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Subject: Zoology

Topic: Tissue fixation

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Teacher: Praveen Deepak

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TISSUE FIXATION

Fixation is the process by which biological tissue is preserved from decaying or altering its ultra-structural detail. In other words, it is the branch of histology which deals with the process by which the natural architecture of the tissue is preserved or locked to make available for ultra-structural study of that particular tissue. It is evident that a tissue or cell is composed of as much as 75% of water varying from tissue to tissue (adipose tissue contains only 10% of water). The carbohydrate moiety of glycoproteins in the extracellular matrices and outer membrane of cells hold a large amount of water by hydrogen bonding due to its hydrophilic nature. Inside the cells, there is also immense water in cytoplasm of the cells to maintain an optimal osmolarity inside the cells. Therefore, as soon as tissue are cut or removed from the body, they immediately began to die and undergo postmortem changes by autolysis and putrefaction. **Autolysis** is the self-decaying of cells or tissue due to the release of autolytic enzymes from lysosomes of the dead cells, while **putrefaction** is the decaying process that is performed by bacteria that invade the tissue after death. Putrefaction is the fifth stage of post-mortem tissue decaying that involves the decomposition of proteins, breakdown of cohesiveness between the cells and/or tissue, and liquefaction of organs.

The main objective of tissue fixation is to preserve cells and tissue specimens in a "life-like state" or as little alteration as possible to the living tissue for ultra-structural detailing. By doing fixation, specimens are prepared or made ready for the slicing in microtome (microtomy) to get thin section so that staining protocol could be applied for visualization under microscope. It is the critical stage of microscopic observation of tissue specimen, avoidance of which may lead irreversible damage of specimen, no matter, how much care have been taken in further processing of the specimen. Fixation is achieved by profusion or immersion immediately after dissection of the tissues for 4 – 24 hours. Fixation for longer period is not generally practiced because it may lead to oversfixation which may mask the antigen making specimen unfit for immunostaining in immunological study.

Fixative

Fixation of tissue is achieved by the use of certain chemicals known as **fixatives**. Therefore, a fixative can be defined as a substance which is meant or designed to preserve biological specimens of cells or tissues by preventing their autolysis and putrefaction, by inhibiting bacterial and fungal growth, and making specimens resistant to damage for long time. There is no perfect fixative yet to be found to make specimen preserve like they are in live condition. However, formaldehyde have been known to be very good fixative and understand as close to the perfect. The choice of fixative may vary from type of specimen and analyses such as light microscopy (LM) or electron microscopy (EM) hat are planned in a study **(Table 1)**. Therefore, aim of the fixative of a good fixative can be drawn as follows:

- It must prevent autolysis and putrefaction of the tissue specimen.
- It must penetrate the specimen and penetration should be even and rapid.

- It must have the property to harden the tissue (must not lead to excessive hardening).
- It must not cause shrinkage or swelling of the cells and tissue of a specimen.
- It must preserve the volume of the tissue.
- It must have the property of increasing the optical differentiation of cells and tissues.
- It must make the cellular component insoluble to the reagents used in tissue processing.
- It must not interfere with the staining procedure and enhance the staining of specimen.
- It must be non-toxic or less toxic or non-allergic for user.
- It must be cheap and easily available.

Table 1: EM processing and imaging demands better tissue fixation

Light Microscopy (LM)	Electron Microscopy (EM)
Visible light	Electron beam (high energy)
No vacuum (1 atm)	High vacuum
Live cells/tissue can be imaged.	Samples must be embedded into plastic
Samples can contain water.	Samples must be dehydrated
Biological tissue has sufficient contrast.	Biological tissues are not electron opaque enough – need heavy metal stains
Formaldehyde fixation is often sufficient to preserve the tissue/cell.	Tissue must be protected against subsequent electron microscope processing and imaging

For fixation of specimen, a good quality of fixative is required. However, there are several factors that affect the fixation and impair the production of satisfactory results. At first, fresh tissue is required for better result. Apart from that the treatment of fixation should be proper so that fixative can penetrate evenly to the tissue. Since, no fixative can penetrate a piece of tissue thicker than 1cm; tissue thickness should be below 1cm (**Table 2** describes how to deal with thicker tissue).

Table 2: Dealing with the thickness of specimen (thicker than 1cm):

Solid organ	Slices should be cut thinner as possible as but not thicker than 5mm.
Hollow organ	Fixative should be filled in the hollow space or pack lightly with wool soaked with fixative and then cut specimen to get it of requisite thickness.
Large specimen	In this type of specimen, a fixative should be injected in the vessels (blood or air) so that entire part of the organ is fed with fixative before cutting.

