Chapter 4

Fluorescence Imaging with One Nanometer Accuracy: In Vitro and In Vivo Studies of Molecular Motors

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Abstract

Traditional microscopy techniques are limited by the wave-like characteristics of light, which dictate that about 250 nm (or roughly half the wavelength of the light) is the smallest distance by which two identical objects can be separated while still being able to distinguish between them. Since most biological molecules are much smaller than this limit, traditional light microscopes are generally not sufficient for single-molecule biological studies. *F*luorescence *I*maging with *O*ne *N*anometer *A*ccuracy (FIONA) is a technique that makes possible localization of an object to approximately one nanometer. The FIONA technique is simple in concept; it is built upon the idea that, if enough photons are collected, one can find the exact center of a fluorophore's emission to within a single nanometer and track its motion with a very high level of precision. The center can be localized to approximately $(\lambda/2)/\sqrt{N}$, where λ is the wavelength of the light and N is the number of photons collected. When $N = 10{,}000$, FIONA achieves an accuracy of 1–2 nm, assuming the background is sufficiently low. FIONA, thus, works best with the use of high-quality dyes and fluorescence stabilization buffers, sensitive detection methods, and special microscopy techniques to reduce background fluorescence. FIONA is particularly well suited to the study of molecular motors, which are enzymes that couple ATP hydrolysis to conformational change and motion. In this chapter, we discuss the practical application of FIONA to molecular motors or other enzymes in biological systems.

Key words: FIONA, Molecular motors, Single-molecule tracking, TIRF microscopy

1. Introduction

In order to conduct single-molecule studies, researchers are confronted with the diffraction limit of light. For visible optical microscopy, this limit is $\lambda/(2 \times \mathrm{NA})$, where λ (the wavelength of the light) is approximately 500 nm and NA (the numerical aperture of the microscope objective) is about 1.4. Diffraction-limited spots in a traditional light microscope are, therefore, generally larger

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than 200 nm in diameter – much bigger than the molecules of interest. This resolution limit means that two identical fluorophores in close proximity can, therefore, not be distinguished using traditional light microscopy unless they are greater than ~250 nm apart. In recent years, some modern techniques, often called "super-resolution" techniques, have to varying degrees bypassed this limit (1, 2). Although extremely useful for some applications, most of these techniques require relatively slow timescales, multiple lasers, the use of photoswitchable dyes, and extensive postprocessing. In this chapter, we discuss *F*luorescence *I*maging with *O*ne *N*anometer *A*ccuracy (FIONA), a simpler but versatile technique for achieving nanometer precision at biologically relevant timescales. Unlike the superresolution techniques mentioned above, FIONA does not improve the resolution of fluorophores in close proximity but instead improves the localization accuracy of a single fluorophore.

We have shown that FIONA enables the localization of a single molecule to within 1.5 nm (3) and with 1–500 ms temporal resolution (4). Data can be taken inside or outside of live cells at a timescale that is physiologically relevant, making FIONA an extremely valuable tool in the toolbox of single-molecule research techniques. The principle behind FIONA is simple. In a typical FIONA measurement, a fluorophore is attached to some biological molecule of interest. As the biological molecule (and the attached fluorophore) moves through space, the center of its emission is continuously localized, making it possible to track single biomolecules with great precision. At the core of the technique is the ability to collect a large number of photons emitted by a single fluorophore. When we plot the number of photons emitted by a fluorophore versus its position in the *x*–*y* plane (Fig. 1), we can localize the center of the resulting Airy function much more accurately than the width of the function. (A 2D Gaussian function is often used to approximate the Airy function, with little error). Theoretically, the accuracy with which it is possible to locate the center is the standard error of the mean, i.e., the standard deviation divided by the square root of the total number of counts (5). Thus, the accuracy of FIONA depends on the collection of large numbers of photons. For 10,000 photons collected, for example, one obtains an accuracy of approximately $250/100 = 2.5$ nm.

To be slightly more quantitative, in practice, the accuracy depends on three factors, as shown in Eq. 1: the number of photons (N), the effective pixel size of the detector, a, which is the pixel size divided by magnification, and the standard deviation of the background, b.

$$\sigma = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}\right)} \tag{1}$$

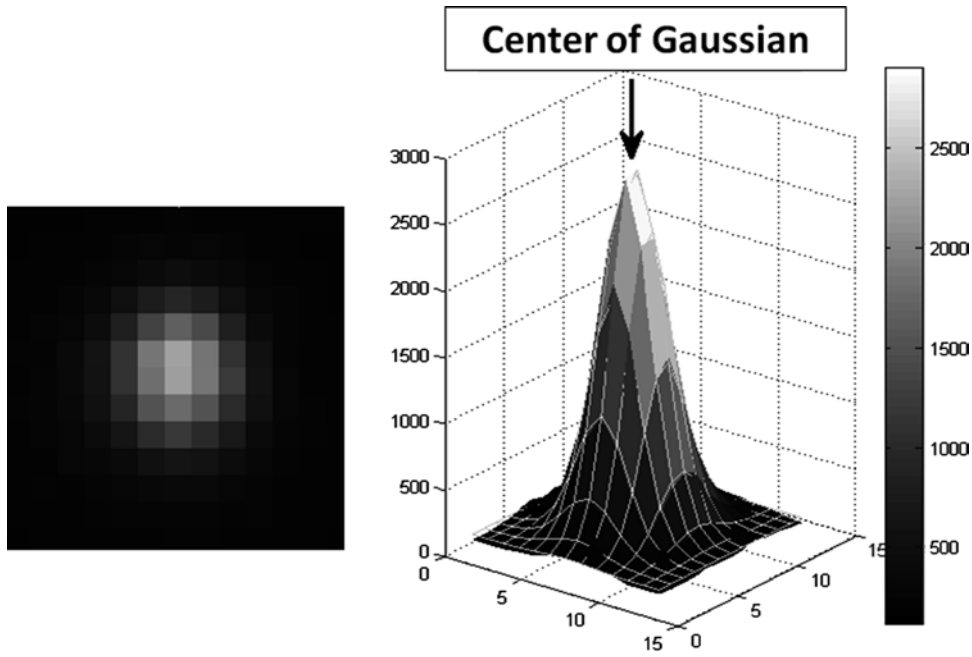

Fig. 1. Sample of a cropped fluorophore PSF captured with a CCD camera (***left***), and the same PSF plotted in three dimensions, intensity as a function of (***x***, ***y***). Note that a good PSF should be circularly symmetric and the peak should be significantly higher than the background. A Gaussian function is fitted to the PSF, shown as a mesh overlay in the plot.

