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Control of Actin Moving Trajectory by Patterned Poly(methylmethacrylate) Tracks

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ABSTRACT Poly(methylmethacrylate) (PMMA), a photoresist polymer, was found to be useful for immobilizing heavy meromyosin (HMM) molecules while retaining their abilities to support the movement of actin filaments. PMMA substrate was spin-coated on a coverslip, and various shapes of PMMA tracks, such as straight lines, concentric circles, and alphabetical letters, were fabricated by UV photolithography. An observation by a Tapping mode atomic force microscope (AFM) shows that the typical circular tracks were 1–2 μm wide and about 200 nm high. In in vitro motility assay, a solution of HMM molecules was applied to immobilize the molecules on the tracks by adsorption, and movement of actin filaments labeled with tetramethylrhodamine-phalloidin were observed in the presence of ATP by using an epifluorescence microscope and an image-intensified CCD camera. Actin filaments were seen to move precisely only on the PMMA tracks, and their traces drew the exact shapes of the tracks. The mean velocity of actin movement on the PMMA was 4.5 mm/s at 25°C, and it was comparable to that on a conventionally used nitrocellulose film.

INTRODUCTION

The recent development of an in vitro actin motility assay has made it possible to study the actomyosin motile system in a simple and controllable model system of muscle contraction (Kron and Spudich, 1986). Individual actin filament labeled with a fluorescent dye, tetramethylrhodamine-phallodin, can be observed to be moving on myosin molecules immobilized on a coated coverslip, such as a siliconized coverslip (Harada et al., 1987, 1990) or a nitrocellulose-coated coverslip (Toyoshima et al., 1987, 1990; Kron et al., 1991), in the presence of ATP with a fluorescence microscope and highly sensitive camera system. This in vitro model system of muscle contraction is used to study force and velocity generation by myosin and its proteolytic fragments (heavy meromyosin (HMM) and subfragment-1 (S-1)) to elucidate the molecular mechanism of the actin-myosin force generation system. Huxley reviewed the problems in muscle research by this in vitro method (Huxley, 1990). The actin filaments in most of the conventional in vitro systems, however, meandered around in random directions on the surface because myosin molecules or their fragments, which could interact with the actin filaments, were distributed uniformly and randomly on the coated coverslip.

The actomyosin system has great potential for application to a novel microsize actuator for the micromechanical system. The in vitro motility assays have elucidated that actin and myosin (or its fragments) are essential for the sliding movement and the force generation. Because these proteins are smaller than mechanical components that we can fabricate, a smaller actuator for the micromechanical system can be composed in an in vitro system using the protein molecules. To apply them to a motor device, it is necessary to find out techniques to regulate the trajectory and the direction of the sliding movement in the in vitro motility system.

The simple idea of the control method is to confine the trajectories of the movement by the motor protein molecules immobilized on patterned substrates. Studies using two kinds of patterned substrates demonstrated this idea. One substrate is a poly(tetrafluoroethylene) (PTFE) thin film (Suzuki et al., 1995). The PTFE thin film was made by rubbing a heated coverslip surface with a PTFE rod while applying pressure (friction transfer method), and it consisted of many linear and parallel running PTFE ridges 10 to 100 nm wide on a coverslip. HMM molecules were immobilized on the ridges by adsorption while retaining their ability to support the actin sliding movement. The actin filaments were seen moving on the ridges linearly. With this method, however, the widths and the heights of the ridges were very difficult to control, and the velocity of the actin movement was slower than that on the siliconized glass surface. The other is an aminosilane-coated glass surface. Turner et al. attempted to regulate the movement of another motor protein system, the kinesin-microtuble motile system, on a patterned aminosilane-coated glass (Turner et al., 1995). Linear silanized regions about 2–8 μm wide were patterned by an excimer laser beam exposure, and many microtubles aligned by fluid flow were selectively adhered to the regions. Kinesin-coated beads could be observed to be moving on the microtubles by using an optical microscope. In this system, however, the moving distance of the kinesin bead strongly depended on the length of a single microtubule. This is because the degree of alignment of microtubles was not sufficient to form a single track, and the polarity of the microtubules could not be regulated.

