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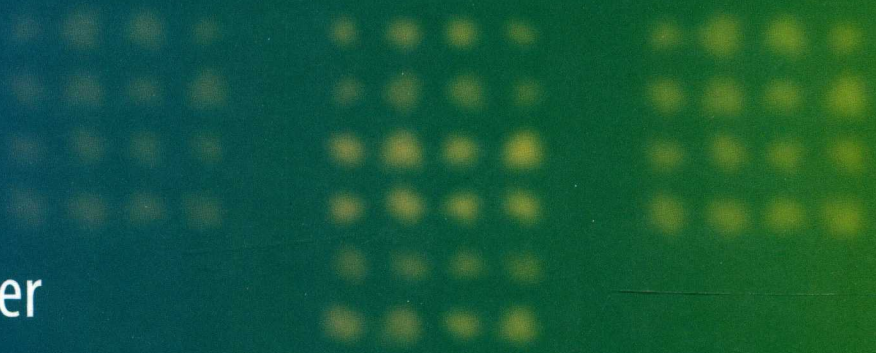

Ella Palmer
Editor

Cell-Based Microarrays

Methods and Protocols

Humana Press

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Edited by

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METHODS IN MOLECULAR BIOLOGY™

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Preface

Cell-based microarrays are a technique first described by the Sabatini group in 2001. They detail the printing of cDNA or siRNAs in a vector construct onto a coated glass slide using a robotic microarrayer. The vector constructs are transfected in defined areas within cells grown over the surface of the slide or microplate. These cell-based microarrays can be used for a variety of high-throughput, downstream functional assays.

Since their development in 2001, they have advanced significantly, and this book, intended for molecular biologists, geneticists, immunologists, and biochemists, covers many aspects of their evolution.

Chapter 1 gives a detailed overview of the whole subject area, including a discussion of the first paper describing the technique and detailed descriptions of the current work in overexpression, RNAi, antibody, and small-molecule cell-based microarrays. The overview also covers the adaptation of cell-based microarrays for a variety of cell types, advances in array surface chemistry and transfection efficiencies, and imaging of cell-based microarrays.

Chapters 2, **3**, **4**, **5**, and **6** describe protocols for overexpression arrays and downstream functional assays. In **Chapters 2** and **3**, Lai et al. and Palmer et al. provide clear protocols for array printing and transfection with standard HEK23T cells. In **Chapter 4**, Redmond et al. describe the use of a novel fluorescent reporter, and in **Chapters 5** and **6**, Hu et al. provide a protocol for high-throughput sub-cellular localization, and Erfle et al. include a protocol for high-throughput organelle imaging.

In **Chapter 7**, Niu et al. provide a protocol for a different cell type to standard mammalian cells: yeast cells (also see **Chapter 11** for blood cells).

Chapter 8 discusses a protocol for shRNAs using adenoviruses, and, in **Chapters 8** and **9**, Konrad et al. and Volkmer et al. both discuss the protocols for infectious disease research.

In **Chapters 10** and **11**, Lin et al. and Roupioz et al. provide protocols for antibody arrays and describe their use with different cell types such as blood.

Chapters 12, **13**, **14**, and **15** discuss protocols for increasing transfection efficiencies on cell-based microarrays. Yamaguchi et al and Hook et al, in **Chapters 12** and **13**, provide protocols for different slide coatings (also discussed in **Chapter 8**). Pernagallo et al., in **Chapter 14**, discuss the use of polymer arrays for functional tissue modelling, and Kato et al., in **Chapter 15**, discuss the use of electroporation to increase transfection efficiency.

In **Chapter 16**, Damoiseaux et al. provide a protocol discussing the development of cell-based array technology by use of microfluidic image cytometry for the analysis of small diagnostic samples with few cells.

Together, the chapters provide an easy-to-use, up-to-date, and comprehensive set of protocols on every aspect of cell-based microarrays.

