensively used in fixed cells to determine the effects of small molecules, although a potential exists for dynamic analysis of living cells. As additional automated and high-resolution microscopes become available, this technology will be invaluable in assessing small-molecule interactions with biological pathways in living cells and organisms. Microarray and cell-imaging technologies will be vital in the exploration of the proteome with small molecules.

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