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Three-dimensional electron microscopical visualization of the cytoskeleton of animal cells: Immunoferritin identification of actin- and tubulin-containing structures

(interconnecting fibrous systems/subplasmalemma actin network/microfilament bundles/microtubules)

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ABSTRACT **Cytoskeletons prepared by Triton X-100 treatment of tissue culture cells appear in stereo electron microscopy as a highly organized and interconnected three-dimensional matrix of different fibrous elements. Microfilament bundles and also tonofilament-like bundles are readily discerned when present in the cell type. In addition thinner fibers, some of which branch (smallest diameter 30–40 Å), as well as fibers of larger diameter, some of which correspond to microtubules, can be seen. Since such cytoskeletons are an open, membrane-free system, individual fibrous organizations can be identified by specific antibodies. An indirect immunoferritin procedure using antibodies to tubulin or actin visualizes microtubules or actin-containing structures. Stereo electron microscopy of cytoskeletons decorated with actin antibody reveals, in addition to the F-actin-containing microfilament bundles, an extended fine actin lattice. This actin net is displayed throughout the cytoplasm not only between the microfilament bundles but also in those regions of the cytoskeleton that in the intact cell correspond to the submembraneous regions. Thus all actin-containing fibrous cytoplasmic structures may be interconnected in the living cell.**

Electron microscopy of thin sections has amply documented the cellular display of microfilaments, microtubules, and intermediate filaments (1). Biochemical studies (2) combined with the results of immunofluorescence microscopy (3, 4) allowed the assignment of certain structural proteins in a specific manner to each of the three major fiber systems. However, it is still difficult to understand how these fibers interact either with each other or with other cellular structures and organelles. Some knowledge of such interactions has been obtained by investigating the fine structural organization of fixed whole cells by stereo normal and high voltage electron microscopy (5–8). Recently it became possible to isolate the whole fibrous system (i.e., the "cytoskeleton"), free of most other cellular components, by the use of a specific nonionic detergent (9–11). In addition, the same cytoskeleton could be examined by both immunofluorescence and low power electron microscopy (12, 13). These studies revealed that the organization of the fibrous matrix present in isolated cytoskeletons is representative of that typical of intact cells and therefore can be used to study the overall fine structure by immunological procedures. Here we show an analysis of cytoskeletons by stereo electron microscopy and identify actin- and tubulin-containing structures by indirect immunoferritin labeling.

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MATERIALS AND METHODS

Growth of mouse 3T3 and rat kangaroo PtK2 cells and the affinity column-purified rabbit IgGs against gizzard actin and porcine brain tubulin have been described (4, 10, 12, 13). Antibodies were stored at 0.05 mg/ml in phosphate-buffered saline at 4°C. Affinity purified sheep anti-rabbit IgGs (13) were conjugated with ferritin (14). The ferritin conjugate was stored in 0.1 M Tris·HCl, pH 7.5, at 0.8 mg/ml at 4°C.

Cells were grown on gold grids sandwiched between a polylysine-coated film of 1.2% Formvar and a glass coverslip (13). Cells were washed twice for 1 min each with 7 ml of S buffer [0.1 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.9/1 mM EGTA/1.5 mM GTP/4% polyethylene glycol 6000 (12)] in a 60-mm dish at room temperature. All subsequent washings were done similarly. Cells were incubated for 4 min in S buffer containing 0.2% Triton X-100 and washed five times for 2 min each with S buffer. For fixation, the wet grid was inverted for 15 min over a 2-ml cavity containing 1 ml of 25% glutaraldehyde. After phosphate-buffered saline and H_2O washes, the grid was removed from the coverslip under water and stained for 20 min in 4% uranyl acetate. Cytoskeletons were dehydrated by using 5-min incubations in 30, 50, 70, 80, 90, 95 and 100% acetone and critically point dried with CO_2.

