

Light microscopic determination of tissue

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ABSTRACT

Tissues to be examined under a light microscope, must pass through certain stages. These are; detection (fixation), washing, dewatering (dehydration), polishing or transparent, absorption, recession or blocking, cutting, coloring and closing the lamella. At the end of this phase examination of the tissues with a light microscope takes place. Mistakes at any stage, may lead to irreparable consequences. For this purpose, the researcher during the examination of biological materials should properly fulfill tissue tracking methods. It is aimed to review the existing information about the first phase of tissue detection and to review and compile new publications. It was aimed to review present knowledge and recent literature on the fixation step which is the first step in tissue determination.

Key Words: Fixation, light microscope, formaldehyde, tissue detection

Introduction

For tissue or intracellular bacteria to prevent degradation by enzymes and to maintain their physiological structure, in the shortest possible time, quickly detection process is known as maintaining fidelity. In other words, in-vivo micro-anatomic imaging is achieved by tapping (1). In order to operate, chemical or physical methods are carried out but are less preferred (2).

Chemical methods include chemical cross-linking agents. They are formaldehyde and glutaraldehyde (3,4). Isotonic buffered 4% formaldehyde solution is one of the best fasteners used in conventional light microscopy. On the other hand in electron microscope widely used primary fixing agent is glutaraldehyde, in which tissue protein amine groups (NH₂) are reacted. Glutaraldehyde, via a dialdehyde crosslinking strongly determines the tissue (2).

While formaldehyde penetrates tissue quickly, it detects proteins slowly. Physical fixation is done by freezing, coagulating, or precipitating components with the aid of heat, organic materials and acid. Examples of fixative solutions used with physical fixation methods include Clark (75 ml of ethanol and 25 ml of acetic acid) and Carnoy (60 ml of ethanol, 30 ml of chloroform and 10 ml of acetic acid) (5).

In histology and pathology labs, formaldehyde is the most widely used detection solution (6). Formaldehyde gas becomes liquid at room temperature. Although it is a colorless gas it has

an irritating smell (7,8). It leads to dermatitis and can cause irritation to the eyes. At the same time due to its carcinogenic properties it also poses a serious threat to environment (6).

Despite the many advantages of formaldehyde in detecting tissues, in some cases this is unsuccessful. Antibody specifically used in immuno-peroxidase staining method; needs to be studied in fresh tissue, which gives no result in formaldehyde fixed tissues. This is because the antigenic epitope by the formaldehyde solution is modified or destroyed (9).

Formaldehyde cross-links molecular groups to prevent immunohistochemical reactions (6). These epitopes can be recovered with "epitope access method" (10). This is one of the methods of antigen retrieval (1). Antigen retrieval applications include enzymes, protein denaturation and temperature. It has been reported that heat is applied to formaldehyde fixation fractions in various solutions, resulting in cross-linking and increased immunostaining properties of many antigens in formalin-fixed and paraffin-embedded tissue sections (5) (Figure 1).

Formaldehyde is the most preferred fixation solution because it changes the antigenic structure of many proteins, resulting in a good structure (11).

Aktan et al. (12) in their study investigated the effects of Carnoy and 10% formaldehyde solution in morphological and immunohistochemical staining of skin tissue. The obtained skin tissue samples were fixed in Carnoy and 10%

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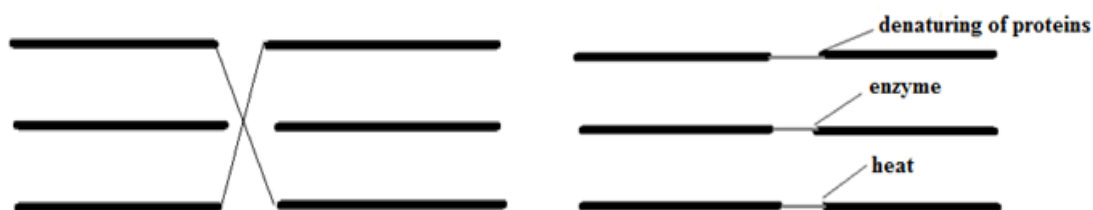


