

Ultrastructural Analysis of Myoblast Fusion in *Drosophila*

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Summary

Myoblast fusion in *Drosophila* has become a powerful genetic system with which to unravel the mechanisms underlying cell fusion. The identification of important components of myoblast fusion by genetic analysis has led to a molecular pathway toward our understanding of this cellular process. In addition to the application of immunohistochemistry and live imaging techniques to visualize myoblast fusion at the light microscopic level, ultrastructural analysis using electron microscopy remains an indispensable tool to reveal fusion intermediates and specific membrane events at sites of fusion. In this chapter, we describe conventional chemical fixation and high-pressure freezing/freeze substitution methods for visualizing fusion intermediates during *Drosophila* myoblast fusion. Furthermore, we describe an immunoelectron microscopic method for localizing specific proteins relative to the fusion apparatus.

Key Words: Cell fusion; myoblast fusion; *Drosophila*; electron microscopy; chemical fixation; high-pressure freezing/freeze substitution; immunoelectron microscopy.

1. Introduction

The development of multinucleated skeletal muscle is a fascinating process that requires fusion of mononucleated myoblasts. Myoblast fusion is a conserved cellular process that occurs in multicellular organisms ranging from insects to humans. The somatic muscle in the fruit fly *Drosophila* is functionally equivalent to vertebrate skeletal muscle, yet the fly musculature is much simpler and takes only a short time to develop (1). These features, together with the great genetic tools available for *Drosophila*, make it an ideal system in which to study myoblast fusion in vivo.

Myoblast fusion in *Drosophila* embryo occurs between two types of muscle cells: muscle founder cells and fusion-competent myoblasts (2,3). Muscle

founder cells determine the position, orientation, and size of the future muscle fibers, whereas fusion-competent myoblasts migrate toward, adhere to, and fuse with founder cells to generate multinucleated muscle fibers. One commonly used technique to monitor myoblast fusion is imaging fixed or live embryos with light microscopy. While antibodies against structural proteins including muscle myosin heavy chain and β 3-tubulin are often used to label either mature muscle fibers in wild-type embryos or fusion-defective myoblasts in mutants (4,5), the sites of fusion in founder cells or fusion-competent myoblasts are marked by antibodies against proteins required for fusion that are localized or recruited to the sites of cell attachment (see Chapter 5; refs. 2,3). These immunohistochemical studies have provided a wealth of information about the function of genes required for myoblast fusion. Besides examining protein expression and localization in fixed embryos, the cellular dynamics of the somatic musculature can be monitored live with fluorescent proteins driven by appropriate mesodermal promoters (see Chapter 5; ref. 6).

The advantages of light microscopy are the simplicity of the technique and the ability to monitor the fusion process live. However, the maximum resolution of light microscopy (200 nm), limited by the wavelength of the light, far exceeds the thickness of cell membrane (3–10 nm). Therefore, another type of microscopy with much higher resolution is required to visualize fusion-related intracellular organelles or ultrastructural events occurring at the plasma membrane. Electron microscopy utilizes an electron beam with a far smaller wavelength than light. As a result, the resolution of a standard transmission electron microscope is about 0.2 nm. To date, electron microscopic analyses have provided significant information on the ultrastructural fusion intermediates leading to myoblast fusion.

1.1. Ultrastructural Analysis of Myoblast Fusion in *Drosophila*

The first electron microscopic study of *Drosophila* myoblast fusion was published in 1997 (7). In this landmark paper, Doberstein et al. revealed several fusion intermediates at the ultrastructural level, including paired vesicles with electron-dense margins, rare electron-dense plaques, and multiple membrane discontinuities (fusion pores) along the apposing myoblast membranes. Although subsequent electron microscopic work from several groups verified the presence of these fusion intermediates (8–11), two issues are worth noting. First, the presence of clusters of prefusion vesicles in wild-type embryos (Fig. 1) is far less frequent than those shown in Figures 2A and 3 of Doberstein et al. (7) and Zhang and Chen, unpublished observation. Second, the number and morphology of fusion pores reported in wild-type embryos prepared by the conventional chemical fixation method require a reevaluation by an independent sample preparation method (see Subheading 1.3.) that better preserves the lipid bilayers of the plasma membrane.

