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# In Situ Surface Plasmon Resonance Imaging Detection of DNA Hybridization to Oligonucleotide Arrays on Gold Surfaces

Andrew J. Thiel, Anthony G. Frutos, Claire E. Jordan, Robert M. Corn, and Lloyd M. Smith*

*Department of Chemistry, University of Wisconsin—Madison, 1101 University Avenue, Madison, Wisconsin 53706*

**A new method for constructing oligonucleotide arrays on gold surfaces has been developed, and these arrays have been used in DNA hybridization experiments with in situ surface plasmon resonance (SPR) imaging detection. The detection technique was able to differentiate between single- and double-stranded DNA regions on the gold surface. The hybridization of both oligonucleotides and PCR-amplified DNA fragments was detectable, with the latter exhibiting slower hybridization kinetics. Temperature control of the in situ SPR cell was used to discriminate between perfectly matched duplexes and single-base-mismatched duplexes. The SPR detection technique requires no label on the DNA, but fluorescently labeled targets were also tested and detected by fluorescence imaging as an independent verification of the hybridization behavior of these DNA arrays. The in situ SPR imaging method for detection of DNA hybridization is expected to complement other existing methods for study of DNA interactions and might find future uses in mutation screening assays and DNA resequencing.**

DNA hybridization is the noncovalent association of two single-stranded (ss) DNA molecules that have complementary nucleotide sequences to form a double-stranded (ds) complex. DNA hybridization is useful as an analytical technique when the nucleotide sequence of one of the members is known; then detection of hybridization allows one to infer the presence of a complementary sequence in a sample of unknown DNA. A common format for the detection of hybridization is to immobilize one strand of DNA on a solid support and then to expose the surface to a solution of the other strand. Often the solution-phase DNA strand is coupled to a label, and the accumulation of the label at the surface is evidence of hybridization. Common labels are radioisotopes, fluorophores, enzyme substrates, or haptens for antibody binding. The most familiar examples of labeled DNA hybridization to surfaces are[1−4] filter hybridization ("dot blots"), gel transfer to membranes ("Southern blotting" and "Northern blotting"), plaque and colony screening, and "sandwich" hybridization in multiwell plates. Recent attempts to improve upon hybridization detection methods have used different substrates or unusual labels, for example, glass microscope slides,[5] light-addressable potentiometric sensing (LAPS),[6] and electrochemiluminescence.[7,8] However the hybridization is implemented, all the methods mentioned above require washing before detection to remove excess unbound label from the surface. Other variations of the labeled DNA hybridization methods allow in situ detection without washing, because they only sense a thin detection region adjacent to the surface. Examples are scanning confocal fluorescence microscopy,[9−11] evanescent wave fluorescence,[12,13] and evanescent wave light scattering.[14,15] In cases where it is undesirable to covalently attach a label to the DNA, it is sometimes possible to use a separate solution-phase label that preferentially binds to dsDNA or has different properties when it is associated with ssDNA versus dsDNA. Examples include the fluorescent intercalating dye ethidium bromide[16] and various redox-active intercalating labels used for detection of DNA hybridization at electrode surfaces.[17−20] In contrast with the large variety of labeled methods, there are relatively few surface-based methods that allow the detection of DNA hybridization without any labels. Changes in mass at the surface when unlabeled DNA hybridizes have been detected using the quartz crystal microbalance (QCM) as a sensor;[21−27] changes

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in surface optical properties when unlabeled DNA hybridizes have been observed using pseudo-Brewster angle reflectometry,[28] the grating coupler,[29,30] the resonant mirror,[31] and surface plasmon resonance (SPR), as discussed below.

The ideal DNA hybridization sensor would be able to detect unlabeled DNA. The avoidance of labeling affords some improvement in versatility and ease of sample preparation and also avoids any possible perturbation by the label of the normal DNA interaction. However, the unlabeled methods are often less sensitive than the labeled ones, so the ideal DNA hybridization sensor would also need to be very sensitive to small amounts of unlabeled DNA. The method must be capable of controlling the stringency of the hybridization, so that perfectly matched duplexes (in which both strands of DNA are perfectly complementary) can be distinguished from mismatched ones (in which the sequences might be almost complementary, but not completely, as in the case of a single-base substitution). An ideal sensor would also be capable of in situ monitoring of hybridization and denaturation as they happen. This is desirable because, in washing techniques, it is often difficult to determine how much washing is necessary to achieve the desired mismatch discrimination. Underwashing can leave a large background of signal on the surface at sites besides the perfectly matched ones, while overwashing can diminish the signal to a point where it is undetectable. Furthermore, the in situ techniques are desirable for thermodynamic studies of DNA hybridization, because they are able to monitor hybridization kinetics and can detect the DNA under equilibrium conditions, in which the dsDNA bound to the surface is in equilibrium with ssDNA in solution above the surface. The ideal hybridization detector would be able to simultaneously monitor many interactions at different locations on the sensor surface. Finally, the ideal hybridization sensor would be reusable, simply constructed, low cost, and easy to operate.

