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Cryoimmobilization and three-dimensional visualization of *C. elegans* ultrastructure

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Summary

Caenorhabditis elegans is one of the most important genetic systems used in current biological research. Increasingly, these genetics-based research projects are including ultrastructural analyses in their attempts to understand the molecular basis for cell function. Here, we present and review state-of-the-art methods for both ultrastructural analysis and immunogold localization in *C. elegans*. For the initial cryofixation, high-pressure freezing is the method of choice, and in this article we describe two different strategies to prepare these nematode worms for rapid freezing. The first method takes advantage of transparent, porous cellulose capillary tubes to contain the worms, and the second packs the worms in *E. coli* and/or yeast paste prior to freezing. The latter method facilitates embedding of *C. elegans* in a thin layer of resin so individual worms can be staged, selected and precisely orientated for serial sectioning followed by immunolabelling or electron tomography.

Introduction

In the past few years, extremely efficient sequencing techniques have provided complete genetic information for several model organisms, including the nematode *Caenorhabditis elegans*. Currently, various techniques, including RNA-mediated interference (RNAi), are applied to answer fundamental questions related to molecular, cell and developmental biology. Importantly, for a description of *C. elegans* biology, the analysis of wild-type and mutant fine structure and the ultrastructural localization of gene products are crucial.

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Using conventional methods of specimen preparation for electron microscopy, *C. elegans* is an organism that is considered to be difficult to fix. When transferred into a buffered fixative, this nematode can respond very quickly and prevent fixative from entering its tissues. By blocking the diffusion of fixatives through the cuticle, *C. elegans* is able to survive up to 7 h in a solution containing 10% glutaraldehyde (Shepard & Clark, 1976). In order to permit penetration of the chemical reagents into the nematode, the worms have to be cut into pieces (Ward *et al.*, 1975; Hall, 1995) or warmed by microwave treatment (Jones & Gwynn, 1992). Compared with whole worms, it is even more difficult to fix isolated early embryos of *C. elegans*. To allow adequate chemical fixation by glutaraldehyde, the eggshell of the embryo has to be either digested enzymatically or treated with bleach (Rappleye *et al.*, 1999) or made porous with a laser beam (Priess & Hirsh, 1986; Kirkham *et al.*, 2003). Such problems related to the diffusion of chemical reagents can be avoided when high-pressure freezing in combination with freeze-substitution is applied. These high-pressure frozen/freeze-substituted samples are suitable for both morphological (Dernburg *et al.*, 1998; Rappleye *et al.*, 1999; Howe *et al.*, 2001; Kirkham *et al.*, 2003) and immunogold studies (Favre *et al.*, 1995; Kirkham *et al.*, 2003).

In this article, we describe and discuss two different methods for cryoprocessing of *C. elegans* samples. The first method takes advantage of transparent, porous cellulose capillary tubes with an inner diameter of 200 μm to contain the worms, which are drawn into the tubes by capillary action (Hohenberg *et al.*, 1994). In the second method, worms are packed in *E. coli* and/or yeast paste prior to high-pressure freezing/freeze-substitution and then embedded in a thin layer of polymerized resin. Using this method, individual worms can

be viewed by high-resolution light microscopy to preselect particular stages of development for precisely orientated sectioning (Rappleye *et al.*, 1999; Lonsdale *et al.*, 2001).

Materials and methods

C. elegans was cultivated on plates or in suspension according to published procedures (Brenner, 1974). Worms were prepared for high-pressure freezing using either the 'capillary tube' or 'thick paste' method.

Capillary tube method

Filling the capillary tubes. The capillary tube method was carried out as described (Hohenberg *et al.*, 1994). Briefly, the tip of a capillary tube (hollow cellulose fibre; type LD OC O2; Microdyn, Wuppertal, Germany) was inserted into a droplet of a nematode suspension and the nematodes were drawn into the tube by capillary action. The capillary was kept in place by a clamping mechanism (Fig. 1). A piece of filter paper was held against the opposite end of the capillary tube, and nematodes were trapped and concentrated in the capillary tube by passing medium out into the filter paper. After about 10 s, 2 mm of the capillary tube was packed densely with the worms. Suitably filled segments of the tubes were submerged in 1-hexadecene and cut with a scalpel. This cutting process sealed the ends of the segments. When there were low nematode concentrations inside the tube, the application of non-penetrating cryoprotectants was necessary. To achieve this, the tubes were filled with nematodes as described above. In addition, either a 10% solution of HES (hydroxy ethyl starch; Echlin *et al.*, 1977) or 10% Dextran (molecular weight Mr~40 000) was drawn into the tube by capillary action, displacing the cultivation medium prior to high-pressure freezing. This process of medium displacement and the preservation of nematode vitality can easily be monitored by light microscopy as a result of the transparency of the capillary tubes.

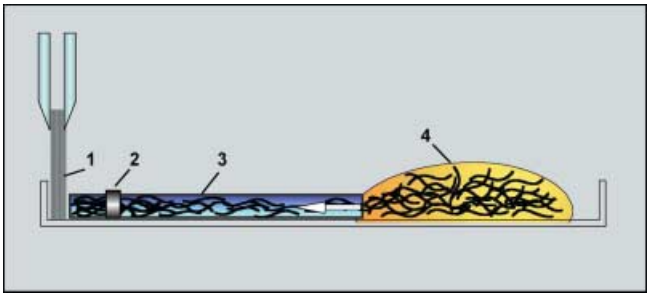

Fig. 1. Using capillary tubes for specimen preparation. The tip of a capillary tube is inserted into a drop of nematode suspension to fill the cellulose tube. Nematodes are drawn into the tube by capillary action. The capillary tube is kept in place by a clamping mechanism. A piece of filter paper is held against the opposite end of the tube to trap and concentrate the specimens. Suitably filled segments of the tube can be cut and sealed prior to freezing: 1, filter paper; 2, clamping mechanism; 3, capillary tube; 4, nematode suspension.

