DNA microarrays were first described in 1995 for simultaneous analysis of a large-scale gene expression patterns. Since then, they have moved to center stage in many areas of biological research and now assuming an increasingly important role in diagnostics, genomics, pharmacology, cancer and other biomedical research, among others. In this article, we discuss the scientific background and principle of microarrays; describe their types, several technical steps needed for obtaining microarray data, and their current applications. The potential applications of DNA microarrays in detection and identification of plant pathogens, especially viruses, viroids and phytoplasmas are presented.

Key words: arrays, cDNA microarrays, detection, diagnostics, DNA chip, DNA macroarrays, DNA microarrays, GeneChip, gene expression, high-density arrays, hybridization, identification, low-density arrays, microarrays, oligo-chip, oligonucleotide microarrays, PCR, phytoplasmas, viroids, viruses.

INTRODUCTION

Plant viruses, viroids, and phytoplasmas are known to cause considerable losses in crop yield, quality of plants, and plant products (Kirkpatrick, 1991; Foster and Hadidi, 1998; Waterworth and Hadidi, 1998; Hadidi et al., 1998, 2003; Barba et al., 2003; Randles, 2003). They pose a particular risk because they are difficult to detect and identify. Many quarantine and certification programs are in place, particularly in the developed countries, to prevent the introduction of these pathogens to a country during the international movement of germplasm by quarantine and to control and reduce their spread within a country by certification. Currently, these pathogens may be detected by biological indexing, immunological methods, molecular hybridization, polymerase chain reaction (PCR), and/or reverse transcription (RT)-PCR. In all these assays, usually one pathogen is detected per assay.

DNA microarrays were introduced in 1995 and since then this technology has attracted great interest among biologists. It has the ability to simultaneously display the expression of thousands of genes at a time, thus it is a powerful tool for genetic analysis. It is currently being applied in the following broad areas: genomics such as sequence analysis, gene expression studies, gene typing, and large scale polymorphism screening; biomedical research of cancer, and infectious diseases and genetic diseases; clinical diagnostics; and drug discovery and development. With the explosion of information arising from sequencing of plant viruses and viroids, Hadidi and Candresse (2001, 2003) predicted the simultaneous detection and identification of many plant viruses, viroids, and other plant pathogens by DNA microarrays. In this article we will discuss the scientific history and principle of microarrays, describe their types and the several steps needed for designing and implementation of a DNA microarrays experiment. We will also describe current applications of microarrays and their potential applications in detection and identification of plant viruses, viroids, and phytoplasmas. Finally, we discuss advantages and disadvantages of microarrays.

HISTORICAL CONCEPT AND DEVELOPMENT OF DNA MICROARRAYS

DNA microarrays exploit the feature of DNA complementarity through base pairing, discovered by Watson and Crick fifty one years ago, and a variety of techniques in order to apply DNA molecules in perpendicular fashion to a solid surface. DNA arrays are a logical extension of the method first described by Gillespie and Spiegelman in 1965, in which DNA immobilized on a membrane can bind a complementary RNA or DNA strand through specific hybridization, and the methods described for applying DNA to a treated cellulose surface (Southern and Mitchell, 1971) and DNA blotting hybridization (Southern, 1975). The later technique has
PRINCIPLE AND TYPE OF ARRAYS

Base-pairing of complementary sequences by hybridization is the underlying principle of DNA microarray. This specific binding of DNA allows a target DNA or RNA to hybridize to a specific complementary DNA probe on the array. Each probe is made of thousands of cDNAs or oligonucleotides, each specific for a gene, DNA sequence, or RNA sequence of interest. An array is an orderly arrangement of samples. It provides a medium for matching known and unknown nucleic acid samples based on base-pairing rules (A-T and G-C for DNA; A-U and G-C for RNA) and automating the process of identifying the unknown. Each array is generated by depositing a few nanoliters of DNA probe on a solid support. The printing is performed by a robot which allows identical spotting serially. In general, arrays are described as macroarrays or microarrays, the difference being the size of the sample spots. Macroarrays contain sample spot sizes of about 300 μm or larger. The sample spot sizes in microarray are typically less than 200 μm in diameters and these arrays usually contain thousands of spots.

