Tissue Processing

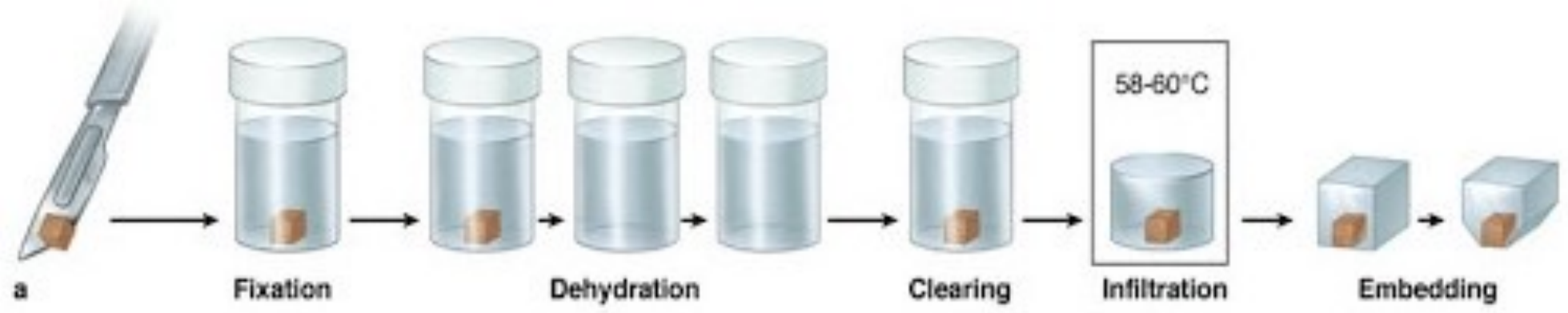
Lecture Objectives

At the end of this lecture you must be able to:

- Describe tissue processing
- Explain stages of tissue processing
- Discuss automatic tissue processing
- Discuss troubleshooting in tissue processing

Introduction

- Tissue processing describes the steps required to take tissues from fixation to the state where it is completely infiltrated (permeated) with a suitable wax i.e. paraffin wax to enable cutting of thin sections.



Principle of Tissue Processing

- Tissue processing is designed to remove all extractable water from the tissue, replacing it with a support medium that provides sufficient rigidity to enable sectioning of the tissue without parenchymal damage or distortion.

Factors Influencing the Rate of Processing

- When tissue is immersed in fluid, fluid exchange occurs between the fluid within the tissue and the surrounding fluid.
- Several factors influence the rate at which the interchange occurs: namely,
    - **Agitation**: Increases the flow of fresh solutions around the tissue. Efficient agitation may reduce the overall processing time by up to 30%.
    - **Heat:** Increases the rate of penetration and fluid exchange. Care must be taken to avoid shrinkage and hardening of the tissue sample.

Factors Influencing the Rate of Processing

- **Viscosity:** Viscosity is the property of resistance to the flow of a fluid. The smaller the size of the molecules in the solution, the faster the rate of fluid penetration (low viscosity).Conversely, if the molecule size is larger, the rate of exchange is slower (high viscosity).

- **Vacuum:** Using pressure to increase the rate of infiltration decreases the time necessary to complete each step in the processing of tissue samples. Vacuum will remove reagents from the tissue, but only if they are more volatile than the reagent being replaced. Vacuum can also aid in the removal of trapped air in porous tissue.

Labeling of Tissues

- It is important that all specimens are properly labeled before processing is started.
- The identification number given to each specimen should be labeled on tissue cassettes which hold specimens when being processed.
- For labeling, pen containing ordinary ink should not be used. Printed, or graphite pencil, are satisfactory.

Stages of Tissue Processing

i. Fixation
ii. Dehydration
iii. Clearing
iv. Infiltration (Impregnation)
v. Embedding

Fixation

- **Preserves cells and tissue components with minimal distortion**
- The most commonly used reagent for the fixation of histological specimens is 10% neutral buffered formalin (NBF).

