

Impact of Histological Processing on Endogenous Elemental Contents and Distributions in Breast Cancer Tissue Sections

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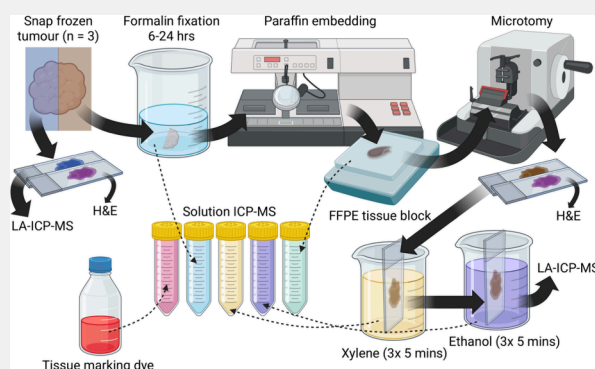
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ABSTRACT: Trace metal homeostasis is critical for cellular function and often disrupted in diseases such as cancer. Spatially resolved techniques enable mapping of metal distributions within complex cellular environments, surpassing the limitations of bulk analyses. Formalin-fixed, paraffin-embedded (FFPE) tissues are widely used in research, yet the impact of FFPE processing on endogenous elemental contents and distributions remains unclear, with conflicting findings across sample types. This study evaluates the suitability of FFPE human breast carcinoma tissue sections for multielemental bioimaging by direct comparison with paired fresh frozen (FF) tissues and assesses potential sources of contamination or elemental losses during processing. Three FF human breast carcinoma specimens were bisected and processed as either FF or FFPE. Serial sections underwent analyses by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), alongside hematoxylin and eosin (H&E)-staining, to compare elemental profiles in whole sections and morphologically distinct regions. In parallel, FFPE processing reagents encompassing tissue-marking dyes, formalin, paraffin wax, xylene, and ethanol were analyzed via solution ICP-MS to contextualize the *in situ* findings. The multielemental imaging results revealed that Zn, Mn, and Fe were largely preserved in FFPE sections, supporting the continued use of such tissues for *in situ* metal analyses. Addition of Cu was observed for all FFPE samples, most likely caused by exposure to blue tissue-marking dye. Formalin induced variable elemental leaching, most notably affecting Mg. Paraffin wax, ethanol, and xylene contained minimal trace metal levels both before and after contact with tissues.

KEYWORDS: elemental imaging, laser ablation ICP-MS, tissue processing, formalin-fixed paraffin embedding (FFPE), breast cancer



INTRODUCTION

Metals play a critical role in maintaining normal cellular functions.¹ For example, metals act as enzyme cofactors, provide structural stability for proteins² and are essential in cell signaling.³ Therefore, in diseases such as cancer, disruption to normal cell function is often reflected in a change in metal homeostasis.^{4,5} These alterations can serve as biomarkers and reveal underlying pathological mechanisms.^{6–9}

Traditionally, bulk sampling techniques have provided valuable insight into the metal contents of healthy and diseased tissues.^{10,11} However, they often fail to capture the spatial heterogeneity observed in such complex cellular environments, and therefore potentially miss significant features that may deepen our understanding of disease processes.^{12,13} Recent advances in instrumentation have guided research focus toward spatially resolved techniques, such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), which enable high-resolution mapping of metal contents across tissue sections.^{14–18}

With the LA-ICP-MS technique, metal concentrations are obtained by ablating tissue samples with a focused laser. Each laser pulse vaporises a defined area of material, which is transported into the ICP, where atomization and ionization occur. The resulting ions are filtered and measured using mass spectrometry. As the laser moves across the sample, signals are collected from each region, which can be compiled to produce 2D elemental maps. This technique achieves both high resolution (down to $\sim 1 \mu\text{m}$) and low limits of detection ($\text{sub } \mu\text{g g}^{-1}$) such that metal accumulation can be localized to regions of differing cellular composition.^{19,20} Moreover, with methodological advancements allowing the production of

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matrix-matched standards, quantitative measurements are now possible. For biological matrices, these include robot-pipetted gelatin microdroplet standards spiked with known metal concentrations.^{21,22}

Within routine clinical practice, tissue samples obtained for diagnostic and research purposes are stored in formalin-fixed, paraffin-embedded (FFPE) form (Figure 1), the gold standard

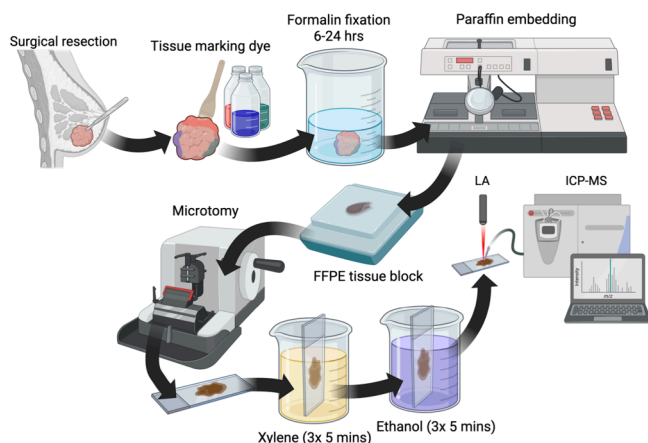


Figure 1. Visual summary of the formalin-fixed paraffin embedding (FFPE) process applied to biological tissues prior to LA-ICP-MS analyses. Created in BioRender. Lovejoy, A. (2026) <https://BioRender.com/uy5df1r>.

preservation method. The process first involves chemically fixing tissues in neutral-buffered formalin, a 1:10 dilution of ~37% aqueous formaldehyde (w/v). Controlling pH minimizes the oxidation of formaldehyde into formic acid which can react with iron-rich species, forming a precipitate.²³ Fixation induces protein crosslinking, inactivates enzymes and kills micro-organisms.²⁴ The tissues are then dehydrated, whereby water is replaced first by increasing concentrations of ethanol and then xylene. This allows full penetration by the liquid paraffin wax into the tissues which once set, stabilizes the tissue architecture and facilitates long-term storage at room temperature.

The resulting FFPE tissue blocks are then mounted onto a tissue-cutting device (microtome) to obtain thin sections for analysis. A broad range of analytical methods, including histological and immunohistochemistry techniques, have been successfully validated on FFPE tissue sections.^{25–27} Post-processing, tissue sections are routinely stained with hematoxylin and eosin (H&E), which enables histopathologists to examine the tissue morphology and produce a report which guides diagnosis and treatment. Other techniques include Ki67 staining, which indicates rate of cell division, and multiplex immunofluorescence (mIF), where antibody-tagged fluorescent probes bind to a panel of target proteins,¹⁵ leveraging the extensive, accessible resources provided by FFPE diagnostic archives. When analyzed in parallel with LA-ICP-MS, metal signatures can be more meaningfully interpreted in a biological context.

Preserved tissues are widely used in biomedical research due to the extensive availability of archived clinical samples. However, the effects of FFPE processing on endogenous metal contents and spatial distribution remain incompletely understood. A clearer understanding of how each processing step affects elemental localization is essential to attribute subtle

spatial variations to biological heterogeneity, rather than artifactual redistribution.

Several studies have compared bulk concentrations of metals in frozen versus FFPE samples from mammalian^{28–30} and human tissues.^{28,30–32} Their results suggest that fixation and embedding can alter various trace element concentrations via leaching or contamination.^{28,29,31,32} However, the same dynamics are not always observed between studies and organ/model type. For example, Zn was reported to have been leached from human prostate, swine liver and rat brain (as well as from rat kidney and liver samples to a lesser extent) during FFPE processing,^{28,30,31} while no significant change was detected for human placenta samples,³² and addition was observed for bovine liver.²⁹ Some data sets also contain unexplained outliers,^{29,32} reflecting a lack of consensus on preservation-induced artifacts. This uncertainty complicates interpretation of spatially resolved elemental data from FFPE tissues, hindering their use in quantitative bioimaging. Prolonged fixation has also been associated with leaching of labile elements such as As, Cd, and Mg from brain tissue.²⁸ This study involved human samples stored in fixative for 19 or 21 years, far exceeding typical clinical protocols (~24 h fixation). Similarly, paraffin embedding may homogenize elemental distributions, particularly for alkali metals, potentially due to solvent-driven diffusion,³³ though biological implications remain unclear. The contrasting results seen for different sample types and fixation procedures highlights the importance of assessing the impact of FFPE processing.

This study investigates the impact of FFPE processing on elemental contents and distributions in a previously uninvestigated tissue type: human breast carcinoma. By comparing *in situ* elemental data from unprocessed and FFPE-matched sections and analyzing processing reagents, we assess potential sources of elemental alteration. The findings indicate key considerations for future elemental bioimaging of breast carcinoma samples, offering insights that could enhance analytical trueness and precision, and inform the development of trace metal-aware protocols for routine histopathology.

