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DNA Microarrays

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Abstract Microarray technology provides new analytical devices that allow the parallel and simultaneous detection of several thousands of probes within one sample. Microarrays, sometimes called DNA chips, are widely used in gene-expression analysis, genotyping of individuals, analysis of point mutations and single nucleotide polymorphisms (SNP) as well as other genomic or transcriptomic variations. In this chapter we give a survey of common microarray manufacturing, the selection of support material, immobilisation and hybridisation and the detection with labelled complementary strands. However, DNA arrays may also serve as the basis for more complex analysis based on the action of enzymes on the immobilized templates. This property gives DNA microarrays the potential for being the template for whole PCR and transcription experiments with high parallelism, as will be discussed in the last section of this chapter.

Keywords Active arrays · DNA microarray · DNA chips · On-chip PCR · On-chip transcription

1 Introduction

Desoxyribonucleic acid (DNA) is the most important biomolecule in nature because of its role as carrier of the genetic information. Many kinds of genetic analysis may be done on the gene level. The analysis may vary from single-nucleotide deviations to whole-genome sequencing. Many methods and inventions have been made in recent years to create analytical tools for the analysis of a huge variety of applications. All the methods invented for DNA analysis are in principle applicable to RNA as well. This is of interest for RNA especially in its role as information carrier, the messenger RNA. Using reverse transcription it is possible to get a DNA copy and therefore all that is said for DNA analysis in this chapter will also be valid for most RNA analysis.

Microarray technology provides new analytical devices that allow the parallel and simultaneous detection of several thousands of probes within one sample. Microarrays, sometimes called DNA chips, are widely used in gene-expression analysis, genotyping of individuals, analysis of point mutations and single-nucleotide polymorphisms (SNP), as well as other genomic or transcriptomic variations.

The high specific base-pair interaction within the DNA or DNA–RNA hybrids with labelled complementary strands makes the microarray technology a powerful analytical tool for monitoring whole genomes. In this chapter we give a survey of common microarray manufacturing, the selection of support material, immobilisation and hybridization, the detection with labelled complementary strands. DNA arrays may also serve as the basis for more complex analysis based on the action of enzymes on the immobilized templates. This property gives DNA microarrays the potential of being the template for whole-PCR and transcription experiments with high parallelism, as will be discussed in the last section of this chapter.

1.1 The Physico-Chemical Properties of Nucleic Acids

A very important feature of DNA is its chemical homogeneous appearance with repeating units, the sugar-phosphate backbone. At neutral pH, DNA is negatively charged via the phosphate groups and attracts positive counterions, i.e. DNA is a strong polyelectrolyte [1]. Depending on the counter-ion concentration, double-stranded DNA has locally a more or less stiff rod-like structure. This feature is relevant for all properties regarding surface contact as it occurs in microarray experiments. The physical parameters of

the surface become relevant for the attraction or repulsion of DNA. This is true for the immobilisation process and its efficiency as well as for the hybridisation.

On the other hand, the structure of DNA is not identically repeating, but due to the four different bases, adenine, guanine, cytosine and thymine (resp. uracile), the sequence is unique depending on composition (see Fig. 1). This feature makes DNA the ideal storage molecule because the blueprint of any organism may be stored within the "bits of the genome". The high similarity and the ease of transformation from a four-letter code to the dual code of information theory and modern computers has inspired much reasoning about "DNA computing" and nucleic-acid-based computers. However, despite the success of Adelman's first demonstration of a molecular biological computing process [2], up to now there is no real computing machine that makes use of DNA as the coding string.

The most prominent interaction of DNA is the base recognition, based on the hydrogen bonds between adenin and thymin, that form two hydrogen bonds, and guanin and cytosin, that form three hydrogen bonds. The binding of two complementary strands is called hybridisation. The stability of the DNA hybrid depends on its GC content. The more GC pairs involved, the higher the stability of the DNA hybrid.

1.2 Microarrays

Since most biological phenomena are within the context of a multitude of parameters and processes, the correlations and interactions of these processes are at the centre of quantitative biological investigations. The interrogation of a broad variety of genes or their transcripts and their activity at one moment is one of the typical questions; and parallel analysis is needed since it can be performed by the use of microarrays.

DNA microarrays are characterised by a structured immobilisation of DNA targets on planar solid supports allowing the profiling of thousands of genes or interactions in one single experiment. An ordered array of these elements on planar substrates is called a "microarray". Usually, for practical reasons, one distinguishes between microarrays and macroarrays, the difference being the size of the deposited spots. Typical spot sizes of macroarrays are featured by a diameter of more than 300 microns, whereas microarray spots are represented in less than 200 microns.

Hybridisation is the underlining principle of DNA chips. According to the nomenclature recommended by Phimister [3] a "probe" is the immobilised or fixed nucleic acid with known sequence, whereas the "target" is the free nucleic acid sample, usually labelled during the preparation process, which interacts with the probe by hybridisation. Commonly used labels for target nucleic acids are fluorescence dyes, radioactive or enzymatic detection labels.

