Current Technologies for Fixation of Biological Material for Immunohistochemical Analysis (Review)

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Based on their own experience and published reports the authors provide an insight into the existing methods of fixation of biological material used in immunohistochemistry. The first quality of an immunohistochemical fixative should be its ability to preserve the tissue structure so that the antigenic properties of macromolecules are minimally affected. Considering this point, the review analyzes the applicability of commonly used fixatives to immunohistochemical staining; among those, aldehydes (formaldehyde, glutaraldehyde, glyoxal), dehydrating (coagulating) agents (ethanol, methanol, acetone), combined fixation solutions (Bouin's solution, Carnoy's solution, methacarn, etc.), as well as the recent zinc-containing fixatives and commercial products. Most of these fixatives inevitably change the tertiary and quaternary structure of many proteins; therefore, the detection of these proteins by immunohistochemistry requires an additional procedure of unmasking the epitopes using proteolytic enzymes or elevated temperatures. When compared for the preservation of antigenic structures, a high quality of the novel zinc-containing fixative — zinc-ethanol-formaldehyde — was noted. It has been concluded that none of the fixatives known to date has such a combination of properties that allow obtaining high-quality histological preparations and, at the same time, allows for detecting of any antigens in the stained tissue.

Key words: fixation of biological material; immunohistochemistry; formalin; ethanol; glutaraldehyde; zinc salts; zinc-ethanol-formaldehyde; heat induced epitope retrieval.

Introduction

Immunohistochemistry is a unique method for studying and analyzing biological objects; it shows the localization of various molecules in the tissue at different levels — in cells, subcellular structures, and the intercellular space. To obtain results of biological or medical significance, the procedure of pre-staining fixation of a biological object is expected to preserve the structure of cellular organelles and extracellular tissue components. Specifically, for the immunohistochemical detection of proteins, it is important to retain their antigenic properties during and after fixation. Thus, the primary quality of the selected fixative, intended for the subsequent immunohistochemical study, should be the ability to ensure the preservation of biological tissue without affecting (or minimally affecting) the antigenic properties of macromolecules.

Among the qualities of any histological fixatives are also the absence of toxicity, simplicity of preparation and a low cost of the components. At present, there is an abundance of methods and technologies for fixation of biological material; those differ between different immunohistochemical protocols and may cause different, sometimes unidentified, artifacts. A reasonable question arises, whether these specific methods of fixation are good enough to carry out an adequate and informative immunocytochemical test. The purpose of this review is to compare the classical and novel technologies of sample processing for immunohistochemistry.

Aldehydes. The most common fixative used in histological and electron microscopic protocols are

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aldehydes, usually formic or glutaric, often called formaldehyde and glutaraldehyde, respectively.

Formaldehyde is a water-soluble gas. The concentration of a saturated solution of formaldehyde in water is 40% (by volume) or 37% (by weight). Aqueous solution of formaldehyde is called formalin, its saturated solution is considered to be 100%; and 10% formalin is used to fix biological tissue [1]. Such a solution contains 4% formaldehyde. In solution, formaldehyde molecules interact with each other over time, forming polymers (with a degree of polymerization of up to 100 units); that is called paraformaldehyde. In concentrated formaldehyde solutions, the polymers gradually form a white precipitate, and its amount depends on the storage conditions. To reduce the polymerization of formaldehyde, manufacturers add 10% methanol to 40% solution of formaldehyde.

In formalin solutions, formaldehyde molecules interact with each other not only during the polymerization reaction, but also in the Cannizzaro reaction, where one formaldehyde molecule is reduced to methanol and the other is oxidized to formic acid [2, 3]. As a result, formic acid gradually accumulates in formalin during storage, which causes acidification of the solution and can adversely affect the quality of fixation. Because of this, to standardize the fixation procedure, it is preferable to use fresh formalin, which can be prepared directly in the laboratory from a commercial Paraform preparation by dissolving it in hot water at a ratio of 4 g of dry paraformaldehyde to 100 ml of distilled water.

Formalin fixes tissues by chemical transformation of macromolecules, forming intra- and intermolecular methylene crosslinks between amino acids (both free and those of proteins), between nucleic acids, and also between amino acids and nucleic acids [2, 4–9]. The crosslinks can be formed only in the presence of uncharged amino groups that exist only at neutral pH values. Therefore, to use formalin as a fixative, it is necessary to prepare it with a buffered solution, usually with 0.1 M phosphate buffer (pH 7.2–7.4). The resulting neutral buffered 10% formalin is used for decades as the standard for tissue fixation in pathology. Such fixation allows for obtaining high-quality histological preparations that completely satisfy the requirements of histological and pathological analysis.