σ is the uncertainty or standard error of the mean, a is the effective pixel size of the detector (pixel size divided by magnification), b includes both the background fluorescence and the detector noise, and s_i is the width of the distribution (which is approximately 250 nm for a diffraction limited spot of visible light) in direction i, where $i = x$ or y. The first term ($\frac{s_i^2}{N}$) is due to photon noise, the second term is the effect of the finite pixel size of the detector, and the third term is the effect of background. Assuming an appropriate effective pixel size (of 80–120 nm, or 16 μm divided by the magnification, e.g., 160×) is used, the second term does not contribute significantly. When appropriate techniques are used to minimize background noise (as discussed below), the first term (photon noise) is the limiting factor in a FIONA measurement.

Thus, it is crucial to both decrease the background and collect as many photons as possible from the single fluorophores to obtain the greatest precision of localization possible. Several considerations must be made: first, an appropriate fluorophore must be chosen – for accurate FIONA measurements to be made, the fluorophore must be sufficiently bright and highly photostable. Oxygen scavenging systems and other chemicals are generally needed to increase the lifetime and stability.

Second, background fluorescence must be minimized. This is typically achieved by the use of a Total Internal Reflection (TIR)

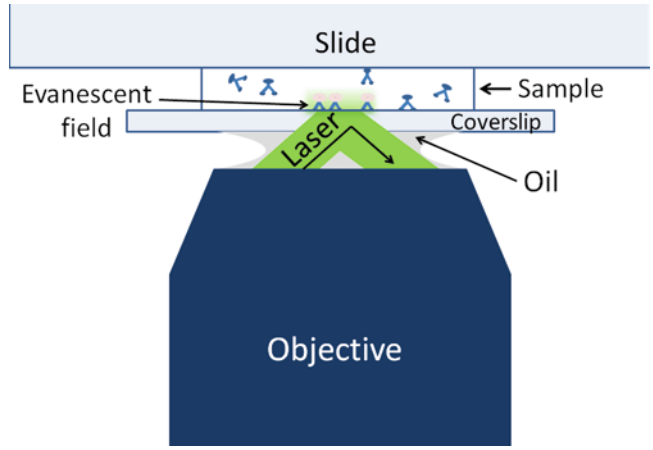

Fig. 2. When the incoming laser is at the correct critical angle for its wavelength, it is totally reflected by at the glass–water interface, and only an evanescent field penetrates into the sample. This evanescent field loses half of its intensity every ~50 nm into the sample, so effectively only fluorophores within ~100 m of the coverslip will be excited. This greatly minimizes background signal and will not photobleach dyes in the bulk of the sample. The oil between the sample chamber and the objective has the same index of refraction as the glass and eliminates refraction of the laser, which would occur if the laser traveled through air.

microscope, which allows the imaging of molecules that are attached to a surface while excluding fluorescence in the solution above the surface. With traditional epifluorescence microscopy, a laser beam is used to excite fluorophores in a sample; however, many fluorophores in solution above or below the focus plane are also excited, leading to high levels of background fluorescence and, therefore, a poor signal-to-noise ratio. Total Internal Reflection (TIR) microscopy solves this problem by sending the laser beam in to the glass–water interface at such a steep angle that TIR is achieved. With TIR, only a thin layer of light (of exponentially decreasing magnitude) called the evanescent wave penetrates into the sample. This evanescent wave excites fluorophores close to the surface of the slide, but will not excite any fluorophores more than about 100 nm away from the surface. Hence, the excellent signal-to-noise ratio and high levels of photon collection required for the FIONA technique are achieved (see Fig. 2). Careful cleaning of sample chambers and efficient surface blocking to avoid nonspecific binding of fluorescent molecules must also be employed to minimize background fluorescence, since impurities on the surface are within the evanescent wave and can decrease the signal-to-noise ratio, even when using TIR microscopy.

Third, sensitive photon detection is required, usually in the form of an electron multiplied charge-coupled device (EMCCD) camera. Back-thinning of EMCCD cameras allows a quantum efficiency of ~90%. In addition, electronic cooling to ~−70°C virtually eliminates dark current, while electron multiplying enables very sensitive detection of photons.

Once a sufficient number of photons can be collected, a researcher can use FIONA data analysis techniques to localize and track the particles in the images that have been recorded. A localization measurement with a standard error of 1–2 nm can be achieved using a laser in the visible spectrum, an effective pixel size of 80–120 nm (see Subheading 3.2), a TIR microscope and an EMCCD camera to minimize background, and a sufficiently bright fluorophore to obtain ~10,000 photons per frame (6). Finally, additional statistical analysis techniques such as Student's *t*-test or Hidden Markov Models can be used to find the steps in the trajectories (if applicable) to determine step size, dwell times, and so forth. These tools allow single-molecule tracking of molecular motors or other proteins in vivo or in vitro excellent spatial and temporal precision.

Although a number of different enzymes can be studied using FIONA, the technique is particularly well suited to the study of molecular motors, which are enzymes that couple ATP hydrolysis into conformational change and motion. Using the techniques described in this chapter, they can be precisely tracked as they move in a cell or in an artificial cell-like environment. For instance, a myosin or kinesin molecule can be tracked as it walks on actin or microtubules inside a living cell, or laid down on a prepared coverslip. The spatial and temporal precision gained by applying the FIONA technique reveals valuable information about the motors, such as the distance traveled with each step, the pause times between steps, or even details such as whether the monomers pass each other with each step (3) – information that cannot be determined using traditional microscopy-based measurements. The study of molecular motors and other types of enzymes can be greatly enriched by the precise localization measurements that FIONA makes possible.

2. Materials

2.1. TIR Setup

TIR microscopes are commercially available from a number of companies (Leica Microsystems, Olympus, Zeiss, and TIRF Technologies). However, for greatest versatility and to minimize cost, a custom-built TIR microscope can be constructed using an inverted microscope on an optical table (see (7)). We list the major components needed here and give a discussion of the major considerations involved in Subheading 3.1.

1. High-gain EMCCD camera: iXon EM+, DV-897E-CS0 (Andor).
2. Inverted microscope: Olympus IX70 or IX71 (Olympus).
3. Laser of desired wavelength (>10 mW power output): 633 nm, He–Ne laser, 35 mW, model #35-LHP-928-249 (Melles Griot) or a 532-nm diode laser, NdYAG, 75 mW, model #GCL-075-L (CrystaLaser).

4. High numerical aperture objective lens: PlanApo 100×, 1.45 NA ∞/0.17 (Olympus) or ApoTIR 100×, 1.49 NA (Nikon).
5. TIR lens: plano-convex lens, AR coated, 30 cm focal length (ThorLabs).
6. Dichroic mirrors and filters: Cy3 – Q565LP dichroic (Chroma) with HQ585/70 M band-pass emission filter (Chroma), Cy5 – Q660LP dichroic (Chroma) with HQ690/90 M band-pass emission filter (Chroma), GFP – Q505LP dichroic (Chroma) with HQ525/50 M band-pass emission filter (Chroma).
7. General purpose optics and mechanics: mirrors, lenses, beam expanders, optical table, etc. (ThorLabs and Newport are good resources).
8. All parts should be set up on an optical table with pneumatically isolated legs: RS4000TM table and I-2000 Lab LegsTM (Newport Corp.).
9. Bead slide to check alignment: prepare a sample with some beads stuck to the surface and some floating in solution. Choose beads that have spectra similar to that of your fluorophore – for instance, if in the experiment Cy3 dye will be imaged, use Nile Red beads (#F-8784, Invitrogen). See Subheading 3.1 for instructions on how to prepare a bead slide. Once prepared, the openings of the sample chamber can be epoxyed over, and the bead sample can be stored at room temperature in the dark for about a month (or until fluid evaporates).

