Methods in Pathology

Histology without formalin?
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Abstract
Because formalin is toxic, carcinogenic, and a poor preserver of nucleic acids, for more than 20 years, there have been numerous attempts to find a substitute, with as many different alternative fixatives, none totally successful. With a fast penetration, formaldehyde is a slow and reversible fixative that requires 24 to 48 hours to completely bind to tissue; thus, any surgical specimen arriving to the laboratory between 8 AM and 4 PM and processed conventionally for the slides to be ready the following day will be only between 30% and 66% bound and even less fixed when the dehydration starts, resulting in an additional and also incomplete alcoholic fixation. This causes infiltration problems and can affect subsequent tests, especially immunohistochemistry. Formaldehyde fixation is tissue thickness independent between 16 µm and 4 mm but is faster at above room temperature, so the fixation of specimens with less than 24 hours in formalin can be improved if the fixing stations in the conventional tissue processors are set at 40°C. If the safety measures are improved to offer a work environment with a time weighted average level of 0.4 ppm, and the contact with formalin is reduced to a minimum by discouraging its neutralization and limiting the recycling practice to filtering methods, formalin could remain as the routine fixative, with modified methacarn for those specimens requiring nucleic acids studies. This is a preferred solution than having to validate all the standard and special procedures, including those US Food and Drug Administration approved, if formalin is replaced by another fixative without its advantages. To the question posed in the title of this article, the answer is “Yes, it can be done, but that is neither likely nor worth it!”
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Keywords: Carcinogenic; Fixation rate; Microwave irradiation; Methacarn

1. The many uses of formaldehyde

During a 1972/74 survey, only 80 years after formaldehyde began to be produced on an industrial scale [1], the National Institute for Occupational Safety and Health estimated that 1.6 million workers in the United States were exposed to formaldehyde in more than 60 industrial categories [2]. Of those workers, about 4% were exposed for 4 or more hours daily, one third were engaged in medical and other health services, and another third worked in chemical and allied industries, printing and publishing, paper and allied industries, machinery, retail, automotive dealers and service stations, funeral homes and crematoria, photographic studies, garment industry, and dry-cleaning plants.

Formaldehyde, usually in the form of a white hydrated solid polymer consisting of 80 to 100 methanal units (polyoxymethylene) called paraform or paraformaldehyde, is used in the manufacture of adhesives, in animal nutrition and agriculture, cosmetics, deodorants, detergents, dyes, explosives, fertilizers, fiberboard, filters, foam insulations, food, friction materials, fuels, fungicides, home building materials, insulation mats, laminates, leather, paints, paper, particle board, pharmaceuticals, phenolic plastics (such as Bakelite), plywood, resins (accounting for more than 50% of its consumption), rubber, surface coatings, textiles, urethane resins, and water softening chemicals [2], making it a substance easy to come in contact with at different concentration levels and environments.

Formaldehyde is also present in the atmosphere as results of incomplete combustion during fires or from hydrocarbon fuels especially from vehicle emissions. The amounts in the air vary from 0.2 to 14 µg/m³ (ppb) and is 4 times higher in the air in manufactured than in conventional homes [3].

During the years, evidence began to mount regarding the health risks posed by formaldehyde exposure, which led to
2. Formaldehyde in the practice of histology

The arrival of formaldehyde in the practice of histology during the last decade of the 19th century, after many fundamental discoveries in microscopic anatomy had already been made, may be regarded as a fortuitous corollary of its first uses as an antiseptic in a 1:10 dilution of the formaldehyde concentrate (37%-40%) aqueous solution manufactured in Germany starting in 1891 under the trade names of Formalin or Formol.

It turned out that some tissue sections prepared after fixation in 4% formaldehyde had a better appearance and staining qualities than with the usual alcoholic fixatives prevalent in histology at that moment. The pathologist Karl Weigert realized the better quality of the sections in 1893, and “the rest is history”; formalin became the fixative of choice in just a few years [1].

Formaldehyde was discovered in 1859 by the Russian chemist Alexander M. Butlerov [1], but from a total of 13 fixatives formulas published between 1841 and 1879, none included formalin; however, between 1880 and 1899, it appeared in 25 of 159 fixative formulas, and the number increased to 192 of 413 new formulas published between 1900 and 1954, for a grand total of 217 different fixatives containing formalin out of 585 created between 1841 and 1954 [14].

Even when other reagents kept appearing in more fixative formulas, formalin became the standard fixative for routine work, and even today, it is the fixative of choice in 81% of US histology laboratories, in almost all laboratories in the UK and in close to two thirds in the rest of the world [15], usually as neutral buffered formalin (NBF), which is a 10% solution of the concentrated formalin, buffered at pH 7 with phosphate salts [16] to prevent its acidification when the aldehyde (methanal) oxidizes into acid (methanoic or formic acid) that can lead to the formation of “formalin pigment” in tissues [17] or to a slower fixation rate [20].

Formaldehyde, the simplest aldehyde (methanal), is obtained by catalytic oxidation of the simplest alcohol (methanol), and, being highly reactive, it is bubbled through water to obtain a concentrated solution (37%-50% wt/wt) to which 7% to 15% methanol is added as a stabilizer to retard its polymerization. Because of its reactivity, formaldehyde reacts with the water and becomes methanediol (methylene glycol), so much so that free methanol (formaldehyde) is present in its solutions at a concentration of 0.1% only [17,19]. The German trade name Formalin was the one selected by the American chemical companies producing the concentrated solution [1], and the name remains in common use.