Ella Palmer

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Contents

Chapter 1

Cell-Based Microarrays: Overview

Ella Palmer

Abstract

Cell-based microarrays were first described by Ziauddin and Sabatini in 2001 as a novel method for performing high-throughput screens of gene function. They reported a technique whereby expression vectors containing the open reading frame (ORF) of human genes were printed onto glass microscope slides to form a microarray. Transfection reagents were added pre- or post-spotting and cells grown over the surface of the array. They demonstrated that cells growing in the immediate vicinity of the expression vectors underwent 'reverse transfection' and that subsequent alterations in cell function could then be detected by secondary assays performed on the array. Subsequent publications have adapted the technique to a variety of applications and have also shown that the approach works when arrays are fabricated using siRNAs and compounds. The potential of this method for performing analyses of gene function and identification of novel therapeutic agents has now been clearly demonstrated. Current efforts are focused on improving and harnessing this technology for high-throughput screening applications.

Key words: Cell-based microarray, reverse transfection, RNAi, siRNA.

1. Introduction

The utility of the microarray format was first effectively demonstrated for gene expression profiling (1, 2). The availability of whole genome sequences, a growing catalogue of genes, better equipment, resources and the increased analytical power of bioinformatic tools, has fuelled the development and application of microarrays for gene expression analysis. As a result, high-throughput, semi-quantitative analyses of gene expression using this platform are now routine in many laboratories. The desirable characteristics of the microarray format platform also led to a diversification in the use of microarray technology in areas other than the study of gene expression. Over the last years, many variations of the microarray format have evolved, including arrays for performing comparative genomic hybridisations (3, 4),

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genotyping (5, 6) and DNA methylation (7), as well as for detecting DNA–protein (8), protein–protein (9, 10), carbohydrate–protein (11) and receptor–ligand interactions (12). Also in the last few years, extensive collections of full-length cDNA resources have been created for key model species such as C. elegans (13) and D. melanogaster (14) and genome-wide clone sets are also comprehensive for human and mouse (16–18). Likewise, genome-wide RNAi reagents are also available for a range of species (19–21), paving the way for cell-based microarray technology. *See* **Fig. 1.1** for an overview of the cell-based transfection methodology.

