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Single-Molecule Dwell-Time Analysis of Restriction Endonuclease-Mediated DNA Cleavage

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TITLE:

Single-Molecule Dwell-Time Analysis of Restriction Endonuclease-Mediated DNA Cleavage

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SUMMARY:

Using quantum-dot-labeled DNA and total internal reflection fluorescence microscopy, we can investigate the reaction mechanism of restriction endonucleases while using unlabeled protein. This single-molecule technique allows for massively multiplexed observation of individual protein-DNA interactions, and data can be pooled to generate well-populated dwell-time distributions.

ABSTRACT:

This novel total internal reflection fluorescence microscopy-based assay describes the simultaneous measurement of the length of the catalytic cycle for hundreds of individual restriction endonuclease (REase) molecules in one experiment. This assay does not require protein labeling and can be carried out with a single imaging channel. In addition, the results of multiple individual experiments can be pooled to generate well-populated dwell-time distributions. Analysis of the resulting dwell-time distributions can help elucidate the DNA cleavage mechanism by revealing the presence of kinetic steps that cannot be directly observed. Example data collected using this assay with the well-studied REase, EcoRV—a dimeric Type IIP restriction endonuclease that cleaves the palindromic sequence GAT↓ATC (where ↓ is the cut site)—are in agreement with prior studies. These results suggest that there are at least three steps in the pathway to DNA cleavage that is initiated by introducing magnesium after EcoRV binds DNA in its absence, with an average rate of 0.17 s^{-1} for each step.

INTRODUCTION:

Restriction endonucleases (REases) are enzymes that effect sequence-specific double-strand breaks in DNA. The discovery of REases in the 1970s led to the development of recombinant DNA technology, and these enzymes are now indispensable laboratory tools for genetic modification and manipulation[1]. Type II REases are the most widely used enzymes in this class as they cleave DNA at a fixed location either within or near their recognition sequence. However, there is a great deal of variation among the Type II REases, and they are divided into several subtypes based on particular enzymatic properties rather than being classified according to their evolutionary relationships. Among each subtype, there are frequent exceptions to the classification scheme, and many enzymes belong to multiple subtypes[2]. Thousands of Type II REases have been identified, and hundreds of them are commercially available.

However, in spite of the diversity among the Type II REases, very few REases have been studied in detail. According to REBASE, the restriction enzyme database established by Sir Richard Roberts in 1975[3], published kinetics data are available for fewer than 20 of these enzymes. Furthermore, while some REases have been directly observed at the single-molecule level while diffusing along the DNA prior to encountering and binding to their recognition sequence[4-7], there are very few single-molecule studies of their cleavage reaction kinetics. The existing studies either do not report adequate statistics to undertake detailed analysis of the variation in the times at which single cleavage events take place[8-10] or are not capable of capturing the full distribution of cleavage times[11]. This type of analysis can reveal the presence of relatively long-lived kinetic intermediates and could lead to better understanding of the mechanisms of REase-mediated DNA cleavage.

At the single-molecule level, biochemical processes are stochastic—the waiting time for a single instance of the process to occur, τ, is variable. However, many measurements of τ can be expected to obey a probability distribution, $p(\tau)$, that is indicative of the type of process taking place. For instance, a single-step process, such as the release of a product molecule from an enzyme, will obey Poisson statistics, and $p(\tau)$ will take the form of a negative exponential distribution:

$$p(\tau) = \frac{1}{\beta} e^{-\tau/\beta}$$

where β is the mean waiting time. Note that the rate of the process, k, will be equal to $1/\beta$, the inverse of the mean waiting time. For processes that require more than one step, $p(\tau)$ will be the convolution of the single-exponential distributions for each of the individual steps. A general solution for the convolution of N single-exponential decay functions with identical mean waiting times, β, is the gamma probability distribution:

$$p(\tau) = \frac{1}{\Gamma(N)\beta^N} \tau^{N-1} e^{-\tau/\beta}$$

where $\Gamma(N)$ is the gamma function, which describes the interpolation of the factorial of N–1 to non-integer values of N. Although this general solution can be used as an approximation when the mean waiting times of individual steps are similar, it must be understood that the presence

of relatively fast steps will be masked by steps with significantly longer waiting times. In other words, the value of *N* represents a lower limit on the number of steps[12]. With an adequate number of waiting-time measurements, the parameters *β* and *N* can be estimated by binning the events and fitting the gamma distribution to the resulting histogram or by using a maximum-likelihood estimation approach. This type of analysis can therefore reveal the presence of kinetic steps that cannot be easily resolved in ensemble assays and requires a large number of observations to estimate parameters accurately[12,13].

This paper describes a method to use quantum-dot-labeled DNA and total internal reflection fluorescence (TIRF) microscopy to observe hundreds of individual REase-mediated DNA cleavage events in parallel. The design of the assay makes it possible to pool the results of several experiments and can create dwell-time distributions containing thousands of events. The high photostability and brightness of quantum dots permit a 10 Hz time resolution without sacrificing the ability to observe cleavage events occurring even many minutes after the start of the experiment. Good temporal resolution and a broad dynamic range, combined with the ability to collect a large data set, allow accurate characterization of the dwell-time distributions to uncover the presence of multiple kinetic steps in the cleavage pathways of REases, which have turnover rates in the 1 min^{-1} range. In the case of EcoRV, three kinetic steps can be resolved, all of which have been identified through other means, confirming that the assay is sensitive to the presence of such steps.

Duplex DNA substrates containing the recognition sequence of interest are produced by annealing a biotinylated oligonucleotide to a complementary strand labeled with a single, covalently attached semiconductor nanocrystal quantum dot. These substrates are introduced into a flow channel built on top of a glass coverslip with a lawn of high molecular weight polyethylene glycol (PEG) molecules covalently attached to its surface. The DNA substrates are captured via a biotin-streptavidin-biotin linkage by a fraction of the PEG molecules that have a biotin at their free end. In TIRF microscopy, an evanescent wave that decays exponentially with distance from the glass-liquid interface provides illumination; the penetration depth is on the order of the wavelength of the light used. Under these conditions, only quantum dots that are tethered to the surface by a DNA molecule that has been captured on the functionalized glass surface will be excited. Quantum dots that are free in solution will not be constrained within the illuminated region and therefore will not luminesce. If the DNA tethering a quantum dot to the surface is cleaved, that quantum dot will be free to diffuse away from the surface, and it will disappear from the fluorescence image.

Although many Type II REases are known to bind DNA in the absence of magnesium[14], all require magnesium to mediate DNA cleavage[15]. These REases can bind the surface-immobilized DNA in the absence of magnesium. When magnesium-containing buffer is flowed through a channel with REase prebound to the DNA, cleavage begins immediately, as indicated by the disappearance of quantum dots. The synchronization achieved by prebinding the REase molecules, and then initiating DNA cleavage by introducing magnesium, facilitates measurement of the lag time to the completion of DNA cleavage independently for each molecule in the population of enzymes observed in an experiment. Fluorescein is included as a tracer dye in the magnesium-containing

buffer to indicate the arrival of magnesium into the field of view. As no enzyme is included in the magnesium-containing buffer, the lag time from the arrival of magnesium-containing buffer to the disappearance of each quantum dot indicates the time it takes for an REase that is already bound to the DNA to cleave the DNA and release the quantum dot from the glass surface. Quantum dot disappearance happens quickly and results in a sharp decrease in the intensity trajectory, providing a clear indication of the time at which a given DNA molecule is cleaved. The determination of event times is accomplished by mathematical analysis of intensity trajectories, and a typical experiment results in hundreds of identifiable cleavage events. The results of multiple experiments can be pooled to provide adequate statistics to allow estimation of the parameters, N and β, by either nonlinear least squares or maximum-likelihood analysis.

PROTOCOL:

1. General information

1.1. Oligonucleotide design

NOTE: The 60 base-pair (bp)-long DNA substrate is formed from a pair of complementary oligonucleotides with a duplex melting temperature of 75 °C in 100 mM NaCl.

1.1.1. Order one oligonucleotide synthesized with a single 5' biotin modification and the other with a 5' thiol modification (with a six-carbon spacer). Place the recognition site in the center of the duplex region.

NOTE: The oligonucleotide sequences for use with EcoRV are shown below (recognition site in bold).
5' biotin – AAA ACC GAC ATG TTG ATT TCC TGA AAC GG**G ATA TC**A TCA AAG CCA TGA ACA AAG CAG CCG – 3'
5' thiol – CGG CTG CTT TGT TCA TGG CTT TGA T**GA TAT C**CC GTT TCA GGA AAT CAA CAT GTC GGT TTT – 3'

1.2. Use ultrapure water with 18 MOhm resistivity for all steps.

1.3. Protect all solutions containing quantum dots from light to prevent photobleaching.

1.4. Use a compressed air source to complete this protocol.

2. Preparation of quantum-dot-labeled DNA substrate materials

NOTE: Besides the oligonucleotides described above, see the **Table of Materials** for other materials and **Table 1** for buffers required for the preparation of quantum-dot-labeled DNA substrates.

2.1. Reduce 5' thiol groups on the oligonucleotide to be coupled to quantum dots.

2.1.1. Resuspend each thiolated oligonucleotide in water at a concentration of 100 µM.

2.1.2. Pipet 650 µL of water into a size-exclusion spin column for each oligonucleotide sample, and vortex for ~15 s. Leave the column to pack for 30 min.

2.1.3. Prepare fresh 100 mM dithiothreitol (DTT) solution immediately prior to each use as DTT degrades in solution rapidly. Carefully open one vial containing 7.7 mg of DTT, and pipet 500 µL of sodium phosphate buffer into the vial; vortex to mix thoroughly.

2.1.4. For each resuspended oligonucleotide, prepare a fresh tube, and add 50 µL of the oligonucleotide and 50 µL of DTT solution to the tube. Pipet up and down to mix. Incubate for 30 min at room temperature to reduce the disulfide bonds between the oxidized thiol groups on the 5' end of the oligonucleotides.

2.1.5. Remove the spin column caps, and place each column into the collection tube. Centrifuge the spin columns at 750 × *g* for 2 min, and discard the eluate.

2.1.6. Transfer the spin columns into fresh centrifuge tubes. Pipet the entire volume (100 µL) of one sample of DNA/DTT mixture gently onto each prepared column. Centrifuge at 750 × *g* for 2 min, and measure the absorbance of the eluate at 260 nm to confirm that the concentration is approximately 40 µM.

2.1.7. Store any samples that will not be used immediately at -20 °C to prevent the oxidation of the thiol groups and the formation of disulfide bonds.

2.2. Coupling of DNA to quantum dots

2.2.1. Pipet one aliquot of 50 µL of the quantum dot stock into a dialysis device for each construct to be made, making sure not to touch the membrane with the pipette tip. Dialyze against a volume of *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer that is at least 1000x the sample volume with stirring at ~100 rpm for 15 min.

2.2.2. Prepare a fresh 6 mM solution of sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) in CHES buffer immediately before each use. Carefully open one vial containing 2 mg of sulfo-SMCC, and pipet 800 µL of CHES buffer into the vial. Vortex to mix thoroughly.

