HISTOLOGY

1ST LEC.

(TISSUE PROCESSING)

SECOND YEAR/COLLEGE OF DENTISTRY
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Tissue processing

Introduction

- **Tissue processing deals with the preparation of TISSUE for MICROSCOPIC examination.**

➢ **Aim of Tissue Processing to:-**

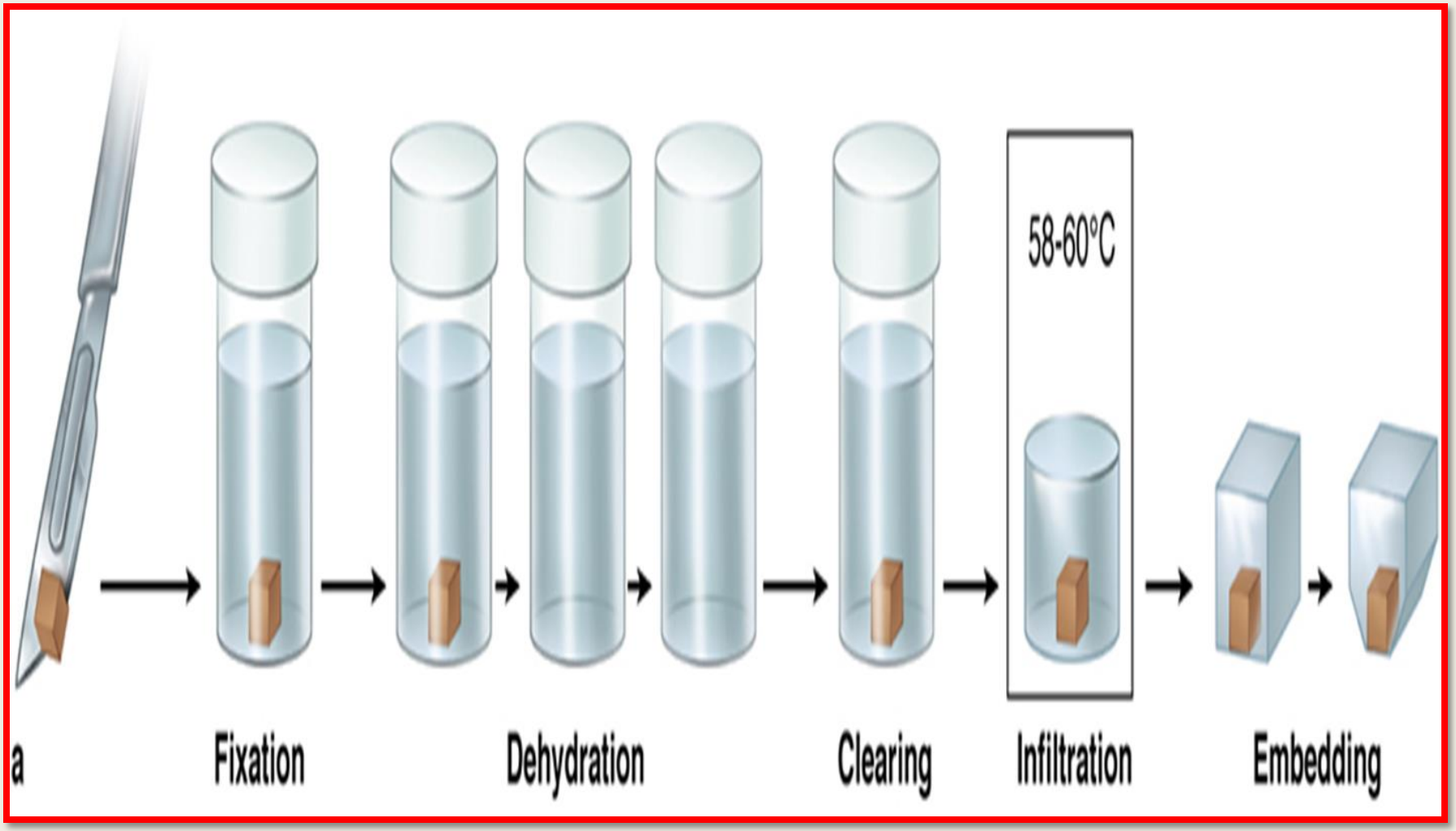
1. **Preserve microscopic components of tissue.**
2. **Make them hard, so that very thin section (3, 4 , 5 micron) can be made.**

- **This is achieved by passing tissue(s) through a sequence of steps.**

- **Tissue processing is a long procedure and required about 24 hours or more depend on the type of tissue.**
- **Tissue processing can be done by manually or mechanically.**

Manual Tissue Processing

In this process, tissue is changed from one container to another <u>by hand.</u>



Mechanical Tissue Processing(Automated):

In this processor, there are different jars containing reagents.

These are arranged in a sequence.

Tissue is moved from one jar to another by a mechanical device.

- Specimen-transfer or "dip and dunk" processors: instruments which transfer cassettes from station to station in a rotary or linear arrangement.
- Fluid-transfer or "enclosed" instruments hold the specimens in a process chamber and the reagents are pumped in and out during processing.

Time controlled by a timer (hours and/or minutes).

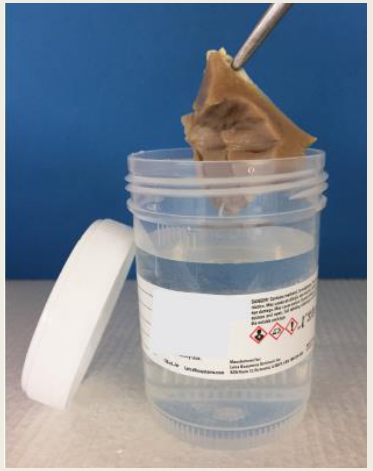
Tissue processing, either manually or mechanically, involves the same steps.

Automated tissue processor

Automated TP.

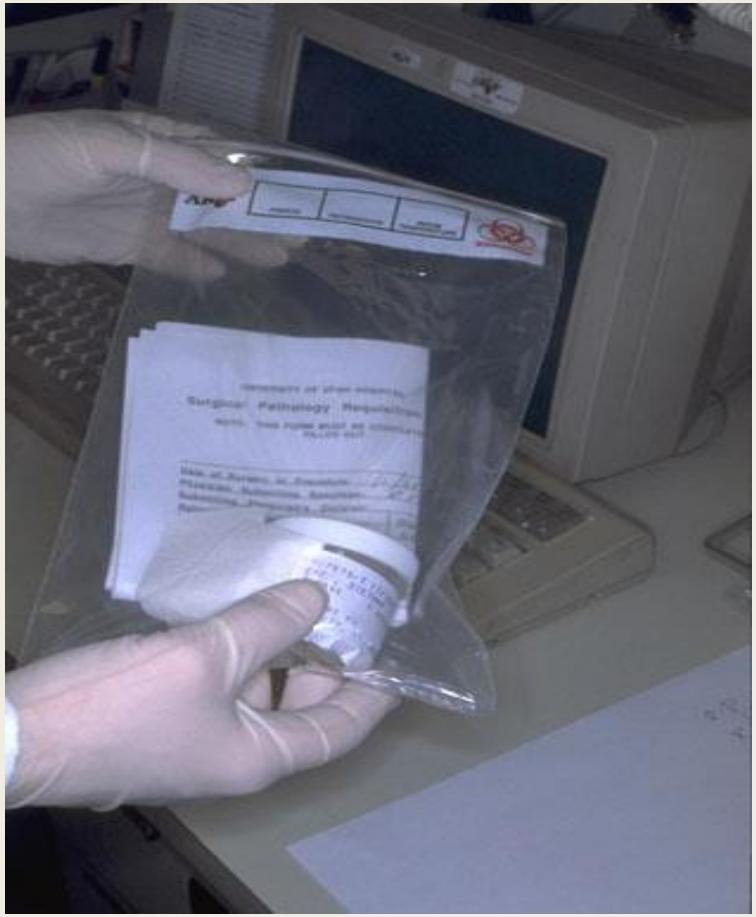
Fixation - types of fixatives

- **The purpose of fixation is to preserve tissues permanently in as life-like state as possible.**
- **Fixation should be carried out (ASAP or promply) after removal of the tissues (in the case of surgical pathology) or soon after death (with autopsy) to prevent autolysis.**
- It is necessary that a specimen remains unfixed for a short period of time, it should be refrigerated at 4 °C.
- **Fixation is the most essential part in histology.**
- **A well fixed tissue is the key for a good slide and therefore a good interpretation for diagnosis.**



