Comparison of Formalin-Free Tissue Fixatives

A Proteomic Study Testing Their Application for Routine Pathology and Research

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• *Context.*—Formalin-fixed, paraffin-embedded tissue is the routine processing method for diagnostics practiced in pathology departments worldwide.

Objective.—To determine the potential value of non–cross-linking, formalin-free tissue fixation for diagnostics in pathology and proteomic investigations.

Design.—We tested 3 commercially available, formalin-free tissue fixatives—FineFIX, RCL2, and HOPE—in lung cancer specimens from 10 patients. The fixatives were evaluated for their effects on tissue morphology, protein recovery, and immunoreactivity for a selected panel of proteins differently expressed in lung cancer, using immunohistochemistry and Western blotting.

Results.—Tumor-cell analysis with hematoxylin-eosin worked equally well for all tested fixatives when compared with the standard formalin-fixed, paraffin-embedded procedure. Movat pentachrome stains showed comparable results for the different matrices and cellular proteins analyzed. The RCL2 ($P = .01$) and HOPE fixatives ($P = .03$) improved protein recovery when compared with formalin-fixed, paraffin-embedded or frozen tissues. Our data clearly show that the fixatives evaluated influenced immunoreactivity to matched, formalin-fixed, paraffin-embedded lung cancer tissue. In particular, membrane-bound proteins, such as epidermal growth factor receptor EGFR, can be detected more efficiently by immunohistochemistry and Western blotting.

Conclusion.—We have demonstrated that formalin-free fixatives have the potential in routine pathology and research to replace formalin in histomorphology and protein preservation.

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In clinical practice biopsies as well as surgical resection specimens are fixed as soon as possible to avoid autolysis and putrefaction, and embedded in paraffin-wax. Formalin-fixed, paraffin-embedded (FFPE) tissue is the routine processing method practiced in pathology departments worldwide. Fixation is a process involving a series of complex chemical modifications of macromolecules present in tissues and cells to preserve the structural and functional components as closely as possible to the living state. Formalin is a cross-linking fixative creating methylene bridges among proteins and, if not adequately buffered, results in nucleic acid fragmentation and degradation due to formic acid.[1,2]

Although formalin-fixed tissues are well preserved for histopathologic evaluation, the quality of the macromolecules is severely compromised, and that has been assumed to render them unsuitable for proteomic studies. However, this view has been challenged in the past decade through the development of innovative methods coupled with heat-induced extraction approaches to achieve solubilized, nondegraded, immunoreactive proteins from FFPE tissues.[3–7]

Formalin-fixed, paraffin-embedded tissue processing represents a highly stable, cheap, and easily storable form of tissue; is an invaluable material for research in molecular medicine; and is archived in hospitals and tissue banks worldwide. However, formalin is a toxic fixative and exposure by nasal, oral, or dermal routes is a human health risk.[8] The International Agency for Research of Cancer recently classified formaldehyde as a human carcinogen (group 1) that causes nasopharyngeal cancer, and it also concluded there is "strong but not sufficient evidence for causal association between leukemia and occupational exposure to formaldehyde."[9(p1206)] For this reason, several European countries already restrict the use and import of formaldehyde because of its carcinogenic properties, and the European Union is considering a complete ban on formaldehyde usage.[10] To overcome the problems of formalin fixation, numerous attempts have been made to find formalin-free tissue fixatives with low toxic properties and a similar quality to preserve nucleic acids and proteins.[11] Numerous formalin substitutes have been examined and seem to be appropriate for DNA and

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RNA analysis,[12–15] but the related procedures have been poorly tested in routine pathology practice and the proteomic field.[16]

Unfixed-fresh or snap-frozen tissue is commonly used as the gold standard for proteomic studies because it preserves proteins excellently. However, the histologic architecture on frozen tissue sections is often disrupted, and it will not work with many special stains. Also, frozen tissues are not widely available because of storage problems. Therefore, it is worth investigating whether formalin-free tissue fixation would improve protein preservation. The aim of our study was to assess the potential value of non–cross-linking, formalin-free tissue fixation for routine diagnostics and proteomic investigations. We compared 3 commercially available, formalin-free fixatives—FineFIX, RCL2, and HOPE—with standard formalin-fixed or frozen, lung cancer tissue samples. The fixatives were evaluated for their effects on tissue morphology, immunohistochemistry, and protein recovery and immunoreactivity by Western blotting.

MATERIALS AND METHODS

Tissue Fixation

Ten unfixed, lung cancer samples submitted for frozen-section diagnosis were chosen arbitrarily. The patients gave informed consent, and the study was approved by the Ethics Committee of the Medical University of Graz (No. 17-247).

From each tumor, 5 tissue samples were taken. One sample was routinely fixed with 10% neutral-buffered formalin, paraffin-embedded, and dehydrated according to the standard protocols. Another sample was immediately embedded in optimal cutting temperature medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, the Netherlands), snap frozen, and stored in liquid nitrogen until use. The remaining 3 samples were parallel-fixed in FineFIX (Milestone Srl, Sorisole, Torre Boldone, Italy), RCL2 (Alphelys, Plaisir, France), and HOPE (DCS Innovative Diagnostik-Systeme, Hamburg, Germany), dehydrated, and paraffin-embedded, according to the respective manufacturer recommendations. To evaluate the effects of prolonged fixation, different fixation times ranging from 21 to 69 hours were analyzed. The resulting FFPE, paraffin-embedded FineFIX, and paraffin-embedded RCL2 tissue blocks were stored at room temperature, whereas the paraffin-embedded HOPE tissue blocks were maintained at 4°C (manufacturer's recommendation). From each case, representative sections were examined to ensure qualitatively similar degrees of cellularity and the absence of necrosis.

Histologic Staining

Sections of FFPE, paraffin-embedded FineFIX, paraffin-embedded RCL2, and paraffin-embedded HOPE tissues (4 µm thick) were dewaxed with xylene and dehydrated with several graded ethanols before routine hematoxylin-eosin (H&E), Movat pentachrome, and immunohistochemical staining were performed.