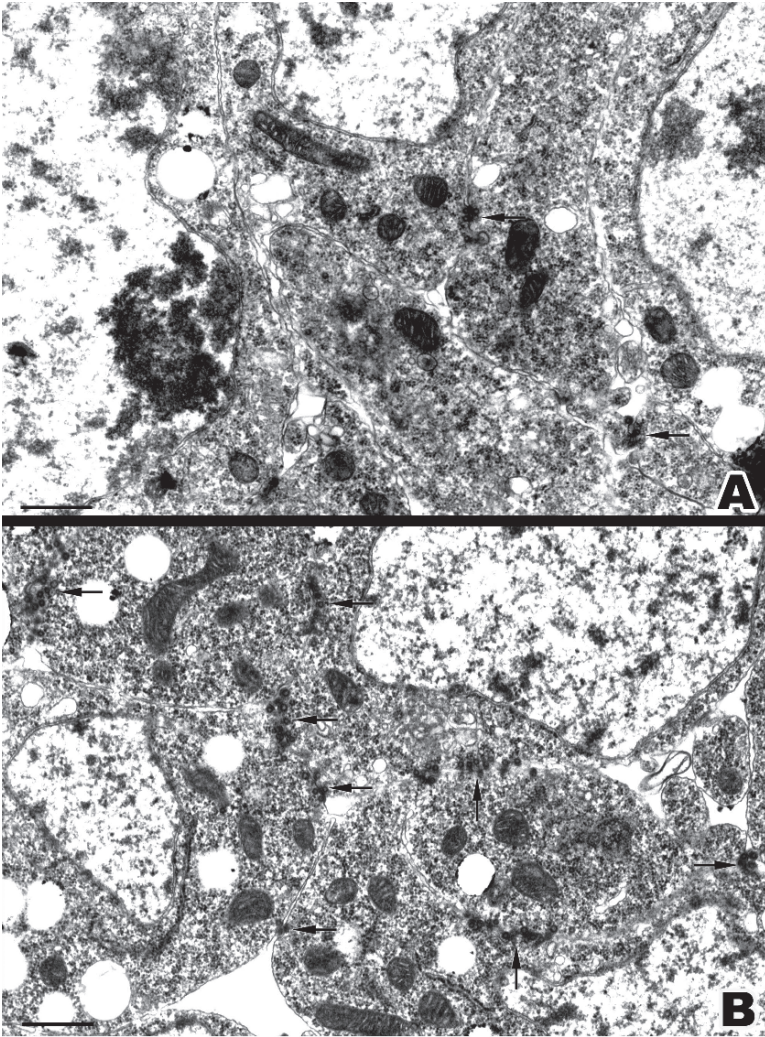


Fig. 1. Prefusion vesicles in wild-type and *sltr* mutant embryos revealed by conventional electron microscopy. A typical cluster of myoblasts in the ventral muscle group VL1–4 is shown in both panels. (A) Prefusion vesicles (arrows) in an early stage 13 wild-type embryo. Note the scarcity and low number of prefusion vesicles at cell contact sites. By the end of stage 13, myoblast fusion in this group of muscles is complete in wild-type embryos, and prefusion vesicles are no longer observed (not shown). (B) Prefusion vesicles (arrows) are accumulated in an early stage 14 *sltr* mutant embryo. Note the increased frequency and number of prefusion vesicles compared with the wild type. Scale bars = 500 nm.

Besides revealing the fusion intermediates localized at the plasma membrane, electron microscopic analyses have also provided information on the origin and trafficking of fusion-related intracellular organelles. For example, prefusion vesicles have been observed budding off from the Golgi apparatus or associating with the microtubules, suggesting that these vesicles are of exocytic origin and are perhaps transported by the microtubule cytoskeleton to the plasma membrane (9).

At the molecular level, fusion-related proteins can be localized relative to the ultrastructural fusion intermediates by immunoelectron microscopy at a resolution that cannot be achieved by light microscopy. To date, there has been only one published immunoelectron microscopic study of *Drosophila* myoblast fusion (9). This study revealed a correlation between actin-enriched foci at cell contact sites and the directional targeting of the Golgi-derived prefusion vesicles.

The significance of these electron microscopic and immunoelectron microscopic studies is underscored by the ultrastructural phenotypes of different fusion mutants that block the fusion process at various stages. For example, some mutants have been found with no prefusion vesicles (*mbc*, *rols7/lants*; refs. 7,12) or accumulated vesicles (*blow*, *sltr*; refs. 7,9). Others form few or no pores (*Drac1^{G12V}*; ref. 7) or multiple pores without completing the fusion process (*Dwip*; ref. 10). Interestingly, while membrane discontinuities along adhering myoblasts have been observed in embryos of both mutant alleles of *sltr/Dwip* prepared by the conventional chemical fixation method (9,10), fusion pores are not seen in *sltr* mutant embryos prepared by the high-pressure freezing/freeze substitution method (9). This apparent discrepancy likely reflects the intrinsic differences between the two sample preparation methods and calls for caution when using the conventional chemical fixation method to examine lipid bilayers during myoblast fusion.

1.2. Conventional Chemical Fixation of *Drosophila* Embryos

Most of the electron microscopic studies of *Drosophila* myoblast fusion to date have relied on the conventional chemical fixation method. Briefly, embryos are fixed at room temperature with a series of chemicals: first with glutaraldehyde to cross-link protein molecules and later with osmium tetroxide to preserve lipids (13). The fixed embryos are then dehydrated with organic solvent such as ethanol before being embedded in a resin, which can be polymerized into a hardened block for subsequent sectioning. Thin sections are cut on an ultramicrotome and later stained with heavy metals such as lead and uranium to give contrast between different cellular structures (13).

Despite the relative ease and cost efficiency of this method, the major limitation is the slow diffusion (from seconds to minutes) of chemical fixatives into

cells, especially cells within thick tissues that have diffusion barriers. The cylindrical *Drosophila* embryos are approximately 200 μm in diameter and 500 μm in length surrounded by the vitelline membrane, which is a natural diffusion barrier. In addition, the embryonic mesoderm is “protected” by the overlaying epidermal cells, making it more difficult for the chemical fixatives to reach. Thus, diffusion of chemicals into the fusing myoblasts can take a considerable amount of time, thus affecting the preservation of cellular structures (14,15).

Another limitation for this method is the selectivity of the cross-linking reactions of chemical fixatives. For example, the primary fixative glutaraldehyde cross-links only proteins, not lipids. As a result, the lipid bilayers of the plasma membrane are less well preserved before the later application of osmium tetroxide (13). Insufficient preservation can cause ultrastructural artifacts, including loss of continuity in the membranes, leakage of some cytoplasmic components, and distortion or disorganization of cytoskeletal organelles (Fig. 2; refs. 14,16,17). These artifacts may confound our interpretation of gene functions during myoblast fusion.

1.3. High-Pressure Freezing/Freeze Substitution of *Drosophila* Embryos

An alternative method to conventional chemical fixation is the high-pressure freezing and freeze substitution method (14–16). Samples are frozen at below -140°C under high pressure to immobilize cellular structures. Subsequently, the frozen water (amorphous at this low temperature) in the sample is substituted with organic solvent containing chemical fixatives at approximately -80°C . The major advantage of the high-pressure freezing/freeze substitution method is the ultrarapid speed (between 20 and 50 ms) of immobilization of all molecules, thus allowing near-native preservation of cellular ultrastructure (14–17). In addition, the high pressure applied during the freezing process makes it possible to preserve thick tissues of up to a few hundred micrometers, circumventing the problem of slow/inadequate fixation of internal tissues by the conventional chemical fixation method (17). Moreover, proteins and lipid molecules are chemically fixed at low temperatures (approximately -50°C by glutaraldehyde and -30°C by osmium) when their thermal energy is low, thus avoiding structural distortions caused by room temperature fixation (13). Taken together, high-pressure freezing/freeze substitution ensures instant and adequate fixation of the entire cellular architecture, with especially marked improvement on the morphology of lipid bilayers of the plasma membrane over chemically fixed samples (see Fig. 2). Thus, it is an optimal method for studying membrane fusion events in whole embryos.

