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Technical Article

Immunocytochemistry of the amphibian embryo - from overview to ultrastructure

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ABSTRACT Amphibian embryos are standard research objects to study pattern formation and morphogenesis. Due to their external development and robust nature, experimental manipulations such as microinjections or transplantations can be easily performed. However, most immunocytochemical approaches addressing the specific localization of proteins are hampered by the fragility of the large and yolky embryonic cells which render high resolution staining difficult. Immunocytochemical data are therefore often restricted to either overall patterns in whole embryo preparations or to immunofluorescent localization with limited resolution on sections. High resolution or ultrastructural protein localization data are rare and can be achieved only with time consuming procedures. Here, a comparative study of immunocytochemical methods suitable for light and electron microscopy using different kinds of plastic resins is presented. Three main approaches are described: preembedding staining of whole embryos, postembedding staining of ultrathin sections and preembedding staining of vibratome sections. All the procedures are designed to study protein expression in early amphibian embryos *en gros* as well as *en detail* and the described techniques are suitable to combine two or three levels of resolution on the very same biological specimen. Examples are presented and advantages and disadvantages of the different protocols are discussed.

KEY WORDS: *embryo, Xenopus laevis, immunocytochemistry, immunofluorescence, immunoelectron microscopy*

Introduction

Amphibians, especially the South African clawed frog *Xenopus laevis* and its near relative *X. tropicalis,* are important model organisms in cell and developmental biology. Embryos can be obtained in large amounts, kept in simple buffer solutions, and development can be easily observed from the outside. In addition, a plethora of experiments such as microinjections of proteins, DNAs and RNAs as well as different microsurgical manipulations are well established since the embryos are relatively large and tolerate a wide range of experimental perturbations. The analysis of such experiments combines phenotype analysis, histology, RT-PCR, Northern blotting, *in situ* hybridization, western blotting or immunocytochemical methods. Detailed examination of *in situ* patterns of protein localization is, however, hampered by several pitfalls. 1. The yolk-rich early embryos are difficult to fix, even for conventional ultrastructural studies (see for example Kalt and Tandler, 1971). In order to obtain appropriate structural preservation for histological and ultrastructural studies strong fixatives are needed. A mixture of formaldehyde and glutaraldehyde has proven to be the best choice to obtain good ultrastructural preservation of early *Xenopus* embryos (Karnovsky, 1965; Müller and Hausen, 1995). The excellent crosslinking of proteins achieved by this fixative, however, is in conflict with antigen preservation, as most antigens tolerate only mild or alcohol based fixatives. Therefore, a compromise between antigen and fine structure preservation must be established empirically for nearly each antigen. 2. The proposed intracellular distribution and the degree of anchorage of the targeted protein to organelles or to the cytoskeleton has to be considered when designing the fixation protocol. Soluble proteins need crosslinking reagents such as formaldehyde in the fixative, whereas membrane or cytoskeleton anchored proteins can be fixed in alcohol solutions. 3. In whole embryo preparations antibody penetration is poor resulting in long incubation times or harsh treatments to facilitate antibody diffusion. Both options are antagonistic to a proper ultrastructural preservation. Taken together, these obstacles prevent the establishment of a general staining protocol suitable for all antigens.

Abbreviations used in this paper: aj, adherens junction; ap, apical; bl, basolateral; nu, nucleus; TEM, transmission electron microscopy; tj, tight junction.

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Therefore, refined immunocytochemical approaches are often neglected in amphibian embryology. Protein localization data are mostly restricted to overall examination in whole-mount preparations using enzyme-coupled detection systems (e.g. notochord or muscle staining (Sive *et al.,* 1999), active MAP-Kinase (Christen and Slack, 1999; Curran and Grainger, 2000), phospho-Smads (Faure *et al.,* 2000), β-catenin (Schneider *et al.,* 1996)), to immunofluorescent staining of cryo-sections (Fagotto, 1999; Schohl and Fagotto, 2002), or to the analysis of sections from embryos stained whole-mount and thereafter embedded in plastic resins and sectioned (e.g. Angres *et al.,* 1991; Gawantka *et al.,* 1992; Schneider *et al.,* 1993; Joos *et al.,* 1995; Kurth *et al.,* 1999). Most of these protocols yield poor structural preservation and limited resolution. High resolution immunofluorescence by postembedding labeling of ultrathin sections, a method successfully applied to adult tissues (Schwarz *et al.,* 1993, Kurth *et al.,* 1996), has so far only been sporadically used for amphibian embryonic tissues (Fagotto *et al.,* 1999; Kurth *et al.,* 1999). Similarly, ultrastructural aspects of protein expression are difficult to obtain and therefore reports on this topic are also rare (e.g. Schwab *et al.,* 1998; Fagotto *et al.,* 1999; Kurth *et al.,* 1999; Fesenko *et al.,* 2000). Although not commonly used, it appears clear that high resolution immunofluorescence and/or immunoelectron microscopy can contribute important aspects to the understanding of protein function in embryonic tissues.

In this article I describe several methods for the immunocytochemical localization of proteins at the light and electron microscopic level. The protocols were designed for *Xenopus* and other amphibian embryos but are also suitable for non-amphibian embryonic systems or for adult tissues. Different approaches were tested and their suitability depends on the proposed localization of the targeted antigen, the questions asked, and the properties of the available antibodies. This is demonstrated with distinct and specific examples using different antibodies and different fixatives. With the proposed approaches the antigens may be observed at different levels of optical resolution, occasionally in the very same specimen. As demonstrated below, the comparison of protein localization data obtained with the stereo microscope, the light microscope and the electron microscope might be essential for the proper interpretation of protein localization.

Results and Discussion

The elucidation of specific spatial and temporal protein localization in early embryos is a key prerequisite for understanding development. It is, however, difficult to obtain protein localization data from early amphibian embryonic tissues with adequate resolution. Different approaches for low to high resolution protein labeling are presented and discussed in this article. Since a complete description of the immunocytochemical methods used in *Xenopus* or other amphibia including e.g. cryo-fixation, cryo-sections or paraffin-sections (see for instance Epperlein *et al.,* 1997, Fagotto, 1999) is beyond the scope of this article, only protocols are considered that are based on sections of plastic embedded specimens.

After some basic considerations about the selection of suitable fixatives and the preferred plastic resins, a brief description of the different protocols and a discussion of their qualities and drawbacks is presented. In general, the immunocytochemical approaches are subdivided into preembedding labeling (staining before embedding into resin) and postembedding labeling (staining of plastic sections after embedding). This report deals in detail with two types of preembedding labeling techniques and with the postembedding labeling of ultrathin sections.

Fixation

Fixation is the critical step in the preparation of histological specimens. Fixation for immunocytochemistry has to yield reasonable preservation of structure on the one hand and of antigenicity on the other. Fixatives that are excellent for preserving tissues and are accordingly used for standard histology and electron microscopy cannot be applied for immunostainings since they are antagonistic to antigen preservation. On the other hand, fixatives used for routine low resolution immunostainings such as mild formaldehyde or alcohol based fixatives yield only poor structural fixation. This is clearly evident when samples are inspected with the electron microscope (see Figs. 1C, 5D and 6B). In the experiments described in this paper different fixation protocols are used: 1) a mixture of methanol/DMSO (Dent *et al.,* 1989) which gives adequate results for immunofluorescent microscopy and can be combined with a prefixation step in a buffered formaldehyde solution for the detection of soluble proteins; 2) a two-step fixation protocol with a weak formaldehyde prefixative and a stronger formaldehyde/glutaraldehyde postfixative, the glutaraldehyde concentration being sufficient for improving the fine structure substantially without affecting antigen recognition or producing autofluorescence; 3) a buffered formaldehyde solution alone. The selection of fixative is dependent on different factors such as the proposed localization and degree of anchorage in the cell, the antigenicity in combination with different fixatives and the required resolution in particular when ultrastructural aspects are to be elucidated.

Plastic Embedding

A number of resins are available for different purposes. For standard light microscopy the glycol methacrylate Technovit 7100 (Kulzer, Germany) was used which is well suited for the immunofluorescent labeling of whole embryos. For transmission electron microscopy (TEM) and preembedding immuno-TEM the first choice is the epoxy resin developed by Spurr (1969). Compared to epon it is less viscous and therefore better suited to the yolk rich early embryos; infiltration, polymerisation and sectioning are handled more easily. The Lowicryl resins HM20 and K4M are excellent for on-section labeling of ultrathin sections (Schwarz, 1994). All the mentioned resins can be cut quite thin (Technovit 7100: 1-2 μm; the others: 50-100 nm).

