

Immuno-Gold Labeling of *Drosophila* Follicles for Transmission Electron Microscopy

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Abstract

Detecting the localization of cellular components using gold nanoparticles has come to offer tremendous advantages in cell biology, allowing for the high resolution imaging of the cellular organization at the subcellular level. This is further aided by the breakthroughs in the cryopreparation of samples, which focus at the retention of antigenicity in efforts to mirror the native state of the tissues and cells as closely as possible. Herein, we describe the methodology for immuno-gold labeling of *Drosophila* follicles, following preparation of the samples using the Tokuyasu method for ultracryosectioning.

Key words Cryosectioning, *Drosophila* eggs, Electron microscopy, Immuno-gold labeling, Oogenesis

1 Introduction

Spearheaded by Faulk and Taylor in 1971 [1, 2], the use of gold particles attached to colloidal sols in immuno-labeling techniques for electron microscopy (EM) has become a routinely utilized method nowadays for imaging the fine structural aspects of cell morphology with great accuracy and specificity. Considered one of the major steps in EM imaging, gold replaced ferritin as a marker of choice for high-affinity cytochemistry as it offers significant advantages for immuno-electron microscopy. Due to its higher electron density and smaller size, not only did the detection of multiple proteins on the same sample become a reality in EM imaging, but also the quality of the acquired images was vastly improved, allowing for better contrast during observation [1, 2]. Moreover, the ability to generate gold nanoparticles of various sizes has favored their usage in a wide array of EM observations [2] with larger particles regarded as well suited for scanning EM, in contrast to smaller gold molecules which are commonly used in transmission EM (TEM) [2]. In the latter case, an additional method known as “silver enhancement” has been developed, in order to further boost the emitted signal to the detection range of the electron microscope [2].

Additionally, of the numerous methods that exist for effectively cryopreparing samples for EM, the most frequently regarded as optimal for subsequent immuno-electron imaging is the “sucrose-infusion and ultra-cryo-sectioning” developed by Taku A. Tokuyasu in 1973, simply referred to as the “Tokuyasu method” [3–5]. In TEM, this method—having undergone a series of improvements over the years—has been successfully applied to image the fine ultrastructure of various mammalian and nonmammalian samples obtained from different sources including *Drosophila* [5]. The main benefits of this technique include the primary brief fixation of the sample using aldehydes, which is the only chemical denaturation step and albeit being rather mild and slow it greatly minimizes the introduction of potential artifacts during observation [3, 4]. Furthermore, it also allows the antigens of interest to retain their antigenicity to a highest degree, which in turn enables their easier recognition and more specific binding by their respective antibodies (Abs) [2–4]. In addition, the infusion by sucrose only partially dehydrates the sample and the molecule itself acts as a cryoprotectant, enabling the more efficient vitrification of the tissue or cells during freezing, and the generation of smoother sections in the follow-up cryosectioning [3, 4]. The initial need for embedding the samples in resin during fixation was also circumvented [3, 4].

Taken together, immuno-gold labeling coupled to the latest advances in cryopreparation of biological specimens has enabled researchers to obtain samples which mirror the native state of the tissue or cells more closely, in regard to the localization, structural integrity, and interactions of the antigens under study each time [2–4]. As a result, they offer some of the best available guarantees so far, for subsequent EM investigation to depict quite reliably the actual interactions taking place at the subcellular level, in turn allowing for more solid conclusions to be drawn. In our lab, we employ the aforementioned methods for TEM, in *Drosophila* follicles (egg chambers). In *Drosophila melanogaster*, the ovary consists of two lobes that contain linear arrays of developmentally ordered follicles (egg chambers) called ovarioles [6, 7]. The follicle is the structural and functional unit of the ovary. Each follicle is composed by three cell types: the oocyte, the nurse cells, and the follicle cells. The germ line cells, nurse cells, and the oocyte are directly connected to each other by intercellular bridges, called ring canals, through which nurse cells transfer their cytoplasm to the oocyte during its maturation [7–9]. Fourteen stages of oogenesis have been described according to morphological criteria, including the appearance of the eggshell coverings, the egg chamber size, and the proportion of the follicle occupied by the oocyte [7, 9, 10]. Stage 1 represents the very first immature follicle, while stage 14 refers to the mature follicle where the nurse cells have degenerated and the eggshell is completed. In this chapter, we will describe a protocol for immune-gold labeling of thawed cryosections of *Drosophila* follicles. We also

underline any important factors for researchers to take under particular consideration, while following these step-by-step guidelines.

2 Materials

2.1 *Drosophila* Flies

Flies are fed on a standard cornmeal/yeast diet and are kept at 24–25 °C on a 12 h light–dark cycle and 70 % humidity.

2.2 Equipment and Reagents for Dissection of the Flies

1. Diffuser pad for CO₂ anesthesia.
2. Stereomicroscope.
3. Fine forceps (style Dumont #5) and tungsten needles.
4. Deep-well glass slide.
5. Ringer's solution: 130 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 10 mM, HEPES, pH 7.0.

