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Measurement and Analysis of *in vitro* Actin Polymerization

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Summary

The polymerization of actin underlies force generation in numerous cellular processes. While actin polymerization can occur spontaneously, cells maintain control over this important process by preventing actin filament nucleation and then allowing stimulated polymerization and elongation by several regulated factors. Actin polymerization, regulated nucleation and controlled elongation activities can be reconstituted *in vitro*, and used to probe the signaling cascades cells use to control when and where actin polymerization occurs. Introducing a pyrene fluorophore allows detection of filament formation by an increase in pyrene fluorescence. This method has been used for many years and continues to be broadly used, owing to its simplicity and flexibility. Here we describe how to perform and analyze these *in vitro* actin polymerization assays, with an emphasis on extracting useful descriptive parameters from kinetic data.

Keywords

Actin polymerization; *in vitro* reconstitution; biochemical assay; quantitative analysis

1. Introduction

Dynamic rearrangements of the actin cytoskeleton underlie key aspects of many cellular functions, including aspects of cell motility, vesicle trafficking, and cytokinesis (1–3). Many of these rearrangements rely on control of when and where actin polymerization occurs. This is regulated at the level of nucleation of new filaments (4–6), elongation of these filaments (5,6), crosslinking of filaments into higher order structures, arrest of filament growth and disassembly of filaments (2). Given the broad cellular importance of these processes, great effort has been put into studying the mechanisms of how these processes are regulated at the biochemical level.

Here we describe a widely used biochemical assay for actin polymerization (7,8) that can be used to answer many important biochemical questions. Although more complex assays are possible (9–12), for questions regarding the activity of actin filament nucleation factors (13–16), often the most direct way to analyze activity is through the use of a bulk kinetic assay. Further, in order to interpret the results from filament mesh reconstitution and bead motility assays, an understanding of the underlying biochemistry is of great utility.

Actin will polymerize into filaments whenever its concentration is greater than its critical concentration. The critical concentration of actin can be modified by type of actin, bound nucleotides (17) and numerous solution conditions (18,19). Thus, purified actin can be maintained as monomers by removing almost all salts present, storing it under mildly alkaline conditions and by including calcium ions rather than magnesium ions (20). This solution can then be induced to form filaments by adding a concentrated buffer mix that alters pH, switches divalent cations, and increases salt concentrations into the physiological range. The polymerization of actin can be tracked in bulk solution by following a variety of observables, including tracking the increase in light scatter or solution viscosity as the polymers grow. Owing to greater flexibility, it is far more common to introduce the environmentally sensitive fluorophore, pyrene, onto an existing cysteine in actin, and follow the increase in fluorescence intensity as the polymers form (8,7).

Part of the great power of the pyrene detected method is that the changes in fluorescence intensity for an actin solution can be measured in real-time with high signal to noise, and thus is well suited to kinetic assays, which are generally of the most interest for this system. We describe this assay in detail, with particular attention paid to constructing quantitative assays.

Analysis of the resulting kinetic profiles is somewhat complicated. The difficulty stems from the fact that polymerization kinetics are a combination of processes: elongation of filaments at both their fast growing ‘barbed’ end and their slow growing ‘pointed’ end, nucleation of new filaments from bulk solution, and nucleation of filaments in a filament dependent manner. These processes all have distinct kinetic effects. There is no general analytic solution for actin polymerization kinetics in the presence of nucleation factors, and thus descriptive metrics for the kinetics are typically used for quantification instead of parameters determined by fitting to the entire dataset (*see* Note 1). Here we describe the calculation of several simple metrics (*see* Note 2): the time at which half of the actin has polymerized ($t_{1/2}$), the actin polymerization rate at $t_{1/2}$, the number of barbed ends present at $t_{1/2}$ (*see* Note 3), and the initial nucleation rate.

¹In a few cases, the system can be set up such that pseudo-first order conditions can be achieved. For example, if the reaction is set up with a known quantity of seed, which is sufficient to overwhelm any nucleation, the system will elongate in a fashion consistent with a first order reaction. This can be fit using analytic equations. Further, if one has a known kinetic model with only a few varied parameters, one could in principle use numeric approaches to solve the rate laws for the model and fit pyrene actin polymerization data directly (21,15,22). Owing to the complexity of the kinetic models for nucleation pathways, this is not a widely used approach. The initial nucleation rate method described in **Section 3.3** / Note 19 is an example of this approach.

²We have found that automating the analysis of the kinetics through the use of an in house developed computer script improves the consistency, reliability and practicality of such analyses. Despite this, we still find it helpful for a human to visually examine the data and extracted numbers (*see* Fig. 3).

³In our experience, the determination of the maximum slope, or maximum barbed ends introduces a fair bit of noise. Examining the polymerization rate or barbed ends as a function of time, a maximum can typically be seen, and the rough center identified by eye. Unfortunately, automatically identifying this maximum in an unbiased fashion, in the context of noisy data, is challenging. Instead, we recommend that the barbed ends or actin polymerization rate be tracked at some easily identified characteristic time, such as $t_{1/2}$. Moreover, for Arp2/3 complex dependent nucleation assays, the time at which barbed ends reach their maximum and $t_{1/2}$ are typically very similar. Thus, the actual numbers found by the two methods tend to be quite similar, but determining the value at $t_{1/2}$ removes the noise and bias associated with finding the maximum.

2. Materials

All buffer and salt stocks are prepared using ultrapure water (>18 MOhm, using a Millipore brand Milli-Q water purification system). Except where noted, all solutions are filtered through a 0.22 µm cellulose acetate membrane. Working buffers are prepared by dilution of buffer stocks into prechilled ultrapure water. Where specific sources are recommended, the manufacturer and part numbers are indicated.

