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DNA Nanoswitches: A quantitative platform for gel-based biomolecular interaction analysis

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Abstract

We introduce a nanoscale experimental platform that enables kinetic and equilibrium measurements of a wide range of molecular interactions by expanding the functionality of gel electrophoresis. Programmable, self-assembled DNA nanoswitches serve both as templates for positioning molecules, and as sensitive, quantitative reporters of molecular association and dissociation. We demonstrate this low cost, versatile, “lab-on-a-molecule” system by characterizing 10 different interactions, including a complex 4-body interaction with 5 discernable states.

Keywords

DNA Origami; Nanoswitch; Multimolecular Interactions; Biomolecular Interaction Analysis; Kinetics; Affinity; Gel Electrophoresis

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 protein[1,2,3].

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Author contributions:

The initial idea was conceived by K.H. and W.P.W. Experiments were designed by all authors. 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.

Competing financial interests: Patent applications have been filed for various aspects of this work by KH, MAK and WPW

However, quantitative kinetic and thermodynamic characterization of molecular interactions on gels remains a challenge. For example, electrophoretic mobility shift assays (EMSA) are primarily used for qualitative analysis of protein-nucleic acid interactions[4]. Quantitative biomolecular interaction analysis typically requires specialized techniques such as Surface Plasmon Resonance (SPR) (e.g. Biacore), radiolabeling, or Isothermal Titration Calorimetry (ITC), with cost, required 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 multi-component complexes are difficult, even with these advanced approaches.

We introduce a new instrument-free platform, based on DNA self-assembly[5,6,7], 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 a single-stranded DNA scaffold (M13mp18, 7,249 nt). These DNA nanoswitches report molecular associations and dissociations through induced topological changes. Exploiting the ability to separate DNA based on topology[8], the different interaction states can be easily resolved as distinct bands on a gel (**Fig. 1a**).

These nanoswitches have several important 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 proteins[5]. Additionally, the size of the DNA allows for the incorporation of thousands of dye molecules, dramatically amplifying the signal per interaction, and making readout of the nanoswitches orders of magnitude more sensitive than most other techniques (**Supplementary Table 1**). Together, these features make this 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, while loop opening over time, in the presence of a competitor, is used to determine the dissociation rate-constant (**Fig. 1b, 1c, and Supplementary Fig. 4**). 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 one-sigma confidence interval), closely matching previously reported values[9]. To demonstrate parallel exploration of a broad range of experimental conditions, we measured off-rates at 16 different conditions, by measuring the fraction dissociated at 6 time points per condition, and running

all 96 samples on a single gel (**Supplementary Fig. 1**). Each condition showed exponential decay over time, yielding 16 uniquely determined off-rates ranging from 0.8 hours to 3 months with an uncertainty typically less than 10% (**Supplementary Fig. 1 and Table 2**). Dissociation kinetics varied nearly 1,000 fold over our temperature range (4-50 °C) but only about 2 fold over our salt range (25-500 mM) (**Fig. 1d and Supplementary Fig. 2**). Based on these results, we present a semi-empirical model for dissociation kinetics between streptavidin and biotin-labeled oligonucleotides from 25 °C to 50 °C and 25 mM to 500 mM NaCl:

$$k_{off} \approx T e^{\left(42.4 - \frac{18300}{T} - 0.033\sqrt{I}\right)}$$

Where k_{off} is the value of the off-rate in s^{-1}, T is the value of the absolute temperature in K, and I is the value of the ionic strength of the solution in mM (**online methods and Supplementary Fig. 2d**). This model does not describe the behavior at 4 °C, presumably due to temperature dependent changes in heat capacity[10].

On-rate kinetics were measured, 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, 1d, online methods, Supplementary Table 2, and Fig. 4). At 150 mM salt we measured a room-temperature 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, ΔG^0, of −17.1 ± 0.1 kcal/mol, and an equilibrium enthalpy change, ΔH, of 26.01 ± 0.05 kcal/mol (**Supplementary Table 2**). In general, our measurements are consistent with values reported in the literature (**Supplementary Table 3**). Specifically, we are within 15% of the reported off-rate of a biotin-labelled oligonucleotide[9], within 30% of on-rate measurements from SPR[11], and within 5% of both equilibrium ΔH measurements by ITC[12] and equilibrium ΔG 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 2**). Although Neutravidin's affinity for biotin is 20 times weaker than avidin's, they surprisingly have similar off-rates (**Supplementary Table 2**), underscoring the limitation of relying solely on affinity measurements to characterize an interaction.