Even though high-pressure freezing/freeze substitution is the preferred method for ultrastructural analysis of myoblast fusion, the cost of a high-pressure freezing

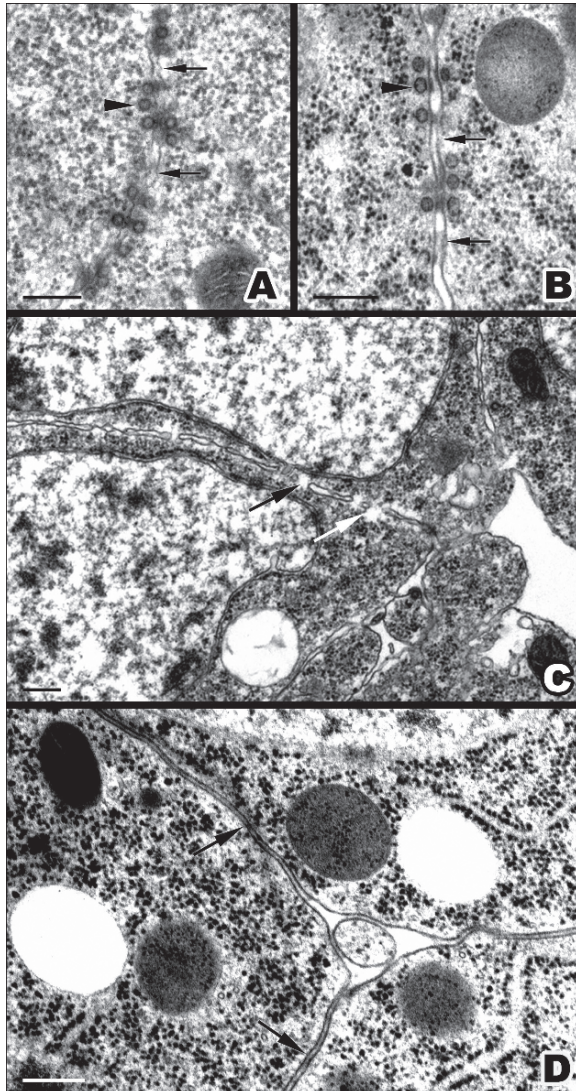


Fig. 2. Comparison of membrane morphology between conventional chemical fixation and high-pressure freezing/freeze substitution electron microscopy. All electron micrographs are taken from *sltr* mutant embryos at early stage 14. Samples in A and C are prepared by chemical fixation, and those in B and D are prepared by high-pressure freezing/freeze substitution. **(A,B)** Prefusion vesicles at myoblast membrane contact sites. Note that prefusion vesicles with electron-dense margins (arrowhead) are mostly paired (a few paired vesicles in B are out of focus). While the plasma membrane (arrows) is not well preserved by chemical fixation **(A)**, it appears smooth and intact in embryos prepared by high-pressure freezing/freeze substitution **(B)**. **(C)** Membrane discontinuities/ruptures (arrow) in a stage 14 embryo prepared by chemical fixation. **(D)** The smooth and intact plasma membrane (arrow) in a stage 14 embryo prepared by high-pressure freezing/freeze substitution. Scale bars = 200 nm.

unit is high, and only a limited number of such units are currently available. For ultrastructural studies on structures other than the plasma membrane, the conventional chemical fixation method remains a valid approach. Therefore, we describe methods for both conventional chemical fixation and high-pressure freezing/freeze substitution of *Drosophila* embryos in this chapter.

1.4. Immunoelectron Microscopy of *Drosophila* Embryos

While light microscopy is routinely used in visualizing fusion-related proteins in the myoblasts, the resolution is insufficient to pinpoint these proteins relative to the fusion intermediates. In addition, it is difficult to unambiguously localize cell type-specific proteins at sites of fusion to either side of the closely apposed plasma membranes with light microscopy. The use of immunoelectron microscopy, in combination with immunohistochemistry at the light microscopic level, will help to circumvent these problems.

Two different approaches are utilized for immunoelectron microscopy, pre-embedding and postembedding labeling (15). The former approach involves staining whole embryos with an antibody prior to processing for electron microscopy, whereas the latter method involves embedding the embryos in a resin, followed by sectioning and antibody staining of the thin sections. The advantage of the preembedding labeling is that, once labeled, the embryos can be processed following standard electron microscopic preparation procedures. This method is most suitable for labeling antigens present on the surface of the embryo, where antibodies can readily detect the antigens without penetrating into deep layers of the embryo (18). Although treatment of embryos with detergent or organic solvent opens up spaces for antibody penetration and is commonly used in immunohistochemistry for light microscopy, such treatment interferes with the preservation of ultracellular structure and thus is not recommended for immunoelectron microscopy (15). Comparatively, the advantage of postembedding is the improved preservation of the ultrastructure of embryos and the possibility of staining internal tissues. However, many thin sections may need to be stained and screened if the antigen is not widely expressed.

In principle, postembedding labeling is more advantageous for studies of myoblast fusion, since myoblasts residing in deeper layers of the embryo are difficult for antibodies to approach. However, because of the limited number of fusion events at a given time point in wild-type embryos (9,19) and the specific and transient expression of many fusion-related proteins at sites of fusion (2,3), hundreds of thin sections may need to be stained to ensure the presence of antigen on at least one or a few sections. An alternative and perhaps more practical approach is to modify the preembedding protocol (e.g., cutting the embryo open to allow antibody penetration to the mesoderm) and subsequently screen for positive signals on thin sections, as demonstrated by Kim et al. (9) in their localization of actin relative to the prefusion vesicles.

No matter which method is used, one should keep in mind that the quality of the antibody is the most critical factor for the success of an immunoelectron microscopic experiment. In general, a higher concentration of antibody should be applied to electron microscopic samples than embryos for immunohistochemistry. However, some antibodies that work well for light microscopy may not do so for immunoelectron microscopy, mainly because of the loss of antigenicity of proteins during the harsh treatment of samples prepared for electron microscopy.

2. Materials

2.1. Conventional Chemical Fixation in *Drosophila* Embryos

2.1.1. Embryo Collection

1. Empty Tri-Stir plastic beaker (Ted Pella, Inc., Redding, CA, catalog number 12904). Punch a few holes in the bottom and along the side with a 20-gauge needle to allow air flow.
2. Egg collection plates: mix 300 mL of H₂O, 100 mL of 50% grape juice, 17 g of agar, and 12 g of sucrose. Microwave the mixture in a flask at 100% power until boiling (~2.5 min). Heat at 10% power and swirl intermittently until all crystals disappear. Cool down to 50°–60 °C and pour into 60-mm Petri dish (BD Falcon, Franklin Lakes, NJ, catalog number 35-3002). Makes approximately 140 plates. Store the plates at 4 °C and warm them up to room temperature before use.
3. Yeast paste: Add water to dry baker's yeast in a beaker and mix with a spatula to make a wet paste. Store at 4 °C.

