

Multifactorial Modulation of Binding and Dissociation Kinetics on Two-Dimensional DNA Nanostructures

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4.2 Materials and Methods

Unless otherwise noted, all oligonucleotides were ordered from Integrated DNA Technologies (IDT).

Preparation of DNA origami scaffolds. Rectangular DNA origami arrays consist of an M13mp18 viral DNA scaffold (Affymetrix) and 202 ssDNA staples as previously described^{1,2}. For all structures assembled here, staples 1-12 and 205-216 were omitted to prevent inter-array base stacking interactions that result in undesirable aggregation. Of the remaining staples, several were modified at their 5'-end with an additional sequence, 5'-CCT CTC ACC CAC CAT TCA TC, to which the probe strand *P* (see below) can bind (positions shown in red, Supporting Figure S2). The arrays were annealed in 1× TA-Mg Buffer (40 mM Tris-HCl, 20 mM acetic acid, 12.5 mM Mg²⁺, pH 7.6) with a 1:3 ratio of M13 to staple strands and a final concentration of 10 nM (M13). The arrays were annealed over 12 hours from 90°C-25°C using a PCR thermocycler (Eppendorf).

Preparation of target and probe oligonucleotides. The probe oligonucleotide *P* (5'-GAT GAA TGG TGG GTG AGA GGT TTT TCA CTA TrAG GAA GAG /3AmMO/) was ordered with a 3'-terminal amine modification ("3AmMO") and HPLC purified by the manufacturer, then labeled with an *N*-hydroxysuccinimidyl ester derivative of Alexa Fluor 647 (Invitrogen) by overnight incubation in 0.1 M NaHCO₃, pH 8.3, followed by ethanol precipitation and thorough washing with 80% ethanol until the supernatant was colorless, yielding *P*-AF647. Denaturing polyacrylamide gel electrophoresis revealed no detectable free dye. The 5'-Cy3 labeled target oligonucleotides *T7+10* (5'-/5Cy3/TCT CTT CTC CGA GCC GGT CGA AAT AGT GAA AA), *T11* (5'-/5Cy3/CTC TTC CTA TA), and *T110* (5'-/5Cy3/TCT CTT CCT ATA CGC TGA AAG GTG ACG GCA AA) were ordered HPLC-purified by the manufacturer and used as-is. Labeling efficiency was quantified by absorbance at 280 nm and either 550 nm (Cy3) or 650 nm (Alexa Fluor 647) using a Beckman DU 640B Spectrophotometer, and was >95% for all ssDNA strands. The strands *P* (5'-GAT GAA TGG TGG GTG AGA GGT TTT TCA CTA TrAG GAA GAG), *P** (5'-GAT GAA TGG TGG GTG AGA GGT AAA TCA TCG AAG ACT CTA), and *P_{comp}* (5'-CCT CTC ACC CAC CAT TCA TC) were ordered gel-purified and used as supplied.

Single-Origami Kinetic Assays. Single-origami kinetic experiments were carried out on an inverted total internal reflection fluorescence (TIRF) microscope with a 1.2 NA 60x water-immersion objective (IX71, Olympus) in a darkened room at an environmentally controlled temperature of 20 ± 3 °C. Fluorescence excitation was provided by a 532-nm green laser (ultra-compact diode-pumped Nd:YAG laser GCL-025-S, CrystaLaser, 1 W/cm²). The Cy3 and Alexa Fluor 647 emission signals were separated by a dichroic mirror with a cutoff wavelength of 610 nm (Chroma) and projected side-by-side onto an ICCD camera chip (iPentamax HQ Gen III, Roper Scientific, Inc.). The Cy3 channel image was passed through a band pass filter (HQ580/60m, Chroma) and the Alexa Fluor 647 channel was passed through a long pass filter (HQ655LP, Chroma). A

Newport ST-UT2 vibration isolation table was used in all experiments. In all measurements, an oxygen scavenger containing oxygen scavenger system³ (OSS \equiv 2.5 mM 3,4-dihydroxybenzoic acid, Sigma P5630; 1 mM Trolox, Acros 218940050; and 25 nM protocatechuate dioxygenase, Sigma-Aldrich P8279) was included in the imaging buffer to reduce photobleaching.

