



Microarray Technologies 2003 – An Overview

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Gene expression analysis has progressed in a relatively short time from the traditional 'one gene at a time' analysis to detailed surveys of complete genomes. Professor Ed Southern is globally recognized for his key insight over 25 years ago that labeled nucleic acid molecules that could be used to interrogate genetic material to determine DNA sequence [1,2]. The Southern blot technique and the many derivatives thereof have led to vast research programs in many different fields of biology, diagnostics and medicine. In the 1980s, with the advent of recombinant cloning, researchers extended this powerful technique to the screening of genomic and cDNA libraries. Filter arrays were generated from bacterial colonies on membrane filters and these initial efforts represented the earliest DNA arrays. Subsequent improvements in laboratory automation enabled the creation of high-density filter arrays containing human, mouse, rat, and yeast genes, which are currently available from ResGen Invitrogen Corporation (Carlsbad, CA, USA), BD Clontech (Palo Alto, CA, USA), and others. As these high-density microarrays were emerging, two different microarray based technologies arose, DNA microarrays from Pat Brown and colleagues at Stanford University (Palo Alto, CA, USA), and GeneChips from Affymetrix (Santa Clara, CA, USA) [3,4]. DNA microarrays have matured considerably over the past few years, and the technology for fabrication and methods for analysis have become more sophisti-

cated. The second 'University of California San Diego Extension, Bioscience Microarray Technologies – An Overview' meeting provided an important forum for reviewing recent advances and future trends in this exciting field (see Box 1).

Microarrays and drug discovery

The identification of a potential drug target relies on the coherent synthesis of a body of biological, physiological, and, at times, clinical data that implicates the target (a specified DNA sequence, RNA species, or expressed protein) in a disease process. Validation of a target does not always have a well-defined end point. The validation is based upon a range of possible degrees of certainty, from a hypothesis driven *in vitro* based disease model or a positive genetic association study, both of which are indirect indices, to a target that has been conclusively defined as a critical molecular mediator of a disease state, for instance the CFTR gene and cystic fibrosis. A drug target such as this one is the exception to the rule, as most of the common diseases that are the current object of drug discovery efforts do not have single gene–single phenotype relationships; but rather are the product of complex genetic backgrounds coupled to significant environmental factors that can robustly affect disease development and manifestation. David M Weiner (ACADIA Pharmaceuticals, San Diego, CA, USA) outlined the complexity of the drug discovery process and highlighted

the role of microarray technologies in target validation and drug discovery efforts [5]. Microarrays have been utilized to address *in vitro* pharmacology and toxicology issues. They are being widely applied to improve the processes of disease diagnosis, pharmacogenomics, and toxicogenomics, and these applications have been reviewed recently [6,7]. Dr Wiener reviewed the role that microarrays have had in providing preliminary evidence for a set of potential new targets in schizophrenia [8–10]. The application of microarrays to the study of the mechanism of action of antipsychotic drugs, such as clozapine, using animal models, and the interrogation of gene expression in schizophrenic brain tissue have yielded surprisingly convergent results that implicate disturbances in membrane lipid biology and synaptic transmission in this disorder.

Mirnic *et al.* carried out one of the first well-controlled gene expression analyses in schizophrenia [10]. They directly compared the expression patterns in the prefrontal cortex from six schizophrenic versus five sex matched control subjects using a matched sample design. They chose the prefrontal cortex for analysis, as this brain region has been extensively studied with respect to cognitive function, and an expanding body of data implicates abnormal prefrontal cortical processes in schizophrenia. In total, 7000 known genes and expressed sequence tags (ESTs) assigned to 250 gene groupings were examined, and 98% of these were found to have no statistically significant differences between the samples. Significant differences were detected in a number of gene groupings, however, including energy metabolism, growth factors, glutamate transmission, GABAergic transmission, and presynaptic secretory machinery.

Oligonucleotide microarrays

When DNA sequence information is available, oligonucleotides can be synthesized to hybridize specifically to each gene. This approach obviates the need for management of large clone libraries as it is guided primarily by sequence data. Furthermore, use of oligonucleotides is favorable for representation of rarely used splicing patterns (which are hard to find as cloned cDNAs even in carefully normalized libraries), and for distinguishing between closely related members of gene families. This approach is particularly well suited to analyzing the expression profiles of organisms with completely sequenced genomes, as all predicted genes can be readily analyzed, and focused custom arrays from genes of interest can be fabricated. As DNA microarray technologies have become more standardized amongst researchers, there has been a trend towards microarrays comprised of long oligonucleotide probes. Microarrays containing long 50- or 60-mer oligonucleotide probes provide several advantages over both traditional cDNA and short oligonucleotide probe microarrays [11].

