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Review

Fabrication of high quality microarrays

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Abstract

Fabrication of DNA microarray demands that between ten (diagnostic microarrays) and many hundred thousands of probes (research or screening microarrays) are efficiently immobilised to a glass or plastic surface using a suitable chemistry. DNA microarray performance is measured by parameters like array geometry, spot density, spot characteristics (morphology, probe density and hybridised density), background, specificity and sensitivity. At least 13 factors affect these parameters and factors affecting fabrication of microarrays are used in this review to compare different fabrication methods (spotted microarrays and in situ synthesis of microarrays) and immobilisation chemistries.
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Keywords: DNA microarrays; Fabrication; Immobilisation chemistry; Optimisation

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1. Introduction

Microarrays of DNA probes were introduced some 10 years ago and there has been a rapid evolution of the technology since then. In 1995, 45 complementary DNA (cDNA) probes were spotted in a microarray on a glass slide, the DNA was immobilised and the resulting microarray was used for gene expression analysis [1] (in this review probe refers to molecules being immobilised and target refers to the molecules in the sample being captured). The technological progress was extremely rapid and 1 year later 1000 probes were arrayed [2–4]. However, impressive these numbers were at the time, an alternative technology based on in situ synthesis of DNA on solid support directed by light [5] allowed fabrication of microarrays of 135,000 probes [6]. It should be pointed out that neither of the technologies was principally new but just improvements of the existing dot blot techniques where DNA is immobilised on membranes and usually probed using radioactively labelled DNA. Some very important improvements compared to the dot blot technique were made. Firstly, *miniaturisation* of the spots allowed for better sensitivity [7] and more genes to be analysed per microgram RNA or DNA. Secondly, *fluorescence* was used for *detection* instead of radioactivity allowing co-hybridisation experiments as well as

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simplifying the use in many laboratories. Thirdly, a *rigid solid support* (glass) was introduced [8,9] that was far more easy to work with than membranes. Miniaturisation is the most important improvement and has allowed ''global'' analysis of the genome or transcriptome in one batch process. Expression of 40,000 different mRNA molecules or 100,000 different single nucleotide polymorphism (SNP) can be analysed on a piece of glass that are between 1 and 8 cm^2 depending on technology used. Beside DNA microarray based assays, the microarray has proven to be an powerful platform for other types of assays like protein-protein interactions [10], antibody microarray as substitute for ELISA [11–13], small molecule measurements [14], high throughput transfections [15] and DNA-protein interaction analysis for genome wide promoter studies [16]. Microarray has also proven to be useful as a diagnostic tool where medium and low numbers of probes are used. The reason is that small sample volumes are required compared with other methods, the sensitivity is adequate, and even as little as 25 probes printed into the bottom of a microtitre plate well result in very high throughput and cost-cuts in diagnostics. For diagnostic microarrays so called spotted microarrays are usually preferred where pre-made and quality controlled DNA probes are dispensed onto the surface. The reason being that these small arrays of up to a few hundreds of probes are too expensive to produce with in situ synthesis techniques. By contrast the large microarrays used primarily in research of genomes and transcriptomes can be produced by spotting or in situ synthesis. This review will investigate the current status for fabricating high quality microarray for both diagnostic use (small microarrays) and research use (large microarrays). Although the review will focus on fabrication of DNA microarray many of the issues discussed here are also relevant for the fabrication of protein microarrays which are rapidly emerging as powerful proteomic and diagnostic tools [17].

Microarray can be divided into two categories: (i) an open standardized platform based on the microscope slide format and (ii) all others. The open platform can be tailored to specific requirement and budget since there are many suppliers of activated slides and hardware accepting the microscope slides like robots for arraying, scanners, washing stations and hybridization station. A further advantage of the open platform is that other types of microarrays (e.g. protein microarrays) printed on microscope slides can be analysed using the same instruments as DNA microarrays. In comparison, non-standardised systems led, very successfully, by Affymetrix, consist of a ''kit'' containing washing-station, hybridisation stations and scanners that fit the Affymetrix DNA microarray chips. An Affymetrix chip cannot be scanned in a microarray scanner for microscope slides. The apparent limitations in the flexibility of the Affymetrix system seem like a drawback but can actually also be an advantage, because it provides a complete system that works without optimization. The pros and cons of the open system versus the Affymetrix platform resemble very much the clear difference in attitude between Macintosh computers and Windows based computers where the Mac stands for user friendliness but traditionally at the cost of flexibility and economics. It is possibly so that part of Affymetrix success is due to its user friendliness; however there are many situations where the open platform is the only choice for both economical and in practical reasons.

2. Microarray performance parameters

Fabrication of spotted microarrays is a multiparameter optimisation problem. The performance of the microarray can be measured by many parameters. In Table 1 these parameters are listed on top and the factors influencing the parameters on the side. Array geometry is the spatial localisation of spots in the microarray. Spot density measured how many (different) spots that can be fabricated in a given area. Spot performance can be divided into three under parameters; morphology, probe density and hybridised density. Morphology regards the shape and homogeneity of the spots. Probe density is defined as the number of probes molecules that are immobilised in a given area and hybridised density is defined as the number of target

