Whole-Mount Immunocytochemistry in Axolotl Embryos

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Immunocytochemistry has become an important technique for detecting specific antigens within cells and tissues. Most protocols for this technique are designed for use on cultured cells and/or sectioned material. However, sectioned material can provide only two-dimensional views of antigen distribution within the plane of the sections. This is particularly limiting when studying development, since an understanding of three-dimensional patterns is of the utmost importance. Previously, the only way of overcoming this difficulty was through tedious serial-sectioning and reconstruction procedures.

A striking improvement in immunodetection has recently been reported by Dent and colleagues (1989), who have developed a protocol for whole-mount immunocytochemistry in Xenopus embryos. The 3-D distribution of antigens can, therefore, be observed directly, and the angle(s) at which a specimen will be sectioned can be chosen (if one wishes to study the tissues in more detail). Indeed, if the marker chosen for the immunolabelling is stable through processing, the same specimen may be sectioned after it has been stained.

In this report, we describe some modifications of this procedure which permit whole-mount immunodetection in larger axolotl embryos and discuss some of the pitfalls which may be encountered. In addition, we describe the use of an alternative to the markers usually used for immunodetection.

Fixation. As a fixative, we have found that "Dent's fixative" (80% methanol, 20% dimethylsulphoxide, v/v) is quite satisfactory for many purposes. Since this is a mixture of two lipid solvents, it has the advantage of permeabilizing the specimen at the same time as it fixes. In addition, it acts so quickly that there is little curling of older embryos and larvae. Overnight fixation at room temperature, as described by Dent et al. (1989) is quite adequate.

There are two disadvantages to the use of this fixative. The first is that embryos become very brittle and tend to shatter. This is particularly true of blastulae, and of gastrulae or early neurulae, where the thin roof of the blastocoel or archenteron, respectively, tends to crumble. In most instances, this can be minimized by avoiding overly vigorous agitation of the embryos.

The second disadvantage is that methanol does not cross-link cellular components like a true fixative, but merely precipitates some of them. Therefore, while Dent's fixative is effective for larger structural proteins, small, soluble ones may be extracted by it. This problem may be overcome by fixing the specimens for a day or so in any of several standard glutaraldehyde- and/or formaldehyde-containing fixatives, to cross-link some of the smaller components, prior to placing them in Dent's solution. The use of these fixatives also seems to lessen the brittleness described above. However, it must be stressed that Dent's solution or some substitute (such as a detergent) must still be used to permeabilize the specimen and allow the antibodies to penetrate.

Bleaching. Bleaching of the embryos allows the stain to be observed without interference from pigment and has the added advantage of destroying endogenous peroxidase activity (Dent et al., 1989). The latter is important if horseradish peroxidase (HRP) is used as a marker. Bleaching is accomplished by placing the specimens in Dent's solution containing 10% (final concentration) H_2O_2 at room temperature. We have found that 2 days, which is sufficient to bleach Xenopus embryos (Dent et al., 1989), is not sufficient to bleach larger axolotl embryos. Therefore, we recommend that axolotl embryos be left in this solution for at least one week, with a change of solution every 3-4 days. Even after a week, the more heavily pigmented areas of wild-type embryos will appear pale brown. Depending on the marker used, this should be sufficient.

If bleaching is undesirable, we suggest that albino or albino/axanthic embryos be used. Note that endogenous peroxidases may remain in unbleached embryos.

Following fixation and bleaching (if desired), embryos may be stored indefinitely at -20°C in absolute methanol, as reported by Dent et al. (1989).

Immunodetection. Staining of the embryos may be performed using a slight modification

of the procedure described by Dent et al. (1989). The major change for axolotl embryos is to increase the time for all steps to correct for the larger size of the embryos. Throughout the procedure, the embryos should be gently agitated at 4°C.