Recently, surface plasmon resonance has begun to be explored as a means to detect DNA hybridization. The SPR technique combines many of the desirable characteristics listed above for a DNA hybridization sensor. A surface plasmon is an electromagnetic wave that propagates along the interface between a metal and a dielectric.[32−37] When a suitable interface is illuminated by p-polarized light under conditions of total internal reflection, at a particular angle of illumination called the surface plasmon angle, the light illuminating the interface can be coupled into surface plasmon modes, causing an observable decrease in reflectivity from the interface at that angle. The exact angle at which surface plasmon resonance occurs is sensitive to the refractive index of the medium immediately adjacent to the metal surface, because the light being reflected at the interface creates an evanescent wave that penetrates a short distance into the dielectric medium. Since refractive index is an intrinsic property of all materials, any analyte that occupies the region of space adjacent to the surface into which the evanescent wave penetrates can be detected, without labeling. Materials with different refractive indices will cause different SPR responses. For a given type of material, the amount present at the surface will also change the SPR response. The SPR technique can be used in situ, since the evanescent wave only senses the environment immediately adjacent to the surface. Thus it is not necessary to wash away the excess analyte solution in order to observe the fraction interacting with the surface. Since the reflected light can be continuously monitored, the technique can observe changes occurring at the surface in real time. While the absolute sensitivity of the SPR technique depends on a number of factors including the refractive index of the analyte and its size, SPR has been shown to have adequate sensitivity to detect small amounts of unlabeled biomolecules. Examples include the SPR detection of submonolayer coverages of avidin on a biotinylated surface,[38] and a study with various radiolabeled proteins that established a SPR detection limit of as little as 50 pg $mm^{-2}$ protein.[39] SPR instruments can be constructed with a variety of optical configurations. For example, when collimated light illuminates the surface, the angle of incidence can be scanned by rotating the sample with respect to the incident beam, and the reflected beam intensity can be monitored with a simple photodiode. Alternatively, the surface can be illuminated with a converging beam including a spread of incident angles, then a linear array detector placed in the diverging reflected beam allows simultaneous monitoring of the entire range of angles. A commercial instrument based on this SPR configuration, called the BIAcore[40−42] (Pharmacia Biosensor, Piscataway, NJ), has been available since late 1990. An alternative configuration, called SPR imaging (or sometimes SPR microscopy) uses collimated illumination of the entire surface, with the reflected beam imaged onto a two-dimensional array detector.[43,44] In SPR imaging, the sample is illuminated at a fixed angle, and spatially localized variations in material at the metal surface cause local variations in reflected intensity, which can be seen as brighter or darker regions in the SPR image. Spatial resolution of features as small as 5 $\mu$m has been reported.[45] To date, most SPR biosensing publications have used the BIAcore instrument, while few have used SPR imaging. SPR imaging would appear to be a particularly attractive format for detection of DNA hybridization, because it would enable


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Table 1. Sequences of Oligonucleotides Used for Experiments Described in This Paper and Arbitrarily Assigned Code Names[a]

| code name | oligonucleotide sequence |
|---|---|
| probe **I** | 5′-amine- TTT TTT TTT TTT TTT CGT CCT CTT CAA GAA 3′ |
| probe **II** | 5′-amine- TTT TTT TTT TTT TTT CTG CTA CAC GTA CAT 3′ |
| probe **III** | 5′-amine- TTT TTT TTT TTT TTT CGT CTT CTT CAA GAA 3′ |
| target **IV** | 3′ GCA GGA GAA GTT CTT GTA TTG GCC CTT AGG 5′ |
| target **V** | 3′ GCA GGA GAA GTT CTT -fluorescein 5′ |
| target **VI** | 3′ GCA GGA GAA GTT CTT CAA ATA GGT CTT CGG TTA CGT GGG TAA CCT -fluorescein 5′ |

[a] The term "probe" is used to denote oligonucleotides that are immobilized on the gold surface, while "target" is used to describe DNA that is hybridized to the surface from solution. All three probes share a common $(T)_{15}$ spacer at the 5′ end, which is used to separate the hybridization sequence from the surface. All three targets have the same 15 bases at their 3′ ends, and it is these bases that are involved in hybridization. The remaining 5′ bases of the targets are just extra "overhang". Probe **I** is a perfect match to all the targets shown; probe **III** is a single-base mismatch. Probe **II** is of the same length and base composition as probe **I**, but the sequence has been scrambled so as to have essentially no complementarity to the target sequences.

simultaneous real time in situ analysis of many DNA hybridizations in parallel on a "DNA chip" without the use of labels. Together with recent developments in oligonucleotide array fabrication technologies, SPR imaging might provide a means to perform large-scale hybridization experiments on unlabeled DNA samples.

Early publications involving SPR biosensing primarily described antibody/antigen, protein/ligand, and protein/protein interactions.[46−56] More recently, the technique has been used to study DNA/protein interactions, for example DNA/repressor or DNA/promoter binding studies[57−59] and DNA/single-stranded DNA binding protein (SSB) studies.[60] Finally, a few publications involving SPR detection of DNA hybridization have appeared.[61−70] Of these, only one[65] reports detection of DNA hybridization using the SPR imaging configuration. This report expands upon the previously reported methods for SPR detection of DNA hybridization by use of a unique surface coupling chemistry, employed in combination with in situ imaging detection and temperature control. The hybridization of unlabeled DNA to an oligonucleotide array is detected using in situ imaging SPR. Important controls are included to test for nonspecific binding of target DNA to the substrate and to verify that the observed DNA/DNA interactions are sequence specific. Temperature control of the in situ cell is shown to enable single-base mismatch discrimination between closely related oligonucleotide sequences. The instrument is tested with synthetic oligonucleotide samples as well as PCR products, and slower kinetics are observed for the PCR product hybridization. Finally, the SPR detection results are shown to agree with an independent fluorescence detection technique.

