BAJOPAS Volume 15 Number 1, June, 2022

http://dx.doi.org/10.4314/bajopas.v15i1.32

Bayero Journal of Pure and Applied Sciences, 15(1): 231 - 243
Received: March, 2022
Accepted: May, 2022
ISSN 2006 – 6996

FIXATION AND FIXATIVES IN HISTOPATHOLOGY: A REVIEW

[1]Ajileye, A. B.* and [2]Esan, E. O.

[1]Department of Biomedical Laboratory Science, College of Medicine, University of Ibadan.
[2]Department of Medical Laboratory Science, University of Medical Sciences, Ondo, Ondo State.
Corresponding Author: E-mail: ayobless05@gmail.com; +2348030445624.
Orcid Number: 0000-0002-1576-6207

ABSTRACT

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/Histoscientist to render a proper diagnosis. This article is aimed at elucidating more on fixatives and fixation process in histopathology. The information presented in this review was gathered primarily from an extensive literature search on PubMed, Scopus and Textbooks. Fixatives in histopathology can be grouped into simple and compound fixatives. Simple fixative is a solution or gas which contains only one active ingredient or that has a single-chemical solution. Examples include; Formaldehyde, Glutaraldehyde, Mercuric Chloride, Potassium Dichromate, Picric Acid, Osmium Tetroxide, Acetic Acid, Ethanol, Acetone and Chromic Acid. When two or more simple fixatives are combined in a solution, the resulting solution is called a compound fixative. This can be further divided into Micro-anatomical and cytological fixatives. Examples of a micro-anatomical fixatives include; 10% formal-saline, Heidenhain's Susa, Boiun's fluid, Formol Sublimate. Cytological fixatives are grouped into cytoplasmic and nuclear fixatives. Fixation terminates any ongoing biochemical reactions, and also increases the mechanical strength or stability of the treated tissues. To accomplish this, tissue samples are usually immersed immediately in a fixative fluid. The fixatives employed prevent autolysis and putrefaction from taking place. Fixatives are integral substances used in histopathology to forestall the actions of autolysis and putrefaction in tissue samples.

Keywords: Compound Fixatives, Fixation, Formaldehyde, Simple Fixatives, Tissues.

INTRODUCTION

Fixation, the first and most important stage in histopathology, is a procedure that uses chemicals to keep tissue from deteriorating and keep the tissue's chemistry and architecture as lifelike as possible after death (Avwioro, 2002).

Tissues and cells start to change shortly after death, which eventually results in their breakdown and destruction. These alterations are known as autolytic activity (or self-destruction) because they may be the result of enzymes that are naturally present in the tissues themselves (Bruce-Gregorios and Faldas 2017). The alterations could also be the result of outside factors, like microorganisms that induce putrefaction and disintegration. These bacteria may be pathogens that are present as a result of a disease process or those that make up the body's normal flora (Ochei and Kolhatkar, 2005). These changes can be slowed down by low tempertures or prevented by fixation. Adequate and complete fixation is the foundation of all good histological preparations (Ochei and Kolhatkar, 2005). Fixation of tissue is regarded as a critical and crucial phase that must be completed as soon as possible following tissue removal from the body after death for the purpose of tissue processing in histopathology (Bruce-Gregorios and Faldas 2017). The tissue should never be allowed to dry as it will shrink. When a tissue is not properly fixed, it will be very difficult to rectify it at the later stage. Fixation alters the physio-chemical state of tissues, which changes how stain-reactive individual cellular components are (Ochei and Kolhatkar, 2005). The amount of time needed for an adequate fixation depends on the size, consistency, and fixative type of the tissue. Whatever the fixative is made of, a lot of fixative is needed. It is advised that the fixative's volume be approximately 50 times that of the tissue (Ochei and Kolhatkar, 2005). Fixation's primary goal is to keep tissues as closely to their natural state as possible by shielding them from harmful changes like autolysis and putrefaction.

Choosing a Fixative

A variety of fixatives are available for use, however, the choice of a fixative depends on the

type of tissue or cells to be preserved and the histological features to be demonstrated. Each fixative preserves morphology differently (Bruce-Gregorios and Faldas 2017).

Duration of fixation

For all ensuing histopathological procedures, adequate fixation is necessary. The morphological interpretation, histochemical or immunohistochemical analysis may be impacted by a delay in placing a tissue in a fixative or by insufficient infiltration of the fixative prior to processing and staining (Ananthanarayanan *et al.,* 2005). The density of the tissue and the rate of fixative penetration determine how long the fixation will last. In comparison to dense fibrous tissues and bone, softer, less dense tissues are penetrated more quickly. The penetration rate of fixatives varies from one to another, e.g. glutaraldehyde and Carnoy's fluid are more rapid in their action than formaldehyde (Burnett and John, 2005). In histopathology laboratories, it is usual practice to describe and then slice big dense specimens to aid in thorough fixation. As an alternative, the fixative can be injected into an entire organ and warmed in an incubator or microwave (Burnett and John, 2005).

Properties of a good fixative

The qualities of a perfect fixative are as follows. However, certain fixatives actually combine these different criteria.

1. The main function of fixatives is to stop the autolysis (attack by enzymes) and putrefaction (attack by bacteria) of tissues.
2. Fixatives maintain the interaction of cells with external substances. Fixatives preserve the cellular and tissue structure in a lifelike condition by rendering the cell components insoluble. This reduces the modification caused by subsequent therapy and also guards against osmotic damage to tissue, which may result in swelling or shrinkage.
3. Fixatives serve to increase the visibility of various tissue components by emphasizing differences in refractive indices and making the tissue more permeable to following reagents (Leong, 1994).
4. Fixatives should stabilize the tissue against the rigors of processing without causing excessive tissue shrinkage, swelling, or hardening.
5. Should not add to or remove from the tissue,
6. Should penetrate the tissue and cells rapidly, evenly and deeply.
7. Prevent distortion by any reagents used subsequently
8. Should impart a suitable hardness and texture into tissues, so that gross cutting becomes easier and subsequently after tissue processing, to allow for easy sectioning.
9. Should allow for long term storage of specimen
10. The fixative should enhance and complement subsequent immunohistochemical, molecular biology, and histological staining methods (Ochei and Kolhatkar, 2005).