Preembedding Staining of Whole Embryos (Fig. 1)

A simple protocol for whole mount fluorescent staining starts with fixation in a methanol/DMSO-mixture (Dent *et al.,* 1989; Fig. 1A). For many membrane associated (adhesion molecules, receptors, etc.) or cytoskeleton-anchored antigens this fixative proved to be adequate (Fig.1 B,D,E displaying the localization of cingulin, cadherin, β-catenin and actin in embryonic tissues). For the detection of soluble antigens, an additional prefixation step with 4% buffered formaldehyde was performed (Fig. 1F, nucleoplasmin in the early *Xenopus* gastrula). This prevents extraction of the targeted protein otherwise caused by the fixation with methanol/DMSO alone. After the formaldehyde fixation the embryos were transferred into methanol/DMSO. In this schedule the methanol/DMSO mixture serves as a permeabilization reagent which im-

proves antibody diffusion in the embryo. The fixation/permeabilization procedure is followed by incubation in solutions containing primary and secondary antibodies, postfixation, dehydration and embedding in Technovit 7100. Double immunofluorescent stainings are performed by simultaneous incubation with the different primary and secondary antibodies, respectively (Fig. 1 E,F). Dependent on the size, density, and composition of the embryos incubation times for antibodies vary from overnight to 3 days. These long incubation times are needed for complete antibody penetration. In particular, fixation in formaldehyde solutions results in prolonged incubation times to cope with the slow antibody diffusion in the cross-linked tissue, even after the subsequent permeabilization with methanol/DMSO.

One major advantage of the procedure is that the serial section analysis of a whole mount stained embryo is possible without the need to stain sections independently as it would be the case for cryo- or paraffin-sections (Fagotto, 1999). Further, sections can be cut from 5 μm down to 1 μm which yields results in good resolution at the light microscopical level (Fig. 1 D,E,F). Fluorescence is stable in the plastic blocks for more than 2 years, and in the sections for about half a year. Plastic sections can additionally be counterstained with DAPI to visualize nuclei (see Fig. 1 E,F).

An alternative option for obtaining appropriate resolution is the inspection of stained whole mounts with a confocal laser scanning microscope. However, due to the limited penetration of the laser beam into the tissue only peripheral aspects of large objects such as whole embryos or even thick vibratome sections can be analysed at high resolution (not shown), a problem which can be partially circumvented by clearing the embryos before examination (Kolker *et al.,* 2000; Marsden and DeSimone, 2001). However, an assess-

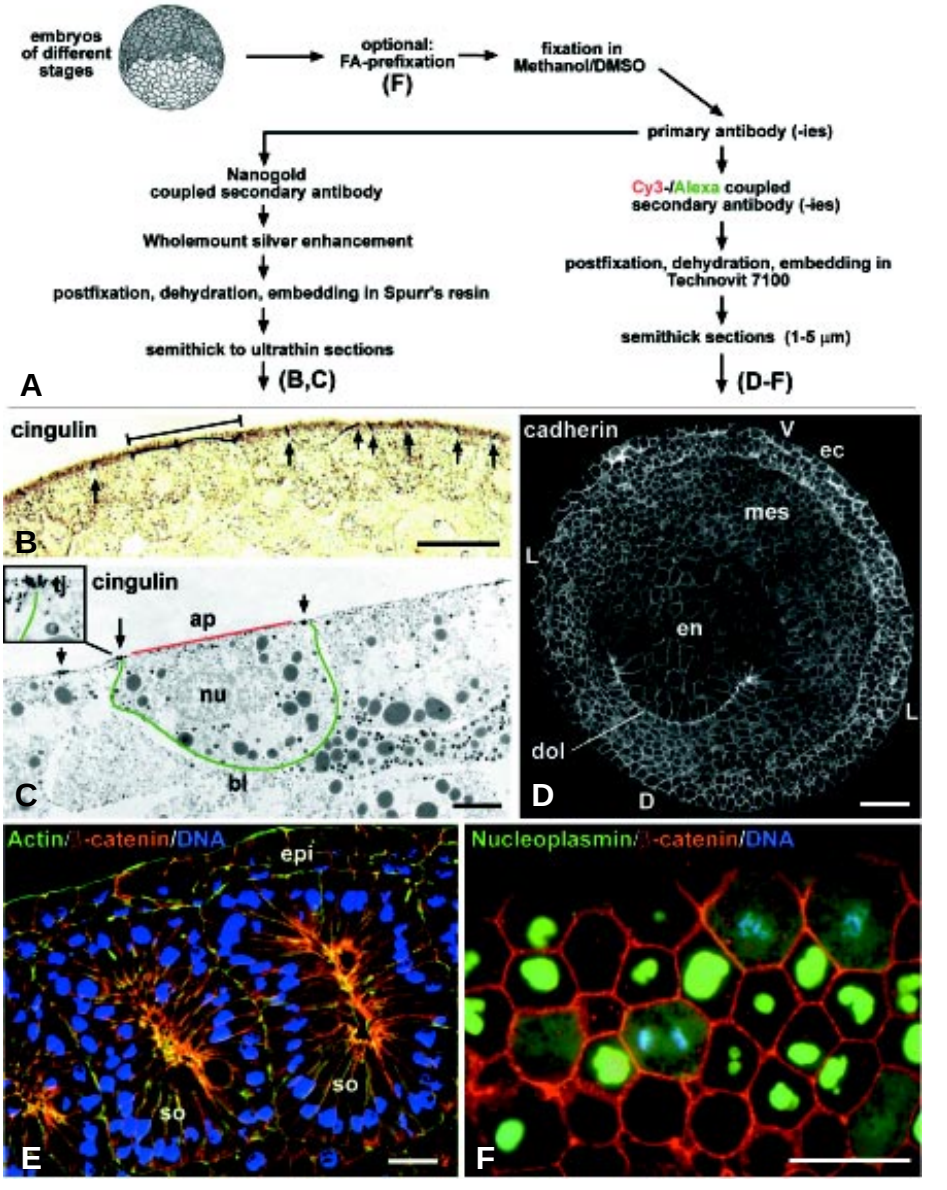


Fig. 1. Preembedding staining of whole embryos for light and electron microscopy. (A) *Schematic summary of the experimental procedures. (B,C) Localization of the tight junction marker cingulin in the outer animal epithelium of a* Xenopus *blastula using an anti-cingulin antibody (C532) and the Nanogold/silver enhancement method. In the light microscope* **(B)***, dark spots (arrows) or strands (black line) demarcate the tight junctions at the borders of the apical and basolateral membrane domains.* **(C)** *Ultrastructural localization of cingulin; tight junctions (tj) are indicated by arrows. The apical (ap) and basolateral (bl) membrane domains are indicated with red and green lines, respectively. One tight junction is enlarged in the insert; nu, nucleus.* **(D)** *Immunofluorescent staining of cell-cell adhesion molecules (cadherins) in a late* Xenopus *gastrula using an anti-cadherin (P35N) and Cy3-coupled secondary antibodies. The section is oriented perpendicular to the animal-vegetal axis and through the marginal zone. As a result, the invagination of the archenteron on the dorsal side is visible as a semicircular arch; D, dorsal; dol, dorsal lip; ec, ectoderm; en, endoderm; L, lateral; mes, mesoderm; V, ventral.* **(E)** *Triple fluorescent micrograph of a sagittal section through a stage 24 axolotl embryo displaying the two-layered epidermis (epi) and somites (so). Red indicates β-catenin (rabbit anti-β-catenin (P14L), Cy3-coupled secondary antibody), green indicates actin (mouse monoclonal anti-Actin, Alexa488-coupled secondary antibody), yellow indicates colocalization, and blue indicates nuclear DNA (DAPI).* **(F)** *Triple fluorescent micrograph of a* Xenopus *gastrula sectioned through the marginal zone. The green fluorescence marks nucleoplasmin (mouse monoclonal anti-nucleoplasmin (b71A9), Alexa488-coupled secondary antibody), a protein involved in a variety of nuclear functions including e.g. nucleosome assembly. Nucleoplasmin diffuses into the cytoplasm when the nuclear envelope breaks down during mitosis. Cell borders are visualized with the antibody against β-catenin (red, P14L, Cy3-coupled secondary antibody). Nuclei are counterstained with DAPI (blue). Bars correspond to 50 μm in (B,E,F), 5 μm in (C) and 200 μm in (D).*

ment of detailed protein distribution in different regions of a given embryo as it can be achieved by serial sectioning of plastic embedded specimens is difficult to attain with this method.

