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Fixation Methods for the Study of Lipid Droplets by Immunofluorescence Microscopy

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SUMMARY The study of proteins associated with lipid droplets in adipocytes and many other cells is a rapidly developing area of inquiry. Although lipid droplets are easily visible by light microscopy, few standardized microscopy methods have been developed. Several methods of chemical fixation have recently been used to preserve cell structure before visualization of lipid droplets by light microscopy. We tested the most commonly used methods to compare the effects of the fixatives on cellular lipid content and lipid droplet structure. Cold methanol fixation has traditionally been used before visualization of cytoskeletal elements. We found this method unacceptable for study of lipid droplets because it extracted the majority of cellular phospholipids and promoted fusion of lipid droplets. Cold acetone fixation is similarly unacceptable because the total cellular lipids are extracted, causing collapse of the shell of lipid droplet-associated proteins. Fixation of cells with paraformaldehyde is the method of choice, because the cells retain their lipid content and lipid droplet structure is unaffected. As more lipid droplet-associated proteins are discovered and studied, it is critical to use appropriate methods to avoid studying artifacts.
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KEY WORDS
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LIPID DROPLETS are found in many different organisms and across many different tissues and cell types. Although the lipid droplets within adipocytes in the adipose tissue of animals are the largest and most easily observed, these specialized structures have been found across the biological kingdom and are now characterized as ubiquitous components of most types of cells (Murphy 2001). Lipid droplets play an important role in lipid storage and trafficking in mammalian cells. These structures provide energy and substrates for synthesis and repair of cell membranes in most types of cells, for synthesis of mediators of the inflammatory response, for steroid hormone biosynthesis in adrenal cortical, testis, and ovarian cells, for surfactant synthesis in lung, and for secretion of the lipid component of milk from mammary epithelial cells (Murphy 2001). In addition, the accumulation of cholesterol esters in lipid droplets in macrophages leads to the formation of foam cells, which play a major role in the development of atherosclerotic lesions. In each cell type, some of the proteins that are associated with the surfaces of lipid droplets regulate the accumulation and mobilization of the lipids that fulfill these functions.

To date, only a few lipid droplet-associated proteins have been identified. Adipophilin is a ubiquitously expressed protein in all mammalian cell types and is found only in lipid droplets and in no other subcellular compartment (Brasaemle et al. 1997b; Heid et al. 1998). Perilipin, a structurally related protein, associates with lipid droplets in adipocytes and steriodogenic cells (Greenberg et al. 1991; Blanchette–Mackie et al. 1995; Servetnick et al. 1995). In these cells it restricts the access of hormone-sensitive lipase and other cytosolic lipases to the lipid droplet under basal conditions (Souza et al. 1998,2002; Brasaemle et al. 2000b; Martinez–Botas et al. 2000; Tansey et al. 2001), and facilitates lipase access to the droplet under lipolytically stimulating conditions (Tansey et al.

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2001; Souza et al. 2002). Moreover, several other lipid droplet-associated proteins have been recently identified, including a third protein related to adipophilin and perilipins, called TIP47 (Wolins et al. 2001; Miura et al. 2002), lipotransin (Syu and Saltiel 1999), a 200-kD capsular protein (Wang et al. 1997), the hepatitis C virus core protein (Hope and McLauchlan 2000) and, under some conditions, caveolins (Fujimoto et al. 2001; Ostermeyer et al. 2001; Pol et al. 2001). Interest in this field of study is growing as more lipid droplet-associated proteins are identified. It is therefore important to establish appropriate methods for the study of lipid droplets and their associated proteins.