Types of fixation

There are two types of fixation according to nature of means of fixation utilized namely physical fixation and chemical fixation. The type of fixation to be utilized in histological studies depends on the type of the study.

Physical fixation: In this type of fixation, mainly heating, micro-waving and freezing of the specimen is performed. Heat fixation is not commonly practiced in tissue specimen, but it is generally utilized in smears of blood and micro-organisms. However, microwave fixation, which is actually a type of heat fixation, is now commonly practiced in routine diagnostic laboratory procedures. In cryo-preservation, freeze-drying is carried out by immersing the specimen in liquid nitrogen and in freeze substitution, water is removed by putting the specimen in vacuum chamber 40°C and is generally practiced some of the applications in histochemistry but not practiced in routine diagnostic laboratory procedures[1].

Chemical fixation: In chemical fixation process, special chemicals are utilized and achieved by immersing the specimen in the fixative solution (***immersion fixation***) or perfusing the specimen with fixative solution through the vascular canal or air canal in case of lung and small animals (***perfusion fixation***). For some specialized histochemical procedures, fixation of specimen is carried out through the use of fixative in vapor form e.g. use of paraformaldehyde and osmium tetraoxide in vapour in vapour-fix-freeze-dried tissue specimen for electron microscopic study. Chemical fixation is achieved by using three types of fixatives viz coagulant fixative, cross-linking fixatives and compound fixatives.

Coagulant fixatives: Coagulant fixatives generally lead to denaturation and coagulation of cytosolic protein molecules. Coagulant fixative does not affect carbohydrates but it leads to extraction of lipids. It is basically a dehydrant and dried the specimens by removing water molecules such as ethanol, methanol and acetone. Methanol is better fixative than ethanol because the specimen is not hardened and is better preserved. It major drawback is that it produce hardening and retraction in the specimen, and lack mordant effect.

Cross-linking fixatives: This type of fixatives cross-links with their target molecules as for example, formaldehyde, gluteraldehyde, acrolein, and osmium tetroxide. Formaldehyde – gluteraldehyde mixture is the most commonly used primary fixative for electron microscopy that is introduced by Karnovkyin in 1965.

But, broadly fixatives are put under two groups; simple fixatives and compound fixatives **(Fig. 1)**. **Simple fixatives:** Simple fixatives are made up of simple chemical compounds and take more time for the fixation of tissues. Example of simple fixatives includes Formaline, Mercuric chloride, Osmic acid, Picric acid, Acetone, Ethyl alcohol, Osmium tetroxide, and Osmic acid. **Compound fixatives:** As described above, compound fixatives are a combination of two or more fixative definite proportion and require a lesser amount of time for fixation. It is broadly studies under two headings; Micro-anatomical and Cytological fixatives.

> ***Micro-anatomical fixatives*** are used to preserve various layers of cells and tissues in relation to one another so that the general structure may be studied. Examples of micro-anatomical fixatives are Formal saline, Neutral buffer formaline, Zenker's fluid and Bouin's fluid.

