Topic Introduction

Single-Molecule Total Internal Reflection Fluorescence Microscopy

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The advent of total internal reflection fluorescence (TIRF) microscopy has permitted visualization of biological events on an unprecedented scale: the single-molecule level. Using TIRF, it is now possible to view complex biological interactions such as cargo transport by a single molecular motor or DNA replication in real time. TIRF allows for visualization of single molecules by eliminating out-of-focus fluorescence and enhancing the signal-to-noise ratio. TIRF has been instrumental for studying in vitro interactions and has also been successfully implemented in live-cell imaging. Visualization of cytoskeletal structures and dynamics at the plasma membrane, such as endocytosis, exocytosis, and adhesion, has become much clearer using TIRF microscopy. Thanks to recent advances in optics and commercial availability, TIRF microscopy is becoming an increasingly popular and user-friendly technique. In this introduction, we describe the fundamental properties of TIRF microscopy and the advantages of using TIRF for single-molecule investigation.

SINGLE-MOLECULE VISUALIZATION USING TIRF MICROSCOPY

Since the development of fluorescence microscopy, a long-standing problem has been the limitation of visualizing single fluorescent molecules. Standard microscopes have a resolution limit near 0.2 μm, much too large to spatially distinguish individual protein complexes. Recent techniques have been developed that greatly enhance resolution, such as deconvolution and confocal microscopy, but these are still confounded by out-of-focus light or rapid photobleaching. One major problem with standard fluorescence imaging is limiting the excitation of fluorescent molecules to a precise focal plane. Out-of-focus fluorescence increases the background noise and detracts from the intensity of true signal, making spatial resolution difficult, if not impossible. The development of TIRF microscopy has effectively eliminated out-of-focus fluorescence by restricting excitation to a very thin section near the coverslip, making it possible to achieve single-molecule or -particle detection (Axelrod et al. 1984). This selective excitation also reduces photobleaching of fluorophores in solution and prevents harmful light damage when imaging live cells.

BASIC PHYSICS OF TIRF MICROSCOPY

The basic concept of TIRF microscopy applies the fundamental properties of optical physics to generate an evanescent field to excite fluorophores instead of using direct illumination. In a typical

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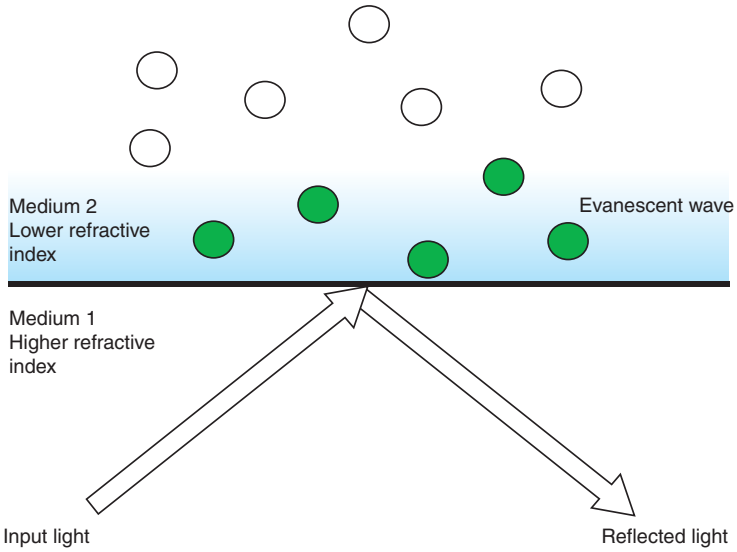

FIGURE 1. Total internal reflection (TIR) microscopy. The proportion of refracted to reflected light can be changed when passing light through two different materials: one of higher refractive index and the other of lower refractive index. A critical angle can be reached that defines the point where all the light is refracted parallel to the boundary between the two mediums. Once this critical angle is passed, light is totally internally reflected and creates an evanescent wave that transmits into the second medium and decays exponentially. In total internal reflection fluorescence (TIRF) microscopy, this phenomenon is exploited to restrict illumination of fluorophores to only those within ~100 nm of the coverslip (green spheres) while eliminating background excitation of fluorophores in solution (white spheres).

TIRF setup, light is transmitted through two adjacent materials, one of a higher refractive index (such as a glass coverslip), followed by another with a lower refractive index (such as an aqueous solution). As light passes through the media, it is partially reflected and refracted depending on the incident angle. By using two materials with different refractive indices, a critical angle can be reached: the point where all the light is refracted parallel to the interface of the two materials. Once this critical angle is passed, the phenomenon of total internal reflection (TIR) occurs (Fig. 1). Although light no longer passes through the second medium, the reflected light creates an electromagnetic field that penetrates into the less refractive material. This electromagnetic field is evanescent and decays exponentially with the depth of penetration. TIRF microscopy exploits this evanescent field, which is typically ~100 nm thick, to exclusively excite fluorophores at the boundary of the two media, that is, very near to the coverslip. This restriction eliminates excitation of molecules away from the coverslip, thereby significantly reducing out-of-focus background fluorescence. Until recently, reaching the proper incident angle to induce TIR was rather challenging due to the limitations of commercially available microscope objectives. However, the development of new objectives with sufficiently high numerical aperture (typically >1.45 Å) to reach the proper incident angle has made TIRF microscopy much more accessible to researchers in recent years (Axelrod 2001). This advancement combined with the increase in commercial availability of TIRF microscopes has greatly enhanced the popularity and usage of TIRF microscopy in today's research.

APPLICATIONS OF TIRF MICROSCOPY

TIRF microscopy is especially useful for studying protein–protein and protein–nucleic acid biochemical interactions. TIRF permits direct visualization of binding events and quantification of kinetic on and off rates. Standard bulk biochemical assays report on the average properties of a population and often miss individual variability and stochasticity that can only be appreciated at the single-molecule level. Visualizing single complexes can reveal sample heterogeneity such as the presence of different

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oligomeric states of a complex. TIRF can be used for stoichiometric quantification of proteins within complexes that are tagged with different fluorescent probes or by quantifying photobleach steps using single probes (Ulbrich and Isacoff 2007). Because the concentration range used in TIRF assays is often on the pM–nM scale, experiments can be performed using minute amounts of protein or DNA. This becomes extremely useful when visualizing native complexes purified from cells because often very little material is recovered. TIRF also makes it possible to watch assembly and disassembly of macromolecular complexes and can reveal hierarchical orders that may be obscured using other techniques (Hoskins et al. 2011). Using TIRF, it is now possible to view complex biological interactions such as cargo transport by a single molecular motor or DNA replication in real time (Axelrod et al. 1983; Vale et al. 1996; Ha et al. 1999; Tanner et al. 2009; Yardimci et al. 2010; Hoskins et al. 2011).