## EXPERIMENTAL SECTION

**Reagents.** All oligonucleotides used in these experiments were synthesized on an ABI DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) at the University of Wisconsin Biotechnology Center. Before use, each oligonucleotide was purified by reversed-phase binary gradient elution HPLC using a Shimadzu SCL-6A HPLC system (Shimadzu Corp., Kyoto, Japan). The sequences of the oligonucleotides used and their code names are given in Table 1. ABI Aminolink II amidite was used for synthesis of 5′-amine-modified oligonucleotides, and ABI 6-FAM amidite was used for 5′-fluorescein-labeled oligonucleotides. Oligonucleotide concentrations and melting temperatures were determined using an HP8452A UV−visible diode array spectrophotometer equipped with an HP89090A peltier temperature control accessory (Hewlett-Packard Co., Palo Alto, CA). PCR products from Exon 4 of the human tyrosinase gene were prepared, purified, and strand-separated as previously described.[5] Unless otherwise specified, solutions used were prepared from standard laboratory-grade reagents. Water used in making solutions and for washing the samples was reverse-osmosis purified (18 MΩ·cm). "2×SSPE/0.2% SDS" is a slightly modified


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version of a standard hybridization buffer[71] and contains 0.3 M NaCl, 20 mM sodium phosphate, 2 mM EDTA, adjusted to pH 7.4 with NaOH, and 0.2% (w/v) (6.9 mM) sodium dodecyl sulfate (SDS). Gold substrates for SPR experiments were prepared as previously described,[72] using vapor deposition to deposit 48 nm thick Au films onto 18 × 18 mm × 0.45 mm thick SF-10 glass ($\eta$ = 1.727) cover slips (Schott Glass Technologies, Durea, PA). 11-Mercaptoundecanoic acid (MUA) was from Aldrich (Milwaukee, WI), poly(L-lysine) (PL) was from Sigma (St. Louis, MO), and 1,4-phenylenediisothiocyanate (PDITC) was from Aldrich.

**Oligonucleotide Probe Immobilization.** MUA monolayers were prepared on clean vapor-deposited gold substrates, and a layer of PL was electrostatically adsorbed onto the MUA surface.[38,73] PDITC was then reacted with the surface by immersion for 2 h in a room temperature solution of 0.2% (w/v) PDITC in 10% pyridine/90% dimethylformamide (DMF), after which the sample was washed (first DMF and then ethanol) and then dried under a stream of nitrogen, leaving an isothiocyanate-derivatized surface. Next, droplets of 5′-amine-terminated oligonucleotide (1 mM "oligo" in 0.1 M sodium bicarbonate buffer, pH 9.0, containing 0.5 M NaCl) were spotted onto the hydrophobic surface and left to react for 2 h at 37 °C in a humid environment to prevent the spots from drying out. To stop the coupling reaction, the samples were immersed for 2 min in room temperature 1% $NH_4OH$, washed two times, 5 min each wash, in room-temperature $H_2O$, and finally blown dry under a stream of nitrogen, at which point they were ready to use for hybridization. A 1.5 $\mu$L droplet of oligonucleotide probe typically resulted in a spot of ~2.0 mm diameter on the hydrophobic PDITC surface.

**SPR Detection.** A Kretschmann-configuration imaging SPR instrument was used, as previously described.[44] The sample was illuminated with p-polarized, collimated light from a 632.8 nm, 1.5 mW HeNe laser (Uniphase Model 1101P, Manteca, CA), coupled through a $MgF_2$ antireflection-coated SF-10 ($\eta$ = 1.727) 30 × 30 × 30 mm equilateral glass prism (Ealing Electro-Optics, Holliston, MA) and $\eta$ = 1.725 index-matching fluid (Cargille, Cedar Grove, NJ) to the sample. The reflected light from the surface was imaged onto a CCD camera [either a Panasonic Model WV-BL200 (Panasonic Broadcast & Television Systems Co., Secaucus, NJ) or an iSight iSC2050 (iSight, Inc., Cedar Knolls, NJ) was used, depending on when the experiment was performed], and the image was digitized by a frame grabber [Video Blaster RT300 (Creative Labs, Inc., Milpitas, CA) or Data Translation DT3155 (Data Translation, Marlboro, MA)] and AdobeCap Video Capture Utility v1.1 (Adobe Systems, San Jose, CA) or Data Translation Acquire To Host Application v1.0 software running on a Gateway 2000 4DX-33 or Dell Dimension P133v computer platform, respectively. In all cases, the image data were recorded as an 8-bit grayscale image. The image acquisition systems were tested and shown to have a linear response to incident light intensity in the range used in the experiments. For the DNA hybridization experiments, a special in situ cell was constructed, in which a Kel-F block (PCTFE, 3M Industrial Chemical Products Division, St. Paul, MN) and $^1/_2$ in. i.d. Viton fluoroelastomer O-ring (Du Pont Dow Elastomers, L.L.C., Wilmington, DE) were sealed against the gold surface, to contain a small volume of liquid in contact with the central region of the gold surface. The cell was fitted with an inlet and outlet for replacing the cell contents by means of a syringe. The total system volume (inlet to outlet) was found to be ~200 $\mu$L. The sample stage was rotatable through a large angular distance to find the optimal observation angle for a given sample but was locked at a fixed angle for the course of a hybridization experiment.

**In Situ Hybridization and Temperature Control.** In all cases except for the PCR hybridization, the hybridization solution consisted of 2.0 $\mu$M target oligonucleotide in 2×SSPE/0.2% SDS buffer. To begin hybridization, a total volume of at least 300 $\mu$L of hybridization solution was injected into the in situ SPR cell using a syringe. Hybridization proceeded at room temperature while the SPR image was continuously monitored. Following hybridization, the cell could be flushed with 2×SSPE/0.2% SDS buffer to remove any unhybridized target DNA if desired. In experiments where it was necessary to remove mismatched hybrids, the cell temperature was gradually raised using resistive thermofoil heating elements (Minco Products Inc., Minneapolis, MN) attached to the cell, with miniature platinum RTD sensors (Minco) embedded in the cell for feedback temperature control with a Love 1600 series self-tuning PID temperature controller (Love Controls, Wheeling, IL). After heating was complete, the sample was passively cooled back to room temperature.

