Scanning Microscopy

Volume 1991
Number 5 *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*

Article 3

1991

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Scanning Microscopy Supplement 5, 1991 (pages S17-S25)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0892-953X/91$3.00+.00

PRESERVATION AND IMMUNOGOLD LOCALIZATION OF LIPIDS BY FREEZE-SUBSTITUTION AND LOW TEMPERATURE EMBEDDING

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Abstract

The success of post-embedding immunocytochemistry depends largely on the preparation methods. The requirements for structural preservation and immunocytochemistry are in some cases contradictory. This is especially the case in the study of lipid-rich structures and the localization of lipid components. Earlier work on freeze-substitution has shown that this method is very promising for the preservation of lipids and the immunocytochemical localization of lipids at the electron microscopical level.

In this study we show that freeze-substitution in combination with low temperature embedding in Lowicryl HM20 has fulfilled this promise. Lamellar bodies in alveolar type II cells contain about 90% lipids and are very difficult to preserve in ultrathin cryosections. Lowicryl sections of freeze-substituted lung tissue shows excellent preservation of lamellar bodies in combination with immunogold localization of a hydrophobic surfactant protein. With an antibody against the Forssman glycolipid we demonstrate a highly reproducible intracellular localization of this glycolipid with high specificity and resolution. This method results in the retention of lipids and glycolipids and allows post-embedding immunogold labeling.

Key Words: freeze-substitution, low temperature embedding, Lowicryl HM20, cryo-ultramicrotomy, immunogold labeling, protein A-gold, Forssman glycolipid, Madin Darby canine kidney cells, lamellar body, lung.

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Introduction

Immunogold labeling has become a very powerful tool in cell biology. Several post-embedding methods such as cryo-ultramicrotomy, low temperature embedding, the use of acrylic resins instead of epoxy resins and freeze-substitution have shown their capability to localize proteins with high accuracy and specificity by immunogold labeling (Verkleij and Leunissen 1989; Hayat 1989). Most of the methods are based on a chemical fixation with aldehydes to immobilize proteins by cross-linking and to retain antigenicity for subsequent immuno-localization. Due to the aldehyde fixation most of the lipids are not preserved in thawed cryosections and lipid-rich structures such as lamellar bodies in alveolar type II cells are not preserved (Walker *et al.* 1986).

The localization of lipids with immunocytochemistry has been hampered in the past mainly by the lack of suitable preparation methods. Glycolipids, to which various antibodies are available (Feizi 1985), have been localized in the past by pre-embedding immunolabeling methods i.e.: fracture label, freeze-etch labeling and labeling followed by plastic embedding (Barbosa and Pinto da Silva 1983; Seybold *et al.* 1989; Tillack *et al.* 1983). Intracellular localization of glycolipids has been restricted mainly to immunofluorescence microscopy using antibodies or fluorescent lipid analogues (Pagano and Sleight 1985; Symington *et al.* 1987; van Meer 1989). In this paper we describe the use of freeze-substitution and low temperature embedding on pre-fixed and cryoprotected cells and tissues in comparison to cryo-ultramicrotomy. Lung tissue is used to show the effect of freeze-substitution on lipid-rich lamellar bodies and immunogold labeling of a hydrophobic surfactant protein. Madin Darby canine kidney (MDCK) cells can be cultured on filters and under these conditions display the properties of a polarized epithelium (Simons and Fuller 1985). A subline, the MDCK strain II cells, contain large amounts of the Forssman glycolipid

(Hansson *et al.* 1986). This subline was therefore used to reveal the intracellular localization of this glycolipid.

Materials and Methods

Cell culture and Immunoreagents

MDCK cells were grown as confluent monolayers on polycarbonate filters ("Transwell" Costar Corp., Cambridge, MA) as described (van Meer *et al.* 1987).

The antibody against the Forssman glycolipid used in this study was a gift of Dr. Arnoud Sonnenberg and is a rat monoclonal antibody (33B12) of the IgG2c subclass which binds to protein A-Sepharose. The antibody reacts with the terminal sugar sequence GalNAcα1-3GalNAc and is specific for Forssman (Sonnenberg *et al.* 1986). The antibody against surfactant protein B (SP-B) was a gift of Dr. Jeffrey Whitsett and is a rabbit polyclonal antiserum against purified cow SP-B and reacts mainly with mature SP-B by immunoprecipitation.

Fixation and cryo-ultramicrotomy

Human lung biopsies were fixed with 2% paraformaldehyde, 0.5% glutaraldehyde, 1% acrolein, 0.1% dimethylsulfoxide in 0.1 M phosphate buffer, pH 7.4. Part of the tissue was cryoprotected with 30% glycerol in 0.1 M phosphate buffer, pH 7.4 for 30 min. Small blocks of approximately 1 mm^3 were frozen in liquid propane (-180°C) and stored under liquid nitrogen for freeze-substitution and low temperature embedding. Another part of the tissue was infiltrated with increasing concentrations of gelatin up to 10% (v/v) in 0.1 M phosphate buffer pH 7.4 at 37°C. After solidification at 4°C small blocks of approximately 1 mm^3 were cut and infiltrated with 2.3 M sucrose overnight at 4°C and frozen in liquid nitrogen.

