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Cytoplasmic Actin: Purification and Single Molecule Assembly Assays

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Abstract

The actin cytoskeleton is essential to all eukaryotic cells. In addition to playing important structural roles, assembly of actin into filaments powers diverse cellular processes, including cell motility, cytokinesis, and endocytosis. Actin polymerization is tightly regulated by its numerous cofactors, which control spatial and temporal assembly of actin as well as the physical properties of these filaments. Development of an in vitro model of actin polymerization from purified components has allowed for great advances in determining the effects of these proteins on the actin cytoskeleton. Here we describe how to use the pyrene actin assembly assay to determine the effect of a protein on the kinetics of actin assembly, either directly or as mediated by proteins such as nucleation or capping factors. Secondly, we show how fluorescently labeled phalloidin can be used to visualize the filaments that are created in vitro to give insight into how proteins regulate actin filament structure. Finally, we describe a method for visualizing dynamic assembly and disassembly of single actin filaments and fluorescently labeled actin binding proteins using total internal reflection fluorescence (TIRF) microscopy.

Keywords

Actin polymerization assay; TIRF; Single molecule imaging; *Acanthamoeba*; Fluorimetry; Pyrene; Cytoskeleton; Phalloidin

1 Introduction

Over the past few decades dozens of actin binding proteins have been discovered, and there is a rapidly growing body of work focused on dissecting how each of these proteins regulates the kinetics and morphology of actin structures [1, 2]. Actin dynamics can be divided into three steps, each of which is specifically regulated. Polymerization is a nucleation-condensation reaction [3], which means that the first step (nucleation) is slow, and the second step (elongation) proceeds quickly once a stable nucleus is formed [4]. Nucleation refers to the assembly of actin monomers into a stable trimer, which is usually an extremely unfavorable reaction. As the filament elongates, incorporated actin monomers hydrolyze their ATP to ADP, making filaments more susceptible to depolymerization, the

third step in the cycle. All steps of this cycle are regulated by actin binding proteins [1, 5]. The rate of nucleation is controlled by actin nucleation factors, including the Arp2/3 complex, formins, and WH2 family nucleation factors (e.g., Spire and JMY) [5–7]. Growth, maintenance and structural integrity of filaments are controlled by different classes of proteins that either elongate, cap, bundle, or sever actin filaments.

The primary in vitro assay used to study the effects of actin binding proteins on actin polymerization is the pyrene actin assembly assay, wherein the environmentally sensitive fluorophore pyrene indicates the polymerization state of actin [8]. Pyrene-labeled actin incorporated in a filament fluoresces significantly greater than a labeled monomer in solution [8]. In the absence of other proteins, a time dependent increase in pyrene fluorescence is directly proportional to the concentration of filamentous actin ([9]; protein binding to actin filaments can also increase pyrene fluorescence, so care must be taken with interpreting these data).

Actin polymerization assays are conducted using buffer conditions similar to those in vivo. Importantly, actin requires magnesium-ATP in its nucleotide pocket, and physiological concentrations of potassium for rapid polymerization at micromolar concentrations. Actin is stored in Buffer A—which contains calcium instead of magnesium, and lacks potassium—to prevent polymerization. Immediately before the assay, calcium is exchanged for magnesium by adding EGTA and magnesium. Polymerization is triggered by the addition of buffer containing potassium [10].

There are two standard sources of actin for biochemical studies: *Acanthamoeba castellanii* and rabbit skeletal muscle. Actin from amoeba is cytoplasmic, and is a good model for cytoplasmic actins (beta-actin isoform) from other organisms [11]. Conversely, skeletal actin is predominantly the alpha-actin isoform, and does not necessarily interact the same with proteins that regulate cytoplasmic actin [12]. Furthermore, *Acanthamoeba* is an excellent source of actin binding proteins, such as the Arp2/3 complex [13, 14]. Although it is best to use actin and actin binding proteins from the same organism and tissue, actin is highly conserved, and in most cases amoeba actin can be used in conjunction with proteins from other organisms [10].

While the pyrene assay is very powerful for measuring kinetics of actin assembly, it does not reveal the structure of the actin filament networks it produces. In addition, pyrene assays are inherently difficult to interpret because the data output is based on an ensemble of protein interactions, and the binding of some proteins to actin filaments can affect pyrene fluorescence. There are two commonly used techniques which address this: fixing and staining polymerization reactions with fluorescently labeled phalloidin [6] and electron microscopy [9, 15]. Fixing actin with labeled phalloidin, detailed in Subheading 3.5, is a straightforward method that can be performed in parallel with pyrene actin experiments.

Recently, innovations in glass surface chemistry, synthesis of fluorescent dye conjugates, and improvements in microscope imaging technology have made it possible to image single dynamic cytoskeletal polymers in vitro using fluorescence microscopy [16–18]. These assays rely on visualizing single filaments adhered to polyethylene glycol (PEG)

functionalized coverslips using total internal reflection fluorescence (TIRF) microscopy. Although more technically challenging than a pyrene assay, single filament TIRF assays offer unparalleled insight into actin filament dynamics and network architecture with single filament resolution. Using this method, several models for actin binding protein function based on pyrene actin polymerization assays have been recently challenged [19, 20]. Since pegylation reduces nonspecific protein absorption to coverslips, these glass surfaces can also be used for single molecule imaging of fluorescently labeled actin binding proteins [21]. These assays are detailed in Subheadings 3.6–3.9.

2 Materials

2.1 Culture of *Acanthamoeba castellanii*

1. Amoeba medium: 7.5 g/L proteose peptone, 7.5 g/L yeast extract, 15 g/L glucose, 0.2 mM methionine, 3 mM KH_2PO_4 , 10 μM CaCl_2 , 1 μM FeCl_3 , 0.1 mM MgSO_4 , 1 mg/L thiamine, 0.2 mg/L biotin, 0.01 mg/L vitamin B12. Autoclave before use.
2. Wash buffer: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl.

2.2 Actin Purification from *Acanthamoeba castellanii*

1. Extraction buffer: 10 mM Tris-HCl, pH 8.0, 11.6 % (w/v) sucrose, 1 mM EGTA, 1 mM ATP, 5 mM dithiothreitol (DTT), 30 mg/L benzamidine, 5 mg/L pepstatin A, 10 mg/L leupeptin, 40 mg/L soybean trypsin inhibitor, 1 mM phenyl-methylsulphonylfluoride (PMSF). The first three ingredients may be combined a day in advance and stored at 4 °C. Add the final ingredients fresh, pH to 8.0 and bring up to volume with cold water (*see* Note 1). Add PMSF immediately before use.
2. Pepstatin A (1,000 $\times$): 5 mg/mL pepstatin A in DMSO; store at -20 °C.
3. Protease cocktail (100 $\times$): 1 mg/mL leupeptin and 4 mg/mL soybean trypsin inhibitor in water; store at -20 °C.
4. PMSF: 200 mM in ethanol or methanol; store at -20 °C. Before use warm to 25 °C with rocking to resuspend. The half life of PMSF in aqueous solution is approximately 30 min, and so it is always added to buffers immediately before use.
5. 100 mM ATP, pH 7.0, dissolved in water.
6. Column buffer: 10 mM Tris-HCl, pH 8.0, 0.2 mM CaCl_2 , 30 mg/L benzamidine, 0.5 mM ATP, 0.5 mM DTT. Add the last three ingredients fresh and bring buffer up to volume with cold water. Adjust buffer to pH 8.0 while at 4 °C, just before use.
7. Diethylaminoethyl cellulose (DEAE) (DE52, Whatman): After use, cycle with 0.5 M NaOH and HCl to wash, and store in Tris base, pH 8.0, 0.2 % (v/v)

¹All solutions should be prepared in ultrapure water with resistivity of 18.2 Ω (e.g., Milli-Q water). Cold water refers to ultrapure water at 4 °C.

benzalkonium chloride at 4 °C indefinitely (*see* Note 2 for details on washing DEAE).

