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IMPROVED METHODS FOR PRESERVING MACROMOLECULAR STRUCTURES AND VISUALIZING THEM BY FLUORESCENCE AND SCANNING ELECTRON MICROSCOPY

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Abstract

To determine the optimal procedures to preserve cytoskeletal and other macromolecular structures for microscopic studies we have evaluated the effects of various methods to extract cultured cells. In this report, we compare results using different fixatives, crosslinking reagents, and permeabilization methods on (1) the labeling of cells for fluorescence microscopy with phalloidin or antibody against tubulin; and (2) the morphological preservation of macromolecular structures for scanning electron microscopy. Maximal labeling of F-actin with phalloidin was obtained by fixing cells in 4% paraformaldehyde (PFA) and labeling the unextracted cells with methanolic phalloidin, whereas maximal labeling of tubulin required prefixation with either PFA or the bifunctional protein crosslinking reagent, dithio*bis* (succinimidylpropionate) (DSP) and extraction with ethanol or Triton in a high salt buffer. However, for both qualitative and quantitative light and electron microscopic studies of intracellular macromolecular structures, prefixation with DSP and extracting with Triton X-100 in a stabilizing buffer is the overall method of choice for both labeling and morphological studies. Although other methods provide maximal labeling or preservation of specific structures, this method provides excellent preservation of morphological structure while allowing proteins to be preserved and labeled by specific probes.

Key Words: Actin, tubulin, phalloidin, dithio*bis*(succinimidylpropionate), fluorescence, scanning electron microscopy, Triton X-100, crosslinking, immunolabeling, cytofluorometry.

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Introduction

Fluorescence light microscopy (FLM) and scanning electron microscopy (SEM) are two of the best methods available to the biologist who wishes to study the relationship between structure and function at the subcellular level. These microscopic methods are especially powerful when they are used in combination with methods that allow specific molecular components to be labeled. Many of the labeling methods also lend themselves to quantification, making it possible both to observe the distribution of specific molecules in cells and to measure their relative amounts. However, for such methods to yield results that are valid or even interpretable, it is important to understand the effects of the methods used to prepare the cells for observation. Otherwise, it is too easy to misinterpret experimental results and fail to recognize artifacts.

We have been engaged in an ongoing effort to optimize procedures for preserving cytoskeletal and other macromolecular structures for qualitative and quantitative microscopic studies. Our own methods of observation include high resolution scanning electron microscopy (Bell, 1981; Bell *et al.*, 1987c, 1988, 1989; Lindroth *et al.*, 1992) and both qualitative and quantitative fluorescence light microscopy (Bell *et al.*, 1987a, 1987b; Safiejko-Mroczka and Bell, 1995a). SEM and FLM provide complementary information about the distribution, organization and composition of subcellular structures, but they are subject to the generation of artifacts from the methods used to prepare the cells for microscopic observation. The methods of preparation are a particularly important source of variation in the results from labeling experiments. To label cells with membrane impermeant probes, such as antibodies or enzymes, the plasma membrane must be either permeabilized or solubilized to permit the probe to enter the cells and bind to their targets. Similarly, to visualize intracellular structures by SEM, the plasma membrane must be solubilized or fractured to permit intracellular structures to be visualized. Permeabilization, solubilization or fracturing of the cell membranes can lead, in turn, to

Table 1. Reagents and solutions.

Abbreviation	Reagent or Solution
BSA	Bovine serum albumin
BSA-TBS	1% BSA in Tris-buffered saline, containing 0.12% Na azide, pH 7.6
Cacodylate-sucrose buffer	0.1 M sodium cacodylate and 0.1 M sucrose, pH 7.4, 300 mOsm
CMEM	Complete MEM: MEM supplemented with 10% fetal calf serum (Gibco, Grand Island, NY)
DSP	Dithio*bis* (succinimidylpropionate), 1 mM: 20 mg/ml stock solution in DMSO, diluted 1:50 just before use (Pierce Chemicals, Rockford, IL)
EtOH	Ethanol, Absolute USP (AAPER, Shelbyville, KY)
Gelvatol	20 g Gelvatol (Monsanto, St. Louis, MO), 40 ml glycerol, 80 ml PBS-A, pH 7 (Sheehan and Hrapchak, 1980)
Glutaraldehyde, GA	25% glutaraldehyde, EM grade (Electron Microscopy Sciences, Ft. Washington, PA) diluted to the final concentration of 2.5% in cacodylate-sucrose buffer (310 mOsm)
Glycine-PBS-A	0.1 M glycine in PBS-A
Glycine-HBSS	0.1 M glycine in HBSS
HBSS	Hanks' balanced salt solution, pH 7.2
MEM	Minimal Essential Medium, Earle's salts (Hazelton, Lenexa, KS), supplemented with 2 mM freshly added glutamine, (JHR Biosciences, Lenexa, KS), MEM vitamins (JHR Biosciences, Lenexa, KS), and 50 μg/ml gentamycin (Sigma)
MeOH	Methanol, Absolute, Acetone-free (Fisher Scientific, Fair Lawn, NJ)
MTSB	Microtubule stabilizing buffer: 1 mM EGTA, 4 % polyethylene glycol 8000, 100 mM PIPES, pH 6.9
Neomycin	Neomycin sulfate, 3.5 mM in MEM
OsO_4	1% osmium tetroxide in cacodylate-sucrose buffer
PFA	4% paraformaldehyde: 20% aqueous stock solution diluted to 4 % in either HBSS or MTSB before use
PBS-A	Phosphate-buffered saline, calcium- and magnesium-free: 137 mM NaCl, 3 mM KCl, 0.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4
Phalloidin	TRITC-labeled phalloidin: 10 μM stock solution in methanol, diluted 1:5 in PBS-A or HBSS before use producing a 2 μM working solution containing 20% methanol
TBS	Tris-buffered saline: 150 mM NaCl, 0.12% Na azide, 20 mM Tris, pH 7.6
Thbss	0.5 % Triton X-100 detergent in HBSS
Tsb	0.5 % Triton X-100 detergent in MTSB

the loss of material from the cells and significantly alter the distribution and relative amounts of different subcellular components.

We have previously evaluated the effects of various methods to extract cultured cells in order to observe intracellular structures by SEM and to label them with antibodies and other fluorescent probes for FLM (Bell, 1981; Bell, *et al.*, 1978, 1987b, 1988, 1989; Lindroth *et al.*, 1992; Safiejko-Mroczka and Bell, 1995a). The general goals of our efforts to prepare biological specimens for microscopic studies are two-fold:

(1) to stabilize the morphological and macromolecular structure of the cytoskeleton and associated structures so that they can be visualized by FLM and

Table 2. Methods used to prepare cells.

Abbreviation	Description of the Method
MeOH	PBS-A, rapid rinse; methanol, -20°C, 5 minutes; PBS-A, 5 minutes.
PFA	PFA in HBSS, 15 minutes, 37°C; HBSS, 5 minutes; glycine-HBSS, 5 minutes; PBS-A, 5 minutes.
PFA-Acetone	PFA in HBSS, 15 minutes, 37°C; HBSS, 5 minutes; glycine-HBSS, 5 minutes; PBS-A, 5 minutes; acetone, -20°C, 5 minutes; PBS-A, 5 minutes.
PFA-EtOH	PFA in HBSS, 15 minutes, 37°C; HBSS, 5 minutes; glycine-HBSS, 5 minutes; PBS-A, 5 minutes; 70% ethanol, 5 minutes; PBS-A, 5 minutes.
PFA-Tsb	PFA in MTSB, 15 minutes, 37°C; MTSB, 5 minutes; Tsb 10 minutes, 37°C; Tsb 5 minutes, 37°C; PBS-A, 5 minutes; glycine-PBS-A, 5 minutes; PBS-A, 5 minutes.
PFA-Thbss	PFA in HBSS, 15 minutes, 37°C; HBSS, 5 minutes; Thbss, 10 minutes, 37°C; Thbss, 5 minutes, 37°C; glycine-HBSS, 5 minutes; PBS-A, 5 minutes.
Tsb	Tsb, 10 minutes, 37°C; Tsb, 5 minutes, 37°C; PFA in MTSB, 15 minutes, 37°C; PBS-A, 5 minutes; glycine-PBS-A, 5 minutes; PBS-A, 5 minutes.
Thbss	Thbss, 10 minutes, 37°C; Thbss, 5 minutes, 37°C; PFA in HBSS, 15 minutes, 37°C; HBSS, 5 minutes; glycine-HBSS, 5 minutes; PBS-A, 5 minutes.
DSP-Tsb	1 mM freshly prepared DSP in HBSS, 10 minutes, 37°C ; 1 mM DSP in Tsb, 10 minutes at 37°C; Tsb, 5 minutes at 37°C; PFA in MTSB, 15 minutes, 37°C; PBS-A, 5 minutes; glycine-PBS-A, 5 minutes; PBS-A, 5 minutes.
DSP-Thbss	1 mM freshly prepared DSP in HBSS, 10 minutes, 37°C ; 1 mM DSP in Thbss, 10 minutes at 37°C; Thbss, 5 minutes at 37°C; PFA in HBSS, 15 minutes, 37°C; HBSS, 5 minutes; glycine-HBSS, 5 minutes; PBS-A, 5 minutes.

high resolution SEM in as close to their native organization as possible; and

(2) to optimize the labeling of specific molecular components for qualitative and quantitative studies.

To achieve these goals, the following conditions must be met:

(1) The structures of interest must be stabilized against extraction, loss, and rearrangement during the various processing steps employed as well as under the rigorous conditions of vacuum, dryness, and high energy radiation encountered inside the electron microscope. The ideal would be to preserve the structures quantitatively and in as close to their native form as possible.

(2) The methods used should not create artifactual structures.