To demonstrate the measurement of weaker interactions, we incorporated desthiobiotin, a biosynthetic precursor to biotin that binds streptavidin with far lower affinity[14]. By optimizing gel running conditions, we resolved the looped and unlooped constructs in as little as 6 minutes, measuring the off-rate of streptavidin-desthiobiotin as 35.3 ± 7.5 minutes at 4 °C and 8.6 ± 1.2 minutes at room temperature (**Supplementary Fig. 3**). We note that while the system is ideal for quantification of long lived interactions, even those out of the range of biacore (**Supplementary Table 1**), the time required to resolve the bands in a gel currently sets the lower limit of detectable dissociation life-times to minutes.

The modularity of the DNA construct facilitates the easy incorporation of different types of molecules. We exploited this feature to measure several biologically relevant interactions including enzymes with time constants of seconds to minutes, DNA, antibodies, small molecules, and even a covalent bond taking weeks to dissociate (**Fig. 2**). As with many techniques including SPR, assay preparation requires the derivatization of at least one molecule of interest. Here, we attach our molecule to a DNA oligonucleotide, which can be accomplished using a variety of techniques. In addition to SMCC-crosslinking[5], 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 readout—even as the molecules, temperatures, and buffer conditions for the interactions change, the “signature” gel readout does not. As an extreme example of this, we characterized the reduction of a disulfide bond at 25°C in 10μM TCEP yielding a time constant of 2.6 ± 0.4 weeks (**Fig. 2a**). Since the signal per molecule is only dependent on the nanoswitch size, this two-atom system yields the same level of signal per interaction as a 150kDa antibody binding to its antigen (**Fig. 2c**).

Additionally, the programmability of these nanoswitches enables the design of multiple topological states that are individually distinguished on a gel, 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 bi-specific receptor. The resulting nanoswitch adopts 5 resolvable states that can be identified with control experiments (**Fig. 3a and Supplementary Fig. 5**). We measured bidirectional transitions for all 5 states, thus determining all rate constants (**Fig. 3 and Supplementary Fig. 6, 7, and Table 6**). 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.

This new approach expands the biomedical researcher's toolbox, enabling 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 (~6 orders of magnitude), for a wide variety of molecular interactions, temperatures and buffer conditions (**Fig. 1d, 2, and Supplementary Fig. 1 and 2**). The signals are 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 (~1ng for a 50kDa protein). In contrast to other techniques that provide one signal to analyze (e.g. SPR, radiolabeling, and ITC), we further differentiate our technique by showing independent measurement of 5 signals simultaneously in a unique multidimensional readout, allowing complete characterization of a complex 5-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, this system could be used as a template-directed synthesis technique to control complex reactions. Overall, this unique lab-on-a-molecule

platform promises to be a powerful research tool, accessible to anyone able to perform gel electrophoresis.

Online Methods

Note that 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.

General nanoswitch formation

The nanoswitches were constructed as previously described in detail[5]. Circular-single-stranded DNA from the 7249 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 10 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 and var 12 representing the last oligonucleotide—see supplemental protocol and supplemental files for sequences). 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 (90 °C to 20 °C at 1 °C/minute unless otherwise noted) produced double stranded DNA. 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 constructs were PEG precipitated after annealing to remove excess oligonucleotides. The PEG precipitation was performed as described in section 3.3 of the supplementary protocol and as previously described in[16].

Key Design Considerations

The nanoswitches were designed with several key design considerations to ensure that they function properly and robustly over a wide range of conditions. The oligonucleotide length was selected to be 60 nt to ensure both site specificity, and to ensure that the oligonucleotides would not spontaneously fall off even at temperatures as high as 50 °C. We show in the paper that at 50 °C even a 20-mer oligonucleotide has a long lifetime of ~18 hours (**Fig. 2d**), and the lifetime of a 60-mer oligonucleotide is predicted to be orders of magnitude longer than the 20-mer oligonucleotide[17].

The ligands were positioned at locations that allow for easy resolution of the looped and unlooped bands. Placement of the oligonucleotides on variable regions 4 and 5 yields two bands that are quite close to one another under our standard gel running conditions. The further apart the ligands are, the more easily resolvable the two bands become. The spacing of ligands on the DNA scaffold also controls their effective concentration, with the effective concentration of one ligand to the other generally decreasing as they are spaced further apart (though if the ligands are brought within one persistence length of the polymer, the effective

concentration may decrease dramatically). We have found that the use of variable regions 4 and 8 provides a nice middle ground.

