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High-pressure freezing and freeze substitution of gravid *Caenorhabditis elegans* (Nematoda: Rhabditida) for transmission electron microscopy

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Summary – Because chemical fixatives have a profound negative influence on tissue morphology and antigenicity, alternative fixation methods must be applied for some purposes. In this work we used high-pressure freezing (HPF) followed by freeze substitution to maximally preserve antigenicity and morphology. We developed a pipette method for bringing living *Caenorhabditis elegans* nematodes into the HPF recipient. Using cellulose tubes, it is possible to select individual nematodes for fixation. We were able to HPF complete adults and preserve the morphology in an enhanced fashion compared to chemically fixed tissue. Cellular organelles, especially mitochondria, were much better preserved. Uterine embryos protected by the intact eggshell were excellently preserved without the need for elaborate techniques. Antigenicity with MH27 and ICB4 antisera was tested. With the MH27 serum, an adequate, reproducible, specific binding pattern with chemically fixed tissue could only be achieved using purified antibodies, whereas with high-pressure freezing, unpurified MH27 antisera was effective. For ICB4 antisera, a reproducible specific binding pattern was achieved at a concentration of primary antiserum 1000× lower than that for chemically fixed tissue.

Keywords – antigenicity, antisera, Cryo-TEM, morphology, ultrastructure.

When specimens are fixed chemically, we observe the reaction of the cell to the fixative (Robards & Sleytr, 1985). For over 50 years, chemically fixed tissue has been studied by transmission electron microscopy and a huge set of data produced concerning many aspects of the cell. The technique is still being refined for several cell types, although it is clear that there are serious disadvantages with chemical fixation. The cross-linking nature of the fixatives has negative effects on the tissue; cell ultrastructure is altered, and proteins are modified due to irreversible binding of epitopes to the fixative, and hence are often no longer sufficiently recognised by probes. All fixatives are selective. They bind only to particular types of molecules, all other types leaching out during dehydration and being lost. This selectivity is inherent to the fixative. Non-selective chemicals, which bind every type of molecule, do not exist and are unlikely ever to be developed. Furthermore, the duration of a fixation process takes considerably longer than most of the fast cellular processes, thus preventing the analysis of many of these processes when using classic fixation methods. The use of chemical fixatives might introduce secondary artefacts that are difficult to link to fixation. In the case of glutaraldehyde, formation of anoxia artefacts is possible due to the consumption of oxygen by this fixative during the fixation process (Gilkey & Staehelin, 1986). An additional problem has been that fixation of the whole animal, even with small invertebrates, is difficult to achieve using chemical fixatives. It is nearly always necessary to permeabilise the animal, mostly by cutting it into pieces, in order to achieve satisfactory results. All these deficiencies are inherent to the reaction mechanisms of the chemical fixatives, and using another fixative will not solve these problems.

From the late 60s onwards, alternative methods have been presented, based on freezing of water in biological objects. A non-crystalline, amorphous state of water is achieved by cooling down the specimens extremely

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rapidly (vitrification). No ice crystals are formed, resulting in the absence of phase-segregation of molecules and ions (Bachmann & Mayer, 1987). These techniques can immobilise the cell contents non-selectively, are very fast and, because there is no cross-linking, epitopes are preserved unaltered, allowing improved binding with probes.

During freeze substitution, vitrified water is diluted and replaced by an organic solvent. During the dehydration, a return to room temperature is achieved. Trial and error experiments of a wide scale of solvents resulted in the recommendation of acetone and methanol as the most suitable substitution solvents (Humbel *et al.*, 1983). Methanol substitutes faster than acetone, but results in a more significant loss of lipids (Nicolas & Bassot, 1993) and cannot be used for plant tissues (Parthasarathy, 1995).

