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High-resolution elemental mapping of human placental chorionic villi using synchrotron X-ray fluorescence spectroscopy

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Abstract

The placenta is the organ that mediates transport of nutrients and waste materials between mother and fetus. Synchrotron X-ray fluorescence microanalysis (SXRF) is a tool for imaging the distribution and quantity of elements in biological tissue, which can be used to study metal transport across biological membranes. Our aims were to pilot placental biopsy specimen preparation techniques that could be integrated into an ongoing epidemiology birth bohort study without harming rates of sample acquisition. We studied the effects of fixative (formalin or glutaraldehyde) and storage duration (30 days or immediate processing) on metal distribution and abundance and investigated a thaw-fixation protocol for archived specimens stored at −80°C. We measured fixative elemental composition with and without a placental biopsy via ICP-MS to quantify fixative-induced elemental changes. Formalin fixed specimens showed hemolysis of erythrocytes. The glutaraldehyde-paraformaldehyde solution in HEPES buffer (GTA-HEPES) had superior anatomical preservation, avoided hemolysis and minimized elemental loss, although some cross-linking of exogenous Zn was evident. Elemental loss from tissue stored in fixative for 1 month showed variable losses (≈ 40% with GTA-HEPES), suggesting storage duration be controlled for. Thawing of tissue held at −80°C in GTA-HEPES solution provided high quality visual images and elemental images.

Keywords

Synchrotron X-ray fluorescence; placenta; sample preparation

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INTRODUCTION

The placenta is a discoid fetal organ approximately 15 cm in diameter and 2 cm thick, which sustains fetal growth and development while hormonally regulating the progression of pregnancy. The quality of the fetal environment during the most rapid period of human growth and development determines future disease risk [1], with an adverse intrauterine environment causing permanent changes in the structure and function of cells, tissues and organs, increasing susceptibility to metabolic syndrome later in life [2]. Fetal growth is determined by nutrient availability, which is in turn determined by the placenta [3]. Given the pivotal role of the placenta, our understanding of nutrient transport, in particular mineral nutrients and environmental metal contaminants is far from complete [4].

Synchrotron X-ray fluorescence mapping is an elemental imaging technique that operates on a micron to sub micron spatial scale [5]. It has been used in the study of membrane-bound metal transport proteins in model plant species, which regulate the cellular and subcellular distribution of essential mineral nutrients and contaminant metals [6] whose expression level can alter metal distribution [7]. Likewise SXRF can be used to image biological tissues in which gene expression can be measured, such as in human tissue collections from large environmental epidemiology cohort studies, to link elemental distribution with the transporters responsible.

Associations between environmental exposure to metals and metalloids and health outcomes rely on cohort studies, where demographic and potential confounding information is available for a large number of study participants who provide biological tissue samples. Biorepositories are critical for accrual of an adequately large study population, often spanning the lifetime of the participants and linking exposure with longer-term health outcomes. Storage conditions for tissue biopsies must maintain tissue in a form amenable to a broad range of techniques, some of which are deliberately not pre-defined. Typically, biological specimens are stored for very long periods of time, often years before analysis. In the case of human tissue biopsies, this typically involves use of formalin or storage at −80°C.

In our study of non-essential and essential metal transport across the placenta, we had access to archived placental biopsy tissues collected as part of a large mother-child cohort study in New England. The New Hampshire Birth Cohort Study recruits pregnant women who obtain their drinking water from private wells, drilled into underground aquifers [8]. In New Hampshire, about 40% of households use private wells, and 9% of those have drinking water arsenic (As) concentrations that exceed the current maximum contaminant limit set by the Environmental Protection Agency (10 μg/L) [9]. The geology of the bedrock in the region also contributes high levels of manganese (Mn), lead (Pb), iron (Fe) and uranium (U) to drinking water [10], of which Mn and Pb are of a particularly concern during fetal development [11, 12]. The collection of placental biopsy tissues in the NHBCS cohort now exceeds 1000 individuals, making it a valuable resource for studying a range of environmental health aspects relating to placental function. Within this cohort, associations have been found between drinking water As and placental As concentrations [9], and between As exposure, expression of membrane-bound metal transporter gene Aquaporin 9

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(*AQP9*) in the placenta and infant birth weight [13]. This study describes pilot experiments for development of sample preparation protocols for elemental imaging on archived and prospective placental specimens using SXRF. While protocols exist for preparation of human biological tissues for SXRF analysis, there are no reports describing their application to placenta. The constraints of method development for an ongoing birth cohort study are the avoidance of major alterations in delivery-room collection protocols; avoidance of additional or unacceptable hazards during specimen collection such as use of liquid nitrogen, or that would preclude sample use for other techniques, such as addition of cryoprotectant solutions to specimens. The specimen collection of any exposure epidemiology study is central to its success, therefore all new analytical techniques are rigorously tested in pilot studies prior to implementation; this measure ensures that specimens are not wasted. Specimen choice for pilot studies also avoids those containing high concentrations of the main contaminants of interest, because these specimens usually rare within the population, and are more valuable. Specimens with elemental abundances more representative of the study population are typically used for pilot tests."

Few methodological studies exist on the multi-elemental distribution of human placental tissue, but numerous studies have examined sample preparation effects on other human and animal tissues. Studying the chemical effects of formalin fixation on brain tissue in mice, Hackett et al [14] compared the molecular and elemental profiles of cryofixed and formalin-fixed specimens. They found that amino acids, carbohydrates, lipids, proteins and ions such as chlorine (Cl) and potassium (K) all leached from fixed tissues. Using particle-induced X-ray emission (PIXE) to image tissue sections, they detected significant changes in K, Ca, Fe abundance and striking changes in Cu and Zn distribution, and concluded that fixation methods involving formalin give only a partial biochemical picture of a tissue sample. Also working on both rat and human brain sections, Gellein et al [15] analyzed the concentration of 19 elements via ICP-MS in both the specimens and the formalin fixative in which the specimens had been stored. Whole unsectioned human brain specimens from a biorepository established between 1979–1983 were dissected at the time of the study. They found that concentrations of arsenic (As), cadmium (Cd), manganese (Mg), rubidium (Rb) and antimony (Sb) increased more than 100-fold upon long-term storage, although materials from which the storage containers were made were not described. Again using brain tissue, Chwiej et al [16] used SXRF to image sections of tissue fixed in formalin and embedded in paraffin. They found decreases in K, Br, P, S, Fe, Cu and Zn, with evidence that some distributional changes were not uniform in different parts of the brain. Al-Ebraheem et al [17] compared formalin-fixed, paraffin-embedded human skin samples with those prepared by cryofixation and freeze drying. They looked specifically at metal distribution between specific tissue layers; the epidermis and the dermis. They saw good agreement between the elemental distributions of the techniques for Ca, Cu and Zn, but some contrasts for Fe. They suggested that Fe remobilization was caused by a compromise in the epidermal layer during sample processing that may have allowed Fe to move to the epidermal junction.

