Imaging Drosophila Embryos and Larvae Using Antibody Probes

(Just About Everything I Know About Immunohistochemistry)

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I. Introduction

The purpose of this chapter is to detail the immunocytochemical procedures used to view specific tissues and cells within whole mount preparations of Drosophila embryos and young larvae. A special emphasis is placed on providing examples of antibodies and techniques used to examine the patterns of neurons and axons within the developing nervous system. These methods have two primary applications in the study of Drosophila development. First, once a gene of interest has been cloned and antibodies raised to the protein product, these immunocytochemical techniques can be used to determine the pattern of protein distribution during embryogenesis and larval development. Second, antibody probes that recognize defined tissues and cells can be used as markers to analyze phenotypes in mutant embryos.

Most immunocytochemical methods for staining Drosophila whole mount embryos utilize techniques first developed by Zalokar and Erk (1977) and Mitchison and Sedat (1983). For the most part, subsequent modifications reflect progress made in the production of primary and secondary antibody reagents, in the techniques developed for visualizing enzymatically and fluorescently coupled antibodies, and in the optical and dissection methods used to examine stained preparations. The procedures outlined in this chapter also work well for immunostaining a wide variety of organisms other than Drosophila (Patel et al., 1989a; Patel et al., 1994).

I have chosen to detail various methods for fixing, staining, and dissecting whole-mount Drosophila embryos and larvae and to discuss parameters that effect the quality of the results. I have not, however, attempted to exhaustively describe the specific tissues, identified neurons, and available antibody reagants because these issues have been dealt with in great detail in several excellent reviews that have been published in the last several years

The recently published volume edited by Bate and Martinez-Arias (1993) presents extensive reviews discussing embryonic pattern formation and the development of all tissues and organs. For descriptions of neural development, the following references may serve as good starting points. For details of neuroblast formation, and a few examples of markers expressed during early neurogenesis, see Campos-Ortega (1993), Goodman and Doe (1993), Gutjahr et al., (1993), Doe (1992), and Skeath and Carroll (1992). The position and axon projections of identified neurons and glia of the central and peripheral nervous system are described in Goodman and Doe (1993), Jan and Jan (1993), Klämbt and Goodman (1991), Sink and Whitington (1991), Jacobs and Goodman (1989), Bodmer and Jan (1987), Dambly-Chaudière and Ghysen (1986), Ghysen et al., (1986), and Thomas et al. (1984). Examples of antibodies that stain specific patterns of neurons or axons within the developing central and peripheral nervous system are described in Nose et al. (1992), Dambly-Chaudière et al. (1992), Grenningloh et al. (1990), Kania et al. (1990), Blocklinger et al. (1990), Patel et al. (1989b), Doe et al. (1988), Patel et al (1987), and White and Vallés (1985). Listed in the next section are a few specific examples of antibody reagents that are useful in imaging specific tissues and cells within developing embryos and larvae.

II. Reagents used to visualize specific tissues and subsets of neurons.

A. Examples of markers for specific tissues.
Not all these antibodies are strictly specific for the tissues listed, nevertheless they are useful for examining these tissues during certain stages of devlopment.

1. Germline cells.
   Anti-vasa antiserum (Lasko and Ashburner, 1988) and monoclonal antibody (MAb 46F11; Hay et al., 1988). Stain pole plasm and germline cells (Fig. 1A).
2. Trachea. See Manning and Krasnow (1993) for a detailed review of tracheal development.
   Monoclonal antibody 2A12. Stains lumen of tracheal tree (N. Patel and C. Goodman, unpublished; described in Manning and Krasnow, 1993; Fig. 1C).
3. Mesoderm. See Bate (1993) for a detailed review of mesoderm and muscle formation.
   Anti-snail antiserum (Alberga et al., 1991). Stains nuclei of mesodermal cells during early embryogenesis.
   Anti-muscle myosin antiserum (Kiehart and Feghali, 1986). Stains cytoplasm of differentiated muscles.
   Anti-fasciclin III monoclonal antibody (MAb 2D5; Patel et al., 1987). Stains cell surface of visceral mesoderm cells (Fig. 1B; also see section B3 below).
4. General nervous system or large subset of nervous system.
   Anti-hunchback antiserum (Tautz et al., 1987). Stains nuclei of all neuroblasts during early neurogenesis.
   Anti-elav antisera (Robinow et al., 1988) and monoclonal antibody (MAb 44C11; Bier et al., 1988). Stain nuclei of all neurons (Fig. 1D-I).
   Anti-HRP (horseradish peroxidase) antiserum (not to be confused with HRP conjugated antibodies; Jan and Jan, 1982). Stains neural cell membranes by recognizing a carbohydrate moiety attached to a number of neural proteins (Snow et al., 1987).
   Monoclonal antibody BP104 (Hortsch et al., 1990). Stains neural cell membranes by recognizing a nervous system-specific isoform of the neuroglian protein (Fig. 2B and C).
   Monoclonal antibody 22C10 (Fujita et al., 1982; Zipursky et al., 1984) Stains cytoplasm and inner surface of cell membrane of all PNS neurons and a subset of CNS neurons (Fig 4A and B, 6A, E, and F).
   Monoclonal antibody BP102 (A. Bieber, N. Patel, and C, Goodman, unpublished; described in Seeger et al., 1993). Stains axons of CNS neurons. All CNS axons within the commissures and connectives appear to be stained by stage 13-14, but many longitudinal axons are not stained during their initial outgrowth in stage 12 (Fig, 3A-D, 5A-F, and 6B, C, and G-I).

B. Examples of antibodies that recognize small subsets of neural precursors and neurons.

1. Subset of cells in the peripheral nervous system.
   Anti-cut antiserum (Blocklinger et al., 1990). Stains nuclei of all external sensory organ precursors.

Anti-pox neuro antiserum (Dambly-Chaudière et al., 1992). Stains nuclei of poly-innervated external sensory cells.

2. Subset of central nervous system neuroblasts.
   Anti-acheate monoclonal antibody (Skeath and Carroll, 1992). Stains nuclei of 4 neuroblasts per hemisegment during the first wave of neuroblast segregation.
   Anti-engrailed antisera (DiNardo et al., 1985) and monoclonal antibody (MAb 4D9; Patel et al., 1989a). Stain nuclei of all row 6 and 7 neuroblasts and a neuroblast of row 1.
   Anti-gooseberry antiserum (Gutjahr et al., 1993). Stains nuclei of all row 5 and 6 neuroblasts.
3. Subset of central nervous system neurons.
   Anti-even-skipped antiserum (Frasch et al., 1987) and monoclonal antibodies (MAb 3C10 and MAb 2B8; Patel et al., 1992 and Patel et al., 1994). Stain nuclei of a small subset of neurons (Fig. 3E-H, 5H-L, 6A, H and I).
   Anti-engrailed antisera (DiNardo et al., 1985) and monoclonal antibody (MAb4D9; Patel et al., 1989a). Stains nuclei of a small subset of neurons (Fig. 2E-H, 4C, D, 6B, E, and F).
   Anti-fasciclin III monoclonal antibody (MAb 2D5; Patel et al., 1987). Stains surface of a subset of neurons and axons (Fig 1B and 2I).
   Anti-fasciclin II antiserum (Grenningloh et al., 1990) and monoclonal antibody (MAb 1D4; G. Helt and C. Goodman, unpublished; described in Van Vactor et al., 1993) . Stain surface of a subset of neurons and axons (Fig. 2D).
   Anti-FMRFamide antiserum (Schneider et al., 1993). Stains cytoplasm of a subset of neurons (cell body and axonal processes).
   Anti-serotonin antiserum and monoclonal antibody (described in White and Vallés, 1985). Stains cytoplasm of a subset of neurons (cell body and axonal processes).

C. Segmentation markers.

Antibodies are available to a wide variety of segmentation gene products and many of these genes are also expressed in specific tissue and neurons later in development. For example, even-skipped and engrailed are both segmentation genes, but are also expressed by specific patterns of neurons (see above and Fig. 2E - H, 3E, and 5J). Further details of segmentation gene expression patterns can be found in Akam (1987), Ingham (1988), Ingham and Martinez-Arias (1992), and Pankratz and Jäckle (1993).

D. Identification of specific tissues and neurons by enhancer trap lines or by non-radioactive in situ hybridization.

A large number of lacZ enhancer trap lines which express betagalactosidase in specific patterns of cells and tissues have been described (see for example Bier et al., 1989; Bellen et al., 1989; Ghysen and O'Kane, 1989; Hartenstein and Jan, 1992). The patterns in these lines can be visualized with a mouse monoclonal antibody against betagalactosidase (Promega; Cat. No. Z3781; use at a 1:200 - 1:1000 dilution) or a rabbit antisera against betagalactosidase (Cappell; Cat. No. 55976; use at a 1:1000 - 1:5000 dilution; Fig.

6G). In cases where antibodies are not available to a gene of interest, the expression pattern can be monitored in whole mount preparations using the in situ procedures described in the chapter by Lehmann and Tautz (also see Patel and Goodman, 1992).

III. Fixation, Methanol Devitellinization, Storage, and Rehydration Protocols

A. Whole mount preparation of 0-17 hr embryos

1. Collect embryos on apple or grape juice agar plates.
2. Rinse embryos with water into a Nitex nylon mesh (Tetko Inc., Cat. No. 3-85/35XX or 3-100/47).
3. Dechorionate with 50% bleach (2.6% sodium hypochlorite) for about 3 min.
4. Rinse embryos thoroughly with dH_2O. Examine embryos under a dissecting microscope to check that they are completely dechorionated, if not, extend time in 50% bleach until fully dechorionated.
5. Place embryos into a 25 ml glass scintillation vial containing 10 ml heptane plus 10 ml of PEM-FA fixative (see Solutions in section XIII). The solutions will form two layers; the lower layer is fixative, the upper layer is heptane, and the embryos will sink in the heptane to lie at the interface of the two layers. The heptane will become saturated with fixative, allowing the fixative to penetrate the hydrophobic vitelline membrane surrounding the embryos.
6. Agitate the vial gently for 10 to 60 min. The optimal fixation time varies depending on the particular antigen and antibody involved. In most cases, fixation time of 10-20 min appears to give good results. See section XII for further comments on fixation.
7. Remove as much of the aqueous (bottom) layer as possible. Swirling the vial will cause the embryos to spin to the center but will leave the final drops of the aqueous phase at the edges, where they can then be easily removed. Make sure that there is still 8-10 ml of heptane remaining with the embryos in the vial.
8. Add 10 ml of methanol to the embryos and shake vigorously for 30 to 60 sec. The vitelline membranes should split open and the fixed embryos will fall to the bottom of the container. The empty vitelline membranes and non-devitellinized embryos will remain at the interface between the heptane and methanol. At least 80% of the embryos should be devitellinized.
9. Remove the heptane, methanol, and all material at the interface. Wash the devitellinized embryos at least three times with methanol. Shake gently between washes. These methanol washes are required to remove the last traces of heptane.
10. Embryos in methanol can be transfered to a polypropylene tube and kept at –20°C for extended storage. I have embryos that have been stored for four years in absolute methanol at –20°C; they still retain excellent morphology and stain well with a wide variety of antibodies.
11. To proceed with antibody staining, rehydrate embryos by removing the methanol and then washing 2 X 5 min and 1 X 30 min with PT (see section XIII). At this point, the embryos will be become somewhat "sticky" and is best to use polypropylene tubes and pipettes (1000 μl "blue" tips) to prevent too many embryos from being lost. If you do use glassware, make sure to coat it with PT before trying to transfer the embryos. Pre-cellularized embryos (<3 hrs) should be rehydrated through a methanol/PT series to

maintain good morphology. Proceed to section IV to continue antibody staining.

General comments:

Keep the embryos at room temperature throughout the procedure because exposure to cold or heat just prior to fixation may create artifacts in morphology. Do not allow the embryos to dry out at any time during the procedure. The protocol can be easily scaled up to process several grams of embryos collected from population cages. Use 250 ml of heptane and 250 ml of PEM-FA in a large glass bottle with a lid impervious to heptane and methanol to fix up to 5 gms of embryos. Store up to 5 ml of packed embryos in 40 ml of absolute methanol at –20°C. Remove aliquots of embryos when needed and rehydrate as descibed above. The fixation protocol described here preserves good cellular morphology at the level of light microscopy; see the protocols in the chapters by B. Theurkauf and K. McDonald for procedures required to preserve cytoskeletal structure and to maintain good morphology at the E.M. level.

B. Whole mount preparation of 17-22 hr embryos.

Embryos older than 17 hrs develop a cuticle that impedes the penetration of antibodies. The following protocol uses the same fixation procedure as described above, but adds a sonication step that allows easy penetration of antibodies through the cuticle. Precedent for sonication comes from a procedure originally developed for staining brine shrimp naupuli (Manzanares et al., 1993).

1. Collect embryos age 17 – 22 hrs (at 25°C). This covers the period from the onset of cuticle deposition to hatching. A wider age collection will make it difficult to determine the optimal sonication period.
2. Follow steps 1-6 of Protocol IIIA above.
3. Embryos just ready to hatch (~ 21-22 hrs) will break out of their vitelline membrane and sink to the bottom of the aqueous layer. Remove and discard the aqueous layer (PEM-FA), but retain both the embryos at the interface and those that have fallen through the interface to the bottom of the aqueous layer. Remove as much fixative as possible. Make sure that there is still 8-10 ml of heptane remaining with the embryos in the container.
4. Follow steps 8-10 of protocol IIIA above.
5. Rehdrate by washing 3X 5 min and 1 X 30 min with PT.
6. Place about 100 - 150 μl of packed embryos into 500 μl of PT in an eppendorf tube.
7. Sonicate with a probe tip sonicator. I use a Branson Sonifier 250 set at 100% duty cycle and the lowest possible output (setting #1). At these settings, 3 seconds of sonication results in almost no visible damage, but antibody penetration improves considerably. After 15 seconds of sonication, about 25 - 50% of the embryos show obvious morphological damage. Total sonication times of 6-9 seconds (two or three pulses of 3 seconds duration) minimizes the percentage of damaged embryos, while maintaining excellent staining with MAb BP102, MAb 22C10, anti-serotonin, anti-fasciclin II MAb, MAb 2B8 (anti-even-skipped), and MAb 4D9 (anti-engrailed). The optimal settings for a given sonicator should be determined empirically by sonicating for a series of different times and then assaying for alterations in morphology and extent of antibody staining. Protect the embryos from excessive heating during the

sonication procedure by placing the tubes on ice for 15-30 seconds between sonication pulses. MAb 22C10 (1:10 dilution of tissue culture supernatant) is an especially useful control antibody when working out the sonication conditions. Remember to include unsonicated embryos as a control. After sonication, keep the embryos on ice until the next step. It does not appear that a post-sonication fixation step is necessary, but this may be useful for some antigens.

8. Wash 2 X 5 min in PT. Proceed to section IV to continue antibody staining.

C. Whole mount preparation of young first instar larvae (0-3 hrs post-hatching)

Larvae have a cuticle that not only prevents antibody penetration but also requires a somewhat harsher fixation procedure.