2.2. Slides and Coverslips

1. Glass slides: 30103X1, 0.93–1.05 mm (Gold Seal Microslides).
2. Coverslips: 12-544-A 22X30-1.5 (Fisherbrand). It has the right thickness for 1.45 NA objective lenses.
3. Glass slide holder: 900570 (Wheaton Scientific).
4. Teflon coverslip holder: C-14784 (Invitrogen).
5. 1 M KOH. Store at room temperature.

2.3. PEGylation of Slides and Coverslips

1. Aminosilane: CAS 1760-24-3 (United Chem. Tech.). This should be stored at –20°C and brought to room temperature before opening.
2. mPEG-Succinimidyl Valerate: # MPEG-SVA-5000-1g, MW 5,000 (Laysan Bio, Inc.). Should be stored under nitrogen or argon at –20°C and brought to room temperature before opening.
3. Biotin-PEG-SC: Biotin-PEG-SC-5000-1g, MW 5,000 (Laysan Bio, Inc.,) – see storage note for **item 2** above. Biotin PEG is used if immobilization of avidin-conjugated biomolecules is desired.
4. 1 M KOH. Store at room temperature.

5. 10 mM Sodium bicarbonate buffer (prepared in distilled water). This must be made fresh for each PEGylation preparation.
6. Acetic acid (glacial).

2.4. Sample Chamber

1. Cleaned slides and coverslips (see above).
2. Double-sided permanent adhesive tape.
3. Electric drill and ¾ mm diamond drill bit (Optional).
4. Quick-drying epoxy resin (such as "5-min" variety) (Optional).

2.5. Preparing a Sample Chamber and Acquiring Data

1. Labeled biological molecule of interest (see Note 1).
2. Axonemes (8) (or other molecular "track" appropriate to the specific experiment).
3. 20 mg/mL casein. Prepare stock by dissolving in motility buffer overnight, centrifuging at 245,000 × *g* for 30 min, and filtering through 0.22-μm syringe filters to remove insoluble components. Measure protein concentration and dilute as necessary to give 20 mg/mL final concentration. Flash-freeze in liquid nitrogen and store at −80°C for 1 year (9).
4. BSA: Bovine Serum albumin. Dissolve at 10 mg/mL BSA in motility buffer and filter with a 0.22 μm syringe filter. Store at 4°C for up to 3 months.
5. Mg-ATP: Adenosine 5′-triphosphate magnesium salt. Prepare 100 mM ATP stock in motility buffer. Aliquot and flash-freeze in liquid nitrogen and store at −20°C or −80°C. Use each aliquot only once (do not refreeze).
6. Motility buffer BRB80: 80 mM PIPES, 1 mM EGTA and 1 mM $MgCl_2$. Prepare 5× solution and filter with a 0.22-μm filter. Store at room temperature for a few months. see Note 2.
7. ATP regenerating system: Creatine kinase (#127566, Roche). Prepare 200 U/mL solution in motility buffer. Aliquot and flash-freeze in liquid nitrogen and store at −20°C for up to a year. Use each aliquot only once (do not refreeze). Creatine phosphate (Sodium creatine phosphate dibasic tetrahydrate, #27920, Sigma). Prepare 200 mM solution in motility buffer. Aliquot and flash-freeze in liquid nitrogen and store at −20°C for up to a year. Use each aliquot only once (do not refreeze).
8. Deoxygenation system – PCA and PCD (10): PCA (3,4-Dihydroxybenzoic acid, #37580, Fluka). Prepare 50 mg/mL solution in distilled water, adding 1 M NaOH as needed to dissolve PCA. Adjust pH to 7.4 with NaOH. Aliquot and flash-freeze in liquid nitrogen and store at −20°C for 6 months. Thaw aliquots as needed. Aliquots can be stored at 4°C for about a week. PCD (Protocatechuate 3,4-dioxygenase, #P8279, Sigma). Prepare 5 μM solution in 50% glycerol, 50 mM NaCl, 100 mM Tris, pH 8.3. Store at −20°C for up to several months.

9. DTT: Dissolve 1 M DDT stock in BRB80 buffer, make 10 μL aliquots, and flash-freeze in liquid nitrogen. Store aliquots at −20°C or −80°C for no more than 6 months.
10. Final imaging solution: 2.5 mM PCA, 50 nM PCD, 1 mM DTT, 1 mM MgATP (pH 7.0), 2 U/mL creatine kinase (optional, see Note 11), 2 mM creatine phosphate (optional, see Note 11), 10 mg/mL BSA, desired concentration of labeled molecular motor, all in motility buffer, such as BRB80. This imaging solution CANNOT be made in advance but must be is mixed during the protocol. See Subheading 3.5 step 1 for the proper timing. All the components of this imaging solution (with preparation and storage instructions) are listed above.

2.6. Data Analysis

1. Image viewing and manipulation program such as ImageJ.
2. Software program to fit a 2D Gaussian to each frame of movie: It can be custom written software, or commercial software. Another option is the free program called Video Spot Tracker, which can be downloaded at http://www.cs.unc.edu/Research/nano/cismm/download/spottracker/video_spot_tracker.html.
3. Step analysis program: Student's *t*-test or HMM custom-written, or contact selvin@illinois.edu for programs (11, 12).

3. Methods

See Note 3, applicable to all methods in this chapter.

3.1. Building a TIR Setup

While the details of building and aligning a TIR microscope are beyond the scope of this chapter, we list the essential components in such a setup and highlight the most important considerations involved. See Figs. 3 and 4 for a basic schematic of an objective-type TIR setup. For detailed instructions on how to align a TIR microscope, please see (7).

