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Chapter 12

Single Molecule FRET Analysis of DNA Binding Proteins

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Abstract

The complex binding dynamics between DNA and proteins are often obscured by ensemble averaging effects in conventional biochemical experiments. Single-molecule fluorescence methods are powerful tools to investigate DNA–protein interaction dynamics in real time. In this chapter, we focus on using single-molecule Förster Resonance Energy Transfer (smFRET) to probe the binding dynamics of individual proteins on single DNA molecules. We provide a detailed discussion of total internal reflection fluorescence (TIRF) instrument design, nucleic acid labeling with fluorophores, flow cell surface passivation, and data analysis methods.

Key words Single molecule, FRET, TIRF, Nucleic acid–protein interaction, DNA binding protein, PEG surface passivation

1 Introduction

Single-molecule spectroscopy has contributed significant insights into the complex dynamics of a wide range of biological systems, including DNA [1–3] and RNA polymerases [4–7], helicases [8–11], DNA binding proteins [12–16], and molecular motors [17–19]. These methods readily allow quantitative characterization of kinetic intermediates and heterogeneous populations that may be obscured by ensemble averaging effects in conventional biochemical experiments.

Single-molecule fluorescence imaging is a particularly useful tool to observe important biological processes in real time. Common fluorescence illumination methods include confocal scanning, epifluorescence, and total internal reflection (TIR). In TIR microscopy, the laser beam penetrates a surface–solution interface at a greater angle of incidence than the critical angle θ_c:

$$\theta_c = \sin^{-1}\left(\frac{n_1}{n_2}\right)$$

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where n_1 and n_2 are the refractive index of the solution and surface, respectively [20]. This generates an evanescent wave with intensity I that penetrates the solution as a function of distance z:

$$I(z) = I_0 e^{\frac{-z}{d}}$$

where I_0 is initial intensity and d is the decay constant [21]. The penetration depth is approximately 100 nm from the surface. TIRF is therefore used to excite fluorophores near the surface–solution interface, which is an illumination method well-suited for single-molecule Förster Resonance Energy Transfer (smFRET).

FRET occurs when energy is transferred from a donor fluorophore to an acceptor fluorophore through dipole–dipole interactions [22]. The FRET pair shares spectral overlap such that donor emission overlaps with acceptor excitation. The energy transfer efficiency E is:

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

where R is the distance between the two fluorophores and R_0 is the characteristic distance at which half of the energy is transferred [23]. This strong distance dependence allows sensitive measurements on the molecular scale, in the range of 2–10 nm [24], which makes FRET a powerful tool for characterizing the structure and dynamics of individual molecules in solution.

In smFRET experiments involving nucleic acids and nucleic-acid-binding proteins, fluorophore-labeled nucleic acids and proteins can be immobilized on a surface, extending imaging lifetimes. The fluorophores are excited by an evanescent wave generated by TIRF at the surface–sample interface. This combined approach has the key advantage of constraining fluorescence excitation to molecules within 100 nm of the surface, sharply reducing background from untethered molecules further in solution. Here, we discuss smFRET with TIRF excitation as a method to study DNA–protein interactions in real time. We present protocols for TIRF instrument design, nucleic acid labeling, surface passivation, sample immobilization, data acquisition, and data analysis.

2 Materials

2.1 TIRF Instrument

TIRF instruments are commonly designed using either a prism or a microscope objective, and each method has its advantages (*see* **Note 1**). Here, we describe an objective-based TIRF instrument with fiber-coupled lasers.

1. Green laser (532 nm, diode-pumped solid state, 80 mW, fiber-coupled, Coherent; *see* **Note 2**).
2. Red laser (640 nm, diode, 75 mW, fiber-coupled, Coherent; *see* **Note 3**).
3. Beam combiner (single mode fiber output, Coherent; *see* **Note 4**).
4. Two lenses for beam expansion (plano-convex, Thorlabs; *see* Subheading 3.1, **step 2**).
5. Lens to converge beam (plano-convex, 5 cm diameter, 350–400 mm focal length, Thorlabs).
6. Mirror (broadband dielectric, 5 cm diameter, Thorlabs).
7. Translation stage (4 cm range, Thorlabs).
8. Microscope frame (inverted, Nikon).
9. Dichroic mirror (longpass, 550 nm, Chroma).
10. Dichroic mirror (longpass, 655 nm, Chroma).
11. Objective lens (air, 40×, Nikon; optional, *see* Subheading 3.1, **step 7**).
12. Objective lens (for TIRF, 1.4 NA, oil immersion, 100×, Nikon).
13. Immersion oil (refractive index 1.5).
14. Dichroic mirror (longpass, 610 nm, Chroma).
15. Emission image splitter (Optosplit II, Cairn; *see* **Note 5**).
16. Electron-multiplying charge-coupled device (EMCCD) camera (iXon Ultra, Andor; *see* **Note 6**).
17. Bandpass filter (680/20 nm, Semrock).

2.2 DNA Construct Labeling

Nucleic acid oligonucleotides are biotin-modified at either terminus for surface immobilization, with amino-modified C6-dT nucleotides incorporated for fluorophore labeling. Here, we describe labeling and purification methods for synthetic, commercially available nucleic acid oligonucleotides.

2.2.1 Reverse-Phase High-Performance Liquid Chromatography (HPLC)

1. Nucleic acid oligonucleotides (biotin-modified, with amino-modified C6-dT at desired label position, desalted, 1 nmol, dry, Invitrogen).
2. Triethylammonium acetate (TEAA) buffer: 15 mM trimethylamine, 5% acetonitrile, pH 7.0. Adjust pH with glacial acetic acid (HPLC grade).
3. Acetonitrile (HPLC grade).
4. DNA storage buffer: 10 mM Tris–HCl, pH 8.0.
5. Vacuum concentrator (DNA SpeedVac, Thermo Scientific).

2.2.2 Fluorophore Labeling

1. Cy3, Cy5 (*N*-hydroxysuccinimide (NHS) ester form, 20 nmol, Amersham CyDye Mono-Reactive NHS Ester, GE Healthcare Life Sciences).
2. Dimethyl sulfoxide (DMSO).
3. Labeling buffer: 0.1 M sodium tetraborate, pH 8.5, −20 °C (*see* **Note 8**).
4. Thermomixer (Thermomixer Comfort, Eppendorf).

