
CHAPTER 4

Correlative Light and Electron Microscopy of Early *Caenorhabditis elegans* Embryos in Mitosis

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The early embryo of *Caenorhabditis elegans* is one of the most powerful model systems in which to study cell division. Here we have developed a correlative light and electron microscopic approach to stage early *C. elegans* embryos prior to high-pressure freezing and electron tomography. We use cellulose microcapillaries to contain the embryos in short transparent tubes. These permit the viewing of early mitotic events under the light microscope. Using the newly developed rapid transfer system (RTS) of the Leica EMPACT2 high-pressure freezer, we have reduced the time between light microscopic observation and cryoimmobilization to about 5 sec. This correlative approach allows systematic structure–function studies in staged early wild-type embryos and those treated with RNA-mediated interference (RNAi) to reduce the level of specific gene products.

I. Introduction

The *Caenorhabditis elegans* early embryo is one of the most powerful model systems in which to study various subprocesses of mitosis, such as nuclear envelope assembly/disassembly, centrosome dynamics, centriole assembly, formation of the mitotic spindle, kinetochore assembly, chromosome segregation, and cytokinesis (for a review see Oegema and Hyman, 2005). For analysis of these processes by correlative microscopy, *C. elegans* offers several advantages. (1) The stereotypic early development of the embryo has been studied and characterized in detail (for reviews see Cowan and Hyman, 2004; Pelletier *et al.*, 2004a; Schneider and Bowerman, 2003). (2) The appearance, migration, rotation, and disappearance of the pronuclei around prometaphase can be easily recognized by noninvasive microscopy, allowing precise staging of embryonic development. (3) Mitotic spindle components, such as microtubules, kinetochores, or centrosomes, can be visualized using GFP-tagged strains. Moreover, strains are available which express multiple-tagged proteins (Oegema *et al.*, 2001). (4) RNA-mediated interference (RNAi) is used routinely to generate oocytes whose cytoplasm is reproducibly depleted of targeted essential gene products. Following fertilization, these oocytes are then analyzed as they attempt to go through the first round of mitosis (reviewed in Oegema and Hyman, 2005). Application of RNAi allows the comparison of wild-type and mutant embryos. (5) The reliability of RNAi technology has it made possible to perform genome-wide screens to identify all the components involved in the early mitoses of *C. elegans* (Sönnichsen *et al.*, 2005). Taken together, these features allow one to study the

dynamics and ultrastructure of cell division in a systematic manner that was not possible until recently.

Correlative approaches in microscopy have been used to obtain complementary information about a given system (Kapoor *et al.*, 2006; Rieder and Cassels, 1999). For example, light microscopy (LM) in combination with GFP-tagging, on the one hand, offers the advantage of studying centrosome dynamics in living systems, but with obvious limitations in the obtained resolution. Electron microscopy (EM), on the other hand, delivers high-resolution "snapshot" images of centrosomes, either purified (Chrétien *et al.*, 1997) *in vitro* (Schnackenberg *et al.*, 1998) or *in vivo* (O'Toole *et al.*, 2003b), but the yield of information about the dynamics of the process under study is low. On the basis of only "snapshot" images, it is not always possible to unambiguously reconstruct a complex process, like the assembly of a cellular structure. For analysis of an organelle like the centrosome in the early *C. elegans* embryo, one would like to be able to observe spindle dynamics by live-cell imaging and stop the process immediately at a specific stage by rapid cryofixation; this would permit subsequent, high-resolution analysis *in situ*, preferably by electron tomography. The prerequisites for such a correlative approach are: (1) to optimize the time resolution of the technique and (2) to use a method that gives optimal preservation of the ultrastructure.

One approach to achieving the first goal (i.e., to stop the developing *C. elegans* embryo at specific stages of early mitosis) has been laser-assisted chemical fixation (Dammermann *et al.*, 2004; Kirkham *et al.*, 2003; Pelletier *et al.*, 2004b; Priess and Hirsh, 1986). Taking advantage of the fact that early embryos are protected by coverings that allow them to continue to divide in the presence of fixative, selected embryos were attached to an Aclar plastic surface and "bathed" in M-9 buffer containing 2% glutaraldehyde. A laser beam was used to perforate the embryo's eggshell, allowing the fixative to diffuse into the embryo through a small (1.5 μm) hole and to stop development. Since this method achieved a rapid cessation of the embryonic development and permitted a staging of the embryo prior to conventional EM, it has been applied to understand the role of specific genes in centriole duplication (Dammermann *et al.*, 2004; Kirkham *et al.*, 2003; Pelletier *et al.*, 2004b). The major disadvantage of laser-assisted fixation, however, is its poor rate of fixation and the resulting insufficient structural preservation of the early embryo (for a general discussion on the disadvantages of chemical fixation on cellular fine structure, the reader is referred to Murk *et al.*, 2003). In order to achieve the kind of resolution necessary for electron tomographic studies, we have previously used high-pressure freezing as the means of primary fixation (O'Toole *et al.*, 2003b). Whole worms were high-pressure frozen and embedded in a thin layer of resin, so individual worms could be remounted and sectioned longitudinally. Embryos at the desired stages can be identified in serial sections of these longitudinally oriented worms. Using electron tomography of such high-pressure frozen material, we have described the three-dimensional (3D) structure of the mitotic *C. elegans* centrosome and identified both open and closed morphologies

of centrosome-associated microtubule ends (O'Toole *et al.*, 2003b). However, a disadvantage of this procedure is that the whole worms contain populations of embryos at many phases of development, so the stage of mitosis had to be determined after sectioning. Here, we describe a method that combines the advantages of: (1) a correlative LM approach that allows us to stage development and fixation times with high time resolution, (2) high-pressure freezing for the best possible preservation of ultrastructure, and (3) electron tomography for visualization and analysis of high-resolution structure in 3D.

II. Rationale

Rationale of the described technique is to combine the observation of live mitotic embryos with high-pressure freezing of clearly defined stages, allowing us to obtain tomographic reconstructions of mitotic spindle components in well-preserved embryos of *C. elegans* as they develop. The methods presented here include procedures to transfer the isolated embryos into capillary tubes, to stage selected embryos by LM, to immobilize these staged embryos by high-pressure freezing, and to fix them by freeze-substitution. Finally, we describe thin-layer embedding and 3D reconstruction of spindle components by electron tomography (Fig. 1). In the following paragraphs, we give step-by-step descriptions of these methods.

