Small molecule microarrays: from proteins to mammalian cells – are we there yet?

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A recent publication by Stockwell and colleagues documents a leap forward toward the continued development of small molecule microarray (SMM) technology. By creating microarrays of small molecules impregnated in a biodegradable polymer, the authors have, for the first time, shown that SMMs can be used in a cell-based format. This technological improvement opens the door for using SMMs to perform high-throughput screens in mammalian cells.

Introduction
Microarray technology is modernizing biomedical research by allowing the simultaneous analysis of tens of thousands of samples and by examining low nanomolar to picomolar amounts of materials. A recent study estimated an annual compound growth rate for the microarray market of 63%, from US$232 million to US$2.6 billion, between 1999 and 2004. An ever-expanding sector of the market of 63%, from US$232 million to US$2.6 billion, an annual compound growth rate for the microarray

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all three caspases. A significant advantage of this strategy was the ability to perform miniaturized high-throughput solution-phase screening of reactions at extremely low reaction volumes (1.6 nL per spot) without physical immobilization of any component.

Getting into the cell – beyond recombinant proteins and lysates

Although these advances have surmounted many technical barriers, drug efficacy and toxicity still cannot be measured in in vitro binding assays, nor are they predictors of cell permeability. Instead, SMMs – such as those discussed above – typically provide information on only one target, and the cellular effects of compounds are not examined. By contrast, cell-based screening provides simultaneous information on multiple parameters for a given target, or even for multiple target proteins in a biologically relevant setting. Improving the quality and quantity of data from primary, cell-based screening will be vital given the increasing number of new targets that are emerging, and for which the pharmaceutical industry has little or no past experience.

To develop a SMM that was compatible with a cell-based format, Bailey et al. [14] created microarrays consisting of small molecules impregnated in a biodegradable polymer that were printed on a standard microscope slide. A monolayer of cells was then placed and cultured over the array, allowing each compound to affect proximal cells. Although the principles underlying this attack seem simple, several barriers had to be overcome before the efficacy of a cell-based SMM could be tested.

Figure 1. Methods for SM immobilization and target presentation. (a) Covalent attachment of SMs to the microarray surface. The target, either in the form of a labeled purified protein or as mixture of proteins in a cell lysate, can be probed for SMM binding. The un-bound material is washed away and active SMs on the arrays are detected by the presence of the label. (b) Nanoliter droplets of glycerol can be used to dissolve and spatially separate the SMs [13]. The method has been used to probe enzymatic reactions and identify enzyme inhibitors. The enzyme and a specific fluorogenic substrate are aerosolized over the array slide containing the glycerol-immobilized SMs. Droplets that contain an inhibitor of the enzymatic reaction will prevent the cleavage of the substrate and consequent release of the fluorophor. (c) A biodegradable polymer can be used to embed and print the SMs on arrays [14]. The polymer solution is printed at 1nM/drop and dries to form a 200-μm diameter spot in a doughnut-like shape. Picomole amounts of each SM are encased into the polymer and then covered with a layer of fibronectin to allow for the attachment of cells onto the slide. The SM diffuses radially from the polymer and affects cells growing within several hundred micrometers of the spot.
The first issue was to identify an optimal polymer. The material needed to be non-toxic and compatible with existing chemical libraries, which are typically dissolved in dimethyl sulfoxide (DMSO). The material also had to have a sufficiently large diffusion barrier such that compounds would release slowly into the media. After assessing more than two dozen polymers, a biodegradable poly-(D), (L)-lactideglycolide copolymer (PLGA) was identified. PLGA and other poly (lactic acid) polymers enable the controlled release of proteins and small molecules through a combination of diffusion and polymer erosion. Next, picomole amounts of each compound on a solid surface had to be encased into the polymer and then covered with a layer of fibronectin to facilitate subsequent cell attachment. The PLGA solution, which was printed at 1nM per drop, dried to form a 200μm doughnut-shaped spot, and contained a thicker edge and a thinner center (Figure 1c). This printing method allowed the spotting of test compounds in any desired configuration.

