

## Direct Detection of Genomic DNA by Enzymatically Amplified SPR Imaging Measurements of RNA Microarrays

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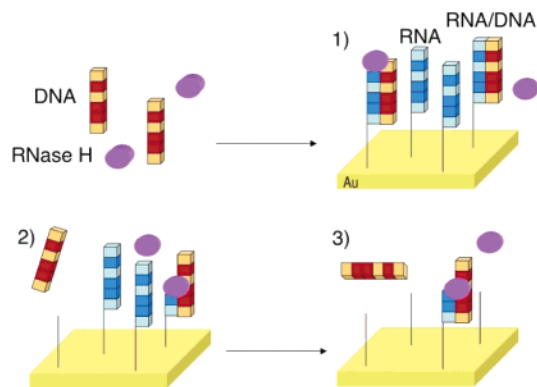
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The direct analysis of genomic DNA and RNA in an array format without labeling or PCR amplification would be extremely useful for the identification of viral<sup>1</sup> and bacterial samples,<sup>2</sup> gene expression analysis,<sup>3–5</sup> and biological warfare detection.<sup>6</sup> The label-free technique of surface plasmon resonance (SPR) imaging is in principle an excellent candidate for these direct detection measurements, but to date has not yet been extensively applied to DNA and RNA analysis due to a nanomolar detection limit;<sup>7</sup> typical genomic DNA samples are approximately 20 fM (35  $\mu\text{g}/\text{mL}$ ). In this paper, we demonstrate a novel surface enzymatic amplification process that utilizes RNase H and RNA microarrays to sufficiently enhance the sensitivity of SPR imaging for the detection of DNA oligonucleotides down to a concentration of 1 fM, corresponding to a remarkable  $10^6$  enhancement in sensitivity. The utility of this method is further demonstrated by the direct detection of the TSPY gene in human genomic DNA samples.

The enzymatic amplification process presented in this Communication is based on the unique selectivity of RNase H, which specifically destroys RNA in RNA–DNA heteroduplexes, but does not digest single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), ssRNA, or dsRNA. An overview of the surface enzymatic amplification process used in these SPR imaging experiments is shown in Scheme 1. A single-stranded RNA microarray is exposed to a solution that contains a complementary ssDNA sequence and the enzyme RNase H. The DNA will bind to its RNA complement on the surface, forming an RNA–DNA heteroduplex (step 1). The enzyme RNase H will then specifically hydrolyze only the RNA strand in this heteroduplex,<sup>8</sup> releasing the DNA molecule back into solution (step 2). This DNA molecule can then bind to another RNA molecule on the surface, causing the hydrolysis of a second RNA probe. This cyclic process results in an amplification effect whereby a very small number of DNA molecules can initiate the hydrolysis of many RNA molecules from the surface (step 3). This loss of RNA probes from the surface results in a significant negative change in percent reflectivity. The decrease in percent reflectivity will become larger with time until all of the available RNA probes on the surface are destroyed.

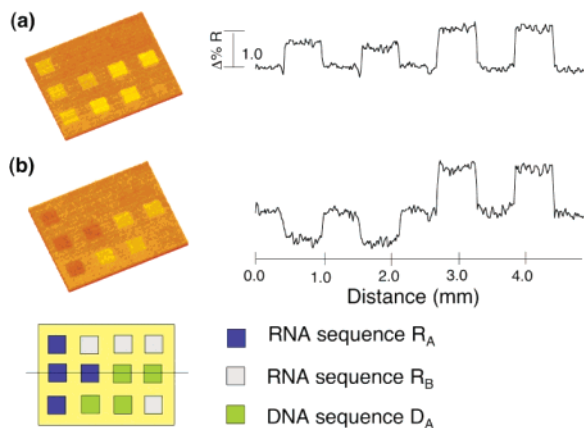
SPR imaging measurements were first performed on a three-component microarray to demonstrate the selectivity of RNase H for the hydrolysis of surface-bound RNA–DNA heteroduplexes. A three-component array (see Figure 1 inset) composed of two non-interacting RNA probe molecules (with sequences denoted  $R_A$  and  $R_B$ ) and a third DNA probe molecule ( $D_A$ ) with the same sequence as  $R_A$  was created by attaching thiol-modified RNA and DNA oligonucleotides onto an alkanethiol-modified gold thin film using a fabrication methodology described previously.<sup>9</sup> This array was exposed to a 500 nM solution of target DNA that was complementary to sequences  $R_A$  and  $D_A$ ; the SPR reflectivity difference image was obtained by subtracting the images taken before and after exposure (see Figure 1a). The DNA target bound specifically to both the  $R_A$  and the  $D_A$  array elements on the surface, and non-specific adsorption was not observed to either the mismatched  $R_B$

