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Tissue Fixation with a Formic Acid-Deprived Formalin Better Preserves DNA Integrity over Time

Enrico Berrino[a, b] Laura Annaratone[a, b] Paolo Detillo[c] Dora Grassini[a, b] Alberto Bragoni[a, b] Anna Sapino[a, b] Benedetta Bussolati[d] Giovanni Bussolati[b] Caterina Marchiò[a, b]

[a]Candiolo Cancer Institute, FPO-IRCCS, Candiolo, Italy; [b]Department of Medical Sciences, University of Turin, Turin, Italy; [c]ADDAX Biosciences srl., Turin, Italy; [d]Department of Molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy

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Tissue fixation · Acid-deprived formalin · DNA integrity · Next-generation sequencing

Abstract
Introduction: Optimization of pre-analytic procedures and tissue processing is a basic requirement for reliable and reproducible data to be obtained. Tissue fixation in formalin represents the extensively favored method for surgical tissue specimen processing in diagnostic pathology; however, formalin fixation exerts a blasting effect on DNA and RNA. ***Methods:*** A formic acid-deprived formaldehyde solution was prepared by removing acids with an ion-exchange basic resin and the concentrated, acid-deprived formaldehyde (ADF) solution was employed to prepare a 4% ADF solution in 0.1 M phosphate buffer, pH 7.2–7.4. Human (n = 27) and mouse (n = 20) tissues were fixed in parallel and similar conditions in either ADF or neutral buffered formalin (NBF). DNAs and RNAs were extracted, and fragmentation analyses were performed. ***Results:*** Besides no significant differences in terms of extraction yield and absorbance ratio, ADF fixation reduced DNA fragmentation, i.e., the largest fragments (>5,000 bp) were significantly more prevalent in the DNAs purified from ADF-fixed tissues ($p < 0.001$ in both cohorts). Moreover, we observed that DNA preservation is more stable in ADF-fixed tissue compared to NBF-fixed tissues. ***Conclusion:*** Although DNA fragmentation in FFPE tissues is a multifactor process, we showed that the removal of formic acid is responsible for a significant improvement in DNA preservation.

Introduction

Archival, formalin-fixed, and paraffin-embedded (FFPE) tissue samples represent a rich source of biological data of diagnostic, prognostic, and therapeutic interest in the management of cancer patients. In this respect, standardization and optimization of pre-analytic procedures and tissue processing are basic requirements for reliable and reproducible data to be obtained [1, 2]. With the tremendous advances of precision medicine, patholo-

Karger@karger.com
www.karger.com/pat

Correspondence to:
Caterina Marchiò, caterina.marchio@ircc.it

gists face the challenge to integrate morphology, and immunophenotyping with genetic and epigenetic analyses [3]. These advances require purification of good-quality nucleic acids, while DNA fragmentation would lead to lower coverage of unique reads in the whole genome and whole exome sequencing approaches [3], and it may also decrease the success rate of amplicon-based methods due to reduced size of DNA templates [4–6]. While uniformity and reproducibility have been achieved in most steps involved in the histological processing (e.g., dehydration, paraffin embedding, sectioning, staining), the fixation process represents the single critical factor with the highest impact on final results.

Basically, two types of fixation mechanisms have been proposed. Coagulative, alcohol-based fixatives (such as PAX fixative) produce a valuable nucleic acid quality [7, 8] but are defective in structural preservation and are thus not popular among pathologists. The cross-linking mechanism produced by aldehyde fixatives provides instead a gold-standard preservation of structural components. In fact, tissue fixation in formalin with the generation of FFPE tissue blocks represents the extensively favored method for surgical tissue specimen processing in diagnostic pathology.

It is widely accepted that formalin fixation exerts a blasting effect on both DNA and RNA, with damages comprising fragmentation and base alterations that show critical proportional consequences related to storage time [9, 10]. Of note, polymerase chain reaction-based next-generation sequencing (NGS) methods are strongly influenced by DNA quality [11]. Moreover, the low quality of DNA from FFPE samples also stems from formalin-induced base artifacts within the sequences, which generate false mutation calls, specifically in sub-clonal scenarios [12, 13].

The reagent currently used for surgical tissue fixation is neutral buffered formalin (NBF), obtained by dissolving 1:10 the saturated (commercial) solution of formaldehyde in 0.1 M phosphate buffer pH 7.2–7.4. The pH of the reagent is critical since it is known that fixation in acidic formalin results in DNA fragmentation and alteration of bases [7, 14–16]. The commercial formaldehyde solution, wherefrom formalin is prepared, is rich in formic acid [17] resulting in acidity in the range of pH 2. In the buffered, slightly alkaline, final solution, the acid is present as sodium formate, which is currently regarded as inactive and irrelevant in the fixation process.

In the present study, we have been checking this assumption by creating a formic acid-deprived formaldehyde solution (ADF), which was achieved by removing acids with an ion-exchange basic resin. The concentrated, acid-free formaldehyde solution was employed to prepare a 4% ADF in 0.1 M phosphate buffer pH 7.2–7.4. Parallel samplings from human and animal tissues were fixed in parallel and similar conditions in either ADF or NBF, then embedded in paraffin, and a detailed analysis of DNA and RNA quantity and quality was performed, which showed remarkable differences in DNA preservation, a finding of biological and potential clinical interest.