8. 1 M Tris base (Trizma).
9. High salt buffer: 600 mM KCl, 10 mM Tris-HCl, pH 8.0, 0.2 mM CaCl₂, 30 mg/L benzamidine, 0.5 mM ATP, 0.5 mM DTT. Prepare as in **step 1** (extraction buffer) above.
10. 200 mM MgCl₂.
11. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Invitrogen): Prepare 0.5 M stock in cold water and pH to 7.0 with KOH.
12. Buffer A (1×): 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.5 mM TCEP, 0.1 mM CaCl₂. pH to 8.0 with KOH and bring up to volume with cold water (*see* Note 3).
13. KMEI buffer (10×): 500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, 100 mM Imidazole HCl, pH 7.0 (*see* Note 4).
14. Gel filtration column: Flex column (25 mm × 100 cm, Fisher Scientific) for packing resin manually. Alternatively, use a Superdex S-75 or S-200 column (GE Healthcare).
15. Sephacryl S-200 HR resin (GE Healthcare): Resin comes stored in 20 % (v/v) ethanol and will swell when fully hydrated. To avoid cracking the resin, equilibrate in buffer before decanting into gel filtration column. Pack the column by flowing 1–2 column volumes of Buffer A at a faster rate than will be used in gel filtration.
16. Parr Bomb (Parr Instrument Company).

2.3 Pyrene-Labeling of Actin

1. Actin: If actin is from *Acanthamoeba*, it must be gel filtered before labeling. Skeletal actin does not have this requirement.
2. KMEI buffer (10×): *see* Subheading 2.2, **item 13**.
3. Dialysis buffer: 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM Imidazole HCl, pH 7.0, 0.2 mM ATP.
4. Pyrenyl iodoacetamide (Invitrogen): 10 mM stock solution in dimethylformamide (DMF), stored at –20 °C in the dark.
5. Buffer A (1×): *see* Subheading 2.2, **item 12**.

²More complete protocol: Dry DEAE resin (vacuum resin until just damp; not white). Transfer to beaker and add water to 4 L. Add solid NaOH to 0.5 M and stir 5 min, 25 °C. Dry and wash with 4 L water. Repeat NaOH wash, this time stir for 30 min. Dry and wash with 8 L water. Resuspend in 2,870 mL water and add 130 mL concentrated HCl. Stir 30 min, 25 °C. Dry and wash with 8 L water. Resuspend in 3–4 L water and add 1 M Tris base until pH is 8.0. Add 0.2 % (v/v) benzalkonium chloride and store at 4 °C.

³Make Buffer A as a 100× stock without CaCl₂—Calcium-ATP precipitates at this concentration—and freeze at –20 °C. Add CaCl₂ during dilution to 1×, and aliquot in 15 mL conical tubes and freeze at –20 °C. Once thawed, one aliquot may be used for up to 1 week.

⁴Imidazole is best stored in the dark, at 4 °C, as a 1–2 M stock solution. Store 10× KMEI buffer for fluorimetry in the dark, at 4 °C. Discard when autofluorescence of the buffer becomes noticeable during fluorimetry. Alternatively, use HEPES buffer in place of imidazole in KMEI.

6. DTT: 1 M stock in water.

2.4 Pyrene Actin Assembly Assay

1. Buffer A (1×): *see* Subheading 2.2, **item 12**.
2. KMEI buffer (10×): *see* Subheading 2.2, **item 13**.
3. ME buffer (10×): 0.5 mM MgCl₂, 2 mM EGTA.
4. Ethanol, HPLC grade for washing cuvettes.
5. Vakuwash Cuvette washer (Fisher Scientific).

2.5 Microscopy of Stabilized, Labeled Actin Filaments

1. Reagents for pyrene actin assembly assay, as in Subheading 2.4.
2. Alexa Fluor 488 Phalloidin (Invitrogen): Make stocks in methanol, to be in molar excess to actin in reaction. For example, 5 µL of a 6.6 µM stock of phalloidin was used to stabilize an equal volume of 4 µM actin [6]. Store stocks at −20 °C for several months. TRITC phalloidin (Sigma) also works well.
3. Wide-bore pipette tips: Cut pipette tips with a sterile razor, and be consistent.
4. Poly-L-lysine: Prepare a 1 mg/mL stock in water.
5. Poly-L-lysine coated coverslips: Clean slides by sonication first in 1 M KOH, then in ethanol. Rinse in water, and air dry. Spot 1 mg/mL poly-L-lysine on parafilm, and float coverslips on drops (500 µL) for a few minutes, rinse with water, and use within a few days.
6. Clear nail polish.

2.6 Silanization and Pegylation of Glass Coverslips

1. Diamond pen (J&M Diamond Tool Inc., HS-110).
2. Glass weigh jars (Fisher, 03-420-5C).
3. Ceramic coverslip racks.
4. 18 mm × 18 mm coverslips, 1.5 mm thickness.
5. Bath sonicator.
6. Sodium hydroxide (Fisher, S318-1).
7. Sulfuric Acid (Fisher, A300-500).
8. Hydrogen peroxide (30 % solution, Fisher H325-500).
9. APTES (3-Aminopropyl)triethoxysilane (Sigma-Aldrich, A3648).
10. mPEG-5000-NHS (JenKem Technology USA, M-SCM-5000).
11. Biotin-PEG5000-NHS (JenKem Technology USA, Biotin-PEG-5000-NHS).
12. Dimethylformamide (anhydrous, Sigma-Aldrich 227056).

13. Isopropanol (Fisher, A451-4).
14. Ethanol (Fisher, A995-4).
15. Acetone (Fluka, 00561).
16. Ultrapure water (*see* Note 1).

2.7 Labeling A. castellani Actin with Maleimide Derivatives

1. Buffer A (1×): *see* Subheading 2.2, **item 12**.
2. Actin labeling buffer: 5 mM Tris-HCl, pH 8, 200 μM ATP, 100 μM CaCl₂.
3. KMEI buffer (10×): *see* Subheading 2.2, **item 13**.
4. Fluorescent dyes: Alexa Fluor 488 maleimide (Invitrogen, A-10254), Cy3 maleimide (GE Healthcare, PA13131), and Cy5 maleimide (GE Healthcare, PA15131).
5. Biotin-PEG2-Maleimide (Fisher Thermo Scientific/Pierce, #21901).
6. PD10 salt exchange column (G25 Sephadex resin, GE Healthcare 17-0851-01).
7. 500 mM TCEP-HCl, pH 7.0 with KOH (Pierce, 20491).
8. 1 M DTT dissolved in water (Roche, 100034).

