A switchable surface enables visualization of single DNA hybridization events with atomic force microscopy

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Supporting information

Materials and methods

The following oligonucleotides were purchased from Integrated DNA Technologies (Iowa,USA):

Sf-Target50	5'-CGTACTGACTGCTCACGAGGTAGCTC TGAACTGTTTAAAGCATTTGAGGG-3'
Sf-Prime105	5'-TTTAGACTGGATAGCGTCCAATACTG-3'
Sf-Prime204	5'-AACACTATCATAACCCTCGTTTACCA-3'
Sf-Prime396	5'-ACCTTATGCGATTTTAAGAACTGG-3'
Sf-Prime3679	5'-TCC TTG AAA ACATAG CGATAG CTT AG-3'
Sf-DraPrime+Tail	5'-CGTACTGACTGCTCACGAGGTAGC/ C3 spacer/ TCTGAACTGTTTAAAGCATTTGAGGG-3'

Sf-ProbeAmine3′ 5'- GCTACCTCGTGAGCAGTCAGTACGTTTTT/ Amine/ -3'

Sf-ProbeThiol3′ 5'- GCT ACC TCG TGA GCA GTC AGT ACG TTT TT/

Triethylene glycol spacer/ C3 spacer + thiol/ -3'

The thiolated oligonucleotides were shipped in their oxidized (disulfide) form, and were reduced by overnight incubation in an aqueous buffer solution containing a 1000-fold excess of the reducing agent dithiothreitol (DTT), and were purified using Illustra NAP-5 columns from GE Healthcare Life Sciences (Pennsylvania, USA). After reduction and purification, thiolated probes were stored in TAE buffer, and the container was backfilled with nitrogen gas and stored at -20°C until use.

DNA target preparation

M13mp18 RF I DNA (M13, New England Biolabs Inc, Massachusetts,USA) was linearized using EcoRI restriction enzyme (New England Biolabs Inc.) to form a template for PCR. To produce double-stranded targets, 50 pg of linearized M13 was combined with OneTaq MasterMix with Standard Buffer (1X, New England Biolabs Inc.), Sf-DraPrime+Tail (200 nM), and Sf-Prime105/204/396/3679 (200 nM) in a 250 μL PCR tube. (The numerals at the end of the second primer name indicate the total length of the target strand produced by PCR with that primer.) Ultrapure water produced by a Barnstead Nanopure Diamond water purification system (Thermo Scientific, North Carolina, USA) was used to bring the solution up to a volume of 50 μL. Solutions were incubated through the following program: an initial melting step of 94°C for 2 minutes, followed by 35 cycles of melting at 94°C for 30 s, annealing at 48-52°C for 45 s, and

extension at 68°C for 30-230 s, followed by a final cycle with an extension of 2-5 minutes (times and temperatures varied for the different primers used). Primers and polymerases were removed using the QIAquick PCR Purification Kit (QIAGEN). Purified DNA targets were kept in TAE buffer at 4°C for short-term, and -20°C for long-term storage.

Preparation of DNA capture probe surface

Substrate preparation: Single-crystal gold bead or single-crystal gold microplate substrates were prepared in house and used for all AFM experiments. The gold beads were prepared following an established protocol.[1] The gold bead substrates were cleaned by thorough rinsing with ultrapure water and organic solvents, followed by immersion in hot nitric acid or piranha solution (3:1 sulfuric acid: hydrogen peroxide. CAUTION— piranha is highly corrosive and reacts violently with organics). Substrates were then rinsed with pure water and briefly annealed under a hydrogen flame, and then placed into the thiol assembly solution. Gold microplates were synthesized following a recently published protocol.[2] After reductively desorbing the iodide adlayer from as-synthesized single-crystalline microplates, the microplate substrates were exposed to piranha for 5 min., rinsed with pure water and then immersed into the thiol assembly solution.[2] Thiol solutions were backfilled with nitrogen gas, sealed and kept in the dark at room temperature to minimize thiol oxidation during monolayer assembly.

