**Submission ID #: 62180**
**Scriptwriter Name: Domnic Colvin**
**Project Page Link:** https://www.jove.com/account/file-uploader?src=18964948

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

## Authors and Affiliations:

Ananya Tripathi[1], Charles Bond1[1,2], James R. Sellers[1], Neil Billington[1]*, Yasuharu Takagi[1]*

[1]Laboratory of Molecular Physiology, Cell and Developmental Biology Center, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, 20892, United States of America
[2]Current affiliation: Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, 19104, United States of America

**Corresponding Authors:**
Neil Billington (neil.billington@nih.gov)
Yasuharu Takagi (takagiy@nhlbi.nih.gov)

**Email Addresses for All Authors:**
ananya.tripathi@nih.gov
sellersj@nhlbi.nih.gov
bondcr@pennmedicine.upenn.edu
neil.billington@nih.gov
takagiy@nhlbi.nih.gov

# Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees self-record interview statements. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

   **Walking distance (across the hallway).**

**Current Protocol Length**

Number of Steps: 18
Number of Shots: 40

# Introduction

**1. Introductory Interview Statements**

**REQUIRED:**

1.1. **Yasuharu Takagi:** This protocol can be used to characterize nonmuscle myosin dynamics at the protein level, which can inform the motile properties that contribute to their roles in cells.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Yasuharu Takagi:** This is a fast, reproducible, and highly controlled method that can be used to study the effects of various regulatory conditions on myosin motility, informing their cellular behaviors.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

**OPTIONAL:**

1.3. **James Sellers:** These techniques can be used to investigate how disease-causing mutations in nonmuscle myosins affect their behaviors at the single molecule and ensemble level.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **James Sellers:** The general approach of this methodology can be applied to various other cytoskeletal systems, such as the characterization of purified kinesins and dyneins with microtubules.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.5. **Ananya Tripathi:** This protocol's versatility can raise questions about which fluorophores and chemical conditions are most appropriate, but the method can be optimized relatively quickly.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.6. **Ananya Tripathi:** Understanding how to set up and coat the flow chambers makes for a strong foundation upon which one can adapt various conditions for these experiments.

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

# Protocol

## 2. Gliding actin filament assay

2.1. To begin, prepare a 1% nitrocellulose solution in amyl acetate **[1]** and place a circular filter paper with a 125-millimeter diameter on the bottom of a tissue culture dish **[2].**

2.1.1. WIDE: Establishing shot of talent preparing nitrocellulose solution.

2.1.2. Talent placing a circular tissue paper to the bottom of a culture dish.

2.2. Load eight square coverslips onto a rack and thoroughly wash them with approximately 2 to 5 milliliters of 200-proof ethanol followed by 2 to 5 milliliters of distilled water **[1-TXT]**. Then, dry the coverslips completely using a filtered air-line or Nitrogen-line **[2].**

2.2.1. Talent washing 8 coverslips with 200-proof ethanol and water. **TEXT: No. 1.5 thickness 22 mm**

2.2.2. Talent drying the coverslips using air-line/nitrogen-line.

2.3. Slowly pipette 10 microliters of 1% nitrocellulose solution along one edge of one coverslip **[1]**. Smear it across the rest of the coverslip using the side of a 200-microliter pipette tip in one smooth motion **[2]**, then place this coverslip on the tissue culture dish with the nitrocellulose side up **[3]**. *Videographer: This step is difficult and important!*

2.3.1. Talent pipetting 1% nitrocellulose solution on the edge of coverslip. Videographer NOTE: 2.3.1 – 2.3.3 are filmed as a single shot in varied angles

2.3.2. Talent smearing the solution on the rest of the coverslip.

2.3.3. Talent placing the coverslip on the tissue culture dish.

2.4. Repeat this for the remaining coverslips and allow them to dry **[1-TXT].** Wipe a microscope slide with an optical lens paper to clean off large debris **[2]**. Cut two pieces of double-sided tape, approximately 2 centimeter in length **[3]**, and place one piece along the middle of the long edge of the microscope slide **[4-TXT].**

2.4.1. Talent smearing another coverslip. **TEXT: use coverslips within 24 h after coating.** Videographer NOTE: **Use 2.3.4.1'**

2.4.2. Talent wiping slide with an optical lens paper.

2.4.3. Talent cutting pieces of double-sided tape. Videographer NOTE: 2.4.3, 2.4.4 and 2.5.1 are filmed as a single shot 2.4.3