Since the commercialisation of HPF equipment, many cell types were HPF processed and subsequently freeze substituted for transmission electron microscopic studies. For each tissue type, amended protocols had to be developed. Most of the previous studies concentrated on one, or only a few, cell types, but none of these approach the complexity of cryo-immobilising and substituting an entire organism comprising many different cell types and varying cell contents. Müller-Reichert *et al*. (2003) developed, using the same equipment, a technique which allows orientation only by using a thin layer of resin. We set out to develop a single protocol to cryo-immobilise *C. elegans* specimens with a superior preservation of morphology and antigenicity for all tissues in the animal and allowing orientation in each direction.

In this work, a HPF and freeze substitution protocol for ultrastructural morphology and for immunohistochemical analysis of *C. elegans* is presented.

Material and methods

Nematode culture

The free-living nematode *Caenorhabditis elegans* N2 was obtained from the Caenorhabditis Genetics Centre (CGC). *Escherichia coli* OP50 was used as a food source. Stock cultures were kept at 15°C. Culturing and handling were as described by Brenner (1974).

Preparation

Since ambient pressure cryo-techniques are limited to a specimen thickness of 30 μm (Sitte *et al.*, 1987), only the hyperbaric cryo-technique is suitable to cryo-immobilise entire *C. elegans* specimens which are *ca* 90 μm thick.

The HPF was carried out in copper tubes (350 μm inner diam., 640 μm outer diam., Leica, Vienna, Austria). At first, the living nematodes were washed off from the culture plate with distilled water and aspirated using a 20 μl Gilson pipette. This, however, resulted in the formation of a plug of specimens at the entrance of the copper tube and, as a consequence, nematodes were absent from the centre of the tube, *i.e*., the best position for HPF. As a result, we subsequently used transparent, porous cellulose tubes with a diameter of 200 μm (Hohenberg *et al*., 1994). One cellulose tube was put in a copper tube so that the cellulose protruded from both ends. A standard mouth pipette equipped with a fine tip (<200 μm diam.) capillary glass, which fits in the cellulose tube (Fig. 1) was used to aspirate, allowing individual selection of nematodes. After aspiration, the copper tube was positioned at the middle of a concentration of nematodes in the cellulose tube. The cellulose tube was then cut at both ends so as to fit in the copper tube. Using a Leica Recycling Kit device (Leica), the copper tube was loaded in the specimen holder and high-pressure frozen in a Leica EMPact.

HPF and transfer to the freeze substitution apparatus

High-pressure freezing was carried out with a Leica EMPact (Leica). Only specimens which were frozen at pressures above 196 MPa were further processed. Because the high pressure and the low temperature occur solely at the centre of the copper tube, specimens at the periphery are not properly high-pressure frozen. For this reason, the centre of the tube is cut out (5 mm long) using a Tube Punch device (Leica). To provide an optimal contact between the specimen and the substitution fluid, the copper tube is peeled open using a Banana Peeler device (Leica). A longitudinal section is made through the copper wall, uncovering the contents of the tube.

Freeze substitution

Freeze substitution was carried out using a Leica AFS (Leica). The freeze substitution process consists of a substitution phase, performed in a medium containing the organic solvent (acetone or methanol) and chemical fixatives (tannic acid and OsO_4) at −90°C. We tested a substitution phase of 60 and 90 h. The warming up phase lasted for 15 h with plateaus at −60°C and −30°C, each lasting 8 h. During the warming up phase, dehydration

