

Immuno-staining with resin embedded tissue

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Material

Tissues obtained by surgery or bioptic materials are fixed with aldehydes, then dehydrated and embedded in epoxy resin. Generally, fixation, dehydration and embedding schedules may largely vary depending on trial experiments with the tissue under study.

I. Method for semithin resin sections

The procedure of indirect immunoperoxidase localization of alpha-1-fetoprotein in sections from epoxy resin embedded rat liver (fetal liver; regenerating liver after CCl₄ intoxication) 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. phosphate buffer, cacodylate buffer) or other fixatives of choice
- Sodium methoxide
- Methanol
- Benzene
- Xylene or xylene substitute
- Ethanol (absolute)
- Phosphate buffered saline at pH 7.4 (PBS), see chapter *Buffer solutions*
- Bovine serum albumin (BSA), 30% solution
- PBS with 1% BSA (PBS/BSA)
- Endogenous peroxidase blocking solution (e.g. 10% H₂O₂ in PBS)
- Peroxidase cytochemical substrate solution, see chapter *Enzyme cytochemical substrate solutions*
- 0.1% OsO₄ in PBS
- Distilled water
- Resinous mounting medium (e.g. Eukitt®)

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

Immunological reagents:

- Primary antibodies: rabbit anti-rat alpha-1-fetoprotein (AFP)
- Secondary antibodies: sheep anti-rabbit IgG antibodies, purified by immunoabsorbents and conjugated with horseradish peroxidase (HRP); conjugated molecules are purified by gel filtration or affinity chromatography. Alternatively, HRP labeled sandwich antibodies are purchased.

Glass slides: glass slides coated with adhesives are recommended for enhanced adhesion of tissue sections to resist the subsequent histological procedures. Useful procedures include the conditioning of glass slides with bovine serum albumin (BSA) and glutaraldehyde (see chapter *Immunofluorescence staining of cryostat sections*) or the conditioning with silane (see chapter *Silane conditioning of glass slides*).

Semithin staining procedure

- 1. Resin removal and rehydration:** semithin sections (0.5-1.5 μm) are cut with an ultramicrotome (see chapter *Microtomy of tissue specimens, collection of sections*) and mounted on coated glass slides
 - Partial removal of resin by the following series of treatments and washes at room temperature (see also chapter *Resin removal from sections for immunohistology*)
 - sodium methoxide 3 min
(in methanol/benzene)
 - methanol/benzene 2 x 1 min
 - acetone 2 x 1 min
 - ethanol (absolute) 1 rinse
 - 95% ethanol 1 rinse
 - 70% ethanol 1 min
 - distilled water 2 x 1 min
 - rehydration is completed when sections are passed into the working buffer
 - PBS 2 x 2 min
- 2. Antigen retrieval:** this step is not always necessary; the right retrieval method must be established by trial (see chapter *Retrieval of antigenic determinants*). In the present case of AFP staining in resin sections from aldehyde fixed liver blocks, antigen retrieval is not necessary. However, conditioning of fixed tissue blocks with inert compounds such as PVP or reversal chemical modification of proteins with ethyl acetimidate prior to embedment improved antigen staining (see chapter *Tissue dehydration and embedment*).
- 3. Inhibition of endogenous peroxidase:** blocking of endogenous peroxidases is optional and can be done by several procedures (see chapter *Blocking solutions*)
 - inhibition of peroxidases by hydrogen peroxide
 - 10% H_2O_2 in PBS 10 min
 - PBS 2 x 5 min
- 4. Principle of indirect immunostaining:** incubation in unlabeled primary antibody is followed by incubation in peroxidase (HRP) labeled sandwich antibodies (directed against IgG immunoglobulins of the species that provide the primary antibodies). In this example, rabbits provide the primary antibodies against rat AFP. The localisation of

AFP is then done by the use of HRP labeled secondary antibodies. These are obtained from sheep immunized with IgG of the primary species (rabbit IgG).