Immunofluorescence microscopic techniques have been used to study the localization of proteins in cells for many years, resulting in the development of an assortment of standard methods. Given the recent expansion of research initiatives investigating lipid droplet-associated proteins, an evaluation of these methods is now imperative. Chemical fixation is used to preserve cell or tissue structure so that the ensuing imaging methods accurately capture the localization of cell components that existed in the cell at the time of fixation (Griffiths 1993). We have noted that researchers have applied a number of fixation protocols that have classically been utilized to study cytoskeletal structure (Osborn and Weber 1977) to the study of lipid droplets, including cold methanol (Jiang and Serrero 1992; Heid et al. 1998; Chen et al. 2001) and cold acetone (Barba et al. 1997; Frolov et al. 2000; Atshaves et al. 2001) fixation protocols. These fixatives have been used before staining for adipophilin in cultured adipocytes (Jiang and Serrero 1992), macrophages (Chen et al. 2001), fibroblasts (Frolov et al. 2000; Atshaves et al. 2001), and various cell lines derived from dogs and cattle (Heid et al. 1998), and for the core protein of the hepatitis C virus expressed in Chinese hamster ovary (CHO) fibroblasts (Barba et al. 1997). Chemical crosslinkers such as paraformaldehyde and glutaraldehyde have also been used as fixatives to study proteins associated with many cell compartments, including lipid droplets (Blanchette–Mackie et al. 1995; Brasaemle et al. 2000a,b). In this study we have investigated the effects of cold methanol, cold acetone, and paraformaldehyde fixation procedures on the imaging of lipid droplets and their associated proteins by fluorescence microscopy, and on cellular lipid and protein composition.

Materials and Methods

Materials

Ham's F12 medium, Dulbecco's minimal essential medium (DMEM), and trypsin were purchased from Mediatech (Herndon, VA). Fetal bovine serum, oleic acid, fatty acid-free bovine serum albumin, saponin, paraformaldehyde, goat IgG, monoclonal anti-β-tubulin antibody clone TUB 2.1, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were purchased from Sigma (St Louis, MO). Lissamine–rhodamine-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch (West Grove, PA). Goat anti-rabbit AlexaFluor 488 and Bodipy 493/503 were purchased from Molecular Probes (Eugene, OR). The BCA Protein Assay Kit was purchased from Pierce Chemical Company (Rockford, IL). Five percent ammonium sulfate-impregnated silica gel thin-layer chromatography plates were purchased from Analtech (Newark, DE). Methanol and acetone were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Culture

CHO fibroblasts were maintained in Ham's F12 medium and 3T3-L1 cells were maintained in DMEM. Media for both cell lines were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37C in a 5% CO_2 atmosphere. The cells were grown in culture flasks and dishes from Corning Life Sciences (Acton, MA). Cells for microscopy experiments were grown on glass coverslips in 100-mm culture dishes.

The differentiation of 3T3-L1 cells to adipocytes was accomplished by incubating confluent monolayers of cells in DMEM supplemented with 10% fetal bovine serum, 0.5 mM isobutylmethylxanthine, 10 μg/ml insulin, and 10 μM dexamethasone with fresh medium changes every 24 hr for 72 hr, followed by DMEM supplemented with 10% fetal bovine serum with medium changes every 24 hr for an additional 72 hr (Brasaemle et al. 1997b).

Lipid loading of CHO fibroblasts was achieved when the cells reached confluence by supplementing cell culture medium with 400 μM oleic acid complexed to fatty acid-free bovine serum albumin (6:1 moles of oleate:mole of albumin) (Brasaemle et al. 2000b) for 24 hr to increase the synthesis and storage of triacylglycerols.

Fixation of Cells

Methanol Fixation. Methanol was stored at −20C before use. The culture medium was removed and the cells on coverslips were washed twice with PBS. The cells were incubated with cold methanol (Jiang and Serrero 1992) in a pre-chilled glass tray kept on dry ice for 5 min, and then were washed four times with cold PBS.

Acetone Fixation. Acetone was stored at −20C before use. The culture medium was removed from the cells, and the cells were incubated with cold acetone for 10 min (Hammond and Glick 2000) in a pre-chilled glass tray kept on dry ice during fixation. Cells were washed four times with cold PBS.

Paraformaldehyde Fixation. Medium was removed from the cells, which were then washed twice with PBS. Cells were incubated with 3% paraformaldehyde in PBS for 20 minutes at room temperature (Blanchette–Mackie et al. 1995) and then washed four times with PBS.

