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DNA microarrays in medicine: manufacturing techniques and potential applications

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Since their first description in 1995, DNA microarrays have moved to centre stage in many areas of biomedical research and are now assuming an increasingly important role in diagnostics and in pharmacology. Such microarrays may be used to detect and quantify nucleic acid and to determine its sequence. In medicine, a major area of use is in detection of changes in the pattern of gene expression in conditions such as cancer. Other uses are in recognition of sequence variation and in the detection of infectious organisms. In the field of pharmacogenetics, microarrays are being used to monitor the presence of polymorphisms in genes encoding cytochromes and other proteins, in an attempt to reduce side effects and tailor treatment more effectively to the patient's genetic background. Not surprisingly, these developments have been accompanied by a burst of patenting activity in all fields relating to DNA microarrays, including methods of manufacture, methods of detecting hybridisation, analysis of array data and concrete applications for use of microarrays in the diagnostic arena. This review will focus largely on manufacturing techniques.

Keywords: cancer, diagnostics, DNA microarray, photolithography

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1. Introduction

DNA microarrays exploit a variety of techniques in order to apply DNA molecules in perpendicular fashion to a solid surface. This idea is not new. In technical terms, DNA arrays are a logical extension of the method first described by Dr Edward Southern at Cambridge University over 30 years ago, of applying DNA to a treated cellulose surface [1]. Since its introduction, Southern blotting has been a workhorse of the field and continues to be used in nearly every molecular biology laboratory up to the present day. However, in recent years, a number of methods have been developed that allow very large numbers of DNA oligonucleotides to be applied to surfaces in ordered two-dimensional arrays, thus allowing the massively parallel analysis of hybridisation events. The starting-gun for this field was sounded in 1995, when a group at Stanford University published the first paper describing DNA microarrays [2]. Since then, the number of published scientific studies employing microarray technology has increased sharply, reaching 110 in 1999, 400 in 2000 and 600 in 2001 [3]. Coupled with the explosion of information arising from the sequencing of the human and other genomes, such arrays hold promise in a wide range of research, diagnostic and therapeutic contexts. This review will describe the types of DNA arrays that exist, how they are made and some of the uses to which they can be put. For reasons of brevity, this discussion be will limited to a selection of applications in the field of human health and disease and will exclude any discussion of other types of arrays, in particular protein arrays, which are currently in development. Arrays

that do not utilise a classical solid support, including those based on microbeads, will also be excluded from discussion in this article. For further details on such arrays, the interested reader is referred to the patents held by Lynx Pharmaceuticals such as [101] or to those of Luminex Corporation such as [102]. This review will not address in detail the technologies related to the labelling, hybridisation and scanning of DNA microarrays, nor to the techniques involved in data analysis. For such information, the reader is referred to the specialist literature or to newer reference works (for example [4]).

2. How DNA microarrays work

All DNA microarrays function in essentially the same fashion, by exploiting the tendency of DNA to undergo Watson–Crick base pairing where adenine binds to thymidine and cytosine to guanine. This specific binding of DNA allows the conclusion to be draw that a target DNA hybridising to a particular probe DNA on the array is complementary in sequence to that DNA probe. This in turn leads to the two main areas of utilisation of DNA microarrays, sequence assessment and measurement of gene expression. 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 thousands of different sequences at once. This is of particular use in the area of gene expression analysis. Whereas, only a few years ago, it took several days to study the expression of a single gene, DNA microarray technology now allows the analysis of the expression of thousands of genes in a single day. Other uses of DNA microarray technology include assessment of DNA copy number [5,6], identification of disease-causing genes by positional cloning [7] and detection of DNA–protein interactions [8,9].

3. Low-density microarrays

The term 'low-density' is generally used to describe microarrays with a density of the order of 100 spots per cm^2. Such low-density arrays are available from a wide range of commercial suppliers or may be produced by research groups in their own laboratories. In one application, nucleic acids are coupled with high affinity to a nylon membrane, with or without the use of ultraviolet light for covalent crosslinking. In many cases, a glass surface is utilised for low-density arrays. Nucleic acids bind poorly to plain glass, which is therefore generally treated before use. Poly-L-lysine, which binds molecules by ionic interaction, is often used for this purpose. Such arrays can be used only once, however, because the salt conditions required to remove the hybridised sample DNA also dissociate the immobilised probes from the surface of the array. In addition, the nucleic acid probes on such coated chips are entwined, reducing the hybridisation kinetics by steric hindrance. This problem may be avoided, or at least reduced, by the use of linker molecules such as derivatives of oligoethyleneglycol to separate the immobilised DNA from the surface of the array. The glass substrate may also be treated with silane, which binds covalently to the probe DNA by means of a Schiff–base reaction, thus preventing removal of the probe DNA during hybridisation and washing steps and allowing the arrays to be reused. Epoxy-coating or acid treatment of the surface may also be used [10,11]. Glass slides that are used to detect radioactively-labelled nucleic acids may be coated with a nylon membrane to which the DNA probes are covalently crosslinked 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 polymerase chain reaction of 100 – 500 base pairs or synthetic oligonucleotides of 20 – 60 base pairs. If synthetic oligonucleotides are used, these may be modified by addition of an amino or thiol group to their 5′ end.

