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Title: Reconstituting Myosin-I-Mediated Branched Actin Assembly in a Bead-Based Actin Comet-Tail Motility Assay

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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. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 22

Number of Shots: 58

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Mengqi Xu:** Our research focuses on studying how myosin-I shapes branched actin networks and drives force generation at membrane interfaces. **Videographer's Notes: Author prefers the 1st attempt in this take.**
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Mengqi Xu/Mike Ostap:** Previous cellular studies make it hard to isolate myosin-I effects; this protocol reconstitutes everything in vitro under well-controlled conditions.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Mengqi Xu:** This protocol allows researchers to study how membrane-bound myosin motors regulate actin network density, growth kinetics, and polymerization-driven bead movement.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Mengqi Xu:** The most important consideration is protein quality. Always validate new preparations against a previously validated batch before quantitative experiments.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Mengqi Xu/Mike Ostap:** Future studies building on this work can test other membrane-associated myosins, disease-associated motor variants, or actin regulators at membrane-like interfaces.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Preparation of Neutravidin-NPF-coated beads

Demonstrator: Mengqi Xu

- 2.1. To begin, gather the required reagents [1]. After preparing the nucleation-promoting factor, or NPF (*N-P-F*), and neutravidin coating mixture, gently mix the coating mixture by pipetting up and down 5 to 10 times, avoiding bubble formation [2-TXT]. **NOTE:** For the removed shot, VO is concisely added in the next step

2.1.1. WIDE: Talent arranging the reagents on the workbench. *Videographer: Please obtain CU shot as well for this shot.*

~~2.1.2. TEXT ON A PLAIN BACKGROUND:~~

~~**NPF and Neutravidin Coating Mixture:**~~

~~GST-VCA 5 μ L~~

~~Neutravidin 10 μ L~~

~~X buffer 35 μ L~~

- 2.1.3. Talent gently pipetting the coating mixture up and down without bubble formation. **TXT: Refer to the text for the coating mixture preparation**
Videographer's Notes: Mistakenly slated as 2.3

C112_C580_0728WH_001

C112_C581_0728GY_001

C112_C582_0728E1_001

- 2.2. Transfer 5 microliters of bead slurry to a microcentrifuge tube [1]. Add 10 microliters of X buffer to the beads and pipette at least 5 times [2]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius [3]. Carefully remove the supernatant by tilting the tube and aspirating from the side opposite the bead pellet [4].

2.2.1. Talent transferring 5 microliters of bead slurry into a microcentrifuge tube.

2.2.2. Talent adding X buffer to the beads and pipetting repeatedly.

2.2.3. Talent placing the microcentrifuge tube into the centrifuge.

2.2.4. Talent tilting the tube and aspirating the supernatant from the side opposite the bead pellet. **Videographer's Notes: Mistakenly slated aa 2.3.3 please use clip**
C112_C597_0728T1_001

- 2.3. Resuspend the washed beads in 50 microliters of the previously prepared coating mixture [1]. Mix the suspension by gentle pipetting, and leave a small air bubble to facilitate mixing during incubation [2]. Incubate the beads on a benchtop rotator at 20 revolutions per minute for 2 hours at 4 degrees Celsius [3]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius and carefully remove the supernatant containing excess neutravidin and GST-VCA [4].
 - 2.3.1. Talent resuspending the washed beads in the coating mixture.
 - 2.3.2. Talent gently pipetting the bead suspension and leaving a small air bubble in the tube. Videographer's Notes: C112_C594_0728LJ_001 -please ignore audio slate which state it's 2.3.1
 - 2.3.3. Talent placing the tube on the benchtop rotator.
 - 2.3.4. Talent placing the tube into the centrifuge
- 2.4. After diluting the 100 milligrams per milliliter BSA stock solution in X buffer, resuspend the coated beads in 200 to 400 microliters of 10 milligrams per milliliter BSA [1]. Incubate the suspension on ice for 30 minutes to block unoccupied binding sites on the bead surface [2]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius and carefully remove the supernatant [3].
 - 2.4.1. Talent resuspending the coated beads in the diluted bovine serum albumin solution.
 - 2.4.2. Talent placing the tube on ice.
 - 2.4.3. Talent placing the tube into the centrifuge. Videographer's Notes: reuse 2.2.3 or clip C112_C588_0728IU_001
- 2.5. Resuspend the beads in 50 microliters of 1 milligram per milliliter BSA prepared in X buffer [1]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius [2]. Remove the supernatant, and repeat the wash twice [3]. Store the coated beads in 1 milligram per milliliter BSA prepared in X buffer on ice and use them within 1 week [4].
 - 2.5.1. Talent resuspending the beads in bovine serum albumin prepared in X buffer.
 - 2.5.2. Talent placing the tube into the centrifuge. Videographer's Notes: reuse 2.2.3 or clip C112_C588_0728IU_001
 - 2.5.3. Talent removing the supernatant. Videographer's Notes: reuse 2.3.3 or clip C112_C588_0728IU_001
 - 2.5.4. Talent storing the coated beads on ice. Videographer's Notes: use 2nd shot in 2.4.2 or clip C112_C599_0728YN_001

