INDIVIDUAL MICROTUBULES VIEWED BY IMMUNOFLUORESCENCE AND ELECTRON MICROSCOPY IN THE SAME PtK2 CELL

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ABSTRACT

PtK2 cells were grown on gold grids and treated with Triton X-100 in a microtubule stabilizing buffer. The resulting cytoskeletons were fixed with glutaraldehyde and subjected to the indirect immunofluorescence procedure using monospecific tubulin antibodies. Grids were examined first by fluorescence microscopy, and the display of fluorescent cytoplasmic microtubules was recorded. The grids were then stained with uranyl acetate and the display of fibrous structures recorded by electron microscopy. Thus the display of cytoplasmic microtubular structures in the light microscope and the electron microscope can be compared within the same cytoskeleton. The results show a direct correspondence of the fluorescent fibers in the light microscope with uninterrupted fibers of diameter ~550 Å in the electron microscope. This is the diameter reported for a single microtubule decorated around its circumference by two layers of antibody molecules. Thus under optimal conditions immunofluorescence microscopy can visualize individual microtubules.

KEY WORDS cytoskeleton · tubulin antibody · immunofluorescence microscopy · electron microscopy · microtubules

Antibodies against tubulin are useful tools to describe the overall distribution and organization of cytoplasmic microtubules and other microtubular structures in tissue culture cells by immunofluorescence microscopy (6, 19). The arguments for identifying the networks stained by the tubulin antibody in interphase cells as cytoplasmic microtubules have included (1, 5, 6, 12, 15, 18, 19) the monospecific nature of the antibodies, the sensitivity of the expression of the networks to mitotic drugs, low temperature and Ca^{++} ions as well as the repolymerization of the network (from the cytocenter acting as a microtubule-organizing center) after removal of the depolymerization influence. In addition the antibodies react with homogeneous tubulin by immunodiffusion (6, 17), immunoelectrophoresis (6) and in radioimmunological assays (7).[1] Furthermore when tubulin antibodies are used either in the immunoperoxidase technique (3, 4), or directly (20), specific decoration of cytoplasmic microtubules is observed when thin sections are studied with the electron microscope.

Although we have recently studied glutaraldehyde fixed cells processed through the indirect immunofluorescence procedure in the fluorescence microscope, as well as thin sections of similarly processed cells in the electron microscope and have been able to show specific decoration of cytoplasmic microtubules (20), it has so far not been possible to examine the same cell by both techniques. For such a purpose cells treated with non-ionic detergents seem especially suitable. Electron microscopy (2, 9) as well as immunofluorescence microscopy (14), has shown that the

[1] Hiller, G. and K. Weber. Radioimmunoassay for Tubulin. Submitted for publication.

resulting membrane-free cytoskeletons contain as major structural elements: the nucleus, bundles of microfilaments, and intermediate filaments. Recently immunofluorescence microscopy has also shown that cytoplasmic microtubules are preserved in cytoskeletons if the detergent treatment is performed in a microtubule stabilizing buffer (15). Therefore it is now possible to try to record within the same cytoskeleton the microtubular organization after decoration with rabbit antitubulin antibody and fluorescein goat anti-rabbit γ-globulins by both immunofluorescence microscopy and by electron microscopy. Here we show such a comparison.

MATERIALS AND METHODS

Cells and Antibodies

The growth of rat kangaroo PtK2 cells has been described (11). Cells were grown on gold grids which were held on 12 mm glass cover slips by a film made with 1.2% Formvar. The Formvar was coated with poly-L-lysine-HBr (0.1% in water).

The rabbit antibody directed against homogeneous porcine brain tubulin free of microtubule associated proteins has been described (12, 21). It was made monospecific by affinity chromatography on tubulin covalently bound to Sepharose 4B (6, 21) and used at a protein concn of 0.05 mg/ml. The fluorescein labeled goat anti-rabbit IgGs were purchased from Miles-Yeda (Israel) and used at 0.5 mg/ml in phosphate-buffered saline (PBS).

