Oligonucleotide Arrays from Aldehyde-Bearing Glass with Coated Background

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Abstract: Presented here is a method for preparing small DNA arrays on aldehyde-bearing glass slides. Immobilization involves reductive amination and employs oligonucleotides with 3′-terminal lysine residues, obtained in high yield from solid phase syntheses. Spot patterns are produced by protecting selected areas of the aldehyde slides with wax, coating the free surface with a methyl triethylene glycol derivative, and removing the wax with dichloromethane. The DNA arrays give better signal to noise ratios in hybridization experiments than slides without passified background.

Key words: oligonucleotides, Schiff bases, solid phase synthesis, DNA, molecular recognition

DNA microarrays (‘DNA chips’) have become indispensable tools in biomedicine.[1,2] Some DNA chips allow the detection of up to ~10^5 different hybridization processes in a single experiment and therefore simultaneous gene expression analysis on the level of entire genomes.[3] Results from these analyses may lead to new targets for drug development.[4] During the preparation of DNA chips, probes are patterned onto a surface, either by photolithographic syntheses[1a,5] or by immobilizing suitably functionalized pre-synthesized oligonucleotides. Read out processes after incubation of the probe studded surfaces with fluorophore labeled or radioactive analyte DNA rely on the formation of stable Watson–Crick duplexes between complementary strands.

Photolithographic syntheses yield high density chips, whose false positive rates in routine hybridization experiments can be reduced to less than 2% by using a large fraction of the spots for probe redundancy and mismatch control purposes,[6] though this level of stringency may lead to very substantial false negative rates.[7] For spotted microarrays of PCR amplified DNA, with chain lengths exceeding those that are currently accessible synthetically, false positive rates (hybridization cross reactivities) are higher, reaching values of up to 57%.[8] Clearly, for applications such as SNP genotyping[9] or sequencing by hybridization, where high stringency is mandatory, higher fidelity chips are desirable. These will most probably require optimization of the chemical structure of the oligonucleotide probes, further optimization of linkers between surface and probes, and modification of surface sites where undesirable binding or reactions can occur.

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Work with modified oligonucleotides as probes, where the modification is something other than a fluorophore or the moiety providing the link to the chip surface, has been limited thus far. Oligomers with a glycine ethylene diamine backbone (PNAs) have been employed in microarrays.[10] Phosphorothioate linked sequences and sequences with terminal modifications were tested and the former shown to bind their DNA targets with lower affinity than their unmodified counterparts.[11] Some modified oligonucleotides were reported to reduce the number of incorrect results in DNA chip hybridization experiments.[12] Structured DNA probes, for whom a three state binding equilibrium may operate, leading to enhanced selectivity,[13] have also been immobilized, though in devices involving optical fibers[14] rather than conventional chips. Finally, modified oligonucleotides whose modifications facilitate electrochemical detection have been employed on surfaces.[15]

We and others have an interest in developing modified oligonucleotide probes whose affinity for target strands is less dependent on their G/C content than that of unmodified DNA,[16,17] and whose ability to bind tightly and selectively at the termini is enhanced.[18,19] Since it is not trivial to design modified oligonucleotides that bind a range of mixed sequence target strands with similar affinity, and modifying several nucleotides in a DNA strand can lead to unexpected cooperativity effects,[20] extensive experimental testing may be required. For this, we became interested in developing a rugged immobilization methodology that would allow testing modified oligonucleotides efficiently at moderate cost. Here we report a method for immobilizing modified and unmodified oligonucleotides on commercial glass slides, where the oligonucleotides used for immobilization are prepared entirely on an inexpensive solid support. Further, we show how surface sites surrounding the DNA strands may be coated such that unspecific adsorption of analyte strands is minimized.

A number of methods have been previously described for the covalent immobilization of DNA strands on chip surfaces. Possibly the simplest of these involve adsorption on polylysine modified surfaces[1c] and UV cross linking of long, otherwise unmodified DNA to surfaces with free amino groups.[21] Other methods employ site selectively modified DNA and activated surfaces, where the covalent link does not involve the nucleobases directly. Examples are surfaces with bromoacetamido moieties reacted with DNA containing phosphorothioate groups,[22,23] epoxide and isothiocyanate modified surfaces reacted with amino modified DNA,[24,25] semicarbazide derivatized surfaces

reacted with benzaldehyde modified DNA,[26] and mercaptopropylsilane modified surfaces allowed to form disulfides with disulfide bearing DNA in disulfide exchange reactions.[27] A number of these and related attachment regiments rely on bifunctional linkers.[28] Some involve self assembled monolayers, on silica[29] or gold substrates.[30] To improve accessibility of the DNA strands, spacers[2b,31] and dendrimer linkers[32] have been employed.