An H&E-stained reference section of each case and each fixative was histologically verified by experienced pathologists (H.H.P. and E.S.), and tumor areas (with >85% of tumor cells) were indicated with a pen mark on the slide. The H&E reference slides were used to locate the tumor area on the unstained sections for further protein extraction. Five consecutive, unstained sections from the same tissue blocks were used for protein extraction.

In all fixed samples, the following details were evaluated separately: nuclear and nucleolar preservation; chromatin pattern and details; mitosis and chromosomes, if present in the metaphase; cytoplasmic details, such as phagolysosomes in the alveolar macrophages; and mucin in mucinous adenocarcinomas. Both pathologists evaluated the cases on a score sheet containing each of these details: nucleoli present/enlarged; chromatin coarse/fine, granular/reticular; mitosis visible; mitotic figure/chromosomes visible; phagolysosomes visible; and mucin visible (using Harris H&E). After both pathologists had evaluated the cases, those cases with disagreements were reviewed using a multiheaded microscope.

Movat Pentachrome Staining

The Movat pentachrome stain was chosen because that staining procedure exposes each tissue section to a variety of acidic and basophilic solutions and also to metal impregnation procedures. In addition, several matrix and cellular proteins are differently colored, which makes this stain very useful in evaluating mesenchymal tissue reactions. The Movat staining was done according to standard protocols.[17] The staining pattern of the matrix proteins—elastin, collagen, reticulin fibers—and the myofilaments of smooth muscle cells was evaluated.

Immunohistochemistry

Immunohistochemical analysis was performed in parallel on 3-µm-thick FFPE, paraffin-embedded FineFIX, paraffin-embedded RCL2, and paraffin-embedded HOPE tissue sections by routine protocols for FFPE tissue sections for the following antibodies: CK7 (cytokeratin 7), E-cadherin, EGFR (epidermal growth factor receptor), PCNA (proliferating cell nuclear antigen), and STAT1 (signal transducer and activator of transcription 1). The EGFR pharmDx kit (Dako Österreich GmbH, Wien, Austria) was used for EGFR immunohistochemistry. The kit's protocol was strictly followed for all samples. Detailed information about antibody source, antigen retrieval, and detection is listed in Table 1. Immunohistochemical analysis was carried out by 2 pathologists (H.H.P. and E.S.) blinded to the type of fixation. Protein expression was recorded semiquantitatively as described recently.[18] Staining scores were calculated by multiplying the percentage of positive cells (0%–100%) and by the staining intensity (1–3). The product scores obtained, ranging from 0 to 300, were used for statistical analyses. Both pathologists scored the slides independently and recorded their scores on a separate sheet. Then, the pathologists together evaluated on a multiheaded microscope those cases with a disagreement.

Protein Extraction From Fixed Tissue Sections

Protein extraction from each case of the parallel-fixed and paraffin-embedded tissue blocks for FFPE, FineFIX, RCL2, and HOPE was performed using Qproteome FFPE tissue kit (Qiagen, Hilden, Germany), as previously reported by Becker et al,[4] for the application with FFPE tissue sections. Briefly, the 10-µm thick tissue sections from each case (n = 5) of the FFPE, paraffin-embedded FineFIX, paraffin-embedded RCL2, and paraffin-embedded HOPE blocks were cut and placed onto glass slides (SuperFrost, Menzel-Gläser, Braunschweig, Germany). After deparaffination of the tissue sections, the tumor areas were scratched from the unstained slide with a needle. The microdissected tissue was transferred into the Qproteome kit buffer, and proteins were extracted according to manufacturer recommendations and stored frozen at −20°C. From the frozen tissue specimens, multiple sections were cut; the first section was used for reference H&E staining, and consecutive 10-µm-thick sections were cut onto glass slides and stored at −80°C until protein extraction was performed as described above.

Protein Yield

Protein concentrations were determined in 3 replicates using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Waltham, Massachusetts) according to the protocol provided by the supplier for 96-well formats. To take measurements of the tumor area, the H&E reference slides with the marked tumor areas were scanned on a flatbed scanner, JPEG files were generated, files were imported into the FixFoto software program (version 2.91, 2008), and the data were used for calculating the tumor area in square millimeters. Protein yield was then

Antibody	Clone (Order No.)	Source, Location	Localization	Dilution	Antigen Retrieval	Detection
CK7	OV-TL 12/30 (M 7018)	Dako Cytomation, Glostrup, Denmark	Cytoplasmic	1:100	P	CM ACE
EGFR	· · · (K1494)	Dako Cytomation, Glostrup, Denmark	Membranous	Ready to use	Pk Dako	Dako Kit DAB
E-cadherin	4A2C7 (18-0223)	Zymed Laboratories, San Francisco, California	Membranous	Ready to use	WB	LSAB AEC
PCNA	PC10 (M0879)	Dako Cytomation, Glostrup, Denmark	Nuclear	1:20	P	CM AEC
STAT1	M-22 (sc-592)	Santa Cruz Biotechnology, Santa Cruz, California	Cytoplasmic and nuclear	1:1000	Ventana	DAB

Table 1. Antibodies Used For Immunohistochemistry

Abbreviations: CK7, cytokeratin 7; CM, ChemoMate, Dako; DAB, DAB ChemoMate Envision kit, Dako; EGFR, epidermal growth factor receptor; HRP, horseradish peroxidase, rabbit; LSAB AEC, LSAB Kit Peroxidase Blocking Solution and AEC Substrate-Chromogen, Dako; P, Protease XXIV, 0.1% in phosphate-buffered saline, Sigma-Aldrich, St Louis, Missouri; PCNA, proliferating cell nuclear antigen; STAT1, signal transducer and activator of transcription 1; WB, water bath for 40 min at 90°C for epitope retrieval solution.

expressed as micrograms per square millimeter of tissue section. The protein yield from all tested tissue fixatives and frozen tissues was evaluated.

