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***In vitro* studies of actin filament and network dynamics**

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Abstract

Now that many genomes have been sequenced, a central concern of cell biology is to understand how the proteins they encode work together to create living matter. *In vitro* studies form an essential part of this program because understanding cellular functions of biological molecules often requires isolating them and reconstituting their activities. In particular, many elements of the actin cytoskeleton were first discovered by biochemical methods and their cellular functions deduced from *in vitro* experiments. We highlight recent advances that have come from *in vitro* studies, beginning with studies of actin filaments, and ending with multi-component reconstitutions of complex actin-based processes, including force-generation and cell spreading. We describe both scientific results and the technical innovations that made them possible.

Actin filament architecture

The actin cytoskeleton plays a role in many different cellular processes, including maintenance of cell shape and mechanics, membrane trafficking, cell division, and locomotion. Assembly of functional actin networks (Figure 1) requires collaboration between a variety of regulators—filament crosslinkers, nucleation and elongation factors, and motor proteins—but the basic unit of these networks is the actin filament.

Recent work has significantly deepened our understanding of actin filament architecture and the ways in which regulatory proteins modulate it. The atomic structure of monomeric actin was solved in 1980 but definitive high-resolution structures of filamentous actin have proven harder to obtain. The best filament models derive from electron microscopy and image reconstruction. New methods of sample preparation and data collection have now pushed the resolution of actin filament models well below 10 Å and provide mechanistic explanations for key features of filament assembly.

Namba and co-workers [1^{**}], for example, made use of energy filtering, optimal ice thickness, and liquid helium cooling to obtain high-contrast images of frozen, hydrated actin filaments. From these they computed a filament model with 6.6 Å resolution, one which clearly resolves protein secondary structures. In their filament, the four subdomains of actin are rotated to flatten the molecule against the filament axis. Longitudinal interactions between protomers along the two-start helices turn out to be very strong, while interactions between the two strands of the filament are weaker than expected. The ‘hydrophobic plug’, for example, which was proposed to connect the two strands, does indeed make cross-strand connections but they turn out to be hydrophilic in nature.

In a similar study, Wakabayashi and colleagues [2^{**}] stabilized actin filaments with high concentrations of phosphate and obtained a reconstruction with 5 Å resolution. These authors identified both Mg⁺⁺ and phosphate ions in their filament and proposed a

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convincing mechanism by which ATP hydrolysis is stimulated by filament formation. They also identified a $6 \text{ \AA} \times 18 \text{ \AA}$ cylindrical 'tunnel' in the heart of the filament, through which phosphates, cleaved from ATP, escape. At the upper end of this 'phosphate escape tunnel' the authors identified a phosphate binding site (Figure 2) that stabilizes the interaction of three adjacent protomers in the filament, providing a molecular explanation for both the slow kinetics of phosphate release and the stability of ADP-Pi actin filaments.

Some cytoskeletal regulators have evolved to make use of the conformational flexibility of actin filaments. When the actin depolymerization factor cofilin, for example, binds a filament, it alters the helical pitch [3] and this alteration propagates for some distance down the filament. The spread of this conformational change enables cofilin to communicate its presence to other nearby actin binding proteins. This promotes the binding of additional cofilin molecules while greatly accelerating dissociation of the Arp2/3 complex, a filament nucleating and branching factor [4]. This effect has been estimated to propagate over a distance of seven monomers, almost 20 nm. It is appealing to imagine actin filaments as conduits for communication between actin binding proteins. Such a mechanism could explain the simultaneous construction of distinct actin networks (*e.g.* 'lamellipodial' and 'lamellar' actin networks near the leading edges of migrating cells), with different architectures and different cadres of binding proteins, in more-or-less the same space.

