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TITLE:

Myosin-Specific Adaptations of In Vitro Fluorescence Microscopy-Based Motility Assays

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KEYWORDS:

myosin, actin filaments, protein purification, fluorescence microscopy, motility assay, single molecule assay, bipolar filaments, total internal reflection fluorescence microscopy.

SUMMARY:

Presented here is a procedure to express and purify myosin 5a followed by a discussion of its characterization, using both ensemble and single molecule in vitro fluorescence microscopy-based assays, and how these methods can be modified for the characterization of nonmuscle myosin 2b.

ABSTRACT:

Myosin proteins bind and interact with filamentous actin (F-actin) and are found in organisms across the phylogenetic tree. Their structure and enzymatic properties are adapted for the particular function they execute in cells. Myosin 5a processively walks on F-actin to transport melanosomes and vesicles in cells. Conversely, nonmuscle myosin 2b operates as a bipolar filament containing approximately 30 molecules. It moves F-actin of opposite polarity toward the center of the filament, where the myosin molecules work asynchronously to bind actin, impart a power stroke, and dissociate before repeating the cycle. Nonmuscle myosin 2b, along with its other nonmuscle myosin 2 isoforms, has roles, including cell adhesion, cytokinesis, and tension maintenance. The mechanochemistry of myosins can be studied by performing in vitro motility assays using purified proteins. In the gliding actin filament assay, the myosins are bound to a

microscope coverslip surface and translocate fluorescently labeled F-actin, which can be tracked. In the single molecule/ensemble motility assay, however, F-actin is bound to a coverslip and the movement of fluorescently labeled myosin molecules on the F-actin is observed. In this report, the purification of recombinant myosin 5a from *Sf9* cells using affinity chromatography is outlined. Following this, we outline two fluorescence microscopy-based assays: the gliding actin filament assay and the inverted motility assay. From these assays, parameters such as actin translocation velocities and single molecule run lengths and velocities can be extracted using the image analysis software. These techniques can also be applied to study the movement of single filaments of the nonmuscle myosin 2 isoforms, discussed herein in the context of nonmuscle myosin 2b. This workflow represents a protocol and a set of quantitative tools that can be used to study the single molecule and ensemble dynamics of nonmuscle myosins.

INTRODUCTION:

Myosins are motor proteins that exert force on actin filaments using the energy derived from adenosine triphosphate (ATP) hydrolysis[1]. Myosins contain a head, neck, and tail domain. The head domain contains the actin-binding region as well as the site of ATP binding and hydrolysis. The neck domains are composed of IQ motifs, which bind to light chains, calmodulin, or calmodulin-like proteins[2,3]. The tail region has several functions specific to each class of myosins, including but not limited to the dimerization of two heavy chains, binding of cargo molecules, and regulation of the myosin via autoinhibitory interactions with the head domains[1].

The motile properties of myosin vary greatly between classes. Some of these properties include duty ratio (the fraction of myosin's mechanical cycle in which the myosin is bound to actin) and processivity (the ability of a motor to make multiple steps on its track before detachment)[4]. The over 40 classes of myosins were determined based on sequence analyses[5–8]. The class 2 myosins are classified as "conventional" since they were the first to be studied; all other classes of myosins are, therefore, classified as "unconventional."

Myosin 5a (M5a) is a class 5 myosin and is a processive motor, meaning that it can take multiple steps along actin before dissociating. It has a high duty ratio, indicating that it spends a large part of its mechanical cycle bound to actin[9–14]. In common with other myosins, the heavy chain contains an N-terminal motor domain that includes both an actin-binding and an ATP hydrolysis site followed by a neck region that serves as a lever-arm, with six IQ motifs that bind to essential light chains (ELC) and calmodulin (CaM)[15]. The tail region contains α-helical coiled-coils, which dimerize the molecule, followed by a globular tail region for binding cargo. Its kinetics reflect its involvement in the transport of melanosomes in melanocytes and of the endoplasmic reticulum in Purkinje neurons[16,17]. M5a is considered the prototypical cargo transport motor[18].

Class 2 myosins, or the conventional myosins, include the myosins that power contraction of skeletal, cardiac, and smooth muscle in addition to the nonmuscle myosin 2 (NM2) isoforms, NM2a, 2b, and 2c[19]. The NM2 isoforms are found in the cytoplasm of all cells and have shared roles in cytokinesis, adhesion, tissue morphogenesis, and cell migration[19–22]. This paper discusses conventional myosin protocols in the context of nonmuscle myosin 2b (NM2b)[23]. NM2b, in comparison to M5a, has a low duty ratio and is enzymatically slower with a V_{max} of 0.2 s^{-1} [23]

compared to M5a's V_{max} of ≈18 s^{-1} [24]. Notably, truncated NM2b constructs with two heads do not readily move processively on actin; rather, each encounter with actin results in a power stroke followed by dissociation of the molecule[25].

NM2b contains two myosin heavy chains, each with one globular head domain, one lever-arm (with one ELC and one regulatory light chain (RLC)), and an α-helical coiled-coil rod/tail domain, approximately 1,100 amino acids long, that dimerizes these two heavy chains. The enzymatic activity and structural state of NM2b are regulated by phosphorylation of the RLC[23]. Unphosphorylated NM2b, in the presence of ATP and physiological ionic strengths (approximately 150 mM salt), adopts a compact conformation wherein the two heads make an asymmetric interaction and the tail folds back over the heads in two places[23]. In this state, the myosin does not interact strongly with actin and has very low enzymatic activity. Upon RLC phosphorylation by calmodulin-dependent myosin light chain kinase (MLCK) or Rho-associated protein kinase, the molecule extends and associates with other myosins through the tail region to form bipolar filaments of approximately 30 myosin molecules[23]. The aforementioned phosphorylation of the RLC also leads to increased actin-activated ATPase activity of NM2b by approximately four times[26–28]. This bipolar filament arrangement, featuring many myosin motors at each end, is optimized for roles in contraction and tension maintenance, where actin filaments with opposing polarities can be moved relative to each other[23,29]. Accordingly, NM2b has been shown to act as an ensemble of motors when interacting with actin. The large number of motors within this filament allow NM2b filaments to move processively on actin filaments, making in vitro filament processivity possible to characterize[29].

While progress has been made in understanding the role of myosins in the cell, there is a need to understand their individual characteristics at the protein level. To understand actomyosin interactions at a simple protein-protein interaction level, rather than inside of a cell, we can express and purify recombinant myosins for use in in vitro studies. The results of such studies then inform mechanobiologists about the biophysical properties of specific myosins that ultimately drive complex cellular processes[12–14,25,29]. Typically, this is accomplished by adding an affinity tag to a full-length or truncated myosin construct and purifying via affinity chromatography[29–31]. Additionally, the construct can be engineered to include a genetically encodable fluorophore or a tag for protein labeling with a synthetic fluorophore. By adding such a fluorescent label, single molecule imaging studies can be performed to observe myosin mechanics and kinetics.

Following purification, the myosin can be characterized in several ways. ATPase activity can be measured by colorimetric methods, providing insight into the overall energy consumption and actin affinity of the motor under different conditions[32]. To learn about the mechanochemistry of its motility, further experiments are required. This paper details two in vitro fluorescence microscopy-based methods that can be used to characterize the motile properties of a purified myosin protein.

The first of these methods is the gliding actin filament assay, which can be used to quantitatively study the ensemble properties of myosin motors, as well as qualitatively study the quality of a

batch of purified protein[33]. Although this paper discusses the use of total internal reflection fluorescence (TIRF) microscopy for this assay, these experiments can be effectively performed using a wide-field fluorescence microscope equipped with a digital camera, commonly found in many labs[34]. In this assay, a saturating layer of myosin motors is attached to a coverslip. This can be accomplished using nitrocellulose, antibodies, membranes, SiO_2-derivatized surfaces (such as trimethylchlorosilane), among others[29,33,35–38]. Fluorescently labeled actin filaments are passed through the coverslip chamber, upon which the actin binds to the myosin attached to the surface. Upon addition of ATP (and kinases in the study of NM2), the chamber is imaged to observe the translocation of actin filaments by the surface-bound myosins. Tracking software can be used to correlate the velocity and length of each gliding actin filament. Analysis software can also provide a measure of the number of both moving and stationary actin filaments, which can be useful to determine the quality of a given myosin preparation. The proportion of stalled filaments can also be intentionally modulated by surface tethering of actin to other proteins and measured to determine the load dependence of the myosin[39]. Because each actin filament can be propelled by a large number of available motors, this assay is very reproducible, with the final measured velocity being robust to perturbations such as alterations in the starting myosin concentration or the presence of additional factors in the solution. This means it can be easily modified to study myosin activity under different conditions, such as altered phosphorylation, temperature, ionic strength, solution viscosity, and the effects of load induced by surface tethers. Although factors such as strong-binding myosin "dead heads" incapable of ATP hydrolysis can cause stalled actin filaments, multiple methods exist to mitigate such issues and allow for accurate measurements. The kinetic properties of myosin vary greatly across classes and, depending on the specific myosin used, the speed of actin filament gliding in this assay can vary from under 20 nm/s (myosin 9)[40,41], and up to 60,000 nm/s (*Characean* myosin 11)[42].

The second assay inverts the geometry of the gliding actin filament assay[12]. Here, the actin filaments are attached to the coverslip surface and the movement of single molecules of M5a or of individual bipolar filaments of NM2b are visualized. This assay can be used to quantify the run lengths and velocities of single myosin molecules or filaments on actin. A coverslip is coated with a chemical compound that blocks non-specific binding and simultaneously functionalizes the surface, such as biotin-polyethylene glycol (biotin-PEG). The addition of modified avidin derivatives then primes the surface and biotinylated actin is passed through the chamber, resulting in a layer of F-actin stably bound to the bottom of the chamber. Finally, activated and fluorescently labeled myosin (typically 1–100 nM) is flowed through the chamber, which is then imaged to observe myosin movement over the stationary actin filaments.

These modalities represent fast and reproducible methods that can be employed to examine the dynamics of both nonmuscle and muscle myosins. This report outlines the procedures to purify and characterize both M5a and NM2b, representing unconventional and conventional myosins, respectively. This is followed by a discussion of some of the myosin-specific adaptations, which can be performed to achieve successful capturing of motion in the two types of the assay.

Expression and molecular biology

The cDNA for the myosin of interest must be cloned onto a modified pFastBac1 vector that encodes for either a C-terminal FLAG-tag (DYKDDDDK) if expressing M5a-HMM, or an N-terminal FLAG-tag if expressing the full-length molecule of NM2b[23,43–46]. C-terminal FLAG-tags on NM2b results in a weakened affinity of the protein for the FLAG-affinity column. In contrast, the N-terminally FLAG-tagged protein usually binds well to the FLAG-affinity column[23]. The N-terminally tagged protein retains enzymatic activity, mechanical activity and phosphorylation-dependent regulation[23].

In this paper, a truncated mouse M5a heavy meromyosin (HMM)-like construct with a GFP between the FLAG-tag and the C-terminus of the myosin heavy chain was used. Note that unlike NM2b, M5a-HMM can be successfully tagged and purified with either N- or C-terminal FLAG tags and in both cases the resulting construct will be active. The M5a heavy chain was truncated at amino acid 1090 and contains a three amino acid linker (GCG) between the GFP and the coiled-coil region of the M5a[47]. No additional linker was added between the GFP and FLAG-tag. M5a-HMM was co-expressed with calmodulin. The full-length human NM2b construct was co-expressed with ELC and RLC. The N-termini of the RLC was fused with a GFP via a linker of five amino acids (SGLRS). Directly attached to the FLAG-tag was a HaloTag. Between the HaloTag and the N-terminus of the myosin heavy chain was a linker made of two amino acids (AS).

Both myosin preparations were purified from one liter of *Sf9* cell culture infected with baculovirus at a density of approximately 2 x 10^6 cells/mL. The volumes of the baculovirus for each subunit depended on the virus's multiplicity of infection as determined by the manufacturer's instructions. In the case of M5a, cells were co-infected with two different baculoviruses—one for calmodulin, and one for M5a heavy chain. In the case of the NM2b, cells were co-infected with three different viruses—one for ELC, one for RLC, and one for NM2b heavy chain. For labs working with a diversity of myosins (or other multi-complex proteins), this approach is efficient since it allows for many combinations of heavy and light chains and commonly used light chains such as calmodulin can be co-transfected with many different myosin heavy chains. All cell work was completed in a biosafety cabinet with proper sterile technique to avoid contamination.

For the expression of both M5a and NM2b, the *Sf9* cells producing the recombinant myosins were collected 2–3 days post-infection, via centrifugation, and stored at -80 °C. Cell pellets were obtained by centrifuging the co-infected Sf9 cells at 4 °C for 30 min at 2,800 x *g*. The protein purification process is detailed below.

PROTOCOL:

1. Protein purification

1.1. Cell lysis and protein extraction

1.1.1. Prepare a 1.5x Extraction Buffer based on **Table 1**. Filter and store at 4 °C.

1.1.2. Begin thawing the cell pellets on ice. While the pellets are thawing, supplement 100 mL of Extraction Buffer with 1.2 mM dithiothreitol (DTT), 5 µg/mL leupeptin, 0.5 µM phenylmethylsulfonyl fluoride (PMSF) and two protease inhibitor tablets. Keep on ice.

1.1.3. Once the pellet has thawed, add 1 mL of the supplemented Extraction Buffer per 10 mL of cell culture. For example, if the cell pellets were formed from 500 mL of cell culture, then add 50 mL of supplemented Extraction Buffer to the pellet.

1.1.4. Sonicate the cell pellets while keeping them on ice. For each pellet, use the following conditions: 5 s ON, 5 s OFF, duration of 5 min, power 4–5.

1.1.5. Collect all the homogenized lysate into a beaker and add ATP (0.1 M stock solution; pH 7.0) such that the final concentration of ATP is 1 mM. Stir for 15 min in a cold room. The ATP dissociates active myosin from actin, allowing it to be separated in the following centrifugation step. It is, therefore, essential to proceed to the next step immediately to minimize the possibility for ATP depletion and rebinding to actin.

1.1.6. Centrifuge the lysates at 48,000 x *g* for 1 h at 4 °C. While this is occurring, begin washing 1-5 mL of a 50% slurry of Anti-FLAG affinity resin (for a pellet formed from 1 L of cells) with 100 mL phosphate-buffered saline (PBS), according to the manufacturer's instructions. For example, for 5 mL of resin, wash 10 mL of a 50% slurry. In the final wash step, resuspend the resin with 1–5 mL of PBS with enough volume to create a 50% slurry.

1.1.7. Following lysate centrifugation, combine the supernatant with the washed resin slurry and rock gently in the cold room for 1–4 h. While waiting, make the buffers described in **Table 1** and keep them on ice.

1.2. FLAG affinity purification preparation

1.2.1. Centrifuge the solution in step 1.7 at 500 x *g* for 5 min at 4 °C. The resin will be packed at the bottom of the tube. Without disturbing the resin, remove the supernatant.

1.2.2. Resuspend the resin in 50 mL of Buffer A as detailed in **Table 1** and centrifuge at 500 x *g* for 5 min at 4 °C. Without disturbing the resin, remove the supernatant.

1.2.3. Resuspend the resin in 50 mL of Buffer B as detailed in **Table 1** and centrifuge at 500 x *g* for 5 min at 4 °C. Repeat this step once more and resuspend the resin in 20 mL of Buffer B. Then, mix the resin and the buffer thoroughly by gently inverting the tube by hand approximately 10 times.

1.3. Protein elution and concentration

1.3.1. Make 30 mL of Elution Buffer as described in **Table 1** and let it chill on ice.

1.3.2. Set up the elution column in a cold room. Gently pour the resin slurry into the column. Wash the column with 1–2 column volumes of Buffer B as the resin packs on the bottom, ensuring that the resin does not dry out.

1.3.3. Flow 1 mL of the Elution Buffer through the resin and collect the flow-through in a 1.5 mL tube. Repeat such that 12, 1 mL fractions are collected.

1.3.4. At this point, perform a crude Bradford test on the fractions to qualitatively determine which fractions are the most concentrated[48]. On one row of a 96-well plate, pipette 60 µL 1x Bradford reagent. As fractions are collected, mix 20 µL of each fraction per well. A darker blue coloration indicates the more concentrated fractions.

1.3.5. In a 50 mL tube, collect the remaining protein by gently pipetting the remaining Elution Buffer through the column, to release any remaining myosin bound to the resin in the column flowthrough. This flow-through will be concentrated in the next step. Ensure that the resin is then regenerated for reuse and stored according to the manufacturer's instructions.

1.3.6. Pool the three most concentrated fractions and further concentrate the flow-through in the 50 mL tube as well as the remaining 1 mL fractions using a 100,000 MWCO concentrating tube. Load the pooled sample onto the concentrating tube and centrifuge at 750 x *g* for 15 min at 4 °C and repeat until all eluted protein has been concentrated to a final volume of approximately 0.5–1 mL.

NOTE: This pore size allows for the retention of the myosin molecules, which have masses several times the molecular weight cutoff. The light chains remain tightly bound to the motor domains during this time course of concentration, as verified by performing SDS-PAGE gel electrophoresis on the final product.

1.4. Dialysis and flash-freezing

1.4.1. Make 2 L of Dialysis Buffer, as described in **Table 1**. Load the sample in a dialysis bag or chamber and dialyze overnight in the cold room. Note that the composition of the dialysis buffers differs for NM2b and M5a.

NOTE: In the case of NM2b, the purpose of this dialysis step is to form myosin filaments in the low ionic strength buffer. Sedimentation of these filaments then provides an additional purification step and allows for the concentration of the sample. There will, therefore, be a visible white precipitate in the dialysis chamber the next day. These filaments will be collected by centrifugation and depolymerized in step 5.1. In the case of M5a-HMM, after the overnight dialysis, the protein will be sufficiently pure for the use in subsequent assays. Further purification steps such as gel filtration or ionic exchange chromatography can be performed, if required. For M5a recovery after dialysis, go to step 5.2.

1.5. Recovering myosin after dialysis

1.5.1 For NM2b, carefully unload the entire sample from the dialysis bag or chamber and centrifuge at 4 °C for 15 min at 49,000 x *g* to collect the myosin filaments. Discard the supernatant and incrementally add the Storage Buffer to the pellet as described in **Table 1** until it has dissolved. Gentle up and down pipetting helps to solubilize the pellet. Normally, this does not require more than 500 µL per tube. After ensuring that the pellet is fully dissolved in the high ionic strength storage buffer, an additional centrifugation step (15 min at 49,000 x *g*) can be performed to remove unwanted aggregates if required, since the myosin will now be unpolymerized and will remain in the supernatant.

1.5.2 For M5a-HMM, carefully collect the entire sample from the dialysis chamber and centrifuge at 4 °C for 15 min at 49,000 x *g* in case any unwanted aggregates are present. Take the supernatant.