2.2.3 Ethanol Precipitation

1. 3 M sodium acetate.
2. 100 mM dNTPs.
3. Ethanol.

2.3 Sample Chamber Preparation

There are a number of slide cleaning and surface modification methods to minimize background fluorescence [25–27]. Here, we describe surface passivation with polyethylene glycol (PEG; *see* **Note 7**).

2.3.1 Slide Assembly

1. Drill with diamond bit (Dremel 3000, Dremel).
2. Glass microscope slides (76 × 26 mm, Thermo Scientific).
3. Glass coverslips (24 × 24 mm, Thermo Scientific).
4. Double-sided tape (Scotch).
5. Epoxy (Devcon).

2.3.2 Slide Cleaning

1. Double distilled water (ddH_2O).
2. Razor blades.
3. Powder detergent (Alconox, VWR).
4. Ethanol (200 proof).
5. Basic piranha solution: 150 mL ddH_2O, 30 mL 30% H_2O_2, 30 mL NH_4OH (ACS grade).
6. Beaker (250 mL).
7. Magnetic stir plate and stir bar (1.6 cm; *see* **Note 8**).
8. Tweezers (*see* **Note 9**).
9. Bunsen burner.
10. Slide rack (Micro Slide Staining Rack, VWR).
11. Coplin staining jars (Wheaton Scientific).
12. Sonicator.
13. 1 M KOH.
14. Methanol (ACS grade).

2.3.3 Aminosilanization

1. Aminosilanization solution: 100 mL methanol, 5 mL glacial acetic acid (ACS grade), 1 mL 3-aminopropyltriethoxysilane (Vectabond, Vector Laboratories; *see* **Note 10**).
2. Nitrogen gas.
3. PEGylation chambers (*see* **Note 11**).

2.3.4 PEGylation

1. PEGylation buffer: 84 mg $NaHCO_3$ dissolved in 10 mL ddH_2O, syringe-filtered (0.2 μm membrane; *see* **Note 12**).
2. PEGylation reaction solution: 7 mg biotinylated PEG succinimidyl carboxymethyl (Bio-PEG-SCM, MW 3400, Laysan Bio), 80 mg methoxy PEG SCM (mPEG-SCM, MW 5000, Laysan Bio), 380 μL bicarbonate PEGylation buffer (*see* **Notes 13** and **14**).

2.4 Sample Immobilization

Nucleic acids and proteins can be tethered to the surface in a variety of ways, which lends flexibility to the design of in vitro FRET experiments. Here, we describe DNA construct immobilization through a biotin–streptavidin linkage (*see* **Note 15**).

1. DNA (biotin- and fluorophore-labeled, *see* Subheadings 2.2 and 3.2).
2. Annealing buffer: 50 mM MOPS, pH 7.4, 5 mM $MgCl_2$, 60 mM NaCl, 2 mM Trolox. (This buffer is for dsDNA constructs; *see* **Note 16**.)
3. Dry block heater (Grant Instruments).
4. T50 buffer: 50 mM Tris–HCl, pH 7.0, 50 mM NaCl.
5. Bovine serum albumin (BSA) solution: 0.2 mg/mL BSA in T50 buffer. (To use BSA for surface passivation in experiments without PEG, *see* **Note 17**).
6. Streptavidin solution: 1 mg/mL streptavidin in T50 buffer.
7. Experimental buffer: 40 mM Tris–HCl, pH 7.9, 5 mM $MgCl_2$, 60 mM NaCl, 0.2 mg/mL BSA, 2 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; *see* **Note 18**), 2.5 mM 3,4-dihydroxybenzoic acid (PCA), 250 nM protocatechuate dioxygenase (PCD; *see* **Note 19**), up to 20 nM protein (*see* **Note 20**).

2.5 Data Acquisition and Analysis

Data is acquired with LabView, using a custom data acquisition (DAQ) card for the EMCCD. Fluorescent beads are used to match donor and acceptor signals using IDL, and single-molecule traces are analyzed using Matlab.

1. Fluorescent beads (FluoSpheres Carboxylate-Modified Microspheres, 0.2 μm, Red (580/605)).
2. 1 M $MgCl_2$.

3. LabView (National Instruments).
4. IDL (Harris).
5. Matlab (MathWorks).

3 Methods

3.1 TIRF Instrument

1. Mount the fiber-coupled lasers (*see* **Note 21**) and plug them into the beam combiner (Fig. 1).
2. Expand the beam output from the single mode fiber so that it is approximately 20 mm in diameter (*see* **Note 22**). Ensure that the beam is well-collimated.
3. Mount the mirror on a translation stage, and place it behind the microscope frame so that beam path enters the back port.
4. Insert the 550 nm and 655 nm longpass dichroic mirrors (for the green and red lasers, respectively) in separate, labeled cubes inside the filter turret. Begin with the 550 nm dichroic mirror first, so that the green laser beam enters perpendicular to the plane of the objective.
5. Converge the beam by placing the lens one focal length (350–400 mm) away from the objective.
6. Insert the beam target in one of the empty spaces in the objective turret. The beam should be clearly visible on the ceiling. If the beam is clipped, correct the beam path with the mirror on the translation stage (*see* **Note 23**).
7. Establish epifluorescence with the 40× objective to check the beam's alignment (optional, *see* **Note 24**). The beam will form a concentric ring pattern on the ceiling. If this pattern is angled or elongated, use the mirror to adjust the beam position laterally and horizontally so that the beam enters the objective at the correct angle of incidence.
8. Move the lens longitudinally along the beam path, minimizing the diameter of this concentric pattern. This precisely focuses the beam on the back focal plane of the objective so that the beam emerges collimated.
9. Insert the 100× TIRF objective and re-establish epifluorescence. This involves a small adjustment of the lens in order to focus the beam on the back focal plane of this particular objective (*see* **step 8**).
10. Establish TIRF by adjusting the lateral position of the beam with the mirror. As the beam moves towards the edge of entrance pupil of the objective, the illuminated pattern on the ceiling slides down the wall and disappears. Adjust the beam to

