CME/SAM

Formaldehyde Substitute Fixatives

Analysis of Macroscopy, Morphologic Analysis, and Immunohistochemical Analysis

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Upon completion of this activity you will be able to:
- list the advantages and disadvantages of formalin as a universal fixative.
- discuss the effects of alcohol-based fixatives on tissue morphology and immunohistochemistry.
- compare the effects and costs of crosslinking and non-crosslinking alcohol-based fixatives in pathology.

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Abstract

Because formaldehyde is toxic and creates cross-links that may hinder immunohistochemical studies, we tested 3 new cross-linking (F-Solv [Adamas, Rhenen, the Netherlands]) and non–cross-linking (FineFIX [Milestone, Bergamo, Italy] and RCL2 [Alphelys, Plaisir, France]) alcohol-based fixatives for routine staining in comparison with neutral buffered formalin (NBF) as the "gold standard." Fresh tissue samples were divided into 4 equal pieces and fixed in all fixatives for varying times. After paraffin embedding, H&E staining, 7 common histochemical stains, and 9 common immunohistochemical stains were performed. RCL2 fixation resulted in soft and slippery tissue, causing sectioning difficulties. F-Solv and FineFIX led to partial tissue disintegration during fixation. F-Solv performed morphologically similar to NBF but needed considerable protocol adjustments before being applicable in daily histologic and immunohistochemical practice. FineFIX did not necessitate major protocol changes but caused shrinkage artifacts, degranulation, and lysis of RBCs. RCL2 generated morphologically overall good results without major protocol changes but caused pigment deposition, degranulation, and RBC lysis. The alcohol-based fixatives had positive and negative attributes and environmental drawbacks, and none was overall comparable to NBF with regard to macroscopy, morphologic evaluation, and immunohistochemical studies.

An optimal fixative should be nontoxic and allow for detailed morphologic analysis, high-quality special histochemical and immunohistochemical staining, and good preservation of DNA and RNA at a reasonable price. Unfortunately, such a universal fixative does not exist, and it is important to assess the advantages and drawbacks of existing and new fixatives for each platform.

In surgical pathology, neutral buffered formalin (NBF, aqueous solution of 4% buffered formaldehyde) has been the "gold standard" fixative for decades. It is cheap, enables long-term storage of surgical material, preserves morphologic features well, allows special histologic stains, and, in combination with antigen retrieval, allows for reliable immunohistochemical analysis. However, formaldehyde was classified as "carcinogenic to humans" (group 1) by the International Agency for Research on Cancer[1] and, therefore, represents a risk to anyone handling the solution. Furthermore, its cross-linking masks antigens, which may hamper immunohistochemical analysis,[2-7] and fragments nucleic acids, which impairs the extraction efficiency and quality of DNA and RNA.[8-11]

Other less toxic alcohol-based cross-linking fixatives (such as the aldehyde-containing F-Solv [Adamas, Rhenen, the Netherlands]) and non–cross-linking fixatives (such as FineFIX [Milestone, Bergamo, Italy] and RCL2 [Alphelys, Plaisir, France]) have been proposed as NBF alternatives. Alcohol-based non–cross-linking fixatives exert their effect by protein precipitation. Reported advantages of this type of fixation include faster fixation; elimination of carcinogenic vapors; better preservation of glycogen, DNA, and RNA;

greater staining avidity; and no need for enzyme predigestion for immunohistochemical analysis. Disadvantages are variability of tissue staining, tissue shrinkage and hardening, artifactual pigment deposition in bloody specimens, partial or complete lysis of erythrocytes, and increased flammability.[12,13] Another component increasingly used in alternative fixatives is acetic acid (such as in RCL2). It complements the action of other ingredients such as alcohol, makes collagen fibers swell, precipitates nucleoprotein, and may have a solvent action on cytoplasmic granules.[14]

Use of formaldehyde substitutes has been restricted to a few laboratories, and publications about them are scarce. We report on the influence of F-Solv (cross-linking), FineFIX (non–cross-linking, non–acetic acid), and RCL2 (non–cross-linking, acetic acid) fixation on tissue morphologic features and the quality of special histologic stains and immunohistochemical stains in comparison with NBF as the gold standard.

Materials and Methods

Fixatives

All fixatives were stored and used at room temperature. NBF 4%, FineFIX, and RCL2 were prepared fresh from stock solutions before use according to the manufacturers' instructions. F-Solv was received in a ready-to-use form.

Specimens and Fixation

Fresh surgical tissue samples from placenta, liver, brain, esophagus, stomach, duodenum, colon, omentum, lung, breast, adrenal gland, kidney, lymph node, thyroid, tonsil, spleen, and gallbladder were collected at the Department of Pathology, University Medical Center Utrecht, Utrecht, the Netherlands. Procedures followed were in accord with the ethical standards established by the University Medical Center Utrecht. Each tissue sample was divided into 4 equal pieces and fixed in one of the fixatives for 24 hours (n = 16), 2 to 4 days (n = 11), 1 to 2 weeks (n = 5), or 1 to 2 months (n = 7).