Based on the protocol described above whole embryos were also stained for electron microscopic observation (Fig. 1 A,B,C). For that purpose primary antibodies are labeled with secondary antibodies conjugated to 1-2 nm gold particles (Nanogold). These ultra-small gold particles are visually enhanced by incubation in a solution containing silver lactate/hydroquinone/gum arabic (Danscher, 1981; Lah *et al.,* 1990; Stierhof *et al.,* 1991; Humbel *et al.,* 1995). Stained and deydrated samples were embedded in Spurrs resin for thin and ultrathin sectioning. Semithick (2-4 μm) sections were inspected in the light microscope (Fig. 1B), ultrathin sections in the electron microscope (Fig. 1C). The resulting images can be directly compared since the corresponding sections were cut from the same block. Fig. 1C also displays, however, the major drawback of this method, since the fine structure preservation is rather poor due to the use of the methanol-based fixative. It is only convenient at low magnification (see also Fig. 6B). Another problem is that the silver enhancement has to be carried out blindly in pigmented embryos so that incubation times for the silver enhancement solution are to be empirically established solely based on the final outcome. This problem can partially be solved by the use of albino embryos or bleaching before staining (Sive *et al.,* 1999). Taken these aspects into account, whole mount Nanogold staining and silver enhancement of embryos can only be regarded as an additional option for those antigens which cannot be visualized with any other protocol.

Postembedding Staining of Ultrathin Sections (Figs. 2,3)

Postembedding labeling is widely used for immunoelectron microscopy of adult tissues and is also suitable for fluorescence microscopy (Schwarz *et al.,* 1993; Schwarz, 1994; Kurth *et al.,* 1996). Only the surface of an ultrathin methacrylate section is stained and accordingly the method yields good tissue preservation and excellent light as well as electron microscopical resolution. In Fig. 2 some examples disclosing the localization of β-catenin (Fig. 2 B-E) and actin (Fig. 2E) in adult epithelial tissues are shown. Due to the circumvention of penetration effects different staining intensities clearly correspond to different amounts of antigen. This is exemplified by the different staining intensities for β-catenin in adherens junctions as compared to the remainder of the basolateral membrane (Fig. 2 C,D). Labeling differences can be quantitatively analysed by counting gold granules (Griffiths, 1993) or measuring fluorescence intensities. This is in contrast to all preembedding protocols, where diffusion barriers can contribute substantially to the differences in staining intensities which imposes severe limitations to quantitation. The major drawback of the postembedding labeling method is the limited sensitivity: only proteins beyond a certain amount or a certain degree of concentration (e.g. β-catenin in adherens junctions, Fig. 2) can be detected. The amount and concentration of proteins in early embryonic stages is very often

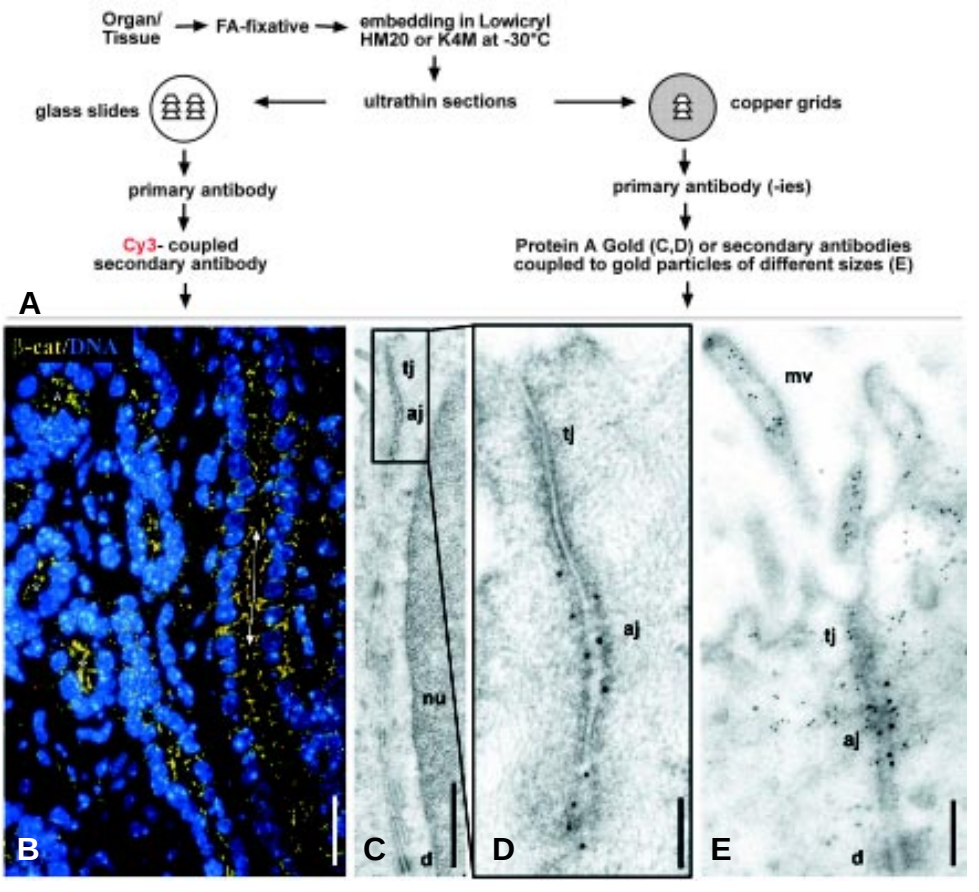


Fig. 2. Postembedding staining of ultrathin sections of adult tissues. (A) *Experimental procedure: ultrathin sections of tissue pieces embedded in methacrylate resins (HM20 or K4M) were stained for immunofluorescence or immunoelectron microscopy.* **(B)** *β-catenin at the cell borders of mouse kidney tubule epithelial cells (anti-β-catenin, Cy3-coupled secondary antibodies; yellow fluorescence due to the use of specific Cy3-filters). Asterisks indicate cross sections through kidney tubules, the arrow lies within the lumen of a longitudinally sectioned tubule. Nuclei are counterstained with DAPI (blue).* **(C,D)** *TEM-micrographs of a cell contact region between two* Xenopus *kidney epithelial cells. The β-catenin signal is concentrated in the adherens junction (aj) and can be seen to a lesser degree at the remainder of the basolateral cell membranes. The region indicated by the square in (C) is enlarged in (D); d, desmosome; nu, nucleus; tj, tight junction.* **(E)** *TEM micrograph of an ultrathin section of* Xenopus *intestinal epithelium simultaneously stained for β-catenin and actin. Primary antibodies are detected by secondary antibodies coupled to small (actin, 6 nm) or large (β-catenin, 12 nm) gold particles, respectively. The actin signal is associated with the tight and adherens junctions (tj, aj), as well as with the apical domains of the cells including the microvilli (mv) of the brush border. β-catenin is confined to the adherens junction; d, desmosome. Bars correspond to 20 μm in (B), to 1 μm in (C) and to 200 nm in (D) and (E).*

not sufficient to be clearly visualized by this method (data not shown). Therefore the method is not sensitive enough to address many questions concerning protein distribution in early embryonic cells. It is, however, a useful tool for the localization of overexpressed proteins (Fagotto *et al.,* 1999; Kurth *et al.,* 1999). This is further illustrated in Fig. 3 which displays localization data of tagged mutant versions of *Xenopus* maternal EP/C-cadherin. The mutant proteins were overexpressed in embryonic cells by injection of the corresponding mRNAs into 2-cell stage embryos. At gastrula stages the injected embryos were fixed in a buffered formaldehyde solution and the extrusions appearing at the injection sites were cut off. These were further processed for postembedding labeling (Fig. 3 A). Ultrathin sections were stained in parallel for immunofluorescence and immunoelectron microscopy (Fig. 3 B-F). To compare the light microscopical resolution achieved with this method to that achieved by the preembedding labeling of whole embryos part of the injected embryos were fixed in methanol/DMSO and stained whole-mount (Fig. 3 G-I).