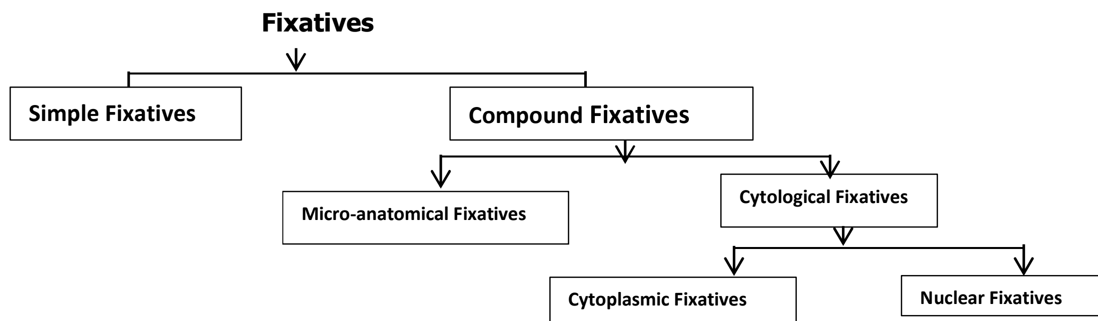

Figure 1.0: Types of Fixatives Used in Histopathology (Baker *et al.,* 2001)

Types of Fixatives Used in Histopathology

- **A. Simple Fixative**
- **B. Compound Fixative**

(A) Simple Fixatives

Simple fixative is a solution or gas which contains only one fixative or that has a single-chemical solution. Some simple fixatives when used may produce artefacts. Examples of simple fixatives include; Formaldehyde, Glutaraldehyde, Mercuric Chloride, Potassium Dichromate, Osmium Tetroxide, Acetic Acid, Picric Acid, Chromic Acid, Ethanol and Acetone (Ochei and Kolhatkar, 2005).

1. Formaldehyde (HCHO)

Formaldehyde is a gas produced by the oxidation of methyl alcohol (methanol), and is soluble in water to the extent of 40% by weight in volume. This solution is generally referred to as "formalin" or "concentrated formaldehyde". Pure stock solution of 40% formalin is unsatisfactory for routine fixation since high concentrations of formaldehyde tend to over-harden and darken the outer layer of the tissue

and affect staining adversely (Avwioro, 2002). For fixation, one part of formalin is usually diluted with nine parts of water or buffer. This produces a 10% formalin solution which contains about 4% formaldehyde by weight in volume, an optimal concentration for fixation (Ochei and Kolhatkar, 2005).

Both proteins and nucleic acids react with formaldehyde, which penetrates between them to create a stable nucleic acid-protein combination (Chang and Loew, 1994). When compared to other fixatives, formaldehyde tends to harden tissue more and causes less tissue shrinkage. Formaldehyde does not fix carbohydrates, but lipids are preserved (Kiernan, 2000).

A small number of formate ions are also present in formaldehyde (Rolls *et al.,* 1994). Through the Cannizzaro reaction, these are obtained. Two formaldehyde molecules combine in this process. Methanol is produced when one molecule condenses, while formic acid is created when a second molecule is oxidized (Henwood, 2010). This lowers the pH of the solution, and the resulting acid formation is detrimental to the success of the fixation process (Thavarajah *et al.,* 2012). To counteract this, add a small amount of magnesium carbonate, or store the formaldehyde over marble chips (Ochei and Kolhatkar, 2005). When neutralizing formaldehyde with magnesium carbonate, caution should be taken because carbon dioxide could release suddenly. It is advised to carry out neutralization in a wide-mouth vessel (Bruce-Gregorios and Faldas 2017).

2. Glutaraldehyde

Three methylene bridges separate the two aldehyde groups that make up glutaraldehyde. When compared to formaldehyde, glutaraldehyde is observed to penetrate more slowly. In addition to having some unreacted aldehyde groups that, unless chemically inhibited, might induce background staining with techniques like Periodic Acid Schiff, glutaraldehyde will be more extensively cross-linked than tissue preserved in formalin (PAS). Although the significant cross-linking has a negative impact on immunohistochemical staining, it does a great job of preserving ultrastructure, which is why it is frequently used as a main fixative for electron microscopy (Ananthanarayanan *et al.,* 2005). Despite its limited penetration, glutaraldehyde provides the best overall cytoplasmic and nuclear detail. Therefore, it is advised that tissues have a thickness of less than 1mm in at least one dimension.

Glutaraldehyde will polymerize to generate cyclic and oligomeric molecules as well as slowly degrade to produce glutamic acid. In order to achieve a 3% glutaraldehyde concentration for use, glutaraldehyde is best obtained in sealed ampoules in a handy form that has been "stabilized for electron microscopy". Primary fixation in glutaraldehyde for electron microscopy is frequently followed by secondary fixation in osmium tetroxide. Normally, glutaraldehyde is not utilized in routine histopathology (Hornickel *et al.,* 2011). Small tissue pieces and needle biopsies are fixed in 2-4 hours at room temperature using a 2.5% solution. A 4% solution is recommended for larger tissues less than 4 mm thick, fixed in 6-8 hours up to 24 hours.

The pH of this fixative falls easily which leads to poor fixation. Contact with the solution or vapour of glutaraldehyde should be avoided since it is poisonous. its exposure could irritate the digestive system, skin, and respiratory tracts (Ochei and Kolhatkar, 2005).

Advantages of Glutaraldehyde

1. It has a more consistent impact on tissues, resulting in a firmer texture and better tissue sections, particularly in the tissues of the central nervous system.
2. Plasma proteins are preserved better.
3. Less tissue shrinking results from it.
4. It preserves cellular structures better; hence, is recommended for electron microscopy (Avwioro, 2002).

Disadvantages of Glutaraldehyde over Formaldehyde:

1. It is more expensive.
2. It is less stable.
3. It penetrates tissues more slowly.
4. It tends to make tissue (i.e. renal biopsy) more brittle (Avwioro, 2002).

3. Mercuric Chloride ($HgCL_2$)

The most popular metallic fixative, soluble to a maximum of 7% in water and 33% in alcohol. Since they are poisonous by nature, metals shouldn't be exposed to them. Mercuric chloride is a common secondary fixative that reacts with a variety of amino acid residues and causes spectroscopic alterations, most likely as a result of reactivity with histidine. They have a limited ability to penetrate and cause some tissue hardening, but they provide superb nuclear detail. The retention of cytological detail, which facilitates simpler morphological interpretation, is one of the main benefits of utilizing these fixatives. These fixatives can be used with other fixatives to create a balanced solution and frequently contain neutral salt to preserve tonicity. Fixation of hematopoietic and reticulo-endothelial tissues is their optimum use (Bruce-

Gregorios and Faldas 2017). Since it has weak penetration after about 2 to 3mm of penetration and causes tissues to shrink, large portions of tissue cannot be fixed in it; hence it is typically combined with other fixative agents. Mercuric chloride pigments are created when tissues are treated with mercuric chloride-containing solutions (apart from Susa). Before staining, these pigments can be removed from deparaffinized sections by treating them with a 0.5% iodine solution diluted in 70% ethanol for 5–10 minutes. Sections are rinsed in water, treated for 5 minutes with sodium thiosulfate solution at 5%, and then washed under running water (Ochei and Kolhatkar, 2005).

