Supporting Information

Improved Free-Energy Landscape Quantification Illustrated with a Computationally Designed Protein-Ligand Interaction

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Materials & Methods:

DNA constructs

We synthesized our DNA via PCR using 5′-labeled primers (Integrated DNA Technologies). The primer sequences used to generate the DNA constructs along with the end labeling are detailed in Table S1. For the template DNA, we used M13mp18 (New England BioLabs). Specifically, the reaction mixture consisted of 30 pmoles of each primer, 2 ng of template, and amplified with KOD-Hot Start DNA Polymerase (Novagen), as recommended. We purified the resulting DNA from the primers using a Qiagen Qiaquick PCR purification kit with 2 additional PE wash steps. Next, we gel purified the DNA using a 1% agarose/TAE gel, which also confirmed the DNA length. The gel was stained with SyBR-Green I nucleic acid gel stain (Life Technologies). The DNA was recovered from appropriately sized bands using a Bio-Rad Freeze 'N Squeeze DNA Gel Extraction Spin Columns, and then concentrated and buffer exchanged using an Amicon ultra-0.5 centrifugal filter 10K with four 400-µl rinses of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Finally, to remove the residual agarose, we performed a second Qiagen Qiaquick PCR purification with 2 additional PE wash steps and eluted the DNA from the column with TE.

Cloning and purification of Cys-DIG10.3

A tag with the amino acid sequence MCGGS was introduced at the N-terminus of DIG10.3 by Kunkel mutagenesis[1] with mutagenic oligonucleotide (Table S2) and DIG10.3/pET29b template[2] to yield Cys-DIG10.3. Following mutagenesis, the Kunkel reaction mixture was transformed into XL1-Blue Supercompetent Cloning Cells (Agilent Technologies) and the DNA was extracted (Qiagen plasmid extraction kit) and verified by Sanger sequencing (Genewiz). Cys-DIG10.3/pET29b was transformed into BL21*(DE3) cells (Invitrogen) for expression. Cells were grown in 1 L of TB at 37 °C to an OD_{600} of 0.8 and then protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Induced cultures were incubated at 18 °C for 18 h and then harvested by centrifugation at 4000xRCF for 15 min. Cys-DIG10.3 was purified as previously reported for DIG10.3.[2] Typical final protein yield for Cys-DIG10.3 ranged from 5 to 6 mg per L of culture.

Single-molecule assays

Coverslip and AFM tip functionalization. We prepared DNA bound to PEG-coated coverslips and DIG10.3 bound to PEG-coated AFM tips, using a previously described protocol.[3] Briefly, glass coverslips were irradiated with UV light in a UV ozone cleaner for 30 min prior to functionalization. We then incubated the coverslips in a solution of 0.15 mg mL^{-1} silane-PEG-azide (Nanocs, Inc) dissolved in toluene at 60 °C for 3 h. We next quickly rinsed the coverslips with toluene, isopropanol and ultrapure water. Coverslips were dried using dry nitrogen and stored dry at 4 °C until use. To attach DNA, we applied 20 µL of 635-nm-long DNA at 60 ng $µl^{-1}$ onto the azide-functionalized coverslip and incubated it in a humidity chamber for 16 h at 4 °C. As described above, the DNA was 5'-labeled at one end with DBCO and DIG via PCR. Coverslips were then rinsed 15 times with 1 ml (15 ml total) of PBS buffer [10 mM phosphate buffer (pH 7.4), 140 mM NaCl, 3 mM KCl] to remove unbound DNA. We stored DNA-labeled coverslips in PBS at 4 °C until use. Such DNA surfaces could be reused over multiple days.

