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Immunolabelling of embryos

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Abstract

The molecular mechanisms controlling *Drosophila* embryogenesis are among the best-studied examples in animal development. While the formation of developmental pattern in embryos was intensely examined in the past three decades, the cell biological basis of morphogenesis is now entering the centre stage of the research on fly embryos. A fundamentally important procedure has always been to determine the sub-cellular localization of proteins in embryos by immunolabelling. The challenge of the commonly used whole mount staining procedures is to balance a good structural preservation during fixation and allow at the same time the penetration of the antibodies through the tissue. Different procedures have been developed that allow the preservation of distinct compartments of the cell and thus optimize for the specific sub-cellular localization of proteins. This chapter provides a general immunolabelling protocol with variations suitable for a broad panel of antigens.

Keywords

antibodies; embryos; immunolabeling; microscopy; whole mount

1. Introduction

One of the most widely used techniques to study the function of proteins during embryogenesis is to determine their localisation in fixed samples using specific antibodies. The specificity of the antibodies, the abundance of the epitopes of the proteins under investigation and the general conservation of the cell and tissue structure are the most important factors to obtain reliable staining results. The choice of the immunolabelling protocol and the interpretation of immunolocalisations will therefore always need to take all of these factors into account.

In nature *Drosophila* embryos develop at the open air and hence need to be protected from dehydration. This protection provides the eggshell, the chorion and the vitelline envelope, which render the embryo inaccessible to aqueous solutions, including fixatives. In particular the outer, waxy layer of the vitelline envelope needs to be permeated by the fixatives (1). The removal of the vitelline envelope often causes problems with the preservation of

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¹The methanol treatment affects both the antigenicity and the localization of proteins. To circumvent these problems, the vitelline envelope can also be removed manually after fixation. Remove embryos from fixative using a wire mesh basket and rinse with PBS several times. Blot semi-dry on paper towels and place into a depression slide with PBS. Attach a piece of double-stick tape in the middle of the lid of a 60 mm petri dish. Transfer embryos using a paintbrush onto the tape and cover with PBS. Rapidly add the PBS to make sure that the embryos do not dry out. Remove vitelline envelopes using a 27-gauge syringe needle under a dissecting scope; if that turns out to be difficult try to slightly over-fix the embryos, as only well-fixed embryos will readily devitellinise.

²For signal enhancement of HRP-conjugated antibodies, the ABC staining kit Vectastain® by Vector Labs has been very successfully applied to *Drosophila* embryos.

antigens and structural conservation. While manual removal of the vitelline envelope reveals the best results with regard to fine structure, low permeability of the tissue may be a problem. Physical removal of the vitelline envelope by methanol treatment is suitable for large amounts of embryos and results in decent staining results because of good tissue permeability; the structural preservation however is severely affected by methanol treatment. Some of these technical hitches have been addressed by the traditional phase partition fixation by Zalokar and Erk (2), where embryos are fixed at the interphase of a heptane/aldehyde aqueous solution. The Zalokar fixation also provides a good structural preservation for electron microscopy (Figure 1A,B,C).

Immunolabelling procedures must consider the accessibility of antigens within the cell. Antibodies have to penetrate into the tissue; a process strongly improved by using detergents. However, different cellular proteins might require diverse fixation procedures, because of distinct requirements to maintain certain sub-cellular structures and to maintain the availability of epitopes. These considerations make it almost impossible to advise a single immunolabelling procedure that will simultaneously fulfil all requirements for different antibodies and antigens; therefore many improvements specific for particular antigens or cellular structures have been developed throughout the years.