Glutaraldehyde fixes the tissue similarly to formaldehyde, forming inter- and intramolecular crosslinks in proteins and nucleic acids, but each molecule of glutaraldehyde contains not one but two aldehyde groups. Therefore, its ability to interact with macromolecules in biological tissue is higher, and its effect on the protein structure is greater than that of formalin [10–12]. The intermolecular crosslinks formed by glutaraldehyde between polypeptide molecules are so strong that glutaraldehyde is used even in cardiovascular surgery to crosslink collagen fibers and enhance their mechanical strength [13]. During storage, molecules of glutaraldehyde form oligomers [11], which penetrate the tissue depth more slowly than monomers. Therefore, for fixation with glutaraldehyde, only small pieces of tissues should be used. After fixation, a significant amount of unreacted glutaraldehyde molecules may be retained in the sample; those are capable of non-specific binding of antibodies as well as histological and histochemical dyes, which necessitates the use of special procedures for removing excess glutaraldehyde before starting the immunohistochemical staining [2].

It is important to note that the tissue fixed with glutaraldehyde has marked spontaneous fluorescence due to the reaction of glutaraldehyde with certain amines, lipids and proteins, especially collagen [11, 14]. This phenomenon significantly limits the use of glutaraldehyde with the material intended for further fluorescence measurements or laser confocal microscopy; in such cases, additional procedures to block autofluorescence are required [14–20]. It should be noted that even after fixation in formalin, some tissue autofluorescence occurs, especially in a material left in the fixing solution for a long time; the signal though is much lower than that after treatment with glutaraldehyde [21–25].

A considerable shortcoming of glutaraldehyde fixation is that due to the increased crosslinking of protein molecules, a dense tissue is formed; this dense tissue is hardly permeable to molten paraffin, which makes paraffin impregnation more difficult. In addition, this fixation procedure causes excessive compaction of the tissue blocks and complicates the preparation of standard paraffin sections. The above factors significantly reduce the value of using glutaraldehyde for fixation with subsequent paraffin embedment of histological objects [2]. Because of that, glutaraldehyde is normally used to fix small-size samples for further examination by electron microscopy or electron immunocytochemistry; in such protocols, the samples are encapsulated in special polymerizable resins capable of penetrating the glutaraldehyde-fixed tissue [16, 26–32].

The modification of the tertiary and quaternary structure of proteins by formalin or glutaraldehyde [4, 10, 11, 33–41] leads to changes in the antigenic properties of these proteins. Specifically, part of the epitopes become masked, which prevents them from interacting with the added antibodies and thus interferes with their immunohistochemical detection [42–46]. Although there is evidence that, not only during fixation but also at other stages of sample processing, the masking of antigens takes place [47–49], fixation is considered the main factor that negatively impacts the detection of tissue antigens [46]; the degree of this impact depends on the concentration of the fixative [50].

The use of sections prepared from unfixed frozen samples allows for a more effective implementation of immunohistochemistry methods; however, preservation of cell structures without fixation remains poor.

The formation of intermolecular crosslinks after

fixation with formalin is a reversible process, at least in part, and the epitopes of detectable antigens can be unmasked with proteolytic enzymes or an elevated temperature [51–53]. For the enzymatic unmasking, trypsin, chymotrypsin, pepsin, pronase, proteinase K, and other proteases are used. For the heat induced epitope retrieval, the samples placed into various buffer media are subjected to heating in a microwave oven, microwave heating under high pressure, autoclaving, boiling under pressure, steam heating, or water bath heating [51, 54–56]. The acidity (pH) of the buffer medium for heat induced epitope retrieval is important [4]. The optimal method of thermal or enzymatic unmasking is determined experimentally for each specific antigen [51, 57]. In a number of cases, after the heat induced epitope retrieval an additional blockage may be needed to suppress non-specific and background reactions in the sample under study [52].

