Cell-biological applications of transfected-cell microarrays

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Cell microarrays are a recent addition to the set of tools available for functional genomic studies. Each cell microarray is a slide with thousands of cell clusters that are each transfected with a defined DNA, which directs either the overproduction or the inhibition of a particular gene product. By using a range of detection assays, the phenotypic consequences of perturbing each gene in mammalian cells can be probed in a systematic, high-throughput fashion. Combining well-established methods for cellular investigation with the miniaturization and multiplexing capabilities of microarrays, cell arrays are a versatile tool that can be useful in many cell-biological applications.

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Recent advances in genomics have challenged cell biologists with the exciting but daunting task of characterizing the *in vivo* functions of the products encoded by tens of thousands of novel genes. Although current methods such as transcriptional profiling [1], high-throughput mass spectrometry [2] and protein microarrays [3] allow the rapid systemic quantitation of mRNAs and proteins in biological samples, there are few high-throughput methods for the analysis of gene function in mammalian cells. In traditional approaches to *in vivo* gene analysis, DNA constructs that direct the overproduction of a gene product or inhibit its synthesis or function are expressed in cells. Using a range of assays, the effects of altering the level of the gene product on cellular physiology is measured and the function of the gene product inferred.

In a high-throughput adaptation of this experimental model, Ziauddin and Sabatini have recently described a novel cell-based microarray system for identifying the cellular functions of gene products [4]. Such 'cell microarrays' have features ('spots') that are clusters of mammalian cells expressing defined DNA constructs, which direct the increase or decrease in the function of specific gene products. By overexpressing cDNAs in expression vectors, investigators can use cell microarrays to identify genes involved in diverse cellular processes, such as apoptosis, cell adhesion and signal transduction. When implemented on a genome-wide scale, cell microarrays should be of broad usefulness to biologists studying a range of phenomena in mammalian cells. Here, we describe the general advantages of cell microarrays and their potential use in loss- and gain-of-function studies.

Microarrays of cells overexpressing defined cDNAs

In their pilot study of cell microarrays, Ziauddin and Sabatini implemented a method of overexpressing genes using cDNAs in plasmid expression vectors (Fig. 1) [4]. Using a commercially available microarrayer robot, nanoliter quantities of cDNA plasmids dissolved in an aqueous gelatin solution are printed onto the surfaces of microscope slides. After the slides are briefly exposed to a lipid transfection reagent, mammalian cells are added on top of the slides and cells landing on the printed areas become transfected with the printed plasmid. The cell microarrays are ready for assaying after a few days in culture, when a confluent lawn of cells forms on the surface of each slide. Each printed spot becomes a cluster of 30–80 transfected cells actively overexpressing a defined gene product and forming, in aggregate, a living microarray of transfected cell clusters within a background of untransfected cells.

To identify cDNAs whose expression affects a cellular property of interest, live cell clusters can be visualized in real time for cellular events such as protein translocation or calcium fluxes. Alternatively, the arrays can be fixed and cell clusters assayed with any technique that is compatible with cells growing on slides, such as immunofluorescence, *in situ* hybridization, chemiluminescence or autoradiography. For large-scale applications in which thousands of cell clusters need to be screened, fluorescent scanners can be used to provide a view of the entire array area. Alternatively, automated-stage microscopes can be used to acquire high-content images of each cell cluster at high resolution, allowing the visualization of subcellular phenotypes such as protein localization. These images can be patched together to form a detailed image of the entire array, as in Fig. 2, which shows various magnifications of a cell microarray with ~5000 cell clusters producing the green fluorescent protein.

The microarray platform has several advantages over well-based cell-assaying methods. The distance between adjacent features on a cell microarray is typically <400 μm, allowing a sample density that is nearly 200 times that of a 384-well plate. Arrayed on slides, the entire set of human genes can be expressed on a total area smaller than a single microtiter plate. Miniaturization has other advantages: only small quantities of potentially rare cell lines or biological samples are needed to assay many genes, affording economies of scale. Furthermore, cell microarrays are highly amenable to multiplexing because hundreds of arrays can be mass produced from a single set of source plates. For small arrays (on the order of 100 features), additional multiplexing can be achieved by printing duplicate arrays on the bottoms of microtiter plate wells to give 'arrays of arrays'. cDNAs printed in gelatin

