Applications of transfected cell microarrays in high-throughput drug discovery

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DNA microarrays and, more recently, protein microarrays, have become important tools for high-throughput genomic and proteomic studies. Transfected cell microarrays are a complementary technique in which array features comprise clusters of cells overexpressing defined cDNAs. Complementary DNAs cloned in expression vectors are printed on microscope slides, which become living arrays after the addition of a lipid transfection reagent and adherent mammalian cells. This article discusses two potential uses of cell microarrays in drug discovery: as a method of screening for gene products involved in biological processes of pharmaceutical interest and as *in situ* protein microarrays for the development and assessment of leads.

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▼ The need to translate genomic data into meaningful insights for drug discovery is an emerging challenge. Identification of novel targets for the treatment of human diseases requires functional characterization of the products encoded by the tens-of-thousands of genes that have been identified by the Human Genome Project and EST (expressed sequence tag) sequencing efforts [1–3]. That many genes generate several transcripts through alternative splicing greatly complicates this challenge by increasing the number of unique proteins that need to be analyzed [4]. Methods such as transcriptional profiling [5], high-throughput mass spectrometry [6] and protein microarrays [7] enable the systematic and rapid quantitation of individual mRNAs and proteins in biological samples. As a complement to these methods, Ziauddin and colleagues have recently described a microarray-driven technique for the high-throughput analysis of gene function in mammalian cells [8]. Upon the addition of cells and a lipid transfection reagent, microscope slides printed with cDNAs become living microarrays, whose features are clusters of cells overexpressing defined gene products. Such transfected cell microarrays (cell microarrays for short) can be used as a powerful and versatile platform during several steps of the drug discovery process. In particular, cell microarrays can be used to: (1) identify potential drug targets by functionally characterizing large numbers of gene products in cell-based assays; (2) evaluate the specificity of drug leads; and (3) identify binding proteins for drugs of unknown mechanism-of-action or for leads identified in phenotype-based assays.

Characterization of gene function

Recent strides in genomics have promised to greatly facilitate the identification of targets for human diseases by making it feasible, in concept, to screen functionally, all human gene products for those with roles in cellular processes associated with diseases. Initiatives by the Mammalian Gene Collection (MGC; http://www.hgmp.mrc.ac.uk) [9] and the Harvard Institute of Proteomics (http://www.hip.harvard.edu/research.html) are already underway to make available full-length cDNAs of all open reading frames in the human genome, paving the way for pan-genomic analyses of gene function within human cells. Traditionally, *in vivo* analysis of mammalian genes is performed one gene at a time by expressing within cells a DNA construct that directs the overproduction of a gene product or inhibits its synthesis or function. Although such cell-based assays are typically performed in culture dishes or, for high-throughput applications, in 96- and 384-well microtiter plates, these formats are not readily conducive to the screening of 10^5–10^6 genes in parallel in large numbers of high-content cell-based assays.