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In this report we chose poly(methylmethacrylate) (PMMA) as a substrate for immobilization of HMM molecules because PMMA is a popular positive photoresist that can be easily patterned photolithographically. In addition, several groups have reported that protein molecules bind to PMMA with great affinity (Hiraoka et al., 1985; Gizeli et al., 1992). We fabricated various patterns of tracks of PMMA on a coverslip by photolithography. After HMM molecules were immobilized on them by adsorption, an in vitro motility assay was carried out. Fluorescently labeled actin filaments were observed, and their trajectories were strictly confined to the PMMA tracks.

MATERIALS AND METHODS

Substrate preparation

The tracks of PMMA on a coverslip were fabricated in three steps: 1) A coverslip (thickness 0.12–0.17 mm) was cleaned with 0.1 N KOH solution and ethanol, rinsed with distilled water, and dried in air. The PMMA film on the coverslip was prepared by spin casting at 2000 rpm for 5 s, then at 4000 rpm for 90 s, and baked at 170°C for 20 min in an oven. PMMA in ethylcellosolveacetate solution (OEBR-1000) was purchased from Tokyo Ohka Kogyo Co. (Tokyo, Japan). 2) The PMMA-coated coverslip was irradiated with UV light by a UV exposure system MJB3 (Karl-Suss, München-Garching, Germany) through a mask for 5 min to a dose of about 1050 mJ/cm^2 at 240 nm. 3) The film was developed at 20°C in a solution of 90% isoamylacetate and 10% ethylacetate (developer for OEBR-1000, Tokyo Ohka Kogyo Co., Tokyo, Japan) with continuous stirring, rinsed with distilled water, and then baked again at 170°C for 20 min to remove the developer solvent because the organic solvent denatures the motor protein. These procedures produced the PMMA tracks on the coverslip. The conditions, such as exposure time and developing time, depend on the temperature and the intensity of the UV light. Lack of UV exposure or a short development time left PMMA residues on the glass surface.

The lithographic mask used in this experiment had 100 sets of patterns; each set contains lines, concentric circles, figure-eight patterns, and alphabetical letters (Fig. 1). The linewidths of the circles and figure-eight patterns were 1 and 3 μm and those of the letters were 3, 5, and 8 μm. The patterns were made of chromium on 0.09-inch quartz glass. The interval between two pattern sets was about 2 mm. About 80 patterns were transferred to a PMMA film on a 24 × 40 mm coverslip by using a UV exposure process.

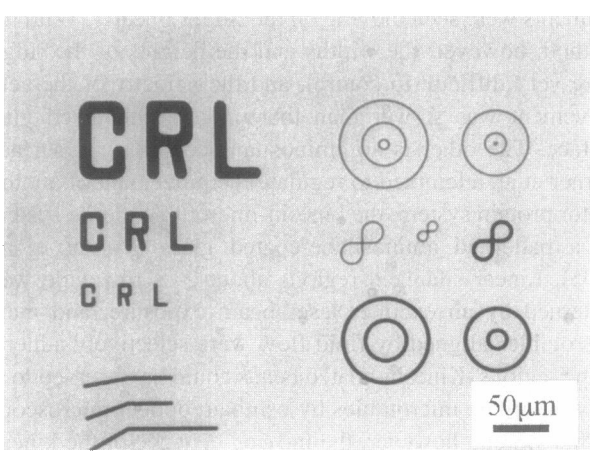

FIGURE 1 Lithographic mask pattern used to fabricate the PMMA tracks. The linewidths of the triple concentric circles were 1 μm, and those of the double concentric circles were 3 μm. The lines of the alphabetical letters were 8, 5, and 3 μm in width.

An atomic force microscope (AFM) image of the PMMA tracks was acquired in air using a Bioscope (Digital Instruments, Santa Barbara, CA). All images were obtained in Tappping mode using silicon cantilevers with a spring constant of 40 ± 11 N/m purchased from Digital Instruments.

A nitrocellulose-coated coverslip was used as the reference substrate for the in vitro motility assay. The nitrocellulose-coated glass was prepared by a method similar to that described by Kron et al. (1991). A drop of 2% (v/v) collodin in amylacetate was placed on the surface of clean distilled water in a glass dish and left, after covering with a larger beaker to prevent dust contamination, to evaporate for 15 min to form a clear film. A coverslip, 24 × 40 mm, was gently placed on the film. The coverslip with the film was grasped by forceps and flipped in the water, and then brought out of water. The coverslip was left on a filter paper to dry.