1. While using a commercial inverted microscope as a starting point is not strictly necessary, it does make the process much easier. We recommend either the Olympus IX70 or IX71, or a Nikon Eclipse TE2000-U.
2. A high numerical aperture objective is critical to getting good TIR images because it allows for the collection of more photons. An objective with 1.4 NA or higher is required to achieve TIR, such as the PlanApo 100× 1.45 NA ∞/0.17 (Olympus) or the ApoTIR 100× (1.49 NA) (Nikon).
3. A light source with which to illuminate the sample is obviously a critical piece of equipment. This must be matched to the absorption spectra of the fluorophore in question. It is best to

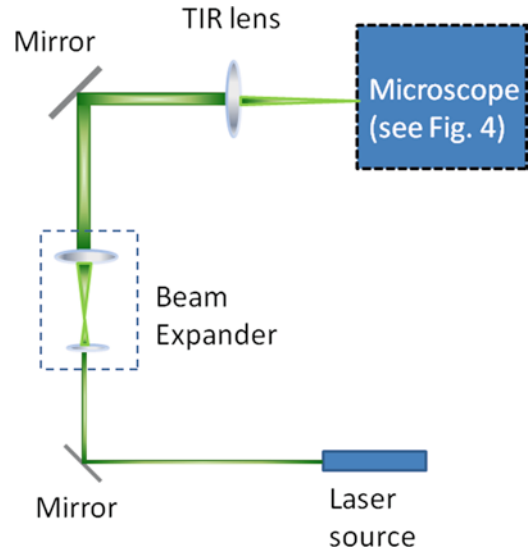

Fig. 3. Schematic of a minimalist TIRF microscopy setup. The laser beam is first expanded and collimated through a beam-expander, then passed through a plano-convex lens, which adjusts the angle with which the beam enters the microscope.

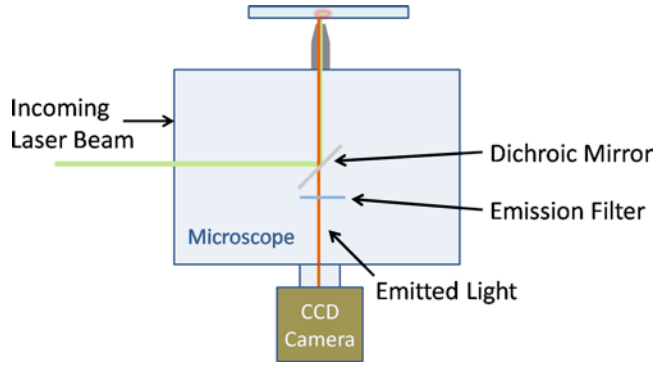

Fig. 4. Schematic of laser path inside the microscope. The incoming excitation laser is depicted (***lighter beam***) as is the outgoing emission signal (***darker beam***). Once inside the microscope, the laser reflects off a dichroic mirror and then through the objective, exciting the fluorophores in the sample. The signal initially travels back down the same path as the laser, but passes through the dichroic mirror chosen to reflect the laser but pass the signal. The signal then passes through an emission filter, which filters out any traces of the laser light that passed through the dichroic. The signal is then sent toward the CCD camera.

use a single mode, TEM_{00} laser. Generally, at least 10 mW of power is required. 532-nm diode lasers and 633-nm He–Ne lasers are perhaps the most common, but the specific type of laser needed will depend on the application. A clean Gaussian laser profile is also an important requirement to consider. See Note 4 for more information on choosing an appropriate laser.

4. A dichroic mirror and emission filter set will be needed inside the microscope. The type of dichroic and filter set

needed will depend on the fluorophores being used. Chroma and Semrock make high-quality filter sets for virtually any fluorophore desired.

5. A highly sensitive camera is absolutely critical for obtaining adequate data for FIONA measurements. Typically, this is a high-gain EMCCD camera, such as the iXon EM + (DV-897E-CS0), Andor. The effective pixel size (once magnification of the objective and microscope are considered) should be 100–150 nm for optimal resolution (6).
6. Other standard optics parts, such as mirrors, lenses, beam expanders, and translation stages will also be needed. At least two mirrors are always required for proper laser alignment to give enough degrees of freedom to get the laser path to be perfectly straight into the microscope. Also, the laser beam generally needs to be expanded about ten times to create a large enough excitation area. This can be achieved using two lenses or by purchasing a commercial beam expander.
7. A large (50 mm diameter) lens of focal length ~30 cm is required to create TIR illumination. The lens must be placed such that the distance between the lens and the back focal plane of the objective equals the focal length of the lens. In other words, the lens focuses the laser down to a point at the back focal plane of the objective, creating a collimated beam that comes out the other end of the objective. We have found 30 cm focal length lenses to be ideal, as it not possible to position the lens much closer than 30 cm from the objective, and because larger focal lengths create smaller illumination areas. Translating the lens in x and y changes the angle that the beam exits the objective (and thus allows for total internal reflection). The lens should be mounted on a 3D micrometer stage to allow for these adjustments. The large diameter of the lens is required because the TIR lens is translated when changing the angle of incidence, meaning the beam (which has been expanded to around 20 mm at this point) will be offset from the center of the lens. Smaller lenses might clip the beam. In addition, the larger diameter decreases the curvature of the lens in the area which the beam passes through, thereby reducing any possible spherical aberrations.
8. After the TIR setup is built and aligned (see Note 5), check the TIR alignment with a bead slide. To prepare a bead slide with carboxylate-modified 0.02-μm Nile Red beads (2% solids), first dilute beads by 1:1,000,000 in distilled water. Pipette 100 μl into each of two 0.5-mL tubes. To one tube, add 1 μL of 0.1 M HCl. Flow the HCl-bead solution through the sample chamber and incubate for 5 min. Then, wash the sample chamber with 100 μL water or buffer. Flow in the second aliquot of beads (without HCl) to provide some beads in solution. Epoxy over

openings if future reuse of bead slide is desired. Place the bead sample on the microscope stage and focus on the surface. Adjust the TIR lens position in either *x* or *y* until the beads on the surface fluoresce brightly, but no beads in solution are excited. Check that the excitation area is centered and has a roughly Gaussian intensity profile.

3.2. Microscope Slide and Coverslip Cleaning

In order to avoid background fluorescence from dust and organic debris, slides and coverslips must be carefully cleaned prior to use. We present a basic protocol for doing so below, but also see Note 6 for additional options.

1. Prerinse a slide holder, Teflon coverslip holder, and glass beaker with distilled water.
2. Place slides in a slide holder and place coverslips in a Teflon holder.
3. Fill slide holder and beaker with acetone. Place the Teflon holder in the beaker.
4. Sonicate for 20 min.
5. Remove Teflon holder from beaker. Carefully pour out acetone from slide holder and beaker and discard.
6. Refill slide holder and beaker with distilled water. Place the Teflon holder in the beaker.
7. Remove Teflon holder from beaker and pour out water from both slide holder and beaker.
8. Repeat **steps 6** and 7 for a total of three rinse steps.
9. Fill slide holder and beaker with 1 M KOH. Place Teflon holder in beaker.
10. Sonicate for 20 min.
11. Remove Teflon holder from beaker. Carefully pour out KOH from slide holder and beaker and discard.
12. Refill slide holder and beaker with distilled water. Place the Teflon holder in the beaker.
13. Remove slides one by one from the slide holder with tweezers, rinsing with copious amounts of distilled water and drying carefully with nitrogen. Repeat with coverslips (see Note 7).
14. Proceed directly to assembling the sample chambers (see below), or store the slides and coverslips carefully covered for a few days before assembling.