Dehydration

- **The removal of ‘free’ unbound water and aqueous fixatives from the tissue components.**
- **Dehydrants**: Ethanol, Methanol, Butanol, Isopropanol, Acetone, industrial methylated spirit (denatured alcohol).
- The most commonly used dehydrant is ethanol. It ensures total dehydration, making it the reagent of choice for the processing of electron microscopy specimens.
- **Additives to dehydrants**: phenol(4%), for softening hard tissue e.g. tendon, nail, dense fibrous tissue etc.

Dehydration

Ethanol

- Fast-acting and reliable.

- Graded concentrations of ethanol are used for dehydration; the tissue is immersed in 70% ethanol in water, followed by 95% and 100% solutions.

- Should be accomplished slowly. If the concentration gradient is excessive, diffusion currents across the cell membranes may increase the possibility of cell distortion. For this reason, specimens are processed through a graded series of reagents of increasing concentration.

- For delicate tissue it is recommended that the processing starts in 30% ethanol.

Dehydration

- Excessive dehydration may cause the tissue to become hard and shrunken.
- Incomplete dehydration will impair the penetration of the clearing reagents into the tissue, leaving the specimen soft and non receptive to infiltration.

Clearing

- The next step alcohol should be replaced by paraffin wax.
  - As paraffin wax is not alcohol soluble, we replace alcohol with a substance in which wax is soluble.

- **Clearing reagents act as an intermediary between the dehydration and infiltration solutions.**

- Clearing leaves the tissues clear and transparent. This term relates to the appearance of the tissues after dehydrating agent has been removed. If the refractive index of the clearing agent is similar to the protein of tissue, the tissue becomes transparent.

- The end point of clearing can be noted by the transparent appearance of the tissue.

Clearing

- Clearing of tissue is achieved by any of the following reagents:
  - Xylene, Chloroform, Benzene, Carbon tetrachloride, Toluene, Cedarwood oil, Chloroform

- Xylene is commonly used.

- Criteria for choosing a suitable clearing reagent includes:-
  —Rapid penetration of tissues, rapid removal of dehydrating agent, ease of removal by melted paraffin wax, minimal tissue damage, low flammability, low toxicity and Low cost.

Infiltration

- After clearing, tissues are transferred to molten paraffin wax for infiltration. During this process clearing reagent diffuses out and molten wax is infiltrated.

- **Infiltration is the saturation of tissue cavities and cells by a suppor ting substance which is generally the medium in which they are finally embedded.**

- The most common agent of choice is paraffin wax which is molten when hot and solid when cold.

- An infiltrating and embedding medium should ideally be molten between 30°C and 60°C and suitable for sectioning.

Infiltration

- The use of vacuum infiltration is often used to help complete impregnation of tissues with wax.

- The aim of vacuum treatment is:

i. To remove air bubbles in the tissue, as may occur in porous tissue such as lung, spleen, decalcified bone and skin.

ii. To remove the clearing agent more rapidly by increasing its vaporisation.

Infiltrating and Embedding Reagents

Paraffin wax

- The most common in histopathology laboratories.
- Permeates the tissue in liquid form and solidifies rapidly when cooled, has a hard consistency so section of 3-4 micron thickness can be cut. Its inexpensive, provides quality sections and easily adaptable to a variety of uses.
- Forms a matrix and prevents distortion of the tissue structure during microtomy.

Paraffin wax

- **Additives:** Beeswax, Rubber, Ceresin, Plastic polymers and Diethylene glycol distearate

- Additives to paraffin wax are used for the following reasons:

i. To increase hardness in order to cut thinner sections

ii. To increase hardness in order to give the necessary support when sectioning harder tissues

iii. Improve ribboning.

iv. Decrease melting point of paraffin wax

v. Improve adhesion between specimen and wax

Alternative Embedding Media

- There are occasions when paraffin wax is an unsuitable medium for the type of tissue being processed including:

1. Sections are required to be thinner, e.g. lymph nodes
2. The use of heat may adversely affect tissues or enzymes
3. The infiltrating medium is not sufficiently hard to support the tissue

- Examples of alternative embedding media: Resin, Gelatin, celloidin etc

Embedding

- The final stations in any tissue processor is infiltration of all specimens with paraffin or another wax-based embedding medium. Thereafter, all the metal or plastic cassettes holding tissue are removed from the tissue processor and taken at the embedding center.

