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Coping with formalin banning in pathology: under vacuum long-term tissue storage with no added formalin

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Abstract

Formalin is toxic and has recently been classified as carcinogenic leading to a proposed European formalin ban. But, the pathology use of formalin has however been completely overlooked, and this is proving to be a relevant issue, as no alternative, reliable, tissue fixative is available. Various systems have been proposed to reduce formalin use and exposure; long-term storage and disposal of formalin is also a problem. With this in mind, under vacuum sealing (UVS) systems have been proposed for transportation/storage, however, for how long tissue retains its characteristics (morphological and molecular) is unknown. This study aims to compare histology specimens stored by formalin immersion (FI) and specimens stored after fixation with UVS technique with no additional formalin, at different time periods. Twenty tissue samples (10FI; 10UVS) were stored for different time periods (15 days, 1–2–3–6–12 months) for a total of 120 samples, compared with regard to their morphology, histochemistry, immunoreactivity (24 specific antibodies) and DNA status. All samples showed well-preserved morphology and overlapping staining quality. A significant reduction in immunoreactivity was however identified in the various time periods, particularly for heat pre-treated nuclear antigens, and this commenced earlier (1 month) for FI. UVS storage showed higher DNA content than FI but slightly poorer DNA integrity. These results add important knowledge to the use of UVS in daily practice, as long-term storage of pre-fixed tissue in UVS is not detrimental to the quality of tissue while having the boon of using very little formalin with less operator exposure and lower disposal costs.

Keywords Formalin ban · Vacuum storage · Safety · Pathology

Introduction

Formaldehyde is a chemical product used for a multitude of purposes in industry, manufacturing and the health care sector. In the latter, formaldehyde is utilized for its preservative and anti-bacterial properties. In particular, formaldehyde (as a 40% by volume aqueous solution named formalin), is extensively used in pathology laboratories for specimen fixation. Formalin is, indeed, the best available tissue fixative in pathology procedures thanks to its numerous qualities, including long-lasting preservation of cell morphology and tissue architecture, antiseptic properties, easy storage and low cost (Grizzle 2009). Histochemistry, immunohistochemistry and molecular processes have therefore been standardized for formalin-fixed tissue.

Over time, formalin has presented criticalities regarding both tissue management (e.g., antigen masking) and, even more important, safety issues (toxicity and carcinogenicity) (Grillo et al. 2015; Shi et al. 2007; IARC Working Group 2006). Extensive formalin exposure has emerged as a major health risk factor, as declared by the 2006 IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. According to this document, the major risk derives from the inhalation of formalin both for its direct toxicity (irritation of nose, throat and eyes), its suspected ability to

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cause genetic defects and for its mutagenic potential (nasopharyngeal cancer). In addition, exposure to formalin causes contact dermatitis and it is probably also a skin sensitizer; other correlations have been evaluated, but they lack strong evidence (e.g., leukaemia). Based on these observations, formalin has been classified as a class 1 carcinogenic agent. According to this, the European Union (European Chemicals Agency; Scientific Committee on Occupational Exposure Limits—SCOEL—Recommendation 125 on Formaldehyde, adopted 30 June 2016) has proposed formalin banning and, in accordance with these suggestions, the new guidelines of the Italian Public Health Ministry, published in 2015 (http://www.salute.gov.it/imgs/C_17_pubblicazioni_2369_allegato.pdf), exhort every formaldehyde user to immediately minimize utilization and, after a 3-year period, to achieve a complete formalin ban (IARC Working Group 2006; European Regulations 2008; Baan et al. 2009).

Medical use of formalin has however been completely overlooked. This is proving a relevant issue for pathology, as no alternative fixative to formalin is as yet available. The European Society of Pathology (ESP), together with the European Union of Medical Specialists (UEMS) have drafted a document (https://www.uems.eu/__data/assets/pdf_file/0005/39641/Draft-statement-Formalin-Banning-UEMS-Council.pdf) detailing the irreplaceability of formalin in pathology as well as lobbying the European Parliament and Council for specific legislation.

Procedures which have been standardized for formalin-fixed tissue and are fundamental in diagnosis, prognostic stratification and therapeutic choice, are immunohistochemistry and molecular biology (DNA and RNA extraction). In immunohistochemistry, formalin fixation creates unspecific cross links between formalin and proteins which may lead to antigen masking depending on time and temperature of fixation (Sompuram et al. 2004). Standardized and automated procedures have been implemented to overcome antigen masking by carrying out antigen retrieval based on heat treatment and/or enzymatic digestion. Epitope masking especially affects formalin-fixed tissue stored for lengthy periods of time and this may pose a serious problem when tissue is over-fixed (e.g., transfer to pathology services in other hospitals or re-sampling of specimen, etc.) (Grillo et al. 2017). In molecular biology, excessive or insufficient fixation may lead to DNA and RNA degradation and poor quality of DNA for subsequent PCR analysis.

Therefore, development of new procedures to reduce formalin quantity and exposure has been proposed and implemented in all stages of the process (e.g., protective procedures, both of individual and collective types). Among the various instruments useful in decreasing the quantity of formalin used in the pathology lab, under vacuum sealing of tissue (UVS) has been shown to be a valid procedure (Bussolati et al. 2008; Di Novi et al. 2010; Veneroni et al. 2016). UVS minimizes formalin use during fixation, as it involves automated machinery which requires less formalin and a safely sealing process; it has also been shown to reduce cold ischemia damage (Bussolati et al. 2015) and reduce costs of formalin-associated waste disposal.

TissueSafe and SealSafe (Milestone, Bergamo, Italy) are commercial UVS instruments. The TissueSafe unit guarantees sealed, formalin-free and temperature-controlled transfer from the operating theatre to the pathology laboratory. SealSafe can work both with a controlled-minimized quantity of formalin and without formalin, permitting transport, handling and storage of histology specimens in safety conditions (Kristensen et al. 2011; Zarbo 2015). For storage purposes in particular, vacuum-sealed, formalin-fixed tissue can be stocked without the necessity of additional formalin. Leftover tissue may be stocked for a variable amount of time depending on turnaround times for diagnosis or specific laboratory procedures (some labs require that all leftover tissue is stocked for at least 1 month following diagnosis). Re-sampling of leftover tissue is sometimes necessary and this may take place even after some time from initial fixation.