3.3. PEGylation of Slides and Coverslips

Some biomolecules are especially "sticky" and require extra blocking to prevent them from binding nonspecifically to the glass surface. In general, a surface blocking agent such as BSA or casein can be used (see Subheading 3.4 for details), but in some cases more rigorous surface passivation is needed. Although much more time-intensive,

PEGylation yields a surface much more resistant to sticking of proteins (13). A mixture of biotinylated PEG and regular PEG can be used to immobilize avidin-conjugated molecules to the surface while blocking nonspecific adsorption of other molecules. If PEGylation is necessary, skip the slide cleaning protocol listed above and proceed straight to the PEGylation technique described below:

1. Clean coverslips in a glass holder by covering with 1 M KOH and sonicating 20 min.
2. Meanwhile, equilibrate aminosilane and PEG – to room temperature.
3. Rinse coverslips at least three times with distilled water.
4. Cover the coverslips with aminosilane solution (see Note 8): 2 mL aminosilane, 5 mL acetic acid (glacial), and 100 mL methanol.
5. Let coverslips sit in aminosilane solution for 20 min, sonicating briefly (1 min) halfway through.
6. Pour off aminosilane solution and rinse with distilled water.
7. Rinse each coverslip with water and dry with nitrogen (see Note 7).
8. Dissolve 80 mg PEG in 320 μL of 10 mM sodium bicarbonate buffer. When using biotinylated PEG, a mixture of mPEG-SV and biotin-PEG-SC should be used. Depending on the application, 10–40% biotin-PEG-SC (by weight) is generally appropriate.
9. Vortex and centrifuge 1 min at 7,200 × *g*.
10. Carefully pipette 50 μL of PEG solution onto the top of one coverslip. Spread out the liquid with pipette tip, then carefully flip over a second coverslip and place it on top of the first to make a "sandwich."
11. Eliminate any air bubbles by tapping with a pipette tip or pressing gently.
12. Repeat with remaining coverslips.
13. Place in sealed container (see Note 9), and place in a dark place protected from light for 3 h.
14. Take each "sandwich" apart and rinse with copious amounts of distilled water, making sure to remember which side was exposed to the PEG solution. Blow dry with nitrogen and place in a container, with PEG-side up.
15. Store at –20°C or –80°C, protected from light, and in a sealed container (face up) until ready to use. Equilibrate to room temperature before opening the container.
16. Proceed to use PEGylated coverslip to build a sample chamber (see Subheading 3.4 below).

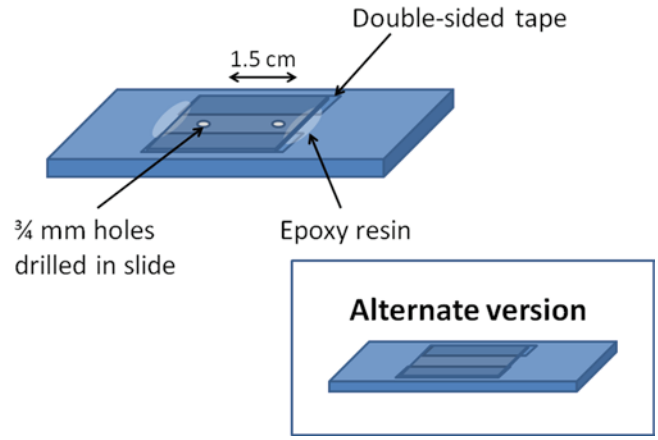

Fig. 5. Schematic of double-sided tape, drilled-hole sample chamber and alternative capillary flow chamber (***inset***). Two holes approximately 1.5 cm apart are drilled halfway between the two long edges of a microscope slide with an electric drill. After cleaning off both the drilled slide and coverslip, the coverslip is attached using double-sided tape. The openings at each end are sealed off with 5-min epoxy. ***Inset***: Alternative chamber with no drilled holes and the openings left unsealed.

3.4. Building the Sample Chamber

1. If pipette holes in slide are desired (see Note 10), use a ¾ mm diamond drill bit to drill two holes about 1.5 cm apart as shown in Fig. 5. Wash the slide with water.
2. Clean the slide and coverslip as described in Subheading 3.2.
3. Place cleaned slide on a kimwipe (clean side up).
4. Cut two pieces of double-sided adhesive tape and place on the long edges of the slide, just above and below the holes. The tape pieces should be a few millimeters apart, leaving a long thin channel of untaped glass in between.
5. Place coverslip on top of tape, cleaned side down (or PEGylated side down, if applicable). Use a pipette tip to gently press the coverslip down, removing any air bubbles between the glass and the tape.
6. Seal the ends of the coverslip using epoxy resin and allow to dry for at least 15 min.
7. Remove excess tape with a razor blade, leaving only the tape beneath the coverslip.
8. The sample chamber volume should be approximately 20 μL. Fluid can be flowed through the chamber by places the pipette tip in one hole and gently expelling the liquid from the pipette through the chamber and out the other hole.

3.5. Preparing the Sample Chamber and Acquiring Data

The specific steps and parameters for this section will vary drastically depending on the biomolecule being studied and the goals of the experiment. Although many of these parameters might be different for other specific applications, we feel the clearest way to

explain this step would be to present a sample experimental protocol here, from which changes can be made as needed for your desired experiment. Below, we present a protocol for a single-molecule tracking of kinesin on axonemes.

1. Get a container of ice to put chemicals in as you are using them at the lab bench.
2. Mix in a small eppendorf 1 μL stock axonemes and 29 μL BRB80. Pipette up and down slowly to mix so that axonemes are not damaged. Flow this mixture into your sample chamber. Wipe off excess fluid that emerges from the opposite end and then store coverslip-side down at 4°C for half an hour so that the axonemes will stick to the surface of the coverslip.
3. (Optional) While you wait for the axonemes, you can do an axoneme affinity purification on the kinesin to select for the active protein. Set centrifuge to 4°C and mix in the tube the following components: 20 μL kinesin (~10 μM stock), 1 μL 100 mM MgATP, 5 μL axonemes, and 24 μL BRB80. Depending on how much of your protein is inactive, the final protein concentration will change after the affinity purification. Also, depending on how concentrated your protein was to start with, this purification might work better if the protein is diluted more with BRB80. Let mixture sit on ice for 10 min. Then spin at 4°C, 15,000 × *g* for 30 min. When it finishes spinning, there should be a visible milky white pellet at the bottom of the tube. This is the axonemes, along with any kinesin that bound to the axonemes but were inactive and, therefore, got stuck rather than walking off the axoneme. Pipette off the top 20 μL, being careful not to disturb the pellet. Proceed through the rest of the sample prep with kinesin taken from this tube.
4. Flow into your sample chamber a 1:10 dilution of 20 mg/mL casein or undiluted 20 mg/mL BSA to block the glass surface that is uncovered by axonemes. Wait for 10 min with coverslip-side down.
5. In a clean eppendorf tube, mix 1 μL kinesin with 1 μL anti-histidine conjugated Quantum dot 655 or other flourophore of your choice (see Note 1). Mix thoroughly by pipetting up and down and then leave on ice for 20 min.
6. Meanwhile, mix final imaging buffer (see Subheading 2.5 **item 11**): 2.5 mM PCA; 50 nM PCD; 1 mM DTT; 1 mM MgATP (pH 7.0); 2 U/mL creatine kinase (optional, see Note 11); 2 mM creatine phosphate (optional, see Note 11); labeled kinesin from step 5. All chemicals mixed in BRB80 with 10 mg/mL BSA.
7. Plug in camera and turn on laser. Wait 5–10 min for laser to stabilize and camera to cool down.

