

Direct Visualization of Fluorescein-labeled Microtubules In Vitro and in Microinjected Fibroblasts

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ABSTRACT Microtubule proteins and tubulin have been purified from brain and labeled with dichlorotriazinyl fluorescein (DTAF). This procedure compromises neither the polymerizability of the proteins nor their affinities for unlabeled proteins. Within 15 min after microinjection of either DTAF-microtubule proteins or DTAF-tubulin into cultured gerbil fibroma cells, there was an evolution of a fluorescent fibrillar pattern with a distribution similar to that of the microtubular network seen after staining with fluorescent antitubulin. These filaments were colchicine sensitive and could be seen to elongate with time. DTAF-labeled microtubule accessory proteins from brain were not incorporated into filaments and appeared to label autophagic vacuoles.

Within the past decade, numerous components of the cytoskeleton have been purified and biochemically characterized. Antibodies have proven to be powerful tools for the study of their disposition in cells and tissues. The major limitation of the immunocytologic approach has been the necessity of working with fixed cells, precluding the possibility of continuous observation of the dynamic behavior of cytoskeletal elements. This problem can be circumvented by the introduction of purified, labeled cytoskeletal components directly into the living cell, using either pressure injection through a glass micropipette or fusion-injection with protein-filled vesicles. Microinjection of fluorescence-labeled α -actinin (6), actin (10, 14), and 130,000-dalton protein (3) into living fibroblasts has allowed study of the mobilization of these components into appropriate cytoskeletal structures (7).

The dynamic changes in microtubule assembly that accompany a variety of cellular processes argue strongly for the use of this approach with labeled tubulin. In the preparation of fluorescent tubulin for such studies we have attempted to meet the following criteria for efficiently self-assembling cytoskeletal components: (a) Labeled material should assemble with normal kinetics and should respond to alterations in assembly conditions in the same manner as unlabeled material. (b) Labeling should be adequately intense to allow direct microscope visualization of the subunits when incorporated into appropriate structures. (c) Labeled material should have the same affinity for the assembly site in the polymer as the unlabeled material. Operationally, this means that labeled material at less than the critical concentration for self-assembly should coassemble proportionately when added to an unlabeled assembly system and

should retain this proportionality through cycles of assembly and disassembly. (d) The fluorochrome chosen should have an excitation wavelength of 450 nm or greater to diminish cellular damage by exciting radiation and to minimize problems of cellular autofluorescence.

These criteria are quite similar to those previously proposed for actin (14). In the experiments described below we will detail the preparation of microtubules and tubulin meeting these criteria and the mobilization of this material into the microtubular network of Triton-extracted cells and living fibroblasts.

MATERIALS AND METHODS

Microtubules were prepared from bovine brain by the method of Shelanski et al. (13). Briefly, brain tissue was minced in 1 ml of reassembly buffer (RB) 0.1 M 2-(*N*-morpholino)ethanesulfonic acid [MES]; 10^{-3} M GTP; 5×10^{-4} M $MgCl_2$; 10^{-3} M EGTA at pH 6.8 per gram of tissue and then homogenized in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) at 4°C (three pulses of 5 s). The homogenates were centrifuged at 40,000 rpm for 30 min in a Spinco 70 Ti rotor (Beckman Instrument Co., Spinco Div., Palo Alto, Calif.). The supernates were pooled, made up to 4 M glycerol by addition of pure glycerol, and incubated at 37°C for 30 min to assemble microtubules. The tubules were then pelleted by centrifugation in the 70 Ti rotor for 30 min at 20°C. After discarding the supernates, we resuspended the pellets in cold RB, using a Teflon-pestle glass homogenizer and allowed them to depolymerize for 30 min at 4°C. Particulates were removed by centrifugation at 40,000 rpm for 30 min at 4°C. The supernates were pooled, adjusted to 4 M glycerol, and assembled at 37°C for 30 min.

Intact microtubules obtained by the procedure described above were labeled by mixing 0.1 ml of a solution of dichlorotriazinyl amino fluorescein (DTAF) (5 mg/ml) in dimethyl sulfoxide (DMSO) (type I; Sigma Chemical Co., St. Louis, Mo.) with each milliliter of microtubule suspension (5 mg/ml) for 10 min at 37°C. DTAF is a fluorescein-bearing ligand that tends to label amino groups more efficiently at low pH than does fluorescein isothiocyanate (FITC) (2).

Control tubules were obtained in the same manner except that DTAF and DMSO were omitted.

The dye-to-protein ratio was determined by the measurement of the protein-bound dye by absorption at 495 nm after separation of the DTAF-PC (phosphocellulose-column-purified)-tubulin by phosphocellulose chromatography and removal of free dye by dialysis against 8 M urea. Free DTAF (molar extinction coefficient = 5.4×10^5) in RB was used as a standard.