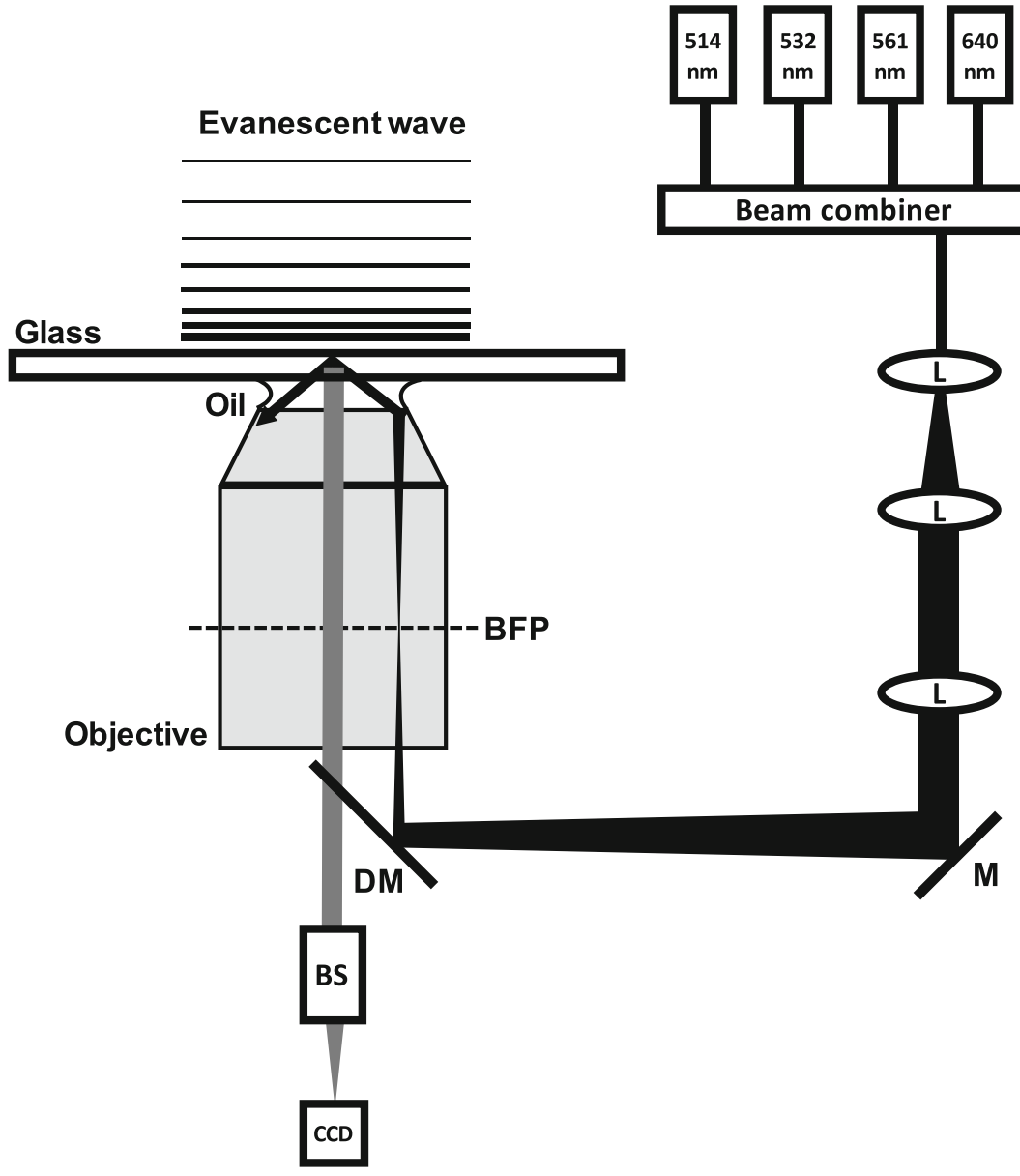

Fig. 1 Schematic representation of an objective-based TIRF instrument. Individual fiber-coupled lasers are combined and coupled into a single excitation beam, which is expanded with a set of lenses (L). The collimated beam is focused on the back focal plane of the objective with a lens, and steered with a mirror (M). A dichroic mirror (DM) directs the excitation beam into the objective. The beam enters at the edge of the objective, and TIR occurs at the glass–water interface, generating an evanescent wave to excite the molecules immobilized on the surface. The emission beam travels through objective, and the dichroic mirror (DM) directs it into the beam splitter (BS), where the donor and acceptor signals are separated and detected by a back-illuminated EMCCD camera

a position just before the vanishing point, so that it propagates along the outer edge of the objective.

11. Mount a fluorescent bead slide (*see* Subheading 3.5, **step 1**) above the objective, careful to ensure that the coverslip surface is in contact with the immersion oil. The beam will no longer be visible on the wall, due to total internal reflection at the interface between the coverslip and the sample chamber.
12. Direct the fluorescence signal through the eyepiece, adjusting the objective height to bring the beads into focus.
13. Insert the 610 nm longpass dichroic mirror in the image splitter. Install the image splitter on a side port of the microscope frame.
14. Install the EMCCD on the image splitter, careful to ensure that the unit is at the same height as the side port in order to avoid geometric aberration.
15. Direct the fluorescence signal through a side port. Adjust the mirror to achieve a uniform field of illumination. Align the short (donor) and long (acceptor) wavelength images on the left and right, respectively.
16. Adjust the lateral position of the beam with the mirror to visually optimize the signal-to-background ratio of the beads using the image on the EMCCD.
17. Remove the beads slide and mount a slide with Cy3-labeled DNA molecules (*see* Subheadings 3.3 and 3.4). Adjust the lateral position of the beam until Cy3 molecules suddenly become brighter as the background simultaneously sharply decreases (*see* **Note 25**).
18. Remove the Cy3-only slide and mount a slide with both Cy3- and Cy5-labeled DNA molecules. The donor and acceptor signals should be well-separated, with minimal cross-talk (*see* **Note 26**). If significant leakage of the donor signal into the acceptor channel is observed, insert the 680/20 nm bandpass filter into the long wavelength slot of the image splitter.
19. To confirm the presence of the acceptor, switch to the 655 nm dichroic mirror in the filter turret and use the red laser to excite Cy5 directly.

3.2 DNA Construct Labeling

The distance sensitivity of FRET requires relatively short nucleic acid oligonucleotides, typically up to 100 bp in length. Such short constructs are commercially available with a number of convenient modifications, such as biotinylation at the terminus for surface immobilization and amino-modification of C6-dT nucleotides incorporated for fluorophore labeling. Here, we describe HPLC analysis to check the purity of synthetic DNA (*see* **Note 27**), followed by a detailed discussion of DNA labeling and purification.

