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Electron microscopy of the early *Caenorhabditis elegans* embryo

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Summary

The early *Caenorhabditis elegans* embryo is currently a popular model system to study centrosome assembly, kinetochore organization, spindle formation, and cellular polarization. Here, we present and review methods for routine electron microscopy and 3D analysis of the early *C. elegans* embryo. The first method uses laser-induced chemical fixation to preserve the fine structure of isolated embryos. This approach takes advantage of time-resolved fixation to arrest development at specific stages. The second method uses high-pressure freezing of whole worms followed by freeze-substitution (HPF-FS) for ultrastructural analysis. This technique allows staging of developing early embryos within the worm uterus, and has the advantage of superior sample preservation required for high-resolution 3D reconstruction. The third method uses a correlative approach to stage isolated, single embryos by light microscopy followed by HPF-FS and electron tomography. This procedure combines the advantages of time-resolved fixation and superior ultrastructural preservation by high-pressure freezing and allows a higher throughput electron microscopic analysis. The advantages and disadvantages of these methods for different applications are discussed.

Introduction

The early *Caenorhabditis elegans* embryo has been studied and characterized in detail and is currently a popular model system to study centrosome formation, kinetochore assembly, spindle formation and positioning, chromosome segregation, and cellular polarization. These embryos offer significant experimental advantages. Cellular events occur at approximately the same time and in the same manner (Sulston *et al.*, 1983; Wood, 1988; Pelletier *et al.*, 2004a). The embryos are suitable for microscopy and transformation with GFP-fusion constructs, allowing intracellular processes in living cells to be monitored. In addition, selective depletion of specific gene products by RNA-mediated interference (RNAi) is an extremely powerful approach in *C. elegans* (Oegema & Hyman, 2005) that has been used in genome-wide screens to identify genes required for early embryogenesis (Sönnichsen *et al.*, 2005).

During early *C. elegans* development, meiotic and mitotic events can be observed in the same embryo, but they are normally temporally and spatially distinct. In the female germline, mature oocytes are developmentally arrested at diakinesis of prophase I. The sperm signals and fertilizes the oocyte as it commences the final stages of meiosis and passes through the spermatheca (Harris *et al.*, 2006). Meiosis then resumes with the formation of two successive acentrosomal spindles to ensure both chromosome segregation and extrusion of polar bodies (Albertson & Thomson, 1993). Completion of meiosis II is followed directly by the first mitotic cell division (Albertson & Thomson, 1982; Albertson, 1984). The female and male pronuclei typically form at the future anterior and posterior sides of the embryo, respectively. The pronuclei migrate towards each other, partly due to microtubules, which emanate from the sperm-derived centrosomes to capture the female pronucleus. After the pronulei meet near the center of the embryo, the whole centrosome–microtubule–nuclear complex aligns with the longitudinal axis of the embryo, the nuclear envelopes break down, and a centrosomal mitotic spindle forms (Albertson & Thomson, 1982; Albertson, 1984). Importantly, all of these movements are highly predictable and can be reliably used as developmental landmarks.

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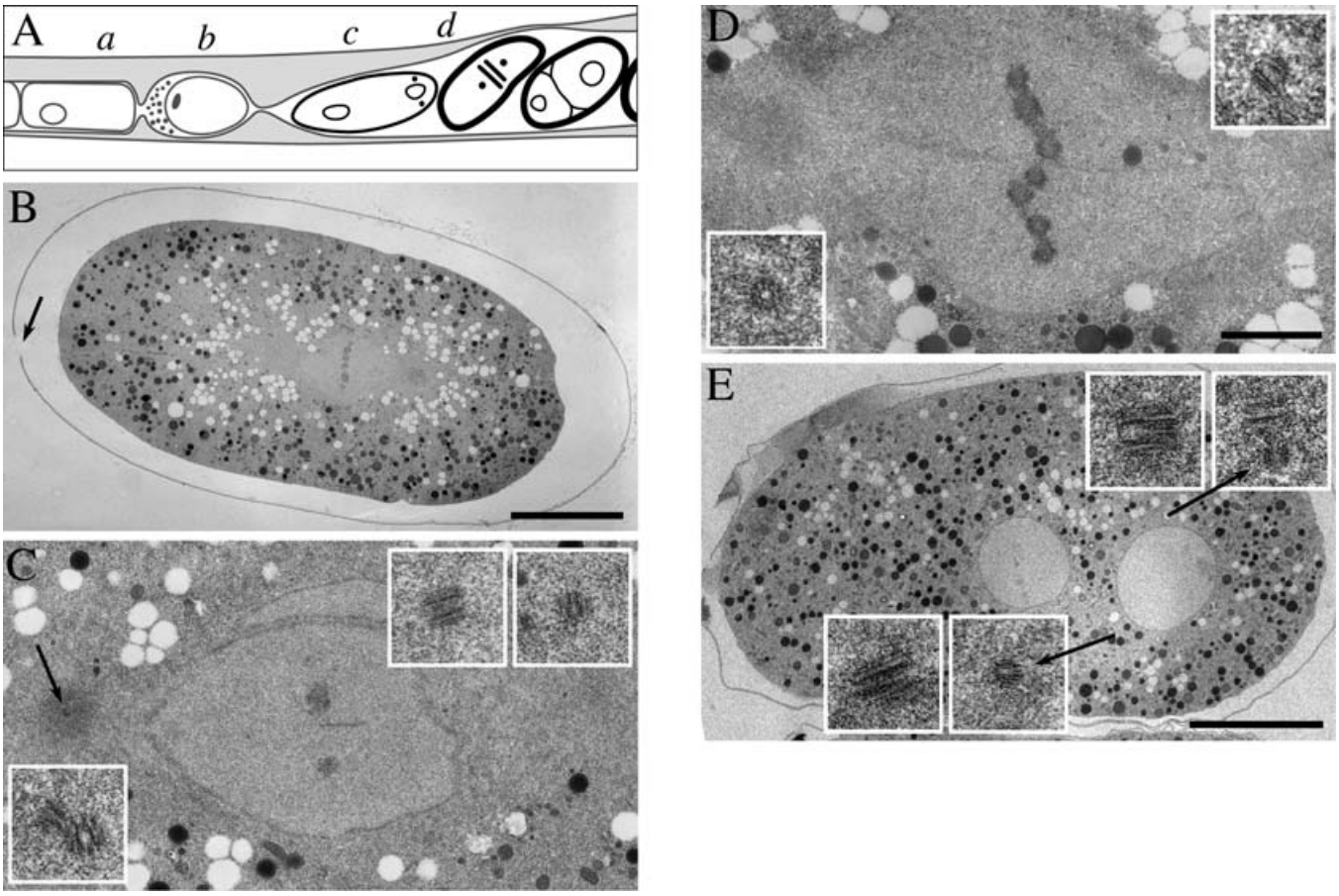