Both labeled and unlabeled microtubule proteins were separated into tubulin and microtubule accessory protein (MAP) fractions by phosphocellulose chromatography in RB by the procedure of Weingarten et al. (18). The PC-tubulin fraction was made up to 2 M glycerol and 10^{-3} M GTP. PC-MAP fractions were desalted by chromatography on Sephadex G-25 in RB. For kinetic studies, DTAF-PC-tubulin was recombined with unlabeled MAPs at a 10:1 (wt/wt) tubulin:MAPs ratio. Controls used unlabeled PC-tubulin and unlabeled PC-MAPs.

Assembly kinetics were determined by turbidimetry in a Beckman ACTA MVI spectrophotometer (Beckman Instruments Inc.) at 350 nm, using a thermostated sample changer (8). Supplementary observations on elongation were made using a Leitz Ortholux epifluorescence microscope and Zeiss 63X planapochromat objective with an RCA 1030H image-intensification television camera.

Labeled PC-tubulin (2–3 mg/ml) in 80 mM PIPES was assembled onto presumptive microtubule-organizing centers (MTOCs) of Triton-lysed PtK2 and 3T3 cells as described by Pepper and Brinkley (11).

Pressure microinjection of labeled components with glass micropipettes into gerbil fibroma cells (CCL146) was carried out as described by Feramisco (6). In preparation for injection, microtubules were assembled from the desired mixture of labeled and unlabeled tubulin and MAPs and pelleted in the ultracentrifuge. The pellet was then disassembled in RB at 4°C without glycerol and adjusted to a final concentration of ~10 mg/ml. The sample was split into 15- μ l aliquots after depolymerization and frozen by immersion in liquid nitrogen. This material was used for injection for periods of up to 1 mo after preparation. Control injections were done with the concentrated supernate from the microtubule-labeling step, which is composed of denatured microtubule proteins and free DTAF. Earlier studies (3) have estimated that between 10^{-13} and 10^{-14} liters of material are injected by this procedure.

Cells were mounted in controlled environment chambers (5) and viewed with a Zeiss photomicroscope III equipped with phase and epifluorescence optics. Photographs were taken on Kodak Tri X film as previously described (7). Time-lapse observations were made with an RCA 1030H camera mounted on the microscope. Data were recorded by photographing the television monitor.

RESULTS

Labeling of Assembly-competent Tubulin and MAPs

Analysis of DTAF-labeled microtubule proteins by polyacrylamide gel electrophoresis in the presence of SDS revealed labeling in both tubulin and accessory proteins. The most intensely fluorescent components are the high molecular weight MAPs (HMW) followed in order of decreasing intensity by tubulin and a minor tau component (Fig. 1). Approximately 75% of the total fluorescence as determined by scanning microfluorometry is in the HMW fraction and 25% in the tubulin fraction. In all cases there is a small amount of fluorochrome moving at the front of the gel. This material appears to be released by the SDS gel sample preparation procedure because free dye is found at the gel front even after extensive dialysis of DTAF-proteins against 8 M urea and RB. The dye-to-protein ratio on PC-DTAF-tubulin after dialysis against 8 M urea followed by RB is ~0.5 mole of DTAF per tubulin dimer.

When the assembly kinetics of DTAF-labeled microtubule proteins were compared to those of unlabeled proteins before phosphocellulose chromatography, almost identical curves were obtained (Fig. 2). Similarly, the kinetics of DTAF-PC-tubulin reconstituted with labeled or unlabeled MAPs and of unlabeled-PC-tubulin with unlabeled MAPs were comparable (Fig. 2).

DTAF-microtubule proteins in increasing amounts, starting below the critical concentration (C_c) for self-assembly and extending to the C_c , were added to decreasing amounts of

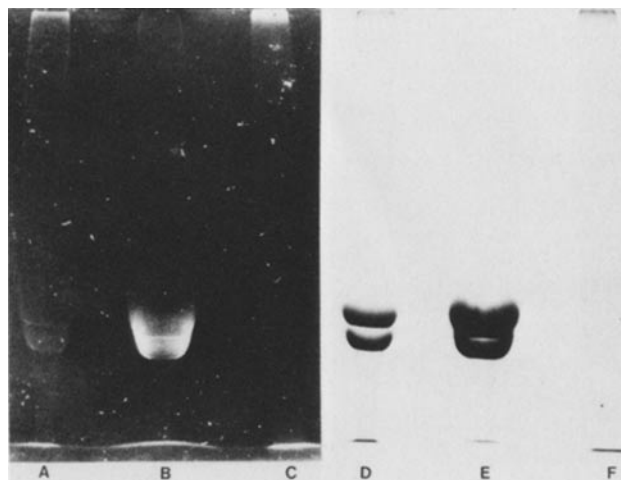


FIGURE 1 Polyacrylamide gel electrophoresis patterns of DTAF-microtubule proteins (A and D), DTAF-PC-tubulin (B and E), and DTAF-PC-MAPs (C and F). Fluorescence photographs taken before Coomassie Blue staining.

