Faculty of Biological Science and Technology
Zoology and Botanical Department
Practical Histology

An introduction to tissue processing and staining

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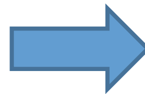
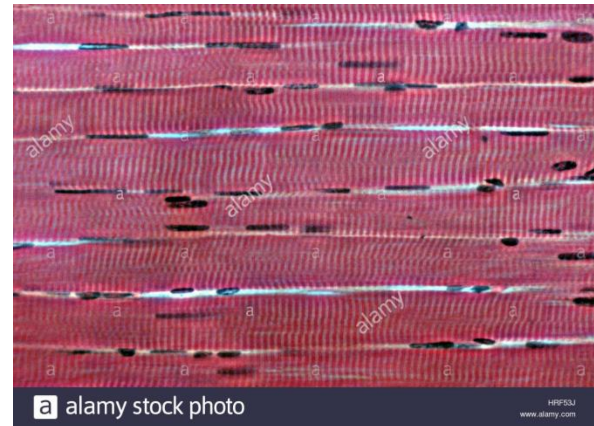
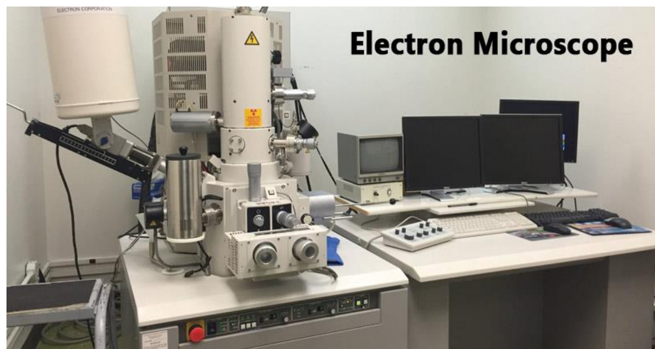
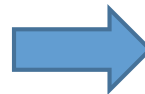
What we need for histological studies?

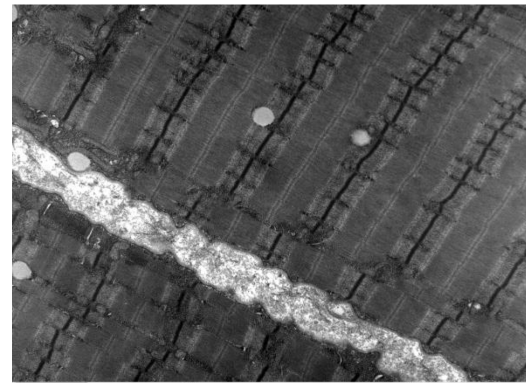
- A method for processing and staining a tissue, so it can be observed by the microscope
- A suitable microscope (optical, electronic, fluorescent, etc.)









