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Expression, purification and *in vitro* analysis of myosin.

Glenn Carrington[1,2,$], Yasuharu Takagi[3,$], Oliver Umney[1,2,5], Alistair Curd[1,2], Jonathan E. Bird[5], Michelle Peckham[1,2*]

1. Astbury Centre for Structural and Molecular Biology, University of Leeds, Leeds, LS2 9JT
2. School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, LS2 9JT
3. Laboratory of Molecular Physiology, Cell and Developmental Biology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA
4. School of Computing, Faculty of Engineering and Physical Sciences, University of Leeds, UK
5. Department of Pharmacology and Therapeutics, University of Florida, Gainesville, FL, USA

$Glenn Carrington and Yasuharu Takagi contributed equally to this work

* *correspondence:* m.peckham@leeds.ac.uk

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Abstract

To understand the mechanics and kinetic properties of cytoskeletal molecular motors such as myosin, typically the motor of interest needs to be expressed and purified and then analysed using a range of *in vitro* based assays. In this chapter, we describe how to express and purify myosin using the insect cell system, how to characterise the purified protein by mass photometry and negative-stain EM to assess its quality, and how to perform *in vitro* assays in which fluorescently labelled myosin walks along actin tracks, including a brief description of adapting these assays for MINFLUX imaging.

Key words: Myosin, actin, *in vitro* motility, negative-stain electron microscopy, mass photometry,

1. Introduction

Myosins are cytoskeletal motor proteins that interact with filamentous-actin (F-actin) to generate movement or contraction, driven by ATP hydrolysis. All eukaryotes express myosin, the isoforms of which can be organised into specific classes, based on their sequence similarity ***(1-3)***. Of the many isoforms of myosin, striated muscle and smooth and non-muscle myosin 2 isoforms are all found in class 2 (the so-called 'conventional myosins'). All the remaining classes comprise unconventional myosin isoforms, the first of which to be discovered was the class 1 myosin isoform from *Acanthamoeba* ***(4)***. There are at least 79 classes of unconventional myosin isoforms across eukaryotes ***(1)*** many of which have not been functionally characterised.

To understand how each myosin is adapted for its specific cellular task, they each need to be expressed, purified and tested experimentally. However, the motor region of myosin typically requires chaperone proteins to fold correctly and function as an ATPase (reviewed in ***(5-7)***), which means that active myosin protein cannot be expressed using *E.coli*. As a result, most myosin isoforms are typically expressed using the insect *Spodoptera frugiperda* (Sf9)/baculovirus cell system, although mammalian cell expression systems are also used ***(8)***. In Sf9 cells, the myosin heavy chain is co-expressed with the correct light chains, to generate a properly folded lever arm. Sometimes, this is as simple as co-expressing calmodulin (CaM) for many of the unconventional myosin isoforms, however, in some cases, specific light chains may be required ***(9)***. In Sf9 cells, co-expression of chaperone proteins such as UNC45 and HSP90 may also be required for successful expression of active protein ***(10-12)***. However, expression of vertebrate class 2 striated muscle myosin isoforms in Sf9 cells remain the exception, in that even with co-expression of the correct light chains and chaperones, fails to generate active protein ***(13)***. Currently, these proteins can only be expressed using cultured muscle cells, such as the C2C12 cell line ***(14, 15)***. Once the myosin is expressed using the insect cell system, it can be purified using an affinity tag of choice, although the FLAG tag (DYKDDDDK) is most commonly used, followed by downstream additional purification steps if required.

The purity of the resulting protein is typically assayed by SDS-PAGE gel electrophoresis, which also reveals if the light chains have been co-purified. In addition, we have recently begun to use mass photometry (MP) to assay the quality of the purified protein ***(16)***. It is a light scattering based technique that detects single unlabelled particles as they adsorb onto a glass microscope slide ***(17)***. MP can be used to rapidly determine mass in the 40 kDa to 5 MDa range, the oligomeric state, and heterogeneity of a wide range of macromolecules and their complexes in solution under equilibrium conditions. The final result of the MP measurement is a molecular mass distribution reflecting the molecular composition of the sample. Using this approach for myosin allows the molecular mass of the intact complex to be measured, allowing an accurate assessment of the numbers of light chain bound to each heavy chain (stoichiometry) and analysis of any heterogeneity ***(18)***.

We also commonly use negative stain EM (nsEM) to assess the quality of the purified protein. This technique is a relatively easy way to visually inspect the expressed protein and assess its purity, concentration, heterogeneity, flexibility and overall structure ***(19)***. Although the resolution limit of this technique is relatively low (~20 Å), a great amount of biologically relevant detail can be revealed ***(20)*** and it is a useful intermediate step prior to performing high-resolution CryoEM. In nsEM, the protein of interest is adsorbed onto the surface of an EM grid, then surrounded by an amorphous matrix of electron dense stain, to provide contrast between the background and the targeted particle with lower electron density. Imaged particles appear lighter than the background stain due to their lower ability to scatter electrons. The resultant images show the overall structure and flexibility of the molecules. They can additionally be processed to generate image classes with higher signal-to-noise ratios and improved detail.

Here we discuss how to express and purify myosin using the Sf9 system, how to use mass photometry and nsEM to assess the quality of the purified protein complex, and finally how to analyse the motile behaviour of the purified myosin using *in vitro* TIRFM and MINFLUX-based motility assays.

2. Materials

All solutions should be prepared using ultrapure water and analytical grade reagents. All reagents can be stored at room temperature (unless indicated otherwise). Follow local guidelines for disposing of waste materials.

2.1 Protein expression and purification

1. pFastBac 1 (Thermo Fisher Scientific) with gene of interest
2. Max Efficiency® DH10Bac Competent E. coli (Thermo Fisher Scientific)
3. Cellfectin® II Reagent (Thermo Fisher Scientific)
4. Plasmid DNA purification kit (Macherey-Nagel)
5. PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000) (Polysciences)
6. Sf9 cells (Thermo Fisher Scientific)
7. Sf900 II or III SFM (Thermo Fisher Scientific)
8. Corning® Polycarbonate Erlenmeyer Flasks (Corning)
9. PYREX® Fernbach Culture Flasks (Corning)
10. Multitron cell shaker with temperature control (INFORS HT)
11. cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche)
12. Adenosine 5′-triphosphate disodium salt hydrate (ATP) - A2383 (MilliporeSigma)
13. KIMBLE® KONTES® DUALL® Tissue Grinder, Size 24, 30 mL (DWK Life Sciences)
14. Sorvall LYNX 6000 Superspeed Centrifuge (Thermo Fisher Scientific)
15. T29-8 x 50 Fixed Angle Rotor (Thermo Fisher Scientific)
16. PBS, pH 7.4 (Gibco™, Thermo Fisher Scientific)
17. Benchtop Centrifuge 5810 R (Eppendorf)
18. ANTI-FLAG® M2 Affinity Gel (MilliporeSigma)
19. DYKDDDDK Peptide - # RP10586 (GenScript)
20. Orbital laboratory mixer VSN-6 (PRO Scientific Inc.)
21. Tube roller MX-T6-S (DLAB)
22. Poly-Prep® Chromatography Columns - #7311550 (Bio-Rad)
23. Protein Assay Dye Reagent Concentrate, 450 ml - #5000006 (Bio-Rad)

24. Spectrum™ Spectra/Por™ 2 RC Dialysis Membrane Tubing 12,000 to 14,000 Dalton MWCO (Thermo Fisher Scientific)
25. Slide-A-Lyzer™ Dialysis Cassettes, 10K MWCO (Thermo Fisher Scientific)
26. Sonicator XL2020 Ultrasonic Liquid Processor (Misonix)
27. Amicon® Ultra Centrifugal Filters, 10K MWCO (MilliporeSigma)
28. HisPur™ Ni-NTA Spin Purification Kit (Thermo Scientific™)

2.2 Additional consumables for Mass Photometry (Refyn Instruments), nsEM and fluorescence imaging (HaloTag) for MINFLUX (Abberior).