Actin filament dynamics

Early studies of actin dynamics relied on interpreting bulk properties of actin-containing solutions, such as flow birefringence, light scattering, or the fluorescence of environmentally sensitive dyes. Assays like these remain important—partly because they are easy—but they provide less information than methods based on imaging individual filaments. The first single-filament studies of actin relied on electron microscopy but, around twenty years ago, improvements in (1) light microscope optics; (2) sensitivity of CCD cameras; and (3) coupling of fluorescent dyes made it relatively easy to image single, dynamic actin filaments. More recent technical innovations have further expanded the power and scope of single filament studies. These innovations include: (1) the use of total internal reflection fluorescence (TIRF) microscopy, (2) improved chemical passivation of glass surfaces, (3) improved methods for immobilizing proteins, and (4) the use of microfluidics to rapidly switch buffer conditions and protein concentrations.

Single-filament TIRF studies were critical in demonstrating that some actin regulators are insertional polymerases, surfing on the ends of growing filaments and accelerating their elongation. Formin-family proteins were the first to be shown to have this activity. Their interaction with actin filaments is remarkably processive, with individual formin molecules remaining attached to a growing end for more than 1000 s [5]. Similar experiments demonstrated that Ena/VASP proteins also accelerate filament elongation, but interact with growing filaments by a different mechanism and fall off much more quickly than formins, after only 1–2 s [6,7,8]. Importantly, these *in vitro* experiments resolved outstanding questions about the activity of Ena/VASP proteins. From *in vivo* experiments, these proteins were known to promote polymerization [9,10] but the molecular mechanism was, for several years, unclear.

New dyes and more sensitive cameras have expanded the color range of fluorescence microscopy beyond the traditional reds and greens. A recent, and beautiful, three-color TIRF experiment, for example, uncovered a remarkable collaboration between two actin regulators: a filament nucleation factor, adenomatous polyposis coli (APC), and a formin-family polymerase, mammalian Diaphanous 1 (mDia1) [11]. These molecules interact to form a complex which both nucleates new filaments and promotes their rapid growth. The

nucleation factor, APC, remains associated with the filament at the site of nucleation while the polymerase, mDia1, dissociates and rides away on the growing barbed end. This has been described as a 'rocket-launcher' mechanism and may explain interactions observed between other actin nucleators and polymerases (*e.g.* the spire-family nucleators and cappuccino-family formins [12]).

In addition to facilitating new discoveries, new experimental techniques can also be used to revisit long-standing questions. Arguably the most sophisticated system for studying single filament dynamics was developed by Jégou *et al.* [13**] to study disassembly of actin filaments and kinetics of phosphate release following ATP hydrolysis. These authors combined TIRF microscopy with microfluidics to enable them to both image single filaments and to rapidly change protein concentrations. They minimized interaction between proteins and glass surfaces by using spectrin-actin complexes to attach their filaments to coverslips only at one end. To measure lengths they used fluid flow to align their filaments and stretch them out parallel to the coverslip. To study depolymerization, they first grew long filaments from attached seeds then initiated disassembly by rapidly diluting the monomeric actin. By carefully monitoring the rate of filament disassembly and correlating it with the nucleotide state of the filament, Jégou and co-workers showed convincingly that phosphate dissociation from actin protomers is a random process that is not influenced by neighboring molecules. This is true for all but the terminal subunits in the filament, which appear to lose their phosphate groups more rapidly than subunits deeper in the filament.

Initiating actin filament assembly

Several factors are now known to nucleate new actin filaments, including the Arp2/3 complex, formin-family proteins, and spire-like proteins. At first glance, the spire-family proteins would seem the easiest to understand: they bind multiple actin monomers and stabilize their interaction [14]. Newly solved atomic structures of spire-actin complexes, however, reveal that spire does not position monomers in the orientation found in an intact filament [15,16*]. This discovery might provide a structural explanation for recent biochemical studies demonstrating that spire can profoundly destabilize preformed filaments [17,16*]. Based on these results, it might be best to think of spire-family proteins as constructing transitional structures, intermediate between monomers and filaments. Like an enzyme stabilizing a transition state, spire might facilitate both forward and reverse reactions: promoting filament assembly in some cases and disassembly in others.

The mechanism of nucleation by formins is still a mystery. In fact, not all formins are capable of nucleating new filaments *in vitro* and, even for the nucleation-competent formins, it is unclear whether their primary role in cells is to create new filaments or enhance growth of filaments nucleated by other mechanisms [11**]. Formin mutations that uncouple nucleation from processive polymerase activity would be extremely useful in sorting out this question.