2.3 Equipment and Reagents for Fixation and Cryosectioning

1. PBS: Phosphate-Buffered Saline pH 7.4.
2. 4 % methanol free formaldehyde in PBS prepared from a 16 % solution stock.
3. 0.1 % glutaraldehyde in PBS prepared from 8 % solution stock.
4. 0.5 % toluidine blue in distilled water.
5. 2.1 M sucrose solution in distilled water.
6. 2 % methyl cellulose in distilled water.
7. Liquid nitrogen.
8. Eppendorf tubes 1.5 ml.
9. Rotator.
10. Aluminum specimen holders.
11. Aluminum plate.
12. Stainless steel loop.
13. Formvar-coated copper grids 100/200 mesh.
14. Petri dish.
15. Ultramicrocryotome.
16. Diamond trim tool and 45° ultradiamond knife.
17. Eyelash attached to a wooden stick (to maneuver sections).
18. Static line ionizer (*see* **Note 1**).

2.4 Tools and Reagents for Immune-Gold Labeling of Thawed Cryosections

1. Parafilm.
2. Petri dish.
3. 0.2 M Glycine in PBS.
4. Blocking solution: 0.2 glycine, 0.8 % BSA (bovine serum albumin) and 1 % FCS (fetal calf serum) in PBS.

5. Primary antibodies diluted in blocking solution.
6. Secondary antibodies or Protein A gold conjugates diluted in blocking solution (*see Note 2*).
7. 0.45% uranyl acetate in 1.7% methyl cellulose.
8. Filter paper.
9. Transmission electron microscope.

3 Methods

3.1 Ovary Dissection

1. Anesthetize the flies under carbon dioxide.
2. Dissect the females in deep-well glass slides in 100 ml of Ringer's solution.
3. For removing the ovaries from the body, hold the fly between abdomen and thorax with forceps and pull out the terminal part of the abdomen with another pair of fine forceps. Then, gently squeeze the abdomen with the forceps to release the ovaries from the abdomen.
4. Separate ovarioles from one another with tungsten needles and/or very fine forceps and isolate the follicles (*see Note 3*).
5. Transfer gently the isolated follicles to the appropriate solutions using glass pipettes or plastic pipettes that have been previously rinsed in PBT (PBS containing 0.1% Triton-X 100). This helps the isolated egg chambers not to stick on the pipette (*see Note 4*).

3.2 Tissue Processing for Ultra-Cryosectioning

1. Fix the follicles with 4% formaldehyde in PBS for 45 min at room temperature or with 4% formaldehyde and 0.1% glutaraldehyde in PBS (*see Note 5*).
2. Wash three times with PBS for 5 min each time.
3. Stain the fixed follicles with 0.5% toluidine blue (*see Note 6*).
4. Incubate the follicles overnight in 2.1 M sucrose at 4 °C. Be sure that the samples are rotated.
5. Mount the follicles on aluminum specimen holders using one drop of 2.1 M sucrose solution. You will need to use a cold plate, in order to put the samples up on it, while you are working with them to avoid thawing (*see Note 7*).
6. Immerse the samples in liquid nitrogen, in order to freeze them.
7. Use an ultramicrocryotome, in order to section the frozen follicles (*see Note 1*).
8. Recover the ultrathin cryosections from the cryochamber of the microtome with a drop of 1:1 mixture of 2.1 M sucrose: 2%

methylcellulose using a stainless steel loop mounted on a wooden stick. Transfer the droplet toward the face of the sections, until you observe that the sections are attached to the droplet.

9. Lay the drop containing the cryosections on a carbon- or formvar-coated copper grid.
10. Until you are ready for the immuno-gold labeling, save the samples in a Petri dish at 4 °C.

3.3 Immuno-Gold Labeling

1. By using a pair of forceps, lift the grids (which contain the cryosections) and put them onto a 50 µl drop of 0.2 M glycine in PBS on a sheet of parafilm.
2. Wash three times, 5 min each on new 50 µl drops of 0.2 M glycine in PBS.
3. Incubate the grids for 10 min with blocking solution.
4. Incubate the grids for 1 h (or more depending on the antibody) with the primary antibody diluted in blocking solution (*see Note 8*).
5. Wash three times, 5 min each time by floating the grids on droplets of blocking solution.
6. When the primary antibody does not interact with protein A (e.g., if it is a mouse monoclonal), incubate the grids with a secondary antibody (e.g., rabbit anti-mouse) that binds protein A, diluted in blocking solution, for 30 min (*see Note 2*).
7. Wash grids three times, each time for 5 min with blocking solution.
8. Incubate grids for 30 min with 10 nm protein A-gold particle suspensions in blocking buffer, usually diluted 1/60 to 1/80.
9. Wash grids five times, each time for 5 min with PBS.
10. Wash grids five times, each time for 5 min with distilled water.
11. Stain the grids in 0.45% uranyl acetate in a 1.7% aqueous solution of methyl cellulose on ice in the dark for 10 min.
12. Retrieve the grids with a stainless steel loop of diameter slightly larger than the grid, attached to a pipette tip.
13. Drain away the excess of methyl cellulose/uranyl acetate solution by touching the loop at an angle of 45 °C to a filter paper.
14. Air-dry the grids at room temperature by placing the pipette tips upside-down in a pipette-tip rack.
15. The sample is ready for electron microscopy observation (Fig. 1).