3.2.1 HPLC Analysis

1. Dissolve DNA pellet in 50–100 µL DNA ddH_2O, varying the volume depending upon reported concentration of the synthetically prepared DNA.
2. Add to an Eppendorf tube, for 100 µL total: (a) 2 µL DNA oligonucleotides, and (b) 98 µL TEAA HPLC buffer.
3. Confirm DNA purity with reverse-phase HPLC on an analytical C18 column. If synthesis products are detected, purify the whole DNA sample before proceeding with the labeling reaction (*see* **Note 28**).

3.2.2 Fluorophore Labeling

1. Add 14 µL DMSO directly to Cy5 tube. Vortex and centrifuge to mix the solution well. Use 7 µL for each labeling reaction (*see* **Note 29**).
2. Add to a 1.5 mL Eppendorf tube, for a total of 100 µL: (a) 25 µL purified DNA, (b) 10 µL 0.1 M Na_2CO_3, pH 8.5, −20 °C, (c) 7 µL Cy5 with DMSO, and (d) 58 µL ddH_2O.
3. Vortex, and then centrifuge quickly to collect any droplets into the solution. Cover with aluminum foil to protect the dye from photobleaching. Shake gently overnight on the Thermomixer Comfort at 27 °C, 500 rpm.

3.2.3 Ethanol Precipitation

1. Add to each Eppendorf tube, for a total of 360 µL: (a) 10 µL (1/10th volume) 3 M NaOAc, (b) 0.5 µL 100 mM dNTPs (for a final concentration of 140 µM, *see* **Note 30**), and (c) 250 µL (2–2.5× volume) 100% ethanol.
2. Shake well and store at −20 °C overnight or −80 °C for 4 h.
3. Centrifuge the sample for 30 min at 4 °C, 19,000 × *g*.
4. Pour off the brightly colored supernatant. This is excess dye, which does not precipitate with the DNA.
5. Add 500 µL 70% ethanol and centrifuge for 15 min at 4 °C, 19,000 × *g*.
6. Repeat **steps 4** and **5** (*see* **Note 31**).
7. Pour off supernatant and vacuum-dry the DNA. A colored pellet will be clearly visible at the bottom of the tube.
8. Dissolve the DNA in 100 µL TEAA HPLC buffer for separation of labeled and unlabeled fractions (*see* **Note 32**).

3.2.4 HPLC Purification

1. Add to an Eppendorf tube, for 100 µL total: 5 µL DNA oligonucleotides (*see* **Note 33**) and 98 µL TEAA HPLC buffer.
2. Run analytical HPLC to determine approximate positions of unlabeled and labeled DNA peaks.
3. Modify the preparation method as necessary to separate peaks and collect fractions.
4. Vacuum-dry the appropriate fractions (*see* **Note 34**).
5. Resuspend labeled and unlabeled DNA in 25 µL DNA storage buffer. Store at 4 °C.

3.3 Sample Chamber Preparation

Sample chambers are prepared using microscope slides with predrilled holes for the purpose of creating a flow channel. Here, we describe how to clean the slides and coverslips in order to remove impurities that contribute to background fluorescence. Then, we describe amino functionalization of the slide surface in order to coat it with NHS ester-labeled linear PEG, which minimizes nonspecific protein interactions (Fig. 2) [25]. Finally, we provide a detailed protocol for sample chamber assembly and storage.

3.3.1 Slide Cleaning

1. Prism-based TIRF methods require the use of expensive quartz slides that can be reused. In this case, boil previously used slides in ddH_2O for 30–40 min. Remove the coverslip and any debris from previous applications using a razor blade. In the case of new slides, omit this step.
2. For new slides, drill holes with a diamond drill bit while the microscope slides are submerged in water.
3. Clean slides using a 1:1 mixture of Alconox and ddH_2O. The slide surface should be scrubbed vigorously with this paste (*see* **Note 35**). Rinse slides thoroughly with distilled water to remove any remaining soap (*see* **Note 36**).

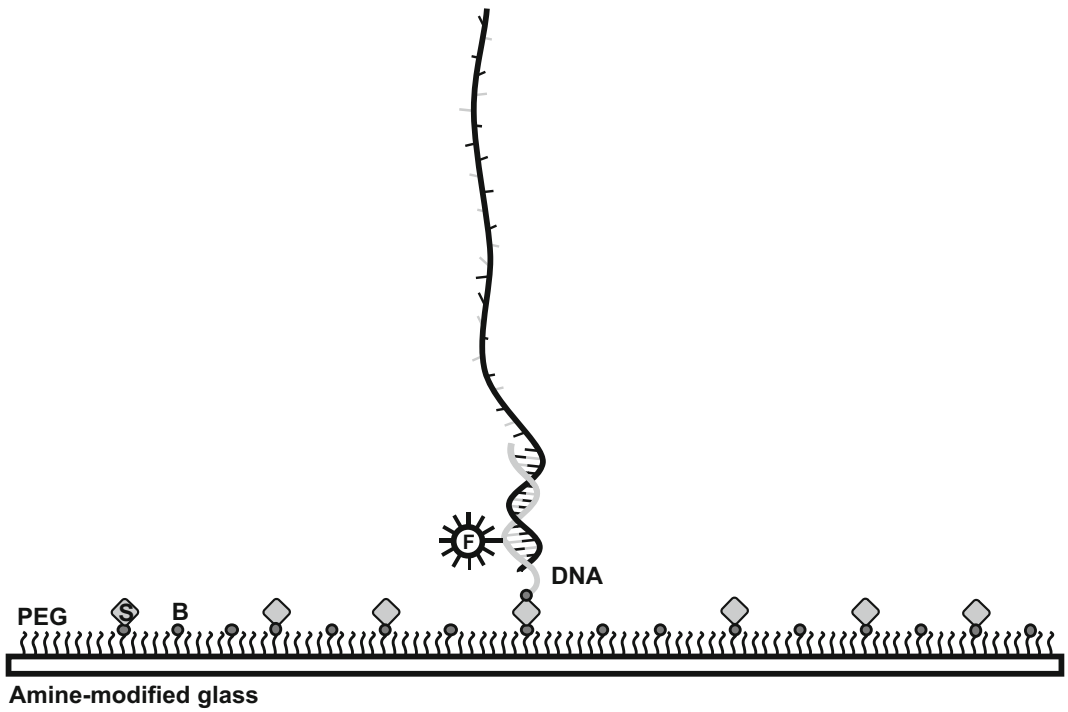

Fig. 2 Schematic representation of sample immobilization. The glass coverslip is amine-modified, and the NHS-ester form of mPEG and biotin-PEG are covalently conjugated to the surface. Streptavidin (S, *squares*) specifically binds to a fraction of the biotin (B, *circles*). DNA molecules labeled with a fluorophore (F) are then tethered to the surface via a biotin–streptavidin linkage