Label Specimens

- Tissue specimens received in the surgical pathological laboratory**. This tissue have request form included (patient information and history along with a description of the site of origin).**
- The specimens are labelled by giving them a **number that will identify each specimen for each patient**.



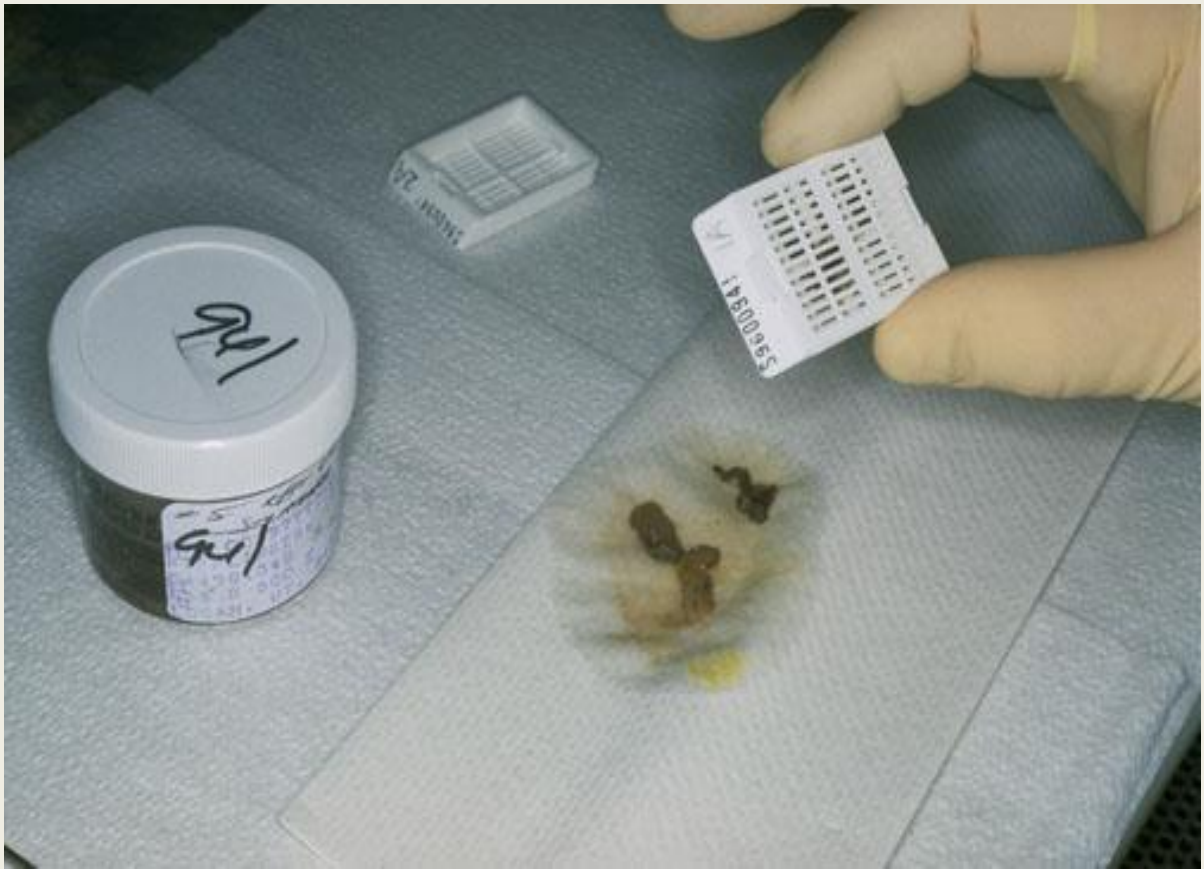
Label Specimens

- Each specimen should be properly identified and all details recorded as soon as possible.

Specimens with incomplete labels such a these, should not be accepted by a laboratory.

Gross examination

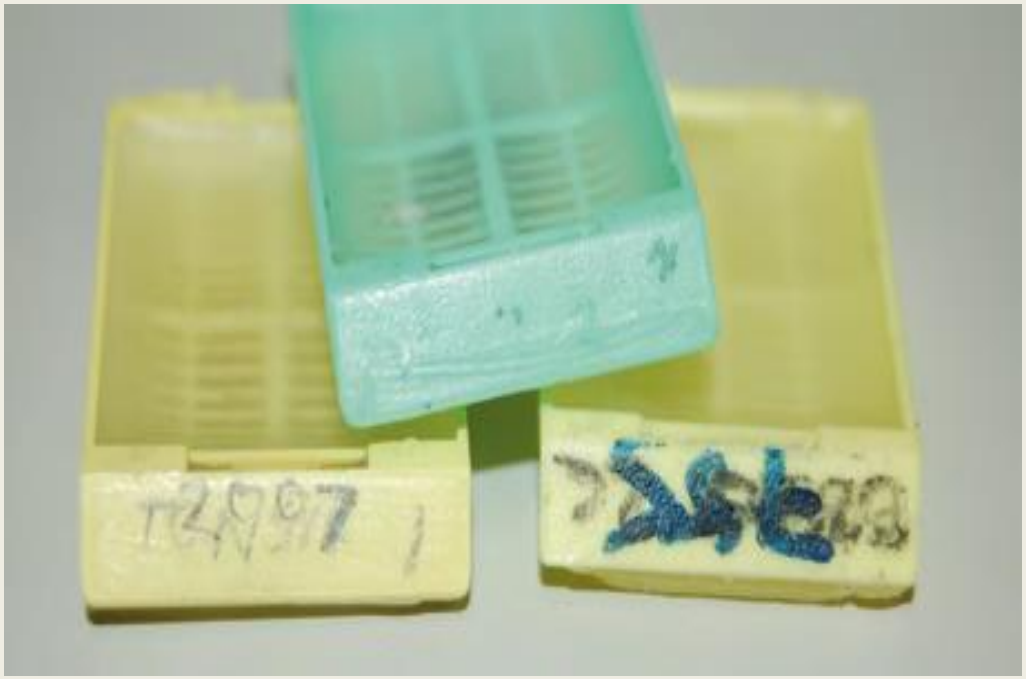
- Gross examination consists of describing the specimen(measurement, consistency) and placing all or parts of it into a small plastic cassette which holds the tissue. Initially, the cassettes are placed into a fixative.