Table 1
Factors affecting DNA microarray performance

	Array geometry	Spot density	Spot performance			Background	Specificity
			Morphology	Probe density	Hybridised density		
Robotics	Yes	No	No	No	No	No	No
Spotter type (pin, inkjet)	No	Yes	Yes	Yes	Yes	No	No
Pin type	No	Yes	Yes	Yes	Yes	No	No
Humidity	No	Yes	Yes	Yes	Yes	No	No
Temperature at spotting	No	Yes	Yes	Yes	Yes	No	No
Probe concentration	No	Yes	Yes	Yes	Yes	No	No
Spotting buffer	No	Yes	Yes	Yes	Yes	No	Yes
Immobilisation chemistry	No	Yes	Yes	Yes	Yes	Yes	Yes
Blocking technique	No	No	No	No	No	Yes	No
Stringency during hybridisation/washing	No	No	No	No	Yes	No	Yes
Hybridisation conditions (diffusion/mixing)	No	No	Yes	No	Yes	Yes	Yes
Probe sequence	No	No	No	No	Yes	No	Yes
Target preparation	No	No	No	No	Yes	No	No

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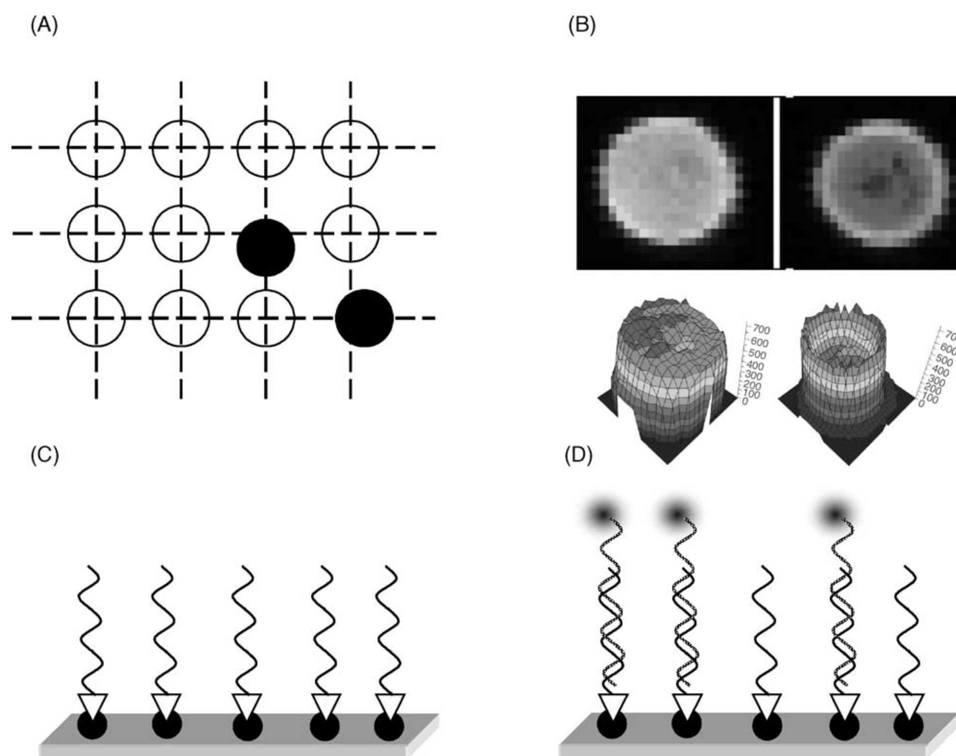

Fig. 1. Definitions of parameters affecting fabrication of microarrays. (A) Spot density and array geometry. Spot density is defined as the number of spots that can be fitted into a given area. Spot density is determined by the distance between the spots centre to centre in both the *X* and *Y* direction. Array geometry is defined as the consistency in which the spots are deposited. The spots should be spotted in a perfect array with equal distances between each spot in the *X* and *Y* direction. Black spots illustrate misalignment during spotting. Beside misalignment, a common error is completely lacking spots or spots containing various amounts of probes. (B) Fluorescent image and 3D illustration of high quality spots (homogenous) and low quality spots (coffee ring). (C) Probe density is defined as number of probes located on a given surface while hybridised density is defined as the number of target located on a given surface (D). Hybridisation efficiency is defined as the ratio between hybridised and probe density.

molecules that can hybridise to a given area (Fig. 1). From this matrix it is apparent that a perfectly optimised microarray experiment is not possible in practice since the number of possible combinations is too large. As a consequence, trouble-shooting microarray fabrication can be complex. Table 1 can be used as guide when trouble-shooting the performance of microarrays produced by spotting. These parameters and factors are discussed below in the respective section.

3. In situ synthesis of DNA microarrays

There are three fundamental ways to fabricate a DNA microarray: (i) contact, (ii) non-contact printing and (iii) in situ synthesis of microarrays. The different fabrication methods have their strengths and weaknesses. In situ synthesised microarrays are very powerful since extremely high spot densities can be reached and the probe sequence can be chosen more or less randomly for each synthesis. Affymetrix for instance produces microarrays routinely with millions of probes on 1.28 cm^2 surface. There is almost no space between the spots and spotsizes are below 10 μm. The drawback is that these microarrays are produced with clean room techniques borrowed from the semiconductor industry. Thus, microarrays are produced by subsequent exposure of the surface with different masks (Fig. 2A). The masks define in which areas of the array the photo labile protective group on the phosphoramidite is destroyed. Destruction of the photo labile group is necessary for the addition of the next building block to the growing DNA chain (Fig. 2A). The probes in these microarrays are limited in size to 25 bases in practice since the yield of full length probes drops rapidly with the length of the synthesised probes. The flexibility of this fabrication process is limited since up to 100 masks are needed for fabrication of one chip layout (sequence of the probes). Changing the chip layout requires development of new masks. A more flexible variant of on chip synthesis uses small mirrors to guide the synthesis avoiding the need for masks (Fig. 2B) [18]. Spot sizes down to 16 μm with 16 μm between spots have been reported meaning that 97,500 spots per cm^2 can be obtained. Initially, the calculated efficiency of the synthesis was quite low (95% in each step) but has improved to 99% using the appropriate photolabile DNA synthesis blocking reagent (2-nitrophenyl propoxycarbonyl (NPPOC)) [19]. As a results 77% of 25 bases long oligonucleotides are synthesised correctly and even 60 bases or longer probes (132 bases) can be synthesised with decent yield [19]. Another alternative, adopted by Agilent, is in situ synthesis of DNA microarrays by spotting four different phosphoramidite nucleosides directly on the spots (Fig. 2C). The order of added phosphoramidite nucleoside determines the probe sequence. The spot densities of the latter technology are