The term “low-density” is generally used to describe microarrays with a density of the order of 100 spots per cm². Such low-density arrays are available from a wide range of commercial supplies or may be produced by research groups in their own laboratories. In many cases, a glass surface is utilized for low-density arrays. Nucleic acids bind poorly to plain glass, which is generally treated before use. Poly-L-lysine, which binds molecules by ionic interactions, is often used for this purpose. The glass substrate may also be treated with saline, which binds covalently to the probe DNA, thus preventing removal of the probe DNA during hybridization and washing steps. Glass slides that are used to detect radioactively-labeled nucleic acids may be coated with a nylon membrane to which the DNA probes are covalently cross-linked using ultraviolet light. The DNA applied to the surface of the array may consist of plasmids of 500-5,000 bases, complementary DNA of several hundred bases in length, products of the PCR of 100-500 base pair, or synthetic oligonucleotides that may be modified by addition of an amino or thiol group on their 5’ end.

The term “high-density” is generally used to describe microarrays with a density of the order 1,000-10,000 spots per cm² or even higher. Such high-density arrays are generally known by the term “DNA chip”. The substrate used for immobilization of high-density DNA arrays is usually chemically modified glass or silicon.

The technical advance represented by DNA microarray technology lies in its massively parallel nature, that is to say, the capacity to apply many thousands of nucleotides in an ordered array to a surface, thus allowing the parallel interrogation of many thousand of different sequences at once.

DESIGNING AND IMPLEMENTATION OF A DNA MICROARRAY EXPERIMENT

There are several steps in the design and implementation of a DNA microarray experiment. These steps include:

DNA probes with known nucleotide sequences. Probes may include short or long oligonucleotides, cDNAs, chromosomes, whole organism, or others with known identity. The probes may be created by using standard technologies, such as restriction enzymes, cloning, and PCR. Alternatively, they can be purchased separately or attached to chips, glass slides, or nylon support. cDNA or oligonucleotide probe libraries can be purchased as well as synthesized. Each probe is made of thousands of these cDNAs or oligonucleotides, each specific for a gene, DNA sequence, or RNA sequence of interest. Arrays containing oligonucleotides representing the known genes have been designed from the baker’s yeast *Saccharomyces cerevisiae*. Oligonucleotide arrays containing more than 65,000 DNA synthesis features were prepared. Each 50×50 μm synthesis feature is composed of more than 10⁷ copies of a specific 25-mer oligonucleotide that is complementary to a portion of a yeast gene. The full set of oligonucleotides includes an average of 40 synthesis features for each of the yeast 6,321 identified genes (Lipshutz et al., 1999). A similar approach has been taken to design oligonucleotides specific for the ~30,000 human genes (http://www.mwg-
biotech.com/html/d_arrays/d_catalog_human_10k.shtml). The oligonucleotides are inherently only of known sequences, while cDNAs can have the “advantage” of being unknown, meaning an experiment using cDNAs can potentially help find the function of previously uncharacterized and un-sequenced genes, including genes of viral genomes.