Protein preparation

Myosin was extracted from the back and leg muscles of a rabbit and purified, and heavy meromyosin (HMM) was prepared from it by using chymotrypsin according to the method of Margossian and Lowey (1982). G-actin was prepared from rabbit skeletal muscle acetone powder (Pardee and Spudich, 1982) and polymerized with a slight molar excess of tetramethylrhodamine-phalloidin (Molecular Probes) in the assay buffer solution. The assay buffer solution used in the experiment consisted of 40 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 10 mM dithiothreitol, and 20 mM HEPES (pH 7.8).

Experimental system

The cell was constructed from a coverslip with PMMA tracks and a nitrocellulose-coated coverslip; the procedure was modified from that described by Kron et al. (1991). Two parallel lines of grease were dispensed from a syringe and placed symmetrically about 20 mm apart on the PMMA coverslip. Then HMM (0.1 mg/ml) in the assay buffer solution was dropped onto the PMMA tracks on the coverslip and the nitrocellulose-coated coverslip was placed on the PMMA coverslip and pressed gently. HMM molecules were adsorbed onto the PMMA tracks and a nitrocellulose-coated surface while the cell was kept still for 5 min. Unbound HMM molecules were washed out by infusing the assay buffer solution from one side of the cell while removing the surplus solution from the other side, and then the assay buffer solution containing actin filaments labeled with tetramethylrhodamine-phallodin, 1 mM ATP, 5 mg/ml glucose, 50 μg/ml glucose oxidase, and 10 μg/ml catalase was introduced into the cell. Bovine serum albumin, added in most of the conventional in vitro motility assay for blocking unoccupied binding sites on the nitrocellulose surface, was not used in this experiment to simplify the system.

Actin filaments moving on the surface were observed at room temperature (24–25°C) with an epifluorescence microscope (Olympus BX-50) and recorded with an image-intensified CCD camera system (Hamamatsu Photonics C2400-87) and a Hi-8 VCR set (Sony EV-NS7000NTSC). Image processing of accumulation for drawing the traces of actin filaments was performed by ARGUS-10 (Hamamatsu Photonics).

The recorded video images were transferred to a rewritable optical disk by an optical disk recorder (Panasonic LQ-4100A) to analyze the images frame by frame. The image was captured from the optical disk with an interval of 10 frames by Image Analyst (Automatix, Billerica, MA) on a personal computer (Macintosh Centris 650). The velocity was calculated from displacement data of the centroid of the randomly selected actin filaments.

RESULTS AND DISCUSSION

AFM observation

An AFM topographic image and a cross section of the concentric circular PMMA tracks are shown in Fig. 2. They

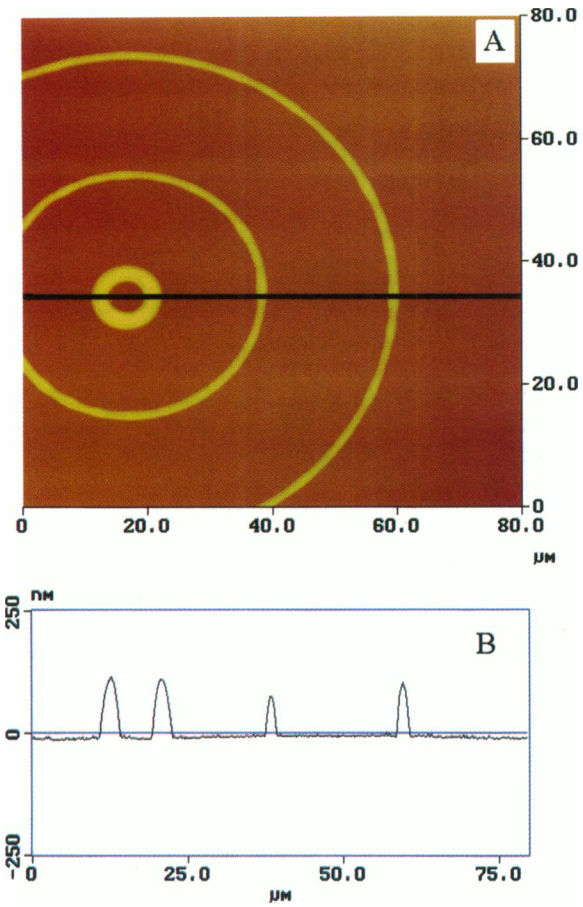

FIGURE 2 (*A*) AFM image of the triple concentric circular PMMA tracks. The radii of the tracks were 40, 20, and 5 μm. The substrate corresponding to the bare glass surface was very smooth. (*B*) Cross section along the black line in *A*. The width and the height of the track were 1–2 μm and 200 nm, respectively.

show that the tracks were 1–2 μm wide and about 200 nm high. The radii of the circles were 40, 20, and 5 μm. As this substrate was sufficiently developed, the PMMA resist was removed from the glass surface on the area except the tracks (corresponding to the flat region of Fig. 2 *B*). Lack of development, however, left wider lines and many residues of PMMA on an unexpected area of the glass surface.