In previous studies using model plant *Arabidopsis thaliana* (mouse-eared penny cress) seeds [18], we used SXRF elemental imaging on both unfixed, unsectioned tissue (via microtomography) and tissue that had undergone sample preparation processing (involving

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glutaraldehyde fixation, dehydration and LR White resin infiltration) and sectioning. We obtained good agreement between elemental distribution, particularly for Fe. In *Arabidopsis* seeds, Fe is bound to phytate [14] and stored within vacuoles, (broadly comparable to the lysosome in animal cells [19]), which may be factors in the stability of Fe during sample processing.

In this study we tested specimen fixation and storage techniques that could be integrated into existing NHBCS protocols without harming rates of sample acquisition, that would allow later analysis of placenta via SXRF and to investigate whether sample preparation protocols could be developed that would to allow use of archival specimens currently stored at −80°C without a cryoprotectant.

In this pilot study, we found that a fixative solution of glutaraldehyde in HEPES buffer at physiological pH provided superior anatomical and elemental preservation compared to formalin, buffered formalin and glutaraldehyde in a phosphate buffer, although binding of exogenous Zn was suspected. Our study looked at specimen storage for far shorter periods than are typically used in biorepositories, and found evidence of elemental loss from the tissues. Thawing of tissue held at −80°C in a solution of glutaraldehyde in HEPES buffer at physiological pH provided the highest quality visual images, minimal visual freeze damage and no evidence of membrane disruption. Imaging areas without visible freeze damage provided elemental distributions similar to those obtained from fresh samples. In general, cells of the syncytiotrophoblast contained elevated abundances of P, Ca, and Zn, and Fe was associated with erythrocytes within the vascular spaces, and macrophages (Hofbauer cells) within the stroma of the villi.

MATERIALS AND METHODS

Statement of Human and Animal Rights

The study protocols for the New Hampshire Birth Cohort Study (NHBCS) were approved by the Committee for the Protection of Human Subjects at Dartmouth College. All study participants provided written informed consent.

Sample preparation

Fresh de-identified human placental tissue was sampled from the Pathology Department of Dartmouth Hitchcock Medical Center. Comparative samples were collected from the same placenta, using immediately adjacent slices of the biopsies, taken as a cross section through the maternal side of the placenta at the base of the cord insertion, avoiding vasculature, calcifications or the margin. The maternal decidua was removed in all cases. Orientation of the biopsies used the easily identifiable blue membrane covering the fetal surface of placenta.

Light Microscopy

To locate areas of interest in the embedded placental tissue (broadly comparable terminal villi), semi-thin sections were collected and stained with 1% toluidine blue, and imaged with a light microscope (Olympus BX43 LM with a DP26CU camera). Once the desired area was

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chosen, semi-thin sections for visual light microscopy (VLM) were collected immediately prior to and following thicker sections used for synchrotron SXRF mapping.

SXRF Study 1: Comparison of fixatives

In all studies, placental biopsies were stored in metal-free polypropylene tubes. Two fixatives were used; formalin, which is commonly used for fixation of pathology specimens, and a solution consisting of 3% glutaraldehyde (electron microscopy grade distillation purified), 1% paraformaldehyde in 0.1 *M* Na_xH_xPO4 (GTA-phosphate buffer) which has been established for use in electron microcopy sample preparation protocols. Initial placenta biopsies were approximately 15 mm × 15 mm × 10 mm, and were trimmed down to approximately 2 mm × 2 mm × 4 mm using a scalpel (observing maternal-fetal orientation to avoid separating maternal tissue from fetal tissue). Samples were fixed for 6–8 hours at room temperature using a rotator, then for 24 hours at 4°C on a rotator. Samples were rinsed in 0.1 *M* HEPES several times over the course of one hour, using a rotator. Specimens were dehydrated in an ethanol series (30%, 50% and 70%) for one hour per step, using a rotator. Specimens were rinsed in 100% ethanol three times over one hour using a rotator. Samples were then placed in a 1:3 LR White medium resin (Electron Microscopy Sciences, Hatfield, PA)-ethanol solution for one hour, before being transferred to a 1:2 LR White medium resin-ethanol solution on a rotator overnight at 4°C. Samples were brought to room temperature before being subject to two rinses in a 1:1 solution of LR White and ethanol. Samples were stored in 1:1 LR White-ethanol solution for one hour, and then transferred to a 2:1 LR White-ethanol solution, with four changes of solution over a period of 2 hours at room temperature. Following this, specimens were then placed in to a solution of 100% LR White resin overnight at 4°C on a rotator. The final stage of embedding involved warming the specimens to room temperature with three changes of 100% LR White resin over the course of two hours. Placenta samples were then transferred to gelatin molds, and re-orientated using a light microscope. Samples were polymerized at 60°C for 24 hours. For this first study, samples were sectioned to a thickness of 0.85–1 μm using a microtome (Leica UC7 ultra microtome) and mounted on silicon nitride (SiN_4) windows (Silson, Northampton, UK) (Frame width 5mm, membrane width 1.5 mm, membrane thickness 200 nm and frame thickness 200 μm), but sectioning thickness was increased to 5 μm for subsequent studies to increase count rates. To mount sections on SiN_4windows, windows were placed flat side uppermost on a clean glass slide. A 10 – 20 μL drop of Millipore Filtered dH_2O was added to the window. The glass slide was then transferred to a slide warmer (Hade VWR 320). Using a dissecting scope, sections on fluid drops were guided toward the center of the drop using an eyelash tool as the water droplet was dehydrating. Sections were mounted one per silicon nitride window. Thinner sections (0.5 μm) were cut before and after sections for SXRF analysis for visual inspection and photographing.