1. Collect larvae that are 0 - 3 hrs post-hatching. When you first try this procedure, make sure to start with this narrow age collection so that you can determine the proper sonication period.
2. Rinse with 50% bleach for 1 min to remove adhering yeast.
3. Rinse well with dH_2O.
4. Place larvae into a scintillation vial containing 10 ml of heptane plus 10 ml of PEM-FA. Rotate container for 10-20 minutes. Heptane may be unnecessary for fixing larvae because there is no vitelline membrane, but I have never left it out.
5. Unlike embryos, larvae will sink to the bottom of the aqueous layer because they do not have vitelline membranes. Transfer those larvae that have sunk to the bottom of the vial to a new scintillation vial and fix them for an additional 5 min in PEM-FA containing 0.1% Tween-20. If you are staining for neurotransmitters, such as serotonin, you will need to fix for as long as 2 hrs. See comments in section XI B 1.
6. Remove the fixative and wash the larvae several times with methanol. Washing stepwise with a dehydration series of methanol/PBS will help minimize morphological damage.
7. As with embryos, larvae can be stored in methanol at –20°C for an extended period of time. As needed, aliquots of larvae can be removed and processed as described below.
8. Remove methanol and replace with PT. Again, a methanol/PT series will help minimize tissue damage .
9. Wash 2 X 10 min in PT
10. Place about 100 - 200 μl of packed larvae into 500 μl of PT in an eppendorf tube.
11. Sonicate with a probe tip sonicator. As described earlier, I use a Branson Sonifier 250 set at 100% duty cycle and the lowest possible output (setting #1). After about 18-20 sec of sonication, about 25 to 50 % of the larvae will show obvious morphological damage. Sonications times of 12-15 seconds result in good CNS staining with the same antibodies described in step 7 of section III B. Also see step 7 of section III B for suggestions on determining the optimal sonication time; again MAb 22C10 serves as a good control antibody for initial attempts at this procedure. After sonication, keep the larvae on ice until the next step.
12. Wash 2 X 5 min in PT. Proceed to section IV to continue antibody staining.

General comments: I have only recently developed the protocols for sonicating and staining older embryos and larvae described in section III B and C. While these protocols have worked well in my hands for staining with a half dozen or so different primary antibodies, it is possible that not all antigens can be detected with these procedures.

D. Hand dissection of larvae

Third instar larvae are easily dissected by hand. With practice, second instar larvae can also be hand dissected.

1. Wash larvae with water to remove adherent yeast.
2. Dissect larvae in PBS or directly into PBS-FA or PEM-FA in a glass depression well slide (precoating the wells and pipettes with PBS+1% BSA will help prevent larval tissue from getting stuck to the glass). To remove brain, ventral nerve cord, salivary gland, and anterior discs, use one pair of forceps to grab the mouth hooks and a second to grab the middle of the larva. Pull apart and then tease away excess tissue. Larvae can also be slit along their entire length and held flat while fixed. Alternatively, they can be cut in half and then turned inside out. If the dissection is done in PBS, transfer the tissue to PBS-FA. Fix for 15 - 30 min. If you are staining for neurotransmitters, you may want to fix for as long as 2 hrs. See discussion of fixation time in section XII B 1.
3. Wash 3 X 5 min with PT. From this point on, you can treat the dissected larval tissue as you would intact larvae. Proceed to section IV to continue antibody staining.

E. Hand dissection of living embryos.

In some situations, it is preferable to hand-devitellinize embryos. For example, some epitopes are denatured by methanol and the methanol devitellinization procedure would eliminate antibody recognition. For protocols for hand devitillinization of fixed embryos, see the chapter by B. Theurkauf. It is also possible to hand dissect living embryos and then fix them to a glass slide.

1. Prepare microscope slides with silicone walls. First, wash slides thoroughly with ethanol and dry. Using silicone aquarium sealant, construct a rectangular wall about 2 cm X 3 cm and about 2-3 mm high. Be certain that there is continuous contact between the silicone and the glass surface. Allow the silicon to dry overnight. Test the slides by adding PT and checking that none leaks out through gaps between the silicone and the slide. Just before use, clean the slides by rubbing the glass in the well vigorously with a Kimwipe soaked in ethanol.
2. Cut a small square of double-stick tape (~ 0.7 cm square) and stick it down on the bottom of one side of the silicone well. Carefully place about one dozen stage 12-15 embryos on the double stick tape.
3. Using blunt forceps, gently roll all the embryos to remove their chorions. Orient the embryos dorsal side up and make sure they are still well stuck to the double-stick tape.
4. Fill the well with 1X PBS.
5. Using a sharpened tungsten needle (see section IX B 1), carefully slit open the vitelline membrane over the dorsal side of one of the embryos. Gently lift

the embryo, always keeping it well below the meniscus of the liquid, and move it to an area of clean glass.

6. Gently push the embryo against the glass. If the glass is well cleaned, the embryo will stick quite strongly to the surface (it may be helpful to coat the surface with poly-L-lysine beforehand if problems are encountered).
7. Use the needle to fold down the body wall and brain region. Lift the gut to the side to expose the underlying CNS. Repeat steps 5 and 6 for as many embryos as possible. Try to keep all dissected embryos in the center of the well. Embryos dissected close to a wall will be difficult to look at later. Eventually the PBS will fill with enough protein to coat the glass and prevent additional dissections from adhering to the glass.
8. Simultaneously use two pastuer pippettes, one to add PBS-FA and another to remove the 1X PBS. In this way, the dissections can be fixed without the the meniscus of the liquid touching the dissections (if the meniscus does come close a dissected embryo, the surface tension will pull the nerve cord from the rest of the embryo or the entire embryo from the glass).
9. After 10 - 30 minutes of fixation, use two pasteur pipettes to wash the embryos 3 X 1 min and then 3 X 10 min with 1X PBS. Stain the embryos as described in sections IV and V. Always use two pastuer pipettes to exchange the wash solutions. To add antibodies, remove enough solution so that the embryos are just below the surface of the liquid, and then add the primary or secondary antibodies at the appropriate concentration.
10. When ready, the embryos can be viewed using a liquid immersion lens. Alternatively, the well can be filled with 50 then 70% glycerol and then the silicone walls can be scraped away with a razor blade and the dissections covered with a coverslip. Use a Kimwipe to blot away the excess glycerol and seal the coverslip with fingernail polish.

General comments: This dissection technique can also be used to prepare embryos for Lucifer fills of neurons (Sink and Whitington, 1991). If no detergents are used up until the time that the secondary antibody is washed off, this technique can also be used to show that an antibody epitope is on the outside of the cell memebrane (i.e, acessible without detergents to permeabilize the cell membrane). MAb 2D5 (anti-fasciclin III) can be used as a positive control and anti-tubulin can be used as a negative control in this type of experiment.

IV. Primary and secondary antibody incubation procedure.

1. Wash fixed, rehydrated embryos, larvae, or larval tissues for 30 min in PT. This and all subsequent steps are preformed at room temperature unlesss otherwise stated and can be carried out in 5 ml polypropylene round-bottom, snap-cap tubes (Falcon No. 2063 culture tubes) or in 1.5 ml microcentrifuge tubes. Use about 25-75 μl of embryos per 5 ml tube or 5-25 μl of embryos per microcentrifuge tube. Washes are improved if the tubes are gently agitated on a rotator (Labquake; Lab Industries) or a rocking platform (Nutator; Clay Adams). Use wash volumes of 4.7 ml with 5 ml tubes and 1.2 ml with 1.5 ml tubes.
2. Incubate 30 min in 100 μl of PT+NGS (see section XIII). The normal goat serum (NGS) and BSA will help to block nonspecific antibody binding sites.

Gently mix and, if possible, place tubes in a rack mounted on a rotary platform shaker set to approximately 100 rpm. Avoid shaking so hard that embryos splash up onto the walls of the tube.

3. Add the appropriate amount of primary antibody to achieve the desired final concentration (see section XI B 3). For example,use tissue culture supernatants of MAb 2B8 (anti-even-skipped) at a dilution of 1:50 by adding 2 μl of MAb 2B8 to the 100 μl of PT+NGS that is already in the tube. For antisera that need to be used at very low concentrations, you can first make up a pre-dilution in sterile 1X PBS containing 5% normal goat serum and 0.02% sodium azide. This pre-dilution should be stable for at least several weeks at 4°C.
4. Gently mix the embryos and antibody solution and incubate overnight at 4°C. If possible, carry out this incubation on a rotary platform shaker set at about 100 rpm.
5. Wash 3 X 5 min with PT. Before these washes are started, it is possible to recover the diluted primary antibody, and this used antibody can often be used several more times.
6. Wash 4 X 30 min with PT.
7. Incubate 30 min in 100 μl of PT+NGS as in step 2 above.
8. Add appropriate secondary antibody to the desired final concentration. Listed below are the concentration ranges I routinely use for several secondary antibodies available from Jackson ImmunoResearch Labs. The antibodies will arrive in a lyophilized form. After they are reconstituted according to the enclosed instructions, centrifuge the solutions to remove any insoluble particles and then store them as 200 μl aliquots at –70°C (or in a non-defrosting –20°C freezer). Repeated freeze-thawing will rapidly lower the titer of many of these secondary antibodies. Aliquots in use can be stored at 4°C for several weeks or months. Alternatively, an excellent way to store secondary antibodies (and primary antibodies that can be used at dilutions of 1:10 or greaater) is to mix them with and equal volume of 100% glycerol (giving a final glycerol concentration of 50%) and keep aliquots at –20°C. At this temperature, the antibodies will not freeze (much like restriction enzymes which also come in glycerol), and are stable for years.
    - Goat anti-mouse IgG conjugated to FITC, RITC, HRP, or alkaline phosphatase (Cat. No. 115-095-003, 115-025-003, 115-035-003, or 115-055-003). Use at 1:300 to 1:1000 final dilution. Although antibodies to IgG do crossreact with IgM, it is best to use secondary antibodies directed against both IgG and IgM if you know that the primary antibody is an IgM monoclonal.
    - Goat anti-rat IgG conjugated to FITC, RITC, HRP, or alkaline phosphatase (Cat. No. 112-095-003, 112-025-003, 112-035-003, or 112-055-003). Use at 1:300 to 1:1000 final dilution.
    - Goat anti-rabbit IgG conjugated to FITC, RITC, HRP, or alkaline phosphatase (Cat. No. 111-095-045, 111-025-045, 111-035-045, or 111-055-04). These anti-rabbit secondary antibodies are subtracted against human serum, which appears to lower the background often found with anti-rabbit secondary antibodies. Use at 1:500 to 1:2000 final dilution.

For most fluorescent applications, we have now swithced over to using Alexaflour conjugates 488 and 546 which are available from Molecular Probes. These show much better signal and less fading than FITC and RITC conjugates.

As a working stock, the secondary antibody should be first diluted to 1:100 in PT+NGS before use. Then, to achieve a final dilution of 1:300, you would add 50 µl of 1:100 diluted secondary to the 100 µl PT+NGS-embryo mix. These 1:100 dilutions of secondary antibody should be stable for at least a week at 4°C.

9. Mix the embryos and secondary antibody solution gently. For embryo staining, incubate for 2-3 hrs at room temperature. For larval staining, incubate overnight at 4°C. If possible, carry out these incubations on a rotary platform shaker set at about 100 rpm.
10. Wash 3 X 5 min with PT.
11. Wash 4 X 30 min with PT. If you used a fluorescently tagged secondary antibody, proceed to the clearing procedures in section VIII. For HRP or alkaline phosphatase conjugated secondary antibodies, proceed to the histochemical development reactions in section V.

General comments:

In this protocol, the secondary antibodies are produced in goats and normal goat serum is used as a blocking agent. This ensures that the secondary antibodies will not recognize any of the immunoglobulins in the normal serum being used as a blocking agent. If you use secondary antibodies produced in other animal species, be certain that the normal serum in steps 2, 3, 7, and 8 does not contain immunoglobulins that will be recognized by the secondary antibody being used.

V. Histochemical development reactions

A. Brown HRP reaction.

1. Incubate embryos in 300 µl of DAB solution (see section XIII) for approximately 2 min. For larvae and larval tissues, extend this to 5-10 min to improve DAB penetration into the tissue.
2. Start the reaction by adding H_2O_2 to a final concentration of 0.01-0.03%. To do this, add 10 to 30 µl of 0.3% H_2O_2 to each tube. For a stock solution, 3% H_2O_2, which is available in any drug store, works well. It is stabilized with 0.001% phosphoric acid and can be stored for many years at room temperature. Dilute a small amount to 0.3% in PBS just before use.
3. Monitor the reaction by looking down into the tube with a dissecting microscope or by removing a few embryos to a depression slide. Brown signal will often be visible within 30 seconds. Depending on the primary antibody being used, and the concentration at which it is used, it can take anywhere from 1-5 min for the embryos to reach an optimal signal-to-noise ratio. Generally, no improvement in signal is seen after about 10 minutes.
4. Stop the reaction by washing 2 X 1 min with PT. Proceed to the glycerol or methyl salicylate clearing protocols in section VIII.

B. Black HRP reaction.

1. Incubate embryos in 300 μl of DAB+Ni solution (see section XIII) for approxiamtely 2 min. For larvae and larval tissues, extend this incubation time to 5 to 10 minutes.
2. Follow steps 2-3 in the protocol for the brown HRP reaction (section V A above) The reaction of the nickel ions and DAB product results in the production of a purple to black signal that will often be visible within 10 seconds. This nickel enhanced procedure is several fold more sensitive than the regular DAB procedure. While this reaction is more sensitive than the brown HRP reaction, it produces a more granular staining that may make fine details harder to visualize.
4. Stop the reaction by washing 2 X 1 min with PT. Proceed to the glycerol or methyl salicylate clearing protocols in section VIII. Silver enhanced HRP reaction provides an even more sensitive stain. Details of this enhancement procedure can be found in Lazar and Taub (1992).

C. Orange and Red HRP reactions.
1. An orange reaction product can be obtained using a DAB plus catechol reagent available from Kirkegaard & Perry Labs (Cat. No. 54-74-00). This reaction is about as sensitive as the regular brown DAB reaction.
2. A red reaction product can be obtained using 3-Amino-9-ethylcarbazole (AEC; available from Sigma Cat. No. AEC-101). Due to the relatively poor sensitivity, however, this reaction is only useful when used with robust primary antibodies. When succesful, however, it provides a very useful second label when combined with the Black HRP reaction. React by washing embryos 2 X 5 min with AEC buffer, then add 300 μl AEC Reaction mix to the embryos, wait 2 min, and then add 20 μl of 0.3% hydrogen peroxide. The reaction mix will be cloudy, so you may neeed to temporarily remove the reaction solution from the tube when montoring the progress of the reaction. Stop the reaction by washing the embryos 1X with AEC Buffer and then PT. The reaction product is soluble in organic solvents, so use only the glycerol clearing procedure of section VIII.

D. Purple Alkaline Phosphatase reaction
1. Wash embryos or larvae 2 X 5 min in A.P. buffer (see section XIII).
2. Add 300 μl of BCIP/NBT solution (see section XIII).
3. Monitor the reaction. An optimal signal-to-noise ratio will usually be reached anywhere from 5-15 min, but the reaction can be allowed to continue for several hours if needed. The sensitivity of this technique is equal or slightly better than a black HRP reaction. Alkaline phosphatase reactions, however, are more prone to background problems than HRP reactions and the reaction products sometimes give diffuse staining around fine structures such as individual axons.
4. Stop the reaction by washing 2 X 1 min with A.P. buffer. Proceed to the glycerol clearing protocol in section VIII.
5. Background alkaline phosphatase activity in cuticular stripes and in the trachea are sometimes observed in embryos past stage 17. Note that this background is not effectively inhibited by Levamisole, which is often used as an inhibitor of endogenous alkaline phosphatase in vertebrate tissues.