While the advantages of the vacuum system, in terms of reduction of cold ischemia damage and formalin use, have been well-documented, little information is available on whether vacuum-sealed fixed tissue, stored without additional formalin, modifies tissue antigenicity in the long term.

Furthermore, little is known about DNA preservation. A recent study (Metovic et al. 2018) explored the extraction of nucleic acids for molecular testing, showing no differences in quality of DNA and RNA between vacuum storage for up to 1 month and standard immersion formalin fixing. No study has however explored at which time point DNA quality becomes a problem for extraction in vacuum-stored tissues.

The aim of this study is therefore to compare histology, histochemistry, immunoreactivity and DNA status of histology specimens stored by formalin immersion and specimens stored after fixation with UVS technique with no additional formalin, at different time periods up to 12 months.

Materials and methods

Study design

The study was prospectively designed using leftover tissue from surgical specimens of patients who were anonymized and had given consent for use of tissue for research purposes. All procedures performed were in agreement with the ethical guidelines of the Declaration of Helsinki and its later amendments. A formal institutional ethics committee approval was not required, as only anonymized leftover tissue with no clinical correlation or implications was used.

Ten surgical specimens, with known cold ischemia time between 2 and 4 h, were selected from the routine workload submitted to the pathology laboratory during one work day. The collected specimens included cases with more than abundant leftover tissue, both from neoplastic (leiomyoma, gastrointestinal stromal tumour - GIST, colon cancer) and non-neoplastic tissues (stomach, colon, skin, uterus with cervix, bladder and breast). All tissue arrived fresh and vacuum sealed. Samples were routinely examined and formalin fixed under vacuum conditions for 24 h using the SealSafe unit (Milestone, Bergamo, Italy), which uses specific vacuum soft plastic bags to contain the specimen. The bag is then placed inside the sealed chamber of the unit; it is automatically weighed and formalin is added in different proportions according to weight. The SealSafe in our work practice is pre-set to a 1:2,5 formalin ratio to fix and a 1:1 or no added formalin to stock. Formalin is added in a safe leak-proof environment, the specimen is placed under vacuum and the bag is thermo-sealed. Bags may be cut open and re-thermo-sealed up to three to four times depending on the bag size. After sampling for diagnosis, further samples were collected from the 10 surgical specimens to represent all aforementioned neoplastic and non-neoplastic tissues (6 couples of matched tissues from each of the 10 surgical specimens, total 120 samples).

Six samples were UVS stored with no additional formalin (using SealSafe), while the other six were routinely stored using the formalin immersion technique, using airtight, plastic containers, filled with a 1:10 formalin ratio.

A further sample from each surgical specimen was immediately routinely processed and paraffin embedded; this sample was used as control (named T0). At set time intervals, 15 days (T1), 30 days (T2), 2 months (T3), 3 months (T4), 6 months (T5) and 12 months (T6), one sample from the UVS-stored batch and a matching sample from the formalin-immersion (FI) batch for each of the ten surgical specimens were routinely processed. At study conception, samples from each time period were sealed in six different bags, one for each time set, so that there would be no need for un-sealing or re-sealing of bags to take out contents. FFPE blocks were stored until the end of sample accrual in closed cardboard boxes. The entire procedure is schematically represented in Fig. 1.

All FFPE blocks were collected at the end of the 12-month time period and a haematoxylin–eosin stained section was performed for every specimen to evaluate sample histology and morphology. This procedure was adopted to standardize the immunohistochemistry run; previous experiences from our group (Grillo et al. 2017) have demonstrated no loss/reduction of immunoreactivity in FFPE blocks preserved for 1 year or more (up to 5–6 years).

On the basis of histologic tissue, evaluated on haematoxylin–eosin stained sections, samples were used to test different antibodies.

Histology, histochemistry and immunohistochemistry

All collected FFPE blocks were microtome sectioned in a single day by a single medical laboratory scientist with the same microtome and mounted on Superfrost slides (Thermo Scientific, Braunschweig, Germany) (Grillo et al. 2015; Gambella et al. 2017).

The first section of each block was stained with haematoxylin and eosin for morphological evaluation of the tissue. Tissue type was annotated; cell morphology and quality of staining were evaluated. Furthermore, on selected samples from set time intervals T1–T2–T3 and T6, histochemical staining for Alcian Blue-Periodic Acid Schiff, Masson's Trichrome and elastic fibre stain was performed.

Immunohistochemistry was carried out with the automated BenchMark Ultra Immunostainer® (Ventana Medical Systems, Tuscon, Arizona, USA) using 24 sample-specific antibodies as shown in Table 1. The system uses the Ultraview universal DAB detection kit (Ventana Medical Systems, Arizona, USA) which is a biotin-free indirect method. Heat and enzymatic pre-treatment is carried out specifically by the automated immunostainer and reactions routinely performed as previously detailed (Grillo et al. 2015).

Antibodies were chosen to display membranous, cytoplasmic and nuclear staining as well as to reflect different diagnostic, prognostic or predictive intents of immunohistochemistry. Chosen antibodies required heat or enzymatic antigen retrieval or required neither of the above.

Histochemical and immunohistochemical evaluation

All histochemical stains and immunostains were evaluated simultaneously by two pathologists (FG and LM) blinded as to the type of formalin fixation and time set except for T0 control sections. Any discrepancies in evaluation were resolved immediately by consensus. Histochemical stains were evaluated for intensity and appropriateness of staining. Immunosections stained at T0 were considered optimal (control sections: +++) and compared with the staining intensity of each antibody at each cutoff time.