Fig. 1. Laser-induced chemical fixation of isolated early *C. elegans* embryos. (A) Schematic drawing of the proximal gonad of the *C. elegans* hermaphrodite. Mitotic embryos are surrounded by an eggshell (indicated by a 'thick line'), a structure that is secreted from the embryo after fertilization (*a*, oocyte; *b*, spermatheca containing a meiotic embryo; *c*, mitotic embryo at pronuclear appearance; *d*, mitotic embryo at anaphase; modified after Wood, 1988). (B) Thin-section electron micrograph of a wild-type *C. elegans* embryo in metaphase of the first mitotic division. Perforation of the eggshell after laser-induced chemical fixation is indicated (arrow). (C) One-cell, single wild-type embryo showing a pair of centrioles at metaphase (inserts). The position of the anterior centrosome is indicated (arrow). (D) SAS-4 depleted embryo at metaphase showing a single centriole per pole (inserts; Kirkham *et al.*, 2003). (E) Laser perforation of an embryo at pronuclear migration. This is the developmental stage when both a mother and a daughter centriole (arrows) can be detected after chemical fixation (inserts; Pelletier *et al.*, 2006). Scale bars = 10 μm (B, E), 2.5 μm (D).

After fertilization, the embryo assembles an eggshell that is impervious to many toxic agents, including 2% glutaraldehyde, illustrating the challenge of preserving these specimens for high-quality ultrastructural analyses (Fig. 1A; Priess & Hirsh, 1986; Kirkham *et al.*, 2003). In contrast to the osmotically sensitive meiotic embryo, early mitotic embryos can be readily obtained by releasing them from dissected whole worms. Here, we review three different techniques to analyze spindle assembly in *C. elegans* embryos: first, laser-induced chemical fixation of isolated mitotic embryos (Kirkham *et al.*, 2003); second, high-pressure freezing of early meiotic and mitotic embryos in whole-mounted hermaphrodites (Müller-Reichert *et al.*, 2003; Srayko *et al.*, 2006); and third, high-pressure freezing of isolated and staged mitotic embryos (Müller-Reichert *et al.*, 2007).

Materials and methods

Laser-induced chemical fixation of isolated C. elegans *embryos*

A single wild-type or RNAi early embryo is transferred to a marked region on an Aclar sheet (Electron Microscopy Sciences, Hatfield, PA, USA), previously cut down to coverslip size, glow-discharged and coated with 1% poly-L-lysine (Sigma, P1524; Sigma-Aldrich, St. Louis, MO, USA) in PBS (Kirkham *et al.*, 2003). Using an inverted microscope (Axiovert 200M, Zeiss, Germany), embryos are imaged through the Aclar using DIC. Excess liquid is removed, and the embryo is surrounded with 20 μL of 1× PHEM (60 mM PIPES [pH 6.9], 25 mM HEPES [pH 6.9], 10 mM EGTA, and 2 mM $MgCl_2$) containing 2% glutaraldehyde and 0.4% tannic

acid (O'Connell *et al.*, 2001). A pulsed solid-state UV laser (PowerChip, JDS Uniphase, Milpitas, CA, USA) focused with a 63× water immersion objective lens (C-Apochromat, Zeiss, Germany) is used for laser permeabilization of the eggshell (for details see Kirkham *et al.*, 2003). About five shots are taken at regions where the vitelline membrane touches the eggshell at the very anterior of the embryo and fixation is monitored by live DIC microscopy. The laser-induced diffusion of the fixative into the embryo instantly stops the developing process. Embryos are then incubated in fixative on ice for at least 30 min, washed 3 to 4 times in 1× PHEM, and postfixed in 1% osmium tetroxide and 0.5% $K_3Fe(CN)_6$ in 1× PHEM buffer (O'Connell *et al.*, 2001). The samples are then dehydrated through a graded acetone series, gradually infiltrated with Epon/Araldite resin (Electron Microscopy Sciences, Hatfield, PA, USA) and thin-layer embedded as described below.

High-pressure freezing of embryos in whole worms

Adult wild-type worms are selected under a stereomicroscope and transferred with a platinum wire "worm pick" to sample holders prefilled with either an *E. coli* suspension (Müller-Reichert *et al.*, 2003) or M9 worm buffer (22 mM potassium phosphate monobasic [KH_2HPO_4], 19 mM NH_4Cl, 48 mM sodium phosphate dibasic [Na_2HPO_4], 9 mM NaCl) containing 20% BSA (Sigma; Müller-Reichert *et al.*, 2007). Up to 50 worms are placed in a 100-μm-deep specimen holder: either a type A planchette, when working with the HPM 010 (BAL-TEC, Liechtenstein), or a membrane carrier, when working with the EM PACT2+RTS (Leica Microsystems, Vienna, Austria). The type A planchettes are covered with the flat side of a complementary planchette (type B; Engineering Office M. Wohlwend, Sennwald, Switzerland). Samples are high-pressure frozen and stored in liquid nitrogen until further use.

Staging of early embryos in whole worms

To stage early embryos in whole worms prior to freezing, loading devices are prepared by cutting a 2-cm-long piece of cellulose capillary tubing (inner diameter of 200 μm; Leica Microsystems), mounting it onto a plastic pipette tip (10 μL volume), and using nail polish to seal (Müller-Reichert *et al.*, 2007). To cut pieces of capillary tubing to appropriate sizes, a crimping tool is 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 (McDonald *et al.*, 2007). Hermaphrodites expressing GFP::β-tubulin/GFP::histone (Oegema *et al.*, 2001) are transferred to M9 worm buffer containing 20% BSA (Sigma) and 50 mM Levamisole (Sigma) on a glass slide. Worms become completely paralyzed within about 2 min. Under a dissecting scope, anaesthetized worms are sucked into the capillary tubes using the loading device. The distal end of the tubing is crimped off using the blunt edge of a scalpel and the other side of the capillary tube is closed by crimping (without cutting the capillary tube). The extra length facilitates manipulation of the tubing after imaging, and the crimped section keeps the worm in a relatively contained section at the end of the tubing. The capillary tube is then positioned along the long axis of the glass slide and a coverslip is placed directly on the dialysis tubing containing the worm to observe meiotic spindle assembly in real time using a spinning disk confocal microscope (DIC and fluorescence microscopy, 200 ms at 2 × 2 binning). The loading device pipette tip remains outside of the coverslip-contained area and can rest on the slide surface. At an appropriate time point, the coverslip is removed and the proximal end of the tubing is crimped off, relatively close to the encased worm. The short capillary tube containing the worm is then loaded into the pre filled membrane carrier and frozen using the RTS of the EM PACT2 high-pressure freezer. The developmental history of every embryo is documented in time-lapse movies.