Regarding the concentrations, it is important to consider that 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 since 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. 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.

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 due 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 based on the speed of mixing, and the solution on-rate of the protein being studied.

Following these design principles and those laid out in[5], is key to the successful use of this platform. To aid in implementing this method in your lab we have written a supplementary protocol which provides information on reagents needed, and detailed step-by-step instructions on how to succesfully 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 0.5x Tris-borate EDTA (TBE) (Biorad). 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 minutes at 4 V/cm, unless otherwise noted, and subsequently stained in 1x SYBRGold stain (Invitrogen) for a minimum of 30 minutes before being imaged with a gel imager (Biorad) 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 non-linear intensity scaling. .gel images can be linearized using the imageJ Linearize gel Data plugin (http://rsb.info.nih.gov/ij/plugins/linearize-gel-data.html). Alternatively the gel image can be saved as a linear .tiff 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 4x molar excess to the scaffold, while all other oligonucleotides were used in a 10x molar excess. The reason for this lesser amount is twofold: 1) to be less wasteful of the more expensive functionalized oligonucleotides, and 2) because excess biotin oligonucleotide in solution could interfere with our measurements. The final DNA construct was then diluted 100x 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 concentrations of ~80 pM and 3nM, 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 1μL 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. It is important to note that for on-rate experiments, using low binding tubes (Eppendorf LoBind) was important for getting repeatable results due 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 based on the HABA assay.

Off-rate measurements were performed by forming looped construct as described above, and letting the solution sit for at least 24 hours 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 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 time scale as the streptavidin experiments.

Desthiobiotin-Streptavidin

Desthiobiotin experiments were conducted in a similar manner as the biotin experiments with slight modifications. The var 4 oligonucleotide was changed to a desthiobiotin-functionalized oligonucleotide while the var 8 oligonucleotide remained biotin functionalized. The off-rate of the desthiobiotin interaction is much faster than the typical 100 minute 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 and 4 °C, and quantified the fraction looped as a function of running time (**Supplementary Fig. 3**). 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 for 10 minutes in pre-chilled 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 3 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, while the other oligonucleotides remained at 10x 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 compliment to this restriction sequence was also added 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 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 crosslinking a free amine on the antibody (Polyclonal Sheep Antibody from Roche) to a thiol labeled oligonucleotide, and purified by electroelution as described previously[5]. 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/minute to facilitate annealing of the antibody-modified var 4 oligonucleotide. Off-rate measurements were performed by quenching with 335nM of antibody at various times at room temperature.

Sortase Catalyzed Peptide Ligation experiments

This construct was created in 3 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[16]. Pra-LPETGHHHHHH, where Pra is a Propargyl glycine which adds an alkyne 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 Tobaco 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[16]. These oligonucleotides were then at a concentration of ~10uM as judged by running on a precast 4-20% gradient polyacrylamide TBE gel (BioRad). Equal volumes (10μL each) of the sortase-compatible oligonucleotides were mixed with 5 μL of 14.1mg/ml sortase (Chen et. al, 2011), and 25 μL of 2x Sortase Reaction buffer (600 mM Tris HCl pH 7.5, 300 mM NaCl, 10 mM $MgCl_2$, and 10 mM $CaCl_2$). This was allowed to sit for 3 hours at room temperature before running on a polyacrylamide gel and purifying 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, as peptide denaturation was not a concern. 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 sortase ligating free GGG-X peptide. To accomplish this a mixture was made with the following concentrations. 2 nM DNA nanoswitches, 10 μM sortase, 40 μM GGG-S-SCH_3, 300 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM $MgCl_2$. Catalysis by sortase is highly calcium dependent thus the transpeptidation could be quenched at different times by adding an equal volume of 100mM EDTA in water. 10 time points were collected over 20 minutes at room temperature.

Disulfide Reduction

This construct was created in 3 steps. 1) Var 4 and a truncated version of var 8 with a 3' and a 5' thiol respectively, were reduced in 50 mM TCEP (BondBreaker Thermo Scientific). 2) These two oligonucleotides were linked by a disulfide. 3) The disulfide-bridged oligonucleotides were hybridized onto the DNA nanoswitch.