Advantages

1. It precipitates all proteins.
2. It is the routine fixative of choice for preservation of cell detail in tissue photography.
3. It permits brilliant metachromatic staining of cells.
4. It is recommended for renal tissue, fibrin, connective tissue and muscle (Bruce-Gregorios and Faldas 2017).

Disadvantages

1. It rapidly hardens the outer layer of the tissue with incomplete fixation of the center.
2. If left in fixative for more than 1-2 days, the tissue becomes unduly hard and brittle.
3. It causes considerable lysis of red blood cells and removes much iron from hemosiderin.
4. It leads to the formation of black granular deposits in the tissues.
5. Compound solutions containing mercuric chloride deteriorate rapidly upon addition of glacial acetic acid to formalin.
6. It is extremely corrosive to metals (Bruce-Gregorios and Faldas 2017).

4. Potassium Dichromate ($k_2Cr_2O_7$)

This is one of the oldest of the simple fixatives. It’s used in a 3% aqueous solution. The effectiveness of this reagent on the substances fixed is pH dependent (Ochei and Kolhatkar, 2005). The pH of potassium dichromate solutions profoundly affects the type of fixation. On the more acid side, potassium dichromate acts like chromic cid, both nucleus and cytoplasm being precipitated with destruction of mitochondria. One of the most important properties of potassium dichromate is its strong fixative action on certain lipids and also fixes chromosomes. This attribute is used particularly in the study of myelinated nerve fibres. If a fixative contains potassium dichromate, tissue preserved in it should be well washed in running water, prior to dehydration to prevent the formation of an insoluble precipitate (Eltoum *et al.,* 2001).

Advantages:

1. It penetrates tissues well.
2. It hardens tissues better and more rapidly than Orth's fluid.
3. It is recommended for the demonstration of chromatin, mitochondria, mitotic figures, Golgi bodies, RBC and colloid-containing tissues (Avwioro, 2002).

Disadvantages:

1. It deteriorates and darkens on standing due to acidity; hence, the solution must always be freshly prepared.
2. Penetration is slow; hence, tissues should not be thicker than 2-3mm.
3. Chromate-fixed tissues tend to produce precipitates of sub-oxide, hence should be thoroughly washed in running water prior to dehydration.
4. Prolonged fixation blackens tissue pigments, such as melanin; this may be removed by washing the tissues in running tap water prior to dehydration (Avwioro, 2002).

5. Osmium Tetroxide (OsO_4) (Osmic Acid; OsO_4)

Usually referred to as osmic acid. This light yellow powder can generate a potent oxidizing solution when dissolved in water (up to 6% at 20°C), and it can also dissolve in non-polar solvents. Typically, this pricey reagent is offered for sale as a crystalline solid enclosed in a glass ampoule. Tiny tissue fragments are fixed with osmium tetroxide for use in electron microscopy research. Additionally, it fixes lipids, mitochondria, and golgi apparatus. Due to the slow rate of reaction or the restricted penetration of osmium tetroxide into tissue, both proteins and vast amounts of carbohydrates are eliminated during fixation by osmium tetroxide. Osmium tetroxide is employed as a secondary fixative for electron microscopic research, and it also works well as a stain and adds contrast when viewed under an electron microscope. Osmium tetroxide is also helpful for staining of lipids in frozen sections. Osmium tetroxide fixing is observed to cause tissue swelling, which can be reduced by adding fixatives with sodium chloride or calcium chloride (Ochei and Kolhatkar, 2005). Osmium tetroxide crystals are observed to change from a solid to a vapour state. Osmium tetroxide vapours can deposit in the cornea over time, which eventually results in blindness. To avoid the development of artefacts, osmic acid-fixed tissues must be rinsed under running water for at least 24 hours (Oliver and Jamur, 2010).

Advantages:

1 It fixes conjugated fats and lipids permanently by making them insoluble during subsequent treatment with alcohol and xylene.

2. It preserves cytoplasmic structures well, e.g. Golgi bodies and mitochondria.
3. It fixes myelin and peripheral nerves well; hence, it is used extensively for neurological tissues.
4. It adequately fixes materials for ultrathin sectioning in electron microscopy, since it rapidly fixes small pieces of tissues and aids in their staining (Avwioro, 2002).

Disadvantages:
1. It is very expensive.
2. It is a poor penetrating agent, suitable only for small pieces of tissues (2-3 mm thick).
3. Prolonged exposure to acid vapor can irritate the eye, producing conjunctivitis, or cause the deposition of black osmic oxide in the cornea, producing blindness.
4. It is extremely volatile (Avwioro, 2002).

6. Acetic Acid (CH_3COOH)

Acetic acid is a colorless liquid, when undiluted is called "Glacial" Acetic Acid. Acetic acid does not have much effect on proteins, other than to enable swelling by the absorption of water (Ochei and Kolhatkar, 2005). Acetic acid is not used alone for fixation but is incorporated into other fixatives to form a compound solution, most commonly at a concentration of approximately 5%. Acetic acid when combined with ethanol, is used as an effective cytological fixative that helps in conservation of nucleic acids, but if it is used singly, it results in swelling of cells. Time required for fixation by acetic acid is less, as penetration of acetic acid is faster into tissues (Bancroft and Gamble, 2002).

Advantages
1. It fixes and precipitates nucleoproteins.
2. It precipitates chromosomes and chromatin materials; hence, is very useful in the study of nuclear components of the cell. It is an essential constituent of most compound nuclear fixatives.
3. It causes tissues (especially those containing collagen) to swell. This property is used in certain compound fixatives to counteract the shrinkage produced by other components (e.g. mercury) (Chang and Loew, 1994).

Disadvantages
1. When combined with Potassium Dichromate, the lipid-fixing property of the latter is destroyed.
2. It is contraindicated for cytoplasmic fixation since it destroys mitochondria and Golgi elements of cells.
3. Concentrated acetic acid is corrosive to skin and must, therefore, be handled with appropriate care, since it can cause skin burns, permanent eye damage, and irritation to the mucous membranes. These burns or blisters may not appear until hours after exposure (Avwioro, 2002).

7. Trichloroacetic Acid (CCL_3COOH)

This is sometimes included in compound fixatives. Although it is a general protein precipitant, many tissues are noticeably swollen as a result of this property, which is used to counteract the shrinkage brought on by other simple fixatives. It can also be employed as a slow decalcifying agent, and because it softens dense fibrous tissue, it makes it easier to prepare sections from blocks of this kind (Baker *et al.,* 2001)

Advantages:
1. It precipitates proteins.
2. Its marked swelling effect on tissues serves to counteract shrinkage produced by other fixatives.
3. It may be used as a weak decalcifying agent.
4. Its softening effect on dense fibrous tissues facilitates preparation of such sections.

