DNA Assays

A Direct Glimpse of Cross-Hybridization: Background-Passified Microarrays That Allow Mass-Spectrometric Detection of Captured Oligonucleotides**

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The generation of DNA microarrays or DNA chips[1,2] has made possible the massively parallel detection of DNA and RNA sequences. The ability to detect DNA or RNA sequence selectivity with DNA chips relies on the formation of Watson–Crick duplexes between immobilized strands (probes) and the target strands in solution. High-density DNA chips feature up to 250 000 spots and allow gene expression to be monitored on the level of entire genomes.[3,4] Duplex formation between DNA strands with only partial complementarity (cross-hybridization) is difficult to suppress at this level of complexity. As conventional DNA chip experiments use the same fluorophore for labeling all sequences from one biological sample, cross-hybridization can lead to false positive results, threatening the reliability of chip experiments. This is particularly true when single-base resolution is required, either because single-nucleotide polymorphisms have to be detected or because the expression of closely related genes has to be monitored.[5] Single-mismatch resolution is also important for control sequences employed on some chips for background correction,[6] an approach that has been challenged.[7]

Several approaches for simulating cross-hybridization computationally have been reported.[8,9] Accurate prediction of duplex stability remains difficult, however,[10] making it desirable to determine cross-hybridization experimentally. MALDI-TOF mass spectrometry[11] offers detection capabilities for short DNA strands that differ in base composition. The tiny amounts of DNA bound to micrometer-sized spots on flat surfaces make it difficult, however, to obtain sufficient signal to evaluate hybridization events. The direct ablation of oligonucleotides captured by complementary strands covalently bound to silicon surfaces has been reported.[12] The method reported in the literature involves a single genotyping target and a polymerase-catalyzed step. It provides sufficient resolution to distinguish between DNA strands of different lengths.

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We reasoned that metal surfaces would be most useful because they are electrically conductive, as required for the desorption process in MALDI mass spectrometers. Our initial attempts to detect directly from gold surfaces were unsuccessful,[13] but the refined approach reported herein allows the direct mass spectrometric detection of cross-hybridization. The approach uses background-passified surfaces[14] to prevent nonspecific adsorption of DNA on gold surfaces.[15]

Our method involves the capture of DNA strands on the surface by hybridization to immobilized probes, a washing step to remove unbound strands, the addition of the matrix 2,4,6-trihydroxyacetophenone in an ethanolic solution to favor dissociation of duplexes and desorption, and mass-spectrometric detection of the captured target strands. Several factors were found to be critical for obtaining DNA signals. One such factor is a smooth surface. For this, gold layers (250-nm thickness) on quartz substrates were smoothed by heating in an H_2 flame. Another factor is the generation of a surface that prevents nonspecific adsorption of probe and target strands. To this end, we generated self-assembled monolayers (SAMs)[16] on smoothed gold surfaces in which the solvent-exposed portions of the assembly chains consisted of tetraethylene glycol units. The ethylene glycol moieties suppress nonspecific adsorption. Similar constructs have previously been employed in studies on cell adhesion.[17] Unlike typical oligoethylene glycol SAMs, our tetraethylene glycol chains terminate in a methyl ether, not a free hydroxy group. This renders the surface less sticky and allows the detection of DNA-bearing spots by their ability to retain water drops on a surface that is moderately hydrophobic (Figure 1). Fluorinated compounds are also known to generate hydrophobic surfaces on DNA chips.[18] The current spot diameter is 2 mm, but spot sizes as small as the laser beam diameter in the MALDI mass spectrometer are conceivable.