(3) The structures of interest must be rendered visible in the microscope. For FLM, this requires labeling of macromolecular structures with fluorescent labels attached to specific probes. For SEM, this requires the application of a metal coating to serve as a source of secondary electrons in the secondary electron mode or to provide scattering contrast in the scanning transmission mode. Newer technologies, such as scanning tunneling, atomic force, and X-ray microscopy may offer the possibility of visualizing structures without coating.

(4) For quantitative studies, the labeling probe(s) must have equal probability of binding to all possible binding sites. Differential access to or differential binding to different populations of target molecules will cause the labeling not to be proportional to the concentration of the target molecules and lead to inaccurate interpretation of the labeling results.

In this report, we present the results of new experiments to evaluate the effects of different fixatives, crosslinking reagents, and permeabilization methods on: (1) the labeling of cells for FLM with either phalloidin conjugated to rhodamine, a low molecular weight (1.3 kD) probe that binds specifically to filamentous actin (F-actin), or antibodies directed against tubulin; and (2) the morphological preservation of macromolecular structures for scanning electron microscopy.

Materials and Methods

Reagents and abbreviations

The reagents (from Sigma Chemicals, St. Louis, MO, unless otherwise indicated) and solutions used are listed in Table 1. They will be referred to subsequently by the abbreviations indicated in Table 1.

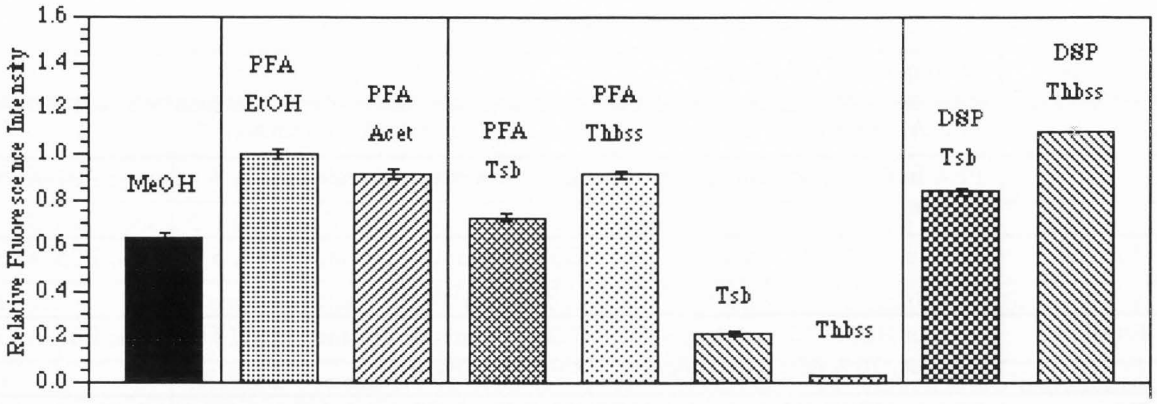


Figure 1. Immunolabeling of tubulin. Histogram showing the fluorescence intensity of glioma cells immunolabeled for tubulin after preparation using nine different procedures. Measurements are the mean for 100 cells, calculated relative to the fluorescence intensity of cells prepared with the PFA-EtOH procedure. Bars are standard error of the mean. For an explanation of the abbreviations, see **Materials and Methods.**

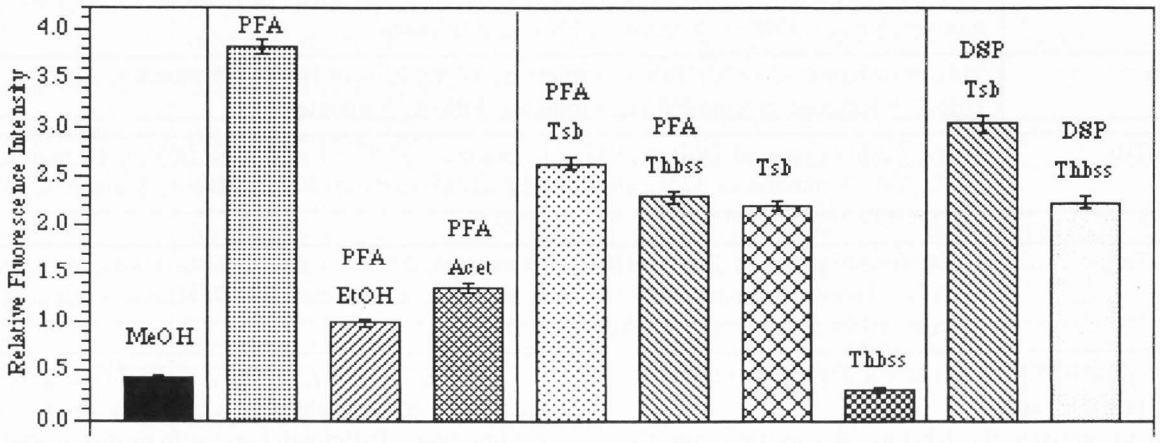


Figure 2. Phalloidin-labeling of F-actin. Histogram showing the fluorescence intensity of glioma cells labeled for F-actin with TRITC-phalloidin after preparation using ten different procedures. Measurements are the mean for 100 cells, calculated relative to the fluorescence intensity of cells prepared with the PFA-EtOH procedure. Bars are standard error of the mean. For an explanation of the abbreviations, see **Materials and Methods.**

Cells and cell culture

Human foreskin fibroblasts, AG-1523 (Human Mutant Cell Repository, Institute for Medical Research, Camden, NJ) and human malignant glioma cells, U-251 MG (Pontén, 1975; Collins, 1983) were grown in CMEM in tissue culture dishes (Corning, Corning, NY) in incubators containing 5% CO_2 in humid air at 37°C. Cells were removed for subculturing with 0.25% trypsin - 2 mM ethylenediaminetetraacetic acid (EDTA). For experimental studies, 2 x 10^4 cells in 3 ml of CMEM were seeded into 6-well plates (Corning, Corning, NY) containing two 18 mm round coverglasses. After culturing overnight to allow the cells to attach and spread, the coverglasses were rinsed with HBSS and processed as described in the following sections. All processing steps took place in the well of the 6-well plate.

Fixation and extraction procedures

Cells on coverglasses were prepared for labeling by one of the procedures listed in Table 2.

Neomycin treatment

Cells growing on coverglasses were incubated in 3.5 mM neomycin in MEM for 10 minutes at 37°C prior to extraction or fixation.

Fluorescent labeling of cells

F-actin was labeled with phalloidin conjugated to rhodamine (TRITC). Except for the 20% methanol present in the working solution, PFA-fixed intact cells were not permeabilized prior to labeling. This procedures provided a very high efficiency of labeling. Cells were incubated in 2 μM rhodamine-phalloidin in PBS-A con-

taining 20% methanol, 20 minutes, room temperature, in a moist chamber in the dark; rinsed twice in PBS-A; and mounted on a glass slide in Gelvatol.

Tubulin was labeled by indirect immunofluorescence as follows. Permeabilized or Triton-extracted, PFA-fixed cells were incubated in BSA-TBS, 5 minutes; incubated with anti-tubulin (murine monoclonal IgM, Tp-TUB1, a gift from V. Peter Collins, Stockholm, Sweden) in BSA-TBS, 45 minutes, 37°C in a moist chamber; rinsed 3 times in BSA-TBS; incubated with TRITC-labeled goat anti-mouse IgM (Calbiochem, La Jolla, CA), 45 minutes, 37°C in a moist chamber; rinsed twice in BSA-TBS; rinsed in PBS; and mounted on a glass slide in Gelvatol.

Cytofluorometry

U-251 cells were used for cytofluorometry because their compact shape allowed them to fit readily into the circular measuring window of the cytofluorometer. The fluorescence intensity of individual labeled cells was measured with a cytofluorometer (Bell *et al.*, 1987b) consisting of a Zeiss Universal microscope equipped with an 100 Watt mercury epi-illuminator, a photometer, and both hardware (Zeiss) and software (Zeiss Manual Photometer Program) for measuring fluorescence. The measurements were made using a 40x oil immersion objective lens (Olympus DApo 40 UV). Fluorescence was measured by using the mechanical stage to move the optical image of a cell into a circular measuring window reflected onto the image plane of the ocular and then triggering the acquisition of a signal by the photometer by pressing the space bar on the computer keyboard next to the microscope. In each experiment, fluorescence was measured relative to control cells, and the average fluorescence and standard error of the experimental samples were calculated as a proportion of the control. Usually, 100 cells were measured in each sample and the mean and standard error calculated.

Photography

For FLM, cells were examined using a Zeiss Universal epifluorescence microscope equipped with Olympus oil immersion DApo UV objectives. Images were recorded on Kodak T-MAX 400 film at 400 ASA. Exposure of the film was controlled by an integrating photometer, which adjusted exposure time automatically to create a properly exposed negative. Therefore, the photographic images are not an accurate representation of relative brightness of the cells, in that darker cells appear relatively brighter than they actually are because of longer exposure times.

Scanning electron microscopy

After the different fixation and extraction procedures described previously, cells on coverglasses were fixed in 2.5% glutaraldehyde in cacodylate-sucrose buffer, room temperature, 15 minutes; stored up to one week at 4°C until further processing. Intact and solvent-extracted cells were rinsed twice in the same buffer; post-fixed in 1% OsO_4 in cacodylate-sucrose buffer, 4°C, 30 minutes; rinsed twice in the same buffer; rinsed once in distilled water; dehydrated in acetone of increasing concentration up to 100%; critical point dried from liquid CO_2 (Bell, 1981, 1984) and coated with 5 nm tungsten in a magnetron sputter coater (Lindroth *et al.*, 1992). Detergent-extracted samples were prepared in the same way, excluding the OsO_4 post-fixation step. Samples were examined in a JSM-840 scanning electron microscope (JEOL) operated at an accelerating voltage of 20 kV.