Transwell filters with a confluent monolayer of MDCK cells were fixed for 1 hour with 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 on ice. Filters were embedded in 10% gelatin in 0.1 M phosphate buffer at 37°C. After solidification at 4°C the gelatin was postfixed with 0.5% glutaraldehyde at 4°C. Small blocks of approximately 1 mm^3 were cut, infiltrated with 2.3 M sucrose, mounted on specimen stubs and frozen in liquid nitrogen. Ultrathin cryosections were cut on an LKB V microtome with cryoattachment.

Freeze-substitution and low temperature embedding

Transwell filters with a confluent monolayer of MDCK cells were rinsed shortly with 0.1 M Pipes buffer (pH 7) and fixed for 1 hour in 2% paraformaldehyde in 0.1 M Pipes on ice. Cells were cryoprotected by immersing the filters in 30% glycerol containing 1% paraformaldehyde in 0.1 M Pipes for 30 min. The filters were cut in small squares of approximately 1 mm^2. Several squares were mounted on top of a LKB specimen stub and were frozen in liquid propane (-180°C) after removing the excess of the glycerol solution. Samples were stored in liquid nitrogen before use.

Frozen MDCK cells and lung biopsies were transferred (under liquid nitrogen) into the Reichert CS-auto freeze-substitution unit. Freeze-substitution was carried out at -90°C in methanol supplemented with 0.5% uranyl acetate for at least 36 hours. After raising the temperature to -45°C at a rate of 5°C/hour and washing several times with pure methanol, the samples were infiltrated with Lowicryl HM20. Infiltration was done in the following graded series of Lowicryl-methanol mixtures: 1:1 for 2 hours, 2:1 for 2 hours, pure Lowicryl for 2 hours and pure Lowicryl overnight. The samples were transferred, inside the Reichert CS-auto, to a flat embedding mold filled with pure Lowicryl and polymerized by UV-light at -45°C for 2 days. Ultrathin Lowicryl sections were cut on an LKB III ultramicrotome.

Immunogold labeling of ultrathin cryosections

Frozen sections were transferred to carbon-coated Formvar grids and immunogold labeled according to Slot *et al.* (1988). Thawed sections of MDCK cells were incubated for 1 hour at room temperature with a rat anti-Forssman monoclonal antibody (diluted 1:2500) and subsequently with 12 nm protein A-gold (OD_{520}=0.2). Sections of human lung tissue were labeled with a polyclonal antibody against the hydrophobic surfactant protein SP-B (diluted 1:225) and subsequently with 10 nm protein A-gold (OD_{520}=0.2). Protein A-gold probes were prepared according to Slot and Geuze (1985). Antibodies and protein A-gold probes were diluted in 1% bovine serum albumin in 20 mM phosphate buffered saline, pH 7.4. Sections were observed in a Jeol 1200EX electron microscope at 80 kV.

Immunogold labeling of Lowicryl sections

Ultrathin Lowicryl sections of MDCK cells and human lung biopsies were placed on Formvar-carbon-coated copper grids and labeled according to the following procedure. Non-specific binding sites were blocked by incubating the sections for 30 min on a drop of 5% fetal calf serum in 20 mM Tris, 130 mM NaCl (TBS). Sections were incubated at room temperature with primary antibody (anti-Forssman diluted 1 to 2500 and anti-SP-B diluted 1 to 225) in 0.1% bovine serum albumin in 20 mM Tris, 130 mM NaCl, pH 8.4 (BSA/TBS) for 1 hour. After rinsing for 3 times 10 min with BSA/TBS the sections were incubated with protein A-gold (12 nm and 10 nm subsequently) for 1 hour and rinsed for 3 times 10 min with BSA/TBS, 2 times 5 min with TBS and 3 times 10 min with distilled water. Sections were stained for 7 min with 3% uranyl acetate and for 4 min with Reynolds lead citrate and observed in a Jeol 1200EX electron microscope at 60 kV.

