# Microfluidics-Assisted TIRF Imaging to Study Single Actin Filament Dynamics

Shashank Shekhar[1]

[1]Department of Biochemistry, Rosenstiel Basic Medical Science Research Center, Brandeis University, Waltham, Massachusetts

Dynamic assembly of actin filaments is essential for many cellular processes. The rates of assembly and disassembly of actin filaments are intricately controlled by regulatory proteins that interact with the ends and the sides of filaments and with actin monomers. TIRF-based single-filament imaging techniques have proven instrumental in uncovering mechanisms of actin regulation. In this unit, novel single-filament approaches using microfluidics-assisted TIRF imaging are described. These methods can be used to grow anchored actin filaments aligned in a flow, thus making the analysis much easier as compared to open flow cell approaches. The microfluidic nature of the system also enables rapid change of biochemical conditions and allows simultaneous imaging of a large number of actin filaments. Support protocols for preparing microfluidic chambers and purifying spectrin-actin seeds used for nucleating anchored filaments are also described. © 2017 by John Wiley & Sons, Inc.

Keywords: Actin dynamics • microfluidics • TIRF • single-filament imaging • actin depolymerization

**How to cite this article:**
Shekhar, S. (2017). Microfluidics-assisted TIRF imaging to study single actin filament dynamics. *Current Protocols in Cell Biology*, *77*, 12.13.1–12.13.24. doi: 10.1002/cpcb.31

## INTRODUCTION

The dynamic nature of the actin cytoskeleton lies at the heart of a number of critical cellular processes including cell migration, phagocytosis, cytokinesis and wound healing (Carlier & Shekhar, 2017; Carlier et al., 2015). Depending on the specific cellular processes, cells organize actin filaments either into dendritic branched structures or into linear bundles. While dendritic filament arrays are predominantly found in the lamellipodia of migrating cells, filament bundles are found in organelles such as filopodia. Branched actin structures are formed by autocatalytic branching of actin filaments by the nucleator Arp2/3, and linear arrays are initiated by proteins of the formin and Ena/VASP family (Carlier & Shekhar, 2017). Both the size and shape of these filament structures are tightly controlled by a large array of regulatory proteins that interact either with actin filaments or actin monomers. Many of these proteins directly bind filament barbed ends and alter their rate of growth. For example, formins bind and processively track barbed ends to accelerate filament growth (Chesarone, DuPage, & Goode, 2010; Kovar, Harris, Mahaffy, Higgs, & Pollard, 2006; Romero et al., 2004), and cappers (e.g., capping protein and gelsolin) bind growing barbed ends to arrest filament growth (Edwards et al., 2014).

While *in vivo* imaging and cellular perturbation methods have been key to identifying important molecular players required for actin assembly in cells, *in vitro* approaches have played a significant role in gaining a mechanistic understanding of how these players control actin assembly. Traditionally, bulk assays (e.g., light scattering, fluorescence intensity, and anisotropy) have been used to characterize the activity of individual actin



*Current Protocols in Cell Biology* 12.13.1–12.13.24, December 2017
Published online December 2017 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpcb.31

regulatory proteins. One of the most common such techniques used by actin biochemists is the "pyrene assay," which exploits the 20-fold increase in the intensity of pyrenyl-labeled actin upon actin polymerization when compared to monomeric actin (Kouyama & Mihashi, 1981). Though bulk approaches have enabled characterization of kinetic parameters of actin regulatory proteins, they suffer from a number of limitations. For example, the pyrene assay only measures polymer formation, and separating actin nucleation, elongation, and annealing can be extremely complicated. Moreover, the parameters measured from most bulk approaches are based on the assumption that all reactions occurring in the sample are identical—i.e., there is no heterogeneity. Interpretation of bulk results can also depend heavily on the mathematical models used. Additionally, some proteins like the actin depolymerizing factor (ADF) can alter the emission properties of fluorophores bound to actin monomers (Carlier et al., 1997). Given these limitations, there exists a need to be able to directly visualize and quantify individual reactions occurring at the level of individual actin filaments.

Daniel Axelrod first used the phenomenon of Total Internal Reflection Fluorescence (TIRF) in a microscope to study cellular focal adhesions in 1981 (Axelrod, 1981). This was then followed by the milestone study by Yanagida and colleagues to detect individual ATP turnover reactions by myosin molecules in an aqueous environment (Funatsu, Harada, Tokunaga, Saito, & Yanagida, 1995). Real-time actin filament branching was first visualized by TIRF in 2001 (Amann & Pollard, 2001), followed by the measurement of kinetic rate constants for assembly and disassembly of pure actin filaments (Kuhn & Pollard, 2005). Individual formins tracking a growing barbed end of an actin filament and (Paul & Pollard, 2009) and Arp2/3 initiating a new filament branch on an existing mother filament (Smith, Daugherty-Clarke, Goode, & Gelles, 2013) have since been observed by combining TIRF with single-molecule imaging. Although, these approaches have enabled direct visualization of individual protein-protein reactions, TIRF imaging requires objects remain at most 150 to 200 nm above the glass surface, in the so-called "evanescent field". Actin filaments are often kept in the evanescent field either by physically anchoring them sparsely along their length to the coverslip surface (e.g., using biotinylated actin monomers or inactivated N-ethylmaleimide-myosin) or by using crowding agents (e.g., dextran and methylcellulose) that push the filaments into the evanescent field. The proximity of anchored filaments to the glass surface can lead to artifacts in filament dynamics and potential limit the availability of anchored subunits that interact with filament sides. Using dextran and methylcellulose can lead to filament bundling and overcrowding of filaments in the evanescent field. Too many overlapping filaments can make the post-acquisition analysis very difficult. To overcome some of these drawbacks, a new approach, inspired by single-molecule studies of DNA (Brewer & Bianco, 2008), has been developed that combines TIRF and microfluidics.

The microfluidics approach was first employed by Antoine Jégou and Guillaume Romet-Lemonne of the Carlier group (Jegou et al., 2011) to study actin dynamics and characterize phosphate release from actin monomers in an actin filament. This approach has since been used to study barbed-end and pointed-end dynamics of single actin filaments, as well as to study mechanosensitivity of formins (Jegou, Carlier, & Romet-Lemonne, 2013; Pernier et al., 2013; Shekhar & Carlier, 2017; Shekhar et al., 2015). Briefly, actin filaments are initiated from filament nucleators anchored to the glass surface exposed to a flow containing actin monomers. Depending upon the reaction of interest, this approach can be used to either grow filaments with free pointed ends (Shekhar & Carlier, 2017) or free barbed ends (Jegou et al., 2011). The main advantages of this method are the ability to rapidly change the biochemical conditions to which filaments are exposed, and filaments align along the direction of the flow (Figure 12.13.1). By pressing the filaments on the coverslip surface, the flow also keeps them in the evanescent field, thus eliminating the need to use crowding agents or to anchor filaments along their length. Changes in the

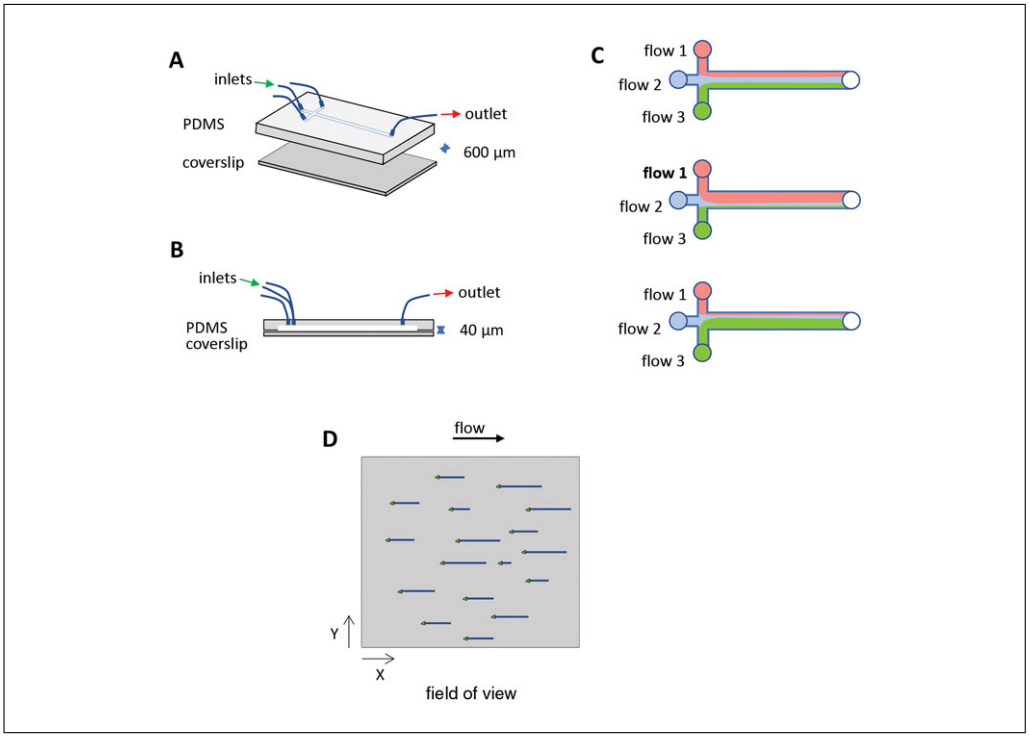


**Figure 12.13.1** Schematic representation of the setup used for microfluidics-assisted single filament TIRF imaging (adapted from Jegou et al., 2011). **(A)** Top-view of the microfluidic flow cell assembled by attaching a PDMS microchamber on a plasma-cleaned glass coverslip. The microchamber has three inlet ports and one outlet port. Liquids of specific biochemical compositions stored in external reservoirs can be introduced into the flow cell via the inlet ports. Actin filaments are initiated from seeds anchored to the coverslip. Width of the main channel is 600 µm. **(B)** Side view of the assembled PDMS flow cell. The internal height of the PDMS microchamber is about 40 µm. **(C)** Depending upon the experimental conditions required, the input flow to which the anchored filaments are exposed can be rapidly altered (<1 s). The amount of pressure applied to the reservoirs defines the dominant flow cell: Top, all equal; Middle, flow 1 is dominant; Bottom, flow 3 is dominant. **(D)** Schematic representation of a field of view of anchored filaments aligned in the flow.