Application of DNA to the array. A wide variety of techniques exist for the immobilization of DNA on the surface of the array, including contact-dip deposition printing, micro contact printing, microfluidics networks and electrocapture. Piezoelectric printing and micro wet printing may be used both for immobilization and for in situ synthesis. The photolithographic technique is used for in situ synthesis alone. Each array is generated by depositing a few nanoliters of purified DNA on a solid support. The printing is performed by a robot which allows identical spotting serially. The size of the support for macroarrays can go from 10-15 cm² with 10 to 100 DNA pieces per cm² and for microarrays can go to 2-3 cm² with several thousands genes per cm² (Bertucci et al., 2001). Oligo-chips also known as GeneChips have been developed by Affymetrix (www.affymetrix.com). Their original approach was to synthesize directly on a rigid support polynucleotides (20-25 mers), single-stranded DNA segments produced sequentially by chemical synthesis (Lipshutz et al., 1999). The production of these arrays requires two techniques: photolithography and solid-phase DNA synthesis. Briefly, synthetic linkers are attached to a glass substrate and coupled to different bases allowing arbitrary DNA probes to be synthesized at each site of the glass substrate. This method allows the construction of arrays with a large number of probes: several hundreds or thousands of polynucleotides in one cm², even one million in some experimental prototypes (Lipshutz et al., 1999).

Target preparation. Poly(A)⁺RNA, purified or as total RNA, is reverse-transcribed to the first single-stranded cDNA. When the support is glass, Cys- or Cy5-fluorescently labeled dCTP is incorporated into the cDNA during the reverse transcription step (Lockhart et al., 1996). Alternatively, reverse transcription is performed with chemically reactive nucleotide analog (amino allyl-dUTP) and the cDNA is “post labeled” with the reactive forms of Cys- or Cy5-NHS esters, which bind to the modified nucleotides. These cDNAs generate even and bright signal levels in microarray hybridization. When the support is nylon membrane, the cDNA is labeled during reverse transcription with radiolabeled nucleotides, such as α²³ P-dCTP, and hybridization is detected by autoradiography or chemiluminescence (Bertucci et al., 1999a, 1999b). Alternatively, the cDNA can be labeled by incorporating biotin-16-dUTP or digoxigenin-11-dUTP; following hybridization, β-galactosidase-conjugated streptavidin or alkaline-phosphatase-conjugated anti-digoxigenin antibody is employed and the hybrids are detected by colorimetry (Chen et al., 1998). Purity of the RNA is a critical factor at this step of the experiment, to avoid interference in the detection of the radioactive as well as the fluorescent signals.

Hybridization. The probe DNA is denatured by heating, and then the labeled target solution is applied. The amount of the original total RNA required for hybridization varies depending on the type of support used: from 300 ng (Bertucci et al., 1999a) to 5 µg of total RNA with the nylon membranes (www.clontech.com) and 20 µg of total RNA on glass (www.affymetrix.com). If the array is hybridized with a single cDNA type, single-dye or radioactive labeling hybridization is sufficient. In the case the array is used to analyze differential gene expression, control cDNA may be labeled with Cy3-dCTP, and experimental cDNA with Cy5-dCTP. In this case, it is important to make sure that equal amounts of cDNA and of dye are used. The slide or chip is moved to a moist 65°C hybridization environment for several hours. Finally, the slide or chip is washed to remove any unbound nucleotides in preparation for the detection stage.

Image detection. Detection is done using either a scanning or direct imaging system. Scanning uses a laser excitation source (for the detection of fluorescently-labeled DNA) that works on each spot one at a time and a detector that measures the resulting light. Direct imaging uses an excitation source that illuminates the entire array at once, and a camera to capture the resulting image. It is used when radioactive hybridization or colorimetry is chosen. Scanning has higher resolution and sensitivity than direct imaging. Graphic computer files (TIFF, etc.) are created based on the detection results.