High pressure freezing. The 2-mm segments of the capillary tubes were fitted into the cavity (depth 200 μm) of the standard aluminium planchette (type A; Engineering Office M. Wohlwend, Sennwald, Switzerland) filled with 1-hexadecene. 1-Hexadecene is a hydrophobic and chemically inert paraffin oil of rather low viscosity. Therefore, cavities are easily filled (Studer *et al.*, 1989). Type A planchettes were sandwiched with the flat side of a complementary type B planchette. The sandwich was then inserted into the holder of the high-pressure freezer (BAL-TEC HPM 010, BAL-TEC, Liechtenstein; Moor, 1987) and frozen. The frozen sandwiches were stored in liquid nitrogen until further use.

Freeze-substitution, embedding and electron microscopy. Freeze-substitution was performed in anhydrous acetone containing 2% osmium tetroxide (van Harreveld & Crowell, 1964). The frozen capillary tubes were freed mechanically from adhering 1-hexadecene at −90 °C in the substitution medium. At −90 °C, 1-hexadecene is not soluble in acetone, methanol or ethanol, thereby possibly preventing the substitution of the aqueous phase. As a consequence, adequately frozen samples may be damaged by subsequent ice crystal growth, if substitution occurs only at elevated temperatures. The capillary tubes, freed from 1-hexadecene, were then transferred to 1.5-mL Eppendorf tubes containing the substitution medium precooled to −90 °C. A miniature 'transfer basket' was used to transfer the capillary tubes and change solutions during freeze-substitution (for details see Hohenberg *et al.*, 1994). The samples were kept at −90, −60 and −30 °C for 8 h at each step and finally brought to 20 °C for 1 h in a freeze-substitution unit (Leica EM AFS, Vienna, Austria). The samples were then washed three times in anhydrous acetone and embedded stepwise in Spurr's resin (30% and 70% resin in acetone) at 25 °C for 4 h at each concentration (Spurr, 1969). The capillary tubes were orientated either parallel or perpendicular to the rim of the embedding form prior to polymerization at 60 °C for 24 h. Thin sections (50 nm) were cut using a Leica Ultracut UCT Microtome (Leica), post-stained with 2% uranyl acetate followed by Reynold's lead citrate and imaged in a Philips CM 120 transmission electron microscope (FEI, The Netherlands) operated at 60 kV.

Thick paste method

Sample loading and high-pressure freezing. In preparation for high-pressure freezing, adult wild-type worms containing embryos were selected under a stereomicroscope and transferred to sample holders containing an *E. coli* suspension. Alternatively, a thick paste of yeast was made from dry bakers yeast and 10% methanol and worms were mixed with this paste and loaded into the high-pressure freezing planchette. About 50–

100 worms were placed in the 100-µm-deep well of a BAL-TEC type A planchette (McDonald, 1999; Engineering Office M. Wohlwend). Samples were covered with the flat side of a complementary planchette (type B) and rapidly frozen using a BAL-TEC HPM 010 high-pressure freezer (BAL-TEC). Frozen samples were stored in liquid nitrogen until further use.

Freeze-substitution and infiltration with resin. For freeze-substitution, specimen holders were split under liquid nitrogen and a single planchette type 'A', containing the nematode worms, was transferred to a precooled cryovial (–90 °C) containing the fixative. For morphological studies, freeze-substitution was carried out in anhydrous acetone containing 1% osmium tetroxide and 0.1% uranyl acetate (McDonald & Müller-Reichert, 2002). For immunolabelling studies, we used a 'cocktail' containing 0.2% glutaraldehyde and 0.1% uranyl acetate in anhydrous acetone (McDonald, 1999). Using a Leica EM AFS, samples were maintained at –90 °C for 3 days. Freeze-substituted samples were then allowed to warm to room temperature at a rate of 5 °C h^{-1}. At room temperature, specimens were removed from the planchettes and washed three times for 1 h in fresh anhydrous acetone. After the acetone washes, samples were then gradually infiltrated with either Epon/Araldite resin (1 part resin : three parts acetone) for 1 h; 1 : 1 for 2 h; 3 : 1 for 2 h, and 100% resin for 1 h, then overnight, then for 1 h), or LR White resin (1 part resin : 1 part acetone for 2 h; 100% resin 4 × 2 h and overnight).

Thin-layer embedding. Worms were embedded in thin, optically clear layers of resin on microscope slides. For Epon/Araldite embedding (McDonald, 1994; Fig. 2A), microscope slides were wiped clean with a soft cloth and coated with Teflon® spray (MS-122DF, Miller-Stephenson Chemical Co., Inc., Danbury, CT, USA). Worms in Epon/Araldite were evenly distributed on the cleanly wiped surface of the glass slide. Two layers of Parafilm® were used as spacers and coated slides were put on top of the resin samples. For LR White embedding (Fig. 2B), we used the methods described in Lonsdale *et al.* (2001). Briefly, a microscope slide was coated with Teflon spray as above, then wiped clean. A piece of Thermanox® 22 mm square from which an 18-mm square had been removed from the centre was glued to the slide with a Krazy Glue® Pen and allowed to dry for at least 1 h. Worms in pure LR White resin were placed in the 18-mm square cavity and covered with a 25-mm square piece of Aclar® plastic such that no air bubbles were trapped in the cavity. The slide was then placed in a container flooded with dry nitrogen gas, which was then sealed and put into a 60 °C oven for 1–2 days. The Aclar plastic was peeled off and selected worms were cut out and remounted for sectioning.

