



HISTOPATHOLOGIC & CYTOLOGIC TECHNIQUES LEC

Module 7: Tissue Preservation: Fixation (Properties and Concepts of Fixatives)

MTHC31-24

Prof. Neil Allen Elquinto

TOPIC OUTLINE

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A.	Goals
B.	Two Mechanisms of Cation
C.	FActors involved in Fixation
D.	Practical Considerations
II.	Types of Fixatives According to Composition
III.	Types of Fixatives According to Action
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B.	Cytological Fixatives
C.	Histochemical Fixatives
IV.	Secondary Fixation
A.	Post Chromatization
V.	Washing Out
VI.	General Precautions In Handling and Fixation of Specimens
VII.	Characteristics of Good Fixatives
VIII.	Difficulties caused by Improper Fixation
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XI.	Specialized Fixation
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B.	Fixation Methods

FIXATION

The first and most critical step in histopathology is **FIXATION**.

A process that preserves tissues from decay, thereby preventing autolysis or putrefaction. The quality of the section on the slide is only as good as the quality of the fixed tissue specimen. If fixation is not adequate, the other processes that follow, such as dehydration, clearing, infiltration, embedding, microtomy and staining, will also be inadequate. A poorly processed tissue will make it difficult for the pathologist to render a diagnosis

It is the process of preserving the cells and tissue constituents in a condition identical to that existing during life.

Goals of Fixation

- Preserve the morphological and chemical integrity of the cell.
- Harden and preserve tissue for further handling.
- Prevent decomposition.

Two Mechanisms of Action

Type	Description	Example
Additive fixation	➤ The fixative becomes part of the tissue by formation of cross link complexes. ➤ Stabilizes the tissue proteins.	Formalin Mercury Osmium Tetroxide

Non-Additive Fixation	➤ Fixative NOT incorporated into tissues. ➤ Alteration of the tissue composition. ➤ Stabilizes the tissue by removing the bound water to stabilize the Hydrogen bonds.	Alcoholic Fixatives
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Factors Involved in Fixation

Factor	Description
pH	➤ 6-8
Temperature	➤ Autotechnicon (+40°C) ➤ Traditionally at Room Temperature ➤ Electron Microscope and Histochemistry at 0 to +4°C ➤ Rapid Fixation: +60°C; ➤ For tissues with Tuberculosis +100°C ➤ Increased Temperature will also Increase Denaturation of Protein.
Thickness	➤ Electron Microscope (1-2 mm²) ➤ Light Microscope (2cm²) ➤ No more than 0.4cm for Light Microscopy.
Osmolality	➤ Slightly Hypertonic Solution and 400-450 mOsm.
Concentration	➤ 10% Formalin ➤ 3% Glutaraldehyde: for electron microscopy ➤ 0.25% Glutaraldehyde: ideal for immunoelectron microscopy.
Time Duration	➤ Primary Fixation (2-6 hours) ➤ Electron Microscopy (3 hrs – placed on buffer)

Practical Considerations

Consideration	Description
Speed	➤ specimens shall transfer quickly to the fixatives right after harvesting to the body or biopsy. ➤ Type of fixative is considered ➤ Ex. Carnoy's Fluid.
Penetration	➤ 1 mm per hour for formalin
Volume	➤ 10-20 times of that tissue
Duration	➤ Primary Fixation (2-6 hours) ➤ EM – fixation of 3 hours
Fixatives shall be changed regularly because of the body fluids.	

TYPES OF FIXATIVES ACCORDING TO COMPOSITION

Type	Description
Simple Fixatives	one (1) type of fixative
Compound Fixatives	two (2) or more type of fixative

TYPES OF FIXATIVE ACCORDING TO ACTION

Microanatomical

Those permit the general microscopic study of tissue structures without altering structural pattern and normal intracellular relationship.



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Example	
10% Formol Saline	➤ Zenker's solution
10% neutral buffered formalin	➤ Zenker-formol (Kelly's solution)
Heidenhain's Susa	➤ Bouin Solution
Formol sublimate (Formol Corrosive)	➤ Brasil's solution.

Cytological Fixatives

Are those that preserve specific parts and particular microscopic elements of the cell itself.