Several immobilization methods for oligonucleotides were recently compared, while using the ability to bind analyte strands as the basis for evaluation.[33] The authors found DNA microarrays prepared via reductive amination from aldehyde modified glass slides and DNA with amino groups ('aminated DNA') to have the most favorable properties.[33] Another study tested for the ability to perform minisequencing reactions with immobilized primers and found that disulfide linkages between DNA and the surface gave the best results.[34] A third comparative study focused on solid phase DNA amplification as the read out technique and recommended 5′-thiol-modified oligonucleotides and attachment to amino silanized glass slides via a heterobifunctional cross linker as the method of choice.[35]

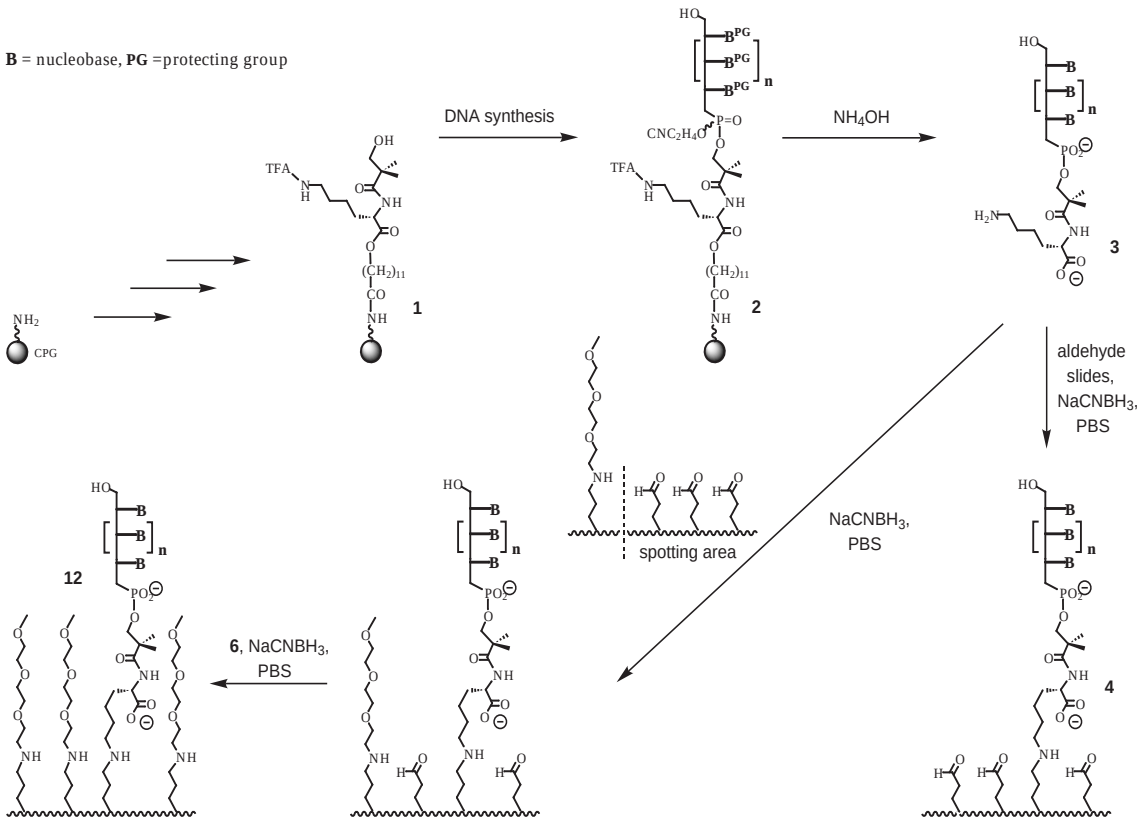
We were interested in a technique allowing immobilization of pre-synthesized oligonucleotides via the 3'-terminus. Immobilization of pre-synthesized strands was preferred over in situ synthesis on the chip surface, since this allows characterization and purification of the strands, a feature considered critical when developing new chemistries. Further, it was desirable to use the 3′-, rather than the 5′-terminus for immobilization, since a number of modifications to the 5′-terminus that enhance target affinity and base pairing selectivity had already been developed.[19,36] These would be non trivial to incorporate in the linker required for anchoring the probe on the chip. It was also desirable to employ a slide surface that was not electrostatically attractive for DNA, to prevent that the DNA lies flat on the chip, which would block other immobilization sites and would reduce the accessibility for hybridization. Finally, we were interested in developing a methodology that was not only versatile and provided good hybridization results, but was also affordable. Many of the techniques routinely employed for preparing DNA arrays require pre-modified CPG, non nucleosidic phosphoramidites, or very expensive slides as starting materials.

For our present work, hybridization with fluorophore labeled target(s) was the required application for DNA microarrays. We therefore used aldehyde slides and the conditions recommended in the recent comparative study by Zammatteo and collaborators[33] as a starting point for our work. Unfortunately, this publication does not give the structure of the appendage to the oligonucleotides that provides the amino groups for immobilization on the aldehyde bearing slides. We decided to choose oligonucleotides with an L-lysine residue at the 3'-terminus[37] as amino modified DNA strands for immobilization. Oligonucleotides with amino groups at their 3'-termini have been used previously for immobilizing oligonucleotides on surfaces,[11] though not via reductive amination, and not with lysine residues as linkers.

The synthesis of the strands to be immobilized started from long chain alkylamine controlled pore glass (LCAA CPG) and involved intermediate **1**, whose preparation follows a method for the preparation of 3′,5′-dipeptidyloligonucleotides,[37] except that a single ω-hydroxylauric acid residue is employed between the controlled pore glass and the L-lysine residue (Scheme 1). The linker includes a 2,2-dimethylhydroxypropionic acid (DP) moiety, which is known to reduce side reactions originating from 3′-appendages.[37] Standard DNA synthesis on support **1** yielded intermediates **2**, whose deprotection with saturated aqueous ammonia gave lysine bearing oligonucleotides (**3**) in high purity, as determined by MALDI–TOF mass spectrometry of crude products (see Figure S4, Supporting Information, for an example).

