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Minireview

Motility assay: achievements and perspectives*

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Since, in 1984, Yanagida and coworkers [1] introduced labeling of actin with rhodamine-phalloidin for direct observation of motion of single F-actin filaments under fluorescent microscope, new possibilities for designing of the systems for actin-myosin interaction analysis appeared. Two years later Kron & Spudich [2] developed an *in vitro* motility assay which relies on the observation of the movement of fluorescently labeled actin filaments on the surface covered with myosin in the presence of ATP as energy source, and allows for measurement of velocity. Therefore, this assay provides some information about mechanochemical properties of contractile proteins.

Muscle contraction and other actomyosin-dependent motility phenomena are based on relative sliding of actin and myosin filaments fueled by chemical energy from the hydrolysis of ATP by myosin heads. However, in spite of numerous investigations many aspects of mechanochemical coupling and its regulation remain unclear. Until the development of the motility assay techniques, two main lines of study of the mechanochemical processes were possible: examinations of enzymatic activity of myosin in solution (providing no information on mechanochemical properties of contractile proteins) and physiological testing of mechanical parameters of contraction of the isolated muscles and muscle fibers (too complex to give detailed information concerning the molecular basis of contraction). The first *in vitro* motility assay system allowed for observation of the movement of myosin-coated beads over an ordered array of actin filaments derived from alga *Nitella axillaris* [3]. However, the present form of motility assay has the advantage of using purified proteins isolated from any source. Within only a few years it has been introduced into many laboratories.

The actin filament velocity in motility assays is analogous to the unloaded shortening velocity measured in muscle fibers [4, 5]. Detailed studies revealed that, under a wide range of conditions, filament velocity is indeed a good analogue of shortening velocity, although it is more sensitive to pH changes and to temperatures lower than 20°C [6].

In this review, microtubule-based motility test systems using video-enhanced light microscopy (e.g. [7]) will not be covered. Only some of the possible applications to solve particular problems concerning the actomyosin system will be discussed.

METHODOLOGY

A typical flow cell usually used for the observations of motion of individual actin filaments propelled by myosin immobilized on a glass surface is built from coverglass separated from the microscopic slide by glass spacers. A detailed description of the experimental procedures has been presented by Kron *et al.* [8].

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Briefly, myosin in an appropriate form (monomeric or filamentous, optionally myosin fragments) is attached to the surface of a glass coverslip, most frequently pre-treated with silicone (e.g. Sigmacote, Sigma) [9] or nitrocellulose [10]. After blocking of any remaining unsaturated protein-binding sites on the glass surface with bovine serum albumin, fluorescently labeled actin is introduced to the cell, excess actin is washed out and ATP is infused to initiate movement of actin filaments bound to myosin.

Photobleaching of the fluorescent label and protein damage by free radicals produced due to irradiation by UV light are the factors limiting the time of observation. To overcome this problem either 0.1 M dithiothreitol [11] or the glucose oxidase-catalase-glucose system [9] is used. In some cases it is convenient to include 0.5 - 1% methylcellulose to the assay buffer. Methylcellulose increases "macroviscosity" and to a large degree reduces the rate of dissociation of actin filaments from the myosin-covered surface while having no effect on the filament velocity [12].

Velocity of actin filaments can be measured manually (e.g. by overlaying a plastic sheet on the video monitor and marking of positions of moving filaments) but such methods are dependent on the observer's objectivity and are laborious and time-consuming. Therefore, computer-based tracking systems recently have begun to be used. The first such system was described by Work & Warshaw [13]. A similar system, with software written by Optotech (Warsaw), is at present being used in the authors' laboratories (Fig. 1). A typical digitized image of fluorescently labeled actin filaments after filtration and background subtraction is presented in Fig. 2.

An interesting alternative monitoring system was proposed by Borejdo & Burlacu [14]. This confocal microscopy- and fluorescence correlation spectroscopy-based system for measurements of velocity and numbers of filaments does not require imaging of filaments; fluctuations in light intensity arising from the change in the number of the actin filaments present in the sample volume (defined by a diaphragm placed in front of the photomultiplier) are analyzed. Velocity is defined as the ratio of the diameter of the diaphragm to the half time of the relaxation of fluctuations measured by an autocorrelated function.