Category	Description
Nuclear Fixatives	➤ Those preserve the nuclear structures (e.g. chromosomes). It must have a pH of 4.6 or less. a. Flemming's fluid b. Carnoy's fluid c. Bouin's fluid d. Newcomer's fluid e. Heidenhain's Susa
Cytoplasmic Fixatives	➤ Those preserve the cytoplasmic structure. Must not contain glacial acetic acid which destroys mitochondrial and golgi bodies of the cytoplasm. a. Flemming's fluid without acetic acid b. Kelly's fluid c. Formalin with "post-chroming" d. Regaud's fluid (Muller's fluid) e. Orth's fluid
Histochemical Fixatives	➤ Those preserve the chemical constituents of the cells and tissues. a. 10% formol saline b. Absolute Ethyl Alcohol c. Acetone d. Newcomer's Fluid

SECONDARY FIXATION

Is the process of placing an already fixed tissue in a second fixative in order to:

- Facilitate and improve the demonstration of particular substances.
- Make special staining techniques possible (with secondary fixative acting as mordant).
- Ensure further and complete hardening and preservation of tissues.

Post-chromatization

Form of secondary fixation whereby a primarily fixed tissue is placed in aqueous solution of 2.5-3% KCr_2O_7 for better staining effects and to aid in cytologic preservation of tissues.

WASHING OUT

Is the process of removing excess fixative from the tissue after fixation in order to improve staining and remove artifacts from the tissues. Several solutions may be used:

1. Tap water is used to remove:
 - a. Excess chromates from tissues fixed in Kelly's, Zenker's, and Flemming's solutions.
 - b. Excess formalin
 - c. Excess osmic acid.
2. 50-70% alcohol is used to wash out excess amount of picric acid (Bouin's solution)
3. Alcoholic iodine is used to remove excessive mercuric fixatives.

GENERAL PRECAUTIONS IN HANDLING AND FIXATION OF SPECIMENS

1. All tissue specimens must be properly labeled and identified.
2. Surgical specimens should be fixed immediately after removal, refrigerated if delayed.
3. Refrigerate the tissue but do not freeze.
4. Universal precautionary measures must apply, fresh tissue may be infectious.
5. Drying should be avoided to prevent shrinkage and distortion of tissue and cell.
6. Distortion, rough handling or other mechanical damage to the tissue will cause permanent morphological changes which can make interpretation difficult.
7. Autolysis may occur if the tissue is placed in NSS prior fixation.
8. Tissues should not be more than 5 mm except in lung edema, with minimum squeezing and handling.
9. Purulent material, exudates, or transudates should be marked and kept for possible cultures, smears and other bacteriologic examination.
10. Fixatives shall be 10-20 times the volume of tissue specimen except OsO_4 which is 5-10 times.
11. For prolonged fixation volume ex. Museum preparation should not be less than 50 times.
12. Contamination of fixed tissues with precipitates should be provided. Wash the fixed tissues thoroughly to remove salts and/or pigments.
13. The required period for fixation should not be exceeded to prevent tissue brittleness and hardening.
14. Hollow organs (e.g. stomach, intestines) should be packed with cotton soaked in fixative or completely opened.
15. Air-filled lungs may float on fixative. The organs shall be covered with several layers of gauze to maintain it under the surface.



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16. Human brains may be suspended by a cord tied under the Circles of Willis to prevent flattening. Intravascular perfusions (washing out of blood with Ringer's lactate) may lead to artifact formation with loss of blood content. Dense tissues such as brains are poorly penetrated, hence require long fixation (usually 2 weeks prior to sectioning).
17. Eyes should not be dissected before they are fixed since this may lead to immediate tissue collapse and wrinkling due to the escape of vitreous humor. Hence, formal alcohol must be injected before immersing the organ in the fixative.
18. Frozen sections may lead to formation of ice crystal artifacts.
19. To avoid rigor contraction and staining of artifacts when fixing muscle, the Fresh biopsy material may be stretched by sutures on each end or laid flat in a moist filter paper before suspending in fixatives.
20. Water should not be used for glycogen-containing tissues because it is soluble in H_2O .
21. To assure rapid access of the fixative to all parts of the tissue, the tissues may be minced, that is, small pieces of the specimen may be divided into small fragments and transferred to the vial of a fixative by means of a tooth pick.
22. Hard tissues (e.g. cervix, uterine, fibroids, hyperkeratotic skin, fingernails, etc.) may be washed out in running water overnight and immersed in 4% aqueous phenol solution for 1-3 days (Lendrum's method). This will soften the tissue and allow easier sectioning without producing any marked distortion of inner structures.
23. Presence of mucus prevents complete fixation, it must be washed away by NSS.
24. Fatty tissues should be cut in thin sections and fixed longer.
25. Tissues containing large amounts of blood should be flushed out with NSS before fixing.
26. The position or shape that is desired should be maintained before fixation.
27. Fixative should penetrate from all sides. Always place specimens into containers that already contain fixatives.
28. Hollow organs or specimens with natural cavities should be opened to allow immediate access to fixative.
29. Gentle agitation (swirling) of the specimen during its first few minutes in fixative will facilitate

penetration. An adequate volume is vital (at least 20:1). An excess of fixative is required as its effective components may be depleted as part of the reactions of fixation.