Microscope slides with a flow channel were prepared using double-sided tape (Scotch) and treated with biotinylated BSA and streptavidin as described^{4,5} to prepare the surface for immobilization of biotinylated DNA origami. A solution containing 20-100 pM origami was incubated in the presence of 1x HBS (50 mM HEPES-KOH, pH 7.2, 150 mM NaCl) supplemented with OSS at room temperature for 10 min, and excess sample was flushed away by two washes with 1x HBS. A solution of 200 nM *P*-AF647 was added to the slide channel and incubated for 15 min before flushing the excess away by two washes with 1x HBS. Fluorescence from the AF647 label of the probe *P* was visible even under 532-nm excitation, enabling us to focus on and locate origami prior to beginning FRET measurements.

Association Kinetics. To limit photobleaching, a shuttered illumination scheme was used: the sample was illuminated for 0.5-s intervals separated by 29.5-s dark periods. After an initial waiting period, a solution of 25, 50, 75, or 100 nM *T7+10*, *T11*, or *T11O* in 1x HBS was added to the slide during the beginning of a dark period with a dead time of 5 s. FRET from Cy3 to AF647 resulted in an approximately 5-fold increase in AF647 fluorescence upon binding of the target to the probe.

Dissociation Kinetics. For dissociation kinetics experiments, the length of dark periods was increased to 119.5 s. The same exposure time of 0.5 s was used. During the dark period after the first measurement, a solution of 500 nM unlabeled *P* was added as a chase.

The fluorescence intensity of each origami was normalized to its maximal value in a given experiment. The mean intensity across all origami was plotted as a function of time and fit to the single exponential decay models $y = C(1 - e^{k'_{obs}t})$ and $y = C_1 e^{k'_{obs,1}t}$ for association and dissociation measurements, respectively.

Solution Kinetic Assays. All measurements were performed at 22 °C on an Aminco-Bowman Series 2 Luminescence Spectrometer at a time resolution of 1 or 6 s, exciting at 520 nm (4 nm bandwidth) and detecting at 690 nm (16 nm bandwidth). As in the single-origami kinetic assays, all measurements were taken in the presence of oxygen scavenger and 1x HBS. Under these conditions, no photobleaching was observed over the course of 1 h.

Association kinetics. To a 99.5- μ L solution of 25, 50, 75, or 100 nM *T7+10*, *T11*, or *T11O* (final concentration) was added 0.5 μ L of a pre-equilibrated solution of 1 μ M *P*-AF647 (final concentration 5 nM) and 4 μ M P_{comp} , and the solution was mixed well by pipetting. P_{comp} was used to block the portion of *P* that normally hybridizes to overhangs on the DNA origami. The increase in A647 fluorescence due to FRET was

monitored until the signal was stable, and subsequently fit with a single exponential function.

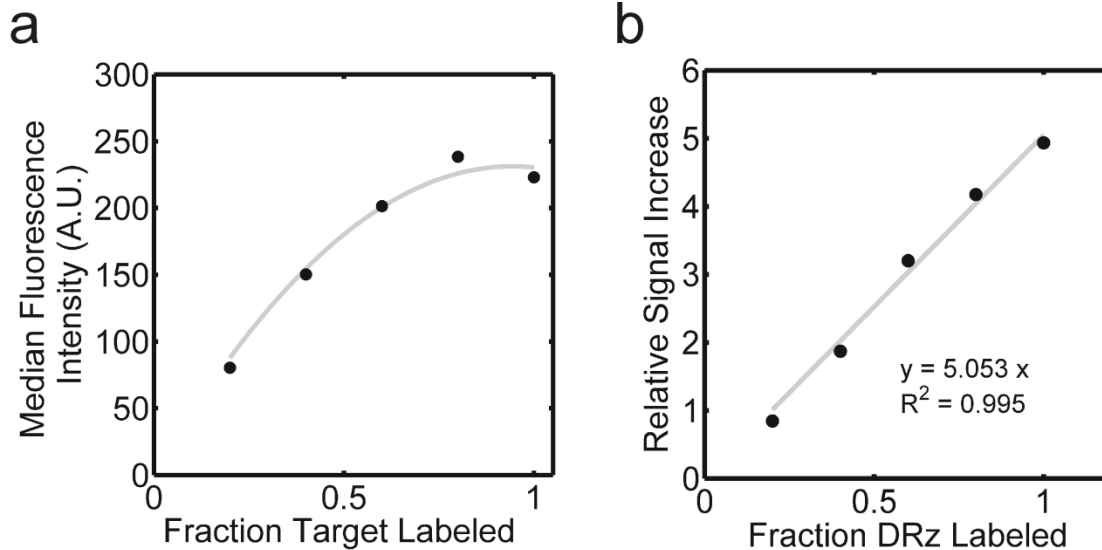
Dissociation kinetics. To a 97.5- μ L solution of 25 nM *T7+10*, *T11*, or *T110* (final concentration) was added 2.5 μ L of a pre-equilibrated solution of 1 μ M *P*-AF647 (final concentration 25 nM) and 4 μ M P_{comp} . After equilibrium was reached, a 25- μ L chase solution of 2.5 μ M unlabeled *P* (final concentration 500 nM) was added to the reaction and mixed well. The decay was fit with a single exponential function.