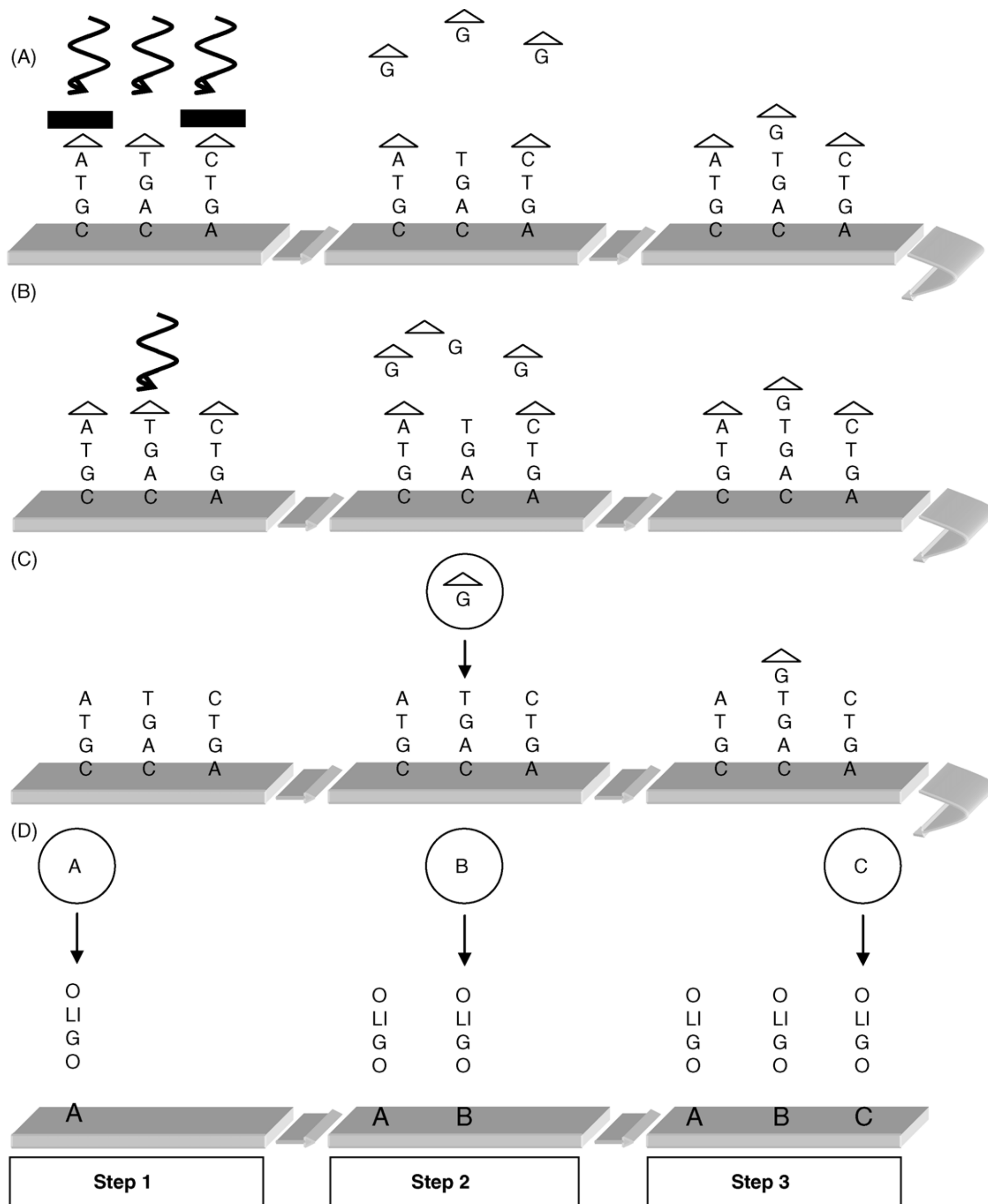

Fig. 2. Methods to fabricate DNA microarrays. (A) In situ synthesis using masks. Extension of DNA oligonucleotides are determined by light that passes through a mask (step 2). The light destroys a photolabile protective group (step 1–2) on the phosphoamidite making selective extension possible (step 3). This process requires 100 masks and thus 100 repetitions of step 1–3 for production of arrays with 25-bases long probes. (B) Light can also be directed to certain parts of the microarray using small mirrors instead of masks (step 1). Also in this case, the light destroys a protective group (step 2) allowing selective extension of certain oligonucleotides (step 3). The process is repeated 4 × length of the oligonucleotides being synthesised meaning that a 60 bp probe requires 240 iterations of step 1–3. (C) Selective extension by nanodispensing of nucleotides. First all the oligonucleotides on the slide are deprotected (step 1). The phosphoramidite nucleoside that is to be extended is dispensed using an inkjet printer (step 2). Only the spots that received the drop of nucleoside will incorporate the nucleoside (step 3). Step 1 and three is repeated until the synthesis is finished. (D) Fabrication of spotted microarray. In each step a pre synthesised DNA is deposited onto the microarray. The number of fabrication steps equals the number of probes on the surface. However, higher rate of processing can be obtained by using printing heads with 48–96 pins allowing 48–96 different oligos to be deposited in each process step which reduces printing time.

