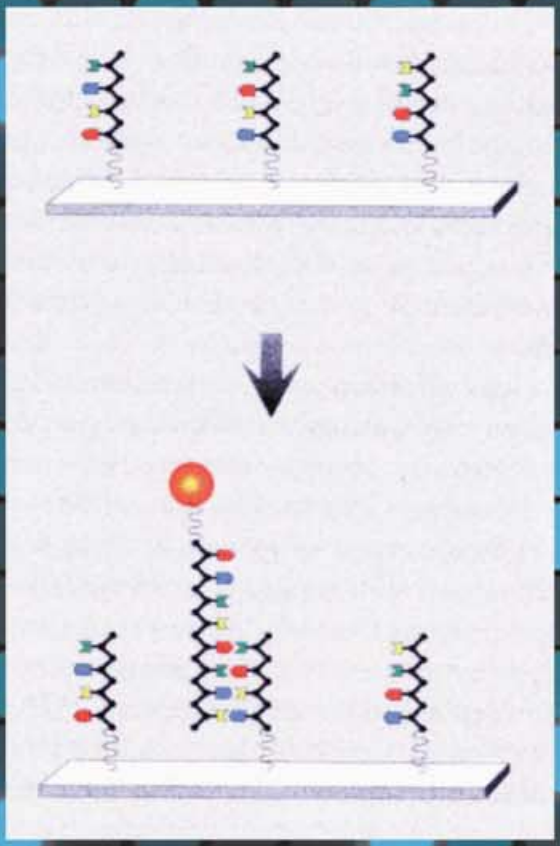
The term DNA chip or DNA microarray refers to the systematic arrangement of biomolecular probes such as DNA molecules on a solid surface (e.g. glass, metal, or silicon wafer).



To identify a particular molecule, characteristic molecular interactions are made use of, for example, the sequence-specific hybridization of nucleic acids. After the hybridization of fluorophore-labeled cRNA or cDNA probes, the resulting hybridization pattern can be detected with a fluorescence scanner.

The middle part of the illustration was reproduced from the Review by C. M. Niemeyer and D. Blohm (ref.[5] cited in this Review).

How to Make a DNA Chip

Michael C. Pirrung*

Microarrays are one of the hottest areas in biological research today. Microarrays have been mostly applied to nucleic acid analysis, specifically to the assessment of which genes are being expressed and at what level. Early microarrays were prepared by using photolithographic methods, which were more commonly used for integrated circuit ("computer chip") production. Hence the colloquial term "DNA chip" came into being. The completion of the sequencing of the human genome and that of many other organisms makes the determination of gene function an important next step in understanding the role of DNA in the processes of life. DNA microarrays are an excellent tool to address this question because their numerous probe sites enable the analysis of many genes simultaneously. With good experience in this initial use, many further applications of microarrays are being developed, including genotyping in research and genetic diagnosis in medicine. DNA microarrays have made abundantly clear the power of vast parallelism in biological analysis, which is raising interest in other types of microarrays (small-molecule, protein). Many applications for DNA microarrays have been developed and clearly many more will emerge through the creativity of the scientists who use them. In early studies, users produced their own microarrays. The apparent power of microarrays has demanded improvements in production methods, and technologies from physical sciences and engineering are now being applied to DNA chips. Many branches of chemistry can contribute to improved methods: from synthetic chemistry (to attach or prepare DNA), to the physical chemistry of surfaces, to analytical chemistry (to assess surface reactions).

Keywords: analytical methods · combinatorial chemistry · microarrays · nucleotides · surface analysis

1. Introduction

Microarrays are defined here as monolithic, flat surfaces that bear multiple probe sites, often hundreds or thousands, and each bear a reagent whose molecular recognition of a complementary molecule can lead to a signal that is detected by an imaging technology, most often fluorescence. Literature references to microarrays before 1995 concerned arrays of electrodes rather than arrays of different molecules. The first molecular microarray, reported in 1991, was composed of peptides, not DNA, and was not even identified as a microarray.[1] The photolithographic technology used to make that array was well discussed in an earlier review.[2] Three books on DNA microarrays have been published,[3] and the area has been reviewed recently,[4,5] and discussed in the popular science press.[6]

DNA arrays are topical owing to their promising ability for obtaining information on nucleic acid levels and sequences faster, simpler, and cheaper than traditional methods. Nucleic acid recognition elements are readily synthesized and can often be reused. Major applications of DNA arrays are projected in large-scale genotyping and gene-expression profiling. Indeed, the parallel processing power of DNA arrays has encouraged a novel approach to science, born from genome sequencing: systematic investigation, in contrast to hypothesis-driven experimentation.

Two key parameters of a microarray are the number of different probe *sites* (spots) per unit area, which reflects its information density (also called complexity), and the number of probe *molecules* per unit area within an individual probe site (density, but often conflated in the literature with complexity). More probes per array means more information and a more powerful bioanalytical tool. To minimize array size, the probe sites (also called features) and their spacing (pitch) should be as small as possible, while still being able to detect reliably molecular recognition (Figure 1). Small arrays reduce the biological sample needed for analysis. A summary of such characteristics for a selection of chip production methods is provided in Table 1. The number of probe molecules in a given spot controls their intermolecular interactions, similar to the loading level on solid-phase synthesis supports. Close probe spacing enables complex interactions among multiple probes and analyte nucleic acids,

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Table 1. Selected Parameters of DNA Chips.

Researcher	Affiliation	Density (molecules cm^{-2})	Surface and Linking Chemistry	Representative Features
Schena[41]	Stanford U.	NR	glass-polylysine to PCR product	2479 × 345 μm
Belosludtsev[31]	Genometrix	3×10^{13}	glass-silane-amine to native DNA	7 × ? μm
Beattie[20]	HARC	1.0×10^{12}	silicon-glass-silane-epoxide to aminolink DNA	NR
Wöhrle[33]	U. Bremen	9.0×10^{12}	glass-silane-amine-PDI-PAMAM-PDI to 5′-aminolink DNA	128 × 140 μm
Zammatteo[45]	U.Notre-D. de la Paix	3.6×10^{11}	glass-silane-aldehyde to 5′-aminolink DNA	9 × 0.4 mm
Boyce-Jacino[27]	Orchid	3×10^{13}	glass-silane-thiol to disulfide DNA	49 × 1 mm
Steel[36]	Gene Logic	6×10^{12}	microchannel glass-silane-thiol to maleimide-3′-aminolink-DNA	256 × 200 μm
Mirzabekov[34]	Argonne	1.8×10^{15}	glass-polyacryl-hydrazide to 3′-dialdehyde-DNA	64 × 100 μm
Smith/Hamer[26]	U. Wisconsin	5.3×10^{12}	silicon-undecanoate-polylysine-maleimide to 5′-thiol-DNA	2 × 2 mm
Hogan[21]	Baylor U.	1×10^{12}	silicon-silane-epoxide to 5′-aminolink DNA	16 × ? μm
Téoule[39]	CIS Bio	1×10^{12}	platinum to 5′-(poly)pyrrole DNA	4 × 0.62 mm
Heller[40]	Nanogen	1.6×10^{12}	platinum-agarose-streptavidin to 5′-biotin DNA	10000 × 30 μm
Smith/Corn[19b]	U. Wisconsin	5×10^{12}	gold-thiol-acid-polylysine-maleimide to thiol-DNA	8 × 3 mm
Corn[24]	U. Wisconsin	NR	gold-thiol/amine-maleimide to thiol-DNA	9 × 750 μm
Corn[25]	U. Wisconsin	1.5×10^{12}	gold-thiol/amine-disulfide to thiol-DNA	16 × 500 μm
Yamamoto[37]	Canon	NR	quartz-silane- amine-maleimide to 5′-thiol DNA	64 × 70 μm
Pirrung[28]	Duke U.	NR	glass-silane-bromoacetamide to 5′-phosphorothioate DNA	64 × 100 μm
Zhao[29]	Amersham	NR	glass-silane-bromoacetamide to internal phosphorothioate DNA	100 × 150 μm
Southern[72]	U. Oxford	4×10^{13} (calc.)	in situ DMTr synthesis on glass - HEG linker	191 × 2.5 mm
Theriault[68]	Combion	1.2×10^{7}	in situ DMTr synthesis	10000 × 0.1 mm
Brennan[67]	Protogene	1.3×10^{12}	in situ DMTr synthesis on glass - HEG linker	? × 50 μm
Blanchard[69]	Rosetta	NR	in situ DMTr synthesis on glass - HEG linker	25000 × 100 μm
Trulson[7]	Affymetrix	1×10^{12}	in situ photochemical synthesis on silica - HEG linker	135000 × 35 μm
McGall[60]	Affymetrix	NR	in situ DMTr/polymer film photoresist synthesis on silica - HEG linker	256 × 100 μm
Matson[71]	Beckman	1.2×10^{10}	in situ synthesis on polypropylene - 5′-phosphoramidate linker	64 × 0.5 mm

NR - not reported.