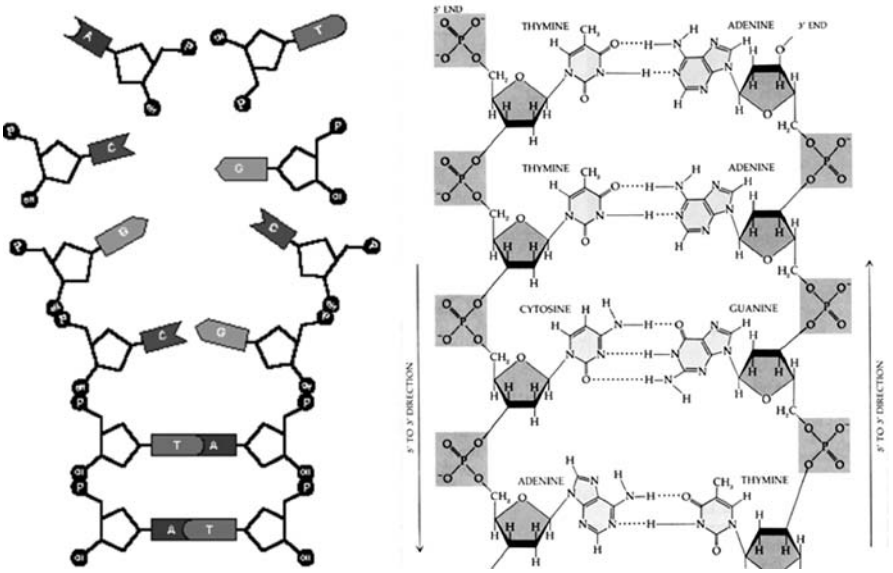

Fig. 1 The building blocks of DNA, base-pairing, the double-helix, and hybridisation

Low expenditure of time, high information content and a minimum of probe volume are attractive features of this technology.

To provide free and accessible functionalities of the DNA strand that is to be immobilised, i.e. which will be the probe, is the essential precondition for a proper immobilisation. The DNA contains three different biochemical components, a base that is substituted on the first carbon of desoxyribose forming a nucleoside, and a negatively charged phosphodiester that connects the sugars to a chain as shown in Fig. 1. In principle, the amines in the bases, the negatively charged backbone, the phosphordiesters within the backbone, and the phosphates on the 5′-end as well as the hydroxyl group on the 3′-end are potential candidates for coupling. However, since hybridisation is the aim of the experiment, it should be noted that in double strands the bases are engaged in hydrogen bonds and thus, are not accessible for the chemical coupling reactions on the surface.

Microarrays can be fabricated with DNA from various sources; the probes may be derived from cDNA, PCR products or synthetic oligomers.

1.3 Enzymes Acting on Immobilised Templates

Biosensors are defined by the close contact of a biomolecular recognition element with a transducer, which converts the biochemically generated signal into an electronic signal. This type of device has been investigated intensively, with

a great variety of recognition elements, like enzymes, antibodies, DNA, aptamers, etc. [4]. Enzymes play a major role in this field, since the activity of an enzyme may be used for signal transduction and amplification in one.

More complex biochemical reactions have been introduced into biosensors by use of coupled enzyme reactions or coupled binding and enzymatic reactions. While this is a theme of its own in biosensor research for metabolic biosensors, there are only a few reports on DNA-modifying enzymes (see Sect. 3 in this chapter). When DNA is immobilised properly on a surface, it still keeps most of its physico-chemical properties. Moreover, it turned out that the capability of correct base pairing, the hybridisation process, is not affected by the immobilisation. This enables immobilised DNA to serve as a possible template for even complex enzymatic reactions. Enzymatic digest by restriction endonucleases [5] as well as synthesis on arbitrary templates by polymerases have been shown in real-time and label-free experiments [6, 7]. A second feature of immobilised DNA on a microarray is to use the spots as addresses to couple something else, for example, some proteins that are tagged with the complementary strand [8, 9]. This concept paved the way for the development of "active arrays".

In microarrays this approach has not been used very often. The mere binding event, the hybridisation, is determined using fluorescent labels, but also microarrays bear the potential activity measurement, and the immobilised DNA may serve as a template for DNA–protein interaction analysis as well as a template for chip-coupled PCR. Moreover, whole genes may be immobilised and used for in vitro transcription and translation.

2 Microarray Technology

Microarray technology allows massive parallel determination and multiple measurements of a variety of binding events that are to be carried out simultaneously. In addition, it has the advantage of requiring a small amount of material and a modest investment of labour; moreover, it might save a lot of time and may easily be automated. Microarrays in general consist of many microscopic spots, each containing identical molecules, i.e. receptors, probes or targets. The numbers of spots may vary from less than 100 to many 100 000. The molecules are attached to a solid support that can be made from glass, silicon or a polymer. In the case of nucleic acids, the receptors are usually oligonucleotides or cDNA, and the binding event is simply the hybridisation of complementary strands. Biochip technology (especially DNA chips) is a rapidly developing field with high commercial potential. Introductions to the principles of the technology as well as to various applications are available in many reviews [10, 11], and in the well-written practical approach by Schena [12].

Thus, the combination of molecular biology, microfabrication and bioinformatics has generated novel tools and has the strong potential to bring more products on to the market for genetic analysis purposes; applications of which can be used in all branches of the life sciences.

2.1 The Typical Microarray Experiment

The central process in all biochip or microarray experiments is a binding event, the hybridisation. One binding partner (either receptor or ligand, probe or target) is immobilised within a small but well-defined area on a flat solid support of glass or polymer; referred to as the "spot" or "feature" of the microarray. There are also examples of prestructured slides, structured for example by microcavities (nanotiter plates) or by chemical structuring as well as by electronic features, such as microelectrodes. The features con-