much lower than light directed synthesis (5000 spots/cm^2) mainly because of the natural limitation of liquid handling. The standard phosphoramidite chemistry [20,21] is very efficient and allows longer probes to be synthesized (60–100 bp). A similar inkjet based in situ DNA system has recently been described allowing construction of a machine that synthesis 9800 DNA probes on a standard microscope slide [22]. Xeotron technology (now Invitrogen) is also based on phosphoramidite chemistry. In this case however, a photogenerated acid is used to deprotect the growing DNA chain. Xeotron uses a microarray of microfluidic chambers where each chamber defines a microarray spot. The process is apparently very robust since

150 bases long DNA oligos have been generated using this technology. Another variant of synthesising DNA is used by Combimatrix. In this technology small electrodes at the spots generates acids locally to initiate DNA synthesis. The technology provides probes up to 40 bases long.

The only commercially available complete system for on chip synthesis is the FEBIT biotech's Geniome One. This system is attractive since it contains everything needed for making a microarray experiment. It contains in-situ DNA synthesis, hybridisation and detection units all within one instrument. Microarrays are synthesised automatically using similar maskless technology as Nimblegen according to the procedure described by Singh-Gasson et al. [18]. Geniome One can synthesise arrays with up to 48,000 features on one chip. However one chip can be divided into eight subarrays each consisting of 6000 features allowing eight separate experiments to be analysed on one chip. Currently the maximum synthesis length of probes is 25 bases but modification of the instrument is underway to allow synthesis of longer probes (personal communication with Peer Stähler, FEBIT Biotech).

4. Spotted microarrays

Custom made microarrays are however to a large extent dominated by the competing technology where presynthesised DNA is spotted onto microscope slides (Fig. 2D). These spotted microarrays can be produced either by contact printing or non-contact printing. Microarray fabrication using contact printing is based on high definition pins that upon contact with the microarray substrate deposits a small amount of probe solution (Fig. 1D) [1]. The pins are attached to a robotic arm that moves the pins between the different probe solutions, the glass slides where the microarray is created and a washing station. Non-contact printing is similar in terms of robotics but instead of pins, small dispensing systems are mounted on the robotic arm [23,24]. The dispensing system can be based on inkjet, bubble-jet or piezo actuation technology and can usually dispense in the range of 100 pL to 2 μL. Contact printing usually results in spot densities of 2000–4000 spots/cm^2 while non-contact printing can have slightly higher spot density.

Although a full transcriptome microarray with 40,000 features can be fitted onto a microscope slide there is however a need to produce microarrays with higher spot densities. The area defining the microarray also defines the amount of sample that is required. A small compact microarray will need less target material than a larger microarray, which is very important since often starting material is scarce. If the microarray is sufficiently small, diffusion is a rapid enough transport mechanism for efficient mixing avoiding the need for additional instrumentation and laboratory steps for mixing [25,26]. Further miniaturisation also allows fabrication of large microarrays in the bottom of microtitre plate wells with an area of 0.6 cm^2. A microarray with 40,000 features in a microtitre plate well would allow for high throughput diagnostics using standard robotics. Fabrication of large microarrays in microtitre well is needed for introducing cancer diagnostics based on gene expression profiles [27–29] and comparative genomic hybridization [30] and assessing the risk of diseases using large panels of SNPs. There are some interesting possibilities to increase the spot density. TOPSPIN Aps provides silicon pins for contact printing where spot densities up to 10,000/cm^2 has been obtained (personal observations) in preliminary experiments. The reason for the higher spot density is that the pins have a smaller footprint, which yields smaller spots than steel pins usually used for contact printing. Spotted microarrays can be produced with nanometer sized features in so called nanoarrays (spot size less than one micrometer) [31–33]. These nanoarrays are fabricated by contact printing where very small cantilevers (atomic force microscopy tips) are used to dispense the biological material. Spot sizes below 100 nm has been obtained spaced 1–2 μm apart [32]. Assuming 1 μm between the spots, nanoarrays could have as many as 100 million spots/cm^2. However, nanoarrays still need to prove themselves in real multiplex assays. Depositing small amount of molecules might be possible but spotting 100 million *different* capture molecules at these densities is difficult since it is difficult to load the cantilevers with different probe solutions.

Decreasing the droplets delivered by non-contact printing devices will result in smaller spot sizes size. Interestingly, a 9600 dot per inch (dpi) inkjet printer dispenses only 1 pL of ink per drop can be bought for less than 200$ in a computer store and since similar technology is used for non-contact printing of DNA microarrays, the density of spots could be increased substantially. One pL drops would result in spot diameter of approximately 20 μm meaning that non-contact printing using smaller droplets could result in microarrays with up to 60,000 spots/cm^2. In situ synthesis by non-contact printing (Fig. 2C) would also benefit from using decreased drop sizes to obtain significantly higher spot densities.

