11 DNA Array

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11.1 INTRODUCTION

DNA arrays are already making their impact felt in all spheres of life. These systems, together with faster, more powerful computer and software platforms, are applied not only in basic science but also in medicine and industry. They are, in principle and practice, extensions of hybridisation-based methods which have been used for decades to identify and quantify biocompounds in samples. The idea of using ordered arrays of probes to carry out parallel hybridisation studies is also not in itself new. However, many parallel advances have occurred to transform the standard methods into much more handy and efficient ways to perform parallel genetic analyses (Cobb, 2006; Jayapal and Melendez, 2006).

The term of 'DNA array' originates either from the nature of immobilised capturing compound, or from the type of material being analysed. In that order, 'DNA array' refers to: (1) a system functionalised with nucleic acid sequences (of different length synthesised ex situ or in situ, such as oligo-/polynucleotides, complementary DNAs (cDNAs), PCR products, bacterial artificial chromosome (BACs), etc.) dedicated for analyses of distinct molecules such as proteins, peptides, nucleic acids, and others; and (2) a system referred to as DNA-oriented array, devoted to analyses of definite molecules which are nucleic acids, nothing else.

DNA arrays are small, solid supports, usually glass microscope slides, silicon chips or membranes with different carriers, on to which a huge number of compounds are immobilised or attached at fixed locations (Gabig-Ciminska, 2006; Southern *et al.*, 1999). The American Heritage Dictionary defines 'array' as 'regular order or arrangement'. This is reflected in the DNA array system, where the capturing compounds are attached to allow support in an orderly and fixed way, and users may utilise the location of each spot in the array to identify a particular target material. The original concept of DNA array comes from a computer chip that can execute millions of mathematical calculations in a short period. Still, it is hard to imagine that the DNA array with a surface area smaller than a fingernail, stands for the collection of a huge number of miniaturised tests, which may be undergone quickly and simultaneously. To generate high-quality and reliable data, proper care must be taken in all steps of a DNA array experiment, i.e. in sample preparation, microarray manufacturing, hybridisation, image scanning, data analysis, mining and

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management. The sensitivity, reproducibility and throughput of DNA arrays are enhanced via standardisation through automation. This eliminates multiple variables of the tests and consequently increases quality and consistency of the results.

The aim of this chapter is to present current, state-of-the-art and recent advances in DNA array technology to readers. The chapter is structured as follows. In the first instance the history and principles of DNA arrays are presented. Next, DNA array structure and operating rules, including their production process and functioning, are described, followed by a section on DNA array handling. Finally, showing trends and directions of future research, practical values and possible fields of its application are put forward.

11.2 HISTORY – FROM DOUBLE HELIX VIA BLOT TO DNA ARRAY

It may be a proper time to write a history of DNA arrays because this technology is no longer new and most likely has more of a past than a future. DNA array episodes in the second half of the 1990s became a new biotechnical revolution of equal importance as the decoding of DNA structures that took place in the 1950s; researching fundamental laws of molecular genetics, such as genetic code and the major dogma of molecular biology, took place in the 1960s; discovering reverse transcription, creating first recombinant constructions took place in the 1970s; and research of enzyme methods manipulations with genetic material *in vitro*, such as amplification technologies, took place in the 1980s. During this period, two major achievements in the field of molecular biology are worthy of note. The first was when in the late 1960s scientists carried out hybridisation reaction on cells fixed to microscopic slides (*in situ* hybridisation, SH) (Southern, 2001); the second, introduced by Ried *et al.* (Grody 1999) and Balding and Ward (Geller *et al.*, 1997), consisted of the multicolour fluorescent labelling techniques for analysis of multiple probes (fluorescent *in situ* hybridisation, FISH).

DNA array technology evolved from Southern blotting, where DNA fragments bound to a solid substrate are probed with a known sequence. However, more relevant to DNA arrays were the methods of dot-blotting, the technique of analysing multiple hybridisation targets in parallel by applying them to a filter in a defined pattern giving an array. The subsequent automation and miniaturisation of the dot-blot method showed how duplexing could be used on a large scale. Saiki and his co-workers introduced a variant, the *reverse dot blot*, in which multiple probes are attached as an array to the membrane and the free-floating target to be analysed is labelled (Saiki *et al.*, 1989).

During this period, organic chemistry has also undergone a revolution, initiated by the introduction of solid-phase synthesis (Merrifield, 1969). This has made an impact on molecular biology by the development by Letsinger *et al.* (1975) and Beaucage and Caruthers (1981) of methods that were suitable for the solid-phase synthesis of nucleic acids. The employment of a collection of distinct DNAs in the format of arrays was first described by Kulesh *et al.* (1987). The arrayed DNAs were used for transcription profiling to identify gene expression modulated by interferon. These premature gene arrays were developed by spotting bacterial cDNAs on to filter paper with the use of a pin-spotter and were read out autoradiographically. We have come a long way since the first official meeting of scientists working in the field of DNA arrays (Cantor *et al.*, 1992), which took place in Moscow in 1991, comprising about 50 scientists from Europe and the United States. The initial aim was the development of a technology for high-throughput sequencing

of the human genome, named sequencing by hybridisation (SH). At that time, first photolithographic printing by Affymetrix (California, USA) was performed.

The field quickly branched out, however, in terms of methodology and application. In 1994 first cDNA collections were developed at Stanford, California, USA (Schena *et al.*, 1995). The use of miniaturised DNA arrays for gene expression profiling was reported in 1995 (Schena *et al.*, 1995). The arrays were prepared by automatic printing of cDNAs on to glass slides. Wide gene expression monitoring was achieved by simultaneous, two-colour fluorescence hybridisation. This work resulted in publishing a complete eukaryotic genome of *Saccharomyces cerevisiae* (Lashkari *et al.*, 1997). Similar cDNA microarrays with higher densities have been used for the gene expression analysis of human cancer cells and on a genomic scale (DeRisi *et al.*, 1996, 1997). At that time, Affymetrix commercialised their high-density oligonucleotide microarrays as a tool for genome-wide characterisation of expression levels (Lockhart *et al.*, 1996; Wodicka *et al.*, 1997). In 2000 molecular profiling of breast cancer was achieved, while three years later DNA arrays were introduced into clinical practices. Whole human genome on one microarray was obtained in 2004. In just a few years advances in the development of alternative techniques to DNA array approach, and also progress in miniaturisation, robotic and informatics of DNA arrays, have permitted the hosting of more than 250 000 or 1 000 000 different features (spots) into a single square centimetre slide (Maughan *et al.*, 2001; Szczepanek *et al.*, 2007). Nowadays, DNA arrays seem to be a common product in biological sciences and in medicine.