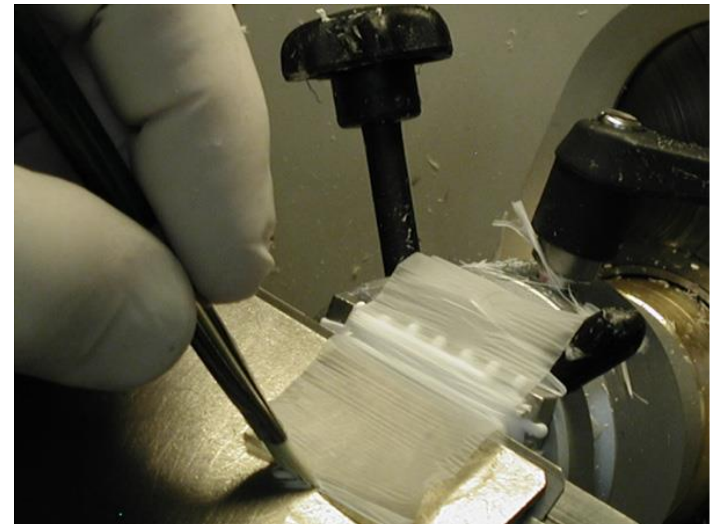


How a sample is prepared for histological studies

- Most of the fresh animal tissues are very soft and are easily torn or damaged, so it is not possible to prepare a thin section from them unless they are chemically preserved or stabilized in such a way they are supported during cutting
- There are generally two ways to provide support during cutting:
- 1) tissues can be cut when they are frozen. These types of sections are called “frozen sections or cryosections”
- 2) A chemical substance is introduced into the tissue in a liquid state, which later turns into a solid with appropriate physical properties and allows thin slices to be taken. Paraffin is an example of these materials. These types of sections are called “paraffin sections”
- Frozen sections have lower quality than paraffin sections and are prepared in cases where a quick examination of the tissue is needed

Cryosection

- Frozen sections have lower quality than paraffin sections and are prepared in cases where a quick examination of the tissue is needed
- To prepare frozen sections, first the tissue is frozen by immersing water-rich tissues in liquid nitrogen
- Then, the samples are cut by frozen microtomes. Samples are immersed in a gel-like environment during cutting, which has a density similar to the density of the frozen tissue, such as polyethylene glycol or polyvinyl alcohol
- Next, the slices are stained and studied under a microscope



Refrigerated microtome is known as cryostat microtome

Tissue processing for paraffin sections

- The steps in which a tissue taken from human or animal is fixed and completely immersed in a suitable wax so that it is ready to be cut by a microtome are called “tissue processing”
- Tissue processing consists of four steps: fixation, dehydration, clearing and infilteration (with wax or paraffin)

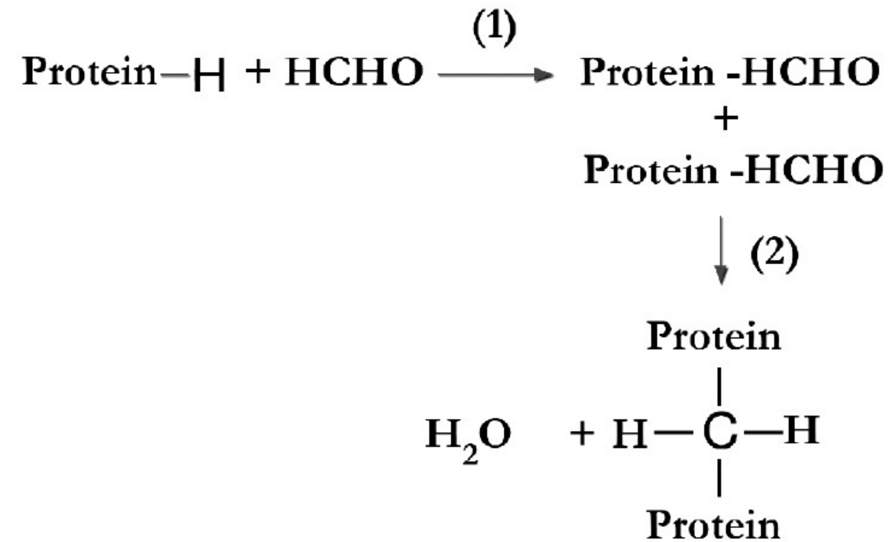
Fixation

- The most important part of all histological and cellular technique is to preserve the tissue or cells in their natural form. To achieve this goal, tissue samples are immersed in a fixation fluid
- Fixation must be perform immediately after taking a sample from a human or laboratory animal at the same place of collection. Otherwise, the sample should be immediately transported to the laboratory and placed in fixative
- Basically, fixatives prevent autolysis by deactivating lysosomal enzymes and stabilize inside and outside cellular structure. Fixatives also make macromolecules to be insoluble in water. Furthermore, fixatives prevent the growth of bacteria and fungi which cause spoilage
- Usually, 4% or 10% formaldehyde is used for fixation
- Fixation is done in room temperature
- Depending on the size of the sample, it takes 6 to 24 hours to fix
- Specimens are placed in special labelled cassettes (small baskets) to separate them from other specimens

The importance of fixation

- The main morphological details of specimen can only be used when the sample is well fixed
- Following weak fixation:
- Cutting specimen is difficult
- The morphology of specimen is not shown even if the rest of tissue processing perform accurately