1.6. Concentration determination and flash-freezing

1.6.1 To determine the concentration of the product, measure the absorbance using a spectrophotometer at wavelengths 260, 280, 290, and 320 nm. Calculate the concentration in mg/mL ($c_{mg/mL}$) with **Equation 1**, where A_{280} represents the absorption at 280 nm and A_{320} represents the absorption at 320 nm. The resulting concentration in mg/mL can be converted into µM of myosin molecules with **Equation 2**, where M is the molecular weight of the entire protein (including the heavy chains, light chains, fluorophores, and all tags).

$$c_{mg/mL} = (A_{280} - A_{320})/\varepsilon \ (1)$$

$$\mu M \ molecules = 1000 c_{mg/mL}/M \ (2)$$

NOTE: If a dilution is necessary, then it must be done in a high ionic strength buffer. The extinction coefficient (ε) can be determined by importing the amino acid sequence of the protein into a program such as ExPASy. Typical yield for the M5a-HMM is approximately 0.5–1 mL of 1–5 mg/mL protein and for the full-length NM2b is 0.5–1 mL of 0.5–2 mg/mL. The extinction coefficient for the M5a-HMM used in this paper was 0.671. The extinction coefficient for the NM2b used in this paper was 0.611.

1.6.2 Store the purified myosin in one of the two ways. Aliquot between 10–20 µL into a thin-walled tube, such as a polymerization chain reaction tube, and drop the tube into a container of liquid nitrogen for flash-freezing. Alternatively, directly pipette between 20–25 µL of myosin into liquid nitrogen and store the frozen beads of protein in sterile cryogenic tubes. In either case, the resulting tubes can be stored in -80 °C or liquid nitrogen for future use.

NOTE: Since both motility assays described below require very small amounts of protein, storage in small aliquots, as described, is economical.

2. Gliding actin filament assay

352 2.1. Coverslip preparation
353
354 2.1.1. Make a 1% nitrocellulose solution in amyl acetate.
355
356 2.1.2. Obtain a tissue culture dish (150 x 25 mm) and add a circular filter paper (125 mm
357 diameter) to the bottom of the dish.
358
359 2.1.3. Load eight No. 1.5 thickness 22 mm square coverslips onto a rack and wash with
360 approximately 2–5 mL of 200-proof ethanol followed by 2–5 mL of distilled water (dH_2O). Repeat
361 this washing step, ending with water. Then, dry the coverslips completely using a filtered air-line
362 or N_2-line.
363
364 2.1.4. Take one coverslip and slowly pipette 10 µL of the 1% nitrocellulose solution along one
365 edge of the slip. Then, in one smooth motion, smear it across the rest of the coverslip using the
366 side of a smooth-sided 200 µL pipette tip. Place this coverslip on the tissue culture dish with the
367 nitrocellulose side up. Repeat for the remaining coverslips and allow them to dry while preparing
368 the remaining reagents and use coverslips within 24 h after coating.
369
370 2.2. Chamber preparation
371
372 2.2.1. Wipe a microscope slide with an optical lens paper to clean off large debris. Cut two pieces
373 of double-sided tape, approximately 2 cm in length.
374
375 2.2.2. Place one piece along the middle of the long edge of the microscope slide. Ensure that
376 the edge of the tape aligns with the edge of the slide. Place the second piece of tape roughly 2
377 mm below the first piece of tape such that the two are parallel and aligned. This creates a flow
378 chamber that can hold approximately 10 µL of solution (see **Figure 1**).
379
380 2.2.3. Take one of the nitrocellulose-coated coverslips from Part 1. Carefully stick the coverslip
381 onto the tape such that the side coated with nitrocellulose is making direct contact with the tape,
382 (see **Figure 1**). Using a pipette tip, gently press down on the slide-tape interface to ensure that
383 the coverslip has properly adhered to the slide. Cut the excess tape hanging over the edge of the
384 slide with a razor blade.
385
386 2.3. Actin preparation
387
388 2.3.1 Make 20 µM F-actin by polymerizing globular actin (G-actin) in polymerization buffer (50
389 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 25 mM MOPS (pH 7.0)) at 4 °C overnight.
390
391 2.3.2 Dilute F-actin to 5 µM in motility buffer (20 mM MOPS, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM
392 DTT (pH 7.4)). Label with at least 1.2x molar excess of rhodamine-phalloidin. Leave (covered in
393 aluminum foil) for at least 2 h on ice. This can be used for up to 1–2 months, stored on ice.
394
395 2.4. Performing the myosin 5a gliding actin filament assay

NOTE: In this section, the details of the myosin 5a (HMM) gliding assay are provided.

2.4.1. Prepare the solutions for myosin 5a described in **Table 2** and keep them on ice.

2.4.2. Flow in 10 µL of the myosin 5a (50–100 nM) through the flow chamber and wait for 1 min.

2.4.3. Flow in 10 µL of the 1 mg/mL BSA in 50 mM MB with 1 mM DTT ("low salt" buffer). Repeat this wash two more times and wait for 1 min after the third wash. Use the corner of a tissue paper or filter paper to wick the solution through the channel by gently placing the corner of the paper at the flow chamber exit.

2.4.4. Wash with 10 µL of 50 mM MB with 1 mM DTT. Repeat this wash two more times.

2.4.5. Flow in 10 µL of the black actin solution (5 µM F-actin, 1 µM calmodulin, and 1 mM ATP in 50 mM MB with 1 mM DTT) to eliminate "dead heads", as discussed in the **Discussion** section.

2.4.5.1. Pipette the solution with a 1 mL syringe and 27 G needle to shear the actin filaments before introducing the solution to the chamber. Repeat this step two more times and wait for 1 min after the third time. Approximately 20 pipetting events are sufficient.

2.4.5.2. To perform the "dead head" spin, add a stoichiometric amount of F-actin to myosin in the presence of 1 mM ATP and 1 mM $MgCl_2$ at a salt concentration of 500 mM. Then ultracentrifuge at 480,000 x *g* for 15 min at 4 °C. The dead myosin will be in the pellet.

2.4.6. Flow in 50 µL of 50 mM MB with 1 mM DTT and 1 mM ATP to deplete the chamber of free actin filaments.

2.4.7. Wash with 10 µL of 50 mM MB with 1 mM DTT. Repeat this wash two more times to deplete the chamber of any ATP.

2.4.8. Flow in 10 µL of 20 nM rhodamine actin (Rh-Actin) solution containing 1 mM DTT in 50 mM MB and wait for 1 min to allow rigor binding of actin filaments to the myosin 5a attached to the surface of the coverslip.

2.4.9. Wash with 10 µL of 50 mM MB with 1 mM DTT to wash away Rh-Actin filaments not bound to the surface. Repeat this wash two more times.

2.4.10. Flow in 30 µL of Final Buffer.

2.4.11. Record images on a fluorescence microscope using an excitation wavelength of 561 nm to visualize Rh-Actin. An appropriate exposure time is 200 ms at 1.4 mW laser power for a total acquisition duration of 0.5–1 min.

NOTE: Ensure that the acquisition rate is scaled appropriately to the speed of the moving filaments. An important consideration before collecting data for use with tracking programs is the acquisition frame rate. Subpixel movements between frames will result in an overestimate of the velocity, and movements of several hundred nanometers are required to obtain accurate values. An optimal acquisition rate features actin gliding for at least one pixel distance between frames. In the case of the TIRF microscope used for the imaging here, this threshold translates to 130 nm; therefore, a myosin expected to travel 1 µm/s must be imaged at a rate of 5 frames/s (0.2 s interval) to achieve 200 nm of movement while a myosin expected to travel 10 nm/s requires 0.05 frames/s (20 s intervals). Data can therefore be downsampled at this stage if necessary (see **Discussion** for more details).

2.5. Performing the nonmuscle myosin 2b gliding actin filament assay

NOTE: In this section, the details of the full-length nonmuscle myosin 2b gliding assay are provided. The nonmuscle myosin 2b gliding actin filament assay protocol is different from the myosin 5a protocol at certain steps. Ensure that the correct buffers are used for each of these steps. For example, the NM2b assay requires attachment of myosin to the coverslip in high salt buffer while the M5a can be attached to the coverslip in high or low salt buffers. Additionally, the M5a gliding actin filament assay uses a lower concentration of myosin to mitigate the frequency of actin filaments breaking apart during acquisition.

2.5.1. Prepare the solutions for NM2b as described in **Table 2** and keep them on ice.

2.5.2. Flow in 10 µL of the nonmuscle myosin 2b (0.2 µM) in 500 mM Motility Buffer (MB) ("high salt" buffer) and 1 mM dithiothreitol (DTT) through the flow chamber and wait for 1 min.

NOTE: The high salt buffers dissociate myosin filaments and allow for the attachment of single myosin molecules to the surface, as nonmuscle myosin 2b can polymerize into filaments at ionic concentration <150 mM.

2.5.3. Flow in 10 µL of the 1 mg/mL bovine serum albumin (BSA) in 500 mM MB with 1 mM DTT ("high salt" buffer) as described in **Table 2**. Repeat this wash two more times and wait for 1 min after the third wash. Use the corner of a tissue paper or filter paper to wick the solution through the channel.

2.5.4. Wash with 10 µL of 500 mM MB with 1 mM DTT as described in **Table 2**. Repeat this wash two more times.

2.5.5. Flow in 10 µL of the black actin solution as described in **Table 2** to eliminate "dead heads," as discussed further in the **Discussion** section. The black actin solution contains 5 µM of unlabeled F-actin, 1–10 nM MLCK, 1 mM ATP, 0.2 mM $CaCl_2$, 1 µM CaM, and 1 mM DTT in 50 mM NaCl motility buffer to phosphorylate the nonmuscle myosin 2b on the surface of the chamber.

2.5.5.1. Pipette the solution with a 1 mL syringe and 27 G needle to shear the actin

filaments before introducing the solution to the chamber. Repeat this step two more times and wait for 1 min after the third time. Approximately, 20 pipetting events are sufficient.

2.5.6. Flow in 50 µL of 50 mM MB with 1 mM DTT and 1 mM ATP to deplete the chamber of free actin filaments.

2.5.7. Wash with 10 µL of 50 mM MB with 1 mM DTT. Repeat this wash two more times to deplete the chamber of any ATP.

2.5.8. Flow in 10 µL of 20 nM Rh-Actin solution containing 1 mM DTT in 50 mM MB and wait for 1 min to allow rigor binding of actin filaments to the nonmuscle myosin 2b attached to the surface of the coverslip.

2.5.9. Wash with 10 µL of 50 mM MB with 1 mM DTT to wash away Rh-Actin filaments not bound to the surface. Repeat this wash two more times.

2.5.10. Flow in 30 µL of Final Buffer. For nonmuscle myosin 2b gliding actin filament assay, the Final Buffer also includes calmodulin, $CaCl_2$, and myosin light chain kinase to provide full phosphorylation of the nonmuscle myosin 2b during video imaging. 0.7% methylcellulose can also be included in the Final Buffer if actin filaments are only loosely bound or are not bound to the surface. This is discussed further in the **Discussion** section.

2.5.11. Record images on a fluorescence microscope using an excitation wavelength of 561 nm. An appropriate exposure time is 200 ms at 1.4 mW laser power for a total acquisition duration of 0.5–3 min.

NOTE: Ensure that the acquisition rate is scaled appropriately to the speed of the moving filaments. An important consideration before collecting data for use with tracking programs is the acquisition frame rate. Subpixel movements between frames will result in an overestimate of the velocity, and movements of several hundred nanometers are required to obtain accurate values. An optimal acquisition rate features actin gliding for at least one pixel distance between frames. In the case of the TIRF microscope used for the imaging here, this threshold translates to 130 nm; therefore, a myosin expected to travel 1 µm/s must be imaged at a rate of 5 frames/s (0.2 s interval) to achieve 200 nm of movement while a myosin expected to travel 10 nm/s requires 0.05 frames/s (20 s intervals). Data can therefore be down-sampled at this stage, if necessary (see **Discussion** for more details).

3. Single molecule TIRF assay

3.1. Coverslip preparation

3.1.1. Divide the stock powder into 10 mg aliquots (in 1.5 mL tubes) of methoxy-Peg-silane (mPEG) and 10 mg aliquots of biotin-Peg-silane (bPEG). Store at -20 °C in a sealed, moisture-free container and use within 6 months.

3.1.2. Load eight No. 1.5H (high precision) thickness 22-mm square coverslips onto a rack and wash with 2–5 mL of 200-proof ethanol followed by 2–5 mL of distilled water. Repeat this washing step, ending with water. Then, dry the coverslips completely using an air-line or N_2 and plasma-clean with argon for 3 min.

3.1.3. Place the clean coverslips on filter paper (90 mm) in a tissue culture dish (100 x 20 mm) and incubate in a 70 °C oven while performing the following steps.

NOTE: The plasma cleaning can be replaced with other chemical cleaning methods[49].

3.1.4. Prepare 80% ethanol solution with dH_2O and adjust the pH to 2.0 using HCl. Add 1 mL of this to a 10 mg aliquot of mPEG and 1 mL to a 10 mg aliquot of bPEG. Vortex to dissolve, which should not take more than 30 s.

3.1.5. Take 100 µL of the bPEG solution and add 900 µL of 80% ethanol (pH 2.0). This solution is 1 mg/mL bPEG. Then, make a solution of both the PEGs as follows, mixing thoroughly.

3.1.5.1. 200 µL of 10 mg/mL mPEG (final concentration: 2 mg/mL).

3.1.5.2. 10 µL of the 1 mg/mL bPEG (final concentration: 10 µg/mL).

3.1.5.3. 790 µL of the 80% ethanol (pH 2.0) solution.

3.1.6. Take the coverslips out of the oven. Carefully dispense 100 µL of the PEG solution onto the center of each coverslip, ensuring that only the top surface is wet. Then, place the slips back in the oven and incubate for 20 to 30 min.

3.1.7. When the coverslips begin to take on a holey appearance, with small circles apparent across the surface, remove them from the oven.

3.1.8. Wash each coverslip with 100% ethanol, dry with an air-line, and place back in the oven. Incubate only for the time required to create chambers in step 2.

3.2. Chamber preparation

3.2.1. Clean a microscope slide for use in making the chamber. Cut two pieces of double-sided tape, approximately 2 cm in length.

3.2.2. Place one piece along the middle of the long edge of the microscope slide. Ensure that the edge of the tape aligns with the edge of the slide. Place the second piece of tape roughly 2 mm below the first piece of tape such that the two are parallel and aligned.

3.2.3. Take one of the functionalized coverslips from the oven (created in 3.1). Carefully stick the coverslip onto the tape such that the side coated with PEG is face down and making direct contact with the tape, as shown in **Figure 1**. Using a pipette tip, gently press down on the slide-tape interface to ensure that the coverslip has properly adhered to the slide.

3.2.4. Cut the excess tape hanging over the slide with a razor blade. These chambers can be used immediately or placed pairwise into a 50 mL tube and stored in a -80 °C freezer for future use. It is important to store immediately or the surface will degrade.

3.3. Performing the myosin 5a TIRF microscopy assay

3.3.1. Prepare the solutions for myosin 5a inverted motility assay described in **Table 3** and keep them on ice.

3.3.2. Wash the chamber with 10 µL of 50 mM MB with 1 mM DTT.

3.3.3. Flow in 10 µL of the 1 mg/mL BSA in 50 mM MB with 1 mM DTT. Repeat this wash two more times and wait for 1 min after the third wash. Use the corner of a tissue paper or filter paper to wick the solution through the channel.

3.3.4. Wash with 10 µL of 50 mM MB with 1 mM DTT. Repeat this wash two more times.

3.3.5. Flow in 10 µL of the NeutrAvidin solution in 50 mM MB with 1 mM DTT and wait for 1 min.

3.3.6. Wash with 10 µL of 50 mM MB with 1 mM DTT. Repeat this wash two more times.

3.3.7. Flow in 10 µL of biotinylated rhodamine actin (bRh-Actin) containing 1 mM DTT in 50 mM MB and wait for 1 min. For this step, use a large-bored pipette tip and avoid pipetting up and down to minimize shearing of the fluorescent actin filaments to ensure that long actin filaments can be attached to the surface (20–30 µm or longer). An effective alternative is cutting the cone of a standard pipette tip (with an opening of ≈1–1.5 mm).

3.3.8. Wash with 10 µL of 50 mM MB with 1 mM DTT. Repeat this wash two more times.

3.3.9. Flow in 30 µL of Final Buffer with 10 nM myosin 5a added, then immediately load onto the TIRF microscope and record after finding the optimum focus for TIRF imaging modality. Exposure times between 100–200 ms are appropriate at 1.4 mW laser power for the actin and GFP-labeled myosin. An appropriate acquisition time for velocity analysis is 3 min.

3.4. Performing the nonmuscle myosin 2b TIRF microscopy assay

NOTE: In this section, the details of the nonmuscle myosin 2b TIRF assay using polymerized and phosphorylated filaments are provided. Detailed protocol (sections 4.1–4.3) for phosphorylating and polymerizing the nonmuscle myosin-2b in a tube is included.

3.4.1. To phosphorylate the purified NM2b, make a 10x kinase mix with the following conditions: 2 mM $CaCl_2$, 1 µM CaM, 1–10 nM MLCK, and 0.1 mM ATP. This can be brought to volume with 500 mM MB with 10 mM DTT. Add the 10x kinase mix to the myosin at a volumetric ratio of 1:10 and allow this to incubate for 20–30 min at room temperature. Typically, the myosin concentration for this step is 1 µM.

3.4.2. To polymerize the phosphorylated myosin into filaments, lower the salt concentration of the NM2b to 150 mM NaCl. To do so, make a 1x motility buffer (1x MB) with no salt by diluting the 4x MB four times in dH_2O. This 1x MB can be used to lower the salt concentration because the NM2b was frozen in a 500 mM salt buffer.

3.4.3. For every 3 µL of stock NM2b, add 7 µL of 1x MB to lower the salt concentration to 150 mM NaCl and incubate on ice for 20–30 min to form NM2b filaments.

NOTE: The order in sections 4.1–4.3 is not crucial as long as the NM2b is phosphorylated and the final salt concentration is 150 mM. Incubation on the order of 30 min–1 h allows enough time for complete phosphorylation and polymerization.

3.4.4. Prepare the solutions for nonmuscle myosin 2b inverted motility assay described in **Table 3** and keep them on ice.

3.4.5. Wash the chamber with 10 µL of 150 mM MB with 1 mM DTT.

3.4.6. Flow in 10 µL of the 1 mg/mL BSA in 150 mM MB with 1 mM DTT Repeat this wash two more times and wait for 1 min after the third wash. Use the corner of a tissue paper or filter paper to wick the solution through the channel.

3.4.7. Wash with 10 µL of 150 mM MB with 1 mM DTT. Repeat this wash two more times.

3.4.8. Flow in 10 µL of the NeutrAvidin solution in 150 mM MB with 1 mM DTT and wait for 1 min.

3.4.9. Wash with 10 µL of 150 mM MB with 1 mM DTT. Repeat this wash two more times.

3.4.10. Flow in 10 µL of bRh-Actin and wait for 1 min. For this step, use a large-bored pipette tip and avoid pipetting up and down to minimize shearing of the fluorescent actin filaments, to ensure that long actin filaments can be attached to the surface (20–30 µm or longer). An effective alternative is cutting the cone of a standard pipette tip.