Western Blot Analysis

Twenty micrograms of total protein from the protein extracts was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride–type membranes (Hybond-P, GE Healthcare Europe GmbH, Freiburg, Germany) using a semidry blotting apparatus (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Blocking was performed in Tris-buffered saline containing 0.1% Tween-20 and the blocking substance for 2 hours at room temperature. The blocked membrane was incubated with the primary antibodies for detecting β-actin, E-cadherin, EGFR, PCNA, and STAT1 for 16 hours at 4°C. Additional information about the source of the antibodies and their blocking and dilution is in Table 2. After incubation with the primary antibody, the membrane was washed 3 times for 10 minutes with Tris-buffered saline containing 0.1% Tween-20 and then incubated with the secondary horseradish peroxidase-conjugated antibody for 1 hour at room temperature. The membrane was washed 3 times for 10 minutes with Tris-buffered saline containing 0.1% Tween-20 and developed using ECL Western Blot Detection Reagents (GE Healthcare Europe) and Kodak film (T-MAT G film, Eastman Kodak Company, Rochester, New York). Quantitative analysis of the Western blot band intensities was performed using Scion Image for Windows (beta 4.0.3, Scion Corporation, Frederick, Maryland). Films were scanned on a flatbed scanner; images were saved as 8-bit TIFF files and imported to the imaging software.

Statistical Analyses

To avoid the problems in testing multiple fixations with multiple immunochemistry and Western blots measurements, we adapted the Goeman global test,[19] after which, it was possible to test whether any of the fixation methods caused any difference in any of the immunohistochemistry or Western blot measurements by 1 P value. To reduce the influence of outliers, each measurement (immunohistochemistry scores, Western blot, protein yield) was rank-transformed. In addition, the mean rank of each specimen was subtracted to remove the block effect of the specimens. The global test was applied using the permutation test criterion with 10 000 replications. Pairwise differences among fixation methods were tested by Wilcoxon signed rank test. Values of $P < .05$ were considered statistically significant. The R 2.9.0 (http://www.r-project.org/) and R-package global test (bioconductor package 4.10.0) software, as well as the R-code provided by the Department of Medical Statistics at the Georg-August-University at Göttingen, Germany (http://www.ams.med.uni-goettingen.de/de/sof/ld/rp/ld.f1.r), were used.

RESULTS

Histologic/Morphologic Findings

Sections stained with H&E and Movat pentachrome were compared in each of the 10 lung cancer cases after fixation and paraffin embedding using either standard formalin or FineFIX, RCL2, and HOPE fixatives. On frozen tissue sections, only H&E staining was performed, but those sections were not used for the morphologic evaluation.

Table 2. Antibodies Used For Western Blotting

Antibody	Clone (Order No.)	Source, Location	Localization	Blocking	Dilution
β-Actin	AC15 (A5441)	Sigma-Aldrich, Inc, St Louis, Missouri	Cytoplasmic	5% MP	1:10 000
E-cadherin	36 (610181)	BD Biosciences, San Jose, California	Membranous	5% MP	1:5000
EGFR	· · · (2232)	Cell Signaling Technology, Danvers, Massachusetts	Membranous	5% BSA	1:2000
PCNA	PC10 (M0879)	DakoCytomation, Glostrup, Denmark	Nuclear	5% MP	1:1000
STAT1	M-22 (sc-592)	Santa Cruz Biotechnology, Inc, Santa Cruz, California	Cytoplasmic and nuclear	5% MP	1:1000

Abbreviations: BSA, bovine serum albumin; EGFR, epidermal growth factor receptor; MP, nonfat dry milk powder; PCNA, proliferating cell nuclear antigen; STAT1, signal transducer and activator of transcription 1.

