# Ultrastructural Analysis of *Drosophila* Ovaries by Electron Microscopy

**Thomas R. Hurd**[1,2], **Carlos G. Sanchez**[1,2], **Felipe K. Teixeira**[1,2], **Chris Petzold**[3], **Kristen Dancel-Manning**[3], **Ju-Yu S. Wang**[1,2], **Ruth Lehmann**[1,2], and **Feng-Xia A. Liang**[2,3]

[1]Howard Hughes Medical Institute (HHMI) and Kimmel Center for Biology and Medicine of the Skirball Institute, New York University School of Medicine, New York, NY 10016, USA

[2]Department of Cell Biology, New York University School of Medicine, New York, NY 10016, USA

[3]Office of Collaborative Science Microscopy Core, New York University School of Medicine, New York, NY 10016, USA

## i. Summary

The *Drosophila melanogaster* ovary is a powerful, genetically tractable system through which one can elucidate the principles underlying cellular function and organogenesis *in vivo*. In order to understand the intricate process of oogenesis at the subcellular level, microscopic analysis with the highest possible resolution is required. In this chapter, we describe the preparation of ovaries for ultrastructural analysis using transmission electron microscopy and focused ion beam scanning electron microscopy. We discuss and provide protocols for chemical fixation of *Drosophila* ovaries that facilitate optimal imaging with particular attention paid to preserving and resolving mitochondrial membrane morphology and structure.

### Keywords

*Drosophila* ovary; germline; transmission electron microscopy (TEM); mitochondria; cristae; focus ion beam scanning electron microscopy (FIB-SEM)

## 1. Introduction

*Drosophila* oogenesis is a widely used model for studying a range of biological processes including adult stem cell self-renewal, maintenance and cell differentiation [1-3]. The wealth of genetic tools available and the ease with which *Drosophila* ovaries can be imaged make it a particularly attractive system to study these processes. Light microscopy of fixed ovaries is the most commonly used approach to study *Drosophila* oogenesis. In addition to examining protein expression and localization in fixed tissues, the dynamics of oogenesis are increasingly also being explored in real time by live confocal imaging, with fluorescent protein markers to follow proteins, and the bacterial phage MS2 coat proteins and its RNA binding motifs to follow RNAs [2, 4]. The simplicity of light microscopy coupled with the fact that it can be used to monitor changes live as they occur, account for its widespread use. However, not all cellular structures can be resolved by light microscopy. Electron

microscopy (EM) in contrast permits much better resolution of most cellular structures by exploiting the far shorter de Broglie wavelength of the electron [5, 6]. To date, EM has proven an essential method for understanding key processes of oogenesis such as polar granule morphology and composition.

### 1.1 Electron Microscopy of *Drosophila* Oogenesis

The first EM studies of the *Drosophila* ovary were published in the late 1960s and early 1970s. These initial studies by Cummings and King [7, 8] and Mahowald [9] characterized many general aspects of the process. However, since then the focus of many ultrastructural studies has been on determining the structure and composition of a specialized cytoplasm, called the germ plasm, that forms at the posterior of the late stage oocytes, and which is both necessary and sufficient to generate germ cells in the developing embryo [10, 11]. Ultrastructural studies have shown that the germ plasm is enriched in mitochondria and large electron dense structures, termed polar granules [12] (Fig. 1). Further studies using EM have characterized when polar granules first form and how their shapes change during development and between species [13]. Immuno-EM has also been indispensible to determining the protein and RNA constituents of polar granules [13] (Fig. 1). The elucidation and characterization of the polar granule structure and composition with EM is just one example of how useful this method can be. Due in no small part to ultrastructural studies, we now have a much better understanding not only of germ plasm and germ granules, but also of many facets of *Drosophila* oogenesis.

### 1.2 Preparation of *Drosophila* Ovaries for Transmission Electron Microscopy

The most often used method to prepare *Drosophila* ovaries for transmission electron microscopy (TEM) is conventional chemical fixation [9, 14, 15]. Typically, ovaries are first fixed at either room temperature or 4°C with glutaraldehyde to cross-link proteins, and subsequently with osmium tetroxide to preserves lipids, especially phospholipid membranes [6, 16-18]. Ovaries are then treated with organic solvents such as ethanol to remove water prior to embedment in a water-insoluble resin for subsequent sectioning [6, 16] (Fig. 2). An ultramicrotome is used to cut thin sections, which are subsequently stained with heavy metals to impart contrast for cell ultrastructure [6, 16] (Fig. 3).