Remounting, sectioning and electron microscopy. Worms containing appropriate numbers of early embryos were selected by light microscopy and remounted on 'dummy' blocks for ultramicrotomy (Fig. 2C–E). Longitudinal sections through the

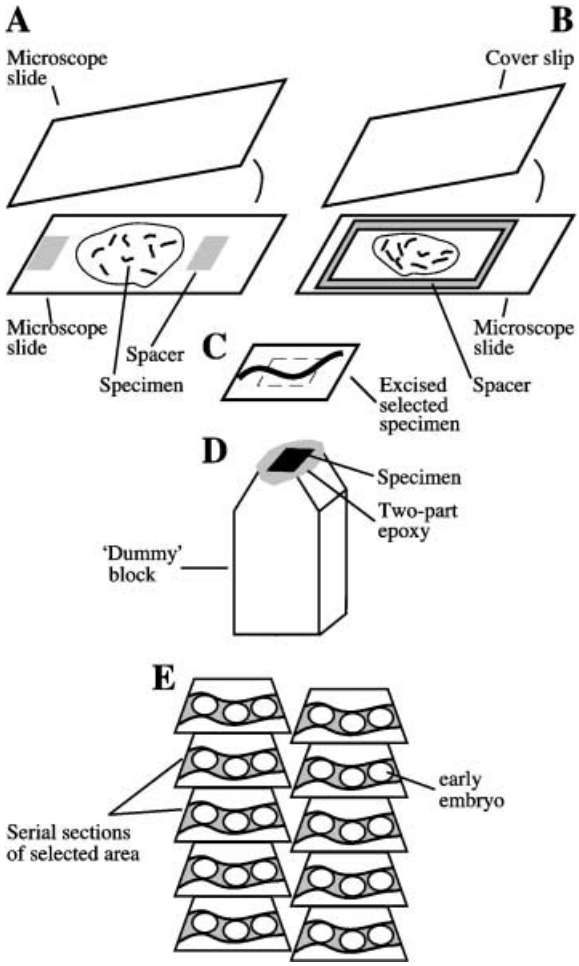

Fig. 2. Selection of thin-layer embedded worms for electron microscopy. (A) Epon/Araldite-infiltrated worms are sandwiched between two Teflon®-coated microscope slides. Spacers are used to hold the slides apart. (B) Worms infiltrated with LR White are placed in slide moulds made of a microscope slide and a Thermanox® spacer. Slide moulds are closed using an Aclar® cover slip. After polymerization of the resin, selected specimens are excised (C) and remounted on 'dummy' blocks (D) for serial sectioning (E).

worms were cut using either a Reichert Ultracut E or a Leica Ultracut UCT Microtome (Leica). Both thin (60–70 nm) and semi-thick (300–400 nm) Epon/Araldite sections were collected on Formvar-coated copper slot grids. Thin sections (50–70 nm) for immunolabelling were collected on Formvar-coated rhodium/copper slot grids. Sections were post-stained with 2% uranyl acetate in 70% methanol followed by Reynold's lead citrate and imaged in a TECNAI 12 transmission electron microscope (FEI) operated at 100 kV.

On-section immunolabelling. All steps of immunolabelling were performed in a humid chamber at room temperature. The samples were floated on blocking buffer containing 0.8% bovine serum albumin, 0.01% Tween 20 and 0.1% fish skin

gelatin (Nycomed, Amersham Inc.) in physiologically buffered saline (PBS) for 20 min. Samples were then transferred to blocking buffer containing the diluted primary polyclonal antibody (1 : 1200). After 45 min the grids were rinsed three times each for 5 min in PBS and transferred to blocking buffer containing the secondary antibody. The secondary goat anti-rabbit IgG antibody (British BioCell, U.K.), coupled to 10-nm colloidal gold, was diluted 1 : 40 in blocking buffer. After rinsing in blocking buffer and PBS (three times each for 5 min), samples were fixed for 5 min using 1% glutaraldehyde in PBS, then rinsed three times each for 1 min in dH_2O, and post-stained as described above.

Tomographic reconstruction of mitotic spindle components in early C. elegans *embryos.* High-voltage electron tomography was carried out essentially as described in O'Toole *et al.* (1999). Briefly, 15-nm colloidal gold particles were affixed to both surfaces of semi-thick (300–400 nm) sections, and the grids were carbon coated to stabilize the sample under the electron beam. Samples were placed in a high-tilt, rotating stage (Gatan model 650, Pleasanton, CA, U.S.A.) and imaged using a JEOL JEM1000 microscope operating at 750 kV. Serial, tilted views were collected over a ±60° range at 1.5° increments about two orthogonal axes. Images were acquired using a Gatan 1k × 1k CCD camera at a pixel size of 1.4 nm using an automated image capture software package developed in the Boulder Laboratory for 3-D Fine Structure. Multiple frames, or montages, were collected in order to reconstruct a larger area of the cell as described in Marsh *et al.* (2001). Image processing was carried out using the IMOD software package (Ladinsky *et al.*, 1999; O'Toole *et al.*, 1999; Marsh *et al.*, 2001). The serial, tilted views were aligned using the positions of the gold particles, and tomograms computed using an R-weighted back projection algorithm. Tomograms from each axis were then aligned to each other and combined (Mastronarde, 1997). The IMOD modelling program was used for image display and modelling (Kremer *et al.*, 1996). The ratio of the microtome setting to the final measured thickness of the reconstructions was used to calculate a thinning factor to correct for the collapse of the section during microscopy.

Results

Cellulose capillary tubes

A transverse section through a high-pressure frozen *C. elegans* hermaphrodite prepared in a capillary tube is shown in Fig. 3(A). Excellent ultrastructural preservation is indicated