Disadvantage:
It is a poor penetrating agent, hence, is suitable only for small pieces of tissues or bones.

8. Picric Acid ($C_6H_2(No_2)_3OH$)

It is sparingly soluble in water (about 1%, at room temperature) but more so in alcohol (nearly 5%) and benzene (10%). It is a powerful protein precipitant. It forms water–soluble picrates with protein and fixes glycogen. Tissue should be taken to alcohol after fixing in picric acid to insolubilise the picrates before treating them with aqueous solutions. This fixative is explosive when dry and therefore must be stored damp, preferably under water. Picric acid is an acidic solution. Therefore, sometimes it gets washed out by alcohol. To avoid this, lithium carbonate is added, which acts as a neutralizer. Brighter staining is seen by picric acid fixatives (Eltoum *et al.,* 2001; Carleton *et al.,* 1980).

9. Chromic Acid

It is a solution of chromium oxide in water, is used in 1-2% aqueous solution, It precipitates all proteins and adequately preserves carbohydrates. It is a strong oxidizing agent and should not be combined with alcohol or formalin. It is a strong protein precipitant and preserves carbohydrates. Tissues fixed in chromic acid should be thoroughly washed in running water to avoid formation of an insoluble precipitate (Baker *et al.,* 2001).

10. Ethanol (C_2H_5OH)

Ethanol is a colourless liquid that is readily miscible with water. It's used at a concentration of 70-100%. If the lower concentrations are used, the Red Blood Cells become haemolyzed and WBC's are inadequately preserved. It may be used as a simple fixative. Ethanol preserves certain enzymes, precipitates proteins and glycogen. It is used for fixing smears. It is

however more frequently incorporated into compound fixatives for better results. Ethanol is a reducing agent and should not be mixed with chromic acid, potassium dichromate or osmium tetroxide. Ethanol produces considerable hardening and shrinkage of tissue. It is also highly flammable (Eltoum *et al.,* 2001).

Advantages:

1. It preserves glycogen and certain enzymes.
2. It fixes blood, tissue films and smears.
3. It preserves nucleoproteins and nucleic acids, hence, is used for histochemistry, especially for enzyme studies.
4. It is ideal for small tissue fragments.
5. It may be used both as a fixative and dehydrating agent (Atwood *et al.,* 2003)

Disadvantages:

1. It is a strong reducing agent; hence, should not be mixed with chromic acid, potassium dichromate and osmium tetroxide which are strong oxidizing agents.
2. Lower concentrations (70-80%) will cause Red Blood Cell hemolysis and inadequately preserve leukocytes.
3. It dissolves fats and lipids, as a general rule. Alcohol-containing fixatives are contraindicated when lipids are to be studied.
4. Tissue left in alcohol too long will shrink, making it difficult or impossible to cut.
5. It causes polarization of glycogen granules (Atwood *et al.,* 2003).

11. Acetone

Acetone is not recommended as morphological fixative for tissue blocks, mainly because of its shrinkage and poor preservation effects. Its use is reserved for the fixation of cryostat sections or for tissues in which enzymes have to be preserved (Avwioro, 2002). Acetone is almost always used alone and without dilution; it fixes by dehydration and precipitation. It is used to fix specimens at cold temperature (0 to 4°C). Fixation time may vary from several minutes (for cell smears, cryostat sections) to several hours (1-24 hours for small tissue blocks) (Eltoum *et al.,* 2001).

Advantages:

1. It is recommended for the study of water diffusible enzymes especially phosphatases and lipases.
2. It is used in fixing brain tissues for diagnosis of rabies.
3. It is used as a solvent for certain metallic salts to be used in freeze substitution techniques for tissue blocks.

Disadvantages:

1. It produces inevitable shrinkage and distortion.
2. It dissolves fat.
3. It preserves glycogen poorly and evaporates rapidly

[B] Compound Fixative

When two or more simple fixatives are combined in a solution, the resulting solution is called a compound fixative (Avwioro, 2002). Such a solution either combines the advantages of the constituent fixatives as in Zenker formol which contains mercuric chloride, potassium dichromate and formalin or act against each other so as to neutralize the disadvantages of the constituent fixative e.g. acetic acid which swells collagen, is combined with picric acid which shrinks tissue in Boiun's fluid (Ochei and Kolhatkar, 2005).

Compound fixative can be further sub-grouped into:

(A) Micro-anatomical fixative

(B) Cytological fixatives (Ochei and Kolhatkar, 2005).

(A) Micro-Anatomical Fixative

This preserves the architecture of tissues and enables the relationship between cells and tissues substances to be maintained. Example of micro-anatomical fixatives include; 10% formal-saline, Heidenhain's Susa, Boiun's fluid, Formol Sublimate (Avwioro, 2002).

1. 10% Formal-Saline

Formal saline is a micro-anatomical fixative, but not a compound one and is described here merely for convenience (Baker *et al.,* 2001). This is made up of saturated formaldehyde (40%, by weight volume) diluted to 10% with sodium chloride (Prento and Lyon, 1997). This mixture of formaldehyde in isotonic saline was widely used for routine histopathology prior to the introduction of phosphate buffered formalin (Kiernan, 2009). It is recommended for fixation of central nervous tissues and general post-mortem tissues for histochemical examinations (Titford and Horenstein, 2005). It is also recommended for the preservation of lipids, especially phospholipids. The period of fixation required is 24 hours or longer, depending on the size of the tissue. It often produces formalin pigment (Ochei and Kolhatkar, 2005).

Formula:

40% formaldehyde	100ml
Sodium chloride	8.5g
Distilled water	900ml

(Avwioro, 2002).

Advantages:

1. It penetrates and fixes tissues evenly
2. It preserves micro-anatomic and cytologic details with minimum shrinkage and distortion.
3. Large specimens may be fixed for a long time provided the solution is changed every three months.
4. It preserves enzymes and nucleoproteins.

5. It demonstrates fats and mucin.
6. It does not over-harden tissues, thereby facilitating dissection of the specimen.
7. It is ideal for most staining techniques, including silver impregnation.
8. It allows natural tissue color to be restored upon immersion in 70% alcohol.
9. Museum specimens are better fixed (Ochei and Kolhatkar, 2005).