■Labeled of Tissue

■For labeling, pen containing ordinary ink should not be used. Printed, graphite pencil used to label tissue.

■Clearly Label Cassettes



These illegible cassette labels are totally unacceptable.

Properties of an Ideal Fixative

- 1. Prevents autolysis and bacterial decomposition.
- 2. Preserves tissue in their natural state and fix all components.
- 3. Preserves tissue volume.
- 4. Avoid excessive hardness of tissue.
- 5. Allows enhanced staining of tissue.

Fixation basics

- **1- Obtain of tissue**
- **2- Size of tissue**
- **3- Size of fixative**
- **4- Period of fixation**

Fixation basic

1- Obtain of tissue

After operation or FNA or killing of experimental animal, emersion tissue by used **normal saline** after that transferred to vial contain fixative.

2- Size of tissue

- The specimen dimensions allow rapid penetration of the fixative. Large specimens should be rapidly transported to the lab for slicing tissue to allow proper fixation to occur.

Fixation basic

- **3- Size of Fixative**
- The size of fixative is always 5-10 times more than tissue.

Use Sufficient Fixative and a Suitable Container

- An adequate volume of fixative (ratio of at least 20:1) is used in a container of an appropriate size. This avoids distortion of the fresh specimen and ensures good quality fixation

This container is too small for the mass of tissue it contains. There is insufficient fixative present and the specimen may well have been distorted as it was pushed into the container.

- **4. Period of fixation**
- Specimens should be fixed for approximately 6 to 72 hours.
- O/V “Overnight” fixation (i.e. 8-12 hours) is generally indicated for 10 mm thick slices of tissues.
- Fixation for **12-24 hours is considered optimal for most immunohistochemistry**. Minimum fixation of 6 hours to a maximum of 72 hours for breast cancer specimens.
- Prolonged fixation i.e. >72 hours in formalin should be avoided because it may produce **nonspecific background staining**.

Formalin or Formaldehyde 10%

- The most widely employed universal fixative particularly for routine paraffin embedded sections.
- It is a gas with a very acute odor, soluble in water to a maximum extent of 40% by weight and is sold as such under the name of formaldehyde (40%) or formalin (a colorless liquid).
- Formaldehyde or 10% buffered formalin is commonly prepared by adding 100 ml of 40% formaldehyde to 900 ml distilled water.

Formalin or Formaldehyde

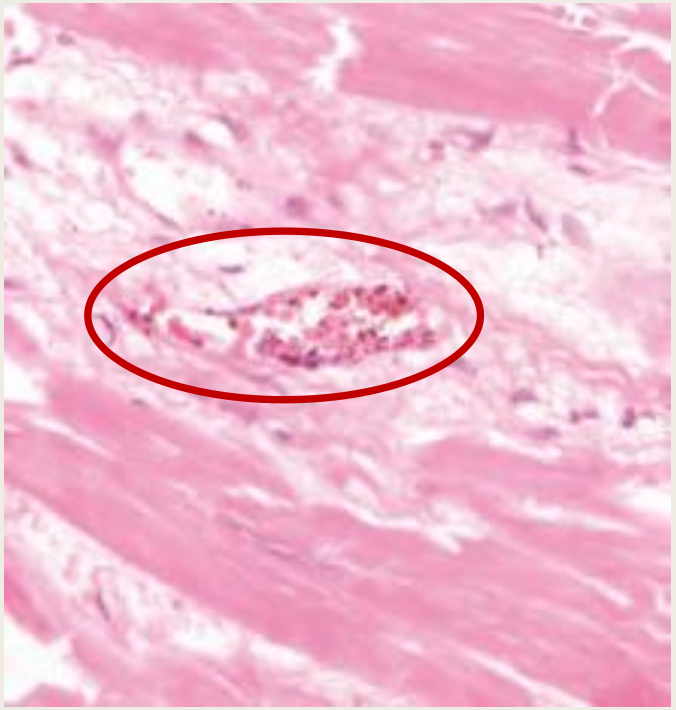
- Tissue is fixed by cross-linkages formed in the proteins.
- This cross-linkage does not harm the structure of proteins greatly, so that antigenicity is not lost. Therefore, formaldehyde is good for IHC techniques.
- Formalin penetrates tissue well, but is relatively slow.
- The standard solution is 10% neutral buffered formalin.
- Its prevents autolysis.

Factors Affecting Fixation

- There are a number of factors that will affect the fixation process:
- Buffering
- Penetration
- Volume
- Temperature
- Time interval

Buffering

- **Fixation is best carried out close to neutral pH, in the range of 6-8.**
- **Acidity favors formation of formalin-heme pigment that appears as black, deposits in tissue.**
- **Commercial formalin is buffered with phosphate at a pH of 7.**

