

DNA microarrays on silicon surfaces through *thiol-ene* chemistry

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1. Materials

The silicon-based chips were provided by the Nanotechnology Center (NTC) at the Universitat Politècnica de València (Spain) as 3- μm -thick silicon oxide layer grown on (100) silicon wafer. Streptavidin, streptavidin-ATTO from Bovine serum albumin (BSA), hydrogen peroxide 35% w/w, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), triethyleneglycol monomethylether, D-biotin and sulfuric acid 95-98% were purchased from Sigma-Aldrich Química (Madrid, Spain). 3-Mercaptopropyl triethoxysilane was from Acros (Barcelona, Spain). Dimethylsulfoxide (DMSO), toluene and dichloromethane were purchased from Scharlau (Madrid, Spain). Oligonucleotides were acquired from Tib Molbiol (Berlin, Germany) and their sequences are shown in Table S1. *Note: All the chemicals should be handled following the corresponding material safety data sheets.*

The buffers employed in this study were phosphate buffer saline (PBS, 0.008 M sodium phosphate dibasic, 0.002 M sodium phosphate monobasic, 0.137 M sodium chloride, 2.7 M potassium chloride, pH 7.5), PBS-T (1 \times PBS containing 0.05% Tween 20), saline sodium citrate (1 \times SSC, 0.9 M sodium chloride, 0.09 M sodium citrate, pH 7) and carbonate buffer (1 \times CB, 0.5 M sodium carbonate, pH 9.6). Washing solutions were filtered through a 0.22 μm pore size nitrocellulose membrane from Whatman GmbH (Dassel, Germany) before use.

2. Instruments.

Microarray printing was carried out with a low volume non-contact dispensing system from Biodot (Irvine, CA, USA), model AD1500. Contact angle system OCA20 equipped with SCA20 software was from Dataphysics Instruments GmbH (Filderstadt, Germany). The measurements (n=5) were done at room temperature with a volume drop of 10 μL employing 18 $\mu\Omega$ water. X-ray photoelectron spectra were recorded with a Sage 150 spectrophotometer from SPECS Surface Nano Analysis GmbH (Berlin, Germany). Non-monochromatic AlK α radiation (1486.6 eV) was used as the X-ray source operating at 30 eV constant pass energy for elemental specific energy binding analysis. Vacuum in the spectrometer chamber was 9×10^{-9} hPa and the sample area analyzed was 1 mm^2 . Automated Mask Alignment System from EVG model EVG620 was employed to perform the thiol-ene coupling. The fluorescence signal of the spots was detected and quantified by a homemade surface fluorescence reader equipped with a high sensitive charge couple device camera Retiga EXi from Qimaging Inc, (Burnaby, Canada) and light emitting diodes Toshiba TLOH157P as the fluorescence excitation light source (SFR).^[1] For standard microarray image analysis, GenePix Pro 4.0 software from Molecular Devices, Inc. (Sunnyvale, CA, USA) was employed.

3. Probes and target oligonucleotides

Table S1. Nucleotide Sequence of Probes and Targets Used.

Name	sequence (5' to 3')	5' end	3' end
Probe A	(T) ₁₅ -CCCGATTGACCAGCTAGCATT	biotin	none
Probe B	(T) ₁₅ -CCCGATTGACCTGCTAGCATT	biotin	none
Probe C	(T) ₁₅ -CCCGATTGATTAGCTAGCATT	biotin	none
Probe D	(T) ₁₅ -CCATATTGACCAGCTATCATT	biotin	none
Probe E	(T) ₁₅ -ATTGCTAGCTAATCAATCGGG	biotin	none
Probe F	(T) ₁₅ -CCCGATTGATTAGCTAGCATT	biotin	Cy5
Target A	AATGCTAGCTGGTCAATCGGG	Cy5	none

4. Experimental procedures

4.1. Thiol-functionalized Si/SiO₂ slides

Si/SiO₂ wafers were cut into pieces of 2×1 cm² and cleaned with piranha solution (H₂SO₄:H₂O₂ 3:1 v/v) for 1 hour at 50 °C to remove organic contaminants. *Caution: Piranha solutions react violently with organic materials and should be handled with extreme care.* The chip was washed with deionized water and air dried. For achieving the surface functionalization, the chip was immersed under an argon atmosphere into a solution of 3-mercaptopropyl trimethoxysilane 1% in toluene for 2 hours at room temperature. Then the chip was washed with CH₂Cl₂ and air dried.