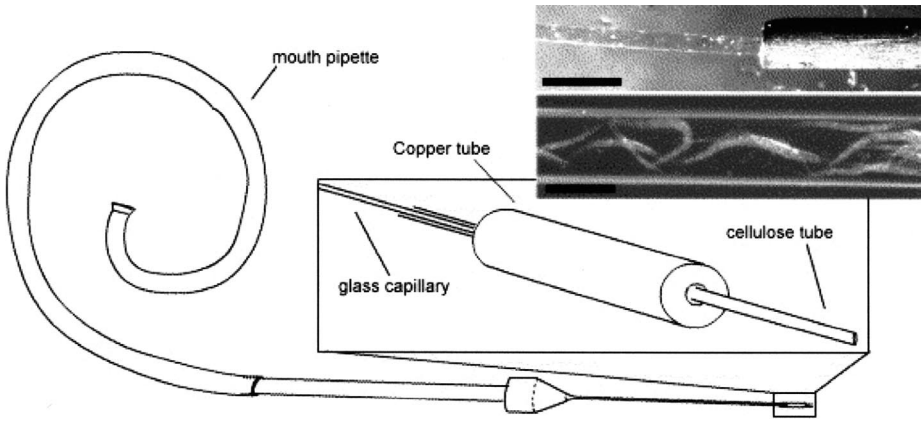

Fig. 1. *Schematic illustration of the pipette technique used to aspirate the nematodes in the high-pressure freezing recipient. The end of the cellulose tube is put over the tip of a glass capillary, which is connected to a standard mouth pipette. A copper tube is positioned over the cellulose tube. Individual nematodes can be selected and aspirated from any vial containing nematodes into the cellulose tube. The nematodes show no change in behaviour when inside the cellulose tube for a long time. Next, the cellulose tube containing the nematodes is positioned in the middle of the copper tube and the protruding remnants of the cellulose tube are cut away. The specimens can now be directly high-pressure frozen. The whole procedure takes a maximum of 45 s. The top photograph shows the cellulose tube fitting into a copper tube, bottom photograph shows* C. elegans *in the cellulose tube. (Scale bar: top = 20 μm, bottom = 200 μm.)*

is achieved. Specimens for immunohistochemical studies were substituted in a medium containing only the organic substituent. The protocols for ultrastructural morphology studies differ depending on the solvent used, the duration of the substitution phase and the relative duration of exposure to chemical fixatives (Table 1).

Embedding

For ultrastructural morphology studies, specimens were embedded in Spurr's resin (Agar, Essex, UK). Because of the cross-linking nature of this epoxy resin, specimens for immunolocalisation studies were embedded in LR White, an acrylic resin.

Trimming was performed using a Leica Ultratrim (Leica). The 70 nm thick sections were cut using a Reichert Ultracut S (Leica) and collected on GILDER single slot grids (Laborimpex, Brussels, Belgium).

Immunocytochemical treatment

The antigenic features of the HPF tissue were evaluated by two monoclonal antibodies raised against *C. elegans* epitopes. ICB4 binds predominantly near the brush border of the intestinal lumen (Okamoto & Thomson, 1985) and MH27 binds on a protein present on the apical edge of desmosomes (Francis & Waterston, 1991). Antibodies were administered as a supernatant and therefore the exact concentration of the antibodies is unknown.

Grids were incubated in PBS (Sigma, St Louis, MI, USA) for 10 min followed by a 2 h incubation in the primary antibody in PBS at 4°C. ICB4 serum was diluted 1/1000, MH27 diluted 1/5. Grids were washed five times in PBS for 10 min and incubated in 1/50 diluted 15 nm gold labelled goat anti-mouse secondary antibody (Sigma) for 30 min at 22°C. The grids were washed five × 10 min in PBS and subsequently five × 10 min with distilled H_2O. Controls were done by omitting the primary antibody to evaluate the degree of non-specific background binding. With both monoclonal antibodies background staining was absent.

Post-staining

For the on-grid contrast staining, a Leica EM stain (Leica) with uranyl acetate and lead citrate (Van Hopplynus, Brussels, Belgium) was used. Further analysis of the tissue was done using a JEOL JEM 1010 electron microscope (Akishima, Tokyo, Japan) operating at 60 kV. Images were processed with Kodak Electron Microscopy Film (Eastman Kodak Company, Rochester, NY, USA) and developed with Kodak Developer D19.