Intensity was semi-quantitatively evaluated using a numerical scale between 0 and 3+ on a 4° scale as follows:

- −: no staining (negative)
- +: very low staining (low intensity and/or irregular) which does not permit diagnostic evaluation
- ++: low staining as compared with control, but evaluable

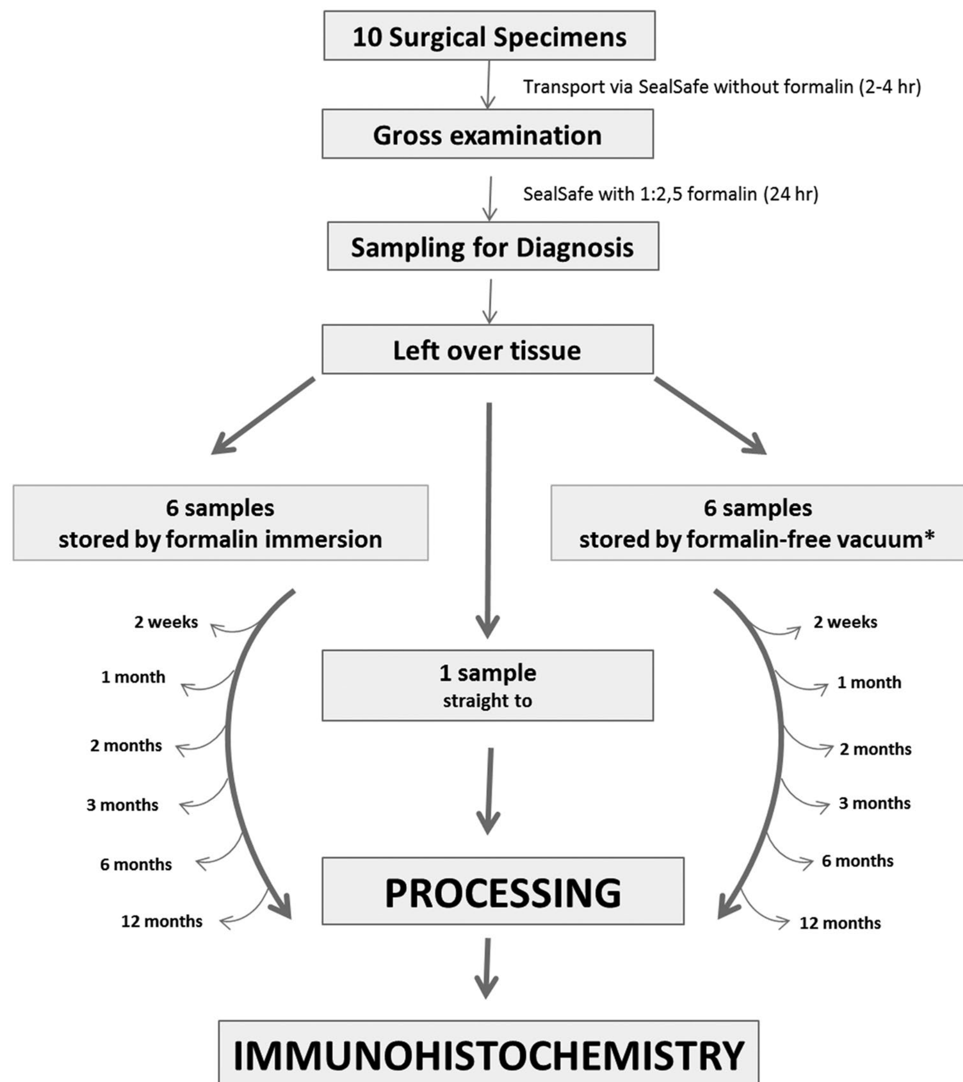

Fig. 1 Schematic representation of study procedures. Asterisk: Under vacuum storage of previously formalin-fixed samples with no added formalin during storage

- +++: normal staining as compared with control

DNA extraction, quantification and quality

Genomic DNA was isolated from FFPE blocks of colorectal cancer at time point 0, and for all time sets (T1–T6) both for UVS stored with no added formalin tissue and FI fixed tissues. From these blocks three, 8-μm-thick sections were microtome cut and placed in sterile Eppendorf tubes (Eppendorf srl, Hamburg, Germany). DNA was extracted using MagCore HF16 Plus (Diatech Lab Line srl, Ancona, Italy) in 1.5 ml centrifuge tube as per protocol given in the kit handbook. DNA quantification was determined by SPECTROstar Nano (BMG Labtech, Ortenberg, Germany) for samples at T0, T1, T2, T5 and T6.

The UV-absorbance method uses a spectrophotometer which depends on light absorption by DNA at 260 nm. The following ratios are used to determine the purity of the nucleic acid:

- ratio A260/A230 = index of contamination from carbohydrates and phenols (solvents); the optimal value of this ratio is about 2.2, lower ratios indicate solvent contamination
- ratio A260/A280 = index of protein contamination; the ratio should be 1.6–1.8 for fresh tissue DNA, however for FFPE tissue, this ratio is lower.

Statistical differences between dsDNA concentration and contamination indexes were tested using the *T* test. Differences were considered significant if $p < 0.05$.

DNA sample degradation status was assessed for all samples in all time sets (T1–T6) and T0 control by evaluating DNA integrity number (DIN) value on TapeStation 2200 (Agilent Technologies, Santa Clara, CA, USA) using

Table 1 List of antibodies (clone, origin, pre-treatment, incubation time and antigen site) tested on specific tissue types