E. Blue Alkaline Phosphatase reaction.
   1. Follow steps 1-4 in the protocol for the purple alkaline phosphatase reaction (section V D above)
   2. After stoping the reaction by washing with A.P. buffer, wash the embryos 2 X 5 min and 1 X 30 min in methanol. The signal will slowly turn from purple to blue. Wash with methanol until the desired level of blue is obtained. The color change occurs because the purple alkaline phosphatase reaction product is actually composed of two different reaction products: an alcohol-soluble purple product and an alcohol-insoluble blue product. Because this procedure lowers the level of signal, it should be used only if the starting purple reaction product is relatively strong. An alternative substrate (Fast Blue Base) system for generating a blue reaction product is available from Kirkegaard & Perry (Cat. No. 55-70-00).
   3. Wash 2 X 5 min with A.P. buffer. Proceed to the glycerol clearing protocol in section VIII.

F. Red Alkaline Phosphatase reaction product.

1. A red alkaline phosphatase reaction product can be obtained using a Fuchsin dye substrate (Kirkegaard & Perry; Cat. No. 55-69-00). The reaction is somewhat weak and slow (30-60 min), but gives very good staining with antibodies that have a strong signal (MAb BP102 for example). The background will look relatively high initially, but much of the background staining will disappear during the glycerol clearing steps in section VIII. This reaction product is soluble in organic solvents, so do not use the methyl salicylate clearing procedure of section VIII.

VI. Labeling with multiple primary antibodies.

Because of the variety of possible color detection schemes, both fluorescent and histochemical, it is possible to visualize multiple antibody staining patterns simultaneously in the same embryo. Double labeling procedures have a number of useful applications. For example, by double labeling, the staining pattern of a newly isolated antibody can be compared to the patterns seen with a well characterized antibody. Double labeling also allows the unambiguous comparison of genotype and phenotype in embryo collections (see section XI). Fluorescent double labeling is possible only if the primary antibodies are from different animal species or if they are conjugated directly to fluorochromes. However, it is possible to use primary antibodies made in the same animal species, such as two mouse monoclonal antibodies, for histochemical double labeling (see C and D below).

In theory, RITC and FITC signals can be collected independently in the microscope, which makes it possible to determine whether and where two staining patterns overlap. Three factors affect the ability to accurately detect overlapping fluorescent signals. First, the secondary antibodies must not recognize the inappropriate primary antibody. Appropriate "subtracted" secondary antibody reagents are now widely available from a number of commercial sources. Second, the microscope's fluorescence filter set must be able to separate the emission spectra of FITC and RITC. Secondary antibodies conjugated to Texas Red rather than RITC can be used to improve color separation. Finally, the microscope must be able to distinguish stained cells that are lying one on top of the other. Confocal microscopy would appear to be the obvious solution to this third consideration, but the small size of many of the cells inside the Drosophila embryo will prove a challenge to many confocal microscopes. This is especially problematic when trying to distinguish the flat glial cells from the neurons they sometimes wrap around.

Using histochemical reactions, it is posssible to use primary antibodies made in the same species (two mouse monoclonal antibodies, for example), because the antibody incubations and histochemical reactions can be done sequentially (see sections C and D below). When two antibodies stain the same cell, the overlap can be easily distinguished as long as the two antibodies stain different cellular compartments (nucleus vs. cytoplasm vs. cell membrane). The example in section C below shows such a case, in which one antibody recognizes a nuclear antigen (purple stain) and the other recognizes a cytoplasmic antigen (brown stain). Staining should be planned so that the darker reaction product does not obscure the product of the lighter reaction product. For example, if the colors were reversed in section C below, the brown nuclear stain would not be visible through the intense purple cytoplasmic staining. With practice, and with the appropriate controls, it is possible in some cases to document

histochemically detected staining overlap even if the antigens reside in the same cellular compartment.

Certain color combinations are not useful. For example, the brown HRP and orange HRP products are not sufficiently different from one another to be used together in double labeling. Usually, a combination of brown staining (DAB reaction) with black (DAB+Ni), purple (alkaline phosphatase), or blue (alkaline phosphatase plus methanol) staining provides the best results. Combinations of black or purple with red also work in some cases. A procedure for fluorescent triple-labeling has been described (Paddock et al., 1993), and I have had some success with a few histochemical triple label combinations.

A large number of procedural combinations are possible for double labeling, but some specific examples will illustrate the basic approaches to double labeling. In each case, embryos should be fixed and devitellinized following the steps in section III A. Antibody incubations and washes are done as described in section IV.

A. FITC detection of mouse monoclonal antibody 22C10 combined with RITC detection of rabbit anti-even-skipped (Fig 6A).

1. Incubate embryos 30 min in 100 μl PT+NGS.
2. Incubate embryos overnight at 4°C in PT+NGS containing a 1:5000 dilution of rabbit anti-even-skipped and a 1:20 dilution of MAb 22C10.
3. Wash 3X 5 min and 4X 30 min with PT.
4. Incubate 30 min in 100 μl PT+NGS.
5. Incubate for 2 hrs at room temperature in PT+NGS containing a 1:300 dilution of goat anti-rabbit IgG conjugated to RITC (Jackson ImmunoResearch Cat. No. 111-025-144; this secondary antibody has been subtracted against human, mouse, and rat sera so that it will not crossreact with immunoglobulins from these species) plus a 1:250 dilution of goat anti-mouse IgG conjugated to FITC (Jackson ImmunoResearch Cat. No. 115-095-146; this secondary antibody has been subtracted against human, bovine, horse, rabbit, and swine sera).
6. Wash 3X 5 min and 4X 30 min with PT.
7. Clear in DABCO solution (see section XIII) as described in section VIII B. Dissect and mount as described in section IX B.

General comments: A fluorescent double-labeling procedure that is especially easy and useful when looking at neural antigens involves the use of goat anti-HRP conjugated directly to RITC. Follow steps 1-7 of section IV. Add the appropriate FITC conjugated secondary antibody but also include goat anti-HRP conjugated to RITC (Jackson ImmunoResearch Cat. No. 123-025-021; see section II A 4) at a dilution of 1:500. Continue with the remaining steps in section IV. The FITC channel will reveal the pattern of the primary antibody while the RITC channel will reveal all neurons and their axons.

B. Alkaline phophatase (purple reaction product) detection of mouse monoclonal antibody 2B8 (anti-even-skipped) combined with HRP (brown reaction product) detection of rabbit anti-paired (Fig. 5J and K).

1. Incubate embryos 30 min in 100 μl PT+NGS.
2. Incubate embryos overnight at 4°C in PT+NGS containing a 1:1000 dilution of rabbit anti-paired and a 1:30 dilution of MAb 2B8.
3. Wash 3X 5 min and 4X 30 min with PT.
4. Incubate 30 min in 100 μl PT+NGS.

5. Incubate for 2 hrs at room temperature in PT+NGS containing a 1:300 dilution of goat anti-mouse IgG-alkaline phosphatase conjugated (Jackson ImmunoResearch Cat. No. 115-055-146; this secondary antibody has been subtracted against human, bovine, horse, rabbit, and swine sera) plus a 1:300 dilution of goat anti-rabbit IgG-HRP conjugated (Jackson ImmunoResearch Cat. No. 111-035-144; this secondary antibody has been subtracted against human, mouse, and rat sera). If non-crossreactive secondary antibodies are unavailable, the sequential procedures of sections C or D below may be used instead.
6. Wash 3X 5 min and 4X 30 min with PT.
7. Proceed with the brown HRP reaction as described in section V A.
8. After the 2X 1 min washes in PT, wash 2X 5 min with A.P. buffer.
9. Proceed with the purple alkaline phosphatase reaction described in section V D. (If desired, steps can be interchanged so that the alkaline phosphatase reaction is carried out before the HRP reaction).
10. Change the purple reaction product to blue using the protocol in section V E. The methanol treatment will not alter the color of the brown DAB reaction product.
11. Clear embryos in glycerol as described in section VIII B. Dissect and mount as in section IX B.

C. Alkaline phosphatase (purple reaction product) detection of mouse MAb 4D9 (anti-engrailed) combined with HRP (brown reaction product) detection of mouse MAb 22C10 (Fig. 6E and F).

1. Incubate embryos 30 min in 100 μl PT+NGS.
2. Incubate embryos overnight at 4°C in PT+NGS containing a 1:3 dilution of MAb 4D9 (100 μl PT+NGS plus 30 μl of MAb 4D9 tissue culture supernatant).
3. Wash 3X 5 min and 4X 30 min with PT.
4. Incubate 30 min in 100 μl PT+NGS
5. Incubate for 2 hrs at room temperature in PT+NGS containing a 1:300 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase.
6. Wash 3X 5 min and 4X 30 min in PT.
7. React the embryos with NBT/BCIP (purple alkaline phosphatase reaction) as described in section V D.
8. After the 2X 1 min washes in PT, wash the embryos 2X 30 min with PT.
9. Incubate embryos in 100 μl of PT+NGS.
10. Incubate overnight at 4°C in PT+NGS containing a 1:10 dilution of MAb 22C10.
11. Wash 3X 5 min and 4X 30 min in PT.
12. Incubate 30 min in 100 μl PT+NGS
13. Incubate for 2 hrs at room temperature in PT+NGS containing a 1:300 dilution of goat anti-mouse IgG conjugated to HRP.
14. Wash 3X 5 min and 4X 30 min in PT.
15. React the embryos with DAB (brown HRP reaction) as described in section V A.
16. Clear in glycerol solution as described in section VIII B. Dissect and mount as described in section IX B.

D. HRP (black reaction product) detection of mouse MAb 2B8 (anti-even-skipped) combined with HRP (brown reaction product) detection of mouse MAb BP102 (Fig. 6H and I).

1. Incubate embryos 30 min in 100 μl PT+NGS.
2. Incubate embryos overnight at 4°C in PT+NGS containing a 1:50 dilution of MAb 2B8.
3. Wash 3X 5 min and 4X 30 min with PT.
4. Incubate 30 min in 100 μl PT+NGS
5. Incubate for 2 hrs at room temperature in PT+NGS containing a 1:300 dilution of goat anti-mouse IgG conjugated to HRP.
6. Wash 3X 5 min and 4X 30 min in PT.
7. React the embryos with DAB+Ni solution (black HRP reaction) as described in section V B.
8. After the 2X 1 min washes in PT, wash the embryos 2X 30 min with PT.
9. Incubate embryos in 100 μl of PT+NGS.
10. Incubate overnight at 4°C in PT+NGS containing a 1:10 dilution of MAb BP102
11. Wash 3X 5 min and 4X 30 min in PT.
12. Incubate 30 min in 100 μl PT+NGS
13. Incubate for 2 hrs at room temperature in PT+NGS containing a 1:300 dilution of goat anti-mouse IgG conjugated to HRP.
14. Wash 3X 5 min and 4X 30 min in PT.
15. React the embryos with DAB (brown HRP reaction) as described in section V., A.
16. Clear in glycerol solution as described in section VIII B. Dissect and mount as described in section IX B.

General comments: The reactions in sections C and D must be done in the order described; the darker (black or purple) reaction must be done before the lighter (brown) reaction. This is because the second histochemical reaction will create some signal over the first reaction. This will not be a problem if the first reaction is much darker than the second (a little brown staining on top of a dark black signal will make no difference). This color overlap can be avoided entirely, however, if the antibodies are eluted after the first histochemical reaction. This can be accomplished by washing the embryos 2 X 5 min in antibody elution buffer (10 mM glycine, pH 2.3; 500 mM NaCl; 0.1% Triton X-100; 0.1% BSA) after the 2 X 1 min washes in PT in step 8 in sections VI C or D. Then wash 2 X 15 min in PT and continue at step 9 in sections VI C or D.

Be certain that there will be no unwanted crossreactions between the primary and secondary antibodies. For example, if a combination of a mouse monoclonal and a rabbit polyclonal primary antibody is used, do not detect them with a combination of rabbit anti-mouse secondary antibody conjugated to FITC and a goat anti-rabbit seconday antibody conjugated to RITC. Also, be certain that the normal serum does not contain immunoglobulins that will be recognized by the secondary antibody being used.

VII. Rapid staining procedure

Following the embryo fixation and staining procedures described in sections III and IV, it will take one to one and one-half days to go from embryo collection to stained embryos. However, with robust antibodies such as MAb BP102, MAb 22C10, and MAb 2B8 (anti-even-skipped), it is possibly to streamline the procedure significantly so that stained embryos are ready only three hours after the embryos are collected. This rapid procedure also uses somewhat simplified solutions. Embryos fixed according to the procedures in section III A and then stored in methanol can enter this rapid procedure at step 6.

1. Collect, dechorionate, and rinse embryos as in steps 1-4 of section III.
2. Place embryos into a scintillation vial containing 10 ml heptane plus 10 ml PBS-FA or PEM-FA. Fix for 10-20 min, agitating the vial 2-3 times during the fixation period.
3. Remove the aqueous layer from the bottom.
4. Add 10 ml methanol. Shake for 10-15 sec.
5. Remove devitellinized embryos from the bottom and wash them 3X 1 min with methanol.
6. Wash 2X 1 min followed by 1X 10 min with PT.
7. Incubate 10 min in 100 μl PT+NGS.
8. Add primary antibody to the appropriate final concentration (MAb BP102 at 1:10; MAb 22C10 at 1:5; MAb 2B8 at 1:30). Primary antibodies should be used at about 1.5 to 2 times the "normal" concentration (i.e., that used for the staining procedure in section IV).
9. Mix and incubate in the primary antibody at room temperature for 30 min.
10. Wash 3X 1 min with PT.
11. Wash 3X 10 min with PT.
12. Add secondary antibody. It is not necessary to pre-block with PT+NGS. For HRP immunohistochemistry, use the goat anti-mouse IgG at a dilution of 1:250. Mix and incubate in the secondary antibody for 30 min at room temperature.
13. Wash 3X 1 min with PT.
14. Wash 3X 10 min with PT.
15. Add 300 μl DAB or DAB+Ni solution.
16. Add 10-30 μl of 0.3% H_2O_2.
17. When ready, stop the reaction by washing 3X 1 min with PT.
18. Wash 1X with 1X PBS.
19. Replace 1X PBS with 70% glycerol solution. After 5 min, mix the embryos gently. Embryos will be cleared sufficiently in about 10-20 min. Proceed section IX.

VIII. Clearing embryos.

After washing in PT, the embryos or larvae may look good under a dissecting microscope, but they will appear relatively opaque under a compound or fluorescence microscope. By "clearing" (equalizing the refractive index of the tissue and surrounding media) the embryos or larvae with methyl salicylate or with glycerol, it is possible to view them at higher magnification. Optically, methyl salicylate clearing produces the best results. However, tissues cleared in methyl salicylate are very brittle and difficult to dissect in a controlled manner. Furthermore, methyl salicylate will solubilize some of the reaction products described in V (see below). Although glycerol clearing does not produce the same level of transparency, the embryos are easy to

handle, and cleared tissue can be dissected and flattened to obtain an improved view of certain internal structures. For embryos stained using DAB or DAB+Ni, it is often useful to clear half of the stained embryos in methyl salicylate and the other half in glycerol. Methyl salicylate should be used with care as it can damage plastic racks as well as cause skin irritation.

A. Methyl salicylate clearing.

Fluorescently (RITC or FITC) and DAB (brown or black reaction) stained embryos can be cleared in methyl salicylate with no loss of signal. Methyl salicylate by itself seems to be efficient at preventing the quenching of both FITC and RITC. Methyl salicylate will entirely or partially extract the other reaction products described in section V.