4. High-density microarrays

The term 'high-density' is generally used to describe microarrays with a density of the order 1,000 – 10,000 spots per cm^2. The upper limit for spot density is constantly being extended; at the time of writing, some companies and research groups have produced microarrays with a density in excess of 20,000 spots per cm^2. Such high-density arrays are what is generally meant by the term 'DNA chip'. The substrate used for immobilisation of high-density DNA arrays is usually chemically modified glass or silicon.

5. Manufacturing techniques

Microarrays of low spot density are usually produced by the application of preformed DNA to the surface of the array. Arrays of high spot density may also be produced by application of preformed DNA but in general tend to exploit methods for *in situ* oligonucleotide synthesis.

5.1 Application of DNA to the substrate

A wide variety of techniques exist for the immobilisation of DNA on the surface of a low-density array, including contact-tip deposition printing, microcontact printing (µCP), microfluidics networks (µFN) and electrocapture. Piezoelectric printing and micro wet printing (µWP) may be used both for immobilisation and for *in situ* synthesis. The photolithographic technique is used for *in situ* synthesis alone. The following provides a brief description of each of these techniques.

5.1.1 *Contact-tip deposition printing*

This technique, developed in 1997 by Synteni (Fremont, CA, USA), is used by a large number of research teams to generate their own DNA arrays. The nucleic acid to be immobilised is first dissolved. A needle is dipped into this solution so as to leave a defined amount of solution at its tip. This solution is

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then applied to the surface of the substrate. In practise, several needles are used simultaneously. In most cases, the needles have a groove that serves as a reservoir for the solution as with a conventional ink pen [2].

Genetic Microsystems (Woburn, MA, USA) introduced a variation of this technique called the pin and ring system in 1998 [12]. A small ring of several millimetres in diameter is dipped into the nucleic acid solution. A membrane or lamella is formed in the ring by surface tension in exactly the same way as a soapy membrane forms in a child's toy for blowing soap bubbles. The ring is positioned over the array and a flat-topped pin is inserted through the lamella until it makes contact with surface of the array. In this way, the small, defined portion of the lamella adhering to the tip of the pin is deposited on the array. Genetic Microsystems have registered a patent for depositing fluid specimens on substrates resulting in ordered arrays and have described techniques for the deposition of arrays [103].

Contact-tip deposition printing may be used to produce arrays of high density. For example, at the time of writing, Synteni produces an array containing ~ 10,000 different cDNAs ranging in size from 500 to 5,000 bases on a glass surface of 3.6 cm^2. Synteni aims to use this technique to produce an array containing up to 100,000 probes on a surface measuring 6.5 cm^2. The reproducible minimal spot diameter is in the range of 50 µm, with an inter-spot distance also in this range.

Due to the limited amount of technical effort required for contact-tip deposition printing, smaller laboratories are using this method to generate their own arrays. Commercial printing machines (spotters) are now available. The research group led by Patrick Brown, at the University of Stanford in California, has even provided instructions for a do-it-yourself printing apparatus on the internet [201]. In addition, this group has disclosed two inventions of a method and an apparatus for forming microarrays of biological samples on a support [104,105]. A method for creating a low-density array by deposition printing is also held by the American company Affymetrix [106].

5.1.2 Microcontact printing

The microcontact printing method (µCP) works in a method analogous to contact-tip deposition printing. In µCP, a polydimethysiloxane (PDMS) stamp is used to transfer the nucleic acids to the carrier surface. The advantage of this material is that it can be used to produce very small structures. At the time of writing, stamps are already being produced in a reproducible fashion with defined structures of < 50 nm. In theory, this opens up a new level of miniaturisation [13]. However, practical results obtained with the µCP method have been disappointing. While µCP has been used to immobilise antibodies on gold substrates [14], this technique has not yet succeeded in producing the density of probes generally required for the manufacture of DNA arrays. The Motorola Corporation has been issued with a patent for a flexible microcontact printing stamp that may be used in biochip manufacture [107].

5.1.3 Microfluidics network

Microfluidics network technology (µFN) is a development of µCP. In µFN, a PDMS stamp containing small channels is placed on a glass, gold, polystyrene or silicone/silicone dioxide surface. These small channels are filled with a solution containing the substrate, which perfuses the surface of the array by capillary attraction. This system has been used to immobilise antibodies following activation of the substrate by hydroxysuccimidylesters, which undergo chemical coupling with the amino group of the proteins [15]. However, occlusion of the capillaries may occur and the number of suitable solvents is limited. As with µCP, the feasibility of µFN for production of DNA arrays has yet to be demonstrated. PDMS stamps measuring 3 mm × 1 mm have been used to generate 100 capillaries with a depth of 1.5 µm, a width of 3 µm and a length of 3 mm on a variety of substrates [16].