Oligonucleotides can be synthesized *in situ* or prefabricated and then printed. Synthesis of oligonucleotides by light-directed, combinatorial solid-phase chemistry or other *in situ* methods offers the advantage of having the oligonucleotide synthesized on the support which will be used in the hybridization, obviating the need to hydrolyze the oligonucleotide from its synthetic support and reattach it to the microarray. Covalent attachment of prefabricated oligodeoxyribonucleotides to the arrays is an alternative approach and the primary concern with a postsynthetic attachment method is whether the chemistry is robust, specific and reproducible. Three commercial oligonucleotide platforms were reviewed from Agilent, Amersham and Metragenix, respectively.

Agilent oligonucleotide microarrays

Agilent Technologies (Palo Alto, CA, USA) has developed an *in situ* synthe-

sized DNA microarray platform, and Chris Hopkins (Agilent) highlighted the various features of this platform. The probes are synthesized directly on the microarray surface using standard DNA phosphoramidite chemistry [12]. The probe design methods were developed by Agilent and Rosetta Inpharmatics (Kirkland, WA, USA). Commercial array products available from Agilent currently include human, mouse, yeast and *Arabidopsis* oligonucleotide microarrays.

- The human array contains 22,000 unique 60-mers representing 17,000 unique human genes derived from Incyte's database.
- The mouse array contains over 20,000 unique 60-mers derived from the National Institute on Aging/ National Institute of Health cDNA mouse clone set.
- The yeast genome array contains over 10,000 unique 60-mers representing more than 6250 open reading frames (ORFs) from the *Saccharomyces* Genome Database 25-May-2002 ORF list.
- The *Arabidopsis* microarray is printed with over 14,800 unique 60-mers representing sequences from The Institute of Genomic Research (TIGR) *Arabidopsis thaliana* Database.

The sensitivity of these arrays was evaluated using control spike targets, which were added to the hybridization reaction and were easily detected over background. Signal reproducibility with the Agilent arrays based on microarray to microarray comparisons of the log ratio for control features on each microarray revealed a standard deviation of < 0.1. Greater than 95% of the microarray features display signal intensity well above background when hybridized with a labeled reference target that hybridizes to all the features on the microarray.

Dr Hopkins discussed some of the recent applications of this platform in the classification of breast cancer tumors [13]. Breast cancer patients with the same stage of disease often have markedly different treatment responses. The strong-

est predictors for metastases (e.g., lymph node status and histological grade) fail to accurately classify breast tumors according to their clinical behavior. Van't Heer *et al.* used Agilent DNA microarrays to analyze primary breast tumors in 117 young patients, and applied supervised classification in order to identify a gene expression signature that was strongly predictive of a short interval to distant metastases ('poor prognosis' signature) in patients without tumor cells in local lymph nodes at diagnosis (lymph node negative). The poor prognosis signature consists of genes regulating cell cycle, invasion, metastasis and angiogenesis and this gene expression profile can be used to predict disease outcome.

CodeLink™ bioarray system

A complete platform of bioarray tools is now available from Amersham Biosciences (Piscataway, NJ, USA) for expression and single nucleotide polymorphism (SNP) analysis. Scott R Magnusson (Amersham Biosciences) outlined the use of the CodeLink™ bioarray system for expression profiling. Data from a rat liver model treated with the hepatotoxic agent carbon tetrachloride, the effects of corticosteroid treatment in a human inflammation model, and a hepatocellular carcinoma model were presented to demonstrate that predicative and reproducible patterns were observed across a small set of genes using this technology.

The core technology consists of a proprietary CodeLink 3D gel surface, an integrated probe design process, a proprietary chamber design, parallel processing tools and open platform system software. The CodeLink 3D gel is a 3D hydrophilic gel which reduces non-specific binding and results in lower background noise. Prequalified oligonucleotides are applied to a 3D gel matrix through a non-contact proprietary piezoelectric dispensing method. Through covalent attachment the oligonucleotides penetrate and are immobilized to active functional groups on the slide surface, resulting in very efficient binding capacity and, consequently, sensitive

Box 1. Recent advances and future trends.