1359-6446/02/$ – see front matter PII: S1359-6446(02)02386-3

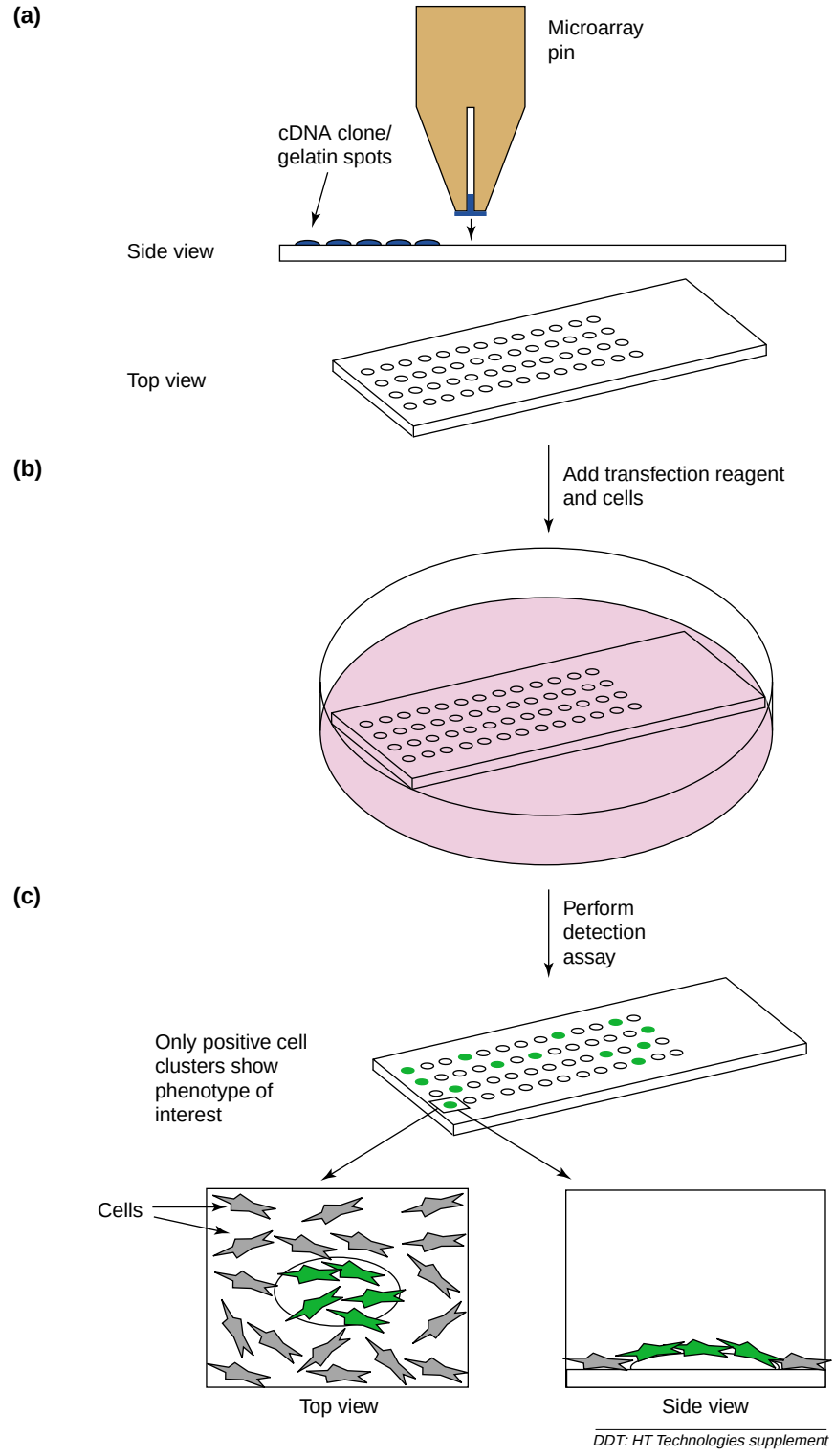

Figure 1. (a) Cell microarrays use a microarray pin transfer device to transfer nanoliter volumes of a gelatin solution containing cDNA clones in expression vectors onto a slide. (b) Transfection reagent and cells are then added, creating a 'cell microarray'. Each array feature is a cell cluster overexpressing a specific cDNA. (c) The array is then removed from media, and downstream detection assays can be performed.

Implementation

Cell microarrays, which offer a format without wells for high-throughput cell-based assaying, are an alternative system for the large-scale analysis of mammalian gene function. In this technique (Fig. 1), nanoliter quantities of cDNA-containing plasmids are dissolved in an aqueous gelatin solution and printed onto the surface of a glass slide using a robotic microarrayer device. The printed arrays are then briefly exposed to a lipid transfection reagent, resulting in the formation of lipid–DNA complexes on the surface of the slide. Mammalian cells in medium are added on top of the arrays in culture dishes, and cells that grow on top of the areas where plasmids were printed take up these plasmids and become transfected. The result is a living microarray in which each feature is a cluster of 30–80 cells overexpressing a particular gene product. To visualize cell microarrays, the array slides can be fixed and a variety of detection assays can be applied, including *in situ* hybridization, immunofluorescence and autoradiography. Alternatively, cell microarrays can be examined when still alive so that cellular processes such as protein translocation or calcium fluxes can be visualized in real-time. Because cell microarrays are printed with the same robotic arrayers as conventional DNA microarrays, they can achieve a similar magnitude of density: 6000–10,000 spots per slide. Thus, a small number of slides should be sufficient to express the entire set of human genes.