5.1.4 Piezoelectric printing

Piezoelectric printers use the technology that was developed for conventional ink-jet printers to dispense small amounts of DNA solution [17]. Using silicon and glass pipette tips of < 100 µm in diameter, volumes of between 15 and 500 pl can be applied with a frequency of up to 2 kHz and a coefficient of variation of less than 1%. Synteni and Incyte Pharmaceuticals offer microarrays produced using piezoelectric printing with up to 10,000 spots/cm^2. Commercially available piezoelectric systems allow production of arrays with spot diameters of about ~ 200 µm, inter-spot distances of 300 µm and a spot density of > 1,400 spots/cm^2. Piezoelectric dispenser systems are also able to target solutions with impressive accuracy. Incyte have been awarded a patent on methods and solvent vehicles for reagent delivery in oligonucleotide synthesis using automated pulse jetting devices [108] and for a jet droplet device for the piezoelectric production of DNA microarrays [109].

Contamination, leading to poor reproducibility, is a problem associated with piezoelectric dispenser systems. This may result from vaporisation of the small probe volumes and by satellite spots, which result from splashing as the drop impacts the surface of the substrate. Attempts are being made to deal with this problem by developing surfaces with a high degree of wetability. Protogene (Palo Alto, CA, USA), for example, uses substrates with hydrophilic areas separated by hydrophobic barriers [18]. Protogene hold a series of patents related to the conducting of chemical reactions on a solid surface [110,111] and for the chemical synthesis using a two-dimensional array [112]. Alternatively, photolithographically-produced polyacrylamide cushions may be used. Polyacrylamide gel has a high capacity for nucleic acids, which reduces the problem of vaporisation [19].

Piezoelectric dispensers may also be used to synthesise oligonucleotides *in situ* [17,20]. Highly specialised nozzles are used, which limits the ability to employ different solutions

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and reagents with various physical properties in the same dispenser. Two strategies may be used to solve this problem, both of which utilise a modified phosphoramidite procedure to synthesise the oligonucleotides. In the single jet system, a single dispenser is used to apply a reagent that de-protects the 5´-hydroxyl group of the nucleotide derivatives at defined positions on the substrate. Coupling of a nucleotide to the unprotected hydroxyl group and phosphorous oxidation is then achieved by moistening the entire substrate surface with the appropriate reagent. In the multiple jet system, five different dispensers are used, each of which each contains one of the four phosphoramidite nucleotides needed for oligonucleotide synthesis. The fifth dispenser is needed for the detritylation reagent.

5.1.5 *Electrocapture*

A completely different procedure to piezoelectric printing has been developed by Nanogen (San Diego, CA, USA) [21]. This active programmable electronic device technology is based on a silicon chip containing an array of 1 cm^2, which is loaded with 25, 64 or 100 platinum electrodes. These chips are manufactured by generating a layer of silicone dioxide on a silicon carrier by means of thermal oxidation. This layer is coated with aluminium and then with photoresist. Using a mask and ultraviolet light, the resist is developed and removed in certain areas, to which a 20 nm layer of chrome is applied, followed by platinum electrodes of 500 nm in thickness. The remaining resist and the aluminium coating are then removed. The whole chip is then covered by a 2 µm dielectric layer of trisilicontetranitride (Si_3N_4) and afterwards with photoresist. This resist is developed and removed exactly above the platinum electrodes, exposing them. The Si_3N_4 layer is then removed so that the electrodes are freely accessible from the top and isolated at the borders by Si_3N_4. Finally, the residual resist is removed and the array is coated with streptavidin-derivatised agarose. The agarose layer supports the immobilisation of biotinylated molecules on the surface of the array.

Nucleic acid arrays are produced by covering the surface with a solution of biotinylated oligonucleotides. These are specifically transported to the streptavidin-containing spots by applying a positive voltage to each of the electrodes in turn. Immobilisation is achieved by the high binding affinity of the streptavidin/biotin interaction (K_D = 10 – 15 M). At the time of writing, chips with up to 400 electrodes are under development for genetic analyses, while for expression analyses there are plans for arrays with 1,000 – 10,000 electrodes.

The Nanogen Corporation holds more than 40 patents related to their system for production of DNA microarrays, including methods for the reading of hybridisation events on the chip surface using planar waveguide technology. Some of the more important patents held by Nanogen include one on methods for fabricating multi-component devices for molecular biological analysis and diagnostics [113], one on multicomponent devices for molecular biological analysis and diagnostics [114] and a patent on active programmable electronic devices for molecular biological analysis and diagnostics [115].

An advantage of these arrays is that the efficiency of hybridisation can be increased by applying an electric potential to the electrodes during addition of the sample solution [116]. Detection is by means of optical systems including planar waveguide technology, as noted above.

5.1.6 *XNA on gold™*

Interactiva Biotechnologie (Ulm, Germany) has developed a biochip suitable for all common biotinylated biomolecules (XNA on gold™). Using thin film immobilisation technology, a self assembling monolayer of long chain thioalcanes is covalently bound via sulfur atoms to a 100 nm layer of 24 carat gold fixed by vapour deposition to a microscope glass slide. Biotin is covalently linked to the thioalcanes and saturated with streptavidin. At the free binding sites, biotinylated molecules, which may be nucleic acids, polysaccharides, peptides, lipids or other biomolecules may be attached to the streptavidin. Prior to immobilisation of the probes, the surface of the chip is covered with a hydrophobic Teflon layer by photolithographic thick film technology in order to avoid cross contamination. This results in a pattern of cavities, with a depth of 50 µm and a diameter of 1.5 mm. The thioalcane layer is applied within these cavities. The aqueous solutions of biomolecules collect in the cavities due to the hydrophobic nature of the surrounding surface of the array.

