



Preservation of plasma membrane ultrastructure in *Drosophila* embryos and larvae prepared by high-pressure freezing and freeze-substitution.

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Abstract

The ultrastructural analysis of biological structures is often critical to the understanding of biological processes. Fixation by freeze-substitution after immobilisation by High-pressure freezing (HPF-FS) is a powerful method to preserve the ultrastructure of small specimens such as the embryo of the fruit fly *Drosophila melanogaster*. But only a small fraction of specimens prepared following this protocol display a visible plasma membrane; therefore, the study of processes including the plasma membrane is often unsatisfactory. In the following paper, we are summarising our achievement to improve the reproducibility of plasma membrane visibility in HPF-FS treated *D. melanogaster* embryo without worsening overall morphology.

Introduction

The fruit fly *Drosophila melanogaster* is a model organism for various biological problems, such as pattern formation, morphogenesis, and cell polarity. Due to its genetic versatility, to address these problems, especially the phenotypic analysis of *D. melanogaster* embryos mutant for a particular gene, has been useful. To evaluate the role of a given gene in a biological process, it is often essential to assess the histological defects associated with respective mutations. For a detailed histological study, the method of choice is electron microscopy. The quality of sample preparation is crucial for the preservation of the tissue and cell ultrastructure. The best routine approach for visualising the ultrastructure of multicellular organisms is the inspection of ultrathin sections of samples, which were first cryo-immobilised by high pressure freezing (HPF) and then freeze substituted and concomitantly chemically stabilised at subzero temperatures for further embedding (McDonald, 2009; McDonald and Muller-Reichert, 2008; McDonald *et al.*, 2007). These combined methods, however, have the drawback that the recognisability of membranes succeeds only in a subset of treated samples (McDonald and Morpew, 1993).

In the past years, we have investigated the ultrastructural phenotype of *D. melanogaster* embryos and larvae that produce a defective exoskeleton, the cuticle, which is formed by the epidermis, the tracheal (respiratory) network, and the fore- and the hindgut (Moussian, 2010). Based on the cellular mechanisms of formation, the *Drosophila* larval cuticle consists of three horizontal layers (Moussian, 2010). The first layer to be formed is the envelope that is composed of three films. The bipartite epicuticle is assembled underneath the envelope. The procuticle, finally, is formed between the apical plasma membrane and the epicuticle. All three layers are histologically distinguishable (Figure 1). During cuticle differentiation, the plasma membrane of epidermal cells changes its topology (Moussian *et al.*, 2006a; Uv and Moussian, 2010). The deposition of the

envelope at stage 15 and early stage 16 occurs at randomly protruding positions of the apical plasma membrane, which subsequently at stages 16 and 17 forms regular microtubule stabilised corrugations, the apical undulae that are the interface of epi- and procuticle assembly. The lateral plasma membrane is straight at stage 15 prior to cuticle formation, and meanders while the epidermal cells flatten until the end of embryogenesis.