rate of elongation or depolymerization of the filaments are used to detect bound ligand, thus eliminating the need to separately label the ligand. Elongation rate changes can be used to characterize the underlying kinetic mechanisms. Microfluidics-assisted TIRF imaging has so far been successfully employed to study: nucleotide hydrolysis when a monomer incorporates into the actin filament (Jegou et al., 2011), the effect of tension on formin-elongation of an actin filament (Jegou et al., 2013), formation of ternary complex between formin and capping protein at the filament barbed end (Shekhar et al., 2015), direct visualization of enhanced filament depolymerization by actin depolymerizing factor (ADF), the funneling effect of capping protein (CP), and direct measurement of steady-state monomer concentration (Shekhar & Carlier, 2017).

Here I describe the detailed protocols for using microfluidics-assisted TIRF imaging for single actin filament studies. I first describe the preparation of the PDMS microchambers used in this assay (Basic Protocol 1), followed by a protocol for initiating surface-anchored filaments with their free barbed exposed to the flow (Basic Protocol 2). Kinetic characterization of a barbed-end binding regulatory protein is described, using capping protein as an example (Basic Protocol 3). Using microfluidics-TIRF approach to directly measure the steady-state concentration of actin monomers due to presence of a specific regulatory protein is then described (Basic Protocol 4). Lastly, I describe the steps for initiating actin filaments with free pointed ends from anchored gelsolin-actin seeds

(Basic Protocol 5), and how the setup is used to study filament severing and rapid depolymerization of free pointed ends by actin depolymerizing factor (Basic Protocol 6).

***BASIC PROTOCOL 1***

## PREPARATION OF PDMS MICROCHAMBERS

Polydimethylsiloxane (PDMS) has been instrumental in the development of microelectromechanical systems for lithography technologies over the past few decades. PDMS can be used to rapidly prepare microchambers by pouring and curing liquid PDMS base solution on a mold containing the desired microchamber design. The molds for PDMS casting can either be commercially ordered or be prepared in house at a cleanroom facility. The design used in this protocol contains three inlets and one outlet (Figure 12.13.1). The channel width is about 600 µm and the channel height is about 40 µm (Figure 12.13.1). These microchambers are then attached to glass coverslips to form a microfluidic flow cell. The 40-µm high roof ensures that the autofluorescence from PDMS does not contribute to background noise while imaging in TIRF. PDMS microchambers can be reused once they are adequately cleaned (See Support Protocol 4), and fresh microchambers only need to be prepared if existing microchambers do not bind coverslips well.

### *Materials*

Sylgard 184 Silicone Elastomer Kit (PDMS base & curing agent)
Molds for PDMS casting (commercially ordered)
100% ethanol

100-ml glass beaker
Stirring spatula
Desiccator (BEL-ART)
Vacuum pump
Plastic Petri dish
Oven (70°C)
Scalpel
PDMS puncher (Elveflow, France)
Kimwipe tissues
Parafilm

1. Place the glass beaker on a balance, and measure 50 g PDMS base into beaker.
2. Add 5 g of curing agent over the PDMS base.

   *The amount of curing agent needs to be about 1/10$^{th}$ of the amount of PDMS base added in the prior step. It is important to maintain this 1:10 ratio between the curing agent and PDMS base. Adding a higher proportion of the curing agent will lead to extremely stiff (and brittle) PDMS microchambers, and a lower proportion of curing agent will lead to inadequate curing of the PDMS.*

3. Mix the solution well by vigorously whisking the mixture with a spatula for at least 10 min.

   *Thorough mixing is needed to make sure that the curing agent is uniformly distributed throughout the PDMS base. This ensures uniformly crosslinked PDMS.*

4. Place the beaker containing the PDMS mixture into a desiccator connected to a vacuum pump, and degas for about one hour to remove the air bubbles created during mixing.
5. Place the mold in the center of a petri dish. Pour PDMS solution into the mold.
6. Place the petri dish in a preheated oven at 70°C, and let the PDMS cure overnight.

7. The next morning, gently press on the surface to make sure the PDMS cured uniformly.
8. Cut out a PDMS microchamber with a scalpel.
9. Place the PDMS microchamber on a clean surface channel-side facing up. Carefully create the inlets and outlets by punching holes in the PDMS using the puncher.
10. Clean the PDMS microchambers with KimWipes tissues dipped in 100% ethanol to remove any contaminants and PDMS debris.
11. Air dry the ethanol from the microchambers, and place the chambers in a new petri dish. Seal the petri dish with parafilm to prevent contamination.

## GROWING ANCHORED FILAMENTS WITH FREE BARBED ENDS

***BASIC PROTOCOL 2***

A majority of proteins that regulate the assembly of actin cytoskeleton interact with filament barbed ends. To study this at the level of individual actin filaments, actin filaments with free barbed ends can be grown under flow while anchored on the coverslip surface of the microfluidic flow cell. Filaments initiated from spectrin-actin seeds grow with their pointed ends anchored to coverslip surface via the spectrin-actin seeds and barbed ends free in the flow (Figure 12.13.2). The rate of elongation of these barbed ends can be used as a readout for the biochemical composition of the flowing liquid.

### *Materials*

1 mg/ml PLL-PEG solution (see recipe)
TIRF buffer (see recipe)
Spectrin-actin seeds (see Support Protocol 3)
10% (w/v) BSA solution (see recipe)
Purified G-actin, 10% Alexa 488-labeled (See Support Protocol 2)

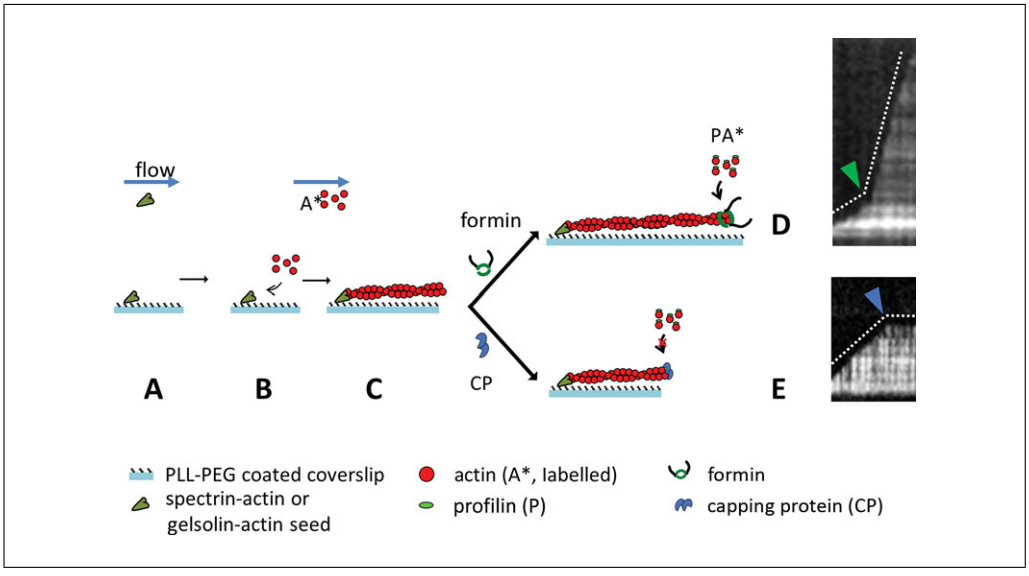


**Figure 12.13.2** Experimental setup for growing filaments from spectrin-actin seeds or Gelsolin-actin seeds anchored on the glass coverslips under flow. **(A)** Spectrin-actin or gelsolin-actin seeds are anchored on a PLL-PEG coverslip. **(B-C)** Actin filaments are initiated by exposing the seeds to actin monomers. Gelsolin-actin seeds initiate filaments with free distal pointed ends, and spectrin-actin seeds initiate filaments with free barbed ends. **(D)** Free barbed ends sequentially exposed to a flow containing formins and profilin-actin exhibit accelerated elongation. A representative kymograph shows the increased elongation upon formin binding (green arrow). **(E)** Free barbed ends sequentially exposed to capping protein and profilin-actin exhibit arrested growth. A representative kymograph shows the arrested elongation upon CP binding (blue arrow).

Purified profilin

Pre-cleaned coverslips (see Support Protocol 1)
Plasma cleaner (oxygen plasma)
PDMS microchambers (Basic Protocol 1)
Compressed nitrogen/air
20-µl pipette
Microscrew reservoir tubes
Maesflow microfluidic flow-control system (Fluigent, France)

***Flow cell preparation***

1. Dry a pre-cleaned coverslip using a stream of compressed nitrogen air.
2. Place the coverslip in a plasma cleaner, and expose to oxygen plasma under vacuum for about 3 min.