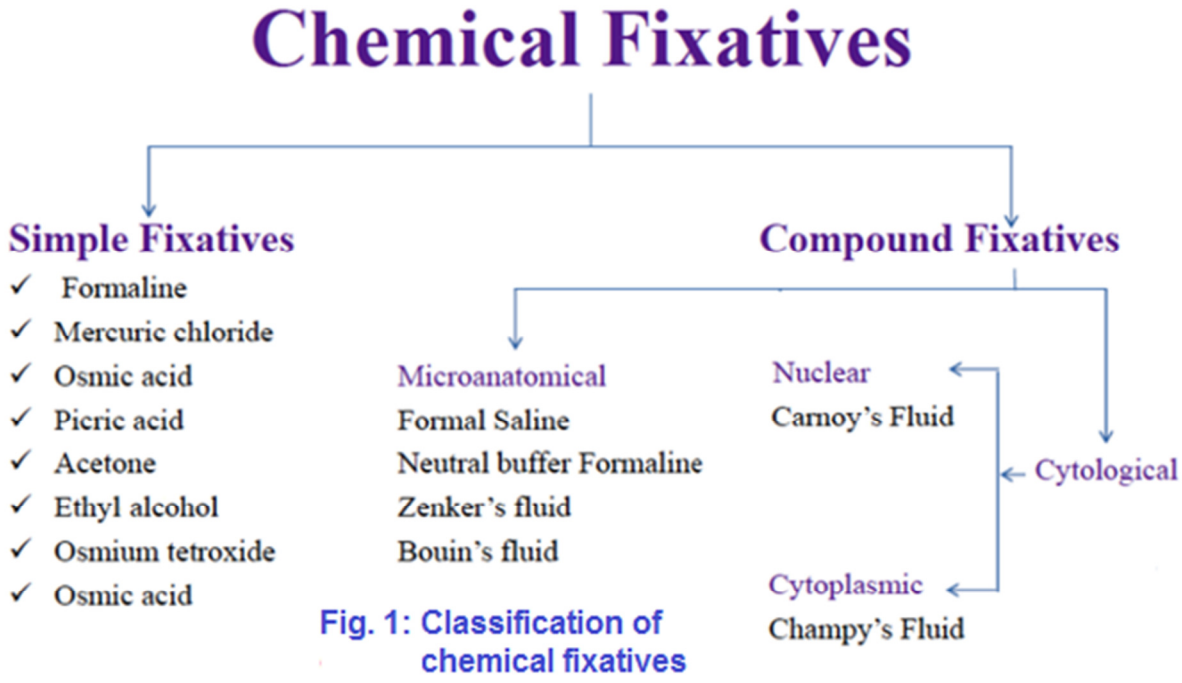


Fig. 1: Classification of chemical fixatives

Cytological fixatives are used to preserve the constituent elements of the cells. It is usually sub-divided into *nuclear* and *cytoplasmic fixatives*. Nuclear fixatives include Carnoy's fluid, while cytoplasmic fixatives include Champy's fluid.

Mechanism of action of a fixative

Fixation is regarded as complex process and considered as "a complex series of chemical events"[2]. Though, what is exactly happening in the fixation process is not completely understood, but most of the fixative works by denaturing or precipitating the protein molecules which then form a sponge or meshwork, tending to hold the other subcellular extracellular constituents of the tissues. At the molecular level, fixative have the property of coagulating proteins in the tissue, through the formation of crosslink's between proteins molecules thereby keeping their relation to each other. There are two major mechanisms which are important in fixation of proteins and protein complexes: denaturation, and addition and cross-link formation.

Denaturation: The denaturation of proteins is carried by the use of dehydrants such as the alcohols or acetone also known as coagulant fixatives. The alcohols or acetone remove and replace the free water in cells and tissues in cells and tissues and cause a change in the tertiary restructure of proteins by reshuffling the hydrogen bonding in the protein molecules (hydrophoblic areas of tertiary proteins commonly residing in the core of proteins released due to repulsion of water and become free to occupy a greater area whereas in hydrophilic areas, water molecules are released due to loose bonding leading to destabilization of hydrogen bonding; **Fig. 2**). This resulting change in conformation causes a change in the solubility of the protein molecules which finally lead to water soluble protein molecules irreversibly insoluble in water[3].