Fixation



Formaldehyde reaction with protein. Formaldehyde is a cross-linking agent that can inactivate, stabilize, or immobilize proteins

Dehydration

- Because paraffin hydrophobic, water must be removed before specimen is placed in paraffin
- This process is done by immersing specimen in ethanol alcohol solutions, which are ordered from low to high concentration, and finally pure alcohol

For example:

- typical dehydration sequence for specimens not more than 4mm thick would be:
  - 70% ethanol 15 min
  - 90% ethanol 15 min
  - 100% ethanol 15 min
  - 100% ethanol 15 min
  - 100% ethanol 30 min
  - 100% ethanol 45 min

Clearing

- Even though the specimen is completely water free after dehydration, paraffin cannot be penetrated in it because alcohol and paraffin are not soluble in each other. So, an intermediate solvent is used, which can be mixed with both alcohol and paraffin. Ethanol is replaced by this solvent and then it is replaced by melted paraffin in the next step
- Some material used in clearing step cause transparency of tissue due to their relatively higher refractive index
- Another important role of clarifying substances is to dissolve and remove fat, which prevents the penetration of paraffin
- Xylol is the most common clarifying agent. It is necessary to pass specimens several times through xylol to completely replace ethanol

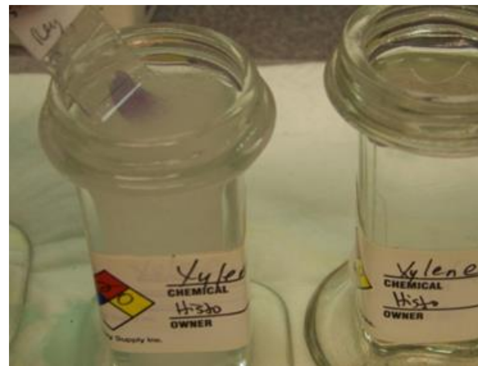
For example:

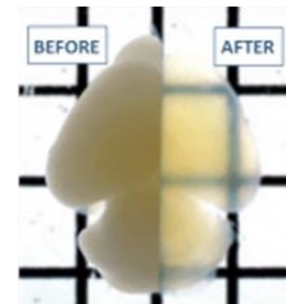
- A typical clearing sequence for specimens not more than 4mm thick would be:

  xylene 20 min

  xylene 20 min

  xylene 45 min





Infiltration with wax

- At this stage, a histological wax is penetrated into specimen. Although, historically various materials have been used for this purpose, paraffin-based histological waxes are more common
- Paraffin is liquid at 60°C, and then cools down to 20°C, where it solidifies and can be made into coherent slices
- Sometimes these waxes are a mixture of pure paraffin and some other additives such as various resins
- The special formulation has suitable physical properties that make the wax penetrate into the specimen and then it is tough enough to cut a thickness of at least 2 µm by microtome. On the other hand, waxes have enough flexibility to melt on the water after floating specimen slices in a hot water bath

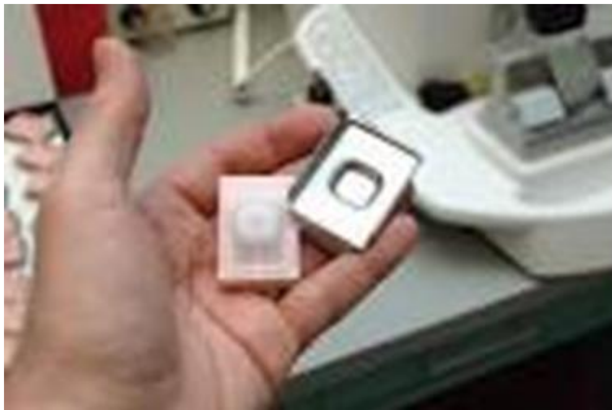
For example:

- A typical infiltration sequence for specimens not more than 4mm thick would be:
  - wax 30 min
  - wax 30 min
  - wax 45 min