3. Tissue fixation with formalin: penetration, binding, and cross-linking

Fixation with formaldehyde is a complex process including a very rapid penetration that stops autolysis, followed by covalent bonding and cross-linking, the 3 happening simultaneously but at very different rates [20,21], penetration being about 12 times faster than binding, which in turn is about 4 times faster than cross-linking. This 3-step process is the fundamental peculiarity of this “universal” fixative.

Also, formaldehyde (methanal) in the fixing solution (NBF) is present in hydrated form (methylene glycol) and has to be regenerated before starting binding and cross-linking with the proteins [1].

The penetration rate of any fixative is governed by the laws of diffusion [17] where the penetration distance (d in millimeters) is a function of a coefficient of diffusibility (the Medawar constant k) times the square root of the time (t) the tissues are in the fixative: d = k√t.

Depending on the subject used for its determination, there are several values of k for formaldehyde: 0.78 for liver, 3.6 for gelatin albumen, and 5.5 for plasma clots [1,17], but it is
thought to be less than 3.6 because of natural barriers [17], such as cell membranes or the resistance of lipids, which explains why specimens such as colon or breasts require more time to be completely fixed.

Confronted with that range and because \( k \) values calculated in gel are from 2.3 to 4.6 times larger than in tissues [17], the author decided to use the value determined for liver \( (k = 0.78) \), but because tissues are penetrated simultaneously from at least 2 surfaces, the penetration rates for slices of tissue of different thicknesses (Table 1) were calculated as follows:

\[
d = 2k \sqrt{t} = 1.56 \sqrt{t}.
\]

In Table 1, the penetration is presented as a percentage of the tissue thickness from 1 to 5 mm, and it can be seen that an “average” slice of tissue, usually 3 mm thick, will be completely penetrated in 4 hours after being placed in the NBF.

Another completely different issue is the binding or covalent bonds formation rate that precedes the actual cross-linking (fixation). The binding step was studied with radioactive carbon \(^{14}\)C labeled formaldehyde [1] using 16-\( \mu \)m frozen sections of rat kidney at 2 different temperatures \((25^\circ C\) and \(37^\circ C\)), and it was found that at \(25^\circ C\), the radioactivity plateau was reached in 24 hours with an additional 44% of binding taking place between 24 and 40 hours of exposure to formalin, when the total count leveled to the 48 hours maximum value of 65 cpm \( \times 1000\).

For \(37^\circ C\), the plateau was reached in 18 hours, with the same count level \((80 \text{ cpm} \times 1000)\) up to 48 hours of exposure. These findings indicate that the ideal temperature for formaldehyde covalent binding seems to be \(37^\circ C\) because no additional binding was observed after reaching an initial maximum value and because the radioactivity counts at \(37^\circ C\) are between 1.8 and 2.5 times the counts at \(25^\circ C\) at any hour of exposure before reaching equilibrium and 1.3 times larger after that point.

Using the available data [1], we calculated the percentage of total covalent binding for different periods at \(25^\circ C\) and \(37^\circ C\). The data show that, for example, even when a piece of 4-mm-thick tissue is completely penetrated in 4 hours, at that moment, it is only 20% bound and even less cross-linked (fixed).

The percentage binding per fixation period at \(37^\circ C\) is between 1.3 and 2.3 times larger than at \(25^\circ C\), similar to the count ratios at these 2 temperatures between 2 and 48 hours of exposure to formalin.

This only partial cross-linking of the proteins implies that the fixation will be completed by the dehydrating alcohols when the tissues start being processed, ending with a mixture of 2 very different fixing agents in their mode of action that can impair many immunohistochemical (IHC) methods because neither one reacted in a complete manner [19].

A piece of tissue will be 50% bound in 10.5 hours at \(25^\circ C\) and in 5.3 hours at \(37^\circ C\), meaning that the minimum of 6 to 8 hours of fixation time recommended by the American Society of Clinical Oncology—College of American Pathologists (ASCO-CAP) regarding breast fixation guidelines are not enough, because at 6 to 8 hours, the samples will be only 30% to 39% bound at \(25^\circ C\) and 56% to 68% at \(37^\circ C\) (Fig. 1).

The ASCO-CAP recommendation of a maximum time of 48 hours seems correct because then is when the tissues reach a 100% binding equilibrium at \(25^\circ C\) and is 100% sustained since the 18 hours of equilibrium binding at \(37^\circ C\) [1].