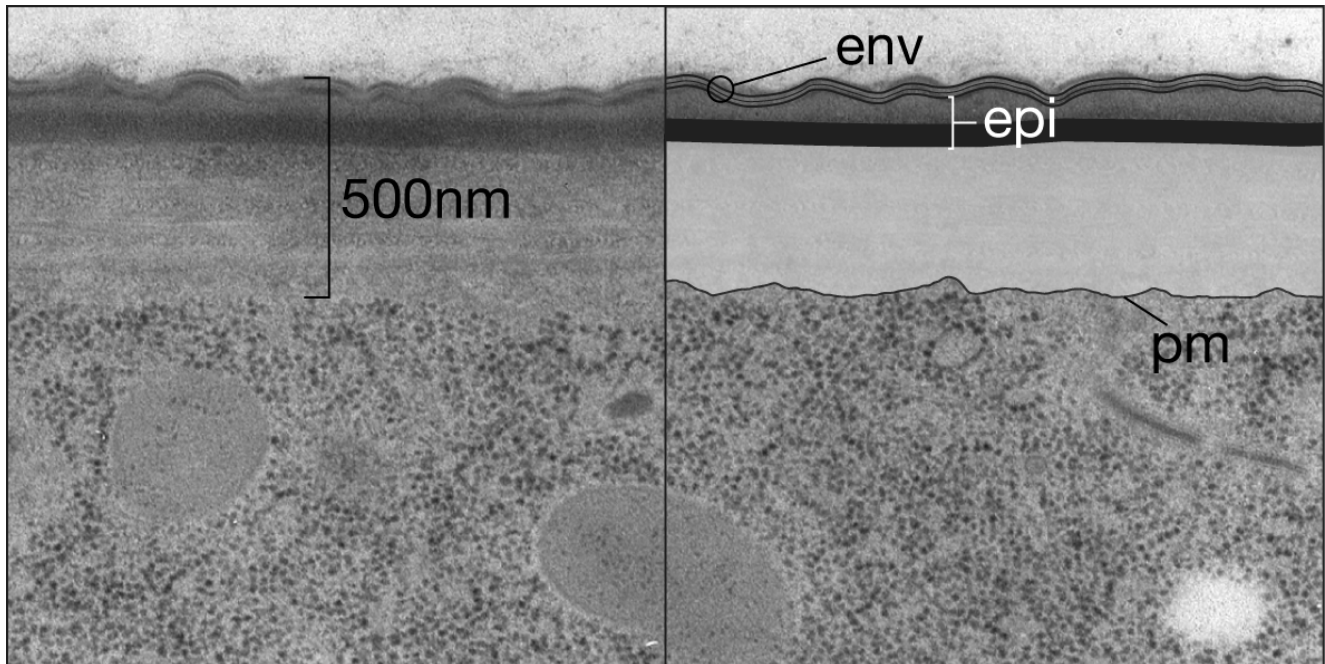


Figure 1. Scheme of the *Drosophila* larval cuticle.

One important observation in these studies is that the topology of the apical plasma membrane is essential for the organisation of the layered cuticle. The visualisation of the plasma membrane succeeded rather by chance and reproducibility required a high number of specimens. Here, we present our attempts to optimize the protocol of HPF followed by chemical fixation at low temperatures to obtain reproducible results of enhanced ultrastructural membrane appearance in the embryo and larva of *D. melanogaster*.

The cuticle is a layered extracellular matrix formed at the apical side of epidermal cells. Based on the cellular mode of construction (Moussian *et al.*, 2006a; Moussian, 2010), three major layers of the *Drosophila* larval cuticle are distinguished. The outermost envelope (env) consists of proteins, lipids and waxes that are organized in a typical array of alternating electron-dense and electron-lucid sheets. The middle epicuticle (epi) is a network of cross-linked proteins distributed in two homogeneous electron-lucid and electron-dense sublayers. The innermost procuticle (pro) is composed of a chitin-protein matrix.

Results and Discussion

Conventional freeze substitution for D. melanogaster embryos

In our previous works to study the histology of *Drosophila* larval cuticle differentiation (Moussian *et al.*, 2005a; Moussian *et al.*, 2006a; Moussian *et al.*, 2005b; Moussian *et al.*, 2006b; Moussian *et al.*, 2007; Tønning *et al.*, 2006), we applied a protocol based on the one developed by

Kent McDonald (McDonald and Morpew, 1993) as described in Moussian and colleagues (Moussian *et al.*, 2006a). This protocol is similar to the one developed by S. Zhang and E. Chen (2008).