2.8 Assembly of TIRF-M Flow Cells for Attachment of Filamentous Actin

1. 15 mm wide double sided tape (Tesa, 05338).
2. 25 mm × 75 mm × 1 mm Fisher Premium Microscope slides (Fisher, 12-544-7).
3. 3 mg/mL Poly-L-lysine PEG ((PLL-PEG) [22]; *see* ref. 18 for grafting protocol).
4. 500 mM TCEP-HCl, pH 7.0 with KOH (Pierce, 20491).
5. Streptavidin (Rockland Immunochemicals, S00-01): Resuspend lyophilized protein in 10 mM Tris-HCl, pH 8 to final concentration of 20–40 μM. Ultracentrifuge at 350,000×g (80,000 rpm with a TLA100.4) for 20 min to remove aggregates. Freeze with liquid nitrogen. Store at –80 °C.
6. Heavy meromyosin (HMM; chymotrypsin cleaved rabbit skeletal muscle myosin, e.g., Cytoskeleton, Inc., MH01). Alternatively use NEM-inactivated myosin II (*see* refs. 19, 20).
7. Biotin-phalloidin (Sigma-Aldrich, P8716).
8. Biotin-PEG11-Maleimide (Fisher Thermo Scientific/Pierce, 21911) for labeling heavy meromyosin (HMM) (*see* Subheading 3.7, **step 19**).
9. 10 mg/mL kappa casein dissolved in 1× PBS, pH 7.2 (Sigma-Aldrich C0406; bovine milk): Ultracentrifuge at 350,000 × g (80,000 rpm with a TLA100.4) for 20 min to remove aggregates. Use within 3 days.
10. Pluronic F-127 (Sigma-Aldrich, P2443): Prepare 10 % (v/v) in water.

11. KMEI buffer (10×): *see* Subheading 2.2, **item 13**.
12. 1× PBS buffer: 2.9 mM NaH₂PO₄, 7.1 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.2 with NaOH.
13. Blocking buffer: 1 % Pluronic F127, 50 µg/mL kappa casein, 1× PBS, pH 7.2.
14. Wash buffer: 20 mM HEPES, pH 7.0, 200 mM KCl, 1 mM TCEP, 1 mg/mL BSA.

2.9 Visualization of Dynamic Actin Filament Assembly

1. Unlabeled monomeric actin stored in Buffer A.
2. Alexa Fluor 488, Cy3, or Cy5 labeled monomeric actin stored in Buffer A.
3. 100 mM ATP dissolved in water, pH 7.0 with KOH (Sigma-Aldrich, A26209-10G). Store aliquots at −20 °C.
4. 50 mg/mL BSA dissolved in water (Sigma-Aldrich, A0281-1G).
5. 1 M D-(+)-Glucose in water.
6. 14.3 M β-mercaptoethanol.
7. 2 % methylcellulose (w/v) (cP400, Sigma-Aldrich M0262): Suspend methylcellulose in 90–100 °C water by vortexing in a microfuge tube. Invert tube and chill on ice to solubilize. Microcentrifuge and resuspend methylcellulose to homogeneity.
8. 10 mg/mL glucose oxidase dissolved in OSS buffer (Biophoretics/Serva Electrophoresis, T#22778.01): Ultracentrifuge at 350,000 × g (80,000 rpm with a TLA100.4) for 20 min to remove aggregates. Use within 3 days.
9. 10 mg/mL catalase dissolved in OSS buffer (Sigma-Aldrich C40-100MG; bovine liver): Ultracentrifuge at 350,000 × g (80,000 rpm with a TLA100.4) for 20 min to remove aggregates. Use within 3 days.
10. Buffer A (1×): *see* Subheading 2.2, **item 12**.
11. ME Buffer (10×): *see* Subheading 2.4, **item 3**.
12. OSS buffer: 20 mM HEPES, pH 7.0, 150 mM NaCl.
13. 2× TIRF Buffer: 40 mM HEPES, pH 7.0, 50 mM KCl, 2 mM MgCl₂, 2 mM EGTA, 0.4 % methylcellulose (cP400), 40 mM glucose, 40 mM beta-mercaptoethanol, 2 mg/mL BSA, 2 mM ATP, 250 µg/mL glucose oxidase, 20 µg/mL catalase. Add oxygen scavenger enzymes (i.e., glucose oxidase and catalase) 5–10 min before combining with actin and imaging.
14. Protein dilution buffer: 20 mM HEPES, pH 7.0, 100 mM KCl, 1 mM TCEP, 1 mg/mL BSA.
15. VALAP (1:1:1 vasoline:lanoline:paraffin).
16. NIKON Eclipse TE2000-E microscope with Nikon Perfect Focus, or equivalent.
17. ANDOR iXon cooled EM CCD camera.

18. 100× Nikon Apo TIRF objective (NA 1.49).
19. 40 mW 488/514 nm Argon Ion laser or 40 mW 542 nm crystal laser.
20. Chroma/Shemrock multibandpass filter.
21. Micromanager 4.0 software (*recommended*; [23]).

3 Methods

3.1 Culture of *Acanthamoeba castellanii*

1. Passage amoeba in 10 mL culture tubes with moderate bubbling of humidified air, at 25 °C [24, 25].
2. Fill beveled Fernbach flasks (3 L) with 1 L Amoeba medium and inoculate with cultures. Grow with moderate shaking at 25 °C, until growth plateaus (*see* Note 5). Use Fernbachs to inoculate 14 L carboys outfitted with bubblers. Practice sterile technique to avoid contaminating the cultures, which grow without any antibiotic.
3. When cultures plateau, harvest by centrifugation at $4,500 \times g$ (4,000 rpm in a Sorvall RC-3B), 5 min. Refill tubes and centrifuge again until all amoeba have been pelleted.
4. Wash amoeba with wash buffer. Resuspend cell pellets in the smallest volume of buffer possible. Pool these, and centrifuge as above. In this way all amoeba are transferred into one or two tubes. During the pairing-down of tubes, weigh amoeba, to get approximately 500 g (enough for actin purification) into a single tube.

3.2 Purification of Actin from *Acanthamoeba castellanii*

1. Resuspend pelleted amoeba in 1 mL of cold extraction buffer per gram of amoeba (example volumes given are for purification from 500 g amoeba. Buffer and resin volumes can be scaled linearly as needed). If amoeba are frozen, thaw in cold extraction buffer (*see* Note 6). After resuspension, conduct all subsequent steps at 4 °C (*see* Note 7).
2. Lyse cells by nitrogen decompression in a Parr Bomb. Rinse the Parr Bomb with water and cool on ice. Transfer cells into the Parr Bomb and pressurize with nitrogen gas at 400 psi for 5 min. To lyse, release cells from the Parr Bomb slowly, while maintaining constant pressure. Alternatively, amoeba can be lysed by rapid freezing with liquid nitrogen and then thawing into cold extraction buffer. This step can be immediately followed by centrifugation.