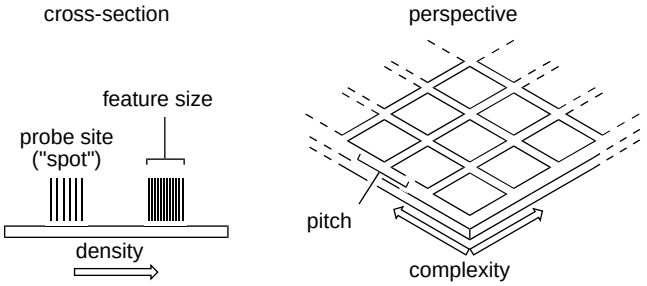


Figure 1. Key microarray parameters.

thus resulting in a nonclassical binding behavior, which can be advantageous.[7] In other cases, a (mostly) normal Langmuir binding isotherm has been reported.[8]

Although many chemical reactions have been developed for DNA attachment to flat surfaces, some have not been used to form multielement microarrays. It seems evident that if one oligonucleotide can be prepared or attached, then, in principle, many can be. In practice, the repetitive steps required to generate highly complex microarrays means that small errors in individual steps accumulate to generate chaos. That the efficiency of a multistep sequence is the product of the yields of individual steps is familiar to chemists. Stamping techniques that may be excellent for creating patterned surfaces may be impractical to deliver ten or more reagents or thousands of DNA probes.

Many aspects of microarray production are common to the different techniques. One aspect is a linker required to create a distance between the surface and the section of oligonucleotide that is to be used in hybridization. This distance avoids steric clashes with incoming molecules and the whole operation more closely resembles conditions in solution. Ethylene glycol oligomers or simply a sequence of thymine

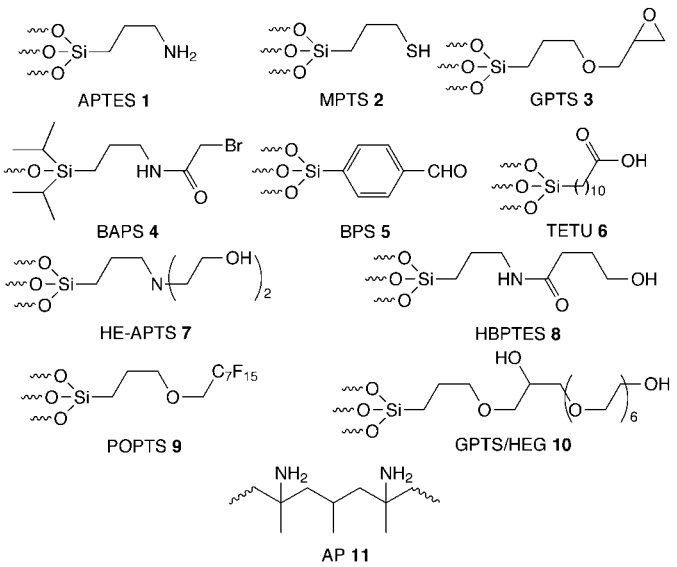
Michael Pirrung began his chemical career at the University of Texas at Austin. He completed his Ph.D. at the University of California at Berkeley with Clayton Heathcock, where his independent research initiated his interest in synthetic photochemistry. After one year at Columbia University, NY, with Gilbert Stork, he took up a position at Stanford. From 1988, he was a member of a group of scientists led by Alejandro Zaffaroni that formed a new company, Affymax, in Palo Alto. His work there focused on light-directed synthesis, which led to the first reported microarray in 1991. He is currently professor of chemistry at Duke University, where his research efforts lie in chemical biology, with an emphasis on microarray technologies and combinatorial chemistry.

(T) bases have been used as linkers. A wide number of protocols for slide preparation/derivatization have been reported. The existence of many solutions to a problem often means that none work very well. However, in an emerging field such as that of DNA chips, workers may not be aware of all contemporary research, and the value of particular technologies may not yet be widely known.

Much effort has been dedicated to the sourcing of the surface on which a microarray is made and its preparation. Owing to accessibility, the standard glass microscope slide has been most widely used, but more sophisticated surfaces, including fused silica, gold, and silicon wafers, are also used. Usually, a cleaning procedure is applied and can involve detergents, strong oxidizers (H_2SO_4/H_2O_2 or "piranha solution", oxygen plasma, NH_3/H_2O_2), sonication, acid, or base. The purpose (or even success!) of cleaning is not always clear, although a generally accepted benefit is the removal of any organic surface contaminants that could interfere with subsequent silane-coating protocols.

For silicon oxide based surfaces, it is necessary to coat the chip with an organosilane. This silane should bear a functionality that can react with a group on the oligonucleotide or on a linker that is suitable for the synthesis of oligonucleotides. Thus, a knowledge of silicon coatings is very relevant to DNA chips. It is generally believed that the formation of siloxane thin films on silicon oxides involves alkoxysilane hydrolysis to a polymeric network that is physisorbed to silanol hydroxy groups in the coating stage. The generation of a stable chemisorbed film occurs upon annealing.[9] Consistent slide derivatization with an organosilane has likely given many novice DNA chip workers significant difficulty. As a macroscopic process, coating is not readily monitored, although one consequence is usually a significant increase in hydrophobicity, which can be observed by the beading of water on the surface and analyzed through the contact angle. Such surfaces are often described as monolayers; *not* because of direct evidence but more likely because an idealized scheme for siloxane bonding does not permit multilayers. A report that oxidized silicon wafers can be coated with a common DNA chip derivatizing agent, APTES (**1**, Scheme 1), to form a true monolayer under very mild conditions (toluene, room temperature, 15 min) has been widely overlooked.[10] Many investigators have used more forcing conditions for coating that can produce multilayers. Use of chemometric tools to optimize the coating of glass slides with MPTS (**2**, Scheme 1) showed that only the coating time and annealing step significantly affect ultimate thiol loading.[11]

The design of the probe sequences that will be placed on a microarray seems straightforward—they must be complementary to the analyte. However, leveling the melting temperature of short probes with different G/C contents by changing their lengths may be necessary. Probes should also have minimal internal structure and should address sites in the analyte with minimal internal structure. These problems should be readily addressed by computation, although predicting the secondary structure of an RNA molecule is computationally intractable. An empirical heuristic that is based on hybridization to probe arrays can be used,[12] and other guidelines have been advanced.[13]



Scheme 1. Reagents for the derivatization of surfaces. APTES = aminopropyltriethoxysilane, MPTS = 3-mercaptopropyltrimethoxysilane, GPTS = glycidoxypropyltrimethoxysilane, HE-APTS = bis(hydroxyethyl)aminopropyltriethoxysilane, HBPTES = hydroxybutyramide propyltriethoxy silane, POPTS = (perfluorooctyloxy)propyltriethoxysilane,

2. Delivery of Presynthesized Oligonucleotides

Microarrays of presynthesized oligonucleotides have been widely produced. The factors that must be considered in their production include derivatization of the slides with functional groups, derivatization of the nucleic acid with complementary functional groups, delivery of tiny volumes of spotting solution, and deactivation of unreacted surface-bound functional groups. A quality issue is the distribution of oligonucleotide within each spot. A common observation is the formation of spots with greater oligonucleotide density at the edges than in the middle. Such a phenomenon is also observed when coffee is spilled and a ringlike stain is produced. This can be readily explained in terms of simple physics: solution flow to the spot edge with evaporation.[14] This is one possible cause of anisotropic spots; contact-based printing methods might also damage the surface. To avoid this morphology, the wetting properties of the solvent must be modified, evaporation must be avoided, and/or surface damage must be limited in the spotting method.

