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Xenopus laevis: Practical Uses in Cell and Molecular Biology

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Chapter 22

Whole-Mount Staining of Xenopus and Other Vertebrates

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I. Introduction

In toto or "whole-mount" imaging of organisms has a long history (see Campbell, 1986). Its advantages over section-based analysis are obvious. Whole-mount imaging provides an immediate and three-dimensional view of the stained components. Three-dimensional information can also be gained by serial section analysis, but only following an indirect, time-consuming, and rather tedious process. In addition, sectioning is subject to many preparation artifacts that can distort the morphology of the tissues under study. Early whole-mount methodology was largely restricted to staining for cartilage or bone. Recently, technical developments have made it possible to also use antibodies and nucleic acid probes in whole-mount preparations. At the same time, advances in light microscopy make it possible to section thick specimens optically. We are therefore now able to describe, at higher resolution and in the three-dimensional context of the intact organism, the temporal and spatial distribution of a wide range of cellular and extracellular components. Our aim here is to present a working guide to the use of these methods in *Xenopus*, other amphibians, and in vertebrate embryos in general. Recipes for stains, fixatives, etc., are provided in Section III.

A. Clearing Agents

The prerequisite for any whole-mount analysis is that we be able to see through the specimen. This means either that the specimen must be naturally transparent, a rare feature among higher metazoans, or that it must be possible to "clear" it. Clearing generally involves two steps: extracting material from the specimen, then matching the refractive index of the bulk of the specimen remaining, thereby rendering it transparent. Primary considerations in selecting a clearing agent are how closely it matches the refractive index (n_D) of the specimen, its inherent toxicity, and its compatibility with the staining reagents used. A wide range of clearing agents have been used over the last century. These range from potassium hydroxide/glycerol (Schultze, 1987; Mall, 1904) to methyl salicylate (artificial oil of wintergreen) (Spalteholz, 1914), methyl salicylate and benzyl benzoate (Ojeda *et al.*, 1970), and carbon disulfide (Lundvall, 1904). With respect to the anuran amphibians *Xenopus*, *Bombina*, and *Eleutherodactylus*, the clearing agent devised by Andrew Murray and Marc Kirschner, a 1:2 mixture of benzyl alcohol (n_D1.54035) and benzyl benzoate (n_D1.5681) (BABB), is a close match to the refractive index of the oocyte and embryo. It is also compatible with fluorescence- and peroxidase-based immunocytochemistry (Dent *et al.*, 1989; Dent and Klymkowsky, 1989) and polarization optics (Chu and Klymkowsky, 1989). BABB is an effective clearing agent for insects (R. B. Cary, personal commu-

nication), chick (J. B. Miller, personal communication), mouse (Wright *et al.*, 1989a), shrimp (Fig. 1A) (P. Hertzler, personal communication), and zebrafish (Metcalfe *et al.*, 1990) embryos. In addition, BABB works effectively on specimens stained for cartilage with Alcian blue (see Fig. 3E).

Benzyl alcohol and benzyl benzoate are both naturally occurring products (see *Merck Index*). Both are irritants, and care should be taken to avoid exposure to skin; we routinely use gloves when handling this reagent. Used BABB must be disposed of by incineration by the appropriate toxic waste disposal service. BABB suffers from two minor technical drawbacks. First, it renders the embryos brittle, and care must be taken when specimens are manipulated. Second, many of the commonly used chromogenic substrates are soluble in BABB (Dent *et al.*, 1989).

Clearing Agents for Cartilage and Bone

A number of procedures for whole-mount staining of bone and/or cartilage call for clearing the specimen by prolonged immersion in potassium hydroxide followed by transfer to glycerol (e.g., Park and Kim, 1984; Wassersug, 1976). In our experience, using trypsin to macerate soft tissues, especially muscle, is more effective and faster, especially with large or dense specimens (Dingerkus and Uhler, 1977; Kelly and Bryden, 1983). Maceration must be done with care; the specimen eventually will decompose if left in the solution too long or if the solution is too concentrated. As with clearing with potassium hydroxide, maceration using trypsin is temperature dependent; the processing time for large or dense specimens can be shortened by placing the trypsin solution in a warm incubator. To determine whether maceration is complete, it often is helpful to advance the specimen to the next step in the staining procedure, that is, into the alizarin red solution; the degree of digestion of muscle is readily gauged against the background provided by the red bones and blue cartilage. If more maceration is needed, the specimen can simply be backed down into a fresh trypsin solution. In cases where specimens are to be stained for cartilage but not bone, BABB can be used to clear the specimens (see below).

B. Bleaching

In addition to their inherent opacity, many embryos are also highly pigmented. The presence of pigment generally interferes with the clear visualization of antibody and histochemical staining. Where naturally occurring albino variants are available, as in *Xenopus* (see Chapters 1 and 3, this volume), they can be used. However, maintaining a colony of albinos is an added burden (particularly as albinos are rather unattractive). In any case, it is possible to bleach most pigments using hydrogen peroxide (see Bechtol, 1948; Evans, 1948; Dent *et al.*, 1989). For immunocytochemistry, we have found

that 1 part 30% hydrogen peroxide in 2 parts Dent fixative bleaches even the darkest embryos within 2 to 7 days. Although some pigment may persist in the retina, it generally does not interfere with viewing the specimen. Bleaching also has the added benefit of destroying endogenous peroxidase activities that interfere with horseradish peroxidase-based secondary reagents. In our experience, bleaching has little affect on the antigenicity of a wide variety of monoclonal antibodies that we have tested. In fact, some antibodies that fail to react with their target molecule in simple methanol-fixed embryos react strongly following bleaching, suggesting that the bleaching step may sometimes uncover hidden epitopes.

Bleaching Bone–Cartilage-Stained Whole Mounts

Because bone–cartilage staining typically involves later embryonic or posthatching stages, naturally occurring pigment can be an even greater problem than in immunocytochemical whole mounts of early embryonic stages. Given that much of the pigment lies in the integument, it can be readily removed by skinning the specimen. Additional pigment, however, frequently lies beneath the dermis. It is not removed by either skinning or evisceration and must be bleached if it interferes with viewing the stained tissues. We use a 3% hydrogen peroxide solution, added during the early stages of glycerol infiltration, after maceration and staining. In our experience, the peroxide does not affect the intensity of Alcian blue- or alizarin red-stained tissues.

C. Fixation

Perfect fixation would immobilize every molecule of the specimen instantaneously. However, a perfect fixation would also pose a serious barrier to the diffusion of molecules into and out of the specimen. In the case of whole-mount immunocytochemistry, the sample must be permeable to molecules of the order of 150,000 (immunoglobulin G, IgG) to 900,000 (IgM) daltons. At the same time, to be useful the fixative must preserve cellular structure. Two basic types of fixatives have been used successfully for whole-mount immunocytochemistry: alcohol-based (Dent *et al.*, 1989) and aldehyde-based fixatives (Patel *et al.*, 1989). Alcohol-based fixatives reduce the dielectric constant of the solvent phase and result in the denaturation of proteins. In particular, the reduced dielectric constant of the solvent tends to disrupt hydrophobic interactions while stabilizing hydrogen bonds. Therefore, alcohol-based fixatives are expected to preserve secondary structure, while destabilizing tertiary interactions (Pearse, 1980). Antibodies directed against linear epitopes should be largely unaffected by alcohol-based fixatives. The denaturation of proteins by alcohol-based fixation is not necessarily

permanent, and some local renaturation presumably occurs when the specimen is returned to aqueous conditions. Therefore, antibodies directed against some three-dimensional (as opposed to linear) epitopes should also be relatively unaffected by alcohol-based fixatives. Antibodies directed against three-dimensional antigenic determinants that cannot reform following alcohol denaturation will probably be lost. In addition, proteins and other components that are soluble in the alcohol fixative will be lost from the specimen and so not detectable.

Aldehyde-based fixatives act by chemically cross-linking proteins. Under the conditions usually employed (near neutral pH, 1–4% w/v formaldehyde) the cross-linking reaction is far from complete (Pearse, 1980). However, molecules that may be soluble in alcohols can be readily rendered insoluble by aldehyde-based fixatives. Aldehyde-based fixatives leave the plasma membrane largely intact, so they must be followed by extraction of membrane lipids with detergent or organic solvents. Antigenic sites that contain reactive groups may be chemically modified during aldehyde fixation and their antigenicity lost.

