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Imaging Immunolabeled *Drosophila* Embryos by Confocal Microscopy

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1. Introduction

Monoclonal and polyclonal antisera raised against recombinant proteins are highly sensitive probes that reveal cellular and subcellular protein localization in developing embryos. For *Drosophila* researchers, the ease of generating such antisera ***(1)*** and the number and widespread availability of existing antibodies make immunofluroescence of embryos an indispensable technique. The use of fluorochrome-conjugated secondary and/or tertiary antibodies on *Drosophila* embryos and detection by confocal microscopy offers two, critical advantages over enzyme-mediated detection methods, such as alkaline phosphatase and horseradish peroxidase. First, the sensitivity achieved with confocal microscopy may be difficult to match with enzyme-mediated detection especially when imaging cells deep within later stage embryos. Second, immunofluorescence and confocal detection allow the selective and simultaneous labeling with up to three different primary antisera. The following protocol contains instructions for the fixation, labeling, and detection of *Drosophila* embryos with one, two, and three different primary antisera.

Considerable forethought must go into designing a multiple labeling experiment. The critical considerations are: (1) the number of primary antibodies to be used, (2) the host species for each of the primary antibodies, (3) the availability of appropriately specific and appropriately conjugated secondary and/or tertiary antisera, and (4) the order and timing of antibody incubation. In general, each of the primary antibodies should be raised in a separate species. Primary antibodies can be labeled directly with fluorescently tagged secondary antibodies, or detected via a biotin-labeled secondary antibody and a

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strepavidin-conjugated fluorochrome. Secondary and tertiary reagents must be non-cross-reactive and able to distinguish between different reagents used in previous incubation.

Fluorochromes that may be used in the terminal labeling step include fluorescein (excites at 498 nm), rhodamine (excites at 568 nm depending on the exact subtype), and Cy5 (excites at 647 nm).

2. Materials

2.1. Embryo Fixation

1. Embryo wash buffer: 0.4% NaCl, 0.3% Triton X-100
2. Clorox
3. Fix buffer: 100 m*M* PIPES (pH. 6.9) 2 m*M* EGTA, 1 m*M* MgSO4
4. Heptane
5. 37% Formaldehyde
6. Methanol
7. Methanol/EGTA: 90% methanol, 0.05*M* EGTA
8. Ethanol

2.2. Immunolabeling

1. Phosphate-buffered saline (PBS): 13.7 m*M* NaCl, 0.27 m*M* KCl, 0.43 m*M* $Na_2HPO_4{\cdot}7H_2O$, 0.14 m*M* KH_2PO_4
2. PBS-Triton: PBS with 0.1% Triton X-100
3. Blocking solution: PBS; 0.1% Triton X-100, 1% BSA. Make fresh and store at 4°C
4. Primary antisera: Raised against the protein of interest in any one of several hosts ***(1)***
5. Secondary and/or tertiary antisera: Commercially manufactured antisera conjugated with specific fluorochromes
6. Mounting medium: 50 m*M* Tris-HCl (pH 8.8), 10% glycerol, 0.5 mg/ml *p*-phenylenediamine

3. Methods

3.1. Embryo Fixation

1. Embryos from the appropriate wild-type or mutant stock are collected on agar-molasses caps for approximately at 25°C ***(2)*** and *see* **Note 2**.
2. Wash the collected embryos in embryo wash buffer.
3. Dechorionate embryos in 50% Clorox for 2 min (*see* **Note 3**).
4. Thoroughly rinse in embryo wash buffer.
5. Transfer the embryos to a 15-mL screwtop tube containing 4 mL of fix buffer.
6. Add 5 mL of heptane and 1 mL of 37% formaldehyde, and shake the tube by inversion for 20–25 min.
7. Devitellinize the embryos by removing the fix buffer and adding 5 mL of 100% methanol to the embryos in heptane and shaking vigorously for 2–3 min.

8. The devitellinized embryos sink to the bottom of the tube and are removed to a 1.5-mL snap-top tube with a Pasteur pipet.
9. Fixed, devittelinized embryos are rinsed 2× in 90% methanol/0.05*M* EGTA.
10. For long-term storage, embryos are dehydrated in five or six washes in 100% ethanol and stored at –20°C. For immediate use, or to recover from long-term storage, embryos are rehydrated in two changes of PBS-Triton (PBT) and then placed in PBT at 4°C for blocking.