Disadvantages

1. It is a slow fixative. The period of fixation is required to be 24 hours or longer.
2. Formal-saline fixed tissues tend to shrink during alcohol dehydration; this may be reduced by secondary fixation.
3. Metachromatic reaction of amyloid is reduced.
4. Acid dye stains less brightly than when fixed with mercuric chloride.
5. It produces formalin pigment in blood containing organs, although this pigment is not found in tissue fixed in buffered formalin.
6. Formalin is a source of hazard to health. The vapour causes eye irritation and sinusitis, when in prolong contact with the skin, it causes dermatitis (Ochei and Kolhatkar, 2005).

2. 10% Neutral-Buffered Formalin

The most widely used fixative for routine histology is 10% neutral buffered formalin (NBF, approximately 4% formaldehyde), buffered to pH 7 with phosphate buffer. This fixative can effectively prevent autolysis and provide excellent preservation of tissue and cellular morphology (Kiernan, 2000). It is considered the fixative of choice for many other procedures that require paraffin embedding, including immunohistochemistry (Eltoum *et al.,* 2001; Dapson, 2007) and Fluorescent In-Situ Hybridization (FISH). It is recommended for the preservation and storage of surgical, post-mortem and research specimens. The period of fixation is 24 hours or longer (Ochei and Kolhatkar, 2005).

Formula:

Mix together:
Sodium Dihydrogen Phosphate (anhydrous) 3.5g
Disodium hydrogen Phosphate (anhydrous) 6.5g
40% formaldehyde 100 ml
Distilled water 900 ml
(Avwioro, 2002).

Advantages

The advantages of 10% neutral buffered formalin are similar to formal-saline with the following additions:
1. It prevents precipitation of acid formalin pigments on post mortem tissue.
2. It is the best fixative for tissues containing iron pigments and for elastic fibers which do not stain well after Susa, Zenker's or chromate fixation.
3. It requires no post-treatment after fixation and goes directly into 80% alcohol for processing.

Disadvantages:

1. It is longer to prepare; hence, is time-consuming.
2. Positivity of mucin to PAS is reduced.
3. Reactivity of myelin to Weigert's iron hematoxylin stain is reduced (Thavarajah *et al.,* 2012).

3. Heidenhain's Susa

It penetrates tissue rapidly and evenly producing little shrinkage and hardening. It is especially recommended for fibrous tissue and biopsies. Time spent during dehydration of tissue is reduced because tissue is transferred directly to 95% alcohol. Heidenhain's Susa combines the advantages of mercuric chloride, formalin, trichloro acetic acid and acetic acid. It dissolves red blood cell and certain cytoplasmic granules and tissues exceeding 1cm thick are poorly fixed (Kiernan, 2000).

Formula:

Mercuric chloride 45 g
Sodium chloride 5 g
Trichloroacetic acid 20 g
Glacial acetic 40 ml
40% Formaldehyde 200 ml
Distilled water 800 ml (Avwioro, 2002).

Advantages:

1. It penetrates and fixes tissues rapidly and evenly.
2. It produces minimum shrinkage and hardening of tissues due to the counter-balance of the swelling effects of acids and the shrinkage effect of mercury.
3. It permits most staining procedures to be done, including silver impregnation, producing brilliant results with sharp nuclear and cytoplasmic details.
4. It permits easier sectioning of large blocks of fibrous connective tissues.
5. Susa-fixed tissues may be transferred directly to 95% alcohol or absolute alcohol, thereby reducing processing time (Carleton *et al.,* 1980).

Disadvantages:

1. Prolonged fixation of thick materials may produce considerable shrinkage, hardening and bleaching; hence, tissues should not be more than 1cm thick.
2. Red Blood Cell preservation is poor.
3. Some cytoplasmic granules are dissolved.
4. Mercuric chloride deposits tend to form on tissues; these may be removed by immersion of tissues in alcoholic iodine solution.

5. Weigert's method of staining elastic fibers is not possible in Susa fixed tissues (Avwioro, 2002).

4. Bouin's fluid

Contains picric acid, formalin and acetic acid. The complementary effects of the three ingredients of Bouin's solution work well together to maintain morphology. Embryos and pituitary biopsies are best fixed in Bouin's fluid. Glycogen is also well preserved. Bouin's fluid imparts a yellow colour on to tissue, which enables easy handling of very small and fragmented tissues. Because of its acidic nature, it will slowly remove small calcium deposits and iron deposits (Grizzle, 2009). Fixation of small pieces of tissue is complete in 12 to 24 hours and these tissues should be transferred directly to alcohol without washing in water. Prolong fixation in this acidic mixture causes hydrolysis and loss of stainable DNA and RNA.

Formula:

saturated aqueous Picric acid	75 ml
40% formaldehyde	25 ml
Glacial acetic acid	5 ml

(Avwioro, 2002).

Advantages:

1. It is an excellent fixative for glycogen demonstration.
2. It penetrates tissues well and fixes small tissues rapidly.
3. The yellow stain taken in by tissues prevents small fragments from being overlooked.
4. It is stable.
5. It is suitable for Aniline stains (Mallory's, Heidenhain's or Masson's methods) (Avwioro, 2002).

Disadvantages:

1. It causes Red Blood Cell haemolysis and reduces the amount of demonstrable ferric iron in tissue.
2. It is not suitable for frozen sections because it causes frozen sections to crumble when cut.
3. Prolonged fixation makes tissues hard, brittle and difficult to section. Tissues should not be allowed to remain in the fluid for more than 12-24 hours (depending on size).
4. It alters and dissolves lipids (Ochei and Kolhatkar, 2005).

5. Formol Sublimate

Is a solution of mercuric chloride and formalin. There is considerable shrinkage and hardening of tissue but without distortion of tissue constituents. Formol-sublimate solution is recommended for secondary fixation and routine post-mortem tissues (Ochei and Kolhatkar, 2005).

Formula:

Sat. Aq. Mercuric chloride	90 ml.
40% Formaldehyde	10 ml

(Avwioro, 2002).

Advantages:

1. It produces minimum shrinkage and hardening.
2. It is excellent for many staining procedures including silver reticulum methods.
3. It brightens cytoplasmic and metachromatic stains better than with formalin alone.
4. Cytological structures and blood cells are well preserved. There is no need for "washing-out".
5. It fixes lipids, especially neutral fats and phospholipids (Ochei and Kolhatkar, 2005).

Disadvantages:

1. Penetration is slow; hence, tissue sections should not be more than 1cm thick.
2. It forms mercuric chloride deposits.
3. It does not allow frozen tissue sections to be made.
4. It inhibits the determination of the extent of tissue decalcification (Ochei and Kolhatkar, 2005).