EP/C-cadherin is a cell-cell adhesion molecule with an extracellular part involved in homophilic binding to another cadherin, a membrane spanning region, and an intracellular domain which binds via linker proteins to the actin cytoskeleton (for reviews see Jamora and Fuchs, 2002; Kühl and Wedlich, 1996). Overexpressed wildtype EP/C-cadherin can be found in plasma membranes, the endoplasmic reticulum, Golgi-fields and in vesicles (Kurth *et al.,* 1999). Mutant versions lacking the extracellular part (EP/C-ΔE/MT, Fig. 3 B,C,H) or

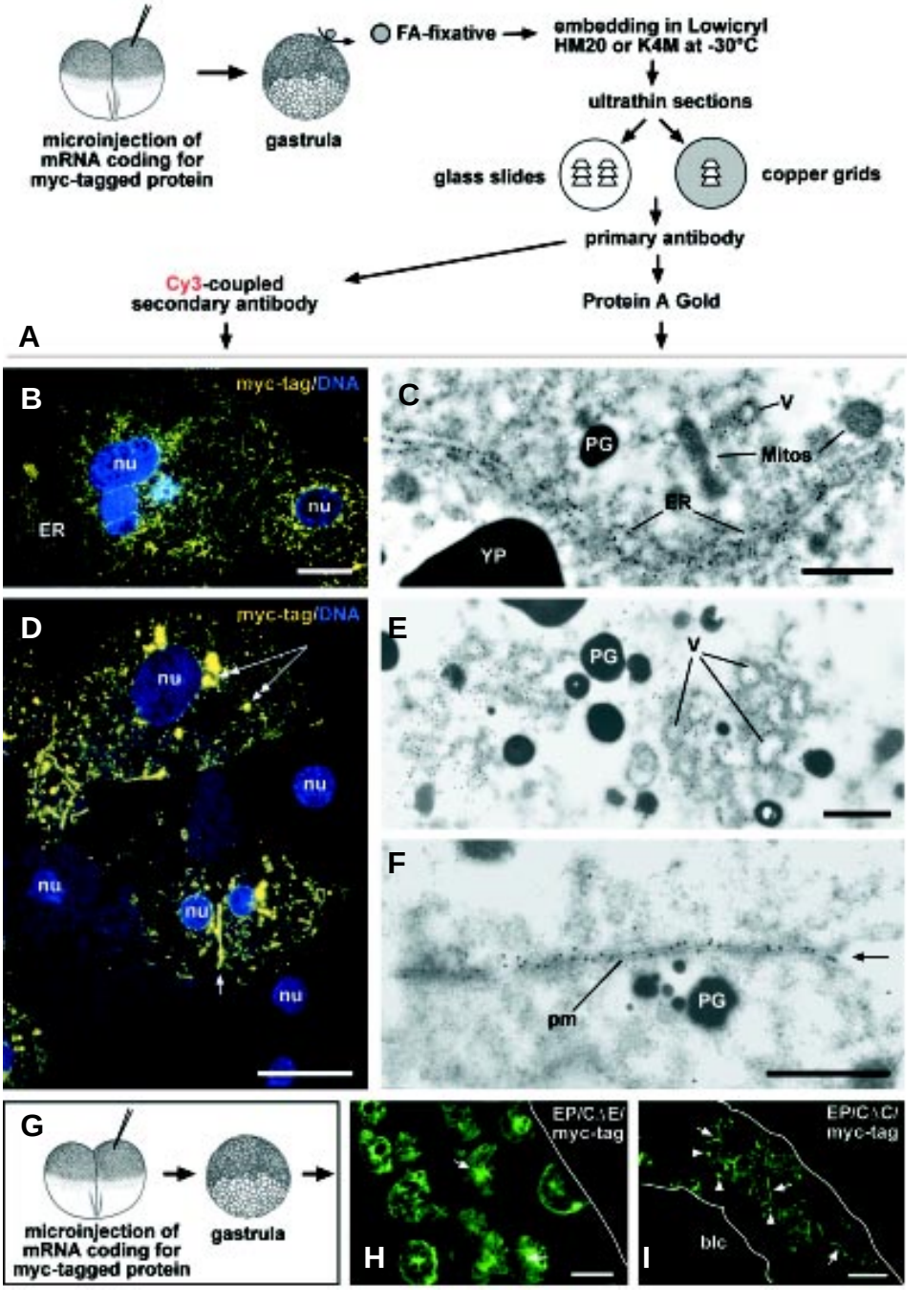


Fig. 3. Postembedding staining of ultrathin sections of embryonic *Xenopus* tissue. (A) *Experimental procedure: 2-8-cell embryos were injected with mRNAs coding for myc-tagged versions of truncated EP/C-cadherin mutants lacking either the extracellular domains (EP/CΔE/MT) or the intracellular c-terminal domain (EP/CΔC/MT). At gastrula stages, the extrusions appearing at the injection sites were cut off, fixed and embedded in Lowicryl HM20. Ultrathin sections were stained independently for immunofluorescence and immunoelectron microscopy. (B-F) The two mutant cadherins are localized to different compartments in the cell and display different transport defects.* **(B,C)** *Overexpressed EP/CΔE/MT localizes to the nuclear membrane, the endoplasmic reticulum (ER) and intracellular vesicles (V) as deduced from immunofluorescent (B) and ultrastructural (C) micrographs.* **(D-F)** *Overexpressed EP/CΔC/MT can be detected in large vesicle fields, which appear as heavily stained spots (double arrows in D) and are displayed at higher resolution in (E). The cadherin-mutant is also found in cell-cell contacts; small arrow in (D). One such cell-cell contact is shown in (F) at the ultrastructural level (arrow). Mitos, mitochondria; nu, nucleus; PG, pigment granule; pm, plasma membrane; YP, yolk platelet. (G-I) For reasons of comparison whole embryos treated likewise were subjected to fixation in methanol/DMSO followed by preembedding labeling of the myc-tag.* **(G)** *Experimental procedure.* **(H)** *EP/CΔE/MT-cadherin in the marginal zone of a gastrula; note the conspicuous intracellular staining indicating the ER-network (arrows, compare to (B)). The outer surface of the embryo is outlined by the white line.* **(I)** *EP/CΔC/MT-cadherin in the animal cap (outlined by the two lines) of a gastrula. The protein is detectable in plasma membranes (arrows) and in intracellular spots (arrowheads, compare to (D)); blc, blastocoel. Bars correspond to 20 μm in (B,D), 1 μm in (C,E,F), and 50 μm in (H,I).*

the intracellular part (EP/C-ΔC/MT, Fig. 3 D-F,I) display different intracellular localization patterns. EP/C-ΔE/MT is localized predominantly in the endoplasmic reticulum or in vesicles and EP/C-ΔC/MT is localized in the plasma membrane and in large intracellular fields, each consisting of many vesicles. The latter structures cannot be resolved by light microscopy alone and can be explained only after examination of gold labeled sections in the electron microscope (compare Fig. 3D and Fig. 3E). Further, the optical resolution obtained after fluorescent staining of ultrathin sections exceeds that obtained from semithin sections after preembedding labeling of whole embryos (compare Fig. 3B with Fig. 3H and Fig. 3D with Fig. 3I). This is due to the fact that the fluorescent signal is restricted to the surface of the ultrathin section and the image is therefore not blurred by out of focus fluorescence.

Taken together, the data presented in Fig. 3 indicate that both mutants display obvious but different transport defects which have to be kept in mind when analysing gross phenotypes of embryos that overexpress the mutant proteins. This example illustrates that the combination of high resolution light and electron microscopic data yields substantial information about subcellular protein distribution and that the rigorous analysis of ectopic protein localization might give important hints for the interpretation of overexpression experiments.