2.2.3. Using a pipettor, carefully retrieve the suspended quantum dots from the dialysis device, and transfer into a fresh tube containing an equal volume of the sulfo-SMCC solution; pipet up and down to mix. Incubate for 1 h at room temperature with shaking at 1000 rpm to allow the sulfo-SMCC to react with the primary amines on the quantum dots.

2.2.4. Using a pipettor, carefully transfer each sample into a fresh dialysis device. To remove

excess sulfo-SMCC, dialyze against a volume of CHES buffer that is at least 1000x the volume contained in the dialysis device(s), with stirring, for 15 min. Exchange the buffer 2x, and perform dialysis with fresh CHES buffer 3x in total, allowing dialysis to proceed 15 min after each buffer exchange.

2.2.5. To exchange the buffer in preparation for the second reaction, transfer the dialysis devices into a beaker containing a volume of phosphate-buffered saline (PBS) that is at least 1000x the volume contained in the device(s). Dialyze with stirring for 15 min, exchange the buffer 2x, and perform dialysis with fresh PBS 3x in total, allowing dialysis to proceed for 15 min after each buffer exchange.

2.2.6. Using a pipettor, carefully recover the solution containing the quantum dots from the dialysis device(s), and transfer each sample into a fresh tube containing an equimolar amount of thiolated oligonucleotide diluted in PBS. Combine an equal volume of PBS and a $1/10^{th}$ the volume of the reduced and purified oligonucleotide sample (approximately 40 µM concentration) as the quantum dot concentration at this point is ~4 µM. Incubate for 2 h at room temperature, with shaking at 1000 rpm.

2.2.7. Add bovine serum albumin (BSA, 10 mg/mL) to each sample to obtain 0.5 mg/mL final BSA concentration. Using a pipettor, carefully transfer each sample into a fresh dialysis device, and dialyze against a volume of storage buffer that is at least 1000x the volume contained in the dialysis device(s). Exchange the buffer 2x, and perform dialysis with fresh storage buffer 3x in total, allowing dialysis to proceed for 15 min after each buffer exchange.

2.2.8. Using a pipettor, carefully recover each sample, place it into a fresh tube, and store at 4 °C.

NOTE: Do not store at -20 °C as quantum dots are damaged by freezing.

2.3. Annealing of quantum-dot-labeled oligonucleotide to biotinylated oligonucleotide

2.3.1. Resuspend each biotinylated oligonucleotide in storage buffer at a concentration of 100 µM. Store unused samples at -20 °C.

2.3.2. Combine a sample of quantum dot-labeled oligonucleotide with a 10-fold molar excess of biotinylated oligonucleotide. As the estimated concentration of DNA in the quantum-dot-labeled sample is 2 µM, add 0.2 µL of biotinylated oligonucleotide for every 10 µL of this sample.

2.3.3. Heat the mixture in a heat block to 75 °C (the melting temperature for the complementary region). Hold at that temperature for 5 min. Turn off the heat block, and allow to cool slowly. Store the completed construct at 4 °C; do not freeze.

3. Surface functionalization of coverslips

NOTE: This process has been described previously in other JoVE video protocols[16,17]. This protocol describes an adapted version of the procedure with minor changes to accommodate a smaller glass slide. See the **Table of Materials** for other materials required for the surface functionalization of coverslips.

3.1. Place 5 coverslips into each coverslip holder. Make sure to skip one space between each pair of coverslips so that they do not stick to one another. Place the coverslips in their holder into a jar with a cover, add ethanol to the jar until the coverslips are covered, and screw the top on securely. Place the entire jar into the water in the bath sonicator, without submerging it completely, and sonicate for 30 min.

3.2. Fill a clean beaker or jar with ultrapure water. Use metal tweezers to remove the coverslips in their holder from the ethanol, and submerge them in water to rinse. Then, transfer the coverslips and holder into a jar containing a 1 M potassium hydroxide (KOH) solution. Screw the top on securely, place the jar in the sonicator bath, and sonicate for 30 min.

NOTE: Use care when handling the KOH solution as it is corrosive and an irritant.

3.3. Repeat the sonication in ethanol and KOH solution, rinsing the coverslips in water as described above between each step.

3.4. Transfer the coverslips in their holder into a clean jar filled with pure acetone. Finish the cleaning by sonicating the jar as described above for 30 min. Do not rinse with water after this step.

3.5. Using metal tweezers, transfer the coverslips in their holder into a clean 100 mL beaker containing 80 mL of fresh acetone and a micro scale stir bar. Place the beaker on a magnetic stir plate set to at least 1,000 rpm. While the acetone is being stirred vigorously, pipet 1.6 mL of 3-aminopropyltriethoxysilane (APTES) into the beaker to make a 2% v/v solution.

NOTE: Use care when pipetting APTES as it is corrosive.

3.6. Allow the coverslips to incubate in the solution for 2 min, then use metal tweezers to transfer the coverslips in their holder into a beaker of water to quench the reaction. Rinse the coverslips two more times, replacing the water in the beaker.

3.7. Cure the silanized glass in the oven at 120 °C for 75 min. If not continuing to the next step immediately, store the coverslips under vacuum for maximum of a few days.

3.8. Dissolve *N*-hydroxysuccinimide (NHS) ester-derivatized polyethyleneglycols (PEGs) in 100 mM sodium bicarbonate (pH 8.2). Use a 10:1 ratio of methoxy-terminated PEG to biotin-terminated PEG, with ~100 mg/mL of methoxy-terminated PEG.

3.9. Pipet 100 µL of the PEG solution onto half of the dry silanized coverslips, and cover each

one with a second coverslip. Use small pieces of parafilm placed at each corner as spacers to prevent the coverslips from sticking to each other.

3.10. Incubate the coverslips in a humid environment for 3.5 h. Separate the coverslip sandwiches. Use a squirt bottle to wash each coverslip with copious water and dry with compressed air.

3.11. Store functionalized coverslips under vacuum. Be sure to keep the PEG-treated side up because the other side will not capture the DNA substrate.

4. Microfluidic device construction

NOTE: See the **Table of Materials** for other materials required for the construction of the microfluidic device.

4.1. Using a handheld rotary multitool fitted with a tapered diamond point wheel bit, drill two holes in opposite corners of the quartz slide to serve as an inlet and outlet. Be sure to secure the slide in place, lubricate the bit, and slide constantly with water while drilling. After each experiment, recover the prepared quartz slide for reuse by soaking a used device in acetone to dissolve the adhesive and epoxy; discard the remaining components.

NOTE: The drilling can be carried out by hand, but the use of a press-style holder for the multitool makes the process easier.

4.2. Combine 25 µL of streptavidin solution with 80 µL of PBS and 20 µL of blocking buffer. Coat a PEG-treated coverslip with this mixture, and incubate in a humid environment for 30 min.

4.3. During the incubation of the coverslip, prepare the imaging spacer to create a flow channel. Cut a 1 inch square piece of imaging spacer material, then mark a 2 mm wide channel aligned to the ends of the holes drilled in the quartz slide (**Figure 2**). Cut the channel out of the spacer material with a scalpel.

4.4. Peel one side of the backing from the image spacer, and carefully place it onto the quartz slide, taking care not to cover the inlet and outlet holes. Be sure to clean the quartz slide thoroughly with acetone to remove any remnants of adhesive from prior experiments.

4.5. Wash the coverslip with water, and dry with compressed air. Remove the other side of backing from the imaging spacer, and sandwich it between the quartz slide and the functionalized, streptavidin-coated coverslip, using the plastic tweezers to press the assembly together and remove air bubbles from the adhesive.

4.6. Insert one 30 cm long polyethylene tubing into each hole in the quartz slide. Be sure to cut the ends of the tubing at an angle to ensure free flow of solution. Use a tube rack or other support to hold the tubes in place, and seal the tubes in place and the edges of the assembled

device with epoxy.

NOTE: It works well to build the device on a piece of parafilm, which can easily be peeled off the bottom when the epoxy has set.

4.7. Once the epoxy has set, insert the blunt needle on the empty syringe into the outlet tube of the device, and submerge the end of the inlet tube in a container filled with blocking buffer. Pull back on the syringe plunger to fill the device with blocking buffer. Leave the device to incubate for at least 30 min prior to use.

5. Surface tethering of quantum-dot-labeled DNA substrate

NOTE: Besides the microfluidic device, DNA substrate, and blocking buffer described above, see the **Table of Materials** for other materials and **Table 1** for buffers required for the surface tethering of quantum-dot-labeled DNA substrates.

5.1. Attach the microfluidic device to the microscope stage plate with tape, bring the objective into contact with the bottom of the device, and position the objective so that the field of view is within the microfluidic channel.

5.2. Flush the microfluidic channel with fresh blocking buffer by pulling back the syringe plunger after connecting the outlet tube to the syringe pump. Check to make sure there are no bubbles trapped in the tubing or in the channel.

NOTE: From this point on, make sure that the inlet tube is in liquid so that no air bubbles are introduced into the device.

5.3. Dilute 1 µL of the prepared DNA substrate into 1 mL of blocking buffer. Place the inlet tube into the diluted DNA substrate, and flow 800 µL of the substrate solution through the channel at a rate of 200 µL/min. Allow the DNA solution to incubate in the channel undisturbed for 15 min after the flow stops.

5.4. Flow at least 800 µL of blocking buffer through the channel at a rate of 200 µL/min to rinse the unbound DNA out of the channel.

5.5. Adjust the laser power, microscope focus, and TIRF angle so that the surface-tethered quantum dots are clearly visible.

NOTE: Although quantum dots do not bleach quickly, it is best to keep the power as low as possible to minimize blinking.

6. REase-mediated DNA cleavage

NOTE: See the **Table of Materials** for materials and **Table 1** for buffers required for REase-

mediated DNA cleavage.

6.1. Make sure that the camera is cooled to optimal operating temperature and set up for high-speed streaming with an exposure time of 0.10 s. Be prepared to collect data for ~4 min.

6.2. Flush the microfluidic device with 800 µL of experimental buffer without magnesium at a flow rate of 200 µL/min.

6.3. Add the REase to an aliquot (1 mL) of experimental buffer without magnesium, and mix gently by pipetting. Use 4 µL of 100,000 units/mL stock, which corresponds to 400 units/mL of EcoRV. Flow 800 µL of the diluted enzyme through the channel at a flow rate of 200 µL/min.

6.4. Start the experiment by flowing experimental buffer containing magnesium and fluorescein at a flow rate of 200 µL/min. Begin capturing data immediately after starting the syringe pump. After flowing 800 µL of buffer, stop data acquisition.

7. Data analysis

NOTE: See the **Table of Materials** for the data analysis software used for this protocol, and make adjustments if using a different analysis platform.

7.1. Zero time-point determination

7.1.1. Subtract background fluorescence from each frame of the experimental movie using the **imtophat** function, a built-in morphological top-hat filtering function in the **Image Processing Toolbox**. Select a **disk with a radius of three pixels** as the structuring element. Obtain the background intensity by subtracting the filtered image from the original image.

NOTE: The filter only retains image features brighter than the background and smaller than the structuring element.