4. Scrub and rinse slides twice with ethanol and ddH_2O on both sides for 1–2 min each.
5. Boil slides for 40 min in freshly prepared basic piranha solution in order to regenerate the slide surface. Use a 250 mL beaker for five slides, which allows sufficient room so that the slides do not touch each other (*see* **Note 37**). Ensure the PEGylation surface faces the solution and both drilled holes are submerged.
6. Carefully remove the slide with tweezers, and rinse thoroughly with ddH_2O. Flame slide on both sides with a Bunsen burner to pyrolyse organic residues on the surface. Ensure that the PEGylation surface is well-sterilized. Place hot slide on the slide rack, with the PEGylation surface facing up. Repeat for remaining slides.
7. Place five slides and five coverslips into two clean, dry Coplin jars, ensuring that the PEGylation surface faces towards the solution at both ends.
8. Fill the jars with 1 M KOH and sonicate for 30 min to remove any remaining debris.
9. Rinse slides with ddH_2O (*see* **Note 38**).
10. Rinse with methanol.
11. Fill the jars with methanol and sonicate for 30 min.

3.3.2 Aminosilanization

1. Rinse a clean 200 mL beaker with methanol. Prepare the aminosilanization solution with Vectabond equilibrated to room temperature (*see* **Note 10**).
2. Mix the solution with the pipette tip, and fill the Coplin jars with it, ensuring that all slide surfaces are submerged. (a) Incubate for 10 min, (b) sonicate for 2 min, and (c) incubate for 10 min.
3. Decant aminopropylsilane solution into the waste container, then rinse the slides with (a) methanol, (b) ddH_2O, and (c) methanol.
4. Dry slides and coverslips with nitrogen (*see* **Note 39**). Place dried slides in PEGylation chambers, prepared with a 1 cm layer of distilled water in the bottom to create a humid environment. (This prevents the coverslips from sticking to the slides as they incubate overnight, as described in **step 5** of Subheading 3.3.3). Place coverslips in a box lined with a clean paper towel. Ensure that the PEGylation surface is always facing upwards.

3.3.3 PEGylation

1. Prepare the PEGylation buffer and use it to prepare the PEGylation reaction solution. Vortex the solution to dissolve the PEG, then centrifuge briefly to remove any bubbles.

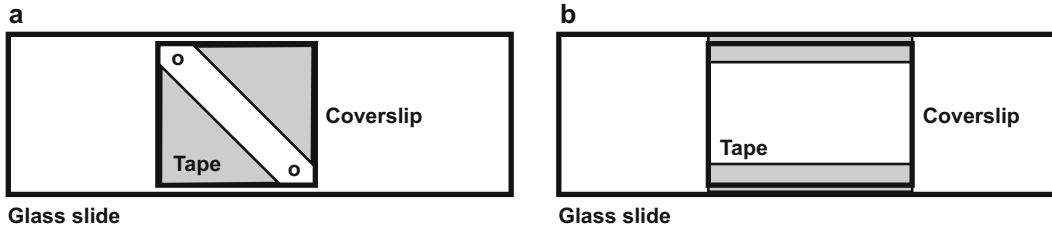

Fig. 3 Schematic diagram of sample chambers for (**a**) single-molecule experiments and (**b**) fluorescent bead calibration. Flow channels are formed with two layers of double-sided tape (*gray*), and the chamber is sealed with epoxy applied between the slide and coverslip

2. Place 80 μL of the PEGylation reaction solution onto the surface of each slide (*see* **Note 40**).
3. Gently place coverslips onto the slides with tweezers, ensuring there are no bubbles trapped underneath coverslip (*see* **Note 41**).
4. Use a marker to mark the PEGylated surface of the slide and the non-PEGylated surface of the coverslip in the lower right corner.
5. Close the PEGylation chambers and incubate them overnight at room temperature in the dark.

3.3.4 Slide Assembly

1. Rinse slides and coverslips gently with ddH_2O and dry with nitrogen (*see* **Note 42**). Place slides on the slide tray and coverslips in a box lined with a clean paper towel.
2. Apply double-sided sticky tape to create a straight, 4 mm wide channel on either side of the holes. Apply a second layer of tape on top of the first one to make the channel depth approximately 100 μm (Fig. 3a).
3. Deposit a coverslip on the top of the sticky layer with caution so that both holes are covered on either side. Gently press the coverslip on the sticky tape to ensure proper adhesion.
4. Remove any excess tape around the coverslip using a razor blade (*see* Note about technique).
5. Dispense epoxy into a small weigh boat, mix with a 200 μL pipette tip and wait 1–2 min for glue to thicken. Use the pipette tip to apply the epoxy, sealing the gap between the coverslip and the tape. The glue will spread upon application, so be careful not to use so much glue that it seals the holes.
6. Cover and allow glue to dry for 10 min.
7. Place sample chambers in 50 mL tubes filled with nitrogen gas. Store in the dark for 1–2 weeks.

3.4 Sample Immobilization

Approximately 8% of PEG molecules used for surface passivation are labeled with biotin in order to enable immobilization of DNA

molecules through a biotin–streptavidin linkage. Here, we describe sample preparation procedures for imaging single DNA molecules on the coverslip surface.

1. Add to an Eppendorf tube, for 10 μL total: (a) 1 μL of 10 μM biotinylated DNA, (b) 2 μL of 10 μM non-biotinylated DNA, and (c) 7 μL annealing buffer (*see* **Note 43**).