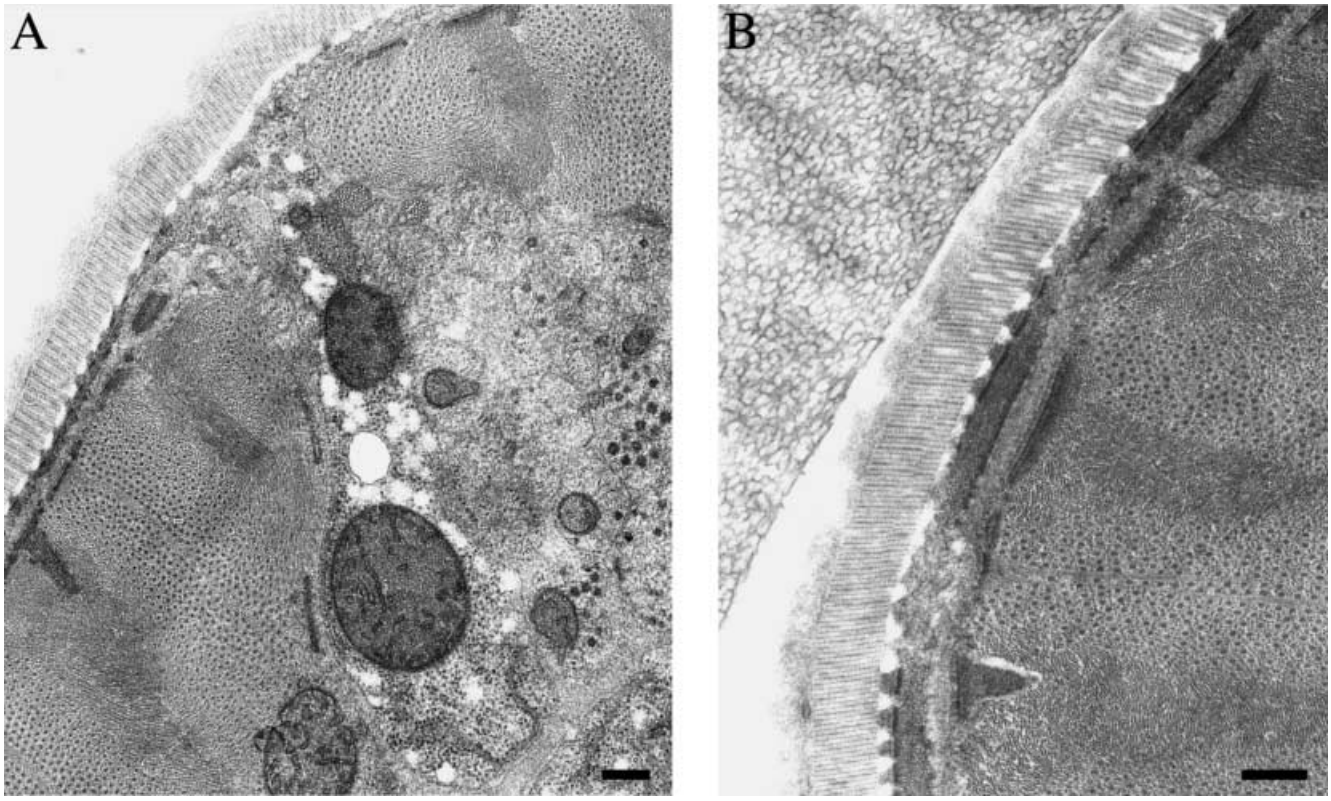

Fig. 3. Nematode worms frozen in capillary tubes. (A) Ultrastructure of a wild-type worm in cross-section. (B) Worm ultrastructure in the presence of 10% dextran. High-pressure frozen worms were freeze-substituted in acetone containing 2% osmium tetroxide. Worms were embedded in Spurr's resin. Scale bars = 0.1 μm.

by the nematode cuticle, which shows the sharply delineated and highly ordered striations (Hohenberg *et al.*, 1994). The outer somatic muscles as well as structures in the centre of the worm are free of detectable segregation patterns. This method adequately cryofixed nematodes, suspended in their natural medium, by high-pressure freezing throughout the entire length of the capillary tubes (diameter of 200 µm, over a length of 2 mm). Even low concentrations of nematodes, frozen in an HES or Dextran solution, showed good freezing results although in some cases more than 80% of the capillary lumen was filled with an aqueous solution of the respective cryoprotectant (see Hohenberg *et al.*, 1994). As shown in Fig. 3(B), a single nematode surrounded by an ice crystal pattern is well preserved and shows no intracellular ice crystal formation of detectible size. Only at the outer part of the cuticle are patterns of structural damage visible.

Thick paste method and thin-layer embedding

To analyse the ultrastructure of early *C. elegans* embryos, an alternative method of specimen preparation was applied. Adult worms were frozen in a 'thick paste' of *E. coli* and/or bakers yeast and subsequently thin-layer embedded after applying high-pressure freezing/freeze-substitution. Embedding of whole worms in a thin layer of Epon/Araldite allowed us to screen for worms with appropriate numbers of early embryos. A low-magnification image of an early embryo, showing cells in interphase as well as in mitosis, is given in Fig. 4(A). At higher magnification, spindle components of a cell in telophase are visualized (Fig. 4B). The condensed chromatin and the centrioles of the spindle poles are indicated. The mitotic spindle apparatus is shown in Fig. 4(C). This electron microscope image shows spindle microtubules, the condensed DNA and a nuclear envelope, which is partially dispersed at the spindle pole.

To localize specific gene products in the *C. elegans* embryo by immunogold labelling, thin-layer embedding in LR White resin was performed. When looking for specific labelling of mitotic spindle components it is crucial to screen a number of labelled embryos. Again, thin-layer embedding was useful because worms with appropriate numbers of embryos could be selected for immunostaining. On-section immunolabelling of spindle components in the early *C. elegans* embryo is illustrated in Fig. 5. The micrograph shows one half of a bipolar spindle with spindle microtubules and condensed DNA. Details of the immunolabelling at higher magnification are shown in Fig. 5(B). The polyclonal primary antibody (gift of Dr K. Oegema, University of California, San Diego), raised against the kinetochore component Knl-1p, recognized antigens along the 'surface' of the chromatin (Desai *et al.*, 2003). Arrows mark the 10-nm gold particles.