An automatic tissue processing machine

Embedding or blocking out

- When the specimen is completely infiltrate with wax, it must be placed into special mold, so it can be connected to microtome for cutting
- For this step, the mold is partially filled with melted wax and then the specimen is carefully placed in the wax in the desired direction. Then, the rest of the mold is filled with melted wax
- It is very important to pay attention to the direction of the specimen, because it will determine the type of cut
- Wax blocks containing specimens are completely stable and can be stored for a long time

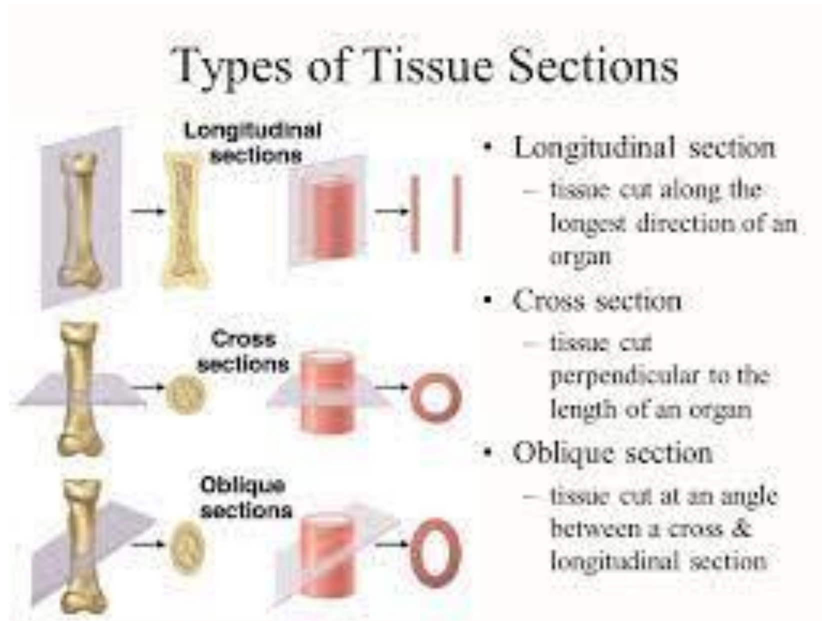


Covering glass slides

- Most tissue sections are placed on glass slides. Different materials are used to cover glass slides. This helps to prevent the specimens being washed off from the surface of slides and causes the sample to stick to the surface of the slide in the following stage
- Egg albumin is the most common substance that is used for covering slides

cutting

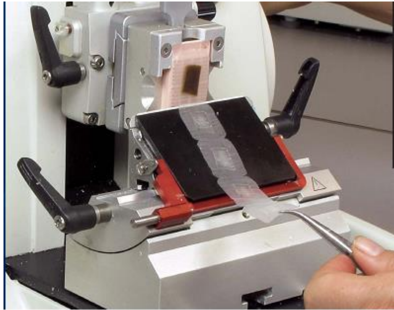
- Type of sections in histology:
- Longitudinal or sagittal sections, in which the cut is made along the long axis of the tissue
- Cross or transverse sections, in which the cut is made along the short axis of the tissue
- Oblique sections, in which the cutting plane is oblique to the short or long axis of the structure



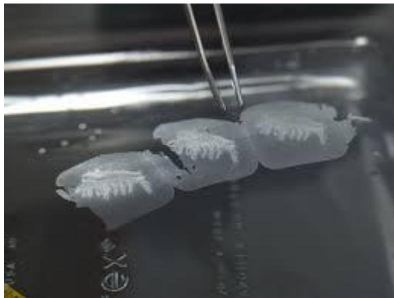
From: https://www.bcchr.ca/sites/default/files/group-bcchr-histology-core/histology-best-practices.pdf

Microtomes for cutting paraffin- based samples

Cutting and mounting



- A microtome and a strip of serial sections from tissue specimen in paraffin



- Floating of slices on hot water

- Transfer of slices from the water surface to the coated slide which is known as mounting

