

# Reorganization of Actin Filament Bundles in Living Fibroblasts

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**ABSTRACT** We investigated how actin bundles assemble, disassemble, and reorganize during cell movement. Living chick embryonic fibroblasts were microinjected with actin molecules that had been fluorescently labeled with tetramethylrhodamine. We found that the fluorescent analogue of actin can be used successfully by both existing and newly formed cellular structures. Using time-lapse photography coupled to image-intensified fluorescence microscopy, we were able to detect various patterns of reorganization in motile cells. Assembly of stress fibers occurred near both the leading and the trailing ends of the cell. The initial structure appeared as discrete spots that subsequently extended into stress fibers. The extension occurred unidirectionally. The site of initiation near the leading edge remained stationary relative to the substrate during subsequent cell advancement. However, the orientation of the fiber could change according to the direction of cell movement. In addition, existing stress fibers could merge or fragment. The shortening of stress fibers can occur from either end of the fiber. Shortening from the proximal end (centrifugal shortening) was accompanied by a decrease in fluorescence intensity, as if the bundle were disassembling, and usually led to the total disappearance of the bundle. Shortening from the distal end (centripetal shortening), on the other hand, is usually accompanied by an increase in fluorescence intensity at the distal end of the bundle, as if this end had pulled loose from its attachment and retracted toward the center of the cell. Besides stress fibers, arc-like actin bundles have also been detected in spreading cells. These observations can explain how the organization of actin bundles coordinates with cell movement, and how stress fibers reach a highly regular pattern in static cells.

Light and electron microscopic studies have clearly demonstrated the presence of actin bundles in nonmuscle cells (2). The most prominent class, stress fibers, contain a number of accessory proteins such as alpha-actinin, tropomyosin, and myosin, arranged in a regular pattern somewhat similar to that in myofibrils (7, 14, 18). At least one end of the stress fiber is usually attached to the membrane, opposite to the site of adhesion plaque on the external surface (10, 12). Besides stress fibers, actin bundles have also been identified in the "arcs" of spreading cells (9, 20) and in microvilli (26).

Although these different types of bundles probably represent different structures and probably serve different functions, they share a common characteristic: the ability to reorganize. For example, "arcs" form near the leading edge of the cell, then move toward the nucleus and disappear (9,

20). Stress fibers exhibit a drastic reorientation when cells are placed in an electric field (16). Even in relatively static cells, changes in the organization of actin bundles have been detected (29). This is not surprising in view of the highly dynamic nature of the actin filaments, which are capable of polymerization-depolymerization, and of interaction with molecules such as myosin and gelsolin (for reviews, see references 5, 17). However, little is known about exactly how the relatively large bundle structures can be effectively reorganized in a cell. For example, how are actin bundles "translocated" during cell movement? Do cells constantly assemble new bundles at the leading edge and disassemble bundles at the trailing end? Or do existing bundles somehow move during cell locomotion?

In this study, we employed fluorescent analogue cytochem-

istry to follow directly changes in the organization of actin bundles in living chick embryonic fibroblasts. The approach involves preparation of functional fluorescent conjugates of cellular components, followed by microinjection of the fluorescent analogue into living cells. Since the introduction of the technique (22), a large number of fluorescently labeled cytoskeletal components, including actin, alpha-actinin, tropomyosin, vinculin, and tubulin, have been successfully injected into living cells and used by normal cellular structures (for reviews, see references 13, 24, 27). Thus this approach offers a unique opportunity to study the reorganization of specific molecules in living cells.

One common problem encountered in this approach is the use of intense excitation light, which causes photobleaching and possible cellular damage, during the detection of weak fluorescence emitted from a single cell (discussed in reference 28). As a result, each injected cell can be studied only for limited period of time. In this study, we have minimized the problem by using a highly sensitive image intensifier coupled

to a sophisticated time-lapse recording technique. The sequences of images taken at short time intervals revealed various ways through which actin filament bundles reorganize in living, motile cells.

## MATERIALS AND METHODS

*Preparation of Fluorescent Analogue:* Actin was purified from rabbit back and leg muscles according to Spudich and Watt (21). Iodoacetamidotetramethylrhodamine (Research Organics, Cleveland, OH) was dissolved in a buffer containing 2 mM Tris, 0.2 mM CaCl<sub>2</sub>. After titrating the pH to 9.0, the dye solution was mixed 1:1 with a solution of 5 mg/ml G-actin in 2 mM Tris, 0.2 mM CaCl<sub>2</sub>, 0.5 mM ATP, 0.05 mM dithiothreitol, pH 8.0, to obtain a final concentration of 0.3 mg dye/mg actin. The solution was centrifuged at 100,000 g for 2 h to remove undissolved dye aggregates. The supernatant was incubated at 0°C for 4 h, followed by addition of 5 mM dithiothreitol to stop the reaction. The solution was then dialyzed overnight against 2 mM Tris, 0.2 mM CaCl<sub>2</sub>, 0.5 mM ATP, 0.2 mM dithiothreitol, pH 7.8. Residual quenched dye molecules were removed by desalting through a G-25 column. Fluorescent fractions in the void volume were collected and carried through two cycles of polymerization and depolymerization. The conjugate, at a con-

