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# Measuring Orientation of Actin Filaments within a Cell: Orientation of Actin in Intestinal Microvilli

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ABSTRACT Orientational distribution of actin filaments within a cell is an important determinant of cellular shape and motility. To map this distribution we developed a method of measuring local orientation of actin filaments. In this method actin filaments within cells are labeled with fluorescent phalloidin and are viewed at high magnification in a fluorescent microscope. Emitted fluorescence is split by a birefringent crystal giving rise to two images created by light rays polarized orthogonally with respect to each other. The two images are recorded by a high-sensitivity video camera, and polarization of fluorescence at any point is calculated from the relative intensity of both images at this point. From the value of polarization, the orientation of the absorption dipole of the dye, and thus orientation of F-actin, can be calculated. To illustrate the utility of the method, we measured orientation of actin cores in microvilli of chicken intestinal epithelial cells. F-actin in microvillar cores was labeled with rhodamine-phalloidin; measurements showed that the orientation was the same when microvillus formed a part of a brush border and when it was separated from it suggesting that "shaving" of brush borders did not distort microvillar structure. In the absence of nucleotide, polarization of fluorescence of actin cores in isolated microvilli was best fitted by assuming that a majority of fluorophores were arranged with a perfect helical symmetry along the axis of microvillus and that the absorption dipoles of fluorophores were inclined at 52° with respect to the axis. When ATP was added, the shape of isolated microvilli did not change but polarization of fluorescence decreased, indicating statistically significant increase in disorder and a change of average angle to 54°. We argue that these changes were due to mechanochemical interactions between actin and myosin-I.

## INTRODUCTION

Actin filaments are involved in maintaining cell shape and movement (e.g., Alberts et al., 1983). It is therefore important to be able to measure continuously orientation of actin filaments in different regions of a cell during alterations in cell shape and during motion. A convenient way to measure orientation of fluorescent molecules is to determine polarization of their fluorescence, because it is possible to monitor fluorescence from a very small area rapidly and with great sensitivity (Weber, 1966). Polarization of fluorescence carries the information about the angular distribution of the absorption (and emission) dipoles of the fluorophore (Tregear and Mendelson, 1975; Borejdo et al., 1982; Wilson and Mendelson, 1983). In the case of F-actin, a simple way to render it intensely fluorescent is to attach external fluorophore-fluorescent phalloidin (Yanagida et al., 1984). This probe is specific for F-actin (Wulf et al., 1979) and does not affect these systems in which polymerization-depolymerization of actin does not play a role in function (Prochniewicz-Nakayama et al., 1983). Nontoxicity of phalloidin is further demonstrated by the fact that labeling does not change the distribution of lengths of actin filaments (Burlacu et al., 1992), stabilizes filamentous structure of actin against depolymerizing conditions (Estes et al., 1981), protects against heat denaturation and proteolytic degradation (Dancker et al., 1975; Low et al., 1975), only slightly changes polymerizability of modified actin (Miki et al., 1987), and has no effect on tension or $Ca^{2+}$ regulation of striated muscle (Prochniewicz-Nakayama et al., 1983).

In this work we present a way to continuously measure polarization of fluorescence of F-actin within a cell which is based on analysis of fluorescent images of phalloidin-labeled actin filaments. We split, by a birefringent crystal, a fluorescent image of actin filaments into two orthogonally polarized images. We recorded the two images on videotape and later calculated polarization of fluorescence at any point from the relative intensity of both images at this point. As an example of application of the method we measured orientation of actin filaments in microvilli (MV)[1] of chicken intestinal cells. Microvilli are small invaginations present on the apical side of brush borders (BB) of intestinal epithelial cells that may be responsible for the transport of electrolytes and nutrients across epithelium (Mooseker and Howe, 1982). Microvilli are good model systems in which to measure polarization of fluorescence, because all actin filaments are parallel to MV axis and fluorescent dye (which is bound to phalloidin) is immobilized on the surface of actin. From the value of polarization we calculated, using a simple model of assembly of fluorophores, the angle of excitation (or emission) dipole of the dye with respect to MV axis and the degree of order with which rhodamine molecules (and hence actin monomers in a filament) were arranged along the microvillar axis.

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[1] *Abbreviations used in this paper:* MV, microvillus; BB, brush border; TW, terminal web; HMM, skeletal heavy meromyosin; BC, birefringent crystal; TRITC-phalloidin, tetramethyl-rhodamine-isothiocyanate-phalloidin; AOI, area-of-interest; DTT, dithiothreitol; BBSS, brush border stabilization solution; PMSF, phenylmethylsulfonyl fluoride.

Using this method we show that the orientation of actin cores in microvilli constituting a part of brush border is not different from orientation of cores in isolated MV, suggesting that "shaving" brush borders during preparation of isolated microvilli did not damage MV structure. We also show that, in isolated MV, actin cores change orientation during activation by ATP and argue that this change is due to mechanochemical interactions between actin and myosin-I.

## MATERIALS AND METHODS

### Imaging BB and MV

Adaptation of the basic video microscope to measure local polarization of fluorescence from small samples such as BB and MV is shown in Fig. 1: mercury lamp- or argon laser-generated excitation light. In the case of lamp illumination, the light was passed through a 546-nm bandpass interference filter (Zeiss BP 546/12) and in the case of laser illumination (at 514.5 nm), the laser beam was passed through the stabilization accessory (Liconix model 50SA, Sunnyvale, CA) which reduced the noise in the light output to less than 0.01% rms. The beam of light was attenuated by neutral density filters and its direction of polarization was defined by a polarizer (PR). The beam was next passed through an adjustable-width slit which replaced excitation field diaphragm (FD). The purpose of the slit was to ensure that the two orthogonal images did not overlap. A dichroic mirror DM (Zeiss FT 580) directed all wavelengths below 580 nm at the sample (S) which was placed on the stage of a Zeiss Photomicroscope III. The fluorescent light was collected by a × 100 Neofluar (numerical aperture (NA) = 1.3) objective and passed through a barrier filter (Zeiss LP 590) onto a birefringent crystal (BC) which created two nonoverlapping images of a sample separated vertically by 120 pixels. The top image was created by emitted light which was polarized horizontally, and the bottom one was created by light which was polarized vertically. When the sample was illuminated with the mercury lamp, a Glan-Taylor PR and a calcite BC (Iceland spar) beam displacing prism were used. When the sample was illuminated with the argon laser, an argon laser polarization rotator PR and a Wollaston prism BC were used. The angle between the incident and the emitted light beams was 360°. A similar arrangement was originally used by Kinosita et al. (1991). The image was formed on a faceplate of an SIT camera (model SIT-68; Dage-MTI, Michigan City, IN). The high sensitivity SIT camera was used to reduce, as much as possible, the incident light intensity (and hence photobleaching). The video signal was recorded on a VCR (Sharp VC6610U, Mahwah, NJ).