- BASE is a web-accessible data management platform. Users interact with a central microarray database and data analysis tools via a web browser.
- Gene expression profiles in cultured HAECs in response to 24 h of laminar shear stress at 12 dyn/cm investigated using microarrays. Genes related to inflammation and EC proliferation were downregulated.
- The Nanogen microelectronic array platform currently facilitates the creation of custom chips for SNP and STR analysis, infectious disease diagnostics and gene expression analysis. The next generation arrays will likely have on-chip integrated amplification capabilities.
- RLS has been applied recently to two-color differential gene expression microarray experiments and provides an accurate determination of differential gene expression with increased sensitivity as compared to fluorescent detection methods. An instrument has been developed for detection of microarrays labeled with RLS particles.
- The current trend is towards microarrays comprised of long oligonucleotide probes. Microarrays containing long 50- or 60-mer oligonucleotide probes provide several advantages over both traditional cDNA and short oligonucleotide probe microarrays.
- Amersham CodeLink biochips have a 3D hydrophilic gel surface, which reduces non-specific binding and results in lower background noise.
- Metrigenix is providing focused arrays using a 'Flow Through Chamber', a 4D microarray technology platform containing relatively few markers for diagnostic and high throughput screening.

BASE: Bioarray Software Environment; EC: Endothelial cell; HAEC: Human aortic endothelial cell; RLS: Resonance light scattering; SNP: Single nuclear polymorphism; STR: Short tandem repeat.

and reproducible hybridization assays. The 3D nature of the slide surface promotes an aqueous biological environment and solution-phase kinetics which enhance assay sensitivity.

The probe design process begins with sequence data from the genes of interest, probe design, selection of three optimal oligonucleotides, synthesis and dispensing of these probes, prototype array validation via actual hybridization with biological samples, determination of the best performing probe, and ultimately array dispensing. The flex chamber replaces the glass cover-slip often associated with microarray experiments. It creates an enclosed array environment, and facilitates uniformity of mixing and enhanced hybridization. A parallel fixture tool allows processing of twelve arrays simultaneously using a reagent bathing or immersion method. The entire process and the engineering of the hardware components results in greater consistency from experiment to experiment, which ultimately translates to very robust data. In addition, the fixtures are provided in microtiter plate format for compatibility and ease of use with programmable robotics. The CodeLink product line currently consists of UniSet mouse, human and rat bioarrays, each containing ~ 10,000 genes and a series of positive and negative controls. Bioarrays with increased

genetic content will be available later this year. The expression protocol starts with target preparation with 1–5 µg of input total RNA, which is subjected to first and second strand cDNA synthesis, *in vitro* transcription based amplification and biotin incorporation. The amplified RNA (aRNA) is fragmented and hybridized to the array. Following overnight hybridization, the arrays are rinsed and washed, and the bound target is detected with streptavidin alexa.

The sensitivity and dynamic range of this system were evaluated by measuring the ability of the probes to detect very low abundance transcripts and generate signal above background. Sensitivity is typically 1:300,000 mass ratio as determined from spiking experiments. Specificity of the arrays was evaluated using arrays containing two and three base mismatches along the entire length of four different oligos (two human probes and two bacterial probes). Signal intensity was greatly diminished when mismatches were present. Dynamic range for these arrays is defined as signal response over three orders of magnitude, with a linear response over two orders of magnitude. Signal reproducibility with this system as measured by percentage coefficient of variation (CV) is in the order of 7–16%. The minimum detectable fold change is typically 95% of array probes within 1.5-fold

when comparing the same tissue on different arrays.

Metrigenix MGX 4D arrays

Metrigenix (Gaithersburg, MD, USA) is providing defined content arrays with relatively few markers compared to Affymetrix, Amersham or Agilent. These arrays are aimed primarily at clinical diagnostics applications or where screening large numbers of samples is required. Currently available arrays from Metrigenix represent the following gene categories: proliferation, inflammation, lung cancer, breast cancer, apoptosis, colon cancer, neural degeneration, cell signaling, and angiogenesis. Dave Tabor from Metrigenix outlined the various features of this Flow-Thru Chip™ (FTC) Technology. The FTC is a 4D microarray technology platform for high-throughput, high-content gene expression assays. The FTC has many advantages over traditional microarrays. Unlike conventional arrays, the molecular interactions occur within the 3D volumes of ordered microchannels rather than on 2D flat surfaces. A fourth dimension is created by microchannels, which connect the upper and lower faces of the chip allowing fluid to flow through the chip. Capture probes are deposited into one or more discrete microchannels creating an array that can multiplex assays in parallel. The

microscopic diameter of each micro-channel results in a much larger surface area:volume ratio than can be obtained with flat surface platforms [14]. Additional advantages of the FTC include:

- improved assay response due to the increased surface area
- reduced assay times due to enhanced mass-transport within the channels
- a more uniform spot morphology due to the wetting properties of the microporous chip
- smaller sample and reagent volume requirements due to the reduction in the reaction volume.