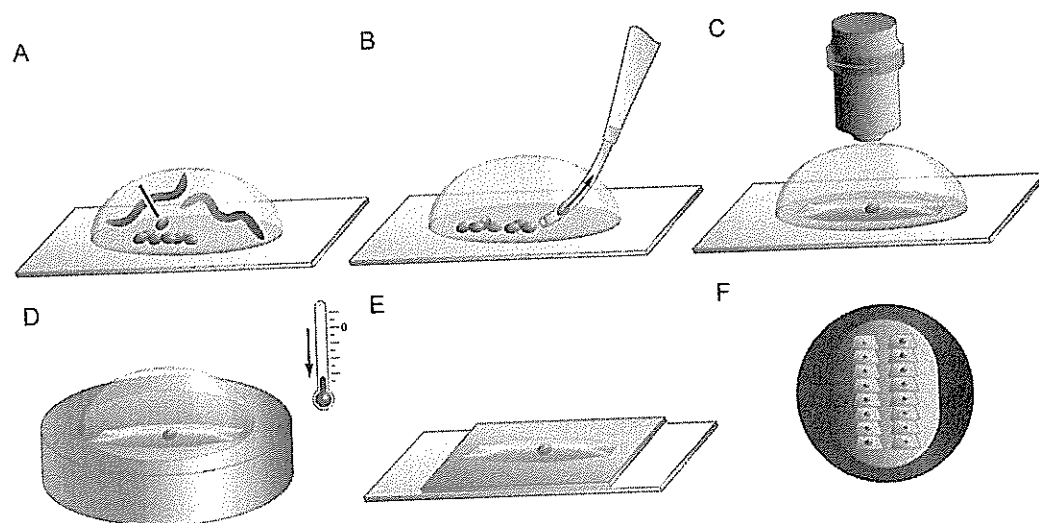


Fig. 1 Diagram illustrating the processing of isolated *C. elegans* embryos for correlative LM/EM. (A) Release of early embryos. (B) Collection of a selected embryo into capillary tubing. (C) Staging of the capillary-contained embryo by LM. (D) Stage-defined fixation by high-pressure freezing. (E) Thin-layer embedding of the freeze-substituted embryo. (F) Serial sectioning for subsequent electron tomography.

III. Methods

A. Staging of Isolated Embryos by LM

This section describes the preparation of isolated *C. elegans* embryos for live-cell imaging under conditions suitable for subsequent high-pressure freezing. The idea is to make use of capillary tubing to contain the embryos in short transparent tubes so that one can observe early developmental events under the light microscope, prior to time-resolved high-pressure freezing (McDonald *et al.*, 2006).

As a first step, we prepare a "loading device" to collect isolated early embryos into capillary tubes and a "crimping" tool to cut pieces of tubes of appropriate sizes. Loading devices were prepared by cutting 2-cm-long pieces of capillary dialysis tubing (Hohenberg *et al.*, 1994), mounting them into gel loader tips, and using nail polish for sealing. Finally, the loading device was mounted on a Pipetman. The crimping tool was made from a scalpel tip by cutting or filing off the final millimeter of the blade, and then shaping it with a whetstone to the shape of a chisel (Fig. 2). The crimping tool was important to enable pieces of tubing of appropriate sizes to be cut and simultaneously closed during the cutting procedure.

Using a dissecting scope, worms were cut open in small Petri dishes using M-9 buffer containing 20% bovine serum albumin (BSA). Embryos were released by cutting the worm in the gonad region with two injection needles. In order to get a high yield of released early embryos, it is preferred to take young adults for cutting. It is also advantageous to choose embryos in early stages of development, for example, when the pronuclei first appear (Fig. 3B). In the wild-type embryo development, from pronuclear appearance to the time of their first meeting takes about 13 min. This time is sufficient to load an embryo into the capillary tube and get ready for live-cell imaging. Thus, an early embryo was selected and sucked into the capillary tube, using the loading device mounted on a Pipetman.

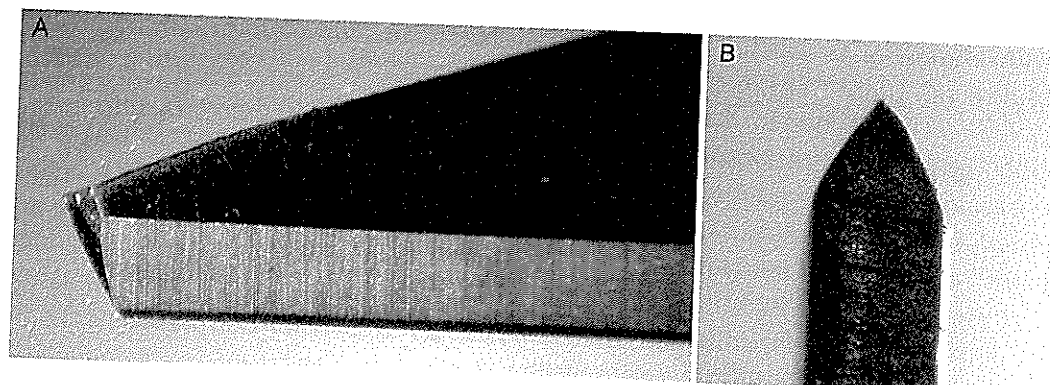


Fig. 2 The "crimping tool" as made from a scalpel tip. The final millimeter of the blade is cut off and then shaped with a whetstone. (A) Side view. (B) Top view.

