Improved Fixation for Immunofluorescence Microscopy Using Light-activated 1,3,5-triazido-2,4,6-trinitrobenzene (TTB)

ELENA McBEATH and KEIGI FUJIWARA
Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT A new fixation method has been developed for immunofluorescent microscopy using the photosensitive compound 1,3,5-triazido-2,4,6-trinitrobenzene (TTB). Our results show that TTB-fixed cells are well preserved morphologically and that the cellular antigens are better preserved than conventionally fixed cells. By altering one condition at a time in the TTB fixation procedure and analyzing resulting fluorescent antitubulin staining patterns in mammalian tissue culture cells, an optimal procedure was developed. Cells fixed with TTB and stained with antitubulin, antiprekeratin, anti-intermediate filament, anti-α-actinin, antimyosin, antiactin, or anticlathrin were compared with cells fixed by conventional methods and stained with the same antibody. The quality of immunofluorescence images of TTB fixed cells was the same as or better than that of conventionally fixed cells. The most dramatic improvement in image quality was seen when using antiprekeratin or antitubulin. In dividing cells, particularly in metaphase, fluorescent staining with antiactin and anti-α-actinin was relatively excluded from the spindle. Antimyosin, on the other hand, stained the spindle and surrounding area more heavily than the subcortical region. We suggest that after TTB fixation, the immunofluorescent patterns of these contractile proteins more closely reflect their relative concentrations in living cells. The exact mechanism for fixation by TTB is not yet known. However, our studies indicated that TTB fixation was not caused by the typical fast photoinduced nitrene diradical mechanism, but rather by some slower, temperature-dependent reaction of a photoactivation product of TTB with the cell.

Since the first immunofluorescence studies were published in 1942 by Coons and his associates (14; for historical background, see 13), numerous technical improvements and innovations have been made to increase the sensitivity and power of immunofluorescence microscopy. For example, in addition to fluorescein and tetramethylrhodamine, several new fluorescent dyes are available (38, 47), and conjugation procedures of these dyes to polypeptides have been worked out (6, 12, 58, 60). The design of microscopes and filters has also been improved (40–42). All these improvements and innovations have contributed to the wide spread use of the immunofluorescent method in cell biology.

While all of these innovations represent important aspects of the technique, the most important concern is specimen preparation. Biologically significant results cannot be obtained from poorly prepared specimens. In the majority of cases, specimens are fixed to stabilize and immobilize cellular antigens, and then they are permeabilized to make the cells' interior accessible to antibody molecules. These procedures, especially the fixation method, must fulfill two requirements: (*a*) they do not alter antigen distribution and (*b*) they do not destroy or mask the antigenic determinants to which antibodies bind. With the fixation methods available to date, however, fulfilment of these two requirements is often inversely related. The better fixed in place antigens are, the more poorly antigenicity is preserved.

For example, glutaraldehyde is a fixative that, morphologically, preserves many biological structures extremely well (26), but it also reduces specific binding of antibodies (11, 37). Cells fixed with formaldehyde, on the other hand, are better substrates for antibodies but morphological distortion during fixation (49) and loss of protein from the fixed cells during antibody staining (23) have been reported. Although it is far from being ideal, formaldehyde is the most commonly

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 DECEMBER 1984 2061–2073

used fixative for immunofluorescence microscopy.

It has been noted, however, that formaldehyde also completely or partially fails to preserve certain antigens. For instance, intermediate filament antigens are sensitive to formaldehyde (19, 20, 44). To avoid destruction of these antigens, cells are fixed using methanol or acetone (3, 39, 55). Although effective for immunofluorescent labeling of intermediate filaments, detectable amounts of proteins, including actin and tubulin, can be extracted into buffered saline from cells treated with these organic solvents (23). Cytoplasmic microtubules of cultured cells are also difficult to preserve using formaldehyde. For this antigen, the additional use of glutaraldehyde at low concentrations improves the image quality of antibody stained microtubules (9, 15, 59). One solution to these problems with fixation is to develop a simple, general fixation method that preserves the antigenicity of many different antigens. Such a method would not only simplify the immunofluorescence technique, but would also be ideal for double antibody staining since it simultaneously preserves more than one antigen. In this paper, we report on the development of a new fixation method for immunofluorescence microscopy, using a photosensitive compound. In the past, both azido and diazo compounds have been used as light activated crosslinking agents (16, 32). We reasoned that a compound with two or more light activated fixing groups of this type would crosslink biological macromolecules effectively. 1,3,5-triazido-2,4,6-trinitrobenzene (TTB)[1] is such a compound and was tested for its ability to act as a fixative. Upon irradiation, this compound preserved both cellular morphology and the antigenicity of many proteins. Our studies on the mechanism of fixation by TTB indicate that fixation is probably not due to a photochemical reaction of TTB with the specimen. Rather, it is due to a nonphotochemical reaction between the biological macromolecules and a photoactivation product of TTB. Preliminary results of this work were reported in abstract form (35).

MATERIALS AND METHODS

Chemicals: 1,3,5-triazido-2,4,6-trinitrobenzene (TTB) was purchased from ICN Pharmaceuticals, Inc. (Plainview, NY). However, it is no longer available from ICN. It now may be obtained from Polysciences, Inc. (Warrington, PA). Since dry TTB can explode (17), it comes as a moist paste containing about 25% water, just as picric acid does. TTB should be handled with the same precautions as for picric acid. When TTB concentrations are given in the text, they refer to the concentration of the paste rather than of TTB itself. Other chemical compounds used were imidazole (Grade 1, Sigma Chemical Co., St. Louis, MO), 2,4,6-collidine (Sigma grade purified, Sigma Chemical Co.), methanol (anhydrous ACS reagent grade, Fisher, Pittsburgh, PA, and MCB Manufacturing Chemists, Inc., Darmstadt, Germany), ethanol (pure dehydrated USP, U.S. Industrial Chemical Company, Tuscola, IL), acetone (certified ACS, Fisher), and chloroform (Baker analyzed, J.T. Baker Chemical Company, Phillipsburgh, NJ). The following stock solutions were made and stored at 4°C: 0.1 M imidazole buffer, pH 7.2–7.4 (ImB); 0.1 M imidazole in methanol (ImM); and 0.5% TTB in methanol. The TTB stock was kept in the dark.

Spectrophotometry: Absorption spectra of TTB and of organic solvents were obtained using a Cary 14 recording spectrophotometer or a Beckman Model 24 spectrophotometer. The TTB concentration and the solvent used are described in the appropriate figure legends.

Cells: Tissue culture cell lines, including HeLa, 3T3, and PtK-2 were grown on circular glass coverslips (12-mm diam). Dulbecco's modified Eagle medium (GIBCO Laboratories, Grand Island, NY) was supplemented with 10% fetal calf serum (GIBCO Laboratories) and 50 U of streptomycin/penicillin (KC Biological, Inc., Lenexa, KS). Cells were routinely grown in a 5% CO_2 incubator for 24–48 h before use.

[1] *Abbreviations used in this paper:* TTB, 1,3,5-triazido-2,4,6-trinitrobenzene; ImB, imidazole buffer; ImM, imidazole in methanol.

Fixation Procedure Using TTB: A detailed description of the TTB fixation procedure will be presented in Results. In this section, we will describe the two procedures that have given the best immunofluorescent pattern of the test antigen, tubulin. Unless otherwise noted, all steps of the fixation were done on ice and all solutions used were at ice temperature. Cells on coverslips were fixed in a plastic tissue culture Petri dish (50-mm diam). Up to 12 round coverslips may be placed in this Petri dish without overlap. The quantities of fluid given in the procedure are suitable for a dish of this size containing 12 coverslips.

DEHYDRATION OF CELLS: Cells were brought out from the incubator and the culture medium was aspirated off. A 1:2 mixture of ImM (2ml) and ImB (4 ml) was quickly poured onto cells and agitated for 4 s. Cells were then washed first with 6 ml ImM and then 6 ml of −20°C methanol. From the aspiration of medium to addition of −20°C methanol, it typically took about a half a minute.

PHOTOACTIVATION: To fix cells, TTB must be photoactivated. However, it was not necessary for cells to be irradiated along with TTB. The following two procedures provided good immunofluorescent staining.

Method A: Under a red safelight (Kodak Wrattin series 1A), the −20°C methanol was replaced with 3 ml of a 0.5% solution of TTB in methanol. TTB was allowed to permeate into the cells for ~1 min in the dark. The coverslips in the Petri dish were then exposed to the semi-collimated light of a 1,000 W mercury-xenon lamp (model 977B-0010, Kratos/Schoeffel Instrument Company, Westwood, NJ) for 5 min. The distance of the light path from the bulb to the coverslip was ~40 cm (Fig. 1). The use of a water filter and of quartz elements are crucial for removing heat from the illuminating beam and for ultraviolet transmission, respectively. After irradiation, the cells were rinsed with −20°C methanol until the methanol appeared clear.

Method B: 3 ml of a 0.5% TTB solution in methanol cooled to −70°C were irradiated for 40 min on dry ice as shown in Fig. 1. The −20°C methanol on the previously dehydrated cells was removed. The preirradiated TTB solution was then added to the cells and they were left for 15 min on ice. They were then rinsed with −20°C methanol until the methanol appeared clear.

REHYDRATION: The methanol in which cells had been washed was replaced with ImM (6 ml) and then with 6 ml of a 1:1 mixture of ImM and ImB. Cells were exposed to the 1:1 mixture for 4 s while agitating the container. Then it was quickly replaced with ImB (6 ml). After 3 min, the ImB wash (6 ml, 3 min) was repeated. Room temperature PBS (containing 0.85% NaCl/10 mM sodium phosphate, pH 7.2–7.4/0.02% NaN_3) was used for the final 3-min wash. It is important that cells are not allowed to dry out during the rehydration steps. TTB-fixed cells may be stained with antibodies immediately after the final PBS wash or, as we found, they may be stored at 4°C in PBS for up to 1 y without considerable deterioration of tubulin immunofluorescent morphology. Cells were usually mounted in formalin (pH 5) or formalin diluted 1:1 with PBS immediately after staining with antibodies.