METHODS

Ethical Approval Statement

Human samples used in this research project were obtained from the Imperial College Healthcare Tissue Bank (ICHTB; Human Tissue Authority license: 12275). The ICHTB is supported by the National Institute for Health Research Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. ICHTB is approved by Wales Research Ethics Committee 3 to release human material for research (22/WA/0214), and the samples for this project (R15001) were issued from the ICHTB main surgical collection.

Reagents

All reagents used to process tissue samples were standard solutions used routinely in histopathological practice. Ten percent neutral-buffered formalin, prepared from 40% formaldehyde (w/v) (Genta Medical) diluted 1:10 with HPLC Gradient grade water (Fisher Scientific), Surgipath Paraplast paraffin wax pellets (Leica Microsystems) and tissue-marking dyes (TMDs; Cancer Diagnostics, Inc.), were all provided by North West London Pathology, Charing Cross Hospital. Analytical grade ≥ 97% xylene (Fisher Scientific) and 96% ethanol (VWR International Ltd.) used for slide deparaffinisation were provided by the London Metallomics Facility (LMF).

In Situ Analyses of Tissue Sections for Elemental Concentrations

Preparation of Tissue Sections. Three surgically resected breast cancer samples, snap-frozen in liquid nitrogen, were selected based on no prior contact with optimal cutting temperature (OCT) solution and appeared visibly undamaged. Samples 1 and 2 were invasive lobular carcinomas and Sample 3 was an invasive mucinous carcinoma, a subtype accounting for less than 5% of all breast cancers.³⁴ Histopathology reports indicated that Samples 1 and 2 were inked with blue, green and black TMDs and Sample 3 with black TMD at resection. However, no TMD was visible on the frozen samples. Further physical and pathological details can be found in Table S1. Prior to analysis, the samples were stored at -80°C for 5 (Sample 1) and 7 years (Samples 2 and 3).

Manual tissue handling and histological staining was performed at ICHTB, Charing Cross Hospital. Metal contamination was minimized by using ethanol-cleaned tools and surfaces, and powder-free, colorless vinyl gloves, which are less enriched in leachable elements than latex or neoprene gloves.³⁵ Each breast cancer sample was divided into two halves using a sterile scalpel. The first fresh frozen halves of each sample, henceforth referred to as “FF”, were mounted onto a chuck using ultrapure $18.2\text{ M}\Omega\text{-cm}$ water from a Milli-Q Advantage system (Merck Millipore), and sectioned to $5\text{ }\mu\text{m}$ thickness using a Leica CM1520 cryomicrotome cooled to -21°C . Adjacent cryosections were collected where possible. In cases where a section was damaged during cutting, minimal trimming took place before obtaining the next section—such tissue section pairs are hereafter referred to as “pseudoadjacent.”

The second halves of each sample, henceforth referred to as “FFPE”, were individually fixed overnight in formalin at room temperature, with the latter sampled pre- and post-fixation. Once fixed, the FFPE sample halves were placed in separate cassettes and oriented so that the cut sides appeared “face up”. The cassettes were submitted to North West London Pathology, Charing Cross Hospital, for embedding, joining up to 300 routine diagnostic breast samples. Embedding was carried out using a Leica HistoCore PELORIS 3 automatic embedder at controlled temperature and pressure, with a built-in stirring mechanism to enhance liquid penetration into the tissues. Briefly, tissues are dehydrated in increasing concentrations of ethanol, then washed in xylene and finally impregnated with liquid paraffin wax at $55\text{--}85^{\circ}\text{C}$, with further details available in the User Manual.³⁶ The resulting FFPE blocks were mounted onto a semiautomatic CUT 5062 microtome (SLEE Medical GmbH) and sectioned to $5\text{ }\mu\text{m}$ thickness.

Both FF and FFPE tissue sections were transferred to Eprelia Superfrost Plus adhesion microscope slides. The remaining paraffin wax adhered to the cassette surrounding each FFPE breast cancer sample was also sampled. The FF and FFPE sections originating closest to the central division of the sample were H&E-stained. After at least 24 h, digital images of the H&E sections were created using a NanoZoomer S360 digital slide scanner (Hamamatsu Photonics, UK) at $40\times$ magnification ($0.23\text{ }\mu\text{m}/\text{pixel}$) to visualize tissue architecture. The pseudoadjacent unstained FF and FFPE sections were stored until further use at -80°C and room temperature, respectively.

In Situ Measurements of Elemental Concentrations by LA-ICP-MS. The unstained FF and FFPE sections were analyzed at the LMF. Prior to LA-ICP-MS, the unstained FFPE sections were dewaxed, which involves three separate 5 min vertical immersions in xylene, followed by three more 5 min immersions in ethanol. *In situ* elemental concentration measurements of Mg, Mn, Fe, Cu and Zn were performed via LA-ICP-MS using an Analyte Excite 193 nm ArF*excimer-based LA system (Teledyne Photon Machines, USA) with a HelEx II two-volume ablation cell (Teledyne CETAC Technologies, USA), coupled to an iCAP TQ ICP-mass spectrometer (Thermo Fisher Scientific, USA) via an Aerosol Rapid Introduction System. Elements were selected based on their established biological relevance to tumorigenesis, differing endogenous cellular concentrations and variable occurrence in protein-bound versus free-ion forms under physiological conditions.

Per run, the ablation chamber held three tissue sample slides and one calibration slide containing gelatin-based microdroplets, used to convert ion counts to elemental concentrations. The calibration slide was obtained from the Institute of Analytical Chemistry, University of Vienna.²¹ Each calibration set included six microdroplets containing 0, 173, 351, 684, 1821, and 3455 fg of each analyte (including Mg, Mn, Fe, Cu and Zn), prepared from 10% w/w fish skin gelatin and a mixture of multi- and single-element stock solutions (LabKings, Hilversum, Netherlands) to closely match the tissue matrix. Calibration was performed prior to ablating each tissue section. Calibration curves generated R^2 values typically >0.98 (one exception listed in Table S2), showing a high degree of linearity across the gelatin microdroplet concentrations.

The instrument settings (Table S3) were tuned using the NIST SRM 612 glass certified reference material (National Institute for Standards and Technology, USA). Analyte gas blanks and a series of SRM 612 standard ablation scans were acquired before and after every section to model background noise and correct for instrumental drift, respectively. The monitored isotopes were carefully chosen based on their natural abundances and anticipated isobaric and polyatomic interferences. Where such interferences could not be avoided, measurements were performed using the collision reaction cell operated in O_2 mass shift mode to ensure effective suppression of relevant species. The main concern is ^{56}Fe , as $^{40}\text{Ar}^{16}\text{O}$ passes the first mass filter at m/z 56. However, ArO reacts readily with O_2 in the collision reaction cell, forming species that do not transmit through the second mass filter. This allows robust quantification of ^{56}Fe , which is preferable because it has the highest natural abundance (91.8%) of all stable Fe isotopes. Possible interferences on $^{24}\text{Mg}^+$ from minor C_2^+ formed in the plasma were not found to be problematic. On the one hand, this reflects the generally high ion beam intensities generated by the samples for ^{24}Mg . On the other, the gelatin-based concentration standards employed in this study matched the C-based matrix of the tissue samples, to provide highly linear calibrations for Mg that adequately account for the presence of minor C_2^+ interferences.

Elemental images were acquired using a square laser spot, with a spatial resolution of $10\text{ }\mu\text{m}$. This resolution was selected to be appropriate for comparing the image output with the adjacent section microscopy images, maintaining low limits of detection by ablating sufficient material in each laser fire, while optimizing time and costs. The number of laser pulses delivered per spot (“dosage”; Table S6), was optimized to achieve total ablation of the sample with the minimum number of pulses, to avoid ablation of the underlying glass slide and maintain efficient acquisition speed.

Positional and ICP-MS ion count data were reconstructed using the HDF-based Image Processing software (HDIP, Teledyne Photon Machines Inc., USA). A bespoke pipeline, written in Python v3.8, was used to generate elemental images from reconstructed data and statistics. In summary, this pipeline calculates a quantification coefficient per element, performs image masking by removing small features, filling holes and erosion/dilatation to correct for instrumental noise, and then allows the user to set the image thresholding values for optimal visualization. Values below $0.0001\text{ }\mu\text{g g}^{-1}$, attributed to instrumental noise, were excluded from subsequent analyses.

Image Analysis and Statistical Treatment of the In Situ Data

New bespoke image and statistical analysis pipelines were designed to enable comparison of elemental concentrations between FF and FFPE sections derived from the same breast cancer samples. The workflow was developed specifically for this study and focuses on comparing elemental concentration distributions extracted from biologically defined regions. The chosen methodologies were used to determine both whether FFPE processing led to (i) any elemental loss/gain to/from the sample, and (ii) biologically relevant redistribution of elements within the sample. Analyses were performed at both whole-section and regional levels to assess elemental variations in relation to tissue composition and cellular density.