Fig. 1. Epitopes in several "epitope retrieval method" and recycling. Such epitopes can be recovered with "epitope access method". This is one of the methods of antigen retrieval. Antigen retrieval applications include enzymes, protein denaturation and temperature.

formaldehyde solution, and after the second day, the skin texture data were obtained with the same routine histological procedures. 5-micron-thick paraffin sections were stained by using hematoxylin-eosin, Masson trichrome and immunohistochemical dyes, F8, Ki67 and anti-actin. Hematoxylin-eosin and Masson trichrome Carnoy and 10% formaldehyde detection in tissue sections provides observation of morphological differences. However, in sections prepared from 10% formaldehyde-detected tissues, loss of tissue and deterioration in cross-sections of antigen, recovered by microwave, was observed. Especially more layer wear has been observed in the reticular dermis. It can be concluded that the sections of skin tissue prepared by Carnoy are more successful than the 10% formaldehyde solution. Tissue sections detected in Carnoy solution gives more successful results than tissue sections detected in formaldehyde solution. It is less toxic than formaldehyde solution; It can be suggested as a powerful alternative (11).

Stability of proteins in tissue detection is the most important step in the process. It is known that fixatives show cross-linking between proteins, bringing them to gel form and protecting everything in a live state. In this regard, it is reported that soluble proteins become insolubly fixed to structural proteins. While there is not much information available on the effect of fixation on lipids, frozen sections are used because conventional fixation and tissue processing lead to the loss of lipids(13).

Factors affecting fixation

These factors are; hydrogen ion concentration (pH), temperature, penetration, osmolality, concentration and time.

Hydrogen ion concentration (pH): PH changes in different fixation methods. In general, the hydrogen ion concentration will generally be in the physiological range by using a suitable buffer for pH adjustment. Best fixing for pH is between 6 and 8. In the course of cell death, the pH in the

cell decreases due to the anoxia. A specific pH range is selected in different studies. For example, the best pH range for gastric mucosa is 5.5. Similarly, in the case of the catecholamine chromophore reaction, storage granules for adrenaline and noradrenalin have been shown to be most stable in the presence of formalin at a pH of 6.5.(13).

Temperature: Routinely, the detection of tissue for light microscopy is performed at room temperature. For electron microscopy, and some histochemical studies temperature range is set between 0-4°C. However, fixation is performed at room temperature for some cells, such as mast cells, for electron microscopy. Hopwood showed that fixatives which are used for the fixation of nucleic acids don't react in any temperature scale (14).

For bacteriology and for tissues such as blood samples which have an increased risk of deterioration, use of heat is chosen. It is known that formalin heated at 60 ° C is sometimes used for rapid detection of very urgent biopsy specimens. Formaldehyde at 100 ° C can be used for the detection of tuberculosis (13).

Penetration: It is an important event for texture. Since this process is quite slow, a block taken should be small (ex 1 mm electron microscopy) or fine to obtain adequate fixation. In this context, it should be guaranteed that the obtained samples should be thin enough for suitable fixing of the samples(13).

The researcher, Medawar, studied the diffusion rates of fixative in tissues. According to his formula; The depth of penetration is constantly proportional to the time square which is formulated as: $d = K\sqrt{t}$ (15) (Table 1).

Osmolality: Cells shrink in hypertonic solutions. To correct this situation, hypotonic fixative process is applied. Criterion was obtained using light hypertonic solutions (400-450 mOsm, isotonic solutions with 340 mOsm). By changing the osmolality, it is possible to change the

Table 1. Concentrations of specific tissues and the fixative gel diffusion rates

Fixative	Fixative concentration (%)	K (tissue)	K (gel)
Acetic acid	5	1.2	2.75
Chromium trioxide	0.5	0.25	1
Formaldehyde	4	0.78	3.6
Ethanol	100	1	--
Glutaraldehyde	6	1	--
Osmium tetroxide	0.5-2	0.29-0.58	0.85

Table 2. Commonly used fixative solutions (16):