Conventional Fixation Procedures: To evaluate the effectiveness of the TTB fixation, we compared immunofluorescence patterns of TTB-fixed cells with those of cells fixed by other procedures. These methods include (*a*) fixation with formaldehyde (3.7% in PBS, 10 min at room temperature) followed by permeabilization with cold acetone or methanol (5 min, −20°C); (*b*) treatment with cold acetone or methanol only (5 min, −20°C); and (*c*) mild

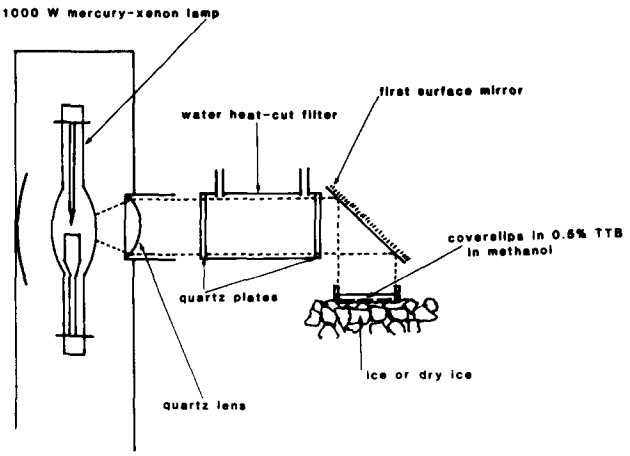

FIGURE 1 Schematic drawing of the arrangement for TTB activation. Light (dotted line) emitted by the lamp is collected by the quartz lens, passed through a 10-cm water filter, and reflected by the first surface mirror down onto the ice or dry ice cooled sample.

glutaraldehyde fixations designed for microtubule immunofluorescence (for details, see 9, 59).

Antibody Staining: Rabbit antisera against sea urchin cytoplasmic tubulin (22), human platelet myosin (21), and chicken gizzard α-actinin (24) have previously been described and characterized. Antiprekeratin is an autoimmune rabbit serum. All the above antibodies were used at a 100 times dilution of the serum in PBS. Rabbit antiactin raised against fish skeletal actin muscle was affinity purified (8) and was used at 15 to 200 μg/ml. Antimyosin was also affinity purified using human platelet myosin bound to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA), and used at 118 μg/ml. Mouse monoclonal anti-intermediate filament antibody raised against human glial fibrillary acidic protein was kindly provided by Dr. R. Pruss (Laboratory of Clinical Science, National Institutes of Mental Health, National Institutes of Health, Bethesda, MD 20205) (44). The spent culture medium was used straight. Anticlathrin against calf brain clathrin was kindly provided by Dr. M. S. Robinson (Laboratory of Molecular Biology, Medical Research Council Centre, Cambridge, United Kingdom) (46). For controls, we used preimmune sera or absorbed IgG (for antiactin and antimyosin). Autofluorescence of the TTB fixed cells and the extent of nonspecific binding of the secondary antibody were also examined. Little or no fluorescence was observed in these control stainings.

Antibody staining was performed using either the direct or indirect method. The rhodamine-labeled secondary antibodies were obtained from Cappel Laboratories (West Chester, PA). Epifluorescence microscopy was done using a Leitz Orthoplan equipped with a Ploemopak 2 containing a Leitz N-2 filter and a Zeiss ×63 planapo phase lens (NA 1.4 oil) and fluorescent images were recorded by a Leitz Orthomat using Tri-X film (Eastman Kodak Co., Rochester, NY) exposed at ASA 1,000 and developed in Acufine (Acufine, Inc., Chicago, IL).

RESULTS

TTB As a Fixative for Immunocytochemistry

A model system was needed to test the ability of TTB to fix cells for immunofluorescence microscopy. Our experiences as well as immunofluorescent images published by other investigators indicate that immunofluorescence morphology of microtubules in mammalian tissue culture cells is sensitive to fixation conditions. In interphase cells, poorly preserved microtubules appear faint, beaded, and sometimes abruptly bent, while well fixed microtubules are seen as brightly fluorescent, continuous, and curving lines. Furthermore, the well preserved mitotic spindle has a morphology similar to the living state as seen with polarizing microscopy in which the spindle fibers gently arch from pole to pole. For these reasons, we used the immunofluorescent morphology of microtubules and spindles as an indicator of antigenic and structural preservation so that various steps in the TTB fixation procedure could be optimized.

A general outline of the steps for TTB fixation and factors that influence the microtubule staining pattern are as follows. (*a*) Cells must first be dehydrated by being transferred to an appropriate organic solvent. Damage to the cells caused by this step can be minimized in two ways. One is to add certain solutes to all the solvents used in the transfer steps. The other is to introduce cells to the organic solvent gradually. (*b*) The dehydrated cells must then be placed in the appropriate concentration of unactivated or preactivated TTB in the appropriate organic solvent. (*c*) TTB solution, with or without cells, should be irradiated using the best available light source for the proper length of time. (*d*) Cells must be transferred back into an aqueous environment (rehydration) to stain them with antibodies. As in dehydration, choice of solvent, solute, and rate of transfer are important factors in affecting the final outcome.

To determine the optimum condition for various factors, we held the conditions constant for all but one factor and varied the conditions of this factor until an optimum was found. Then that optimum condition was used for all the remaining experiments in which the rest of the factors were each separately tested in the same way. The five major factors found to affect the immunocytochemical preservation of microtubules were: (*a*) Organic solvents used; (*b*) solutes used; (*c*) introduction to and removal from organic solvents; (*d*) TTB concentration in organic solvents; and (*e*) TTB photoactivation. The following detailed descriptions of each category should be helpful in understanding the various steps of the TTB fixation procedure described in Materials and Methods.

ORGANIC SOLVENTS: We used acetone and methanol most extensively, although chloroform, ethanol, and tetrahydrofuran were also tested. To compare the effects of acetone and methanol on fixation, we used the same concentration (0.5%) of TTB and light activated them identically. Immunofluorescence results showed that although microtubules qualitatively appeared equally well preserved using either solvent, they stained more brightly when using methanol. Acetone absorbs ultraviolet radiation while methanol, from 230 nm and up, does not. Since TTB is activated by ultraviolet radiation (see Mechanism of Fixation by TTB), the brighter staining intensity of cells fixed by TTB in methanol may indicate more efficient activation of TTB in methanol than in acetone. Chloroform containing 0.5% TTB and ethanol containing 0.3% TTB (i.e., saturated) were also tested. Chloroform produced more discontinuous microtubules and ethanol, less intense microtubule staining than that found with methanol or acetone. Since chloroform is fairly noxious and TTB is less soluble in ethanol than in methanol, these solvents were not used further. Tetrahydrofuran severely damaged the cellular morphology and no microtubular staining was apparent.

SOLUTES: When cells were plunged into organic solvents, considerable morphological damage resulted. Such damages could be minimized if certain compounds were dissolved in all the solvents. A dramatic improvement in the immunofluorescent microtubule image quality was seen by using imidazole buffer (10–100 mM, pH 7.2–7.4) or collidine buffer (0.1 M, pH 7.2–7.4) and organic solvents containing the same concentration of solute.

Both imidazole and collidine are (*a*) highly soluble in both water and organic solvents; (*b*) alkaline in nature with a good buffering capacity; and (*c*) reduce the difference in surface tension, measured using capillary tubes (10), between water and methanol. We studied if any or all of the above three qualities of imidazole and collidine were necessary for preserving microtubule morphology. Ethylene glycol is soluble in both water and the organic solvents, but has no buffering capacity and does not reduce the difference in the surface tension between water and methanol. Immunofluorescent microtubule morphology did not improve by using 0.1 M ethylene glycol. Both Tris (10 mM) and sodium phosphate (0.1 M) are good buffers at pH 7.4 but are insoluble in the organic solvents. They also failed to improve microtubule morphology. Phenylphosphonic acid is soluble in both water and the organic solvents, is an acidic buffer, and slightly reduces the difference in surface tension. Microtubule morphology was poor in cells dehydrated and rehydrated in the presence of 0.1 M phenylphosphonic acid. These observations, when taken together, suggest that all three properties of imidazole and collidine listed above are important for preserving immunofluorescent microtubule morphology.

INTRODUCTION TO AND REMOVAL FROM SOLVENT: When living cells are plunged into methanol or ace-

tone, the violent mixing at the solvent-water interface can damage cellular morphology, including microtubules. Good microtubule immunofluorescent patterns were consistently obtained by transferring cells through a graded series of solvent/water mixtures. The most satisfactory results for tissue culture cells were obtained using the procedure described in Materials and Methods. Cells were plunged into a 2:1 mixture of ImB and 0.1 M imidazole in the organic solvent, and agitated for 4 s. Cells were then transferred to 0.1 M imidazole in the pure organic solvent. Cells left in the 2:1 mixture for a shorter time than 4 s displayed microtubules that were often sharply bent, and beaded or broken. Cells kept in the mixture longer than 4 s also had more beaded and broken microtubules.

The TTB fixed cells must then be rehydrated before being stained with antibodies. To consistently preserve microtubule morphology, fixed cells were plunged into a 1:1 mixture of ImB, and 0.1 M imidazole in the organic solvent, and agitated vigorously for 4 s. Shorter agitation times tended to cause oddly bent microtubules while prolonged agitation caused cytoplasmic background staining to increase and microtubule staining to decrease.

TTB CONCENTRATION: When TTB is purchased, it contains 25% water and appears as a somewhat sticky yellow crystalline powder. A 10% solution can be readily made in acetone, while only ~0.5% is soluble in methanol. Since there is 25% water in the purchased TTB, the true TTB concentrations are 7.5% in acetone and 0.375% in methanol.

Using acetone as the solvent, we investigated the effects of TTB concentration (0.1, 0.5, 1, and 10%) on the staining of microtubules. The best microtubule image was obtained with 1% TTB. Cells fixed with 0.1 and 10% TTB had fainter, more beaded microtubules than those treated with 1% TTB. At 10%, TTB may be denaturing the tubulin to the point where it significantly loses antigenicity. Alternatively, the cell may be too highly crosslinked to allow sufficient penetration of the staining antibodies.

We also tested 0.1 and 0.5% TTB in methanol. While 0.1% TTB preserved microtubules reasonably well, cells fixed with 0.5% TTB showed extremely well preserved microtubules that were very bright and that appeared continuous most consistently from specimen to specimen. The morphology and the brightness of these microtubules were noticeably better than those seen in cells treated with either 0.5 or 1% TTB in acetone.

The presence of TTB in acetone or methanol during irradiation was necessary to obtain good morphological preservation of microtubules. Cells treated with acetone or with acetone followed by illumination with light showed poorly defined microtubules. Interestingly, however, when cells were treated with ethanol or methanol and illuminated by light (as shown in Fig. 1), their microtubules and prekeratin intermediate filaments became clearly detectable by immunofluorescence microscopy although the image quality was not nearly as good as when TTB was present. Methanol or ethanol treatment without light irradiation did not produce this effect. This observation indicates that fixation of microtubules or prekeratin intermediate filaments by methanol or ethanol can be improved by simply irradiating the specimen with light. This effect may be due to the breaking and reforming of bonds in macromolecules by ultraviolet irradiation (see for example 50 and 57). Acetone, unlike the alcohols, absorbs a significant amount of ultraviolet irradiation and may suppress this type of ultraviolet effect.