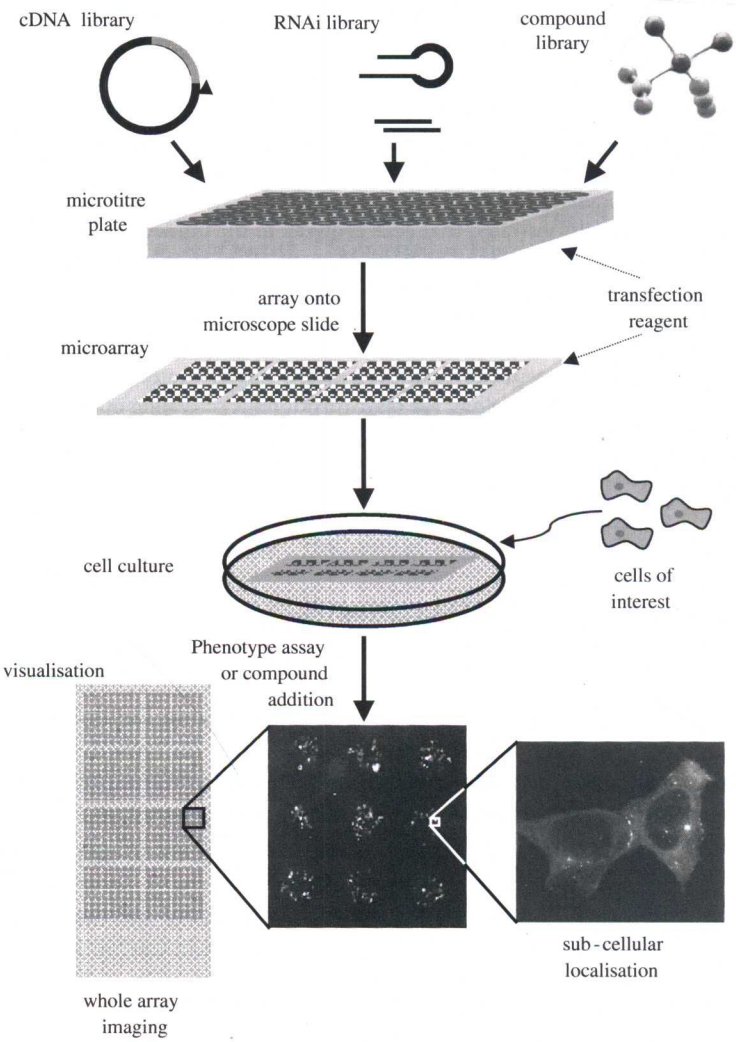

Fig. 1.1. Cell-based microarray methodology. Plasmids are prepared from cDNA or RNAi clone expression libraries, alternatively compound libraries can be used directly. Transfection reagent used to transport DNA/RNA into the cell can be either added directly to the plates prior to printing or used to treat the array just prior to cell culture. For compound screening, a surface chemistry must be used that is compatible with the retention and controlled release of the compounds. After printing, microarrays are cultured with cells until a confluent monolayer covers the surface of the slide. If reagents are tagged, transfection events can be visualised at the slide or cellular level, or the cells stained to detect cells with altered phenotype.

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2. Developments in Cell-Based Microarrays

2.1. Contents and Conclusions of the First Cell-Based Microarray Paper

The first paper describing cell-based microarrays powerfully illustrated the salient features of the technology (22). In initial studies, the Sabatini group printed 192 genes in a V5-epitope-tagged expression vector. The arrays were probed with Cy3-labelled anti-V5 antibody as a transfection control and then with Cy3-labelled anti-phosphotyrosine antibody. Six genes were found to have increased phosphotyrosine activity, five of which were known tyrosine kinase proteins and the sixth gene encoded a protein of unknown function. The cells were also observed for abnormal morphologies, the apoptosis-inducing protein, TNFRSF10B was associated with cells that appeared fragmented and was positive for the TdT-mediated dUTP nick-end labelling reaction. As well, cells growing over the cell surface protein CD36, were found to be in close contact. Sub-cellular localisation studies were also performed on the arrays, many matched localisations that had already been described for the proteins and sub-cellular localisations were also demonstrated for proteins that had not been studied previously. The authors concluded that the advantages of the cell-based microarray technology were that the proteins were translated within the environment of a mammalian cell and were therefore likely to fold correctly and undergo molecular interactions similar to the native protein. Furthermore, the assays were quick compared to other over-expression strategies; the signal was concentrated in small well-defined areas and the arrays could be used to screen live cells. Finally, the arrays were compact, easy to handle, economical and in principle the entire set of human genes could be printed on a small number of slides.

2.2. Downstream Functional Assay-Based Microarrays

2.2.1. Over-Expression Cell-Based Microarrays

Over-expression arrays of cDNAs expressing the gene of choice, as published by the Sabatini group, were the first format of cell-based microarrays, prior to the development of RNAi, and a number of groups still use arrays in this format for functional studies.

In initial studies, Webb et al. co-transfected expression vectors containing the serum response element (SRE) reporter (which activates MAPK and JNK pathways) coupled to GFP with five known upstream activators of SRE. Each of the five SRE activators generated patches of cells with a significantly higher GFP signal than the control vector demonstrating that members of

signalling pathway initiation can be determined using cell-based transfection microarrays (23). Mishina et al. demonstrated that cell-based transfection arrays could be used to identify novel therapeutic targets. G-protein coupled receptors (GPCRs) have a role in mediating signalling in cellular metabolism and are therefore prime candidates for drug targets. Nine hundred GPCRs were printed onto a 96-well plate. GPCR agonists and a fluorescent calcium indicator dye were added and 15 positive interactions were discovered (24). In studies using the cAMP-response element (CRE) activated by cAMP-dependent protein kinase (PKA) coupled to GFP, GFP levels were also used to detect genes important in activating this pathway (25).