Staining

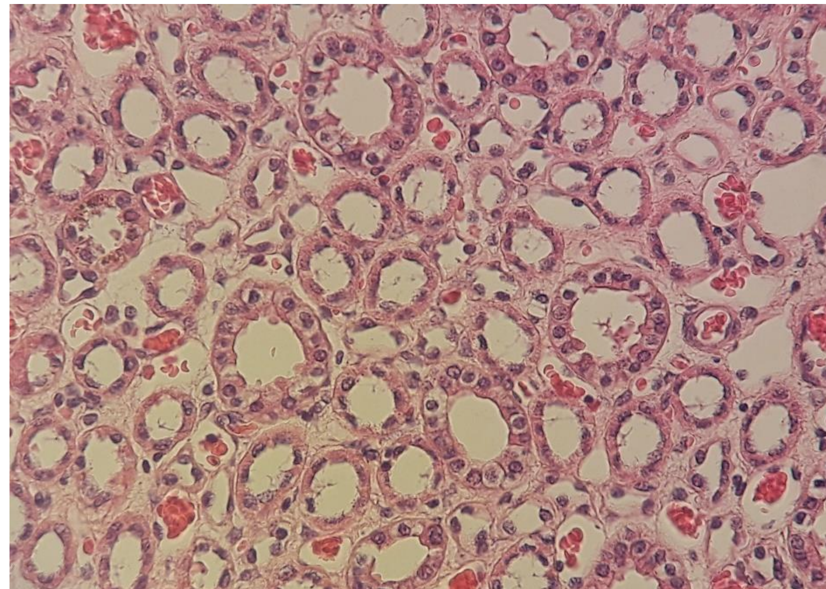
- Most cells are colourless or transparent, so they must be stained to be visible. There are two types of staining technique:
- 1) Non-specific staining: in this case, all types of cells and tissues are stained in the same way
- 2) specific staining: in this case, specific chemical groups or molecules are stained in the cells or tissues
- Staining is usually done by mixture of dyes the make some parts of the cells lighter and some part of cells darker

Acidic and basic dyes

- Acidic dyes react with basic (or cationic) components of the cell. Proteins or aother components of cytoplasm have alkaline properties, so they can bind to acidic dyes (cell components that take these dyes are called acidophilic components)
- Basic dyes react with acidic (or ionic) components of the cell. Nucleic acids are acidic and therefore bind to basic dyes (cell components that take these dyes are called basophil components

Hematoxylin and eosin (H&E) staining

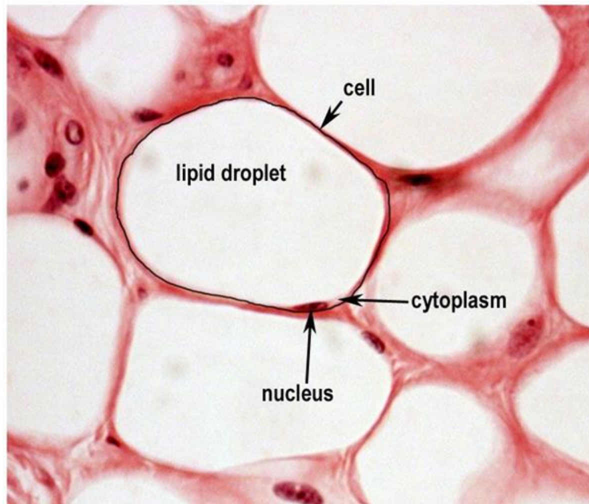
- H&E is a common non-specific staining, in which hematoxylin and eosin are used
- Eosin is an acidic dye with a negative charge. This dye turns the alkaline (or acidophilic) structures into red or pink. These structures are sometimes called as eosinophilic structure
- Hematoxylin is considered as a basic dye. It is used to stain acidic (or basophilic) structure into purple. So cell structures such as DNA in the nucleus and RNA in ribosomes turn purple