To compare shorter and standard fixation times, 10 additional tissue samples were cut and divided into 8 pieces (4 for 4-hour fixation and 4 for 24-hour fixation). After fixation, tissues were dehydrated and paraffin impregnated using an automated Peloris Rapid Tissue Processor (Leica, Valkenswaard, the Netherlands). In short, the Peloris ran overnight starting with 3 dehydrating ethanol 70% baths (60°C for a total of 1 hour, 30 minutes), followed by two 80/20 ethanol/isopropyl alcohol (a xylene replacement) baths at 60°C for a total of 1 hour, 30 minutes), 3 isopropyl alcohol (100%) baths (at 60°C for 3 hours), and paraffin impregnation (2 baths at 85°C for 2 hours and 1 bath at 65°C for 40 minutes). After paraffin embedding, blocks were stored at room temperature.

H&E Staining and Histochemical and Immunohistochemical Studies

H&E Staining and Microscopic Evaluation

Paraffin sections were cut at 2 μm, mounted on glass slides, dried for at least 10 minutes on a hot plate, and processed with an HMS740 (for F-Solv-, FineFIX-, and RCL2-fixed tissue sections) and an HMS760 (for NBF-fixed tissue sections, along with diagnostics) Robot Stainer (Microm, Walldorf, Germany). H&E staining protocols for F-Solv, FineFIX, and RCL2 were adjusted to avoid bias of the pathologist in microscopic evaluation because staining according to the protocol for NBF specimens was generally darker. For F-Solv, the hematoxylin step was reduced from 2 minutes, 15 seconds to 1 minute, 15 seconds; eosin staining was reduced from 1 minute to 5 seconds; and the subsequent alcohol steps were increased from 2 minutes, 30 seconds to 4 minutes, 30 seconds. For FineFIX and RCL2, the hematoxylin step was also reduced from 2 minutes, 15 seconds to 1 minute, 15 seconds; eosin staining was reduced from 1 minute to 10 seconds; and the subsequent alcohol steps were increased from 2 minutes, 30 seconds to 3 minutes. All other steps were kept identical to those for the NBF protocol.

A blinded evaluation of H&E staining was carried out by an experienced pathologist (F.J.tK.). The physical quality of the sections (disruption, adhesion, cracking, and section thickness), the quality of tissue preservation (nucleus, cytoplasm, extracellular components, special tissue-specific features, and zonal fixation), and the quality of staining (uniformity, nuclear, cytoplasmic, and extracellular components or muscle) were separately evaluated. The staining quality for these aspects was graded as 0 (inadequate for diagnostics), 1 (quality reasonable for diagnostics, but adjustments in protocol needed), or 2 (quality good for diagnostics). For each fixative, a total percentage was calculated based on the ratio of the sum of all scores (given to different tissue samples) relative to the maximum score possible.

Histochemical Evaluation

Paraffin blocks were sectioned at 2 to 4 μm for staining with periodic acid–Schiff (PAS) without and with diastase (PASD), alcian blue, azan, elastin van Gieson (EvG), and Gordon-Sweet (G&S) and Jones silver stains. Alcian blue, PAS, and PASD stains were done in an HMS740 Robot Stainer. The other stains were done manually.

For alcian blue, slides were deparaffinized in xylene, rehydrated through graded ethanol, stained with alcian blue for 15 minutes, rinsed, stained with nuclear fast red for 10 minutes, rinsed again, and dehydrated through graded ethanol and xylene.

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For PAS, slides were deparaffinized in xylene, rehydrated through graded ethanol and incubated in periodic acid (1%) for 15 minutes. After a short rinsing step, slides were incubated in Schiff reagent for 30 minutes, rinsed, incubated in hemalaun for 8 minutes, rinsed again, and dehydrated to xylene.

For PASD, slides were deparaffinized up to 96% alcohol, then incubated for 5 minutes in a 1/1 mixture of 37% formalin/96% alcohol, rinsed thoroughly, and incubated in 0.15% diastase for 90 minutes at room temperature. After rinsing, slides were further processed as described for PAS.

For EvG, slides were deparaffinized and rehydrated up to 70% alcohol, incubated for 45 minutes in Lawson solution, differentiated in 100% and 96% alcohol, rinsed, stained with Mayer hemalaun for 5 minutes, rinsed for 10 minutes, incubated with van Gieson picrofuchsin for 5 minutes, and dehydrated in graded ethanol and xylene.

For G&S staining, slides were deparaffinized and rehydrated, oxidated in acidified potassium permanganate for 5 minutes, rinsed, bleached in 1% oxalic acid for 2 minutes until colorless, and rinsed and treated with 2% iron alum for 15 minutes. After rinsing in distilled water and impregnation with ammoniacal silver solution, slides were rinsed and reduced in 3.7% aqueous formalin, rinsed again, incubated in 0.1% gold chloride for 5 minutes, and fixed in 5% aqueous sodium thiosulfate for 5 minutes. Finally, slides were counterstained with nuclear fast red for 5 minutes and dehydrated.