Fixations that allow for whole-mount immunolabelling of fly embryos are generally not sufficient to provide good preservation of the fine structure of the cell (Figure 1D-L). Cellular structures are often destroyed or extracted by detergents or organic solvents used during fixation and labelling. In particular extraction of cytosolic proteins and membranes have to be considered when interpreting results of whole-mount stained embryos. On the other hand, protocols that maintain the fine structure of the cells – for example in transmission electron microscopy – will usually not allow for whole mount immunolabelling of embryos. It is therefore pivotal to keep in mind that the localisation of antigens by whole mount immunolabelling might not reflect the precise sub-cellular localization, simply because the particular cellular compartment or its normal cellular position has been destroyed or altered (3). The problem is most evident in the case of membrane proteins; for example to get access in a whole mount preparation to a luminal epitope within the ER, the ER membrane itself needs to be at least partially destroyed, because antibodies will need to permeate the ER membrane. In such cases co-staining with established sub-cellular markers and/or electron microscopy is essential to provide some evidence for specificity. Although whole-mount procedures might therefore often not provide the exact localization of proteins in most cases these procedures are still valid when immunolabelling in wild type is directly compared with mutant embryos. The problem of the exact sub-cellular localization is less important, because the fixation artifacts that are being produced will usually be identical in both cases.

The present chapter will provide protocols for immunolabelling of embryos that take into consideration some of the problems described. Additional procedures have been developed for the staining of particular cellular components and will not be described here. For example staining of microtubules or the actin cytoskeleton require specific fixation procedures and are described elsewhere (4-6). In this chapter, I will provide a protocol for immunolabelling of embryos, which can be broadly used with a particular emphasis on different fixation procedures with respect to tissue preservation and permeability.

2. Materials

2.1. Materials for harvesting and dechorionisation of embryos

1. Egg collection: Flies are placed in egg collection cups as advised by Wieschaus and Nüsslein-Volhard (7). Briefly, 100 ml plastic beakers (TRI-Pour® polypropylene)

are prepared to contain tiny holes. Each beaker will hold a 60 mm petri dish attached with rubber band.

2. Apple juice plates: Preparation of apple juice plates is described elsewhere (8). The medium contains 17.5% agar, 12.5 g/l sucrose, 25% apple juice and 0.2% Nipagin M. Apple juice plates are covered with a little smear of yeast (from dry yeast or bakers yeast). Halocarbon oil 27 (Sigma) is used for making the chorion transparent for microscopic inspection.
3. Dechorionisation: A basket that fits regular depression slides can be prepared of a stainless steel wire mesh; alternative devices have been successfully used, like Nitex® mesh used as a sieve with a cut-off 15 ml Falcon tube. For 3% sodium hypochlorite, commercial bleach can be used.

2.2. Fixatives

1. Formaldehyde fixative: 4% formaldehyde (p.a. grade, 37% stock solution, methanol-free) in PBS or 0.1 M sodium phosphate buffer pH 7.4.
2. Modified Stefanini's fixative (9, 10). 4% formaldehyde (p.a. grade, 37% stock solution, methanol-free), 15 ml of a saturated aqueous solution of picric acid, 75 mM PIPES pH 7.4.
3. Heat/methanol fixation (11). 10x Triton-X-Salt solution (TSS): 3 ml Triton-X-100, 40 g NaCl on 1 l distilled H₂O.
4. Heptane and Methanol should be p.a. grade only.

2.3. Solutions for immunolabeling procedures

Incubations are performed in 1.5 ml test tubes, if volumes between 350 µl and 500 µl are used. If smaller volumes are required - for example to save primary antibody - 0.5 ml test tubes are used to incubate embryos in 150 µl to 300 µl. Alternatively, embryos can be stained in 24-well COSTAR® plates. All surfaces that come into contact with fixed embryos, in particular glass and plastic pipettes need to be pre-absorbed with excess protein solution (e.g. blocking solution) to avoid sticking of embryos.

1. Blocking solution: 10% Serum (Normal goat, horse or donkey) in 1× PBS (130 mM NaCl, 7 mM NaHPO₄; 3 mM NaH₂PO₄; pH 7.4) with 0.1% Tween 20. Instead Tween 20, Triton-X-100 can be used, which is a stronger detergent and thus promotes permeability of the tissue by extraction of membranes.
2. PBT: 1× PBS with 0.05% Tween 20.
3. Antibody solutions: in blocking solution (see 1.). A collection of excellent monoclonal antibodies for various *Drosophila* antigens is available through the Developmental Studies Hybridoma Bank (DSHB) in Iowa City, USA (<http://www.uiowa.edu/~dshbwww/>).
4. Tubes/plates are incubated on a nutating shaker or Rocker Plate.