Single-molecule imaging has been vital to the characterization of molecular movements along a substrate, especially polymers such as actin, microtubules, or DNA (Funatsu et al. 1995; Vale et al. 1996; Harada et al. 1999). In addition, visualization of cytoskeletal structures and dynamics at the plasma membrane, such as endocytosis, exocytosis, and adhesion, has become much clearer using TIRF microscopy (Mashanov et al. 2003; Cai et al. 2007). Molecular diffusion and movement can be difficult to see within cells due to the complexity of structures within the cytoplasm. Recapitulating these events in vitro is a powerful method used for understanding biochemical function. Visualizing molecular events in real time provides key information about their temporal regulation, such as the stalling and reinitiation that helicases undergo while unwinding DNA (Ha et al. 2002). Single-molecule analysis is an excellent method for elucidating the function of enzymes and characterizing the timing of individual steps in catalytic reactions. TIRF has been instrumental in understanding the precise function of molecular machines such as the hand-over-hand motion of myosin V along microtubules (Yildiz et al. 2003), GroEL-assisted protein folding (Yamasaki et al. 1999), the dynamics of actin polymerization (Amann and Pollard 2001), and the analysis of the dynamic assembly of the spliceosome (Hoskins et al. 2011).

Coupling the properties of TIRF microscopy with other optical and biophysical techniques has been quite successful for the development of new combinatorial technologies. TIRF has been used to develop single-pair fluorescence resonance energy transfer (spFRET), giving even higher spatial resolution to single molecular colocalization (Weiss 1999). spFRET can reveal conformational changes within single enzymes during their interactions with substrates. TIRF combined with super-resolution methods, such as stochastic optical reconstruction microscopy (STORM), has been useful for refining molecular localization to within a few nanometers. Coupling TIRF with optical tweezer microscopy has also been tremendously powerful. Simultaneous use of both technologies allows for direct correlation of mechanical movement with structural changes (Ishijima et al. 1998; Lang et al. 2003). These techniques have lead to important and sometimes surprising findings, such as the demonstration that ATP hydrolysis and mechanical movement are not always simultaneous (Ishijima et al. 1998).

PROTOCOLS FOR TIRF MICROSCOPY

In the accompanying protocols, we provide step-by-step procedures for preparing and imaging samples for TIRF and for analyzing the resulting data. See Protocol: **Coverslip Cleaning and Functionalization for Total Internal Reflection Fluorescence Microscopy** (Kudalkar et al. 2015a), Protocol: **Preparation of Reactions for Imaging with Total Internal Reflection Fluorescence Microscopy** (Kudalkar et al. 2015b), and Protocol: **Data Analysis for Total Internal Reflection Fluorescence Microscopy** (Asbury 2015).

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REFERENCES

Amann KJ, Pollard TD. 2001. Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. *Proc Natl Acad Sci* **98:** 15009–15013.

Asbury CL. 2015. Data analysis for total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085571.

Axelrod D. 2001. Selective imaging of surface fluorescence with very high aperture microscope objectives. *J Biomed Opt* **6:** 6–13.

Axelrod D, Thompson NL, Burghardt TP. 1983. Total internal inflection fluorescent microscopy. *J Microsc* **129:** 19–28.

Axelrod D, Burghardt TP, Thompson NL. 1984. Total internal reflection fluorescence. *Annu Rev Biophys Bioeng* **13:** 247–268.

Cai D, Verhey KJ, Meyhofer E. 2007. Tracking single Kinesin molecules in the cytoplasm of mammalian cells. *Biophys J* **92:** 4137–4144.

Funatsu T, Harada Y, Tokunaga M, Saito K, Yanagida T. 1995. Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature* **374:** 555–559.

Ha T, Ting AY, Liang J, Caldwell WB, Deniz AA, Chemla DS, Schultz PG, Weiss S. 1999. Single-molecule fluorescence spectroscopy of enzyme conformational dynamics and cleavage mechanism. *Proc Natl Acad Sci* **96:** 893–898.

Ha T, Rasnik I, Cheng W, Babcock HP, Gauss GH, Lohman TM, Chu S. 2002. Initiation and re-initiation of DNA unwinding by the *Escherichia coli* Rep helicase. *Nature* **419:** 638–641.

Harada Y, Funatsu T, Murakami K, Nonoyama Y, Ishihama A, Yanagida T. 1999. Single-molecule imaging of RNA polymerase–DNA interactions in real time. *Biophys J* **76:** 709–715.

Hoskins AA, Friedman LJ, Gallagher SS, Crawford DJ, Anderson EG, Wombacher R, Ramirez N, Cornish VW, Gelles J, Moore MJ. 2011. Ordered and dynamic assembly of single spliceosomes. *Science* **331:** 1289–1295.

Ishijima A, Kojima H, Funatsu T, Tokunaga M, Higuchi H, Tanaka H, Yanagida T. 1998. Simultaneous observation of individual ATPase and mechanical events by a single myosin molecule during interaction with actin. *Cell* **92:** 161–171.

Kudalkar EM, Deng Y, Davis TN, Asbury CL. 2015a. Coverslip cleaning and functionalization for total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085548.

Kudalkar EM, Davis TN, Asbury CL. 2015b. Preparation of reactions for imaging with total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085563.

Lang MJ, Fordyce PM, Block SM. 2003. Combined optical trapping and single-molecule fluorescence. *J Biol* **2:** 6.

Mashanov GI, Tacon D, Knight AE, Peckham M, Molloy JE. 2003. Visualizing single molecules inside living cells using total internal reflection fluorescence microscopy. *Methods* **29:** 142–152.

Tanner NA, Loparo JJ, Hamdan SM, Jergic S, Dixon NE, van Oijen AM. 2009. Real-time single-molecule observation of rolling-circle DNA replication. *Nucleic Acids Res* **37:** e27.

Ulbrich MH, Isacoff EY. 2007. Subunit counting in membrane-bound proteins. *Nat Methods* **4:** 319–321.

Vale RD, Funatsu T, Pierce DW, Romberg L, Harada Y, Yanagida T. 1996. Direct observation of single kinesin molecules moving along microtubules. *Nature* **380:** 451–453.

Weiss S. 1999. Fluorescence spectroscopy of single biomolecules. *Science* **283:** 1676–1683.

Yamasaki R, Hoshino M, Wazawa T, Ishii Y, Yanagida T, Kawata Y, Higurashi T, Sakai K, Nagai J, Goto Y. 1999. Single molecular observation of the interaction of GroEL with substrate proteins. *J Mol Biol* **292:** 965–972.

Yardimci H, Loveland AB, Habuchi S, van Oijen AM, Walter JC. 2010. Uncoupling of sister replisomes during eukaryotic DNA replication. *Mol Cell* **40:** 834–840.

