Old-School Actin Biochemistry

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We will cover some basic concepts in the analysis of actin polymerization kinetics and actin bundling.

1) Basic properties of actin
2) Actin preparation – key points
3) Actin polymerization kinetics – basics
4) Viscometric assays of actin – the falling ball assay
5) Spectrofluorometric assay of actin polymerization kinetics – the pyrene-actin assay

Note – many of the things covered fairly informally here have been covered in more detail (and with references provided) in some of my publications. Specifically, see the Methods in Enzymology 2006 article by Harris & Higgs. My 2005 TiBS review gives a pretty good account of the differences between "bulk" assays like pyrene-actin and single-filament assays such as TIRF. Some of my papers go into pretty thorough detail about kinetic assays, and give references for unlabeled actin and pyrene-actin preparation (Higgs et al 1999 Biochemistry, Li & Higgs 2005 JBC are two good ones). Of course, papers by Faix or Blanchoin or Bugyi could probably be used, but mine are much, much better.

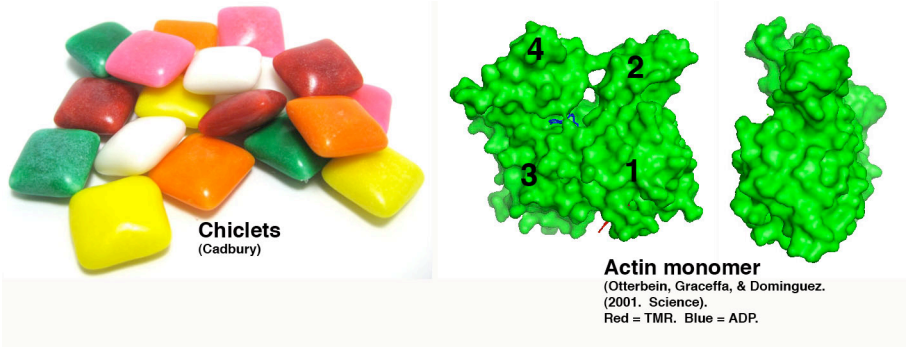
For falling ball assays, a review by Tom Pollard goes into great mathematical detail about its uses (T. D. Pollard (1982) Methods in Cell Biology. Volume 24, 301-311). The classic paper by Schleicher, Gerisch and Isenberg (1984, EMBO J.) shows its uses for purifying capping protein and alpha-actinin.

It is definitely worth reading the paper on the original polymerization studies on actin (Straub F. B. & Feuer G. (1950) Biochim. Biophys. Acta. Volume 4, 455-470).

Unless otherwise indicated, schematics of actin and actin polymerization are from the Pollard & Earnshaw textbook.

BASIC PROPERTIES OF ACTIN

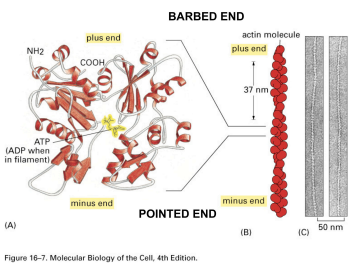
Actin is a 43 kDa globular protein with the relative dimensions approximately of a Chiclet. Do you know what a Chiclet is? It is (or, perhaps was) a type of chewing gum that's sort of square-shaped and reasonably flat (you might need to use your imagination, but the front and side views of an actin monomer are shown below).

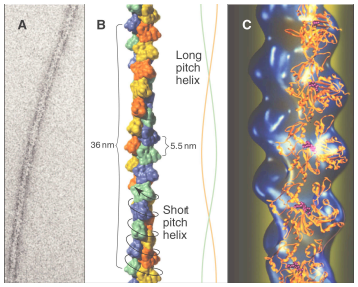


A few basic things about actin:

1) It has a tightly bound nucleotide deep down in the cleft between sub-domains 2 and 4 (the blue thing you can barely see in the actin chiclet above). That nucleotide is usually adenine-based (ATP or ADP), but actin can bind guanosine-based nucleotides as well. In cells, there's much more adenine nucleotide around and, in a healthy cell, there's much more ATP than ADP around. We'll learn later that actin is an ATPase, but I just point that out here.
2) If actin isn't bound to a nucleotide, the protein is pretty unstable and won't stay correctly folded for long (although the properties of nucleotide-free actin have been studied, see De la Cruz et al (2000) J. Mol. Biol. 295 (3), 517-526).
3) In order to be tightly bound, a divalent cation must be present to coordinate the nucleotide and the protein. In cells, magnesium is the divalent cation, because there's way more of it (about 0.5 mM) than any other (calcium's at about 100 nM in a resting cell, and spikes to <1000 nM upon activation). In the lab, we use calcium when we want to keep the actin monomeric (more on this later).

