Tissue Fixation

Lecture Objectives

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

- Define fixation and state its principle
- Discuss aims of fixation
- State ideal properties of fixatives
- Discuss factors that affect fixation
- Describe methods of tissue fixation
- Know the classification of fixatives and give examples
- State advantages and disadvantages of various fixatives

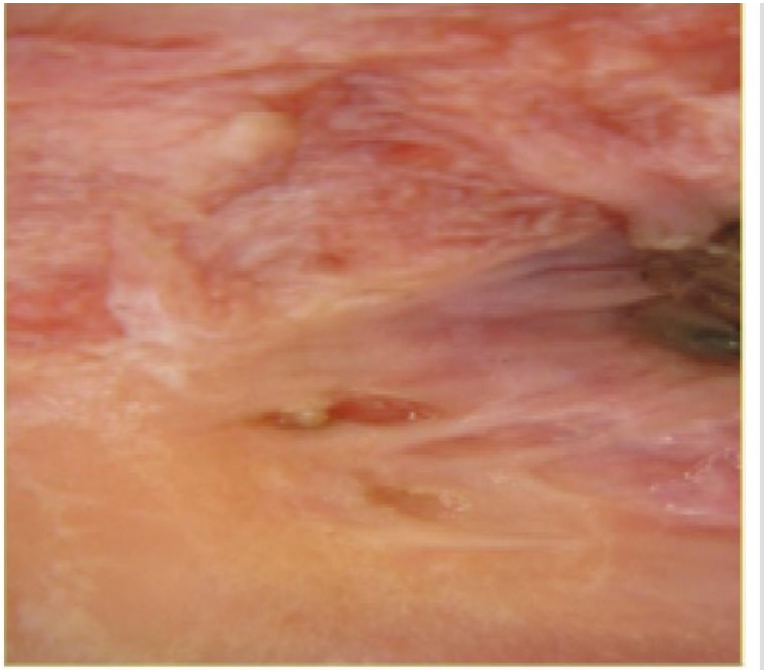
Introduction

- Fixation is a chemical process by which biological tissues are preserved from decay by exposing the tissue to chemical compounds, called fixatives.

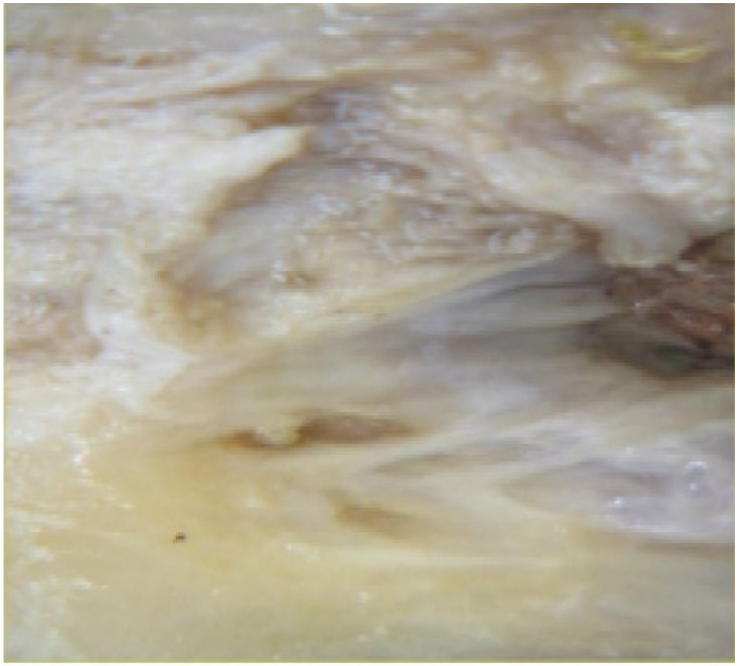
- **Principle of fixation**

The fixative brings about crosslinking, denaturation or coagulation of proteins so that the semifluid state is converted into semisolid state; so that it maintains everything in vivo in relation to each other. Thus semisolid state facilitate easy manipulation of tissue.