   *Plasma cleaning has three effects. Firstly, it etches away any contaminants that might be present on the coverslip surface. Secondly, it enhances the surface binding of long-chain PLL-PEG molecules that form a hydrophilic brush over the surface and reduce non-specific protein binding. Thirdly, plasma cleaning enables good sealing of the PDMS microchambers on the glass coverslip. This ensures that the seals do not leak during the experiment.*

3. Rinse the PDMS microchamber with 100% ethanol, and dry it with a nitrogen air stream. Immediately place the plasma-cleaned coverslip on the PDMS microchamber, and press gently to seal the gap between the two. This microchamber-coverslip system is referred to as the "flow cell."

   *If the PDMS is too old, the microchamber won't seal well against the coverslip. New PDMS microchambers should then be prepared.*

4. Filter all buffers that will be introduced into the flow cell (with a 0.2 µm filter). Degas all filtered buffers (except PLL-PEG) for at least 30 min in a desiccator connected to a vacuum pump.
5. Insert the tip of a pipet containing 20 µl PLL-PEG solution into the outlet of the flow cell, and inject the solution into the flow cell.
6. Gently remove the pipet tip from the outlet, leaving behind a small drop of PLL-PEG at the outlet.
7. Incubate 15 min to let the PLL-PEG passivate the surface.
8. Place the microfluidic flow cell on a TIRF microscope stage.
9. Fill 4 reservoir tubes with about 300 µl of TIRF buffer, and tighten them to the Maesflow flow system.

   *We use a commercially available integrated flow system to control the flow in the microfluidic flow cell. Alternatively, traditional syringe-based flow systems can also be used, but they are much more cumbersome in coordinating and controlling multiple flows.*

10. Connect the reservoir tubing into the outlet channel, and push the liquid through.
11. Continue flowing TIRF buffer into the flow cell through the outlet channel for about 3 min to rinse away unbound PLL-PEG in the chamber.
12. Connect the reservoir tubing to the three inlets of the flow cell, making sure not to introduce air bubbles into the flow cell.

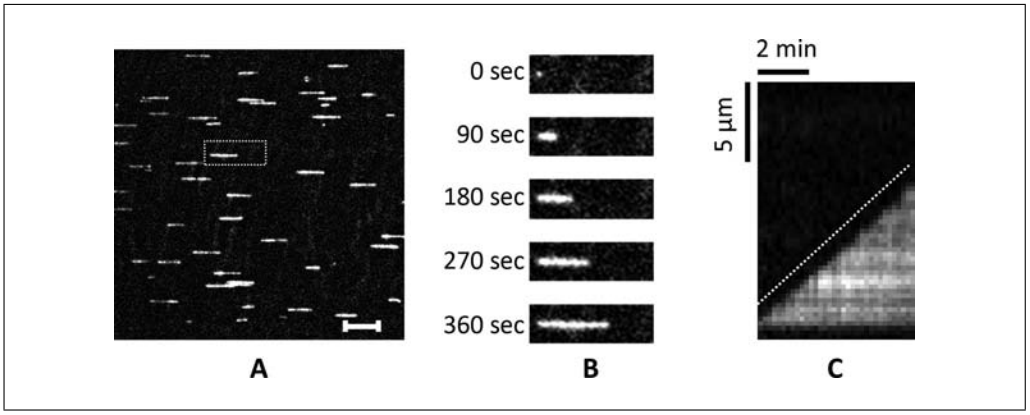


**Figure 12.13.3** Direct visualization of actin filaments growing in the microfluidic chamber. **(A)** TIRF image of actin filaments initiated from spectrin-actin seeds with their freely growing barbed ends aligned in the flow. Scale bar, 10 µm. **(B)** Time-lapse images of a filament (highlighted with the dotted white rectangle in **A)** growing under flow containing 1 µM actin (10% Alexa 488-labeled) and 4 µM profilin in TIRF buffer. **(C)** Kymograph of the same filament. The dotted line indicates the slope from which the rate of elongation of the filament can be determined.

13. Prepare a 50 pM solution of spectrin-actin seeds in TIRF buffer and inject into the flow cell using one of the inlet channels. Continue injection for 5 min.

    *Spectrin-actin seeds bind PLL-PEG-coated surfaces non-specifically.*

14. Rinse the tubing and the flow cell with TIRF buffer for 5 min to wash away unbound spectrin-actin seeds.

15. Prepare 2% BSA in TIRF buffer, and inject into the flow cell for 5 min.

    *BSA solution is used to passivate any remaining unbound sites on the glass surface.*

16. Rinse again with TIRF buffer for 5 min.

17. Prepare a profilin-actin solution containing 1 µM G-actin (10% Alexa 488-labeled) and 4 µM profilin in TIRF buffer and 0.1% BSA.

    *Profilin binding to actin monomers prevents spontaneous nucleation of filaments in the tube. Profilin-actin monomers can only bind free barbed ends. Therefore, the presence of profilin ensures that no polymerization occurs in the reservoir tubes and that the monomeric actin pool is not depleted.*

18. Inject profilin-actin solution into the flow cell via one of the inlet channels, and start imaging in TIRF immediately.

    *Depending upon the length of the tubing between the reservoir tubes and the microfluidic flow cell, the liquid takes a few minutes to reach the chamber. The dead volume is about 50 µl for each meter of tubing.*

19. Profilin-actin monomers that bind anchored spectrin-actin seeds will produce a large number of actin filaments growing aligned in the flow (Figure 12.13.3A).

20. Continue injecting profilin-actin until filaments reach the required length. Rate of filament elongation is defined by (Figure 12.13.3B,C):

$$V = k_+^B.\left([PA] - C_C^B\right)$$

where $k_+^B = 10\ s^{-1}\ \mu M^{-1}$ is the on-rate of profilin-actin monomers at the barbed end; $C_C^B = 0.08$ µM is the critical concentration at the barbed end; and [PA] is the concentration of profilin-actin monomers.

*BASIC PROTOCOL 3*

## CHARACTERIZING THE INTERACTION OF REGULATORY PROTEINS AT THE BARBED ENDS

The rate of elongation of an actin filament is defined by the regulatory proteins bound to its barbed end. For example, formins like mDia1 can bind and processively track barbed ends (Figure 12.13.2D). In the presence of profilin-actin, formins greatly accelerate the rate of filament elongation (Chesarone et al., 2010). Capping protein on the other hand tightly binds filament barbed ends ($K_D$ = 0.1 nM) and blocks further monomer addition and resulting growth (Figure 12.13.2E; Edwards et al., 2014). The rate of filament elongation can therefore provide very useful hints about the nature of the ligand bound to the barbed end. Formins and capping protein have recently been shown to simultaneously bind barbed ends to form a ternary complex (Bombardier et al., 2015; Shekhar et al., 2015). Microfluidics-assisted TIRF imaging enables relatively rapid and direct measurement of kinetic parameters of filament elongation by barbed-end interacting proteins. As a simple example, this protocol describes how to determine the association rate constant ($k_{on}$) and dissociation rate constants ($k_{off}$) of capping protein to filament barbed ends. From these rates, the equilibrium dissociation constant ($K_D$) can easily be determined.

***Materials*** *(also see Basic Protocol 2)*

Purified capping protein

The association rate constant ($k_{on}$) of capping protein for barbed ends is determined as follows.

1. Prepare a PDMS flow cell, passivate the surface with PLL-PEG, and anchor spectrin-actin seeds on the coverslip surface, as described in Basic Protocol 2.
2. Prepare two solutions for different biochemical conditions.
   a. Reservoir 1: Prepare a solution containing 1 μM actin (10% Alexa 488-labeled) and 4 μM profilin in TIRF buffer.
   b. Reservoir 2: Prepare a solution containing the same profilin-actin concentration as in reservoir 1, but supplement with 500 pM (final) capping protein.
3. Initiate surface-anchored actin filaments with free barbed ends by flowing in solution from reservoir 1.

   *Free barbed ends can now be exposed to a solution containing a barbed-end regulator at a given concentration. If the protein of interest changes the rate of elongation of the barbed end (i.e., either increases or pauses filament elongation), the change in elongation rate can be considered as a phenotype of protein binding. Alternatively, the protein of interest can be fluorescently labeled, and its appearance at the barbed end can be visualized directly by single-molecule imaging.*
4. Switch pressure to expose the filaments to flow from reservoir 2 containing profilin-actin and capping protein.

   *In presence of capping protein, growing filaments will begin to arrest.*
5. Continue flowing reservoir 2 until capping protein caps all the filaments in the field-of-view and stops their growth.
6. Draw a kymograph for all filaments, and identify the time point at which each filament arrests (see Support Protocol 5).
7. Graph the fraction of filaments paused as a function of time (with $t = 0$ being the time capping protein flow was turned on). Include at least 80 to 100 filaments in the analysis.

*This time-based representation of the rate of occurrence of individual events (capping in this case) is referred to as the Cumulative Distribution Function (CDF).*

8. Fit the CDF to a single-exponential function $y = 1-\exp(-k_{obs}\cdot t)$, where $t$ is the time and $k_{obs}$ is the rate measured from the fit and is referred to as the observed association rate constant.
9. Repeat steps 1 to 8 for at least 3 other concentrations of capping protein.
10. Plot $k_{obs}$ as a function of capping protein concentration, and determine the association rate constant $k_{+C}$ as the slope of the linear increase in $k_{obs}$ with capping protein concentration.