7.1.2. Average the subtracted background over each movie frame. Use a trajectory of this value to determine the zero time-point for the experiment (**Figure 3A**). Determine the start time by finding the first frame at which the rate of increase exceeds 3x the standard deviation of the rate of change during the dead volume flow.

NOTE: A sharp increase in the rate of change of the average value of the subtracted background for each movie frame indicates the time at which the final experimental buffer containing the tracer dye entered the flow cell (**Figure 3B**).

7.2. Quantum dot intensity trajectory calculation and analysis

7.2.1. Calculate a maximum-intensity projection for the set of background-corrected movie frames recorded prior to the arrival of the tracer dye. Determine the locations of quantum dots

to one-pixel accuracy in this projection image using a peak-finding function such as **pkfnd**.

7.2.2. Generate intensity trajectories for individual quantum dots by calculating the average intensity in each movie frame of a square region three pixels per side surrounding the location returned by the peak-finding function.

NOTE: The background correction described above removes the artefacts introduced by the tracer dye. The resulting intensity trajectories should be relatively flat, but still include naturally occurring intensity fluctuations (**Figure 4**).

7.2.3. Identify quantum dot disappearance events by statistical analysis of the intensity trajectories. For each quantum dot intensity trajectory, calculate a threshold intensity that is above the minimum value by an appropriate fraction of the difference between the maximum and minimum values for that trajectory, depending on the noise observed in the trajectories.

NOTE: The intensity fluctuation for the quantum dot is typically much greater than the background fluctuation. An appropriate value can be determined by comparing these values: the threshold selected must be higher than the highest background value, but ideally be lower than the lowest value for quantum dot fluorescence. For the data presented, the threshold used was calculated to be above the minimum value for each trajectory plus one third of the difference between the maximum and minimum values for that trajectory. A quantum dot can be deemed to disappear at the last time point that the observed intensity at its location was above this threshold.

7.2.4. Confirm putative disappearance events. Only include events in the final analysis if the observed intensity is above the midpoint between the minimum and maximum intensity values for the trajectory for over half of the movie frames prior to the time of disappearance and the standard deviation of the intensity trajectory decreased after the event.

NOTE: Other statistical tests may be employed to ensure that only valid disappearance events are logged.

REPRESENTATIVE RESULTS:

The flow cell is directly coupled to a high numerical aperture oil-immersion 60x magnification objective on an inverted microscope equipped with laser illumination for through-objective TIRF imaging (**Figure 5A**). After introducing the DNA substrate and washing away excess DNA and quantum dots, there are typically thousands of individual quantum dots in a field of view (**Figure 5B**). These quantum dots are stably attached to the glass surface, and they do not undergo noticeable dimming or significant bleaching over the time scale of the experiment. However, if a buffer containing both magnesium and an appropriate REase is flowed through the flow channel, at the end of a typical four-minute observation period, at least 30% of the quantum dots present at the beginning of an experiment will have disappeared from the field of view. Confirming the magnesium requirement, when the REase is flowed through the channel in the absence of magnesium, at least 95% of quantum dots present at the beginning of the experiment can still be

seen at the end of the observation period (**Figure 5C**). However, when magnesium-containing buffer is flowed immediately after allowing the REase to bind the surface-bound DNA in the absence of magnesium, up to half of the quantum dots will have disappeared by the end of the observation period (**Figure 5D**), similar to what is observed when REase and magnesium were flowed through the channel together. The exact yield of events will depend on the efficiency of the enzyme under the conditions used. When magnesium-containing buffer is flowed without having previously introduced the appropriate REase into the flow channel, less than 5% of quantum dots disappear during the observation period, and there is no discernible peak for a histogram of the events. This result indicates that prebound REase molecules mediate cleavage of the DNA that tethers the quantum dots to the glass surface, and this DNA cleavage is responsible for the vast majority of observed quantum dot disappearance events in these experiments.

Quantum dot disappearance happens quickly and results in a sharp decrease in the intensity trajectory, providing a clear indication of the time at which a given DNA molecule is cleaved (**Figure 5E**). The determination of event times is accomplished by mathematical analysis of intensity trajectories. Single quantum dots generate intensity trajectories with significantly higher variance than the background under the image conditions used, so putative events are confirmed when the variance of the intensity trajectory decreases to a level comparable to the variance of the background after the observed intensity drop. In addition, trajectories that include a large degree of blinking prior to a putative disappearance event are excluded from the final analysis. However, a typical experiment results in hundreds of events that meet these criteria, and the results of multiple experiments can be pooled to provide adequate statistics to allow estimation of the parameters N and β by either nonlinear least-squares curve-fitting or maximum-likelihood parameter estimation.

The representative data presented were collected by performing this experiment with the well-studied Type IIP REase, EcoRV (**Video 1**). The 60-bp-long duplex DNA substrate is constructed with a biotin molecule on the 5' end of one strand of the duplex and a quantum dot covalently attached to the 5' end of the other strand. The DNA substrate contains a single copy of the recognition sequence, GAT↓ATC, which is cleaved by EcoRV in the middle, as indicated by the downward arrow (↓). EcoRV was prebound to the DNA substrate in the absence of magnesium, and then magnesium-containing buffer was flowed to initiate DNA cleavage. The representative data include the pooled results of five separate experiments, yielding a total of 3451 observed cleavage events. After excluding events that occurred before the zero time-point or outside of the prominent activity peak, 2987 events remained, which were enough to populate a histogram with one-second bins. Both nonlinear least-squares curve-fitting and maximum-likelihood parameter estimation were used to extract the values of N and β from the data. The two parameter estimation methods were in agreement (**Figure 6A**), with N = 3.52 (95% confidence interval: 3.32–3.71) and β = 5.78 s (95% confidence interval: 5.41–6.15 s) for the nonlinear least-squares fit and N = 3.41 (95% confidence interval: 3.25–3.58) and β = 6.06 s (95% confidence interval: 5.75–6.39 s) for the maximum-likelihood estimation.

The estimate of at least three kinetic steps is reasonable given what is known about the

mechanism of EcoRV. This enzyme is known to bind DNA non-specifically in the absence of magnesium[14]. Bulk solution phase observations of the changes in the intrinsic fluorescence of tryptophan residues in EcoRV under stopped flow conditions suggested that EcoRV that has bound to DNA in the absence of magnesium must undergo a conformational change prior to the entrance of magnesium into the active site[18]. This observation is corroborated by crystal structure data[19]. The results of tryptophan fluorescence studies also indicate that DNA cleavage and product release are separate steps that occur with similar rates[18]. Therefore, a reasonable reaction mechanism for EcoRV-mediated DNA cleavage in this experiment is:

$ES \rightarrow ES^* \rightarrow EP \rightarrow E+P$

In this mechanism, ES, the initial enzyme-substrate complex, is formed when EcoRV is prebound to the DNA in the absence of magnesium. When magnesium enters the flow cell, the enzyme-substrate complex undergoes a conformational change to become ES*, the activated enzyme-substrate complex. This activated complex then cleaves DNA, but does not immediately release the product molecules, becoming an enzyme-product complex (EP). Finally, the product, P, is released in the final step. This mechanism requires three steps, which is in agreement with the data presented. The resulting estimate for the average waiting time for each step is ~6 s, equivalent to a rate of 0.17 s^{-1} for each step. This calculation is in general agreement with previous estimates of the rates for these processes—on the order of 0.3–0.4 s^{-1} for DNA cleavage and product release and ~0.5 s^{-1} for the conformational rearrangement.

FIGURE AND TABLE LEGENDS:

Figure 1: Labeled DNA and enzyme reaction schematic. Quantum-dot-labeled DNA molecules are tethered to the functionalized glass surface via biotin-streptavidin-biotin linkage and observed using TIRF microscopy. The DNA molecules contain the recognition site for the REase of interest. When a DNA molecule is cleaved by the REase, the quantum dot is free to diffuse away from the surface and out of the illumination zone. Abbreviations: REase = restriction endonuclease; PEG = polyethylene glycol; TIRF = total internal reflection fluorescence.

Figure 2: Microfluidic flow cell device. (**A**) Exploded view showing the three layers used to create the device: the functionalized glass coverslip on the bottom, the quartz slide with inlet and outlet holes on the top, and the double-sided adhesive imaging spacer with a channel cut into it sandwiched in the middle. (**B**) A completed device with polyethylene tubes sealed in place and with edges coated with epoxy.

Figure 3: Zero time-point determination. (**A**) Average background intensity as determined using the morphological top-hat filtering function over each frame of the movie. The background intensity increases markedly when the experimental buffer containing fluorescein enters the field of view. The results from three different experiments are shown here. There is substantial variation in the lag time from experiment to experiment. (**B**) The sharp increase in the rate of change of the background fluorescence intensity trajectory facilitates accurate determination of

the zero time-point.

Figure 4: Background correction. The increase in the background intensity due to the fluorescein tracer dye indicating the arrival of magnesium can be seen in the raw fluorescence intensity trajectories for individual quantum dots (grey trajectory). After subtracting background using the morphological top-hat filtering function, the artefacts introduced by the tracer dye have been removed from the trajectory (black trajectory).

Figure 5: Single-molecule TIRF experiment for parallelized observations of DNA cleavage events. (**A**) A flow cell constructed on a functionalized glass surface designed to capture quantum-dot-labeled DNA is directly coupled to a high numerical aperture 60x oil immersion microscope objective for TIRF imaging. (**B**) Representative image from the beginning of an experiment. Quantum-dot-labeled DNA has been loaded into the flow cell, and excess unbound quantum dots have been washed away. Individual DNA-tethered quantum dots can be resolved from one another. (**C**) The same field of view after buffer containing a REase capable of cleaving the DNA tethers has been flowed through the flow channel in the absence of magnesium for four minutes. There has been no significant loss of DNA-tethered quantum dots. (**D**) The same field of view at the conclusion of an experiment. Magnesium-containing buffer was flowed through the flow channel immediately after flowing REase in a magnesium-free buffer. This image was acquired after approximately four minutes of buffer flow. Prebound REases have cleaved many of the DNA tethers, releasing the quantum dots from the surface. For ease of viewing, only the central quadrant of the objective field of view is shown in each image. Ten movie frames (equivalent to one second of observation time) were averaged to diminish the effects of quantum dot blinking. Brightness and contrast settings are identical for all three images. (**E**) Representative fluorescence intensity trajectories from image locations where a quantum dot was present at the beginning of the experiment. Trajectories obtained from image locations corresponding to quantum dots that are not released from the surface (grey) may display brief dips to a lower intensity level, but they begin and end at a high intensity level. Trajectories obtained from image locations, corresponding to quantum dots that are released from the surface (black), display a rapid decrease in intensity to a low background level that is instantaneous with respect to the time resolution of the experiment (10 Hz). Released quantum dots do not reappear within the four-minute observation period. Abbreviations: REase = restriction endonuclease; TIRF = total internal reflection fluorescence.

