Actin Antibody: The Specific Visualization of Actin Filaments in Non-Muscle Cells

Elias Lazarides, and Klaus Weber

PNAS 1974;71;2268-2272
doi:10.1073/pnas.71.6.2268

Proc. Nat. Acad. Sci. USA
Vol. 71, No. 6, pp. 2268–2272, June 1974

Actin Antibody: The Specific Visualization of Actin Filaments in Non-Muscle Cells

(immunofluorescence/microfilaments/sodium dodecyl sulfate gel electrophoresis)

ELIAS LAZARIDES AND KLAUS WEBER

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Communicated by J. D. Watson, March 11, 1974

ABSTRACT **Actin purified from mouse fibroblasts by sodium dodecyl sulfate gel electrophoresis was used as antigen to obtain an antibody in rabbits. The elicited antibody was shown to be specific for actin as judged by immunodiffusion and complement fixation against partially purified mouse fibroblast actin and highly purified chicken muscle actin. The antibody was used in indirect immunofluorescence to demonstrate by fluorescence light microscopy the distribution and pattern of actin-containing filaments in a variety of cell types. Actin filaments were shown to span the cell length or to concentrate in "focal points" in patterns characteristic for each individual cell.**

Eucaryotic cells contain three basic fibrous structures: filaments, microfilaments, and microtubules. These three structures are thought to be intimately involved in the maintenance of cell shape, in cell movement, and in other important cellular functions (1). Microfilaments are thought to contain actin. This assumption is based on the observation that these structures can be selectively decorated with heavy meromyosin, a specific proteolytic fragment of muscle myosin known to interact with muscle actin (2). Furthermore, actin is now now known to exist as a major protein component of a variety of non-muscle cellular types and in each case it has properties markedly similar to those of its muscle counterpart (3–7)*. The major protein of the microtubular system, tubulin, has been isolated and well characterized (9). The basic protein subunit of the filament structure, however, has not so far been identified. Presumptive muscle proteins like myosin (10–13) and tropomyosin (14) have been found in some non-muscle cells. However, their exact distribution within the cell, as well as their specific localization in one of these fibrous structures, is as yet undetermined.

We have developed a relatively simple way of selectively visualizing filamentous structures in the cell by using antibodies made against different structural proteins. The problem of purifying each antigen separately was circumvented by using sodium dodecyl sulfate (SDS) gel electrophoresis. The denatured proteins are antigenic, and the antibody obtained cross reacts with the native protein. Once specificity has been demonstrated, the antiserum obtained can be used in indirect immunofluorescence to visualize the structures in the cell with which the protein is associated.

In this paper we have used mouse fibroblast actin to test this approach. The actin, purified by SDS gel electrophoresis, was used to obtain an antibody in rabbits. The antiserum obtained was shown by immunodiffusion and complement fixation to be specific for actin. This antibody was then used in indirect immunofluorescence to show that microfilaments are polymers of actin. This technique also enabled us to demonstrate the complex network of actin filaments in a variety of cell types.

Abbreviations: SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

* The authors apologize for not referring to all the contributors in this field. The reader is referred to a recent detailed review of actin and myosin in non-muscle cells for complete references (8).

MATERIALS AND METHODS

Growth of Cells. Actin was isolated from the cell line SV101, a clone of mouse fibroblast 3T3 cells transformed by Simian virus 40. This transformed cell line was chosen because it grows to a higher saturation density than the parent 3T3 cell line (15). The cells were grown in roller bottles (Vitro Corp.) in Dulbecco's modified Eagle's medium containing 10% calf serum and 50 μg/ml of gentamycin. At confluency, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). The cells were then scraped off the bottles, collected by low speed centrifugation, and stored at −70°.

Preparation of Actin. The cells were thawed and homogenized in 20 volumes of 95% ethanol. The precipitate was collected by low speed centrifugation, washed immediately with ether, and air dried. The yield from 10 bottles was approximately 1.2 g of ethanol-ether powder. The ethanol-ether powder was stirred at 4° in 0.01 M sodium phosphate buffer (pH 6.8), 10 mM $MgCl_2$, and 1 mM dithiothreitol (15 ml of buffer per g of powder) for 3–5 hr. The supernatant was made 30% in ammonium sulfate by adding 0.17 g of ammonium sulfate per ml of extract. After stirring for 30 min at 4°, the precipitate was collected by centrifugation and dissolved in and dialyzed against 0.01 M Tris·HCl (pH 7.5), 10^{-4} M $CaCl_2$, 1 mM dithiothreitol, and 10^{-4} M ATP. The actin could be further purified by a second precipitation at 30% ammonium sulfate saturation. Under these conditions, 1 g of ethanol-ether powder yields approximately 1 mg of actin.