2.4.4. Talent placing one piece of the tape along the middle edge of the slide. **TEXT: Align edge of the tape with the edge of the slide.** Videographer NOTE: **Use 2.4.3**

2.5. Place the second piece of tape roughly 2 millimeters below the first piece of tape such that the two are parallel, creating a flow chamber that can hold approximately 10 microliters of solution **[1]**. Carefully stick one of the nitrocellulose coverslips onto the tape, so that the side coated with nitrocellulose is making direct contact with the tape **[2].** *Videographer: This step is important!*

2.5.1. Talent placing second tape on the slide below the first tape. *Video editor show Figure 1B in the inset.* Videographer NOTE: **Use 2.4.3**

2.5.2. Talent sticking nitrocellulose coverslips in contact with the tape. Videographer NOTE: 2.5.2 and 2.6.1 are filmed as a single shot

2.6. Using a pipette tip, gently press down on the slide-tape interface to ensure that the coverslip has properly adhered to the slide **[1]**, then cut the excess tape hanging over the edge of the slide with a razor blade **[2].**

2.6.1. Talent pressing the tape with a pipette tip. Videographer NOTE: **Use 2.5.2**

2.6.2. Talent cutting the excess tape with blade.

2.7. Prepare the solutions for myosin 5a and keep them on ice **[1].** Flow in 10 microliters of the myosin 5a through the slide flow chamber and wait for 1 minute **[2-TXT]**, then flow in 10 microliters of 1 milligram per milliliter BSA in motility buffer with DTT *(D-T-T)*, repeat this twice and wait a minute after the third wash **[3-TXT]**

2.7.1. Talent preparing solutions for myosin 5a.

2.7.2. Talent flowing the myosin 5a protein through slide flow chamber for a minute. **TEXT: 50 – 100 nM**

2.7.3. Talent flowing BSA in MB and DTT solution through flow chamber slide. **TEXT: 50 mM MB with 1 mM DTT**

2.8. 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 **[1]**. Then, wash with 10 microliters of motility buffer with DTT **[2].**

2.8.1. Talent wicking the extra solution using a tissue paper.

2.8.2. Talent washing the slide with MB-DTT.

2.9. Pipette the black actin solution with a 1 milliliter syringe and 27-gauge needle to shear the actin filaments before introducing the solution to the chamber **[1].** Add a stoichiometric amount of F-actin to myosin in the presence of 1 millimolar ATP and 1 millimolar magnesium chloride at a salt concentration of 500 millimolar **[2]**. *Videographer: This step is important!*

2.9.1. Talent pipetting black actin solution through the syringe.

2.9.2. Talent adding F-actin solution to myosin.

2.10. Next, flow in 50 microliters of motility buffer with DTT and 1 millimolar ATP *(A-T-P)* to deplete the chamber of free actin filaments **[1].** Wash with 10 microliters of motility buffer with DTT three times to deplete the chamber of any ATP **[2]**.

2.10.1. Talent flowing solution to wash away free actin. Videographer NOTE: **Use 2.8.2+1**

2.10.2. Talent flowing solution to deplete ATP.

2.11. Flow in 10 microliters of 20 nanomolar Rh-actin *(R-H)* solution containing motility buffer with DTT and wait for 1 minute to allow rigor binding of actin filaments to the myosin 5a attached to the surface of the coverslip **[1-TXT]**.

2.11.1. Talent flowing Rh-actin solution to assist actin and myosin binding. **TEXT: Rh-rhodamine**

2.12. Wash with 10 microliters of 50 millimolar motility buffer with DTT twice to remove unbound Rh-Actin filaments **[1]**. *Videographer: This step is difficult and important!*

2.12.1. Talent flowing solution to wash away unbound rhodamine actin filaments.

2.13. Flow in 30 microliters of Final Buffer **[1].** Record images on a fluorescence microscope using an excitation wavelength of 561 nanometers to visualize the Rh-Actin **[2-TXT]**. *Videographer: This step is important!*

2.13.1. Talent flowing final buffer through the slide chamber.

2.13.2. Talent recording images under fluorescence microscope. **TEXT: exposure time-200 ms at 1.4 mW laser power for 0.5-1 min**

## 3. Performing the myosin 5a TIRF microscopy assay

3.1. Prepare the solutions for myosin 5a inverted motility assay and keep them on ice **[1]** and wash the chamber with 10 microliters of motility buffer with DTT **[2].**

3.1.1. Talent preparing the solutions for inverted motility assay.

3.1.2. Talent washing the chamber with solution.

3.2. Flow in 10 microliters of 1 milligram per milliliter BSA in motility buffer with DTT. Repeat this twice, waiting a minute after the third wash **[1].** Use tissue or filter paper to wick the solution through the channel by gently placing the corner of the paper at the flow chamber exit **[2]**.