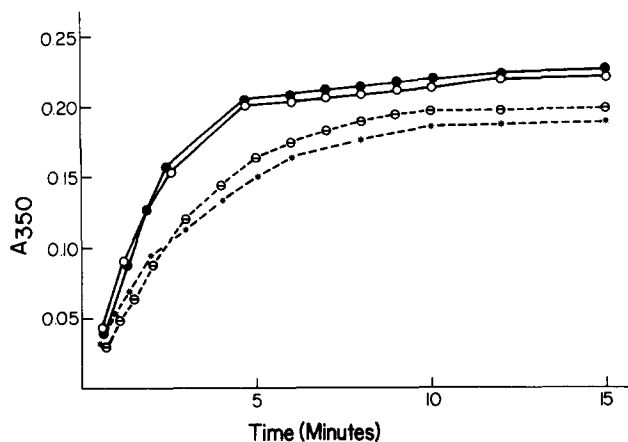


FIGURE 2 Polymerization of labeled and unlabeled microtubule proteins (1.2 mg/ml) and labeled and unlabeled PC-tubulin (1.6 mg/ml) reconstituted with unlabeled PC-MAPs at 37°C. ●, DTAF microtubule proteins; ○, control microtubule proteins; *, DTAF-PC-tubulin + unlabeled MAPs; ⊖, control-PC-tubulin + MAPs.

unlabeled microtubule proteins so that total protein concentrations remained constant. After polymerization, the microtubules were pelleted and the pellets were analyzed fluorometrically for their content of labeled microtubule proteins. The amount of fluorescent microtubule protein pelleted was dependent on the amount of fluorescent protein added in an approximately linear manner (Fig. 3). The total amounts of tubulin pelleted at each point were comparable, subject to a variation of 5%. Mixing experiments in which PC-DTAF-tubulin was added to the assembly mixture in the same manner also showed quantitative copolymerization.

The fluorescent signal allows observation of polymerization of DTAF-labeled microtubule proteins by fluorescence microscopy of tubulin solutions on microscope slides at 4°C and after warming to 37°C. Data were recorded on videotape in real time, and measurements were made on playback. Statistical analysis of the resulting measurements gave a mean rate of microtubule growth of $0.28 \mu\text{m}/\text{min}$ at 28°C, and $-0.45 \mu\text{m}/\text{min}$ at 5°C. Using the reported data on the thermodynamic behavior of tubulin solutions (8), we may convert these rates, respectively, to 9.2×10^{-7} and -1.5×10^{-6} mol/min, which

are lower but within a factor of three of those that can be derived from the literature (8, 9). Electron microscopy showed that these preparations are primarily single microtubules.

Polymerization of DTAF-PC-Tubulin onto Lysed Cell Models

When PtK2 or 3T3 cells were preincubated with colchicine at 2×10^{-5} M for 4 h in a modification of the procedure of

Pepper and Brinkley (11) and then lysed in 0.05% Triton X-100, few microtubules were detectable within lysed cells. Addition of DTAF-PC-tubulin to the models under conditions identical to those of Pepper and Brinkley gave rise to the rapid evolution of a complex pattern of fibrillar staining as well as extremely intense fluorescence over the nucleus and in the perinuclear area (Fig. 4). Controls with FITC-labeled bovine serum albumin showed no fibrils and a diffuse staining in the

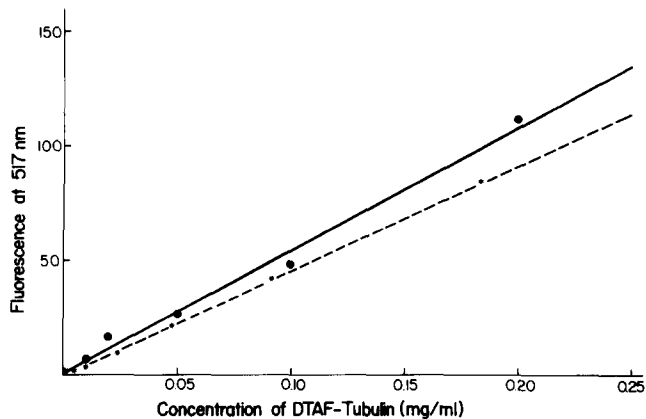


FIGURE 3 Fluorescence in resuspended microtubule pellets as a function of the concentration of DTAF-PC-tubulin (*) or DTAF-microtubule proteins (●) in the assembly mixture ([final protein concentrations labeled and cold: solid trace = 0.5 mg/ml; dashed trace = 2.0 mg/ml]).

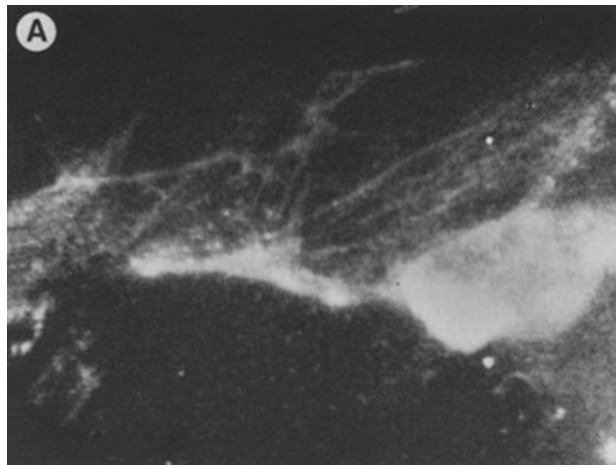


FIGURE 4 Fluorescence micrograph of colchicine-treated, Triton-lysed Swiss 3T3 fibroblast postincubated with DTAF-PC-tubulin. $\times 1,000$.