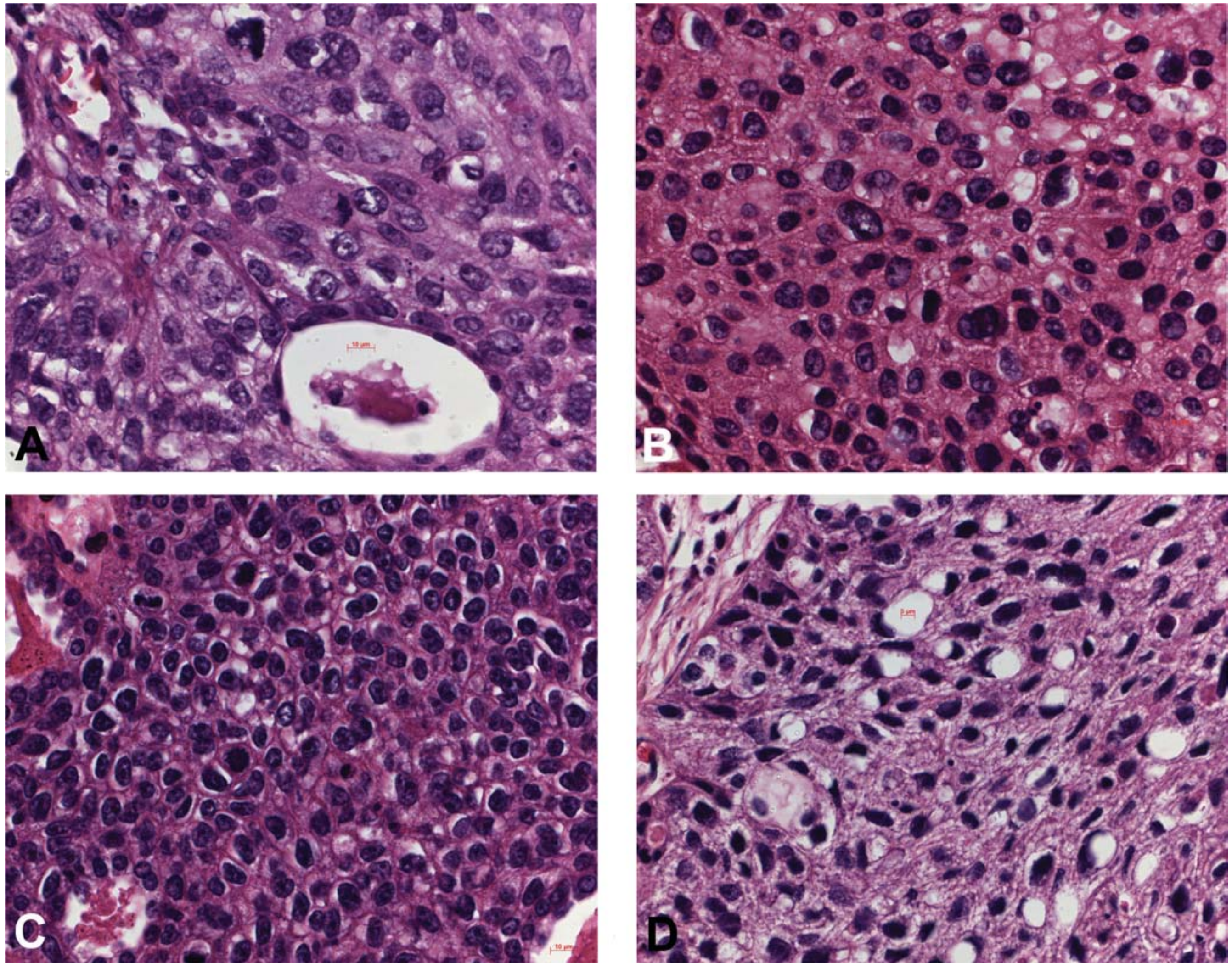


Figure 1. *Hematoxylin-eosin stains from parallel formalin-fixed, paraffin-embedded tissues (A) and from paraffin-embedded FineFIX (B), paraffin-embedded RCL2 (C), and paraffin-embedded HOPE (D) fixed tissues from lung cancer cases (original magnifications ×400).*

All fixatives yielded a comparable, good H&E-staining quality (Figure 1, A through D). All nuclear details were clearly visible, and cytoplasmic differentiation products could also be seen. However, there were a few differences; FFPE, FineFIX, and RCL2 were found to be excellent at preserving nuclear and cytoplasmic details, whereas the HOPE-stained sections were ranked visible but not clear regarding the nuclear features. Also mucin and surfactant inclusions were more difficult to see in the HOPE-stained sections than in the FFPE-, FineFIX-, and RCL2-stained sections. However, for tissue analysis of complex tissue reactions, such as desmoplastic stroma or vascular abnormalities, all tissues fixed by different processes were equally well suited. Movat stains showed comparable results for the different matrix proteins (not shown). Tumor cell analysis was equally possible for all 3 formalin-free fixatives, as compared with the routine, standard formalin fixative.

Immunohistochemical Analyses

Representative immunohistochemical stains for antibodies detecting CK7, STAT1, PCNA, E-cadherin, and EGFR for parallel-stained FFPE, paraffin-embedded FineFIX, paraffin-embedded RCL2, and paraffin-embedded HOPE tissue sections are shown in Figure 2 (A through T). Each antibody measurement from the 3 formalin-free fixatives and the standard FFPE process were matched, and the resulting differences are listed in Table 3; all antibody measurements are summarized in Figure 3 (A and B). Cytoplasmic staining with CK7 showed no differences among the standard FFPE and the formalin-free tissue fixatives, and no differences could be detected among the formalin-free fixatives, respectively. Some proteins like STAT1 (cytoplasmic and nuclear) and PCNA (nuclear) could not be detected in the formalin-free fixatives when the staining intensity was low in the FFPE tissue sample. Interestingly, STAT1 was better preserved in FFPE ($P = .005$) than it was with the FineFIX- and RCL2-fixed lung cancer tissue. On the contrary, formalin-free fixatives were superior to formalin in preserving surface proteins, such as the membrane-receptors EGFR and E-cadherin we used. We found significantly higher staining for EGFR with FineFIX ($P = .005$) and RCL2 ($P = .005$), whereas E-cadherin could be more strongly detected in paraffin-embedded RCL2 ($P = .007$) and paraffin-embedded HOPE ($P = .02$) than in FFPE tissues.

The comparison among the formalin-free fixatives (Table 3) revealed that CK7, STAT1, and PCNA could be