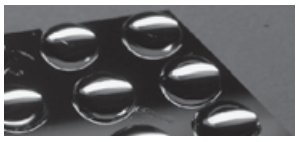
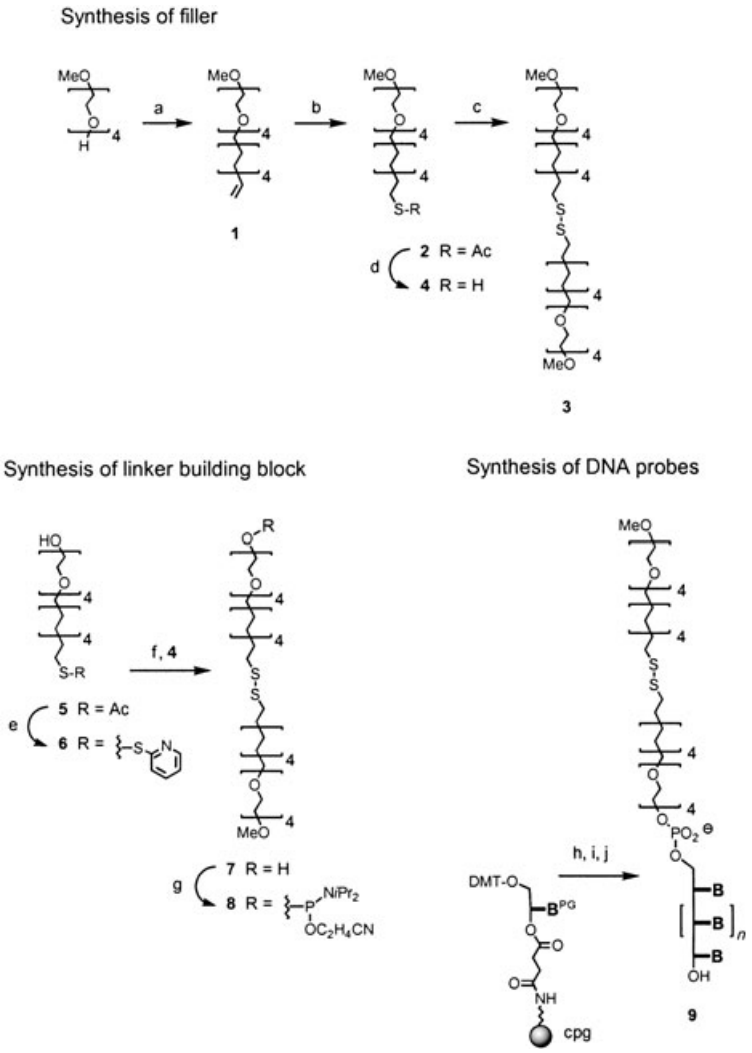


Figure 1. Photograph of a chip with immobilized DNA and filler after washing and inverting the chip. DNA-bearing spots retain the aqueous solution preferentially over the surrounding areas. During hybridization, a cover slip may be used to ensure contact between all spots and the solution.

We decided to favor the formation of as tight a SAM as possible by employing the same chains for DNA immobilization that were used for passifying the background surface. This was done by preparing oligonucleotides of general structure **9** (Scheme 1). These feature DNA linked to a tetraethylene glycol unit, which in turn is linked to the alkylthiol that forms the bottom portion of the SAM. Key to the successful generation of highly DNA-sensitive surfaces was the protecting-group scheme used to generate the oligonucleotide probes. Our immobilization method uses disulfides instead of thiols. The very filler for passifying the surface is also used as a disulfide-forming protecting group. As the removal of these protecting groups produces additional filler molecules without the generation of disruptive protecting group fragments, it favors particularly homogeneous surfaces. The absence of protecting and deprotecting steps also leads to a shorter route than those employing trityl-protected thiols.

The passifier or filler molecules were prepared as shown in Scheme 1. Starting from **1**, thioacetate **2** was prepared in a thermal addition of AcSH[19] and either converted into symmetrical disulfide **3** or deprotected to thiol **4**, whose reaction with 1 equivalent of activated disulfide **6** gave



Scheme 1. a) NaH, THF, 11-bromo-1-undecene (52%); b) AcSH, toluene, AIBN, Δ (79%); c) NH_4OH (quant.); d) HCl in MeOH (quant.); e) dithiodipyridine; NH_4OH, MeOH (84%); f) **4**, THF (78%); g) DIEA, chloro-*N*-(diisopropylamino)-2-cyanoethoxyphosporamidite, CH_2Cl_2 (75%); h) standard DNA synthesis, including extension cycle with **8**; i) I_2, py, THF, H_2O; j) NH_4OH; B = nucleobase, PG = protecting group, cpg = controlled pore glass, AIBN = azobisisobutyronitrile, DIEA = diisopropylethylamine.

alcohol **7**. The alcohol was phosphitylated to **8**, which was directly coupled to DNA oligomers conventionally prepared on controlled pore glass. Disulfides **9**, obtained upon treatment with aqueous ammonia, are more readily purified by HPLC than symmetrical disulfides featuring two DNA strands or oxygen-sensitive free thiols. Their separation from failure sequences of DNA syntheses is quite facile, because the disulfide tail increases the elution time from reversed phases. Furthermore, removal of the thiol protecting group, another step often found to give low yields in our hands, is not required with oligonucleotides of general structure **9**, as they can be directly added to gold surfaces. After spotting the oligonucleotide probes for surface attachment, remaining exposed gold surface regions were filled by treating with a solution of the passifier disulfide **3** (Scheme 2).