2.4. Antibody detection

1. Fluorescence-conjugated antibodies: Continue with 2.5.
2. Fluorescent DNA staining: DAPI (4',6'-Diamidino-2-phenylindol) can be used at a concentration of 1 mg/ml. Alternatively, other DNA stains can be used, e.g. YOYO®-1 iodide (Molecular Probes INC.); note that many fluorescent nucleic acid dyes (except DAPI or Hoechst 33342) will also stain RNA and thus embryos must be pre-treated with RNase (at 0.2 mg/ml for 1 hour at room temperature).

3. Alkaline Phosphatase (AP) detection; AP buffer: 100 mM Tris pH 9.5; 50 mM MgCl_2 , 100 mM NaCl, 0.1% Tween 20. AP buffer should be stored without Tween 20 at 4°C. 1 ml AP buffer + 4.5 μl NBT (nitroblue tetrazolium chloride; stock solution at a concentration of 18.75 mg/ml) + 3.5 μl BCIP (5-bromo-4-chloro-3-indolyl-phosphate, toluidine; stock solution at a concentration of 9.4 mg/ml).
4. Horseradish-Peroxidase (HRP) detection: For detection commercially available enhancement systems can be used following the given protocols. DAB-solution: 0.5 mg/ml DAB (3,3' diaminobenzidine); DAB staining solution: per 1 ml DAB solution add 2 μl H_2O_2 (from 3% stock-solution).

2.5. Mounting of specimen

1. Fluorescently labelled specimen: Mowiol/DABCO: 1.5% Mowiol 4-88 (Polysciences), 33% glycerol, few crystals (1-2 per ml) of DABCO (1,4 - diazabicyclo (2.2.2) octane). To prepare Mowiol: dissolve 1.5% (w/v) Mowiol in PBS (pH 7.4) and stir overnight. Spin down non-dissolved Mowiol particles by centrifugation and add 33% (v/v) glycerol and stir overnight. Mowiol without DABCO can be stored at -20°C for months. DABCO is added to Mowiol solution, gently mixed and centrifuged. This working solution can be kept for a few days at 4°C.
2. AP/HRP color precipitates: Ethanol series: 30%, 50%, 70%, 90%, absolute, 100% ethanol or acetone (maintained on molecular sieve). Araldite/Durcupan (Sigma).

Methods

2.6. Embryo collection and dechorionisation

1. Collect 3-5 day old female and male flies to set up at a ratio of 1:3 (male to female) in egg collection cup to which a yeasted apple juice plate has been attached (place a small amount of yeast on the centre of the plate and spread with a spatula).
2. Change apple juice plates every 8 hours (25°C) or 18 hours (18°C). Flies will require 2 to 3 days to adjust to the cage until egg production is best.
3. For staged embryo collections change plate after 30 min and incubate at a constant temperature to the desired developmental stage. Alternatively, a 0 to 3 hour collection of embryos can be obtained and embryos will then be staged after treatment with halocarbon oil under a dissection microscope equipped with transmitted light (for staging see (7, 12)). Upon covering embryos with a drop of halocarbon oil, the chorion will become transparent and the embryo will be visible using a stereomicroscope with transmitted light.
4. For dechorionisation, embryos are collected in a wire mesh basket that fits to a depression slide. Gently rinse embryos off the apple juice plate with tap water and using a cut shorthair paintbrush. Rinsing in tap water to remove yeast and excess water by blotting dry on a paper towel. Transfer embryos within the mesh into 3% sodium-hypochlorite solution. A 3 min treatment at room temperature removes the chorion; dechorionated embryos will float on the surface of the solution. Microscopic observation of the efficiency of the bleaching procedure is advised, because different batches and age of sodium-hypochlorite as well as the genotype of the embryos might affect the timing. Over-bleaching might produce abnormal morphology.
5. If embryos were hand-selected under halocarbon-oil, staged embryos will be collected using watchmaker forceps and transferred to a wire mesh basket in water.