Staging and high-pressure freezing of isolated mitotic C. elegans *embryos*

Worms are cut open in small Petri dishes using M9 buffer containing 20% BSA (Sigma). Embryos are released by cutting the worm in the gonad region with two injection needles. An early embryo is then selected and sucked into the capillary tube, using the loading device mounted on a Pipetman (Müller-Reichert *et al.*, 2007). Afterwards, the tubing is submerged into the BSA-containing M9 buffer in the Petri dish and the crimping tool is then used to cut the region of the tubing containing the early embryo to a final length of about 1 mm. The closed tube is then transferred from the Petri dish to a droplet of BSA-containing M9 buffer on a glass slide to observe the further development of the embryo in real time using a light microscope equipped with phase contrast or DIC. The developmental history of the selected embryo is documented by video microscopy. The 1-mm segment of the capillary tube is then fitted into the cavity of a 100-μm-deep membrane carrier (Leica) pre filled with M9 buffer containing 20% BSA (Sigma). The specimen carrier is then inserted into the rapid transfer system of the EM PACT2 (Leica) and frozen. The frozen samples are stored in liquid nitrogen until further use.

Freeze-substitution and infiltration with resin

For freeze-substitution, sample holders (i.e. either type A planchettes or membrane carriers) are transferred to pre-cooled cryovials (–90°C) containing the freeze-substitution 'cocktail'. Freeze-substitution for morphological studies is performed in anhydrous acetone containing 1% osmium and 0.1% uranyl acetate (McDonald & Müller-Reichert, 2002). Using a Leica EM AFS, samples are maintained at –90°C from 6 to 48 h. Freeze-substituted samples are then allowed to warm to room temperature at a rate of either 5 or 10 °C h^{-1}. At room

temperature, specimens are washed three times for 1 h in fresh anhydrous acetone. After the washes, samples are gradually infiltrated with Epon/Araldite resin (one 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) as described (Müller-Reichert *et al.*, 2003).

Thin-layer embedding and remounting

After infiltration, either whole worms or specimen-containing capillary tubes are embedded in thin, optically clean layers of Epon/Araldite on microscope slides (McDonald, 1994; Müller-Reichert *et al.*, 2003; Müller-Reichert, 2007). Slides are wiped clean with a soft cloth and coated with Teflon® using either a spray (MS-122DF) or a solution (MS-143V, Miller-Stephenson Chemical, Danbury, CT, USA). Worms or capillary tubes in Epon/Araldite are gently pressed down to the surface of the glass slides. Two layers of Parafilm are used as spacers and coated slides are put on top of the resin samples. After polymerization in an oven at 60°C for 1–2 days, one glass slide is removed and worms and capillary tubes are relocated by light microscopy. Samples are remounted on 'dummy' blocks for ultramicrotomy as published (Müller-Reichert *et al.*, 2003).

Serial sectioning and electron microscopy

Longitudinal sections through whole worms or isolated embryos are cut using an Ultracut UCT Microtome (Leica). Both thin (70 nm) and semi-thick (300–400 nm) Epon/Araldite sections are collected on Formvar-coated copper slot grids and post-stained with 2% uranyl acetate in 70% methanol followed by Reynold's lead citrate. Thin sections are imaged in a TECNAI 12 transmission electron microscope (FEI) operated at 100 kV.

Tomographic reconstruction and 3D modelling

In preparation for electron tomography, 15-nm colloidal gold particles are attached to both surfaces of the semi-thick (300- or 400-nm) sections. Samples are placed in a high-tilt stage (Gatan) and imaged using a TECNAI F30 microscope (FEI, The Netherlands) operated at 300 kV. Serial, tilted views are collected over a $\pm 60^\circ$ range at 1° increments in two orthogonal axes. Images are acquired using a Gatan Ultrascan 895 CCD camera at a pixel size of 1 nm using an automated image capture software package (SerialEM, Boulder Laboratory for 3-D Fine Structure). For larger areas, multiple frames or montages are collected as described (Ladinsky *et al.*, 1999; Marsh *et al.*, 2001; O'Toole *et al.*, 2003b). Image processing is carried out using the IMOD software package (Kremer *et al.*, 1996). The serial, tilted views are aligned using the positions of the gold fiducial markers, and tomograms are computed using an R-weighted back-projection algorithm (Gilbert, 1972). Tomograms from each axis are then aligned to each other and combined (Mastronarde, 1997). The 3dmod program (Kremer *et al.*, 1996) is used for image display and 3D modelling of cellular components, such as microtubules, pole-proximal or -distal microtubule ends, centrioles and chromatin.

Results

Laser-induced chemical fixation of isolated embryos

Isolated mitotic *C. elegans* embryos continue to divide in the presence of 2% glutaraldehyde. To stop the early development of the *C. elegans* embryo at specific stages and fix its ultrastructure for electron microscopy, we have used laser-assisted chemical fixation (Priess & Hirsh, 1986; Kirkham *et al.*, 2003). Perforation of the embryo's eggshell allows a diffusion of the fixative into the embryo and stops the mitotic division. The local destruction of the eggshell with a laser beam is shown by thin-section electron microscopy (Fig. 1B; arrow). This method gives good ultrastructural preservation to answer some questions, such as whether centriole duplication has occurred under certain mutant conditions. As an example, in *sas-4(RNAi)* embryos the first mitotic division is normal, but embryos develop a monopolar spindle in the second mitotic division (Kirkham *et al.*, 2003). Electron microscopy can show that each centrosome of the one-cell wild-type embryo at metaphase consists of a pair of centrioles surrounded by pericentriolar material (PCM; Fig. 1C, insets). In contrast, centrosomes of one-celled *sas-4(RNAi)* embryos show a single centriole organizing the surrounding PCM (Fig. 1D, insets). Electron microscopy can therefore establish whether a specific protein is involved in centriole duplication. Another application of this method is the analysis of nuclear envelope breakdown (NEBD) during the first mitotic division. Application of laser perforation is sufficient to visualize the polar fenestration of the two pronuclei during the first mitotic division. Using this method, however, it is difficult to resolve the ultrastructural details of the centriole itself. Along this line, the developing daughter centriole can first be observed at pronuclear migration (Fig. 1E, insets). In conclusion, this method is sufficient for rapidly arresting cellular processes to obtain specimens for routine thin-section electron microscopy, but these specimens are usually inadequate for high-resolution 3D structure analysis by electron tomography.