The major limitation of chemical fixation methods is the slow rate of diffusion of fixatives into tissues [6, 16, 17]. This is particularly problematic for thick and/or poorly permeable tissues. While *Drosophila* germarium and early stage egg chambers are relatively small (15 μm in width) and permeable, diffusion of fixative into later stage egg chambers is more problematic as they are larger (140 μm in width and 450 μm in length) and surrounded by a poorly permeable vitelline membrane [19]. An alternative method often used to circumvent these problems is high-pressure freezing and freeze-substitution (HPF-FS). In this method, cellular structures are rapidly immobilized prior to chemical fixation by freezing to –140°C or below under high pressure [20, 21]. Although the rapid rate of immobilization often yields optimal near-native sample preservation, it requires expensive instrumentation, advance skills, and is not necessary to address many scientific questions. In general, for most biological applications, regular conventional chemical fixation yields satisfactory results [14, 15].

Standard chemical fixation methods preserve most structures in the ovary, however inner mitochondrial membrane morphology is often not well maintained (Fig. 3A). To improve resolution of the inner mitochondria membrane we have modified the standard chemical fixation protocol. We have found that decreasing the dehydration time and performing most processing steps at 4°C greatly improves mitochondria membrane morphology (Fig. 3B). We have also found that including 0.1% ruthenium red in the initial fixation reaction significantly improves the overall contrast of mitochondria (data not shown). The modified chemical fixation protocol described in detail below yields improved resolution of intracellular membranous structures without the hassle and expense of HPF-FS sample preparation-based approaches.

### 1.3 Preparation of *Drosophila* Ovaries for Focused Ion Beam Scanning Electron Microscopy

EM is increasingly being used to determine the three-dimensional ultrastructure of cells and tissues. Traditionally this has been done by reconstruction of serial TEM sections or TEM tomography. Recently, however, focused ion beam scanning electron microscopy (FIB-SEM) has become a valuable technique to provide broad-spectrum resolution of large tissue volumes with high-resolution three-dimensional imaging. In this method, a focused ion beam is used to abrade the surface of the specimen exposing a new surface that is then imaged with a scanning electron beam. The repetition of this process generates a stack of successive images that can be compiled into a three-dimension representation of the tissue being imaged. Automated ion milling and image acquisition make this method an increasingly powerful and efficient way to assess the three-dimension structure of biological specimens at the ultrastructure level [22-24].

For FIB-SEM analysis, specimens are normally prepared using the chemical fixation OTO method [25]. In the last section of this chapter, we describe a modification of the OTO method with *en bloc* lead staining that improves preservation of *Drosophila* ovaries. With this method we are able to preserve and resolve cell membranes and mitochondrial structure (Fig. 4). This method should allow for detailed assessment of the three-dimension ultrastructure of *Drosophila* ovaries.

## 2. Materials

All reagents should be made using ultrapure water (dH$_2$O, 18.2MΩ•cm at 25°C). Solutions should be stored at room temperature unless otherwise indicated.

1. 0.2M Sorenson's phosphate buffer (PB) stock solutions [16]:

   • 0.2M Monobasic stock solution: 24g sodium phosphate monobasic (NaH$_2$PO$_4$, MW 120g/mol) dissolved in 1 liter of dH$_2$O. Filter the solution using filter paper, autoclave and store at room temperature.

   • 0.2M Dibasic stock solution: 53.65g sodium phosphate dibasic heptahydrate (Na$_2$HPO$_4$·7H$_2$O, MW 268.07g/mol) dissolved in 1 liter of dH$_2$O. Filter the solution using filter paper, autoclave and store at room temperature.

- 0.2M Sorenson's PB: mix 9.5 ml 0.2M monobasic stock and 40.5 ml 0.2M dibasic stock solution to make 50 ml of total volume.

- 0.1M Sorenson's PB: 25 ml 0.2 M Sorenson's PB and 25 ml $dH_2O$ to make 50 ml final volume.

2. Fixative working solution: 2.5% glutaraldehyde and 2% paraformaldehyde in PB, pH 7.3, 10 ml in total volume (1 ml EM grade 25% glutaraldehyde, 1.25 ml EM grade 16% paraformaldehyde, 5 ml 0.2M Sorenson's phosphate buffer, 2.75 ml $dH_2O$). Adjust pH to 7.3 if necessary.