    *To measure the dissociation rate constant ($k_{off}$) of capping protein from capped barbed ends, the evolution of a large population of capped filaments is recorded. The rate at which these filaments resume elongation is referred to the as the dissociation rate constant ($k_{off}$).*

11. Fragment all filaments in the field of view by continuously exposing the field of view to the TIRF laser.

    *New filaments can now be initiated.*

12. Prepare 2 different solutions.

    a. Reservoir 1: Prepare a solution containing 1 μM actin (10% Alexa 488-labeled) and 4 μM profilin in TIRF buffer.
    b. Reservoir 2: Prepare a solution containing the same profilin-actin concentration as in reservoir 1, but supplement with a high concentration of capping protein (~10 nM) to ensure rapid capping of barbed ends.

13. Grow surface-anchored actin filaments with free barbed ends by flowing in the profilin-actin solution from reservoir 1.
14. Choose a field of view containing 80 to 100 filaments. Switch flows to the solution in reservoir 2 to expose growing ends to a high concentration of capping protein.

    *The high concentration of capping protein should rapidly cap all filaments in the field of view and stop growth.*

15. Once all filaments stop growing, switch the flow back to reservoir 1 (profiling-actin without capping protein).
16. Record the time point at which each paused filament resumes elongation.
17. Graph (CDF) the fraction of paused filaments that resume elongation as a function of time (with $t = 0$ being the time at which flow 1 was re-introduced).
18. Determine the dissociation rate constant ($k_{off}$) for capping protein release from barbed ends by fitting the CDF to an exponential fit.
19. Determine the equilibrium dissociation constant, $K_D = k_{off}/k_{on}$.

    *This protocol can be used to measure the rate constants for binding of other proteins to the barbed ends if binding changes actin filament elongation or depolymerization or if a labeled ligand binds the barbed end.*

## MEASURING THE CONCENTRATION OF STEADY-STATE ACTIN MONOMERS

***BASIC PROTOCOL 4***

A high concentration of polymerizable actin monomers is the key determinant in the ability of actin filaments barbed ends to grow rapidly and apply force against a surface

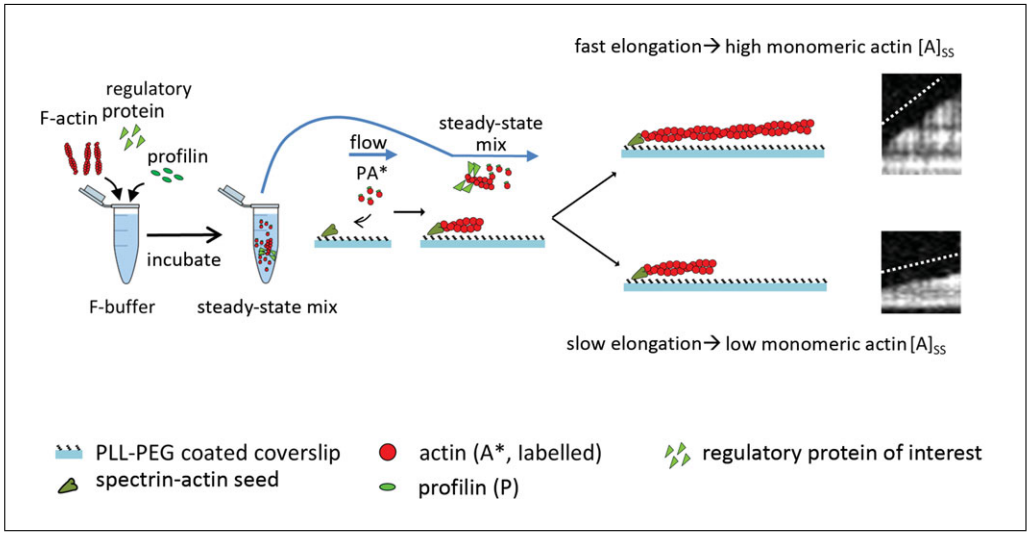


**Figure 12.13.4** Experimental setup for measuring the amount of steady-state actin monomers using microfluidics-assisted TIRF imaging. Tracer barbed ends grown from surface-anchored spectrin-actin seeds are exposed to a flow containing solutions of pre-assembled F-actin, profilin, and varying amounts of the protein of interest at steady state. The rate of elongation of tracer barbed ends indicates the amount of steady-state actin monomers. Representative kymographs for high and low steady-state monomer concentration are shown. The dotted lines indicate the slope from which the rate of elongation of the filaments can be determined (reproduced from Shekhar & Carlier, 2017).

(e.g., plasma membrane). A number of proteins have been implicated in the cell's ability to maintain a large pool of polymerizable monomers, including actin depolymerizing factor (ADF), capping protein (CP), and thymosin β4. So far, the effect of a protein on the steady state monomer concentration has mainly been studied by bulk solution assays—i.e., SDS-PAGE of supernatants from high speed centrifugation of F-actin. In these assays, polymerized actin is expected to pellet, but actin monomers should remain in the supernatant. Very short filaments might not pellet during high-speed sedimentation, however, causing an over-estimation of monomeric actin in the supernatant.

Microfluidics-assisted TIRF imaging has recently been exploited to directly measure the change in concentration of polymerizable monomers due to actin regulatory proteins (Shekhar & Carlier, 2017). A solution containing F-actin along with the protein of interest is allowed to reach steady state and then flowed in over "tracer" filament barbed ends anchored on the surface. The rate of elongation of these tracer filament barbed ends can be used to determine the amount of polymerizable monomers in the flow—i.e., the effect of the regulatory protein on the steady-state concentration of polymerizable monomers (See Figure 12.13.4).

***Materials*** *(also see Basic Protocol 2)*

F-actin solution (10% Alexa 488-labeled)
Profilin
Regulatory proteins of interest (e.g., ADF, CP), purified as described in Shekhar and Carlier (2017) or purchased from Cytoskeleton Inc.

1. Mix 5 μM F-actin (10% Alexa 488-labeled) with 3 μM profilin with a given concentration of protein of interest (e.g., ADF or CP) in TIRF buffer.

   *Actin monomers complexed with profilin (i.e., profilin-actin) can only bind filament barbed ends.*

2. Incubate the solution at 4°C for until it reaches steady state.

*The time required to reach steady-state differs depending upon the regulatory protein added to the mix. In presence of capping protein, overnight incubation is required. In presence of ADF, however, steady-state is reached much faster (~2 hr).*

3. Prepare a PDMS flow cell, passivate the surface with PLL-PEG, and anchor spectrin-actin seeds on the coverslip surface, as described in Basic Protocol 2.
4. Prepare two flow solutions:
   a. Reservoir 1: Prepare a solution containing 1 μM G-actin (10% Alexa 488-labeled) and 4 μM profilin in TIRF buffer.
   b. Reservoir 2: Pre-incubated steady-state solution of F-actin. Pipette carefully with a cut-pipette tip to prevent too much fragmentation of the filaments as this would disturb the steady-state.
5. Expose spectrin-actin seeds to profilin-actin by flowing the solution in reservoir 1. Filaments thus initiated are referred to as "tracer barbed ends".
6. Acquire time-lapsed images of the elongation of tracer barbed ends.
7. Switch the flow reservoir to expose tracer barbed ends to flow solution in reservoir 2 (steady-state solution).
8. Prepare kymographs of individual tracer barbed ends and measure the rate of elongation, *V*, for each filament (see Figure 12.13.3C).
9. Calculate the total concentration of polymerizable monomers ($[A]_{SS} + [PA]_{SS}$) using the following equation (Shekhar & Carlier, 2017):

$$[A]_{SS} + [PA]_{SS} = \frac{V}{k_+} + C_C$$

10. Calculate steady-state G-actin and profilin-actin concentrations using the following equations:

$$[A]_{SS} = \frac{-\left(K_P + P_{total} - C_C - \frac{V}{k_+}\right) \pm \sqrt{\left(K_P + P_{total} - C_C - \frac{V}{k_+}\right)^2 + 4.K_P.\left(C_C + \frac{V}{k_+}\right)}}{2}$$

$$[PA]_{SS} = P_{total}.\frac{[A]_{SS}}{[A]_{SS} + K_P}$$

Where $k_+ = 10$ $s^{-1}$ $\mu M^{-1}$ is the on-rate of free actin monomers and profilin-actin at the barbed end; $[P]_{total} = 3$ μM is the total concentration of profilin; $C_C^B = 0.08$ μM is the critical concentration for assembly at the barbed end of a filament; $K_P = 0.1$ μM is the equilibrium dissociation constant for profilin-actin complex.

## GROWING ANCHORED FILAMENTS WITH FREE POINTED ENDS

***BASIC PROTOCOL 5***

To study the effect of certain proteins on the dynamics of actin-filament pointed ends, filaments anchored at their barbed ends need to be grown. This ensures that filament pointed-ends are free to be exposed to a solution containing protein of interest. This method has recently been used to study the effect of ADF on filament pointed ends (Shekhar & Carlier, 2017; Wioland et al., 2017).

Filaments with free pointed ends are nucleated from gelsolin-actin seeds. Gelsolin binds filament barbed ends with high affinity and blocks barbed-end growth. In presence of free

actin monomers gelsolin forms a gelsolin-actin ($GA_2$) complex, in which each gelsolin molecule is bound to two actin monomers. Actin monomers can bind the pointed-end of the $GA_2$ complex and elongate filaments from their pointed ends. The critical concentration for pointed end growth ($C_C^P = 0.6\ \mu M$) is much higher than that for barbed-end growth ($C_C^P = 0.08\ \mu M$), however, so higher concentration of actin monomers is required for fast filament elongation at the pointed ends. $GA_2$ seeds can be attached to the coverslip surface, similar to spectrin-actin seeds (Figure 12.13.2).