Detergent Treatment

The detergent treatment has been described (15). Cells were treated with Stabilization buffer: 0.1 M piperazine-*N*,*N*′-bis[2-ethane sulfonic acid] sodium salt adjusted to pH 6.9 with KOH, 1 mM ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 2.5 mM GTP and 4% Polyethylene glycol 6000 (Serva Feinbiochemica, Heidelberg, Fed. Rep. of Germany), twice 30 s at room temperature, and then incubated for 4 min at room temperature in the same buffer containing 0.2% Triton X-100. The cytoskeletons were then washed twice (30 s each) with Stabilization buffer at room temperature, and then fixed for 10 min at room temperature in the same buffer containing 1% glutaraldehyde (Serva Feinbiochemica). The resulting, glutaraldehyde fixed cytoskeletons were then treated with 0.5 mg/ml sodium borohydride (20) in PBS twice 4 min at room temperature, and then washed with PBS twice 3 min at room temperature. They were then processed for immunofluorescence microscopy by our usual procedure (18). Briefly rabbit monospecific tubulin antibody was added and the cells incubated for 45 min at 37 C. After washing well with PBS, fluorescein labeled goat anti-rabbit γ-globulins were added, and the cytoskeletons again incubated for 45 min at 37°C. After further washings with PBS the cover slips were mounted with 0.04 M Tris-HCl, pH 8.5, 0.14 M NaCl and immediately photographed using epifluorescent optics. Then the cover slip was carefully removed and washed twice with the Tris-NaCl buffer, twice with 0.3 M KCl, and then treated with 1% aqueous uranyl acetate for 45 s. The grid was then detached from the cover slip and screened in the electron microscope. Cells which had been photographed in the fluorescence microscope were located and photographed.

RESULTS AND DISCUSSION

In order to correlate directly the organization of cytoskeletal structures as revealed by immunofluorescence microscopy and by electron microscopy cytoskeletons were selected since fibrous systems can be visualized in selected areas by low power electron microscopy of the whole cytoskeleton. Thus the need for thin sectioning and three dimensional reconstruction is avoided, and the immunofluorescent and the electron microscope image can be directly compared. Cytoskeletons of PtK2 cells prepared by the action of non-ionic detergents in the buffers normally used (2, 9, 14) contain nuclei, bundles of microfilaments, and intermediate filaments. In addition they show the system of wavy tonofilamentous fibers previously documented for this cell type (11). A full account of these fibrous structures viewed in the same cytoskeleton by immunofluorescence and by electron microscopy will be given elsewhere.[2]

To look specifically at the microtubular organization as viewed by the two methods, advantage was taken of our previous immunofluorescence studies on PtK2 cells (15) which suggested that microtubules could be preserved if the detergent treatment was performed in a buffer known from in vitro studies (22) to stabilize microtubules. Thus PtK2 cells were treated with Triton X-100 in this buffer, and were then subjected after glutaraldehyde fixation to decoration with rabbit monospecific tubulin antibody followed by fluorescent goat anti-rabbit γ-globulins. The cytoskeletons of 100 PtK2 cells treated in this manner were photographed in the fluorescence microscope. The

[2] Webster, R. E., M. Osborn, and K. Weber. Visualization of the same PtK2 cytoskeletons by both immunofluorescence and low power electron microscopy. Submitted for publication.