TTB PHOTOACTIVATION: For TTB to fix cells for immunofluorescence microscopy, it must be photoactivated. When TTB paste was irradiated with ultraviolet radiation containing light, it turned yellow, suggesting that TTB had been activated. The color change was monitored to determine the best light source for photoactivating TTB. A drop of TTB was dried on glass slides and then exposed to different light sources. It took a few minutes to yellow in direct sunlight, in the light of a hand held, short wavelength ultraviolet lamp, and in the light of a 100-W xenon lamp (with glass collecting lens). However, a 100-W mercury lamp (with glass collecting lens) caused the color to change within 10 s.

We then determined the best length of activation time for TTB fixation. Because photofixation of microtubules occurred with methanol and light irradiation alone, differences in the effect of fixation due to TTB are more clearly demonstrated using acetone. Cells in 1% TTB in acetone were irradiated with several light sources for varying lengths of time and immunofluorescent staining with antitubulin was analyzed qualitatively. When a 100-W mercury lamp (HBO-100, Osram, Berlin, West Germany) with a glass collecting lens was used for 3, 15, 30, or 60 min, the best microtubule preservation was seen with cells irradiated for 30 min or longer. Cells must be irradiated for 10 min or longer (1, 3, 5, 10, and 30 min were tested) with a 1,000-W xenon lamp (976C-0010, Canrad Hanovia, Newark, NJ) with a quartz collecting lens, to obtain similar results. The most efficient irradiation was with a 1,000-W mercury-xenon lamp which took 3 min (1, 5, and 20 s and 1, 3, 5, and 10 min were tested) to give good results. Since longer irradiation did not damage the image quality, to insure full activation, we routinely irradiated cells for 5 min using this lamp.

When we used methanol as the solvent, similar observations were made. Because the intensity of immunofluorescent images was brightest in cells irradiated for 5 min in 0.5% TTB in methanol, this was determined to be the optimum condition for TTB photoactivation, and is described as method A.

Cells were also irradiated while in 0.5% TTB in methanol with a hand held, 4 W, short wavelength ultraviolet lamp (model SL-2537, Spectronics, Westbury, NY) with its filter removed. An irradiation time of 40 min or more (5, 10, 20, 40, and 90 min were tested) produced good microtubule preservation.

In all the instances described above, activation times shorter than the minimum needed for the best preservation produced fainter, sometimes beaded microtubules against a diffuse background staining. By phase contrast microscopy, these cells appeared less dense and many of them were broken open. The shorter the activation time, the more obvious these defects became and the more frequently broken cells were encountered.

These optimum fixation times are much longer than typical photoactivated production and reaction times for free radicals. Furthermore, we had some evidence that adequate fixation took longer in colder TTB solutions. Photoactivated production of free radicals, on the other hand, is an essentially temperature independent process (32). The long photoactivation times and the temperature dependence of TTB fixation are not characteristic of typical free radical reactions. Therefore, we investigated if the fixing component, with its relatively longer life, might persist after irradiation ceases and still be capable of fixation. Cells immersed into already irradiated TTB in an organic solvent (method B) did give good microtubule staining. This result confirmed that it was not the TTB

itself reacting with and fixing the cells, but some photoactivation product of TTB.

To study how stable that product was, TTB in methanol was irradiated for 5 min and then left in the dark at 4°C for 18 and 108 h. These TTB solutions preserved microtubules poorly, especially when the 108-h old sample was used. Cold temperature slowed down the decay of the activated TTB product. For example, it took several days at −70°C before the activated TTB lost a noticeable amount of its ability to fix. The instability of the activated product implies that it is very reactive.

To determine how fast this light activated TTB product could fix microtubules, cells were immersed for 1 s, 15 s, 1, 3, 5, or 10 min in 0°C preirradiated TTB in dim light. The immunofluorescent quality (the staining intensity and continuity of the microtubules) improved as cells were left longer in the preirradiated TTB, up to 3–5 min. No apparent improvement was seen with more than 5 min of fixation.

We then determined how long TTB must be irradiated to get the maximum amount of the fixing product. TTB was irradiated with the 1,000-W mercury-xenon lamp for 3, 5, 10, 20, 40, 90, or 180 min and used to fix cells for 5 min. The best preserved microtubules were seen in cells fixed with TTB preirradiated for 40 min. Microtubules did not stain as brightly if preirradiation time was more or less than 40 min. For example, cells fixed in 5-min preirradiated TTB or in 180-min preirradiated TTB showed similar microtubule staining intensities. The decrease in microtubule staining intensity when using TTB irradiated for longer than 40 min suggests that the TTB fixing compound is being destroyed.

TTB Fixation vs. Conventional Fixation

MICROTUBULES: The effectiveness of the present method could be appreciated when immunofluorescence patterns of TTB-fixed cells were compared with those of cells fixed by the different procedures listed in Materials and Methods. Microtubules in formaldehyde-fixed cells appeared faint and often beaded (Fig. 2*A*). Individual microtubules were often difficult to resolve, and the quality of the image varied from region to region within one coverslip and from coverslip to coverslip. It was particularly difficult to resolve microtubules in the perinuclear region. Contrary to this situation, microtubules in TTB fixed cells over the entire coverslip were seen as bright continuous, gently curving lines (Fig. 2, *B* and *C*). Individual microtubules were clearly visible, and the dense accumulation of microtubules around the perinuclear cytoplasm was seen. HeLa cells contain a large number of microtubules that often cannot be preserved by conventional fixation procedures for immunofluorescence (Fig. 2*A*) but are preserved by the present method (Fig. 2*C*).

Reasonable microtubule staining patterns (Fig. 2*D*) were obtained using a previously published fixation method (9) with a modification. The procedure was modified by incubating the fixed cells in 0.15 M Tris buffer, pH 7.4, overnight (J. Tomasek, personal communication) to quench glutaraldehyde. However, the staining intensity was substantially lower than that seen with TTB fixed cells. Furthermore, there was nonspecific background fluorescence seen, especially in the thicker part of the cell.

Chromosomal spindle fibers and microtubules of the mitotic spindle were also clearly demonstrated in TTB-fixed cells (Fig. 3, *A* and *B*). The ability to resolve single microtubules allowed us to detect the presence of astral rays in tissue culture cells in all mitotic phases. The number and length of the astral microtubules were large in prophase. Although the aster became smaller in metaphase, we frequently found a few microtubules radiating out from the spindle pole toward the cell margin (Fig. 3*B*). The aster then increased in size during anaphase. In late telophase when the midbody is present, it was clearly seen that the microtubules of the midbody were not part of those connected to the centriole (Fig. 3*C*).

INTERMEDIATE FILAMENTS: A dramatic improvement was noted when the anti-intermediate filament staining patterns of TTB-fixed cells were compared with those of conventionally fixed cells. PtK-2 cells fixed with formaldehyde or glutaraldehyde (9) and stained with the antiprekeratin antibody showed no fibrous staining patterns (Fig. 4*A*). However, when TTB-fixed PtK-2 cells were stained, a bright fluorescent pattern of prekeratin intermediate filament distribution was seen (Fig. 4*B*). Using the monoclonal intermediate filament antibody with conventional formaldehyde fixation, intermediate filaments were rarely seen in 3T3 or PtK-2 cells. Using the fixation procedures recommended by Pruss (44) for each intermediate filament type, some intermediate filament staining could be obtained but it was faint and often discontinuous. With photoactivated TTB fixed cells, a bright intermediate filament pattern was always seen (Fig. 4*C*).

OTHER ANTIBODIES: Interphase cells: The photoactivated TTB-fixed tissue culture cells also stained brightly with antiactin, antimyosin, and anti-α-actinin. When immunofluorescence results between conventionally fixed cells and TTB fixed cells using these antibodies were compared, the staining patterns were either the same or crisper and better defined in TTB fixed cells. For example, formaldehyde fixed PtK-2 cells stained with antimyosin showed dull fluorescent patterns of stress fibers and cortical staining (Fig. 5*A*). TTB fixed cells, on the other hand, showed a crisper image (Fig. 5*B*). Cross-reactivity of a given antibody was also better when TTB fixed cells were used. For example, with formaldehyde fixation, our anti-α-actinin stains chick cells well but 3T3 cells poorly. TTB fixed 3T3 cells, however, stained brightly with this antibody.

The organic solvents used in the TTB procedure extract membranes. Therefore, we also tested the ability of TTB to fix a membrane-associated antigen, clathrin. Cells fixed by method A and stained with anticlathrin demonstrated a staining pattern and intensity comparable with those of conventionally fixed cells.

Dividing cells: When mitotic cells were stained with affinity-purified antiactin used at concentrations of >50 μg/ml, the fluorescent intensity of the spindle was lower at all stages of mitosis than the staining intensity of the nonspindle region. This spindle staining pattern was most noticeable at metaphase (Fig. 6, *A* and *B*). This reduced staining of the spindle depended on the antiactin concentration used. For example, at 15 μg/ml, mitotic cells stained uniformly throughout the cell, but at 50 μg/ml, relatively reduced spindle staining could be found in some metaphase cells. With 200 μg/ml of antibody (Fig. 6*A*), the reduced spindle staining was easily detected. When the staining patterns of interphase cells, which contain stress fibers, were studied using 15, 50, and 200 μg/ml antiactin, the staining intensity of the stress fibers and certain other parts of the cytoplasm increased as more antibody was used, but we detected no change in the staining pattern.

Anti-α-actinin staining intensity in the spindle area relative to nonspindle cytoplasm was also lower (Fig. 6*C*). As with antiactin, the reduced spindle staining seen with anti-α-actinin

was found at all stages of mitosis. Unlike antiactin and anti-α-actinin, antimyosin staining was brighter in the spindle region than outside of it. A typical metaphase cell stained with antimyosin showed bright fluorescence in and around the area occupied by the mitotic spindle and relatively low fluorescence in the subcortical region (Fig. 6*D*). Affinity-purified antimyosin also revealed a similar staining pattern. During anaphase, a fibrous antimyosin staining running parallel to the spindle axis was observed between the separating chromosomes in some cells (Fig. 6, *E* and *F*). During cytokinesis, antiactin, anti-α-actinin, and antimyosin staining was found in the cleavage furrow. Fig. 6*E* illustrates antimyosin staining of the cleavage furrow.