In a proof-of-principle screen of 192 distinct cDNAs in expression vectors, the researchers demonstrated how cell microarrays can be used as an expression cloning system to identify genes that regulate phenotypes of interest [8]. Ziauddin and colleagues screened for three phenotypes: (1) genes that increase the activity of kinase signaling pathways; (2) genes that induce apoptosis; and (3) genes that affect cell–cell adhesion. In the first screen for kinase activity, the array was probed

with an anti-phosphotyrosine antibody to detect gene products that increase tyrosine phosphorylation. Of the six cell clusters that displayed an above-background level of phosphorylation, five overexpressed known tyrosine kinases whereas the sixth overexpressed a gene of unknown function. To identify genes that induce apoptosis or changes in cell–cell adhesion, each cell cluster was visually inspected at high magnification. One cluster of cells overexpressing the apoptosis-inducing TRAIL receptor 2 exhibited signs of apoptosis, which was confirmed by TdT-mediated dUTP nick end-labeling [10]. Another cluster, in which the plasma membranes of cells were in close contact with each other, overexpressed the cell adhesion molecule CD36. This study showed that cell microarrays can be used to rapidly uncover gene function and could facilitate the discovery of proteins involved in biological processes of potential pharmaceutical interest.

Advantages, limitations and future work

Cell microarrays have several advantages over traditional methods of expression cloning. Because known cDNAs are printed at defined locations, it is unnecessary to isolate the cDNA when a phenotype of interest is discovered. This circumvents the substantial work typically required for fluorescence-activated cell sorting or successive rounds of 'sib' selection (in sib selection, clones conferring the phenotype of interest are isolated from clone libraries by iteratively subdividing the clone population) [11]. The microarray format also has several general advantages over any cell-based assay. Through miniaturization, cell microarrays achieve economies of scale because only small quantities of potentially scarce biological samples or rare cell lines are necessary to assay large sets of genes. Hundreds of microarrays can be printed from a single set of source plates for multiplexing purposes, and printed cDNAs can be stored for months before transfection. Finally, cell microarrays are more amenable to high-content assaying of subcellular phenotype because cells can be individually analyzed. By contrast, current microtiter-plate readers typically detect signals, such as fluorescence intensity, averaged over all cells in a well. Because cell microarrays are implemented on microscope slides, they are accessible to a broader range of detection methods.

Some important limitations of cell microarrays are intrinsic to the process of transfection. Transient transfections result in the strong overexpression of individual genes, and their induced cellular phenotypes might not always represent true *in vivo* gene function. Furthermore, only transfectable cell lines such as HEK and COS have been shown to be compatible with current cell microarray technology. This is because each array spot, ~150 μm in diameter, accommodates ~100 cells, and so most primary cell lines, which can have transfection efficiencies of <1%, are unlikely to be compatible with the microarray format. Hence, cell microarrays would benefit from new methodologies that increase primary cell transfection efficiencies.

So, cell microarrays using cDNA transfection enable the study of phenotypes that result from gene overexpression. In addition, cell microarrays might be adaptable to emerging techniques that facilitate the rapid study of gene underexpression. RNA interference (RNAi) is the recently discovered phenomenon of dsRNA-induced sequence-specific post-transcriptional gene silencing in animals and plants [12,13]. The mechanism of RNAi is still under investigation, but it is currently thought that the dsRNA is cleaved by cellular machinery into small fragments, 21–25 base-pairs in length, which then mediate degradation of their complement mRNAs. Although initial attempts to use RNAi in mammalian systems were unsuccessful, it was subsequently shown that synthetic 21–23-base-pair RNA forms duplexes with 3′-dinucleotide overhangs (called siRNAs), which can then efficiently mediate mammalian RNAi [14]. RNAi can quickly knock-down the protein product of any gene of known sequence. It therefore offers a rapid way of studying the effects of underexpression of genes in mammalian systems, and will probably be adaptable to the cell microarray format. Recently, plasmid-based systems have been described that, when transfected into mammalian cells, generate *in vivo* siRNAs [15]. These siRNA-generating plasmids should be compatible with existing cell microarray technology, enabling the creation of arrays of cell clusters with knock-downs in the levels of defined proteins. Cell microarrays could be used for pan-genomic RNAi studies that knock-down all genes in parallel, which could be a powerful tool for biological investigation.

