Supporting information

High Density Single-Molecule-Bead Arrays for Parallel Single Molecule Force Spectroscopy

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S1. General approach to fabrication of single-molecule-bead arrays.

Figure S1 shows a schematic for our general approach for creating a high density array for force spectroscopy. It details the DNA preparation and modification (Figure S1a), the attachment and blocking layer chemistry (Figure S1b), as well as the interaction forces between the probe and the surface (Figure S1c).

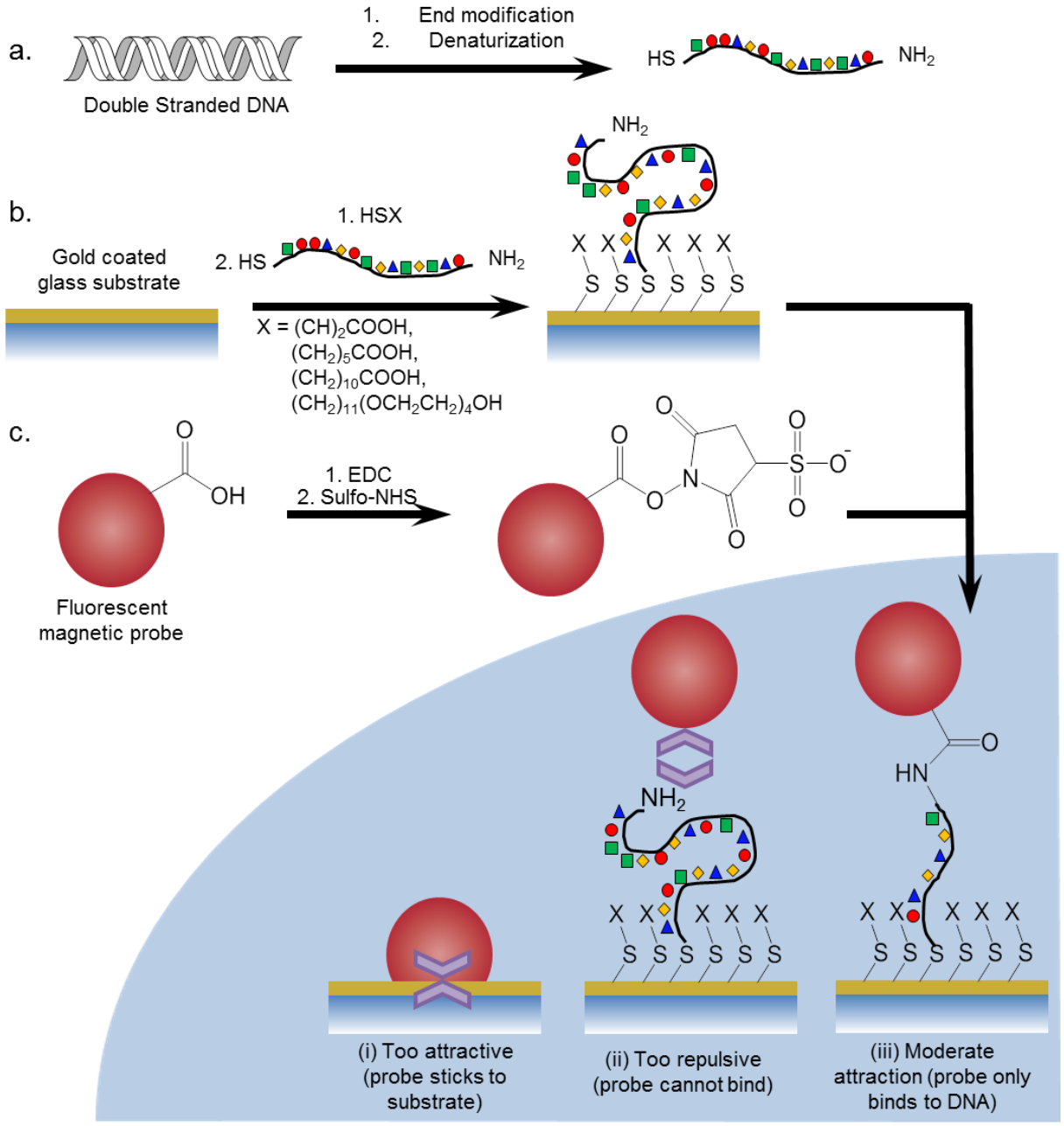

Figure S1. *Chemistry of attachment of a force probe to DNA oligomers anchored at a solid surface. A double stranded DNA (a) is end-modified, denatured, and combined with a thiol blocking molecule for reaction with a gold coated substrate (b). A carboxyl terminated probe is then activated and allowed to come in contact with the probe (c). When in the vicinity of the DNA molecules one of three outcomes may occur (shaded area): (i) the probe and surface will be too attractive and the probe sticks to the substrate, (ii) the probe and surface will be too repulsive and the probe is unable to bind, or (iii) the probe will come close enough to the DNA and covalently link, while not adhering to the surface.*

S2. Detailed experimental methods.

Substrate preparation. We prepared the substrate by depositing a 45 Å layer of titanium followed by a 110 Å layer of gold onto a clean glass slide using an electron beam evaporator vapor deposition apparatus (Eddy Co. SYS-24, SC-20-Digital System Controller). The slides were stored under vacuum for later use. To prepare the self-assembled monolayers (SAMs), a thiol blocking molecule [mercaptopropionic acid (MPA) (Alfa Aesar, CAS 107-96-0), mercaptohexanoic acid (MHA) (Aldrich CAS 17689-17-7), mercaptoundecanoic acid (MUA) (Aldrich CAS 71310-21-9), mercaptohexadecanoic acid (MHDA) (Aldrich CAS 69839-68-5), or mercaptoundecyl tetraethylene glycol (MutEG) (Aldrich)] was dissolved to produce 10 mM solutions in either a pH 7.4 phosphate buffer with 1 M sodium chloride or in ethanol. The solvent was chosen based on the solubility of the all the thiols in the series, i.e. the phosphate buffer was used as a solvent unless MUA or f were needed in a series, in which case ethanol was used as a solvent for all the thiols.