   Gently flick to mix, and briefly centrifuge to collect the liquid. Wrap the tube in aluminum foil to protect the fluorophores and heat on a dry block heater at 90 °C for 45 s. Remove the block and allow it to slowly cool the sample to room temperature.
2. In two serial dilutions, prepare a 200 μL solution of the DNA in annealing buffer, for a final concentration of 25–50 pM. Store both solutions on ice and protect them from ambient light to minimize photobleaching.
3. Cut a 200 μL pipette tip to fit exactly inside one of the holes that form the channel in the slide. Inject 85 μL BSA solution (*see* **Note 44**) and incubate for 10 min at room temperature (*see* **Note 45**).
4. Inject 200 μL T50 buffer to wash out excess BSA.
5. Inject 200 μL streptavidin solution and incubate for 10 min (*see* **Note 46**).
6. Inject 200 μL annealing buffer to wash out excess streptavidin.
7. Inject 200 μL of the final DNA dilution (25–50 pM DNA, *see* **step 2**) and incubate for 10 min (*see* **Note 47**).
8. Inject 200 μL experimental buffer to wash out excess DNA.
9. Inject 200 μL of the final imaging solution in experimental buffer (*see* **Note 48**).

3.5 Data Acquisition and Analysis

To accommodate minor optical alignment issues, the position of the donor and acceptor signal on the image splitter is matched using a fluorescent bead reference. Here, we describe this calibration procedure, and briefly discuss data analysis methods.

1. Prepare a calibration sample chamber using a clean glass slide, without pre-drilled holes, by applying double-sided sticky tape across the top and bottom of the slide. Apply a second layer of tape on top of the first one to make the channel depth approximately 100 μm (Fig. 3b).
2. Deposit a coverslip on the top of the sticky layer. Gently press the coverslip on the sticky tape to ensure proper adhesion.
3. Remove any excess tape around the coverslip using a razor blade.
4. Make a 500-fold dilution of the fluorescent beads in ddH_2O. Use a 200 μL pipette tip to inject the bead solution into the square sample chamber. Incubate for 10 min.

5. Wash out excess beads with 1 M $MgCl_2$. Incubate for 10 min.
6. Dispense epoxy into a small weigh boat, mix with a 200 μL pipette tip and wait 1–2 min for glue to thicken. Use the pipette tip to apply the epoxy around the coverslip, sealing the sample chamber closed.
7. Cover and allow glue to dry for 10 min. The sealed slide will last 1–2 months.
8. Mount the calibration slide on the microscope. The fluorescent beads will be clearly visible through the eyepiece. Adjust the objective position to bring them into sharp focus (*see* **Note 49**).
9. Direct the beam through the side port. Two mirror images will be visible on the camera. Adjust the mirror to achieve roughly uniform illumination across the field of view. Record a short calibration movie (approximately 1000 frames is sufficient).
10. Remove the beads slide and mount the DNA sample on the microscope, careful to ensure that the coverslip faces the immersion oil. Single molecules will be visible on the camera. Bring them into focus with minor adjustments to the objective position.
11. Movies are typically recorded from different areas of the sample chamber on the scale of minutes to hours, depending upon experimental design (*see* **Note 50**). Record the laser power after each movie (*see* **Note 51**).
12. Glass slides may be discarded after use. Expensive quartz slides required for prism-based TIRF may be cleaned and reused (*see* Subheading 3.3.1, **step 1** and **Note 52**).
13. Using a custom script written in IDL (available upon request), select three beads from the calibration movie to obtain a polynomial map of the overlay between donor and acceptor image (*see* **Note 53**).
14. For each single-molecule movie, use the IDL script to extract the intensity over time for each spot. The IDL script uses an optimized threshold to identify molecules, which are individually confirmed with manual analysis.
15. Analyze individual traces using a script written in MatLab (available upon request). A single molecule is identified by single-step photobleaching of the dye. The script calculates the apparent FRET efficiency:

$$\text{FRET} = \frac{I_A}{(I_D + I_A)}$$

where and I_D is the donor intensity and I_A is the cross-talk corrected acceptor intensity (*see* **Notes 54–56**).

16. FRET traces can be used to build FRET histograms for a range of solution conditions (*see* **Note 57**). The kinetics of a two-state system can be extracted with dwell-time analysis (*see* **Note 58**). It is often useful to synchronize molecules to an initial binding event to quantify the distribution of FRET states over time (*see* **Note 59**). In the case of a multiple-state system, more advanced analysis tools, such as hidden Markov modelling (HaMMy) may be helpful (*see* **Note 60**).

4 Notes

1. Although we describe an objective-based TIRF instrument, the methods discussed here are also applicable for prism-based TIRF. A prism-based instrument is simpler to build, align, and maintain, and it easily accommodates flow-based experiments. However, matching the index of refraction of the prism to the imaging surface requires expensive quartz slides that need to be thoroughly cleaned before reuse. Furthermore, the sample chamber surface is covered with a prism in this instrument design, making it difficult to accommodate sample chambers commonly used for live cell imaging.
2. A number of lasers are typically used to achieve TIR, including Nd:YAG solid state lasers (Coherent, Newport) or diode lasers (Vortran).
3. A red laser is useful for direct excitation of the acceptor dye. Diode lasers (Coherent, Vortran), diode-pumped solid state lasers (Cobolt), and HeNe lasers (Melles Griot) are commonly used for this purpose.
4. A beam combiner provides alignment stability, which is often a priority in biological laboratories. We couple multiple lasers into our single mode fiber, and recommend a long (2 m), reinforced steel fiber for this purpose (Coherent, Cobolt). However, it is common, and relatively straightforward, to use dichroic mirrors to combine the excitation beams.
5. We use an emission image splitter (Cairn, Hamamatsu, Photometrics) for alignment stability, since the dichroic mirror may simply be exchanged between experiments with different dyes. However, it is relatively straightforward to use dichroic mirrors to split the emission beam.
6. Single-molecule experiments commonly use EMCCD cameras for detection (Andor, Hamamatsu, Photometrics). Their advantages include uniform amplification, low noise, and high quantum yields achieved by back-thinning. Thermal noise is reduced by cooling the chip, typically to −80 °C.

