Preparation of thin slices for light microscopy

Optical light microscopy course
23.10.2012
Kirsi Rilla

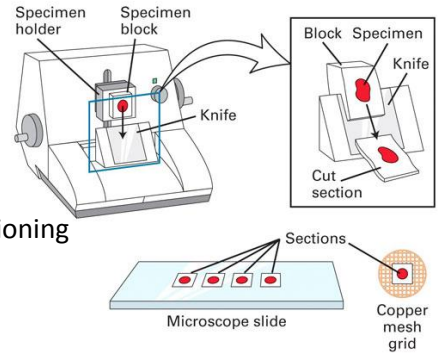
Shortly: Histological sample preparation for microscopy

1. Fixation:
To fix the tissue components in place, and its preservation.

2. Embedding:
Paraffin supports the tissue during sectioning

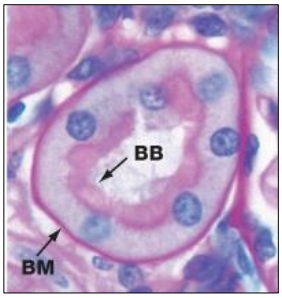
3. Sectioning:
With microtome (5-15 micrometers)

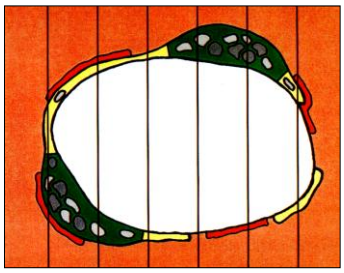
4. Staining:
Thin unstained sections do not absorb light, and are not visible in normal light microscopy



Tissue sectioning

In the traditional microscopy we see a two-dimensional image of the original three-dimensional structure!!!





Histological sample preparation

- In order to work at the microscope the sample block has to be cut into thin sections (typically 5 µm). In this case, the contrast of cells and tissues is very weak, so normally sections have to be stained to achieve more contrast.

-The most common ways to produce tissue sample is to make paraffin or frozen sections. Paraffin advantage is that tissue structures are remained very natural.

- Frozen sections are used primarily when you need quick information about the tissue (e.g. cancer tissue obtained from patients may be analyzed during surgery), or when, for example antibody used in immunostainings does not work in paraffin sections.

Why fixation?

- The purpose of fixation is tissue solidification and preserving the structure of the sample as natural as possible.

- Fixation is the first and most important step of the sample preparation. None of the following work steps can correct the failure of the fixation.

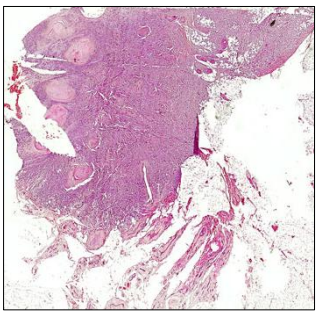
There are two main ways of chemical fixation:

1. Immersion = a small piece of tissue is immersed in high volume of fixative.

2. Perfusion = fixation via blood flow: The fixative is injected into the animal's bloodstream.

Properties of a good fixative

1. Penetrates the tissues and cells quickly
2. Kills the cells quickly and prevents post-mortem changes
3. Maintains the structure of cells and tissues as normal as possible
4. Prevents cells and tissue breakdown
5. Increases the mechanical strength of the sample
6. Increases the contrast of the sample



Fixatives

Typical fixatives:
Cross-linking reagents: form cross-links between proteins
- Formalin (10% formaldehyde)
- Paraformaldehyde
- Glutaraldehyde

Organic solvents: remove lipids and dehydrate the cells, while precipitating the proteins
- Ethanol
- Methanol
- Acetone

- Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components.
- Cross-linkers fix proteins, but not properly fats and sugars.
- A suitable fixative for all samples does not exist.