High-pressure freezing of whole worms

Alternatively, we have performed high-pressure freezing of whole worms to get superior preservation of embryonic ultrastructure. Hermaphrodites were frozen in M9 worm buffer containing 20% BSA and subsequently thin-layer embedded after freeze-substitution. Embedding of whole worms in a thin layer of Epon/Araldite allowed us to screen for worms with appropriate numbers of embryos (Müller-Reichert *et al.*, 2003). A low-magnification image of a worm

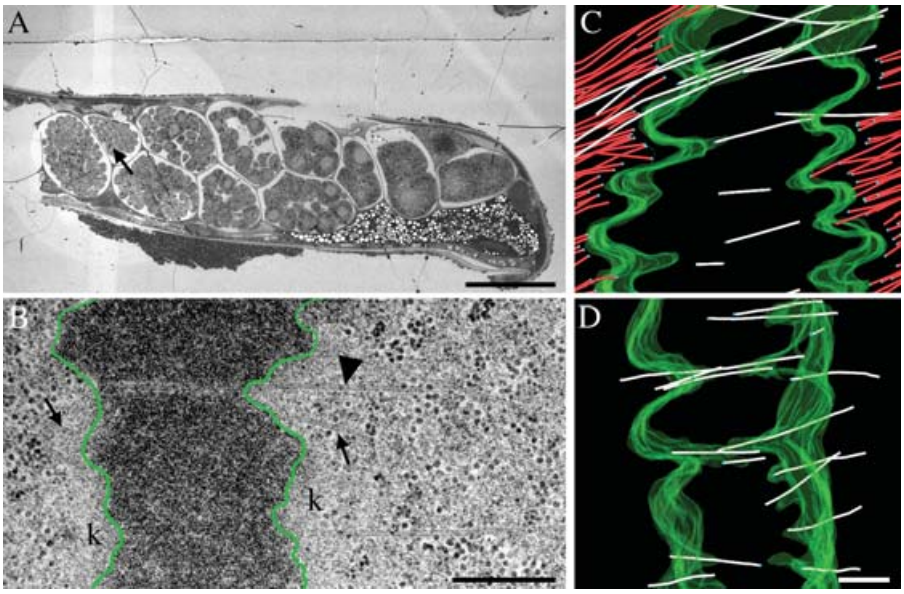

Fig. 2. High-pressure freezing of mitotic embryos in whole worms. (A) Low-magnification view of a serial section showing a worm in longitudinal orientation. A cell in metaphase in a multi-cell embryo was selected (arrow). (B) Thin section through two kinetochore regions from cell in metaphase. Kinetochores (k) on the pole-facing sides of the chromatin (chromosome surface outlined in green) show a characteristic ribosome-free zone (Howe *et al.*, 2001; O'Toole *et al.*, 2003b). Most microtubules terminate in this zone (arrows), but some microtubules pass through the chromatin (arrowhead). (C, D) 3D models of microtubules in the central region of metaphase spindles. Microtubules terminate at the kinetochore region (red lines, microtubule plus ends shown as blue spheres) or pass through the chromatin (white lines). Scale bars = 50 μm (A), 500 nm (B, D).

containing multiple embryos is shown in Fig. 2A. The image shows one section out of a series that was screened for an embryonic cell in mitosis (Fig. 2A, arrow). Two kinetochore regions of a mitotic cell in metaphase are shown at higher magnification (Fig. 2B). This thin-section electron micrograph shows the condensed DNA, a ribosome-free zone around the chromatin and spindle microtubules either terminating in the ribosome-free zone or passing the condensed chromatin. We used such prepared specimens to analyze the 3D distribution of microtubules at the metaphase plate. A 3D model obtained after tomographic reconstruction of a portion of a metaphase plate is shown in Fig. 2C and D. A prominent zone of interdigitating microtubules at this stage is not obvious from light microscopic data. We therefore sought to determine whether bundles of microtubules penetrate the condensed chromatin. Three-dimensional modelling revealed that there are many microtubules that pass through the chromatin (Fig. 2C, D; white lines). In essence, high-pressure freezing of whole worms delivers samples with excellent ultrastructural preservation, but the stage of the mitotic embryos must be determined after preparation for electron microscopy.

We sought to use EM to reveal structural details of the *C. elegans* meiotic spindle. In contrast to mitotic embryos, meiotic embryos are osmotically sensitive and fragile, and cannot easily be cultivated after release from whole worms. One possibility is to freeze meiotic embryos in whole worms and cut through the hermaphrodite to identify and reconstruct meiotic spindles in serial semi-thick sections (Fig. 3; Srayko *et al.*, 2006). Using this approach, we were able to partially reconstruct meiotic spindles (Fig. 3A) and show morphologies of both pole-proximal (Fig. 3B, top) and pole-distal microtubule ends (Fig. 3B, bottom). In addition, we identified katanin-dependent microtubule lattice defects in meiotic wild-type spindles (Fig. 3C). These lattice defects were absent in katanin-depleted embryos and support a mechanism of microtubule severing that involves an initial perforation of the protofilament wall (Srayko *et al.*, 2006).

In this study, the stage of the meiotic embryos was determined after freezing by identifying the position of the embryos within the spermatheca/gonad and by determining the number of extruded polar bodies (Srayko *et al.*, 2006). We therefore wanted to develop a method to stage developing meiotic embryos prior to cryoimmobilization. To achieve this goal, we anaesthetized whole hermaphrodites using Levamisole and collected them into capillary tubes (Fig. 4A). Taking advantage of a GFP::tubulin/GFP::histone strain to visualize meiotic spindle assembly, we were able to identify fertilized oocytes of various stages in these whole worms and record their early development (Fig. 4B–F). Stills of a QT movie show an oocyte passing through the spermatheca. Chromosomes are condensing and aligning (Fig. 4B–F, arrows). As a disadvantage, this method also requires serial sectioning of whole worms to locate the video-recorded meiotic cells of interest.