Several oligonucleotides of general structure **3** were immobilized on aldehyde bearing glass microscope slides, following the protocol recommended by Zammatteo and collaborators.[33] When solutions of fluorophore bearing DNA were spotted onto the resulting DNA bearing spots, it was found that one step reductive amination with $NaCNBH_3$ (**3** to **4**, Scheme 1), rather than the recommended two step procedure (Schiff base formation, followed by reduction with $NaBH_4$)[33] gave the strongest fluorescence signal upon hybridization. However, when several DNA spots were incubated collectively, with exposure of the non DNA bearing background surface between the spots to the fluorophore bearing target DNA, strong background signals were observed, sometimes to the extent that the background fluorescence was stronger than that from the DNA bearing spots. Unacceptably strong background signals of up to 100 intensity units on a scale with a maximum of 200 units were also detected when the remaining aldehyde groups of the surface were reduced with $NaBH_4$ prior to hybridization,[33] indicating that the signals were the result of adsorption, not reactions with the aldehyde groups. Both the cyanine dye Cy3 and fluorescein as the fluorophore gave this result, and neither of the numerous washing protocols tested, including those recommended in reference 33 alleviated the problem. To further exclude side reactions between nucleobases and aldehyde groups, the reactivity of mixed sequence DNA oligomers of general structure **3** towards aldehydes was tested in solution. Monitoring of the conversion under the chosen reductive amination conditions with several equivalents of benzaldehyde by MALDI–TOF mass spectrometry showed that the reactivity was similar to that observed for amino terminal, but otherwise protected DNA strands,[36a] indicating that the exocyclic amino groups of the nucleobases were not susceptible to Schiff base formation.

The problem of unspecific background adsorption of target strands on surfaces has been known since the early days of DNA microarrays. A detailed study published in 1994 reported that efforts to reduce or eliminate this prob-



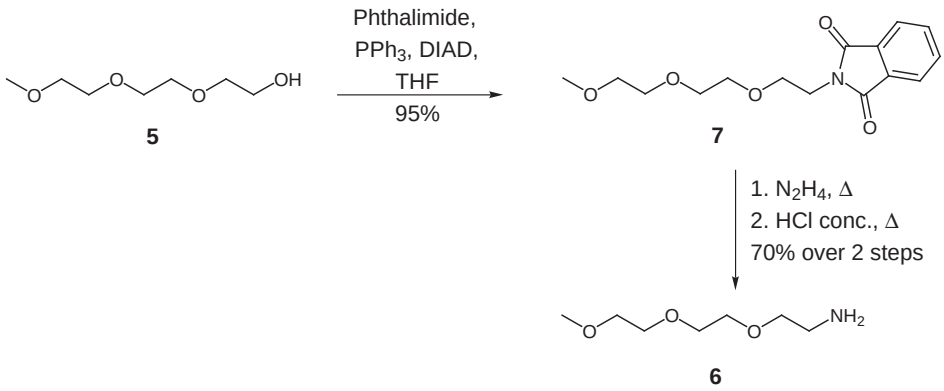
Scheme 1

lem by using non specific blocking DNA or various detergents were unsuccessful.[25] For 'aminated' surfaces and PCR amplified DNA, some improvement may be achieved with betaine as an additive to the spotting solution,[38] but no general solution for aldehyde- or alcohol-bearing surfaces has emerged. We therefore decided to develop a 'passifying' or coating approach to decreasing the non specific adsorption on the slide surface. It has been shown that poly- and oligoethyleneglycol chains can prevent non specific adsorption of biomacromolecules.[39] Further, oligoethylene chains have been used in the construction of linkers between DNA and surface.[2b,40] An alternative approach, the use of fluorinated compounds to create non adsorptive areas surrounding DNA spots,[41] was not pursued due to higher costs and possible difficulties of working with solvophobic surfaces.

To ensure a system that is chemically well defined, we chose oligoethylene chains that are available as single compounds, rather than the longer chains that are produced by polymerization and have to be employed as mixtures with a distribution of chain lengths. Triethyleneglycol monomethylether (**5**) is available at low cost and has about the same contour length as the 3′-appendage of the oligonucleotide probes of general structure **3**, thus avoiding blocking the base pairing of the 3′-terminal region of the oligonucleotides. To be suitable for immobilization on the aldehyde modified slide surface, **5** had to be converted to amine **6**.[42,43] We decided to employ the synthetic route shown in Scheme 2, which is one step shorter than the known methods for the preparation of **6**.[42,43] Alcohol **5** was converted to phthalimide **7**[42] in a Mitsunobu reaction, and **7** was converted to **6** via hydrazinolysis. Both reactions proceeded uneventfully, though distillation of **6** lowered the yield for the second step to 70%. Exploratory experiments showed that undistilled **6**, obtained by our procedure in 84%, gave coating results that are indistinguishable from those obtained with distilled **6**.

With passifying amine **6** in hand, a method was developed for coating the background surface of the slides prior to the immobilization of the DNA probe strands. Paraffin wax was chosen as a protecting agent for the spots where DNA was to be placed later. This led to the procedure shown in Scheme 3. Melted wax was applied to slide **8** via a multichannel pipette, using an inexpensive drill press for positioning the pipette, producing 'spot protected' **9**. The remaining exposed aldehyde groups of **9** were then reacted with **6** in a reductive amination to give **10**. The protective wax layers were then removed by dissolving the wax in dichloromethane, which gave slide **11**, whose aldehyde bearing spots were then used for immobilizing lysine modified DNA strands of general structure **3**. A final reductive amination with **6** on the entire slide surface sealed remaining aldehyde sites between DNA chains on the spots, as well as possibly providing additional tightening of the passifying coat in the background area, resulting in DNA arrays of general structure **12** (Schemes 1 and 3).