8. Use a bead sample to optimize TIR lens position (thereby changing the TIR angle) for TIR imaging of the plane of the coverslip just inside the sample chamber (see Subheading 3.1 step 8 for how to make a bead slide; see the troubleshooting section in Note 16 if you cannot see the beads after placing a bead sample on the microscope).
9. Adjust ND filters in front of your laser to obtain the desired laser intensity. Generally with a bright dye like Alexa or quantum dots, an ND of 1.5 in front of a 75 mW laser is sufficient. Dyes that are less bright may require a higher laser power – this must be optimized for each set of experimental conditions (see Note 18).
10. Set camera software to acquire 500 frames at 100 ms exposure, with EM gain of 200. (These settings can be adjusted to suit the needs of the experiment).
11. Flow the imaging buffer into the sample chamber and clip the sample chamber securely onto the microscope stage. Turn off room lights and other lights.
12. Unshutter the laser and check that the signal is not saturating the camera (see Note 19). Adjust EM gain, Pre-amp gain, and/or the ND filter in front of the laser so that the brightest part of the image is close to but less than saturation. Move to a new location in the sample with a large number of fluorophores and reshutter laser.
13. Wait for 2 min for the stage to stabilize (see Note 20). When ready, unshutter the laser and quickly start the acquisition on the camera.
14. When the aquisition is done, make sure that file is saved, open it in a program such as ImageJ, and scroll through to see if there is movement. You may need to try a few different areas in the sample before finding a place where you see motility, as it is not uncommon for some of the motors to be inactive. If there is still no motion, see troubleshooting in Note 17.

3.6. Data Analysis

1. To apply FIONA to single-molecule tracking data, one must first select a spot and fit a two-dimensional Gaussian function to the point-spread function of the photons emitted (see Note 12). Using an Andor EMCCD camera, export data in TIF format, which is then easily viewed using the freely available program ImageJ, which can be downloaded at http://rsb.info.nih.gov/ij/. Using this program, select a fluorophore exhibiting motility and the crop the movie images to exclude other fluorophores.
2. Fit a 2D Gaussian function to each frame of the movie. We use a custom program written in IDL for this step, but many other programming platforms can be used. see Note 13 for more options. The center of the 2D Gaussian gives the localization of the fluorophore (once pixels are converted to nanometers), which, along with the frame number (once converted to seconds), provides the trajectory of the fluorophore over time.

3. Use Eq. 1 to determine the uncertainty of each localization measurement: convert the number of counts measured by the camera to photons (this conversion will vary for each camera and the settings used for that camera during this particular movie). The number of photons emitted by the fluorophore for this frame is *N*. Also, measure the standard deviation of the background of the image (an area not including the spot), which is *b*. To determine *a*, calculate the effective pixel size of the detector (actual pixel size divided by magnification). Finally, the width of the Gaussian function (in the *x* or *y* direction) that was used to fit the frame is *sx* or *sy*, respectively.
4. Once the trajectory and fitting error have been found, the step size of the trajectory can be determined (if desired). Several different methods can be used to determine this. Steps can often be seen simply by eye, but it is best to use an analytical method to determine the step locations to avoid error due to human bias. The two most common methods used in our lab are the application of Student's *t*-test (see Fig. 6) or the use of a Hidden Markov model method (HMM). see Note 14 for a discussion about the circumstances in which each of these methods would be more appropriate (11, 12). Contact selvin@illinois.edu to obtain these program packages.

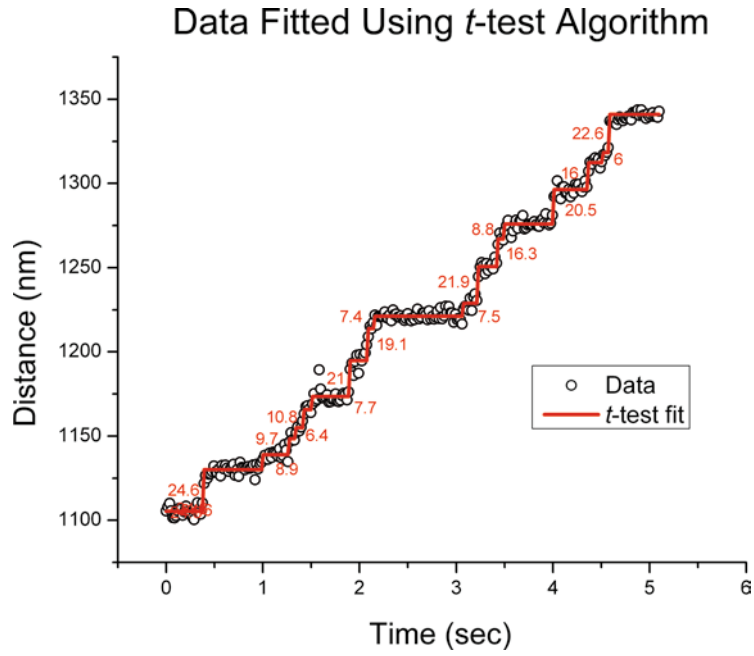

Fig. 6. An example of FIONA stepping data of kinesin motors attached to a fluorescent bead, walking on axonemes in sample chamber. ***Open circles*** represent the center of the Gaussian functions fitted to the PSF of the bead's emission for each frame. The ***solid line*** represents a Student's ***t***-test fit to the trajectory, showing where steps occurred. The average of the step size distribution for the full trace as determined by the ***t***-test fit is 8.7 ±0.5nm.