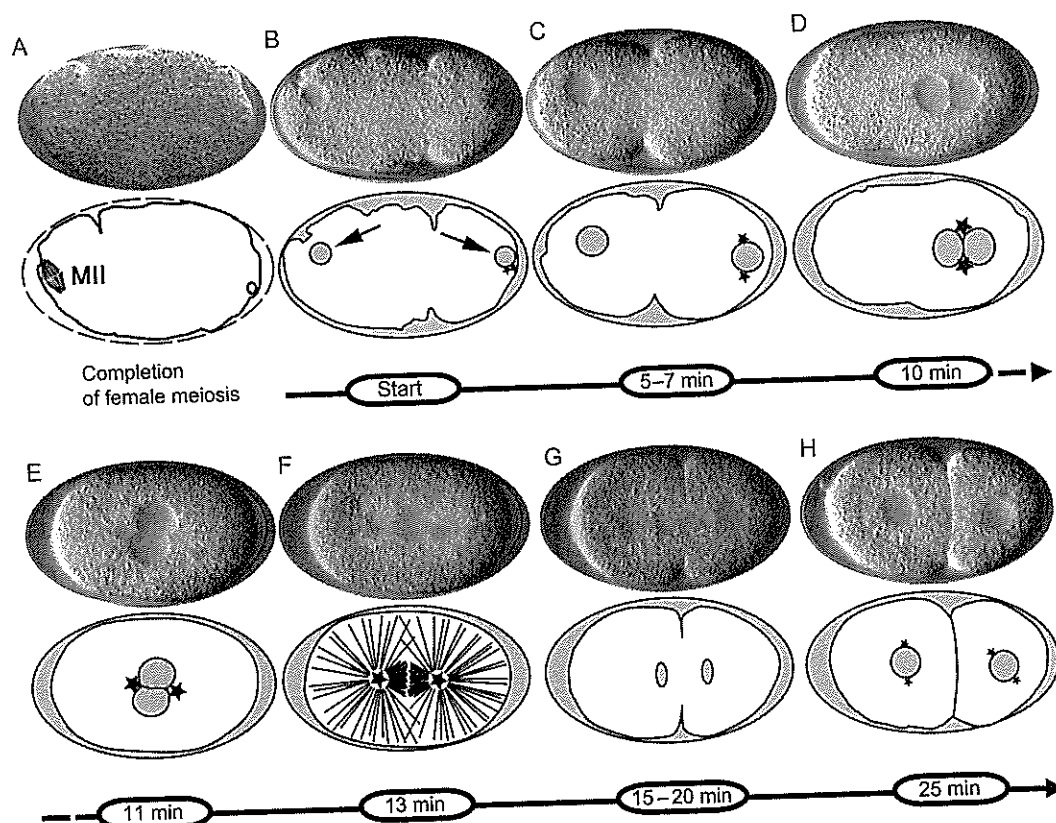


Fig. 3 Early development of the *C. elegans* embryo. Stages are illustrated by differential interference contrast (DIC) images and corresponding schematic drawings. (A) Completion of female meiosis II. (B) Pronuclear appearance. (C) Pronuclear migration. (D) Pronuclear meeting. (E) Pronuclear rotation. (F) Metaphase of the first mitotic division. (G) Telophase. (H) Two-celled embryo. The position and the disappearance of the two pronuclei (arrows) are indicative of specific developmental stages. Asterisks indicate the position of the centrosomes.

The tubing was submerged into the BSA-containing M-9 buffer in the Petri dish, then the "crimping tool" was used to cut the region of the tubing containing the early embryo to a final length of about 1 mm. It is important that this piece of tubing is about this length because it has to fit into the specimen carrier used later for high-pressure freezing. The ends of the tube should be closed during the crimping in order to avoid leaking out of the early embryo. Therefore, the tube should be cut and simultaneously pressed down with the flattened side of the crimping tool.

The closed tube was then transferred from the Petri dish to a droplet of BSA-containing M-9 buffer on a glass slide to observe the further development of the embryo in real time using a light microscope equipped with phase contrast, differential interference contrast (DIC) or fluorescence optics (Fig. 4). In the latter case, a line expressing either GFP:: β -tubulin GFP:: γ -tubulin or GFP:: γ -tubulin and GFP:: γ -tubulin can be used to observe the stages of mitotic spindle assembly. It is

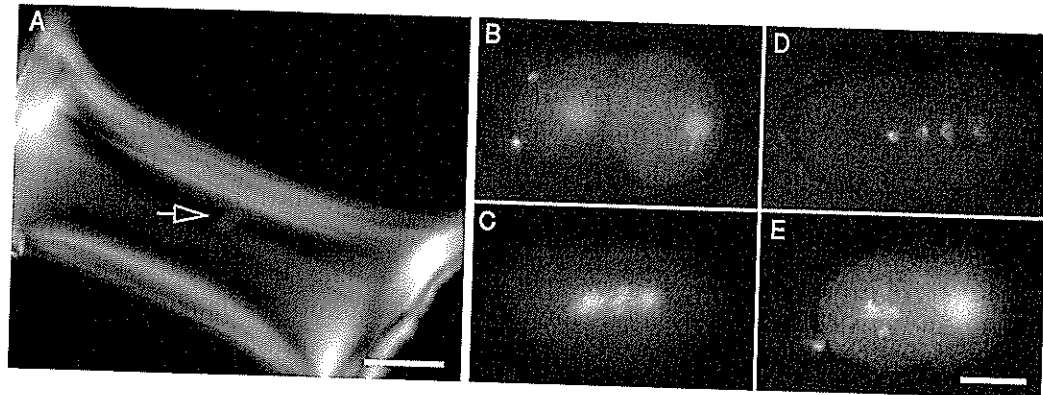


Fig. 4 LM of the developing *C. elegans* embryos. (A) Early *C. elegans* embryo (arrow) contained in a closed capillary tube. (B–E) Staging of contained embryos by fluorescence microscopy. (B) Pronuclear appearance. (C) Metaphase. (D) Anaphase. (E) Two-celled embryo. (C) Strain expressing GFP:: β -tubulin and GFP::histone and (B, D, and E) strain expressing GFP:: γ -tubulin and GFP::histone were used to visualize spindle components. Scale bar in (A) = 100 μ m and in (E) = 10 μ m.

also possible to observe the developing embryo with phase contrast because the appearance, position, and migration of the two pronuclei are good indicators of the developmental events during the first mitotic division. The developmental history of every selected embryo should be documented. A time-lapse movie, however, is not absolutely required for documentation.