For probe attachment, we have used two different methods to anchor single-stranded DNA probes to the SAM surface. For the results in Figures 1, 2, and S2, thiolated probes were inserted into a pre-assembled monolayer (method 1). In all other experiments, an amide coupling reaction was used (method 2). While both approaches are effective in immobilizing probes that are isolated from each other, amide coupling yields more reproducible probe densities and hence is more suitable for kinetics studies, as discussed below.

Method 1-- thiolated probe insertion: In this method the monolayer was assembled from a 0.5-1.0 mM ethanolic solution of 11-mercaptoundecanoic acid (MUDA) containing 10% acetic acid by volume. The assembly time was reduced to 10 minutes in order to ensure the presence of some monolayer defects, into which probes could insert and link to the substrate *via* the gold-sulfur bond.[1] After monolayer assembly, the substrates were removed from the solution and immediately rinsed and sonicated for 10 s in a 9:1 ethanol:acetic acid solution, then rinsed with pure ethanol and gently blow-dried with filtered air. They were then placed in a custom-built PTFE fluid cell and rinsed several times with a TAE buffer. The insertion was then carried out by immersing the carboxylic acid-terminated gold substrates in an aqueous TAE buffer solution containing 2.0 μM thiolated DNA probe (sequence Sf-ProbeThiol3') and 50 mM NaCl for 20-30 minutes. Following the insertion step, the surfaces were repeatedly rinsed with TAE and then placed under an aqueous Ni(II) imaging buffer consisting of 5 mM Ni(II) acetate, 4 mM Tris acetate, and 0.1 mM EDTA.

Method 2-- amide coupling reaction: In this method the monolayer was assembled from a 0.5-1.0 mM ethanolic solution of 16-mercaptohexadecanoic acid (MHDA) containing 10% acetic acid by volume. Assembly times were typically between 16 and 20 hours, with no observable difference in monolayer quality for longer growth times. After monolayer assembly, the substrates were removed from the solution and immediately rinsed and sonicated for 10 s in a 9:1 ethanol:acetic acid solution, then rinsed with pure ethanol and gently blow-dried with filtered air. They were then placed in a custom-built PTFE fluid cell and rinsed several times with 1X PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 11.9 mM phosphate buffer, pH 7.4). DNA probes were then attached to the surface using an EDC-activated amide coupling reaction.[3] The carboxylic acid-terminated gold substrates were immersed in an aqueous solution containing 20 mM EDC activator (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), 20 nM amine-labeled DNA probe (sequence Sf-ProbeAmine3'), and 0.1X PBS for a reaction time of 5-10 minutes. The probe coverage was found to increase roughly linearly with reaction time in this range. Following the coupling reaction, the surfaces were repeatedly rinsed with PBS, then TAE (1X = 40mM Tris acetate, 1 mM EDTA, pH 8.3), and finally placed under an aqueous Ni(II) imaging buffer consisting of 5 mM Ni(II) acetate, 4 mM Tris acetate, and 0.1 mM EDTA.

In comparison to the amide coupling reaction, the insertion method resulted in surfaces that were less reproducible in terms of the probe surface coverage. Surfaces prepared by the insertion method were also found to be more susceptible to organic contamination during experiments, *e.g.*, small point protrusions in Figure 2. These

protrusions are 2 -3 nm high, *i.e.*, significantly higher than ssDNA probes. The protrusions are mobile and may disappear between frames. Sequential images (Figure 2) show that the DNA targets are not attached to the locations of such protrusions. Hence, these features are contamination from adventitious adsorption on the relatively disordered monolayer, which is needed for the insertion of probes. In contrast, surfaces prepared using the 'amide coupling reaction' method (*i.e.*, Method 2), were generally free of such features and allow identification of probe molecules. This is likely due to the much higher quality of the monolayer, which was typically assembled for a period of 16-20 hours. These observations prompted us to use surfaces prepared with Method 2 for all further hybridization experiments.