Yildiz A, Forkey JN, McKinney SA, Ha T, Goldman YE, Selvin PR. 2003. Myosin V walks hand-over-hand: Single fluorophore imaging with 1.5-nm localization. *Science* **300:** 2061–2065.

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Protocol

Coverslip Cleaning and Functionalization for Total Internal Reflection Fluorescence Microscopy

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Total internal reflection fluorescence (TIRF) microscopy allows visualization of biological events at the single-molecule level by restricting excitation to a precise focal plane near the coverslip and eliminating out-of-focus fluorescence. The quality of TIRF imaging relies on a high signal-to-noise ratio and therefore it is imperative to prevent adherence of molecules to the glass coverslip. Nonspecific interactions can make it difficult to distinguish true binding events and may also interfere with accurate quantification of background noise. In addition, nonspecific binding of the fluorescently tagged protein will lower the effective working concentration, thereby altering values used to calculate affinity constants. To prevent spurious interactions, we thoroughly clean the surface of the coverslip and then functionalize the glass either by applying a layer of silane or by coating with a lipid bilayer.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Biotinylated antibody (200 μM stock solution)
Biotinyl Cap PE (0.1 mg/mL in 2:1 chloroform/methanol) (Avanti Polar Lipids 870277X)
BRB80 (or any buffer with pH 5–9 and <200 mM ionic strength) <R>
Butylamine
Chloroform
Concentrated HCl (37%)
Concentrated sulfuric acid (95%–98%)
1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; 10 mg/mL in chloroform) (Avanti Polar Lipids)
Ethanol
Hydrogen peroxide (30%)
Imaging buffer <R>

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κ-casein (5 mg/mL stock solution; filter-sterilized)
Methanol
2-Methoxy(polyethyleneoxy)propyltrimethoxysilane (Gelest SIM-6492.7)
Nitrogen gas (dry)
Purified protein of interest
Streptavidin (1 mg/mL stock solution)
Toluene
Wash buffer (BRB80 containing 1 mg/mL κ-casein)

Equipment

Beaker (2-L, with Teflon lid and gas inlets)
Beakers (glass; 1- and 2-L)
Coverslips (glass; 22 × 60 mm)
Double-sided tape
Drying oven (set at 50°C)
Fume hood
Hot plate (set at 50°C)
Mercury lamp
Metal bar to hold Teflon racks
Nail polish
pH strips (range 2.0–7.0)
Pipettes (glass)
Plasma cleaner
Quartz cuvette
Razor blade
Safety gear (laboratory coat, goggles, gloves, and UV eye protection)
Slides (glass; 3 × 1 in)
Sonicating water bath
Sonifier (with microtip probe)
Spray bottle
Syringes (glass; 10-µL and 100-µL)
Teflon racks (custom-fabricated; see Fig. 1)
Teflon straw
Test tubes (glass)
Vacuum pump and desiccator
Vials (glass; for storing lipids)
Use Teflon tape for sealing the vials. Do not use rubber cap liners.

FIGURE 1. Custom-fabricated Teflon coverslip rack. Our coverslip racks hold 13 glass coverslips (22 × 60 mm) with minimal contact and four units can be linked together to clean and silanize 52 coverslips in one preparation. Teflon can withstand all the chemicals used in the protocol and the racks can be reused for several years.

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Vortexer
Water bath (set at 50°C)

METHOD

Coverslips are thoroughly cleaned and the glass surface is functionalized either by applying a layer of silane (Steps 1–21) or by coating with a lipid bilayer (Steps 22–42).

Functionalizing Glass Coverslips by Silanization

The silanization method (adapted from Cras et al. 1999 and Walba et al. 2004) involves a harsh acid wash to clean the glass followed by application of a functionalized layer of silane, which adheres to the glass and creates a uniform, covalently linked layer on the coverslip surface. The silane is linked to a short polyethyleneglycol molecule at one end, creating a hydrophilic layer pointing away from the surface. The hydrophilic layer attracts water and helps prevent proteins from adhering to the coverslip.

Acid-Washing the Coverslips

1. Load glass coverslips (22 × 60 mm) into four custom-fabricated Teflon racks (Fig. 1).

   A typical rack contains 13 slots and four units can be utilized together to clean and silanize 52 coverslips in one preparation. The racks are handled by inserting a horizontal metal bar into the top loops of each rack. All subsequent manipulations are performed with the coverslips loaded into the racks.

2. Holding the racks by the metal bar, briefly rinse the coverslips by dipping the racks into a 1-L beaker filled with distilled water.
3. Mix 500 mL of methanol with 500 mL of concentrated HCl in a 2-L beaker in the fume hood. Cool on ice for ~10 min (the reaction is exothermic) to allow time for the fuming to stop. Place the methanol/HCl beaker into a sonicating water bath.
4. Remove the coverslips from the distilled water and gently shake off excess water.
5. Carefully lower the coverslips into the methanol/HCl solution and gently dip up and down several times. Sonicate for 30 min in the sonicating water bath.
6. Prepare five wash beakers by filling five 2-L beakers with 1 L of distilled water each.
7. After sonication, gently shake off excess methanol/HCl solution and dip the coverslips several times into the first wash beaker. Repeat with the second wash beaker.
8. Place the second wash beaker into the sonicating water bath and sonicate for 3 min.
9. Thoroughly rinse the coverslips sequentially in the remaining wash beakers. Carefully dip the racks up and down several times in each wash solution. Check the pH of the water in each wash beaker using pH strips to ensure that the HCl is being washed away. (By the fourth or fifth beaker, the pH should equal to that of distilled water.) Thoroughly, but gently, shake off excess water after the final wash.
10. Pour 1 L of concentrated sulfuric acid into a clean 2-L beaker in the fume hood. Place the beaker into the sonicating water bath.
11. Lower the coverslips into the sulfuric acid, gently dipping up and down several times as in Step 5. Sonicate for 30 min.
12. Prepare seven wash beakers by filling seven 2-L beakers with 1 L of distilled water each.

    These additional washes are required to fully rinse off the sulfuric acid.

13. Rinse the coverslips sequentially in the wash beakers and check the pH of the rinse water. Sonicate the wash beakers for 10 min during the second and third washes. (By the seventh wash, the sulfuric acid should be removed.) Shake off excess water following the final wash.
14. Fill a spray bottle with 300 mL of ethanol. Spray the coverslips extensively with ethanol and place them in the drying oven for 30 min at 50°C.