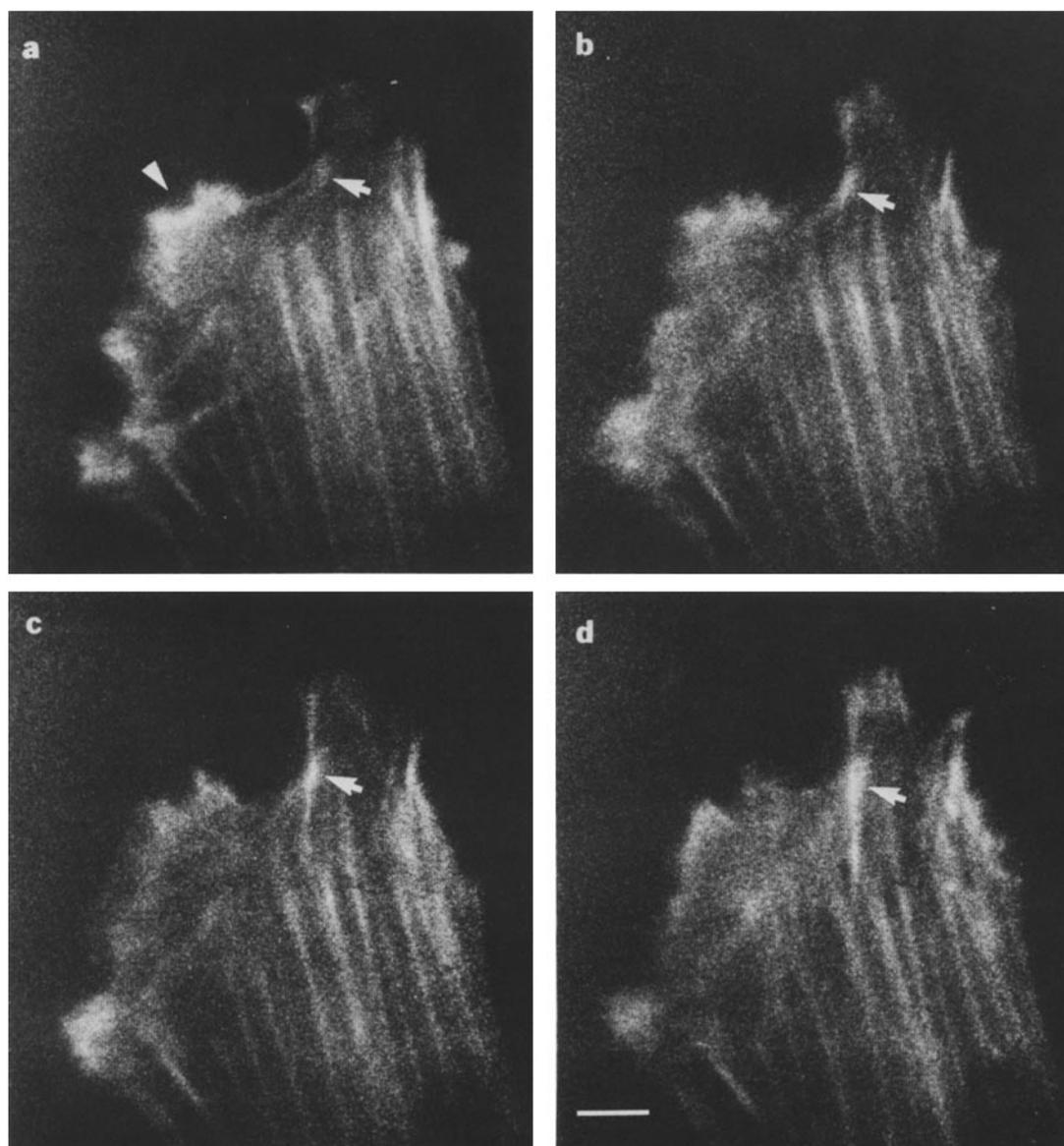


FIGURE 1 Assembly of a stress fiber (arrows) at the leading edge of the cell. Ruffle region was marked (arrowhead). Photographs taken at  $t = 0$  (a), 6 (b), 9 (c), and 15 min (d). Bar, 5  $\mu\text{m}$ .  $\times 1,925$ .