For the bulk solution assays of *T110* dissociation from *P* displayed on origami in free solution (Supporting Figure S9), a 20- μ L solution containing 9.3 nM origami bearing probe sites spaced by 22 nm (Supporting Figure S2c) and 670 nM *P*-AF647 was incubated in the annealing buffer for 10 min at room temperature. Excess staples and probes were removed by threefold filtration with an Amicon Ultra-0.5 centrifugal filter (Ultracel-100 Membrane, 100 kDa MWCO, Millipore) according to the manufacturer's instructions. The resulting 20 μ L of purified origami-probe complex was diluted to a volume of 97.5 μ L with HBS (final concentration 1x) and oxygen scavenger, and 2.5 μ L solution of *T110* was added. After the association reaction reached completion as measured by AF647 fluorescence, a 25- μ L chase solution of 2.5 μ M unlabeled *P* (final concentration 500 nM) was added to the reaction and mixed well. The decay was fit with a single-exponential function.

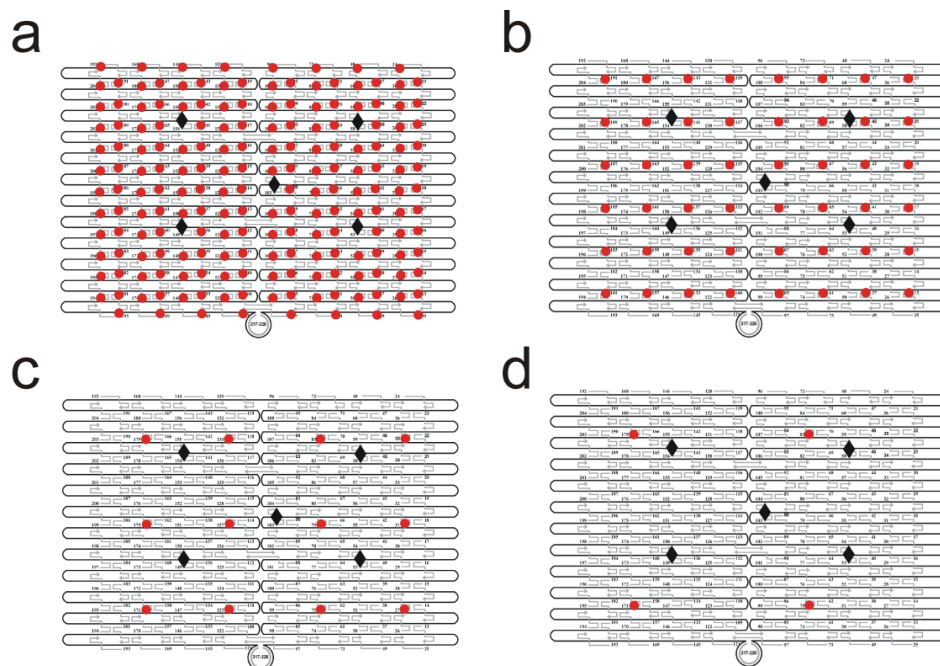
Assay of probe cleavage by T7+10. To assess the possible influence of probe cleavage by the deoxyribozyme target *T7+10*, four 100- μ L reaction mixtures were prepared in 1x HBS containing oxygen scavenger. Reactions were carried out as in the Solution Kinetics Assays (above), with [*P*] adjusted to 25 nM in each 100- μ L reaction. After a 10 min hybridization reaction between *P* and 100 nM *T7+10*, 1 μ L of chase strand (unlabeled *P*) was added to a final concentration of 500 nM. Reactions were incubated for 5 h and stopped with 100 μ L of formamide loading buffer (90% formamide, 89 mM Tris-borate, 2 mM EDTA). The reaction mixtures were heated to 70 $^{\circ}$ C for 2 min, cooled to room temperature, and separated on a 20% polyacrylamide gel containing 8 M urea. The bands were imaged by Alexa Fluor 647 fluorescence using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences) and quantified in ImageQuant 5.2 (Molecular Dynamics). Results are shown in Supporting Figure S4.