15. When the coverslips are thoroughly dry, continue with silanization (Steps 16–21).

Silanization

16. Mix the silanization solution in a 2-L beaker by combining 960 mL of toluene, 16.8 mL of 2-methoxy(polyethyleneoxy)propyltrimethoxysilane, and 6 mL of butylamine.
17. Transfer the acid-cleaned, dry coverslips into the silanization solution and cap with a Teflon lid with N_2 gas inlets. Incubate for 90 min under N_2 gas at a flow rate of 2–3 mL/min.
18. Prepare two wash beakers by filling two 1-L beakers with 500 mL of toluene each. After incubating for 90-min, remove the coverslips from the silanization solution and gently shake to remove excess liquid. Dip the coverslips sequentially into the toluene washes, thoroughly shaking off the liquid between each wash.
19. Place the coverslips into a clean 2-L beaker with a Teflon lid and gas inlets. Fit two Teflon straws through the gas inlets and cover the beaker with the lid. The straws should reach the bottom of the beaker to achieve the best results. Dry thoroughly with N_2 gas at a flow rate of 5 mL/min for 90 min.
20. Cure overnight with N_2 gas at a flow rate of 1 mL/min.
21. Turn off the N_2 gas the following morning and cap the inlets. Store the coverslips at room temperature under N_2.

To use silanized coverslips for TIRF microscopy experiments, see Protocol: ***Preparation of Reactions for Imaging with Total Internal Reflection Fluorescence Microscopy*** *(Kudalkar et al. 2015).*

Functionalizing Glass Coverslips by Lipid Passivation

Besides silanization, coating surfaces with a lipid bilayer provides an alternative approach for specifically immobilizing molecules of interest, while eliminating nonspecific interactions between the surface and background molecules. In general, lipid bilayers appear to be inert to most biomolecules, including nucleic acids and most soluble proteins. For the purpose of TIRF imaging, lipid bilayers can be created by the spontaneous deposition of small unilamellar vesicles (SUVs) on a flat hydrophilic surface such as a clean glass coverslip (Sackmann 1996; Cremer and Boxer 1999). Lipids that carry modified head groups can be mixed with regular phospholipids to introduce specific immobilization of the molecule of interest on the surface. We use biotinylated phosphoethanolamine (Biotinyl Cap PE) as the affinity anchor. The density of the active linkers can be conveniently adjusted by altering the fractions of modified and regular lipids. In addition, the extensive variety of lipid species allows one to tune the physical properties of the lipid bilayer such as its diffusivity and phase.

Preparation of Small Unilamellar Lipid Vesicles

22. In the fume hood, transfer 1 mL of chloroform into three glass test tubes for washing the glass syringes.
23. Wash a fourth test tube briefly with chloroform and drain. This tube will be the dry lipid test tube.
24. Wash a 100-µL glass syringe three times with the washing chloroform in each of the three test tubes before use. Transfer 70 µL of chloroform to the lipid test tube.
25. Transfer 30 µL of the DPPC lipid solution to the lipid test tube. Wash the 100-µL glass syringe nine times with the washing chloroform.
26. Wash the 10-µL glass syringe nine times with the washing chloroform. Transfer 4 µL of Biotinyl Cap PE to the lipid test tube. Wash the 10-µL syringe nine times.
27. Vortex to mix the lipid.
28. Evaporate the chloroform from the mixture in the lipid test tube by gently blowing N_2 gas above the mixture for at least 5 min while slowly rotating the tube.

The dried lipid mixture will form a white patch at the bottom of the test tube.

29. Place the lipid test tube in the desiccator and further dry under vacuum for at least 15 min.

A plasma cleaner can also be used to dry the lipid. Keep the plasma off if drying with plasma cleaner.

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30. Add 200 µL of BRB80 (or other desired buffer) to the dried lipid and vortex briefly. Place the test tube in a water bath set at 50°C. Vortex every 5 min until the lipid patch at the bottom of the tube is completely rehydrated into giant unilamellar vesicles (GUVs), which appear as a white opaque suspension.

    This step typically takes <15 min.

31. Transfer the rehydrated lipid to a 1.5-mL microcentrifuge tube. Immerse the sonifier microtip probe into the suspension and sonicate for 3 min, while keeping the test tube in a water bath set >50°C. To prevent spurious bubbling, lower the sonication duty cycle and the output power.

    The white GUV suspension should turn into a clear SUV suspension.

32. (Optional) If low fluorescence background is required, add 100 µL of warm 30% hydrogen peroxide to the liposome suspension and transfer the mixture to a quartz cuvette. Place the cuvette under a mercury lamp and irradiate for 30 min to bleach the fluorophores in the liposome.

    This step can be performed at room temperature. Proper eye protection is required to prevent UV damage.

    UV irradiation and peroxide treatment have no observable effect on the passivation ability of saturated phospholipid and the biotin functional group, but they significantly lower the fluorescence of the contaminants in the liposome.

33. Keep the SUV preparation on a hotplate or in a water bath set at 50°C. Use the liposomes within 1 d.

Coating Glass Coverslips with a Lipid Bilayer

34. Clean coverslips and glass slides with a plasma cleaner.
35. Apply several pieces of double-sided tape crosswise to a plasma-cleaned glass slide, leaving ~1–3 mm gaps in between the pieces to create chambers.
36. Place a plasma-cleaned coverslip on the slide. Gently press down to ensure a tight and even seal between the coverslip and the tape. Place on a hot plate set for 5 min at 50°C and periodically press down on the coverslip to secure sealing.
37. Using a razor blade, trim off excess tape from either side of the coverslip so it is flush with the edge.
38. Transfer the slide to a warm humidity chamber, such as a pipette tip box half-filled with hot water. Flow in BRB80 and prewet the chamber to prevent lifting of the double-sided tape. Flowthrough SUV in warm buffer (from Step 33). Incubate for 5 min to allow the lipid bilayer to form. Before or during the incubation, prepare a 0.25 mg/mL solution of streptavidin in wash buffer.
39. Wash the chamber with 70 µL of wash buffer. Move the slide to the humid chamber at room temperature. Flowthrough the streptavidin solution and incubate for 5 min. Before or during the incubation, prepare a 20 µM solution of biotinylated antibody solution in wash buffer.
40. Wash the chamber with 70 µL of wash buffer. Flow through the antibody solution. Incubate for 5–15 min. During the incubation, prepare the protein solution in wash buffer.
41. Wash the chamber with 70 µL of wash buffer. Flowthrough the purified protein solution and incubate for 5 min. Before or during the incubation, prepare the imaging buffer.
42. Wash the chamber with 70 µL of wash buffer. Flowthrough the imaging buffer, seal the chamber with nail polish. Observe the reaction under the microscope (see Protocol: **Data Analysis for Total Internal Reflection Fluorescence Microscopy** [Asbury 2015]).

DISCUSSION

As with silanization, the lipid coating method provides a way to block nonspecific interactions between the glass surface of the coverslip and the solute while keeping a particular molecule of interest

attached to the surface. We find the lipid coating procedure to be effective against nonspecific adsorption of DNA oligonucleotides, single protein molecules such as green fluorescent protein (GFP), and large protein complexes such as purified kinetochore particles. However, we do not recommend the lipid coating method when using microtubules because the lipid bilayer has an extremely high affinity for microtubules. (See Protocol: **Preparation of Reactions for Imaging with Total Internal Reflection Fluorescence Microscopy** [Kudalkar et al. 2015] for further details on using silanized coverslips in TIRF microscopy experiments for studying the binding of kinetochore proteins to microtubules.) Fine-tuning the ratio between functionalized and regular lipids can accurately control the density of the active functional groups on the bilayer surface. Each DPPC lipid occupies 50 $Å^2$ and 0.1% of the lipid carries a biotin group according the ratio used in the protocol. Thus, the density of biotin on the bilayer is 2000 μm^{-2}.

Lipid bilayers have complex phase behavior according to temperature and composition. Above the transition temperature, the bilayer forms a two-dimensional fluid and the lipid molecules are diffusive. For TIRF imaging applications, the diffusion will cause motion blur, and the severity of this depends on the exposure time. This can be avoided by using lipids with high phase-transition temperature (such as DPPC) that can form a two-dimensional gel on the surface. However, the passivation efficiency is impaired if the lipid is in the gel phase. Supplementing the incubation buffer with κ-casein restores the passivation, presumably by blocking flaws in the gel-phase bilayer. When lateral constraint is not required, we recommend using low transition temperature lipids such as κ-POPC. In the latter case, all procedures can be performed at room temperature without κ-casein. The passivation efficiency of POPC is higher, and the POPC liposomes can be stored for 3–5 d at 4°C.

RECIPES

BRB80 (5×)

Reagent	Quantity (for 100 mL)	Final concentration (1×)
K-PIPES	12.1 g	80 mM
$MgCl_2$ (1 M)	0.5 mL	1 mM
EGTA (0.5 M)	1 mL	1 mM

Dissolve the K-PIPES and 2.7 g of KOH pellets in 85 mL of Milli-Q purified water. Add the $MgCl_2$ and EGTA. Stir until all the K-PIPES has dissolved. If necessary, add the KOH pellets one at a time until the K-PIPES goes into solution, but be careful not to exceed pH 6.8. Do not put a pH probe into the solution until all the PIPES is dissolved. Measure the pH and bring up to pH 6.8 using 5 M KOH. Adjust the volume to 100 mL with Milli-Q water and divide into 15-mL aliquots. Store the aliquots at −20°C. A working aliquot may be stored for up to 1 mo at 4°C. Prepare fresh 1× BRB80 (diluted in Milli-Q water) each day.

Imaging Buffer

Reagent	Final concentration
Glucose oxidase (oxygen scavenger; 10 mg/mL)	200 mg/mL
Catalase (oxygen scavenger; 1.75 mg/mL)	35 mg/mL
Glucose (1.25 M)	25 mM
Dithiothreitol (DTT; 250 mM)	5 mM
κ-casein (5 mg/mL)	1 mg/mL

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REFERENCES

Asbury CL. 2015. Data analysis for total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085571.

Cras JJ, Rowe-Taitt CA, Nivens D, Ligler FS. 1999. Comparison of chemical cleaning methods of glass in preparation for silanization. *Biosens Bioelectron* **14:** 683–688.

Cremer PS, Boxer SG. 1999. Formation and spreading of lipid bilayers on planar glass supports. *J Phys Chem B* **103:** 2554–2559.

Kudalkar EM, Davis TN, Asbury CL. 2015. Preparation of reactions for imaging with total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085563.

Sackmann E. 1996. Supported membranes: Scientific and practical applications. *Science* **271:** 43–48.

Walba DMLC, Korblova E, Farrow M, Furtak T, Chow BC, Schwartz DK, Freeman AS, Douglas K, Williams SD, Klittnick AF, Clark NA. 2004. Self-assembled monolayers for liquid crystal alignment: Simple preparation on glass using alkyltrialkoxysilanes. *Liquid Crystals* **31:** 481–489.

Protocol

Preparation of Reactions for Imaging with Total Internal Reflection Fluorescence Microscopy

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Here we present our standard protocol for studying the binding of kinetochore proteins to microtubules as a paradigm for designing single-molecule total internal reflection fluorescence (TIRF) microscopy experiments. Several aspects of this protocol require empirical optimization, including the method for anchoring the polymer or substrate to the coverslip, the type and amount of blocking protein to prevent nonspecific protein adsorption to the glass, the appropriate protein concentration, the laser power, and the duration of imaging. Our method uses bovine serum albumin and κ-casein as blocking agents to coat any imperfections in the coverslip silanization and thereby prevent protein adsorption to the coverslip. Protein concentration and duration of imaging must be optimized for each experiment and protein of interest. Ideally, a range is determined that allows for resolution of single complexes binding to microtubules to ensure proper measurement of kinetic off rates and diffusion along microtubules. Excessively high concentrations may lead to overlapping binding of proteins on microtubules, making it impossible to resolve single binding events. The duration of imaging must be long enough to capture very low off rates (long residence time on microtubules) and we typically image at 10 frames/sec for 200 sec. The laser power can be adjusted to prevent photobleaching, but must be high enough to achieve a sufficient signal/noise ratio.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Alexa-568-labeled GMPCPP seeds (Hyman et al. 1991)
Alexa-568-labeled, paclitaxel-stabilized microtubules (Hyman et al. 1991)
BB80 (BRB80 <R> containing 8 mg/mL BSA)
BB80T (BB80 containing 10 μM taxol)
Bovine serum albumin (BSA; 40 mg/mL stock solution; filter-sterilized)
Catalase (1.75 mg/mL stock solution)
Dithiothreitol (DTT; 250 mM stock solution)
Ethanol

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Glucose (1.25 M stock solution)
Glucose oxidase (10 mg/mL stock solution)
GTP (100 mM stock solution, pH 7.0)
κ-casein (5 mg/mL stock solution; filter sterilized)
Microtubule growth buffer (GB; BB80 containing 1 mM GTP)
Purified protein of interest
Rigor kinesin (purified in our laboratory)
Tubulin (bovine; 1:100 Alexa-568-labeled to unlabeled) (purified and labeled in our laboratory)

Equipment

Adaptors for peristaltic pump (custom-made)
Adhesive transfer tape (3M F9473PC)
Coverslips (glass; 22 × 60 mm; silanized) (see Protocol: **Coverslip Cleaning and Functionalization for Total Internal Reflection Fluorescence Microscopy** [Kudalkar et al. 2015])
Drill (with diamond bit)
Double-sided tape
Nail polish
Razor blade
Slides (glass; 3 × 1 in)
Vacuum grease

METHOD

This protocol for using TIRF microscopy to study the binding of kinetochore proteins to microtubules at the single-molecule level is adapted from Gestaut et al. (2010).