2.1. Stock Materials and Solutions

1. Rabbit muscle acetone powder (Pel-Freez #41995-2).
2. N-(1-pyrene) iodoacetamide (Invitrogen #P-29): 10 mM stock in anhydrous dimethylformamide (DMF). The solution is stored at −20°C in 1 mL aliquots.
3. Dithiothreitol (DTT): 1 mM stock in ultrapure water, filtered, and stored at −20°C in 1 mL aliquots. When thawing, place in cool water until solution is liquid, then transfer to ice until needed. Once added to buffers, assume DTT is no longer functional after 48 hours when stored at 4°C.
4. Adenosine 5' triphosphate disodium salt (ATP; Sigma #A7699): 100 mM stock in 100 mM Tris-HCl pH 8.0, and titrated to pH 7.4 to 7.5 with sodium hydroxide. The solution is filtered and stored at −20°C in 1 mL aliquots.
5. 0.5 M EGTA: Prepared using ethylene glycol tetraacetic acid powder and titrated to pH 8.0 with sodium hydroxide. Initially, EGTA is insoluble, but comes into solution as the pH is adjusted.
6. 1 M Tris-HCl pH 8.0: Prepared using tris base, and titrated to pH 8.0 at room temperature with concentrated hydrochloric acid.
7. 1 M Imidazole pH 7.0: Titrated with hydrochloric acid to pH 7.0 at room temperature.
8. 2 M KCl.
9. 5 M NaCl.
10. 1 M MgCl₂.
11. 1 M CaCl₂.
12. 1 M Sodium Azide.

2.2. Working Buffers

1. KMEI: 50 mM KCl, 10 mM imidazole pH 7.0, 1 mM MgCl₂, 1 mM EGTA.
2. 10x KMEI: 500 mM KCl, 100 mM imidazole pH 7.0, 10 mM MgCl₂, 10 mM EGTA.
3. Buffer G: 2 mM Tris-HCl pH 8, 200 µM ATP, 0.5 mM DTT, 0.1 mM CaCl₂, 1 mM sodium azide.
4. Buffer G-Mg: 2 mM Tris-HCl pH 8, 200 µM ATP, 0.5 mM DTT, 0.1 mM MgCl₂.

5. 10E/1M: 10 mM EGTA, pH 8, 1 mM MgCl₂.

2.3. Chromatography Columns

1. Superdex 200 pg column: This is a prepacked HiLoad 26/600 Superdex 200 pg column, with 320 mL of resin in XK26/600 column hardware (GE# 28-9893-36). The maximum flow rate is 4.25 mL/min with a maximum pressure of 0.5 MPa. We run this column run at 1–2.5 mL/min at less than 0.4 MPa back pressure.

2.4. Hardware and Equipment

1. Two centrifuges are needed: (1) An ultracentrifuge (Beckman Optima or equivalent) equipped with Type 45 Ti, Type 70 Ti and SW 41 Ti or other appropriate rotors (*see* Note 4). (2) A refrigerated centrifuge (Beckman Coulter Avanti Centrifuge), equipped with JA-25.50 rotors. Any refrigerated centrifuges capable of spinning 50 mL tubes at ~2,000 x *g* are equivalent for this purpose.
2. A liquid chromatography system capable of delivering linear gradients and operating columns at the described flow rates and pressures.
3. A 40 mL and 15 mL dounce homogenizer.
4. 14 kDa dialysis tubing and clamps.
5. A large stir plate and a magnetic stir bar.
6. Cold room or cold box with space for the stir plate.
7. Fluorometer: We recommend a system with monochromator based excitation and emission wavelength selection, and some degree of bandpass control. Fluorescence light source must be capable of illuminating at 365 nm.
8. Fluorescence cuvettes: Many styles of cuvette may be used for this assay, but we use a 3 mm × 3 mm microvolume cuvette. This cuvette ends up being a good compromise between sample volume (200 µL), cost and ease of complete cleaning.
9. Micro-volume pipettes: P-200, P-20, P-10 or equivalent.

3. Methods

3.1. Purify and Pyrene Label Actin

1. Ensure that centrifuges and rotors are cold.
2. Weigh out 4 to 5 g rabbit muscle acetone powder.
3. Place 20 mL of cold buffer G per gram of rabbit muscle acetone powder in a beaker, add muscle acetone powder. Stir at 4°C for 30 minutes.
4. Filter solution through four layers of cheese cloth. Recover and resuspend the muscle acetone powder residue in 20 mL of cold buffer G per gram of starting weight (*see* **step 2**). Stir at 4°C for 30 minutes.

⁴Fill tubes according to manufacturer's instructions. Generally, at the speeds used here, tubes should be very nearly full. In scaling the preparation up and down, try to anticipate the changes in volume that will result and change the rotor and tube choices accordingly.