**Fluorescence Detection.** For detection of hybridization with fluorescently labeled targets, the hybridization was performed by pipetting a small volume (~20 $\mu$L) of hybridization solution onto the gold surface and "sandwiching" the droplet between the gold surface and a plain glass cover slip, which was laid on top. The sandwich assembly was enclosed in a petri dish on a platform above several milliliters of water, in order to provide a humid atmosphere to prevent drying of the sample during the hybridization period. After hybridization, the cover slip was removed and the gold surface washed by immersion in a beaker of 2×SSPE/0.2% SDS. Finally, after washing, the sample was placed face down on top of the glass scanner tray provided with a Molecular Dynamics FluorImager 575 (Sunnyvale, CA) with a droplet of 2×SSPE/0.2% SDS between the gold surface and the glass scanner tray to provide an aqueous environment during the scan. The sample was scanned in the FluorImager using the standard Scanner Control v.1.1 software provided with the instrument, using a 530 ± 15 nm band-pass interference filter to select for the fluorescein label's fluorescence emission.

**Data Analysis.** The SPR images were imported into NIH Image v.1.61 software,[74] where they were filtered and cropped as necessary, and "Plot Profile" analysis was performed on selected regions. In a plot profile, the average pixel value for each column of pixels within a rectangular region is calculated and plotted against that column's position. Then numerical values from each plot profile were exported as a text file which was subsequently opened in Igor v.1.25 graphing software (WaveMetrics, Lake Oswego, OR), where plot profiles could be overlaid, offset, ratioed, etc. For analysis of fluorescence images, ImageQuaNT v.4.1 software (provided with the FluorImager) was used.

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(74) NIH image is a public domain computer program written by Wayne Rasband at the U.S. National Institutes of Health and available from the internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, Part No. PB93-504868.

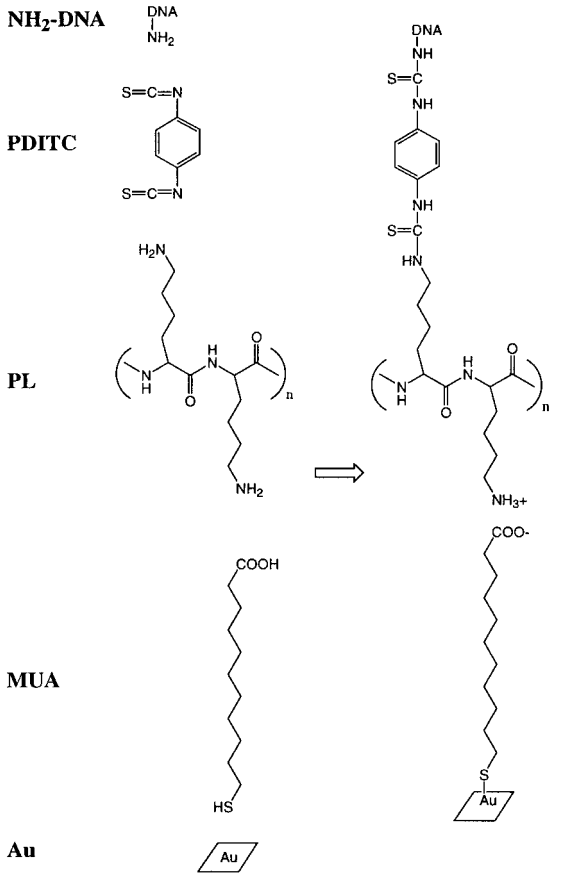


Figure 1. Schematic of method used for coupling 5′-amine-terminated oligonucleotide probes to a gold surface.

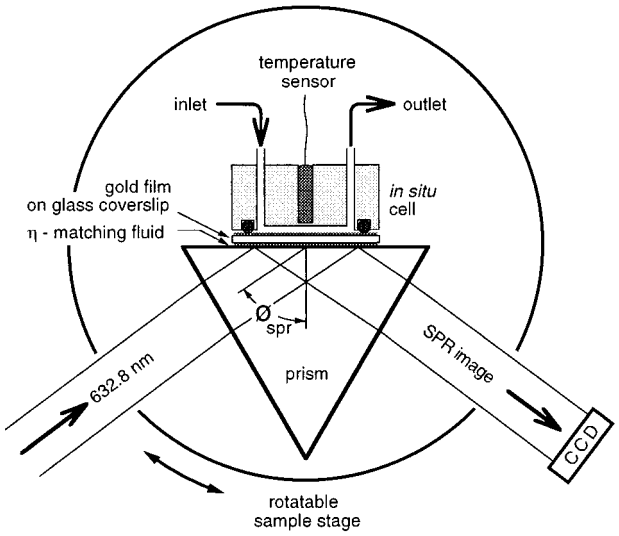


Figure 2. Diagram of temperature-controlled in situ SPR imaging apparatus. A spatially-filtered, expanded, collimated, p-polarized HeNe laser beam illuminates the gold sample through a TIR prism coupler. The gold film is in contact with a small volume of solution contained in a flow cell, which is sealed against the gold film with an O-ring. Reflected light from the gold surface, containing the SPR image, is monitored with a CCD camera. The angle of incidence ($\phi_{spr}$) can be changed by rotating the entire sample assembly. The temperature of the cell is monitored with a miniature temperature sensor. The entire assembly is clamped together against a large metal block (not shown), the temperature of which is adjusted using resistive foil heaters and passive cooling. Optics for forming the beam and for transferring the image to the CCD camera are not shown for simplicity.

