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TITLE

Decreasing formalin concentration improves quality of DNA extracted from formalin-fixed paraffin-embedded tissue specimens without compromising tissue morphology or immunohistochemical staining.

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ABSTRACT

Genomic technologies are increasingly used clinically for both diagnosis and guiding cancer therapy. However, formalin fixation can compromise DNA quality. This study aimed to optimise tissue fixation using normal colon, liver and uterus (n=8 each) by varying neutral buffered formalin (NBF) concentration (1-5% w/v) and fixation time (24-48h). Fixation using 4% NBF improved DNA quality (assessed by qPCR) compared to routine (4% unbuffered formal saline-fixed) specimens ($P<0.01$). Further improvements were achieved by reducing NBF concentration ($P<0.00001$), whereas fixation time had no effect (P=0.110). No adverse effects were detected by histopathological or QuPath morphometric analysis. Immunohistochemistry for multi-cytokeratin and α-smooth muscle actin revealed no changes in staining specificity or intensity in any tissue other than on liver multi-cytokeratin staining intensity, where the effect of fixation time was more significant (P=0.0004) than NBF concentration (P=0.048). Thus, reducing NBF concentration can maximise DNA quality without compromising tissue morphology or standard histopathological analyses.

INTRODUCTION

The increasing use, accessibility and cost-effectiveness of genomic analyses for both clinical and research purposes promise to revolutionise cancer medicine. However, the routine histopathological tissue from which DNA is extracted is typically formalin-fixed and paraffin embedded (FFPE) and notoriously variable in the quality of the nucleic acids extracted.[1] DNA extracted from FFPE tissue is fragmented, crosslinked and contains abasic sites. This can impact on PCR-based sequencing assays, where the stochastic effects of low template copy number can lead to false mutation calls, particularly when combined with low tumour cell content/low percentage mutation.[2] Furthermore, there are the additional concerns of toxicity and carcinogenicity associated with formalin use.[3] Endeavours have focused on finding alternative, "molecular" fixatives but widespread use of these agents has broadly failed to gain any traction over standard formalin-based methods which are used globally in clinical laboratories. This is partly due to expense and the fact that re-optimisation of immunohistochemistry protocols is usually required and, in general, formalin outperforms other fixatives for most antibodies tested.[4]

While it is well established that longer formalin fixation times reduce DNA yield/quality, the effects of reducing formalin concentration on DNA quality or tissue morphology have received scant attention.[1] This study aimed to optimise formalin fixation protocols with a view to maximising extracted DNA quality by varying both formalin concentration and fixation time, as well as investigating the effects on tissue morphology and immunohistochemical staining.

MATERIALS AND METHODS

Specimen collection and fixation

Anonymised specimens were collected immediately after surgery and included colon, liver and uterus (*n*=8 for each). All material collected was normal background tissue from resections surplus to diagnostic requirements. Tumour tissue was deliberately excluded in order to obviate the impact of variable tumour necrosis across malignant specimens. Buffered formalin (NBF) solutions were made by diluting 40% w/v formaldehyde (Solmedia, Shrewsbury, UK) in PBS. Tissue samples were divided into 10 pieces (<5mm thickness) and fixed under the following conditions: 5%, 4%, 3%, 2% and 1% (w/v) formaldehyde in PBS for 24h and 48h at room temperature, after which they were placed into 70% ethanol prior to routine embedding in paraffin.[5] Given the estimated penetration constant of formalin[6], complete fixation would have been achieved at both time points. In parallel, tissue-matched specimens (*n*=4 each) fixed according to our routine clinical service protocol (10% unbuffered formal saline; 4% w/v formaldehyde at room temperature for 24-48h) were collected in order to assess the adequacy of our routine tissue fixation regimens.

DNA extraction and assessment of DNA quality

DNA was extracted from tissue sections using a QIAamp DNA FFPE Tissue Kit (Qiagen, Manchester, UK) and included an RNase step. DNA concentration was determined on a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Loughborough, UK). Quantitative PCR using primers amplifying a 180bp region of the *FTH1* gene (TaqMan assay ID hs01694011-s1) was used to assess amplifiable copy number. Duplicate reactions were performed using 50ng input DNA in TaqMan Gene Expression Master Mix on a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific). Amplifiable copy number was

92 determined using a standard curve prepared from fresh human genomic DNA (extracted from
93 whole blood), serially diluted 2-fold (50 to 0.78ng/reaction).