Cell microarrays as substitutes for protein microarrays

As arrays of cells expressing high levels of individual proteins, cell microarrays can be used as protein microarrays. Protein arrays, in which purified proteins are immobilized onto a glass slide, are particularly useful for the quantitation of protein–small-molecule and protein–protein interactions [16]. Protein microarrays are potentially a tremendous asset for the process of drug discovery if they can be produced at the pan-proteomic scale. Uses of proteomic arrays include: (1) the screening of leads for target specificity; (2) the identification of binding partners for small molecules whose targets are unknown (such binding partners include some current drugs as well as leads identified in phenotype-based screens); and (3) the characterization of small molecules on the basis of their interaction profiles with numerous proteins, enabling an understanding of how small-molecule structure relates to protein structure. However, technical limitations currently prevent the construction of protein arrays that can represent the magnitude and diversity of mammalian proteomes. Cell microarrays, which can be

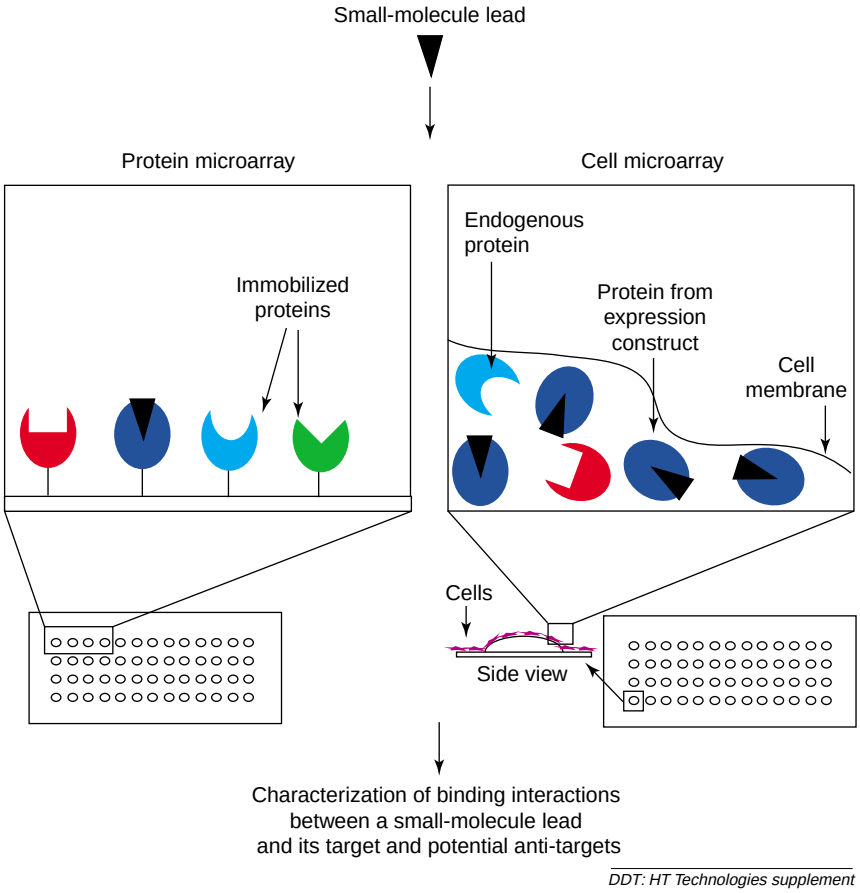

Figure 2. Cell microarrays as protein microarrays. Small molecule leads can be used to probe protein- or cell-microarrays for binding interactions. Protein arrays use protein that is immobilized directly on a surface, whereas cell microarrays overexpress specific proteins in cell clusters *in situ*. The binding patterns of labeled small-molecules on the arrays can provide information regarding target specificity and mechanism-of-action.

regarded as arrays of cellular 'factories' for the localized production of defined proteins (Fig. 2), circumvent many of these difficulties and might be useful as substitutes for highly representational protein arrays.