Materials and Methods

Cohort Assembly and Experimental Design

This study involved two cohorts: (i) the patient cohort including 27 tissue samples (10 from normal "N" tissues, 17 from tumoral "T" lesions) from lung, breast, colon, pancreas, kidney, and fat tissue; (ii) the mouse cohort including 20 tissue samples (15 from N tissues, 5 from T lesions) sampled from tumor xenografts and the normal liver, kidney, and spleen (shown in Fig. 1).

With respect to the patient cohort, 27 tissue samples were collected from surgical specimens of 23 patients having a lesion of at least 2 cm in size to allow proper parallel sampling. The study was approved by the Ethics Institutional Review Board (IRB) responsible for "Biobanking and use of human tissues for experimental studies" – Department of Medical Sciences, University of Turin.

For the mouse cohort, tumor generation was performed by subcutaneous injection of 1×10^6 HER2-positive breast cancer TUBO cells as described in [18] and tissues from 5 mice were collected. Guidelines for the Care and Use of Laboratory Animals were followed during the investigation. All animal studies were conducted in accordance with the national guidelines and regulations and were approved by the Italian Ministry of Health (protocol number: 959/2018-PR). Each of the 47 collected samples was fixed in parallel as follows: (i) fixation in formalin, i.e., NBF (Diapath, Bergamo Italy), which represents the fixative used in daily practice, and (ii) fixation in ADF (acid-deprived formalin prepared by Addax Biosciences srl, Turin, Italy).

Preparation of Fixative Solutions

For NBF, the aqueous solution of 4% formaldehyde in buffer phosphate (the pH value of the NBF was checked, found in a range between 6.8 and 7, and therefore corrected to neutrality with a few

Fig. 1. Cohort assembly and experimental design. **a** Schematic representation of NBF and ADF fixatives. NBF represents the standard formalin solution, routinely used in daily practice. All analyses were performed by comparing the impact of ADF and NBF on nucleic acids. **b** Diagram of the two cohorts involved in the study: patient cohort (tissue = 27) and mouse cohort (tissue = 20). Each of the 47 samples was fixed in parallel as follows: (i) fixation in phosphate-buffered formalin (NBF); (ii) fixation in acid-deprived formalin (ADF). DNA and RNA were extracted at baseline. A second DNA extraction was conducted on all patient cohort samples after 6 months of storage.

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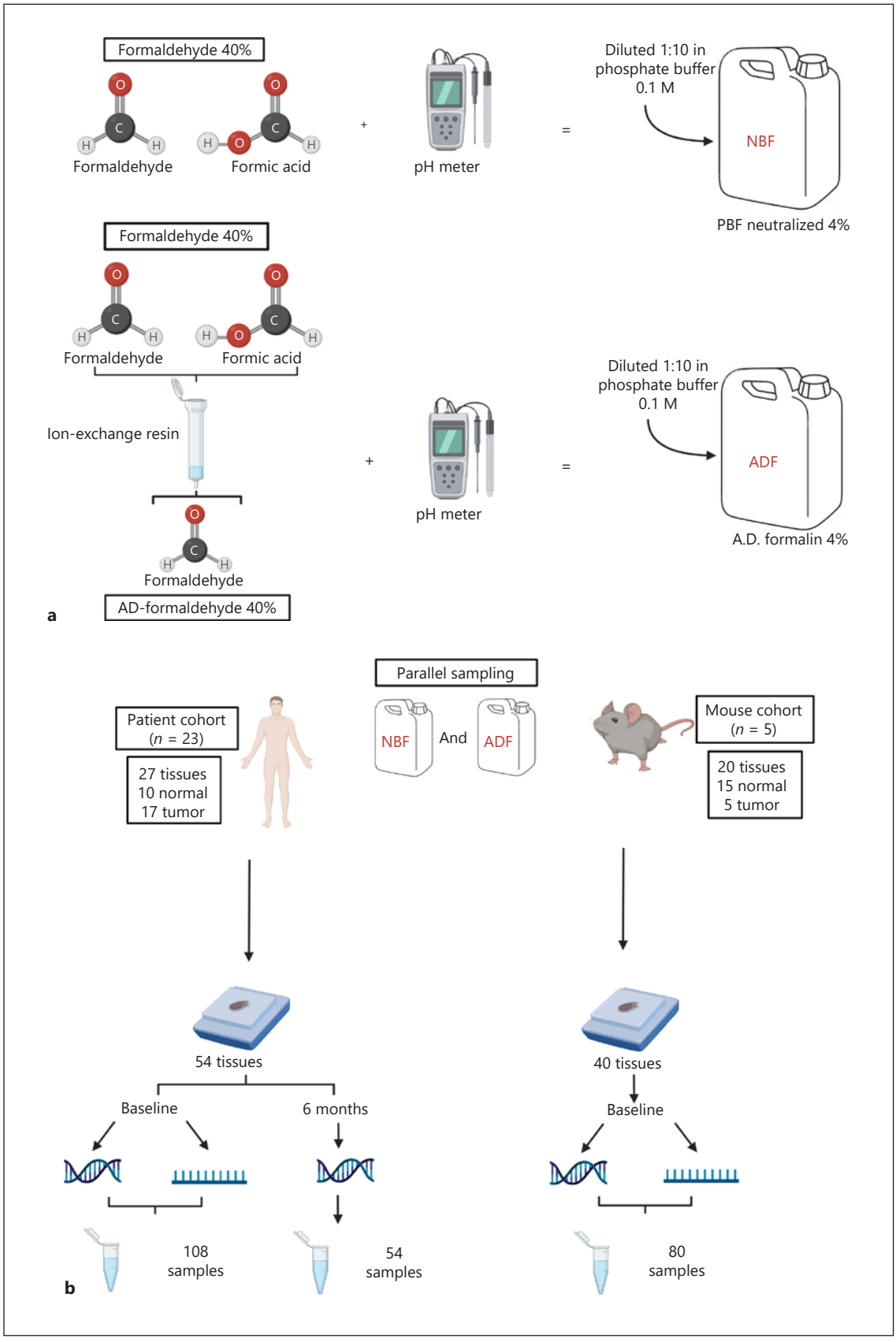

