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The effect of paraformaldehyde fixation and sucrose cryoprotection on metal concentration in murine neurological tissue

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Aldehyde preservation of tissue and cryoprotection by sugars and polyols has traditionally been used in histology to preserve structural integrity of samples. The effect of paraformaldehyde (PFA) fixation and sucrose cryoprotection on murine brain metals was studied by examining the changes in total brain metal levels throughout a complete fixation/cryoprotection protocol. Blank-corrected metal levels in 4% PFA and two changes of 30% sucrose solutions following brain immersion were determined by ICP-MS. Fixed and cryoprotected tissue was lyophilised, digested in HNO_3/H_2O_2 and analysed using the same ICP-MS protocol. The sum of metal loss or gain in each solution and total digest was used to determine the percentage of total brain metal present in fixed, cryoprotected tissue. Almost all K (99.78%) and Mg (78.01%) leached from brain tissue during fixation and cryoprotection, whilst Ca and Sr either retained or absorbed additional metal ions from the sucrose solution. Less water-soluble transition metals displayed smaller degrees of metal leaching, with Fe, Cu and Zn losing 26.61–31.62% of their total metal content during preparation for cryosectioning. These results suggest that metal leaching is highly metal specific, and that preparatory steps for cryosectioning of brain tissue should be approached according to this specificity.

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Introduction

Fixation and cryoprotection are essential steps in typical histological protocols where tissue is sectioned at a low temperature, applications for which include immunohistochemistry, electron microscopy, and imaging mass spectrometry. Fixation ensures that further post mortem putrefaction by either enzymatic proteolysis or external bacteria is halted and structural integrity is maintained, allowing long term storage of harvested tissue.[1] Cryoprotection reduces the effects of freezing artifact arising from the formation of ice crystals. Rapid freezing in iso-pentane cooled to just above freezing in liquid nitrogen can be used to limit freeze-fracturing,[2] though larger samples like whole brains are best cryoprotected by extended immersion (*i.e.* until the sample sinks) in sugar solutions, such as sucrose[3] or glycerol-DMSO[4] prior to freezing and sectioning.

Increased interest in quantitative imaging of trace metals in cut tissue sections has necessitated that tissue preparatory methods be revisited to ensure contamination and metal loss is kept to a minimum.[5,6] Whilst contact with trace metal contaminants is almost inevitable in the standard histology laboratory, numerous steps can be taken to ensure the influence is negligible. However, the requirement for fixation and cryoprotection to retain cellular structure and integrity becomes just as necessary as contamination control as trace metal imaging techniques move closer to the single cell level.[7–10]

Relatively little data is available on the effects of fixation on tissue metals levels, and those that have been reported are either contradictory,[11,12] or discuss long term fixation effects[13] that are not necessarily relevant to fixation and freezing of tissue after a relatively short post-mortem interval. Regardless, it is apparent that the length of fixation period does have an effect on leaching of metals in to or out of tissue.[14] Even less information is available regarding the effects of freezing on tissue metal levels. Slow freezing of fish tissue at −20 °C found a depression (10–30%) in heavy Zn, Cd and Pb,[15] though the significance of this finding on the rapid freezing of neurological tissue is difficult to ascertain.

We aimed to determine the effects of paraformaldehyde (PFA) fixation and sucrose cryoprotection (the two most commonly used reagents for the task) on the metal concentrations of whole mice brains. The data presented provides an important guide for the preparation of neurological tissue for

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cryosectioning and its effect on metal concentration and distribution.

Experimental

Animals

All animal experiments conformed to the Australian National Health and Medical Research Council standards of animal care and were carried out in accordance with the requirements of the Howard Florey Animal Ethics Committee. 16-month old male C57BL/6 mice ($n = 20$) were raised according to standard animal care protocols and fed normal chow and water *ad libitum*. Animals were killed with an overdose of sodium pentobarbitone (100 mg kg^{-1}) and perfused with 30 mL of warmed (37 °C) 0.1 M phosphate buffered saline (PBS), pH 7.4.

Inductively coupled plasma-mass spectrometry

All metal analyses were performed on an Agilent Technologies 7700x ICP-MS with a standard sample introduction system consisting of a concentric nebuliser (Burgener) and Scott-type double-pass spray chamber (Glass Expansion). Helium was used as a collision gas for interference removal. Typical instrument parameters are shown in Table 1. An external calibration was used with 0, 5, 10, 50, 100 and 500 μg L^{-1} of certified multi-element ICPMS standard calibration solutions (ICP-MS-CAL2-1, ICP-MS-CAL-3 and ICP-MS-CAL-4, Accustandard) for a range of elements. Full instrument tuning was performed prior to all experiments using a 1 μg L^{-1} solution of Li, Co, Y, Cs and Tl in 1% HNO_3, and a pulse/analogue (P/A) factor to correct for dual-mode detection[16,17] was determined using a 500 ppb multi-element standard. A certified standard solution containing 200 μg L^{-1} of ^{89}Y (ref. 18) was added online *via* a T-piece and was used as an internal control. Data for Li, Cr, Co, Se, Rb, Ru, Mo, Rh, Pb, Cd and Ba were excluded due to ICP-MS measurements routinely falling at or below instrument sensitivity for all measured samples. Limits of detection (LOD) and background equivalent concentrations (BEC) for measured elements are given in Table 2. BEC was used in place of LOD as the threshold concentration for detection, as it provides a more accurate assessment of the contribution of the matrix to measured concentrations.[19]