Figure 6: Dwell-time distribution analysis of EcoRV-mediated DNA cleavage. (**A**) Histogram and predicted envelopes for the 2987 cleavage events that occur within the main activity peak in a set of five pooled experiments with EcoRV. The two predicted curves are nearly identical, and the fit residuals (below the histogram) do not indicate systematic error in the nonlinear least squares fit. (**B**) Histogram of the entire set of 3393 cleavage events that occurred after the zero time-point. The curve predicted by the maximum-likelihood estimation of the parameters (unbroken line, MLE curve) assuming a gamma probability distribution fails to enclose the histogram. The curve predicted by a nonlinear least-squares fit of the formula for the gamma probability distribution to the bin heights (dashed line, NLS curve) is superior, but the residuals of the fit (below the histogram) reveal systematic error. Abbreviations: MLE: maximum-likelihood

614 estimation; NLS = nonlinear least-squares.
615
616 **Table 1: Table of Buffers.**
617
618 **Video 1: Example single-molecule movie.**
619
620 **DISCUSSION:**
621 The DNA substrate for this assay is labeled with a quantum dot using a two-step reaction scheme
622 using sulfo-SMCC. This bifunctional crosslinker consists of an NHS ester moiety that can react
623 with a primary amine, and a maleimide moiety that can react with a sulfhydryl group[20]. The
624 thiolated oligonucleotides used to prepare the substrate are shipped in their oxidized form. It is
625 important to reduce and purify them, as described, before proceeding with the coupling
626 procedure, or the efficiency of the coupling reaction will be reduced. It is critical to prepare fresh
627 DTT and sulfo-SMCC solutions immediately before use. These molecules hydrolyze rapidly in
628 aqueous solution. Use care when transferring solutions into and out of the dialysis devices—if
629 the pipette tip touches the membrane, it will cause a rupture, and the sample will be lost. It is
630 ideal to complete all the steps in section 2 without interruption to avoid sample loss.
631
632 The coupling reaction used to generate the quantum-dot-labeled oligonucleotide is inefficient.
633 After combining and annealing the quantum-dot-labeled oligonucleotide with the biotinylated
634 oligonucleotide, there will be excess unlabeled DNA present in the prepared substrate sample.
635 Although these species can bind to the functionalized surface, they are not visible under the
636 imaging conditions recommended and do not contribute to the readout of the experiment. To
637 confirm that cleavage of the completed construct results in the release of quantum dots from
638 the surface, carry out the surface tethering as described in section 5 of the protocol, and image
639 a region of the surface to determine how many quantum dots are present. Then, flow 800 µL of
640 a 1 mL aliquot of 10 U/mL of DNase I in DNase buffer (2.5 mM $MgCl_2$; 10 mM Tris-HCl; 0.1 mM
641 $CaCl_2$, pH 7.6) through the microfluidic channel. Allow the DNase I to incubate in the channel for
642 15 min, wash with 800 µL of DNase buffer (without enzyme), and image the same region of
643 interest. Approximately 90% of the quantum dots should be released from the surface if the
644 construct and surface are properly prepared.
645
646 To covalently attach a mixture of methoxy-terminated and biotin-terminated PEG molecules,
647 glass surfaces must be treated to capture the quantum-dot-labeled DNA substrate on the surface
648 for imaging. It is critically important to carry out all cleaning steps rigorously. Multiple cleaning
649 steps are required to remove surface impurities and to expose hydroxyl groups on the glass
650 surface that will react with the alkoxy groups on the silane to form covalent bonds. While it is
651 important to rinse the slides with ultrapure water between the clean steps using ethanol and
652 KOH solutions, no water should be used after the final cleaning with acetone. The presence of
653 excess water during the silanization step will encourage multimerization of the silane in solution
654 at the expense of surface binding. In addition, the silane solution must not be prepared in
655 advance. To prolong its useful life, the silane stock should be stored in a desiccator cabinet.
656
657 The single-molecule cleavage assay is designed to be carried out in a microfluidic device (**Figure**

2). Fabricate a new device prior to each experiment, reusing the drilled quartz slide that forms the top of the device. This procedure has been described previously[16,17] and its variations are widely used for TIRF microscopy. The procedure described herein has been adapted to accommodate 1 inch square slides. It is important to work quickly to build the device so that the functionalized surface does not degrade while it is exposed to air. Epoxy is fluorescent, so take care to avoid covering the channel with it. The drilled quartz slide can be recovered for reuse by soaking the used device in acetone and disassembling it.

This assay is designed to be carried out on an inverted microscope that has through-objective total internal reflection illumination capabilities fitted with an electron-multiplying charge-coupled device (EMCCD) camera for data collection. Use a 60x, high numerical aperture objective, and a 488 nm laser excitation source, and use a filter cube that includes a single-bandpass emission filter and single-edge dichroic suitable for the laser, and an emission filter that permits both the fluorescence of the quantum dots (emission peak at 655 nm) and at least some of the fluorescein fluorescence (emission peak at 512 nm) to pass through. For example, the data presented here was collected with a filter cube fitted with a 482/18 nm single bandpass excitation filter, a 488 nm single edge dichroic, and a 675/67 nm single bandpass emission filter. The assay can be carried out by imaging in a single spectral channel, as described in this protocol. If desired, the assay can also be carried out by splitting the fluorescence image into two spectral channels, one for the quantum dots, another for the fluorescein emission. An image splitter fitted with a 640 nm single edge dichroic mirror would be appropriate for this purpose. However, multichannel imaging is not required.

It is important to wash out any untethered DNA substrates from the channel before beginning the experiment. Fluorescein is included at a concentration of 50 µM in the final buffer to signal the arrival of the magnesium into the field of view. Depending on the microscope setup, it may be necessary to adjust the concentration of fluorescein so that its emission does not obscure the quantum dots. Be sure to start acquiring data immediately after starting the flow of the magnesium-containing buffer. Record data during the lag time before the arrival of the magnesium-containing buffer so that the mean and standard deviation of the rate of change in the background intensity can be calculated to facilitate accurate determination of the zero time-point. Note that the most accurate zero time-point is the first frame at which the rate of change of the background fluorescence exceeds the natural fluctuations in this measurement. The background fluorescence intensity continues to increase after this point as fluorescein diffuses across the boundary layer established by the laminar flow in the channel. Magnesium, which is much smaller than fluorescein, diffuses more rapidly, and its concentration can be expected to reach equilibrium much more rapidly.

For the example data presented, a histogram of all 3393 events that occurred after the zero time-point displays a prominent peak within the first 60 s of the experiments. Many bins corresponding to longer lag times contain no events, and those bins that contain events contain only a few, similar to what is observed when no active REase is present. A nonlinear least-squares curve-fit of the gamma probability distribution to all the binned data resulted in parameter estimates of N = 3.06, with a 95% confidence interval of 2.94–3.18, and β = 7.04 s, with a 95% confidence

interval of 6.72–7.36 s. However, the maximum-likelihood estimates for the parameters were N = 1.30 (95% confidence interval: 1.24–1.35) and β = 26.7 s (95% confidence interval: 25.3–28.1 s), and by visual inspection, the curve predicted by the maximum-likelihood parameter estimate does not match the data well (**Figure 6B**). Furthermore, although the curve predicted by the parameters estimated by nonlinear least-squares fitting follows the shape of the histogram of observed event frequencies relatively well, the residuals (difference between observed and predicted event frequencies) become consistently positive for time-points beyond about 60 s (**Figure 6B**).

As the frequency of events in the later time-points is comparable to the frequency of quantum dot disappearance observed in the absence of active enzyme, most of these later events are likely to be part of a low background level of quantum dot loss that is not caused by REase-mediated DNA cleavage. It is therefore reasonable to exclude those bins from fitting. When all events that occurred more than 60 s after the zero time-point of each experiment were excluded, the two parameter estimation methods were in agreement (**Figure 6A**). Therefore, to achieve the best parameter estimates, exclude events outside of the activity peak, and confirm that the maximum-likelihood parameter estimate and the nonlinear least-squares curve-fit results have converged.

The experimental approach outlined in this paper permits real-time observations of REase-mediated DNA cleavage at the single-molecule level. Hundreds of events can be observed in each individual experiment, and the results of several experiments can be pooled to obtain the large number of observations required to characterize the distribution of waiting times by fitting the data to the gamma probability distribution. While this assay can only be used as described with REases that bind but do not cleave DNA in the absence of magnesium or another cofactor, many REases fall into this category. It is important to recognize that the exact details of all intermediate steps will not be revealed by this assay. Steps that are significantly faster than the rate-limiting step will not impact the dwell-time distribution and will not figure into the value of N extracted from the data[12]. Likewise, the value of β that can be extracted from the data is an approximate average of the waiting time for the steps that do impact the dwell-time distribution. The actual waiting time for each step may vary from the value of β[12]. However, the single-molecule cleavage assay described in this paper can provide evidence of the presence of multiple intermediate steps along the reaction pathway, even if the reaction intermediates themselves cannot be directly observed.

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DISCLOSURES:

The authors have no competing financial interests or other conflicts of interest

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795

Figure 1

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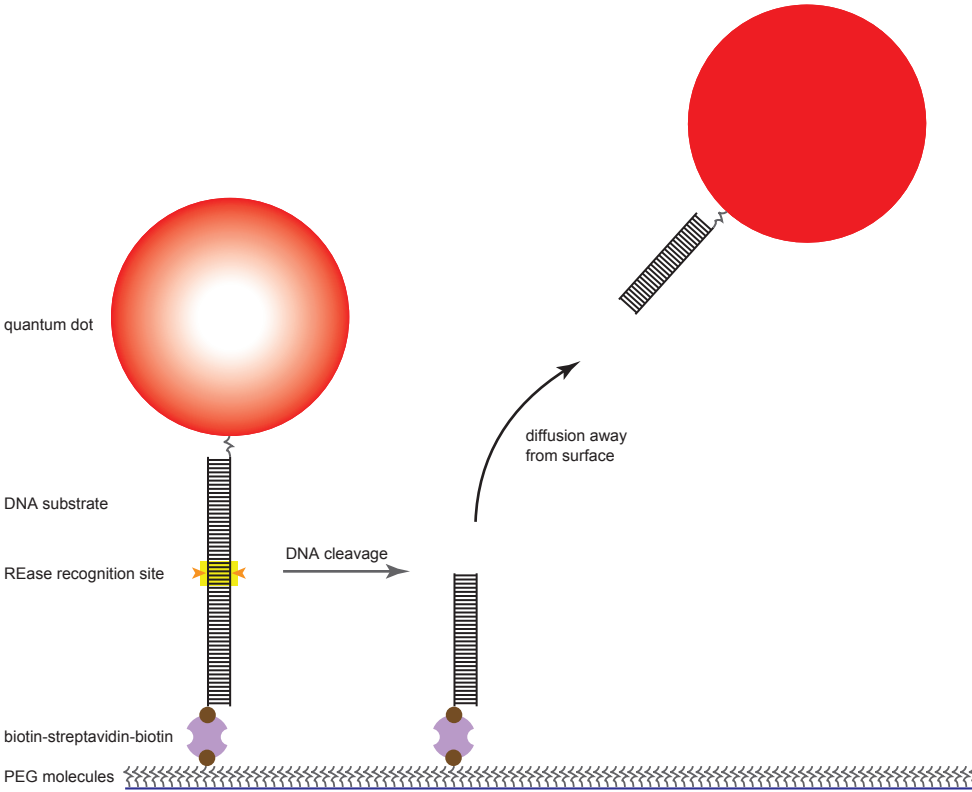