Formalin vs. paraformaldehyde

Formalin:
- A 10% formalin is actually only a 4 % solution of formaldehyde.
This is because 10% formalin is a 10% solution made from a stock bottle of 40% formaldehyde. Formalin contains about 1% methanol.

Formalin is slowly increasing the concentration on methanol, which promotes clumping of proteins, instead of the cross-linking.
→ formalin should not be stored for too long times !

Paraformaldehyde:
- A powder of polymerized formaldehyde
- Paraformaldehyde is depolymerized to formaldehyde in solution.
- Pure but unstable (methanol!)

Factors affecting fixation

pH
-Should be physiological, between pH 4-9.
Osmolarity
- Hypertonic solutions result in cell shrinkage.
- Hypotonic solutions result in cell swelling.
Size of the Specimen
- Optimal thickness is 1-4 mm
Volume of the Fixative
- At least 15-20 times greater than tissue volume
Temperature
-Increasing the temperature increases the rate of fixation.
-Fixation is routinely carried out at room temperature.
Duration
- As a general rule 1 hour per 1 mm
Time interval between death and fixation
- The time interval between death and fixation should be minimized

What is a suitable fixative for my sample?

Best fixative of choice when you want to preserve:

Proteins: Formalin, Paraformaldehyde
Lipids: Frozen Sections
Enzymes: Frozen Sections
Nucleic acids: Alcoholic fixatives
Mucopolysaccharides: Frozen Sections
Glycogen: Alcoholic fixatives

Embedding

Dehydration:
- Since paraffin is insoluble with water, samples need to be dehydrated by using the rising alcohol series: For example 70%, 80%, 95% and 100%

Clearing:
-A clearing agent (usually xylene) is used to remove the ethanol, because paraffin wax in insolube in ethanol

Infiltration:
- Molten paraffin wax infiltrates the sample and replaces the xylene.

Infiltration

-The tissue pieces are transferred into metal or plastic embedding cassettes.
-Write the sample code with pencil
(pen and ink are soluble in the following stages).

1. Select the mold, there should be sufficient room for the tissue with at least a 2 mm surrounding margin of wax.
2. Fill the mold with paraffin wax.
3. Place the tissue in the mold according to the side to be sectioned.
This side should be facing down against the mold.
4. Add more paraffin into the mold to fill the cassette and mold.
5. Cool the block on the cold plate.
6. Remove the block from the mold.

Tips for proper embedding

Careless embedding can make microtomy much more difficult.
- Avoid under-filling the cassette as this can allow unstable clamping in the microtome and lead to cutting “thick then thin” sections.
-Avoid over-filling cassettes as this can interfere with the correct alignment of the block face for sectioning.
-Any excess wax on the outside of a cassette should be removed before clamping to ensure the block is firmly held during sectioning.
- Specimen orientation is very important !

Sectioning

Microtome is utilized for making 5-15 µm sections of the paraffin block

1. Place the paraffin block in the block holder and advance it closer to the blade.
2. Start the rough trimming of your paraffin section until a complete section is seen in the block.
3. Start obtaining a ribbon, and pick the ribbon with a paint brush and transfer the ribbon to your water bath.
4. Lay the ribbon on your water bath and allow the sections to stretch for a few seconds.
5. Carefully separate the sections and pick each section on a glass slide.
6. Pick the section on an angle to allow the water to exit the slide and section.
7. Allow the slide section to drain for a few minutes.
8. The glass slides are then placed in a warm oven for about 15 minutes to help the section adhere to the slide.