![Fig. 1. Transformation of Fig. 2 from Fox et al. [1] to percentages of equilibrium binding from 0 to 24 hours in formalin at 25°C and 37°C.](image)
Table 2
Relation of formaldehyde and glutaraldehyde binding times to the thickness of the experimental objects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Thickness</th>
<th>50% binding</th>
<th>Binding plateau</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide “spots”</td>
<td>Peptide molecules with “zero” thickness</td>
<td>&gt;6 h</td>
<td>14C-containing formaldehyde [23]</td>
<td></td>
</tr>
<tr>
<td>Rat kidney</td>
<td>16-μm frozen sections</td>
<td>11 h at 25°C</td>
<td>24 h at 25°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.5 h at 37°C</td>
<td>18 h at 37°C</td>
<td></td>
</tr>
<tr>
<td>Rabbit liver</td>
<td>4-mm cubes</td>
<td>ca. 2 h</td>
<td>25 h at 25°C</td>
<td>14C-containing formaldehyde [20]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>20 h</td>
<td></td>
</tr>
<tr>
<td>Rabbit brain and kidney</td>
<td>8-mm slices</td>
<td>4 h</td>
<td>50 h</td>
<td>3H-buffered glutaraldehyde [24]</td>
</tr>
</tbody>
</table>

* Plateau reached faster at 37°C than at 25°C, and at pH 7 than at pH 4.

In a study for the detection of the nuclear proliferation-associated antigen Ki-67 after different fixation times [22], the best results were found in tissues fixed between 24 and 48 hours, when compared with others fixed for 4 hours.

4. Fixation rate and specimen thickness

Using an antigen-antibody reaction [23], radioactive carbon (14C)-labeled formaldehyde [1,20] and tritium (3H)-labeled glutaraldehyde [21,24] specimens of thickness ranging from “zero thickness” peptide spots [23] to 8-mm-thick tissue slices [21] have been studied (Table 2), and evidence indicates that there is no effect of the specimen thickness on the binding rate between 16 μm and 4 mm, but that it is slower with 8-mm-thick tissue slices. This finding goes against the common belief that the thinner the pieces of tissue are, the faster they are fixed.

The binding plateau was reached in 24 hours for the 16-μm frozen sections and in 25 hours for the rabbit liver 4-mm cubes, both at 25°C with similar methodologies [1,20], and again, binding was faster at 37°C and, additionally, faster at pH 7 than at pH 4 [20].

The experiment with 4-mm liver cubes exposed to 3H-glutaraldehyde [24] showed a binding plateau in 20 hours, similar to the one for 14C-formaldehyde [20] (25 hours) with the same type of experimental subject. It seems that glutaraldehyde, a much larger molecule but with 2 aldehyde groups (1,5-pentane dial), reacts at a similar rate than the much smaller formaldehyde.

5. Fixation rate and temperature

Data show [1] that binding rates are greater at 37°C than at 25°C, and this could justify the common practice of heating tissues up to 60°C to accelerate fixation [17] or even placing tissues in boiling formalin before freezing with CO2 as was common practice before the advent of the cryostat [14], but those high temperatures fix the tissues by heat coagulation rather than by accelerating the formaldehyde cross-linking.

Increasing the formaldehyde fixation temperature to 50°C ± 5°C using microwaves (MWs) has been advocated for some years for both light [25-27] and transmission electron microscopy [28] as a means to accelerate fixation, but the actual effect is still the subject of some discussion and requires the tissues to be completely penetrated before being exposed to the MW action [29].

In the absence of MW irradiation, tissue processors with heat capabilities should have the fixation stations set at 40°C, with agitation to contribute to the fixation of those tissues with less fixation time than the 24 to 48 hours required [30].

6. The quest for formalin substitutes

The quest for formalin substitutes has been motivated by 2 fundamental developments: the OSHA regulation standard declaring it hazardous and advocating its substitution with less dangerous chemicals [5] and the fact that formalin does not assure a complete DNA and messenger RNA (mRNA) recovery, essential to many tests of molecular biology now under continuous development [27].

Under those circumstances and the large potential market involved for any successful substitute, many chemical companies and private individuals alike have developed many new fixatives all aimed at substituting formalin.

The alternate fixatives are alcoholic fixatives, fixatives for nucleic acids, nonalcoholic substitutes, and fixatives with less than 10% of formalin.

7. Alcoholic formalin substitutes

All alcoholic fixatives act by coagulation and do not mask antigenic sites not requiring heat-induced epitope retrieval (HIER) as formalin does when performing IHC tests [27].

Many of the alcoholic fixatives (Table 3) are of secret nature, either in their components or in their formulation, and all are more expensive than formalin.

Three of them (Boon-Fix, Fix All, and Uni-Fix) have not been evaluated independently, and Neo-fix substitutes both Kryo Fix and Micro Fix. The tests with F 13 were limited to morphological comparisons with formalin that, for all the potential substitutes, has been the “gold standard.”

The benefits of Fine Fix claimed by the manufacturer were not sustained by an independent test [18], which ranks it worse than NOTOXhisto that is a nonalcoholic dialdehyde–containing fixative.