First, *in situ* elemental concentrations were uploaded to Fiji ImageJ2 (v2.14.0/1.54f) and folded areas were outlined, based on optical images taken before LA-ICP-MS. Folding, also termed “scoring” (Figure S1), is caused by microtome-induced shear forces, resulting in regions that are no longer a single layer in thickness. The data from these areas were excluded from further analysis. Concentration data from each section were $\log_{10}$ -transformed to mitigate right skew and facilitate outlier detection and removal via the interquartile range (IQR) method.³⁷ Values were subsequently back-transformed for further analysis.

Tissue shrinkage was then assessed using QuPath v0.5.0, an open-source software platform for visualizing, annotating, and quantitative analysis of whole-slide pathology images.³⁸ Representative areas of invasive carcinoma ($1 \times 1 \text{ mm}^2$ each) were annotated on H&E-stained breast cancer tissue sections and the “Cell Detection” function was applied to estimate cell counts. These areas contained between 3000–10,000 cells (Table S7) and covered about 30–60% of the samples’ cross-sectional tumor cell-dense areas. The FFPE sections permitted multiple nonoverlapping annotations, whereas FF sections typically allowed only a single annotation to be drawn because of smaller section areas. Due to unequal sample sizes, a Welch’s *t*-test was used to compare FF and FFPE results.

Regions of interest (ROIs) were manually drawn in Fiji based on cellular phenotype. Regions of interest densely populated with tumor epithelial cells were annotated as “tumour”, while ROIs composed of mostly normal-appearing stromal cells, were annotated as “stroma”. Elemental concentrations and their corresponding coordinate locations within these ROIs were extracted for downstream spatial analyses.

Concentration data were analyzed using Microsoft Excel v16.89 and R programming language v4.4.2. Normality was assessed using the Shapiro–Wilk test, or the Anderson–Darling test for sample sizes >5000. For two-group comparisons, Student’s or Welch’s *t*-tests were used for parametric data, depending on variance homogeneity, which was assessed with Bartlett’s test. The *P* values from multiple comparisons were adjusted using the Benjamini–Hochberg method. Statistical significance was defined as *p* < 0.05.

Given the nonparametric nature of elemental concentration data within ROIs and the presence of large, spatially correlated data sets, a repeated random subsampling approach was used to estimate robust mean concentrations, based on a form of Monte Carlo resampling.^{39,40} In this approach, repeated random subsamples of fixed size (100 values) were drawn without replacement from each ROI across 200 iterations, and the mean concentration was calculated for each subsample. This subsampling strategy was chosen to obtain stable estimates of central tendency while reducing the influence of spatial correlation and local clustering, providing a robust alternative to classical nonparametric tests such as the Wilcoxon rank-sum test by maintaining statistical power and not relying on assumptions of normality or homoscedasticity. This approach is aligned with subsampling and patch-based random sampling strategies previously applied in biological imaging contexts to improve robustness.⁴¹ The resulting subsample means were used for independent *t*-tests.

To compare differences in elemental concentration distributions between FF and FFPE samples within tumor and stroma ROIs, quantile–quantile (Q–Q) relative differences were computed. For each element, concentration values were extracted at quantiles between the fifth and 95th percentiles for both FF and FFPE data. At each quantile level (*p*), the relative difference was defined as

$$\Delta(p) = \frac{Q_{\text{FFPE}}(p) - Q_{\text{FF}}(p)}{Q_{\text{FF}}(p)}$$

The distribution of $\Delta(p)$ values was summarized as boxplots showing the 25th, 50th, and 75th percentiles, with whiskers corresponding to the fifth and 95th percentile limits.

Jensen–Shannon distances (JSDs) were calculated following the statistical framework described in Mesa et al.⁴² Recent applications in neurobiological image analysis have demonstrated the utility of Jensen–Shannon divergence for comparing complex biological

distributions,⁴³ supporting its use in this context. The JSD offers a symmetric, bounded and continuous (i.e., “fuzzy”) measure of divergence between two probability distributions, allowing quantification of the magnitude of distributional differences rather than a binary hypothesis-testing outcome, such as the Kolmogorov–Smirnov test.^{44–46} In this study, probability distributions of elemental concentrations in each section were estimated using Kernel Density Estimation. The JSD ranges from 0 (indicating identical distributions) to 1 (maximal divergence). As a true mathematical distance, the JSD has several useful properties, including symmetry and compliance with the triangle inequality,^{44–46} which make it a robust and interpretable metric for comparing compositional similarity across sections.

Determination of Elemental Concentrations for the Sample Processing Reagents

The elemental concentrations of relevant sample processing reagents were determined by solution ICP-MS to evaluate if they could be responsible for mobilizing metals from the tissue samples or introduce metal contamination. These encompass (i) TMDs, (ii) formalin, (iii) paraffin wax, (iv) xylene and (v) ethanol.

In detail, leachates from red, orange, yellow, green, blue and black TMDs routinely used for sample orientation following surgical resection, fresh paraffin wax pellets, paraffin discard from the embedder and paraffin surrounding the embedded breast cancer samples, were analyzed. Formalin, xylene and ethanol reagents were sampled at two time points, before and after coming into contact with tissue, and are hence categorized as “baseline” and “post-fixation” or “post-immersion,” as appropriate. All xylene and ethanol aliquots were 5 mL and formalin aliquots up to 15 mL.

Sample Preparation

The sample preparation for reagent analyses was carried out in the metal-free ISO Class 6 clean rooms of the MAGIC Laboratories, Imperial College London, with all critical steps completed in ISO Class 4 laminar flow workbenches.

For reagent handling and storage prior to ICP-MS analysis, acid-cleaned 22 mL Savillex PFA vials or trace metal grade 15 mL high-performance centrifuge tubes (VWR International Ltd.) were used. Reagents for digestion included distilled concentrated HNO_3 (15.4 M, prepared in-house from analytical grade; Fisher Scientific), and Suprapur H_2O_2 (Merck). Ultrapure 18.2 M Ω -cm water from a Milli-Q Advantage system (Merck Millipore) was used for all preparations.

The formalin, xylene and ethanol samples were dried on a hot plate at 150 °C. Then 2 mL of concentrated HNO_3 was added to each sample, followed by 10 min ultrasonication to promote dissolution of the residue. Subsequently, the samples were subjected to a closed-system hot plate reflux, where the temperature was gradually increased to 150 °C. After drying at 160 °C, any residual organic material was digested by refluxing overnight with a mixture of 0.8 mL concentrated HNO_3 and 0.2 mL 30–32% H_2O_2 at 150 °C.

A liquid–liquid extraction procedure was carried out with the paraffin to release any trace metal contaminants. To this end, the paraffin samples were placed in 50 mL centrifuge tubes and after 15 mL xylene had been added, the mixture was vortexed until the paraffin was fully dissolved. Subsequently, 15 mL of 2 M HNO_3 was added to the tubes and shaken vigorously for 2 min and left to settle for 5 min; the shaking and settling was repeated three times, leaving the trace metals in the inorganic nitric acid. A pipet was then used to extract the xylene, and the remaining nitric acid was transferred to Savillex vials and evaporated to dryness on a hot plate at 120 °C, overnight. While metal yields of the procedure were not verified, the yields of similar simple extraction systems for trace metals were found to be nearly quantitative.^{47,48} As such, the extraction method enables a reasonable assessment of the mobile metal contents of the paraffin, but likely overestimates the amounts that are readily leached during FFPE processing, as the latter is more mild than the acid extraction that was employed here.

The dried formalin, xylene and ethanol digests were reconstituted in 2 mL 0.5 M HNO_3 and the dried paraffin sample digests in 1.98 mL of 0.1 M HNO_3 . The solutions were then refluxed overnight on a