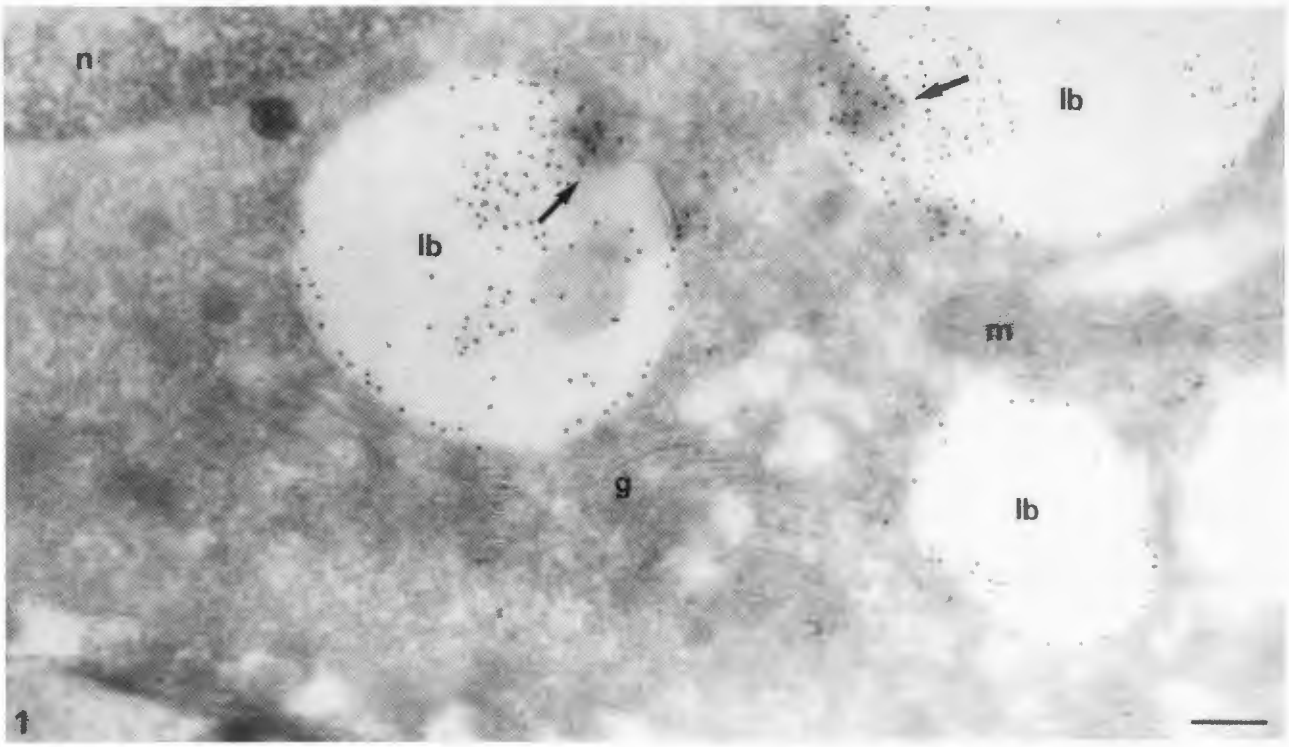


Fig. 1. Ultrathin cryosection of human lung labeled for SP-B. The content of lamellar bodies (lb) has disappeared but remnants of lamellar bodies (arrows) are clearly labeled. Mitochondria (m), the nucleus (n) and the Golgi complex (g) are negative. Bar = 0.2 μm.

Results

Lung tissue

The overall morphology of ultrathin cryosection of lung tissue shows good structural preservation of cellular organelles except for lamellar bodies. Most of the content of lamellar bodies is disappeared after thawing of the cryosections, leaving some remnants of lamellar structures and collapsed lipidic structures (Fig. 1). Due to the very loose and inhomogeneous structure of lung tissue, flat ultrathin cryosections are very difficult to obtain. Most ultrathin cryosections show compression and many wrinkles. The remnants of lamellar bodies and some multivesicular structures were heavily labeled using the antibody against SP-B (Fig. 1). Other cellular structures such as mitochondria, endoplasmic reticulum, the Golgi complex and the nucleus showed no label above background.

Freeze-substitution and Lowicryl embedding of prefixed and cryoprotected lung tissue results in a very good overall morphology. Especially the content of lamellar bodies is well preserved, revealing the closely stacked membrane structures (Fig. 2). Low magnification overviews are easily obtained since large flat sections can be made routinely without problems. Immunogold labeling with the antibody against SP-B reveals the same sub-cellular distribution as on cryosections. However, in the well preserved lamellar bodies it can now be observed that the SP-B protein is homogeneously distributed over the lamellar content of lamellar bodies (Fig. 2). The nucleus, endoplasmic reticulum, the Golgi complex and mitochondria again show no labeling above background. Control experiments in which the primary antibody was omitted were devoid of labeling both on cryosections and Lowicryl section.

MDCK cells

The preparation of ultrathin cryosections of MDCK cells grown on filters often resulted in rupture of the cells from the filters and wrinkled sections. Labeling of ultrathin cryosections with the antibody against Forssman and protein A-gold results in a sparse labeling