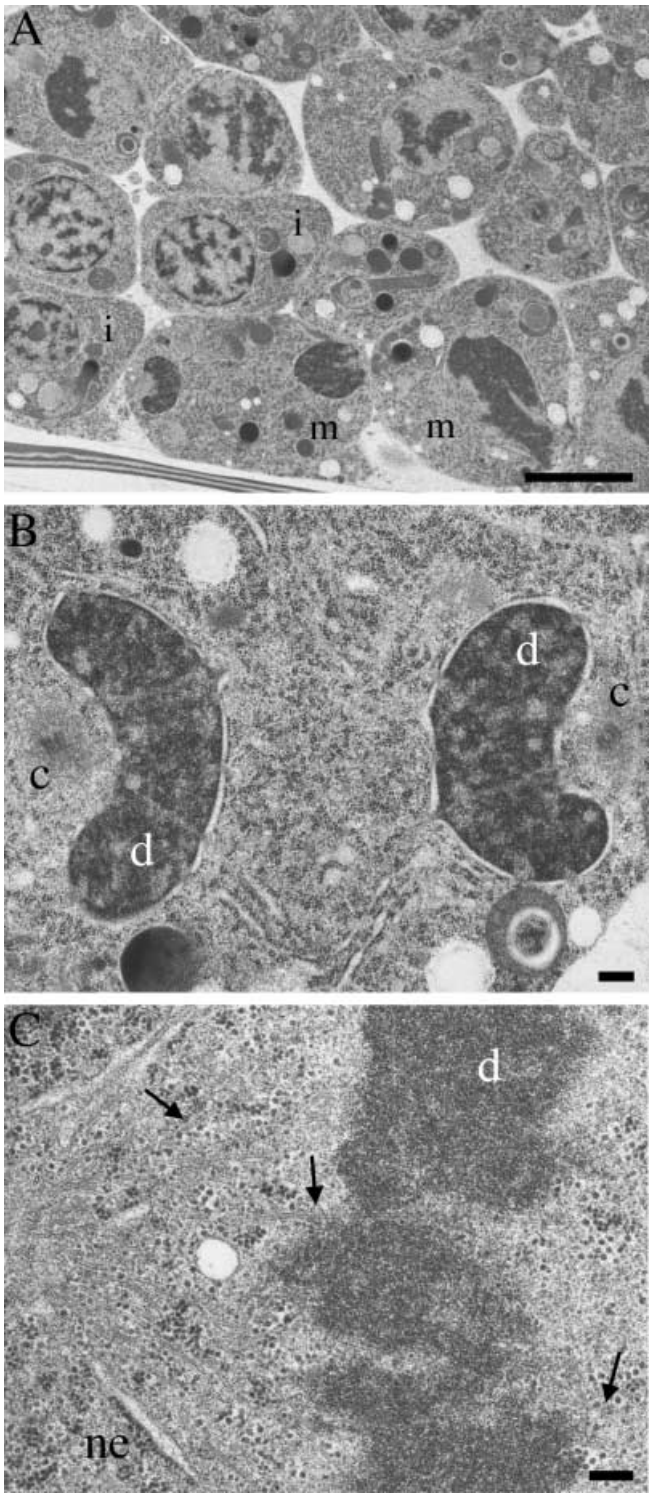

Fig. 4. Early *C. elegans* embryo in a thin-layer embedded whole-mounted wild-type worm. (A) Electron micrograph showing cells in interphase (i) and in mitosis (m). (B) Mitotic cell in telophase. Two centrioles (c) and the condensed DNA (d) are visible. (C) Ultrastructural details of a cell in metaphase. The partially dispersed nuclear envelope (ne) surrounds the DNA (d). Spindle microtubules (arrows) are visible. Wild-type hermaphrodites were high-pressure frozen. Specimens were freeze-substituted in acetone containing 1% osmium tetroxide and 0.1% uranyl acetate and embedded in Epon/Araldite. Scale bars = 0.25 µm.

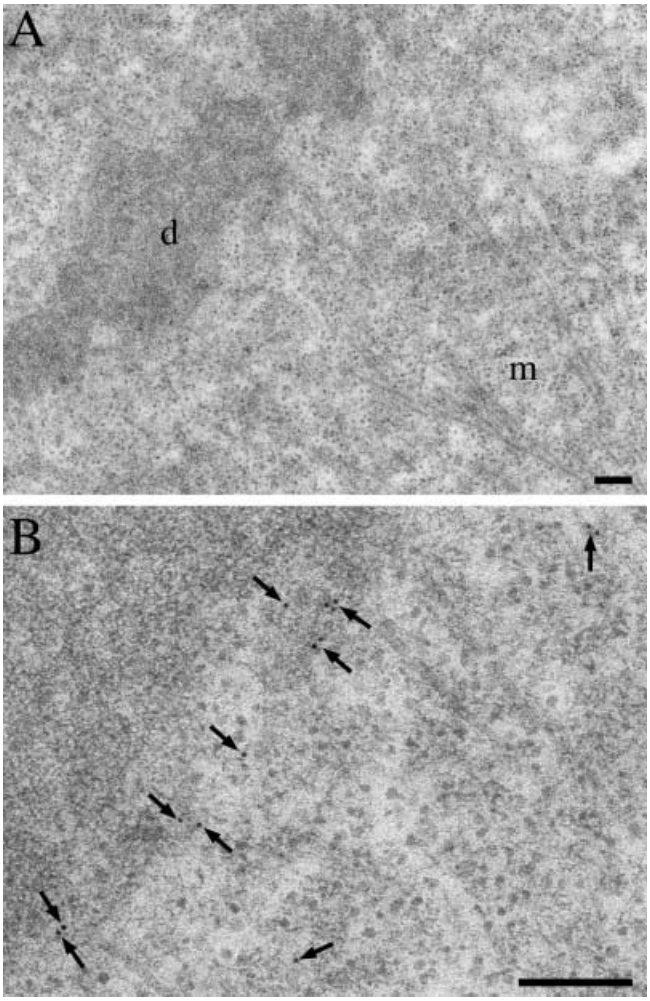

Fig. 5. Immunogold labelling in the early *C. elegans* embryo. (A) Longitudinal section through a metaphase spindle showing condensed DNA (d) and microtubules (m). (B) Details of the immunolabelling at higher magnification. The antibody recognized a kinetochore protein along the 'surface' of the condensed DNA. Wild-type worms were cryofixed by high-pressure freezing, freeze-substituted in acetone containing 0.2% glutaraldehyde and 0.1% uranyl acetate, and infiltrated with LR White. Sixty- to 70-nm sections were immunolabelled with antibody to Knl-1p and 10-nm gold secondary antibody (arrows). Scale bars = 0.25 μm.