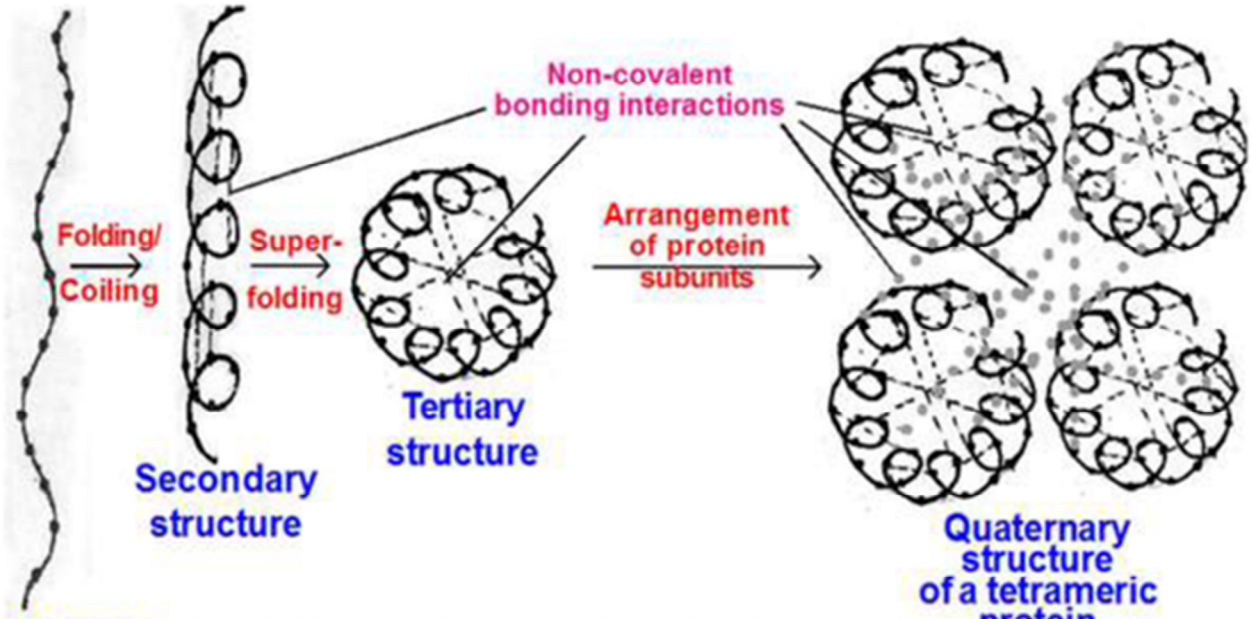


Fig. 2: Structural organization of protein showing different levels of organization by coiling and folding through rearrangement of hydrogen bonding.

Addition and cross-linking: Addition and cross-linking of protein molecules are carried by non-coagulant fixatives such as formaldehyde by being bound to them and forming inter-molecular and intra-molecular cross-links. Since this type of fixative is reactive in nature, therefore they bind to a variety of different molecules in the tissue leading to alteration in molecular conformation and thus solubility of molecules. Formaldehyde reacts with amino groups of molecular constituents to form methylene bridges due to which amino groups of the molecules are no longer available for negatively charged dye molecules such as eosin to bind. However, gluteraldehyde is found to be more effective at forming cross-links methylene bridges than formaldehyde[4] and therefore specimen fixed with gluteraldehyde stain poorly with conventional-dye staining method.

Fixative solution: It is a fixative dissolved in a solvent such as water or alcohol. It may contain a single fixative agent dissolved in a solvent such as water or alcohol or more commonly, a buffer solution to stabilize pH. Some popular fixative solutions contain several different fixing agents in combination, the rationale being that the defects in one agent can be compensated for by the addition of another e.g. acetic acid is present in some formulations to counter the shrinkage caused by other agents such as ethanol.

Factors affecting fixation

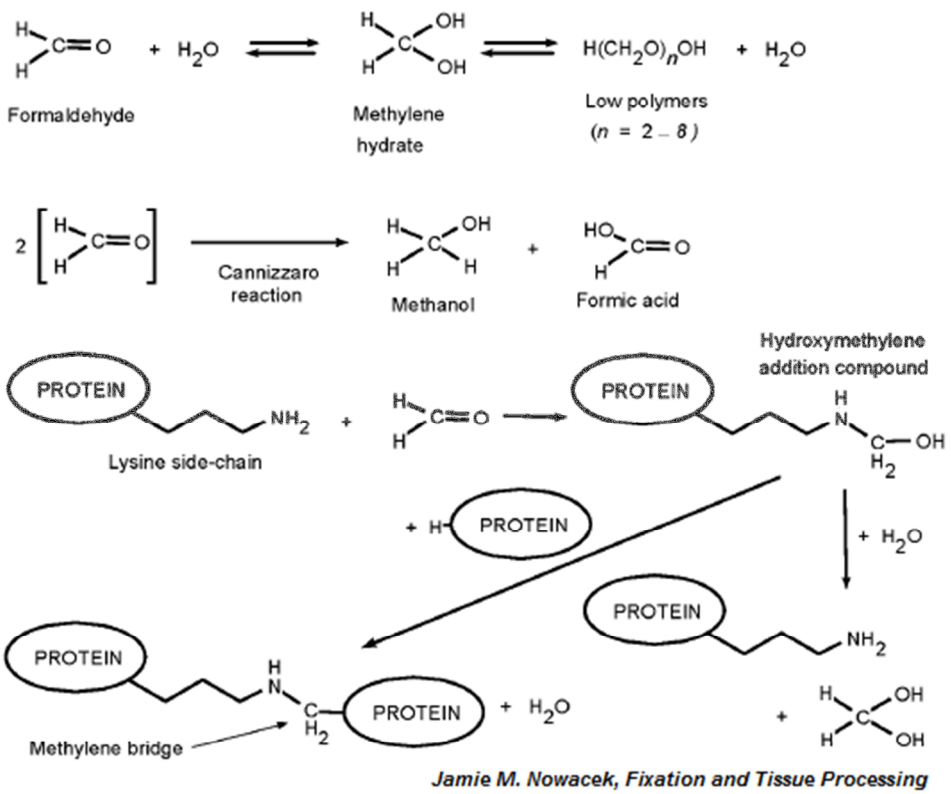
1. **Temperature:** In general, an increase in temperature increases the rate of fixation but also increases the rate of autolysis and diffusion of cellular elements. Traditionally, 0 to 4°C has been considered the ideal temperature or the fixation of specimens. Now, fixation is routinely carried out at room temperature.