### Measuring polarization of fluorescence

Images were transferred from videotape to a computer by a frame grabber (Data Translation 2861, Marlboro, MA) connected to a DT 2868 auxiliary frame processor. The relative intensities of the two images carry the information about polarization properties of the sample. The intensities were analyzed by an Image Pro Plus image analysis program (Media Cybernetics, Silver Spring, MD). Image Pro allows one to measure the average intensity with a better than 0.5% accuracy. Only those MVs which happened to be oriented horizontally were analyzed. Let the difference in intensity of the light emanating from the object of interest and of the background immediately adjacent to the object be indicated by $I$. Let the direction of an excitation polarization be indicated by a subscript v (for vertical or perpendicular to the axis of MV) or h (for horizontal or parallel to the axis of MV) *before* the symbol $I$. Let the direction of emission polarization be indicated by a subscript v or h *after* the symbol $I$. Since birefringent prism created two images of MV, we measured four components of polarization of fluorescence (two for each of the two orientations of the excitation polarizer). Thus when the excitation polarizer was vertical, the intensity of a MV in an image created with vertically polarized light was ${}_{v}I_{v}$. The intensity of the same MV in an image created with light which was horizontally polarized was ${}_{v}I_{h}$. When the excitation polarizer was horizontal, the corresponding intensities were ${}_{h}I_{v}$ and ${}_{h}I_{h}$. Horizontal polarization of fluorescence was defined as $P_h = ({}_{h}I_{h} - {}_{h}I_{v})/({}_{h}I_{h} + {}_{h}I_{v})$ and vertical polarization of fluorescence as $P_v = ({}_{v}I_{v} - {}_{v}I_{h})/({}_{v}I_{v} + {}_{v}I_{h})$.

### Corrections for polarization of fluorescence

A major error in calculating $P$ is caused by the fact that the dichroic mirror depolarizes light, which results in uneven splitting of polarization components by the birefringent crystal. Also, the crystal is intrinsically inhomogeneous which further increases uneven splitting of light. To correct for this effect, we measured ${}_{h}I_{h}$, ${}_{h}I_{v}$, ${}_{v}I_{v}$, and ${}_{v}I_{h}$ of a solution of a rhodamine dye. In theory, the polarization of free rhodamine in solution must be 0 and must not depend on the polarization of the excitation beam. Therefore ${}_{v}I_{v}$ must be equal to ${}_{v}I_{h}$, and ${}_{h}I_{v}$ must be equal to ${}_{h}I_{h}$. Instead, in our optical system we had $c_v = {}_{v}I_{v}/{}_{v}I_{h} = 1.57$ and $c_h = {}_{h}I_{v}/{}_{h}I_{h} = 1.24$. Thus the corrected values

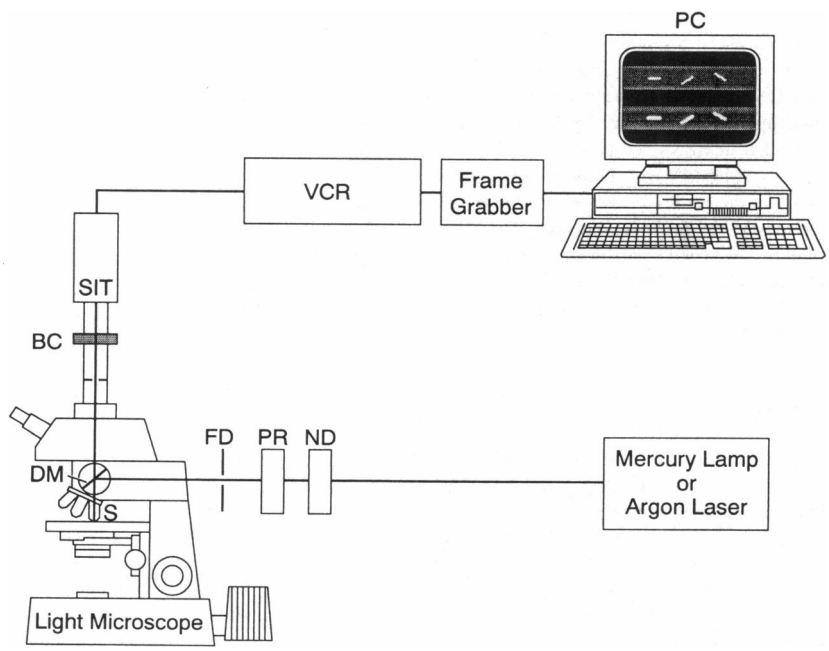


FIGURE 1 Experimental apparatus used to collect polarization data from isolated microvilli. ND, neutral density filters; PR, polarization rotation assembly; S, sample mounted on stage of fluorescence microscope; DM, dichroic mirror; FD, adjustable slit in place of excitation field diaphragm; SIT, SIT video camera; VCR, video cassette recorder; PC, personal computer.

of polarizations were as follows.

$$P_v = ({}_vI_v/c_v - {}_vI_h)/({}_vI_v/c_v + {}_vI_h) \quad (1)$$

$$P_h = ({}_hI_h - {}_hI_v/c_h)/({}_hI_h + {}_hI_v/c_h) \quad (2)$$

The errors due to photobleaching were small, because all solutions contained 1% β-mercaptoethanol and because the light intensity was low (incident intensity was typically 0.15 mW). The quantum yield for photobleaching, which was measured by following the decrease in emitted light intensity of rhodamine-phalloidin-labeled actin filaments attached to a coverslip, was smaller than $10^{-11}$.

## Materials

Tetramethyl-rhodamine-isothiocyanate-phalloidin (TRITC-phalloidin), DTT, ATP, and ADP were from Sigma Chemical Co. (St. Louis, MO). Iodoacetamido-tetramethyl-rhodamine was from Molecular Probes (Eugene, OR). Sephadex G-150 Superfine was from Pharmacia LKB (Uppsala, Sweden).