2.1.2. Embryo Fixation

1. Scintillation vial.
2. Primary fixative:
Solution A (total volume is 10 mL; see [Note 1](#)):
 - a. 5.0 mL 50% glutaraldehyde in water (Sigma, St. Louis, MO, catalog number G7651).
 - b. 2.5 mL 0.4 M sodium cacodylate, pH 7.4 (Sigma, catalog number C4945).
 - c. 1.0 mL 100% acrolein (PolySciences, Warrington, PA, catalog number 00016).
 - d. 1.5 mL distilled H₂O.

Add 10 mL heptane to 10 mL of Solution A and shake vigorously. Let the phases separate and shake again. Repeat intermittently for about 10 min. Withdraw the top heptane phase for embryo fixation. The actual amount of glutaraldehyde and acrolein that goes into the heptane phase is fairly low.

Solution B (total volume is 10 mL; see [Note 2](#)):

- a. 1.6 mL 50% glutaraldehyde in water (Sigma, catalog number G7651).
- b. 5.0 mL 16% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, catalog number 15710).

- c. 2.5 mL 0.4 M sodium cacodylate, pH 7.4 (Sigma, catalog number C4945).
- d. 0.9 mL distilled H₂O.

Add 10 mL heptane to Solution B and follow the procedure described above to make equilibrated heptane with fixatives.

3. Small paintbrush.
4. Dechorionating basket: use a 50-mL centrifuge tube (BD Falcon, catalog number 35-2070) from which the conical end has been cut off and a large hole cut in the cap. Place a square of Nitex mesh between the threads of the tube and screw on the cap.
5. 50% bleach diluted in water.
6. Fluorescent dissecting microscope (for picking green fluorescent protein [GFP]-negative homozygous embryos if the balancer has a GFP marker).
7. Nutator or shaker.
8. 0.1 M sodium cacodylate (pH 7.4).
9. Double sticky tape.
10. Microscope slide (Fisher Scientific, Pittsburg, PA, catalog number 12-550-14).
11. Microscope slide with a clear silicone rubber well (*see Note 3*).
12. Pasteur pipettes (Fisher Scientific, catalog number 13-678-6A).
13. Dissecting microscope with light source from the bottom (this type of microscope makes the amnioserosa clearly visible).
14. Sharpened tungsten needle (Electron Microscopy Sciences, catalog number 62091-01).
15. Eppendorf tubes.
16. Secondary fixative: 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4).

2.1.3. Embryo Postfixation

1. 1% reduced osmium tetroxide (*see Note 4*):

Tube A:

- a. 0.5 mL 0.4 M sodium cacodylate buffer (pH 7.4).
- b. 1.5 mL distilled H₂O.
- c. 40 mg potassium ferrocyanide (Sigma, catalog number 3289).
- d. Vortex to mix.

Tube B:

- a. 0.5 mL 0.4 M sodium cacodylate buffer (pH 7.4).
- b. 0.5 mL distilled H₂O.
- c. 1.0 mL 4% osmium tetroxide (Ted Pella, catalog number 18459).

Right before use, mix the contents of tube A and tube B, add 80 μ L 0.3 M CaCl₂, and rotate the mixture slowly on a Nutator. The solution will turn dark brown.

2. 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.4).
3. Ice.
4. Foil.

5. Nutator or shaker.
6. 0.25% tannic acid diluted in distilled water.
7. 1% uranyl acetate diluted in distilled water (*see Note 5*).

2.1.4. Embryo Dehydration

1. Ethanol series: 10%, 30%, 50%, 70%, 95%, and 100%.
2. Nutator or shaker.

2.1.5. Embryo Embedment

1. Glass scintillation vials.
2. Propylene oxide (Ted Pella, Cat. # 18601).
3. Disposable plastic pipettes: 5 mL and 25 mL.
4. Two EPON stock solutions (*see Note 6*):

EPON A:

- a. 71.92 g Eponate 12 resin.
- b. 100 g DDSA.

EPON B:

- a. 116 g Eponate 12 resin.
- b. 121 g NMA.

The reagents are available in the Eponate 12 kit (Ted Pella, catalog number 18010).

5. 1:1 EPON:propylene oxide (3 mL EPON A, 3 mL EPON B, 6 mL propylene oxide).
6. EPON: Mix the needed volume of EPON A and EPON B at a ratio of 1:1 and add 1.5% DMP-30 as a catalyst. DMP-30 is also available in the Eponate 12 kit.
7. Nutator or shaker.
8. Waste container.
9. Fine forceps (Electron Microscopy Sciences, catalog number 72700-D).
10. Embedding mold (Ted Pella, catalog number 105).
11. Oven.

2.1.6. Sectioning

1. Ultramicrotome.
2. Knife maker.
3. Ultra glass knife strips (Electron Microscopy Sciences, catalog number 71012).
4. GKB plastic troughs (Ted Pella, catalog number 123-3).
5. Nail polish (Electron Microscopy Sciences, catalog number 72180).
6. Transmission electron microscopy razor blades (Tousimis, Rockville, MD, catalog number 7250).
7. Microscope slides (Fisher Scientific, catalog number 12-550-14).
8. Syringe.
9. Hot plate.

10. Staining solution: 1 g of toluidine blue O, 1 g of sodium tetraborate decahydrate, 30 mL of 100% ethanol, 70 mL of distilled H₂O.
11. Inverted microscope.
12. Diamond knife (Diatome, Biel, Switzerland, ultra 45°).
13. Fine forceps.
14. Dust-Off FGSA kit (Electron Microscopy Sciences, catalog number 70705).
15. Filter paper (Whatman, catalog number 1001 090).
16. Slot grids (Ted Pella, catalog number 1GC12H) or slot with formvar/carbon (Electron Microscopy Sciences, catalog number FCF2010-Cu).
17. Grid storage box.

2.1.7. Staining

1. 5% uranyl acetate diluted in distilled water (*see Note 5*).
2. Syringe (Becton Dickinson, Franklin Lakes, NJ, catalog number 309644).
3. Syringe driven filter unit (Millipore, catalog number SLGV033RS).
4. Glass Petri dishes.
5. Parafilm.
6. Fine forceps.
7. Small weighing boat.
8. Sato's lead (Sato, 1968): Weigh out 0.1 g of lead nitrate, 0.1 g of lead citrate, 0.1 g of lead acetate, and 0.2 g of sodium citrate. Add 8.2 mL of degassed distilled water to the above mixture of chemicals in a 15-mL Falcon tube and shake vigorously for 1 min. The solution looks very milky. Add 1.8 mL of freshly made 4% NaOH. The solution becomes clear except for some large white grains at the bottom of the tube. Filter the solution with a 0.22- μ m syringe driven filter unit. It is ready for use (*see Note 7*).