Monte Carlo simulations of target binding to and dissociation from probes on DNA origami. Origami nanopogboards were modeled as a collection of 187, 48, 12, or 4 probes, each capable of binding one target. Target association was modeled as a single-exponential increase with a pseudo-first-order rate constant k'_{obs} taken from experiment. Target dissociation was modeled as a single-exponential decrease, again with a rate constant taken from experiment for a given origami construct. In association experiments, bound targets were allowed to dissociate, whereas in dissociation experiments, dissociation was considered irreversible. Each run was divided into 1,000 timesteps spanning five half-lives of the reaction. During each timestep, each probe has an opportunity to bind and/or release a target according to the probability of reaction

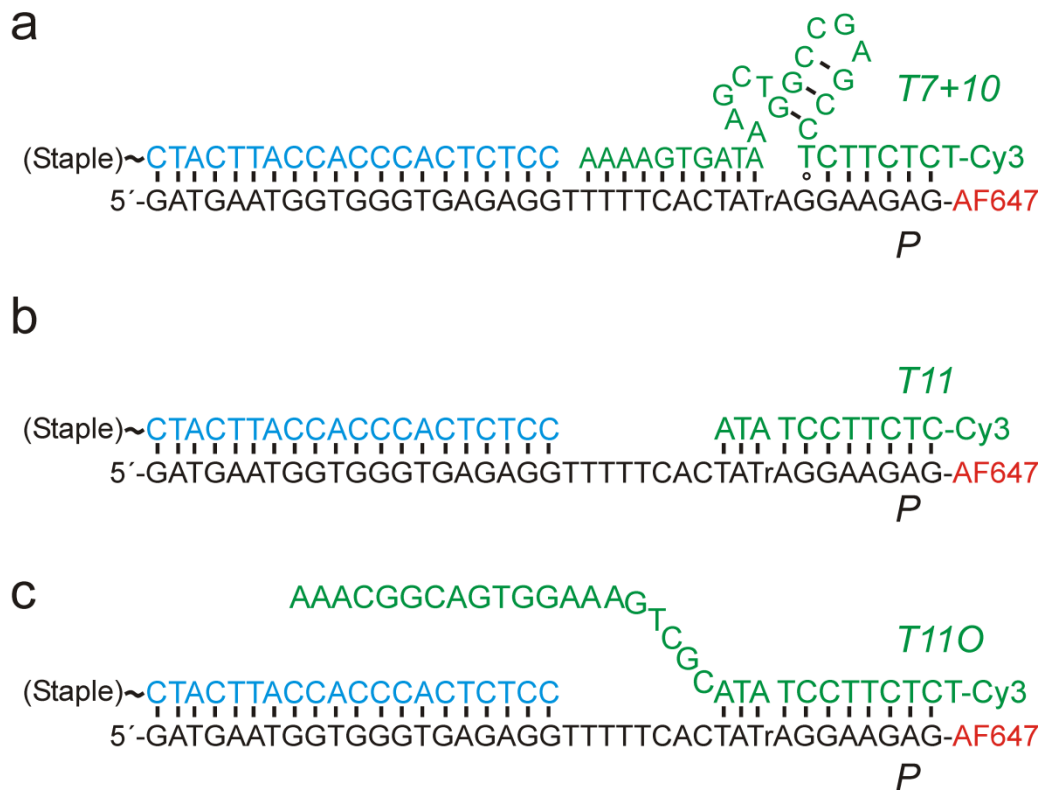
$P(r) = (1 - \exp(-k \Delta t))$, where k is the rate constant and Δt is the timestep. For each condition, 1,000 runs, each representing a single origami trajectory, were performed.