The Arp2/3 complex contains what appears to be a cryptic filament barbed end, constructed from the actin-related proteins, Arp2 and Arp3. It binds preexisting filaments and creates new filaments that grow as branches off the older ones. The activity of the complex is controlled by upstream regulators, called nucleation promoting factors (NPFs). Rather than being a simple switch, however, the complex is more like a Chinese Puzzle Box that requires binding of two nucleation promoting factors [18], and a handoff of actin monomers from the activators to the complex [19,20*], that induces a conformational change [21,19]. The two NPF-binding sites on the complex have significantly different affinities and each appears to execute a distinct step in the nucleation reaction [22,23] but the details of the activation mechanism are still under study.

The activity of nucleation promoting factors is regulated, in turn, by upstream signaling molecules, including small G-proteins. N-WASP was the first NPF whose regulation was understood at the molecular level [24]. The regulation of WAVE, which helps generate actin filaments near the leading edges of motile cells, has proven more complicated. WAVE proteins exist as part of a five-subunit complex (the WAVE Regulatory Complex or WRC). Recent work using recombinant WRC is now beginning to fill some of the gaps in our understanding. Like N-WASP, the WRC is auto-inhibited and integrates multiple upstream inputs, including Rac1 and Arf1 GTPases [25**], PI(3,4,5)P₃, and IRSp53 [26,27,28**]. Phosphorylation of the native WAVE2 complex is also required for Rac2-GTP and PIP₃ to function as activators [27].

Assembling complex, biomimetic structures in vitro: protrusive networks and spreading cells

Over a decade ago the Carrier laboratory succeeded in reconstituting assembly of motile, dendritic actin networks from purified components [29]. They constructed branched filament networks with the Arp2/3 complex, activated by immobilized NPFs, and modulated by accessory proteins, including capping protein. Since that time, the field has developed increasingly sophisticated reconstitutions and used them to study many different aspects of actin network assembly and function. One important difference between reconstitution systems is the method by which they control the localization of active nucleation promoting factors. Substrates used to localize nucleation promoting factors include (Figure 3): (1) rigid particles, (2) lipid-coated particles, (3) lipid vesicles with external actin networks, (4) lipid vesicles encapsulating internal actin networks, and (5) encapsulating vesicles interacting with solid surfaces. Each localization method has advantages and disadvantages and the choice of method is generally dictated by the question being asked.

Rigid particles, uniformly coated with covalently attached nucleation promoting factors are excellent analogs for pathogens that hijack the actin cytoskeleton to move from cell to cell. Such particles are relatively simple to produce and have been used to study many aspects of network formation, including mechanical properties of Arp2/3-generated actin networks. Kawska *et al.* [30] recently followed the growth of small, dendritic subnetworks on the surfaces of polystyrene microspheres and watched them merge into a single, entangled structure. Interestingly, the final, unified network appears to retain weak spots at the boundaries between the original subnetworks that can fracture under mechanical loading.

Nucleation promoting factors covalently attached to rigid surfaces can activate the Arp2/3 complex but, because they are not free to move, their own dynamics cannot be studied. Coating a solid surface with a lipid bilayer, enables membrane-anchored nucleation promoting factors to diffuse and rearrange in response to network growth. Lipid-coated microspheres are also amenable to reconstitution of more complicated upstream signaling events that control actin assembly and in studying the role of lipids in the regulation of actin assembly. In a beautiful recent study, Koronakis *et al.* [25**] mapped the signaling landscape for actin networks assembled from lipid bilayers of different compositions. By constructing and isolating actin networks from lipid-coated microspheres in brain extracts, they discovered that two small G proteins, Rac1 and Arf1, synergize to activate the WAVE regulatory complex. Consistent with previous reports, they also found that N-WASP- and WAVE-dependent actin assembly are promoted by different phospholipids: PIP₂ and PIP₃, respectively. PIP₃ has been specifically implicated in regulating WAVE complex activity but it remains unclear whether this effect is owing to localization or allosteric activation.