Introduction



Fresh tissue



Fixed tissue

Aims of Fixation

- To preserve the tissues as close to their living state as possible
- To prevent autolysis and bacterial attack
  - Fixatives are toxic to microorganisms or bacteria which may be present or attack the tissue. They also disable intrinsic biomolecules e.g. proteolytic enzymes which may digest the cell.
- To harden the tissues
  - The hardening effect of fixatives allows easy manipulation of soft tissue like brain, intestines etc.)
- To prevent tissues from changing their shape and size during processing

Aims of Fixation

- To allow clear staining of sections subsequently
  - Certain fixatives like formaldehyde intensifies the staining character of tissue especially with haematoxylin
- To improve the optical differentiation of cells & tissues
  - Fixatives alter to varying degrees the refractive indices of the various components of cells and tissues so that stained components are more easily visualized than when unfixed

General Properties of fixatives

1. Coagulation and precipitation.
2. Penetration
   - Fixation is done by immersing the tissue in fluid containing the fixative. The faster a fixative can penetrate the tissue the better it is. penetration power depends upon the molecular weight e.g. formalin fixes faster than osmic acid
3. Solubility
   - All fixatives should be soluble in a suitable solvent, preferably in water so that adequate concentrations can be prepared

General Properties of fixatives

4. Concentration

- It is important that the concentration of fixative is isotonic. Excessively hypertonic or hypotonic solutions can lead to cell membrane damage

5. Reaction

- Most fixatives are acidic. It may help in fixation but can affect staining so has to be neutralized e.g. formalin is neutralized by adding of calcium carbonate. [breakdown of formaldehyde to form formic acid produces an acidic solution which in turn reacts with hemoglobin to produces an artefact pigment (acid formaldehyde hematin]

Properties of an ideal fixative

- Prevents postmortem changes.
- Preserves tissue in their natural state and fix all components.
- Make the cellular components insoluble to reagent used in tissue processing.
- Preserves tissue volume.
- Avoid excessive hardness of tissue.
- Allows enhanced staining of tissue.
- Should be non toxic and non allergic for user
- Not very expensive.
- Allow cell parts to become clear visible by means of dyes and improved refractive indices .

Properties of an ideal fixative

- Should be readily disposable or recyclable
- Have a shelf life of at least one year
- Be compatible with modern automated tissue processors.
- Being useful for a wide variety of tissues.
- Support specialized procedures such as histochemical, immunohistochemical, *in situ* hybridization, etc.
- Should permit the recovery of macromolecules including proteins, mRNA, and DNA without extensive biochemical modifications from fixed and paraffin embedded tissues

Factors Affecting Fixation

- Volume of the Fixative
- Duration of fixation
- Time interval from removal of tissues to fixation
- The rate of penetration of the fixing fluid
- Choice of fixative
- Nature of tissue
- Size and thickness of piece of tissue
- Agitation
- pH
- Osmolarity
- Temperature

Factors Affecting Fixation

- **Volume of the Fixative**
  - It is important to have an excess volume of fixative in relation to the total volume of tissue because with additive fixatives (cross linking fixatives) the effective concentration of reagent is depleted as fixation proceeds and in a small total volume this could have an effect on fixation quality. At least 10-20 times greater than tissue volume.

- **Duration of fixation**
  - The optimal time for fixation will vary between fixatives. general rule 1hr per 1mm (e.g prolonged fixation in formaldehyde causes shrinkage & hardening of tissue).

Factors Affecting Fixation

- **Time interval from removal of tissues to fixation**
  - Artefacts will be introduced by drying, so if tissue is left out it should be kept moist with saline.
- **The rate of penetration of the fixing fluid**
  - Penetration into a thin section occur more rapidly than for a thick section (one way to get around the problem is to section tissues thinly e.g. 2-3mm)
- **Choice of fixative**
  - Depends on the treatment a tissue is going to receive after fixation e.g. what is the chemical structure that needs to be stained?

Factors Affecting Fixation

- **Nature of tissue**
  - Nature of tissue (Fatty tissues, tissue covered by blood or organ containing very large amount of blood and Tissue covered by large amount of mucous fix slowly)
- **pH**
  - Should be kept in the physiological range, between pH 4-9. The pH for the ultrastructure preservation should be buffered between 7.2 to 7.4
- **Osmolarity**
  - Hypertonic solutions give rise to cell shrinkage. Hypotonic solutions result in cell swelling and poor fixation.