7. Surface passivation is commonly performed with PEG, particularly for experiments involving fluorophore-labeled proteins that contribute to high background signal when they collapse on the imaging surface. However, it may be sufficient to use BSA to passivate the surface for experiments without such stringent requirements.
8. Magnetic stir bars should be sufficiently large for stability while spinning in a 250 mL beaker, but sufficiently small not to disturb glass slides standing along the edges.
9. Tweezers with wide, flat ends to grip the slide by one end are preferred.
10. Store Vectabond sealed with nitrogen gas at 4 °C to avoid oxidation. (It should be a colorless solution, and signs of yellowing indicate oxidation.) Allow Vectabond to equilibrate to room temperature for 30 min before use. Make aminosilanization solution immediately prior to use. Exchange air in the Vectabond bottle with nitrogen gas, seal with parafilm, and store at 4 °C.
11. PEGylation chambers can be made from old pipette tip boxes by machining out a rectangular section in the plastic inserts to hold five vertically arranged slides. Clean boxes thoroughly with ddH_2O before each use.
12. Make PEGylation buffer immediately prior to use.
13. Aliquot biotin-PEG and mPEG in 1.5 mL Eppendorf tubes, and store at −20 °C. Allow aliquots to equilibrate to room temperature for 30 min prior to use. Store unopened PEG bottles wrapped in foil at −20 °C. For partially used bottles, exchange air with nitrogen gas and seal with parafilm prior to wrapping in foil and storing at −20 °C.
14. Make PEGylation reaction solution immediately prior to use.
15. Surface attachment may also be achieved using a biotin–neutravidin linkage, with little to no discernible differences in immobilization efficiency.
16. If using an ssDNA construct with no dsDNA region, omit annealing buffer and dilute DNA directly in experimental buffer.
17. Molecules can also be immobilized in the absence of PEG, using BSA for surface passivation (*see* **Note 7**) and molecule immobilization. In this case, use a biotinylated BSA solution: 0.2 mg/mL BSA in T50 buffer. Aliquot biotin-BSA in 1.5 mL Eppendorf tubes and store at 4 °C.
18. Trolox enhances fluorophore photostability, reducing blinking and photobleaching. Store Trolox at 4 °C. Instead of ddH_2O, a saturated solution of Trolox may be used to prepare reagents

for single-molecule experiments. To prepare the Trolox solution, dissolve 5 mg Trolox in 10 mL ddH_2O. Shake at room temperature for 30 min. Syringe filter the solution, and store at 4 °C for up to 2 weeks.

19. PCA/PCD is an oxygen scavenging system that decreases dissolved oxygen levels in the experimental buffer. This increases fluorophore photostability, reducing blinking and photobleaching. Aliquot PCA and PCD and store at −20 °C.
20. Single-molecule experiments are performed at low protein concentrations, particularly in the case of labeled proteins, in order to minimize background signal.
21. We use a power supply box (Coherent) to facilitate mounting multiple fiber-coupled lasers.
22. A 2 mm output from a single mode fiber can be expanded to a 20 mm beam using lenses of 10 and 100 mm focal length.
23. If the beam is not visible on the ceiling, ensure the correct dichroic mirror is in place in the filter turret. If so, reduce the laser power, ensure appropriate safety goggles are in place, and look directly down at the target to locate the beam. If a bright spot is not visible, or partially visible, use the mirror to steer the beam so that it enters the objective and a spot is clearly visible in the middle of the target.
24. It may be tricky to go directly to working with the 100× TIRF objective, where the concentric ring pattern is larger and more difficult to visualize on the ceiling when the beam is not focused on the back focal plane of the objective. We therefore recommend beginning with the 40× objective to adjust the lens position, but this step is optional.
25. Single molecules will be visible in epifluorescence mode, albeit with a high background signal. Upon visual estimation, the background may appear higher relative to the background in the prism-based TIRF method. However, quantitative signal-to-noise comparisons do not exhibit any appreciable differences.
26. Cross-talk decreases FRET resolution, as donor signal is observed in the acceptor channel and vice versa. The donor signal is more likely to leak into the acceptor channel due to the long wavelength tail of fluorescence emission spectra. Crosstalk is therefore usually measured using the donor-only sample to quantify the signal in the acceptor channel. This leakage will typically be 5–10%, and should be no more than 15%. Inserting bandpass emission filters reduces cross-talk, albeit at a small cost to the overall signal. Cross-talk will vary for different sets of dyes, and is therefore measured and corrected based on each

experimental design (*see* Subheading 3.5, **step 15** and **Note 55**).

27. Although we discuss DNA labeling, the methods described here are applicable for nucleic acids in general, and therefore include RNA.
28. To purify the commercially prepared DNA, vacuum-dry the sample to obtain a pellet again, and dissolve it in 100 μL TEAA HPLC buffer. Modify the HPLC collection method based on the peaks in the initial analysis spectrum, and isolate the peak representing the full synthesis product. Vacuum-dry the DNA and dissolve the pellet in ddH_2O.
29. Seal tube with remaining dye, cover with foil, and store at 4 °C.
30. The nucleotides increase the yield of labeled DNA in the ethanol precipitation, and provide a clear reference peak for HPLC analysis and purification.
31. Handle the tube carefully to avoid shaking the pellet loose. If the solution is accidentally mixed in the process, repeat the centrifugation step to reform the pellet.
32. For constructs with amino-modification of internal C6-dT nucleotides, it may be helpful to repeat the fluorophore labeling reaction in order to achieve at least 80% labeling efficiency. In this case, dissolve the DNA in 25 μL ddH_2O and repeat Subheadings 3.2.1–3.2.3.
33. The increase in DNA sample for HPLC analysis after labeling accommodates for DNA loss during the purification steps in ethanol precipitation.
34. Labeling efficiency is approximately 80%, which yields sufficient DNA for single-molecule experiments. However, store the purified, unlabeled fraction at −20 °C in case it becomes useful for a future labeling reaction.
35. Quartz slides used in prism-based TIRF are brittle, and will easily break with too much force. Clean them thoroughly, but maintain a gentle grip.
36. To save time, use distilled water directly from the tap instead of ddH_2O from a squeeze bottle, for all but the final rinse.
37. First set up the stir bar to mix the solution at a stable rate. Then add the slides one at a time, carefully leaning them against the sides of the beaker. Ensure that their bottom edge is not in the path of the stir bar, which can cause the slides to tip over into one another and crack (*see* **Note 8**).
38. Cover the Coplin jar and use the lid to carefully decant the solution into the waste beaker.
39. Affix a 1 mL pipette tip to the end of the nitrogen gas tube coming from the regulator valve. Hold the slide by the bottom

edge, angled towards you. Use your dominant hand to hold the pipette near the top edge of the slide and use the force of the gas to systematically slide the droplets towards the bottom edge.