Mechanism of Fixation by TTB

Free radical reactions are usually very fast, on the order of milliseconds (27, 32). We found, however, that the fixing component of irradiated TTB had a life expectancy in the hours at room temperature and days at −70°C. Long life is more characteristic of non-free radical compounds, although some free radicals are very stable (45). Because it is relatively stable, the formation and decay of the activated TTB fixing component could be studied by spectrophotometry. Such studies may provide insights into the mechanism of fixation.

Using a dilute (0.01% or less), TTB solution in methanol, we characterized TTB and its photoactivated form by spectro-

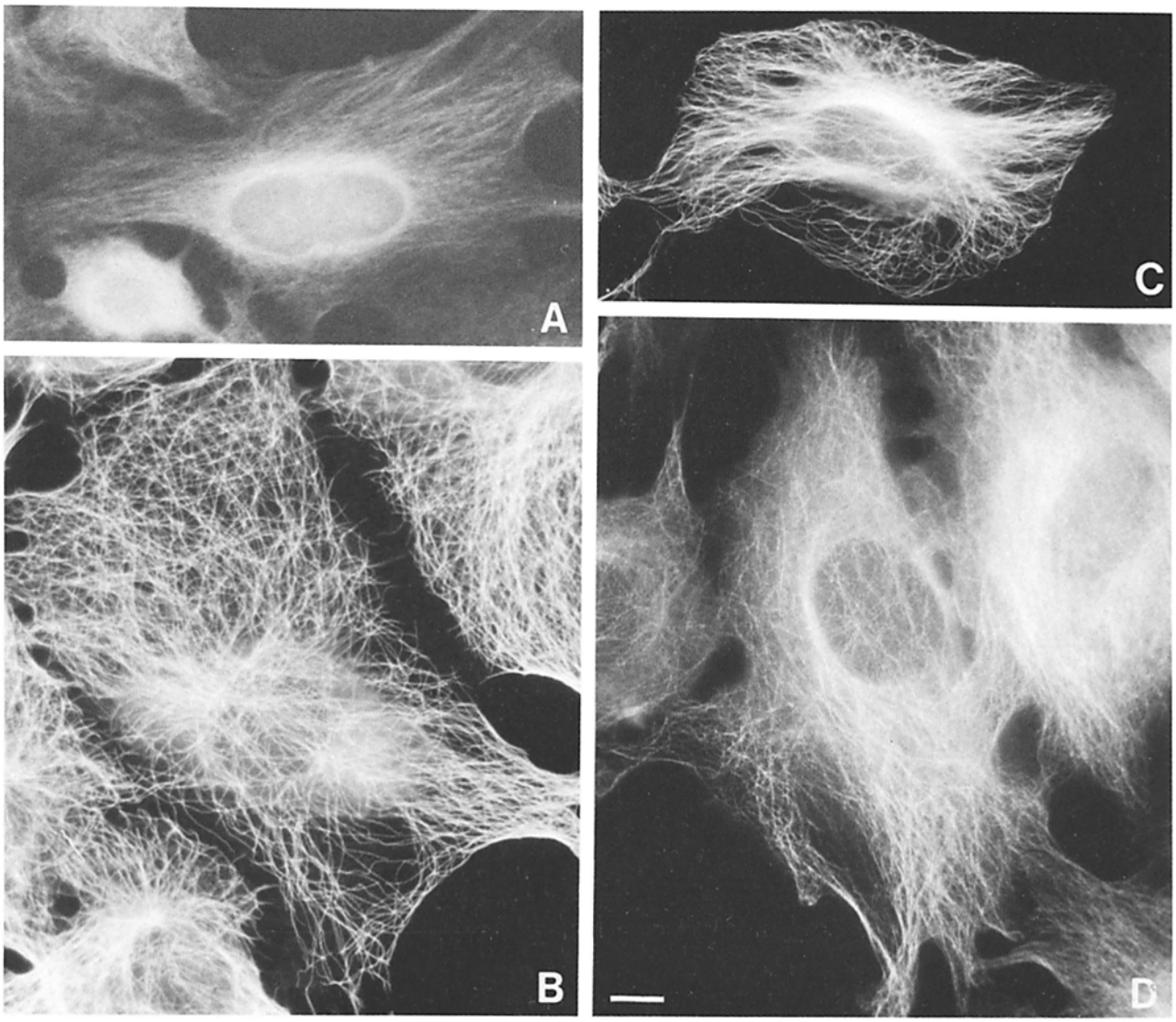

FIGURE 2 Comparison of antitubulin immunofluorescent image quality using different fixation methods. To indicate fluorescent intensity, the photographic exposure time is provided for each micrograph. (*A*) HeLa cells fixed with formaldehyde and permeabilized with acetone, then stained directly with fluorescein-labeled antitubulin. Individual microtubules are not clearly visible. Exposure time: 1.5 min. (*B*) 3T3 cells fixed using TTB (method B) then stained indirectly with antitubulin. Microtubules are seen as continuous lines. The large cell in the center is in early prophase and its two MTOCs can be detected. Exposure time: 8 s. (C) A HeLa cell fixed using TTB (method B) and stained indirectly with antitubulin. Compared with the cells in *A*, this cell shows densely accumulated individual microtubules. Exposure time: 8 s. (*D*) 3T3 cells fixed with formaldehyde/glutaraldehyde in microtubule stabilizing buffer (9) and stained indirectly with antitubulin. Some individual microtubules are visible, but they are beaded. General background fluorescence is present. Exposure time: 1 min. Bar, 10 μm.

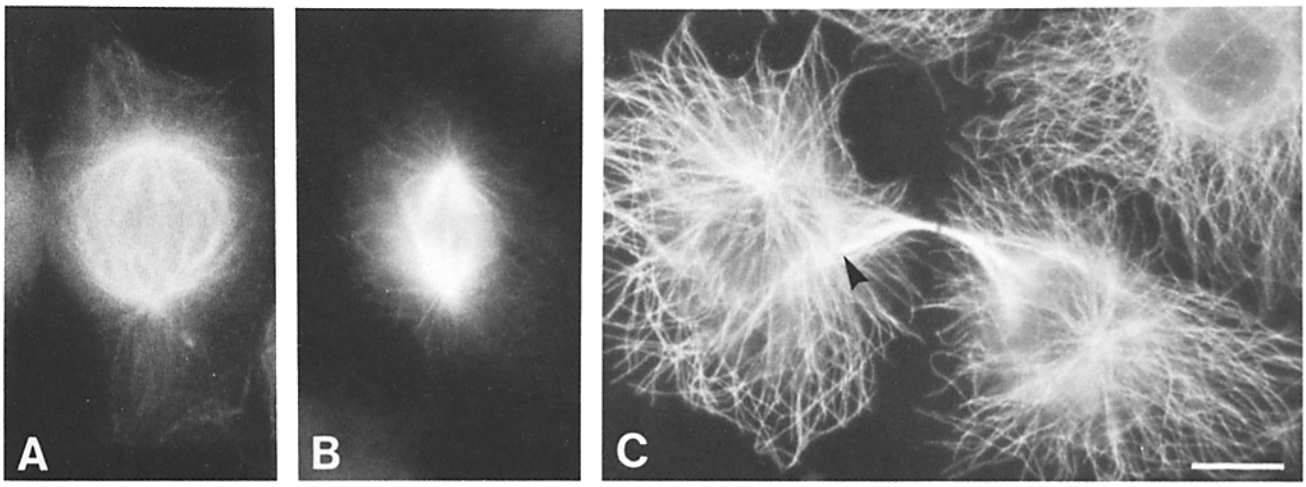

FIGURE 3 Dividing tissue culture cells fixed using TTB (method B) and indirectly stained with antitubulin. (*A*) 3T3 cell in mid-anaphase. Note astral microtubules extending from the spindle pole to the cell margin. Chromosomal fibers are easily observable. (*B*) PtK-2 cell in metaphase. Both asters and chromosomal fibers are seen. (C) PtK-2 cell in late telophase. The arrowhead points to one of the terminations of the midbody microtubules.

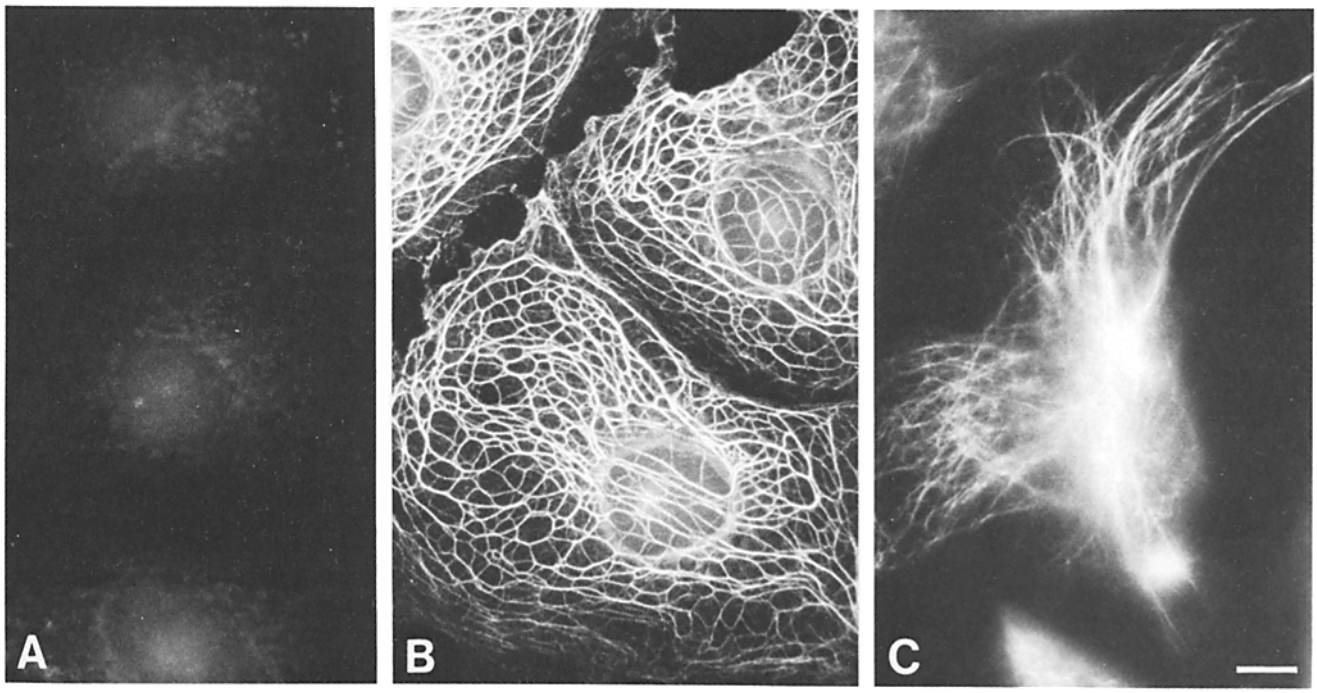

FIGURE 4 Comparison of intermediate filament staining in tissue culture cells fixed by different methods. (*A*) PtK-2 cells fixed with formaldehyde and permeabilized with methanol, then stained indirectly with antiprekeratin. Faint fluorescence is associated with the nucleus and the perinuclear region. The absence of the prekeratin pattern in these cells indicates that the antigen is not preserved by this fixation procedure. (*B*) PtK-2 cells fixed using TTB (method A) and stained indirectly with antiprekeratin. Note the extensive network of prekeratin fibers. (*C*) 3T3 cells fixed using TTB (method A) and stained indirectly with monoclonal anti-intermediate filament. Vimentin is the type of intermediate filament expressed by this cell line. Bar, 10 μm.