It should be noted that in the motility assay system, it is possible to measure the rate of the ATP hydrolysis by immobilized myosin or myosin fragment, in parallel with measurements of filament velocity, in the same experimental cell. This possibility of simulta-

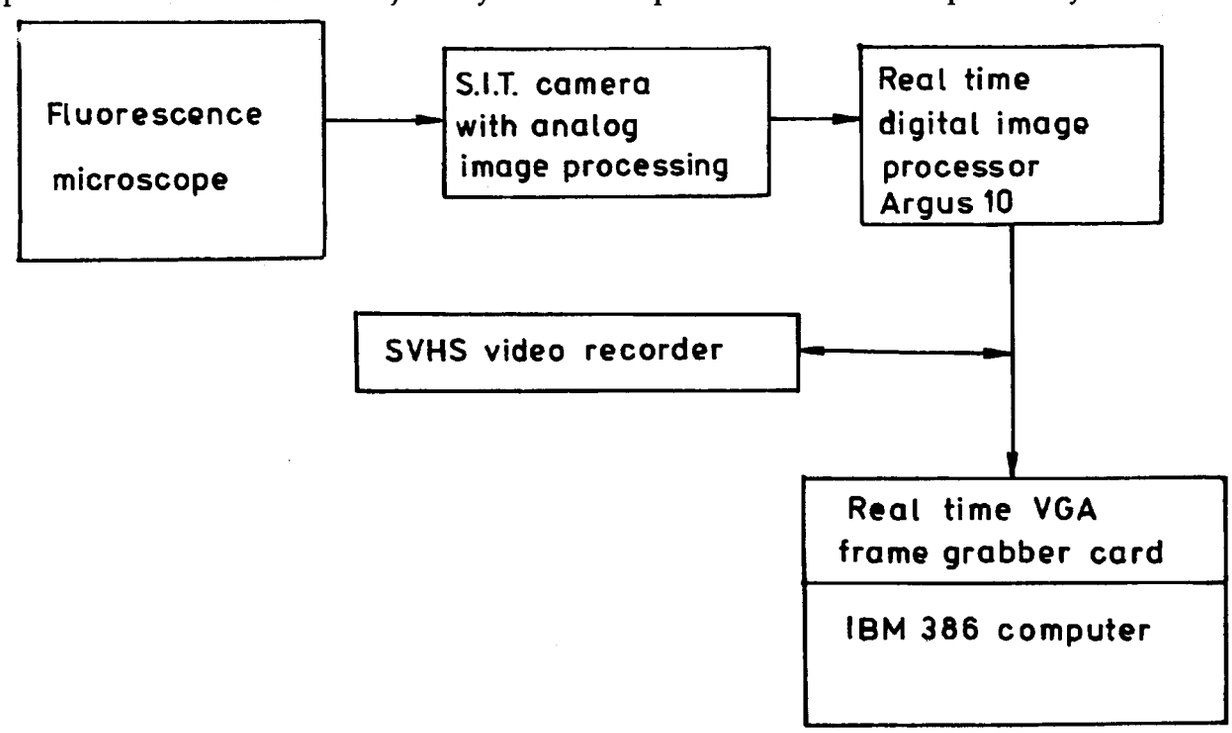

Fig. 1. Block diagram of instrumentation.