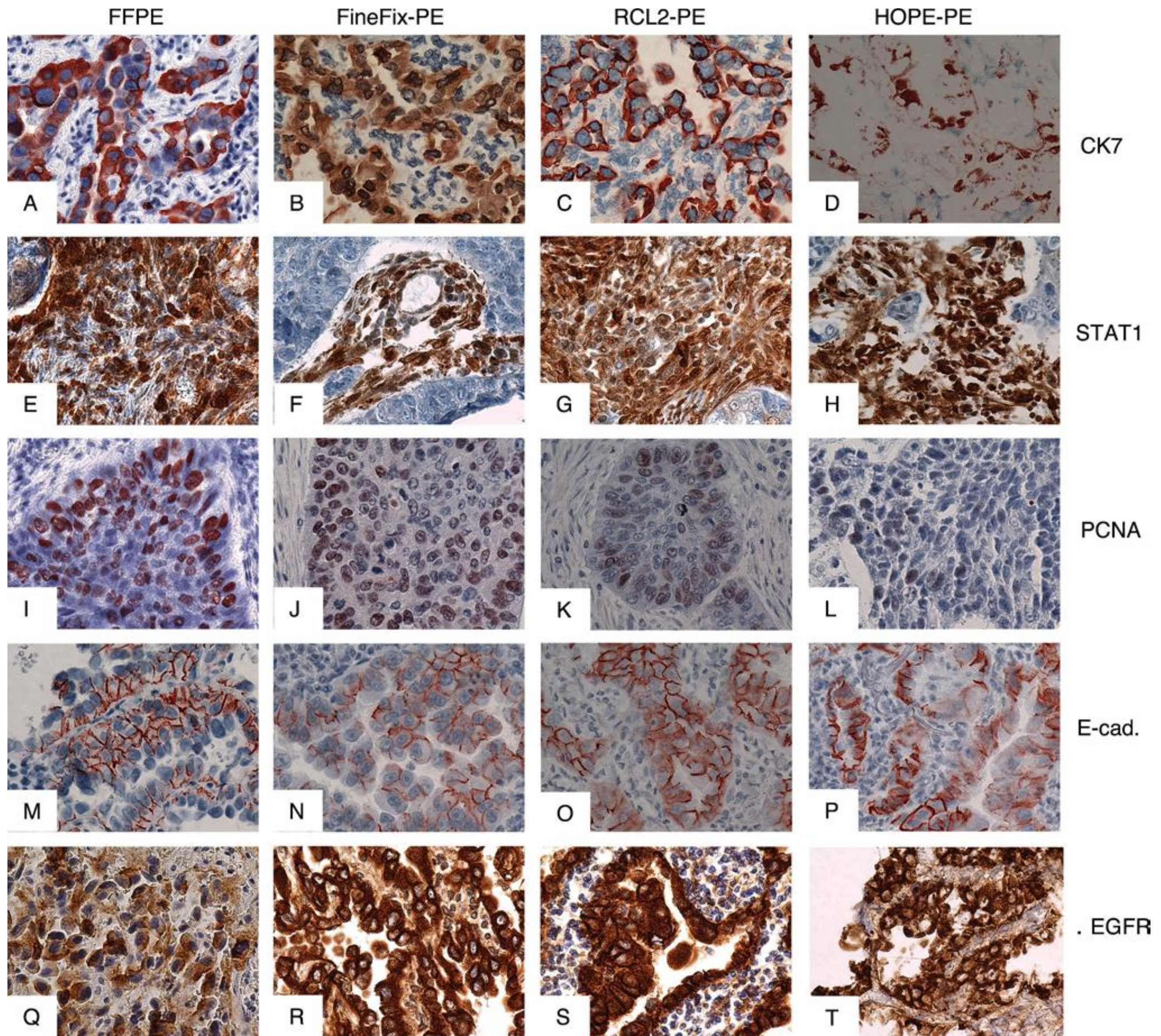


Figure 2. *Immunohistochemical staining of parallel formalin-fixed, paraffin-embedded (FFPE) tissues and paraffin-embedded FineFIX (FineFix-PE), paraffin-embedded RCL2 (RCL2-PE), and paraffin-embedded HOPE (HOPE-PE) fixed lung cancer tissue sections. A representative overview of immunohistochemical staining is shown for cytoplasmic cytokeratin 7 (CK7; A through D), cytoplasmic and nuclear signal transducer and activator of transcription 1 (STAT1; E through H), nuclear proliferating cell nuclear antigen (PCNA; I through L), membranous E-cadherin (E-cad.; M through P), and epidermal growth factor receptor (EGFR; Q through T) (original magnifications ×600).*

detected equally well in paraffin-embedded FineFIX, paraffin-embedded RCL2, and paraffin-embedded HOPE, whereas paraffin-embedded RCL2 and paraffin-embedded FineFIX gave excellent staining results for membranous E-cadherin ($P = .01$) and EGFR ($P = .03$). Overall, there was less protein preservation in paraffin-embedded HOPE tissues, likely because of digestion by the proteinase pretreatment and the retrieval procedures. In general, FineFIX and RCL2 worked equally well and were most often comparable to formalin, although they yielded less-intense stained cells with STAT1 and PCNA; the HOPE fixative yielded the least-satisfying results.

The global comparison among the fixatives (includes all antibody measurements) showed that paraffin-embedded FineFIX, paraffin-embedded RCL2, and paraffin-embedded HOPE highly influenced the efficiency of the protein preservation for the selected antibody panel ($P = .02$) when compared with standard FFPE tissue (global test; Table 3).

Western Blot Analyses

To evaluate different tissue fixatives for possible effects on protein detection, proteins with different cellular localizations and molecular weights, such as β-actin (42 kDa), STAT1 (84/91 [β/α isoforms] kDa), PCNA (36 kDa), E-cadherin (120 kDa), and EGFR (175 kDa), were selected for Western blot analyses (Figures 4 and 5, A). The quantitative image analyses of the Western blot band intensities were evaluated globally for the influence of the fixatives and for individual proteins by Wilcoxon pair testing (Table 4). The latter indicated no difference in the immunoreactivity for β-actin and PCNA between FFPE and alternative tissue fixatives, whereas STAT1 could be more strongly detected in the paraffin-embedded HOPE

Fixatives	CK7	STAT1	PCNA	E-Cadherin	EGFR	Global Test[b]
	P Value					
FFPE : FineFIX-PE	.35	**.005**	.08	.28	**.005**	**<.001**
FFPE : RCL2-PE	.46	.06	.79	**.02**	.06	**.02**
FFPE : HOPE-PE	.73	**.005**	.47	**.007**	**.005**	**<.001**
FineFIX-PE : RCL2-PE	.85	.17	.07	**.049**	**.03**	**.001**
FineFIX-PE : HOPE-PE	.89	.55	.17	**.01**	.50	**<.001**
HOPE-PE : RCL2-PE	.78	.49	.46	.05	**.01**	.32

Table 3. Comparison of Fixatives by Immunohistochemistry[a]

Abbreviations: CK7, cytokeratin 7; EGFR, epidermal growth factor receptor; FFPE, formalin-fixed, paraffin-embedded tissue; FineFIX-PE, paraffin-embedded FineFIX fixed tissues; HOPE-PE, paraffin-embedded HOPE fixed tissues; PCNA, proliferating cell nuclear antigen; RCL2-PE, paraffin-embedded RCL2 fixed tissues; STAT1, signal transducer and activator of transcription 1.

[a] Resulting *P* values from Wilcoxon pair testing for each antibody and fixative comparison and from global testing are shown; values of $P < .05$ were considered statistically significant and are marked in bold. Frozen tissue was included only for Western blot investigations; see Table 4.

[b] Global tests were performed on rank-transformed variables with 10 000 random permutations.