6. Zenker's solution

Zenker's solution is excellent for tissue which is to be stained by the trichrome methods for connective tissue fibres. This is recommended for the fixation of pituitary tissue and bone marrow. The period of fixation required is from 12-24 hours. Tissues must be washed out thoroughly in running water to permit good staining (Avwioro, 2002).

Formula

Mercuric chloride	5 g
Potassium dichromate	2.5 g
Sodium sulphate (optional)	1g
Distilled water	100 ml
Glacial acetic acid	5ml (to be added just before use)

(Avwioro, 2002).

Heat, cool, filter in brown bottle. Wash sample for 24 hours with distilled water after fixation.

Advantages:

1. It produces a fairly rapid and even fixation of tissues.
2. It is recommended for trichrome staining.
3. It permits brilliant staining of nuclear and connective tissue fibers.
4. It is recommended for congested specimens (such as lung, heart and blood vessels) and gives good results with PTAH and trichrome staining.
5. It is a stable fixative that can be stored for many years (Carleton *et al.,* 1980).

Disadvantages:

1. Penetration is poor.
2. It is not stable after addition of acetic acid.
3. Prolonged fixation (for more than 24 hours) will make tissues brittle and hard.

4. It causes lysis of red blood cells and removes iron from hemosiderin.
5. It does not permit cutting of frozen sections.
6. It has the tendency to form mercuric pigment deposits or precipitates.
7. Tissue must be washed in running water for several hours (or overnight) before processing. Insufficient washing may inhibit or interfere with good cellular staining (Carleton *et al.,* 1980).

7. Helly's Fluid (Zenker Formol)

This is widely used both as micro-anatomical and cytological fixative. It is recommended for the fixation of pituitary tissue, bone marrow and extramedullary hematopoiesis and intercalated discs of cardiac muscle. Fixation of tissue is slower than in zenker's fluid. However, it produces mercury pigment which should be removed from sections prior to staining and it can produce chrome pigment if tissue is not washed in water prior to processing. After water washing, fixed tissue should be stored in 70% ethanol. Because of the low pH of this fixative, formalin pigment may also occur. Never use metal forceps to handle tissue (Ochei and Kolhatkar, 2005).

Formula

Mercuric chloride	5 g
Potassium dichromate	2.5 g
Sodium sulphate (optional)	1g
Distilled water	100 ml
40% formaldehyde	5 ml (to

be added immediately before use) (Ochei and Kolhatkar, 2005).

Heat, cool, filter in brown bottle. Wash sample for 24 hours with distilled water after fixation.

Advantages:

1. It is an excellent micro-anatomical fixative for pituitary gland, bone marrow and blood containing organs such as spleen and liver.
2. It penetrates and fixes tissues well.
3. It preserves cytoplasmic granules well (Ochei and Kolhatkar, 2005).

Disadvantages:

The disadvantages of Helly's solution are similar to Zenker's solution except that brown pigments are produced if tissues (especially blood containing organs) are allowed to stay in the fixative for more than 24 hours due to Red Blood Cell lysis. This may be removed by immersing the tissue in saturated alcoholic picric acid or sodium hydroxide (Ochei and Kolhatkar, 2005).

8. Gendre's Fluid

This is a general micro-anatomical fixative which is also widely used for the preservation of glycogen (Ochei and Kolhatkar, 2005).

Formula:

95% Ethyl alcohol saturated with picric acid = 80 ml.

40% formaldehyde solution = 15 ml.

Glacial acetic acid = 5 ml.

Advantages:

1. Fixation is faster.
2. It can be used for rapid diagnosis because it fixes and dehydrates at the same time, e.g., in the frozen section room.
3. It is good for preservation of glycogen and for micro-incineration technique.
4. It is used to fix sputum, since it coagulates mucus (Ochei and Kolhatkar, 2005).

Disadvantages:

1. It produces gross hardening of tissues.
2. It causes partial lysis of RBC.
3. Preservation of iron-containing pigments is poor (Ochei and Kolhatkar, 2005).

(B) Cytological Fixatives

These are fixatives which preserve cell constituents and maintain nuclear and cytoplasmic substances for histochemistry and other cytological procedures (Avwioro, 2002).

There are 2 types:

(A) Nuclear fixative

(B) Cytoplasmic fixative

(A) Nuclear fixative: this is a cytological fixative which preferentially fixes the nucleus, sometimes to the detriment of the cytoplasm. The pH is usually less than 4.4 and may contain an acid, usually acetic acid. Examples are Fleming's fluid and Carnoy's fluid (Avwioro, 2002).

1. Fleming's Fluid

This contains chromic acid, osmium tetroxide and acetic acid. It fixes chromosomes very well and permanently preserves fat resulting in the blackening effect on lipids by osmium tetroxide, which it contains. It penetrates very slowly. Solution is prepared immediately before use because the prepared solution deteriorates when stored Ochei and Kolhatkar, 2005).

Formula:

1% Aqueous chromic acid = 15 ml.

2% Aqueous osmium tetroxide = 4 ml.

2% Glacial acetic acid

1 ml (Ochei and Kolhatkar, 2005).

Advantages:

1. It is an excellent fixative for nuclear structures, e.g. chromosomes.
2. It permanently fixes fat.
3. Relatively less amount of solution is required for fixation (less than 10 times the volume of the tissues to be fixed) (Ochei and Kolhatkar, 2005).

Disadvantages:

1. It is a poor penetrating agent; hence, is applicable only to small pieces of tissues.
2. The solution deteriorates rapidly and must be prepared immediately before use.
3. It has a tendency to form artifact pigments; these may be removed by washing the fixed

tissue in running tap water for 24 hours before dehydration.

4 It is very expensive (Ochei and Kolhatkar, 2005).

2. Carnoy's Fluid

This is recommended for fixing chromosomes, lymph glands and urgent biopsies. The period of fixation required is from 30 minutes to 3 hours (Avwioro, 2002).

Formula

Absolute alcohol	60ml
Chloroform	30ml
Glacial Acetic Acid	10ml

Advantages:

1. It is considered to be the most rapid fixative and may be used for urgent biopsy specimens for paraffin processing within 5 hours.
2. It fixes and dehydrates at the same time.
3. It permits good nuclear staining and differentiation.
4. It preserves Nissl granules and cytoplasmic granules well.
5. It preserves nucleoproteins and nucleic acids.
6. It is an excellent fixative for glycogen since aqueous solutions are avoided.
7. It is very suitable for small tissue fragments such as curettings and biopsy materials.

Disadvantages:

1. It produces Red Blood Cells haemolysis, and can produce excessive hardening
2. It causes considerable tissue shrinkage.
3. It is suitable only for small pieces of tissues due to slow penetration.
4. It tends to harden tissues excessively and distorts tissue morphology.
5. It dissolves fat, lipids, and myelin.