Actin filaments are right-handed, two-start helices. As far as we know, this is the only way actin polymerizes in cells. All the subunits in the filament face in the same direction (in the example below, all the groovy sides face down). This makes the filament polar (one end's different from the other). Some people call the ends "plus" and "minus", but I call them "barbed" and "pointed". ***Do you know why?***





Actin Preparation

For better or worse, pretty much 95% of everybody obtains their actin from the skeletal muscle of a vertebrate (rabbit or chicken mostly). Why? Because there's a lot of actin in muscle. There are procedures for purifying actin from lots of other places (red blood cells, yeast, etc) but they're pretty tedious and the yield is low, so you have to want it pretty badly. Skeletal muscle actin is >90% identical to non-muscle actin, and something like 85% identical to budding yeast actin. On the other hand, some properties differ between actins (notable differences have been found between yeast and muscle actin in nucleotide exchange), and some organisms have multiple isoforms expressed in the same cells (there are two isoforms of mammalian non-muscle actin, for example. I believe Drosophila has many actin isoforms, and Dictyostelium is a single-celled organism with a bunch of actin genes).

I'll just give an overview of how actin's isolated from muscle, so that you have a feel for the process. More detailed procedures are included separately. Here are the basic steps:

1) Find yourself a muscle source. I use rabbits, and make rabbit skeletal muscle actin (RSM-actin). Last time we did this (last June), we found a guy named Jim, about 40 miles away, to supply us with rabbits. Jim's a real New Englander, living by hunting, fishing, and I don't know what else. He told me on the phone he was going to charge $15/rabbit but, when we got there, somehow the price got raised to $25. We negotiated down to $20, but I didn't want to press it because we were way out in the woods and this is America, where guns roam free. Jim definitely looked like he might own several.
2) Sacrifice the animal and immediately bring its body temperature down. Why? Because you don't want rigor mortis to set in. Rigor mortis occurs when muscle myosin uses up all the ATP, releases its bound nucleotide, then binds extremely tightly to the actin filament (the "rigor" state or nucleotide-free state). So, you need to cool the muscle fast and keep it cool. I know that some people now use chicken breasts that have been rapidly frozen. I still use fresh rabbit (as Feuer and Straub did all those years ago) because I like interacting with people like Jim. So, what happened last June was that we brought coolers of ice with us, Jim sacrificed the animal (by hitting it on the back of the head with a baseball bat. Sounds bad, but it's really quick. The rabbit died immediately), his daughter cut off the head and drained the blood, then we plunged the rabbit's body into ice/water. We drove five rabbit carcasses back to the lab, and spent the rest of the day working away.
3) Take as much muscle as you can (from rabbits, most of the muscle is in the hind legs, but we take front leg and back muscle too). Last time we did this, we also skinned the rabbits and tried to cure the pelts in sulfuric acid/NaCl, for fun. We then forgot about them for 2 months (they were sitting in a bucket in a corner in the microscope room), and then my grad student, Ernest, made off with them. I think he made slippers for his girlfriend. They're no longer seeing each other, so what a waste. We should have made a rabbit fur loincloth, to be worn as a sacred ritual on subsequent preparation days.
4) Homogenize the muscle, then extract with 500 mM KCl, 100 mM di-basic potassium phosphate. This high-salt solution depolymerizes the myosin thick filament (not the actin thin filament). Because you've kept the muscle nice and cold, the myosin is largely not bound to the actin.

5) Centrifuge. The actin goes into the pellet, and the myosin into the supernatant.
6) Wash the pellet with water lots of times, to get rid of all the salt.
7) Extract with acetone, then with chloroform, to get rid of most of the lipids. You can now store this "acetone powder" for a long time in the freezer.
8) When you need some actin, you will "cycle" the actin. This means you will:
-depolymerize it by mixing some acetone powder in "G-buffer"
-centrifuge (with the actin monomers in the supernatant)
-save the sup. and repolymerize the monomers by adding polyerization buffer
-centrifuge (actin filaments now in the pellet)
-save the pellet and depolymerize by dialyzing in G-buffer
-centrifuge (actin now in supernatant)
-save the supernatant