B. High-Pressure Freezing and Freeze-Substitution

Next, the staged embryo must be cryoimmobilized to “freeze” the observed developmental process in time. The key here is to minimize the time window between observation and freezing. For this, we applied the newly developed EMPACT2+RTS (Leica Microsystems, Vienna, Austria) high-pressure freezer, a portable machine that can be easily moved to the site where staging of the embryo is performed. In addition, the rapid transfer system (RTS) allows a fast loading of the specimen into a preloaded high-pressure freezer under standardized conditions (McDonald *et al.*, 2006). Following high-pressure freezing, the isolated embryos have to be processed for freeze-substitution.

In preparation for freezing, the RTS has to be preloaded with a freezing “pod” (Fig. 5). In addition, a specimen carrier in which the sample will be placed at an appropriate time point must be premounted in the so-called rapid loader (for details see McDonald *et al.*, 2006). Routinely, we used 100- μ m-deep “membrane carriers,” designed to separate the compartment holding the capillary tube from the pressure by a thin “membrane” of copper. Shortly before freezing, the membrane carrier was filled with M-9 buffer containing 20% BSA (see the preceding section). To avoid “overfilling” of the holder, excess buffer can be sucked away with strips of filter paper. At an appropriate developmental stage, the

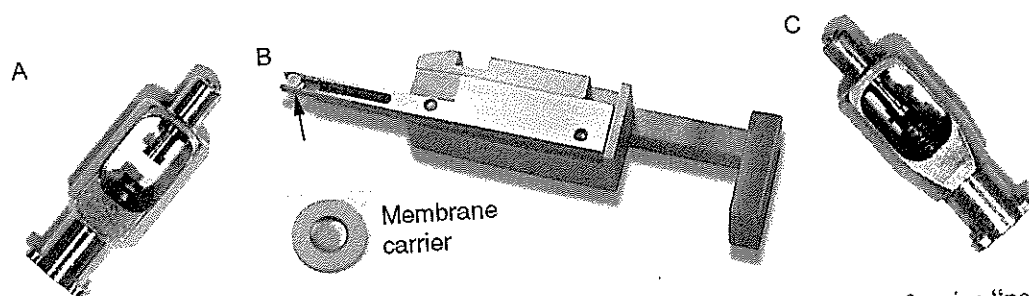


Fig. 5 Loading devices for the RTS of the EMPACT2 high-pressure freezer. (A) The freezing "pod." (B) "Rapid loader" with premounted specimen carrier (arrow). A "membrane carrier" is shown at higher magnification. (C) Closed freezing "pod" with specimen carrier (McDonald *et al.*, 2006).

embryo-containing capillary tube was quickly transferred from the microscope slide to the cryoprotectant-filled specimen carrier using tweezers. As soon as the capillary tube was loaded in the membrane carrier, the rapid loader was transferred to the RTS. The rapid loader fits exactly into the pod. Sliding the rapid loader into the RTS triggers the pod to close automatically and shoot into the EMPACT2, ultrarapidly freezing the sample. Applying this approach, the isolated embryo was transferred from the light microscope to the high-pressure freezer within about 5 sec. After freezing, the membrane carriers were released from the pod and stored in liquid nitrogen until further use.

The membrane carriers containing the tubes were then transferred to precooled cryovials filled with the freeze-substitution "cocktail." Freeze-substitution was carried out in anhydrous acetone containing 1% osmium tetroxide and 0.1% uranyl acetate (for detailed information on the composition and preparation of the freeze-substitution "cocktail", the reader is referred to McDonald and Müller-Reichert, 2002). Using a Leica EM AFS, we maintained the samples at -90°C for a minimum of 8 h and a maximum of 24 h. Freeze-substituted samples were then allowed to warm to room temperature at a rate of $5^{\circ}\text{C}/\text{h}$. Specimens were washed three to five times in fresh anhydrous acetone. After these acetone washes, samples were gradually infiltrated with Epon/Araldite resin (1 part resin:3 parts acetone for 2 h, 1:1 for 3 h, 3:1 for 4 h, and 100% resin for 4 h, then overnight, then for 4 h). Importantly, throughout all these steps, the capillary tubes remained in the BSA-filled membrane carriers and were not removed prior to the final flat-embedding step to avoid the loss of the small capillary tubes during rinsing and resin infiltration.

C. Thin-Layer Embedding, Serial Sectioning, and Prescreening of Samples

After resin infiltration, the embryo-containing capillary tubes are further processed for flat embedding. The objective here is to embed the tubes in thin, optically clean layers of resin on microscope slides, allowing an easy and routine handling of

the embryo for serial sectioning. Thin-layer embedding and serial sectioning of whole worms have been described previously (Müller-Reichert *et al.*, 2003). Here, the protocol is adapted to the needs of processing embryo-contained cellulose capillary tubes.

Routinely, labeled microscope slides were wiped clean with a soft cloth, submerged in a Teflon® solution, and allowed to dry. The slides were then polished to get a clean transparent surface, and two layers of Parafilm® were placed on the margins of the glass slide to serve as spacers. About 200–300 μ l of Epon/Araldite were placed on the microscope slide and allowed to disperse on the surface of the slide. Subsequently, the membrane carrier containing the capillary tube was placed in the resin droplet and held tightly with appropriate tweezers. The capillary tube was then removed from the specimen carrier with a sharpened tungsten needle. This needle was also used to remove excess BSA from the tubing. Importantly, the tubing tends to be brittle after the freeze-substitution steps, and care must be taken to avoid any mechanical damage to the embryo during these procedures. We embedded two capillary tubes on a single microscope slide with proper labeling indicating the specimen type. After removal of the empty membrane carrier, the embryo in the tube has to be checked so that the orientation is the same as that seen in the light microscope. Orientation of the embryo in the tube can best be checked by simultaneously rotating the tube while looking at the position of the embryo within the tube. This step is important later when the polymerized sample must be remounted and trimmed for serial sectioning. Tubes in their final orientation were then softly pressed down to the surface of the glass slide, and a second, coated and cleaned glass slide was put on top of the resin samples. This “sandwich” was placed on an appropriate support in the oven (about 48 h at 60°C) to polymerize the resin. After resin polymerization, one of the microscope slides was removed by pressing a razor blade between the resin and one of the glass slides. At this stage, the tubes were on a microscope slide suitable for view in a light microscope and embedded in a thin layer of resin, ready to be remounted on “dummy” blocks for microtomy.