Results

Table 1 summarises the results of the different substitution protocols tested. Using complete, uncut adults of

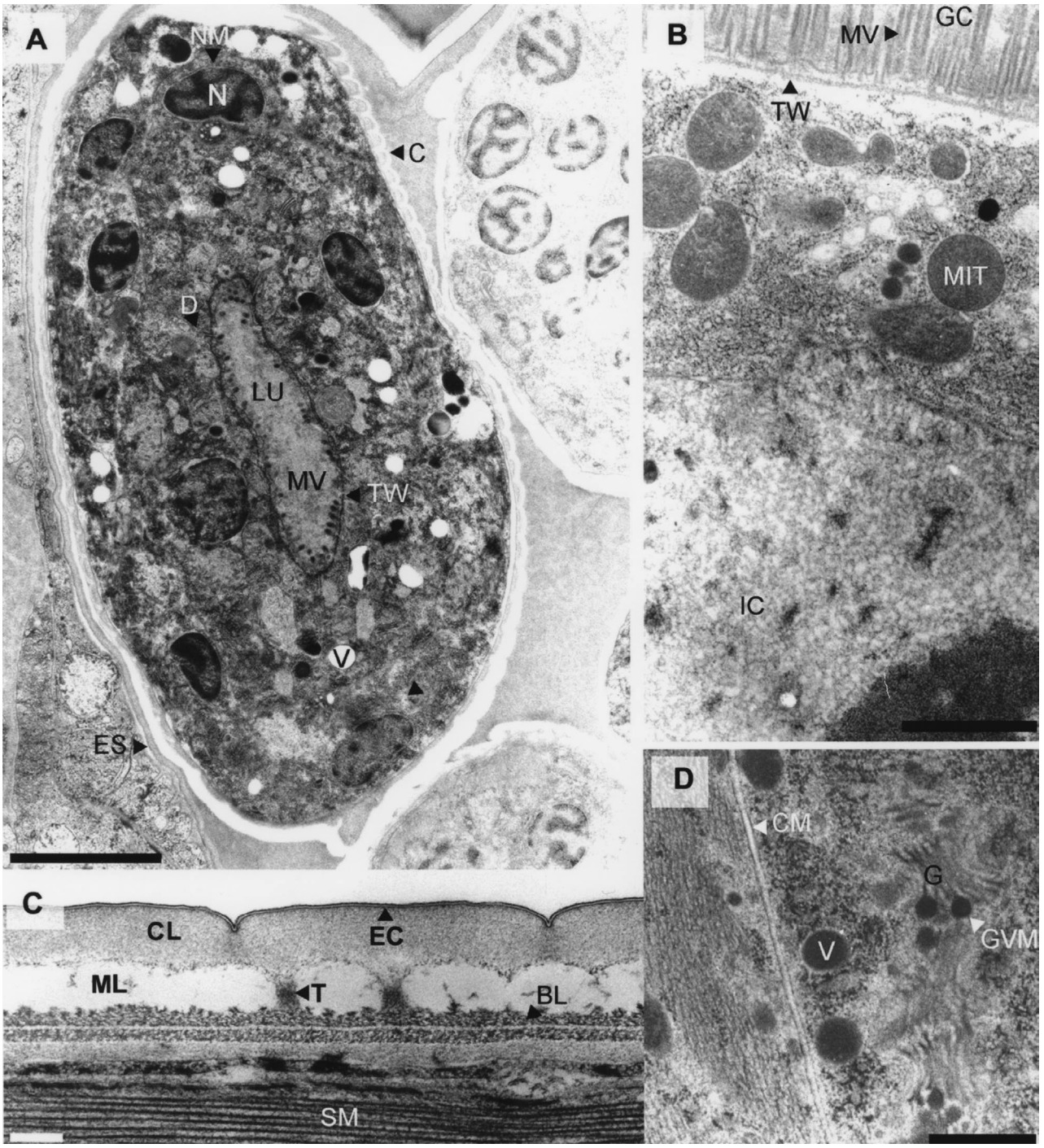

Fig. 2. Caenorhabditis elegans. *High-pressure freezing and freeze substitution with OsO_4, Spurr resin. TEM observations. A: Longitudinal section through uterus of hermaphrodite displaying a developing embryo. In the embryo, the formation of the cuticle, microvilli in the embryonic intestine, a terminal web and desmosome can be seen; B: Longitudinal section of adult tissue, situated near intestinal lumen. The mitochondria, as observed with high-pressure freezing, are abundant near the terminal web. These structures are generally round, with a dark content, concealing the cristae. The microvilli-terminal web-glycocalyx complex is clearly discernible;*