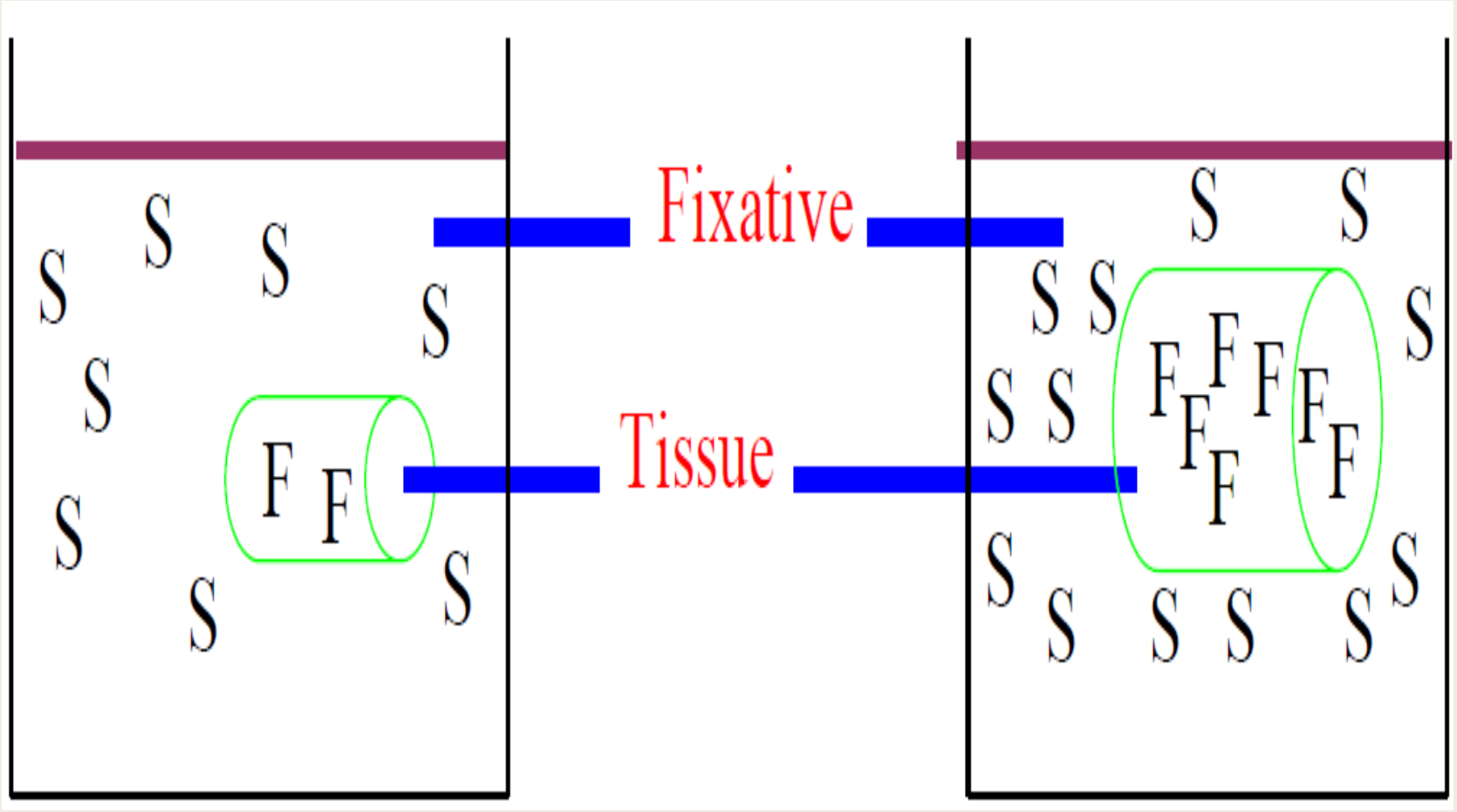


Penetration

- Penetration of tissues depends upon the diffuse ability of fixative as well the size of sample.
- Penetration into a thin section will occur more rapidly than for a thick section.

Volume of fixative

- The recommended ratio of the tissue volume to the fixative volume is at least 15 to 20 times greater than the tissue volume.


Fixative
Tissue

Temperature

- The fixation can be carried out at room temperature.
- **Increasing the temperature, as with all chemical reactions, will increase the speed of fixation, as long as you don't cook the tissue.**
- **Hot formalin will fix tissues faster, and this is often the first step on an automated tissue processor.**

Concentration

- Concentration of fixative should be adjusted down to the lowest level possible, because you will expend less money for the fixative. Formalin is best at 10%.

Time interval (drying &moisture)

- Also very important is time interval from removal of tissues to fixation. The faster you can get the tissue and fix it, the better.
- Artifact will be introduced by drying, so if tissue is left out, we can moist with saline.
- The longer you wait, the more cellular organelles will be lost and the more nuclear shrinkage .

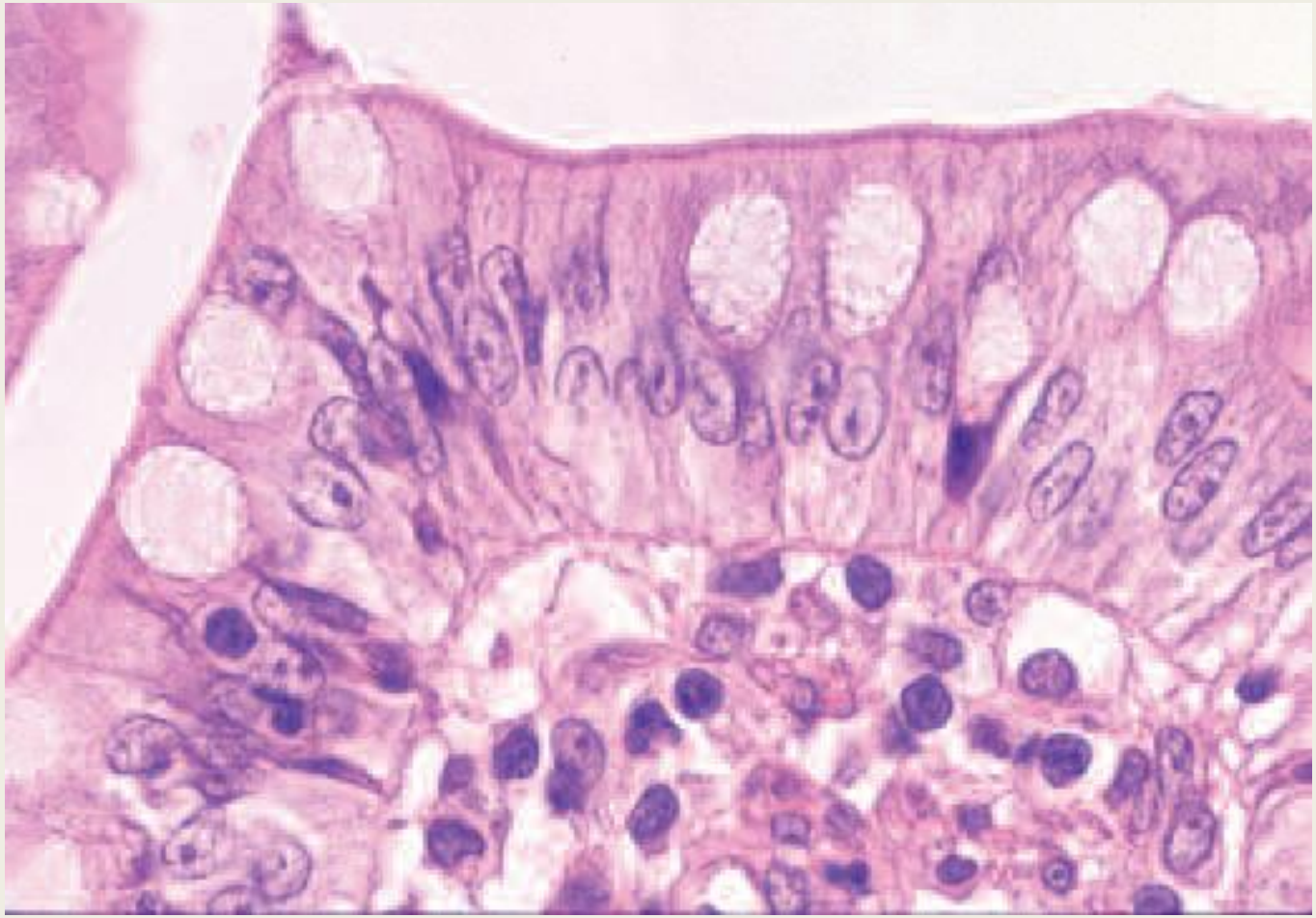
What Should Be Seen in a Well-Fixed, Well-Processed Specimen Stained With Hematoxylin and Eosin

Nuclei should show clearly with blue satin.

The cell cytoplasm should be well preserved and should stain well with eosin (PINK).

There should not be any artifactal spaces between the individual cells.

There should not be any cell shrinkage.



A well-fixed and well-processed section of small intestine is demonstrated in this image. Nucleoli can be seen in the nuclei. No cell shrinkage is noted.