At the time of writing, 2 sets of 96 probes can be immobilised on a glass slide measuring 75 mm × 25 mm. Arrays containing 2 sets of 384 probes are currently under development. Detection is by means of chemiluminescence, fluorescence or autoradiography.

5.1.7 *Photolithographic removal of protective groups using chrome/glass masks*

Affymetrix (Santa Clara, California, USA) has developed a procedure [22] for synthesising oligonucleotides *in situ* based on the techniques used in semiconductor production. Glass is used as a carrier surface and is first covered by a photo-instable protective layer. Using classical photolithographic techniques, a mask is used to define areas on the surface of the array that are chemically activated by ultraviolet light. The activated areas are incubated with a selected nucleotide that reacts with hydroxyl groups that have now become accessible at the surface of the array. A second mask is then used to produce activation of other areas, which are then free to react with other nucleotides. The nucleotides added contain a photosensitive protective group at the 3´-position of the ribose subunit. Thus, by repeated covering with different masks, followed by photoactivation and incubation with protected nucleotides a compact lawn of defined oligonucleotides is produced on the surface of the chip. More than 65,000 different oligonucleotides with a length of 8 nucleotides can be produced by this procedure within 32 cycles of synthesis by skilful selection of the 32 masks. The

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synthesis of a complete chip with oligonucleotides of 25 bases requires the use of 80 different chrome/glass masks in 80 synthesis cycles. The arrays can only be used once and are relatively expensive. A surface of 1.3 × 1.3 cm contains up to 300,000 oligonucleotides.

The photosensitive protective groups, which have been developed by Affymetrix for the synthesis of oligonucleotides are characterised by the following formula Ar-C(R1)(R2)-O-C(O)-X. Ar is an optional substituted, condensed polycyclic aryl-group, a heteroaromatic group or a vinyl-like derivative of those groups. R1 and R2 are independent hydrogen groups, an optional substituted alky-, alkenyl-, alkynyl-, aryl-group or vinyl like derivative of those. X represents a group to be split off, a chemical fragment that will be coupled to the Ar-C(R1)(R2)-O-C(O)-group by a heteroatom or a solid surface. The most suitable arrangement for oligonucleotide synthesis is when Ar is a condensed polycyclic aromatic hydrocarbon and R1 and R2 are electron donors. Affymetrix prefers to use pyrenyl-methyl-oxy-carbonyl- (PYMOC) with an aromatic pyrenyl group or α-methyl-*o*-nitro-piperonyl-oxy-carbonyl (MeNPOC) and hydrogen substituted R1 and R2 groups [117]. The Affymetrix company holds an impressive 142 US, and numerous international, patents granted in the last 5 years in relation to the process of photolithography, array manufacturing, hybridisation detection and analysis including bioinformatic technology. In this review, we can therefore only highlight some of what we consider to be among the more important patents from this company. These include the following:

- patents awarded for photolithographic and other means for manufacturing arrays [118-123]
- a patent for support-bound probes and methods for their analysis [124]
- a patent for a method to detect gene polymorphisms and monitor allelic expression using a probe array [125]
- a patent on a method for computer-aided nucleic acid sequencing [126]
- a patent on methods and compositions for the multiplex amplification of nucleic acids using predetermined ratios of primer pairs in the polymerase chain reaction [127]
- a patent on a method for synthesis of immobilised polymer on a very large scale [128]
- a method for comparing and identifying differences in nucleic acid sequences using surface-bound sequence-specific recognition probes [129]
- a patent for a query system for a gene expression database [130]
- a patent on a method for mapping the regulatory relationship among genes by massively parallel monitoring of changes in gene expression [131]
- several patents relating to the development of a functional microfluidic station [132-134] and a miniaturised nucleic acid diagnostic device [135-137]
- general patents relating to the principle of high-density microarray technology [138,139]

Beier and Hoheisel reported that 2-(2-nitro-phenyl)propyloxycarbonyl (NPPOC) residues may be used as rapidly detachable photo-protecting groups with higher coupling efficiencies than conventional MeNPOC groups [23-25]. The enhanced photocleavage of the NPPOC-groups takes place via base-assisted β-elimination. This group has disclosed a patent for DNA sequencing by hybridisation [140].

The main disadvantages of the photolithographic method is the high cost of masks and the coupling efficiency of 95% per cycle, which places an effective limitation of the size and quality of the oligonucleotides. With 25-mers, for example, the yield of correctly synthesised oligonucleotides is only ~ 30%. In companies such as Affymetrix, much effort is currently being devoted to improving the efficiency of the coupling reaction.