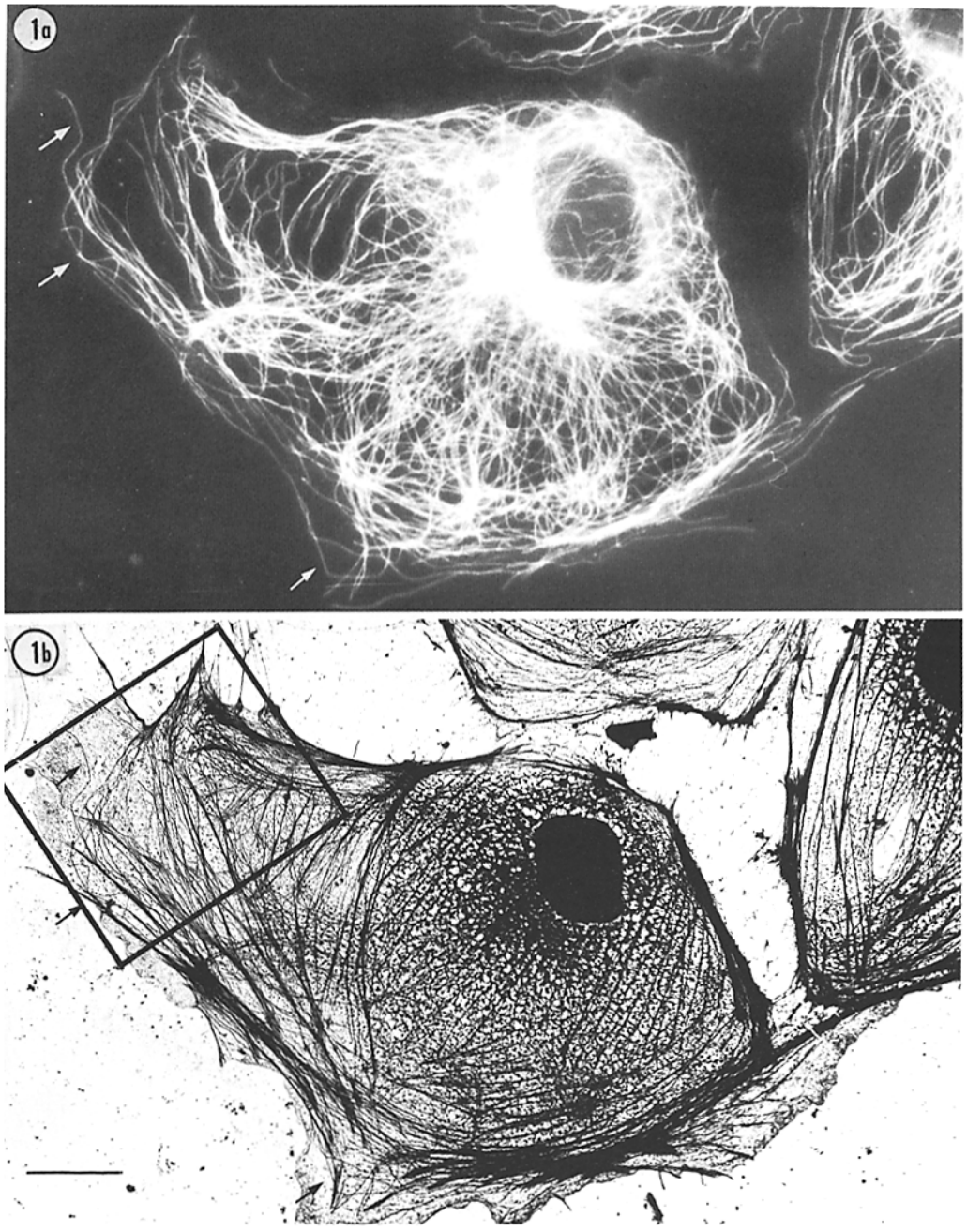


FIGURE 1 Fluorescent micrograph (*a*) and electron micrograph (*b*) of the same PtK2 cytoskeleton. The cell was treated with detergent, fixed in glutaraldehyde, and treated with monospecific tubulin antibody followed by fluorescent second antibody before being photographed in the fluorescence microscope (*a*). The cytoskeleton was then stained with uranyl acetate, and photographed in the electron microscope (*b*). Fig. 1*a* shows a typical display of fluorescent fibers running between the perinuclear area and the outer cell margin. Even at this magnification a correspondence can be seen between some of the fluorescent fibers and some of the fibers in the electron micrograph (arrows). The area within the rectangular outline is enlarged in Fig. 2. Bar, 10 μm. (*a*) × 1,140, (*b*) × 1,270.

