DNA nanoswitches: a quantitative platform for gel-based biomolecular interaction analysis

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We introduce a nanoscale experimental platform that enables kinetic and equilibrium measurements of a wide range of molecular interactions using a gel electrophoresis readout. Programmable, self-assembled DNA nanoswitches serve both as templates for positioning molecules and as sensitive, quantitative reporters of molecular association and dissociation. We demonstrated this low-cost, versatile, ‘lab-on-a-molecule’ system by characterizing ten different interactions, including a complex four-body interaction with five discernible states.

Gel electrophoresis has been a workhorse of biological research for over 50 years, providing a simple way to determine size, topology and quantity of DNA, RNA and protein1–3. However, quantitative kinetic and thermodynamic characterization of molecular interactions on gels remains a challenge. For example, electrophoretic mobility shift assays are primarily used for qualitative analysis of protein–nucleic acid interactions4. Quantitative biomolecular interaction analysis typically requires specialized techniques such as surface plasmon resonance (SPR) (for example, Biacore), radiolabeling or isothermal titration calorimetry (ITC), with cost, necessary technical expertise and material requirements sometimes posing barriers to their use (Supplementary Table 1). Furthermore, quantitative analysis of long-lived interactions, small-molecule interactions and multicomponent complexes are difficult even with these advanced approaches.

We introduce an instrument-free platform, based on DNA self-assembly5–7, that meets these challenges by enabling quantitative analysis of molecular interactions using standard gel electrophoresis, for pennies per sample (Supplementary Table 1). DNA oligonucleotides (60 nt) are functionalized with interacting molecules and hybridized to specific locations on an ssDNA scaffold (M13mp18, 7,249 nt) to form DNA nanoswitches.

These nanoswitches report molecular associations and dissociations through induced topological changes. Because DNA can be separated on the basis of topology8, the different interaction states can be easily resolved as distinct bands on a gel (Fig. 1a).

These nanoswitches have several useful features. Their programmable nature enables precise control over relative concentrations and stoichiometries on a per-molecule basis. The large DNA construct causes interaction-triggered topological changes to yield distinct and repeatable gel shifts, even with the integration of large proteins5. Additionally, the large size of the DNA allows for the incorporation of thousands of dye molecules, thereby dramatically amplifying the signal per interaction and making read-out of the nanoswitches orders of magnitude more sensitive than with most other techniques (Supplementary Table 1). Together these features make DNA nanoswitches a versatile, accessible and inexpensive tool for studying multimolecular interactions.

By monitoring changes in the nanoswitch states over time, we can determine equilibrium and kinetic rate constants for a variety of molecular systems using standard gel electrophoresis. Loop closure over time is used to determine association rate constants, whereas loop opening over time, in the presence of a competitor, is used to determine the dissociation rate constant (Fig. 1b,c and Supplementary Fig. 1). These kinetic processes take place in solution and are ‘quenched’ to halt kinetics at various time points, with the gel acting as a post-experiment readout, enabling experimental conditions that are independent of gel running conditions. Ease of readout and other nanoswitch characteristics can be optimized by tuning key design parameters, including oligonucleotide length, ligand positioning, reaction concentrations and temperatures (Online Methods).

We first assessed the nanoswitch platform using the ubiquitous biotin–streptavidin system. At physiological salt conditions and 25 °C, we measured a dissociation time of 9.7 ± 0.4 days (all values are reported as the error-weighted fit parameter ± its 1σ confidence interval), closely matching previously reported values9. To demonstrate parallel exploration of a broad range of experimental conditions, we obtained off-rates at 16 different conditions by measuring the fraction dissociated at six time points per condition and running all 96 samples on a single gel (Supplementary Fig. 2). Each condition showed exponential decay over time, yielding 16 uniquely determined off-rates ranging from 0.8 h to 3 months with an uncertainty typically <10% (Supplementary Fig. 2 and Supplementary Table 2). Dissociation kinetics varied nearly 1,000-fold over our temperature range (4–50 °C) but only about twofold over our salt range (25–500 mM) (Fig. 1d and Supplementary Fig. 3). On the basis of these results, we present