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drops of NaOH [0.1 M]). For ADF, commercially available concentrated formaldehyde solution (37%; pH = 3.5, Sigma Aldrich, Milano, Italy) was passed through a column of Amberlite (Carlo Erba, Milan, Italy), a basic ion-exchange resin. The collected eluate, with a pH ranging between 6.8 and 7.3, was dissolved in a ratio of 1:10 in phosphate buffer (0.1 M; pH 7.2), thus obtaining a solution of 4% acid-deprived formaldehyde in phosphate buffer. To monitor the stability of the pH, we added phenol red 0.001% as an indicator. The solution was found to remain stable, at room temperature, for at least 2 years (shown in Fig. 1a, b).

Immunohistochemistry

Three-micron-thick FFPE sections of 4 tissue pairs were stained with 14 markers, as reported in online supplementary Table 1 (see www.karger.com/doi/10.1159/000525523 for all online suppl. material). Scores and results were assessed by two independent pathologists (A.B., C.M.).

Nucleic Acid Extraction and Quantification

At baseline, eight sections (8 μm thick) were cut from FFPE tissue blocks of all the samples processed in parallel (fixation in NBF and ADF). Four slides were subjected to DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), following an overnight incubation at 56°C with proteinase K to maximize tissue digestion, according to the manufacturer's protocol. A total of 94 DNA samples were obtained.

A second set of four slides were subjected to automated RNA extraction, by using the Maxwell® RSC RNA FFPE Kit (Promega, Madison, WI, USA) as previously described [19]. A total of 94 RNA samples were obtained (shown in Fig. 1b). Subsequently, after 6 months of storage, a second DNA extraction was performed on all patient cohort samples and additional 54 DNA samples were collected (shown in Fig. 1b).

To evaluate whether the two different types of fixative have any impact on DNA and RNA, we evaluated the extraction yield (variable "Quantity," in μg) by using both fluorometry (Qubit 3.0 Fluorometer, Life Technologies, OR, USA) and spectrophotometry on the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Absorbance ratios (260/230 nm: R230 and the 260/280 nm: R280, variable "Quality") were investigated by NanoDrop 1000 Spectrophotometer to assess the purity of the eluate (shown in online suppl. Fig. 1a, b).

Nucleic Acids Fragmentation Analysis

We performed fragmentation analysis to evaluate DNA and RNA preservation. DNA integrity was evaluated with Agilent 2100 Bioanalyzer using High Sensitivity DNA Analysis Kit (Agilent Technologies, Santa Clara, CA, USA) setting a sizing range of 0–30.000 bp for analysis. The curve resulting from the detected fluorescence intensity was split into discrete regions (bins) with scalable ranges in size, based on the instrument sensibility. Size distribution analysis was performed by calculating the fraction of the total area under the curve (AUC) for each bin (example of Bioanalyzer trace with bins in online suppl. Fig. 2). RNA integrity was evaluated with Agilent 2100 Bioanalyzer using Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA, USA) by assessing the RNA Integrity Number (RIN) and the percentage of RNA fragments >200 nucleotides (DV200) (shown in online suppl. Fig. 1b).

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism statistical software v8.0 (GraphPad Software, San Diego, CA, USA). After the normality test, we applied both the unpaired and paired distribution test to evaluate significant differences in quality controls between the two fixatives. The unpaired distribution test was used to assess differences as independent measurements of uncorrelated samples, whereas the paired distribution test evaluated the statistical distribution considering the different fixation of the same sample as repeated and correlated data. Fisher's exact test assessed differences among contingency variables. We first analyzed the total cohort by merging the data obtained from the two sets and then by separately considering NBF-fixed samples and ADF-fixed samples, p values of <0.05 were considered statistically significant.

Results

Evaluation of Tissue and Nucleic Acid Quality

Immunohistochemistry run for 14 markers revealed overlapping results between parallel samples fixed with ADF or NBF (online suppl. Fig. 3). Fluorometric quantification of DNAs purified from the FFPE blocks of the patient cohort at the baseline revealed a superimposable yield of extraction for both NBF- and ADF-fixed samples (mean NBF: 3.6 μg and ADF: 3.9 μg, p value = 0.87, paired t test, shown in Fig. 2a). The lack of influence of the two different fixatives over the DNA purification quantity was confirmed by the spectrophotometric analysis (mean NBF: 15.52 μg and ADF: 15.18 μg, p value = 0.56, paired t test, shown in Fig. 2a), which also returned information about the absorbance quality. In this context, no significant differences were identified comparing the R230 and R280 of DNAs extracted from NBF-fixed tissues and ADF-fixed tissues (mean R230 NBF: 2.07 and ADF: 2.00, p value = 0.85, mean R280 NBF: 1.87 and ADF: 1.88, p value = 0.99, paired t test, shown in Fig. 2b).