There are several problems with ultra miniaturisation of the microarrays. Part of the success of the microarray technology has been the relatively simple detection method using fluorescence. As a consequence of decreasing spot sizes, new fluorescent scanners with higher resolution must be developed. Many of the commercial scanners today have a resolution of 2.5–5 μm meaning that the smallest spotsize still giving robust quantification (25–50 pixels/spot) is approximately 30 μm in diameter corresponding to spot densities of about 30,000/cm^2 assuming 30 μm between each spot. Higher resolution scanners will have the drawback that the fluorescent signal will be weaker for each pixel since fluorescence is collected from a smaller area. Noise from for instance electronics will therefore play a larger and larger role when resolution of the scanner increases. Other detection methods may be applied like atomic force microscopy (AFM) [32,34] or scanning electron microscopy (SEM) [35,36]. AFM is label free while SEM requires an electron dense particle like a gold nano-particle as a label on the target. Particle as labels are however less fortunate because they are relatively large as comparison with an organic fluorochome. For instance, only 25 nanogold particles with diameter of 10 nm can be fitted in a spot that is 50 nm in diameter. The dynamic range would thus be maximally 25-fold and probably much less since gold particles

are difficult to pack to perfect monolayers [35]. A very sensitive detection method relies on two step process where a gold nanoparticle functions as a nucleus for a silver reduction reaction [37,38]. The resulting particle is in the range of 1 μm [38]. This detection system is sensitive enough to detect SNP in unamplified human DNA [39] and gene expression from small amount of total RNA [40]. Although sensitive, this detection method appears to be incompatible with small spot sizes since the resulting silver particle is very large compared to a spot. For instance it is impossible to get a quantitative readout of a 1 μm^2 spot using this detection technique since only one nano gold particle would result in a silver particle that is equally large as the spot.

Decreasing spot-sizes also means that the number of probe molecules can be reduced to such low numbers so the dynamic range a spot can sense may be impeded. The maximum density of double stranded DNA is 50 pmol/cm^2 or 3×10^{13} molcules/cm^2 [41]. There would therefore be 300,000 DNA per μm^2 and a spot with 50 nm diameter would contain only 600 molecules. In order to sense a dynamic range of 10^5-fold which is desired for gene expression analysis, the spots must contain at least 10^5 molecules. Considering that the maximum hybridised density observed is often significantly less that 50 pmol/cm^2 (see later section) a spot that is sensitive to a large dynamic range of incoming target molecules must be larger than about 1 μm^2. For these reasons, it is likely that the optimal spots size will be significantly larger than 1 μm in diameter but most likely be smaller than 50 μm depending on assay.

Beside high spot density the microarray must be well defined geometrically meaning that each spot should be at exactly the same distance from each other within an array (Fig. 1A). The reason for this is the need to simplify quantification which requires that a grid is overlayed the graphic image. The grid is determined by a computer and is very precise since it is based on pixels where one pixel typically represents 5 or 10 μm depending on scanning resolution. An offset in the spotter will results in problem with automatic quantification and manual adjustment of the grid is needed. For a few samples this can be acceptable but if microarrays are to be used as diagnostic tools, automation of the quantification procedure is necessary. It is therefore desired that the spots are located at exactly the same position on each slide. Microarrays fabricated using in situ synthesis guided by light should have very high precision of the array geometry (within micrometers) allowing automated quantification and analysis The array geometry of spotted microarray mainly depends on robotics (Table 1). The linear magnetic linear drive of modern spotters have precision of 1 μm suggesting that the precision is adequate for reproducible microarray fabrication. However, a 20,000 spot array can be spotted as a 100 × 200 array where each spot is 300 μm apart. Even an offset of 1 μm per spot will result in a 100–200 μm misalignment over the microarray which may impede automatic quantification.

The most troublesome array geometry problem is probes that are lacking entirely on the surface, which can be caused by failures to deliver the drops from the printing tool to the surface during printing. Contact printing on hydrophobic surfaces can be problematic and in the worst case the liquid may not be delivered properly to the surface since the droplet is not anchored to the surface when the pin hits the surface and therefore cannot be dragged off the pin when the pin is retracted. The main advantage of non-contact printing is that a defined droplet is delivered to the surface without touching it. The amount of liquid deposited is therefore not dependent on surface properties of the slide. Significant better spots morphology has been observed on hydrophobic surfaces using non-contact printing compared to contact printing [48,49]. Furthermore, many non-contact printers comes with drop control that verifies that a droplet is fired from the nuzzle which would limit the number of missing spots. Another reason for missing spots is that a full transcriptome microarray takes hours to days to print depending on the robot used and the few microlitres of probe solution in the microtitre plates can evaporate during this period. This can have profound effect on the quality of the microarray since in worst case the well can dry out resulting in no probe being deposited. The counteraction is to use betaine or DMSO based buffers which retain water. Alternatively, some spotters come with cooled microtitre plate holders allowing the probes to be kept cool. This decreases evaporation but can also protect sensitive probes like proteins. An elegant way to account for any type of spotting failure is to use fluorescently labelled probe molecules. Spots without or with little DNA can be excluded from analysis which result in better microarray analysis [42,43]. Alternatively, the probes can be synthesized to contain a constant region to which a synthetic fluorescently labelled target can hybridise (Dufva et al., unpublished results). The drawback of these approaches is that the probes are more expensive to produce.