5. Filter solution through four layers of cheese cloth and combine with the filtrate from **step 4**.
6. Centrifuge the filtrate for 30 minutes at 16,000 rpm at 4°C in a JA25.50 rotor or equivalent.
7. Carefully decant the supernatant into a graduated cylinder (pellet is soft) and measure the volume.
8. Slowly add 2 M KCl to a final concentration of 50 mM. Slowly add 1 M MgCl₂ to a final concentration of 2 mM. Continue to stir for 2 hours at 4°C.
9. After two hours has passed, slowly add solid KCl to a final concentration of 800 mM (add 5.6 g per 100 mL of solution volume). Stir for 30 minutes at 4°C.
10. Collect actin filaments from **step 9** by centrifugation. Centrifuge at > 100,000 x g for 2 hours at 4°C (35,000 rpm in a Type 70 Ti rotor) (*see* Note 4).
11. Discard supernatant. Rinse pellet (containing filamentous actin) with ~500 µL of buffer G.
12. Transfer the pellet into a dounce homogenizer. Place 8 mL per gram of starting rabbit muscle acetone powder weight into the body of a 40 mL dounce homogenizer. Lightly scrape the pellets from the centrifuge tube using a curved spatula and transfer to the homogenizer. The pellet will stick to the spatula, and can be transferred by scraping it onto the pestle, and then dislodging from the pestle into the buffer in the homogenizer. Gently passage the homogenizer pestle up and down about 50 times to resuspend the pellet, trying not to incorporate air. If the solution remains highly viscous as it is processed, dilute it slightly with buffer G.
13. Rinse 12 to 15 cm lengths of 14 kDa MWCO dialysis tubing in water and equilibrate a few minutes in buffer G.
14. Place resuspended actin from **step 12** into 14 kDa MWCO dialysis tubing. Dialyze against 2 L buffer G overnight.
15. Change the buffer on the second and third nights, using buffer G made without DTT.
16. On the morning following the third night of dialysis, collect the solution in the dialysis bags. Retain a small amount of dialysis buffer. Remove actin filaments from the dialyzed solution by centrifugation at > 100,000 x g for 2 hours, at 4°C (using a Type 70 Ti rotor at 35,000 rpm or a SW 41 Ti rotor at 40,000 rpm) (*see* Note 4).
17. Equilibrate a Superdex 200pg gel filtration column with buffer G.
18. Carefully remove the centrifuge tubes from the rotor, minimizing jostling. Collect the upper 1/2 of the supernatant and inject onto the equilibrated Superdex 200pg gel filtration column, collecting in 5 mL fractions. Decant remove the remaining supernatant solution into a small glass beaker and reserve at 4°C.

19. Choose gel filtration fractions to save (*see* Fig. 1 and Note 5). Measure the concentration of actin in each fraction by UV absorbance (*see* Note 6). Transfer fractions into separate small pieces of equilibrated 14 kDa MWCO dialysis tubing (*see* Note 7), and store in buffer G. Store actin in a large stirred beaker at 4°C, in 2 L of buffer G; change the buffer twice per week. Seal the top of the beaker with plastic wrap and aluminum foil. Stored in this fashion, actin can typically be kept for two months.
20. Add a clean magnetic stir bar to the glass beaker with the actin supernatant in it (*see* **step 18**), and begin stirring the solution slowly at 4°C. Note the volume of supernatant. Measure the concentration of actin by UV absorbance (*see* Note 6), using the dialysis buffer reserved in **step 16** as the absorbance blank.
21. Slowly add 2 M KCl to a final concentration of 50 mM. Slowly add 1 M MgCl₂ to a final concentration of 2 mM. Continue to stir for 2 hours.
22. From the concentration and volume measured in **step 20**, calculate the total actin present. Add a 5- to 10-fold molar excess of N-(1-pyrene) iodoacetamide. Close off the top of the beaker with parafilm or saran wrap. Then wrap the entire beaker with aluminum foil to protect it from light (*see* Note 8). Stir over night at 4°C.
23. The next morning, centrifuge the labeling reaction at 4°C, at ~2,000 x g (5,000 rpm the Beckman Avanti JA25.5 rotor) for 5 minutes to remove excess dye. Remove the supernatant with a pipet (the pellet is very soft).
24. Collect actin filaments from the supernatant from **step 23** by centrifugation. Centrifuge at > 100,000 x g for 2 hours at 4°C (using a Type 70 Ti rotor at 35,000 rpm or a SW 41 Ti rotor at 40,000 rpm) (*see* Note 4).
25. Collect centrifuge tubes and discard supernatant. Rinse pellets (containing pyrene labeled filamentous actin) with ~500 µL of buffer G.
26. Transfer the pellet into a Dounce homogenizer. To do this, place 8–12 mL of buffer G into the homogenizer body. Lightly scrape the pellets from the centrifuge tube using a 3–7 mm wide, slightly curved spatula. The pellet will stick to the spatula, and can be transferred by scraping it onto the pestle, and then dislodging from the pestle into the buffer in the homogenizer. The pellet will typically be somewhat opaque with residual pyrene iodoacetamide. Gently passage the homogenizer pestle up and down about 50 times to resuspend the pellet, trying not to incorporate air.

⁵The actin and pyrene-actin gel filtration peaks have a characteristic asymmetric shape, with a leading tail (*see* Fig. 1). This may stem from the low salt conditions under which the column is run, or may be related to oligomerization. For the 320 mL Superdex 200 column, we collect 5 mL fractions. We keep only the three most concentrated fractions, which should be greater than 10 µM actin and, for the pyrene labeled actin, be greater than 35% labeled, and discard fractions from the leading edge of the peak.

⁶To calculate the actin concentration, measure the absorbance at 290 nm to reduce the interference from ATP. Use of absorbance at 290 nm instead of 280 nm reduces the interference from nucleotide. Actin has an extinction coefficient at 290 nm of 26,600 M⁻¹ cm⁻¹.

⁷Conventional dialysis tubing is recommended for the actin preparation because our experience shows that it is the least prone to actin losses during the prolonged dialysis steps.

⁸To reduce photobleaching of pyrene actin we take several steps to minimize light exposure. We wrap columns in aluminum foil to prevent light penetration while the column is running. Similarly, we shield fractions with another piece of aluminum foil during and after collection. We turn off lights in the cold box containing the chromatography system.

Once resuspended the solution will appear slightly turbid. If the solution remains highly viscous as it is processed, dilute it slightly with buffer G.