Thereafter, polymerized samples were observed with a light microscope to relocate the capillary tubes on the microscope slide. An objective marker with a diamond tip was used to mark a circle around the tube. The purpose of this circling is twofold: first, tubes are clearly marked during subsequent steps (i.e., during remounting) and second, the circle is used to determine the position of the embryo within the resin. When both embryo and circle are in about the same focus level then the embryo is positioned at the upper surface of the resin. In contrast, when the focus level of the embryo is not identical with the circle then the embryo is positioned close to the lower surface of the resin layer. In the latter case, the sample has to be remounted upside down on the “dummy” block. The tubes with surrounding BSA are usually not stained homogeneously during the freeze-substitution step and the resulting “landscape” can be used to sketch the position of the embryo in the tube before remounting. Such a sketch is helpful later during trimming of the specimen. Using a scalpel, small quadrates

of $1 \times 1 \text{ mm}^2$ resin containing the capillary tube were cut out under a stereo microscope. A rapidly setting Epoxy glue was used to remount the samples on "dummy" blocks with the early embryos facing up. Polymerization of the glue at 60°C was completed within about 30 min. At this step, the sketch (see above) was helpful to relocate the embryo in the remounted piece of resin.

After polymerization, the remounted sample was trimmed for serial sectioning. The trapezoid block face should have a height slightly larger than the embryo ($\sim 0.1 \text{ mm}$). The width of the block face has to be large enough to facilitate serial sectioning ($\sim 0.3 \text{ mm}$). Semithick (300–400 nm) sections were then collected in ribbons (with about 10–13 sections per ribbon) and 2–3 ribbons were collected on Formvar-coated copper slot grids. At this stage, it is important to keep the ribbons and the EM grids in sequence. Sections were routinely poststained with 2% uranyl acetate in 70% methanol followed by Reynold's lead citrate.

Stained specimens were then viewed by low-voltage EM (100 kV) to identify features of interest in the serial sections. At this stage, it is advantageous to start screening at low magnification to store the position of each section. By recalling the stored positions when at higher magnification, the sections that show features of interest can be identified. It was important here to document the position of the selected sections (e.g., left ribbon, third section from the top) for further electron tomography.

D. Electron Tomography

Next, we record electron micrographs of a series of tilted views of selected features on the prescreened grids. This tilt series is used to compute the tomogram from which we can generate 3D models (Müller-Reichert *et al.*, 2003; O'Toole *et al.*, 2003b). For details on this method, the reader is referred to Frank (1992).

In preparation for electron tomography, 15-nm-colloidal gold particles were affixed to both surfaces of the semithick sections. Samples were placed in a high-tilt, tomography stage and imaged with an intermediate-voltage electron microscope operated at 300 kV. Serial, tilted views were collected over a $\pm 60^\circ$ range at 1° increments about two orthogonal axes. Images were acquired using a $2\text{K} \times 2\text{K}$ CCD camera at a pixel size of 1.01 nm using software for automated image capture (SerialEM). Multiple frames, or montages, were collected to reconstruct an area larger than could be seen as a single CCD frame (Marsh *et al.*, 2001; O'Toole *et al.*, 2003b). Image processing was routinely carried out using the IMOD software package (Kremer *et al.*, 1996). The serial, tilted views were aligned using the positions of the fiducial markers (i.e., the gold particles), and tomograms were computed using the R-weighted back-projection algorithm. Tomograms from each axis were then aligned to each other and combined (Mastronarde, 1997). The IMOD modeling program was used for image display and 3D modeling of mitotic features such as centrioles, microtubules, microtubule ends, and chromatin (Fig. 6).

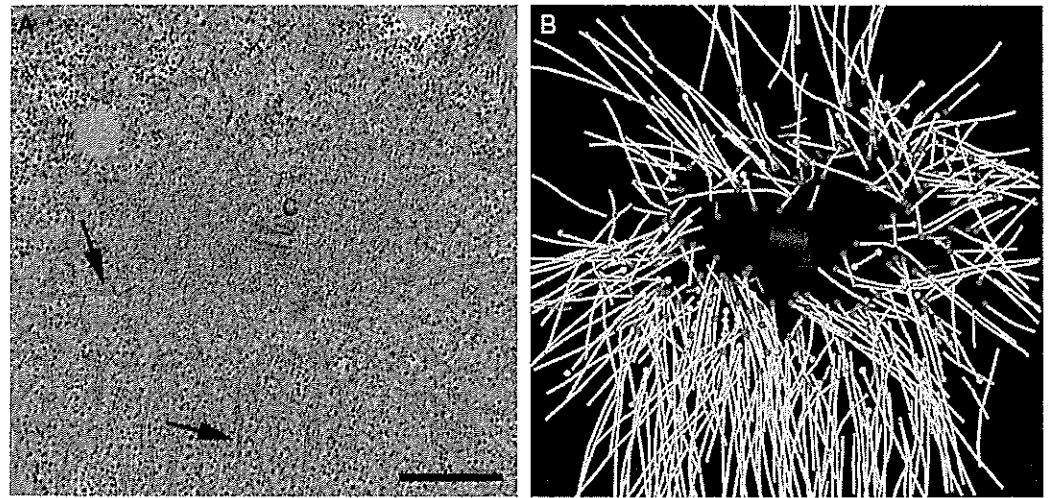


Fig. 6 Electron tomography and 3D modeling of mitotic spindle components in an isolated, eight-celled *C. elegans* embryo. (A) Tomographic slice showing the organization of microtubules (arrows) surrounding the centrioles (c). (B) Corresponding 3D model illustrating microtubules (white lines), centrioles (blue cylinders), and microtubule minus ends (closed, red spheres; open, yellow spheres) and microtubule plus ends (blue spheres). Scale bar = 1 μm . (See Color Insert.)