Since the initial studies, Yamauchi et al., using the vascular endothelial growth factor receptor (FLK1) promoter attached to GFP on mouse embryonic stem cells lines and expressing a combination of transcription factors relevant to differentiation, demonstrated that the level of GFP was an indicator of differentiation, and they discovered a number of potent activators of differentiation (26). Hu et al. have undertaken a high-throughput screen of the sub-cellular localisation of genes on chromosome 21 using organelle markers (27).

Various studies on apoptosis have been undertaken; Ziauddin and Sabatini used a nick-mediated TUNEL assay to identify any pro-apoptotic effects of over-expression (22), and in a study by Palmer et al., TUNEL and caspase 3 assays were used to determine pro-apoptotic genes (28); Mannherz et al. also undertook a screen for pro-apoptotic genes using EYFP attached to the genes as a readout for apoptotic bodies (29).

2.2.2. RNAi Cell-Based Microarrays

RNA interference (RNAi) is an enormously powerful tool for investigating gene function. The process was first discovered in *Caenorhabditis elegans* (30); it was demonstrated that double stranded RNA (dsRNA) can direct the silencing of gene targets in a sequence-specific manner. In invertebrates such as *C. elegans* and *Drosophila melanogaster* (31), when long dsRNA is introduced into their cells it is processed by a dsRNA-specific endonuclease, Dicer (32), into short interfering RNAs (siRNAs) 21–24 nucleotides in length. siRNAs are then incorporated into an RNA-induced silencing complex (RISC) which cleaves mRNAs homologous to the dsRNA originally introduced (33, 34). In mammalian cells, dsRNAs longer than 30 bp trigger the antiviral/interferon pathways, which result in global shutdown of protein synthesis (35). RNAi-mediated gene silencing is however possible in mammalian cells either by delivery of chemically synthesised short (less than 30 bp) double stranded siRNA molecules (36) or by expression of short hairpin RNAs (shRNAs) bearing fold-back stem–loop structures (37).

In initial studies on Drosophila cells, Sabatini's group developed a prototype microarray with 384 different dsRNAs against the majority of tyrosine kinases annotated in the *D. melanogaster* genome and all predicted serine/threonine protein phosphatases. The nucleus and actin were stained and the arrays scanned using automated microscopy and image analysis software, which quantified the number and size of nuclei in cells growing over each dsRNA spot. Forty-four RNAis were identified that resulted in features with at least two standard deviations below the mean number of nuclei as compared to control dsRNA. These, therefore, represented genes likely to be essential for normal cell proliferation, survival or adhesion. The group also found that it was possible to knockdown two genes simultaneously, which opened up the possibility of performing large-scale screens for synthetic or epistatic genetic relationships (38).

Since then, several groups have described RNAi studies in mammalian cells coupled with cell-based microarray technology. A commonly used positive control is to co-transfect GFP expression vectors with vectors containing siRNAs or shRNAs targeting GFP and demonstrate decreased GFP expression (39, 40). Silva et al. printed an array of eight different shRNAs to EG5, a gene involved in spindle formation. Using cells expressing a tubulin–GFP fusion protein, they were able to show that cells growing over two of the shRNAs had spindle defects (41). Erfle et al. optimised a two-step procedure, where the transfection reagent and siRNA are mixed before being printed onto the array. They used siRNAs to knockdown the expression of three genes in the secretory pathway, *COP1*, *GM130* and *Sec31*, and also used a marker assay to show that the *COP1* gene was dysfunctional (42). A further group developed a cell-based array system for screening RNAi reagents, as not all siRNAs/shRNAs selected for targeting a gene result in efficient gene silencing. They printed MyoD, Lamin A/C and P53 siRNAs, and shRNAs onto a slide and then added cells plus expression vectors containing the target gene attached to GFP for visualisation. They were able to gauge the efficacy with which the siRNA and shRNA knocked down the target gene by measuring the levels of GFP fluorescence (43).