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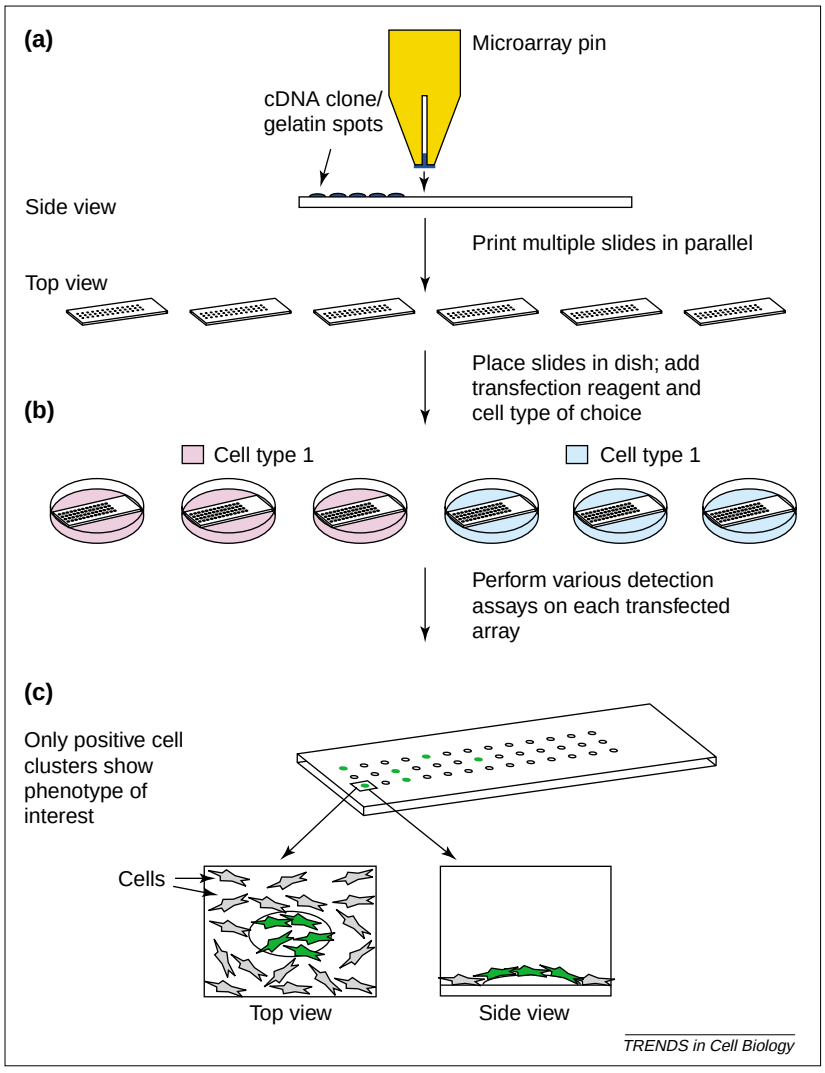

Fig. 1. The fabrication and analysis of cell microarrays. (a) cDNAs in gelatin are transferred to a solid surface using a robotic transfer device. Multiple slides can be printed in parallel. (b) After slides are overlaid with transfection reagent and placed in dishes, different cell types can be added. Only cells that land on areas printed with DNA become transfected. (c) Analysis can then be performed on each array of transfected cell clusters using a variety of assays, including high-content characterization of subcellular phenotypes.

are stable for months [4], allowing arrays to be stored and converted into cell microarrays only when needed. Finally, because thousands of cell clusters can be simultaneously assayed on well-less slides with minimal repetitive labor, the screening of very large sets of genes need not require significant automation.

Loss-of-function studies using plasmid-based siRNAs

A powerful strategy for probing the biological roles of gene products is the study of the phenotypes that result from the loss of gene function. Although knockouts or hypomorphic alleles in mammalian cells and animals can be created using homologous recombination or antisense oligonucleotides, the former is too time consuming and the latter too unreliable to be suitable for studying large sets of genes. For this reason, RNA interference (RNAi) has become attractive as a genetic tool for rapidly and systematically silencing genes of interest in mammalian cells.

RNAi, which is thought to have evolved as a defense mechanism against viruses harboring double-stranded RNA, is the process by which double-stranded RNA initiates the sequence-specific degradation of its homologous mRNA [5–7]. RNAi has been successfully applied to mammalian cells by the use of chemically synthesized small interfering RNAs (siRNAs) [8], which are 21-bp RNA duplexes with 3′ dinucleotide overhangs that mimic the cleavage products of the Dicer enzyme (Dicer is a nuclease that cleaves double-stranded RNA precursor molecules into small temporal RNAs and short interfering RNAs). Several studies have shown that synthetic siRNAs can transiently suppress the expression of specific genes in a wide range of mammalian cell lines [9].