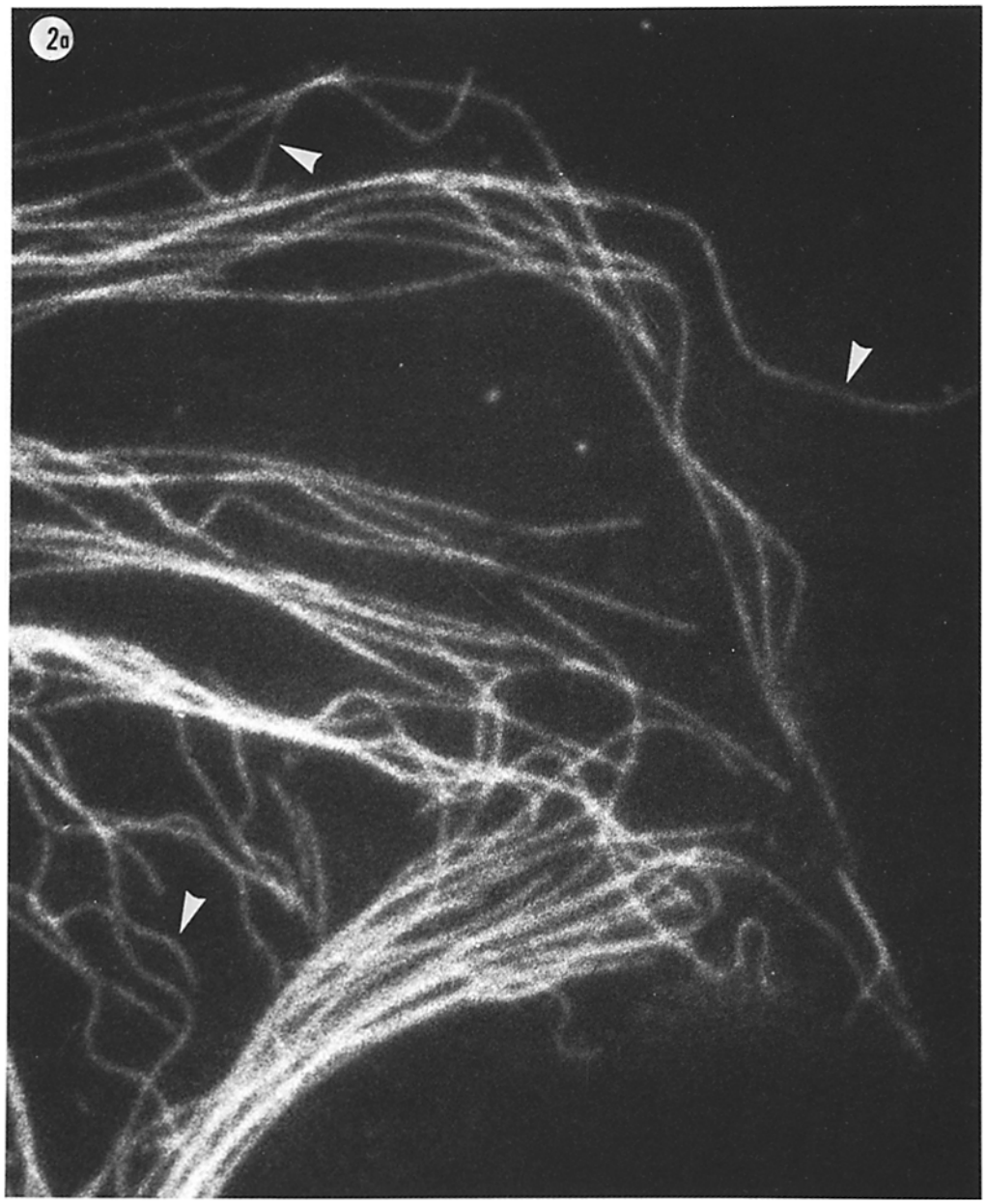