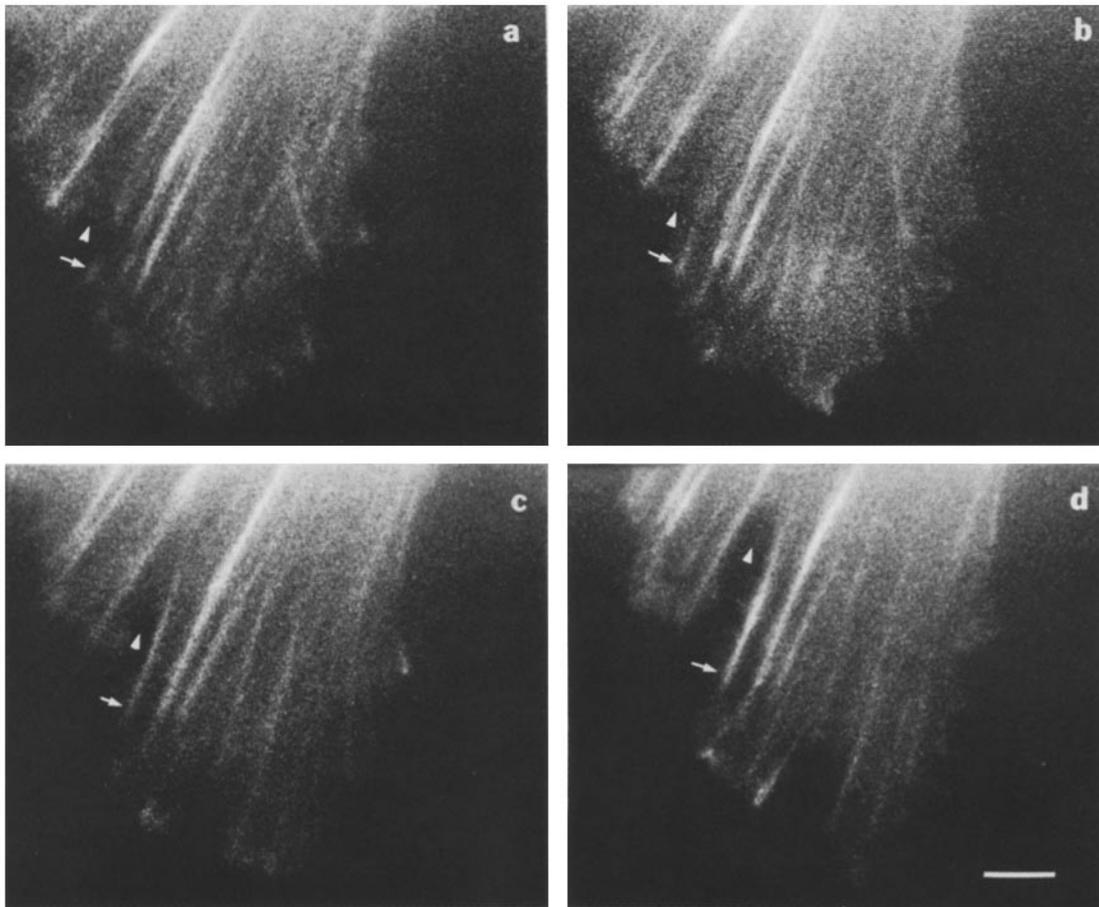


FIGURE 2 Assembly of a stress fiber (arrows) at a retracting end. Photographs taken at  $t = 0$  (a), 6 (b), 15 (c), and 24 min (d). The retraction of cytoplasm was indicated by arrowheads. Bar, 5  $\mu\text{m}$ .  $\times 1,875$ .

centration of 5 mg/ml, was stored by adding 2 mg of sucrose per milligram of actin, and immersed in liquid nitrogen. Before microinjection, an aliquot of the solution was dialyzed against injection buffer containing 0.5 mM PIPES, 0.05 mM  $\text{MgCl}_2$ , 0.2 mM ATP, 0.1 mM dithiothreitol, pH 6.95. The solution was then clarified in a Beckman type 42.2Ti rotor (Beckman Instruments, Palo Alto, CA) at 100,000  $g$  for 20 min. Each aliquot of labeled actin was used within 4 d after removal from liquid nitrogen.

The labeled actin had a dye to protein molar ratio of 0.7–0.9, measured according to Taylor et al. (23). Gel electrophoresis revealed a single fluorescent band with a mobility corresponding to that of actin.

**Cell Culture and Microinjection:** Embryonic chick heart fibroblasts were obtained according to Clark (3). The cells were plated on a number two coverslip (Fisher Scientific, Pittsburgh, PA), which was attached with High Vacuum Grease (Dow Corning, Midland, MI) to the bottom of a plexiglass plate, covering a 35-mm-diameter hole. F12K medium (KC Biological, Lenexa, KS) containing 5% fetal calf serum (Irving Scientific, Santa Ana, CA) and 50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin was used for cell culture. Microinjection was performed after 12–24 h of incubation, on a Zeiss IM35 inverted microscope equipped with phase-contrast optics, a stage heater, and a  $\text{CO}_2$  stream. The stage heater was similar to that described by Willingham and Pastan (30). An open dish of water placed near the inlet of warm air maintained the humidity in the environment. Procedures for microinjection were essentially as described by Graessmann et al. (8). Cells were incubated for 1 h after microinjection to allow complete dispersion and incorporation of injected molecules.

**Image Recording:** Cells were maintained on the IM 35 microscope stage in warm, humid air supplemented with  $\text{CO}_2$ . A stable temperature is crucial for maintaining the constant focal plane during image recording. Fluorescence images were detected using a  $100\times/\text{N.A.}1.30$  neofluar phase objective, and a Venus DV-2 two stage TV image intensifier. A 100-W quartz-halogen lamp was used as the light source. An electronic shutter (Ealing, S. Natuck, MA) was attached to the lamp to control the duration of excitation. Images on the TV monitor were photographed with an Olympus OM-2 camera, equipped

with a 250 exposure film magazine and a motor winder. The operation of the shutter and the camera was controlled by a Commodore 64 microcomputer with a self-constructed relay circuit board attached to the user port. The program, written in a combination of the 6502 machine language and BASIC, allows precise control of the duration of each exposure (up to 1/60 s) and the interval of recording. At each fixed time interval (usually 3 min), the shutter was opened briefly and the camera was triggered. We used Kodak Tri-X film with  $\frac{1}{2}$ -s exposure, while keeping the intensity of excitation light to a minimum. The relatively long exposure allows 15 consecutive frames of TV images to accumulate on the film and improves the signal to noise ratio. The exposure time during printing was varied slightly to balance the slight photobleaching occurred during image recording and to obtain prints of similar contrast against the background.