photometry. Unactivated TTB had a characteristic single absorption peak (λmax = 254 nm) in the ultraviolet region (Fig. 7*A*) and no absorption in the visible range. When the TTB solution was activated by a 1,000-W mercury-xenon lamp as shown in Fig. 1, the 254-nm peak quickly disappeared (Fig. 7*A*). Fig. 7*B* illustrates the kinetics of the disappearance of the 254-nm peak and shows that, for 0.0005% TTB in methanol, >90% of the peak was gone within 10 s. While the ultraviolet absorption peak was disappearing, a new absorption peak (λmax = 432 nm) was appearing in the visible region (Fig. 7*C*). As shown in Fig. 7, *C* and *D*, this peak was also transient. The development of the 432-nm peak was rapid, reaching its maximum absorbancy in 30 s for a 0.01% solution; but its decay was relatively slow (Fig. 7*D*). When this concentration of TTB was activated longer than 5 min, it showed no absorption peak in the ultraviolet or visible regions (Fig. 7*C*).

It should be noted that these spectroscopic changes can be monitored only in very dilute TTB solutions. When similar experiments were conducted using the TTB concentration

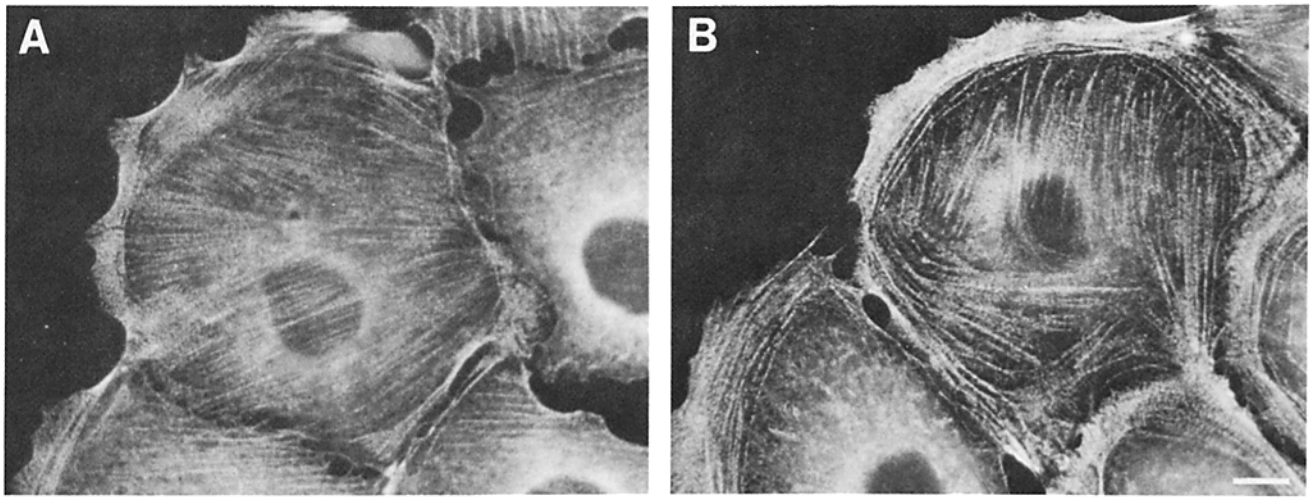

FIGURE 5 Comparison of antimyosin immunofluorescence image quality using different fixation methods on PtK-2 cells. (*A*) Cells fixed with formaldehyde and permeabilized with methanol. The immunofluorescent pattern appears dull. (*B*) Cells fixed using TTB (method B). Unlike the micrograph in *A*, the immunofluorescent image is crisp and the finer details are evident in the stress fibers and the cortex. Bar, 10 μm.

used to fix cells, the pale yellow, 0.5% TTB solution first turned an intense yellow and within 5 min, it became amber. The amber color continued to deepen with up to 40 min of irradiation. Although this color then slowly faded during further irradiation (up to 3 h), it never entirely disappeared. The ultraviolet absorption spectrum of the 40-min irradiated 0.5% TTB in methanol was different from the spectrum of similarly irradiated 0.0005% TTB in methanol, indicating that the products of the photochemical reaction of TTB depended on its concentration. After irradiation, two peaks, at 206 nm and at 240 nm, and two shoulders, at ~285 and 375 nm, were present (Fig. 8). During storage over time, absorption gradually increased while the profile of the spectrum changed only slightly. However, 1-d-old irradiated TTB (whose absorption spectrum is shown in Fig. 8) lost its ability to fix. The 375-nm shoulder present in 5-min old irradiated TTB was not detectable in this 1-d-old sample. This suggests that the loss of this shoulder is related to the loss of the fixing ability of the irradiated TTB solution. In support of this hypothesis, we found that both processes were temperature dependent. The colder the irradiated TTB solution was kept, the slower was the alteration in the spectra and the slower the TTB solution lost its ability to fix.

DISCUSSION

Advantages of TTB Fixation

We have demonstrated that the immunofluorescent images of TTB-fixed cells are much brighter and better defined than those of conventionally fixed cells. We have also shown that TTB fixed cells crossreact with many more antisera than formaldehyde fixed cells. These observations suggest that TTB fixation better preserves the antigenic sites and/or the antigen's position in the cell. The TTB fixation procedure may maintain a high local concentration of the antigen by rapidly precipitating proteins, upon exposure of the cells to methanol, followed by the chemical fixation of the precipitated protein with TTB. Since antigen redistribution is more likely to occur in an aqueous environment than in methanol (23), the TTB method has the advantage of chemically fixing the cells with TTB, presumably by crosslinking, before they are re-exposed to aqueous solutions. The sharpness and brightness of the microtubule images of TTB fixed cells indicates that the redistribution of tubulin is below the level of detection by the light microscope.

Formaldehyde, the most commonly used fixative, does not effectively preserve microtubule or intermediate filament antigenicity so that special fixatives must often be used to get reasonable antibody labeling. For example, glutaraldehyde-based fixatives have been used to fix microtubules for immunofluorescence microscopy (9, 59). When we tested the effectiveness of these fixation procedures using our particular tubulin antibody, we failed to obtain satisfactory results using one method (59), while the other (9) did not give as clear an image as did the TTB fixed cells. We conclude that, of all these methods, the TTB method most consistently gives the best microtubule immunofluorescence pattern.

Certain fixatives are effective only for certain antigens. For example, the glutaraldehyde method (9) is good for microtubules but not for intermediate filaments. Methanol fixation sometimes provides excellent immunofluorescence images of intermediate filaments (19, 20) but it is not a good fixative for microtubules. Acetone treatment is good for myosin localization but not for microtubules and actin (23). When two or more antigens must be localized simultaneously, the fixative used must preserve the localization and antigenicity of more than one antigen. The TTB method is an ideal fixation method since it superbly preserves many different types of antigens.

A monoclonal antibody is made against one specific antigenic site of a molecule. Thus, any fixation method employed for immunofluorescence using monoclonal antibodies must preserve that particular antigenic site. Although our experience with monoclonal antibodies is limited, the present method preserves the antibody binding site of a monoclonal antibody that normally requires special fixation procedures (44). Given the superb antigenic preservation demonstrated in TTB-fixed cells, this new fixation procedure may become useful for immunofluorescence using monoclonal antibodies.

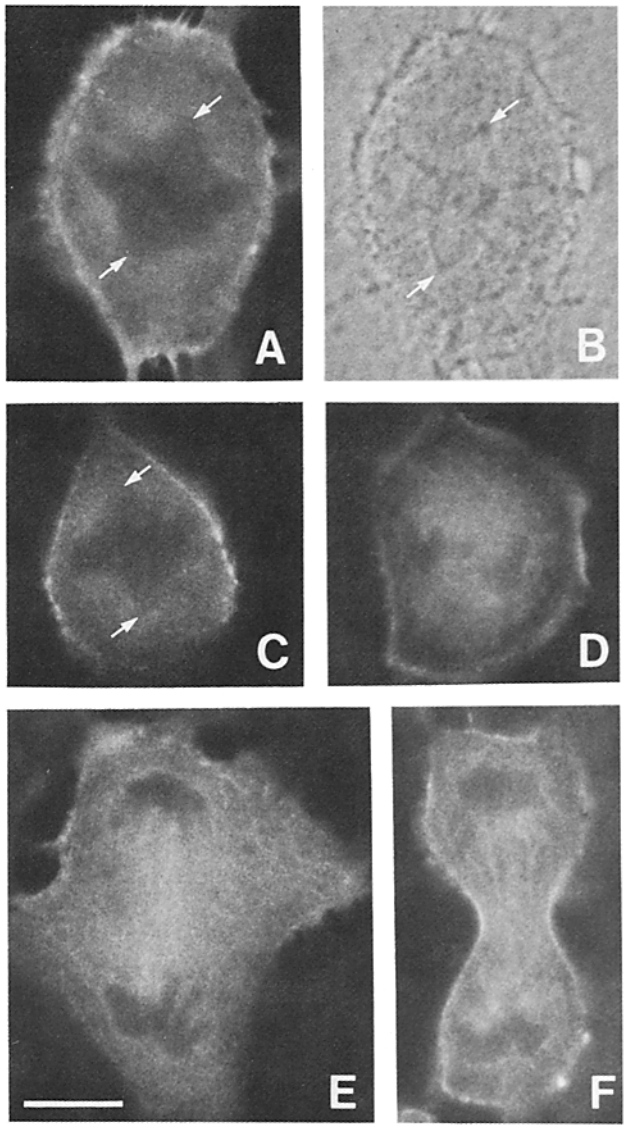

FIGURE 6 Dividing PtK-2 cells fixed using TTB (method B) and stained with various antibodies. The arrows point to the spindle poles. (*A*) A metaphase cell indirectly stained with 200 μg/ml affinity-purified antiactin. The fluorescent intensity is lower in the spindle and the area occupied by the chromosomes. (*B*) Phase contrast micrograph of the same cell as in *A*. (*C*) A metaphase cell indirectly stained with anti-α-actinin. The spindle and chromosome mass are darker than the rest of the cell. (*D*) A metaphase cell indirectly stained with antimyosin serum. The area occupied by the spindle and the surrounding area show brighter fluorescence. The staining does not extend into the subcortical region. (*E*) A late anaphase cell indirectly stained with antimyosin serum. Note the fluorescent fibrous structures between the separating chromosomes. (*F*) A telophase cell indirectly stained with 118 μg/ml affinity-purified antimyosin. The cleavage furrow is stained and fibrous structures can again be seen between the separated chromosomes. Bar, 10 μm.