27. Rinse 12 to 15 cm lengths of 14 kDa MWCO dialysis tubing in water and equilibrate a few minutes in buffer G.
28. Place resuspended actin from **step 26** into 14 kDa MWCO dialysis tubing. Dialyze against 2 L buffer G for three nights, changing the buffer on the second and third nights.
29. On the morning following the third night of dialysis, collect the solution in the dialysis bags. Remove actin filaments from the dialyzed solution by centrifugation at $> 100,000 \times g$ for 2 hours at 4°C (using a Type 70 Ti rotor at 35,000 rpm or a SW 41 Ti rotor at 40,000 rpm) (*see* Note 4).
30. Equilibrate a Superdex 200pg gel filtration column with buffer G.
31. Carefully remove the centrifuge tubes from the rotor, minimizing jostling. Collect the upper 2/3 of the supernatant. Inject supernatant onto equilibrated Superdex 200pg gel filtration column.
32. Choose gel filtration fractions to save (*see* Fig. 1 and Note 5). Measure the concentration of actin and degree of pyrene labeling in each fraction (*see* Note 9). Transfer fractions into separate small pieces of equilibrated 14 kDa MWCO dialysis tubing (*see* Note 7), and store in buffer G. Store pyrene labeled actin in a large stirred beaker at 4°C, in 2 L of buffer G; change the buffer twice per week. Seal the top of the beaker with plastic wrap and cover the whole beaker with aluminum foil to prevent light from reaching the labeled actin, which will photobleach the pyrene. Stored in this fashion, pyrene actin can typically be kept for two months.

3.2. Pyrene Actin Polymerization Assays

Below is our protocol for preparing actin polymerization assays. The exact timing and addition order is not necessarily critical, but for best data reproducibility as specific order and timing should be planned. For clarity we also include an example of the process (*see* Note 10).

1. Turn on fluorometer, ignite the lamp and start the software (*see* Note 11).
2. Optionally re-measure the concentrations of actin and pyrene labeled actin by UV absorbance (*see* Notes 6 and ⁹).
3. Prepare actin stock. Calculate the number of reactions needed, including controls (*see* Note 12). Calculate the volume of actin and pyrene labeled actin needed for the entire 'actin stock' at the desired actin concentration and labeling (*see* Note 13).

⁹To calculate the concentration and labeling efficiency of pyrene actin we measure the absorbance at 290 nm and at 344 nm. Pyrene concentration is determined by absorbance at 344 nm, using an extinction coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$. To calculate the actin concentration we first account for the contribution from pyrene at 290 nm, which is found by multiplying the absorbance at 344 nm by 0.127. The remaining absorbance at 290 nm comes from actin. The fraction of pyrene labeling is found by dividing the concentration of pyrene by the concentration of actin. The fractional pyrene labeling should be relatively consistent across the different fractions, but different preparations may vary between 35% and 100% labeled.

Check that the concentration of actin in the planned actin stock is at least 2.2 fold greater than the desired assay concentration. Following this check, mix the calculated volumes of actin and pyrene labeled actin. Store the actin stock in a lightproof tube on ice.

4. Calculate the volumes of all materials needed in the assay. The assay solution is divided into two parts. The first is an actin containing mixture (Mix A) and the second contains all additional proteins in a salt containing solution (Mix P). For our assay, Mix A and Mix P are both 100 μ L in volume. The components of Mix A are typically kept constant throughout the experiment; Mix P is usually different for each reaction, as the experiment dictates. Before starting to mix the components for

¹⁰For clarity, we provide an example of the calculations used to run a hypothetical assay (analogous to the Actin + Arp2/3 complex + VCA sample in Fig. 2).

First we make an actin stock for twenty five 200 μ L reactions with 4 μ M of 5% pyrene labeled actin. We will make these from a 30 μ M actin stock and a 20 μ M 50% labeled pyrene actin stock. The stock needs a total of 1 nmole of pyrene and 20 nmoles of actin. Thus, we find that 100 μ L of the pyrene labeled actin is needed (1×10^{-9} mole/ 1×10^{-5} M pyrene). As the actin is 50% labeled, this introduces 2 nmoles of actin, and the remaining 18 nmoles of actin is provided with 600 μ L of the 30 μ M actin stock. We want at least an 8.8 μ M actin concentration ($4 \mu\text{M} \times 2.2$) in the actin stock, and our stock is $\sim 28.6 \mu\text{M}$.

Next we plan out an actual reaction. Our reaction will contain 4 μ M actin (5% pyrene labeled), 10 nM Arp2/3 complex and 250 nM VCA. We assume stock concentrations of 400 nM Arp2/3 complex in KMEI and 10 μ M in KMEI. We plan Mix A as 28 μ L from the above prepared actin stock, and 2.8 μ L of 10E/1M. The remaining volume is made up with 69.2 μ L of buffer G-Mg. Mix P will need 5 μ L each of the Arp2/3 complex stock and the VCA stock. As Arp2/3 complex and VCA are in KMEI, we only need to add 10x KMEI to account for the volume of Mix A (which we treat as not contributing to either total salt or the "MEI" concentration) and the volume of 10x KMEI added. This is 11.1 μ L of 10x KMEI, and 78.9 μ L of KMEI to bring the Mix P volume to 100 μ L.

With the calculations complete, we prepare the actual reaction. We setup the fluorometer to acquire one data point every other second, illuminating only during the 0.5s time that data is acquired. The system is initialized and left waiting to start the kinetic acquisition. We prepare Mix A by placing 28 μ L of the actin stock in a fresh tube and adding 2.8 μ L of 10E/1M. Shortly after, we add 69.2 μ L of buffer G-Mg, mix by pipetting and begin our timer counting up. We prepare Mix P by placing 78.9 μ L of KMEI in a separate fresh tube. We add 5 μ L of the 400 nM Arp2/3 complex stock, then we add 5 μ L of the 10 μ M VCA stock. When the timer reads 1 minute 50 seconds, 11.1 μ L of 10x KMEI is added to Mix P. Then all of Mix P is picked up with a P-200. When the timer reads 2 minutes exactly, Mix P is added to Mix A. The contents are mixed, transferred to a cuvette and data acquisition is started when the timer reads 2 minutes 20 seconds. The dead time is recorded as 20 seconds.

¹¹We have also adapted the assay to a plate reader (Thermo Scientific Varioskan Flash) with good results. In this case, the instrument uses monochrometers for wavelength selection, but allows very little control over the width of the excitation and emission bandpass, with only 5 nm and 12 nm options. As a result we found that the overall change in signal was only six- to eight-fold. While the resulting dynamic range is not as large as is theoretically possible, the plate-reader based assay is still very useful. In principal, filter based excitation and emission methods are also possible, but the filters will need to be selected carefully.