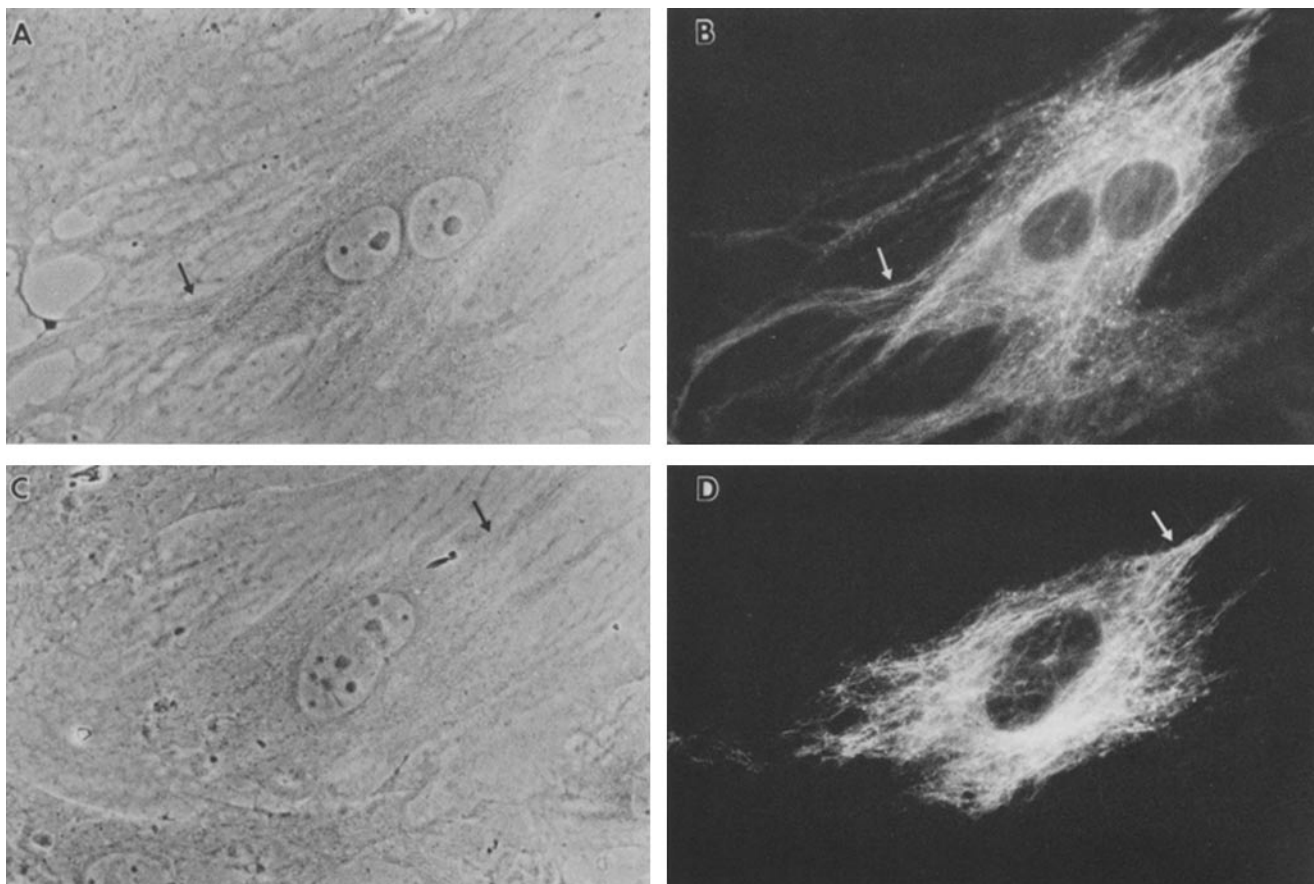


FIGURE 5 (A and C) Phase-contrast and (B and D) fluorescence micrographs of living gerbil fibroma cells microinjected with DTAF microtubule proteins. $\times 650$.

nuclear area. Formation of the microtubular network was blocked by colchicine and cold. DTAF-PC-tubulin was incapable of forming microtubules under these conditions in the absence of the extracted cells.

Microinjection of DTAF-Tubulin into Living Fibroblasts

Within 10–20 min after microinjection of DTAF-microtubule proteins (cycled, unseparated tubulin and MAPs) into fully spread fibroblasts, a few fluorescent filaments were seen near the periphery extending from an area of diffuse fluorescence near the nucleus. 1 h after labeling, the filamentous pattern had become more extensive and networks of fluorescent fibres were seen encircling the nucleus and swirling away to the cell borders (Fig. 5). The injected cells also showed widespread dots of fluorescence. This punctate pattern increased

with the time of tubulin storage at 4°C and, most likely, was the result of accumulation of denatured tubulin and labeled MAPs (*vide infra*) in autophagic vacuoles, a process that has been observed for several other fluorescence-labeled proteins microinjected in a denatured form (6, 16).