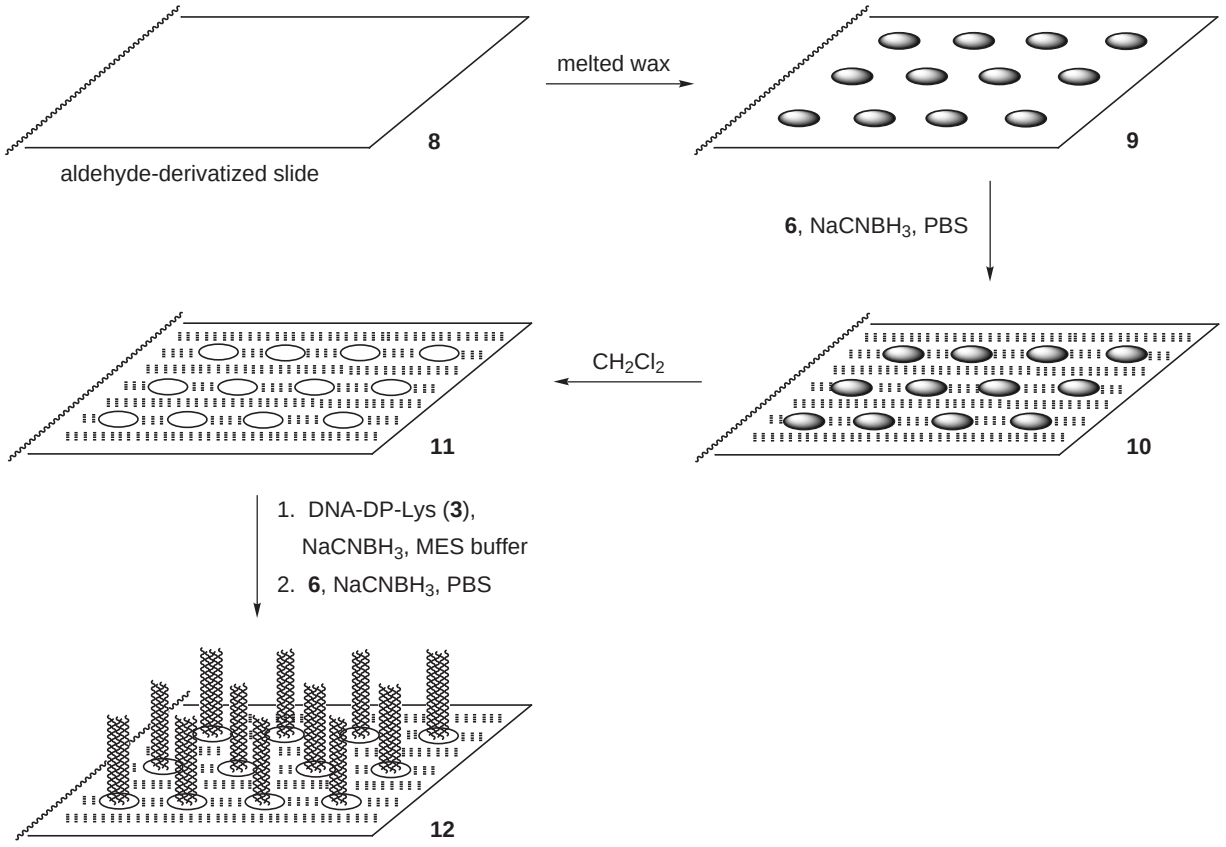
DNA microarrays with sequences **13**, **14**, and **15** (Figure 1) were prepared following the protocol shown in



Scheme 2

Scheme 3, with spotting every DNA probe strand in duplicate. Oligonucleotide **13** contains a cholic acid moiety at its 5′-terminus, a modification shown earlier to increase target affinity and base pairing fidelity at the terminus of a duplex.[19] Sequence **14** is made up of unmodified DNA, except for the 3′-linker to the surface, and so is sequence **15**, but **15** contains a mismatched base pair at the penultimate position of the duplex with Cy3 labeled target strand **16**. When the slide presenting **13**, **14**, and **15** was incubated with **16**, under a cover slip, i.e. with exposure of the background surface to the fluorophore labeled DNA, selective hybridizations at the sites of DNA immobilization were observed (Figure 2). The mismatched probe **15** gave less hybridization signal than fully matched **14**, even though the mismatch is very close to the terminus, and 5′-capped **13** gave the strongest signal.

The DNA array also proved to be reusable. Removal of bound target DNA with water at 90 °C gave back a slide with no or virtually no fluorescence signal upon scanning. When used three times in a row, less than 25% loss of hybridization signal was observed between the first and the last experiment. Together, these results demonstrated that the slide with coated background operates successfully as