## RESULTS AND DISCUSSION

SPR occurs only at specific types of metal surfaces. For this work, thin gold films were chosen as the desired surface, and so, in order to study DNA hybridization via SPR, it was necessary to devise a chemical method for constructing oligonucleotide arrays on gold supports. Several methods were tested, with an emphasis being placed on creating a surface that avoids nonspecific binding problems while providing a functional surface for hybridization. This work will be described in detail elsewhere.[75] The method that was chosen for the experiments reported here is shown in Figure 1. In brief, 5′-amine-modified oligonucleotides were coupled to an amine-derivatized gold surface via the homobifunctional cross-linker PDITC. The fabrication of MUA/PL bilayers on gold substrates has been described in detail,[38,73,76] and such surfaces are well-characterized. Starting with these amine-derivatized gold surfaces, a coupling method similar to the one described by Guo et al.,[5] in which amine-terminated oligonucleotides were immobilized on (3-aminopropyl)trimethoxysilane glass supports, could be used. The amine-terminated surface was converted to an isothiocyanate-terminated surface by reaction with PDITC, and then small droplets of 5′-amine-terminated oligonucleotides were immobilized on the PDITC surface. For the proof-of-principle experiments described here, the physical placement of the droplet on the surface dictated the position and boundaries of the oligonucleotide-derivatized region, and no effort was made to minimize the size of the oligonucleotide probe regions beyond use of a small volume in creating the droplet. Future efforts will be directed toward miniaturization of the probe regions, through use of photopatterning techniques.[44]

A temperature-controlled in situ SPR imaging apparatus was constructed for the DNA hybridization experiments, as pictured in Figure 2. The in situ region of the gold surface was a $^1/_2$ in. diameter circle, which was large enough to monitor several immobilized oligonucleotide probe spots simultaneously. The use of this instrument for studies of protein adsorption has been previously reported.[44]

As a first test of the SPR hybridization detection method, an oligonucleotide/oligonucleotide hybridization was done, as shown in Figure 3. In the experiment, four ss oligonucleotide probe spots were attached to the gold substrate as shown in Figure 3a. Probe **I** and probe **II** (see Table 1 for oligonucleotide sequences) were each applied in duplicate on opposing diagonals. After attachment of the oligonucleotide probes, the surface was placed in the in situ SPR imaging apparatus, the cell was filled with 2×SSPE/0.2% SDS buffer, and the SPR image shown in Figure 3b was acquired. The four bright spots in this image show the regions where the single-strand probe DNA is attached to the surface. Plot profiles through these spots, displayed in Figure 3d (solid lines), show the quantitative signal intensity of the spots. The contrast between the spots and the background is due to the additional material present on the surface where the DNA has been attached.

Next, unlabeled DNA Target **IV** was hybridized to the surface by injecting a solution of **IV** into the in situ SPR cell. The SPR image evolved rapidly (within 5 min of injection of the target DNA) into the image shown in Figure 3c (with plot profiles shown as dashed lines in Figure 3d). The brightening of the upper left and lower right spots (these are the probe **I** spots) in the surface plasmon image relative to their starting intensities indicates the

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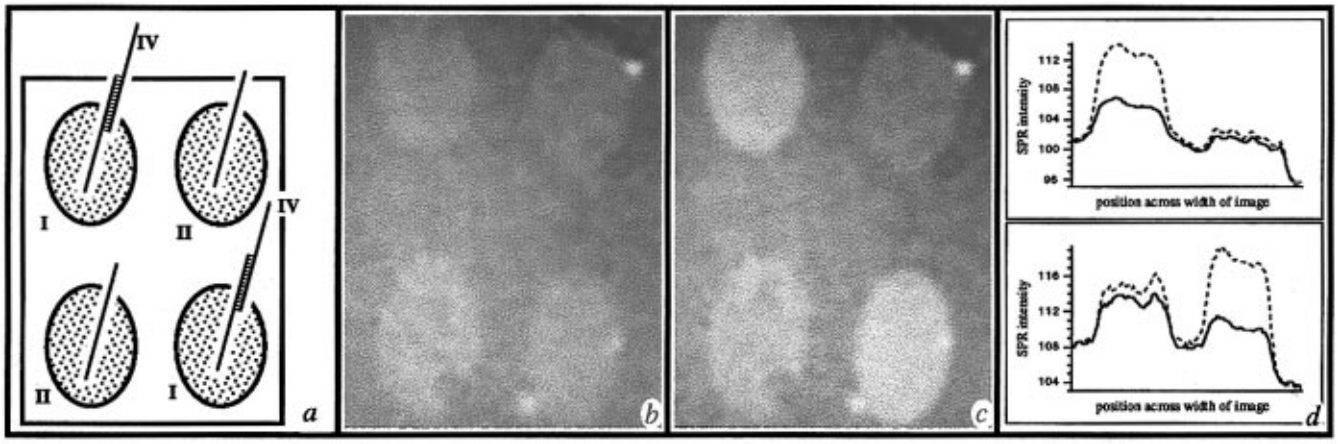


Figure 3. (a) Schematic diagram showing the locations where oligonucleotide probes **I** and **II** were immobilized on a gold surface and where target oligonucleotide **IV** should hybridize to that surface. (b) In situ SPR image of surface shown in (a) before hybridization. (c) In situ SPR image of same surface after hybridization to unlabeled DNA target **IV**. (d) Plot profiles through SPR images shown in (b) (solid lines) and (c) (dashed lines). The profiles through the top two spots of images b and c are overlaid in the upper graph; similarly, the profiles through the bottom spots are overlaid in the lower graph. In each graph, the horizontal axis represents position from left to right across the width of the SPR image and the vertical axis shows SPR response in arbitrary units of "SPR intensity". Brighter regions in the SPR image represent additional material on the surface and correspond to larger values of SPR intensity.