Typically, the choice of fixative is determined empirically. During the development of our whole-mount method, Joseph Dent examined a number of different alcohol-based fixatives, beginning with 100% methanol, a fixative used routinely for visualizing cytoskeletal systems in cultured cells. He found that antibody staining was largely restricted to exposed surfaces. By adding dimethyl sulfoxide (DMSO) to the methanol, he was able to arrive at a fixative (Dent fixative: 1 part DMSO to 4 parts methanol) that preserved cellular and tissue structure, while at the same time allowing antibody molecules to penetrate throughout the embryo. Dent fixative appears quite good for a range of antibodies against cytoskeletal, nuclear, and extracellular matrix components (Figs. 1A,B and 2) (see also Chu and Klymkowsky, 1989; Wright *et al.*, 1989a,b; McMahon and Moon, 1989; Jones and Woodland, 1989; Hanken *et al.*, 1990). However, Dent fixative is not always adequate.

The experiences of those who have tried to visualize the organization of microtubules in the *Xenopus* oocyte are particularly instructive. Palecek *et al.*, (1985) used Bouin–Hollande fixative and section-based immunocytochemistry and found a substantial accumulation of tubulin in the region of the mitochondrial mass (Balbiani body) of the early stage oocyte. Similar results were reported by Wylie *et al.* (1985). In contrast, we found no such accumulation of tubulin immunoreactivity in Dent-fixed oocytes (Dent and Klymkowsky, 1989). Electron microscopic analyses of glutaraldehyde-fixed oocytes reveal relatively few microtubules, particularly considering the known abundance of tubulin within the oocyte (see Dent and Klymkowsky, 1989, for a review). In contrast, Gard (1991) has found that fixation of *Xenopus* oocytes with either a formaldehyde/glutaraldehyde/taxol or formaldehyde/

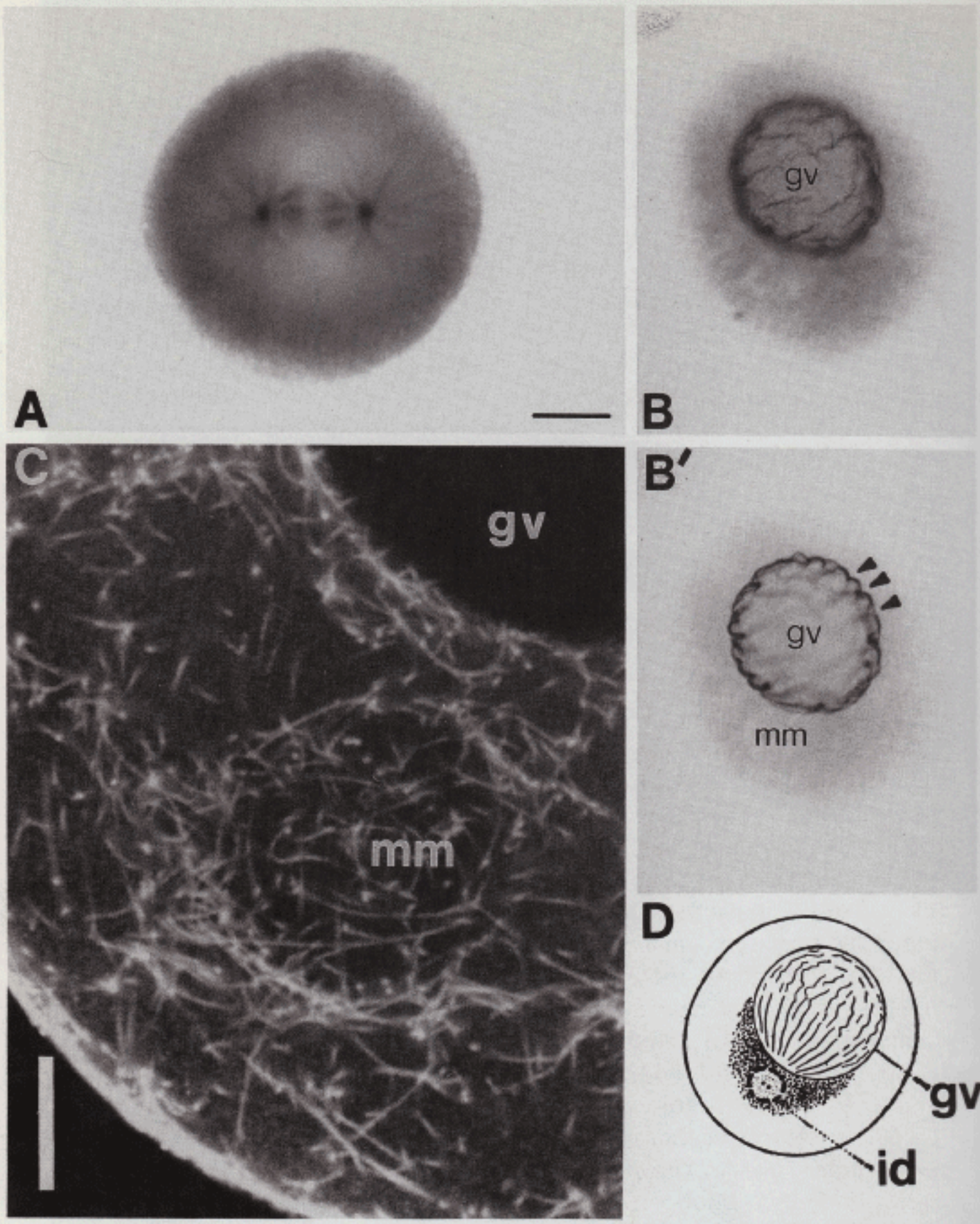

FIG. 1. Dent fixative and immunoperoxidase reagents provide clear low-resolution images of cytoskeletal components. In (A) the mitotic spindle of a brine shrimp egg is visualized using an antitubulin antibody. In small *Xenopus* oocytes, it is often possible to section the specimen optically using a standard microscope. Such "low-tech" optical sectioning of a stage I *Xenopus* oocyte stained with an antilamin antibody is shown in (B) and (B′); arrowheads point to indentations in nuclear envelope visible in one, but not the other "section." The mitochondrial mass (mm) can be made out as an unstained region adjacent to the nucleus. Higher resolution imaging requires confocal microscopy; (C) is such a confocal image of the microtubules of a stage I *Xenopus* oocyte. The interaction between microtubules and the mitochondrial mass is particularly evident (gv, germinal vesicle). The interaction between germinal vesicle and mitochondrial mass, known as the "idiozome" (id) in earlier literature, is illustrated diagrammatically in (D). [(A was supplied by Phil Hertzler; (C) by Dave Gard; and (D) is from Wilson, 1925.]