4.2. Synthesis of biotin probes

Detailed synthesis procedures and chemical characterization of D-biotin allyl ester (**1**), D(+)-biotin-3,6,9,12-tetraoxapentadec-14-enyl ester (**2**), D(+)-biotin-6,9,12,-trioxapentadec-14-enyl ester (**3**) and D(+)-biotin-3,6,9-trioxadecyl ester (**4**) have been described elsewhere (Chart 1).^[3]

4.3. Biotin-functionalized Si/SiO₂ slides

Biotin probes dissolved in DMSO were drop cast with a noncontact dispenser in a 90% humidity environment (50 nL/drop) or spread under a coverslip onto thiol-functionalized slides and subsequently exposed to UV-light at 365 nm (6 mV/cm²) for 10 min using a photolithography apparatus (Mask Alignment System from EVG model EVG620) to induce the photoreaction and immobilization. After exposure to UV light, slides were washed with PBS-T (3×10 min), deionized water and finally air dried.

4.4. Biotin microarrays analysis

Biotin probes were serially diluted (concentrations ranging from 0.05 to 5 μM) in DMSO. Solutions were immediately microarrayed on the thiol-functionalized slides as 40 nL drops with a noncontact dispenser in a 90% humidity environment and subsequently exposed to UV-light at 365 nm for 10 min through a photolithography apparatus. After exposure to UV light and washing, a solution of BSA 1% in PBS 1 \times was deposited on the sensing surface of the chip for 30 min to block against unspecific protein adsorption. After washing with PBS-T (3 \times 10 min), deionized water and air drying, immobilized biotin was allowed to bind ATTO-conjugated streptavidin (50 ppm) by dispensing 50 μL on each slide, spread out under a coverslip. Finally, slides were washed with PBS-T (3 \times 10 min), deionized water and finally air dried. The fluorescence was measured with the SFR and microarray images were analyzed with the GenePixPro 4.0 software.

4.5. Oligonucleotide immobilization

In order to demonstrate the viability of the developed chemical modifications for the covalent attachment of oligonucleotide probes on biotin-functionalized surfaces, silicon-oxide slides were treated following the above-described procedure to obtain the corresponding biotin-functionalized slides. To perform this study, oligonucleotide probe F (Table 1), consisting of 5' biotin- 3'-Cy5 oligomer of sequence (T)₁₅-(CCC GAT TGA TTA GCT AGC ATT) was used. For that, probe F solutions (1 and 10 μM) containing streptavidin (ranging from 0.3 to 1 molar ratio) were prepared by employing different solvents: SSC (1 \times , pH = 7), CB (1 \times , pH = 9.6) and PBS (1 \times , pH = 11). Afterward, 40 nL of each solution were printed onto the biotin-functionalized surface and incubated in a wet, dark chamber overnight at room temperature. Finally, slides were thoroughly rinsed with PBS-T and water and air dried. Immobilization results were read using a homemade SFR detector. Measurements were made by accumulation of emitted light by the samples during 30 seconds with a device gain of 10.