1. Wash 2 X 1 min with 1XPBS. Make sure that the embryos or larvae do not get stuck to the sides of the tube.
2. Wash 1 X 5 min with 50% ethanol (ethanol series is in dH_2O).
3. Wash 1 X 5 min with 70% ethanol.
4. Wash 1 X 5 min with 90% ethanol.
5. Wash 2 X 5 min with 100% ethanol. Make certain that all traces of water are removed from the tube and cap of the tube.
6. Add 500 µl of methyl salicylate (Sigma Cat. No. M 6752). Do not disturb the tube for the first 1-2 min or the embryos will end up stuck to the walls of the tube. After a 5-10 min, the embryos should settle to the bottom. Fluorescently stained specimens will be so clear that they will only be visible as blue ghosts. Draw off the methyl salicylate and replace with a fresh 500 µl of methyl salicylate.
7. DAB-stained tissue can be stored in sealed tubes containing methyl salicylate for several months at room temperature or indefinitely at 4°C. Proceed to section IX to mount the embryos.

General comments: DAB stained (brown or black reaction) tissue can also be prepared for sectioning. At the end of step 5, imbed the dehydrated embryos or larvae in LR White acrylic resin (medium grade) and section with a diamond knife.

B. Glycerol clearing.

All fluorescently or histochemically stained embryos and larvae can be cleared safely with glycerol.

1. Wash 1 X 1 min with 1X PBS.
2. Add 500 µl of 50% glycerol (v:v with 1XPBS) to the embryos or larvae. To conterstain nuclei so that they fluoresce blue, include DAPI (diaminophenylindole) at a concentration of 1.0 - 0.1 µg/ml in the 50% glycerol solution. Allow the tissue to settle. After about 30 to 60 min, remove the 50% glycerol.
3. For histochemically stained preparations, add 500 µl 70% glycerol (v:v with 1XPBS). Allow the embryos to sit at room temperature for 60 min or overnight at 4°C. The embryos can be stored indefinitely in 70% glycerol in sealed tubes in the dark at either 4°C or –20°C. Proceed to step IX when you are ready to mount the embryos. Even clearer preparations can be produced by placing the embryos in 90% glycerol, but the tissue is more difficult to dissect and mount than when in 70% glycerol.

4. For fluorescently stained preparations, add 500 μl of DABCO solution (anti-fade agent in 70% glycerol). Allow the embryos to sit at room temperature for 60 min or overnight at 4°C. The embryos can be stored at least a week in the DABCO solution at –20°C in the dark. Proceed to step IX when you are ready to mount the embryos.

General comments: If histochemically stained tissues are counterstained with DAPI in step 2 above, the stained cells will appear to be unstained by DAPI when viewed under fluorescence. This quenching of the DAPI signal is especially prominent if the histochemical staining is nuclear. The negative staining effect is quite striking and is very useful when examining expression patterns at the blastoderm stage (Karr and Kornberg, 1989).

IX. Dissecting and mounting stained embryos.

Proper mounting of embryos is essential for seeing internal tissues clearly and for obtaining high quality photographs.

A. Mounting cleared embryos and larvae.

1. Allow the embryos or larvae in methyl salicylate or 70% glycerol (with or without DABCO) to warm to room temperature. Place them into a glass depression slide and examine under a dissecting microscope. Select and transfer anywhere from a single embryo to two dozen embryos in a volume of about 25-30 μl to a clean glass slide. Place two 18X18 mm #1 thickness coverslips on both sides of the drop, about 1 cm apart. These act as support coverslips to keep the embryos from being crushed by the coverslip that will go on top. For larvae, #2 thickness coverslips should be used.
2. Place a single 18X18mm #1 thickness coverslip over the embryos. The glycerol or methyl salicylate should just fill about 80-90% of the space bounded by the slide and three coverslips. Additional methyl salicylate or glycerol can be added from the side, or any excess can be removed by wicking it away with a Kimwipe.
3. While looking at the embryos under the compound or dissecting microscope, you can roll the embryos by gently moving the top coverslip. Excessive rapid rolling will cause the methyl salicylate-cleared embryos to fracture into pieces and the glycerol-cleared embryos to twist up. If a single embryo is mounted, it is possible to photograph all orientations of the embryo by gently rolling it between photographs.
4. It is also possible to slightly flatten an embryo so that more of it is in a single focal plane. First orient the embryo by sliding the top coverslip, then use one hand to hold the top coverslip steady and another hand to pull away one or both of the support coverslips. The top coverslip will now press down on the embryo, flattening it somewhat. The quantity of glycerol or methyl salicylate under the coverslip (and whether one of both support coverslips have been removed) will control the extent to which the specimen is flattened. If there is insufficient glycerol or methyl salicylate, the embryo will crack or become badly distorted. If the specimen is not flat enough, a Kimwipe can be used to wick away excess methyl salicylate or glycerol
5. To save a slide of methyl salicylate-cleared specimens, seal all the edges of the coverslips with Permount. Alternatively, the embryos can be placed in a 1:4 mixture of methyl salicylate: Permount (v:v) after step VI., A., 6. and then

mounted under a coverslip. Slides should be allowed to dry for one or two days. If you want to save the slides of glycerol cleared specimens, completely fill the space underneath the coverslip with glycerol and seal all the edges of the coverslips with fingernail polish. Some batches of fingernail polish contain solvents that will either cause the staining to fade or cause the embryos to become discolored after a few days, so first check your brand of fingernail polish on some less important specimens. Slides of histochemically stained preparations will last at least several years when stored in the dark at room temperature. Slides of fluorescently stained preparations will last at least several days if stored at 4°C in the dark.

B. Dissecting glycerol cleared embryos and larvae.

With a bit of practice, it is possible to dissect glycerol-cleared embryos and larvae before mounting. Larvae that have been stained as whole mounts can be dissected to obtain a better view of the stained tissues. Dissecting germband extended embryos makes it possible to visualize both the future dorsal and ventral ectoderm of the embryo at the same time. Most of the identified neurons sit on the dorsal surface of the CNS, and by dissecting open the embryo, it is possible to look directly down onto the dorsal surface of the nerve cord.

1. For the dissections, you will need a sharpened wire needle made from a 2-3 cm long piece of tungsten wire (0.13 mm diam.; Ted Pella Inc. Cat No. 27-11). Put a kink near one end of the wire and insert this end into the beveled opening of a 26 G syringe needle. The kink will create enough friction so that the base of the wire will remain securely in place within the syringe needle. The wire can then be sharpened electrically using a 1M solution of NaOH and a direct current power supply (I use one that supplies 6.3 amps at an adjustable voltage between 2-6 V). Wear face and hand protection to avoid being splashed by droplets of NaOH that might bubble up from the solution. Place one electrode in a small beaker of 1M NaOH and clip the other onto the syringe needle. Dipping the needle in and out of the solution will cause the needle to taper to a point. Start at a setting of 4-5 volts to taper the end of the wire to a blunt point, and then switch to 2-3 volts to put a sharp point just at the end of the needle. Avoid creating a long, thin end as this will be bent too easily when you use it to dissect embryos.
2. Transfer embryos to a depression well slide and pick out an embryo to dissect. Transfer a single embryo in 2-4 μl of 70% glycerol to a microscope slide. Use the wire needle to move the embryo out of the drop of glycerol. The surface tension of the glycerol still coating the embryo will hold the embryo down to the slide and allow you to manipulate it without it rolling around uncontrollably. The glycerol also makes it possible to dissect the embryo slowly without it drying out. For mid-stage 12 to late stage 15 embryos, use the wire tool to roll the embryo onto its ventral side. Make slits along the amnioserosa, at the base of the head, and near the posterior end of the embryo. Fold down the body walls and brain region. Lift out the gut and remove it entirely or slide it next to the embryo. For stage 8 to early stage 12 embryos, roll the embryo on its side, make a slit along the amnioserosa, and the use the wire tool to "unfold" the embryo. Scrape out the mesoderm if you are going to be looking at ectodermal or neural

patterns. Turn the embryo so that the ectoderm faces up (away from the slide) to get the best view of ectodermal or early neuroblast patterns.

3. Place a 10-15 μl drop of 70% glycerol on a coverslip. Invert the coverslip and hold it directly over the slide so that the drop is above the dissected embryo. Drop the coverslip onto the slide. The drop should spread out and fill the area under the coverslip. If the dissected embryo flattens too much, use a larger volume of glycerol in the drop on the coverslip next time. Alternatively, place support coverslips (#1 or #0 thickness) to both sides of the dissected embryo, place a coverslip on top, introduce glycerol from the side, and then remove one or both supports to somewhat flatten the preparation (this support technique is advisable when looking at neuroblast patterns). With some practice, it should be possible to dissect multiple embryos on a single slide. Never let the coverslip move sideways once it has been placed on the slide – this will destroy the dissected embryos. To flatten the dissected embryos a bit more, wick away excess glycerol from the side of the coverslip with a Kimwipe. To seal the slide, first place a small drop of fingernail polish at each corner of the coverslip. Wait for these drops to dry (about 5 min), and then use the fingernail polish to seal all the edges of the coverslip.

X. Hints for photographing histochemically stained specimens.

A. Optics

1. Brightfield optics will show the highest contrast between staining and background, but little detail will be visible in the unstained regions. Nomarski (DIC) optics will allow unstained tissue to be visualized and stained regions will appear sharper than in brightfield, but the contrast between stained and unstained regions will be lessened. By adjusting the DIC slider, it should be possible to find a good optical balance that gives both good contrast and visualization of unstained structures.
2. Magnification in the range of 200-250X will allow the entire field to be filled by a whole embryo. I generally use 20X and 40X dry lenses and a 100X oil immersion lens for photography. Oil immersion 40X and 63X lenses are also useful in many instances.
3. Closing the aperture diaphragm of the condenser will help eliminate excessive "glow" in the center of glycerol cleared embryos, but if it is closed too much, unstained tissues will take on a grainy appearance.
4. Before taking a photograph, make sure that you have Köhler illumination. Keep the luminous field diaphragm closed as far as possible (to the edge of the photographic field) to minimize light scattering.

B. Color balance

1. Most microscopes come with a dark blue filter that is designed to convert tungsten illumination to a daylight color balance (3200K to 5500K conversion filter). Even more useful than this filter, however, are light and medium blue filters (CB3 and CB6; Zeiss Cat. No. 467851 and 467852). They can be placed directly on top of the luminous field diaphragm and do not necessarily need to be installed into the filter magazine.

2. Brightfield photographs will usually require the dark blue filter so that the background does not come out too yellowish. If this background is too blue, the CB6 or CB3 filters should be used instead.
3. Specimens with brown or red reaction products viewed with DIC optics look best when photographed with a medium blue background. Either the CB3 or CB6 filter should be used for this.
4. Black, purple, and blue reaction products look best when photographed on a white or even slightly grey background. Use the CB3 filter or no filter at all.

C. We now do all our photography using either a Kontron 3012 digital camera or a Spot camera attached to a Zeiss Axiophot microscope.

XI. Hints for looking at mutants that effect central nervous system development.

A. Identifying genotype of embryos.

When looking at the embryos collected from a balanced lethal stock, only one-forth of the embryos will be homozygous for the mutation being studied. To unambiguosly identify the mutant embryos, balancer chromosomes carrying lacZ constructs can be used. For example, mutants in gooseberry (gsb) can be balanced with a second chromosome balancer containing a lacZ gene under the control of the fushi tarazu promoter (CyO-ftz lacZ). When embryos from this stock are collected, they can be stained for both betagalactosidase (see section II D) and an antibody of interest (MAb 2B8 for example) using the double labeling protocols of section VI. Three fourths of the stage 8-12 embryos will show the ftz-betagal pattern (large subset of the CNS) and the MAb 2B8 pattern, and one-fourth of the embryos, those homozygous for the gsb mutation, will only show the MAb 2B8 pattern. In this way, the MAb 2B8 pattern of gsb mutants can be studied. A wide variety of lacZ markers are available for the commonly used balancer chromosomes. Remember to pick a balancer whose betagalactosidase pattern will be clearly visible for the stages of embryogenesis that are under examination.

If the mutation being studied is a protein null allele, the genotype can also be determined by staining with an antibody to the protein product. For example, if the embryos for the gsb stock described above are double-labeled with an antibody of interest plus an antibody against gsb, the gsb mutants will fail to show any staining with the gsb antibody.

B. Examples from known mutants.

1. zipper mutant. The zipper gene encode the heavy chain of non-muscle myosin (Young et al., 1993). This product is supplied maternally, but as the maternal contribution is exhausted, several morphological defects become visible in a variety of tissues. In the nervous system, axon outgrowth is altered and commisures are "fused" together (Fig. 3D).
2. cyclin A mutant. Cyclin A is provided maternally, but as this maternal product runs out, cell division is disrupted (Lehner et al., 1989). In the nervous system, this causes several changes in the normal lineage patterns. For example, the ganglion mother cell that forms aCC and pCC neurons fails to undergo its division to produce these two neurons (Fig. 3H).
3. gooseberry mutant. The gooseberry gene (gsb-d) encodes a transcription factor containing both a homeodomain and a paired domain (Baumgartner et

al., 1987). Mutations at this locus cause changes in neural differentiation. For example, RP2 neurons become duplicated and changes in cell differentiation also lead to alterations in the pattern of axonogenesis (Patel et al., 1989b; see Fig. 6I).

4. General comments; two notes of caution. First, when analyzing mutants with neural markers, be suspicious of axonal defects which only appear in the third to fifth abdominal segments. These defects are often related to pleitropic defects that cause the process of germband extension to be delayed. Second, use a variety of non-neural and segmentation markers to examine the development of other organs to be certain that neural defects are not secondary to defects in adjacent tissues.

XII. Trouble-shooting

A. Low percentage of devitellinized embryos.

1. If there are too many embryos in the vial, a smaller percentage will be devitellinized. Optimally, the embryos should form no more than a monolayer at the interface between the heptane and fixative. When fixing large quantities of wild-type embryos in a large container (5 gm of embryos in 250 ml heptane plus 250 ml PEM-FA), only about half the embryos will become devitellinized, but this is usually satisfactory since enough embryos for hundreds of staining experiments will still be obtained.
2. Devitellinization efficiency can be improved by a rapid temperature change. After step 7 of section III A, remove most of the remaining heptane (do not let the embryos dry out). Add 10 ml of heptane that has been chilled to –20 to –50°C. Then quickly add 10 ml of room temperature methanol and shake the vial vigorously while holding it under a warm tap water stream. Although this procedure is not necessary when processing Drosophila embryos, it is very useful for devitellinizing many other insect embryos (such as Tribolium embryos).
3. If the methanol has absorbed significant quantities of moisture from the air, it will be less efficient at devitellinizing embryos. Keep methanol containers tightly closed.
4. If necessary, the non-devitellinized embryos from the interface can be salvaged to some extent. At step 9 of section III A, remove the non-devitellinized embryos from the interface to a new vial containing 2-3 ml of methanol. Remove any heptane that was transfered with the embryos (it will be floating on top of the methanol). Fill the vial with methanol and shake gently. The embryos should now sink in the methanol. Wash 3X 5 min with methanol and then proceed to step 11 of section III A. Continue staining as if the embryos were devitellinized. Anywhere from 10-50% of these non-devitellinized embryos will be stained, because although the vitelline membrane has not been removed, sufficiently large holes are present to allow antibodies to enter. These non-devitellinized embryos will not, however, sink into glycerol. The remaining vitelline membranes can be dissected away while the embryos are in 50% glycerol and the embryos can then be transfered to 70% glycerol.