SXRF Study 2: Effect of Short-term Storage

For this study placental biopsies were collected from the fetal side, avoiding vasculature and calcification and split in to three sub-biopsies. Specimens were fixed in either 10% Millionigs phosphate-buffered formalin or in 3% glutaraldehyde (electron microscopy grade distillation purified) and 1% paraformaldehyde in 0.1 *M* HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: $C_8H_{18}O_4N_2S$) at pH 7.0–7.2, a fixative compatible with

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mammalian tissue, buffered to physiological pH (GTA-PF-HEPES) solution, and were subjected to immediate embedding (0 days) or embedding after a period of 30 days storage in fixative solution. Sample preparation once again used LR White resin, using the processing steps described above. For this study sections were cut to 5 μm thick, to enhance fluorescence count rates for synchrotron analysis. To avoid wrinkles while 5μm-thick sections were dried onto the SiN_4 window, protocol steps were altered slightly. A 5μm section was placed onto a 10–20 μl drop of 40% ethanol which had been pipetted on to the center of the SiN_4 window. As the drop evaporated, a 5 μL drop of 70% ethanol was added, followed by additional drops as evaporation continued; using a third drop if necessary. The sectioned was kept in the middle of SiN_4 window as it dried. Once again, semi-thin sections were cut prior to and after thick sections, stained with 1% toluidine blue and inspected for appropriate regions using a light microscope.

SXRF Study 3: Thaw-fixation of samples held at −80°C

To mimic placental tissue biopsies stored at −80°C for extended periods, placental biopsy tissue was collected from the same tissue source as that used in the storage study. One biopsy was held at −80°C for one week, and the other fixed immediately using the GTA-HEPES protocol described in Study 2. Frozen tissue was subject to a modified sample preparation protocol, entailing an initial step of defrosting frozen tissue in the GTA-HEPES solution. Approximately 12–13 ml of fixative solution was added to each frozen specimen in a 15 ml metal-free centrifuge at room temperature, using hands to warm tube surface. Frozen samples came away from the tube walls within 10–20 seconds. If not, a small spatula was used to gently touch the sample, to dislodge it from tube wall. Samples were cut in half to allow fixative to penetrate deeper into the specimen. Samples were swirled gently in GTA-HEPES for 3 hours at room temperature, and were then cut into smaller pieces (7 mm × 7 mm × 5 mm), after which they were placed on a rotator at 4°C overnight. Samples were then placed in to fresh fixative and transferred to a cutting dish, and cut to approximately 2 mm^3, before being returned to fixative and placed on a rotator for 8 hours at room temperature and then 4°C overnight. Tissues were then rinsed in 0.1 *M* HEPES, with several washes over a one hour period. Specimens were then subject to the dehydration series, LR White embedding, and section mounting on SiN_4 windows as described in Study 2 above.

Synchrotron X-ray Fluorescence Microscopy

For all samples analyzed via SXRF, the immediately adjacent stained semi-thin section was inspected for comparable regions of terminal chorionic villi, and target areas were marked for analysis. Placental chorionic villi were imaged at two hard X-Ray fluorescence microprobe beamlines at the Argonne National Laboratory Advanced Photon Source; the Bionanoprobe (21-ID-D) and 2-ID-E. The choice of beamlines used was based on our requirement for several imaging resolutions; encompassing both fine-scale analysis to look for evidence of elemental remobilization across membranes of individual chorionic villi, and alternately larger scale to check characteristic elemental distributions across multiple villi. Samples for the fixative and storage were imaged at the Bionanoprobe, and for the thaw-fixation test at beamline 2-ID-E. The Bionanoprobe (BNP) operates at an undulator beamline at the Advanced Photon Source at the Argonne National Laboratory, as one of several instruments at the Life Sciences Collaborative Access Team. The beamline provides

hard X-rays with photon energies (E) in the range of 4.5–35 keV, and an energy resolution ($\Delta E/E$) of 2×10^{-4}. This energy range allows mapping of most elements in the periodic table using *K*- and *L*-edge excitation [20]. For the fixative study, villi were mapped for 350–400 milliseconds dwell time per point using a 30 nm spot size, and 0.25 μm steps between points.

Beamline 2-ID-E has a nominal energy range of 7–17 keV, and energy resolution of 1.4×10^{-4}, and provides a focused beam of 0.25–0.5 μm. The samples imaged at this beamline were excited using 10.1keV incident energy X-rays (which provides information on elements of atomic number from Z=15 (P) to Z=30 (Zn)) focused to a 0.5 μm spot with a zone plate, through which the samples were raster-scanned and fluorescence emission spectra collected with a 4-element Vortex-ME4 Silicon Drift detector. Overview images of the samples at 2.5 μm resolution with 30 milliseconds per pixel dwell time that provided about 1.2 mm field of view were collected first. Using these images, higher resolution images of regions of interest, about 0.5mm in size, were selected for imaging with 0.5 μm resolution and 50 milliseconds per pixel dwell time.

Quantification of fluorescence counts: Data fitting

X-ray fluorescence data were analyzed using *MAPS* software [19], including per-pixel spectrum fitting and elemental content quantification. The spectrum collected from each scanning step was fitted using modified Gaussians. The elemental concentration (typically in $\mu g/cm^2$) was derived by comparing the fluorescence counts per pixel from the sample and a calibration curve generated from measurements of a thin-film X-ray fluorescence standard (RF8-200-S2453, AXO DRESDEN GmbH, Germany).

ICP-MS elemental analysis of fixatives and placenta

To understand whether elements are either lost from or gained by placental biopsies during fixation, we used inductively coupled plasma mass spectroscopy (ICP-MS) for the analysis of all fixative solutions used. We compared their elemental profiles (P, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sn, Sb, Hg, Pb and U) with an aliquot of fixative from the same prepared batch in which placental specimens (adjacent biopsies collected from the same placenta) had been stored for 1 week.