Type of tissue	Antibody	Clone	Origin	Heat pre-treatment (min)	Enzymatic pre-treatment	Incubation time (min)	Antigen site
Normal colonic wall	Smooth muscle actin	1A4	Cell marque	N	N	60	C
Colon cancer	Pan cytokeratin	AE1/AE3	Ventana	N	Y	20	C
Normal breast	Cytokeratin 7	SP52	Cell marque	60	Y	32	C
Colon cancer	Cytokeratin 20	SP33	Cell marque	60	Y	32	C
Normal skin	Cytokeratin CAM 5.2	B22.1 and B23.1	Cell marque	N	Y	32	C
Colon cancer	CEA	CEA31	Cell marque	30	N	20	C
Colon normal mucosa	CDX2	EPR2764Y	Cell marque	60	N	32	Nu
Normal stomach mucosa	Chromogranin	LK2H10	Cell marque	30	N	8	C
Gastrointestinal stromal tumour	CD117	POLICL	Cell marque	30	N	32	C
Gastrointestinal stromal tumour	DOG 1	SP31	Cell marque	60	N	32	C
Normal stomach wall	CD3	2GV6	Ventana	60	N	32	M
Normal stomach wall	CD20	L26	Ventana	60	N	24	M
Uterine leiomyoma	CD31	JC70	Cell marque	30	N	32	M
Normal uterine wall	Factor VIII	POLICL.	Cell marque	N	Y	24	C
Colon cancer	Ki67	30 – 9	Ventana	30	N	16	Nu
Colon cancer	MLH 1	M1	Ventana	60	N	80	Nu
Colon cancer	MSH 2	G219-1129	Cell marque	30	N	60	Nu
Colon cancer	MSH 6	44	Ventana	30	N	20	Nu
Colon cancer	PMS 2	EPR3947	Cell marque	30	N	40	Nu
Normal breast	p63	4 A4	Ventana	30	N	32	Nu
Normal skin	S100	4C4.9	Ventana	30	N	16	C
Normal breast	ER	SP1	Ventana	60	N	16	Nu
Normal breast	PR	1E2	Ventana	30	N	32	Nu
Normal uterine wall	Vimentin	V9	Ventana	30	N	16	C

N no, *Y* yes, *C* cytoplasmic, *M* membrane, *Nu* nuclear

the Genomic DNA ScreenTape assay (Agilent Technologies). DIN was represented as a DIN scale—DIN 1–10. A high DIN indicates highly intact DNA, and a low DIN a strongly degraded DNA sample. Statistical differences between DINs for UVS and FI were tested using the *T* test. Differences were considered significant if $p < 0.05$.

Results

Histology

All samples, at all time intervals, presented well-preserved morphology with clearly recognizable tissue components (Fig. 2a). No microtome cutting artefacts were identified nor formalin precipitates in both UVS stored and FI batches. Comprehensively, haematoxylin and eosin-stained slides were equivalent with regard to colour, intensity, contrast and sharpness at all time periods and for both storage methods.

Urinary bladder samples did not show superficial epithelium due to diffuse ulceration and for this reason these samples were not used for immunohistochemistry evaluation.

Histochemistry

No problems in histochemical staining were identified in all samples for the tested stains which showed same intensity, appropriateness and definition of tissue component staining both for UVS (Fig. 2b, c) and the formalin immersion batches, equal to the control sample.

Immunohistochemistry

All immunohistochemical evaluations are summarized in Table 2.

No differences in immunoreactivity were seen at the first 15-day (T1) checkpoint. Starting from T2 (1 month), a reduction in immunostaining intensity was noted for some nuclear antigens (MSH2, PMS2 and p63) and this was slightly more evident in the FI samples. From time point T3 (2 months) on

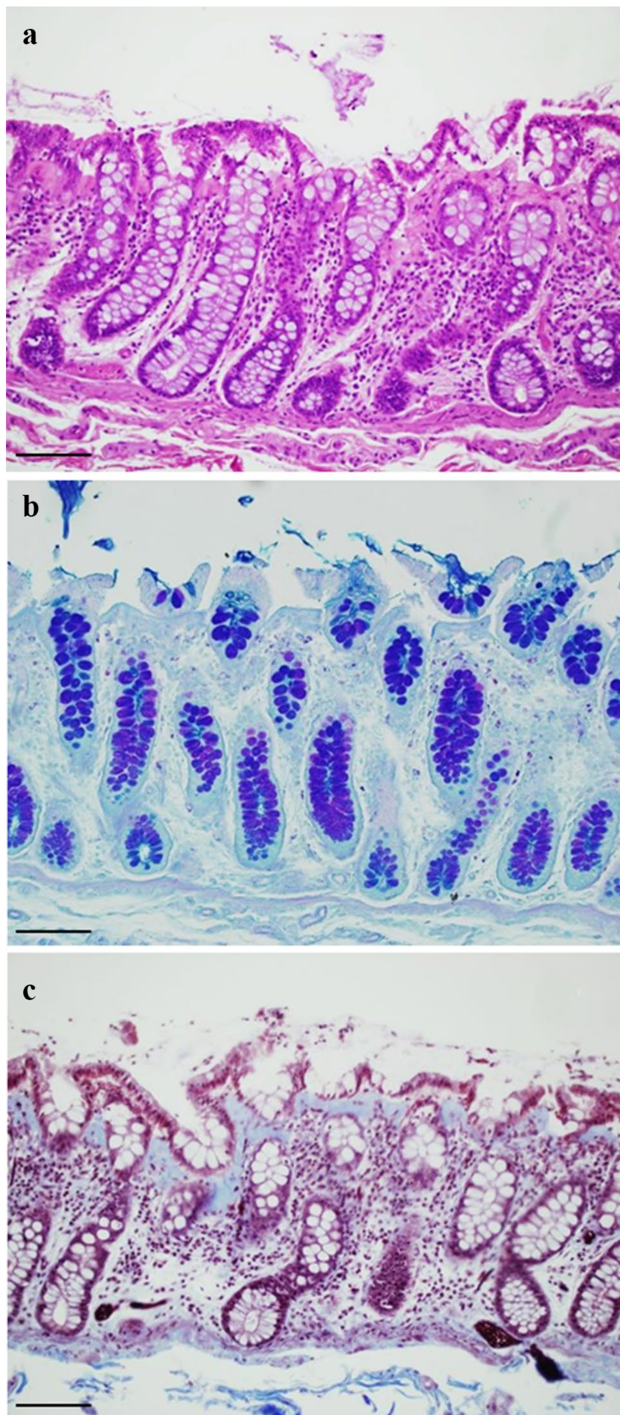

Fig. 2 Preservation of morphology and cyto-architectural details in samples stored under vacuum (UVS) for 12 months. **a** Haematoxylin and eosin stain, magnification ×20; **b** Alcian Blue-Periodic Acid Schiff stain, magnification ×20; **c** Trichrome stain, magnification ×20. Scale bar represents 100 µm