4.6. Hybridization assays

To study hybridization kinetics, silicon-oxide slides were functionalized with 3-mercaptopropyl triethoxysilane and subsequently with biotin compound **2** as described above. Afterwards, mixtures containing streptavidin at different concentrations (10, 5 and 1 μM) and oligonucleotide 5' biotin-labeled (probe A) diluted in 1:1, 1:2 and 1:3 (streptavidin: probe ratio) in carbonate buffer (pH 9.6) were spotted (40 nL) onto the functionalized slides creating the microarrays. Then slides were stored in a dark chamber at room temperature overnight. The slides were washed with PBS-T (15 min) and water (5 min) and air-dried. After drying, 50 μL of BSA 1% in PBS 1 \times was spread under a coverslip for 30 min to block the remaining active

sites. Then 50 μL of the complementary oligonucleotide solution 5' Cy5-labeled (target A) at different concentrations in SSC 1 \times was dispensed on each slide, spread out under a coverslip and incubated for performing the hybridization. The temperature of hybridization was 37 $^{\circ}\text{C}$, incubating in a dark and humidified chamber for 1 h. After rinsing with PBS-T (15 min) and water (5 min) and drying, the fluorescence intensity of the spots was registered and quantified. Measurements were made by accumulation of emitted light by the samples during 30 seconds with a device gain of 10.

4.7. Oligonucleotide density estimation

The oligonucleotide immobilization density was estimated by fluorescence detection using the probe tracer (probe A). Hybridized target density was calculated through fluorescence signal of target after hybridization with probe A immobilized on functionalized surface at different densities. Calibration curves were made using Cy5-labeled oligonucleotide (probe tracer and target), detecting the fluorescence of 40 nL solutions dispensed on activated surfaces at concentrations ranging from 1 to 100 nM. The fluorescence signal of the spots was detected and quantified by SFR. Measurements were made by accumulation of emitted light by the samples during 30 seconds with a device gain of 10. The amount of immobilized and hybridized DNA was obtained from the respective calibration curves. The yield of DNA hybridization was calculated as the percentage between target and probe density.

4.8. Detection of mismatches

To investigate the sensitivity of the system, five oligonucleotides, *viz.*, Biotin-(T)₁₅-CCC GAT TGA CCA GCT AGC ATT) (probe A), Biotin-(T)₁₅- CCC GAT TGA CCT GCT AGC ATT) (probe B), Biotin-(T)₁₅- CCC GAT TGA TTA GCT AGC ATT) (probe C), Biotin-(T)₁₅- CCA TAT TGA CCA GCT ATC ATT) (probe D) and Biotin-(T)₁₅-ATT GCT AGC TAA TCA ATC GGG) (probe E) having zero, one, two and three base mismatches, and a noncomplementary sequence, respectively, were spotted (40 nL/spot) with a noncontact dispenser in a 90% humidity environment at 5 μM onto an biotin-functionalized slide followed by blocking and usual washings. Subsequently, the microslides were subjected to hybridization experiment with a labeled oligomer (target A), Cy5-(AAT GCT AGC TGG TCA ATC GGG), and washed as mentioned above. After drying, the fluorescence signal of the spots was detected and quantified by SFR.

5. Figures.

Figure S1. Results obtained after spotting (40 μL) compound **2** in DMSO on thiol-functionalized Si/SiO₂ slides, irradiation and ATTO-streptavidin incubation.

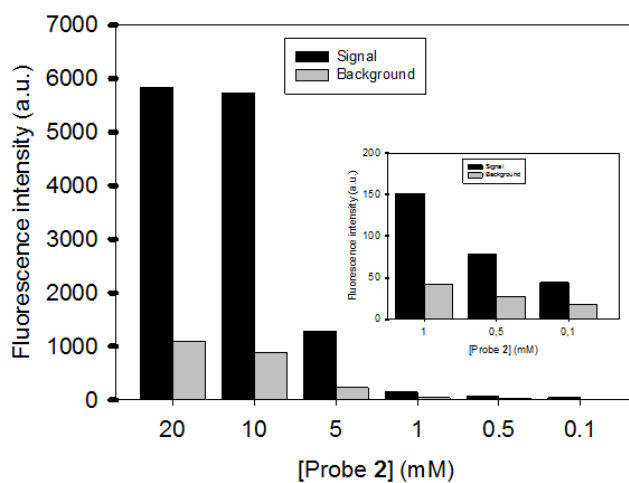


Figure S2. Influence of pH on the immobilization of ATTO-streptavidin.