Preembedding Staining of Vibratome Sections (Figs. 4,5,6)

The major drawbacks of preembedding staining of whole embryos and postembedding staining of ultrathin sections are hindered diffusion and a poor sensitivity, respectively. A compromise

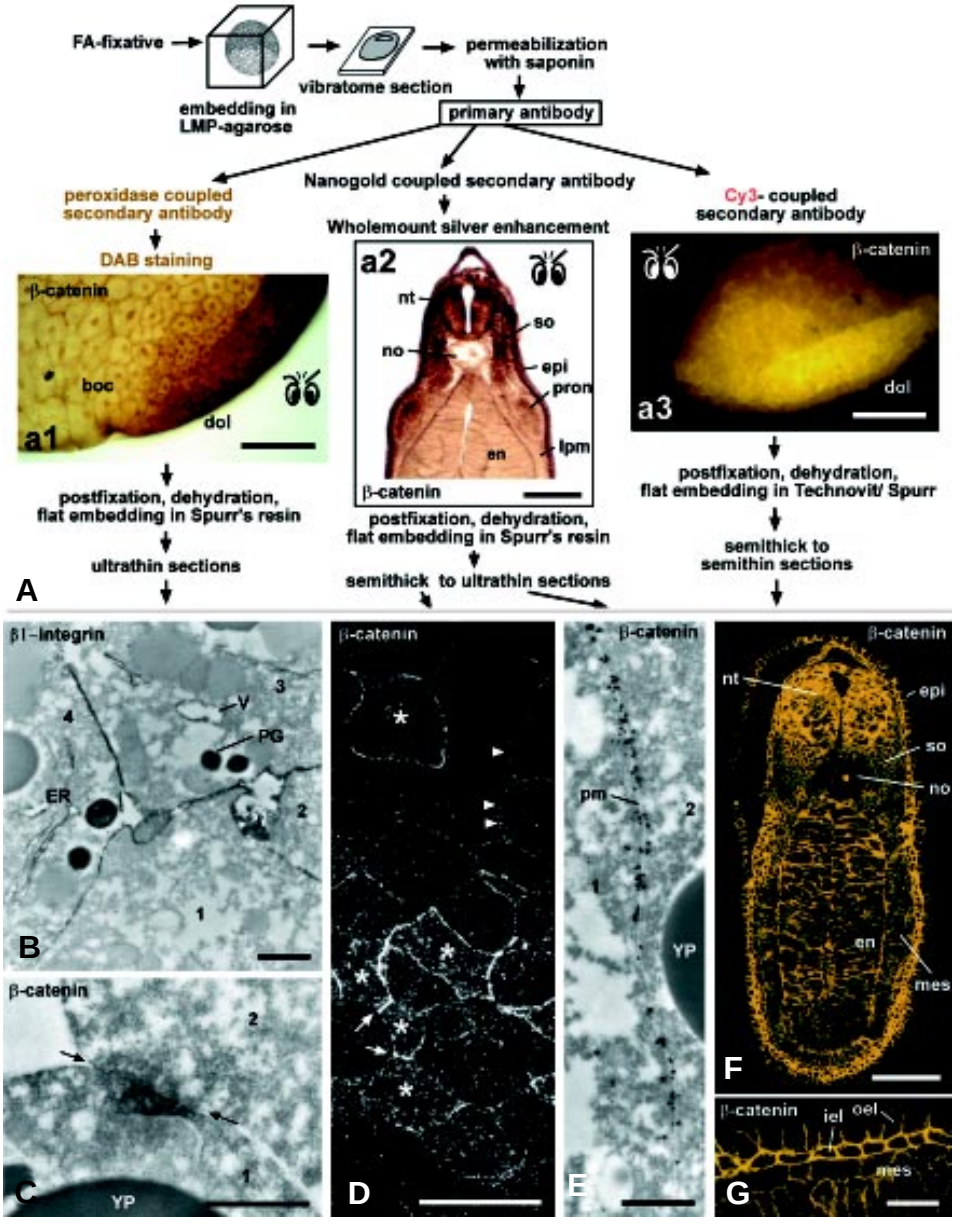


Fig. 4. Preembedding staining of vibratome sections through *Xenopus* embryos. (A) *Experimental procedures. After the preparation of sections, permeabilization, and incubation with primary antibodies, there are three options to continue: DAB-staining, Nanogold + silver enhancement, or fluorescent staining. All methods give the opportunity to inspect successful staining before embedding and processing for further light and electron microscopic examination (indicated by the eyes in a1-a3); FA, formaldehyde; LMP-agarose, Low Melting Point agarose.* (B,C) *Ultrastructural localization of* β_1*-integrin* **(B)** *and β-catenin* **(C)** *in early gastrulae using the DAB method. Integrin is localized in the endoplasmic reticulum (ER), in vesicles (V) and along the plasma membranes (pm) separating the cells 1-4 (B). (C) The DAB-signal indicating β-catenin in a cell-cell-contact region appears as increased contrast (arrows).* **(D)** *Detection of silver enhanced Nanogold particles under polarized light. An early gastrula overexpressing EP/C-cadherin after injection of the corresponding mRNA was stained for β-catenin. Cadherin expressing cells (some of them are indicated by asterisks) display stronger β-catenin staining than do other cells (arrowheads). Further, cell-cell contacts between the former cells are heavily stained (arrows).* **(E)** *Ultrastructural localization of β-catenin in a midgastrula using the Nanogold/silver enhancement method. Staining is confined to the plasma membranes (pm) connecting two adjacent cells (1,2).* **(F)** *Immunofluorescent staining of a transverse vibratome section through a tailbud embryo. The section was embedded in resin and resectioned (5 μm thick).* **(G)** *Similar semithin plastic section but at higher magnification: inner and outer epithelial layers (iel, oel), the underlying mesoderm (mes) and the different labeling intensities of the tissue layers are visible. Further abbreviations: boc, bottle cells; dol, dorsal lip; en, endoderm; epi, epidermis; lpm, lateral plate mesoderm; no, notochord; nt, neural tube; PG, pigment granule; pron, pronephros; so, somite, YP, yolk platelet. Bars correspond to 200 μm in (a1-a3), 1 μm in (B,C,E), 50 μm in (D), 100 μm in (F) and to 20 μm in (G).*

between the two methods is the preembedding staining of thick vibratome sections and their subsequent flat-embedding into plastic and resectioning, the new sectioning plane being parallel to the original vibratome sectioning plane. This reduces the penetration problems and offers the use of better fixatives which leads to better ultrastructural tissue preservation. On the other hand staining intensity is similar to the level achieved by preembedding labeling of whole embryos since the staining is not confined to the surface of the vibratome section. Vibratome sections are cut 50-100 μm thick. Without any further treatment such sections are penetrated only for about 20 μm on both sides (Ding *et al.,* 1993). Therefore, the mild detergent saponin was added to the blocking reagent to permeabilize the tissue and get complete antibody penetration. Since the saponin permeabilization is reversible, traces of this detergent were also added to the antibody and washing solutions. Despite the saponin treatment long incubation times are still necessary to get reasonable staining.

The preembedding labeling of vibratome sections is the most versatile approach described in this report. Primary antibodies can be detected with secondary antibodies coupled to enzymes such as peroxidases, to gold particles or to fluorescent dyes (Fig. 4A). Successful staining can be inspected at low magnification before embedding in plastic resins, which is helpful for the „early" recognition of failed experiments or the selection of promising specimens (Fig. 4a1-a3). Further, the same vibratome section can be analysed with different optical resolutions. The direct observation of the stained vibratome section with stereo or conventional light microscopes yields low resolution surveys (Fig. 4 a1-a3). Medium to high resolution can be obtained by embedding and resectioning of the vibratome sections and the subsequent inspection of semithin sections with conventional or fluorescence microscopes (Fig. 4 D,F,G). Finally, ultrastructural resolution is obtained by the inspection of ultrathin sections in the TEM (Fig. 4 B,C,E).

a) DAB-Peroxidase Staining (Fig. 4 a1,B,C)

The DAB staining of vibratome sections for immunoelectron microscopic investigations has been described previously (Ding *et al.,* 1993). After DAB-development the stained sections were flat-embedded in Spurrs resin (Spurr, 1969), mounted on a new block and sectioned for further light and electron microscopic examinantion. I have tried the protocol with two different antibodies and the following examples demonstrate the influence of the subcellular localization of the targeted protein on the quality of labeling. Stainings with antibodies against the extracellular part of the fibronectin-receptor β_1-integrin (Gawantka *et al.,* 1992) display labeling on plasma membranes as well as in the endoplasmic reticulum and in vesicles. Obviously, the corresponding antigen is freely accessible and the electron dense DAB-precipitate is clearly visible (Fig. 4B). Sometimes, however, the DAB-precipitate seems to diffuse away substantially from the antibody-enzyme complex and that confuses the specificity of staining (data not shown). This problem with the DAB-procedure has been observed previously in other systems (Courtoy *et al.,* 1983). The second antibody recognizes β-catenin which is localized intracellularly directly underneath the plasma membranes (see also Fig. 2). Here it is complexed with the intracellular tails of cadherins and with the cytoskeletal protein α-catenin, the latter binding either directly or through further linker proteins to the actin cytoskeleton (for review see Jamora and Fuchs, 2002). DAB-staining of this protein is also positive but appears in the TEM only as increased submembrane contrast in certain regions of cell-cell contact (Fig. 4C). This could be due to hindered diffusion of antibodies and enzyme complexes in the submembrane cortex. To distinguish specific signal from mere contrast ultrathin sections of DAB-stained specimens were not contrasted with uranyl acetate and lead citrate. But even then embryonic tissues of *Xenopus* still displayed a contrast that obscured DAB-staining in many cases. Therefore, the usefulness of the DAB-method for ultrastructural protein localization in early *Xenopus* embryos is limited.