Preparation of cryo-sections

1. Freezing of a piece of tissue
- CO2 (-70 ° C)
- The liquid nitrogen (-195 ° C)

2. Sectioning
- Cut into 5-40 µm sections, utilizing cryostat
- Sections are settled on coverglasses in room temperature, to which it adheres by itself

3. Staining
- Histochemical and immunohistochemical stainings

4. Fixation
- If a permanent slide is desired, it can be fixed with formaldehyde
- After fixation, an additional staining can be done (Hematoxylin)

5. Mounting

Cryo-sections

Advantages:
• High-speed technology: suitable for use in surgical pathology, which clip, and the diagnosis is made in a few minutes
• demonstrate the lipids
• many enzymes and antigens remain active in frozen sections

Disadvantages:
• The details of the cell structures are lost
• Frozen blocks are difficult to maintain for long periods of time
• More difficult to make serial sections than from paraffin blocks

Staining

Histochemistry
- Hematoxylin-Eosin staining

Historadiography
-Radiolabeled molecules

In Situ Hybridization
- DNA or RNA localization in their cellular environment.

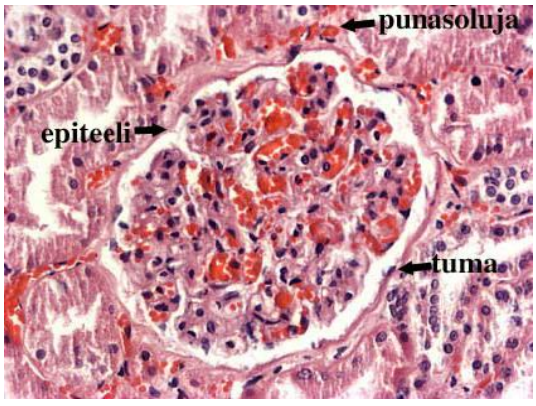
Immunohistochemistry
- Antibodies to detect specifically proteins, carbohydrates or lipids

Immunofluorescence
- The stain is a fluorescent molecule

HE-staining – tissue section

HE-staining is the most common basic staining, utilizing hematoxylin to stain nuclei violet and eosin to stain cytoplasm and the extracellular matrix filaments pink.

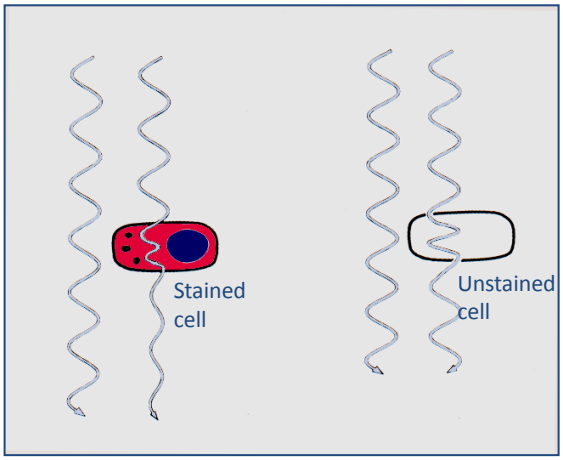
Kidney glomerulus



Why staining ?

Thin sections from biological tissue have little contrast.

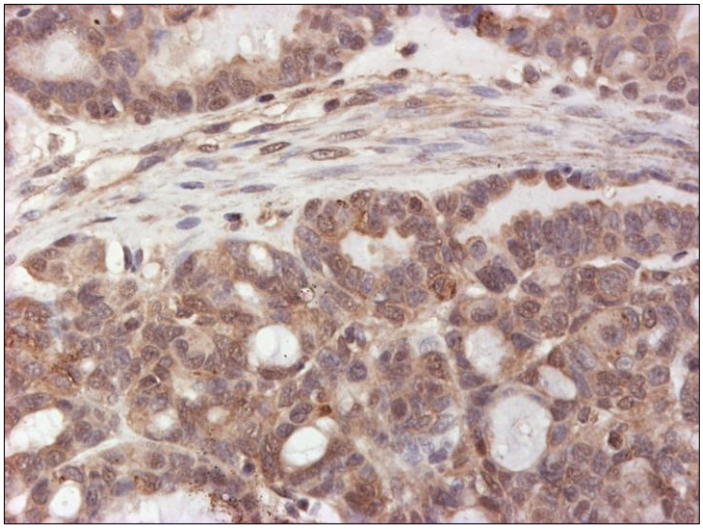
Dyes influence the amplitude (height) of light waves traversing through the sample → more contrast



