2010

Chapter 26 – Multiple Color Single Molecule TIRF Imaging and Tracking of MAPs and Motors

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Multiple color single molecule TIRF imaging and tracking of MAPs and motors

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Running Title: Multi-color single molecule TIRF imaging

Abstract:

Microtubules are part of a complex mechano-chemical network inside cells. In order to understand how the components of these systems work together, careful *in vitro* experiments must be performed with added complexity. These experiments can ideally image all the interacting species. In order to image these molecules, multiple-color fluorescence imaging can be performed. In this chapter, we describe some methods for performing multiple-color single molecule fluorescence imaging using total internal reflection fluorescence microscopy. We give several specific examples of species of microtubule-associate proteins and motors that can be examined with detailed protocols for labeling, purification, and imaging.

I. Introduction

Within the last decade, the ability to visualize and quantify the actions of single proteins has given researchers a novel view into the working lives of proteins. This has been especially true for enzymatic motors, which tend to translocate on their linear substrates. For microtubules, many molecular motor species have been studied in this way (Dixit et al., 2008; Reck-Peterson et al., 2006; Ross et al., 2006; Vale et al., 1996; Yildiz et al., 2004). These breakthrough studies have revealed details about motors such as step size, back steps, and stepping style. The strength of single molecule studies is the ability to determine the entire distribution of actions that a type of protein can perform – instead of only obtaining the average value for many molecules. The distributions can reveal rare events and non-normal distributions (e.g., bimodal distributions). In addition, single molecule fluorescence experiments can probe multiple, differentially labeled species simultaneously, which many bulk studies cannot (Dixit et al., 2008). This makes single molecule fluorescence studies extremely powerful for looking at interactions between proteins in more and more complex systems *in vitro*.

In order to see single molecules, most use total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy was first used to study microtubule motors in 1996 to study single kinesin motors walking along a microtubule (Vale et al., 1996). The brilliance of TIRF is that it uses the evanescent wave of decaying photons at the glass-water interface to illuminate the fluorophores (Figure 1). This results in illumination only very close to the cover glass surface, about a 100 nm within the sample (Axelrod et al., 1984). Only fluorescent molecules at this surface will absorb photon energy to become excited and ultimately emit a photon for detection. This ability greatly increases signal to noise that allows for detection of single fluorescent molecules even though many fluorophores may be in the sample. For microtubule-motor systems, the microtubules are usually affixed to the cover glass, and are therefore within the

evanescent field. Motor proteins are freely diffusing in a 3-dimensional volume and only those that bind to the microtubules are illuminated and imaged.

Single molecule TIRF imaging can easily be modified to allow multiple color imaging. Simultaneous imaging of multiple species can allow interactions between these molecules to be imaged and quantified. For instance, binding affinities can be measured, but also specific localization (e.g., the microtubule plus-tip) and cooperative binding can be assessed through direct visualization. Motion and molecular complex lifetimes can be measured with these methods. Moreover, a visual stop-flow experiment can be performed by flowing in and exchanging buffers in the chamber as the system is imaged.

II. TIRF optics

A. Commercial TIRFs

The popularity of single molecule imaging has led to multiple commercial options for purchasing a TIRF illumination system. Most major microscope companies sell a TIRF system and claim single molecule abilities. For single molecule applications, a laser needs to be coupled to the TIRF system via a fiber optic. Some models have separate input fibers for each color. Others have a single fiber for all colors. For multiple colors lasers, one can purchase a laser sled that utilizes an acousto-optical modulator (AOM) to pick the wavelength that is fed into the fiber optic. The AOM can also enable fast switching between colors for almost simultaneous imaging. Typically, the commercial vendors will have a computer interface for controlling the laser sled, which your microscope vendor should install properly for you. Otherwise a dual-wavelength filter system can be utilized to image two colors simultaneously on the same camera or different cameras, and the lasers can be controlled with relatively fast shutters.

B. Home-built TIRFs

Although many groups that are new to single molecule assays may wish to purchase the TIRF system, these systems can be less versatile than a home-built system. In a home-built TIRF, the optics are quite easy to set up around a commercial microscope body using either a prism or through the objective. With the prism, you can get higher intensity light through to the sample, since there is less glass in the light path. In addition, some groups use quartz prisms, which require quartz slides to image through, which can be expensive, and must be washed for re-use. Objective TIRF has become quite popular recently, since it is relatively easy to build around a conventional microscope body. In addition, TIRF objectives are commercially available from major microscope vendors and they use typical glass cover slips. On the down side, you lose light intensity and typically image with the same objective you use to illuminate the sample. The objectives for TIRF are oil-coupled to the cover glass, which needs to be a number 1.5, which is approximately 0.17 mm in thickness. They come in 60x or 100x with correction collars for temperature. Their most important feature is their numerical aperture, which must be higher than 1.38 for TIRF illumination from glass to water (see Figure 1). Most are NA 1.45 or NA 1.49 with glass objectives. An objective with NA 1.65 is available, but it is composed of sapphire, and needs special cover slips. These high NA objectives are also very good for obtaining a large solid angle of emission light, allowing much of the fluorescence to be captured to the camera.

For building a home-built TIRF system, the most important rules are 1) focus the laser light on a back focal plane conjugate and 2) place the steering mirror at a front focal plane conjugate. The first allows the laser to come out of the objective relatively collimated. The second allows the steering mirror to change the angle without changing the location of the illumination spot in the sample. Figure 2 shows a very simple diagram of a TIRF system. For multiple colors, the beams from each laser can be combined into one path using dichroic filters (Figure 3). Or, if a high power laser with multiple wavelengths (e.g., Argon-Krypton) is being used, a prism can be used to disperse the wavelengths. An achromatic spatial filter will help

clean up the laser shape and intensity smoothness as well as expand the beam, if desired. An AOM can be added for fast switching of wavelengths.

Other variations of home-built TIRF systems exist. For instance, one does not have to build the system around a commercial microscope stand, but could build the entire system from scratch. Each has pros and cons. For instance, commercial systems typically have nice integrated focus-correction systems that allow for stable long-term imaging, which may be nicer. Building these focus-drift correction systems can be long and arduous. On the other hand, stands made from bricks are often more stable and may need less focus-drift correction.

In order to perform TIRF, one must first pass the laser light through the microscope in epi-illumination. This will be a daunting task if you do not use a second light source. On a TIRF built around a commercial microscope, this is very easy because the fluorescence and transmitted light paths point in opposite directions. Thus, by turning on the transmitted light through the condenser, you will be able to see the light path on which to place your laser. In addition, the condenser can help you identify conjugate front and back focal planes that will help you place the focusing lens and steering mirror. For this, your condenser must be in Köhler illumination so that the slightly closed iris is visible and centered in the sample/focal plane. Making sure that the dichroic for TIRF is in place, with the transmitted light intensity maximized, the location of the iris in focus will denote the position to place the steering mirror. The center of the iris is the center-line of the light path, and this is where the laser should go.

Before attempting to pass the laser light into the microscope, lower the power to <1 mW. This will enable visualization of both laser and transmitted lights simultaneously, limit accidental stray laser beams, and keep the laser from damaging your microscope objective. Using the transmitted light, you can adjust the laser mirrors to align the laser through the objective (Figure 3). Perform this alignment for only one laser, which is your reference laser. Typically, this should be the laser that is farthest from the microscope. In order to align the laser on the center-line,

you only need to adjust two mirrors: the steering mirror (SM) and the first mirror after combining the wavelengths (see Figure 3, CM1). First, place a paper on the side of the steering mirror closest to the focusing lens (Figure 3) to visualize the laser and transmitted light at the same time. Center the laser light on the iris image perfectly on top of each other by adjusting the knobs on mirror CM1. Once that is done, place the paper on the side of mirror CM1 closest to the microscope to visualize the transmitted light and laser light at the same time. Center the transmitted light (it may look like the iris still, or it may look like the filament or other back focal plane image) on the laser light by adjusting the knobs of the steering mirror (SM). Repeat this process of adjusting the CM1 and SM mirrors one at a time until the transmitted light and laser light are perfectly on top of one another. If the condenser is moved away, the laser light should now be visible to come straight out of the microscope in a collimated beam. The final adjustments to make the laser come perfectly straight out of the objective can be performed by adjusting the steering mirror. Using a plumb bob (plummet weight) to find the exact location that the light should hit the ceiling if coming out perfectly will help. It should be noted that a recent protocols manuscript describes a similar procedure for aligning lasers into a microscope for direct illumination of samples (as opposed to oblique illumination used for TIRF) (Gould et al., 2009).