More recently, with the availability of genome-wide RNAi reagents for a range of species (19–21), a number of groups have reported high-throughput RNAi screens using cell-based micorarrays.

Neumann et al. in studies led by Pepperkok, developed an array-automated platform for high-content RNAi screening using time-lapse fluorescence microscopy of live HeLa cells expressing histone–GFP to determine chromosome segregation and structure using siRNA (44–46). More recently, Walter et al. have

described a high-throughput RNAi screen of chromosome phenotypes (47).

2.2.3. Antibody Cell-Based Microarrays

A variation on the theme of over-expression arrays was the development of antibody cell-based microarrays. The potential to screen single-chain antibody fragments using cell-based microarrays was first demonstrated by Delehanty et al. They expressed a wild-type fluorescein antibody fragment and three mutants on HEK293T cell membranes on a cell-based microarray and demonstrated that fluorescein had a higher affinity for the wild-type fluorescein antibody fragment than the antibody mutants (48). Suranati et al. and Roupioz et al. have demonstrated the use of antibody arrays for the detection of blood cells, in particular lymphocytes on cell-based micorarrays (49, 50).

2.2.4. Drug Screening on Cell-Based Microarrays

The groups of Sabatini and Stockwell have explored the possibility of combining RNAi and compound screens on cell-based microarrays. To facilitate the retention and slow diffusion of arrayed compounds, they first printed discs of a polymer matrix onto the slide. They then printed 70 known active compounds in triplicate at three concentrations on top of the polymer discs. Seven siRNAs that knocked down proteins involved in cell death, P53, PTEN, MDM2, EGFR, TSC2, BCL2 and BRCA1 were transfected into the cells growing over the bioactive compounds. Clusters of cells associated with three of the compounds were observed to change in density, indicating that the drugs were counteracting the effect of the genes that had been knocked down (51).

2.3. Adaptation of Cell-Based Microarrays for a Variety of Cell Types

The initial studies on cell-based microarrays were carried out in HEK293T cells, as they are an easy to transfect cell line. However, the Sabatini group sought to circumvent this issue by printing lentiviruses onto arrays. Lentiviruses have a high take-up rate in a variety of cells including primary cells, and the group showed that lentiviruses pseudotyped as vesicular stomatitis virus glycoprotein were taken up by primary human BJ fibroblasts and primary mouse dendritic cells as well as HeLa, A549, HEK-293T and DU145 cells (52).

Other groups also developed systems for less easy to transfect cells. Oehmig et al. demonstrated the use of adenovirus for cell-based microarrays; the transfection step is not necessary when using adenovirus and this enables less easily transfected cells to take up the gene of interest. The group demonstrated the approach by the transfection of primary human umbilical vein cells (HUVEC) (53).

Narayanaswamy et al. demonstrated the use of cell-based microarrays with yeast cells; they applied 4,800 yeast deletion

strains to arrays to establish genes controlling the response of yeast cells to mating pheromone (54).

Kato et al. coated the surface of a glass culture dish with a cell membrane anchoring reagent, biocompatible anchor for membrane (BAM), with an oleyl chain as a lipid anchor. They demonstrated that non-adherent human erythroleukemic K562 cells and liposomes could attach to the BAM (55, 56). Another approach by Yoshikawa et al. was to use surface-deposited fibronectin on the surface of the microarray, which enhanced transfection efficiency and allowed transfection of primary human mesenchymal stem cells (57).