Scheme 3

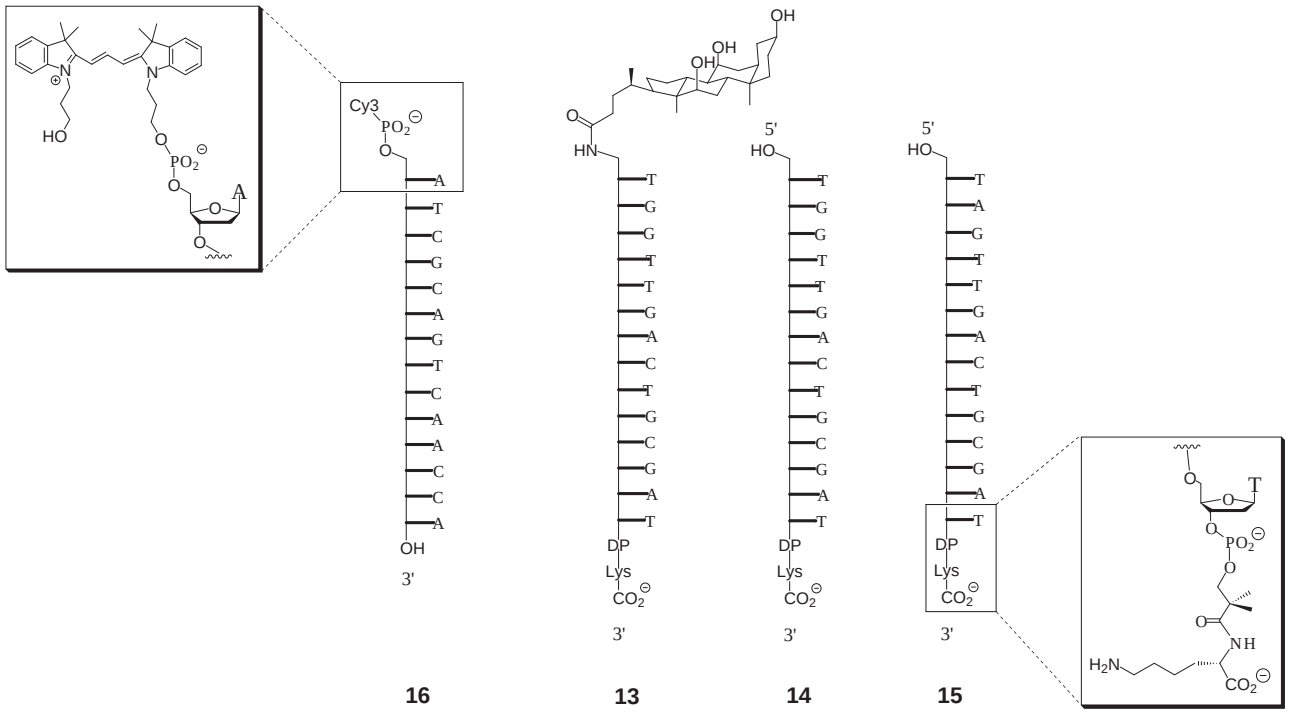


Figure 1 Oligonucleotides employed in hybridization experiments with DNA microarrays. Fluorophore-labeled **16** was employed in solution. The three lysine bearing sequences **13**, **14**, and **15** were immobilized on the glass surface in separate regions. Every letter to the right of a bold horizontal bar represents a nucleobase in a DNA sequence. The highlighted boxes show structural details of modified termini.

DNA microarray, and that the hybridization is selective, both in terms of sequence selectivity and in terms of stronger binding when an affinity-enhanced, modified probe is used.

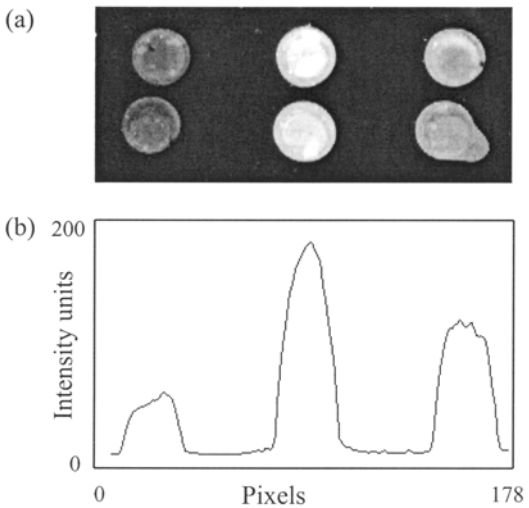


Figure 2 Results of a hybridization experiment with a model microarray displaying oligonucleotides **15** (left lane), **13** (center lane), and **14** (right lane), immobilized on a background coated glass surface, prepared as shown in Scheme 3. Each oligonucleotide was spotted in duplicate (upper and lower row) to test reproducibility. Fluorescent oligonucleotide **16** (10 μM) in buffer solution was incubated with the surface of the array, as described in the Experimental Part. (a) Fluorescence image, (b) fluorescence intensity profile for the upper row of spots, generated with NIH Image/Scion Image.[48]

Several extensions of the current scheme can be imagined. One would facilitate automation of the hybridization and washing steps by introducing a fixed container around the slide, though the engineering effort for this and a circulating flow system with a pump may be substantial, without offering major advantages for moderate throughput studies. Further, one could perform the immobilization step with oligonucleotide duplexes, rather than single strands, as reported for DNA immobilized on gold surfaces.[44] The probe strand, engaged in Watson–Crick duplex formation with a complementary strand, would have any residual reactivity of the exocyclic amino groups of the nucleobases masked, and the presence of the complementary strand would ensure that enough space is available for complementary strands to bind in later hybridization experiments. However, the cost and effort for synthesizing the complementary strands are not negligible, and complete removal of the complementary strands after immobilization is not trivial to establish either. Thirdly, it may be worthwhile replacing Cy3 as the fluorophore of the target strands. The phosphoramidite with which this cyanine dye is introduced is currently the most costly chemical employed in our procedure. Labeling strands of general structure **3** with fluorescein isothiocyanate or rhodamine B isothiocyanate has already been performed successfully, though the fluorescence intensity upon hybridization of the labeled strands to the microarrays is currently lower than that achieved with the corresponding Cy3 labeled strands, possibly because the surface was not wetted sufficiently prior to scanning.[25]