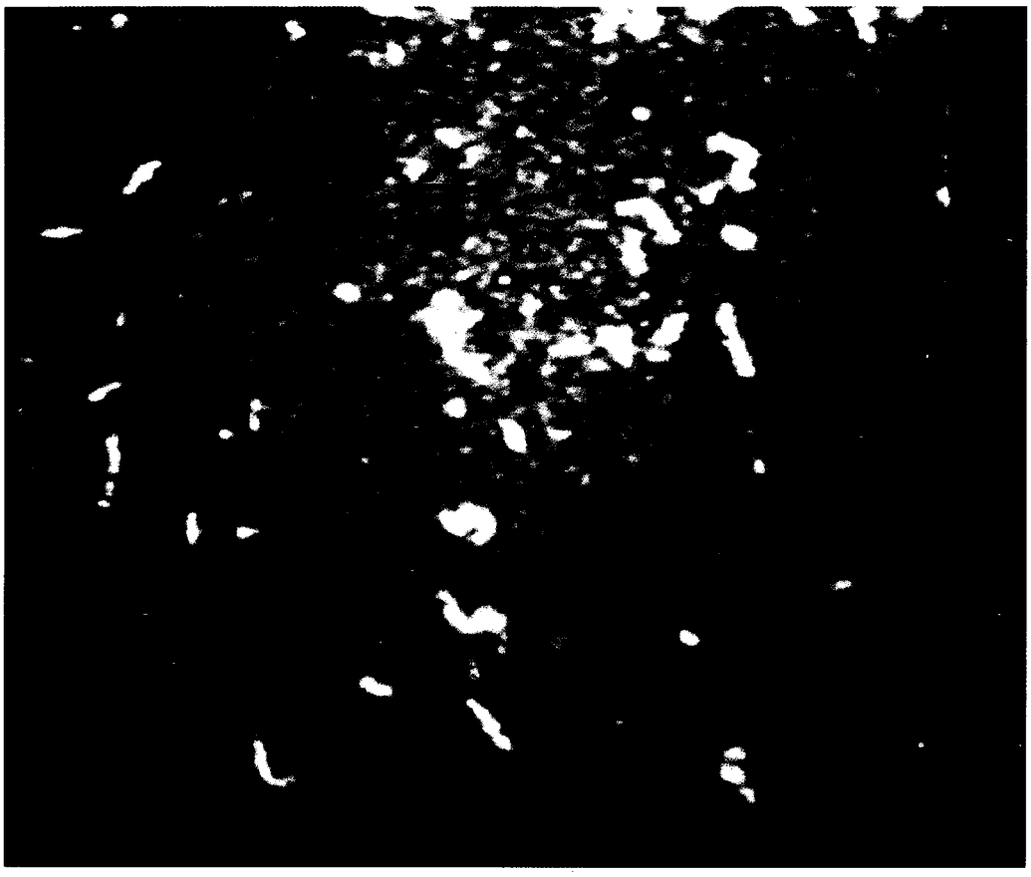

Fig. 2. Typical digitized image of fluorescently labeled actin filaments after filtration and background subtraction.

neous measurement is important for two reasons: it enables simple determination of the amount of immobilized myosin (after determining the ratio of specific activity of myosin in solution to that of immobilized myosin; the activity is proportional to the amount of immobilized protein [15]) and secondly it makes possible to test the correlation between actin filament velocity and actomyosin ATPase activity under various conditions.

Finally, the motility assay system has the advantage of the possibility to develop various modifications in order to solve particular problems. Some of these modifications are mentioned in the following sections.

APPLICATIONS OF THE MOTILITY ASSAY METHOD

Structure-function analysis of myosin

One obvious application of the motility assay method is the comparison of the motile properties of contractile proteins isolated from different species and tissues. Results of these investigations performed for vertebrate muscle myosins are summarized in Table 1. From these data one can conclude that the well known rough correlation of actin-activated myosin ATPase activity with the velocity of contraction of source muscle is also reflected in the results of the motility assay.

In skeletal muscle cells myosin is assembled into filaments. It was shown that both filamentous and monomeric myosin can propel actin filaments in a motility assay [10]. The filaments of one-headed myosin (with one head digested off) were also able to produce movement [16]. Even reducing the number of heads in the filaments formed from one headed myosin by "shaving off" about 90% of the heads by subsequent papain digestion did not prevent the motility of actin filaments [16]. One can conclude that neither assembly into filaments nor the presence of both heads in myosin molecules is necessary for motility.

Digestion of the myosin molecule into proteolytic fragments, light meromyosin and heavy

Table 1

Comparison of velocities of actin filaments moving over surfaces coated with various myosins

Myosin	Velocity μm/s	Temperature °C	Reference
Skeletal, filamentous	3 - 4	24	[2]
Skeletal, filamentous	5.4	24	[9]
Skeletal, filamentous	3.25	22	[21]
Skeletal, filamentous	3.9	30	[10]
Skeletal, monomeric	3.5	30	[10]
Skeletal, monomeric	11.2	30	[31]
Skeletal, monomeric	5.5	22	[31]
Smooth, filamentous*	0.3	22	[21]
Smooth, filamentous*	0.35	24	[22]
Smooth, filamentous*	0.3	22	[21]