***Materials*** *(also see Basic Protocol 2)*

Purified gelsolin (for details, see Shekhar and Carlier, 2017)
Modified TIRF buffer
G-actin (Support Protocol 2)
1 M magnesium chloride solution
250 mM EGTA solution

1. Plasma clean a pre-cleaned coverslip and attach it to a PDMS microchamber, as described in Basic Protocol 2.
2. Prepare gelsolin-actin $GA_2$ seeds by mixing 2.5 molar equivalents of G-actin to gelsolin in G-buffer (e.g., mix 100 pM gelsolin and 250 pM G-actin to get 100 pM $GA_2$ seeds).

   *Gelsolin needs calcium for its activity. Therefore, none of the solutions used in this experiment should contain EGTA as its calcium sequestering ability will inactivate gelsolin.*
3. Inject gelsolin-actin seeds into the PDMS flow cell at a concentration of 100 pM using a pipet. Incubate for 5 min to allow the seeds to bind the plasma-cleaned surface of the coverslip.
4. Connect the microchamber flow cell to a Maesflow control unit.
5. Rinse the chamber with G-buffer for 5 min.
6. Inject 10% BSA solution into the chamber to passivate the free surface of the coverslip (i.e., not covered by gelsolin-actin seeds).

   *Given the higher viscosity of the 10% BSA solution (as compared to buffer), a higher pressure will need to be applied to maintain the same flow rate.*
7. Rinse the chamber with G-buffer to remove unbound excess BSA.
8. Convert the remaining of Ca-ATP-G-actin to Mg-ATP-G-actin by adding 20 μM $MgCl_2$ followed by 0.2 mM EGTA.
9. Prepare 5 μM G-actin (10% Alexa 488-labeled) in modified TIRF buffer (i.e., without $MgCl_2$ and EGTA), supplemented with 0.1% BSA.

   *No profilin is added for pointed-end elongation of filaments as profilin binding to actin monomers prevents them from associating at the pointed end of actin filaments.*
10. Inject the G-actin solution into the flow-cell via one of the inlet channels, and start imaging in TIRF immediately.
11. Continue injection of actin monomers until filaments reach the required length. Rate of filament elongation is defined by:

$$V = k_+^P \cdot \left([A] - C_C^P\right)$$

where $k_+^P = 1.3\ s^{-1}\ \mu M^{-1}$ is the on-rate of free actin monomers at the pointed end, and $C_C^P = 0.6\ \mu M$ is the critical concentration at the pointed end.

## STUDYING FILAMENT DEPOLYMERIZATION FROM POINTED ENDS AND SEVERING BY ADF

*BASIC PROTOCOL 6*

Although pure actin filaments depolymerize extremely slowly from their pointed ends (~0.2 subunits per second), their rate of depolymerization can be enhanced by ancillary proteins. Actin depolymerizing factor, for example, cooperatively binds the sides of actin filaments and enhances the rate of depolymerization of monomers from filament ends. Microfluidics-assisted TIRF imaging has recently been used to measure the effect of ADF on the rate of filament depolymerization and severing (Shekhar & Carlier, 2017).

***Materials*** *(also see Basic Protocol 5)*

Purified actin depolymerizing factor (ADF; for details, see Shekhar and Carlier, 2017)

1. Incubate gelsolin-actin seeds on the surface in the microchamber and passivate the free coverslip surface with BSA (as described in Basic Protocol 5).
2. Prepare flow solutions in three reservoirs:
   a. Reservoir 1: 5 µM G-actin (10% Alexa 488-labeled) in TIRF buffer without $MgCl_2$ and EGTA, supplemented with 0.1% BSA, as described in Basic Protocol 5.
   b. Reservoir 2: TIRF buffer only.
   c. Reservoir 3: 1 µM actin depolymerizing factor in TIRF buffer.
3. Nucleate actin filaments from anchored gelsolin-actin seeds by flowing in the solution from reservoir 1.
4. Continue elongation until filaments reach ~10-µm length.

   *All filaments in the field of view should now be anchored at their barbed ends and have their pointed ends free in the flow.*
5. Expose the filaments to the solution from reservoir 2 for 15 min.

   *This step is carried out to ensure complete hydrolysis of ATP bound to actin monomers in the filament and release of the bound phosphate.*
6. Expose the filaments to the solution from reservoir 3 with ADF, and start acquiring TIRF images.
7. Prepare a kymograph of individual filaments (see Support Protocol 5) and determine the mean rate of depolymerization from at least 50 filaments (Figure 12.13.3C).
8. Repeat steps 6 and 7 for varying ADF concentrations to determine the concentration-dependent effect of ADF on the rate of filament depolymerization and severing.

## CLEANING MICROSCOPE COVERSLIPS

*SUPPORT PROTOCOL 1*

High-resolution TIRF imaging requires very low background noise. The three most common sources of noise linked to coverslip surface include: autofluorescence in the coverslip glass, contaminants stuck on the coverslip surfaces and fluorescent proteins sticking non-specifically to the coverslip during imaging. The inherent autofluorescence of the glass can differ between manufacturers and sometimes even between batches from the same company. The contaminants on the coverslip surface can be removed via a set of cleaning steps using organic and inorganic solvents. Non-specific protein binding can be reduced by chemically passivating the surface with long-chain molecules like PLL-PEG that form a hydrophilic brush over the surface and reduce non-specific interactions.

***Materials***

Deionized distilled water
Acetone
Ethanol
2 M potassium hydroxide solution

Glass coverslips (40 × 24-mm #1.5, Fisher Scientific)
Multi-coverslip holder
Water bath sonicator

1. Place coverslips in the coverslip holder. For proper cleaning, ensure that coverslips don't stick to each other.
2. Fill the coverslip holder with deionized distilled water.
3. Place the holder in a water bath sonicator and sonicate for 20 min at room temperature.
4. Decant the water carefully without disturbing the coverslips, fill the coverslip holder with acetone, and sonicate for another 20 min.

   *Ensure that the holder remains covered during all sonication cycles to prevent contaminants from settling down on the coverslips.*

5. Decant the acetone, fill the holder with ethanol, and sonicate another 20 min.
6. Remove the ethanol, rinse twice with water, and sonicate in water another 20 min.

   *Acetone and ethanol remove the organic contaminants bound to the coverslips.*

7. Replace distilled water with 2M potassium hydroxide solution, and sonicate 20 min at room temperature.

   *Hydroxide removes all inorganic contaminants from the coverslips.*

8. Rinse twice with distilled water.
9. The cleaned coverslips can be stored up to a month in water. Prior to the TIRF experiment, dry the coverslips under a nitrogen stream and passivate with PLL-PEG or BSA, depending upon the experimental requirements.

***SUPPORT PROTOCOL 2***

## PURIFICATION AND FLUORESCENT LABELING OF SKELETAL MUSCLE ACTIN

Skeletal muscle actin is purified from acetone powder using established protocols (Ebashi & Maruyama, 1965; Hertzog & Carlier, 2005; Spudich & Watt, 1971). For fluorescence imaging, purified actin monomers need to be covalently functionalized with fluorescent dyes. The protocol below describes purification and labeling of G-actin. Actin monomers are labeled on their lysine residues using dyes conjugated to a succinimidyl ester functional group. The protocol for actin purification has been reproduced from (Hertzog & Carlier, 2005).

***Materials***

Rabbit muscle acetone powder (Pel-Freez Biologicals/Cytoskeleton Inc.)
Extraction X buffer (see recipe)
Dialysis buffer D1 (see recipe)
Potassium chloride (KCl), powder and 4 M solution
G-buffer (see recipe)
Modified F-buffer (see recipe)
Alexa 488 succinimidyl ester (Thermo Fisher Scientific)

Glass beakers
Glass rod
Glass wool
Glass funnel
Water bath, 20° to 25°C
Thermometer
Dialysis membrane (12 to 14,000 kDa)
Potters (5- and 30-ml)
Tip sonicator
2.5 × 100-cm gel filtration column (GE Life Sciences)

***Purify G-actin***

1. Measure 9 g of acetone powder into a 500-ml beaker and place on ice. Add 270 ml of extraction X buffer and gently stir with a glass rod to completely wet the powder.
2. Let the powder swell on ice. Stir gently with the glass rod every 10 min for a total of 40 min.

   *Gentle mixing minimizes the solubilization of α-actinin. Actin should now be in the solution.*

3. Centrifuge the solution in a Beckman Coulter 45 Ti rotor and tubes for 45 min at 25,000 × *g*, 4°C.
4. Filter the supernatant by pouring it over glass wool placed in a funnel. Measure the volume.

   *Glass wool is used to filter the solution, because the liquid is too viscous to pass through a membrane filter.*

5. To the filtered solution, add potassium chloride powder (KCl) to a final concentration of 3.3 M KCl. Note that KCl addition increases the volume about 10%, so this should be considered while calculating the amount of KCl to be added.
6. Place the beaker in a warm water bath between 20° and 25°C, and mix by magnetic agitation at room temperature until the temperature of the solution returns to 15°C. Place the solution on ice without mixing until the temperature reaches 5°C.