Staged *D. melanogaster* embryos and larvae were dechorionated in 50% bleach. Living embryos within the vitelline membrane and larvae were transferred to aluminium platelets of 100 or 150 μm depth containing 1-hexadecene (Studer *et al.*, 1989). The platelets were sandwiched with platelets without cavity and frozen with a high-pressure freezer (Bal-Tec HPM 010, Balzers, Liechtenstein). The frozen embryos were freed from extraneous hexadecene under liquid nitrogen and transferred to 2 ml microtubes with screw caps (Sarstedt) containing the substitution medium pre-cooled to -90°C . Samples were incubated in 2% osmium tetroxide, 0.5% uranyl acetate (prepared from a 20% stock solution in methanol) and 0.5% glutaraldehyde (prepared from 10% anhydrous glutaraldehyde in acetone, Electron Microscopy Sciences, Ft. Washington, USA) in anhydrous acetone at -90°C for 32 h, at -60°C and -40°C for 4 h at each step in a home-made freeze-substitution unit. After washing with acetone the samples were transferred into an acetone-Epon mixture at -30°C (1:1 for 4 h, 1:2 for 12 h), warmed up to room temperature, infiltrated in Epon (3 changes within 30 h) and polymerised at $+60^{\circ}\text{C}$ for 48 h. Ultrathin sections (50 to 70 nm) were contrasted with 2% uranyl acetate in 70% methanol for 10 min and in 0.4% lead citrate in 0.1 N NaOH for 2 min.

Applying this conventional protocol for cryo-immobilization and freeze substitution, the visualisation of the plasma membrane of cuticle producing epidermal cells has been difficult at the ultrastructural level (Figure 2). Only about 10% of the specimen showed a distinct plasma membrane. Indeed, to trace the apical plasma membrane often the presence of ribosomes served to distinguish between the cytoplasm and the extracellular matrix. The identification of lateral cell-cell contacts was even more dubious.

Modified freeze substitution for D. melanogaster embryos

Membrane recognisability probably correlates with membrane denaturation through protein and lipid rearrangements that occur during fixation. To enhance plasma membrane denaturation, we modified our conventional freeze-substitution protocol, which was established for the first time by A. Steinbrecht in 1980, at two different steps. First, 1.5% water was added to the freeze-substitution cocktail according to the protocol of Walther and Ziegler (2002) and Buser and Walther (2008), who have demonstrated that in various cell types including yeast cells and 3T3 mouse fibroblast cells addition of water results in a better visibility of the membrane bilayers. Our freeze substitution cocktail hence consists of 1.5% water, 0.5% uranyl acetate, and 0.5% glutaraldehyde (prepared from a stock solution of 25% glutaraldehyde in water, which is actually the source of the final 1.5% water in the substitution medium) at the beginning of freeze-substitution at -90°C . Second, osmication was increased as the freeze substitution medium was removed from the samples at 0°C instead of at -90°C to enhance lipid denaturation by osmium tetroxide. In the following, samples were washed at 0°C with acetone and infiltrated with acetone-epon mixtures as mentioned above.

Compared to the conventional processing of the samples, the visibility of the plasma membrane of larvae and embryos prepared by the modified version of the freeze-substitution protocol was markedly and reproducibly improved (Figure 2). Indeed, the membrane unit is as presented in textbooks and is composed of two thin electron-dense lines separated by an electron-lucid thicker line together about 20 nm thick (Figure 2G). At the same time, the modifications did not worsen the overall histological quality of HPF treated samples.