Factors Affecting Fixation

- **Temperature**
  - Increasing the temperature will increase the speed of fixation. Fixation is accelerated by maintaining temperature around 60^{o}C
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- **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%; glutaraldehyde is generally made up at 0.25% to 4%. Too high a concentration may adversely affect the tissues and produce artifacts.

Factors Affecting Fixation

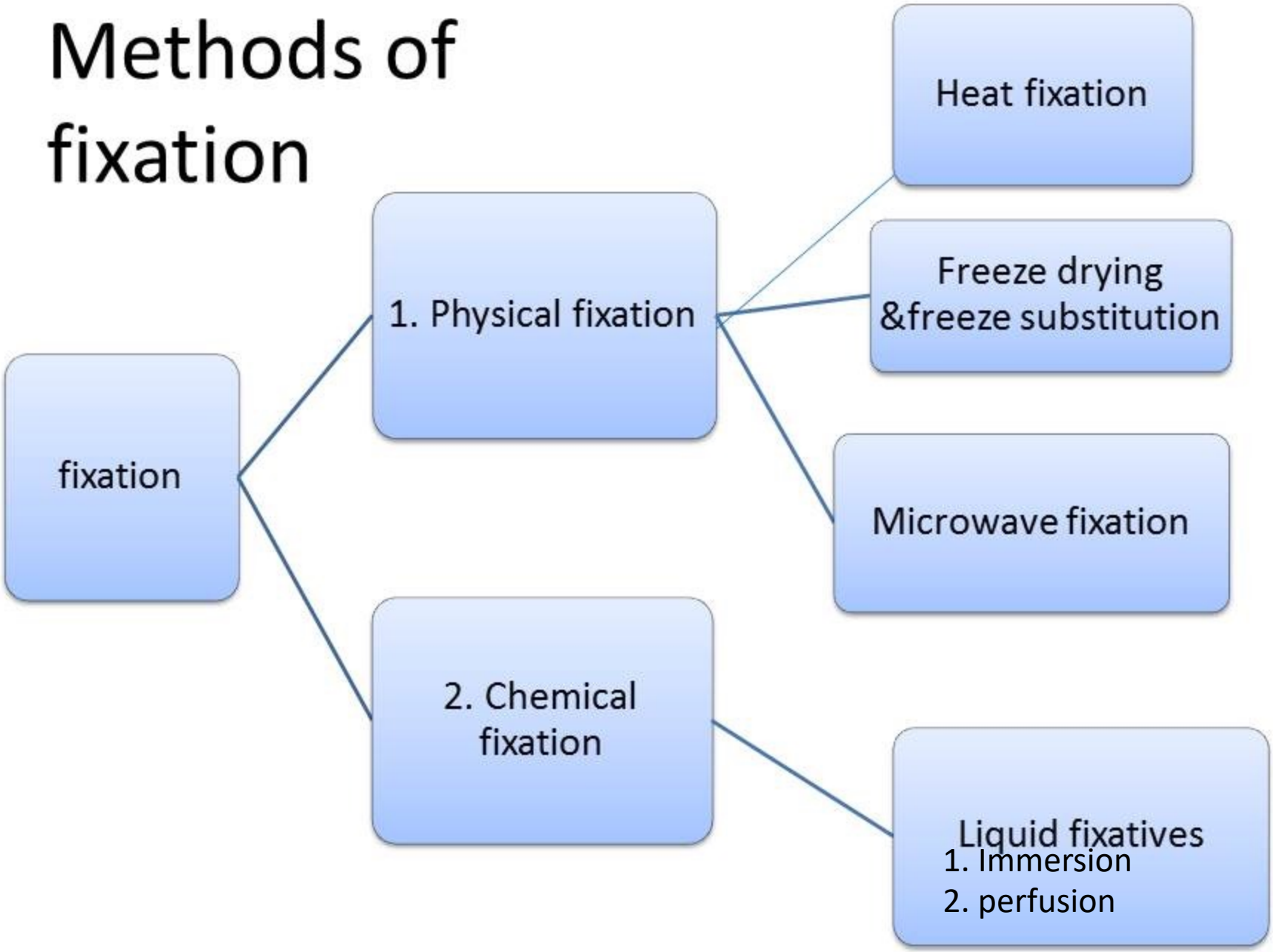
Figure 1: A whole heart specimen that has been “toast-racked” (sliced) to allow proper penetration of fixative. For fixation to occur the fixative has to penetrate, by diffusion, to the centre of the specimen.

