Immunostaining with paraffin embedded tissue sections

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Material

Tissue specimens obtained by surgery or bioptic materials are used. Fixation is done by aldehydes or other schedules according to results from preliminary experiments.

Method of immunostaining

The procedure of indirect immunoperoxidase localization of alpha-1-fetoprotein in sections from paraffin embedded 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. phosphate buffer, cacodylate buffer) or other fixatives of choice
- 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. 1% H_2O_2 in PBS), see chapter *Blocking solutions*)
- Peroxidase cytochemical substrate solution, see chapter *Enzyme cytochemical substrate solutions*
- Osmium tetroxide(OsO4), haematoxylin for counterstaining, see chapter *Haematoxylin staining methods*
- Distilled water
- Resinous mounting medium (e.g. Eukitt®)

Immunological reagents:

- Primary antibodies: rabbit anti-rat alpha-1-fetoprotein (AFP)

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

- Secondary antibodies: sheep anti-rabbit IgG antibodies, purified by immunoadsorbents 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 by silane (see chapter *Silane conditioning of glass slides*).

Staining procedure for paraffin sections

1. **Deparaffinization and rehydration**: sections mounted on coated glass slides are used
   - deparaffinize in the following series of washes at room temperature
     - xylene or xylene substitute 2 x 5 min
     - absolute ethanol 2 x 5 min
     - 95% ethanol 2 x 1 min
     - 70% ethanol 2 x 1 min
     - distilled water 2 x 1 min
   - rehydration is completed when sections are passed into the working buffer
     - PBS 2 x 5 min
2. **Antigen retrieval**: this step depends mainly on the fixation employed and is not always necessary; the right retrieval method must be established by trial (see chapter *Retrieval of antigenic determinants*. In the case of AFP staining in paraffin sections from ethanol-acetic acid fixed liver blocks, no antigen retrieval is necessary. Especially proteolytic digestion (PIER) can result in heavy cell damage and loss of reactivity. However, when tissue specimens have been submitted to prolonged fixation in formaldehyde (as will occur in histopathological routine), antigen retrieval is an important measure (see chapter *Retrieval of antigenic determinants*). In this case we prefer a heat-induced epitope retrieval technique
   - water bath or steamer: preheat the system with COPLIN jar containing sodium citrate buffer (0.01 M sodium citrate pH 6.0 supplemented with 0.05% Tween 20) until 95-100°C are reached,
   - slides in the staining rack are placed into the COPLIN jar and incubate for 20-30 min (optimal time can be different for other antigens),
   - water bath/steamer is turned off, remove the COPLIN jar and allow the slides to cool down to room temperature (about 20 min),
   - rinse the slides with PBS.
3. **Inhibition of endogenous peroxidase**: blocking of endogenous peroxidases can be done by several procedures (see chapter *Blocking solutions*)
   - in the case of more sensitive antigens, it is adviced to perform this step after incubation in primary antibodies,
   - inhibition of peroxidases by hydrogen peroxide
     - 1% H_2O_2 in PBS 30 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. **General remarks**: management of incubation and washings steps
   - prior to incubation in antibodies, a water repellent barrier is applied on the slide by encircling each tissue section with Apiezon® grease by means of a syringe. This measure keeps reagents localized on the tissue sections and, thus, allowing the use of only small volumes. Furthermore, mixing of reagents is prevented when staining more than one section on the same slide . At the end of all incubations, Apiezon is wiped away with a cleansing tissue. Alternatively, Apiezon is removed during dehydration in the xylene baths,
   - incubations with immunological reagents are done in a humid chamber,
   - washings are performed in large volumes of buffer by the use of staining jars (e.g. COPLIN, HELLENDAHL or SCHIEFFERDECKER).

6. **Incubation and washing schedules**: do not let dry the sections during all procedures
   - pretreatment of sections with PBS/BSA supplemented with 5% sheep normal 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 12-24 hours at 4°C,
   - 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 µg/mL,
   - sections are incubated with diluted HRP conjugates for 30 min at room temperature or at 37°C,
   - washings in PBS/BSA for 3 x 5 min.

7. **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/H_2O_2 substrate mixture for 20 min,
   - washings in PBS for 3 x 5 min
   - washings in distilled water for 2 x 1 min.

8. **Post-chromogenic intensification, counterstaining**: the staining intensity of DAB reacted slides can be improved by metal salts (see chapter *Enzyme substrate solutions*). Furthermore, counterstains (f.e. haematoxylin or other histological stains) can influence the final result because supplementary information to that of the immunostain can be obtained
   - osmium tetroxide: the final DAB reaction is osmiophilic, and postfixation in OsO_4 enhances the contrast. This step is optional. Treatment of sections 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,
   - counterstains: counterstains are also optional, but very useful for histological details. We prefer a haematoxylin counterstain. Examples are given in the section “Laboratory

Methods", f.e. *Haematoxylin staining methods*, *Methyl green counterstaining* and other articles.

9. **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% sheep normal serum for 5 min in order to block nonspecific bindings (background),
   - primary antibodies: non tissue relevant antibodies from same species providing the primary antibodies, e.g. rabbit anti-glucose oxidase (IgG); rabbit non immune (normal)
     IgG globulins,
   - sandwich antibodies same as described above,
   - incubation schedules and washings same as described above.

10. **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 (e.g. Eukitt®).

Selected publications for further readings

Kuhlmann WD (1975)
Kuhlmann WD (1978)
Wurster K *et al*. (1978)
Kuhlmann WD and Wurster K (1980)
Brandtzaeg P (1981)
Kuhlmann WD (1984)
Wolf CR *et al*. (1984)
Kuhlmann WD and Peschke P (1986)

Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

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