3. Preparation of myosin-decorated and fluorescent dye-decorated beads

- 3.1. To exchange the storage X buffer with the M buffer before myosin coating, divide 50 microliters of neutravidin-NPF-coated beads equally into two microcentrifuge tubes [1]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius and carefully remove the supernatant [2]. Wash the beads with 25 microliters of 1 milligram per milliliter BSA prepared in M buffer [3]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius, carefully remove the supernatant, and repeat the wash twice [4].
 - 3.1.1. Talent dividing the neutravidin-nucleation-promoting factor-coated beads equally into two microcentrifuge tubes.
 - 3.1.2. Talent placing the microcentrifuge tubes into the centrifuge. Videographer's Notes: reuse 2.2.3 or clip C112_C588_0728IU_001
 - 3.1.3. Talent adding bovine serum albumin prepared in M buffer to the beads.
 - 3.1.4. Talent placing the tubes into the centrifuge for the wash steps. Videographer's Notes: reuse 2.2.3 or clip C112_C588_0728IU_001
- 3.2. Resuspend the control beads in 25 microliters of 1 micromolar biotin-carboxyfluorescein 640 or CF640 (*C-F-six-forty*) fluorescent dye diluted from the stock solution in M buffer [1].
 - 3.2.1. Talent resuspending the control beads in the biotin-carboxyfluorescein 640 fluorescent dye solution. Videographer's Notes: use the CU version or take 2 C112_C604_0728DX_001
- 3.3. Resuspend the myosin beads in 25 microliters of 500 nanomolar to 1 micromolar biotinylated myosin supplemented with 1 to 5 micromolar calmodulin [1].
 - 3.3.1. Talent resuspending the myosin beads in the biotinylated myosin solution supplemented with calmodulin. Videographer's Notes: reuse 3.2.1 or clip C112_C604_0728DX_001
- 3.4. After covering the tubes with aluminum foil, incubate the bead suspensions on ice for at least 30 minutes [1]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius and carefully remove the supernatant containing excess biotinylated myosin or fluorescent dye [2].
 - 3.4.1. Talent placing the aluminum foil-covered tubes on ice.
 - 3.4.2. Talent placing the tubes into the centrifuge. Videographer's Notes: reuse 2.2.3 or clip C112_C588_0728IU_001
- 3.5. Wash the decorated beads with 25 microliters of 1 milligram per milliliter BSA prepared in M buffer [1]. Pellet the beads by centrifugation at 16,000 g for 2 minutes at 4 degrees Celsius, carefully remove the supernatant, and repeat the wash twice [2].
 - 3.5.1. Talent adding bovine serum albumin prepared in M buffer to the decorated beads. Videographer's Notes: reuse 3.2.1 or clip C112_C604_0728DX_001

3.5.2. Talent placing the tubes into the centrifuge. Videographer's Notes: reuse 2.2.3 or clip C112_C588_0728IU_001

3.6. Supplement the myosin-decorated beads with 1 to 5 micromolar calmodulin [1]. Protect all decorated beads from light by covering the tubes with aluminum foil [2]. Store the far-red fluorescent dye-decorated beads or myosin-decorated beads in 1 milligram per milliliter BSA prepared in M buffer on ice [3].