Figure 2: A specimen of fatty breast tissue that has been squashed into a small container holding an inadequate volume of fixative. In this case it is likely that the specimen will be poorly fixed and mechanically distorted.

Methods of Fixation

- Fixation of tissues can be accomplished by physical and/or chemical methods.
- **Physical methods** are not used commonly in the routine practice
- Most methods of fixation used in processing of tissue for histopathological diagnoses rely on **chemical fixation** carried out by liquid fixatives.

Methods of fixation



1. Physical Methods of Fixation

Heat fixation

- Simplest form of fixation
- Attaches the section to the slide and partially fixes it by heat and dehydration.

Microwave fixation

- Speeds fixation and can reduce times for fixation of some gross specimens and histological sections from more than 12 hours to less than 20 minutes.
- Heat is considered to be the major factor responsible for the effects of microwaves during tissue fixation. Apart from increasing diffusion rates heat will increase molecular kinetics and speed up chemical reactions.

Freeze-drying and freeze substitution

- Are used primarily in the research environment and are rarely used in the clinical laboratory setting
- **In Freeze-drying**
  - tissues are immersed in liquid nitrogen, and the water is removed in a vacuum chamber at −40°C.
- **In freeze substitution**
  - specimens are immersed in dehydrant at −40°C, i.e. acetone or alcohol, which slowly remove water through dissolution of ice crystals, and the proteins are not denatured; bringing the temperature gradually to 4°C will complete the fixation process

2. Chemical Methods of fixation

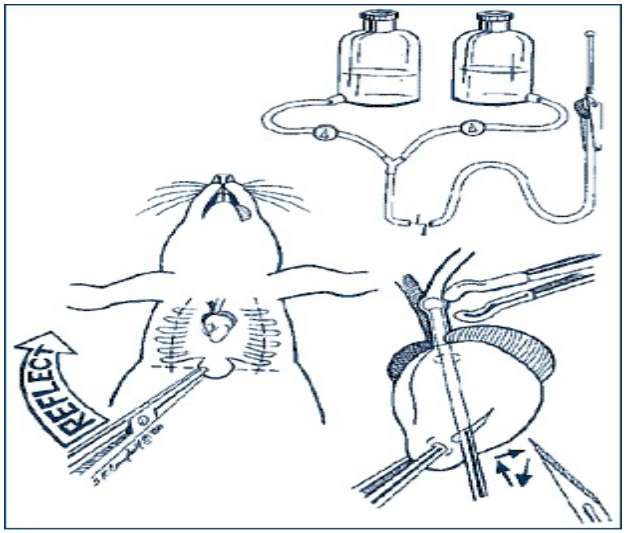
There are generally two types of fixation process:

1. Immersion:

The sample of tissue is immersed in fixative of volume 10-20 times greater than the volume of the tissue to be fixed.

2. Perfusion:

Fixation via blood flow. The fixative is injected into the heart with the injection volume matching cardiac output.



