PROCEDURES OF PROCESSING TISSUES FOR HISTOPATHOLOGICAL EXAMINATION

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CONTENTS

- Fixation
- Processing
- Section making
- Staining - H&E
- Mounting

Whole process in tissue processing can be divided into following steps

- Fixation
- Processing
- Section cutting
- Staining and mounting

FIXATION

- Chemical process
- By which biological tissues are preserved from decay, either through autolysis or putrefaction
- Tissue specimens are fixed or preserved

  - to determine its micro-anatomy

  - allow localization of its various chemical constituents
- Tissues should be fixed as soon as possible after death or removal from the body

FIXATION

- Terminates any ongoing biochemical reactions
- Increase the mechanical strength or stability of the treated tissues
- Amound of fixative in jars should be 20-50 times the bulk of tissues to be fixed

PURPOSE OF FIXATION

- Preserve a sample of biological material as close to its natural state
- To achieve this several condition should be met :
    1. Fixative usually acts to disable intrinsic biomolecules -proteolytic enzymes
    2. Protect a sample from extrinsic damage
    3. Toxic to most common microorganisms -exist in a tissue sample

4. Will chemically alter the fixed material to make it less palatable to oppurtunistic micro organisms

5. Alter the cells or tissues on a molecular level to increase their mechanical strength or stability

TYPES OF FIXATION

- 3 types

1. <u>Heat fixation</u>

- Smear has been allowed to dry at room temperature

- Passed through flame of a Bunsen burner several times to heat kill and adhere the organisms to a slide

2.PERFUSION

- Fixation via blood flow
- Volume should match cardiac output
- Advantage - preserving perfect morphology
- Disadvantage-subject dies and cost is high

3.IMMERSION

Sample of tissue is immersed in fixative of volume at a minimum of 2/3rd greater than the volume of the tissue to be fixed

Fixative must diffuse through the tissue in order to fix, so tissue size[0.5 cm] and density, as well as type of fixative must be taken into account

TYPES OF FIXATIVES

- **Cross linking fixatives**

  Aldehydes -formaldehyde [40%neutral buffered formalin],gluteraldehyde [2%]

- **Oxidising agents**

  Osmium tetroxide –electron microscopy

- **Precipitating fixatives**

  ethanol,methanol,acetone

CROSSLINKING FIXATIVES

- Act by creating covalent bond between proteins in tissue
- Anchors soluble proteins to the cytoskeleton
- lends additional rigidity to the tissue

1. Aldehydes –

- Formaldehyde-gold standard of fixatives for routine histology
- Preserves protein,peptides,nucleic acids and cellular components

- In presence of calcium good preservative for lipids also
- Basic mechanism- formation of methylene bridges between reactive sites
- Change of quaternary and tertiary structures into secondary and primary structures

2.OXIDISING AGENTS

- React with various side chain of proteins and other biomolecules-formation of crosslinks
- Not used in light microscopy-penetration to thick sections poor

3.PRECIPITATING FIXATIVE

- Act by reducing the solubility of proteins
- Disrupting hydrophobic interactions
- Acetic acid-denaturant –sometimes used along with this

2.PROCESSING

- Aim of Tissue Processing

  -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
- 4-6 µm thick - light microscopy
- 80-100 nm thick - electron microscopy

PROCESSING....

- For light microscopy, paraffin wax is most frequently used

- Paraffin wax –fairly hard in consistency, allows sections of only 4-6 microns in thickness to cut

WHOLE PROCESS CAN BE DIVIDED INTO

A. Dehydration

B. Clearing

C. Impregnation

D. Block making

WASHING

- Specimens are kept in stainless steel in running tap water to remove formalin - overnight

A. DEHYRATION

- To remove water from the washed tissues
- Paraffin immissible in water
- Samples are transferred through baths of progressively more concentrated ethanol to remove the water
- Ascending grades of ethanol [70,80,90%, absolute ethanol I,II,III] 1 hour each
- Isopropyl alcohol also using
- Tissues should not be left in absolute ethanol for long -brittleness

B.CLEARING

- To replaces ethanol with some agent that are miscible in paraffin
- Commonly used clearing agents-

  xylene,turpentine oil, chloroform,cedar wood oil
- Cleared tissue has a translucent appearance
- Usually takes 1-1.5 hours

C.IMPREGNATION

- Process in which clearing agents is completely removed and substituted with melted paraffin [MP-56-58^{o}C]
- Achieved in a paraffin oven kept at the temperature slightly more than the MP of paraffin
- Decalcification should be done before embedding

- Paraffin wax does not provide a sufficiently hard matrix for cutting very thin sections for electron microscopy
- Instead, resins are used
- Epoxy resins are the most commonly used, but acrylic resins are also used if immunohistochemistry is required
- Thicker sections (0.35μm to 5μm) of resin-embedded tissue can also be cut for light microscopy