Table 1. *Summary of different freeze substitution protocols with effects on tissues, structures, organelles and antigenicity. (+) indicates a preservation of morphology that is better compared to chemical fixation; (±) indicates a preservation of morphology that is comparable to chemical fixation; (−) indicates a preservation of morphology that is worse than chemical fixed tissue (multiple signs are an indication of the improvement or otherwise).*

Substitution solvent	Morphology				Antigenicity	
	acetone		methanol		acetone	methanol
Duration of substitution phase	60 h	90 h	60 h	90 h	76 h	76 h
Duration of fixative exposure						
Tannic acid	24 h	72 h	24 h	72 h		
Osmium tetroxide	36 h	18 h	36 h	18 h		
General tissue condition						
Tissue tearing	−−	+	++	−		
Contrast	±	±	±	±		
Tissue perforations and leakage	−−	+	+	−		
Structures, tissues and organelles						
Epidermis and cuticle	−	+	+	+		
Ovum and encased embryo	+++	+++	+++	+++		
Cell membrane	±	+++	+	+		
Membrane of the nucleus	+	++	++	++		
Nuclear content	±	++	++	++		
RER	−−	+	++	±		
Mitochondria	−	+	++	+		
Golgi apparatus	++	++	++	++		
Microvilli and terminal web	−−−	+	+	−		
Vacuoles (lipid, yolk and lysosomes)	+	+	+	+		
Antigenicity						
MH27 antiserum						+++
ICB4 antiserum						+++

the nematode *C. elegans*, inadequate morphology preservation was obtained using short substitution times (60 h) with acetone as solvent. When substitution lasted up to 90 h a considerable improvement in morphology was achieved. Using methanol as substitution solvent resulted in excellent morphology after 60 h, comparable to 90 h acetone. A longer substitution with acetone or methanol substantially degraded the morphology. When comparing the acetone and methanol substituted tissues, the faster substitution with methanol resulted in a slightly better preservation of morphology than a longer substitution with acetone. In all tests, intact specimens could be recovered for embedding and sectioning. Several adult hermaphrodites carried embryos in their uterus which were already encased in an eggshell. As seen in Figure 2A, the eggs, with fully formed eggshell, were excellently preserved in the uterus with no visible artefacts. In all cases, tissue preservation of the embryos was excellent. In older

Fig. 2. *(Continued). C: Longitudinal section of the adult integument. The cuticle consists of a thin epicuticle, a cortical layer, a median layer and a basal layer. Trabeculae can be seen between the cortical and the basal layer. Somatic muscle fibres run parallel with the cuticle; D: Longitudinal section of adult tissue. Cell membranes are flat, and not undulating. Vesicles are not leached out and are typically round. Budding of vesicles at the Golgi apparatus can be arrested by this process. (BL = basal layer; C = cuticle; CL = cortical layer; CM = cell membrane; D = desmosome; EC = epicuticle; ES = eggshell; G = Golgi apparatus; GC = glycocalyx; GVM = Golgi vesicle membrane; LU = lumen; IC = intestinal cell; MIT = mitochondrion; ML = median layer; MV = microvillus; N = nucleus; NM = nuclear membrane; SM = somatic muscle; V = vesicle; T = trabeculae; TW = terminal web.) (Scale bar: A = 2 μm; B = 1 μm; C = 500 nm; D = 500 nm.)*

hermaphrodites, eggs are further developed and, regardless of developmental stage reached, tissue preservation was identical. Structures easily identifiable in the embryos include the pharynx, intestine with microvilli and desmosomes. The formation of an embryonic cuticle is evident, and annuli are present (Fig. 2A). Organelles such as rough endoplasmatic reticulum, nuclei with nucleoli, mitochondria and vacuoles containing lipid or yolk are present and clearly delineated.