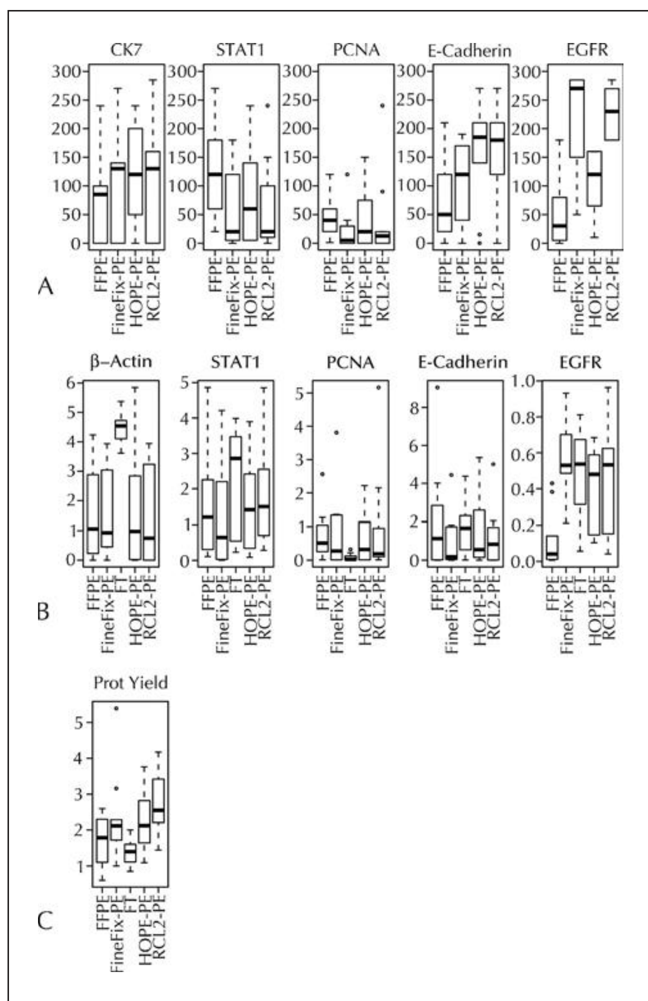


Figure 3. *Box and whisker plots for all antibody measurements resulting from immunohistochemistry (A) and Western blot (B) analyses, and the protein yield (C) from the protein extractions is also shown. A, The evaluation (scoring) for membraneous, nuclear, and cytoplasmic markers are shown for the different fixatives. The scoring sums are on the vertical axis, and mean and standard deviation are shown. B, On Western blots, the protein concentration is given as band intensities (×10^{6}); for comparison, frozen tissue (FT) was included but was restricted to the Western blots. C, Protein yield is expressed in micrograms of protein per square millimeter of tissue section. Whiskers indicate the standard error of the means. Abbreviations: FFPE, formalin-fixed, paraffin-embedded tissue; FineFix-PE, paraffin-embedded Fine-FIX fixed tissues; HOPE-PE, paraffin-embedded HOPE fixed tissues; RCL2-PE, paraffin-embedded RCL2 fixed tissues.*

tissue samples ($P = .01$). In addition, we found β-actin and STAT1 were better preserved in frozen tissues than in fixed tissue, whereas PCNA seemed better preserved in the fixed tissue samples (Table 4).

The EGFR and E-cadherin, 2 important protein markers for pathologic diagnosis and often involved in therapeutic decisions, gave different results. We found EGFR immunoreactivity significantly increased by the formalin-free tissue fixation ($P = .007$), when compared with FFPE tissue section, whereas by Western blotting, E-cadherin showed no difference among fixation methods. The global comparison indicated a strong improvement for the paraffin-embedded FineFIX ($P < .001$), paraffin-embedded RCL2 ($P < .001$), paraffin-embedded HOPE ($P < .001$), and the frozen tissues ($P < .001$) as compared with the FFPE tissue processing method (Table 4).

Protein Yield From Fixed and Frozen Lung Cancer Tissue

Proteins can be efficiently extracted from fixed and unfixed lung cancer tissues by applying the same extraction method. Alternative fixation methods improve protein recovery when compared with FFPE tissues or frozen tissue (Table 5). In detail, the comparison of the protein yield from matched FFPE, paraffin-embedded FineFIX, paraffin-embedded RCL2, paraffin-embedded HOPE, and frozen lung cancer tissue samples demonstrated (Figure 3, C) the highest protein-recovery results were from paraffin-embedded RCL2 tissues ($P = .01$), when compared with FFPE tissues and when compared with frozen-tissue, paraffin-embedded RCL2 ($P = .02$), and the paraffin-embedded HOPE tissues ($P = .03$) showed higher protein yields. Interestingly, the protein yield obtained from frozen tissues samples was lower than that from the fixed and paraffin-embedded tissues, regardless of which fixative was used (Figure 3, C).

COMMENT

The potential of archival, fixed- and paraffin-embedded tissue collections as an alternative to frozen tissues for biomarker discovery is only just beginning to be recognized. Frozen tissue banking for tissue archiving represents a cost-intensive practice and is, therefore, usually restricted to large pathology departments or universities. The aim of our study was to evaluate the suitability of non–cross-linking, formalin-free tissue fixation for routine pathology diagnostics and proteomic investigations.

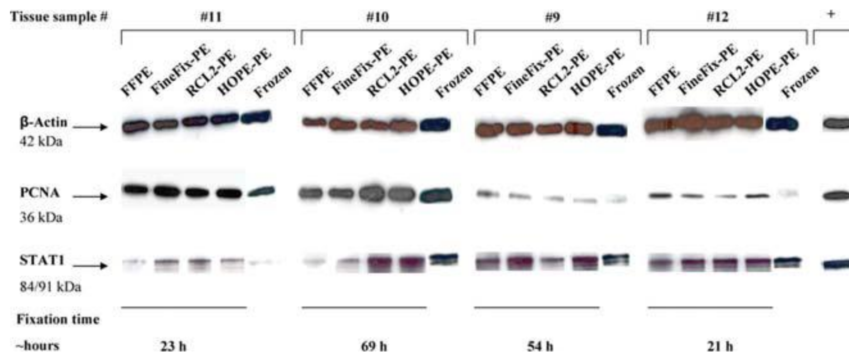
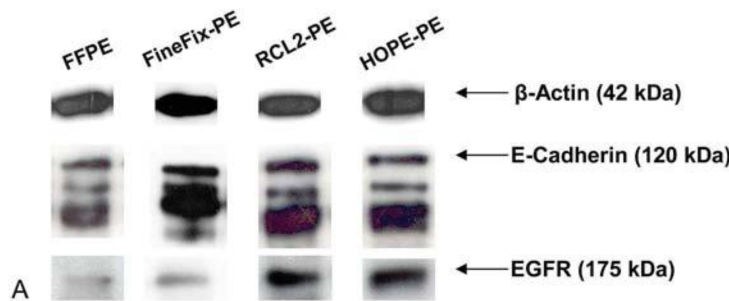