Immunofluorescence Microscopy

All incubations were done in antibody diluent (PBS with 0.1 mg/ml saponin and 0.5 mg/ml goat IgG); all washes were done with PBS. Fixed cells were incubated for 45 min at RT in antibody diluent with 0.2 M glycine (Blanchette–Mackie et al. 1995) to block nonspecific antibody binding and to quench background fluorescence due to aldehydes. Cells were incubated with primary antibody in antibody diluent overnight at 4C and warmed at RT for 30 min before four 10-min PBS washes. Primary antibodies used were as follows: (a) rabbit polyclonal anti-mouse adipophilin antibody (Wolins et al. 2001; donated by Dr. Charles Schultz, National Institutes of Health); (b) rabbit polyclonal anti-rat perilipin amino-terminal antibody (Brasaemle et al. 1997a; donated by Dr. Constantine Londos, National Institutes of Health); or (c) monoclonal anti-β-tubulin antibody clone TUB 2.1. Cells were incubated with secondary antibody in antibody diluent for 1 hr at RT in the dark and washed four times for 10 min in PBS. Secondary antibodies used were (a) lissamine–rhodamine-conjugated goat anti-rabbit IgG, (b) goat anti-rabbit AlexaFluor 488, or (c) FITC conjugated goat anti-mouse IgG. Neutral lipids were stained with Bodipy 493/503 (Gocze and Freeman 1994) during the secondary antibody incubation. Coverslips were mounted on glass slides with *p*-phenylenediamine in glycerol (Blanchette–Mackie et al. 1995). Cells were viewed with a Nikon Eclipse E800 fluorescence microscope (Nikon Instruments; Melville, NY) equipped with a Hamamatsu Orca digital camera (Hamamatsu Photonic Systems; Bridgewater, NJ) interfaced with a Power Macintosh G4 computer (Apple Computers; Cupertino, CA). Images were processed using Improvision Openlab software (Improvision; Lexington, MA).

Protein Assay

To test the effects of chemical fixation on the protein content of cells, total protein mass was assessed after the fixation protocols. Densely subconfluent CHO cells were incubated with fixatives and rinsed with PBS, as described above. For control (no fixative) conditions, cells were washed six times with PBS before harvest to mimic the wash conditions of the fixed cells. Cells were harvested by scraping into PBS. Protein content of the collected cells was measured by the bicinchoninic acid method (Smith et al. 1985).

Lipid Extraction and Analysis

To test the effects of chemical fixation on the lipid content of cells, total lipids were extracted from cells after the fixation procedures in 2:1 chloroform:methanol (Folch et al. 1957). Lipid extracts were spotted onto 5% ammonium sulfate-impregnated silica gel thin-layer chromatography plates. Plates were developed in 90:10:1 hexane:ethyl ether: formic acid for 35 min, dried under a fume hood, and then charred at 160C in a Fisher Scientific Isotemp oven for 30 min (Brasaemle et al. 2000b). Charred spots corresponding to various lipid classes in the samples were quantified by densitometry using a Molecular Dynamics Personal Densitometer (Amersham Biosciences; Piscataway, NJ) and were compared to lipid standards resolved on the same plates using ImageQuant 5.1 software. Masses of triacylglycerols and phospholipids were expressed relative to total cell protein content. The experiment to quantify cell lipids and proteins was conducted four times on sets of two to five samples per treatment. Because lipid loading conditions varied slightly for each experiment, yielding different total lipid content relative to cell protein, data were normalized relative to unfixed cells. Pooled data were analyzed by a one-way ANOVA followed by Dunnett's multiple comparison test.

Results

We tested three fixation procedures that have been used before microscopy of lipid droplets and their associated proteins for their effects on cellular lipid content and lipid droplet structure. Cold acetone and cold methanol fixatives have traditionally been effective for subsequent viewing of cytoskeletal elements. Paraformaldehyde crosslinks proteins to preserve structure in cells and has been used for many applications. CHO cells were grown to confluence and then loaded with oleic acid complexed to bovine serum albumin for 24 hr to increase the storage of triacylglycerols in lipid droplets (Brasaemle et al. 1997a). Cells were then fixed using each of the three fixation procedures and prepared for immunofluorescence microscopy. The cells were stained with antibodies raised against adipophilin and with Bodipy 493/503, a fluorescent hydrophobic molecule that localizes to structures containing neutral lipids (Gocze and Freeman 1994).