Motility

Fig. 3 shows fluorescence microscope images of actin filaments on the triple concentric circular PMMA tracks: white filaments are fluorescently labeled actin filaments. In the snapshot (Fig. 3 *A*), white arrows show the direction of actin movement, and both clockwise movement and counterclockwise movement were observed, i.e., the direction of the movement was not regulated. Fig. 3 *B* was obtained by accumulation for 256 frames (8.5 s) to observe the traces of actin movement. In Fig. 3 *B*, a vague white shadow (*a*) represents the Brownian motion of an actin filament in the solution, and a black filament surrounded by a white region (*b*) is an actin filament anchored to the surface. The fluorescence intensity of the anchored filament was accumulated at the same point, and it caused overflow of intensity during the image processing of frame accumulation. This image, in which the traces drew concentric circles, shows that actin filaments were restricted to move only on the triple circular tracks. The linewidth, however, was wide enough (1–2 μm) for actin filaments to turn around; therefore the actin filaments drew winding traces on the tracks, and some filaments did U-turns on a single track. Some filaments that moved clockwise were observed to be passing by other filaments moving counterclockwise without changing their velocities.

This circular track had the advantage of rough estimation of the sliding velocity; the rough velocity was given by dividing the known circumference of the track by the measured time for an actin filament to go around along the track. According to this estimation, there is no definite difference in velocities between the clockwise movement and the counterclockwise movement and between that on an inner track and that an outer track. This result means that the

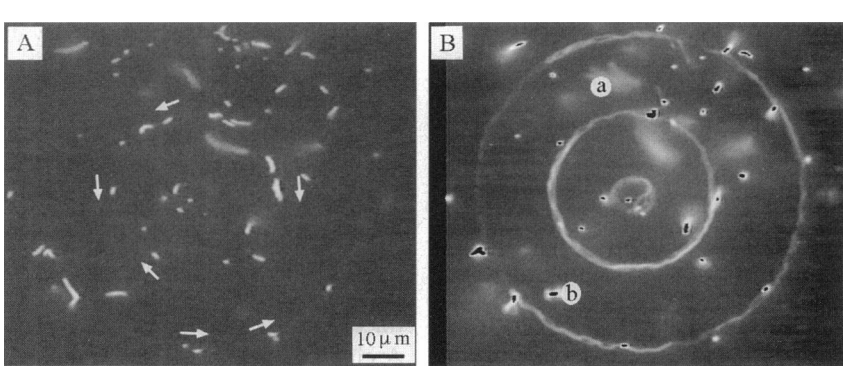

FIGURE 3 Fluorescence microscope images of tetramethylrhodamine-phalloidin-labeled actin filaments on the triple concentric circular PMMA tracks coated by HMM molecules. (*A*) Snapshot and (*B*) accumulated image of 256 frames (8.5 s). In the accumulated image (*B*), (*a*) vague white shadows represent the Brownian motions of actin filaments and (*b*) black filaments surrounded by white were caused by the overflow of the intensity during the accumulation process because of anchored actin filaments. Traces of actin filaments drew the circular tracks exactly.

polarity of the HMM head was not regulated, unlike a synthetic myosin filament, on which actin velocity strongly depends on the polarity of the myosin filament (Sellers and Kachar, 1990; Yamada et al., 1990).

Another shape of PMMA track could easily be fabricated: for example, a figure-eight PMMA track (Fig. 4). The actin filaments moved on the figure-eight track, and their traces drew the figure clearly. At the central cross point, some filaments moved straight forward and some occasionally turned to the right or left. Some filaments crossed over another filament at the cross point without diffusing away. This observation also shows that there were enough HMM molecules to support the movement of two actin filaments on the tracks and the cross point.