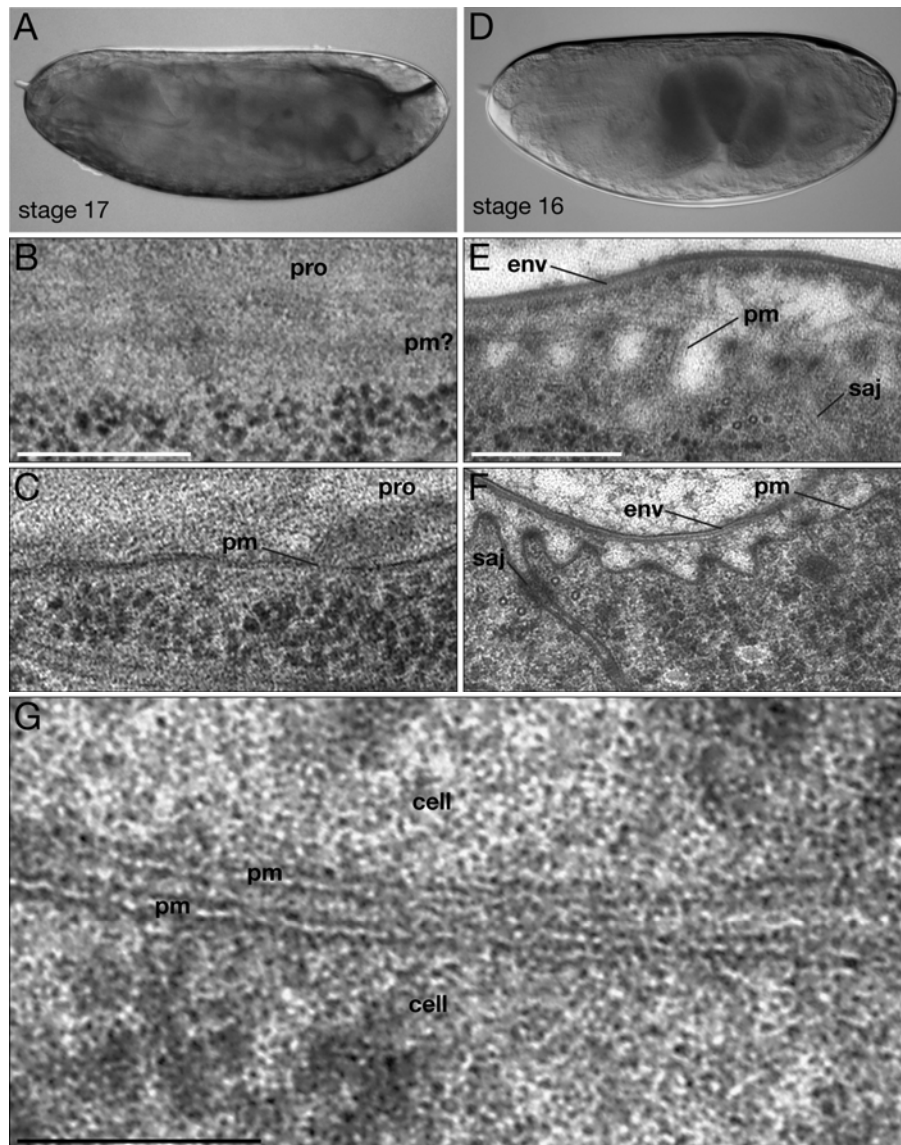


Figure 2. The plasma membrane of *Drosophila* embryos during cuticle differentiation.

The plasma membrane of epidermal cells of ready-to-hatch larva (A) fixed by the conventional method is hardly discernible (B), while fixed with our modified method it is clearly distinguishable as a smooth electron-dense double-line (C). Earlier, during massive cuticle deposition in stage 16 and 17 embryos (D), the apical plasma membrane forms extensive corrugations called *apical undulae* (und) above the sub-apical junctions (saj) at the conjunction of the lateral and apical plasma membranes (E, F). These corrugations are rather faintly contrasted in conventionally treated samples (E), whereas those of embryos prepared following our improved protocol distinctly trace the apical surface of the epidermal cell (F). The refined view on the plasma membrane indicates that the shape of the apical undulae may be rather variable probably reflecting certain dynamic behaviour, a conclusion that cannot be drawn by the study of the apical undulae obtained conventionally due to their fuzzy appearance. As shown in textbooks, two outer electron-dense and one inner electron-lucid sheets constitute the plasma membrane fixed by our modified method (G).

Sections were viewed in a Philips CM10 electron microscope at 60 kV. Scale bars 500 nm in B and E, and apply to C and F, respectively. Scale bar 200 nm in G.

Conclusion

Through two simple changes in the composition of the fixative for freeze-substitution of cryo-immobilized specimens, we are able to ameliorate the reproducible visibility of the plasma membrane in *D. melanogaster* embryos and larvae. The ultrastructural analysis of membrane-defective phenotypes of embryos fixed by our modified method should allow us a refined view on the plasma membrane and give us new insight in the function of the respective factors in particular and in membrane biology in general.

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Affinity purification of FLAG-tagged protein complexes: A cautionary tale.

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Introduction

Tagging proteins with a FLAG peptide epitope (DYKDDDDK) or a variant thereof has become a standard laboratory technique both to detect proteins for which there are no available