and up to T6 (12 months), a variable and sometimes marked decrease in staining intensity was noted for all nine tested nuclear antibodies (Fig. 3); in particular complete or marked loss, sufficient to render diagnostic evaluation impossible, was observed for Ki67, MSH2, MSH6, PMS2, p63 and ER, starting from 2 to 3 months. No significant differences in immunostaining were noted between UVS and FI samples, even though the UVS samples seem to maintain immunoreactivity for longer. Immunosignal decrease or loss did not necessarily follow a constant reduction gradient with some antibodies (e.g., Ki67 and ER) showing fluctuating staining at consecutive time points.

Only in three of the cytoplasmic/membranous antigens tested, namely CD117, DOG1 and CD3, was reduction of immunosignal observed, and this was generally mild and limited to FI fixed samples.

On the whole, all antigens for which reduction in immunoreactivity was observed require heat pre-treatment as an antigen retrieval system; no antibody requiring enzymatic pre-treatment or no pre-treatment showed immunostaining reduction.

DNA extraction and quality

Comparing DNA extraction between UVS and FI samples (Table 3), UVS samples showed higher dsDNA concentration at all time intervals and this was statistically significant ($p = 0.0288$). While no significant differences in protein contamination index ($p = 0.4$) were seen at all time intervals between UVS and FI, lower solvent contamination was present in UVS samples until 6 months (but this was not statistically different to FI—$p = 0.94$). In all samples, DNA was sufficient for molecular analysis.

Statistical differences ($p < 0.05$) in DIN were seen between UVS samples and FI samples at different time points (see Fig. 4). FI seems to show marginally less DNA fragmentation compared to UVS.

Discussion

Modern pathology requires optimal preservation of tissues, so that morphology and molecular status are maintained and quantifiable. The most used fixative is indeed formalin (the 4% neutral buffered aqueous solution of formaldehyde), which is inexpensive and widely used even though concerns regarding its toxicity/cancerogenicity have come into the limelight in the last decade.

In the last 30 years, many fixatives have been tested to find viable alternatives to formalin. Microwave treatment coupled with UMFIX (universal molecular fixative), a mixture of methanol and polyethylene glycol (Vincek et al. 2003), ice-cold methanol (Tanaka et al. 2010), RCL2 (a French proprietary formula based on acetic acid), ethyl alcohol, non-reducing carbohydrates (Zanini et al. 2012), glutaraldehyde (Smith and Reese 1980), and other

Table 2 Differences in immunoreactivity (as compared with control at time 0) related to fixation procedures, time interval and type of antibody

Antibodies	Fixative procedure	T1	T2	T3	T4	T5	T6
Smooth muscle actin	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Cytokeratin AE1/AE3	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Cytokeratin 7	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Cytokeratin 20	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Cytokeratin CAM5.2	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Carcinoembryonic antigen (CEA)	FI	+++	+++	++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
CDX2	FI	+++	+++	+++	++	++	+
	UVS	+++	+++	+++	++	+	+
Chromogranin-A	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
CD117	FI	+++	+++	++	++	+	+
	UVS	+++	+++	+++	+++	+++	+++
DOG1	FI	+++	+++	+++	++	++	++
	UVS	+++	+++	+++	+++	+++	+++
CD3	FI	+++	+++	+++	+++	+++	++
	UVS	+++	+++	+++	+++	++	++
CD20	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
CD31	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Factor VIII	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Ki67	FI	+++	+++	+	−	−	+
	UVS	+++	+++	+	−	−	+
MLH1	FI	+++	+++	+++	++	++	+++
	UVS	+++	+++	+++	++	+++	+++
MSH2	FI	+++	++	++	−	−	−
	UVS	+++	++	+	−	−	−
MSH6	FI	+++	+++	++	−	+	+
	UVS	+++	+++	+	−	+	++
PMS2	FI	+++	+	++	+	+	+
	UVS	+++	++	++	+	++	++
p63	FI	+++	++	−	−	−	−
	UVS	+++	+++	−	−	−	−
S-100 protein	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++
Oestrogen receptor	FI	+++	+++	++	+	+	+
	UVS	+++	+++	+++	+	++	+
Progesterone receptor	FI	+++	+++	++	++	++	+
	UVS	+++	+++	++	++	++	+
Vimentin	FI	+++	+++	+++	+++	+++	+++
	UVS	+++	+++	+++	+++	+++	+++

FI formalin immersion of tissue, *UVS* under vacuum sealing of tissue, *T1* 15 days, *T2* 1 month, *T3* 2 months, *T4* 3 months, *T5* 6 months, *T6* 12 months, − no staining (negative), + very low staining, ++ low staining, +++ intense staining