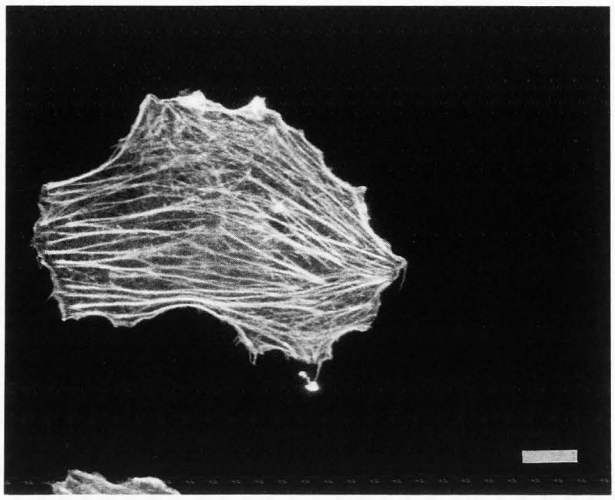
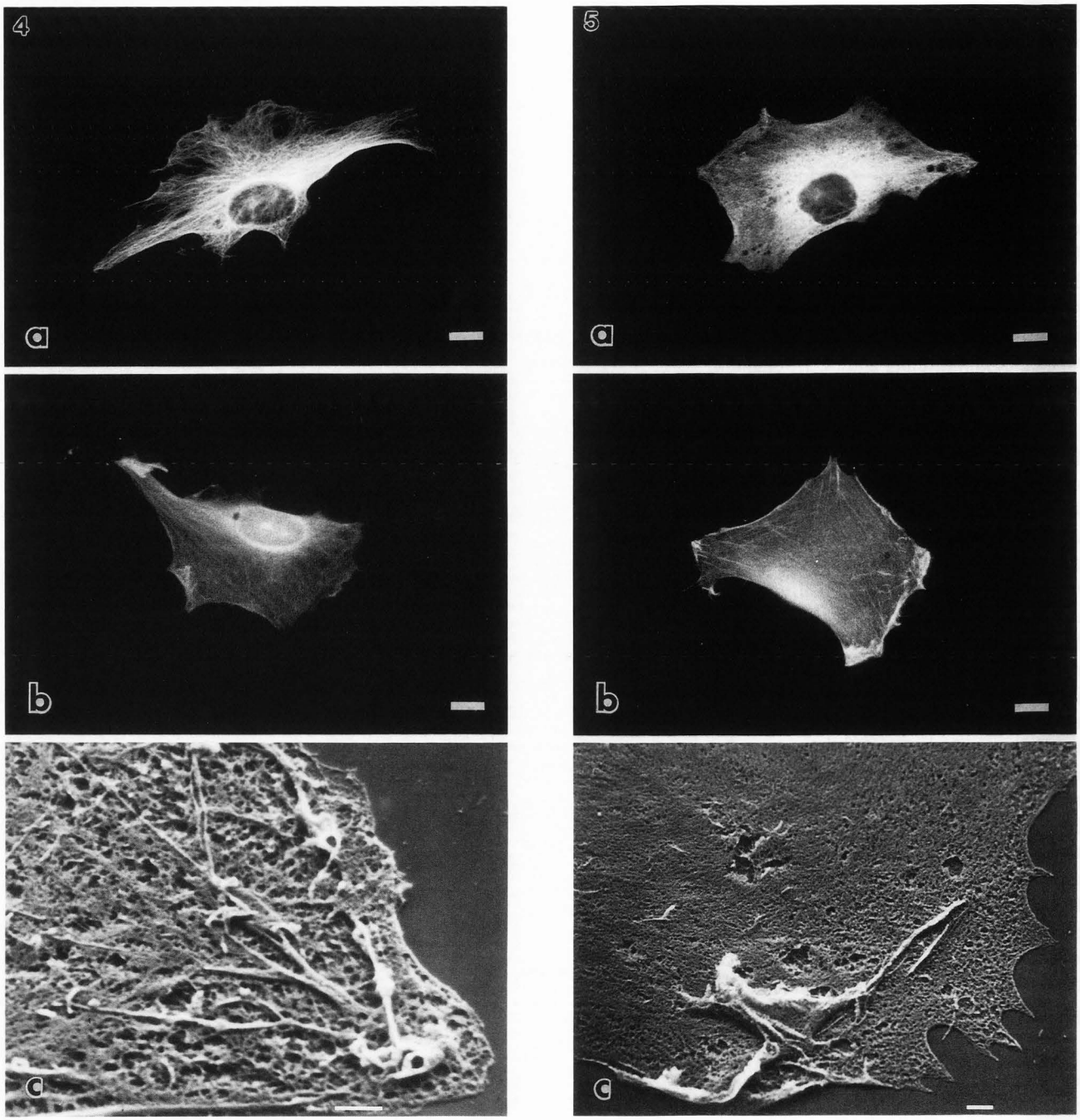


Figure 3. PFA. Fluorescence light micrograph (FLM) of a glioma cell fixed in PFA and labeled with TRITC-phalloidin. F-actin is concentrated at the cell periphery and in bundles (stress fibers) that stand out against the diffusely labeled background. Bar = 10 μm.

Results

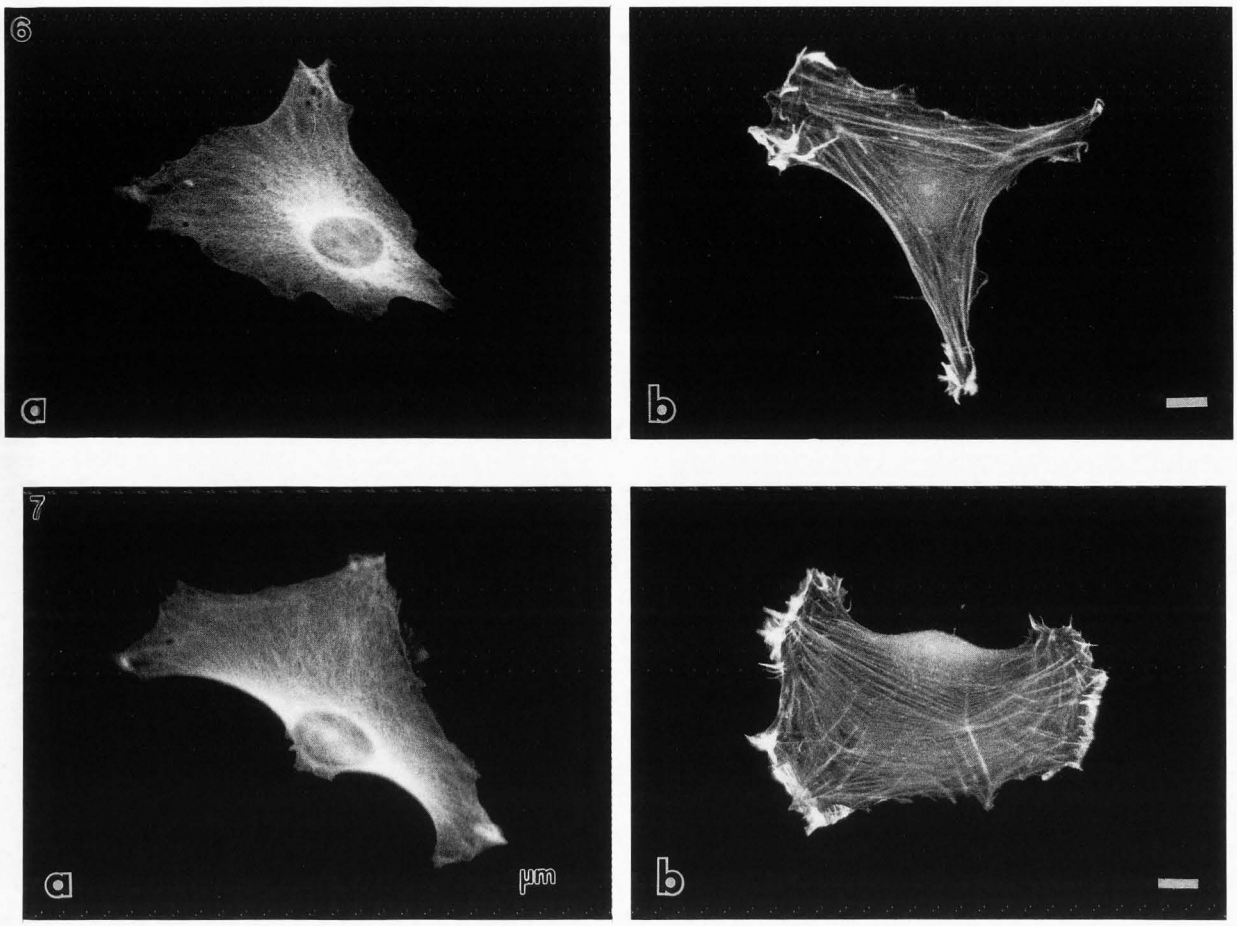
Comparison of different extraction methods on fluorescence labeling

Using human glioma cells, we have compared the effects of different permeabilization and extraction procedures on the fluorescent labeling of tubulin and F-actin and on the preservation of cytoskeletal structures for SEM. Figures 1 and 2 show measurements of the relative fluorescence intensity of labeling for tubulin and F-actin, respectively, in cells prepared by different procedures. Figures 3 through 14 show fluorescent and scanning micrographs of cells prepared using the same methods. In the following sub-sections, the effects of each method on labeling and preservation will be discussed and compared.