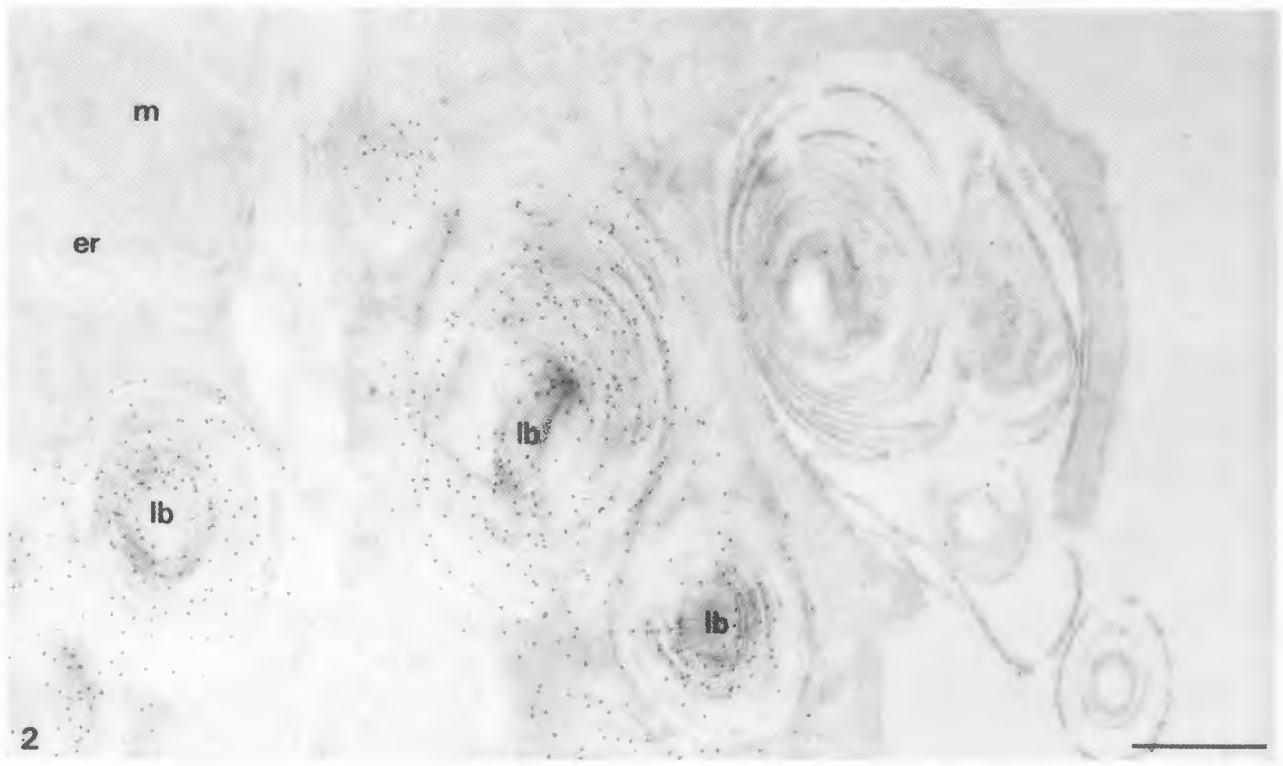
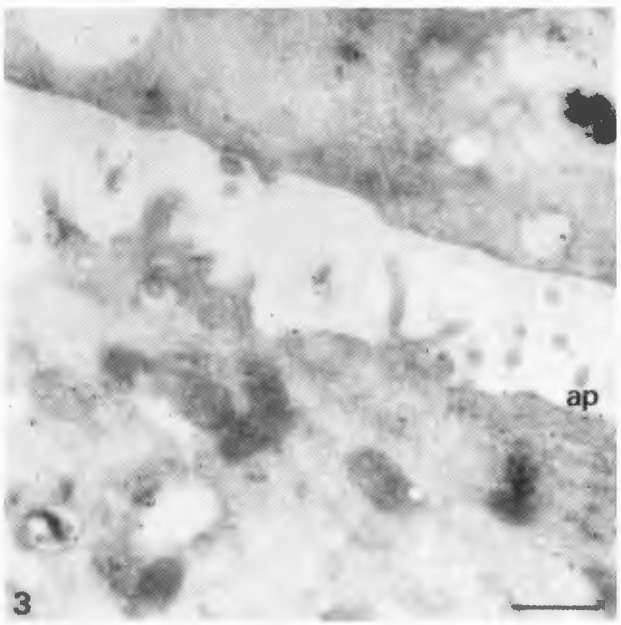


Fig. 2. Lowicryl HM20 section of freeze-substituted human lung labeled for SP-B. Lamellar bodies (lb) are very well preserved and label densely. The gold probe is distributed over the stacked lamellae. Mitochondria (m) and endoplasmic reticulum (er) are negative. Bar = 0.5 μm.

of the apical plasma membrane (Fig. 3). Besides the plasma membrane many intracellular organelles are labeled (Fig. 4). The Golgi complex is clearly labeled. Also electron lucent, electron dense and multivesicular vesicles are labeled. Besides this organelle-associated labeling we often observe a smear of gold labeling over the cytoplasm and the nucleus on cryosections of MDCK cells (Fig. 4). This labeling pattern over the cytoplasm and the nucleus is probably due to relocation of the Forssman glycolipid after section thawing and during the immuno-incubations.

In contrast, prefixed and cryoprotected MDCK cells show an improved morphology after freeze-substitution and low temperature embedding in Lowicryl HM20 compared to ultrathin cryosections (Fig. 5). MDCK cells are clearly polarized with microvilli on the apical plasma membrane, tight junctions with neighboring cells, a more basal located nucleus and clear Golgi complexes located mostly at the apical site of the nucleus. Many large and smaller vacuoles show internal vesicles and membrane structures.