B. Poor signal and/or high backround

1. Overfixation often results in low signal and underfixation often results in high background. For most of the antibodies mentioned in these protocols, fixation times of 10-20 minutes produce good results. Some antigens, however, require harsher fixation. For example, the neurotransmitters serotonin and FMRFamide are small molecules that require longer periods of fixation or harsher fixatives such as Bouin's fixative (see Schneider et al. (1993) for a discussion of fixation parameters for FMRFamide staining).
2. Methanol also acts as a fixative. In fact, several antibodies, such as MAb 2D5, will produce acceptable results even if no formaldehyde is used in section III A. Methanol, however, may extract and/or denature some antigens. If staining is methanol sensitive, embryos will have to be devitellinized by hand (see section III E).
3. Adjust concentration of the primary antibody. It is important to try a range of concentrations to see what concentrations work best for any given primary antibody. On occasion, even two- to three-fold changes in antibody concentration produce dramatic changes in signal to background ratios. For antisera and ascites fluid, usable dilutions may range anywhere from 1:200 to 1:50,000. Monoclonal antibodies in the form of tissue culture supernatants may work well anywhere in the range of dilutions from 1:1 to 1:200. Oddly enough, sometimes increasing antibody concentrations will actually result in a decrease in signal (without any apparent increase in background), especially ith histohemical detection. Thus, it is really critical to test over a wide range of dilutions.
4. Preabsorp the primary antibodies. Crude antisera, especially from rabbits, often contain random immunoglobulins that bind to various epitopes in Drosophila embryos or larvae, leading to high background. This can often be eliminated by preabsobing the antiserum with fixed Drosophila embryos. First, dilute the antiserum 1:50 with PT+NGS. In a 500 μl microfuge tube, combine fixed embryos (i.e., processed as far as the end of step 11 in section III A) with the diluted antiserum using approximately 50 μl of packed embryos per 200 μl of diluted antiserum. Mix gently at 4°C overnight. Recover the supernatant, centrifuge to remove any remaining particulate material, and store the preabsorbed antisera at 4°C. Sodium azide can be added to a final concentration of 0.02% to inhibit bacterial growth.
5. Adjust concentration of the secondary antibody. It is useful to try a range of concentrations to see what concentrations work best for any given primary antibody and detection system. If the secondary antibodies are affinity purified, preabsorption with fixed embryos makes very little difference. For example, embryos that are not incubated with a primary antibody but are incubated for 2 hrs with a 1:300 dilution of goat-anti mouse IgG conjugated to HRP (Jackson ImmunoResearch Cat. No. 115-035-003) and then washed, will not show any color change after 10 min of reaction with DAB+Ni containing 0.01% H_2O_2. If preabsorption is needed, follow the procedure for preabsorbing primary antibodies, but do not add sodium azide to HRP conjugated antibodies (see XII B 11).
6. Prepare fresh substrate solutions. The various substrates used for histochemical detection will degrade with time.
7. Adjust histochemical reaction times. Staining will often appear suitable when viewed with a dissecting microscope but will seem weak when observed with

the intense transillumination of a compound microscope. If this occurs, allow histochemical reactions to proceed longer to intensify the staining reaction. If histochemical reactions proceed too rapidly, high background may result. In this case, either reduce the concentrations of the antibodies or reduce the concentration of the reaction substrates.

8. Replace directly conjugated secondary antibodies with biotin-conjugated secondary antibodies and follow with HRP, FITC, RITC, or alkaline phosphatase conjugated streptavidin. Biotinylated secondary antibodies and streptavidin conjugates are available from a variety of sources. A system using streptavidin-biotin complexes is available from Vector Labs (VectaStain Elite Kit). Compared to directly conjugated secondary antibodies, these avidin/biotin reagents may provide increased sensitivity in situations where the concentration of primary antibody is limiting. In my own experience, the vast majority of antibody staining of whole mount preparations is not improved significantly by switching to biotin-streptavidin complex detection systems.
9. Increase the stringency of the washes. In steps 6 and 11 of section IV, replace the first of the 30 min washes in PT with a 30 min wash in PT in which the NaCl concentration has been increased to 500 mM. This increased salt concentration (from 175 mM to 500 mM) will help elute potential low affinity binding to cross-reactive epitopes. Increasing the length of the washes beyond 4X 30 min will generally not make much difference because these wash times are probably already excessive.
10. Endogenous HRP activity can be destroyed by incubating tissue for 30 min in 70% methanol containing 0.5% H_2O_2. This potential background source is rarely a problem in embryos, but it does occur in some larval tissues.
11. Make certain that HRP conjugated reagants are not mixed with solutions containing sodium azide. Sodium azide will reduce or completely destroy the activity of the HRP enzyme. Sodium azide in primary antibody solutions will not be a problem because it will be washed away from the tissue before the addition of HRP-coupled secondary antibodies.
12. Some antigens are removed from fixed tissue by prolonged exposure to Triton X-100. The replacement of the Triton X-100 in PT with 0.02% saponin will improve staining intensity in such cases.
13. Adjust antibody incubation times. Increasing or decreasing incubation times in primary and secondary antibodies may increase signal or reduce background.

XIII. Solutions

10X PBS

18.6 mM	NaH_2PO_4	(2.56 g $NaH_2PO_4 \cdot H_2O$ per 1000 ml dH_2O)
84.1 mM	Na_2HPO_4	(11.94 g Na_2HPO_4 per 1000 ml dH_2O)
1750.0 mM	NaCl	(102.2 g NaCl per 1000 ml dH_2O)

Adjust pH to 7.4 with NaOH or HCl. Prepare 1X PBS by diluting 1:10 with dH_20. Both 1X and 10X PBS can be kept indefinitely at room temp.

PT

1X	PBS
0.1%	Triton X-100

Mix 100 ml 10X PBS, 899 ml dH_2O, and 1 ml Triton X-100. Store at 4°C or at room temp.

Some protocols us PBT in place of PT in all antibody washes and incubations. The addition of BSA is supposed to reduce background further, but in my hnd I hve found it unecessary, and in a few cases it even leads to a slight loss of signal.

PBT

1X	PBS
0.1%	Triton X-100
0.1%	BSA (Sigma Cat. No. A-7906)

Mix 100 ml 10X PBS, 800 ml dH_2O, 1 ml Triton X-100, and 1 g BSA. Adjust volume to 1000 ml. Store at 4°C. Solution will usually last at least one month. Discard if bacterial growth is detected (solution will start to turn cloudy).

PT+NGS

1X	PBS
0.1%	Triton X-100
0.1%	BSA (Sigma Cat. No. A-7906)
5.0%	Normal Goat Serum (Gibco-BRL Cat. No. 200-6210AG)

Heat inactivate the serum at 56°C for 30 min. Filter through a 0.22 μm filter while still warm. Aliquot into sterile tubes. Store aliquots at –20°C. Once thawed, aliquots are stable for several months at 4°C. To prepare the PT+NGS solution, mix 4.75 ml PT with 0.25 ml Normal Goat Serum and store at 4°C. Solution will usually last at least two or three weeks. Discard if bacterial growth is detected (solution will start to turn cloudy).

PEM (pH 7.0)

100.0mM	PIPES (Disodium salt, Sigma Cat. No. P-3768)
2.0mM	EGTA
1.0mM	$MgSO_4$

Weigh out the solid PIPES, EGTA, and $MgSO_4$ into a beaker, add the appropriate volume of dH_2O, mix for 20 min., and then adjust the pH to 7.0 with concentrated HCl. The free acid form of PIPES is more difficult to get into solution and the pH will need to be adjusted with NaOH instead of HCl. PEM can be stored for at least one year at 4°C.

PEM-FA

9.0 ml	PEM
1.0 ml	37% formaldehyde (Fisher Cat. No. F79-500; this 37% stock solution can be stored at room temperature for at least one year)

This PEM-FA solution should be made just before use. For the immunohistochemical and immunofluorescence whole-mount procedures outlined in this chapter, it is unnecessary to start with solid paraformaldehyde. The 37% formaldehyde solution is stabilized with 10-15% methanol, however, so when using the protocols in section III D or E to detect antigens sensitive to methanol, the fixative should be prepared from solid paraformaldehyde.

PBS-FA

Same as PEM-FA above, except use 9.0 ml 1X PBS instead of 9.0 ml of PEM.

DAB solution

1X	PBS
0.05%	Tween-20 (Sigma Cat. No. P-1379)
0.3 mg/ml	DAB (3,3'-diaminobenzidine; Sigma Cat. No. D-5905)

The 10 mg DAB tablets sold by Sigma are very convenient and help minimize the risk of exposure. Note that DAB is a potential carcinogen and should be handled and disposed of in accordance with University regulations. Add one 10-mg DAB tablet to a 50 ml tube containing 33.0 ml PBS and 16.5 μl Tween-20. Rock gently in the dark for about 30 min. Filter through a 0.22 μm filter to remove particulate matter. Store aliquots at –70°C or in a non-defrosting –20°C freezer. Aliquots should be used immediately after thawing.

DAB+Ni solution

Prepare an 8% solution of nickel chloride ($NiCl_2 \bullet 6H_2O$; Fisher Cat. No. N54-500) in dH_20. This 8% solution can be stored indefinitely at room temperature. Prepare the DAB+Ni solution by combining 1 ml of the 0.3 mg/ml DAB solution described above with 8 µl of 8% nickel chloride. Mix well and use immediately. It is not advisable to store DAB containing nickel chloride because the nickel will precipitate out of solution (as nickel phosphate) after a few hours.

AEC Buffer

23.75 mls H_2O + 400 µl 3M NaAcetate pH 5.2 + 50 µl 20% Tween-20

AEC Reaction Mix

475 µl AEC Buffer + 25 µl AEC in DMF (add AEC/DMF slowly to buffer while mixing).
To prepare AEX in DMF, dissolve 20 mg AEC in 2.5 ml of DMF (dimethylformamide), aliquot and store out of light at room temperature.

A.P. (alkaline phosphatase) buffer

5.0 mM	$MgCl_2$
100.0 mM	NaCl
100.0 mM	Tris, pH 9.5
0.1%	Tween-20 (Sigma Cat. No. P-1379)

Prepare just prior to use. The solution will become cloudy after a few hours and then will not work as well for the enzymatic reaction.

BCIP/NBT solution

1.0 ml	A.P. Buffer
4.5 µl	NBT (50 mg/ml in 70% DMF)
3.5 µl	BCIP (50 mg/ml in 70% DMF)

Mix just before use. The NBT and BCIP solutions can be purchased together from Promega (Cat. No. S3771).

Glycerol solutions

Some batches of glycerol contain contaminants that cause nickel-enhanced DAB reactions to fade within a day or two. To avoid this, use ultrapure glycerol (Boehringer Mannheim, Cat. No. 100 647). Prepare 50%, 70%, and 90% glycerol solutions by mixing the appropriate volumes of glycerol with 1X PBS. Use pH paper to make certain that the pH of the glycerol solutions is around 7.4. Low pH will cause rapid fading of DAB reaction products. Glycerol solutions can be stored at room temperature. Glycerol solutions containing DAPI should be stored in the dark at 4°C.

DABCO solution

70.0% glycerol containing 2.5% DABCO (Sigma Cat. No. D-2522)

Quenching of fluorescently stained preparations can be minimized by the addition of free radical scavengers such as DABCO (1,4-diazobicyclo-[2.2.2]-octane). DABCO is more stable and soluble than other commonly used anti-fade reagents. Prepare this solution by dissolving 1.25 gms of DABCO in 15 ml 1X PBS. Add 35 ml of ultrapure glycerol and mix gently for 1-2 hrs. Store at –20°C.

XIV. Suppliers

Middlesex Sciences
100 Foxborough Blvd. Suite 220
Foxborough, MA 02035

Jackson ImmunoResearch Laboratories
P.O. Box 9
872 W. Baltimore Pike
West Grove, PA 19390-0014

Kirkegaard and Perry Laboratories, Inc.
2 Cessna Court
Gaithersburg, MD 20879

Gibco/BRL
P.O. Box 68
Grand Island, NY 14072-0068

Tetko Inc.
333 South Highland Blvd.
Briarcliff Manor, NY 10510

Ted Pella, Inc.
P.O. Box 492477
Redding, CA 96049-2477

Source Digital Systems
1420 Springhill Rd.
McLean, VA 22102

Fisher Scientific
50 Fadem Rd.
Springfield, NJ 07081

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FIGURE LEGENDS

Figure 1. (A) Anti-vasa monoclonal antibody staining of stage 7 embryo (using protocols described in sections IIIA, IV, VB, VIIIB, and IXA). Pole cells (arrow) are moving dorsally with the posterior midgut invagination. (B) MAb 2D5 (anti-fasciclin III) staining of stage 12 embryo (protocols IIIA, IV, VA, VIIIA, and IXA). Arrow points to prominent staining of the visceral mesoderm. Segmentally repeated staining is in the neuroepithelium and in the developing nervous system. (C) MAb 2A12 staining of developing trachea in a stage 15 embryo (protocols IIIA, IV, VA, VIIIA, and IXA). The trachea entering the nervous system (arrow) are visible in this ventral view. (D-I) Embryos stained with MAb44C11 (anti-elav; using protocols IIIA, IV, VB, VIIIB, and IXA). D-F are lateral views and G-H are corresponding ventral views at stage 10 (D and G), stage 13 (E and H), and stage 15 (F and I). All neurons are stained as they differentiate. Some of the isolated neurons of the peripheral nervous system are indicated by the arrows.

Figure 2. (A) Anti-achaete MAb staining of stage 9 embryo (protocols IIIA, IV, VB, VIIIB, and IXB). At this time, expression is in 4 neuroblasts per hemisegment; the column l and m neuroblasts of rows b and d). The arrowhead indicates the l column neuroblast of row d and the arrow indicates the m column neuroblast of row d (also called NB 7-1 and 7-4 respectively by Doe, 1992). (B) MAb BP104 staining at stage 11 (protocols IIIA, IV, VA, VIIIA, and IXA). (C) MAb BP104 of the peripheral nervous system at stage 15 (protocols IIIA, IV, VA, VIIIB, and IXB). (D) Anti-fasciclin II MAb staining of central nervous system at mid stage 12 (protocols IIIA, IV, VB, VIIIB, and IXB). At this stage, fasciclin II is highly expressed by the cell bodies of aCC (arrowhead) and pCC (arrow) and the growth cone of pCC (triangle). Expression is also beginning on vMP2/MP1 (not visible in this focal plane). (E) MAb 4D9 (anti-engrailed) staining of stage 11 embryo (protocols VII, VIIIB, and IXA). The embryo is slightly flattened so that more of each stripe is in a single focal plane. Arrow points to the stripe of the mandibular segment, arrowhead points to the stripe of the first abdominal segment. (F) MAb 4D9 (anti-engrailed) staining of stage 9 embryo (protocols IIIA, IV, VB, VIIIB, and IXB). The germband has been "unfolded", the hindgut removed, and the head folded out and flattened. The three arrowheads indicate the three head segments that express engrailed at this stage (pre-antennal, antennal, and intercalary segments; expression in the clypeolabrum will start after this stage). Arrow and arrowhead indicates the mandibular and first abdominal stripe respectively. (G and H) MAb 4D9 (anti-engrailed) staining of a subset of CNS neurons at stage 15 (protocols IIIA, IV, VB, VIIIB, and IXB). At this stage, prominent medial and lateral clusters of engrailed expressing neurons are located slightly ventral to the dorsal surface of the CNS (G). At a slightly more ventral focal plane (H), engrailed-positive progeny of the median neuroblast (arrow) and some of the engrailed-positive neurons arising from engrailed-negative neuroblasts (arrowhead) are visible. (I) MAb 2D5 (anti-fasciclin III) staining of the CNS at stage 14 (protocols IIIA, IV, VA, VIIIA, and IXB). Fasciclin III is expressed on three bundles of the anterior commissure (arrows) and two pathways in the posterior commissure (arrowheads).