Problems Encountered With Fixation and Processing

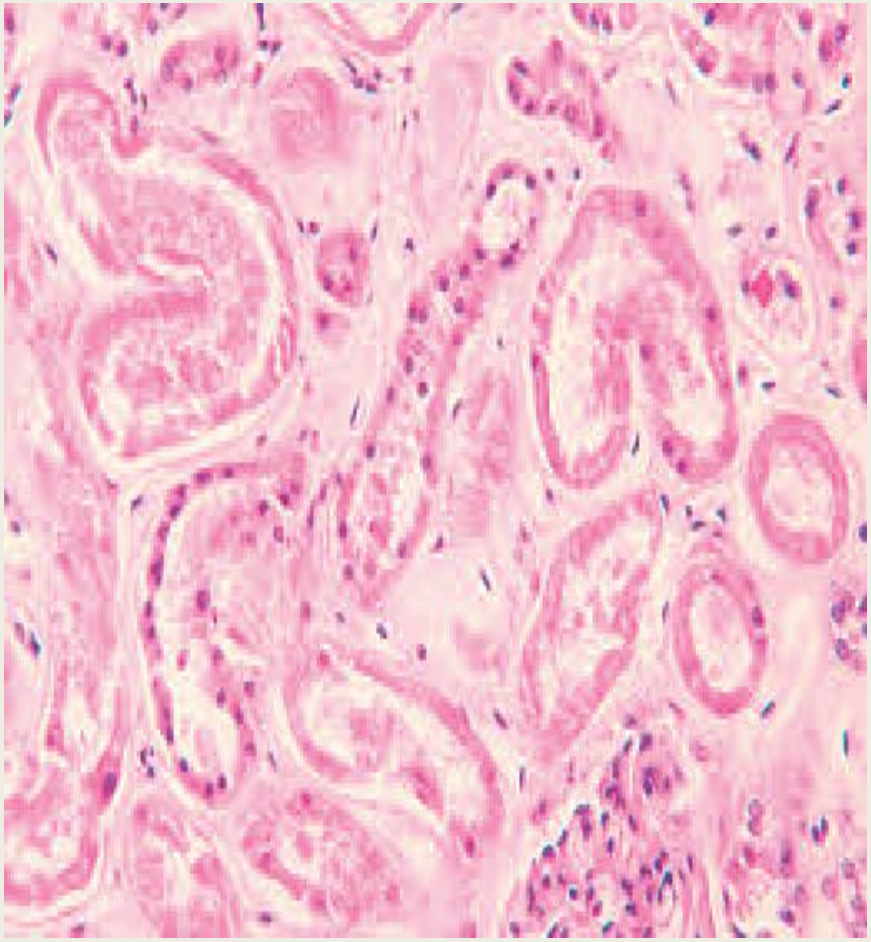
- **PROBLEM:** Fixation Delayed
- **APPEARANCE:** Nuclei may show blue halo, fading, or complete disappearance (fig 1). There may be cell shrinkage, disruption of the cytoplasm, and artifact spaces around cells (fig2). If delay is prolonged, some cells may completely disappear, such as the epithelial cells in intestinal specimens obtained at autopsy (fig3).

■CAUSES:

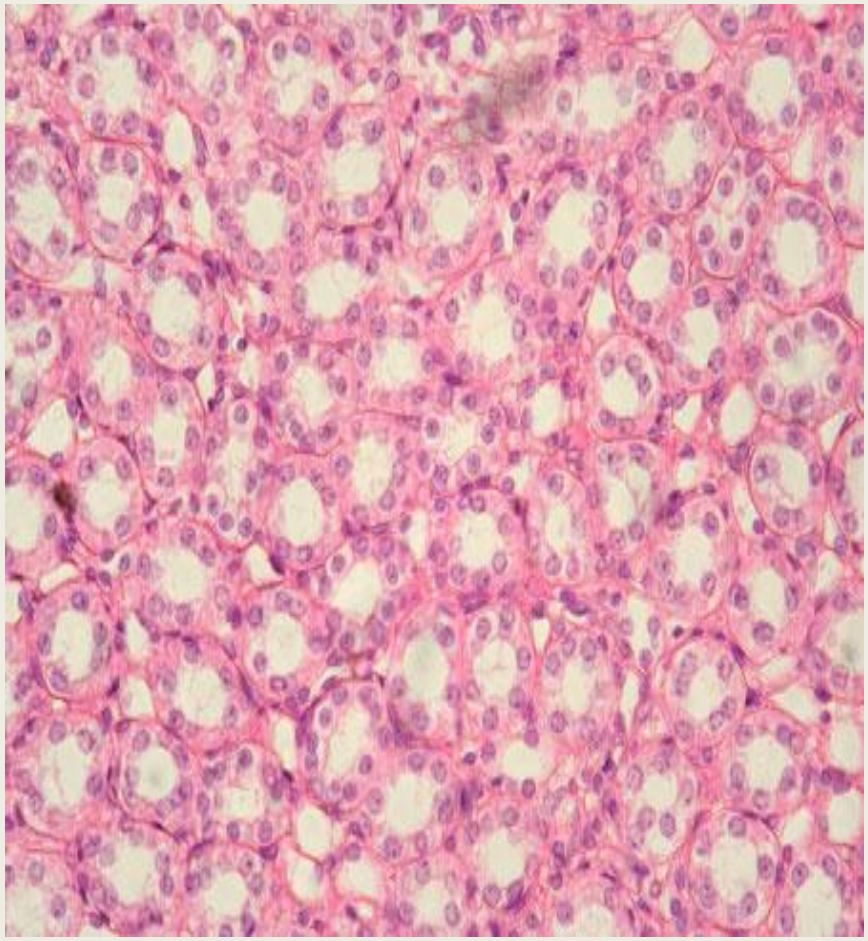
- **Specimens are obtained long after the blood supply has been compromised (eg, autopsy).**
- **The specimen is not opened so that fixative can come in contact with all surfaces (eg, uterus, small intestine, colon).**
- **The specimen is not thinly cut so that fixatives can penetrate more easily (eg, spleen, breast, organ resections, large tumors).**
- **Inadequate volume of fixative relative to the amount of tissue.**

SOLUTIONS:

- Place specimens in fixative as soon as possible after the blood supply has been interrupted.
- Open specimens wherever possible. Gastrointestinal specimens should be opened and placed in fixative. Uterus specimens should also be opened and placed in fixative.
- Slice specimens, such as spleen, breast, kidney, any organ resection, or large tumor, into thin slices and place in fixative.