2. **Size:** 1–4 mm thickness is needed for better visualization under microscope (LM & EM).
3. **Volume ratio:** At least 15-20 times greater than tissue volume is essential.
4. **Time:** Fixation needs 24 – 48 hours.
5. **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.

Different types of fixatives and their composition

Formaldehyde

Formaldehyde is a water soluble gas when dissolved to water. It is a widely used fixative and is known to provide good structural preservation for tissue for long periods of time with small retractions. It is said to be compatible with most techniques of histological staining procedures, including immunostaining and *in situ* hybridization of mRNA. When dissolved in water, it forms methylene hydrate which reacts with one another to forms polymers. It reacts to functional groups of proteins such as enzymes to give rise to hemiacetal groups making most of the enzymes non-functional and thus prevents it from autolytic degradation. Formaldehyde reacts with several other groups like ammino, sulphhydral, guanidyl, and aliphatic hydroxyl to form hydroxymethyl compound, which reacts with other groups, either located in the same or different groups, to from methylene bridges. On adding calcium, it is capable of preserving lipids also (Calcium minimizes the solubility of phospholipids).



Reaction of formaldehyde with water and proteins

It takes 24 to 5o h to fix tissue specimen, however, in certain condition last up to 1 to 2 weeks. For immunohistochemistry, a 12 to 24 h fixation at 4°C is recommended. Formaldehyde is usually prepared from the solid paraformaldehyde *(polymer of formaldehyde with N is up to 100)* the fixatives prepared from formaldehyde includes buffered

formaldehyde, Bouin's fluid, Formalin – Acetic acid – Alcohol (FAA) fixative, Periodate – Lysine – Paraformldehyde (FLP) fixative, etc.

Formalin: It is prepared by mixing 40% formaldehyde in 100 w/v of distilled water. It is routinely used in preserving specimen because it penetrates tissue very rapidly. Frozen section can also be prepared with formalin. However, it has some limitations in using such as irritation, formation of precipitate of paraformaldehyde *(can be prevented by adding 11-16% methanol)*, formation of black formalin pigment and acid formaldehyde hematin, and causing collagen shrinkage.

Neutral Buffered Formaldehyde: It is a formalin solution added with buffer such as sodium dihydrogen orthophosphate (monohydrate) and disodium hydrogen orthophosphate (anhydrous). The formula for preparing 1 lit of neutral buffered formaldehyde is following; pour100ml distilled water into a suitable container → add 4g sodium dihydrogen orthophosphate (monohydrate) → add 6.5 g disodium hydrogen orthophosphate (anhydrous) → add 100ml formalin → finally, add 800ml distilled water for use. It takes 6-12 h for fixation.

Bouin's Fluid: It is a combining fixative of formaldehyde. It is prepared by adding following chemicals; add 75ml saturated aqueous solution of picric acid in a suitable container → add 25ml formalin to give 100ml total volume → add 5ml glacial acetic acid. The main use of Bouin's fluid is the fixation of soft tissue with delicate structure. It takes 6h for fixation and colours the tissue in yellow which can be eliminated by washing with ethanol or PBS (phosphate buffered saline)-Tween but it is not used for electron microscopy. It is known to damage the nucleic acid and hence it is not used in RNA detection. Upon addition of formic acid, it is used for decalcifying bones and upon adding 25% ethanol, it is used for the fixation of adipose tissue rich in fat such as breast tissue.

Combining acid fixatives FAA and FPA: These are the good fixatives for general purposes. However, it has not fast tissue penetration, and additionally due to presence of alcohol, it leads to tissue shrinkage, and therefore amount of acid in solution may vary from 2 to 6% to modulate the shrinkage. It results in the fixation of tissue in 18-24h. Preparation method of FAA and FPA is given below in table.