3.6.1. Talent pipetting calmodulin into the microcentrifuge tube containing the myosin-decorated beads.

3.6.2. Talent covering the tubes with aluminum foil.

3.6.3. Talent storing the decorated bead suspensions on ice.

4. Preparation of the Actin Motility Assay and Sample Imaging

4.1. After protein purification, prepare a 50-microliter motility mixture [1-TXT].

4.1.1. Representative shot of talent adding BSA and bead slurry into a container to prepare the motility mixture. **TXT: Refer to the text for the motility mixture preparation**

4.2. Initiate the reaction by adding 4 microliters of 50 micromolar G-actin containing 5% to 10% fluorescently labeled G-actin [1]. Mix the reaction immediately and designate this time point as time equals 0 [2].

4.2.1. Talent adding fluorescently labeled globular actin to initiate the reaction.

4.2.2. Talent mixing the reaction. Videographer's Notes: Added a Pickup shot of clock

4.3. Clean the glass slide and coverslip with 70% ethanol, followed by deionized water [1]. Plasma-clean the glass surfaces using ambient-air plasma for 10 minutes [2]. Apply 2.3 microliters of the reaction mixture to the glass slide [3]. Place a 22-millimeter-by-22-millimeter coverslip over the sample to form a squeeze chamber with an approximate chamber height of 4.3 micrometers [4]. Seal the chamber with vacuum grease [5].

4.3.1. Talent cleaning the glass slide and coverslip with ethanol followed by deionized water.

4.3.2. Talent placing the glass slide and coverslip into the plasma cleaner.

4.3.3. Talent dispensing 2.3 microliters of the reaction mixture onto the glass slide.

4.3.4. Talent placing the 22-millimeter × 22-millimeter coverslip onto the sample to form the squeeze chamber.

4.3.5. Talent applying vacuum grease around the coverslip edges.

4.4. Using an epifluorescence microscope, acquire fluorescence images [1].

4.4.1. Talent placing the chamber under microscope.

Results

5. Results

5.1. Surface-bound NPFs stimulated Arp2/3 (*Arp-two-three*) complex-mediated branched actin assembly around the bead surface, and actin first appeared as a sparse shell surrounding the bead [1].

5.1.1. LAB MEDIA: Figure 1C. *Video editor: Under the control row, in the image 175s, highlight the white cloud-like shape between the round bead and its outside border.*

5.2. Continued actin assembly at the bead surface drove outward network growth, which led to symmetry breaking [1] and the formation of a polarized comet tail [2].

5.2.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the white outer line in the control row in 275s, 375s, and 475s.*

5.2.2. LAB MEDIA: Figure 1C. *Video editor: Highlight the white tail line in the control row in 275s, 375s, and 475s.*

5.3. Once a comet tail was established, ongoing actin nucleation and polymerization at the bead surface supported sustained bead movement [1].

5.3.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the image in the control row in 475s, 575s and 675 seconds.*

5.4. Under standard assay conditions, myosin-coated beads typically produced dimmer, sparser, or more diffuse actin comet tails than control beads [1].

5.4.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the myosin row showing the actin comet tails. LAB MEDIA: Figure 1C. Video editor: Highlight the white outer line in the myosin row, 175s, 275s, 375s, 475s, 575s and 675s.*

Pronunciation guides:

1. Nucleation

Pronunciation link: <https://www.merriam-webster.com/dictionary/nucleation>

IPA: /ˌnuːkliˈeɪʃən/, /ˌnjuːkliˈeɪʃən/

Phonetic Spelling: noo-kee-AY-shuhn (or nyoo-kee-AY-shuhn)