b) Nanogold-Staining (Fig. 4 a2,D,E)

As an alternative to the DAB-staining a different approach was tried using the Nanogold/silver enhancement method already described for the preembedding of whole embryos (see above). The antibodies coupled to 1 nm gold particles are small enough to penetrate the saponin permeabilized tissue and the enhanced

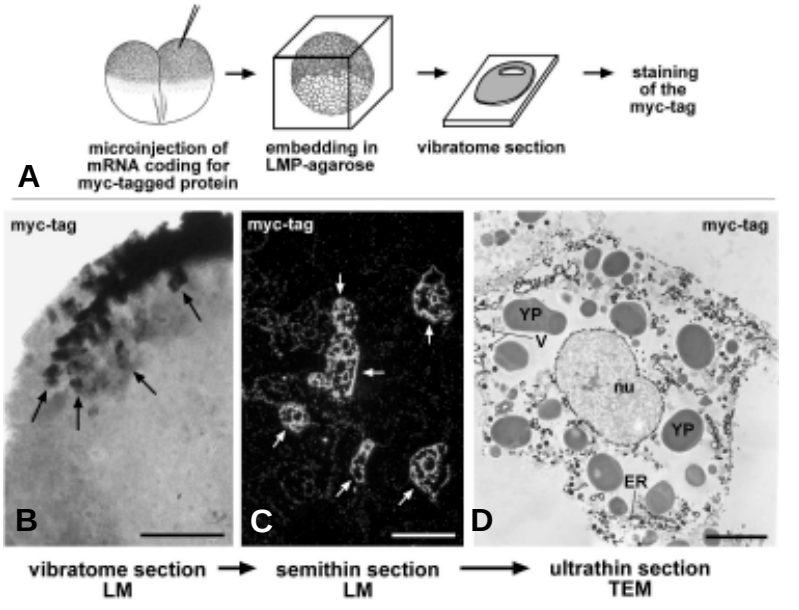


Fig. 5. Different levels of optical resolution of protein localization can be revealed from one vibratome section. (A) *Experimental procedure: 2-4-cell embryos were injected with mRNA coding for a myc-tagged EP/C-cadherin mutant (EPΔE/MT, see also Fig. 3 B,C). After fixation of gastrulae, vibratome sections were prepared and stained with the anti-myc antibody (monoclonal 9E10) using the Nanogold/silver enhancement method.* **(B)** *Vibratome section inspected at low magnification in the light microscope. Dark staining indicates EP/CΔE/MT-expressing cells (arrows). After embedding in Spurrs resin, the vibratome section was resectioned for light and electron microscopy.* **(C)** *A 5 μm thick plastic section reveals normal light microscopical resolution under polarized light. Some cells are stained (arrows) and surrounded by cells not expressing the mutant cadherin.* **(D)** *Inspection of an ultrathin section in the transmission electron microscope (TEM) reveals the mutant cadherin intracellularly in the endoplasmic reticulum (ER), in vesicles (V) and in the nuclear envelope. LM, light microscope; nu, nucleus; YP, yolk platelet. Bars correspond to 200 μm in (B), 50 μm in (C), and 5 μm in (D).*

staining is clearly distinguishable from normal tissue contrast. In addition, the silver enhancement procedure performed on vibratome sections can be visually controlled under the stereo microscope (Fig. 4a2). As in the DAB-method the stained vibratome sections were flat-embedded into Spurrs resin and resectioned for light and electron microscopy. The light microscopical analysis of Nanogold-stained specimens can be performed either in the bright field microscope (not shown here but see also Fig. 1B) or under polarized light as shown in Fig. 4D (β-catenin in an embryo overexpressing wild type EP/C-cadherin, semithin section). At the ultrastructural level the Nanogold staining procedure yields clear cut labeling of β-catenin along the membranes in cell-cell contacts. In contrast to the DAB-peroxidase method the signal cannot be mixed with normal tissue contrast.

c) Immunofluorescent Staining (Fig. 4 a3,F,G)

Immunofluorescently labeled vibratome sections were subsequently embedded in the glycolmethacrylate Technovit 7100. After polymerisation of the resin they were resectioned (2-4 μm thick) parallel to the sectioning plane of the vibratome section. A transverse view through a tailbud embryo of *Xenopus laevis* prepared this way and stained for β-catenin is shown in Fig. 4 F. The whole vibratome section is homogenously stained and at higher magnifications intensity and resolution of the staining in such specimens appear quite satisfying (Fig. 4G) and comparable to results obtained after preembedding staining of whole embryos. Double immunofluorescent labellings can be performed analogous to the whole embryo preparations (data not shown).

Among the three described approaches using vibratome sections the Nanogold/silver enhancement method yields the best combination of light and electron microscopic aspects. To demonstrate this further embryos were injected with a mRNA coding for the myc-tagged cadherin mutant lacking the extracellular domains (EP/CΔE/MT, compare with Fig. 3) and fixed at gastrula stages. Vibratome sections were prepared and stained for the tag using Nanogold and silver enhancement (Fig. 5). Stained vibratome sections were analysed as a whole in the light microscope (Fig. 5B) then subsequently embedded in Spurrs resin and resectioned semithin for light microscopy (Fig. 5C) or ultrathin for electron microscopy (Fig. 5D). The results confirm the intracellular localization of the mutant protein in the endoplasmic reticulum and in vesicles as already inferred from the data obtained by preembedding labeling of whole embryos (Fig. 3H) and postembedding labeling of ultrathin sections (Fig. 3 B,C). They further demonstrate that three optical levels of resolution can be drawn from one vibratome section. Moreover, since the Spurr resin can be cut from semithick (5 μm) to ultrathin (50 nm) one may even "zoom" through several resolution levels at the light microscopical level simply by analysing sections of different thickness (not shown). This is also true for the fluorescently labeled sections as has already been described for adult tissues (Kurth *et al.,* 1996).

The ultrastructural preservation of Nanogold-stained vibratome sections is much better than that of Nanogold-stained whole embryos (Fig. 6). This is due to the use of formaldehyde based fixatives. When traces of glutaraldehyde were present in the fixative the fine structure of the samples may even be comparable to samples fixed for conventional fine structure analysis. However, due to the long incubation times parts of the cytoplasm are extracted, especially in early embryonic cells (compare Fig. 4E with Fig. 6A) or when the glutaraldehyde is omitted from the fixative (compare Fig. 5D with Fig. 6A). The extraction is not as pronounced in later embryonic stages (compare Fig 6A with Fig. 6 C,D).