Omni Fix is the most studied with 8 independent evaluations [23,31,34,43-47], many with totally contradictory morphology results and usually poor nucleotides preservation.
Table 3
Alcoholic formalin substitutes

<table>
<thead>
<tr>
<th>Substitute</th>
<th>Components</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boon-Fix</td>
<td>Ethanol, PEG200, acetic acid</td>
<td>Good for IHC (no HIER required) preserves DNA 1.8 times more than F and 1.3 times more than Kryo Fix after 24 h of fixation [27] (no independent evaluation)</td>
</tr>
<tr>
<td>EthMeth</td>
<td>95% ethanol and methanol (3:1)</td>
<td>Better morphology and DNA preservation than F [31]</td>
</tr>
<tr>
<td>F13</td>
<td>Ethanol, methanol, PEG300, water</td>
<td>The one formula out of 15 tested [32] with better results as compared with F (no other information available)</td>
</tr>
<tr>
<td>Fix All</td>
<td>Alcohol base</td>
<td>Good for IHC, DNA, and mRNA (claim by manufacturer). Can be used with MWO</td>
</tr>
<tr>
<td>Fine Fix</td>
<td>Patented formula diluted with ethanol to a 70% concentration</td>
<td>Worse than NOTOXhisto [18]</td>
</tr>
<tr>
<td>Kryo Fix (discontinued)</td>
<td>Ethanol, PEG300, water</td>
<td>Good for IHC [25], no effect after prolonged fixation [33]. Worse morphology than F [34]</td>
</tr>
<tr>
<td>Methacarn</td>
<td>Methanol, acetic acid, with or without chloroform (modified formula)</td>
<td>Morphology similar [35] or better than F [36], not recommended for most surface antigens [37]. Good for IHC, DNA, and mRNA [28,35,38,39]; better morphology and similar mRNA preservation than UMFix [36] (this claim has been challenged [40]) (replaced Kryo Fix, but is no longer available)</td>
</tr>
<tr>
<td>Micro Fix</td>
<td>Ethanol, PEG, water</td>
<td>Detects β-galactosidase activity better than F [41], morphology not better than with zinc F [42] (replaces Kryo Fix)</td>
</tr>
<tr>
<td>Neo-fix</td>
<td>1,2-Propanediol, ethanol, polyvinyl alcohol, water</td>
<td>From better morphology after 48 h of fixation [43] to 80% [31] or 89% [44] to worse quality than F [34]. From no good preservation of DNA and RNA [43] or yielding less amount [23,31,45] to yielding larger DNA fragments than with F [46] or less ISH staining intensity than with F [47]</td>
</tr>
<tr>
<td>Omni Fix (II and 2000)</td>
<td>Ethanol, ethylene glycol, sodium and zinc salts, acetic acid, water</td>
<td></td>
</tr>
<tr>
<td>RCL 2</td>
<td>Ethanol, acetic acid, complex carbohydrate</td>
<td>Morphology and penetration similar to F, needs less HIER; larger protein yield than F; good fixative [35]</td>
</tr>
<tr>
<td>UMFix</td>
<td>Propanol, acetone, PEG, DMSO, acetic acid</td>
<td>Morphology 85% of F [36] to similar to F [48], good for IHC (70 antibodies tested), better DNA and RNA preservation than F [48,49] (no other information available)</td>
</tr>
<tr>
<td>Uni-Fix</td>
<td>Ethanol, 2-propanol, methanol, glycerol, polyvinyl alcohol, water</td>
<td></td>
</tr>
</tbody>
</table>

F indicates formalin; MWO, MW over; PEG, polyethylene glycol.

The 2 other proprietary fixatives (RCL 2 and UMFix) are considered good for both morphology and nucleic acids preservation [48,49], but both have been ranked similar to methacarn [35,36]. Also, a simple fixative that can be prepared fresh in the laboratory (95% ethanol and methanol at a 3:1 proportion) produces better morphology and DNA preservation than formalin [31].

Finally, the one with better ranking is methacarn with 6 independent evaluations [28,35,39], although its superiority to UMFix has been challenged by the developers of the latter [40].

Carnoy’s fixative (from 1887) is a mixture of 100% or 95% ethanol (6 parts), chloroform (3 parts), and acetic acid (1 part) and is the choice when nuclear fixation is especially required [16]. When ethanol in the formula is substituted with methanol (6 parts), the fixative is called methacarn and is a good fixative but not recommended for most surface antigens [37].

When chloroform is eliminated and the formula becomes 100% ethanol (3 parts) and acetic acid (1 part), it is called “modified Carnoy’s fixative” or Clarke’s fluid [17].

The modified methacarn with better results [36] is made with 8 parts of methanol and one of acetic acid. All these components (ethanol, methanol, and acetic acid) have coefficients of diffusibility higher than formaldehyde (1.0, 1.5, and 1.2, respectively) [17] and assure an even faster penetration than with formalin.

8. Preservation of nucleic acids

There is information on nucleic acids preservation with 5 proprietary alcoholic substitutes, with 2 different ethanol concentrations, and for Carnoy’s and methacarn fixatives (Table 4).

One (Boon-Fix) has not been independently evaluated, Kryo Fix preserves DNA less than Boon-Fix [27], and both RCL 2 and UMFix present same results than with modified methacarn [35,36].

Both ethanol at 70% and Carnoy’s fixative have the same mRNA preservation [36] and better than with formalin [31], and absolute (100%) ethanol, although better than formalin, is worse than 70% ethanol [43].

If methacarn, modified or not, cheap and readily manufactured in the laboratory, is as good as or better for both general morphology and nucleic acids preservation than the 2 proprietary and expensive fixatives (RCL 2 and UMFix), the choice evidently favors methacarn.