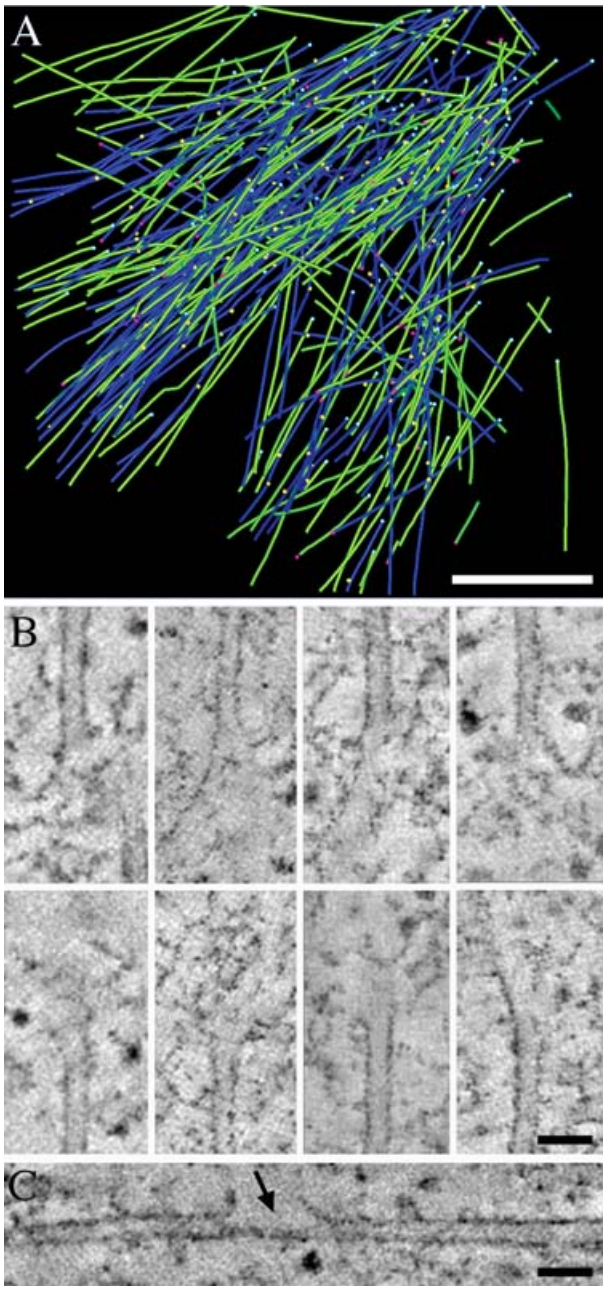

Fig. 3. Arrangement and structure of spindle microtubules in meiotic embryos contained in whole worms. (A) Three-dimensional distribution of microtubules in a meiotic half spindle. The model was built from three serial tomograms. Microtubules (blue, green lines) form a short barrel-shaped spindle. Microtubule pole-proximal ends (blue spheres) are not focused at a distinct pole (p). Microtubule pole-distal ends (purple spheres) were also found throughout the spindle. A subpopulation of microtubules (blue lines) showed points of fragmentation, as indicated by yellow spheres. (B) The majority of pole-proximal (top) as well as pole-distal microtubule ends (bottom) are open. (C) Selected 2-nm tomographic slice showing a microtubule along its length. Evidence of lateral disruption of the microtubule is evident (arrow). This fragmentation is caused by MEI-1, the *C. elegans* katanin microtubule severing protein (Srayko *et al.*, 2006). Scale bars = 1 μm (A), 50 nm (B, C).

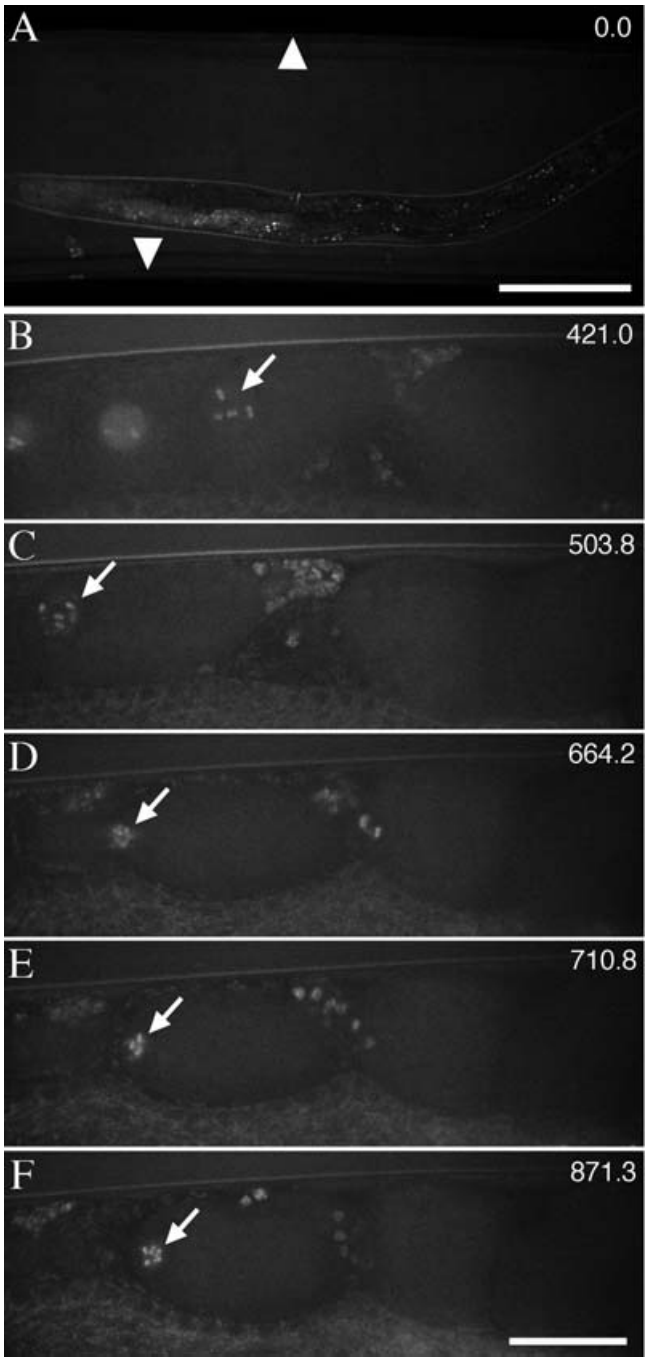

Fig. 4. Light microscopic observation of developmental events in whole worms. (A) Fluorescence microscopy of a worm expressing GFP::β-tubulin/GFP::histone. The capillary tube is indicated (arrow heads). (B–F) Proximal gonad of the same worm at higher magnification. An embryo (left) is pushed through the spermatheca. Chromosomes are condensing and aligning (arrows). This embryo is approaching the first meiotic metaphase (F). Using time-lapse microscopy, the meiotic divisions can be recorded continuously. Scale bars = 250 μm (A), 25 μm (F).