In the adult animals, the double membrane of the nucleus can be observed. The chromatin is dispersed and not aggregated (Fig. 2B). Mitochondria appear round and filled with a darkly stained material that largely obscures the cristae. In some instances some elongation of the mitochondria was observed, but no branching was present (Fig. 2B). The fine structure of the terminal web and microvilli is evident (Fig. 2B). The fuzzy glycocalyx emanating laterally from the villi is prominent.

In the adult tissue, the layers of the cuticle are readily discernible *viz*, a thin epicuticula, a dark stained cortical layer, a lighter stained median layer and a double basal layer (Fig. 2C). Trabeculae were observed as darker bars in the median layer. Somatic muscle fibres run parallel with the cuticle and show dark-light stripes in a transverse direction.

Rough endoplasmatic reticulum (RER) is bordered by ribosomes and the lumen of RER and Golgi vesicles is darker stained than the surrounding cytoplasm (Fig. 2D). Prominent budding vesicles could be observed containing almost near-black contents. Other vesicles in the cryofixed tissue are typically round, with a clearly visible membrane (Fig. 2D).

With the MH27 serum, an adequate, reproducible, specific binding pattern with HPF processed tissue could be achieved using a non-purified antiserum (Fig. 3A). With MH27 we observe binding solely at the desmosomes of the intestine. A specific binding pattern can be distinguished, running parallel with the membranes of the desmosome. In a control incubation, deprived of the primary antibodies, no binding could be observed.

An analogous, specific binding pattern could be achieved with HPF, not osmificied tissue, using a 1000-fold lower serum concentration then with chemically fixed tissues containing the ICB4 antibody (Fig. 3B). Uniformly distributed binding with gold probes can be observed in the lumen of the entire intestine. This binding pattern suggests the epitope to be present, not only at or near the membrane and microvilli, but also in the centre of the lumen. In the control, deprived of primary antibody incubation, no binding at all can be observed in the lumen.

Discussion

Methodology

The technique employed in this study is comparable to that developed by Müller-Reichert *et al*. (2003). However, there are some notable differences. Instead of using a paper filter driven accumulation technique, we used a mouth pipette to suck in worms in the cellulose tubes. This gives us greater flexibility in choosing particular nematode stages of interest. Müller-Reichert *et al.* (2003) submerged the tubes in 1-hexadecene before HPF and, when the tube was filled with several nematodes, used a non-penetrating cryoprotectant. In our technique the nematodes were directly HPF in the distilled water in which they were collected, regardless of the quantity. This allows for a very short period of stress for the specimens before freezing, an essential prerequisite for high-pressure freezing of organisms as detailed by Moor (1987).

In the study of Müller-Reichert *et al.* (2003) it is stated that HPF nematodes in tubes has disadvantages when: *i*) selecting individual worms; and *ii*) orientating the specimens for embedding. In our technique we circumvented the first problem by sucking individual nematodes in the tube using a mouth pipette. In our protocol the nematodes float out of the tubes during freeze substitution into the Eppendorf tube. The nematodes are individually picked out and orientated as required in the resin before polymerisation. Using this technique there is no need to embed the tube.

Morphology

The improved morphology using longer acetone incubations can be explained due to the slower exchange rate of acetone (Humbel *et al*., 1983; Robards & Sleytr, 1985). Because of the faster exchange rate of methanol (Humbel *et al.*, 1983; Robards & Sleytr, 1985), a shorter methanol protocol resulted in morphology comparable to the long acetone protocol. The negative effect of prolonged incubations with methanol is most likely due to excessive lipid extraction by the methanol leading to tissue damage.