9. Nonalcoholic formalin substitutes

Except for DMA and ILs, all others are dialdehyde-containing fixatives (Table 5), 6 containing the nonvapors
Table 4
Preservation of nucleic acids

<table>
<thead>
<tr>
<th>Fixative</th>
<th>DNA preservation</th>
<th>mRNA preservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bouin-Fix [27]</td>
<td>3.2 times more than with F, 1.2-2.0 times more than with Kryo Fix</td>
<td>Up to 4000 nucleotides (majority, 25-2000 nucleotides)</td>
</tr>
<tr>
<td>Carnoy [36]</td>
<td>More than with F, enough for PCR</td>
<td>Up to 4000 nucleotides (majority, 25-2000 nucleotides)</td>
</tr>
<tr>
<td>EthOL 70% [36]</td>
<td>More than with F, enough for PCR</td>
<td>More than with F in quality and quantity</td>
</tr>
<tr>
<td>EthOL 100% [47]</td>
<td>38% less than with F (with 4-6 h fixation)</td>
<td>41% less than with F (with 4-6 h fixation)</td>
</tr>
<tr>
<td>EthOL 100% [43]</td>
<td>+/- after 100 h of fixation with EthOL</td>
<td>+/- after 1 wk of fixation with EthOL</td>
</tr>
<tr>
<td>Methacarn [39]</td>
<td>+/- after 6 h of fixation with F</td>
<td>+/- after 48 h of fixation with EthOL</td>
</tr>
<tr>
<td>Methacarn [35]</td>
<td>Amount as in frozen tissue, the DNA is degraded in F</td>
<td>Enough mRNA for PCR</td>
</tr>
<tr>
<td>Modified</td>
<td></td>
<td>ER mRNA, PR mRNA, and mRNA amounts as in</td>
</tr>
<tr>
<td>methacarn [36]</td>
<td></td>
<td>frozen tissue</td>
</tr>
<tr>
<td>Omni Fix [47]</td>
<td>81% of ISH staining intensity with F (with 4-6 h of fixation)</td>
<td>70% of ISH staining intensity with F (with 4-6 h of fixation)</td>
</tr>
<tr>
<td>OmniFix [43]</td>
<td>+/- ISH staining after 48 h of fixation</td>
<td>+/- ISH staining after 1 wk fixation</td>
</tr>
<tr>
<td>Omni Fix [46]</td>
<td>Same base amplification as with F with up to 24 h of fixation, and more when fixed more than 24 h</td>
<td></td>
</tr>
<tr>
<td>RCL 2 [35]</td>
<td>Amounts similar to frozen tissue and more than with F</td>
<td>Similar to frozen tissue and equivalent to results with methacarn</td>
</tr>
<tr>
<td>UMFix [36]</td>
<td></td>
<td>Up to 6000 nucleotides (majority, 400-3000 nucleotides)</td>
</tr>
<tr>
<td>UMFix [48,49]</td>
<td>Similar to frozen tissue contents</td>
<td>(same as with modified methacarn)</td>
</tr>
</tbody>
</table>

Methacarn: Carnoy prepared with methanol. ER indicates estrogen receptor methacarn; EthOL, ethanol; F, formalin; PCR, polymerase chain reaction; ISH, in situ hybridization; PR, progesterone receptor.

producing Glyoxal (ethanediol) that has been hailed as the best alternative to formalin [54].

Two of them (Prefer and Preserve) have not been independently evaluated, and 4 others (HistOCHOICE, HistoFix, Mirsky’s fixative, and NOTOXhisto) produce worse morphology than formalin [34,44].

Glyo-Fixx produces a morphology close to formalin [44] but, along with Prefer, require modifications of the IHC protocols, and the only one with better morphology than formalin and Prefer (Safe Fix II) has worse nucleic acids recovery than formalin [31].

Summing up, all Glyoxal-containing substitutes, although they eliminate the dangers posed by formalin, are no match to its morphological and preserving qualities and, except for Safe Fix II, have not been evaluated regarding the nucleic acids preservation, the second problem to be solved by the substitutes.

These facts leave the other 2 nonalcoholic fixatives of which DMS cross-links and insolubilizes proteins as