## Solutions

Saline solution contained 0.15 M NaCl, 4 mM $NaN_3$, 10 mM imidazole buffer, pH 7.4. Cell dissociation solution contained 0.2 M sucrose, 12 mM EDTA, 0.076 M $Na_2HPO_4$, 0.019 M $KH_2PO_4$. Homogenization buffer contained 4 mM EDTA, 1 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF, 4 mM $NaN_3$, 10 mM imidazole buffer, pH 7.0. Brush border stabilization solution (BBSS) contained 75 mM KCl, 5 mM $MgSO_4$, 1 mM EGTA, 4 mM $NaN_3$, 0.1 mM DTT, 0.1 mM PMSF, 10 mM imidazole buffer, pH 7.0. Phalloidin solution was prepared by dissolving 0.1 mg of TRITC-phalloidin in 0.1 ml of 50% methanol and diluting it 10 times with BBSS.

## Preparation of brush borders and microvilli

BB and MV were prepared as described by Mooseker and Howe (1982) with minor modifications. Briefly, chickens were sacrificed, and two intestinal segments approximately 20 cm each were flushed with cold saline and filled with cell-dissociation solution. Filled segments were massaged vigorously and, after 30 min, were drained and rinsed with 40 ml of cold saline containing 0.1 mM PMSF. Dissociated cells were collected by centrifugation in a Sorvall centrifuge at 200 rpm for 5 min (small rotor), resuspended in homogenization buffer, and homogenized on ice in an Omni Mixer (Waterbury, MA) at a 7.5 setting in four bursts of 5 s each. The resulting BBs were washed with BBSS, and contaminating nuclei were removed by centrifugation in a sucrose step gradient as described by Mooseker and Howe (1982). Sucrose was replaced by a stabilization solution (BBSS) by repeated centrifugation at 1000 rpm for 10 min. To prepare isolated MVs, BB were suspended in BBSS containing in addition 15 mM $Mg^{2+}$ to increase the rigidity of MV. MV were prepared by vigorous homogenization of BB (100 strokes of the pestle) in a Kontes (Vineland, NJ) homogenizer with stainless steel pestle and ground glass tube. The "shaved" BB were collected as a pellet in low speed centrifugation. The MV remained in a supernatant and were collected by centrifugation at 35,000 *g* for 15 min. All measurements were done in BBSS containing 1% β-mercaptoethanol which was included to reduce photodegradation.

## Proteins

Myosin was prepared from rabbit skeletal muscle by the method of Tonomura et al. (1966). Heavy meromyosin (HMM) was obtained by a chymotryptic digestion of myosin according to Weeds and Pope (1977), respectively. Skeletal actin was prepared according to Spudich and Watt (1971). The concentrations of proteins were measured by absorbance using: G-actin, $A^{1\%}_{290} = 6.3$; F-actin, $A^{1\%}_{290} = 6.7$. Proteins were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by the ability to produce motions in vitro in a motility assay.

# RESULTS

## Polarization of fluorescence of MV which formed a part of BB

Suspension of BBs was first incubated with 5 μM TRITC-phalloidin for 1 h at room temperature. The sample was next applied to a microscope slide, covered with no. 1 coverslip, and placed on a stage of a video microscope. Excess phalloidin was removed by washing BBs with BBSS containing 1% β-mercaptoethanol by applying a drop of a solution to one side of a coverslip and sucking it with a filter paper on the other side. BBs were illuminated first with horizontally polarized light, and its two orthogonally polarized fluorescent images were recorded on a VCR. A phase-contrast image of a typical BB is shown in Fig. 2 *A*. The image is of poor quality because it was obtained with a SIT camera in which spatial resolution was sacrificed in order to obtain high light sensitivity. Top and bottom images in this frame were created with light which was polarized horizontally and vertically, respectively. Fig. 2 *B* is the fluorescence image of the same BB obtained with excitation light polarized horizontally. To measure polarization of fluorescence, the image of BB was magnified, and a rectangular area-of-interest (AOI) was defined within a region in which MVs were oriented horizontally, as shown in Fig. 3. The average intensity within AOI

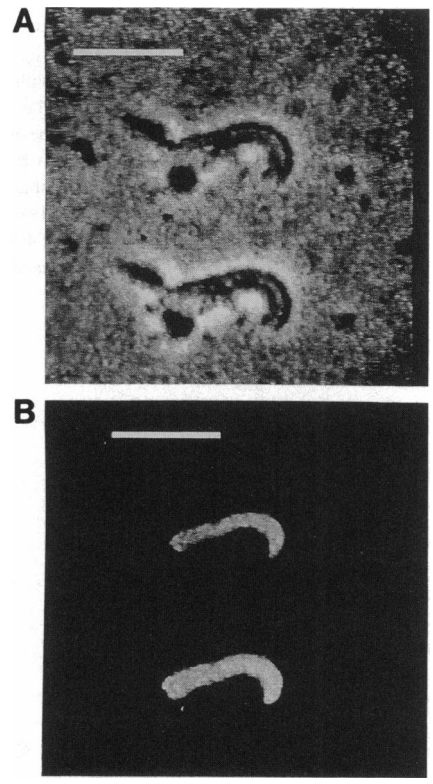


FIGURE 2 (*A*) Phase-contrast image of a brush border. The top image was created with light polarized horizontally, the bottom one with light polarized vertically. The image is of poor quality, because, to reduce as much as possible incident light intensity, a SIT camera with high light sensitivity but poor spatial resolution was used. (*B*) Fluorescence image of the same brush border. BBs were incubated with TRITC-phalloidin as described in the text. Excitation polarization was horizontal. Bar is 10 μm.

was measured by the Image Pro Plus program. The average intensity of the background immediately adjacent to the BB was measured using *the same* AOI and subtracted from the measured intensity. When the BB was illuminated with horizontally polarized light, the intensity within AOI defined in the lower image (such as shown in the lower part of Fig. 2 *B*) was ${}_{h}I_{v}$. When the BB was illuminated with horizontally polarized light, the intensity within AOI defined in the upper image (such as shown in the upper part of Fig. 2 *B*) was ${}_{h}I_{h}$. The BB was next illuminated with vertically polarized light (perpendicular to the MV axis). The intensity within AOI defined in the lower image was then ${}_{v}I_{v}$, and the intensity within AOI defined in the upper image was ${}_{v}I_{h}$. Polarization of fluorescence was calculated according to Eqs. 1 and 2. The data is plotted in histogram form in Fig. 5 *B*; i.e., polarizations are plotted versus the percentage of measurements which gave the particular value of polarization. Table 1 (row 1) summarizes the results of 16 experiments. The polarizations are corrected for depolarization of light due to high numerical aperture of the objective using the method of Axelrod (1979).