Once the TIRF system is working for one laser, the second laser (and dichroic cube inside the microscope, if using different ones, see below) can be put in place for alignment. The second, third, fourth lasers, should be aligned as much as possible on top of the reference laser using the mirrors that adjust only the direction of that laser – not the mirrors where the lasers are combined. Usually this will include adjusting some aligning mirrors (AM, Figure 3), the laser height or position, and the dichroic that is combining the lasers. Once the second laser is on top of the reference laser, it should point directly out of the microscope just like the first. If everything is the same in the path between the laser combination and the microscope, but the

second laser is coming out of the microscope at an angle, it is likely that your dichroic cube is in a different position than the one for the reference laser. Special dichroic cube holders where the angle of the dichroic can be adjusted can be used to fix this problem, although it is arduous. It is recommended to use a multiple wavelength cube so that the same dichroic can be used for multiple colors (see below).

C. Filters and dichroics

Some of the best advances for multi-color fluorescence imaging have come in filter sets that allow multiple types of fluorophores to be imaged with the same set. For multiple-colors, one can use different filters in the turret. Some commercial microscopes can rapidly turn the turret (in the msec realm), if fast imaging is needed, but most are very slow (1-2 sec). This can slow down the imaging of multiple colors. In order to illuminate with multiple colors simultaneously, there are now commercially available dichroics that are notched to allow for a variety of excitation wavelengths to be reflected up to the sample while allowing emission wavelengths through. If using one of these filters, a second, fast filter changer just before the camera(s) will allow the correct wavelength to be picked. Or, a dual-wavelength filter system could be used for simultaneous imaging on one or multiple cameras. The dual-wavelength system can be purchased from a commercial vendor, or constructed from a dichroic filter and two excitation filters just before the camera(s).

Another particularly ingenious variation on a home-built TIRF is to use a specially engineered optics with two small, mirrored surfaces at the edges. By bouncing the lasers off this mirrored surface, all the light goes into the sample and none is lost at the dichroic (Friedman et al., 2006). Since the mirrored surface is rather thin, very little excitation light is lost to the reflecting area during collection compared to the dichroic. Since the reflecting surfaces reflect all wavelengths, the cube does not have to be changed ever – only the excitation filters in front of the camera, allowing for particularly easy two-color fluorescence excitation and imaging.

D. Cameras and pixel size

The cameras themselves are a concern for single molecule imaging. The current camera of choice is an electron multiplier charged coupled device (EM-CCD) camera. There are a couple of brands that are most used that have different options, but all cost between $35,000-$40,000 each. Most people use 512x512 size chip, although 1024x1024 are available. The pixel sizes of these chips are different, so make sure you demonstrate them both to determine what is best for your application.

Most important when imaging single molecules is making sure you are expanding the diffraction-limited spot over two pixels for optimal localization (Gould et al., 2009; Thompson et al., 2002). Typically expanding your image so that each pixel is 80-100 nm will give enough resolution to fit the spot with a two-dimensional Gaussian to tracking and high resolution localization. Beam expansion can be done using a two-lens beam expander/telescope. This is perhaps the simplest of all optical systems where the two lenses are placed apart by a distance equal to the sum of the focal lengths (Figure 3). The expansion power is given by the ratio of the focal lengths of the two lenses.

For image expansion, the first lens with a shorter focal length must be placed so that the lens is one focal length from the location of image formation coming out of the microscope. This should be estimated as well as possible. After the lens is correctly placed, the image light will be collimated. The second lens with a longer focal length must be placed so that the focal length lands perfectly on the CCD to create an image. This can be determined empirically by placing the lens in front of the CCD and moving it toward and away from the camera while imaging a dim object over ten feet away (effectively at infinity). When the far away object is perfectly in focus, the lens is correctly placed. The distance between the two lenses is variable because the light is collimated in that space, so it should be set to whatever works well for your system.

III. Labeling molecules

In order to image molecules of various species in two-color fluorescence, they must be labeled with fluorescent molecules. For *in vitro* microtubule systems, it is good to label the tubulin that composes the microtubule (around 5% labeled dimers). Tubulin can be labeled in house (there are several good methods articles about this (Desai and Mitchison, 1998; Wadsworth and Salmon, 1986)) or purchased from a commercial vendor (Cytoskeleton, Denver CO), although the options for fluorophores are quite limited). Most motor proteins and microtubule-associated proteins (MAPs) can be expressed in bacteria or insect cell culture. These proteins can be labeled with genetically encoded fluorescent proteins, such as green fluorescent proteins (GFP), and its analogs. Further, small molecule fluorophores can be used to label the proteins either randomly on the surface or specifically to sites built into the protein sequence (e.g., Cysteine-lite mutants). We used a cysteine-labeling kit (Molecular Probes/Invitrogen) to label single tau molecules with Alexa546 (Dixit et al., 2008). Finally, if the protein can be biotinylated – either specifically or randomly – the molecule can have a streptavidin-conjugated quantum dot bound (Ali et al., 2008). Quantum dots work well for motors as cargo substitutes, but for other MAPs, you run the risk of having a relatively large (5-10 nm) quantum dot interfere with binding. Since many of these procedures are published, we will defer further elaboration in general, and give a couple examples below.

IV. Examples and protocols

There are numerous examples of single molecule work in the literature (Ali et al., 2008; Dixit et al., 2008; Reck-Peterson et al., 2006; Ross et al., 2006; Vale et al., 1996; Yildiz et al., 2004). Only recently have two color techniques become more common to study the interactions between multiple molecular species *in vitro*. In this section, we detail some protocols for two-

color single molecule imaging. Also, please see the chapter in this volume by Dixit and Ross for a fourth application of two-color imaging *in vitro*.

A. Flow chambers

All *in vitro* samples are made in some sort of flow chamber. These typically consist of one slide and one cover slip or two cover slips assembled together in a sandwich separated by two pieces of double-sided permanent tape. The cover glass through which you are imaging needs to be very clean, and there are several acceptable methods to do this. One can clean with treatments of acids and/or bases, one can wash with detergents, or one can plasma clean – much like you would to clean electron microscopy sample grids. Different labs use different procedures and most are equivalent. There is one particular procedure outlined in the chapter of this volume by Dixit and Ross.

In addition to cleaning the glass, there are several methods to coat the glass to enable specific binding or repulsion of biological molecules. These techniques are covered in other locations (Bieling et al., 2007; Bieling et al., 2008; Brouhard et al., 2008; Dixit et al., 2008; Dixit et al., 2009; Helenius et al., 2006; Roll-Mecak and Vale, 2005; White et al., 2007), including this volume, so we will only cover the specific examples below. In general, for microtubule-based assays, it is important to specifically bind the microtubules to the surface to keep them still while imaging. This can be done with antibodies, biotin-streptavidin, or even non-motile motors, depending on your application. In many applications, biotin-streptavidin is preferred because it is very strong (K_D value ~ 10^{-15} M), and you can flow multiple times over the microtubules without them lifting from the surface. On the down side, the biotin-streptavidin linker may be too strong or two short for specific applications. In particular, if the microtubule needs to be propped-up off the cover slip surface, it may be better to use a non-motile kinesin or antibody.

B. Daily TIRF management

Everyday, the TIRF system should be double checked for proper alignment and illumination. It is recommended to do this on a sample of fluorescent beads that have multiple fluorophore types on each bead (e.g., Tetraspeck beads from Invitrogen). A chamber with these beads can be imaged in epi-fluorescence to check focus first. Make sure you are focused on the correct surface (e.g., the bottom cover slip if using objective TIRF). Finding an area with a few beads stuck to the cover slip will allow you to check that the TIRF is working. Illuminate the beads with each laser in TIRF and adjust the angle to optimize the fluorescence intensity while maintaining total internal reflection. If the alignment has changed drastically on your home-built system, repeat the alignment of the steering and combination mirrors outlined above. Center the illumination of each laser. If your lasers are coupled at some point, but the illumination area is different between two wavelengths, make sure you are adjusting the mirrors in the area where only the single wavelength is affected. These adjustments should be able to be performed on either a home-built or commercial TIRF system. Once the TIRF is set-up for the wavelengths you desire, take an image of your beads in each illumination color. This will allow you to correlate your images between the two colors. If you are using a dual-wavelength imaging system, use it to take simultaneous images of the beads to correlate the locations on the camera(s).