## RESULTS

The tetramethylrhodamine conjugate of actin has been used previously by Glacy (6) to study the incorporation of actin into living chick fibroblasts. This conjugate has a sufficient stability under the excitation light beam, so that when appropriate recording techniques are used (see Materials and Methods), a single cell can be recorded for many hours without significant deterioration of the image quality. In addition, under these conditions living cells showed no apparent response to or damage by the pulses of excitation light, and the motile activities were not affected (see, for example, Fig. 3). We recorded each injected cell at an interval of 3 min for up to 1 h. The relatively short interval allowed specific structures in different frames to be traced reliably.

Apparent redistribution of injected actin was detected both

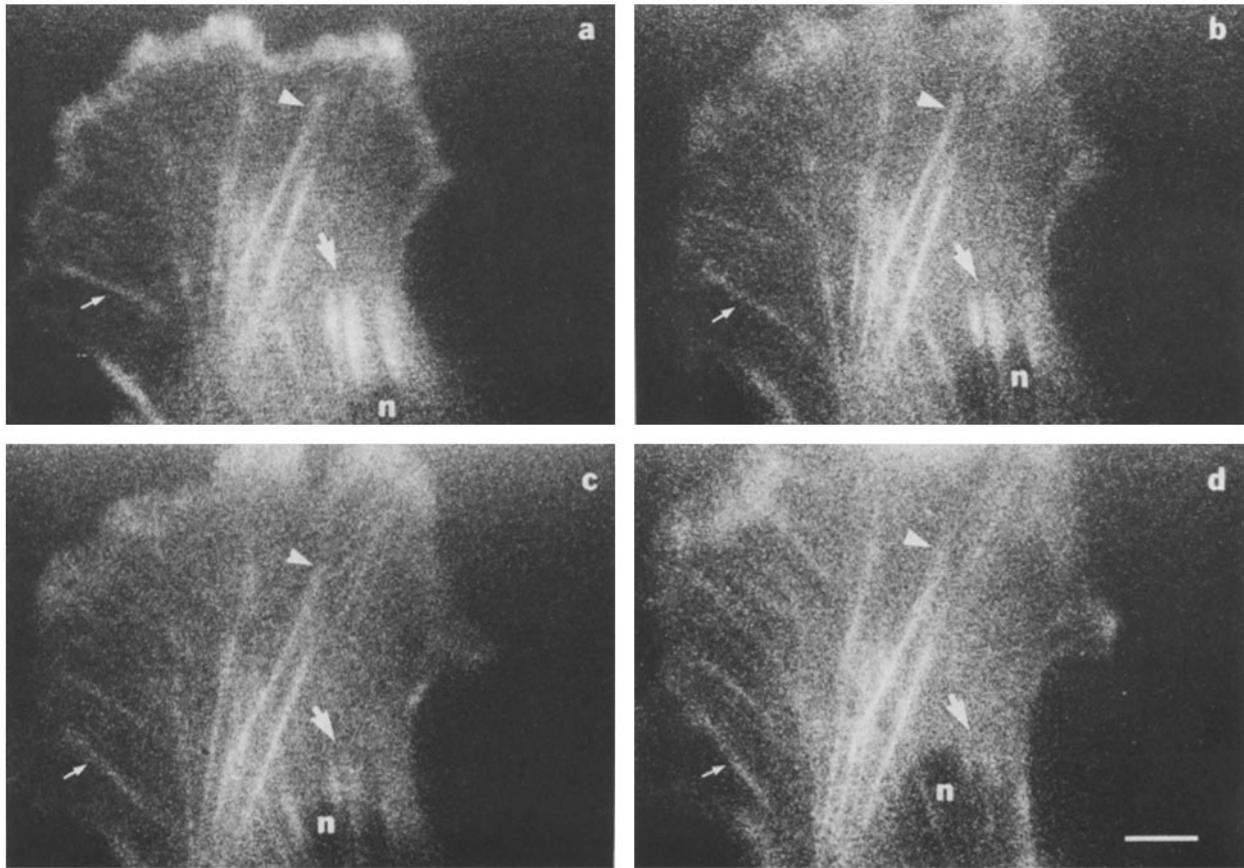


FIGURE 3 Stress fibers in a rapidly advancing cell. A fiber (small arrows) was initially misaligned with the axis of the cell but later tilted to assume a more parallel orientation. As the cell advanced during the period of observation, so did the nucleus (*n*). However, the distal ends of stress fibers remained stationary (arrowheads). Two neighboring stress fibers (large arrows) underwent centrifugal shortening and disassembled during the period. Photographs taken at  $t = 0$  (a), 3 (b), 6 (c), and 9 min (d). Bar,  $5 \mu\text{m}$ .  $\times 1,875$ .

in the ruffle area and along stress fibers. Accumulation of actin occurred in the ruffle during active advancement (Fig. 1). When the ruffle activity ceased, the fluorescence intensity also decreased. However, since injected control molecules such as bovine serum albumin also accumulate in the ruffle (15), definitive interpretation of the distribution of actin in relation to the ruffle activity was difficult.