   *The addition of KCl results in a highly endothermic reaction.*

7. Centrifuge for 30 min at 25,000 × *g*, 4°C.
8. Filter supernatant through glass wool.
9. Dialyze overnight in the cold room against 32 volumes of dialysis buffer D1.
10. Add KCl to a final concentration of 0.8 M, and mix with magnetic agitation for 1.5 hr at 4°C.

    *This step releases tropomyosins into the solution.*

11. Centrifuge for 3.5 hr at 100,000 × *g*, 4°C. Discard the supernatant.
12. Gently remove the pellet with a spatula and place it in the 30-ml potter. Carefully wash the actin from the walls of centrifuge tubes with 25 ml extraction X buffer. Homogenize the solution with the potter.
13. Add 75 µl 1 M $MgCl_2$ and 375 µl 4 M KCl, adjust the volume to 38.6 ml with extraction X buffer, and homogenize.
14. Add 9 ml 4 M KCl and adjust the volume to 50 ml with extraction X buffer. Mix with magnetic agitation for 1.5 hr at 4°C.

15. Centrifuge for 3.5 hr at 100,000 × *g*, 4°C.
16. Suspend the pellet with 30 ml of extraction X buffer and homogenize with the potter.
17. Transfer the solution to a dialysis bag, and dialyze against 2 liters of G-buffer for 48 hr at 4°C.
18. Change the buffer, and dialyze overnight against 1 liter of G-Buffer.
19. Using a tip sonicator, sonicate the solution three times with 30 sec pulses by dipping the tip in the dialysis bag.
20. Dialyze again overnight in fresh 1 liter G-buffer.
21. Centrifuge the solution for 1.5 hr at 400,000 × *g*, 4°C.
22. Load the supernatant onto a pre-equilibrated 2.5 × 100-cm gel filtration column. Collect 5 ml fractions.
23. Based on the absorbance at 290 nm, pool the fractions containing G-actin.
24. Record the spectra between 240 and 340 nm and calculate the actin concentration.

    *The molar extinction coefficient of actin at 290 nm is 0.617 mg/ml, and the molecular weight is 42 kDa. The typical yield of this preparation is 40 ml at 50 μM (2.1 mg/ml) G-actin.*

25. Store G-actin on ice for up to 3 weeks.

***Label actin with Alexa 488***

26. Dialyze 2 ml of G-actin overnight in modified F-buffer.

    *In this step, G-actin is dialyzed to remove Tris and to polymerize the G-actin to F-actin.*

27. Add Alexa 488 dye to a final concentration of 0.25 mM in 2 ml F-actin solution, and incubate at room temperature for 2 hr on a rotator.
28. Centrifuge the reaction mixture for 40 min at 450,000 × *g*, room temperature.
29. Suspend the pellet in 2 ml G-buffer, and homogenize in a 5-ml potter.
30. Incubate on ice for two hours to depolymerize filaments.
31. Polymerize G-actin by adding KCl to a final concentration of 100 mM and $MgCl_2$ to final concentration of 1 mM. Incubate on ice for 1 hr.
32. Centrifuge for 40 min at 450,000 × *g*, 4°C.
33. Suspend the pellet in 2 ml G-buffer, homogenize in a 5-ml potter, and dialyze overnight in 1 liter G-buffer at 4°C.
34. Centrifuge for 40 min at 450,000 × *g*, 4°C, and collect the supernatant containing labeled actin.
35. Measure protein concentration and labeling efficiency by measuring the absorbance at 280 nm ($A_{280}$) and at 495 nm ($A_{495}$). Calculate the concentration of actin and dye:

$$[Actin] = \frac{(A_{280} - A_{495} * CF_{280})}{\varepsilon_{P280}}$$

$$[Dye] = \frac{(A_{495})}{\varepsilon_{D280}}$$

$$\% \text{ labeling} = \frac{[Dye]}{[Actin]} * 100$$

*Molar extinction coefficient for actin, $\varepsilon_{P280} = 45{,}840\ M^{-1}cm^{-1}$ and for Alexa 488 fluorophore, $\varepsilon_{D280} = 71{,}000\ M^{-1}cm^{-1}$ and the correction factor Alexa 488 fluorophore at 280 nm, $CF_{280} = 0.11$.*

## PURIFICATION OF SPECTRIN-ACTIN SEEDS

***SUPPORT PROTOCOL 3***

Anchored actin filaments with free barbed ends are initiated from spectrin-actin seeds attached to the glass coverslip in the microfluidic flow cell. Spectrin is abundantly present in red blood cells and can easily be purified from human blood (Casella, Maack, & Lin, 1986). During purification, spectrin tetramer dissociates into heterodimers containing two spectrin subunits. Spectrin purifies as a complex with actin monomers, and the purified protein is therefore referred to as spectrin-actin. The molar ratio of spectrin dimer to actin monomer in the purified complex is 1:2 (Brenner & Korn, 1980). These seeds can be used to nucleate actin elongation in both pyrene assays and single-filament imaging methods.

### *Materials*

Packed human red blood cells, ~100 ml (acquired from a blood bank)
Washing buffers A and B (see recipes)
Lysis buffer (see recipe)
Spectrin extraction buffer (see recipe)
10% pyrene-labeled CaATP-G-actin
G-buffer (see recipe)
20× KME solution (see recipe)
$MgCl_2$
EGTA
F-buffer
DTT
Protease inhibitors
Glycerol

50-ml conical tubes
Measuring cylinders
Fluorimeter

### *Wash the red blood cells*

1. To a 50 ml falcon tube, add 20 ml packed red blood cells and 25 ml cold washing buffer A. Prepare about 5 such tubes.
2. Centrifuge for 15 min at 2000 × *g*, 4°C and discard the supernatant.
3. Repeat the steps 1 and 2 twice.

### *Lyse the cells*

4. Combine the washed cells and suspend them in about 700 ml (approximately 10 times the volume of washed cells) ice-cold lysis buffer in a 1-liter beaker. Incubate 40 min with magnetic stirring at 4°C.

   *This step lyses the cells.*

5. Centrifuge the lysed cells in a Beckman Coulter 45 Ti rotor and tubes for 15 min at 45,000 × *g*, 4°C.
6. Carefully pipet away the supernatant without disturbing the snowy pellet.

*The red pellet at the bottom contains unlysed cells and should be left untouched and discarded. The thick snowy pellet (membrane ghosts) close to the bottom are lysed membranes that contain the protein.*

7. Transfer the snowy pellet to a 500-ml measuring cylinder.
8. Adjust the total volume to 360 ml with washing buffer B, and mix manually by swirling.
9. Centrifuge the solution using multiple centrifuge tubes for 15 min at 45,000 × *g*, 4°C.
10. Discard the supernatant and suspend the snowy pellets in a combined volume of ~180 ml of washing buffer B.

    *There might be a smaller deep red colored pellet formed. These are unlysed red blood cells. Discard this pellet.*

11. Centrifuge the solution in a Beckman Coulter 45 Ti rotor and tubes for 15 min at 45,000 × *g*, 4°C.
12. Repeat steps 10 to 11 once more.

***Spectrin-actin seeds extraction***

13. Gently remove the supernatant, leaving behind the translucent snowy pellet.
14. Suspend each pellet in 5 ml spectrin extraction buffer by vortexing, combine the suspended pellets into a single tube, and adjust the volume to 60 ml with spectrin extraction buffer.
15. Centrifuge in a Beckman Coulter 45 Ti rotor and tubes for 30 min at 60,000 × *g*, 4°C.
16. Repeat steps 13 to 15.
17. Suspend the final pellet with equal volume of spectrin extraction buffer in a 15-ml tube and vortex to mix thoroughly.
18. Incubate the suspension for 40 min in a water bath at 37°C, manually inverting the tubes at regular intervals. The spectrin-actin seeds should now be in the solution.

    *Maintaining the temperature is very important to prevent the dissociation of spectrin dimers into monomers.*

19. Ultracentrifuge in a Beckman Coulter TLA 100.3 rotor and tubes for 30 min at 450,000 × *g*, 4°C.

    *Spectrin-actin seeds will remain in the supernatant.*

20. Analyze the purified protein by SDS-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining.

    *Expect spectrin-actin to show two prominent bands at ~225 and 260 kDa for spectrin subunits, and one at ~42 kDa for actin subunits (Figure 12.13.5A).*

***Measure the concentration of spectrin-actin seeds***

*Spectrin-actin seed concentration is measured from a functional assay of assembly of pyrenyl-actin (Fig* 12.13.5*B). See (Hertzog & Carlier,* 2005*) for detailed instructions on pyrenyl-actin assembly assay.*