⁵Assay growth by absorbance at 600 nm (A_{600}). A typical A_{600} of amoeba for purifying actin is 6. A_{600} values over 1.0 are out of the linear range in most cases, so dilute the amoeba before measuring. Amoeba settle out of solution, so mix before reading absorbance (amoeba that do not settle or media that remains turbid after ~10 min may indicate that the amoeba are dying in culture). Alternatively, amoeba can be grown in a fermenter.

⁶We have noticed inconsistencies with actin purified from amoebae that have been frozen for longer than 6 months.

⁷The first day of the actin purification protocol is significantly long, and is aided by making buffers and gathering equipment and reagents the day before. Once started, the entire purification takes 5–7 days, including 2–3 days for the actin to depolymerize.

3. To remove cellular debris, centrifuge lysate at $>5,000 \times g$ (7,000 rpm in a GS-3 rotor), 10 min, 4 °C. Centrifuge supernatant from this spin at $>100,000 \times g$ (38,000 rpm in a Beckman Ti45 rotor), 2 h, 4 °C.
4. Equilibrate 500 g DEAE resin in 2 L column buffer. To equilibrate large volumes of resin; mix resin and buffer in beaker, then pour over a Buchner funnel fitted with a Whatman filter 541 (ashless) and pull vacuum until buffer has passed through but the resin is still damp. It is essential that the pH of the resin is 8.0 before use.
5. After the second spin, siphon off the lipid layer at the very top of each tube. Decant supernatants into a rinsed glass beaker, on ice. Mix supernatant with ~450 mL equilibrated DEAE resin, and stir for 30–60 min at 4 °C, to batch bind.
6. In the cold room, pour remaining ~50 mL equilibrated DEAE resin into a glass column (diameter 5 cm; height 60 cm) and let resin settle and excess liquid drip through. Gently pour DEAE slurry above this, and collect flow-through (*see* Note 8). Wash resin with 1 L column buffer, pumped at 6 mL/min.
7. To elute actin, use a gradient maker to run a linear gradient from 0 to 600 mM KCl using 1 L column buffer (low salt buffer) and 1 L high salt buffer, pumped at 3 mL/min. Actin normally elutes as a broad (~200 mL) peak between 300 and 400 mM KCl. Keep actin fractions in the cold room overnight, covered. (*See* Note 2 regarding recycling of DEAE resin.)
8. The next day, move fractions to 25 °C to speed up polymerization. Actin will polymerize in a few hours. Detect polymerized actin fractions by tipping fraction tubes sideways; if there is sufficient polymerized actin, the fraction will be a gel.
9. Pool actin fractions into a beaker. Add MgCl_2 to 2 mM and ATP to 1 mM. Stir actin gently for 30 min at 25 °C to complete polymerization.
10. Centrifuge actin at $1,000 \times g$ (3,000 rpm in a Sorvall GSA rotor), 5 min, 4 °C. Pour off supernatant, and wash with $1 \times$ KMEI buffer. Centrifuge at $>100,000 \times g$ (38,000 rpm in a Ti45 rotor), 2 h, 4 °C. Discard supernatant and rinse the pellet (polymerized actin) quickly with Buffer A. Scrape actin into a Dounce homogenizer. Homogenize 5–10 times with a “loose” pestle in Buffer A, to 40–80 mL final volume, without creating air bubbles.
11. Dialyze into Buffer A for 2–3 days at 4 °C to depolymerize actin. Change dialysis buffer daily. Actin can be stored in dialysis at 4 °C for several weeks.
12. Equilibrate an S-75 or S-200 gel filtration column with Buffer A. Centrifuge 15–20 mL depolymerized actin at $>100,000 \times g$ (38,000 rpm in a Ti45 rotor), 2 h, 4 °C. Load actin onto gel filtration column and gel filter in Buffer A, collecting ~3 mL fractions.
13. Determine the concentration of unlabeled actin by measuring absorbance at 290 nm, using an early fraction from the column to blank the spectrophotometer (*see*

⁸To eliminate bubbles when decanting resin slurry into the column, pour it slowly down the outside of a Pasteur pipette or glass stir rod, onto the inside wall of the column. Do not let the resin drip directly into the column.

Note 9). The concentration of actin is 38.5 μM per absorbance unit at 290 nm (i.e., $A_{290} = 1.0$ when actin is at 38.5 μM), assuming a 10 mm pathlength.

14. Gel filtered actin can now be pyrene labeled or used unlabeled in polymerization assays (Fig. 1a). For pyrene labeling, use the entire second (major) peak. Dark actin for fluorimetry is taken from the back end (later fractions) of this peak. The front end of the peak contains actin dimers, which will interfere with kinetic measurements [10].
15. Assay the purity of actin by SDS-PAGE and Coomassie staining. Actin is 42 kDa and should appear as a single band with little to no break-down (Fig. 1b).

3.3 Pyrene-Labeling of Actin

1. Pool 30–80 mg of actin and measure concentration. Dilute actin to 1.1 mg/mL (26.4 μM ; due to the limited solubility of pyrene iodoacetamide, the final concentration of actin should be 1 mg/mL) in Buffer A.
2. Polymerize actin by adding one-tenth the final volume of 10 $\times$ KMEI buffer and let stand at 25 $^{\circ}\text{C}$. Actin will be completely polymerized within a few minutes; look for bubbles in the actin solution that do not move.
3. To remove reducing reagent for labeling, carefully transfer polymerized actin into dialysis tubing, and dialyze into dialysis buffer for a few hours at 4 $^{\circ}\text{C}$.
4. Transfer actin to a beaker and measure the volume. Add 4–7 mol pyrenyl iodoacetamide per mol actin, while stirring. Cover the beaker to keep dark, and gently stir overnight at 4 $^{\circ}\text{C}$.
5. Quench labeling reaction by adding DTT to 10 mM. (This immediately renders excess pyrene iodoacetamide unreactive, thus preventing it from reacting with the chromatography resin used in **step 11**.)
6. Centrifuge labeled actin at $2,000 \times g$ (5,000 rpm in a Beckman JA-20) rotor, 5 min, 4 $^{\circ}\text{C}$, to pellet precipitated dye.
7. Centrifuge supernatant at $>100,000 \times g$ (38,000 rpm in a Ti45 rotor), 2 h, 4 $^{\circ}\text{C}$, to pellet actin filaments.
8. Homogenize the pellet with a Dounce homogenizer as above, in Buffer A, into a final concentration of about 2–6 mg/mL (48–144 μM) (*see* Note 10).
9. Dialyze into Buffer A, at 4 $^{\circ}\text{C}$, using at least two changes of buffer over at least 2 days, to depolymerize actin.
10. Centrifuge depolymerized pyrene actin at $>100,000 \times g$ (38,000 rpm in a Ti45 rotor), 2 h, 4 $^{\circ}\text{C}$, to remove remaining actin filaments.

⁹Actin requires ATP, which absorbs significantly less at 290 nm than at 280 nm. To limit the time that actin fractions are out of the cold room, it is practical to reuse pipettes and not wash the cuvette between each reading. To avoid contaminating monomeric actin with actin dimers, start at the highest fraction and take readings backwards.

¹⁰The critical concentration of calcium-actin is 150 μM [10]. Actin will not fully depolymerize above this concentration.