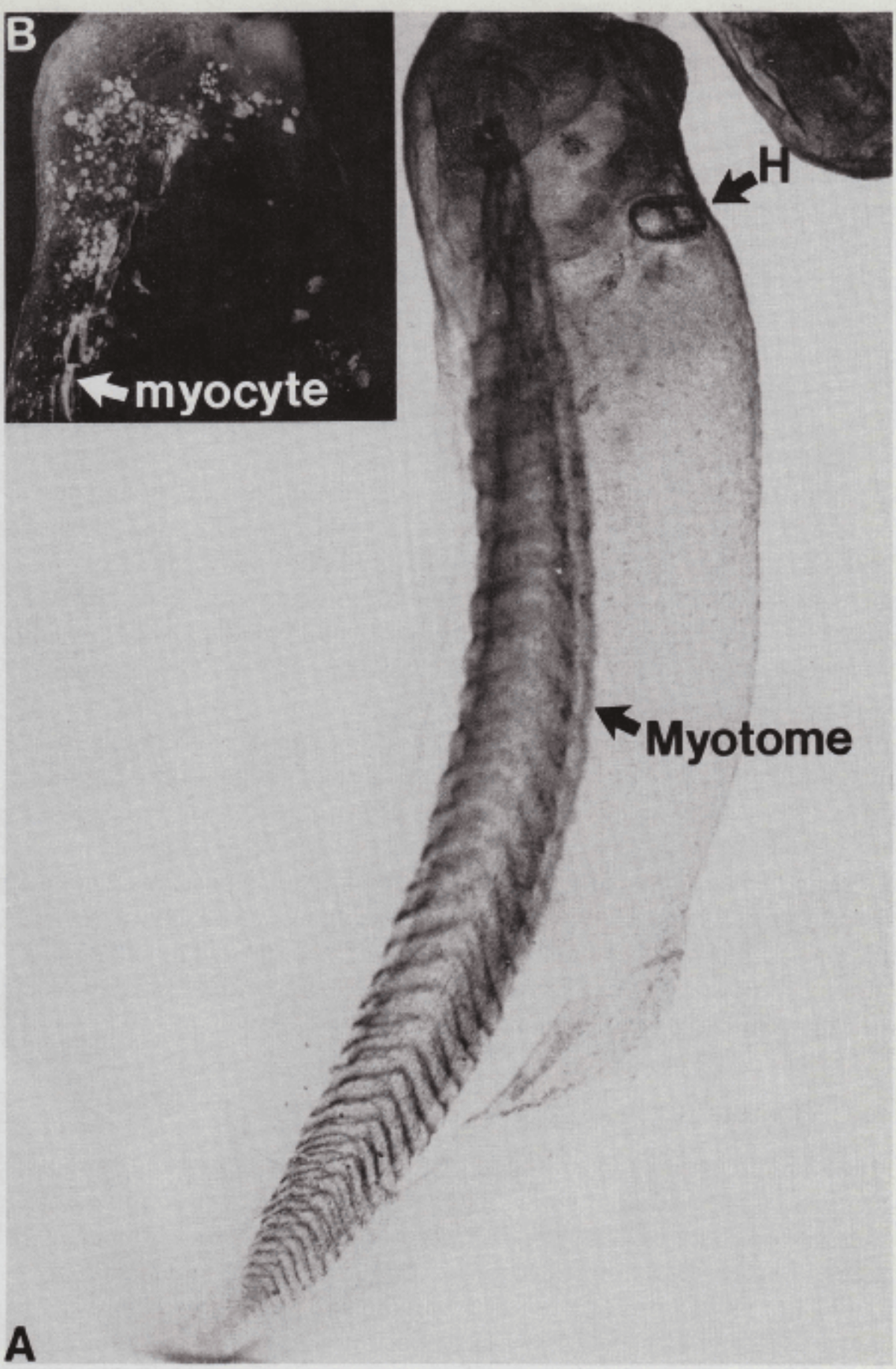

FIG. 2. Whole-mount immunocytochemistry can be used to visualize the specificity of an antibody. In this case, a commercial antidesmin antibody was tested. The pattern of staining (A) indicates that at the dilution used, the antibody was rather specific for myotomal (Myotome) and heart (H) muscle. Whole-mount methods can also easily be used to visualize the pattern of expression of various exogenous DNA or RNA molecules. For example, in (B) a fertilized egg was injected with DNA in which a *Xenopus* cytoplasmic actin promoter drove the expression of the bacterial protein β-galactosidase. Using an antibody against β-galactosidase, the pattern of cellular expression of the exogenous DNA can be easily visualized (head is oriented up). (B) was printed directly from a "positive" color slide and so is a negative image: stained cells are white.

glutaraldehyde fixative preserves a substantial microtubule system in all stages of oogenesis (Fig. 1C). In contrast to previous reports, microtubules appear to course around the mitochondrial mass but do not appear to be particularly concentrated there (Fig. 1C). So care must be taken, as dramatically different conclusions can arise from the use of different fixation protocols. Determining which reflects the "true" organization is often difficult.

Fixation for Bone Cartilage Staining

The stability of bone and cartilage make the choice of fixatives more straightforward. For whole-mount staining, the most effective and convenient fixative is buffered formalin. It is also possible to stain specimens fixed in buffered formalin and preserved in 70% ethanol, as is standard for many museum specimens, although with generally less favorable results. Cartilage staining, in particular, is often less intense following alcohol preservation. Nevertheless, we have found that the cartilages of specimens fixed in Dent fixative stain well (Fig. 3E).

Embryos, larvae, and adults whose integument is relatively porous to formalin may simply be immersed in the solution. In posthatching stages with a relatively impervious integument, for example, most amniotes, the fixative may have to be injected or perfused into the specimen, or the specimen may be skinned to ensure proper fixation. It is possible to bypass fixation and begin the staining procedure by immersing the freshly killed (and eviscerated) specimen directly into Alcian blue (Table II, Step 3). The efficacy of both staining and clearing are about the same in fixed and unfixed specimens, although unfixed specimens tend to curl.

D. Visualizing Binding of Antibodies

The binding of antibodies to specific components in fixed tissues can be visualized using colloidal gold, enzymatic, or fluorescent-conjugated secondary reagents. The highest possible resolution would be obtained with Fab fragments and electron microscopy, but it is often impossible to unambiguously recognize unlabeled Fab fragments. Therefore, immunogold conjugates are generally used. The resolution of these reagents is limited by the physical size of the primary and secondary reagents. At the level of light microscopy resolution is limited not by the reagents, but by the microscope itself. Fluorescent conjugates, chromogenic enzymes, and immunogold particles are all theoretically capable of generating images from structures below the resolution limit of the light microscope. They differ primarily in sensitivity. Because they create an image by emitting rather than absorbing light, fluorescent reagents are theoretically the most sensitive. Fluorescein-,

rhodamine-, and Texas red-based fluorophores are the most widely used, and a wide range of secondary antibody reagents are commercially available. Fluorescein-based reagents are more sensitive to photobleaching under conditions of microscopic examination and photography than are rhodamine/Texas red-based reagents. However, the bleaching of fluorescein can be markedly reduced by the addition of "antifade" agents to the mounting media (Giloh and Sedat, 1982). We concoct our own mounting media for fluorescently stained specimens (see Section III).

Although extremely sensitive, fluorescent secondary reagents suffer from a number of practical limitations when used in whole-mount immunocytochemistry. First, their effective sensitivity is dependent on the numerical aperture (N.A.) of the lens used to examine the specimen. Unfortunately, most high N.A. lenses have very short working distances, making it impossible to focus completely through a thick specimen. Lower N.A., higher working distance lenses can be used, but these are effective only on rather robust signals. A second problem with fluorescent reagents arises from the fact that they form an image by emitting light. In *Xenopus*, there is a high level of autofluorescence, primarily from yolk platelets. This autofluorescence is not affected by clearing and obscures the signal from specific fluorescent reagents in most parts of the embryo, with the exception of thin, nonyolky regions, such as the tail (see Kay *et al.*, 1988). In addition, the excitation light illuminates more than just the focal plane currently being examined, and fluorophores both above and below the focal plane are excited and emit light. This generates out-of-focus fluorescence that decreases the signal-to-noise ratio and degrades the image. Autofluorescence from above and below the plane of focus also degrades the signal from the true focal plane.

These problems may be overcome either through the use of computational correction, which effectively removes the contaminating, out-of-focus noise (Agard *et al.*, 1989), or through the use of a confocal microscope. Gard (1991) used confocal microscopy to image the microtubule organization of *Xenopus* oocytes (Fig. 1C). The high signal-to-noise ratio of the confocal microscope provides a level of detail previously obtained only in relatively thin (5 to 10 μm thick) cultured cells. In addition, because confocal microscopes store data digitally, full serial reconstructions of the specimen can be generated, manipulated, and displayed. However, there still are serious limitations. Again, the working distance of high magnification/N.A. lenses generally is less than the thickness of the specimen (plus the cover glass). Under these conditions, the rather drastic measure of cutting the specimen in half (or into even smaller sections) must be taken. In addition, the high intensity of the excitation light that is used in many confocal microscopes tends to bleach the specimen (even in the presence of antifade reagents) in advance of its examination.