1. Reusable gaskets (CultureWell) 50 mm (diameter) depth 3 mm x 1 mm (Grace Bio-Labs, GBL103250) for Mass Photometry (consumables can also be purchased directly from Refyn)
2. High Precision Cover glass (# 1.5H), 24 X 50 mm (Thorlabs)
3. Olympus IMMOIL-F30CC (Olympus)
4. Rabbit Skeletal Muscle G-Actin (>99% Pure) (Cytoskeleton, Inc)
5. Biotinylated Rabbit Skeletal Muscle G-Actin (>99% Pure) (Cytoskeleton, Inc)
6. Carbon coated copper grids (400 mesh) (AGAR Scientific)
7. Glass Pasteur pipettes
8. 1% Uranyl acetate solution
9. Whatman® Quantitative filter paper, ashless, Grade 42 (Cytiva)
10. Janelia Fluor® 646 HaloTag® Ligand (Promega)
11. Rhodamine-phalloidin (Thermo Fisher)
12. 99% Pentyl acetate (Sigma)
13. 2% Nitrocellulose (collodion) solution in amyl acetate (Sigma)
14. Scotch Double Sided Tape (Permanent) 1/2 in x 250 (3M: Cat# 3136)
15. Kimwipe (KimTech)
16. Calmodulin (prepared in house – for details see ***(21)***)
17. Catalase (Cambridge Bioscience)
18. Glucose (Sigma)
19. Glucose oxidase (Sigma)
20. Adenosine 5′-Triphosphate, Disodium Salt (Merck)

2.3. Standard buffers for purification

These buffers can be made in advance and stored for up to 1 month at 4 °C. In addition, we often make these buffers as 1.5X or 4X stocks. If buffers are to be stored for some time, we recommend that the stocks are either filter sterilized using a 0.45 µm filter, or that NaN_3 (final concentration of 3mM) in buffers is included (as shown below) to avoid bacterial growth.

1. Extraction buffer: 10 mM MOPS (pH 7.3), 200 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 3 mM NaN_3, 0.1 mM DTT. 0.1 mM PMSF, 1 mg/L leupeptin, cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche) (1 tablet in 50ml), 2 mM ATP (pH 7.5).
2. Wash buffer with salt: 10 mM MOPS (pH 7.2), 0.5 M NaCl, 0.1 mM EGTA, 3 mM NaN_3, 0.1 mM DTT, 0.1 mM PMSF, 1 mg/L leupeptin.
3. Wash buffer with salt and ATP: Wash buffer above with 1 mM ATP and 5 mM $MgCl_2$.
4. Wash buffer without salt: 10 mM MOPS (pH 7.2), 0.1 mM EGTA, 3 mM NaN_3, 0.1 mM DTT, 0.1 mM PMSF, 1 mg/L leupeptin.
5. Elution buffer: 10 mM MOPS (pH 7.2), 150 mM NaCl, 0.1 mM EGTA, 3 mM NaN_3, 0.1 mM DTT, 0.1 mM PMSF, 1 mg/L leupeptin, 0.3 mg/ml FLAG-peptide.
6. Dialysis buffer: 10 mM MOPS (pH 7.2), 500 mM KCl, 0.1 mM EGTA, 3 mM NaN_3, 1 mM DTT.

2.4. Buffers for functional assays

1. Myosin base (MB) motility buffer: 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EGTA.
2. Polymerisation buffer: 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 25 mM MOPS (pH 7.0)
3. MB150+: 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EGTA + 150 mM KCl + 1mM DTT
4. MB50+: 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EGTA + 50 mM KCl
5. MB50+DTT: 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EGTA + 50 mM KCl + 1mM DTT

6. MB50+ ATP: 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EGTA + 150 mM KCl + 1mM DTT + 2 mM ATP
7. Final buffer: 20 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EGTA + 50 mM KCl + 20 nM myosin6-HMM + 1 mM ATP + 50 mM DTT + 1 μM calmodulin + 2.5 mg/ml glucose + 100 μg/ml glucose oxidase + 40 μg/ml catalase

3. Methods

3.1 Protein expression, plasmid assembly, bacmid and virus generation

The design of the required protein construct for purification and its ability to be used in multiple experimental assays is an important first step, before cloning the gene of interest into a plasmid for expression. For protein purification, there are many affinity chromatography methods that utilize different tags (*i.e.* FLAG, 6x His, GST, or Strep-Tag Peptide Systems) and there are multiple ways of fluorescently labelling the protein from including genetically encodable fluorophores, to using a self-labelling moiety such as the Halo tag ***(22)***. The Halo-tag is a 33 kDa protein domain (297 amino acid residues) that covalently and specifically binds synthetic ligands ***(22)***. Multiple commercial fluorescent ligands (dyes) are available enabling the purified protein to be labelled for imaging at the wavelength of choice. We recommend that you carefully considering all these options, and their usefulness in a range of downstream techniques, to enable the use of the purified proteins in multiple assays. Here we give the example of our process for the human myosin 6 (Myo6) construct (Met^{1} – Ala^{1021}).

1. GFP-M6HMM: The human Myo6 construct was similar to a previously published construct design ***(23)***, but contained a C-terminal GCN4 leucine zipper, as a dimerization sequence, eGFP was added as a fluorophore for visualising the purified protein and a FLAG sequence was included for affinity purification.

2. Halo-M6HMM: A second Myo6 construct with a N-terminal Halo-tag, and a C-terminal GCN4 leucine zipper, eGFP, and a 6x His-tag was also constructed.
3. Expression was performed using the Sf9/baculovirus expression system.
4. Plasmid design and production can be found in the literature published by our laboratories ***(10, 24-28)***.
5. pFastBac1 is used as our general plasmid to generate most of our clones, designed to incorporate affinity tags, any genetically encodable fluorophores ***(29, 30)*** or even specific attachment of myosin to surfaces ***(10)***.
6. Chimeric proteins using protein domains from different myosin isoforms can also be generated ***(24)*** to understand how protein sub-domain substitution can affect myosin mechanics and kinetics.
7. Attention to plasmid design is clearly important to not disrupt the native function of these proteins.
8. After subcloning and analysis of the pFastBac1 construct with the gene of interest *via* PCR, the construct should be sequenced to confirm that the coding sequence is in the correct orientation, error free, and to verify that it is in frame with any tags.
9. Alternatively, we commonly use commercial companies such as Genscript to make constructs. If you do this, careful checking of the final planned sequence is essential, and independent sequencing of the generated clone is recommended.
10. We recommend that you generate an *E.coli* glycerol stock of the pFastBac construct after identification of the correct clone for long term storage at -80 °C.
11. Once the correct pFastBac plasmid has been identified, the plasmid is transformed into DH10Bac Competent *E. coli* cells, to enable transposition of the polyhedrin promoter, open reading frame and SV40 polyA sequence into the proviral backbone DNA to generate recombinant bacmid DNA, following protocols recommended by the manufacturer (Thermo Fisher Scientific; https://tools.thermofisher.com/content/sfs/manuals/bactobac_man.pdf).