5.1.8 *Photolithographic removal of protective groups using digital micromirrors*

Scientists of the University of Wisconsin, USA, reported a photolithographic technique that no longer requires expensive masks [26,141,142]. Using the same technology found in liquid crystal display projectors (Texas Instruments, Dallas, Texas, USA) the investigators manufactured a virtual mask with 480,000 digitally steered micromirrors with an edge length of 16 µm. These mirrors can be used to exactly photosensitise a predefined point on the array by ultraviolet light. In this way, exact areas of deprotection can be achieved in order to allow exact *in situ* oligonucleotide synthesis. With this procedure, tens of thousands of 16 µm × 16 µm features can be produced. In theory, up to 480,000 different nucleotides can be synthesised on an array measuring 1 cm^2. Arrays with up to two million features have been developed using Texas Instruments high-definition television mirror arrays. If we assume that ~ 40 features are needed to effectively measure the expression of a single gene, then the expression of ~ 50,000 different genes could be simultaneously measured using a single array. This micromirror technology is also implemented in the Geniom 1 device (Febit AG, Mannheim, Germany). Febit have disclosed over 40 patents in this field including one for a novel strategy for synthesising polymers on surfaces [143], a method and device for the integrated synthesis and analysis of analytes on a support [144] and several inventions relating to the chemistry of photolabile protective chemical groups [145,146].

5.1.9 *Photoresist wet masking*

Prompted by the relatively low 95% coupling efficiency of conventional photolithographic methods using solid-phase synthesis chemistry with photosensitive protection groups, Affymetrix and the IBM research centre in Almaden have cooperated in developing a procedure using the so called photoresist technique that uses conventional DNA solid-phase chemistry while achieving a resolution in the range of optical lithography (< 1 µm) [27]. In this single resist process, a surface coated with N,N-bis(hydroxyethyl)aminopropyltriethoxysilane is protected by the phosphoramidite

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group 4,4′-dimethoxytritylhexaethylenglycol(2-cyanoethyl-N,N-diiso-propyl)phosphoramidite and then completely covered by a photoresist layer similar to that used in semiconductor and microsystem technology. Immediately after exposure to ultraviolet light through a photolithographic mask, the resist in the exposed areas is developed and removed. This generates a pattern of openings with an edge length of 1 µm, through which the reagents can reach the surface and remove the phosphoramidite groups. In the next step, preformed oligonucleotides are allowed to bind to the free hydroxyl groups on the chip. Re-exposure, development and removal of the resist uncovers new areas on the chip, which are available for subsequent reactions with newly added oligonucleotides.

The use of a photoresist increases the contrast between the exposed and protected areas by reducing the border effects caused by scattered light, thus increasing the efficiency of oligonucleotide synthesis in these areas.

Nevertheless, photoresist procedures exhibit only moderate rates of synthetic efficiency. This is because some of the reagent used to remove the protection groups reacts with a part of the phosphoramidite residues embedded within the resist. In addition, the chemicals used for development and removal of the resist may interfere with subsequent synthetic steps.

One way around this has been to insert an inert polymer layer between the surface of the substrate and the resist in order to reduce the possibility of adverse reactions with the oligonucleotides embedded in the polymer (bilayer resist procedure). The polymer layer reduces optical resolution but allows total recoveries of ~ 90%. A liquid crystal display projector may also be used here to avoid the need for masks when removing the photo-resist [28].

5.1.10 *Micro wet printing*

The micro wet printing (µWP) technique was developed by Ermantraut *et al.* based on a wet masking technique [29] and is used by the German chip company ClonDiag Chip Technologies in Jena. A printing cartridge with a silicon frame is used to position a mask on the surface of the array. The combination of the silicon frame and glass manifold creates a system of channels connecting areas on the surface of the array to the mask in the printing cartridge. The correct alignment of cartridge and chip surface is achieved by the presence of complementary position markers on each surface. After alignment, the areas on the surface of the substrate are accessible only to reagents located below the openings in the mask. The reagents and a washing solution are passed through a meander to the array surface. The mask is then removed and a new one is positioned for a further cycle of synthesis. By alternating addition of nucleotide derivatives and the use of new masks with varying patterns of the openings, oligonucleotides can be synthesised at defined positions on the surface with a spot size in the range of 1 µm. This technique may also be used to deposit preformed biomolecules including nucleic acids and proteins at particular locations on the surface of the substrate. ClonDiag Chip Technologies have claims on a method for producing structured, self-organised molecular monolayers of individual molecular species [147] and also claim a method for the detection of molecular interactions based on the detection and quantification of molecular interactions, using for example, silver staining [148].

The phosphoramidite technique used for the synthesis of oligonucleotides with the µWP procedure and dispenser-mediated *in situ* synthesis was first described by Robert Letsinger and developed further by Marvin Caruthers as a solid-phase technique more than 20 years ago [30,31]. It supports an automated fast oligonucleotide synthesis and is used by many companies.

6. Resequencing by hybridisation

Use of high-density oligonucleotide arrays for resequencing by hybridisation allows the identification of mutations and single nucleotide polymorphisms (SNPs) in known gene sequences. Oligonucleotides complementary to certain parts of the gene are fixed to the DNA chip. After amplification and labelling, the sample to be investigated is hybridised to the oligonucleotides on the chip. Use of an array that contains oligonucleotides complementary to each of the four bases at each position effectively allows an unknown DNA sequence in the sample to be determined based on the hybridisation pattern alone. At present, sequencing by means of DNA arrays is limited by difficulties in optimising hybridisation conditions, so that confirmation of any mutations found is necessary before this technique can be used for medical diagnostic purposes. Moreover, this technique is prone to error if multiple mutations or complex sequence deviations such as insertions or deletions are present. For these reasons, conventional sequencing is currently a more efficient method. In the future, of course, this situation may change with advances in array design and progress in bioinformatics. One of the many patents held by the Affymetrix corporation relates to the array-based sequencing of the cystic fibrosis gene [149].