Commonly used fixative solutions	Formulation	Recommended Applications
Phosphate buffered formalin	100 ml 40% formaldehyde 900 ml Distilled water 4 g Sodium dihydrogen phosphatemonohydrate 6.5 g Disodium hydrogen phosphate anhydrous The solution should have a pH of 6.8 Fixation time: 12 – 24 hours	For routine histopathology this is the most widely used formaldehyde based fixative. Phosphate buffer is chosen to avoid formation of formalin pigment. Following its administration many epitopes require antigen uptake for successful IHC.
Formal calcium	100 ml 40% formaldehyde 10 g Calcium chloride 900 ml Distilled water Fixation time: 12 – 24 hours	Recommended for the preservation of lipids especially phospholipids.
Formal saline	100 ml 40% formaldehyde 9 g Sodium chloride 900 ml Distilled water Fixation time: 12 – 24 hours	Formaldehyde mixture in isotonic saline has been widely used for routine histopathology prior to administration of phosphate buffered formalin. It usually produces formalin pigment.
Carnoy's solution	60 ml Ethanol absolute 30 ml Chloroform 10 ml Glacial acetic acid Fixation time: 1 – 4 hours	Due to its rapid penetration properties it can be used for detection of all tissues. Fine structure of nucleus is well protected. It fixes glycogen and plasma cells well. In order not to cause shrinkage or hardening, fixation which exceeds 4 hours should be avoided.
Zinc formalin (unbuffered)	1 g Zinc sulphate 900 ml Deionized water (Stir until dissolved then add) 100 ml 40% formaldehyde Fixation time: 4 – 8 hours	Zinc formaldehyde solutions were developed as an alternative to mercury chloride formulations. They are supported by the work which states that they give better results with IHC.
Zenker's fixative	950 ml Distilled water 50 gr Mercuric chloride 25 g Potassium dichromate 50 ml Glacial acetic acid Fixation time: 4 – 24 hours	The acetic acid-free stock solution can be preserved. Zenker is an effective fixative for microanatomic structures and is used because of its potent effect on cytoplasmic and fibril dyes. In fresh material it is more efficient compared to post-mortem tissues. However with this fixative red blood cells are not protected sufficiently. Blocks are fixed for 3-8 hours and washed with tap water to remove excess dross. Mercury pigment is removed by the previous method.
Gendre's solution	800 ml 95% Ethanol saturated with picric acid 150 ml 40% formaldehyde 50 ml Glacial acetic acid Fixation time: 4 - 18 hours	Like the Bouin solution this fixative affects tissues positively. In order to protect glycogen and other carbohydrates this is the recommended fixative. After fixation, the tissue should be placed in 70% ethanol. For the removal of yellow stains washing should be administered.