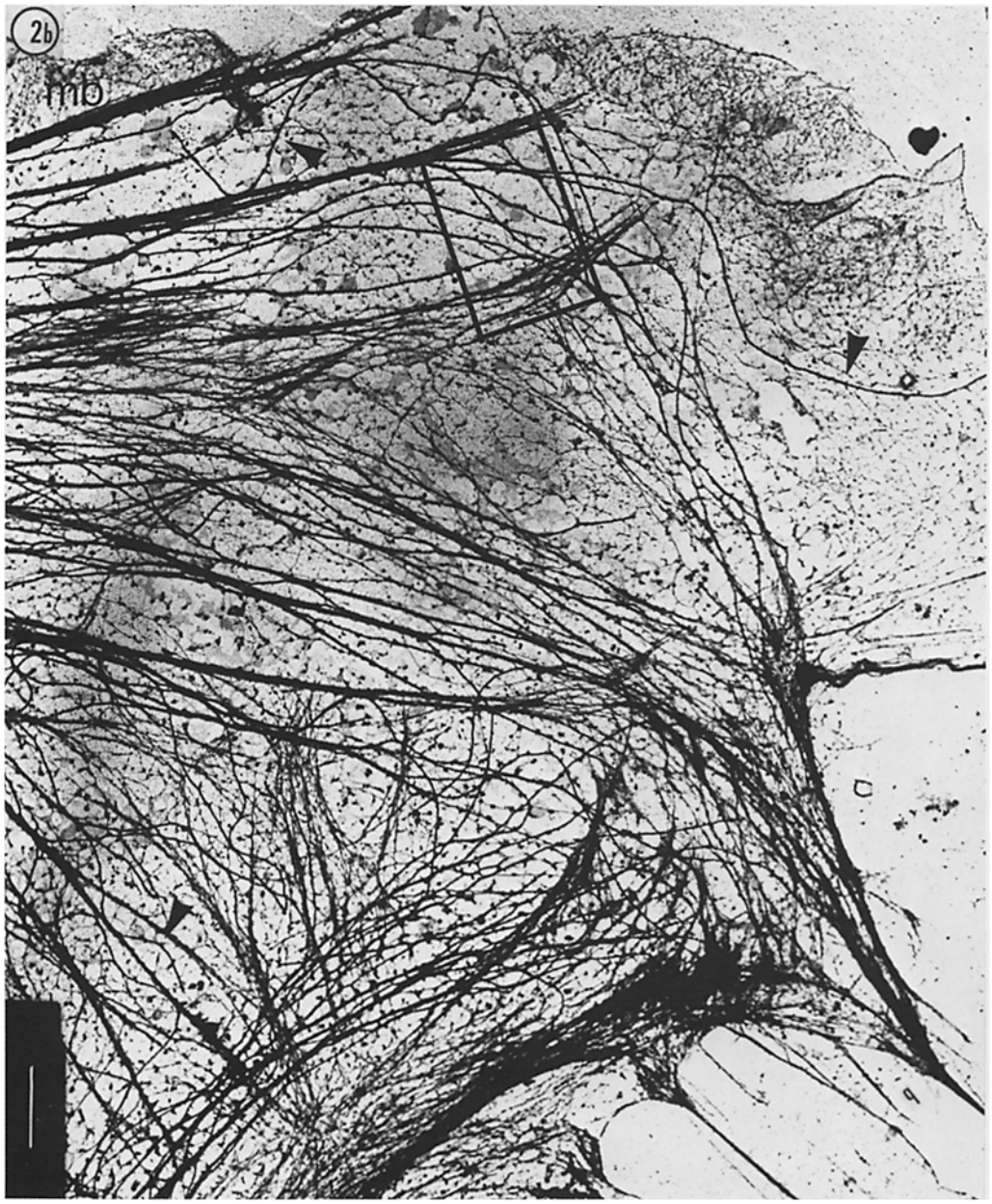