Two instruments have been developed by Metragenix, an MGX 2000 for running the hybridization assay, and an MGX 1200CL for chemiluminescence detection and automated imaging. Total hybridization time per assay is 4 h with an overall assay time of ~ 8 h. The sensitivity of the 4D gel is typically 1:100,000 mass ratio or better as determined from spiking experiments. An mRNA concentration of approximately 5 pM or 1–10 CPC (copies per cell) is detectable and the dynamic range for this system has a linear response over two orders of magnitude. Intrachip signal reproducibility with this system as determined by percentage CV is usually < 15% and the interchip correlation is typically > 0.9.

Resonance light scattering technology

Juan Yquerabide (Genicon Sciences) discussed the simplicity and ease of use of the resonance light scattering (RLS) method, and outlined recent developments with this technology. RLS Technology is a reproducible, ultra-sensitive signal generation and detection technology based on nanometer-sized metal colloidal particles – the RLS particles. They can be used as ultra-sensitive labels for a wide variety of analytical bioassays, and have been applied to DNA and protein microarray studies. When illuminated with configured white light, spherical gold and silver RLS particles of uniform dimension (40–120 nm diameter) generate intense,

scattered light signals [15,16]. The color and intensity of the RLS signal can be predicted from the composition of the particle and its size and shape. The signal obtained from an individual RLS particle is up to 10^4 – 10^6 times greater than that obtained with fluorescent molecules. This improved signal intensity translates at the assay level to greater sensitivity with RLS as compared to fluorescence. Unlike fluorescent signals, the RLS signal is stable and does not photobleach, quench or decay, thus providing researchers with an achievable record and more robust experimental results.

Multiple measurements of RLS-based assays can be made, facilitating the use of different exposure times to obtain the optimal image without the risk of signal degradation. RLS has been applied recently to two-color differential gene expression microarray experiments, and provides an accurate determination of differential gene expression with increased sensitivity as compared to fluorescent detection methods. An instrument has been developed for detection of microarrays labeled with RLS particles. The GSD-501 (Genicon Sciences Corp.) instrument uses a 10 W metal halide arc lamp, a cooled CCD digital camera with 16-bit digital resolution and a high precision XY stage for positioning the sample in the illuminating and detection paths. A computer controlled lens system provides optical resolution options of 5 or 10 μ . The GSD-501 records images of 500 \times 500 pixel square tiles that are stitched together to form a complete composite image. Software automatically controls the illuminating light pattern according to resolution and array type. Optical filters on a wheel can be selected for a particular particle type.

Microelectronic arrays

Active microelectronic array systems, such as those developed by Nanogen (San Diego, CA, USA), have been developed for applications in DNA diagnostics and pharmacogenetic research. These devices allow traffic of charged molecules, including DNA,

RNA, oligonucleotide probes, amplicons, antibodies, proteins, enzymes, nanostructures, cells and semiconductor structures, to and from electronic test sites present on the device surface. The Nanogen platform currently facilitates the creation of custom chips for SNP and STR analysis, infectious disease diagnostics and gene expression analysis. Michael Heller (University of California, San Diego, USA) discussed the future of microelectronic arrays. The next generation arrays will have on-chip integrated amplification capabilities. Both amplification and genetic analysis will take place on a single platform, and multiple reactions will be carried out on individual sites. Heller outlined the challenges associated with the development of miniaturized integrated systems for point of care diagnostics and as biosensors for pathogen detection. There is currently a need for miniaturized sensor or detector devices that are inexpensive, rapid and reliable, and allow the assessment of both genotype and phenotype. Such instruments would have to be continuous real-time low-level detectors, permitting robust determination of microorganism subtypes, virulence factors and antibiotic resistance. Devices of this nature will require high performance specifications and false positive or false negative calls cannot be tolerated. The wide variation that exists in samples (i.e., air, water, food, animals, contaminated surfaces) will entail that these instruments are versatile. For micro- and nanofabrication applications, microelectronic array devices have the potential for use in combinatorial selection processes. The challenges that exist for nanotechnology and nanofabrication today are a better understanding of scaling problems when dealing with assays on a miniaturized scale. Better synthetic methods are required to create the nano building blocks, a problem that is compounded by the enormous complexity and heterogeneity in materials.