Figure 3. (A) MAb BP102 staining at early stage 13 shows that the commissures have just begun to seperate from one another (protocols IIIA, IV, VA, VIIIB, and IXA) (B) Same embryo as in A, but now dissected and photographed at higher magnification.

(C) MAb BP102 staining at stage 14 (protocols VII, VIIIB, and IXA). (D) MAb BP102 staining of a zipper mutant (protocols VII, VIIIB, and IXA). The head has failed to involute, leaving the brain in an abnormal anterior position (arrow) and the commissures show some fusion. (E) MAb 3C10 (anti-even-skipped) staining of stage 15 embryo (protocols IIIA, IV, VB, VIIIB, and IXB). This embryo has been dissected flat so that the entire CNS and body wall are visible in a single focal plane. The brain has been unfolded so that it lies flt and the arrowhead points to one of the pairs of even-skipped expressing neurons in the brain. Arrow indicates the anal pad and the triangle points to the dorsal mesoderm expression of even-skipped. (F and G) Higher magnification views of the CNS of the embryo shown in E. (F) Ventral focal plane showing even-skipped expression in the EL neurons (arrow), the CQ neurons (triangle) and the U neurons (arrowhead). (G) Dorsal focal plane with aCC (arrow), pCC (triangle), and RP2 (arrowhead) neurons. (H) Dorsal focal plane of the CNS of an MAb 3C10 (anti-even-skipped) stained cyclin A mutant (compare to G). Instead of three even-skipped expressing neurons per hemisegment, the mutant has only two eve-expressing neurons per hemisegment on the dorsal surface of the CNS. This is because the ganglion mother cell that produces the siblings aCC and pCC fails to complete its division (this same failure to divide may be happening for the RP2 ganglion mother cell, but there is no data on the fate of the sibling of RP2).

Figure 4 (A) MAb 22C10 staining of a newly hatched larva (protocols IIIC, IV, VB, VIIIB, IXA) Ventral view. Arrow points to the condensed CNS. (B) MAb 22C10 staining of a 21 hr embryo (protocols IIIB, IV, VB, VIIIB, IXA). Arrowhead indicates the forming denticles of the ventral cuticle. Arrow points to some of the sensory neuron projections into the cuticle. (C) Third instar CNS stained with MAb 4D9 (anti-engrailed; protocols IIID, IV, VA, VIIIA, IXB). During larval development, a prominent increase in the number of engrailed expressing neurons is seen in the median neuroblast lineages of the three thoracic segments and the first abodominal segment (arrow points to T2). There are also three prominent columns of engrailed expressing cells in each brain lobe (arrowhead indicates the middle column of one lobe). (D) Imaginal disc stained with MAb 4D9 (anti-engrailed; protocols IIID, IV, VA, VIIIA, IXB). Staining is clearly seen in the posterior compartment of the disc.

Figure 5. (A-F) Stage 14 embryos stained with MAb BP102 (protocols IIIA, IV, VIIIB, IXB; each embryo reacted differently at step V). (A) Brown HRP reaction of section VA. (B) Black HRP reaction of section VB. (C) Orange HRP reaction of section VC. (D) Purple alkaline phosphatase reaction of section VD. (E) Blue alkaline phosphatase reaction of section VE. (F) Red Alkaline phosphatase reaction of section VF. (G-I) Stage 5 embryo double labeled with rabbit anti-Krüppel (RITC) and MAb 2B8 (anti-even-skipped; FITC) using the procedure described in VIA. (G) anti-Krüppel staining. (H) MAb 2B8 staining. Arrowhead indicates eve stripe 2. (I) Overlap between the two patterns. (J and K) Stage 5 embryo stained with MAb 2B8 (anti-even-skipped; purple) and rabbit anti-paired (brown) as described in section VIB. (L) Another stage 5 embryos stained with the same combination of primary antibodies as in J and K, but now detected fluorescently (even-skipped in green and paired in red).

Figure 6. (A) CNS of stage 13 embryo stained with MAb 22C10 (green) and rabbit anti-even-skipped (red) as described in section VIA. U1 neuron (small arrowhead): aCC (small traingle); pCC (arrow); SP1 (large arrowhead); RP2 (large triangle). (B) CNS of a

stage 15 embryos stained with MAb 4D9 (anti-engrailed; purple alkaline phosphatase detection) and BP102 (red HRP detection). (C) Anti-fasciclin II MAb (Black HRP reaction) and MAb BP102 (Brown HRP reaction) staining of a stage 16 CNS using the basic procedure of section VID. At this stage, two prominent fasciclin II expressing bundles are present near the dorsal surface of the bilaterally symmetric longitudinal connectives. Additional fasciclin II expressing bundles are located at more ventral focal planes. (D) MAb 4D9 (anti-engrailed; brown HRP reaction) and MAb 2B8 (anti-even-skipped; black HRP reaction) staining of a stage 15 CNS using the basic procedure of section VID. (E and F) MAb 22C10 (brown HRP reaction) and MAb 4D9 (anti-engrailed; purple alkaline phosphatase reaction) staining of the peripheral nervous system of a stage 14 embryo as described in section VIC. Small arrow points to the engrailed positve neuron in the dorsal PNS cluster. Arrowhead points to the chordotonal cells of the lateral cluster which express engrailed. Large arrow indicates the engrailed-positive neuron of the dorsal cluster that is located more internally within the embryo. Stripes of engrailed-positive cells are in the ectoderm. (G) Anti-betagalacosidase staining (black HRP reaction) of an embryo carrying an P-lacZ enhancer trap in a gene expressed in longitudinal glia (*repo*; Xiong et al., 1994) combined with MAb BP102 staining (brown HRP reaction). The numerous longitudinal glia lie on the dorsal surface of the axonal connectives. (H) Stage 14 embryo CNS stained with MAb 2B8 (anti-even-skipped) and BP102 (brown HRP reaction) as described in section VID. Arrowhead points to aCC, large arrow to RP2, small arrow to pCC. (I) gooseberry mutant embryo stained with the same combination of antibodies as in H. Posterior commissure of each segment is reduced or eliminated. RP2 neurons (arrows) are duplicated.

Localization of Horseradish Peroxidase with 3,3',4,4' Diaminobenzidine

by W.H. Starkweather

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Introduction

When viewed under high magnification, isolated cells and thin tissue slices are virtually transparent, and cellular detail is invisible. To visualize cellular structures, the microscopist employs selective stains that enhance molecular details. For example, these stains may react with complex carbohydrates in cell walls or dissolve in lipid bilayers. Specific stains insert into DNA. Other stains adhere to highly charged polyanions or polycations to delineate the observed ultrastructure.

Some enzymes will deposit an indelible stain when supplied with an appropriate substrate. Cells containing one of these enzymes will become selectively stained when exposed to the corresponding substrate. Examples of signalling enzymes include: peroxidases, catalases, oxidases, phosphatases and esterases.

If an antibody that recognizes a cell-specific marker is coupled with one of the signalling enzymes noted above, the antibody will guide the enzyme to the antigens on the target cell. If the cell is subsequently reacted with a stain producing substrate, the cell becomes selectively stained. Thus, enzyme-antibody conjugates are considered universal immunostaining systems where specificity is defined by available antibodies.

Principles

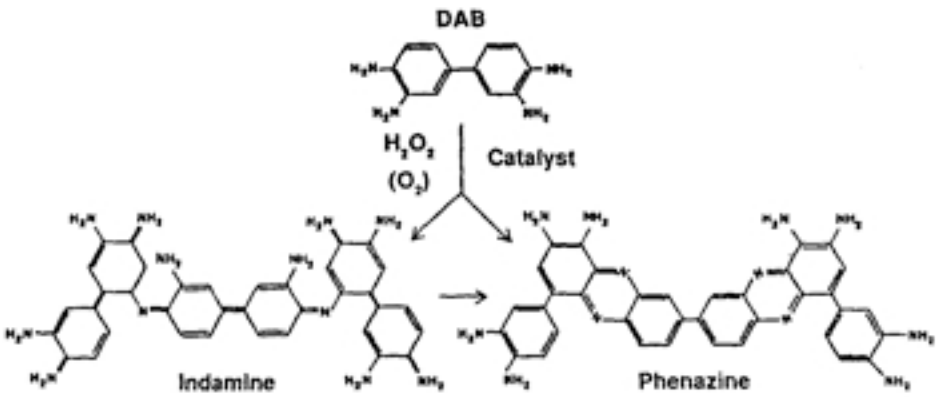
Peroxidase labeled antibodies, together with the substrate 3,3'4,4'diaminobenzidine (DAB), constitute a widely used immunostaining system. Thoughtfully devised immunoenzyme protocols using DAB allow a broad spectrum of color combinations which are much more diverse than procurable fluorochromes. As a result, the histologist has practical alternatives to fluorescent antibody procedures that suffer from short-lived signals.

Ideally the immunostaining system is applied to tissues and cells that are treated using conventional procedures. The histologist can then visualize marked cells in the context of a familiar landscape. The effectiveness of the immunostaining system depends upon the specificity of the antibody conjugate, the chemistry of DAB and the background biochemistry presented by the tissue.

Colorless DAB becomes brown when oxidized by peroxidase or other

DAB Reactions

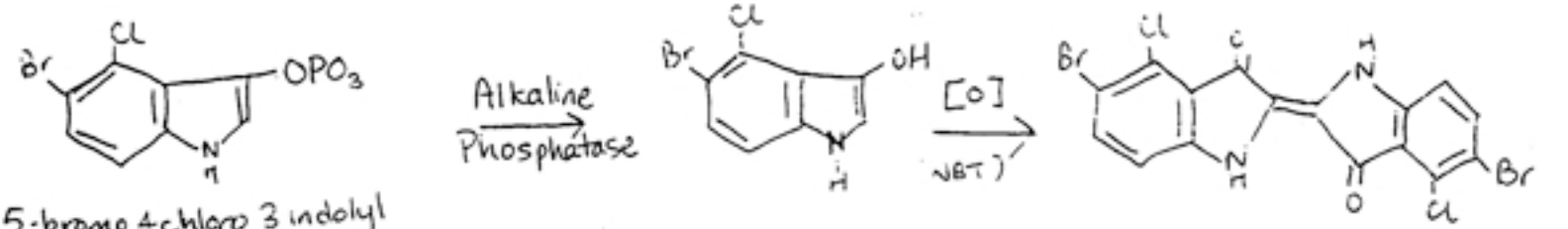
The application of DAB for ultrastructural localization dates back to the work of Graham and Karnovsky[1]. The oxidation of DAB is a 4-electron process that involves free radical intermediates[2] which subsequently polymerize to indamine or phenazine polymers[3].



The oxidation of DAB occurs with the conversion of oxygen (O_2) or hydrogen peroxide (H_2O_2) to water. Without enhancement DAB oxidation proceeds slowly. Metallic or enzymatic catalysts amplify the reaction rate. Hemoglobin, myoglobin, cytochrome c, catalase and myeloperoxidase are examples of these catalysts.

Alkaline Phosphatase / X Phosphate / NBT Reaction

Methods of Enzymology: 121 p. 851 / 132 p. 113



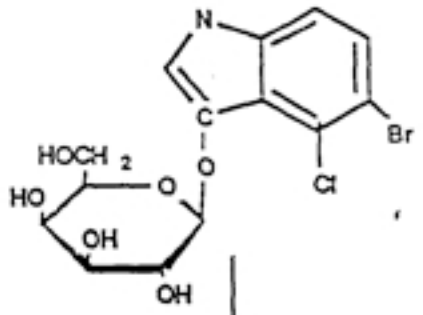
5-bromo 4-chloro 3 indolyl phosphate

5,5' dibromo-4,4' dichloro indigo – blue insoluble precipitate

Nitroblue tetrazolium:

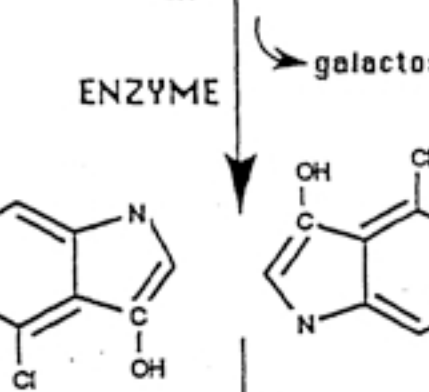
NBT - soluble yellow redox dye, which in presence of superoxide anions, is reduced to yield formazan, an insoluble purple compound (oxidation catalyst)

(2 molecules)