End modification of DNA. Here, a different procedure, also using ligation, was used to produce our model DNA molecule. To create a 142-mer ssDNA terminated with an amine on the 5' end and a thiol group on the 3' end, a 5'-amine terminated 71-mer with a phosphorylated 3' end, a 3' thiol terminated 71-mer, and a 30-mer complementary to 15 bases at non-modified ends of each 71-mer (all purchased from Integrated DNA Technologies, Coralville, IA) were annealed, and ligated together. First, 4.5 μL of 1 mM aqueous solutions of each DNA oligo were mixed with 10 μL of an annealing buffer (100 mM Tris HCl, 1M NaCl, 10 mM EDTA) and diluted to 100 μL with autoclaved deionized water. The solution was denatured by undergoing a heat cycle of 2 min at 95° C in a thermocycler (Techne TC-3000) followed by five cycles of 95° C for 15 s, 40° C for 15 s, and 72° C for 60 s. At the end of the last cycle the system was annealed for 5 min

at 72° C. This procedure resulted in 45 μM of annealed DNA in a 100 μL of solution. The two strands of DNA were then ligated by combining 50 μL of the DNA solution with 14 μL of 10× T4 ligase buffer (New England Biosciences) and 18.75 μL of T4 DNA ligase (7500 units) (New England Biosciences) and finally diluting to 150 μL with autoclaved deionized water. The solution was again denatured by undergoing a heat cycle of 2 min at 95° C in the thermocycler and purified using a MinElute column (Qiagen) and eluted with 10 μL deionized water. The DNA was purified from the 30-mer and other side-products by a 6× TBE Urea gel (Invitrogen). After excising the band, the sample was eluted with 1× TBE buffer at 37 °C overnight. The final product was purified with a MinElute column and eluted with 20 μL of DI water to a concentration of ~50 ng/μL.