Table 5
Nonalcoholic formalin substitutes

<table>
<thead>
<tr>
<th>Substitute</th>
<th>Components</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMS</td>
<td>Diamideester</td>
<td>Produces cross-links. Insolubilize 92% of proteins (similar to glutaraldehyde), for TEM and light microscopy [50]</td>
</tr>
<tr>
<td>Glyo-Fixx</td>
<td>10%-25% Glyoxal sol., 2-propanol, methanol or ethanol (5%-10%), acetic acid (&lt;5%)</td>
<td>Aldehyde type fixation, 94% of F quality [44]</td>
</tr>
<tr>
<td>HistOCHOICE</td>
<td>40% Glyoxal and 9 other components</td>
<td>Anecdotal data indicate image different to that of F, and IHC requiring adjustments</td>
</tr>
<tr>
<td>HistoFix</td>
<td>15%-26% Glyoxal sol., methanol, ethanol (5%-8%), acetic acid (1%-5%)</td>
<td>Unknown mode of action. Worse morphology than F [34] and quality significantly (P &lt; .001) worse than F [44]</td>
</tr>
<tr>
<td>IIs</td>
<td>Isonic liquid *</td>
<td>Better morphology than F, more IHC intense staining [51,52]</td>
</tr>
<tr>
<td>Mirsky’s fixed</td>
<td>“dialdehyde” at 35%-50% conc. (probably Glyoxal)</td>
<td>Causes irritation of the respiratory tract; very slow with poor preservation, similar IHC than F for some antigens [37], worse results than F [34]</td>
</tr>
<tr>
<td>NOTOXhisto</td>
<td>Complex dialdehyde in 70% ethanol</td>
<td>Combination of F and alcohol fixation patterns, good for IHC and general fixation [18,53], worse than F [34]</td>
</tr>
<tr>
<td>Prefer</td>
<td>Glyoxal in buffer and ethanol</td>
<td>Anecdotal data indicate that all IHC and HC protocols need to be modified. Similar to Glyo-Fixx (no independent evaluation)</td>
</tr>
<tr>
<td>Preserve</td>
<td>Glyoxal; ethanol</td>
<td>For MWO fixation, acid (pH 3.75-4.25) (no independent evaluation)</td>
</tr>
<tr>
<td>Safe Fix II</td>
<td>10% Glyoxal, ethanol, methanol, water</td>
<td>Better staining than F and Prefer, better morphology (1.4) and 88% of DNA recovery than with F [31]</td>
</tr>
</tbody>
</table>

* indicates formalin; HCl, histochemistry; MWO, MW oven; TEM, transmitted electron microscopy; IHC, immunohistochemistry.

1-Methyl-3-octyl-oxymethyl-imidazolium-tetrafluoroborate.
glutaraldehyde does, but it has not been evaluated for nucleic acids preservation [50].

The other substitute (ILs), although producing better morphology and more intense IHC staining than formalin [51,52], has not been evaluated for nucleic acids preservation and, with a price tag for the basic material of €19.45/g, cannot compete costwise with NBF (at $3.25/gal).

In general, nonalcoholic formalin substitutes are less likely to replace formalin to solve the 2 fundamental reasons behind the need for its substitution, namely, safety and nucleic acid preservation for the new breed of molecular pathology tests.

10. Fixatives containing less than 10% formalin

Their lower formalin contents could qualify them as “less toxic” than the regular NBF, but 2 in Table 6 (Pen Fix and Stat Fix) have undisclosed amounts of formalin, and neither has been independently evaluated, with one (Pen Fix) having anecdotal reports of drying small biopsies, because of its alcoholic components.

IBF, alcoholic and with less than 3% formalin, has been reported as producing excellent morphology in prostate biopsies [55], and STF has been evaluated as producing from better [31], to comparable [44], to worse [34] morphology than formalin, which is, to say the least, conflicting. It seems that its main problems are with routine hematoxylin and eosin (H&E) and trichrome parasite staining [56]. With undisclosed amounts of formalin and other components, its unknown mode of action, and the associated quality problems it presented, it is no longer available.

Evidently, the 4 commercial fixatives with less than 10% formalin in Table 6 are not good alternatives to NBF.

At this juncture, it is necessary to take a hard look at formalin and what would it mean to find a substitute because, before changing course, we need to know where we stand.

Table 6

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Components</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBF</td>
<td>&lt;3% formaldehyde, methanol, 2-propanol, barium chloride</td>
<td>Excellent cell morphology in prostate biopsies [55]</td>
</tr>
<tr>
<td>Pen Fix</td>
<td>&lt;10% formaldehyde, methanol, ethanol, 2-propanol</td>
<td>Anecdotal reports of overfixing and drying small biopsies (no independent evaluation)</td>
</tr>
<tr>
<td>Stat Fix</td>
<td>Undisclosed small amount of formaldehyde, ethanol, PEG, water, with or without acetic acid</td>
<td>Unknown mode of action. Anecdotal reports of tissue softer than with F, nice IHC, poor H&amp;E, worse trichrome parasites staining [56]. Also better morphology than with F [31] to 92% of the quality with F [44] to worse than with F [34]</td>
</tr>
<tr>
<td>STF</td>
<td>Undisclosed small amount of formaldehyde and 4 other components in proprietary amounts (no longer available)</td>
<td></td>
</tr>
</tbody>
</table>

F indicates formalin; PEG, polyethylene glycol.

11. Advantages of formalin

The fundamental advantages stem from its continuous and almost universal use for at least 100 years and all the accumulated scientific knowledge on it. The formalin-fixed paraffin-embedded tissue (FFPET) stained with H&E is the “gold standard,” and it has been said [57] that there is no other histopathology technique that provides so much information so quickly and for such little cost.

This long-term use has made it the fixative of choice for almost all histotechniques; all antibody manufacturers have optimized their products for FFPET, and even the US Food and Drug Administration (FDA) has approved some procedures only for this type of processed specimens.

Formalin preserves the secondary structure of proteins [58], insolubilizing them in more than 90% [59], and even its cross-linking is reversible [1,19,22,60] using hypotonic buffers at 37°C for 2 days, as was done before the advent of the more recent HIER techniques using different pH buffers [30].

All blocks and slides archives, all the diagnostic images learnt during pathology training, and most of the scientific articles on histopathology are based on FFPET.