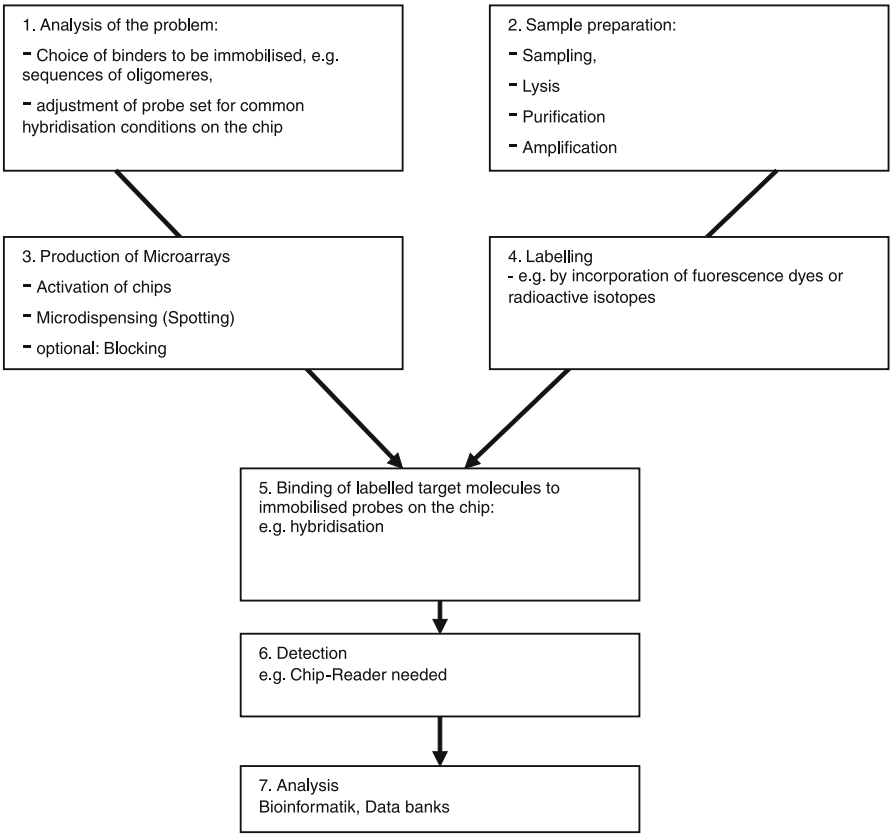

Fig. 2 Workflow of a microarray experiment

tain identical molecules, while the whole array contains arbitrarily chosen or systematically varied compounds.

Once the microarray has been produced, the sample that contains the ligands or targets to be investigated is added and binding occurs at several features on the chip, which results in a characteristic pattern representing the sample. The procedure of a typical biochip experiment is schematically represented in the workflow below.

Fluorescence labels are by far the most often used labels to detect the binding event. The sample itself is labelled in a step prior to incubation, i.e. the hybridisation. After incubation, the chip is washed and then read out by means of a scanning or imaging device, usually in a dry state, giving a snapshot picture of fluorescence intensities. Internal standards for comparison purposes are often incorporated into the procedure.

2.2 Manufacturing of Microarrays

For microarray production, two different approaches are used:

1. Synthesis on the chip; and
2. bulk synthesis with subsequent deposition on the chip.

The first method is applicable to generate chemical libraries, for example, of short oligonucleotides or peptides; the second method may also be adapted to long polynucleotides or proteins, or any of the many receptors. Detailed description of these methods may be found in [13–15].

2.2.1 Synthesis on the Chip

Fodor et al. reported as early as 1991 for the first time the notion of synthesising an array of oligomers on a chip surface by use of step-wise local photo-deprotection [16]. In their first paper, peptide synthesis was in the focus, however, it was soon recognized that oligonucleotide arrays would be much more needed and due to only four bases instead of 20 amino acids would be easier to implement. The process of production is similar to wafer production in microelectronics: a chemically activated silicon wafer surface is covered with photo-labile protecting groups. After UV irradiation through a mask with high spatial resolution, the activated groups are reactive at certain localities and a nucleotide, that again bears the same photo-labile protecting group at its 3′-end, may couple with its 5′-end. Repetition of this procedure with all four bases will end up with defined sequences in each feature of the array, $4n$ steps (and masks) are needed for n nucleotides. Standard microarrays have features of $0.1\ \text{mm} \times 0.1\ \text{mm}$, but the process is optimised down to a feature size of $10\ \mu\text{m} \times 10\ \mu\text{m}$, enabling several thousand up to