IV. Instrumentation and Materials

A. Staging of Early Embryos

Instrumentation: Stereo dissecting microscope with light source, light microscope equipped with either DIC or epifluorescence (for example, a Zeiss Axioplan 2 with a 20×0.5 NA Plan-Neofluar Apochromat dry objective, Hamamatsu Orca 12 bit digital camera controlled by MetaView Software, time-lapse intervals of 10–15 sec).

Materials: *C. elegans* strain XA3501 expressing GFP:: β -tubulin and GFP::histone to visualize microtubules and chromatin, strain TH31 expressing GFP:: γ -tubulin and GFP::histone to visualize centrosomes and chromatin, dialysis tubing with an inner diameter of 200 μm (Leica Microsystems), a micropipette (0.5- to 10- μl size), two syringe needles, small plastic Petri dishes, number 11 scalpel tip, whetstone, gel loader tips, nail polish, glass slides, fine-tipped tweezers.

Reagents: Worm buffer M-9 [22-mM potassium phosphate monobasic (KH_2PO_4), 19-mM NH_4Cl , 48-mM sodium phosphate dibasic (Na_2HPO_4), 9-mM NaCl], BSA (Sigma-Aldrich, Steinheim, Germany).

B. High-Pressure Freezing and Cryoprocessing of Staged Embryos

Instrumentation: Stereomicroscope with light source, high-pressure freezer (Leica EMPACT2+RTS), freeze-substitution machine (Leica EM AFS, Leica Microsystems).

Materials: Membrane carriers (100- μ m deep).
Reagents: Anhydrous acetone (EM grade), osmium tetroxide, uranyl acetate.

C. Thin-Layer Embedding, Serial Sectioning, and Prescreening of Samples

Instrumentation: Objective marker with a diamond tip (Leica), ultramicrotome (Leica UCT, Leica Microsystems), electron microscope operated at 100 kV (TECNAI 12, FEI, Eindhoven, The Netherlands) equipped with a compustage.

Materials: Glass slides, Parafilm[®], "dummy" blocks.

Reagents: Teflon[®] solution (MS-143V TFE Release Agent, Miller-Stephenson Chemical Co., Inc., Danbury, CT), Epon/Araldite, fast glue, Formvar, copper slot grids, methanol, uranyl acetate, lead citrate.

D. Electron Tomography

Instrumentation: Intermediate-voltage electron microscope operated at 300 kV (we use a TECNAI F30 FEG), high-tilt rotating stage (Gatan model 650, Pleasanton, CA), 2K \times 2K CCD camera (e.g., Gatan), image capture software package (SerialEM), and 3D reconstruction software (IMOD). Details can be found at <http://bio3d.colorado.edu>

Materials: Parafilm[®], fine-tipped tweezers.

Reagents: 15-nm-colloidal gold (Ted Pella, Redding, CA).

V. Discussion

We are interested in the 3D analysis of spindle components in early *C. elegans* embryos that have a known developmental history. Our method for correlative LM/EM has enabled us for the first time to select and stage mitotic *C. elegans* embryos prior to high-pressure freezing and subsequent electron tomography. In the next sections, we discuss various methodological aspects of this technique and, briefly, its potential for the analysis of other systems.

A. Live-Cell Imaging

Live-cell imaging prior to EM provides a short developmental history of the specimen that can help with accurate interpretation of ultrastructural information (Kapoor *et al.*, 2006; Rieder and Cassels, 1999). This is especially relevant for studies of mitosis, where it is important to determine the precise cell-cycle stage of a developing embryo and/or compare wild-type and mutant structures at identical mitotic stages. In previous studies, whole worms were cryoimmobilized and the stage of embryonic cell was determined later (O'Toole *et al.*, 2003b). Here, we have

staged single embryos prior to high-pressure freezing. For the purpose of stage determination, live-cell imaging, using either conventional phase contrast or DIC, can be applied. In the case of the early *C. elegans* embryos, phase contrast appears to be sufficient for most applications, as the early development of the embryo offers morphological criteria indicative of specific stages of the first mitotic division (Fig. 3). For instance, the appearance, migration, rotation, and disappearance of the pronuclei have routinely been followed by this method for embryonic staging. However, metaphase and early anaphase are more difficult to determine, with either phase or DIC optics. Although the application of video microscopy is not a prerequisite for documenting mitotic stages, it might be important when a specific mitotic event (e.g., onset of mitotic spindle assembly) warrants ultrastructural analysis. Imaging live embryos expressing GFP-tagged proteins that mark specific cellular components, such as centrosomes, kinetochores, histones, or spindle microtubules, provides a clear advantage for the documentation of early development, although the quality of the obtained images is not optimal (see below).

In general, the samples must be recovered during the imaging process; given that embryonic cell division in *C. elegans* is rapid, one must work quickly. For this reason, the embryos (encapsulated in dialysis tubing) are submerged in small volumes of BSA-containing M-9 buffer on the slide, but are imaged with "high-dry" objectives; this facilitates rapid recovery of the embryos. The BSA serves as a cryoprotectant but, unfortunately, it further reduces image quality. Despite the poor quality of the images, we found that most major cellular events can be distinguished (Fig. 4), and freezing of the sample can proceed within seconds after the end of imaging by LM.

B. Capillary Tubes

Nematodes have previously been studied in capillary tubes, which allow them to be incubated, gently concentrated, and continually observed in their cultivating medium until their cryoimmobilization in this state by high-pressure freezing (Hohenberg *et al.*, 1994). Here, short pieces of capillary tubes were used to "handle" isolated single *C. elegans* embryos during preparation for both light and electron microscopic observations. The advantages of cellulose capillary tubes for the described approach can be summarized as follows (Müller-Reichert *et al.*, 2003). (1) Capillary tubes can easily be cut into short pieces. (2) Development of the embryo can be continually monitored by LM because the capillary tubes are transparent. (3) Freezing in capillary tubes gives a high yield of adequately and reproducibly high-pressure frozen samples when combined with the use of an adequate cryoprotectant (see below). (4) The high porosity of the cellulose tubes means that freeze-substitution and embedding procedures are not prolonged. The tubes have pores with various molecular weight cutoffs, ranging from 5 to 20 kDa; these allow free passage of all substitution media and resin monomers. (5) The resin-embedded tubing is suitable for serial thin and semithin sectioning.