With method 1, it was found that the spacing between probes was dependent on the salt concentration of the insertion buffer, with higher salt concentrations resulting in aggregation of the probes on the surface. It is hypothesized that this salt dependence of probe spacing is a result of electrostatic repulsion between the negatively charged probes, which provides an energetic barrier for a second probe to insert in close proximity to a probe which has already attached, but becomes negligible at higher ionic strength. Reducing the NaCl concentration to 50 mM produced surfaces with mostly isolated probes.

AFM imaging

All imaging was carried out using an NTEGRA Vita Atomic Force Microscope, manufactured by NT-MDT (Moscow). Images were acquired while operating in semi-

contact (tapping) mode under liquid, using silicon tips mounted on silicon nitride cantilevers with a nominal spring constant of 0.3 N/m and a resonant frequency of approximately 16 kHz in liquid (model SNL-10, manufactured by Bruker, California).

DNA surface hybridization

Prior to all hybridization experiments, the monolayer surfaces were repeatedly rinsed with an STAE buffer (saline Tris-acetate-EDTA: 200 mM NaCl, 40 mM Tris acetate, 5 mM EDTA, pH 8.3) to remove any Ni(II) ions that were bound to the surface. Substrates were then exposed to the target DNA in a hybridization buffer containing 1.0 M NaCl, 1X TAE, and 1.0 mM SDS (sodium dodecyl sulfate) for a predetermined amount of time, after which the substrate was gently rinsed three times with STAE and placed under Ni(II) imaging buffer.

AFM image analysis

Heights of individual DNA molecules were obtained after first flattening AFM images line by line, then highlighting molecules using Gwyddion image analysis software (http://gwyddion.net/). The molecular features were identified and selected in a semi-automated procedure using Gwyddion as follows. After flattening the image line-by-line, a mask was generated of all features that are over a minimum height threshold. Prior to mask generation, a median filter was applied to the image in order to filter out single-pixel noise; however, this filtered image was used only to create the mask, and the unfiltered image was used for actual height analysis. We found it

necessary to perform some minor editing of the mask by hand in order to separate closely-spaced or overlapping features that could clearly be identified as discrete objects. At the low probe surface coverage, the fraction of closely spaced probes that are difficult to distinguish is estimated to be less than 1.2%.

The maximum heights of the individual probes, z, were recorded relative to the mean height of a ten pixel halo immediately surrounding that molecule obtained using MATLAB for features between 0.4 and 2.4 nm tall. The finite width of the height histogram is likely the result of several factors. First, the DNA molecules likely adopt slightly different conformations on the monolayer surface, leading to small variations in the feature heights. Second, changes in the imaging set point during imaging will vary magnitude of the tip-sample interaction, which may cause minor changes in the apparent heights of features in different images. Finally, the feature height is measured relative to the average height of a ten pixel halo surrounding the feature, and some variability in the background height is expected due to nanoscale inhomogeneity, such as gold adatoms and molecular defects within the monolayer, atomic steps in the gold substrate, and counterion condensation at the interface.

To determine Γ, the fraction of probes that had hybridized with a target at a given time point, the molecule heights from all images for each time point were aggregated, and a threshold height was set such that any molecule whose height exceeds the threshold is considered to be hybridized at that time, and any remaining molecules are considered to be unhybridized. This analysis was applied to a minimum of N = 750

molecules for each time point to determine Γ(t). The time points were then plotted, and the best fit of equation 1 for a simple Langmuir adsorption model was used to determine the rate constant k.