Highly purified chicken muscle actin was a generous gift from Dr. Susan Lowey.

Antibody Preparation. The actin used as an antigen in rabbits was purified through SDS slab gel electrophoresis from the high speed supernatant (100,000 × *g*) of a mouse fibroblast cell homogenate (see *Results*). Approximately 400 μg of the antigen was injected in complete Freund's adjuvant and 2 weeks later the rabbits were boosted with an additional 400 μg. Blood was collected 6 weeks after the last injection and the serum was clarified by centrifugation at 10,000 rpm. The gamma globulin fraction was partially purified using precipitation with half saturated ammonium sulfate. It was

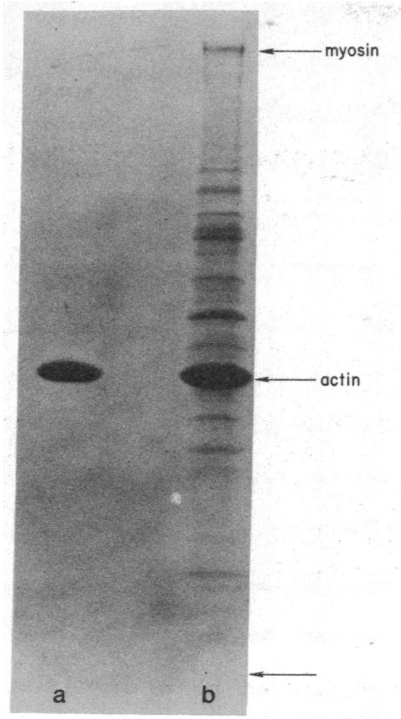

FIG. 1. SDS gel electrophoresis of actin. SDS polyacrylamide slab gel electrophoresis was performed in 12.5% slabs according to Studier (19). (*a*) Highly purified chicken muscle actin (10 μg). (*b*) A 30% ammonium sulfate cut of a low-salt extract from an ethanol powder of mouse fibroblasts (15 μg). A small amount of myosin copurifies with actin under the extraction conditions used (see *Materials and Methods*). The *arrow* at the bottom of the figure shows the dye front of the gel.

then dialyzed into 0.15 M NaCl, 0.01 M Tris·HCl (pH 7.5), and stored at −20° at a concentration of approximately 30 mg/ml.

Indirect Immunofluorescence. Cells were grown on glass coverslips in the appropriate medium (see figure legends). The coverslips were washed briefly in PBS to remove excess medium and fixed in PBS containing 3.5% formaldehyde for 20 min at room temperature. They were subsequently washed thoroughly in PBS, treated with absolute acetone at −10° for 7 min, and air dried. An appropriate dilution (1:20 in PBS) of the rabbit antibody was applied to the cells. After incubation in a humid atmosphere at 37° for 1 hr, the coverslips were washed 3 times in PBS and incubated for 1 hr with a 1:10 dilution of goat anti-rabbit globulins coupled to fluorescein made in PBS (Miles). The coverslips were washed 3 times in PBS and once in distilled water and mounted in Elvanol on a glass slide. Coverslips were viewed in a Zeiss PM III microscope with ultraviolet optics. Photographs were taken using Plus-X film (Kodak).

RESULTS

A protein purified from an ethanol powder of SV101 cells has several properties which identify it as mouse fibroblast actin: (*i*) it coelectrophoreses on SDS gels with highly purified chick muscle actin with a molecular weight of 45,000 (Fig. 1); (*ii*) it precipitates characteristically at a low ammonium sulfate saturation (12) (see below); (*iii*) it binds ATP on millipore filters; (*iv*) it is precipitable by 3 mM vinblastine sulfate

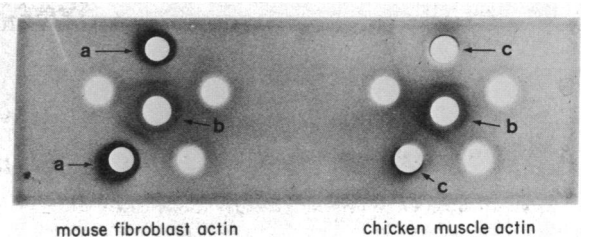

FIG. 2. Immunodiffusion of actin antiserum. (*a*) 4 μg (upper hole) and 2 μg (lower hole) of a 30% ammonium sulfate cut from mouse fibroblasts. The heavy precipitate around the holes is due to aggregated actin. (*b*) 20 μl of partially purified rabbit antiserum. (*c*) 5 μg (upper) and 2 μg (lower) of purified chicken muscle actin. Immunodiffusion was performed at 37° for 24 hr. The plates were washed for 24–36 hr at room temperature in 0.15 M NaCl, 0.01 M Tris·HCl (pH 7.5). They were subsequently stained in 0.25% Coomassie brilliant blue–50% methanol–7.5% acetic acid for 1 hr, destained in 7.5% methanol–7.5% acetic acid, and photographed.