Perfusion Setup Diagram

Classification of Fixatives

Based on mechanism of action, fixatives are classified into 5 major groups

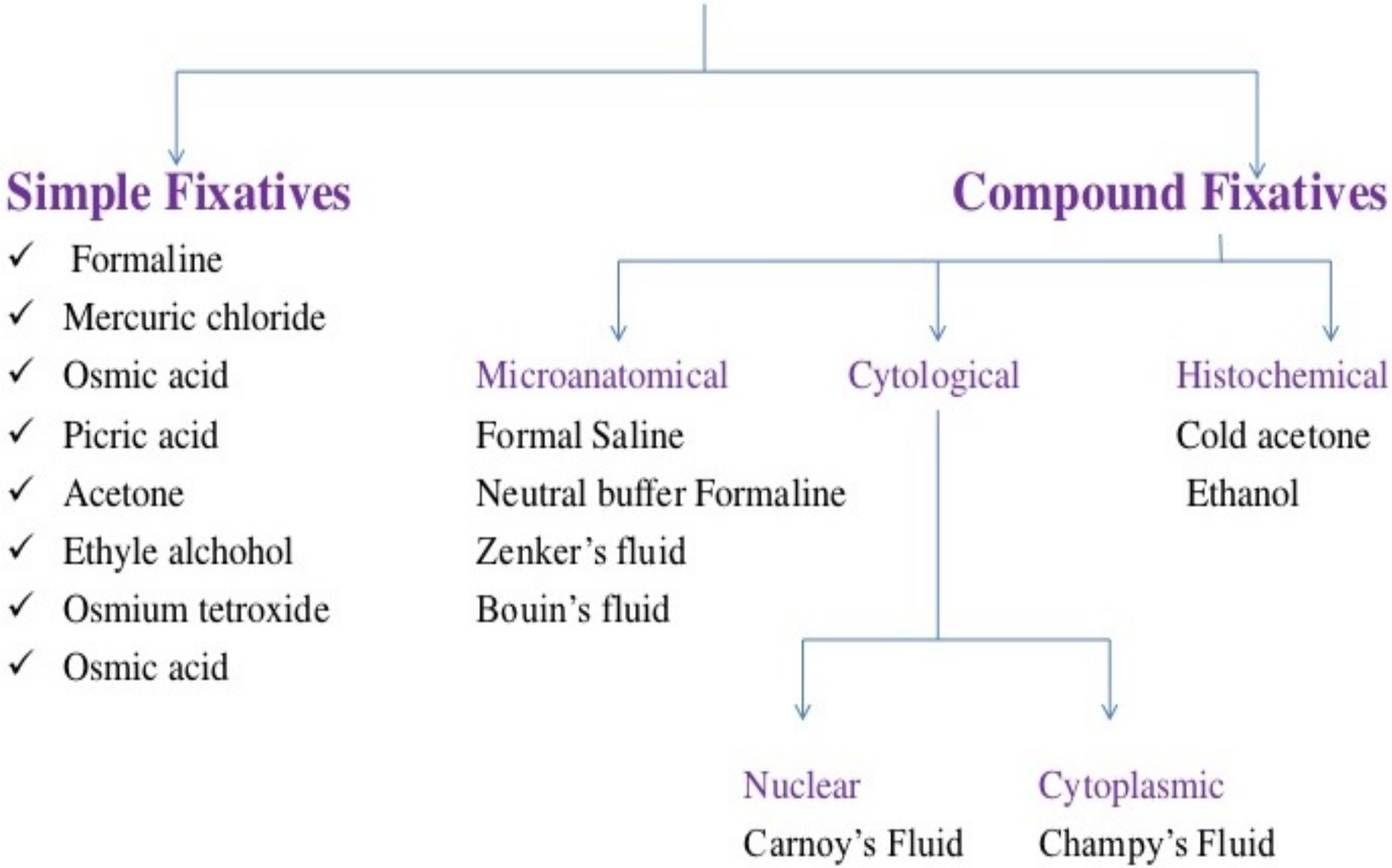
Fixative group	Examples	Fixation mechanism
Aldehydes	Formaldehyde, glutaraldehyde	Cross-link proteins
Oxidizing agents	Osmium tetroxide, potassium permanganate Potassium Dichromate	Cross-link proteins
Alcohol-based fixatives	Methanol, ethanol, acetic acid	Denature proteins
Mercurials	B-5 and Zenkers	Reacts with amines, amides, amino acids and sulphydryl groups and hardens tissue
Picrates	Bouin's solution	Precipitates proteins

Classification of Fixatives

Based on their use, fixatives are classified into 3 major groups

	Use	Examples
1.Microanatomical (tissue) fixatives	Preserves the anatomy of the tissues	10% formalin in 0.9% normal saline, Formal calcium, Buffered formal sucrose, B5 stock solution, Heidenhain Susa, Zenker's fluid, Zenker formal (Helly's fluid), Gender's fluid and Bouin's fluid
2.Histochemical fixatives	Preserve the chemical nature of the tissue	Formal saline, cold acetone, absolute alcohol
3. Cytological fixatives i. Nuclear & ii. Cytoplasmic fixatives	Preserves intracellular structures or inclusion	• Nuclear-Carnoy's fluid, Clarke's fluid and New Comer's fluid • Cytoplasmic-Champy's fluid and Formal saline and formal Calcium

Chemical Fixatives



Aldehydes (Cross-linking fixatives)

Act by creating covalent chemical bonds between proteins in tissues. This anchors soluble proteins to the cytoskeleton, and lends additional rigidity to the tissue.