Since recently, another aldehyde fixative, ***glyoxal***, has been used for immunohistochemical studies [58]. This simple dialdehyde has an advantage over formaldehyde — it is able to fix tissues faster and with less intermolecular crosslinks between proteins. Thus, its interference with polypeptides and their antigenic structure is less than that of formaldehyde, which allows for running immunohistochemical staining without prior unmasking [58, 59]. Glyoxal, however, reacts with arginine residues in polypeptide chains to form imidazole; as a result, the arginine-rich antigens may be distorted, which prevents their immunohistochemical detection.

Dehydrating fixatives. In addition to aldehydes, an important group of fixatives is represented by dehydrating (coagulating) agents. They include ethanol and methanol, as well as acetone. These substances have long been used to fix biological material — alone or, more often, in various combinations with other chemicals. Alcohols and acetone are capable of dehydrating the tissue; the replacement of water molecules with alcohol or acetone destroys hydrophobic and hydrogen bonds, leading to modification of the tertiary structure, protein denaturation and a change in the antigenic structure [35, 60]. However, the decrease in the protein antigenicity after using of dehydrating fixatives is less pronounced than after fixation in formalin [61]. Therefore, even a material fixed in formalin was proposed to be placed in ethanol or a mixture of ethanol-acetic acid (2:1) before the subsequent use for immunohistochemistry [33, 62, 63]. Along with that, a poor preservation quality of cellular structures, especially membranes, and a significant deterioration in detectability of some proteins was found in monolayer cell culture after fixation in acetone or methanol as compared to formalin or glutaraldehyde [64].

It should be noted that using coagulating fixatives may negatively affect, first of all, the detection of low-molecular compounds and haptens; those can escape into the solution since they are not strongly bound to the cell compartments. In contrast, high-molecular compounds not bound to cell membranes (for example, intermediate filament proteins) are well identified after treatment by coagulating fixatives [65].

Other fixatives. In addition to the above major fixatives, there are a large number of fixing compositions that incorporate the major fixing agents at different proportions with the addition of other components. In the histological techniques, such fixatives as the Bouin's reagent (mixture of a saturated aqueous solution of picric acid, formalin and glacial acetic acid at a ratio of 15:5:1) are widely used. Among other examples: the Carnoy's solution (absolute alcohol, chloroform and glacial acetic acid, 6:3:1); methacarnoy fixative, or methacarn, in which ethyl alcohol is replaced by methyl (methanol, chloroform and glacial acetic acid, 6:3:1); alcohol-formol (96º ethanol and formalin, 9:1); alcohol-formaldehyde-acetic acid, 85:10:5); periodate-lysine-paraformaldehyde-PLP (3% paraformaldehyde, 75 mM L-lysine, 10 mM $NaIO_4$ in 0.1 M phosphate buffer), etc. In addition, solutions of heavy metal salts — mercury, chromium or osmium (Zenker's fixative, chromic acid and potassium dichromate, osmium tetroxide) are used for fixation.

Some of these fixatives have been tested for preparation of sample for immunohistochemical staining. For example, it was noted that after fixation in the Carnoy's solution, many antigens can be well detected, even better than after the standard fixation in neutral formalin [66–69]. Fixation with methacarn also allowed for visualization of certain epitopes better than after fixation with formalin [70]. The comparison of fixation with acetone, ethanol, neutral formalin and neutral formalin + calcium chloride produced mixed results. Thus, different antigens were identifiable better or worse after the treatment with different fixative reagents; according to some authors [71, 72], after fixation with neutral formalin most of the sought antigens could be visualized upon immunohistochemical staining. However, other studies [60, 73] showed poor detectability of antigens after fixation in formalin as compared with other tested fixatives: ethanol, methanol, acetone, the Bouin's reagent, alcohol-formol, or zinc-formalin. The detection of epitopes after prolonged fixation in neutral formalin was particularly poor [7, 74, 75].

The PLP fixative was shown to preserve the intact antigenic structure much better than formalin; for a number of proteins fixed with PLP, there is no need in the subsequent antigens unmasking [61]. However, when the biological sample preservation was compared between different fixative agents, PLP was found to cause the strongest compression of tissues upon fixation. In this respect, formalin was less aggressive, and the zinc-containing fixatives were the least compressive fixatives [76]. There is a report that the antigens of invertebrate tissue remained well-preserved after fixation in a mixture of uranyl acetate, trehalose, and methanol [77].