Conclusion (Table 1)

Many immunocytochemical methods are applicable to embryonic amphibian tissues. The choice of the appropriate protocol depends on factors such as the properties of the available antibodies and the antigenicity profile of the antigen, desired resolution, and finally the proposed localization and the anchorage of the antigen in the cell. An important aspect is the time which has to be invested for the different protocols. Preembedding stainings of whole embryos and vibratome sections are quite time consuming (7-14 days) the latter being in addition rather complex whereas the postembedding staining of ultrathin sections could be finished in a few days, the staining itself taking only 1 day. As a result, the

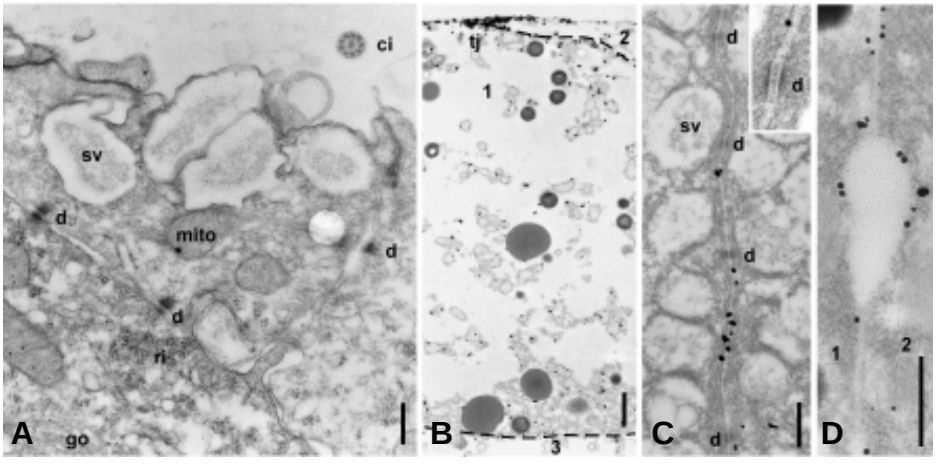
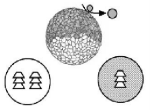
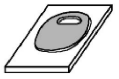


Fig. 6. Two step fixation in combination with preembedding staining of vibratome sections yields an adequate compromise between antigen and ultrastructure preservation. (A) *Outer epithelial cells of a* Xenopus *tailbud stage after fixation with modified Karnovskys fixative for conventional electron microscopy. Intracellular structures such as slime loaden vesicles (sv), mitochondria (mito), Golgi-field (go), ribosomes (ri), a cilium (ci) and desmosome (d) associated intermediate filaments (if) are clearly discernible.* **(B)** *Cingulin in a gastrula after preembedding staining of the whole embryo. Major portions of the cytoplasm are extracted and overall ultrastructural preservation is poor. The basolateral membranes separating cells 1-3 are indicated by the dashed lines.* **(C)** *Vibratome section of a stage 30 embryo (early tadpole) fixed in two steps, stained for β-catenin, and subsequently processed for electron microscopy. A contact region between two cement gland cells is shown. Staining and fine structure are of reasonable quality, desmosomes e.g. display their typical ultrastructural features (intracellular plaques, electron dense line between the membranes, see insert); sv, slime containing vesicle.* **(D)** *Vibratome section of an early gastrula (stage 10) processed as in (C). At higher magnification, β-catenin staining can be detected intracellularly directly underneath the plasma membrane (compare with Fig. 2 D,E). Bars correspond to 0.5 μm in (A,C,D) and to 1 μm in (B).*

TABLE 1

SUMMARY OF THE DESCRIBED IMMUNOCYTOCHEMICAL METHODS

	Detection systems	Resolution	Combination of different levels of optical resolution	Double staining	Advantages/ Disadvantages
Preembedding staining of whole embryos 	Nanogold/ Fluorescence	medium (LM) to ultra (TEM)	yes, 2 levels with the same sample (Nano-gold, Figure 1 B,C) no (Fluorescence)	Immuno-Fluorescence: yes (Figure 1E,F) Immuno-EM: no	+ easy and uncritical for many antigens, medium resolution, serial section analysis possible - poor fine structure, time consuming
Postembedding staining of ultrathin sections 	Gold/ Fluorescence	high (LM) to ultra (TEM)	yes, 2 levels with the same sample (Figure 3)	Immuno-Fluorescence: yes (not shown) Immuno-EM: yes (Figure 2E)	+ high resolution, semiquantitative, good fine structure, quick - high amount or concentration of antigen needed (critical in early stages)
Preembedding staining of vibratome sections 	Nanogold/ DAB/ Fluorescence	low (vibratome section) medium (semithin section) ultra (ultrathin section)	yes, 2-3 levels with the same vibratome section (Figures 4 and 5)	Immuno-Fluorescence: yes (not shown) Immuno-EM: no	+ the most versatile option, reasonable fine structure, up to 3 levels of reso-lution comparable - complex and time consuming

All protocols are characterised by advantages as well as by drawbacks. Dependent on the antigen and the question addressed the most convenient procedure has to be selected. Sometimes it may be even helpful to combine different protocols for a better understanding of protein localization.

procedures are used for different purposes: a first overall impression of protein localization with reasonable resolution can be obtained with the preembedding staining of whole embryos. For a closer examination of intracellular localization preembedding staining of vibratome sections yield 1) the best compromise between antigenicity and ultrastructural preservation and 2) the largest spectrum of optical resolution levels which can be compared on the very same biological sample. The best resolution and fine structural quality is achieved by the postembedding labelling of ultrathin methacrylate sections. Their suitability is, however, hampered by the low sensitivity of this method which is due to the fact that only the surface of an ultrathin plastic section is stained. Its application is therefore restricted to the analysis of highly concentrated or of overexpressed proteins.

The characteristics, advantages and drawbacks of the different immunocytochemical methods are summarized in Table 1. None of the protocols yields the perfect solution to all problems and for some antigens it may be even convenient to compare results from 2 or 3 of the described approaches to get a better understanding of the localization data. Such a comparison of data from all three methods is presented for the localization of a tagged cadherin mutant (EP/CΔE/MT) in Figs. 3 and 5.

The combined methodology described in this report allows us to analyse protein localization even in the early amphibian embryo with high accuracy and may help to reveal aspects of protein function so far unaccessible.

Experimental Procedures

Animals, Embryos and Microinjections

Adult individuals of *Xenopus laevis* were purchased from the African *Xenopus* facility C.C. (South Africa). Embryos were obtained by *in vitro* fertilization as described in Fey and Hausen (1990), cultured in 0.1 MBSH (MBSH: 88mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 10 mM HEPES (pH 7.4), 10 μg/ml streptomycin sulphate and penicillin) and staged according to Nieuwkoop and Faber (1967). Mutant EP/C-cadherin-cDNAs cloned into the pCS2-MT expression vector were used for overexpression experiments (Kurth *et al.,* 1999). Synthesis of capped mRNAs and microinjection was performed as described previously (Kurth *et al.,* 1999).

Antibodies

The following primary antibodies were used: polyclonal rabbit antibodies are P14L (against β-catenin, Schneider *et al.,* 1993,1996), P35N (directed against the β-catenin binding site in the intracellular domain of EP/C-cadherin, Kurth *et al.,* 1999), and C532 (serum, against chicken cingulin, Cardellini *et al.,* 1996); mouse monoclonal antibodies are CL9001 (Cedarlane, against actin, Lessard, 1988), 9E10 (against the myc-epitope, Evan *et al.,* 1985), 8C8 (against β_1-integrin, Gawantka *et al.,* 1992) and b7-1A9 (against nucleoplasmin, Wedlich *et al.,* 1985). Secondary antibodies were the goat-anti-rabbit and goat-anti-mouse IgGs coupled to fluorescent dyes (Cy3, Dianova; Alexa488, Molecular Probes) or to 1 nm gold particles (Nanogold; Nanopobes). For DAB stainings goat-anti-rabbit or goat-anti- mouse IgGs and the corresponding peroxidase-anti-peroxidase complexes (Sternberger) were used. 13 nm Protein-A gold or secondary antibodies conjugated to gold particles of different sizes (12 nm goat-anti-rabbit IgG and 6 nm goat-anti-mouse IgG, Jackson) were applied to detect primary antibodies on ultrathin sections for transmission electron microscopy (TEM).

Preembedding Staining of Whole Embryos

Embryos were fixed in 20% dimethylsulfoxide (DMSO) in methanol overnight at -20°C (Dent *et al.,* 1989) or prefixed in 4% formaldehyde/PBS overnight at 4°C followed by postfixation in the DMSO/methanol mixture. Embryos can be stored in methanol at -20°C for several weeks. Specimens were rehydrated in a graded series of methanol/water (100%, 90%, 70%), in methanol/PBS 50%, 30%, 15 min each and in PBS, 2 x 15 min, blocked in 20% normal goat serum in PBS (NGS/PBS) for 2 hrs at room temperature and incubated in 20% NGS/PBS containing the primary antibody (-ies). Incubation times varied from overnight to 3 days at room temperature, depending on the size of the specimen and whether or not formaldehyde was used as a prefixative. After washes in PBS (short, 5, 10, 15, 30 min, 4 x 1 h, 1 x 2hrs) secondary antibodies in 20% NGS/PBS were applied, again from overnight to 3 days at room temperature. Samples were washed in PBS, postfixed in 4% formaldehyde/PBS for 1-2 hrs, dehydrated in a graded series of ethanol/water (30%, 50%, 70%, 80%, 90%, 100%, 15 min each) and infiltrated in the glycolmethacrylate Technovit 7100 (Kulzer, Germany). The number of infiltration steps is dependent on size, density and composition of the samples. The standard procedure is as follows: 1 part resin/1 part ethanol, 1 h; 2 parts resin/1 part ethanol, 1h; resin (used), 1h; resin (new), 1.5 hrs; resin new, overnight. Finally specimens were embedded according to the manufacturers instructions. Sections were cut (1-5 μm) and (optionally) counterstained with DAPI to visualize the nuclei. Double immunofluorescent staining was performed likewise but the samples were incubated simultaneously with the 2 primary and later with the 2 secondary antibodies.