(16, 17) and by 50 mM Mg^{+2} ions (18); and (*v*) it undergoes monomer to polymer transformations. Similar properties characterize skeletal muscle actin and the protein extensively studied and identified as actin-like (13, 16–18) in a variety of nonmuscle cellular types including cultured chick embryo fibroblasts (3) and human platelets (6). Mouse fibroblast actin purified as described in *Materials and Methods* is approximately 70% pure and shows one major polypeptide on SDS polyacrylamide gels and on polyacrylamide gels at pH 4.5 run in the presence of 8 M urea. Fig. 1*b* shows the purity of mouse fibroblast actin used in the experiments below. The gel is purposely overloaded to reveal all minor contaminants.

Approximately 10% of the total cellular actin precipitates at the 30% ammonium sulfate cut of a low-salt extract of an ethanol powder. Another 50% is recovered in the 30–60% ammonium sulfate cut. The remainder can be extracted as an actomyosin-like complex from the powder in the presence of 0.6 M NaCl. This differential fractionation appears to depend in part on the state of the polymerization of the actin under the extraction conditions used. In order to obtain sufficient actin to use as antigen, we therefore decided to purify this protein by SDS gel electrophoresis from the high-speed supernatant of homogenized SV101 cells.

SDS polyacrylamide gels separate proteins according to the molecular weights of their polypeptide chains (27) and therefore all actin will move with a uniform molecular weight on the gel regardless of its original state of polymerization. This method has the further advantage that SDS denatured proteins often make good antigens and allow one to use many different proteins as antigens from the same high-speed supernatant. The procedure is of general applicability and will be described in detail elsewhere (E.L. and K.W., manuscript in preparation).

The proteins moving as a major band at molecular weight 45,000 on SDS slab gels were recovered from the gels by elution. Approximately 800 μg of protein were obtained and this material was used as an antigen. Previous experiments involving ion exchange chromatography of the high-speed supernatant had shown that actin constituted more than 85% of the total protein moving in this molecular-weight range. The remaining 15% was shown to have different chromatographic properties than actin and showed a small number of minor species on polyacrylamide gels at pH 4.5 in 8 M urea.