To corroborate these data, the same quality controls of quantity and absorbance ratios were applied to the DNAs purified from samples of the mouse cohort. In line with the patient cohort results, the two fixatives did not affect extraction yield (mean μg fluorometer NBF: 2.50 and ADF 2.39, p value = 0.64, mean μg spectrophotometer NBF: 18.44 and ADF: 16.54 p value = 0.15, paired t test, shown in Fig. 2c) and absorbance quality values (mean R230 NBF: 1.78 and ADF: 1.83, p value = 0.33, mean R280 NBF: 1.87 and ADF: 1.89, p value = 0.14, paired t test, shown in Fig. 2d).

Finally, we wondered if the different fixatives could affect the absorbance ratios and the quantity of the RNA obtained from both human and mouse samples at baseline. The results of the analysis on human RNAs are in line

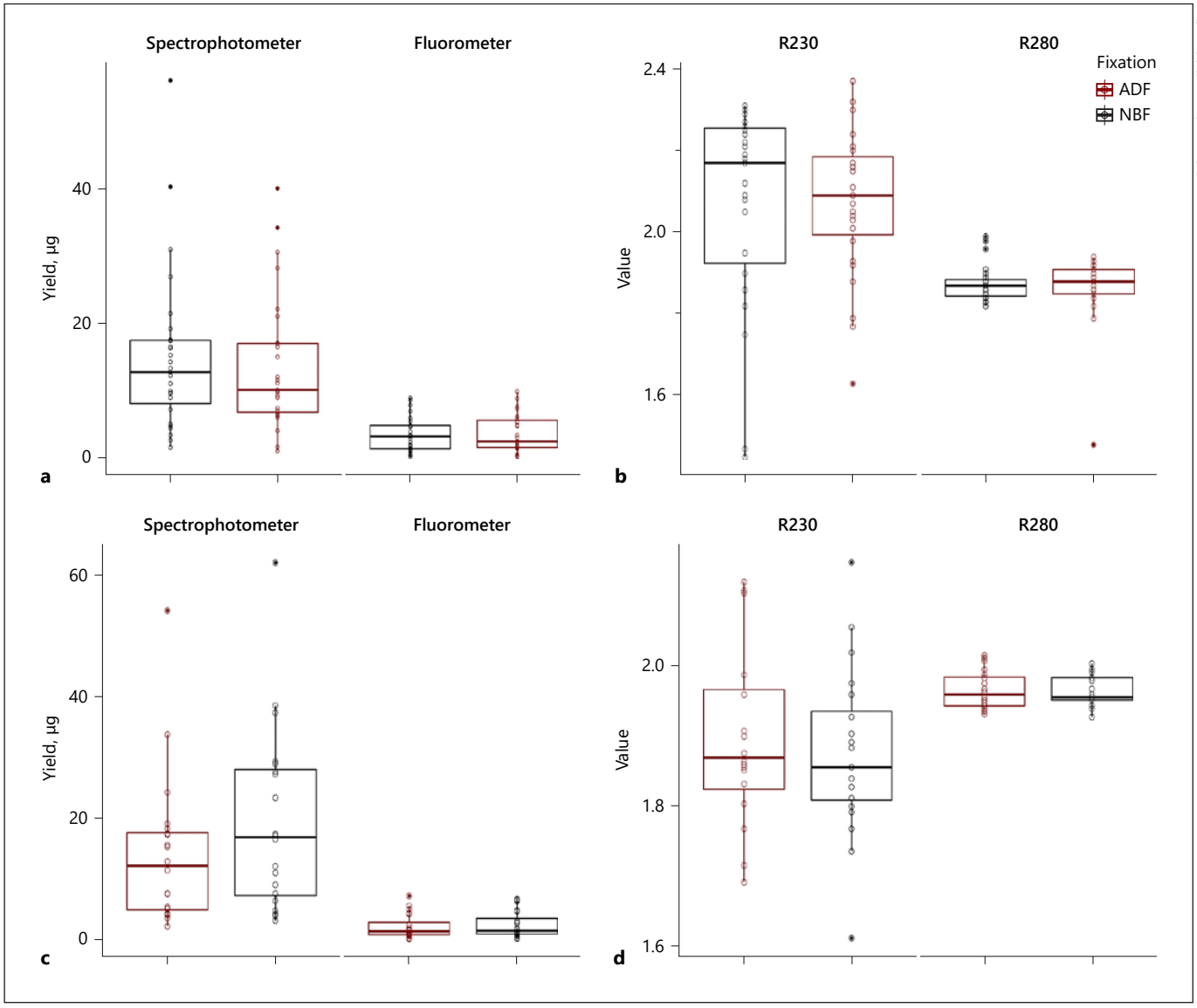

Fig. 2. DNA quality and quantity. **a** Box plots reporting fluorometric and spectrophotometric quantification (µg) of DNAs purified from FFPE blocks of the patients cohort. **b** Box plots reporting the distribution of the absorbance ratios (R230 and R280) of DNAs purified from FFPE blocks of the patients cohort at baseline. **c** Box plots reporting fluorometric and spectrophotometric quantification (µg) of DNAs purified from FFPE blocks of the mouse cohort. **d** Box plots reporting the distribution of the absorbance ratios (R230 and R280) of DNAs purified form FFPE blocks of the mouse cohort at baseline.

with those reported for the DNA: fluorometric and spectrophotometric quantification of RNA showed that the two fixatives lead to similar amounts (µg) of nucleic acid. In addition, the distribution of the absorbance ratios (R230 and R280) of RNAs showed no significant differences among NBF-fixed and ADF-fixed samples. Quantity and absorbance ratios were also assessed on the RNAs purified from samples of the mouse cohort, with superimposable results. RNA distribution plots are displayed in online supplementary Figure 4 and average data with *p* values are reported in online supplementary Tables 2A–B.