11. Gel filter supernatant over S-75 or S-200 gel filtration column equilibrated with Buffer A, as before.
12. Determine the concentration and percent labeling by measuring absorbance at 290 and 344 nm. To correct for pyrene absorption at 290 nm.

$$A_{290(\text{corrected})} = A_{290(\text{measured})} - 0.127 \times A_{344} \quad (1)$$

The concentration of total actin is thus 38.5 μM per corrected A_{290} . The concentration of pyrene actin is 45.0 μM per A_{344} . To calculate percent labeling.

$$\text{percent labeled} = \frac{[\text{pyrene actin}]}{[\text{total actin}]} \quad (2)$$

13. Store pyrene actin fractions on ice, in the dark, in the cold room. Pyrene actin is more stable than unlabeled actin, and can be used for several months.

3.4 Pyrene Actin Assembly Assay

1. Mix unlabeled actin, pyrene actin and Buffer A to achieve a working stock that is 5–10 % labeled and 5–20 times the concentration to be used (*see* Note 11). Store on ice, in the dark.
2. Set up the fluorimeter. Set excitation wavelength to 365 nm, and emission wavelength to 407 nm.
3. Assemble reagents by the fluorimeter. (All volumes listed are based on a 100 μL total reaction volume.) Pre-aliquot buffers into labeled microfuge tubes to ensure that concentrations of each reagent are exactly the same for each experimental condition: (1) 10 μL aliquots of 10 $\times$ KMEI buffer and (2) aliquots of 10 $\times$ ME

¹¹Concentration of dark actin and pyrene actin stocks must both be measured. Because some proteins do not bind as tightly to pyrene actin as dark actin, the actin used in fluorimetry is normally only 5–10 % pyrene actin [10]. To calculate how much pyrene actin and dark actin to combine to yield a given percent labeling and a given concentration, use the equations:

$$\begin{aligned} L_i &= \text{starting percent labeling of pyrene actin (100 \% labeled=100)} \\ C_p &= \text{starting concentration of pyrene actin } (\mu\text{M}) \\ C_d &= \text{starting concentration of dark actin } (\mu\text{M}) \\ L_f &= \text{final percent labeled (i.e., 5–10)} \\ C_f &= \text{Final concentration of actin } (\mu\text{M}) \\ V_f &= \text{Final volume of actin } (\mu\text{L}) \end{aligned}$$

Bring up to volume (V_f) with Buffer A.

$$\text{volume pyrene actin } (\mu\text{L}) = \frac{L_f C_f V_f}{L_i C_p} \quad (3)$$

$$\text{volume dark actin } (\mu\text{L}) = \left(V_f - \frac{\text{volume pyrene actin } C_p}{C_f} \right) \frac{C_f}{C_d} \quad (4)$$

buffer (one tenth the volume of actin added to the reaction). Make a dilution series of the protein to be tested in Buffer A and store on ice (*see* Note 12). Clean cuvettes extensively with water, ethanol, water, and wipe dry with lens paper.

4. Set a stop watch to 3 min and start time. Immediately, add actin to pre-aliquoted 10× ME buffer to exchange Ca^{2+} for Mg^{2+} . Exchange for 2 min exactly.
5. During this 2 min incubation, assemble the rest of the reagents: Add proteins to be tested to 10 μL pre-aliquoted 10× KMEI buffer (*see* Note 13). Add Buffer A to bring the total reaction volume (including actin and ME buffer) to 100 μL . Set a pipetteman to 110 μL and pipette up the KMEI/protein mixture.
6. After 2 min exchange, pipette KMEI/protein mixture into the actin. Triturate the reaction 1–3 times (be consistent) to mix thoroughly, then pipette into the cuvette, being careful to avoid bubbles. Load the cuvette into the fluorimeter, and activate data collection. Record dead time: the time from addition of the KMEI/protein mixture to actin until the second the first data point is collected. Always collect complete data sets, until pyrene fluorescence plateaus. Try the same condition at least three times to ensure reproducibility. (*See* Note 14 for tips on improving data.)
7. Every time the fluorimeter is turned on, or a different concentration of actin is used, optimize settings to maximize signal while eliminating photobleaching (*see* Note 15). Polymerize actin as described, and monitor the plateau over the course of 1 h, maximizing the signal that causes no appreciable drop in plateau fluorescence over

¹²Because ATP absorbs significantly at 280 nm, proteins other than actin should not be in Buffer A for quantification by spectrophotometry.

¹³It may be desirable to pre-mix proteins before starting actin exchange, to allow them to come to equilibrium. Although not recommended for detailed kinetic analysis, it is possible to add *E. coli* lysate containing expressed protein (or impure eluates from affinity chromatography) to pyrene actin assembly assays as a first-pass functional test. Uninduced (control) lysate should have no effect on actin polymerization.

¹⁴There are many factors to consider for collecting meaningful and reproducible fluorimetry data. To list key points.

- a. Always do an actin alone (control) reaction to test the actin first.
- b. The assay is very sensitive to the concentration of monovalent salt (e.g., potassium in KMEI), so keep that constant in all reactions. In the event that a protein component is stored in a buffer containing salt, adjust the molarity of KCl in 10× KMEI buffer to keep the final concentration of monovalent salt in the reaction at 50 mM. If a higher salt concentration is required for protein stability, make sure that all reactions contain the same salt concentration.
- c. Exchange in 10× ME buffer for exactly 2 min and be consistent.
- d. Eliminate photobleaching.
- e. Always collect complete data sets: until pyrene fluorescence plateaus.
- f. Wash cuvettes extensively between each reaction, as polymerized actin remaining in the cuvette will nucleate new filaments in the next run.
- g. Avoid bubbles, which both scatter light and cause oxidative damage to proteins.
- h. Store proteins on ice, but ensure all components are at 25 °C at the start of the reaction. Pre-incubating tubes in a water bath works well.
- i. When expressing Glutathione-S-Transferase (GST)-tagged recombinant proteins, it is critical to remove the GST, as GST will mediate the dimerization of the fusion protein. For example, Arp2/3 activators display faster kinetics when the GST remains uncleaved [27, 28].
- j. Pyrene is an environmentally sensitive fluorophore, and binding of proteins to the side of a labeled actin filament can affect fluorescence. Therefore pyrene fluorescence alone cannot be used to definitively conclude that a given actin binding protein increases or decreases actin assembly; visualization of actin filaments is an essential complementary approach to pyrene assembly assays.

time. This is usually achieved by adjusting the diaphragm between the light source and the cuvette (*see* Note 16).

8. Figure 2 illustrates polymerization data and how they are analyzed. Plot fluorescence (arbitrary units) as a function of time (seconds). Add back dead time to x-axis data (time). Subtract baseline fluorescence from all y-axis data. Baseline fluorescence is fluorescence at time zero, assuming the reaction is slow enough to catch. If the conditions used do not alter the critical concentration of actin (i.e., protein being tested does not cap actin filaments or sequester monomers), normalize data by dividing all y-axis data by the fluorescence at plateau. *See* [10] for methods to determine critical concentration change.
9. Reaction half times are convenient metrics to measure (Fig. 2c inset). Replot the linear region of the curve surrounding half-maximal, and fit a linear equation to these points. Use this equation to solve for half time (the value of x where $y = 0.5$, if data are normalized).

