

# A New Method to Prepare Sections from Amphibian Embryos for Immunohistology

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The method involves impregnation of fixed embryos with a solution of plexiglass. The solvent is evaporated slowly. Blocks of plexiglass containing the specimen are thus formed. Sections (1–2  $\mu\text{m}$ ) are stretched and attached by an unconventional method. Immunohistological techniques allow antigen localization in a combination of high resolution, excellent quality of the sections and low background.

## Introduction

The developing amphibian embryo has long been an object of intensive investigations and the field has gained a vigorous revival in the last years. This was, besides a general rise of interest in embryological questions, primarily due to new techniques, which allow the easy tracing of specific molecules in the developmental processes. Proteins are mainly assayed by immunological methods and the introduction of the monoclonal antibody technique has greatly facilitated this approach.

Since morphological studies are a precondition of embryological investigations, antibody staining of histological sections is a standard technique. The application of this method to amphibian embryos is notoriously difficult for several reasons.

The material is, due to the high yolk content, very brittle and it is therefore not easy to obtain sections with adequate preservation after paraffine or polyester wax embedding. Plastic embedding gives much better results but in most cases the plastic cannot be removed from the sections in order to render them accessible to the antibodies. After fixation with aldehydes the yolk material exhibits a high autofluorescence, which disturbs the unequivocal tracing of the specific fluorescent dye. Furthermore a specifically fluorescent structure becomes a localized source of light emission upon illumination with the excitation light. The high turbidity of the surrounding material generates a Tyndall effect, which limits the microscopic resolution.

To handle these obstacles we have searched for a plastic embedding technique, which allows sectioning at 1–2  $\mu\text{m}$  thickness. The aim was to enhance resolution and minimize the disturbing autofluorescence. Good stretching and adhesion of the sections had to be elaborated, under conditions which disturb the morphological preservation to the least possible extend. The plastic should be soluble in solvents which preserve the antigenic activity of the proteins under study. This would allow removal of the plastic prior to staining. Jamrich *et al.* [1] have used methylmethacrylate (plexiglass) as embedding agent, which was polymerized within the specimen. This could be removed from the section by chloroform treatment, allowing to use the section for *in situ* nucleic acid hybridization studies. We have found, however, that the polymerization process, which generates a transient occurrence of free radicals, largely reduces the antigenicity of proteins. Gorbisky and Borisy [2] have used the method of infiltrating a prepolymerized plexiglass solution into fixed tissue culture cells which, after slow evaporation of the solvent, resulted in embedded material which could be adequately sectioned. After removal of the plexiglass, staining of the sections with fluorescent antibodies was possible.

This communication describes an adaptation of this method to amphibian embryos. After a number of serious obstacles had been surpassed, a method evolved which gives very satisfactory results avoiding the difficulties and drawbacks described above.

## The Method

Embryos are fixed at the desired stages with Romeis fixative (20 ml of 5% trichloroacetic acid,

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15 ml of 37% formaldehyde and 25 ml of saturated mercury chloride) [3] for 2 h and then directly transferred to absolute ethanol. The application of graded series of ethanol concentrations causes undesirable swelling of the specimen and is therefore not recommended. This fixative gives a good gross preservation of tissue morphology although the fine structure appears fairly coarse. Depending on the aim of the study other fixatives may be applied with similar success.

After thorough dehydration 3 times for 2 h in water-free ethanol, the embryos are transferred to ethanol–dichloromethane 1:1 and then to dichloromethane, 2 h each. The procedure may be interrupted at any stage after the fixation for overnight storage.

A solution of 200 g plexiglass granules in 1 l dichloromethane is prepared by stirring overnight. The embryos are transferred to a half strength plexiglass solution, which is exchanged for the quite viscous full strength solution after 2 h.

The specimen are then placed into petridish-like polypropylen vials filled up to a height of 1 cm with plexiglass solution and the vials are tightly closed. The plexiglass is allowed to infiltrate the tissue for three days. A 3 mm hole is then punched into the cover allowing a very slow evaporation of the solvent dichloromethane. This slow evaporation is required to ensure that the slowly concentrating plexiglass solution is permanently in equilibrium with the interior of the embryo. In spite of this caution is might happen that in the very sensitive late blastula and gastrula stages the blastocoel roof collapses. A small hole pierced into the embryo after fixation helps to avoid this minor drawback.