The above approach likely results in some error in Γ due to the incorrect assignment of some molecules whose height is close to the threshold. Therefore, an alternative approach which utilizes a maximum likelihood estimate was also used to ascertain the level of error.[4] To assign the probability that an observed molecule was hybridized, two Gaussian curves, $N_t(z)$ with taller mean and $N_s(z)$ with shorter mean, were fit to the histogram of the heights of the probes observed for each time or concentration value; probes with height above the mean of $N_t(z)$ were labeled as hybridized (hybridization probability $p_h = 1$), probes with height shorter than the mean of $N_s(z)$ were labeled unhybridized ($p_h = 0$), and probes with height h between the means of the two Gaussians were given hybridization probability $p_h = N_t(z)/(N_t(z) + N_s(z))$. Hybridization was assumed to behave as an irreversible Poisson process (probability of a probe remaining unhybridized at time t $S(t) = \exp(-k*t)$), and the hybridization rates obtained by minimizing the negative log-likelihood function $-\log \mathcal{L}(k\,|\Theta)$ with MATLAB given data Θ (for each observed probe i with hybridization probability $p_{h,i}$ at the time the probe was observed t_i) for rate k:

$$-\log \mathcal{L}(k\,|\Theta) = \sum_i \left(1 - p_{h,i}\right) kt_i - p_{h,i} \log(1 - \exp(-kt_i)).$$

When this second approach was applied to a large subset of the data, the rate constant found was nearly identical to the constant found using the first approach.

Additional AFM images

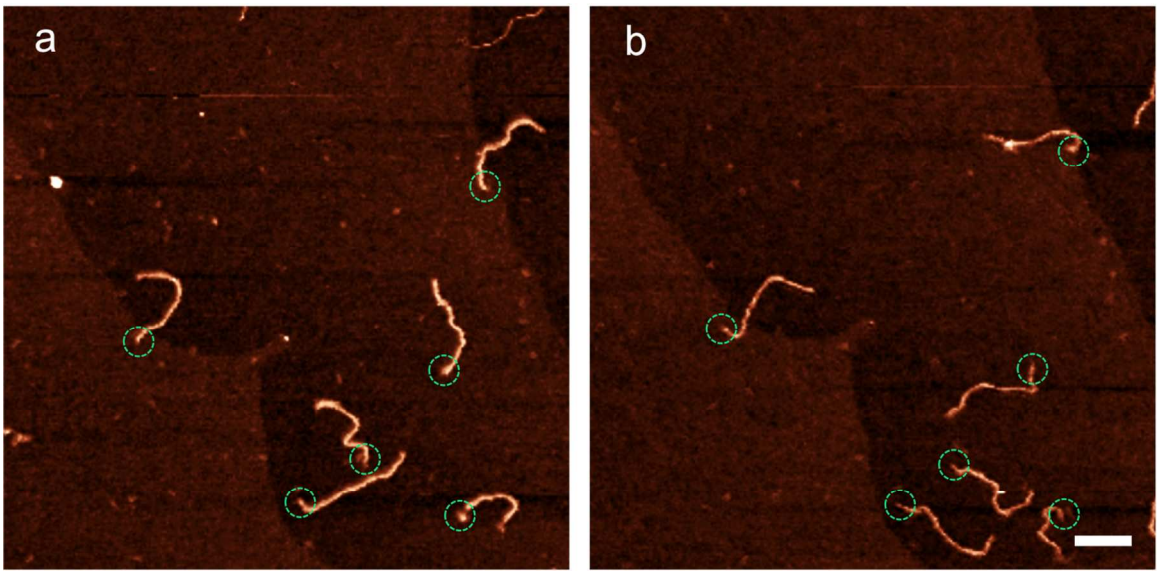

Figure S1: Switching of DNA-surface interaction. AFM images acquired under Ni(II) imaging buffer show the same 500 nm area containing 400 bp dsDNA targets hybridized to 24 nt surface probes both before (a) and after (b) rinsing the surface with an STAE buffer solution that did not contain DNA. During the rinsing step, the DNA strands are no longer electrostatically bound to the surface and are free to rotate around the end that is hybridized to the surface probes. When the Ni(II) buffer is replaced, the DNA readsorb on the surface in new conformations. Scale bar is 50 nm. The green circles highlight the anchors that remain stationary.