3. Araldite 502 (Luft) [26]: Warm both Araldite 502 and DDSA in a 60°C oven for 5 min, pour 27 ml Araldite 502 and 23 ml DDSA into a 50 ml Falcon tube and mix on rotator for 30 min. Then add 1.5 ml BDMA slowly and continue mixing for 30 min.

4. 1% Toluidine blue staining solution: 1% toluidine blue in 1% sodium borate

- Prepare 1% sodium borate solution: dissolve 1.0g sodium borate into 100 ml $dH_2O$, protect from light.

- Weight 1.0g toluidine blue O C.I. 52040 on top-loader balance being careful to avoid dispersing the stain. Add the stain to above sodium borate solution. Label, date and shake daily for a week.

- Filter the staining solution using filter paper before use it.

5. Durcupan ACM hard recipe: weight 11.4g single component A, 10g single component B, 0.3g single component C, and 0.05-0.1g single component D, mix up thoroughly using rotator.

## 3. Methods

### 3.1 Dissection

1. 12 to 24 hours prior to analysis, fatten female *Drosophila melanogaster* in polystyrene vials (28.5 mm diameter) containing standard cornmeal molasses medium with active, granular yeast at 25°C (*see* Note 1).

2. Anaesthetize *Drosophila* using carbon dioxide.

3. Remove the ovaries by grabbing the lower thorax of the fly with forceps and with a second set of forceps grab the lower abdomen and pull until the ovaries have separated from the rest of the fly (*see* Fig. 2B).

4. Remove any addition cuticle or organs from the ovaries.

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[1]*Drosophila melanogaster* often contain intracellular bacterial endosymbionts such as *Wolbachia.* We have found that it can be difficult to distinguish these from membrane-bound intracellular organelles such as mitochondria. We suggest either using strains that do not contain intracellular bacteria or removing them with antibiotics such as tetracycline prior to analysis.

### 3.2 Conventional Fixation Method for TEM

#### 3.2.1 Conventional Fixation and Embedding

1. Transfer ovaries to 2 ml small-capped glass vials or eppendorf tubes containing 1 ml fixative working solution, and fix 1 hour at room temperature and then continue overnight at 4°C.

2. Wash 3 × 5 min each with 1 ml of 0.1 M PB at 4°C.

3. Postfix for 1 h with 200 μl of 1% osmium tetroxide in 0.1 M PB at 4°C.

4. Wash 3 × 5 min each with 1 ml dH$_2$O at 4°C.

5. *En bloc* stain 1 h with 200 μl of 1% uranyl acetate in dH$_2$O at 4°C.

6. Wash 3 × 5 min with 1 ml of dH$_2$O at 4°C.

7. Dehydrate using 1 ml of each serial ethanol solution, starting by incubating with 30% ethanol for 5 min at 4°C.

8. Remove medium and incubate with 50% ethanol for 5 min at 4°C.

9. Remove medium and incubate with 70% ethanol for 5 min at 4°C.

10. Remove medium and incubate with 85% ethanol 5 min at room temperature.

11. Remove medium and incubate with 95% ethanol 5 min at room temperature.

12. Remove medium and incubate 2 × 5 min each with 100% ethanol at room temperature.

13. Incubate 2 × 5 min each with 100% absolute ethanol at room temperature.

14. Incubate 2 × 10 min each with a mixture of 1 part 100% absolute ethanol to 1 part of Araldite 502 (without BDMA) at room temperature.

15. Incubate for 10 min with a mixture of 1 part 100% ethanol to 2 parts Araldite 502 (without BDMA) at room temperature.

16. Incubate 3 × 10 min each with pure Araldite 502 (without BDMA) at room temperature.

17. Infiltration overnight with pure Araldite 502 (without BDMA) at room temperature.

18. Tease apart ovarioles carefully using two insect pins (*see* Note 2).

19. Infiltration for 1 h with pure Araldite 502 (with BDMA) at room temperature.

20. Transfer each ovary into a pyramid embedding mold (Fig. 5A) or flat embedding with BEEM® capsule (Fig. 5B). The polymerized pyramid block (Fig. 5A arrows) will be mounted onto the specimen stub with a specimen label affixed to it using epoxy glue (Fig. 5A star) (*see* Note 3). For flat embedding, place one or two

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[2]Ovarioles are easily damaged if ovaries are separated during sample processing. We therefore separate each ovary at the last step in pure resin in order to avoid damage due to physical manipulation prior to the tissue being properly fixed.
[3]Embedment using pyramid mold may require the removal of small air bubbles. After transferring the ovary into the embedding mold, make sure the whole egg chambers are in the center and parallel to the bottom of the mold.

ovaries onto glass slides covered with ACLAR embedding film, which makes it much easier to detach the block after polymerization. Place a size 0 BEEM® capsule that is half filled with Araldite upside-down over the samples (Fig. 5B). Include a small label containing detailed sample information inside the BEEM® capsule for sample identification (*see* Note 4).