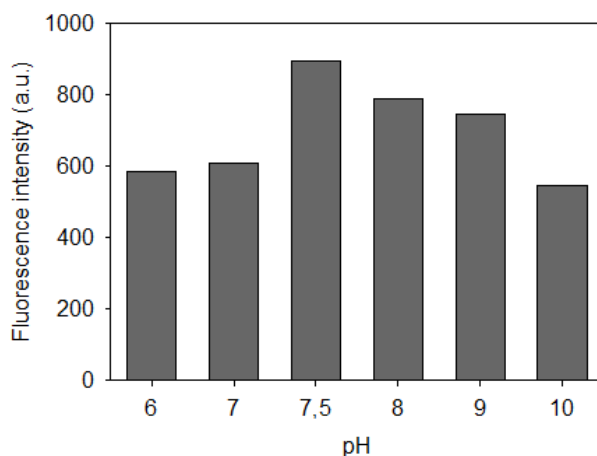
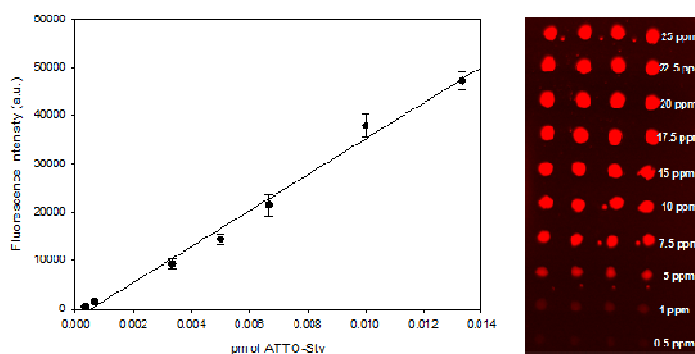


Figure S3. Standard calibration curve for ATTO-streptavidin.



$$\text{Coverage of immobilized protein} = \frac{\text{amount of immobilized ATTO - Stv (pmol)}}{\text{area of the spot (cm}^2\text{)}}$$

Where, diameter of the spot = 220 μm , spotting volume = 40 nL, and the amount of immobilized ATTO-Stv was obtained from the calibration curve.

Figure S4A. XPS spectra of the raw silicon surface (black), thiolated surface (red) and biotin-functionalized surface (blue).

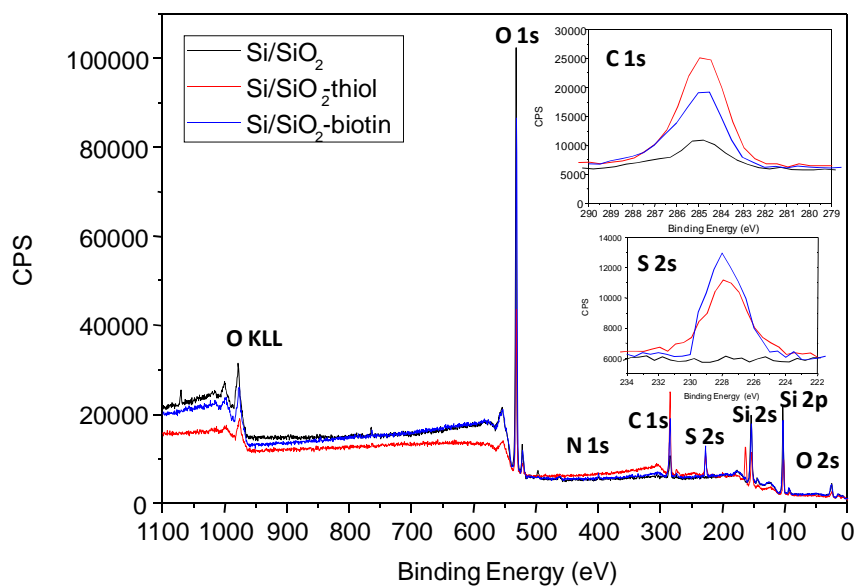


Figure S4B. C1s XPS spectra of thiol- and biotin-functionalized slides.