Figures 4 (MeOH) and 5. (PFA-EtOH). Fluorescence and scanning electron micrographs of: glioma cells extracted with methanol (**Fig. 4**), and glioma cells fixed in PFA and permeabilized with EtOH (**Fig. 5**): (**4a** and **5a**) immunolabeled for tubulin (bar = 10 μm); (**4b** and **5b**) labeled with phalloidin (bar = 10 μm); (**4c** and **5c**) SEM (bar = 1 μm).

PFA. Fixing cells in PFA without a separate permeabilization step provides the maximum fluorescence labeling for F-actin with phalloidin (Fig. 2) and gives excellent morphological preservation and labeling of F-actin-containing structures (Fig. 3). The presence of 20% methanol in the phalloidin working solution provides sufficient permeabilization to permit the phalloidin to enter the cells without extracting much, if any, of the actin filaments from the cell. Non-methanolic phalloidin also penetrates the membrane of PFA-fixed cells, but the intensity of staining is lower than with methanolic phalloidin (Safiejko-Mroczka and Bell, 1995a).

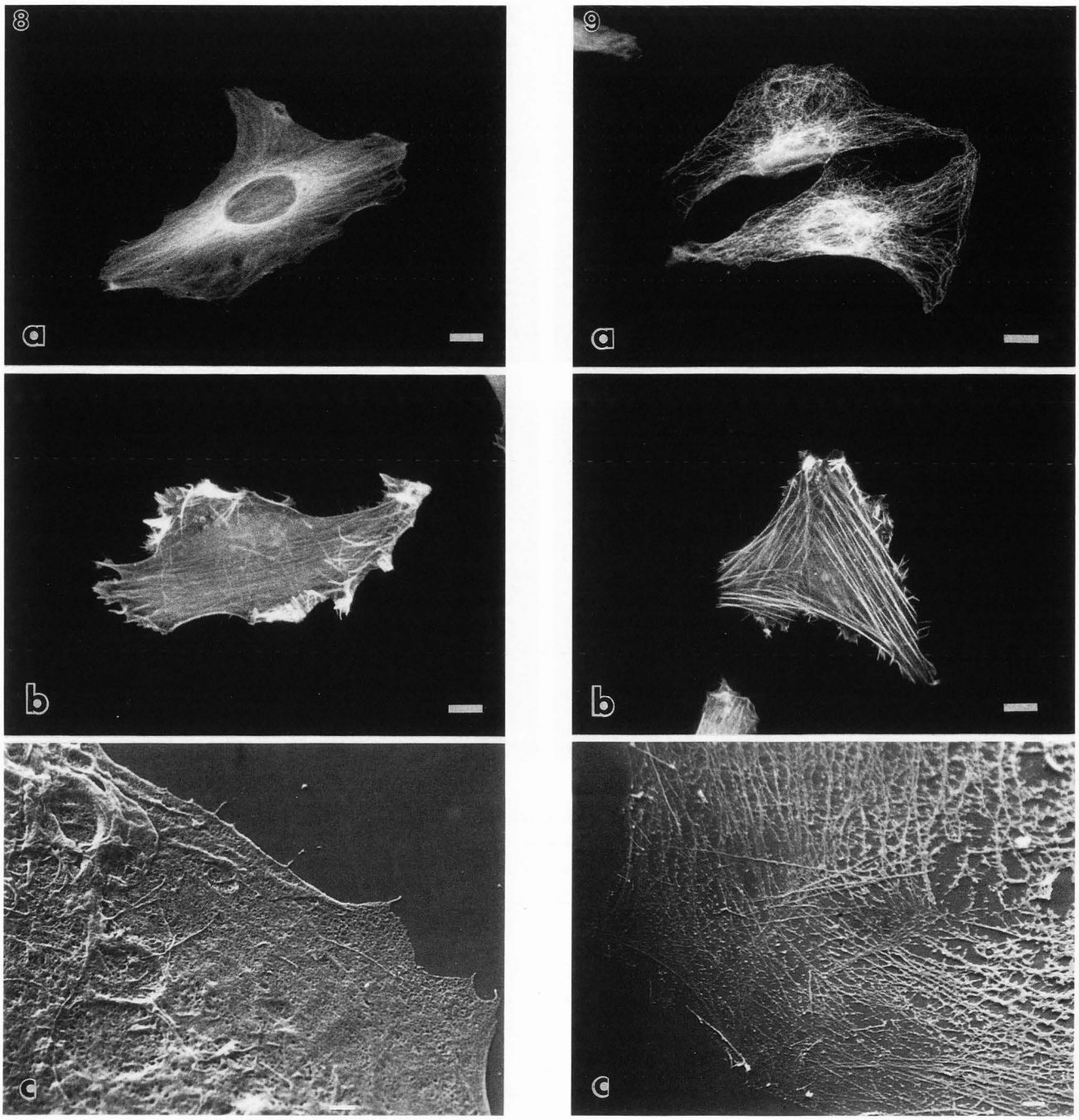


Figures 6 (PFA-Acetone) and 7 (PFA-Tsb). Fluorescence micrographs of glioma cells: fixed in PFA and permeabilized with acetone (**Fig. 6**), and glioma cells fixed in PFA and extracted with Tsb (**Fig. 7**): (**6a** and **7a**) immunolabeled for tubulin (bar = 10 μm); (**6b** and **7b**) labeled with phalloidin (bar = 10 μm).

This procedure is unsuitable for labeling with tubulin, because the antibodies do not penetrate the intact plasma membrane. Although this method is suitable for viewing intact cells in SEM, if followed by GA and osmium-fixation, it does not allow visualization of internal cell structures.

MeOH. Although, simultaneous fixation and extraction in methanol is a commonly used procedure for preparing cells for immunolabeling of tubulin (Füchtbauer *et al.*, 1985; Gurland and Gundersen, 1993; Kapeller *et al.*, 1993; Lieuvin *et al.*, 1994)) and other proteins, the intensity of labeling is significantly less than that provided by other methods tested (Fig. 1). Moreover, microtubules are not as clearly delineated in FLM (Fig. 4a) as with other methods. Methanol also provides poor quantitative labeling of F-actin (Fig. 2) and actin-containing structures are not well labeled (Fig. 4b). Actin filaments may not be well preserved by this procedure, and/or they may have become coated with other denatured proteins that block access of the label. In SEM, the membrane appears to be heavily extracted but a lot of material remains that covers the underlying cytoskeleton (Fig. 4c). This suggests that methanol extracts the lipid portion of the plasma membrane, leaving denatured membrane proteins behind. These observations support the conclusion that a denaturing fixative, such as methanol, provides suboptimal preservation and visualization of macromolecular structures.

PFA-EtOH. Fixation in PFA followed by extraction in EtOH is one of the best procedures for quantitative immunolabeling of tubulin (Fig. 1; Bell *et al.*, 1987b). FLM shows extensive diffuse labeling of cells (Fig. 5a), probably owing to labeling of unpolymerized tubulin, which partially masks the network of microtubules seen with other methods. This method provides poor quantitative labeling of F-actin (Fig. 2) and poor visualization of F-actin-containing structures (Fig. 5b). In SEM, cells are covered with an extensive meshwork



Figures 8 (PFA-Thbss) and 9 (Tsb). Fluorescence and scanning electron micrographs of: glioma cells fixed in PFA and extracted with Thbss (**Fig. 8**) and glioma cells extracted with Tsb prior to fixation (**Fig. 9**): (**8a** and **9a**) immunolabeled for tubulin (bar = 10 μm); (**8b** and **9b**) labeled with phalloidin (bar = 10 μm); (**8c** and **9c**) SEM (bar = 1 μm).

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that masks the underlying cytoskeleton and which may be remnants of the non-lipid components of the plasma membrane (Fig. 5c), making this a poor method for visualizing the cytoskeleton.

PFA-Acetone. Cells fixed in PFA and permeabilized with acetone are similar to PFA-EtOH cells. The intensity of tubulin labeling is slightly, but not significantly, lower (Fig. 1) than with PFA-EtOH. Tubulin

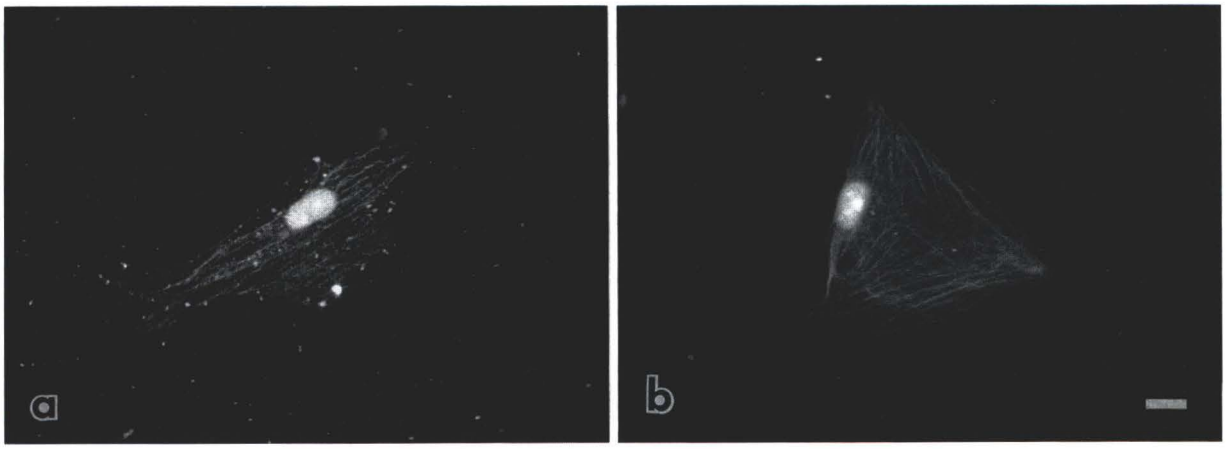


Figure 10. Thbss. Fluorescence micrographs of glioma cells extracted with Thbss prior to fixation: (a) immunolabeled for tubulin (bar = 10 μm); (b) labeled with phalloidin (bar = 10 μm).