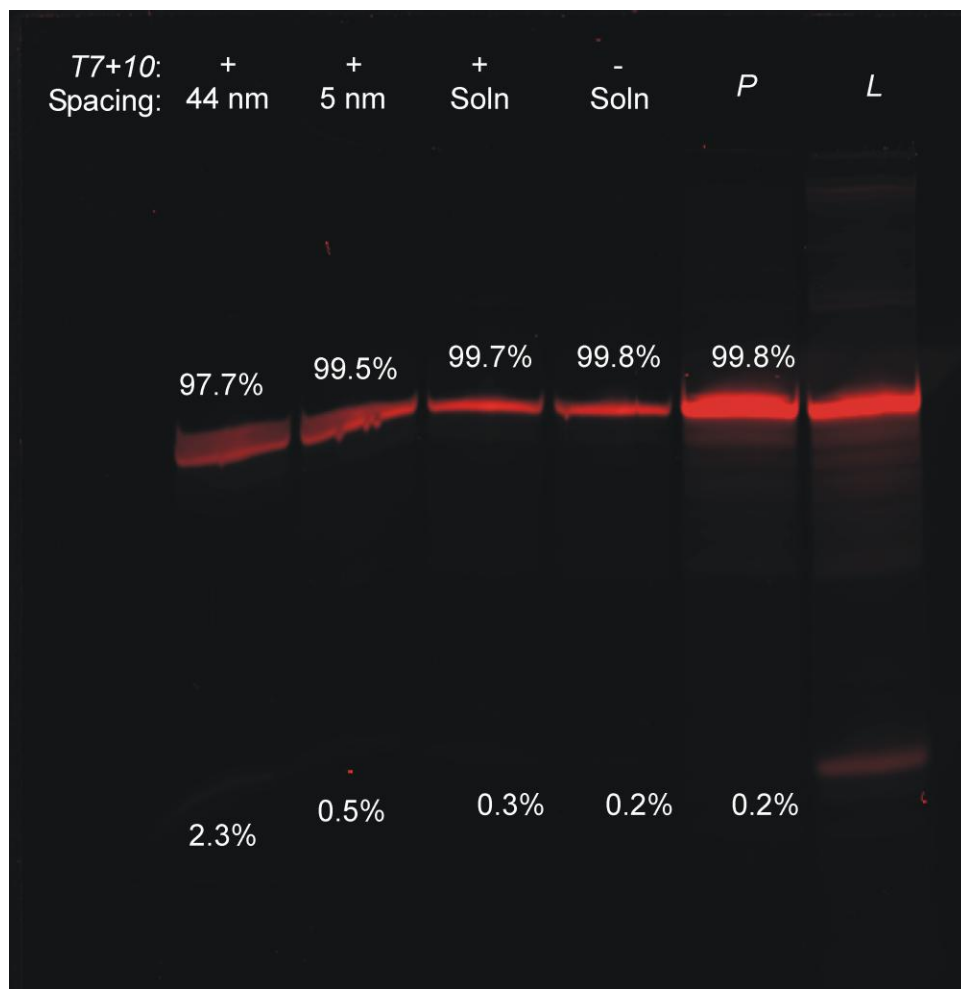
Supporting Figure S1. (a) Median fluorescence intensity per origami tile as a function of the fraction of *P* labeled with AF647. The gray curve is a best-fit quadratic polynomial. (b) Fold increase in AF647 fluorescence from FRET upon *T7+10* binding as a function of the fraction of *T7+10* labeled with Cy3. The gray line is a linear regression fit with the y-intercept constrained to the origin.