Electron tomography of selected embryos

We used selected high-pressure frozen, thin-layer embedded worms to demonstrate the three-dimensional (3-D) reconstruction of early embryo ultrastructure by electron tomography (O'Toole *et al.*, 2002, 2003). Tomography is based on the use of serial, tilted views of a semi-thick section to create a computer-generated volume that can be sliced and imaged in any orientation. Figure 6(A) shows a selected, 2.14-nm-thick tomographic slice through a portion of a mitotic spindle from an early *C. elegans* embryo. The cell is well preserved and free of ice crystal damage. The DNA is dense and uniform, and portions of the nuclear envelope are evident. The centrosome is also well preserved, and features such as the centriole pair and the surrounding pericentriolar material are intact. Numerous microtubules can be seen embedded in this pericentriolar matrix. Structures of interest can be modelled within the complete tomographic volume and their positions displayed in three dimensions (Fig. 6B,C). The trajectories of microtubules, the position of the nuclear envelope and the surface of the condensed chromatin were modelled. Two different views of the model are displayed. As shown in these models, the spindle pole sits outside a large opening in the nuclear envelope. Microtubules radiate out from the centrosome, with the majority facing the chromosomes. Microtubule ends were identified and their positions marked with a blue sphere. Although this tomographic reconstruction contained approximately 2.8 μm × 1.4 μm × 250 nm of cell volume, only a portion of this spindle was visualized. Serial section tomography of adjacent sections would be needed to reconstruct this entire spindle.

Discussion

The most critical part of high-pressure freezing is probably loading of the specimens. Some practical guidelines for specimen loading are clearly presented by Moor (1987) and some of the key points are briefly repeated here. (1) Specimens should be in optimal physiological conditions at the moment of freezing. (2) Most samples need to be loaded quickly to minimize physical and physiological disruptions. (3) The entire cavity of the specimen holder has to be filled. If there are air spaces, these will collapse and possibly crush the specimen and/or interfere with effective heat transfer. (4) If the planchettes available do not seem to fit the sample, special planchettes that do fit may have to be made. (5) If existing planchettes have to be used, non-penetrating cryoprotectants can be used to fill air spaces and facilitate heat transfer. In summary, if the sample is not freezing well, it is probably not the fault of the high-pressure freezer, but rather the condition of the specimen as it goes into the freezer. It is generally known in high-pressure freezing laboratories that poor freezing is frequently correlated with the state of the water in and/or around the sample. Specimens with high water content, such as some plant cells and cartilage, are difficult to freeze. Likewise, samples that are surrounded by aqueous solutions such as cell suspensions tend to freeze poorly. The two methods we have presented here help to overcome some of the difficulties associated with loading of *C. elegans* into the freezing planchettes. Choosing either one of these two methods, however, will largely be based on the type of analysis following specimen preparation.

Using capillary tubes for freezing

Nematodes were incubated, gently concentrated and continually observed in their natural medium until rapidly frozen in their living state by high-pressure freezing. Direct contact of the nematodes with 1-hexadecene or other cryoprotectants