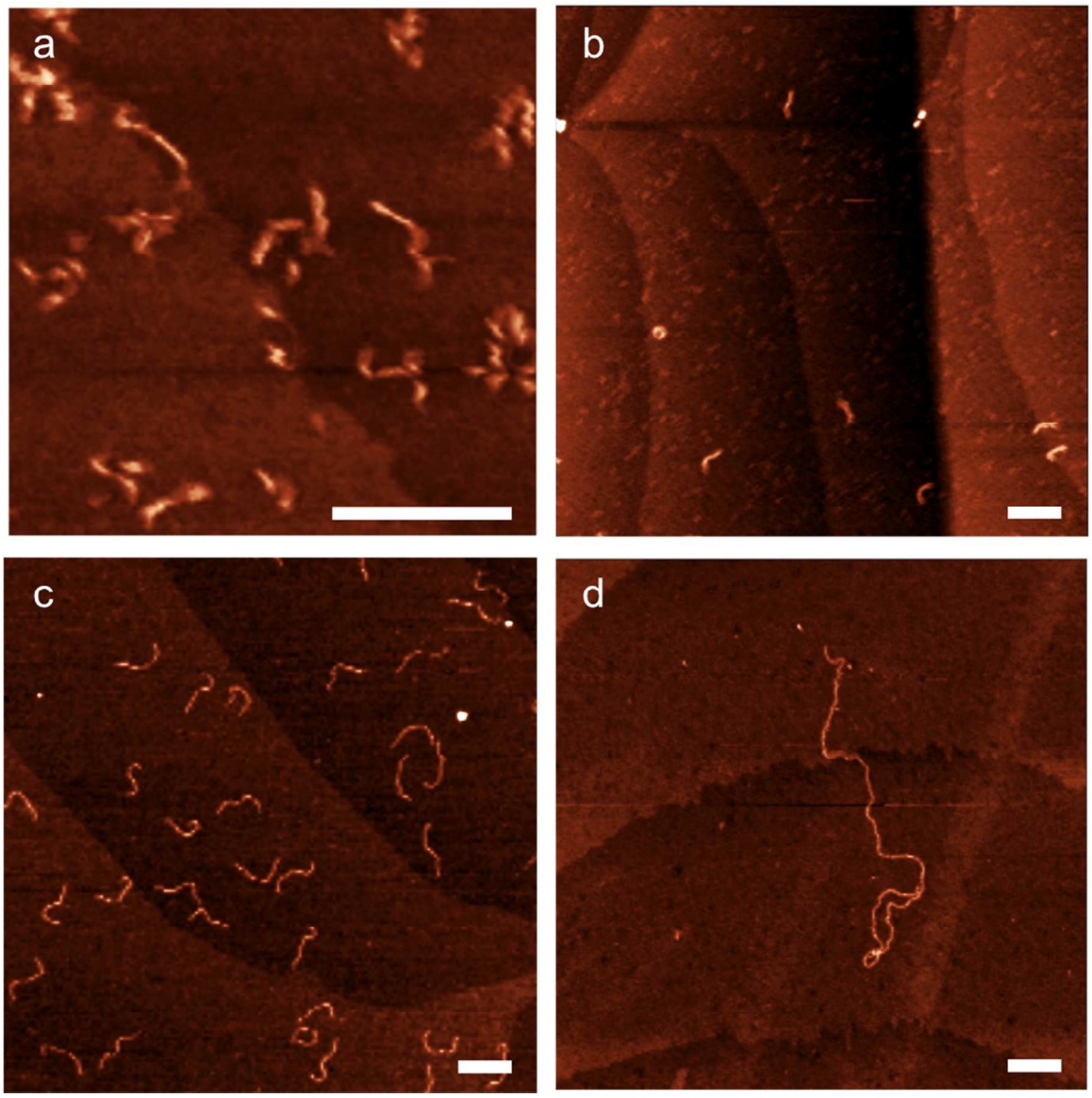

Figure S2: AFM images of hybridized DNA targets of different lengths. Images show dsDNA targets that are approximately (a) 100 bp, (b) 200 bp, (c) 400 bp, and (d) 3700 bp, all hybridized to 24 nt ssDNA surface probes. Scale bars are 100 nm in all images.