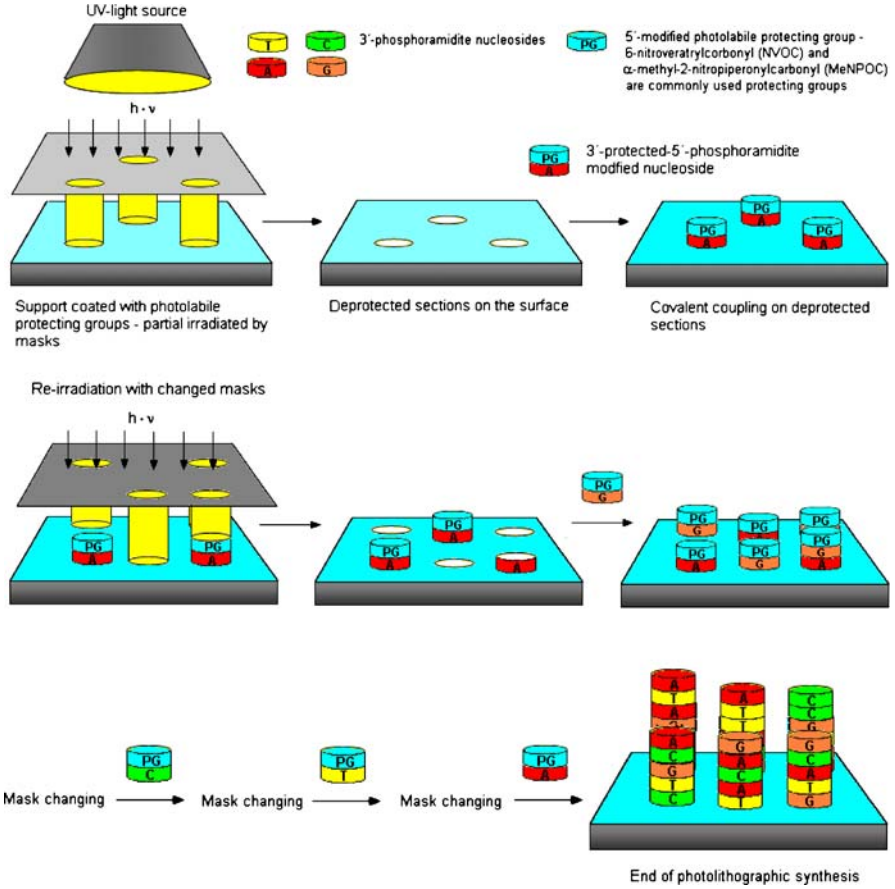

Fig. 3 Photolithographic surface structuring and oligonucleotide synthesis [17]

hundreds of thousands of features on one chip. This method is therefore optimised for high parallel synthesis. The only limitation in absolute number of features comes from feature size and mask resolution. With this type of array, whole-genome screening is possible. This method is very much linked to the name of the company Affymetrix who industrialised the method and currently produces a new series of high-density microarrays for several applications.

The method, however, is limited to oligonucleotides. The length of the oligonucleotides on the chip surface is limited to about 25 nt, since there is no "100%"-chemical reaction and thus the addition of incomplete reactions results in a loss of sequence precision within one feature. Assuming a 99% chemical reaction, a feature with a 25 nt oligomer contains 33% of molecules with an incomplete, i.e. wrong, but nevertheless similar sequence.

2.2.2 Spotting Techniques

An alternative way of creating a microarray is to deposit small amounts of pre-activated oligonucleotides by microdispensing. An advantage compared to on-chip synthesis is the possibility of cleaning up the material that will be deposited. While on-chip synthesis is limited in sequence length by the efficiency of each coupling step during the synthesis, pre-synthesised material may be cleaned up even from a mixed environment like fermentation broth. Moreover, the dispensing methods are not limited to any chemical species, but may be used for any kind of array, including PCR products or complete genomes. On the other hand, pre-synthesised probes have to be processed sequentially and thus the whole procedure (and in consequence the number of features) may be limited by the processing time.

Two different techniques are currently used: contact and non-contact printing.

The first technique uses pin tools, or needles, that are dipped into the probe solution and dispense a certain amount of material by contacting the support material. An advantage of pin tools is that parallelisation is easily

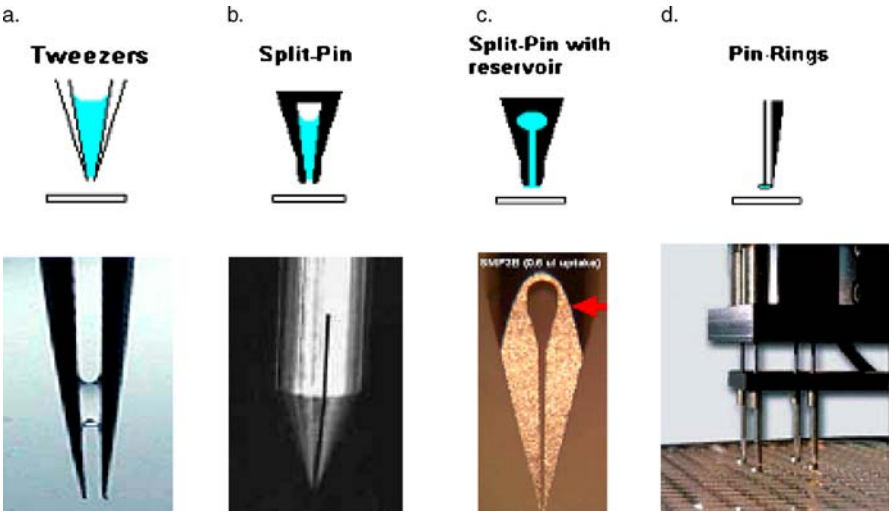

Fig. 4 Pin tools for contact printing: **a** *Tweezers*: Micro-tweezers are loading the sample by capillary action and expels defined spot volumes on the surface by tapping forces. **b** and **c** *Split-Pins*: Through capillary action, a defined target volume is loaded into the split or other cavities (per surface-tapping small spots were deposited). Depending on the amount of spots to be set in one spotting run, the cavaties are variously shaped **d** *Pin rings*: Pin rings load and hold the sample in a ring like a soap-bubble. For spot-deposition, a needle is propelled through the ring while the sample is carried by the needle to contact the surface [12]

done by adding more needles to the printhead. However, reproducibility of this method is limited, since the tiny tips of the pin tools change their shape during repeated contacts to the support. Therefore for high and middle dense arrays for research use, pin tools is the method of choice.

Figure 4 shows a pin tool printer (4d) and some examples of pin tools (4a–c) that have been developed for optimal and repeated deposition of probe material. The slits and holes in the pin tools are reservoirs for repeated printing of the same substance. The print step may be repeated 20–40 times. Typically, pin tools are used for in-house production of a limited number of high specific genomic slides. Reproducibility of this method is limited.