We collected placental specimens as described above, from the fetal side of the placenta, cleared of obvious vasculature, calcification and maternal decidua, and cut them into cubes measuring 0.5 cm^3. We placed specimens in 15 ml of either a 10% formalin solution (FOR), a neutral buffered 10% formalin solution (BFOR), a solution of glutaraldehyde in phosphate buffer (3% glutaraldehyde (electron microscopy grade distillation purified) and 1% paraformaldehyde in 0.1 *M* $Na_xH_xPO_4$) (GTA-PB), or a solution of 3% glutaraldehyde, 1% paraformaldehyde in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: $C_8H_{18}O_4N_2S$) buffer at a physiological pH of 7.0–7.2 (GTA-HEPES) in a trace-metal free polypropylene tube, with five replicates of each treatment. Fixatives with and without placental biopsies were maintained at 4°C for one week, after which a 1 ml aliquot of fixative solution was collected for ICP-MS analysis. Solutions were analyzed via ICP-MS at the Trace Element Analysis Core facility at Dartmouth College. ICP-MS Analysis followed

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EPA 6020A procedure and used NIST-traceable calibration standards, internal standards, initial and continuing blank and calibration verification, and analytical duplicates and spikes.

Statistical Analysis

For comparison, images for were scaled to the highest maxima obtained for each element and each particular study. Bulk ICP-MS data from the fixatives with and without a placental biopsy, were analyzed using Wilcoxon ranked sum test to establish significant difference between fixatives without and with a placental biopsy specimen. This provided a test statistic for within-pair differences (with and without placenta) and among pairs (different fixatives).

RESULTS

Fixative comparison study

Elemental maps for P, S, Ca, Fe, Zn, Co and Cu in fetal chorionic villi fixed in either formalin (FOR) or a solution of glutaraldehyde in phosphate buffer (GTA-PB) are shown in Figure 1. Elemental abundances are shown on the same scale for each element, expressed at μg/cm^2, alongside visual light microscope (VLM) images of the corresponding villi obtained from an immediately adjacent thin section. Abundances of Mn, Pb and As were below detection limits. We were able to distinguish the syncytiotrophoblast (sc), cytotrophoblast cells (c), fetal vasculature (v), and Hoffbauer cells (hc) from elemental images of chorionic villi (VLM, Figure 1). In both formalin and GTA-PB fixed specimens, P was localized to the syncytiotrophoblast and cytotrophoblast, and to the endothelial cells of the fetal vasculature. Sulfur was distributed diffusely throughout the stroma of the villi, vascular spaces and syncytiotrophoblast. Calcium localized to the syncytiotrophoblast at low abundances. Formalin caused hemolysis; evident in Fe maps, where erythrocytes in the vascular spaces of the FOR specimen had ruptured and fused into a single mass, which had then contracted and cracked during dehydration. In the GTA-PB fixed specimens erythrocytes were intact. Further, a Hoffbauer cell was evident in the GTA-PB fixed specimen, and contained locally elevated abundances of Fe. Differences in the abundance range of Cu and Zn were striking between formalin and GTA-PB fixed specimens. Although natural variation within the sample is a likely cause, it prompted further measurement of the elemental composition of fixatives with and without a placental biopsy (Fixative study). In formalin fixed specimens, Cu abundances were close to background levels; whereas Cu abundances were an order of magnitude higher in GTA-PB fixed specimens. In GTA-PB fixed specimens Zn was localized discretely to cells of the syncytiotrophoblast, whereas in the formalin fixed specimens Zn was not solely associated syncytiotrophoblast cells, but with the hemolyzed blood within the vascular spaces and cytotrophoblast cells, and appeared to be present at the margins of the villus in a continuous layer. Aside from natural variations in Zn distribution, contrasting distribution may suggest elemental mobilization, or (since we did not observe membrane rupture) binding of exogenous Zn during sample processing.

Short-term storage Study

Figure 2 shows representative VLM images of chorionic villi in each of the four conditions; tissues fixed with either buffered formalin or GTA-HEPES fixative immediately before

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processing, or after a period of 30 days. Buffered formalin did not cause erythrocyte hemolysis as observed in non-buffered formalin fixed specimens, although there was slight membrane contraction, seen by the wide margins between neighboring erythrocytes. Again anatomical structure preservation was superior in the GTA-HEPES fixed specimen, and visual inspection of specimens showed that storage for 30 days did not affect anatomical structure.

Elemental images from the storage study are shown in Figure 3. Elemental images were collected from two individual specimens prepared for each condition. Randomly chosen single chorionic villi are shown, and elemental images are scaled to the same maximum abundance for each element, expressed as μg cm^{-2}. Abundances of Mn, Pb and As were below detection limits. Elemental distributions observed in the Fixative Study; such as P, Ca, Cu and Zn in the cells of syncytiotrophoblast, S throughout the stroma, and Fe associated with erythrocytes and fetal vasculature held true for the image set in the Storage Study. A longer dwell time of 500 milliseconds was used in the fine scale maps shown in Figure 3, compared with 350–400 milliseconds in the Fixative Study (Figure 1). Discrete localization of elements with anatomical structures was evident for S and Zn (Figure 3) in the Storage study. Additionally, Fe was elevated in a region of the stroma surrounding the fetal vasculature in this image series, and not solely in erythrocytes and Hoffbauer cells. To determine whether and to what extent elemental loss was taking place, we conducted a user-defined region of interest analysis using the MAPS software program [21] to compare mean sample abundances between fixatives and storage durations (Supplemental Figure 1). Comparison of SXRF images from each condition (N=2) indicated loss of P and S from GTA fixed specimens (with an average difference in abundance of 43% and 49% respectively), but no loss in formalin fixed specimens. Similar Ca loss for FOR and GTA were observed at 52% and 43%, and no appreciable loss of Fe occurred. We observed a loss of Cu in GTA only (31%) and loss of Zn of 68% and 46% respectively from FOR and GTA fixed specimens.