Enzyme-based secondary reagents work by generating a light-absorbing product from soluble substrate. Because the signal is formed by absorbing rather than emitting light, they are less sensitive than fluorescent reagents. Nevertheless, in many cases the lower sensitivity is more than compensated for by other characteristics. First, they are not affected by autofluorescence. Second, where the signal is relatively strong or tightly localized, such as within the nucleus, they are easy to visualize even at very low magnification (Fig. 1A,B, 2A, and 4). At low magnification it is possible to exploit the relatively narrow depth of field of light lenses to section the specimen optically (Fig. 1B,B'). At higher magnification/resolution, however, many of the same problems that plague fluorescent-based imaging affect enzyme-based imag-

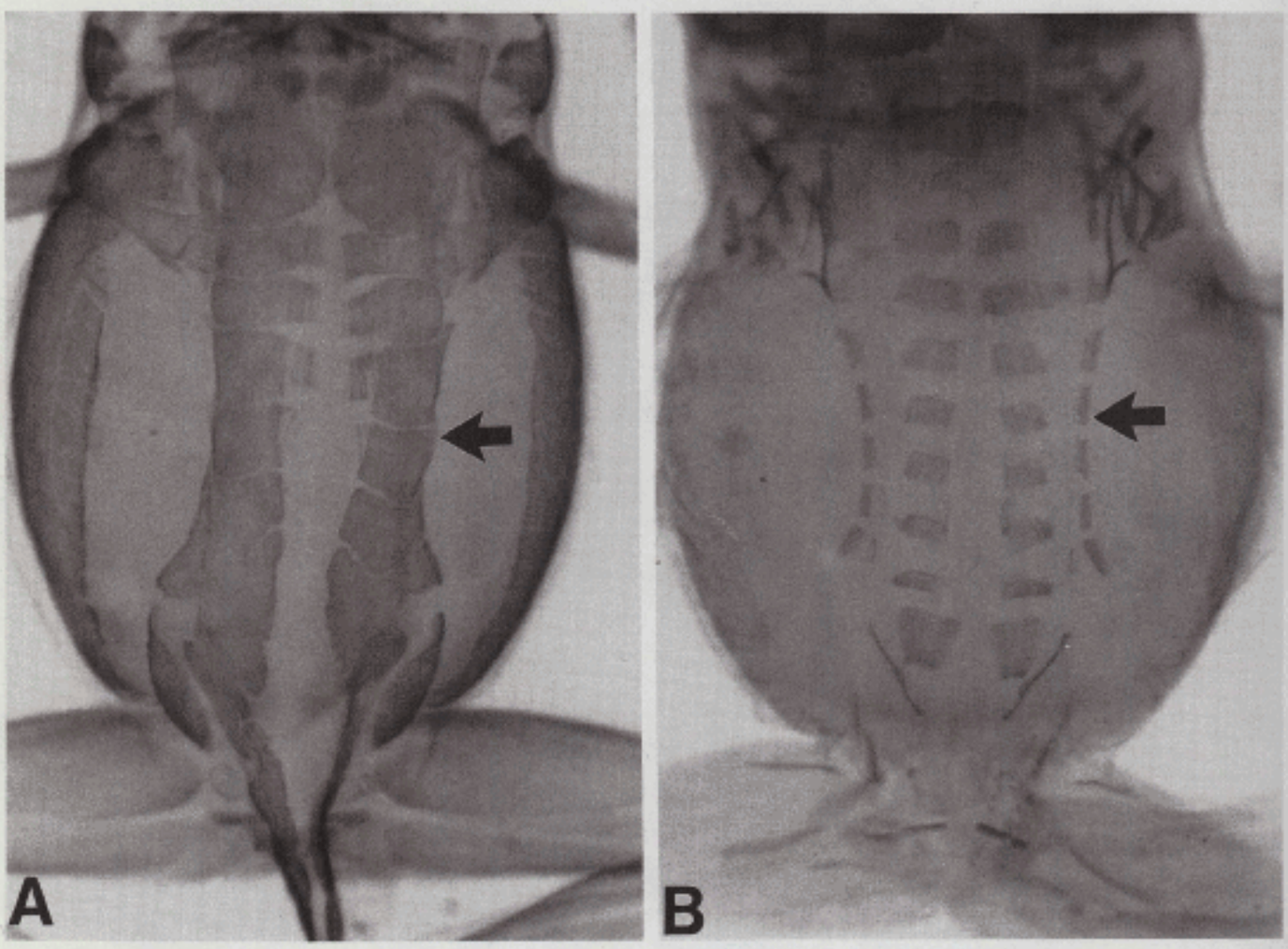

FIG. 4. Whole-mount images can dramatically illustrate both the specificity of particular antibodies and the differential distribution of specific molecules and anatomical components. Here, two posthatching *E. coqui* are stained with antibodies against either (A) fast-twitch or (B) slow-twitch fiber striated muscle myosins using monoclonal antibodies supplied by J. B. Miller (Massachusetts General Hospital) and Frank Stockdale (Stanford University). Note, for example, the different distribution of slow-versus fast-twitch fibers in the axial musculature (arrows).