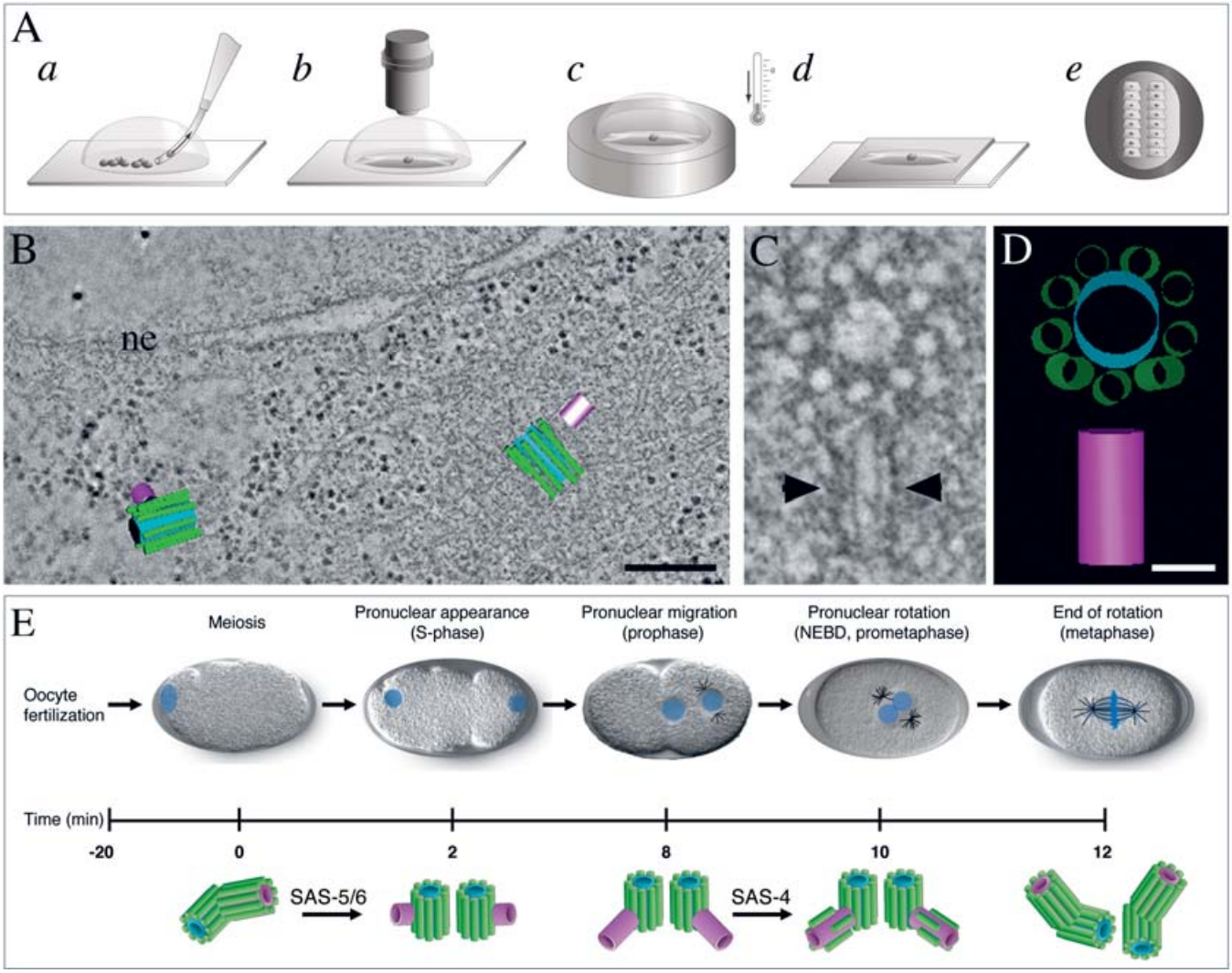

Fig. 5. Correlative light- and electron microscopy of isolated *C. elegans* embryos. (A) The preparation of single embryos for high-pressure freezing and electron tomography involves: *a*, collecting isolated embryos into capillary tubing; *b*, observation of early embryos in capillary tubing by light microscopy; *c*, transfer of tubing to specimen carriers for high-pressure freezing; *d*, thin-layer embedding of single embryos; and *e*, serial thick-sectioning of the remounted embryo for subsequent electron tomography (modified from Müller-Reichert *et al.*, 2007). (B) Tomographic slice illustrating the ultrastructure of centrioles at pronuclear appearance. The mother centrioles (blue) and the developing daughter centrioles (purple) are visible; ne = nuclear envelope. (C) Tomographic slice at higher magnification showing the assembly of the daughter centriole at pronuclear migration. The 'naked' tube of the developing daughter centriole is marked (arrow heads). (D) Three-dimensional model of the developing daughter centriole. (E) Structural pathway of centriole assembly in the context of the early development of the one-cell *C. elegans* embryo. Daughter centriole assembly begins with the formation and elongation of a central tube followed by the peripheral assembly of nine singlet microtubules. Tube formation and elongation is dependent on the SAS-5 and SAS-6 proteins, whereas the assembly of the nine singlet microtubules onto the central tube depends on SAS-4 (adapted from Pelletier *et al.*, 2006). Scale bars = 250 nm (B), 50 nm (D).