Make sure to remove remaining oil by blotting the basket on paper towels or briefly rinse and blot using bleach. Continue with 4.

6. After dechorionisation, rinse embryos with distilled water in the wire mesh basket and keep in depression slide on distilled water. Note that dechorionated embryos are very sensitive to dehydration, which will cause severe artifacts. Prolonged treatment with sodium hypochlorite will increase the frequency of such problems.

2.7. Formaldehyde-based fixation procedures

1. Preparation of fixatives: As fixative, buffered formaldehyde solution or modified Stefanini's fixative can be used; the latter exhibiting a better preservation of both structure and antigenicity (9, 13). 0.05% Tween 20 can be added to the formaldehyde fixative to improve permeabilization through an increased extraction of cytosolic proteins and membrane lipids. Do not add Triton-X-100 to fixing solutions as it will interfere with phase separation. Fixatives can be generally be stored at 4° C for at least a week.
2. Add 4 ml of fixative and 4 ml of heptane to a glass scintillation vial. Mix the solution by vigorously shaking for 30 seconds to allow the formaldehyde to partition into the heptane phase.
3. Transfer the embryos to the vial using the wire basket or a paintbrush. Embryos will float between the two phases. Remove the wire basket after transfer of the embryos.
4. Incubate embryos for 25 min at room temperature on a nutating mixer device.
5. After fixation remove the lower, formaldehyde layer of the fixative and add 4 ml of methanol. Close the vial quickly and immediately shake the solution for 15 seconds. Embryos will partition into the lower, methanol phase and can be collected using a glass Pasteur pipette. Embryos do not stick to the glass while in methanol.
6. Embryos from multiple fixations can be pooled in one test tube and stored in methanol at -20°C for months. The success of immunolabelling of stored embryos depends on the antigen under examination. For several antigens, immunostaining of embryos was successful after storage in methanol over several years.

2.8. Heat-Methanol fixation

This fixation procedure essentially represents a methanol fixation. The advantage of this method is that the preservation of epitopes is generally very good, because methanol provides a mild fixation. The disadvantage is that the preservation of the structure is very poor (Figure 1J,K,L). In particular cytosolic antigens will be extracted during the procedure.

1. Fill 5 ml of 1x Triton-X-Salt solution (TSS) into a scintillation vial and close lid loosely. Place into boiling water bath.
2. Prepare embryos as described in 3.2.) and dump dechorionated embryos on wire mesh basket into the hot TSS.
3. Immediately add ice-cold TSS to completely fill the vial and remove wire mesh basket. Place vial on ice and let sit for at least 5 min. If several fixations are to be carried out it might be convenient to store different samples on ice at this stage.
4. Carefully remove TSS and add 4 ml of heptane. Then add 4 ml methanol (p.a. grade is essential) quickly close cap and vigorously shake the solution immediately. Fixed embryos will sink to the lower, methanol phase.

5. Collect embryos with Pasteur pipette, rinse 2 times with Methanol and store at -20°C .

2.9. Staining procedures

Immunolabelling procedures start by rehydration of fixed embryos. Following rehydration, unspecific binding sites are blocked by incubation with excess protein. The best results are obtained by using serum protein, preferably from the same species as the secondary antibody was obtained. Antibody incubations are being performed at 4°C overnight or 1.5 hrs at room temperature (25°C) in blocking buffer.