Many new practitioners in this field, our research group included, likely have the initial rewarding experience of spotting a functionalized oligonucleotide onto a derivatized surface and detecting a nice bright spot, only to be chagrined to find that a control oligonucleotide without the functional group also gives a bright spot, either when a label is attached or in the subsequent hybridization. It is not surprising that native DNA can adhere strongly to glass surfaces; this is the basis of many kits used to purify plasmid DNA from minipreps. Indeed, it has been reported that derivatization of the oligonucleotide and the glass surface is unnecessary for assays, even under fairly stringent conditions.[15]

The equipment for spotting oligonucleotides onto arrays were initially homemade, but are now commercially available.

The first such system was developed at Stanford University (see Section 2.2). Rose has provided an extensive analysis of the methods and equipment that can be used to deliver tiny volumes of oligonucleotide solution.[16] Spotting can involve either bringing a pin or needle into contact with the surface or projecting a liquid droplet from a jet nozzle under pressure. The liquid volume that can be delivered is 50 pL – 100 nL, and the resulting spot size is 75 – 500 μm. These two critical factors do not differ much between contact and noncontact methods, thus the choice of spotting technology must be based on other considerations. The design of "pins" for the delivery of pL volumes is ingenious (Figure 2). Many pins are essentially

Figure 2. Pin and stylus designs for the delivery of pL volumes.

highly miniaturized stainless-steel fountain-pen nibs with a gap that draws up the oligonucleotide solution by capillary action. These are commercially available[17] in a wide range of dimensions and lead to a variety of spot sizes and densities. They can load up to ~1 μL of solution so that multiple deliveries can be made from one loading. "Preprinting" several waste spots removes excess solution and enables consistent spotting. Other pins are solid, adhere solution to their faces (flat or concave), and deposit their entire load in one spot. In a modification of this concept, the solution is loaded into a metal ring through which a solid pin is pushed to first pick up and then deliver oligonucleotide, thus enabling multiple deliveries per load.[18] Upon contact of either type of pin with the surface, a ~1-nL spot is delivered, the diameter of which is determined by the surface energies of the pin, liquid, and slide. A universal concern when using delivery systems with multiple oligonucleotides is carryover between spotting a first oligonucleotide and returning to a source plate to pick up a second for spotting. Washing steps are required between samples. Multiple pins or jets in a printhead speed up array production, and up to 64 have been used. Automated spotting systems include fixed locations for the slides to be spotted and for the "source plates", which are microtiter plates that contain the oligonucleotides. Both can be addressed by the printhead by means of a multiaxis positioning stage or gantry. The hydrophobicity of the silane film is useful to maintain (through surface tension) the spot size once it is delivered; the spot does not spread as it would on a hydrophilic surface. The environment must be controlled during deposition to maintain cleanliness and limit evaporation after spotting (relative humidity >70%), so that oligonucleotide attachment, which can take hours with some methods, has sufficient time to occur. High humidity may also improve spot homogeneity.

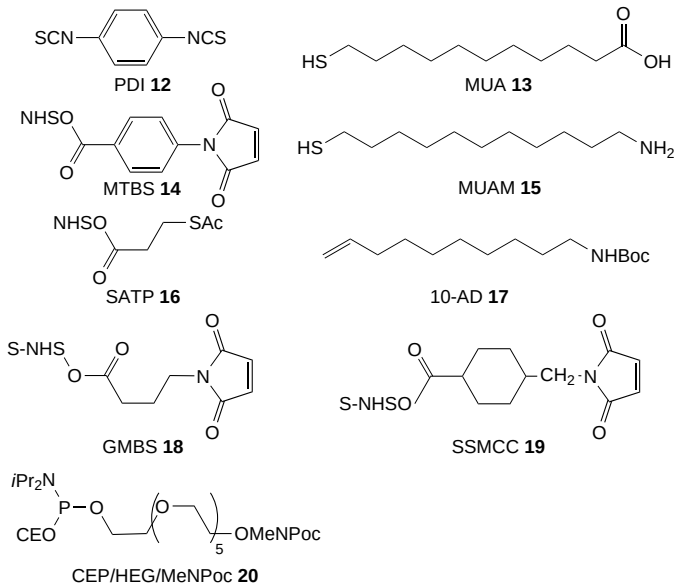
2.1. Synthetic Oligonucleotides

To immobilize synthetic oligonucleotides, chemical functionalities that can react with complementary functionalities on the surface are introduced during conventional automated synthesis. Generally, linking units can be added at the 3′ end (beginning of the synthesis), internal positions, or 5′ end (end of the synthesis). For hybridization assays, the position of attachment should be irrelevant. For other assays in which enzymatic transformations (polymerase- or ligase-based[19]) on the surface-bound oligonucleotide must occur, a free 3′ end is required; thus the linkage must be internal or at the 5′ end.

2.1.1. Spotting

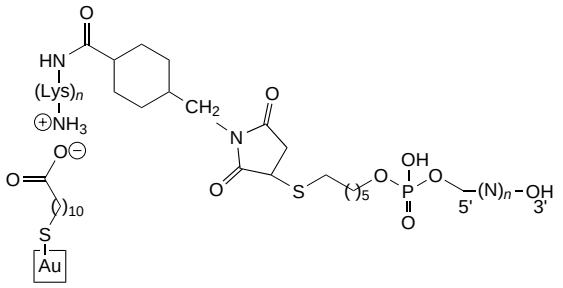
Early work on this production method came from Baylor University and Houston Advanced Research Center (HARC). Glass slides[20] or SiO_2-coated silicon wafers[21] were treated with GPTS (**3**, Scheme 1) to introduce epoxide groups. Synthetic oligonucleotides (3′- or 5′-aminopropyl 9-mers and 36-mers, **21** and **22**, Scheme 6) were spotted in KOH (0.1M, 5 μL). The epoxide-opening reaction is facilitated at relatively alkaline pH values that maintain the amine as the free base, but is still relatively slow and inefficient (~1%). An increase in the number of triethyleneglycol phosphate spacer units between the DNA molecule and the surface attachment increases subsequent hybridization to long DNA targets, but has no effect with shorter targets.

DNA arrays also were prepared on glass by Corn and coworkers at the University of Wisconsin.[22] Treatment of APTES slides with PDI (**12**, Scheme 2) enables the immobilization of 5′-amino oligonucleotides with a T_{10} spacer. About 20% of the deposited oligonucleotides are available for hybridization. A more sophisticated attachment chemistry was developed based on self-assembled monolayers (SAMs) of thiols on gold. A multilayer structure was created, starting with MUA (**13**, Scheme 2), which adsorbs polylysine electro-



Scheme 2. Linking reagents for DNA attachment. S-NHS = sulfo-succinimidyl, Boc = *tert*-butoxycarbonyl, MeNPoc = ((α-methyl-2-nitropiperonyl)-oxy)carbonyl, PDI = 1,4-phenylenediisocyanate, MUA = 11-sulfanylundecanoic acid, 11-MUAM = mercaptoundecylamine, 10-AD = 10-amino-1-decene.

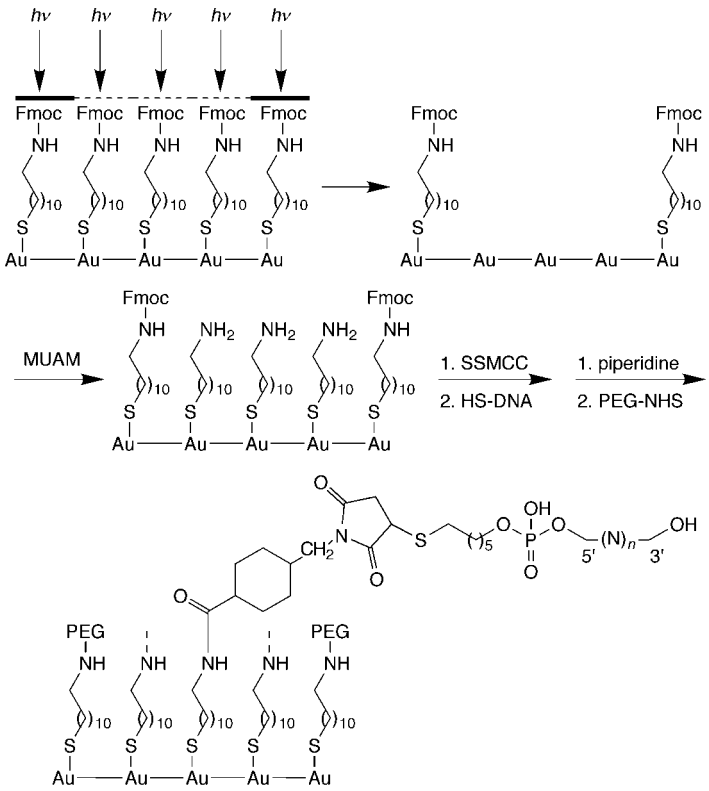
statically (Scheme 3).[23] This surface is derivatized with the cross-linking agent SSMCC (**19**, Scheme 2), which enables it to react with oligonucleotides that are modified at the 5′ end. The delivery of 0.8-μL drops results in 3-mm spots. The gold substrate allows analysis by means of surface plasmon resonance (SPR), thus permitting the direct observation of each production step (based on measurements of film thickness) as well as binding events. SPR imaging[8] offers an alternative to confocal fluorescence microscopy, thus far the workhorse of the array field.



