

Preembedding immuno-staining for electron microscopy

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Material

Fresh tissue specimens are obtained by surgery or by biopsy and fixed in aldehydes. Optimal fixation has to be established by appropriate trials. In most cases, neutral cacodylate buffered formaldehyde freshly prepared from paraformaldehyde and used either alone or in combination with glutaraldehyde are favored.

Annotation to tissue sampling for preembedding immunostaining

Apart from loss of immunoreactivity due to fixation, negative staining results are to be expected if immunohistological reagents are not able to reach their corresponding sites. Hence, localization of intracellular antigens by labeled antibodies is governed by their penetrability. Our studies with aldehyde fixed cell suspensions and various marker molecules of known molecular weight have shown that molecules of 80,000 daltons and more do not penetrate cell membranes or do so only sluggishly (KUHLMANN WD and MILLER HRP, 1971; KUHLMANN WD et al. 1974; KUHLMANN WD, 1977). Enzymatic predigestion of cell surface material (e.g. by trypsin, neuraminidase, papain, hyaluronidase) improved penetration, however, the total number of stained cells remained low.

When examining the penetration of macromolecules into fixed tissue blocks, we observed that small hand-cut fragments were penetrated poorly if at all. As a rule, only cells at the edge of the block came into contact with reagents. In order to overcome penetration problems, treatments of aldehyde fixed cells f.e. with saponin, Tween and other detergents were examined (KUHLMANN WD, 1977; KUHLMANN WD, 1984). Yet, no convincing results could be obtained with hand-cut tissue fragments. Hitherto, the best results have been obtained with thick frozen sections from well-fixed specimens. Indeed, careful fixation is necessary to avoid as much as possible diffusion artefacts and cell damage by the freeze-thaw cycles and all the manipulations in the further incubation steps (KUHLMANN WD and MILLER HRP 1971; KUHLMANN WD 1978; KUHLMANN WD 1984). Even if these insights are known at least since the 1970s, problems of tissue fixation and penetration enhancement of reagents are still a subject of interest to be further studied (ELDRED WD et al. 1983).

Our original method of preembedding immunostaining for electron microscopy, i.e. the use of thick frozen sections from aldehyde fixed tissue specimens, is still the method of choice in our laboratory. The adoption of this preembedment immunohistology to a large variety of tissues is quite easy, and a typical protocol will be described in detail.

Method of immunostaining

The procedure of direct immunoperoxidase detection of alpha-1-fetoprotein in rat liver (fetal liver; regenerating liver after injury; hepatoma bearing liver) is described. *

Reagents

Chemicals *p.a.* are used according to the recommendations of the manufacturer and the respective safety protocols:

- Formaldehyde fixative freshly prepared from paraformaldehyde (extra pure) in neutral buffer (e.g. 0.2 M cacodylate pH 7.2); glutaraldehyde of high purification degree according to chapter *Fixatives*
- Dimethylsulfoxide (DMSO)
- Embedding medium for tissue freezing (Tissue-Tek® OCT™ Compound)
- Isopentane (2-methylbutane)
- Liquid nitrogen (N₂)
- Hydrogen peroxide (H₂O₂)
- Bovine serum albumin (BSA), 30% solution
- 0.2 M cacodylate buffer pH 7.2
- 0.01 M sodium citrate buffer pH 8.5
- 0.1 M phosphate buffer pH 7.4
- PBS/BSA solution
- Enzyme substrate DAB and H₂O₂ in Tris-HCl buffer according to chapter *Enzyme cytochemical substrate solutions*)
- Distilled water
- Osmium tetroxide (OsO₄)
- Ethanol (absolute)
- Epoxy resin embedding medium according to chapter *Dehydration and resin embedment of tissues*

Immunological reagents: rabbit anti-rat alpha-1-fetoprotein (AFP) antibodies (IgG) conjugated with horseradish peroxidase; conjugated molecules are purified by gel filtration or affinity chromatography.

Immunoperoxidase staining of thick frozen sections

1. **Tissue sampling:** freshly taken liver is cut into blocks of about 2 mm in a drop of fixative and fixed at 0-4°C in cacodylate buffered 6% formaldehyde for 5 h followed by 6% formaldehyde plus 0.25% glutaraldehyde for 60-90 min. Specimens are then washed overnight in buffer.
2. **Anti-freeze pretreatment:** tissues are soaked with 10% DMSO/cacodylate buffer for 1 h, placed with a drop of water soluble embedding medium such as Tissue-Tek®) on a

* Chemicals used for immunohistology can be toxic. They must be handled with care

piece of aluminium foil (or directly onto the tissue holder of the cryomicrotome) and frozen in liquid nitrogen cooled isopentane; frozen tissue blocks are transferred to the cryo-chamber for sectioning. Prior to cutting, frozen tissue blocks should equilibrate to the temperature of the cryostat.