9) After cycling, you will conduct size exclusion chromatography on the actin. Yes, you will!!! Some people don't do this, but you're a good scientist and, well, you will. Here are some reasons why:

a) oftentimes, there seems to be a low percentage of actin oligomers that persist after depolymerization/centrifugation. I'm not sure why these oligomers don't depolymerize, but they don't apparently. If one gel filters and uses the "back" half of the actin peak (in other words, the smaller stuff), one removes the majority of these.
b) Depending on how the actin is prepped, there might remain a small amount of capping protein. Muscle has lots of capping protein. It only takes a little bit of capping protein (less than 1:1000 ratio to actin) to change kinetics. Again, taking the back half of the peak greatly reduces CP contamination (CP is a heterodimer of two 30 kDa proteins, so it migrates at about 60 kDa as opposed to the 43 kDa actin monomer). *The presence of a little bit of capping protein actually speeds up actin polymerization (why?)*
c) When we have needed non-muscle actin, we have bought it (platelet actin) from a company who sells such things (I won't name the company, but it's located in the same region that's famous for a guy who "Thanks God He's a Country Boy"). The company freely admits that this actin is contaminated by a certain amount of gelsolin, if prepared the way they recommend. What they don't admit (or know) is that there is also a significant amount of Arp2/3 complex present (see Harris & Higgs Methods in Enzymology 2006 for a western blot). Who knows what other actin-binding proteins are present! Gel filtration does a good job at removing Arp2/3 complex (and gelsolin) from the actin bought from this company.

The first two things (stable nuclei and capping protein) will cause a decrease in the "lag" of actin monomer polymerization, because they provide nuclei for polymerization (oops, I gave the answer). You don't believe me? Test for yourself. Gel filter some actin, then test the polymerization of fractions from the beginning, middle, and end of the actin peak. Lazy people in my lab who have wanted to skip the gel filtration step (including me) have done this test and have found that the early fractions polymerize faster.

10) Actin is stored in the refrigerator. Don't freeze it if you want to do good kinetics. Many people (including me) have tried many methods of freezing and, to my knowledge, they all compromise the protein. Even "flash" freezing in liquid nitrogen doesn't work (incidentally, there's nothing "flash" to freezing in liquid N2 in a protein's world. At best, it takes a second,

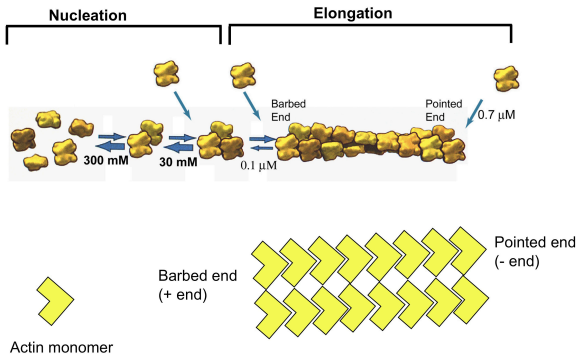
which is something on the order of a few thousand seconds in protein-time). Ideally, you keep the actin stored in a dialysis bag, dialyzing against fresh buffer, which renews the DTT and ATP. The DTT is the most important thing (actin has a very labile cysteine). In one of the Feuer & Straub papers, they use ascorbic acid for this purpose. Some people might use TCEP now, but I haven't tried it. Some people store actin at -20 degrees C in 50% glycerol. If you do this, you need to be aware that increasing viscosity changes actin polymerization kinetics (see Drenckhahn & Pollard (1986) J. Biol. Chem.) and you better keep a constant amount of glycerol in all your assays.

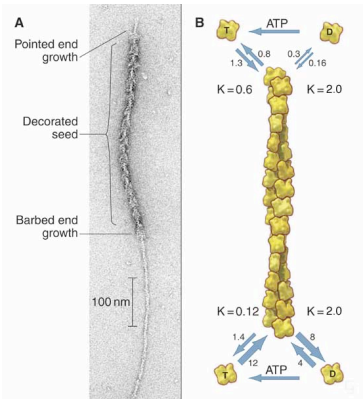
Actin Polymerization Basics

OK, enough about gel filtration. Above, I talk about "polymerizing" and "depolymerizing" the actin, but how do you do that? Here are some basic principles of actin polymerization:

1) Three major influences on actin polymerization are pH, ionic strength, and the nature of the divalent cation present.
2) Why is pH important? Actin is a fairly anionic protein (RSM-actin has a pI of 5.07), and its surface carries a net negative charge. Decreasing the pH increases polymerization rate by progressively neutralizing acidic residues. Much to the annoyance of my post-doc advisor, I tested this and found it indeed to be true ("why are you wasting time re-inventing the wheel?").
3) Why is ionic strength important? Actin is a fairly anionic protein (see above). At low ionic strength, the actin monomers repel each other to a greater extent than at high ionic strength. However, there's a balance, because if the ionic strength is too high it weakens the electrostatic interactions that contribute to actin polymerization (even though actin has a net negative charge, it still has 37 basic residues). Some papers by either Tom Pollard or Marie France Carlier back in the 1980s (I forget who showed this, probably both) show that the optimal ionic strength for polymerization of RSM-actin is somewhere around 50 mM KCl (or NaCl if you prefer).
4) Why is divalent cation important? Actin is an ATPase, and binds a nucleotide. As with almost all nucleotide-binding proteins, it requires a divalent cation for high affinity nucleotide binding. When Mg2+ is used as the divalent cation, actin is much more readily polymerizable than when Ca2+ is used (some sort of conformational change). Since the cytoplasm has much more Mg2+ than Ca2+ (by about 500-fold, even in the most hyper-stimulated cell in the universe (or, at least, any cell I know of)), actin is in the Mg2+-bound form in the cytoplasm.
5) So, given those three factors, we store our actin monomers in "G-buffer". The composition of G-buffer that my lab uses (it can vary a bit from lab to lab) is: 2 mM Tris-HCl pH 8.0 (at 23 degrees C), 0.5 mM DTT, 0.2 mM ATP, 0.1 mM CaCl2, 0.01% sodium azide. The buffer has low salt, relatively high pH, and Ca2+. We include azide to keep microbes from growing in the actin (don't freeze your actin if you want to do good kinetics). It is a mistake to think that actin cannot polymerize in G-buffer. It can. If you add some pre-formed filaments to high concentrations of actin monomers in G-buffer, they will add to the filaments. It's just that polymerization is extremely unfavorable (critical concentration above 100 uM).
6) Polymerization is induced by changing the buffer to one which simultaneously lowers the pH, raises the salt concentration, chelates the Ca2+, and adds Mg2+. In my lab, we typically use K50MEI, which is 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, and 10 mM imidazole-HCl pH 7.0 at 23 degrees. We add this to our actin monomers from a 10x stock, to give the final concentrations indicated above.

Actin has two "phases" of polymerization:
-nucleation, which consists of the dimerization and trimerization steps
-elongation, subsequent addition of monomers





Nucleation is really unfavorable, with equilibrium constants in the mM range (the constants given above are rough estimates, determined from kinetic modeling studies). Intuitively, one might imagine that these steps are unfavorable because the binding interface isn't as extensive as in the elongation phase.

Remember that actin filaments are polar, with barbed and pointed ends. Monomer addition is much more favorable at the barbed end.

It is a mistake to think that these are one-way paths! Actin monomers come on and off filaments even in the polymerizing state (in fact, they come off the barbed end faster than they come off the pointed end). In other words, the diagram above is incomplete (should have arrows in both directions at every step). For any binding reaction, the on-rate is concentration-dependent, whereas the off-rate is concentration independent. Therefore, actin will polymerize until the concentration of monomers reaches a point at which the on-rate and off-rate is equal. This is called the **critical concentration (Cc)**.

Look at the two graphs to the right, showing polymerization time courses for actin. In the top graph, actin starts as monomers. For the first phase of polymerization, not much happens. This is the nucleation phase, where nuclei are forming then falling apart before they can reach the stable tetramer stage. Even so, a few nuclei do form, and start to elongate. When enough nuclei build up, elongation becomes robust. When the concentration of monomers reaches the critical concentration (about 100 nM for actin), there is no more net polymerization. However, monomers continue to add to and subtract from both the barbed and pointed ends. ***Even when there is no net polymerization, there is a dynamic equilibrium at both ends of the filament.***