## Polarization of fluorescence of isolated MV

In order to see whether actin cores were disrupted while homogenizing BBs during preparation of MVs, we compared polarization of fluorescence of MV forming a part of BB and of isolated MVs. Any disruption is likely to cause disorganization of actin within cores, and this must have an effect on polarization of fluorescence because polarization is a sensitive indicator of symmetry of arrangement of actin. Suspension of microvilli was incubated with 5 $\mu$M TRITC-phalloidin for 1 h at room temperature. The sample was then applied to the microscope slide, covered with a coverslip, and placed on a stage of a video microscope. Excess phalloidin was removed as described before. A typical preparation of MV illuminated with horizontally polarized light is shown in Fig. 4. Polarization of fluorescence was measured only for MV which were oriented horizontally, such as MV pointed out by the arrow in Fig. 4. Intensities were measured as described before, i.e., by enclosing entire MV in AOI and subtracting from the result the average intensity of the background. For each microvillus, two pairs of orthogonal intensity components were measured as described above. The data is plotted in histogram form in Fig. 5 *A*. Table 1 (row 2) summarizes the results of 36 measurements of $P_v$ and 38 measurements of $P_h$. Again, the polarizations have been corrected for depolarization of light due to high numerical aperture of the objective using the method of Axelrod (1979). We can state with high degree of confidence that $P_v = P_h$ ($t = 0.08$, $P = 0.93$).

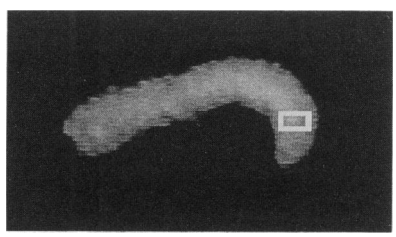

FIGURE 3 Brush border of Fig. 2 *B* magnified two times. The rectangular box encloses microvilli oriented horizontally.

**TABLE 1 Comparison of polarized fluorescence of actin cores in microvilli which either form a part of BBs or are isolated from it (rows 1 and 2) and comparison of polarized fluorescence of actin cores in isolated microvilli in the absence and presence of ATP (rows 2 and 3)**

| | | $P_v$* | $P_h$* | $n$‡ |
|---|---|---|---|---|
| 1 | Part of BB§ | 0.156 ± 0.028 | 0.108 ± 0.022 | 16,16 |
| 2 | Isolated¶ | 0.097 ± 0.026 | 0.096 ± 0.011 | 36,38 |
| 3 | Isolated + ATP | 0.185 ± 0.021 | 0.116 ± 0.012 | 52,58 |
| 4 | Filaments | 0.025 ± 0.018 | 0.246 ± 0.017 | 9,20 |

* Mean ± SE was corrected by 41% for depolarization of light due to high numerical aperture of the objective using the method of Axelrod (1979).
‡ Number of measurements of $P_v$, $P_h$.
§ Microvilli formed a part of BB.
¶ Isolated microvilli.

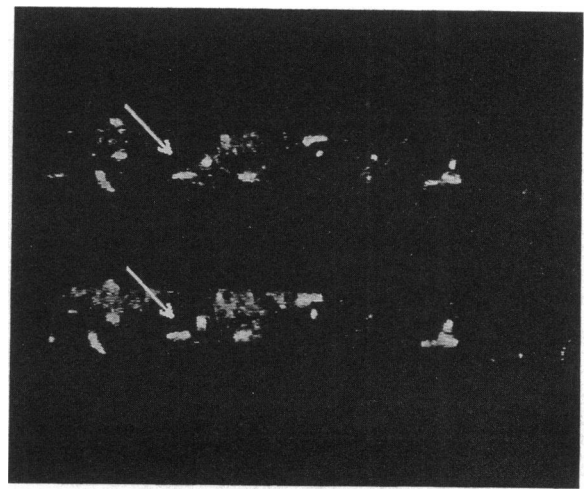

FIGURE 4 Fluorescence image of isolated microvilli labeled with TRITC-phalloidin. Excitation polarization was horizontal. Arrows point to a microvillus which happened to be oriented horizontally. The average intensity of this microvillus in the top image (which was created with light polarized horizontally) after subtracting the average intensity of the background, was ${}_{h}I_{h}$. The average intensity of the same microvillus shown in the bottom image (which was created with light polarized vertically) after subtracting the average intensity of the background, was ${}_{h}I_{v}$. Horizontal polarization of fluorescence, $P_h$, is related to both intensities by Eqs. 1 and 2. The outline of the adjustable slit FD can be seen. Magnification as in Fig. 2.

## Comparison between BB and MV

The distribution of polarizations of MV actin cores, forming part of BB and isolated MV, is shown in Fig. 5, and the means are summarized in Table 1. The difference in mean polarizations was not statistically significant. For $P_v$ the $t$ test gave $t = 1.26$, $P = 0.21$. The difference in $P_h$ was not statistically