presence of additional DNA associated with the surface due to hybridization in these regions. The contrast between the probe **II** ("mismatch") spots and the probe **I** ("match") spots can be thought of as the difference between single-stranded DNA and double-stranded DNA on the surface, as shown schematically in Figure 3a. When the plot profiles are overlaid as shown in Figure 3d, it can be seen that the signal from the background does not change, illustrating that nonspecific attachment of target DNA to the PDITC surface is not occurring. The signal from the mismatch spots does increase slightly, indicating that there might be a small affinity of the target DNA for the mismatch spots relative to the background, but this interaction is much less than that observed for the perfectly matched spots. It is unknown why the duplicate spots did not show the same amount of adsorption; perhaps this reflects variations in the underlying probe surface density (notice that not all of the ss probe spots started out with equal intensities).

A similar experiment, using fluorescently labeled target oligonucleotide and fluorescence detection instead of SPR, was performed as an independent verification of the hybridization. A gold substrate with four oligonucleotide probe spots identical to the one used for the SPR experiment was prepared (as in Figure 3a). In this experiment, fluorescent target **V** was hybridized to the surface, and after hybridization and washing, the sample was scanned in a Molecular Dynamics FluorImager 575 to detect fluorescence on the surface. The fluorescence image, shown in Figure 4, agrees with the SPR image, showing specific hybridization to the probe **I** match spots, and no detectable nonspecific binding to other regions of the surface.

As an expanded test of the SPR DNA hybridization detection technique, hybridization of a more complex DNA target was attempted. Rather than hybridizing a synthetic oligonucleotide, a PCR product was amplified from exon 4 of the human tyrosinase gene.[5,77−79] The resulting amplicon was 347 base pairs long and contains target sequence complementary to probe **I** at the "wild-type 406" position. (See Figure 2 of ref 5 for the sequence; GenBank accession number M63238.) The amplicon was purified and strand-separated before hybridization, as previously described.[5] A gold substrate identical to that shown in Figure 3a was prepared and assembled into the in situ SPR imaging cell. The starting image shown in Figure 5a was recorded, and then the hybridization solution (48 nM single-stranded 347 nucleotide PCR product in 2×SSPE/0.2% SDS) was injected. In contrast with the oligonucleotide hybridizations, no change in the image was detectable within the first 5 min. However, after 15 h, the image shown in Figure 5b had developed. The kinetics of the hybridization are shown in Figure 5c, in which the background-corrected spot intensities from a series of intermediate images are plotted

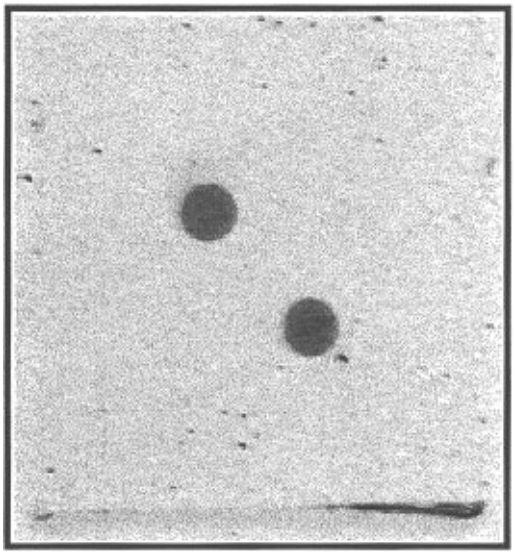

Figure 4. Fluorescence image of target **V** hybridized to a four-spot array of oligonucleotide probes **I** and **II** on a gold surface. The locations of the probe spots are identical to the sample shown in Figure 3a.

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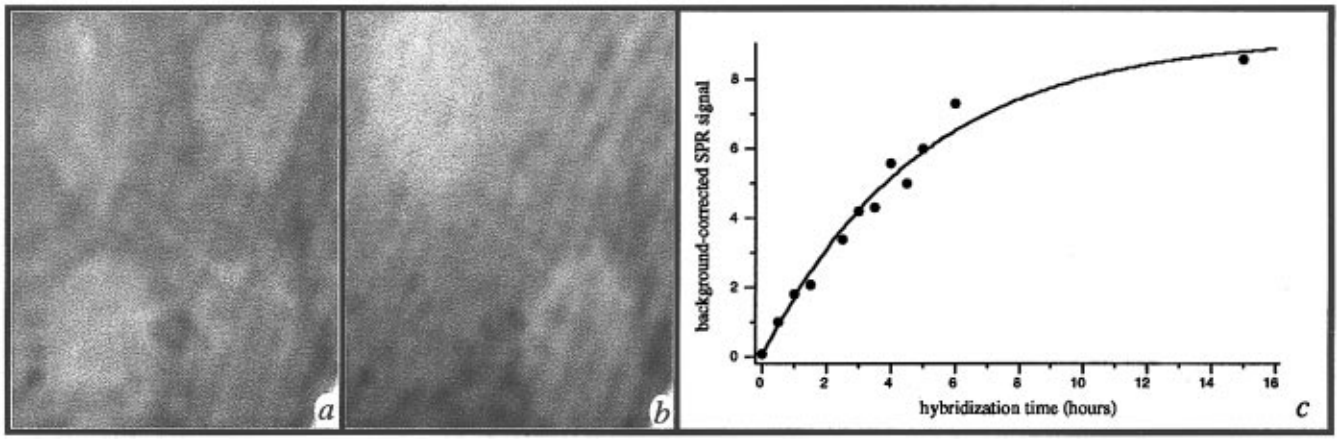