Probe Preparation. Microspheres were prepared by emulsification of polymer solutions that contained ~10 nm diameter magnetite nanoparticles (20 mass %) and organic fluorescent dye (experimental details on fabrication of the force probes will be provided elsewhere). Smooth probes of low polydispersity had a diameter of 4.5 ± 0.5 μm (error is FWHM as determined by scanning electron microscopy) and displayed a superparamagnetic response as verified by a vibrational sample magnetometer. Zeta potential measurements were taken on the probes by preparing a 1 mg/mL solution of probes in 10 mM phosphate buffer with a controlled pH and using a Brookhaven zetaPALS instrument. For DEP tweezers, the probes were fabricated without magnetite.

Probe mounting. To mount the force probe onto the AFM cantilevers, the capabilities of the Asylum Research MFP-3D mounted on an inverted microscope was exploited. The magnetite-doped fluorescent probes were attached to a tipless triangular silicon nitride cantilever (Nanoworld PNP-TR-TL) with an approximate spring constant of 30 pN/nm. To do so, the

suspension of probes in water was first dried on a clean glass microscope slide alongside microdroplets of a two component epoxy (Epoxy Technology 377 kit). The cantilever was lowered onto a microdroplet by manually adjusting the micropositioner. The relative lateral alignment of the probe and the droplet was monitored by optical microscopy and adjusted simultaneously with the lowering of the cantilever. Once the cantilever was loaded with a small amount of epoxy it was touched to the glass surface 1-5 times to remove the excess. The cantilever was then raised and positioned over top of a probe and gently touched to the top of it. The cantilever with the probe attached was then raised and removed from the tip holder of the AFM. Finally the epoxy on the tip was cured at 45° C overnight.

AFM measurements. Force spectroscopy was conducted using an open fluid cell setup of the Asylum Research MFP-3D Bio atomic force microscope. Buffer solutions were pipetted directly onto the sample. The force curves were recorded with a 2 μm/s cantilever velocity and 10 nN trigger force for turnaround point. Data were analyzed and fitted for the electrostatic/van der Waals parameters using custom software coded in IGOR Pro 6.2 (Wavemetrics Inc, OR).

Probe binding assays. The probe binding assays were performed in samples comprising a 4 mm thick polydimethylsiloxane (Sylgard 184 Silicone Elastomer kit) mask with 4 mm diameter wells on top of a gold substrate. In each well, 20 μL of the desired thiol blocking molecule solution was added and allowed to react for 2 h to ensure a complete coverage of the SAM. Upon completion of the reaction, the well was rinsed twice with the solvent that the thiol was dissolved in and then twice in the final buffer solution meant to be studied. During the preparation of the substrate, the probes were prepared for the assay. 20 μL of a 2 mg/mL (~2.5 million probes/mL) stock solution in 1% SDS were added to a microcentrifuge tube and washed in deionized water three times. The probes were then dried and reconstituted with 500 μL of

buffer being studied. Once reconstituted, the buffer was removed from the well and replaced with the solution of probes. The probes were allowed to settle for 15 min and a fluorescent image of TAMRA labeled probes was taken at 100× total magnification using a filter set designed for the TRITC chromophore. A permanent magnet was placed on top of the wells, producing an approximate force of 200 pN to detach the probes from the surface, and a fluorescent image was taken after five minutes. On these images, the population of probes was sufficiently low that the probes could be individually counted using the particle analysis package in Igor Pro.

Mapping of fluorescent labeled DNA. A short ssDNA oligo labeled with carboxytetramethylrhodamine (TAMRA) at the 3'-end and a protected thiol at the 5'-end (39-mer – 5'-$HOCH_2CH_2S$-S-$(CH_2)_6$-TTT TTT TTT TTT TTT TCA TCG CAC ATC GTA GCA CAA GAC-TAMRA-3') was purchased from Integrated DNA Technologies. A 100 μM stock solution of DNA was made in autoclaved Millipore DI-H_2O. Before each experiment, an aliquot of the labeled DNA was diluted by a factor of 5 with a solution of 5 mM TCEP (tris(2-carboxyethyl)phosphine) in 6x SSC buffer (pH 7.4) and left standing for 30 minutes to reduce the disulfide. All subsequent dilutions were by a factor of ten in 10 mM phosphate buffer with 1 M NaCl at pH 7.4. Solutions of MutEG were diluted from a 1 mM stock solution in 10 mM phosphate buffer with 1 M NaCl at pH 7.4. For the two-step binding process, we allowed fluorescently labeled DNA (1 μM) to adsorb onto a surface for 10 minutes followed by incubation in a 1 mM solution of MutEG for 1 hour. The competitive binding employed a 1:100 ratio of DNA to MutEG (500 nM DNA:50 μM MutEG) in a 2 hour reaction and was also followed by incubation in a 1 mM solution of MutEG for 1 hour to ensure formation of a