Even without additional extensions and improvements, it is hoped that the method presented here will prove useful for routine testing of modified oligonucleotides as hybridization probes in microarrays, and possibly for the preparation of arrays for biomedical applications themselves. Since syntheses on lysine bearing solid support **1** produce few side products, spotting without purification should be possible, making the preparation of multispot arrays feasible. These should enable the identification of hybridization probes, whose mismatch discrimination is better than that of unmodified DNA, particularly when the mismatch is close to one of the termini of the duplexes, where breathing and fraying prevent high fidelity recognition in natural DNA and RNA.[45]

The following solvents were of the best commercial quality and were used as received: DMF, THF and TFA (Fluka/Riedel deHaen, Taufkirchen, Germany), CH_3CN (Acros, Geel, Belgium), and AcOH (Roth, Karlsruhe, Germany). Also used as received were the following chemicals: Ac_2O and DIAD (Fluka), NH_4OH and PPh_3 (Fluka/Riedel deHaen), sodium dodecylsulfate (SDS), sodium citrate, hydrazine monohydrate, and $NaCNBH_3$ (Acros), triethylenglycol monomethylether (Aldrich, Taufkirchen, Germany), Boc-Lys(TFA)-OH (NovaBiochem, Läufelfingen, Switzerland), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexaflourophosphate (HBTU), and hydroxybenzotriazole monohydrate (HOBT) (Advanced ChemTech, Louisville, KY). The phosphoramidite building blocks for DNA synthesis were from Proligo (Hamburg, Germany) for dA^{Bz}, dC^{Bz}, and T; and from Glen Research (Sterling, VA) for dG^{dmf}. The controlled pore glass (CPG) for DNA synthesis was long chain alkylamine (LCAA) CPG from Controlled Pore Glass Inc. (Lincoln Park, NJ) and had the following specifications: mean pore diameter 1088 °A, surface area 34.4 m^2/g, particle size 80–120 mesh, loading 93.3 μmol/g. The Cy3 phosphoramidite building block for 5′-fluorophore labeling was from Glen Research (catalog No. 10–5913–95). Oligonucleotides were purified by RP HPLC C4 columns (Vydac, Hesperia, CA or Macherey–Nagel, Düren, Germany), using a gradient of CH_3CN (solvent B) in 0.1 M triethylammonium acetate (TEAA) at pH 7. Yields of oligonucleotides are based on the intergration of HPLC traces of crudes. Paraffin wax (purum, mp 50–52 °C) for protecting slide areas was from Fluka. Aldehyde bearing slides were from Cell Associates, (Houston, TX) and were ordered via GenPak Limited (New Milton, UK), who sells them as 'silylated slides'. According to the supplier, there is a three carbon linker between the silicon atom that is part of the surface network and the aldehyde group. Spotting was performed with a multichannel micropipette (Roth), using drill press Bohrständer Basic*** (Emil Lux GmbH, Wermelskirchen, Germany) for positioning. Saline sodium citrate buffer for hybridization experiments (SSC, 150 mM NaCl, 15 mM sodium citrate) was prepared as a 20–fold concentrated stock solution and diluted to the desired concentration when needed. Phosphate buffered saline solution (PBS, pH 7) was prepared using a standard protocol.[46] MALDI–TOF mass spectra were acquired on a Bruker BIFLEX III mass spectrometer in negative mode at 19 kV with a delayed extraction voltage of 17.15 kV. A mixture of 2,2,6-trihydroxy acetophenone (0.3 M in EtOH) and diammonium citrate (0.1 M in H_2O) was used as matrix and comatrix for the mass spectrometer. A Perkin Elmer Lambda 10 spectrophotometer was used to acquire UV spectra. NMR spectra were acquired on a Bruker AC 250 spectrometer. The fluorescence images of DNA arrays with bound analyte strand were obtained with a Molecular Imager Fx (BioRad, Hercules, CA) using software Quantity One, version 4.2.

***N*-(3,6,9-Trioxadecyl)phthalimide (7)**

Phthalimide (3.53 g, 24 mmol) and PPh_3 (6.3 g, 24 mmol) were dried for 1 h at 0.1 Torr, placed under argon, and dissolved in THF (100 mL). The solution was treated with triethylenglycol monomethylether **5** (3.2 mL, 20 mmol). After 15 min, DIAD (4.73 mL, 24 mmol) was added dropwise at r.t., resulting in a slight warming of the solution. After 12 h at r.t., TLC showed that the reaction was complete (petroleum ether (PE)–EtOAc, 1:1, educt R_f 0.1, product R_f 0.45). The reaction was quenched by addition of EtOH (40 mL). The solvent was evaporated in vacuo and the residue dried at 0.1 Torr for 1 h. The residue was treated with PE–EtOAc (1:1, 20 mL) and stirred at 40 °C for 1 h. The white residue was filtered off and washed with the same solvent mixture (10 mL). The filtrate was evaporated to dryness, dried at 0.1 Torr, and chromatographed on silica gel (PE–EtOAc , 4:1), yielding the title compound as colorless oil (5.57 g, 19 mmol, 95%).