**Screening small molecule libraries in cells – almost there**
Once the SMMs were prepared, two proof-of-principle experiments were performed. The first was a synthetic lethal screen that measured podophyllotoxin (PTX) and phenylarsine oxide (PAO) cytotoxicity in an A549 non-small cell lung cancer line. After being treated with the compounds, the cells were fixed, permeabilized and stained with phalloidin–fluorescein isothiocyanate (FITC, which binds filamentous actin) and Hoechst (which binds DNA). Images of all slides were recorded using both light and fluorescence microscopy and the number of Hoechst-stained nuclei was divided by the area in a region of interest. An initial, important observation was that cell density was lowest at a site containing one of the cytotoxic compounds, but rose with increasing distance from the spot. Indeed, cell growth was affected several hundred micrometers from the site at which the compounds were delivered. This phenomenon reflected compound release from the polymer matrix and was a measure of the dose-dependent response of the compounds. Six other cell lines were also examined (HeLa, BJ, BJELR, MEF, 293T and DU145), and as anticipated from microtiter analyses, small-molecule activity in this system was cell type-dependent.

In a second proof-of-principle experiment, the authors examined whether phenotypes other than cell death could be screened. To this end, the effect of rapamycin, a natural product, on S6 phosphorylation was tested in A549 cells. Rapamycin inhibits the mammalian target of rapamycin (mTOR) pathway, a pathway that normally culminates in ribosomal S6 protein phosphorylation. After rapamycin treatment, the A549 cells were fixed and permeabilized, and the arrays were stained with Hoechst and an anti-pS6 (phosphorylated S6) antibody coupled to the fluorescent dye, cy3. As anticipated, Bailet et al. found that rapamycin-sensitive cells contained lower levels of S6 phosphorylation and exhibited decreased fluorescence.

Finally, a small-scale synthetic lethal screen was performed to test whether the small-molecule cell microarrays could be used to identify compounds that are not lethal to cells on their own, but that display increased or decreased activity in the presence or absence of specific cancer-related genes. A549 and HeLa cells were transfected with one of seven different siRNAs that targeted the messages encoding p53, PTEN, MDM2, EGFR, TSC2, BCL2, and BRCA1 for destruction. Next, 70 biologically active compounds selected from Sigma, Calbiochem and the National Cancer Institute were tested for their effects on cell density in triplicate at three concentrations. Of the 980 compound siRNA-treated cell combinations examined, four conditions were found in which knockdown of a specific mRNA altered tumor cell-sensitivity to a specific compound. Of these four, one was confirmed in 384-well plate format by a conventional viability assay. These data validated the screening of small molecule cell-based microarrays.

**Conclusions and future directions**
An advantage of the method presented by Stockwell and colleagues is that low concentrations of compound and cell numbers are necessary to generate high-content information. The platform also has the potential to screen many compounds with minimal automation. However, several concerns must still be addressed before this strategy can be applied to high-throughput screens. One is that the suitability of the PLGA polymer for the release of a large array of chemical libraries remains to be determined. Although the authors have tested 12 compounds, ranging from 167 to 1255 atomic mass units, compound hydrophobicity or polarity will also have a role in the kinetics and extent of release. In addition, the analysis time needs to be improved to be applicable for high-throughput screening. The authors reported that it takes 7 min to acquire the images for each slide, and an additional 40 min to analyze one slide of images using a standard Pentium 4, 2-Ghz processor with 2 gigabytes of random access memory. Finally, technical advances on printing densities might be necessary to prevent one spot from affecting cells on another, nearby spot. Future evaluations will be required to address these concerns. Nevertheless, and in spite of current concerns and limitations, microarrays that enable the screening of small-molecule libraries in mammalian cells will prove valuable to researchers who are performing small-molecule screens to discover new chemical tools and therapeutically-active agents. Undoubtedly, the work discussed in this review represents only the first of many new technologies that will augment these endeavors.

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**References**

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