Correlative light and electron microscopy of isolated embryos

Next, we combined the advantage of excellent ultrastructural preservation with the possibility to stage-isolated embryos prior to freezing for electron microscopy (Müller-Reichert *et al.*, 2007). The steps involved are illustrated in Fig. 5A. Early embryos are obtained by cutting whole worms and collecting them in cellulose capillary tubes. The contained embryos are then imaged by light microscopy to follow the developmental events during the first mitotic division. Staging was easily achieved by phase contrast, because the formation, migration and disassembly of the pronuclei are indicative of specific stages of the first mitotic division (Pelletier *et al.*, 2004a). At specific stages we transferred the cellulose tubing to specimen holders for high-pressure freezing and processed the samples for electron microscopy. This method provided staged specimens

with excellent ultrastructural preservation. Serial sectioning through whole embryos then allowed us to identify those thick sections containing the centrosomes with developing daughter centrioles. Mapped sections were then imaged by intermediate-voltage electron tomography to obtain a series of tilted views. Calculated tomograms were used to analyze the ultrastructure of centrioles at specific stages (Fig. 5B, C) and to generate 3D models (Fig. 5D). Using this approach, we have analyzed the structural pathway of centriole assembly in early wild-type *C. elegans* embryos as summarized in Fig. 5E (Pelletier *et al.*, 2006). Electron tomography revealed that daughter centriole assembly begins with the formation and elongation of a central tube followed by the peripheral assembly of nine singlet microtubules. In contrast to chemically fixed embryos, formation of the central tube could be observed as early as pronuclear appearance (see above). In addition, we have used RNAi in combination with electron tomography. Using this approach, we showed that tube formation and elongation is dependent on the SAS-5 and SAS-6 proteins, whereas the assembly of the nine singlet microtubules onto the central tube depends on SAS-4 (Pelletier *et al.*, 2006). Previously, we could demonstrate by immunoelectron microscopy that the SAS proteins localize to the *C. elegans* centriole (Kirkham *et al.*, 2003; Dammermann *et al.*, 2004).

Discussion

Chemical fixation vs. high-pressure freezing

The first mitotic division of the developing *C. elegans* embryo is attracting much attention, because the early embryonic development can be followed elegantly by non-invasive live-cell light microscopy and is subject to molecular manipulation by RNA-mediated interference (RNAi). Thus, there is an increasing demand to analyze the ultrastructure of wild-type and mutant early *C. elegans* embryos. Using conventional methods of specimen preparation for electron microscopy, however, *C. elegans* has traditionally been considered difficult to fix (Müller-Reichert *et al.*, 2003). The thick cuticle surrounding *C. elegans* acts as a natural diffusion barrier to chemical fixatives and worms are able to survive up to 7 h in a solution containing 10% glutaraldehyde (Shepard & Clarke, 1976). Compared with whole worms, it is even more difficult to fix early embryos of *C. elegans*. When submerged in buffer containing 2% glutaraldehyde, early embryos continue to go through the first mitotic divisions normally (Kirkham *et al.*, 2003). To allow adequate chemical fixation by glutaraldehyde, the eggshell of the embryo has to be either digested enzymatically or treated with bleach (Vancoppenolle *et al.*, 2000). These methods are not suitable to fix a developing embryo rapidly. We have therefore applied laser-assisted perforation of the eggshell to stop and fix embryos at defined stages (Kirkham *et al.*, 2003; Dammermann *et al.*, 2004; Pelletier *et al.*, 2004b).

Chemical fixation is fraught with a number of problems (for a review see Murk *et al.*, 2003). The quality of chemical fixation is mainly dependent on the diffusion rate of the fixing agent. For *C. elegans*, glutaraldehyde can diffuse into the embryo through a laser-induced hole in the eggshell (Fig. 1). The quality of this laser-induced chemical preservation is surprisingly good and some questions can be answered, such as whether centriole duplication has happened or whether the nuclear envelope is intact at embryonic spindle poles. It is clear, however, that the preservation is not sufficient to analyze intermediate products of centriole duplication in 3D (Pelletier *et al.*, 2006). The alternative to conventional chemical fixation by diffusion is cryoimmobilization, and numerous reports have shown that high-pressure freezing followed by freeze-substitution is the method of choice for the preservation of *C. elegans* ultrastructure (Fig. 2 and 3; for additional references see Müller-Reichert *et al.*, 2003; Weimer, 2006; McDonald, 2007; McDonald *et al.*, 2007). It should be mentioned here briefly, that HPF-FS is also the method of choice for on-section immunolabelling studies in *C. elegans*. A detailed protocol has been published previously (Müller-Reichert *et al.*, 2003).

Loading strategies for high-pressure freezing

Loading of the sample into the specimen carrier is one of the most important steps in high-pressure freezing. Cryoprotectants, used for sample loading, serve to fill the entire volume of the inner cavity of the specimen holder and simultaneously prevent the formation of ice crystals during the freezing process. As an example, 1-hexadecene, a hydrophobic and chemically inert paraffin oil of rather low viscosity, was used in other studies (Dahl & Staehelin, 1989; Studer *et al.*, 1989). For this, whole worms were enclosed within the buffer-containing capillary tubes and submerged in 1-hexadecene-filled sample holders, thereby avoiding direct contact of the worms with the paraffin oil (Hohenberg *et al.*, 1994). We have reported the use of *E. coli* and/or yeast paste to fill sample holders for HPF (Müller-Reichert *et al.*, 2003). Another loading strategy is to reduce the volume of the sample holder by employing a slot-grid as a 'spacer' between the flat sides of Type B specimen carriers used for the BAL-TEC HPM 010 high-pressure freezer (McDonald *et al.*, 2007). This method gives excellent specimens for ultrastructural studies and avoids a possible darkening of the *E. coli*/yeast paste during freeze-substitution (see also below).

Recently, we have introduced the use of BSA-containing M9 buffer for routine specimen preparation and loading (McDonald *et al.*, 2007). BSA in buffer gives excellent freezing results for both whole worms and isolated *C. elegans* embryos and a number of additional other specimens, including marine sponges, marine clam eggs, choanoflagellates, wasp embryos, *Drosophila*, and MDCK II cells grown on filters (Manninen *et al.*, 2005; McDonald *et al.*, 2007; Müller-Reichert *et al.*, 2007; Verkade, 2008). Disadvantages, however, are related to sample

handling after freeze-substitution. The BSA-containing 'filler' turns into a viscous material after freeze-substitution. Worms might therefore break during removal of the specimens from the sample holder. In addition, when working with isolated embryos, BSA may turn dark, thus preventing an easy identification of embryo in the cellulose capillary tube prior to remounting (Müller-Reichert *et al.*, 2007).