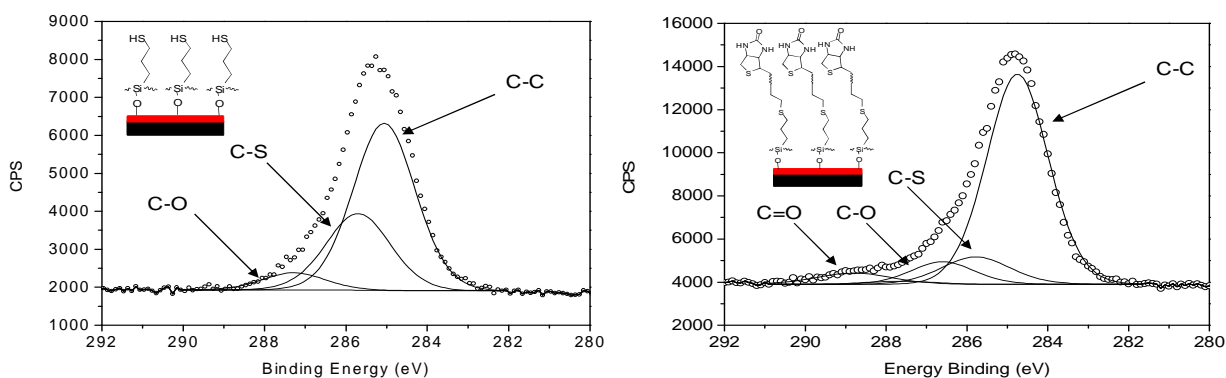


Figure S5. Fluorescence intensities for different streptavidin:probe F molar ratios.

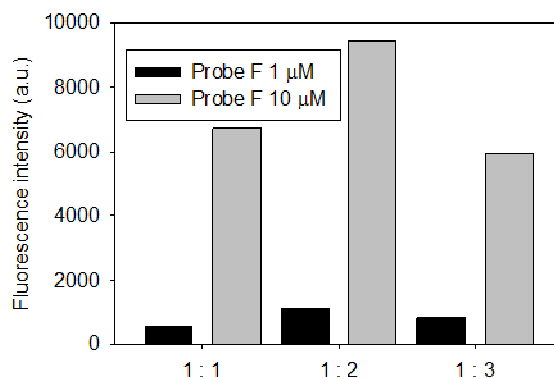


Figure S6. Fluorescence emission intensities for 1 μM and 10 μM of oligonucleotide probe F immobilized using different buffer conditions.

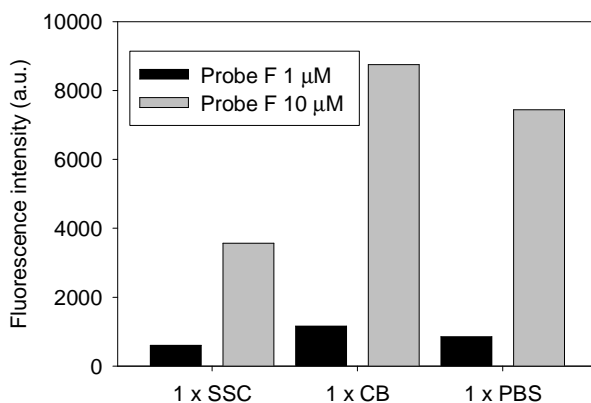


Figure S7. Standard calibration curve for immobilization of probe F.