Paraformaldehyde Fixation Preserves Lipid Droplet Structure Most Effectively; Methanol Fixation Facilitates Fusion of Lipid Droplets

Paraformaldehyde-fixed CHO cells showed the most consistent staining of adipophilin surrounding lipid droplets of all sizes (Figures 1A–1C). Methanol-fixed CHO cells generally showed stronger staining of neutral lipids than acetone or paraformaldehyde-fixed cells (Figure 1). However, methanol-fixed cells appeared to lack adipophilin staining on the smallest lipid droplets, and the adipophilin staining appeared discontinuous around the larger lipid droplets so that there were visible gaps in the surface staining (Figures 1D and 1F). In general, the droplets of the methanol-fixed cells looked larger than those of paraformaldehyde-fixed cells, suggesting that fusions of the lipid droplets may have occurred within these cells. To quantify the observation that the methanol-fixed cells had larger lipid droplets than the paraformaldehyde-fixed cells, lipid droplets from 50 cells per treatment, fixed by either the methanol or the paraformaldehyde protocols, were sized and counted. The results showed that methanol-fixed cells have significantly more lipid droplets larger than 2.5 μm and fewer droplets smaller than 1 μm per cell compared to paraformaldehyde-fixed cells (Figure 2).

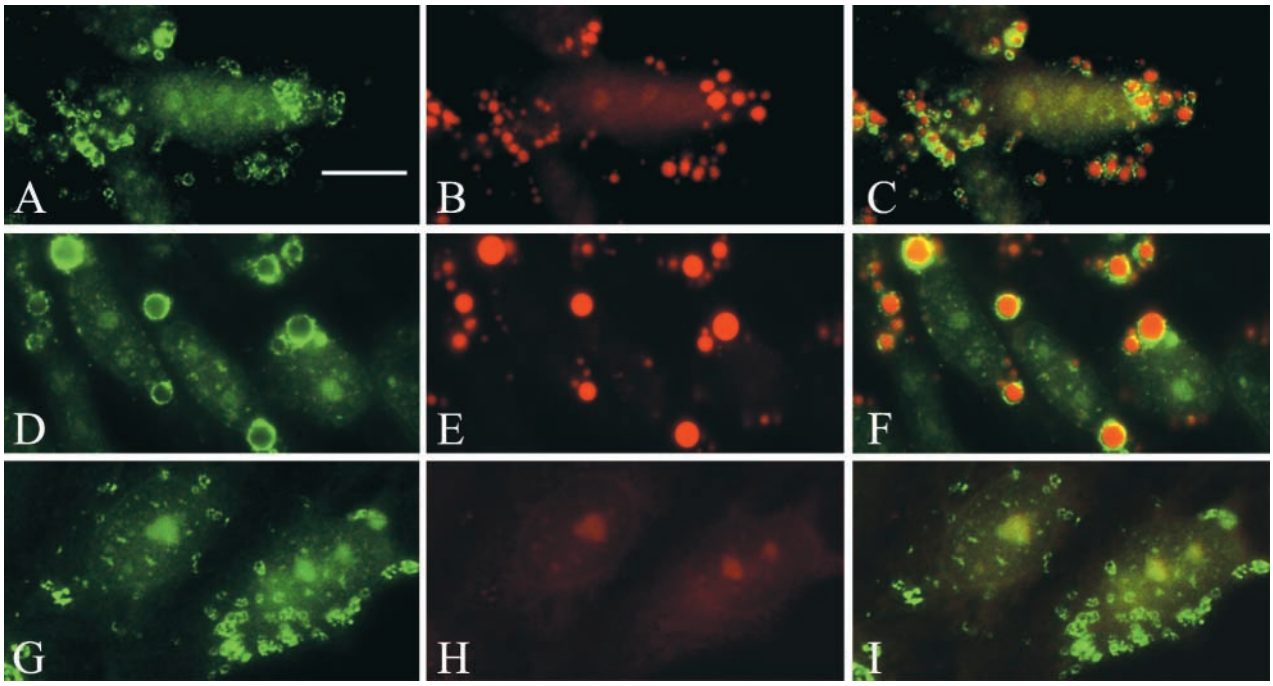

Figure 1 Fixation of CHO cells with paraformaldehyde preserves lipid droplet structure better than fixation with cold methanol or cold acetone. Lipid-loaded CHO cells were fixed with paraformaldehyde (**A–C**), cold methanol (**D–F**), or cold acetone (**G–I**) and then simultaneously stained for adipophilin (**A,D,G**) and neutral lipid with Bodipy 493/503 (**B,E,H**). Images were captured in black and white, colorized, and merged to show the relative distribution of both stains (**C,F,I**). Bar = 10 μm.