After one week at room temperature the plexiglass has hardened to a 3 mm thick disc which is removed from the vial by cutting off its polypropylene walls. It is advisable to store the plexiglass disc for a few more days before sectioning.

Blocks containing the specimen are cut from the disc by means of a saw and mounted in proper orientation on plastic microtome holders with a mounting resin.

Sections at 1–2  $\mu$ m thickness are obtained using Ralph's glass knives.

Stretching and attachment of the sections to the glass slide has to be carried out in the following rather unconventional way since all other attempts had failed to give satisfactory results.

A 10  $\mu$ l drop of water is placed on a 1 cm round coverslip, coated with chromalumn gelatine, and a section is floated on top of it. The plexiglass, being completely water insoluble, does not allow stretching of the section. If, however, the coverslip is submerged in dichloromethane in a petridish, the water drop adheres to the coverslip due to its surface tension and the embedding plexiglass floating on the water is rapidly dissolved. The tissue section is very evenly stretched at the dichloromethane/water interface.

The preparations are kept under dichloromethane overnight; many sections may be collected in one petridish. During this time the water is slowly taken up by the dichloromethane, while the sections very gently attach themselves firmly to the coverslips.

After removal of the dichloromethane from the sections by passing them through a graded series of ethanol into phosphate buffered saline, the sections are ready for antibody staining using conventional methods.

The results, presented below as examples, were obtained by using mouse monoclonal antibodies and FITC labelled goat antimouse antibodies as secondary antibodies. Biotinylated lectins were used to stain the sections for glycoconjugates and lectin binding was visualized with FITC conjugated avidin.

## Products

Plexiglass granules were: Plexiglass Formmasse, Röhm Chemische Fabrik, D-6100 Darmstadt 1, F.R.G. Polypropylene vials were obtained from Brand, D-6890 Wertheim, Cat. Nr. 61815. Mounting resin was K-Mount from Medim, Medizinische Diagnostik-Methoden, Gießener Straße 116, D-6300 Gießen, F.R.G. Biotinylated lectins were purchased from Sigma and FITC-goat-anti-mouse antibodies and FITC-streptavidin from Amersham, U.K.

## Results and Discussion

Fig. 1 shows the phase contrast image of a section through a *Xenopus laevis* oocyte. Higher magnification of such a section reveals the excellent preservation of structural details. Staining of this section with the monoclonal antibody b7-1D1 [4] which is directed against the nuclear protein nucleoplasmin is shown in Fig. 1b. The antigenic protein is strictly localized in the nucleus, in accordance with many other reports. The absence of virtually any back-