Also, formalin is readily available, cheap, fairly convenient to store, allows long-term storage, preserves lipids well, and has been accepted [61] as the closest thing there is to the perfect fixative, with no clear “all-purpose” alternative found to date.

12. Disadvantages of formalin

Formaldehyde, besides being a carcinogen, is a slow fixative requiring from 24 to 48 hours to completely bind, which determines that surgical cases arriving between 8 AM and 4 PM and processed conventionally to have the slides ready the next day will start dehydration when they are only between 30% and 66% bound and much less cross-linked with often dire consequences for the paraffin infiltration and the subsequent histology. Tissues in these conditions benefit if placed in the fixing stations of the tissue processors at above room temperature but never at more than 40°C to avoid heat denaturation [62].

Although the use of MW irradiation has been advocated to accelerate formaldehyde fixation rate, this practice has 2 fundamental disadvantages: the production for dangerous formaldehyde fumes requiring additional precautions to limit the exposure and evidence that this procedure causes thermal coagulation, rather than an increment in the formaldehyde diffusion or reaction rates [63].

Nucleic acids in the cells are closely associated with proteins, and formaldehyde will produce mRNA-protein and DNA-protein cross-links similar to those between proteins alone determining smaller free fragments the more time the tissues are in formalin [60], increasing the possibilities of fluorescent in situ hybridization of human epidermal growth factor receptor 2 (HER2) failures [22,64] and limiting its effectiveness as a fixative for the molecular tests using FFPET.
13. Advantages of an alcoholic formalin substitute

The documented characteristics of nonalcoholic substitutes (Table 5) eliminate this group as plausible substitution options, leaving only the group of alcoholic substitutes.

Alcohols are fast fixatives, but always produce some tissue shrinkage and hardening, act by coagulation of proteins, and collapse nucleic acids that substantially reverse to their original size when rehydrated; also, the inclusion of methanol and acetic acid in any fixing formula will allow the fixation of larger specimens. The tissue appearance will be different to that fixed with formalin, and because the dehydration will start simultaneously to the fixation, the processing protocols can be shorter, allowing a faster turn around time.

The data in Table 3 point to modified methacarn (8 parts of methanol and 1 part of acetic acid) [36] as the best option because it gives the best nucleic acid extraction and results in good general morphology.

14. Disadvantages of any formalin substitute

Any formalin substitute will have a common set of disadvantages especially concerning all the validation tests that will be required when changing from formalin to any substitute.

CAP guidelines (ANP22997) require simultaneously running at least 25 (ideally from 25 to 100) parallel samples with formalin and the substitute, and this validation refers to any procedural change, either in part or as a whole, for all the IHC and special procedures, and any FDA tests approved to be completed with FFPET, which, to a busy laboratory, is a daunting, costly, and discouraging perspective.

All the IHC procedures and antibodies working dilutions will also have to be validated; the whole set of controls used will have to be renewed; and if any block processed with the substitute has to be sent out for consultation, the validity of the results from the consultant becomes questionable.

In the case of a law suit, if the challenged results were obtained with a tissue fixed with a formalin substitute, it could increase the chances for a liability ruling against the laboratory and the pathologist, unless the changed validation process was accepted by CAP during a regular inspection.

These have been the challenges faced by 19% of US laboratories and 36% of laboratories of other countries [15] that fix their tissues with formalin substitutes, with some preference for alcoholic substitutes.

15. What to do?

It is the opinion of this author that, in spite of its health risks and its limited usefulness for nucleic acids preservation, the advantages of formalin, added to the intrinsic problems to be created by its substitution, outweigh its disadvantages. Formalin can be used safely, and when the diagnostic pressures result in more frequent and varied molecular diagnostic tests, it is sure that some special automated instruments for the efficient DNA and mRNA extraction using fresh tissue samples will be developed, and that those tests will be done not as part of the histopathology laboratory but as part of a molecular pathology section of the clinical laboratory.

16. Using formalin safely

Presently, 81% of US laboratories use NBF, but 15% do not monitor for its presence in the work areas, and the same percentage recycles it. The airflow is not tested in 13% of the laboratories, and 6% do not have formalin spills neutralizing substances [15], and all these deficiencies have to be eliminated.

The contact between personnel and formalin should be kept to a minimum, and although recycling can be a cost-effective practice, distillation not only is less efficient (80%-90% recovery), but also requires adding the buffer salts to the distillate increasing the exposure; so if