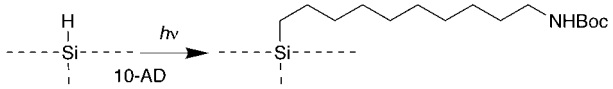
Scheme 3. Thiol/gold multi-layer DNA array.

Corn and co-workers also developed a similar method to generate a prepatterned array whose feature size is not limited by spreading (Scheme 4).[24] A first layer of 11-MUAM (**15**, Scheme 2) was protected with Fmoc. Photolithography (750-μm features) removed thiols from exposed areas of the gold by oxidation, thus creating array elements that were again exposed to MUAM to form areas of amine surface surrounded by hydrophobic Fmoc amine groups ("streets" or nonwetting regions). These array elements were derivatized with SSMCC (**19**; delivery of 0.1 μL by micropipette) followed by an oligonucleotide 5′ thiol. The Fmoc groups were removed, and the resulting amines were treated with a polyethylene glycol NHS ester to render the streets resistant to adsorption. This method (Scheme 4) is more complex than the direct modification of gold surfaces with thiolated DNA sequences, but decreases the amount of nonspecific DNA attachment and permits the density of surface-bound DNA to be varied by dilution with simple thiols. A further extension of this chemistry is the formation of disulfide surfaces for attachment to thiol-derivatized DNA (**23**, Scheme 6).[25] The patterned MUA surfaces are treated with SATP (**16**, Scheme 2), which is deacetylated to give a thiol. The thiol is converted with $(PyrS)_2$ into a disulfide that reacts with 5′-thiol-DNA. All these surfaces were extensively characterized by using IR spectroscopy and SPR imaging.

Novel methods for the modification of silicon surfaces for DNA attachment were also investigated at the University of Wisconsin (Scheme 5).[26] Silicon with terminal hydrogen atoms is prepared by treatment of oxidized silicon wafers with HF. The Si–H bond undergoes a photochemical addition to alkenes to form a Si–C bond. When this method is applied to Boc-protected 10-AD (**17**, Scheme 2) a Boc amine surface is produced. Deprotection permits coupling with SSMCC for attachment of thiolated DNA, as described before. The amine



Scheme 4. Thiol/gold predefined DNA array. Fmoc = 9-fluorenylmethoxycarbonyl, PEG = polyethyleneglycol, NHS = hydroxysuccinimide.

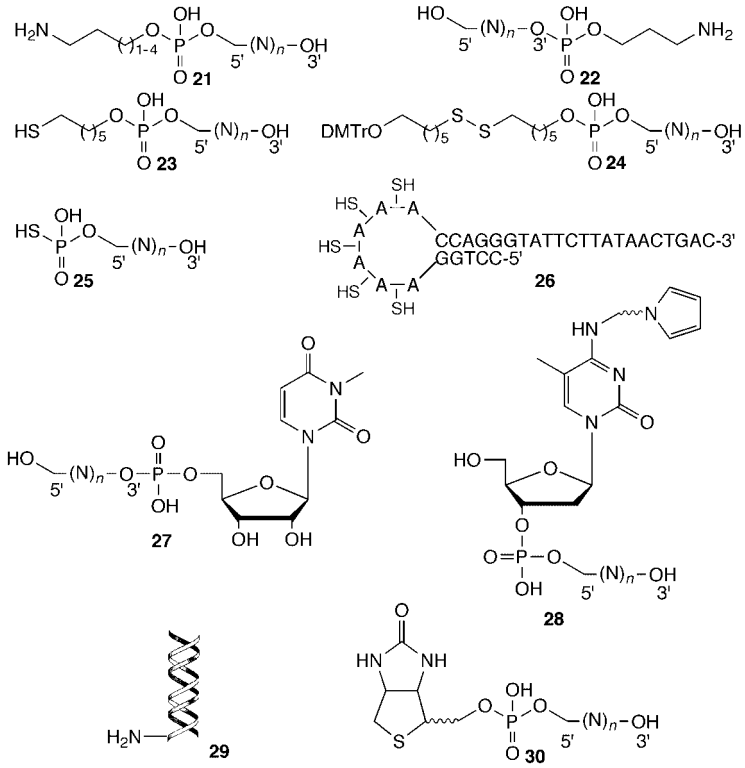


Scheme 5. Si/C DNA array attachment.

sites can be diluted by including dodecene in the initial surface functionalization. These surfaces have been well characterized by X-ray photoelectron spectroscopy.

Researchers at Orchid developed the immobilization of disulfide-modified oligonucleotides on sulfanylsilane-derivatized glass by thiol–disulfide exchange.[27] Slide coating is performed in a solution of MPTS in ethanol/acetic acid, followed by annealing. Oligonucleotides **24** derivatized with a 5′-disulfide modifier (Scheme 6) are deposited in carbonate buffer. The efficiency of attachment is directly related to oligonucleotide concentration up to 20 μM, when it becomes saturated; the half-time for attachment is approximately 10 min. Hybridization solutions do not damage the disulfide linkage, and approximately 16% of the deposited oligonucleotides are available for hybridization. This method was used to generate arrays with a syringe robot and the MicroFab jet (Section 3.1.2), which produced 140-μm spots.

Difficulties with the reproducibility of silane coatings were addressed by our group[28] by using a strategy drawn from chromatography, in which silanes that bear only one alkoxy group give superior sorbents because they cannot form siloxane polymers. However, simple monoalkoxysilane surfaces are also more labile than trialkoxysilanes to a nucleophilic attack at silicon that removes the silane from the surface. Two strategies address this problem: introduction of bulky groups



Scheme 6. Functionalization of oligonucleotides for coupling with the slide. DMTr = dimethoxytrityl.

at the silicon atom (e.g. BAPS, **4**) and mild DNA attachment conditions based on the known reaction of bromoacetamides with 5′-phosphorothioate DNA. Researchers at Amersham extended this method by incorporating phosphorothioates into internal positions of an oligonucleotide hairpin **26** (Scheme 6) that could prime a DNA polymerase extension.[29]

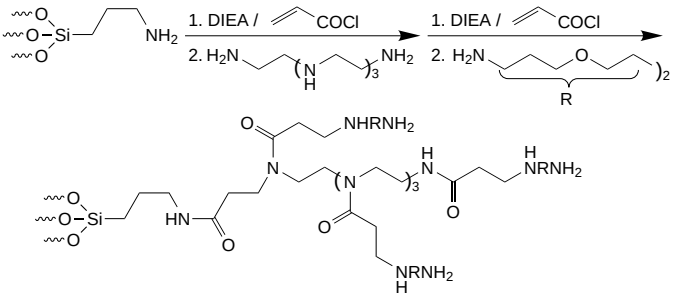
The noncovalent interactions of positively charged polylysine surfaces with negatively charged polymerase chain reaction (PCR) products are adequate to immobilize them as probes (see Section 3.2). It is therefore logical that the charge of shorter oligonucleotides might also be adequate for immobilization. This offers the advantage that no chemical modification of the synthetic probe is required. Operon uses polylysine slides to immobilize synthetic 70-mers by essentially the same methods reported by the Stanford University groups.[30] Genometrix makes use of APTES slides to immobilize dodecamers.[31] After drying, the amines are capped, first with acetic anhydride and then with succinic anhydride to produce a slightly anionic surface that exhibits low nonspecific adsorption of analyte DNA. The high initial affinity of the positively charged surface for probe DNA and the engineered reduced affinity for target DNA through acylation is an essential feature of microarray methods based on aminated surfaces.