The perfect spots have reproducible morphology. Since most custom made microarray systems rely on dispensing liquid the perfect spot is round. However, using other fabrication methods like the one used by Affymetrix in which DNA is synthesised using masks, the spots can have any shape but usually a square is used. The spots should also have the probes evenly distributed so that no region of the spots have higher probe densities compared to others (Fig. 1B). Factors affecting spot morphology are the spotting buffer [44–46], temperature and humidity (Table 1). Producing spots with low intra-spot probe density variation is not trivial since during drying there will be a movement of material from the centre of the spot to the periphery which results in coffee spot phenomena [47]. The movement of material from the centre of the spot to the periphery can be lessened if the liquid has high viscosity [47]. Therefore, salt and polymers (‘‘spotting buffer’’) are usually mixed with the probes prior to spotting. Other additives have other functions. Formaldehyde, betaine and DMSO reduce the evaporation rate and denature DNA. At least for betaine this increases the binding of PCR products to poly-L-lysine coated and aminosilane coated slides [46] which is also reflected by higher hybridisation signals [45,46]. Betaine also results in more homogenous spots. The reason is not clear but most likely addition of betaine makes the spotting solution more viscous and reduces the evaporation rate. As mentioned above, higher viscosity leads to reduced mass transport by capillary actions

while the reduced evaporation rate let the DNA react with the surface in an even fashion leading to more homogenous spots. That the evaporation rate is important for spot homogeneity is also supported by the fact that the temperature in the laboratory affects the spot morphology. We have observed that the spots on our microarrays produced during winter time are much better than those produced during summer. During summer time, the spots have profound coffee spot morphology and the only apparent difference is that the temperature in the laboratory is 5–7 °C higher. It is therefore logical to place the spotters in environmentally controlled rooms where the temperature and also the humidity are stable. However such rooms are expensive to build and maintain and it would be desirable to have temperature as well as humidity control within the spotter.

5. Optical microarrays

Instead of spatially separating the probes as described above, the probes can be separated optically in so called optical arrays. They are not microarrays as such but instead, the different probes are immobilised on beads that have different combination of fluorescent dyes on them. The dyes make up a unique ''barcode'' and a particular probe is associated with one particular barcode. The beads are analysed in a flow cytometer to resolve the identity of the bead using the barcode and thus the probe bound to the bead [50,51]. The bead array is in suspension and is therefore suitable for automation using standard equipment leading to extremely high throughput. In addition, hybridisation kinetics are better for beads than planar microarray since the beads can easily be moved around in solution. There is a limit to how many different barcodes that can be resolved by flow cytometery and currently 100 different beads can be bought and functionalised. This is probably enough for a foreseeable future in diagnostics. However, bead can be functionalised with quantum dots that are spectrally very well defined allowing that 10^6 different beads can theoretically be produced [50]. Optical arrays can thus be almost as large as in situ synthesised microarray. However, functionalising 10^6 different beads with specific probes will not be economically possible. Instead of using a flow cytometer, hybridised fluorescently encoded beads can be detected on bundles of optical fibres where the end of each fibre is fabricated to bind only one bead [52,53]. In contrast to optical microarrays detected with flow cytometry, these random microarrays can use larger numbers of different beads since each bead can be decoded using a series of hybridisation reactions after immobilisation of the beads to the optical fibres. This increases the multiplex capacity to several thousands of different beads [54]. The random microarrays have excellent sensitivity (in the zeptomolar range) since hybridisation takes place in solution [55] which led to the development of PCR free genome wide genotyping [56]. The fibre optical microarray has been commercialised by Illumina.

6. Electrical driven functionalisation

DNA can also be immobilised on microfabricated electrodes without robotics in a technology used by Nanogen. An array of electrodes are integrated into a microfluidic system and DNA which is negatively charged is attracted to the electrodes having a positive change while being repelled by the negatively charged electrodes [57,58]. A microarray is fabricated by adding the different DNA in series which makes the procedure very slow. Therefore only small microarrays can be produced by this technology. However, the electrodes can subsequently be used to improve hybridisation kinetics as a DNA sample can be hybridised in just a few minutes by applying a positive charge on the electrodes. Reversing the field results in an extremely rapid ''electronic washing'' where mismatch hybrids are removed in seconds [59]. The rapid kinetics of hybridisation and washing make the Nanogen systems ideal in cases where rapid answers are required like in point of care diagnostics.

7. Immobilisation of DNA to surfaces

One of the first choices to make when fabricating microarrays is which chemistry to use to immobilise the DNA to the solid support. Another choice is whether or not to fabricate the slides yourself. Home made slides are much less expensive at least in reagents costs than the commercial slides and still provide equal or better performance characteristics than the commercial slides [60,61]. Fabricating your own slides can be a cumbersome process that may actually not pay off economically despite commercial slides being expensive. Commercial slides are usually made of glass and if some other material is needed for an application, special immobilisation protocols might be required. However, DNA has been immobilised on various relevant materials like silicon [62–64], oxidised silicon and fused silica [65], poly (methyl-meta acrylate) (PMMA) [48,49,66,67], poly(dimethylsiloxan) (PDMS) [68,69] and SU-8 [70].

Factors that influence the fabrication of DNA modified surfaces is the immobilisation chemistry, spotting buffer, probe concentration and physical factors like spotter type, pins used and environmental conditions (Table 1). The goal is to have the probes evenly spaced over the surface at optimal distance from each other to allow for high hybridisation efficiencies and highest possible hybridisation signals. Maximum hybridisation signal and maximum hybridisation efficiency is not necessarily obtained at the same probe density [48]. The reason is that DNA probes that are too closely packed cannot participate in hybridisation reaction due to steric hindrance or electrostatic interactions [71–73]. However, in the vast majority of the immobilisation chemistries described (Table 2), the optimisation process is designed to find condition that gives maximum hybridisation signal rather than maximum hybridisation efficiency. Finding optimal probe densities for spotted microarrays is a simple experiment where a dilution series of probe solution is printed and subsequently hybridised using excess target. After quantification of probe density and hybridized density (Fig. 1C and D) the efficiency of hybridisation can be calculated. Despite the use of excess target, hybridisation efficiency is often far less than 100%. This indicates that not all probe molecules on a microarray spot can participate in hybridisation reactions. Steric (Fig. 3A and B) or electrostatic factors can be the reason as mentioned above.