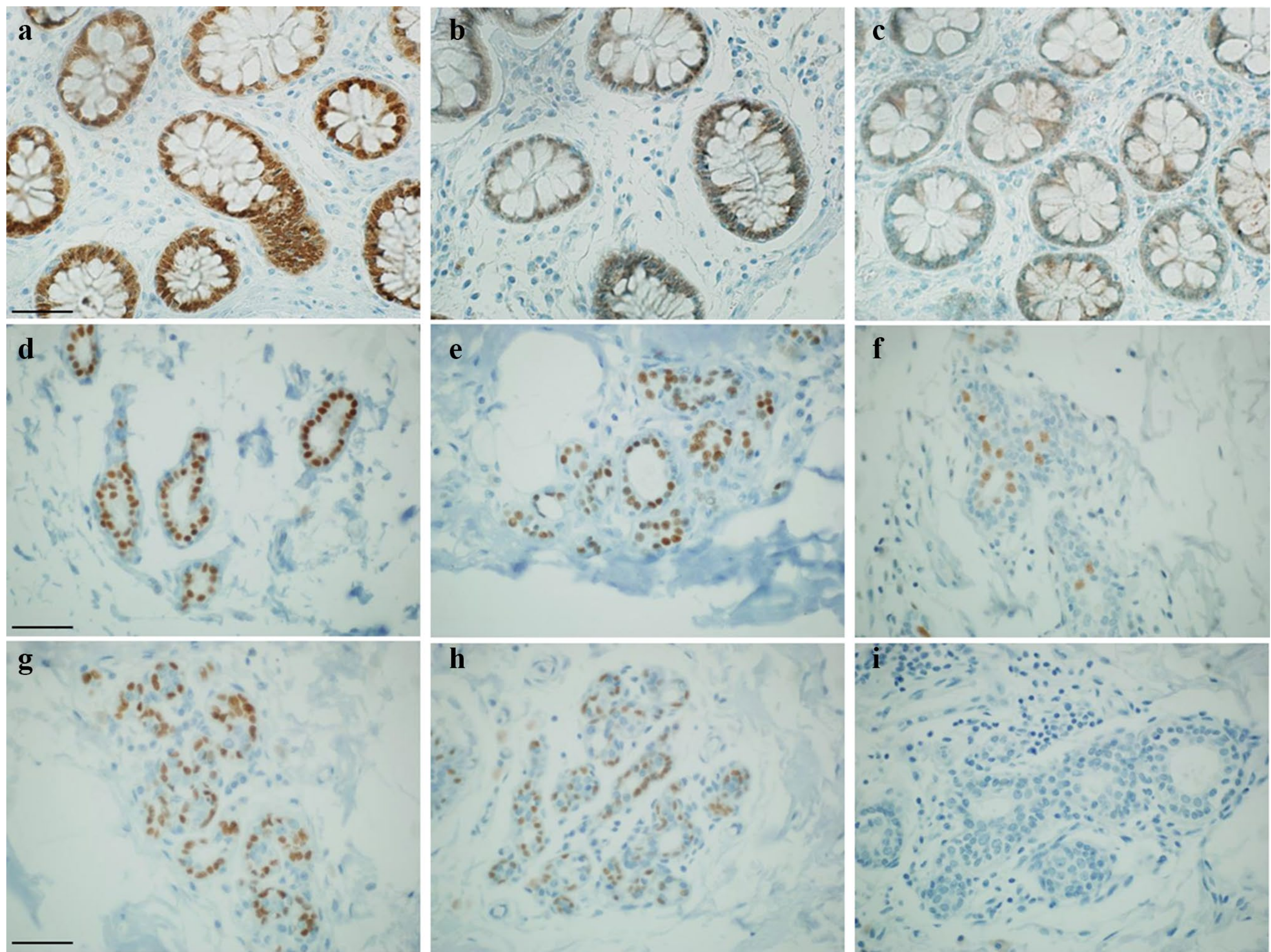

Fig. 3 Reduction of immunostaining intensity in both under vacuum (UVS) and formalin immersion (FI) stored samples at different time sets. CDX2 immunostaining, magnification ×60 at: T0, score 3+ (**a**); T4 UVS, score 2+ (**b**); T6 UVS, score 1+ (**c**). Oestrogen receptor immunostaining, magnification ×60 at: T0, score 3+ (**d**); T5 UVS, score 2+ (**e**); T6 UVS, score 1+ (**f**). p63 immunostaining, magnification ×60 at: T0, score 3+ (**g**); T1 FI, score 2+ (**h**); T3 FI, score 0 (**i**). Scale bar represents 50 µm. *FI* formalin immersion of tissue, *UVS* under vacuum sealing of tissue

Table 3 DNA extraction, concentration and quality in colorectal cancer samples, related to fixation procedures and time intervals

Sample	dsDNA concentration: 50× (ng/µl)	Ratio 260/230	Ratio 260/280
T0	59.7	1.0822	1.6372
T1 (UVS)	34.4	1.0115	1.6192
T2 (UVS)	39.8	0.8529	1.5197
T5 (UVS)	43.3	1.1035	1.5298
T6 (UVS)	21.3	0.6167	1.3100
T1 (FI)	22.5	0.7233	1.6247
T2 (FI)	25.9	0.7483	1.5266
T5 (FI)	24.5	0.7817	1.4616
T6 (FI)	17.5	0.6887	1.3464

UVS under vacuum sealing of tissue, *FI* formalin immersion of tissue, *T0* tissue control at time 0, *T1* 15 days, *T2* 1 month, *T5* 6 months, *T6* 12 months

aldehydes have been used, pure or mixed with paraformaldehyde, but none of these have shown sufficient preservation of both tissue morphology and antigenicity, and none are comparable to the gold standard formalin. Moreover, some of the commercially available products are "secret mixtures", as registered in proprietary formulas, with severe limitations to reproducibility studies (Kiernan 2008). Among the various compounds, glyoxal (Richter et al. 2018) seems to provide the best performance in terms of suitability and efficiency, in particular for tissue morphology, immunohistochemistry and molecular biology. In spite of these promising results, a wider spectrum of antibodies and molecular tests needs to be performed, especially considering that the handling of glyoxal is challenging (Bussolati et al. 2017). Finally, the use of these substances is as yet not widely present in pathology labs