An increase in oligonucleotide density within probe sites should increase signals and therefore sensitivity. A versatile dendrimeric linker for oligonucleotide attachment was developed for this purpose at the German Cancer Research Center, Heidelberg (Germany).[32] Aminated glass or polypropylene surfaces were subjected to a four-step divergent dendrimer synthesis (Scheme 7) that resulted in a fivefold increase in the primary amine loading. The amines were activated by means of the diisothiocyanate method for the immobilization of 5′-aminolink probes or PCR products (see Section 3.2) in volumes of 1 – 50 nL to create 0.2 – 0.8-mm spots. The residual isothiocyanate groups were deactivated with 6-aminohexanol.

An alternative method involves the attachment of a preformed dendrimer to the surface.[33] Workers at the University of Bremen bound PAMAM dendrimers to an isothiocyanate-activated aminated glass surface, then further activated the amine groups with PDI for the binding of a capture oligonucleotide (in 1 nL or less). The hybridization signal from these arrays was twice as strong as that from conventional aminosilane glass, and was more uniform and reproducible.

The density of sites on a functionalized surface should be directly proportional to surface area. A flat, nonporous glass/silicon surface must have a surface area close to the macroscopic surface area of the object. The formation of a three-dimensional structure on the surface should increase the area and therefore the oligonucleotide density. Two approaches to this goal have been taken.

Drawing on gel-making methods widely used in molecular biology, workers at Argonne National Lab polymerize acrylamide/bis-acrylamide into predefined 10 – 100-μm square pads by using photolithography (Figure 3).[34] The resulting 5 – 20-μm thick



Scheme 7. In situ synthesis of a dendrimeric linker. DIEA = diisopropylethylamine.

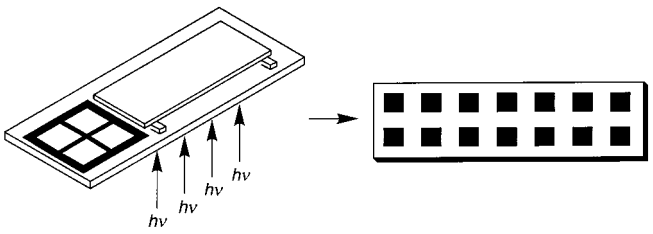


Figure 3. Production of a gel-pad array.

polyacrylamide pads have pL to nL volumes and are separated by 20 – 200-μm hydrophobic streets. Amine immobilization sites are added to the gel pads by transamidation with diamines. Because the 4% cross-linking limits the porosity of the gel, a limit of 150 – 200 nt is imposed on the DNA to be immobilized. Aldehydes are introduced into this DNA by synthesis with a 3-methyluridine at the 3′ end (e.g. **27**,

Scheme 6), which can be cleaved with periodic acid. Functionalized oligonucleotide solutions are delivered to the gel-pad array with a pin or a piezoelectric capillary jet.[35] The dialdehyde is irreversibly attached to the gel by reductive amination (pyridine · BH_3).

Workers at Gene Logic have developed three-dimensional "flow-thru" chips that theoretically increase the surface area 100-fold over that of planar substrates.[36] Commercially available glass array plates (48×48 mm^2) with 10-μm hexagonal-packed microchannels provide the starting point. Whereas the channels are readily filled with liquid by capillary action, exchange with liquids outside is very slow. Pressure or ultrasonication are used to force liquid through the microchannels, which are emptied by wicking. Silanization with a solution of MPTS (**2**, Scheme 1) gives a thiol surface. Aminolink oligonucleotides that are modified with the maleimide cross-linking agent GMBS (**18**, Scheme 2) are deposited in volumes of 5 – 10 nL with a piezoelectric capillary jet[35] because glass array plates cannot tolerate contact printing methods. The resulting spots (~200 μm) are about 60 % of the size of those obtained with planar chips and do not exhibit the "coffee-ring" effect. The functionalization level reported for these chips is comparable to that for planar surfaces, thus their three-dimensional structure offers no advantage in increasing the total amount of immobilized oligonucleotide. They also have a somewhat higher fluorescence background than slides, but still give a 44-fold enhancement of the hybridization signal over that of planar chips. This enhancement was attributed to a larger surface area per probe site, which means a lower density of probes with equivalent probe loading.

2.1.2. Jets

The delivery of reagents for microarray production by means of jets has been reported by several groups. Contrary to the case in spotting methods, no direct contact is made with the surface. Bubble jets are simple semiconductor devices developed for printers; fluid is ejected from a nozzle by rapid heating. Researchers at Canon have reported DNA ejection in a solvent that includes glycerin, urea, and thiodiglycol as wetting agents.[37] Thermal and shearing stresses of the bubble jet do not damage DNA of 10-300 nt. Up to 12 different oligonucleotide solutions could be dispensed in volumes of 24 pL, and 70-μm spots were created. Ejected 18-mer 5′ thiol oligonucleotides were immobilized on maleimide-modified aminated glass slides.

A drop-on-demand jet system with ten independent fluid channels in an integrated piezoelectric block printhead (see Section 3.2) was developed by researchers at MicroFab.[38] Jet openings are 40 μm in diameter and 2 mm apart and deliver oligonucleotides in 20 % aqueous ethylene glycol. The printhead must be brought to within 200 μm of a GPTS-derivatized slide for printing.

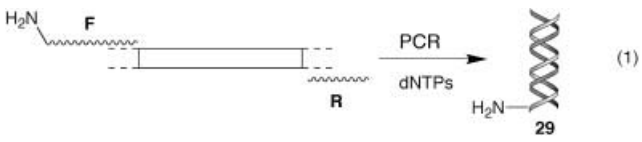
2.1.3. Other Methods

CIS Bio have reported the electropolymerization of oligonucleotides directly onto platinum minielectrodes.[39] The oligonucleotides are synthesized with a 5-methylcytidine moiety at the 5′ end; a pyrrole group is linked to N4 (**28**, Scheme 6). Oxidation by repeated potential sweeping in the presence of approximately 10000-fold excess pyrrole produces a 50-nm layer of polypyrrole, with fractional incorporation of oligonucleotide-bearing pyrrole groups.

Nanogen brings an additional variable, electric field, into the parameters that can be manipulated in microarray production and use.[40] This method is conceptually similar to electrophoresis, except that molecules move freely in solution. It relies on an array of as many as 10000 platinum microelectrodes, each as small as 25 μm, on a silicon wafer substrate produced by common techniques for semiconductors. The electrodes are coated with a 1 – 10-μm permeation layer of streptavidin – agarose that serves to insulate the DNA from deleterious electrode reactions and to immobilize biotin-derivatized 12- to 26-mer oligonucleotide probes **30** (Scheme 6) that are concentrated at the electrode pad by a positive electric field. The movement of DNA in the field requires a low conductivity buffer such as histidine. For simple 5×5 and 10×10 arrays, external electrical connections can be made directly to each electrode. For more complex arrays, the array may have onboard CMOS-FET (complementary metal oxide semiconductor-field effect transistor) logic to control each electrode so that the number of external connections is minimized. The active area of the array is 2 – 4 mm^2. The ability to control the field at the electrode-immobilized probe enables analyte to be brought to the electrode from dilute solution and adds an additional type of control, "electronic stringency", during the hybridization step.

2.2. Biologically-Derived Nucleic Acids

Many biological studies into microarrays have as their starting point a set of clones whose functions and sequences may be unknown. Thus, the probe array is not designed based on a sequence, but the clones themselves are converted into probes that can be used to detect cellular RNA. Generally, this is accomplished by PCR. PCR not only allows the production of quantities of a particular nucleic acid by amplification, but also enables specific reactive chemical groups or labels to be introduced during the PCR [Eq. (1)] because all product molecules **29** are derived either from the



(1)

primers or from deoxynucleotide triphosphates (Scheme 6). These microarray production technologies are the most widely used in biology laboratories today and have created a demand for supplies that is now commercially available.