2.1.8. Microscopy

1. Transmission electron microscope.
2. Liquid nitrogen.

2.2. High-Pressure Freezing and Freeze Substitution in *Drosophila* Embryos

2.2.1. Embryo Dechoriation and Staging

1. Small paintbrush.
2. Dechorionating basket (*see Subheading 2.1.2.*).
3. 50% bleach diluted in water.
4. Embryo collection plates (*see Subheading 2.1.2.*).
5. Fluorescent dissecting microscope for staging embryos.

2.2.2. High-Pressure Freezing

1. High-pressure freezing device and accessories.

2. Yeast paste: add 10% methanol to dry baker's yeast in the beaker and mix well with a spatula (see **Note 8**).
3. Liquid nitrogen.
4. Filter paper.
5. Specimen holders: the top holder and the bottom holder.
6. Forceps.

2.2.3. Freeze Substitution

1. Freeze substitution device.
2. Aluminum foil.
3. Cryovials.
4. Pencil for labeling cryovials.
5. Osmium fixatives in acetone: measure 25 mL of dry acetone in 50-mL disposable centrifuge tube and chill on dry ice. Quickly dissolve 0.25 g of crystalline OsO_4 in the chilled acetone using glassware with a screw cap. Uranyl acetate crystal (25 μg) is dissolved in 0.25 mL dry methanol to make a 10% uranyl acetate solution. Add this solution to the OsO_4 /acetone container and mix. As a result, this fixative contains about 98% acetone, 1% methanol, 1% OsO_4 , and 0.1% uranyl acetate. Aliquot this fixative into cryovials. Cap the cryovials and immediately immerse them in liquid nitrogen to freeze the solution. Keep the cryovials upright during freezing so that most of the fixative stays at the bottom of the tubes. Store until ready to use (see **Note 9**).

2.3. Immunoelectron Microscopy in *Drosophila* Embryos

2.3.1. Embryo Prefixation

1. 50% bleach diluted in water.
2. Small paintbrush.
3. Dechorionating basket (see **Subheading 2.1.2.**).
4. Embryo collection plates.
5. Fluorescent dissecting microscope.
6. 0.1 M sodium phosphate buffer (pH 7.2): mix 68.4 mL of 1 M Na_2HPO_4 (141.96 g Na_2HPO_4 per liter) and 31.6 mL of 1 M NaH_2PO_4 (119.98 g NaH_2PO_4 per liter). Dilute the mixture with distilled H_2O up to 1 L. This solution can be stored at room temperature for months.
7. Scintillation vials.
8. Fixation solution:
 - a. 5 mL 16% paraformaldehyde.
 - b. 25 μL 8% glutaraldehyde.
 - c. 5 mL phosphate buffer (pH 7.2).
 - d. 10 mL heptane.
 - e. Mix right before use.
9. Nutator or shaker.
10. PBS+: PBS contains 1% normal goat serum, 50 mM glycine, 1 mg/mL BSA, 0.02% NaN_3 , 0.1% gelatin.

11. Double sticky tape.
12. Microscope slide.
13. Microscope slide with a clear silicone rubber well (*see Note 3*).
14. Pasteur pipettes.
15. Dissecting microscope with light source from the bottom.
16. Sharpened tungsten needle.
17. Eppendorf tubes.

2.3.2. Antibody Staining

1. PBS++: PBS contains 1% normal goat serum, 50 mM glycine, 1 mg/mL BSA, 0.02% NaN₃, 0.1% gelatin, 0.01%–0.02% saponin.
2. Nutator.
3. Primary antibody.
4. Immunoelectron microscopy grade secondary antibody (Nanogold).

2.3.3. Embryo Postfixation

1. 0.1 M sodium phosphate buffer (pH 7.2).
2. Fixation solution: 2% formaldehyde, 1% glutaraldehyde in phosphate buffer.
3. Nutator.

2.3.4. *N*-Propyl-Gallate (NPG) Silver Enhancement

1. High-speed centrifuge.
2. 15 mL Falcon centrifuge tubes.
3. Gum Arabic stock: Dissolve 50 g of Gum Arabic (Sigma, catalog number 51200-250G) in 100 mL of distilled water under constant agitation over several days. Centrifuge at 12,000 *g* for 4–5 h. Aliquot the solution into Falcon centrifuge tubes (5 mL each tube) and store at –20 °C (*see Note 10*).
4. Medium-sized weighing boat.
5. Nutator.
6. PH meter.
7. Stirring plate.
8. Freshly prepare the following solutions:
 - a. 1 M Hepes (Sigma, catalog number 54457-50G-F), pH 6.8. (Adjust pH with NaOH. If too much NaOH has been added, remake the solution instead of readjusting with HCl.)
 - b. 50 mM Hepes, pH 5.8, containing 200 mM sorbitol (Sigma, catalog number 85529).
 - c. 20 mM Hepes, pH 7.4 containing 250 mM sodium thiosulfate (Sigma, catalog number S7026).
 - d. 10 mg of NPG (Sigma, catalog number 02370) dissolved in 250 μL of 100% ethanol first. Bring volume up to 5 mL with distilled water.
 - e. 36 mg of silver lactate (Sigma, catalog number 85210) in 5 mL of distilled water. Make this solution right before use and store in the dark.
9. 24-well plate.

2.3.5. Embryo Postfixation

1. 0.1 M sodium phosphate buffer (pH 7.2).
2. 0.1% osmium tetroxide in phosphate buffer.

2.3.6. Embryo Dehydration

1. Ethanol series: 50%, 70%, 95%, and 100%.
2. Nutator.

2.3.7. Staining

1. 5% uranyl acetate diluted in distilled water (*see Note 5*).
2. Syringe (Becton Dickinson, catalog number 309644).
3. Syringe driven filter unit (Millipore, catalog number SLGV033RS).
4. Glass Petri dishes.
5. Parafilm.
6. Fine forceps.

3. Methods

3.1. Conventional Chemical Fixation in *Drosophila* Embryos

The protocol for conventional chemical fixation in *Drosophila* embryos is modified from Lin et al. (20) and McDonald et al. (15).

3.1.1. Embryo Collection

1. Set up the collection beakers 2–3 days ahead of use.
2. Start a fresh collection plate in the late afternoon of the day before harvest. On the day of harvest, collect all embryos (0–16 h) laid overnight with a small paintbrush to the dechorionating basket.