**21.** Move the embedded samples to 60°C oven and polymerize for 48 h.

### 3.2.2 Sectioning and Staining

**1.** Trim ovarioles around the area of interest, cut semi-thin sections (500 nm) on an ultramicrotome, and collect the sections using a beveled wooden applicator stick (*see* Note 5).

**2.** Transfer samples to a drop of water on glass slide.

**3.** Dry the glass slide on a 60°C slide warmer and stain with 1% toluidine blue to identify the area of interest, for example the germarium (Fig. 2C). The terminal filament structure (Fig. 2D) can be used to orient the germarium to facilitate the identification the germline stem cells it necessary.

**4.** Collect serial ultrathin sections (60 nm) on 0.25% formvar [27] coated $1.0 \times 2.0$ mm slot copper grids (*see* Note 6).

**5.** Stain with 3% uranyl acetate in 50% methanol for 20 min in the dark.

**6.** Stain with Reynold's lead citrate for 5 min to further increase the contrast of the sample.

**7.** Acquire micrographs using a transmission electron microscope, in our case a Phillips CM-12 transmission electron microscope with Gatan $4k \times 2.7k$ CCD camera and digital micrograph software.

## 3.3 FIB-SEM Sample Preparation

**3.3.1 FIB-SEM Fixation and Embedding**—The sample preparation for FIB-SEM was modified from Ellisman's OTO method [28]. Dissection and primary fixation (step 1 and 2) are the same as in conventional chemical fixation. An increase in sample contrast required for SEM is accomplished by the following steps:

**1.** Post-fix for 1 h with freshly made 1% $OsO4$ containing 1.5% potassium ferrocyanide in 0.1 M PB at 4°C.

**2.** Rinse $5 \times 3$ min each with $dH_2O$ at 4°C.

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[4]Separating polymerized Epon, Araldite or Durcupan from glass can be troublesome. ACLAR film separates very easily from polymerized blocks and is therefore used as the sample surface, on top of which the BEEM® capsule is inverted. The glass slide serves only as a support for the ACLAR film, i.e., slide, ACLAR, sample, BEEM® capsule from bottom to top respectively.
[5]To facilitate identification of an area of interest, it is often easiest to find an ovariole with a straight germarium and egg chambers (Fig. 2).
[6]The shiny side of a slot grid is usually the flat side. However, this may vary by manufacturer. Grids should be placed with the flat side down on the formvar film to help prevent the formvar membrane from breaking. Also parafilm should be used to pick up and store the grids. Newspaper or filter paper may break the film because of water absorption.

3. Stain with TCH solution (0.01%) for 20 min at room temperature (*see* Note 7) for TCH solution preparation.

4. Rinse 5 × 3 min each with dH$_2$O at 4°C.

5. 1% OsO4 in dH2O for 30 min at 4°C.

6. Rinse 5 × 3 min each with dH$_2$O at 4°C.

7. *En bloc* stain with 1% uranyl acetate in dH2O (freshly diluted from 3% uranyl acetate stock) at 4°C overnight.

8. Wash 5 × 3 min each with dH2O at 4°C.

9. Dehydrate using ice-cold 30%, 50%, 70%, 85%, 95%, 100%, 100% ethanol for 5 min each at 4°C.

10. Incubate with ice cold 100% acetone for 5 min.

11. Incubate with 100% acetone for 5 min at room temperature.

12. Incubate with Durcupan:100% acetone mix 1:3 (25%) at room temperature for 2 h.

13. Incubate with Durcupan:100% acetone mix 1:1 (50%) at room temperature for 2 h.

14. Incubate with Durcupan:100% acetone mix 3:1 (75%) at room temperature for 2 h.

15. Incubate with 100% Durcupan overnight at room temperature.

16. Incubate with fresh 100% Durcupan at room temperature for 2 h, and embed at 60°C for 48 h.

### 3.3.2. FIB-SEM Sectioning

1. Trim and thin section samples on slot grids to identify the area of interest.

2. Mount sample block on a SEM sample holder using double-sided carbon tape.

3. Use colloidal silver paint to electrically ground the exposed edges of the tissue block.

4. Sputter coat the entire surface of the specimen with a thin layer of gold/palladium.

5. Image the sample using backscattered electron (BSE) mode in an FEI Helios Nanolab650 dual beam SEM. Record image after each round of ion beam milling using the SEM beam at 2 keV and 50pA with a working distance of 4mm.