The assembly of new stress fibers was detected in most motile cells (Figs. 1 and 2). The first detectable structures were numerous small actin-containing aggregates. At least some of these aggregates subsequently developed into discrete bundles. The elongation of stress fibers occurred only in the centripetal direction (toward the nucleus), and was usually accompanied by an increase in fluorescence intensity, indicating a possible increase in the number of filaments within the bundle. Assembly of bundles has been detected near both the leading (Fig. 1) and the retracting ends (Fig. 2) of the cell.

Once assembled, the end of the stress fiber near the leading edge remained stationary relative to the substrate despite the forward movement of the cell (Fig. 3, arrowheads). Some fibers were misoriented, with regard to the direction of cell movement, at the initial stage of assembly. However, at least some of them later tilted to assume an orientation more or less parallel to the direction of cell movement (Fig. 3, small arrows). The opposite, tilting away from the direction of cell movement, has never been observed.

Existing stress fibers can merge (Fig. 4). Therefore, even though they did not elongate directly in the forward direction, they can nevertheless extend in the forward direction by merging with bundles which were assembled in a more distal location. The converse, fragmentation, has also been observed. In Fig. 5, a stress fiber has fragmented into three fibers of slightly different orientations.

The shortening of stress fibers can occur at either the proximal or the distal end of the fiber. However, the two modes of shortening probably represent two different processes. Shortening from the distal end (centripetal shortening) was usually coupled to an increase in fluorescence intensity at that end of the bundle (Fig. 6). Shortening at the proximal end (centrifugal shortening), on the other hand, was coupled to a simultaneous decrease in fluorescence intensity along the length, and eventually resulted in the total disappearance of the bundle (Fig. 3, large arrows).

We have also observed the reorganization of bundles that were perpendicular to the axis of the cell. Such bundles, previously referred to as "arcs" (9, 20), were commonly observed in spreading cells. In Fig. 7, the formation of arcs was first detected in regions  $5\text{--}10 \mu\text{m}$  from the leading edge. Unlike the assembly of stress fibers, the formation of these bundles occurred without the involvement of discrete initiation sites. They appeared directly as short bundles, and, as the bundles moved toward the nucleus, both the fluorescence

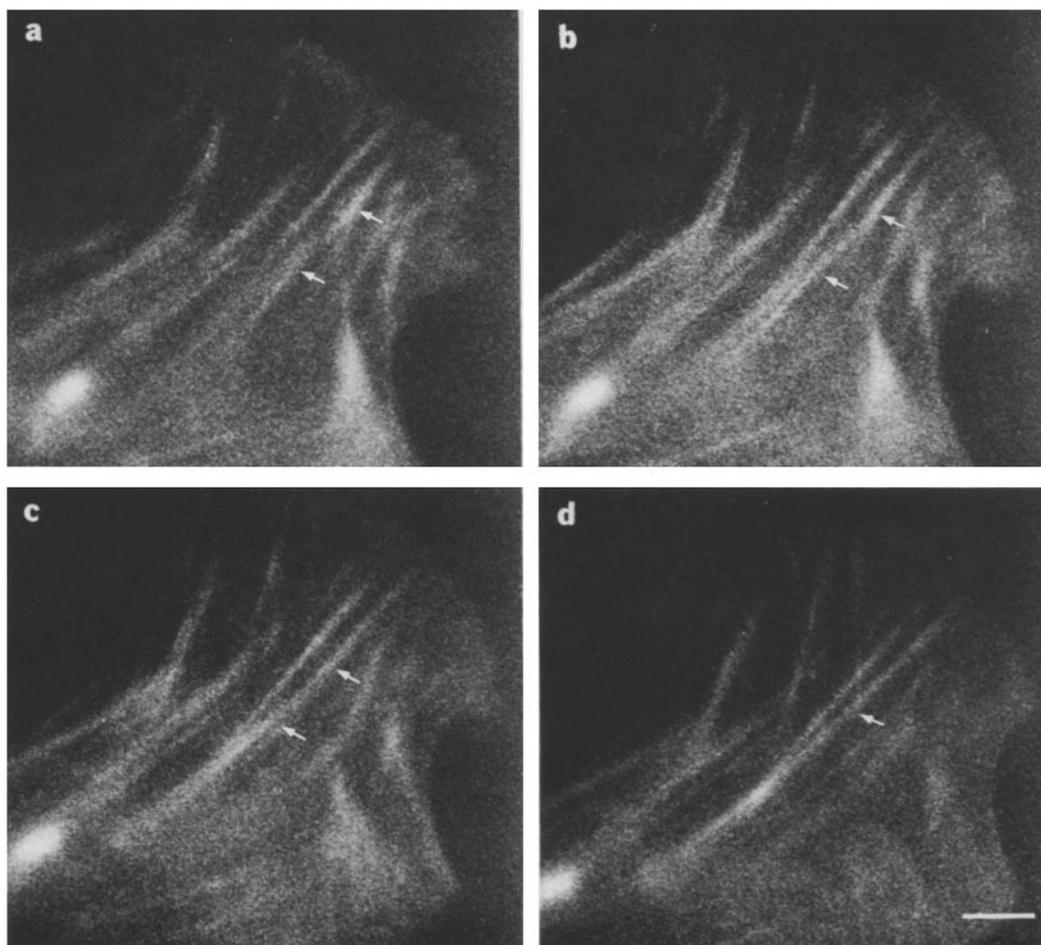


FIGURE 4 Merging of two stress fibers (arrows). Photograph taken at  $t = 0$  (a), 9 (b), 18 (c), and 30 min (d). Bar, 5  $\mu\text{m}$ .  $\times 1,925$ .