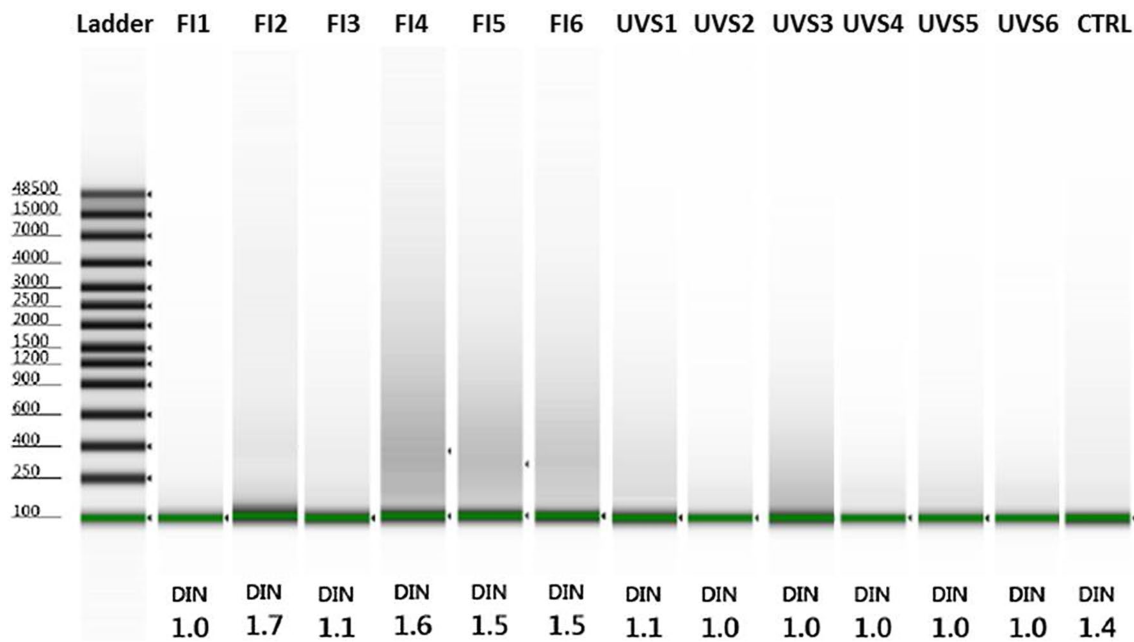

Fig. 4 DNA integrity number for each sample. DIN scale is from 1 to 10 with 1 showing most degraded DNA and 10 with highest DNA integrity. *DIN* DNA integrity number, *FI* formalin immersion, *UVS* under vacuum storage, *CTR* T0 control

probably due to the absence of precise protocols, validation of methods and cost analysis.

The group from Turin have shown that under vacuum transport and storage (Bussolati et al. 2008, 2015; Metovic et al. 2018) are effective and comparable to routine quality assured immersion formalin fixation. Advantages of the UVS technique are important; UVS uses reduced formalin volumes for primary fixation of samples, it allows storage of sampled, leftover specimens in plastic bags with no additional formalin and requires less storage space compared to plastic containers as well as reducing formalin exposure risks. Furthermore, it reduces the quantity of formalin waste which requires specific, and expensive disposal.

Metovic et al. (2018) have shown that formalin-fixed tissue preserved for up to 1 month in UVS, liquid-free environment, retains antigenicity for immunohistochemistry and DNA and RNA integrity for extraction and molecular analyses. This study details the example of tissue transfer to referral or centralized pathology laboratories which requires longer time periods, often, but not always, within the observed 1 month time period. Furthermore, in some cases, for example, in the research setting, tissue banking or samples with medico-legal implications, tissue may be stored for more than 1 month. Indeed, our study reproduces and completes the Turin groups observations, extending the number of antigens evaluated, lengthening the time intervals and comparing with traditional FI storage.

So what happens when tissue is fixed for longer than 1 month, with no added formalin, and does this type of fixation show advantages with respect to the routine FI technique? Overfixation in formalin has been shown to determine reduction in antigenicity in numerous studies and this is one of the main reasons why the pre-analytical phase has been greatly emphasized in the recent literature. Our results demonstrate that morphology and histochemistry are completely preserved and undistinguishable from routine fixation with no differences between UVS and FI long term fixation techniques. On the other hand, both UVS and FI cause a severe reduction in immunoreactivity, in particular but not limited to nuclear antigens which require heat pre-treatment and this phenomenon becomes apparent starting from 1 month and worsening with age.

Quantity of extracted DNA is reduced in stored tissues both with UVS and FI compared to T0 control, however, vacuum storage performs better in as much as dsDNA concentrations are higher and less solvent contamination is seen in these samples compared to equal time interval FI samples. With regards to DIN, both fixation systems show fragmentation and were both similar to the DIN identified in the control tissue (T0). Unexpectedly, FI seems to preserve DNA integrity marginally better than UVS. We have no clear explanation for these findings, however, the limited number of samples analysed may play a role. What must be underlined is that the differences in DIN are minimal when one considers that the overall DIN scale is from 1 to 10 and that T0 control had similar results. Both preservation methods, while not perfect, still permit molecular analyses on DNA.

With regards to costs, UVS systems permit expense cutting in various areas. A cost comparison was performed when the UVS system was first introduced in our pathology lab (not as part of this study). Briefly, a saving of 0.34 € per container (vacuum bags compared to rigid plastic buckets) for specimen transport and storage and approximately a 33% reduction in formalin costs associated with the UVS system were identified. Neither the reduction in expenditure connected with less formalin waste nor the reduction in operator costs (in terms of easier transport, space-saving and less formalin exposure) have yet been quantified.

In conclusion, UVS is a well-performing technique for safe tissue transport and fixation. Quality of histology, histochemistry and immunohistochemistry is comparable with standard FI procedure with the well-known ageing-related reduced immunoreactivity for nuclear antigens requiring heat pre-treatment. DNA quantity is better in UVS samples up to 1 year of storage, while DNA integrity is fair in all samples. These results add important knowledge to the use of UVS in daily practice, as long-term storage of pre-fixed tissue in UVS with no added formalin is not detrimental to the quality of tissue (or at least not more so than the usual formalin immersion storage), both with regard to its morphology and molecular characteristics, having however the boon of using very little quantity of formalin with less operator exposure and lower disposal costs.

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Compliance with ethical standards

Conflict of interest No conflict to disclose.