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a semi-empirical model for dissociation kinetics between streptavidin and biotin-labeled oligonucleotides from 25 to 50 °C and 25 to 500 mM NaCl:

$$k_{\text{off}} = T e^{\left(\frac{18,300}{T} - 0.033 \frac{f}{T}\right)}$$

where $k_{\text{off}}$ is the value of the off-rate in s$^{-1}$, $T$ is the value of the absolute temperature in kelvin, and $f$ is the value of the ionic strength of the solution in micromolar concentration (Online Methods and Supplementary Fig. 3d). This model does not describe the behavior at 4 °C, presumably owing to temperature-dependent changes in heat capacity$^{10}$.

We measured on-rate kinetics for the biotin-streptavidin interaction, at a variety of temperatures, by monitoring loop formation over time. Loop closure occurs through two separate binding events: the binding of a molecule from solution to the nanoswitch and then the closing of the loop. Thus, we fit loop-closure data to a two-step kinetic model to extract these rates (Fig. 1b,d, Online Methods, Supplementary Table 3 and Supplementary Fig. 1). At 150 mM salt, we measured a room-temperature (25 °C) on-rate of $(4.0 \pm 0.7) \times 10^6$ M$^{-1}$ s$^{-1}$. Combining our on-rate and off-rate measurements, we calculated a dissociation constant of $(2.94 \pm 0.51) \times 10^{-13}$ M, an equilibrium free energy change, $\Delta G^0$, of $-17.1 \pm 0.1$ kcal mol$^{-1}$ and an equilibrium enthalpy change, $\Delta H$, of $26.01 \pm 0.05$ kcal mol$^{-1}$ (Supplementary Table 3). In general, our measurements are consistent with values reported in the literature (Supplementary Table 2). Specifically, we are within 15% of the reported off-rate of a biotin-labeled oligonucleotide$^{8}$, within 30% of on-rate measurements from SPR$^{11}$ and within 5% of both equilibrium $\Delta H$ measurements by ITC$^{12}$ and equilibrium $\Delta G$ measurements made by monitoring kinetics of radiolabeled biotin$^{13}$.

Without modifying the DNA construct, we were also able to measure kinetic and equilibrium properties for avidin and NeutrAvidin (Supplementary Table 3). Although NeutrAvidin's affinity for biotin was 20 times weaker than avidin's, they surprisingly had similar off-rates, a result underscoring the limitation of relying solely on affinity measurements to characterize an interaction.

To demonstrate the ability to measure weaker interactions, we incorporated desthiobiotin, a biosynthetic precursor to biotin that binds streptavidin with far lower affinity, into our nanoswitch$^{14}$. By optimizing gel running conditions, we resolved the looped and unlooped constructs in as little as 6 min, measuring the off-rate of streptavidin-desthiobiotin as $35.3 \pm 7.5$ min at 4 °C and $8.6 \pm 1.2$ min at room temperature (Supplementary Fig. 4). We note that although the system is ideal for quantification of long-lived interactions, even those out of the range of SPR, the time required to resolve the bands in a gel currently sets the lower limit of detectable dissociation lifetimes to minutes.

The modularity of the DNA nanoswitch facilitates the easy incorporation of different types of molecules. We exploited this feature to measure several biologically relevant interactions including a covalent bond taking weeks to dissociate (Fig. 2a), a protein–small-molecule interaction dissociating over days (Fig. 2b), an antibody-antigen interaction taking hours to dissociate at room temperature (Fig. 2c) and a 20-bp DNA oligonucleotide dissociating over hours at 50 °C (Fig. 2d). Additionally, we were able to study peptide ligation kinetics with a time constant of minutes (Fig. 2e) and restriction enzyme cleavage over seconds (Fig. 2f). As with many techniques including SPR, assay preparation requires the derivatization of at least one molecule of interest. Here, we attached our molecules of interest to a DNA oligonucleotide, which can be accomplished using a variety of techniques. In addition to sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate cross-linking$^{4}$, we previously described the use of click chemistry to attach peptides to oligonucleotides and the use of the enzyme sortase$^{15}$ to rapidly and efficiently attach proteins to our nanoswitches while preserving protein function$^{16}$.