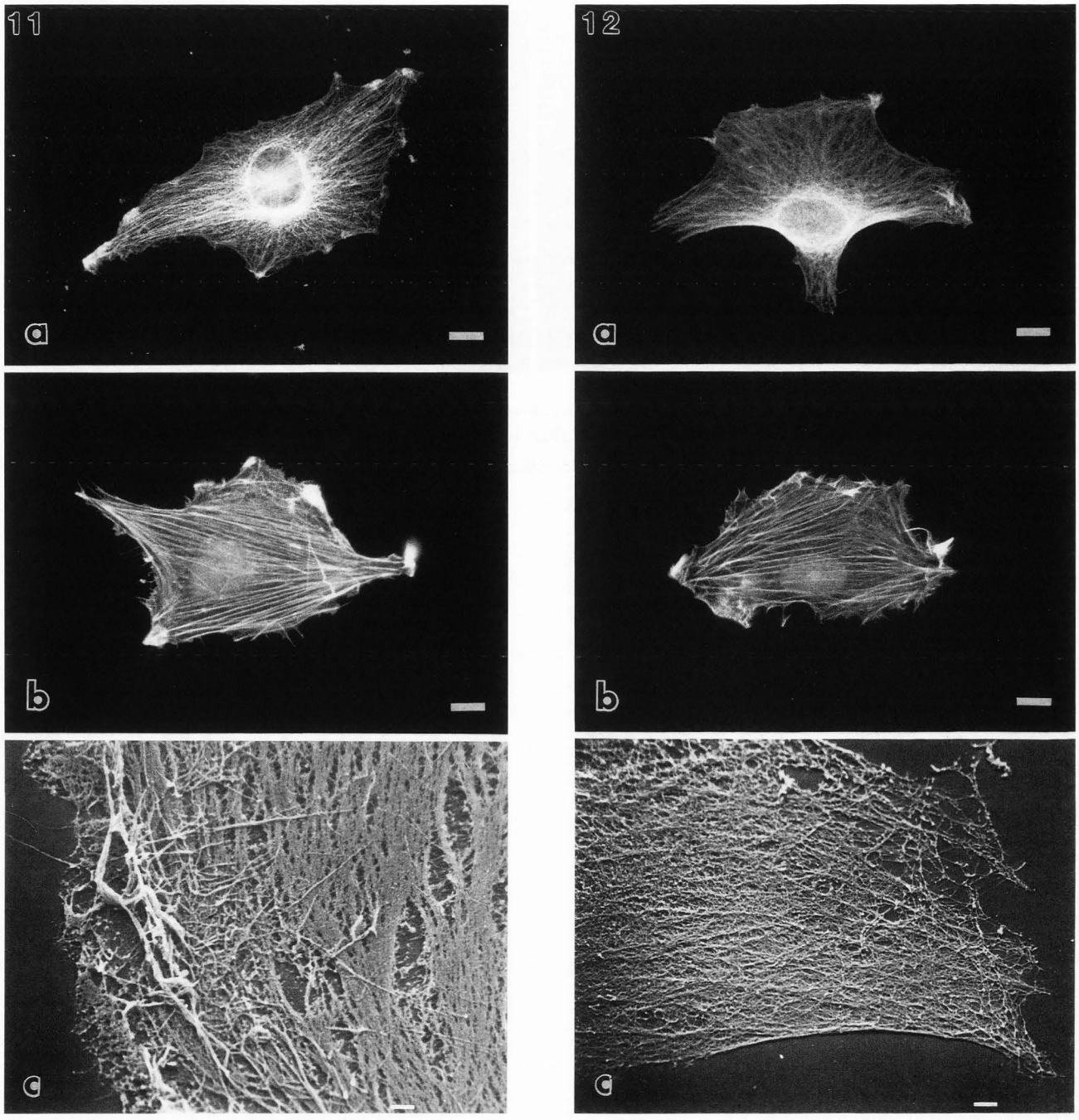
staining is diffuse and the microtubule network is not well visualized (Fig. 6a), similar to PFA-EtOH. In contrast, the intensity of actin labeling is higher than with PFA-EtOH, but still substantially lower than with PFA alone (Fig. 2). F-actin-containing structures are better labeled than in PFA-EtOH (Fig. 6b), but not as well as with some other methods. SEM (not shown) is also similar to PFA-EtOH.

PFA-Tsb. Fixation with PFA followed by extraction with Tsb gives lower intensity of tubulin labeling than seen in PFA-fixed cells permeabilized with EtOH or acetone (Fig. 1). Tubulin staining is diffuse and very similar to that seen with PFA-Acetone (Fig. 7a). In contrast, PFA-Tsb gives substantially better intensity of labeling of F-actin over that obtained with solvent-permeabilized cells (Fig. 2). Overall, this method ranks third (after PFA alone and DSP-Tsb) in the intensity of phalloidin-labeling. This method also provides good visualization of F-actin-containing structures (Fig. 7b).

PFA-Thbss. The intensity of immunolabeling of tubulin in cells fixed in PFA and extracted in Thbss is higher than in PFA-Tsb and equal to PFA-Acetone (Fig. 1). The pattern of tubulin labeling is also similar to that seen in cells fixed with PFA and permeabilized with acetone. Microtubules can be seen, but they are masked by diffuse cytoplasmic staining, probably due to labeling of unpolymerized tubulin (Fig. 8a). The intensity of F-actin labeling is slightly lower that with PFA-Tsb (Fig. 2), and, although F-actin-containing structures are intensely labeled, stress fibers are masked by diffuse cytoplasmic staining (Fig. 8b). In SEM, the cytoskeleton is masked by covering material, similar to that seen in solvent-permeabilized PFA-fixed cells (Fig. 8c).

Tsb. Extraction of cells in Triton in stabilizing buffer gives a very low intensity of tubulin labeling (Fig. 1). However, individual microtubules are more clearly seen because there is a significant loss of cytoplasmic fluorescence, probably due to the extraction of unpolymerized tubulin (Fig. 9a). There appear to be fewer microtubules labeled than with the better methods and the pattern of microtubules is more wavy than normally seen, indicating that the native structure is not being preserved. The intensity of F-actin labeling is moderately high (Fig. 2) (higher than with either PFA-Acetone or PFA-EtOH). Stress fibers and other F-actin-containing structures are labeled with phalloidin (Fig. 9b), and look similar to those in cells fixed in PFA and not extracted. This is one of the best methods for visualizing F-actin by FLM. SEM of Tsb-extracted cells shows a well-exposed cytoskeleton consisting of a loose network of filaments and filament bundles of various sizes with large spaces in between (Fig. 9c).

Thbss. Extraction with Triton under non-stabilizing conditions causes a loss of most of the tubulin and F-actin from the cells. This method gives the lowest immunolabeling of tubulin and F-actin among all those tested in this work (Figs. 1 and 2). Labeling of microtubules (Fig. 10a) as well as of F-actin-containing stress fibers (Fig. 10b) is very weak. The cell nuclei are labeled with both anti-tubulin and phalloidin (Figs. 10a and 10b), consistent with previous reports that both tubulin (Sharma *et al.*, 1992) and actin (Verheijen *et al.*, 1986; Fukuda *et al.*, 1987; Parfenov and Galaktionov, 1987; Valkov *et al.*, 1989) are associated with the nuclear matrix. During this procedure, a high percentage of the cells detach from the substratum and are lost from the preparations. The overall morphology of the few remaining cells is poorly preserved, making this method unsuitable for SEM.



Figures 11 (DSP-Tsb) and 12 (DSP-Thbss). Fluorescence and scanning electron micrographs of glioma cells crosslinked with DSP and: extracted with Tsb (**Fig. 11**) or extracted with Thbss (**Fig. 12**): (**11a** and **12a**) immunolabeled for tubulin (bar = 10 μm); (**11b** or **12b**) labeled with phalloidin (bar = 10 μm); (**11c** or **11d**) SEM (bar = 1 μm).

DSP-Tsb. Cells prefixed with the homobifunctional protein crosslinking reagent DSP (Lomant and Fairbanks, 1976; Staros, 1982, 1988) before extraction and extracted in Tsb in the presence of DSP are very well preserved for both qualitative and morphological studies. Tubulin labeling is lower than in PFA-EtOH (Fig. 1) but the microtubule network is clearly visualized and one can trace individual microtubules. There is also some diffuse cytoplasmic staining indicating that some unpolymerized tubulin is also preserved and labeled (Fig. 11a). Phalloidin labeling is very high (Fig. 2) (second after PFA alone). This method also provides excellent

morphological preservation of stress fibers and F-actin containing structures (Fig. 11b). SEM of DSP crosslinked and Tsb extracted cells shows the cytoskeleton as a dense network of individual filaments and bundles with non-filamentous material bound to them (Fig. 11c).

DSP-Thbss. Crosslinking of cells with DSP and extraction in Thbss containing DSP provides the highest intensity of immunolabeling of tubulin of all of the methods tested (Fig. 1). Tubulin staining is diffuse and the microtubule pattern is not as clearly visualized as in DSP-Tsb (Fig. 12a). The intensity of labeling of F-actin with this method is lower than in DSP-Tsb (Fig. 2), but the staining of F-actin-containing structures is still excellent (Fig. 12b) and similar to these seen in cells prepared by DSP-Tsb. The cytoskeleton as seen in SEM is denser that in Tsb alone but the morphology is less well preserved than with DSP-Tsb (Fig. 12c).