Thaw-fixation of frozen placental samples

Inspection of VLM images subject to thaw-fixation showed isolated regions of damage from the freezing process, but membranes were intact and image quality was excellent. Where damage was evident, it took the form of detachment of the syncytiotrophoblast away from the stroma, and occurred more often in the center of larger biopsies. There was also the presence of what was assumed to be condensate from the freezing process in the thaw-fixed specimen. Elemental images of samples fixed from fresh or thaw-fixed directly from storage at −80°C are shown in Figure 4. Elemental distributions showed good agreement with those obtained from the above fixative and storage studies and also between fresh and frozen tissue. Looking specifically at the chorionic villi, P, Ca and Fe were observed in the syncytiotrophoblast, S was evident throughout the stroma, and Zn localized both to the syncytiotrophoblast and stroma. We were able to image a much larger field of view at APS beamline 2-ID-E where these images were collected, and noted that Fe was present in the syncytiotrophoblast and stroma, in addition to being present as a component of the erythrocytes within the fetal vessels. The signal-to-noise ratio was too high to obtain images of Cu at this beamline, and abundances of Mn, Pb and As were below detection limits.

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ICP-MS analysis of fixatives with and without placental biopsy

Results from fixative comparison and storage studies suggested differences in Cu as a result of fixative use, prompting an additional study of the elemental composition of the fixatives, and how incubation of a placental biopsy changed this composition (Supplemental Figure 2).

GTA-HEPES fixative had the lowest P concentration (2.48 SD 3.03 ppm), whereas formalin, buffered formalin and GTA-PB all contained over 2,000 ppm P. Formalin contained significantly more Ca: 3.5 (SD 0.16) ppm compared to < 1 ppm for the other fixatives (P<0.0001). Concentrations of Fe in fixatives ranged from 16–55 ppm, with formalin containing the highest Fe concentration and GTA-HEPES the lowest (P<0.0001). Cu concentration was an order of magnitude higher in formalin (P<0.0001) with a mean concentration of 32.9 (± SD 0.8) ppm compared to 4.5–5.1 ppm for the other fixatives, which might suggest that absorption of exogenous Cu into the biopsy is less likely to occur in GTA-HEPES, suspected in the Fixative study. Zinc concentration was higher in the two GTA-based fixatives (P<0.0001) at approximately 145 ppm each, compared to less than 10 ppm in the formalin-based fixatives.

For the Fixative Study, we assumed that significant decreases in fixative elemental concentrations in the presence of a placental biopsy relative to concentrations in native fixative inferred absorption of metals by or adsorption to the placental biopsy. Likewise we interpreted increases in fixative metal concentration to indicate elemental loss from the biopsy into the fixative. Concentrations of elements of interest for SXRF in fixative solutions without and with incubation of a placental biopsy are shown in box and whisker plots in Supplemental Figure 2. As expected, metals were primarily lost from the biopsy into the fixative solutions. As well as containing less P, the amount of P leaching from the biopsy into GTA-HEPES was less than the other fixatives. Calcium, Mn and Fe were also lost from the biopsy, with the general pattern being FOR > BFOR > GTA-PB > GTA-HEPES. Our results did not support the hypothesis that binding of exogenous Cu to the biopsy occurred in GTA-HEPES buffer. There was Cu enrichment of fixative consistent with loss of Cu from the biopsy (P =0.0036), but this response had greater variability. Zinc concentrations of fixatives suggested a net gain into solution from the biopsy for FOR, BFOR and GTA-PB, but GTA-HEPES incubated with placenta appeared to have a more variable response consistent with loss of Zn from fixative solution. We did not collect bulk elemental profiles from placental biopsies in the Fixative Comparison study because natural tissue heterogeneity would obscure changes caused by exposure to the various fixatives.

DISCUSSION

A comparison between the maximum elemental abundances obtained from the individual studies showed a good agreement on a per-element basis. For P, abundances range from 1.2 μg cm^{-1} in Study 2, to 3.55 μg cm^{-2} in Study 1. For S, however, much higher abundances were detected in the thaw-fixation test of Study 3, where abundances were 2.05 μg cm^{-2} in comparison with 0.8 and 0.28 μg cm^{-2} in Studies 1 and 2 respectively. This is likely to be due to the analysis of a much larger field of view in Study 3, which included a large portion of maternal decidua, whereas the first two studies were higher resolution analysis of single terminal chorionic villi. Likewise, we observed higher concentrations of Ca in Study 3

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(2.32 μg cm^{-2}) than in Studies 1 and 2 (0.10 and 0.15 μg cm^{-2} respectively). Abundance ranges of Fe and Zn were also consistent between studies, with Fe in the range of 0.1–0.49 μg cm^{-2}, and Zn in the range of 0.02–0.07 μg cm^{-2}). We observed a large variation in Cu abundance between Study 1 and 2 (the signal to noise ratio was too high to detect Cu in Study 3), 0.58 and 0.01 μg cm^{-2} respectively.

Biospecimen archives from pathology collections or from existing cohort studies can be a valuable resource for conducting various types of analysis, and are an essential component of environmental epidemiological studies where sample accrual occurs over long periods of time. We conducted studies to determine how fixation and periods of storage affected the distribution of elements in archived human placenta as imaged by SXRF, and whether protocol modifications could be made that would allow elemental imaging of specimens previously stored at −80°C. The results we present suggest that the elemental distribution of both archival and prospective biospecimens can be analyzed via SXRF to obtain good quality data. However, because SXRF is not a high-throughput technique, use of this technique would out of necessity be contingent upon careful selection of samples shown to have an elevated metal content.

In agreement with the literature, formalin was inferior to glutaraldehyde and paraformaldehyde-based fixatives in the preservation of both anatomical structure and elemental integrity. The use of a buffered formalin prevented ultrastructural changes associated with hemolysis and could be easily substituted in most biorepository protocols. In general, GTA-HEPES caused the least elemental disruption among the fixatives investigated here, with the exception of Zn. ICP-MS data from analysis of fixatives with and without a placental biopsy indicated inconsistent binding of exogenous Zn, which may suggest interaction between Zn binding and heterogeneous features of the placenta. Although a consideration of why Zn in particular was suspected to bind to placental biopsy tissue is beyond the scope of this study, it is clearly a topic that should be explored further.