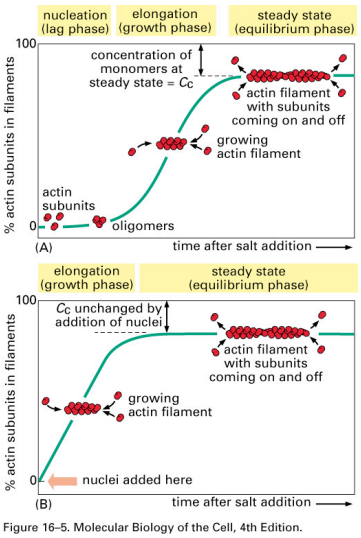


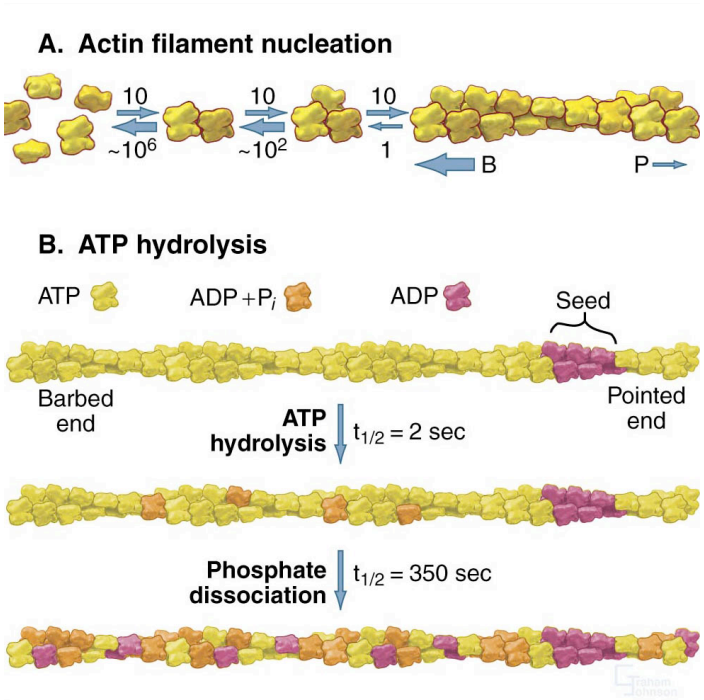
Figure 16–5. Molecular Biology of the Cell, 4th Edition.

Looking at the graph on the bottom, one can see that there is no lag. There is no lag because a few pre-polymerized actin filaments were included in the assay. These filaments served as nuclei, effectively by-passing the nucleation step.

An interesting and significant phenomenon during polymerization reactions is "annealing", or the end-to-end association of two filaments (pointed end of one filament to barbed end of another). Annealing occurs much more rapidly for short filaments than for longer ones, thus is a significant factor in solutions with high densities of short filaments. Proteins bound to barbed or pointed ends (capping proteins, formins, Arp2/3 complex) prevent annealing. See the following reference for detailed information and cool images of annealing (Andrianantoandro, Blanchoin, Sept, McCammon, & Pollard (2001) J. Mol. Biol.).

ATP hydrolysis

Remember, actin is an ATPase. Actin polymerizes most readily when it's ATP-bound, and the vast majority of actin monomer in cells is ATP-actin. When actin's a monomer, it hydrolyzes ATP slowly, very slowly. Upon adding to a filament, the hydrolysis rate increases dramatically. But, it is still quite slow compared to polymerization. Even slower is release of the phosphate after hydrolysis. This differential in speeds of polymerization, ATP hydrolysis, and phosphate release causes the filament environment to be different from one end to the other. ***Many things change once this phosphate is released!*** The polymerization dynamics change, and the ability of certain proteins (cofilin is a very important example, but a formin we work with, called INF2, is another) to interact with the filament changes.



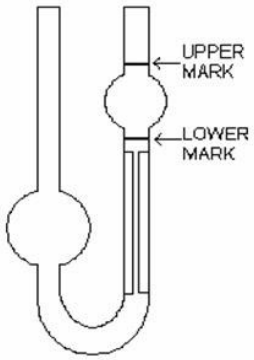
Measuring Changes to Actin by Viscometry: The Falling Ball Assay

In the beginning, there was the word. And the word was with viscometry and the word was…viscometry.

Before other assays came along, before pyrene-actin assays, before TIRF, even before decent spectrophotometers to measure light scattering, there was viscometry.

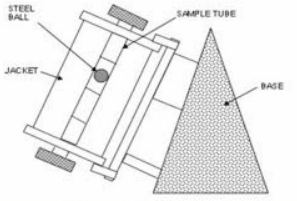
The basic principle is that actin filaments increase the viscosity of the solution to a much greater extent than do actin monomers. So, one can measure actin polymerization by the change in viscosity. That's how Feuer and Straub measured actin polymerization for the first time (take a look at there seminal paper: Straub FB & Feuer G (1950) Biochim. Biophys. Acta. "Adenosinetriphosphate. The functional group of actin).

The first viscometers used for this purpose were called Ostwald viscometers. Check out the picture. You filled this glass apparatus with fluid (in this case, your actin in polymerization buffer) until it was filled up to above the upper mark. Then, you sucked on the opening on the left side until the liquid got to the lower mark. Then, you released the suction and timed how fast it took the liquid to get back to the upper mark. The more viscous the solution, the longer the time. The problem with the Ostwald viscometer (apart from the sucking) is that it requires a lot of actin! Several mL for each experiment.



An Ostwald Viscometer

The advent of the falling ball assay was a significant advance. In this assay, you fill a capillary tube with your solution (we use 200 uL), wait for a while for the actin to polymerize, then drop a small steel ball into the tube. You time how fast it takes the ball to fall a specific distance in the tube, and that gives you a relative idea of the viscosity of the solution.



Falling Ball Viscometer

While one can use this assay to study proteins that affect actin polymerization, it's even more sensitive to proteins that crosslink actin. Falling ball viscometry was used to great effect in the early 1980s to purify several proteins:

1) Purification of several crosslinking proteins from Acanthamoeba extracts (MacLean-Fletcher & Pollard (1980) J. Cell Biol.).
2) Purification of capping protein and alpha-actinin from Dictyostelium (Schleicher, Gerisch, & Isenberg (1984) EMBO J.).