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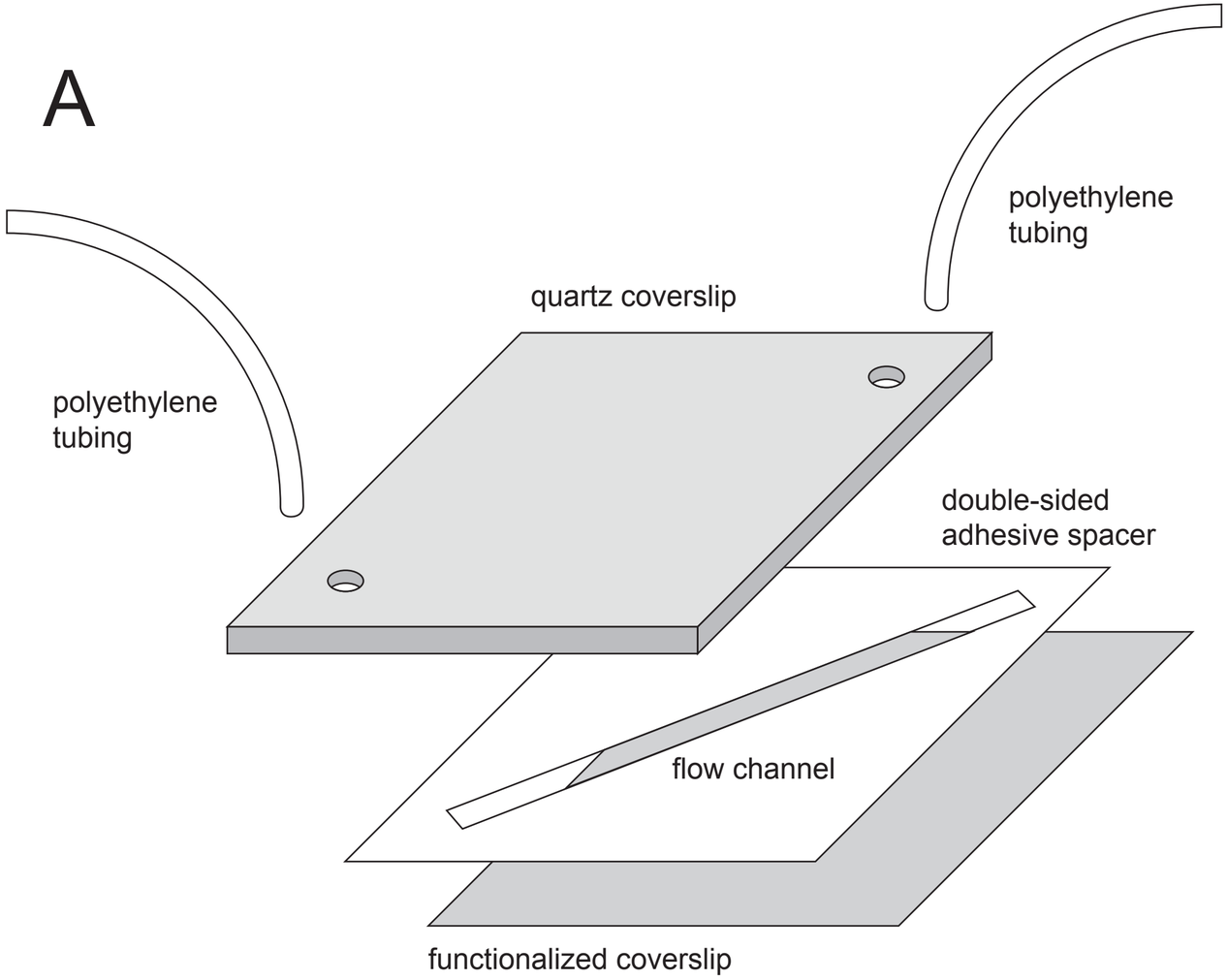

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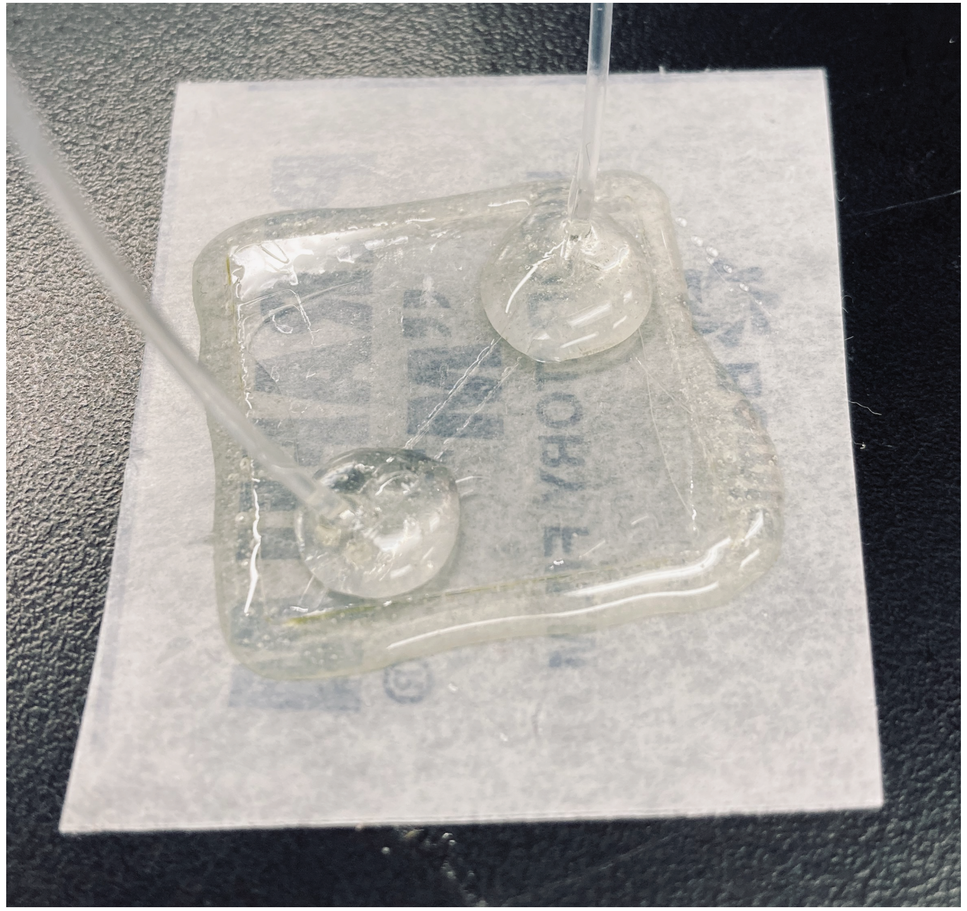
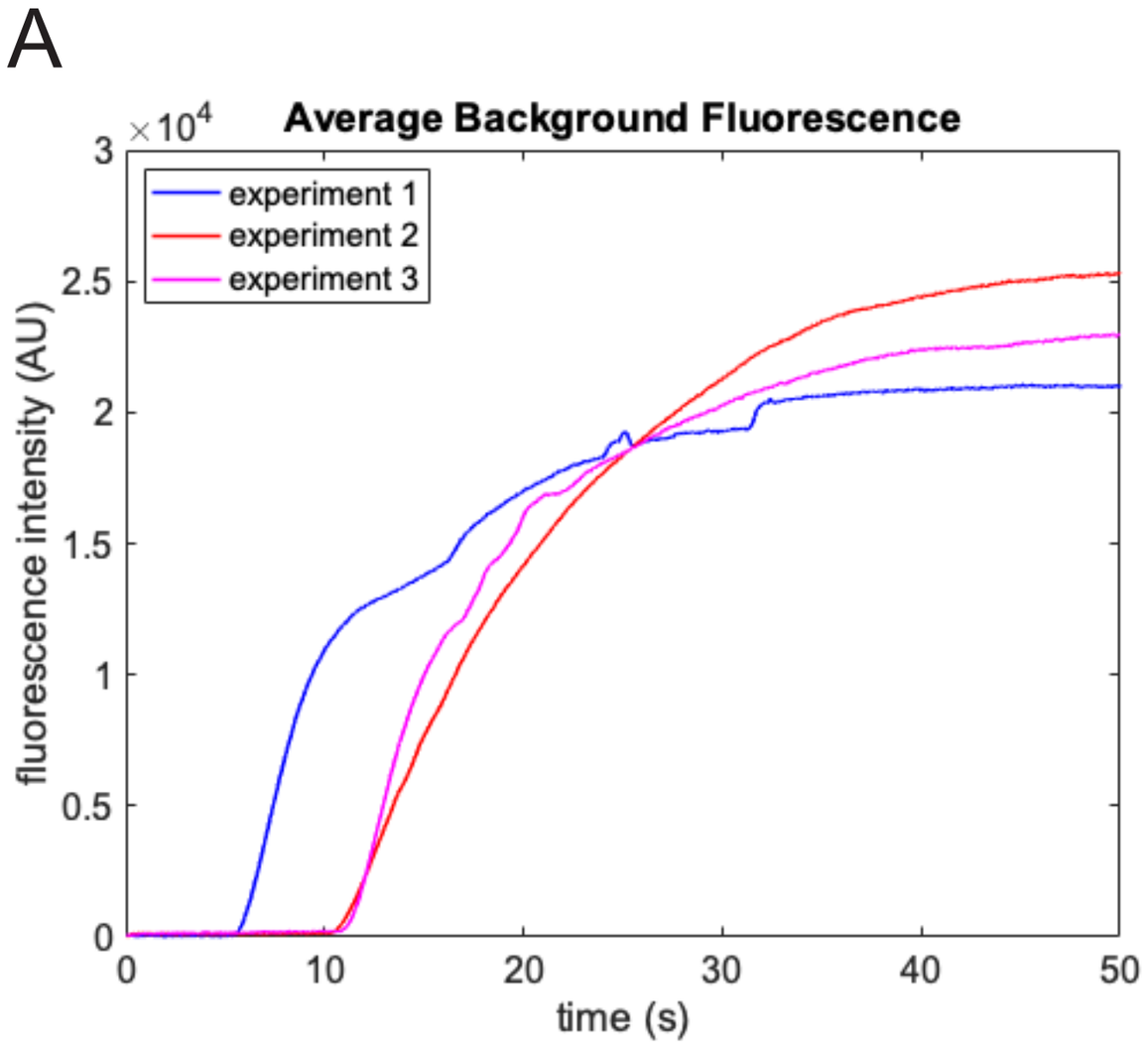