Cellulose capillary tubes for live-cell imaging and high-pressure freezing

Capillary tubing was first used in electron microscopy to incubate, concentrate and observe nematodes in their cultivating medium prior to high-pressure freezing (Hohenberg *et al.*, 1994). We have used short pieces of cellulose capillary tubes to stage isolated mitotic early *C. elegans* embryos (Müller-Reichert *et al.*, 2007). There are many advantages to using capillary tubes for correlative light and electron microscopic studies. (1) Early embryos can easily be collected in these tubes and short pieces of appropriate sizes are easily obtainable. (2) Transparency of the cellulose tubes allows a continuous observation of the early embryonic development prior to freezing using a variety of microscopy techniques. (3) A high yield of adequately frozen samples is obtained when using BSA in M9 buffer. (4) The pores of the tube allow passage of substitution media and resin monomers. (5) Serial sectioning of plastic-embedded tubes can be achieved routinely.

Live-cell imaging of meiotic embryos is hindered by the fact that the development of the eggshell is not complete at these early stages of development. Thus, the meiotic embryo is osmotically sensitive and does not tolerate 20% BSA after release from whole worms. We therefore decided to use cellulose capillary tubing to stage meiotic embryos in whole worms contained in BSA-filled capillary tubes (Fig. 4). A fast transfer of the capillary tubing from the light microscope to the sample holder, as developed for single embryos (see below), allows a good correlation of live-cell imaging and electron microscopy. When using this method, it is an advantage that the meiotic stage of embryos can be determined prior to freezing. It is a disadvantage, however, that one has to collect serial sections through whole worms. When working with worms in tubes, it is important to note that the tubing cannot always be sealed permanently. Although hermaphrodites can be anaesthetized for video microscopy with Levamisole, worms might occasionally "escape" because they are not completely immobilized. In addition, the use of BSA as a cryoprotectant may reduce image quality and impede the visualization of fine structures such as microtubules containing GFP-labeled tubulin (Müller-Reichert *et al.*, 2007) or growing microtubule ends obtained with a GFP-EB1 marker (Srayko *et al.*, 2005). Despite this disadvantage, the cellular events can be distinguished to allow staging of the meiotic embryo, and GFP-histone allows sufficient detail in the fluorescence channel. Interestingly, a similar correlative approach has been published by Sims and Hardin (2007), who have used fluorescence light microscopic images as overlays to identify specific structures in micrographs obtained from transmission electron microscopy.

High-pressure freezing for cellular electron tomography

C. elegans embryos, contained in capillary tubing, are rapidly frozen using an EM PACT2+RTS (Leica) high-pressure freezer, a portable machine that can be easily moved to the site where staging of the embryo is performed. The rapid transfer system (RTS) allows fast loading of the specimen into a pre-loaded high-pressure freezer under standardized conditions (McDonald *et al.*, 2007; Verkade, 2008). At an appropriate developmental stage, the cellulose capillary tube is quickly transferred from a microscope slide to the BSA-filled specimen carrier. As soon as the capillary tube is loaded in the membrane carrier, the rapid loader is transferred to the RTS. Sliding the rapid loader into the RTS triggers the pod to close automatically and start the ultra rapid freezing procedure. Specimens are thus transferred from the light microscope to the high-pressure freezer within about 5 seconds. As mentioned elsewhere, the use of the EM PACT2 machine is not a prerequisite for applying a correlative microscopic approach (Müller-Reichert *et al.*, 2007). However, it is recommended to use the rapid transfer system (RTS) of the EM PACT2 to minimize the time window between light microscopic observation and freezing.

Superior preservation of ultrastructure is the first important step in undertaking a 3D study (Müller-Reichert *et al.*, 2003). Electron tomography is currently the method of choice for the 3D visualization of cellular fine structure with a resolution of approximately 6 nm in 3D (reviewed in McEwen & Marko, 2001; Frank, 2002; McIntosh *et al.*, 2005). Conceptually similar to CT scans in medical imaging, this method is based on the use of serial, tilted views of a semi-thick section (300–400 nm) to create a computer-generated volume that can be sliced and imaged in any direction. Using this method, the spindle pole body (Bullitt *et al.*, 1997; O'Toole *et al.*, 1999) and mitotic spindle organization (O'Toole *et al.*, 1999; Yoder *et al.*, 2005) in *Saccharomyces cerevisiae*, the organization of interphase microtubules in fission yeast (Höög *et al.*, 2007), 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 (VandenBeldt *et al.*, 2006; Dong *et al.*, 2007, in press) have been reconstructed in 3D. We have recently applied electron tomography to analyze the role of katanin in female *C. elegans* meiosis (Srayko *et al.*, 2006) and the structural pathway of centriole assembly in early *C. elegans* embryos (Pelletier *et al.*, 2006).

Previously, worms containing random populations of embryos were frozen and the stages of embryos determined after the fact by collecting serial plastic sections and traversing

through entire worms (O'Toole *et al.*, 2003b; Özlü *et al.*, 2005). Following the same specimen in the light and electron microscope has eliminated this time-consuming effort and allowed the identification of many embryos of known developmental history (Fig. 5; Pelletier *et al.*, 2006; Schlaitz *et al.*, 2007). For the structural analysis of centriole assembly in *C. elegans* embryos, we were able to record five double-tilt data sets of wild-type poles at pronuclear appearance, five at pronuclear migration, five at pronuclear rotation, and six during mitosis. In addition, we acquired four data sets of *sas-4(RNAi)* embryos, two of *sas-5(RNAi)*, and three of *sas-6(RNAi)* embryos (Pelletier *et al.*, 2006). We consider this to be an important step towards a higher throughput EM analysis of *C. elegans* mitosis.

In summary, we believe that the correlative LM-HPF approach in tandem with electron tomography of serial resin sections offers a number of significant advantages for the ultrastructural analysis of the early *C. elegans* embryo. These include (1) exact staging of single embryos by light microscopy; (2) consistent identification of cellular components, such as centrosomes and kinetochores in plastic-embedded material; (3) higher throughput analysis by electron microscopy; and (4) systematic comparison of wild-type and RNAi-treated or mutant embryos for structure/function studies. All these advantages will contribute to a full microscopic exploitation of a genetic system, such as *C. elegans*, and we are certainly only at the beginning of doing so.

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