B-5 fixative	<p>Stock solution 12 g Mercuric chloride 2.5 g Sodium acetate anhydrous 200 ml Distilled water Working solution, prepare immediately before use B-5 stock solution: 20 ml 2 ml 40% formaldehyde Fixation time: 4 – 8 hours</p>	<p>With this fixative morphological detail can be obtained at a high level. It is used in the fixation of hematopoietic tissues. Nuclear details are provided excellently. With this fixative IHC gives good results. This, however, creates a rigid fixation that will cause the tissue to disintegrate. Mercury pigment must be removed before dyeing. The tissue should not be stored in B5 fixative, it should be taken in 70% ethanol.</p>
Helly's fixative	<p>1000 ml Distilled water 25 g Potassium dichromate 10 g Sodium sulphate 50 g Mercuric chloride 50 ml Immediately before use add – 40% formaldehyde Fixation time: 4 – 24 hours</p>	<p>It is added immediately before using 5 cc formalin instead of acetic acid in Zenker. Although Helly contains a liquid oxidizing agent (potassium dichromate) and a reducing agent (formalin), it is an excellent fixative. Helly is especially useful for bone marrow, spleen, lymph glands, pituitary gland and pancreas. Blocks should be fixed for 6-24 hours and the mercury pigment should be removed as in Zenker. Helly can be used both as a micro-anatomic and cytological (cytoplasmic) fixative and as a second fixative after 10% formal-saline, such as formal-sublimate.</p>
Methacarn	<p>60 ml Methanol absolute 30 ml Chloroform 10 ml Glacial acetic acid Fixation time: 1 – 4 hours</p>	<p>Carnoy solution has similar properties. Less shrinkage and hardening occurs.</p>
Bouin's solution	<p>Picric acid saturated aqueous soln. (2.1%): 750 ml 250 ml 40% formaldehyde 50 ml Acetic acid glacial Fixation time: 4 – 18 hours</p>	<p>It gives very good results with tricrom painted tissue. It protects glycogen well but usually it lyses erythrocytes. Sometimes it is recommended for gastrointestinal biopsies, animal embryos and endocrine gland tissues. The stains due to picric acid are bright yellowish color. Excessive picric must be washed from the tissues before dyeing with 70% ethanol. Since the tissue may become brittle, it shouldn't be fixed more than 18 hours. Because picric acid may cause DNA degradation, intact DNA should not be used in studies that require it.</p>
Hollande's solution	<p>25 g Copper acetate 40 g Picric acid 100 ml 40% formaldehyde 15 ml Acetic acid 1000 ml Distilled water Dissolve chemicals in distilled water without heating. Fixation time: 4 – 18 hours</p>	<p>With this solution fixation of gastrointestinal canal specimens and of endocrine tissues are advised. It produces less fragmentation than Bouin. It has some decalcification properties. Since a solubilizing phosphate precipitate will form, the fixative must be washed from the tissues with a phosphate buffered formalin solution in the processing machine.</p>
Clarke's solution	<p>75 ml Ethanol (absolute) 25 ml Glacial acetic acid Fixation time: 3 – 4 hours</p>	<p>This solution is used in frozen and smear samples. It can produce fair results by conventional treatment in a very short time, which provides better fixing time. Preserves the nucleic acids but eliminates the lipids. Tissues can be directly transferred to 95% ethanol.</p>
Alcoholic formalin	<p>100 ml 40% Formaldehyde 900 ml 95% Ethanol 0.5 g calcium acetate can be added to ensure neutrality Fixation time: 12 - 24 hours</p>	<p>It combines the effect of formalin with the additional fixative and cross-linking effects. Sometimes, the incomplete primer is used during processing to secure the fixation after formalin fixation. It can be used for fixation or correction of large fat specimens (especially chest) because it allows lymph nodes to be detected more easily when cleansing and removing lipids. When used for primer fixation, tissue samples can be placed in 95% ethanol for direct processing.</p>
Formol acetic alcohol	<p>85 ml Ethanol absolute 10 ml 40% formaldehyde 5 ml Acetic acid glacial Fixation time: 1 – 6 hours</p>	<p>It is also a substance that is faster than alcohol formalin due to the presence of acetic acid, which can produce formalin pigment. Sometimes used in Cryostat diagnostics to correct insufficiently fixed regions. When used for primer fixation, the samples can be placed directly into 95% ethanol.</p>

structure of membrane systems in various cells. It is known that fixative changes the size of extracellular compartments of various adjuvants (13).

Concentration: The concentrations used in fixative for particular function is determined based on the effectiveness and solubility factors. In the experiments performed, it was determined that the effect of different concentrations of fixatives on the morphology obtained is different. Glutaraldehyde is normally used as a 3% solution in the determination of electron microscope, but in the studies performed it has been found to be effective at 0.25% when the appropriate pH is provided. Aldehyde in the presence of buffers in the polymerization, has resulted in a reduction in effective concentration. Small concentrations of glutaraldehyde (0.25%) have been found suitable (13).

Fixation time: For fixation the most commonly used agent is formalin. This is allowed to stand for 2-6 hours for sublimation in the fixation process. For electron microscopy, the chopped tissue is allowed to sit for 3 hours and then a buffer is recommended. In the case of formalin, the detection and washability of the tissues for 24 hours is checked. Formaldehyde and tissue component reacts quickly with each other. This long-term process of fixing with a fixative causes hardening of the tissue and causes shrinkage.

For electron microscopy, using glutaraldehyde as a fixing solution is more advantageous. Long-term fixations made with aldehydes would seriously affect enzyme activity. Limitations on this substrate and crosslinking between protein molecules cause diffusion (13).

Other factors affecting fixation: Volume change, substance addition, the use of detergent during fixation (13).

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