2. **Cryomicrotomy:** sections are cut at 10-40 μm thickness with a C-profile steel knife at -25°C to -30°C and using an anti-roll plate. Frozen cut sections are dropped straight into small vials containing 10% DMSO/cacodylate buffer, transferred outside the cryo-chamber and rinsed with cacodylate buffer. Sections are kept free floating in these vials during the whole incubation procedure until final resin embedment is reached.
3. **Antigen retrieval:** antigen retrieval can be an important measure and will depend on the molecules studied. In the case of AFP staining, antigen retrieval in cryosections is not essential (with aldehyde fixation as described above) but will certainly improve the staining efficiency when higher and longer fixation schedules are employed,
 - water bath: preheat a glass vial containing 0.01 M sodium citrate buffer pH 8.5 in a water bath to 80°C and maintain this temperature,
 - transfer the free floating sections into this buffer vial,
 - keep the sections in this solution and at this temperature for 30 min,
 - Remove the vial from water bath and allow to cool down to room temperature,
 - Sections are rinsed three times for 5 min each in PBS.
4. **Principle of antigen staining:** The method of immunostaining is outlined above. Briefly, purified HRP labeled rabbit anti-rat alpha-1-fetoprotein (AFP) antibodies are used for direct antigen detection.
5. **Incubation and washing schedules:** all incubation steps are performed at room temperature. Do not let dry the sections during the procedures. After each step, vials are cautiously drained and blotted on filter paper (do not dry),
 - inhibition of endogenous peroxidases with 1-2% H_2O_2 for 60 min, followed by washings in PBS for 3 x 5 min,
 - pretreatment of sections with PBS/BSA for 15 min in order to block nonspecific bindings (background),
 - HRP labeled antibodies, appropriately diluted in PBS/BSA (f.e. 0.5 mg/mL),
 - sections are floated in conjugates for at least 2 h; overnight incubation proves also useful,
 - washings in cacodylate buffer for 3 x 10 min,
 - fixation (this step is optional) in 0.5% glutaraldehyde for 5 min followed by rinses and washings in cacodylate buffer for 3 x 10 min.
6. **Enzyme cytochemical staining:** DAB cytochemistry is used for the detection of HRP activity (= cellular antigenic site). The enzyme substrate is prepared according to chapter *Enzyme cytochemical substrate solutions*
 - incubation in DAB/ H_2O_2 substrate mixture for 20 min,
 - washings in cacodylate buffer
 - postfixation in 1% OsO_4 in cacodylate buffer for 30 min.
7. **Control sections:**
 - principles of quality control as described in chapter *Specificity and standardization of immunohistology*,

- HRP labeled rabbit anti-glucose oxidase antibodies (IgG); HRP labeled rabbit non immune (normal) IgG globulins,
- control reactions as described in chapter *Artefactual staining in immunohistology*,
- incubation schedules and washings same as described above.

8. Dehydration and resin embedment:

- sections are dehydrated (sections are always in the same vials as they have come from the microtome) in ascending series of ethanol and embedded in Epon (principle, see chapter *Dehydration and resin embedment of tissues*),
- single sections are placed into polyethylene lids (from Beem capsules or equivalent) containing a droplet of fresh Epon mixture,
- from polymerized Epon molds (prepared in advance in lids of Beem capsules), Epon is removed and put with the flat surface on top of the tissue sections,
- final polymerization as usual.

9. Selection for ultramicrotomy:

- after subsequent polymerization a desired part of the section is selected by light microscopy,
- tissue and excess of resin are cut away with a razor blade. The selected part is then placed with a drop of fresh resin on top of a gelatin capsule prepared in advance with polymerized Epon,
- the selected area of the flat embedded tissue sections is trimmed and cut with an ultramicrotome.

Selected publications for further readings

Kuhlmann WD and Miller HRP (1971)
 Kuhlmann WD *et al.* (1974)
 Kuhlmann WD (1977)
 Guillouzo A *et al.* (1978)
 Kuhlmann WD (1978)
 Kuhlmann WD (1979)
 Eldred WD *et al.* (1983)
 Kuhlmann WD (1984)

Full citation of publications is given in chapter *References*

link: <https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf>