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Tissue fixation and the effect of molecular fixatives on downstream staining procedures

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ABSTRACT

It is impossible to underplay the importance of fixation in histopathology. Whether the scientist is interested in the extraction of information on lipids, proteins, RNA or DNA, fixation is critical to this extraction. This review aims to give a brief overview of the current "state of play" in fixation and focus on the effect fixation, and particularly the effect of the newer brand of "molecular fixatives" have on morphology, histochemistry, immunohistochemistry and RNA/DNA analysis. A methodology incorporating the creation of a fixation tissue microarray for the study of the effect of fixation on histochemistry is detailed.

1. Introduction

Fixation is the foundation step behind the study of pathology and essentially exists to prevent the autolysis and degradation of the tissue and tissue components such that they can be observed both anatomically and microscopically following sectioning. A number of fixatives exist, either having being in use for decades, or in the case of formaldehyde over a century, whilst others have only been created in the last 10 years. To attempt to classify this chaos, fixatives can be placed into two categories; denaturing fixatives and cross-linking (or addition) fixatives. Table 1 details some of the standard histological fixatives used and their classification.

The alcohol-based fixatives, for example Carnoy's and Methacarn, are denaturing fixatives. The action of the alcohol present in the solution acts to cause protein denaturation through the removal of water from the free carboxyl, hydroxyl, amino, amido and imino groups of the proteins [1] which results in protein coagulation and tissue shrinkage. Carnoy's fixative adds chloroform and acetic acid to the mixture which counteracts the shrinkage effects of ethanol and engenders tissue fixation through hydrogen bonding of the constituents to the tissue [2]. Similarly, Methacarn, where ethanol in the Carnoy's solution is replaced by methanol, appears to work by the same method [3].

The mercuric-containing fixatives, for example B-5 and Zenker's, are little-used in current practice and are thought to act through binding to sulphydryl and amino groups in an additive reaction [1,4].

Bouin's, like Carnoy's, was first described in the late 19th Century by Pol Andre Bouin. Consisting of picric acid, acetic acid and formaldehyde, it has both a coagulative as well as cross-linking effect on proteins. In particular, the penetration of picric acid into the tissue is slow and it coagulates proteins but has no known chemical interaction with them. Whereas, acetic acid penetrates relatively quickly and opposes the tissue shrinkage caused by the picric acid. With formaldehyde present in a higher concentration than for formaldehyde alone, approximately 10% formaldehyde, the actions of formaldehyde over time cross-link the tissue, although this may be inhibited by the low pH of the solution, pH 1.3–1.6 [1].

Undoubtedly the most investigated fixative for its mechanism of action is formaldehyde. First discovered in 1859, its use in pathological applications was characterised by the work of Ferdinand Blum (for review see [5]). Formaldehyde is a small molecule (MW = 30) existing as a gas, which is commonly in the form of a 37% formaldehyde solution created by bubbling formaldehyde gas through water until saturation point. Its most common form in histological laboratories is as a 10% solution, thus ~4% formaldehyde, either diluted in water (originally termed formalin) or in a buffered solution (termed neutral buffered formalin or NBF). However, while the principle component of formaldehyde solution is formaldehyde, the researcher should be aware that oxidisation of formaldehyde will produce an unknown amount of formic acid, hence the reason why unbuffered formalin is acidic, and that 10–15% methanol may be present in the solution and act as a stabilising agent.

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Table 1
Details of the breakdown of the different fixatives.

Fixative	Method of fixation	Contents
B5	Denaturing	5.4% Mercuric Chloride (w/v), 1.1% Sodium Acetate (w/v), 4% Formaldehyde (v/v), Water
Bouin's	Denaturing, cross-linking	25% of 37% formaldehyde solution, 70% picric acid, 5% acetic acid
Carnoy's	Denaturing	60% ethanol, 30% chloroform, 10% Glacial acetic acid
Glutaraldehyde	Cross-linking	Generally, 2% v/v of glutaraldehyde to water/PBS
Methacarn	Denaturing	60% methanol, 30% chloroform, 10% Glacial acetic acid
Neutral buffered formalin (NBF)	Cross-linking	10% of 37% formaldehyde solution, in a neutral pH
Paraformaldehyde (PFA)	Cross-linking	Generally, 4% w/v of paraformaldehyde to Water/PBS
Zenker's	Denaturing	5% Mercuric Chloride (w/v), 2.5% Potassium Dichromate (w/v), 5% Glacial acetic acid (v/v), Water

The active ingredient in any formaldehyde solution is methylene glycol, the hydrated form of formaldehyde and the two chemicals co-exist within the solution in an equilibrium favouring methylene glycol. It has been proposed that the known paradox between the rate of penetration of formaldehyde and its rate of fixation may be due to the fast penetration speed of methylene glycol and the slow fixation rate of formaldehyde [5].