For functionalizing the AFM cantilevers, we first removed the gold and chromium layers from our AFM cantilevers [Olympus BioLevers, $k = 6$ pN nm^{-1} (nominal)] to improve force stability.[4] AFM tips were then irradiated with a UV lamp (BondWand, Electro-Lite Corporation) for 1 h and then placed in a solution of 0.15 mg mL^{-1} silane-PEG-maleimide (Nanocs, Inc) dissolved in toluene at 60 °C for 3 h. We next quickly rinsed the cantilevers with toluene, isopropanol and ultrapure water. We then immersed the cantilevers into a 50-µl drop of 150 µM cysteine-modified DIG10.3 in labeling buffer [10 mM phosphate buffer (pH 6.75), 140 mM NaCl, 3 mM KCl, 1 mM TCEP] for 3 h at room temperature and stored at 4 °C overnight. Next, the DIG10.3-coated cantilevers were rinsed in three separate 10-mL beakers filled with PBS for 30 s each. We finally stored the cantilevers in PBS at 4 °C prior to use and between uses across multiple days.

AFM measurements. All AFM measurements were performed in a temperature controlled, closed fluidic chamber on an Asylum Research Cypher ES. We calibrated the sensitivity and the spring constant of each cantilever using standard methods.[5] To initiate the assay, we brought the AFM tip into relatively gentle contact with the surface (100 pN) for 2 s. Next, we retracted the tip from the surface at constant speed (1 µm s^{-1}). To detect if the tip was bound to a DNA molecule, we used a triggering routine that stopped retraction if $F > 20$ pN when the cantilever was at least 150 nm from the surface. If a successful attachment was detected, we then executed an automated alignment routine correction.[6] This routine ensured a vertical stretching geometry by positioning the DNA's attachment point to the coverslip directly underneath its attachment point to the cantilever. After such alignment, we either brought the tip back into gentle contact then retracted at constant pulling speed or initiated the constant force feedback loop from an extension of 150 nm above the surface. After rupture in either of these data-acquisition protocols, we then brought the tip back into gentle contact to initiate another connection. Data was digitized at 50 kHz.

The above data-acquisition protocol was fully automated and could run for more than a day unsupervised (Fig. S1). To do so, we centered the detection laser on the quadrant photodiode every 10 attempts at surface attachment using an automated procedure available on our commercial AFM. Sensitivity calibrations were checked every 50 attempts to account for any drift in sensitivity. Such long runs also required the aforementioned sealed, temperature controlled fluidic chamber that minimized buffer evaporation. Finally, the data-acquisition protocol probed many different lateral surface locations. All AFM experiments were done in PBS at 25 °C. The activity of a DIG10.3-coated tip was not observed to decrease with time, in contrast to mechanically induced degradation of an oxime linkage where such decline in activity was observed.[3] This result implies the DIG10.3 was not irreversible unfolded during DIG10.3-Dig dissociation (if it unfolded at all).

To deduce landscape parameters, we fit the data in Fig. 2B via Mathematica's NonlinearModelFit function using the Levenberg-Marquardt algorithm. The reported uncertainties correspond to the standard error in the fitting parameters. To check the robustness of the deduced parameters, we varied the starting parameters. For instance, we started the fit with v ranging from 0.2 to 0.8 and the fitting routine consistently returned $v = 0.67 \pm 0.02$, implying a robustness in the determination of v.

Optical-trapping measurements. We prepared epoxy-stabilized flow chambers from KOH-cleaned yet unfunctionalized coverslips, slides, and double sticky tape with an approximate volume of 15 µl. We then incubated the flow cells with 1 µM cysteine-modified DIG10.3 in labeling buffer for 1 h at room temperature in a humidity chamber. In this process, the DIG10.3

was passively adsorbed to the surface as is widely used in many surface-coupled DNA assays.[7,8] Afterwards, we rinsed the flow cells four times with 200 µL of PBS. Next, we incubated the flow cells with a blocking buffer [20 mM Tris (pH 7.5), 10 mg mL^{-1} BSA, 0.4% v/v Tween-20] for 1 h to passivate the surface. Next we washed the flow cells with 200 µL of working buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 0.4% Tween-20] and then added 0.3 nM of 2000-nm long DNA that was 5'-labled with Dig and biotin for 2 h. We then rinsed the flows cells twice with 200 µL of working buffer (400 µL total) and added 50 pM of streptavidin-coated polystyrene beads (750-nm diameter) suspended in working buffer for 1 h. Finally, the flow cells were washed twice with 200 µL of working buffer, and mounted into the optical trap.