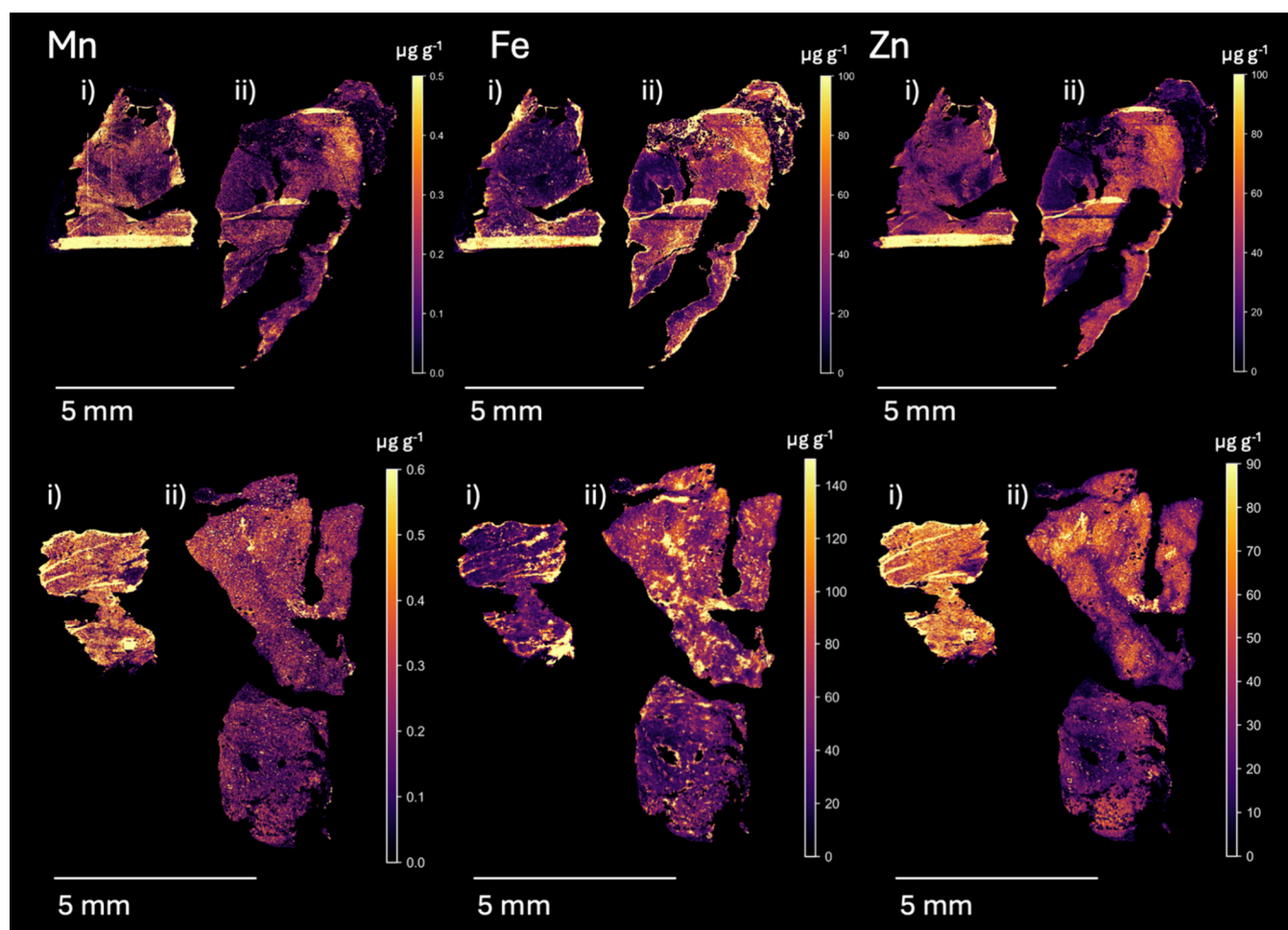


Figure 2. LA-ICP-MS elemental maps of Mn, Fe, and Zn obtained from breast cancer tissue sections. Sample 1 (top row) and Sample 2 (bottom row). (i) FF and (ii) FFPE sections.

hot plate at 100 °C and transferred to 15 mL centrifuge tubes. Approximately 0.5 mL of each TMD was pipetted into centrifuge tubes and 12 mL 2 M HNO₃ was added. After 72 h at room temperature, 100 μL aliquots were taken from each tube for analysis. For the subsequent analyses, the formalin, xylene and ethanol analytes, and the extracted paraffin and TMD leachates were diluted to obtain 2–5 mL of solution in 0.04 to 0.5 M HNO₃.

ICP-MS Analyses of Reagents

The multielemental concentration measurements of the solutions were conducted at the LMF using a PerkinElmer NexION 5000 ICP-MS. Samples were introduced using a Cetac ASX-560 autosampler coupled to a SeaSpray glass nebulizer fitted to a quartz cyclonic spray chamber. Dynamic reaction cell mode was used to measure the concentrations of biologically relevant macronutrients (Mg, K and Ca), micronutrients (Mn, Fe, Co, Ni, Cu, Zn, Se and Mo) and nonessential metals (V, Cr, Cd and Pb). Isotopes monitored and additional instrumental parameters are listed in Table S4. Selenium and Mo were analyzed using an oxygen mass shift from mass numbers 80 to 96 and 95 to 127, respectively. All other isotopes were analyzed on-mass using ammonia as a reaction gas to suppress polyatomic interferences. Calibration standards were run every 15 samples. The calibrants were made from a TraceCert multielement standard (Sigma-Aldrich) and had elemental concentrations of between 0.05 and 100 μg L⁻¹. Samples and standards were doped with Ga (Teledyne Leeman Laboratories) to a concentration of 10 μg L⁻¹.

Quality control of measurements was ensured through repeated analyses of acid blanks, a 1 μg L⁻¹ calibrant and the certified reference material Trace Metals in Drinking Water (CRM-TMDW) following dilution by a factor of 10. Analyte measurements were normalized to

the internal Ga standard to account for instrument drift and matrix effects, and the data were subsequently blank corrected by removing the average analyte intensity of repeated blank analyses (every 15 samples). The corrected analyte intensities were converted to concentrations using the linear equation generated by the calibration curve defined by the standard solutions.

Limits of detection (LOD) and quantification (LOQ) were defined as three and ten times the standard deviation of procedural blanks, respectively (Table S5). These blanks were used to correct sample concentrations. Most blanks were below 8 ng g⁻¹, except for one elevated Zn value (12.5 ng g⁻¹) and variable Ca levels (7.31–223 ng g⁻¹; median = 26.8 ng g⁻¹). After blank correction, the elemental concentrations measured for CRM-TMDW were within 10–15% of the certified values (Table S6), except for the Mo abundance determined in the third batch of analyses, which exceeded the reference value by about 36%. Overall, the data for CRM-TMDW hence show acceptable agreement between measured and reference values.

RESULTS AND DISCUSSION

Assessment of Tissue Section Quality

Prior to elemental analysis, notable differences could be observed in FF versus FFPE tissue section quality. First, the FF and FFPE sections had different shapes and sizes (Figures 2 and 3 and Figures S2–S4), as sections were taken from two separate halves of each sample which, by nature, are amorphous in shape and composition. Second, the FFPE

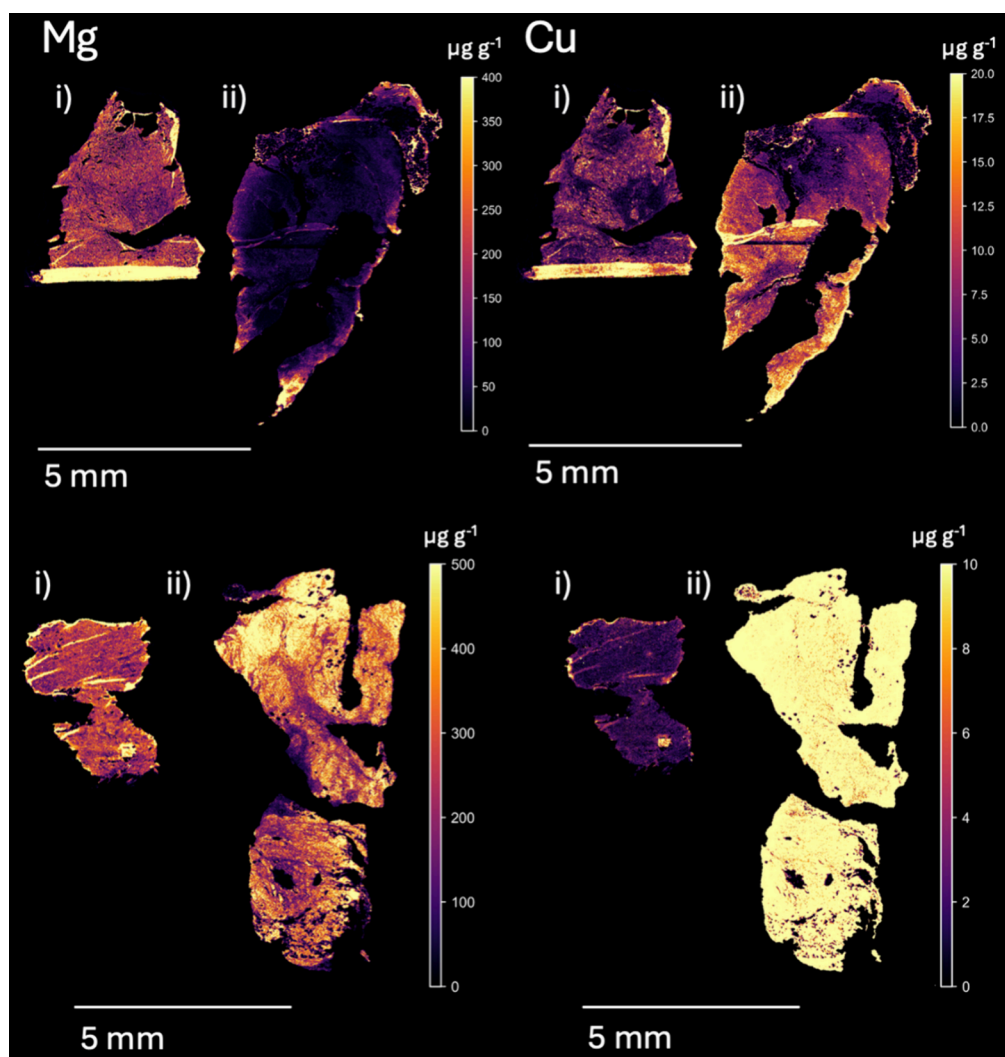


Figure 3. LA-ICP-MS elemental maps of Mg and Cu obtained from breast cancer tissue sections. Sample 1 (top row) and Sample 2 (bottom row). (i) FF and (ii) FFPE sections.

