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Supporting Material

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Supplementary Information : Salt concentration effects on equilibrium melting curves from DNA microarrays

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Materials and Methods

The materials and methods used for this study are described in details in this Supplementary Information file.

DNA sequences and chip preparation

The probes of 16 bases correspond to the SNP G/A A870G of codon 242 on exon 4 of the cyclin D1 gene. The analyzed mutation is placed in the middle of the probes to decrease the affinity for mismatched duplexes to a greater extent. A second mutation is placed adjacent to the analyzed SNP to enhance mutation detection (1, 2). The probe denoted PC is used as a positive control for DNA hybridization on-chip. Oligonucleotide probes with a pyrrole moiety are synthesized on an EXPEDITE^{TM} - nucleic acid synthesis system (Applied Biosystems, Foster City, CA, USA) using standard phosphoramidite chemistry. Thiols modified probes and oligonucleotide targets are purchased from Eurogentech (Angers, France). The DNA chips are prepared on a gold coated glass prism for SPR applications purchased from GenOptics (Orsay, France).

For immobilization purposes with electro-polymerization, probes bear a 10 thymidine spacer chain and a pyrrole moiety on their 5' end. The prism is rendered hydrophobic by submerging in 2.5mM 1-dodecanethiol ($C_{12}H_{26}S$) in ethanol 96.2°. The excess of thiols is eliminated by rinsing with

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ethanol. The oligonucleotides are immobilized spot by spot with electro-polymerization of poly-pyrrole (3–5). Pyrrole-modified oligonucleotides and pyrrole monomers are mixed at a 1/2000 ratio in the solution containing 0.5% glycerol, 20% DMSO, 30% acetonitrile, 0.15M $LiClO_4$ and ultrapure water. Spotting is carried out in a modified micropipette tip in which a platinum counter electrode is inserted. The tip can be precisely placed on the prism by a home-made pipette guiding device. A 2V impulsion for 100ms between the platinum wire in solution and the gold surface leads to poly-pyrrole formation and, thereby, located oligonucleotide immobilization. *The grafting process is carried out using a difference of potential of 2 V between the counter electrode (tip) and the gold surface in presence of pyrrole monomers. Using a more classical 3-electrode system including a reference electrode, the pyrrole monomer oxidization occurs around 0.7V vs SCE. At this potential, the oxidization of DNA bases cannot occur.* The tip is rinsed between the polymerization of different spots. The poly-pyrrole spots have a diameter of about 400μm and a thickness of about 5nm. Since poly-pyrrole is absorbing, spots are easily located by SPRi. The probe density obtained is estimated to be about $2 - 10\text{pmol.cm}^{-2}$ (4).

The procedure for thiols self-assembly was adapted from (6). The gold coated prisms are cleaned in piranha solution for $5 - 10$min, then, spotted with 10 and 50μM solutions of thiolated oligonucleotides in K_2HPO_4 at 1M using an OmniGrid automated spotter (GenOptics, Orsay, France) with X-tend microarray pins and left for 90min at 85% humidity and another 90min under the extraction hood. After incubation, the prisms are rinsed with deionized water and dried under argon or nitrogen stream then immersed for 90min in a 1mM solution of 6-mercapto-1-hexanol (MCH) which serves as blocking thiol, replacing oligonucleotides that are not adsorbed by its thiol moiety and filling in imperfections of the layer (7). Since only parts of the surface are functionalized by oligonucleotides, MCH also serves to block the remaining gold surface thereby rendering it insensitive to non specific DNA adsorption.

Temperature scan experiments on thiol microarrays by SPRi showed a temperature stability of the hybridization signal of up to $75°C$*, when temperature scans of* $2°C$*/min are applied and the end temperature is maintained for 5min. This thermal stability is in agreement with findings by R. Meunier-Prest and collaborators (8). However, upon the first heating above* $50°C$*, every chip showed a signal loss of* $5 - 10\%$ *of the initial hybridization signal. Furthermore, when temperature scans are limited to* $75°C$*, no important signal variation is detected over at least 18 heating cycles at* $2°C$*/min (9). This proves the possibility to employ thiol functionalized gold surfaces for SPRi*

studies using temperature scans.