* Phosphorylated

meromyosin containing two heads or subfragment-1 containing only one head and rod fragment, does not destroy actin-activated ATPase activity which is retained in head-containing fragments. The possibility that these fragments could also retain the ability to produce movement has been investigated in a series of experiments. The heavy meromyosin and subfragment-1 were tested [10] and proved to be active in producing motility, however with smaller velocity than intact filamentous myosin. One of the reasons for this difference may be the reduced amount of light chains observed in proteolytic fragments. Lowey *et al.* [17] found that the presence of light chains in a myosin molecule is essential for the conversion of chemical energy into movement. It has been proved that, to cause the movement of actin filaments, an intact myosin head is sufficient. Therefore, the hypothesis of Harrington [18] that force generation requires the swivel part of the myosin molecule involving the rod, has been questioned. However, it is still an open question whether the force generated by a one-headed fragment (subfragment-1) is comparable to that generated in the muscle.

As opposed to skeletal muscle myosin, smooth muscle and non-muscle myosins require calcium/calmodulin-dependent myosin light chain kinase-mediated phosphorylation of the regulatory light chain to show actin-activated ATPase activity and to be active in the motility assay (e.g. [5, 19 - 22]). Phosphorylation of the light chains of these myosins was also shown to be necessary to initiate contraction of smooth muscle or, as an example of the non-muscle system, activation of platelets *in vivo* [19, 20, 23]. It was reported [24] that not only phosphorylated smooth muscle and platelet myosin filaments, but also monomers of these phosphorylated myosins, propel actin filaments, demonstrating that myosin filament formation is not necessary for movement. On the other hand, recently it was shown that a monoclonal antibody against the rod portion of smooth muscle myosin interferes with filament assembly of phosphorylated myosin, reducing also actin-activated ATPase activity and actin filament sliding velocity [25]. Thus, further studies are necessary to clarify the requirement of proper myosin filament assembly for myosin activity.

Myosin-Is, a class of monomeric myosins with a single head that, as in "conventional" myosins, transduce chemical energy into mechanical work upon interacting with actin, are the subject of increasing interest (see [26, 27]). Without going into details, it is worth noting that myosin-Is are believed to be responsible for movements of actin relatively to the cell membrane *in vivo*. Tails of myosin-Is contain an acidic phospholipid-binding site and, in some isoforms, an ATP-insensitive actin-binding site. It was demonstrated that *Acanthamoeba*

myosin-I with a phosphorylated heavy chain, bound to lipid-coated glass surface, propells actin filaments in the motility assay [28]. Thus, it appears that this experimental system is also suitable for studying properties of molecular motor molecules supported by lipid surfaces.

Myosin step size

One of the basic questions concerning the interaction between myosin and actin in the presence of ATP is the question about the distance which actin filaments slide per one molecule of ATP split by myosin. In other words, what is the "step size" of the myosin molecule? Assuming that filament sliding, which occurs while myosin is bound to a single site on actin, is coupled to ATP hydrolysis on a one-to-one basis, a natural limit for a myosin step would be the doubled chord length of myosin head, i.e. about 40 nm. This assumption stems from the model of contraction in which the myosin head rotates. Several experiments involving mechanical measurements have resulted in estimation of the step size being well below this limit (10 - 20 nm) [29]. Studies including the sliding of fluorescently labeled actin in contracting myofibrils [30] and *in vitro* motility assay [31] have suggested that the step size of myosin may be in the range of 60 - 200 nm, much higher than the structural limit of 40 nm. These values of step size would require a new model of coupling between the ATPase cycle and mechanical events. One would have to accept a "one-to-many" fashion of interaction between ATP, actin and myosin, i.e. one molecule of ATP hydrolysed would provide energy for multiple cycles of interaction between actin and myosin. However, Spudich and colleagues [12, 32, 33] using a similar motility assay reported step sizes close to the values given by conventional theory. This discrepancy led to new investigations of the problem involving muscle mechanics [34, 35]; these investigations did not exclude the possibility of multiple interactions between actin and myosin per one ATP molecule. There are certain differences in the experimental conditions used by Yanagida and colleagues and the Spudich group. These differences have been discussed by these authors and also by Burton [29]. Perhaps further experiments that would relate the different conditions applied by these two groups would lead to explanation of these discrepancies.