Nucleic Acids Fragmentation Analysis

Bioanalyzer provided information about the size range of DNA fragments. In terms of DNA size profile, the unsupervised clustering of the patient cohort identified three clusters: (i) highly fragmented DNA; (ii) moderately fragmented DNA; (iii) conserved DNA (shown in

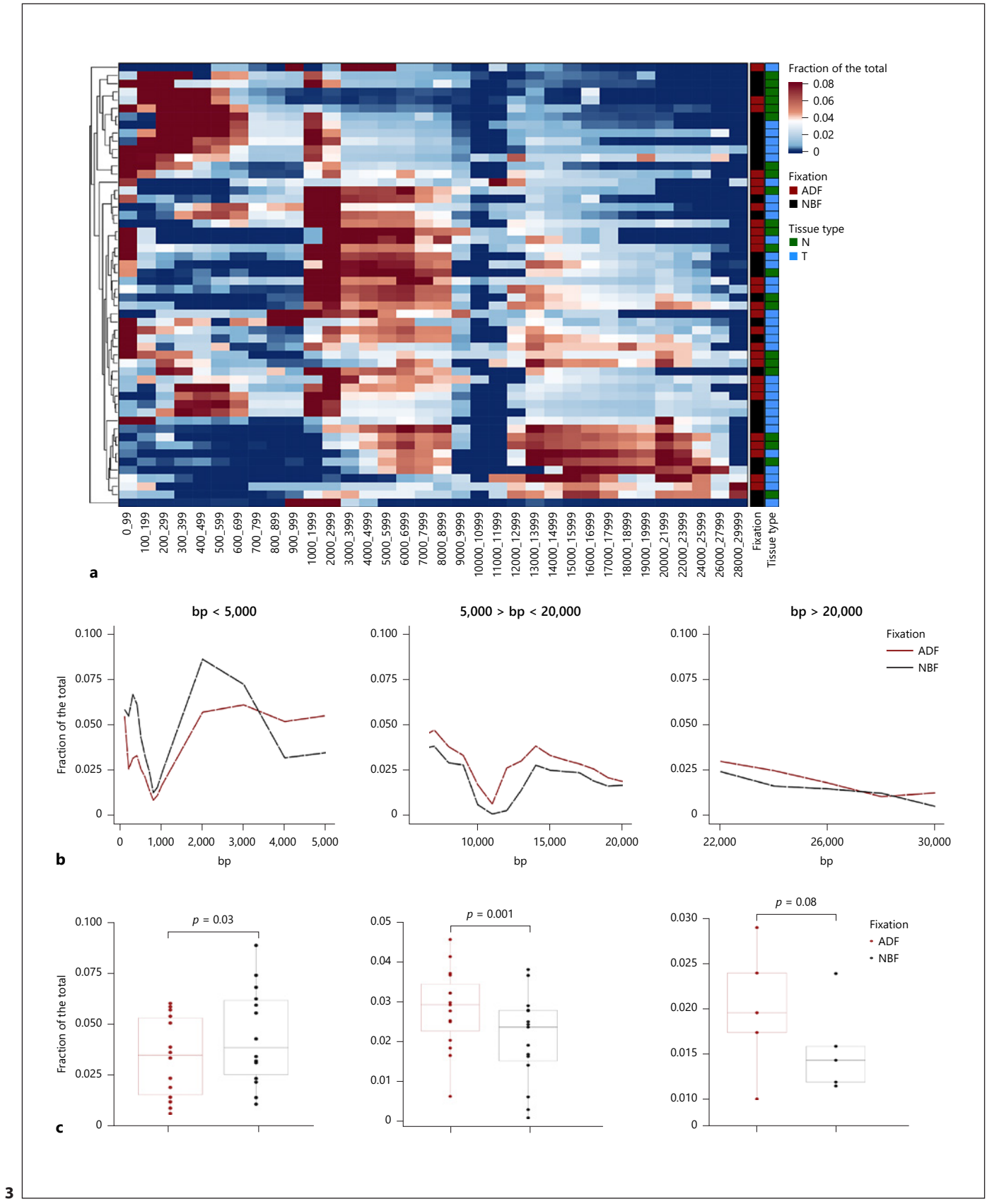

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Fig. 3a). Of note, the size distribution is independent of the tissue type (T and N). The evaluation of the mean values of the fraction of the total for each bin demonstrated that short fragments (<5,000 bp) were significantly more prevalent in NBF-fixed samples, whereas ADF-fixed samples were enriched in 5,000 > bp < 20,000 fragments. Last, fragments >20,000 bp were equally distributed across samples, regardless of the type of fixation. Line plots with mean values are displayed in Figure 3b.