We stretched the DNA using a previously described optical trap.[9] Briefly, we first visually located a single bead-DNA complex and then performed a 2D lateral centering routine that aligned the DNA's anchor point to the surface with the vertical axis of the optical trap. This routine also screened for single attachments by measuring the elasticity of the DNA and positioned the bead at ~400 nm over the coverslip. For the constant-velocity overstretching experiments, we moved the coverslip at 2 µm s^{-1}. For constant force experiments, we used stage-based force clamp running at 40 Hz. The trap stiffness was 0.7 pN nm^{-1} and 0.4 pN nm^{-1} for the constant-velocity and the constant-force assays, respectively.

Control experiment for specific attachment via DIG10.3:

We wanted to verify that attachment to the AFM cantilever occurred via the specific DIG10.3•Dig interaction, given the prevalence of nonspecific attachment in AFM assays. We minimized the potential for non-specific sticking by using PEG-coated AFM tips and coverslips as well as applying a low force by AFM standards (100 pN) during surface contact. To verify that DIG10.3 proteins on the cantilever tip were selectively binding to the Dig-labeled DNA on the surface, we performed an anti-Dig passivation experiment, a standard control.[10]

A 20-µL drop of 635-nm-long DBCO-DNA-Dig (60 ng $µL^{-1}$) in TE buffer] was deposited on an azide functionalized surface and incubated in a humidity chamber for 16 h at 4 °C. Samples were then rinsed 15 times with 1 ml of buffer (15 mL total of PBS) to remove unbound DNA. Before anti-Dig passivation of the DBCO-DNA-Dig, a DIG10.3 functionalized AFM tip was brought into contact with the surface 100 times. Each surface contact exerted 100 pN on the cantilever for 2 s and occurred at a separate point on the surface as dictated by a search grid. The search grid was a 1×1 $µm^2$ square with 100 nm horizontal and vertical spacing between search points. After each attempt, the tip was retracted from the surface at 2 µm s^{-1}. The resulting force-extension curves were then categorized as no attachments, single attachment, or multiple attachments according to the known mechanical behavior of DNA (Fig. S2A,B). After the initial 100 attempts, 20 µl of 200 µg ml^{-1} anti-Dig was added to the sample and allowed to incubate for 1 h at room temperature. The surface was then probed 100 times again with the same procedure. The resulting force-extension curves were categorized (Fig. S2C). The significant decrease in DNA attachments indicates that AFM tip was selectively binding to the DNA via the Dig moiety.