If imaging fluorescence intensity is important, the fluorescence on the CCD can be calibrated by imaging a constant light source to calibrate the shot noise of the camera. This technique has been described several times before with the theoretical justifications for the measurement (Gould et al., 2009; Thompson et al., 2002). As a practical method, all one has to do is measure the average number of counts (gray values, <C>) and the standard deviation of those counts (δC) over time for each pixel. These two values are related by a coefficient, β, such that: $\delta C^2 = \beta b \langle C \rangle$. This constant, βb, is the same constant that relates the counts on the camera, C, to the number of detected photons, N_{det}. In this method, the exposure, EM gain, and

light must be noted and constant throughout the imaging. By taking 10,000 frames, the statistics should be quite good to determine the value of β□. This value should be constant for your camera's CCD chip, so this calibration should not need to be repeated daily.

C. Dynein-dynactin on microtubules

In this section, we will describe in detail techniques for imaging single motor proteins labeled with GFP on single rhodamine-labeled microtubules as described in (Ross et al., 2006).

i. Purifying fluorescent dynein-dynactin from mouse brains.

Here, we detail the procedure to purify dynein-dynactin-GFP from mouse brains as described in (Ross et al., 2006). In order to purify and use a fluorescently labeled dynein-dynactin complex, the Holzbaur Lab created a genetically modified mouse that expressed a GFP-labeled dynamitin (p50) subunit of dynactin (Ross et al., 2006). Figure 4 shows a schematic of the protocol to purify the dynein-dynactin from mouse brains. The mice brains are harvested fresh from anesthetized mice by cutting through the spinal chord at the neck and then using scissors to cut through the back of the thin skull. Small tweezers are used to remove the brain and place it into a chilled glass Dounce homogenizer. For each brain, 400 μl of chilled vesicle-stabilizing buffer (10 mM HEPES-KOH, 175 mM L-aspartic acid, 65 mM Taurine, 85 mM Betaine, 25 mM glycine, 6.5 mM $MgCl_2$, 5 mM EGTA, 0.5 mM D+glucose, 1.5 mM $CaCl_2$) with protease inhibitors (200 μM PMSF, 20 μg/ml Leupeptin, 1 μg/ml Pepstatin-A, 2 mM DTT, and 20 μg/ml TAME) are used. After all the brains and buffer are in the glass dounce, the teflon plug is rotated by a motorized homogenizer and passed back and forth three times (typically, 4 brains are sufficient for the preparation). Homogenized brains are transferred to 1.5 ml tubes and centrifuged at 30,000 g for 30 min at 4°C. After this low speed spin, the supernatant is transferred to micro-ultra centrifuge tubes and centrifuged at 100,000 g for 30 min at 4°C. The high-speed supernatant has the soluble protein used for the rest of the protocol. The high-speed

pellet has the membrane-bound proteins used for the vesicle preparation below. Gel samples are taken of the supernatant and pellet for later analysis.

To separate the dynein-dynactin from the rest of the free proteins in the supernatant, we use a microtubule affinity step. First, dynein-dynactin is tightly bound to microtubules in rigor in the apo state by depleting ATP with hexokinase (0.1 unit/ml of hexokinase is used with 1 mg/ml glucose). In addition, 20 μM taxol is used to polymerize microtubules in the supernatant to which the dynein-dynactin will bind. For mouse brains, which are small, a small amount of exogenous microtubules can be added to the supernatant, about 40 μl of 5 mg/ml stock per 250 μl. The supernatant is incubated with the microtubules at 37°C for 15 min and then at room temperature for an additional 15 min. The supernatant with microtubules are centrifuged in a micro ultra centrifuge for 40 min at 66,000 g at 25°C. After this step, the dynein-dynactin should be in the pellet with the microtubules. Gel samples of the supernatant and pellet should be taken to confirm that dynein-dynactin is enriched in the pellet. The microtubule pellet with dynein-dynactin should is resuspended in PEM-100 (100 mM K-Pipes, 1 mM EGTA, 1 mM MgSO4, 6.8 pH) with 0.5 mM DTT and 10 μM taxol. The microtubule pellet is gently homogenized with a pipette tip and then passed through a Hamilton syringe 2-3 times to break up any large chunks. Add ATP to 20 mM to release the dynein-dynactin from the microtubules. The microtubules, dynein-dynactin, and ATP are incubated 30 min at room temperature. Microtubules are centrifuged at 82,000 xg for 30 min at 25°C. After this second spin, the dynein-dynactin should be in the supernatant and the pellet should have the microtubules and dynein-dynactin that is incapable of releasing with ATP.

The supernatant is layered on top of a sucrose gradient in a thin-walled plastic SW41 centrifuge tube (or equivalent). Sucrose gradients are made from 5% sucrose and 25% sucrose in PEM-100 buffer with protease inhibitors, as above. To make sucrose gradients, the gradient maker is first cleaned with ddH_20 at least four times with all the valves open and the stir bar

spinning in the chamber closest to the outlet valve. After the last cleaning, some air is allowed to leave the machine and go into the tubing as a small bubble. This bubble marks the start of sucrose. The valves are closed between the two chambers and the outlet value. We place 6.5 ml of 5% sucrose in the chamber farthest from the outlet. We place 6.5 ml of 25% sucrose in the chamber closest to the outlet. The outlet valve is opened to allow through the 25% sucrose into the tubing and out to the centrifuge tube. After the 25% sucrose has flowed for approximately five seconds, the valve between the two chambers is opened. The stir bar mixes the 5% into the 25% to create a linear gradient. The gradient is flowed into the centrifuge tube through a small plastic tube with a bent needle on the end to hook it onto the centrifuge tube. The flow is continued until there is only about 1 ml of space left at the top of the gradient. Although only one gradient is needed, at least two are made so that one can balance the other. The gradients are placed into a swinging bucket rotor and use all the buckets during centrifugation – even if the extra buckets are empty. The gradients are centrifuged at 126,000 g for 16 hr at 4°C.

After 16 hr, the gradients are removed and carefully placed on ice without agitation. Using a 27 gauge needle, the bottom of the thin-walled centrifuge tube is punctured and drops are collected into 1.5 ml tubes. We collected 1 ml fractions and made gel samples from each fraction for a diagnostic gel. The dynein-dynactin typically comes out in the same fractions around 5, 6, and 7. Although an SDS-PAGE gel should be run, the quickest test for active dynein-dynactin enriched fractions is a microtubule-gliding assay. For GFP-labeled dynein-dynactin, the identification is made even easier by inspecting the green fluorescence channel. Microtubule gliding assays are performed as follows: a chamber is made with a regular slide and cover slip and permanent double stick tape to define the flow path. First, anti-dynein intermediate chain solution [0.1 mg/ml antibody to dynein intermediate chain 74.1 (Chemicon), 10 μM taxol, 1 mg/ml BSA in Motility Assay Buffer (10 mM Na-PIPES, 50 mM Potassium Acetate, 5 mM MgSO4, 1 mM EGTA, pH to 7.0)] is flowed into the chamber. The first solution is

incubated in the chamber for 5 min to allow the antibody to bind to the cover slip surface. The second solution is a BSA wash (1 mg/ml BSA and 10 μM taxol in Motility Assay Buffer). The BSA wash is incubated in the chamber for 5 min to allow the BSA to bind to the open areas on the cover slip. Next the fraction to be tested is flowed into the chamber and allowed to incubate for 5 min. Typically, the concentration is low, so addition of the fraction can be repeated 2-3 times to enhance binding to the antibody. Next, 50 μg/ml rhodamine-labeled microtubules, which can be polarity marked, are added to the chamber and allowed to incubate for 1 min. Finally, the activation mix is added to the chamber, which contains 1 mM ATP, 10 μM taxol, 36 μg/ml catalase and 220 μg/ml glucose oxidase, 4.5 mg/ml glucose in Motility Assay Buffer. The chamber is imaged immediately in the red channel and a five min movie is taken to determine if the motors are active. Intervals between frames are 2-5 sec and a shutter should be used to stop illumination while the camera is not imaging. Fluorescence images from the green channel will inform about the amount of GFP-labeled dynactin is in the fraction.