Figure 5. (a) In situ SPR image of ss oligonucleotide probe surface similar to that shown in Figure 3a, before hybridization. (b) In situ SPR image of same surface after 15 h of hybridization to a single-stranded 347 nucleotide PCR product DNA target, complementary to probe **I**. The optical interference bands apparent in the images are discussed in the text. (c) Graph showing kinetics of PCR product hybridization. SPR images were acquired at $t = 0$, 0.5, 1, 1.5, 2.5, 3, 3.5, 4, 4.5, 5, 6, and 15 h during the hybridization. For each image, the difference between the average SPR intensity of the perfect match spot and the background was calculated, and this background-corrected SPR signal was plotted vs hybridization time.

vs elapsed hybridization time. A fit of the data to an exponential curve as would be predicted for pseudo-first-order kinetics of surface hybridization using single-stranded DNA[2] gives $t_{1/2} = 3.4$ h for the hybridization. The slowness of the kinetics compared to the oligonucleotide hybridization (where $t_{1/2}$ was estimated to be < 0.1 h; data not shown) is probably due to three factors:[2−4] the lower solution concentration of target, the longer length of target, and the greater complexity of the target. Resolution of the relative contribution to kinetics from each of these factors was not attempted but would be a good subject for future study by this technique.

Figure 5 also illustrates several current experimental complications. First is the degradation of the surface over long time periods. The surface can deteriorate by loss of probe molecules over time, as well as by nonspecific binding of unwanted material to the background. Some evidence of probe loss can be seen in Figure 5b, where the mismatched probe spots appear to have decreased in intensity relative to the background, compared to their starting intensities in Figure 5a. Probe loss may also account for the apparent difference in amount of material gained by the two match spots. Various alternative coupling chemistries are being investigated in attempts to find one that is more stable over longer time periods. Additional difficulties are caused by optical interference phenomena. Figure 5 shows the presence of optical interference fringes giving the image a "corrugated" appearance. For reasons not completely understood, some cover glass−prism assemblies resulted in interference-free images such as Figure 3, while others resulted in interference patterns. Vapor deposition of the gold film directly onto the prism itself might eliminate the problem, but was not done because of the convenience of working with disposable coverglass samples. Finally, in the simple single-beam, unreferenced instrument design used here, any laser power, laser pointing, or camera sensitivity drift would be detrimental to quantitative analysis of the kinetics. Because of these complicating factors, the kinetic data reported above for the PCR hybridization should be viewed as tentative at best, serving mainly to illustrate the potential of in situ SPR imaging to evaluate kinetics with additional future refinements.

Another important aspect of the SPR DNA hybridization detector is the ability to distinguish between closely related sequences. In the experiment described in Figure 3, the mismatch probe (probe **II**) had very little complementarity to the target oligonucleotide. In essence, it was a "complete mismatch". But for most diagnostic applications, the ability to distinguish very closely related sequences such as single-base mismatches is necessary. To accomplish this, a resistive heating device with feedback control was integrated into the in situ SPR cell. The idea was to perform hybridizations at a temperature below the "selective temperature" and then to gradually raise the cell temperature until the mismatches "melt off". An experiment to test this was performed, with the results shown in Figures 6 and 7. A four-spot oligonucleotide array similar to that pictured in Figure 3a was prepared, except that in this array, probe **III** was used for the mismatch instead of **II**. Therefore, the array consisted of two oligonucleotide probes differing by only 1 base out of 15 involved in the hybridization. The spotted surface was assembled into the in situ SPR cell, and the starting image shown in Figure 6a was recorded. Plot profiles of these ssDNA spots are shown in Figure 6d. Next, target **VI** was hybridized to the array at room temperature (~23 °C), and after the hybridization, the cell was washed by flushing room-temperature 2×SSPE/0.2% SDS solution through. [Separate spectrophotometric melting experiments in 2×SSPE/0.2% SDS buffer found the melting temperature ($T_m$) for the perfectly matched **I**/**VI** pair to be 58.8 °C, while the single-base mismatched **III**/**VI** pair was found to have $T_m = 48.0$ °C (data not shown). Therefore, the hybridization and wash at room temperature were known to be well below the solution-phase $T_m$ for both the match and the single-base mismatch.] As observed in Figure 6b and corresponding plot profiles in Figure 6d, all four spots show an increase in material due to the hybridization of the target at this nonselective temperature. Then, as shown in the series of images in Figure 7, the temperature was gradually raised. This "melting movie" has two interesting features. First is that the intensities of the perfect match and mismatch spots appear to change relative to one another as the temperature is raised. This shows the "melting off" of the mismatches while the perfect matches are retained. However, the second feature is that the overall intensity of the images, including the background, darkens. This is due to the decrease in bulk refractive index of the aqueous solution as the