In their preliminary study of cell microarrays, Ziauddin and colleagues demonstrated that cell microarrays can be used to detect the specific interaction of small molecules with target proteins [8]. For this purpose, the investigators used FK506 (a small molecule used clinically for its potent immunosuppressive effects [17]) and its well-known mammalian target protein FKBP12 (FK506-binding protein 12) [18,19]. An array of expression constructs for either FKBP12 or green fluorescent protein (GFP) was printed and, after transfected cell clusters formed, the array was probed with radiolabeled FK506. Cells transfected with the cDNA for FKBP12 bound more FK506 than cells transfected with that for GFP, and specificity of the interaction was shown by elimination of the signal by the addition of rapamycin, a competitive antagonist of FK506. This study showed that the detection of a strong and highly specific small-molecule–protein interaction such as FK506–FKBP12 is possible in the background of endogenous cellular proteins.

The use of cell microarrays to select leads that are specific to a target protein of interest in an environment of mammalian cells is a general strategy that can be extended to leads other than small molecules. For example, monoclonal antibodies (mAbs) such as trastuzumab (Herceptin), which targets the cancer-associated ErbB2 receptor [20], have demonstrated significant therapeutic efficacy. Cell microarrays could be used to screen mAbs in a fashion similar to small molecules. Furthermore, cell microarrays can be used to express any gene product as long as the corresponding cDNA is available, making it possible to produce arrays of proteins from any organism of interest. For example, cell microarrays expressing proteins from pathogens can be created as platforms for monitoring antibody immune responses to vaccines. This approach could be especially useful for the study of clinically significant but poorly understood organisms such as *Plasmodium falciparum*, the protozoan that causes malaria.

Comparision to protein microarrays

As substitutes for protein arrays, cell microarrays side-step significant technical hurdles in protein array fabrication: the purification, maintenance and attachment of proteins. Because the range of protein half-lives spans many orders-of-magnitude, it is unclear whether arrays of numerous heterogeneous proteins can maintain uniform stability. Methods used to immobilize proteins on a solid support, which can involve van der Waals forces [21], covalent linkage [22] or polyacrylamide gels [23], might further compromise protein stability. Cell microarrays are one of several methods that address such issues. Tanaka *et al*. have developed a western-like approach (drug–western), in which protein produced from cDNA libraries is immobilized on a nitrocellulose membrane and directly probed with small molecules [24]. In a separate study, He *et al*. described protein arrays created *in situ* (PISA) from PCR-generated DNA fragments by cell-free protein expression and immobilization

onto beads or microtiter plates [25]. Cell microarrays, which express desired proteins *in situ* in adherent cells, share some advantages of both systems, while also benefitting from the miniaturization and multiplexing capabilities of the microarray format. Cell microarrays avoid problems associated with protein stability because they are printed arrays of cDNA and are converted to cell arrays only when needed. Furthermore, synthesis of mammalian proteins in mammalian cells increases the likelihood that proteins receive appropriate post-translational modifications. By activating or inhibiting appropriate biological pathways, levels of post-translational modifications could be changed as necessary.

Cell microarrays have the characteristic that proteins are synthesized in their endogenous cellular environments, which can be important for many protein array applications. This attribute is particularly useful for creating microarrays of membrane proteins, a common class of drug targets, which are unlikely to be properly folded in the absence of the cell membrane. Fang *et al.* recently demonstrated that membrane proteins can be stably arrayed by immersing them in a lipid medium that models cell surfaces [21], but it is unclear whether such supportive media can be extended to all membrane proteins. To show that cell microarrays are compatible with the study of membrane proteins, Ziauddin and colleagues created cell arrays expressing the dopamine D1 receptor or the seratonin 1A or 3A receptors [8]. The arrays were probed with a labeled dopamine antagonist and only cell clusters expressing dopamine receptors bound the antagonist, suggesting that the receptors were synthesized in manner that retained their native binding capabilities (Fig. 3).