complete monolayer. The epi-fluorescent images were taken with inverted microscope (Olympus IX 71) and CCD camera (Andor Technologies, iXon DU888, Belfast, Ireland).

Force spectroscopy demonstration. To conduct the force spectroscopy experiments, we fabricated a microwell array as described elsewhere.[6,31,32] A glass substrate was coated with a 10 nm layer of titanium followed by a 130 nm layer of gold using electron beam evaporation. Using photolithography, this gold substrate was patterned with SU-8 photo resist producing 7 μm diameter, 4 μm deep round wells on a square 15 μm × 15 μm lattice. The opaque gold film at the bottom of the wells was carefully etched away using a standard gold etchant (4 g KI, 1 g I_2, and 80 mL DI-H_2O) for approximately 30 s to 2 min. The required etching time varied and was evaluated for each substrate using transmission microscopy of the substrate. Finally, another deposition of 4 nm Ti followed by 11 nm of gold was placed on top of the pattern. DNA was added to the surface in a 1 μM solution with 10 mM phosphate buffer (pH 8) with 100 mM NaCl followed by a surface modification with MutEG (chosen as the best blocker from the probe binding assays) followed by the conjugation of the probes. The two step binding process was chosen to maximize the probe DNA binding efficiency. The force probes were base washed, followed by a wash in MES buffer. Sulfo-NHS and EDC were then added to the probe solution to activate the carboxylic acid groups on the surface of the probes for reaction with the amine groups at the free end of the DNA. A 100 μm thick silicon gasket was sandwiched between patterned and flat gold electrode to form a fluid cell. The activated probes were flushed in and allowed to settle inside the microwells and react with the DNA. Variable AC voltage applied to the top and bottom electrodes supplied the force necessary to conduct force spectroscopy.[6] To generate stretching curves for individual DNA molecules, a modulating dielectrophoretic (DEP)

force was applied to the probes via a 1 kHz AC potential modulated between 0 and 10 V by means of a function generator (645-G, Berkeley Nucleonics Corp., CA).

S3. Control of DNA surface density.

An important aspect of a magnetic/DEP tweezers is the ability to set up an experiment where only one molecule is interrogated by any one probe and sufficient spacing exists between the probes to differentiate them with optical microscopy. Therefore, one must control both the DNA aggregation and spacing. We compared two methods of attaching DNA and forming a blocking layer on a gold surface: a two-step (sequential) binding process and a competitive (parallel) binding process. The two-step process includes a deposition of the DNA at low surface coverage from a dilute DNA solution for a controlled time followed by the completion of the monolayer by a small molecule thiol during exposure to the solution of the blocking thiol. The competitive binding method involves mixing the DNA together with the thiol forming a blocking layer in a solution of predefined composition, causing the two species (DNA and thiol) to compete for the binding sites on the gold surface and, thus, restricting the amount of bound DNA. Both procedures yielded, through empirical optimization, a controlled population of DNA with all areas of the gold between oligos blocked with the inert molecule of our choosing.

We compared the two types of binding by directly mapping the density of a shorter (39-mer) fluorescently labeled DNA. Fluorescent images (taken in the air with NA = 1.45 objective) for each deposition method revealed the distribution of the individual DNA molecules (Figure S2). While both methods achieve controlled densities of the DNA over the entire sample surface, the presence of numerous oversized spots created in the course of the two-step process suggests that there is aggregation of the DNA immobilized on the surface with this method. These bright spots

are not as pronounced in the process of competitive binding with high MutEG-to-DNA ratio, suggesting a more even distribution of DNA and reduced aggregation than in the sequential process. Since the spacing of the probes is critical to the preparation of a highly parallel force spectroscopy platform, and it is advantageous to prevent the probes from binding to more than one molecule, it is clear that for a controlled force spectroscopy platform we must use a competitive binding scheme (manuscript in preparation).