11.3 PRINCIPLE

The core principle behind DNA arrays is the feature of the DNA duplex resulting from a process named *hybridisation*. The 'key and lock' principle of hybridisation utilised in DNA array analysis exploits the property of matching nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. It is notable that a molecule of such large structural complexity can reassemble with perfect fidelity from the separated sequences (Schena *et al.*, 1995; Southern, 2001).

In a typical application, *probe* chains are attached to a solid substrate and nucleic acid *target* sequences of interest are isolated from a biological sample using standard molecular biology protocols. The term *probe* (also *capture* or *capturing probe*) is used for the nucleic acids of known sequence, which is attached to the matrix, and the term *target* describes the sequence or collection of sequences to be analysed (Southern *et al.*, 1999). The target sequences are fragmented and labelled with reporter molecules for detection, and the mixture of labelled sequences ('key') is applied to the array, under controlled conditions, for hybridisation with the surface probes ('lock'). Sequence complementarity leads to the hybridisation between two single-stranded nucleic acid molecules, one of which is immobilised on a matrix (Southern *et al.*, 1999). However, a high number of nucleotide sequences results in noncovalent binding between the two strands; therefore after washing off nonspecific bonding sequences, only strongly paired strands will remain hybridised. As the labelled target sequences bound to probe chains generate a signal, the array is then imaged with a reader to locate and measure the binding of target sequences from the sample to complementary probe on the array, and software collects the data and presents them in a format determined by the application. In this way, highly parallel hybridisation assays on miniaturised flat substrate are possible.

There are different names for the DNA arrays, such as DNA chips, bio-arrays, DNA-tables, gene-arrays, etc. The array, described as a macro-array or micro-array, can be defined as an ordered set of macrospots or microspots, respectively, with every spot (feature) containing single defined species of a nucleic acid chain. Large collections of these nucleic acid probe sequences are immobilised in addressable locations on the surface of a solid support capable of accessing large amounts of genetic information from biological samples in a sole hybridisation assay. Each feature corresponds to conventional analysis in a test tube. The matrix of spots applied to the array reproduces each of these analyses several times and thus increases the reliability of the test results.

There are two types of arrays, in terms of the property of arrayed nucleic acid probe sequence with known uniqueness: Format I – DNA arrays and Format II – oligo-/polynucleotide arrays (Lockhart *et al.*, 1996). As already stated in the Introduction, these two types of arrays are often described as DNA arrays and are dedicated for analyses of distinct molecules. Fundamental differences exist between these two variants of arrays. Moreover, there are two basic directions of array creation. Chronologically the first one was the technique of housing on arrays nucleic acid probes that are previously synthesised oligo-/polynucleotides or DNA fragments (ex situ synthesis). Further direction was in situ synthesis of nucleic acid probes (i.e. oligo-/polynucleotides) using photolithography or piezoelectric printing that employed inkjet printer-like technology (Southern, 2001).

The nucleic acid arrays (NA arrays) range from small custom arrays (*low-density arrays*) designed, for example to monitor the expression of a few hundred genes or to examine the presence of sequences of interest, to comprehensive arrays (*high-density arrays*) that represent tens of thousands of genes or entire genomes.

11.4 DNA ARRAY STRUCTURE AND OPERATING RULES

11.4.1 Array fabrication technology

An integrated circuit (IC), in electronic technology commonly referred to as a chip or microchip, is a miniaturised array of electronic circuits constructed of a *semiconductor* substrate, usually one of single-crystal silicon. It is called an integrated circuit because the components, circuits and base material are all made together, or integrated, out of a single piece of silicon (Malone, 1995). The circuit is packaged in a hermetically sealed box or a nonhermetic synthetic container with leads extending from it for input, output, power-supply connections, and for other links that may be needed when the device is put to use. A different scale of integration of such circuits exists. More than 1000 transistors are present in IC with very-large-scale integration. It is worth mentioning that the number of transistors per semiconductor chip doubles every 18–24 months. Nowadays, the microprocessors chips consist of more than 100 millions of transistors per several square centimetres. The resulting enormous integration scale makes modern computers very fast, compact and relatively inexpensive.

This phenomenon may be observed not only in computer technology but in molecular biology. The use of miniaturised tools for the construction of a smart and portable device such as the spotted DNA array system offers the pharmaceutical, biotechnology and agriculture industries more efficient and economical solutions. The DNA arrays have been fabricated using photolithography on an analogous pathway as integrated circuits. In 1998, an Affymetrix array contained fewer than 1000 genes; by 2000, it boasted 12 000; while

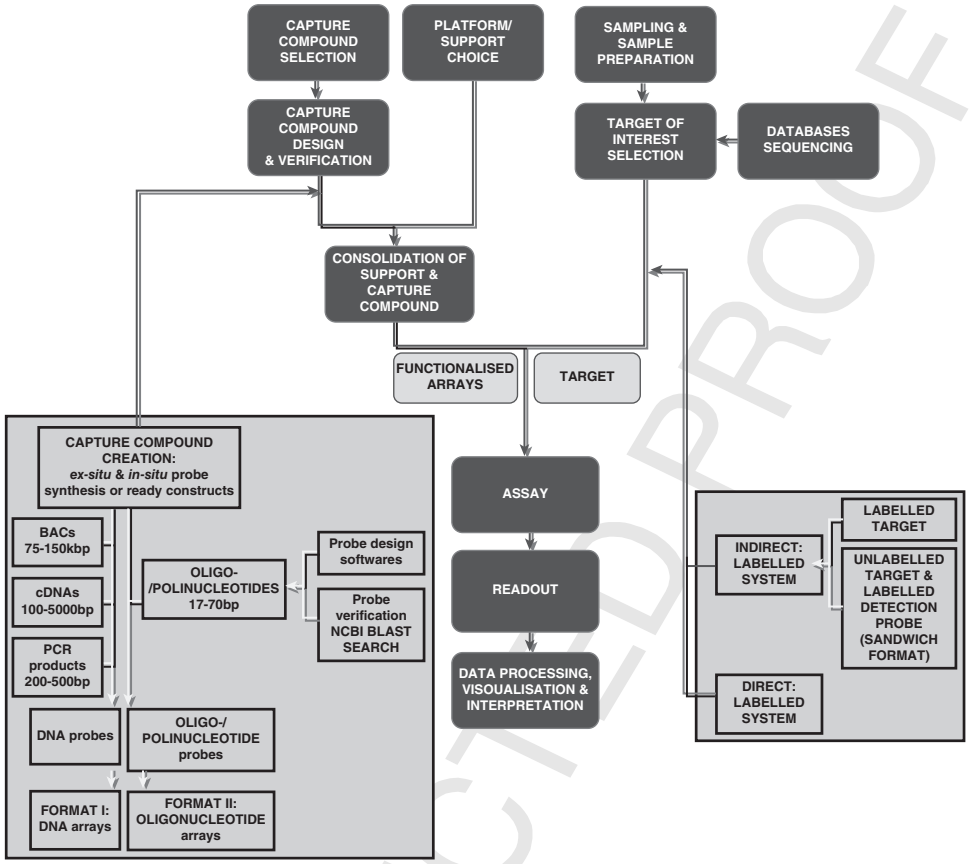

Fig. 11.1 The workflow in the implementation of a DNA array technology.

the latest product in the family of Affymetrix expression arrays offers whole-genome coverage (i.e. of the order of 28 000 genes and expressed sequence tags (ESTs)).