From absolute methanol, the specimens must first be rehydrated to an appropriate buffer, such as Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.85% NaCl, pH 7.6). While Dent et al. (1989) do this directly, we feel that 15 minute washes in 75%, 50%, and 25% methanol:TBS, followed by three 15 minute washes in TBS is less drastic. The specimens are then incubated in the primary antibody diluted with TBS. An exogenous protein source is usually also added to minimize non-specific adsorption of the antibody. Dent et al. (1989) used 20% calf serum, but we have found that 0.2% bovine serum albumin (BSA) will usually suffice. A 24-hour incubation period is sufficient to stain the tissues of even small larvae.

The embryos should be washed for 24 hours in at least 5 changes of TBS/BSA. Even longer washing periods do not seem to affect the staining. This incubation and washing procedure should be repeated for the secondary antibody and marker (if necessary). Finally, embryos stained with enzymatic markers are immersed in a developing solution for the appropriate time, the reactions are stopped by dehydrating them in methanol, and they are cleared in a benzyl alcohol:benzyl benzoate mixture (1:2, Dent et al., 1989).

Markers. In their study, Dent et al. (1989) use secondary antibodies conjugated to HRP and alkaline phosphatase (AP). However, we have found it useful to use a biotinylated secondary antibody. Although it necessitates further incubation (with streptavidin conjugated to a marker) and washing steps, this has two major advantages. First, a variety of markers linked to streptavidin (SA) are commercially available, allowing one to change markers without changing secondary antibodies. The second is that the highly specific biotin-SA binding allows one to magnify the signal, if necessary, by incubating in successive washes of SA and biotin prior to the marker-SA conjugate. Since the binding is highly specific, little or no background staining is seen.

The choice of marker is a personal one, and depends on the type of signal desired. However, we wish to report that striking results can be obtained using a commercially available (BRL) streptavidin-β-galactosidase (SA-β-gal) conjugate. This is simply diluted in TBS-BSA and the embryos are incubated in it for 24 hrs at 4°C, and washed for 24-48 hours as described above. The colour is then developed by incubating the embryos at room temperature or 4°C in the buffer described in the accompanying product information (0.42 mg/ml X-gal in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 3.0 mM potassium ferricyanide, 3.0 mM potassium ferrocyanide, 1.0 mM $MgCl_2$).

The use of β-gal as a marker has several important advantages over more the commonly used HRP, AP, and fluorescent markers. First, β-gal does not require the specialized optics which fluorecsent markers require.

Second, the reaction product formed by X-gal (in the presence of β-gal) has a distinct, dark-blue colour which is easily visible (even in embryos which have not been cleared). This is a particularly important advantage over HRP and AP, which tend to have paler reaction products. Indeed, the pale brown reaction product formed by HRP acting on diaminobenzidine and H_2O_2 could easily be confused with the pale brown colour which remains after bleaching.

Third, the reaction occurs quickly. The blue reaction product usually begins to appear within 15-30 min, compared with reaction times of 2-6 hrs (HRP) and 1-3 hrs (AP) reported by Dent et al. (1989).

Fourth, β-gal is a microbial enzyme which is not present in the embryos. This eliminates the need to inhibit endogenous enzyme activity, as with HRP and AP, without worrying about background activity. Therefore, albino embryos may be stained immediately after fixation without bleaching.

Finally, the reaction product formed by β-gal acting on X-gal is highly stable and insoluble. This means that the embryos may be dehydrated and embedded in glycol methacrylate (M. Whiteley, pers. comm.) or paraffin without loss of the colour. The embryos may, therefore, be stained first and sectioned later. Indeed, the colour is so distinct that unbleached wild-type embryos may be stained and sectioned. While heavily-pigmented embryos may obscure some of the stain in whole-mounts, it is clearly visible in the sections. As well, the stain is stable enough to allow bleaching of the embryos after they have been stained (M. Whiteley, pers. comm.).

While the product information supplied

with the SA-β-gal conjugate warn that its large size may prevent it from reaching some intercellular components, we have have been able to stain a variety of larger cytoplasmic proteins, even in larval axolotls of 1-1.5 cm in length.

Reference:

Dent, J.A., A.G.Polson, and M.W. Klymkowski. 1989. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in Xenopus. Development *105*:61-74.