30. Fixatives should be carefully made from reagents of suitable quality, and fresh if so specified. Poor quality reagents can produce poor quality fixation. Some formulated fixatives should be prepared from stock solutions immediately before use because they are unstable (ex. Helly's fluid).
31. If a specimen is received in fixative of dubious quality, it must be replaced with fresh fixative.
32. Fixatives should be used only once. Other specimens shed cells and tissue fragments.
33. Avoid metal lids. Some fixatives are highly corrosive and will attach metals.
34. Some fixatives require the specimens be washed in water prior to processing (ex. Zenker's or Helly's) or some other requirements may exist (phosphate may precipitate from buffer in concentrations of alcohol of greater than 70%).
35. All fixatives are toxic and irritant. Anyone using fixatives should be aware of their potential hazards.

CHARACTERISTICS OF GOOD FIXATIVES

1. It must be readily available/cheap.
2. It must be stable.
3. It must be safe to handle.
4. It produces minimum distortion of cell constituents.
5. It must inhibit bacterial decomposition.
6. It must produce minimum shrinkage of tissue.
7. It must permit rapid and penetration of tissues
8. It must harden tissues to cut sections easier.
9. It must make cellular components insoluble.

DIFFICULTIES CAUSED BY IMPROPER FIXATION

1. Failure to arrest early autolysis of cells. Cause: Failure to fix immediately/insufficient fixative.
2. Removal of substances soluble in fixing agent. Cause: Wrong choice of fixatives.
3. Presence of artifact pigments on tissue sections. Cause: Incomplete washing of fixatives.
4. Tissues are soft and feather-like in consistency. Cause: Incomplete fixation.
5. Loss or inactivation of enzymes needed for study. Cause: Wrong choice of fixatives.
6. Shrinkage and swelling of cells and tissue structures. Cause: Overfixation



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7. Tissue blocks are brittle and hard. Cause:
Prolonged fixation.

FIXATION OF ARTIFACTS

Artifact	Description
Formalin Pigment	- Well known artifact that may be produced under acid conditions. - The pigment may be eliminated or reduced under acid conditions. It can be eliminated by the phenol-formalin. - Neutral buffered formalin and Phenol formalin almost completely stops the formation of formalin pigments.
Crush Artifact	- May be found in surgical specimens. - Due to partial coagulation of partially fixed protein by ethanol or by incomplete wax impregnation during subsequent histological processing.

FIXATION BY TISSUE COMPONENT

Lipid Fixation	- Fixatives containing HgCl_2 and KCr_2O_7 can be effective for preservation of lipids in cryostat sections. - Aldehydes – for phospholipids which contain amino acids however, formalin reacts to Unsaturated Fatty Acids during histological fixation, so that less lipid can be demonstrated histochemically. - Baker's formal-calcium may be used in phospholipids.
Carbohydrate Fixation	- Alcoholic fixatives are generally used for glycogen fixation. - Alcoholic formaldehyde is better fixative in human skin compared with neutral buffered formaldehyde. - Most useful fixatives – Rossman's fluid or cold absolute alcohol. This is essential when processing tissues from patients with glycogen storage disease.
Protein Fixation	Neutral buffered formal saline or formaldehyde vapor are the most commonly used fixatives for amino acid histochemistry.

SPECIALIZED FIXATION

Fixation for Electron Microscopy	Most useful primary fixatives for electron microscopy are the osmium tetroxide, glutaraldehyde and paraformaldehyde with the whole procedure performed at $+4^\circ\text{C}$. For routine purposes, glutaraldehyde or osmium tetroxide is adequate.
Fixation for Enzyme Histochemistry	- Aim of fixation of enzyme histochemistry is to preserve the maximum enzyme activity at its original localization while also preserving structural integrity. - The tissue for study may be fixed in 4% formaldehyde or

	formal-saline overnight, or fresh frozen cryostat sections may be fixed in acetone or formaldehyde and washed in distilled water prior to enzyme staining.
Fixation for Immunofluorescence	- Immunofluorescence techniques are commonly used in pathology for the demonstration of various antibodies. The preservation of antigenicity has become a very important consideration when choosing fixative. - Formalin fixed and paraffin embedded sections may be used. In more sensitive cases, the tissue must be prepared as a cryostat section and fixation limited to a few seconds in absolute methanol or acetone.
Fixation for Immunohistochemistry and Immunocytochemistry	- Fixation is often an important consideration when preserving tissue for immunohistochemistry. Some antibodies cannot recognize antigens if they are cross-linked in a certain manner. Antigen retrieval may be used to uncover antigens after fixation. - Immunocytochemistry, sample preparation essentially entails fixing the target cells to the slides. Perfect fixation would immobilize the antigens, while retaining authentic cellular and subcellular compartments. - To ensure free access of the antibody to its antigen, the cells must be fixed and permeable. In general, fixation strength and times are considerably shorter for cells than on the thicker, structurally complex tissue sections. Two classes of Fixatives: - Organic Solvents - Cross-linking Reagents