X-GAL

5-Bromo, 4-chloro-
indole-ß-D-galactoside

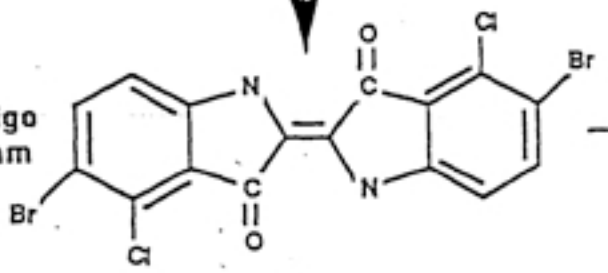


galactose

ENZYME

BromoChloro-indoxl

NONENZYMATIC
DIMERIZATION
AND OXIDATION

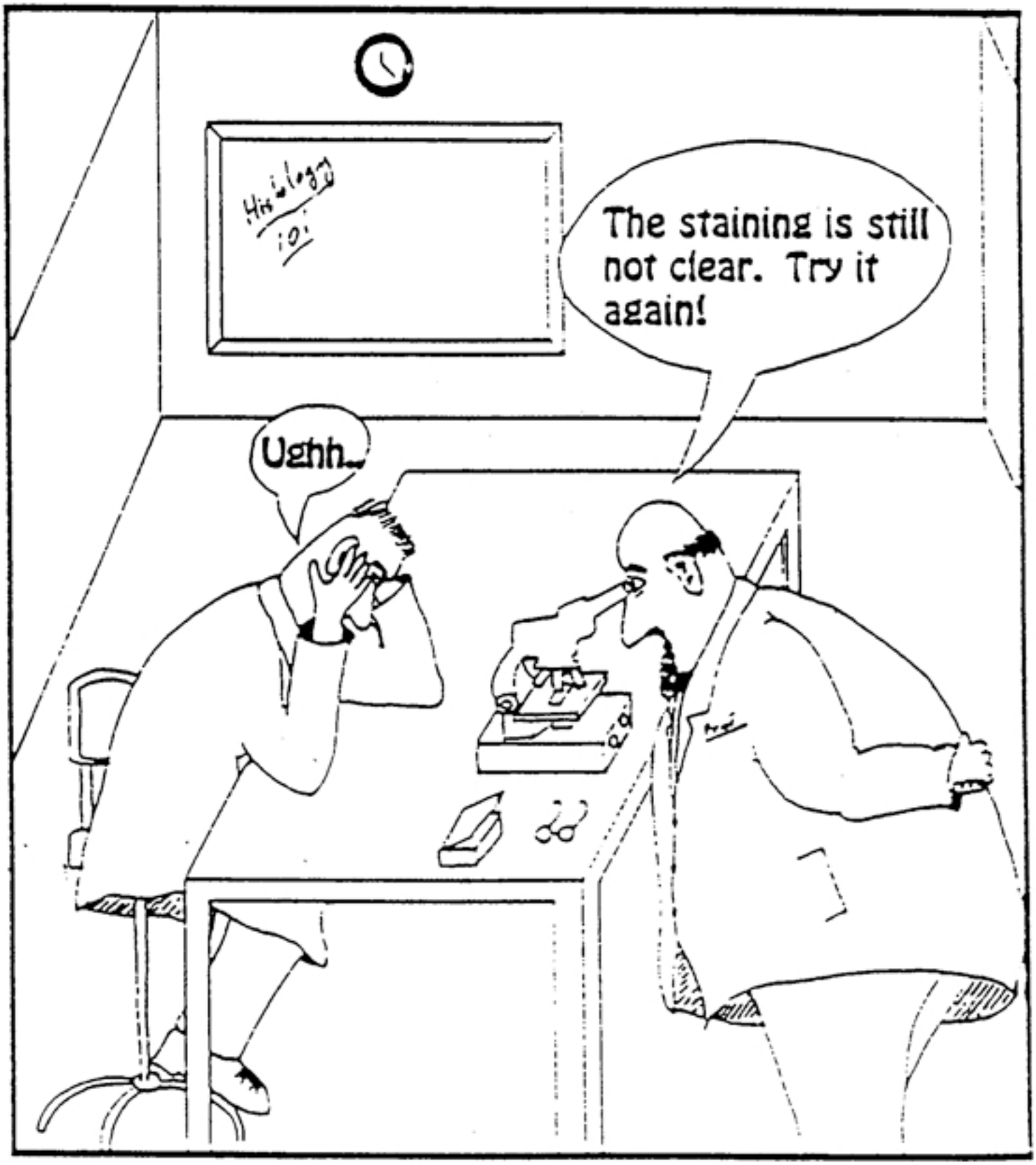


ChlorBromindigo
abs max 615 nm

BLUE
PRECIPITATE

Substrate availability

A key parameter determining the sensitivity of the staining seems to be the availability of the substrate. X-gal is a very insoluble molecule in pure aqueous solutions. This is not solely due to hydrophobic nature of the indole group; X-gal becomes much more soluble at higher temperatures, while hydrophobic molecules tend to become less soluble at higher temperatures [11]. Apparently the poor solubility of X-gal is due to a crystal structure which is very stable. The thermal coefficients for solubility are apparently very large, so that a dramatic increase in solubility is observed between 4°C and 42°C. In increasing the solubiiity of the substrate (X-gal) it is important not to solubilize the final indigo reaction product. Normally a low concentration of DMF (in which the X-gal is initially dissolved) is present to increase solubility. DMSO (and less