Assembly of Flow Chamber

The design of the flow chamber depends on whether the experiment is to be performed with the peristaltic pump method (see Steps 1–8) or the sealed slide method (see Steps 9–12). The peristaltic pump method is used to introduce reactants to the flow chamber during imaging on the microscope and the sealed slide method is used to introduce reactants before imaging. Both methods use the same procedure for washes, adherence of rigor kinesin, and introduction of microtubules and proteins. If the reactants are introduced before imaging, the entire protocol is performed at the bench using an aspirator to draw liquid through the chambers and then the slide is sealed with either clear nail polish or vacuum grease.

Flow Chamber for Peristaltic Pump Method

1. Drill eight holes into a 3 × 1 in glass slide using a diamond bit—two sets of four holes on the long axis of the slide, directly across from one another (Fig. 1A).
2. Clean the drilled glass slide with ethanol. Place double-sided tape crosswise between the drilled holes leaving ~3 mm between each piece of tape to create four equivalently sized flow chambers.
3. Place a silanized coverslip lengthwise over the middle of the slide. Ensure all eight holes are covered to create four perfusion chambers. Gently press down on the surface of the coverslip to ensure a tight seal (Fig. 1B).
4. Remove the excess tape on the sides of the coverslip using a razor blade.
5. Using a cotton-tipped applicator, seal the chambers by gently pressing vacuum grease into each opening on the outer edges of the slide until it reaches the holes. Wipe off excess grease with ethanol.
6. Flip the glass slide over so the coverslip is underneath. Apply adhesive tape to four of the holes on this side of the slide and, using forceps, remove circles of tape that cover each hole (Fig. 1C).

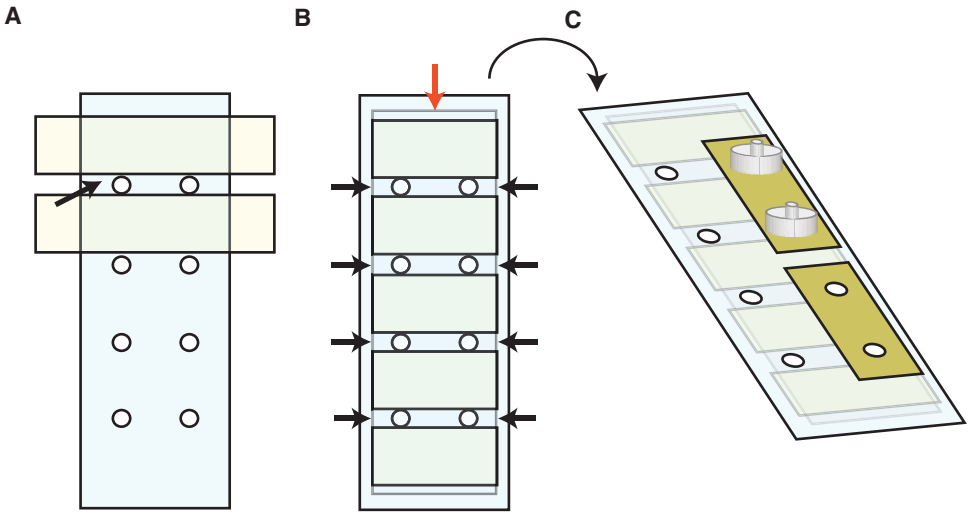

FIGURE 1. Assembly of flow chamber for peristaltic pump method. (*A*) Apply double-sided tape to a glass slide predrilled with two sets of four holes in a lengthwise arrangement. Position the tape to create four 3-mm-wide chambers (black arrows). (*B*) Position a cleaned coverslip (red arrow) in the *middle* of the slide and press firmly to seal against the tape. Trim the tape on both sides using a razor blade so it is flush with the coverslip edge. Push vacuum grease into the sides of chambers (black arrows) to seal. Do not cover the holes. (*C*) Flip the slide over so coverslip is face down. Apply 3M adhesive tape to cover the holes on one edge of slide (yellow). Using forceps, remove the tape covering each hole to create an open channel. Center custom-made flow adaptors (gray) on top of each hole and press firmly to seal.

7. Place custom-made adaptors on the tape, centering each one with the holes in the slide. Press gently to ensure a good seal (Fig. 1C).
8. Make a small circle around each remaining hole using vacuum grease to create a pool for buffers.

Flow Chamber for Sealed Slide Method

9. Clean a 3×1 in glass slide thoroughly with ethanol.
10. Apply several pieces of double-sided tape crosswise to the slide, leaving a gap of ~3 mm in between the pieces to create chambers.
11. Place a silanized coverslip crosswise on the slide leaving an equal-sized ledge on either side of the slide. Gently press down to ensure a tight and even seal between the coverslip and tape.
12. Using a razor blade, trim off excess tape from either side of the coverslip so it is flush with the edge.

Binding Interactions Using Paclitaxel-Stabilized Microtubules

Direct adsorption of microtubules to the coverslip can interfere with their ability to grow and shorten (if using non-stabilized microtubules) and could potentially hinder protein binding. To attach microtubules to the coverslip without direct adherence to the glass, we use an established method that uses a mutated kinesin ("rigor kinesin") that is competent to bind, but not release, microtubules (Rice et al. 1999). We optimize the concentration of rigor kinesin to ensure that microtubules are stably tethered to the coverslip, but the kinesin does not interfere with experimental protein binding.

13. Flow 100 µL of Milli-Q purified water through the chamber twice.
14. Flow 25 µL of rigor kinesin (diluted in BB80T) through the chamber and incubate for 5 min. Determine the dilution factor for rigor kinesin empirically for each coverslip preparation to ensure proper anchoring and coverage of microtubules. Apply 50 µL of BB80T to the edge or hole of the flow chamber during incubation to prevent the chamber from drying.
15. Flow 50 µL of BB80T through the chamber.

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16. Flow 15 µL of Alexa-568-labeled, paclitaxel-stabilized microtubules diluted in BB80T through the chamber. Determine the dilution factor empirically to achieve an appropriate amount of microtubule coverage (typically about three to seven nonoverlapping microtubules per field is optimal for analysis). Incubate for 5 min. Apply 50 µL of BB80T to the edge or hole of the flow chamber during incubation to prevent the chamber from drying.
17. During the microtubule incubation, prepare the experimental reaction, which typically contains 10–100 pM protein, 25 mM glucose, 5 mM DTT, and oxygen scavengers 200 µg/mL glucose oxidase and 35 µg/mL catalase. Adjust the volume to 50 µL with BB80T.