5. **Incubation and washing schedules:** do not let dry the sections during all procedures,
 - pretreatment of sections with PBS/BSA supplemented with 5% normal sheep serum for 5 min in order to block nonspecific bindings (see chapter *Blocking solutions*),
 - primary antibodies (as well as control antibodies) are appropriately diluted in PBS/BSA, f.e. 1-5 μg antibodies per mL,
 - incubation with diluted primary antibodies for 24 hours at 4°C under humidified atmosphere,
 - washings in PBS/BSA for 3 x 5 min,
 - labeled secondary antibodies: HRP conjugated sheep anti-rabbit IgG antibodies are appropriately diluted in PBS/BSA, f.e. 10-50 $\mu\text{g}/\text{mL}$,
 - sections are incubated with diluted HRP conjugates for 30 min at room temperature or at 37°C under humidified atmosphere,
 - washings in PBS/BSA for 3 x 5 min.
6. **Enzyme cytochemical staining:** DAB cytochemistry is used for the detection of HRP activity. The enzyme substrate is prepared according to chapter *Enzyme cytochemical substrate solutions*
 - incubation in DAB/H₂O₂ substrate mixture for 20 min,
 - washings in PBS for 3 x 5 min
 - washings in distilled water for 2 x 1 min.
7. **Counterstaining:** the final reaction mixture is osmiophilic, and postfixation in OsO₄ enhances the contrast, but this step is optional and not necessary. Osmium treatment for 1 min with 0.1% OsO₄ in distilled water or PBS is sufficient. Excess of osmium is then washed off with several rinses in 70% ethanol.
8. **Control sections:**
 - principles of quality control as described in chapter *Specificity and standardization of immunohistology* and in chapter *Practical aspects in quality control of immunohistology*,
 - pretreatment of sections with PBS/BSA supplemented with 5% normal sheep serum for 5 min in order to block nonspecific bindings (background),
 - primary antibodies: specific primary antibodies are replaced by non tissue relevant antibodies from same species providing the primary antibodies, e.g. rabbit anti-glucose oxidase (IgG). Also, rabbit non immune (normal) IgG globulins are used instead of primary antibodies,
 - sandwich antibodies same as described above,
 - incubation schedules and washings same as described above.
9. **Mounting under coverglass:**
 - dehydrate in the following series of washes
 - distilled water 2 x 1 min
 - 70% ethanol 2 x 1 min
 - 95% ethanol 2 x 1 min
 - absolute ethanol 2 x 5 min
 - xylene or xylene substitute 2 x 5 min

- sections are mounted under coverglass with a drop of resinous medium.

II. Method for ultrathin resin sections

The procedure of indirect immunoperoxidase localization of IgG molecules in sections from epoxy resin embedded rat popliteal lymph nodes 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. phosphate buffer, cacodylate buffer) or other fixatives of choice
- 0.5% Glutaraldehyde in PBS
- Sodium hydroxide stock solution (2 g NaOH in 100 mL absolute ethanol)
- Ethanol (absolute)
- Phosphate buffered saline at pH 7.4 (PBS), see chapter *Buffer solutions*
- Tris-HCl buffer pH 7.4
- Bovine serum albumin (BSA), 30% solution
- PBS with 0.2% BSA (PBS/BSA)
- Peroxidase cytochemical substrate solution, see chapter *Enzyme cytochemical substrate solutions*
- 0.1% OsO₄ in distilled water
- Distilled water

Immunological reagents:

- Primary antibodies: rabbit anti-rat IgG for the localization of rat IgG in lymph node cells
- Secondary antibodies: rabbit anti-sheep IgG and sheep anti-rabbit IgG antibodies purified by immunoabsorbents, respectively. Rabbit anti-sheep antibodies conjugated with horseradish peroxidase (HRP) and purified by gel filtration and affinity chromatography.

Nickel grids (200 mesh): ultrathin sections (gold interference color) are collected on Formvar and carbon-coated grids.

Incubation steps: grids are floated with sections downwards on droplets of the various solutions; droplets are placed on sheets of Parafilm®. Grids are transferred with forceps. After each step, a given grid is blotted by touching its edge onto filter paper (do not dry).