Labeling and morphological preservation of tubulin at the edges of neomycin-induced lamellipodia: FLM and SEM

The sensitivity of fluorescent labeling and morphological preservation is well illustrated by our efforts to label and visualize the cytoskeleton as part of our experiments to study the mechanism of cell motility. Fibroblasts treated with 3.5 mM neomycin are rapidly stimulated to protrude new lamellae from the cell edge (Hedberg *et al.*, 1993). The leading edge of the new lamellae is characterized by the presence of a band of cytoplasmic material that labels brightly with TRITC-phalloidin in unextracted cells, indicating the presence of F-actin. However, our results show that the nature of the labeling and the degree of preservation of this marginal band depends upon the method used to prepare the cells.

Figure 13 shows the degree to which the immunolabeling of tubulin in the marginal band of 1523 human fibroblasts is sensitive to the extraction procedures. In cells prepared by PFA-EtOH the marginal band labels with anti-tubulin (Fig. 13a). Microtubules are also well labeled, but they are partially masked by cytoplasmic labeling, which is apparently due to unpolymerized tubulin. If, instead, the cells are extracted in Tsb alone (Fig. 13b), the marginal band is not labeled with anti-tubulin, although microtubules are clearly labeled throughout the rest of the cell. If the cells are first crosslinked with DSP and then extracted with Tsb containing DSP, tubulin labeling of the marginal band is preserved along with tubulin-labeling of the microtubules (Fig. 13c). Interestingly, there is little diffuse cytoplasmic labeling, indicating either that the unpolymerized tubulin is lost from the cells or that it is not accessible to the antibodies. Therefore, preservation of tubulin in the marginal band depends on crosslinking prior to detergent extraction.

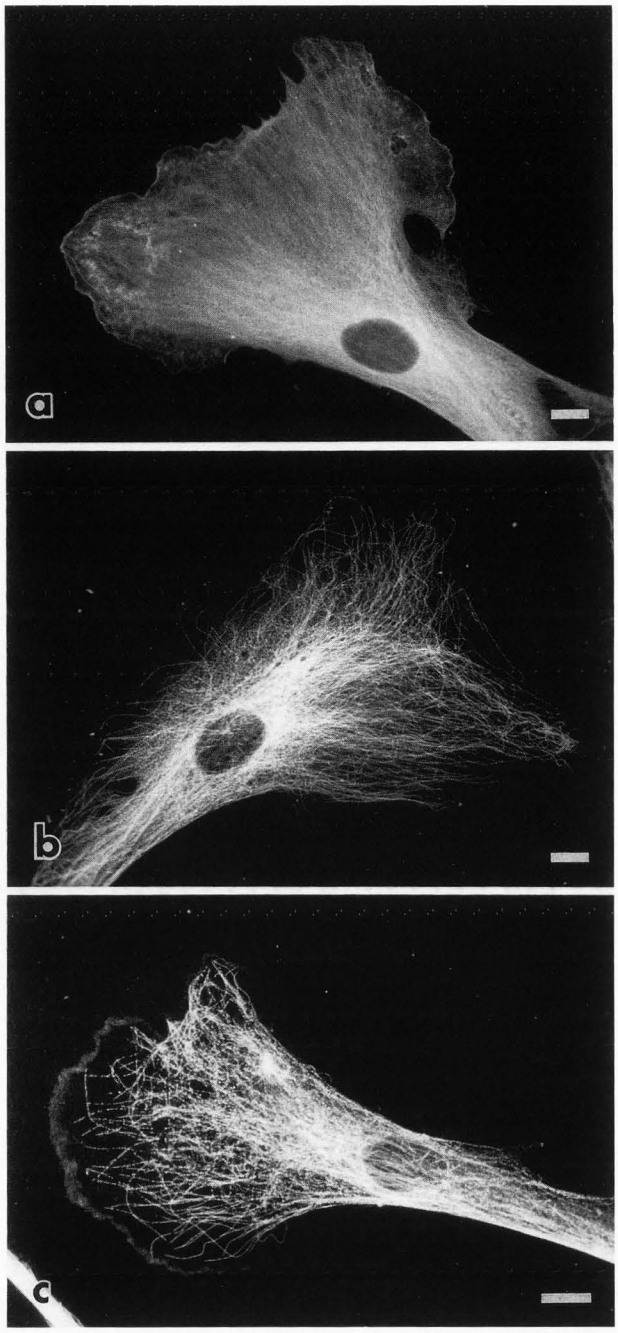


Figure 13. Preservation of tubulin. Fluorescence micrographs of human fibroblasts incubated in neomycin and immunolabeled for tubulin after one of the following three preparative procedures: (a) PFA-EtOH, (b) Tsb, and (c) DSP-Tsb. Bar = 10 μm.

Morphological preservation of the marginal band in detergent-extracted cells (Fig. 14) parallels the results with immunolabeling. After extraction with Tsb alone (Figs. 14a and 14b), the marginal band as visualized by

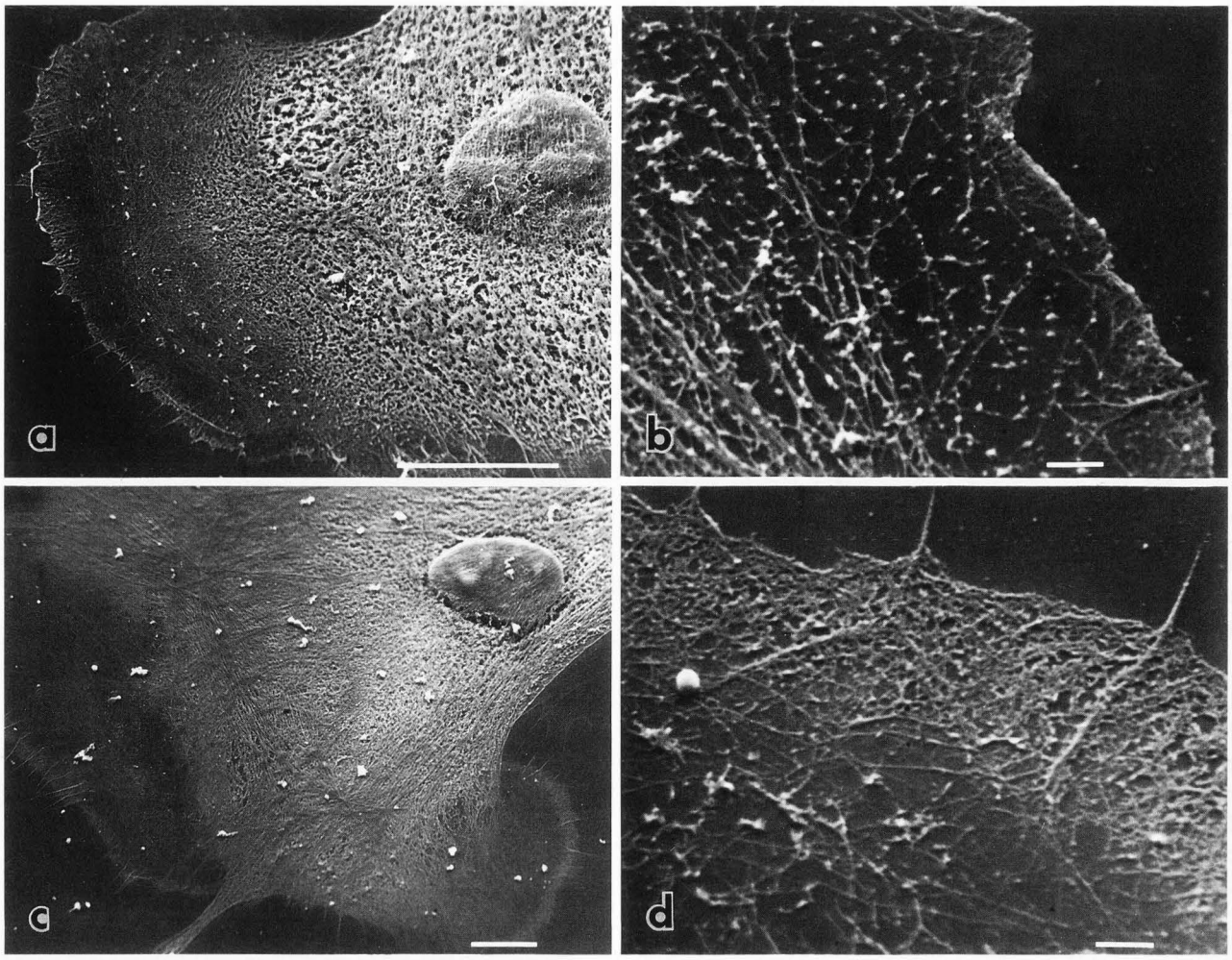


Figure 14. Preservation of actin cytoskeleton. Scanning electron micrographs of human glioma cells incubated in neomycin and extracted using one of the following preparative procedures: (a) and (b) Tsb, and (c) and (d) DSP-Tsb. Bars = 10 μm (a and c) or 1 μm (b and d).

SEM is heavily extracted. Microspikes, which label with phalloidin and are composed of "naturally" crosslinked actin filaments, are preserved, but the marginal band is otherwise sparse and relatively narrow. In contrast, the marginal band material is extensively preserved in cells crosslinked with DSP prior to Tsb-extraction (Figs. 14c and 14d). In these cells, the band is relatively broad and composed of a dense meshwork of filamentous material. Clearly, the crosslinking of actin filaments, whether the natural result of interactions with actin binding proteins or introduced by chemical crosslinkers, increase the preservation of actin filaments in detergent-extracted cells.