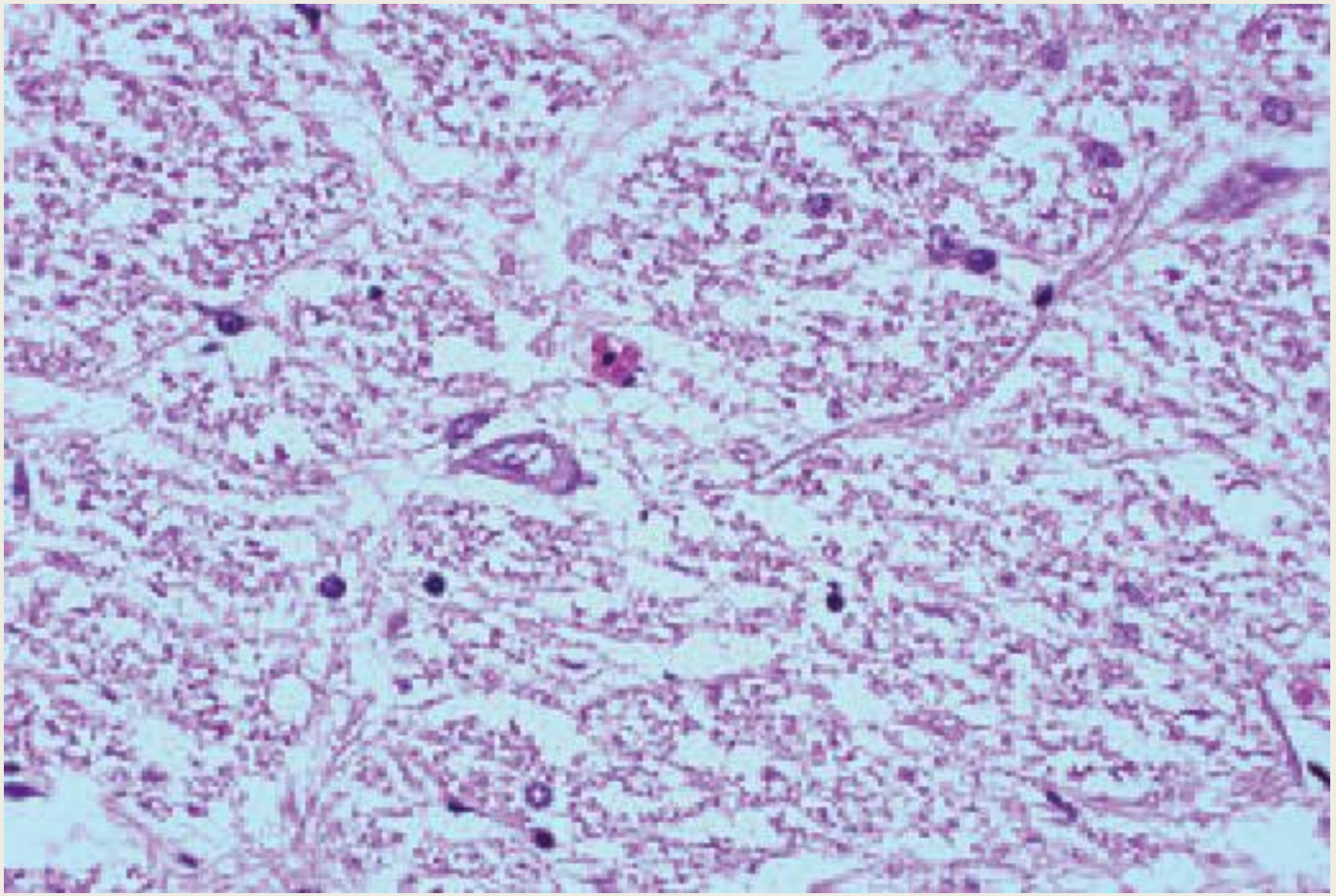


The effects of mild autolysis can be seen in this section of kidney. No nuclei remain in some of the tubules



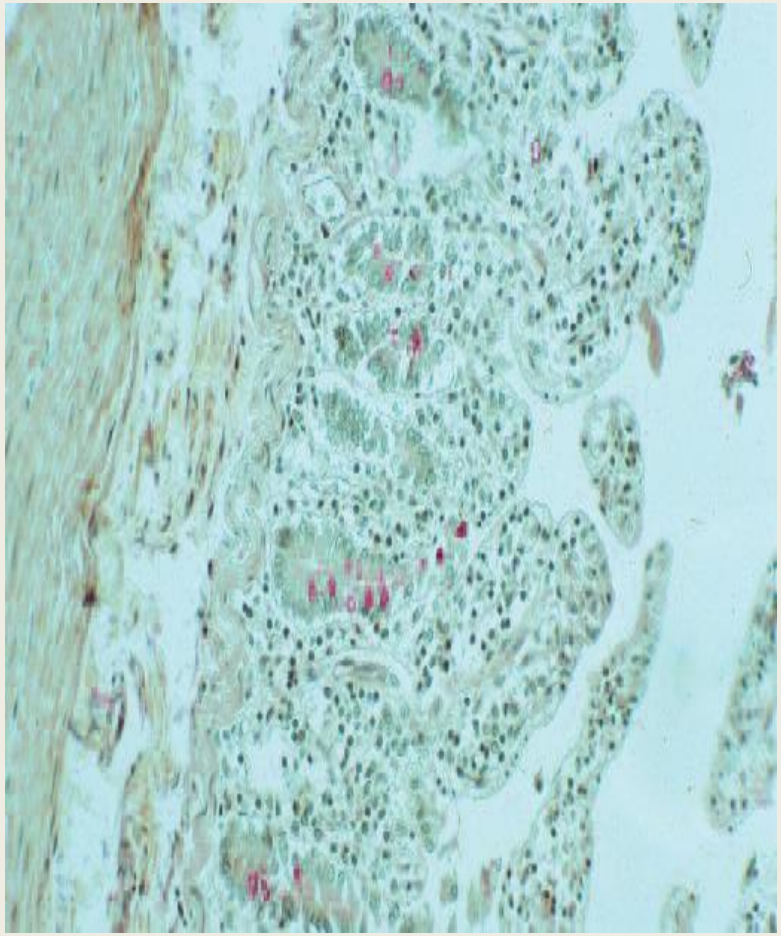
Excellent fixation (perfect fixation)

Fig 1



In this section we are demonstrates the results of delayed fixation, with marked disruption of the normal morphology.

Fig 2



The epithelial layer has totally disappeared in this very autolyzed section of small intestine. This is typical of delayed fixation of autopsy tissues, and sections of this type should not be used as control tissue.