H&E and pseudoadjacent elemental section images more closely resembled each other, enabling clearer regional segmentation according to cellular phenotype, alongside reduced folding (Figures S2–S4), when compared to the FF counterparts. Paraffin infiltration stabilizes FFPE tissues, enhancing their resistance to the mechanical shear force of microtomy. Although minor folding may occur, FFPE sections are floated on a water bath, where surface tension aids in flattening them before mounting onto slides. In contrast, FF samples are inherently more brittle, making it difficult to obtain intact, flat sections. These sections cannot be floated, increasing the likelihood of incomplete contact with the slide surface. Bands of elevated concentrations observed in the *in situ* elemental data of FF sections reflect folding, whereby multiple tissue layers were ablated. Further complications arise for FF sections in the ablation chamber during gas evacuation and instrument tuning, where they thaw, lose moisture and shrink. Combined, these factors result in artifacts visible in both histological and elemental FF section images (Figures 2 and 3 and Figures S2–S4).

The artifacts that impact FF tissue sections introduce two major analytical challenges. First, regions of tissue folding cannot be reliably quantified. Quantitative calculations assume

a uniform section thickness and a consistent ablation volume per laser pulse, both of which are compromised in folded FF sections. The concentration data obtained for folded regions were hence manually assigned a value of 0 and then removed.

Moisture loss during thawing can also alter the nominal cutting thickness ($\sim 5 \mu\text{m}$ in this study). Currently, no standardized method exists to correct for local variations in section thickness during ablation. However, in this study, a $10 \mu\text{m}$ spot size was selected to achieve high resolution but this is also more tolerant to minor topographical variations than ultrafine spots ($1\text{--}2 \mu\text{m}$). Furthermore, all slides were examined via light microscopy postimaging to confirm that all material had been removed.

Discrepancies in tissue section morphology caused by folding or shrinkage can hinder accurate spatial correlation of histological and molecular features since alignment between serial sections is compromised, essential for complementary analyses such as H&E and mIF.

While the structural integrity of the FFPE sections outperformed the FF counterparts, FFPE has been recognized to cause cytoplasmic shrinkage.^{49–51} Therefore, it was imperative to determine the extent of the section morphing at a cellular level, before carrying out like-for-like quantitative

comparisons between FF and FFPE section pairs. Samples 1 and 2 exhibited no significant differences in the number of cells detected per region of the same area (Table S7). Therefore, disparities in area between FF and FFPE section pairs, with up to 3-fold differences (Table S1), are likely due to FF section folding, moisture loss, or an orientation change of the breast cancer sample during processing. For embedding, the samples were placed in individual cassettes which do not necessarily secure the sample from movement during reagent introduction and removal. Therefore, the sample orientation can change such that the side of the sample that is “face up” when the FFPE block is sectioned, is not the originally cut side. A combination of these factors is likely responsible for the difference in shape and size in all three sample FF–FFPE pairs (Figure 2), even though they were cut from a similar area of the sample.

Sample 3 displayed mucin-rich, gel-like secretions throughout the H&E sections (Figure S4). The loss of stable cell-to-cell adhesions in mucinous breast cancers may enhance susceptibility to elemental leaching during all stages of sample preparation, including tissue embedding and section dewaxing, compared to more common ductal and lobular carcinoma subtypes. Other imaging challenges were apparent for both the FF and FFPE sections of Sample 3. For example, the cell detection algorithm performed poorly (Figure S5), recognizing mucin strands as cell nuclei and consequently overestimating the cell count. The FF section for LA-ICP-MS displayed folding throughout and the FFPE section elemental data appeared noisy due to the lower elemental concentrations obtained for Sample 3 versus Samples 1 and 2. Despite exhibiting adequate fixation quality, as confirmed by a specialist histopathologist, the inherent morphological appearance of the FFPE H&E image (Figure S4) prohibited tumor/stroma regional annotation. As a result, it was decided that Sample 3 would not be used for regional FFPE–FF comparisons.

LA-ICP-MS Results

Tissue Section Elemental Distributions. The concentrations of Mg, Mn, Fe, Cu and Zn determined for pairs of breast cancer tissue sections that originated from the same sample but were processed differently are compared in the following, to explore whether FFPE alters the endogenous distributions of these elements in terms of gain, loss or redistribution.

The overall concentration distributions per element for each section are shown in Figure 4. The median obtained Mn, Fe and Zn concentrations range within previously reported values (Table 1).^{4,10,18,52–61} Most median Cu concentrations are also in accord with literature values, except for one value for an FFPE section ($51 \mu\text{g g}^{-1}$; Table 1), which exceeds the highest previously reported value by nearly a factor of x2. Most median Mg concentrations ($\sim 46\text{--}1625 \mu\text{g g}^{-1}$, mean = $456 \mu\text{g g}^{-1}$) exceed the range reported in the literature ($56 \pm 30 \mu\text{g g}^{-1}$), but the latter is only based on a single study, which may not be representative of the full natural range of values (Table 1).

Comparing each FF–FFPE section pair, the IQRs overlap for Mn, Fe and Zn, preliminarily indicating that no substantial elemental loss or gain occurred during processing (Figure 4). This is reflected in the elemental images (Figure 2), where the concentration ranges and distributions of Mn, Fe and Zn appear similar between FF–FFPE section pairs. This suggests that the concentrations and distributions of these three elements in FFPE sections likely reflect endogenous tissue

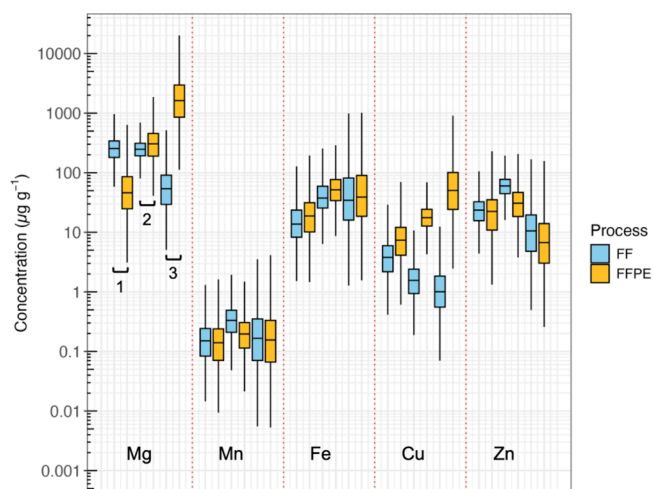


Figure 4. Boxplots of Mg, Mn, Fe, Cu, and Zn concentrations across FF and FFPE breast cancer sections determined using LA-ICP-MS. The numbers indicate the sample from which the section pairs originated.

levels. For Zn, a mouse study using FFPE sections found significant increases in Zn across all studied mouse organs.³³ This was attributed to potential contamination during sample processing, highlighting the importance of trace metal clean procedures during FFPE processing for samples that will be analyzed for elemental concentrations.

In contrast, the images clearly show that elemental distributions and/or concentrations changed for Mg and Cu (Figures 3 and 4), whereby multiple redistribution patterns can be observed when comparing FF–FFPE sections. For Sample 1, Cu appears to accumulate at the base of the FFPE section (Figure 3). This apparent gravity-driven accumulation may have occurred during paraffin impregnation as the cassettes are lifted to drain the excess. Unlike Cu, Mg appears to have been lost from the center of Sample 1, accumulating peripherally (Figure 3). This “rind” effect⁶² could be due to elemental mobilization during embedding or dewaxing,^{33,63} or uneven tissue fixation, causing the specimen periphery to harden. The Sample 2 FFPE section displays striking Cu addition, such that thresholding the images according to the FF Cu concentration range results in complete saturation of the FFPE image (Figure 3). The more divergent IQRs across each FF–FFPE pair for Cu and Mg (Figure 4) align with these qualitative observations, with Cu elevated in all FFPE sections compared to FF and Mg exhibiting both gain (Sample 2) and loss (Sample 1). The loss can likely be attributed to leaching during FFPE processing, as observed elsewhere,²⁸ as Mg is largely mobile (unbound) in most tissues.