Pyrene-actin fluorescence intensity is prone to photobleaching. Photobleaching can be reduced to acceptable levels by either attenuating illumination intensity through decreased bandpass or with neutral density filters, or decreasing the time of illumination. In using a narrow bandpass (1 nm), we have strongly attenuated our excitation intensity, which partly improves photobleaching. For longer kinetics, we further decrease photobleaching by illuminating and measuring for 0.5 s and then closing an excitation shutter for 2.5 s. This is then repeated for as many as 1,000 cycles, depending on the timing of the reaction.

Pyrene emission intensity is slightly sensitive to events in addition to polymerization. In faster kinetics, a slight 'overshoot' is frequently observed. This is not due to photobleaching, but instead is commonly ascribed to events of ATP hydrolysis and/or phosphate release (23). For slower kinetics the overshoot is not observed at all, as the later step will occur as filament is formed. We have also observed a few cases where the proteins added to a polymerization reaction have changed the intensity of pyrene fluorescence, presumably by binding filament and changing the environment of the pyrene. The exact solution to such a problem will depend on the system (increasing salt concentrations has helped for our systems), but it is worth paying attention to any anomalous pyrene fluorescence intensity that is observed.

¹²Actin polymerization is very sensitive to small differences in concentration, and to very small quantities of seed fibers that may be present. Thus, we run a set of controls each day that detects any problems in the actin stock, or in the nucleation factors. The controls run for a series of actin nucleation assays in the presence of Arp2/3 complex are: actin in the absence of Arp2/3 complex, actin with Arp2/3 complex and actin with Arp2/3 complex in the presence of saturating N-WASP VCA (see Fig. 2). Together, comparison of these data to analogous data acquired on other days will detect problems with buffers, actin or Arp2/3 complex. For the best results, compare kinetics collected on the same day using a single actin mix. We have generally had good results comparing between actin mixes, but this eliminates all variability concerns.

¹³Up to 10% pyrene labeling of actin is routinely used. For actin polymerization assays in the absence of profilin, total actin concentrations between 1 and 4 μ M are routinely used. For single reactions, we generally use 4 μ M actin (5% pyrene labeled) in a 200 μ L reaction. When working in a multi-well plate, we generally use 2 μ M actin (10% pyrene labeled) in a 200 μ L reaction. We generally make a single actin mix sufficient for a day's worth of assays, accounting for controls and including $\sim 10\%$ extra. The mix is stored, protected from light, on ice. Typically, we use a mix within 16 hours of preparation, but have not seen evidence of changes in behavior up to about 36 hours.

a reaction, calculate all the volumes needed in both Mix A and Mix P. Mix A contains the actin stock prepared above, 10E/1M buffer and buffer G-Mg (*see steps 6–10* below). Mix P components include buffer KMEI, nucleation factors, additional proteins, 10X KMEI and additional KCl, if needed (*see steps 11–14* and Note 14).

5. Initialize the fluorometer for data acquisition. On most fluorometer systems, positioning monochrometers, setting slit widths, and initializing the data set can be performed prior to starting data collection. This helps to minimize the delay between combining Mix A and P and beginning data collection. Pyrene-actin fluorescence is excited at 365 nm and emission is detected around 407 nm. The overall excitation and emission of pyrene is actually rather broad, but the enhancement used to detect filament formation occurs only in narrow bands. We use 1 nm bandpass for excitation, and 5 nm bandpass for emission when using a PTI QuantaMaster fluorometer with monochromator based wavelength selection for both excitation and emission (*see* Note 11).
6. Begin preparing Mix A by pipetting the volume of actin stock needed to give a 4 μ M final concentration in 200 μ L into a 1.5 mL tube.
7. One tenth of the volume (of the actin stock) of 10E/1M is added to Mix A to exchange the Ca^{2+} for Mg^{2+} .
8. Finally, add the volume of buffer G-Mg needed to give a final volume of 100 μ L.
9. Mix by pipetting up and down 3 times and start a timer counting up.
10. Mix A is incubated at room temperature ($\sim 22^{\circ}\text{C}$) for 2 minutes. Two minutes is enough time for the exchange of calcium and magnesium bound to actin to come to complete. Any time between two minutes and one hour of exchange time will be fine, but the timing should be consistent between samples. If a different exchange time is desired, adjust the timing in **steps 14** and **15** accordingly.
11. Begin preparing Mix P by pipetting the volume of KMEI needed into a new 1.5 mL tube.
12. Add the appropriate volumes of the nucleation factors and additional proteins (*see step 4*), beginning with the nucleation factor (if used). For improved assay reproducibility, add proteins and additives in the same order and with the same timing and pipetting operations.

¹⁴The protocol presented here is for assay of actin polymerization in KMEI, a buffer with 50 mM KCl. Different pHs, concentrations of salts, or osmolytes can be used, but will result in substantial differences in actin polymerization kinetics. Thus, particular attention should be paid to normalizing the buffer across a given set of assays, and comparisons should typically only be made between samples under identical buffer conditions.

Despite this, some proteins may need to be prepared under different buffer conditions, and then corrected back to a standard condition in the assay. We address this problem by tracking the total concentrations of KCl, and of MgCl_2 , EGTA and imidazole pH 7.0. Do not account for differences in salt concentrations just by changing the volume of 10x KMEI added. Doing so will usually result in higher or lower concentrations of the rest of the KMEI components, and will perturb the kinetics. Instead we prepare all proteins in buffers with different concentrations of KCl, but matched concentrations of all other components. Then the final protein mixes are supplemented with 2 M KCl, or a KMEI buffer lacking any KCl, such that the final salt and other component concentrations are matched. Where necessary, a similar procedure is used for matching glycerol concentrations.