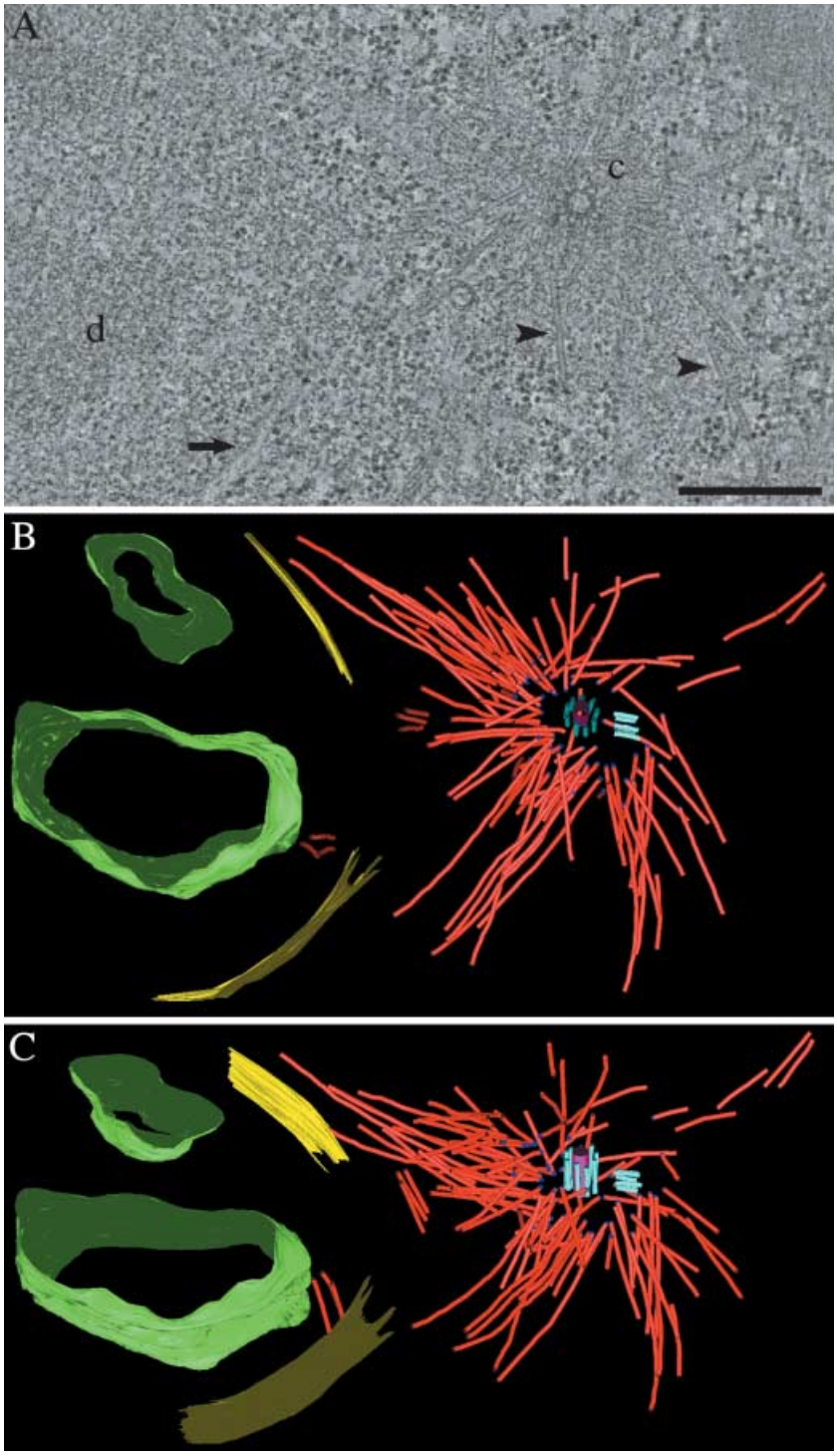

Fig. 6. Electron tomography of *C. elegans* ultrastructure. (A) Selected tomographic slice showing mitotic features in an early embryo. The centrosome (c), the condensed DNA (d) and a partially dispersed nuclear envelope (arrow) are visible. Microtubules are indicated by arrowheads. (B) Three-dimensional model of the same data set showing the complex array of microtubules (red) around the centrioles (blue). The 'surface' of the DNA is modelled in 'green' and the nuclear envelope is modelled in 'yellow'. Minus ends of microtubules are indicated by blue spheres. (C) Horizontal rotation of the same 3-D model. Specimens for tomographic reconstruction were cryofixed by high-pressure freezing and freeze-substituted in acetone containing 1% osmium tetroxide and 0.1% uranyl acetate. Samples were embedded in Epon/Araldite. Scale bar = 0.5 µm.

(Dahl & Staehelin, 1989; Studer *et al.*, 1989) was avoided. Nematodes, enclosed within cellulose capillary tubes and submerged under 1-hexadecene, remain alive for 7 h (observed by light microscopy). Upon direct contact with 1-hexadecene, nematodes stretch out and die in less than 5 min. Importantly, the hydrophobic 1-hexadecene is only marginally soluble in acetone at subzero temperatures. Ice crystals may grow and distort the sample, if the substitution medium is prevented from dissolving the frozen sample at very low temperatures. The frozen 1-hexadecene therefore must be removed from the specimen prior to freeze-substitution. The capillary tube pores are water-filled and prevent the entry of 1-hexadecene.

Electron diffraction of cryosections of such prepared samples revealed vitrification of the nematodes and hexagonal ice

in the surrounding medium in most cases (Hohenberg *et al.*, 1994). It is important to note that the freezing quality was correlated with the concentration of the nematodes inside the capillary tubes; 40–50% of the capillary lumen should be filled with the worms. In the case of low nematode concentrations or the preparation of selected individuals or embryos, the application of solutions containing non-penetrating cryoprotectants (i.e. HES or Dextran) gave good results. The pattern of ice crystal formation in the frozen solution enclosing the nematodes is directly visible.

The advantages in using porous cellulose capillary tubes for the preparation of nematodes for high-pressure freezing are as follows. (1) The samples are cryoimmobilized in their natural environment, a procedure which retains structural information that can be correlated with the physiological state of the worms. Viability and vitality of the nematodes can be continually checked by light microscopy until freezing because capillary tubes are transparent. (2) The suspensions can be processed within a microcontainer of confined volume (about 0.5 μL), avoiding repeated centrifugation during cryoprocessing. Loss of material is impossible because the containers are sealed. Nematodes of a single batch can be used for cryoimmobilization, cryosectioning and freeze-substitution. (3) This method ensures a very high and predictable yield of adequately and reproducibly high-pressure frozen samples. (4) Freeze-substitution and embedding procedures are not prolonged owing to the high porosity of the cellulose tubes. The pores have a variable molecular weight cut-off from 5 to 20 kDa, allowing the free passage of all substitution media and resin monomers. The disadvantages of this method can be summarized as follows. (i) It is not possible to select individual worms after high-pressure freezing/freeze-substitution. (ii) Specimens cannot be orientated before polymerization of the resin. In summary, the capillary tube method gives good freezing results. The use of this method, however, is limited when individual worms have to be selected for further analysis.

Thick paste/thin-layer embedding method

Using *E. coli* as the filler is very convenient because the worms are usually crawling through it on the food plate. This is their natural state and one can assume that the physiological conditions are excellent for the worms. However, the viscosity of the *E. coli* paste being used as filler in the planchettes is critical and on the food plates it is often not viscous enough. The same viscosity that is ideal for picking is good for filling. When using *E. coli* paste, a separate plate of viscous cells as the filler instead of what is on the plate with the worms should be used. If one has to work with worms in liquid, they have to be gently centrifuged first, before putting them on an agar plate. The agar will absorb the excess liquid and the worms can be picked off the plate as if they had been growing there. The overall requirement is to have intimate contact between the paste and the worms during freezing. If the worms are surrounded by aqueous solutions, the transfer of heat will be impeded and they will not freeze well. Alternatively, a thick paste of bakers yeast can be used to fill the cavity of the freezing planchettes.

The advantages of the thick paste/thin-layer embedding method can be summarized as follows. (1) A large number of wild-type or mutant worms can be processed. Typically, 50–100 worms can be loaded into a specimen planchette (type A, 100 μm deep) for every freezing event. This method works best when worms are being 'picked' off food plates rather than being grown in liquid cultures. (2) Both types of pastes are easy to freeze well. In our experience, the yeast is usually better than the *E. coli* because in a thick paste the yeast probably act as tiny 'sponges', absorbing excess liquid from around the worms. (3) Specimen loading is fast. With a little practice, it is easy to load 50–100 worms into a planchette in 2 min or less. When working on an agar food plate, the paste will not dry out in that time. (4) Once through the freeze-substitution and resin infiltration procedures, it is possible to separate individual worms from the thick paste using a pair of fine tungsten needles to tease away the filling material during thin layer embedding. Worms should be spread on the slide so they are far enough apart to be cut out individually. (5) Single worms can be selected for (serial) microtomy. Following resin polymerization the worms are in a thin layer of resin on a microscope slide. This slide can be mounted in a high-quality light microscope and individual worms can be checked for stage of development (see Lonsdale *et al.*, 2001). This means that it is possible to work with mixed populations and select individuals for sectioning.