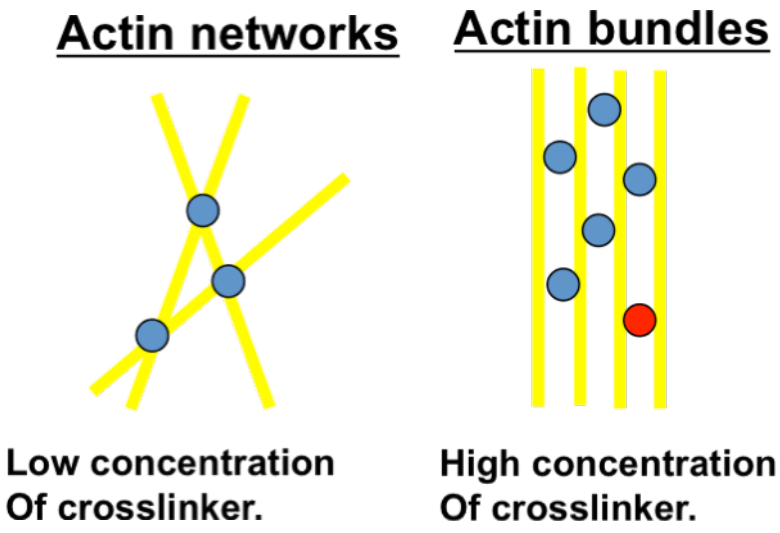
It's even been used more recently to characterize the bundling activity of proteins, by people who might be in the room with you right now! See Faix et al (1996) Cell.

Falling ball – the good, the bad, and the ugly:

The thing about falling ball viscometry is that it's sensitive to EVERYTHING! This is good and bad:

1) The good. If you are trying to purify actin binding proteins from an extract, falling ball is awesome. Pretty much anything that binds to actin will change the viscosity of it. Here are some examples of proteins that change viscosity. For a challenge, I want you to tell me what they will do to the viscosity (increase it above the viscosity of actin filaments or decrease it below the viscosity of actin filaments) and why:
   a) Thymosin beta4
   b) Capping protein
   c) Alpha-actinin
   d) Fascin
   e) Arp2/3 complex (active)
   f) mDia1 (active)
2) The bad. It is impossible to figure out what factor caused the viscosity to change. Here are a number of factors that will cause the same concentration of actin filaments to have a varying viscosity:
   a) Change in the number of nuclei
   b) cross-linking into a network.
   c) Cross-linking into bundles.
3) The ugly. Not sure there is an ugly here. Except if you're using an Ostwald viscometer and somebody surprises you when you are sucking on the tube.

In one of our falling ball experiments, we will demonstrate a phenomenon that occurs for most crosslinking proteins, and is especially clear with alpha-actinin. At low ratios to actin filaments, a-actinin causes network assembly, resulting in increased viscosity. At high concentration, a-actinin causes bundling, resulting in decreased viscosity. We will test the ability of another protein, the formin FMNL3, to do this.



Kinetic Analysis Of Actin Polymerization: The pyrene-actin assay

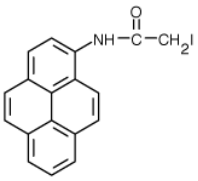
In comparing the differences between the actin and the microtubule fields, I find it striking that there is a lot more knowledge of biochemical mechanisms for actin binding proteins. Some may argue this, but I believe it to be more-or-less true. A major reason for this difference in knowledge is, I believe, the availability of a simple and robust method for assessing actin polymerization/depolymerization kinetics: the pyrene-actin assay. When used properly, variations on this assay can allow one to elucidate a huge amount of information on effects of a particular actin-binding protein. The pyrene-actin assay is free from many of the confounding features of viscometric assays (like falling ball) or from assays such as light scattering. As with any assay, however, it is not perfect, and knowledge of its attributes and limitations is important for its correct use. Nowadays, a combination of pyrene-actin assays and other assays (TIRF microscopy examination of single actin filaments) gives one even more analytical ability.

Basic attributes of the assay

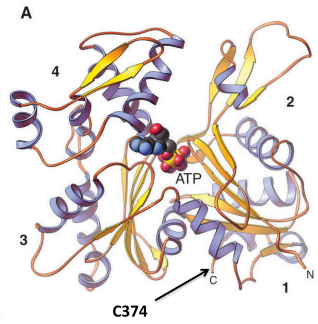
In short, here's how the assay works:

1) Actin monomer is labeled with the fluorophore, pyrene.
2) Pyrene fluorescence is highly sensitive to environment. It fluoresces much more in hydrophobic environments than when exposed to aqueous solvent.
3) A low percentage of pyrene-actin monomers is mixed with unlabeled actin monomers.
4) Polymerization is induced.
5) As the actin polymerizes, pyrene-actin monomers incorporate into the filament.
6) When a pyrene-actin monomer adds to the filament, the pyrene gets somewhat buried within the filament, and its fluorescence increases.
7) So, by monitoring the increase in pyrene fluorescence, one can monitor polymerization kinetics.