1. Rehydration: After fixation, rinse embryos twice with methanol and ensure that traces of heptane have been removed, as it will interfere with the rehydration. Discard the methanol and add PBT. Let embryos settle and rinse three times for 5 min with PBT. It is also possible to rehydrate in a methanol or ethanol series, but we did not see any major differences in the staining results.
2. Blocking: Remove PBT and add PBT containing 10% serum (e.g. goat, donkey). Incubate for at least 1 hour at room temperature to overnight at 4°C .
3. Primary antibody incubation: Dilute primary antibody to desired concentration in blocking buffer. Incubate 1.5 hrs at room temperature or overnight at 4°C . When using a new batch of antibodies, test runs of different dilutions are often critical to obtain specific labelling. In the case of purified antibodies a concentration between 1 and $5\text{ }\mu\text{g/ml}$ IgG are likely to show specific staining. Some antibodies might require a pre-clearing step. Dilute primary antibody in PBT at 1:5 up to 1:20 and incubate with fixed and rehydrated embryos overnight at 4°C . Spin down embryos for 5 min in a cooled micro-centrifuge and store supernatant at 4°C with a preservative (e.g. 0.02% sodium azide).
4. Wash step: Remove primary antibody (in some cases this solution can be retained and used for another staining). Rinse twice with PBT by letting embryos sediment and re-suspend in PBT. Wash three more times for 15 min at room temperature using a nutating mixer device.
5. Secondary antibody incubation: Prepare secondary antibody mix in blocking buffer. Dilute antibodies after the supplier's protocol. For double- or triple-immunolabelling multiple secondary antibodies can be applied at the same time. Although we do not find it necessary to pre-clear secondary antibodies, unspecific binding of secondary antibodies might be a potential problem of specificity (for pre-clearing secondary antibodies, incubate antibodies at a 1:10 dilution in PBT with fixed embryos at a ratio of 1:10 (v/v) overnight at 4°C). Incubate secondary antibody overnight at 4°C on a nutating mixer device.
6. Wash step: The wash step is identical to 4.

2.10. Antibody detection

1. Immunofluorescence: In the case of fluorescently conjugated secondary antibodies, embryos are mounted as advised under 3.6. and examined under an epi-fluorescence or confocal laser scanning microscope. Moreover a number of fluorescent substrates for enzyme-conjugated antibodies have been developed and can be applied according to the manufactures' protocols. One method that works well in fly embryos is signal amplification of HRP-conjugated antibodies with fluorescently labelled tyramide conjugates (14).

2. Colour detection: various methods have been described to detect enzyme conjugated secondary antibodies in embryos. Horseradish Peroxidase (HRP) and alkaline Phosphatase (AP) remain the most commonly used enzyme conjugates. The traditional detection of HRP is the colourless substrate diamino-benzidine (DAB), which will turn into a brown precipitate by the HRP activity in the presence of H_2O_2 . This signal can be enhanced using metal ions, for example 0.5% NiCl solution. For staining, rinse embryos in PBT once and replace with DAB solution. Stop reaction by adding PBT, 0.02% sodium azide or excessive rinsing with PBT. For AP staining, embryos have to be adjusted to AP - buffer to amend appropriate pH and salt conditions for AP. For colour detection NBT and BCIP are dissolved in AP-buffer. Stop colour reaction at desired point by adding excess PBT. For each staining procedure transfer embryos to a depression slide and monitor staining under a stereomicroscope. AP and HRP staining reaction can be performed sequentially to obtain a double immunolabelling. Although both ways are possible, it is advised to stain HRP first followed by AP detection because the DAB precipitate tends to be more stable.

2.11. Mounting of specimens

1. Fluorescently labelled specimen: After rinsing in PBT the stained embryos are being transferred to a drop of Mowiol/DABCO onto a glass slide using a cut-off yellow pipette tip. Mix the embryos carefully with the mounting medium and place a cover slip onto the sample. Let the sample settle for an hour before microscopy. Over time the Mowiol/DABCO will solidify and the samples can be kept for several months at 4°C.
2. Light microscopy: After colour reaction rinse with PBT and dehydrate with a graded ethanol series (see 2.5.2.) followed by 2 incubations with 100% acetone (each incubation 5 min). For infiltration with araldite incubate with acetone/Araldite (50:50) for 3 hours up to overnight at 4°C. Although some of the NBT/BCIP precipitates will be extracted during infiltration, the amount is usually minimal. If this poses a problem increase AP staining reaction time and reduce the incubation in acetone/Araldite solution. After infiltration place embryos onto a slide and orient the embryos using an eyelash mounted on a holder, like a glass pipette or syringe needle. Let the acetone evaporate for 3 hours at room temperature and incubate at 60°C overnight. Let slide cool down and add few droplets of pure araldite solution, just enough to completely cover the specimens after placing a cover slip onto the slide. Carefully place a cover slip onto the slide and incubate 24 hours at 60°C.