Non-contact techniques rely on the piezo effect. Like in an ink-jet printer, a microfluidic nozzle is activated to release droplets of a size less than 1 nl. The distance of the features can be minimised down to 100 μm. This type of dispensing allows for high accurate manufacturing of low- and medium-density microarrays (up to a few thousand features). Figure 5 shows a one-nozzle non-contact pipette releasing a drop on demand. This kind of pipette may also be used for a variety of different pipetting procedures where small

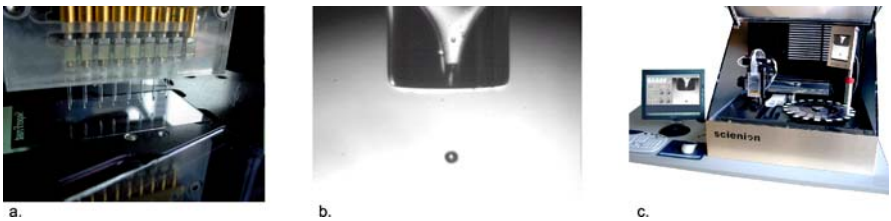

Fig. 5 Non-contact spotting via piezo technique. **a** Printhead with eight piezo nozzles. **b** Close-up of a piezo nozzle releasing a sample drop. **c** SciFlex Piezo-spotter (Scienion, Berlin)

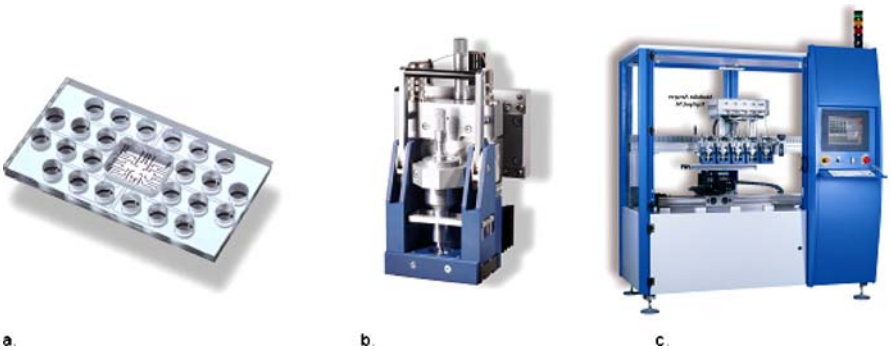

Fig. 6 TopSpot technology. **a** TopSpot Printhead with 24 nozzles. **b** Printing module with actuator that holds the printing head. **c** TopSpot Arrayer with five printing modules for series production of microarrays

volumes as low as 100 pL (pico litre = 10^{-12} L) have to be applied. While machines of this type are capable of producing a lot of spots (features) of the same kind with one loading, reloading and change to the next probe needs intensive washing. Currently, parallelisation of nozzles is limited, suppliers of such machines offer up to 16 parallel nozzles.

A microsystem approach has been made by Zengerle and coworkers [18, 19] which they named "TopSpot". An array of nozzles is produced in a monolithic manner using Si-wafer technology. This array is combined with a glass microfluidic system that incorporates reservoirs and microchannels connecting these reservoirs with the nozzle and thus form a printhead. By external actuation, the nozzles release simultaneously one droplet of each probe loaded in the reservoirs. Printheads with 24 and 96 nozzles are available with a fixed array format, but in principle, any number up to several hundred may be produced. Figure 6 shows printhead and machine for small series production (up to several thousand arrays per hour). The reservoirs can collect up to 5 μL, and thus depending on feature size and support material, more than 20 000 print processes may be done with a single loading.

2.2.3 Performance and Quality Control

Quality control is of great importance for microarray fabrication, especially if diagnostic applications are in the focus of interest. The quality control has to follow each production step, but finally the overall assay performance is the sole criterion for a well-manufactured microarray.

After each spotting step a control is needed, whether all features are properly generated and within defined accuracy limits. The definition of such limits depends on the application. Usually, the diameter of the spot gives a first indication about the quality of the printing process. Figure 7 gives an example from a production series with the TopSpot technology. At this stage, misaligned arrays or arrays with missing or misprinted features may be ex-

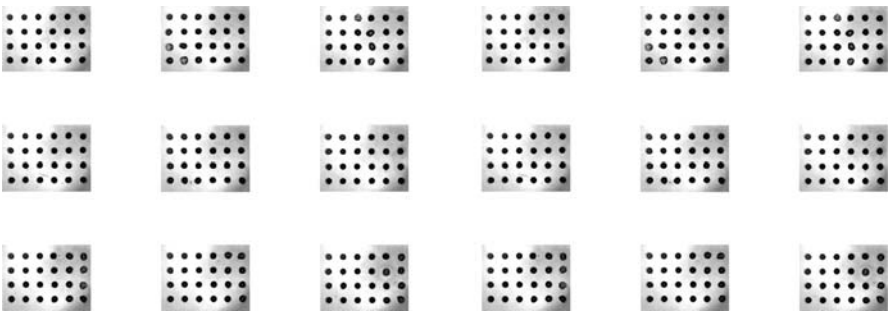

Fig. 7 Production-scale control of sequential printing results

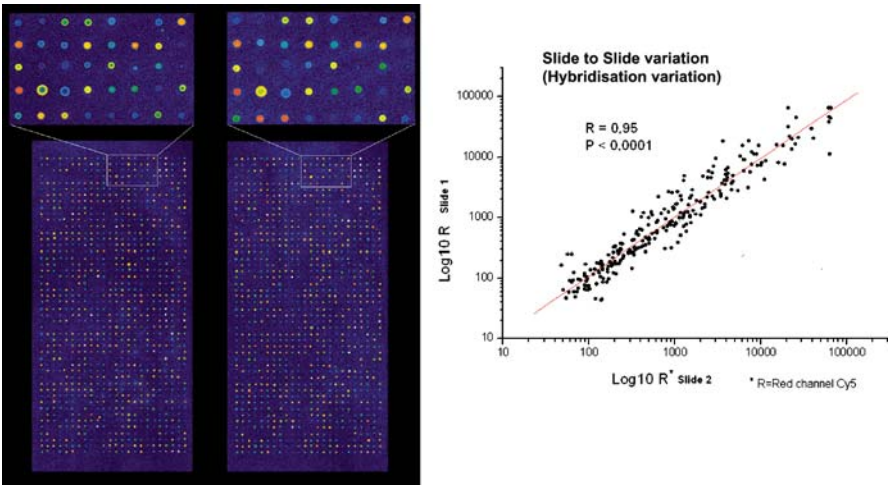

Fig. 8 Comparison between two microarrays for determination of slide-to-slide variation