In vivo, the assembly of actin filaments around unsupported vesicle occurs during many processes, including late stages of endocytosis. Unilamellar vesicles are more difficult to

produce in vitro, but, in addition to mimicking physiological processes, the release of mechanical constraints makes them useful for answering basic questions that cannot be addressed using lipid-coated particles. For example, actin-dependent deformation of vesicles in vitro has been used to calculate mechanical forces generated by growing actin networks [31]. Also, by growing actin networks on the surfaces of giant unilamellar vesicles, the Fletcher laboratory discovered that actin assembly can drive large-scale phase separation of lipids [32]. This group also used giant unilamellar vesicles to reconstitute formation of filopodia that project into the vesicle lumen [33].

To fully reconstitute the actin-membrane interactions that control cell shape, mechanics, and motility requires reproducing the geometry of the cell itself. That is, we must encapsulate actin and its regulatory factors inside closed, membrane-bound compartments. Controlled encapsulation of soluble and membrane-associated proteins inside well defined, unilamellar vesicles remains difficult but several new technologies now make this process easier. Reverse emulsion techniques can produce vesicles with well defined contents, surrounded by lipid bilayers of a specified composition [34]. Encapsulation techniques that utilize microfluidic jetting are even more technically challenging but have many important advantages [35,36,37]: (a) they produce vesicles of a highly uniform size and (b) they can mix multiple solutions at the moment of encapsulation to initiate a biochemical reaction inside the vesicle. Several groups have encapsulated actin and observed shape changes in vesicles upon polymerization. Recently, however, the Sykes group used reverse emulsion techniques to encapsulate membrane-associated nucleation promoting factors along with actin and other factors required to generate dynamic, Arp2/3-nucleated actin networks [38]. They used these internal networks as a model for the cell cortex and they characterized the physical and geometrical constraints controlling the thickness of the actin network and the rate at which filaments in the network turn over. This is an important first step toward reconstituting more dramatic cell-like behaviors, including: (a) localized control of mechanical properties; (b) regulated shape change and; ultimately, (c) directed migration. One requirement to reconstitute, or at least mimic, migration on a two-dimensional surface is adhesion. In a recent, and very clever, study the Sykes group encapsulated a minimal system for reconstituting Arp2/3-dependent actin assembly inside lipid vesicles, made with nickel-conjugated lipids [39]. After allowing time for the actin inside the vesicles to polymerize, the authors dropped them onto glass surfaces coated with poly-histidine. Tight interactions between nickel and poly-histidine drove spreading of the vesicles across the glass, enabling the Sykes group to study how the mechanics of the underlying actin network affects spreading dynamics. A next, obvious step along this line will be to use actin assembly itself to generate forces that drive spreading.

In addition to regulated polymerization and network assembly, several groups are now reconstituting and studying the behavior of motor-powered, contractile actin networks. In one set of studies [40,41], actin filaments were tethered, with random orientations, to rigid microspheres, which were, in turn, attached to an elastic, poly-acrylamide substrate (Figure 4a). Actin filaments attached to adjacent particles often overlapped and, in the presence of myosin and ATP, these overlapping filaments became crosslinked into mixed-polarity bundles. Motor activity of the myosin molecules caused the bundles to contract and contractile forces were measured by displacement of the actin-associated particles. Remarkably, the myosin motors also self-organized into discrete clusters, similar to those observed in vivo. In a recent and very elegant study, Reymann *et al.* [42] patterned nucleation promoting factors on a glass surface and used them to create Arp2/3-generated actin networks (Figure 4b) in which different fractions of filaments overlapped in parallel or anti-parallel orientations. When these authors added myosin motors, the networks became contractile but their behavior depended strongly on local network architecture. When they altered the pattern to increase the number of antiparallel filaments they observed stronger

contraction and pronounced filament disassembly. This represents the most satisfying reconstitution to date of a type of myosin-dependent actin network disassembly thought to occur *in vivo*.