3.4.11. Wash with 10 µL of 150 mM MB with 1 mM DTT. Repeat this wash two more times.

3.4.12. Flow in 10 µL of the nonmuscle myosin 2b solution (approximately 30 nM) and wait for 1 min.

3.4.13. Wash with 10 µL of 150 MB with 1 mM DTT. Repeat this wash two more times.

3.4.14. Flow in 30 µL of Final Buffer, then immediately load onto the TIRF microscope and record after finding the optimum focus for TIRF imaging modality. Exposure times between 100–200 ms are appropriate at 1.4 mW laser power for the actin and GFP-labeled myosin. An appropriate acquisition time for velocity analysis is 3 min.

4. Image analysis

4.1. Image analysis for gliding actin filament assay

NOTE: The images can be analyzed using the software and manuals linked in the **List of Materials**. It is important to note that the program described here requires TIFF-stacks for analysis. The process for analyzing the gliding actin filament assay is as follows[50].

4.1.1. Upload raw movie stacks into a specified folder structure and input the top-most directory of the movie folders into the program.

NOTE: The program analyzes the files throughout this directory and subdirectories, treating the lowest-level directories as replicates. Average statistics for each group of replicates will be produced. In this case, a single movie was used for each myosin. When characterizing a novel myosin or investigating a new experimental condition, it is recommended to analyze movies from three field-of-views (FOV) per chamber for a total of three chambers and to repeat this workflow for three preparations of the myosin being investigated.

4.1.2. Use the script “stack2tifs” in conjunction with the user-inputted frame rate to convert each TIFF stack into a folder containing a series of individual TIFF files and a corresponding metadata.txt file containing the start time of each frame. For data not in the TIFF stack format, a conversion must first be applied using software such as those listed in the **Table of Materials**.

NOTE: This script is the part of the software package. The information of the script can be found here: https://github.com/turalaksel/FASTrack/blob/master/bin/stack2tifs

4.1.3. Use the -px parameter, which is the pixel size (in nm) during acquisition. In this case, the pixel size is 130 nm. Use the -xmax and -ymax parameters for scaling the axes for the scatter plot outputs. These correspond to the longest plotted filament length and the maximum plotted velocity (in nm/s).

NOTE: These are estimated values and can be set to higher-than-expected values to ensure data are contained in the plot. Following analysis, the raw data can also be exported for use in other

statistical or graphing software for viewing and analysis.

4.1.4. Use the -minv parameter, which is a minimum velocity cutoff parameter, to define the filaments that are not moving, and can, therefore, be excluded from the analysis. For a slow myosin such as NM2b, this parameter must be low (in this example, 5 nm/s) to avoid cutting out true gliding movements. For a fast myosin such as M5a, this parameter can be higher (in this example, 100 nm/s) to apply a more stringent filter, while retaining the true gliding speed distribution.

4.1.5. Usethe -pt cutoff parameter to identify smooth movement. For each sampling window, a value is calculated equivalent to 100 x Velocity Standard Deviation/Mean velocity. Tracks with higher values than the cutoff, have more variable velocities and are excluded from further analysis. In this example, a cutoff value of 33 was used. Tracks with higher values have more variable velocities and are excluded from further analysis.

4.1.6. Use -maxd to set a maximum allowed linkage distance between frames. This is a calculated frame-to-frame distance moved by the centroid of the filament in units of nm. It can be useful for excluding sporadic movements or incorrect linkage between filaments. In the examples here, the parameter was left on the default value of 2,000 nm.

4.2. Image analysis for TIRF microscopy assay

NOTE: The process for analyzing the single molecule TIRF assay on the imaging software specifically listed in the **Table of Materials** is as follows[29].

4.2.1. Click and drag the recorded microscopy video to the software's workspace to open it[51]. Then, split the acquisition channels. Click on **Image** > **Color** > **Split Channels**.

NOTE: In the event of appreciable stage drift during the acquisition, images must be stabilized to correct instrumental drift on the imaging plane. In this case, no compensation for Z-axis drift was used as the microscope used to obtain this data stabilizes the Z-focus well. To stabilize the image on the image analysis program, install the appropriate stabilizer plug-in that is linked on the **List of Materials**. The image stabilizer assumes fixed positions for the objects in the image and uses a rolling average of the previous frames as a reference. The recommended procedure is therefore to begin with the channel containing images of labeled actin, since this is in a fixed position.

4.2.2. Click on **Plugins**, then find **Image Stabilizer**; ensure that **Translation** is selected and keep the default settings. Check the box next to **Log Transformation Coefficients**. Applying this Log step allows for the calculated shift parameters to be applied to the other channel in the next step. Allow for the process to complete.

4.2.3. Then, open the channel with labeled myosin and apply the stabilization by clicking on **Plugins** > **Image Stabilizer Log Applier**. If images of actin cannot be acquired during the same acquisition due to a requirement for higher rate imaging in a single channel, drift stabilize the

stack of images by selecting a region that contains static objects such as a biotinylated fiducial marker or fluorophores bound non-specifically to the biotin-PEG surface. This region can be cropped from the original stack and stabilized, followed by application of the resulting shift values to the original stack.

NOTE: In practice, the drift observed in motility experiments will be negligible relative to the motion of myosins which move at several hundred nm/s, but for the slowest myosins this becomes an important consideration.

4.2.4. Then, open TrackMate, click on **Plugins**; then, in its dropdown menu click on **Tracking** and finally on **TrackMate**. At this point, the image analysis is subject to optimization based on the parameters of the fluorophore and assay conditions. However, ideal starting parameters are as follows.

4.2.4.1. Calibration settings: keep all of the default values.

4.2.4.2. Detector: LoG detector.

4.2.4.3. Estimated blob diameter: 0.5–1.0 micron.

4.2.4.4. Threshold: 25–200. (This can be determined by clicking on **Preview** after choosing a number to see whether the detected spots match up to the movie and adjusting appropriately.)

4.2.4.5. Initial thresholding: not set.

4.2.4.6. View: HyperStack Displayer.

4.2.4.7. Set filters on spots: not set.

4.2.4.8. Tracker: Simple LAP tracker.

NOTE: These depend on frame rate and myosin velocity and must be large enough to connect subsequent positions while excluding unwanted connections between different particles.

4.2.4.9. Linking max distance: 1.0 micron.

4.2.4.10. Gap-closing max distance: 1.0 micron.

4.2.4.11. Gap-closing max frame gap: 1.

4.2.4.12. Set filters on tracks: Track Displacement (>0.39—to include only spots moving more than 3 pixels), Spots in tracks (>3—to include only tracks with at least 3 spots). Other filters such as Minimal Velocity may be introduced to exclude spots that stall for long periods. The results of filtering must be checked by visual inspection of tracks to ensure that spurious tracks

(i.e., myosin movement in the background that is not along an actin track) are removed while retaining the tracks associated with actin.

4.2.5. Once the **Display Options** screen comes up, click on **Analysis** for the relevant outputs. Save the three tables produced (Track Statistics, Links in Track Statistics, and Spots in Track Statistics). The **Track Statistics** table will contain the velocity and displacement data that can then be subsequently analyzed to characterize a novel protein or the effects of a certain experimental condition, for example.

REPRESENTATIVE RESULTS:

The purification of myosin can be evaluated by performing reducing sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel-electrophoresis as shown in **Figure 2**. While this figure represents the final, post-dialyzed myosin, SDS-PAGE can be performed on aliquots from the various stages of the purification procedure to identify any products lost to the supernatant. Myosin 5a HMM has a band in the 120–130 kDa range and the full-length nonmuscle myosin 2b has a band in the 200–230 kDa range, corresponding to heavy chains[29,44]. The myosin 5a also has a band near the 17 kDa mark, marking calmodulin. The nonmuscle myosin 2b has a band at approximately 17 kDa, denoting the ELC. Because a GFP-tagged RLC is present in this NM2b preparation, the RLC appears at approximately 47 kDa; however, an unlabeled RLC will be present at approximately 20 kDa if not tagged with a GFP.

The gliding actin filament assay shown in **Video 1** and **Figure 3** represents the characteristics of an ideal and trackable movie. This gliding actin filament assay features the smooth movement of labeled actin filaments. The black actin wash ensures that the dead myosin heads are removed from the measurement field, further contributing to the overall smooth movement of the actin filaments. The fluorescently labeled filaments are short enough that a single filament does not cross over on itself, which is more optimal for the tracking program. Actin filaments that are too long will cross over other filaments, which can present difficulties to the gliding actin filament assay tracking program. This problem can be avoided by pipetting up and down 10–20 times to shear the actin filaments before loading onto the coverslip.

In the case of NM2b, the use of methylcellulose can significantly improve the quality of the recorded movies as it reduces the diffusion of the actin away from the imaging surface. This is not necessary for M5a because its higher duty ratio allows for a stronger attachment of the actin to the myosin-coated surface. If methylcellulose is used, wicking the solution through the chamber is necessary to ensure the solution flows through. As shown in **Video 2**, when all other conditions are identical except for the exclusion of methylcellulose, the actin filaments do not remain as closely associated with the myosin-coated surface.

Conversely, the goal for the inverted motility assay shown in **Video 3** and **Figure 4** is to introduce surface-tethered fluorescent actin filaments upon which myosin movement can be observed. An important requirement of the inverted assay is to ensure that the myosin movement is consistently observed across the FOV, as shown. The use of a mixture of DTT, glucose, catalase, and glucose oxidase can minimize photobleaching to allow for longer measurements[52].

Furthermore, if the acquisition rate for the assay is low, shuttering the illumination light off between acquiring frames can help with excessive photobleaching. Shuttering of the excitation light can be done via a mechanical shutter, or an acousto-optic tunable filter (AOTF).

FIGURE LEGENDS:

Figure 1: Preparation of functionalized flow-cell chambers. (**A**) Begin with a cleaned microscope slide, two pieces of double-sided tape cut to approximately 2 cm, and a functionalized coverslip. (**B**) Add the tape to the center of the microscope slide. (**C**) Attach the coverslip to the tape with the coating (i.e., nitrocellulose) facing down and gently press on the overlapping regions with the tape using a plastic pipette tip to ensure that the coverslip has adhered to the chamber.

Figure 2: SDS polyacrylamide gel electrophoresis of expressed NM2b and M5a-HMM. (**A**) A representative SDS PAGE gel image for a full-length NM2b heavy chain (≈230 kDa) and GFP-RLC (≈47 kDa) and ELC (≈17 kDa). Gel image reproduced and modified from Melli et al. (2018)[29]. (**B**) A representative SDS PAGE gel image for an M5a-HMM-like heavy chain (≈120 kDa) and calmodulin (≈17 kDa). Note that the gel in this image does not have a GFP inserted to the C-terminal end. A GFP inserted in the myosin heavy chain increases the molecular weight by ≈27 kDa. Gel image reproduced and modified was originally published in the Journal of Biological Chemistry[44].

Figure 3: Gliding actin filament assay results acquired via TIRF illumination. (**A**) Example frame from a movie showing translocation of rhodamine-phalloidin labeled actin filaments (in red) on 0.2 µM NM2b in the presence of 0.7% methylcellulose at 30 °C. Scale bar = 10 µm. (**B**) Filament tracking image output from the FASTrack program for NM2b for the same FOV as shown in (**A**) Scale bar = 10 µm. (**C**) Representative histogram of the acto-NM2b gliding velocity, showing that this sample of NM2b can generate an actin gliding velocity of 77 ± 15 nm/s (mean ± standard deviation; number of tracks = 550). (**D**) Example frame from a movie showing translocation of rhodamine-phalloidin labeled actin filaments (in red) on 75 nM M5a-HMM. Scale bar = 10 µm. (**E**) Filament tracking image output by the FASTrack program for M5a-HMM for the same FOV as shown in (**D**) Scale bar = 10 µm. (**F**) A representative histogram of the acto-M5a-HMM gliding velocity, showing that this sample of M5a can generate an actin gliding velocity of 515 ± 165 nm/s (mean ± standard deviation; number of tracks = 25098).

Figure 4: Inverted assay results acquired via TIRF illumination. (**A**) A representative FOV from a two-channel merged image stack showing the movement of NM2b filaments (displayed in green) on biotinylated actin filaments labeled with AF647-phalloidin (displayed in blue) at 30 °C. Polymerized filaments of recombinantly expressed and purified NM2b co-expressed with ELC and GFP-RLC are observed as the green, elongated particles in the FOV. Scale bar = 10 µm. (**B**) Representative histogram of the velocity of NM2b filaments. Analysis was performed using the image analysis software described in the **Table of Materials**. Single NM2b filaments have a velocity of 84 ± 22 nm/s (mean ± standard deviation; number of particles tracked = 133), when moving along single actin filaments. (**C**) Example kymograph of the NM2b filament motion along a single actin filament. Note that some of the regions of the particle shows a “rotation” of the NM2b filament, along the actin filament, which most likely represents the time when one side of the bipolar NM2b filament detaches from the actin filament, as shown previously in Melli et al.[29].

(**D**) A representative FOV of the single molecule movement M5a-HMM (displayed in green) on biotinylated actin filaments labeled with rhodamine-phalloidin (displayed in red). Scale bar = 10 µm. (**E**) Representative histogram of run length of M5a-HMM, fit to a single exponential. Analysis was performed using the image analysis software described in the **List of Materials**. The characteristic run length is 1.3 µm with a 95% confidence interval of 1.23–1.42 µm in this example. (**F**) Representative histogram of single molecule M5a-HMM velocity on single actin filaments. Analyzed data output from image analysis shows a mean velocity of 668 ± 258 nm/s (mean ± standard deviation; number of particles tracked = 684). (**G**) Example kymograph of single molecules of M5a-HMM motion along a single actin filament.

Video 1: Comparison of NM2b and M5a-HMM gliding actin filament assay. The NM2b gliding actin filament assay was performed in the presence of methylcellulose (left; video panel A) and the M5a-HMM in the absence of methylcellulose (right; video panel B) at 30 °C. Note that the time stamp advances faster in the NM2b video panel, compared to the M5a-HMM video panel to show the movement of the rhodamine-labeled actin filaments (red) that is approximately the same. This is since the actual actin translocation velocity of NM2b is close to 7 times slower than that for M5a-HMM (77 nm/s, versus 515 nm/s, respectively, extracted from the Gaussian peak fit to the histogram in **Figure 3**). Scale bar = 10 µm in both video panels. NM2b data acquired at 0.33 frames per second with 200 ms exposure. M5a-HMM data acquired at 5 frames per second with 200 ms exposure (continuous) and subsequently down-sampled to 1 frame per second. Timestamps were added using the plugin described in the **List of Materials**.

Video 2: Gliding actin filament assay of NM2b in the absence of methylcellulose. When all other conditions are the same except for the absence of methylcellulose, the actin filaments sometimes do not stick well to the coverslip coated with 0.2 µM NM2b, leading to lower-quality movies with actin filaments "flopping" close to the surface of the NM2b coated coverslip. Scale bar = 10 µm. This can be resolved by introducing methylcellulose to show the smooth motion of the actin filaments, as shown in the left video panel of Video 1 (NM2b gliding actin filament assay). Another alternative is to increase the NM2b concentration to ≈1 µM. This movie was acquired at 0.33 frames per second with 200 ms exposure.

Video 3: Comparison of NM2b and M5a-HMM inverted motility assay. The NM2b inverted motility assay was performed in the presence of methylcellulose and recorded at a rate of 0.33 frames per second with the use of a shutter (left; video panel A), Video panel C shows the same FOV as A, but with particles are identified and tracked using image analysis software. Similarly, the inverted motility assay for M5a-HMM in the absence of methylcellulose was recorded at a rate of 5 frames per second (right; video panel B). Video panel D shows the same FOV as B, but with particles identified and tracked using image analysis software. Scale bars = 10 µm in all video panels. The two lasers were toggled back and forth with the use of a single camera for acquisition.

Table 1: Buffers used in protein purification.

Table 2: Buffers used in gliding assay.

Table 3: Buffers used in TIRF assay.

DISCUSSION:

Presented here is a workflow for the purification and in vitro characterization of myosin 5a and nonmuscle myosin 2b. This set of experiments is useful for quantifying the mechanochemical properties of purified myosin constructs in a fast and reproducible manner. Although the two myosins shown here are just two specific examples out of the many possibilities, the conditions and techniques can be applied, with some tailoring, to most myosins and to many other motor proteins.

The protocols discussed here are subject to variations depending on the individual needs of the lab and experiments. For example, as discussed in the Expression and Molecular Biology section, the proteins used in this paper were generated from a co-infection of two or more viruses; however, successful protein expression can also be achieved with multi-expression vectors such as the p-FastBac dual expression vector or the BiGBac expression system[53].

Several factors can hamper the successful production of the recombinant protein. To prevent protein degradation, it is imperative that every step of the protein purification is completed at the appropriate temperature, with all centrifugation steps occurring at 4 °C and all other steps being performed on ice. On inspecting a gel of the final, post-dialysis protein product, excess bands may be apparent. This could be indicative of degradation or contamination. Subsequent purification via size-exclusion or ionic-exchange chromatography can enhance the purity of the samples[54]. To that effect, it is recommended to save aliquots at each step of the protein purification process for troubleshooting, should there be a low yield of myosin or suspected protein contamination. Sometimes, there can be inadequate binding of the lysate to the resin in step 1.8, which can result in the loss of the myosin to the supernatant in subsequent centrifugations. This can be resolved by varying the duration of resin binding during this step, even leaving it to bind overnight, if necessary. Longer incubation times introduce a greater risk of protein degradation if contaminant proteases are not sufficiently inhibited, and myosins with proteolytically sensitive regions will be adversely affected. Additionally, improper washing of the resin both before and after use may result in the elution of an undesired protein product, so it is imperative that the proper protocols are followed before and after using the FLAG-affinity resin. If the resin is washed immediately after use and stored appropriately, it can be reused up to 20 times.

It is important to note that protein degradation also occurs during the expression stage and shortening expression times may be advantageous in terms of degradation, although this can be at the expense of the total yield. Following the procedures outlined here to monitor the protein sample at different stages of the protocol (i.e., before, during, and after purification) will help to determine the stages necessary for optimization. For myosins that repeatedly resist successful expression and purification, common problems can be co-expression with insufficient or inappropriate light chains as well as improper folding during overexpression. Appropriate light chains must be selected based on known interactions when possible and heavy chain to light chain baculovirus ratios must be tested in small-scale experiments to determine the optimum.

For myosins that aggregate or yield little or no soluble active product, co-expression with chaperones can aid in successfully obtaining active protein[54,55].