ii. **Single molecule motility experiments.**

To perform single molecule experiments, we first make a flow chamber with permanent double-stick tape to for a flow path. The cover glass should be cleaned, if needed, by sonication, plasma discharge, or other means, which have been worked out previously. First microtubules need to be bound to the surface. Depending on the glass and cleaning procedures, microtubules will sometimes stick directly to the cover glass. If not, charged polymers, such as DEAE-dextran or poly-lysine can be used to bind microtubules to the cover glass via their highly negatively charged carboxy-terminal tails of tubulin. After microtubules (50 μg/ml stabilized with 20 μM taxol) are added, the chamber incubates about 5 min to allow the microtubules to bind. Then the chamber is washed with a blocking solution. Since we have found that dynein can stick to BSA, we opt to use casein to block the exposed surfaces and prevent further binding of GFP-labeled molecules. The casein block solution (5 mg/ml casein in motility assay buffer with 20 μM

taxol) is washed through and helps to remove unbound microtubules in solution. Before motors are flowed in, the chamber needs to be checked in epi-fluorescence to make sure that the amount of microtubules on the surface is adequate and that the microtubules are stuck down well. If there are too many microtubules, bundling of microtubules will occur and you will not be able to tell on which microtubules the complexes are walking. If there are too few microtubules, it will be difficult to find locations to image. If the microtubules are not bound well to the surface, they will diffuse out of the evanescent field of the TIRF system, and any single molecules binding or moving in that area will not be visible.

After a chamber with a good number of microtubules that are well adhered is made, the fluorescent complexes can be added. The motility mix has motors at some known dilution, 1 mM ATP, 36 µg/ml catalase and 220 µg/ml glucose oxidase, 4.5 mg/ml glucose into casein wash. It is best to set up the TIRF ahead of time with a bead sample, as described above. Very little alignment or adjustment should be needed when the first sample goes onto the microscope stage. Rhodamine-labeled microtubules can be imaged in TIRF using the 532 nm laser or in epi-fluorescence, since there should be no rhodamine microtubules in solution to give a high background. Typically, an image of the microtubules can be taken with epi-fluorescence before and after a fast movie of dynein-dynactin motility is captured. The amount of complexes on the microtubule is important. It cannot be too many, because they will get too close and be impossible to distinguish. If there are too few, it will take a very long time see an event when a single molecule binds and is motile. The amount of motors can be adjusted by changing the amount of motors added into the motility mix.

For true two-color in this system, a dual-wavelength imaging system is recommended. The rhodamine microtubules can be excited with 488 nm laser light, and then the emission wavelengths for GFP and rhodamine can be split before the image is put onto the CCD. Fast

movies can be captured continuously to the computer at 10-30 frames per second. If the motility is slower, longer exposure times or even shuttering can be used to enable long movies.

iii. **Single molecule motility analysis.**

For analysis, the fastest method is to make kymographs (space-time plots) from the movies (Figure 5B). The region of interest should be chosen from the image of the microtubules. The same location can be found in the green channel images using the alignment correlation based on the images of multi-fluorophore coated beads taken during the daily alignment. In ImageJ, there is a kymograph plug-in available written by J. Reitdorf and A. Seitz. The plug-in makes kymographs from stacks of movies after a segmented line selection is created. It can create several kymographs from a single movie simultaneously. From the kymograph images, the velocity and run length and run time can be deduced by measuring the number of pixels a single complex moves in space and time. In these kymographs, the vertical direction is time advancing forward as you go downward. Using the time stamp per frame and the size of a known pixel the actual duration, length, and velocity of any motion can be deduced.

Another method to analyze the motion of single fluorescent complex is to track and fit the intensity profile to a two-dimensional Gaussian. This is very popular method because the fitting allows for better localization than the diffraction limit usually allows. The goodness of fit is proportional to the number of photons collected on the single complex, so brighter objects can theoretically be tracked with high precision. In general, one can measure the accuracy of this method to be about 10-30 nm, if you track an unmoving particle and determine the accuracy of the fitting empirically. But, with very good imaging and many photons with long exposure times, a slow-moving molecule can be tracked with 2 nm accuracy to reveal step sizes and stepping mechanisms (Yildiz et al., 2004). In general, there are home-programmed algorithms for IDL, MatLab, and ImageJ that can perform this tracking and fitting.

iv. Photobleaching measurements.

Often in single molecule imaging, it is necessary to measure the fluorescence intensity of individual molecules over time to ensure that you truly have a single molecule or complex. This can be performed by quantitative photobleaching experiments. Although molecules that are motile will photobleach, it is often difficult to distinguish a molecule that photobleaches from one that simply dissociates from the microtubule. Thus, for quantitative photobleaching, imaging a field of affixed complexes has the least confusion. In addition, a single molecule can be imaged and the light intensity can be placed onto a photodiode or other photon-counting device. This is very accurate, but time consuming, since only one molecule can be recorded/measured at a time. A time-efficient measurement is to record the fluorescence of many molecules in an area and take a movie as the molecules are continuously illuminated and go dark. To analyze the intensity over time, one can use ImageJ to measure the average intensity of a 7x7 pixel^2 region that enclosed the fluorescent spot. The mean intensity can be plotted vs. time to visualize steps in the fluorescence intensity (Figure 6). Each time the intensity reading steps down, a single GFP is photobleached. For complexes with very few fluorescent molecules, there should be countable number of bleaches with approximately equal jumps in intensity for each one. Using the measured value of β to relate the gray scale level on the CCD (C) to the number of photons (N): $C = \beta N$, you can actually quantify the number of photons emitted by the complex over time. If the plot of the intensity over time has jumps but also has an overall decrease in fluorescence over time – even after the molecule has completely photobleached, it is likely that there is background fluorescence that is being bleached. This can be accounted for by repeating the measurement of the intensity in a 7x7 pixel^2 area without a single complex to measure the background fluorescence over time. This background can be subtracted out from the measurement of the complex intensity to correct for the overall bleaching.

By counting the number of bleaches, you can effective count the number of fluorophores on each complex. For the dynein-dynactin, we have GFP-labeled dynamitin as well as

endogenous unlabeled dynactin in the same complexes. The maximum number of steps we should see is four, which would imply that the complex has all four dynamitin subunits that are GFP-labeled. The probability that any one of the four subunits is a GFP-labeled one is proportional to the percentage of GFP-labeled subunits that are available to be incorporated. In our case, we had about 55% of the dynamitin is GFP-labeled. Thus, the probability that any particular dynamitin in a particular complex is labeled is 55/100 ~ 3/5. The probability that all four dynamitins are GFP-labeled is: 3/5 x 3/5 x 3/5 x 3/5 = 81/625 = $(3/5)^4$ = 0.1296. The likelihood that three are GFP-labeled and one is unlabeled is higher: 0.3456. This is very close to the percentages of bleaches that we found in our dynein-dynactin (Ross et al., 2006).

D. Vesicles on microtubules

In this section, we describe detailed methods for working with purified vesicles in imaging assays, as previously described in (Caviston et al., 2007; Ligon et al., 2004; Perlson et al., 2009)**.**

i. Purifying fluorescent vesicles.

To purify vesicles labeled with GFP-dynactin, dynein, and kinesin-1, we start with the same protocol as above for purifying dynein-dynactin from mouse brains. Figure 4 shows a schematic of the protocol for purifying the vesicles. After the high-speed centrifugation step, the pellet will have membrane fractions. Resuspend the high-speed pellet in 200 μl vesicle-stabilizing buffer with protease inhibitors. Remove the pellet from the centrifuge tube and place in a small glass tube for homogenizing with a glass ball homogenizer. Take a gel sample for a later diagnostic gel. Add 400 μl of 2.5 M sucrose and mix by stirring. Place the vesicles in the high concentration sucrose in the bottom of a thin-walled 13 ml SW41 tube. Add 1.6 ml of 2.5 M sucrose on top. Stir gently to mix. Try to avoid and remove bubbles. Layer 6 ml of 1.5 M sucrose on top gently so as not to disturb the high sucrose portion. Layer 4 ml of 0.6 M sucrose on top – gently. The layers should have very clear index of refraction changes between them at changes

in sucrose concentration. Balance the buckets. Centrifuge in swinging bucket rotor in the ultra centrifuge for 2 hr at 198,000 g in 4°C. Carefully take out the centrifuge tubes. Inspect the layer between the 0.6 M and 1.5 M sucrose. There should be a white fluffy layer of membranes there. Carefully remove the white fluffy layer by pipetting. Remove some of each layer and make gel samples for diagnostic gel. Save the vesicles on ice for use over the next several days. The experimental and analysis methods for vesicles are virtually identical to those for single dynein-dynactin complexes.

ii. Photobleaching measurements.