Two Classes of Fixatives

Class	Description
Organic Solvents	Such as alcohols and acetones, remove lipids and dehydrate cells, while precipitating the proteins on the cellular architecture.
Cross-linking reagents	- Form intermolecular bridges, normally through amino groups, thus creating a network of linked antigens. - Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components, and require the addition of a permeabilization step, to allow access of the antibody to the specimen. - The mechanism of fixation of fixation is dependent on the reagent used. Alcohol based fixation dehydrates cells/tissues, causing proteins to denature and precipitate in situ. - Paraformaldehyde causes covalent cross-links between molecules,



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	effectively gluing together into an insoluble meshwork. Many researchers believe that tissue may be stored indefinitely in 10% neutral buffered formalin without harm. ➤ Do not over-fix the tissue if it is intended to be used for immunostaining. Fixation method, time, and temperature are variables that need to be considered when developing immunohistochemistry protocols.
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Fixation Methods

1. Acetone Fixation
2. Methanol Fixation
3. Ethanol Fixation
4. Methanol-Acetone Fixation
5. Methanol-Acetone Mix Fixation
6. Methanol-Ethanol Mix Fixation
7. Formalin Fixation
8. Paraformaldehyde-Triton Fixation
9. Paraformaldehyde-Methanol Fixation



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- I. Chemical Fixatives
 - A. Type of Chemical Fixatives
- II. Aldehyde Fixatives
 - A. Formalin
 - B. 10% Formol-Salide
 - C. 10% Neutral Buffered Formalin (10% NBF) or Phosphate Buffered Formalin (pH7)
 - D. Formol-Corrosive (Formol Sublimate)
 - E. Gendre's Fixatives
 - F. Glutaraldehyde
 - G. Paraformaldehyde
- III. Metallic Fixatives
 - A. Mercuric Chloride
 1. Zenker's Solution
 2. Zenker Formol (Helly Solution)
 3. Heidenhain's Susa Solution
 4. B-5 Fixative
 - B. Chromate Fixatives
 1. Potassium Dichromate
 2. Regaud's (Miller's) Fluid
 3. Orth's Fluid
 - C. Lead Fixatives
- IV. Picric Acid Fixatives
 - A. Aqueous Picric Acid
 - B. Bouin's Solution
 - C. Brasil's Alcoholic Picroformol Fixative
- V. Glacial Acetic Acid Fixatives
- VI. Alcohol Fixatives
 - A. Absolute Alcohol
 - B. Methyl Alcohol 100%
 - C. Isopropyl Alcohol 95%
 - D. Ethyl Alcohol (70-100%)
 - E. Carnoy's Fluid
 - F. Newcomer's Fluid
- VII. Osmium Tetroxide / Osmic Acid
 - A. Flemming's Solution
 - B. Flemming's Solution without Acetic Acid
- VIII. Trichloroacetic Acid (TCA)
- IX. Acetone Fixatives

CHEMICAL FIXATIVES

Type of Chemical Fixatives

1. Cross Linking – Aldehyde Fixatives
2. Precipitating – Alcoholic Fixatives
3. Metallic Fixatives
4. Glacial Acetic Acid Fixatives
5. Picric Acid Fixatives
6. Osmium Tetroxide Fixatives
7. Trichloroacetic Acid
8. Acetone fixatives

ALDEHYDE FIXATIVES

- **Formaldehyde** - A gas produced by oxidation of methyl alcohol and is soluble in H₂O

- **Glutaraldehyde** - made up of 2 formaldehyde residues, linked by 3 carbon chains.

A. Formalin

- 10% Formaldehyde commonly used from a stock solution of 40% Pure Stock Solution and it is usually buffered with Phosphate to pH 7.
- 40% of pure stock solution is unsatisfactory as it over hardens the outer layer of tissue.
- Fixation time: 24 hrs.