Given the unique biphasic structure of placental tissue; specifically the presence of contiguous intercommunicating channels of the intervillous space filled by the ebb and flow of maternal blood, a portion of the elemental loss observed in the Fixative Study is likely to have been associated with loss of maternal blood from the intervillous space rather than from within the vascular tree itself. More studies are needed to determine the extent of elemental loss across the syncytiotrophoblast.

Although limited by the low number of replicates of a heterogeneous tissue specimen, storage of specimens in fixative suggests elemental loss (P, S, Ca and to varying extents Cu and Zn) occurs over a relatively short period of time, and suggests either that storage duration be controlled for in elemental imaging studies on archived specimens, or that if possible specimens be embedded prior to long term storage. Where easily lost elements such as P are the focus of the study, P-containing fixatives and buffers should be avoided. For archived specimens held at −80°C, we present a simple technique modification that can provide good quality visual and elemental images. Further studies are needed to look at the stability of elements and organic component in specimens stored in fixative over longer time

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periods, such as those typical of biorepositories, however, development of thaw-fixation techniques may obviate this kind of storage for samples destined for elemental imaging.

Elemental imaging showed that cells of the syncytiotrophoblast were enriched with P, Fe, Cu and Zn, and that vascular spaces were filled with Fe-rich erythrocytes. Sulfur, Fe and Zn were also observed the stromal tissue. Our observation of Fe localization in macrophages (Hofbauer cells) is in agreement with the literature; Bastin et al [22] conducted fluorescence staining of Fe transporting proteins Transferrin receptor-1 and ferroportin and saw localization to the syncytiotrophoblast and macrophages. Ferritin has been identified in the syncytiotrophoblast in term placenta [23], and we observed this distribution of Fe in lower resolution imaging of multiple chorionic villi (Figure 4).

Taken together with tissue responses to sample preparation reported in the literature, we hypothesize that elemental disruption in response to fixation, dehydration and resin infiltration varies both by tissue and by element. The majority of studies carried out on the potential changes in elemental distribution and abundances during sample preparation have used brain tissue from either humans or rodents. Neural tissue has a contrasting anatomical structure and a particularly high lipid content in comparison with placental tissue, which is a highly vascularized network of fetal chorionic villi adapted for bi-directional membrane transport. It may not be accurate to assume that sample preparation-induced elemental movements occur in a similar manner in different types of biological tissue. The molecular composition of the tissue, the structure and permeability of the membranes, and the specific metal binding ligands may result in widely differing chemical interactions with fixatives and thus their response to tissue processing.

Despite the advantages of cryo-fixation over chemical fixation in general, where preserving the sample in an amorphous glass of ice ideally preserves elemental content [24–26], employing cryopreservation creates important constraints. For instance, in hospital delivery rooms where our placental biopsy samples are obtained, implementing protocols that use hazardous materials and require additional training risk reducing sample acquisition rates and impacting numerous other studies that rely on archived biospecimens. Comparisons of the kinds of techniques used here to cryopreservation have been made elsewhere [27–29], and should important mechanistic hypothesis arise from elemental imaging work conducted on fixed biopsies, validation would clearly require analysis of a cryo-fixed specimen. Nonetheless, it remains worthwhile to determine whether archived specimens hold the potential to be used for these types of analytical techniques, and how this potential might be safeguarded in the future as elemental imaging techniques become more commonplace.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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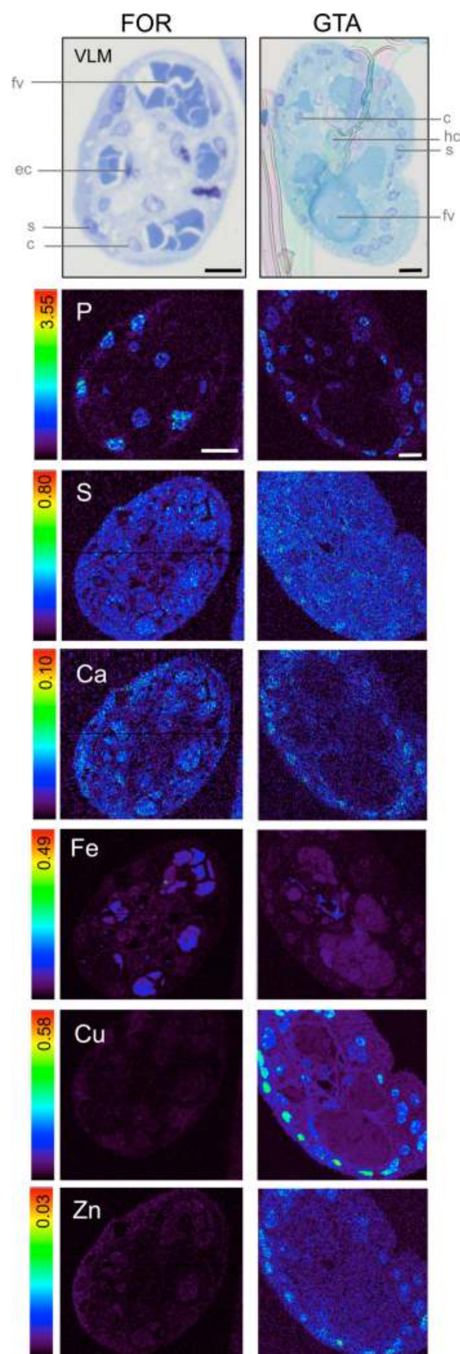

Figure 1.

Visual light micrographs (VLM) and elemental maps of P, S, Ca, Fe, Cu and Zn in fetal chorionic terminal villi, fixed in either formalin (FOR) or a solution of 3% glutaraldehyde, 1% paraformaldehyde in sodium phosphate buffer (GTA) prior to resin embedding as part of the fixative comparison study. VLM sections are immediately adjacent to those on which elemental imaging data was collected. fv: fetal vasculature, ec: endothelial cell, s: syncytiotrophoblast, c: cytotrophoblast, hc: Hoffbauer cell. Elemental data is expressed as μg cm^{-2}. Scale bars are 10 μm.