Purified myosin products inevitably contain a small population of damaged myosin, referred to as “dead heads,” which can be addressed in two ways. One method, outlined in this protocol, involves flowing unlabeled, or “black”, actin through the chamber in the gliding actin filament assay. Subsequently washing with ATP causes functional myosins to dissociate from the black actin while dead heads will remain bound to this unlabeled actin due to their inability to hydrolyze ATP and due to their high affinity. While performing the black actin wash, a syringe can be used to shear the actin effectively. Additional shearing can be accomplished by vortexing, provided that any resultant bubbles are removed by centrifugation. An alternative method is to selectively pellet the dead heads from the myosin sample by mixing myosin with F-actin and Mg-ATP at high salt (0.5 M) concentrations and sedimenting in a table-top ultracentrifuge. The myosins capable of hydrolyzing ATP under these conditions do not stay bound to the actin due to their low affinity for actin under these high salt conditions and are found in the supernatant, whereas the myosin dead heads remain bound to the actin in the pellet[56]. Similarly, a sedimentation with actin and resuspension of the pellet can also be used to remove myosins which are incapable of binding to actin in the absence of ATP. Note that a small proportion of this type of dead heads will have less of an impact on these types of assays. By doing a nucleotide-free sedimentation and resuspension followed by an ATP-bound centrifugation and resuspension, myosins that are competent for both actin-binding and ATP-dependent release from actin can be isolated.

Motility assays can also be modified in several ways. For example, in the gliding actin filament assay for the NM2b, the NM2b is phosphorylated in the chamber via the addition of MLCK, calmodulin, calcium, and ATP in the black actin step, as well as in the Final Buffer. However, the NM2b can also be phosphorylated in a tube, before performing the assay. By doing so, the percent of phosphorylated NM2b can be quantified by running a native gel with a urea-containing sample buffer or performing mass spectrometry[57,58]. The effect of temperature on myosin activity can also be investigated. This can be accomplished by employing an objective heating system on the microscope or an environmental enclosure, so that the flow cell is maintained at constant temperature. Ionic strength is another important consideration. For many myosins, actin affinity and enzymatic activity will be increased at lower ionic strength; for others, higher ionic strength is necessary[59]. In addition to providing valuable information about the myosin mechanism, lowering the ionic strength can enhance motility and make myosin more accessible to investigation with many assays. In contrast, some motors will exhibit electrostatic tethering effects, which will slow motility at lower ionic strengths. Finally, when assaying the movement of NM2b filaments, it is crucial to maintain ionic strength within a narrow range (150–200 mM ionic strength), approximating those found in most cell types. The use of lower ionic strengths results in aggregation of the myosin filaments, while the filaments depolymerize at higher ionic strengths.

With many myosins, particularly those with low duty ratios, the conditions of the final buffer given for M5a would result in the fluorescently labeled actin filaments being only loosely bound

to the surface or dissociating altogether. This results in erratic movements that complicate quantification. Better quality movement can often be obtained using methylcellulose (0.7%) in the Final Buffer. Methylcellulose is a viscous crowding agent and forces actin filaments to remain close to the surface even when the density of attached myosin motors is sparse[60]. Similarly, it has been observed that the inclusion of methylcellulose in the final buffer of the single filament motility assay is necessary to observe movement with NM2a, and the same phenomenon was reported for smooth muscle myosin filaments[29,61]. This also increases the processivity of NM2b filaments. One potentially unwanted side effect of using methylcellulose in this assay is that the crowding agent properties can promote the lateral association of myosin filaments into bundles. Alternatives to methylcellulose when troubleshooting a lack of movement or loosely bound actin filaments in the gliding actin filament assay are to lower the salt concentration in the motility buffers or to increase the myosin surface density. As stated above, a high ionic strength in the motility buffers has been shown to lower the ability of some myosins to bind to actin[29,34,62].

Another variation of the gliding actin filament assay is the use of antibodies to anchor the myosin onto the glass coverslip. For example, if a GFP is present at the C-terminal end of the myosin construct, an anti-GFP antibody can be used to fix the GFP-myosin to the coverslip[36,63,64]. This can aid with obtaining successful motility in situations where the geometry of the system may otherwise hamper actin translocation, such as in the case of testing artificial or short lever arms[54,64]. Additionally, the effect of load on translocation velocities can be investigated in the gliding actin filament assay by employing actin-binding proteins such as α-actinin or utrophin[39,50,65]. Such a measurement can be useful to compare the effect of load on an ensemble of myosins versus the load-dependent kinetics of a single myosin that can be measured using an optical trapping assay[66,67]. This can be accomplished by adding increasing amounts of an actin-binding protein along with the myosin in the initial step. The actin-binding protein binds to the surface and exerts a frictional load on the actin filaments that are being moved by myosins, which results in a graded velocity as the concentration of actin-binding protein on the surface is increased[39].

The single molecule/ensemble motility assay can also be adapted to investigate the effect of various actin structures on myosin movement. For example, rather than observing myosin movement on top of single actin filaments, fascin- or α-actinin-mediated actin bundles can be studied as an in vitro reconstitution of the actin filament network found in cells[68,69]. The effect of actin-binding proteins such as tropomyosin can also be studied[65,70–72].

Of note is the versatility in choosing a label for the single molecule/ensemble motility assay. In this report, a GFP label was used on both the M5a-HMM and NM2b; however, many other labels can be used. Examples include HaloTag or SNAP-tag, which can be genetically fused to the myosin and covalently bind a synthetic dye. The benefit of HaloTag technology lies in its versatility for several experimental adaptations, such as labeling with different colors or adding a biotin affinity tag[29,73]. Additionally, the use of quantum dot technology can be employed to improve the resolution of single molecule fluorescence tracking, which also addresses the limitation of GFP's low brightness and the tendency for photobleaching[74]. Tags can be successfully attached to light chains as well as the heavy chain[11,75,76].

For achieving success in the single molecule TIRF motility assay, a key factor is using a well blocked and functionalized surface. A simple method to achieve moderate blocking is to use biotinylated-BSA bound to a nitrocellulose surface. Although this will work well enough to characterize many motors, including M5a, the level of nonspecific binding on such a surface is prohibitive for reproducing clean movement with samples such as NM2b. A key breakthrough in this regard was the transition to PEGylated surfaces doped with biotin-PEG for functionalization[77]. The PEG surfaces provide a far superior level of surface blocking and a defect-free PEGylated surface can remain free of nonspecific binding for very long periods of time. The specific protocol detailed here allows the production of biotinylated PEG surfaces in a matter of hours and if immediately stored as described, the surfaces can be used for several weeks with only a marginal decline in quality.

A key consideration before collecting data for tracking is the acquisition frame rate. Movement between subsequent frames must be large enough to avoid oversampling errors. High sampling rates will yield overestimated velocities due to the division of localization errors by a small time interval and increase the apparent error of the measurement. In cases where the raw data is too finely sampled, the data can be down-sampled by taking every Nth frame to create a new stack and considering the change in frame rate that results. Subpixel movements between frames must be avoided and movements of several hundred nanometers are required to obtain accurate values. In all cases where a new sample is being characterized, the results generated by automated analysis must be compared to a small dataset of manually tracked filaments for consistency.

When analyzing data from single molecule motility experiments, care must be taken when choosing which parameters to measure, how to filter data, and how to fit data. As stated above, the sampling rate can be an important factor when analyzing velocity data. For many myosins, processive runs will be short and well approximated by a straight line. In such cases, the start to endpoint distance of the track may provide a good measure of the run length and this can be divided by the duration of the track to yield a good estimate of velocity. In cases where the tracks are very long and follow curved paths around bent filaments, this type of analysis will yield inaccurate results and a total distance traveled must be used, using an acquisition rate that allows for successive localization points to be sufficiently well spaced to avoid oversampling errors as described above, while being close enough together that the straight line distance between them remains a good approximation of the curve between those points. In addition, for motor proteins with long run lengths in relation to the length of the track, additional statistics such as the Kaplan-Meier estimator must be made when calculating run lengths[78]. The same is true for situations in which photobleaching is sufficiently likely to occur before the end of a processive run. Another phenomenon that can be observed in single molecule fluorescence studies is photoblinking, in which fluorophores switch between the on and off state rapidly and appear to blink. This typically does not occur in these motility experiments; however, if this does occur, the laser intensity and exposure times can be decreased which should minimize the effect. Several chemicals, including β-mercaptoethanol, Trolox, cyclooctateraene, n-propyl gallate, 4-nitrobenzyl alcohol, and 1,4-diazabicyclo[2.2.2]octane can be utilized to mitigate this as well[79].

In summary, this article presents detailed protocols that are robust in their ability to quantify mechanochemical properties such as actin translocation velocity, myosin translocation velocity, and myosin run length. These assays are reproducible and can be used to determine the quality of the purified myosin even in situations where the motile characteristics are not the specific end goal of the study. In addition, changes such as pH, temperature, and chemical regulators can be introduced to these assays to examine how the mechanochemistry of the studied myosin is affected. Taken together, the actin gliding and inverted motility assays can allow for a better understanding of myosin ensemble behavior and intermolecular variations in molecular motor mechanics and kinetics. The fluorescence microscopy-based assays described here support a reductionist's approach to cytoskeletal research and can be a powerful tool to understand protein-protein dynamics in vitro. Together, data collected from these highly controlled experiments can be used to advise mechanobiologists of key actomyosin behaviors that may be relevant at the cell biological level, and beyond.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

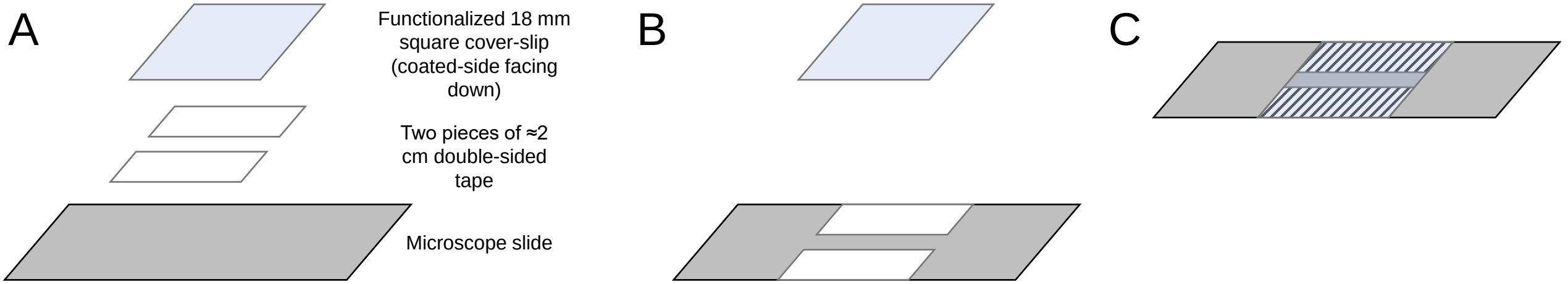


Figure 2

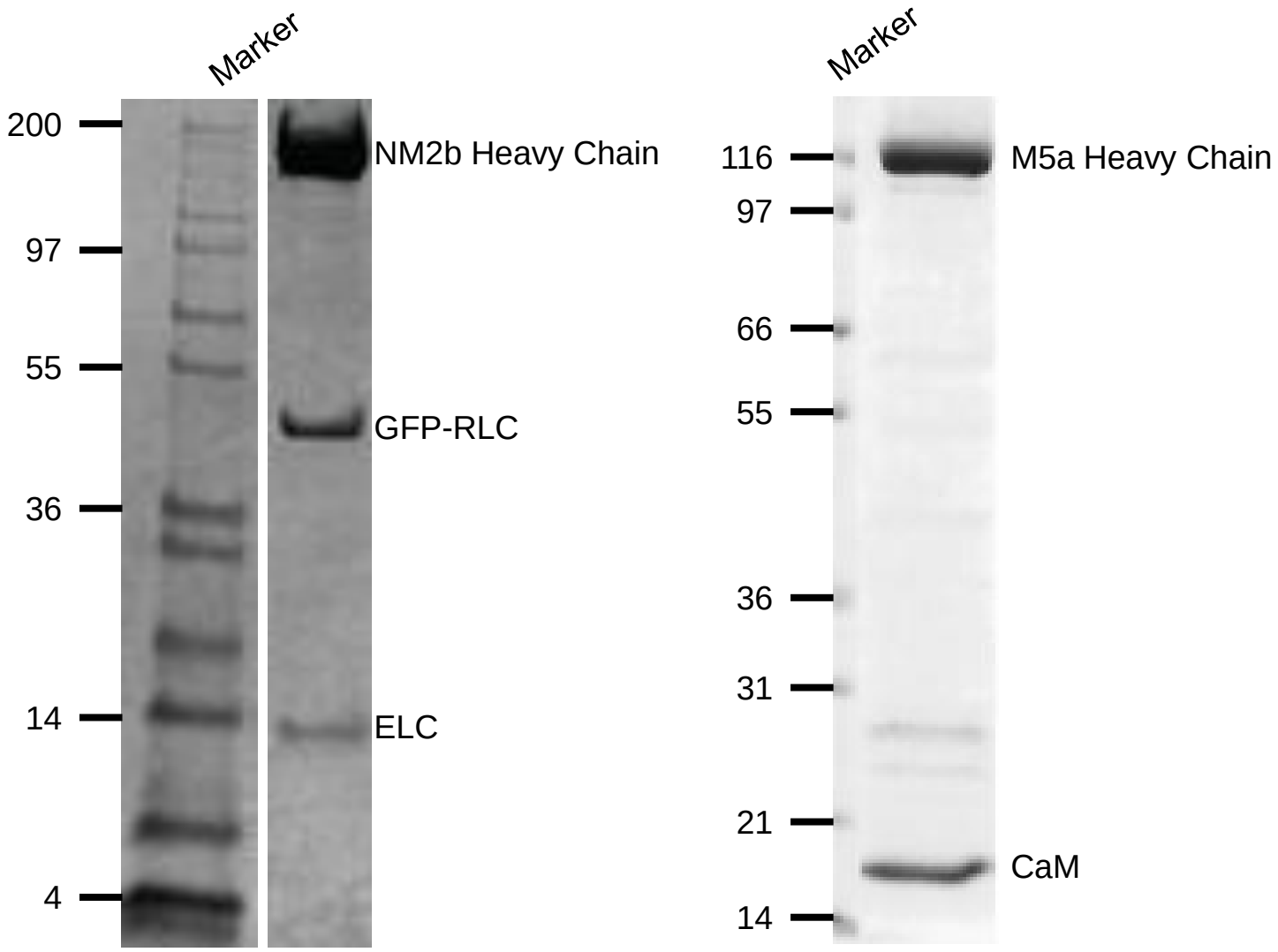


Figure 3

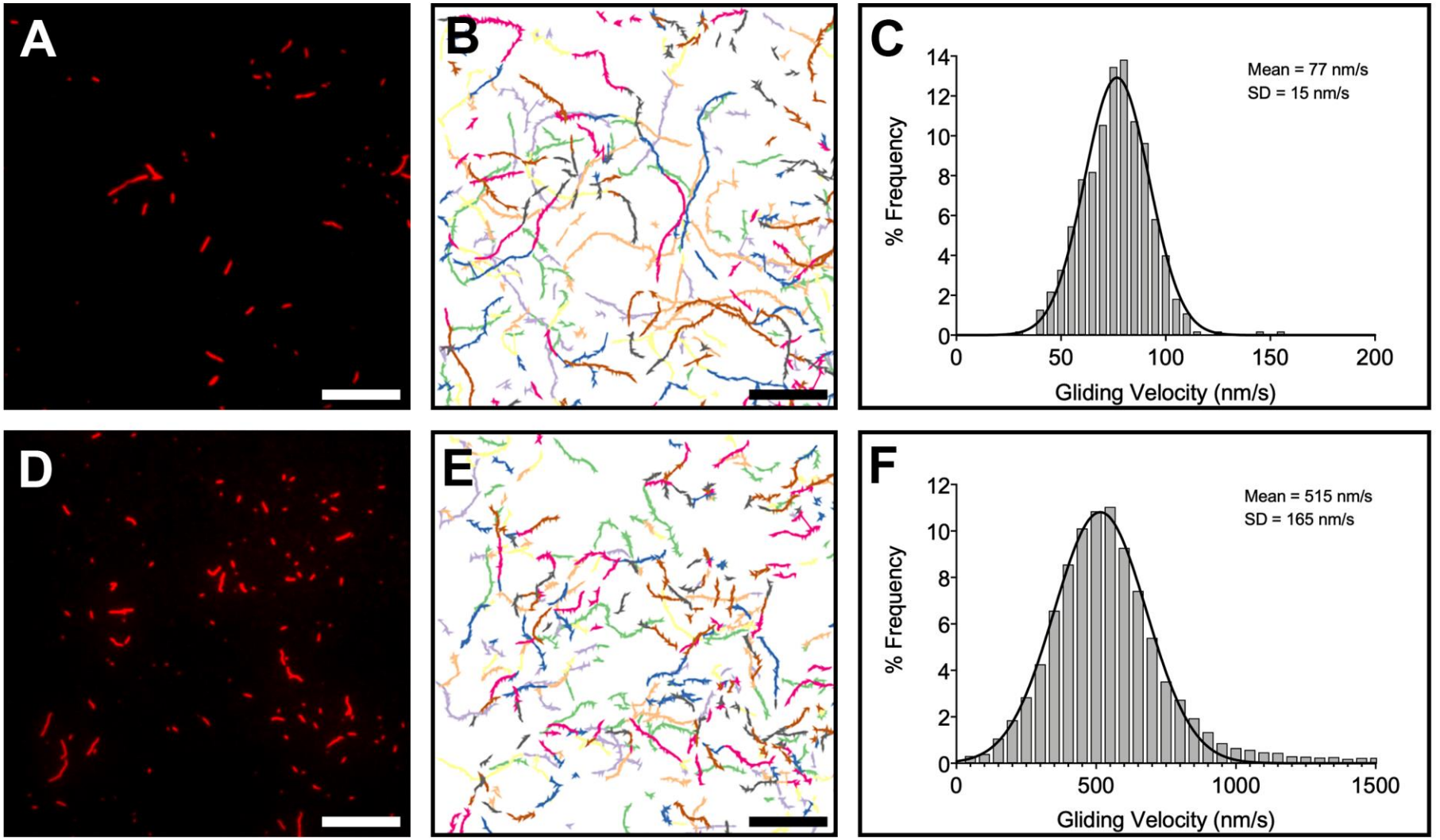
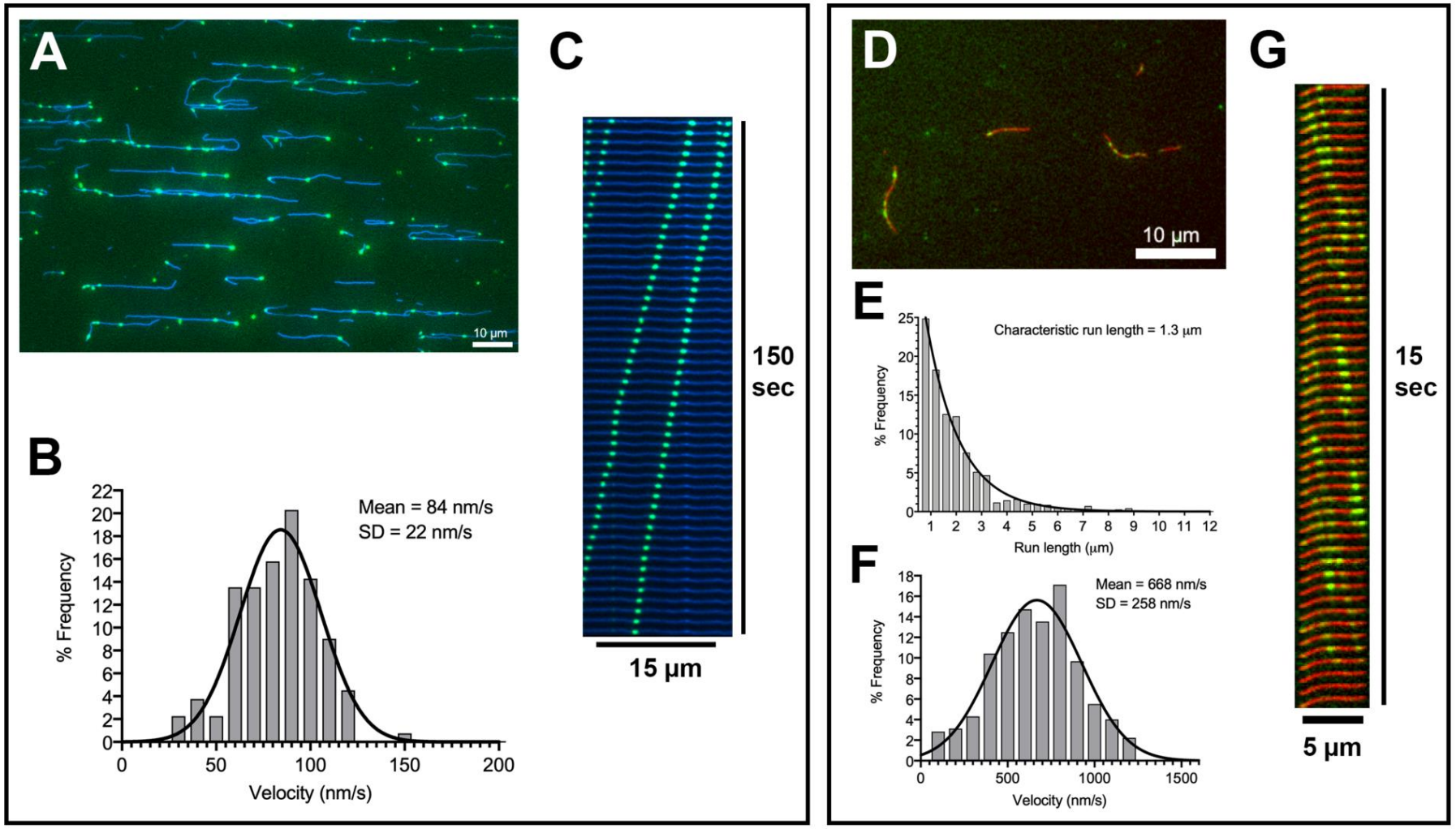