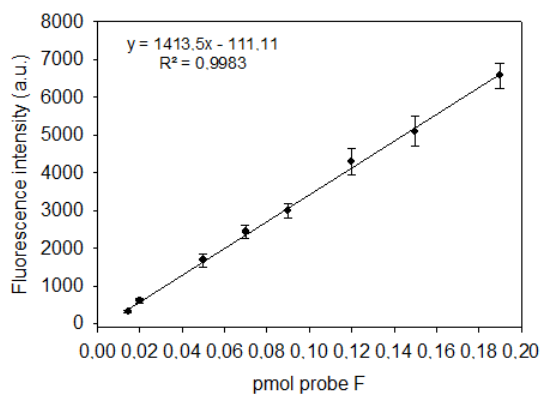
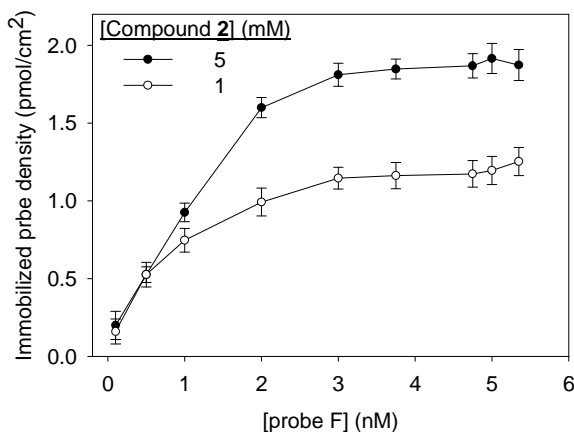


Figure S8. Oligonucleotide immobilization density for probe F.



$$\text{Immobilized probe density} = \frac{\text{amount of immobilized probe F (pmol)}}{\text{area of the spot (cm}^2\text{)}}$$

Immobilization efficiency (%) was calculated from the immobilized probe density in comparison with the one calculated for close-packed ssDNA strands.³

Table S2. Comparison of immobilization and hybridization yields on SiO₂ (*) and on other surfaces.

Surface	Immobilized probe (pmol/cm ²)	Hybridization yield	ref
(*) SiO ₂	2	46-93%	---
SiO ₂	2	76%	4
Si	5		5
Glass	2	40%	6
PMMA	4	10%	7
Gold	3	50	8
PC	5	47%	9

Figure S9. Hybridization results of target A at different Stv:probe concentrations.

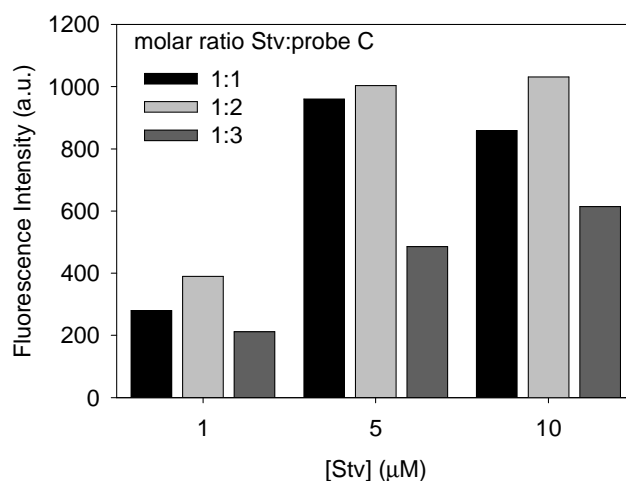


Figure S10. Standard calibration curve for hybridization of target A.

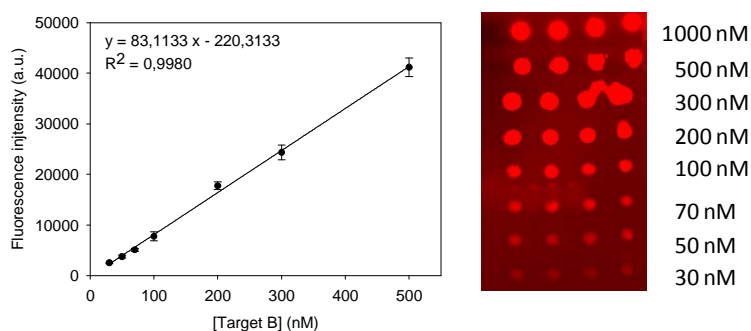


Figure S11. Hybridized target density calculated from fluorescence intensity values of Cy5 labeled target obtained at different probe and target concentrations.