A disadvantage of the method, however, is that the tubing cannot always be sealed permanently, so the embryos "leak" out during the light microscopic observation. To prevent this, it is important to press the crimping tool down carefully during cutting.

C. Use of BSA as a "Filler"

The most critical part of high-pressure freezing is probably the loading of samples into the sample holder of the high-pressure freezer (McDonald *et al.*, 2006). It is absolutely necessary to fill the entire volume of the capillary tube, as well as the inner cavity of the specimen holder (e.g., the membrane carrier), with an appropriate medium. If there are air spaces, these will either interfere with effective heat transfer or collapse as the pressure rises, leading to the possibility that the specimen is crushed. In addition, the water content in the surrounding medium should be as low as possible. Usually, addition of an extracellular cryoprotectant serves to prevent the formation of ice crystals during the freezing process. To serve both purposes, people have previously used 1-hexadecene, a hydrophobic and chemically inert paraffin oil of rather low viscosity; it works both as "filler" and cryoprotectant (Dahl and Staehelin, 1989; Studer *et al.*, 1989). For the previous preparation of whole worms, nematodes were enclosed within buffer-containing cellulose capillary tubes and submerged in 1-hexadecene-filled sample holders, thereby avoiding direct contact between the worms and the paraffin oil (Hohenberg *et al.*, 1994). But because 1-hexadecene remains solid at freeze-substitution temperatures (Thijssen *et al.*, 1998), it is important to manually remove frozen 1-hexadecene from around the tubing under liquid nitrogen prior to freeze-substitution (Hohenberg *et al.*, 1994). Here, we have used BSA-containing M-9 buffer for routine specimen preparation and loading.

Our strategy has been to dissect whole worms in the future freezing medium, load the resulting embryos into capillary tubes, image mitotic divisions, and rapidly freeze the living embryos, all in the same medium. Importantly, when transferring the embryo-containing tube from the microscope slide to the prefilled membrane carrier, dilution effects, due to the presence of different media, can be excluded. Recent results have shown that 20% BSA gives good freezing for a variety of specimens, such as marine sponges, choanoflagellates, *Drosophila*, and wasp embryos, *C. elegans* hermaphrodites (McDonald *et al.*, 2006), murine skin cells (Reipert *et al.*, 2004a), PtK2 cells (Reipert *et al.*, 2004b), and MDCK II cells grown on filters (Manninen *et al.*, 2005). Importantly, BSA caused no problems during later steps of preparation (i.e., during serial sectioning).

D. High-Pressure Freezing

The EMPACT2 offers a variety of specimen holders (Chapter 2 by McDonald, this volume; McDonald *et al.*, 2006). The general holder is 1.2- or 1.5-mm wide and 200- μ m deep (with a central hole for the pressure tube). We routinely use the newly developed 1.5-mm-wide and 100- μ m-deep "membrane" carrier (without a

central hole). The copper "membrane" prevents direct contact of the specimen with the pressurizing fluid. In general, this specimen holder gives excellent and reproducible freezing results with isolated early embryos.

The described approach was developed for the tomographic analysis of staged *C. elegans* embryos. A prerequisite for this correlative LM/EM technique, however, is that the time between staging by LM and fixation for EM is as short as possible. The "standard" loading procedure for the EMPACT2 (i.e., when using the high-pressure freezer without the RTS) involves loading of the capillary tube into the sample holder, manually closing the sample holder after insertion into the "pod," insertion of the sample holder into the high-pressure freezer, and starting the freezing process. To minimize the time required for specimen loading, the use of the newly developed RTS associated with the EMPACT2 high-pressure freezer (Leica) is advantageous (McDonald *et al.*, 2006). When the rapid loader is used for automatic insertion of the sample holder into the preloaded pod (Fig. 5), the RTS reduces the time between observation and freezing to about 5 sec. Moreover, the automatic closure of the pod allows standardization of the loading/freezing procedure, thereby contributing to the high reproducibility of our method. It should be noted here that freezing of the isolated embryos is by no means restricted to the EMPACT2+RTS. The question, however, is whether very fast loading times can be achieved with the other commercially available high-pressure freezers. In summary, the freezing method described here gives excellent and reproducible results for ultrastructural studies.

E. Serial Sectioning

Obtaining superior preservation of cellular fine structure is a significant step in undertaking a 3D structure study (McIntosh *et al.*, 2005). It is also critical, however, to be able to localize and/or identify specific structures in a sample both quickly and repeatably (Müller-Reichert *et al.*, 2003). As an example, when one is interested in the structure and duplication of the centriole, one has to be able to routinely find centrosomes in different mitotic stages of either wild-type or RNAi embryos (Dammermann *et al.*, 2004; Kirkham *et al.*, 2003; Pelletier *et al.*, 2004b). Currently, the method of choice for this identification procedure is serial sectioning of plastic embedded samples. With good ribbons of serial sections on slot grids, one can sequentially image slices of the entire *C. elegans* embryo and identify the particular sections that contain the centrosome. Critically, for the identification of the embryonic centrosome, it is absolutely necessary to collect all the serial sections of a series because the small *C. elegans* centrioles are contained within only two to three thin sections of about 70 nm.