The platform’s versatility is facilitated by its universal gel readout. An extreme example of this is our characterization of the
reduction of a disulfide bond at 25 °C in 10 μM TCEP (Tris(2-carboxyethyl)phosphine) yielding a time constant of 2.6 ± 0.4 weeks (Fig. 2a). Because the signal per molecule is dependent on only the nanoswitch size, this two-atom system yielded the same level of signal per interaction as a 150-kDa antibody binding to its antigen (Fig. 2c).

Additionally, the programmability of these nanoswitches enables multiple topological states to be individually distinguished on a gel, thereby facilitating the analysis of complex multicomponent interactions (Fig. 3). We engineered nanoswitches with three integrated ligands, placed strategically to form two asymmetric loops when simultaneously bound by a bispecific receptor. The resulting nanoswitch adopted five resolvable states that could be identified with control experiments (Fig. 3a and Supplementary Fig. 5).

We measured bidirectional transitions for all five states, thus determining all rate constants (Fig. 3, Supplementary Figs. 6 and 7 and Supplementary Table 4). This ability to monitor the fraction of molecules populating each state over time would be difficult or impossible to achieve with most other measurement techniques.

Our approach enables low-cost, accessible and parallel multicomponent biomolecular interaction analysis using a basic laboratory technique, gel electrophoresis. We have demonstrated our platform’s ability to characterize interactions with time constants ranging from seconds to months (approximately six orders of magnitude), for a wide variety of molecular interactions, temperatures and buffer conditions. The signals were robust and highly amplified, giving detection limits in the range of attomoles and...
allowing quantitative kinetic and thermodynamic analysis of proteins as shown here with femtomoles of material (~1 ng for a 50-kDa protein). In contrast to other techniques that provide one signal to analyze (for example, SPR, radiolabeling and ITC), our technique has the ability to perform independent measurement of five signals simultaneously, allowing complete characterization of a complex five-state system. The modularity and programmability of the nanoswitches affords control over the relative concentrations and stoichiometries of interacting components, independent of the nanoswitch concentration. This feature suggests that, in addition to monitoring reactions, nanoswitches could be used as a template-directed synthesis technique to control complex reactions. Overall, this unique lab-on-a-molecule platform will be a powerful research tool, accessible to anyone able to perform gel electrophoresis.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
The initial idea was conceived by K.H. and W.P.W. Experiments were designed by all authors. The method was expanded by M.A.K. and A.W., and experiments were carried out by K.H., M.A.K. and A.W. All authors participated in data analysis, critical discussion and writing of the manuscript.

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The authors declare competing financial interests: details are available in the online version of the paper.

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1. Thorne, H.V. Virology 29, 234–239 (1966).
2. Bishop, D.H.L., Claybrook, J.R. & Spiegelman, S. J. Mol. Biol. 26, 373–387 (1967).
3. Smithies, O. Biochem. J. 61, 629–641 (1955).
4. Hellman, L.M. & Fried, M.G. Nat. Protoc. 2, 1849–1861 (2007).
5. Halvorsen, K., Schaak, D. & Wong, W.P. Nanotechnology 22, 494005 (2011).
6. Saccà, B. & Niemeyer, C.M. Angew. Chem. Int. Ed. Engl. 51, 58–66 (2012).
7. Seeman, N.C. Annu. Rev. Biochem. 79, 65–87 (2010).
8. Aaij, C. & Borst, P. Biochim. Biophys. Acta 269, 192–200 (1972).
9. Levy, M. & Ellington, A.D. Chem. Biol. 15, 979–989 (2008).
10. Prabhu, N.V. & Sharp, K.A. Annu. Rev. Phys. Chem. 56, 521–548 (2005).
11. Qureshi, M.H., Yeung, J.C., Wu, S.C. & Wong, S.L. J. Biol. Chem. 276, 46422–46428 (2001).
12. Kumb, L.A., Chu, V. & Stayton, P.S. Biochemistry 37, 7657–7663 (1998).
13. Chivers, C.E. et al. Nat. Methods 7, 391–393 (2010).
14. Florin, E.L., Moy, V.T. & Gaub, H.E. Science 264, 415–417 (1994).
15. Chen, I., Dorr, B.M. & Liu, D.R. Proc. Natl. Acad. Sci. USA 108, 11399–11404 (2011).
16. Kousa, M.A., Sotomayor, M. & Wong, W.P. Methods 67, 134–141 (2014).
ONLINE METHODS