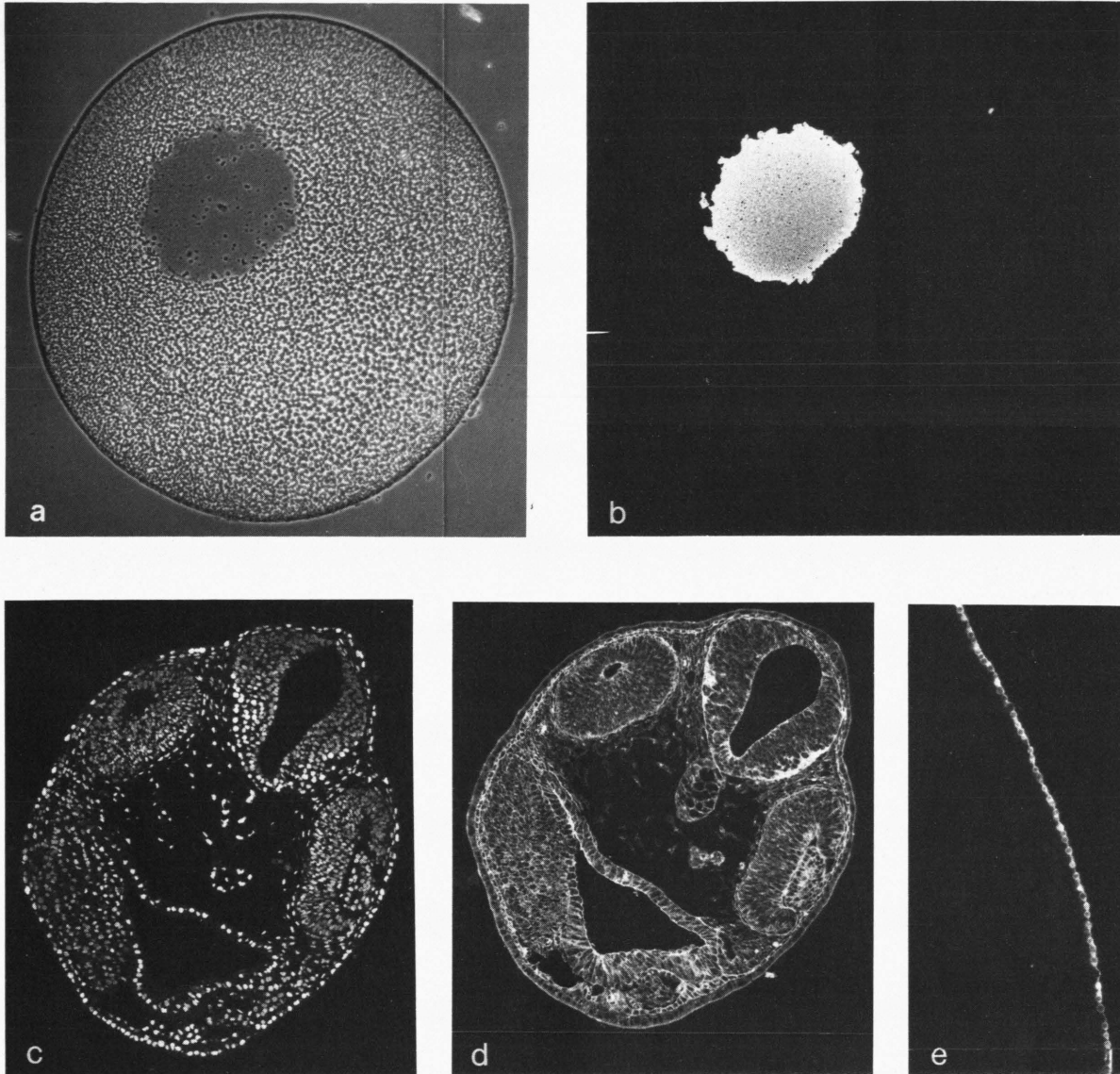


Fig. 1. a) and b) Section through a *Xenopus* oocyte; a) phase contrast; b) staining with monoclonal antibody b7-1D1 against nucleoplasmin. Magnification  $65\times$ . c) Section through the head of a stage 38 tadpole stained with monoclonal antibody b7-1D1. d) Adjacent section stained with phytohemagglutinin (*Phaseolus*). Magnification  $65\times$ . e) Section through the cortical region of an oocyte stained with monoclonal antibody directed against cortical granule membranes. Magnification  $400\times$ .

ground-staining or autofluorescence in the cytoplasm should be emphasized. The sharp rim of the stained nuclear area follows the convoluted outline of the nuclear lamina in fine detail.

The quality of structural preservation can also be recognized in Fig. 1c and Fig. 1d. Monoclonal anti-

body b7-1D1 was used to stain the nuclei of a section through the head of a stage 38 tadpole, (Fig. 1c) and the lectin from *Phaseolus vulgaris* was used to stain the glycoconjugates of the cell surfaces and the extracellular matrix (Fig. 1d) in an adjacent section. As may be seen from the latter picture, individual cell

coats and the basal laminae are well decorated by the lectin.

The high resolution of the technique is demonstrated in Fig. 1e. A monoclonal antibody with unknown specificity was found to bind specifically to the envelopes of the cortical granules positioned in the cortical cytoplasm of the oocyte. The 100 $\times$  lens resolves details in these bodies without interference by the otherwise disturbing Tyndall effect.

The illustrations demonstrate that the difficulties of antibody or lectin staining which are described in the introduction are mostly solved by this new method.

A serious drawback of the method is the time-consuming way of preparation of embedded speci-

men. However, once embedded, the embryos may be stored for a long time, at least several months, without recognizable loss of antigenic activity.

It depends on the antigen whether its binding epitope retains its affinity for the antibody throughout fixation and in the strongly hydrophobic environment of the plastic solvent. Thus only a fraction of the antibodies tried gave good results. In some cases the antigen may be renatured by exposing the section, after removal of the plastic to 3 M area overnight as has been described earlier [5].

We have used the method described with invariably good success for over one year now. We expect that it may also be useful for other embryos or tissue preparations.

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