F. Electron Tomography and 3D Modeling

In principle, a series of thin sections can be used for 3D reconstruction by computationally "stacking" images of all successive sections and then modeling features of interest in 3D. However, this method has the limitation that the resolution

perpendicular to the plane of the section is no better than twice the average section thickness (McDonald *et al.*, 1996). Electron tomography is therefore the method of choice for visualizing the fine structure of cellular organelles with high *z*-resolution (reviewed in McEwen and Marko, 2001; Frank, 2002). Conceptually similar to CT scans in medical imaging, this method is based on the use of serial, tilted views of a semithick section (300–400 nm) to create a computer-generated volume that can be sliced and imaged in any direction. As examples, the spindle pole body (Bullitt *et al.*,

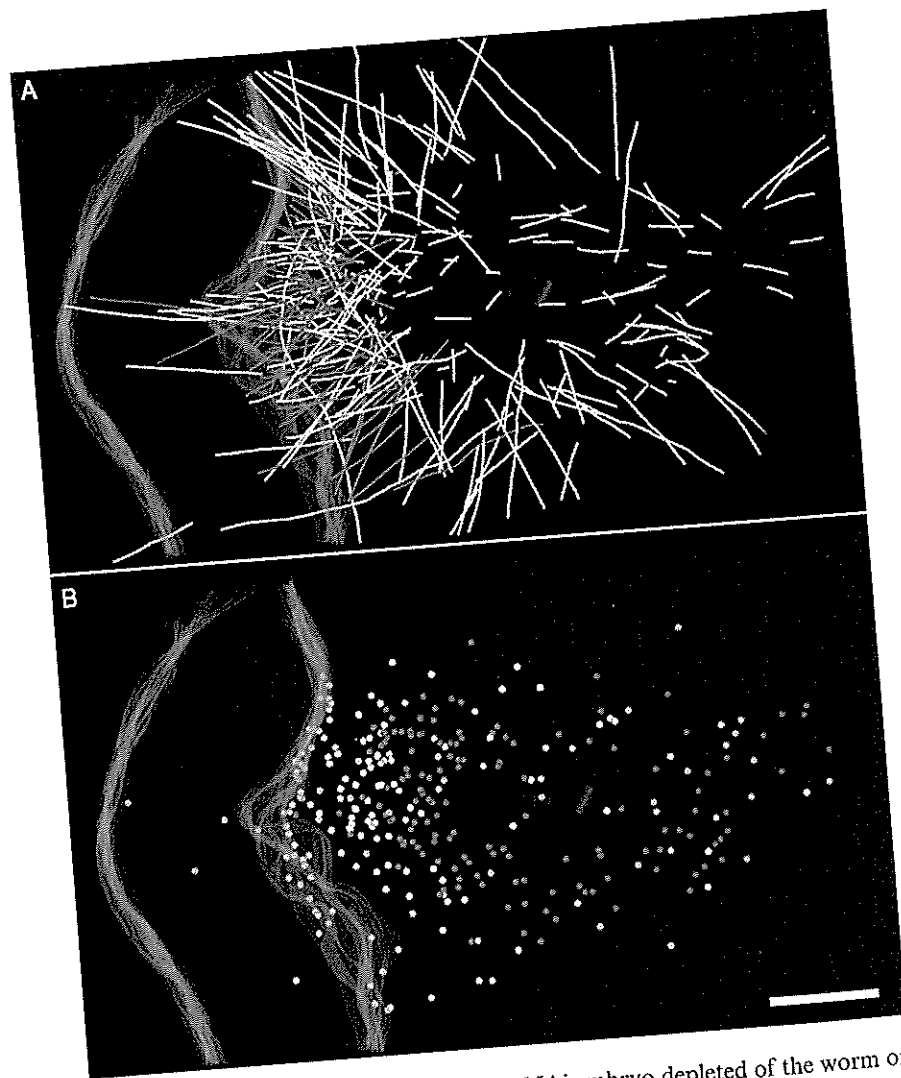


Fig. 7 Reconstruction of a spindle collapse in an RNAi embryo depleted of the worm orthologue of TPX-2. (A) 3D model computed from a 2×1 montage, showing boundary of a chromosome (blue), the position of spindle microtubules (red, white lines), and centrioles (blue cylinders). Microtubules that ended on the chromosome were defined as kinetochore microtubules (red). (B) Position of closed (red spheres) and open (yellow spheres) microtubule minus ends in the vicinity of the centrosome. Microtubule plus ends are marked with blue spheres. A high percentage of open microtubule minus ends were associated with kinetochore microtubules, and these ends were situated in a cluster at the outer edge of the centrosome (Özlu *et al.*, 2005). Scale bar = 1 μ m. (See Color Insert.)

1997; O'Toole *et al.*, 1999) and the mitotic spindle organization (O'Toole *et al.*, 1999; Yoder *et al.*, 2005) in *Saccharomyces cerevisiae*, the basal body structure in *Chlamydomonas* (O'Toole *et al.*, 2003a), the centrosome in *C. elegans* (O'Toole *et al.*, 2003b), *Drosophila* (Moritz *et al.*, 1995) and *Spisula* (Schnackenberg *et al.*, 1998), and the mammalian kinetochore (McEwen *et al.*, 1998) have been reconstructed in 3D by electron tomography. Illustrating the potential of the technique, we have successfully applied electron tomography to analyze the ultrastructure of an aster in a blastomere of an isolated eight-celled embryo (Fig. 6). Recently, we have also performed electron tomography on *C. elegans* embryos found in whole worms treated with RNAi; these show a spindle that collapse in metaphase due to depletion of the worm orthologue of TPX-2 (Fig. 7; Özlü *et al.*, 2005). 3D models of the depleted embryos revealed an obvious decrease in kinetochore microtubule length and an interesting distribution of open microtubule minus ends (i.e., a cluster of open minus ends situated on the chromosome-facing side of the centrosome). Tomography has currently been used to analyze the role of katanin in *C. elegans* meiosis (Srayko *et al.*, 2006) and to investigate centriole formation in wild-type and RNAi-treated *C. elegans* embryos in mitosis (Pelletier *et al.*, 2006).

G. Concluding Remarks

The correlative LM/EM approach described here is certainly not limited to the study of early embryos from *Caenorhabditis*. The early *Drosophila* embryo offers similar possibilities for structure–function studies (Raff, 2004). Staging early *Drosophila* embryos can be achieved by using GFP-tagged strains, and depletion of targeted gene products is routinely achieved by RNAi. Capillary tubes and the EMPACT2+RTS have been used successfully to freeze *Drosophila* embryos in the presence of 20% BSA (McDonald *et al.*, 2006). It will be challenging, however, to adapt our correlative approach to the analysis of *Drosophila* Schneider S2 and mammalian cells in culture.

We are certainly at the beginning of exploiting the use of model systems such as *C. elegans* and *Drosophila* for systematic structure–function studies. There is no doubt that it will be a challenge to take advantage of the data obtained from genome-wide screens in order to understand the mechanics of mitosis by correlative LM/EM.

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