Excellent fixation

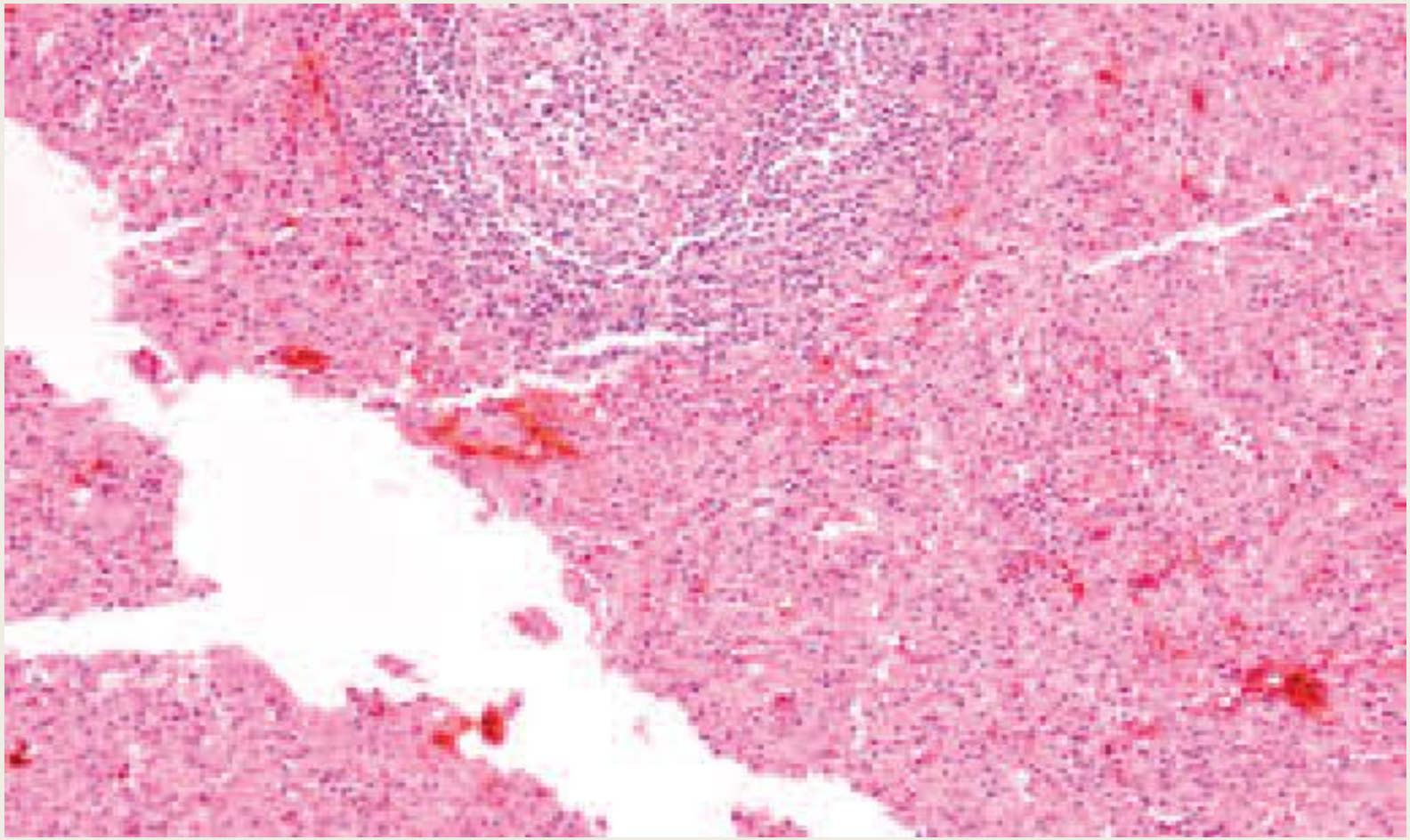
Fig 3

PROBLEM: Fixation Incomplete

- **APPEARANCE:** Tissue morphology is not well maintained (Figure 4).

- **CAUSES:**
- Tissue sections not allowed enough time in fixative.
- Inadequate amount of fixative relative to tissue volume.
- Sections grossed too thick for good penetration.
- Formalin solution is depleted.

- **SOLUTIONS:**
- Ensure that enough time is allowed for good fixation.
- Ensure that the fixative volume is 15 to 20 times the tissue volume.
- Ensure that the grossed sections are thin, preferably no more than 3 mm thick.
- Change formalin solutions frequently throughout the process to prevent depletion

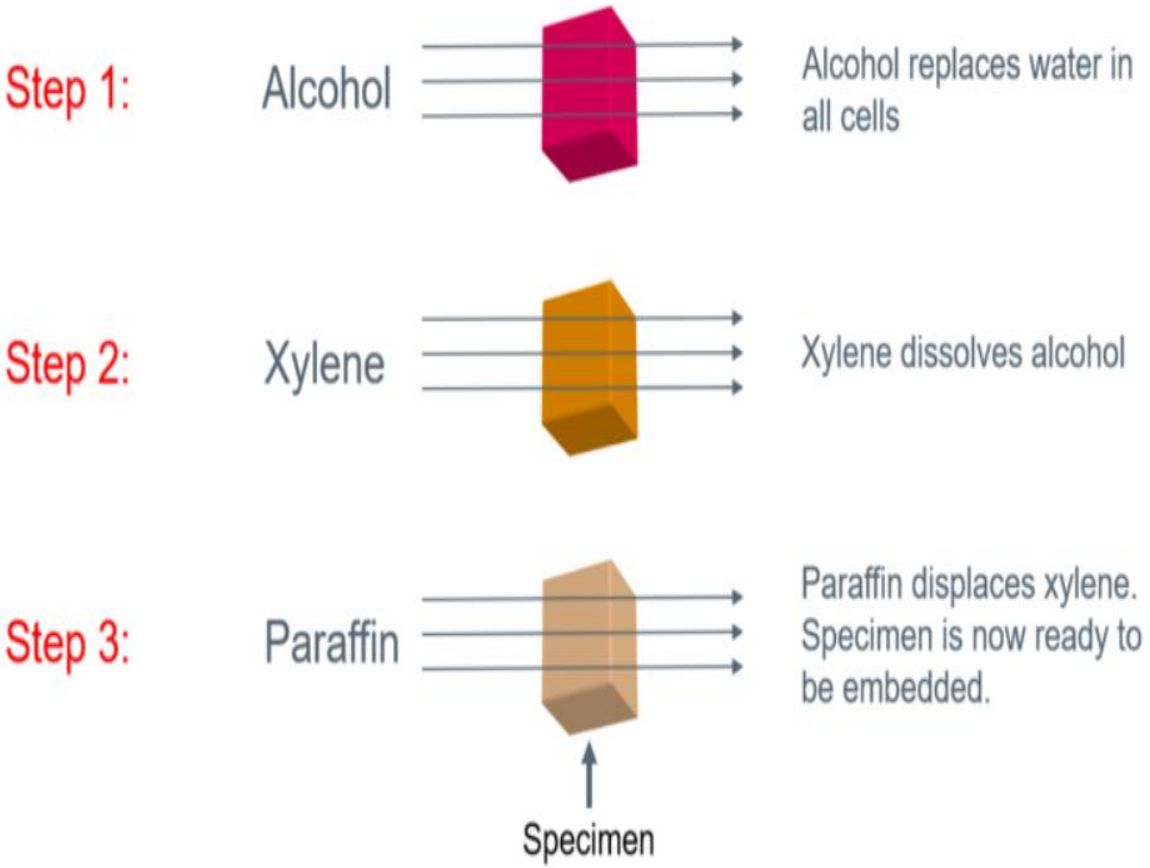


A section of spleen demonstrating the results of incomplete fixation. There is a large crack that occurred due to the incomplete fixation.

Washing, dehydration and clearing

- After fixation, the tissue should be washed by water or buffer solutions to removed the fixatives from tissue.
- For scientific processing, this procedure achieved continuously or directly to get up a good slides.

Overview of the steps in tissue processing for paraffin sections



Dehydration

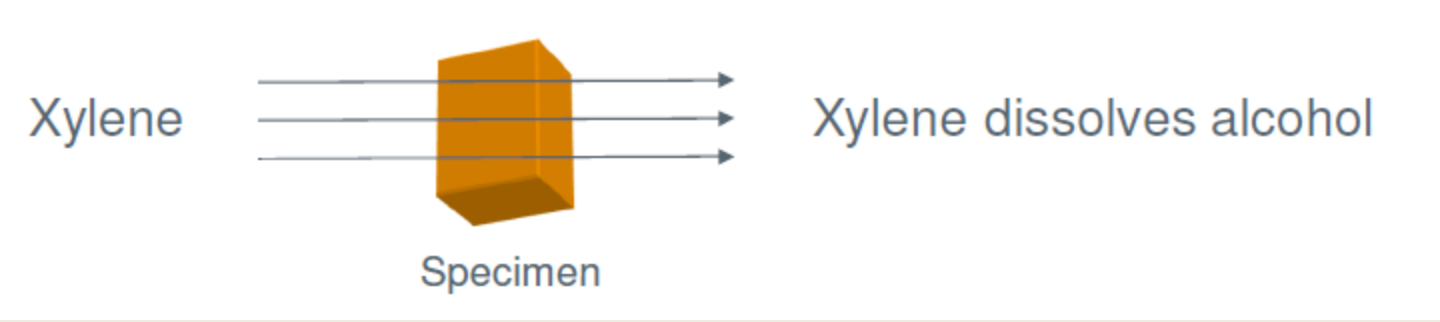
- Paraffin is hydrophobic (immiscible i.e. not mixable with water), water inside a specimen must be removed before it can be infiltrated with paraffin. This process is carried out by immersing specimens in a series of alcohol.
- Alcohol **progressively replaces** water in all the cells of the specimen.
- A series of increasing (typically from 70% to 100%) alcohol concentrations are used to avoid excessive distortion of the tissue.