Yanagida and his colleagues have developed recently a new system for measuring forces produced by a small number of heads interacting with a single actin filament [36, 37]. The conclusion of these experiments was that a single head can produce a force which is comparable with that estimated from mechanical measurements [36], and that heads interacting with an actin filament produce fluctuations of force. The analysis of these fluctuations led to the conclusion that the mechanical to chemical coupling ratio is one-to-one in isometric conditions but many-to-one during filament sliding [37]. The authors proposed that, when work is done at higher velocity, the myosin head directly uses the free energy liberated by the hydrolysis of a single ATP molecule for many working strokes.

Factors determining direction of movement

Both actin and myosin filaments have well organized structures with polarity imposed by the arrangement of actin and myosin molecules. The relative movement of filaments in muscle occurs in a definite direction from Z-line toward the center of sarcomer. In a series of experiments it was shown that the direction of movement is determined by actin filament polarity. Toyoshima *et al.* [38] has formed tracks of myosin heads on a nitrocellulose surface by placing actin filaments decorated with heavy meromyosin. Actin was then released by treatment with ATP and washed out. Rhodamine-phalloidin labeled actin filaments were introduced to the flow cell and their movement on the prepared tracks of heavy meromyosin was observed. Movement occurred in both directions along the tracks with approximately the same velocity. Sellers & Kachar [39] used large native thick filaments isolated from clam adductor muscles, bound to glass surface to observe movement of fluorescently labeled actin filaments, both toward the center of the thick filament and away from the center. The actin polarity determined the direction of movement also in this case. However, the velocity of movement in the two directions was different. The direction of the movement of actin filaments away from the center of the filament was opposite to the direction of movement which occurs in the muscle and the speed observed in

this case was much lower than in the direction toward the center. The authors concluded that the actin filament polarity determines the direction of movement but the speed of movement depends on proper arrangement of myosin molecules in the filament, i.e. myosin molecules have more freedom to propel actin in the direction toward the center of thick filament. The difference between the results of Toyoshima *et al.* [38] and Sellers & Kachar [39] may result from the fact that in the first case the heavy meromyosin molecules were less ordered than molecules of myosin organized in filament. The observations of Sellers & Kachar [39] were confirmed by Yamada & Wakabayashi [40] on reconstituted rabbit myosin filaments; however, the difference between velocities of movement away from the center of filament and toward the center was smaller than in the case of native filaments from clam adductor muscle. This may result from differences in structure of reconstituted and native filaments.

Motility assay in investigating the actin structure role in the motility phenomenon

Sequences of actins derived from different species are very similar. However, actin isoforms from different tissues of vertebrates are slightly different. The comparison of actin from skeletal muscle and smooth muscle did not reveal any differences in the velocities of filaments formed from the two kinds of proteins [24, 41]. This result is in agreement with the opinion that the speed of contraction depends on the type of myosin, and differences in actin isoform sequences seem to be related to specific interactions of actin with different actin-binding proteins from various cells or cell compartments. Moreover, it appeared that actin of an organism as divergent from vertebrates as yeast is also able to move over rabbit muscle myosin-covered surface, although with only half of the velocity of rabbit skeletal muscle actin [42].

For a long time actin has been considered as only a passive cable on which the active myosin heads are moving. It has been known that actin activates the MgATPase of myosin and interactions between these proteins are associated with conformational changes of the actin molecule [43 - 45] that are restricted in the presence of thin filament-associated inhibitory proteins [46]. Some authors [47, 48] have proposed that the conformational changes within actin protomers in the filament may contribute to the muscle contraction. The motility assay allows for more direct correlation of these conformational changes of actin with the movement. An interesting approach to the investigation of this problem is to modify actin molecules, copolymerize them with intact actin and study their motility behavior. Thus, Shwyter *et al.* [49] used actin molecules cleaved with subtilisin (nicked between Met-47 and Gly-48), and Próchniewicz & Yanagida [50] employed crosslinking of monomeric and filamentous actin. Subtilisin-digested actin forms filaments and retains several properties of intact actin, such as binding of heavy meromyosin, dissociation from heavy meromyosin by ATP and activation of the MgATPase of heavy meromyosin (although with significantly lower K_{app}). Copolymers of intact and nicked actins, up to approx. 50% of cleaved actin, moved with the same velocity as native actin filaments. At higher contents of modified actin, the average velocity and percentage of moving filaments decreased [49]. Similarly, copolymers of unmodified and cross-linked actin monomers showed reduced motility [50]. So, it is possible to inhibit the motile function of actin and simultaneously to retain its ability to bind to myosin heads and to activate myosin ATPase. This may lead to the conclusion that actin is not only the activator of myosin MgATPase but that the conformational changes occurring within its molecule and/or filament are also required for motility.