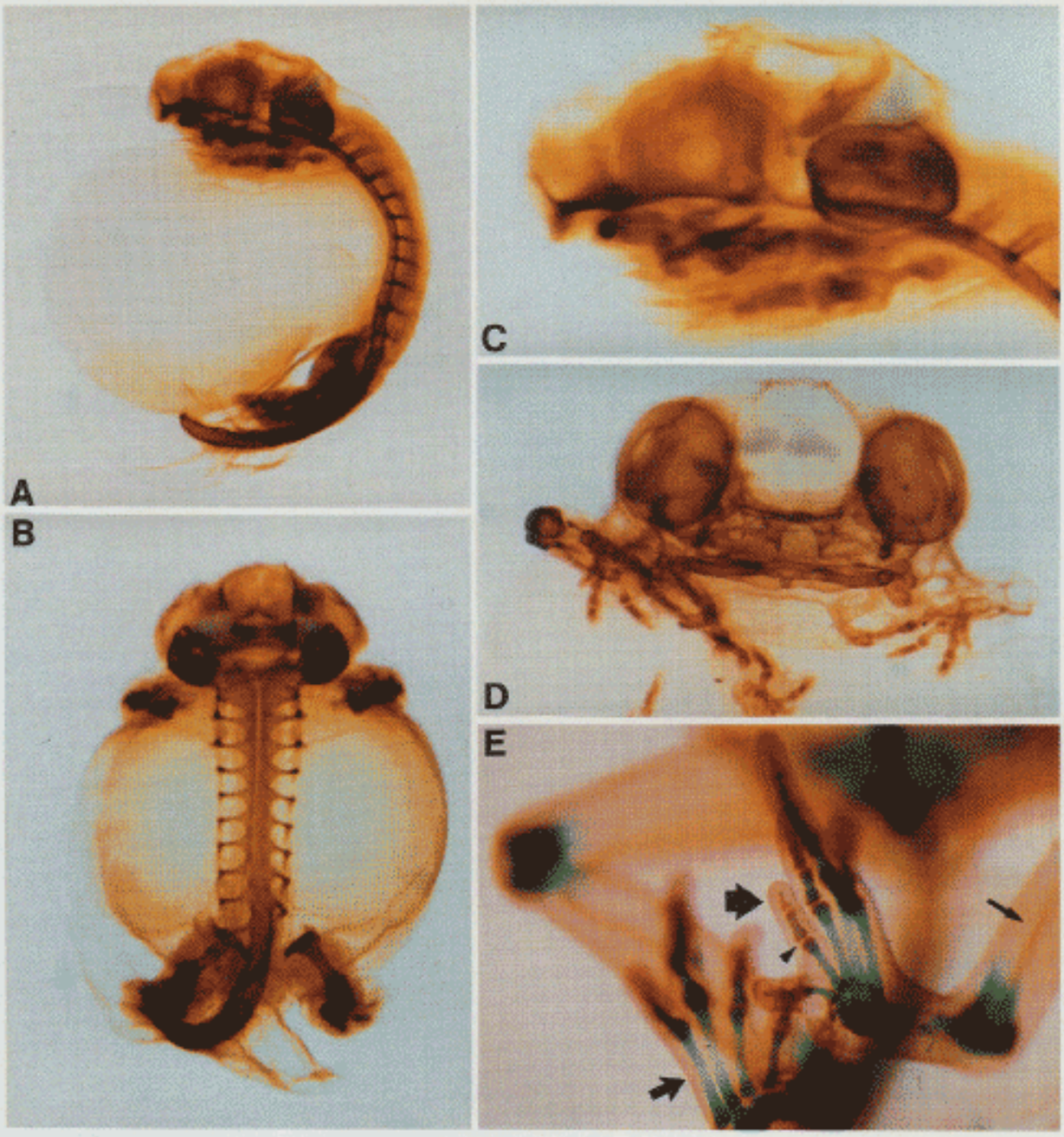

FIG. 3. Whole-mount methods work on a range of organisms, in addition to *Xenopus*. In this case, we have used a monoclonal antibody against type II collagen (provided by T. Linsenmayer, Tufts University) to describe cartilage development in the direct-developing frog *Eleutherodacytlus coqui*. Illustrated here are collagen-immunostained embryos, Townsend-Stewart (1985) stages 9 (A–C, different views) and 12 (D), as well as a posthatching specimen stained both immunocytochemically and with Alcian blue (E). The latter preparation effectively distinguishes cartilage in early stages of development (e.g., collagen-stained terminal phalange, large arrow) from mature cartilage (Alcian blue-stained metatarsal, medium arrow). As skeletogenesis proceeds, most of the cartilage is replaced by bone (small arrow), although some collagen is retained at joint surfaces (arrowhead). Color photographs were taken using Kodak Ektachrome (tungsten) ISO 50 or 160 film on a Wild M5A or M8 dissecting microscope equipped with fiber optic illumination. Black and white photographs were taken using Kodak TMAX 400 film.

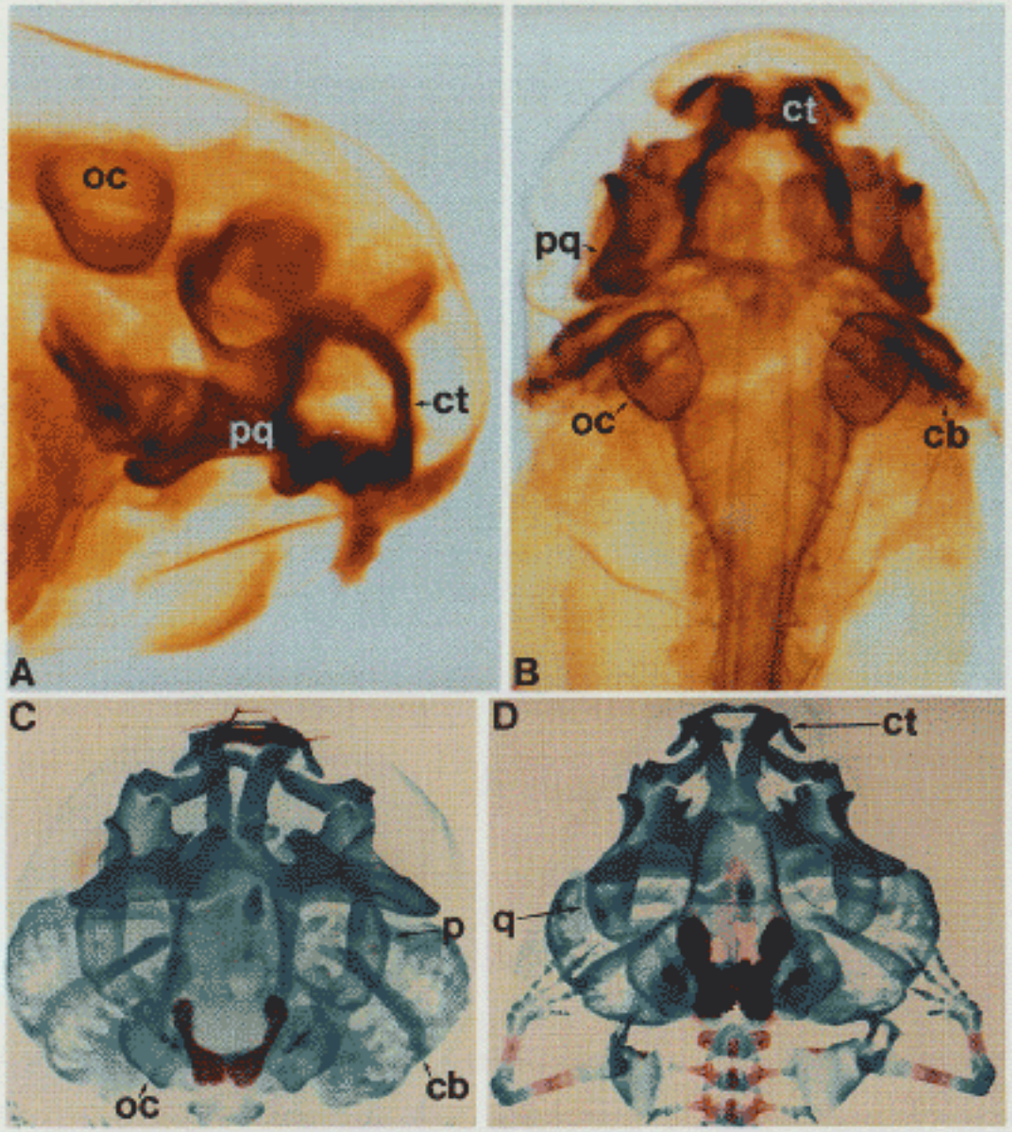

Fig. 5.

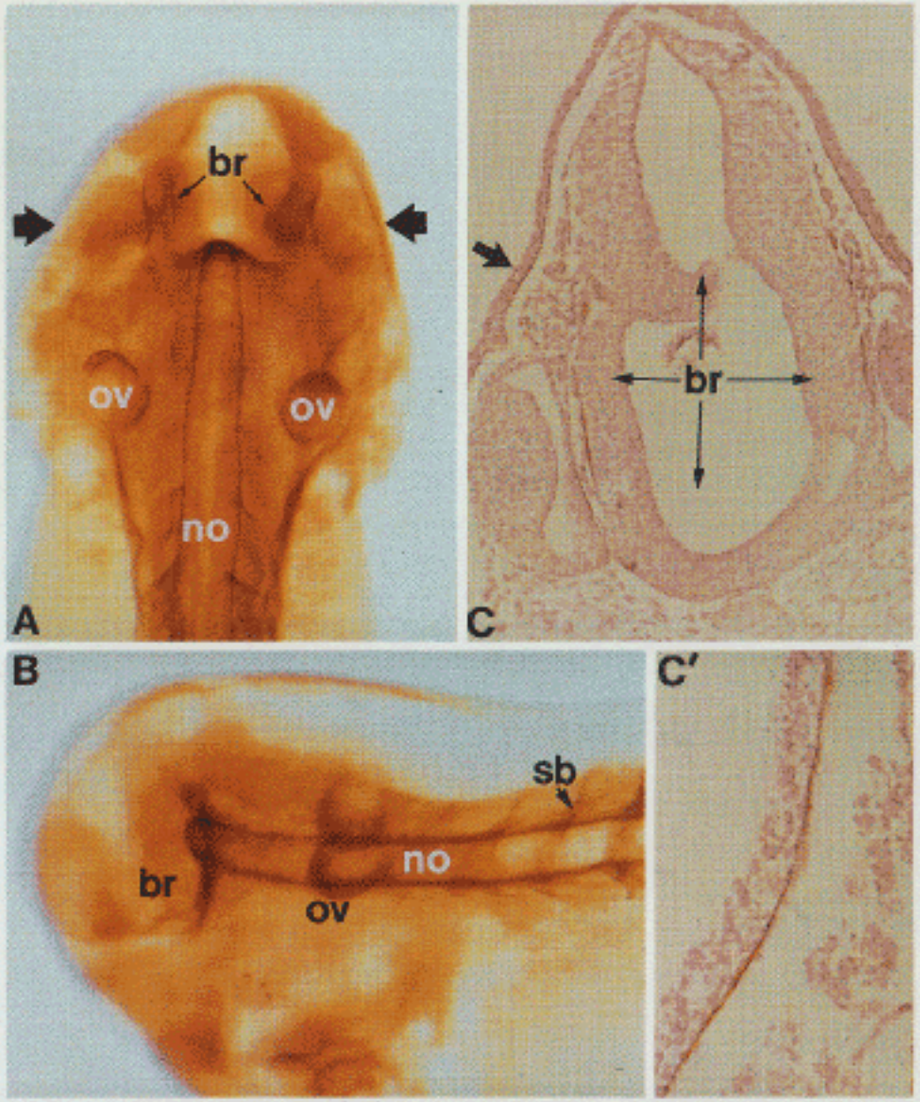

Fig. 6.

ing, namely, the need for high N.A., long working distance objectives and the degradation of the signal by out-of-focus information (Fig. 3A–C and 5A,B). Nevertheless, at present, enzyme-based visualization appears to be the best choice for most studies of protein/RNA localization during development.