<table>
<thead>
<tr>
<th>Country</th>
<th>Name</th>
<th>Year</th>
<th>PEL (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TWA</td>
</tr>
<tr>
<td>Australia [66]</td>
<td>TLV</td>
<td>2007</td>
<td>1.0</td>
</tr>
<tr>
<td>Austria</td>
<td>MAK</td>
<td>2005</td>
<td>0.2</td>
</tr>
<tr>
<td>Belgium [67]</td>
<td>TLV</td>
<td>1989</td>
<td>1.2</td>
</tr>
<tr>
<td>Brazil</td>
<td>AL</td>
<td>1982</td>
<td>0.4</td>
</tr>
<tr>
<td>Canada [68] (federal)</td>
<td>ELV</td>
<td>2006</td>
<td>0.3</td>
</tr>
<tr>
<td>Canada (provinces)</td>
<td></td>
<td></td>
<td>0.3-1.0</td>
</tr>
<tr>
<td>Czechoslovakia [67]</td>
<td>MAC</td>
<td>1985</td>
<td>0.4</td>
</tr>
<tr>
<td>European community</td>
<td>OEL</td>
<td>2005</td>
<td>0.3-0.5</td>
</tr>
<tr>
<td>Finland [67]</td>
<td>MPC</td>
<td>1989</td>
<td>1.1</td>
</tr>
<tr>
<td>Germany [68]</td>
<td>MAK</td>
<td>2005</td>
<td>0.3</td>
</tr>
<tr>
<td>Hungary [67]</td>
<td>MAC</td>
<td>1985</td>
<td>0.8</td>
</tr>
<tr>
<td>Iceland [69]</td>
<td></td>
<td>1999</td>
<td>0.3</td>
</tr>
<tr>
<td>Italy</td>
<td>TLV</td>
<td>1985</td>
<td>1.0</td>
</tr>
<tr>
<td>Japan</td>
<td></td>
<td>2001</td>
<td>0.3</td>
</tr>
<tr>
<td>Netherlands [67]</td>
<td>MXL</td>
<td>1987</td>
<td>1.2</td>
</tr>
<tr>
<td>New Zealand</td>
<td>TLV</td>
<td>2007</td>
<td>1.0</td>
</tr>
<tr>
<td>Poland [67]</td>
<td>MPC</td>
<td>1985</td>
<td>1.6</td>
</tr>
<tr>
<td>Romania [67]</td>
<td>CLV</td>
<td>1985</td>
<td>3.2</td>
</tr>
<tr>
<td>Russia [70]</td>
<td>MAC</td>
<td>2003</td>
<td>0.4</td>
</tr>
<tr>
<td>Sweden [68]</td>
<td>HLV</td>
<td>2005</td>
<td>0.5</td>
</tr>
<tr>
<td>Switzerland [67]</td>
<td>MAK</td>
<td>1987</td>
<td>1.0</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>WEL</td>
<td>2002</td>
<td>1.6</td>
</tr>
<tr>
<td>United States</td>
<td>PEL</td>
<td>1998</td>
<td>0.75</td>
</tr>
<tr>
<td>Yugoslavia [67]*</td>
<td>MAC</td>
<td>1985</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Conversion factors: 1 ppm = 1.23 mg/m³ and 1 mg/m³ = 0.81 ppm (at 20°C). AL indicates allowable limit; CLV, ceiling limit value; ELV, exposure limit value; HLV, hygienic limit value; MAC, maximum allowable concentration; MAK, maximale Arbeitsplatz-konzentration (workplace concentration); MXL, maximum limit; MPC, maximum permmissible concentration; OEL, occupational exposure limit; TLV, threshold limit value; WFL, work exposure level. STEL, for 15 minutes; TWA, for 8 hours of exposure.

* Official name before 1990.
recycling is to be practiced, it should be by filtration that is 99% efficient and requires only a final pH adjustment [65].

Being a very hazardous substance to the environment, EPA regulations forbid the direct disposal of formalin into any sewer system, resulting that it has to be disposed off by specialized contractors at a very high cost or has to be “neutralized” in the laboratory with sometimes very expensive neutralizing agents (costing up to more than $70/gal), all causing additional personnel exposure. Opting to neutralize based on cost analysis only, without considering the additional exposure, is similar to putting a price on the personnel’s health and well-being.

The existing formaldehyde TWA level (0.75 ppm) places the US behind 9 other countries with lower limits, and our STEL (2.0 ppm) is behind 8 countries with only 4 having similar or higher values (Table 7).

Both US levels are 20 years old and are based on even older data, but it is almost hopeless to expect that they will be lowered any time soon. On the other hand, because any well-managed laboratory can operate with an average TWA of 0.3 ppm or less [14], it is not impossible for any one to implement all the required safety precautions and operate with a TWA level of at least 0.4 ppm, which is the value adopted by the European community in 2005 and the official limit for some countries, such as Brazil, the Czech Republic, and Russia (Table 7).

Another step that can be taken is to reduce the formaldehyde contents in the NBF from 10% to 8% as is practiced in some European laboratories.

To summarize, formalin can be used more safely than at present if laboratory safety is improved to allow an operation at a TWA level of 0.4 ppm or less, which will require additional personnel training. The formaldehyde contents in the NBF should be lowered to 8%, and if recycling is practiced, it should be by filtration methods, with all neutralization procedures discouraged.

17. Histology without formalin?

This is the title question and the answer is “Yes, it can be done, but it is neither likely nor necessary if adequate measures to work with formalin safely are implemented!”

Acknowledgments

The author thanks John A. Kieman (Department of Anatomy and Cell Biology, The University of Western Ontario, London, Canada), Bryan R. Hewlett, MLT ART (Retired Consultant, Hamilton, Ontario, Canada), Richard Edwards, BSc (University of Leicester, UK), and Hadi Yaziji, MD (President, Vitro Molecular Laboratories, Miami, Fla), for commenting on and reviewing a draft of the manuscript. Five colleagues (R. Edwards, M. Heimann, G. Lang, M. Peshkov, and A. Werno) provided information about PEL values from the UK, Iceland, Austria, Russia, and New Zealand, respectively.

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