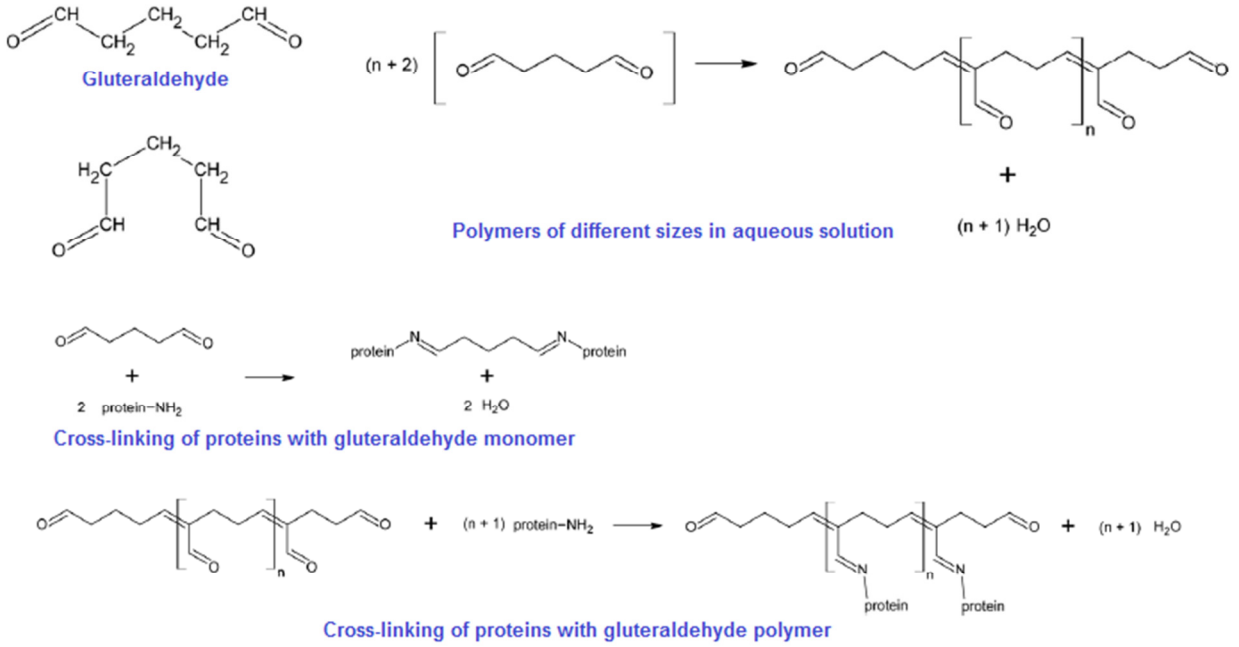
Formalin–Acetic acid–Alcohol (FAA)		**Formalin–Propionic acid–Alcohol (FPA)**	
Ethanol (95%)	50ml	Ethanol (95%)	50ml
Glaceial acetic acid	5ml	Propionic acid	5ml
Formalin (37% formaldehyde)	10ml	Formalin (37% formaldehyde)	10ml
Distilled water	35ml	Distilled water	35ml

Glyoxal

It is the simplest dialdehyde with the formula of OHC – CHO. It readily forms hydrates and polymers in water and therefore, it is generally supplemented with buffer compound at pH 4. It also contains a small proportion of ethanol, which catalyzes its reaction with proteins. It is decomposed in neutral or alkaline solution through Cannizzaro reaction1[1]. Fixation of small specimen achieved in one hour with glyoxal. It is generally sold as pre-made solution which is usefull in general histology and immunohistochemistry. It is found to better fixatives than formaldehyde for some experiment such as (i) staining is suppressed in arginine-rich proteins by anionic dyes after fixation with formaldehyde (Paneth cells, eosinophil, leukocytes or tails of spermatozoa), and (ii) antigen retrieval is frequently required after fixation with formaldehyde.

Gluteraldehyde

It is a widely used fixative generally for electron microscopy purposes. It is first introduced by Sabatini et. al. in 1963 as a fixative for EM. Due to presence of two aldehyde groups per molecule, with a longer, flexible hydrocarbon chain, it is more efficient in cross-linking. However, it slowly penetrates the tissue as compared to formaldehyde. In aqueous solution, it is present as monomers and polymers of variable sizes.



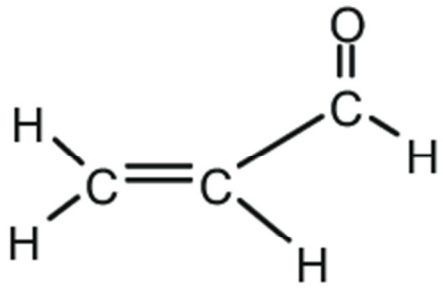
Aldehyde groups located inside the polymerized molecule react with the amino groups of amino acid, linking proteins by making cross-linkage or molecular bridges. The outer aldehyde groups, however, remain unbound, which should be blocked to prevent false positive results such as in

[1] The Cannizzaro reaction (named after its discoverer Stanislao Cannizzaro) is when a non-enolizable aldehyde (an aldehyde molecule whose molecule has no alpha hydrogen) reacts with itself in a strong base, such as sodium hydroxide (NaOH), to form a carboxylic acid and an alcohol.