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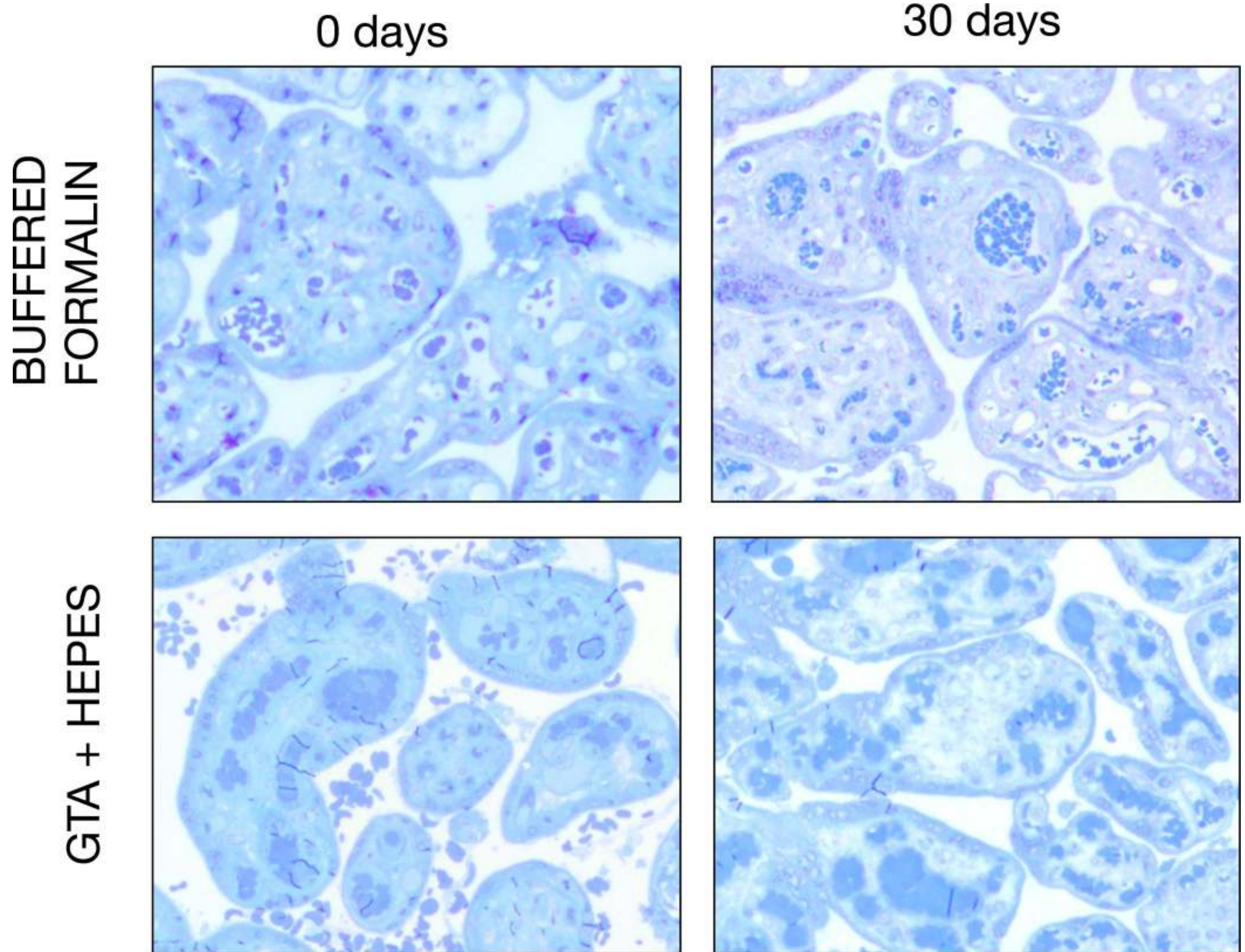

Figure 2.

H&E stained visual light micrographs (×40) of regions of terminal villi fixed with either buffered formalin or GTA-PB and subject to resin-embedding either immediately (0 days) or after 30 days.

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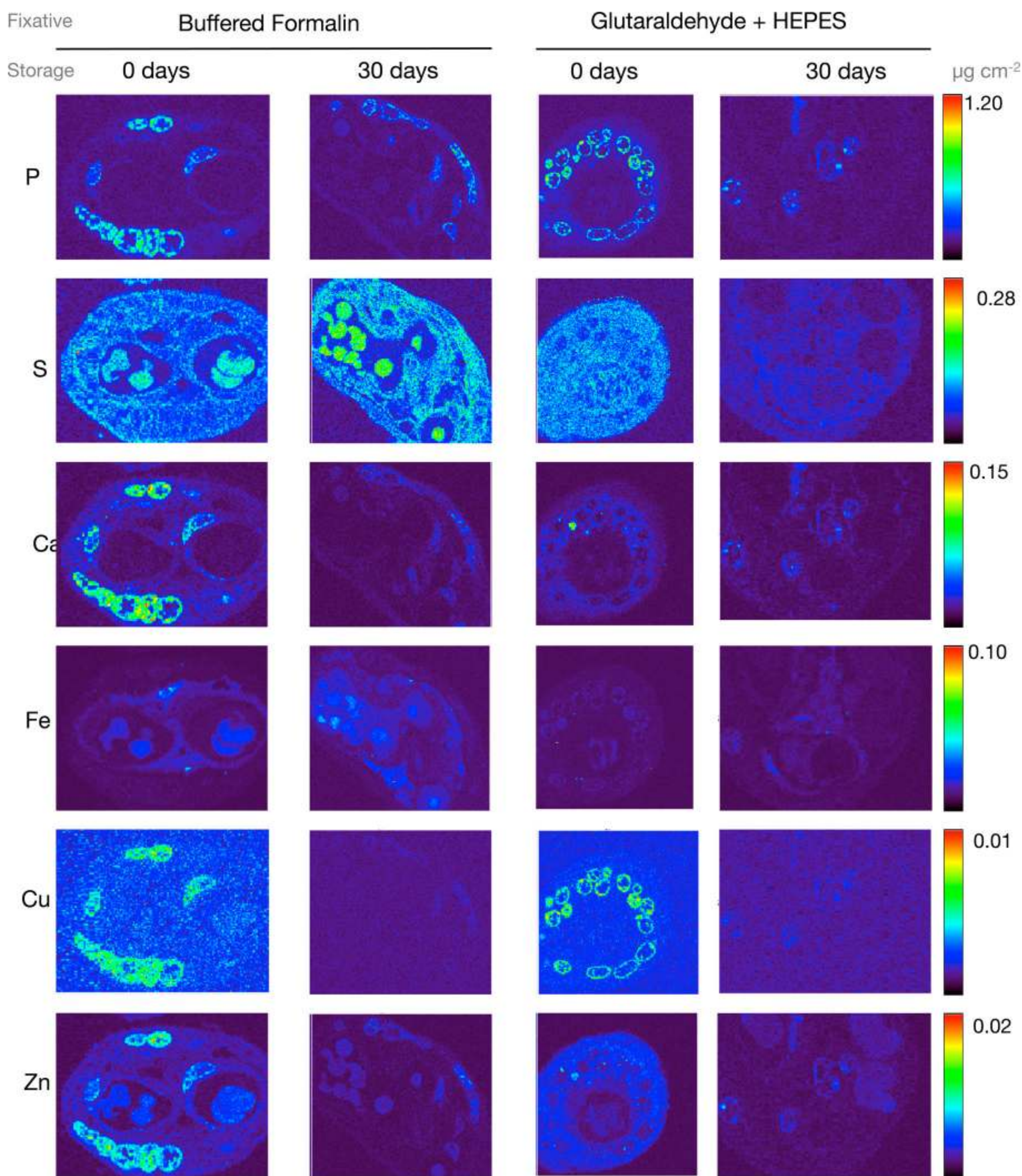