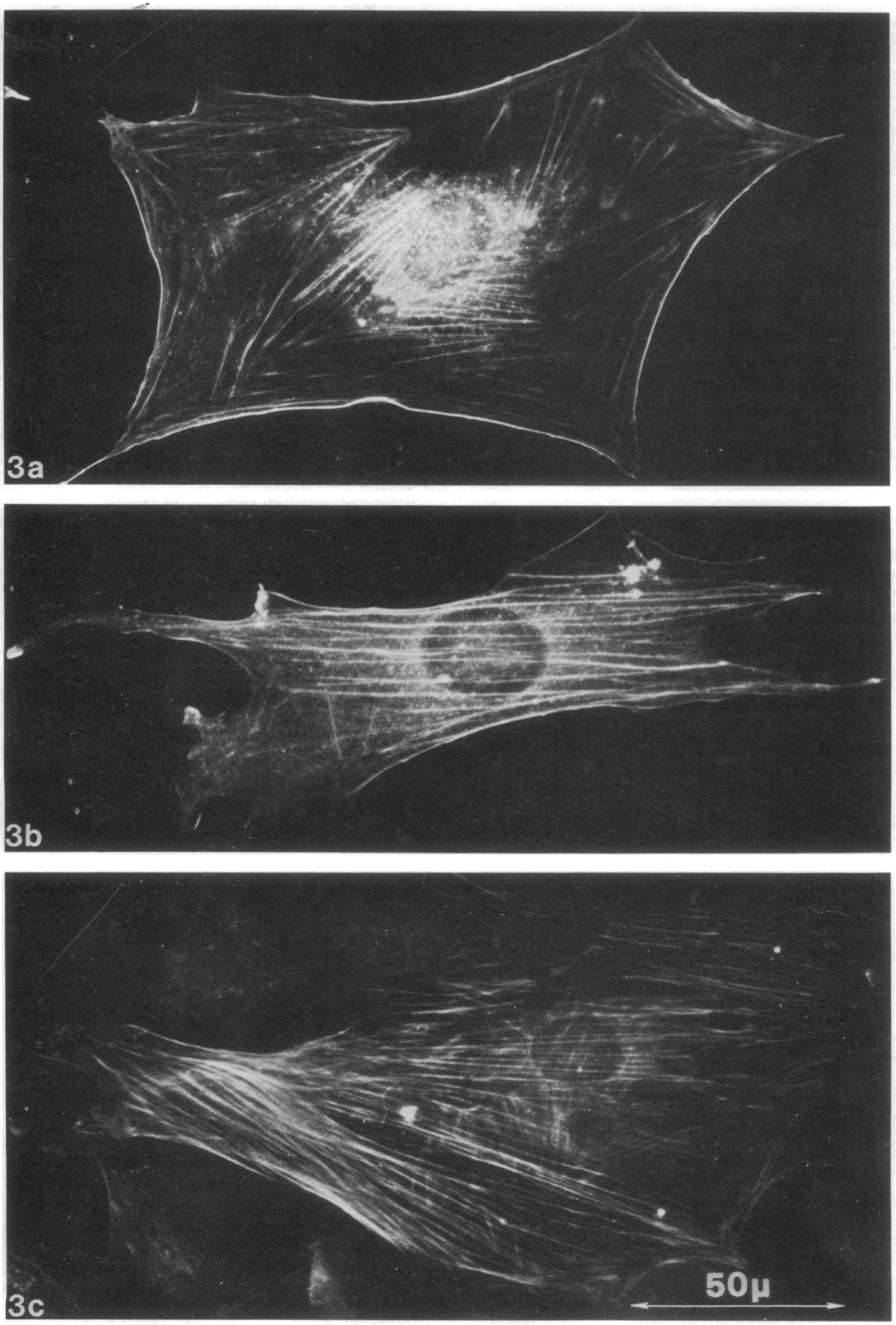

FIG. 3. (*Legend appears at bottom of the next page.*)

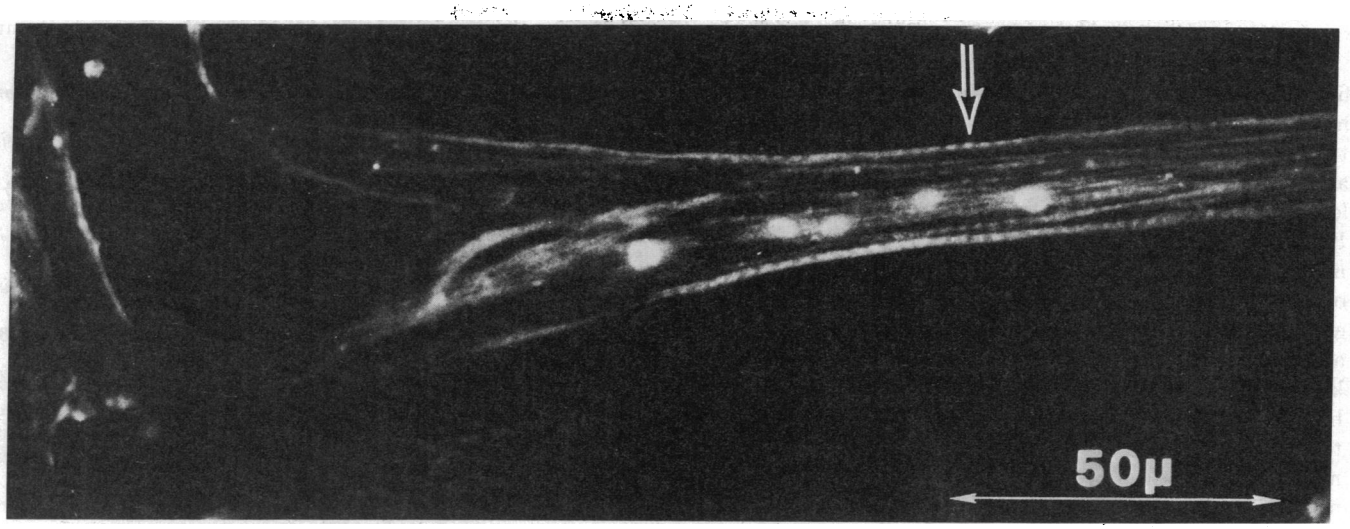

FIG. 4. Indirect immunofluorescence of actin antibody with primary chick embryo myoblasts. The *arrow* indicates the actin striations characteristic of myofibrils. The nuclear staining seen in this figure seems to be nonspecific. Purification of the antibody used in this experiment by 50% ammonium sulfate fractionation removes the nuclear fluorescence. The culture of primary chick embryo myoblasts was prepared for us by Dr. C. M. Chang using 5% chick embryo extract, 7% horse serum, and 3% fetal-calf serum in F-12 medium.