The methods for photobleaching vesicles are the same as for single molecules. If you have examined the vesicles by electron microscopy, and know that they are larger than 100 nm in diameter, make sure that you perform photobleaching with the laser in the epi-illumination configuration instead of the TIRF illumination scheme. Since the TIRF laser only penetrates the sample well up to 100 nm, larger vesicles will have less illumination on their entire surface, and thus not all the vesicle will become photobleached. The analysis is almost identical except that vesicles have more than one dynein-dynactin on them. When many fluorophores are all together, it can be difficult to count individual bleaches, as the probability that two very close GFPs will photobleach almost simultaneously is high. On the other hand, after most of the fluorophore bleaches, individual steps of photobleaching will be easily seen for the last 2-3 molecules. These bleaches should look identical to those of single molecules with a characteristic intensity drop during bleaching. By taking the average intensity drop for the last several bleaches, one can determine the fluorescence intensity of a single fluorophore, called ΔI. Since fluorescence is additive within the linear dynamic range of the CCD, the initial intensity, I_0, is proportional to the number of original fluorophores on the vesicle. By dividing the initial intensity by the change in intensity when one bleaches, you can deduce the number of fluorescent molecules in the complex: $n = I_0/\Delta I$ (Figure 6). For vesicles, we found that the

number of fluorescent molecules on the vesicles varied between 3 and 14, implying that 2-6 dynein-dynactins were bound to each vesicle.

E. Kinesin, dynein-dynactin and tau

In this section, we detail procedures to image tau and motor proteins in two-color fluorescence imaging, as described in (Dixit et al., 2008).

i. Purifying and labeling tau.

Tau can be purified from bacterial cultures (BL21 strain). A small starter culture (5 ml) can be made from a single colony or glycerol stock and incubated overnight at 37°C. Use the overnight starter culture to inoculate a 200 ml culture (LB) in a 1 L flask. Let the 200 ml culture grow for 2 hr at 37°C rotating at 250 rpm until the Absorbancy at 600 nm is 0.6 – 1.0. Save 200 μl of culture as uninduced sample for a diagnostic gel. Induce the culture with 2 ml of 100 mM IPTG (1 mM final concentration). Incubate the induced culture at 37°C, 250 rpm for 2-3 hr. Save 200 μl sample for diagnostic gel. Chill the culture at 4°C and harvest bacteria by centrifugation in a chilled GSA bottle at 5,000 rpm for 10 min at 4°C in a SLA-1500 rotor. Discard the media in the supernatant and resuspend in 5 ml of PEM-100 (100 mM K-Pipes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.8) and protease inhibitors (same as above). Freeze the pellets in a minus 20°C freezer to help lyse the cells. Thaw the cell pellets on ice and lyse twice in a cold French Pressure Cell using high duty, 1150 psi pressure. The lysate should look clear after passing through the press. Centrifuge the lysate at 8,000 rpm for 10 min at 4°C in an SS34 rotor to remove cell debris. Save a 50 μl sample for a diagnostic gel. Add NaCl to a final concentration of 500 mM and boil for 20 min. The solution should get quite cloudy as most proteins will denature and aggregate when boiled. Centrifuge and remove the pelleted denatured and aggregated proteins at127,000 xg for 30 min at 4°C in a 70 Ti rotor. Take samples of the supernatant and pellet for gel samples. The supernatant will contain the tau protein.

To further purify the tau, we use a phosphocellulose (PC) column. This column is identical to the ones used to purify tubulin (Weingarten et al., 1975). First we need to charge the phosphocellulose. Put 300 ml of 0.5 M NaOH in Sodium Phosphate Buffer (43.6g dibasic, 26.9g monobasic per liter) into a 600 ml beaker. Add 25 ml x 0.22 g/ml = 5.5 of PC into the beaker by stirring in a little at a time. Allow the PC to settle to the bottom. Aspirate off the liquid to leave 1 cm of liquid on top of the solid. Add 300ml of Sodium Phosphate Buffer to the PC and stir. Allow the PC to settle. Check the pH of the liquid. If it is > 7, then, aspirate the liquid and add more Sodium Phosphate Buffer. Repeat the Sodium Phosphate Buffer until the pH is 7. After reaching neutral pH, add 300 ml of 0.5 M HCl in Sodium Phosphate Buffer and stir. Allow the PC to settle to the bottom and aspirate excess liquid off top. Add 300 ml of Sodium Phosphate Buffer to the PC and stir. Allow the PC to settle. Check the pH of the liquid. If it is < 7, the, aspirate the liquid and add more Sodium Phosphate Buffer. Repeat this procedure until the PC is at neutral pH.

Once the PC is charged, add the PC to the column. Allow the buffer to run through the column until there is only 1 cm of liquid above the top of the settled PC in the column. Flow through 100 ml of PEM-100 (100 mM Na-Pipes, 1 mM EGTA, 1 mM MgSO4, pH to 6.8) with 1% β mercaptoethanol (β Me). Allow the 100 ml of PEM-100 buffer to flow through. When most has run through and there is 1 cm of liquid on top of the PC, clamp the top and bottom. Next, add the entire supernatant sample with the tau to the column using a glass Pastuer pipette. Be careful not to disrupt the PC at the top of the column. When the sample has only 1 cm of liquid left to run through, start to add 25 ml of PEM-100 1% β Me with the glass pipette to wash. Collect the wash in 12.5 ml fractions. When PEM-100 1% β Me has only ≈ 1 cm of liquid left, add 25 ml of 0.2 M NaCl in BRB-80 1% β Me. Collect 12.5 ml elutions. Repeat with 25 ml of 0.4 M NaCl, 0.6 M NaCl, and 1.0 M NaCl (each in PEM-100 with 1% β Me). Take 20 µl of each aliquot for running the SDS-Page gel. Typically 0.4 M NaCl has the peak tau fractions.

Once the fractions with tau are identified, the tau can be desalted and buffer exchanged

into Motility Assay Buffer using SpectraPor regenerated cellulose MWCO = 15,000. If necessary, protein can be concentrated using Centricon columns. Tau is not quantified accurately using Bradford or other colorimetric assays. One reliable method to quantify tau concentration is densitometric analysis using commercially available tau40 and tau23 (Sigma) as standards.

We label tau using an Alexa-546 reactive dye using a malemide thiol-reactive linker to bind surface cysteines. The benefits of the malemide is that the reaction occurs at pH 7.0 and does not react with histidines or methionines. Tau in Motility Assay Buffer, pH 7.0, is diluted to 50 μM. A 10 mM stock of dye is prepared in Motility Assay Buffer immediately prior to use. To label the protein, a 15x molar excess of dye compared to protein is used. The dye reagent is added to the protein drop-wise as it is stirring. The reaction is allowed to proceed for two hr at room temperature. The reaction is stopped using 100 mM reduced glutathione and incubated at room temperature for 10 min to quench the unused dye. The excess free dye is removed from the dyed protein using a Sephadex G-25 gel filtration column.

ii. Performing experiments

Single molecule experiments with GFP-kinesin (purified according to Pierce and Vale (Pierce and Vale, 1998)) or dynein-dynactin-GFP in the presence of Alexa-546 tau are performed similarly to the single molecule experiments described above. First, 50 nM microtubules are incubated for 10 min at room temperature in the presence of varying amounts of tau protein. During the incubation, flow chambers are made from cover slips silanized as described in the next chapter by Dixit and Ross (similar to (Helenius et al., 2006)). Anti-tubulin, TUB2.1 antibodies (Sigma) are flowed into the chamber at a 0.08% dilution from a stock of 2 mg/ml and incubated for 5 min. Next, 5% F-127 pluronic block co-polymer is added to the chamber and incubated for 5 min. The microtubules with tau mixture are flowed in next and incubated for 5 min to allow the microtubules to bind to the antibodies on the surface. Finally,

the motility mix with tau (dynein-dynactin-GFP or GFP-kinesin at a known working dilution is added to 0.5% F-127 pluronic, 1 mM Mg-ATP, 1 mg/ml BSA, 100 mM DTT, 40 μM taxol, 36 μg/ml catalase and 220 μg/ml glucose oxidase, 4.5 mg/ml glucose, and the final concentration of tau that is incubated with the microtubules) is added to the chamber. The chamber is visualized in TIRF immediately and experimental and data analysis procedures are identical to those described above for dynein-dynactin-GFP alone except the images of the Alexa-546-labeled tau protein are taken instead of microtubules (Figure 6).