11.4.2 DNA array concept

One important achievement in the biological, biotechnical and biomedical area is the development of DNA array technology. Basically, DNA arrays are just parallelised blotting modules, where, in contrast to earlier techniques, the status of blotted and free-floating components is turned on its head in the analysis in order to obtain parallelism. The workflow in the implementation of a DNA array technology involves: DNA array fabrication with the prior support selection and nucleic acid probe creation, target preparation, assay performance, image analysis and data handling (Fig. 11.1). Many strategies have been investigated at each of these steps (Cobb, 2006; Gabig-Ciminska, 2006).

11.4.2.1 *Foreword – support and probe*

Construction of DNA arrays begins with the support selection. There are some factors such as accurate location of the positions of the spots, dimensional stability and rigidity of

substrate, being crucial for automated analyses with the use of DNA arrays. Permeable membranes with fragility and flexibility commonly used in standard blotting assays swell in solvents and tend to shrink when dried, which in consequence makes it impossible to use them and to draw spots with the high precision that can be achieved on a rigid supporting material. Thus, the introduction of impermeable substrates was a major step forward that offered a number of advantages.

Several materials are likely to be suitable as substrates for making arrays. Glass-based, polypropylene and silicon-based arrays are the materials of choice due to their good characteristics and properties (Southern, 2001). It was found that the nature of the support, and particularly the nature of a *linkage* between the support and the probe, has great effect on performance of the assay with the use of DNA array. In addition, an extra part present between linkage and the probe, namely *spacer*, is used. It helps to overcome steric interference, when the ends of the probes closest to the surface are less accessible than the ends further away. Nucleic acid probes on long spacers extend away from their neighbours and from the substrate surface, and thus they allow more efficient contact with the target. It has been shown that the spacer's length has a marked effect on hybridisation yield and that the optimal spacer length gives up to 150-fold increase in the yield of hybridisation (Southern *et al.*, 1999).

Silanol groups of silicate glass cannot couple nucleic acid probes directly to the surface or to most plastics. It is necessary to create the surface with a group from which the growth of the nucleic acid chain can be initiated. The glass-based arrays, most often made on microscope slides, are coated with poly-lysine, amino silanes or amino-reactive silanes, which enhance both the hydrophobicity of the slide and the adherence of the deposited nucleic acid fragment (Schena *et al.*, 1996).

At present, in the manufacturing process of DNA arrays, semiconducting materials among others are widely used. A semiconductor is a solid material whose electrical conductivity at room temperature is between that of a conductor and that of an insulator (Muller and Kamins, 1986; Sze, 1981, 2006). While at high temperatures its conductivity approaches that of a metal, it acts as an insulator at low temperatures. Semiconductors may be elemental materials such as silicon and germanium, or compound semiconductors such as gallium arsenide and indium phosphide, or alloys such as silicon germanium or aluminium gallium arsenide. Although various materials can be used in DNA array production, they are usually fabricated on wafers of single-crystal silicon, in which the silicon orientation of all of the crystal is the same. The major steps in fabricating the process of semiconducting material-based arrays include: film formation, impurity doping, photolithography, and packaging. In the film-formation step, silicon oxide, also called silica, is grown on the surface of the silicon. Patterning the film with the desired micro-features, and removing (or etching) portions of the film is obtained in the next phases. In this, a layer of photoresist is added to the oxide layer and then exposed to ultraviolet light through a mask. After exposure an etching process removes the unwanted areas of the oxide and impurity doping ads charge carriers to the silicon. Additional layers of silicon are deposited to create for instance bipolar transistors. By this means, in memory chip fabrication there are some 30 lithography steps, i.e. 10 oxidation, 20 etching and 10 doping steps are performed (Muller and Kamins, 1986; Sze, 1981, 2006).

Except for the flat-surface glass, polypropylene or silicon arrays, supporting materials such as microscopic beads, microtiter plates, microelectrode array, nanochannel glass or phototransistor arrays are also used for hosting nucleic acid probes (Gabig-Ciminska, 2006). An interesting development in the field of DNA array technology was reported by

Illumina (California, USA) (Cheng *et al.*, 2009). They used microscopic polystyrene bead-based arrays instead of the large solid support, thus offering high sensitivity, flexibility, and many replicates in one assay. Another promising approach was the use of nanochannel glass slides for array printing. Nanochannel glass materials are unique glass structures containing a regular geometric array of parallel holes or channels as small as 33 nm in diameter or as large as several micrometers in diameter (Watson *et al.*, 2000). In this case, the surface area of nanochannel glass is much greater than that of regular glass, enabling larger amounts of nucleic acid material to be deposited in each spot. The hybridisation kinetics is also significantly improved.

Nucleic acid sequence fixed to a solid substrate, a target specific compound of DNA array, is described as a *probe*. Sometimes it is also stated as a *capture* or *capturing probe* as it picks out the target of interest from a sample enabling its detection. Two commonly used types of arrays, Format I and Format II, differing in the size of the arrayed nucleic acid probe exists (Southern *et al.*, 1999). In Format I, DNA probes (e.g. cDNAs, BACs, PCR products) with relatively long molecules (100 bp–150 kb long) are placed on a substrate. The deposition of a few nano litres of purified material usually at 100–500 µg/ml generates each array dot. Based on the spotting principle, a robot arrayer uses contact or noncontact printing methods. The printing is also carried out by utilising ink-jet technology that spots a sample of DNA on to a number of matrices in a serial operation or printouts, respectively. This type of arrays is used mostly for large-scale screening and expression studies. In Format II, an array of oligo-/polynucleotide probes are fabricated either by conventional synthesis followed by on-surface immobilisation or by *in situ* chemical synthesis of oligo-/polynucleotide building up nucleotide by nucleotide each element of the array (McGall *et al.*, 1996). These arrays are designed and produced on the basis of sequence information alone, without the need for any clones, PCR products, cDNA and so on. Sequences may be longer, polynucleotides (up to 70-mer such as the Agilent (California, USA) and Illumina design), or shorter, oligonucleotides (up to 25 nt produced by Affymetrix) depending on the desired purpose. While oligo-/polynucleotides can be either in situ synthesised or *spotted* on to an array, large DNA molecules (cDNA, BAC, PCR product) can only be *spotted*. It is important to mention that arrays, both with oligonucleotide and polynucleotide probes, are commonly named throughout the literature as *oligonucleotide arrays*.