Immunogold labeling on Lowicryl sections of freeze-substituted MDCK cells results in a dramatic increase in the density of labeling of both the apical and the basolateral plasma membrane (Fig. 5). The increased labeling density on the plasma membrane, as compared to cryosections, suggests that the freeze-substitution procedure preserves this glycolipid much better than the cryo-ultramicrotomy method. Control experiments in which the primary antibody was omitted show no gold labeling. The nuclear matrix is always free of gold label for Forssman which shows that there is no redistribution of the Forssman glycolipid during the preparation procedure and subsequent labeling steps. Mitochondria, which do not contain glycolipids, show hardly any gold label for Forssman (Fig. 6). These unlabeled mitochondria indicate the high specificity of labeling and the very low background labeling. Unlabeled mitochondria also indicate that in vivo nor during the whole procedure there is no exchange of glycolipids between membranes. Intracellular labeling is found in small vesicular and tubular structures underneath the plasma membrane, in



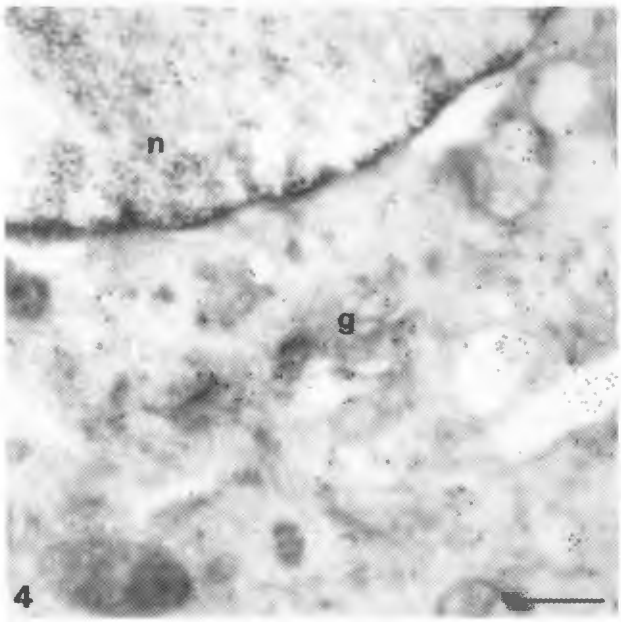


Fig. 3. Ultrathin cryosection of a MDCK cell showing sparse labeling of the apical plasma membrane (ap) for the Forssman glycolipid. Bar = 0.5 μm.

Fig. 4. Labeling for the Forssman glycolipid on an ultrathin cryosection of a MDCK cell. Label is found over the Golgi complex (g), electron lucent vesicles, the nuclear matrix (n) and the cytoplasm. Bar = 0.5 μm.

larger vacuoles associated to small vesicles and membranes, in the Golgi complex, in vesicular structures throughout the cytoplasm and in the nuclear envelope. Apparent cytoplasmic labeling turned out to be associated to small vesicular or tubular structures at high magnifications (Fig. 7).

Discussion

The benefits of freeze-substitution in combination with Lowicryl HM20 embedding at low temperature are demonstrated for the preservation of lipidic structures and the localization of glycolipids. Freeze-substitution has resulted previously in the retention of lipids. Dehydration at low temperature in combination with uranyl acetate, which probably complexes with lipids, prevents the extraction of lipids (Weibull and Christiansson 1986; Humbel 1984; Humbel and Müller 1984). Morphological studies have also shown that freeze-substitution is capable of preserving pure lipidic structures (Verkleij *et al.* 1985). Lamellar bodies of lung alveolar type II cells contain about 90% lipids and 10% proteins (van Golde *et al.* 1988). These lamellar bodies can not be preserved in ultrathin cryosections probably due to their high lipid content. Freeze-substitution in combination with embedding in Lowicryl HM20 at low temperature results in an improved preservation of the lamellar bodies compared to ultrathin cryosections. The appearance of stacked membranes is in agreement with the morphology of freeze-fractured lamellar bodies (Williams 1978) and classical Epon sections (Kalina and Pease 1970). Berryman and Rodewald claimed in their study (Berryman and Rodewald 1990) that post-fixation with uranyl acetate, dehydration in aceton at -20°C and low temperature embedding in LR-Gold increased the retention of membrane phospholipids. When we applied their method to our prefixed lung tissue we found no retention of the stacked lamellar content of lamellar bodies. Therefore it is probably the dehydration in methanol with uranyl acetate at -90°C which preserves these stacked lamellar membranes. Sparse data in literature have indicated that lipids can be retained to various extends using different substitution media and dehydration protocols. During dehydration by progressive lowering of temperature and Lowicryl HM20 embedding at -70°C about 89% of the lipids were retained in ethanol, 86% in acetone and 54% in methanol (Weibull and Christiansson 1986). During freeze-substitution in acetone 95% of the lipids were retained and in methanol 55 to 85% (Weibull *et al.* 1984). The addition of 0.5% uranyl acetate to methanol reduced the extraction of lipids during freeze-substitution to 2% at -70°C and 4% at -30°C compared to 9% at -70°C and 15% at -30°C in pure methanol (Humbel 1984). Taken