Acetone Fixation Extracts the Core Neutral Lipids

Acetone-fixed CHO cells had minute structures that stained for adipophilin but not for neutral lipid (Figures 1G–1I). The staining pattern for adipophilin was fragmented at the surfaces of the structures and also appeared in globular shapes that were not representative of the circular and continuous adipophilin staining pattern observed in the paraformaldehyde-fixed cells (Figures 1A–1C).

Methanol Fixation of 3T3-L1 Adipocytes Confirms Lipid Droplet Fusion Events

To confirm our initial findings from experiments with CHO cells, the methanol and paraformaldehyde fixation procedures were tested on differentiated 3T3-L1 adipocytes, which have much larger lipid droplets. Pre-adipocytes were seeded onto glass coverslips, grown to confluence, and then treated with isobutylmethylxanthine, dexamethasone, and insulin to induce differentiation. Fully differentiated adipocytes were fixed and stained for immunofluorescence microscopy with antibodies raised against perilipins, the major lipid droplet-associated proteins in adipocytes, and with Bodipy 493/503 to detect neutral lipids. Methanol-fixed cells showed larger droplets, obvious fusion

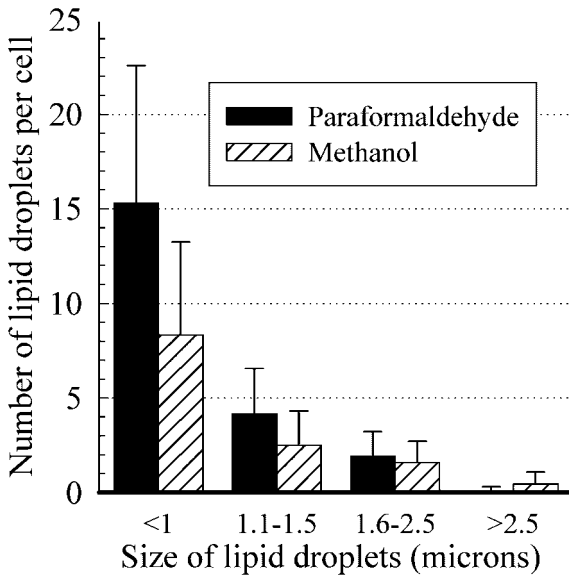

Figure 2 Methanol fixation of CHO cells leads to more numerous large lipid droplets and fewer small lipid droplets per cell. Lipid droplets were measured, counted, and grouped into four size ranges for 50 CHO cells from paraformaldehyde-fixed cells (filled bars) and for 50 CHO cells fixed with cold methanol (open hatched bars). Data were analyzed by a two-way ANOVA. Sizes of lipid droplets were different between the two treatments at $p<0.0001$.