(B) Cytoplasmic Fixative

This preferentially fixes the cytoplasm, sometimes to the detriment of the nucleus. The pH is always above 4.6 and usually does not contain an acid. Examples are Helly's fluid and Fleming's Fluid without acetic acid (Ochei and Kolhatkar, 2005).

1. Helly's Fluid

Is a good cytoplasmic and micro-anatomical fixative

Advantages and Disadvantages: same as Helly's fluid.

2. Flemming's solution without acetic acid

Is made up only of chromic and osmic acid, recommended for cytoplasmic structures particularly the mitochondria. The removal of acetic acid from the formula serves to improve the cytoplasmic detail of the cell (Avwioro, 2002).

Advantages and Disadvantages: same as Flemming's solution. The omission of acetic acid improves the cytoplasmic detail (Avwioro, 2002).

Washing Out

This 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 (Bruce-Gregorios and Faldas 2017).

Secondary Fixation

Tissues removed from 10% formol saline, is sometimes advantageous to refix the tissue for a further 4 hours in a second fixative (Avwioro, 2002). The fixatives usually selected for this purpose are Formol Sublimate, Zenker-Formol and Heidenhain's Susa. This have the advantage of imparting a firmer texture to the tissue and in many instances, improves the subsequent staining result.

Post-Fixation

When cells and proteinous substances are enveloped in a fatty part of the tissue, they are not adequately fixed because fixatives which are aqueous solutions do not penetrate fats effectively. During tissue processing, the clearing agent removes the fat. Hence an unfixed region of cells and protein rich substances are exposed. To prevent degradation of tissue which may occur, tissue should be transferred from the clearing agent back to absolute alcohol for about 2 hours where it is post fixed. Tissue is then returned to the clearing agent (Ochei and Kolhatkar, 2005).

Post- Chromatisation

This can also be called post-chroming or post-mordanting. It is the treatment of tissue with 3% potassium Dichromate after the primary fixative which is usually 10% formol saline. Post-chromatisation is done to mordant tissue when demonstrating mitochondria in the Altmann's method and for myelin in the weigert-pal's technique. Post-chromatisation can either be performed on tissue before processing or on section before staining (Baker *et al.,* 2001).

Factors Affecting Fixation

1. Length of Fixation (Time)

Different fixatives have different ideal fixation times. The fixative must diffuse into the specimen's center for fixation to take place, and then enough time must be allowed for the fixation reactions to take place. The optimal time will change depending on the fixative being used, as both diffusion time and reaction time

depend on the specific reagent used. There is a lot of pressure in busy diagnostic laboratories to speed up turnaround times, and this can lead to the processing of incompletely-fixed tissues. Because poorly fixed tissue does not process effectively, this might result in low quality sections revealing tissue distortion and poor quality staining. Ethanol will continue to fix the tissue if it is removed from formalin and placed in it during processing. As a result, the morphological image in the specimen's center will reflect ethanol fixation. Longer fixation times, however, lead to over-cross-linking, which makes samples fragile. If the fixation period is too brief, there won't be enough penetration into the tissues to cause cross-linking (Burnett and John, 2005).

2. Temperature

Increasing the temperature of fixation will increase the rate of diffusion of the fixative into the tissue and speed up the rate of chemical reaction between the fixative and tissue elements. It can also potentially increase the rate of tissue degeneration in unfixed areas of the specimen (Ajileye and Adeyemi, 2020). Microwave fixation may involve the use of higher temperatures, up to 65°C, but for relatively short periods. For electron microscopic studies, 0°to 4°C is appraised as ideal temperature (Bruce-Gregorios and Faldas 2017).

3. Concentration

Fixative agents need prolonged time for fixation if concentration is low. If concentration of fixing agent is high, it results in damaging of cellular structures as well as obliterated enzyme activities (Titford and Horenstein, 2005). Too high a concentration may adversely affect the tissues and produce artifact similar to excessive heat. Different fixatives have different ideal concentration that is determined experimentally; for example, ideal fixative for oral soft tissue is formalin used in 10% concentrated solution (Bruce-Gregorios and Faldas 2017).

4. Size

Tissue thickness is one of the important factors for fixation. If the sample size is large, it is unfavorable for the fixative to penetrate and reach to the deeper part of the tissue, which would result in autolysis of epithelium. Ideally, 3mm to 5mm thick specimen is best suited for complete penetration by fixatives (Ochei and Kolhatkar, 2005).

5. Osmolality

If cells are fixed in a hypertonic solution, the cells may shrink. If the cells are fixed in a hypotonic solution, the cells may swell and burst. For that reason, we recommend using a normal phosphate buffered saline (PBS) based fixative (Titford and Horenstein, 2005). Hypertonic solutions give rise to cell shrinkage. Isotonic as well as hypotonic fixatives cause cell swelling and poor fixation. The best results are usually obtained using slightly hypertonic solutions (Bruce-Gregorios and Faldas 2017).

6. Penetration rate

The penetration rate of a fixing agent depends on its diffusion characteristics and varies from agent to agent. Formalin and alcohol penetrate the best and glutaraldehyde the worst (Ajileye et al., 2018). Mercurials and others are somewhere in between.

7. Volume ratio

It is important to have an excess volume of fixative in relation to the total volume of tissue because with additive 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. A fixative to tissue ratio of 20:1 is considered the lowest acceptable ratio but it would be better and faster if one can have a target ratio of 50:1. Agitation will also enhance fixation of the specimen. The use of small volumes of fixation fluids for larger specimens is the most frequent cause of poor tissue preservation (Baker *et al.,* 2001).

General Precautions in Handling Fixation of Specimens

1. Each and every tissue sample needs to be correctly labelled and identified.
2. If fixation is not achievable right away, keep refrigerated but do not freeze. Avoid slowly freezing unfixed tissues close to 0°C as this could encourage the formation of ice crystal artefacts. On the other hand, frequent freezing and thawing will damage cellular organelles, release enzymes, and diffuse soluble parts of the cell.
3. Fresh tissue may be infectious. Consider any fresh or incompletely-fixed tissue as potentially infectious one and other workers in the Medical Laboratory.
4. Avoid drying to prevent tissue shrinkage and distortion with loss of cellular detail. Drying of specimen surfaces will cause permanent damage and may mask any pathological change. Small endoscopic specimens are particularly susceptible to this type of damage. Small tissue biopsies may be placed in a petri dish with moistened filter paper to prevent drying.
5. Tissues should not be more than 5mm thick except in lung edema (in which case tissue slices may be 1-2cm. thick), with minimum squeezing and handling. Thin sections allow complete penetration by fixative in a short time.