II. Methods

A. Cortical Whole-Mount Immunocytochemistry

Fluorescence-based imaging of the cortical region of *Xenopus* oocytes and embryos is straightforward (Klymkowsky *et al.*, 1987). In the last few years, we have made only minor changes, simplifying the process. After being dejellied in 2% cysteine, specimens are fixed in Dent fixative overnight. They are rehydrated in Tris-buffered saline (TBS). Typically, we incubate 10 to 20 oocytes/embryos in 0.25 to 0.3 ml of primary antibody for 1 hour at room temperature or overnight at 4°C. During incubation, the samples are gently rocked; excessive movement can destroy the specimen completely, or remove its outer cortical layers. The specimens are then washed five times with TBS. Washing consists of aspirating the previous solution and adding TBS. When specimens have been stained for only an hour or two, the washes can be as short as 10 to 30 seconds each. For samples incubated overnight, each wash is for 2 to 5 minutes. Secondary antibody is then added and incubated (with rocking) for 2 hours at room temperature (or overnight at 4°C). Late stage *Xenopus* ooctyes and early embryos are approximately 1 to 1.2 mm in diameter. To examine cortical staining, we use 0.8-mm-deep depression slides

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FIG. 5. Skeletogenesis may be followed in whole mounts from early stages of chondrogenesis through later stages of ossification, as in the development of the skull in the Oriental fire-bellied toad *Bombina orientalis*. (A, B) Hatchling tadpole, Gosner (1960) stage 24 (lateral and dorsal views, respectively), stained for type II collagen, which is particularly effective in early stages of cartilage development. (C) Mature tadpole (stage 36, ventral view) and (D) midmetamorphic tadpole (stage 39, dorsal view) differentially stained with alizarin red and Alcian blue; the latter stain is preferable for visualizing larger, mature cartilages. Note the changing morphology of several cartilages, including the otic capsule (oc), palatoquadrate (pq), ceratobranchials (cb), and cornu trabeculae (ct). Bone (red) is visible in the skull, vertebrae, and forelimbs in (D).

FIG. 6. Analysis of serial sections may be required to determine the complete distribution of specific molecules assayed initially in whole mounts. (A, B) In immunostained whole mounts of embryonic *B. orientalis* (Gosner stage 19; dorsal and lateral views, respectively), type II collagen is apparent in the notochordal sheath (no), otic vesicles (ov), segmental boundaries (sb), and the brain (br). (C, C′) In cross sections at the level of the developing eye (arrowheads in A), an additional subepidermal layer of collagen is also visible (C′ is higher a magnification view of the region denoted by the arrow in C).

(obtainable from most suppliers). Late stage oocytes, eggs, and embryos are placed in the center of these slides; earlier stage oocytes can be placed at the periphery. Mounting media (see Section III) is added, and a coverslip placed over the depression slightly flattens the specimens, bringing large expanses of their surface into a single focal plane (see Klymkowsky *et al.*, 1987; Klymkowsky and Maynell, 1989) that can be viewed using high-resolution lenses. This approach has also worked well for microtubules (Elinson and Rowing, 1988; Dent and Klymkowsky, 1989).

B. Total Whole-Mount Immunocytochemistry

For complete whole-mount imaging we use peroxidease-conjugated secondary reagents and diaminobenzidine (DAB) (Table 1). Our current protocol includes some minor changes that reduce background and improve the penetration of antibodies into the specimen. In particular, the addition of DMSO to both the primary and secondary antibody incubations was suggested by Ben Szaro (National Institutes of Health). An important point to remember in all immunohistochemical staining is to control for the specificity of primary and secondary antibodies. Different antisera and secondary reagents can vary dramatically in titer and specificity. Each antibody *must* be titred to determine the most effective concentration with the lowest nonspecific, background staining (see below).

We have examined the usefulness of alkaline phosphatase-conjugated secondary reagents and various alternatives to DAB, such as cobalt/nickel intensification of DAB and the Histo-mark orange and black reagents from Kirkegaard & Perry, Inc., in the hopes of developing a workable double immunocytochemical staining method. Unfortunately, we have little success in this direction. The only alkaline phosphatase substrate we have found that is stable in BABB is black and difficult to distinguish from the brown DAB reaction product. In addition, we consistently find that alkaline phosphatase-conjugated secondary reagents have much higher nonspecific background staining than horseradish peroxidase-conjugated reagents, even in the presence of the phosphatase inhibitor levimisole (Dent *et al.*, 1989). We also find very high backgrounds when the cobalt/nickel-intensified DAB reaction is used. The Histo-mark orange reagent is stable to BABB but produces relatively weak labeling in our hands.

C. Staining for Bone and Cartilage

A large number of whole-mount procedures for staining bone or cartilage have been published, beginning almost 100 years ago (Campbell, 1986). Only in the last 20 years, however reliable methods been available for dif-

TABLE I

WHOLE-MOUNT IMMUNOPEROXIDASE STAINING[a]

1. If a jelly coat is present, dejelly the specimen either chemically (2% cysteine, pH 8.0, for *Xenopus*) or maturally. Wash with Ringer's solution.

2. Fix overnight at room temperature in Dent fixative.

Alternatively, specimens can be fixed using aldehyde-based fixatives for 2 hours at temperature (Section III). Where aldehyde-based fixatives are used, extract the speciment overnight with 100% methanol or with Dent fixative following fixation.

3. Bleach pigment in 10% hydrogen peroxide (diluted from a 30% stock into Dent fixative).

Bleaching may take from 1 to 4 days; be patient!

4. Wash specimen in TBS for 15 minutes.

5. Incubate specimen overnight in primary antibody diluted into 95% calf serum/5% DMSO. (We add 0.1% thimoserol to serum as a preservative.) Rock gently.

Rocking should be carried out with rocker tilted to minimize turbulence.

6. Wash 5 times in TBS, 1 hour each.

7. Inclubate overnight in secondary antibody, diluted as for primary antibody.

8. Wash 5 times in TBS, 1 hour each.

9. React for 1 to 2 hours in 0.5 mg/ml DAB diluted into TBS plus 0.02% hydrogen peroxide. *Wear gloves*: DAB is carcinogenic!

We routinely make up stocks of DAB (10 mg/ml in water) and store them at −20°C, at which temperature they are stable indefinitely. Once diluted, unused DAB should be destroyed with bleach and discarded.

10. Stop reaction by dehydration with methanol (2 changes, 15 to 30 minutes each).

11. Clear in BABB (benzyl alcohol/benzyl benzoate).

[a] All steps can be carried out in either glass vials or microcentrifuge tubes. If clearing is not complete, specimens were not adequately dehydrated. They can be returned to 100% methanol and then cleared again. BABB causes specimens to become brittle; take care when manipulating them.

ferential and stimultaneous staining of bone cartilage in the same specimen (e.g., Dingerkus and Uhler, 1977; Park and Kim, 1984; Wassersug, 1976) (Table II). Several dyes have been used to stain cartilage, including Alcian blue, Alcian green, and toluidine blue. All have an affinity for acid mucopolysaccharides, one of the principal components of cartilage extracellular matrix (Bloom and Fawcett 1975; Ham and Cormack, 1979; Humason, 1979). The most reliable and effective of these stains for whole-mount double-staining techniques appears to be Alcian blue 8GX (e.g., Polysciences, Inc., Warrington, PA, Cat. No. 234). Because of the affinity of the stain for acid mucopolysaccharides, cartilage that has a poorly developed extracellular

TABLE II

WHOLE-MOUNT STAINING FOR BONE AND CARTILAGE[a]

1. Skin and eviscerate the specimen. During evisceration, be careful not to damage or even remove ventral skeletal structures, such as the limb girdles or the hyobranchial skeleton.

Skinning and evisceration may be omitted for embryonic, larval, and early posthatching stages in which the integument is poorly developed.