Discussion

Our results (summarized in Table 3) are consistent with previous conclusions that the preservation and labeling of cytoskeletal proteins is highly dependent on the methods used to prepare the cells for observation. However, the current results also show that the choice of methods is constrained by various considerations, including the method that will be used to visualize the cells and the nature of any probe that will be used to label specific structures and molecules of interest. For example, to visualize the cytoskeleton by SEM, the interior of the cell must be exposed, either by solubilizing the plasma membrane with detergent or by physically opening the cells with a method such as freeze-fracture. In addition, to allow large probes, such as antibodies to enter cells, the plasma membrane must be permeabilized or removed. In turn, the procedures used to permeabilize, solubilize, or fracture the cell membrane may lead to the loss or rearrangement of molecular and structural

Table 3. Comparison of the effects of different methods of preparation, using DSP as the crosslinker, on the fluorescence intensity, pattern of fluorescent labeling and morphological preservation of actin filaments and tubulin. The range is from excellent (+++++) to very poor (0). Treatments not done indicated by "nd".

	LABEL				
	Antitubulin		Phalloidin		SEM
METHOD	Fluorescence	Pattern	Fluorescence	Pattern	Pattern
MeOH	+++	+++	+	+	0
PFA	nd	nd	+++++	+++++	nd
PFA-Acet	++++	++++	++	+++	nd
PFA-EtOH	+++++	++++	++	++	0
PFA-Tsb	+++	+++	++++	++++	nd
PFA-Thbss	++++	++++	+++	++++	0
Tsb	+	+++	+++	++++	+++
Thbss	0	0	+	0	nd
DSP-Tsb	++++	+++++	++++	++++	+++++
DSP-Thbss	+++++	+++++	+++	++++	++++

components. Therefore, the choice of an optimum method for preparing cells is a compromise between the conflicting goals of maximizing preservation and maximizing labeling and visualization.

Labeling F-actin with phalloidin

If the goal is to maximize the fluorescent labeling of F-actin with phalloidin, our results show that the best method is simply to fix them in PFA. TRITC-phalloidin is able to penetrate the PFA-fixed plasma membrane without an additional permeabilizing procedure (Safiejko-Mroczka and Bell, 1995a). Permeabilizing with absolute acetone, 70% EtOH, or Triton X-100, after fixation in PFA, actually cause a decrease in the intensity of fluorescent labeling with phalloidin, indicating that some F-actin is extracted along with the plasma membrane. However, although fixing and extracting in absolute methanol alone is a poor method for preserving F-actin, methanolic phalloidin (containing 20% MeOH) stains cells more intensely than aqueous phalloidin (Safiejko-Mroczka and Bell, 1995a). This may be because the concentration of methanol is high enough to facilitate the penetration of the phalloidin into the PFA-fixed cell, but sufficiently low that little F-actin is extracted. In addition, the simultaneous presence of phalloidin during the methanol permeabilization may help to stabilize the F-actin against loss.

Two alternative methods, PFA-Tsb and DSP-Tsb, give an relatively high intensity of phalloidin-labeling. This indicates that chemical crosslinking with either PFA or DSP, followed by permeabilizing with Triton X-100 under condition that stabilize microtubules is a good choice for labeling F-actin for quantitative studies, especially if the cells need to be permeabilized for other reasons. Examples include double labeling with a large, membrane-impermeant probe or parallel preparation for SEM.

Although they provide less intense actin labeling, several additional methods provide good morphological preservation of the F-actin pattern. These include PFA-Thbss, Tsb, and DSP-Thbss. The decreased intensity of labeling with these methods could be due to either a loss of F-actin or decreased accessibility of the actin filaments to phalloidin. SEM observations, showing that cells prepared by Tsb and DSP-Thbss are more extensively extracted than cells prepared by methods that give more intense labeling, support the conclusion that the decreased intensity of labeling is due to a decrease in the amount of F-actin that is preserved.

Immunolabeling of tubulin

In the case of immunolabeling of tubulin, the considerations are quite different than with F-actin. Antibodies are large probes, and the best labeling of tubulin was obtained under conditions that facilitate the penetration of the antibodies into the cells. Maximal labeling was obtained with DSP-Thbss. The intensity with PFA-EtOH was only slightly less. Thus, maximal labeling of tubulin required chemical crosslinking, with either DSP or PFA, followed by extraction, with Triton X-100 or

EtOH, under conditions that promote loss of material. This approach appears to stabilize microtubules, which are particularly labile and sensitive to loss during extraction, while extracting enough other cytoplasmic material to allow the antibodies to gain access to their epitopes. Among the material extracted is F-actin, and the two methods that maximize labeling of tubulin give less than maximal labeling of F-actin.

Our results also show that maximal labeling is not always optimal. Our studies of tubulin labeling illustrate quite well the point that what constitutes optimal morphological preservation depends on what one is looking for. Because tubulin exists in cells both in a polymerized and an unpolymerized form and because the antibody binds to both forms, labeling of one form may mask the labeling of the other. Thus, the more unpolymerized tubulin that is labeled, the more difficult it is to visualize the microtubules. Methods that use the bifunctional crosslinker DSP to stabilize tubulin prior to extraction increase the diffuse cytoplasmic staining, presumably because they preserve more unpolymerized tubulin. In contrast, extraction only in Tsb, which is based on a buffer originally designed to stabilize microtubules, preserves individual microtubules quite well, while allowing much of the unpolymerized tubulin to be extracted. Thus PFA-EtOH and DSP-Thbss permit more accurate quantification of tubulin, DSP-Tsb and Tsb gives a clearer visual image of the distribution of microtubules. The DSP-Tsb method also gives excellent morphological preservation of F-actin, making this the method of choice for SEM of the cytoskeletal filaments.

Double labeling

When it is desired to double label cells with two different probes or to compare the labeling of similarly prepared samples with different probes, it may be necessary to select a method that gives adequate but less than maximal labeling by the individual probes. Thus, to label both tubulin and actin in the same preparation, DSP-Tsb proved to be the optimal method. Fortunately, this method gives excellent morphological preservation of both F-actin and microtubules and only slightly less labeling than the respective maximal methods.

Scanning electron microscopy

As previously discussed, to visualize internal structures by SEM, they must be exposed to the electron beam, either by removing the cell membrane or fracturing the cells in some way. We have focused our efforts on the former approach, using the non-ionic detergent Triton X-100. Of the various methods used in this paper to prepare cells for SEM, three are useful for visualizing internal cell structures (Table 3). In decreasing order of quality, these methods are: DSP-Tsb, DSP-Thbss, and Tsb.

Crosslinking cells in DSP and then extracting in Tsb is the optimal method for preserving cytoskeletal filaments, as well as many other macromolecular structures (Lindroth *et al.*, 1992) for visualization by SEM. Although many cytoplasmic components are extracted (Bell *et al.*, 1989; Lindroth *et al.*, 1992), actin filaments and microtubules are well preserved, as shown by quantitative labeling. In addition the internal skeleton of such delicate structures as ruffles, microspikes and microvilli are preserved in a form that closely resembles the native form seen in living cells by light microscopy. The efficacy of DSP-Tsb for preserving subcellular structure is demonstrated by our finding that this method preserves tubulin at the leading edge of neomycin-induced lamellipodia. As shown by labeling studies, this population of tubulin is lost if cells are permeabilized without crosslinking. DSP also increases the preservation of F-actin, but F-actin is less sensitive to being lost than tubulin.

DSP-Thbss is a method that stabilizes intracellular structures by crosslinking but extracts more material, including F-actin, than DSP-Tsb. Because this method appears to facilitate the access of antibodies to their epitopes, it might be particularly useful for SEM studies involving labeling with probes such as colloidal gold. Some loss of actin and other cytoplasmic molecules may be acceptable to obtain good labeling of the remaining structures.

Tsb alone provides acceptable preservation of actin filaments and structures containing F-actin, but microtubules are less well preserved than with the previous two methods. The results of extracting with Tsb are also somewhat less consistent than those obtained when cells are stabilized with DSP before extracting. We would only use Tsb to prepare cells for SEM when fluorescent labeling studies confirm that the molecules or structure of interest are well preserved.

Conclusions

For qualitative and quantitative microscopic studies of intracellular macromolecular structures, the method DSP-Tsb is the overall method of choice for both labeling and morphological studies. Although other methods provide maximal labeling or preservation of specific structures, this method provides excellent preservation of morphology while allowing proteins to be labeled by specific probes.

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Discussion with Reviewers

W. Bohn: How do these cytoskeletons appear in the transmission electron microscope (TEM) after shadowing with carbon, which may give better resolution? Is there any experience with carbon or heavy metal/carbon-shadowed cytoskeletons obtained with the Tsb and DSP-Tsb methods?

Authors: TEM images of cytoskeletal preparations prepared by the Tsb and DSP-Tsb methods have been published (Bell *et al.*, 1989; Lindroth *et al.*, 1992). In general, these images provide excellent high resolution images at high magnification of the cytoskeletal filaments and associated structures, such as ribosomes, suitable for magnification. They are equivalent to the lower resolution images available with SEM. In TEM, the structure of the metal film used to coat the biological structures can be resolved. The primary drawback with TEM is that the high energy beam causes the samples to melt and vaporize, unless they are coated with a thick coat of carbon, and even this may not be adequate to protect the samples completely.

W. Bohn: Has any labeling technique been combined with any of these extraction procedures in order to identify individual filament classes in the EM?

Authors: We have used colloidal gold-conjugated antibodies to label microtubules and intermediate filaments and visualized the images with SEM, in both secondary electron and scanning transmission mode, and TEM (Bell *et al.*, 1988). The results show that colloidal gold is an excellent label for use in combination with the methods described in this paper.

W. Bohn: Labeling of tubulin was done with IgM antibodies. IgM antibodies are rather big molecules and it may be difficult to get rid of unbound antibodies, especially when working with cells prefixed with aldehyde. Please discuss this, especially in light of the diffuse labeling with this antibody seen in cells prefixed in PFA.