12. After transposition, we verify the phenotype and analyze the recombinant bacmid DNA via PCR following protocols recommended by the manufacturer (Thermo Fisher Scientific; https://tools.thermofisher.com/content/sfs/manuals/bactobac_man.pdf).
13. The recombinant bacmid DNA can then be transfected into Sf9 insect cells using Cellfectin and Cellfectin II reagent, using Sf9 cells seeded on a 6-well or 35 mm tissue culture plate.
14. Alternatively, we use alternative DNA:lipid complexes for transfection ***(10)*** as follows
15. Grow up Sf9 cells in suspension to a density of ~0.8 x 10^6 cells/ml; in ~100 ml volume of medium,
16. Freshly prepare PEI MAX reagent (Polysciences) from powder to a final concentration of 1 mg/mL
17. Use molecular biology grade water and filter the solution using a 0.2 µm filter.
18. Make two solutions, Solution A and B, in separate 1.5 ml Eppendorf tubes.
19. In solution A, mix 25 µg of bacmid with 300 µL of PBS.
20. In solution B, mix 300 µg of PEI in water with 300 µL of PBS.
21. Add solution A dropwise into solution B and then incubate at room temperature for 10 minutes.
22. Add the DNA:lipid complex to the flask of Sf9 cells and medium (~0.8 x 10^6 cells/ml; ~100 ml volume),
23. Place into an incubator, with a rotation speed of ~125-150 RPM, temperature of 27 °C, for ~2-3 days to generate the P1 viral stock.
24. Collect the P1 stock into 50 ml plastic conical tubes
25. Centrifuge at ~1000 RCF for 3-5 minutes,
26. Collect the supernatant
27. Stored in a 4 °C refrigerator.
28. To amplify the virus and generate P2 stock, prepare Sf9 cells in suspension as in step 15.
29. Use a multiplicity of infection (MOI) of ~ 0.05-0.1 (ratio of virus to cells) to infect 100 ml cultures of Sf9 cells and media, and culture as before (step 23).

30. The higher-titre P2 virus stock can be used subsequently to generate even higher-titre stocks or used immediately for protein expression.
31. Usually, a P2 virus stock has a high enough titre for protein expression (>> 1 x 10^8 pfu (plaque forming units)/ml).
32. Virus stocks decrease in titre if kept at 4 °C for extended periods (>6 months). If necessary, virus stocks can be stored at -80 °C for longer term storage to minimise loss of titre ***(31)***
33. To measure the titre of the generated viruses, the plaque assay as described in the manufacturer manual can be followed.
34. Alternatively, we use the end-point dilution method and the Sf9-ET cell line as detailed ***(32)***.

3.2. Protein expression

After measuring and ensuring that the titre of the manufactured stock viruses is sufficiently high, protein expression can be performed. The protocol for protein expression that we follow is based on the Sf9/baculovirus expression system manual provided by the manufacturer.

1. Briefly, Sf9 cells (cell count = 1 – 1.5 x 10^6 cells/mL; volume = 1 L) are grown in plastic 250 ml Erlenmeyer flasks
2. prior to expression, the cells and medium are transferred to a 2.8L Fernbach flasks.
3. Fernbach flasks are prepared the day before by washing thoroughly and sterilization, with loose aluminium foil used as a “cap”.
4. Virus titres in the range of 1 – 5 MOI (per baculovirus used) are used to transduce the Sf9 cells.
5. Multiple viruses, such as calmodulin together with the myosin heavy chain virus, can be used for simultaneous expression.
6. After addition of the viruses, Fernbach flasks are left stationary for ~1 hour,
7. Flasks are then transferred to a temperature-controlled incubator, such as temperature-controlled cell culture shaker (Multitron, INFORS HT, or equivalent) used in our laboratories,
8. The rotation of the orbital rotator is adjusted to ~85 – 90 RPM and the temperature set to 27 °C.

9. Expression depends on the transfection efficiency of the viruses, but usually cells are harvested after 48 – 60 hours post-transfection.
10. Cells should be harvested before their viability drops significantly as measured using the trypan blue assay.
11. Cell pellets can be collected using a plastic 250 ml conical tube, flash frozen using liquid nitrogen and stored in the -80 °C freezer until purification.
12. We use affinity chromatography to purify proteins, depending on which tag is present (e.g. using either FLAG or His-tags (see Note 1))
13. Cell pellets from ~500 ml of culture are used for a single purification to yield a high enough protein concentration and sample volume for experiments to follow.
14. In general, we follow instructions in the protein purification manual associated with the Sf9/baculovirus expression system from the manufacturer, as discussed and cited in the previous section, with slight modifications.

3.3 Protein Purification

1. Add ~100 ml of extraction buffer to a thawed cell pellet from ~500 mLlof culture
2. Using a manual glass homogenizer, homogenize the cell pellet with the extraction buffer.
3. Pool all cells in extraction buffer in a 250 ml glass beaker and set on ice.
4. Check the homogenized product using a microscope to check for complete cell lysis.
5. Apply sonication if lysis of cells is incomplete. With our Misonix sonicator with a standard probe, we used a power setting of ~ 4 for 2.5 minutes. Cycle time – 1 second ON, 1 second OFF. Container with homogenized product should ALWAYS be on ice during sonication.
6. Spin at 20k RPM (~47850 RCF) for 20 – 30 minutes using T29-8 x 50 Fixed Angle Rotor and a Sorvall LYNX 6000 Superspeed Centrifuge.
7. Save the supernatant.
8. Prepare FLAG resin by washing with PBS (~5 – 7x volume), as detailed in the manufacturer's protocol.

9. Add ~3 ml 50% FLAG resin, prepared as in the previous step (8), into the supernatant,
10. Rock or rotate this sample using either an orbital laboratory mixer or tube roller for ~3 – 4 hours. This step can also be done overnight (<10 hours).
11. Using a 50 ml conical tube, spin down the resin (1000 RPM (~210 RCF), 3 – 5 minutes) using a centrifuge (Eppendorf 5810/ 5810 R tabletop centrifuge).
12. Remove the solution using a pipette without disturbing the resin on the bottom of the tube. The protein should be bound to the resin.
13. It may be useful to save a small amount (10 – 20 μL) of the supernatant and subsequent solutions for SDS-PAGE analysis
14. Repeat steps 11 and 12 on the supernatant and FLAG resin mixture until all of the resin (~1.5 ml) is collected in a 50 ml conical tube. Removed solution can be discarded.
15. Add wash buffer containing salt and ATP (see section 2.3) into the 50 ml conical tube with only the resin. This step is carried out to remove any actin filaments attached to the myosin and any proteins that are non-specifically bound. The protein of interest is still bound to the resin. Gently shake to disperse the resin. Repeat steps 11 and 12.
16. Add wash buffer with salt (section 2.3) into the 50 ml conical tube with the resin. This step is carried out to further remove proteins that may be non-specifically bound.
17. Gently shake to disperse the resin. Repeat steps 11 and 12.
18. Add wash buffer without salt (section 2.3) into the 50 ml conical tube with the resin. This step removes any contaminants from the resin, if any.
19. Gently shake to disperse the resin.
20. Repeat steps 11 and 12.
21. Repeat step 16 and 17.
22. Load the supernatant containing resin and protein into a plastic chromatography column (Bio-Rad).
23. Allow the resin to settle to the bottom of the column and then wash with ~5 – 10 resin volumes of wash buffer without salt.
24. Cap off the column after buffer has flowed through.
25. Add ~1 ml elution buffer, uncap the column and collect a 1 ml fraction.