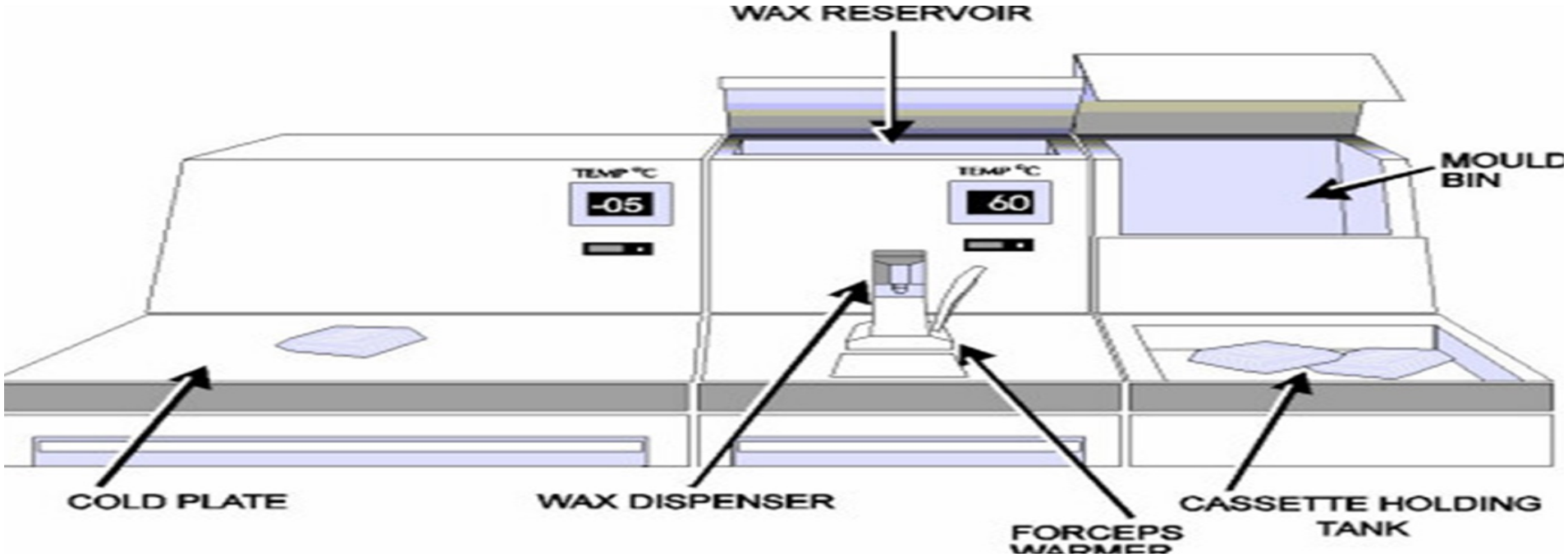
Embedding

- **Embedding involves the enclosing of properly processed, correctly oriented specimens in a support medium that provides external support during microtomy.**

- The medium should provide elasticity, resisting section distortion while facilitating sectioning.

- Paraffin embedding is the standard method used in histology laboratories to produce blocks of tissue for section cutting (microtomy)

- Allowed to occur at melting point temperature of paraffin wax, which is 54 ^{o}C -60^{o}C.
- Volume of wax should be about 25-30 times the volume of tissues.