3.2. Single Immunofluorescent Labeling of Embryos

1. Primary antisera should initially be used at a variety of concentrations to determine the optimal concentration for detection by the specific confocal imaging system (*see* **Note 4**). Typical final concentration ranges are from 0.5 to 2 ug/mL.
2. Fixed, blocked embryos are incubated in a 1.5-mL snap-top tube with primary antibodies in a volume of 200 µL overnight at 4°C.
3. Wash the embryos 10 times in PBT over a period of 1 h.
4. Incubate with the secondary antibody in a 1-mL volume for 2 h at 4°C with rocking.
5. Embryos are again washed 10 times in PBT over a period of 1 h.
6. After the addition of fluorescent conjugates, the embryos are protected from light as much as possible (i.e., wrapped in aluminum foil). If needed, incubation with tertiary antibodies takes place again in a volume of 1 mL for 1 h at 4°C with rocking.
7. After a final series of 10 washes, the embryos were equilibrated in mounting medium and then mounted on slides in this same solution (*see* **Notes 5** and **6**).

3.3. Double and Triple Immunofluorescent Labeling

A sample labeling scheme using antibodies against three *Drosophila* segmentation proteins is shown in **Table 1**, and the resulting single- and triple-labeled images are shown in **Fig. 1**.

1. For multiple labeling, embryos are incubated in a 1.5-mL centrifuge tube with primary antibodies in a volume of 200 µL overnight at 4°C.
2. Embryos are then washed 10 times in PBT over a period of 1 h.
3. Incubation with secondary antibodies takes place in a 1-mL volume for 2 h at 4°C with rocking.
4. Embryos are again washed 10 times in PBT over a period of 1 h.
5. Incubate with tertiary antibodies in a volume of 1 mL for 1 h at 4°C with rocking.
6. Again, after the addition of fluorescent conjugates, the embryos are protected from light as much as possible (i.e., wrapped in aluminum foil).
7. After a final series of washes, the embryos are equilibrated in mounting medium and then mounted on slides in this same solution.

3.4. Confocal Microscopy

Fluorescent images were collected using a Bio-Rad MRC600 Laser Scanning Confocal Microscope equipped with a krypton/argon laser as a light source

Table 1
A Sample Labeling Scheme for the Simultaneous Detection of Three Different *Drosophila* Segmentation Proteins

Primary antibody	α-Hairy	α-Krüppel	α-Giant
Host species for primary antibody	Mouse	Rat	Rabbit
Secondary antibody	Cy5 α-Mouse IgG	α-rat IgG	Biotinylated α-rabbit IgG
Host species for secondary antibody	Donkey	Goat	Donkey
Tertiary antibody or conjugate or conjugate	None	Rhodamine α-goat IgG	Fluorescein streptavidin
Host species for tertiary antibody	Donkey	N/A	

The primary antisera are each raised in a different host species. Secondary antibodies are directed specifically against the primary host species to avoid cross-hybridization. In the case of the mouse a hairy antibody, a Cy5 conjugated secondary antibody is used in a two-step detection. For both Rat a Krüppel and Rabbit a Krüppel, a three-step detection was used.

(*see* **Note 7**). This light source has three major lines that emit at 488 nm (for detection of fluorescein), 568 nm (for detection of rhodamine), and 647 nm (for detection of Cy5). Two or three single optical planes are Kalman averaged and collected at a resolution of 8 bits (**Fig. 1**). The images are assigned specific colors and then merged into a single two or three color image using methods described previously ***(3,4)*** and in Chapter 21.

4. Notes

1. Embryos may be stored for several hours at 4°C prior to fixation, as development is essentially arrested at this temperature.
2. The timing of embryo collection depends upon the desired developmental stages. For example, if the desired antigen(s) is expressed during hours 4–8 of development, then embryos should be collected for 4 h, and then aged for 4 h prior to fixation.
3. Dechorionation (removal of the chorion shell) and devitellinization (removal of the vitelline membrane) are essential to allow probes to penetrate the embryos.
4. During the initial stages of staining embryos with novel antibody probes a series of antibody concentrations should be tested on embryos to fine tune the protocol to the confocal imaging system.
5. Coverslips should be sealed with clear nail polish, and slides should be clean and dry before viewing. Make sure to select the correct coverslip thickness for the objective lens.