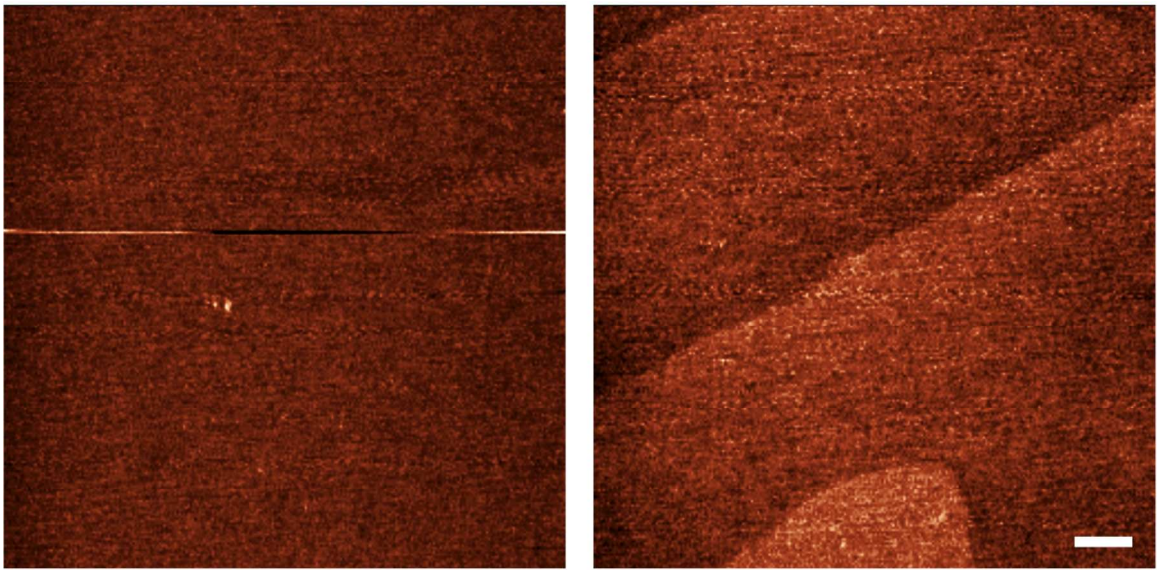

Figure S3: Control hybridization experiment—no probes. Both images are representative of an MHDA surface without any DNA probes after it has been exposed to a 10 nM concentration of 50 nt ssDNA targets in hybridization buffer for 10 minutes. The almost total lack of molecular-sized surface features indicates that nonspecific adsorption of DNA is negligible. Scale bar is 50 nm.