Embedding Stages

https://www.youtube.com/watch?v=Nab97hfUVPc

Embedding stages

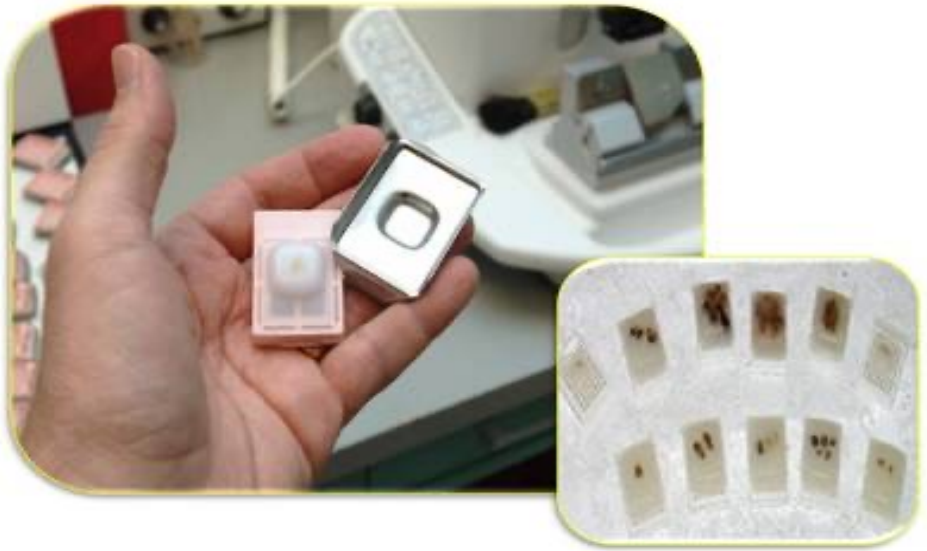
Remove tissue from cassette

Fill mould with wax and orientate tissue

Cool and flatten as required

Add cassette, fill with wax and put on cold plate

Remove cassette from mould



Block now ready for sectioning (microtomy)

Embedding stages

1. Open the tissue cassette, check requisition form entry to ensure the correct number of tissue pieces is present.

2. Select the mould; there should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax.

3. Fill the mould with paraffin wax.

4. Using warm forceps select the tissue, taking care that it does not cool in the air; at the same time.

5. Place the tissue in the mould according to the side to be sectioned. This side should be facing down against the mould. A small amount of pressure may be used in order to have more even embedding.

Embedding stages

6. Chill the mould on the cold plate, orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.

7. Insert the identifying label or place the labelled cassette base onto the mould.

8. Add more paraffin into the mould to fill the cassette and mould.

9. Cool the block on the cold plate.

10. Remove the block from the mould.

11. Cross check block, label and requisition form.

Orientation of Tissues

- Important for the demonstration of proper morphology.
- Incorrect orientation → diagnostic tissue elements being damaged during microtomy or not being evident for pathology review.
- Orientation of the tissue should offer the least resistance of the tissue against the knife during sectioning.

Tissue Orientation

- There are many different kinds of tissue that have specific embedding requirements.

- Some few examples are:

i. If the tissue is fairly homogeneous (like a piece of liver) and roughly equal in vertical and horizontal dimensions, it doesn't really matter how it is oriented it in the block.

ii. If the shape of the tissue is much longer in one dimension than the other, it is better to embed it parallel to the microtome knife edge, or diagonal in the block, rather than perpendicular to the knife edge.

Tissue Orientation

iii. If the tissue has layers of different consistency e.g. skin, the knife should pass through the softer layers first. For example, in a cross section of skin, the knife should cut through the subdermal fat first, then the collagenous dermis, then the epidermis and finally the keratinous layer.

iv. Tough tissues like bone and cartilage should not be oriented parallel to the knife edge, but diagonally, so that the knife does not contact the entire length of the specimen simultaneously.

v. When multiple small specimens are embedded in one block, they should be spread out horizontally as much as possible, so that they are not all cut by the exact same area of the knife edge.

Tissues requiring special orientation

Type of Tissue	Orientation
Tubular structures	cross section of the wall and lumen should be visible; arteries, veins, fallopian tube and vas deferens samples
Skin biopsies	shave punch or excisions, cross section of the epidermis, dermis and subcutaneous layers must be visible.
Intestine, gallbladder, and other epithelial biopsies	cut in a plane at right angles to the surface, and oriented so the epithelial surface is cut last, minimizing compression and distortion of the epithelial layer.
Muscle biopsies	sections containing both transverse and longitudinal planes.
Multiple pieces of a tissue	oriented side by side with the epithelial surface facing in the same direction

Choose an Appropriate Mould

- A mould of suitable size is always chosen for each specimen.
- If the same mould size is used for every specimen some tissue may touch the edge of the mold.

NOTE:

- **The mould used for the above specimen was too small. The specimen is in contact with the edges of the block and may therefore be difficult to section.**

Do Not Over-fill Moulds

- Moulds are filled to an optimum level and do not overflow.
- If over-filled can lead to:

✓ scraping of the back and edges of the cassette before microtomy.