References

Baan R, Grosse Y, Straif K, Secretan B, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V, WHO International Agency for Research on Cancer Monograph Working Group (2009) A review of human carcinogens—part F: chemical agents and related occupations. Lancet Oncol 10:1143–1144. https://doi.org/10.1016/S1470-2045(09)70358-4

Bussolati G, Chiusa L, Cimino A, D'Armento G (2008) Tissue transfer to pathology labs: under vacuum is the safe alternative to formalin. Virchows Arch 452:229–231. https://doi.org/10.1007/s00428-007-0529-x

Bussolati G, Annaratone L, Maletta F (2015) The pre-analytical phase in surgical pathology. Recent Results Cancer Res 199:1–13. https://doi.org/10.1007/978-3-319-13957-9_1

Bussolati G, Annaratone L, Berrino E, Miglio U, Panero M, Cupo M et al (2017) Acid-free glyoxal as a substitute of formalin for structural and molecular preservation in tissue samples. PLoS ONE 12(8):e0182965. https://doi.org/10.1371/journal.pone.0182965

Di Novi C, Minniti D, Barbaro S, Zampirolo MG, Cimino A, Bussolati G (2010) Vacuum-based preservation of surgical specimens: an environmentally-safe step towards a formalin-free hospital. Sci Total Environ 408:3092–3095. https://doi.org/10.1016/j.scitotenv.2010.04.022

European Parliament and Council of European Union (2008) Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Off J Eur Union L 353:1–1355

Gambella A, Porro L, Pigozzi S, Fiocca R, Grillo F, Mastracci L (2017) Section detachment in immunohistochemistry: causes, troubleshooting, and problem-solving. Histochem Cell Biol 148:95–101. https://doi.org/10.1007/s00418-017-1558

Grillo F, Pigozzi S, Ceriolo P, Calamaro P, Fiocca R, Mastracci L (2015) Factors affecting immunoreactivity in long-term storage of formalin-fixed paraffin-embedded tissue sections. Histochem Cell Biol 144:93–99. https://doi.org/10.1007/s00418-015-1316-4

Grillo F, Bruzzone M, Pigozzi S, Prosapio S, Migliora P, Fiocca R, Mastracci L (2017) Immunohistochemistry on old archival paraffin blocks: is there an expiry date? J Clin Pathol 70:988–993. https://doi.org/10.1136/jclinpath-2017-204387

Grizzle WE (2009) Special symposium: fixation and tissue processing models. Biotech Histochem 84:185–193. https://doi.org/10.3109/10520290903039052

IARC Working Group on the Evaluation of Carcinogenic Risk to Humans (2006) Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol IARC monographs on the evaluation of carcinogenic risks to humans, vol 88. International Agency for Research on Cancer, Lyon

Kiernan JA (2008) Theory and practice. Histological and histochemical methods. Scion Publishing, Bloxham, pp 12–44, 431–437

Kristensen T, Engvad B, Nielsen O, Pless T, Walter S, Bak M (2011) Vacuum sealing and cooling as methods to preserve surgical specimens. Appl Immunohistochem Mol Morphol 19:460–469. https://doi.org/10.1097/PAI.0b013e318214e523

Metovic J, Bertero L, Musuraca C, Veneziano F, Annaratone L, Mariani S, Cassoni P, Bussolati G, Papotti M (2018) Safe transportation of formalin-fixed liquid-free pathology specimens. Virchows Arch 473:105–113. https://doi.org/10.1007/s00428-018-2383-4

Richter KN, Revelo NH, Seitz KJ, Helm MS, Sarkar D, Saleeb RS, D'Este E, Eberle J, Wagner E, Vogl C, Lazaro DF, Richter F, Coy-Vergara J, Coceano G, Boyden ES, Duncan RR, Hell SW, Lauterbach MA, Lehnart SE, Moser T, Outeiro TF, Rehling P, Schwappach B, Testa I, Zapiec B, Rizzoli SO (2018) Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy. EMBO J 37(1):139–159. https://doi.org/10.15252/embj.201695709

Shi SR, Liu C, Taylor CR (2007) Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: from experiments to hypothesis. J Histochem Cytochem 55:105–109. https://doi.org/10.1369/jhc.6P7080.2006

Smith JE, Reese TS (1980) Use of aldehyde fixatives to determine the rate of synaptic transmitter release. J Exp Biol 89:19–29

Sompuram SS, Vani K, Messana E, Bogen SA (2004) A molecular mechanism of formalin fixation and antigen retrieval. Am J Clin Pathol 121:190–199. https://doi.org/10.1309/BRN7-CTX1-E84N-WWPL

Tanaka KA, Suzuki KG, Shirai YM, Shibutani ST, Miyahara MS, Tsuboi H, Yahara M, Yoshimura A, Mayor S, Fujiwara TK, Kusumi A (2010) Membrane molecules mobile even after chemical fixation. Nat Methods 7(11):865–866. https://doi.org/10.1038/nmeth.f.314

Veneroni S, Dugo M, Daidone MG, Iorio E, Valeri B, Pinciroli P, De Bortoli M, Marchesi E, Miodini P, Taverna E, Ricci A, Canevari

S, Pelosi G, Bongarzone I (2016) Applicability of under vacuum fresh tissue sealing and cooling to omics analysis of tumor tissues. Biopreserv Biobank 14:480–490. https://doi.org/10.1089/bio.2015.0093

Vincek V, Nassiri M, Nadji M, Morales AR (2003) A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples. Lab Investig 83:1427–1435. https://doi.org/10.1097/01.LAB.0000090154.55436.D1

Zanini C, Gerbaudo E, Ercole E, Vendramin A, Forni M (2012) Evaluation of two commercial and three home-made fixatives for the substitution of formalin: a formaldehyde–free laboratory is possible. Environ Health 11:59. https://doi.org/10.1186/1476-069X-11-59

Zarbo RJ (2015) Histologic validation of vacuum sealed, formalin-free tissue preservation, and transport system. Recent Results Cancer Res 199:15–26. https://doi.org/10.1007/978-3-319-13957-9_2

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