Advantages	Disadvantages
➤ Cheap, readily available, easy to prepare ➤ Compatible with many stains ➤ Does not overharden the tissues even with prolonged period of fixation ➤ Preserves fat, mucin and glycogen ➤ It does not precipitate proteins ➤ Has anti-fungal effect	➤ Irritating to nose, eyes and skin ➤ Does not harden cytoplasmic structures ➤ Fixing for excessively long periods of time may produce bleaching of specimens and loss of natural tissue color. ➤ Unbuffered formalin will slowly oxidize to formic acid resulting in a fall in pH. Under these conditions the formic acids will react with hemoglobin forming acid formaldehyde hematin, a brown black granular artifact pigment which is deposited in blood-rich tissues.

B. 10% Formol-Saline

- Microanatomical fixative (Silver Impregnation Technique). It is made up of saturated formaldehyde (40%) diluted to 10% NaCl. It is also used in Fixation of CNS tissues and General Post-Mortem Tissue for Histochemical Examination.
- Fixation time: 24 hours at +35°C (+95°F); 48 hours at +20 to +25°C (+65 to +77°F)

Advantages	Disadvantages
➤ It penetrates and fixes tissues evenly ➤ It preserves microanatomic and cytologic details with minimum distortion and shrinkage. ➤ Preserves fat, enzyme, mucin and nucleoproteins	➤ Slow fixative ➤ Formol-saline fixed tissues tend to shrink during alcohol dehydration.

C. 10% Neutral Buffered Formalin (10% NBF) or Phosphate Buffered Formalin (pH7)

- It is the best fixative for Iron Containing Pigments and Elastic Fibers. Inert to Lipids and longer to prepare.
- "Best general fixatives" with sodium dihydrogen phosphate (pH 7).



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- Fixation time: 4-24 hours.

Advantages	Disadvantages
➤ Recommended for preservation and storage of surgical, post-mortem and research specimens. ➤ For tissues containing iron pigments and elastic fibers. ➤ Prevents precipitation of acid formalin pigments on tissues	➤ Longer to prepare, time consuming. ➤ It is inert towards lipids esp. neutral fats and phospholipids.

D. Formol-Corrosive (Formol Sublimate)

- It contains mercuric chloride and is recommended for post-mortem examinations.
- Excellent for stains like Silver Reticulin Methods. No washing out.
- Fixation time: 3-24 hours.

Advantages	Disadvantages
➤ Fixes lipids, especially neutral fats and phospholipids. ➤ Brightens cytoplasmic and metachromatic stains	➤ Penetration is slow. ➤ Forms mercuric chloride deposits (brown)

E. Gendre's Fixatives

- Has 95% Ethyl Alcohol with Picric Acid and Glacial Acetic Acid. Good for Glycogen, Microincineration Techniques and it fixes sputum. It is composed of 95% ethyl alcohol saturated with picric acid; Glacial Acetic Acid; Strong formaldehyde.
- It fixes and dehydrates at the same time.

Advantages	Disadvantages
➤ Faster fixation rate. ➤ Can be used for rapid diagnosis ➤ Good for preservation of glycogen ➤ Use to fix sputum.	➤ Produce gross hardening of tissues

F. Glutaraldehyde

- Buffered glutaraldehyde followed by 2nd fixation in osmium tetroxide is satisfactory for "electron microscopy". Acts similarly to formalin.
- 2.5% solution is used for small tissue fragments and needle biopsies.
- Fixation time: 2-4 hours at Room Temperature. 4% solution is for Large Tissue.
- Fixation time: 6-8 hours up to 24 hours.
- Recommended for enzyme histochemistry.

Advantages	Disadvantages
➤ Gives firmer texture with better tissue sections especially of CNS tissue ➤ More pleasant and less irritating. ➤ Does not cause dermatitis. ➤ Less tissue shrinkage	➤ Penetrates tissue slowly ➤ Tends to make tissue brittle ➤ Expensive ➤ Reduces PAS positivity to mucin

G. Paraformaldehyde

- A polymer of formalin and in white powder form in prolonged storage, it is also for thin and ultrathin sections for plastic embedding.

METALLIC FIXATIVES

A. Mercuric Chloride

- Most common metallic fixative; 5-7% ; included in compound fixatives. May produce black granular deposits except Heidenhain Susa. Removal can be done by addition of saturated iodine solution of 96% alcohol. Penetrates and hardens tissue rapidly.
- Routine fixative choice for preservation of cell detail in tissue photography.

Advantages	Disadvantages
➤ Trichrome staining is excellent. ➤ Penetrates and hardens tissue rapidly. ➤ Permits brilliant metachromatic staining. ➤ Nuclear components are shown in fine detail. ➤ Recommended for renal tissues, fibrin, connective tissues and muscles.	➤ Marked shrinkage of cells. ➤ Inert to fats and lipids. ➤ Leads to formation of black deposits.