Actin is labeled on cysteines using pyrene-iodoacetamide. One remarkable thing is that only one cysteine on actin is modified by pyrene-iodoacetamide. This is cysteine 374, or the penultimate amino acid in rabbit skeletal muscle (RSM) actin. RSM-actin has five cysteines, but the other four are sufficiently buried so as to remain unlabeled in the procedure. The procedure is to label the actin under polymerizing conditions, then to pellet the filaments, then to depolymerize the filaments and gel filter them. This way, one is maximizing the odds that the labeled actin is polymerizable.



Pyrene-iodoacetamide (pyrene-IAA)



Pyrene-actin polymerizes with very similar kinetics as unlabeled actin, even if 100% of the actin is pyrene-labeled. This is remarkable, since labeling with other reagents at cysteine 374, such as tetramethylrhodamine (TMR) maleimide causes an almost complete loss of polymerization ability. In fact, a crystal structure of TMR-actin monomers has been solved by Roberto Dominguez (see figure on page 1), under conditions that would have polymerized unlabeled or pyrene-actin.

Here are some pointers on doing actin polymerization assays (with pyrene-actin or using other techniques):

1) One can induce actin (stored in G-buffer) to polymerize by adding K50MEI (see previous discussion).
2) However, to really measure kinetics accurately, it is advisable to convert actin monomers to the Mg2+ salt BEFORE inducing polymerization. This is because the Mg2+/Ca2+ exchange step is relatively slow, so the first 30 seconds or more of your polymerization assay will actually be slower, due to this exchange (I believe Marie France Carlier showed this in the 1980s). So, before adding the K50MEI, we mix our monomers with 0.1 volumes of something called "10E/1M". This is 10 mM EGTA/1 mM MgCl2. We add this to the actin in G-buffer, to make a final concentration of 1 mM EGTA/0.1 mM MgCl2. The EGTA chelates the Ca2+, and the Mg2+ then binds the actin. We do this for 2 min at room temp before starting the reaction.
3) Facts to remember when doing pyrene-actin assays in K50MEI:
    a. the barbed end critical concentration is about 0.1 uM actin.
    b. The pointed end critical concentration is about 0.5 uM actin.

    So, you will obtain no actin polymerization if you start with less than 0.1 uM, or less than 0.5 uM if capping protein is present (and you have no formins around, to compete with capping proteins). Typically, we conduct polymerization assays with 1-4 uM starting concentration of actin (depending on what we are testing). If your actin is in proper condition, you should see a noticeable lag in polymerization at these concentrations. If your actin hasn't been gel filtered, you might see little or no lag at any of these concentrations. In my hands, the lag becomes too short to measure at >5 uM actin.
4) We use a mix of pyrene-actin and unlabeled actin in most of our assays. Why? To minimize the undesirable effects of the pyrene label (see below, "The Bad"). Ideally, we use 5% pyrene-actin, but often we need a higher % to get good signal. We try to keep it at 20% or below. It is often a good idea to test the effects of the % pyrene (see Li & Higgs 2003 Curr. Biol., where we do this for mDia1).
5) Pay attention to buffer compositions when adding other components to pyrene-actin assays. What is the pH of the buffer? What is its ionic strength? Does it contain EDTA? How much volume of this component are you adding? For example, if you have a 1 uM solution of protein X that you want to test, and you want to test it at 0.5 uM, 50% of your reaction volume will be from protein X. If protein X is in a buffer containing 50 mM Tris pH 8.0, 250 mM NaCl, and 5 mM EDTA, you will totally alter the buffer composition of the polymerization reaction (raising the pH, raising the ionic strength, and chelating all the Mg2+). If your buffer contains a large amount of glycerol or sucrose, the increased viscosity will alter actin polymerization kinetics (see Drenckhahn & Pollard 1986 JBC). If your buffer contains high concentrations of

phosphate, this will alter kinetics of depolymerization as well as the ability of cofilin (and other proteins such as INF2) to bind to filaments and conduct their fantastic actions. This is because free phosphate can force the actin into the ADP-Pi state if at high enough concentration (my rule is that you should worry if you have over 1 mM, depending on pH. See Carlier & Pantaloni 1986 JBC for a nice titration of phosphate binding to filaments). There are several ways around these buffer issues: get your proteins into K50MEI, through dialysis or gel filtration; have your proteins at very high concentrations, so that you are diluting their buffers so much that they don't perturb the polymerization reaction; or save an aliquot of the buffer alone, and make sure your control (actin alone) reactions are conducted with this buffer added instead of your protein.