94

95 **Haematoxylin-eosin staining and immunohistochemistry**

96 Tissue sections (5µm) were mounted on Superfrost Plus slides and haematoxylin-eosin (H&E)
97 stained using standard protocols. Immunohistochemistry (IHC) was carried out with antibodies
98 against multi-cytokeratin (MCK; AE1/AE3; PA909) and α-smooth muscle actin (SMA; αSM-
99 1; PA0943), both Leica Biosystems, Milton Keynes, UK, using the Leica Bond III autostainer
100 IHC validated protocol F, with the Bond Polymer Refined Detection (DS9800). Slides were
101 digitised at 20x magnification on Aperio XT slide scanners (Aperio Technologies, Vista, CA,
102 USA) with a compression rate of 70% and the images hosted on the University of Leeds
103 servers. Review of H&E digital images was performed blind by independent scorers (NMO,
104 KA) and assessed against predetermined criteria including nuclear morphology, architectural
105 integrity, presence of retraction artefact and appropriateness of H&E staining. Slides were
106 scored 2, 1 and 0 indicating good, sub-par and compromised morphology for diagnostic
107 purposes, respectively.[7] Morphometric assessment of H&E images was carried out using
108 QuPath[8], using the Watershed Nuclear Detection algorithm to measure both number of nuclei
109 per unit area and mean nuclear area, selecting a minimum of 1 million μm^2 per slide for analysis
110 (glandular and myometrial areas were selected for colon and uterus, respectively). In order to
111 obviate interobserver variability in IHC scoring, staining intensity and specificity were also
112 measured objectively using QuPath. Colour deconvolution was applied followed by automated
113 tissue detection. Simple Linear Iterative Clustering superpixels were then calculated for the
114 annotations for each image. 3,3'-Diaminobenzidine (DAB) Optical Density (OD) Intensity
115 Features (mean, minimum, maximum, median) were then added to the superpixels at a width

of 25µm. A thresholding script was then used to delineate positive (stained) from negative (unstained) superpixels based on the mean DAB OD for each antibody-tissue combination. Median DAB OD value for each categorised superpixel was exported and the median values of all positive and negative superpixels per slide calculated.

Statistics

Statistical analysis was performed using *R* or IBM SPSS (Version 21, Armonk, NY, USA). Comparisons were made by non-parametric tests (Mann-Whitney *U*, Kruskall-Wallis, Scheirer-Ray-Hare, and chi-squared tests), as appropriate. Dunn *post-hoc* tests with Benjamini-Hochberg False Discovery Rate (FDR) correction for multiple comparisons were also performed, as appropriate.

RESULTS

Effect of formalin fixation conditions on DNA quality

Formalin fixation causes a number of artefacts which decrease the effective (PCR-amplifiable) DNA copy number, potentially leading to stochastic effects on NGS sequencing results. We therefore chose to assess DNA quality using a qPCR-based approach[9].We began by comparing DNA quality from our experimentally fixed colon, liver and uterus tissue samples (fixed for 24h in standard 10% NBF; 4% w/v formaldehyde) with contemporaneous samples fixed in the routine histopathology lab using unbuffered formal saline (4% w/v formaldehyde). There were no significant differences in DNA quality between the different tissue types in either fixation condition. However, for each tissue type, the quality of DNA was significantly higher in experimentally fixed compared to routinely fixed samples, where none of the former and half of the latter had fewer than 100 amplifiable copies per 50ng DNA input (equivalent to <0.6% of the amplifiable copy number of fresh genomic DNA). We next examined the effect of varying NBF concentration on the quality of extracted DNA (Figure 1B). Analysis of pooled copy number data from all tissues (n=24 for each fixation condition) showed the overall effect of NBF concentration on DNA quality was highly significant (P=1.63x10^{-9}) whereas the effect of fixation time was not (P=0.110). There was no significant interaction between the two variables (P=0.609); Scheirer-Ray-Hare test. *Post-hoc* testing revealed significant increases in amplifiable copy number in samples fixed with 1% and 2% formaldehyde compared with those fixed with the standard NBF solution (4% formaldehyde) at both time points. Additionally, there was a significant difference between samples fixed in 3% *vs*. 4% formaldehyde at 48h (Figure 1B). While a third of samples fixed for 48h in the standard NBF solution had <10% of the amplifiable copy number of fresh DNA, all of the samples fixed with 1% formaldehyde for the same length of time were above this threshold.

Effect of formalin fixation conditions on tissue morphology and immunohistochemical staining

When H&E sections were reviewed blind by two histopathologists, no significant differences in tissue morphology scores were identified between different fixation conditions in any tissue (Figure 2 and Table 1).