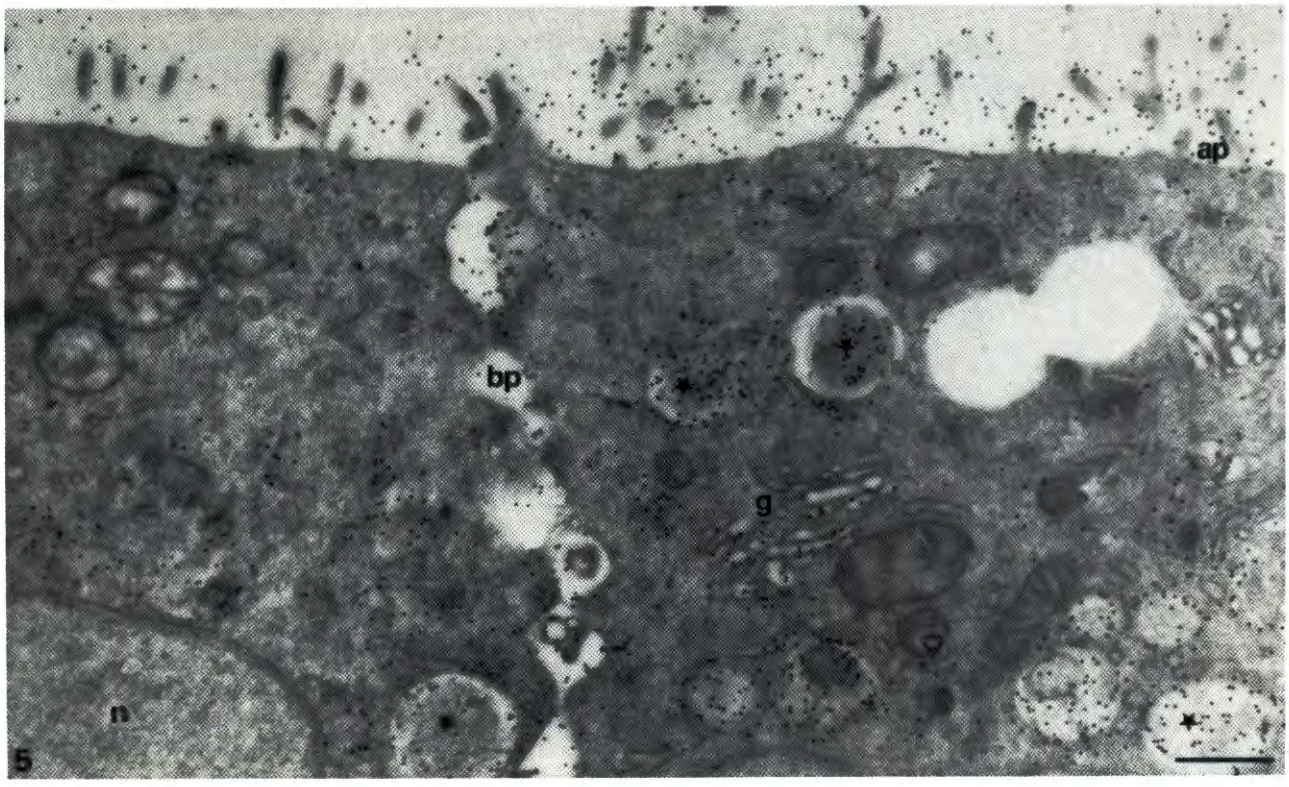


Fig. 5. Lowicryl HM20 section of freeze-substituted MDCK cells labeled for the Forssman glycolipid. Both the apical (ap) and basolateral (bp) plasma membrane are labeled heavily. Various intra-cellular organelles such as the Golgi complex (g), small vesicular structures, electron lucent and electron dense vesicles (*) are clearly labeled. The nuclear matrix (n) and mitochondria (m) are negative. Bar = 0.5 μm.

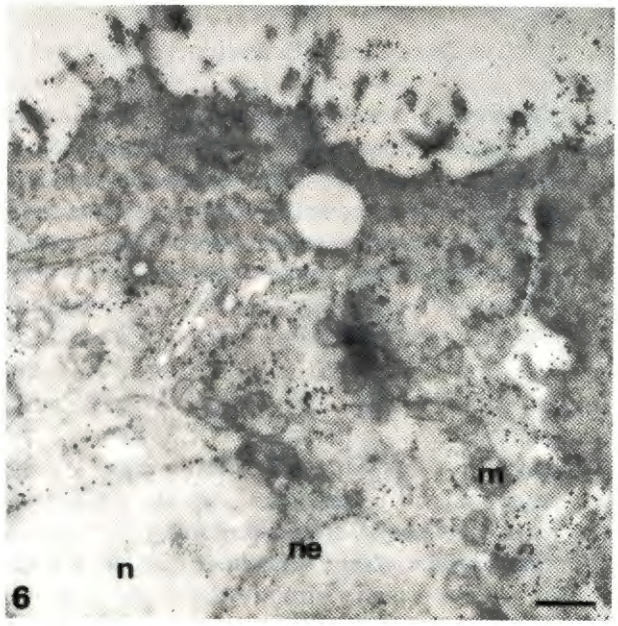
into account both these data and our practical experience with different substitution protocols we preferred methanol with uranyl acetate over acetone for freeze-substitution. Whether freeze-substitution in pure acetone or progressive lowering of temperature will give comparable results we do not know.