Two groups at Stanford University made the first widespread use of DNA chips in studying gene expression.[41] The protocols for chip production have been extensively described

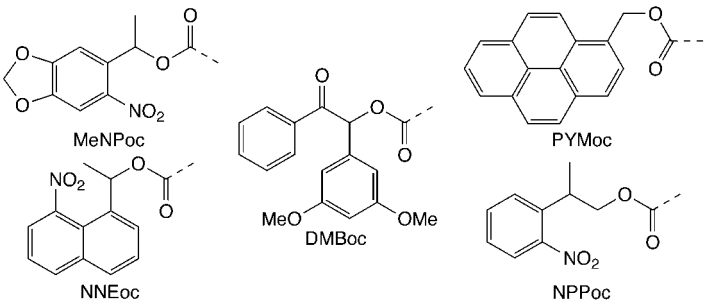
and widely adopted and adapted. Slides coated with polylysine provide a good starting point for this technology and are commercially available. Spotted probe DNA adheres to this surface through, at least initially, electrostatic interactions, and hybridization with analyte DNA does not disrupt this adhesion. A stylus (Figure 2) is used to deposit 5 nL of a PCR product (>1 kb) and gives spots of <350 μm. The addition of 1.5 M betaine in the spotting buffer improves spot homogeneity (no "coffee ring"), increases spot viscosity, slows spot evaporation without affecting the surface tension needed to keep it from spreading, and results in a 2.5-fold increase in the signal.[42] The stylus moves on a gantry over an X,Y table. After hydration in a humid environment, the spots are quickly dried. The spotted DNA can also be photochemically cross-linked to the surface. This process has a long history in the manipulation of DNA on membranes; attachment may be based on nucleophilic substitution of the lysine amine group on T.[43] Residual amine groups on the polylysine are capped with succinic anhydride in borate buffer or in an organic solvent[42] that prevents the spreading of spotted DNA on the slide as is sometimes seen in aqueous solvents. To prepare probes for hybridization, the array is heated to denature the double-stranded PCR product.

An alternative covalent linking chemistry has also been introduced by the groups at Stanford University. PCR products from primers that bear 5′-aminolink groups (**29**, Scheme 6) enable the use of another commercially available slide coated with a silane aldehyde **5** (Scheme 1). After 1056 PCR products were spotted as described above, the array was incubated with $NaBH_4$ for 5 min to reduce the imine group.[44] The attraction of this protocol is a tenfold increase in sensitivity as a result of reduced background fluorescence, thus enabling the detection of mRNA that represents 1/500 000 of the total mRNA in a sample. One disadvantage of aldehyde slides is that they can oxidize in air to the acid. Other workers have shown that 5′-aminated PCR products (1-nL drops give 400-μm spots with a 1000-μm pitch) can also be coupled to carboxylic acid coated glass (TETU, **6**, Scheme 1) with carbodiimides.[45]

Electrostatic binding of PCR products to APTES slides in the presence of sodium thiocyanate (3–5 M) has been reported by researchers at Molecular Dynamics.[46] Despite the fact that no further treatment or capping is used, a significant fraction of the deposited DNA is retained during hybridization and wash steps. Their Gen III spotter deposits nanoliter volumes of DNA from 12 matched pens created from stainless steel capillaries with a slot cut in one wall (Figure 2).

3. Synthesis In Situ

An earlier review on spatially addressable combinatorial chemistry encompasses synthetic methods for preparation of DNA chips in situ.[2] A major attraction of in situ synthesis is that it is a combinatorial method that enables the preparation of many more compounds than the number of chemical steps. However, serial methods of synthesis that proceed very rapidly have advanced with the benefit of engineering technology to the stage that they may be time-competitive with some parallel-synthesis methods. Two strategies exist for the in situ synthesis of oligonucleotides. Each synthesis cycle has two stages, the deprotection step and the coupling step, and either can be spatially directed to the individual probe sites, while the other step can be performed at all sites simultaneously. An important consideration is the direction of DNA synthesis, because some assays require DNA with free 3′ ends (see Section 2.1). In the case of the deposited arrays described above, locating the attachment anywhere other than at the 3′ end is readily accomplished by automated synthesis. Conventional DNA synthesis proceeds from 3′ →5′, thus the DNA is attached at its 3′ end for arrays synthesized in situ. Amidites with reversed orientation (5′-phosphoramidite, 3′-dimethoxytrityl) are commercially available and can be used in the jet and in physical masking methods. However, they must be prepared with appropriate photochemically removable groups for photolithographic synthesis (Scheme 8). It is also conceivable to synthesize the DNA normally and then to reverse the attachment, but this has thus far only been reported on bead supports.[47] The direction of synthesis can be a vital issue, as it has been shown that hybridization assays with the GeneChip (see Section 3.1) cannot detect insertion and deletion mutations in the p53 tumor suppressor gene,[48] whereas primer extension assays readily do.[49]



Scheme 8. Photochemically removable protecting groups for the synthesis of DNA microarrays.

3.1. Photolithography

The Affymetrix method of microarray production is by now a very familiar process. A surface is coated with linker molecules that bear photoremovable protecting groups. The pattern of irradiation (dictated by masks) deprotects certain regions, which are coupled to monomer units that are exposed to the whole surface. The process is repeated to build up different sequences at different sites. The combinatorial synthesis enables 4^l different sequences of length l to be prepared in $4 \times l$ chemical steps. This method was described, primarily for peptide synthesis, in an earlier review,[2] and a good summary of its application to DNA chips is available.[50]

The first report by the research group at Affymetrix on the application of this technology to DNA array production introduced the MeNPoc photoremovable protecting group (Scheme 8) in the preparation of DNA probe sequences

(>200).[51] The protecting groups for the nucleobases are phenoxyacetyl (Pac) for A and isobutyryl for G and C, the so-called "easy-off" groups[52] that can be removed with ethylenediamine or ethanolamine in ethanol in 4 h at room temperature. The HE-APTS (**7**, Scheme 1) surface is prepared for synthesis by treatment with **20** (Scheme 2).

Subsequent work at Affymetrix[53] made use of fluorescein derivatization and imaging to determine the number of 5′ hydroxy groups produced in each step. This showed that the photochemical deprotection reaction is strictly first order and gave half-times of 9–13 s at 27.5 mW cm^{-2} for each monomer unit. The influence of the solvent on the rate of surface photodeprotection was studied, and a nonpolar solvent or no solvent proved best. Whereas some previous users of nitrobenzyl photochemistry have included reagents to scavenge nitrosoaldehyde by-products, this does not seem necessary with the MeNPoc group. The average yield of a MeNPoc-based DNA synthesis cycle (91–98%) was measured by using a similar fluorescence-staining technique. The basis of the low cycle yields on microarrays is unclear, as solution-based photochemical deprotection and coupling proceed in very high yields. Low cycle yields mean that the purity of these sequences synthesized in situ is fairly low, but the number of full-length, accurate probes is still significant and evidently adequate for use. The spin coating of substrates before derivatization and synthesis with a ~0.4-μm film of 70–100-nm colloidal silica particles increases their surface area sevenfold. A presumed increase in probe density accounts for a corresponding increase in hybridization signal.[54]

The effect of sequence defects on the hybridization performance of microarrays synthesized in situ has been studied with synthetic mixtures. It is not surprising that higher probe purity is projected to improve the performance of the microarrays.[55] A variety of photochemically removable protecting groups have been investigated to address the shortcomings of the MeNPoc group in DNA synthesis, and their performance has been studied in simple photochemical solid-phase synthesis as well as on arrays (Scheme 8). These include the DMBoc,[56] PyMoc,[57] and NNEoc groups,[57] all of which give 96% cycle yields. It was recently reported that the NPPoc group gives quantitative cycle yields in photochemical synthesis on arrays, thus representing a significant advance.[58] The mechanism of deprotection differs among these groups. The MeNPoc and NNEoc groups exhibit conventional nitrobenzyl photochemistry, and undergo oxygen migration from the nitro group to the benzylic position to form a nitroso hemiketal. Its further conversion into the nitrosocarbonyl produces a hemicarbonate ester that decarboxylates to the alcohol. The DMBoc and PyMoc groups are thought to photosolvolyze to their cations and the hemicarbonate. NPPoc is a relatively new group that is cleaved by a light-promoted beta-elimination process in the presence of mild bases. It is converted into the nitrostyrene and the hemicarbonate.

The manufacture of Affymetrix Genechip microarrays is performed on 5-inch fused silica substrates that bear 50–400 replicate arrays, each of up to 1.6 cm^2 and with up to 400 000 probes. The deprotection of MeNPoc groups in each cycle requires less than 1 min and a filtered I-line (365 ± 10 nm, 1 kW) exposure system is used. The solvent-free cleavage of MeNPoc proves advantageous, as a contact printing process can be used in which the masks are placed directly against the wafer. Completed wafers are diced into individual arrays and packaged into a plastic carrier with ports that permit the addition of hybridization solutions. The feature size on current commercial chips is 50 μm, but the next generation technology will decrease that to about 25 μm.