For all elements, the concentration distributions across the entire sections exhibit pronounced right-skewed tails (Figure 4; note that the data are plotted on a logarithmic scale). These infrequent but high-magnitude values are unlikely to result from instrumental error, which typically manifests as linear artifacts aligned with the raster movement of the laser, or tissue folds, which were systematically removed. Furthermore, the calibration curves generated from the picogel standards exhibited high linearity, with R^2 values ≥ 0.98 (Table S2), supporting reliable extrapolation to obtain these high concentrations. Instead, these values may represent areas of tissue that have naturally higher concentrations of metals (e.g.,

Table 1. Elemental Concentrations of Breast Cancer Tissues Measured by LA-ICP-MS in This Study and in Comparison with Literature Values^a

element	process	sample	median ($\mu\text{g g}^{-1}$)	Q1 ($\mu\text{g g}^{-1}$)	Q3 ($\mu\text{g g}^{-1}$)	concentration range reported in literature ($\mu\text{g g}^{-1}$)
Mg	FF	1	255	180	341	55.7 $\pm$ 30.1 ^b
		2	248	193	312	
		3	53.8	29.4	90.9	
	FFPE	1	46.0	24.8	85.8	
		2	305	190	455	
		3	1625	856	2960	
Mn	FF	1	0.15	0.08	0.24	0.12–1.07 ^c
		2	0.33	0.21	0.49	
		3	0.17	0.07	0.35	
	FFPE	1	0.14	0.07	0.24	
		2	0.20	0.11	0.31	
		3	0.16	0.07	0.33	
Cu	FF	1	3.79	2.18	5.97	0.2–27 ^d
		2	1.56	0.94	2.40	
		3	1.00	0.55	1.84	
	FFPE	1	7.36	4.12	12.1	
		2	17.6	12.7	24.3	
		3	50.5	24.2	101	
Fe	FF	1	13.7	8.26	23.6	1.4–216 ^d
		2	37.5	25.6	59.3	
		3	34.5	16.0	81.4	
	FFPE	1	18.8	10.1	31.4	
		2	51.7	34.1	76.8	
		3	39.1	18.5	90.1	
Zn	FF	1	23.6	15.7	32.5	0.47–64 ^e
		2	60.0	44.6	77.3	
		3	10.6	4.80	196	
	FFPE	1	22.4	10.9	35.1	
		2	30.8	18.4	46.6	
		3	6.7	3.03	14.0	

^aMedian, first quartile (Q1), and third quartile (Q3) elemental concentrations of breast cancer tissue sections, as determined by LA-ICP-MS in this study, are presented alongside reported ranges from the literature. Reference ranges are from tissues that were either freshly resected and dried or fresh frozen, analyzed without preservation or additional processing. ^bLiterature source: ref 55. ^cLiterature sources: refs 55 and 56. ^dLiterature sources: refs 52–55. ^eLiterature sources: refs 52–55 and 10.

blood vessel walls, red blood cells). Alternatively, they may be caused by exogenous metal deposition.

Contribution of Processing and Biological Heterogeneity to Changes in Elemental Distribution. The elemental relationships between ROIs consisting of mostly (i) tumor cells or (ii) stroma (Figure 5), were compared across FF and FFPE samples. By reducing biological variability, any differences in elemental distribution can more likely be attributed to processing rather than innate heterogeneity. Despite Mn, Fe and Zn distributions appearing well-conserved and Cu and Mg appearing to be affected by processing, bootstrapped *t*-tests for FF–FFPE stroma and FF–FFPE tumor pairs reveal significant differences in concentration for most comparisons (adjusted *p* < 0.001; Table S8). Discrepancies between differently processed sections originating from the same sample were anticipated, as the sections were pseudoadjacent and morphological analysis of H&E sections confirmed compositional differences. This highlighted an additional challenge: distinguishing elemental variations caused by endogenous biological heterogeneity versus tissue processing.

To ascertain biological relevance of these concentration differences, a commonly used threshold in molecular biology

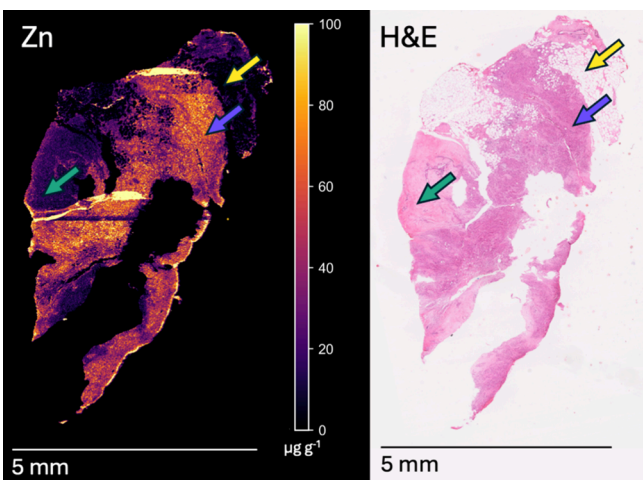


Figure 5. Elemental distribution of Zn in an FFPE breast cancer tissue section determined by LA-ICP-MS (left) and a pseudoadjacent H&E-stained FFPE section (right). Arrows indicate regions of differing cellular compositions: yellow = lipid-rich, green = stroma-rich, and purple = tumor-rich.

was used: absolute $\log_2$ fold change >1.5 , corresponding to a $>50\%$ change.⁶⁴ Among the elements analyzed, only Cu and Mg exhibited FF–FFPE differences above this threshold between bootstrap means (Table S8). Thus, while most elemental distributions were statistically different between FF–FFPE ROIs, the magnitude of the differences were only biologically significant for Mg and Cu.

To better compare differences in elemental concentration distributions between FF and FFPE samples within tumor and stroma ROIs, quantile–quantile relative differences were calculated (Figure 6). As shown, Mn and Zn exhibited the

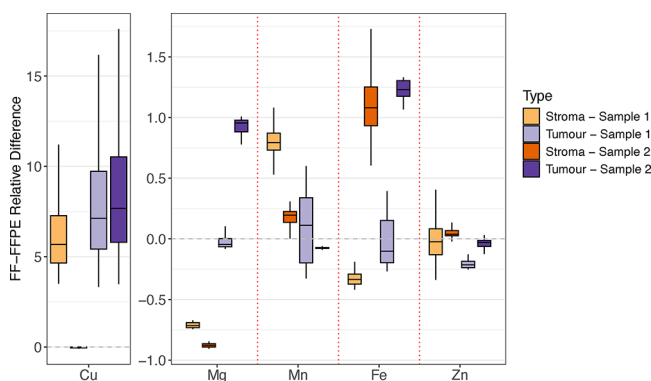


Figure 6. Quantile–quantile relative difference of elemental concentrations between FF and FFPE samples. Copper is shown on a separate axis due to the large relative differences. Boxplots are grouped by element and colored by tissue type and sample.

least variation with $\Delta(p)$ ranging from -0.5 to 1.0 . Magnesium and Fe showed larger differences, with $\Delta(p)$ ranging from -1.0 to 1.5 , while Cu displayed a substantially larger relative difference, between 5- and 10-fold in three of the four ROIs analyzed.

No systematic effect was observed based on the cellular composition of the ROI. For example, tumor ROIs did not exhibit consistently greater differences between FF and FFPE sections than stroma. Moreover, the direction of relative differences for Mg, Mn, Fe, and Zn was inconsistent, as both elemental losses and gains were indicated, regardless of the element. In accord with our results, another study reported that Mn and Fe concentration distributions and maxima for mouse kidney samples were similar.³³ However, the latter comparison was based largely on visual rather than numerical assessments. Given the limited sample size in our study, these observations warrant validation with larger data sets.

To assess differences in elemental distributions between FF and FFPE sections, JSDs were calculated for tumor and stroma ROIs. A JSD of 0 thereby denotes identical distributions, while 1 indicates complete divergence. Within tumor ROIs, JSD values for Mn and Zn were between 0.09 and 0.15, indicating a high degree of similarity in elemental concentration distributions. The stroma ROIs exhibited slightly higher JSD values (0.21–0.31), suggesting a mild divergence in distributions. These results reinforce that despite FFPE processing, the elemental distributions of Mn and Zn, particularly within tumor tissue, remain notably consistent. In contrast, Mg, Fe and Cu reached maximum JSDs of 0.95, 0.65 and 0.99, respectively (Table 2). Iron showed a moderate divergence overall, with higher JSDs in tumor ROIs (0.50 and 0.65) compared to stroma (0.31 and 0.21), a pattern that was not replicated by the other elements. Tumour neovasculature is

Table 2. FF versus FFPE ROI Jensen–Shannon Distances^a

ROI	element	sample	JSD _{FF–FFPE}
tumor	Mg	1	0.95
		2	0.78
	Mn	1	0.15
		2	0.09
	Fe	1	0.50
		2	0.65
	Cu	1	0.06
		2	0.99
	Zn	1	0.11
		2	0.09
stroma	Mg	1	0.80
		2	0.17
	Mn	1	0.30
		2	0.22
	Fe	1	0.31
		2	0.21
	Cu	1	0.90
		2	0.99
	Zn	1	0.21
		2	0.31

^aConcentration values per element per ROI were each separately converted into a probability distribution in R. The Kernel Density Estimation method was used to generate 1000 values by inverse transform sampling from the cumulative distribution function. Each FF–FFPE pair of probability distributions were then compared to calculate a Jensen–Shannon divergence (using the “philentropy” R package), which is then square rooted to calculate the Jensen–Shannon distance (JSD). Values of 0 indicate that the distributions are identical while 1 indicates that they are very different.

well-documented to be structurally and functionally abnormal, particularly with respect to blood flow and vascular permeability.⁶⁵ This characteristic may account for the higher JSD values observed for Fe in tumor compared to stroma ROIs, perhaps reflecting a more heterogeneous elemental distribution driven by erratic perfusion. This may suggest that Fe is more readily mobilized in tumor tissue.