To determine whether the fibrillar labeling was attributable to tubulin or to MAPs, we injected DTAF-PC-tubulin reconstituted with unlabeled MAPs, DTAF-PC-MAPs reconstituted with unlabeled tubulin, and DTAF-PC-MAPs alone into cells in separate experiments. Within 25 min of the introduction of DTAF-PC-tubulin, a well-established pattern of fibrillar fluorescence was apparent. These filaments were concentrated in the perinuclear area with numerous bundles of filaments extending to the cell border (Fig. 6*a* and *b*). 1 h after injection, an extensive fluorescent microtubular network extended throughout the cell and out into cell processes (Fig. 6*c* and *d*). A late anaphase cell observed 4 h after injection revealed an

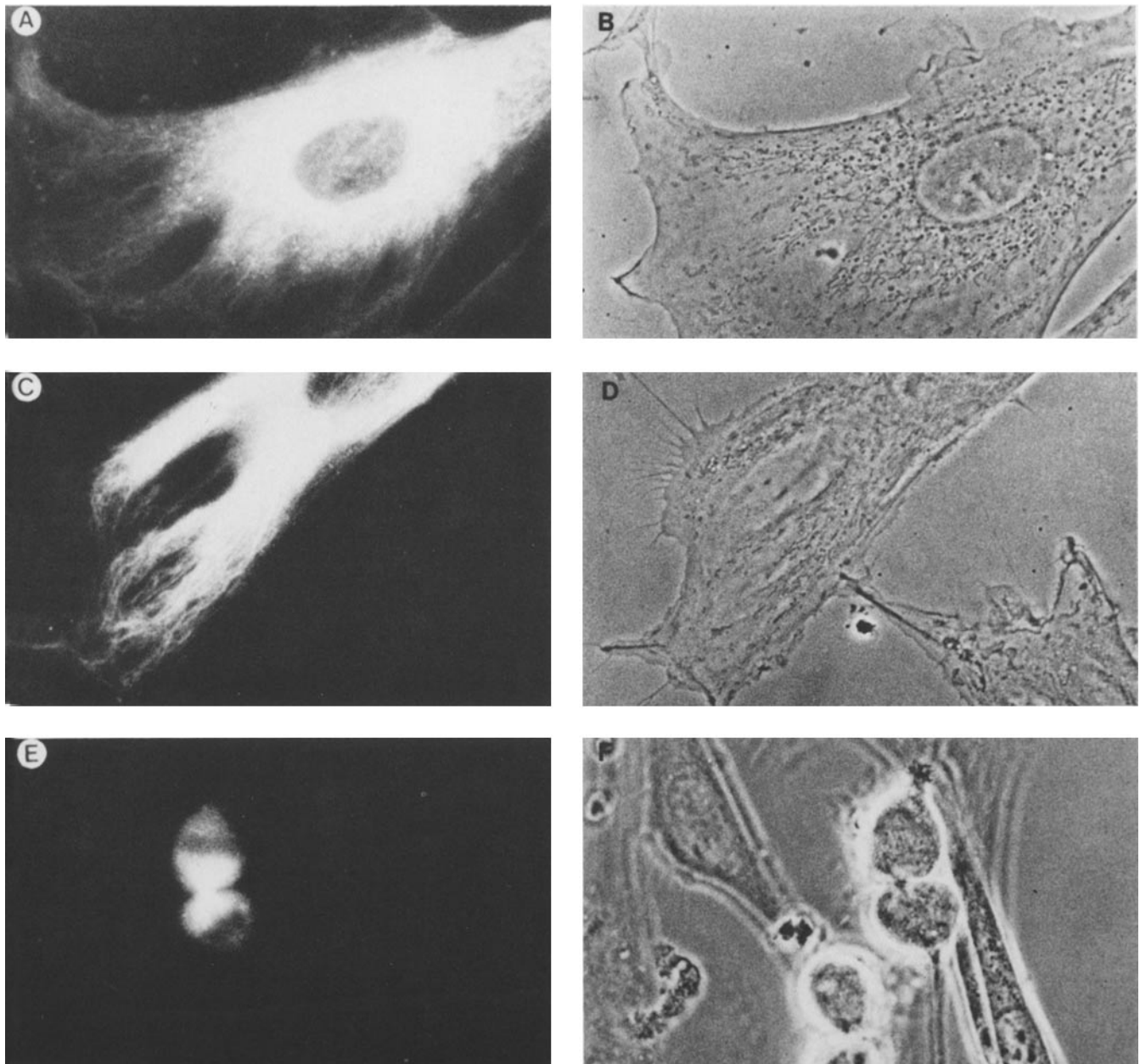


FIGURE 6 Phase-contrast and fluorescence micrographs of living gerbil fibroma cells injected with DTAF-PC-tubulin. (A and B) 20 min after injection; (C and D) 1 h after injection; (E and F) late anaphase cell 4 h after injection. $\times 700$.

extremely bright signal in the midbody fanning out toward the daughter-cell nuclei (Fig. 6 *e* and *f*). The rounded phenotype of the cell made the resolution of individual fibers impossible.

