

## Technology for microarray analysis of gene expression

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The past year has demonstrated the versatility of microarrays for the analysis of whole model-organism genomes and has seen the development of chips to measure the expression of 40,000 human genes. Microarray technology has also become considerably more robust and sensitive. Technology enhancements include the use of noncontact printing methods, improved 2-color sample preparation, and statistically based software for data analysis.

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### Introduction

Examination of gene expression levels can provide insights into the temporal changes that occur in induced cells and into the molecular differences between different cells or tissue samples. Microarrays detect gene expression levels in parallel by measuring the hybridization of mRNA to many thousands of genes immobilized on a glass surface (the ‘chip’), providing a sensitive and high-throughput method well suited to take advantage of the sequence and clones produced in genome sequencing efforts. The technology of microarrays has advanced from reverse Northern blots on filters detected using radioactive probes to a highly technical field involving miniaturized synthesis, multi-color fluorescent labelling, and database driven sample and data management. Recently, whole genomes have been analyzed [1•,2•] and specific gene families have been studied [3•]. Microarray analyses typically follow the steps of gene selection, microarray synthesis, sample preparation, array hybridization, detection, and data analysis with appropriate controls required for each. Each of the steps in a microarray analysis is considered in this review. (Please note that in this review we define the genes attached to the chip as ‘targets’, the complex sample in solution as the ‘probe’, and each individual DNA spot on a microarray as an ‘element’.)

### Oligonucleotide arrays

When gene sequence information is available, oligonucleotides can be synthesized to hybridize specifically to each gene. Oligonucleotides can be synthesized *in situ*, directly on the surface of the chip, or can be pre-synthesized and then deposited on to the chip. This approach obviates the need for management of large clone libraries as it is sequence information driven. This approach is particularly suited to

analyzing the expression profiles of organisms with completely sequenced genomes [1•] as all predicted genes can be analyzed.

In the *in situ* synthesis methods, oligonucleotides are built directly on the solid support. Affymetrix (Santa Clara, CA) generates arrays using a photolithographic approach where a mercury lamp is used to shine light through a photolithographic mask on to the surface of the chip, selectively removing photo-labile deprotecting groups from the growing oligonucleotide chain [4] in a stepwise fashion to create oligonucleotides. Individual elements can be as small as 10  $\mu\text{m}$ , with further miniaturization being embodied using non-linear semiconductor photore-sist technology [5]. No oligonucleotide purification is possible and, as the stepwise synthesis yield is only about 95%, oligonucleotides no longer than 25 bases can be synthesized. For gene expression analyses, this can dramatically reduce sensitivity and specificity, although specificity can be addressed by comparing perfectly complementary targets with single base mismatched sequences, and by synthesizing many oligonucleotides for each gene to be analyzed [1•]. Oligonucleotides can also be synthesized *in situ* by robotically delivering the appropriate phosphoramidite (base synthesis reagent) to specified locations on the array in successive rounds of synthesis, thereby creating the desired sequence at each array element. Arrays of oligonucleotides may be synthesized [6,P1•] with a high step-wise yield. The use of a hydrophobic surface with hydrophilic wells [P2] helps in the creation of uniform spots during synthesis. This method is highly flexible as from one run to the next, the robot can be easily configured to synthesize different oligonucleotide sequences at each array element. For this reason, it is anticipated that this synthesis method will become very widespread for creating microarrays for research use. Southern [P3] describes a method that deprotects the growing oligonucleotide chains in chosen locations using miniature arrays of electrodes. Reaction of the array with a base synthesis reagent causes addition of the base only to the oligonucleotide chains that were previously deprotected. Stepwise deprotection and base addition allows the chosen sequences to be synthesized at each array element. Other methods for *in situ* synthesis [7–9] allow the creation of arrays of oligonucleotides of different lengths that are likely to hybridize to a single known contiguous sequence and are useful for finding DNA polymorphisms or for selection of anti-sense reagents [10]. These methods are not useful for gene expression monitoring due to the inability of the methods to create independent oligonucleotide sequences at each array element.

Printing of oligonucleotides on to 20  $\mu\text{m}$  thick gels [11] creates arrays with 40  $\mu\text{m}$  elements. The high loading capacity of the gel relative to glass results in enhanced hybridization kinetics. The oligonucleotides must diffuse into the gel matrix and so only short oligonucleotides can be used. Nanogen (San Diego, CA) uses controlled electric fields to immobilize presynthesized, biotinylated oligonucleotides on microelectrodes [12] and have integrated this with sample preparation [13].