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

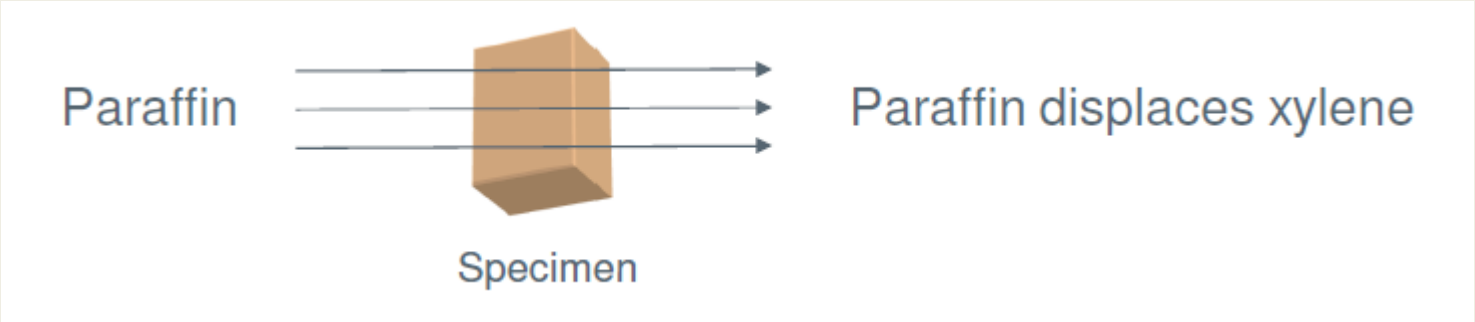
- **Alcohols and paraffins are not miscible**, an intermediate solvent that is fully miscible with both (such as xylene), must be used.
- This solvent displaces the alcohol in the tissue through the process called "**clearing**.
- Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to paraffin infiltration.
- To make sure that all traces of alcohols are removed from tissues being processed, multiple changes of fresh xylene are required.



- A typical clearing sequence for specimens not more than 4mm thick would be:
- xylene 20 min
- xylene 20 min
- xylene 45 min

Infiltration

- The specimen can now be infiltrated with paraffin. Molten paraffin infiltrates tissues and when cooled solidifies to a consistency that allows sectioning on a microtome.
- This is allowed to occur at melting point temperature of paraffin wax, which is 54-60oC.
- Volume of wax should be about 25-30 times the volume of tissues.



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

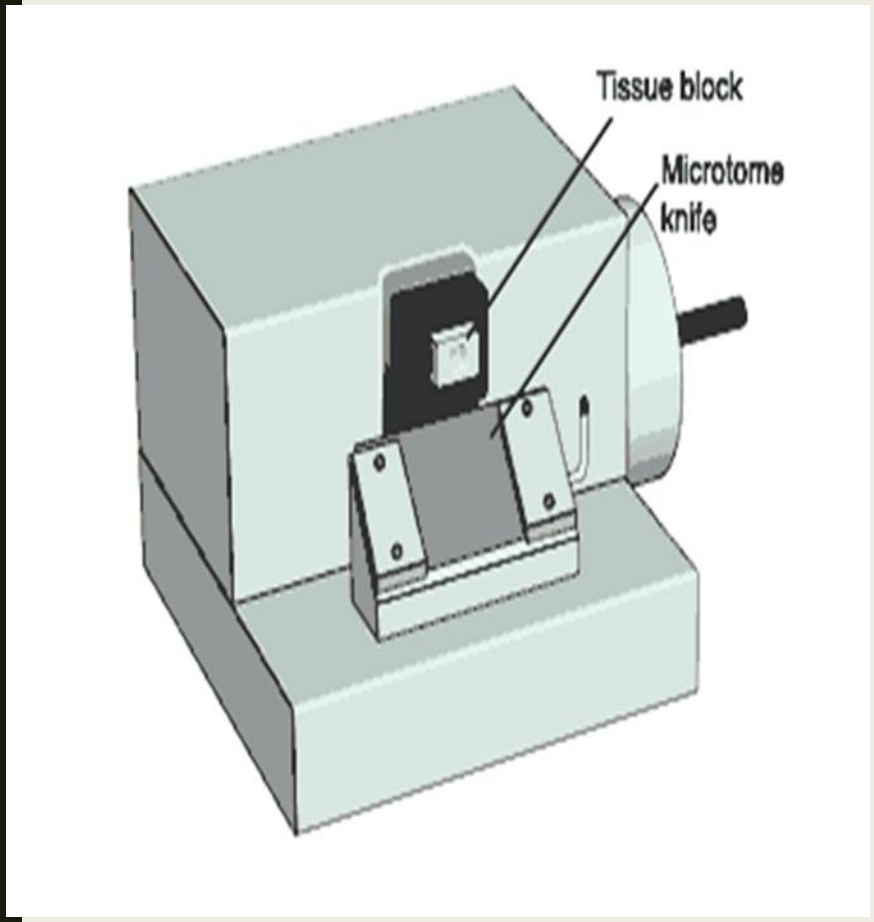
wax	30 min
wax	30 min
wax	45 min

Blocking

- Tissues that come off the tissue processor are still in the cassettes and must be manually put into the blocks by a technician who must pick the tissues out of the cassette and pour molten paraffin over them.
- This "embedding" process is very important, because the tissues must be aligned, or oriented, properly in the block of paraffin.

Sectioning

- Once the tissues have been embedded, they must be cut into sections that can be placed on a slide. This is done with a microtome. (1) a very sharp knife, (2) a very sharp knife, and (3) a very sharp knife.


Tissue block
Microtome knife


TBS
TBS

Microtome

Procedure

- 1.Fixation 6-24 or 72 hrs.
- 2.Dehydration
- 70% ethanol 15 min
- 90% ethanol 15 min
- 100% ethanol 15 min
- 100% ethanol 15 min
- 100% ethanol 30 min
- 3. clearing
- xylene 20 min
- xylene 20 min
- xylene 45 min

- 4. Wax infiltration
- wax 30 min
- wax 30 min
- wax 45 min
- 5. blocking and sectioning

H and E staining protocol

- **Deparaffinization**: flame the slide on burner and place in the xylene. Repeat the treatment to remove the wax.
- **Hydration**: Drain xylene and hydrate the tissue section by passing through decreasing concentration of alcohol baths (100%, 90%, 80%, 70%) and water.
- **Nuclear Staining**: Stain in hematoxylin for 3-5 minutes.
- Wash in running tap water until sections “blue” for 5 minutes or less.
- **Differentiation**: selective removal of excess dye from the section). Dip in 1% acid alcohol (1% HCl in 70% alcohol) for a few seconds.
- **Blueing**: Rinse in running tap water. Dip in ammonia water until the sections become blue, followed by tap water wash.
- **Counterstain**: Stain in 1% Eosin Y for 10 minutes.
- Wash in tap water for 1-5 minutes.
- **Dehydration**: Dehydrate in increasing concentration of alcohols.
- **Clearing**: Put slides in two xylene baths for clearing.
- **Mounting**: Mount in DPX or other mounting media.
- Observe under microscope.

Results and Interpretation

- Nuclei : blue, black
- Cytoplasm : Pink/purplish pink
- Muscle fibres : deep red
- RBCs : orange red

Conclusion

- The aim of Tissue Processing is to remove water from tissues and replace with a medium that solidifies to allow thin sections to be cut.
- Biological tissue must be supported in a hard matrix to allow sufficiently thin sections to be cut, typically 5 µm (Micro metres; 1000 micro metres = 1 mm) thick for light microscopy.
- For light microscopy, paraffin wax is most frequently used.

Regards

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