Injection of DTAF-PC-MAPs, whether or not they were reconstituted with unlabeled PC-tubulin, failed to give rise to a fibrillar pattern of fluorescence even after 2 h. These samples did, however, give rise to punctate fluorescent structures very similar to those seen underlying the fluorescent filaments in cells injected with DTAF-microtubular proteins (Fig. 7 *a* and *b*). Injection of DTAF (0.5 mg/ml) and a mixture of DTAF-labeled assembly-incompetent microtubule proteins resulted in a diffuse fluorescence over the entire cell as well as punctate labeling but no labeling of linear structures (Fig. 7 *c* and *d*).

The fluorescent filamentous pattern showed the expected response of microtubules to Colcemid. After exposure of cells to Colcemid (1 $\mu\text{g}/\text{ml}$), there was a rapid loss of the filamentous arrays and a transition to a diffuse, nonfibrillar pattern (Fig. 8). Many of the Colcemid-treated cells showed accumulation of fluorescence into phase-dense areas near their edges.

The utility of this procedure for studying microtubule dynamics was tested over a brief time period, using intermittent illumination and video image intensification to minimize bleaching of the fluorochrome. A series of observations over a span of 12 min, 4 h after injection of a fully spread fibroblast with DTAF-microtubule proteins, showed peripheral extension of labeled filaments (Fig. 9, arrows) into cell processes. Measurement of the lengths of these extensions as a function of time gave a rate of growth of 1 $\mu\text{m}/\text{min}$ at 37°C.

DISCUSSION

A number of cellular events including mitosis, certain types of prey-catching, and a variety of movements are thought to be

associated with microtubules and with their polymerization and depolymerization. Although immunohistological and electron microscope studies have provided much valuable information on microtubule-related functions, there is a need for methods that allow the direct observation of these organelles in living cells. To accomplish this, methods for (*a*) labeling microtubule proteins and (*b*) introducing these proteins into cells in a manner that permits their mobilization into the cytoskeleton must be defined.

Fluorescence-labeled microtubules have been prepared previously (1), using FITC conjugation at elevated pH. We have been able to repeat these experiments, and we find that, although this material will form microtubules *in vitro*, it is not incorporated into the polymer in detectable amounts when mixed with unlabeled microtubule proteins under assembly conditions. It would, therefore, appear to be a weak candidate for microinjection experiments in which the exogenous tubulin will be diluted by tubulin in the endogenous pool. Tubulin has also been labeled using fluorescamine and reported to self-assemble (15). The utility of this procedure may be limited by the short excitation wavelength of fluorescamine and the rapid degradation of fluorescamine-protein conjugates. The use of DTAF, as described here, avoids the pitfalls of the earlier procedures and yields labeled microtubule proteins that are indistinguishable from unlabeled protein in all tested assembly parameters.

The data show clearly that individual labeled proteins of the microtubules (tubulin and MAPs), when reconstituted with appropriate unlabeled components, assemble with kinetics that do not differ significantly from those of unlabeled controls (Fig. 2). The linearity of incorporation of DTAF-tubulin into microtubules as a function of concentration, at and below the critical concentration for self-assembly, when mixed with an