For actin-decorated cytoskeletons, cells were treated as above, washed three times for 1 min each with S buffer, but not fixed. Instead, they were incubated for 30 min at 37°C with actin antibody diluted 1:2 with S buffer. After they were washed three times for 1 min each with S buffer, cytoskeletons were incubated for 30 min at 37°C with ferritin conjugate diluted 1:5 with S buffer. The decorated cytoskeletons were washed three times for 2 min each with S buffer, fixed for 20 min with 1.5% glutaraldehyde in S buffer, washed, stained, dehydrated, and critically point dried as above.

For tubulin-decorated cytoskeletons, cells were treated with Triton, washed, and vapor fixed as above. Fixed cytoskeletons were treated with $NaBH_4$ (0.5 mg/ml) for 3 min in S buffer, washed once with phosphate-buffered saline, and treated for 4 min more with $NaBH_4$ in phosphate-buffered saline (15). After they were washed three times for 1 min each with phosphate-buffered saline, cytoskeletons were incubated at room temperature for 30 min with tubulin antibody in phosphate-buffered saline, washed three times for 1 min each with phosphate-buffered saline, and incubated for 30 min at room temperature with ferritin conjugate diluted 1:5 with phosphate-buffered saline. Cytoskeletons were washed three times for 2 min each in phosphate-buffered saline, fixed for 20 min with 1.5% glutaraldehyde in phosphate-buffered saline, stained, dehydrated, and critically point dried as above.