intensity and the length increased. After reaching the perinuclear region, the bundles dispersed and resulted in an accumulation of diffuse fluorescence in the region (Fig. 7f).

## DISCUSSION

Although stress fibers are most prominent in static cells (11), they are nevertheless capable of undergoing dynamic reorganization in motile cells. The observations made in this study clearly illustrate how the organization of stress fibers can change and thus coordinate with the translocation of the cell. However, since the assembly of stress fibers occurs at both the leading and trailing ends of the cell, it is unlikely that the assembly process is directly related to the mechanism of the forward advancement of the cell.

Various mechanisms for the assembly of stress fibers have been proposed, including lateral aggregation of membrane-associated filaments (4), mechanical stretching of an actin network (10), and net polymerization (19). Although the present observations do not allow us to distinguish among these possibilities, they do reveal important constraints any valid mechanism must satisfy. First, the initial stage of assembly involves discrete actin-containing sites near the edge of the cell. These sites could consist of nucleation sites or "profilactin" (25), could represent the initial patch of membrane-associated actin filaments, or could represent sites where a stretching force will be exerted. The elongation process is

unidirectional and propagates from the site of initiation over a period of  $\sim 20$  min. Thus, if the stretching mechanism is correct, the transmission of force and/or the response to the force probably occur at a relatively slow rate. In addition, since a clear increase in fluorescence intensity is detected along the length of the fiber, polymerization or association of additional filaments probably occurs during elongation. This would explain why filaments near the membrane have a uniform polarity (19), whereas filaments in the central region of stress fibers exhibit mixed polarities (1).

Various interesting, yet unsolved, questions exist regarding the arrangement of stress fibers. For example, stress fibers are constantly detected near the leading edge of motile cells. Is this achieved by moving existing fibers forward, by constantly assembling new fibers and disassembling those trailing behind, or by adding extensions to existing fibers? In addition, stress fibers with both ends anchored to the membrane have been detected in static cells (10). Yet no explanation has been offered as to how this configuration was reached. Does a single stress fiber extend across the cytoplasm and establish a second attachment point when it reaches the other end of the cell? Heath and Dunn (10) have also observed stress fibers with multiple focal contacts along the length, as well as single fiber branching into multiple fibers. On the basis of the mechanism of assembly alone, it is difficult to explain how these various types of organization are achieved.

Although the merging of stress fibers could represent superficial superimposition of actin bundles, it does offer an attrac-