We provide a Supplementary Protocol that includes lists of reagents needed and detailed step-by-step instructions for performing on-rate and off-rate experiments. Additionally, to facilitate getting started, materials will be available upon request as detailed in section 2 of the Supplementary Protocol.

General nanoswitch formation. The nanoswitches were constructed as previously described in detail. Circular ssDNA from the 7,249-nt bacteriophage M13 (New England BioLabs) was linearized by enzymatic cleavage of a single site using BtsCI (New England BioLabs) and a site-specific oligonucleotide. Oligonucleotides (from Bioneer or Integrated DNA Technologies (IDT)) were designed to complement the linearized M13 DNA along the backbone, resulting in 120 60-nt oligonucleotides and a single 49-nt oligonucleotide. The first and last oligonucleotide along with ten evenly distributed oligonucleotides are intended to be interchangeable and will be referred to as variable oligonucleotides (var 1–12, with var 1 representing the first oligonucleotide var 12 representing the last oligonucleotide) — see Supplementary Protocol, Supplementary Data and Supplementary Tables 5 and 6). These variable oligonucleotides were stored separately from the remaining 109, referred to as backbone (bb) oligonucleotides, which were mixed in equimolar concentration in a single tube. Mixing a molar excess of the oligonucleotides (10:1 unless otherwise noted) with the ssDNA scaffold and subjecting the mixture to a temperature ramp (from 90 to 20 °C at 1 °C min−1 unless otherwise noted) produced dsDNA. Final constructs were spiked with a low concentration of DNA ladder (BstNI digest of pBR322 DNA, New England BioLabs) to aid in quantification. For many experiments the DNA ladder was linearized by enzymatic cleavage of a single site using BtsCI (Invitrogen) for a minimum of 30 min before being imaged with 0.7% agarose gels, cast from LE agarose (Seakem) or Ultrapure Agarose (Life Technologies) dissolved in 0.5× Tris-borate EDTA (TBE) (Bio-Rad). Before loading, samples were mixed with a Ficoll-based loading solution (Promega), which we found to give sharper bands than glycerol-based loading dyes, simplifying quantification. Gels were run for 90–100 min at 4 V cm−1, unless otherwise noted, and subsequently stained in 1× SYBGRGold stain (Invitrogen) for a minimum of 30 min before being imaged with.

There are three concentrations that can be independently tuned in an on-rate experiment. There is the concentration of the scaffold, the concentration of the receptor, and the effective concentration between the two ligands on the polymer. If these concentrations are adjusted carefully, many problems can be avoided. For example, if the effective concentration between the two tethered ligands is significantly higher than the concentration of the receptor, then one can minimize capping (the binding of two receptors to a single scaffold resulting in an unloopable construct). We note, however, that because our model accounts for capping, the values obtained outside this optimal regime will still be correct; the looped-band intensities will simply be weaker, resulting in a lower signal-to-noise ratio. Although not usually a problem, one can avoid higher order aggregation by ensuring that the scaffold concentration is significantly lower than the effective concentration between the two ligands on the scaffold. One can also simplify the analysis by selecting a receptor concentration that is significantly higher than the scaffold concentration so that the receptor concentration stays effectively constant over the course of the experiment. Following these experimental design principles, in our experiments using variable oligonucleotides 4 and 8, the effective concentration between the two ligands on the loop is ~30 nM, the scaffolds are used at a concentration of 80 pM, and the receptor is used at a nominal concentration of 3 nM (Supplementary Table 8).