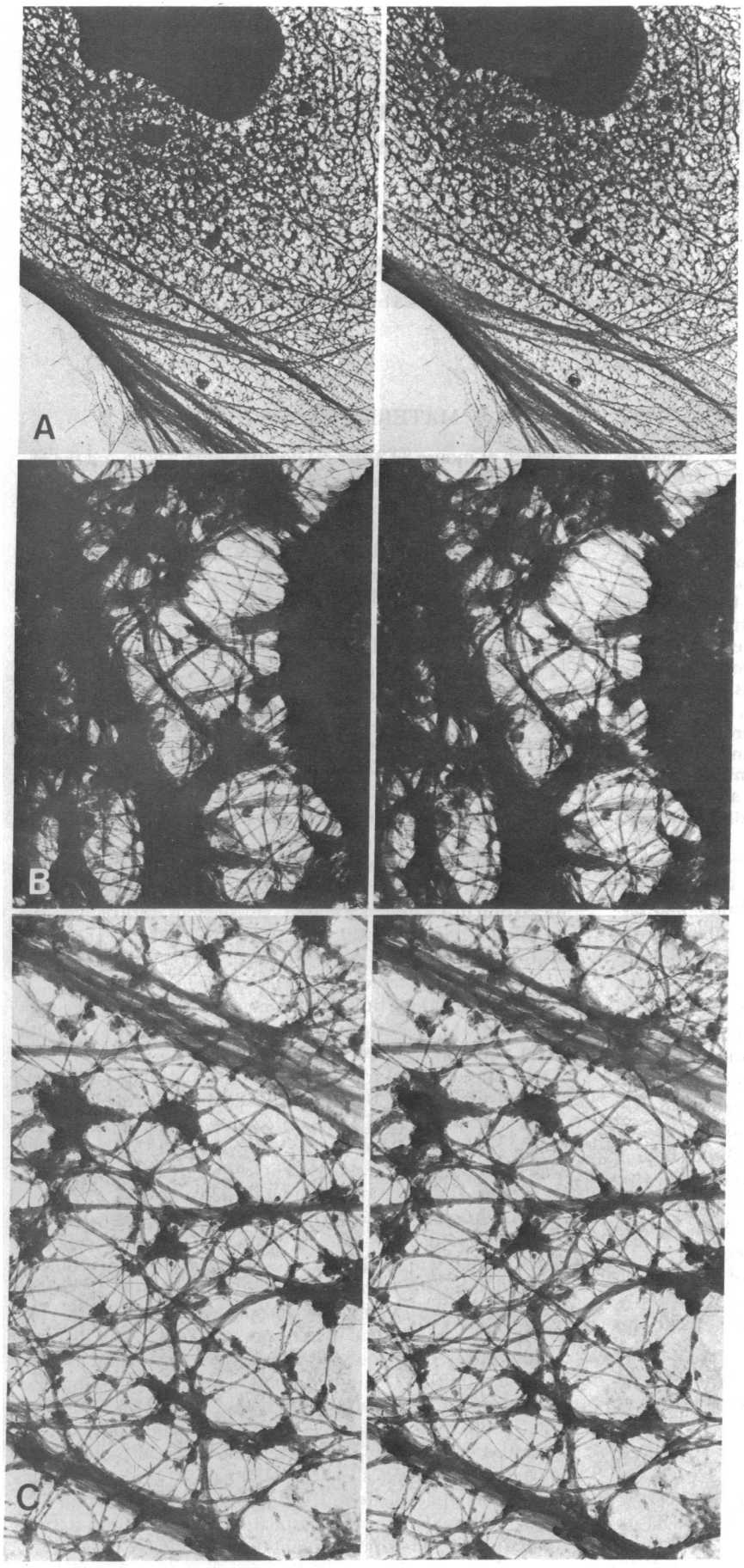

FIG. 1. Stereo electron micrographs of PtK2 cytoskeletons. (*A*) Low power: magnification ×2800, tilt ±15°. (*B*) Region near nucleus: magnification ×34,000, tilt ±2°. (*C*) Region near periphery: magnification ×34,000, tilt ±4°. Figs. 1–3 should be viewed with a stereo viewer.