Figure 4



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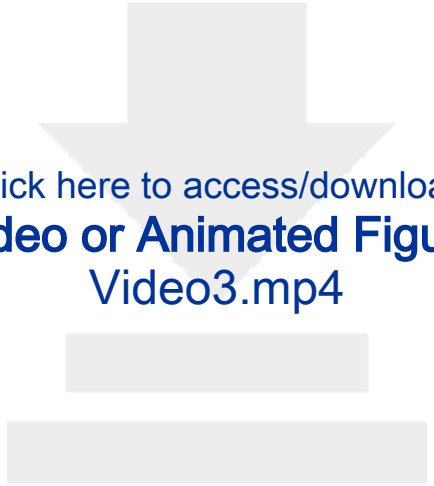

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Table 1 Click here to access/download;Table;Table 1.xlsx

Buffer Name	Composition	Step(s) Used
M5a Extraction Buffer	0.3 M NaCl	1.1
	15 mM MOPS, pH 7.2	
	15 mM $MgCl_2$	
	1.5 mM EGTA	
	4.5 mM NaN_3	
NM2b Extraction Buffer	0.5 M NaCl	1.1
	15 mM MOPS, pH 7.2	
	15 mM $MgCl_2$	
	1.5 mM EGTA	
	4.5 mM NaN_3	
Buffer A	0.5 M NaCl	2.2
	10 mM MOPS, pH 7.2	
	0.1 mM EGTA	
	3 mM NaN_3	
	1 mM ATP	
	1 mM DTT	
	5 mM $MgCl_2$	
Buffer B	0.5 M NaCl	2.3
	10 mM MOPS, pH 7.2	
	0.1 mM EGTA	
	3 mM NaN_3	
	1 mM DTT	
Elution Buffer	0.5 M NaCl	3.1
	0.5 mg/mL FLAG peptide	
	10 mM MOPS, pH 7.2	
	0.1 mM EGTA	
	3 mM NaN_3	
	pH 7.2	
M5a Dialysis Buffer	500 mM KCl	4.1
	10 mM $MgCl_2$	
	10 mM MOPS, pH 7.2	
	0.1 mM EGTA	
	1 mM DTT	
NM2b Dialysis Buffer	25 mM NaCl	4.1
	10 mM $MgCl_2$	
	10 mM MOPS, pH 7.2	
	0.1 mM EGTA	
	1 mM DTT	
NM2b Storage Buffer	0.5 M NaCl	5.1
	10 mM MOPS, pH 7.2	
	0.1 mM EGTA	
	3 mM NaN_3	

Comments
Keep on ice.
Keep on ice.
Keep on ice.
Keep on ice.
Keep on ice.
Use cold dH_2O to bring to volume.
Use cold dH_2O to bring to volume.
Keep on ice.

Table 2 Click here to access/download;Table;Table 2.xlsx

Buffer Name	Composition (M5a)
4X Motility Buffer (4X MB)	80 mM MOPS, pH 7.2
	20 mM $MgCl_2$
	0.4 mM EGTA
	pH 7.4
50 mM Salt Motility Buffer (50 mM MB)	25% v/v 4X MB
	50 mM KCl
	Raise to volume with dH_2O
500 mM Salt Motility Buffer (500 mM MB)	N/A
Myosin	0.05-0.1 µM myosin
	1 mM DTT
	Dilute in 50 mM MB
1 mg/mL Bovine Serum Albumin (BSA)	1 mg/mL BSA
	Dilute in 50 mM MB
	1 mM DTT
5 µM Unlabeled F-actin in 50 mM MB (black actin)	5 µM unlabeled F-actin
	1 µM calmodulin (CaM)
	1 mM ATP
	Dilute in 50 mM MB
MB with 1 mM DTT and 1 mM ATP	1 mM DTT
	1 mM ATP
	Dilute in 50 mM MB
MB with DTT	1 mM DTT
	Dilute in 50 mM MB
20 nM Rhodamine-Phalloidin F-actin (Rh-Actin)	20 nM Rhodamine-phalloidin F-actin
	1 mM DTT
	Dilute in 50 mM MB
Final Buffer	50 mM KCl
	20 mM MOPS, pH 7.2
	5 mM $MgCl_2$
	0.1 mM EGTA
	1 mM ATP
	50 mM DTT
	1 µM calmodulin
	2.5 mg/mL glucose
	100 µg/mL glucose oxidase
	40 µg/mL catalase

Composition (NM2b)	Step(s) Used (M5a/NM2b)
80 mM MOPS, pH 7.2	
20 mM $MgCl_2$	
0.4 mM EGTA	
pH 7.4	
25% v/v 4X MB	
50 mM NaCl	
Raise to volume with dH_2O	
25% v/v 4X MB	
500 mM NaCl	
Raise to volume with dH_2O	
0.2 µM myosin	4.2/5.2
1 mM DTT	
Dilute in 500 mM MB	
1 mg/mL BSA	4.3/5.3
Dilute in 500 mM MB	
1 mM DTT	
5 µM unlabeled F-actin	4.5/5.5
1 mM ATP	
0.2 mM $CaCl_2$	
1 µM CaM	
1–10 nM myosin light chain kinase (MLCK)	
Dilute in 50 mM MB	
1 mM DTT	4.6/5.6
1 mM ATP	
Dilute in 50 mM MB	
1 mM DTT	4.4, 4.7, 4.9/5.4, 5.7, 5.9
Dilute in 50 mM MB	
20 nM Rhodamine-phalloidin F-actin	4.8/5.8
1 mM DTT	
Dilute in 50 mM MB	
0.7% methylcellulose (optional)	4.10/5.10
50 mM NaCl	
20 mM MOPS, pH 7.2	
5 mM $MgCl_2$	
0.1 mM EGTA	
1 mM ATP	
50 mM DTT	
1–10 nM MLCK	
0.2 mM $CaCl_2$	
1 µM calmodulin	
2.5 mg/mL glucose	

100 µg/mL glucose oxidase	
40 µg/mL catalase	

Comments
Vacuum filter and store in 4°C
Vacuum filter and store in 4°C
Vacuum filter and store in 4°C
Keep on ice.
Keep on ice.
Keep on ice. Sheer actin by pipetting up and down 5-10 times, or by using a syringe.
Keep on ice.
Keep on ice.
Keep on ice. Do not vortex.
Add in the glucose, glucose oxidase, and catalase immediately before performing the experiment. Keep on ice.

Table 3 Click here to access/download;Table;Table 3.xlsx

Buffer Name	Composition (M5a)	Composition (NM2b)
4X Motility Buffer (4X MB)	80 mM MOPS, pH 7.2	80 mM MOPS, pH 7.2
	20 mM $MgCl_2$	20 mM $MgCl_2$
	0.4 mM EGTA	0.4 mM EGTA
	pH 7.4	pH 7.4
50 mM salt Motility Buffer (50 mM MB)	25% v/v 4X MB	25% v/v 4X MB
	50 mM KCl	50 mM NaCl
	Raise to volume with dH_2O	Raise to volume with dH_2O
150 mM salt Motility Buffer (150 mM MB)		25% v/v 4X MB
		150 mM NaCl
		Raise to volume with dH_2O
Myosin		30 nM myosin
		1 mM DTT
		Dilute in 150 mM MB
2 mg/mL NeutrAvidin	2 mg/mL NeutrAvidin	2 mg/mL NeutrAvidin
	1 mM DTT	1 mM DTT
	Dilute in 50 mM MB	Dilute in 150 mM MB
1 mg/mL bovine serum albumin (BSA)	1 mg/mL BSA	1 mg/mL BSA
	1 mM DTT	1 mM DTT
	Dilute in 50 mM MB	Dilute in 150 mM MB
200 nM rhodamine-phalloidin biotinylated F-actin (bRh-Actin)	200 nM rhodamine-phalloidin biotinylated F-actin	200 nM rhodamine-phalloidin biotinylated F-actin
	1 mM DTT	1 mM DTT
	Dilute in 50 mM MB	Dilute in 150 mM MB
MB with DTT	50 mM DTT	50 mM DTT
	Dilute in 50 mM MB	Dilute in 150 mM MB
Final Buffer	50 mM KCl	0.7% methylcellulose (optional)
	20 mM MOPS, pH 7.2	50 mM NaCl
	5 mM $MgCl_2$	20 mM MOPS, pH 7.2
	0.1 mM EGTA	5 mM $MgCl_2$
	1 mM ATP	0.1 mM EGTA
	50 mM DTT	1 mM ATP
	1 µM calmodulin	50 mM DTT
	2.5 mg/mL glucose	1–10 nM MLCK
	100 µg/mL glucose oxidase	0.2 mM $CaCl_2$
	40 µg/mL catalase	1 µM calmodulin
	10 nM myosin	2.5 mg/mL glucose
		100 µg/mL glucose oxidase
		40 µg/mL catalase

Step(s) Used (M5a/NM2b)
See "Final Buffer" Recipe/4.12
3.5/4.8
3.3/4.6
3.7/4.10
3.2, 3.4, 3.6, 3.8/4.5, 4.7, 4.9, 4.11, 4.13
3.9/4.14

Comments
Vacuum filter and store in 4°C
Vacuum filter and store in 4°C
Vacuum filter and store in 4°C
Keep on ice.
Keep on ice.
Keep on ice.
Avoid sheering by not vortexing or pipetting up and down. To mix, gently invert.
Keep on ice.
Add in the glucose, glucose oxidase, and catalase immediately before performing the experiment. Keep on ice.

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name	Vendor/Company
REAGENTS	
1 mL Syringe	BD
2 M $CaCl_2$ Solution	VWR
2 M $MgCl_2$ Solution	VWR
27 Gauge Needle	Becton Dickinson
5 M NaCl Solution	KD Medical
Acetic Acid	ThermoFisher Scientific
Amyl Acetate	Ladd Research Industries
Anti-FLAG M2 Affinity Gel	Millipore Sigma
ATP	Millipore Sigma
Biotinylated G-Actin	Cytoskeleton, Inc.
Bovine Serum Albumin	Millipore Sigma
bPEG-silane	Laysan Bio, Inc
Bradford Reagent Concentrate	Bio-Rad
Calmodulin	
Catalase	Millipore Sigma
Cell Line (*Sf9*) in SF-900 II SFM	ThermoFisher Scientific
Circular Filter Paper - Gliding Assay	Millipore Sigma
Circular Filter Paper - Inverted Assay	Millipore Sigma
cOmplete, EDTA-Free Protease Inhibitor Tablets	Millipore Sigma
Concentrating Tubes (100,000 MWCO)	EMD Millipore Corporation
Coomassie Brilliant Blue R-250 Dye	ThermoFisher Scientific
Coverslip Rack	Millipore Sigma
Coverslips: Gliding Acting Filament Assay	VWR International
Coverslips: Inverted Motility Assay	Azer Scientific

Dialysis Tubing (3500 Dalton MCWO)	Fischer Scientific
DL-Dithiothreitol	Millipore Sigma
Double-Sided Tape	Office Depot
DYKDDDDK Peptide	GenScript
EGTA	Millipore Sigma
Elution Column	Bio-Rad
Ethanol	Fischer Scientific
G-actin	
Glucose	Millipore Sigma
Glucose Oxidase	Millipore Sigma
Glycine Buffer Solution, 100 mM, pH 2-2.5, 1 L	Santa Cruz Biotechnology
HaloTag	Promega
HCl	Millipore Sigma
KCl	Fischer Scientific
Large-Orifice Pipet Tips	Fischer Scientific
Leupeptin Protease Inhibitor	ThermoFisher Scientific
Mark12 Unstained Standard Ladder	ThermoFisher Scientific
Methanol	Millipore Sigma
Methylcellulose	Millipore Sigma
Microscope Slides	Fischer Scientific
MOPS	Fischer Scientific
mPEG-silane	Laysan Bio, Inc
Myosin Light Chain Kinase	
NaN_3	Millipore Sigma
NeutrAvidin	ThermoFisher Scientific
Nitrocellulose	Ladd Research Industries
NuPAGE 4 to 12% Bis-Tris Mini Protein Gel, 15-well	ThermoFisher Scientific
NuPAGE LDS Sample Buffer (4X)	ThermoFisher Scientific
Phosphate-Buffered Saline, pH 7.4	ThermoFisher Scientific

PMSF	Millipore Sigma
Razor Blades	Office Depot
Rhodamine-Phalloidin	ThermoFisher Scientific
Sf9 Media	ThermoFisher Scientific
Tissue Culture Dish - Gliding Assay	Corning
Tissue Culture Dish - Inverted Assay	Corning
Smooth-sided 200 µL Pipette Tips	Thomas Scientific

EQUIPMENT	
Centrifuge	ThermoFischer Scientific
Microscope	Nikon
Microscope Camera	Andor
Microscope Environmental Control Box	Tokai HIT
Microscope Laser Unit	Nikon
Mid Bench Centrifuge	ThermoFischer Scientific
Misonix Sonicator	Misonix
Optima Max-Xp Tabletop Ultracentrifuge	Beckman Coulter
Plasma-Cleaner	Diener electronic GmbH + Co. KG
Sonicator Probe (3.2 mm)	Qsonica
Standard Incubator	Binder
Waverly Tube Mixer	Waverly

SOFTWARE	
ImageJ FIJI	https://imagej.net/Fiji/Downloads
FAST (Version 1.01)	http://spudlab.stanford.edu/fast-for-automatic-motility-measurements
Image Stabilizer Plugin	https://imagej.net/Image_Stabilizer

ImageJ TrackMate	https://imagej.net/TrackMate
Imaging Software	NIS Elements (AR package)
	http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html
	File:TrackMate-manual.pdf
	https://github.com/turalaksel/FASTrack
	https://github.com/turalaksel/FASTrack/blob/master/README.md

Reference or Catalog Number	Additional Information
309628	
10128-558	
10128-298	
309623	
RGE-3270	
984303	
10825	
A2220	https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/a2220bul.pdf
A7699	
AB07	
5470	
Biotin-PEG-SIL-3400-1g	
5000006	
PMID: 2985564	
C40	
11496015	http://tools.thermofisher.com/content/sfs/manuals/bevtest.pdf https://tools.thermofisher.com/content/sfs/manuals/bactobac_man.pdf
WHA1001125	
WHA1001090	
5056489001	This should be stored at 4 °C. The tablets can be used directly or can be reconstituted as a 25x stock solution by dissolving 1 tablet in 2 mL of distilled water. The resulting solution can be stored at 4 °C for 1-2 weeks or at least 12 weeks at -20 °C.
UFC910024	The MWCO of the tube is not necessarily "one size fits all," as long as the MWCO is less than the total molecular weight of the protein being purified. The NM2b herein was concentrated with a 100,000 MWCO tube and the M5a was concentrated with a 30,000 MWCO tube.
20278	
Z688568-1EA	
48366-227	
ES0107052	

08-670-5A	The diameter of the dialysis tube can vary, but the MWCO should be the same. The NM2b used herein was dialyzed in an 18 mm dialysis tube. The tubes can be stored in 20% alcohol solution at 4 °C.
D0632	
909955	
RP10586	This can be dissolved in a buffer containing 0.1 M NaCl, 0.1 mM EGTA, 3 mM NaN_3, and 10 mM MOPS (pH 7.2) to a final concentration of 50 mg/mL. This can be stored at -20 °C as 300 µL aliquots.
E4378	
761-1550	These can be reused. To clean, rinse the column with 2-3 column volumes of PBS and distilled water. Chill the column at 4° C before use.
A4094	
PMID: 4254541	G-actin stock can be stored at 200 µM in liquid N_2.
G8270	
G2133	
sc-295018	
G100A	
320331	
P217-500	
02-707-134	
78435	
LC5677	
MX0482	
M0512	
12-553-10	
BP308-100	
MPEG-SIL-2000-1g	
PMID: 23148220	FLAG-tagged MLCK can be purified the same way that the FLAG-tagged myosin was purified herein.
S8032	
31050	
10800	
NP0323PK2	
NP0007	
10010023	

78830	PMSF can be made as a 0.1 M stock solution in isopropanol and stored in 4 °C. Isopropanol addition results in crystal precipitation, which can be dissolved by stirring at room temperature. Immediately before use, PMSF can be added dropwise to a rapidly stirring solution to a final concentration of 0.1 mM.
397492	
R415	Stock can be diluted in 100% methanol to a final concentration of 200 µM.
12658-027	This should be stored at 4° C. Its shelf life is 18 months from the date of manufacture.
353025	Each tissue culture dish can hold approximately nine coverslips.
353003	Each tissue culture dish can hold approximately four coverslips.
1158U38	

75006590
Model: Eclipse Ti with H-TIRF system with 100x TIRF Objective (N.A. 1.49)
Model: iXon DU888 EMCCD camera (1024 x 1024 sensor format)
Custom Thermobox
LU-n4 four laser unit with solid state lasers for 405nm, 488nm, 561nm,and 640nm
Model: CR3i
XL2020
393315
System Type: Zepto
4418
Model: 56
TR6E

FAST is available for Mac OSX and Linux based systems.