For a given organism under study, nucleic acid probes can be designed as soon as a sequence of genomic region or transcript from that species becomes available. As whole genomes are already sequenced and millions of expressed sequence tags (ESTs) are deposited into public records, nucleic acid probes representing genes or genomic regions of an organism are chosen directly from the databases including mainly GeneBank (Benson *et al.*, 1997) and UniGene (Schuler *et al.*, 1996) at the National Center for Biotechnology Information (NCBI). However, sometimes the nucleic acid sequences utilised in probe design process are achieved through direct sequencing made by users. In general, the probe sequence is selected on the basis of gene, EST or sequenced data according to a number of criteria. Most importantly, they should be unique (verification is made with basic local alignment tool such as NCBI BLAST) and relatively uniform in their hybridisation properties, which are determined by a similar overall guanine-cytosine (GC) content, melting temperature (T_m), and tendency to form a secondary structure (Southern *et al.*, 1999). It is obvious that DNA arrays aim to provide accurate measurements of true values of the phenomenon under study. This is achieved by a high specificity (reduced false-positive rate) and a high sensitivity (reduced false-negative rate) of arrayed probes. Preferentially, probes

should be available as segment lengths that are sufficiently short to allow for fast hybridisation kinetics and limited steric hindrance, while sufficiently long enough to ensure specificity. Basically, long nucleic acid probes provide greater sensitivity at the expanse of reduced specificity. In case of oligonucleotide arrays the probe is designed by using the oligonucleotide design programs. A number of oligonucleotide design software packages that may help in probe selection and analysis are available.

A probe that has also gained an application is peptide nucleic acid (PNA) (Aoki and Umezawa, 2003; Lucarelli *et al.*, 2004; Park, 2007; Pellestor and Paulasova, 2004). Probes made of peptide nucleic acid demonstrate very strong affinity for complementary DNA sequence, resulting in the specificity improvement. In fact, these probes more effectively discriminate sequences at the level of single-base mismatches. In addition, PNA/DNA duplexes are resistant to nuclease attack, due to inability of nucleases and proteases to identify the peptide backbone. They have higher thermal stability and melting temperature than the equivalent DNA/DNA hybrid, while relative insensitivity to ionic strength by the fact of the neutral charge of PNA.

11.4.2.2 *Consolidation of support and probe – DNA array fabrication*

Once the target specific nucleic acid probes are selected, the array fabrication can be performed. DNA arrays can be produced using a variety of technologies. Robot arrayers so called 'spotters' are used for contact (printing with pins) and noncontact (deposition with piezoelectric inkjet) printing; while photolithography (with pre-made masks or dynamic micro-mirror devices) and piezoelectric inkjet printing are employed for in situ fabrication. These two major paths, for *spotted* nucleic acid arrays (i.e. oligonucleotide or DNA arrays) and in situ synthesised nucleic acid arrays (i.e. only oligonucleotide arrays) are depicted in Fig. 11.2. From the technological point of view, functionalisation, i.e. the fixation of different nucleic acid sequences on to different positions, is an important issue and in fact the several different DNA arrays present on the market today are mainly characterised by the different functionalisation technologies employed.

In the first manner, that is for *spotted* NA arrays, nucleic acid sequences (previously chemically synthesised oligo-/polynucleotides or single strained DNA fragments, i.e.

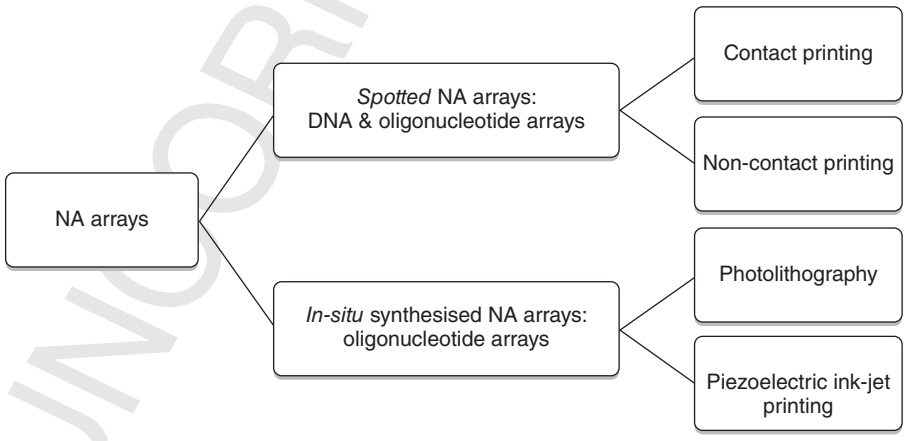

Fig. 11.2 Methods for creating a nucleic acid array.

cDNA, small fragments of PCR products) are deposited on the array surface of a glass slide or semiconducting material, and then spotted via contact or noncontact printing on to it sequentially to form capturing probes (Fig. 11.2). In this approach, an array of fine-pointed pins, needles or nozzles controlled by a robotic arm that is dipped into wells containing nucleic acid probes and then depositing each probe at designed location on the array surface is utilised. Thus, droplets containing many copies of nucleic acid sequence can be stuck to a substrate. Contact printing involves wetting a printing pin with the nucleic acid probe solution and tapping it to the array surface, while noncontact piezoelectric ink-jetting ejects uniform droplets of solution on to the substrate. In general, the latter printing method generates small, homogenous spots, whereas the result of contact printing depends largely on the quality of the printing pins or needles.

Adapting semiconductor photolithography to create oligo-/polynucleotide probes directly on glass or other substrate has produced in situ synthesised oligonucleotide arrays. This approach, alternative to the above, to manufacture nucleic acid arrays employs the in situ fabrication which builds the oligo-/polynucleotide sequence at each site one nucleotide at a time. Production requires more sophisticated and costly equipment. By applying photolithographic methods, driven from the semiconductor industry, to the fabrication of in situ synthesised oligonucleotide arrays, Affymetrix pioneered this field and has dominated for many years.