Table 1. Histopathological assessment of the effects of buffered formalin fixation conditions on colon, liver and uterine tissues.

H&E stained sections (*n*=8 cases for each tissue) were reviewed blind by two histopathologists and given scores (0-2; (no scores of zero were given). *P*-values for each tissue type are indicated (chi-squared test).

Tissue	**Colon (*P*=0.628)**		**Liver (*P*=0.609)**		**Uterus (*P*=0.427)**	
Fixation	1 (*n*)	2 (*n*)	1 (*n*)	2 (*n*)	1 (*n*)	2 (*n*)
1%, 24h	3	5	1	7	0	8
2%, 24h	4	4	1	7	0	8
3%, 24h	5	3	0	8	1	7
4%, 24h	5	3	0	8	0	8
5%, 24h	3	5	0	8	0	8
1%, 48h	5	3	1	7	0	8
2%, 48h	4	4	0	8	0	8
3%, 48h	1	7	0	8	0	8
4%, 48h	3	5	0	8	0	8
5%, 48h	4	4	0	8	0	8
	Sum of scores					

Morphometric assessment using QuPath revealed no differences in either mean nuclear area or number of nuclei per unit area between different fixation conditions (as a measure of tissue shrinkage) (Figure 3). Sections were also stained with the antibodies to the epithelial marker MCK and to SMA (Figure 4). Given that not all uterine sections contained endometrium, these were stained with SMA only. When reviewed blind, no difference in staining intensities or specificity were observed in any of the tissues. In order to objectively assess immunohistochemical staining intensity we employed QuPath to measure median DAB intensity (OD) in both positive and negative superpixels (Figure 5). No significant difference in specific (positive superpixels) or background non-specific (negative superpixels) staining intensities were observed in any antibody-tissue combination with the exception of liver MCK staining intensity. In this case, fixation time had a significant effect (P=0.0004), whereas the effect of formaldehyde concentration was just significant (P=0.048); Scheirer-Ray-Hare test. *Post-hoc* testing revealed increased MCK positive staining intensity in samples fixed for 24h in 1% formaldehyde compared to those fixed for 48h in either 4% or 5% formaldehyde (adjusted P=0.046; Figure 5).

CONCLUSIONS

The experimental FFPE samples fixed for 24h with the standard concentration (4% w/v) of neutral buffered formalin yielded significantly better quality DNA than those fixed in the routine diagnostic histopathology lab in the standard concentration of unbuffered formal saline (a practice no longer used in our clinical laboratory). This agrees with the majority of studies employing a PCR-based assay to estimate DNA quality.[1, 10] A likely cause is the oxidation of formaldehyde to formic acid, which in unbuffered fixative leads to reduced pH, resulting in DNA depurination[11] and consequently reduced PCR amplification efficiency.[12]

This study showed that reducing the formaldehyde concentration in buffered formalin fixative further improved DNA quality, whereas varying time had no significant effect for the time points used in this study (24h and 48h). The majority of PCR-based studies agree that fixation times of <72h are preferable for maximising DNA integrity.[1] While the improvements afforded by reducing formaldehyde concentration are less dramatic than those observed when switching from unbuffered to buffered formalin fixative, they are still meaningful from a clinical perspective. For example, a third of samples fixed for 48h in standard (4%) buffered formaldehyde have a copy number of <10% that of fresh DNA, equivalent to 333 copies per 10ng input, whereas all of the samples fixed for the same time in 1% and 2% formaldehyde exceeded this threshold. Reported threshold copy number input for accurate next generation sequencing-based mutation quantitation or detection were 379 and 95, respectively for mutation frequencies of ~30-40%.[13] While it is recognised that thresholds are arbitrary and depend on various factors including mutation frequency and amplicon length, the data herein suggest reducing formaldehyde concentration has the potential to increase the number of

222 samples amenable to accurate mutation testing, especially those with low cellular
223 content/percentage tumour cells/mutation frequency.

224

225 Reducing formaldehyde concentration had no appreciable effect on histological architecture,
226 nuclear morphology or quality of H&E staining. Morphometric analysis using QuPath revealed
227 no significant effects on either mean nuclear area or the number of nuclei per unit area (as a
228 measure of tissue shrinkage). Surprisingly, very few studies addressing the effect of
229 formaldehyde concentration on tissue morphology/morphometry have been published.
230 Nonetheless, these have shown that reducing formaldehyde concentration to 1-2% had no
231 substantial effect on morphology[14] or morphometry[15], although these studies lacked statistical
232 analysis.