In summary, these results imply that FFPE processing strongly alters the concentration distributions of Cu and Mg, and to a lesser degree, of Fe. In contrast, the concentration distributions of Mn and Zn, particularly in tumor ROIs, remain largely unaffected. Notably, Mg and Cu exhibited a wide range of JSD values (0.17–0.95 and 0.06–0.99; Table 2), highlighting the need for further investigation that employ larger sample sizes and independent replicates to evaluate reproducibility and generalizability. Overall, the findings support the rationale for not only examining the element concentrations of bulk samples but also interrogating elemental distributions more deeply within different tissue compartments when evaluating the effects of tissue processing.

Reagent Results

Trace metal analyses were performed on TMDs, formalin, paraffin wax, xylene and ethanol to assess their potential to contaminate breast cancer tissues or induce elemental leaching during FFPE processing and slide dewaxing. These data were used to evaluate whether elemental addition or losses observed in reagents correspond to *in situ* elemental differences between paired FF and FFPE tissue sections, thereby indicating which processing stages contribute to observed elemental redistributions. For elements that appeared unaffected by processing,

such as Zn, reagent analyses were used to confirm whether minimal or no elemental mobilization had occurred. In the following, the results obtained for the reagents are discussed in the order they are used in sample processing.

Addition of Exogenous Metals to the Samples

Tissue-Marking Dyes. Tissue-marking dyes were leached to evaluate trace metal contamination potential. Following leaching with 2 M HNO₃, only the blue TMD displayed any visible solubility, with the acid immediately turning transparent blue. The red, orange, yellow, green and black TMDs did not visibly dissolve in the acid, which remained colorless even after 72 h, while the TMDs immediately solidified against the tube walls. Solution ICP-MS results revealed minimal leaching of Mn and Zn from TMDs, with elemental concentrations consistently below 15 $\mu\text{g g}^{-1}$ for the TMDs (Figure 7 and Table S9). Iron leaching was greater, with orange, red, green, and blue TMDs leaching between 100 and 445 μg per gram of dye (black only 1.37 μg per gram).

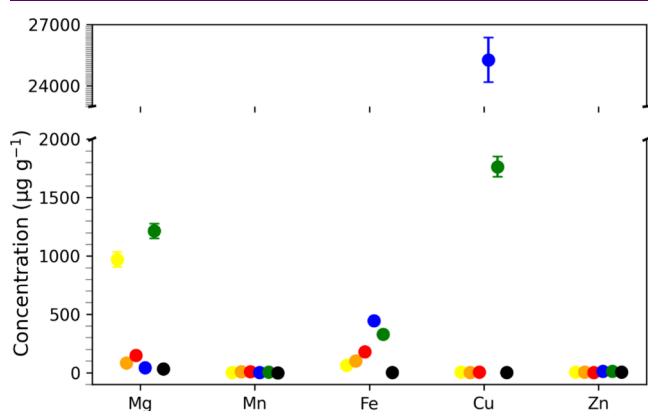


Figure 7. Elemental concentrations leached from tissue-marking dyes of varying colors (yellow, orange, red, green, blue, and black). Data points represent the mean concentrations, with error bars indicating ± 2 SD. Error bars may not be visible when their magnitude is smaller than the size of the data points.

Notably, 25 mg of Cu was leached per gram of blue TMD (Figure 7 and Table S9), suggesting that a Cu compound was used in the production of this dye. Despite not showing any visible signs of Cu leaching, 1.8 mg Cu was leached per gram of green dye. All other colors leached $\leq 5 \mu\text{g}$ of Cu per gram of TMD. Since both Samples 1 and 2 had been marked with blue and green TMDs, it is plausible that Cu from the dyes was mobilized during the FFPE process, potentially contaminating the FFPE samples. This may account for the Cu saturation observed in Sample 2 (Figure 3), but a similar effect was not evident for Sample 1.

As TMDs are typically applied to tissues with a paintbrush, it is unlikely that a whole gram is used per tissue sample. Therefore, when contextualized by endogenous tissue concentrations (Table 1), it is recommended that researchers exercise caution when analyzing tissues with low endogenous Fe concentrations ($\leq 10 \mu\text{g g}^{-1}$) that have been annotated with TMD. Most importantly, Cu levels in tissues annotated with green and particularly blue TMDs are likely to be artificially elevated.

Paraffin Wax. The 2 M HNO₃ extracts obtained from fresh paraffin wax pellets dissolved in xylene showed low elemental concentrations: 1 ng g⁻¹ Mn, 78 ng g⁻¹ Fe, 6 ng g⁻¹ Cu and 18

ng g⁻¹ Zn (Mg was < LOQ). The extracts of the paraffin sampled from around the embedded samples exhibited higher elemental concentrations, but the levels were still negligible compared to the concentrations in the FFPE tissue sections (Table 3 and Table S10). For example, the Cu present in the

Table 3. Comparison between Elemental Concentrations Extracted from Paraffin Wax Surrounding FFPE Breast Cancer Samples versus the Median Elemental Concentrations Obtained for the FFPE Breast Cancer Sections^a

element	paraffin block concentration range ($\mu\text{g g}^{-1}$)	FFPE tissue section median range ($\mu\text{g g}^{-1}$)
Mg	0.10–0.24	46–1600
Mn	0.01–0.02	0.14–0.20
Fe	0.33–0.67	19–52
Cu	0.01–0.02	7.4–51
Zn	0.26–0.36	6.7–31

^aThe paraffin block and FFPE tissue section median concentration ranges are based on analyses of three breast cancer samples.

paraffin wax (0.01–0.02 $\mu\text{g g}^{-1}$) would have contributed less than 0.5% of the median Cu FFPE tissue section concentration (7–51 $\mu\text{g g}^{-1}$). It was considered that the Cu enrichment observed at the base of one FFPE section (Figure 3) may reflect gravitational settling during paraffin embedding, as tissues are repeatedly lifted to drain excess paraffin. However, this pattern was not observed for other samples or elements, indicating that redistribution may arise from other steps during the FFPE process. Furthermore, the extracts analyzed here were obtained via acid extraction of the dissolved paraffin, which equates to harsher extraction conditions than samples would experience during paraffin embedding. Therefore, the elemental concentrations of the acid extracts are likely overestimates, and it is thus unlikely that paraffin wax is responsible for elemental contamination of FFPE samples.

Leaching of Endogenous Metals from the Samples

Formalin Fixation. Formalin fixation and sampling of Sample 1 took place about 2 months before Samples 2 and 3 were processed. The baseline formalin elemental concentrations revealed distinct differences between the batches. The baseline Mg, Mn, Fe, Cu and Zn concentrations of the formalin used for Samples 2, 3 were all less than 5 ng g⁻¹, except 10 ng g⁻¹ Cu for the Sample 2 formalin. In contrast, the baseline element concentrations of the Sample 1 formalin were considerably higher, at 346, 4, 106, 16, and 98 ng g⁻¹ for Mg, Mn, Fe, Cu and Zn, respectively. The variability in the baseline trace metal contamination levels may be due to storage conditions, as histopathology laboratories prioritise sterility over preventing trace metal contamination in day-to-day procedures. However, the exact source of contamination for these elements remains unclear.

After sample fixation, the concentrations of the major elements Mg, K and Ca, and the trace elements Fe, Cu and Zn increased in all formalin batches, suggesting that the elements were leached from the tissues. The magnitude of concentration change appeared to vary according to sample, whereby the formalin concentrations of Mg and Fe increased by 163 ng g⁻¹ and 18 ng g⁻¹, respectively, for Sample 1, but only by 4 ng g⁻¹ and 0.28 ng g⁻¹, respectively, for Sample 3. Elemental mobilization during formalin fixation, with movement of metals from the tissue into the formalin, was reported

previously.^{28,66} One of the main clinical purposes of formalin fixation is to cause protein denaturation. This results in a decreased or lost capacity for protein-bound metal ions to maintain stable complexes, facilitating their movement and therefore loss from tissues. The magnitude of leaching of the major elements Mg, K and Ca from the samples was generally 10-fold greater than for the trace elements Mn, Fe, Cu and Zn (Table S11), reflecting the higher abundances and mobilities of the former in biological tissues.^{28,30,33,67,68} In this study, the leaching of Mg, the least protein-bound of our target elements, into the formalin appeared to align with the *in situ* results (Figure 3). In detail, during fixation, the increase in the Mg concentration of the Sample 1 formalin exceeded that measured for the formalin used to fix Samples 2 and 3 by at least an order of magnitude (Table S11). Correspondingly, the Sample 1 section and tumor/stroma ROI analyses revealed loss of Mg from the FFPE section but Mg addition to the Sample 2 and 3 FFPE sections, when compared to paired FF data (Figures 4 and 6 and Table 1).