For Jones staining, slides were deparaffinized and rehydrated, treated with 1% periodic acid for 15 minutes, rinsed, and incubated for 40 to 60 minutes in a methenamine silver working solution at 56°C. After rinsing, the slides were incubated in 0.1% gold chloride for 5 minutes and in 2% sodium thiosulfate for 5 minutes, followed by a 5-minute Mayer hemalaun stain and bluing. Finally, slides were stained with eosin and dehydrated.

For azan (Heidenhain), slides were deparaffinized and rehydrated, stained with azocarmine G for 5 minutes, quickly rinsed, and differentiated in aniline-ethanol. After rinsing in acetic acid–ethanol, slides were incubated in a 5% phosphotungstic acid solution for 15 minutes, rinsed, and stained with an aniline blue–orange G solution for 5 minutes before dehydration.

A blinded evaluation of the histologic stains was carried out by an experienced pathologist (F.J.tK.). Staining quality was graded as 0 (inadequate for diagnostics), 1 (quality reasonable for diagnostics, but adjustments in protocol needed), or 2 (quality good for diagnostics). For each fixative, a total percentage was calculated based on the ratio of the sum of all scores (given to different tissue samples) relative to the maximum score possible.

Immunohistochemical Evaluation

Paraffin sections were cut at 4 μm, mounted on silane-coated slides, dried for 10 minutes or more on a hot plate, and processed with a Bond-Max automated staining machine (Vision Biosystems, Newcastle, England) using the Bond polymer refine detection kit (catalog No. DS9800, Vision Biosystems), as previously described.[15] **Table 1** shows the 9 primary antibodies tested, sources, dilutions, and antigen retrieval methods. For each antibody, stains were done as usual for NBF, and 2 samples were tested with and without heat-induced antigen retrieval or pepsin pretreatment. A blinded evaluation of the immunostaining was carried out by an experienced pathologist (F.J.tK.). Staining quality was graded as 0 (inadequate for diagnostics), 1 (quality reasonable for diagnostics, but adjustments in protocol needed), or 2 (quality good for diagnostics). For each fixative, a total percentage was calculated based on the ratio of the sum of all scores (given to different tissue samples) relative to the maximum score possible.

Results

Macroscopy

All fixatives had different penetration speed and resulted in different tissue color and texture. Tissue color after F-Solv fixation was much darker than with NBF. In contrast, the

Table 1
Data for Primary Antibodies Used in Immunohistochemical Evaluation of Alcohol-Based Fixatives in Comparison With Neutral Buffered Formalin

	Source	Catalog No.	Clone	Antigen Retrieval	Dilution
CD45	DAKO, Glostrup, Denmark	M0701	2B11+PD7/26	Citrate	1/400
CAM5.2	BD Biosciences, San Jose, CA	345779	CAM5.2	Pepsin	1/80
Vimentin	DAKO	M0725	V9	Citrate	1/600
AE1/3	NeoMarkers, Fremont, CA	MS-343-P	AE1/AE3	Citrate	1/200
Chromogranin A	DAKO	A0430	—	EDTA	1/800
Estrogen receptor	DAKO	M7047	1D5	EDTA	1/80
Progesterone receptor	DAKO	M3569	PgR636	Citrate	1/100
p63	NeoMarkers	MS-1081-P	4A4	EDTA	1/400
S-100	DAKO	Z0311	—	Citrate	1/4,000

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non–aldehyde-fixed tissue specimens (FineFIX and RCL2) were paler than NBF-fixed specimens. In a case of cholestasis, NBF and RCL2 clearly showed green for the liver, but after F-Solv and FineFIX fixation, this color was barely visible.

Tissue fixed by F-Solv and FineFIX was more rigid, whereas tissue fixed in RCL2 was much softer and more slippery, causing difficulty in cutting. At standard fixation times, recognition of structures in different organs was good for all fixatives, although FineFIX and RCL2 showed some exceptions. After prolonged (2 months) fixation, structures became generally more difficult to recognize, especially after F-Solv fixation.

Penetration speed for FineFIX and RCL2 was similar but somewhat faster than NBF. However, for larger tissue samples, F-Solv fixation seemed incomplete in the center, even after 2 months' fixation. Lymph nodes were more difficult to detect in all alternative fixatives. F-Solv smells like melon, FineFIX is odorless, and RCL2 smells very acidic. Both of the odors were experienced as unpleasant. F-Solv and FineFIX but not RCL2 resulted in a considerable amount of tiny tissue fragments **Image 1** floating in the fixative solution.

Image 1 Example of FineFIX fixation resulting in a considerable amount of tiny tissue fragments floating within the fixative solution.

Table 2
Results of Microscopic Evaluation of 50 H&E-Stained Tissue Samples Fixed in Different Alcohol-Based Fixatives in Comparison With NBF*

	NBF	F-Solv	FineFIX	RCL2
Physical quality of the section	94	90	88	89
Quality of tissue preservation	89	82	77	82
Quality of staining	96	92	92	92
Total (average)	93	88	86	88

NBF, neutral buffered formalin,
* Values are given as percentages. Staining quality was graded as 0 (inadequate for diagnostics), 1 (quality reasonable for diagnostics, but adjustments in protocol needed), or 2 (quality good for diagnostics). For each fixative, a total percentage was calculated based on the ratio of the sum of all scores (given to different tissue samples) relative to the maximum score possible (50 tissues × maximum score 2/tissue = 100).