✓blocks may sit unevenly in the microtome chuck causing instability that may lead to the tissue becoming damaged during microtomy

Blocks that have resulted from over-filling the molds during embedding.

Automatic Tissue Processing

- Tissue processing may be performed manually (hand processing) or with the help of an automated tissue processor.

- Manual tissue processing is rarely used in routine laboratory. The advantages include:
  I. Small number of samples can be processed in a small laboratory.
  II. Careful monitoring in each step is possible.
  III. In case of emergency when the automated tissue processor is not working, one can take the help of the manual processing.
  IV. In case of manual processing, it is possible to select the reagents of choice with flexibility in time duration.

Automatic Tissue Processing

- Advantages of automated tissue processor –

i. Saves time
ii. Decreases human error
iii. Effective fluid circulation
iv. Temperature can be adjusted and vacuum/pressure can also be incorporated.

Automatic Tissue Processing

- Most tissue processing machines have a 12 container processing cycle, which gives flexibility in designing a wide range of processing cycles to suit different tissues.

- Some machines apply heat and vacuum to the tissues during tissue processing. This increases the rate of processing, but care must be taken in the selection of tissue for this type of processing. There is tendency of some tissues e.g. spleen, muscle, skin, those containing blood to become unduly hardened.

Automatic Tissue Processing

- The rate of processing can be increased by:

1. The use of warm (40-50^{o}C) fixative to ensure that fixation is complete before commencing dehydration
2. Dehydration commencing at 95% alcohol stage, though this should not apply to delicate tissue.
3. The use of a fast clearing agent, e.g. xylene
4. The use of vacuum at all wax stages
5. Agitation at all stages, even during fixation
6. The minimum of time at all stages

Replacement of Processing Fluids

- The frequency with which fluids should be changed depends on the number and size of the tissues processed. An odour of clearing agent in the final wax indicates that a change is required.

- Points worth of note:

1. Beakers and wax bath must be filled to the correct fluid level and located in their fasteners.
2. Any spillage of fluid must be wiped away.
3. Accumulations of wax must be removed, particularly from beaker covers.

Replacement of Processing Fluids

4. Particular attention should be paid to the fastening of the process baskets (which hold cassettes) on the machine- some machines are prone to shed baskets with disastrous results.

5. The paraffin wax bath should be checked to ensure that that the wax is molten.

6. Most tissue processing machines have facilities for setting a delay period before commencing; this period should be carefully checked.