The substitution method does not only result in an improved structural preservation but also allows the localization of the hydrophobic surfactant protein SP-B. Although many membrane associated proteins give lower labeling levels on Lowicryl HM20 sections (unpublished results) this protein gives an abundant labeling over lamellar bodies.

Immunogold labeling of the Forssman glycolipid shows a drastic increase of labeling on Lowicryl HM20 sections of freeze-substituted MDCK cells (prefixed in 2% paraformaldehyde) compared to ultrathin cryosections. Ultrathin cryosections of 2% glutaraldehyde fixed MDCK cells often showed a smear of labeling over the sections resulting in labeling over the nuclear matrix and mitochondria. MDCK cells fixed in 2% paraformaldehyde showed no increase of labeling on ultrathin cryosection whereas the morphology decreased dramatically. Despite the increased labeling on Lowicryl HM20 sections, labeling was absent over the nuclear matrix and mitochondria. These results suggest that glycolipids are better preserved during freeze-substitution, since the increase in labeling after freeze-substitution is not due prefixation with paraformaldehyde instead of glutaraldehyde fixation and no redistribution of this glycolipid takes place during the preparation procedure and subsequent immunolabeling.

Conclusion

We have shown that this method results in improved structural preservation in combination with a highly reproducible intracellular localization of a hydrophobic



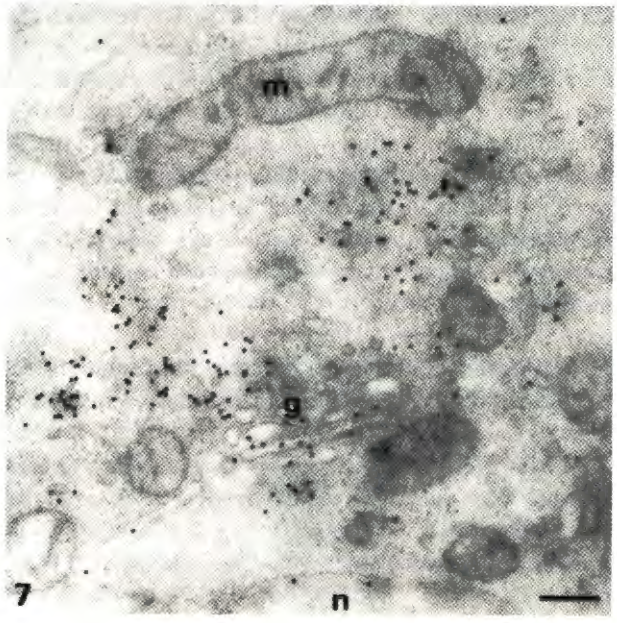


Fig. 6. MDCK cell labeled for the Forssman glycolipid after freeze-substitution and Lowicryl HM20 embedding. Besides the apical and basolateral plasma membrane and other cytoplasmic organelles the nuclear envelope (ne) shows specific labeling. Mitochondria (m) and the nuclear matrix (n) are negative. Bar = 0.5 μm.

Fig. 7. Higher magnification of a freeze-substituted MDCK cell labeled for the Forssman glycolipid. Small vesicular structures in the cytoplasm near to the Golgi complex (g) are labeled. Mitochondria (m) are negative. Bar = 0.2 μm.

membrane associated protein and of a glycolipid at the electron microscopical level. This powerful preparation method for post-embedding immunocytochemistry will extend our present insights in the intracellular traffic of membrane lipids.

Acknowledgements

The authors thank Tineke Veenendaal for the excellent technical assistance, and Tom van Rijn and René Scriwanek for preparing the photographs.

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Discussion with Reviewers

L. Edelmann: Although the method described is most likely to become a very powerful routine method in immunocytochemistry it would be interesting to know whether similar results can be obtained without using chemical fixatives and cryoprotectants before freezing and without using uranyl acetate in the substitution fluid. According to the work of Weibull *et al.* (J. Microsc. **134**, 213-216, 1984) it appears feasible to preserve lipids during freeze-substitution in pure acetone and low temperature embedding. Do you have some preliminary results?

Authors: The omission of chemical prefixation and cryoprotection will be one more step closer to the *in vivo* situation and will therefore probably give similar or even better results. There is however a technical problem in achieving this goal since the depth of proper freezing after rapid freezing without cryoprotection is limited to 5-10 μm. Especially for lung tissue it is almost impossible to freeze without ice crystal formation. To overcome this problem the strategy of prefixation and cryoprotection was chosen which resulted in a marked improvement compared to ultrathin cryosections. Concerning the use of uranyl acetate, we included uranyl acetate in our substitution medium, which was methanol, since it is known from several studies that it improves the retention of lipids. We have never tried aceton or methanol without uranyl acetate as a substitution medium.

L. Edelmann: Did you try other fixation solutions for fixing lung biopsies? Is it necessary to use acrolein and dimethylsulfoxide?