^{1}H NMR ($CDCl_3$, 250 MHz): δ = 3.28 (s, 1 H), 3.40 (m, 2 H), 3.51–3.62 (m, 6 H), 3.67–3.71 (t, *J* = 5.5 Hz, 2 H), 3.83–3.84 (t, *J* = 5.5 Hz, 2 H), 7.65–7.69 (m, 2 H), 7.77–7.81 (m, 2 H).

^{13}C NMR ($CDCl_3$, 62.9 MHz): δ = 37.09, 58.84, 67.74, 69.93, 70.37, 70.39, 71.67, 123.05, 131.98, 133.76, 168.07.

EI-MS (70 eV, 105 °C): *m*/*z* (%) = 293, 234, 218, 204, 190, 174, 59.

3,6,9-Trioxadecylamine (6)

The *N*-(3,6,9-Trioxadecyl)phthalimide precursor **7** (5.75 g, 20.08 mmol) was taken up in EtOH (55 mL) and treated with hydrazine monohydrate (1.16 mL, 22.4 mmol). The resulting mixture was refluxed at 100 °C for 5 h, whereupon a white precipitate formed. TLC showed full conversion (EtOAc, educt R_f 0.6, product R_f 0). The slurry was allowed to cool and then treated with concd HCl (4.8 mL), followed by refluxing again for 1 h. The slurry was allowed to cool to r.t. and the white solid was filtered off. The filtrate was evaporated in vacuo and the residue taken up in H_2O (30 mL), and the solution brought to pH 11 with 1 N NaOH (20 mL). The aqueous phase was saturated with NaCl and extracted with CH_2Cl_2 (8 × 30 mL). The combined organic phases were dried over Na_2SO_4, evaporated to dryness and briefly dried at 0.1 Torr, yielding a slightly brownish liquid (2.77 g). Kugelrohr distillation (0.1 Torr, heating chamber at 110 °C) gave the title compound as colorless liquid (2.3 g, 14 mmol, 70%).

R_f 0.55 (MeOH–NH_4OH, 9:1).

^{1}H NMR in agreement with the literature.[13,43]

^{13}C NMR ($CDCl_3$, 62.9 MHz): δ = 41.59, 58.88, 70.12, 70.39, 70.45, 71.80, 73.21.

Support for synthesis of DNA to be immobilized (1)

The preparation of the controlled pore glass on which the DNA to be immobilized was synthesized followed largely a methodology reported earlier.[37] Briefly, coupling of 12-trityloxylauric acid (100 μmol, 45.6 mg) with HBTU (90 μmol, 34.6 mg), HOBT (100 μmol, 15.5 mg), and *N*,*N*-diisopropylethylamine (DIEA) (233.6 μmol, 40 μL) in DMF (600 μL) to LCAA CPG (45 mg) for 1 h was repeated once, followed by a capping step, which was performed using Ac_2O (1 mL) for 3 min. Detritylation with CH_2Cl_2–TFA (1:1, 6 mL) was followed by coupling of Boc-Lys(TFA)-OH (35.3 mg, 100 μmol) with the same activation mixture as for trityloxylauric acid for 20 min. After another capping step and Boc-removal with CH_2Cl_2–TFA (1:1, 2 mL), 2,2-dimethyl-3-trityloxypropionic acid (36.1 mg, 100 μmol) was coupled for 1 h under the same activation conditions as for the other two acids. Capping with Ac_2O (2 mL, 3 min) and detritylation, followed by the usual rinsing with CH_3CN (12 mL) and drying at 0.1 Torr gave the support ready for DNA synthesis.

DNA Synthesis

Oligodeoxyribonucleotides were synthesized on an ABI 380B DNA synthesizer on a 1 μmol scale, following the manufacturer's recommendations. Extended coupling times of 15 min were used for all non standard phosphoramidites. After completion of the DNA synthesis, using the trityl off mode, the DNA bearing CPG was placed in Eppendorf-type polypropylene vessels in portions of 10 mg/vessel, and concentrated aqueous ammonia ('NH_4OH', 1.5 mL) was added, followed by vortexing. The vessels were tightly sealed. After 16 h at r.t., excess ammonia was blown off with a gentle stream of air directed on the surface of the solutions. The supernatants were removed, the CPG washed with H_2O (0.5 mL), and the combined solutions lyophilized to dryness. The residues were purified by HPLC.

5′-TGGTTGACTGCGAT-DP-Lys (14)

Yield 58%.

HPLC gradient of 0% B for 5 min, to 30% B in 35 min, to 100% B in 10 min.

R_t 20.4 min.

MALDI–TOF MS: *m*/*z* [M – H]$^-$ calcd for $C_{149}H_{193}N_{53}O_{91}P_{14}$, 4615.8; found: 4617.5.

5′-TAGTTGACTGCGAT-DP-Lys (15)

Yield 62%.

HPLC gradient of 0% B for 5 min, to 30% B in 40 min, to 100% B in 10 min.

R_t 34.3 min.

MALDI–TOF MS: *m*/*z* [M – H]$^-$ calcd for $C_{149}H_{193}N_{53}O_{90}P_{14}$, 4599.8; found: 4597.6.

5′-chl-T*GGTTGACTGCGAT-DP-Lys (13)

Following standard DNA synthesis on support **1**, except that the last coupling step was performed with the phosphoramidite building block of 5′-amino-5′-deoxythymidine,[47] a mixture of cholic acid (40.9 mg, 100 μmol), HBTU (35.1 mg, 90 μmol), and HOBT (15.5 mg, 100 μmol) in DMF (600 μL) was treated with DIEA (40 μL, 233.6 μmol), the solution was vortexed and pushed into a synthesis cartridge containing the loaded CPG (15 mg) using two syringes. After 1 h, the support was rinsed with DMF (6 mL) and CH_3CN (6 mL), and dried at 0.1 Torr for 1 h. Deprotection and purification were the same as described above.