Although it should be feasible to modify cell microarrays to use synthetic siRNAs directly, RNAi can be implemented on cell microarrays with current array-fabrication techniques, by using plasmid-based siRNA expression systems. Several recent reports have described mammalian RNA polymerase III promoter systems that can express functional siRNAs *in vivo* [10–13]. Some of the reported constructs produce a single small RNA transcript containing both sense and antisense siRNA sequences flanking a short spacer sequence [10,11]. This transcript is predicted to fold back into a hairpin structure, which can then be cleaved by Dicer into an active siRNA duplex. In other plasmid systems, sense and antisense strands are transcribed from separate RNA polymerase III promoters within the same construct and spontaneously hybridize *in vivo* [12,13]. Both types of siRNA plasmid system have demonstrated potent, sequence-specific gene silencing in mammalian cells.

By printing siRNA-plasmid constructs on slides in a manner analogous to cDNA constructs, it has been possible to create cell microarrays in which each cluster of cells is deficient in a defined gene product (R.Z. Wu and D.M. Sabatini, unpublished). Such ‘siRNA microarrays’ complement the functionality of overexpression microarrays by facilitating the systematic ‘knock-down’ of large numbers of genes using RNAi. Because similar printing and transfection procedures can be used to create both cDNA and plasmid-based-siRNA microarrays, these two categories of cell microarray can co-exist on the same array. Furthermore, the two species of constructs can be co-transfected to yield cell clusters that both overexpress and underexpress genes of interest in any combination.

Potential uses of cell microarrays

Cell microarrays have advantages over traditional methods of expression cloning because the identity of each cDNA is known from its array coordinates. This eliminates the need to isolate the cDNAs responsible for phenotypes of interest, a process that often requires substantial work involving fluorescence-activated cell sorting or repeated rounds of sib selection (in sib selection, clones conferring the phenotype of interest

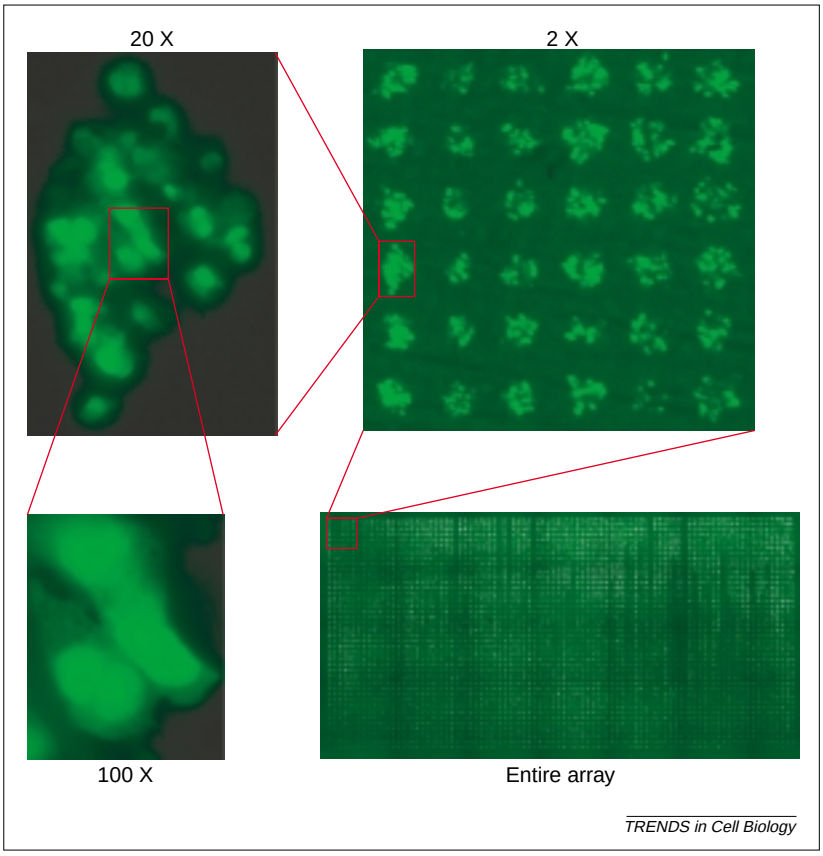

Fig. 2. Increasing magnifications of an array consisting of ~5000 cell clusters producing green fluorescent protein show different levels of detail that can be obtained from cell microarrays. At 100× magnification, subcellular features are clearly visible within cells of each transfected cluster.