26. Repeat step 25 for ~10 – 15 ml.
27. For a quick test, perform a simple protein dye/Bradford reagent check. We use the Bio-Rad Protein Assay Dye Reagent to quickly estimate which fractions contain more, or less, protein. Add ~10 µL of each collected fraction to 50 µL drops of the protein assay dye reagent on either parafilm or a 96 well plastic plate. This is for a quick check to see if the protein of interest is being eluted and in which fraction(s). A stronger "blue" colour means more protein is present in the fraction.
28. Take samples (10 – 20 µL) from each fraction to analyze by SDS-PAGE later.
29. The quick protein dye test usually makes it clear which collected 1 ml fractions have high enough concentrations for dialysis.
30. Pool the 1ml fractions with the highest protein concentrations and add into pre-wet dialysis tubing or into a dialysis cassette. Dialyse samples (usually <10 ml) against 2 – 4 litres of dialysis buffer and exchange the buffer after 6 hours. Dialysis should be performed in a 4 °C refrigerator or cold room and on a magnetic stirrer. This dialysis step can be performed for ~10 - 12 hours (overnight)
31. Collect the dialyzed samples and concentrate the protein using a commercial centrifugal filter such as the Ultra Centrifugal Filter (Amicon) using the recommended protocol with a benchtop centrifuge (Centrifuge 5810/ 5810 R - Benchtop Centrifuge). We typically concentrate samples by approximately 10 times the original collected sample volume, to a final volume of approximately 1 ml. Protein concentration can be measured using a nanodrop using the estimated extinction co-efficient.
32. After this step, samples can be tested for assays (ATPase or *in vitro* motility/actin gliding assays), mass photometry or nsEM.
33. For long term storage, flash freeze aliquots of ~10-20 µL sample per tube, using thin-walled PCR tubes by dropping the tubes into liquid nitrogen.
34. Store samples at a -80 C freezer, or in a liquid N_2 tank.

4. Mass Photometry analysis using a commercial REFYN TwoMP instrument.

Mass photometry ***(18)*** can be used to quickly assess the homogeneity and quality of the purified protein using the commercial mass photometer developed by Refeyn, Ltd. We follow the protocol recommended by the manufacturer at (https://www.refeyn.com/twomp-mass-photometer). Below is a brief description outlining how we use the instrument.

4.1 Initial set up and calibration

1. Turn on the Refeyn TwoMP instrument and allow it to warm up for at least 45 mins.
2. Open the AquireMP software to perform the measurements.
3. Clean #1.5H high precision glass cover slides with sequential washes of MilliQ water and isopropanol (2x) followed by a final MilliQ water wash. Dry the coverslip using either filtered air or nitrogen line.
4. Using soft-tipped tweezers, place a clean re-usable silicon gasket on the cleaned coverslip.
5. Using a sheet of white tissue paper, apply gentle pressure to ensure the gasket makes uniform contact with the coverslip.
6. Place a small drop of immersion oil on the objective lens.
7. Mount the prepared coverslip so that the red autofocus laser is roughly centred within one of the wells.
8. Secure the coverslip with the magnets provided.
9. Prior to beginning data collection, we perform the following checks.
10. Switch to the ratiometric view to assess the buffer and coverslip cleanliness. A clean aqueous buffer and slide should be free of bright or dark spots that could be dirt or dust. If necessary, use the lateral control arrows to adjust the field of view to dust free area, then find focus by setting the image view to “Native” in the toolbar and use “droplet dilution” to find focus. The focus settings will lock automatically using the ‘droplet dilution’ button.
11. Examine quality metrics, ensuring they are all within the normal limits (signalled by the ‘blue’ colouration). Quality metrics will turn orange if they are outside the optimal range. The baseline signal should be less than 0.06 if there is no detergent in your buffer. Sharpness should be between 4.5-6.5%.

12. (optional) Go to File>Preferences and check the ‘advanced mode’. Go to Image>Acquisition and change the ‘binning rate’ to 5.
13. Measurements can be affected by a number of factors including temperature, buffer composition, and instrument drift. To negate these factors, we calibrate the instrument with fresh protein standards (see above) in the desired buffer before taking experimental measurements.
14. Calibrate the instrument by creating a standard curve using known mass standards (Cytiva gel filtration calibration kits [part No# 28403841]) in the same buffer that the protein of interest is in (see Note 2).

4.2 Experimental measurements with the protein of interest:

1. Using a fresh coverslip with applied gasket prepared as in steps 4.3-8, apply 6 µL of myosin motility buffer (2.4.1) containing 150 mM KCl and 1mM DTT to the well, as a buffer blank, taking extra care to not let the pipette tip touch the carrier slide.
2. If necessary, adjust the image size (located in the tool bar) according to the estimated sample mass. Most dimeric myosin samples (~200 – 600 kDa) will be measured using the default window size (10 x 2 µm).
3. Set the image view to “Native” in the toolbar and use the “droplet dilution” to find focus. The focus settings will lock automatically.
4. Add 6 µL myosin diluted in myosin motility buffer (2.4.1) containing 150 mM KCl and 1 mM DTT (aim for a final concentration of 10 – 100 nM) and mix by pipetting 3 – 4 times, before removing ~6 µL of diluted sample. The final concentration of the myosin on the coverslip will thus be half the prepared dilution concentration. We aim for a final concentration of between 5 – 50 nM.
5. Acquire a movie (0.5 – 3 minutes; depending on the number of events observed; see Note 3). Click ‘enable analysis preview’ to watch data collection in real time.
6. Analyse the data using the Refeyn DiscoverMP software.
7. Playback the movie to check for any large aggregates or dirt events. These can be excluded by clicking and dragging on the movie field of view to select the problem area. All events within the selected area will be excluded for the entirety of the movie.

8. Upload the calibration curve and export events, figures and tables using the export function.
9. Exporting tables generates a corresponding mass for each event which can then be analysed in your data visualisation software of choice, allowing you to set bin width and fit a Gaussian distribution to the data (Figure 1).

5. Negative stain EM

Negative stain EM (nsEM) provides a quick and efficient way to visualize the structure of the purified protein. We typically use nsEM to quickly assess the quality of our proteins after purification to determine if they are correctly folded (have the expected appearance) and are free from contaminants or aggregates.

5.1 Preparing actin and myosin protein samples for negative stain EM.

G-actin can be extracted from rabbit muscle ***(34)*** and stored in the liquid nitrogen storage tank for years. Our stock concentration of G-actin is in the ~200 - 300 µM range. Alternatively, both commercially available G-actin and biotinylated-G-actin is available from Cytoskeleton (https://www.cytoskeleton.com/) or Hypermol (https://www.hypermol.com/) and should be reconstituted as described by the manufacturers' protocols. We include NaN_3 in the polymerisation buffer to avoid bacterial growth.

1. Reconstitute (or dilute) G-actin (depending on the source of the actin) to 20 µM in polymerization buffer: 50 mM KCl, 25 mM MOPS (pH 7.0) 2 mM $MgCl_2$, 0.2mM ATP, 0.5mM DTT, 0.5 mM NaN_3. Leave the F-actin to polymerise at room temperature for 30 min – 1 hr, or at 4 °C overnight.
2. Dilute F-actin to 1 µM in motility buffer plus 50 mM KCl (MB+50). This can be stored at 4°C and used for up to 1 week.
3. Dilute purified myosin (e.g. GFP-M6HMM purified as above) to a final concentration of 100nM in motility buffer containing 50 mM KCl and 2 µM ATP.