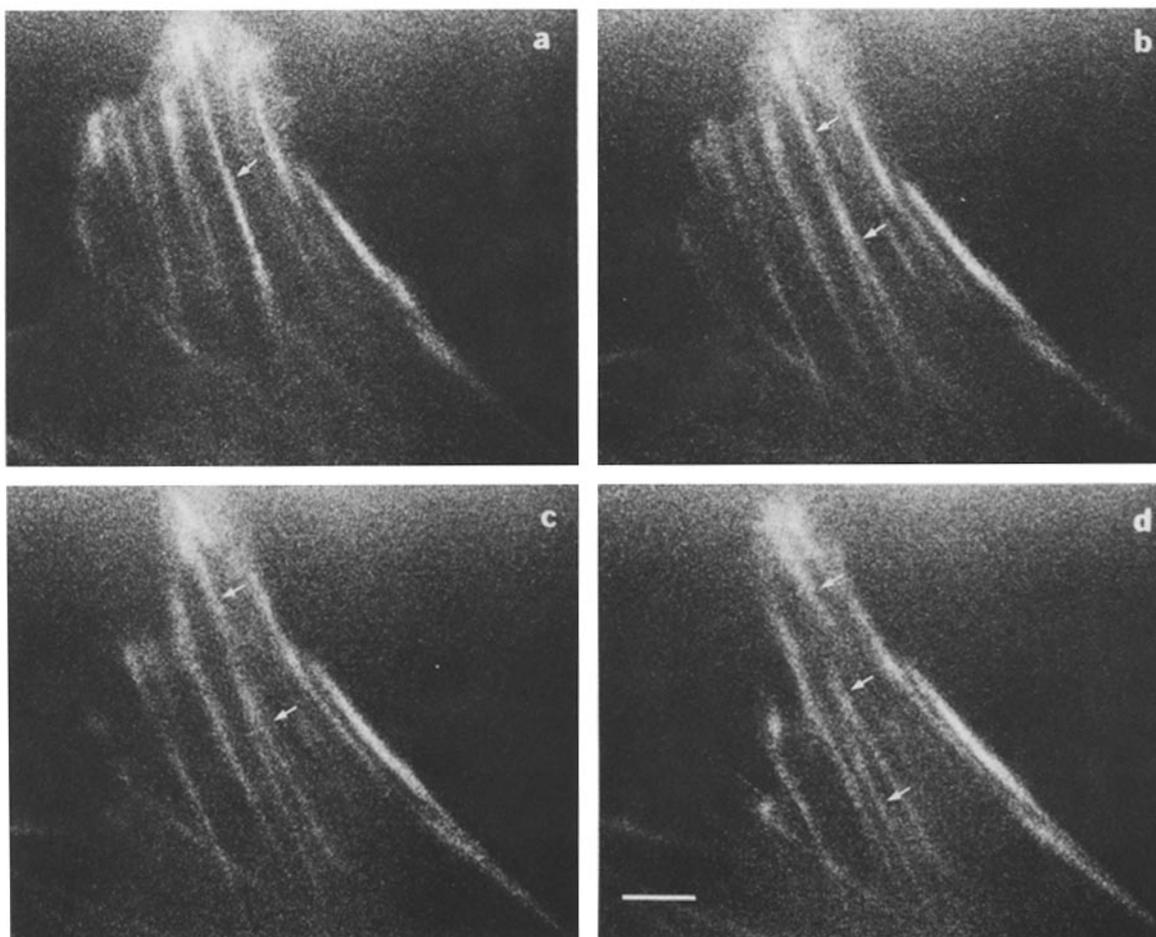


FIGURE 5 Fragmentation of one stress fiber into three fibers (arrows). The region of the cell was retracting during observation. Photographs taken at  $t = 0$  (a), 6 (b), 12 (c), and 18 min (d). Bar,  $5 \mu\text{m}$ .  $\times 1,875$ .

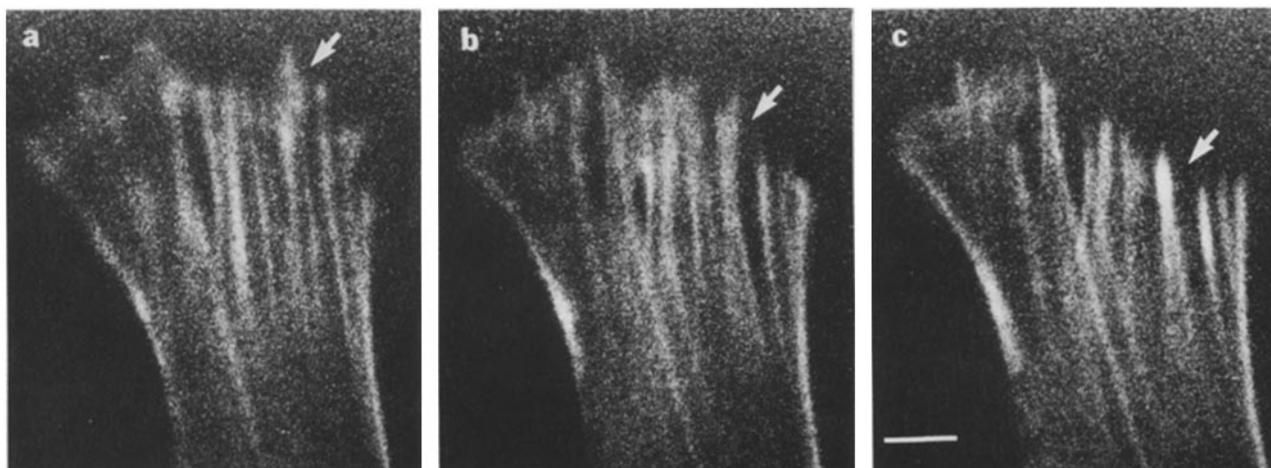


FIGURE 6 Centripetal shortening of stress fibers. A group of stress fibers (arrows) underwent centripetal shortening during the retraction of the cell. Photographs taken at  $t = 0$  (a), 15 (b), and 24 min (c). Bar,  $5 \mu\text{m}$ .  $\times 1,925$ .

tive answer to all these questions. First, merging would result in fibers with multiple and/or branching focal contacts, as described by Heath and Dunn (10). In addition, it allows existing fibers to “extend” along the direction of cell movement, simply by merging with new fibers initiated at a more distal location. In static cells, stress fibers initiated in different regions of the cell could extend and merge near the center of the cell, forming fibers with both ends attached. The mecha-

nism of merging could be similar to that involved in elongation, during which additional actin filaments appear to be incorporated into the stress fiber. Both processes presumably would involve longitudinal associations of actin filaments through interactions with bundling factors and/or myosin.