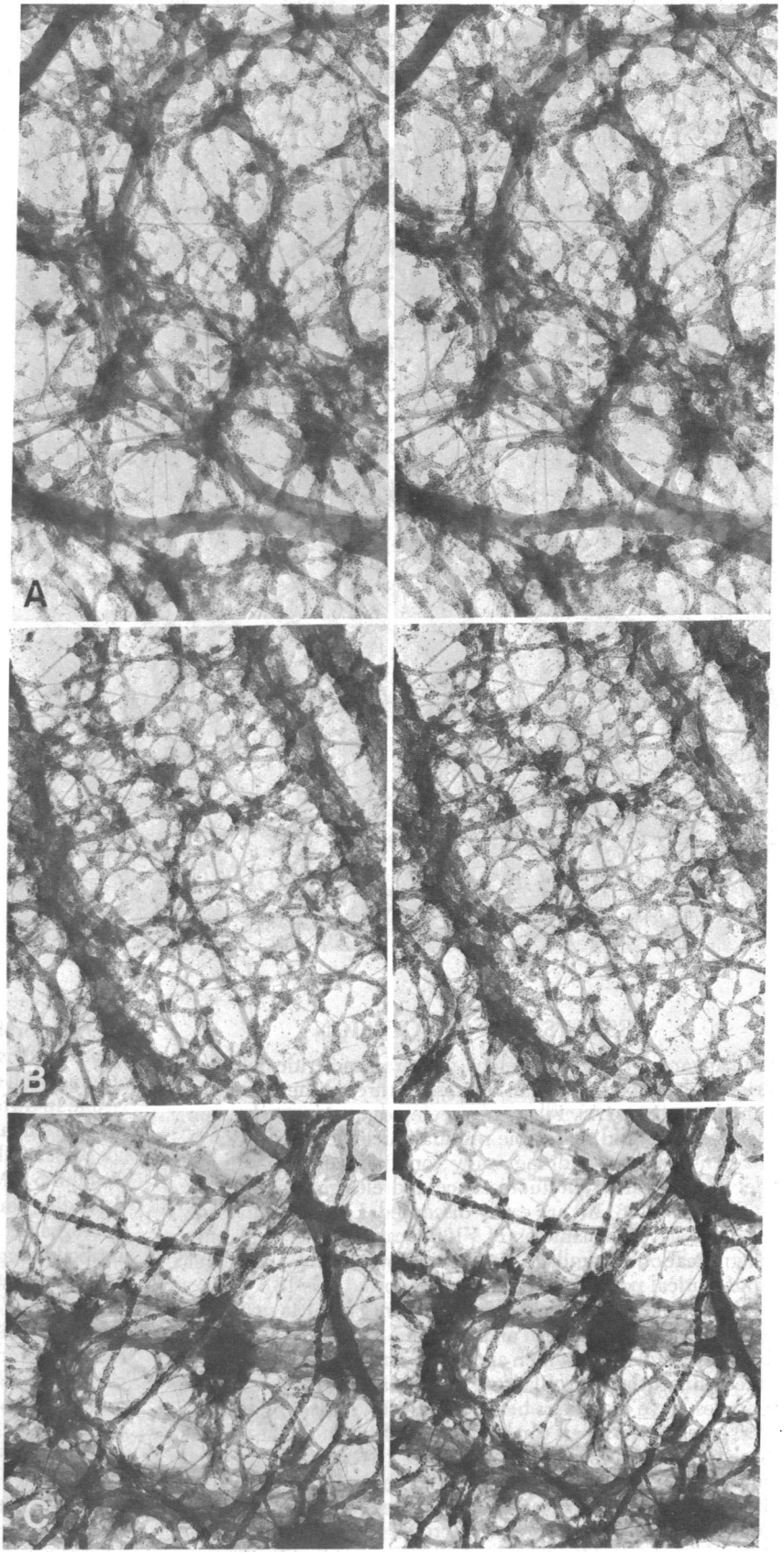

FIG. 2. Stereo electron micrographs of PtK2 cytoskeletons subjected to indirect immunoferritin decoration. (*A*) Interior and (*B*) periphery of cytoskeletons decorated with actin antibody. (*C*) Cytoskeleton decorated with tubulin antibody. All magnifications ×34,000, tilt ±4°.