An alternative photolithographic method developed by workers at Affymetrix in collaboration with IBM uses traditional semiconductor photoresists as a two-layer physical barrier to conventional chemical reagents for DNA synthesis (Figure 4).[59] A standard DMTr-protected, derivatized glass

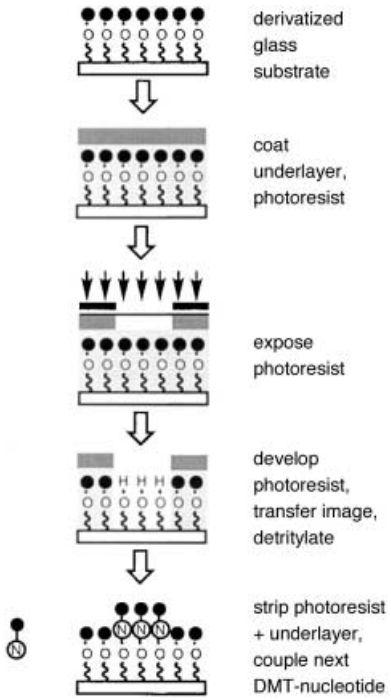
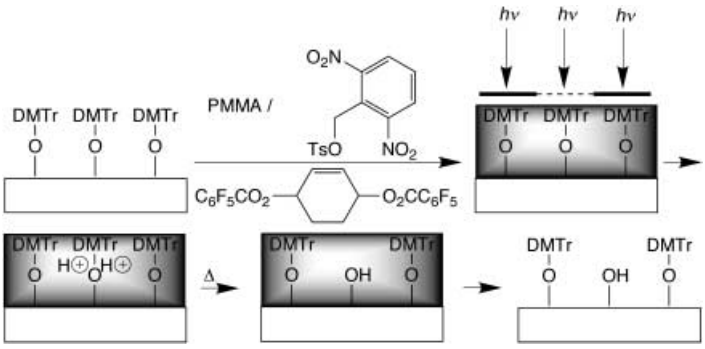


Figure 4. Photolithographic DNA synthesis with a bilayer resist system (reproduced from ref.[59] with permission, Copyright (1996) National Academy of Sciences, USA).

surface was coated with a soluble polyimide (~0.5 μm) to protect the molecules from resist chemistry. The overlying negative-tone resist layer (~0.5 μm) is an epoxy resin combined with a photochemical acid generator that cross-links the resin, thus making it insoluble. This strategy draws on substantial experience in acid-generating chemically amplified resist systems that are currently used in submicron lithography for computer chips.[60] Here, photogenerated acid (PGA) enabled the ready production of 4-μm features. The epoxy was developed from unexposed areas with cyclohexanone, and the underlying polyimide was stripped off with anisole. Detritylation of 5′-DMTr protecting groups used dichloroacetic acid in cyclohexanone to avoid dissolving the polyimide. Dissolution of all polymers in chlorinated solvent prepared the surface for phosphoramidite coupling, which proceeded in 90 ± 5% yield per step. Microarrays of decanucleotides in 100-μm features that were prepared by using this

protocol showed comparable performance with those prepared with MeNPoc.

A different approach to light-directed DNA synthesis uses conventional acid-sensitive hydroxy-protecting groups with chemically amplified, PGA polymer films (Scheme 9).[61] Initial *p*-toluenesulfonic acid production then generates two equivalents of pentafluorobenzoic acid, which is sufficiently



Scheme 9. DNA synthesis by photogenerated acid. Ts = toluene-4-sulfonyl, PMMA = polymethylmethacrylate.

acidic to deprotect DMTr groups without affecting the purine nucleosides. Since the deprotection of DMTr groups is catalytic in acid, which is diffusible, it might be feared that PGA could cause indiscriminate deprotection. However, a polymethyl methacrylate film limits lateral diffusion and permits the production of spots that are smaller than 10 μm. The cycle yields thus far reported with this method are about 90 %, still much lower than desirable. An attractive aspect of this method is that the chemical amplification in the acid generation step results in a nonlinear dependence of deprotection on photon dose. This nonlinearity of photochemical response is key to the production of small features in conventional lithographic methods.[62]

The reverse direction of synthesis (see Section 2.1) to give DNA with free 3′ ends can be addressed by exchanging the protecting group and the phosphoramidite group in the monomer units, and usually by lengthening coupling cycles (Scheme 10). Workers at Affymetrix recently reported methods for the preparation of monomer units that bear 5′-phosphoramidites and 3′-MeNPoc groups (**32**, Scheme 10) for

Scheme 10. Amidite reagents for reverse synthesis of DNA with free 3′ ends. PRG = MeNPoc, NPPoc.

this purpose.[50] This strategy has also been applied to the NPPoc group to give, with high fidelity, sequences that can be used in enzymatic processing.[63] In principle, the newer methods of acid-based photochemical deprotection should also make DNA arrays with free 3′-ends more accessible, as the 3′-DMTr-5′-phosphoramidites (**31**, Scheme 10) are commercially available. This also makes clear the advantage of using conventional monomer units, which can be purchased for many nucleotide analogues with a wide variety of base and sugar modifications.

Two groups have sought to replace the static masks used in the Affymetrix method with dynamic programmable devices that are somewhat similar to computer overhead projection systems. A digital micromirror array was used at the University of Wisconsin[64] in a projection optics system that has been commercialized by NimbleGen (Figure 5). A similar

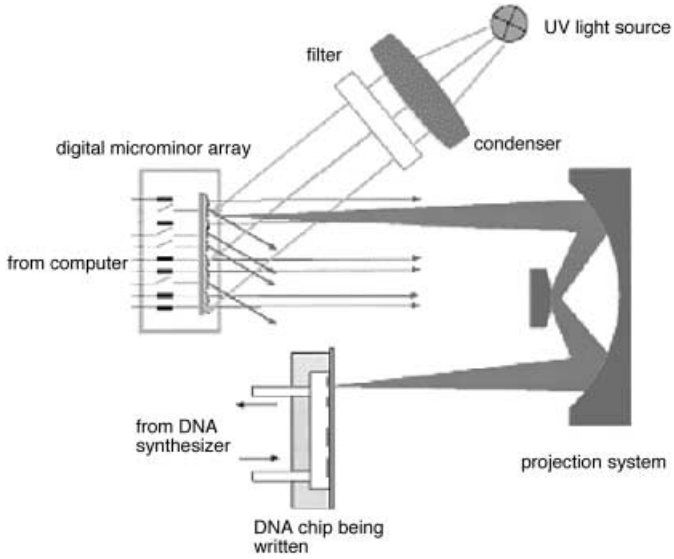


Figure 5. Reflective optics for photolithography with micromirror arrays (reproduced with permission from ref.[64]).

technique was used at the University of Houston[65] and is being commercialized by Xeotron. The primary patterning device employed in this method is actually a 1.5×1.5 cm^2 glass surface with fluorocarbon streets surrounding up to 48×48 synthesis regions with microwells as small as 300 μm. These substrates are made by using the Protogene methods described in Section 3.2, followed by derivatization with HBPTES (**8**, Scheme 1). The synthesis begins the coupling of two T bases by using conventional acid deprotection chemistry. Immersion of the chip in a solution of photogenerated acid precursor leaves liquid droplets behind in the microwells after draining. Photochemical DMTr deprotection, which was demonstrated in solid-phase DNA synthesis (> 92 % cycle yields),[66] is directed in particular microwells with a 480×640 array of 16×16 μm^2 micromirrors to project the light. Optimization of deprotection efficiency based on the number of 5′-hydroxy groups created (measured by fluorescein derivatization by using the Affymetrix method) showed that the superior photoacid cocktail was composed of a diaryl iodonium salt (with a short wavelength λ_{max}) and a thioxanthenone sensitizer (that absorbs the radiation) in dichloromethane. With this method, oligonucleotides with up to 30 residues have been prepared.