Dear Editor and Reviewers,

Thank you for your thorough review of our manuscript "*Myosin-specific adaptations of in vitro fluorescence-microscopy based motility assays*". We are grateful to resubmit a revised version of the manuscript in response to your comments. We particularly would like to thank reviewers #2 and #3, for their time and effort for a thorough review, addressing issues with our manuscript. We believe that the manuscript is now improved, after incorporating the reviews and comments by all reviewers.

We have addressed all of the concerns of the reviewers and a "point-to-point" response to the reviewer's concerns are attached at the end of this letter. In this response letter, our response to each question is in *Green font* and have also included the text changes that we made in the article in ***bold, italicized text***.

We are very grateful for your time and effort in handling our revised manuscript and we hope that you will find that it has been suitably revised.

Sincerely,

Ananya Tripathi, Charles Bond, Jim Sellers, Neil Billington and Yasuharu Takagi

++

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
    - Thank you. We have corrected this.
2. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
    - Thank you. We have corrected for this throughout the article.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.
    - Thank you. We have corrected for this throughout the article .

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: FAStrack, ImageJ/Fiji (even though these are open-source, please mention in the Table of Materials) etc
    - Thank you. We have corrected for these points to remove the commercial language in the manuscript.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be

written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

- Thank you. We have corrected this throughout the protocol.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
    - Thank you. We have corrected for this, especially in the context of the image analysis section. Specifically, please note that numerical values are variable depending on fluorophores and microscope used, so while we provide our numerical values and thought-processes as an examples for the two types of myosin with different kinetic and mechanical activity, this is not a "one size fits all" solution. However, we have addressed how such parameters are determined throughout the article for easier replication of the protocol.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
    - Thank you. We have corrected this and highlighted the protocol text for the video.

8. As we are a methods journal, please briefly discuss limitations of your technique in the Discussion.
    - Thank you. We have corrected for inclusion of limitations/problems for these techniques. We believe that we have included enough limitations and problems for both protein purification and fluorescence microscopy assays in the ***DISCUSSION SECTION***. The following are examples of limitations/problems that has been included. We have highlighted below the start of the section discussing these limitations, as well as some specific problems.
        - Line 916: ***Several factors can hamper successful production of the recombinant protein.***
        - Line 935-937: ***It is important to note that protein degradation also occurs during the expression stage and shortening expression times may be advantageous in terms of degradation, although this can be at the expense of total yield.***
        - Line 939-941: ***For myosins which repeatedly resist successful expression and purification, common problems can be co-expression with insufficient or inappropriate light chains as well as improper folding during overexpression.***
        - Line 947-948: ***Purified myosin products inevitably contain a small population of damaged myosin, referred to as "dead heads," which can be addressed in two ways.***
        - Line 986-989: ***With many myosins, particularly those with low duty ratios, the conditions of the final buffer given for M5a would result in the fluorescently***

labeled actin filaments being only loosely bound to the surface or dissociating altogether. This results in erratic movements that complicate quantification.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript presents the detailed procedures for three key techniques which are widely used for characterizing myosin motor function in vitro: 1) expression and purification of recombinant myosins using baculovirus-Sf9 system; 2) the in vitro actin-gliding assay (the ensemble motility assay); and 3) the single-molecule motility assay. Except for a few minor issues, this is a well-written technique article.

Major Concerns:
No

Minor Concerns:

1. P5, line 179, "Step 1.8" should be "Step 1.7"
    - Thank you. We have now corrected this. Please see Line 247.

2. P15, line 573, "Figure 5" should be "Figure 4"
    - Thank you for pointing this out. We have now corrected this error. Please see Line 810.

3. P15, line 613, please define the abbreviation "VOF" at its first use.
    - Thank you. We have now defined the abbreviation on Line 677.

4. There are two Figure 3.
    - Thank you for finding this error. We have corrected for this and re-uploaded one figure each for the document labeled “Figure 3” and “Figure 4”.

Reviewer #2:

Manuscript Summary:
The manuscript by Tripathi et al. titled "Myosin-specific adaptations of in vitro fluorescence-based motility assays," provides an experimental protocol for the quantification of myosin 5a and myosin 2b in vitro motility. In general, the manuscript is well-written and will provide a newcomer to the field an excellent resource to perform such measurements. I appreciated the pitfalls and potential solution to problems highlighted in the discussion. I particularly enjoyed the acknowledgment of issues surrounding framerate because this is often overlooked. I have minimal concerns that are meant to improve the readability and impact of the manuscript for non-experts.

Major Concerns:

1. The Introduction should be reorganized to improve access. I would start with a general description of what is common to all myosin molecules (motor domain, lever arm, light chains, calmodulin, tails, ATP hydrolysis etc.) Next introduce the classes of myosin, talk about processivity and duty ratio, and then

move into myo 5a and NM2b. Spend a bit of time talking about how they operate and are regulated, it will come up in the protocol. Finally introduce how we know this knowledge (purified proteins, reductionists in vitro motility assay). Provide experimental rationale for the use of the actin sliding versus inverted assays. You can also use this same logic in the Abstract.

- Thank you for the suggestions. We have rearranged the introduction as advised and believe that now, the introduction provides better access to the readership.

Minor Concerns:

1. Line 143 - why are the tags on different termini? Please provide rationale.
    - Tags are placed either at the N- or C-terminus depending on what it will be used for. Numerous studies have shown that N-terminal tags, in general, do not affect enzymatic properties. Placing a tag on the N-terminus allows one to specifically track the position of the motor domain on actin during single molecule IVM studies. Placing a tag on the C-terminus allows for specific attachment of the myosin to the surface. Note, that our initial attempts to place a FLAG tag on the C-terminus of NM2A resulted in an inability to bind to a FLAG affinity column.
    - We have now addressed these points in the article and included the following sentence (Lines 176-182): ***The cDNA for the myosin of interest must be cloned onto a modified pFastBac1 vector that encodes for either a C-terminal FLAG-tag (DYKDDDDK) if expressing M5a-HMM, or an N-terminal FLAG-tag if expressing the full length molecule of NM2b. C-terminal FLAG-tags on NM2b result in weakened affinity of the protein to bind to the FLAG-affinity column. In contrast, the N-terminally FLAG-tagged protein usually binds well to the FLAG-affinity column. The N-terminally tagged protein retains enzymatic activity, mechanical activity and phosphorylation-dependent regulation.***

2. Line 249 - which molecular weight should you use? If you use the MW for the heavy chain, I don't believe you are determining the concentration of dimers. Please clarify.
    - Thank you for this question. We have now clarified this point about molecular weight of the myosin and corrected the sentence as (Lines 326-329): ***The resulting concentration in mg/mL can be converted into µM of myosin molecules with Equation 2, where M is the molecular weight of the entire protein (including the heavy chains, light chains, fluorophores and all tags).***

3. Line 291 and elsewhere - should you add the method for the dead head spin down? I realize you talk about this in the Discussion but it might be useful in the method because it can be a critical step in the protocol.
    - Thank you for the suggestion to include some details about a "dead head" spin. We have included the following sentence as step 4.5.2 (Lines 415-418): ***To perform the "dead head" spin, a stoichiometric amount of F-actin can be added to myosin in the presence of 1 mM ATP and 1 mM $MgCl_2$ at a salt concentration of 500 mM. This can then be ultracentrifuged at 480,000 x g for 15 min at 4 °C. The dead myosin will be in the pellet.***

4. Line 317 and elsewhere - please prove some examples for frame rates, particularly under your conditions. How many frames total?
    - Thank you. We addressed frame rates here:
        - Lines 439-448: ***Ensure that the acquisition rate is scaled appropriately to the speed of the moving filaments. An important consideration before collecting data for use with tracking programs is the acquisition frame rate. Subpixel movements between frames will result in an overestimate of the velocity, and movements of several hundred nanometers are required to obtain accurate values. An optimal acquisition rate features actin gliding for at least one pixel distance between frames. In the case of the TIRF microscope used for the imaging here, this threshold translates to 130 nm; therefore, a myosin expected to travel 1 µm/s must be imaged at rate of 5 frames/s (0.2 s interval) to achieve 200 nm of movement whilst a myosin expected to travel 10 nm/s requires 0.05 frames/s (20 s intervals). Data can therefore be down-sampled at this stage if necessary (see Discussion for more details).***
        - Lines 880-881: ***NM2b data acquired at 0.33 frames per second with 200 ms exposure. M5a-HMM data acquired at 5 frames per second with 200 ms exposure (continuous) and subsequently down-sampled to 1 frame per second.***
        - Lines 891-892: ***This movie was acquired at 0.33 frames per second with 200 ms exposure.***
        - Lines 894-896: ***The NM2b inverted motility assay was performed in the presence of methylcellulose and recorded at a rate of 0.33 frames per second with the use of a shutter.***
        - Lines 897-899: ***Similarly, the inverted motility assay for M5a-HMM in the absence of methylcellulose was recorded at a rate of 5 frames per second***

5. Line 322 - please provide a few quick lines highlighting the differences and rationale for the differences.
    - Thank you for the suggestion. We have now addressed some of these differences and rational for image acquisition (Lines 453-458): ***The nonmuscle myosin 2b gliding actin filament assay protocol is different from the myosin 5a protocol at certain steps. Ensure that the correct buffers are used for each of these steps. For example, the NM2b assay requires attachment of myosin to the coverslip in high salt buffer whilst the M5a can be attached to the coverslip in high or low salt buffers. Additionally, the M5a gliding actin filament assay uses a lower concentration of myosin to mitigate the frequency of actin filaments breaking apart during acquisition.***

6. Line 335-337 - does not make sense as written.
    - Thank you. We have reviewed the sentence, and rephrased this (Lines 478-482): ***Flow in 10 µL of the black actin solution as described in Table 2 to eliminate "dead heads," as discussed further in the Discussion section. The black actin solution contains 5 µM of unlabeled F-actin, 1-10 nM MLCK, 1 mM ATP, 0.2 mM $CaCl_2$, 1 µM CaM, and 1 mM DTT in 50 mM NaCl motility buffer to phosphorylate the nonmuscle myosin 2b on the surface of the chamber.***

7. Line 661 delete "purified".
    - Please refer to Lines 905-906 for this change.

8. Title - Should the title be "…adaptations of fluorescence-based in vitro fluorescence-based motility assays"?
    - Thank you for this suggestion. Respectfully, we would like to keep our current title: ***Myosin-specific adaptations of in vitro fluorescence microscopy-based motility assays***

Reviewer #3:

Manuscript Summary:

The protocol provide very useful description of step by step execution of important biochemical and biophysical assays in the field of mechanochemistry to successfully characterize conventional and unconventional molecular motors. It provides useful assays adaptation to motor specificity as demonstrated on two kinetically very different myosins. Although the assays are well known by some labs working with molecular motors it can be great source of knowledge to any new in the field. With this in mind I suggest several small but useful details to be addressed in order to further clarify the protocol to be quickly and most importantly correctly adapted to any new research environment with the hope to bring greater consistency and reproducibility in our modern science.

Major and Minor Concerns:

1. Title: It would be clearer and more specific to either say fluorescence microscopy -based (as in summary) or just skip (without fluorescence-based)
    - Thank you for this suggestion. Respectfully, we would like to keep our current title: ***Myosin-specific adaptations of in vitro fluorescence microscopy-based motility assays***

2. 30: walk "along" or better "on"?
    - We have made the suggested change and used the word, “on”. Please refer to Lines 32-33.

3. 33: please add a sentence of some cell function for myosin 2b also (similar to myo5a above)
    - We have now included some cellular function of nonmuscle myosin 2b:
        - Lines 36-38: ***Nonmuscle myosin 2b, along with its other nonmuscle myosin 2 isoforms, has roles including cell adhesion, cytokinesis, and tension maintenance.***
        - Lines 82-83: ***The NM2 isoforms are found in the cytoplasm of all cells and have shared roles in cytokinesis, adhesion, tissue morphogenesis, and cell migration.***

4. 39: you use "fluorescence-based", "fluorescence microscopy-based" and here "fluorescence imaging based". Please chose one term and use it through the entire paper consistently.
    - Thank you for the suggestion. We have changed these different terms to all be “fluorescence microscopy-based” for consistency. These are changed in Lines 25-26, 44, 125, and 1089.

5. 45: why myosin 2s? Is not 2b?
    - We were trying to communicate the plural form of nonmuscle myosin 2, to discuss the general use of the assays to study all nonmuscle myosin 2 *isoforms*, but we have corrected

this to be less confusing. Changes have been made in the abstract to reflect this as (Lines 47-49): ***These techniques can also be applied to study the movement of single filaments of the nonmuscle myosin 2 isoforms, discussed herein in the context of nonmuscle myosin 2b.***

6. 47-48: I think overall is a protocol for both an ensemble (motility assays) and single particle/molecule (and not just that). Please modify accordingly.
    - Thank you for pointing this out. We have modified this sentence to clarify that we address/cover both ensemble and single particle/molecule/filaments in our protocol. We have modified the sentence as (Lines 49-51): ***This workflow represents a protocol and a set of quantitative tools that can be used to study the single molecule and ensemble dynamics of nonmuscle myosins***

7. 54: any specific reason to write Class (also throughout the text) with capital letter?
    - Thank you for picking up on this error. We have changed this to lower case.

8. 59: so several step per encounter…when it dissociates?
    - We have clarified this in the text. The sentence was changed to (Lines 69-70): ***Myosin 5a (M5a) is a class 5 myosin and is a processive motor, meaning that it can take multiple steps along actin before dissociating.***

9. 76: "slow enzymatically" please define/describe/relate what that means
    - We have now addressed this including actual ATPase rates (Lines 85-86): ***NM2b, in comparison to M5a, has a low duty ratio and is enzymatically slower with a V_{max} of 0.2 s^{-1} compared to M5a's V_{max} of ≈18 s^{-1}.***

10. 76: if NM2b is hexamer what is the M5a in this sense?
    - M5a will be a 14-mer, but we have adjusted our language to avoid the use of "polymer terminology" such as monomer, dimer, etc. to reduce confusion. Please see Line 90.

11. 76-79: structure of NM2b: perhaps can be written more clearly and to separate description dimer and description of monomer (you have head domain (singular) but lever-arms (plural), etc.
    - We have adjusted our language to avoid the use of "polymer terminology" such as monomer, dimer, etc. to reduce confusion and have instead used terminology such as "molecules". In this case, we have changed the sentence to (Lines 90-92): ***NM2b contains two myosin heavy chains, each with one globular head domain, two lever-arm regions (each with one ELC and one regulatory light chain (RLC)), and an α-helical coiled-coil rod/tail domain, approximately 1,100 amino acids long, that dimerizes these two heavy chains.***

12. 79: "long" coiled coil: please define/describe/relate what that means
    - We have edited this sentence to clarify the length of the coiled-coil (Line 92): **[…]** ***and an α-helical coiled-coil rod/tail domain, approximately 1,100 amino acids long, that dimerizes these two heavy chains.***

13. 81: activation of what?

- We have removed the word “activation” in this sentence and replaced as follows (Lines 97-100): ***Upon RLC phosphorylation by calmodulin-dependent myosin light chain kinase (MLCK) or Rho-associated protein kinase, the molecule extends and associates with other myosins through the tail region to form bipolar filaments of approximately 30 myosin molecules.***

14. 83: "increased" please provide some approximate quantification e.g. like x-fold
    - We have now clarified this (Lines 100-102): ***The aforementioned phosphorylation of the RLC also leads to increased actin-activated ATPase activity of NM2b by approximately four times.***

15. 83: "filaments" (throughout the text): since the text talks about myosin and actin filaments would be wise to consistently use that two words together so we always easily know about what filaments we are talking
    - We have now clarified this in another sentence (Lines 102-104): ***This bipolar filament arrangement, featuring many myosin motors at each end, is optimized for roles in contraction and tension maintenance, where actin filaments with opposing polarities can be moved relative to each other.***

16. 93: perhaps myosins (plural) better word here
    - We have now addressed this (Lines 110-112): ***To understand actomyosin interactions at a simple protein-protein interaction level, rather than inside of a cell, we can express and purify recombinant myosins for use in in vitro studies.***

17. 110: "robust to perturbations" define/list of some such perturbations? On the other hand it is not that this particular assay is quite sensitive to myosin dead heads at least in comparison to solution kinetics?
    - The gliding actin filament assay is more sensitive to deadheads than is the measurement of the ATPase activity. For example, if you have 5% deadheads the gliding assay would be a mess… There will be (1) lots of immobile actin filaments, (2) many “stop and go” movements, slower overall rates of movement due to the effect of (1) and (2) and furthermore, lots of “shearing” of actin filaments. On the other hand in the ATPase assay you would merely see a rate that is 95% of that of a “perfect” prep and this is probably within the error of your measurement. We have added some discussion on this as well as perturbations to the assay here (Lines 144-152): ***Because each actin filament can be propelled by a large number of available motors, this assay is very reproducible, with the final measured velocity being robust to perturbations such as alterations in the starting myosin concentration or the presence of additional factors in the solution. This means it can be easily modified to study myosin activity under different conditions, such as altered phosphorylation, temperature, ionic strength, solution viscosity, and the effects of load induced by surface tethers. Although factors such as strong-binding myosin “dead heads” incapable of ATP hydrolysis can cause stalled actin filaments, multiple methods exist to mitigate such issues and allow for accurate measurements.***

18. 112: "nitrocellulose" some short discussion about myosin attachment to different surfaces may be of value…i.e. is nitrocellulose the most optimal way for nonmuscle myosins? Are there any other applicable (e.g. TMCS, glass, silica, gold, hydrophobicity …)?
    - We have now addressed this (Lines 134-136): ***This can be accomplished by using nitrocellulose, antibodies, membranes, SiO_2-derivatized surfaces (such as trimethylcholorosilane), amongst others.***

19. 112: "dense" layer: please define what that means? Is there an optimal density to achieve or one can just saturate as much as possible the surface with myosin molecule, or?
    - We have now addressed this (Lines 133-134): ***In this assay, a saturating layer of myosin motors is attached to a coverslip.***

20. 116: velocity is one parameter which can be derived. What about the fraction of motile filament? Can we learn anything from that?
    - We have now addressed this (Lines 139-142): ***Tracking software can be used to provide to correlate the velocity and length of each gliding actin filament. Analysis software can also provide a measure of the number of both moving and stationary actin filaments, which can be useful to determine the quality of a given myosin preparation.***

21. 119: please provide some myosin class examples for both extreme velocities listed.
    - We have now addressed this (Lines 152-154): ***The kinetic properties of myosin vary greatly across classes and, depending on the specific myosin used, the speed of actin filament gliding in this assay can vary from under 20 nm/s (myosin 9), and up to 60,000 nm/s (Characean myosin).***