Analysis of results. Results obtained have to be presented in the most informative way for interpreting the DNA array data. This requires sophisticated bioinformatics methods. When using dyes, the resulting image files are analyzed with special software to determine the intensity of light at each of the wavelengths measured. When hybridizations are done with a mixture of cDNAs, labeled for example with Cy3 and Cy5, the slide is scanned to detect Cy3 and Cy5 fluorescence and the ratio Cy3/Cy5 is determined. A false-color overlay is obtained depicting spots in which more or less of one population of cDNA hybridizes as red or green, and equal amounts of each population hybridize as yellow. Thus the relative abundance of each cDNA (experimental vs. control) is reflected by the ratio of “red” to “green” measured at the spot representing that gene. These measurements are then combined by the software to create a graphical representation of the relative expression levels of the experimental and controlled samples. A non
exhaustive list of image analysis software can be found at http://ihome.cuhk.edu.hk/~b400559/arraysoft_image.html. The output can usually be seen in different formats. This include a two-dimensional grid that shows red, green, and yellow spots of varying shades that correspond to the spots in the array. Alternatively, the output can be a scatter plot that better shows the difference in expression levels between different genes. Complex data sets need to be analyzed using sophisticated softwares such as GeneSpring (http://www.silicongenetics.com) and Spotfire (www.spotfire.com).

**APPLICATIONS OF DNA MICROARRAYS**

The microarray system is a powerful tool for genetic analysis. It is currently being applied in the broad areas of biomedical, pharmacological, and genomic research as well as diagnosis and food safety and quality. The major applications of microarrays include:

**Analysis of gene expression.** Microarrays have proven to be a very powerful means of analyzing the simultaneous expression of multiple genes. cDNA-based microarrays is the method of choice for analyzing the simultaneous expression of up to 15,000 expressed sequence tags (ESTs) at once, especially when they have not been sequenced. Only those ESTs which show differential patterns of expression are subsequently sequenced. Specifically-designed oligonucleotide-based microarrays have advantages over cDNA-based microarrays in terms of sensitivity and specificity. Gene expression profiling using DNA microarrays have been extensively used from developmental biology of model organisms such as Drosophila (White et al., 1999), Saccharomyces cerevisiae (Wodicka et al., 1999) and Arabidopsis (Beisson et al., 2003).

**Diagnostic and clinical applications.** Microarrays have been applied to human cancer research (Thieblemont, 2000; Cullen and Lorkowski, 2002). Microarrays have also been used in human prognosis. Samples from biopsies and peripheral blood lymphocyte from patients hybridized with whole genome array allowed to classify patients based on their risk for kidney transplant rejection (Koppar, 2004).

**Drug discovery and development.** One of the major areas of research in pharmacology is in the field of pharmacogenomics (Debouck and Goodfellow, 1999). Its goal is to find a correlation between therapeutic response or side effect profile of drugs to an individual genetic background. Several approaches using the DNA microarray technology are currently being exploited to providing more individualized pharmacological treatment. For example the microarray analysis of human neurons treated with antidepressants, antipsychotics, or opioid receptor agonists, pointed to new directions for research into depression and psychosis, by identifying the responsive genes which in turn may serve as markers for each of the drug families.

**Toxicological research.** The goal of toxicogenomics, a major area of research in toxicology, is to find a correlation between toxic responses to toxicants and changes in the genetic profiles of the objects exposed to such toxicants (Waring et al., 2001).

**Agricultural applications.** Currently the microarray technology is being utilized to better understand plant genetics and gene functions. Very recently, the first DNA chip aimed at testing the integrity of genetically modified food has been developed by GeneScan Europe and introduced to the market (Lübbeck, 2002). The chip screens and identifies GMOs in raw materials, processed food, and animal feed. It is able to detect viral DNA (CaMV), selection genes (bar, resistance to antibiotics), gene fragments (Nos-terminator), as well as specific gene fragments (Br, EPSPS).

**Food quality.** The presence of unwanted or unknown animal species in food is of great concern for public health, economic, religious and legal reasons. For the first time, a DNA chip (FoodExpert-ID Array) which contains 80,000 different oligonucleotide probes could be used to detect 33 species of animals, making the array a technology to distinguish between animal species that might be present in food (http://www.affymetrix.com/corporate/media/genechip_essentials/foodexpert/FoodExpert_ID_Array.affx).