These results indicate that only HMM molecules adsorbed on the surface of the PMMA track could support the movement of actin filaments. Although HMM molecules could be adsorbed on the bare glass surface beside the PMMA tracks, those on the bare glass surface were thought to lose their ability to support actin movement. Actin filaments were not observed to be moving on HMM molecules applied on a bare glass surface (data are not shown).

HMM molecules on the PMMA tracks retained their ability to support actin movement, which was comparable to that on nitrocellulose without bovine serum albumin. Fig. 5 shows a histogram of the velocity of the actin movement on PMMA tracks. These data were obtained from the actin filaments moving on a linear PMMA track because it was easy to measure their displacements. The mean velocity on the PMMA track was about 4.5 ± 1.3 μm/s (n = 42, T = 24–25°C), which agreed with the velocity roughly estimated from the circular track. On the other hand, the mean velocity on the nitrocellulose-coated glass in the same condition was about 4.6 ± 1.2 μm/s (n = 47). These values are within the error of the actin velocity observed on HMM molecules bound to the nitrocellulose surface (Anson, 1992).

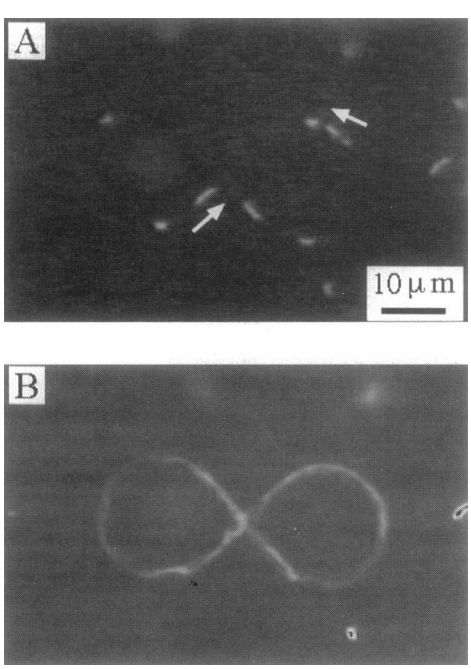

FIGURE 4 Fluorescence microscope images of actin filaments on the figure-eight PMMA track coated by HMM molecules. (*A*) Snapshot. (*B*) Accumulated image of 256 frames (8.5 s), showing that the trace of actin filaments drew the figure of eight.

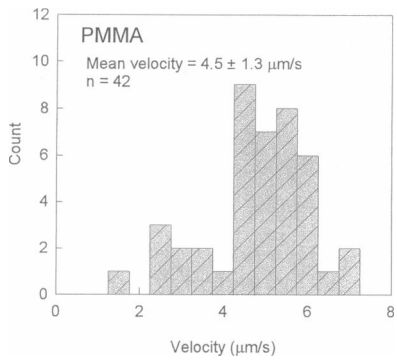

FIGURE 5 The velocity histogram of movement of actin filaments on the PMMA tracks. The velocity was measured from actin filaments on the linear PMMA track. The mean velocity on the PMMA track was about 4.5 ± 1.7 μm/s (n = 42).

The facility of patterning suggests the possibility of observing a trajectory of a single actin filament, until its fluorescence bleaches out. In most in vitro assay systems, most of actin filaments moved out of the vision field under the microscope, and it was difficult to follow a single actin filament for a long time. The circular PMMA track made by the photolithography enables us to follow a single actin filament without shifting the vision field, because the filament's trajectory is confined to the track.

The narrower pattern of the PMMA substrate can reduce the number of HMM molecules interacting with actin filaments. Because a deep UV exposure system was used for UV lithography in this experiment, the linewidth of the PMMA track depended on the linewidth of the mask pattern, and the narrowest line was 1 μm wide, which is on the order of 20–30 heads of a myosin molecule. This was wide enough for actin filaments to draw their winding traces on a single track. This resolution of the pattern can be improved using an electron-beam exposure system (EB system). The patterning process by an EB system can provide the pattern with a linewidth of under 100 nm. Such a narrow pattern is comparable to about a few myosin heads; i.e., decrement of the myosin head number on the surface can be achieved by controlling the linewidth. The narrower line can prevent actin filaments from doing U-turns and drawing winding traces, so the trajectories can be controlled more precisely. A circular island of PMMA with a diameter under 100 nm can restrict the number of myosin molecules interacting with actin filaments. This technique will provide a new experimental system with a restricted number of HMM molecules.