Preservation of the embryos was excellent in all the protocols used. The cuticle, and especially the eggshell covering the embryos, are strong barriers for chemicals, but not for pressure or temperature. A procedure for

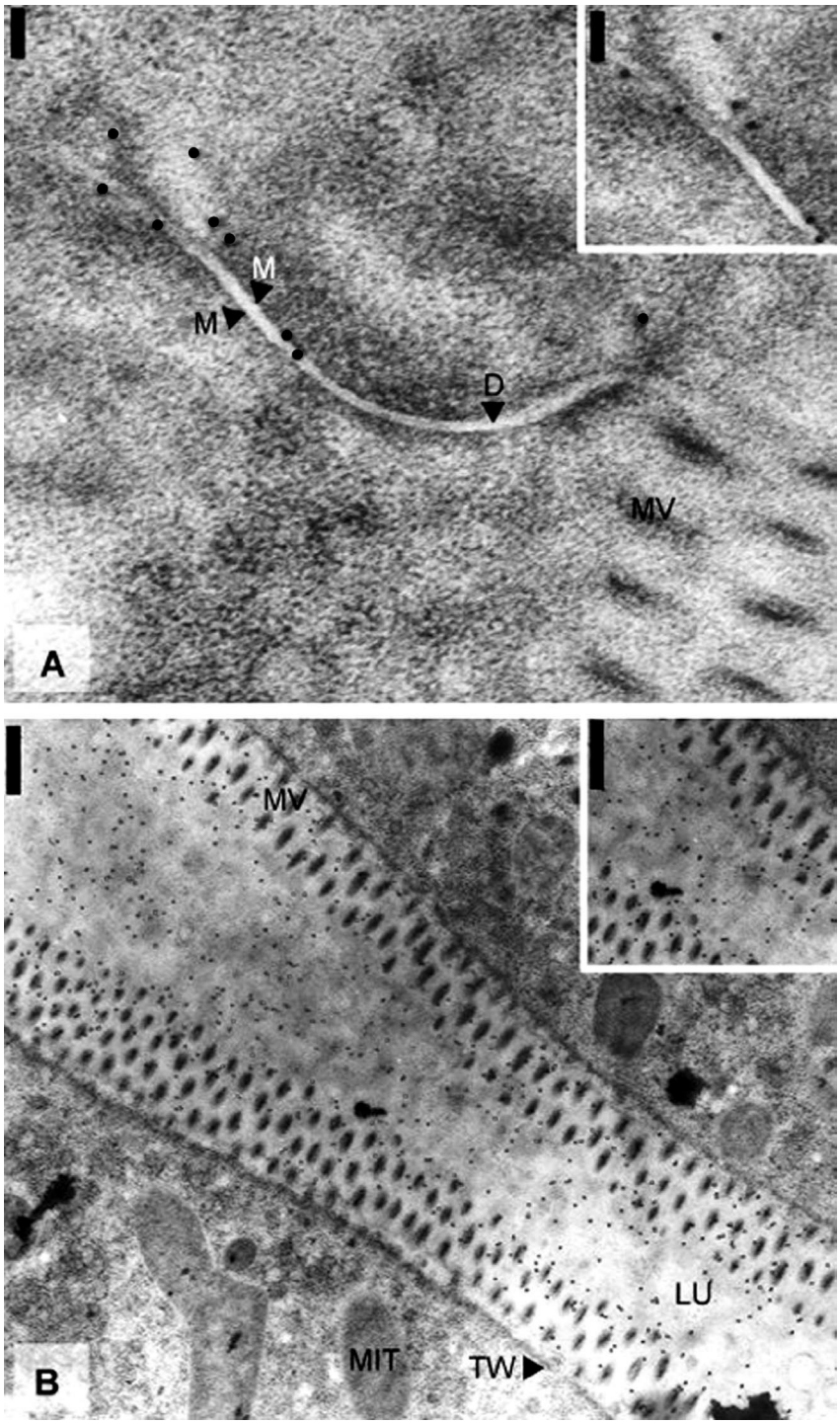

Fig. 3. Caenorhabditis elegans*. TEM observations (fixation by means of high-pressure freezing and freeze substitution without chemical fixative, embedded in LR white, immunolabelling of thin sections). The gold probes are enhanced with black dots with the original picture as an inset. A: MH27 binding pattern, longitudinal section; B: ICB4 binding pattern in longitudinal section of the intestinal region. (D = desmosome; LU = lumen; M = membrane; MIT = mitochondrion; MV = microvillus; TW = terminal web.) (Scale bar: A = 200 nm; B = 1 μm.)*

chemical fixation of nematode embryos was described by Vancoppenolle *et al.* (2000), but it is difficult and time-consuming and the resulting embryo, dissected from the hermaphrodite, is missing its eggshell and vitelline membrane due to enzyme and acid treatments. Using HPF techniques, embryos are present in the uterus of an intact adult and are arranged in the way they are in the living nematode.