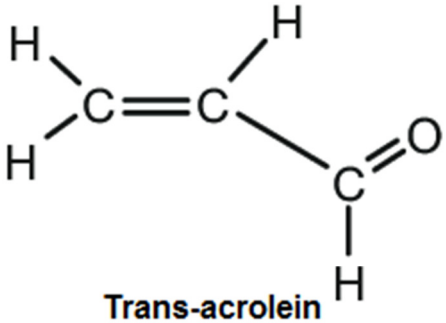
immunohistochemical reactions or PAS (**P**eriodic **a**cid-**S**chiff) histochemistry or in reaction with phospholipids containing free amino groups e.g. phosphatidylserine, phosphatidylethanolamine, etc. by pre-treatment with 1% sodium borohydride. It is avoided when paraffin embedding of specimen is necessary because obtaining section is more difficult after gluteraldehyde fixation.

Acroolein (2 – propenal)

It is an aldehyde commonly known as acrylic aldehyde (H2C=CH.CHO) which reacts with macromolecules in a similar ways as is done by formaldehyde that forms a cross-link. However, acrolein forms more cross-linkage than the formaldehyde. It reacts with fatty acids through its double bonds. Due to its lacrimatory activity, it is not commonly used despite of being fast tissue penetration. Furthermore, it is unstable at alkaline pH, and readily forms polymers. It is commonly used for enzyme histochemistry and for the fixation of plant specimen. It is an alternative fixative to formaldehyde in immunocytochemical localization of neuropeptides by perfusing briefly with 5% acrolein. Following fixation, residual carbonyl groups in the tissue will cause background staining with PAS staining procedure.



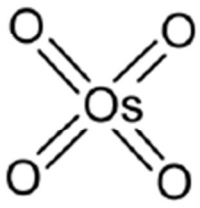
Cis-acrolein



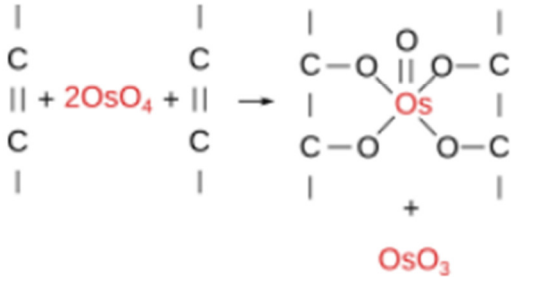
Trans-acrolein

Osmium tetroxide (OsO4)

It is said to be a multi-tasker, introduced in 1948 by Claude as a fixative agent. It can exist in nine oxidative states, five of which are reasonably stable. It is soluble in both polar (aqueous; about 7% at room temperature) and non-polar media. It easily penetrates into the cells or tissue



Osmium tetroxide (OsO4)



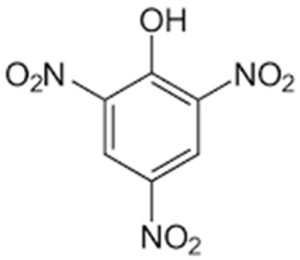
Chemical bonds between OsO4 and unsaturated fatty acid chain

and reacts with hydrophobic regions of cell or tissue and unsaturated fatty acids of a cell to make cell membrane dark, insoluble and electron dense to which only electron can pass. Since Osmium tetroxide is electron opaque, it is used in electron microscopy where it acts as both fixative as well as a stain. It also acts as mordant in enhancement of leas staining. It has low tissue penetration (< 100μm of thickness) and is highly toxic and therefore, a stringent care is needed in handling.

Acid fixatives

Acetic acid: It has molecular formula CH3 – COOH. It does not fix cells or tissue directly but its treatment results in change in the physical state of proteins into colloidal. It is not a good fixative and does not fix cell membrane and cytoplasm, and in addition, it destroys mitochondria. It is used at a concentration of 1 to 5% and is commonly combined with other fixatives like Bouin's fluid and FAA. Sometimes, it is used to counteract the effect of artifacts that might be caused by ethanol or picric acid.

Picric acid: It is an organic compound with the formula (O2N)3C6H2OH. Fixation of tissue by picric acid is mediated by the coagulation of proteins produced by picrate salts. It is used in a concentration of 2 to 15% and is used generally in combination of other fixatives like formaldehyde. It is thought to be a good fixative for preserving the cellular structure, as well as glycogen and lipids. It has mordant effect which is needed for some dyes of several embedding staining methods. However, before paraffin embedding, specimens need to be thoroughly washed to prevent impediment in paraffin embedding due to picric acid.



Zinc salts

Zinc salts such as zinc chloride and zinc sulfate were earlier used as a fixative agent. However, it has now become a component of several fixative solutions. Currently, they are combined with paraformaldehyde for the fixation of tissue meant for immunohistochemical antigen localization. They minimize the antigen masking effect of paraformaldehyde. However, it needs to be removed after fixation by thorough washing in distilled water. Earlier, mercury salts were used in place of zinc salts.