Regulatory thin-filament proteins

The *in vivo* composition of actin filaments differs between organisms, tissues and cell compartments, both with respect to actin and tropomyosin isoforms and presence of regulatory proteins. In general, in all tissues, the actin-myosin interactions leading to movement are regulated by intracellular calcium ion concentration. In vertebrate skeletal muscle the thin filaments component, the troponin complex, is the factor responsible for conferring the calcium sensitivity, whereas in smooth muscle and non-muscle cells regulation is provided mainly by phosphorylation of myosin light chains (see above). Caldesmon and calponin, the proteins present in actin filaments of smooth muscle and non-muscle cells, are be-

lieved to be involved in additional, thin filament-linked regulation. Non-vertebrate regulatory systems will not be discussed in this review.

To date, little attention has been paid to the behavior of the regulated skeletal muscle actin (a ternary complex of F-actin, tropomyosin and troponin) in the motility assay. As one could expect, for unregulated actin the measured velocity is Ca^{2+}-independent. Regulated actin filaments move with a velocity that is slightly dependent on Ca^{2+} concentration within pCa between 3 and 5.5. At pCa 5.8 and higher filaments do not show movement. All these results are essentially in agreement with the data obtained in the ATPase assay under the same conditions, although ATPase activity is less dependent on pCa and temperature than filament velocity [51]. Similar results were reported in [31].

Both skeletal and smooth muscle tropomyosin were reported to increase actin filament velocity to a similar extent (about 2-fold stimulation) [22], although these two proteins exhibit opposite effects on actomyosin ATPase activity [52]. The mechanism of action and even the regulatory role of caldesmon and calponin, components of the smooth muscle and non-muscle cell actin filaments, are presently a matter of dispute. Results of biochemical studies (in particular inhibition of actomyosin ATPase activity; see [53]) make these proteins good candidates for thin-filament linked regulators of smooth muscle contraction. Unfortunately, results so far obtained in the motility assay are inconsistent. It was reported that caldesmon inhibits the velocity of actin filaments in a dose-dependent fashion [54], that it has a concentration-dependent dual effect (stimulatory at low concentrations and inhibitory at high ones) [55], and that caldesmon-induced inhibition is displayed only with oxidized, disulfide bridge-crosslinked form of caldesmon but monomeric caldesmon is able to induce motility of actin filaments under conditions in which it does not occur due to weak binding of actin to myosin [56]. In the one report concerning the effect of calponin which has appeared to date, this protein was shown to inhibit filament movement in an "all or none" fashion, i.e. by decreasing in a dose-dependent manner not the average filament velocity but the number of moving filaments [54].

CONCLUDING REMARKS

The *in vitro* motility assay has been used to solve many problems which were outlined in this review. The possibility to measure quantitatively the motile function of proteins at the molecular level is the major advantage of this method. The method can be used in wide areas of research on motility. Since the time of introducing the motility assay the number of laboratories using it has grown remarkably; it is slowly becoming a standard method for studying the properties of myosin and actin from different sources. Combination of the motility assay with recombinant DNA techniques offers a powerful tool for studying the structure and motile function interrelationships [57]. Especially interesting are some of the modifications of the method designed to solve particular problems. The system designed for measuring the force produced by a single actin filament interacting with myosin heads attached to a glass surface [36, 37] was described above. Further progress in the field of measurements of force produced by a small number of myosin molecules probably will be facilitated by the use of "optical tweezers" [58]. Another modification has been developed recently by Nishizaka *et al.* [59]. These authors, in an excellent experiment, showed that the sliding force has a right-handed torque component causing right-handed rotation of the actin filament around its long axis. Certainly, in the next few years more modifications of the motility assay will appear, including other labeling methods than by rhodamine-phalloidin, and other methods of detecting moving filaments.

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