Figure 4. *Western blot analyses using antibodies against β-actin, signal transducer and activator of transcription 1 (STAT1), and proliferating cell nuclear antigen (PCNA). Whole protein lysates from formalin-fixed, paraffin-embedded (FFPE) tissues, paraffin-embedded FineFIX (FineFix-PE) fixed tissues, paraffin-embedded RCL2 (RCL2-PE) fixed tissues, paraffin-embedded HOPE (HOPE-PE) fixed tissues, and frozen lung cancer tissue sections (Frozen), were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to the membrane. Representative Western blots from selected lung cancer samples (samples 9 through 12) are shown. The time of fixation indicates that there was no influence on the quality of the extracted proteins over time. The plus (+) indicates the cell line protein lysates for methodologic control.*

The pathologist Karl Weigert realized the better quality of the tissue sections after formalin fixation in 1893, and the rest is history. Most tissues stored in hospitals and pathology departments across the world are fixed in formalin and embedded in paraffin wax. Almost all histology techniques and antibody manufacturers have optimized their products for FFPE tissues, and even the US Food and Drug Administration has approved some procedures only for FFPE tissue processing. The disadvantage of formalin—besides being toxic and carcinogenic—is its limited effectiveness as a fixative for molecular tests, which has driven the quest for formalin substitutes for tissue fixation. Under those circumstances, and because of the large potential market involved, many scientists and companies have developed numerous new formalin-free fixatives.[20]



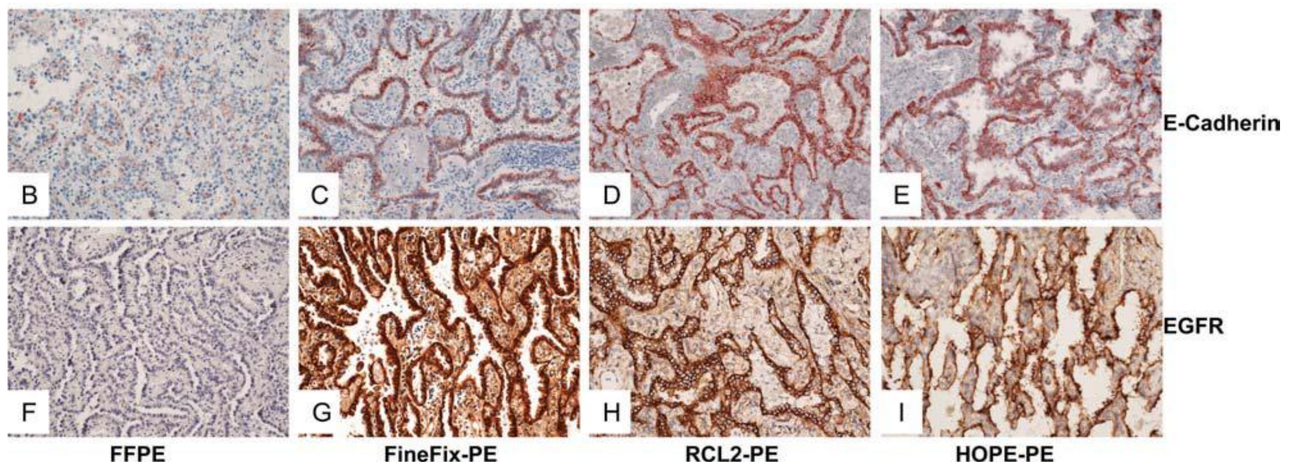


Figure 5. *Membranous E-cadherin and epidermal growth factor receptor (EGFR) are better preserved with the paraffin-embedded FineFIX (FineFix-PE), the paraffin-embedded RCL2 (RCL2-PE), and the paraffin-embedded HOPE (HOPE-PE) than with the formalin-fixed, paraffin-embedded (FFPE) lung cancer tissues. E-cadherin and EGFR results from tissue sample 2 analyzed by Western blot (A) and immunohistochemistry (B through I) are shown. A, β-actin is shown for loading control. Whole protein lysates from FFPE, FineFix-PE, RCL2-PE, and HOPE-PE lung cancer tissue sections were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then detected by EGFR and E-cadherin antibodies. For validation immunohistochemistry was performed on tissue sections from the same tissue blocks. E-cadherin (B through E) and EGFR (F through I) immunohistochemistry for FFPE, FineFix-PE, RCL2-PE, and HOPE-PE lung cancer is shown (original magnifications ×200).*

Table 4. Comparison of Fixatives by Western Blot[a]

Fixatives	*P* Value					
	β-Actin	STAT1	PCNA	E-Cadherin	EGFR	Global Test[b]
FFPE : FineFIX-PE	.44	.37	.77	.08	**.007**	**<.001**
FFPE : RCL2-PE	.68	.08	.77	.21	**.007**	**<.001**
FFPE : HOPE-P	.44	**.01**	.89	.26	**.007**	**<.001**
FineFIX-PE : RCL2-PE	.59	.07	.89	.26	.51	.16
FineFIX-PE : HOPE-PE	.31	.51	.89	.11	.31	.08
HOPE-PE : RCL2-PE	.21	.31	.95	.51	.68	.16
FFPE : FT	**.02**	**.02**	**.02**	.31	**.02**	**<.001**
FineFIX-PE : FT	**.02**	.06	**.04**	.50	.50	**<.001**
RCL2-PE : FT	**.02**	.73	**.02**	.87	.50	**<.001**
HOPE-PE : FT	.09	.31	**.02**	.73	.18	**<.001**

Abbreviations: EGFR, epidermal growth factor receptor; FFPE, formalin-fixed, paraffin-embedded tissue; FineFIX-PE, paraffin-embedded FineFIX fixed tissues; FT, frozen tissue; HOPE-PE, paraffin-embedded HOPE fixed tissues; PCNA, proliferating cell nuclear antigen; RCL2-PE, paraffin-embedded RCL2 fixed tissues; STAT1, signal transducer and activator of transcription 1.

[a] Resulting *P* values from Wilcoxon pair testing for each antibody and fixative comparison and from global testing are shown for the Western blot evaluations; values of $P < .05$ were considered statistically significant and are marked in bold. FT was included only for Western blot investigations.

[b] Global tests were performed on rank-transformed variables with 10 000 random permutations.