Other factors that may potentially influence elemental leaching during fixation include sample mass and surface area, temperature and fixing duration.^{67,69} Sample 3, with the largest tumor by mass (Table S1), showed the smallest increase in formalin elemental concentrations across all measured elements except for Cu (Table S11), perhaps due to a reduced surface area-to-volume ratio. Formalin fixation is typically carried out at room temperature for convenience, however, variability in fixation temperature has been recognized as a source of methodological inconsistency,⁷⁰ which may affect fixation rate and potentially increase the risk of autolysis before fixation is complete. Autolysis is the process of enzymatic cellular degradation that leads to loss of membrane integrity, which can impede histological and elemental evaluations due to the release of intracellular contents. Fixation duration also remains poorly standardized, with guidelines ranging from 6 to 72 h.⁷¹ Prolonged fixation may induce sample hardening and shrinkage, further complicating analysis. In this study, minimal cell shrinkage was observed which is encouraging, as excessive shrinkage can artificially increase elemental signals due to increased cellular density. To improve the reproducibility of both histological and elemental data acquisition, standardization of fixation parameters such as temperature, duration and the formalin-to-tissue mass ratio is critical, particularly for mobile, unbound elements.

In this study, prior to being loaded into the automatic tissue embedder, all breast cancer samples were placed in a holding formalin bath along with hundreds of other samples, as is standard histological practice. This formalin bath had a clearly visible transparent blue color. Therefore, in addition to the TMD results discussed earlier, there are two possible scenarios that may induce Cu contamination: (i) any TMD present releases Cu into the formalin bath which then penetrates the tissues; and/or (ii) any Cu deposited on the periphery of a sample by TMD is smeared across the sample as the paraffin is drained or when cut by a microtome. The former option is more likely, as smearing would also be expected to occur during sectioning of FF samples.

Slide Dewaxing with Xylene and Ethanol. Most elements were present at concentrations below the LOQ in the baseline and post-immersion xylene and ethanol aliquots. The consistently low baseline concentrations are expected, as the reagents are stored and used in a facility where metal

blanks are minimized, compared to histopathology laboratories, which focus on biological rather than metal cleanliness. In summary, Zn was measured at below 5 ng g⁻¹ for 35 of 36 solvent analyses, Fe at below 5 ng g⁻¹ for 34 of 36 analyses and Mn, Ni, Se, Mo, Cd and Pb at below 1 ng g⁻¹ for all analyses (Table S12). Copper and Co were < LOQ for all analyses. Notably, the low Cu concentrations post-immersion indicate that this processing step did not remobilise TMD from the periphery of the sectioned samples. The negligible metal concentrations of the solvents suggest that formalin fixation is the most likely process to contribute to elemental loss, with any easily mobilized ions and contaminant ions from the TMDs released at this stage rather than during slide deparaffinisation with xylene and ethanol. This observation confirms the assumption previously made by Sarafanov et al.³¹ Furthermore, no consistent patterns were found to differentiate the Sample 1 batches of xylene and ethanol from those used for Samples 2 and 3, demonstrating similar elemental levels over time.

Study Implications and Limitations

The results of this study contribute new insights into the complex interplay of technical and biological factors which can alter metal distributions in tissues during FFPE processing. While the investigation employed the atypical practice of embedding tissue samples that were previously frozen, the fixation and staining of our FFPE sections were confirmed to be of standard quality by a specialist histopathologist.

Our findings demonstrate primarily element-specific susceptibility to remobilisation or contamination, with Mn, Fe and Zn less affected than Mg and Cu. This study is, however, limited by the small sample size ($n = 3$) and the fact that the samples represent rarer breast cancer subtypes rather than the more common invasive ductal carcinoma. Consequently, the results do not necessarily reflect how FFPE processing affects *all* breast cancer types, or indeed all soft biological tissues.

However, the comprehensive nature of the experimental design, whereby each processing reagent is examined both pre- and postcontact with the samples, and integration of these measurements with spatial metal distributions, would not be feasible for a large cohort. This study therefore offers depth rather than breadth to inform sample preparation and experimental design for future *in situ* elemental studies. Moreover, the availability of FF tissue samples is limited, as surgically resected tissues are typically FFPE processed, making responsible and ethical use of these materials essential.

The TMD and paraffin wax results have implications for researchers utilizing FFPE materials to study Cu distributions. Crucially, the infiltration of paraffin wax into the tissues is unlikely to cause significant elemental contamination. This is significant because it confirms the legitimacy of using FFPE samples for studying elemental distributions, on the condition that the tissues are screened to avoid those annotated with blue and, to lesser extent, green TMDs (Figure 7). Information on whether TMDs were used on tissue samples is detailed in standard histopathology reports which can be accessed by researchers planning such experiments following ethical approval. Therefore, to mitigate the risk of elemental contamination, samples should first be selected based on the TMD use, then fixed in a fresh formalin bath to prevent contamination from TMDs released from other samples.

At the time of resection, guidelines stipulate that surgeons should orientate all breast cancer resection specimens using

sutures, metal staples and/or TMDs.⁷² Currently, there are no histopathology guidelines for selecting TMD colors for annotating dissected tissue specimens, with the choice of TMD instead determined by availability or personal preference. This practice presents an opportunity for improvement, as a simple modification, of avoiding blue and green colors, could mitigate a significant source of elemental contamination for tissues.

One variable not addressed in this study is the possible effect of OCT compounds. Such compounds are used to embed frozen tissues to provide structural support, allowing thin cryosectioning, and reducing the risk of sample shattering. Frozen samples embedded in OCT compounds were excluded from our experiments to avoid potential trace metal contamination of the FF controls. Future work should assess whether OCT compounds contribute to elemental contamination since, if negligible, the enhanced tissue stability provided by the compounds may justify the preferential use of FF over FFPE samples for elemental analysis. Furthermore, advances in technology such as the Cryo Cobalt ablation cell (Teledyne Photon Machines, USA) allow users to maintain the ablation cell temperature at -20°C . With this technology, LA-ICP-MS could be used to compare elemental results between thawed and frozen samples. These options may be particularly valuable when investigating more mobile elements, such as Mg, which are particularly susceptible to contamination and leaching during formalin fixation. However, FFPE is still the preferred sample storage technique for studies that aim to compare elemental distributions with data from biological analyses, such as mIF, which requires epitope preservation.

CONCLUSIONS

This investigation employed elemental data for reagents and materials used in the FFPE procedure, and *in situ* multielement concentrations for human breast cancer tissues to evaluate the utility of FFPE sections for imaging of key elements. While FF tissues are generally regarded as the gold standard for bulk elemental analyses, our findings support the continued utility of FFPE sections for elemental imaging studies. This reflects that the use of FFPE tissue offers several key advantages. First, the extensive availability of archival FFPE materials provides researchers with access to diverse sample sets. Second, tissue architecture is better preserved in FFPE sections, with reduced risk of folding and no risk of shrinkage during analysis, such as from thawing in the ablation chamber. This structural integrity facilitates easier serial sectioning and enables cross-platform imaging comparisons, critical for providing biological contextualisation. Third, both paraffin wax and standard deparaffinisation procedures introduce minimal trace metal contamination or leaching. Finally, while formalin fixation can impact mobile elements (e.g., Na, Mg, K, and Ca), its effect on less mobile elements (e.g., Mn, Fe, Cu, and Zn) remains limited.

Among the elements studied, Zn demonstrated the greatest resilience to FFPE processing, as evidenced by whole-section qualitative and quantitative analyses, as well as more in-depth analyses of separate tissue compartments. Manganese and Fe distributions also showed acceptable preservation at a whole-section level, though Fe, in particular, exhibited increased variability within tumor regions. Despite minor concentration changes detected through reagent and *in situ* analyses, these variations are unlikely to confound biologically relevant comparisons, for example between healthy and diseased

tissues, making these elements suitable candidates for elemental analyses using FFPE sections.

In contrast, Cu distributions were distorted by FFPE preparation. Therefore, an adapted trace metal-free FFPE procedure is recommended, which avoids blue and green TMDs during resection, as these pose a significant contamination risk. For capturing the distributions of mobile elements, such as Mg, FF tissues remain preferable, as our whole-section and regional *in situ* data revealed inconsistent changes, whereby both elemental additions and losses were observed. These changes were likely caused by leaching during formalin fixation rather than slide deparaffinisation.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/cbmi.5c00217>.

Additional figures and tables to support the Methods and Results sections, including further H&E and elemental images, detailed sample information and ICP-MS instrument settings (PDF)

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Notes

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