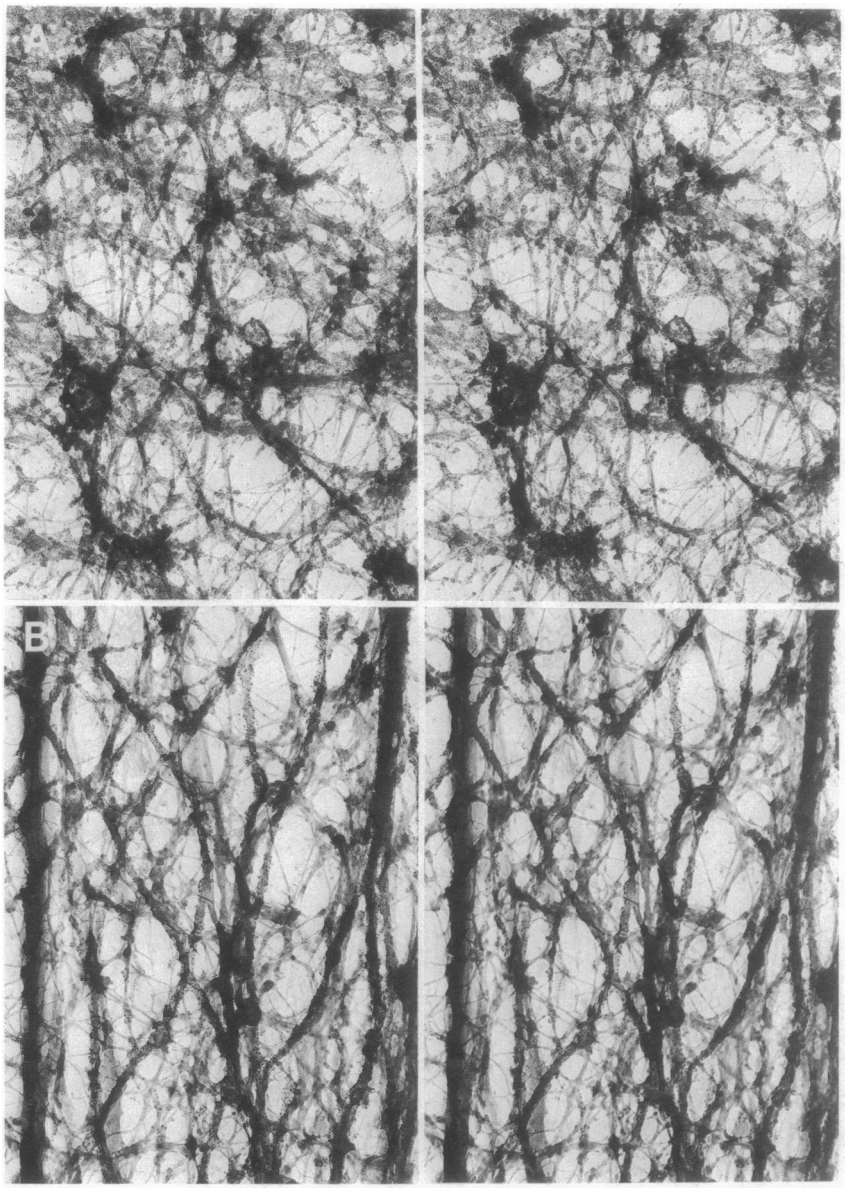

FIG. 3 Stereo micrographs of 3T3 cytoskeletons subjected to indirect immunoferritin decoration. Cytoskeleton decorated with (*A*) actin antibody or (*B*) tubulin antibody. All magnifications ×34,000, tilt ±4°.

RESULTS AND DISCUSSION

Rat kangaroo PtK2 cytoskeletons, prepared and critically point dried as described, retain an impressive three-dimensional organization (Fig. 1*A*). Fibers of varying diameter can be seen over, around, and under the residual nuclear matrix. Microfilament bundles and the wavy tonofilament-like bundles identified by immunofluorescence and electron microscopy in these cells (13, 16) are easily discerned. Long unbranching microtubules with diameters of ≈130 Å can be noticed at higher magnification. A similar reduction of microtubular diameter upon critical point drying has been reported (17, 18).

Higher magnification shows that thinner fibrillar structures are also preserved (Fig. 1 *B* and *C*). These fibers, many of which branch and many of which appear to emanate from and even interconnect the microfilament bundles and tonofilament-like bundles, have diameters between 30–40 Å and well over 100 Å. These thinner fibers can be detected throughout the cell but are more numerous near the nucleus (Fig. 1*B*) than near the cell margin (Fig. 1*C*). The open vertical channels between such fibers vary between 300 and 800 Å in diameter when measured at a single plane in the stereo micrographs (e.g., lower right-hand part of Fig. 1*B*). The dimensions suggest that the thinner fibers are probably part of the fibrous microtrabecular system described by stereo normal or high voltage electron microscopy of whole cell mounts (5, 6, 8). The dense amorphous material visible in different areas of the cytoskeleton presumably represents remnants of the endoplasmic reticulum. Ribosomes are not present, although they can be preserved when the S buffer contains 5 mM $MgCl_2$.

Studies comparing the immunofluorescence and electron micrographs of the same cytoskeleton decorated with actin antibody suggested that, in addition to microfilament bundles, actin was contained in a lattice-like structure present in the cell body (13). When unfixed cytoskeletons of PtK2 are decorated with actin antibodies followed by the ferritin-conjugate, a lattice-like structure can be discerned which is decorated by ferritin molecules (Fig. 2 *A* and *B*). The reaction of the actin antibody appears to stabilize this network, thus eliminating the need for fixation prior to decoration. However, the same structures are decorated, although to a lesser extent, when actin antibodies are added to cytoskeletons fixed first with glutaraldehyde and then reduced with sodium borohydride.