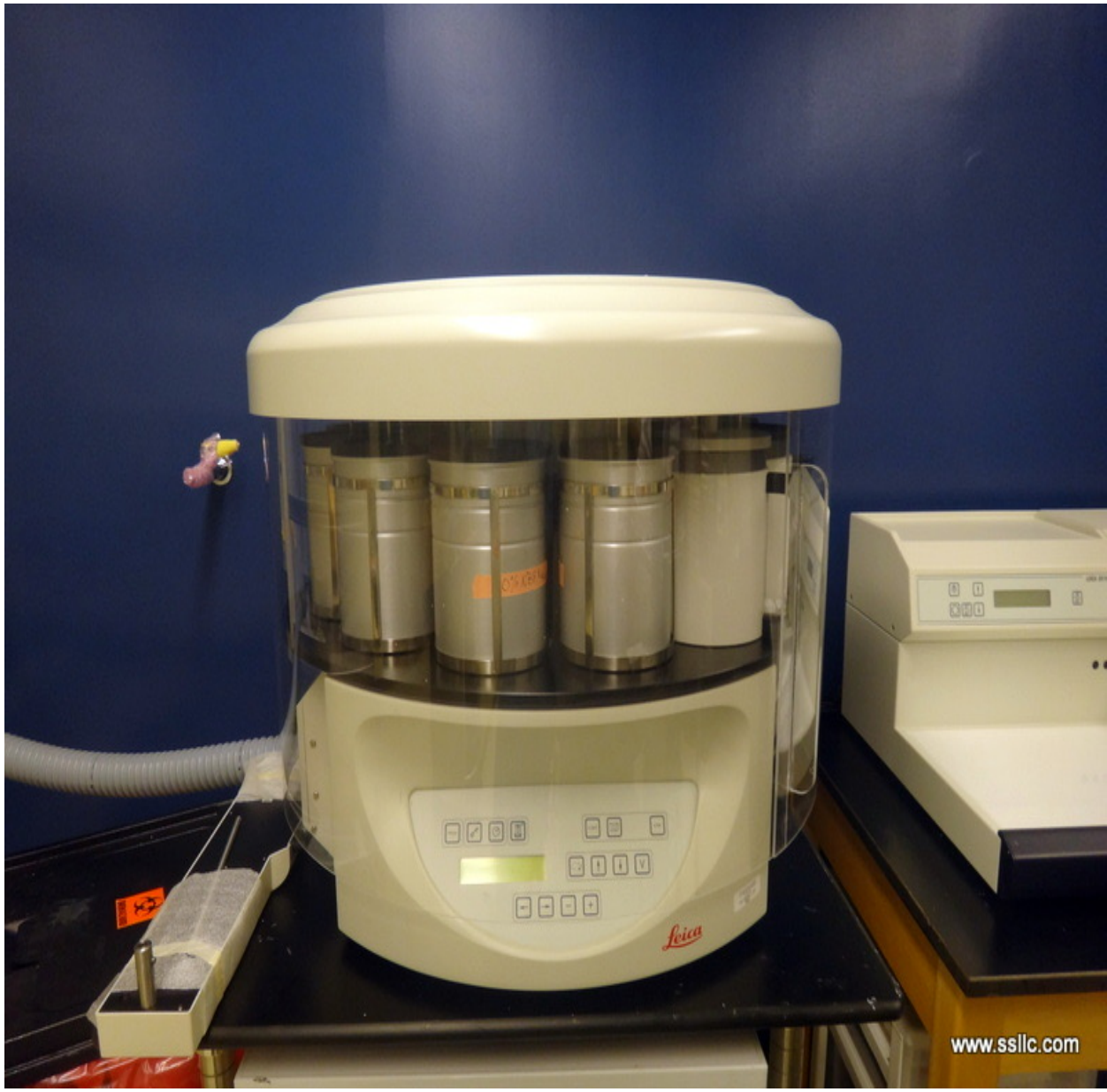
Tissue processors

- There are two main types

1. Tissue transfer (dip and dunk)

- Specimens are transferred from container to container to be processed.

- Vertical oscillation or the mechanically raising and lowering of the tissue into the reagent containers provides the agitation needed for the processing of the tissue.



Tissue processors

2. Fluid transfer (enclosed) types

- Specimens are held in a single process chamber or retort and fluids are pumped in and out as required.

- Most modern fluid-transfer processors employ raised temperature, effective fluid circulation and incorporate vacuum/pressure cycles to enhance processing and reduce processing time.

Troubleshooting in Processing

Problem	Cause	Remedy
Tissue is soft during embedding	• Tissue is too thick during grossing • Reagents are saturated with water • Paraffin is saturated with clearing agent e.g. Xylene	• Tissue should be properly cut • Change the reagents • Change paraffin
Tissue is coming out from the block during sectioning	• Dehydration process is inadequate, so there is defective paraffin infiltration in the tissue	• Change the processing program and give adequate time for dehydration
Microchattering effect around the edges of the tissue section	• Excessive dehydration	• Change the dehydration time. It is better to process the smaller biopsy tissue separately from the larger tissue to have proper dehydration
Cracked and folded tissue section	• Excessive dehydration	• The block is soaked with a wet gauze piece before sectioning
Irregular staining of haematoxylin and eosin stain	• Dehydration process is suboptimum	• Change the processing program, and give adequate time for dehydration

Summary

- Stages of tissue processing:

1. **Fixation** – stabilizes and hardens tissue with minimal distortion of cells.
2. **Dehydration** – removal of water and fixative from the tissue.
3. **Clearing** – removal of dehydrating solutions, making the tissue components receptive to the infiltrating medium.
4. **Infiltrating** – permeating the tissue with a support medium.
5. **Embedding** – orienting the tissue sample in a support medium and allowing it to solidify

- Automatic tissue processing is more convenient and much more efficient to use, especially with multiple specimens.