**Microbial detection.** DNA microarrays can be used for rapid and simultaneous detection of hundreds of microorganisms from virtually any sample. Application areas include, amongst others, detection of:

1. **Human pathogens in clinical samples and waste waters**

   The following human pathogens have been reported to be detected by microarrays: orthopoxviruses, rotaviruses, pathogenicity factors in Escherichia coli, antibiotic-resistance determinants in Staphylococcus (Chumakov, 2003). An improved method for producing microarrays that detected pathogens such as anthrax or small pox virus has also been reported (http://www.eu-reakalert.org/features/doc/2002-01/dnml-mfd061702.php). Potential bio-warfare agents such as bacteria and viruses were successfully detected by portable microarray analysis (Jackman, 2003). With microarray technology it is possible in less than two hours to rapidly identify human bacterial pathogen species based on their ribosomal RNA sequences (http://www.jhuapl.edu/programs/rpc/Pathogens/RapidDetectionAndId.html).
A DNA microarray has been developed for use in testing key parasite and bacterial contaminants in surface and ground water which include: Cryptosporidium parvum, Giardia intestinalis, Cyclospora cayetanensis, Escherichia coli, Salmonella, Listeria, and Campylobacter (http://www.gov.on.ca/OMAFRA/english/research/special_research/2001/sr9087.htm).

2. Food-borne pathogens

The following key food-borne bacterial pathogens were detected by microarrays: Shiga toxin-producing E. coli (STEC), E. coli serotype O157:H7, Campylobacter, Salmonella, Salmonella typhimurium DT 104, and Listeria monocytogenes (http://www.gov.on.ca/OMAFRA/english/research/special_research/2001/sr9087.htm).

3. Methanotropic bacteria

Microarrays were designed with a nested set of many probes targeting different phylogenetic subgroups of methanotrophs from the strain level up to general probes with the broadest possible specificity (Bodrossy et al., 2002; http://www.arcs.ac.at/ul/ulb/bt/p).

4. Animal pathogens in veterinary samples

Microbial microarrays were developed for the detection of pathogens in veterinary samples as well as in food (Bodrossy et al., 2002).

5. Fish pathogens

A DNA microarray was developed for the simultaneous detection and differentiation among fish fastidious pathogens based on 16 S rDNA polymorphisms (Warsen et al., 2004). The pathogens included Aeromonas salmonica, A. hydrophila, Mycobacteria spp., Yersinia ruckeri, and others.

6. Human viruses

Wang et al. (2000) developed DNA microarray capable of simultaneously detecting of about 140 human and animal viruses which included double-and single-stranded DNA viruses, retroviruses, and both positive- and negative-stranded RNA viruses. In their system, they were able to efficiently detect and identify many diverse viruses, such as picorna, rhino, entero, orthomyxo, paramyxo, retroid, herpes, adenov, papillima, hepatitis B, hepatitis C, etc.. Members of each viral family were detected by selecting probes with the most highly conserved regions within the family. This is a viable strategy for detecting un-sequenced or uncharacterized viruses and it may also prove to be a useful approach to novel virus discovery. Members of each genus were detected by selecting short regions of high nucleotide conservation such as the 5’ untranslated region for members of the enterovirus genus. Related viral serotypes were distinguished by the unique pattern of hybridization generated by each virus. Thus microarray-based viral detection may offer a powerful alternative for determination of viral subtypes.

**POTENTIAL APPLICATIONS OF DNA MICROARRAYS FOR THE DETECTION AND IDENTIFICATION OF PLANT VIRUSES, VIROIDS AND PHYTOPLASMAS**