F. Crossing microtubules.

In this section, we detail methods for creating crossing microtubules, as described originally in (Ross et al., 2008). Flow chambers are assembled from two cover slips bound at the corners by double-stick tape to make two perpendicular, crossed flow paths (Figure 7 A). To bind the microtubules to the cover slips, a biotin-streptavidin system is employed. First, 10 μl of biotinylated-BSA solution (1 mg/ml) is flowed in to coat the cover glasses, incubated for two min, and washed out with three chamber volumes of wash buffer (5 mg/ml BSA, 20 μM Taxol, 10 mM DTT in Motility Assay Buffer). Second, 10 μl of streptavidin solution (2 mg/ml streptavidin in wash buffer) is flowed in, incubated for two min, and washed out with three chamber volumes of wash buffer. Third, 10 μl of biotinylated-rhodamine-labeled microtubules (1:50 biotin-tubulin, 1:50 rhodamine-tubulin, final concentration 0.45 μM tubulin, 20 μM taxol in Motility Assay Buffer) is flowed into the chamber in the direction of the y-axis (Figure 7 A), allowed to incubate for 30 seconds, and washed out with wash buffer. Fourth, 10 μl of biotinylated-rhodamine-labeled microtubules is flowed into the chamber in the direction of the x-axis (Figure 7 A), allowed to incubate for 30 sec, and washed out with wash buffer. The result is that microtubules flowed along the y-axis are bound closer to the cover slip, and we refer to them as “underpass” microtubules. The microtubules flowed along the x-axis are held away from the cover slip at crossovers; they are termed “overpass” microtubules (Figure 7 B, C). Experiments with single

GFP-kinesins, dynein-dynactin-GFPs, or other molecules can be performed on this system of crossed microtubules using similar techniques as those described above.

V. Conclusions and outlook

Two-color single molecule imaging is gaining popularity because researchers are interested in how multiple molecular species interact while bound to microtubules. The cell is composed of multiple overlapping chemo-mechanical networks, which include or intersect with the microtubule cytoskeletal system. In vitro experiments that “build complexity” are very important to understand the interactions between the numerous biological molecules. In this section, we describe some specific new multiple-color techniques that will be vital for understanding how single microtubule-associated proteins interact in a complex network with other species.

One particular technique that will prove useful for imaging multiple species in very complex systems is Fluorescence Photactivatable Localization Microscopy (FPALM) (Gould and Hess, 2008; Gould et al., 2009). FPALM is a technique that uses photoactivatable or photoconvertable fluorescent dyes or proteins to switch on individual molecules of a group. In this system, very few activation photons are used to activate 5-10 molecules in the illumination region. Taking images at rates from 2-30 frames per sec, as usual for single molecule, one could acquire images of hundreds of molecules in the same network for up to 30 min, which is the life-span of most oxygen scavenging systems.

From the acquired images, single molecules could be identified and tracked using in-house generated MatLab code. The single molecules are accurately localized with ~20-50 nm accuracy by fitting a two-dimensional Gaussian to the intensity profile of each spot. The fitting and tracking routines can reveal information about position and velocity, but also correlations between multiple components in the same network.

Multiple colors of photoactivatible dyes can be used to measure the position, velocity and correlations between different species in different colors. Most photoactivatable or photoconvertable fluorophores use UV light (405 nm) to convert, which allows the same laser to activate different colors simultaneously for imaging. In addition, the microtubules could be labeled with a different fluorophore to image them simultaneously, using the same optics described above.

Although the PALM technique have been touted most for their ability to give ultra-structural information in fixed cellular samples, we believe this techniques has an even greater potential for revealing the dynamics of complex systems *in vitro*. As these techniques are used more frequently, they will uncover detailed information to allow the building of bottom-up networks that resemble cellular compartments and organelles – especially those involving microtubules (e.g, the axon or cilia).

Ackowledgements:

We thank both Drs. Erika L. F. Holzbaur and Yale E. Goldman in whose labs these protocols were developed and performed. We also thank Mariko Tokito, Lee Ligon, and Juliane Caviston for their expertise on protein expression and purification. We thank Karen Wallace for her care and dissection of the mice used in this study.

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Figure Captions:

Figure 1. TIRF Theory. (A) The process of total internal reflection is quite basic, and can be explained with simple ray optics. At any interface, such as that between glass and water, light is bent. At incident angles, θ_1, that are smaller than the Brewster's angle, θ_B, some light reflects and most transmit at an angle, θ_2 that is determined by the indicies of refraction of the two materials at the interface, n_1 and n_2. The relation is determined by Snell's Law. As θ_1 increases, θ_2 also increases. When $\theta_2 = 90°$, $\theta_1 = \theta_B$, and the refracted wave propagates parallel to the interface. When θ_1 is larger than θ_B, the majority of the light reflects. (B) The resulting evanescent wave is less straight-forward to understand since it is a fundamental property of wave nature of propagating light, which is made from oscillating electric and magnetic fields. Using the wave mechanics solutions to describe the propagating wave, we find that the boundary conditions that causes reflection of most of the light actually results in a wave propagating parallel to the surface with a decaying exponential intensity (see Jackson's Classical Electrodynamics, 3rd ed. pp.302 - 309 for the full mathematical details of the derivation). If you calculate the Poynting vector to determine the amount of energy that crosses the barrier, you will find that it is zero. This is the same phenomenon that makes optical fibers work to transport light without losses. On the other hand, there are photons within the evanescent wave. If these photons are absorbed by a fluorophore, then there is loss of energy in the reflected wave, since energy must be conserved.

Figure 2. Simple TIRF system. The required components to construct a TIRF system through a high numerical aperture objective are quite simple. The collimated laser is passed through one lens that focuses onto the back focal plane of the objective. By placing the steering mirror at a location that is the conjugate to the front focal plane, you can redirect the beam through the

objective at a high angle while keeping the illumination location the same while steering. (A) When the laser is focused on the back focal plane and goes into the objective in the center-line, the light is collimated and comes straight out along the center-line. (B) By turning the steering mirror, the light is focused at the back focal plane off the center-line. The light coming out of the objective is collimated coming out at an angle.

Figure 3. Mutli-color TIRF. Multi-color optical schematic for TIRF system built around a commerical microscope. M: mirror, CM: combination mirror, SM: steering mirror located at front focal plane conjugate, AM: aligning mirror, O: objective, S: sample, C: condenser, TL: transmitted light lamp, DM: dichroic mirror, FL: focus lens, BFL: back focus lens, FM: flip mirror, AOM: acousto-optical modulator, FS: fast shutter, BE: beam expander, DW: dual-wavelength splitting system, EM-CCD: electron multiplier CCD camera. To align the laser, we pass the transmitted light through the objective and out through the laser port. Using only the steering mirror (SM) and the first combination mirror (CM1), you can iterate back and forth to align the reference laser onto the light coming out from the transmitted lamp. After aligning the first laser to bring the laser out the top collimated, the other lasers can be aligned on top of the reference laser using the aligning mirrors (AM).

Figure 4. Protocol schematic for dynein-dynactin and vesicle purification.

Figure 5: Single motors with tau. (A) Using TIRF microscopy, we can image the fluorescence of single protein molecules in two colors. Time series from a movie showing GFP-kinesin cannot pass Alexa-546-tau23 patches. (B) Single molecules imaging can be analyzed quantitatively to determine the number of molecules in each spot. Movement and fluorescence changes over

time are easily assessed by kymographs. Scale bar, 3 μm. Reproduced with permission from Science Dixit, et al, 2008.