Morphologic Analysis

Table 2 shows the results of microscopic evaluation of 50 H&E-stained tissue sections. The physical quality of the sections (disruption, adhesion, cracking, and section thickness) was similar for all fixatives, with NBF showing the highest score. Quality of tissue preservation (nucleus, cytoplasm, extracellular components, special tissue-specific features, and zonal fixation) was highest for NBF and lowest for FineFIX. RCL2 and F-Solv performed equally. Quality of staining (uniformity, nuclear, cytoplasmic, and extracellular components or muscle) was good for all fixatives, with NBF showing the highest score. Overall, NBF performed best (93%), followed by F-Solv, RCL2 (both 88%), and FineFIX (86%).

As shown in **Image 2**, several important differences could be noted between NBF and the other fixatives. FineFIX and RCL2 fixation resulted in RBC lysis within 4 hours. Also, eosinophilic cytoplasmic granules (eg, in the Paneth cells of the intestine and granulocytes) were lost after fixation with FineFIX and RCL2, even when shorter fixation times were used. FineFIX and, to a lesser extent, RCL2 led to considerable amount of tissue shrinkage that did not increase with longer fixation. RCL2 (acidic, pH 3.10) often showed a brownish granular pigment comparable to the well-known formalin pigment, even at standard fixation times of 24 hours. Neutralization of RCL2 was fairly difficult (maximum pH 6.35) and resulted in loss of the pigment, but RBC lysis remained. On the other hand, RCL2 showed excellent nuclear detail, in contrast with NBF and F-Solv in most cases.

The effect of fixation time (4 hours vs 1-4 days vs 1-2 weeks vs 1-2 months) was limited for all fixatives. In almost all cases, NBF outperformed the other fixatives, regardless of fixation time. For small tissue pieces (1-2 cm^2), a very short fixation time (4 hours) was sufficient for all fixatives and morphologic features did not clearly differ from 24-hour fixation. F-Solv was most sensitive to longer fixation times (≥2 weeks).

Histochemical and Immunohistochemical Analyses

Histochemical Evaluation

PAS, PASD, azan, alcian blue, EvG, G&S, and Jones stains were scored 0 to 2 (0, insufficient; 1, intermediate; 2, optimal). NBF performed best with a total score of 100% as expected, followed by FineFIX (93%), RCL2 (89%), and F-Solv (57%). F-Solv was given 1 insufficient score for Jones stain in the kidney and scored intermediate for most other

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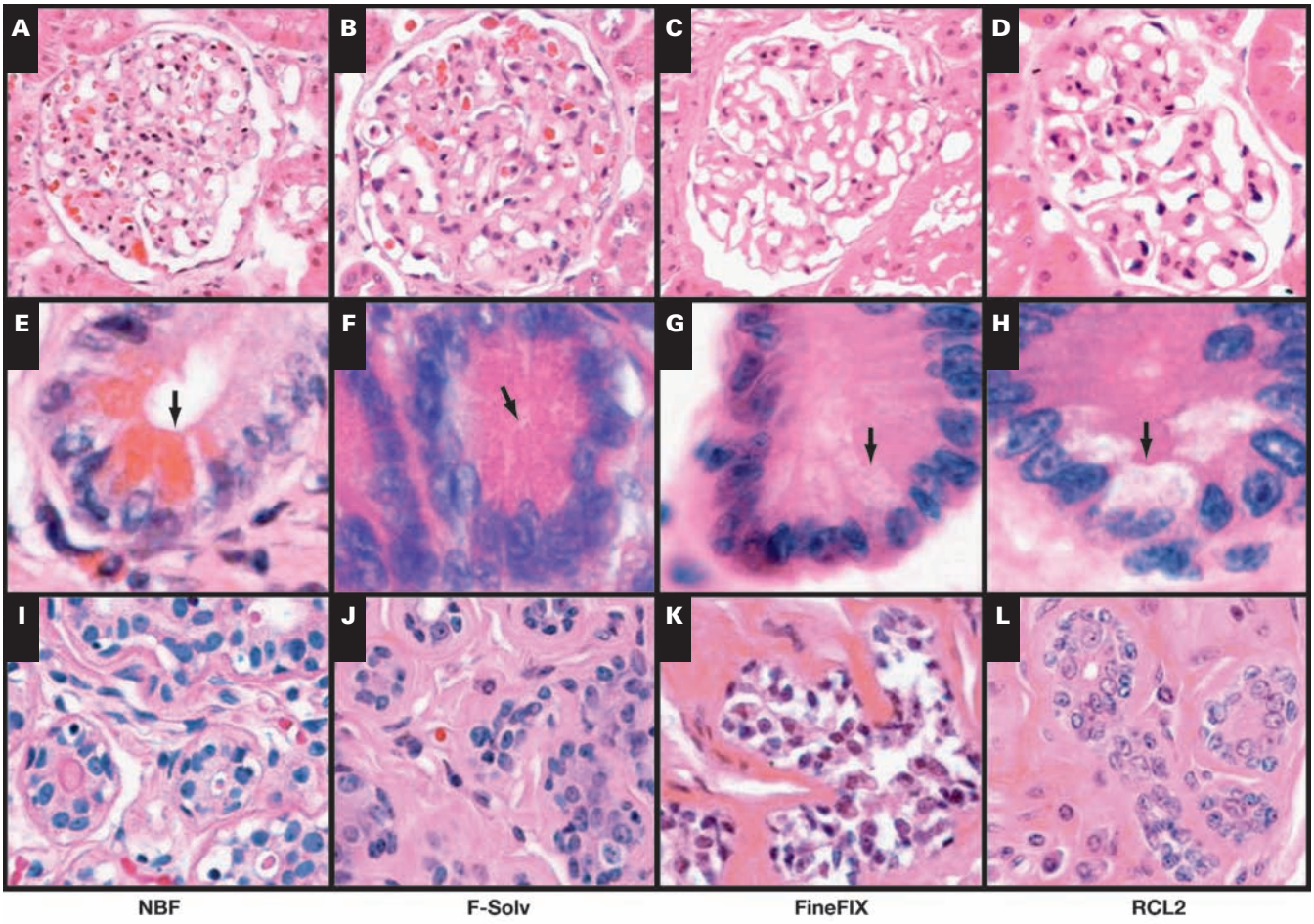

Image 2 H&E staining after neutral buffered formalin (NBF) fixation and fixation with 3 alternative fixatives (F-Solv, FineFIX, and RCL2). **A**-**D**, Kidney tissue sample. Note that erythrocytes are absent in **C** and **D** (**A**, ×5; **B**, ×5; **C**, ×5; **D**, ×5). **E**-**H**, Paneth cells in the small intestine. Note that **G** and **H** contain no granules (arrows) (**E**, ×40; **F**, ×40; **G**, ×40; **H**, ×40). **I**-**L**, Normal breast ducts. Note the shrinkage artifacts in **K** and the clear nuclear structure in **L** (**I**, ×20; **J**, ×20; **K**, ×20; **L**, ×20).

stains. **Image 3** shows representative examples for some of the stains.

Immunohistochemical Evaluation

Cytokeratin (CK) AE1/3, CAM5.2, CD45, estrogen receptor (ER), progesterone receptor, p63, chromogranin A, S-100, and vimentin were scored 0 to 2. NBF performed best with a total score of 100% as expected, followed by RCL2 (70% without pretreatment and 58% with pretreatment), FineFIX (68% and 60%, respectively), and F-Solv (60% and 53%, respectively).

Chromogranin A staining was optimal for all fixatives, with or without pretreatment. CD45, CK AE1/3, and ER stains were inadequate (score 0) for F-Solv, with or without pretreatment. (CD45 stained solely perivascularly, CK AE1/3 did not stain complete ducts but individual cells, and ER staining was not nuclear but cytoplasmic.) RCL2 and FineFIX also resulted in inadequate ER staining (cytoplasmic or too weak) regardless of pretreatment. None of the 3 alternatives reached optimal (but did reach intermediate) staining for progesterone receptor and p63 without further adjustments to the staining protocol other than omitting pretreatment. S-100 staining was suboptimal for all alternatives (especially FineFIX) in the colonic nerve plexi, but it performed generally better in a neurilemmoma in which optimal staining was obtained for F-Solv without pretreatment.

In most cases, when using these alternatives, pretreatment could be omitted. However, in some cases it was necessary to adjust the type of pretreatment. For example, F-Solv did not show any CK AE1/3 staining with the standard NBF protocol, but when citrate antigen retrieval was replaced by pepsin, the staining quality was equal to that of all other fixatives. **Image 4** illustrates the effect of pretreatment on CK AE1/3 staining.

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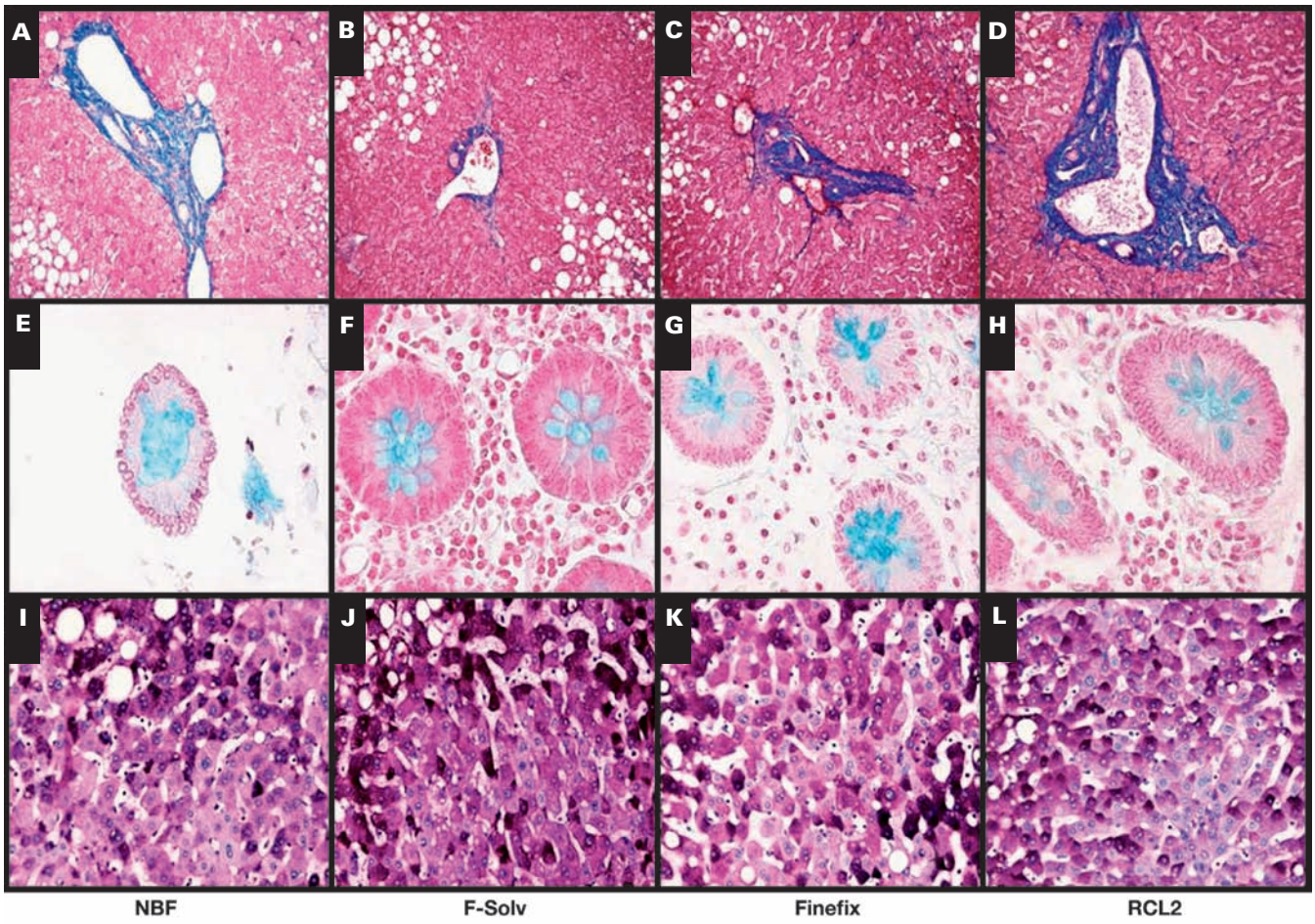

Image 3 Histologic staining after neutral buffered formalin (NBF) fixation and fixation with 3 alternative fixatives (F-Solv, FineFIX, and RCL2). **A**-**D**, Liver sample stained with azan (**A**, ×2; **B**, ×2; **C**, ×2; **D**, ×2). **E**-**H**, Goblet cells in the small intestine stained with alcian blue (**E**, ×10; **F**, ×10; **G**, ×10; **H**, ×10). **I**-**L**, Liver sample stained with periodic acid–Schiff (**I**, ×5; **J**, ×5; **K**, ×5; **L**, ×5).

Discussion

In surgical pathology, formaldehyde has been the "gold standard" fixative for decades. It enables long-term storage of surgical material and preserves the detailed morphologic features necessary for microscopy. Formaldehyde is, however, toxic and was classified as "carcinogenic to humans" (group 1) by the International Agency for Research on Cancer, although there is only strong but insufficient evidence for a causal association with myeloid leukemia and a limited association with nasopharyngeal carcinoma.[1]

The aim of the present study was to test the suitability of 3 new, presumably less toxic, alcohol-based fixatives for routine diagnostics. Formaldehyde is generally used in a 4% aqueous solution (10% formalin) and buffered at pH 7 by acetate or phosphate. In this form, it is named NBF, and fixation is achieved through cross-linking of amines, amides, aromatic rings, hydroxyls, guanidine groups, sulfhydryl groups, and reactive hydrogen atoms.[3,4]

F-Solv (Yvsolab NV, Beerse, Belgium, pH 6.34) was originally sold as an aqueous alcohol solution with a lower toxicity profile than NBF, without any danger symbols (2007). However, in 2009, the safety information was adjusted (according to Supplement I of Guideline 67/548/EEG, group 605; http://europa.eu/legislation_summaries/consumers/product_labelling_and_packaging/l21276_en.htm); F-Solv was from then on sold as an aqueous alcohol solution (with stabilization components) with an aldehyde derivative and was, therefore, harmful (R23/25, R42/43, and S26-37-45). So, within 2 years, the safety sheet of this fixative was drastically changed based on newer guidelines, which further highlights the fact that one can never be sure of the exact content and, thus, the toxicity profile of these commercial (and, in most cases, patented) alternatives.