Methods for the detection and identification of plant viruses, viroids, and phytoplasmas are needed for epidemiological and genetic studies, comparison of pathogens, for quarantine and certification purposes, and as a follow-up to pathogen therapy (Hadidi et al., 1998, 2003; Pasquini et al., 1999; Ragozzino et al., 2004). During the last several decades, methods have been developed for the specific detection of each member of the above classes of pathogens. These methods fall into four broad categories. The traditional biological indexing which includes the use of herbaceous and woody indicators. This method has deficiencies in its ability to reliably identify the species and strain of pathogens and it is most often designed to identify one pathogen per assay. Immunological techniques, such as gel diffusion, enzyme-linked immunosorbert assay (ELISA), immunoblotting, etc., all designed to identify one pathogen per assay and all employ the antibody-antigen interaction as the prime determinant of specificity and sensitivity. These techniques, in case of viruses, are limited to genetic information of coat protein; thus, they cannot be applied to viroids because viroids are infectious naked RNAs that lack coat proteins (Hadidi et al., 2003). Immunological techniques have been applied to phytoplasmas (Loi et al., 2002). The other two categories include targeted nucleic acid-based techniques, such as molecular hybridization assays using cDNA or cRNA probes, which are designed to identify one pathogen per assay, and PCR or RT-PCR assays. The PCR provides excellent sensitivity and selectivity in most applications. A limitation is that, even with advanced multiplexing, generally only one organism is targeted per reaction tube. For reviews on detection of plant viruses and viroids, see Hadidi et al. (1998, 2003). For PCR detection of phytoplasmas, see Lorenz et al. (1995), Zhu et al. (1998) and Pasquini et al. (2000).

Recent reports on initiating research on the detection of plant viruses and other plant pathogens by DNA microarrays have been limited, generally unspecific and in the form of short abstracts. Research works have been initiated toward detection of plant viruses which included potato viruses (Boonham et al., 2002; Perez-Ortin, 2002; Sip, 2002; Bystricka et al., 2003) and “viruses” (Bonants et al., 2002; Nicolaision, 2002). Similarly, investigations have been initiated toward detection of “bacteria” (Bonants et al., 2002; Perez-Ortin, 2002), mycotoxin-producing Fusarium spp. (Nicolaision, 2002), aflatoxin-producing Aspergillus spp. (Scherm et al., 2002), “fungi and nematodes” (Bonants et al., 2002), and “plant pathogens” (Bonants et al., 2002; Schoen et al., 2002, 2003).

Agilent technologies of Palo Alto, California, in col-
Advantages and Disadvantages of Microarrays

**Advantages:** (i) Simultaneous detection and quantification of thousands of hybridization events; (ii) great scope for miniaturization, for high-throughput applications and for development of integrated, automated systems (lab on a chip).

**Disadvantages:** DNA array instruments, DNA chip production, probes, and bioinformatics are fairly expensive; however, a rapid fall in prices is expected in the coming years.

Future Directions

DNA microarray technology holds a great promise for genomic research and diagnostics, biomedical research of cancer and infectious and genetic diseases, and improving the effectiveness and reducing the side effect profile of pharmaceuticals. DNA microarrays will increasingly be utilized in integrated analytical and diagnostic systems, where all steps for sample preparation through assay analysis and interpretation will be performed in cheap and disposable cartridges or in high-throughput microfluidic devices (lab on a chip). If current trends continue, it is expected that DNA microarrays to become part of the routine human, animal, and plant pathogen diagnostics.

It is possible that in the future, the large-scale microarrays will be replaced by small biosensors which contain the nucleic acid signature of a unique or a small number of pathogens deposited on an electronic platform. Since DNA carries an electric charge, the formation of target-probe complex could elicit the binding of specially designed micron-size magnetic beads, which could be detected by very sensitive thin film magnetic detectors. In theory the sensor should be able to detect the presence of a single pathogen DNA molecule in a very short period of time. Recently, the electronic detection of point mutations in DNA has been achieved using a transistor network (Pouthas et al., 2004). Similarly, fluorescently-labeled antibodies raised against a given pathogen could be linked to a solid support. In this case, quenching of fluorescence due to the binding of a proper antigen could be detected by a fluorimeter. These devices would be extremely useful in the fight against bio-terrorism (Da Silva, 1999; Parker et al., 2003; Zahavy et al., 2003). Such sensors are developed by a number of companies and institutions.

**References**


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