In addition to the ratio of concentrations, there are some important lower and upper limits of concentration to keep in mind. We have found that working with protein concentrations below 1 nM can be unreliable owing to losses of protein to the walls of the tubes. We have performed on-rate experiments with streptavidin concentrations as low as 0.3 nM, but losses of protein can be as high as 80% even in protein LoBind tubes (Eppendorf technical data sheet). Unless a means of eliminating protein loss to tubes and pipette tips is implemented, we do not recommend working below 1 nM. The upper limit is not a hard limit. We have found that the on-rate for streptavidin is very fast at 30 nM, making it difficult to pipette fast enough to take multiple time points before the plateau. If one has a means of more rapidly mixing solutions (i.e., microfluidics), or a protein with a slower on rate, higher protein concentrations can be used. We have found that 3 nM provides a nice middle ground, though one may wish to optimize the protein concentration used according to the speed of mixing and the solution on-rate of the protein being studied.

Following these design principles and those laid out in ref. 5 is key to the successful use of this platform. The Supplementary Protocol provides information on reagents needed and detailed step-by-step instructions on how to successfully perform on-rate and off-rate experiments.

Electrophoretic conditions. All looped constructs were run in 0.7% agarose gels, cast from LE agarose (Seakem) or Ultrapure Agarose (Life Technologies) dissolved in 0.5× Tris-borate EDTA (TBE) (Bio-Rad). Before loading, samples were mixed with a Ficoll-based loading solution (Promega), which we found to give sharper bands than glycerol-based loading dyes, simplifying quantification. Gels were run for 90–100 min at 4 V cm−1, unless otherwise noted, and subsequently stained in 1× SYBGRGold stain (Invitrogen) for a minimum of 30 min before being imaged with.
a gel imager (Bio-Rad) or laser gel-scanner (GE Typhoon). It is important to note that the standard output file of this imager is often set to a .gel file, which has a nonlinear intensity scaling. Such .gel images can be linearized using the ImageJ “Linearize GelData” plug-in (http://rsb.info.nih.gov/ij/plugins/linearize-gel-data.html). Alternatively, the gel image can be saved as a linear.tif file off of the imager. We would like to point out that these expensive imagers are not required for quantification, and we obtained similar results using a blue transilluminator (Invitrogen) and a point-and-shoot camera (Canon S95).

**Biotin-streptavidin nanoswitch experiments.** This construct used biotinylated versions of two oligonucleotides (var 4 and var 8), which were used in 4× molar excess to the scaffold, and all other oligonucleotides were used in a 10× molar excess. The reason for this lesser amount is twofold: (i) to be less wasteful of the more expensive functionalized oligonucleotides and (ii) because excess biotin oligonucleotide in solution could interfere with our measurements. The final DNA construct was then diluted 100× from its original concentration of ~16 nM (to 160 pM) and mixed in equal volumes with streptavidin (Rockland) at 6 nM nominal concentration to form the loops, yielding final nominal scaffold and streptavidin concentrations of ~80 pM and 3 nM, respectively.

On-rate experiments were performed by mixing equal volumes of 160 pM DNA construct with a nominal 6 nM streptavidin concentration, followed by taking 10-µL aliquots of the mixture at various times and mixing them with 1 µL of a saturated biotin solution to quench the formation of loops. The 25 °C experiment was performed at room temperature, the 4 °C experiment was performed in a cold room, and the 37 °C and 50 °C experiments were performed using a thermal cycler. For on-rate experiments, using low-binding tubes (Eppendorf LoBind) was important for getting repeatable results owing to significant streptavidin adsorption to the tubes when incubated at 6 nM. Actual concentrations used to determine the on-rates were measured using spectrophotometry and a HABA assay to determine streptavidin activity. We found that the actual streptavidin concentration was within 10% of the nominal concentration, and over 85% of the protein was active on the basis of the HABA assay.

Off-rate measurements were performed by forming looped construct as described above and letting the solution sit for at least 24 h to allow the system to reach equilibrium. Aliquots of the looped construct were mixed at various times with a quenching solution consisting of biotin and sodium chloride to achieve the proper experimental salt concentrations and were immediately put at the experimental temperature. The 4 °C condition was done in a refrigerator, the 25 °C sample was done in a water bath, and the 37 °C and 50 °C temperatures were done in a thermal cycler. To run all the samples on a single gel, the quenching times were determined relative to the predetermined gel running time.