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Figure 1.

Preservation of fine structure by different fixations for whole mount immunolabelling of embryos.

Embryos (post-gastrulation stages, extended germ band) were fixed by standard methods for transmission electron microscopy (Zalokar, A-C) or by methods described in this chapter: formaldehyde fixation (FA; D-F), modified Stefanini fixation (Stefanini; G-H) or heat-methanol fixation (J-L). After primary fixation all samples were treated the same way and then processed for transmission electron microscopy as described elsewhere (9) (A,D,G,J) overviews show general structural preservation and evidence of extraction in FA and heat-fixed samples (J) did not exhibit much recognizable structure. (B,E,H,K) structure of nucleoplasm (Nu) and nuclear membranes (arrowheads). FA-fixed samples (E) show evidence of extraction of chromatin and nuclear membrane. Stefanini-fixed embryos (H) show more evenly distributed chromatin as well as heat-methanol treated embryos (K). In heat-fixed embryos, nuclear membranes were not observed. (C,F,I,L) Adherens junctions (AJ) between epidermal cells are well preserved in FA and Stefanini-fixed samples, but in heat-fixed samples ZA structure cannot be resolved. Bars represent 1 μm in (J,K) and 0.5 μm in (L).

Figure 2.

Comparison of fluorescent immunolabelling of heat-fixed and formaldehyde-fixed embryos. Embryos at post-gastrulation stages (extended germband) were fixed by heat-methanol (A,B,C) or by FA (D,E,F) and labelled with anti-Neurotactin (NRT), anti-Armadillo (ARM) antibodies and DAPI for DNA. Secondary antibodies were Cy2®- and Cy3®-conjugated antibodies. (A,D) Plasma membranes are labelled with NRT; note that labelling of NRT is very similar regarding the two fixation methods, although plasma membranes were not preserved by heat-fixation on the fine-structural level (see Figure 1). (B,E) Arm protein is present at plasma membranes, in the cytoplasm and - in response to wingless signalling - in the nucleus. Heat-fixation results in loss of most of the cytoplasmic Arm (compare B to C) and to a pronounced staining of Arm associated with adherens junctions (arrowheads in B). (C,F) Nuclear staining is more pronounced in formaldehyde-fixed samples compared to heat-fixed embryos.

Figure 3.

Double-immunolabelling followed by colour detection.

Embryos at different developmental stages were fixed with modified Stefanini's fixation and stained for Twist (Twi) (brown, HRP-DAB precipitate) and Even Skipped (Eve) (blue, AP-NBT/BCIP precipitate). A: stage 5 - blastoderm; B: stage 7 - gastrula; C: stage 8 - germ band extension; D: stage 11 - extended germ band; E: stage 13 - germ band retraction; F: stage 15 - retracted germ band. Eve is first expressed in seven stripes up until after germ band extension (the posterior of these stripes is labelled with an arrow (in A-C). After germ band extension Eve is expressed in 13 segmental clusters of dorsal mesoderm cells (arrow in D,E,F marks the posterior three clusters) as well as in the developing nervous system (arrowheads in F). Twi is expressed in all mesoderm precursor cells from early on; initial expression is seen in the ventral cells of the blastoderm (arrowhead in A). During morphogenesis, the mesoderm cells are being internalised (B,C) and later redistribute in the interior of the embryo; note segmental accumulation of mesoderm cells in late stage embryos (arrowheads in E).