Immunofluorescence staining of cryostat sections

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Material

Tissue specimens obtained by surgery or bioptic materials are used. Fixation is optional, f.e. by aldehydes and according to results from previous experiments. When working with cell suspensions, then prepare cryo-sections from cell suspenions encapsulated with bovine serum albumin (BSA).

Method of immunostaining

The procedure of indirect immunofluorescence localization of collagen types and laminin in frozen cut sections from oral mucosa 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)
- Embedding medium for tissue freezing (e.g. Tissue-Tek® OCT™ Compound)
- Isopentane (2-methylbutane)
- Ethanol (absolute)
- Acetone (dry)
- Bovine serum albumin (BSA), 30% solution
- Phosphate buffered saline at pH 7.4 (PBS)
- PBS plus 1% BSA (PBS/BSA)
- Distilled water
- Glycerol (anhydrous pure)
- p-Phenylenediamine
- Propidium Iodide (PI)
- DAPI (4’6,-diamidino-2-phenylindole)

Immunological reagents: primary antibodies and conjugates (e.g. FITC labeled) either self prepared or purchased.

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

Glass slides: *super frost plus* glass slides or glass slides coated with adhesives. Enhanced adhesion of tissue sections to resist the subsequent histological procedures can be obtained f.e. with bovine serum albumin (BSA) and glutaraldehyde conditioned slides. Also, glass slides being coated by other means may be used. Especially silane coated slides are recommended (see chapter *Silane conditioning of glass slides*). Frozen sections also adhere very well to glass slides when irradiated by microwaves.

Preparation of BSA/aldehyde coated slides

Chemicals	**Chemical solution**
Bovine serum albumin (BSA, 30% solution, Millipore filtrated) Glutaraldehyde (25%, purified) Acetone p.a. Extran Merck MA 01 Distilled water	• 1% BSA solution: 3.0 mL BSA stock solution *plus* 87.0 mL distilled water • 2% glutaraldehyde: 4.0 mL glutaraldehyde stock *plus* 96.0 mL distilled water

Coating procedure

Glass slides should be cleaned prior to coating. This can be done with acetone. Alternatively, slides can be cleaned with 10% Extran solution (overnight) followed by intensive washing. Slides are placed in a vertical glass slide holder (staining rack) and the holder is placed in a glass tray (Coplin jar):

–	Acetone	overnight
–	rub and dry slides with lint-free cotton cloth	
–	alternative: Extran solution	overnight
–	wash in running hot water	90 min
–	distilled water	several rinses
–	drying at 100°C	60 min
–	1% BSA solution	5 min
–	drain the slide holder	30 sec
–	dry at 100°C (store at room temperature)	2 hours
–	2% glutaraldehyde at 4°C	overnight
–	distilled water	3 x 10 min
–	slides are air dried	

Coated slides can be used for at least 24-48 hours

Immunofluorescence staining of sections

1. **Tissue sampling**: specimens from freshly taken tissue (and aldehyde fixed or not), are placed on a piece of aluminium foil (or in a special cryomold), covered by a drop of water soluble embedding medium such as Tissue-Tek®) and immediately frozen in liquid nitrogen cooled isopentane; frozen tissue blocks are tranferred to the cryo-chamber for sectioning or are stored in firmly closed vials at -70°C until use. Before cutting sections, frozen tissue blocks should equilibrate to the temperature of the cryostat.

2. **Cryomicrotomy**: sections of 4-8 µm thickness are cut with the cryo-microtome (C-profile steel knife) at -25°C to -30°C) using an anti-roll plate and collected on glass slides, preferably coated as described above for improved adherence of tissue sections. Slides with tissue sections are immediately used or stored frozen until needed at -70°C in a sealed slide box.

3. **Handling of tissue sections**: prior to immunostaining, sections are warmed at room temperature and air-dryed. Also, frozen sections can be defrosted and dried in a microwave oven. Optionally, frozen sections may be fixed at -20°C for some minutes in precooled fixative prior to warming.

4. **Fixation**: frozen sections from unfixed tissue blocks are ususally fixed on the slide by organic solvents such as precooled acetone (-20°C). The optimal fixation depends on the antigen under study and must be determined in previous experiments. Typical solvent fixations for routine are

   - precooled acetone (dry) for 5 min at 0°C or at -20°C, followed by rinsings in PBS,
   - precooled 80% ethanol for 5 min at 0°C, followed by rinsings in PBS.

   Other useful fixatives include aldehydes and *p*-benzoquinone.