cluded from further processing. As one measure of quality at this stage the diameter and deviation from the ideal circle may be employed. Since the surface of the slide should be homogeneous and the deposited droplet should have the same amount in each feature, an ideal circle with a well-defined size should occur after each spotting process. Up to now, using this measure the reproducibility of the spots was found to be less than 8% in the ideal case using the TopSpot technology. However, functionality of the features, as measured by hybridisation efficiency, might be even better, since the absolute number of immobilised probe molecules and their accessibility to the sample is the decisive entity, and not the geometric appearance of the feature.

Since a non-invasive test of functionality of all features is not possible, batch control must serve as a quality measure. For this it is necessary to know about the deviations that occur typically within one series. Figure 8 shows a comparison of two microarrays from one batch and the deviation from the mean value after hybridisation. The state of the art is a standard deviation of about 12%.

2.3 Hybridisation

As mentioned before, hybridisation conditions have to be optimised, i.e. buffer conditions, like ionic strength and pH, and temperature have to be chosen in such a way that only complete matching strands form stable hybrids. Since these conditions depend on strand length and on composition, usually different sequences do not bind simultaneously in an equal stringent way. This situation has to be regarded during the microarray design and some bioinfor-

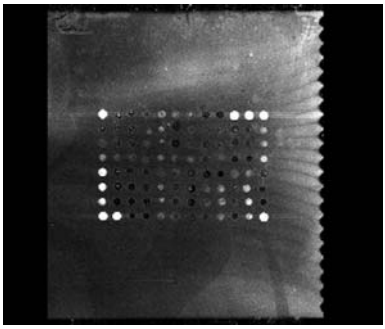

Fig. 9 Microfluidic hybridisation chamber: the special design with multiple inlets allows for a homogeneous sample stream to guarantee equal hybridisation conditions in all places of the microarray

matics tools have been generated to help to find the desired set of sequences for one microarray (e.g. ArrayDesigner 3; Premier Biosoft International).

Hybridisation has to be done under controlled conditions; often incubation over a long period of several hours at elevated temperature is required. Typical are temperatures between 45 and 65 °C. To guarantee the correct incubation, often hybridisation stations are used, some of those speed up the protocol by actuating the reagents during incubation. An example of such actuation is to facilitate gentle and homogeneous sample stream by special design of a flat fluidic chamber. The microfluidic challenge is to cover a plane of relative big size homogeneously with a small volume of fluid. For this purpose, chambers have been developed that solve this problem by a proper design; Fig. 9 gives an example from our own lab (not published).

2.4 Detection and Analysis

Detection fluorescence is by far the most often used method. Usually, fluorescent dyes are incorporated into the sample during preparation, e.g. during amplification. The use of intercalators or similar DNA-specific dyes is also common. Since most instruments for detection are applicable only to dry microscopic slides, fluorescence dyes that work in the dry state are preferred, e.g. dyes from the cyanin family like Cy3 or Cy5. For transcription analysis, two different stages are compared on one slide and therefore two different dyes are used.

Two types of instruments are used: imaging and scanning devices. The principle of the optical arrangement is very similar to a fluorescence microscope in which the fluorescence illumination (excitation) runs the same optical path as does the emitted light. The imaging device takes a picture from the whole or part of the array, while the scanning device works as a laser

scanning microscope with a focused excitation beam to enhance performance by reducing the background signal. In both cases, a resolution of about 5 μm is achieved that allows for a high accurate analysis of each single feature. Software tools are usually employed to further analyse the acquired images.

3
The Microrray Experiment

3.1
Transcription Analysis

By far the most often used application of DNA microarrays is transcription analysis. Due to the tenets of molecular biology, the information flow runs from the storage medium, the genomic DNA, via transcription to the messenger RNA (mRNA) and by translation in the ribosome machinery to the protein. Many details of the regulation of gene activity have been discovered in recent years. It is of high scientific (and also medical) interest to learn more about this mechanism and the analysis of transcribed genes as the first step of gene activity is currently in the focus of all life sciences. The "transcriptome", i.e. the manifold of all genes transcribed in a specific cell (cell type) at a given time under defined conditions, can effectively be analysed using DNA microarrays. Usually, a comparison is made between a cell type in status A and an altered state, status B, which might be linked for instance to a disease. Both samples, material extracted from cells of status A and B, are labelled with two different fluorochromes. The difference in gene activity in both cell types can easily be recognised wherever one colour exceeds the other. The comparison method compensates for many shortcomings of the array method, since each feature of the array is referenced in itself. The only information drawn is with regard to the binding of species A or B to the same probe on the surface. Different hybridisation conditions for different features on the array result in various absolute signal values (fluorescence intensities), however, the relative amount of the two compared samples are still valid. Therefore most applications of microarray technology reported today are in the realm of transcription analysis.

3.2
Genotyping with Oligonucleotide Arrays

The first driving force for microarray development was the Human Genome Project (HUGO) conducted as an international exercise to decode the whole human genome for a first example [20].