When layering gold surfaces thus prepared with a MALDI matrix, we were able to detect traces of disulfide **3** upon irradiation with the MALDI laser.[20] This signal disappeared, however, after washing with THF, suggesting that the disulfides desorbable by the MALDI process had not been covalently linked to the surface, but were noncovalently incorporated in the SAM. Brief exposure of SAMs to THF is known to lead to minimal loss of covalently held chains.[21] When surfaces of general structure **10** (Scheme 2) were incubated with mixtures of strands with full or partial complementarity, and washed with ammonium acetate solution, spectra such as those shown in Figure 2 were obtained. At high laser power, each spot gave signal for the target strands both in linear and in reflectron mode, when analyzed with an unmodified commercial spectrometer. No signal for the immobilized strands or their disulfides were detected, suggesting that they were stably immobilized.

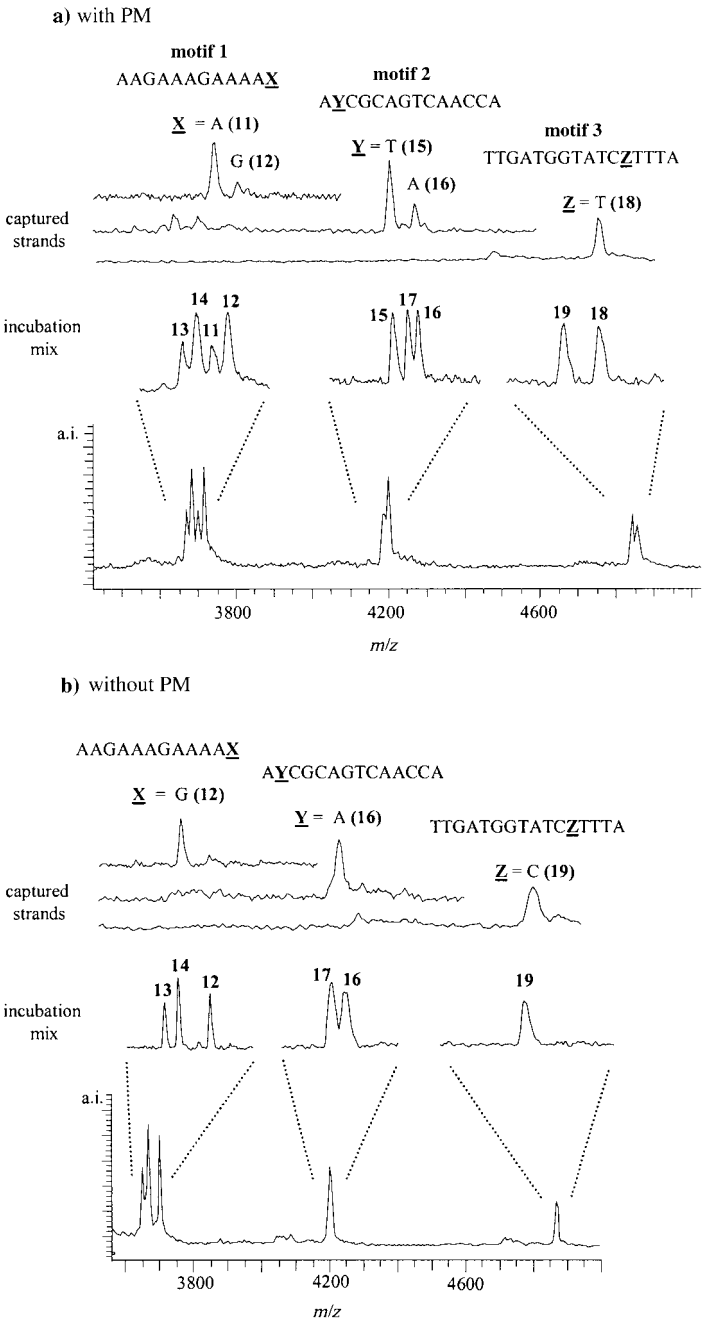
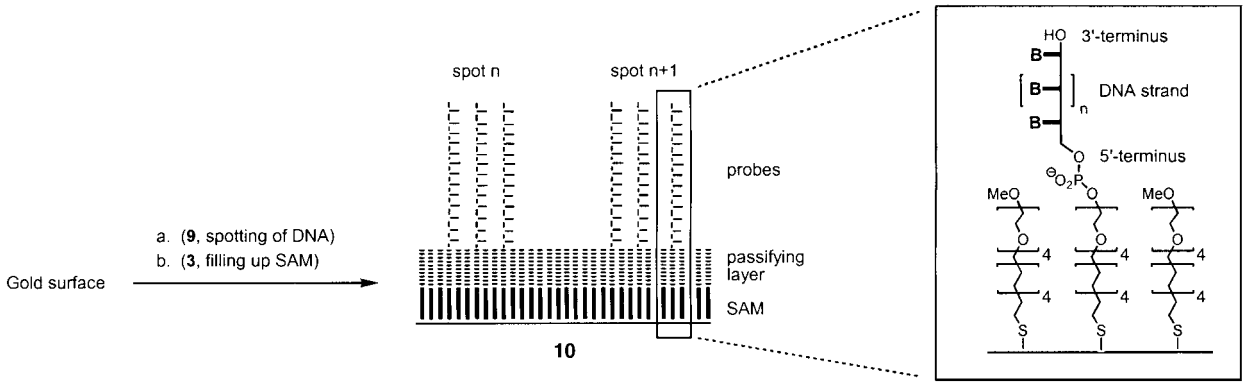