The mechanism of action of formaldehyde has been researched extensively and occurs through the formation of intra and intermolecular cross-links. The principal cross-links occur between side chain amino group of lysine which over time results in the formation of methylene bridges [6]. However, cross-linking can also occur between the aminomethylol groups and phenol, indole and imidazole side chains by a form of the Mannich reaction [7]. Consequently, the variety of amino acids affected by formaldehyde includes lysine, arginine, tyrosine, asparagine, histidine, glutamine and serine [8]. Through work by Rait et al. [9] and Mason & O'Leary [10], who have modelled formaldehyde fixation using ribonuclease A, it has been determine that the secondary and tertiary structure of proteins are unaffected, and thus preserved, by formaldehyde fixation. Moreover, that structure can be revealed, and the activity of the enzyme recovered, by heating [9]. Following this work, Sompuram et al. [11] have sought to classify proteins, based on their staining using immunohistochemistry following fixation of a small peptide with formaldehyde vapour. Using this model, peptides fall into three groups, based on (1) the presence of tyrosine at the antibody-binding site and an arginine elsewhere, (2) a tyrosine, but no arginine and (3) no tyrosine present.

A final consideration applying to all fixatives is the rate of penetration, temperature and length of time in fixative, which are all interlinked and following from this, the tissue processing method. Medawar [12] initially proposed the formula $d = K\sqrt{t}$, where d is depth, K is the coefficient of diffusion and t is time. As the coefficient of diffusion is different for each fixative, different fixatives and chemical mixtures will have different properties, as demonstrated by Dempster [13] where acetic acid shows the quickest penetration followed by formaldehyde. As noted above, this does not necessarily reflect the rate of fixation which will proceed at a slower rate and may reflect the often seen observation of differential staining on the outside of a large specimen, compared to the middle. A general rule of thumb to apply for penetration is 1 mm/h and a fixation time of 24 h is recommended for NBF-fixed specimens.

The temperature at which the sample in fixative is stored is relevant since basic chemical principles dictate that the speed of any reaction can be increased with heat, and consequently slowed when chilled. This can be demonstrated experimentally, as Fox et al. [5] showed that tissue sections fixed in formaldehyde reached equilibrium in 24 h at 25 °C and <18 h at 37 °C while Sompuram demonstrated loss of antigenicity, as a model for fixation, in peptides fixed at 42 °C compared to room temperature [11]. However, as fixation in a clinical environment is often through the processing unit being used, the method of processing is also important. In support of heating as a method for speeding tissue penetration and fixation, the introduction of automated microwave tissue processing has shown that using microwaves can shorten processing times without any effect on morphology or other downstream processes [14,15] presumably through heating. However, heating may not necessarily be required, as ultrasound methodologies have also been demonstrated to shorten fixation times, but heating is not implicated [16].

2. Molecular fixatives

It has been known for many years that formaldehyde fixation represents not only a biohazard for the laboratory [17] but also limits the quality of RNA & DNA available for extraction from the block. With the increasing use of molecular testing in the clinical arena, this limitation could be a severe restriction to the use of these tests. Thus, a number of "molecular fixatives" have been created and applied to histology with the aim to replace formaldehyde for experimental or health reasons.

The majority of molecular fixatives use alcohol or acetone as a base solution, with the addition of other stabilising agents to overcome the well-documented shrinkage effects of alcohol in a straight-forward replacement of formalin in the tissue processing procedure. However, in some cases, the procedure requires modification, for example, the HOPE technique, first introduced in 2001, which consists of Hepes-glutamic acid-buffer mediated Organic solvent Protection Effect combined with an acetone fixation step [18]. It is claimed that the amino acid components of the HOPE solution protect the tissue from the deleterious effects of the acetone. In other cases, specialised equipment is recommended to maximise the efficiency of the fixative. For example, UMFIX (Universal Molecular FIXative) first published in 2003 [19], which has been used extensively in the Department of Pathology, University of Miami. It is methanol-based with the addition of polyethylene glycol and is most effective in combination with a rapid tissue processing system [20]. Finally, not all solutions include conventional denaturing liquids. Z7 [21], and similar Zinc-based buffers such as ZBF [22,23], are a mixture of Zinc salts, thus are inexpensive to make, non-toxic and should slot into established processing protocols.

3. Effect of molecular fixatives on downstream processes

3.1. Morphology

The preservation of morphology is a central tenet of histopathology and therefore it follows that the manufacturers of the commercially-prepared molecular fixatives ensure that the morphological criteria of the staining is similar to formaldehyde fixation. However, some subtle differences do occur, which are principally associated with the ethanolic base of the fixatives used [19,24,25]. It has been reported that Paxgene-fixed tissue [26] increases eosinophilia, but not sufficiently to limit diagnosis. Shrinkage of tissues and swelling or lysing of erythrocytes has been