3.1.2. Embryo Fixation

1. Rinse the embryos well with distilled water and dechorionate the embryos for 2 min with 50% of fresh bleach.
2. Rinse the embryos with PBS or distilled water to remove the residual bleach. Try to remove as much liquid as possible by blotting the bottom of the Nitex mesh with Kimwipes, but do not let the embryos completely dry out. If you need to pick up homozygous mutant embryos, follow **step 3**. Otherwise, move to **step 4**.
3. Transfer the bleached embryos from the mesh onto a fresh egg collection plate. Try to distribute the embryos evenly on the plate. If the balancer chromosome is marked by GFP, homozygous mutant embryos can be identified with a fluorescent dissecting microscope by their absence of GFP expression.
4. Move the embryos with a small paintbrush into a scintillation vial containing heptane previously equilibrated with fixatives. Fix for 20–30 min with rotation. Depending on which cellular ultrastructure you are interested in visualizing, different fixatives (Solution A or Solution B) can be used for optimal preservation (*see Notes 1 and 2*).

5. Rinse the embryos three times with fresh heptane to remove residual glutaraldehyde, paraformaldehyde, and/or acrolein.
6. Transfer the embryos with a glass pipette and slowly place them on a glass slide so that a monolayer of embryos forms. The embryos should eventually clump together into a single tight monolayered group. Wait until almost all the heptane has evaporated before starting the next step.
7. Gently lay a piece of double sticky tape down on the embryos (*see Note 11*).
8. Carefully peel the tape off the glass slide. The embryos are now attached to one side of the tape. Invert the tape with embryos on top, and place the tape in the previously made silicone rubber well on a glass slide.
9. Immediately cover the embryos with 0.1 M sodium cacodylate buffer (pH 7.4).
10. Carefully poke at one end of the fixed embryos with a sharpened tungsten needle. Once the embryos are popped out of the vitelline membrane, they will float on the aqueous solution. During this step, you can pick embryos at the desired stage based on the shape of the amnioserosa (**Fig. 3**; *see Note 12*).
11. Transfer the embryos at desired stages with a glass pipette to an Eppendorf tube containing fresh 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Fix the embryos for 1 h with rotation at room temperature.

3.1.3. Embryo Postfixation

1. Rinse the embryos two to three times for 5 min each with 0.1 M sodium cacodylate buffer (pH 7.4).
2. Postfix the embryos with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) or 1% reduced osmium tetroxide for 1 h at 4 °C with rotation in the fume hood (*see Note 4*). Keep the Eppendorf tubes covered with foil.
3. Rinse the embryos two to three times for 5 min each in 0.1 M sodium cacodylate buffer (pH 7.4).
4. Rinse the embryos two to three times for 5 min each in distilled H₂O.
5. *Optional*: Fix the embryos for 5 min in 0.25% tannic acid (*see Note 13*). Rinse two to three times for 5 min each with distilled water.
6. At room temperature stain the embryos with 1% uranyl acetate in distilled water for 1 h in the dark (*see Note 5*).

3.1.4. Embryo Dehydration

1. Rinse the embryos two times for 5 min each with distilled H₂O.
2. Dehydrate the embryos in an ethanol series for 5 min each: 10%, 30%, 50%, 70%, 95%, and 100% (three times). Use a *new* unopened bottle of 100% ethanol for each experiment (*see Note 14*).

3.1.5. Embryo Embedment

1. Transfer the embryos to a glass scintillation vial. Transit through propylene oxide from 100% ethanol by three changes of 100% of propylene oxide, 5 min each.
2. Add 1:1 EPON:propylene oxide, and rotate 30 min at room temperature.

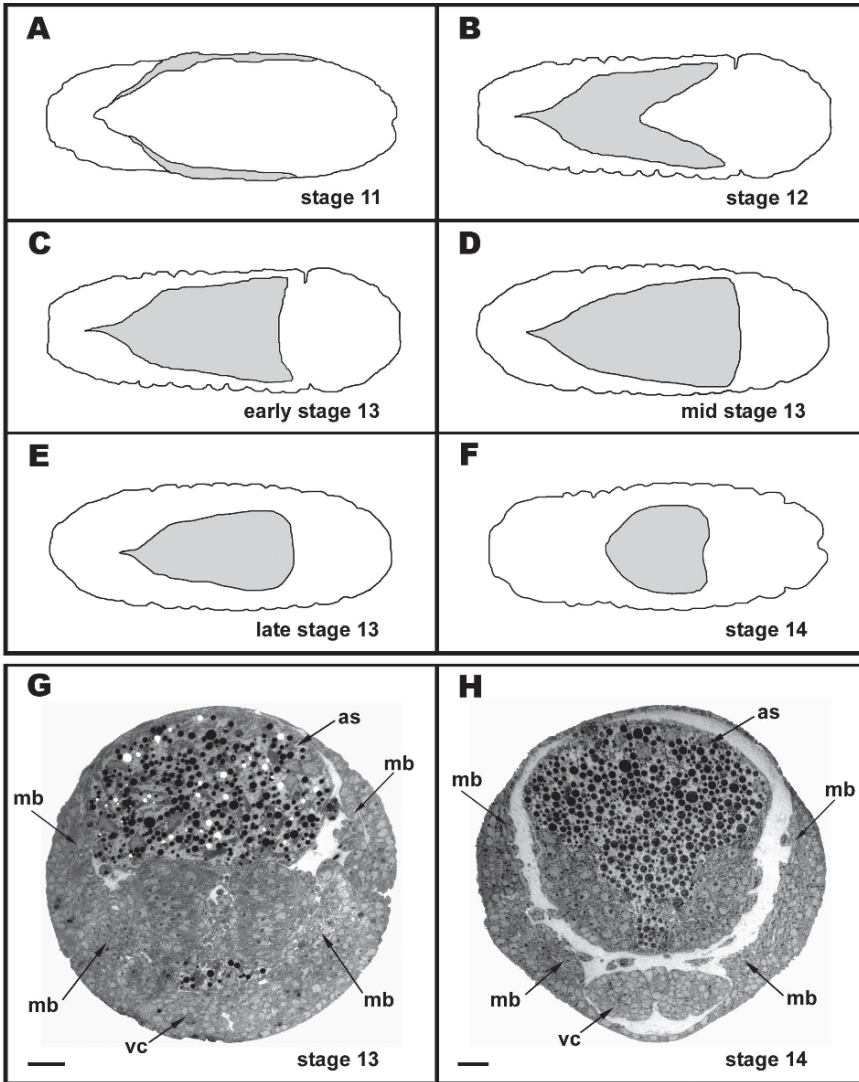


Fig. 3. Embryo staging during myoblast fusion. (A–F) Schematic drawings of stages 11–14 wild-type embryos. In all panels, a dorsal view of the embryo is shown, and anterior is to the left. The amnioserosa is marked in grey. Note that the specific shapes of the amnioserosa can be used to further stage embryos during stage 13. (G,H) Transverse sections of the abdominal segments of stages 13 and 14 wild-type embryos. as, amnioserosa; vc, ventral cord; mb, myoblasts. Scale bars = 20 μ m.