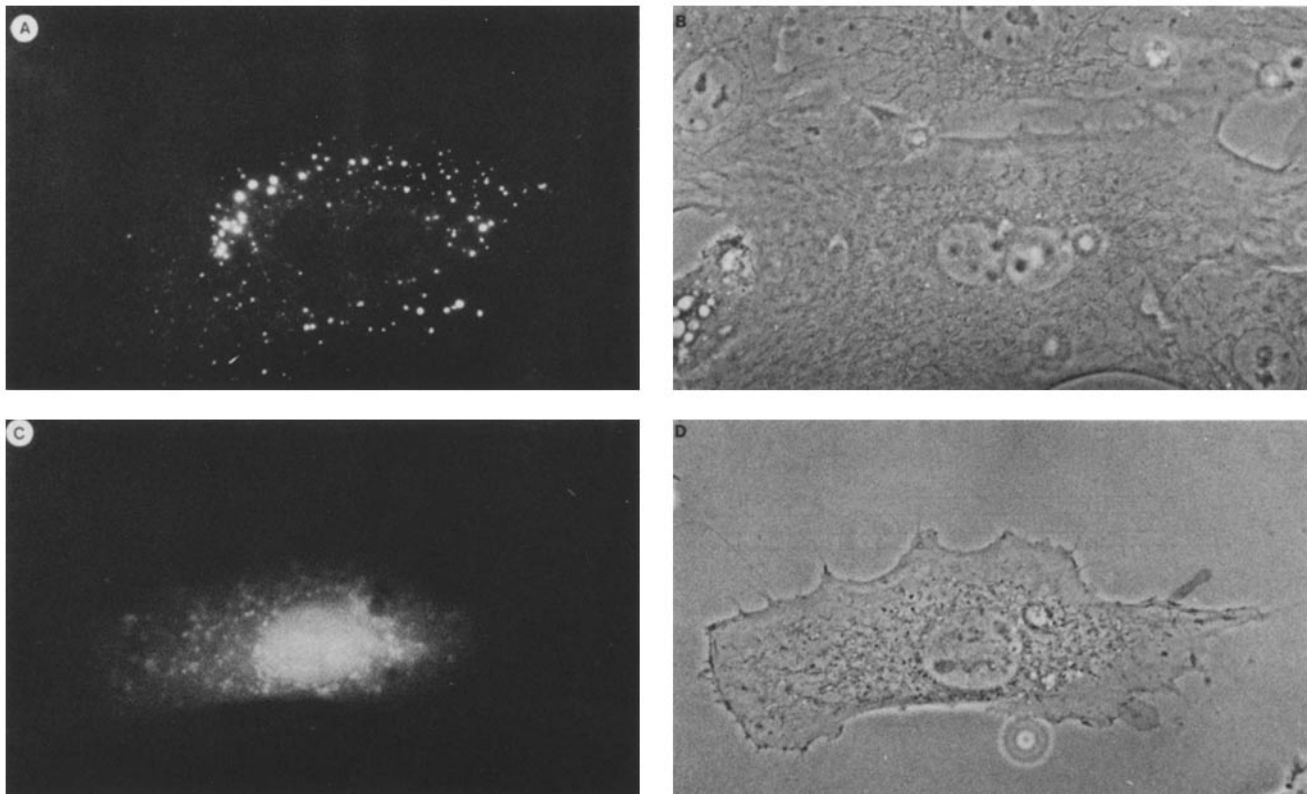


FIGURE 7 (A and B) Fluorescence and phase micrographs of living gerbil fibroma cells 2 h after injection of DTAF-PC-MAPs and (C and D) 2 h after injection of denatured DTAF-microtubule proteins and free DTAF. $\times 650$.

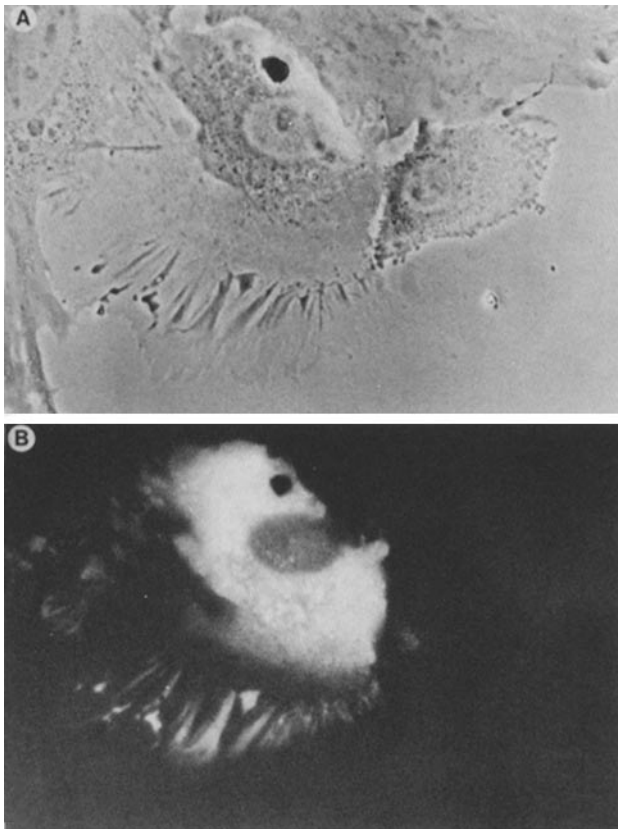


FIGURE 8 (A) Phase-contrast and (B) fluorescence micrographs of cells microinjected with DTAF-microtubule proteins and posttreated with Colcemid. $\times 620$.

unlabeled microtubule assembly system provides convincing evidence for copolymerization and a lack of alteration in assembly affinity.

Furthermore, the behavior of DTAF-PC-tubulin in the lysed-cell model system is identical to that predicted by Pepper and Brinkley (11). This demonstrates the ability of DTAF-PC-tubulin to assemble into microtubules in a model of the cellular environment. As reported, assembly occurs in this system without the need for accessory proteins and stops when the tubules reach a defined length, which appears to coincide with the limits of the "cytoskeleton" of the extracted cell. These results are consistent with the idea that positional information and assembly-promoting activity are embedded in the cellular ground substance, perhaps as part of the microtrabecular system (4, 12). The results of these kinetic and cell model studies demonstrate that DTAF-tubulin meets the criteria outlined in the introduction and that the labeling is adequate to allow direct visualization in the microscope.

The injection of DTAF-labeled tubulin into living fibroblasts results in the time-dependent appearance of a network of fluorescent fibrils. Neither DTAF alone nor DTAF-labeled denatured microtubule protein gives rise to a similar pattern. The labeling occurs only with assembly-competent tubulin or microtubule proteins, is sensitive to colchicine, and is strikingly similar in appearance to the arrays of microtubules seen by immunofluorescence techniques (17). We have, therefore, concluded that the fluorescent filamentous pattern is the result of incorporation of exogenous DTAF-tubulin into cytoplasmic microtubules.