5.2 Preparing grids for negative stain EM.

Negative stain grids can be prepared by different methods, with a variety of heavy metal stains and can have very different outcomes on the appearance of the molecule within the stain which have been summarised by ***(35)***. Detailed below is the method that we use to prepare negative stain grids.

1. Place the carbon coated grid face up on a microscope slide in a glow discharge unit. Treat for a minimum of 30 s at 10 mA.
2. Centrifuge 1ml 1% uranyl acetate (UA) in a 1.5ml Eppendorf tube at 17k x g for 2 mins to pellet any debris prior to use in f. (N.B. UA is radioactive and appropriate safety requirements should be followed).
3. Grip the edge of the freshly glow discharged grid with a pair of negative pressure tweezers.
4. Mix equal volumes of diluted F-actin and myosin samples (prepared in steps 5.1) apply 3 – 5 µL to the grid and allow the sample to attach for no more than 10 s to avoid ATP depletion. (see Note 4).
5. Holding the grid at an approximate 45° angle facing away, rapidly flick off excess liquid from the top of the grid.
6. Using a glass Pasteur pipette, apply a drop of 1% uranyl acetate to the grid surface and 'flick off' immediately as in 5.2.5. Repeat 2-5 times dependent on stain depth required for visualization of specimen. The number of drops added and the contact time of the stain with the grid affects the depth of the stain. Alter as necessary.
7. Remove excess stain by touching the torn edge of a piece of WHATMAN ashless, grade 42 filter paper (Cytiva) to the edge of the grid.
8. Allow the grid to air dry or dry under an incandescent lamp for 2 minutes.
9. Visualise samples using a transmission electron microscope at a nominal magnification of ~29000x. Capture micrographs using a 2 – 5 µm defocus. Micrographs (Figure 2) can then be visualised using ImageJ and further processed using image processing software such as RELION ***(36)*** or Imagic (https://imagescience.de/imagic.html) ***(37)*** to assess protein integrity. Micrographs containing particles of interest on F-Actin can be manually picked and subjected to iterative rounds of 2D classification and

alignment. This data can be used to examine to step size of individual motors along with other structural features relating to the myosin, such as the positions of the lever, as has been done previously ***(38)***.

6 Preparing proteins for molecular motor assays.

6.1 Fluorescently labelling myosin

Recombinantly expressed myosin can be labelled fluorescently using a number of different methods, such as introducing a genetically encodable fluorophore (GFP/eGFP) engineered into the construct ***(30, 39)*** or by labelling a myosin-interacting accessory protein, such as calmodulin labelled with a synthetic organic fluorophore and exchanged onto the myosin heavy chain ***(40-42)***. These techniques allow the visualisation of these motors directly in fluorescence imaging assays. Here, we used the genetically encodable HaloTag, which allows the purified protein (here, Halo-M6HMM) to be labelled with a range of synthetic organic fluorophores for use in *in-vitro* motility assays.

1. Purified Halo-tagged myosin can be labelled using a range of commercial HaloTag compatible dyes available from Promega or Tocris. Some Janelia dyes are available directly from Janelia (https://www.janelia.org/open-science/janelia-fluor-dyes).
2. To label Halo-tagged myosin, Janelia Fluor 646 HaloTag Ligand was reconstituted in myosin motility buffer to a final concentration of 100 μM, added in 10x molar excess to the Halo-tagged myosin and incubated overnight at 4˚C.
3. Excess dye was removed by dialysis into myosin dialysis buffer overnight using a suitable molecular weight cut-off as described previously (Section 3.3.30.) Alternatively, the dye can be quickly removed by multiple passes (2 – 3) through Zebra Spin Desalting Columns (7K MWCO) (Thermo Fisher Scientific) according to the manufacturers' instructions.
4. Labelled proteins can either be snap frozen in liquid N_2 or used immediately.

6.2 Fluorescently Labelling actin

To visualize actin filaments (F-actin) in motility assays, it needs to be fluorescently labelled. Typically, we use fluorescent phalloidin to label F-actin. Biotinylated G-actin can additionally be incorporated into the F-actin, to enable attachment of F-actin to the surface of the coverslip via streptavidin or Neutravidin ***(40)*** (see Note 4).

1. G-actin and biotin-G-actin (Cytoskeleton), reconstituted in polymerisation buffer, are mixed in a 9:1 ratio to form 20 µM F-actin, comprised of 10% biotin G-actin as described in step 5.1 and allowed to polymerise overnight. Typically, we would make a 100 µL volume containing 2 µM biotinylated G-actin and 18 µM G-actin.
2. Actin is labelled with rhodamine-phalloidin, the day after polymerization. Biotin-F-actin can be labelled using a 1.2x molar excess of phalloidin. Usually, the polymerized sample is diluted to ~2 – 5 µM in the assay buffer of choice and labelled for at least 2 hrs before use.

7. Fluorescence TIRFM motility assays

In vitro fluorescence imaging of myosins and actin filaments near the surface of the sample (<100 nm from the glass coverslip) can be imaged using total internal reflection fluorescence (TIRF) microscopy and has been commonly used for dynamic studies of myosin ***(29, 40, 43)***. TIRF microscopes are commercially available from many microscope companies or can be homebuilt. In our labs, we have access to a Nikon TIRF microscope that can be used for *in vitro* fluorescence assays. Specifically, we have an Eclipse Ti with H-TIRF system equipped with a 100x TIRF Objective (N.A. 1.49) and an iXon DU888 EMCCD camera. Four different laser emission (405nm, 488nm, 561nm,and 640nm) can be achieved using a LU-N4 solid state laser unit. More information about the microscope can be found on the manufacturer's website (https://www.microscope.healthcare.nikon.com/). Settings for imaging using the Nikon TIRF system for single molecule actomyosin imaging can be found in our previous methods article ***(44)***.

7.1 Coverslip preparation

Coverslips can be functionalised in a number of ways which will be outlined below. These include functionalisation to work with either biotin-BSA, biotinylated-PEG, or PLL-PEG-Biotin.

7.1.1 Coverslip preparation with nitrocellulose for biotin-BSA

1. Prepare a 1% nitrocellulose (colloidal) solution in pentyl acetate.
2. Place desired number of #1.5 thickness 22 mm square coverslips on a rack. Wash them with excess 100% ethanol, followed by distilled water. Repeat the washing step, concluding with water. Subsequently, thoroughly dry the coverslips using a filtered air-line or N2-line. There are alternative ways to wash coverslips (e.g. see ***(44-46)***, but the key point here is that the final coverslips should be free from dust.
3. Select one coverslip and gently pipette 10 µL of the 1% nitrocellulose solution along one edge. Then, smoothly spread it across the entire coverslip using the side of a 200 µL pipette tip. Place this coverslip on a piece of WHATMAN filter paper in a petri dish with the nitrocellulose side facing up. Repeat the process for the remaining coverslips, allowing them to dry while preparing the remaining reagents. Ensure the coverslips are used within 24 hours after coating.

