Lectures of
Histological Microtechniques

(Master Stage)
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2. Washing

After fixation, the tissue should be washed to remove the excess fixative. Either by using water, buffer solutions or ethanol, according to the type of fixative. For example:

* Tissue that fixed by **formalin** should be washed with **water** three times, for a duration of (1/2-1) hour each, and this is done by removing the fixative from the vial in which tissue was fixed and replaced it with water.
* Tissue fixed with **Bouin's solution**, should be washed (3-5) times with concentrated **alcohol 70%** until the yellow color is removed from the tissue.
* While fixation by using **mercuric chloride**, tissue should be washed first by **running water** for a duration of (8-10) hours, then by **alcohol 50% or 70%**, finally, tissue treated with a **saturated iodine solution in alcohol 70%** (because iodine removes excess mercuric chloride deposited in tissues).

After washing, tissue can be kept in an alcohol 70% for several weeks, but it's better to perform the subsequent operations (dehydration, clearing, and infiltration) directly because long-term preservation reduces the susceptibility of the tissues to dye/staining.

3. Dehydration

Because paraffin wax is hydrophobic, most of the water in a specimen must be removed before it can be infiltrated with wax. This process is commonly carried out by using material that mix with water, so the most common and good material is ethanol alcohol. This step is performed by immersing specimens in a series of ethanol alcohol of increasing concentration until absolute (30, 50, 70, 80, 90, 95, 100)%, so that the water in specimen is progressively replaced by alcohol, to avoid excessive distortion of tissue (It is important to include two absolute alcohol (i.e., 100%) steps to ensure that all remaining water has been removed).

A higher concentration of alcohol initially is in inadvisable because this may cause very rapid removal of water may produce cell shrinkage. An exception to this is in case of alcoholic fixative like Carnoy, tissue transferred from can placed in higher grades of alcohol or even in absolute alcohol. For routine biopsy and postmortem tissue of (4-7) mm thickness (70, 90)% and absolute alcohol (2-3 changes for 2-4 hours each) are sufficient to give reasonably satisfactory result.

If the tissue is incompletely dehydrated, it is not possible to "clearing" it, when it is exposed to a subsequent clearing agent (e.g., xylene) the tissue remains opaque and appears milky. This will necessitate re-dehydration again. Dehydration will also remove some of the lipoidal material in the tissue. If the lipids are supposed to be visible, it will be necessary to use an appropriate fixative that will preserve the lipids prior to the dehydration step (e.g., osmium tetroxide).

*** There are various other dehydrating agents used:**

1. Acetone:

- It is clear, colorless volatile inflammable fluid.
- It has a rapid action in dehydrating the tissue but produces shrinkage and distortion to the tissue.
- Low cost is also an advantage.
- It usually dehydrate within (20-30)minutes, 4 changes of it should be used.

2. Dioxane:

- It dehydrates and clears at the same time.
- It is miscible with paraffin and with water and alcohol, tissue from dioxane can be transferred straight to paraffin.
- Causes less shrinkage of tissues.
- Tissue can be left in it without danger of hardening for longer period.
- Disadvantage: It is toxic to human. It is more expensive than alcohol.

3. Isopropyl alcohol:

- It is miscible with water and other organic solvents.
- It does not harden the tissue like alcohol.
- It is expensive.

Note: all water dehydrating agents should be characterized by mixing with water and clearing solutions.

4. Clearing

Although tissue is now water-free, we still cannot infiltrate it with wax (paraffin) because wax and ethanol do not mix. Therefore, we must use an intermediate solvent that is fully mixing with both ethanol and wax. This solvent will displace the ethanol in tissue, then this in turn, will be displaced by paraffin wax.

The aim of this step (clearing) is to make specimen transparency. Another important role of clearing agent is to remove a substantial amount of fat from tissue, which otherwise presents a barrier to wax infiltration.

A popular clearing agent is xylene, and multiple changes are required to completely displace ethanol. The immersion of the sample in the intermediary liquid is recommended not to be very long because the samples are hardened and getting sections might be more difficult.

Clearing agents make tissue transparent, because they raise the refractive index of its components, and remove some of them fats, they are added at a ratio of (10:1) of tissue volume.

Incomplete clearing increases cutting failure, such as incomplete dehydration, where tissue appears opaque, and appears milky in color, in which case the dehydration process must be repeated before. If the dehydration process is complete, it is sufficient to change the clearing agent twice, duration of each is between (half an hour and an hour), depending on the size and type of tissue, while the sections are clearing twice, duration of each of them ranges from (3–5) minutes.

* Good Clearing Agents

There are several types of clearing agents, and the good ones are characterized by the following characteristics/features:

1. Rapid removal of the dehydrator.
2. Slow volatilization.
3. Fast clearing without affecting tissue.
4. Moderate price.

* Types of Clearing Agents

Although there are several clearing agents available, xylene is the most common. This is due to its moderate price and satisfactory performance of the clearing process. Other agents: cedarwood oil, chloroform.

(a) Xylene (Xylol)

It is commonly used in clearing. Its boiling point is about 140℃, cheap pricey, fast-acting, makes tissue transparent, and can be removed easily during infiltration step with paraffin wax, but it has some disadvantages, as it causes tissue shrinkage and hardening if leave it for a long time (small tissue with a thickness of 3 mm need a period for clearing 15 to 30 minutes). It is also not suitable for clearing of brain and lymphatic tissue, because it makes them fragile.

(b) Cedarwood Oil

Its boiling point ranges between (165 and 237)℃. It does not cause tissue hardening, even if left in it for a long time, and it's permeating quickly. This agent is expensive, and it takes a long time to get it out of the tissue in the wax oven, the wax must be changed 3 times to get rid of its residue.

(c) Chloroform

Its boiling point is 61℃, and it has less effect on tissue, in terms of shrinkage or hardening when compared to xylene. It is an excellent clearing for fetuses, nervous system, and lymphatic organs, it also evaporates quickly in a wax oven, and it does not ignite, but it is rather expensive.