Localization of Contractile Proteins in Dividing Cells

IMMUNOFLUORESCENCE STUDIES: Results of immunochemical localization of various cytoplasmic contractile proteins in the mitotic spindle are controversial as demonstrated by the conflicting immunofluorescence patterns reported by different laboratories (1, 2). Since the present fixation method gave qualitatively much better immunofluorescence images, we reinvestigated spindle immunocytochemistry in TTB-fixed dividing cells.

Since results published by various laboratories on antitubulin staining of spindle are in good agreement, we first studied whether or not antitubulin staining of TTB fixed dividing cells could confirm previously published results. We found that, while the image quality of the TTB-fixed spindle was superior to many previously published immunofluorescent spindle images, our results confirmed old data except that with our new fixative, astral rays were seen throughout various phases of mitosis.

TTB fixed mitotic cells stained with antiactin or anti-α-actinin showed immunofluorescent patterns different from published results: both antibodies stained the spindle region much less brightly than the nonspindle cytoplasm. Previously, spindle staining with antiactin was reported as staining either more brightly than the general cytoplasm (11, 30) or at the same intensity as the rest of the cytoplasm (2, 31). Reduced antiactin staining intensity in the spindle region has not previously been reported. Similarly, anti-α-actinin staining was previously reported to be uniform throughout the mitotic cell (24) but a relative exclusion of anti-α-actinin staining from the spindle has not been reported.

It is possible that the results we obtained are an artifact of the new fixation procedure. For example, TTB may fail to fix actin and α-actinin when they are in the spindle, or their antigenic sites may be inaccessible in TTB fixed cells. Although these possibilities cannot be eliminated, we regard them as unlikely since the results of our other studies reported in this paper have not indicated that these possibilities exist. Rather, we attribute these differences between our present results and others' to the improved fixation of cells by TTB. As we discussed earlier, certain changes in the spindle morphology and the extraction of cellular proteins occur in formaldehyde-fixed cells (23, 49). The greatly improved immunofluorescence images seen in TTB-fixed cells (Figs. 2–5) suggest that cellular structure and antigens are better preserved in TTB-fixed cells than those fixed conventionally. It follows, then, that the localization of antigen observed in TTB-fixed cells reflects more closely the distribution of the antigen in living cells than do already published observations.

Both antimyosin and affinity-purified antimyosin stained the spindle region of TTB-fixed cells more intensely than the cytoplasm. Our results are in agreement with the conclusion of one report in which acetone-fixed cells were used (21), but do not support the results seen with formaldehyde fixed, antimyosin-stained cells (2). Since acetone-precipitated myosin is known to be insoluble in aqueous solution, myosin localization in acetone treated cells is likely to be preserved. TTB-fixed cells had antimyosin spindle staining patterns similar to those of acetone-fixed cells, suggesting that myosin is indeed concentrated in the spindle.

Actin Localization Using Probes Other Than Antibodies

In addition to specific antibodies, there are other molecular probes that can localize actin filaments in fixed cells. Using fluorescently labeled HMM or S1, some workers found actin filaments to be highly concentrated in the spindle region (29, 30, 48, 51), while one group (2) found uniform fluorescence throughout the dividing cell. Fluorescently-labeled phallotoxin has also been used. Whereas uniform staining was

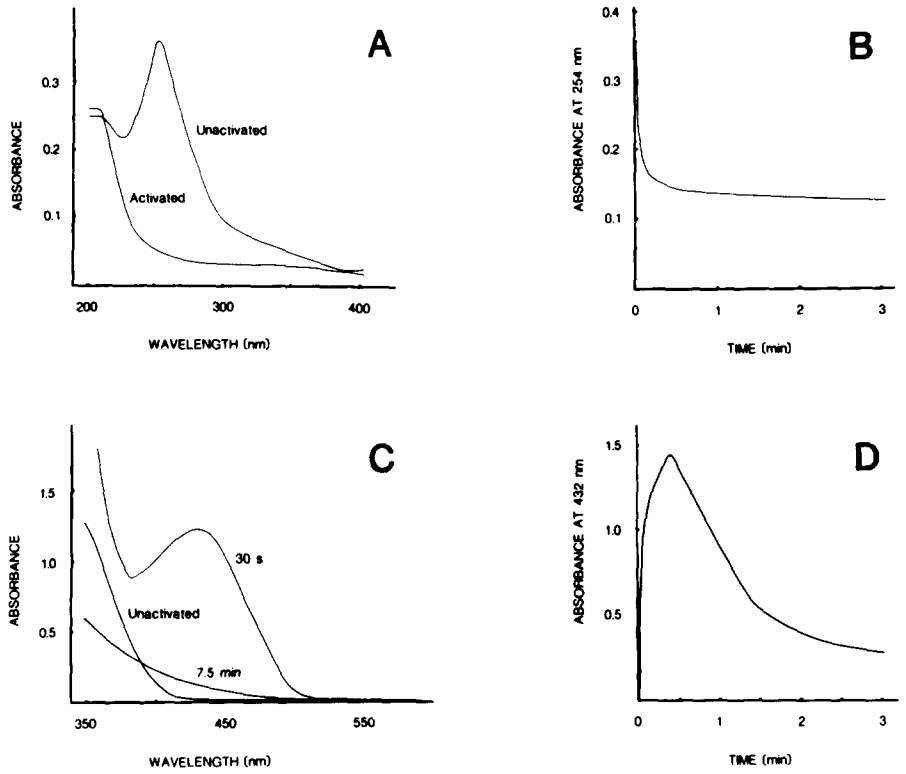

FIGURE 7 Spectrophotometric analyses on TTB before and after irradiation with 1,000-W mercury-xenon lamp light. (*A*) Ultraviolet absorption spectra of 0.0005% TTB in methanol before irradiation (*Unactivated*) and after 30 min of irradiation (*Activated*). Irradiation causes the 254-nm absorption peak to disappear. (*B*) Time course of the loss of the 254-nm absorption peak. This curve was constructed by plotting absorbance at 254 nm measured after a 0.0005% TTB solution was irradiated for the following lengths of time: 1, 2, 3, 5, 10, 20, 50, and 180 s. (*C*) Visible wavelength region absorption spectra of 0.01% TTB in methanol before irradiation (*Unactivated*), after 30 s of irradiation (30 s), and after 7.5 min of irradiation (7.5 min). Note the transient nature of the 432-nm peak. (*D*) Time course of absorption at 432 nm of 0.01% TTB in methanol after increasing lengths of irradiation time. Data points were collected after irradiating for the following lengths of time: 1, 2, 4, 6, 10, 15, 25, 40, 60, 90, and 450 s. The $t_{1/2}$ of 432-nm peak formation is ~2 s while for its decay, $t_{1/2}$ is on the order of 50 s.

observed by Wulf et al. (61) using this probe, a lack of fluorescence in the spindle region was reported by Barak et al (5).

It is difficult to evaluate how reliable each of the above findings is. It is intriguing that such specific probes to actin filaments as HMM (or S1) and phallotoxins yield such varied results. Undoubtedly, the various fixation procedures used contribute to these differences. In addition, the reagents used by different investigators may contain varying amounts of denatured polypeptides or inactive drugs that could effect the level of background staining. Nevertheless, these reports all suggest that actin filaments are present in the spindle. It is the relative amounts of actin filament within vs. without the spindle that is in dispute.

Actin filaments have been found in the spindle by electron microscopy (for review, see 18, 28). Although no morphometric data are available to determine the proportion of actin filaments found inside vs. outside the spindle, Schroeder (52) felt that actin filaments were more frequently encountered outside the spindle region. Although not stated explicitly, others have published electron micrographs showing more actin filaments outside the spindle than inside (for example, 25, 51). Problems with the fixation of actin filaments for electron microscopy are known (18, 34). Thus, it is possible that actin filaments in the spindle are more sensitive to fixation than those in the nonspindle region. However, until this possibility if proven, we will assume that actin filaments inside and outside the spindle are equally affected by the fixative. Therefore, we conclude from ultrastructural studies that more actin filaments are present in the nonspindle area than within the spindle.

Presence of Actomyosin in the Spindle

Of all the conventional fixation methods, the glutaraldehyde based fixation method for electron microscopy provides

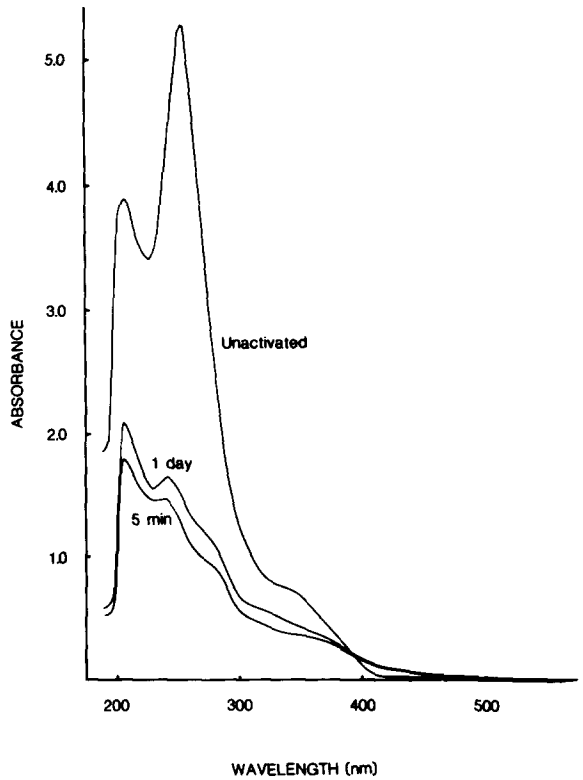

FIGURE 8 Absorption spectra of TTB activated at a concentration of 0.5% in methanol before and after irradiation with 1,000-W mercury-xenon lamp light. The TTB solution was irradiated for 40 min on dry ice and left in the dark at room temperature. 5 min and 1 d later, a portion of the solution was diluted 100 times with methanol and its spectrum determined. The absorption spectrum for unactivated TTB (*Unactivated*) was measured at a concentration of 0.0005% TTB in methanol and the value was multiplied by a factor of 10 to facilitate comparison of the three spectra.

the best preservation of biological structures. Thus, we suggest that the actin filament distribution in vivo is best shown by electron micrographs which indicate that actin filaments are present in the spindle region but are much more abundant in the nonspindle region. Our antiactin results on TTB-fixed dividing cells supports this type of actin distribution. Actin comprises ~10% of the total protein of a nonmuscle cell (7, 56). It is possible that the spindle could contain a considerable amount of actin and still have a lower concentration of actin than the region outside the spindle. The lower concentration of actin in the spindle relative to the cytoplasm could imply either that a high concentration of actin is required for some function outside of the spindle and/or a high concentration of actin gets in the way of spindle function. Therefore, actin might be actively excluded from the spindle, leaving within only a small amount of actin which may be necessary for spindle function.