❖ Above processes are almost always automated for the large volumes of routine tissues processed

❖ Automation consists of an instrument that moves the tissues around through the various agents on a preset time scale

Stage	Time Required
Washing	Overnight
70% Ethanol	1 hour
80% Ethanol	1 hour
90% Ethanol	1 hour
95% Ethanol	1 hour
100% Ethanol-I	1 hour
100% Ethanol-II	1 hour
100% Ethanol-III	1 hour
Benzene + Ethanol	1 hour
Benzene-I	1 ½ hour
Benzene-II	1 ½ hour
Paraffin wax-I	1 hour
Paraffin wax-II	1-6 hour

D.BLOCK MAKING

- External embedding
- Tissue samples are placed into moulds along with liquid embedding material (such as agar, gelatine, or wax)

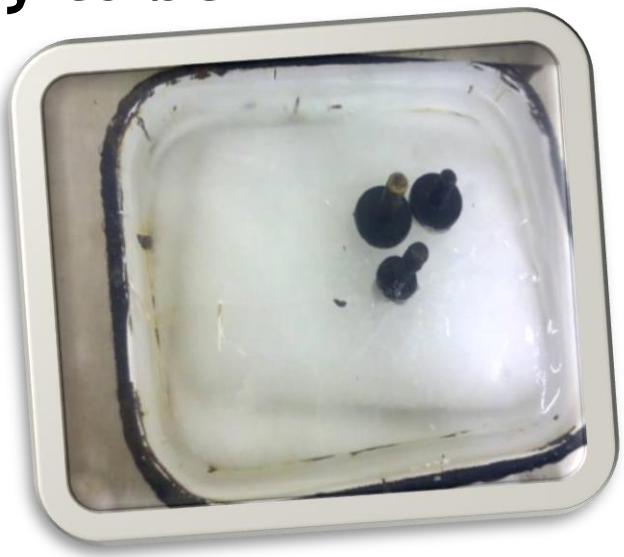
- Apply glycerine on slab
- Put paraffin in moulds /tissue capsule and remove air bubble
- Put tissues and press with forceps
- Apply specimen number and collect blocks after 30mts

HARDENING

- This is achieved by

  - cooling in the case of paraffin wax
  - heating (curing) in the case of the epoxy resins

- Hardened blocks containing the tissue samples are then ready to be sectioned.



- Alternatives to paraffin embedding include various plastics that allow thinner section

- Plastics include methyl methacrylate, glycol methacrylate, araldite, and epon

FROZEN SECTIONS

- Embedding can also be accomplished using frozen, non-fixed tissue in a water-based medium.
- Pre-frozen tissues are placed into moulds with the liquid embedding material
- Usually a water-based glycol, Cryogel, or resin
- Then frozen to form hardened blocks

3.SECTION MAKING

- Cutting of good section depends upon thorough knowledge of equipments used and practical experience
- Vertical sectioning perpendicular to the surface of the tissue is the usual method
- Horizontal sectioning - evaluation of the hair follicles and pilosebaceous units

A **microtome** is a sectioning instrument that allows for the cutting of extremely thin slices of material, known as sections

- For light microscopy, a steel knife mounted in a microtome is used to cut 5-micrometer-thick tissue sections which are mounted on a glass microscope slide
- For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections which are mounted on a 3-millimeter-diameter copper grid.

- Frozen tissue embedded in a freezing medium is cut on a microtome in a cooled machine called a cryostat

IMPORTANT STEPS IN SECTION CUTTING

- Sharpening knife or honing
- Stropping -process of polishing an already fairly sharp edge of section cutting knife
- Trimming the block

  wax is removed with a sharp knife until 1/8th inch remain in all sides of the tissues
- Cutting the section -4-6micron thick

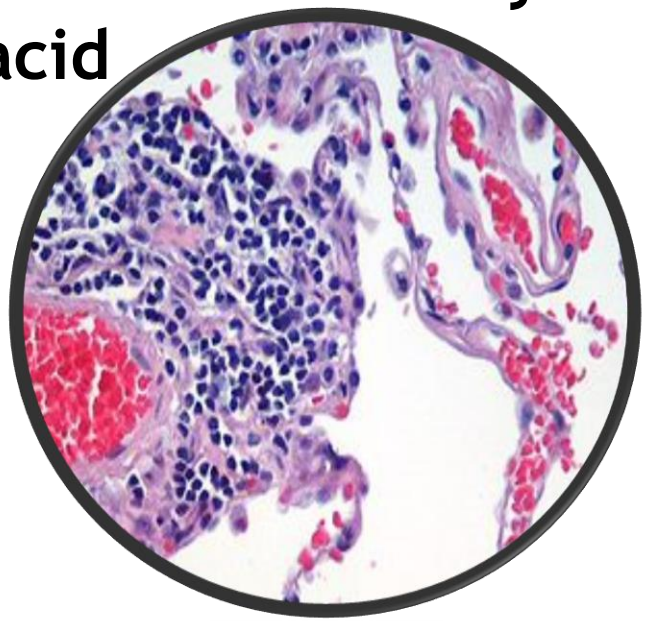
- Once sections are cut, they are floated on a warm water bath that helps to remove wrinkles
- Then they are picked up on a glass microscopic slide
- Glass slides are then placed in a warm oven for about 15 minutes to help the section adhere to the slide