Although the exact contents of FineFIX (pH 7.98) and RCL2 (pH 3.10) are also proprietary, they consist mainly of ethanol (both ~70%). RCL2 working solution also contains

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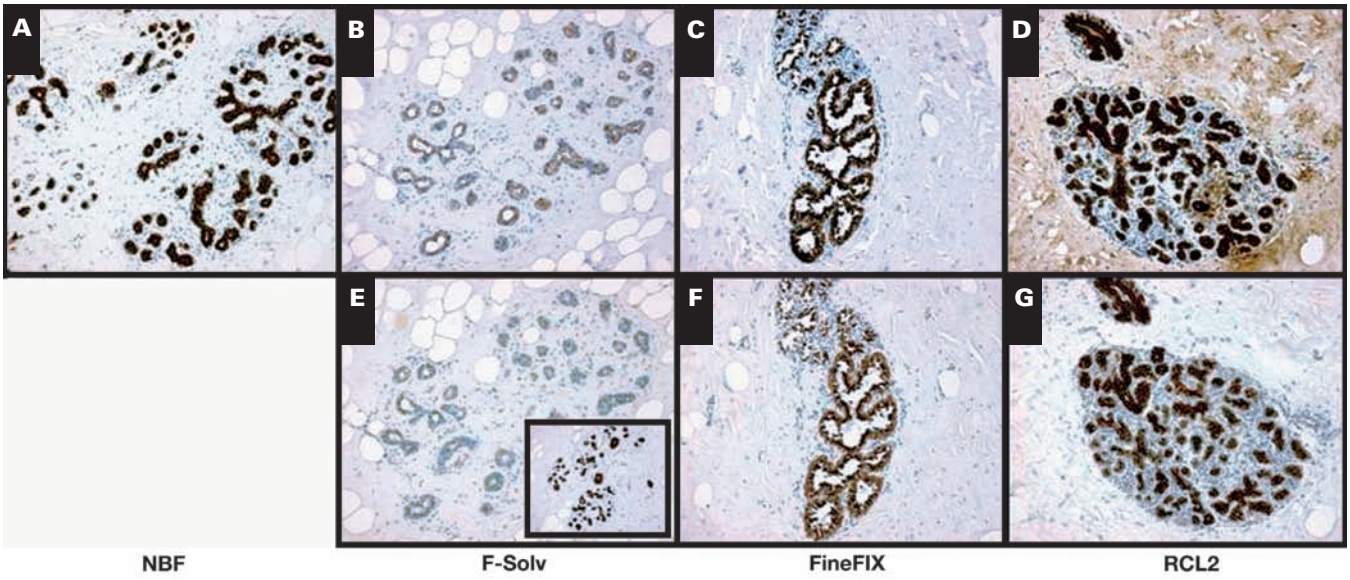

Image 4 The effect of pretreatment on cytokeratin AE1/3 immunohistochemical staining. **A**-**D**, Breast ducts stained by AE1/3 using citrate heat pretreatment (**A**, neutral buffered formalin [NBF]) (**A**, ×5; **B**, ×5; **C**, ×5; **D**, ×5). **E**-**G**, The same breast ducts stained by AE1/3 omitting the pretreatment for F-Solv, FineFIX, and RCL2 (**E**, ×5; **F**, ×5; **G**, ×5). Note the reduced background staining in RCL2 without pretreatment. The F-Solv inset shows staining intensity after pepsin pretreatment (**E**).

a considerable amount of acetic acid (~7%) and a "complex carbohydrate." RCL2 is, therefore, an irritant (risk and safety profiles R36/38 and S24/25), and FineFIX (R11, S7-16) and RCL2 are flammable after dilution in ethanol. In-house analysis of FineFIX showed that it also contains propylene glycol and small amounts of methanol, acetone, and propanol. An in-house Schiff test for aldehydes showed purple/magenta coloration of NBF, F-Solv, and RCL2 but not FineFIX, indicating that it does not contain a complex aldehyde. According to the patent (European patent N. EP 1 455174B1, December 2004; https://data.epo.org/publication-server/rest/v1.0/publication-dates/20041215/patents/EP1455174NWB1/document.pdf), FineFIX contains a mixture of ethanol, distilled water, glycerol, polyvinyl alcohol, and monomeric carbohydrates.

Macroscopically, none of the alternative fixatives was able to compete with NBF in a broad range of tissue samples (placenta, liver, brain, esophagus, stomach, duodenum, colon, omentum, lung, breast, adrenal gland, kidney, lymph node, thyroid, tonsil, spleen, and gallbladder). It was, however, impossible to evaluate macroscopy blindly because of the characteristic smell of most of the fixatives. Some of the findings such as smell, color, and texture are characteristics to which one could become accustomed. However, incomplete fixation and difficulties in cutting, recognizing structures (eg, kidney cortex vs medulla), palpation of lymph nodes, or recognizing disease characteristics such as liver cholestasis or cirrhosis could seriously impact the diagnostic process. The presence of many tiny tissue fragments in some of the fixative solutions (F-Solv and FineFIX) indicates that these fixatives lead to partial tissue disintegration, which could be problematic, especially for small biopsy specimens. Furthermore, disposal procedures for the alternative fixatives are unclear, and standard Septodry absorption material (HW-Logistics BV, Nijkerk, the Netherlands) (used for NBF) was not able to absorb the alternative fixatives. Overall, it seems that, at the macroscopic level, none of these alternatives can adequately replace NBF in daily pathology practice.