R.M. Albrecht: What measures are taken to avoid or control for non-specific binding of label?

Authors: Although it is always possible that some of the IgM is trapped in the PFA-crosslinked cells, we believe that the variations in the amount and pattern of labeling follow preparative procedures too closely to be due simply to the non-specific trapping of antibodies; and although we cannot do a tubulin-negative control with these cells, all other negative controls show extremely low levels of non-specific binding of antibodies. Moreover, the fluorescence intensity of DSP-crosslinked, Thbss-extracted cells, which are extensively extracted, is greater than that of PFA-crosslinked, ethanol-extracted cells. Yet, both show diffuse staining with anti-tubulin. Therefore, we believe that the diffuse labeling with anti-tubulin is indicative of the continued presence of unpolymerized tubulin. The steps we take to avoid non-specific labeling include blocking the crosslinkers and PFA with an excess of glycine; using 1% BSA to block non-specific protein-binding sites; and including 1% BSA in all antibody solutions to limit non-specific binding.

W. Bohn: What is the basis for the authors' interpretation that the higher intensity of tubulin fluorescence in PFA-treated cells results from stabilization of unpolymerized tubulin and that better visibility of individual microtubules is due to the extraction of unpolymerized tubulin? Do any biochemical experiments exist that would support this notion?

Authors: There is a clear correlation between the brightness of the anti-tubulin fluorescence and the presence of diffuse cytoplasmic anti-tubulin staining (compare DSP-Thbss with DSP-Tsb in Figures 1, 11 and 12), which obscures the microtubules. Secondly, the amount and variety of proteins, including tubulin, retained with the cytoskeleton has been shown by SDS-PAGE to increase with increased crosslinking (Bell *et al.*, 1987b, 1989). Therefore, crosslinked cells have more tubulin, more diffuse cytoplasmic anti-tubulin staining and less visible microtubules.

M. Malecki: Spatial rearrangements of the cytoskeletal architecture are involved in vital functions of cells. Your results could be well applied in studies of those rearrangements. Can you share your comments on the preservation of the three-dimensional organization using protocols you described?

Authors: The methods we describe are ideally suited to study changes in the three-dimensional architecture of the cytoskeleton associated with functional changes. Our own experience with such studies includes examination of the effects of cyclic AMP (Bell *et al.*, 1978) and cytochalasin B on the cytoskeleton (Bell and Revel, 1979) and changes in the organization of the peripheral cytoskeleton associated with various steps in the process of cell motility (Safiejko-Mroczka and Bell, 1995b). In the first, we showed that cAMP-induced elongation of CHO cells was associated with reorganization of the cytoskeleton. In the second, we demonstrated that the tendency of CB-treated cells to lose their nucleus is the result of a reorganization of the cytoskeleton. In the third, we showed that protrusion of the cell margin is associated with both depolymerization and polymerization of actin filaments at the cell margin.

M. Malecki: Have you considered using fluorescent analogs of proteins or derivatized antibodies delivered into cells through microinjection for imaging cells with

laser confocal microscopy followed by your procedures for scanning microscopy as the means to evaluate preservation of the cytoarchitecture?

Authors: We have not attempted this, but what you propose would be an excellent approach to gaining a better and more dynamic understanding of the cytoskeleton.

M. Malecki: Your results indicate different protocols for the good preservation of microfilaments and microtubules. In many studies, multiple labeling is of advantage. What would be the best compromise for double- or triple labeling?

Authors: For procedures in which multiple labels are to be used, we would begin with those procedures that provide acceptable, although perhaps suboptimal, labeling for each of the labels one desires to use. For actin and tubulin, the DSP-Tsb procedure provides excellent double labeling.

I.D. Burdett: What does the cytofluorometer measure? How is the measurement related to labeling efficiency (percentage of epitopes labeled), a concept used more generally among those concerned in post-embedding labeling procedures for EM? It is difficult to relate the values given in Figures 1 and 2 to any concept of efficiency partly because we have no idea of the maximum number of molecules or epitopes per cell or what factors are truly responsible for masking or otherwise obscuring the molecules/structures to be labeled, although cell permeability would seem to be important. The technique would seem only to be saying something about the relative accessibility or retention of label between different treatments without knowing how much of the plasma membrane of other barriers remain. Although the word "quantification" is used, it should be made rather clear what is being measured.

Authors: The method measures the fluorescence of cells relative to each other. For further discussion, please see, Bell *et al.* (1987b). In the absence of an absolute standard, there is no way to translate these measurements into absolute numbers of epitopes or micrograms of protein nor is it possible to calculate labeling efficiency. Of course, there are very few methods that can measure absolute amounts of biological molecules, especially *in situ*. However, because the cells were labeled under identical conditions, the method is useful for its intended purpose of studying the relative preservation and labeling of epitopes in cells prepared by different procedures.

I.D. Burdett: Do the cells collapse after any of these fixation protocols and is cytofluorometric measurement influenced by the thickness of the cell? Have the authors examined their preparations by thin section EM, or biochemically by detection of labeled plasma membrane, to see how much membrane remains after permeabilization.

Authors: Transmission electron micrographs of thin sections and scanning electron micrographs of glutaraldehyde-fixed intact cells and crosslinked, detergent-extracted cells show that the three dimensional morphology of the cells is similar (Bell, 1981). Dehydration and drying produce overall shrinkage, but the optimal extraction protocols do not appear to cause the cells to collapse. Although we have not labeled the plasma membranes for biochemical studies, transmission electron micrographs of thin sections show that the lipid bilayer of the plasma membrane is completely extracted by Triton X-100, even after crosslinking (Bell, 1981). Membrane proteins, such as receptors, may survive extraction and remain associated with the cytoskeleton.

I.D. Burdett: How do the authors select the most suitable labeling protocol? I think it a pity that not more is made of the neomycin-induced lamellipodia (Figs. 13 and 14) or other situations in which drugs or other means have been used to examine a biological phenomenon. Changes in the dynamic features of the cytoskeleton could be used to test the suitability of one or two of the most useful protocols.

R.M. Albrecht: What was the purpose of using neomycin?

Authors: As we have consistently tried to stress, the suitability of a labeling protocol depends on what one is trying to study. In the current paper, we have used maximum fluorescence intensity, the pattern of fluorescence and the preservation of structure in the SEM as criteria. In other studies, we have used density and specificity of colloidal gold labeling as criteria for optimal preparation (Bell *et al.*, 1988). As part of our research to investigate the mechanisms of cell motility, we have found that neomycin induces cells to protrude cytoplasmic lamellae from their margins, which is the first step in the motility of metazoan cells. We are very interested in how neomycin induces the formation of lamellipodia and have shown that it stimulates an initial decrease in F-actin, followed by an increase in F-actin (Safiejko-Mroczka and Bell, 1995b). In the current paper, we show the importance of crosslinking to preserve tubulin in the marginal cytoskeletal band formed at the leading edge of the neomycin-induced lamellae.

I.D. Burdett: On a specific level, a previous paper by Bell *et al.* (1987b) seems to recommend a different optimal protocol (DSP-Tsb-PFA) than that given in the present paper. Have the criteria for visualization and/or maximum labeling changed?

Authors: The DSP-Tsb-PFA protocol recommended in the 1987 paper is the same as the DSP-Tsb protocol in the current paper. Note that all samples in the current paper were fixed in PFA prior to labeling with antibody. It was the best of the methods we tested at that time and it is still an excellent method. It is just that for tubulin, we get increased labeling by extracting in Thbss (DSP-Thbss protocol).

I.D. Burdett: What is the rationale for using protocols such as PFA-acetone or PFA-EtOH? How unextractable do tubulin or actin (especially in the unpolymerized forms) become, once fixed in PFA, even by means of ethanol or acetone?
Authors: PFA-ethanol and PFA-acetone, as well as methanol extraction, are among the most commonly used protocols in the literature to prepare cells for fluorescent labeling for light microscopy. Therefore, to determine the optimal methods for visualization and preservation of the cytoskeleton we used these procedures as the "base line" for evaluating the new procedures. Although we cannot measure how much actin or tubulin is actually extracted, our findings indicate that covalent crosslinking of tubulin and actin, either with aldehydes or bifunctional crosslinking reagents, reduces the amount of protein that is extracted following permeabilization with solvent or detergent.

R.M. Albrecht: What was the composition of the storage medium and length of time specimens were in storage between the primary fixation and the post-fixation prior to critical point drying? Were there any negative effects attributable to longer term storage?

Authors: We routinely store samples to be critical point dried in 2.5% glutaraldehyde in cacodylate-sucrose buffer, overnight, prior to critical point drying (CPD). In the set of experiments described in this paper, the samples were stored for up to a week. We have not seen any differences between samples stored for a few hours or a few days. We have never looked at samples stored in glutaraldehyde for longer than a week to ten days.

R.M. Albrecht: Are any noticeable structural changes induced by the 1% osmium tetroxide post-fixation step? Previous studies have cautioned against using high concentrations of OsO_4 and often suggest 0.1% OsO_4 or less to avoid damage to certain filamentous structures.
Authors: We used OsO_4 only on samples that were not extracted with detergent. The OsO_4 was used in these samples to stabilize the membrane against any further solvent-extraction during dehydration in acetone. Otherwise the membrane is poorly preserved and it is impossible to distinguish the structural effects of the initial extraction from those caused during dehydration. Detergent-extracted samples were not post fixed in osmium, to avoid damaging the cytoskeleton.

R.M. Albrecht: Could additional benefit be derived through the use of confocal light microscopy or low, intermediate, and high voltage transmission electron microscopy of whole mounts or sections of cells to evaluate preservation of cytoskeletal integrity?
Authors: Yes, although the high energies associated with some forms of TEM may directly damage the structural integrity of whole mount preparations.