The embryo does not touch the eggshell as there is a small gap between the embryonic cuticle and the inner wall of the eggshell. This might be an indication for a little shrinkage of the embryo, although this is also observed in living specimens. The space between the embryo and the eggshell is required to allow movement and cell migrations during embryogenesis. The quality of the morphology of HPF embryos is comparable with the preservation of HPF adult tissue and is much improved compared to embryos chemically fixed according to the protocol by Vancoppenolle *et al.* (2000).

In the somatic cells of the adult hermaphrodite, the absence of chromatin at the positions of the nuclear pores is also a confirmation of the non-appearance of push and pull forces during fixation.

The darker stained content of the RER and the Golgi vesicles suggests a good preservation of these organelles, their membranes, and their content. It also implies that during HPF the membranes are kept intact. Due to the ultra-short fixation time, the process of budding vesicles at the Golgi apparatus can be halted. The budding vesicles are, in comparison to the Golgi apparatus, larger in HPF processed tissues than in chemically fixed tissues. This difference in size in chemically fixed tissue might be due to leaching or a gradual inhibition of transport as the transport processes come to a halt as a result of gradual chemical fixation. This last possibility is based on the observation that adult *C. elegans* do not die instantly when put in the fixative. The Golgi derived vesicles are stained black, an indication of condensed, not leached out, content.

Antigenicity

Besides excellent preservation of morphology, there is an improved preservation of antigenicity. The cross-linking features of chemical fixatives have a very negative influence on the configuration of proteins, and hence alter epitopes. After HPF, no cross-links are formed, and antigenicity is preserved. The preservation of the tissue depends solely on the physical fixation; no chemical cross-linking fixatives are added during cryosubstitution. Compared to osmicated tissue, membranes are more blurred and there is less contrast. Despite these limitations, all structures could easily be identified. With chemical fixation, this was only possible using purified antibodies (Köppen *et al.*, 2001).

Because we could use an antiserum instead of an affinity-purified antibody (MH27) and a 1000 times lower primary antibody concentration (ICB4) to realise a reproducible binding pattern, we conclude that the preservation of the epitopes of both antibodies is, compared to chemically fixed tissues, much better. These two antibodies were chosen because in the past problems occurred in achieving a reproducible binding pattern with non-cryofixed tissue.

Mitochondria

The most substantial difference between chemically fixed and high-pressure frozen *C. elegans* tissue is the appearance of the mitochondria. In chemically fixed tissues they appear rather devoid of content, elongated and are often branched (Borgonie, 1995). We believe the HPF, dark appearing, oval mitochondria are most likely the authentic representation of the morphology of *C. elegans* mitochondria. First, because the fixation technique used so far, *i.e.*, chemical fixation, is not ideal, we cannot predict how structures really appear. New techniques can result in a surprisingly different morphology for certain structures compared to their chemically fixed, widely accepted morphology. Second, mitochondria are organelles with an important metabolic function. They produce a massive amount of ATP and are packed with a large quantity of proteins, especially in the membranes and the cristae. High-pressure frozen mitochondria are dark-stained, because the proteins did not leach out. By contrast, chemically fixed mitochondria are not darkly stained as extraction of solutes may occur during the slow fixation process.

The smooth oval outline of the HPF mitochondria is a confirmation of a normal turgor pressure. We are confident that there was no shrinkage, extraction or other forces that had a detrimental effect on the native state of organelles and that a natural turgor pressure was preserved. In addition to these arguments, Van Harreveld *et al.* (1965) made similar observations when comparing chemical and HPF rat brain tissue. Therefore, we conclude that this method reflects better the native state of the mitochondria than the chemically fixed techniques.

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