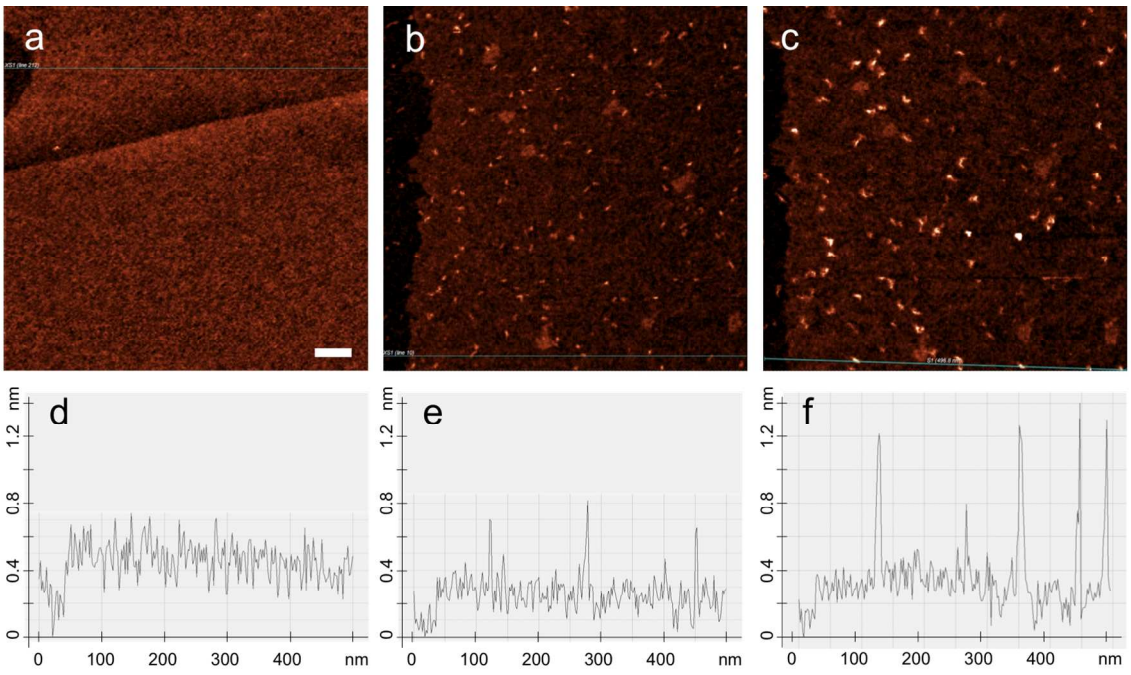

Figure S4: Representative AFM images and height profiles of DNA capture probe surfaces (a,d) prior to probe attachment, (b,e) after probe attachment, and (c,f) after exposure to DNA targets. The probes are visible as ~0.6 nm tall protrusions prior to hybridization with the targets. After hybridization, the height increases significantly to over 1 nm. Scale bar is 50 nm.