are isolated from clone libraries by iteratively subdividing the clone population). To show that cell microarrays can be used as an expression-cloning system for the high-throughput identification of genes of biological interest, Ziauddin and Sabatini conducted a proof-of-principle screen of 192 epitope-tagged cDNA clones in expression vectors [4]. Clones were printed and transfected without prior knowledge of the identities of each gene and the resulting cell arrays were probed in a range of ways to identify genes that can affect particular cellular processes. By simple visual inspection of cell clusters, they could identify genes involved in cell adhesion (close juxtaposition of cell membranes) and apoptosis (cell fragmentation, confirmed by positive results for TdT-mediated dUTP nick end-labeling [14]). Using anti-phosphotyrosine antibodies, known tyrosine kinases and novel genes with similar functions were detected by looking for cells with elevated levels of phosphorylation. By examining high-resolution, high-magnification images of individual cells, nuclear and cytosolic subcellular locations could be distinguished for each epitope-tagged protein.

This preliminary study suggests the diversity of cellular phenomena that are accessible to investigation on cell microarrays. Live cells could be visualized for calcium waves, protein translocation, vesicular trafficking or the subcellular distribution of organelles. Genes that alter cytoskeletal structure can be identified by staining actin, tubulin or nuclear-lamina proteins. Similarly, genes that affect mechanisms of cell division can be detected by looking for variations in chromatin condensation and mitotic-spindle formation. Genes that affect cell proliferation can be detected using BrdU or tritiated-thymidine incorporation assays. These and other assays should be usable on cell microarrays to identify gene products that regulate cell cycle and cell growth.

Cell microarrays offer tremendous versatility because several experimental parameters can be varied in combinatorial fashion to give a nearly endless variety of potential applications. Variables include the substance arrayed, the cell line, the cell culture conditions (including agents that can be added to the growth medium), external stimuli such as ultraviolet irradiation and the assaying method. Arrays representing particular categories of genes can be produced, such as all known transcription factors or transmembrane receptors. Alternatively, large collections of mutants of a particular gene could be expressed on a cell microarray to elucidate structure–function relationships. The size of cell microarrays would also allow the combinatorial expression of sets of genes products known to interact in biological processes of interest. For example, cell microarrays expressing combinations of cDNA and siRNA constructs for candidate oncogenes and tumor-suppressor genes might be used to determine genetic criteria for tumor formation [15]. Also, studies of G-protein-coupled receptors (GPCRs) have shown that the overproduction of a GPCR and its interacting G-protein can be sufficient to produce an active cellular response in the absence of ligand [16]. Hence, an arrayed matrix of GPCRs produced with their G-proteins might be useful for signal-transduction studies. For studies of cell adhesion, heterologous cell types or extracellular matrix proteins can be added to the culture medium. Finally, cell microarrays should be adaptable to accommodate assays not yet directly compatible with slides. For example, studies of cell migration or cellular chemotaxis might use modified forms of the Boyden chamber or the three-dimensional-collagen-matrix assay.

Although cell-microarray technology has very many possible experimental uses, several technical limitations need to be addressed before its full potential can be fulfilled. The most challenging step in the implementation of pan-genome cell microarrays is the creation of the DNA constructs from which slides are printed. Currently, efforts are under way by both public and private sources to create collections of full-length cDNA clones for all human and mouse genes [17] [see Harvard Institute of Proteomics (Boston, MA, USA) http://www.hip.harvard.edu/research.html for details of HIP's cDNA clone repository]. With the rapid adoption of siRNA technology by the research community, there will probably soon be comparable efforts to produce large collections of siRNAs in both

synthetic and plasmid-based forms. A second limitation is that the lipid-based transfection methods currently used to deliver plasmids into cells vary substantially in efficiency for different cell lines. Because a minimal number of transfected cells per cluster is often required to attain a statistically accurate sampling of altered cells, in practice, only easily transfectable cell lines (e.g. HEK293T cells or COS fibroblasts) are used. For primary cell lines that are difficult to transfect, the use of alternative vectors such as retroviruses could be necessary.

Concluding remarks

Cell microarrays are a nascent technology and the full scope of applications that can benefit from their use is still under active investigation. For instance, cell microarrays could be adapted to screen other biologically active substances that affect cellular phenotype, such as small molecules and peptides. With their ability to assay many genes in parallel under a range of experimental conditions, cell microarrays complement existing high-throughput technologies while remaining compatible with a wide range of high-content cell-based assays. As cell-microarray technology matures, it could contribute to the integration of traditional cell-biological investigation and genomic data by providing a platform for the systematic investigation of genes affecting cellular processes of biological interest.

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