Applications of microarrays

S Chien (Department of Bioengineering and the Whitaker Institute of Biomed-

cal Engineering, University of California, San Diego, USA) discussed work from his laboratory on the analysis of gene expression in endothelial cells in response to 24-h shear stress. Using a DNA microarray approach, they investigated gene expression profiles in cultured human aortic endothelial cells (HAECs) in response to 24 h of laminar shear stress at 12 dyn/cm. This relatively long-term shearing of cultured HAECs led to the modulation of the expression of a number of genes. Several genes related to inflammation and endothelial cell (EC) proliferation were downregulated, suggesting that shearing maintains ECs in a relatively non-inflammatory and non-proliferative state compared with static cells. A series of genes were observed to be significantly upregulated by 24-h shear stress treatment, including genes involved in EC survival and angiogenesis (Tie2 and Flk-1), and vascular remodeling (matrix metalloproteinase 1).

Genetic alterations in tumor cells often lead to the emergence of growth-stimulatory autocrine and paracrine signals, involving overexpression of secreted peptide growth factors, cytokines, and hormones. Increased levels of these soluble proteins may be exploited for cancer diagnosis and management, or as points of therapeutic intervention. Garret M Hampton (Genomics Institute of the Novartis Research Foundation, San Diego, CA, USA) discussed work from his laboratory, which combined the use of controlled vocabulary terms and sequence-based algorithms to predict genes encoding secreted proteins from amongst 12,500 sequences present on oligonucleotide microarrays [18]. Expression of these genes was queried in 150 carcinomas from 10 anatomic sites of origin and compared with 46 normal tissues derived from the corresponding sites of tumor origin and other body tissues and organs. Of 74 different genes identified as overexpressed in cancer tissues, several were found to encode proteins which demonstrated clinical diagnostic application (e.g., α -fetoprotein in liver carcinoma, and kallikreins 6

and 10 in ovarian cancer) or therapeutic utility (e.g., gastrin-releasing peptide/bombesin in lung carcinomas). Many of the candidate genes encode proteins with high levels of tumor-associated expression by immunohistochemistry on tissue microarrays.

Bioarray software environment – BASE

Richard Rouse (BIOGEM, University of California, San Diego, USA) discussed the use of BASE, a comprehensive and free web-based database solution available for managing the massive amounts of data generated by microarray analysis. Collecting, managing, and analyzing the deluge of microarray data are undoubtedly the greatest challenge to researchers in this field. Although both commercial and non-commercial solutions exist, in the last 12 months there has been steady growth in the body of freely available, open source software that allows users to analyze data using a host of existing techniques, and facilitates development of their own analysis tools and subsequent integration within the system. The wealth of open source software currently available has been reviewed recently by Dudoit *et al.* [19]. BASE was developed by Peterson and colleagues at Lund University, Sweden [101]. BASE is essentially a web-accessible system, which uses standard browsers to interact with a central microarray database and data analysis tools [20]. This approach has the advantage that users of the system need not concern themselves with whether they have the latest version of the software running on their computer or not. Furthermore, the processor intensive calculations underlying microarray data analysis can be effortlessly shifted to powerful central servers, which is generally more effective than running the analyses locally on less powerful client desktop computers. BASE was designed with the goal of supporting a variety of microarray platforms. Underlying this system is a minimal information about a microarray experiment (MIAME)-supportive, customizable database implemented in MySQL. This tracks the

elements used to construct the arrays and their annotations, the layout, map or design of the array itself, the biological samples used in each hybridization assay, and both the raw and transformed data. The users have the option of including other laboratory information management systems (LIMS) components for tracking samples and reagents. The software that interacts with this database was developed under the Linux operating system in PHP and uses a freely available Apache Web server [102] to provide web access to its functionality. The interface uses Java[®], JavaScript[®], and HTML to provide added utility, and some of the more computationally intensive analysis methods that are carried out on the server have been implemented in C++. The flexible design of BASE allows analysis of one- and two-color systems on a variety of substrates, cDNA and oligonucleotide arrays, and Affymetrix GeneChips[™]. BASE has a plug-in architecture that allows new modules to be easily added as they become available for data transformation, analysis, or visualization.

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