21. Prepare 1 ml 10 μM CaATP-G-actin (10% pyrene-labeled) solution in G-buffer on ice.

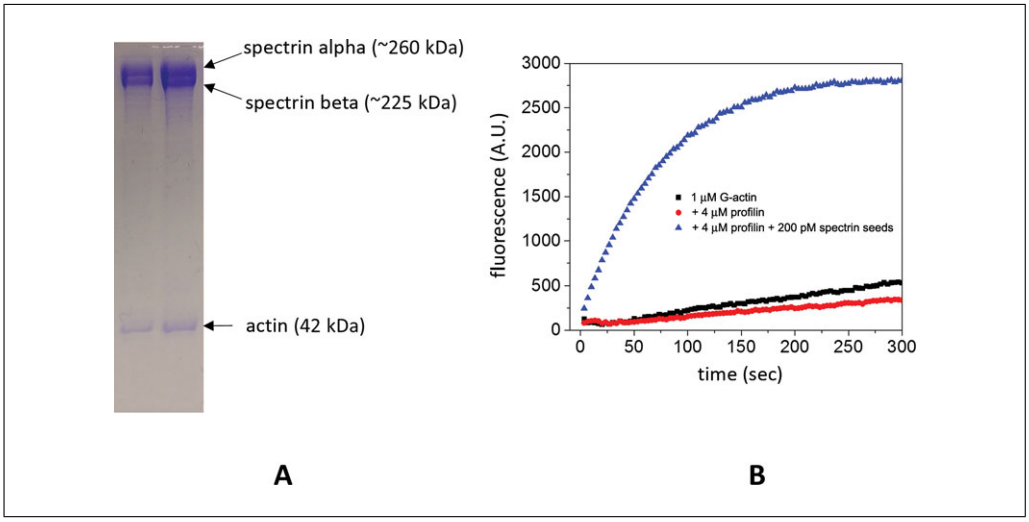


**Figure 12.13.5** Purification and characterization of spectrin-actin seeds from human red blood cells. **(A)** SDS polyacrylamide gel electrophoresis of purified spectrin-actin seeds shows three prominent bands: two for spectrin (alpha and beta subunits) and one for actin. Both lanes are identical samples, except that twice the amount of protein was loaded in the second lane. **(B)** Actin polymerization in the presence of 10% pyrenyl-labeled 1 µM G-actin alone (black), with 4 µM profilin (red), or with 4 µM profilin and 200 pM spectrin-actin seeds (blue).

22. Polymerize 0.2 ml of the G-actin solution into F-actin by adding 10 µl 20× KME (1× final).

23. Convert the remaining of CaATP-G-actin to MgATP-G-actin by adding 20 µM $MgCl_2$ followed by 0.2 mM EGTA. Incubate 10 min.

24. Prepare multiple solutions by mixing increasing amount of G-actin (from step 23) in the range of 0 to 1 µM total actin concentration in F-buffer. Pipet thoroughly to rapidly reach steady state.

25. Measure the pyrene fluorescence of each sample on a fluorimeter, and prepare a critical concentration curve by plotting Pyrene fluorescence at steady state versus actin concentration.

26. Calculate the pyrene fluorescence per µM F-actin from the above graph (denoted by *f*).

27. Conduct a seeded barbed-end growth assay by mixing MgATP G-actin (final concentration 0.5 µM) and KME (final 1×) in G-buffer with variable concentration of spectrin-actin seeds (vary volume between 0 to 20 µl).

28. Measure the initial rate of increase in fluorescence upon addition of spectrin-actin seeds.

29. The initial rate of barbed end assembly is:

$$V = k_+ \cdot [F] \cdot (C - C_C) \cdot f$$

Where $C = 0.5$ µM is the initial actin concentration, $C_C = 0.1$ µM is the critical concentration of the barbed end (i.e.,), $k_+ = 10$ $\mu M^{-1}s^{-1}$ is the association rate constant of actin monomers at the barbed end, and $f$ is the fluorescence measured per µM F-actin.

30. From this equation, the concentration of barbed ends [F]—i.e., the concentration of spectrin-actin seeds—can be determined. A linear increase in $V$ as a function of seed concentration should be observed.

31. Add DTT (2 mM final) and protease inhibitors to the supernatant. Add an equal volume of cold glycerol (50% final concentration) and mix.

32. Spectrin-actin seeds thus prepared can be stored at –20°C and remain stable for at least two years.

*SUPPORT PROTOCOL 4*

## CLEANING AND MAINTENANCE OF THE FLOW SYSTEM AND PDMS MICROCHAMBERS

Good cleaning practices prevent blockage of microfluidic tubing and ensure that no damage occurs to the system or to the sensors. The tubing can get clogged due to organic growth, contaminant blockage, and salt crystallization. PDMS microchambers also need to be cleaned after each experiment to remove any bound protein. Cleaned microchambers can be re-used.

### *Materials*

0.5 M sodium hydroxide (NaOH)
Deionized, distilled water
100% ethanol
Compressed air/$N_2$

### *Clean the flow system*

1. Flow 1 ml 0.5 M NaOH at full pressure (1 bar) through the tubing of the system to remove any protein bound non-specifically to the tubing.

2. Flow 1 ml deionized, distilled water at full pressure (1 bar) through each channel to rinse the tubing.

3. Dry all tubing by flowing $N_2$ compressed air at 1 bar for 5 min.

### *Clean the PDMS microchambers*

4. After completion of an experiment, detach the PDMS microchamber from the coverslip and discard the coverslip in the glass disposal.

5. Rinse the PDMS microchamber with NaOH to remove any protein bound to the chamber surface.

6. Thoroughly wash the PDMS microchamber with deionized, distilled water.

7. Rinse the microchamber with ethanol and dry the PDMS microchamber under a stream of compressed air or $N_2$.

8. The microchamber thus cleaned can be reused for up to 10 experiments.

*SUPPORT PROTOCOL 5*

## IMAGE ANALYSIS AND PREPARATION OF KYMOGRAPHS

Images acquired from TIRF microscopy were processed using ImageJ image processing software.

### *Materials*

ImageJ image analysis software

1. Carry out background subtraction of the images using the built-in rolling-ball background subtraction algorithm.

   *Rolling-ball radius of 5 pixels is used for actin filaments imaged under a 60× objective.*

2. Draw a line on the filament of interest.

3. Make a kymograph for the filament using the ImageJ kymograph plugin with line thickness of 3 pixels (Figure 12.13.3C).
4. Measure the slope of elongation/depolymerization from the kymograph (units of pixels/frame).
5. Convert the units of the slope from pixels/frame to subunits/second. An actin subunit is about 2.7 nm.
6. Repeat the above steps for at least 50 filaments and calculate the mean slope.

## REAGENTS AND SOLUTIONS

*Prepare all solutions with deionized distilled water, unless otherwise mentioned. All solutions that are to be introduced in the microfluidic flow system should be filtered through a 0.2-µm filter to remove any debris that might block the microfluidic channels and flow velocity sensors. Solutions for the microfluidic flow cell should also be degassed. Sodium azide ($NaN_3$) is added to prevent any bacterial growth in the solutions and therefore enables long-term storage and use.*

### *BSA solution, 10%*

10 g bovine serum albumin powder
100 ml final volume of G-buffer
Allow to mix on a rotator in cold room
Filter through 0.2-µm filter to remove undissolved BSA aggregates
Store at –20°C

### *Dialysis buffer D1*

2 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
1 mM $MgCl_2$
1 mM dithiothreitol (DTT)

### *G-buffer*

5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.2 mM ATP
0.1 mM $CaCl_2$
1 mM DTT
0.01% (w/v) $NaN_3$

### *KME solution, 20×*

1 M KCl
20 mM $MgCl_2$
4 mM EGTA

### *Lysis buffer*

5 mM sodium phosphate, pH 7.7 (*APPENDIX 2A*)
1 mM PMSF (add just before use)

*Final pH is set by phosphate buffer; do not re-adjust.*

### *Modified F-buffer*

20 mM PIPES, pH 6.9
0.2 mM $CaCl_2$
0.2 mM ATP
100 mM KCl

***Modified TIRF buffer***

5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.2 mM ATP
50 mM KCl
10 mM DTT
1 mM DABCO (1,4-diazabicyclo[2.2.2]octane)
0.01% (w/v) $NaN_3$

***PBS (phosphate buffered saline, pH 7.4)***

1 mM $KH_2PO_4$
155 mM NaCl
3 mM $Na_2HPO_4$,·$7H_2O$

***PLL-PEG solution, 1 mg/ml***

5 mg PLL(20)-g[3.5]- PEG(2) powder (SuSoS, Switzerland)
5 ml PBS (see recipe)
Mix well and filter with 0.2-µm filters
Prepare single-use 25 µl aliquots
Store at –20°C

***Spectrin extraction buffer***

0.3 mM sodium phosphate, pH 7.6 (prepared by mixing monobasic and dibasic sodium phosphate solutions; *APPENDIX 2A*)
0.1 mM PMSF (add just before use)

***TIRF Buffer***

5 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.2 mM ATP
50 mM KCl
1 mM $MgCl_2$
0.2 mM EGTA
10 mM DTT
1 mM DABCO
0.01% (w/v) $NaN_3$

***Washing buffer A***

5 mM sodium phosphate, pH 7.7 (prepared by mixing monobasic and dibasic sodium phosphate solutions; *APPENDIX 2A*)
150 mM NaCl
1 mM EDTA

***Washing buffer B***

5 mM sodium phosphate, pH 7.7 (prepared by mixing monobasic and dibasic sodium phosphate solutions; *APPENDIX 2A*)
0.1 mM PMSF (add just before use)

***X-buffer***

2 mM Tris·Cl pH 7.8 (*APPENDIX 2A*)
0.5 mM ATP
0.1 mM $CaCl_2$
1 mM DTT
0.01% $NaN_3$

## COMMENTARY

### Time Considerations

Preparation of PDMS chambers and coverslip cleaning require one half day each (Basic Protocol 1 and Support Protocol 1). Basic protocols 2, 3, 4, and 5 each require a maximum of one day. Purification of G-actin requires 6 days and its fluorescent labeling requires 2 days (Support Protocol 2). Purification of spectrin seeds requires 1 day (Support Protocol 3). Image analysis takes about 30 min to measure the elongation/depolymerization rate of about 50 filaments (Support Protocol 5).