In general, the construction of in situ synthesised oligonucleotide arrays is done using either photolithography (with pre-made masks or dynamic micro-mirror devices), or piezoelectric inkjet printing (Fig. 11.2), and begins with chemical priming of the substrate array surface, such as a glass slide or semiconducting material, for sites ready to bind nucleotides. In photolithography with pre-made chromium masks, a photosensitive chemical that detaches under illumination caps the sites. Light at 365 nm is shone through a patterned mask on to the array, causing the capping chemical to break away from the areas it strikes, thus exposing the primed spots. A solution containing one of the four types of nucleotides (each molecule of which is itself attached to a capping molecule) is then washed over the array. The nucleotides bond only to the areas that have been exposed, and add a capping layer themselves. As the procedure can be repeated with another mask and different nucleotide, a variety of nucleic acid sequences can be built on the array. Multiple probe arrays are synthesised simultaneously on a large wafer. This parallel process enhances reproducibility and helps achieve economies of scale (Southern *et al.*, 1999). One weakness to the current photolithography method is that a new set of mask must be produced for every new type of array. More recently, Maskless Array Synthesis from NimbleGen Systems GmbH (MAS system) (Waldkraiburg, Germany) has been utilised (Stengele *et al.*, 2005). In this system, a collection of micro-mirrors that reflect on to the appropriate spots on the array is used.

On the whole, the MAS system is a solid-phase oligonucleotide array fabrication instrument comprised of a maskless light projector, a reaction chamber, a personal computer, and a DNA synthesiser. A digital micro-mirror device (DMD) employs a set of miniature aluminium mirrors to pattern hundreds of individual pixels of light. The DMD creates 'virtual masks' that replace the physical chromium masks used in traditional arrays. They reflect the desired pattern of UV light with individually addressable mirrors controlled by the computer software. The DMD controls the outline of UV light projected on the surface in the reaction chamber, which is coupled to the oligonucleotide synthesiser. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next nucleotide will be attached. The patterns are coordinated with the oligonucleotide synthesis

chemistry in a parallel, combinatorial manner such that up to hundreds of unique probes are synthesised in a single array (Singh-Gasson *et al.*, 1999). Using the DMD, this MAS technology allows the functionalisation of small series of custom high-density arrays at an affordable price.

In piezoelectric ink-printing for in situ fabricated NA arrays, solutions of nucleotides are ejected from the nozzle on to the substrate, and then chemically fixed to the surface. The next set of nucleotides are jetted on to the first and chemically fixed to those. The process is repeated until the desired set of nucleic acid is complete.

Summing up, the technology for spotting arrays is undoubtedly simpler than that of in situ fabrication. Simultaneous production of many arrays with the same set of probes makes the deposition more economical than in situ synthesis. However, comparing the two types of nucleic acid arrays, arrays of prefabricated oligo-/polynucleotides or DNAs (where probes are ex situ synthesised) and in situ (on-array) synthesis of oligo-/polynucleotides, the latter has some disadvantages over deposition of presynthesised nucleic acid probes. It is difficult to assess the quality of the oligonucleotides made on a surface. In contrast, the presynthesised oligonucleotides can be assessed before they are attached to the surface.

Several effects have to be taken into account with functionalisation of the array with capture probes resulting from array fabrication process (Steel *et al.*, 2000). It is important that the capture probe anchor is stable during subsequent assay steps and that the probes have to be functional after attachment or its in situ synthesis. The captures must be available with an appropriate orientation and configuration, so that base pairing is not restrained. No steric barriers or lack of accessibility due to the dense packing of the probes should be of difficulty here. At last, it is necessary to characterise the efficiency of probe immobilisation or its in situ synthesis, along with probe functionality.

11.4.2.3 *Front end – from sample to target*

It is obvious that sample handling has a large effect on the analysis, likewise selection of proper sequences as targets. As already described, there is still some confusion in the nomenclature of the *target* and *probe*. Here we stress once again that it is commonly accepted that *target* determines the analysed, mostly labelled material (in the case of nucleic acid-oriented arrays that is: DNA or RNA); while immobilised nucleic acid sequence such as oligonucleotide, cDNA or PCR product, etc., is the *probe*. However sometimes, probe is also defined as a piece of labelled DNA or RNA which is used in a hybridisation assay.

Various types of nucleic acid targets are selected for detection of objects of interest. It is known that DNA provides evidence for the presence/absence of cells, rRNA is an indicator of cell activity or viability, and mRNA provides evidence for specific activity and expression of functions in the cell. From a procedural point of view the preparation of the analytes for examination based on DNA arrays means preparation of samples containing either complex and unpurified, or extracted and purified compounds being verified. To date, only a few researchers worldwide have reported array-based detection of nucleic acids in cell extracts (Gabig-Ciminska *et al.*, 2004a, 2004c, 2005; Basselet *et al.*, 2008; Liu *et al.*, 2008; Metfies *et al.*, 2005; http://www.alderonbiosciences.com). In their studies DNA analysis of microorganisms was carried out directly on cell homogenates, without prior nucleic acid purification or amplification. The utilisation of isolated and purified nucleic acid material as target is also uncommon matter in the DNA array field (Gabig-Ciminska *et al.*, 2004b). Due to the sensitivity limitations and the fact that targeted molecules, such as DNAs, are present only in very low amounts, usually just as a single copy per cell, or

they are unstable substances, such as mRNAs, it is reasonable to employ their artificial analogues being representations of cellular DNA or RNA pools in the assays for DNA array measurements. From another point of view, work with synthetic nucleic acid analogues is required when developing novel DNA array-based methods, where sensitivity and multiplexing have to be achieved. The main drawback of assays using for example PCR amplicons as targets is that they are not quantitative. Due to saturation of the amplification reaction, the amount of targets provided for DNA array analysis differs from the initial material level. On the other hand, the quantification may be improved with a low cycle number PCR approach, for example (Gabig-Ciminska *et al.*, 2005).

The synthetic analogues being representations of cellular nucleic acid fractions in the assays for DNA array measurements are usually labelled analytes. When the target stays unlabelled supplementary probes are used, namely *detection probes*. Nucleic acid examination, where both capture and detection probes are involved in the detection of a target is described as a *sandwich* format assay (Rautio *et al.*, 2003).

In general, it is preferable to decrease target sequence complexity to produce high-quality hybridisation signal within a reasonable time. RNA as a target has a stable secondary structure, which can interfere with hybridisation. To reduce this effect, RNA can be fragmented, preferably to a size close to that of the capture probes on the arrays. Secondary structure is less of a problem with DNA targets and PCR products. Still, human genomic DNA applied for lots of analyses represents complex target, thus, it has to be processed before assayed. It has also been found that the short targets can interact more efficiently with DNA arrays than large ones, so the target fragmentation is often a proper choice for the assay performance. Preferably, target and probe should have the same length.