1. Zenker's Solution

- Contains Glacial Acetic Acid. Recommended for Small Pieces of Liver, Spleen, CT Fibers and Nuclei, it is recommended for Trichrome Staining. Dezenkerization: removal of mercury deposits using Lugol's Iodine and Sodium Thiosulfate.
- Fixation time: 12-24 hours

Advantages	Disadvantages
➤ It acts as a mordant to make certain special staining reactions possible. ➤ For fixing small pieces of liver, spleen, connective tissue fibers and nuclei.	➤ Tissue must be washed in running water several hours before processing. ➤ Insufficient washing may inhibit or interfere with good cellular staining.



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2. Zenker's Formol (Helly Solution)

- Contains Potassium Dichromate and Formalin. Excellent microanatomical fixative of Pituitary Gland, Bone Marrow, and Blood Organs. Preserves Cytoplasmic Granules. Produces brown pigments (remove using picric acid or NaOH).
- Fixation Time: 12-24 hours.
- Remedy: Removed by immersing the tissue in alcoholic picric acid or sodium hydroxide

Advantages	Disadvantages
➤ For pituitary gland, bone marrow, spleen and liver	➤ Brown pigments are produced if tissues are allowed to stay in fixative for more than 24 hours.

3. Heidenhain's Susa Solution

- Has Trichloroacetic acid (TCA), Glacial Acetic Acid, Formalin. It is recommended for skin biopsies.
- Fixation Time: 3-12 hours

Advantages	Disadvantages
➤ Produces minimum shrinkage and hardening of tissues. ➤ Can be transferred directly to 95% alcohol reducing processing time.	➤ Some cytoplasmic granules are dissolved. ➤ Mercuric chloride deposits.

4. B-5 Fixative

- For bone marrow biopsies.
- It has Anhydrous Sodium Acetate.

Advantages	Disadvantages
➤ Fixation is faster.	➤ Over fixation hardens the tissues and makes cutting difficult. ➤ Mercuric chloride deposits.

B. Chromate Fixatives

- 1-2% aqueous solutions; strong oxidizing agents and should not be combined with reducing agents. It preserves carbohydrates.

1. Mercuric Chloride

- 3% aqueous solution. It preserves lipids and mitochondria at pH 4.5-5.2.
- It fixes 3C's cytoplasm, chromatin and chromosomes are fixed.

2. Regards (Miller's) Fluid

- Recommended for: Chromatin, Mitochondria, Mitotic figures, Golgi bodies, RBC, Colloid-containing tissues.
- Fixation Time: 12-48 hrs.

Advantages	Disadvantages
➤ Penetrates tissues well.	➤ Deteriorates and darkens on standing due to acidity. ➤ Prolonged fixation blackens tissue pigments. ➤ Contraindicated for carbohydrates and does not preserve fats.

3. Orth's Fluid

- Recommended for early degenerative processes and tissue necrosis. Demonstrates rickettsiae and other bacteria. It also preserves myelin.
- Fixation time: 12-48 hours.

C. Lead Fixatives

- 4% aqueous solutions and recommended for acid mucopolysaccharides. It fixes mucin also.
- Remedy: Filtration or adding acetic acid drop by drop.

Advantages	Disadvantages
➤ For acid mucopolysaccharides ➤ Fixes connective tissue mucin	➤ Forms insoluble lead carbonate on prolonged standing

PICRIC ACID FIXATIVES

- It is usually used in strong or saturated solutions. Chemically it is TNT!! (2,4,6-Trinitrophenol). Explosive when dry!

A. Aqueous Picric Acid

- For glycogen demonstration.
- Can be used as a stain (yellow) and small tissue fragments can be seen.
- Remedy: Tissues must be placed in 70% ethyl alcohol followed by 5% Sodium thiosulfate and then wash with running water.

Advantages	Disadvantages
➤ The yellow stain taken in by tissues prevents small fragments from being overlooked.	➤ It produces excessive staining of tissues. ➤ Picrates are formed upon protein. ➤ Highly explosive when dry, therefore must be kept moist.



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B. Bouin's Solution

- For fixation of embryos and pituitary biopsies. Excellent for preserving soft and delicate structures. Preferred fixative or tissues to be stained by Masson's trichrome stain.
- Not for Kidney fixation.
- Fixation time: 6-24 hours.

- Fixation time: 28-48 hours.

C. Isopropyl Alcohol 95%

- For fixing touch preparations and special staining procedures such as Wright-Giemsa.