Supporting Figures & Tables

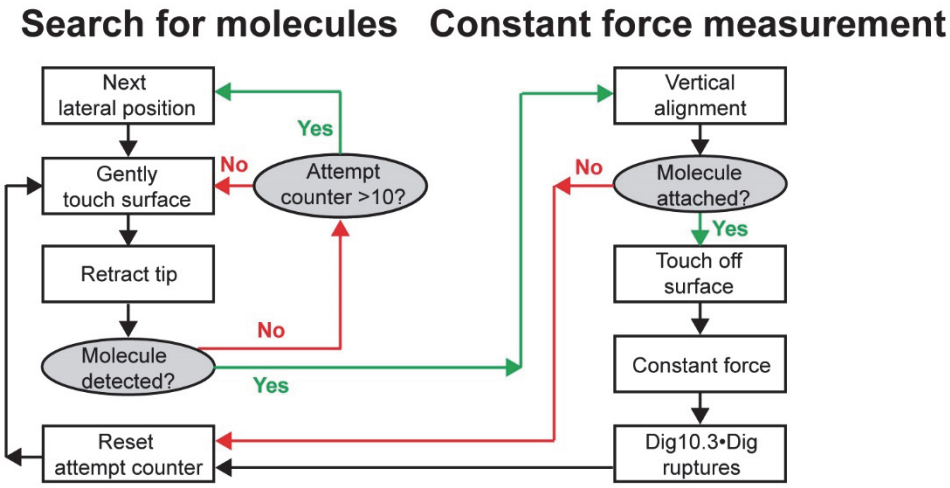

Figure S1. Process diagram for the algorithm automating the acquisition of high-quality constant-force data. The algorithm consists of two sub-routines: (*i*) searching for molecules on the surface and (*ii*) acquiring a vertically aligned constant-force measurement. When searching for molecules, many different lateral locations on the surface are probed in a grid pattern. At each surface location, the assay is initiated by gently pressing the AFM tip into the surface at a low force (~100 pN) and then retracting the tip. Each surface location is probed for a molecular attachment up to 10 attempts before moving onto another location. If a molecule is detected based on a sufficient force at a given extension (*e.g.*, $F > 20$ pN at extension $x > 150$ nm), a vertical alignment algorithm[6] is initiated. If the molecule is still attached to the AFM tip after vertically alignment, the tip is gently pressed into the surface again and then retracted until the Dig10.3•Dig is held under constant force. A drop in force to $F \approx 0$ pN signals dissociation of the Dig10.3•Dig. Successful attachment resets the attempt counter and the same surface location will be probed again up to an additional 10 times in an attempt to reattach to the same DNA molecule. As before, if a surface location is probed 10 times without attaching to a molecule, the sample surface is moved to the next lateral surface position.