Preparations with avidin and NeutrAvidin were prepared in the same way, but protein concentrations were sometimes altered to enable on-rate measurements over a similar timescale to that of the streptavidin experiments.

**Desthiobiotin-streptavidin.** Desthiobiotin experiments were conducted in a similar manner to the biotin experiments with slight modifications. The var 4 oligonucleotide was changed to a desthiobiotin-functionalized oligonucleotide, whereas the var 8 oligonucleotide remained biotin functionalized. The off-rate of the desthiobiotin interaction is much faster than the typical 100-min gel run time. Noting that once a loop opens in the gel, the reptation of the DNA prevents the loop from closing again, we ran samples for different amounts of time in the gel at 15 V cm⁻¹ and 4 °C and quantified the fraction looped as a function of running time (Supplementary Fig. 4). In addition to allowing the determination of the desthiobiotin-streptavidin off-rate, this gel also allowed us to determine the minimum amount of time required to achieve separation of the looped and unlooped bands in the gel. This enabled the use of the standard quenching technique for measuring desthiobiotin off-rates as described in the previous section; these gels were run at 15 V cm⁻¹ for 10 min in prechilled electrophoresis buffer.

**DNA hybridization experiments.** This construct used a 50-nt ‘bridge’ oligonucleotide to span the last 30 nt of the var 4 region and the first 20 nt of the var 8 region. Thus, the normal var 4 and var 8 oligonucleotides were omitted from the mixture and replaced with three oligonucleotides: the aforementioned bridge oligonucleotide and two small ‘filler’ oligonucleotides to fill the remaining bases so that the M13 scaffold would be fully hybridized. In this case, the bridge oligonucleotide was added in equimolar concentration with the scaffold strand, whereas the other oligonucleotides remained at 10× molar excess. Off-rate measurements were quenched with 500 nM 20-nt oligonucleotide corresponding to the loop-closure site. Kinetics were accelerated by performing the measurement at 50 °C.

**Enzyme cleavage experiments.** These constructs were made as described above but with a bridge oligonucleotide containing an inserted sequence recognized by the XhoI enzyme (New England BioLabs). The complement to this restriction sequence was also used to ensure that this region was double-stranded. Cleavage measurements were performed by adding enzyme to the loops (with final concentrations of 2.2 nM and 1,000 units mL⁻¹ for the loops and enzyme, respectively) in the recommended buffer (New England BioLabs) and quenching the enzyme activity with 75 mM EDTA at various times at room temperature.

**Antibody-antigen experiments.** This construct used a 3’ digoxigenin–labeled version of the var 8 oligonucleotide (Integrated DNA Technologies) and a 5’ anti-dig–labeled version of the var 4 oligonucleotide. The antibody-labeled oligonucleotide was made by chemically cross-linking a free amine on the antibody (polyclonal sheep antibody from Roche) to a thiol-labeled oligonucleotide and purified by electroelution as described previously. The construct was made with two annealing steps. First, all the oligonucleotides with the exception of the antibody-labeled oligonucleotide were mixed with the scaffold strand and annealed following our standard protocol described above (except a 1:1, rather than 10:1, molar ratio was used for the digoxigenin oligonucleotide). Second, the purified antibody oligonucleotide was added in a 1:1 molar ratio and annealed from 37 °C to 4 °C at 0.5 °C min⁻¹ to facilitate annealing of the antibody-modified var 4 oligonucleotide. Off-rate measurements were performed by quenching with 335 nM of antibody at various times at room temperature.
Sortase-catalyzed peptide ligation experiments. This construct was created in three steps. (1) Var 4 and var 5 oligonucleotides with a 3′ and a 5′ azide, respectively, were functionalized with sortase-compatible peptides. (2) These two oligonucleotides were linked together with sortase. (3) The peptide-bridged oligonucleotides were hybridized onto the DNA nanoswitch. All custom peptides were purchased from NeobioLab.