D. Ethyl Alcohol (70-100%)

- It is the simplest fixative and used for enzyme studies. It doesn't fix glycogen.
- Fixation time: 18-24 hours.

E. Carnoy's Fluid

- It is the most rapid fixative. It contains Absolute Alcohol, Chloroform and Glacial Acetic Acid. It fixes and dehydrates at the same time. It is recommended for chromosomes, lymph glands, nissl granules and cytoplasmic granules. Used to fix brain tissue for the diagnosis of rabies.
- Fixation time: 6-24 hours

C. Brasil's Alcoholic Picroformol Fixative

- Excellent fixative for glycogen. It has trichloroacetic acid. It is better and less messy than Bouin's solution.

GLACIAL ACETIC ACID FIXATIVES

- It is usually incorporated in compound fixatives. It solidifies at +17°C. It precipitates nucleoproteins and chromatin material but is not recommended for cytoplasmic fixation as it may swell the tissues and cells.

ALCOHOL FIXATIVES

- Rapidly denatures and precipitates protein by destroying hydrogen and other bonds. It must be used in concentration ranging from 70-100%.
- Acts as a fixative and Dehydrating Agent.
- Excellent for glycogen. Preserves the nuclear stains.

A. Absolute Alcohol

- Used to fix and preserve glycogen, pigments, blood, tissue films and smears.

Advantages	Disadvantages
➤ Used as a fixative and dehydrating agent. ➤ Preserves nuclear stains.	➤ It dissolves fats and lipids. ➤ It causes polarization of glycogen granules.

B. Methyl Alcohol 100%

- It is used for fixing dry and wet smears, blood smears and Bone Marrow Tissues. Precautions must be applied as it might cause blindness and toxicity.

Advantages	Disadvantages
➤ Fixes and dehydrates at the same time. ➤ Rapid fixative and used for urgent biopsies	➤ Slow penetration ➤ Causes tissue shrinkage

F. Newcomer's Fluid

- Recommended for mucopolysaccharides and nuclear proteins. It can act as both a nuclear and biochemical fixative. It produces better reactions in Feulgen stains.
- Fixation time: 12-28 hrs at +3°C.

OSMIUM TETROXIDE / OSMIC ACID

- It's a pale yellow powder that dissolves in water (6% at 20°C) to form a strong oxidizing solution. It is suitable only for 2-3 mm thick. Peripheral nerve fixed with osmium tetroxide. Myelin sheaths are stained black.

Advantages	Disadvantages
➤ Fixes conjugated fats and lipids ➤ Fixes myelin sheath and peripheral nerves well.	➤ Very expensive ➤ Poor penetrating agent ➤ Forms black precipitate which settles at the bottom of container ➤ Extremely volatile.



HISTOPATHOLOGIC & CYTOLOGIC TECHNIQUES LEC

Module 7: Tissue Preservation: Fixation (Chemical Fixatives)

MTHC31-24

Prof. Neil Allen Elquinto

A. Flemming's Solution

- Most common chrome-osmium acetic acid fixative. Excellent fixative for nuclear structures. It needs a lesser amount of fixatives. Wash with running tap water for 24 hours.
- It does not require a 1:10 ratio.

Advantages	Disadvantages
➤ Excellent fixative for nuclear structures. ➤ Fixes fat ➤ Less amount of solution is required.	➤ Very expensive ➤ Depress the staining power of hematoxylin. ➤ Has tendency to form artifact pigments.

B. Flemming's Solution without Acetic Acid

- For cytoplasmic structures especially mitochondria and golgi bodies. Removal of acetic acid is improving the cytoplasmic detail of cell.
- Fixation time: 24-48 hours.

TRICHOLOROACETIC ACID (TCA)

- Sometimes incorporated into compound fixatives.

Advantages	Disadvantages
➤ Marked swelling effect counteracts the shrinkage produced by other fixatives. ➤ May be used as weak decalcifying agent. ➤ Softening effect on dense fibrous tissue	➤ Poor penetrating agent.

ACETONE FIXATIVES

- It is used at ice cold temperatures ranging -5°C to +4°C. It is also used in fixing brain tissues for diagnosis of rabies. Evaporated rapidly.
- For diffusible enzymes such as Phosphatases and Lipases.



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Module 7: Tissue Preservation: Fixation (Other Methods of Fixation)

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TOPIC OUTLINE

- I. Heat Fixation
- II. Microwave Irradiation
 - A. Two Ways in which Microwave Technology is used for Tissue Fixation
- III. Ultrasound
- IV. Vapor Fixation
- V. Vacuum Fixation
- VI. HOPE-technique (Hepes-Glutamic Acid Buffer Mediated Organic Solvent Protection Effect)

HEAT FIXATION

- Involves thermal coagulation of CHONs for rapid diagnosis. Usually for frozen tissue sections and bacterial smear.