3.2.1. Talent flowing BSA in DTT solution through flow chamber slide.

3.2.2. Talent wicking the extra solution using a tissue paper.

3.3. Then, wash the chamber with 10 microliters of motility buffer with DTT three times **[1].** Flow in 10 microliters of the NeutrAvidin solution in motility buffer with DTT and wait for 1 minute **[2]**, then wash with 10 microliters of the solution three times **[3]**.

3.3.1. Talent washing the slide with MB in DTT solution.

3.3.2. Talent flowing NeutrAvidin solution.

3.3.3. Talent flowing washing solution.

3.4. Flow in 10 microliters of bRh-Actin *(B-R-H)* containing motility buffer with DTT using a large-bored pipette tip and wait for 1 minute **[1-TXT]**. Then, wash with 10 microliters of motility buffer with DTT three times **[2].**

3.4.1. Talent flowing bRh-actin solution. **TEXT: bRh- biotinylated rhodamine**

3.4.2. Talent flowing washing solution.

3.5. Flow in 30 microliters of Final Buffer with 10 nanomolar myosin 5a **[1]** and immediately load onto the total internal reflection fluorescence microscope. Begin recording once the optimum focus for TIRF imaging is found **[2-TXT]**. *Videographer: This step is important!*

3.5.1. Talent flowing final buffer.

3.5.2. Talent loading the slide to TIRF microscope for image recording. **TEXT: exposure time- 100-200 ms, 1.4 mW laser power, velocity analysis for 3 min**

# Results

**4. Gliding activity assay of the NM2b and M5a-HMM filaments.**

4.1. The purification of myosin heavy chain 5a, non-muscle myosin 2b, calmodulin**,** essential light chains, and regulatory light chains was evaluated by performing SDS-PAGE *(S-D-S-page)* **[1].**

4.1.1. LAB MEDIA: Figure 2. *Video editor focus on all the individual bands.*

4.2. The gliding actin filament assay shows the characteristics of an ideal and trackable movie, featuring smooth movement of labeled actin filaments. The black actin wash ensured that the dead myosin heads were removed from the measurement field, further contributing to the overall smooth movement of the actin filaments **[1]**.

4.2.1. LAB MEDIA: Video1.mp4.

4.3. Filament tracking output images form the FASTrack program were obtained for non-muscle myosin 2b and heavy chain myosin 5a **[1].** A representative histogram shows that the actin-myosin2b gliding velocity is 77 nanometers per second **[2]** and that pf actin-myosin 5a is 515 nanometers per seconds **[3].**

4.3.1. LAB MEDIA: Figure 3B and E.

4.3.2. LAB MEDIA: Figure 3C.

4.3.3. LAB MEDIA: Figure 3F.

4.4. In the absence of methylcellulose, the actin filaments do not remain as closely associated with the myosin-coated surface, causing its flopping close to the surface of the non-muscle myosin 2b coated coverslips **[1].**

4.4.1. LAB MEDIA: REVISED Video2.mp4.

4.5. Myosin movement was observed upon surface-tethered fluorescent actin filaments using the inverted motility assay **[1].**

4.5.1. LAB MEDIA: Video3.mp4.

# Conclusion

**5. Conclusion Interview Statements**

5.1. **James Sellers:** It is important to sheer the unlabeled actin before introducing it to the flow cell for the black actin wash, which will allow for the smooth translocation of actin.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.9.*

5.2. **Yasuharu Takagi:** Additional experiments can investigate how myosin motility is affected by myosin mutations, load-inducing proteins, ionic strengths, and regulatory proteins. Various cellular conditions can be reconstituted for future study.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

5.3. **Ananya Tripathi:** These methods allowed for biochemical and biophysical investigations into how various myosin isoforms vary in their motile and mechanical properties at the single molecule and ensemble level.

5.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.