3.7. In Vivo Considerations

In general, the same FIONA techniques can be used to image motors or other biomolecules inside live cells (see Note 15). This poses extra challenges, however. We list some examples of such challenges and solutions we have used successfully in our lab:

1. Challenge 1: Cells have a huge amount of autofluorescence. This can be reduced by using a fluorophore that can be excited with a red laser. Generally, increases in the excitation wavelength towards the red end of the spectrum result in less autofluorescence. This can also be improved by using cells that tend to be flatter (the smaller thickness means less autofluorescence per surface area). We have used cells such as HEK, HeLa, or Cos7 successfully, for instance.
2. Challenge 2: The areas of interest inside the cell are greater than 300 nm from the coverslip surface, meaning the evanescent wave does not penetrate far enough into the sample. Sometimes (when imaging proteins in the cell membrane, for instance) this can be addressed by adjusting the TIR lens slightly out of TIR so that more of the sample above the surface is excited. The laser should largely be reflected, just not completely. In other cases, the use of TIR is not possible. If the fluorophore is bright enough, achieving a good fit with FIONA may still be possible, but the background will be higher, since the volume above and below the region of interest is also exposed to laser light.
3. Challenge 3: The signal-to-noise ratio is still not high enough, even after following the suggestions in step 1. This can sometimes be solved by attaching multiple dyes to a motor or choosing a better fluorophore (a Qdot, for instance).
4. Challenge 4: In some cases, the photobleaching of the fluorophores can damage the cell that is being imaged due to the formation of reactive oxygen species. Using the smallest laser power possible to achieve the desired results can lessen the photobleaching, as can the use of oxygen scavenging systems. Also, in some cases it is desirable to illuminate the cell in pulses – for instance, taking one frame every 10 s. The laser can be shuttered in between frame acquisitions, thus reducing the amount of time the laser must hit the specimen.
5. Challenge 5: Getting the protein labeled or getting the labeled protein inside the cell. This is a very difficult problem, the solution to which will depend on the type of biomolecule being studied. The easiest solution is to create a fluorescent protein fusion with the protein of interest. However, as discussed earlier, fluorescent proteins are in general not photostable nor bright enough to be practical for FIONA imaging. It is sometimes possible to use multiple fluorescent proteins attached to the molecule of interest to get a brighter signal. For instance, we have successfully imaged large numbers of GFP inside

peroxisomes being transported inside a cell (4). Pigmented organelles such as melonsomes can be imaged in bright-field and successfully analyzed with FIONA (14). Various other strategies have been and continue to be explored to specifically label a biological molecule with a bright fluorophore inside a cell. Attempts to solve the problem of transporting a Q-dot or other fluorophore across the cell membrane include the use of hydrophobic counterions and cell-penetrating peptides (15), endocytosis (16), pinocytosis (17), and microinjection (18). The issue of specific labeling of a protein once the fluorophore has crossed the membrane must also be addressed (unless one is able to successfully transport an already-labeled purified protein in the cell). This can be done using covalent binding of site-specific small molecule probes, for instance (19). Other techniques include ligand–receptor interactions, intein-mediated processes, and enzyme-catalyzed protein modifications (20, 22).

4. Notes

1. Many different fluorophores can be attached to the molecule of interest to track it using FIONA. Qdots or even polystyrene beads are good choices, as they are extremely bright and, therefore, give very good signal-to-noise ratios. Our lab has used a variety of fluorophores for this experiment, two of which are Penta-His Alexa Fluor 532 Conjugate (Qiagen 35330) and Quantum dot 655 conjugated to anti-his with invitrogen's Qdot cconjugation kit (Cat. No. Q22021MP). If Qdots or polystyrene beads are used, make certain to use enough of the fluorophore such that there are more fluorophores than kinesin by at least 2 to 1. This makes it unlikely for multiple kinesin to be attached to the same fluorophore, which is critical if single-molecule traces are desired. Alternatively, organic dyes or even fluorescent proteins can be attached to the molecule using cysteine–maleimide linkages, Halo-tags, His-tags, or other specific labeling methods (or, in the case of fluorescent proteins, by creating a fusion protein). In general, fluorescent proteins do not give sufficient numbers of photons nor have long enough lifetimes before photobleaching to make it feasible to track them using FIONA. If using an organic dye, be certain to choose one that is as bright as possible while still absorbing and emitting at the wavelengths required for the experiment. Dyes that our lab have found especially useful include Cyanine dyes (especially Cy3), Alexa series dyes (Invitrogen), and Atto647 (ATTO-TEC GmbH). An excellent resource for viewing the spectra of dyes and choosing

appropriate filter sets is Invitrogen's Fluorescence Spectra Viewer, found at http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html.

2. Note that for some molecular motors, such as kinesin, the concentration of salt in the buffer can have a significant effect on motility and run length. Thus, it is important to be careful to choose product forms that eliminate any extra sources of salt. For instance, purchase regular PIPES (such as Fluka #80635) rather than PIPES sesquisodium salt. The exact buffer conditions used will, however, greatly depend on the type of biomolecule being studied and the specific demands of the experiment.
3. All methods in this chapter should be done wearing appropriate personal protective equipment – in most cases, nitrile gloves and safety glasses. Additional precautions for some steps are noted below.
4. Prices for a 100 mW laser range from several hundred dollars (USD) for a cheap red diode laser (633 nm) to over 10,000 for a very nice green or blue laser (532 nm or 488 nm). It is not necessary to buy a top-of-the line laser, as long as the laser profile is Gaussian. Cheaper lasers tend to output a larger wavelength range, for example emitting 633 ± 10 nm for a red laser. In most situations, this wider range will not affect your data, but if you are using multiple fluorophores with different absorption/emission spectra and are exciting the wrong ones because of the laser, the problem can be solved by simply placing an appropriate excitation filter in front of the laser to block off the unwanted wavelengths.
5. Laser alignment should be done while wearing laser safety goggles appropriate for the wavelength of the light, and while observing general laser safety practices.
6. Instead of using KOH and sonication to clean slides, plasma cleaning can also be used. We have used both techniques successfully, although plasma cleaning is faster. To use this method, rinse slides and coverslips briefly with isopropanol, dry with nitrogen, and place in plasma cleaner for 5 min under argon plasma.
7. In all steps that involve rinsing a slide (or coverslip), care must be taken to always use tweezers to hold the slide at the bottom edge. Apply rinsing fluid (water, isopropanol, etc.) using a squirt bottle by squirting the liquid as a stream on the top of the slide, allowing the liquid to run down the length of the slide, with the tweezers being the last thing the fluid touches before dripping off. Otherwise, the dirt or residue on the tweezers will flow onto the slide and it will not be left clean. This is also true when blowing dry a slide or coverslip with nitrogen – the tweezers should always be the last thing the liquid touches as it drips or is blown off the slide or coverslip.

8. Aminosilane and acetic acid are both harmful by inhalation, ingestion, or skin absorption. The steps involving these chemicals should be performed in a fume hood and while wearing appropriate gloves and goggles.
9. We prefer to use a plastic box designed to hold 1.5-mL plastic tubes to hold the coverslips during the incubation steps. We squirt distilled water into the bottom of the box so that each hole in the grid is about half full of water. We then place the coverslips on the top of the grid and close the box. This ensures that the box remains humid enough to not dry out the PEG solution as it incubates.
10. In many cases, it is actually more desirable to use capillary action to pull fluid through the sample chamber rather than forcing the liquid through with a pipette. In this case, the slides can be assembled similarly to what is described here, but with slides that do not have any drilled holes, and omitting the epoxy step. Then, rather than flowing the liquid through by placing the pipette tip in the hole, simply expel a small volume of fluid at the edge of the coverslip. As it is pulled through the chamber by capillary action, gradually add more fluid to that end, while applying a kimwipe to the other side to absorb the fluid. We have found this technique to be more reproducible in certain cases, as the rate of flow is not dependent upon the amount of pressure applied to the pipette.
11. Creatine kinase and creatine phosphate together function as an ATP recycling system, which is necessary for sustaining a low, unsaturated ATP concentration. If a low ATP concentration is not required, such as saturated levels of ATP, i.e., >1 mM, omit the creatine kinase and creatine phosphate.
12. The programs used to achieve this can vary depending on the equipment used to acquire images and the desired programming platform. We present the steps of analysis based on the software generally used in our lab; however, other software can be used to achieve the same goals.
13. A free NIH-sponsored program called Video Spot Tracker (which can be downloaded at http://www.cs.unc.edu/Research/nano/cismm/download/spottracker/video_spot_tracker.html) can be used instead of a custom program. Instructions for this program can be found at the same URL. The program provides the x–y position of the molecule being tracked (in pixels) at every frame. If nanometer precision is desired using this program, make sure to reduce the tracking pointer so that it is as small as possible (radius = 1), reduce the precision to the minimum value (0.0001 pixel), and choose the interpolate option. Several commercial programs are also available that