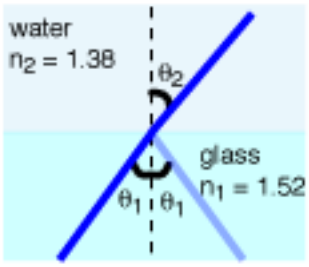
Figure 6: Photobleaching. Example traces of photobleaching from single dynein-dynactin (black) and vesicles (red and blue). To determine the number of GFP molecules for a single dynein-dynactin, one can simply count the number of bleach steps. They varied from 1-4 for each complex. To determine the number of GFP molecules for each vesicle, one must first deduce the average value for the change in intensity, ΔI, for the final few bleaches. Then determine the maximum intensity, I_{max}, and calculate the number of GFP that could produce the maximum intensity.

Figure 7: Crossing microtubules. Specialized flow chamber to create crossing microtubules. (A) The flow chamber is created with four small squares of double stick tape to create two perpendicularly intersecting flow paths. (B) Biotinylated microtubules are specifically stuck to the cover glass via biotin-streptavidin. (C) In the center, where the two paths intersect, microtubules are aligned into perpendicular patterns from the flow.

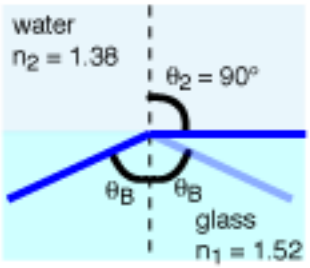
Fig. 1
Ross and Dixit
MCB

A. Ray Optics

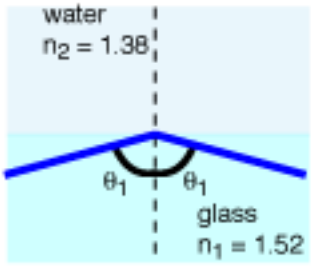
Snell's Law of Refraction:
$n_1 \sin\theta_1 = n_2 \sin\theta_2$
Law of Reflection:
$\theta_1 = \theta_2$



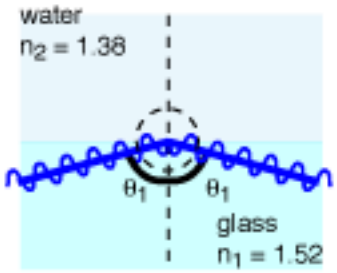
Brewster's Angle:
$\sin\theta_B = n_2 / n_1$



Total Internal Reflection:
$\theta_1 = \theta_1$
θ_2 does not exist



B. Wave Optics



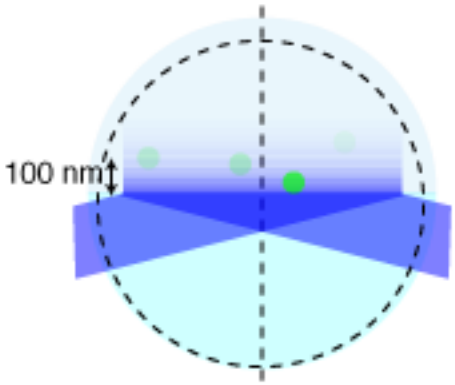


Fig. 2
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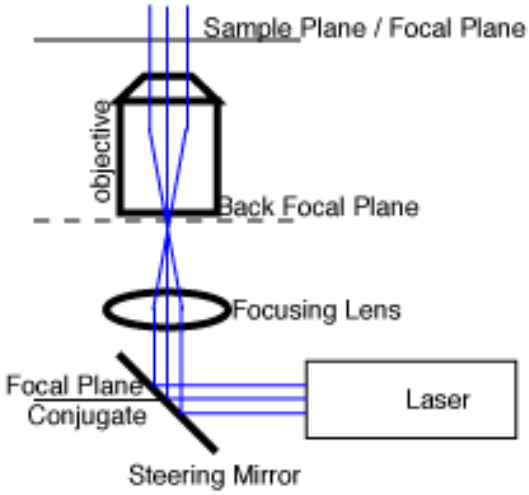
MBC

A. Simplified TIRF system (straight)



B. Simplified TIRF system (angled)

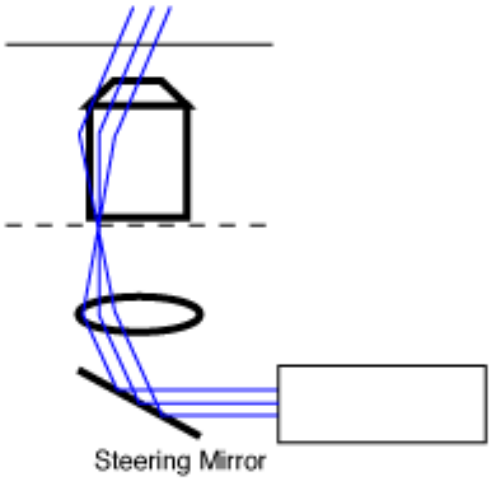


Fig. 3
Ross and Dixit
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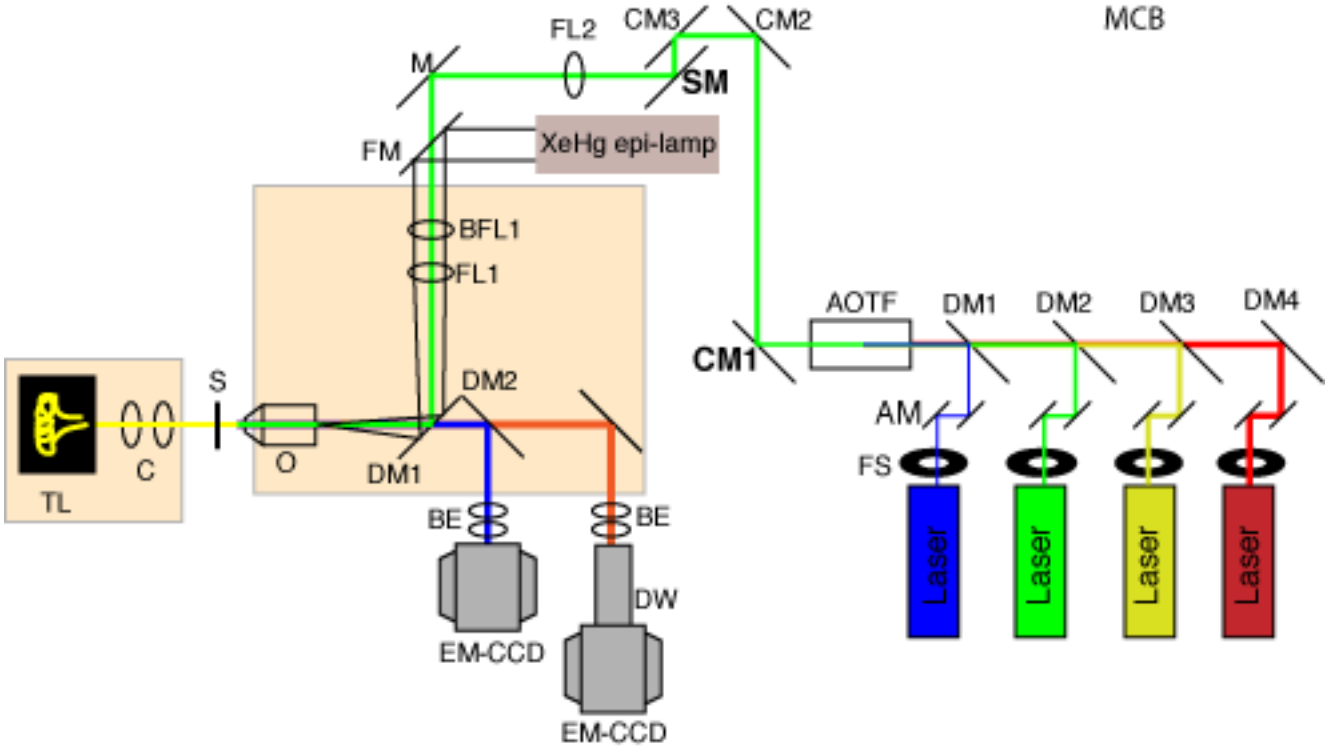