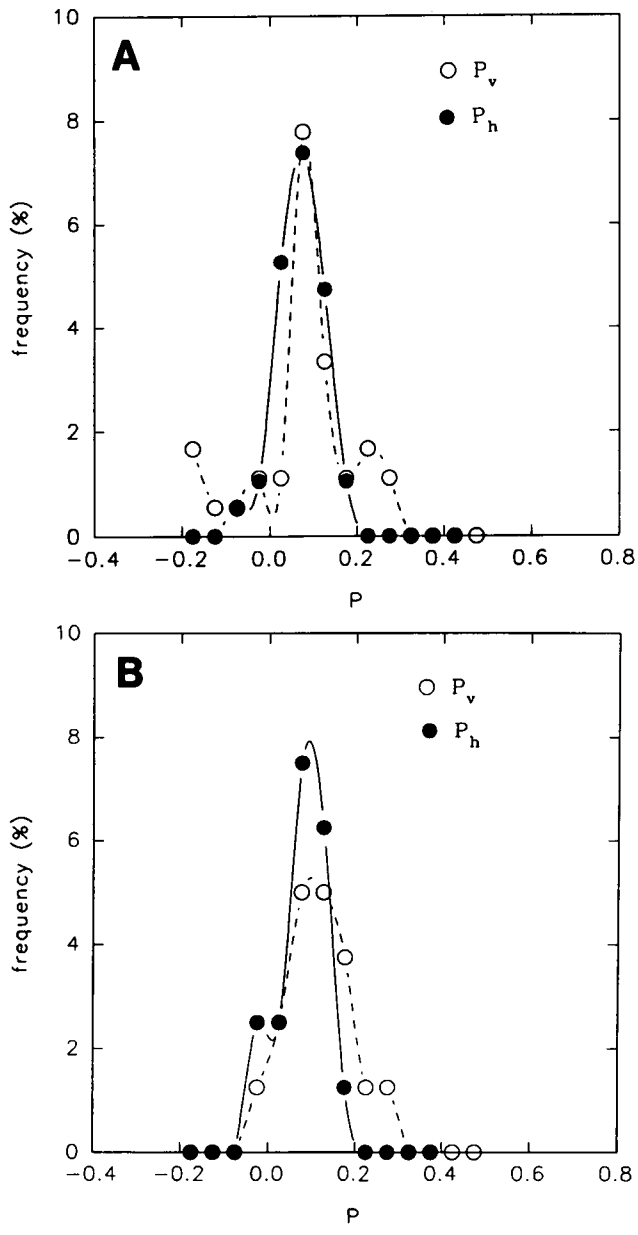


FIGURE 5 Fraction of the total number of measurements which gave particular value of polarization. (*A*) Isolated MV. (*B*) MV forming a part of BB. The polarizations were not corrected for high numerical aperture of the objective. The data points were fitted with a spline function. ●, $P_h$; ○, $P_v$.

significant with even more confidence ($t = 0.54$, $P = 0.59$). The implications of this lack of difference is dealt with in the Discussion section.

## Effect of ATP

We compared conformation of F-actin in the core in the presence and in the absence of ATP. We performed the experiments only in isolated MV, because addition of ATP to BB induced the contraction of terminal web with consequent reorientation of microvilli. 1 mM ATP was added to MV bathed in BBSS. After 5 min ATP produced no visible changes in the appearance of MV. Fig. 6 and Table 1 (row 3) compare the results of 52 measurements of $P_v$ and 58 measurements of $P_h$. ATP affected both the mean and the distribution of polarizations. For $P_v$, the distribution became biphasic, and there was a highly statistically significant difference between means when $P_v$ values were measured in the absence and in the presence of ATP ($t = 2.39$, $P = 0.02$). For $P_h$ there was no statistically significant change in the mean but the shape of distribution changed. In contrast to the polarizations of MV in the absence of ATP, mean $P_v$ was significantly different from $P_h$ ($t = 2.58$, $P = 0.01$).

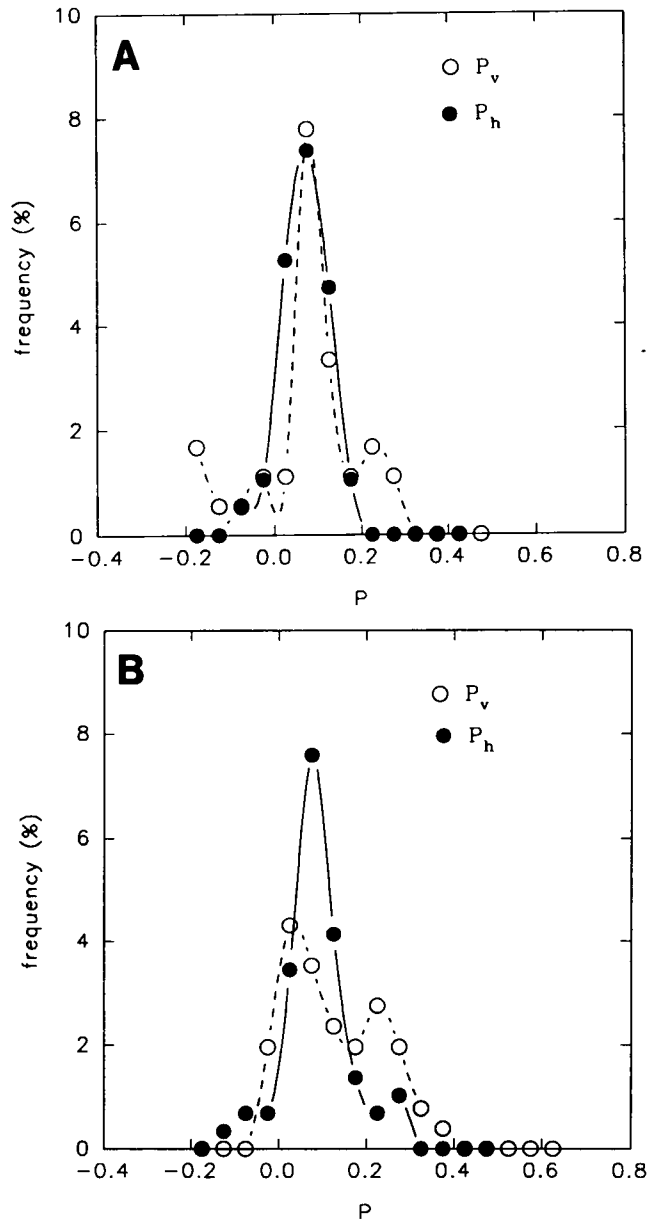


FIGURE 6 Fraction of the total number of measurements which gave particular value of polarization. The polarizations were not corrected for high numerical aperture of the objective. (*A*) Isolated MV. (*B*) Isolated MV + 1 mM ATP. The data points were fitted with a spline function. ●, $P_h$; ○, $P_v$.