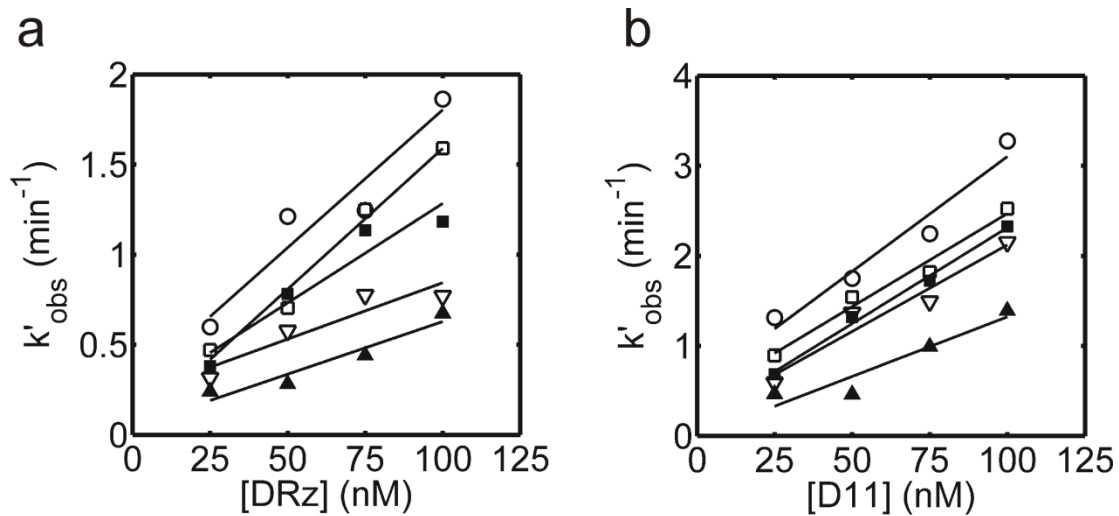
Supporting Figure S2. Schematics of the origami-templated probe arrays used in this study, with spacings of approximately 5 nm (a), 11 nm (b), 22 nm (c), and 44 nm (d) between adjacent probes. Red circles correspond to probe positions, and black diamonds indicate positions of biotinylated staples for immobilization prior to fluorescence microscopy. Biotin moieties and probe molecules project from opposite faces of the rectangular tile. The continuous black line represents the circular M13 viral genome and the gray lines correspond to unmodified staples.



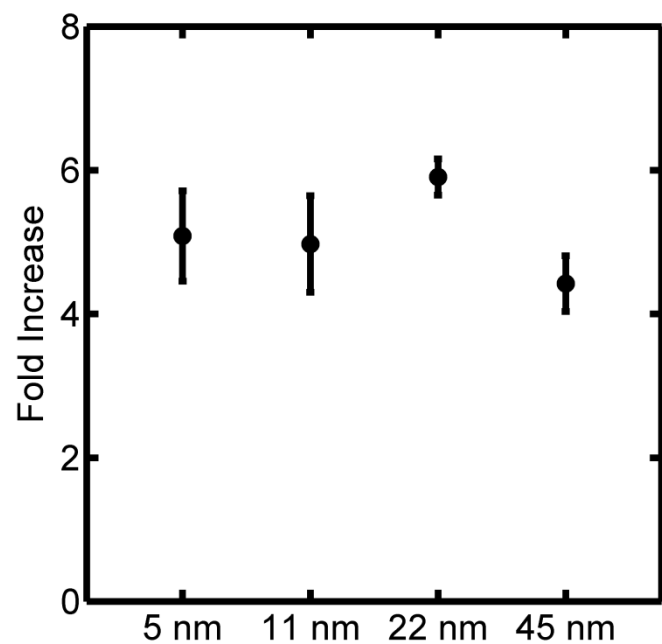
Supporting Figure S3. Secondary structure of probe complexes with targets *T7+10* (a), *T11* (b), and *T11O* (c). The probe (*P*) is immobilized on origami via hybridization to 3' overhangs of staples (blue) that project from one face of the tile. For measurements in free solution, the 3' overhang of the origami staples was replaced by *P_{comp}* (5'-CCT CTC ACC CAC CAT TCA TC).