The antiserum is specific for actin as judged both by immunodiffusion and complement fixation, using the purified mouse fibroblast actin preparation (Fig. 1*b*) and the homogeneous preparation of chicken muscle actin (Fig. 1*a*). The results of the immunodiffusion assay are shown in Fig. 2. Both mouse fibroblast actin and chicken actin show a single precipitin line when diffused against the antiserum. In complement fixation the antibody shows complement fixing ability at a dilution of 1:150 both with the mouse fibroblast actin and the chicken actin. Complement fixation was performed by a modification of the micro method of Sever (20) (Osborn and Weber, in preparation).

Since the antibody appeared to be specific for actin, we attempted to use it for indirect immunofluorescence (21) in the hope of revealing the intracellular distribution of actin. Cells grown on coverslips were fixed in formaldehyde for optimal preservation of fibrous material and were then stained with antibody using the indirect immunofluorescent technique (see *Materials and Methods*). Fig. 3 shows the fluorescence pattern of the mouse fibroblast 3T3 cell line and a baby hamster kidney cell line (BHK) stained with the actin antibody. The fluorescent staining reveals a multitude of actin-containing filaments marking clearly the cell periphery and spanning the interior of the cell frequently parallel to each other. A multitude of patterns have been observed with varying degrees of complexity and each cell appears to have its own individual way of portraying its actin filamentous network. However, after observing hundreds of cells, two major patterns appear to prevail in the cell types tested so far. One is that shown in Fig. 3*b* and *c* where the fibers run parallel to each other along the long axis of the cell. The other is that shown in Fig. 3*a* where actin filaments seem to converge at what we have named "focal points." Control experiments with rabbit antiserum obtained before immunization reveal no fluorescent fibers. Furthermore, antibodies obtained against other cellular structural proteins reveal a very different fluorescent staining pattern.

As shown above (Fig. 2), the antibody cross reacts with chicken muscle actin. We therefore studied the staining pattern of cultured primary chick embryo myoblasts in early myogenesis (Fig. 4). Besides the usual actin-containing filaments, the fluorescence reveals the characteristic banding striations of actin in newly formed myofibrils.

DISCUSSION

It is known that actin is a major component of eucaryotic cells. It accounts for some 10% of the cells' proteins. Mouse fibroblast actin coelectrophoreses with chick muscle actin and has a molecular weight of 45,000. Not surprisingly, therefore, actin purified from SV101 fibroblasts shows the same properties as the protein extensively studied from a variety of different cell types.

SDS-denatured actin obtained by preparative gel electrophoresis of a cell extract after high speed centrifugation has proven to be a good antigen in rabbits. The antibody obtained reacts with native actin from SV101 cells and with highly purified chick muscle actin both by immunodiffusion and complement fixation. The antibody was directed against a specific class of proteins coelectrophoresing with actin and having a molecular weight of 45,000. While the possibility of obtaining antibodies against other minor proteins in the same molecular-weight range is likely, the possibility of obtaining antibodies to proteins with different polypeptide molecular weights is excluded. Thus the previous difficulty of obtaining an actin specific antibody due to the presence of contaminating tropomyosin is circumvented (22). Furthermore, actin is the only major component constituting more than 85% of the proteins migrating with a molecular weight of 45,000. We therefore believe that although the original antigen was not completely homogeneous, the small amounts of con-

FIG. 3 (*on preceding page*). Indirect immunofluorescence using actin antibody. (*a, c*) A sparse mouse fibroblast cell line (3T3). (*b*) A sparse hamster established cell line (BHK). Cells were grown on coverslips in Dulbecco's modified Eagle's medium containing 10% calf serum.

taminating proteins would not interfere with the final analysis.