Immunohistochemical staining protocol with avidin-biotin peroxidase method

1. Deparaffinization (xylene –ethanol –water)
2. Treatment for 5 min with 1% H_2O_2 to block endogenous peroxidase
3. Blocking of the unspecific staining (with 1% BSA)
4. Primary antibody diluted in 1% BSA in PB, incubate overnight at +4°C.
5. Washing with phosphate buffer (PB) 3x5 min, room temperature
6. Secondary antibody (biotinylated), dilute in PB, 1 h, RT.
7. Washing 3 times with phosphate buffer for 5 min
8. Incubate with ABC (kit)
9. Washing
10. Incubation with DAB (Diaminobenzidine) for 5 min.
11. (Counterstaining with Mayer's hematoxylin to stain nuclei.)
12. Dehydration and mounting

Immunohistochemical staining of tissue (Ovarian cancer)

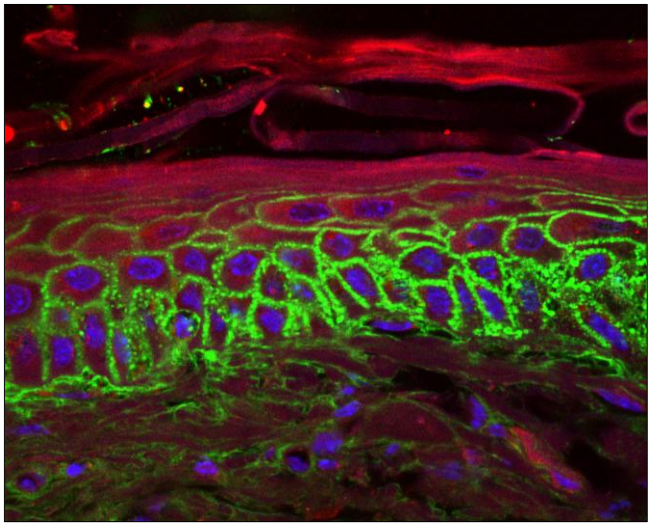


Immunofluorescence staining protocol

1. Deparaffinization (xylene –ethanol –water)
2. Removal of autofluorescence (glycine)
3. Blocking of the unspecific staining (with 1% BSA)
4. Primary antibody diluted in 1% BSA in PB, incubate overnight at +4°C.
5. Wash the sections with phosphate buffer (PB) 3x5 min, room temperature
6. Secondary antibody (labeled with fluorescent marker, dilute in PB, 1 h, RT.
7. Wash 3 times with phosphate buffer for 5 min
8. Mount with mounting media suitable for immunofluorescence (Vectashield)
9. (Countersatin with DAPI to stain nuclei)

Note! If the mounting media is liquid, you have to seal the edges of the coverslip with nail polish.

Immunofluorescence staining of tissue (skin section)



Why mounting?

- Cover slips hold samples in place
- Protects sample from inadvertent movement and contamination.
- It also protects the microscope, preventing direct contact between the sample and lens as well as accidental leakage of water-based preparations

Properties of a good mounting medium:
- A refractive index close to that of glass (1.518)
- Non-reactivity with the specimen
- Stability without crystallizing, darkening, or changing refractive index
- Solubility in the medium the specimen was prepared in
- Not causing the specimen stain to fade or leach

Mounting media

Types of mounting media:
- Water-insoluble mounting media that solidify (Canada Balsam, Depex)
- Water-insoluble mounting media that remain liquid (Paraffin oil)
- Water-soluble mounting media that solidify (Glycerol jelly)
- Water-soluble mounting media that remain liquid (Glycerol, Vectashield)

-For media remaining in a liquid state, it is necessary to prevent the liquid from flowing out by sealing the sides of the cover slip (Nail polish)

Learn to recognize artefacts !