Ultrathin staining procedure

1. **Resin removal and rehydration:** ultrathin sections being cut with an ultramicrotome (see chapter *Microtomy of tissue specimens, collection of sections*) and collected on 200 mesh Nickel grids

- Partial removal of resin in the following series of treatments and washes at room temperature
 - sodium hydroxide solution 5-10 min
(NaOH stock solution diluted 1:2 with distilled water)
 - 50% ethanol 1 rinse
 - distilled water 2 rinses
 - rehydration is completed when sections are passed into the working buffer
 - PBS/BSA 2 x 2 min
2. **Antigen retrieval:** this step is not always necessary; the right retrieval method has to be established by trial (see chapter *Retrieval of antigenic determinants*. In the present case of IgG staining, antigen retrieval by one of the described methods was not tried.
 3. **Inhibition of endogenous peroxidase:** blocking of endogenous peroxidases can be done by several procedures (see chapter *Blocking solutions*)
 - inhibition of peroxidases by hydrogen peroxide
 - 10% H₂O₂ in PBS 5 min
 - distilled water 1 rinse
 4. **Principle of indirect immunostaining:** incubation in unlabeled primary antibody is followed by incubation in unlabeled sandwich antibody and in the 3rd step by peroxidase (HRP) labeled antibodies (directed against IgG immunoglobulins of the species that provide the sandwich antibodies).

First step: rabbit anti-rat IgG (1 µg/mL),
 second step: sheep anti-rabbit IgG (10 µg/mL),
 third step: rabbit anti-sheep IgG labeled with HRP, purified conjugates (10 µg/mL).
 5. **Incubation and washing schedules:** do not let dry the sections during all procedures,
 - pretreatment of sections with PBS/BSA (2 x 2 min) in order to block nonspecific bindings (see chapter *Blocking solutions*),
 - all antibodies are appropriately diluted in PBS/BSA,
 - first step antibodies: incubation with diluted primary antibodies for 30 min (room temperature) or 8 hours at 4°C under humidified atmosphere,
 - washings with PBS/BSA for 3 x 2 min,
 - second step antibodies: incubation with diluted sheep anti-rabbit IgG for 10 min at room temperature,
 - washings with PBS/BSA for 3 x 2 min,
 - third step antibodies: HRP conjugated rabbit anti-sheep IgG for 10 min,
 - washings with PBS/BSA for 3 x 2 min,
 - postfixation with 0.5% glutaraldehyde/PBS for 1 min followed by 3 rinses with Tris-HCl buffer
 6. **Enzyme cytochemical staining:** DAB cytochemistry is used for the detection of HRP activity. 2.5 mg DAB are dissolved in 10 mL Tris-HCl buffer and passed through 0.22 µm filter, then 25 µL of 1% H₂O₂/distilled water are added
 - incubation in DAB/H₂O₂ substrate: grids are held with forceps into gently stirred mixture for 3-5 min,
 - grids are rinsed with distilled water
 7. **Postfixation:** the final reaction mixture is osmiophilic and postfixation in OsO₄ enhances the contrast;

- 0.1% OsO₄ in distilled water 1 min,
- distilled water 2 rinses
- air-drying

8. Control sections:

- pretreatment of sections with PBS/BSA for 5 min in order to block nonspecific bindings (background),
- primary antibodies: specific primary antibodies are replaced by unrelated antibodies from same species providing the primary antibodies, e.g. rabbit anti-glucose oxidase (IgG). Also, rabbit non immune (normal) IgG globulins are used instead of primary antibodies,
- control reactions as described in chapter *Artefactual staining in immunohistology*,
- sandwich antibodies same as described above,
- incubation schedules and washings same as described above.

Selected publications for further readings

Mayor HD *et al.* (1961)
 Lane BP and Europa DL (1965)
 Nakane PK (1970)
 Moriarty GC (1973)
 Sternberger LA and Petrali JP (1977)
 Roth J *et al.* (1978)
 Erlandsen SL *et al.* (1979)
 Sternberger LA (1979)
 Rodning CB *et al.* (1980a, 1980b)
 Takamiya H *et al.* (1980)
 Kuhlmann WD and Krischan R (1981)
 Kuhlmann WD and Viron A (1981)
 Kuhlmann WD (1984)
 Stirling JW and Graff PS (1995)
 Kuhlmann WD and Peschke P (2006)

Full citation of publications is given in chapter *References*

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