7.1.2 Coverslip preparation with biotinylated-PEG

1. Divide the stock powder of methoxy-Peg-silane (mPEG) and biotin-Peg-silane (bPEG) into 10 mg aliquots each, stored in 1.5 mL tubes. Keep them at -20 °C in a sealed, moisture-free container and use within 6 months.
2. Place cleaned coverslips (as in 7.1.1.2) on filter paper (90 mm) in a tissue culture dish (100 x 20 mm) and incubate in a 70 °C oven for 30-60 minutes to dry.
3. Prepare an 80% ethanol solution with ddH_2O, adjust the pH to 2.0 using HCl, and add 1 mL of this solution to a 10 mg aliquot of mPEG and bPEG. Vortex to dissolve.

4. Prepare a 1ml solution containing mPEG to a final concentration of 2 mg/mL and bPEG to a final concentration of 10 μg mL^{-1}, in 80% ethanol (pH 2.0) solution.
5. Dispense 100 μL of the PEG solution above onto the centre of each coverslip and incubate for 20 to 30 minutes at 70 °C. When the coverslips show a 'holey' appearance caused by the solution evaporating, remove them from the oven, wash with excess 100% ethanol, dry with an air-line, and proceed immediately to the flow cell chamber preparation step (step 6.2).

7.2 Chamber Preparation:

1. Take a clean microscope slide and place two narrow pieces of double-sided scotch tape (3M) along its long edge.
2. Take either a functionalized nitrocellulose or bPEG coverslip prepared previously and apply facedown onto the tape (functionalised surface facing down) to form the flow through chamber and press gently with a P200 pipette tip to ensure proper adhesion.
3. Cut the excess tape with a razor blade. Chambers can be used immediately or stored in a -80 °C freezer for future use. Store immediately to prevent surface degradation.
4. Wash the chamber with 30 μL MB50+ buffer using a Kimwipe tissue gently pressed up against the exit of the flow cell; capillary action will aid in drawing the solution through the flow cell. It is necessary to fill with chamber with ~1.5x the volume of the flow cell with each subsequent wash step, typically around 20 – 40 μL.
5. Steps required to apply the biotinylated F-actin to the coverslip surface depend on how the coverslip has been functionalised (7.1.1 or 7.1.2) as described below. (See Note 4).
6. For **nitrocellulose** functionalised coverslips flow 30 μL 1 mg/ml BSA-biotin in MB50+ which adheres to nitrocellulose. Repeat this step 2 more times.
7. Wash the chamber with 30 μL MB50+ buffer (Section 2.4). Repeat this wash step 2 more times.
8. For **biotinylated-PEG** functionalised coverslips, skip forward to step 7.2.8.

9. Flow 30 µL 2 mg/ml Neutravidin in MB50+ buffer into the flow cells and allow it to adhere for 1 minute.
10. Wash the flow cell three times again as in step 7.2.4
11. Flow in 30 µL of rhodamine-phalloidin stabilised biotinylated F-actin (prepared in Step 6.2) at a concentration of 200 nM in MB50+ DTT buffer. Allow the F-actin to adhere for 1 minute.
12. Perform one final wash step with MB50+ DTT buffer.
13. Flow in the final buffer containing 20 nM HaloM6-HMM, 1 mM ATP, 50 mM DTT, 1 µM calmodulin, 2.5 mg/ml glucose, 100 µg/ml glucose oxidase and 40 µg/ml catalase in MB50 buffer. Then quickly place the chamber onto the TIRF microscope, focus and image. We typically use an exposure time of between 100 - 200 ms (5 – 10 frames per second) using 1.4 mW laser power for approximately 3 min ***(44)***.

8. Adapting the motility assay for MINFLUX.

MINFLUX (minimal photon fluxes ***(47)***) is a form of super-resolution microscopy originally derived from STED (stimulated emission depletion) microscopy ***(48, 49)*** and single molecule localisation techniques. In STED, the point source excitation confocal beam is accompanied by a donut shaped stimulated emission depletion beam, which effectively shrinks the size of the fluorescent excitation spot, by depleting fluorescence of all the fluorophores in the donut. In MINFLUX, molecules are excited using a donut-shaped excitation beam which is rapidly moved over the field of view to find single emitters and then scanned around the single emitter with the zero-intensity position of the donut close to the position of the emitter. Another way to think about this using a 1D example, is to imagine a standing wave of excitation intensity translated across a fluorescent emitter; the emitted fluorescence will depend on the intensity of the excitation, and at some point will reach zero ***(47)***. Keeping the emitter close to the zero position enables its position to be monitored precisely using a very small number of photons from a small number of positions of the excitation pattern. This approach can result in very precise localisations (to approximately 2 nm) of a fluorescent tag to a target molecule, and can be

used to investigate the distribution of target molecules across a region of interest, or to track single molecules ***(47)***. For tracking, multiple donuts are scanned across the field of view and can be used to track several molecules at the same time in motility assays as demonstrated for kinesin using in vitro motility assays or in cells ***(50, 51)***. As with many super-resolution techniques, the choice of fluorophore is critical, and the best fluorophore is currently JF646.

The steps used to make the flow cell chambers for the MINFLUX assay are very similar to those described in 7.2, with some minor adjustments as described below. In particular, concentrations of myosin need to be low (sub 100 nM) to ensure that stepping events are well separated spatially. Two stepping molecules close together on a single F-actin may not be discriminated as separate events in MINFLUX. Using low MgATP concentrations (~100 μM) can also be beneficial, as it can take some time for MINFLUX to identify stepping events. For myosin isoforms, such as the Myo6 dimers, which only take a small number of steps (3-5) before detaching, decreasing the ATP concentration means that the time between steps is increased, and they are more easily detected. Slowing this step increases the frequency at which MINFLUX is able to capture several steps. Using Halo-tagged myosin, labelled with JF646 is optimal for imaging. Finally, gold nanoparticles are required, for drift correction. The procedure for making the flow cells for MINFLUX was as follows.

1. Plasma cleaned coverslips were used.
2. Flow cells were assembled as described, and the coverslip coated with PLL-Biotin for 15 minutes.
3. After 1x wash with phosphate buffered saline (PBS), 40 μL resuspended gold nanoparticles (200 nm gold nanoparticles (Nanopartz, Cat# A11-200-CIT-DIH-1-10) were added to the flow cell, and left in a humid chamber for 15 minutes, with the coverslip on the bottom, to promote adherence of the gold particles.
4. Flow cells were washed x1 with PBS, and then 1x with MB50+DTT

5. Flow cells were then washed x 3 with 1mg/mL BSA (diluted in MB50+DTT from 20mg/mL stock), with final wash left for 1 minute.
6. Flow cells were washed x 1 with MB50+ DTT
7. Flow cells were incubated with 40 µL of Neutravidin (5mg/mL stock diluted to 2mg/mL in MB50+DTT) for 1 minute, then washed x1 with MB50+DTT
8. 1 µM fluorescent, biotinylated actin (diluted from 5 µM stock) was further diluted to 40nM in MB50+DTT, using a cut-off yellow tip for pipetting steps, immediately added to the flow cell, and incubated for 2 minutes.
9. Flow cell was then washed x1 with MB50+DTT
10. Myosin was added. The myosin stock solution was diluted into MB50+DTT to a final concentration of between 0.75 nM and 100 nM, and ATP to a final concentration of 100 µM. Purified calmodulin (to a final concentration of ~0.2 µM) can also be added at this point. (See Note 5).
11. The flow cell is then quickly mounted into the MINFLUX microscope for imaging.