Authors: We have tried various fixatives for the lung biopsies including Karnovsky fixative, 2% paraformaldehyde with 0.2% glutaraldehyde and also aldehyde fixation followed by uranyl acetate block staining. All fixatives resulted in loss of the lamellar bodies after cryosectioning but the described fixative gave the best overall morphology although acrolein and dimethylsulfoxide can be omitted without considerable loss in structural preservation.

L.Edelmann: Suske and Acker (Can J. Bot. **67**, 1768-1774, 1989) have shown that biological material may be cryoprotected by 0.6 M sucrose (as in the Tokuyasu technique) before freezing and freeze-substitution in methanol. Did you try sucrose as a cryoprotectant?

Authors: We have used 2.3 M sucrose and 1.6 M sucrose as a cryoprotectant and found no difference in comparison to 30% glycerol.

L.Edelmann: Ikeda *et al.* (J. Electron Microsc. **33**, 242-247, 1984) have shown that "lamellar" bodies reveal a homogeneous interior when freeze-substituted in acetone+4% OsO_4. This finding suggests that lamellar arrays of phospholipid molecules are probably masked in the living cell by dense homogeneous material which is difficult to retain even during freeze-substitution. What is your opinion about this problem?
Authors: We do not believe in masking of the lamellar arrays in living cells. The use of different preparation techniques for electron microscopy has shown the lamellated structure of lamellar bodies in our hands. Freeze-fracture replicas of non-fixed fast frozen lung tissue, classical Epon sections and even ultrathin cryosections (although very exceptional) have depicted the typical lamellar structure of lamellar bodies. The observations of Ikeda, who finds a dense homogeneous interior of lamellar bodies is most probably a result of the use of 4% osmium in aceton and therefore an artefact of the preparation method.

R.M. Albrecht: Has surfactant been released from the large multivesicular body in the upper right of figure 2?
Authors: The lamellar body in the upper right of figure 2 is captured during secretion of its content in the alveolar subphase.

J.A. Hobot: What are the authors' views on acetone, not used in their methods? Weibull and co-workers showed that acetone is most efficient in retaining lipid during cryosubstitution. What are the data for lipid retention in uranyl acetate (UA)/methanol in comparison to acetone? What is the possible role of UA in preserving lipid? Could not UA in some cases interfere with immunolocalization? What procedures would the authors recommend in this instance?
Authors: The aim of this study was to have a method which combines structural preservation of lipid-rich structures, retention of glycolipids and still allows the immuno-localization of proteins and glycolipids. From the literature there are sparse data available on the retention of lipids during dehydration with several substitution media. The data show that both pure acetone and methanol supplemented with uranyl acetate preserve lipids to about 95% during freeze-substitution. For practical reasons we have been using methanol supplemented with uranyl acetate since methanol substitutes the ice very efficient due to its high possible water content at -90°C (29%) whereas acetone has to be completely dry for an efficient substitution since it can contain only 2% water at -90°C. Whether acetone gives similar results as methanol/uranyl has not been tested. The retention of lipids in the methanol/uranyl mixture is probably due to electrostatic interactions of the uranyl ions with phospholipid headgroups. We cannot exclude that uranyl acetate will interfere with the immunolocalization. When it does so we don't know whether this is a direct effect of the uranyl acetate on the antigenic site or due to the retention of the lipids which then mask the antigenic site as can be the case for example with integral membrane proteins. When it is a direct effect of the uranyl acetate itself substitution in acetone can be an alternative.

J.A. Hobot: Low temperature embedding at -50°C (PLT) in Lowicryl HM20 can preserve lipids practically to the levels of cryosubstitution (89% vs 95%, Weibull and coworkers). Why didn't the authors use this technique or credit in their discussions?
Authors: The PLT method was not chosen for 2 reasons. Firstly, from a theoretical point of view freeze-substitution is better than PLT since dehydration starts at the lowest possible temperature and finally ends at the embedding temperature whereas PLT starts at 0°C cooling down to the embedding temperature increasing the concentration of dehydration liquid. When extraction of lipids takes please it probably will be more at the higher temperatures. Secondly, which is more of practical reasons, we have a good freeze-substitution machine for freeze-substitution in a very controlled and reproducible way and in which we can do the embedding and polymerization at low temperature.

J.A. Hobot: The difference in labelling observed in MDCK cells after ultracryomicrotomy or cryosubstitution could be due to different fixation regimes used. Why didn't the authors use the same fixation protocols for MDCK cells? What is the structure of the Forssman glycolipid which shows that it would be totally unaffected by fixation?
Authors: The difference in labeling in MDCK cells after cryo-ultramicrotomy or freeze-substitution is not due to different fixations. There was no increase in labeling on cryosections after fixation with 2% paraformaldehyde compared to MDCK cells fixed with 2% glutaraldehyde. However the structural preservation was markedly reduced after fixation with 2% paraformaldehyde. Since the Forsmann glycolipid consists of a ceramide backbone with five sugar residues, lacking amino groups which can react with aldehyde groups, one does not expect an effect of the aldehyde fixation on the retention or antigenicity of this glycolipid.