5. **Principle of immunostaining**: incubation in unlabeled primary antibody is followed by incubation in fluorescein (FITC) labeled sandwich antibodies (directed against the immunoglobulins of that species which provide the primary antibodies). Here is given an example with mouse monoclonal antibodies for the localisation of collagen structures in human oral mucosa (Table).

   Note: do not let dry the sections during all incubation procedures. Do not use antigen retrieval methods for epitope demasking (heat, enzymatic digestion etc.) as may be applied for paraffin sections

Primary antibody	**Specificity**	**Species**	**Format**	**Manufacturer**
Anti-collagen type VII (human)	collagen type VII, carboxy terminal peptide	mouse mono-clonal IgG	ascites, purified	e.g. Chemicon MAB 1345
Anti-collagen type IV (human)	amniotic type IV collagen, α2 chain	mouse mono-clonal IgG1	ascites, liquid	e.g. Chemicon MAB 1910
Anti-laminin (human)	human laminin, B2 chain	mouse mono-clonal IgG1	ascites, not purified	e.g. Chemicon MAB 1920
Anti-lambda chain (human)	human Ig lambda chain	mouse mono-clonal IgG2a	ascites, not purified	e.g. Chemicon MAB 1306

6. **Pretreatment, incubation, washing schedule**:

   - pretreatment of sections with PBS/BSA for 5 min in order to block nonspecific bindings (background),

- primary antibodies (as well as control antibodies) are appropriately diluted in PBS/BSA (see recommentation of the manufacturer), f.e. 1 µg antibodies per mL,
- sections are incubated with diluted primary antibody for 30 min at 37°C under humidified atmosphere,
- washings in PBS/BSA for 3 x 5 min,
- labeled antibodies: e.g. FITC conjugated goat anti-mouse IgG/IgM antibodies purified by affinity chromatography and diluted according to the manufacturer, f.e. 1:100 with PBS/BSA or 5-10 µg/mL
- sections are incubated with diluted FITC conjugates for 30 min at 37°C under humidified atmosphere.
  Note: it is advised to protect slides from light starting from this step until the end
- washings in PBS (without BSA) for 3 x 5 min,
- counterstain if desired, e.g. Propidium Iodide or DAPI
- sections are mounted under coverglass with a drop of glycerol/PBS (glycerol with 10% PBS) or with an anti-fade medium such as *p*-phenylenediamine/glycerol mixture (200 mg of *p*-phenylenediamine dissolved in 10 mL of 0.1 M phosphate buffer pH 7.4 plus 90 mL glycerol). Sections are stored in the dark.

7. 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*,
- handling and pretreatment of sections as described above,
- primary antibodies: non tissue relevant antibodies from same species providing the primary antibodies, e.g. mouse anti-human lambda chain monoclonal antibodies (IgG); mouse non immune (normal) IgG globulins,
- sandwich antibodies same as described above,
- incubation schedules and washings same as described above.

DAPI counterstain

The blue nuclear fluorescence of DAPI gives at bright contrast to green, red or yellow fluorescent probes. DAPI stains nuclei with little or no cytoplasmic labeling and is a useful counterstain for immunofluorescence with FITC (green) or Texas Red (red) as markers.

Chemicals	**Chemical solution**
4'6,-Diamidino-2-phenylindole (DAPI) Dimethylformamide (DMF) PBS	• DAPI stock solution (5 mg/mL): 10.0 mg DAPI *plus* 2.0 mL DMF mix to dissolve and store aliquots at -20ºC • DAPI working solution (100 ng/mL): 2.0 µL DAPI stock *plus* 100.0 mL PBS store at 4ºC in brown bottle to protect from light

Staining procedure Incubate sections in the dark for 30 minutes at room temperature
Nuclei stain bright blue

Propidium Iodide (PI) counterstain

The red nuclear fluorescence of PI gives at bright contrast to green or blue fluorescent probes. PI stains nuclei with little or no cytoplasmic labeling and is a useful counterstain when FITC (green) or AMCA (blue) are used as markers.

Chemicals	**Chemical solution**
Propidium Iodide (PI) RNase Distilled water PBS	• PI stock solution (1 mg/mL): 1.0 mg PI *plus* 1.0 mL distilled water mix to dissolve and store at 4ºC or store aliquots at -20ºC protected from light • RNase stock solution (1.0 mg/mL): 1.0 mg RNase *plus* 1.0 mL distilled water store aliquots at -20ºC • PI working solution (1 µg/mL PI and 10 µg/mL RNase): 2 µL PI stock *plus* 20 µL RNase stock are added to 2 mL PBS
Staining procedure Incubate sections in the dark for 30 minutes at room temperature	
Nuclei stain red	

Selected publications for further readings

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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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