11.4.2.4 *Middleware – assay considerations*

The assay basically consists of hybridisation process. Sensitive and discriminating recognition of the duplex formation event and an understanding of hybridisation kinetics is an important feature in determining DNA array performance and competence, as any improvement of these properties may increase the efficiency and broaden the scope of DNA array-based analyses.

Different experiments reveal that probes projected on the array surface can lead to hybridisation kinetics significantly altered from those found in bulk solution (Hagan, 2004). Mainly, the observed rates depend on factors such as probe density (Peterson *et al.*, 2001; Zeng *et al.*, 2003), probe length (Okahata *et al.*, 1998), location of the complementary sequence on the probe (Peterson *et al.*, 2002) and rates of diffusion from bulk solution (Georgiadis *et al.*, 2000). The effects of different charged groups in the spacer were also examined in the hybridisation process (Shchepinov *et al.*, 1997). It was shown that both positively and negatively charged groups in the spacer diminish the yield of duplexing. Also, the base composition and sequence of the probes have a large effect on duplex formation. Due to the lower stability of A:T than G:C pairs, the probes of the same length have respectively different T_m values (Southern *et al.*, 1999).

Parameters of the duplexing procedure, like salt-concentration in the hybridisation buffer, temperature, adding of a helper probes, or a fragmentation of the nucleic acid prior to the hybridisation are known from literature to influence the result of a hybridisation reaction and should always be optimised (Liu *et al.*, 2008; Park, 2007). It is obvious that the electrostatic repulsion between the two segments of the nucleic acid duplex decreases with increasing salt concentration, i.e. higher salt concentration increases the stability of

the hybrid, while the maximum rate of hybridisation is from about 16–32°C below T_m. The addition to the assay of helper oligonucleotides that bind in close proximity to capture (and detection if used) probes along target strand may solve problems resulting from higher-order structure of nucleic acid being analysed. It was reported that the helpers have a synergistic effect for the probe binding (Barken *et al.*, 2004; Fuchs *et al.*, 2000; Liu *et al.*, 2008). Additionally, the difficulties caused by higher-ordered structures of nucleic acid targets (Lane *et al.*, 2004), especially in RNA analysis, may be overcome by the fragmentation of the nucleic acid, as mentioned in the previous section. By the generation of smaller sized molecules a fragmentation of the nucleic acid could antagonise negative effects on the hybridisation efficiency caused by steric hindrances (Liu *et al.*, 2008). The reproducibility of this process is critical since it affects the overall reliability of DNA array testing. Both, the enzymatic and non-enzymatic fragmentation protocols applicable to any form of nucleic acids are a significant concern in the design and use of DNA arrays. Nucleic acids are enzymatically fragmented by using for example a hydroxyl radical-based reaction, while in non-enzymatic protocols fragmenting via ultrasound is common. Exploitation of ultrasound to provide nucleic acid fragments suitable for detection by DNA arrays was presented (Gabig-Ciminska *et al.*, 2005; Liu *et al.*, 2008; Mann and Krull, 2004; Priego-Capote and Luque de Castro, 2007).

The main limiting factor for the development and application of DNA arrays is the sensitivity. Analysis of genetic material from single or from rare cells, such as trophoblasts in maternal circulation, means a single-copy gene detection. To reach low detection limits reliably, it is often essential to increase the number of target molecules in the sample or to amplify the signal. Many of the detection systems do both at the same time. The most commonly used method to increase the amount of target in the sample are PCR and related amplification strategies. Nevertheless, a trend towards nonamplification approaches (e.g. branched chain, dendrimer type assay, or multilabelling) is observed. Because a nucleic acid does not have intrinsic properties that are useful for direct detection, a labelling step is required. Actually, the use of improved labels or multiple labelling is a good detection choice, as it may increase the sensitivity, and therefore has been adapted for array-based nucleic acid assays.

11.4.2.5 *Back end – data analysis and handling*

The recognition of the nucleic acid hybridisation relies on the signal generated by the binding event. In order to obtain the complete hybridisation pattern, scanning or imaging the DNA array surface is essential. The most widely used readout method is based on scanners which implement the detection of fluorescence. In this, fluorophores to be examined are excited with the help of a laser. After a few nano- to microseconds the excited species de-excite and emit light at a wavelength larger than the excitation wavelength. In order to be suitable for this method, either the analysed targets need to be labelled with a fluorescent marker, or supplementary labelled detection probes are used. One- or two-colour detection (single- or two-channel detection, respectively) is used in DNA array-based analyses. One-dye, also called single-channel, DNA arrays are designed to give estimations of the absolute levels of gene. Therefore, the comparison of the two sets of conditions requires two separate single-dye hybridisations. Since only a single dye is used, the data collected represent absolute values of gene level. These may be compared to other genes within a sample or to reference normalising probes used to calibrate data across the entire array and across multiple arrays (relative data). Among several single-channel arrays

the most popular systems are the Affymetrix 'GeneChip', Illumina 'Bead chip', Agilent single-channel arrays, and the Applied Microarrays (Tempe, USA) 'CodeLink' arrays. On the contrary, in two-dye or two-channel detection DNA arrays are commonly hybridised with target prepared from two samples to be compared (e.g. diseased tissue vs. healthy tissue) and that are dyed with two distinct fluorophores (Shalon *et al.*, 1996). Fluorescent labels usually used for target labelling are cyanine 3 (Cy3) with a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light range), and cyanine 5 (Cy5), which has a fluorescence emission wavelength of 670 nm (corresponding to the red fraction of the light spectrum). First, the two Cy-dyed samples are mixed, then co-hybridisation to a single DNA array is made. Afterwards, it is scanned in an array scanner to imagine fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength. In order to identify up-regulated and down-regulated genes, relative intensities of each dye are used in ratio-based analysis. The two-channel DNA arrays are offered by Agilent in form of Dual-Mode platform, Eppendorf (Hamburg, Germany) as DualChip platform for colorimetric Silverquant dying, and by TeleChem International as Arrayit (California, USA).