13. Add 10X KMEI to Mix P. The 10x KMEI accounts for the different buffer conditions in Mix A. Add enough 10x KMEI to bring the final salt concentration (after Mix A and P are combined) to 50 mM KCl, while maintaining the final imidazole, magnesium and EGTA concentrations at those of KMEI. If different salt conditions are used, it may also be necessary to add 2 M KCl and or KMEI lacking KCl to correct the salt while keeping the other buffer components constant. In that case, correct the 10x KMEI addition to account the volume of 2M KCl, which lacks imidazole, magnesium and EGTA (*see* Note 14).
14. With P-200 pipet set on 200 μ L pipet Mix P up and down 3 times being careful not to blow bubbles into the liquid. When the timer shows 1 minute 50 seconds, pull all of Mix P into the pipet tip.
15. At exactly 2 minutes (*see* Note 15) add all of Mix P (100 μ L) to the tube containing Mix A. Pipet up and down one time to mix, and then transfer total volume to clean cuvette (*see* Note 16).
16. Place the cuvette into the fluorometer and start data collection. Record the time elapsed between addition of Mix P to Mix A and the time data collection begins (*see* Note 15).
17. Record the time course for pyrene intensity (*see* Note 11 and Fig. 2) until a maximum intensity is reached and begins to descend again. Depending on the reaction conditions, this may take less than 100 s (expected for our example) or longer than 10000 s (*e.g.*, 1 μ M actin alone). For reactions with such different time scales, it may be necessary to alter the illumination method to reduce photobleaching. While short kinetic reactions can be illuminated continuously, longer time courses may benefit from illumination in 0.1–0.5 s intervals, separated by as long 10 s (*see* Note 11).
18. Repeat **steps 4** through **17** for each reaction desired.

3.3. Analysis of Pyrene Actin Polymerization Assays

Here we describe the calculation of several simple metrics: the time at which half of the fluorescence intensity change has occurred ($t_{1/2}$) (*see* **steps 1–6**), the actin polymerization rate at $t_{1/2}$ (*see* **steps 1–8**), the number of barbed ends present at $t_{1/2}$ (*see* **steps 1–10**), and the initial filament nucleation rate (*see* **steps 1–5, 7, 11**). The calculation of $t_{1/2}$ is the most resilient with noisy data, and does not require knowledge of actin critical concentration or

¹⁵As mentioned in **Section 3.2 steps 9, 10, 14** and **15** we perform actin polymerization assay pipetting events at exact times. This is not due to a 2 minute incubation timing being essential (although we have found having a carefully planned pipetting schedule does improve reproducibility), but because it facilitates tracking the delay between combining Mix A and P and beginning the data collection without requiring additional equipment. With the timer counting up, combine Mix P with Mix A when it reads 2 minutes (after preparation of Mix A), and record the delay time as the time shown on the timer when monitoring of the kinetics begins, minus the 2 minute delay.

¹⁶A clean cuvette is essential for high quality data. Before performing actin polymerization assays, clean the cuvette by filling it with concentrated nitric acid and allowing it to sit for at least 10 minutes. Discard the nitric and wash the cuvette thoroughly (7–10 times) with ultrapure water. Rinse the cuvette with ethanol and rapidly dry inside and outside the cuvette with compressed gas. After each reaction we rinse the cuvette 7 times with ultrapure water and the 3 times with ethanol before drying. Every second or third assay we also find it useful to treat with 30% bleach before the water wash. We recommend using just one cuvette for a set of assays, as different cuvettes will have different background scattering.

growth rate constants. The other three metrics inform more directly on the underlying biochemical activities, but require additional knowledge about actin biochemistry. Typically, only one of these metrics will be presented for a dataset. To simplify automation of the analysis process, our method emphasizes a work-flow that reduces human intervention (*see* Note 2).

1. Import the data into a spreadsheet or other computational environment (*see* Note 2).
2. Correct the time values for any known delay between the mixing of Mix A and Mix P and the first time point. This is the recorded time elapsed in **Section 3.2, step 16**. Time corrected fluorescence intensity data will be referred to as $f(t)$ below.
3. Plot the intensity data as a function of time, ideally comparing it to a series of control datasets to ensure that the data appears sensible, and does not show any obvious pathologies (excessive noise, spurious peaks etc.) (*see* Note 17).
4. Find the minimum intensity, $I_{\min}$, for the data set (*see* Fig. 3). In cases of slow kinetics, short dead-times, and high sampling rates, choose ten points that are near the minimum and average them. For faster kinetics and hand mixed samples with dead-times of greater than 30 s, any group of points will overestimate the minimum. Instead we use the minimum intensity data point. Alternatively, we make use of the fact that the minimum intensity should agree between datasets collected in the same session (Fig. 2). This allows us to use the slowest dataset in a group (typically actin alone) to estimate the minimum intensity for all datasets collected in a given session.
5. Find the maximum intensity, $I_{\max}$, for the data set (*see* Fig. 3). Find the ten highest intensity data points and record the associated times. Take the average of these times to estimate the time when the maximum intensity occurs (this reduces the cases where a single anomalous data point leads to incorrect identification of the maximum), retain this time at $t_{\max}$. Find the ten datapoints closest to $t_{\max}$, and take the average of their intensity. This average intensity is used as the maximum intensity, $I_{\max}$.
6. Calculate $t_{1/2}$ for the data set (*see* Fig. 3). First, exclude all datapoints at times greater than $t_{\max}$. Choose the fraction of the intensity change used to find $t_{1/2}$, which we term δ . δ is typically between 0.04 and 0.2. Collect the data points with intensities between $(0.5 - \delta/2) * (I_{\max} - I_{\min}) + I_{\min}$ and $(0.5 + \delta/2) * (I_{\max} - I_{\min}) + I_{\min}$. Using linear least squares, fit a line to these data points. Record the slope, $m_{t1/2}$, and intercept, $b_{t1/2}$, of the line. Visually inspect where these points fall in the dataset, and how the line fits to the considered points. $t_{1/2}$ is then found using:

$$t_{1/2} = (0.5 * (I_{\max} - I_{\min}) + I_{\min}) / m_{t1/2} \quad (\text{Equation 1})$$

¹⁷Random noise in these assays is on the order of 5% or less of total signal. Noise of this order is easily addressed using the methods described. Nonrandom, systematic noise will be poorly handled.