The diameters of individual fibers in the actin lattice range from 200 to 600 Å after decoration (Fig. 2 *A* and *B*). This would

suggest the presence of only a small number of F-actin molecules per fiber, the exact number depending on whether one or both sides are decorated with the two antibodies used in the immunoferritin procedure. These thin actin fibers form a branching interconnected three-dimensional network in the thicker parts of the cell and appear most dense in an area which in the intact cell would be just under the upper plasma membrane (Fig. 2*A*). The area corresponding to the bottom of the cell is also rather dense in such structures. Tonofilament-like bundles and most other undecorated fibrous structures including microtubules appear to reside between these two layers (see also ref. 4). However, as can clearly be seen in Fig. 2*A*, the actin networks in the upper and lower regions are connected via thin fibers decorated by the actin antibody. Near the periphery of the cytoskeletons, the lattice is above the ferritin-decorated large microfilament bundles (Fig. 2*B*). Furthermore the fibers of the lattice are connected with the microfilament bundles indicating that all the actin-containing structures of the cytoskeleton may be interconnected.

The indirect immunoferritin procedure using actin antibodies is specific for the microfilament bundles and lattice-like structure. The wavy tonofilament-like bundles and many fibers of smaller diameter appear relatively free of ferritin (Fig. 2 *A* and *B*). When antitubulin is used, essentially only microtubules are decorated with ferritin (Fig. 2*C*), as expected from our earlier studies (12, 15). In this case, cytoskeletons were glutaraldehyde fixed before antibody decoration, because the binding of tubulin antibody appeared to render microtubules less stable. The average diameter of decorated microtubules is 330 Å, possibly because critical point drying reduces the diameter of microtubules subjected to double antibody decoration.

Cytoskeletons of mouse 3T3 cells were also examined after decoration by using actin or tubulin antibodies and the ferritin conjugate. In this case, the lattice appears denser and seems to branch more than in PtK2 cells (Fig. 3*A*). However, as with PtK2, the lattice fibers are most abundant in the region that would be found just below the upper plasma membrane in the untreated cell. Again microtubules of ≈130 Å in diameter and undecorated structures, which may correspond to intermediate filaments, to part of the microtrabecular system or to both, can be seen (Fig. 3*A*). Microtubules can be clearly identified in Fig. 3*B* for which 3T3 cytoskeletons had been subjected to immunoferritin decoration by using tubulin antibody.

The detergent-resistant cytoskeleton retains the cytoplasmic fibrous architecture very well, because its three-dimensional organization is similar to that found in whole fixed cells by stereo electron microscopy (5–8, 17, 18). The cytoskeleton as a membrane-free open system is suitable for immunoelectron microscopy as shown by the results in this paper. The procedure reveals a three-dimensional fine lattice interconnecting microfilament bundles. This lattice is displayed throughout the interior of the cell and is rather dense in the area just below the plasma membrane. The three-dimensional organization of this lattice suggests that it interconnects many parts of the cytoskeleton, indicating that it may be a vehicle for transmission of contractile and morphological changes in the living cell. It is easy to envision that this lattice interconnects with the membrane and may be changed or modulated by the action of transmembrane receptors as often discussed (see for instance refs. 19 and 20).

The preparation of the cytoskeletons is accompanied by a loss of ribosomes and membranous organelles. Some of the finer fibers of the three-dimensional matrix, which resemble trabeculae (8), are probably also lost. However, some are preserved and of these some can be decorated by actin antibody whereas others, which may have components in common with one of the different subclasses of intermediate filaments, cannot. Manipulations of the composition of the working solutions indicate that at least some organelles can be retained, and thus in the future it may be possible to determine how they interact with the cytoskeleton. In addition it should be possible to assess by immunoelectron microscopy the distribution and organization of those proteins that immunofluorescence microscopy has designated as microfilament- and microtubule-associated proteins. The profiles seen by stereo electron microscopy can also be compared with those seen by stereo immunofluorescence microscopy (4). We believe that such experiments will provide additional evidence for the importance of fibrous structures in preserving the structural integrity of the cytoplasm in living cells.

Note Added in Proof. Due to the difficulty of publishing stereo pictures containing ferritin at this magnification, a very limited number of photographs are available for those with special need.

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