Formaldehyde

- Most commonly used fixative
- Is a gas that easily dissolves in water→ Formalin
- Usually used as a **10% Neutral Buffered Formalin (NBF).**
- It does not harm the structure of proteins greatly, so that antigenicity is not lost. Therefore, good for immunohistochemistry techniques.
- Fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid lysine.

Formaldehyde

- **Advantages**

- Easy availability and cheap
- Does not overharden tissue
- Penetrate rapidly
- Penetrate and preserves fatty tissue
- Permits the use of a variety of staining methods

- **Disadvantages**

- Formation of formalin pigment
- Irritant to the nose, eyes, skin
- Toxic by ingestion and inhalation

Aldehydes (Cross-linking fixatives)

Gluteraldehyde

- Operates in a similar way to formaldehyde. However, it's a larger molecule and so its rate of diffusion across membranes is slower than formaldehyde.
- It causes deformation of alpha-helix structure in proteins so its not good for immunohistochemistry.
- It fixes quickly, hence good for transmission electron microscopy.
- Penetrates poorly but gives best overall cytoplasmic and nuclear detail.
- The important drawback of gluteraldehyde is that its rapid reaction causes loss of enzymes and immunological activity.

Oxidizing Agents

- Cause extensive denaturation hence used rarely.

Potassium dichromate

- Valuable in mixtures for the fixation of lipids especially phospholipids.
- Used for fixing phosphatides and mitochondria.
- **Note** - Thorough washing (running tap water) of the tissue fixed in dichromate is required to avoid forming an oxide in alcohol which cannot be removed later.

E.g. Orth's fluid for highlighting chromaffin granules in adrenal glands

Oxidizing Agents

Osmium Tetroxide

- It gives excellent preservation of details of a cell, therefore exclusively used for electron microscopy.
- It is not used for light microscopy as it penetrates thick sections of tissue very poorly.
- It fixes fat and lipid containing material e.g. myelin sheaths of nerve fibers.
- It also demonstrates fat (can be used as a stain) when 0.5-2% aqueous solution is used it gives a black colour to fat

Alcohols (precipitating or Denaturing fixatives)

Act by reducing the solubility of protein molecules and by disrupting the hydrophobic interactions that give many proteins their tertiary structure.

- Not used routinely for tissues; cause too much brittleness and hardness.
- Good for cytologic smears because they act quickly and give good nuclear detail.
- Penetrates rapidly in presence of other fixative

✓ e.g. Carnoy's fixative is used to increase the speed of tissue processing which preserves RNA and glycogen.

- Ethanol can be used for immunofluorescence or some histochemical methods to detect certain enzymes since it preserves some proteins in relatively undenatured state.

Mercurials

- Contain mercuric chloride e.g. B5, Helly and Zenker's
- Poor penetration, cause tissue hardness and shrinkage but fixes quickly and give good nuclear detail.
- Their best application is for fixation of hematopoietic and reticuloendothelial tissues.
- Brown to black precipitate can be formed referred to as mercury pigment (wash in iodine solutions).