2.4. Advances in Array Surface Chemistry and Transfection Efficiencies

A number of groups have tried to improve transfection efficiency on cell-based arrays. One group has developed slides with cationic polymers on the surface, so that cells can be added without the need for a transfection reagent (58). A further group developed a surface transfection and expression protocol (STEP) with recombinant proteins designed to enhance transfection when in a complex with expression vector DNAs prior to spotting on glass slides (25). Kato et al., as previously mentioned, coated the surface of a glass culture dish with a biocompatible anchor for membrane (BAM) (59). In a further study by the same group, Kato et al. demonstrated that a liposome:plasmid expressing GFP mix spotted onto the BAM surface was capable of transfecting cells. They showed that an RNAi to GFP caused the knockdown of GFP in a non-adherent K562 cell line stably expressing GFP (60). Delehanty et al. compared glass slides coated with different substrates to determine which gave the best transfection efficiencies. They compared polystyrene, two types of aminosilane coating and two types of polylysine-coated slides. They concluded that spot size was proportional to substrate hydrophobicity, i.e. the polylysine slides were the least hydrophobic and had the largest spot size. However, the transfection rates were highest with the most hydrophobic coating and polystyrene and lowest on the polylysine slides (61). Yamauchi et al. used micro-patterned, self-assembled monolayers (SAM) of alkanethiols formed on a gold-evaporated glass plate for cell-based microarrays. They demonstrated that by repeating layers of plasmid DNA and liposome:plasmid DNA mixes, improved transfection efficiencies could be achieved (62). How et al. have described the efficient formation of complexes between plasmid DNA and dendrimers on cell-based microarrays that transfect efficiently into the cell after the addition of lipoplexes (63). Isalan et al. achieved transfection in a variety of cell lines in a cell-based microarray format using magnetically defined positions and PCR product-coated paramagnetic beads (64). To increase transfection efficiency further, Yamauchi et al. described an electroporation method in which electric pulses were used to detach plasmids from the microarray surface to introduce

them into cells grown on the microarray (65). More recently the Iwata group have developed this method for siRNA (66) and have also prolonged the durability of the electroporation microarrays by adding saccharides to nucleic acids (67).

Another approach by Yoshikawa et al. was to use surface-deposited fibronectin on the surface of the microarray, which enhanced transfection efficiency (57).

Hook et al. described a high-density poly (ethylene glycol) coating on glass slides with phenylazide-modified polymers and irradiation by UV to result in cross-linking of the polymer spots to the surface and printing of plasmids for strong attachment; they demonstrated that this coating provides a very adherent substrate for DNA, protein and cell-based arrays (68).

Pernagallo et al. have investigated the use of polymer arrays which allow non-adherent cell lines to adhere and proliferate; they demonstrated that K562 human erythroleukemic cells, which normally grow in suspension, adhered and proliferated on several different polymers coated on slides for cell-based microarrays (69).

2.5. Imaging of Cell-Based Microarrays

High-throughput imaging systems are necessary to systematically record cell-based microarray readouts for fixed and live imaging, and methods are being developed for cell-based microarrays and the Pepperkok group is the forefront of the development of high-throughput RNAi screens (44–47).

A cell image analysis software called CellProfiler has been developed and is freely available to allow automatic quantitative measurements to be made from thousands of images (70).

3. Conclusions

Cell-based microarrays are very powerful analysis tools. Their utility in exploring gene function through both over- and knock-down expression studies has now been clearly demonstrated due to libraries of siRNA and cDNAs for different organisms becoming comprehensive. Robust methods for attachment of cDNA to glass slides have been implemented, transfection techniques have been improved through electroporation and adeno- and lentivirus work allowing a variety of different types of cells such as non-adherent stem cells to be analysed in a high-throughput fashion. Automated platforms for cell imaging have been developed and image software is freely available. The compact format of cell-based arrays and the ability to carry out thousands of independent assays in parallel with the minimum reagent requirements make the cell-based microarray approach a very attractive proposition where routine high-throughput screening is required.

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