6. Acquire data using the Auto Slice and View G3 software collecting 2 areas per sample simultaneously, with XY pixel size of 3.1 nm and Z step size of 10 nm, resulting in typical volumes of 6.3 μm by 6 μm by 0.5 μm.

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[7]Prepare TCH solution: weight 0.1g TCH (thiocarbohydrazide) and put it into 15 ml Falcon tube that containing 10 ml dH2O. Place the tube in a 60°C oven for 1 h agitate by swirling gently every 10 min to facilitate dissolving. Filter this solution through a 0.22um Millipore syringe filter before use. The TCH solution will precipitate at 4°C.

## Acknowledgements

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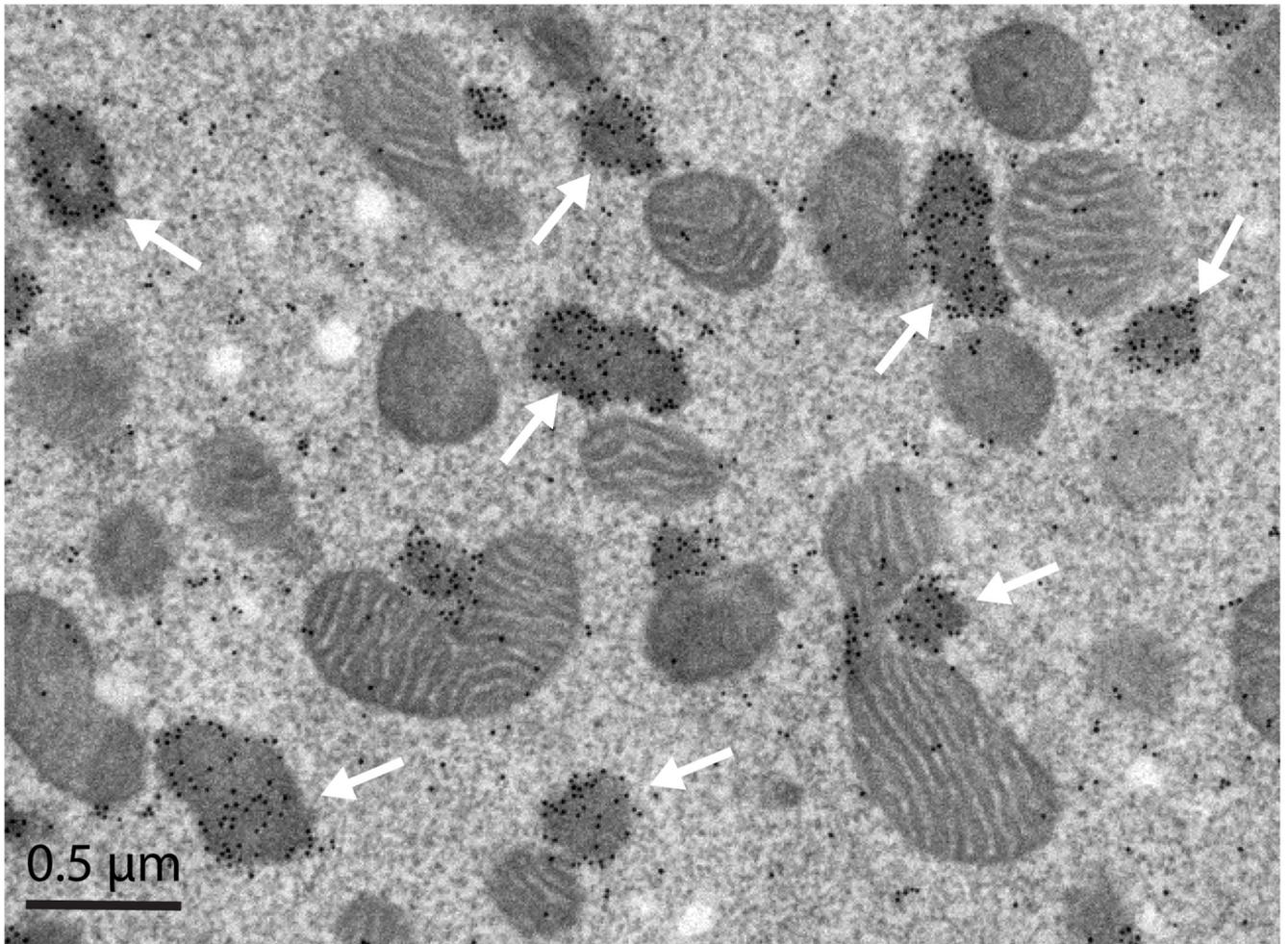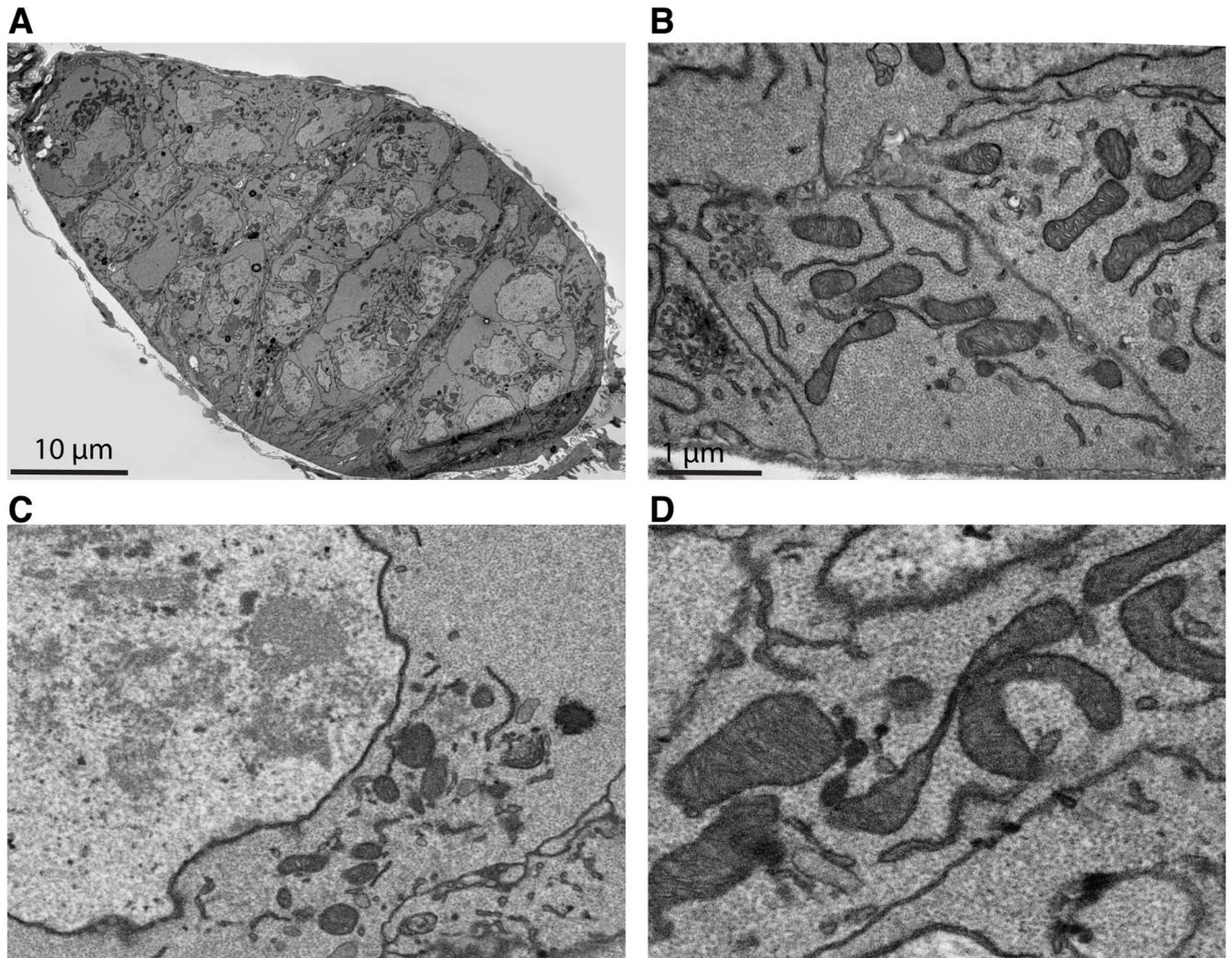
**Figure 1.**
Immuno-electron micrograph of germ plasm from a *Drosophila* embryo. Embryos were labeled with anti-Vasa serum. Polar granules are indicated with arrows. 15nm gold particles are enriched in polar granules.
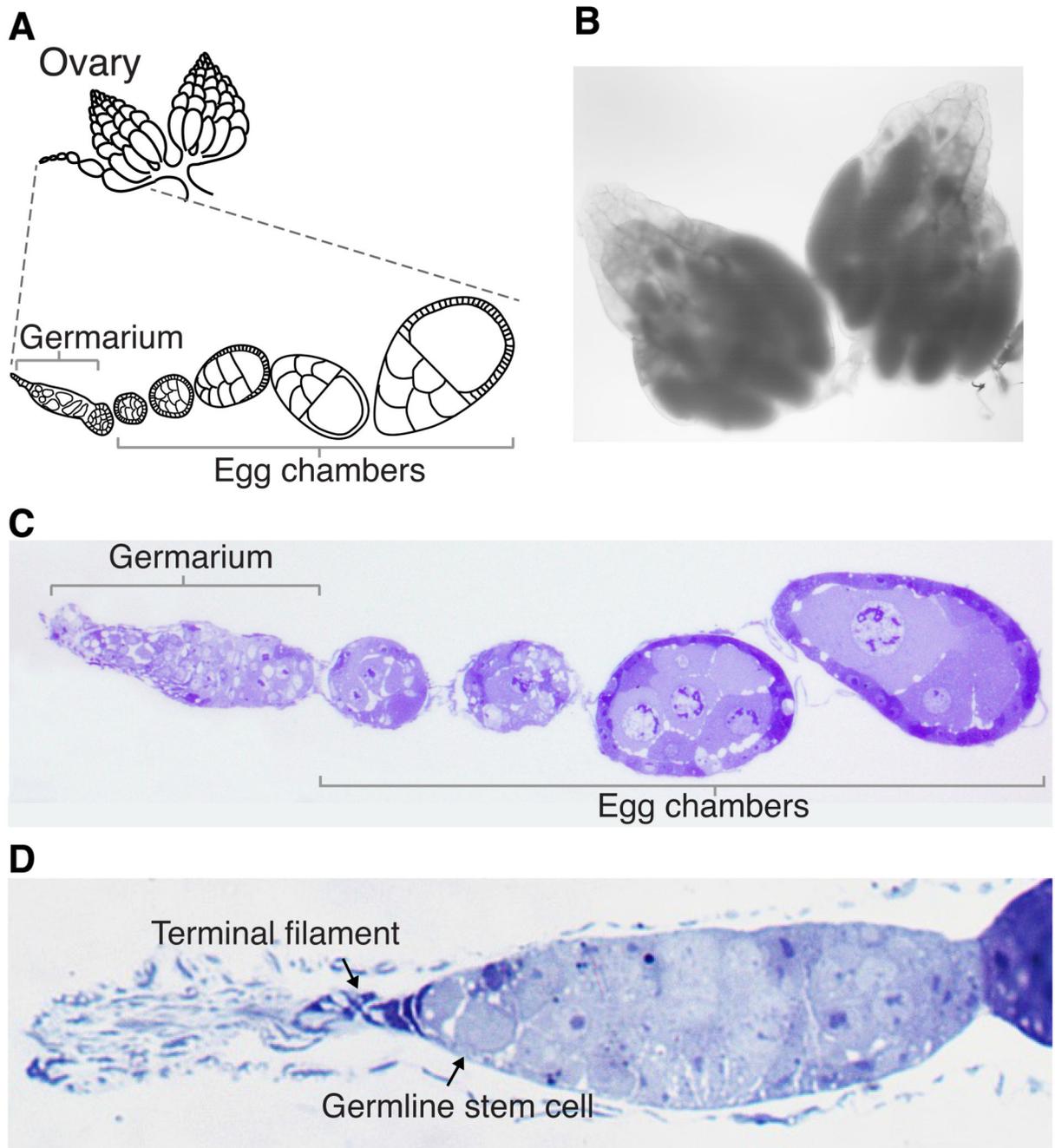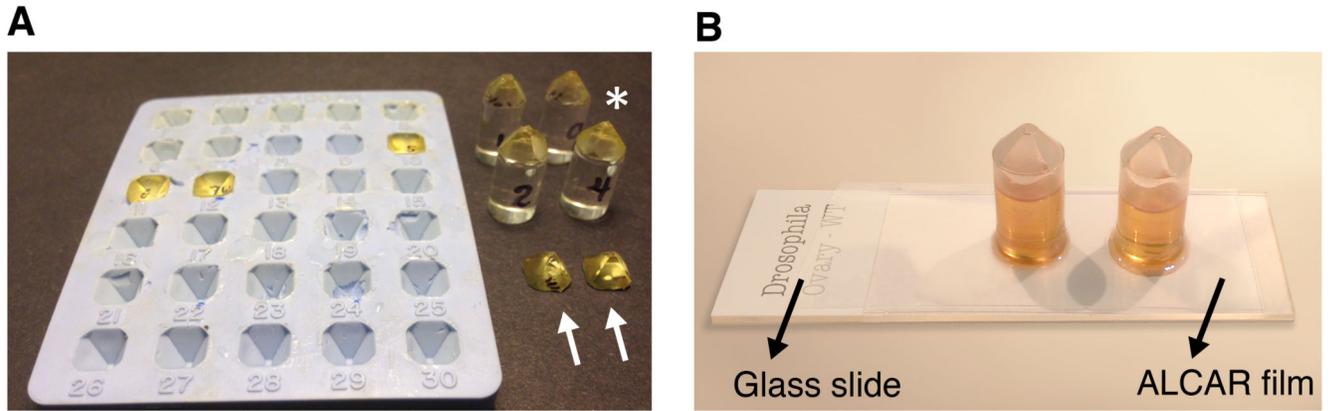