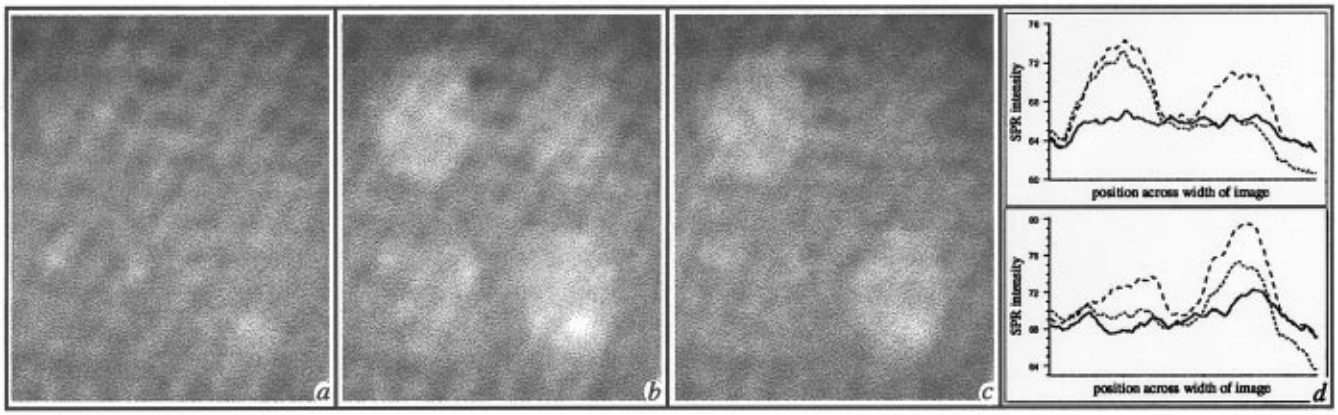


Figure 6. (a) In situ SPR image of ss oligonucleotide probe surface similar to that shown in Figure 3a, except that probe **III** has been substituted for probe **II**. The two types of probes differ by only a single-base substitution. (b) In situ SPR image of the same surface after hybridization to target **VI** at room temperature and washing to remove nonhybridized target DNA from the cell. (c) Same, after heating to 37 °C, washing, and cooling to room temperature. (d) Plot profiles through the top and bottom spots in images a (solid lines), b (longer dashed lines), and c (shorter dashed lines).

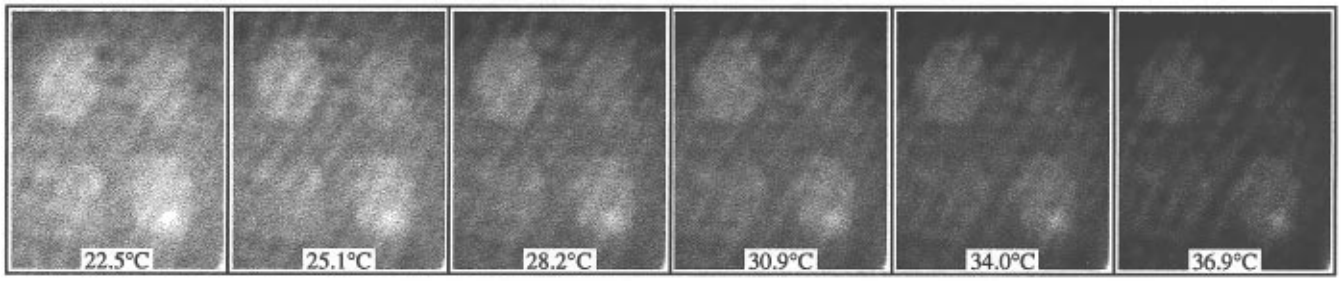


Figure 7. "Melting movie" showing changes in the in situ SPR image of the hybridized surface from Figure 6b as temperature is gradually raised. The cell temperatures at which the images were acquired are listed in the figure.

temperature increases.[80] Theoretical in situ SPR curves confirm that in an imaging SPR instrument operated at a fixed observation angle smaller than the surface plasmon angle, a decrease in bulk refractive index should cause a reduction in percent reflectivity similar to that observed in Figure 7 (data not shown). In separate experiments, when the temperature was raised even further, the image eventually went completely dark. The temperature-related variation in the image is a drawback for use of the instrument for continuous monitoring of the denaturation process. The darkening could be compensated by readjusting the stage angle, but this was not done for the experiment in Figures 6 and 7. To prove that the perceived change in mismatch spot intensities at elevated temperature was real, the cell was again washed while still at the elevated temperature (irreversibly removing any dissociated target from the cell, so that it could not rehybridize with the surface), the sample was cooled to room temperature, and the image shown in Figure 6c was recorded. Comparison of panel c of Figure 6 to panels a and b (which were all taken at room temperature) shows that the apparent mismatch discrimination at elevated temperature was in fact real. The hybridized material from the single-base-mismatched spots appears to have been completely removed by the heating and washing cycle. Note that the perfect match spots have lost some material in the heating process as well, indicating that, even at temperatures considerably below the solution $T_m$, a significant fraction of dsDNA dissociates into ssDNA and is removed from the surface in a washing procedure such as the one used here. This is consistent with known characteristics of matched and mismatched duplex dissociation kinetics.[64,81] An interesting area of further study for this technique would be to investigate the surface melting behavior of oligonucleotide hybrids, to see what differences between surface behavior and solution behavior are exhibited. In doing so, it would be important to make the distinction between true melting experiments, in which ssDNA in solution above the surface exists in equilibrium with dsDNA bound to the surface, vs "washing"-type experiments such as the one performed here, where the ssDNA in solution is irreversibly removed by washing. Washing perturbs the equilibrium, thereby enhancing the dissociation of the double-stranded hybrids, as implied in the distinction between melting temperature ($T_m$) and dissociation temperature ($T_d$).[82] Temperature-controlled in situ SPR would be capable of performing both types of experiments.

## CONCLUSIONS

Oligonucleotide arrays on gold surfaces were constructed and used for hybridization experiments in which in situ SPR imaging was used to detect the hybridization. The SPR imaging detection technique was shown to be able to distinguish between single-stranded DNA and double-stranded DNA regions on the surface, with single-base-mismatch discrimination. The detection method can be used for unlabeled DNA targets and is capable of real-time monitoring of hybridization kinetics. The hybridization behavior has been independently verified using fluorescence

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imaging. In future work, the in situ detection of DNA hybridization might be used to study the biophysics of DNA interactions, or possibly for mutation screening assays and DNA resequencing.

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