40. Hold the pipette tip just above the middle of the slide, careful not to touch the surface. Dispense the solution onto the middle of the sample chamber, and watch it spread evenly over the surface, forming a pool covering both holes. If the solution begins to spread unevenly, due to small imperfections on the surface or the slide sitting unevenly in the PEGylation chamber, adjust your position over the slide to compensate. Pipette slowly and steadily to avoid the formation of bubbles.
41. Grip the coverslip by the bottom edge, and gently place the top edge onto the slide. Release the coverslip gradually in order to minimize bubble formation. Any small bubbles that do form will move towards the edge of the coverslip and escape. If large bubbles form and do not move on their own, gently move the coverslip around to encourage them towards the edges. An even, bubble-free layer is necessary to coat both surfaces. Wait 10 min to ensure that the coverslips do not move out of position. If they do, use the tweezers to gently guide them back into place.
42. Rinse slides and coverslips at the bench over a waste beaker. It typically takes 2 L of ddH_2O to rinse five slides and coverslips.
43. Use a twofold excess of the non-biotinylated strands to ensure complex formation with the labeled strand. This yields an annealed DNA complex concentration of approximately 1 μM in the 10 μL sample volume.
44. If using BSA for surface passivation and sample immobliliza-tion (*see* **Notes 7** and **17**), inject biotin-labeled BSA instead.
45. Ensure that the pipette tip fits tightly inside the chosen hole, or the solution will leak out instead of flowing through the sample chamber. During injection, hold the slide vertically, solely with the pipette tip, such that the injection hole is at the bottom of the slide, and the solution flows upwards into the chamber. Pipette slowly and carefully, so that injection takes at least 30 s. It is crucial to avoid introducing bubbles into the sample chamber.
46. It is important not to vortex the streptavidin to avoid damage to the protein.
47. These incubation times are a guideline, but may be further optimized for each construct. The surface density of the molecules should be sufficiently high to conduct an efficient experiment, but still allow image processing algorithms to establish a separate ROI around each molecule. This limit is

approximately 500 molecules per field of view, which is 512 × 512 pixels for the EMCCD chip discussed here (actual area in μm^2 depends upon magnification).

48. The buffer can be adjusted depending on the biological system under investigation. For example, dNTPs may be added for DNA polymerization experiments, or rNTPs added for transcription experiments.
49. If the fluorescent beads are not easily visible through the eyepiece by moving the vertical position of the 100× TIRF objective to bring them into focus, switch to the 40× objective to roughly identify the correct objective position. The beads will then be slightly out of focus with 100× objective, requiring only minor adjustments to the objective position.
50. At 1 byte (8 bits) per pixel on a 512 × 512 EMCCD chip, a 2 min movie at 17 ms/frame occupies 1.72 Gigabytes (GB). Longer integration times, such as those useful for observing slower biological processes, yield smaller file sizes in addition to decreasing the laser power required to achieve a good signal-to-noise ratio (S/N). Image processing of movies significantly reduces data volumes.
51. Use the lowest laser power necessary to achieve a good S/N in order to minimize dye photobleaching.
52. Quartz slides last for approximately 50 uses, or 6 months. After that, scratches, chips, or other surface imperfections appear. Keep a dated record of the slide used for each experiment, and discard slides that give a consistently high background.
53. Due to minor fluctuations in optical alignment, perform this calibration on the day of the experiment. Do not reuse polynomial maps generated on previous days, since even minor shifts in image overlap will skew intensity profiles.
54. The apparent FRET efficiency reports upon relative fluorescence intensities for each molecule, which mitigates the effects of non-uniform illumination across molecules. Molecules can therefore be compared, both within and across samples, despite minor variations in excitation intensity during data acquisition.
55. The measured intensities are generally corrected for cross-talk by a factor α, which is the percent of the donor signal detected in the acceptor channel. This is empirically measured from a donor-only sample by determining the value of α where the acceptor intensity is zero. The corrected acceptor intensity I_A is therefore:

$$I_A = I_{A0} - \alpha I_{D0}$$

where I_{A0} is the raw acceptor intensity and I_{D0} is the raw donor intensity. It is possible, if not particularly widely practiced, to correct the donor intensity I_D by adding the subtracted signal back:

$$I_D = I_{D0} + \alpha I_{D0}.$$

Additional correction factors may also apply, such as a correction for acceptor signal observed in donor channel, or corrections to compensate for small differences in the detection efficiencies and quantum yields of the two fluorophores. In the case of the Cy3-Cy5 FRET pair discussed here, these additional corrections are negligible, such that the apparent FRET efficiency is effectively the absolute FRET efficiency.

56. In principle, FRET values range between 0 and 1. However, when the signal intensities are very small or large (at zero and maximum FRET, respectively), the background noise becomes relatively high, leading to correction effects (*see* **Note 55**) that yield values slightly below 0 and slightly above 1.
57. A FRET histogram is a collection of the average FRET efficiency for $N > 100$ molecules. This analysis method can identify the FRET value of a single state with a statistical uncertainty of ± 0.1 FRET. To avoid sampling bias due to the length of FRET traces, use the first 100 frames for each molecule.
58. A dwell time histogram is a collection of the dwell times dt in each state for $N > 100$ molecules, where the trace may be categorized into each state manually or automatically using thresholding. A fit to this histogram yields a characteristic lifetime τ, which can be used to obtain the kinetic transition rates k_{on} and k_{off}.
59. A post-synchronization histogram (PSH) is a collection of time-binned FRET traces synchronized at the initial binding event. This analysis method reveals changes in the distribution of FRET states upon initial binding as a function of time.
60. Hidden Markov modelling is a method to distinguish multiple FRET states within a single FRET trajectory that is more stable and reproducible than standard thresholding algorithms. The model generates a predicted FRET trajectory that is typically overlaid on top of the original trace to confirm visually identified FRET states that are not easily quantifiable. These fits can be used to calculate interconversions between multiple discrete FRET states. FRET trajectories that visit all the proposed FRET states multiple times within a single trajectory are good candidates for HaMMy analysis.

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