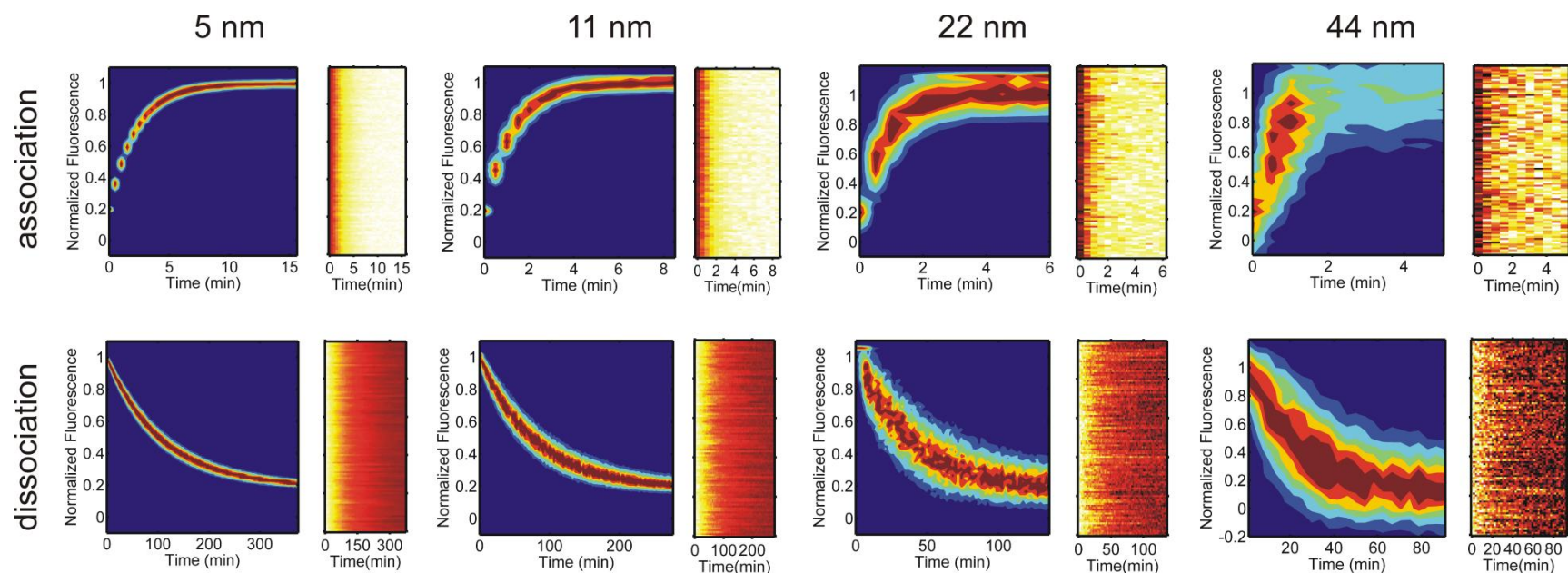
Supporting Figure S4. Denaturing PAGE analysis of *P* cleavage during *T7+10* binding and dissociation reactions at the surface of origami with 44 nm and 5 nm probe spacings, as well as in free solution ('Soln'). Association reactions were carried out for 10 min, followed by 5 h dissociation reactions in presence of the chase strand. Unreacted *P* and its alkaline cleavage ladder (*L*) are shown in the two rightmost lanes for size comparison. Bands were quantified using Alexa Fluor 647 fluorescence.



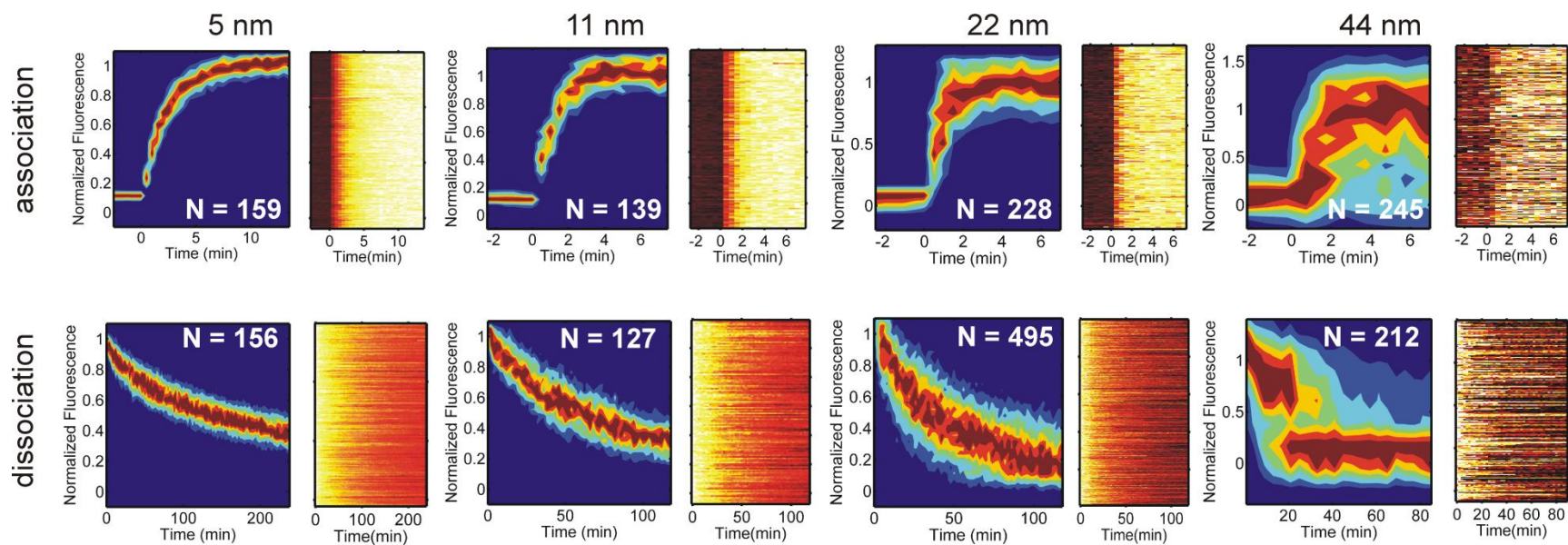
Supporting Figure S5. Pseudo-first-order kinetics for the binding of *T7+10* (a) and *T11* (b) to origami with probes spaced by 5 nm (filled triangles), 11 nm (open triangles), 22 nm (filled squares), 44 nm (open squares), or to probe molecules in free solution (open circles). Best-fit linear regression lines are shown, the slope of which yields the bimolecular binding rate constant.