FIGURE 2 This is an enlargement of the area shown in the box in Fig. 1*b* viewed both in fluorescence (Fig. 2*a*) and in electron microscopy (Fig. 2*b*). Note that the fluorescent fibers seen in Fig. 2*a* correspond to uninterrupted fibers of diameter ~550 Å visualized in Fig. 2*b*. Each 550-Å fiber is considered to be a single antibody decorated microtubule (see text). The arrows indicate areas where it is particularly easy to see the correspondence, but almost all the fluorescent fibers seen in Fig. 2*a* can also be traced in Fig. 2*b*. *mb*, microfilament bundles. The area within the rectangular outline is enlarged in Fig. 3*a*. Bar, 2 μm. (*a*) × 4,820. (*b*) × 5,500.



grids were then removed, stained with uranyl acetate, and the same cytoskeletons located in the electron microscope. Of the 100 cytoskeletons photographed in the fluorescence microscope, 20 were selected and photographed in the electron microscope. The fluorescent and electron microscope profiles of one complete cytoskeleton are shown in Fig. 1 *a* and *b*. An enlargement of part of this cytoskeleton is shown in Fig. 2 *a* and *b*. The fluorescent profile is similar to that already documented for this cell line (11, 15). Comparison of Fig. 2 *a* and *b* shows that the fluorescent fibers previously considered to be antibody-decorated microtubules (19) correspond to fibers stained by

uranyl acetate in the electron microscope. The direct relationship of these two structures is excellent and was present in all cells studied. Higher magnification (Fig. 3*a*) as well as inspection of other similar micrographs, shows that the diameter of the fibers is ~550 Å, and in addition that fibers with the diameter of normal microtubules, i.e., ~250 Å, are conspicuously absent, although microfilament bundles and fibers with the diameter of intermediate filaments remain. It is important to note that if PBS is substituted for the tubulin antibody, not only are the 550 Å diameter fibers not seen but the cytoskeletons contain instead undecorated microtubules with a diameter of ~220 Å (Fig. 3*b*).

The data presented here show that wherever the immunofluorescent structures are not obviously joined in bundles they are seen in the electron microscope as fibers of approx. 550 Å diameter. This diameter is reasonable for a microtubule of diameter 220 Å decorated around its circumference by two layers of antibody molecules since a single IgG molecule has a diameter of ~90 Å. These 550-Å fibers do not bifurcate or decrease sharply in diameter again suggesting that each fiber contains only one microtubule. In addition we have shown (20) that individual microtubules identified in thin sections of 3T3 cells after glutaraldehyde fixation and decoration in the indirect procedure with the same monospecific anti-