Immunofluorescence clearly shows the presence of actin filamentous structures. Although limited by resolution, it has the advantage over electron microscopy in revealing the two dimensional mosaic of actin filaments of a whole cell. It also demonstrates that these fibers frequently span the whole length of the cell or converge to characteristic "focal points." Furthermore, the actin filament pattern observed with immunofluorescence corresponds to the microfilament pattern observed by electron microscopy. These latter structures exist as well organized bundles running in close association with and parallel to the plasma membrane, both in baby hamster kidney cells (23) and in 3T3 cells. In 3T3 cells, microfilament bundles are also seen frequently to converge together in patterns very similar to the "focal points" observed by immunofluorescence (unpublished observations with R. Goldman). Since the fluorescence is seen also in close association with the plasma membrane, the actin filaments observed with immunofluorescence correspond to the microfilaments observed by electron microscopy. This conclusion is further substantiated by the finding that only microfilaments are decorated with heavy meromyosin (2).

The technique of immunofluorescence is convenient and fast, and allows the screening of a large number of cells under a variety of experimental conditions. The availability of antibodies to other major structural proteins will enable us to use this technique to study the intracellular localization of these proteins. This experimental approach has been previously used successfully to localize myosin (24), the light chains of myosin (25), and troponin (26) in myofibrils.

The immunofluorescent demonstration that actin exists in filamentous structures gives us a tool to compare structural differences between normal and transformed cells during various stages of their cell cycle. We hope that the convenience of this technique will aid in answering major questions of cellular structure and movement.

We thank Dr. F. Miller at Stonybrook University for the use of his animal facilities and his help in the preparation of the antibodies. We thank Dr. R. Pollack for the use of his laboratory; Drs. R. Goldman and C.-M. Chang and Art Vogel for their help with the fluorescent microscopy; and Drs. M. Osborn, C. W. Anderson, R. Goldman, and R. Pollack for their comments on the manuscript. We also thank Dr. Susan Lowey for a generous gift of purified chicken muscle actin. This investigation was supported by the National Institutes of Health (Research Grant CA-13106 from the National Cancer Institute).

1. Goldman, R. D. & Knipe, D. M. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 523–534.
2. Ishikawa, H., Bischoff, R. & Holtzer, H. (1969) *J. Cell Biol.* **43**, 312–328.
3. Yang, Y. & Perdue, J. G. (1972) *J. Biol. Chem.* **247**, 4503–4509.
4. Tilney, L. G. & Mooseker, M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2611–2615.
5. Bettex-Galland, M. & Lüscher, E. F. (1965) *Advan. Protein Chem.* **20**, 1–35.
6. Probst, E. & Luscher, R. (1972) *Biochim. Biophys. Acta* **278**, 577–584.
7. Puskin, S. & Berl, S. (1972) *Biochim. Biophys. Acta* **276**, 695–709.
8. Pollard, T. D. & Weihing, R. R. (1973) "Cytoplasmic actin and myosin and cell movement," in *Critical Reviews in Biochemistry*, Vol. 2, pp. 1–65.
9. Weisenberg, R., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry* **7**, 4466–4478.
10. Adelstein, R. S., Pollard, T. D. & Kuehl, W. M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2703–2707.
11. Adelstein, R. S., Conti, M. A., Johnson, G. S., Pastan, I. & Pollard, T. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3693–3697.
12. Stossel, T. P. & Pollard, T. D. (1973) *J. Biol. Chem.* **248**, 8288–8294.
13. Adelstein, R. S. & Conti, M. A. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 598–605.
14. Cohen, I. & Cohen, C. (1972) *J. Mol. Biol.* **68**, 383–387.
15. Todaro, G. J., Green, H. & Goldberg, B. D. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 66–73.
16. Bray, D. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 567–571.
17. Wilson, L., Bryan, J., Ruby, A. & Mazia, D. (1970) *Proc. Nat. Acad. Sci. USA* **66**, 807–814.
18. Spudich, A. J. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 585–593.
19. Studier, F. W. (1972) *Science* **176**, 367–376.
20. Sever, J. L. (1962) *J. Immunol.* **88**, 320–329.
21. Coons, A. H. & Kaplan, M. H. (1950) *J. Exp. Med.* **91**, 1–13.
22. Holtzer, H., Sanger, J. W., Ishikawa, H. & Strahs, K. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 549–566.
23. Goldman, R. D. (1971) *J. Cell Biol.* **51**, 752–762.
24. Pepe, F. A. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 97–108.
25. Lowey, S. & Holt, J. C. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 19–28.
26. Perry, S. V., Cole, H. A., Head, J. F. & Wilson, F. J. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 251–262.
27. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.