## Polarization of fluorescence of skeletal actin filaments

We have compared the orientation of microvillar actin core and isolated skeletal actin filaments. 50 μg/ml of skeletal actin filaments were labeled with equimolar concentration of TRITC-phalloidin overnight in ice. Actin was polymerized with 50 mM potassium acetate, 2 mM $MgSO_4$ for 3 h at room temperature and incubated for 15 min at room temperature with an equimolar concentration of TRITC-phalloidin. To immobilize actin filaments, 0.8 mg/ml of skeletal muscle heavy meromyosin (HMM) was applied to a microscope coverslip coated with nitrocellulose. 3 μl of actin-phalloidin complex at 10 nM a was applied to a coverslip. In this system, routinely used in vitro in motility assay (Kron and Spudich, 1986), HMM is bound to nitrocellulose, and actin filaments are immobilized by binding to HMM. Polarization was measured like for isolated MV. In nine experiments, the vertical polarization of fluorescence (after correction for high N.A. of the objective) was $P_v = 0.025 \pm 0.018$, and, in 20 experiments, the horizontal polarization of fluorescence was $P_h = 0.246 \pm 0.017$ (Table I, row 4). These values were significantly different from polarizations of the microvillar actin core. The possible reasons for this difference are examined in the Discussion section.

## Polarization imaging

The resolution of the method is best visualized by constructing a polarization image of an MV, i.e., by performing operations specified in Eqs. 1 and 2 directly on the polarized images of MV. In performing the arithmetic operations on a pair of images it is important to ensure that the pixel coordinates in one image correspond exactly to the pixel coordinates in the second image. To assure this, both images must be carefully registered. Spatial misalignment of only a few pixels can lead to a "ghost" appearance of MV. Since both ${}_hI_h$ and ${}_hI_v$ were exact replicas of the same object (except for the emission polarizations) and were obtained simultaneously, the errors due to the focusing, to uneven spatial distribution of the exciting light and to fluctuations in the power of the mercury source, were not present.

Fig. 7 is a result of the above procedure applied to the MV pointed to in Fig. 4: To obtain horizontal polarization image, every pixel of the lower image of Fig. 4 was divided by $c_h$ and subtracted from the corresponding pixel in the upper image, giving $P_{numerator} = ({}_hI_h - {}_hI_v/c_h)$. Next, every pixel of the lower image of Fig. 4 was divided by $c_h$ and added to the corresponding pixel in the upper image, giving $P_{denumerator} = ({}_hI_h + {}_hI_v/c_h)$. A small integer (usually 10) was added to the $P_{denumerator}$ to avoid dividing by 0. $P_{numerator}$ was divided to $P_{denumerator}$ to obtain spatial distribution of horizontal polarizations. The polarization image was obtained by multiplying the result by 1000 to bring the polarizations within 8-bit gray level range ($0 \leq$ gray level $\leq 255$) and is shown in Fig. 7 (polarizations are shown before applying correction for N.A.). A three-dimensional representation of the polarization image is shown in Fig. 8. The $x$ axis is the distance along the microvillus of Fig. 7, with pixel 0 representing the left edge. The $y$ axis is the width of the microvillus, with 0 representing the upper edge. The characteristic feature of microvilli was that, within it, polarization was not constant. The average horizontal polarization within the microvillus shown in Figs. 7 and 8 was 0.067, with the smallest value of 0.047 (~23% of the area) and the largest value of 0.127 (~10% of the area).

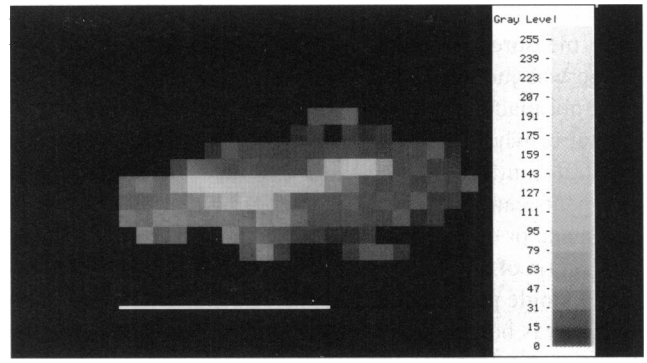


FIGURE 7 Horizontal polarization image of microvillus pointed to by arrows in Fig. 4. The arithmetic manipulations of the orthogonal images of MV are described in the text. Bar is 1 μm.

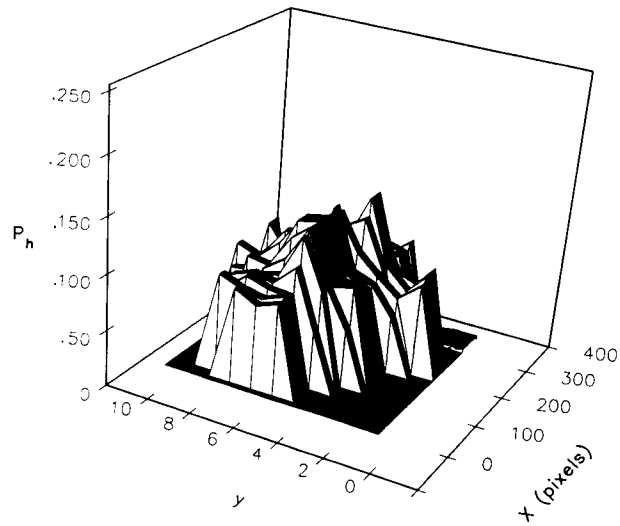


FIGURE 8 Three-dimensional representation of microvillus of Fig. 4. Polarization is plotted along the $z$ axis. Distance along MV is plotted along the $x$ axis, with 0 corresponding to the leftmost edge of the microvillus. The $y$ axis is distance across MV, with 0 corresponding to the upper edge of the microvillus.