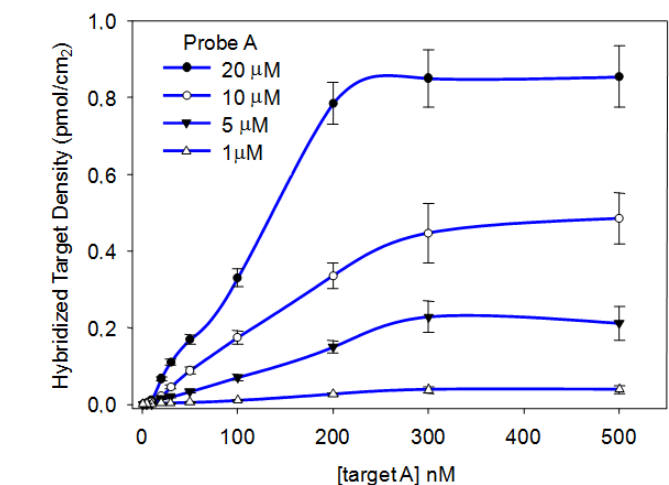


Figure S12. Effect of buffer ionic strength (SSC) in the detection of SNPs: (left) optical density image of DNA hybridization assays; (right) match/mismatch discrimination ratio.

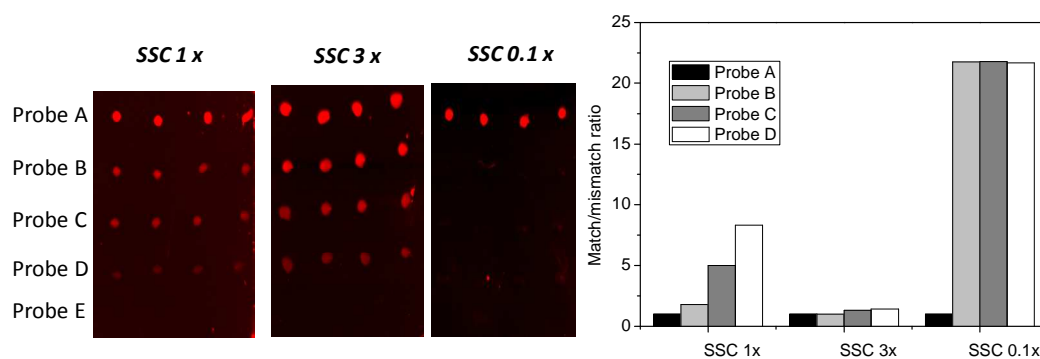
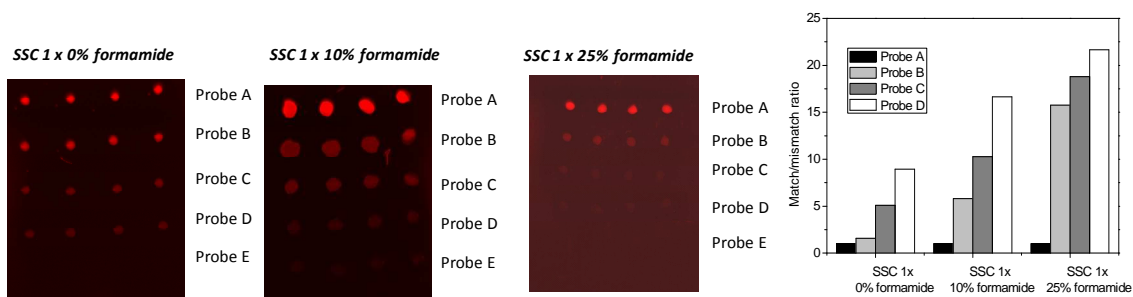


Figure S13. Effect of formamide in the detection of SNPs: (left) optical density image of DNA hybridization assays; (right) match/mismatch discrimination ratio.



6. References

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