Table 2
Surface modification to bind modified and unmodified DNA

DNA modification	Substrate modification	Ref.
None	Polylysine	[77]
	Amine	[78]
	Epoxy	
	Diazonium ion	[80]
	SU-8	[70]
	Unmodified glass	[79]
	Agarose film	[61]
	Membrane	[87]
Silanes	Unmodified glass	[94]
Thiols (−SH)	Gold	[34,95]
	Mercaptosilanes	[96]
	Maleimide	[48,65]
	Iodoacetyl	[65]
Amines ($-NH_2$)	Aldehydes	[3,48,60]
	Epoxy	[97]
	Isothiocyanate	[75,76]
Phosphates (PO_3)	Aminated surfaces	[98]
Biotin	Avidin	[59,64]

Binding DNA to surfaces is a multistep process. In each step the surface is modified and the goal is to have a surface that can efficiently accept and bind the DNA probes being spotted. In most cases the substrate is modified chemically to bind DNA (Table 2). Furthermore the DNA is also modified with a functional group that specifically reacts with the functional group on the solid support (Fig. 3, Table 2). A linker can be used between the DNA and the modified solid support [48,62,65,74–76] to change the functional group extending from the surface to another functional group (Fig. 3). For instance, glass can be aminated by treating glass with an aminosilane. In the next step the resulting amine surface can be changed into an aldehyde surface using glutaraldehyde which binds DNA by a Schiff base reaction [48]. A linker can also, if sufficiently long, function as a spacer to lift up the DNA probes from the surface making it more accessible for the incoming target [71] (Fig. 3).

The simplest chemistry utilises electrostatic interaction between the solid support and the DNA. A positively charged poly-L-lysin layer is grafted onto microscope slides and the negatively charged DNA is bound to the solid support electrostatically. This was one of the first chemistries used to immobilize PCR products [77]. In order to keep the DNA on the solid support the DNA is cross-linked prior to denaturation of the PCR products. It is clear that the DNA is not in its best conformation for hybridisation and most likely is immobilised through internal bonds (Fig. 3A (I)). PCR products can also be immobilised on aldehyde coated surfaces [1]. In this case it is preferred to use amino modified primers during PCR fragment generation. Since double stranded DNA is printed, the amino group is necessary to covalently link the DNA to the aldehyde groups prior to denaturation. The DNA is presumably linked to the glass only through its end modification (Fig. 3B (I)) The combination of epoxy and tertiary amine on the surface allows for binding of unmodified PCR products [78]. The exact binding is not clear but the tertiary amino group attracts the negatively charged backbone of the DNA while the epoxy group covalently links the DNA via amino groups in the bases.

In contrast to double stranded PCR products, single stranded DNA contains many free amine groups provided by the bases A, C and G. This suggests that the unmodified DNA can be spotted directly onto aldehyde or epoxy surfaces. However, it is believed that for efficient immobilisation on aldehyde surfaces requires modification of DNA with a primary amine and many manufacturers of microarray substrates recommend that such

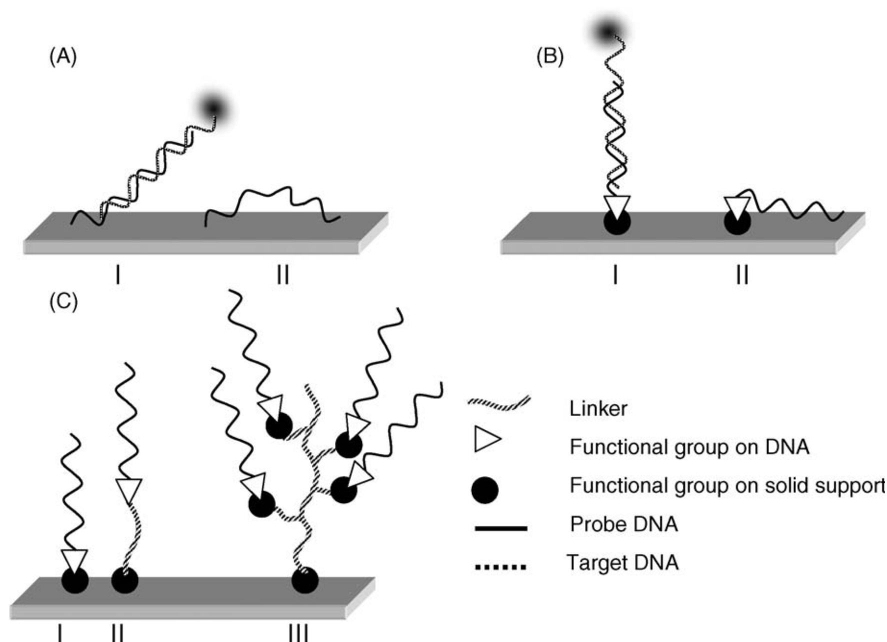

Fig. 3. Immobilisation of DNA to surfaces. (A) Unmodified DNA is randomly immobilised to surfaces meaning that some DNA strands can participate in hybridisation (I) while other cannot (II). (B) Immobilisation of DNA using end modifications (I) can also result in intra chain bonds (II). (C) Molecular organisation of end modified probes directly immobilised to the active groups on the solid support (I) or displaced from the surface using a linker (II) or a dendrimeric linker (III).