3.2. Jets

As an alternative to directing the chemistry to microscopic sites with light, reagents for conventional DNA synthesis can be directed by using the same methods described in Section 2.1.2 for the delivery of complete oligonucleotides. Early contributions to this area were made by researchers at Combion by using coaxial piezoelectric/glass capillary jets (Figure 6).[67] A polyethylene glycol linker that bears a DMTr group is attached to the surface. A jet dispenses the

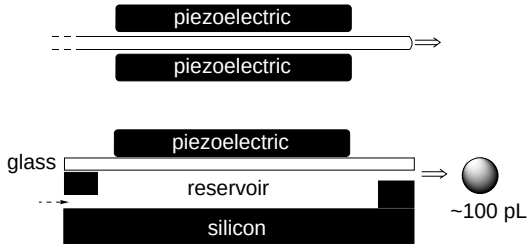
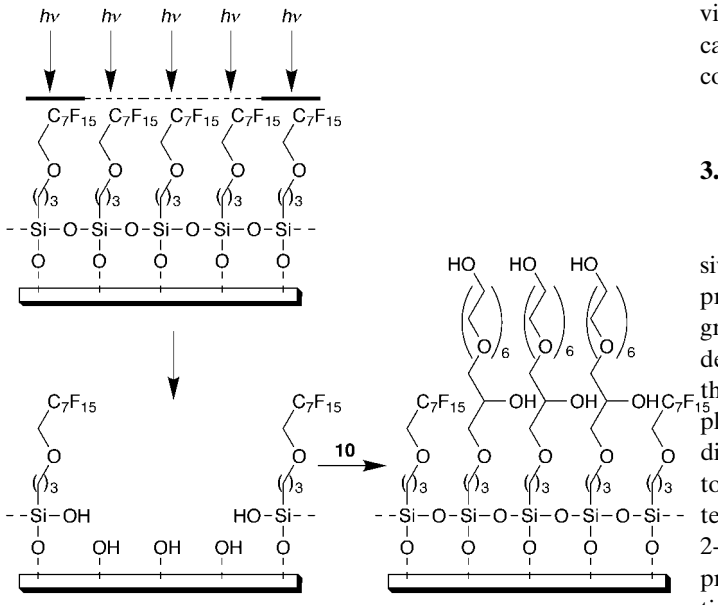


Figure 6. Piezoelectric glass jets for reagent delivery.

deprotecting reagent upward to sites intended for synthesis on the horizontally held substrate. It is moved to a vertical position for rinsing and mounted in a flow cell attached to a DNA synthesizer for coupling reactions. The overall cycle lasts 10 min. A conservative estimate of the yields in each of the steps in this process is 91 %. These workers also identified a difficulty in using volatile solvents such as acetonitrile with jets, and instead used $ZnBr_2$ in CH_2Br_2/isopropanol as a less volatile yet equally effective alternative deprotectant.

The Protogene method isolates the synthesis areas from each other by using nonwetting regions or streets that bear fluoroalkanes (Scheme 11).[68] Several steps are involved in the production of a glass microarray plate: gas-phase coating



Scheme 11. Preparation of a surface with synthesis regions surrounded by nonwetting "streets".

with POPTS (**9**, Scheme 1), exposure through a mask to a CO_2 laser to ablate the silane, preparation of the patterned regions for synthesis by derivatizing with GPTS, and opening of the epoxide with hexaethylene glycol (HEG). Piezoelectric impulse jet pumps produced as a microelectromechanical system (MEMS) are the second novel aspect of this method. A reservoir, inlet, and nozzle are produced as three-dimensional features in a ceramic (Figure 6). To form a chamber, the ceramic is bonded to a flexible top plate, to which a piezoelectric element is attached. When the piezoelectric is energized, it deforms the reservoir like a bellows, which ejects fluid at a velocity of $1-10\,m\,s^{-1}$. A nozzle size of 40 μm produces acetonitrile droplets of about 33 pL (±5 %), of which three will fill a 50-μm diameter synthesis site. These pumps operate at up to 3 kHz, can hit a 250-μm target at a distance of 2 cm, and can be ganged together in printheads (up to 64 nozzles with a 400-μm pitch) to create independently controllable jets that deliver the same reagent from a common reservoir. The printheads are stacked to provide each base as its phosphoramidite or H-phosphonate monomer, along with the appropriate activator for oligonucleotide synthesis. The pumps remain stationary during synthesis and the microarray is moved below them with an X,Y table. With a repetition rate of 1 kHz, the pumphead can deliver monomer units to a 512 × 512 array (262 000 sites) in only 32 seconds! The synthesis sites have a surface tension of 47 dyne cm^{-1}, and more closely match that of acetonitrile (29) than that of the streets (18), thus preventing mixing between adjacent sites. The common synthesis, washing, capping, DMTr deprotection, and final phosphite oxidation/deprotection steps, can be performed as batch operations across the whole microarray simultaneously. Cycle times are 6 min per residue.

Workers at Rosetta use a similar technology to microfabricate monolithic jets that will deliver 100-pL volumes from glass, silicon (10–100-μm nozzle), and piezoelectric ceramic.[69] Another aspect of their method is the use of propylene carbonate as solvent for the synthesis because of its high surface tension (> 30 dyne cm^{-1}), low volatility, and high viscosity.[70] Microarrays of more than 25 000 different 60-mers can be made with this technique and are now available commercially from Agilent.

3.3. Physical Masking

These techniques are similar to stencils and were extensively described in an earlier review.[2] They are produced primarily at the University of Oxford and at Beckman; these groups have also cooperated. Researchers at Beckman developed a unique surface attachment **11** (Scheme 1)[71] by the treatment of a polypropylene sheet with ammonia in a plasma. This introduces tertiary amine functions that can be directly treated with nucleoside phosphoramidite monomers to produce phosphoramidates after oxidation. A sequence of ten T residues is used as a linker. The sheets can be cut into 2-mm strips to create "dipstick" arrays. Protocols have been provided by the group at Oxford University for the preparation of arrays on aminated polypropylene (AP) or glass,[72] including the GPTS/HEG-derivatized glass **10** (Scheme 1)

that does not permit the oligonucleotide to be detached. Polypropylene must be mounted on a glass slide to make it rigid enough to seal against the stencil and for reliable translation relative to it. Polypropylene arrays have been prepared by using 3′-DMTr-5′-phosphoramidites **31** (Scheme 10) that create free 3′ ends for enzymatic processing.[73] A drawback of this method is that the hybridization data are not as unambiguously and as easily interpreted as conventional microarray data.

4. Summary and Outlook

With many methods available to produce microarrays, the strengths and weaknesses of each should be considered. Arrays spotted from cDNA and those derived from synthetic oligonucleotides (either synthesized and spotted or synthesized in situ) have significantly different uses. One requires a cDNA in one's own lab to generate the former. This method is common in biology laboratories that are interested in generating custom microarrays, and also offers longer probes, which some workers prefer. The use of synthetic oligonucleotides means that probes can be designed based only on a sequence found in the genetic databases. However, the efficiency of the synthesis limits their length, currently around 60 nucleotides by any method. A significant question is whether there is a preferred method for microarray production and analysis, and this cannot be answered without knowing the individual needs and resources. The performance of 50-mer oligonucleotide arrays versus arrays of cDNAs/PCR products with more than 300 base pairs has shown no difference.[13]

An area for continued investigation is quality control and monitoring methods for chemical reactions at/on microarray surfaces. Many different techniques have been applied to the chemically based research on DNA microarrays, but most often these are not fast enough to be used as part of routine laboratory operations by nonspecialists, or they require quite sophisticated instrumentation that is not available to most chemists and biologists. Because of the success of SPR techniques in studying other types of biological recognition events (e.g., in BiaCore instruments), SPR imaging offers great promise for future microarray use. The novel technique of reflectomeric interference spectroscopy may be likewise applicable.[74]

Surprisingly, another area in which continued improvements are needed for DNA arrays is in oligonucleotide synthesis, usually considered to be a "perfected" technology. Whether the issue is cycle efficiency in photolithographic synthesis or the purity of an oligonucleotide prepared by conventional automated synthesis with some novel functionality for attachment, many workers find that synthesis failures still cause problems in microarray production.

There is certainly a drive to produce smaller probe sites for reasons described in Section 2. Even femtoliter volumes can now be delivered to make spotted arrays with 20-μm features.[75] However, there is a limit to which making smaller sites will assist microarray analysis, because the detection methods for the products are optical and diffraction-limited. Even if 1000-nm probe sites could be made, a statistically valid number of data points from interrogation of such a site with 650-nm radiation would be very difficult to obtain. Density improvements in production will require commensurate improvements in the spatial resolution of detection.

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