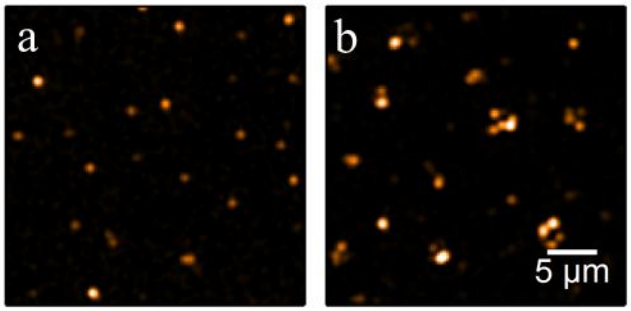

Figure S2. *Images of TAMRA-tagged DNA chemisorbed onto gold surface by the competitive binding process (a) and the two-step process (b). Sample in (a) resulted from the reaction of 50 µM MutEG and 500 nM DNA solution in 1M NaCl phosphate buffer (pH=8) with the Au surface for 2 hours. The two-step binding process in (b) reacted 1µM DNA with Au surface from a 1 M NaCl phosphate buffered (pH=8) solution for 10 min.*

S4. Probe and substrate roughness.

When determining the minimum separation range in our model can be used in it is important to determine the roughness of the probe and substrate. Images of both the probe and the substrate are shown in Figure S3. Since our model also oversimplifies the real systems by ignoring roughness of the surface (R1) (estimated as approximately 0.5 nm over 1μm^2 area) and the bead (R2) (2 nm over 1μm^2 area) (Figure S3). This approximation is true when $z \gg \frac{R_1 R_2}{R_1+R_2}$, or, in our case, when z >> 0.4 nm. This factor, combined with the omission of steric forces, suggests that our model should be fairly accurate at distances greater than 5 nm, but is incomplete at distances under 5 nm.

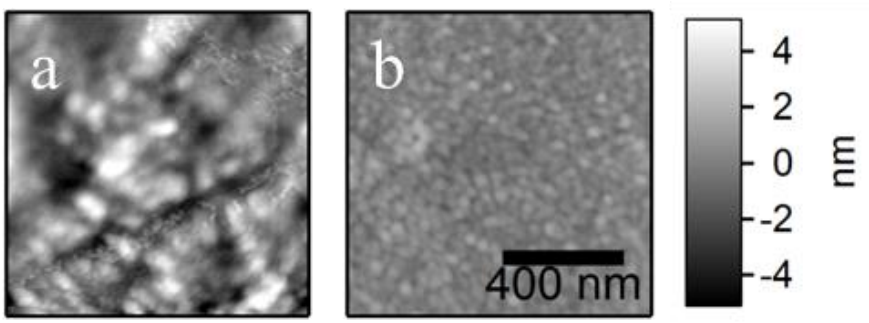

Figure S3. *AFM images of surfaces of the probe (a) and the gold substrate (b). The curvature of the probe in (a) was subtracted from the image using a third order polynomial fit to show the roughness on the same scale as that of the surface.*

S5. Probe binding assay.

To illustrate the probe binding assay, we present in Figure S4 images of probes bound to a substrate both before and after a magnet is applied. We are then able to accurately count the change in the number of probes from one set of images/conditions to the next.

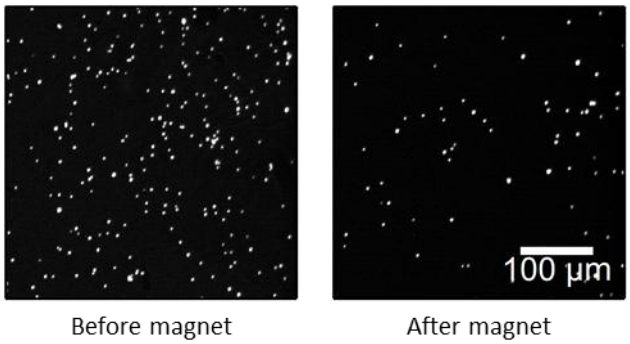

Figure S4. *Representative images from bead binding assay. The probes were allowed to settle onto the surface via gravity (left image) and a permanent magnet was used to remove the probes (right image). This particular experiment was conducted on a SAM of MHDA on gold in a solution of pH 7.0 phosphate buffer with an ionic strength of 1 mM.*

S6. The effects of surfactant on the non-specific adhesion.