1. To create the sortase-compatible oligonucleotides, sortase-compatible peptides were covalently attached using click chemistry as previously described. Pra-LPETGHHHHHHH, where Pra is a propargyl glycine, which adds an alkyn functionality, was coupled to var 4–azide using copper-catalyzed click chemistry. Azide–var 5 was then functionalized with a Flag-TEV-GGG-Pra peptide, where Flag denotes a Flag tag and TEV denotes a cleavage site for the tobacco etch virus protease. After the click chemistry the oligonucleotides were processed with a Qiagen nucleotide-removal kit and run on a polyacrylamide gel. The bands corresponding to the peptide-oligonucleotide chimeras were cut out, and the products were extracted via electroelution as previously described.

2. Once purified, the Flag-TEV-GGG-var 5 was treated with TEV (Sigma) and the two oligonucleotides were concentrated as previously described. These oligonucleotides were then at a concentration of ~10 μM as judged by running on a precast 4–20% gradient polyacrylamide TBE gel (Bio-Rad). Equal volumes (10 μL each) of the sortase-compatible oligonucleotides were mixed with 5 μL of 14.1 mg mL⁻¹ sortase, and 25 μL of 2× sortase reaction buffer (600 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM MgCl₂, and 10 mM CaCl₂). This was allowed to sit for 3 h at room temperature before we ran it on a polyacrylamide gel and purified the dimer band via electroelution, yielding var 4-LPETGGG-var 5. (Note that the GGG indicates the amino acid string Gly-Gly-Gly.)

3. The var 4-LPETGGG-var 5 was used instead of the normal var 4 and var 5. This was annealed onto the linear M13 backbone at a 1:1 ratio and was added along with the other oligonucleotides at the beginning of the annealing. This yielded loops with the peptide LPETGGG bridging variable regions 4 and 5.

With these loops in hand, we could observe loop opening as a result of TCEP reduction of the disulfide bond. To accomplish this, we mixed equal volumes of 20 μM TCEP and 160 nM loops, both of which were diluted in NEB buffer 2, at different time points before running the gel. Seven time points were collected over 10 d at room temperature before running the gel.

Multistate loops. The bispecific receptor was formed by using a lightning link kit (Innova Biosciences) to attach streptavidin to sheep polyclonal anti-dig (Roche 11333089001). The anti-dig, suspended in PBS, was added in a 1:1 ratio to the streptavidin, and the kit protocol was followed exactly. This was then diluted 1:1.250 into NEB buffer 2 with added 150 mM NaCl before use in forming multistate loops. The multistate loop was formed by using var 4 with a 3′ biotin, var 8 with a 5′ digoxigenin, and var 12 with 3′ digoxigenin in place of the normal var 4, 8, and 12 oligonucleotides. On-rate and off-rate measurements were performed using the same procedure used for the biotin-streptavidin experiments with slight modifications. Rather than adding streptavidin, we added the diluted bispecific receptor, and samples were quenched with 2 μL of 5 μM digoxigenin-functionalized oligonucleotide (an oligonucleotide was used, as digoxigenin is not water soluble) suspended in a saturated biotin solution. Gels were run at 6.25 V cm⁻¹ for 125 min with buffer chilled to 4 °C before running.

Gel image analysis. We analyzed gel images in one of two ways.

1. All non-multistate gels (with only two bands) were analyzed in the following way. The amount of material in each gel band was quantified by analyzing the scanned gel images with the gel analysis tool in the freely available ImageJ software package. Using rectangular regions of interest that capture just the width of the gel bands, this toolbox produces intensity profiles whose area can be measured to quantify the total brightness in each band. We applied the same rectangular window size to each lane within a single gel. In many gels the highest–molecular

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weight band of the added ladder was used as a normalizing reference lane. This relaxed the constraints of pipetting perfectly across all lanes.