Microscopic evaluation of tissues fixed in F-Solv, FineFIX, and RCL2 showed that in many cases, depending on the organ evaluated and the need to be able to visualize special cellular or intracellular structures (eg, RBCs, Paneth granules), the alternative fixatives performed largely similar but partly inferior to NBF. Quality of tissue preservation was highest for NBF and lowest for FineFIX. RCL2 and F-Solv performed equally in this respect. Nuclear structure was generally superior in RCL2-fixed tissue (probably because of the acetic acid component), but this type of fixation was associated with RBC lysis, as previously reported,[16] and often resulted in pigment deposition.

FineFIX showed a considerable amount of tissue shrinkage, which was especially apparent in breast and kidney samples, and also resulted in RBC lysis. F-Solv, in contrast, showed RBCs even better than with NBF. This finding is as expected because ethanol, the main component of FineFIX and RCL2, has been shown to be a major cause of RBC lysis.[13,14] The loss of eosinophilic granules and granulocytes in FineFIX and RCL2 was seen as a major

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disadvantage. It has been shown that alcohol and acetic acid can have a role in dissolving these cytoplasmic granules.[13,14] In line with our results, 1 FineFIX-based study was unable to perform mast cell count (on Giemsa-stained slides) owing to degranulation of mast cells.[17] The fact that FineFIX shows more tissue shrinkage than RCL2 and both fixatives contain the same amount of ethanol indicates that the RCL2 acetic acid component compensates for a large part of this shrinkage. Tissue shrinkage by FineFIX has been reported previously,[17,18] and, although undesirable, it generally did not interfere with diagnostics. Detachment from the basement membrane and epithelium has also been reported to be more accentuated in FineFIX-fixed material.[19]

In previous studies, RCL2-fixed specimens showed no loss of morphologic detail and no protein degradation by microscopic evaluation and immunohistochemical studies when stored at room temperature for 8 years, supporting its potential use in long-term storage.[20] At present and according to our current knowledge, no reports for F-Solv have been published. The quality of H&E staining was good for all fixatives. It has to be mentioned, however, that H&E staining intensity for the alternatives was generally stronger than for NBF, especially in the case of F-Solv. Thus, H&E staining protocols had to be adjusted before scoring to avoid bias of the observing pathologist.

Overall, NBF performed best (93%), followed by F-Solv, RCL2 (both 88%), and FineFIX (86%), indicating that aldehyde-containing fixatives perform morphologically better than non–cross-linking alcohol-based fixatives. However, the fact that the quality of F-Solv tissue preservation deteriorated with longer fixation times (≥2 weeks) is regarded as a major disadvantage because tissue samples are kept in fixative up to 3 months for additional resampling when required. Overall, at the microscopic level, each alternative has its pros and cons, but all fixatives were generally inferior to (the more accustomed) NBF.

At the histochemical and the immunohistochemical levels using the usual NBF-based protocols, NBF performance was superior, followed by FineFIX/RCL2 and F-Solv. The fact that tissues fixed in non–cross-linking alcohol-based fixatives (FineFIX and RCL2) could successfully be histochemically and immunohistochemically (for most antibodies) stained by following the usual NBF-based protocols indicates that when switching to these alternative fixatives, no major changes in the daily routine for immunohistochemical studies are to be anticipated. Further diluting antibodies and modifying or even omitting antigen retrieval may be possible, thus reducing immunohistochemical costs and labor. Our results for FineFIX and RCL2 are in line with previous studies investigating the effects of both fixatives on immunohistochemical stains.[16,18,21-24] F-Solv, on the other hand, another cross-linking but less toxic NBF substitute, would need major changes before being applicable in daily histologic and immunohistochemical practice. Furthermore, the switch from NBF to any other type of fixative would require a reevaluation of all markers currently used in diagnostic immunohistochemical studies because the information that is now available about their sensitivity and specificity has been obtained using NBF-fixed, paraffin-embedded tissue specimens.

Before replacing formalin with another fixative in a surgical pathology laboratory, all levels of pathology must be examined thoroughly. In this study, we examined 3 alternative fixatives at the morphologic, histologic, and immunohistochemical levels. Preservation of histomorphologic features and proteins after prolonged storage (eg, 1, 5, and 10 years) needs to be further evaluated. The suitability of FineFIX and RCL2 for DNA and RNA extraction seems good,[16,21,23-26] but F-Solv needs further evaluation for suitability, and electron microscopy also needs further study for these three fixatives. Furthermore, we need to keep in mind that even though most of these substitutes are potentially less toxic than formalin, they always contain components that are potentially toxic for humans, and most of them are also inflammable. The fixation and embedding procedure (handling of tissue specimens and fixation solution, recommended fixation duration, paraffin-embedding process), the infrastructure and logistics needed for fixation and storage, and the associated costs can be different depending on the composition of the fixatives. At our center, the purchasing costs for formalin fixation are approximately €0.88/L, whereas the costs for F-Solv, FineFIX, and RCL2-fixation are approximately €6.5/L, €2.47/L, and €4.83/L, respectively.

The alcohol-based fixatives tested had positive and negative features and their own environmental drawbacks, and none was comparable overall to formaldehyde with regard to macroscopy, morphologic examination, and immunohistochemical studies.

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