1. To reduce the thiols on the oligonucleotides they were incubated in 50 mM TCEP for 1 hour at RT.
2. Equal volumes of the two oligonucleotide-TCEP mixtures were then combined. The TCEP was removed using a QIAGEN nucleotide-removal kit. The oligonucleotides were then allowed to form disulfides in the absence of reducing agent in PBS for 1 hour before running the products on a precast 4-20% gradient polyacrylamide TBE gel. As the oligonucleotides were different sizes (60 and 30nt) the appropriate hetero dimer could be easily identified and purified using electroelution as previously described[5].
3. The var 4-S-S-var 8 was used instead of the normal var 4 and var 8. 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 a disulfide bridging variable regions 4 and 8.

With these loops in hand we could observe loop opening as a result of TCEP reduction of the disulfide bond. To accomplish this equal volumes of 20 μM TCEP and 160 nM loops, both of which were diluted in NEB buffer 2, were mixed at different time points before running the gel. 7 time points were collected over 10 days 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 antidig (Roche 11333089001). The antidig, suspended in PBS, was added in a 1:1 ratio to the streptavidin, and the kit protocol was followed exactly. This was then diluted 1:1250 into NEB Buffer 2 with added 150mM 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, the diluted bispecific receptor was added, 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 6.25 V/cm for 125 minutes with buffer chilled to 4 °C before running (See supplementary data for additional details).

Gel Image Analysis

We analyzed gel images in one of two ways:

1. All non-multistate (only two bands) gels 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 just capture 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 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 (with 5 bands) gels were analyzed as follows

A custom MATLAB interface was developed for fitting the intensity profiles of the imaged gel bands. The software interface was modeled after the ImageJ interface. Rectangular boxes are 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 the background was subtracted using a 4-6 point piece-wise 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 ~−2.5 (**Supplementary Fig. 3**). Thus, the entire multistate median-intensity profile (from just above the highest band to just below lowest) was fit using a sum of 5 skewed Gaussians. A common skew parameter was used for all 5 bands, and a common initial guess of band width was used with a fitting range of ±10 pixels. These input parameters allowed for converging fits across all lanes, and resulted in fits that closely matched the observed intensity profiles (**Fig. 3b**). 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 one-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 identity of the bands were validated by analyzing gels in which individual loop sizes were formed (**Supplementary Fig. 3**). Accuracy of band quantification was confirmed by mixing these individual loops in known ratios—the measured values of the individual bands were found to be within 10% of their true values.

Equation for a skew normal/skewed Gaussian distribution:

$$A \cdot e^{-\left(\frac{x-b}{c}\right)^2} \cdot \left(1+eff\left(a\frac{x-b}{c}\right)\right.$$

Data Analysis

Based on a gel we ran to establish repeatability of pipetting and imaging, we conservatively estimate the error per lane at ± 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 based on 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 seconds, 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:

$$Unbound \quad Linear \rightarrow Singly \quad Bound \quad Linear \rightarrow Looped$$

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 the supplementary discussion.

Thermodynamic Analysis

The dissociation constant K_D was determined by the ratio of the off- and on-rates, and the equilibrium free energy ΔG^0 was determined by:

$$\Delta G^0 = -RTln\left(\tilde{K}_{\mathrm{D}}\right)$$

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. $\tilde{K_{\mathrm{D}}} = K_{\mathrm{D}}/(1M)$. 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 Boltzman constant, h is Plank's constant, and ΔH and ΔS are the enthalpy and entropy of activation, respectively.

For the salt dependence, we used the kinetic salt relationship:

$$log\,(k) = log\,(k_0) + 2A \cdot Z_{\mathrm{A}} \cdot Z_{\mathrm{B}} \sqrt{I}$$

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 Material

Refer to Web version on PubMed Central for supplementary material.

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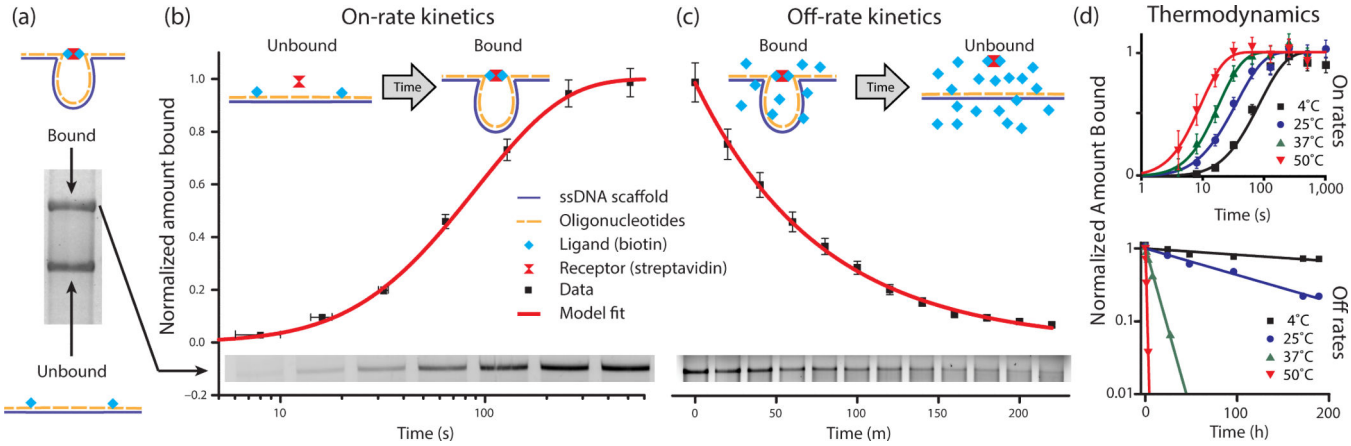