Exploitation of this approach in the elucidation of cellular

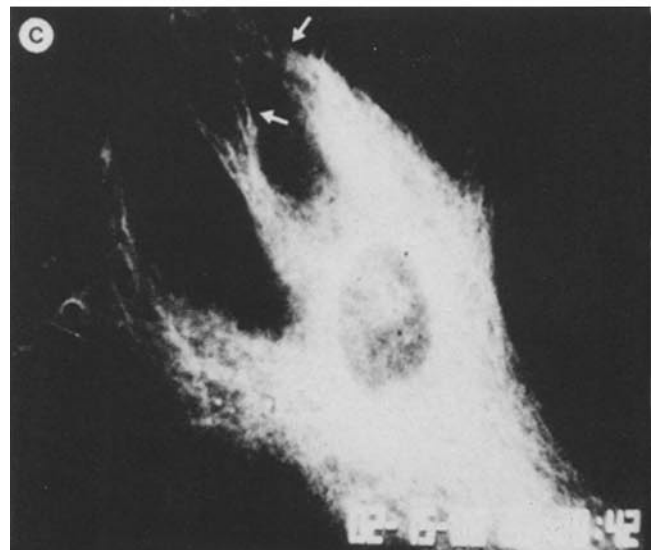
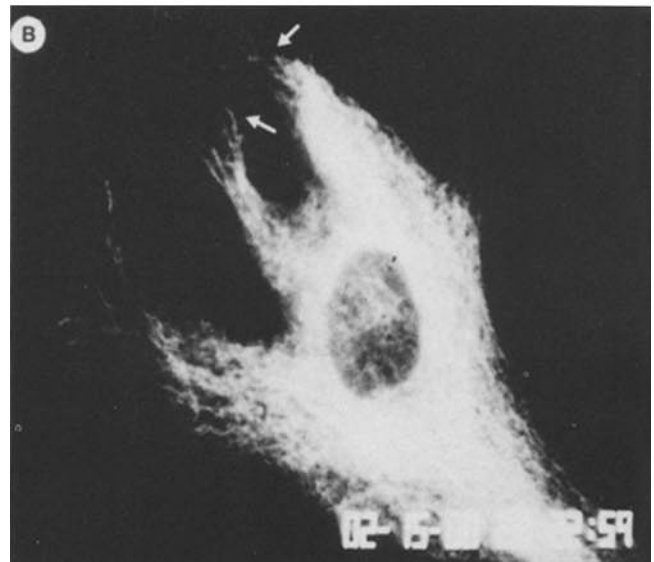


FIGURE 9 Time-course fluorescence micrographs of living gerbil fibroma cells microinjected with DTAF-tubulin. $\times 520$.

processes requires the ability to make sequential observations over appropriate timespans. Unfortunately, the rapid bleaching of fluorescein with intense exciting illumination imposes limits on such studies with even the most sensitive photographic emulsions. These problems can be minimized by the use of a silicon image intensification video camera and intermittent illumination controlled by a shutter in the light path of the fluorescence illuminator. The high sensitivity of the camera allows the use of reduced excitation levels, while exposures can be limited to $\frac{1}{15}$ of a second, the time required for two complete scans of the video raster. In this manner, 900 discrete images can be taken during 1 min total of exposure to exciting radiation. Preliminary studies in our laboratory indicate that up to 3 min of total low-level illumination can be used before bleaching becomes a significant problem in data acquisition. At an exposure rate of 1 frame/min, this would allow 45 h of recording. In the experiments shown in Fig. 9, the high resolution video monitor was photographed with a conventional 35-mm camera. Although there is loss of detail compared with direct photomicrography (Fig. 5), the microtubular network is readily resolved. Use of a conventional $\frac{1}{2}$ -inch video time-lapse tape recorder results in a further loss of resolution but still might be useful in some cases.

The apparent coincidence of the fluorescent fibrils and the phase-dense structures is consistent with the concept of microtubule organization along paths determined by other cytoskeletal elements (18). The initial diffusion of label after injection and the rapidity of labeling over wide regions of the cell within a short time is consistent with the idea that the proteins first enter a water phase in which they are freely diffusible and, later, are mobilized into the more structured cytoskeletal compartment of the cell (12).

Injection of DTAF-MAPs has failed to reveal labeling of fibrillar organelles in the fibroblasts. These studies have been carried out with DTAF-MAPs alone and with DTAF-MAPs reconstituted with cold PC-tubulin. In both cases, the fluorescence was initially diffuse and then confined to bright dots in the cytoplasm, presumably autophagic vacuoles. These data suggest that the gerbil fibroma cell is incapable of utilizing exogenous microinjected bovine brain MAPs. Whether this is attributable to a specificity of this cell type for different accessory proteins or whether microinjection is not a suitable mode of administration for the MAPs is not answered by these experiments.

In summary, these experiments show the utility of DTAF-

labeled tubulin for direct visualization of microtubular arrays in cell models and in living cells. Application of the methods presented here, together with immunocytochemistry and electron microscopy, promises to extend our knowledge of mitosis and other events associated with microtubules.

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