Those who do not know histology are doomed to repeat it

Figure 1

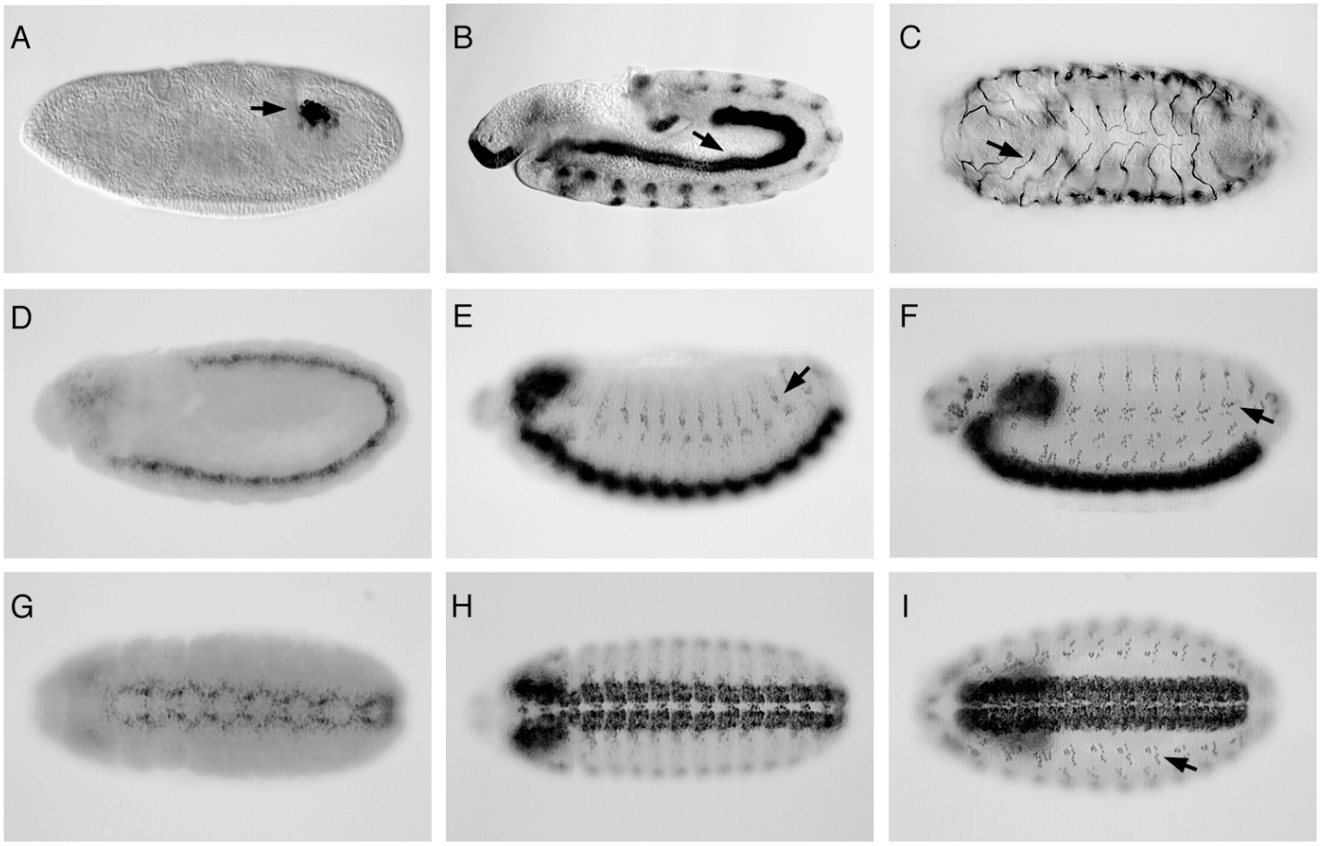


Figure 2

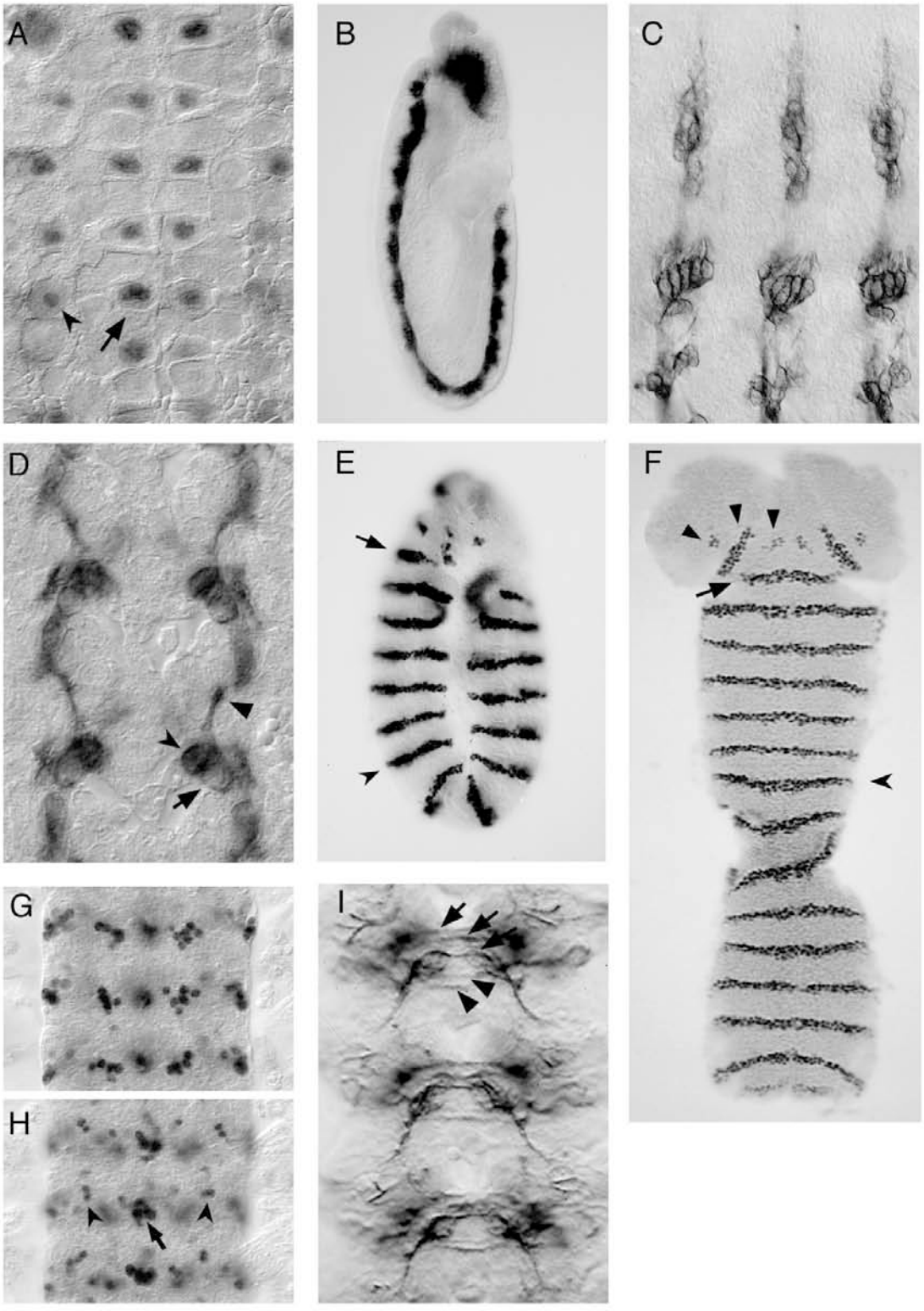


Figure 3

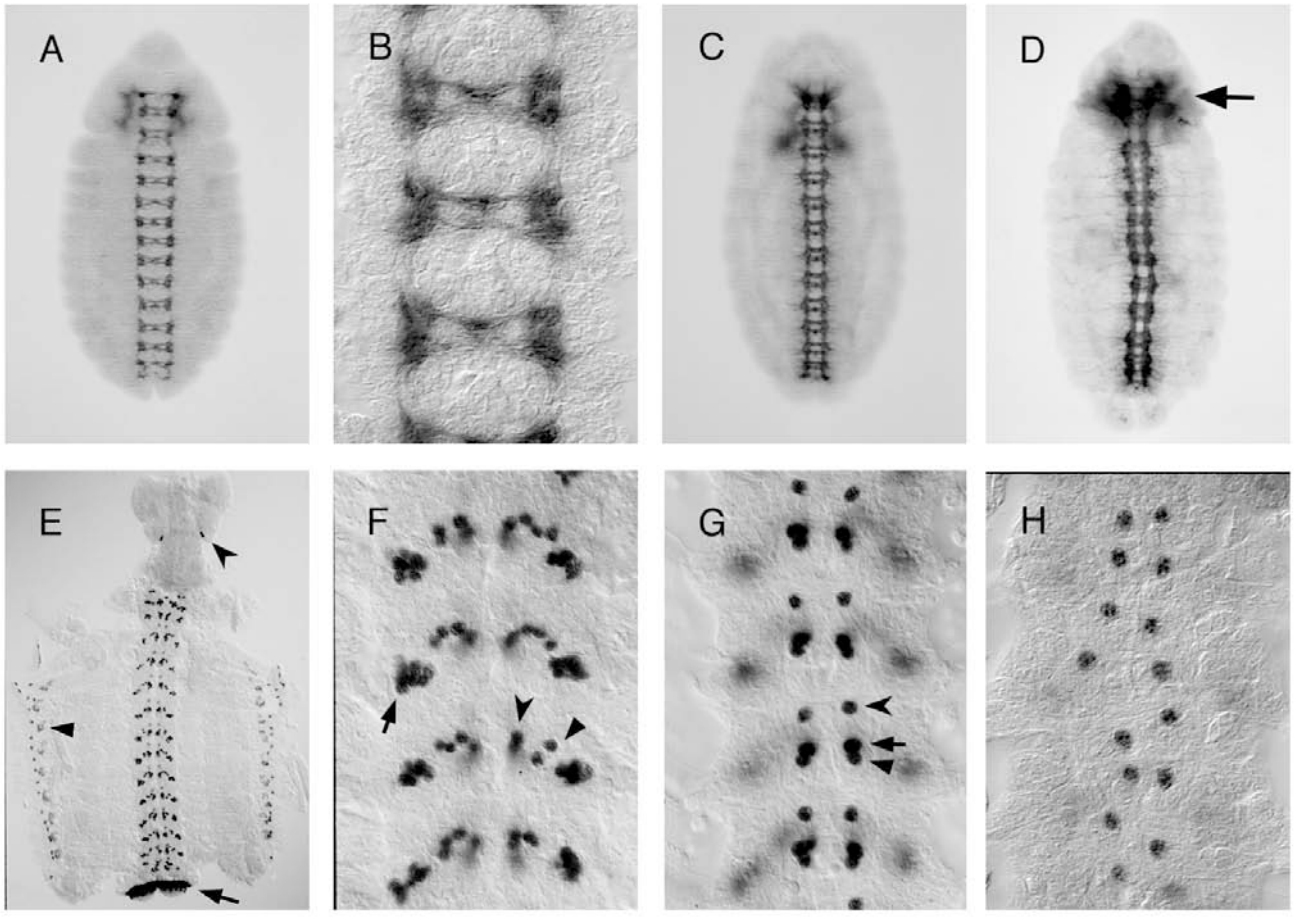


Figure 4

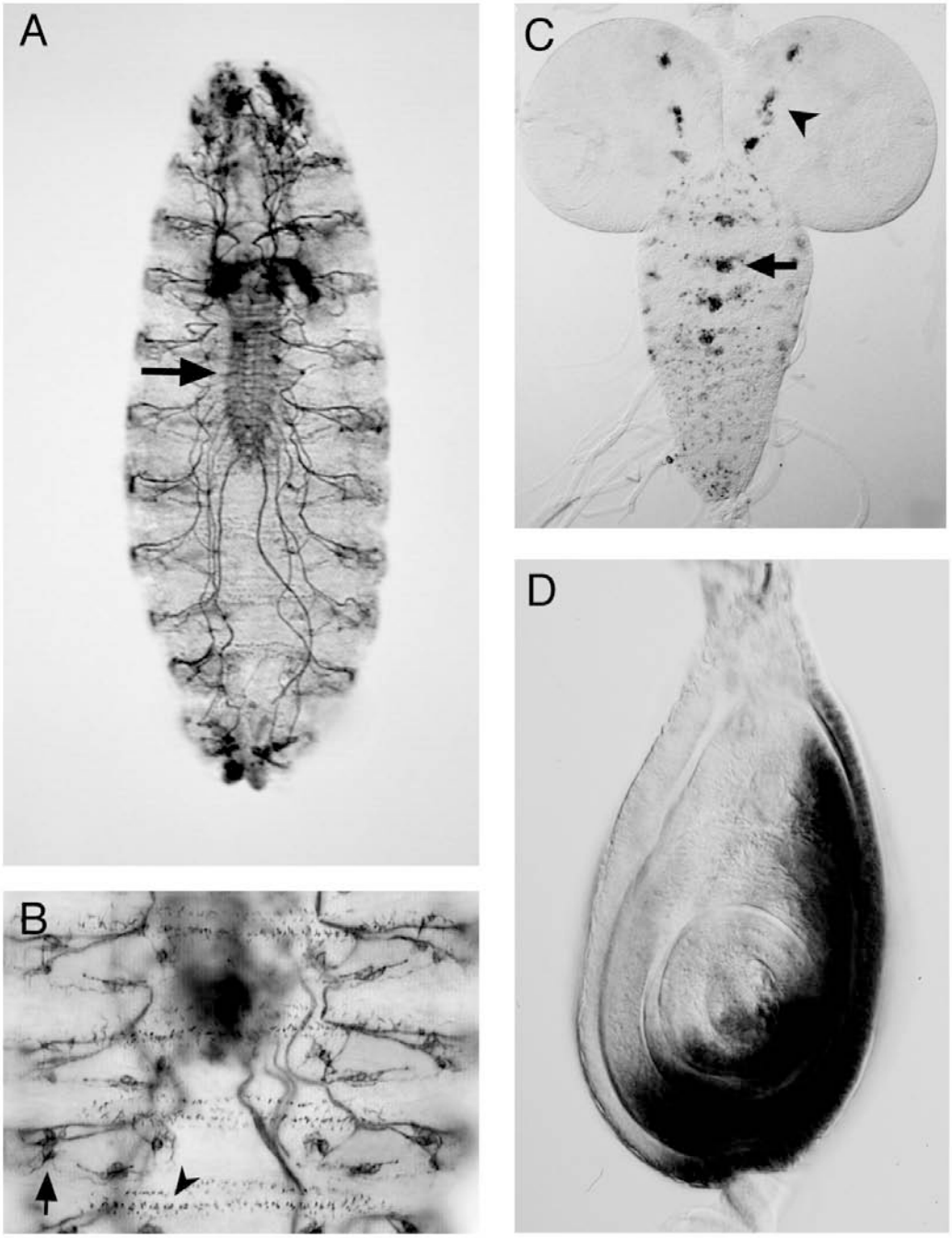


Figure 5

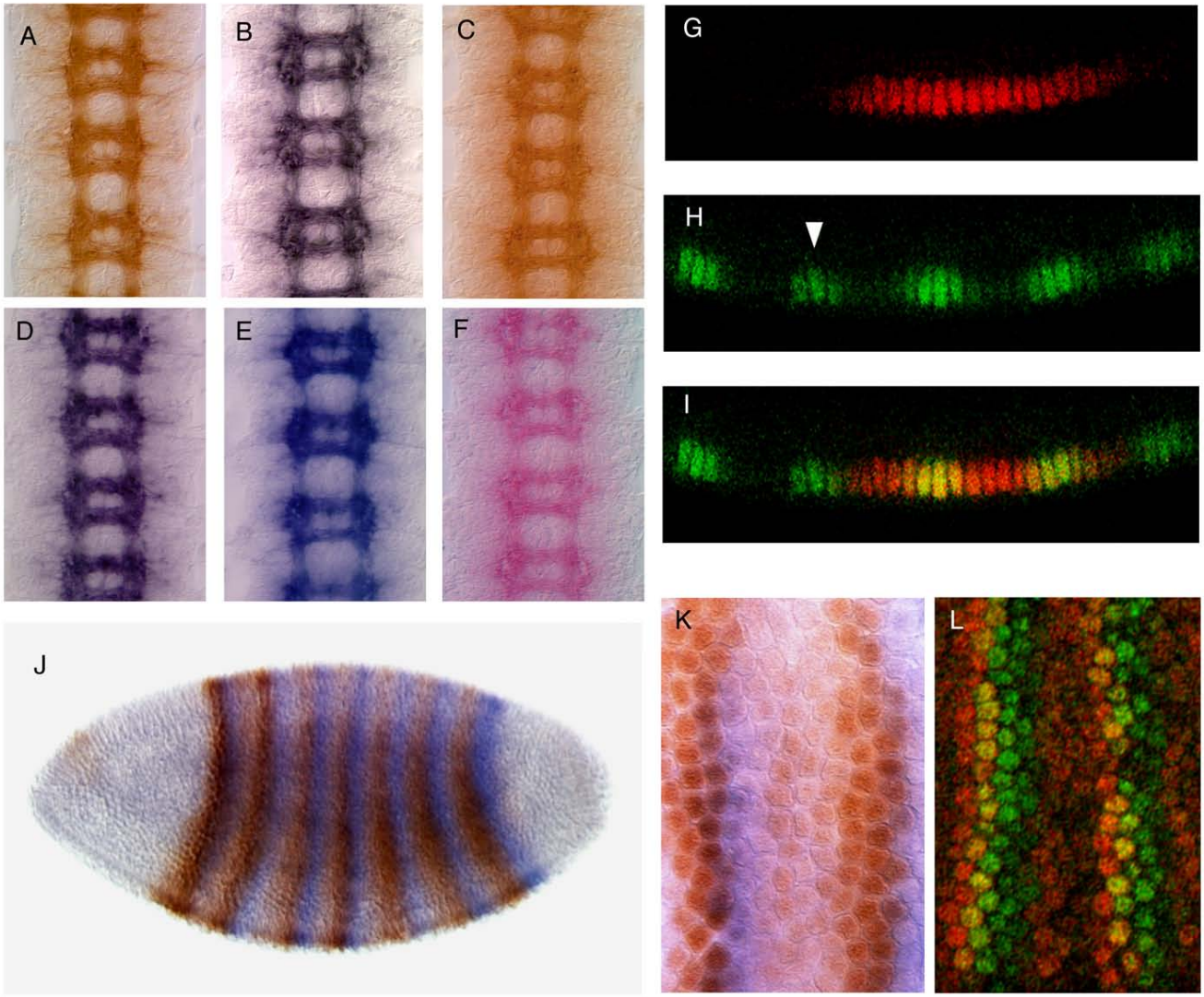
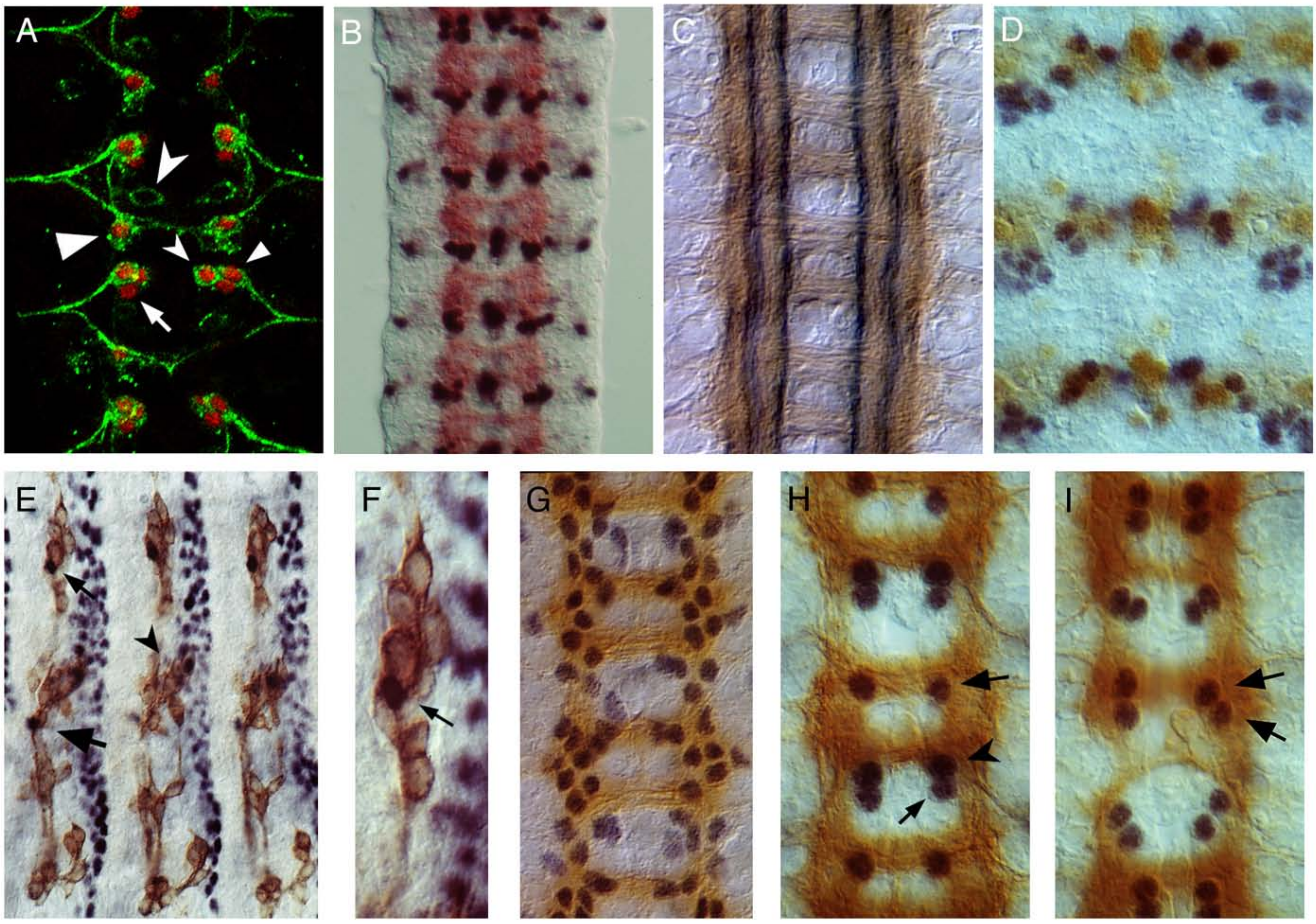


Figure 6



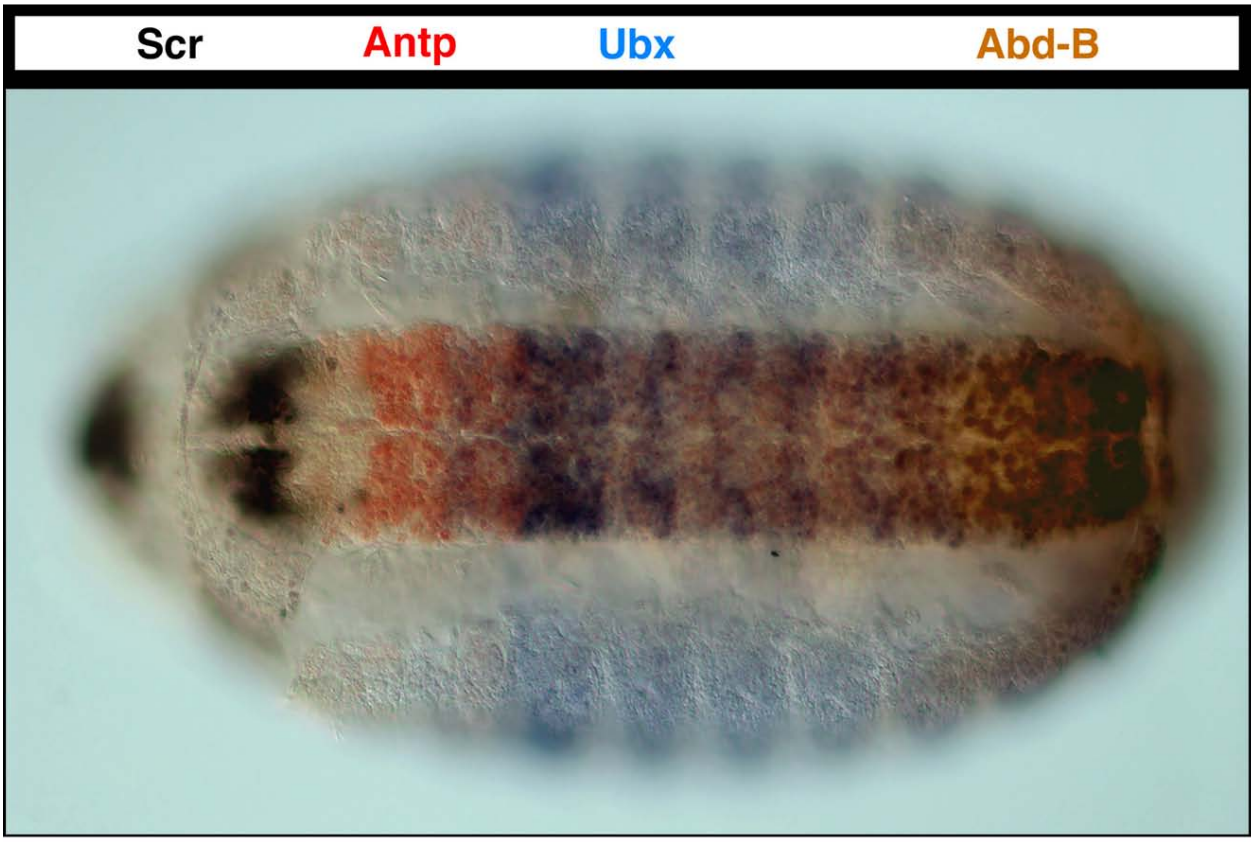
Supplemental Figure 1

Grasshopper embryos (23%, 26%, and 45%)

Left and right embryos are stained for engrailed (black) and Dll (red)

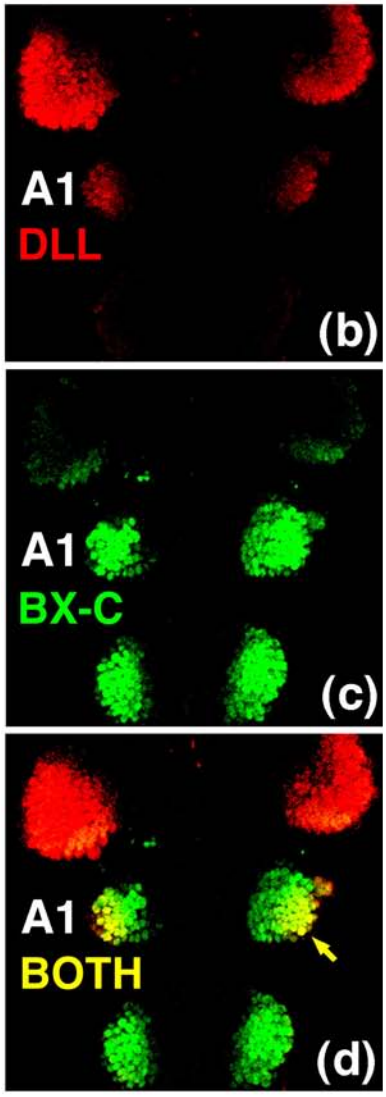
Middle embryo is Ubx (black) and Dll (red)

Supplemental Figure 2



Drosophila embryo (stage 15) labeled with Mouse anti-Scr (black), Mouse anti-AbdB (brown), Mouse anti-Ubx (blue), Mouse anti-Antp (red). All are mouse monoclonal antibodies, staining done using sequential staining procedure - black (DAB+Ni), brown (DAB), blue (BCIP+NBT - taken through methanol), and finally red (AEC). Note that the BCIP+NBT must be converted to blue before the AEC reaction as the AEC product is soluble in methanol.

Supplemental Figure 3



Grasshopper embryos

Ubx - green
Dll - red

Confocal images