Figure 3



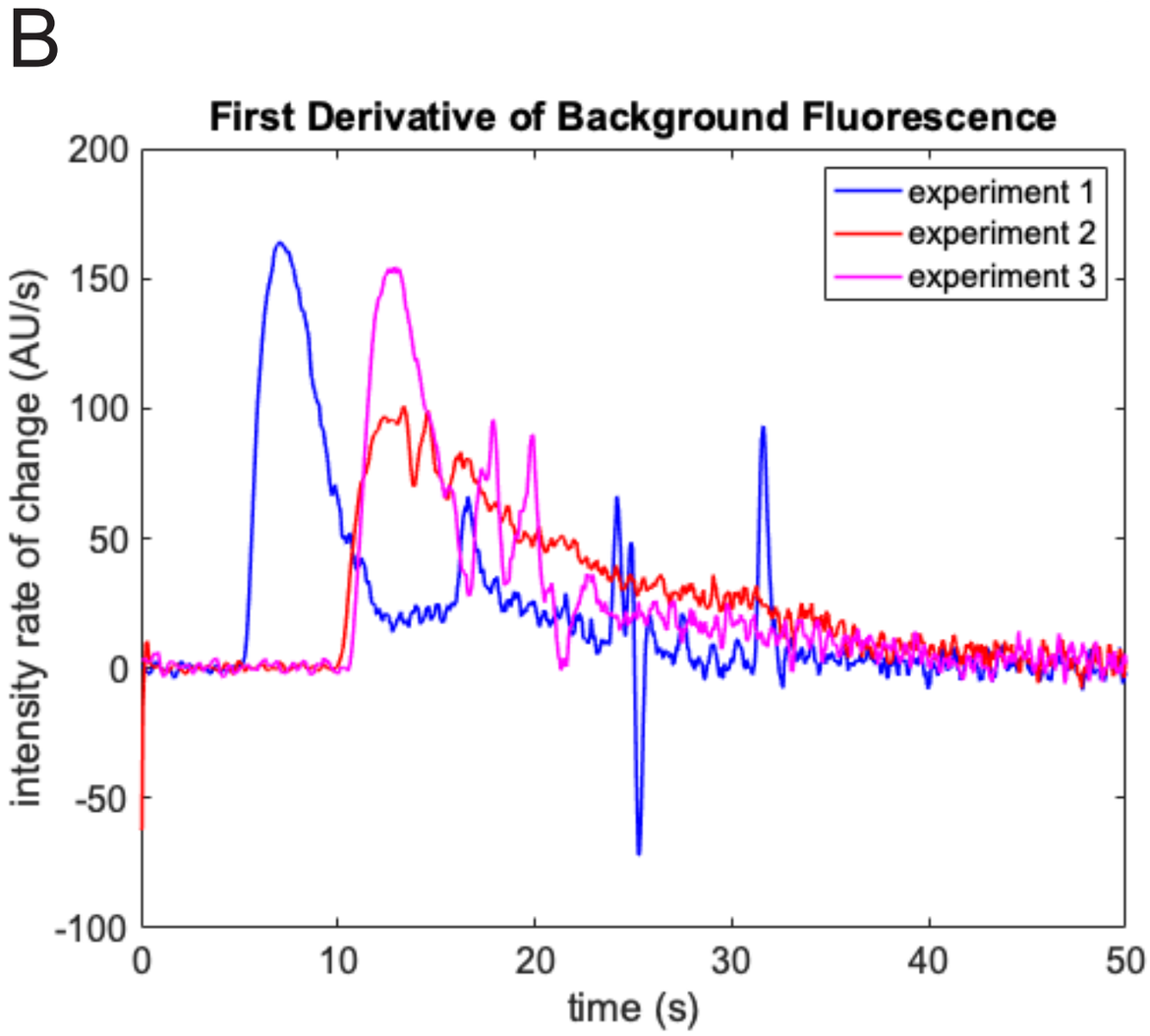


Figure 4

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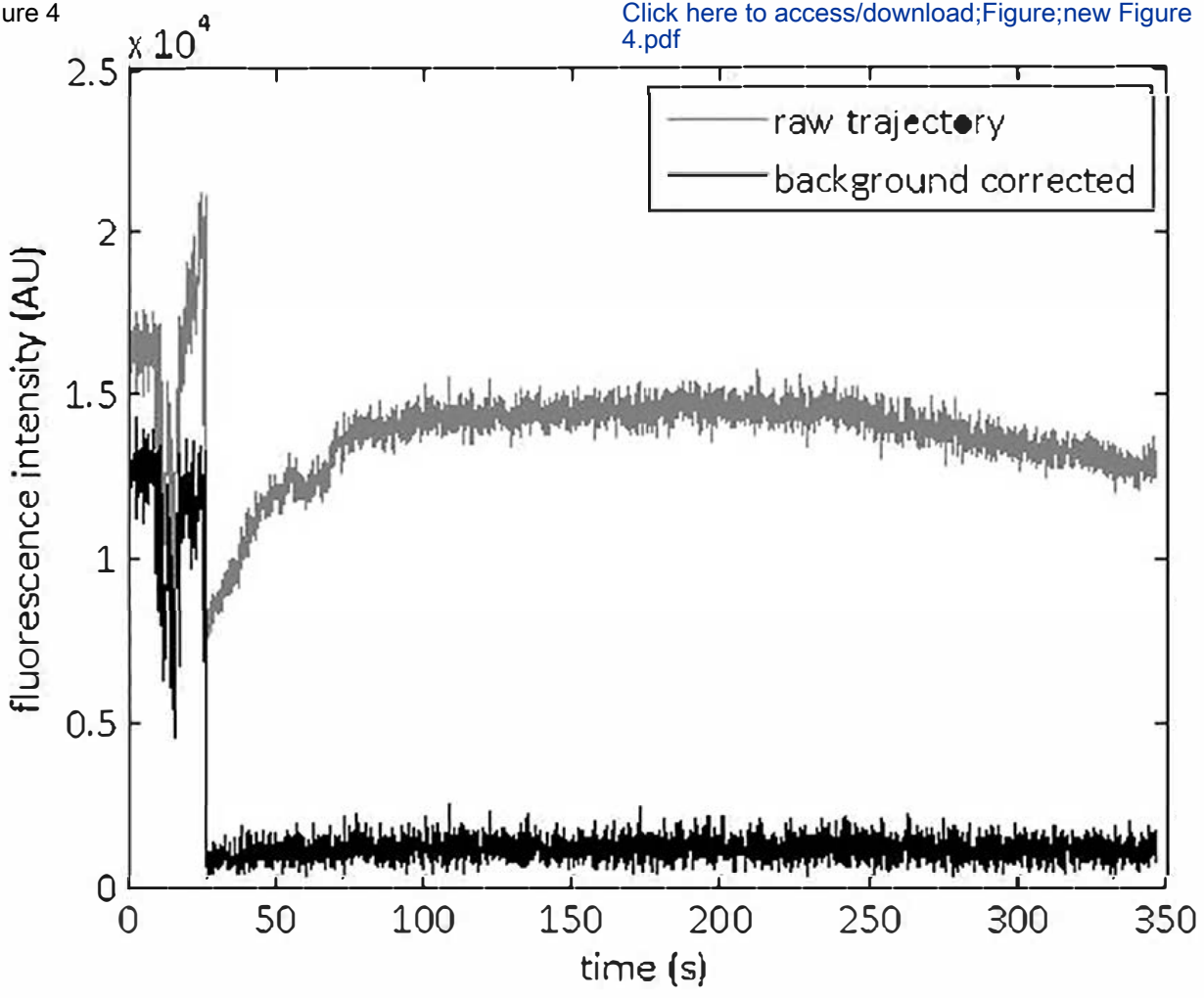