Figure 2. MALDI-TOF mass spectra of oligonucleotides prior to (bottom spectra labeled 'incubation mix') or after capture by surface-bound probes (upper spectra labeled 'captured strands'). The spectra at the very bottom were acquired in linear mode; expansions immediately above are from spectra in reflectron mode. Spectra of captured strands are from the individual spots of the chip. The probe on each spot was complementary to the motif indicated above the spectra, with positions of the variable bases indicated. a) Hybridization experiment with all DNA strands (**11**–**19**), including the perfectly matched (PM) targets. b) Hybridization experiments with DNA strands **12**, **13**, **14**, **16**, **17**, and **19**, that is, without the PM targets.



Scheme 2. a) Spotting of 2 μM solutions of DNA probes of general structure **9** in 1 M KH_2PO_4 (pH 7) at individual locations; b) incubation of entire surface with 1 mM solution of disulfide filler (**3**) in $EtOH/H_2O$ (1:1).

For all three DNA sequence motifs, the signal for the fully complementary DNA strand was the strongest when employing equimolar mixtures of all strands. The extent to which strands with a single mismatch were captured differed for the different sequences. Whereas **motif 3** gave little binding of the mismatched (MM) strand, **motifs 1** and **2** showed a substantial degree. Furthermore, in the absence of perfectly matched (PM) targets, all spots gave strong false-positive signals (Figure 2b). When PM targets were added to hybridization mixtures preincubated with MM strands only, the PM targets appeared in the MALDI-detectable signals, indicating that strand exchange can be induced. Exchange kinetics can also be studied. For example, a 15 h incubation of a mixture that contains only MM strands followed by a 9 h incubation with a solution containing both MM and PM sequences results in 40% cross-hybridization for **motif 3**.

Previous studies involving DNA strands with single and double mismatches had to be carried out with separate solutions, and showed strong binding of each in the absence of fully complementary target.[22] Our directly monitored competition experiments now show that the presence of the fully matched strand partially suppresses the binding of the mismatched strands, and that the extent of the suppression depends on the individual binding constants. Table 1 gives UV melting data for DNA duplexes formed in solution. Table 2 compiles the results from the on-chip hybridization experiments involving MALDI detection, and the expected distributions based on simulation with the computer program ChipCheck.[9] Target strand distributions on the spots calculated by ChipCheck with thermodynamic values determined from UV melting curves in solution are in good agreement with the experimental values for binding of mismatched target **16** for **motif 2** (Table 1 and Table 2). For **motif 1**, however, the cross-hybridization predicted based on database[24] thermodynamic values is closer to that calculated with thermodynamic values determined experimentally for the strands in solution. Furthermore, the mismatch in the hexadecamer of **motif 3** did not lead to as stable a duplex as

Table 1: UV Melting data for DNA duplexes studied in solution.