2. Neutravidin

Pronunciation link: No confirmed link found

IPA: /nuːˈtrævɪdɪn/

Phonetic Spelling: noo-TRAV-ih-din

3. Microcentrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>

IPA: /ˌmaɪkroʊˈsɛntrɪˌfjuːdʒ/

Phonetic Spelling: MY·kroh·SEN·trih·fyooj

4. Centrifugation

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifugation>

IPA: /ˌsɛntrɪfjəˈgeɪʃən/

Phonetic Spelling: sen·trih·fyuh·GAY·shuhn

5. Supernatant

Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>

IPA: /ˌsuːpərˈnɛtənt/

Phonetic Spelling: soo·per·NAY·tuhnt

6. Resuspend

Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>

IPA: /ˌriːsəˈspɛnd/

Phonetic Spelling: ree·suh·SPEND

7. Benchtop

Pronunciation link: <https://www.merriam-webster.com/dictionary/benchtop>

IPA: /ˈbɛntʃˌtɒːp/

Phonetic Spelling: BENCH·top

8. Neutravidin

Pronunciation link: No confirmed link found

IPA: /nuːˈtrævɪdɪn/

Phonetic Spelling: noo·TRAV·ih·din

9. Bovine

Pronunciation link: <https://www.merriam-webster.com/dictionary/bovine>

IPA: /ˈboʊˌvaɪn/

Phonetic Spelling: BOH·vine

10. Albumin

Pronunciation link: <https://www.merriam-webster.com/dictionary/albumin>

IPA: /ælˈbjuːmɪn/

Phonetic Spelling: al·BYOO·min

11. Myosin

Pronunciation link: <https://www.merriam-webster.com/dictionary/myosin>

IPA: /ˈmaɪəsɪn/

Phonetic Spelling: MY·uh·sin

12. Biotinylated

Pronunciation link: No confirmed link found

IPA: /ˌbaɪoʊˈtɪnəleɪtɪd/

Phonetic Spelling: bye·oh·TIN·uh·lay·tid

13. Calmodulin
Pronunciation link: <https://www.merriam-webster.com/medical/calmodulin>
IPA: /ˌkælˌmɑːdʒəˈliːn/
Phonetic Spelling: kal·mah·juh·LEEN
14. Carboxyfluorescein
Pronunciation link: No confirmed link found
IPA: /kɑːrˌbɑːksiˌflʊrəˈsiːɪn/
Phonetic Spelling: kar·BOK·see·floor·uh·SEE·een
15. Micromolar
Pronunciation link: <https://www.merriam-webster.com/medical/micromolar>
IPA: /ˌmaɪkroʊˈmoʊlər/
Phonetic Spelling: MY·kroh·MOH·ler
16. Epifluorescence
Pronunciation link: No confirmed link found
IPA: /ˌɛpiˌflʊrˈɛsəns/
Phonetic Spelling: eh·pee·floor·ESS·uhns
17. Polymerization
Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerization>
IPA: /pəˌlɪməraɪˈzeɪʃən/
Phonetic Spelling: puh·LIM·er·uh·ZAY·shuhn
18. Symmetry
Pronunciation link: <https://www.merriam-webster.com/dictionary/symmetry>
IPA: /ˈsɪmətri/
Phonetic Spelling: SIM·uh·tree
19. Polarized
Pronunciation link: <https://www.merriam-webster.com/dictionary/polarized>
IPA: /ˈpoʊləˌraɪzd/
Phonetic Spelling: POH·luh·ryzd
20. Nucleation-promoting
Pronunciation link: No confirmed link found
IPA: /ˌnuːkliˈeɪʃən prəˈmoʊtɪŋ/, /ˌnjuːkliˈeɪʃən prəˈmoʊtɪŋ/
Phonetic Spelling: noo·klee·AY·shuhn proh·MOH·ting (or nyoo·klee·AY·shuhn proh·MOH·ting)
21. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flʊˈrɛsənt/, /flɔːˈrɛsənt/
Phonetic Spelling: floor·ESS·uhnt (or flor·ESS·uhnt)
22. Globular
Pronunciation link: <https://www.merriam-webster.com/dictionary/globular>

IPA: /'glɑːbjələr/

Phonetic Spelling: GLOB·yuh·ler

23. Plasma

Pronunciation link: <https://www.merriam-webster.com/dictionary/plasma>

IPA: /'plæzmə/

Phonetic Spelling: PLAZ·muh

24. Deionized

Pronunciation link: <https://www.merriam-webster.com/dictionary/deionize>

IPA: /diːˈaɪəˌnaɪzd/

Phonetic Spelling: dee·EYE·uh·nyzd