Figure 5

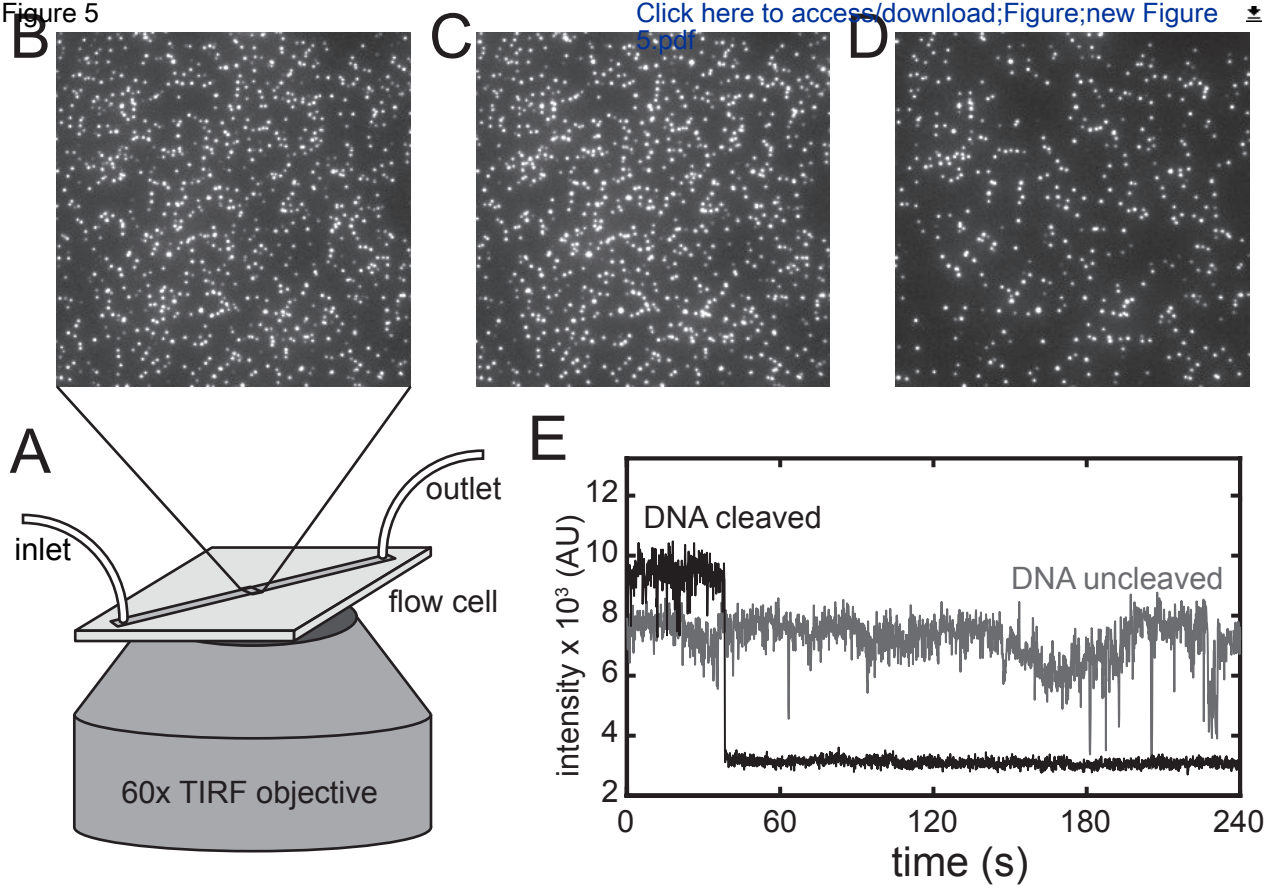
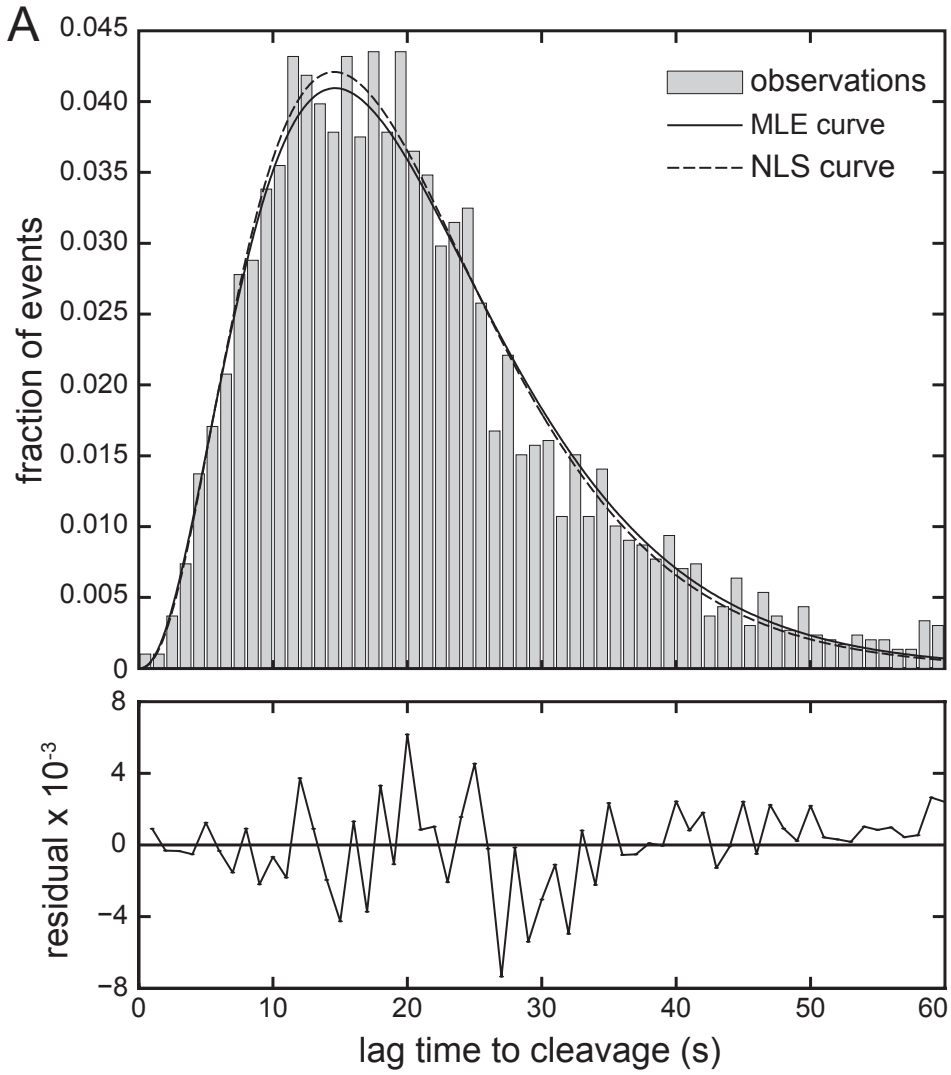
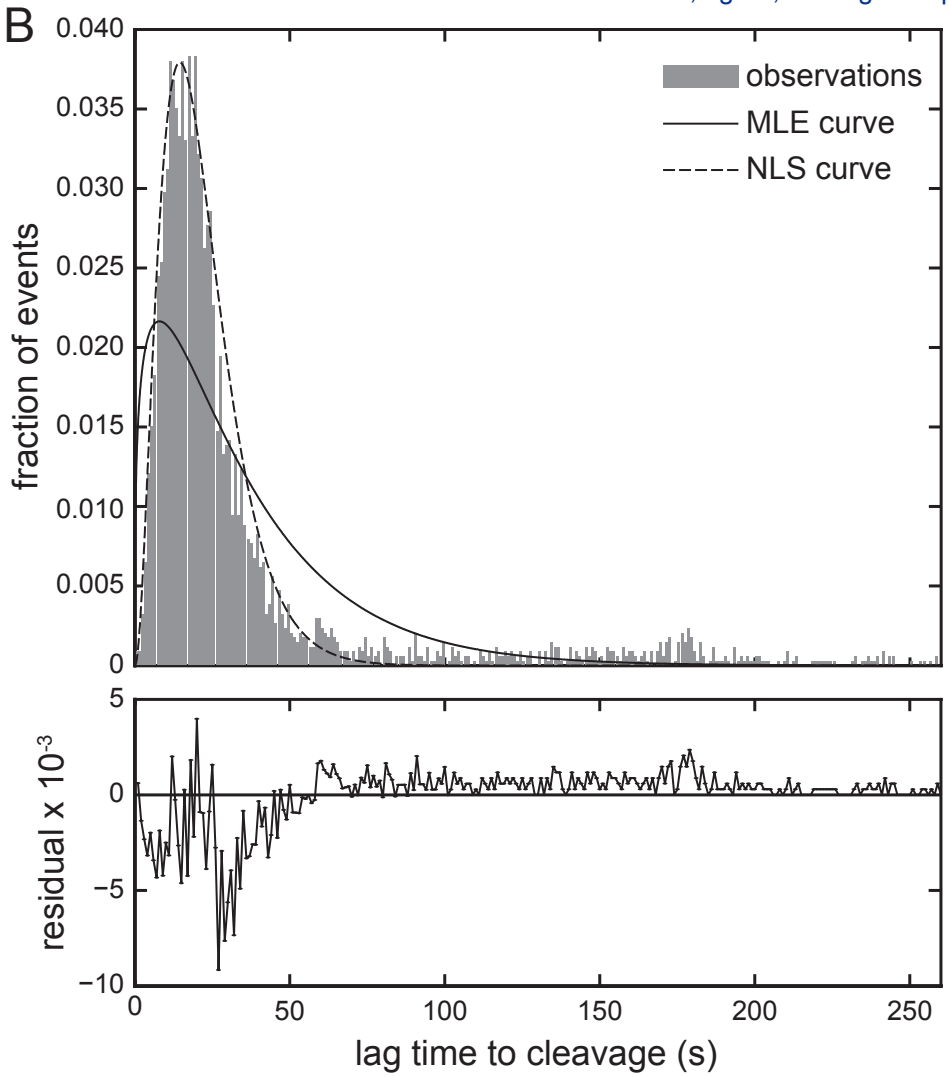


Figure 6





Example Single-Molecule Movie

Click here to access/download

Video or Animated Figure

example.mp4

Table 1 Click here to access/download;Table;Table of Buffers.xlsx

Name of Buffer	Component	Concentration
sodium phosphate buffer	sodium phosphate	100 mM
CHES buffer	N-cyclohexyl-2-aminoethanesulfonic acid (CHES)	10 mM
PBS	sodium phospate	3 mM
	sodium chloride	150 mM
	potassium phosphate	1.05 mM
storage buffer	sodium chloride	100 mM
	Tris-HCl	50 mM
	bovine serum albumin (BSA)	0.5 mg/mL
sodium bicarbonate	sodium bicarbonate	100 mM
blocking buffer	Tris-HCl	20 mM
	Ethylenediaminetetraacetic acid (EDTA)	2 mM
	sodium chloride	50 mM
	Tween-20	0.005% (v/v)
	bovine serum albumin (BSA)	0.2 mg/mL
experimental buffer without magnesium	sodium chloride	100 mM
	Tris-HCl	50 mM
	dithiothreitol (DTT)	1 mM
experimental buffer with magnesium	sodium chloride	100 mM
	Tris-HCl	50 mM
	magnesium chloride	10 mM
	dithiothreitol (DTT)	1 mM
	fluorescein	adjust according to imaging conditions
DNase buffer	Magnesium chloride Tris-HCl calcium chloride	2.5 mM 10 mM 0.1 mM

pH at 25 ℃
8.3
9.0
7.2 - 7.6
8.0
8.2
7.5
7.9
7.9
7.6

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3-aminopropyltriethoxysilane (APTS)	Sigma Aldrich	440140-100ML	Store in dessicator box
5 Minute Epoxy	Devcon	20845	use for sealing the microfluidic device
acetone	Pharmco	329000ACS	use for cleaning coverslips
bath sonicator	Fisher Scientific	CPXH Model 2800	catalog number 15-337-410
Beaker, glass, 100 mL			
Benchtop centrifuge			
Biotin-PEG-Succinimidyl Valerate (MW 5,000)	Laysan Bio	BIO-SVA-5K	Succinimidyl valerate has a longer half-life than succinimidyl carbonate
biotinylated oligonucleotide	Integrated DNA Technologies	custom - see protocol for design considerations	Request 5' Biotin modification and HPLC purification
Bovine Serum Albumin (BSA)	VWR	0903-5G	prepare a 10 mg/mL solution (aq) and heat to 95 ℃ before using
Centri-Spin-10 Size Exclusion Spin Columns	Princeton Separations	CS-100 or CS-101	used to purify thiolated oligonucleotides after reducing the disulfide bond
Centrifuge tubes 1.5. mL			
Coverslips, 1-inch square glass			
coverslip holders			
diamond point wheel	Dremel	7134	use for drilling holes in quartz flow cell topper
dithiothreitol (DTT)	Thermo Scientific	A39255	No-Weigh Format, 7.7 mg/vial
drill press rotary tool workstation stand	Dremel	220-01	facilitates quartz drilling