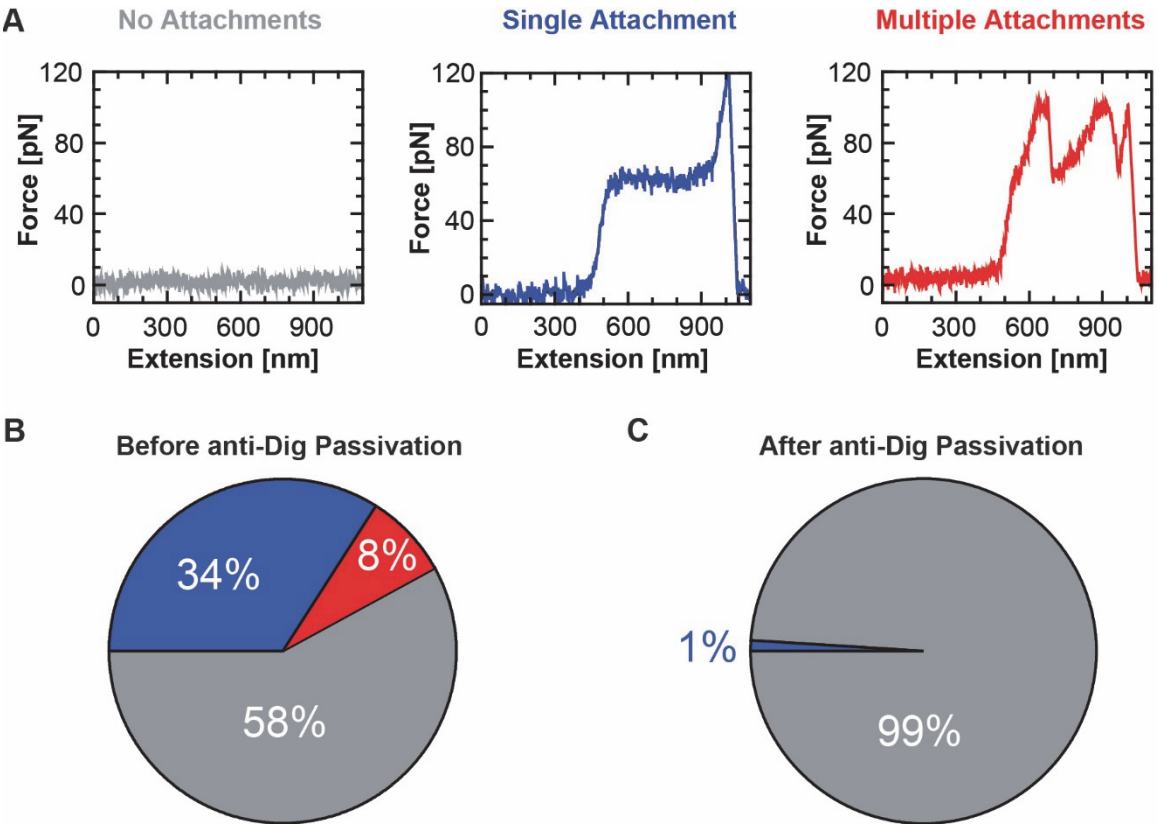

Figure S2. Control experiment demonstrating site-specific attachment via the DIG10.3•Dig interaction. (A) Force-extension curves were categorized into one of three groups: no attachment (grey), a single attachment (blue), and multiple attachment (red). (B) Pie chart of the types of molecular attachment to the AFM tip before the anti-Dig passivation experiment ($N_{attempts}$ = 100). (C) Pie chart of the types of molecular attachment to the AFM tip after the passivation with anti-Dig ($N_{attempts}$ = 100).

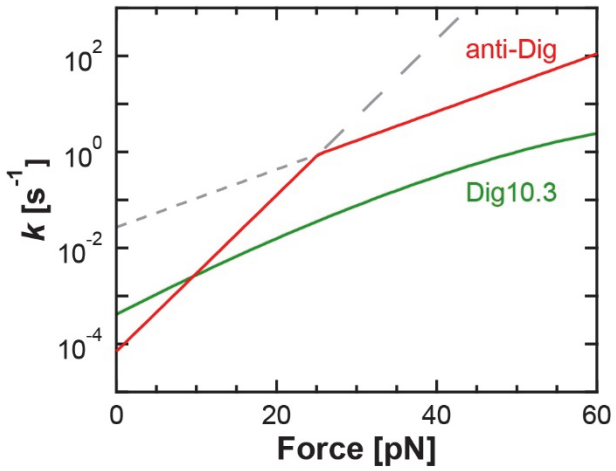

Figure S3. Comparison of the dissociation rate constant (k) as a function of force between previous results for anti-Dig bound to Dig (red)[11] and our results with DIG10.3 (green, solid line). In the anti-Dig work, unbinding over two barriers were determined via a dynamic force spectroscopy assay. We therefore color code the rate limiting step in this two-step process (red). For completeness, we show the projected kinetics of non-rate limiting step for anti-Dig dissociation (gray). Summarizing, below 10 pN, anti-Dig is predicted to have a longer interaction lifetime. Above 10 pN, DIG10.3 should have the longer interaction lifetime.

Primer	**Sequence**
PL4-DBCO	5′-[DBCOTEG]AGT TGT TCC TTT CTA TTC TCA CTC CGC-3'
PR4-Dig	5′-[DigN]AAA CCA AGT ACC GCA CTC ATCG-3'
PL9-Bio	5'-[BioTEG]AAA GCA TTT GAG GGG GAT TC-3'
PR1-Dig	5'-[DigN]TCG GTG CGG GCC TCT TCG CTA TTAC-3'

Table S1. Primers used for 650-nm and 2000-nm DNA constructs.

Primer	**Sequence**
Cys-DIG10.3/pET29b	5′GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG TGC GGT GGT TCC ATG AAT GCT AAA GAA ATT GTT GTC CAC GCT CTC CG-3′

Table S2. Mutagenic oligonucleotide for cysteine modification of DIG10.3.

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