## DISCUSSION

### Method

The main advantage of the method is that it allows measurement of polarization of fluorescence from small organelles. In fact, as shown in Fig. 7, distribution of polarization of within single microvillus could be measured. Measurements are more difficult when they are done with a photomultiplier, but organelles have been imaged using circularly polarized light by this method (Keller et al., 1985;

Tinoco et al., 1987). In the present method the intensities were measured only in the areas where the quality of the image was judged to be good. For example, measurements were not made in the areas where MV was not exactly horizontal or where there was evidence that phalloidin did not penetrate uniformly. The third advantage is that the measurements can be done continuously, e.g., during changes in cell shape or during motion. This is done by using only one orientation of excitation polarizer in which case one obtains only a single polarization value (e.g., $P_h$ when the excitation polarizer is horizontal), but it is enough to measure relative changes in orientation. The measurements can then be done quickly, because only the recording has to be done in real time while analysis can be done later. Further advantage of using a single excitation polarization is that orientational photobleaching, which arises because the quantum yield for photobleaching is different when the excitation polarizer is oriented vertically and horizontally, is rendered irrelevant. This is because both emission intensities are affected by photobleaching to the same extent. (Incidentally, orientational photobleaching has been exploited to measure rotational diffusion of acetylcholine receptors (Velez et al., 1990), DNA reorientation in nuclei (Selvin et al., 1990), and, recently, rotational motion of cross-bridges in single muscle fibers (Burghardt and Ajtai, 1992).) Furthermore, simultaneous recording of the two images eliminates errors due to fluctuations in the power of the mercury source (laser power fluctuations are smaller then 0.01% rms). The final advantage is that, for each orientation of the polarizer, both emission components are exact replicas of the same object (except for the polarization) thus eliminating errors due to the imperfect focusing and to the uneven spatial distribution of exciting light. The primary disadvantage of the method is the limited dynamic response of the SIT camera: when polarization of fluorescence is high, one image may be so bright that it saturates the camera, while the other may be so weak that it cannot be seen.

## Calculating the disorder and angle of absorption dipole from polarization of fluorescence

Polarization of fluorescence indicates symmetry within the core of actin filaments. It is possible to quantitize this symmetry by calculating the amount by which the absorption (or emission) dipole of the dye departs from a perfect helical arrangement along the axis of microvillus, and to compute the angle between the dipole and the axis. To do this, it is necessary to know the angle $\lambda$ between the absorption and emission dipoles of rhodamine, the rigidity of the attachment of TRITC-phalloidin to actin (characterized by an angle $\alpha$ which is an average angle defining a cone within which the dye is able to rotate on the surface of actin), and the type of order of fluorophores. The value of $\alpha$ can be estimated from Perrin plots of the rhodamine-phalloidin-F-actin system as follows: at high viscosity phalloidin cannot rotate (even in the absence of F-actin) and its polarization of fluorescence is high. In the limit of infinite viscosity the polarization extrapolates to "true" limiting polarization $p_o = 0.367$ (obtained from Perrin plots in Borejdo and Burlacu (1991)). (Incidentally, fluorescein-phalloidin gave much smaller value of true limiting polarization of 0.189 (Borejdo and Burlacu, 1991).) At lower viscosities, the overall rotation of F-actin is inhibited, but the rotation of phalloidin is not. The polarization of fluorescence at these lower viscosities extrapolates the "false" limiting polarization of fluorescence $p_x = 0.345$ (Fig. 9 *B* in Borejdo and Burlacu (1991)). The ratio of these limiting polarizations is related to the angle $\alpha$ by $p_x/p_o = (3\cos^2\alpha - 1)/2$ (Weber, 1966). Therefore $\alpha$ is 11°. $\lambda$ is equal to $\arccos\{\sqrt{[(3p_o + 1)/(3 - p_o)]}\}$ and is 26°. For simplicity, both were assumed to be zero even though, in the case of $\lambda$, this assumption is a significant oversimplification.

The type of order which we use here for microvilli is the same as the one used for myosin in skeletal muscle fiber by

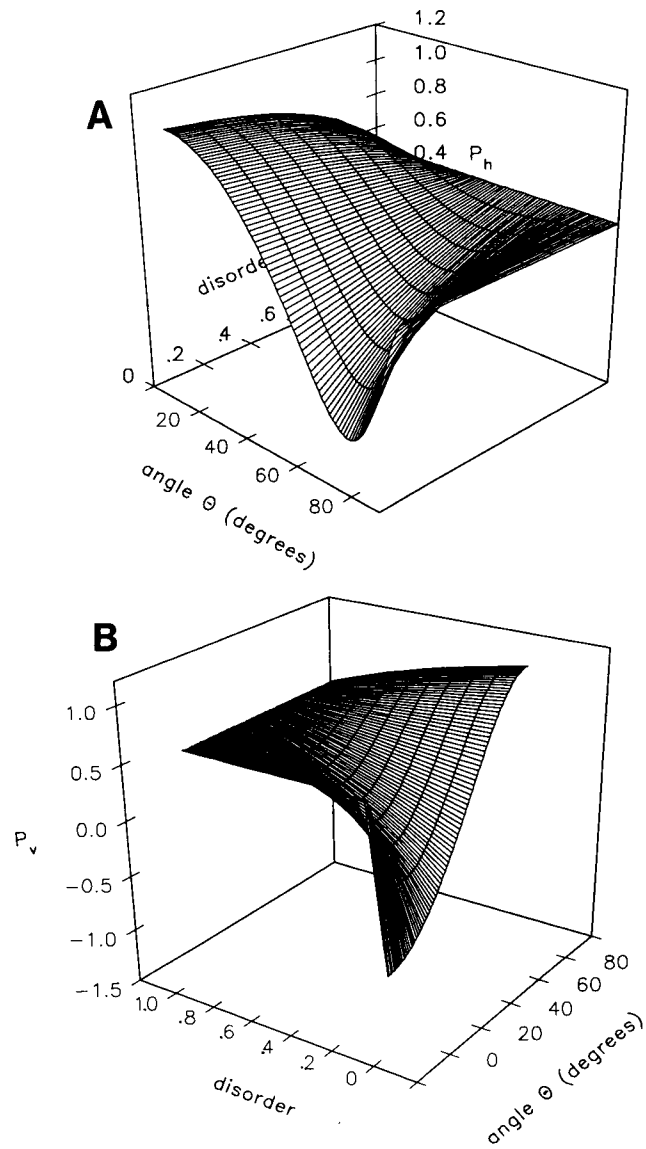


FIGURE 9 (*A*) Theoretical dependence of horizontal polarization of fluorescence on the angle that the absorption (or emission) dipole makes with the actin axis, and on the degree of disorder $(1 - \sigma)$. (*B*) The same for vertical polarization of fluorescence.