22. 128: perhaps is useful to provide some myosin concentration range already here for this type of assay
    - We have now addressed this (Lines 163-165): ***Finally, activated and fluorescently labeled myosin (typically 1 – 100 nM) is flowed through the chamber, which is then imaged to observe myosin movement over the stationary actin filaments.***

23. 142: a short reasoning (why different) regarding tag position (C vs N terminal)… optimal position?, myosin specific?, assay specific?, expression specific?...one would intuitively say that it is always good to keep any tag and fusion protein away from catalytic domain to avoid any kinetic consequence. Is any extra aa linker needed between tag and heavy chain?
    - Our discussion revolves around what we used for our protein, but it is important to note that protein design is not necessarily "one size fits all" and will depend on the experimental needs.
    - We have now addressed these points in the article and included the following sentence (Lines 176-182): ***The cDNA for the myosin of interest must be cloned onto a modified pFastBac1 vector that encodes for either a C-terminal FLAG-tag (DYKDDDDK) if expressing M5a-HMM, or an N-terminal FLAG-tag if expressing the full length molecule of NM2b. C-terminal FLAG-tags on NM2b result in weakened affinity of the protein to bind to the FLAG-affinity column. In contrast, the N-terminally FLAG-tagged protein usually binds***

well to the FLAG-affinity column. The N-terminally tagged protein retains enzymatic activity, mechanical activity and phosphorylation-dependent regulation.[7]

24. 142-143 please provide species of those example myosins (human?, etc).
    - We have now addressed these points:
        - Lines 184-185: ***In this paper, a truncated mouse M5a heavy meromyosin (HMM)-like construct with a GFP between the FLAG-tag and the C-terminus of the myosin heavy chain was used.***
        - Lines 190-191: ***The full-length human NM2b construct was co-expressed with ELC and RLC.***

25. 146: if there any extra amino acid linker needed between different fusion proteins (myosin&GFP) and tag (FLAG)?
    - Discussion on linkers can now be found here:
        - Lines 187-189: ***The M5a heavy chain was truncated at amino acid 1090 and contains a three amino acid linker (GCG) between the GFP and the coiled-coil region of the M5a.***
        - Lines 191-194: ***The N-termini of the RLC was fused with a GFP via a linker of five amino acids (SGLRS). Directly attached to the FLAG-tag was a HaloTag. Between the HaloTag and the N-terminus of the myosin heavy chain was a linker made of two amino acids (AS).***

26. 147: please provide ratio between calmodulin and myosin viruses for co-infection.
    - We have now addressed this here (Lines 197-199): ***The volumes of the baculovirus for each subunit depended on the virus' multiplicity of infection as determined by the manufacturer's instructions.***

27. 148: both light chains fused with GFP? Any extra linker needed between light chain aa and GFP? Is this specific for this myosin to use fluorescent light chains or can be done also as in myosin 5 with gfp on heavy chain? Please discuss. Please provide ratio for co-infection with three different viruses.
    - An N-terminal GFP on the heavy chain is also possible for NM2 isoforms, but we use the HaloTag because it offers more flexibility in the fluorophore color. A discussion of various tags can be found here (Lines 1028-1031): ***Examples include HaloTag or SNAP-tag, which can be genetically fused to the myosin and covalently bind a synthetic dye. The benefit of HaloTag technology lies in its versatility for several experimental adaptations, such as labeling with different colors or adding a biotin affinity tag***.
    - We have also fused a GFP to the NM2 tail group as well[9].
    - We have now addressed these points here:
        - Lines 191-192: ***The N-termini of the RLC was fused with a GFP via a linker of five amino acids (SGLRS).***
        - Lines 197-199: ***The volumes of the baculovirus for each subunit depended on the virus' multiplicity of infection as determined by the manufacturer's instructions.***

28. 149: Is there any specific (beside historical) reason that mammalian? myosin are still expressed in insect cells? For as it know posttranslational modifications in insect cells is quite different that in mammalian ones (e.g. glycosylation) and one can expect that expression host can have some effect on recombinant mechanoenzyme activity. Was ever any "expression host effect" tested? Please discuss expression host selection and possible alternatives
    - In our laboratory, we have relied on the insect cell expression for much of our recent work and since this protocol focuses on the description of our laboratory method, we will discuss only the insect cell expression system.
    - We have two cases in our laboratory, where we have tissue purified myosins and compared that to ones that have been recombinantly expressed with the baculovirus/Sf9 cell expression system – nonmuscle myosin 2a and myosin 5a. We have noted no significant differences in regulation, enzymatic activity or rate of motility for these.
    - Respectfully, we will not include any other "possible" methods for protein expression, since this will include much more information than the scope of this article.

29. 152: are pellets first snap frozen in liquid nitrogen and then put on -80? please clarify.
    - We can either snap freeze in liquid nitrogen or put them straight into the -80 °C freezer. We have tested both methods, and there seems to be no difference in yield and enzymatic activity.

30. 157: please provide some storage time. Beside the Tables are not numerated at all (at least in this overall pdf file) and it seems they do not appear in order or/and are scattered on several pages (they were made too big I guess for one page) as in text…very confusing and please make table numerated and that appear in order as in text and as one table per page…or at least all column in one page (landscape paper orientation is ok). So lets say that table 1 is on page 37 (pdf) then it seems by looking only the table that NM2b buffer consist only of cooking salt…is that correct?
    - We apologize for the complication. Indeed, some of the tables that have been uploaded to the JoVE editorial website was too large to be formatted properly.
    - To ensure that the editors and reviewers can see the tables/lists as we would like to be viewed, we have also attached a PDF version of the Excel files which we converted prior to uploading the files, as supplementary files for review. These documents are: Preconverted - Buffer Table - 12-16-2020.pdf and Preconverted - Material List - 12-16-2020.pdf. Preconverted - Buffer Table - 12-16-2020.pdf includes the protein purification buffer table (page 1), gliding assay buffer table (page 2), and the inverted assay buffer table (page 3). Preconverted - Material List - 12-16-2020.pdf includes the reagents list (page 1), equipment list (page 2) and the software list (page3).
    - The storage time for the extraction buffer, containing 15 mM MOPS, 15 mM $MgCl_2$, 1.5 mM EGTA, 4.5 mM NaN_3, and either 0.3 M NaCl (for M5a) or 0.5 M NaCl (for NM2b), which does not contain such reagents such as DTT, PMSF, leupeptin, protease inhibitor tablets, and ATP (i.e. reagents that can "go off") can be quite long. However, we usually need to prepare more of this buffer since it is all consumed (~ 2 liter bottle/preparation) in the laboratory within ~3 months.

31. 161: perhaps some PMSF stock preparation tips and warning about precipitation and inactivation in aqueous solution is useful here, so that once added the protocol must continue, etc. Is PMSF stable alternative Pefabloc compatible with your protocol?
    - We have added the PMSF preparation tips to the List of Materials.

32. 166: is one ice or water-ice mixture better for cooling during sonicating?
    - We use just ice for this sonication process.

33. 166: please provide specific type of the sonicator (not just vendor) which probe or tip (diameter) is usually used for certain volume (e.g. 50 ml) and if possible power/amplitude and frequency in some international units so that sonication can be readily transferred to different sonicator device.
    - We have added the sonicator probe information to the List of Materials.

34. 169: for any new on the filed it is good to provide short reasoning why is good to add ATP here and why just before this centrifugation. Also it is good to mentioned that this centrifugation is done in ultra centrifuge.
    - We have now addressed this. In step 1.5, we included the sentence (Lines 229-232): ***The ATP dissociates active myosin from actin, allowing it to be separated in the following centrifugation step. It is therefore essential to proceed to the next step immediately in order to minimize the possibility for ATP depletion and rebinding to actin.***

35. 172: 5 ml of resin slurry? Or dry resin? If slurry what is the slurry %? 5 ml of resin per how much of cells (volume)?
    - We have now addressed this (Lines 234-238): ***While this is occurring, begin washing 1-5 mL of a 50% slurry of Anti-FLAG affinity resin (for a pellet formed from 1 L of cells) with 100 mL phosphate-buffered saline (PBS), according to the manufacturer's instructions. For example, 5 mL of resin, wash 10 mL of a 50% slurry. In the final wash step, resuspend the resin with 1-5 mL of PBS with enough volume to create a 50% slurry.***

36. 172: Is any difference in final contaminating level of Pi if you use PBS here and not TBS? Let's say you want to do phosphate release measurements…
    - Since we are not performing transient state kinetics in this paper, we are not worried about Pi contamination. Furthermore, during the following steps for the purification, we thoroughly wash with buffers which do not contain PBS. We do not consider the use of PBS at this step to be a problem.
    - As for the use of TBS instead of PBS, we have not used TBS, so we do not have knowledge of commenting about this query.

37. 179: I do not see step 1.8
    - Thank you for pointing this out. We have now corrected for this error.

38. 187: must the resuspension of resin in steps 2.1 2.2 2.3 always be done my inverting the tube as in last part of 2.3? Is this done by hand or a tube mixer? If so what is the time if mixing. Please clarify.

- We have now addressed this (Lines 254-255): ***Then, mix the resin and the buffer thoroughly by gently inverting the tube by hand approximately ten times.***

39. 183: Buffer A, buffer B… the protocol is started by a step of buffer preparation referencing the table. Please do so for the rest of the buffers when they are mentioned by the first time. –
    - We have now addressed this.

40. 190: please provide volume of elution buffer to make (i.e. certain ml per beads volume used or similar metrics.)
    - We prepare approximately 30 ml of the elution buffer (~12 resin volumes since we use ~2.5 ml resin). This is sufficient for completely eluting the protein for a FLAG affinity purification. The volume of the elution buffer is now included in step 3.1 (Line 259).

41. 191: provide type, vendor etc. of elution column
    - We have now added this information to the List of Materials.

42. 192: please provide the volume of buffer B to wash resin
    - We have provided the volume of buffer B (1- 2 column volumes) to wash the resin. Please see Line 262.

43. 201: reaming elution buffer B: please provide some volume estimation. What is the function/use of this remaining elution in 50 ml tube?
    - We recommended making 30 mL of the Elution Buffer, 12 mL of which is used in collecting the 1 mL fractions (which end up being the most concentrated fractions) meaning that there should be approximately 18 mL of Elution Buffer remaining that is used to collect any remaining protein for further concentration. This is addressed here (Lines 273-276): ***In a 50 mL tube, collect the remaining protein by gently pipetting the remaining Elution Buffer through the column, to release any remaining myosin bound to the resin in the column flowthrough. This flow-through will be concentrated in the next step. Ensure that the resin is then regenerated for reuse and stored according to the manufacturer's instructions.***

44. 206: please provide the type/vendor of concentrator with type of membrane used. Is any membrane preferable to work with myosins? Please also state here the kDa of the purified proteins to relate it with the concentrator cut off size. Do you use any specific ratio between membrane size and size of the purified myosin? Curious: Does not use of such large MWCO lead to the great loss of (added) light chains? Is any danger of overconcentration the myosins or to store them too diluted?
    - The vendor/type is in our List of Materials. The light chains remain tightly bound to the heavy chain and do not pass through the membrane. In our laboratory, myosins stored at less than 0.2 mg/ml did not retain activity very well, but this was never done in a controlled manner. We have not run into "overconcentration" problem in our laboratory using the amount of protein purified from the in the baculovirus/Sf9 cell expression system. The pore size specified in the text (100,000 MWCO) allows for retention of the myosin molecules since their molecular masses are in the range of 500,000 Da. We have written the following on the "Additional Information" column of our List of Materials: ***The MWCO of the tube is***

not necessarily "one size fits all," as long as the MWCO is less than the total molecular weight of the protein being purified. The NM2b herein was concentrated with a 100,000 MWCO tube and the M5a was concentrated with a 30,000 MWCO tube.

45. 212: please provide the type/vendor/membrane material of dialysis bag and dialyzer
    - We have now added this information to the List of Materials.

46. 215: step 4.1.1. so it good to say that for NM2b use dialyzing chamber?
    - We use a dialyzing tube because it is easy to collect the myosin filaments with this set-up.
47. 220: is it proper step 5.1?
    - We have clarified the dialysis section as follows (Lines 291-317):

4.1 Make 2 L of Dialysis Buffer, as described in Table 1. Load the sample in a dialysis bag or chamber and dialyze overnight in the cold room. Note that the composition of the dialysis buffers differs for NM2b and M5a.

NOTE: In the case of NM2b, the purpose of this dialysis step is to form myosin filaments in the low ionic strength buffer. Sedimentation of these filaments then provides an additional purification step and allows for concentration of the sample. There will therefore be a visible white precipitate in the dialysis chamber the next day. These filaments will be collected by centrifugation and depolymerized in step 5.1. In the case of M5a-HMM, after the overnight dialysis, the protein may be sufficiently pure for use in subsequent assays. Further purification steps such as gel filtration or ionic exchange chromatography can be performed if required. For M5a recovery after dialysis, go to step 5.2.

5. Recovering Myosin after Dialysis

5.1 For NM2b, carefully unload the entire sample from the dialysis bag or chamber and centrifuge at 4 °C for 15 minutes at 49,000 x g to collect the myosin filaments. Discard the supernatant and incrementally add the Storage Buffer to the pellet as described in Table 1 until it has dissolved. Gentle up and down pipetting helps to solubilize the pellet. Normally, this does not require more than 500 µL per tube. After ensuring that the pellet is fully dissolved in the high ionic strength storage buffer, an additional centrifugation step (15 minutes at 49,000 x g) can be performed to remove unwanted aggregates if required, since the myosin will now be unpolymerized and will remain in the supernatant.

5.2 For M5a-HMM, carefully unload the entire sample from the dialysis chamber and centrifuge at 4 °C for 15 minutes at 49,000 x g in case any unwanted aggregates are present. Take the supernatant.

48. 230: please provide which tips work best and if it is better to cut the tip cone…

- We have added this to the List of Materials. We have addressed the tip considerations in Lines 601-602.

49. 235: single molecule: like a dimer or monomer?
    - We have modified the language to avoid confusion by changing "single-molecules" to "unpolymerized" (Lines 310-313): ***After ensuring that the pellet is fully dissolved in the high ionic strength storage buffer, an additional centrifugation step (15 minutes at 49,000 x g) can be performed to remove unwanted aggregates if required, since the myosin will now be unpolymerized and will remain in the supernatant.***

50. 247: when using calculated (ExPASy determined) extinction coefficient one would suggest that proteins must first be denaturated by using e.g. guanidine hydrochloride…is this also the case here and with myosins in general?
    - We do not denature the myosin with GdHCl, mainly because we cannot afford the loss of protein to do these measurements in a spectrophotometer in our lab that requires ~200 ml of specimen.

51. 250: short reasoning why to subtract 1.5 A320
    - This is a formula that some labs have used. Others just subtract A320. We have modified to the latter to avoid confusion:

$$c_{mg/mL} = (A_{280} - A_{320})/\varepsilon \quad (1)$$

    - Please refer to Line 330.

52. 251: please provide extinction coefficients for protein in this protocol of example here
    - We have now included the extinction coefficient values for these proteins (Lines 333-336): ***Typical yield for the M5a-HMM is approximately 0.5 – 1 mL of 1 – 5 mg/ml protein and for the full-length NM2b is 0.5 – 1 mL of 0.5 – 2 mg/mL. The extinction coefficient for the M5a-HMM used in this paper was 0.671. The extinction coefficient for the NM2b used in this paper was 0.611.***

53. 255: I see only two ways here
    - We have now changed the wording to (Line 338): ***There are two ways to store the purified myosin.***

54. 267: please provide the appropriate size of Petri dish per coverslips used
    - We have now the Petri dish information in the List of Materials.

55. 268: coverslip rack: model/type/reference missing
    - We have now included the coverslip rack information in the List of Materials.

56. 269: is volume and time of washing steps important?
    - The precise volume of washing does not matter, but we estimate this to be around 2-5 mL and have modified the text accordingly (Lines 354-355): ***Load eight No. 1.5 thickness 22-mm***

square coverslips onto a rack and wash with approximately 2-5 mL of 200-proof ethanol followed by 2-5 mL of distilled water (dH_2O).

57. 270: drying the coverslips: simply on air, RT? with the help of blowing air? Specify
    - Coverslips are dried using a compressed air-line (filtered) at room temperature (RT).

58. 279: please specify some appropriate microscope slides
    - This is defined in our List of Materials.

59. 294: myosin 5a is dissolved here in which buffer?
    - This is defined in our buffers table.

60. 299: is BSA solution here and below for NM2b actin gliding assay without DTT? Is this healthy for myosin?
    - This was a writing oversight. The BSA should include DTT. This has now been corrected on our buffers table.

61. 302: black actin solution: F-actin, calmodulin, etc…please provide final concentrations. Also for NM2b case.
    - We have made the corrections as follows:
        - Lines 407-409: ***Flow in 10 µL of the black actin solution (5 µM F-actin, 1 µM calmodulin, and 1 mM ATP in 50 mM MB with 1 mM DTT) to eliminate "dead heads", as discussed in the Discussion section.***
        - Lines 478-482: ***Flow in 10 µL of the black actin solution as described in Table 2 to eliminate "dead heads," as discussed further in the Discussion section. The black actin solution contains 5 µM of unlabeled F-actin, 1-10 nM MLCK, 1 mM ATP, 0.2 mM $CaCl_2$, 1 µM CaM, and 1 mM DTT in 50 mM NaCl motility buffer to phosphorylate the nonmuscle myosin 2b on the surface of the chamber.***

62. 303: narrow bored plastic pipette tip: please provide example (model, vendor, size) or if it is with syringe the diameter of needle. How many pipetting events to sufficiently break the filaments?
    - We have now addressed this by specifying that a syringe works best (Lines 411-413, 484-486): ***Pipette the solution with a 1 mL syringe and 27 G needle to shear the actin filaments before introducing the solution to the chamber. Repeat this step two more times and wait 1 minute after the third time. Approximately 20 pipetting events is sufficient.***
    - This information is now on the List of Materials.

63. 310: Rh-actin: please use full name when you refer to the item for the first time (also on other occasions). What is the solution? Since labeled F-actin is one of the key reagent would be useful to describe labeling procedure
    - The solution is in the buffers table and we have also written it out here (Lines 426-427): ***Flow in 10 µL of 20 nM rhodamine actin (Rh-Actin) solution containing 1 mM DTT in 50 mM MB and wait 1 minute [..]***

- We have now added our actin preparation procedure – thank you for this suggestion. Please see here (Lines 381-389):

  Actin Preparation

  Make 20 µM F-actin by polymerizing G-actin in polymerization buffer (50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 25 mM MOPS (pH 7.0)) at 4 °C overnight.

  Dilute F-actin to 5 µM in motility buffer (20 mM MOPS, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT (pH 7.4)). Label with at least 1.2x molar excess of rhodamine-phalloidin. Leave (covered in aluminum foil) for at least 2 h on ice. This can be used for up to 1 – 2 months, stored on ice.