6) We routinely add 0.05% (about 850 uM) of the detergent called "thesit" (or nonaethylene glycol monododecyl ether, Sigma P9641) to our polymerization assays. The reason is that we find many of the proteins we work with lose activity if diluted too much (below 1 uM for many of them). This might be due to simple sticking to eppendorfs or to the 96-well plate. Thesit does a great job preventing that. We find no apparent effects of the thesit on actin dynamics. The reason we use thesit instead of Triton X-100 is that thesit does not have an aromatic ring, so it doesn't have significant spectroscopic properties. Triton's OK for pyrene-actin assays, but is no fun if you're doing FPLC runs and you like to see an OD280 trace.

Pyrene-actin assays: the good, the bad, and the ugly

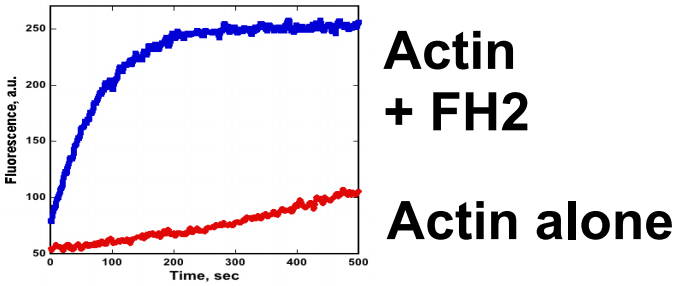
The Good:

There are several good things about the pyrene-actin assay:

1) High throughput. One can do a lot of assays quickly. Especially now, with good fluorescence plate readers (we have a Tecan M1000, which I love and highly recommend. Of course it's good. It's Swiss! Oh, I forgot, I'm in Germany. You guys like the Swiss, right?). It's a lot higher throughput than TIRF assays. We can do about 100 assays in a day, easily. This means we can test lots of conditions (concentration curves of several proteins, etc).
2) Versatility. One can test a number of things with the pyrene-actin assay: polymerization from monomers, elongation from pre-formed filaments, and depolymerization. See Harris & Higgs 2006 Methods Enzymol for polymerization and elongation assays, and Chhabra & Higgs 2006 JBC for depolymerization assays. This isn't unique to pyrene-actin assays (TIRF is actually even more versatile in many ways), but one can test all these effects with high throughput, which is nice.
3) Relative lack of perturbation of the pyrene label. Again, it is remarkable that the pyrene label does not affect polymerization or depolymerization kinetics very much at all. This is less true of some of the Alexa dye labeling procedures used for labeling actin for TIRF. Pyrene-actin isn't completely innocuous, though. We'll discuss that next.

The Bad:

1) The pyrene-actin assays are population-based assays. In other words, you're examining the ensemble changes to the entire population. In the curve shown below, you can't tell whether the effect of the formin tested here is on nucleation rate or on elongation rate. In the blue curve, did one filament elongate 20x faster than the single filament in the red assay, or did 20 filaments elongate at the same rate as the one red filament (there isn't only one red filament, but you get my point). You can figure this out by doing a combination of assays, but you can't determine this from only the assay shown. In TIRF assays, you are examining single filaments, and you can see how fast they are elongating, in addition to how many new filaments are created, which is very powerful.



2) Profilin's affinity for actin monomers is strongly reduced by the pyrene label. It's about a 10-fold decrease in affinity. If you are using 5% pyrene label, the effect on bulk actin polymerization is a lot less, but one needs to remember this affinity difference. Since profilin is a key player in actin dynamics, this is an important point! As a related point, one should not assume that the actin-binding protein being studied has the same affinity for pyrene-actin as it does for unlabeled actin. I must add that many of the labeled actins used for TIRF suffer from the same problem. In fact, this property has been used as a tool in some studies (see Kovar et al Cell 2006).

3) Some proteins change the fluorescence of pyrene-actin when they bind to it. Cofilin is a good example. Cofilin completely quenches the actin filament fluorescence, when bound 1:1 with the filament. Again, you should not assume that your protein does not have this effect. This is an easily testable effect.

The Ugly:

There isn't really an "ugly" to pyrene-actin, just good and bad. I just threw that in there because of the Clint Eastwood movie by that name. BTW, did you see Clint Eastwood make an ass out of himself by talking with an empty chair? Empty is a word that links well with the organization he was speaking to.