3. Add EPON (6 mL EPON A, 6 mL EPON B, 180 μ L DMP-30) and rotate overnight at room temperature.
4. Change EPON twice on the next day before embedding. Allow at least 2 h between each change.
5. Embed the embryos with fresh EPON in embedding mold. Orient and line up the embryos at one end of the block. Bake in a 70°–80 °C oven for 24–48 h.

3.1.6. Sectioning

1. Use a glass knife maker to make glass knives following the manual. Make a “boat” for each glass knife with a GKB plastic trough and nail polish.
2. Perform coarse sectioning with the glass knife. Sections will float on the water within the boat. Put a drop of water on a glass slide. Transfer the sections from the “boat” to the water drop on the slide. Place the slide on a hotplate. Stain the sections with 1% toluidine blue O and 1% sodium tetraborate decahydrate.
3. Examine the sections under an inverted microscope to gauge the quality of fixation and infiltration of the resin. Verify the stage of the embryo and determine which segments have been sectioned.
4. Continue with serial thin sectioning with a diamond knife if the sample is from the desired embryo segment and stage. The ideal thickness for the section is about 50–70 nm. Carefully transfer the sections onto a slot with supporting film. Store the slot in the grid storage box.

3.1.7. Staining

1. Line the bottom of glass Petri dish with parafilm. Add drops of 5% filtered uranyl acetate onto the parafilm. Stain the grid for 10–15 min with 5% uranyl acetate.
2. Gently rinse the grid with degassed distilled H₂O and place it back to the grid storage box until dry.
3. Stain with Sato’s lead as described by Sato (21) for 3 min. This is done in a covered glass Petri dish in the presence of NaOH pellets. Do not stain more than five grids at one time.
4. Carefully rinse the grid with degassed distilled H₂O and place it back into the grid storage box.

3.1.8. Microscopy

1. Screen the sections under a transmission electron microscope (see **Note 15**). Find the myoblasts or myotubes at low magnification (see **Fig. 3**).
2. Change to higher magnification to observe the fusion intermediates during myoblast fusion.

3.2. High-Pressure Freezing and Freeze Substitution in *Drosophila* Embryos

The protocol for high-pressure freezing and freeze substitution in *Drosophila* embryos is modified from McDonald (14) and McDonald et al. (15).

3.2.1. Embryo Dechoriation and Staging

1. Follow **Steps 1–3** in **Subheading 3.1.2**.
2. Stage both wild-type and mutant embryos by the shape of their amnioserosa under the fluorescent dissecting microscope (see **Fig. 3**).

3.2.2. High-Pressure Freezing

1. Place about five embryos at the desired stage on a slot containing a small amount of yeast paste in 10% methanol. Remove the excess moisture with filter paper. Mix the embryos with just enough yeast paste, and place embryos with yeast into the bottom of a specimen holder containing a small amount of yeast paste. Place the top holder on the bottom, and squeeze gently together with forceps (see **Note 16**).
2. Carefully place this sandwich into the specimen carrier to be inserted into the high-pressure freezing device. Make sure the specimen carrier and specimen holders match very well.
3. Freeze the samples in the high-pressure freezer and maintain the frozen sandwich in liquid nitrogen until processing with freeze substitution. Separate top and bottom specimen holders if you are ready for freeze substitution.

3.2.3. Freeze Substitution

1. Remove the frozen samples from the storage container in liquid nitrogen and transfer them to a cryovial containing frozen fixatives. Leave the cap of the cryovial slightly loose to release the liquid nitrogen that got in the tube during transfer. Avoid warming up the cryovials during the entire procedure.
2. Move all individual cryovials to the freeze substitution device and run the appropriate program.

3.2.4. Embryo Embedment

1. Remove the cryovials from the device when the freeze substitution program is complete. Rinse each sample two to three times with fresh 100% acetone at room temperature and embed in the EPON resin. See **Subheading 3.1.5** for the subsequent steps of embryo embedment.

3.3. Immunoelectron Microscopy in *Drosophila* Embryos

The protocol of immunoelectron microscopy in *Drosophila* embryos is modified from Burry et al. (22), Jongens et al. (23), and Tepass (18).

3.3.1. Embryo Prefixation

1. Follow **Steps 1–3** in **Subheading 3.1.2**.
2. Fix the embryos in a scintillation vial containing 10 mL of heptane and 10 mL of fixatives in phosphate buffer (pH 7.2) at room temperature with vigorous shaking for 30 min (see **Note 17**).
3. Briefly wash the embryos twice with phosphate buffer (pH 7.2). Transfer the embryos with a plastic pipette to a glass slide. Use a piece of double sticky tape

to transfer to another slide with a silicone rubber well. Immediately cover the embryos with PBS+.

4. Devitellinize the embryos with a sharpened tungsten needle under a dissecting microscope with light source from the bottom. Stage the embryos (*see Fig. 3*), and cut off the anterior 20% of the embryos to help the penetration of antibodies to the myoblasts.

3.3.2. Antibody Staining

1. Transfer the devitellinized embryos with a glass pipette to an Eppendorf tube. Wash the embryos three times for 5 min each with PBS++.
2. Dilute the primary antibody in PBS++ at a desired concentration, and leave the embryos in the antibody solution at room temperature for 2 h. Shake lightly on a Nutator or shaker.
3. Wash the embryos five times for 20 min each in PBS++.
4. Incubate the embryos in PBS++ containing the secondary Nanogold antibody at 1:200 dilution at room temperature for 2 h. Shake lightly on a Nutator or shaker.

3.3.3. Embryo Postfixation

1. Wash the embryos three times for 5 min each with phosphate buffer (pH 7.2).
2. Postfix the embryos in a fixative containing 2% formaldehyde and 1% glutaraldehyde in phosphate buffer overnight at 4°C with constant shaking.