For both chemistries, the amount of probes is very small compared to the targets in solution, thus, we can assume that the target DNA concentration is constant throughout the experiments. Spots bearing different DNA probes are grafted in multiples in order to control the reproducibility of the signal obtained by SPRi. On each prism, additionally to probes used in the analysis, a positive control sequence (PC) and a negative control (polypyrrole spots without DNA or MCH covered gold for thiol chips). The chips are stocked desiccated under argon atmosphere at 4°C between experiments.

SPR imaging setup with temperature control

Interactions on the DNA spots coated on the gold surface of the prism can be detected using surface plasmon resonance. In short, surface plasmon excitation occurs at a metal/dielectric interface when the thin metal layer is illuminated by transverse magnetic (TM) polarized light through a glass prism under an angle of total internal reflection. When the resonance angle is reached the evanescent wave created by the reflected light couples to oscillation modes of the free electron gas in the gold layer, thus leading to absorption and decreasing the reflected intensity. Since the resonance angle depends strongly on the local refractive index of the dielectric medium in a range of about 100nm from the gold surface, biological interactions on the spots result in a small angle shift of the coupling angle. In surface plasmon resonance imaging, detection is carried out with a CCD camera (Pixelfly VGA, PCO.Company, Kelheim, Germany) under fixed angle. Thus, the shift of the absorption angle leads to a change of intensity of the reflected light (reflectivity). In this way, local adsorption can be followed in real time by image analysis (LabView software from GenOptics, Orsay, France) without any need for target labelling.

All experiments are carried out on a home-made SPRi apparatus coupled to a temperature regulation system as described in (10). Surface plasmons are excited by a light emitting diode (LED) at $\lambda = 635$nm. The angle of incidence is chosen at the maximum slope of the reflected intensity versus angle in order to obtain maximal sensitivity for interactions on the chip. Hybridization reactions on the prism take place in a home-made heated flow cell of 4μl. Regulation is achieved by the ITC temperature controller (OXFORD instruments) controlling the temperature by a negative temperature coefficient resistor and heating via an electric resistance. Linear temperature scans are possible from $20 - 85$°C with a precision of 0.05°C using a home-made LabView interface (National Instruments, USA).

Buffer is injected using an automatic syringe pump (Cavro XLP 6000, TECAN, Cavro scientific instruments, Sunnyvale, CA, USA) and targets are injected using a 6 way injection valve. Careful degassing of the buffer to avoid bubble formation is ensured using an Alltech Elite Degassing System (Deerfield, Illinois, USA). The whole SPRi apparatus is placed in a temperature regulated incubator (France Etuve, Chelles, France).

DNA hybridization and melting curves

Experiments on salinity effects on DNA hybridization are carried out in buffer solutions based on phosphate buffered saline (PBS, 10mM sodium phosphate buffer, 2.7mM potassium chloride and 137mM sodium chloride, pH 7.4, at 25°C) prepared with ultrapure water. The salt concentration is adjusted with NaCl giving final concentrations of monovalent cations from 157 to 620mM (the sodium phosphate buffer is contributing to $[Na^+] \simeq 1.77[PO_4] \simeq 17$mM at $pH = 7.4$ and the potassium chloride to $[K^+] \simeq 3$mM). The buffer is filtered at 0.2μm before use. All reagents were purchased from Sigma.

The flow rate was set to 50μl/min and DNA hybridizations are carried out for 10min at 25°C with a target concentration of 250nM. During hybridization, the absence of non specific signal on negative and positive control spots is controlled. When the chip is not regenerated with a temperature scan, regeneration is done by injection of 10mM sodium hydroxide (NaOH) for one minute. Linear temperature scans are performed at 2°C/min while keeping the DNA concentration constant in the reaction cell (equilibrium DNA melting). The targets solution is maintained in the hybridization chamber while pushing and aspiring alternatively to keep the liquid agitated.

It has to be clearly noted that heating up to 85°C induces large refractive index changes in the buffer leading to reflectivity changes $\Delta R \sim 10 - 14\%$. Most of the reflectivity change is due to the temperature dependence of the dielectric constant of water. It is so large that it is necessary to acquire and subtract a reference curve for each spot in absence of target DNA, while keeping the same buffer, heating and flow conditions in order to obtain the change due to DNA hybridization. The reflectivity remaining in the linear regime, the resulting reflectivity difference is proportional to the fraction of DNA targets hybridized on the chip. Thus, melting curves can be obtained by subtraction of the reference scan without targets from the scan with targets. Negative control spots allow us to obtain a proper base line.

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