STAINING METHOD

Basic steps in staining and mounting paraffin section are as follows:

- Removal of wax with xylene
- Hydration with graded alcohols
- Staining
- Dehydration through graded alcohol
- Clearing in xylene
- Mounting under a cover slip

- Haematoxylin and eosin stain is one of the most commonly used stains in histology
- Permanent stain
- Other common stain is phosphotungstic acid haematoxylin, a mix of haematoxylin with phosphotungstic acid



HEMATOXYLIN

- Hematoxylin - extracted from the heartwood of the logwood tree [*Hematoxylon campechianum]*
- When oxidized it forms hematein (Ripening of hematoxylin –keeping for few months or ripened hematoxylin commercially available)forms strongly coloured complexes with certain metal ions, fe(iii) and al(iii) salts[mordents]
- Metal-hematein complexes are used to stain cell nuclei

BLUEING

- During staining, alum haematoxylin-stained sections are usually passed on to a neutral or alkaline solution (e.g. hard tap water or 1% ammonium hydroxide)
- In order to neutralize the acid
- Form an insoluble blue aluminium haematin complex

HEMATOXYLIN SOLUTIONS

- Ehrlich's hematoxylin
- Weigert's Iron Hematoxylin
- Harris's hematoxylin
- Mayer's hematoxylin
- Gill's Hematoxylin

MAYER'S HEMATOXYLIN

- 5 minutes

CHEMICAL	AMOUNT	FUNCTION
Aluminium potassium sulphate [alum]	50gm	mordant
Distilled water	1000ml	solvent
hematoxylin	1gm	dye
Sodium iodate	0.2 gm	Oxidising agent
Glacial acetic acid	20ml	Ph control

HARRIS HEMATOXYLIN

-produces a deep, purple-blue stain

-does not contain mercury.

COMPOSITION OF EHRLICH HEMATOXYLIN

Haematoxylin -6 gm

Ammonium alum -40 gm

Glycerol,ethyl alcohol,distilled alcohol -300ml

Glacial acetic acid -30ml

EOSIN

- Fluorescent red dye resulting from the action of bromine on fluorescein
- It can be used to stain cytoplasm, collagen and muscle fibers
- Structures that stain readily with eosin - eosinophilic
- Alcoholic eosin

  Eosin -1 gm

  Ethyl alcohol -99ml

VARIANTS

- Eosin Y (also known as eosin Y ws, eosin yellowish, acid red 87, C.I. 45380, bromoeosine, bromofluoresceic acid, D&C red no. 22)- slightly yellowish cast

- Eosin B (eosin bluish, acid red 91, C.I. 45400, saffrosine, eosin scarlet, or imperial red)-faint bluish cast

- Eosin Y is a tetrabromo derivate of fluorescein

- Eosin B is a dibromo dinitro derivate of fluorescein

- Eosin - acidic dye - basic parts of the cell, i.e. the cytoplasm

- Haematoxylin - basic dye - acidic part of the cell like the nucleus

- For staining, eosin Y is typically used in concentrations of 1 to 5 percent weight by volume, dissolved in water or ethanol
- Prevention of mold growth in aqueous solutions- thymol
- 0.5 % acetic acid usually gives a deeper red stain to the tissue

MOUNTING

- Put 1 or 2 drop DPX (distyrene plasticizer xylene) on cover slip
- Earlier used –canada balsam
- Wipeout excess xylene from stained slide
- Mounting is done avoiding presence of air bubble

RESTAINING OF SLIDES

- Dip old slide in xylene - clearing agent
- Repeat the staining procedures again

step	Time [minute]
REMOVAL OF WAX	
xylene -I	10
xylene -II	5
HYDRATION	
Absolute alcohol -I	1
Absolute alcohol II	1
90% alcohol	1
80 %alcohol	1
70%alcohol	1
Washing in tap water	5

step	Time [minute]
STAINING	
haematoxylin	20
Washing in tap water	10
1%acid alcohol(remove excess stain)	1
Washing in the running tap water	5
Rinsing section in ammonia water[5%](blueing)	1
70%alcohol	1
Eosin [alcoholic]	1
DEHYDRATION	
Absolute alcohol-I	1
Absolute alcohol -II	1
CLEARING	
Xylene +ethanol [50:50]	1
Xylol -I	1
Xylol- II	1hr or leave until sections clear

THANKS TO ALL…