7. Detection of DNA binding to microarrays

Much effort has been applied to methods for the detection of hybridisation events on DNA microarrays. For example, the ViaLogy Corporation has disclosed a neural-network based method for identifying mutations based on the so called resonance between the pattern of fluorescence that is expected when a particular set of mutations is present and that which is actually obtained. They claim that this method is robust even when the signal to noise ratio is low [150,151]. NeXstar Pharmaceuticals have disclosed an ingenious method for detection of binding on a microarray or other surface by means of so-called aptamers, which bind to molecular beacons only in the presence (or absence) of target molecules [152,153]. In a further interesting application, Clinical Micro Sensors, Inc. have disclosed a patent for the detection of target sequences by use of nucleic acids that have been modified by addition of electron transfer

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moieties to act as detection electrodes [154]. Several patents relating to nucleic-acid mediated electron transfer have also been awarded to the California Institute of Technology [155,156].

8. Analysis of gene expression using DNA microarrays

To analyse gene expression, nucleic acids that match the sequences of the mRNAs to be measured are applied to the surface of the array. In general, it is not useful to directly apply the mRNA from the tissue under investigation to the chip, as no satisfactory detection system is available for unlabelled nucleic acids. For that reason, the mRNAs are usually reverse transcribed to cDNA. Because of the low efficiency of the reverse transcription process and the small amount of starting material that is usually available from sources such as tissue biopsies or blood samples, the amount of cDNA obtained is too low for most applications. For this reason, the cDNA must be amplified. As far as possible, amplification methods should be used that do not alter the mutual ratios of the nucleic acid species present in the sample. Two techniques are commonly used, either a polymerase chain reaction (PCR) with a low number of cycles or *in vitro* transcription. In the case of *in vitro* transcription, a promoter for RNA polymerase is added to the cDNA strand during reverse transcription of the mRNA from the sample. The cDNA is then amplified by a factor of ~ 1000 using RNA polymerase. Finally, the RNA molecules produced are reverse transcribed once more to labelled cDNA prior to hybridisation with the array.

Two procedures are available for the estimation of gene expression and, in particular, for the estimation of differences in gene expression between two different samples. In the first procedure, labelled nucleic acids from the two samples are hybridised to two identical arrays. In order to normalise the signal intensity of single genes, internal standards and complex statistical methods are used. Usually, housekeeping genes that have a steady rate of expression are used as internal standards. In addition, the mean signal intensity is calculated and used for normalisation. Differences in normalised signal intensities indicate different rates of expression. The second procedure for comparison of gene expression requires only one chip. In this case, the two samples are labelled differently, usually by means of two different fluorophores, Cy3 with an excitation wavelength of 548 nm and an emission wavelength of 562 nm and Cy5 with an excitation wavelength of 646 nm and an emission wavelength of 664 nm. Hybridisation on the chip is performed using a 1:1 ratio of labelled nucleic acid probes. Genes with identical levels of expression are represented by a mixed colour, while with genes with differing rates of expression, one of the fluorophores dominates over the other.

The procedure described above is usually performed for arrays consisting of polymerase chain reaction products or plasmids. The simple production process is an advantage of this method. However, the inhomogeneity of hybridisation kinetics in a mixture that contains such a variety of different nucleic acid molecules is a disadvantage, the reason being that the conditions for hybridisation, such as temperature and salt concentration, are never optimal for all molecules. As a result, unspecific or incomplete hybridisation may occur leading to incorrect results. In addition, since the nucleic acids affix to the substrate in random configuration, many will not be available for hybridisation with DNA in the sample for steric reasons.

A solution to this dilemma lies in the use of oligonucleotide microarrays comprising nucleic acid probes ~ 25 bases in length. These oligonucleotides can be selected for their chemical properties so as to ensure optimal hybridisation. Affymetrix have developed one solution to this problem. At the time of writing, their array used 20 different oligonucleotides for each mRNA to be monitored. These 20 oligonucleotides on the chip are exactly complementary to 20 different sequences within the mRNA and are therefore called perfect match oligonucleotides. Since the melting temperature of the hybrid of the chip oligonucleotide and the sample DNA can be calculated on the basis of the sequence, oligonucleotides with the best hybridisation properties can be selected based on the mRNA sequence of the gene whose expression is to be studied. Thus, even if certain oligonucleotides hybridise poorly or not at all, sufficient remain to enable the results of the analyses with the chip to be interpreted correctly. In addition to the 20 perfect match oligonucleotides, the Affymetrix chip also contains 20 mismatched oligonucleotides that differ from the perfect match sequences by the presence of a single mismatched base in the centre of the oligonucleotide. As a negative control, in addition to each of the perfect match oligonucleotides, a mismatched oligonucleotide is bound to the chip. The mismatched oligonucleotide differs by one base in the middle of the oligonucleotide. Ideally, there is no hybridisation under optimal conditions to the mismatched oligonucleotide so that signals must derive from unspecific hybridisation. The differences in signal intensity allow evaluation of nonspecific binding. An algorithm calculates the corrected intensities for the perfect match oligonucleotides that are compared to the intensity of a second chip hybridised to another probe. The final reported difference in the amount of an mRNA between two samples results from integration of the results of comparison of all 20 oligonucleotides representing that mRNA.