The differences observed among fragments <20,000 bp were statistically significant (fragment <5,000: NBF: 62% vs. ADF: 48%, p value = 0.03; fragment 5,000 > bp < 20,000: NBF: 30% vs. ADF: 42%, p value = 0.001, unpaired t test), while difference for fragments >20,000 bp were not statistically significant (NBF: 8% vs. ADF: 10%, p value = 0.08, paired t test), as shown in the box plots in Figure 3c and in online supplementary Table 2A.

When analyzing the samples pertaining to the mouse cohort, we did not detect the exact reproducibility of the clusters identified in the patient cohort (shown in online suppl. Fig. 5a). In particular, there was a much lower prevalence of conserved DNA samples. However, ADF-fixed samples still showed a significantly higher prevalence of fragments >5,000 bp compared to NBF-fixed samples (NBF: 22% vs. AD-FORM: 39%, p value = 0.001, unpaired t test) (shown in online suppl. Fig. 5b; Table 2B).

After DNA evaluation, we wondered if the two different types of fixative had any impact on RNA fragmentation. Data from human RNAs in terms of RIN (mean NBF: 2.19 and ADF: 2.21, p value = 0.99, paired t test) and DV200 (mean NBF: 42.11 and ADF: 42.56, p value = 0.94, paired t test) showed superimposable results between NBF-fixed and ADF-fixed samples, suggesting no impact on RNA preservation (shown in online suppl. Fig. 6a; Table 2A). Fragmentation analyses were also applied to the RNAs purified from samples of the mouse cohort and results were in line with ones obtained on the patient cohort (shown in online suppl. Fig. 6b; Table 2B).

Fig. 3. DNA fragmentation analysis. **a** Heat map of DNA fragmentation in the patient cohort, reporting the fraction of the total with a blue-to-red map describing the fragment length distribution among the samples. Raw dendrogram groups patients by similar fragmentation distribution. Type of fixative and type of tissue annotation on the right-hand side. **b** Line plots reporting the mean values of the fraction of the total for each bin considering fragments <5,000 bp; fragments comprised between 5,000 > bp < 20,000 fragments and fragments larger than 20,000 bp. **c** Box plots reporting mean values of the fraction of the total for each bin with p values of the distribution of the fragments between the two types of fixative.

DNA Preservation over Time

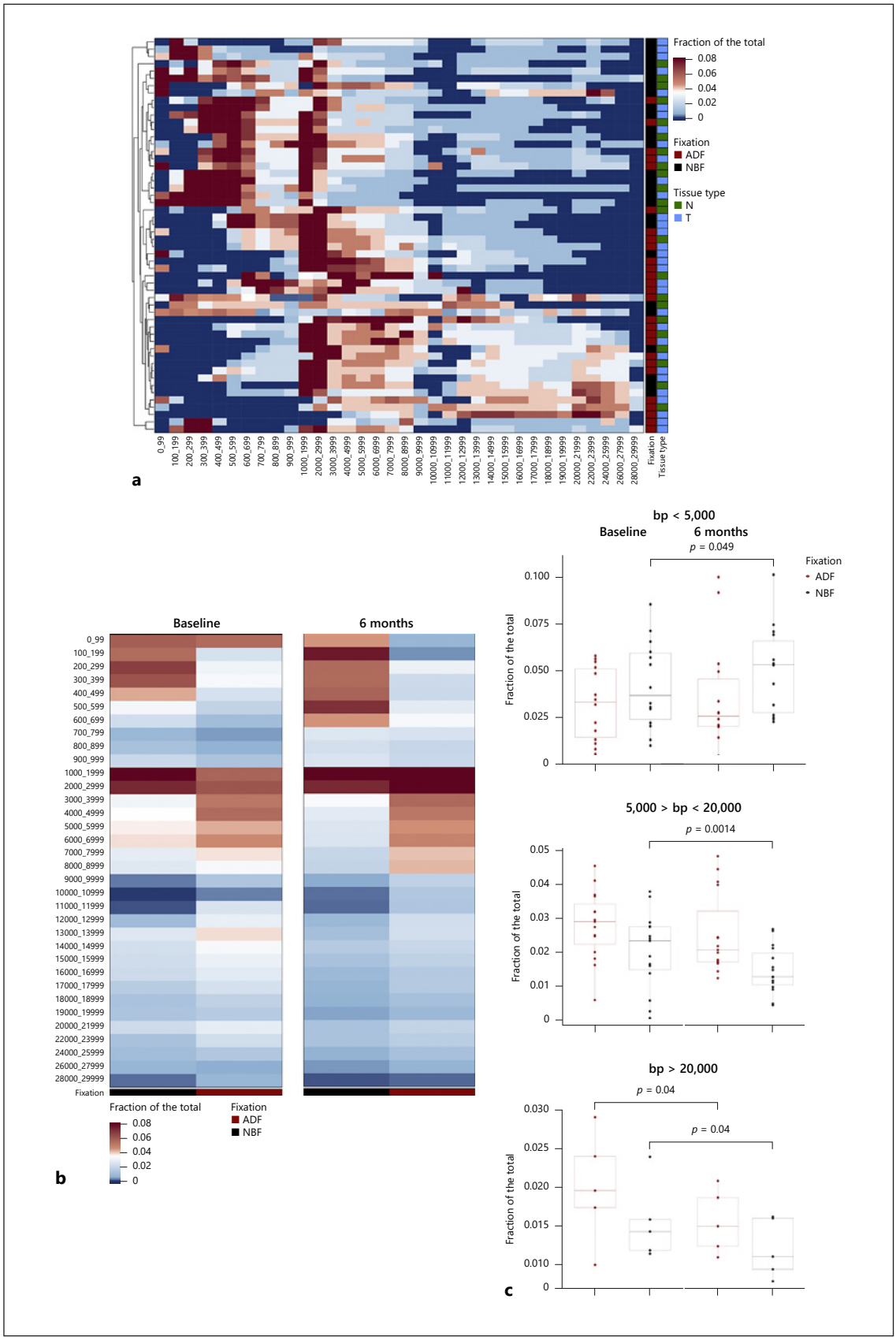
To investigate whether the type of fixative may affect DNA preservation over time, we performed quantity, quality, and fragmentation analyses following 6 months of storage, thus mimicking the initial archival process in pathology laboratories. The DNA quantity variation from baseline to 6 months was evaluated by fluorometry. The variation (delta, %) in yield of extraction (μg) from baseline showed that DNA quantity did not significantly vary over time, independently from the type of fixative (12/27 NBF and 14/27 ADF with reduced yield, p = 0.78, Fisher's exact test, shown in online suppl. Fig. 7a). Similarly, no differences were identified in terms of spectrophotometer quantity (13/27 NBF and 13/27 ADF with reduced yield), R230 (20/27 NBF and 14/27 ADF with reduced R230, p = 0.2), and R280 values (17/27 NBF and 16/27 ADF with reduced R280, p = 0.9) (shown in online suppl. Fig. 7a, b).