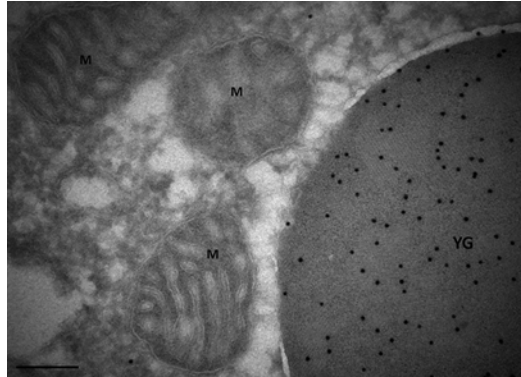


Fig. 1 Representative transmission electron micrograph of *Drosophila* oocytes. Immuno-labeling with 10 nm gold particles on cryo-sections for yolk protein. *M*: mitochondria, *YG*: yolk granule. Scale bar = 150 nm

4 Notes

1. Ultrathin cryosections can easily stick to the knife edge during sectioning. This makes it very difficult to retrieve them. The static line ionizer makes it possible to have perfect ribbons of ultrathin cryosections that will no longer stick to the knife edge or bunch up one at the top of the other. The sections can float in a nice ribbon over the surface of the knife and can be picked up easily.
2. The reactivity of protein A is very good for primary antibodies raised in rabbit, guinea pig, pig, and cow. On the contrary it is poor for primary antibodies raised in mouse, rat, goat, and sheep.
3. It is important to remove the muscle sheath from the ovarioles. To achieve this, gently squeeze the egg chambers until they exit the epithelial sheath.
4. Be gentle with pipetting because rapid pipetting of the egg chambers may cause mechanical stress which may induce phenotypic artifacts [11].
5. Duration of fixation is depended on antigenicity. If the antigen is sensitive to fixation, fixation time can be shorter or be done at 4 °C.
6. Staining with toluidine blue will give a blue color to the egg chambers. This will help to observe them easily during the following steps and will prevent tissue loss.
7. The part of the sample that you want to observe should be at the top, so make sure that you have adjusted the samples properly before you start.
8. The appropriate antibody dilution for immuno-gold labeling is usually ten times less diluted compared to the one used for immunofluorescence, or 100 times less diluted compared to the one used for Western blot analysis.

Acknowledgements

This work is supported by a BBSRC grant (BB/L006324/1) awarded to Dr. Ioannis P. Nezis.

References

1. Faulk WP, Taylor GM (1971) An immunocolloid method for the electron microscope. *Immunochemistry* 8(11):1081–1083
2. Posthuma G, van Donselaar E, Griffith J, Oorschot V, van Dijk S, Slot J (2001) Ultrathin cryo-sectioning and immuno-gold labeling. A practical introduction. Department of Cell Biology, Institute of Biomembranes, University Medical Center Utrecht, The Netherlands
3. Mobius W (2009) Cryopreparation of biological specimens for immunoelectron microscopy. *Ann Anat* 191(3):231–247. doi:[10.1016/j.aanat.2008.11.004](https://doi.org/10.1016/j.aanat.2008.11.004)
4. Mielanczyk L, Matysiak N, Michalski M, Buldak R, Wojnicz R (2014) Closer to the native state. Critical evaluation of cryotechniques for transmission electron microscopy: preparation of biological samples. *Folia Histochem Cytobiol* 52(1):1–17
5. Tokuyasu K (1973) A technique for ultracryotomy of cell suspensions and tissues. *J Cell Biol* 57(2):551–565
6. Giorgi F, Postlethwait JH (1985) Development of gap junctions in normal and mutant ovaries of *Drosophila melanogaster*. *J Morphol* 185(1):115–129. doi:[10.1002/jmor.1051850109](https://doi.org/10.1002/jmor.1051850109)
7. Horne-Badovinac S, Bilder D (2005) Mass transit: epithelial morphogenesis in the *Drosophila* egg chamber. *Dev Dyn* 232(3):559–574. doi:[10.1002/dvdy.20286](https://doi.org/10.1002/dvdy.20286)
8. Mahajan-Miklos S, Cooley L (1994) The villin-like protein encoded by the *Drosophila* quail gene is required for actin bundle assembly during oogenesis. *Cell* 78(2):291–301
9. King RC (1970) Ovarian development in *Drosophila melanogaster*. Academic, New York
10. Margaritis LH (1986) The eggshell of *Drosophila melanogaster*. II. New staging characteristics and fine structural analysis of choriogenesis. *Can J Zool* 64(10):2152–2175
11. Haack T, Bergstralh DT, St Johnston D (2013) Damage to the *Drosophila* follicle cell epithelium produces “false clones” with apparent polarity phenotypes. *Biol Open* 2(12):1313–1320. doi:[10.1242/bio.20134671](https://doi.org/10.1242/bio.20134671)