Oligonucleotide-based microarrays have proven to be a very powerful means of analysing gene expression and are able to analyse the expression of a very large number (~ 10^3 – 10^4) of genes at once. Nevertheless, cDNA-based microarrays have advantages over oligonucleotide-based arrays in terms of sensitivity and specificity and are the method of choice for analysing the simultaneous expression of a smaller number (~ 10^2) of genes.

9. Use of microarrays for gene expression profiling in cancer

The heaviest use of gene expression profiling by means of DNA microarrays has, to date, been in the field of cancer research. A search of PubMed using the phrases ‘cancer’ and

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'expression profiling' yields more than 600 hits. In most of these studies, DNA microarrays, serial analysis of gene expressions (SAGE) or differential display reverse transcriptase PCR (RT-PCR) were used. Due to its ease of use and the high technical demands of SAGE and differential display RT-PCR, DNA microarrays become more and more popular for gene expression analysis. So far, gene expression profiling using DNA microarrays have been used to study, for example, breast [32-35], colon [36], prostate [37,38] and pancreatic [39,40] cancers and lymphomas and leukaemias [41-44]. In no other field of cancer research has so much progress been made in using gene expression profiling for classification and predicting outcome than in the case of lymphoid malignancy. For this reason, the following section will concentrate on applications of microarray technology limited to lymphomas and leukaemias.

9.1 The need for improved classification of leukaemia and lymphoma

The prognosis and response to therapy of leukaemias and lymphomas can vary widely between classes, but classification is often difficult, if not impossible, on the basis of morphology alone. Thus, a classification of these illnesses based on their gene expression therapy may be expected to improve survival rates and patient care.

9.1.1 Human acute leukaemias

In pioneering work, Golub *et al.* described a generic approach to classification of human acute leukaemias based on gene expression monitoring by DNA microarrays [45]. A class discovery procedure was developed that automatically detected the distinction between acute myeloid leukaemia and acute lymphoblastic leukaemia without previous knowledge of these classes. In addition, an automatically-derived class predictor was able to determine the class of new leukaemia cases. These results demonstrated the feasibility of cancer classification based solely on gene expression monitoring and suggested a general strategy for discovering and predicting cancer classes for other types of cancer, independent of previous biological knowledge.

9.1.2 Large B cell lymphomas

In further pioneering work by Alizadeh *et al.*, a systematic characterisation of gene expression in B cell malignancies was conducted using DNA microarrays [46]. Gene expression was found to be diverse among large B cell lymphomas, reflecting differences in tumour proliferation rate, host response and differentiation state of the tumour. Two molecularly distinct forms of diffuse large B cell lymphoma were identified with gene expression patterns indicative of different stages of B cell differentiation. One form expressed genes characteristic of germinal centre B cells (germinal centre B-like diffuse large B cell lymphoma) and the second expressed genes normally induced during *in vitro* activation of peripheral blood B cells (activated B-like diffuse large B cell lymphoma). This classification aided in predicting clinical outcome, patients with germinal centre B-like diffuse large B cell lymphoma had a significantly better overall survival than those with activated B-like diffuse large B cell lymphoma [46,47].

Hastie *et al.* developed a statistical method known as gene shaving [48]. This method identifies subsets of genes that vary widely in expression under different circumstances, but nevertheless display a coherent pattern of expression. Gene shaving differs from hierarchical clustering methods in that the genes identified may belong to more than one cluster. In addition, the clustering of genes by the gene shaving method may be supervised by an outcome measure. In diffuse large B cell lymphoma, gene shaving identified a small cluster of genes whose expression is highly predictive of patient survival.

In a more recent study, Khan *et al*. developed a method for classifying cancers based on their gene expression signatures using artificial neural networks [49]. The authors trained neural networks using small, round blue-cell tumours of childhood as a model. These types of cancer can be divided into the four distinct categories of neuroblastoma, rhabdomyosarcoma, non-Hodgkin's lymphoma and the Ewing family of tumours, but are often difficult to diagnose in clinical practice. The artificial neural network correctly classified all samples and identified the genes most relevant to the classification. In order to test the ability of the trained artificial neural networks models, the authors analysed blinded samples that had not been used for training; in all cases, tumours were correctly classified.

In a further recent approach, Shipp *et al*. analysed the expression of 6,817 genes in diagnostic tumour specimens derived from diffuse large B cell lymphoma patients who received cyclophosphamide, adriamycin, vincristine and prednisone (CHOP)-based chemotherapy [50]. A supervised learning prediction method was developed to classify samples in the groups cured and fatal or refractory disease. The algorithm was able to identify 2 categories of patients with very different 5 year survival rates of 70% and 12%, respectively.