### Troubleshooting

1. *Filaments are too dim to observe*. Keep in mind that filaments elongated by formins are dimmer than those growing with a free barbed end. Nevertheless, if the filaments growing with free barbed ends are also too dim to observe, it might indicate one of the following:

    a. Very low labeling of actin. Check that at least 10% of the actin monomers are labeled.

    b. Inadequate surface passivation. Fluorescently labeled actin monomers binding the glass surface either directly or indirectly via another protein that binds the surface and sequesters actin monomers can drastically increase the background fluorescence and can therefore lead to low signal-to-noise. This often happens due to inadequate surface passivation.

    c. Spectrin-actin seed density too high. Too many filaments growing on the surface from a high density of spectrin-actin seeds can cause high background fluorescence.

2. *Leakage in the flow cell*. If a PDMS flow cell is seen to repeatedly leak, this might indicate bad sealing between the PDMS microchamber and the coverslip. This often indicates ageing of the PDMS microchamber due to repeated reuse. PDMS microchambers should therefore be discarded after 10 uses.

3. *Very slow rate of filament elongation*. This normally happens when the reservoir with profilin-actin has been incubating for a long time and a large amount of monomeric actin has been depleted due to actin polymerization occurring in the reservoir. Profilin-actin solution in the reservoir should be replenished.

4. *Abnormal polymerization* can also indicate extraordinary changes in the temperature of the microscopy room. All protocols reported here have been conducted at room temperature.

5. *Sudden reduction of flow rate in the microfluidic chamber*. This is often caused either by a leakage in the sealing between the PDMS microchamber and the coverslip or due to the blockage of the main flow cell channel by an air bubble or another contaminant. To prevent this, it is important to filter and degas all buffers that are introduced into the flow system.

5. *Gradual drop in flow rate*. When a solution of higher viscosity than water is introduced (e.g., 10% BSA solution, polymerized actin or a solution containing methyl cellulose) the flow rate is seen to gradually reduce followed by stabilization. This is normal.


### Acknowledgements

I thank Jeff Gelles and Bruce L. Goode for their mentorship, continued support, and encouragement. I acknowledge financial support from the NIH (grant GM098143 to J.G. and B.L.G.). I would like to thank Marie-France Carlier for her mentoring and for encouraging me to pursue studies in actin dynamics. I thank Antoine Jégou and Guillaume Romet-Lemonne for introducing me to microfluidics and single-filament imaging. I also thank Silvia Jansen for help with purifying spectrin-actin seeds.


### Literature Cited


Amann, K. J., & Pollard, T. D. (2001). Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 15009–15013. doi: 10.1073/pnas.211556398.

Axelrod, D. (1981). Cell-substrate contacts illuminated by total internal reflection fluorescence. *The Journal of Cell Biology*, *89*, 141–145. doi: 10.1083/jcb.89.1.141.

Bombardier, J. P., Eskin, J. A., Jaiswal, R., Correa, I. R., Jr., Xu, M. Q., Goode, B. L., & Gelles, J. (2015). Single-molecule visualization of a formin-capping protein 'decision complex' at the actin filament barbed end. *Nature Communications*, *6*, 8707. doi: 10.1038/ncomms9707.

Brenner, S. L., & Korn, E. D. (1980). Spectrin/actin complex isolated from sheep erythrocytes accelerates actin polymerization by simple nucleation. Evidence for oligomeric actin in the erythrocyte cytoskeleton. *The Journal of Biological Chemistry*, *255*, 1670–1676.

Brewer, L. R., & Bianco, P. R. (2008). Laminar flow cells for single-molecule studies of DNA-protein interactions. *Nature Methods*, *5*, 517–525. doi: 10.1038/nmeth.1217.

Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., ... Pantaloni, D. (1997).

Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: Implication in actin-based motility. *The Journal of Biological Chemistry*, *136*, 1307–1322.

Carlier, M. F., Pernier, J., Montaville, P., Shekhar, S., Kuhn, S., Cytoskeleton, D., & Motility, G. (2015). Control of polarized assembly of actin filaments in cell motility. *Cellular and Molecular Life Sciences*, *72*, 3051–3067. doi: 10.1007/s00018-015-1914-2.

Carlier, M. F., & Shekhar, S. (2017). Global treadmilling coordinates actin turnover and controls the size of actin networks. *Nature Reviews. Molecular Cell Biology*, *18*, 389–401. doi: 10.1038/nrm.2016.172.

Casella, J. F., Maack, D. J., & Lin, S. (1986). Purification and initial characterization of a protein from skeletal muscle that caps the barbed ends of actin filaments. *The Journal of Biological Chemistry*, *261*, 10915–10921.

Chesarone, M. A., DuPage, A. G., & Goode, B. L. (2010). Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nature Reviews. Molecular Cell Biology*, *11*, 62–74. doi: 10.1038/nrm2816.

Ebashi, S., & Maruyama, K. (1965). Preparation and some properties of alpha-actinin-free actin. *Journal of Biochemical*, *58*, 20–26. doi: 10.1093/oxfordjournals.jbchem.a128159.

Edwards, M., Zwolak, A., Schafer, D. A., Sept, D., Dominguez, R., & Cooper, J. A. (2014). Capping protein regulators fine-tune actin assembly dynamics. *Nature Reviews. Molecular Cell Biology*, *15*, 677–689. doi: 10.1038/nrm3869.

Funatsu, T., Harada, Y., Tokunaga, M., Saito, K., & Yanagida, T. (1995). Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature*, *374*, 555–559. doi: 10.1038/374555a0.

Hertzog, M., & Carlier, M. F. (2005). Functional characterization of proteins regulating actin assembly. *Current Protocols in Cell Biology*, *26*, 13.6.1–13.6.23. doi: 10.1002/0471143030.cb1306s26.

Jegou, A., Carlier, M. F., & Romet-Lemonne, G. (2013). Formin mDia1 senses and generates mechanical forces on actin filaments. *Nature Communications*, *4*, 1883. doi: 10.1038/ncomms2888.

Jegou, A., Niedermayer, T., Orban, J., Didry, D., Lipowsky, R., Carlier, M. F., & Romet-Lemonne, G. (2011). Individual actin filaments in a microfluidic flow reveal the mechanism of ATP hydrolysis and give insight into the properties of profilin. *PLoS Biology*, *9*, e1001161. doi: 10.1371/journal.pbio.1001161.

Kouyama, T., & Mihashi, K. (1981). Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labeled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *European Journal of Biochemistry*, *114*, 33–38. doi: 10.1111/j.1432-1033.1981.tb06167.x.

Kovar, D. R., Harris, E. S., Mahaffy, R., Higgs, H. N., & Pollard, T. D. (2006). Control of the assembly of ATP- and ADP-actin by formins and profilin. *Cell*, *124*, 423–435. doi: 10.1016/j.cell.2005.11.038.

Kuhn, J. R., & Pollard, T. D. (2005). Real-time measurements of actin filament polymerization by total internal reflection fluorescence microscopy. *Biophysical Journal*, *88*, 1387–1402. doi: 10.1529/biophysj.104.047399.

Paul, A. S., & Pollard, T. D. (2009). Energetic requirements for processive elongation of actin filaments by FH1FH2-formins. *The Journal of Biological Chemistry*, *284*, 12533–12540. doi: 10.1074/jbc.M808587200.

Pernier, J., Orban, J., Avvaru, B. S., Jégou, A., Romet-Lemonne, G., Guichard, B., & Carlier, M. F. (2013). Dimeric WH2 domains in Vibrio VopF promote actin filament barbed-end uncapping and assisted elongation. *Nature Structural & Molecular Biology*, *20*, 1069–1076. doi: 10.1038/nsmb.2639.

Romero, S., Le Clainche, C., Didry, D., Egile, C., Pantaloni, D., & Carlier, M. F. (2004). Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. *Cell*, *119*, 419–429. doi: 10.1016/j.cell.2004.09.039.

Shekhar, S., & Carlier, M. F. (2017). Enhanced depolymerization of actin filaments by ADF/Cofilin and monomer funneling by capping protein cooperate to accelerate barbed end growth. *Current Biology*, *27*, 1990–1998.e5. doi: 10.1016/j.cub.2017.05.036.

Shekhar, S., Kerleau, M., Kuhn, S., Pernier, J., Romet-Lemonne, G., Jegou, A., & Carlier, M. F. (2015). Formin and capping protein together embrace the actin filament in a menage a trois. *Nature Communications*, *6*, 8730. doi: 10.1038/ncomms9730.

Smith, B. A., Daugherty-Clarke, K., Goode, B. L., & Gelles, J. (2013). Pathway of actin filament branch formation by Arp2/3 complex revealed by single-molecule imaging. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 1285–1290. doi: 10.1073/pnas.1211164110.

Spudich, J. A., & Watt, S. (1971). The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *The Journal of Biological Chemistry*, *246*, 4866–4871.

Wioland, H., Guichard, B., Senju, Y., Myram, S., Lappalainen, P., Jégou, A., & Romet-Lemonne, G. (2017). ADF/Cofilin accelerates actin dynamics by severing filaments and promoting their depolymerization at both ends. *Current Biology*, *27*, 1956–1967.e7. doi: 10.1016/j.cub.2017.05.048.

## Internet Resources

https://rsb.info.nih.gov/ij/
*ImageJ website.*

https://imagej.net/Multi_Kymograph
*ImageJ kymograph plug-in.*