**Scheme 1.** Schematic Presentation of a Surface Enzymatic Reaction for the Amplification of SPR Signal by Selective RNA Probe Removal (See Text for Details)

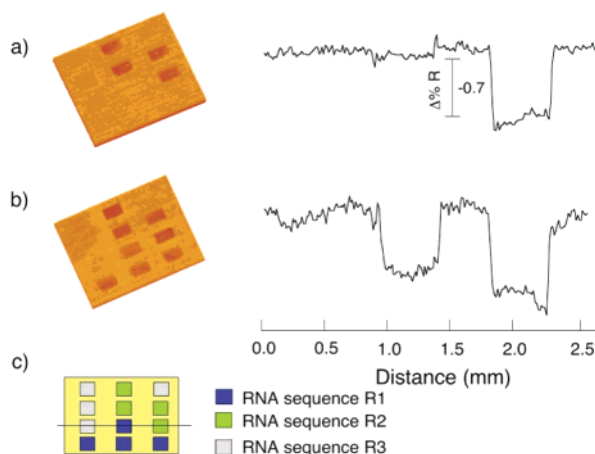


sequence or the array background. This array then contained array elements with heteroduplexed RNA–DNA, dsDNA, and ssRNA. Next, the array was exposed to a solution of RNase H, and Figure 1b shows the SPR reflectivity difference image obtained after approximately a 5-min reaction time. A large decrease in percent reflectivity was obtained only for the  $R_A$  array elements, which were RNA–DNA heteroduplexes prior to exposure to RNase H. This change in SPR signal is attributed to the selective removal of these RNA probes from the surface. Note that both the dsDNA and the ssRNA array elements show no change in SPR signal after exposure to RNase H, demonstrating the specificity and selectivity of this enzyme.

To demonstrate the sensitivity of this enzymatically amplified SPR imaging method, a second three-component RNA microarray was constructed and exposed to target DNA. The three sequences chosen for this array, R1, R2, and R3, are 20mers, two of which (R1 and R2) were selected to bind to the human TSPY gene (testis-specific protein, Y-encoded) located on the Y chromosome, and the other is a negative control. Figure 2a shows an SPR reflectivity difference image of the three-component array obtained after approximately a 4 h exposure to 0.5 mL of a 1 fM solution of a ssDNA 20mer complementary to R2 in the presence of RNase H. A change in percent reflectivity of  $-0.7\%$  was observed for RNA array element R2. The detection of a 1 fM solution of target DNA with this enzymatically amplified SPR imaging technique is a great improvement on the previous SPR imaging detection limit of 1 nM for oligonucleotides based solely on hybridization adsorption.<sup>7</sup> A rough estimate of the degree of enzymatic amplification achieved in this experiment can be obtained from stoichiometry. The complete hydrolysis of RNA from the surface leads to a  $\Delta\%R$  of  $-1.8\%$  (data not shown), so that the  $\Delta\%R$  of  $-0.7\%$  shown in Figure 2 corresponds to a loss of approximately 40% of the probe molecules on the surface. Using a probe surface density of  $1 \times 10^{12}$  molecules/ $\text{cm}^2$  determined previously from fluorescence wash off experiments,<sup>7</sup> we estimate that the 0.5 amol of complementary DNA in solution removed a total of 6 fmol from four 500  $\mu\text{m}$