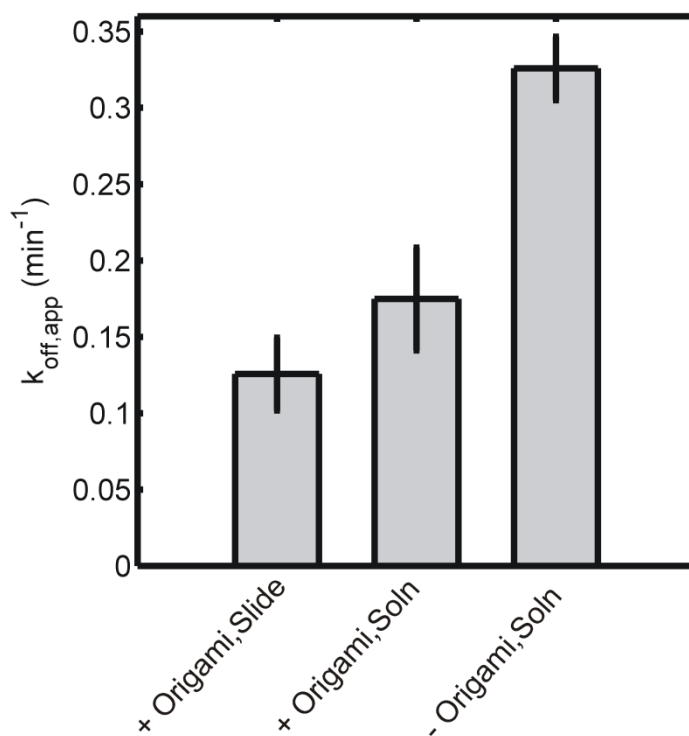
Supporting Figure S6. Fold increase in AF647 signal upon probe binding for origami with different spacings between neighboring probe molecules. Error bars represent one SEM from at least three trials.



Supporting Figure S7. Monte Carlo kinetic simulations showing the expected variation in reaction trajectories of individual origami tiles based on the number of probes per tile. Probability density maps of intensity versus time (left panels) and single origami trajectories (right panels) from Monte Carlo simulations of *T7+10* binding to, and dissociation from, origami with 5, 11, 22, or 44 nm between neighboring probe molecules (i.e., with 187, 48, 12, or 4 probes per origami) are shown. Rate constants were set to the values from runs shown in Supporting Figure S8. Association simulations were carried out according to the predicted pseudo-first-order rate constant at 75 nM *T7+10*. $N = 1,000$ for all simulations.



Supporting Figure S8. Probability density maps of intensity versus time (left panels) and corresponding experimental single origami trajectories (right panels) of *T7+10* binding to, and dissociation from, origami with 5, 11, 22, or 44 nm between neighboring probe molecules. The association reactions shown here were carried out in the presence of 75 nM *T7+10*. The number of origami observed in each reaction (N) is shown in the respective panel.



Supporting Figure S9. Comparison of *T110* dissociation from *P* in free solution, in solution with origami, and at the surface of a microscope slide with origami. All apparent rate constants were determined from fitting with a single-exponential decay function.

Supporting References

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