    To prevent protein loss to the tube (a problem that can occur when working in the pM concentration range), dilute the protein of interest immediately before adding to the reaction mixture.
18. Flow 50 µL of BB80T through the chamber and then 50 µL of reaction mixture. Image immediately on both 488 and 561 nm channels (see Protocol: **Data Analysis for Total Internal Reflection Fluorescence Microscopy** [Asbury 2015]).

Binding Interactions Using Dynamic Microtubules

19. Follow Steps 13 and 14 of the protocol using stabilized microtubules.

    The concentration of rigor kinesin may be increased to ensure proper anchoring of growing microtubule extensions.
20. Flow 50 µL of GB through the chamber.
21. Flow 15 µL of Alexa-568-labeled GMPCPP seeds diluted in BB80 through the chamber. Incubate for 1 min. Optimize the concentration of seeds to ensure proper coverslip coverage.
22. Flow 50 µL of GB through the chamber.
23. Prepare the tubulin mix, which contains 2 mg/mL bovine tubulin (1:100 Alexa-568-labeled to unlabeled), 25 mM glucose, 5 mM DTT, and oxygen scavengers 200 µg/mL glucose oxidase and 35 µg/mL catalase. Adjust the volume to 50 µL with BB80. Flow 50 µL of tubulin mix through the chamber. Incubate for 15 min to allow microtubules to extend off the Alexa-568 GMPCPP seeds.
24. During the incubation, focus the microscope on the channel.

    Once the reaction mix is added (Step 25), the microtubules will immediately begin to depolymerize. Therefore, it is essential to have the field of view already in focus so as to begin imaging promptly on addition of the reaction mix.
25. Prepare the reaction mix containing 10 pM to 1 nM protein, 200 µg/mL glucose oxidase, 35 µg/mL catalase, 25 mM glucose, and 5 mM DTT. Adjust the volume to 50 µL with BB80. Flow 50 µL of reaction mix through the chamber. Begin imaging immediately (see Protocol: **Data Analysis for Total Internal Reflection Fluorescence Microscopy** [Asbury 2015]).

RECIPE

BRB80 (5×)

Reagent	Quantity (for 100 mL)	Final concentration (1×)
K-PIPES	12.1 g	80 mM
$MgCl_2$ (1 M)	0.5 mL	1 mM
EGTA (0.5 M)	1 mL	1 mM

Dissolve the K-PIPES and 2.7 g of KOH pellets in 85 mL of Milli-Q purified water. Add the $MgCl_2$ and EGTA. Stir until all the K-PIPES has dissolved. If necessary, add the KOH pellets one at a time until the K-PIPES goes into solution, but be careful not to exceed pH 6.8. Do not put a pH probe into the solution until all the PIPES is dissolved. Measure the pH and bring up to pH 6.8 using 5 M KOH. Adjust the volume to 100 mL with Milli-Q water and divide into 15-mL aliquots. Store the aliquots at −20°C. A working aliquot may be stored for up to 1 mo at 4°C. Prepare fresh 1× BRB80 (diluted in Milli-Q water) each day.

REFERENCES

Asbury CL. 2015. Data analysis for total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085571.

Gestaut DR, Cooper J, Asbury CL, Davis TN, Wordeman L. 2010. Reconstitution and functional analysis of kinetochore subcomplexes. *Methods Cell Biol* **95:** 641–656.

Hyman A, Drechsel D, Kellogg D, Salser S, Sawin K, Steffen P, Wordeman L, Mitchison T. 1991. Preparation of modified tubulins. *Methods Enzymol* **196:** 478–485.

Kudalkar EM, Deng Y, Davis TN, Asbury CL. 2015. Coverslip cleaning and functionalization for total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085548.

Rice S, Lin AW, Safer D, Hart CL, Naber N, Carragher BO, Cain SM, Pechatnikova E, Wilson-Kubalek EM, Whittaker M, et al. 1999. A structural change in the kinesin motor protein that drives motility. *Nature* **402:** 778–784.

Protocol

Data Analysis for Total Internal Reflection Fluorescence Microscopy

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In the microscopes we use to analyze total internal reflection fluorescence (TIRF), the emitted fluorescence is split chromatically, using dichroic filters, into either two or three different colors ("channels"). In our two-color instrument, the green emission wavelengths (405–488 nm; for imaging green fluorescent protein [GFP]-tagged proteins) and far-red emission wavelengths (650–800 nm; for imaging Alexa-647-labeled microtubules) are projected onto the upper and lower halves, respectively, of a single camera. A single filter can be swapped to collect near-red wavelengths (561–640 nm; for imaging mCherry, or Alexa-568-labeled microtubules) instead of far-red. Our three-color instrument is very similar except that the green, near-red, and far-red color ranges are projected onto three separate cameras. In either case, the different colors can be imaged simultaneously. Typically, we collect images at 10 frames/sec for ~200 sec. We have developed a series of semiautomated image analysis programs, written in LabView, to obtain the brightness, residence time, and mobility of individual particles bound to single microtubules. The basic analysis steps are straightforward and could also be implemented using ImageJ or Matlab. For convenience, this protocol describes the analysis of a single microtubule. Data from many microtubules across many experimental trials are needed to obtain robust conclusions that are independent of stochastic and trial-to-trial variability.

MATERIALS

Equipment

Data analysis program (e.g., Igor Pro, Matlab, or Microsoft Excel; see Step 7)
EMCCD camera (iXon DV887 from Andor)
Image analysis programs custom written in LabView (see Gestaut et al. 2010)

Copies of our programs are available for free upon request.

Microscope (Ti-U from Nikon or equivalent)

METHOD

For appropriate sample preparation procedures, see Protocol: ***Coverslip Cleaning and Functionalization for Total Internal Reflection Fluorescence Microscopy*** *(Kudalkar et al. 2015a)* and Protocol: ***Preparation of Reactions for Imaging with Total Internal Reflection Fluorescence Microscopy*** *(Kudalkar et al. 2015b).*

1. Trace the contour of a microtubule by hand using a multisegment line tool (Fig. 1A).

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The filaments in the experiments described here should remain stationary on the coverslips. Assuming negligible drift, the contours traced in an early image will apply to the entire duration of the time-lapse experiment.

2. Map the microtubule contour automatically onto the other color channel(s), and use it to generate a kymograph(s) showing the arrival of particles on the microtubule, their movement along the filament, and their release (Fig. 1B).

   Accurate mapping from one color channel to another usually requires coordinates to be translated (i.e., moved along x and y directions) and also slightly rotated. Depending on the microscope setup, slight magnification changes might also be needed. The required mapping operation can be determined empirically using reference particles that fluoresce in all the channels.

3. For each particle visible on the kymograph, estimate its location along the microtubule by finding the brightest pixel at each time point.