2. Rinse in distilled water (several changes) overnight. Blot on paper towels.

3. Immerse in Alcian blue for 6–24 hours, according to size (shorter interval for smaller specimens). Blot on paper towels.

4. Run the specimen through ethanol series (100, 100, 95, 70, 40, 15%) and into distilled water, 1–2 hours in each step (longer for large specimens). Specimens may be held overnight or longer in 70% ethanol.

5. Immerse in a solution of trypsin dissolved in 2% sodium tetraborate. Remove the specimen when it is limp and the muscles are translucent; it will eventually decompose if left too long. Begin with a 1% trypsin solution. and adjust the amount as necessary; the maceration rate is proportional to trypsin concentration. Embryos and early posthatching stages may be done after 1–2 hours; larger specimens may take up to several days. Change the trypsin solution daily.

6. Immerse in alizarin red working solution (10–15 drops alizarin red stock solution per 100 ml of 0.5% potassium hydroxide) overnight.

7. Run through a 0.5% potassium hydroxide/glycerol series (3 : 1, 1 : 1, 1 : 3) and then into pure glycerol. Allow at least 2 hours for each step; the specimen is ready to be moved when it sinks to the bottom of the container. Bleaching of pigment (e.g., integument, mesenteries) should be done in the first step (3 : 1), by adding a small amount of 3% hydrogen peroxide. Begin with a few drops of peroxide per 100 ml of solution; if the peroxide concentration is too high, oxygen bubbles will form within the specimen. Bleaching may require several days.

8. Store the specimens in pure glycerol. Thymol or phenol can be added to retard bacterial growth.

Cartilage stains deep blue. Bone, teeth, and other calcified tissues (e.g., calcified cartilage, calcified endolymph) stain red.

[a] Steps 5–8 are reversible, that is, specimens in glycerol may be backed down into alizarin for more intense bone staining or into trypsin for additional maceration, and then back up to pure glycerol. Small specimens should be scored for bone within a few days of completing the procedure, as extremely small or thin bones may destain and become invisible with time; restaining is now always effective. The cartilage stain (Alcian blue) is relatively stable.

matrix (e.g., fibrocartilage) or that is otherwise deficient in mucopolysaccharides will not stain well. This, however, is rarely a serious problem; typically, Alcian blue 8GX beautifully visualizes virtually the entire cartilaginous skeleton (Fig. 3E and 5C,D). Alizarin red S (alizarin sodium sulfonate; Sigma Chemical Co., St. Louis, MO, Cat. No. A 5533) is the preferred stain for bone; it stains calcium in the extracellular matrix, although it will react with

other metals (Humason, 1979) (Fig. 5C,D). For obvious reasons, decalcified specimens will not give a positive reaction to the stain, nor will precalcified bone matrix (osteoid). Also extremely thin or otherwise small bones may be difficult to see in whole mounts because of the faintness of the red color.

D. Combining Immunocytochemistry with Alcian Blue Staining

BABB does not solubilize Alcian blue. It is therefore possible to combine immunocytochemistry with Alcian blue staining. This has the advantage, particularly in later stage embryos, where staining of cartilage can provide a reference with which to compare the pattern of antibody staining (Fig. 3E). Our procedure for this is simple (Table III) and essentially consists of the first part of the standard Alcian blue staining procedure (Table II) followed by standard whole-mount immunocytochemistry (Table I). The combination of these methods allows a direct bridge between immunocytochemistry and more classic cartilage and bone staining methods (Fig. 3). In our experience, however, specimens must first be fixed in Dent fixative.

E. Using Whole-Mount Methods Effectively

1. Importance of Sectioning

Whole-mount methods must be used in conjunction with section-based methods to produce a complete and accurate view of the specimen. Judicious sectioning provides two types of information (Fig. 6). First, it reveals

TABLE III

Alcian Blue/Immunocytochemistry Double Staining

1. Fix specimens in Dent fixative (Table I).
2. Rinse in distilled water (several changes) overnight. Blot on paper towels.
3. Immerse in Alcian blue (Table II). Blot on paper towels.
4. Bleach overnight in Dent bleach (Table I).
5. Rehydrate in TBS for 15 minutes.
6. Incubate in primary antibody, wash, and incubate in secondary antibody (Table I).
7. Wash and react for 1 to 2 hours in 0.5 mg/ml DAB diluted into TBS plus 0.02% hydrogen peroxide.
8. Stop the reaction with methanol and clear.

the exact position of the stained material with respect to surrounding cells and tissues (Dent *et al.*, 1989; Chu and Klymkowsky, 1989). This type of information can be quite difficult to extract from whole-mount images. Second, weakly or diffusely stained components are more easily recognized in sections versus whole mounts. A particularly dramatic example of this comes from our work on the distribution of type II collagen in the fire-bellied toad, *Bombina orientalis* (Seufert *et al.*, 1990). Whole-mount images of anti-type II collagen-stained embryo reveal the major collagen type II-containing regions associated with the notochord, axial segments, brain, and otic vesicles (Fig. 6A,B). Sectioning reveals additional type II collagen immunoreactivity in specific regions of the dermis (Fig. 6C,D). This dermal collagen was completely overlooked during the initial examination of whole-mounts specimens (Fig. 6). A similar caveat applies to the interpretation of bone- and cartilage-stained whole-mounts. Thin layers of calcified bone matrix that characterize the initial stages of ossification, and which are readily visualized in sections, often are invisible in whole mounts. For this reason, whole mounts cannot be used reliably to describe certain aspects of the early stages of bone development, such as the absolute timing of osteogenesis (Hanken and Hall, 1988). However, the combination of whole-mount and section-based analysis easily generates a complete description of the embryo.

Preparation of sections sometimes can be enhanced, and always simplified, by staining the specimen in whole mount. The specimen can also be examined first in whole mount and then sectioned if necessary, to provide a complete description (Table IV). It is difficult to counterstain sections once they have been cleared. If counterstaining is desired, the specimen can be stained in whole mount and then sectioned without prior clearing (Table IV). Originally we used a version of Steedman's polyethylene glycol:cetyl alcohol-based embedding medium for sectioning analysis (Dent *et al.*, 1989). Such sections were fragile and difficult to handle. We have therefore switched to using Paraplast as an embedding medium (Table IV).

2. Whole-Mount Labeling in Practice

Once an antibody against a protein has been generated it must be characterized with respect to specificity. This is generally done by Western blot analysis, preferably of impure samples. If an antiserum is not completely specific, it can be made specific by affinity purification, either on nitrocellulose blots (Olmstead, 1981) or using antigen columns (Hudson and Hay, 1980). The specificity of monoclonal antibodies that are not originally monospecific cannot easily be improved. Moreover, the specificity of an antibody in Western blots does not necessarily guarantee its specificity in cytochemistry. Therefore, cytochemical specificity must be assayed directly. In addition,

TABLE IV

SECTIONING WHOLE-MOUNT IMMUNOSTAINED EMBRYOS[a]

1. Follow the protocol for whole-mount immunohistochemistry (Table I) through Step 9. Stop the reaction by washing embryos in TBS.

Embryos that have been cleared in BABB can be transferred directly into Histoclear (Step 3).

2. Wash specimens in 70% ethanol for 1 hour and then in 95% ethanol for 1 hour, then wash twice in absolute ethanol for 30 minutes each time.

3. Wash twice in Histoclear, 1 hour each.

4. Immerse in molten Paraplast for 1 hour, replace paraplast and let sit for another hour, and then embed in Paraplast.

5. Cut as 6 to 10-μm serial sections using a microtome and mount on glass slides. We typically mount sections on albumin-coated slides.

6. Wash slides twice with Histoclear, each wash 3 minutes or longer.

Note: BABB-cleared specimens do not stain with eosin, so Steps 7–10 can be omitted and the specimens mounted with cover glass following Step 6. Even in uncleared specimens, one can omit eosin staining if desired. In that case go directly to Step 11.

7. Wash slides twice in absolute ethanol, each wash 3 minutes or longer.

8. Wash slides in 95% ethanol and then 70% ethanol, each wash 3 minutes or longer.

9. Incubate in eosin for 20 seconds and then rinse in 70% ethanol for 2–3 seconds, followed by a 2- to 3-second rinse in 95% ethanol.