3.5 Microscopy of Stabilized, Labeled Actin Filaments

1. Polymerize unlabeled actin using conditions described above, in an eppendorf tube. Allow reaction to come to plateau (*see* Note 17).
2. In the meantime, desiccate Alexa Fluor 488 phalloidin in a speed vacuum, to remove all methanol. Labeled phalloidin should be superstoichiometric to actin.
3. Pipette 5 μ L polymerized actin—using a wide-bore pipette tip—into the tube containing desiccated phalloidin to stabilize actin filaments.
4. Add 45 μ L 1 $\times$ KMEI buffer to dilute the reaction. Dilute to low nanomolar concentrations to resolve individual filaments.
5. Pipette 10 μ L of the dilutions onto slides and cover with poly-L-lysine coverslips. Seal slides with clear nail polish.
6. Once nail polish dries, image filaments with an epifluorescence microscope.

3.6 Silanization and Pegylation of Glass Coverslips

1. Use a diamond pen to unambiguously mark individual 18 mm $\times$ 18 mm coverslips (e.g., mark the bottom right-hand corner of coverslip with an “e”). Decide which surface of the coverslips will be silanized and pegylated, and be consistent.
2. Place marked coverslips in a 1 L glass beaker containing 200 mL of 3 M NaOH (*see* Note 18 for alternative cleaning strategies).

¹⁵Photobleaching is a complex phenomenon that cannot be corrected for mathematically [10]. If the overall signal is too low after eliminating photobleaching, try widening emission slits, increasing the voltage of the PMT, or increasing percentage of pyrene-labeled actin, and again measuring photobleaching after the change.

¹⁶We polymerize the concentration of actin that will be used in the experiments in the presence of Arp2/3 and an activator (like *Listeria* ActA or Scar/WAVE VCA), which comes to plateau very quickly. It is also possible to polymerize a high concentration of actin, then dilute down to working concentration and measure in the fluorimeter.

¹⁷First, to determine when a given condition will plateau, perform pyrene actin assembly assay as above. For microscopy, reaction volumes can be scaled down to less than 100 μ L.

3. Sonicate in NaOH for 30–40 min. Swirl every 10 min. Save this base solution for washing glassware in later steps.
4. Decant NaOH and rinse coverslips with copious amounts of ultrapure water.
5. Add 200 mL of 100 % isopropanol and sonicate for 15 min.
6. Meanwhile, make 200 mL of 5 % APTES in isopropanol (containing 1 % water; *see* Notes 19–²¹). APTES is toxic and corrosive. Use glass pipets when aliquoting APTES.
7. Remove coverslips individually from isopropanol and transfer them to a new beaker containing the 5 % APTES solution. Swirl each coverslip in the solution before dropping into the beaker. Bath sonicate the coverslips for 20–30 min.
8. Pour silane solution into waste container. Dispose of waste following appropriate health and safety guidelines. Do not pour APTES and isopropanol down the drain.
9. Rinse coverslips 3× with 100 % isopropanol to remove excess silane.
10. Remove coverslips one-by-one. Dip into a series of three beakers containing isopropanol to wash off excess APTES. In each beaker, move forceps back and forth in isopropanol to thoroughly remove excess silane from each coverslip. Rack coverslips in ceramic holders.
11. Bake for 2 h at 80–90 °C. The purpose of this step is to drive moisture off the glass surface which coordinates interactions between silane and water molecules. Dehydration of the coverslip surface causes the silane molecules to form a covalently linked monolayer on the glass surface. Excess APTES on the glass surface will form an insoluble white precipitate when the glass is heated above 70 °C. If this occurs, discard the coverslips; this is a sign that the silanized glass needs to be washed more thoroughly with isopropanol before baking.
12. After baking the APTES coated glass, bath sonicate the coverslips in 100 % ethanol for 15–30 min.
13. Using forceps, remove individual coverslips from the ethanol. Dip and waft coverslips in a beaker of ultrapure water. Slowly pull the coverslip out of the water. If the glass is properly silanized, water will bead-up and pull away from the coverslip. Oven dry to remove residual water.

¹⁸If the functionalized glass contains a high fluorescent background, Piranha etch the glass before silanizing. Piranha is a 3:1 mixture of sulfuric acid and hydrogen peroxide. Be sure to wear the proper protective gear. Alternative to 3 M NaOH or Piranha etching, 2 % Hellmanex (Fisher, 14-385-944), heated to ~60 °C, can be used to efficiently strip glass of fluorescence and organic matter.

¹⁹The addition of a small amount of water is required for the amino-silane molecules to polymerize and form weak electrostatic interaction with each other. Most reagent grade alcohol will contain a sufficient trace amount of water.

²¹Depending on the biochemical properties of actin binding proteins being characterized, the APTES silanization and NHS-PEG might not sufficiently passivate the glass. Instead, a more dense PEG functionalized glass surface may be required to efficiently reduce nonspecific protein absorption to the glass in the TIRF assay. Alternatively (3-Glycidyloxypropyl)-trimethoxysilane (GOPTS; Sigma-Aldrich 440167) and NH₂-PEG derivative can be used to silanize and pegylate coverslips, respectively. Note that GOPTS is destroyed by aqueous solvents. Therefore, it is necessary to wash the glass with anhydrous acetone after silanizing. After silanizing glass to GOPTS, Hydroxy-PEG-NH₂ 3000 Da (Rapp Polymere, 10-3000-20) and Biotin-CONH-PEG-NH₂ 3000 Da (Rapp Polymere, 13-5000-25-20) can be used to functionalize the coverslips. For more detail, refer to ref. 18.

14. Weigh 45 mg of mPEG-5000-NHS and 5 mg Biotin-PEG5000-NHS (*see* Note 22). The ratio can be varied depending on the desired % of biotin-PEG. Add anhydrous DMF to make a 50 mg/mL final concentration. If necessary, gently warm the solution to completely solubilize the PEG reagents. Be sure to use high quality/new DMF. Some solvents contain a large amount of fluorescent contamination which will bind to the glass. Scale up the amount of PEG-DMF solution as required.
15. Place six coverslips in each glass weigh jar with the diamond etched side up.
16. Spot 75 μ L of solution onto each coverslip.
17. Place a second coverslip on top of first to make sandwich. Repeat, making 6 sandwiches/12 coverslips per weigh jar.
18. Incubate at 75 °C for 2 h.
19. Transfer weighing jars to a fume hood. Cool slightly before opening.
20. Separate coverslip sandwiches with forceps and place them in a large beaker of ultrapure water. Avoid touching the coverslips with hands or gloves. Wash coverslips with copious amounts ultrapure water.
21. Remove coverslips one-by-one with forceps and swirl in a large beaker of water. Dry and store coverslips in a covered container at 4–25 °C. Pegylated glass will last 2 months.

3.7 Labeling A. castellani Actin and Heavy Meromyosin with Maleimide Derivatives

1. Make actin labeling buffer.
2. Equilibrate PD10 column with 3 column volumes of actin labeling buffer.
3. Buffer exchange 2.5 mL of monomeric actin, 25–50 μ M, using a PD10 column to remove the reducing agent (*i.e.*, TCEP).
4. Determine the new actin concentration by measuring the absorbance at 290 nm. An A_{290} of 1.0 is equal to 38.5 μ M amoeba actin.
5. Add 3–5 M excess Alexa Fluor 488, Cy3, or Cy5-maleimide. Maleimide dyes should be dissolved in DMSO to a concentration of 10–20 mM. These reagents should be stored in the –20 °C freezer and used within a year.
6. Incubate on ice for 5–10 min.
7. Quench the labeling reaction by adding DTT to 10 mM (1/100 dilution of 1 M stock). Incubate for 5 min on ice.

²²PEG-NHS and biotin-PEG-NHS reagents are quickly destroyed by moisture. In aqueous buffer, water acts as a nucleophile and reacts irreversibly with the NHS (half-life ~15 min). NHS reagents should be stored at –20 °C in a sealed desiccator. When using these reagents, allow the storage vial to warm to room temperature before opening the bottle. If the bottle is cold, water will condense on the lyophilized chemical and gradually reduce the activity. We recommend storing pre-weighed quantities of each NHS reagent in microfuge tubes that are sealed with parafilm and stored in a desiccator in the –20 °C freezer. This will minimize the number of times any particular reagent is exposed to moisture.

8. Ultracentrifuge the labeled monomeric actin at $350,000 \times g$ (80,000 rpm with a TLA100.4) for 20 min. This will help remove aggregated proteins and insoluble fluorescent dye.
9. Transfer supernatant to a new tube. Add $10\times$ KMEI to polymerize actin for 2–3 h at 20–25 °C. Final KMEI buffer concentrations should be 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM imidazole, pH 7.0. Add ATP to a final concentration of 1 mM.
10. Pellet labeled filamentous actin by ultracentrifugation at $195,000\times g$ (60,000 rpm with a TLA100.4) for 30 min.
11. Remove supernatant and gently wash the labeled actin pellet with 0.5 mL of Buffer A to remove free dye. *Note:* when labeling actin with tetramethyl-rhodamine, labeled actin will not polymerize. Monomeric TMR-labeled actin must be separated from free dye with a desalting or gel filtration column.
12. Gently resuspend the actin pellet in 250–500 μ L of Buffer A. Pipet up and down to shear actin filaments. Transfer labeled actin to a microfuge tube. Allow actin filaments to depolymerize for 3–5 days at 4 °C.
13. After depolymerization, ultracentrifuge the labeled actin at $350,000\times g$ (80,000 rpm with a TLA100.4) for 20 min to remove aggregates and small actin filament seeds (*see* Note 23).
14. To remove the last trace of unconjugated fluorescent dye, buffer exchange the depolymerized actin into Buffer A using a NAP-5 (or PD10) desalting column. Determine the new actin concentration and labeling efficiency using a spectrophotometer.
15. Measure the A_{290} of labeled actin and absorbance at the dye excitation peak. Since fluorescent dyes contribute to the A_{290} signal, subtract this contribution from the A_{290} of the labeled actin to accurately calculate the concentration and labeling efficiency (Table 1).
16. To determine how much an unlisted dye contributes to the A_{280} , make a 1/1,000 dilution of the dye and perform a wave scan. Divide the A_{290} by the $A_{ex\ peak}$ of the dye; this is the A_{290} correction factor.
17. For actin labeled with Alexa488, $A_{290}(\text{actual}) = A_{290}(\text{labeled protein}) - (A_{290}(\text{labeled protein}) \times 0.132)$.
18. Labeling efficiency = $[\text{protein}]_{290} / [\text{protein label}]_{ex\ peak}$.
19. This protocol can also be used to biotinylate actin with maleimide-PEG2-Biotin or heavy meromyosin (HMM) labeling with maleimide-PEG11-Biotin. In the case of

²³Without gel filtration, some actin dimers and trimers will be present. This is really only a problem if you want to measure nucleation rates (as in pyrene assembly assays, above). If you find that spontaneous nucleation is too rapid in the actin TIRF assay, centrifuge ($350,000 \times g$ or 80,000 rpm in a TLA100.4 for 20 min) or gel filter the labeled actin with a Superdex 75 column (as in Subheading 3.2, **step 12**). Alternatively, use profilin to suppress spontaneous actin nucleation. Note that equal molar concentrations of profilin relative to the actin monomer concentration will reduce the barbed end elongation rate of single actin filaments by ~50 %.

biotinylating HMM, buffer exchange reduced protein into 10 mM HEPES (pH 7.0) with 100 mM KCl. Add 5 M excess maleimide-PEG11-Biotin. Label for 15 min on ice. Quench with 10 mM DTT. Microcentrifuge to remove aggregates. Finally, remove free biotin-maleimide by gel filtration or desalting column. Freeze biotin-PEG11-HMM in buffer containing 10 mM HEPES (pH 7.0), 100 mM KCl, 1 mM TCEP, and 25 % glycerol.

3.8 Assembly of TIRF-M Flow Cells for Attachment of Filamentous Actin

1. Clean 25 mm × 75 mm × 1 mm glass slides with 3 M NaOH, 100 % ethanol, or oxygen plasma, as above (Subheading 3.6, **steps 2–4**).
2. Cut double sided tape and attach to glass slides (Fig. 3). Firmly rub across the tape with a capped Sharpie pen to form a tight seal with the slide. Although not essential for visualizing dynamic actin filaments, it's worth passivating the slides with poly-L-lysine PEG to minimize protein depletion effects (Fig. 3).
3. To passivate the slides, spread 5–10 µL of 3 mg/mL PLL- PEG on the space between the double sides tape. Allow the PLL-PEG to dry and then wash the glass under a stream of ultrapure water. Allow the slide and tape to dry before attaching the functionalized coverslip. After attaching a functionalized 18 mm × 18 mm coverslip to the double sided tape, gently press on the surface (e.g., using a capped Sharpie pen) to create a tight seal between the coverslip and tape.
4. Flow 40 µL blocking buffer into the flow chamber (*see* Note 24).
5. Incubate blocking solution for 1 min.
6. Wash with 40 µL wash buffer.
7. Flow 40 µL of 50 nM streptavidin diluted in wash buffer. Incubate for 30–60 s. Flush chamber with 40 µL wash buffer.
8. Flow 40 µL of 50 nM biotin-PEG11-HMM diluted in wash buffer. Incubate for 30–60 s. Flush chamber with 40 µL wash buffer. Alternatively, low concentration of biotin-phalloidin can be used to attach static or dynamic actin filaments. These methods of attachment may not be compatible with all actin binding proteins. Maleimide labeling of HMM can be performed as described in Subheading 3.7, **step 19**.
9. With myosin attached to the coverslip surface, phalloidin stabilized or dynamically elongating actin filaments can be attached to the functionalized glass surface (*see* Fig. 4 and Note 25).

²⁴Passivating the slide surface with PLL-PEG will minimize protein depletion effects caused by nonspecific absorption of protein to the glass surfaces. Protein depletion can lead to inconsistent results, especially when characterizing an actin binding protein that is present at a nanomolar concentration in the assay.

²⁵It is important to optimize conditions such that filaments are not so strongly adhered to the glass as to prevent them from polymerizing. Pauses in elongation/polymerization should be infrequent by kymograph analysis. If the filaments are pausing too much or growing slowly, use less streptavidin and Biotin-HMM. Alternatively, reduce the percentage of conjugated biotin-PEG.

3.9 Visualization of Dynamic Actin Filament Assembly

1. Combine the appropriate volumes of dark actin and fluorescently labeled actin in Buffer A to make a working stock of 4.44 μM (10–30 % Alexa488; *see* Note 26).
2. To exchange the Ca^{2+} for Mg^{2+} , combine 1 μL 10 $\times$ ME and 9 μL of 4.44 μM actin. Incubate for 2 min.
3. Meanwhile combine 18 μL 2 $\times$ TIRF buffer (lacking OSS) with 1 μL glucose oxidase (40 $\times$) and 1 μL catalase (40 $\times$) (*see* Note 27). Be sure to calculate buffer concentrations so that the addition of the OSS brings all of the TIRF buffer reagents/ enzymes to 2 $\times$ the desired final concentration.
4. Add 10 μL of protein dilution buffer to the 2 $\times$ TIRF buffer ($V_{\text{f}} = 30 \mu\text{L}$). Typically this portion of the reaction mixture contains additional actin binding proteins (i.e., profilin, VASP, etc.).
5. To initiate the actin polymerization reaction, transfer the 30 μL TIRF buffer mix into the tube containing 10 μL of ME to exchange monomeric actin. Pipet up and down twice. Flow all 40 μL into the chamber using an aspirator and Whatman paper to wick the solution through the channel.
6. Seal the edges of the flow cells with VALAP, apply immersion oil, and mount on the TIRF microscope. In the presence of 1 μM actin, filaments will spontaneously nucleate and start elongating immediately. By the time the slide is mounted on the microscope and focused on the imaging surface (30–60 s), small actin filament seeds should be visible (Fig. 5a). Imaging with a frame rate of 5–10 s is sufficiently fast to capture the dynamics of actin filament assembly (Fig. 5b). Under these conditions, actin filament barbed ends should elongate at a rate of ~ 10 subunits/second (Fig. 5c).
7. For image analysis of microscopy data, we use ImageJ. By tracing the elongation path of a single actin filament, a kymograph can then be generated using the MultipleKymograph ImageJ plugin (J. Rietdorf, FMI Basel; A. Seitz, EMBL Heidelberg; <http://rsbweb.nih.gov/ij/>) available for free online. This will create an image similar to the one shown in Fig. 5c. The x -axis corresponds to filament length (1 μm of actin filament contains 370 subunits; [26]) and the y -axis represents time (i.e., seconds). The slope of kymograph equals the change in filament length as a function of time. This value is typically expressed as subunits/second.

²⁶For polymerization assays, it is best to use 10 % labeled actin. The higher the percentage labeled actin, the slower filaments will elongate. If more than 10 % labeled actin is required to visualize filaments, something could be wrong with your reagents. Check the quality of your actin by SDS-PAGE; actin that is partially proteolyzed will give the appearance of slower kinetics. In this event you can re-enrich for full-length actin by one cycle of polymerization and depolymerization: add 10 $\times$ KMEI to polymerize, then centrifuge at $100,000 \times g$ to pellet filaments, discard supernatant (containing non-polymerizable actin), depolymerize, and gel filter as in Subheading 3.2, **steps 10–12**. Alternatively, trouble-shoot actin polymerization by visualizing phalloidin-stabilized filaments as in Subheading 3.5. Other common sources of error include: lack of camera sensitivity, too low or high laser power coming through the objective, or improperly functionalized glass.

²⁷Make glucose oxidase and catalase stocks fresh and ultracentrifuge at $350,000 \times g$ (80,000 rpm with a TLA100.4) for 20 min to remove aggregates. There is typically a large dark brown pellet after centrifuging catalase. Keep the supernatant and do not resuspend this pellet. Use within 3 days. If the glucose oxidase and catalase activity are not properly balanced in the TIRF assay buffer, the reaction can acidify. This may result in slower filament elongation rates and changes in photostability of the fluorophores. Be sure to use fresh lyophilized catalase that is not clumpy due to prolonged exposure to moisture.

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Fig 1.

Gel filtration of actin from *Acanthamoeba castellanii*. **(a)** Depolymerized actin was gel filtered over a 323 mL Superdex 75 column (GE Healthcare) in Buffer A. Absorbance at 290 nm was measured, and concentration of actin plotted for each fraction. The *first peak* contains contaminants, including alpha-actinin. The *second* (major) *peak* is actin. It is important to test fractions for activity as early fractions may be contaminated with dimers, which will speed up assembly kinetics [29]. **(b)** Fractions of actin eluted from the Superdex 75 column **(a)** and visualized by SDS-PAGE. Peak fractions should contain less than 1 % proteolyzed actin

Fig 2.

Pyrene actin assembly assay. **(a)** Actin was polymerized as described; curves show spontaneous nucleation of 2 μM (*black*), 4 μM (*dark grey*) and 8 μM (*light grey*) actin. These data have been zeroed by subtracting baseline fluorescence from all fluorescence values. **(b)** Data from above were normalized by dividing all fluorescence values by average fluorescence at plateau. **(c)** 2 μM actin was polymerized in the presence of 10 nM Arp2/3 complex and 5 nM *Listeria* ActA. *Inset*: To calculate time to half-maximal fluorescence ($t_{1/2}$), the linear region of the curve in (c) spanning 0.5 were replotted, and a linear trendline was fit to the data. Solving for time (x) where $y = 0.5$ yields $t_{1/2}$

Fig 3.

Architecture of TIRF microscopy glass imaging chamber. Functionalized 18 mm × 18 mm coverslips are adhered to a glass slides using double sided tape with a spacing of ~5 mm per channel. Before attaching the PEG/biotin-PEG coverslips, coat the spaces between the double sided tape with PLL-PEG to reduce nonspecific absorption of proteins to the glass surface (*see* Note 24)

Fig 4.

Schematic for attachment of filamentous actin to biotin-PEG functionalized coverslips. Biotin-PEG functionalized glass can be coated with streptavidin and biotin-myosin. Pre-polymerized actin filaments can then be immobilized via the biotin-myosin linkage. Free actin monomers in solution can freely associate with the ends of actin filaments

Fig 5.

Visualization of single actin filaments by TIRF microscopy. **(a)** Image showing a field of actin filaments generated by spontaneous nucleation of 1 µM actin (10 % Cy3 labeled) after about 1 min of mixing reactants. Actin filaments are attached to a biotin-myosin coated glass surface. **(b)** Montage of images showing a single actin filament elongating. The *white arrow* indicates the barbed end. **(c)** Kymograph showing the change in length as a function of time for a single elongating actin filament

Table 1

Cysteine reactive maleimide fluorophores used for labeling actin

Fluorophore	Extinction coefficient ($M^{-1}cm^{-1}$)	Absorbance peak (nm)	Contribution to A_{290} (%)
Alexa 488	71,000	494	13.2
Cy3	150,000	550	8
Cy5	230,000	649	3