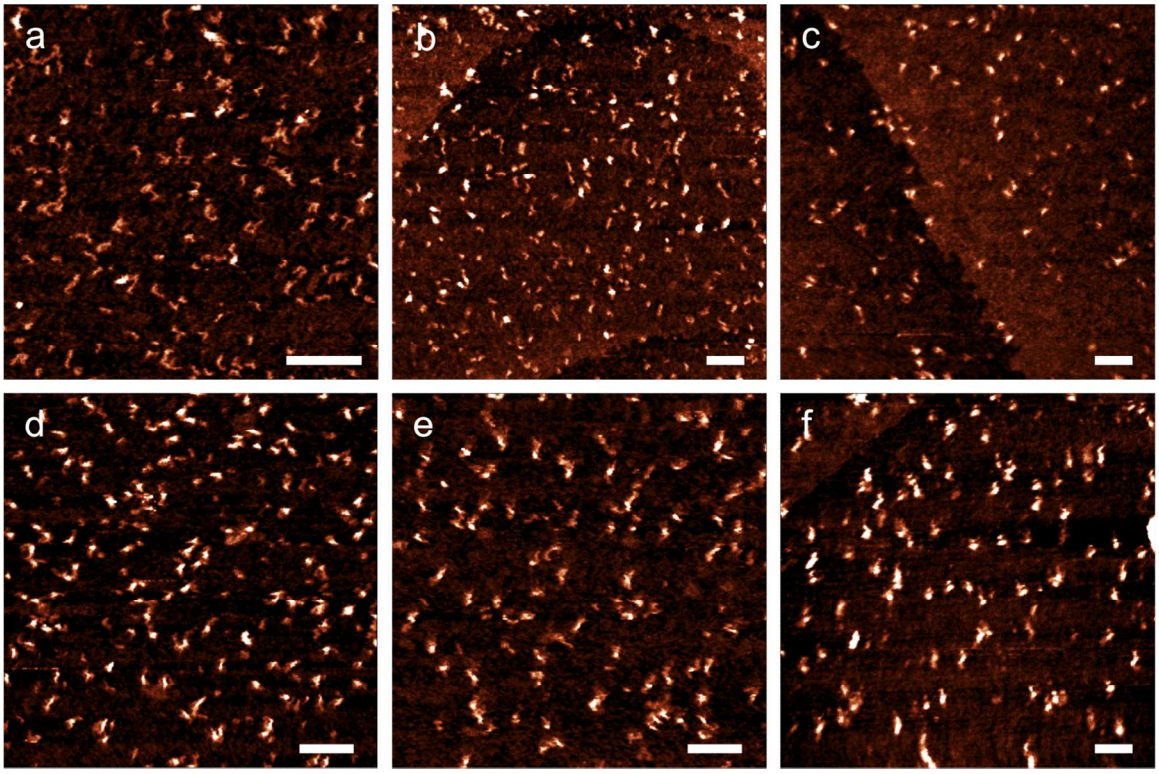

Figure S5: Representative AFM images from the data sets used to extract fractional coverages in Figure 5b. The DNA capture probe surfaces were exposed to a 10 nM solution of 50 nt ssDNA targets in the hybridization buffer. Images show different surfaces after exposure times of (a) 6 min, (b) 12 min, (c) 30 min, (d) 60 min, (e) 90 min, and (f) 180 min. All scale bars are 50 nm.

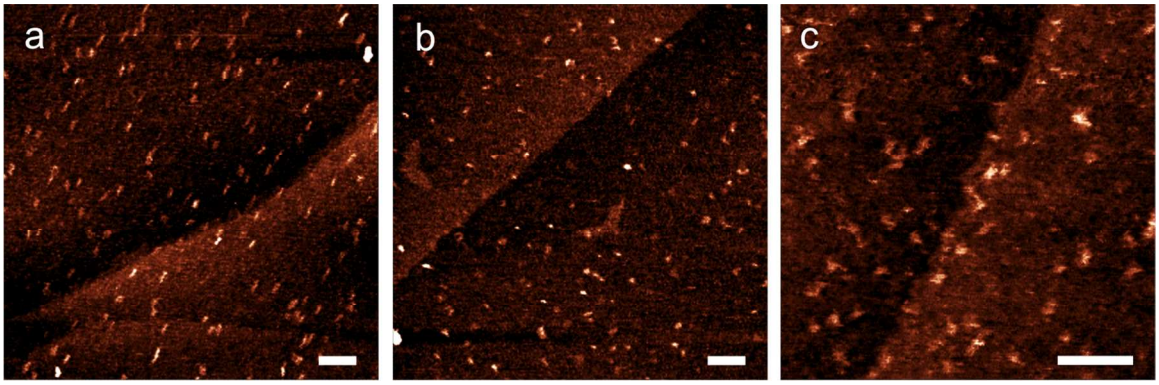

Figure S6: Representative AFM images from the data sets used to extract the target-concentration-dependent fractional coverages in Figure 5c. The DNA capture probe surfaces were exposed to a solution of 50 nt ssDNA targets at varying concentrations in hybridization buffer for a fixed hybridization time of 180 min. Images show different surfaces after exposure to DNA concentrations of (a) 10 pM, (b) 100 pM, and (c) 1.0nM. All scale bars are 50 nm.

1. Josephs, E.; Ye, T. *J. Am. Chem. Soc*. **2012***, 24,* 10021–10030.

2. Lee, C.; Josephs, E. A.; Shao, J.; Ye, T. *J. Phys. Chem. C* **2012***, 33,* 17625–17632.

3. Kufer, S. K.; Puchner, E. M.; Gumpp, H.; Liedl, T.; Gaub, H. E. *Science* **2008***, 5863,* 594-596.

4. Andrec, M.; Levy, R. M.; Talaga, D. S. *J Phys Chem A* **2003***, 38,* 7454-7464.