The printing methods described below can be used to deliver pre-synthesized oligonucleotides. Aminophenyl-trimethoxysilane treated glass surfaces can bind 5' amino-modified oligonucleotides using a homobifunctional crosslinker to attach the aminated oligonucleotide to the aminated glass [14], or by reacting the oligonucleotide with succinic anhydride and attaching the resulting carboxylate group via an ethyldimethylaminopropylcarbodiimide-mediated coupling reaction [15]. 5' phosphate-modified oligonucleotides, reacted with imidazole to produce a 5'-phosphorimidazolidine, can bind to the surface amino groups via a phosphoramidate linkage [16]. The linker must be long enough to eliminate steric hinderance caused by the solid surface to ensure the efficiency of the subsequent hybridization reaction. Shchepinov *et al.* [17] have demonstrated that an optimal spacer length is at least 40 atoms long and can increase hybridization yields by 150-fold. The chemistry should produce no non-specific binding of labeled probes to the surface.

### Clone printing

cDNA clones can be used on microarrays, obviating the need for up front, expensive DNA sequencing of uninteresting clones. Compared to oligonucleotide arrays, cDNA arrays have the advantages of being less susceptible to hybridization changes caused by gene polymorphisms, and of potentially improved sensitivity due to the availability of more target sequence to which to hybridize. A relatively non-stringent subtraction approach can be used to normalize the cDNA library, prior to generating the targets [18]. This has the advantage of reducing representation of the more abundant genes, thereby allowing a more diverse set of genes to be analyzed on the microarray. Incyte (Palo Alto, CA) has produced a set of four microarrays containing 40,000 clones, derived from the IMAGE sequencing project [19]. Although this approach provides maximal information content, clone mix-ups between sequencing and microarray creation mean that 10–30% of clones may be incorrectly identified. This means that interesting clones must be sequenced again to be assured of their identity. Considerable effort is being put into providing sequence-verified cDNA microarrays, where more attention has been paid to ensure clone identity between sequencing and microarray creation.

A considerable disadvantage of the printing approach versus the *in situ* synthesis approach is that amplification of the DNA by PCR must be carried out for each gene of

interest. This is relatively straightforward when vector-specific primers can be used to amplify cDNA clones but when a clone is not in hand, or the clone contains undesirable repeat elements, PCR from genomic DNA or RT-PCR from mRNA with gene-specific primers must be carried out. Approaches to reduce the cost of oligonucleotide synthesis [20] and improve success of PCR [21] enable this to be done on a large scale.

Piezoelectric pipetting for microarray creation has been reported [22] and an arraying robot which utilizes four printing tips has been constructed [23]. Molecular Dynamics (Sunnyvale, CA) has developed a system for array printing based on capillary action printing pens [P4]. This arrayer has an automated microplate retrieval system to allow extended, unmanned operation, and bar code reading capability to check that the correct microplates have been loaded (Carmack C, Albertsen H: Application of High-Density DNA MicroArray Technology. Presented at Genome Mapping, Sequencing and Biology Meeting, Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 14 May 1998). An adaptation of a commercially available laboratory robot has also been described [24].

Attachment of cDNA targets to the glass is usually carried out using non-covalent charge interactions — this does not create an irreversible attachment of the DNA targets to the glass surface, resulting in decreased sensitivity due to loss of the target from the glass. Also, during hybridization, detached target molecules bind to labelled probe molecules in solution, preventing them from hybridizing to the complementary target molecules attached at the microarray element. Glass slides can be coated with poly-L-lysine on to which PCR products resuspended in 3X SSC are printed [2]. A boiling step denatures the target and removes loosely bound molecules. Using this method, mRNA species present at a ratio of 1:100,000 (w/w) in the complex sample, have been detected. Non-covalent attachment to aminopropylethoxysilane-treated slides can be carried out by resuspending the PCR product in 6 M sodium thiocyanate [25] (Penn SG, Dolnik V, Johnston RF: Interaction Of Nucleic Acids And Polysaccharides, For Use In Array Technologies. Hilton Head Genome Sequencing and Mapping Conference 1997). Schena *et al.* [26] have reported a covalent attachment scheme. Amino-modified PCR products are arrayed onto silylated slides containing surface aldehyde groups followed by treatment with sodium borohydride to reduce the imine to the chemically stable amine, allowing detection of 1 part in 500,000 (w/w).