Parallel-fixed and paraffin-embedded lung cancer specimens were evaluated for differences in histomorphology staining, immunohistochemistry, protein recovery, and Western blotting. The use of formalin-free tissue fixation can be recommended in the processing of routine pathology specimens and should give good results for routine H&E stains as well as for special stains. However, for the immunohistochemical laboratory, the protocols for each antibody in use need to be optimized. In particular, the proteinase pretreatment should be abolished completely or used only for seconds, and the heat, steam, and buffer-retrieval methods need adaptations. Because proteins in non–cross-linked tissues might more easily dissolve and leak out, false-negative values could result, but the better preservation of membranous proteins by these alternative fixatives opens up new opportunities in diagnostic pathology because these molecules, such as EGFR, HER2, and interleukin receptors, are hard to evaluate in FFPE tissues. The findings of our study clearly show that membrane-bound proteins, like EGFR and E-cadherin, can be detected more efficiently by immunohistochemistry and Western blotting (Figures 3, A and B, and 5, A through I) when the samples were fixed in FineFIX, RCL2, or HOPE. We, therefore, believe that increased immunoreactivity is a consequence of better protein or antigen preservation and easier release of the proteins through the non–cross-linking characteristics of the fixatives tested.

Table 5. Protein Yield From 10 Parallel, Fixed and Frozen, Lung Cancer Tissue Samples[a]

Sample No.	Protein Yield From Tissue Sections,[b] μg/mm²				
	FFPE	FineFix-PE	RCL2-PE	HOPE-PE	Frozen tissue
1	1.85	2.26	2.32	2.12	NA[c]
2	0.60	1.72	1.97	1.63	1.43
3	2.01	3.16	3.42	1.64	1.40
4	1.72	1.75	4.17	2.82	NA
5	1.10	1.13	1.44	1.09	NA
6	1.60	1.00	2.21	1.70	1.77
7	2.60	2.29	2.50	3.40	1.36
8	2.30	5.39	3.60	3.76	0.85
9	0.80	2.10	2.62	2.39	0.86
10	2.47	2.13	2.60	2.12	2.00

Abbreviations: FFPE, formalin-fixed, paraffin-embedded tissue; FineFIX-PE, paraffin-embedded FineFIX fixed tissues; H&E, hematoxylin-eosin stain; HOPE-PE, paraffin-embedded HOPE fixed tissues; RCL2-PE, paraffin-embedded RCL2 fixed tissues.

[a] Representative H&E reference stains are shown with the marked tumor areas for protein extraction from tissue sample 10 with matched FFPE, FineFIX-PE, RCL2-PE, HOPE-PE, and frozen tissue.

[b] Protein yield was expressed in micrograms of total protein per square millimeter of tissue section. Protein extraction was performed by the same protocol for all tissue sections. The means of the protein yields from the fixed and frozen samples are shown in Figure 2, C.

[c] NA indicates tissue samples with a tumor cell content <85%; these samples were not included in the analysis.

The overall staining intensity of EGFR using the formalin-free fixatives was greater than in FFPE fixatives in lung cancer samples (Tables 3 and 4). A recent study by Nassiri et al[21] showed a similar discrepancy for HER2/*neu* immunohistochemistry among matched UM-FIX (a universal molecular fixative marketed as Tissue-Tek Xpress Molecular Fixative; Sakura Finetek USA Inc, Torrance, California) and FFPE breast cancer samples, whereas they found no difference in the immunohistochemical reaction for the estrogen receptor. Based on our data and recent studies with the same or closely related methods, we are in favor of immunohistochemistry for different therapy-associated markers, such as EGFR, HER2/*neu*, and estrogen receptor, which will need to be optimized and completely validated regarding the definition of scores when those tissue samples are fixed in formalin substitutes.

Protein extraction (performed with the same extraction protocol as used for FFPE tissue blocks) with paraffin-embedded RCL2, paraffin-embedded FineFIX, paraffin-embedded HOPE, and frozen lung cancer tissue enabled us to detect a selected panel of 4 proteins differently expressed in lung cancer, including cytoplasmic STAT1, nuclear PCNA (Figure 4), membranous EGFR, and E-cadherin (Figure 5, A through I) proteins by Western blotting. Furthermore, the protein yield from the formalin-free fixatives was higher than that from FFPE and frozen tissues (Figure 3, C; Table 5). In this respect, our results differ from a previously published report,[22] in which the

authors found that protein yield and immunoreactivity by Western blotting in paraffin-embedded RCL2 tissue samples were clearly decreased when the protein extraction was performed with the same protein-extraction method we used in this study. At present, we do not know why the protein yield in the fixed tissue samples was significant higher than that from the frozen tissue samples. One explanation could be that the tissue or protein density was increased in fixed tissue samples by the tissue shrinking and dehydrating, whereas in the frozen tissue samples, the lower protein yield was a result of the much greater aqueous phase diluting the protein content. The protein yield from frozen tissue sections, ranging between 0.9 and 2 $\mu g/mm^2$ (Table 5), was in close agreement with those obtained by Ahram et al,[23] who calculated 1 to 1.8 $\mu g/mm^2$ for protein extraction from frozen tissue sections.

Our study, like others, was limited by evaluations that were mostly focused on one tissue type, such as fixation using FineFIX for colon cancer,[12] effusions and fine-needle aspirates,[24] HOPE in placenta,[25] lung tissue,[26] soft tissues,[27] RCL2 for normal colonic mucosa samples,[22] and breast carcinomas.[14] Therefore, further comparisons (ring trials, multicenter studies) among formalin substitutes and standard formalin for several tissue types are needed urgently. It would be of great interest to optimize and compare archival tissue fixation linked with extraction and current leader methodologies for clinical tissue specimens processed in different laboratories to rule out the less viable and cost-effective options for a replacement of good old formalin.

The present study (which was not supported by any commercial company) leads us to the conclusion that the formalin-free fixatives tested are capable of being integrated into routine pathology procedures and research. If, however, formalin is to be replaced by another fixative, all established standard processing methods will have to be adapted because they are currently all optimized for the use of FFPE tissue. Furthermore, for most formalin substitutes, the companies will not disclose the ingredients, components, or formulations, and all are more expensive than formalin. Nevertheless, according to the results presented here and in previously published data, we believe that the formalin-free fixatives tested have the potential to replace formalin in histomorphology and protein preservation.

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