Regarding the toxicity of heavy metals, there were attempts to avoid their use for fixation. As it was reported, mercury chloride could be replaced with zinc salts (chloride, sulfate) so the resulting reagent contained zinc ions as the only fixing agent [78]. Although the mechanism of fixation by zinc ions is not fully understood, biological samples fixed with zinc showed a good preservation quality of cells and extracellular material; notably, the zinc-containing fixative was advantageous for the preservation of antigens in comparison with neutral formalin [78–82] or periodate-lysine-paraformaldehyde [83]. Nevertheless, as noted by the authors, aqueous solutions of zinc salts penetrate the depth of a fixed sample not fast enough, which can cause an uneven immunoreactivity of antigens located on the surface or in deep layers of the tissue.

In the Laboratory of Morphology at the Institute of Experimental Medicine (Saint Petersburg), a new zinc-containing combination fixative — zinc-ethanol-formaldehyde (1 g of zinc chloride in 96% ethanol and concentrated formalin, 9:1) — was developed [84]; that reagent demonstrated good preservation of the brain tissue and internal organs of humans and laboratory animals [85, 86]. Immunohistochemical study of nervous tissue (definitive and embryonic) and peripheral organs fixed in zinc-ethanol-formaldehyde allowed for detecting a large number of proteins: calbindin, calretinin, choline acetyltransferase, glutamate decarboxylase, glial fibrillary acidic protein, Iba-1 and NeuN proteins, neuron-specific enolase, synaptophysin, tyrosine hydroxylase, alpha-tubulin, vimentin, nestin and others [87–98]. In addition, immunohistochemical visualization of some antigens did not require heat induced epitope retrieval.

Available commercial offers. In a search for fixatives that would ensure good preservation of every morphological detail and minimally change the antigens, commercial companies offer new types of fixing solutions for immunohistochemical analysis. Among them, CytoSkelFix, F-Solv, FineFIX, Sensofix, RCL2, LN-FIX, FineFIX, UMFIX, Glyo-Fixx, FineFIX, HOPE, NEO-FIX, Cell-Block, ExcellPlus, Greenfix, UPM, CyMol, etc. [73, 99–109]. These trade names often conceal combinations of well-known aldehyde or alcohol fixatives, sometimes added with original admixtures. For example, the UPM fixative is a mixture of ethanol, methanol, 2-propanol, and formalin; CyMol — ethanol, methanol, and 2-propanol; Greenfix — ethanol and ethanediol. However, the precise composition of these fixatives is not disclosed to the public; therefore, it is difficult to assess the effects of individual components of a commercial product on the preservation and antigenic properties of biological samples.

Fixation of biological material for electron microscopic immunocytochemistry requires special conditions. Usually the fixation is made with glutaraldehyde, osmium tetroxide or uranyl acetate; the latter two are also used for contrasting the sample, which is needed for viewing it by means of an electron microscope.

For a combined light and electron microscopy immunocytochemical examination, a mixture of glutaraldehyde and paraform — the so-called Karnovsky's fixative and its variants — is used [10, 110–112]. Acrolein (acrylic acid aldehyde) is also used thanks to its relatively mild masking impact on the tissue antigens; however, acrolein is extremely toxic, therefore it is used rarely or in a combination with glutaraldehyde or formaldehyde [10].

Osmium tetroxide has been shown to mask antigens [10, 113, 114], which makes its use in immunocytochemistry problematic. Due to this, tannic acid has been suggested to replace osmium; tannic acid produces a well-contrasted material for electron microscopy and also provides for the detection of antigens at the ultrastructural level [115–117]. Another option is to use glutaraldehyde alone and dissolve it in a buffer with a balanced ionic composition [118]. Good preservation of the ultrastructure and the antigens was achieved after fixing the material in periodate-lysine-paraformaldehyde [119].

Conclusion

According to the literature, over the recent 25 years of immunohistochemical studies, important improvements in the techniques of biological fixation have been achieved. Yet, none of the fixatives known to date has an ideal combination of properties that enables to obtain high-quality histological preparations and that does not interfere with identification of any antigens in the subsequent immunostaining. The only conclusion from this review is that the optimal fixation protocol should be selected or developed for any specific protein or antibody, considering the experience of others with related proteins. It is not advisable to focus on commercial fixatives with unknown compositions, since their production may be stopped one day for the commercial or technical reasons, and the researcher may be unable to accurately reproduce the conditions required for successful fixation.

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Conflict of interest. There are no conflicts of interests related to this study.

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