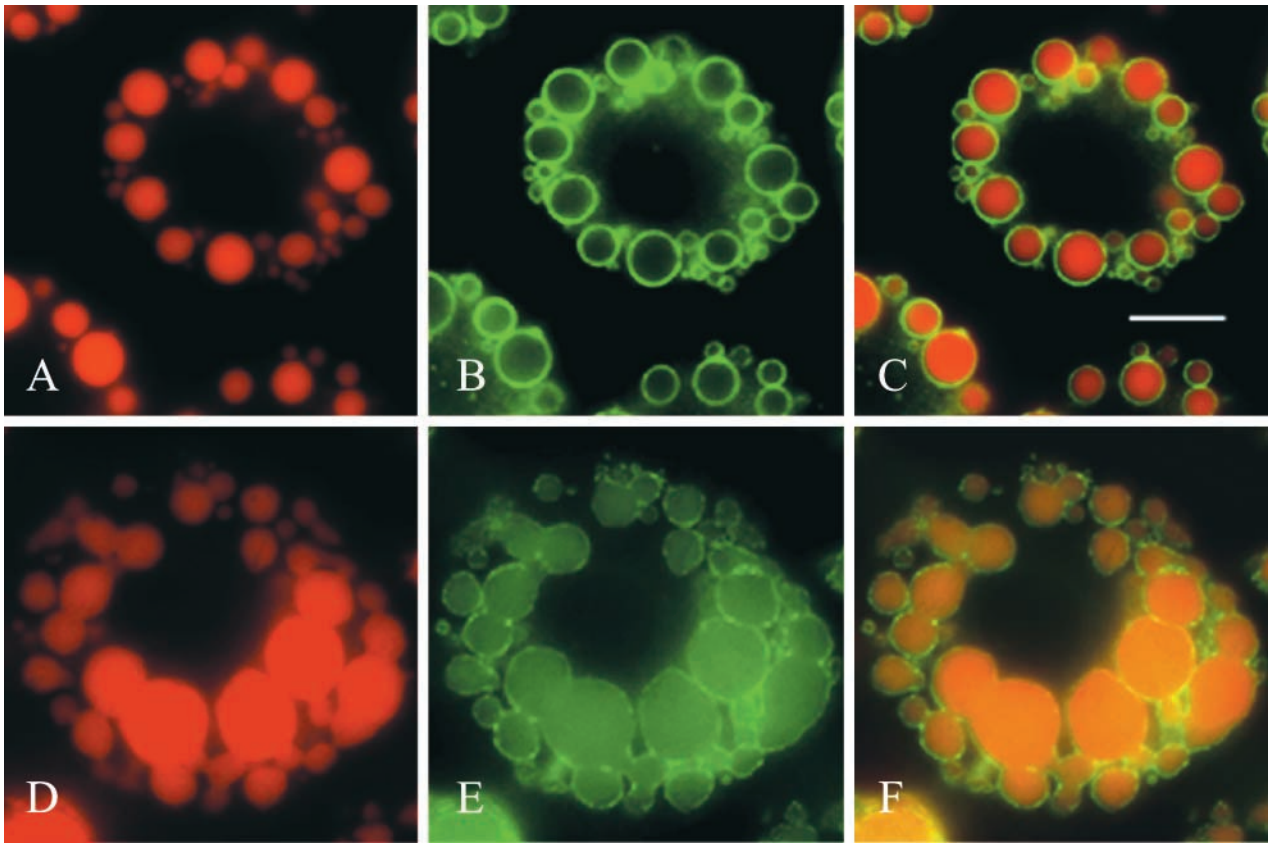

Figure 3 Partial fusion of lipid droplets is visible when 3T3-L1 adipocytes fixed with cold methanol are imaged. Differentiated 3T3-L1 adipocytes were fixed with paraformaldehyde (**A–C**) or cold methanol (**D–F**) and were stained for perilipin (**B,E**) and neutral lipid with Bodipy 493/503 (**A,D**). Merged images depict both stains (**C,F**). Bar = 10 μm.

of lipid droplets with evidence of partially fused droplets clearly visible, and less defined perilipin staining at the edges of the droplets, with higher levels of diffuse perilipin staining in the droplet interiors (Figures 3D–3F) when compared to 3T3-L1 adipocytes fixed with paraformaldehyde (Figures 3A–3C). Paraformaldehyde-fixed adipocytes had smaller droplets, no visible fusion of droplets, clearer distinct staining of neutral lipid, and heavy continuous perilipin staining around all of the lipid droplets.

Methanol and Acetone Fixation Procedures Were Most Effective Before Visualization of Microtubules

Because the cold methanol and cold acetone procedures have been most frequently used to visualize cytoskeletal elements, CHO cells fixed by the three procedures were stained for tubulin, a cytoskeletal protein, and were observed with a fluorescent microscope. Visualization of microtubules was much clearer in cells fixed with cold methanol (Figure 4A) or cold acetone (Figure 4B) than with paraformaldehyde (Figure 4C). Tubulin fluorescence was weaker in paraformaldehyde-fixed cells, and the captured images lacked clarity and definition.

Relative to Unfixed Cells or Cells Fixed with Paraformaldehyde, Cells Fixed with Acetone Lacked the Majority of Neutral and Polar Lipid Content; Methanol-fixed Cells Lacked the Majority of Phospholipids

Because methanol and acetone are used routinely in lipid extraction procedures, we suspected that the visible alterations in lipid droplet structure were due to the extraction of droplet lipids. To determine the effect of the fixation procedures on the cellular lipid content, lipid analysis was performed on lipid-loaded CHO cells that were fixed by the three procedures. Four conditions were tested: unfixed control cells and cells fixed with either paraformaldehyde, cold methanol, or cold acetone. Total lipids were analyzed by quantitative thin-layer chromatography, and the lipid content was expressed relative to cellular protein content to control for differences in cell numbers due to the fixation procedures. We observed significant lift-