The reduced spindle staining with anti-α-actinin could simply mean that α-actinin is associated with actin filaments, thus having a similar localization as actin. Another possibility is that this protein is not actively recruited into the spindle and consequently it is excluded by the presence of other proteins in the spindle. Antimyosin staining was concentrated in and around the mitotic spindle, leaving a ring of less bright subcortical staining in metaphase cells. This could imply that myosin is actively excluded from the subcortex. However, we favor the interpretation that myosin is recruited into the spindle and the surrounding area.

Although the functional significance of our findings is difficult to determine without further experimentation, several possible implications will be considered. The presence of actin and myosin in the spindle could mean that these contractile proteins play a role in chromosome movement and there is some experimental evidence suggesting that this may be the case (53). However, one can also find a considerable amount of experimental evidence suggesting that actomyosin is not involved in this process (for review, see 54). Because it is theoretically possible to move chromosomes using only a small number of myosin molecules (43), the possibility of actomyosin involvement in chromosome movement is not yet disproved. Since most of the actin was excluded from the spindle while the majority of myosin was found in the spindle, the molar ratio of myosin to actin in this area of the cell is greatly increased. This condition may allow myosin and strategically placed actin filaments to interact efficiently in the spindle. Actomyosin may also play a structural role in the spindle and surrounding area. The interpretation that actomyosin plays a structural role in the mitotic apparatus is further supported by the fact that myosin localization goes beyond the boundary of the spindle. The observed fibrous antimyosin staining between separating chromosomes, where myosin probably cannot contribute directly to the poleward movement of the chromosomes, also supports this view.

Mechanism of Fixation

When cells are fixed by method A, it is possible that the radicals formed by photolysis of TTB react with cellular macromolecules and preserve cellular structure by crosslinking. However, the contribution of this scheme is not the major mechanism of TTB fixation since cells can be fixed in preirradiated TTB solution (method B). This suggests that the molecular species which reacts with the antigen is exceedingly long lived compared with the life time of true free radicals. Since fixation is accomplished only if TTB is photoactivated, we will consider what may happen to TTB when it is irradiated by light. Such a consideration may help identify the molecule(s) responsible for fixing cells.

It is known that irradiation of other aryl azides can cause the two terminal nitrogens of the azide to leave (32). The remaining nitrogen is left as a diradical called a nitrene. It is reasonable to suppose that the same can occur with TTB. Detailed studies on TTB photolysis have not been done but Korsunskii and Apina (33) have studied the thermal decomposition of TTB. Their results may give us insights into the photolysis of TTB. TTB contains three azido groups alternating with three nitro groups on benzene (Fig. 9). Upon heating, the two terminal nitrogens of one of the azide substituents leave as nitrogen gas. They hypothesized that the remaining nitrogen (nitrene) will quickly react with one of the adjacent nitro groups to form a furoxan (Fig. 9). Their evidence suggests that the formation of this first furoxan in TTB is the rate limiting step. Once the first furoxan is formed, the remaining two azide and two nitro groups very quickly form two more furoxans so that the final product is benzotrifuroxan (BTF) (Fig. 9). One might suppose that the molecule absorbing at 432 nm is BTF. Unfortunately, the published absorption spectrum of BTF has one major peak at 255 nm and none at 432 nm (4). Another possibility is that, unlike what occurs upon thermal decomposition, photolysis of TTB may lead to mono or difuroxan intermediates. In support of this idea, Baily and Case (4) have shown that the 4-nitrobenzofuroxans always have a prominent absorption peak in the 420-430-nm range, as is seen with the partly activated TTB.

The appearance and disappearance of the 432-nm peak also indicate that TTB changes its molecular structure at least twice during the first 1 min of illumination. The rate of decrease in the original 254-nm peak (Fig. 7*B*) is roughly similar to the rate of increase, upon activation, of the yellow 432-nm peak (Fig. 7*D*). This suggests that the molecular species absorbing at 254 nm, TTB, may be converting, by free radical reactions, into the molecular species absorbing at 432 nm, possibly the mono and difuroxans. Upon further irradiation, the 432-nm peak disappears, suggesting a second alteration in TTB structure.

It is important to note that the 432-nm peak appears only when very dilute solutions of TTB are photoactivated. In fact, when a solution of much higher concentration of TTB is irradiated, the absorption spectrum of the photoactivation products is quite different from that of a dilute solution. When a 0.5% TTB solution was irradiated, an amber color developed and this color deepened with continued irradiation for up to 40 min. The development of the amber color suggests that a different chemical(s) is forming in the 0.5% irradiated

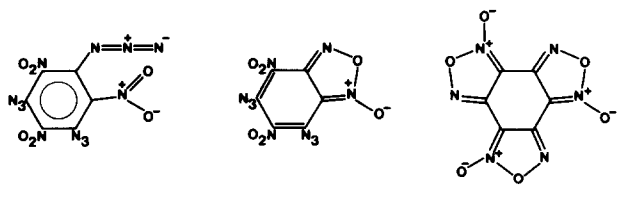

FIGURE 9 Chemical structure of TTB and its derivatives. The bonding for one of the azido and one of the nitro groups on TTB is shown.

TTB. Under these conditions, it is possible that TTB molecules react with each other and/or with solvent molecules to form long polymers of activated TTB. Polymers formed from resonating structures usually absorb at longer wavelengths than the monomer (36). Indeed, we found that 40-min activated 0.5% TTB absorbs more at the longer wavelengths than low concentrations of activated TTB. Such a polymer may be responsible for cellular fixation.

The chemical mechanism of fixation by TTB appears to be complex. Our studies indicate that TTB does not fix as a rapidly reacting free radical but rather through a more ordinary temperature-dependent reaction or very stable free radical mechanism. Upon irradiation, TTB must isomerize, interact with itself, and/or interact with the solvent to form a thermally unstable, short-lived, and therefore very reactive intermediate. We have not yet identified this TTB photoactivation product, however. As an approach to understanding the mechanism of TTB fixation and to identifying the compound responsible for the fixation of cells, we have investigated the possibility of TTB related molecules (i.e., other benzene derivatives) acting as fixatives upon irradiation. Our studies (submitted for publication elsewhere) revealed that many benzene derivatives became excellent fixatives once activated by light. It is hoped that a systematic analysis of these results will give us further insights into the mechanism of fixation by photoactivated TTB.

We would like to thank John Tuckerman for modifying the electrical system of the mercury-xenon lamp and Peter Ley for photography. This work was supported by National Institutes of Health training grant GM 07226-07 to E. McBeath and National Science Foundation grant PCM-81-19171 to K. Fujiwara.

Received for publication 23 September 1983, and in revised form 14 February 1984.