Unsupervised clustering of the size distribution confirmed the three bp-based clusters observed at baseline with a reduction of high molecular weight DNA fragments (shown in Fig. 4a). Tissue type (T vs. N) and tissue of origin did not affect these results (shown in Fig. 4a).

The comparison of the two fixatives at baseline and after 6 months revealed that NBF-fixed samples showed an increase in DNA fragmentation, whereas ADF-fixed samples displayed a stable DNA preservation (shown in Fig. 4b). When considering fragments <5,000 bp in size, the difference was statistically significant only for NBF-fixed samples (baseline NBF: 62% vs. ADF: 48%; 6 months NBF: 73% and ADF: 54%; NBF comparison p value = 0.04, unpaired t test, shown in Fig. 4c). Consequently, we identified a significant reduction of fragments comprised between 5,000 and 20,000 bp in samples fixed with NBF and stored for 6 months (baseline NBF: 30% and ADF: 42%; 6 months NBF: 22% and ADF: 38%; NBF comparison p value = 0.0014, unpaired t test, shown in Fig. 4c). Finally, both NBF- and ADF-fixed tissues showed a reduced quantity of fragments >20,000 bp in size (baseline NBF: 8% and AD-FORM: 8%; 6 months NBF: 5% and ADF: 10%; NBF p value = 0.04, ADF p value = 0.04 unpaired t test, shown in Fig. 4c).

Discussion

The correct handling of FFPE archival tissues plays a pivotal role in the efficiency of nucleic acid-based deep sequencing experiments, such as NGS that has become critical in ensuring "precision medicine" for several malignancies [3, 6, 11, 20]. Unfortunately, the practice of



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specimen preparation is not uniform across laboratories, and a variety of factors impact the quality of the analyte source, i.e. FFPE tissues. As a consequence, a significant number of samples to be analyzed (according to Kuwata and Coworkers [21], up to 54% of the cases, depending on the test employed) may result as unfit for molecular analysis because of the poor quality of nucleic acids.

The large variety of formaldehyde solution formulations used in the past as tissue fixative, proposed and practiced by pathology hubs [17, 22], have been shown to influence both the quantity and quality of nucleic acids. Specifically, acidic solutions were seen to cause an unacceptable degree of nucleic acid fragmentation, and this led to the universal adoption of buffered formalin preparations. Interestingly, among the different buffers (e.g., calcium carbonate, magnesium carbonate, citrate, Tris), phosphate-based buffers appear to be superior to other common formulations for RNA recovery [1], but the reason for such superiority remains unexplained.

For the reasons above, standard formalin (4% formaldehyde) in 0.1 M phosphate buffer, pH 7.2–7.4 (so-called NBF) is currently adopted as the fixative of choice in pathology laboratories, and it was used in the present article to compare the impact of ADF on nucleic acid preservation. The reagent is relatively stable, depending on storage conditions, including light and temperature, but it is subjected to time-dependent formaldehyde-to-formic acid oxidization, believed to contribute to nucleic acid fragmentation [23].

In the present study, we have been challenging the current belief that formic acid, present in the commercial preparation of formaldehyde, can be made ineffective by buffering the solution up to the formation of sodium formate. Our reasoning was that tissues are not a homogeneous "soup" of reactants, since cell and nuclear membranes build up a reality of secluded micro-environments. Specifically, inside the nuclei, the predominance of the DNA phosphate groups might play a role in the fixation reaction. Our assumption that complete removal of formic acid from the NBF might lead to better preservation of DNA integrity was confirmed by the data we provide.