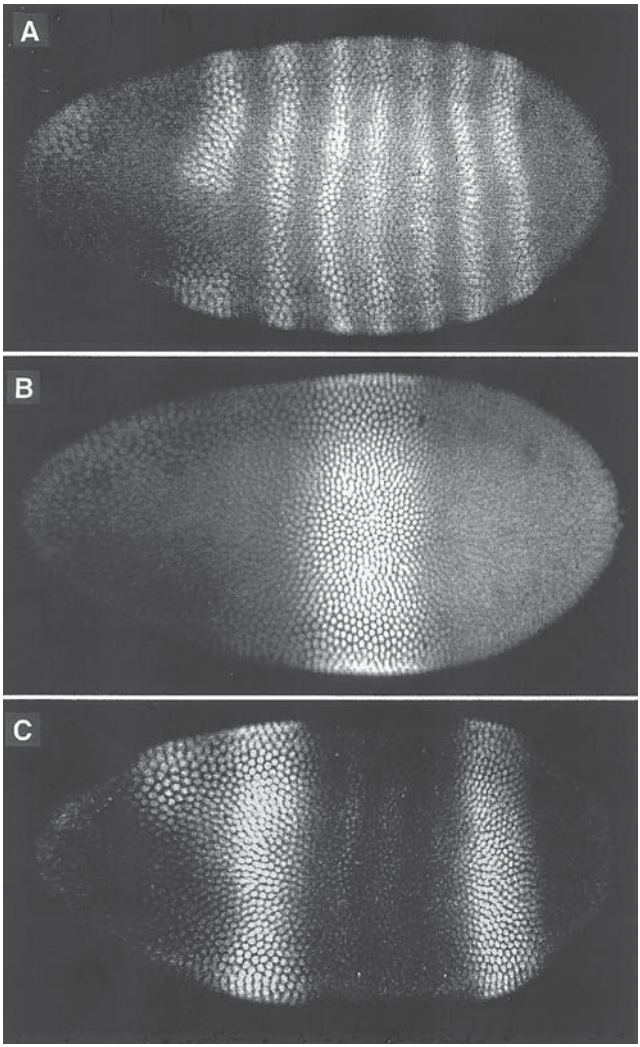

Fig. 1. Simultaneous localization of three segmentation proteins in a *Drosophila* embryo. **(A)** Detection of the seven stripes of hairy protein using α-mouse α-hairy primary antibody and a Cy5-conjugated donkey α-mouse IgG. The image was taken at 647 nm, and pseudocolored in green. **(B)** Detection of the block of Krüppel protein using α-rat α-Krüppel primary antibody, α-goat α-Rat IgG secondary antibody, and a rhodamine-conjugated donkey α-goat IgG. The image was taken at 568 nm and pseudocolored in red. **(C)** Detection of giant protein, using α-rabbit α-giant primary antibody, a biotinylated donkey α-rabbit IgG, and a fluorescein-conugated streptavidin. The image was taken at 498 nm and is pseudocolored in blue. For versions of the three-color merged image of A, B, and C, *see* Color Plate I C,D.

6. Slides should be viewed in a timely fashion after their preparation, as the fluorochrome signals tend to degrade over time. Fluorescein is the most labile and should preferably be viewed within a few hours after mounting. Signal lifetime can be preserved by storing the slides in the dark and in a fridge.
7. The choice of objective lens is important for gaining the best resolution. A Zeiss 16× oil lens or a Nikon 10× dry lens is good for viewing whole embryos and a Nikon 40× NA 1.2 or a Nikon 60× NA 1.4 lens is good for viewing individual cells and cell nuclei. It is always better to choose a higher magnification/NA lens than to zoom using the microscope or by cropping the image to gain the best resolution.
8. The following problems can cause the failure to detect one or more of the fluorochromes. The most common, and most readily curable, is in waiting too long after mounting before viewing (*see* **Note 6**). Another problem can be old and degraded antibodies. Antisera typically have a life of 1–2 years at 4°C. Old wash reagents, particularly old solutions with bovine serum albumin (BSA) can also degrade signal. Make up reagents fresh at least weekly. Another problem can be crossreactivity of secondary and tertiary antibodies; this will result in the same signal on two or more channels. It is essential to choose host species carefully, and to use reagents specifically formulated for minimal cross-reactivity.

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