Acetone

It has molecular formula CH3COCH3 and has a similar action to alcohol, and therefore it is used a fixative and dehydrant in fixation and tissue processing. It is widely used for fixation in a study where histochemical localization of enzymes needs to be dome because of low temperature (4°C) requirement. However, it leads tissue very brittle.

Alcohol fixatives

Alcohols such as ethanol (CH3CH2OH) and methanol (CH3OH) are generally used as fixative agent for some of the histochemical procedure because of their dehydrant property. They work as

coagulant to cellular proteins. Ethanol interacts more strongly with hydrophobic areas than methanol. Ethanol at the concentration of 50 – 60% and methanol >80% are used for fixation of tissue specimen. Ethanol is sometimes used to preserve glycogen but it causes distortion of nuclear and cytoplasmic detail. Methanol is commonly used as a fixative for blood films whereas 95% ethanol is used as a fixative for cytology smears.

Carbodiimides

It is a functional group named as methanediimine with the formula RN=C=NR. It is very reactive compound that reacts with a number of other functional groups. It is a good fixative in histamine immunohistochemistry and already used for the preparation of immunogens (Eltoum, 2001), and some routine histology like fixation of fluorescent and non-fluorescent calcium indicators (Tymianski, 1997).

Potassium dichromate

Potassium dichromate (K2Cr2O7) is used as fixative agent at <3.4 to 3.8 due to its coagulant property at this specific pH. The fixation reactions are thought to involve the oxidation of proteins with the interaction of reduced chromate ions forming some cross-links. It is generally used as a component of several compound fixatives such as Zenker's and Helly's solution, and is generally not used alone. Chromium ions react with carboxyl and hydroxyl side chains of proteins. Since, it leaves amino group free, therefore it is a favored fixative for acid dyes. It is generally used as an ideal fixative agent for mitochondria. However, it is, traditionally, used for the fixation of amine containing "chromaffin" granules of endocrine tissues.

Zenker's solution: Because of mercuric chloride as one of its component, it is also known as mercuric chloride fixative. It rapidly penetrates the tissue and permit excellent staining of nuclei and connective tissues. However, it causes hardening of tissues. It has fixation time of 4 to 24 h depending upon type of specimen. It is prepared by adding following chemicals; add 950ml distilled water in a suitable container → add 25g potassium dichromate → 50g mercuric chloride → 10g sodium sulfate → and finally, 50ml glacial acetic acid.

Helly's solution: It is a good routine fixative solution which leads to excellent cytoplasmic preservation but it is not frequently used now-a-days due to mercuric chloride. It is almost identical to that of the stock Zenker's base solution. It needs 3 to 14 h for optimal fixation depending upon the thickness of specimen.

Stock Helly's base		Working Helly's solution	
Mercuric chloride	49.2g	Stock Helly base	48ml
Potassium dichromate	20g	Formalin, concentrate	2.5ml
Sodium sulfate	10g	Make just before use	
Distilled water	960ml		

Champy's fluid: This fixative solution is mainly used for the fixation of specimen where cytological preservation is needed. However, it needs comparatively thin about 1-2mm thick tissue which never more than 2mm and takes about 24 h for fixation. It is prepared by adding 1g chromium trioxide, 3g potassium dichromate, and 1g osmium tetroxide in 250ml of distilled water[2].

Other combining fixatives

Clarke solution: It contains ethanol and acetic acid in a 3:1 proportion and was one of the first fixatives used for fixation of tissues. It penetrates tissue very rapidly and fixes small tissue in minutes. It contains chloroform and therefore delicate tissue gets damaged when transferred from aqueous solution to this due to extreme hydrophobicity of chloroform and hence results in rapid tissue dehydration. It is generally used in fixation for cytological structure. Fixation of small tissue pieces requires approximately 1 h of incubation and several times washing in absolute alcohol in tissue processing. For 100ml of Clarke solution, 6oml of absolute ethyl alcohol is take in suitable container, then 10ml glacial acetic acid is added, and finally, 30 ml chloroform is added.

Farmer's fixative: It is an anhydrous alcoholic combining fixative that causes rapid dehydration and fixation of tissue. It is also known as acetic alcohol. Like Carnoy's fixative, fixation with Farmer's solution results in rapid exchange of tissue water that causes extreme cellular disruption. It is an excellent fixative for cytological investigations. It is prepared by combining 3 parts of glacial acetic acid with 1 part of absolute ethanol. It is believed to stabilize the structures and prevent chemical and structural changes during staining and mounting.

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[2] Stains File: http://stainsfile.info/prepare/fix/fixatives/champy.htm