Advantages	Disadvantages
➤ Preserves nuclear and cytoplasmic detail. ➤ Accelerates fixation	➤ Destroys RBCs and dissolves starch and glycogen.

MICROWAVE IRRADIATION

- Form of non-ionizing radiation produced by the magnetron in domestic and scientific microwave ovens.

Two Ways in which Microwave Technology is used for Tissue Fixation

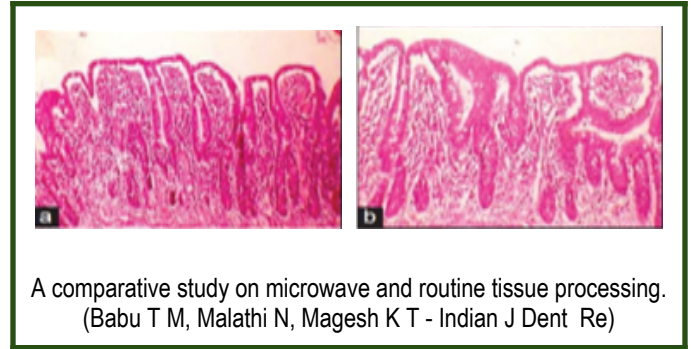
Microwave Fixation

- No chemical fixative is used at this stage. Fresh tissue, placed in saline or other isotonic solution, can be irradiated to produce primary fixation.

Microwave-assisted Fixation

- Microwaving may be carried out while the tissue is in fixatives.

Advantages	Disadvantages
➤ Accelerates fixation time.	➤ There may be some hazard from toxic fumes produced. ➤ The tissue is transferred back to saline or buffer for the microwave step.



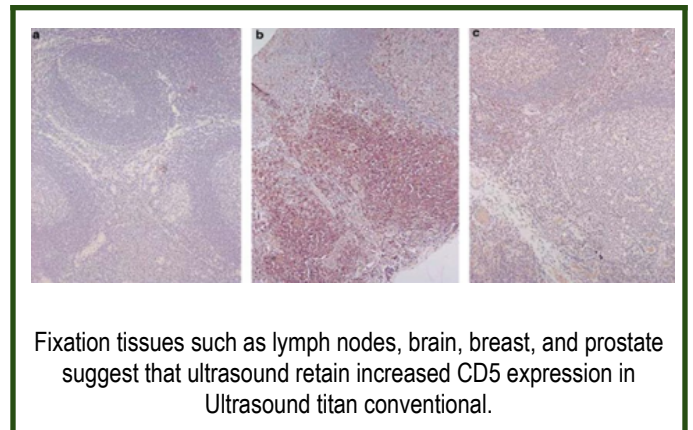
A comparative study on microwave and routine tissue processing. (Babu T M, Malathi N, Magesh K T - Indian J Dent Re)

ULTRASOUND

- Applying high-frequency, high-intensity ultrasound to the formalin fixative cuts fixation time to 5–15 min.

Advantages

- Superior and more uniform tissue morphology preservation.
- Improved molecular preservation to better accommodate both traditional and molecular diagnoses.



Fixation tissues such as lymph nodes, brain, breast, and prostate suggest that ultrasound retain increased CD5 expression in Ultrasound titan conventional.

VAPOR FIXATION

- It is used to avoid liquid fixatives. Heating liquid fixatives to get their vapor.

Formaldehyde Vapor	Acetaldehyde Vapors
Δ at +50°C to +80°C	Δ at about +80°C

VACUUM FIXATION

- It is sealing of tissues in the plastic bags and keeping it cooled at +4°C.

Advantages

- No more formalin
- No spilling
- No fumes
- Color, form and consistency are preserved
- Structures (RNA antigens) preserved for days
- Light and easy to carry



HOPE-TECHNIQUE (HEPES-GLUTAMIC ACID BUFFER MEDIATED ORGANIC SOLVENT PROTECTION EFFECT)



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- When the specimens are submerged in the protection –solution, it penetrates the tissues by diffusion, in the manner of immersion fixatives.
- a. It starts with an incubation of fresh tissue specimens in an aqueous protection-solution overnight at low temperatures of 0 to +4°C.
- b. It is followed by incubation in acetone at 0 to +4°C. Tissues treated in this manner can be directly embedded with paraffin.

Advantages	Disadvantages
➤ HOPE-fixed specimens yielded sufficient amounts of good quality DNA and RNA even after a period of 5 years.	➤ Sections are somewhat difficult to handle because of their fragility.