Stress fibers have been observed to shorten in both centripetal and centrifugal directions. However, the two modes probably represent different processes. Centripetal shortening

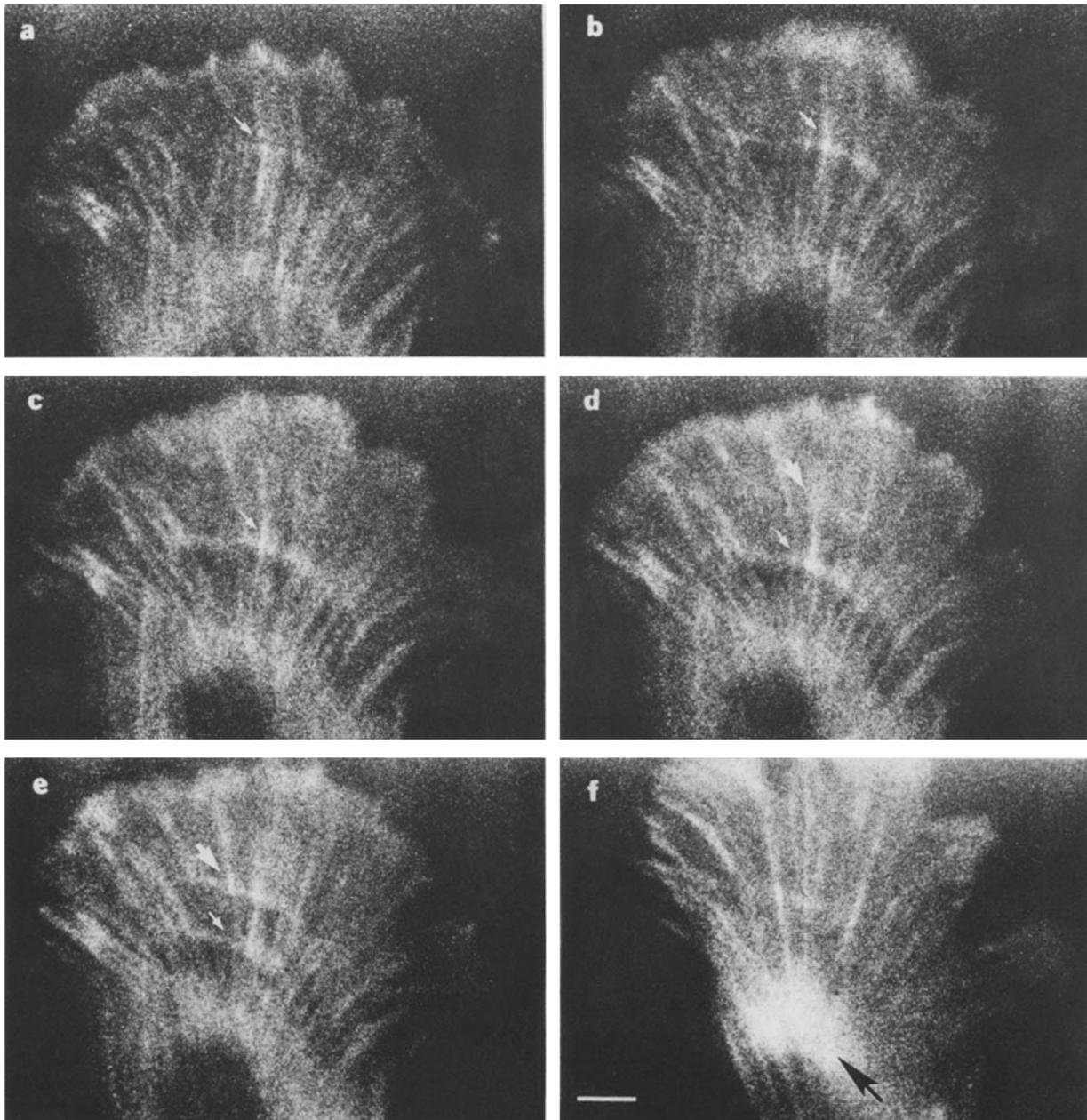


FIGURE 7 Formation and movement of arcs in a spreading cell. Movement of an arc was clearly depicted (small arrows). A new arc formed in *d* and became more prominent 3 min later (large arrows). Photographs taken at  $t = 0$  (*a*), 3 (*b*), 6 (*c*), 9 (*d*), and 12 min (*e*). Accumulation of diffuse fluorescence in the perinuclear region was apparent at a later time point (33 min [*f*], black arrow). Bar, 5  $\mu\text{m}$ .  $\times 1,750$ .

occurs during retraction of the cell. Since no apparent decrease in fluorescence intensity is detected, there is probably no significant disassembly of the bundle. Most likely, centripetal shortening represents retraction or contraction of actin bundles presumably through sliding of actin filaments. This process could be related to the mechanism of cell retraction. Centrifugal shortening, on the other hand, is coupled to a decrease and an eventual disappearance of fluorescence, and probably represents disassembly of stress fibers.

The formation of arcs in spreading cells, as shown in Fig. 7, parallels closely observations made previously using phase-contrast and polarization optics (9, 20). Unlike the formation of stress fibers, the formation of arcs does not involve discrete sites of initiation. It has been proposed that arcs represent

compression waves traveling through a medium of actin meshwork, without actual net translocation of the medium (20). If this is the case, there should be no accumulation of diffuse actin in the perinuclear region. Since we observe an increase in diffuse fluorescence in the perinuclear region following the dispersion of arcs, it is more likely that arcs represent actual translocating actin bundles.

This study describes various modes of reorganization exhibited by actin bundles in motile cells. Using a number of sophisticated techniques, such as photobleaching and image analysis in combination with the present approach, many important aspects of each activity can be probed. For example, during the assembly of stress fibers, does net polymerization of actin occur? If so, at which end? The information is crucial

for understanding how actin achieves its amazing functions in eucaryotic cells.

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