REFERENCES

1. Aubin, J. E. 1981. Immunofluorescence studies of cytoskeleton proteins during cell division. *In* Mitosis/Cytokinesis. A. M. Zimmerman and A. Forer, editors. Academic Press, New York, NY. pp 211–244.
2. Aubin, J. E., K. Weber, and M. Osborn. 1979. Analysis of actin and microfilament-associated proteins in the mitotic spindle and cleavage furrow of PtK-2 cells by immunofluorescence microscopy. *Exp. Cell Res.* 124:93–109.
3. Aubin, J. E., M. Osborn, W. W. Franke, and K. Weber. 1980. Intermediate filaments of the vimentin-type and the cytokeratin-type are distributed differently during mitosis. *Exp. Cell Res.* 129:149–165.
4. Baily, A. S., and J. R. Case. 1958. 4:6-dinitrobenzofuroxan, nitrobenzodifuroxan and benzotrifuroxan: A new series of complex forming reagents for aromatic hydrocarbons. *Tetrahedron.* 3:113–131.
5. Barak, L. S., E. A. Nothnagel, E. F. DeMarco, and W. W. Webb. 1981. Differential staining of actin in metaphase spindles with 7-nitrobenz-2-oxa-1,3-diazole-phallocidin and fluorescent DNase: is actin involved in chromosomal movement? *Proc. Natl. Acad. Sci. USA.* 78(5):3034–3038.
6. Blakeslee, D., and M. G. Baines. 1976. Immunofluorescence using dichlorotriazinylaminofluorescein (DTAF). I. Preparation and fractionation of labelled IgG. *J. Immunol. Methods.* 13:305–320.
7. Bray, D., and C. Thomas. 1975. The actin content of fibroblasts. *Biochem. J.* 147:221–228.
8. Byers, H. R., and K. Fujiwara. 1982. Stress fibers in cells *in situ*: Immunofluorescent visualization with anti-actin, anti-myosin, and anti-alpha-actinin. *J. Cell Biol.* 93:804–811.
9. Byers, H. R., K. Fujiwara, and K. R. Porter. 1980. Visualization of microtubules *in situ* by indirect immunofluorescence. *Proc. Natl. Acad. Sci. USA.* 77:6657–6661.
10. CRC Handbook of Chemistry and Physics, 60th Edition, 1979–1980. R. C. Weast and M. J. Astle, editors. CRC Press, Inc., Boca Raton, FL. 128 pp.
11. Cande, W. Z., E. Lazarides, and J. R. McIntosh. 1977. A comparison of the distribution of actin and tubulin in the mammalian mitotic spindle as seen by indirect immunofluorescence. *J. Cell Biol.* 72:552–567.
12. Cebra, J. J., and G. Goldstain. 1965. Chromatographic purification of tetramethylrhodamine-immune globulin conjugates and their use in the cellular localization of rabbit γ-globulin polypeptide chains. *J. Immunol.* 95:230–245.
13. Coons, A. H. 1971. Introduction: the development of immunohistochemistry. *Ann. NY Acad. Sci.* 177:5–9.
14. Coons, A. H., H. J. Greech, R. N. Jones, and E. J. Berliner. 1942. The demonstration of pneumococcal antigen in tissue by the use of fluorescent antibody. *J. Immunol.* 45:159–170.
15. DeBrabander, M., J. DeMey, M. Joiau, and G. Geuens. 1977. Ultrastructural immunocytochemical distribution of tubulin in cultured cells treated with microtubule inhibitors. *Cell Biol. Int. Rep.* 1:177–183.
16. Dentler, W. L., M. M. Pratt, and R. W. Stephens. 1980. Microtubule-membrane interaction in cilia. II. Photochemical cross-linking of bridge structures and the identification of a membrane associated dynein-like ATPase. *J. Cell Biol.* 84:381–403.
17. Fauth, M. I., and G. W. Roecker. 1961. Reduction of gem-dinitro and trinitro compounds with titanium (III) chloride. *Anal. Chem.* 33:894–896.
18. Forer, A. 1978. Chromosome movements during cell division: possible involvement of actin filaments. *In* Nuclear Division in the Fungi. I. B. Heath, editor. Academic Press, Inc. New York, NY. pp 21–88.
19. Franke, W. W., E. Schmid, M. Osborn, and K. Weber. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. Natl. Acad. Sci USA.* 75:5034–5038.
20. Franke, W. W., K. Weber, M. Osborn, E. Schmid, and C. Freudenstein. 1978. Antibody to prekeratin. Decoration of tonofilament-like arrays in various cells of epithelial character. *Exp. Cell Res.* 116:429–445.
21. Fujiwara, K., and T. D. Pollard. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. *J. Cell Biol.* 71:848–875.
22. Fujiwara, K., and T. D. Pollard. 1978. Simultaneous localization of myosin and tubulin in human tissue culture cells by double antibody staining. *J. Cell Biol.* 77:182–195.
23. Fujiwara, K., and T. D. Pollard. 1980. Techniques for localizing contractile proteins with fluorescent antibodies. *In* Current Topics in Developmental Biology. Vol. 14. Immunological Approaches to Embryonic Development and Differentiation. Part II. M. Friedlander, editor. Academic Press, New York, NY. pp 271–296.
24. Fujiwara, K., M. E. Porter, and T. D. Pollard. 1978. Alpha-actinin localization in the cleavage furrow during cytokinesis. *J. Cell Biol.* 79:268–275.
25. Gawadi, N. 1974. Characterization and distribution of microfilaments in dividing locust testis cells. *Cytobios.* 10:17–35.
26. Handgen, M., D. Schafer, and N. Weissenfels. 1971. The fixation effect of eight different aldehydes on the ultrastructure of cultured cells. II. The structural state of the cytoplasm. *Cytobiolgie.* 3:202–214.
27. Havinga, E., and J. Cornelisse. 1976. Aromatic photosubstitution reactions. *Pure Applied Chemistry* 47:1–10.
28. Heath, I. B. 1981. Mitosis through the electron microscope. *In* Mitosis/Cytokinesis. A. M. Zimmerman and A. Forer, editors. Academic Press, New York, NY. pp 245–275.
29. Herman, I. M., and T. D. Pollard. 1978. Actin localization in fixed dividing cells stained with fluorescent heavy meromyosin. *Exp. Cell Res.* 114:15–25.
30. Herman, I. M., and T. D. Pollard. 1979. Comparison of purified antiactin and fluorescent heavy meromyosin staining patterns in dividing cells. *J. Cell Biol.* 80:509–520.
31. Izutsu, K., K. Owaribe, S. Hatano, K. Ogawa, H. Komada, and H. Mohri. 1979. Immunofluorescent studies on actin and dynein distribution in mitotic cells. *In* Cell Motility: Molecules and Organization. S. Hatano, H. Ishikawa, and H. Sato, editors. University Tokyo Press, Tokyo. pp. 621–638.
32. Ji, T. H.. 1979. The application of chemical crosslinking for studies on cell membranes and the identification of surface reporters. *Biochim. Biophys. Acta.* 559:39–69.
33. Korsunskii, B. L., and T. A. Apina. 1971. Kinetics of the thermal decomposition of 1,3,5-triazido-2,4,6-trinitrobenzene in solution. *Bulletin of the Academy of Sciences of the USSR. Division of Chemical Science.* 21:1971–1973.
34. Maupin-Szamier, I. P., and T. D. Pollard. 1978. Actin filament destruction by osmium tetroxide. *J. Cell Biol.* 77:837–852.
35. McBeath, E., and K. Fujiwara. 1981. An improved fixation method for light level antigen localization using a photoactivatable crosslinker. *J. Cell Biol.* 91:339*a*. (Abstr.)
36. Murrell, J. N. 1963. Conjugated Hydrocarbon Chains. *In* The Theory of the Electronic Spectra of Organic Molecules. Spottiswoode, Ballantyne and Co. Ltd, London and Colchester, Great Britain. pp. 67–90.
37. Nakane, P. 1975. Recent progress in the peroxidase-labeled antibody method. *Ann. NY. Acad Sci.* 254:203–210.
38. Namihisa, T., K. Tamura, K. Saifuku, H. Imanari, H. Kuroda, Y. Kanaoka, Y. Okamoto, and T. Sekine. 1980. Fluorescent staining of microfilaments with heavy meromyosin labeled with N-(7-dimethylamino-4-methylcoumarinyl) maleimide. *J. Histochem. Cytochem.* 28:335–338.
39. Osborn, M., W. W. Franke, and K. Weber. 1980. Direct demonstration of the presence of two immunologically distinct intermediate-sized filament systems in the same cell by double immunofluorescence microscopy. Vimentin and cytokeratin fibers in cultured epithelial cells. *Exp. Cell Res.* 125:37–46.
40. Page Faulk, W., and W. Hijmans. 1972. Recent developments in immunofluorescence. *Prog. Allergy.* 16:9–39.
41. Ploem, J. S. 1967. The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. *Z. Wiss. Mikrosk. Mikrosk. Tech.* 68:129–142.
42. Ploem, J. S. 1971. A study of filters and light sources in immunofluorescence microscopy. *Ann. NY Acad. Sci.* 177:414–429.
43. Pollard, T. D., K. Fujiwara, R. Niederman, and P. Maupin-Szamier. 1976. Evidence for the role of cytoplasmic actin and myosin in cellular structure and motility. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 689–724.
44. Pruss, R. M., R. Mirsky, and M. C. Raff. 1981. All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell.* 27:419–428.
45. Pryor, W. A. 1966. Introduction. 1.5 Spectra. *In* Introduction to Free Radical Chemistry. K. L. Rinehart, Jr., editor. Prentice-Hall, Inc., Englewood, NJ. pp. 6–7.
46. Robinson, M. S., and D. F. Albertini. 1980. Immunofluorescence localization of clathrin in cultured rat ovarian granulosa cells: lack of effect of surface receptor occupancy. *Eur. J. Cell Biol.* 22:194*a*. Abstr.
47. Rothbarth, P. H., H. J. Tanke, N. A. J. Mul, J. S. Ploem, J. F. G. Vliegenthart, and R. E. Ballieux. 1978. Immunofluorescence studies with 4-acetamido-4′-isothiocyanato stilbene-2,2′-disulphonic acid (SITS). *J. Immunol. Methods.* 19:101–109.
48. Sanger, J. W. 1975. Presence of actin during chromosome movement. *Proc. Natl. Acad. Sci. USA.* 72:2451–2455.
49. Sato, H., Y. Ohnuki, and K. Fujiwara. 1976. Immunofluorescent anti-tubulin staining of spindle microtubules and critique for the technique. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 419–433.
50. Schaich, K. M. 1980. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation. Part II: Ultraviolet radiation and photolysis. *CRC Crit. Rev. Food Sci. Nutr.* 13:131–159.
51. Schloss, J. A., A. Milsted, and R. D. Goldman. 1977. Myosin subfragments binding for the localization of actin-like microfilaments in cultured cells: A light and electron

microscope study. *J. Cell Biol.* 74:794–815.
52. Schroeder, T. 1976. Actin in dividing cells: evidence for its role in cleavage but not mitosis. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 265–277.
53. Sillers, P. J., and A. Forer. 1981. Analysis of chromosome movements in crane fly spermatocytes by ultraviolet microbeam irradiation of individual chromosomal spindle fibers. II. Action spectra for stopping chromosome movement and for blocking ciliary beating and myofibril contraction. *Can. J. Biochem.* 59:777–792.
54. Snyder, J. A. 1981. Studies of mitotic events using lysed cell models. *In* Mitosis/ Cytokinesis. A. M. Zimmerman and A. Forer, editors. Academic Press, New York, NY. pp. 301–325.
55. Starger, J. M., W. E. Brown, A. E. Goldman, and R. D. Goldman. 1978. Biochemical and immunological analysis of rapidly purified 10nm filaments from baby hamster kidney (BHK-21) cells. *J. Cell Biol.* 78:93–109.
56. Stossel, T. P., and J. H. Hartwig. 1976. Phagocytosis and the Contractile Proteins of Pulmonary Macrophages. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 529–544.
57. Wagenmakers, A. J. M., R. J. Reinders, and W. J. Van Venrooij. 1980. Crosslinking of m-RNA to protein by irradiation of intact cells with ultraviolet light. *Eur. J. Biochem.* 112:323–330.
58. Wang, Y.-L., and D. L. Taylor. 1980. Preparation and characterization of a new molecular cytochemical probe: 5-iodoacetamidofluorescein labeled actin. *J. Histochem. Cytochem.* 28:1198–1206.
59. Weber, K., P. C. Rathke, and M. Osborn. 1978. Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells viewed by electron microscopy and by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA.* 75:1820–1824.
60. Wood, B. T., S. H. Thompson, and G. Goldstein. 1965. Fluorescent antibody staining. III. Preparation of fluorescein-isothiocyanate-labeled antibodies. *J. Immunol.* 95:225–229.
61. Wulf, E., A. Deboben, F. A. Bautz, H. Faulstich, and T. Wieland. 1979. Fluorescent phallotoxin, a tool for the visualization of cellular actin. *Proc. Natl. Acad. Sci. USA.* 76:4498–4502.