are capable of performing these tasks. Some of these include Andor iQ (Andor Technology), MetaMorph (Universal Imaging Corporation), Image Pro (Media Cybernetics), and 3I's Workbench (Intelligent Imaging Innovations).

14. The Hidden Markov Model technique is very robust and can accurately find steps even with data that is quite noisy. However, it is somewhat model-dependent, requiring the input of some parameters that may not be known a priori. It also tends to be computationally more expensive for long traces. For data with minimal noise, the Student's *t*-test method finds steps accurately but is model-independent. Therefore, for reasonably "clean" data, a Student's *t*-test should be tried first. If the fitting is not satisfactory, the HMM method should then be tried.
15. Working with live cells requires additional safety precautions. Follow the local governmental and institutional biosafety regulations that apply to the type of cells being used.
16. Troubleshooting Problem 1: there is no signal (besides background noise)
    (a) Make certain that you have chosen a dichroic mirror and emission filter appropriate to the emission spectra of the fluorophore and the wavelength of the laser.
    (b) Put a bead calibration slide on the microscope. Do you see a signal with your bead slide? If not, skip to step (f).
    (c) Does your fluorophore emit in the same wavelength range as the beads you used to calibrate? If not, make a bead slide with a more appropriate wavelength of beads and check that you see a signal with those beads (if you see no signal with the new bead slide, skip to step (f)).
    (d) Does turning up the gain on the camera/increasing exposure time/increasing laser intensity fix the problem?
    (e) Does your laser wavelength correspond to the peak (or close to it) of the excitation curve of the fluorophore? If not, your fluorophore will not be efficiently excited by this laser.
    (f) Focus upward into the middle of your chamber. Do you see a lot of fluorophores diffusing in solution? If yes, then the motors did not attach to the surface. This could indicate a problem with the motors or the "track." If the motors are walking on axonemes, look for the axonemes with bright-field illumination to confirm that they are stuck on the coverslip. If not using axonemes, label your track with dye, preferably of different emission spectra from the tag of the motor (e.g., rhodamine on actin or microtubules) and check that the filaments are adhering to

the slide's surface. A different surface attachment scheme may be necessary (perhaps using biotin-conjugated tubulin monomers, for instance).

(g) Check that you are focused on the right plane. If you were using exposure time higher than 100 ms, try decreasing exposure time to ~100 ms and changing the focus to find the correct plane again. If exposure is too long it is easy to completely miss the correct plane while scanning for it.

(h) Do you see some laser light coming out of the objective at all? If not, the laser is misaligned. See step (i).

(i) The laser may be misaligned. Check the laser path going backward from the microscope to the laser itself. Does it look like the beam is clipped somewhere, or strangely shaped? This indicates that the laser needs realignment.

17. Troubleshooting Problem 2: The motors are not walking

(a) First, try a bulk assay such as a gliding assay, to make certain the motor proteins are still active. Over time (even when stored at −80°C) the proteins can degrade and lose activity. A fresh protein prep may be required.

(b) Check that all chemicals are fresh and that the pH of the motility buffer is appropriate for the protein (generally around pH 7).

(c) Make sure that the salt concentration is appropriate for the motor. Some motors are quite sensitive to salt concentrations – for instance, full-length kinesin requires a sufficient level of salt to avoid folding over, which prohibits motility.

(d) Check that there is no nonspecific binding of the proteins to the glass surface. If you have fluorescently labeled the "tracks" (which we recommend) or are using axonemes (which are large enough to with bright-field illumination), check that the motors colocalize with the tracks. If a significant number do not, you will need to take steps to prevent this nonspecific adsorption onto the surface. This can be achieved using PEGylation or other coating techniques.

18. Troubleshooting Problem 3: The dyes photobleach too quickly

(a) First try decreasing the laser power and increasing the gain of the camera. The higher the laser power, the more quickly the dyes will photobleach. Always use the smallest laser intensity possible to obtain sufficient signal-to-noise ratios.

(b) Make sure that you are using an efficient deoxygenation system (see Subheading 3.5). Also, the emission of some dyes

can be improved by including reducing agents (for instance, Cy5 is greatly enhanced by including Trolox (21)).

(c) Be sure that the motors are not simply detaching from the tracks (which sometimes can be confused with dye photobleaching). Adjusting salt concentrations can often affect run lengths of motors. With very small run lengths, the motors may detach from the track too frequently to obtain adequate traces.

(d) Try a different dye. Some fluorophores, especially fluorescent proteins, simply do not last long enough to yield satisfactory FIONA data.

19. Troubleshooting Problem 4: There is too much background fluorescence

(a) Make sure that the slides and coverslips are carefully cleaned according to the directions in Subheading 3.2.

(b) Check that you are imaging the correct surface. It is sometimes possible to focus on the bottom of the glass slide rather than the top of the coverslip. Check that this is not occurring. One trick that is useful is to translate the sample chamber until the double-sided tape is over the objective. Focus on the bottom edge of the tape and then move the chamber back into position to image the sample.

(c) Fluorophores could be binding nonspecifically to the surface (see step (d) of Note 17 above). This can be reduced by changing the surface blocking technique – using PEG coverslips, for instance.

(d) Check that the immersion oil on the objective is not dirty and that there are no air bubbles in the oil. If in doubt, clean the oil off the objective and apply new.

20. Troubleshooting Problem 5: Drift

(a) It is normal to see drift up to 1 nm/s on a microscope. Check to see if the measured location of a stationary fluorophore drifts only in one direction or randomly oscillates in all directions. Drift in one direction can be minimized by upgrading stage (to a micrometer controlled precision stage), clamping down microscope (for example, by removing the legs of the microscope and custom-fitting legs that can be clamped down onto the optics table as is done with optics table posts), or simply waiting longer before taking data for the stage to stabilize after moving it. Random oscillations can be due to an unstable laser (long path lengths traveled by laser before going into microscope, thus magnifying the instability) or air currents in the room, which can be minimized by putting a box around the setup.

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