**Figure 2.**
The *Drosophila melanogaster* ovary. **A**, Cartoon of oogenesis. **B**, a pair of freshly dissected ovaries. **C** and **D,** 500 nm toluidine blue stained thick sections of an ovariole (**C**) and a germarium (**D**).
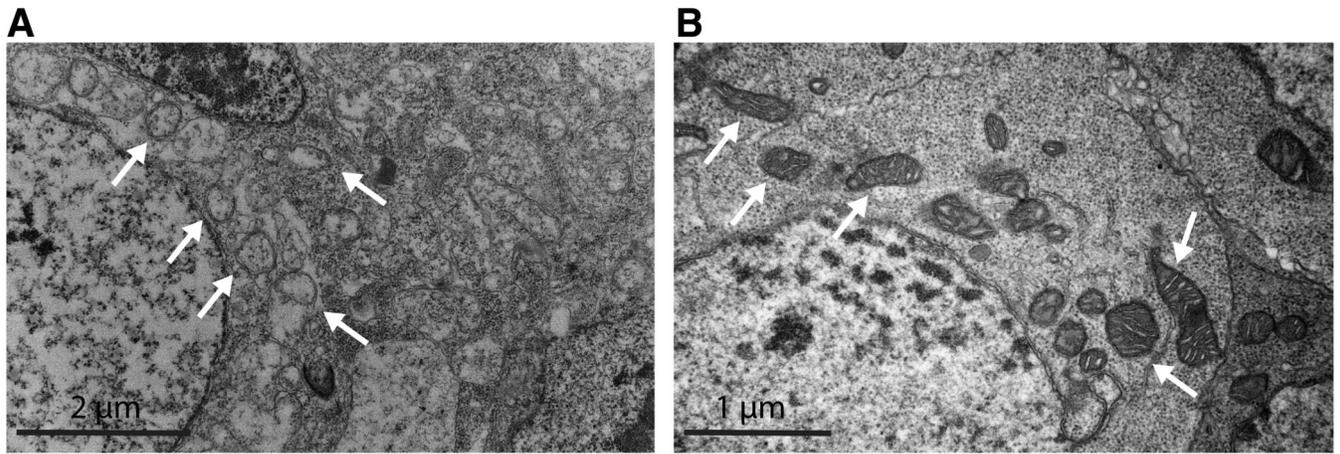
**Figure 3.**
Transmission electron micrographs of *Drosophila* ovaries prepared using different chemical fixation methods. **A**, conventional chemical fixation does not resolve inner mitochondrial membrane structure well. **B**, a modified chemical fixation protocol greatly improves inner mitochondrial membrane structure. Mitochondria are indicated with arrows.

**Figure 4.**
OTO method dramatically improves the contrast of *Drosophila* ovary cell membrane structure. **A** and **B**, TEM images of a *Drosophila* germarium without post-section staining at low (**A**) and high (**B**) magnifications. **C** and **D**, comparable SEM micrographs.

**Figure 5.**
*Drosophila* ovary embedding method. **A**, embedment with pyramid mold. **B**, flat embedment.