EcoRV (REase used to generate example data)	New England Biolabs	R0195T or R0195M	Use 100,000 units/mL stock to avoid adding excess glycerol Check REBASE for suppliers of other REases
ethanol	various	CAS 64-17-5	denatured or 95% are acceptable, use for cleaning coverslips
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	EDS	BioUltra, anhydrous, store in dessicator box
Flea Micro Spinbar	Fisherbrand	14-513-65	3 mm x 10 mm size to fit beneath coverslip rack
fluorescein	Acros Organics	17324	use to make experimental buffers
gravity convection oven	Binder	9010-0131	
handheld rotary multitool	Dremel	8220	use for drilling holes in quartz flow cell topper
ImagEM X2 EM-CCD Camera	Hamamatsu	C9100-23B	air cooling is adequate for this experiment, use HCImage software or similar to control
Imaging spacer, double-sided, adhesive			
Jar, glass with screw cap, (approximately 50 mm diameter by 50 mm high)			
magnesium chloride hexahydrate	Fisher Bioreagents	BP214-500	use to make experimental buffer with magnesium
MATLAB software			Data analysis
metal tweezers	Fisher Brand	16-100-110	
methoxy-PEG-Succinimidyl Valerate (MW 5,000)	Laysan Bio	M-SVA-5K	Both PEGs should have the same NHS ester so that the rate of reaction is consistent
microcentrifuge	Eppendorf	5424	

multiposition magnetic stirrer	VWR	12621-022	
N-cyclohexyl-2-aminoethanesulfonic acid (CHES)	Acros Organics	AC20818	CAS 103-47-9, use to make CHES buffer
orbital shaker and heater for microcentrifuge tubes	Q Instruments	1808-0506	with 1808-1061 adaptor for 24 x 2.0 mL or 15 x 0.5 mL tubes
Parafilm			
PE60 Polyethylene tubing (inner diameter 0.76 mm, outer diameter 1.22 mm)	Intramedic	6258917	22 G blunt needles are a good fit for this tubing size
Phosphate-Buffered Saline (PBS) 10x	Sigma Aldrich	P7059	Use at 1x strength
potassium hydroxide	VWR Chemicals BDH	BDH9262	use a 1 M solution to clean coverslips
Qdot 655 ITK Amino (PEG) Quantum Dots	Invitrogen	Q21521MP	
Quartz Slide, 1 inch square, 1 mm thick	Electron Microscopy Sciences	72250-10	holes must be drilled in the corners for inlet and outlet tubing insertion
reinforced plastic tweezers	Rubis	K35a	use for handling coverslips and building microfluidic device
SecureSeal Adhesive Sheets	Grace Biolabs	SA-S-1L	cut to form spacer for microfluidic device
Single channel syringe pump for microfluidics	New Era Pump Systems	NE-1002X-US	fitted with a 50 mL syringe and a 22 G blunt needle
Slide-a-Lyzer MINI Dialysis Devices, 10 kDa MWCO, 0.1 mL	Thermo Scientific	69570 or 69572	used for buffer exchange during quantum dot coupling to DNA
sodium bicarbonate	EMD Millipore	SX0320	use to make buffer for surface functionalization; 100 mM, pH 8

sodium chloride	Macron	7581-12	use to make experimental buffers
Sodium phosphate dibasic solution (BioUltra, 0.5 M in water)	Sigma Aldrich	94046	use to make 100 mM sodium phosphate buffer
Sodium phosphate monobasic solution (BioUltra, 5M in water)	Sigma Aldrich	74092	use to adjust pH of 100 mM sodium phosphate buffer
Streptavidin from *Streptomyces avidinii*	Sigma Aldrich	S4762	dissolve at 1 mg/mL and store 25 mL aliqouts at -20 ℃
Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC)	Thermo Scientific	A39268	No-Weigh Format, 2 mg/vial
Syringe fitted with blunt 21 G needle			
Syringe pump			
thiolated oligonucleotide	Integrated DNA Technologies	custom - see protocol for design considerations	Request 5' Thiol Modifier C6 S-S and HPLC purificaiton
TIRF imaging system with 488 nm laser illumination	various		custom built
Tris -HCl	Research Products International	T60050	use to make experimental buffers
Tris base	JT Baker	4101	use to make experimental buffers
Tween-20	Sigma	P7949	use to make blocking buffer
Ultrapure water			
vortex mixer	VWR	10153-842	
Wash-N-Dry Coverslip Rack	Electron Microscopy Sciences	70366-16	used for surface functionalization of coverslips

To the editor and reviewers:

Thank you for the opportunity to revise and resubmit this manuscript, and thank you for the careful review and helpful suggestions. We have attempted to address all of the concerns brought to our attention. Please see our responses to your comments in italics below.

Reviewer #1 Concerns:

1) The abbreviation "TIRF" is introduced multiple times (e.g. lines 19, 26, 86). This could be reduced to the first mention of the word.

TIRF is introduced once in the abstract, once in the summary, and once in the main body of the paper, assuming that some readers will engage with only some of these sections.

2) In the abstract in line 36, "in the absence of magnesium" is mentioned. Isn't magnesium added to measure the DNA cleavage? Or are the three steps only observed in the absence of magnesium? Perhaps a slightly different wording could be used.

The binding occurs in the absence of magnesium, and the cleavage mechanism is initiated by introducing magnesium. The wording has been adjusted to better reflect this.

3) Points 2.2.4, 2.2.5, and 2.2.7 of the protocol could potentially be clarified by adding a sentence about the total number of rounds of dialysis, such as "Dialysis is performed with fresh CHES/PBS buffer three times in total".

Thank you for this suggestion for clarification. The change has been made.

4) Regarding point 2.3.2 of the protocol, when annealing with a 10-fold excess of biotinylated oligonucleotide, do the authors observe many single-stranded DNAs bound to the surface?

There are likely a large number of single-stranded DNAs bound to the surface, as well as many duplex DNA molecules that are not labeled with a quantum dot. These species are not visible in the experiment, and do not contribute to the readout of the assay. Discussion of this point has been added to the manuscript.

5) For point 4.3 of the protocol, can the sentence be clarified?

That passage has been revised for clarity.

6) For Figure 4E, a legend could be added to avoid confusion with the legend from Figure 3.

We have added a label to figure 4E as requested.

7) In line 502 and 507, "systemic" or "systematic" error?

Thank you for alerting us to this incorrect word usage. It has been corrected.

Reviewer #2 Concerns:

Major Concerns:

1. A schematic diagram of the labeled DNA substrate and the enzyme reaction is needed in manuscript.

Thank you for this suggestion. A new figure has been included with the resubmission.

2. Gel electrophoresis is recommended to perform to demonstrate the DNA cleavage.

We have found that a good control experiment is to flow a nonspecific nuclease through the channel. We typically see up to 95% of qdots disappearing after such treatment. Description of this control has been added to the discussion.

3. The distributions of dwell times of the following group are needed to present: 1) in the absence of enzyme, 2) in the absence of Mg2+.

These experiments yield less than 5% of quantum dots disappearing. There is no discernable activity peak, therefore there is no need to show these distributions. We have added details on this matter to the discussion.

Minor Concerns:

1. Single-molecule microscopy movies should be provided.

An example movie has been included in the resubmission.

2. The video of experiments are needed.

Due to restrictions imposed to prevent the spread of COVID-19, the video of the experiments will be produced at a later date.

Reviewer #3 Concerns:

One of the main results of the protocol is the estimation of the number of steps in the catalytic cycle of restriction enzyme. This was demonstrated only with one example of RE - EcoRV, where N was found to equal 3. To prove the discriminating power of the measurement and at the same time its utility, examples of other molecular systems with different expected N should be presented.

While we appreciate this comment, we feel that this request is beyond the scope of this manuscript, which is focused on explaining how to carry out the assay. Our current ongoing work involves using this assay to uncover mechanistic details of other Type II REases. In these experiments, we do see different values of N and k. Those results will be presented in journals that are focused on new findings.

The protocol contains sufficient detail for the biochemical and flow-cell preparations. At the same time, the description of the opto-mechanical setup that is an important part of the experiment is only provisional. The authors could complement the protocol with more detail in that regard.

We have assumed that the reader will have access to a TIRF setup. Additional details regarding the specifications of the filters used for imaging were added to the manuscript.

Parts of the description could be made less verbose and easier to understand if the authors used the commonly accepted terminology. For example, the wordy explanation 7.2.2 could be

summarized by that the intensity trajectories were extracted through aperture photometry with square ROI size = 3.

While that terminology may be familiar to the reviewer, we prefer to leave our descriptions as they are. We feel that this work will be of interest to researchers from a broad range of fields, and we do not want to incorporate terminology that might not be familiar to anyone with general scientific training.

Some procedures in the protocol seem not to have been carefully tried and thought through.

The assembly of the microfluidic device is based on a protocol developed over a decade ago. This device has been heavily used and presented in the literature since its development, including multiple examples in JoVE. We have included citations to those video protocols.

For example, in 4., what is the purpose of using an expensive and hard to drill quartz slide if the flow chamber is not going to be used in the prism-type TIRF setup? Instead an ordinary glass slide or even a commercial multichannel flow cell top could be used.

A glass slide is much more difficult to drill than a quartz slide, and the expense for the quartz slide is not prohibitive, considering that the prepared slides can be reused many times. We have edited the protocol to emphasize that the quartz slides are meant to be reused.

We are not aware of a commercially available flow cell top that has the dimensions we are using. The smallest dimension for the channel in this device is approximately 100 um, which, at the flow rates used, results in laminar flow.

The authors could also emphasize which parameters in the protocol are essential. For example, is the long and repetitive sonication in 3 really required? Or would a single and shorter step be sufficient thus economizing on the operators resources?

We consider all of the cleaning steps to be essential. Insufficient cleaning will result in a poor quality of surface functionalization. In general, we have tried to present this protocol in a way that is most likely to result in a good outcome. Omission of steps we have described is not recommended. We have added some clarification to the text to reflect this.

Specific remarks

Lines 182-183, 2.1.7 - the purpose of 2.1 was to reduce the oxidized thiols, so how could the purpose of freezing be to prevent the reduction?

Thank you for alerting us to this misstatement. We have revised the manuscript to correct it.

Line 284-285, 4.1 - drilling glass is not a very common operation in the lab: specify the method and parts used to do that.

Thank you for alerting us to this oversight. We have added more information to the protocol about the glass drilling.

Line 350, 6.3 - experimental buffer is with magnesium: must be an error since this is supposed to be the RE binding step, whereas 6.4 is where the digestion takes place.

Thank you for alerting us to this typographical error. It has been corrected.

Line 368, 7.1.2 - how is this subtracted background extracted?: the imtophat Matlab function returns the filtered image and not the background.

Thank you for alerting us to this lack of detail. We have added a sentence to clarify this.

A long (a few 10s of seconds) rise of the curve in Figure 2A indicates slow mixing of the buffer in the channel and the buffer flown in. Doesn't that introduce a significant uncertainty in the starting point in the restriction reaction?

The starting point is best determined by identifying the first point at which the rate of increase of the background fluorescence exceeds three times the standard deviation of the background rate of change. There is very little uncertainty associated with this approach. We have clarified the procedure for determining the zero time-point and included discussion of this issue.

Lines 384-390, 7.2.3 - "appropriate fraction", "depending on the noise observed in the trajectories": the manner of the dependence of the fraction on noise level should be specified quantitatively.

Additional clarification has been added to this section.

Lines 399-419 - the presentation of the results is qualitative, based on the visual impressions of the observer: line 407 - "many", line 409 - "the majority", line 416 - "a large number". Instead, quantitative analysis should be presented: what is the percentage of the quantum dots that remain after the different controls and the actual experiment?

Additional quantitative information has been added to the manuscript.

The Materials table is incomplete: catalogue numbers and company names of chemicals and equipment are missing.

We have endeavored to fill in any missing information.

Editorial Comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have carefully proofread the manuscript and made corrections, including ensuring that all abbreviations are defined at first use.

2. Please provide an email address for each author.

All authors email addresses have been provided.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have revised the manuscript so that it has the requested style, and we have added safety procedures where needed.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the manuscript so that there is no longer any use of personal pronouns.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added additional detail throughout the protocol.

6. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

We have selected the parts of the protocol that we feel should be included in the protocol video. We have also directed the reader to other JoVE video protocols where that might be helpful.

7. As we are a methods journal, please revise the Discussion to add the following points with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods

We have attempted to address these points in the Discussion. We did this in part by moving some text from other sections, and we also added more text describing modifications and troubleshooting approaches.

8. Please sort the Materials Table alphabetically by the name of the material.

We revised the Materials Table as requested.