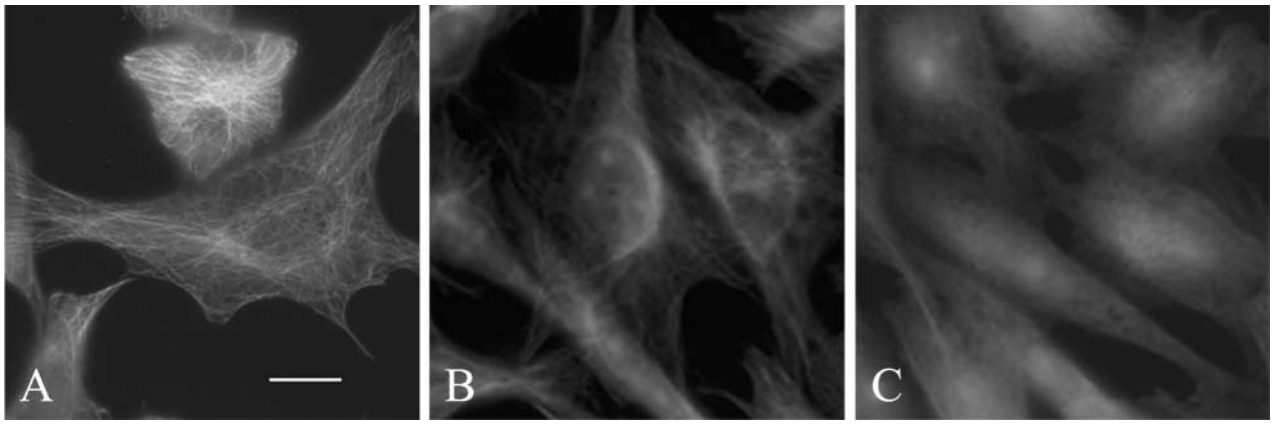

Figure 4 Cold methanol and cold acetone fixation methods are superior to paraformaldehyde fixation of CHO cells for imaging microtubules. CHO cells were fixed with cold methanol (**A**), cold acetone (**B**), or paraformaldehyde (**C**) and were stained with a monoclonal antibody raised against tubulin. Bar = 10 μm.

ing of cells from the growth surface when cold acetone or cold methanol fixatives were used. To compare four separate experiments in which the initial total lipid content varied due to small differences in lipid loading conditions, all data were expressed as percentages relative to the unfixed control cells. Cells fixed with cold acetone had no detectable triacylglycerol and only 25% of the total phospholipid content compared to paraformaldehyde-fixed and control cells (Figure 5). Methanol-fixed cells were depleted of more than 80% of their phospholipid. Paraformaldehyde-fixed cells had levels of triacylglycerols and phospholipids comparable to the unfixed control cells.

Discussion

The most important finding of this study is that paraformaldehyde is the most effective fixative to use for studying the structure of lipid droplets in cells. Cold methanol and cold acetone fixation procedures extracted lipids from the cells and altered the appearance of lipid droplets visualized by immunofluorescence microscopy. Cold acetone fixation procedures quantitatively extracted cellular triacylglycerol, thus eliminating potential Bodipy 493/503 staining of droplets, while altering the staining pattern of adipophilin associated with the outer surfaces of lipid droplets in cultured CHO cells. Many of the lipid droplets displayed a collapsed appearance, possibly reflecting the removal of the core lipid. Cold methanol fixation, on the other hand, extracted the majority of phospholipids. Partial or complete fusion of lipid droplets was observed in most cells, and staining for adipophilin or perilipins on the lipid droplets of CHO cells or adipocytes, respectively, became weak and discontinuous. Paraformaldehyde was the least destructive fixative, yielding clear images of distinct spherical lipid droplets bounded by continuous rings of adipophilin or perilipin staining and with lipid content comparable to control cells. These findings demonstrate that the choice of fixative is critical in studies of lipid droplet structure, because the extraction of cellular lipids by the fixative may alter the appearance of surface-associated proteins and the shape and size of the droplet.

Cold acetone and cold methanol procedures were initially developed to study the structure of cytoskeletal elements in cells. From this study, it is clear that these fixation methods are superior to paraformaldehyde fixation for visualizing microtubules. The effectiveness of these methods in visualizing the cytoskeletal network is likely due to the removal of phospholipids from cell membranes, because these membranes hinder the access of antibodies to the cytoskeletal proteins.