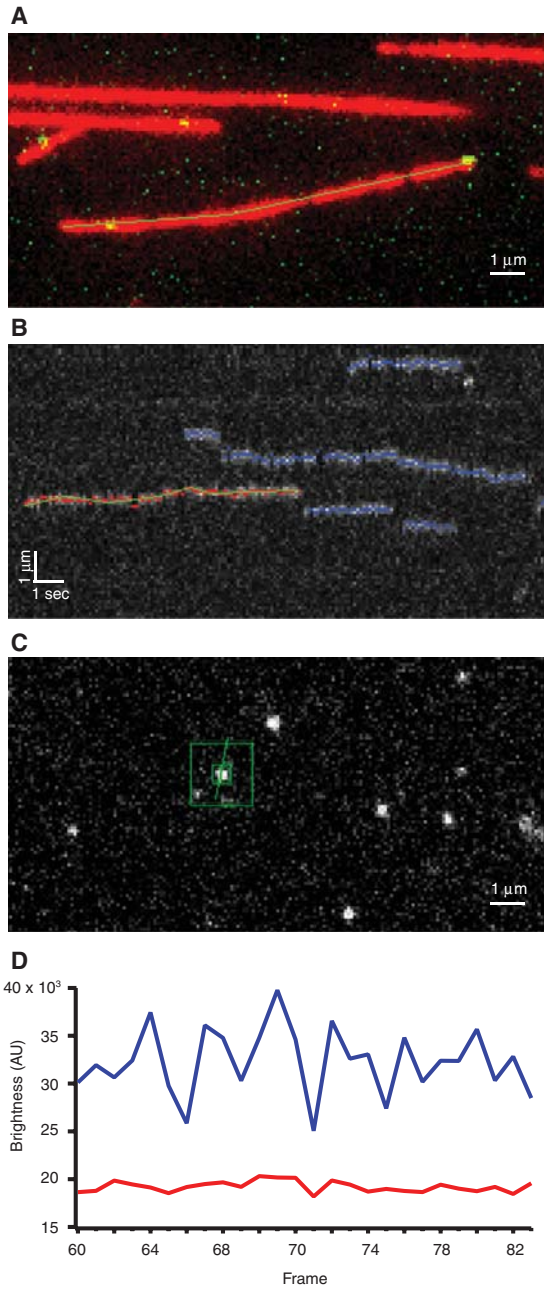

FIGURE 1. TIRF data analysis. (*A*) Overlapping green and red channels depict a microtubule (red) that was traced by hand using a multisegmented tool. Yellow spots show GFP-tagged proteins binding to the microtubule. (*B*) Kymograph created from a traced microtubule as in *A*. Red dots and green line indicate the currently selected binding event, blue lines show previously selected events, and untraced events are shown in gray. (*C*) Particle brightness is computed at each time point of event from *B*. Integrated brightness is measured using small green box surrounding particle and larger green box is used to calculate background intensity. (*D*) Example plot of pixel brightness (blue trace) and background level (red trace) over time generated from *C*.

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4. Map the one-dimensional estimate of location versus time from Step 3 back onto the original, two-dimensional particle image using the contour from Step 2.
5. Obtain the pixel coordinates of the particle in each image by using a two-dimensional search for the brightest pixel within a small square (7 × 7 pixels) centered on the estimated position from Step 4.

   Alternatively, fit a two-dimensional Gaussian function to the intensity distribution within the small square. A Gaussian fit is more time-consuming and generally does not improve localization accuracy for single GFP molecules imaged at 10 frames/sec in our microscopes. However, in cases where more photons are collected per frame, Gaussian fitting may improve localization accuracy.
6. After coordinates are obtained in Step 5, compute particle brightness at each time point by integrating pixel intensities over a small square, centered on the particle position. Estimate background levels by integrating over a larger concentric square area, excluding the central small square (Fig. 1C).
7. For every particle, save a file containing the pixel coordinates, integrated brightness, and background level at each time point (Fig. 1D).

   Once the particle data are saved, we use another graphing and data analysis program, Igor Pro, to carry out the subsequent analysis steps. These steps could also be performed using Matlab or Microsoft Excel.
8. Examine plots of brightness versus time for each event to confirm the arrival and release times, and to identify photobleaching steps.
9. Compute the residence times for each event from the start and end times obtained in Steps 7 and 8. Plot a distribution of residence times, either in the form of a histogram (e.g., see Gestaut et al. 2008 or Powers et al. 2009) or as a cumulative survival probability versus time (e.g., see Sarangapani et al. 2013).

   Histograms are more intuitive, but cumulative distributions avoid the need for binning and facilitate comparisons because many can be overlayed onto a single graph without loss of clarity.
10. Note that the residence time distribution is often (but not always) well described by a single exponential decay, except that the lowest bins (corresponding to the shortest residence times) may be underpopulated due to the finite time resolution of the instrument. Invert the mean from the best-fit exponential (excluding the lowest bins) or, equivalently, its time constant, τ, to give the off rate, $k_{off} = \tau^{-1}$ (e.g., in units of sec^{-1}).
11. For each event, also calculate a mean squared displacement along the microtubule long axis, $\langle x^2 \rangle$, for every possible time lag, Δt. Average the $\langle x^2 \rangle$ values across many events to generate a plot of $\langle x^2 \rangle$ versus Δt for a population (each individual binding event contributing equally to the population average).
12. Compute the one-dimensional diffusion coefficient, D, from the slope, m, of a linear fit to the plot according to $D = (1/2)m = (1/2) \langle x^2 \rangle \Delta t^{-1}$.

REFERENCES

Gestaut DR, Graczyk B, Cooper J, Widlund PO, Zelter A, Wordeman L, Asbury CL, Davis TN. 2008. Phosphoregulation and depolymerization-driven movement of the Dam1 complex do not require ring formation. *Nat Cell Biol* **10:** 407–414.

Gestaut DR, Cooper J, Asbury CL, Davis TN, Wordeman L. 2010. Reconstitution and functional analysis of kinetochore subcomplexes. *Methods Cell Biol* **95:** 641–656.

Kudalkar EM, Deng Y, Davis TN, Asbury CL. 2015a. Coverslip cleaning and functionalization for total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085548.

Kudalkar EM, Davis TN, Asbury CL. 2015b. Preparation of reactions for imaging with total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot085563.

Powers AF, Franck AD, Gestaut DR, Cooper J, Gracyzk B, Wei RR, Wordeman L, Davis TN, Asbury CL. 2009. The Ndc80 kinetochore complex forms load-bearing attachments to dynamic microtubule tips via biased diffusion. *Cell* **136:** 865–875.

Sarangapani KK, Akiyoshi B, Duggan NM, Biggins S, Asbury CL. 2013. Phosphoregulation promotes release of kinetochores from dynamic microtubules via multiple mechanisms. *Proc Natl Acad Sci* **110:** 7282–7287.