The microarray approach of sequencing was the so-called method of sequencing by hybridisation (SBH) [21]. It is based on the notion to combine

the complete sequence of a sample by presenting all possible sequences as a complement on the chip, these are for 12 bases 4^{12} (=16 777 216) oligomers have to be presented on the chip. This goal was not only difficult to achieve technically in the early state of microarray fabrication technology, but also some basic features of natural sequences have been overlooked. For instance, it would be impossible to get homogeneous hybridisation conditions for all sequences on one single chip since the melting temperature depends strongly on the ratio of GC to AT pairs within this sequence. But even worse, there was no way of overcoming the problem of biologically meaningful redundancies within the genome originating from similar proteins, so it turned out to be impossible to get all the short probed sequences unambiguously linked together.

Today SBH can be used in all those contexts were the sequence of interest is already known to a certain extent and deviations are sought.

4
The Concept of "Active Arrays"

Recently the concept of biosensors to measure binding events and other biomolecular activities using surface-bound molecules has been adapted to microarray technology [22]. By use of time and spatial resolved measurement, the kinetics of biomolecular interactions may be disclosed for a set of immobilised receptors in just one single experiment. A few examples are given in the next two paragraphes.

4.1
Enzymes Acting on Immobilised DNA

In the early 1990s it was shown by several authors that DNA-modifying enzymes are capable of acting on immobilised DNA templates or primers [7, 23, 24]. Especially primers, oligonucleotides of less than 30 bases, are of interest in the context of microarrays.

In our laboratory we achieved the parallel measurement of enzyme activities on several templates by virtue of a microfluidic chamber mounted on top of the microarray slide. As an example, the restriction endonuclease (EcoRI) was chosen acting on different templates simultaneously. In this latter case, a usual microarray covered with immobilised oligonucleotides is exposed to a variety of complementary sequences, each of which is labelled with a fluorochrome (FITC in the actual case). By hybridisation in various spots the endonuclease's cleaving site is formed, forcing the applied enzyme to bind. By addition of the cofactor Mg^{2+} the enzymes starts to cleave and releases the short oligonucleotide with the fluorochrome.

Already in 1996 Buck and Buckle et al. published the first results on the observation of polymerase acting on an immobilised template by use of an SPR device (Biacore) in a single channel. This approach could be extended to the enzyme telomerase that is responsible for the elongation of chromosomal ends during cell proliferation. Since its activity is regulated down in differentiated cells, the amount of activity has been established as a significant tumour marker in recent years [25]. Schmidt et al. succeeded to demonstrate that telomerase acts also on artificial telomeres immobilised on a sensor surface [26, 27].

The use of immobilised templates for polymerase processing was also successful with long DNA including whole genes.

Also, for the analysis of single nucleotide polymorphisms (SNPs), enzymes acting on immobilised templates have been employed. This was first demonstrated by Erdogan et al. who used DNA polymerases to elongate surface-bound primers [28]. These authors also showed the reaction on an oligonucleotide microarray. They were using immobilised allele-specific oligonucleotide primers on a glass slide. Single-stranded PCR products serve as a template that hybridises to the corresponding oligonucleotide probes on the microarray. The match and mismatch primer differ at their 3′-end by a variable base, which is discriminated by the DNA polymerase during elongation process and the incorporation of Cy3-labelled dUTP due to the corresponding signal intensity [28]. In this work, a limit for the template length was stated at 5.7 kb. Recent work by von Nickisch-Rosenegk et al. could not find such a limit and showed on-chip elongation up to 16.5 kb, i.e. the whole mitochondrial genome [29].

4.2 PCR on the Chip

While all reactions described up to now were one-step reactions, a complete PCR on the chip needs reproduction of the polymerisation step several times, and heating up the whole chip to 95 °C for denaturation between every polymerisation step.

A complete microarray-based amplification (on-chip PCR) was first described by Adessi et al. [30]. For the amplification the authors proposed the specific covalent attachment of oligonucleotide primers via their 5′-end to the glass slide, allowing the free 3′-end to prime the DNA synthesis. Template DNA can hybridise to these free 3′-ends and will be available for elongation by a DNA polymerase. After elongation, the resulting amplification product is covalently attached to the surface via the primer. Detection of the immobilised amplificates can be achieved by direct labelling with fluorescently labelled primers or dUTPs. Another possibility may be the use of intercalators like SYBR Green, but due to the resulting background problems, this is not yet successful. The same is true for fluorescent dendrimer labels, e.g. 3DNA La-

bels from Genisphere, which are available for microarray hybridisations. For further sequence verification, the amplificates can be denatured with alkali and subsequently hybridised with a sequence-specific fluorescent probe.

Primers used for on-chip PCR have to fulfil three criteria besides the usual properties like specificity, G/C-content and melting temperature. They have to be immobilised in a density that allows the detection of the resulting amplificates. Adessi et al. states that for the immobilised primer, a concentration of 50 μM is best suited for on-chip PCR experiments [30], however, they fail to determine the actual primer density on the glass slide they could achieve by their immobilisation procedure. The linkage of the primer to the surface must be stable enough to withstand the extreme temperature conditions during the cycling process. Therefore covalent attachment (e.g. EDC coupling) is the best option. The last important criterion to be considered is the attachment of the primer at the 5′-end and the introduction of a spacer sequence for superior hybridisation efficiency. The length and composition of the spacer sequence can be variable. An optimal length is described to be between 10 and 18 nucleotides, preferentially as polyT-spacer [30, 31].

The amplification process takes place in two different phases: In the liquid and on the solid phase. Figure 10 represents a schematic overview of the general principle of on-chip PCR at the solid-phase state.