A further analysis procedure for classifying human tumour samples and predicting disease outcome, based on microarray-based gene expression data, were presented by Nguyen & Rocke [51]. Their procedure involved dimension reduction using partial least squares and classification using logistic discrimination and quadratic discriminant analysis. They applied these methods to five different microarray data sets involving various human tumour samples:

- normal versus ovarian tumour
- acute myeloid leukaemia versus acute lymphoblastic leukaemia
- diffuse large B cell lymphoma versus B cell chronic lymphocytic leukaemia
- normal versus colon tumour
- non small cell lung carcinoma versus renal samples [52]

Rosenwald *et al*. analysed gene expression profiles in biopsy samples of diffuse large B cell lymphoma derived from 240 patients using DNA microarrays [42]. Subgroups with distinctive gene expression profiles were defined on the basis of a

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hierarchical clustering procedure. Three subgroups of gene expression profiles were identified:

- germinal centre B cell-like
- activated B cell-like
- Type 3 diffuse large B cell lymphoma.

Two common oncogenic events in diffuse large B cell lymphoma, *bcl-2* translocation and *c-rel* amplification, were detected only in the germinal centre B cell-like subgroup. Patients in this subgroup had the highest 5 year survival rate. To identify molecular determinants of disease outcome, the authors searched for individual genes with expression patterns that correlated with survival in the preliminary group of patients. Most of these genes fell within four gene-expression signatures characteristic of germinal centre B cells, proliferating cells, reactive stromal and immune cells in the lymph node or major-histocompatibility-complex class II complex. Of these, 17 were used to construct a predictor of overall survival following chemotherapy [53].

These studies impressively demonstrated the potential of gene expression profiling using DNA microarrays combined with new bioinformatic approaches. In the field of lymphoma, a number of second-generation chips for investigation of lymphoma-specific gene expression have now been developed [54,55].

Rosetta Inpharmatics have disclosed a number of methods for monitoring disease states and therapies using gene expression profiles [157] and have also disclosed a method to enhance detection and classification of patterns of gene expression on the basis of detecting coregulated gene subsets [158].

9.1.3 *T cell lymphomas*

Using cDNA microarrays, Li *et al.* compared the expression of ~ 8000 genes between two unique, clonally related T cell lines derived from different stages of a progressive T cell lymphoma involving skin [56]. One hundred and eighty genes were found to be differentially expressed at the RNA level by a factor of fivefold or greater. Compared with the cells from the earlier, clinically indolent stage of lymphoma, 56 genes were upregulated, whereas 124 genes were downregulated in the cells from the advanced, clinically aggressive lymphoma. The functions of ~ 65% of these genes are currently unknown. The 22 genes with a known function that were upregulated in the advanced lymphoma cells included several genes involved in promotion of cell proliferation and survival as well as in drug resistance. The 42 functionally characterised genes that were downregulated in the advanced lymphoma cells included negative regulators of cell activation and cell cycle and mediators of cell adhesion, apoptosis and genome integrity. The differential expression identified by the cDNA microarray analysis was confirmed for selected genes by RT-PCR and Northern blotting. The identified differences in gene expression may be related to the differences in behaviour between the early and advanced stages of the T cell lymphoma and point to directions for further investigation into mechanisms of lymphoma progression.

10. Use of DNA microarrays in pharmacology

One of the major areas of research in pharmacology at the present time is in the field of pharmacogenomics, the correlation of therapeutic response or side effect profile to an individuals genetic background. Several approaches are currently being exploited. One involves the collection and annotation of large numbers of single nucleotide polymorphisms spread throughout the genome and correlation of the presence of sets of polymorphisms with therapeutic response. A second approach involves the correlation of therapeutic response or side effect profile with the presence of polymorphisms in the genes encoding the cytochromes that are involved in drug metabolism. A third approach is the measurement of gene expression patterns as part of drug discovery programmes. DNA microarrays now play a major role in all these approaches to providing more individualised pharmacological treatment.

11. Future trends

In some respects, DNA microarrays may be regarded as a biological equivalent to computer chips. Thus, the future of microarray analysis will be characterised by the increase in information density and the fall in price seen with their silicon cousins. In addition, DNA microarrays will increasingly be utilised in integrated analytical and diagnostic systems, where all steps from sample preparation through assay to analysis and interpretation will be performed in cheap disposable cartridges or in high-throughput microfluidic devices (lab on a chip). If current trends continue, it is possible that DNA microarrays will soon leave the research arena to become part of the routine medical diagnostic armamentarium.

12. Expert opinion

The use of DNA microarrays holds great promise for genomic research and diagnostics and for improving the effectiveness and reducing the side effect profile of pharmaceuticals. The key to the success of this technology is its massively parallel architecture, enabling it to detect and quantify thousands of hybridisation events at the same time. Further advantages are the great scope for miniaturisation, for high-throughput applications and for development of integrated, automated systems (lab on a chip). Although still fairly expensive, there is likely to be a rapid fall in cost in the coming years, similar to that seen in the semiconductor industry.

At the time of writing, the main area of application of this technology has been in the field of analysing gene expression in the research setting, although clinical applications may be developed in the near future. These will include the determination of

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genetic polymorphism profiles as a marker of disease susceptibility, customised low and medium-density gene expression arrays for diagnostic purposes and pharmacogenomic arrays to better target pharmacological treatment as part of the general trend towards more individualised medicine. Other important applications are likely to be in the field of diagnosis of infectious disease.

The very high degree of patenting activity in this field is a reflection of the diversity of use of this exciting technology.

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