Once the sample is mounted, a field of view is selected in which actin filaments and myosin molecules are visible (Fig 3A,B). Data is recorded for up to ~20 minutes, and each individual track is colour coded (Fig. 3A,C,D). Data can then be analysed using SMAP (https://github.com/jries/SMAP)***(52)***, which requires MATLAB. In this analysis, individual tracks can be selected and analysed (Fig. 3D–G) in a variety of ways as needed. This type of analysis was reported for kinesin motility both in vitro ***(51)*** and in cells ***(50)***. With localisation precision of ~2 nm and temporal resolution of ~1 ms ***(48, 49)***, using approximately 20 photons per localisation, steps of 4 nm were resolvable for the labelled kinesin stalk ***(49)***. Thus, MINFLUX analysis of myosin motor stepping along actin filaments should develop into a useful tool for understanding myosin behaviour.

Notes

Note 1: If the protein also incorporates a His-tag, samples that are dialyzed and concentrated, can also followed by His affinity purification, such as a commercially available His-tag purification kit and protocol. This tandem

affinity purification is also an extremely powerful technique to use when the myosin constructs required need to have two different myosin heavy chains in the final dimeric construct ***(33)***. The purified product can then be dialyzed as in step 3.3.30 and further stored or immediately used for specific assays.

Note 2: The mass calibration relies on the measurement of proteins of known mass, with each particle that lands on the glass surface producing a contrast signal that is linearly related to its molecular mass. We use the following standards at the following concentrations: BSA (25 nM; molecular weight 66 kDa), apoferritin (16 nM; molecular weight 443 kDa), and thyroglobulin (16 nM; molecular weight 669 kDa), all available from Sigma-Aldrich. After obtaining a movie of each known mass standard, open the resulting movie in the DiscoverMP software. Once loaded, use the cursor to select the peaks to determine the ratiometric contrast values. Next, press the 'Calibrate' button and input the molecular mass of the corresponding peak(s) in the resulting window. If calibrants are saved in separate results files, open a second DiscoverMP window (while keeping the original window open) and load the remaining calibrant result files, and again use the cursor to select the peak(s) to obtain the ratiometric contrast value(s). Returning back to the 'Calibration' window, enter the ratiometric contrast value and molecular mass of the standard as described previously. Repeat these steps for any remaining calibrants. DiscoverMP will fit a straight-line calibration through the points entered in the table. After all calibrants have been entered, name and save the calibration file. A summary of the steps described can be found at the official Refeyn YouTube channel (https://www.youtube.com/watch?v=VLdXHA0BSDw). We typically perform this immediately before our mass-measurement session and then again at the end to make sure there has been no change over time.

Note 3: For samples at a concentration of 10 – 50 nM, we aim for between counts on the order of 100s – 1000s landing events in a movie lasting 1 minute.

Note 4. We use a yellow pipette tip, which has the end cut-off using a scalpel or razor blade, when handling F-actin, to prevent shearing.

Note 5: if using Halo-tagged myosin labelled with the JF646 dye, it is important to remove as much of any 'free' dye as possible from the Halo-tagged myosin, to remove background arising from the binding of free dye to the coverslip by dialysis or spin column purification steps.

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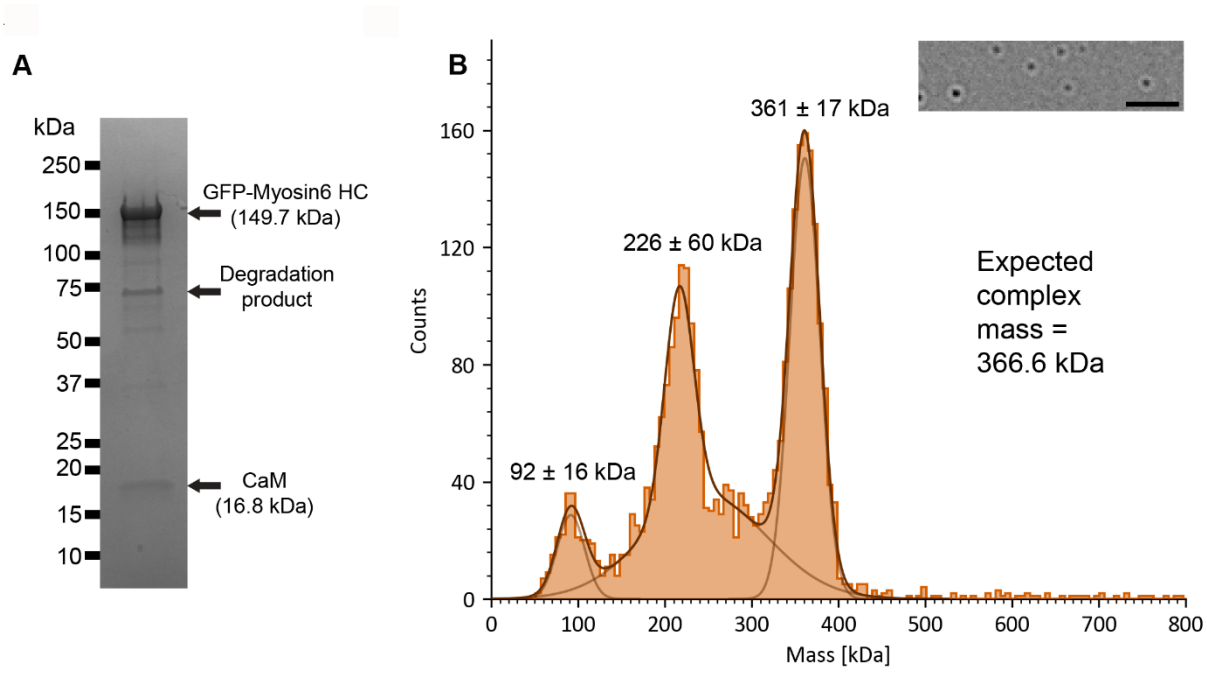
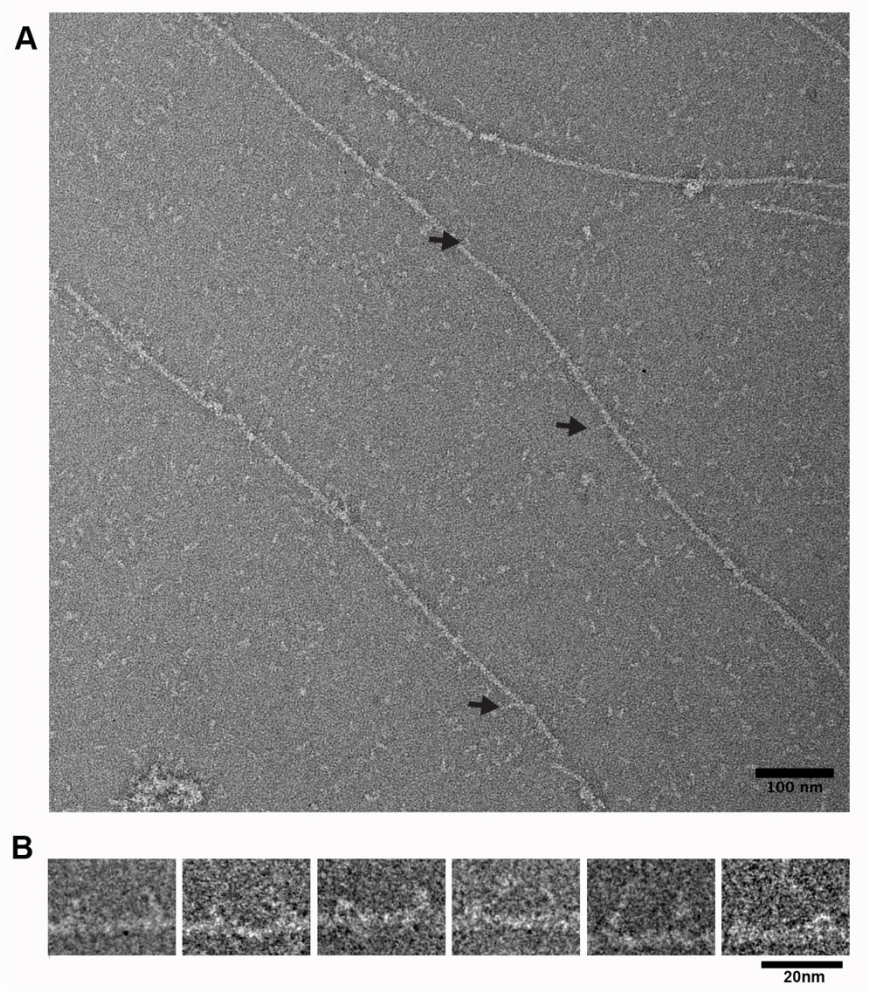
Figure Captions