Fixation of cells with cold methanol resulted in fusion of lipid droplets and a greater intensity of the staining of neutral lipids with Bodipy 493/503. Extraction of the surface monolayer of phospholipids on the surfaces of the lipid droplets probably facilitates the entry of the fluorophore into the neutral lipid core. Furthermore, removal of the surface phospholipids may remove a barrier to lipid droplet fusion. Because methanol fixation extracts almost all of the cellular phospholipids and alters the staining pattern of surface proteins on lipid droplets, this study raises the question of whether the visualization of proteins associated with membranous compartments other than the limiting phospholipid monolayer of lipid droplets, such as lysosomes, endocytic vesicles, Golgi, and endoplasmic reticulum, may also be adversely affected by methanol fixation procedures.

We hope that the results of this study will lead to the re-evaluation and standardization of fixation meth-

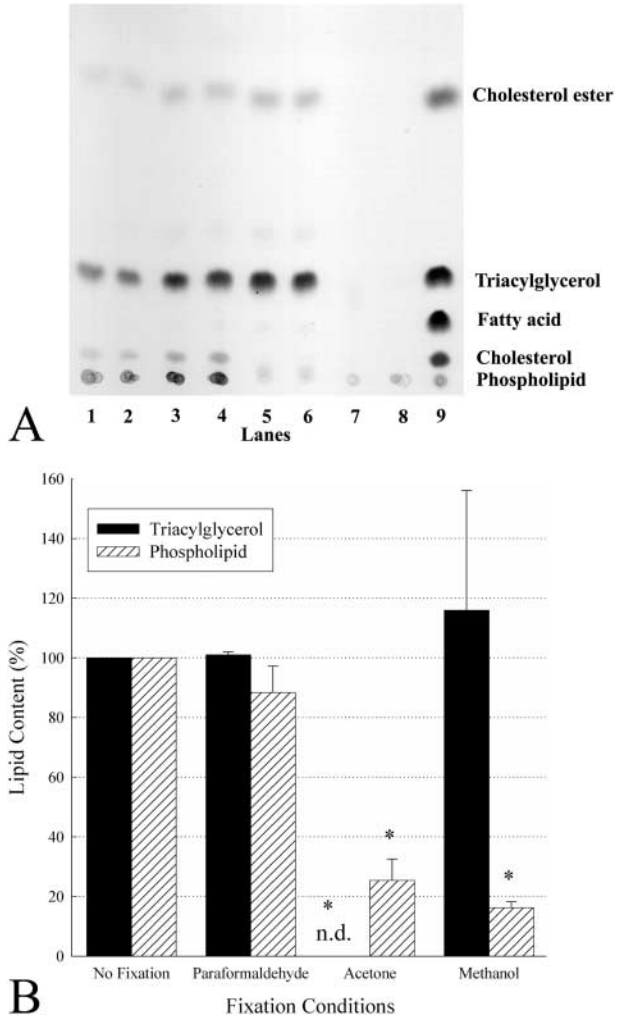

Figure 5 Cold methanol and cold acetone fixation methods extract cellular lipids. (**A**) Thin-layer chromatography plate of lipid extracts from CHO cells. Lanes 1 and 2, lipid extracts from unfixed cells (1.17 μg total cell protein/lane); Lanes 3 and 4, paraformaldehyde-fixed cells (2.6 μg total cell protein/lane); Lanes 5 and 6, cold methanol-fixed cells (2.6 μg total cell protein/lane); Lanes 7 and 8, cold acetone-fixed cells (2.85 μg total cell protein/lane). Lane 9, pure lipid standards for phospholipid (egg lecithin, at spotting origin), cholesterol, fatty acid (oleic acid), triacylglycerol (triolein), and cholesterol ester (cholesteryl oleate), as indicated. (**B**) Triacylglycerol content (filled bars) and phospholipid content (open hatched bars) of CHO cells treated with the various fixation methods relative to unfixed cells. n.d., not detectable. Data were analyzed by a one-way ANOVA followed by Dunnett's multiple comparison test. *Significantly different from unfixed cells at $p<0.01$.

ods for the study of lipid droplet-associated proteins and lipid droplet structure. We have found that aldehyde fixation methods are more effective than alcohol fixation methods in preserving lipid content and lipid droplet structure. Furthermore, it would be useful to develop an adequate fixation procedure to permit the subsequent visualization of both lipid droplets and cytoskeletal elements.

Acknowledgments

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