Figure 1. Kinetic measurements using DNA nanoswitches

a) The two states of the DNA nanoswitches can be distinguished by gel electrophoresis. b) With two integrated biotins, loop formation begins when unlabeled streptavidin is introduced, and progresses over time as evidenced by increasing brightness in the bound (looped) band across different lanes of a gel. The growth curve is fit with a kinetic model to determine the on rate. c) Addition of excess biotin blocks loop formation, making bond rupture irreversible, which leads to the exponential decay of nanoswitches from the bound state into the unbound state. d) Temperature dependence of on rates and off rates at 150mM NaCl. Horizontal error bars represent uncertainty in mixing time (± 2 seconds), and vertical error bars indicate ± 7% uncertainty in the intensity (this is the one-sigma confidence interval determined from 48 repeated measurements of the same construct—see Data Analysis section of online methods for more detail).

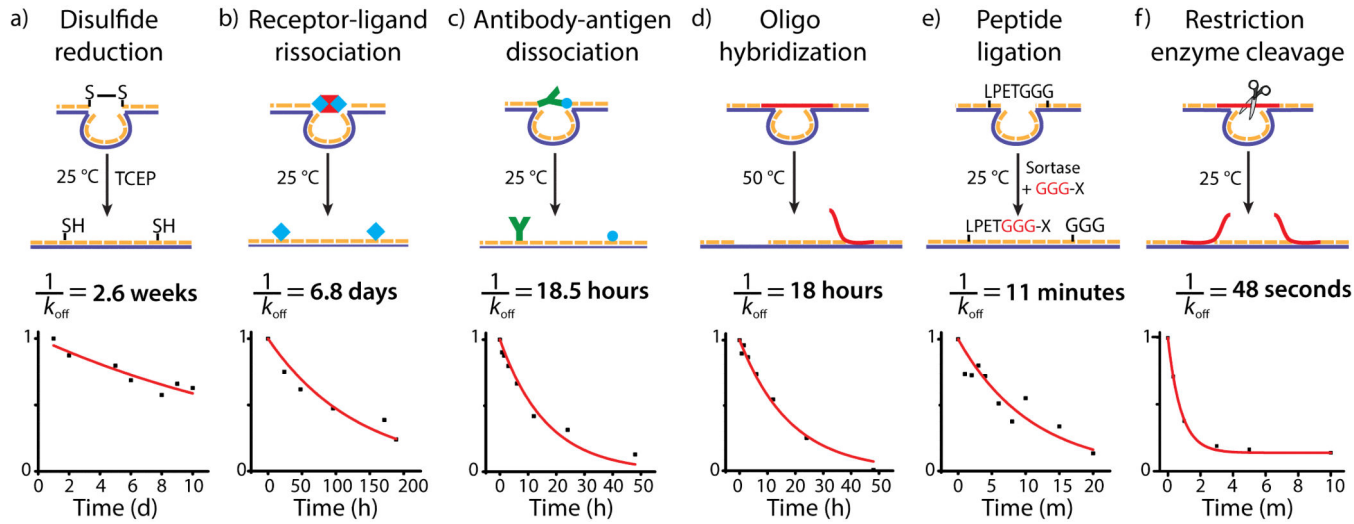

Figure 2. Various biological measurements using the nanoswitch platform

Schematic representation of each measurement, with data and model fit shown beneath. a) Disulfide bond reduction in 10μM TCEP at room temperature (k_{off} = 2.6 ± 0.4 weeks). b) Biotin-streptavidin dissociation in 300mM NaCl at room temperature (k_{off} = 6.8 ± 0.8 days). c) Dissociation kinetics of digoxigenin and its antibody at room temperature (k_{off} = 18.5 ± 2.0 hours). d) Melting kinetics of a 20nt oligonucleotide at 50°C (k_{off} = 18 ± 1.6 hours). e) Sortase-catalyzed transpeptidation at room temperature ($k_{ligation}$ = 11 ± 1.3min). f) XhoI restriction-enzyme kinetics at room temperature (k_{cut} = 48 ± 2 sec). Each value is reported as an error-weighted fit parameter ± its one-sigma confidence interval).