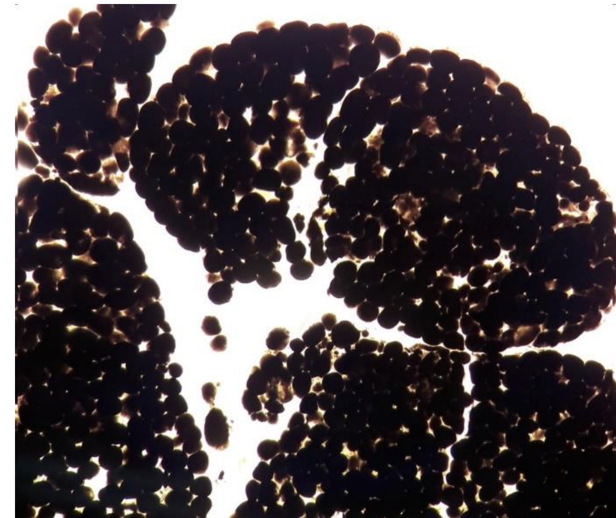
Cross sections of kidney; H&E staining

Fat tissue staining

- The use of alcohol and xylol in the common methods of paraffin section processing leads to the removal of fat from tissue. Therefore, to preserve fat, freezing sections and special staining by lipophilic dyes are usually used
- Black sudan is an example of such dyes that causes neutral fats, phospholipids and sterols stain. By using these colours, rich fat structures such as myelin and fat droplets, turn black



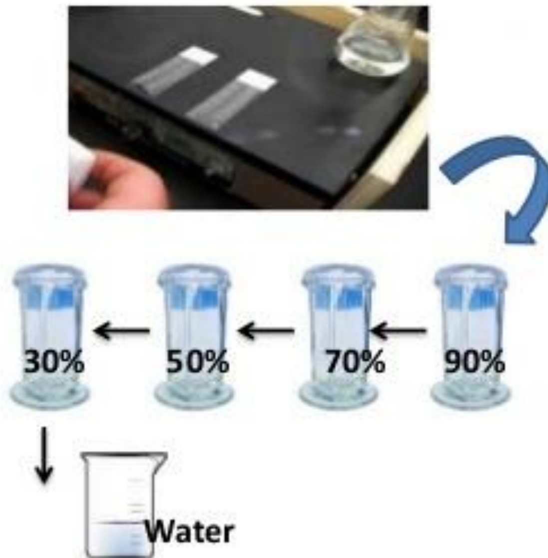
White fat tissue; H&E staining



White fat tissue; black sudan staining

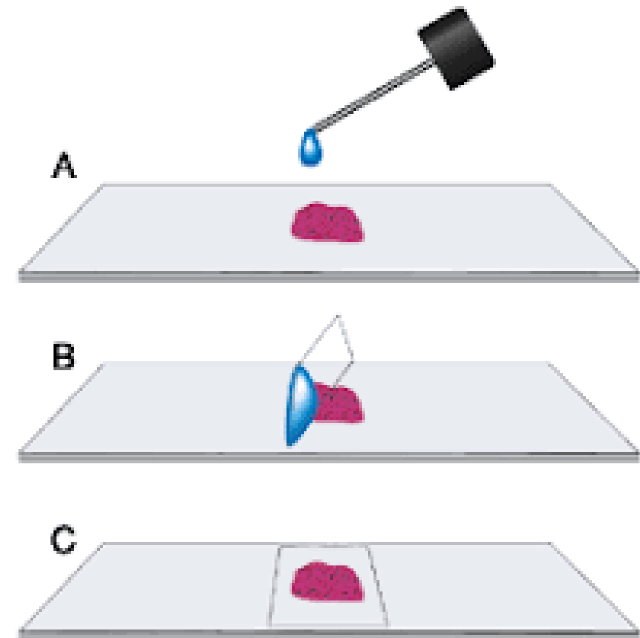
Steps for staining

- Before staining, the slide along with the specimen is placed at a temperature of 60°C to remove paraffin as much as possible
- Then, the specimens are placed in xylol to completely remove the paraffin
- In next step, the specimens are dehydrated by passing mounted specimens through the alcohols that are ordered from high to low concentration
- Water must be removed from specimens after staining by using alcohol gradient from low to high concentration
- Also, xylol is used for more clearing



apply coverslip

- Entellan or canada balsam is used to stick coverslip to the surface of sample
- Small drops of these substances is enough
- Care should be taken not to get air bubbles under coverslips



Prepared slides can be stored for several years