Fig. 4
Ross and Dixit
MCB

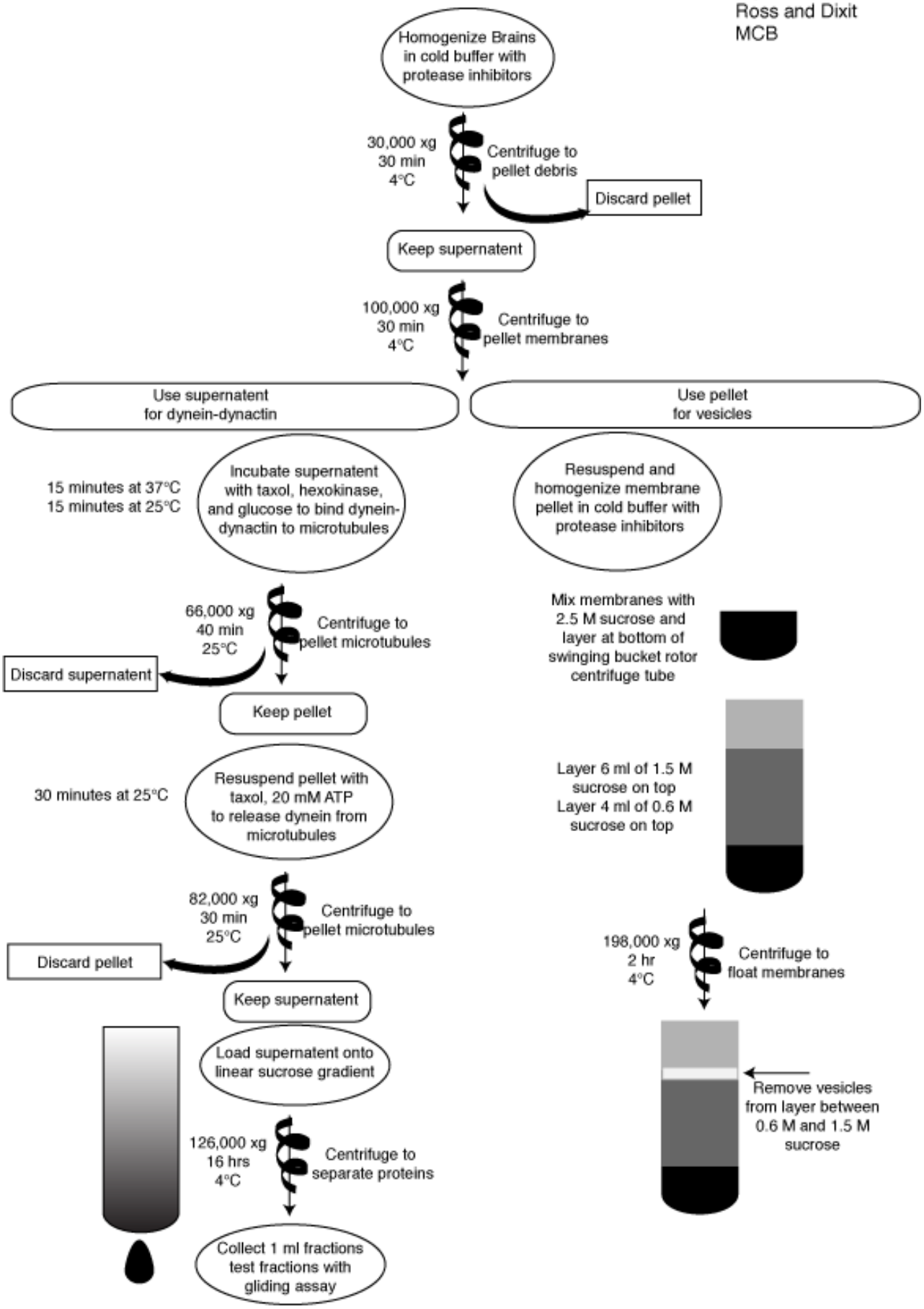

Fig. 5
Ross and Dixit
MCB

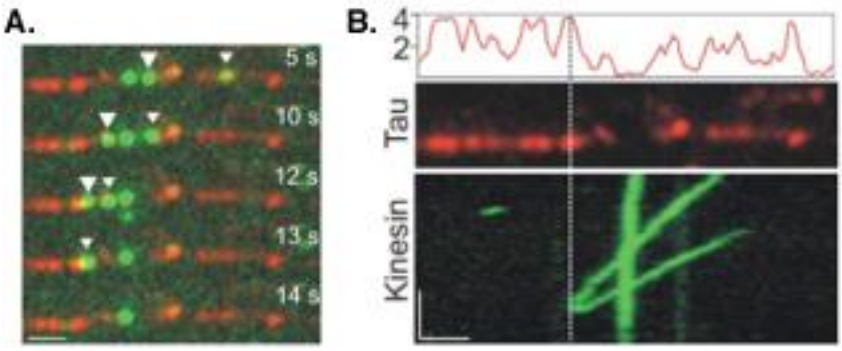

Fig. 6
Ross and Dixit
MCB

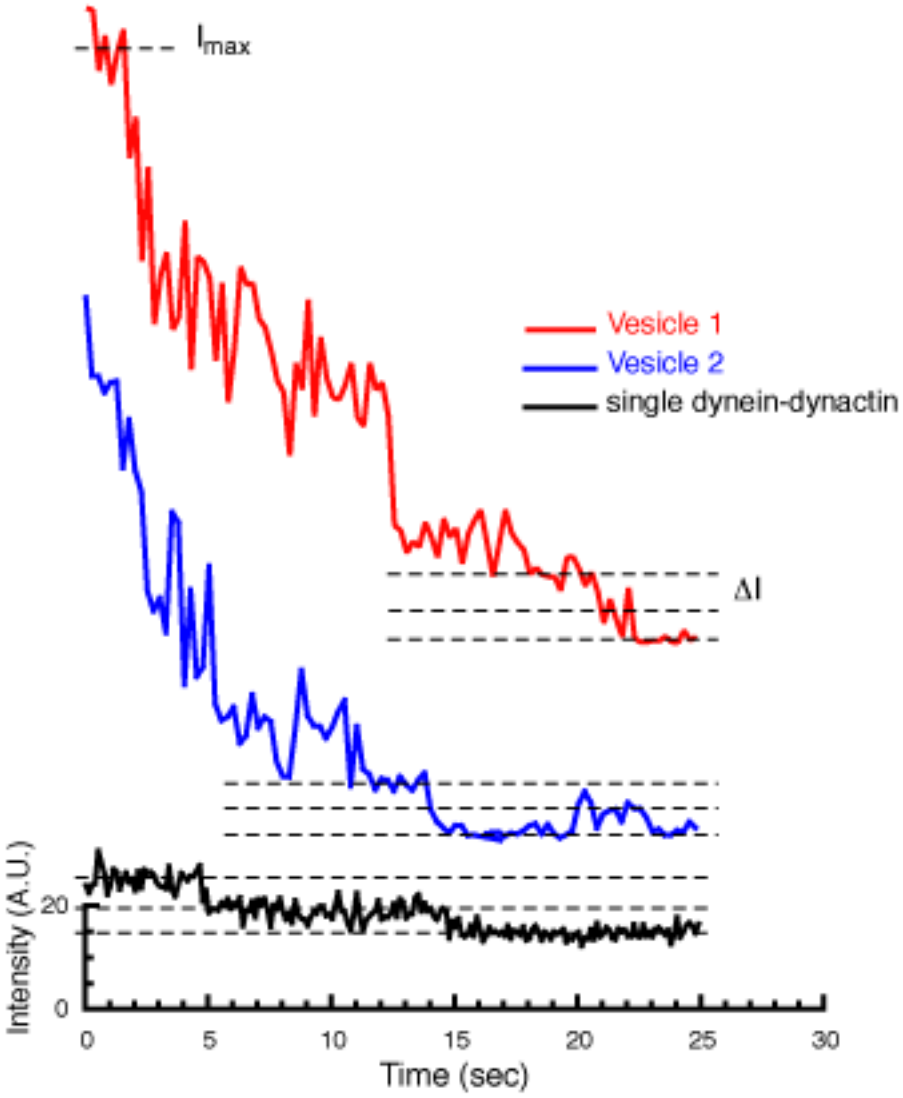

Fig. 7
Ross and Dixit
MCB