64. 315: please provide some useful range/starting point for exposure time and total length of the one recording
    - We have now addressed this (Lines 435-437, 507-509): ***Record images on a fluorescence microscope using an excitation wavelength of 561 nm to visualize Rh-actin. An appropriate exposure time is 200 ms at 1.4 mW laser power for a total acquisition duration of 0.5 – 1 min.***

65. 327: is any particular reason to use 2x more NM2b for the same assay? One would intuitively expect that to compare gliding velocity between myosins the motor heads density on surface should be constant.
    - This is addressed in the Discussion (Lines 988-1003): ***With many myosins, particularly those with low duty ratios, the conditions of the final buffer given for M5a would result in the fluorescently labeled actin filaments being only loosely bound to the surface or dissociating altogether.*** [...] ***Alternatives to methylcellulose when troubleshooting a lack of movement or loosely bound actin filaments in the gliding actin filament assay are to lower the salt concentration in the motility buffers or to increase the myosin surface density. As stated above, a high ionic strength in the motility buffers has been shown to lower the ability of some myosins to bind to actin.***

66. 359: please provide useful range/starting point for exposure time and length of video
    Single molecule TIRF assay
    - We have now addressed this (Lines 608-609): ***Exposure times between 100-200 ms are appropriate at 1.4 mW laser power for the actin and GFP-labeled myosin. An appropriate acquisition time for velocity analysis is 3 min.***

67. 374: several second: please provide approx. number
    - We have now addressed this (Lines 540-541): ***Vortex to dissolve, which should not take more than 30 sec.***

68. 383: to ensure only the top surface is wet: so you pipette the PEG solution like a big drop in the middle of the surface or…?

- Yes. We have rephrased this sentence to say (Lines 552-553): ***Carefully dispense 100 µL of the PEG solution onto the center of each coverslip, ensuring that the only the top surface is wet.***

69. 387: filtered air line as above? How is actually that filtering done?
    - We have modified the text as the air filtration at our institution may be different from that of other institutions.

70. 417: bRH actin: specify
    - We have now addressed this and have added more detail to this step (Lines 598-602): ***Flow in 10 µL of biotinylated rhodamine actin (bRh-Actin) containing 1 mM DTT in 50 mM MB and wait for 1 minute. For this step, use a large-bored pipette tip and avoid pipetting up and down to minimize shearing of the fluorescent actin filaments to ensure that long actin filaments can be attached to the surface (20 – 30 µm or longer). An effective alternative is cutting the cone of a standard pipette tip (with an opening of ≈1 – 1.5 mm).***

71. 418: large bore pipette tip: please list some example…is cutting the tip cone useful alternative?
    - We have now included the example in the List of Materials and that cutting the tip of a plastics pipette is a good alternative/ thank you for this suggestion. Please see Lines 597-598 for the changes.

72. 423: please provide some typical exposure time and recording duration
    - We have now addressed this (Lines 608-609)*:* ***Exposure times between 100-200 ms are appropriate at 1.4 mW laser power for the actin and GFP-labeled myosin. An appropriate acquisition time for velocity analysis is 3 min.***

73. 435: add some useful or typical myosin concentration range
    - We have now addressed this by adding this statement (Line 621): ***Typically, the myosin concentration for this step is 1 µM.***

74. 446: if the order is changed is it ionic strength still the same at the end?
    - This is a good point. We have reworded this section to (Lines 631-633): ***The order in sections 4.1 – 4.3 is not crucial as long as the NM2b is phosphorylated and the final salt concentration is 150 mM. Incubation on the order of 30 min – 1 h allows enough time for complete phosphorylation and polymerization.***

75. 456: here it seems that BSA solutions contains DTT in contrast to motility…
    - The gliding assays should also have DTT in the BSA solutions. This was a writing oversight. Thank you for pointing this error. This has now been corrected on our buffers table.

76. 470: please provide some typical exposure time and recording time
    - We have now addressed this (Lines 664-666): ***Exposure times between 100-200 ms are appropriate at 1.4 mW laser power for the actin and GFP-labeled myosin. An appropriate acquisition time for velocity analysis is 3 min.***

77. 476: please specify for which OS is FASTrack available or if it need any other software to run
    - We have now addressed this in our List of Materials.

78. 478: since acquisition time rate seems to be very important and related to speed of myosin would be nice to provide some general simple rule relating acquisition time and (expected) velocity of actin filaments or myosin.
    - We have now addressed this (Lines 439-448): ***NOTE: Ensure that the acquisition rate is scaled appropriately to the speed of the moving filaments. An important consideration before collecting data for use with tracking programs is the acquisition frame rate. Subpixel movements between frames will result in an overestimate of the velocity, and movements of several hundred nanometers are required to obtain accurate values. An optimal acquisition rate features actin gliding for at least one pixel distance between frames. In the case of the TIRF microscope used for the imaging here, this threshold translates to 130 nm; therefore, a myosin expected to travel 1 µm/s must be imaged at rate of 5 frames/s (0.2 s interval) to achieve 200 nm of movement whilst a myosin expected to travel 10 nm/s requires 0.05 frames/s (20 s intervals). Data can therefore be down-sampled at this stage if necessary (see Discussion for more details).***

79. 482: the replicates are videos of the same condition? From the same chamber or new chamber? Please specify.
    - We have attempted to further clarify this (Lines 677-679): ***When characterizing a novel myosin or investigating a new experimental condition, it is recommended to analyze movies from three field-of-views (FOV) per chamber for a total of three chambers and to repeat this workflow for three preparations of the myosin being investigated.***

80. 490: are xmax and ymax (educated) estimates? Or how you specify them?
    - We have now addressed this (Lines 687-691): ***The -xmax and -ymax parameters scale the axes for the scatter plot outputs and correspond to longest plotted filament length and the maximum plotted velocity (in nm/s). These are estimated values and can be set to higher-than-expected values to ensure data are contained in the plot.***

81. 501: linkage distance: a short definition needed
    - We have now addressed this (Lines 703-706): ***The -maxd can be used to set a maximum allowed linkage distance between frames. This is a calculated frame-to-frame distance moved by the centroid of the filament in units of nm. It can be useful for excluding sporadic movements or incorrect linkage between filaments. In the examples here, the parameter was left on the default value of 2000 nm.***

82. 512: short description what/how image stabilizer correct instrumental drift (i.e. what is the reference point for correction, do you need to use some non-movable object like fluorescent beads)?
    - We have now addressed this (Lines 716-726): ***In the event of appreciable stage drift during the acquisition, images must be stabilized to correct instrumental drift on the imaging plane. In this case, no compensation for Z-axis drift was used as the microscope used to***

obtain these data stabilizes the Z-focus well. To stabilize the image on the image analysis program, install the appropriate stabilizer plug-in that is linked on the List of Materials. The image stabilizer assumes fixed positions for the objects in the image and uses a rolling average of the previous frames as a reference. The recommended procedure is therefore to begin with the channel containing images of labeled actin, since this is in a fixed position. Click on "Plugins," then find "Image Stabilizer," ensure that "Translation" is selected, and keep the default settings. Check the box next to "Log Transformation Coefficients." Applying this Log step allows for the calculated shift parameters to be applied to the other channel in the next step. Allow for the process to complete.

83. 539: minimal velocity here and minimal velocity in line 540 is that unnecessary repetition?
    - We have now addressed this by modifying the diction as follows (Lines 768-770): ***Set filters on tracks: Track Displacement (>0.39 - to include only spots moving more than 3 pixels), Spots in tracks (>3 – to include only tracks with at least 3 spots). Others filters such as Minimal Velocity may be introduced to exclude spots which stall for long periods***.

84. 542: spurious track: please describe
    - We have now addressed this (Lines 770-773): ***The results of filtering must be checked by visual inspection of tracks to ensure that spurious tracks (i.e., myosin movement in the background that is not along an actin track) are removed whilst retaining the tracks associated with actin.***

85. 545: three documents: can we name them?
    - We have named them accordingly (Lines 776-777): ***Save the three tables produced (Track Statistics, Links in Tracks Statistics, and Spots in Tracks Statistics).***

86. 546: some description of "analysis" i.e. what can we do with this data from this step is welcome
    - We have now addressed this within the same sentence (Lines 777-779): ***The "Track Statistics" table will contain the velocity and displacement data that can then be subsequently analyzed to characterize a novel protein or the effects of a certain experimental condition, for example.***

87. 564: how can one assure short enough actin filaments?
    - We have now addressed this (Lines 800-801): ***This problem can be avoided by pipetting up and down 10-20 times to shear the actin filaments before loading onto the coverslip.***

88. 569: short reasoning why actin filaments does not move away in case of myo5
    - Thank you for this suggestion. We have modified accordingly (Lines 804-807): ***This is not necessary for M5a because its higher duty ratio allows for a stronger attachment of the actin to the myosin-coated surface. If methylcellulose is used, wicking the solution through the chamber is necessary to ensure the solution flows through.***

89. 573: There is no Figure 5…the one on the page 33 where Fig5 I supposed should be is identical to Figure 3. Figure 4 probably was meant….?

- We have now addressed this, thank you for pointing out this oversight (Line 811): ***Conversely, the goal for the inverted motility assay shown in Video 3 and Figure 4 is to introduce surface-tethered fluorescent actin filaments upon which myosin movement can be observed.***

90. 577: glucose oxidase: the substance by removing O2 from solution acidify the solution. So it allows longer measurements but not too long. To slow dawn the process assay solutions are additionally degassed and stored in syringe during the experimental day. Is this the approach used also here?
    - We find that our combination of DTT, glucose oxidase, catalase, and glucose allows for long enough imaging (approximately an hour), but we are interested in trying this presented method prior to TIRF imaging as we have not attempted this yet. Additionally, we use shuttered lasers, which means we are not bleaching our samples as much as we would be if we had continuous illumination.

91. Are all images presented acquired under TIRF illumination?
    - Yes. All of the images presented in this article were all acquired under TIRF illumination. We have now addressed this in the article. Please see Lines 837 and 850.

92. 1.A: If this is a composition of not-associating lanes from the same gel in original publication (lane1 and 4?) is better to make that clear by putting a space in between. Is 200 mark lane really so up? Really nice prep I must say.
    - We have reformatted this figure to make it clearer. We have made the gel clearer as suggested.

93. 1B. If any extra background subtraction/correction was used here please provide that info.
    - We did not do any extra background correction (other than image stabilization, which is described in Lines 716-735).

94. 596: the citation of copyright should probably be done a bit different/shorter
    - We have now addressed this and changed the citation. Please see Lines 834-835.

95. 601&606: snapshot image: like first frame of video or some random time frame?
    - We have rephrased this (Lines 837-839): ***Example frame from a movie showing translocation of rhodamine-phalloidin labeled actin filaments (in red) on 0.2 µM NM2b in the presence of 0.7% methylcellulose at 30 °C.***

96. 606&611: number of tracks: is this just from that video or several videos combined?
    - Number of tracks shown is the total number of tracks identified, just from this particular video attached to this article.

97. Please provide temperature at which motility was made. ?
    - We have now provided the temperature for these assays (30 ˚C).

98. 2A: please specify that it is actually two channel (merged) fluorescence image of NM2B and AlexaFluor647-phaloidin labeled actin filaments (blue is probably a pseudo color, is not AF647 red?)
    - We have edited to text to make it clear that it is a two channel (merged) fluorescence image (Lines 850-852): ***A representative FOV from a two-channel, merged image stack showing the single particle movement of NM2b filaments (displayed in green) on biotinylated actin filaments labeled with AF647-phalloidin (displayed in blue) at 30 °C.***

99. 2D: it seems again two channel (merged) fluorescence image. Is this the same magnification/objective as in A?
    - The magnification and objective are the same in each case (during acquisition). The images shown here are cropped to allow individual particles to be seen on the actin. In the case of Myo5a the cropped region is smaller (as shown by the scale bar) since the individual particles are more difficult to see in a large field of view, unlike the larger and brighter myosin filaments.

100. 625: single molecule or single particle as in 629?
    - We have changed this to (Lines 867-868): ***Representative histogram of single molecule M5a-HMM velocity on single actin filaments.***

101. 628: is run length obtained by fit? If so +/- error of the fit can be provided.
    - In the text, we indicate that the histogram for run length is fit to a single-exponential fit in Line 862. A confidence interval can also be deduced from these statistics. In our case, the characteristic run length is 1.32 microns (1.23-1.42 at 95% CI, R^2=0.99).

102. Please provide temperature at which experiment was performed
    - We have now provided the temperature for these assays (30 ˚C).

103. Video Legend 1: provide exact fps/exposure time and perhaps a tool where such nice double timelaps videos can be made
    - We have added the requested information (Lines 880-883): ***NM2b data acquired at 0.33 frames per second with 200 ms exposure. M5a-HMM data acquired at 5 frames per second with 200 ms exposure (continuous) and subsequently down-sampled to 1 frame per second. Timestamps were added using the plugin described in the List of Materials.***

104. Video2: provide time stamps
    - We have included the time stamp with the video for Video 2 now. Thank you for pointing out this oversight.

105. Video legend 2: provide exact fps/exposure time
    - We have now included the exact FPS and exposure time (Lines 891-892): ***This movie was acquired at 0.33 frames per second with 200 ms exposure.***

106. Video legend 3: provide short description how such double channel fluorescence video was made (split camera?, double band filter cube?, etc)….if such equipment must be needed please specify in table
    - We did not use any special equipment for acquisition, but we wrote the following (Lines 901-902): ***The two lasers were toggled back and forth with the use of a single camera for acquisition.***

107. 654: specify more precisely the exposure time, fps, and if the illumination was closed by a shutter in between for NM2b…
    - We have now included the exact FPS, exposure time, and that shuttering of the illumination is deployed.

108. 676: step 1.8 missing
    - Thank you for pointing this out. We have now addressed this. Please see Line 246 for the change.

109. 683: how many times we can reuse resin?
    - We have now addressed this (Lines 931-935): ***Additionally, improper washing of the resin both before and after use may result in the elution of an undesired protein product, so it is imperative that the proper protocols are followed before and after using the FLAG-affinity resin. If the resin is washed immediately after use and stored appropriately, it can be reused up to twenty times.***

110. 753: please define/provide example of short lever arm
    - We have rephrased this language (Lines 1008-1010): ***This can aid with obtaining successful motility in situations where the geometry of the system may otherwise hamper actin translocation, such as in the case of testing artificial or short lever arms.***

111. 766: photobleaching is one issue however and especially in single molecule assay fluorophore blinking is another phenomena usually observed. Please discuss how (if) is this issue demonstrated/present in described single molecule assays and if so what are the approaches commonly used to minimize it.
    - We have now addressed this (Lines 1073-1079): ***Another phenomenon that can be observed in single molecule fluorescence studies is photoblinking, in which fluorophores switch between the on and off state rapidly and appear to blink. This typically does not occur in these motility experiments; however, if this does occur, the laser intensity and exposure times can be decreased which should minimize this. Several chemicals including β-mercaptoethanol, Trolox, cyclooctateraene, n-propyl gallate, 4-nitrobenzyl alcohol, and 1,4-diazabicyclo[2.2.2]octane can be utilized to mitigate this as well.***

112. 790: accessible: well, you still need to have plasma cleaner so one can wonder if piranha solution is not more accessible. ?
    - The piranha solution may be more accessible, but requires a fume hood and is more dangerous to the user than plasma cleaners. We also have used the piranha solution in our

laboratory, but had better success with coverslip cleaning using the plasma cleaner compared to harsh chemicals.

113. 811: following well-spaced out localization points: can you say/describe more what do you mean by that?
- We have edited the language to reduce confusion (Lines 1066-1070): ***[…] using an acquisition rate that allows for successive localization points to be sufficiently well spaced to avoid oversampling errors as described above, whilst being close enough together that the straight line distance between them remains a good approximation of the curve between those points.***

114. please check reference 8 and 65
- Thank you for finding this duplicate error. We have resolved this oversight.

115. As mentioned tables were hard to navigate due to strange appearance in combined pdf and I think need to be redone.
- Again, we apologize for the complication. Indeed, some of the tables that have been uploaded to the JoVE editorial website was too large to be formatted properly.
- To ensure that the editors and reviewers can see the tables/lists as we would like to be viewed, we have also attached a PDF version of the Excel files which we converted prior to uploading the files, as supplementary files for review. These documents are: Preconverted - Buffer Table - 12-16-2020.pdf and Preconverted - Material List - 12-16-2020.pdf. Preconverted - Buffer Table - 12-16-2020.pdf includes the protein purification buffer table (page 1), gliding assay buffer table (page 2), and the inverted assay buffer table (page 3). Preconverted - Material List - 12-16-2020.pdf includes the reagents list (page 1), equipment list (page 2) and the software list (page3).
- Thank you for pointing this out.

116. -store at 4C: please provide some shelf life
- This has been added to the List of Materials.

117. -HaloTag: was not used here so can be skipped
- The NM2b we used in this paper did indeed have a HaloTag even though we did not employ this for the presented data.

118. -calmodulin, actin, kinase, light chains: please provide cat. number or reference to publication protocol by which were purified
- We have now addressed this in the List of Materials.

119. -lasers: please provide laser power and working illumination intensity (W, W/cm2, etc.) if possible
- We have now addressed this (Lines 436-437): ***An appropriate exposure time is 200 ms at 1.4 mW laser power for a total acquisition duration of 0.5 – 1 min.***

Reviewer #4:

Manuscript Summary:

The authors present a protocol for purification of myosin 5a and non-muscle myosin 2b. They use these motor proteins to demonstrate two fundamental techniques for motor characterization: the actin gliding filament assay and 2) the single-molecule translocation/motility assay. They also present established, automated analysis techniques for extracting quantitative data on run length and velocity.

Major Concerns:
None

Minor Concerns:

1. Line 33: I assume the authors mean asynchronously when they write "asynchronistically"
    - Thank you for pointing out this error. We have now corrected this in the article. Please see Line 35.

2. The authors refer to the assay where motors are adhered to the surface and filaments are visualized moving as the "actin gliding assay". While it is admittedly a pedantic point, I believe this assay should be referred to as the "actin gliding filament assay". Because actin can exist in both the g and f forms this eliminates any confusion for the novice reader.
    - We have now addressed this throughout the article and replaced this with “gliding actin filament assay.”

3. Line 148: was the GFP tag fused to NM2b, the light chains, or all? The wording is unclear.
    - We have now addressed this by rewording the sentence (Lines 191-192): ***The N-termini of the RLC was fused with a GFP via a linker of five amino acids (SGLRS).***

4. Line 482: what file formats are acceptable? Are tiff stacks required, as implied by line 486/7, or are other formats (Nikon ND, etc) acceptable? If specific formats are required that should be stated.
    - Tiff stacks are required, and this has been clarified (Lines 669-671): ***The images can be analyzed by using the software and manuals linked in the List of Materials. It is important to note that the program described here requires TIFF-stacks for analysis.***

5. I suggest adding a note about the version of each software package that was used in the creation of each analysis workflow, as future software updates may necessitate workflow changes.
    - We have included the version information of the software packages in the List of Materials.

6. 616: field of view (FOV). Only used twice (616,626), so consider forgoing abbreviation.
    - We have now addressed this throughout the article, since there are a number of instances we used the term “field-of-view”. We have used the abbreviation FOV, after defining this abbreviation on Line 678.

7. Video 2 should have time stamp to allow direct comparison with NM2b in video 1

- We have included the time stamp with the video for video 2 now. Thank you for pointing out this oversight.