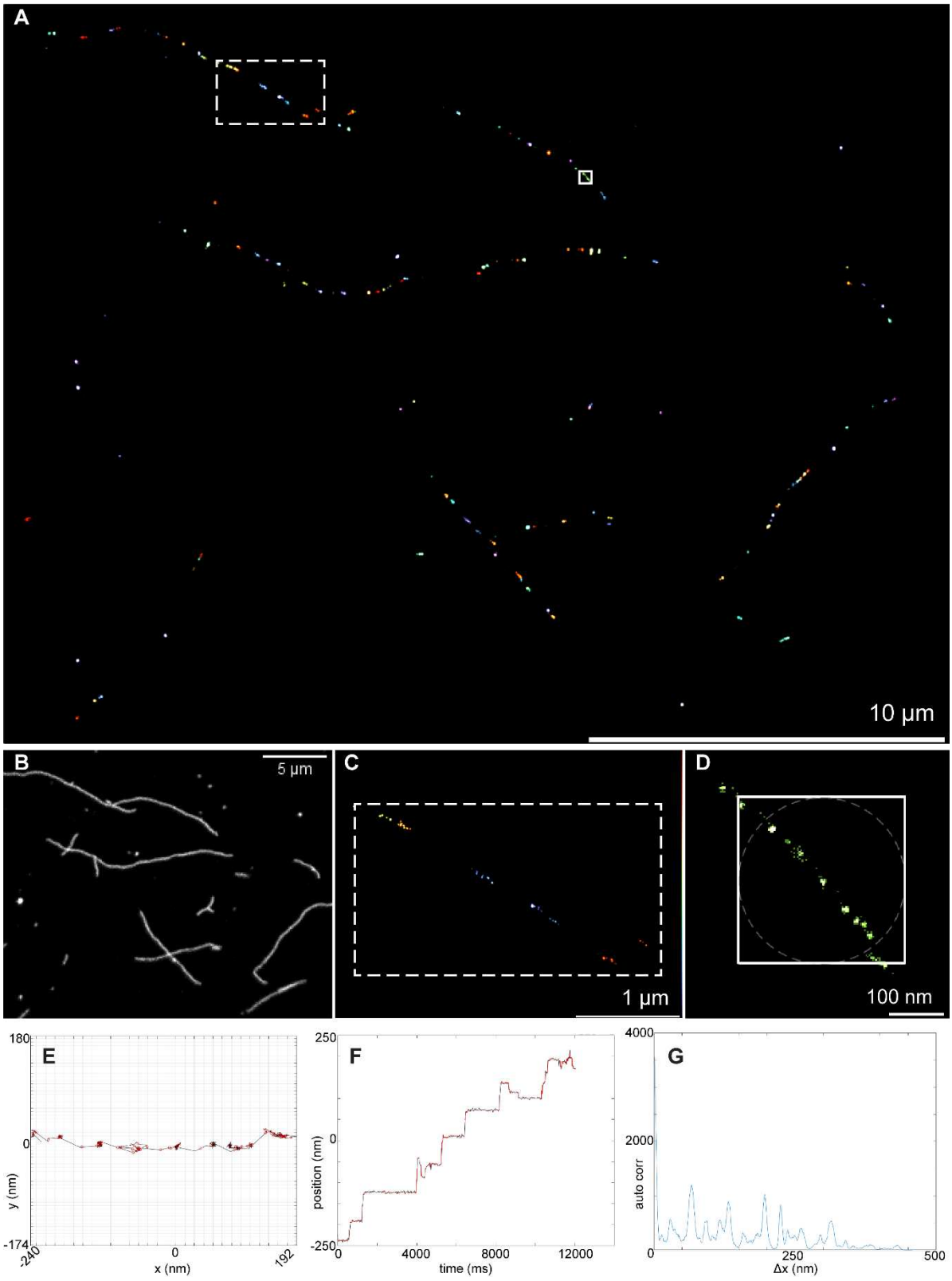
Figure 1: Mass Photometry results for Myo6a dimers. A: shows the SDS gel for purified Myo6, with bands for the heavy chain and light chain observed. B: mass photometry data shows the major peak has a molecular mass of 361 kDa, which is equivalent to a dimer (~300 kDa) and 4 calmodulin light chains (~60 kDa). A second peak with a mass of 226 kDa is likely a dimeric degradation product form with two heavy chains (150 kDa) and 4 calmodulin light chains (60 kDa).

Figure 2. Negative stain images of dimeric Myo6a bound to actin. A: representative field-of-view of M6HMM bound to F-Actin in the presence of ATP (black arrows). Unbound proteins can be seen in the background. B. Montage of **M6HMM molecules bound to F-Actin.**

Figure 3. MINFLUX example data. **A:** MINFLUX FOV with colour-coded individual tracks of dimeric HALO-M6HMM labelled with JF646, recorded after finding F-actin filaments labelled with rhodamine phalloidin. Mg.ATP concentration was 100μM (**B**). **C,D:** Higher magnification views of tracks in boxed regions in **A**. A small number of tracks in the dashed box (**C**) and a single track in the solid box (**D**). **E-G:** Example data obtainable with SMAP ***(52)***. **E:** XY plot of the track in **D**, showing steps between binding sites with coordinates transformed so that X is the average direction of the track. **F:** Plot of distance along the track (X) against time with a fit to the stepping model (red line), allowing the extraction of step distances and times. Autocorrelation of the track in X (G), with the positions and shapes of peaks also containing data on the distribution of step sizes between more stable positions.


A
kDa
250
150
100
75
50
37
25
20
15
10
GFP-Myosin6 HC
(149.7 kDa)
Degradation
product
CaM
(16.8 kDa)
B
361 ± 17 kDa
226 ± 60 kDa
92 ± 16 kDa
Expected
complex
mass =
366.6 kDa
Counts
Mass [kDa]
0
40
80
120
160
0
100
200
300
400
500
600
700
800


A
100 nm
B
20nm


A
10 µm
B
5 µm
C
1 µm
D
100 nm
E
180
0
-174
y (nm)
-240
0
192
x (nm)
F
250
0
-250
position (nm)
0
4000
8000
12000
time (ms)
G
4000
2000
auto corr
0
250
500
Δx (nm)