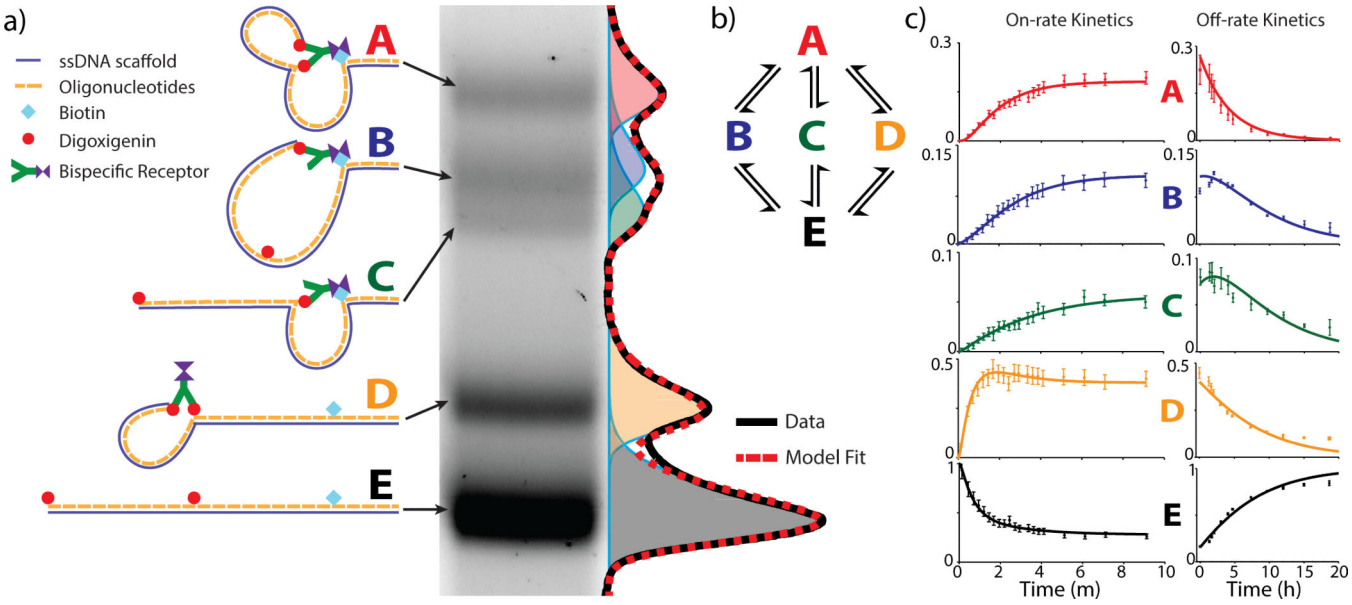

Figure 3. Multistate kinetic analysis

a) A nanoswitch functionalized with two digoxigenin molecules and one biotin molecule can adopt 5 discernable states upon addition of a bispecific receptor. All 5 topological states, **A-E** (**Supplementary Fig. 6**), can be resolved within a single lane of an agarose gel. These bands can be fit globally with a single fit of a sum of skewed Gaussian curves. The black curve represents the median pixel intensity, the dashed red curve represents the fit which is the sum of 5 skewed Gaussians, and the individual skewed Gaussians are shaded by state. b) A reaction diagram illustrating the possible transitions between each of the 5 states. c) (left) on-rate measurements indicating the value of each state at 20 different time points. Solid curves indicate the result of a global fit of all states to the kinetic model illustrated in c. (right) off-rate measurements indicating the value of each state at 12 different time points. Solid curves indicate the result of a global fit of all states to the kinetic model illustrated in b. These fits taken together allowed for the determination of all rate constants from 32 lanes which can be run on a single gel (**Supplementary Fig. 7 and Table 6**). Error bars are based on one-sigma confidence-intervals of the least squares fit to each band (see Gel Image Analysis in Online Methods for details). The on-rate model was fit using 100 measurements while the off-rate model was fit using 60 measurements.