Figure 3.

Elemental maps of P, S, Ca, Fe, Cu and Zn collected from regions of terminal villi fixed with buffered formalin immediately (0 days) or after 30 days. Elemental data is expressed as µg cm^{-2}. Scale bars are 10 µm.

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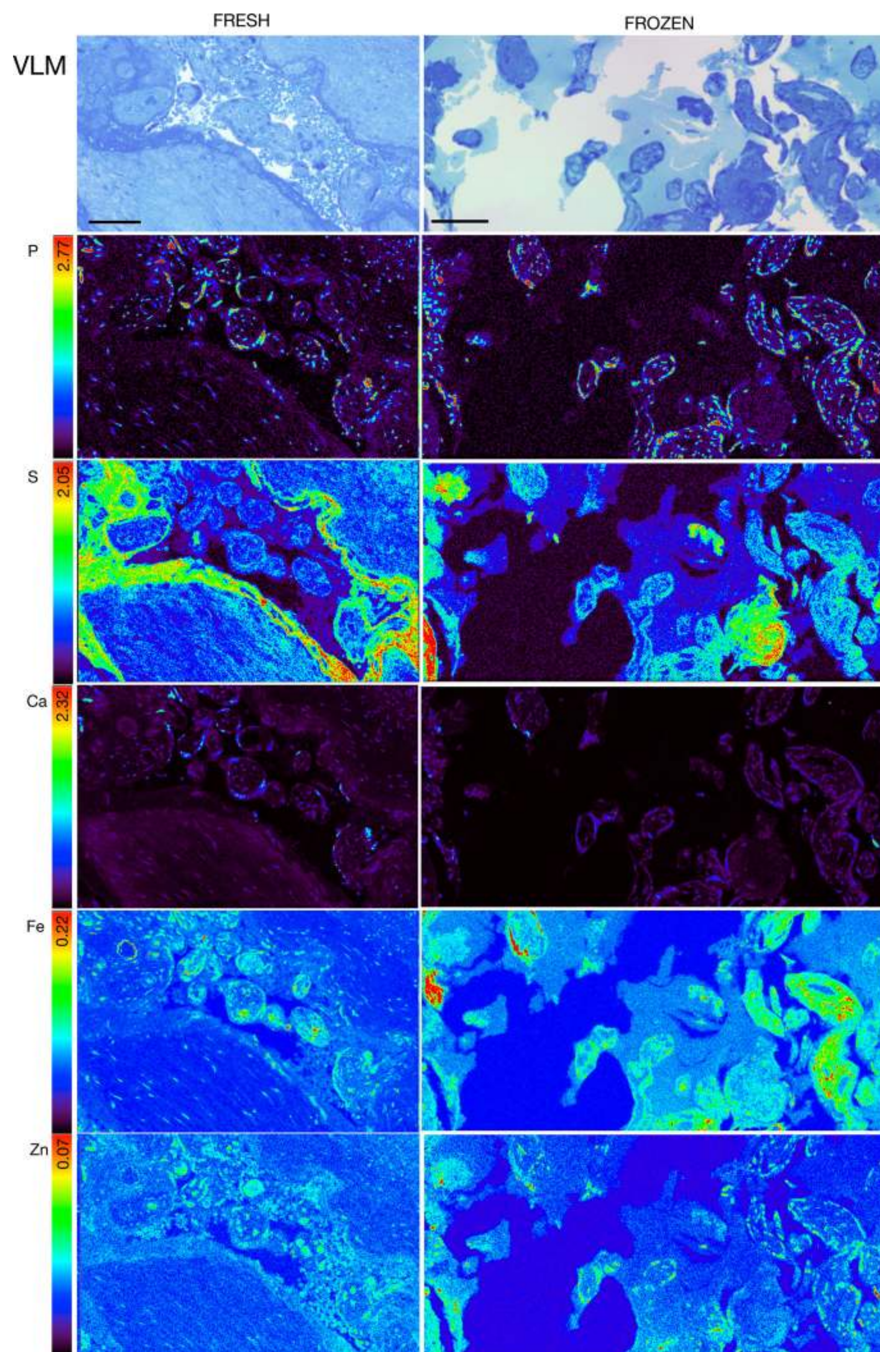

Figure 4.

Visual light micrographs (top row panels) and elemental images of fresh or frozen placental tissue from the same placenta. Frozen tissue was stored at −80°C and thawed in fixative solution. The scale bar is 100 μm, and tissues are shown on the same abundance scale for each element, expressed as μg cm^{-2}.