**Figure 1.** SPR imaging data showing the specificity of RNase H hydrolysis. A three-component array created using two thiol-modified RNA sequences and one thiol-modified DNA sequence (see inset). (a) An SPR difference image for complementary DNA binding to array elements  $R_A$  and  $D_A$ . (b) An SPR image after the induction of RNase H shows a decrease in percent reflectivity for the RNA–DNA duplex, corresponding to the removal of RNA probes from the surface. Array elements are  $500 \mu\text{m} \times 500 \mu\text{m}$  square and contain approximately 4 fmol of oligonucleotide.<sup>7</sup>



**Figure 2.** SPR difference images obtained for the detection of a 1 fM solution of a 20mer oligonucleotide (a) and a solution of male genomic DNA (b) in the presence of RNase H. The inset (c) shows the pattern of the three-component array created using thiol-modified RNA sequences; R1 and R2 were designed to selectively bind the TSPY gene on the Y chromosome, and R3 is a negative control. The line profiles show a decrease in percent reflectivity for array elements where hybridization adsorption and subsequent RNA probe hydrolysis occurred.

squares of RNA on the surface, corresponding to the enzymatic hydrolysis of 12 000 probes for every target molecule.

As expected, lower concentrations of DNA required more time to reach a given change in percent reflectivity due to the fact that more binding–hydrolysis cycles per DNA molecule were required. To reach a change in percent reflectivity of 0.2% required approximately  $12 \pm 2$  min for a DNA concentration of 10 pM,  $50 \pm 10$  min for a DNA concentration of 100 fM, and  $120 \pm 25$  min for a DNA concentration of 1 fM. It may be possible to detect DNA concentrations lower than 1 fM with this technique; however, external variables such as thermal drift make this long-term measurement difficult with the current SPR imaging apparatus.

In a final set of experiments, the direct detection of genomic DNA without PCR amplification was observed by enzymatically amplified SPR imaging measurements. A three-component RNA microarray was constructed with the same RNA sequences: R1 and R2, designed to bind to the TSPY gene, and a third negative control RNA sequence R3. Commercially obtained male and female human genomic DNA samples (Promega) were diluted in buffer,

heated to 95 °C for 5 min, and then mixed with RNase H. The SPR reflectance difference image in Figure 2b was taken approximately 4 h after the injection of a  $5.8 \mu\text{g}/\text{mL}$  solution of male genomic DNA and RNase H. Fragmentation of the genomic DNA was induced by vortexing and sonication. Using an approximate genomic molecular weight of  $9 \times 10^{11}$  and the fact that the target sequence only occurs on the Y chromosome, we estimate the solution concentration of the target DNA fragment to be 7 fM. A decrease in the percent reflectivity for the two probes specific for the TSPY gene can be seen, while negligible nonspecific adsorption is observed to the negative control probe or background. No change in the percent reflectivity was seen for any of the array elements when exposed to female genomic DNA under the same condition.

The enzymatically amplified SPR imaging method described in this paper lowers the limits of detection for ssDNA with SPR imaging from  $10^{-9}$  to  $10^{-15}$  M. This remarkable  $10^6$  enhancement is in part due to the fact that one target DNA molecule can remove approximately  $10^4$  RNA probe molecules from the surface. The second reason for the lower limits of detection is that the enzymatically amplified SPR signal is due to an irreversible RNA probe loss process that is integrated over time instead of the direct binding of DNA target molecules to the microarray. The amount of DNA target molecules on the surface is linked at equilibrium to the solution concentration by the adsorption isotherm; the sensitivity of a normal surface bioaffinity measurement depends on this adsorption coefficient. In contrast, the loss of RNA probes from the surface can be created over time by a very small surface coverage of bound DNA, and therefore can be used to detect a much lower DNA solution concentration.

In addition to SPR imaging, this novel method of enzymatic signal amplification can in principle be used with other techniques (e.g., fluorescence quenching, nanoparticle labeling) and with other surfaces. The ability to detect genomic DNA with the label-free technique of SPR imaging should greatly accelerate its application in the areas of genetic testing, bacterial and viral recognition, and gene expression analysis. In addition, this methodology represents just the first of many possible surface enzymatic-processing reactions that can enhance the sensitivity, selectivity, and applicability of SPR imaging to nucleic acid identification, manipulation, and detection.

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**Supporting Information Available:** Details of materials and methods used in this study (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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