3.3.4. *N*-Propyl-Gallate Silver Enhancement

1. On the second day, perform NPG silver enhancement before osmium postfixation.
2. Pour 5 mL of Gum Arabic stock and 2 mL of 1 M Hepes (pH 6.8) into a medium-sized plastic weighing boat. Slowly mix them with a stir bar on a stirring plate for at least 15 min and make sure no bubbles appear.
3. Wash the embryos three times for 5 min each with 50 mM Hepes (pH 5.8) containing 200 mM sorbitol. During the washes, prepare the enhancement solution as described in **steps 4** and **5**.
4. Add 1.5 mL of freshly made NPG solution to the weighing boat under constant agitation.
5. Add 1.5 mL of freshly made silver lactate solution to the above weighing boat under constant agitation. After adding silver lactate, solution in the weighing boat should be kept in the dark and used as soon as possible.
6. Transfer the washed embryos from **step 3** to a new 24-well plate. Remove the Hepes buffer, and add 1 mL of NPG silver solution from **step 5**. Cover the plate and keep it in the dark for 10 min.
7. Stop the reaction by washing three times for 5 min each with 20 mM Hepes (pH 7.4) containing 250 mM sodium thiosulfate.

3.3.5. Embryo Postfixation

1. Rinse the embryos in phosphate buffer three times for 5 min each. Transfer the embryos to an Eppendorf tube.

2. Postfix the embryos in 0.1% OsO₄ in phosphate buffer for 30 min at 4 °C with constant shaking.
3. Rinse the embryos three times for 5 min each with phosphate buffer.

3.3.6. Embryo Dehydration

1. Dehydrate the embryos in an ethanol series for 5 min each: 50%, 70%, 95%, and 100% (three times). Use a *new* unopened bottle of 100% ethanol for each experiment (see **Note 13**).
2. Transfer the embryos to a glass scintillation vial. Transit through propylene oxide from the 100% ethanol by two changes of 100% of propylene oxide 5 min each.

3.3.7. Staining

1. Line the bottom of a glass Petri dish with parafilm. Add drops of 5% filtered uranyl acetate onto the parafilm. Stain the grid for 10–15 min with 5% uranyl acetate.
2. Gently rinse the grid with degassed distilled H₂O, and place it back to the grid storage box until dry.
3. No lead staining is applied for immunoelectron microscopy (see **Note 18**).

3.3.8. Microscopy

Screen the sections under a transmission electron microscope (see **Note 19**).

4. Notes

1. Solution A is sufficient for visualizing prefusion vesicles. Acrolein is used as an intermediate in the manufacture of acrylic acid. It is extremely toxic to humans from inhalation and dermal exposure. It is supplied in vials capped by a rubber stopper and must be taken out from the vial with a syringe and needle. All these fixatives must be used in a fume hood.
2. Follow Solution B if more detailed intracellular structures, such as the Golgi, microtubules, or actin filaments, need to be visualized.
3. The silicone rubber well should be prepared at least 1 day ahead, because the acetic acid present in the commercially available liquid silicone rubber prevents its polymerization. Once the liquid is out of the container, it takes about 1 day for acetic acid to evaporate from the hardening silicone rubber. Acetic acid can degrade the ultrastructure if it gets into the aqueous solution used in the next step to cover embryos.
4. Reduced osmium tetroxide can result in better preservation of the ultrastructure. Osmium tetroxide is highly toxic and is a rapid oxidizer. Exposure to the vapor can cause severe chemical burns to the eyes, skin, and respiratory tract. Wear nitrile gloves (osmium can penetrate latex gloves) and eye protection. Do not open any vials of osmium tetroxide outside of the fume hood. Place unused 4% stock in a dark glass container with a screw cap. Make sure the container is closed tightly and sealed with parafilm to prevent vapor leakage. Keep the stock at 4 °C.

5. Uranyl acetate is a heavy metal poison. For better staining, filter the uranyl acetate with a 0.22- μm filter before use. Uranyl acetate helps to increase membrane contrast.
6. Store EPON A and EPON B in separate bottles. Keep them dry and store at 4°C until the day of use. Warm up to room temperature before opening the bottles.
7. Usually, the staining solution is ineffective after 3 days. For good staining, always use freshly made lead staining solution in each experiment.
8. Yeast paste made up with H_2O is also usable. However, 10% methanol gives embryos some additional cryoprotection without negative side effects.
9. Acetone and methanol are the most popular fixatives for high-pressure freezing/freeze substitution. Methanol replaces water much faster than acetone. However, McDonald (14) reported that acetone dehydration results in better preservation of the ultrastructure for *Drosophila* embryos.
10. Prepare Gum Arabic stock several days before use. The aliquots can be stored in -20°C for months.
11. Do not exert excessive pressure the embryos so that they are not squished. If too much heptane is left on the slide, some embryos may not stick to the tape well, and it would be difficult to devitellinize them. However, if the embryos are left too dry, the quality of fixation will decrease.
12. To visualize fusion intermediates, embryos at stages 12, 13, and/or 14 should be used. The easiest way to distinguish these stages is to observe the shape of the amnioserosa through a dissecting microscope with a light source from the bottom (see Fig. 3). It is also possible to distinguish the stages under a fluorescent dissecting microscope (see Subheading 3.2.1.).
13. Tannic acid fixation can improve the resolution of the ultrastructure, especially for microtubule and actin filaments.
14. If water is present after dehydration, the resin will not polymerize properly and the embedded samples will be impossible to section.
15. Get properly trained before using the transmission electron microscope. Take care not to burn the supporting film by abruptly increasing the voltage of the filament or switching from high to low magnifications without decondensing the electron beam.
16. Any excess yeast squeezed out needs to be carefully but quickly removed. Dry yeast paste around the embryos will affect the freezing process.
17. These fixatives contain a low concentration of glutaraldehyde. Equilibrated heptane with these fixatives does not fix the embryos well. Thus, we choose to fix the embryos with a mixture of heptane and fixatives with high-speed shaking.
18. Lead staining causes increased contrast in the sample. Thus, it may be difficult to distinguish between lead staining versus gold staining if the former were applied.
19. Because low osmium tetroxide and no lead staining are applied to the samples, it would be a bit harder to identify the myoblasts under the transmission electron microscope. In general, the resolution of the ultrastructure is not as high for immunoelectron microscopy as it is for regular electron microscopy.

Acknowledgments

We thank Michael Delannoy, Richard Fetter, Kent McDonald, and Guofeng Zhang for valuable advice on conventional chemical fixation and high-pressure freezing/freeze substitution; Ulrich Tepass for immunoelectron microscopy; and members of Chen's laboratory for comments. Work in Chen's laboratory is supported by the NIH, the Packard Foundation, and the Searle Scholars Program.

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