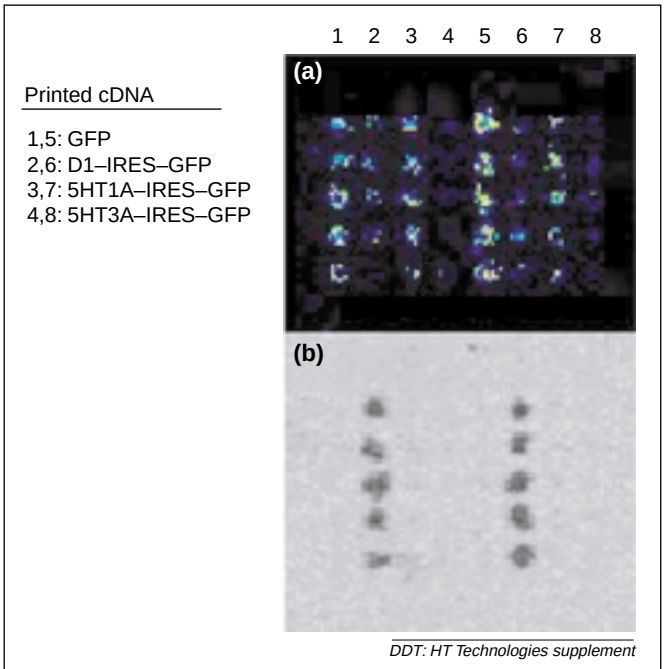

Figure 3. Cell microarray expressing GFP- and IRES–GFP-tagged dopamine D1 receptor or seratonin 1A or 3A receptor. (a) Fluorescent microscopy shows that all clusters expressed GFP. GFP is expressed at lower levels if derived from the IRES-containing plasmids rather than from the standard GFP expression vector. (b) Film autoradiography of the same array after probing with ^{3}H-SCH23390, a dopamine antagonist. The probe bound to cell clusters expressing dopamine D1 receptor but not seratonin 1A or 3A receptors or pure GFP. Abbreviations: GFP, green fluorescent protein; 5HT1A, seratonin 1A receptor; 5HT3A, seratonin 3A receptor; IRES, internal ribosome entry site.

Potential applications

Although we have categorized cell microarrays as expression cloning systems or as protein microarrays, aspects of both roles can be integrated in many potential applications. The study of G-protein-coupled receptors (GPCRs), targeted by many clinically significant drugs, is one such example. Cell microarrays can be used to screen small molecules for their capacity to specifically interact with GPCR targets. This information could be useful as a predictive tool to identify chemical structures with potential to associate with particular GPCRs. Alternatively, cell microarrays could be used to study cellular responses to ligand binding, such as protein phosphorylation, calcium waves, gene transcription and protein translocation. Finally, cell microarrays are not limited to microscope slides but can be formed on any solid surface that supports the attachment of mammalian cells. As the number of GPCRs is limited, it would be possible to create 'arrays of arrays' of GPCRs at the bottom of microtiter plates, enabling simultaneous assaying of numerous small molecules using the plate-based robots common to many HTS operations.

Concluding remarks

Cell microarrays are a potentially powerful addition to the biologist's arsenal of high-throughput tools for the post-genomic era. Although the technology is still in its infancy, cell microarrays might eventually find broad use in the biopharmaceutical industry by accelerating two pivotal steps in drug development: target identification and lead assessment. As a high-throughput expression cloning system, cell microarrays can be used to rapidly uncover all gene products that functionally correlate with human diseases of interest. As protein microarrays, cell microarrays can be used to screen leads for target specificity by characterizing patterns of protein–small-molecule interactions, and have potential uses in characterizing protein–enzyme and protein–antibody interactions. Finally, although current implementations of cell microarrays use printed cDNAs that direct gene overexpression, it might be possible to create other classes of cell microarrays by printing substances such as proteins, small molecules or oligonucleotides.

Acknowledgements

We would like to thank Brent R. Stockwell and Siraj M. Ali for their helpful comments during the preparation of this manuscript.

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High-throughput Technologies: A Supplement to Drug Discovery Today

How to cite an article in this supplement:
Inglese, J. (2002) Expanding the HTS paradigm. *Drug Discov. Today* 7 (Suppl.), S105–S106

How to cite the whole supplement:
Owens, J.M., ed. (2002) *High-throughput Technologies: A Supplement to Drug Discovery Today* Vol. 7, No. 18, Elsevier Science

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