modifications are added. This is less fortunate as this increase the probe costs between 100 and 300%. There is however some observation suggesting that unmodified probes can be used efficiently which would reduce the probe costs considerably. Unmodified probes can be directly linked to unmodified glass but the interaction is not stable at high pH or high temperatures suggesting that the DNA is not covalently bound to glass [79]. A better choice is immobilisation of unmodified DNA to epoxy coated slides since the epoxy groups can bind the amine groups provided by bases A, C and G. Unmodified DNA can be immobilised in an aldehyde modified 3D matrix of agarose with similar efficiency as amino modified probes [61]. Non-modified single stranded DNA can also form bonds with aldehyde coated planar glass surfaces (unpublished observation), but unlike the agarose film substrate the hybridised signals are often weaker from unmodified probes than from amino modified probes. Interestingly a chemistry that specifically forms covalent bonds with aromatic amines in the DNA chain (provided by bases A, G and C) has been developed [80]. Glass is modified using *p*-aminophenyl trimethoxysilane which is subsequently converted into the diazobenzyl form that undergoes reaction with the DNA. However, the diazonium ion is very unstable and requires that spotting and washing is performed at 4 °C. Spotting at 4 °C is not trivial and requires a temperate regulated room or other solutions.

Utilizing the internal amines in the DNA has some drawbacks. Firstly shorter DNA molecules are likely less efficiently immobilised since fewer potential reactive groups are present in the DNA molecules. Secondly, DNA immobilised through internal amines are likely to be less available for hybridisation since some of the bases are linked very closely to the surface which may destroy the hybridisation properties of the probes (Fig. 3A). This indicates that the DNA should be linked through specific functional groups added in the 3′ or 5′ end of the probes for optimal hybridisation. Since the DNA contains internal amines these are likely to react with many possible functional groups on the substrate (see Table 2). This could lead to modified DNA being attached to the surface by several bonds; one terminal determined by the added and assumed specific chemical group and some by intra-chain amines (Fig. 3B). This could explain why as little as 10–20% of the immobilised probes are involved in hybridisation reactions. The risk of multiple bonds between the DNA and the surface is high on amino reactive surfaces suggesting that a better approach would be to use non-amino reactive chemistries (Table 2). Gold for instance should specifically react with SH groups. However, Steel et al. demonstrated that significant amounts of unmodified DNA are absorbed onto gold surfaces while thiolated DNA bound gold only 10-fold better [81]. Thiolated DNA less than 24 bases tended to bind the gold though the thiol group attached at the end of the DNA leading to high surface probe densities while longer probes in addition bound through intra-chain bonds.

8. 3D support to increase probe and hybridised density and assay performance

Although it was a great improvement to go from fragile membranes to glass, the latter has a very limited probe binding capacity compared to membranes since membranes have a 3D structure. Furthermore, glass is not optimal as hybridisation support since it is negatively charged at pH values usually used in DNA microarray experiments leading to repulsion of target DNA [73]. Several attempts have been made to increase the probe density and thereby the hybridised density. Molecular 3D-structures [60,71,74–76], acrylic gels pads [82–85], dried gels [61,85,86] and membranes [87,88] have been grafted or covalently bound to glass surfaces or the glass itself has been structured into pores [89] to improve hybridisation signals compared to 2D surfaces. The probe amount is increased due the larger surface area provided by the 3D structure (Fig. 3C) and is probably the main reason for the increased hybridised density. The 3D structures are also providing a solution like environment for hybridisation by lifting the DNA up into the solution like a long linker (Fig. 3C) [17,74,82,90]. The 3D matrixes also enables solid phase enzyme assays on microarrays [91,92] as well as molecular beacon assays [93].

9. Conclusions

The fabrication of microarrays has matured over the past ten years and has become an industry of its own. The choice of strategy for producing microarrays involves a large number of decisions since fabrication of microarrays is a multi-parametric problem (Table 1). In many cases factor like spotter type, temperature, humidity and pin type can be kept constant leaving ‘‘only’’ the chemistry of immobilising DNA to the surface to be optimised. Finding suitable immobilisation chemistry that work together with a particular spotting buffer can be a demanding task. Fortunately, commercially activated slides are available and results in satisfactory DNA microarrays in terms of selectivity and sensitivity without much optimisation. It is difficult to conclude from the literature which chemistry or slide type that is currently superior. However, it appears that the 3D structured surfaces provide better performance than planar substrates. Fabrication of microarrays with a large number of features is probably best done using in situ synthesis while smaller microarrays used in diagnostics is preferably done using spotting techniques. However, powerful as in situ synthesised microarrays are in terms of flexibility and number of features they are expensive to produce even with maskless technology. In situ synthesised microarray fabricated by spotting cost approximately 50$ per slide for 9800 probes [22]. It is likely that the cost of fabrication of 9800 spots or 50 spots is quite similar and rather it is the number of synthesis cycles that determines the costs for in situ synthesised microarray. In comparison, the probe costs for a 50 probe spotted microarrays are far less than 1$. The largest cost of small diagnostic microarrays is the microarray substrate which varies in cost from less that 1$ to over 15$. There is only one commercial in situ synthesis machine on the market which limits the use of in situ synthesised microarrays considerably. What would be really appealing would be commercial alternative to the build it your self system described by Lausted et al. [22] that produces in situ synthesised microarrays on microscope slides. Besides fabrication of large microarrays,

such a machine could also be used to simplify development of diagnostic microarrays for SNP and other genetic analysis by allowing screening of optimal probes. There will be a development to fabricate microarray with smaller spots to accommodate microarrays in microfluidic structures and in microtitre plate wells. This allows use of microarrays in point of care devices and high throughput diagnostics. One of the larger hurdles will be fabrication of microarray with such a consistency that microarray analysis can be more reliable. However, even though microarrays are perfectly fabricated according to the factors indicated in this review, other factors like probe choice, hybridisation conditions (Table 1) etc. play an equally important role in the final assay.

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