We demonstrated the effects of surfactant on non-specific binding in our probe-substrate system by force-distance measurements and probe binding assays. We found that the surfactant

doesn't change the overall interaction forces of the system (Figure S5a), but it does greatly reduce adhesion and nonspecific binding (Figure S5b).

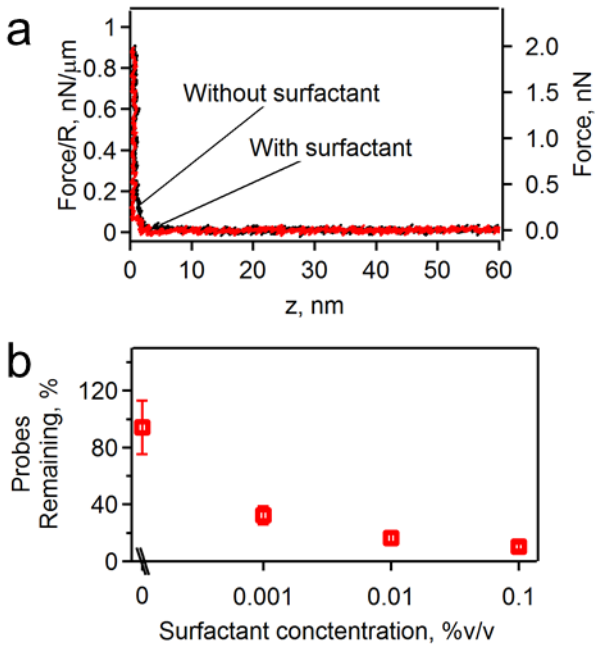

Figure S5. *Effects of introducing surfactant to the system. Representative force curves are shown (a) as well as results from the probe binding assays (b). The force/distance range in part (a) corresponds with the ranges in Figure 7a, d, and g. Note: For clarity, the surfactant concentration is plotted on a split axis with the zero concentration point plotted on a linear scale and the other points plotted on a log scale.*

S7. Dielectrophoretic tweezers experimental details.

Here, we have chosen to conduct our experiments in an array of 7 µm diameter round wells, whose role is to ensure a dielectrophoretic force of sufficient magnitude as well as to simplify probe counting and to facilitate exchange of solutions in the fluid cell. The bottoms of the wells are coated with gold and carry ssDNA bound to the surface, with space in between blocked by MutEG (Figure S1). We delivered the probes inside the microwells by gravity in a low ionic strength solution (pH 8 phosphate buffer with an ionic strength of 24 mM and 0.1 % Tween 20 surfactant) in order to keep the probes mobile and to minimize their adhesion to the walls of the microwells. With the probes in place, a centering negative DEP force (1 MHz, 10 V) was applied

to the probes to optimally position them inside the wells. To bind the probes to the immobilized DNA, we minimized the probe-surface distance by increasing the ionic strength of the solution. While the centering force was still being applied, a 10 mM pH 8 phosphate buffer containing 40 mM NaCl with 0.1 % TWEEN 20 (final ionic strength 64 mM) was flushed through to allow the probes to approach the surface and react with the amino groups at the free end of the DNA molecules (Figure S1). To overcome the residual adhesion of some probes to the substrate, we filled the fluid cell with deionized water to allow the electrostatic forces to lift the probes from the surface. Upon completion of the probe-DNA binding, the original pH 8 buffer with an ionic strength of 24 mM was flushed through to conduct DEP force spectroscopy experiments.

S7. Dielectrophoretic tweezers force curves.

We are able to demonstrate the full extent of the successful assembly of the bead array for force spectroscopy by showing the force curves of all 57 active probes (Figure S6).

Figure S6. *Force spectroscopy curves of each of the labeled probes in Figure 5e in the main text.*