7. Rescale the deadtime corrected data from fluorescence intensity values into units of filament concentration. First, find the fluorescence intensity to actin filament concentration scaling factor, SF, for the data set. For this, one needs knowledge of the initial actin concentration, [Actin](0) and the critical concentration, c_0 . c_0 can be calculated from the on-rate constant, k_+ , and off rate constant, k_- , for filament assembly using:

$$c_0 = k_- / k_+ \quad (\text{Equation 2})$$

In KMEI at room temperature, the barbed ends of rabbit muscle actin elongate and shrink with values of $11.6 \mu\text{M}^{-1} \text{s}^{-1}$ and 1.4s^{-1} for k_+ and k_- , respectively (17). These rate constants are highly sensitive to solution conditions, particularly to the presence of salt and changes in viscosity (19). This allows calculation of the SF:

$$\text{SF} = ([\text{Actin}](0) - c_0) / (I_{\text{max}} - I_{\text{min}}) \quad (\text{Equation 3})$$

8. Calculate $AP_{t1/2}$, the rate of actin polymerization at $t_{1/2}$. Use $m_{t1/2}$, the slope of the line used to find $t_{1/2}$ in **step 6** (see Note 3), and SF, to calculate $AP_{t1/2}$:

$$AP_{t1/2} = \text{SF} * m_{t1/2} \quad (\text{Equation 4})$$

9. Find [Actin]($t_{1/2}$), the remaining free actin at $t_{1/2}$. This can be determined from values already calculated:

$$[\text{Actin}](t_{1/2}) = [\text{Actin}](0) - \text{SF} * ((m_{t1/2} * t_{1/2} - b_{t1/2}) - I_{\text{min}}) \quad (\text{Equation 5})$$

10. Find $BE_{t1/2}$, the number of filament barbed ends at $t_{1/2}$. This calculation recognizes that actin filaments grow at their ends and the rate constants for these processes are known in certain cases (*e.g.*, rabbit skeletal muscle actin polymerizing in KMEI buffer). The calculation ignores growth at the pointed end, as the barbed end grows much faster than the pointed end, and for Arp2/3 complex nucleated reactions, many pointed ends are capped by Arp2/3 complex. Thus, barbed end growth dominates the kinetics, and the rate law for actin polymerization reduces to:

$$d[\text{Filament}](t)/dt = k_+ * [\text{Actin}](t) * [\text{Barbed Ends}](t) - k_- * [\text{Barbed Ends}](t) \quad (\text{Equation 6})$$

Considering the rate law at $t_{1/2}$ we can solve for $BE_{t1/2}$ and substitute in the known values of actin polymerization and actin concentration, we then find $BE_{t1/2}$ using:

$$BE_{t1/2} = AP_{t1/2} / (k_+ * [\text{Actin}](t_{1/2}) - k_-) \quad (\text{Equation 7})$$

11. Find NR_0 , the initial nucleation rate. If there is a constant rate of nucleation of actin filaments through the initial portion of the reaction, then filament end concentration will grow linearly with time. This is true in some systems, and may be more directly related to the nucleation factor activity than the other metrics (see Note

18). By limiting our analysis to times where the concentration of free actin has not changed much from the initial concentration, we can directly fit the data with an approximate solution to the time dependence of the system (*see* Note 19).

Choose a fraction of the intensity change to use in determining the nucleation rate, which we refer to as γ . From the time corrected dataset (*see* **step 2**), select those data points that have intensity values between $I_{\min}$ and $\gamma * (I_{\min} - I_{\min}) + I_{\min}$, where γ is between 0.05 and 0.2 (typically 0.1 is used). Using linear least squares fitting, fit the selected data to:

$$f(t) = A * t^2 + B * t + C \quad (\text{Equation 8})$$

Visually inspect the quality of the fit to the data (*see* Fig. 3). B should be near zero and C should be near $I_{\min}$ (*see* Notes 18 and 19). Repeating the fit with slightly different values of γ (*e.g.*, 0.06, 0.08, 0.1 and 0.12) should not result in substantially different values of A, B or C. The nucleation rate is then calculated as:

$$NR_0 = (2 * A * SF) / (k_+ * [\text{Actin}](0) - k_-) \quad (\text{Equation 9})$$

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¹⁸If B is not near zero and C is different from $I_{\min}$, examine the fit. If it is of poor quality a fixed nucleation rate may be a poor metric for analysis. If the fit is of good quality, check the results for an actin alone control dataset from the same day, ensure there is not extensive evidence of seed filament.

We note that this approximation is not expected to hold once a significant portion of actin monomer has been consumed to form filaments, nucleation should slow as actin monomers are depleted from solution. Thus, only a small fraction of the signal change should be fit in this way. The fraction used will depend somewhat on the signal to noise in the data. When analyzing Arp2/3 complex dependent actin polymerization reactions, we also note that Arp2/3 complex requires binding to an existing filament in order to nucleate a new filament. Thus, depending on the affinity of Arp2/3 complex for filament, the nucleation rate may accelerate as filament accumulates. This latter effect is noticeable in Fig. 3, the curve extrapolated through the data underestimates the intensity at longer times.