This is an exciting time in the field. Complex reconstitutions are now enabling us to answer important, and previously inaccessible, questions in basic cell biology. Soon they may enable us to engineer new biomimetic devices that may find applications in nanotechnology and/or medicine.

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Figure 1.

Levels of complexity in the study of the actin cytoskeleton. **(A)** Eukaryotic cells rely on the actin cytoskeleton to maintain their shape, to control their mechanical properties, to shuffle internal membranes, and to move. **(B)** Dynamic actin networks, such as the lamellipodial actin network under the leading edge of an advancing cell must be assembled and disassembled on a rapid time scale. **(C)** The cellular functions of many actin filament binding proteins have been inferred from in vitro biochemical and biophysical studies. Specific activities that have been reconstituted in vitro include actin filament nucleation, capping, crosslinking, depolymerization, and monomer recycling. One frontier of modern in vitro studies is the reconstitution of complex network behaviors from combinations of actin and its associated regulators.

Figure 2.

Model for polymerization-dependent ATP hydrolysis in an actin filament and the subsequent dissociation of the cleaved phosphate. **(A)** Based on high-resolution filament models derived from electron microscopy Wakabayashi and co-workers [2^{**}] proposed that the three terminal subunits at the fast-growing barbed end of the filament are not in the proper conformation to hydrolyze ATP. Binding of an additional subunit (labeled 0) to the end of the filament alters the position of the terminal monomer (labeled 1), shifting a helix from subdomain 2 of monomer 1 into a pocket between subdomains 1 and 3 of the protomer above it (labeled 3). According to the model, this brings a catalytic water into close proximity with the gamma phosphate in monomer 3 and stimulates ATP hydrolysis. **(B)** Release of cleaved phosphate from the actin filament. Wakabayashi and colleagues [2^{**}] identified a 'cavity' in near the center of the actin filament through which they propose cleaved gamma phosphate groups could exit the filament. In their high-resolution filament model they discovered a phosphate bound to a site near the exit of this cavity. The bound phosphate makes contact with three adjacent protomers in the filament is proposed to stabilize the filament structure.

Figure 3.

Substrates and attachment geometries used for in vitro reconstitution of dynamic actin networks. **(a)** Nucleation promoting factors attached to solid particles, including glass beads and polystyrene microspheres [30]. This system mimics certain pathogens that have evolved to exploit the host cell cytoskeleton [29]. **(b)** Lipid-coated particles, including glass microspheres, are useful to maintain mobility of nucleation promoting factors and to study the contribution of lipids to the control of actin assembly [25**]. **(c)** Lipid vesicles have been used to assemble external actin networks. These systems can be used to probe forces actin on the surface and to mimic actin-dependent movement of endomembranes [31,32,33]. **(d)** Lipid vesicles encapsulating internal actin networks are more difficult to make and control but they mimic the geometry of actin networks that form the cell cortex [38]. **(e)** A lipid vesicle encapsulating a dynamic actin network and adhering tightly to a glass substrate mimics some of the features of a spreading cell [39*].

Figure 4.

Reconstitution of contractile actin networks. **(a)** Thoresen *et al.* [40^{*}] attached actin filaments to rigid microspheres using biotin-neutravidin linkages. The microspheres were attached to a flexible, poly-acrylamide substrate. When they added myosin II thick filaments (red particles) at sufficiently high concentrations they bundled overlapping filaments from adjacent particles. In the presence ATP, the bundles contracted, displacing the microspheres from their original positions (dashed circles). Knowing the stiffness of the substrate, Thoresen *et al.* were able to calculate force-velocity curves for their contractile bundles. **(b)** Reymann *et al.* [42^{**}] generated actin filaments by immobilizing nucleation promoting factors to microscale patterns on a glass surface and then using the Arp2/3 complex to nucleate filament assembly from the pattern. When two NPF patches of were placed in proximity, they produced a zone of anti-parallel filament overlap. When the authors added the processive, minus end-directed motor protein, Myosin VI, it crosslinked the bundles and produced strong contractile forces that eventually detached the network from the micropatterned surface and caused it to disassemble.