Yield 36%.

HPLC gradient of 0% B for 5 min, to 30% B in 40 min, to 100% B in 10 min.

R_t 44.7 min.

MALDI–TOF MS: *m*/*z* [M – H]$^-$ calcd for $C_{173}H_{230}N_{53}O_{95}P_{14}$, 5007.4; found: 5008.3.

5′-Cy3-ATCGCAGTCAACCA-3′ (16)

The synthesis of this fluorophore labeled target sequence was performed on standard CPG (Proligo) loaded with the *N*6-benzoyl-2′-deoxyadenosine building block for the 3′-terminal residue using the DNA synthesis conditions given above. For the labeling step, the phosphoramidite of the dye Cy3 (Glen Research) was coupled with an extended coupling time of 10 min. The trityl group protecting the dye moiety was removed on the DNA synthesizer. Cleavage from the support and purification were performed as given for the other oligonucleotides.

Yield 38%.

HPLC gradient of 0% B for 5 min, to 30% B in 30 min, to 100% B in 10 min.

R_t 32.5 min.

MALDI–TOF MS: *m*/*z* [M – H]$^-$ calcd for $C_{164}H_{207}N_{56}O_{83}P_{14}$, 4722.5; found: 4725.3.

Coating the slide background.

Melted paraffin wax (7 μL per spot, at approx. 60 °C) was applied to selected areas, using a multi channel pipetman attached to a drill press. For this the pipet tips were heated for a few seconds using a heat gun before being placed into the hot wax. The wax was taken up, and quickly applied to the aldehyde bearing slide surface in rows of 6 spots. The wax spots were allowed to solidify to create protected areas that would be unreactive in the subsequent step. The slide was then photocopied on a standard paper copier to record the placement of the wax spots. To coat the free surface, a mixture of triethyleneglycol amine **6** (50 mM in PBS buffer, 5 mL) and Na BH_3CN (31 mM in PBS, 150 μL) was applied to the aldehyde slide and spread, using a glass pipet. The reductive amination was allowed to proceed for 2.5 h at r.t. The slide was then rinsed with SSC/0.2% SDS by dipping it into a 25 mL Erlenmeyer flask and then rotating it therein for 2 min. H_2O was rinsed over the slide to remove excess salt, using a squirt bottle. The slide was then spun dry in cuvette dryer (Zentrax 280, Heildolph, Kelheim, Germany) adapted to hold microscope slides. To remove the wax, the slide was placed in a beaker with CH_2Cl_2 until the wax was entirely dissolved. The slide was then rinsed with fresh CH_2Cl_2 and dried under a stream of argon.

Spotting DNA.

A solution of the 3′-lysine bearing DNA (**3**) (2 μL, 2 μM) in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (0.1 M, pH 6.8) was applied to the aldehyde slide at a site previously protected by wax, following the pattern recorded on the photocopy, which was placed underneath. A solution of $NaBH_3CN$ (0.5 μL, 31 mM in PBS) was added to the droplet on the slide and mixed by pipetting up and down, without touching the glass surface. The remaining spots were generated in the same manner. The slide was placed in a polypropylene storage/transport chamber for individual microscope slides (Merck Eurolab, Darmstadt, Germany), with H_2O drops placed on the ends of the slide to ensure humidity and avoid evaporation losses. The reaction was allowed to proceed for 16 h at r.t. in the closed chamber. Then, spots were removed with a paper towel, and the surface washed in SSC/0.2% SDS for 2 min. After a brief H_2O rinse, the slide was spun dry as described above. Unreacted aldehyde groups were then reacted with **6** in a reductive amination, performed as described above, except that the entire surface of the slide was allowed to react for 1 h. After removal of the reaction solution, soaking in SSC/0.2% SDS for 2 min, and washing with filtered H_2O (0.2 μm pore size filter) to eliminate dust, the slide was spun dry.

Hybridization.

A solution of fluorophore labeled DNA **16** (10 μL, 10 μM) in ten fold concentrated SSC/0.2% SDS buffer was applied to the aldehyde slide and spread with a glass coverslip in the fashion typical for microscope slides. Drops of H_2O were applied on the edges of the slide to prevent evaporation losses. The slide was transferred to a plastic slide chamber and incubated for 16 h at r.t. (22 °C). Afterwards, the coverslip was removed while dipping the slide in a wash solution of SSC/0.2% SDS. The slide, while in the wash solution (in a 25 mL Erlenmeyer flask), was sonicated in a bath sonicator (USR 18 Qualilab, Merck Eurolab, Bruchsal, Germany) for 10 second per washing step. The washing solutions were SSC/0.2% SDS, ten fold diluted SSC/0.2% SDS, and ten fold diluted SSC without SDS. After the final washing step, the slide was rinsed with filtered H_2O and spun dry. To determine the extent of hybridization, the slide was scanned in the imager, using the filter setting for Cy3 provided with the instrument at a resolution of 100 μm. The intensity profiles were

generated from scans using the public domain NIH Image program developed at the U.S. National Institutes of Health.

Supporting Information Available: NMR spectra of compounds **6** and **7**, and MALDI–TOF mass spectra of compounds **13**–**16** (7 pages total).

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