To check the degree of time-related degradation known to occur in FFPE tissues, analysis was made of nucleic acids extracted from human and mouse samples soon after processing and 6 months later. The different tissue cohorts underwent the same extraction procedure and comparative analysis. We observed that the two fixative procedures did not affect the extraction yield of DNA or RNA. The nucleic acid fragmentation was instead remarkably different, exclusively for the DNA. In fact, both in human and mouse samples ADF-fixed samples showed a significantly higher prevalence of DNA fragments >5,000 bp compared to NBF-fixed samples. Notably, a similar, and even enhanced difference was observed in the same tissue samples, when nucleic acids were extracted 6 months after the processing since ADF-fixed samples displayed a more stable DNA preservation. At variance, basically superimposable results between AD formalin and NBF-fixed tissues were observed as far as RNA preservation is concerned. We have no direct explanation for the difference between DNA and RNA preservation degree in alternatively fixed tissue and can only suggest that cytoplasmic versus nuclear location making the difference. The latter micro-environment, being rich in phosphoric acid residues, might link sodium ions and thus liberate formic acid in its active form.

When deepening our analysis by studying the integrity of 108 human DNAs (54 NBF and 54 ADF-fixed samples), we observed that half of the NBF samples (10/27 of the baseline and 17/27 of the 6-month extractions) showed a diffuse fragmentation, with most of the DNA molecules comprised between 100 and 600 bps. On the contrary, only 4/27 at baseline and 6/27 at 6 months of the ADF-purified DNAs were characterized by this type of extensive degradation. Of note, by using a superimposable approach to evaluate DNA fragmentation distribution, McDonough and coworkers [24] showed that increased fragmentation reduced the molecule's "adequate" for subsequent NGS analysis. Indeed, the interest in our data is linked to extensive observations that DNA integrity is the key factor determining the success of NGS [11] and polymerase chain reaction [5] and may prevent false-positive copy number variation results [12].

Improvement and sustainability of precision medicine planning are bound to pass through an improvement of the quality of DNA to be extracted from FFPE tissue blocks. Although substitution of formalin with alcohol-based fixatives can lead to improved integrity of nucleic

Fig. 4. DNA preservation after 6 months of storage time. **a** Heat map of DNA fragmentation in the patient cohort, reporting the fraction of the total with a blue-to-red map describing the fragment length distribution in the samples. Raw dendrogram groups patients by similar fragmentation distribution. Type of fixative and tissue type annotation on the right-hand side. **b** Heat map of the comparison of DNA fragmentation between the two fixatives at baseline and after 6 months. Reporting the fraction of the total with a blue-to-red map describing the fragment length distribution in the samples. Type of fixative annotation below the heat map. **c** Box plots reporting the comparison of the fraction of the total between the two fixatives at baseline and after 6 months.

acids [1] the superior preservation of both, structural and antigenic (for immunohistochemical characterization) guaranteed by the aldehyde fixation of surgical specimens leaves no alternative for this type of tissue preparation.

In conclusion, DNA fragmentation in FFPE tissues is produced by a multiplicity of factors and its improvement requires to focus on the different steps involved in the process, i.e. specimen transfer and grossing; length, type, and temperature of the fixative embedding process [1, 10, 23, 25]. In the present study, we dealt with the single, but critical factor of a better type of phosphate-buffered formalin and showed that the removal of formic acid results in a significant improvement in DNA preservation. Although ADF is not used in diagnostic practice yet, by being a formalin-based solution it holds the potential to easily enter clinical practice, in particular if the features we highlighted related to better DNA preservation will impact the performance of molecular diagnostic assays. On one side, it would mean a minor adaptation of standard protocols in pathology laboratories, on the other side it would guarantee superior quality of FFPE tissue samples.

Statement of Ethics

The study was approved by the Ethics Institutional Review Board responsible for "Biobanking and use of human tissues for experimental studies" – Department of Medical Sciences, University of Turin. All animal studies were conducted in accordance with the national guidelines and regulations and were approved by the Italian Ministry of Health (Protocol Number: 959/2018-PR).

Conflict of Interest Statement

Caterina Marchiò has received personal consultancy fees from Bayer, Roche, Daiichi Sankyo, and AstraZeneca outside the scope of the present work. Caterina Marchiò Served as Associate Editor for the Journal. Paolo Detillo is an employee of Addax Biosciences srl. Gianni Bussolati serves as CEO of Addax Biosciences srl. Enrico Berrino, Dora Grassini, Alberto Bragoni, Laura Annaratone, Anna Sapino, and Benedetta Bussolati have no conflict of interests to declare.

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Author Contributions

Conceptualization: Gianni Bussolati. Data curation: Enrico Berrino, Dora Grassini, Alberto Bragoni, Laura Annaratone, and Paolo Detillo. Formal analysis: Enrico Berrino, Dora Grassini, Alberto Bragoni, and Laura Annaratone. Funding acquisition: Caterina Marchiò and Anna Sapino. Investigation: Enrico Berrino, Benedetta Bussolati, Dora Grassini, Alberto Bragoni, Gianni Bussolati, and Caterina Marchiò. Methodology: Enrico Berrino, Dora Grassini, Benedetta Bussolati, Alberto Bragoni, and Laura Annaratone. Resources: Caterina Marchiò, Anna Sapinio, Benedetta Bussolati, and Gianni Bussolati. Software: Enrico Berrino. Supervision: Benedetta Bussolati, Gianni Bussolati, and Caterina Marchiò. Validation: Enrico Berrino. Visualization: Enrico Berrino. Writing – original draft: Enrico Berrino, Caterina Marchiò, Gianni Bussolati, and Benedetta Bussolati. Writing – review and editing: Enrico Berrino, Dora Grassini, Caterina Marchiò, Gianni Bussolati, Alberto Bragoni, Laura Annaratone, Paolo Detillo, Anna Sapino, and Benedetta Bussolati.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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