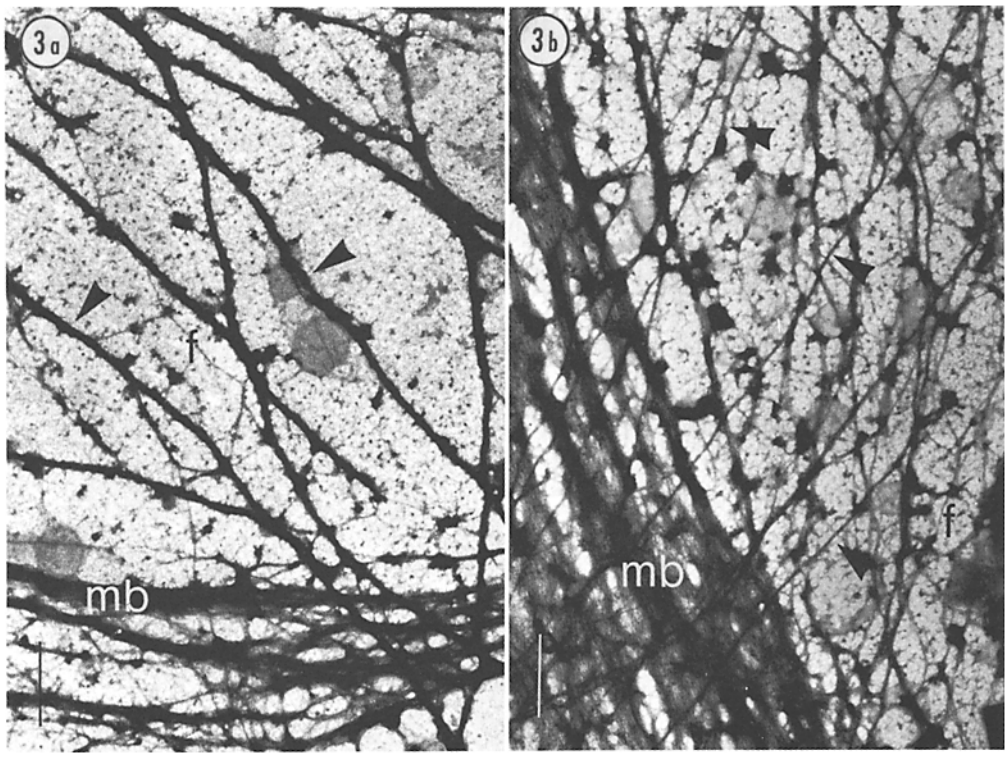


FIGURE 3 This is an enlargement of the area of the PtK2 cytoskeleton shown in the rectangular box in Fig. 2*b*. Measurement of the diameter of those fibers which correspond to fluorescent fibers (arrows) from this and similar micrographs gives a value of ≈550 Å. This is approximately the diameter expected for a microtubule decorated by two successive layers of γ-globulin molecules in the indirect procedure (see text). The decorated microtubules appear to lie on top of the microfilament bundles (*mb*). Although fibers of approximate diameter of intermediate filament can be seen note that fibers with the diameter of normal microtubules ~250 Å are not seen. Bar, 0.5 μm. × 22,200. (*b*) PtK2 cytoskeleton treated as in the legend to Fig. 1 except that PBS was substituted for the monospecific tubulin antibody. Fibers of diameter 550 Å are not seen, but instead fibers of diameter ~220 Å can be distinguished. These are considered to be microtubules because of their diameter, and their distribution in the cell. Note that as in Fig. 3*a* they appear to lie on top of microfilament bundles (*mb*) and the presence of intermediate filaments (*f*). Bar, 0.5 μm. × 22,200.

body have a similar diameter (600 Å). These arguments collectively demonstrate that the 550 Å fibers correspond to individual antibody-decorated microtubules.

Although structures with diameters less than the resolution of the light microscope have been visualized in both dark-field and phase-contrast microscopy (see, for instance, references 8, 10) the proposal that single microtubules could be visualized by immunofluorescence microscopy (19) although supported by some authors (1) has been questioned by others (see, for example, reference 3). However a comparison of Fig. 2*a* and *b* directly confirms by electron microscopy that decorated microtubules can be visualized by immunofluorescence microscopy even though their diameter of 550 Å is theoretically below the limit of resolution of the light microscope. These figures also show that as predicted, such structures are not revealed at their correct diameter, i.e., ~550 Å, but at a diameter approximately equal to the limit of resolution of the light microscope. Measurement of the fluorescent fibers seen in Fig. 1*a*, as well as of other immunofluorescent pictures of single microtubules give values of approx. 2,000 Å, a value close to the calculated limit of resolution when apertures of 1.4 and light of wavelength 5,150 Å is used. Thus as shown in Fig. 2*a* microtubules which are closer together than this distance will not be resolved in the fluorescent picture. Since electron microscope studies have shown that in structures such as the mitotic spindle, the intracellular bridge, or the fish melanophore, neighboring microtubules can be separated by as little as 4–500 Å; this explains why these structures are seen in the fluorescence microscope as masses of fluorescence (6, 16, 18) rather than as individual fibers.

In the well-spread tissue culture cells used here the electron micrographs show that in general each fluorescent fiber corresponds to an individual microtubule. In addition inspection of fluorescent micrographs including Figs. 1*a* and 2*a*, shows that where fluorescent fibers cross over one another, or come close to one another as judged by electron microscopical analysis of the same cytoskeleton a visible increase in light intensity can be seen. This observation provides a new criterion to ascertain single vs. multiple microtubules. Thus even if only the fluorescent image of a given cell stained with tubulin antibody is available, it seems clear that in good micrographs of interphase tissue culture cells which are well spread over the substratum, the majority of fluorescent fibers in regions such as that shown in Fig. 2*a* will correspond to single microtubules. The problems involved with more rounded cells have been discussed elsewhere (13, 15).

The demonstration of a direct relationship between the fluorescent fibers visualized specifically by the tubulin antibody and the electron dense fibers seen in the electron microscope strongly favors the assumption that the organization of cytoplasmic microtubules is well revealed by the technique of immunofluorescence microscopy. Previous studies (1, 5, 12, 13, 15, 17, 19, 20) have emphasized that microtubules traverse the cytoplasm for very long distances without interruption, that they are especially strongly displayed between the perinuclear area and the plasma membrane, and that they can bend gently and on reaching the membrane sometimes follow the margin of the cell for many micrometers.

We thank T. Born for help with the photography. R. Webster was supported by a Macy Foundation Fellowship during a sabbatical leave from Duke University.

Received for publication 14 February 1978, and in revised form 17 March 1978.

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