233

234 Changing the NBF fixation conditions had no visible effect on immunohistochemical staining
235 intensity or specificity with MCK and SMA antibodies when reviewed blind by two
236 histopathologists. While QuPath analysis detected a significant effect of formaldehyde
237 concentration on liver MCK staining intensity, the effect of fixation time was more significant
238 overall. Moreover, there were no measurable differences in staining specificity in any antibody-
239 tissue combination tested. These observations are in line with the fact that the basic chemistry
240 of fixation is unchanged unlike with alternative (non-crosslinking) fixatives, where antibodies
241 require systematic re-optimisation of antigen-retrieval protocols and in some instances only
242 work on FFPE specimens.[4, 16] Thus, reducing the formaldehyde concentration in buffered
243 formalin fixatives has the potential to increase DNA quality and reduce occupational exposure
244 to formaldehyde while avoiding these substantial barriers to clinical implementation.

245

TAKE HOME MESSAGES

Reducing the formaldehyde concentration in buffered formalin fixative to 1-2% significantly increases DNA quality without compromising tissue morphology or immunohistochemical staining.

Implementation by diagnostic histopathology laboratories would be relatively straightforward and would increase the number of samples amenable to genomic analysis while also reducing occupational exposure to formaldehyde.

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FIGURE LEGENDS

Figure 1. Effect of formalin fixation conditions on DNA quality.

(A) Comparison of quality of DNA extracted from experimentally fixed FFPE tissues (*n*=8 each, fixed using neutral buffered formalin (4% w/v formaldehyde) for 24 h; closed circles) with that extracted from routinely fixed specimens (*n*=4 each, fixed using unbuffered formal saline (4% w/v formaldehyde); open circles). Graph depicts amplifiable copy number (median, and interquartile range) of a 180 bp *FTH1* fragment, measured by qPCR, both in absolute terms (right y axis) and relative to fresh genomic DNA (left y axis). Significant differences are indicated by asterisks (Mann-Whitney *U* test). (B) Effect of varying NBF concentration and fixation time on DNA quality. Graphs depict pooled amplifiable copy number data (median, interquartile range) for experimentally fixed colon (blue), liver (green) and uterine (red) FFPE samples. For the sake of clarity, only comparisons with tissues fixed using the standard NBF solution (containing 4% formaldehyde) for each time point are indicated (Dunn *post-hoc* test with FDR correction); * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 2. Effect of formalin fixation conditions on H&E staining and tissue morphology.

Representative images (10 x magnification) of sample-matched experimentally fixed colon, liver and uterus samples. The left hand panel corresponds to samples fixed using the minimum NBF concentration and time (1% formaldehyde, 24h), whereas the right hand panel corresponds to samples fixed using the maximum NBF concentration and time used in this study (5% formaldehyde, 48h).

Figure 3. Effect of formalin fixation conditions on tissue morphometry.

Digitised H&E colon (A, B), liver (C, D) and uterus (E, F) slides (*n*=8 each) were analysed using QuPath, selecting glandular and myometrial areas for colon and uterine slides, respectively. Graphs depict mean +SEM of nuclei per unit area (A, B, C) and mean nuclear area (D, E, F). No significant differences within any tissue type were identified.

Figure 4. Effect of formalin fixation conditions on immunohistochemical staining. Representative images (10 x magnification) of sample-matched experimentally fixed colon, liver and uterus tissue sections stained with multi-cytokeratin (MCK) and α-smooth muscle actin (SMA) antibodies, as indicated. The left hand panel corresponds to samples fixed using the minimum NBF concentration and time (1% formaldehyde, 24h), whereas the right hand panel corresponds to samples fixed using the maximum NBF concentration and time used in this study (5% formaldehyde, 48h).

Figure 5. Effect of formalin fixation conditions on immunohistochemical staining quantified using QuPath.

Images of experimentally fixed colon (A, B) liver (C, D) and uterus (E) (*n*=8 each) tissue sections stained with multi-cytokeratin (A, C) and α-smooth muscle actin (B, D, E) were analysed using QuPath as described in *Materials and Methods*. Graphs depict mean +SEM of the DAB intensity (median OD) of positively and negatively staining regions (superpixels) calculated for each tissue replicate. Significant differences in staining intensities (Dunn *post-hoc* test with FDR correction) are indicated by asterisks (* P <0.05).