2. All multistate gels (with five bands) were analyzed as follows. A custom Matlab interface, available upon request, was developed for fitting the intensity profiles of the imaged gel bands. The software interface was modeled after the ImageJ interface. Rectangular boxes were drawn around each lane to define a region of interest. Median filtering is a common technique used to remove speckle noise in images. Rather than filtering the entire image, each individual lane was median filtered by row to remove speckle noise without sacrificing resolution in the direction of band migration. After plotting the median-intensity profile, we subtracted the background using a 4- to 6-point piecewise linear function to outline the background. The background was found to be very similar across lanes, and often the same background profile could be subtracted from the majority of the lanes. Once the profiles were extracted, least-squares fitting of each profile to the model was performed in Matlab. Individual bands run on their own show a skewed Gaussian profile, also known as a skew normal distribution, with a skew parameter of \( \pm 2.5 \) (Supplementary Fig. 5). Thus, the entire multistate median-intensity profile (from just above the highest band to just below lowest) was fit using a sum of five skewed Gaussians. A common skew parameter was used for all five bands, and a common initial guess of band width was used with a fitting range of \( \pm 10 \) pixels. These input parameters allowed for converging fits across all lanes and resulted in fits that closely matched the observed intensity profiles (Fig. 3a). The areas of the individual bands were calculated by integrating the individual skewed Gaussians. Error in the fitted areas was estimated by calculating the areas within the 1\( \sigma \) confidence interval of the fit parameters. These areas were all normalized by the total area (the sum of all of the skewed Gaussian areas). The identities of the bands were validated by analyzing gels in which individual loops in known ratios—the measured values of the individual bands were found to be within 10% of their true values.

We used the following equation for a skew normal/skewed Gaussian distribution

\[
A e^{-\left(\frac{x-b}{c}\right)^2 \left(1 + \text{erf}\left(\frac{x-b}{a/c}\right)\right)}
\]

**Data analysis.** On the basis of a gel we ran to establish repeatability of pipetting and imaging, we conservatively estimate the error per lane at \( \pm 5\% \) plus the detection limit (which will vary by imager). For lanes that used a reference band to normalize brightness, the 5\% error per band was propagated to yield roughly 7\% error per measurement. Error bars were produced on the basis of this analysis, and all fitting procedures used an error-weighted least-squares fit. Timed pipetting for on-rate experiments was conservatively assumed to have an error of \( 2 \) s, which was propagated to overall \( y \)-error by multiplying by the derivative of a preliminary fit.

**Model.** The time evolution of DNA nanoswitch states are modeled using multistep reaction kinetics. On rates are modeled as a two-step process

Unbound linear \( \rightarrow \) Singly bound linear \( \rightarrow \) Looped

Step 1 represents the binding of a free receptor in solution to a ligand on the scaffold (yielding the solution on-rate); step 2 represents the subsequent binding of this receptor to another ligand on the same scaffold to form a loop (yielding the loop-closure rate). On-rate and off-rate models for both the two-state and five-state systems are detailed in Supplementary Note 1.

**Thermodynamic analysis.** The dissociation constant \( K_D \) was determined by the ratio of the off- and on-rates, and the equilibrium free energy \( \Delta G^0 \) was determined by

\[
\Delta G^0 = -RT \ln(K_D)
\]

where \( R \) is the gas constant, \( T \) is the absolute temperature, and the dissociation constant, which is determined by dividing the off-rate by the on-rate and is made dimensionless by dividing it by a reference concentration, i.e., \( K_D = K_{D0}/(1M) \). We additionally used Eyring analysis to fit the temperature dependence of the kinetic rates

\[
\ln\left(\frac{k}{T}\right) = -\frac{\Delta H}{R} \left(\frac{1}{T}\right) + \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S}{R}
\]

where \( k \) is the kinetic rate constant, \( k_B \) is the Boltzmann constant, \( h \) is Planck’s constant, and \( \Delta H \) and \( \Delta S \) are the enthalpy and entropy of activation, respectively.

For the salt dependence, we used the kinetic salt relationship

\[
\log(k) = \log(k_0) + 2A \times Z_A \times Z_B \sqrt{I}
\]

where \( k \) is the kinetic rate constant, \( k_0 \) is the rate constant without the salt, \( A \) is the Debye-Hückel constant, \( Z_A \) and \( Z_B \) are the charges on the two interacting species, and \( I \) is the ionic strength of the solution (Supplementary Note 2).

17. Strunz, T., Orozlan, K., Schäfer, R. & Güntherodt, H.J. Proc. Natl. Acad. Sci. USA 96, 11277–11282 (1999).