10. Wash twice with 100% ethanol (3 minutes each), then rinse twice in Histoclear (3 minutes each).

11. Mount cover glasss using Permount. Immunostained areas will appear dark brown. Nonstained regions will appear faint orange or pink (in eosin-stained specimens). Slides appear permanent; we have stored them for over 1 month without apparent deterioration of the specimens.

[a]Eosin is used as a counterstain to visualize background tissues and more effectively localize immunostained regions. Histoclear (National Diagnostics, Manville, NJ) is a nontoxic, low-odor histological clearing agent that may be substituted for traditional agents such as toluene and xylene. Permount is sold by Fisher Scientific, Paraplast by Monoject Scientific (St. Louis, MO). Additional details concerning procedures for infiltration, embedding, sectioning, and mounting can be found in standard histology manuals (e.g., Humason, 1979).

fixation conditions that preserve the immunoreactivity of the target protein and its normal distribution within the specimen must also be determined. Both of these goals are largely a matter of trial and error.

In the best case, the distribution of the target molecule in at least one stage of organismic development is already known. That stage can then be used to define appropriate fixation conditions and antibody specificity. In addition, this stage can be used to determine the appropriate working dilutions for both

primary and secondary antibodies. It is particularly helpful if one has available an antibody whose staining properties are already fairly well defined. This antibody can be used to determine the appropriate working dilution for the secondary antibody. Determining the working dilution for the secondary antibody is particularly important; in our experience most difficulties in the successful use of whole-mount staining arise from the use of secondary antibodies at inappropriate dilutions. The optimal working dilutions of commercially available secondary reagents (from the same source) can differ by over 10-fold. Using a secondary reagent at too low a dilution will generate excessive background staining; too high a dilution will result in a weak or nonexistent signal. Once the working dilution of the secondary antibody has been determined, it is then critical to determine that the secondary antibody does not react with the specimen in the absence of primary antibody. Although fortuitous reactions of primary (Gordon *et al.*, 1978) and secondary (Strome and Wood, 1982) antibodies can occasionally be quite helpful, unexpected reactions could be embarrassing if not recognized.

The working dilution of a particular secondary antibody will be the same for each primary antibody/antiserum used. This makes it possible to titer the primary antibody. Typically, we use monoclonal antibody supernatants at dilutions from 1 : 5 to 1 : 100; ascites fluids from 1 : 100 to 1 : 10,000; and rabbit antisera from 1 : 50 to 1 : 10,000. Again, as in the case of the secondary antibody, too low a dilution will generate excessive background, whereas too high a dilution will fail to stain the target tissue adequately.

When the distribution of a particular target molecule is known for a particular specimen, the staining pattern observed actually provides a further measure of antibody specificity. It is important to remember when comparing Western blots and immunocytochemical assays for antibody specificity that target molecule/antibody reactions can be quite different under these different assay conditions, and different classes of molecules may well react with the antibody. For example, a number of antibodies have been characterized that appear to be specific in immunocytochemistry but fail to react with their expected target molecule on Western blots. The reverse situation can also occur; in other words, a molecule can react on blots but fail to react in immunocytochemistry. Failure to react in immunocytochemistry can be due to destruction of the target epitope by fixation, loss of the target molecule from the specimen owing to poor fixation, or inaccessibility of the target epitope in the fixed specimen. Where unexpected reactivities occur, it is best to test other antibodies known to react with the same target molecule. If these are not available, care in interpretation of results must be taken, as it is possible that the target molecule is expressed in the unexpected position or that the reactivity is due to an unexpected but immunoreactive molecule. If sufficient material is available, the presence or absence of the target molecule can be determined directly by Western blots or immunoprecipitation. Unfortunately, when using monoclonal antibodies or affinity-purified antisera,

absorption with the target molecule provides little additional information, since we know *a priori* that such absorption will remove all immunoreactivity.

For most studies, we find immunoperoxidase staining quite effective. It has very low background and easily reveals proteins of moderate abundance (Fig. 2–6). Low-power lenses can be used to provide a global overview of the distribution of the target molecule (Figs. 2A, 3A,B, and 4), whereas higher power lenses (Figs. 4C,D, 5A,B, and 6A,B) and sectioning (Fig. 6C,D) can be used to further define the exact distribution of the target molecule. When we were originally working on whole-mount immunocytochemistry, we were concerned about whether the size of the specimen would pose an insurmountable obstacle to the diffusion of antibodies into and out of the specimen. Over the years, however, we have applied the method to larger and larger specimens. In our latest work, we have examined the distribution of type II collagen and fast- and slow-skeletal muscle myosins in the direct-developing frog *Eleutherodactylus coqui* (Hanken *et al.*, 1990). These embryos are 4–5 mm across, yet antibodies appear to penetrate throughout quite effectively (Figs. 3 and 4). We have not yet reached the upper limit for specimen size in whole-mount immunostaining.

3. Double Staining in Whole Mount

Reliable methods for double staining cartilage and bone are available (Fig. 5), and it is possible to double stain embryos with antibodies and Alcian blue (Fig. 3E). However, double-antibody labeling remains problematic. We have used DAB staining together with polarization optics to follow the interaction between outgrowing neurites and the muscle of the lateral myotome (Chu and Klymkowsky, 1989). However, only select structures can be visualized using polarization optics. Fluorescent-based secondary reagents may be quite useful, but in most cases they will need to be analyzed using a confocal microscope. Even with a confocal microscope, however, true *in toto* imaging of the embryo at the cellular and subcellular level will await the development of high-resolution, long-working distance lenses. When available, double fluorescence imaging of whole-mount stained specimens will open the oocyte and embryo as a readily accessible system in which to study the wide range of cell functions that underlie embryonic development.

III. Formulations

Mounting Medium (for fluorescently labeled speciemens)

Dissolve 10 g airvol 205 (polyvinyl alcohol; Air Products, Inc, Allentown, PA) in 40 ml 50 m*M* Tris, pH 8.0. This takes 24 to 48 hours at 37°C. Add

20 ml glycerol and 1.2 g *n*-propyl gallate. Aliquot and store at 4°C. This mounting medium will dry within 2 to 4 hours. The propyl gallate will reduce the rate of bleaching for fluorescein-conjugated antibodies.

Dent Fixative

1 part DMSO, 4 parts 100% methanol

Aldehyde Fixative

0.1 *M* MOPS, 2mM EGTA, 1 m*M* $MgSO_4$, 3.7% formaldehyde, pH 7.4 (Hemmati-Brivanlou and Harland, 1989).

Dent Bleach

1 part 30% hydrogen peroxide, 2 parts Dent fixative

BABB Clearing Agent

1 part benzyl alcohol, 2 part benzyl benzoate

Alcian Blue Working Solution

20 mg Alcian blue 8GX (C.I. 74240), 70 ml absolute ethanol, and 30 ml glacial acetic acid. Use at room temperature; store refrigerated. Discard after 6 months.

Alizarin Red Stock Solution (Humason, 1979)

5.0 ml alizarin red S (C.I. 58005; alizarin sodium sulfonate) saturated in 50% acetic acid, 10.0 ml glycerol, and 60.0 ml chloral hydrate, 1% aqueous. Keeps indefinitely at room temperature.

Buffered Formalin (Humason, 1979)

100 ml concentrated formalin (40% formaldehyde-saturated water), 900 ml distilled water, 4.0 g sodium phosphate, dibasic (monohydrate), and 6.5 g anhydrous sodium phosphate, monobasic.

Eosin Stock Solution (Humason, 1979)

1.0 g eosin Y (C.I. 45380), 1 liter 70% ethanol, and 5.0 ml glacial acetic acid. Dilute with an equal volume of 70% ethanol before use and add 2–3 drops of acetic acid.

IV. Conclusion

Whole-mount staining makes the analysis of normal and experimentally manipulated embryos much simpler. It can be used in the assay of cellular differentiation in induction and tissue recombination experiments (see Chapters 17 and 18 this volume). It should be possible not only to assay for the indication of specific tissues, but to characterize the three-dimensional relationships between the tissue types. Whole-mount staining greatly simplifies the characterization of the expressions patterns of exogenous DNAs. Similarly, the effects of injected antibodies, antisense reagents, or the ecto-

pic expression of specific molecules (Chapter 23, this volume) on development can be analyzed rapidly.

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