The disadvantages of this method may be summarized as follows. (i) It takes some practice to get the consistency of either the *E. coli* or yeast paste right and this is one of the major keys to success with the thick paste method. If it is too thick, it is difficult to mix with the worms. If it is too thin, it is not effective at heat transfer. One suggestion is to practise making different thickness of paste and use the thickest one that will cover the worms. It sometimes helps to mix the worms and paste on the agar food plate and load them together into the planchette, rather than adding them separately. It is also possible to add them separately, but the worms should be put in first, and then covered with *E. coli*/yeast paste. Working on an agar plate such as the one the worms are growing on can help to keep the paste hydrated. If the paste seems to be drying out, a little liquid medium can be added to rehydrate it. When done correctly, worms should be moving in the paste while putting on the covering planchette. (ii) Worms can be lost during steps of dehydration and resin infiltration. In contrast to the capillary method, specimens are not contained in sealed microtubes. Centrifugation steps are necessary to concentrate worms after each dehydration and infiltration step. (iii) It is easy to introduce 'handling traumas'. After freeze-substitution, worms have to be separated from the surrounding paste prior to flat embedding. This manipulation has to be done with care,

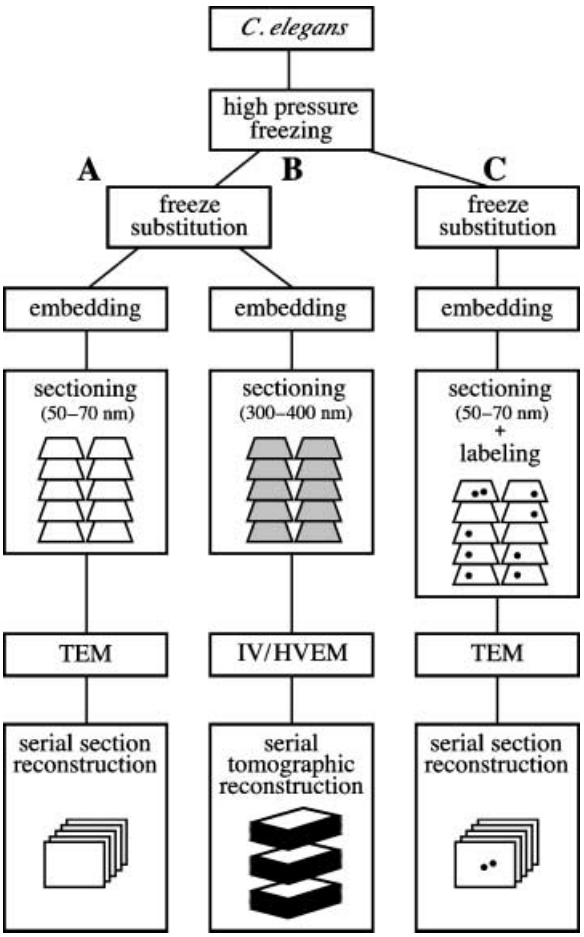

Fig. 7. Using selected, thin-layer embedded specimens for 3-D reconstruction and immunolabelling. To achieve an optimal preservation of *C. elegans* fine structure, high-pressure freezing is applied. (A) Thin sections are used for serial section reconstruction. (B) Semi-thick sections are cut for serial tomographic reconstruction. (C) Serial thin sectioning can be combined with immunolabelling to analyse the distribution of a specific protein in three dimensions.

because the freeze-substituted worms tend to be brittle and therefore 'break' easily. In conclusion, the thick paste method also gives good freezing results. As discussed below, it is recommended to use this method for example when cellular structures have to be identified in serial sections of embryos selected for electron tomography.

Three-dimensional reconstruction of spindle components in selected early C. elegans *embryos*

We have described the application of high-pressure freezing/freeze-substitution to immobilize, identify and reconstruct cellular features in early *C. elegans* embryos. Importantly, superior preservation of cellular fine structure is the first critical step in undertaking a 3-D structure study. The use of poorly fixed material can lead to artefacts, resulting in errors in the final model and, ultimately, the interpretation of the 3-D geometry of cellular organelles. In principle, the third dimension can be reconstructed from serial sections with the limitation that the resolution perpendicular to the plane of the sections is no better than twice the average section thickness (McDonald *et al.*, 1996; Fig. 7). When combined with immunogold labelling, the distribution of a specific antigen can be demonstrated in three dimensions (Müller-Reichert *et al.*, 2003). As an alternative technique, electron tomography has evolved in recent years as the method of choice for a 3-D visualization of cellular fine structure. For example, the spindle pole body (Bullitt *et al.*, 1997) and the early mitotic spindle (O'Toole *et al.*, 1999) in *Saccharomyces cerevisiae*, the mammalian kinetochore (McEwen *et al.*, 1998), the centrosome in *Drosophila* (Moritz *et al.*, 1995) and the actin cytoskeleton in *Dictyostelium* (Medalia *et al.*, 2002) have been analysed by tomography. For a genetic system such as *C. elegans*, the combination of techniques described here has several advantages. First, this method allows serial sectioning of selected, resin-embedded whole mounted worms. Second, cellular components can be visualized *in situ* and modelled in three dimensions using appropriate software (Ladinsky *et al.*, 1999; O'Toole *et al.*, 1999; Marsh *et al.*, 2001). Third, montaging of data sets and serial tomography can be applied to cover larger areas and bigger volumes, respectively (Ladinsky *et al.*, 1999; Marsh *et al.*, 2001). In the future, 3-D reconstruction by electron tomography will be important for characterizing wild-type and mutant ultrastructure in genetic systems such as *C. elegans* and, definitively, high-pressure freezing will continue to play a key role in obtaining excellent ultrastructural preservation for these types of 3-D studies.

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