CONCLUSION

The PMMA surface was found to adsorb HMM molecules while allowing them to retain their abilities. Trajectories of actin filaments were confined by using the PMMA tracks patterned by UV photolithography. The velocity on the PMMA was comparable to that on the nitrocellulose-coated glass.

This technique for controlling the movement generated by motor protein will provide us not only with a new observation system in which the number of myosin heads is restricted, but also with a new device technology that uses biomolecules. If mechanical parts could be attached to actin filaments, such circular movement could be applied to actuators using motor proteins for micromechanical systems. Unidirectional movement of a single actin filament depending only on the polarity of the actin will be required to apply the method to such a micromechanical system. Reduction of the linewidth by the improvement of the patterning process can overcome the problem because a narrower patterned track can prevent actin filaments from doing U-turns. The method using patterned tracks is thought to have great potential for the application of motor protein to a microactuator device.

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REFERENCES

Anson, M. 1992. Temperature dependence and Arrhenius activation energy of F-actin velocity generated in vitro by skeletal myosin. *J. Mol. Biol.* 224:1029–1038.

Gizeli, E., N. J. Goddard, and C. R. Lowe. 1992. A love plate biosensor utilizing a polymer layer. *Sensors Actuators.* B6:131–137.

Harada, Y., A. Noguchi, A. Kishino, and T. Yanagida. 1987. Sliding movement of single actin filaments on one-headed myosin filaments. *Nature.* 326:805–808.

Harada, Y., K. Sakurada, T. Aoki, D. D. Thomas, and T. Yanagida. 1990. Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. *J. Mol. Biol.* 216:49–68.

Hiraoka, J., T. Ogihara, S. Sakurai, J. Suemitsu, and T. Akaike. 1985. Control of immunoglobulin G adsorbed by designing the characters of polymer surface. *Kobunshi Runbunshu.* 42:693–697 (in Japanese).

Huxley, H. E. 1990. Sliding filaments and molecular motile systems. *J. Biol. Chem.* 265:8347–8350.

Ishijima, A., Y. Harada, H. Kojima, T. Funatsu, H. Higuchi, and T. Yanagida. 1994. Single-molecule analysis of the actomyosin motor using nano-manipulation. *Biochem. Biophys. Res. Commun.* 199: 1057–1063.

Kron, S. J., and J. A. Spudich. 1986. Fluorescent actin filaments move on myosin fixed to a glass surface. *Proc. Natl. Acad. Sci. USA.* 83: 6272–6276.

Kron, S. J., Y. Y.. Toyoshima, T. Q. P. Uyeda, and J. A. Spudich. 1991. Assays for actin sliding movement over myosin-coated surfaces. *Methods Enzymol.* 196:399–416.

Margossian, S. S., and S. Lowey. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85:55–71.

Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *Methods Enzymol.* 85:164–181.

Sellers, J. R., and B. Kachar. 1990. Polarity and velocity of sliding filaments: control of direction by actin and of speed by myosin. *Science.* 249:406–409.

Suzuki, H., K. Oiwa, A. Yamada, H. Sakakibara, H. Nakayama, and S. Mashiko. 1995. Linear arrangement of motor protein on a mechanically deposited fluoropolymer thin film. *Jpn. J. Appl. Phys.* 34:3937–3941.

Toyoshima, Y. Y., S. J. Kron, E. M. McNally, K. R. Niebling, C. Toyoshima, and J. A. Spudich. 1987. Myosin subfragment-1 is sufficient to move actin filaments in vitro. *Nature.* 328:536–539.

Toyoshima, Y. Y., S. J. Kron, and J. A. Spudich. 1990. The myosin step size: measurement of the unit displacement per ATP hydrolyzed in an in vitro assay. *Proc. Natl. Acad. Sci. USA.* 87:7130–7134.

Turner, D. C., C. Chang, K. Fang, S. Brandow, and D. B. Murphy. 1995. Selective adhesion of functional microtubules to patterned silane surface. *Biophys. J.* 69:2782–2789.

Yamada, A., N. Ishii, and K. Takahashi. 1990. Sudden increase in speed of an actin filament moving on myosin cross-bridges of "mismatched" polarity observed when its leading end begins to interact with cross-bridges of "matched" polarity. *J. Biochem.* 108:341–343.