Tregear and Mendelson (1975), Mendelson and Morales (see Borejdo and Putnam (1977)), and Borejdo et al. (1982), because the arrangement of actin monomers in filaments, like myosin in thick filaments, exhibits helical symmetry. In this model a fraction of probe molecules describes perfect helical array around F-actin, and the remaining fraction is randomly oriented. Let the total disorder be characterized by a parameter, $\sigma$, which is the sum of disorder introduced by nonspecific labeling, skew of actin filaments (Yanagida and Oosawa, 1978), dye motion on the surface of actin, and the presence of free phalloidin. Then it can be shown, using the expressions given by Mendelson and Morales (see Borejdo and Putnam (1977)) with $\lambda = 0$ and $\psi = 360°$ that

$$P_v = a/b \tag{3}$$

$$P_h = c/d \tag{4}$$

where

$$a = 15\sigma\,(3\sin^4\Theta - 4\cos^2\Theta\sin^2\Theta) + 16(1 - \sigma) \tag{5}$$

$$b = 15\sigma\,(3\sin^4\Theta + 4\cos^2\Theta\sin^2\Theta) + 32(1 - \sigma) \tag{6}$$

$$c = 15\sigma\,(\cos^4\Theta - 0.5\cos^2\Theta\sin^2\Theta) + 2(1 - \sigma) \tag{7}$$

$$d = 15\sigma\,(\cos^4\Theta + 0.5\cos^2\Theta\sin^2\Theta) + 4(1 - \sigma) \tag{8}$$

Eqs. 3 and 4 are plotted in Fig. 9 for different values of $\sigma$. In the limit $\sigma = 0$ (complete disorder) $P$ is constant and equal to 1/2 (immobilized probes, absorption, and emission dipoles parallel). For the case of no disorder and $\Theta = 0°$ for $P_v$ and $\Theta = 90°$ for $P_h$, the limiting polarization is $-1$, even though no light is absorbed by MV at all. In these cases even a small amount of disorder causes the MV to absorb infinitely more light than for $\sigma = 1$ and polarization becomes 0.5.

The solution of Eqs. 3 and 4 was obtained by the Mathematica program (Wolfram Research, Champaign, IL, Version 2) with the aid of FindRoot function which uses Newton's method to find solution to within six digits of accuracy with 15 iterations. Starting values were $\Theta = 20°$ and $\sigma = 0.6$. For the case when microvilli formed a part of brush border (Table 1, row 1) the unique solution was $\Theta = 52°$ and $\sigma = 1.0$. We conclude that in the absence of ATP all fluorophores are arranged with helical symmetry relative to actin axis and that absorption (or emission) dipole of the dye forms an angle of 52° with respect to the axis. A high degree of order was also observed in skeletal muscle in rigor (Wilson and Mendelson, 1983; Borejdo et al., 1982).

## Comparison of actin arrangement in brush borders and in isolated MV

With a high degree of confidence, polarizations of fluorescence were the same in microvilli of brush borders and in isolated microvilli. Application of Mathematica gives $\Theta = 52°$ and $\sigma = 1.0$, the same as when microvilli were connected to a brush border (Table 1, row 2). The simplest interpretation of this observation is that neither the angle of attachment of phalloidin to actin core nor its degree of order has been affected during preparation of isolated microvilli. This lack of difference is reassuring considering that, during preparation of isolated microvilli, actin filaments are subject to severe stress by being cut off from the terminal web.

## Effect of ATP

ATP caused statistically significant change in $P_v$ and a change in distribution of both $P_v$ and $P_h$. Such change must be due to either an increase in disorder and/or to a change in orientation of the dipole of the dye. Application of Mathematica gave the unique answer that, in the case when isolated microvilli were incubated in the presence of ATP (Table 1, row 3), $\Theta$ was 54° and $\sigma$ was 0.88.

ATP most likely did not loosen binding of phalloidin to actin (because it does not do so in skeletal muscle actin (Burlacu and Borejdo, unpublished observation)). Therefore a change in $\sigma$ and/or $\Theta$ indicates transformation in the polymer structure of actin. Such transformation could result if ATP increased the flexibility of actin cores due to a direct effect on filaments or due to solubilization of portion of myosin-I (Matsudaira and Burgess, 1979). Alternatively, the change could be due to the mechanochemical interactions between actin core and myosin-I induced by ATP.

We think that it is unlikely that ATP had a direct effect on filaments, because ATP made skeletal actin filaments more stiff (Janmey et al., 1990). We cannot exclude the possibility that ATP increased flexibility of actin cores by removing myosin-I and causing a decrease in the number of myosin-I molecules interacting with actin. In many instances, however, binding of single-headed myosin subfragment-1 did not affect flexibility of actin filaments (e.g., Oosawa et al., 1973). Moreover, binding of double-headed HMM affected flexibility only at small molar ratios of HMM:actin (Oosawa et al., 1973): at high molar ratios the flexibility was not affected at all (Burlacu and Borejdo, 1992). Finally, recent electron paramagnetic resonance results of Ostap et al. (1992) show that binding of S1 to F-actin does not affect orientation of actin monomers within filaments. We therefore think that the removal of single-headed myosin-I is unlikely to have a major affect on flexibility of the core and conclude that at least some change in polarization was contributed by the mechanochemical activity of MV caused by addition of ATP.

It is not surprising that mechanochemical interactions could lead to changes in conformation of actin. The fact that in vitro myosin-I has actin-activated ATPase activity (Mooseker et al., 1989) and is capable of producing movement (Mooseker and Coleman, 1989) suggests that also in situ it may interact with the actin core to produce contractile force and lead to conformational changes. In striated muscle, a conventional model system where actin and myosin interact mechanochemically, development of force or motion is intimately associated with conformational changes in both myosin head (Huxley, 1969; Huxley and Kress, 1985; Morales et al., 1982; Eisenberg and Hill, 1985) and actin filaments

(Oosawa et al., 1961; Oosawa, 1977). Changes in conformation of myosin head (Dos Remedios et al., 19723; Thomas and Cooke, 1980; Borejdo et al., 1982) and of actin filament (Yanagida and Oosawa, 1978; Yanagida and Oosawa, 1980; Prochniewicz-Nakayama et al., 1983) have been observed in active muscle.

## Comparison with actin filaments from striated muscle

Table 1 (row 4) shows that polarizations were significantly different in microvillar cores and in single skeletal actin filaments. This is in agreement with earlier results on actin labeled with fluorescein-phalloidin (Prochniewicz-Nakayama et al., 1983). Fit to the striated actin data yielded values of $\Theta$ and $\sigma$ which differed by more than 5% from these obtained from microvillar actin. The difference is most likely due to the differences in binding of phalloidin to skeletal and to microvillar actins.

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