Advantages and disadvantages of $HgCl_2$ fixatives (Zenker's & Helly's fluids)

Advantages	Disadvantages
Is a good mordant (chemical which increases the staining potential of tissue).	Is extremely poisonous
Helly's fluid is excellent fixative for bone marrow and blood containing organs	Prolonged treatment in Zenker's fluid will render the tissue brittle, making sectioning difficult
Better staining of nuclei and connective tissue	Mercuric chloride crystals will precipitate on the tissue

Picrates

- Include fixatives with picric acid e.g. Bouin's & Hallande solution.
- Preserves glycogen, connective tissue.
- Picric acid is an explosion hazard in dry form & causes extreme shrinkage of tissues.
- All the tissues fixed in picric acid containing fixatives should be thoroughly washed (50-70% alcohol) to remove the yellow discoloration to ensure proper staining of tissue sections.
- It can be used as a fixative or as a dye

Advantages and disadvantages of picric acid fixatives (Bouin's & Gender's fluids)

Advantages	Disadvantages
It provide good penetration and fixation	Prolonged fixation can Hydrolyse DNA
It is stable and can be made up as a stock solution	
It does not interfere with staining qualities of the tissue if the fixative is washed out thoroughly	
Fix tissue for 4-18 hrs depend on the size and density of the tissue	It causes much shrinkage
Is an excellent **mordant** (chemical which increases the staining potential of tissue).	

Tissue Fixative of choice & Time for fixation

Fixative	Use	Time(hrs)
Formalin	Routine	10-12
Buffered formaldehyde	GIT biopsies Liver biopsies	4-6 4-12
Bouin's	Testicular biopsies Bone marrow biopsy	4-6 2½ then wash in running water overnight
Zenker's fluid	Spleen & blood filled cavities	1-6
B-5	Lymph node	12-18
Carnoy's fluid	Mitochondria, phosphatides and Nissil substance	1-2
Clarke's fluid	Chromosome / cell culture	1-2

Composition of Fixatives

Solution	10% Formalin	10% Formal Saline	Neutral Buffered Formalin	Formalin Ammonium Bromide
Reagents	Formaldehyde 37-40%	Formaldehyde 37-40%	Formaldehyde 37-40%	Formaldehyde 37-40%
	Distilled Water	Sodium Chloride	Sodium Phosphate, Mono	Ammonium Bromide
			Sodium Phosphate, Dibasic	
		Distilled Water	Distilled Water	Distilled Water
Colour	Clear	Clear	Clear	Clear
Tissue	Routine	Routine	Prevent Pigments	Brain Tissue

Composition of Fixatives

Solution	Zenker's	Helly's	B-5	Bouin's
Reagents	Mercuric Chloride	Mercuric Chloride	Mercuric Chloride	Picric Acid
	Potassium Dichromate	Potassium Dichromate	Sodium Acetate	Formaldehyde 37-40%
	Acetic Acid	Formaldehyde 37-40%	Formaldehyde 37-40%	Acetic Acid
Colour	Orange	Orange	Transparent	Yellow
Tissue	Bone Marrow Biopsies	Bone Marrow Aspirates	Bone Marrow Cores, Tumours	Gastrointestinal tract Biopsies

Composition of Fixatives

Solution	Hollande's	Orth's	Zamboni's	Carnoy's
Reagents	Copper Acetate	Sodium Sulfate	Picric Acid	Absolute Alcohol
	Picric Acid	Potassium Dichromate	Sodium Phosphate, Mono	Chloroform
	Acetic Acid	Formaldehyde 37-40%	Sodium Phosphate, Dibasic	Acetic Acid
	Formaldehyde 37-40%		Formaldehyde 37-40%	
Colour	Green	Orange	Yellow	Clear
Tissue	Small Decals, Bones	Adrenal Medulla	EM Fixative	Lyse Red Blood Cells

Composition of Fixatives

Solution	**10% Formal Alcohol**	**Flemming's**	**Gluteraldehyde**	**Schaudinn's**
Reagents	Formaldehyde 37-40%	2% Osmium Tetroxide	Cocodylate Buffer	Mercuric Chloride in Water
	95% Alcohol	1% Chromium Hydroxide	Gluteraldehyde Stock	Absolute Alcohol
		Acetic Acid	Distilled Water	
Colour	Clear	Clear	Clear	Clear
Tissue	EM Specimen	EM Specimen	EM Specimen	EM Specimen

References

- Carson FL. **Histotechnology: a self-instructional text**. 2nd ed. Chicago: American Society of Clinical Pathologists Press; 1996.
- Suvarna, S.K, Christopher L, Bancroft J.D, **Bancroft`s Theory & Practice of Histological Techniques**, 7th edition, Churchill Livingstone, NY, U.S.A.