6. Cotton soaked in fixative should be put inside hollow organs (such as the stomach and intestines).
7. 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. They are not, however, easily penetrated due to tough sclera. Formal-alcohol must be injected before immersing the organ in the fixative.
8. 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 the inner structures.
9. Presence of mucus prevents complete penetration of fixative; hence, tissues that contain mucus are fixed slowly and poorly. Excess mucus may be washed away with normal saline solution.
10. Fatty tissues should be cut in thin sections and fixed longer.
11. Tissues containing large amount of blood (e.g. blood vessels and spleen) should be flushed out with saline before fixing.
12. The fixative should penetrate from all sides. Always place specimens into containers that already contain fixative. This will prevent adhesion of the specimen to the container.
13. Where possible, hollow organs or specimens with natural cavities should be opened to allow immediate access to the fixative.
14. 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.
15. Fixatives should be used only once. Specimens shed cells and tissue fragments into the fixative solution which could contaminate any subsequent specimen.
16. Avoid metal lids. Some fixatives are highly corrosive and will attack metals (e.g., mercury salts).
17. All fixatives are toxic and irritant. Anyone using fixatives should be aware of their potential hazards (Thavarajah *et al.,* 2012).

CONCLUSION

Fixatives are integral substances used in histopathology to forestall the actions of autolysis and putrefaction in tissue samples. Each and every fixative has its own advantages and disadvantages. Most routinely used fixative in histopathology is 10% buffered formal saline. It is considered the fixative of choice because of its stable pH. Adequate fixation is essential for all subsequent histopathological techniques. Various factors such as length of fixation, temperature, concentration, size, osmolality, penetration rate and volume ratio can affect fixation process.

Source of Funding

All the authors funded this review study. No funding was received for the study.

Conflict of Interest

The authors do not have any conflict of interest or financial disclosure. The article or part of the content is not under consideration and has not been published by any other Journal.

Ethical Approval

Not Applicable

Consent

Not applicable

Aknowledgment

Nil

Authors' Contributions

AAB and EEO conceived and designed the work, conducted the literature review, provided research materials. AAB, and EEO wrote initial and final draft of article, and provided logistic support. AAB and EEO critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

REFERENCES

Ajileye, A. B. and Adeyemi, O. A. (2020). Museum Techniques and Practices in Histopathology; A Compendium. J Med Lab Sci. 30 (3): 18-34.

Ajileye, A. B., Esan, E. O. and Adeyemi, O. A. (2018). Human Embalming Techniques; A Review. American Journal of Biomedical Sciences. 10 (2): 82-95.

Ananthanarayanan, V., Pins, M. R., Meyer, R. E. and Gann, P. H. (2005). Immuno-histochemical assays in prostatic biopsies processed in Bouin's fixative. J Clin Pathol. 58(3):322–324.

Atwood, K., Farmilo, A. J., Stead, R. H. and Boenisch, T. (2003). Fixation & tissue processing. From: Handbook for Immunochemical Staining Methods. 3rd ed. Carpinteria, CA: DAKO. 18–22, 44–46.

Avwioro, O. G. (2002). Histochemistry and Tissue Pathology. First Edition, Ibadan: Claverianum Centre. Pp. 51 - 65.

Baker, F. J., Silverton, R. E. and Pallister, C. J. (2001). Introduction to Medical Laboratory Technology. London, UK: Bounty Press limited (Seventh Edition). 182–193.

Bancroft, J. D. and Gamble, M. (2002). Theory and Practice of Histological Techniques. 5th ed. Philadelphia, PA: Churchill Livingstone Elsevier. 63–108.

Bruce-Gregorios, J. H. and Faldas, M. M.(2017). Histopathologic Techniques

Carleton, H. M., Drury, R. A. B. and Wallington, E. A. (1980). Carleton's Histological Technique. 5th ed. New York, NY: Oxford University Press. Pp. 33-47.

Chang, Y. T. and Loew, G. H. (1994). Reaction mechanisms of formaldehyde with endocyclic imino groups of nucleic acid bases. J Am Chem Soc. 116 (8):3548–3555.

Dapson, R. W. (2007). Glyoxal fixation: how it works and why it only occasionally needs antigen retrieval. Biotechnic & Histochemistry. 82(3): 161-166.

David Burnett., and John Crocker. (2005). The Science of Laboratory Diagnosis, 2nd Edition. Pp. 05-08.

Eltoum, I., Fredenburgh, J. and Grizzle, W. E. (2001). Advanced concepts in fixation: 1. Effects of fixation on immunohistochemistry, reversibility of fixation and recovery of proteins, nucleic acids, and other molecules from fixed and processed tissues. Developmental methods of fixation. J Histotechnol. 24:201-210.

Grizzle, W. E. (2009). Special symposium: fixation and tissue processing models. Biotech Histochem. 84(5):185-193.

Henwood, A. (2010). What is the Best Procedure to Remove Formalin Pigment from Formaldehyde-Acetic Acid-Alcohol Fixed Tissues. J Histotechnol. 33(3):109-111.

Hornickel, I., Kacza, J., Schnapper, A. et al. (2011). Demonstration of substances of innate immunity in the esophagus epithelium of domesticated mammals. Part I—methods and comparative fixation evaluation. Acta Histochem. 113(2):175–188.

Kiernan, J. A. (2000). Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do. Microscopy Today. 1(1). 08-12.

Kiernan, J. A. (2009). Histological and Histochemical Methods. Theory and Practice. 4th ed. Bloxham, UK: Scion. Pp. 12-73.

Leong, A. S-Y. (1994). Fixation and fixatives. In Woods AE and Ellis RC eds. *Laboratory histopathology*. New York: Churchill Livingstone. 4.1-1 - 4.1-26.

Ochei, J. K. and Kolhatkar A. (2005). Medical Laboratory Science Theory and Practice. London. Pp.399 – 406.

Oliver, C. and Jamur, M. C. (2010). Fixation and embedding. Methods Mol Biol. 588:353-362.

Prento, P. and Lyon, H. (1997). Commercial formalin substitutes for histopathology. Biotech Histochem. 72(5):273-282.

Rolls, G. O., Farmer, N. J. and Hall, J. B. (1994). Artefacts in histological and cytological preparations. In Woods A and Ellis R eds. *Laboratory histopathology*. New York: Churchill Livingstone. 5.3-1 - 5.3-29.

Thavarajah, R., Mudimbaimannar, V. et al. (2012). Chemical and physical basics of routine formaldehyde fixation. J Oral Maxillofac Pathol. 16(3): 400–405.

Titford, M. E. and Horenstein, M. G. (2005). Histomorphologic assessment of formalin substitute fixatives for diagnostic surgical pathology. Arch Pathol Lab Med. 129(4):502–506.