We have seen an increase of around twofold in signal intensity when single stranded targets, prepared using asymmetric PCR [27] or selective digestion [28], are used compared to double stranded, denatured targets (Mazumder A, unpublished data). This improvement is probably due to an increase in the amount of the probe-specific strand attached to the array and prevention of re-annealing of the target PCR products after printing.

This is at the expense of more sample preparation effort and the need to ensure that the strand of the cDNA complementary to the probe is selected.

### Other array methods

Array encoding techniques other than the positional encoding used for microarrays are possible [29]. Luminex (Austin, TX) describe a method for encoding microspheres according to their fluorescence [30\*]. A different oligonucleotide can be covalently attached to each type of microsphere. The microspheres are then mixed together and hybridized to a fluorescently labelled sample. Reading in a flow cytometer allows each microsphere to be identified and the corresponding hybridization signal to be read [P5]. 512 different assays can be analyzed in a single well in a few seconds [31]. Others report success in synthesis and hybridization of oligonucleotides on microspheres [32,33]. Single microspheres immobilized in wells etched from optical fibre bundles [34\*] have the potential for array elements to be in the sub-micrometer size range.

Kononen *et al.* [35\*\*] describe a method for attaching up to 1,000 tissue sections to a slide. Standard fluorescent *in situ* hybridization techniques are used to probe each of the sections for genomic DNA deletions and amplifications, and to test for mRNA expression differences. Significantly, the high resolution techniques used can also differentiate between different types of cells in the same tumor biopsy.

### Sample processing

A critical step in probe preparation is the isolation of mRNA from the cells or tissues of interest. As the expression pattern in a cell can change quite rapidly following perturbations, such as heat shock or activation with lipopolysaccharide or other reagents, it is essential that the material is snap-frozen in the desired state, and all subsequent steps for isolation of the mRNA be carried out with the utmost care and speed. Laser capture microdissection [36] has been used to separate single cells of interest from the surrounding tissue to improve sample purity.

Primary visualization using fluorescently-labeled RNA or cDNA as the probe is the most common technique. Biotinylated RNA [37] may also be visualized in a second step using streptavidin (which binds biotin) conjugated to a fluorophore such as phycoerythrin. cDNA probes are prepared from the polyA RNA by reverse transcription using murine leukemia virus reverse transcriptase, with incorporation of the fluorophores as dye-labelled nucleotides; for increased signal, the primers used can also be labelled with the same dye (S Balasubramanian, unpublished data).

Even the most sensitive methods require about 1–2 µg of clean polyA+ mRNA, which can be hard to obtain when working with single cells or human biopsy material. Linear sample amplification can be carried out by *in vitro* transcription [38] yielding 20–50-fold amplification with reports that there are no biases introduced by this process

[1\*\*], although this has not been rigorously studied. Primer extension preamplification is a PCR-based method originally developed for amplification of genomic sequences by repeated extensions using a random 15-mer [39]. The method is applicable to gene expression by first converting the mRNA to first strand cDNA and then using PCR with the random primer for amplification [40].

Hybridization of the labelled probe is ideally linear (i.e. proportional to the amount of probe), sensitive so that low abundance genes are detected, and specific so that targets hybridize only to the desired gene in the complex probe mixture. Consideration of the melting temperature equation for polynucleotides [41] dictates that reactions performed in 4-5X SSC are performed at 60–65°C. Hybridization reactions employing 50% formamide are typically performed at 42°C as the duplex melting temperature is lowered by 0.63°C per percentage formamide for DNA–DNA hybrids. This ensures adequate stringency and reduces the temperature induced detachment of non-covalently attached target molecules. The fluorescent dyes in the probes also lower the duplex melting temperature so hybridization and washing conditions need not be as stringent as with non-fluorescently labelled probes. Salmon sperm DNA, poly[dA], tRNA, SDS, and Cot1 DNA are added to the hybridization to eliminate nonspecific hybridization due to repetitive sequences [42].

The hybridization yield depends on the concentrations of immobilized target and probe so the intensity of the hybridization signal can be used to estimate the relative abundance of the transcript. Diffusion rates at solid surfaces are lower than in solution [43], so a particular cDNA may not be able to diffuse to the location where its cognate target is arrayed. Mixing or recirculating the hybridization reaction in a sealed hybridization chamber would ensure a homogeneous probe concentration rather than concentration gradients, which would skew the hybridization signals.