Probe	Target sequence[a]	No.	T_m [°C][b]	ΔT_m	Hyperchromicity [%][c]	$\Delta G°$ [kcal mol^{-1}][d]
motif 1						
TTTTTCTTTCTT (**9a**)	AAGAAAGAAAAA	**11**	33.7 ± 0.4		22.1 ± 1.2	−11.1
	AAGAAAGAAAA**G**	**12**	29.9 ± 0.7	−3.8	21.1 ± 0.5	−10.1
	A**C**GAAAGAAAT**A**	**13**	22.9 ± 1.3	−10.8	22.9 ± 1.5	−8.6
	TTGAAAGAAAA**A**	**14**	27.1 ± 0.5	−6.6	16.9 ± 0.5	−9.5
motif 2						
TGGTTGACTGCGAT (**9b**)	ATCGCAGTCAACCA	**15**	58.7 ± 0.3		21.7 ± 0.9	−16.8
	A**A**CGCAGTCAACCA	**16**	56.4 ± 0.8	−2.3	16.1 ± 0.3	−16.1
	ATCGCAGTCAAC**T**A	**17**	53.2 ± 0.4	−5.5	18.6 ± 1.1	−15.8
motif 3						
TAAAAGATACCATCAA (**9c**)	TTGATGGTATCTTTTA	**18**	49.4 ± 0.3		29.7 ± 3.3	−17.2
	TTGATGGTATC**C**TTTA	**19**	37.6 ± 0.8	−11.8	25.8 ± 2.7	−12.3

[a] Bases underlined and bold indicate mismatches with probe sequences. [b] Average of four melting points ± SD (standard deviation) at 1.1 μM strand concentration and 0.25 M NH_4OAc buffer. [c] Hyperchromicity accompanying duplex dissociation. [d] At 298 K.

Table 2: Target strands captured on chip surfaces.[a] (For sequences **11**–**19**, see Table 1.)

	11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Spot 1: TTTTTCTTTCTT **9a** (**motif 1**)									
MALDI[b]	86	14 ± 5	< 1	< 1	< 1	< 1	< 1	< 1	< 1
calcd (td data)[c]	92.1	4.2	3.3	< 1	< 1	< 1	< 1	< 1	< 1
calcd (Mfold)[d]	88.9	11.1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Spot 2: TGGTTGACTGCGAT **9b** (**motif 2**)									
MALDI[b]	< 1	< 1	< 1	< 1	81	19 ± 11	< 1	< 1	< 1
calcd (td data)[c]	< 1	< 1	< 1	< 1	62.5	19.6	17.9	< 1	< 1
calcd (Mfold)[d]	< 1	< 1	< 1	< 1	98.6	< 1	< 1	< 1	< 1
Spot 3: TAAAAGATACCATCAA **9c** (**motif 3**)									
MALDI[b]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	> 99	< 1
calcd (td data)[c]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	> 99	< 1
calcd (Mfold)[d]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	> 99	< 1
Spot 4: ATCGCAGTCAACCA **9d** (negative control)									
MALDI[b]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
calcd (Mfold)[d]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1

[a] Values in percent of the sum of MALDI peak heights for all targets. [b] Mean of different hybridization experiments with incubation times between 5 h and 144 h. [c] Calculated by ChipCheck with thermodynamic (td) data from UV-melting curves analyzed with MeltWin[23] (compare Table 1). [d] Calculated by ChipCheck with theoretical thermodynamic data obtained from Mfold.[24]

one might have expected.[10] Still, it gives strong cross hybridization when there is no PM target strand (Figure 2b). An example of cross-hybridization between strands with internal mismatches is provided in the appendix to the Supporting Information.

These results illustrate how difficult it is to predict cross-hybridization, and underscore the need for directly measuring this process experimentally with methods that provide strand-specific signals. We are currently developing methods that provide increased dynamic range and increased sensitivity for the detection of longer DNA sequences. Longer sequences are more likely to cross-hybridize, as partial annealing to capture probes may suffice to form a stable duplex. We also plan to employ internal standards in our work to allow absolute quantitation of the strands captured.[25] It has not escaped our attention that even with the current level of our technique, it should be possible to select oligonucleotides with chemical modifications that increase target affinity or base pairing fidelity[26] from small chemical libraries. Thus, this technique should offer a valuable alternative to spectrometrically monitored selection experiments (SMOSE).[27] Furthermore, directly monitoring DNA captured on surfaces could be useful in the context of DNA computing[28] and the generation of arrays for nanotechnology.[29]

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