Although the predominant technique for DNA array signal detection is based on fluorescence, several other modes also show promise. One example is chemiluminescence, which is related to the detection of the emission of a photon of light from a chemical reaction by the release of energy from a chemically excited compound when it returns to the normal unexcited state. However, the chemiluminescent method did not receive attention for long time, until the development of enzyme-enhanced chemiluminescent process and extreme sensitive cooled CCD-camera, able to detect single photon emissions, took place. An alternative to fluorescence and chemiluminescence is radioactivity. Radioactive species have the advantage of high incorporation efficiency, high sensitivity and low cost, though they were not utilised in high-density arrays due to the lack of high-resolution imaging techniques. Other detection systems that implement for example resonance light scattering, oxidation-reduction reaction, electrical changes after hybridisation, resonance-ionisation mass spectrometry, etc., have also been reported in literature (Gabig-Ciminska and Ciminski, 2004).

Here we would like to stress that high-quality samples and high-tech instrumentation alone in DNA array-based analyses are not enough to guarantee reliable and accurate results. A proper approach should consist of experimental design, standardisation, pre-processing of raw data, data normalisation and the appropriate treatment of data with statistical tools for quality control and data analysis. The use of databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) or Gen Ontology (GO) where the function and activities of individual genes have been accurately defined and annotated are of interest (Eisenstein, 2006).

Due to the biological complexity of genetic analyses, the considerations of experimental design are fundamental if statistically and biologically valid conclusions are to be drawn from the data. The multiple levels of replication are to consider when designing a DNA array experiment. Fundamentally, there are two types of replicates: biological replicates and technical replicates. Biological replications are replications taken at the level of the condition being studied. Replication of biological samples is essential for drawing conclusions from the experiment and to be certain that the effects we see can generalise to the condition. On the other hand, technical replicates are taken at the level of experimental apparatus. The purpose of technical replicates is to account for variability in the experimental setup. Technical replicates can be at many levels, such as: replicate features on the

array, which can account for differential printing or hybridisation; replicate arrays hybridised with the same samples; or replicate sample preparation. Basically, they can help to average out nuisance effects, such as spatial variation or feature effects.

Currently, there is an increasing global interest in making DNA array data sets publicly available in a standardised format. Standardisation is a common demand of any DNA array-based work, with special concern for gene expression arrays, being most popular purpose of DNA array technique. Results obtained from DNA array experiments are difficult to exchange due to the lack of standardisation in platform fabrication, assay protocols, and analysis methods. Therefore, standardisation of the methods and materials allowing intra- and inter-comparison of DNA array data collected is a challenge. It has caused dilemma in bioinformatics, so that big attention was devoted to projects developing tools enabling the exchange and analysis of data produced with nonproprietary DNA arrays. Standardisation would allow other investigators to reproduce published DNA array experiments, to thereby independently verify them, and also to evaluate datasets across different array platforms. Standard for DNA array databases must define: a common set of information, a common representation, and a common meaning. The Microarray Gene Expression Data Society (Ball *et al.*, 2002), also known as MGED, has taken the lead in establishing standards, which begins to make data sharing easier. MGED developed a set of guidelines called the MIAME (Minimum Information About a Microarray Experiment) standards (Brazma *et al.*, 2001). The two main public repositories for DNA array data are the Gene Expression Omnibus (GEO), run by the National Center for Biotechnology Information at the National Institutes of Health (Edgar *et al.*, 2002), and ArrayExpress (AE), run by the European Bioinformatics Institute (Brazma *et al.*, 2003). A third repository in Japan, called CIBEX (Center for Information Biology gene EXpression database), has been running since 2004 (Ikeo *et al.*, 2003). The standards for the information provided with DNA arrays include all the data that scientists should provide so that others can interpret and understand their array results. Still, efforts are made to develop improved standards for DNA array data that aimed at making data sharing easier and easier.

The use of a low-density, middle-level and highly parallel approach via employment of DNA array technology enables researchers to achieve vast quantities of experimental data, thus transmitting the complexity of the data into useful information bioinformatics being utilised. Three main processes are included in the data reading: statistical analysis, data interpretation and presentation, which makes the analysis accessible to human thinking. Due to the huge amount of information, only statistical procedures allow an assessment and filtering of array data. Algorithms that affect statistical analysis include: firstly, conversion of the raw signal into values that can be used for knowledge discovery; secondly, data processing, which is background noise subtraction and appropriate normalisation of the data; and finally, identification of statistically significant changes through engagement of different types of tests based on the variation present in the data and the number of experimental replicates.

Post-analysis follow-up, which is discovering knowledge from the DNA array data, can be pursued on several different levels. In the case of gene expression arrays, at the simplest level, the up- and down-regulated genes can be easily identified from array-based experiments. However, although the basic fold-of-change analysis is valuable in many situations, it does not touch the wealthy information occurring behind the DNA array data. As DNA array experiments determine expression levels of thousands of genes, it is reasonably expected to discover higher order associations or hidden patterns among these genes. Data mining methods usually fall into one of two classes: unsupervised and supervised. In

unsupervised analysis, the data are being organised without the benefit of external sorting information.

A method of disclosing a gene relationship is to cluster together genes that exhibit similar expression patterns across multiple experiments. This can help to understand different regulatory mechanisms, as well as to obtain an idea about the functional role of unidentified genes/sequences by the known genes in the same cluster. In 1998 the first cluster analysis of DNA array data was made by Eisen *et al*. (1998). After that, clustering has become a routine way for grouping co-regulated or functionally similar genes or sequences. At present, many variations of clustering algorithms are being utilised in DNA array data analysis; among them the most popular are hierarchic clustering, K-means clustering and self-organising maps. In contrast to unsupervised methods, supervised analysis utilises some external information about datasets in terms of sample classification when entering the data mining. These methods include k-nearest neighbour classification, support vector machines and neural nets.

11.4.3 Bird's eye view of DNA array technology – today and tomorrow

The range of application of DNA array technology is enormous. The implementation of this technology for diverse detection requirements was possible through the exploration of a number of different techniques originating from distinct live spheres. Current directions in the development of nucleic acid-based arrays concern a transformation process of systems from optical to electrical, from label-dependent to label-free approaches, from single to multi-analyte and array formats, towards miniaturised and fully automated (Fig. 11.3).