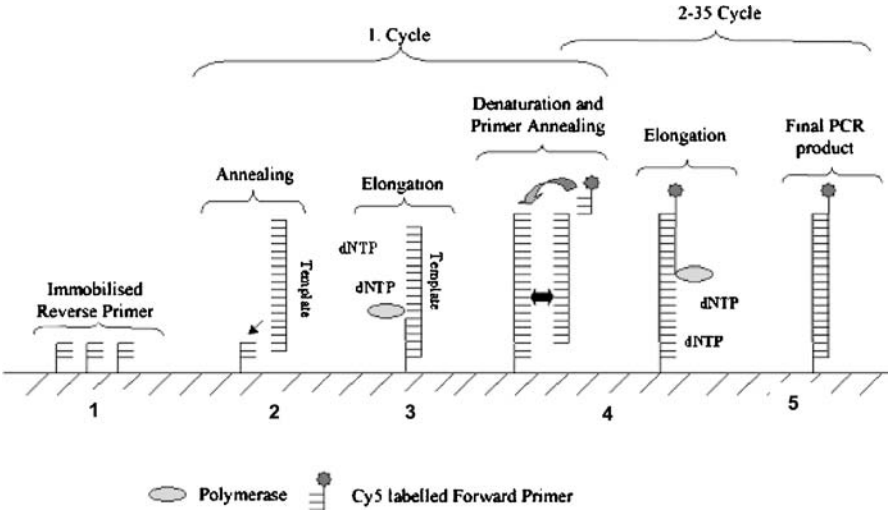

Fig. 10 Principle of the on-chip PCR: solid-phase amplification. In the solid-phase PCR the template DNA, which can be either from the liquid phase or the original template, hybridises to the specific 5′-bound primer (*1*) during the annealing step (*2*). After the annealing, the elongation step by the polymerase follows (*3*). In the next cycle, after denaturation and annealing of the Cy5-labelled primer (*4*), we will find the final Cy5-labelled PCR amplificate 5′-bound to the surface (*5*) and ready for subsequent analysis. This process is repeated during every cycle of the on-chip PCR.

Because the cycling takes place directly on the slide surface, a special containment for the PCR mix is needed. To prevent vaporisation of the PCR mix, this containment has to be absolutely leak-proof even at elevated temperature. For this purpose, there are two different options currently available. The first option is the usage of adhesive reaction chambers, like SealFrame (MJ Research, USA) or HybriWells (Sigma-Aldrich, USA). Another option is the use of the Self-Seal Reagent (MJ Research, USA), which is added directly to the PCR mix and builds a tight seal at the edges of a coverslip by polymerisation during the first denaturation cycle. Both options have been successfully applied for the on-chip PCR [28, 30, 32–34].

The thermocycling of the on-chip PCR is carried out in in situ PCR blocks. Usually, the 16×16 Twin Tower in situ block for the PTC 200 thermocycler from MJ Research is used [28, 30, 32–34] for this purpose. Sensitivities for the on-chip PCR are reported to be in the range of pico to nanomolar DNA template concentrations [30] or between 30–100 ng genomic DNA [28, 32, 33].

Due to the fact that on-chip PCR has been developed quite recently, only a few reports are given on applications. Huber et al. were focussing on the detection of single base alterations in genomic DNA (SNP analysis). In advancing this method, the authors focus on sequence-specific genotyping by solid-phase amplification [32, 33]. Because there is no need of template preparation, advantages like time and cost savings are obvious when using the on-chip PCR. Huber et al. could show that their experimental setup is suitable for the detection of SNPs in the human tumour suppressor gene *p*53 [32]. Further integration could be achieved if the single on-chip PCR is extended for parallel detection of several SNP's in one reaction: a multiplex on-chip PCR. Huber et al. were able to show the multiplex on-chip PCR for accessing SNP's in genomic DNA [33]. In this paper the genotyping of ten different polymorphic sites within seven human genes by direct on-chip multiplex PCR has been shown.

Mitterer et al. reports on-chip PCR in combination with universal primer pairs targeting for the Helix 43 and 69 region of the 23s rDNA and species-specific primers immobilised on the chip surface were used to detect 22 common bacteria causing infertility and abortion in mares [34]. Although false-positive signals were obtained in rare occasions, this work shows possible future perspectives for the on-chip PCR in clinical diagnostics.

Recently we demonstrated the multiplex on-chip PCR with a variety of markers for food ingredients [35, 36].

The area of diagnostic applications is still under development in the field of chip-based assays. A technique that could give fast and reliable answers about, e.g. the current state of an individual's viral or pathogen load, would be of great benefit. With further optimisation, the sensitivity of the PCR and the miniaturisation and parallelism of microarrays could join in form of the on-chip PCR and build a core part in diagnostic BioMEMS [37]for future point-of-care devices.

4.3 Transcription on Chip

The immobilised DNA might be amplified, as described above. Recently we demonstrated that even more functionality may be gained on the chip when the sequence immobilised or produced on the surface is a complete gene. When associated with a proper binding site and a promoter, i.e. a complete gene, the action of a transcriptase can be triggered and a messenger RNA was generated [38]. Moreover, it could be shown in these experiments that the mRNA was functional and served as a template for translation. The encoded protein could be produced with an in vitro assay.

Also it was possible to use microarrays the other way around, namely to gain amplified DNA signals from low amounts of mRNA. We demonstrated recently that the reverse transcription PCR (RT-PCR) could be combined with microarray technology for the detection of low abundant mRNA. The experiment runs similar to the above described on-chip PCR, now starting with the reverse transcription in the same incubation chamber.

4.4 Future Prospects

The examples given here show that microarrays are useful tools for molecular biology, especially for transcription analysis. But there are many more possibilities to make use of immobilised arrayed DNA in small dimensions: using microarrays as a template for enzymes helps to detect and investigate a lot of interactions with DNA, to gain insight into novel features and to develop more sophisticated and time-saving tools of increasing complexity. In this way, we gain insight into the processing steps that usually are imbedded into the complex environment of the whole cell.

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