9. Reymann AC, Boujemaa-Paterski R, Martiel JL, Guerin C, Cao W, Chin HF, De La Cruz EM,

¹⁹Assuming that growth is dominated by growth at the barbed ends, we have the rate law for the increase of actin polymerization from **Section 3.3 step 10**:

$$d[\text{Filament}](t)/dt = k_+ * [\text{Actin}](t) * [\text{Barbed Ends}](t) - k_- * [\text{Barbed Ends}](t) \quad (\text{Equation 6})$$

We limit our analysis to the region of the portion of the data where little of the actin has been consumed. Thus:

$$[\text{Actin}](t) \sim [\text{Actin}](0) \quad (\text{Equation 10})$$

Interpreting the data in terms of a single nucleation rate means that the concentration of barbed ends grows linearly with time, with the rate of nucleation being NR_0 .

$$[\text{Barbed Ends}](t) = NR_0 * t + [\text{Barbed Ends}](0) \quad (\text{Equation 11})$$

By substituting this growth of barbed ends (Equation 11), and the actin concentration approximation (Equation 10) into the rate law for filament growth (Equation 6) we find:

$$d[\text{Filament}](t)/dt = (k_+ * [\text{Actin}](0) - k_-) (NR_0 * t + [\text{Barbed Ends}](0)) \quad (\text{Equation 12})$$

To simplify, we substitute:

$$\kappa = (k_+ * [\text{Actin}](0) - k_-) \quad (\text{Equation 13})$$

into Equation 12 and find:

$$d[\text{Filament}](t)/dt = \kappa * (NR_0 * t + [\text{Barbed Ends}](0)) \quad (\text{Equation 14})$$

Integrating Equation 14 with respect to time we find that filament concentration (in monomer actin units incorporated) over time is:

$$[\text{Filament}](t) = 1/2 * \kappa * NR_0 * t^2 + \kappa * [\text{Barbed Ends}](0) * t + [\text{Filament}](0) \quad (\text{Equation 15})$$

Assuming the fluorescence intensity reports directly on the total concentration of monomers incorporated into filament, the data will follow:

$$f(t) = [\text{Filament}](t) / SF + I_{\min} \quad (\text{Equation 16})$$

We have fit the data (*see Section 3.3 step 11*) where the actin concentration is near the initial concentration to:

$$f(t) = A * t^2 + B * t + C \quad (\text{Equation 8})$$

We substitute Equations 16 into Equation 8, removing $f(t)$:

$$[\text{Filament}](t) / SF + I_{\min} = A * t^2 + B * t + C \quad (\text{Equation 17})$$

Substituting the expression for $[\text{Filament}](t)$ in Equation 15 into Equation 17 and find:

$$(1/2 * \kappa * NR_0 * t^2 + \kappa * [\text{Barbed Ends}](0) * t + [\text{Filament}](0)) / SF + I_{\min} = A * t^2 + B * t + C \quad (\text{Equation 18})$$

By subtracting the righthand side from the left hand side of Equation 18, then grouping the constant, t , and t^2 terms, and solving for A , B and C we find:

$$A = 1/2 * \kappa * NR_0 / SF \quad (\text{Equation 19})$$

$$B = \kappa * [\text{Barbed Ends}](0) / SF \quad (\text{Equation 20})$$

$$C = [\text{Filament}](0) / SF + I_{\min} \quad (\text{Equation 21})$$

If we assume that upon dilution of actin into salt, there are no barbed ends or filament initially (that $[\text{Barbed Ends}](0) = [\text{Filament}](0) = 0$) we find that B should be 0 and C should be $I_{\min}$

Substituting for κ (using Equation 13) in Equation 19, and solving for NR_0 , we find:

$$NR_0 = (2 * A * SF) / (k_+ * [\text{Actin}](0) - k_-) \quad (\text{Equation 9})$$

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Fig. 1.

Gel filtration chromatogram for pyrene labeled actin. An example of the UV absorbance detected chromatogram for pyrene labeled actin purification using a 320 mL Superdex 200 pg column. UV absorbance is shown with a thick black line. Fractions collected are shown with short vertical lines. Relevant fraction numbers are shown. The shape of the chromatographic peak is characteristic for actin purified using Superdex 200, and running the column in buffer G. For this purification, fractions 12, 13 and 14 were retained, and fractions 13 and 14 were used preferentially.

Fig. 2.

Pyrene actin polymerization assay example. 4 μ M actin, 5% pyrene labeled is brought from buffer G-Mg conditions to KMEI conditions to trigger polymerization of actin. Pyrene fluorescence intensity (Y-axis) as a function of time (X-axis) are shown for actin alone (thin line), actin in the presence of 10 nM bovine Arp2/3 complex (thick line) and actin in the presence of 10 nM bovine Arp2/3 complex and 250 nM N-WASP VCA (dashed line). These three curves are the controls for the basic behavior of actin and Arp2/3 complex.

Fig. 3.

Graphical representation of the analysis of pyrene actin polymerization data. 4 μ M rabbit muscle actin (5% pyrene labeled) is induced to polymerize by shifting to KMEI conditions (*see* **Section 3.2**) and adding 10 nM bovine Arp2/3 complex and 40 nM of N-terminally modified WASP VCA. This results in nucleation of actin filaments. Raw data is shown as a thin black line.

Shown are the aspects of the data used to calculate $t_{1/2}$, actin polymerization rate at $t_{1/2}$, barbed ends at $t_{1/2}$ and the initial nucleation rate. The minimum and maximum intensities are shown as horizontal lines. Enlarged purple points were averaged to determine the maximum intensity, these are also bracketed for clarity. Enlarged green points were used to find $t_{1/2}$ and to find the slope at $t_{1/2}$, these are also bracketed for clarity. Enlarged black points were used to find the initial nucleation rate, these are also bracketed for clarity. The determined value for $t_{1/2}$ is shown with a vertical line. The line fit to find $t_{1/2}$ and the slope at $t_{1/2}$ is shown as a thick, dashed, cyan line. The parabola fit to find the initial nucleation rate is shown as a thin, dashed, red line. Note that the parabola has a slight negative slope at $t = 0$, and undershoots the raw data at later time points, both are indications that a single nucleation rate is not a good metric for this type of data.