### Detection

The most common detection method is laser-induced fluorescence, detected using confocal optics. One scanner reads a 1 cm square chip in a single color at a resolution of 11.25 µm in 15 minutes [37]. General Scanning (Watertown, MA) produce a scanner with dual excitation lasers (GHeNe at 543 nm, and RHeNe at 633 nm) that has been reported for use in genomic mismatch scanning [44]. Molecular Dynamics (Sunnyvale, CA) produce a dual color (RHeNe at 633 nm and doubled Nd:YAG at 532 nm excitation) scanner with a microarray feeder and barcode reader to allow unmanned scanning with microarray identification of up to 12 slides.

Unlabelled probe can be detected using surface plasmon resonance [45], although sensitivity and array density are currently poor. Hybridization can be monitored in real time using a charge-coupled device imaging camera [11]. Synthesis of arrays on optical fibre bundles allows easy and

sensitive reading [46]. In another method, use of the evanescent wave effect excites only fluorophores that are bound to the surface, thereby allowing real time hybridization detection on microarrays without washing [47].

## Controls

As microarrays allow the simultaneous analysis of many thousands of data points, use of some of these for controls is advisable. Messenger RNA can be synthesized from cDNA clones by *in vitro* transcription and spiked into each sample. This mRNA should be from a gene that hybridizes only to its complement on the microarray and is not present in the sample: genes from different organisms are ideal for this [26]. If several different genes are spiked into the sample at different concentrations, monitoring of these genes provides confidence that sensitivity and stringency are adequate and allows these spots to be used as sample to sample normalizing factors. Spot to spot variability is most marked in cDNA printing. Simple comparison between two samples using 2-color analysis removes this factor so long as spectrally distinct dyes, such as Cy-3 and Cy-5 (Amersham Plc, Amersham, UK), with equal intensities are used [2\*\*]. When more complex analyses are required over more than two samples, 2-color analysis can be used by including a dye-labelled, vector-specific oligonucleotide or by selecting one sample as the reference to which all others are compared. High confidence in spot to spot reproducibility allows single color analyses to be carried out with confidence. Correction for cross hybridization can be carried out using oligonucleotide arrays by synthesizing single base mismatched probes and verifying that these do not hybridize to the sample [1\*\*].

## Software

Image analysis software to extract the fluorescence data from the scanned image is essential. This software ideally will identify array elements, subtract background, deconvolute multi-color images, flag or remove artifacts, verify that controls have performed properly, and normalize the signals [48\*]. The difficulty of carrying out this task is dependent on the quality of the printing. The spotting pattern and spot shape of *in situ* synthesized oligonucleotide arrays are very regular, allowing simple registration and application of a mask to identify array elements. For printed arrays this is more difficult due to uneven spot morphology caused by the printing method used, and irregular spacing caused by mechanical positioning errors. In 2-color analyses, where two samples are being compared, the software identifies genes that are significantly changed with respect to the normal variation [48\*]. Other software packages are available: LifeArray and GEMTools from Incyte (Palo Alto, CA) integrate image analysis, sample database, and data analysis; Bio-Image (Ann Arbor, MI) provide a modification of their filter analysis software for microarray analysis; NIH Image, a public domain program (developed at the US National Institutes of Health and available on the Internet at

<http://rsb.info.nih.gov/nih-image/>), can be used to extract spots and correct for background.

A database that tracks genes, samples, and results is also required for all but the most simple analyses. Querying of this database can be done using tools that display data in a graphical form, for example, a bar-chart to identify obvious effects. Use of techniques developed for trend analysis in large financial datasets may allow more subtle coordinated expression changes to be identified [49], as will mathematical clustering methods for the identification of genes expressed in similar patterns under changing conditions [50\*].

## Conclusion

Microarray technology is the method of choice for quantitative analysis of the expression levels of many thousands of genes in parallel. Its use in biological systems will identify genes involved in many disease and developmental processes. Enforcement of key patents will protect the innovations made by the inventors. When manufactured in volume, microarrays have the potential to be very cheap — a situation that will hopefully not be hindered by the patent holders. Other developments in multiplexed analysis technology may provide an alternative platform for simultaneous analysis of gene expression.

## Acknowledgements

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