DNA array is simple in concept, nevertheless generating reliable results when using them still requires considerable expertise and technical tricks. Key matters in creating or

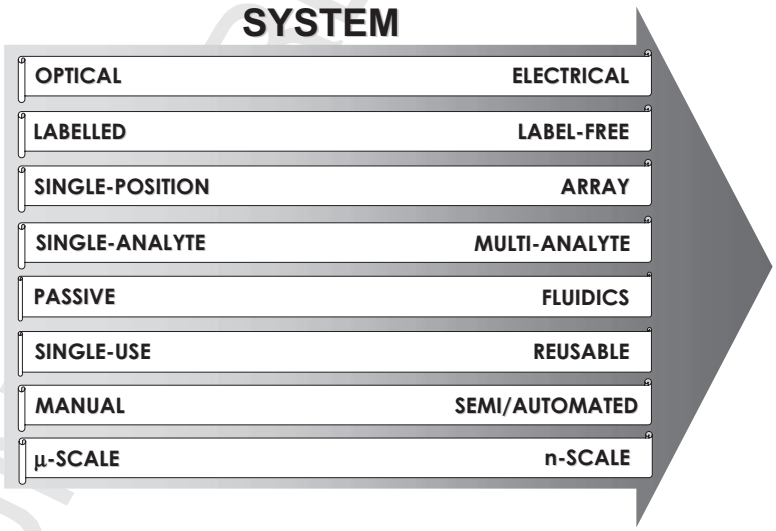

Fig. 11.3 Trends in a DNA array technology.

selection of DNA arrays include: fidelity, reproducibility, flexibility, shelf life and cost. Based on their evaluation method, optical or electric DNA arrays are distinguished (Wang, 2003). Most of the DNA arrays available on the market are based on external or internal optical systems with detection of fluorescence (Marchand *et al.*, 2005). This way of readout is reproducible, but is limited in sensitivity. Therefore, alternatives to optical detection methods such as direct electrical charge recognition are being developed. When compared to optical detection methods, electrical-based methods are advantageous in that they are more applicable to miniaturisation, mobile in terms of being portable, and less expensive (Gabig-Ciminska, 2006). Additionally, electrical DNA arrays are able to conduct detection of both label-free and labelled objects (Drummond *et al.*, 2003; Marchand *et al.*, 2005). The property of label-independent detection of electrical DNA arrays makes them even more attractive over optical systems, as it correlates with an increased attention recently given to label-free detection systems. Also, a highly parallel array format rather than low-density platform, as well as multi-sample approach are of interest nowadays. However, for some bioanalytic applications highly parallel DNA arrays do not offer the best recognition system.

High-density DNA arrays are tools in high throughput analyses (whole genome gene expression analysis with a single array), whereas medium- and low-density array mainly demonstrate the application for clinical diagnoses and personalised medical care (typing of microorganisms, esp. pathogenic bacteria) (Gabig-Ciminska, 2006). Additionally, miniaturisation and automation of DNA array-based systems are another vision for nucleic acid analyses. Trend in miniaturisation (from micro- to nano-miniaturised arrays) and the pursuit of functional integration into a single programmable array system have resulted in the construction and the use of DNA arrays that contributed to microfluidics, instead of passive form of nucleic acid detection systems (Elsholz *et al.*, 2006; Liu *et al.*, 2004). The advantages of miniaturisation and automation are mainly bound to parallelism, reduced reagent consumption, speed and functional integration, while one may ask: is smaller ever better, or may smaller become too small? It seems likely that this tendency may cause problems in certain situations in terms of statistical significance of verified samples (Gabig-Ciminska, 2006).

11.5 APPLICATIONS AND POTENTIAL USE OF THE DNA ARRAYS

The enthusiasm about the potential of DNA arrays has been intensive. Thereby different approaches and potential applications of DNA array technology are broadly discussed throughout literature and by other information resources. Users of this unique platform for nucleic acid analysis realise significant profits compared to other available conventional detection techniques.

The technology finds increasing applications in fundamental and applied research. The key attribute of this technique is that it allows one to perform a simultaneous analysis of a great number of nucleic acid sequences generating databases with information about living processes. Except for research, nowadays DNA arrays serve to wide range of practical use and are classified as remarkable tools in diagnostics and surveillance. Global transcription patterns, nucleotide polymorphisms, mutations, gene discovery, mapping and other matters in bacteria, plant, yeast, animal and human samples can now be readily evaluated using DNA arrays (Gabig-Ciminska and Ciminski, 2004; Iyer *et al.*, 1997; Khan

et al., 2010). Unique patterns associated with particular physiological state of a cell or group of cells are discovered in this way. Knowledge of gene expression profiles can from one side help researchers to identify new drug targets, while from another point of view it provides data on organisms' response to particular medicaments. In consequence, the drug's efficacy or toxicity can be determined, and an understanding of complex diseases obtained (Khan *et al.*, 2010; Yoo *et al.*, 2009). The power of this method is such that it may not only lead to disease diagnosis, but also to prognosis and therapy. DNA array-based comparative genome hybridisation (CGH) produces patterns of amplification or loss of genomic regions, which are indicative of disease development, therefore being useful to prognostic purposes (Bashyam *et al.*, 2005).

DNA arrays are also making a great impact on the development of rapid and sensitive assays for the detection of pathogenic microorganisms (Lin *et al.*, 2006; Lodes *et al.*, 2007; Uttamchandani *et al*., 2008; Yoo and Lee, 2008). They have extensively been applied as a research tool towards understanding the etiology and pathogenicity of dangerous pathogens, as well as in diagnostics (Wong *et al.*, 2007) and vaccine improvement. As food safety is becoming an important objective of public health these days, a lot of investments are put into development of efficient screening made with DNA arrays. It is assumed that NA arrays, nowadays broadly used in the area of biomolecule typing, in the near future may become an extremely important tool for pathogen detection accelerating the ability to respond in an epidemic or crisis.

DNA arrays used in biology for a multitude of differing applications, from the study of gene regulation and microorganism response to environmental changes, genome organisation, and evolutionary questions, up to taxonomic and environmental studies, are described in a huge number of publications to which it is impossible to refer for reasons of space.

Perhaps even more other applications of this technology will be achievable in the near future. However, the variety of approaches described above already illustrates the enormous potential for DNA arrays in almost every aspect of human existence, thereby making them the engine of the twenty-first century.

11.6 CONCLUSIONS

Once set into scientists' hands, DNA array technology was and is still evolving constantly and rapidly. Several years ago, no practical technique could do what DNA arrays are able to conduct in terms of high throughput of nucleic acid analyses. The power of DNA arrays makes them exceptionally important tools for investigation in a wide range of genetic queries. In almost two decades since their introduction, DNA arrays have permeated a various application areas, on the one hand yielding useful insights into basic biology, and on the other being used in highly advanced medicine. However, the early unimpeded enthusiasm became tempered with a dose of reality over time, as it turned out that progress was slower than predicted and some results in highly impacted journals proved hard to reproduce (Cobb, 2006). In this way, a solid intellectual input is still needed in order to improve the overall quality of the analyses, especially when considering their commercialisation, so that no validation of the DNA array data by, for example, quantitative reverse transcriptase real-time PCR assays or blotting will be required.

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