For immunoelectron microscopy primary antibodies were localized using Nanogold coupled secondary antibodies (Nanoprobes) and postfixed in 2% glutaraldehyde/PBS for 2 hrs at room temperature. Following washes in PBS (4 x 10 min) and distilled water (3 x 10 min) specimens were treated with a

silver enhancement solution (Danscher, 1981; Lah *et al.,* 1990). This solution is a mixture of gum arabic, hydroquinone, and silver lactate in a citrate buffer. Aliquots of the silver lactate solution (0.11 g in 15 ml distilled water, 150 µl aliquots) and a mixture (850 µl aliquots) of gum arabic (600 µl, stock: 33% solution in distilled water), hydroquinone (150 µl, stock: 0.85 g in 15 ml distilled water) and citrate buffer (100 µl, stock: 2.55 g citric acid and 2.35 g sodium citrate in 10 ml distilled water) can be stored separately at –20°C and mixed immediately before application. 1-2 hrs incubation was performed in the dark room, the specimens were washed several times in water to get rid of the gum arabic, and then *en bloc* contrasted in 1% aqueous uranyl acetate for 2 hrs on ice. After that the embryos were washed in water, dehydrated in ethanol up to water free ethanol and infiltrated in Spurrs resin (Spurr, 1969): 1 part resin/2 parts ethanol, 1h; 1 part resin/1 part ethanol, 1h; 2 parts resin/ 1part ethanol, 1.5 hrs; resin, 1.5 hrs; resin, overnight. Finally, the specimens were embedded and polymerized at 60-65°C and sections were cut with a pyramitome (5-10 µm) for light microscopy and with an Ultramicrotome (LKB, Ultrotome III, 50 nm) for transmission electron microscopy.

Postembedding Staining of Ultrathin Sections

Pieces of adult or embryonic tissues were fixed in 4% formaldehyde in 50 mM HEPES, 2 mM $CaCl_2$ with 0.1% glutaraldehyde (adult tissues in Fig. 2) or without glutaraldehyde (embryonic tissues in Fig. 3). Specimens were washed, dehydrated in ethanol at progressively lower temperatures and infiltrated in Lowicryl K4M or HM20 (Polysciences, Germany). Polymerization occurred at -35°C by UV light (Carlemalm *et al.,* 1982). Ultrathin sections were cut and used for immunofluorescent and immunogold stainings (Schwarz, 1994). Sections were blocked with 0.2% gelatine/0.5% bovine serum albumin in PBS and incubated with primary antibodies. As detection systems Cy3-conjugated secondary antibodies, antibodies coupled to 6 nm or 12 nm sized gold particles or 13 nm Protein-A gold complexes in PBS/gelatine were used. After the labeling of sections nuclei were counterstained with DAPI. Labeled sections for transmission electron microscopy (TEM) were stained with 1% aqueous uranyl acetate (Schwarz *et al.,* 1992).

Preembedding Staining of Vibratome Sections

Embryos were fixed by a two step fixation procedure 1) prefixation in 2% formaldehyde, 50 mM HEPES, 2 mM $CaCl_2$ for about 1.5 hrs at room temperature. 2) postfixation in 4% formaldehyde, 50 mM HEPES, 2 mM $CaCl_2$ and 0.1% glutaraldehyde for about 2.5 hrs at room temperature or overnight at 4°C (Ding *et al.,* 1993). After that, the specimens were washed 6 x 10 min in 100 mM HEPES and 2 x 30 min in PBS, embedded in 3% Low Melting Point Agarose in PBS and glued to the sectioning block of a vibratome (Vibratome series 1000, Pelco). 50-100 µm thick sections were cut and blocked in 20%NGS/PBS containing 0.1% Saponin. Saponin permeabilizes the vibratome section to allow antibody diffusion. After incubation in a solution containing the primary antibodies in 20% NGS/PBS/0.05% Saponin for 2-3 days at 4°C different routes were taken to continue:

a) Fluorescent staining: washes in PBS (short, 5, 10, 15, 30 min, 4 x 1 h, 1 x 2hrs) and incubation with fluorescent secondary antibodies in 20%NGS/ PBS at least overnight at room temperature. Sections were washed, postfixed in 4% formaldehyde/PBS, dehydrated in ethanol, infiltrated and embedded in Technovit 7100 as described above. Sections (parallel to the vibratome section plane) for light microscopy were cut about 2-4 µm thick.

b) DAB staining: washes in PBS (short, 5, 10, 15, 30 min, 4 x 1 h, 1 x 2hrs), incubation with secondary antibodies and tertiary peroxidase-anti-peroxidase complexes (each 2-3 hrs at room temperature or overnight at 4°C) followed by washes in PBS. Peroxidase generated DAB-staining was performed using a Metal enhanced DAB Kit (Pierce). Staining was controlled under the stereo microscope and stopped by adding an excess of PBS. After several washes in PBS, samples were postfixed in 1% glutaraldehyde/PBS, washed and treated with 1% OsO_4/PBS for 1-2 hrs on ice. After 4 x 5 min washes in PBS and water specimens were dehydrated in ethanol, infiltrated in Spurrs resin and embedded between two plastic foils. After polymerisation overnight at 65°C vibratome sections were cut out and mounted on epon blocks for ultrathin sectioning.

c) Nanogold + silver enhancement: washes in PBS (short, 5, 10, 15, 30 min, 4 x 1 h, 1 x 2hrs), incubation with secondary antibodies conjugated to 1 nm gold particles for overnight to 2 days at 4°C, followed by washes in PBS. After that samples were postfixed in 2% glutaraldehyde in PBS for 2 hrs at room temperature, washed in PBS and water and incubated in the dark for 1-2 hrs with the silver enhancement solution according to Danscher (1981) and Lah *et al.* (1990) (see above). After silver enhancement and washes in water the samples were *en bloc* contrasted in 1% aqueous uranyl acetate for 1h on ice, washed, dehydrated in ethanol and infiltrated and embedded in Spurrs resin between two plastic foils. Following polymerisation at 60-65°C vibratome sections were cut out, mounted on epon blocks and cut on a pyramitome for light and on an Ultramicrotome (LKB, Ultrotome III) for transmission electron microscopy.

Conventional Transmission Electron Microscopy

For conventional ultrastructural analysis embryos were fixed in a mixture of 2.5% glutaraldehyde and 2% formaldehyde in 100 mM HEPES (pH 7.4) overnight at 4°C, washed in 100 mM HEPES and PBS and postfixed in 1% OsO_4 in PBS overnight on ice. After washes in PBS and water they were *en bloc* contrasted with 1% aqueous uranyl acetate, washed in water, dehydrated in a graded series of ethanol/water and embedded in Spurrs resin. Ultrathin sections were consecutively stained with 1% aqueous uranyl acetate and 1% aqueous lead citrate.

Microscopy, Image Acquisition and Processing

Samples were inspected on a Zeiss stereo microscope, a Zeiss Axioplan photomicroscope equipped with epifluorescence optics and a Philipps CM10 transmission electron microscope. Some of the light microscopy images were taken using the MC 100 camera with Kodak Elite-400 color films. Therefore, in those images the Cy3-fluorescence appears yellow (see Figs. 2B, 3 B,D and 4 F,G; this is also due to the use of the Cy3-specific filters (AF Analysentechnik, Pfrondorf). Alternatively, fluorescence data were acquired using a digital camera (Sony) and the Analysis program (Soft Imaging Systems). Here, the yellow Cy3 fluorescence is automatically transferred to red during the process of image aquisition (see Fig. 1 E,F). Picture processing and figure mounting was performed with the Photoshop (Adobe, version 5.0) and Freehand (Macromedia, version 8) programs.

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