Table 1 Typical operating parameters for the Agilent 7700x ICP-MS

RF power	1550 W
Sample depth	8.0 mm
Carrier gas	0.95 L min^{-1}
Makeup gas	0.20 L min^{-1}
Spray chamber temperature	2 °C
Extracts 1, 2	−12, −200 V
Omega bias, lens	−95, 8.3 V
Deflect, plate bias	2.2, −60 V
Cell entrance, exit	−38, −68 V
Ocotople bias, RF	−18.0, 200 V
Collision gas	He, 3.4 mL min^{-1}

Table 2 Limit of detection (LOD) and background equivalent concentration (BEC) values for all measured elements. All data reported as μg L^{-1}

Al	0.304	0.294
B	0.898	1.10
Ba	0.0166	0.0142
Ca	0.722	0.838
Cd	0.0078	0.0077
Co	0.0044	0.0031
Cr	0.0049	0.0334
Cu	0.0125	0.069
Fe	0.0751	0.593
K	0.816	38.8
Li	0.459	0.886
Mg	0.148	0.256
Mn	0.0071	0.0251
Mo	0.210	3.78
Ni	0.0129	0.0641
Pb	0.0110	0.136
Rb	0.0015	0.0157
Rh	0.0045	0.0150
Ru	0.0043	0.0034
Se	0.222	0.353
Sr	0.006	0.0093
Zn	0.109	0.704

Tissue fixation and cryoprotection

The tissue fixation and cryoprotection method used is depicted in Fig. 1. The brains were removed and placed in a 4% (w/v) solution of PFA (Sigma Aldrich) in 0.1 M PBS (4 °C), pH 7.4 overnight (Step 1). Following fixation, brains were transferred to a 30% (w/v) sucrose solution in 0.1 M PBS for 72 hours (Step 2), then transferred to a fresh sucrose solution for an additional 72 hours (Step 3) before snap freezing in iso-pentane cooled in liquid nitrogen and were then stored at −20 °C before digestion and analysis. Each individual solution was retained and blanks ($n = 3$) for each step collected. Solutions were diluted by a factor of 10 with 1% HNO_3 for ICP-MS analysis (metal concentrations in blank 0.1 M PBS, 4% PFA and 30% sucrose are given in Table 3).

Tissue digestion

A subset of brains ($n = 6$) were accurately weighed (wet weight), bisected and lyophilized overnight. 200 μL of concentrated

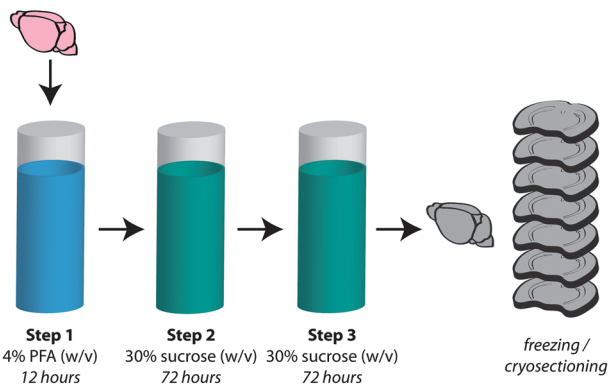

Fig. 1 Fixation and cryoprotection workflow.

Table 3 Concentration (μg L^{-1}) of metals (±1 standard deviation) in blank 0.1 M PBS, 4% PFA and 30% sucrose solutions

	0.1 M PBS	4% PFA	30% sucrose
Al	<BEC	60.70 ± 4.18	4.40 ± 5.80
B	<BEC	158.1 ± 7.87	68.62 ± 5.15
Ca	4.87 ± 2.86	6.34 ± 30.62	815.5 ± 39.3
Cu	< BEC	3.34 ± 0.17	4.12 ± 0.21
Fe	<BEC	17.9 ± 4.66	61.56 ± 1.82
K	2165 ± 71	648.0 ± 73.1	5864 ± 7
Mg	<BEC	26.52 ± 2.42	554.1 ± 6.7
Mn	<BEC	0.36 ± 0.10	1.74 ± 0.31
Ni	<BEC	2.09 ± 0.15	1.80 ± 0.11
Sr	0.03 ± 0.01	0.70 ± 0.10	9.81 ± 0.37
Zn	<BEC	11.54 ± 6.84	4.60 ± 4.23

HNO_3 (Merck) was added to the tissue, which was then allowed to digest at room temperature overnight. The digest was heated to 90 °C for 20 minutes, then cooled to room temperature before the addition of 200 μL H_2O_2 (BDH Chemicals). Samples were left at room temperature for a further 30 minutes, and then heated to 70 °C for 15 minutes in a heating block. The reduced digest volume was determined and diluted by a factor of 21 in with 1% HNO_3. Each sample was measured in triplicate.

Data analysis

Data was exported as comma separated value files (.csv) and analysed in GraphPad Prism. Significance (p value) was determined according to post-hoc Bonferroni's multiple comparison test (appropriate for five comparison groups or less); accepted significance level $p < 0.05$ (95% confidence interval).

Results and discussion

Leaching of metals into the fixative (PFA) and cyroprotective (sucrose) solutions was determined by measuring the difference in metal content prior to and following tissue immersion. A representative subset ($n = 6$) of whole brains was digested and analysed for total metal content. The sum of total leached metal in each solution (*i.e.* metal content in solution following blank correction) and the mean total metal in digested tissue was used to determine the original total metal content of each whole brain. Table 4 shows the total metal loss or gain from the immersed brains following each change in solution, as well as the total metal content and concentration of fixed, cryoprotected tissue. Generally, all metals appeared to leach into the fixative/cryoprotective solutions to varying degrees (Fig. 2 and Table 5). The percentage of total metal in each brain remaining in the digested tissue varied from element to element (Fig. 3), ranging from near-total loss of K (0.218 ± 0.006%) to complete retention of Ca (100.5 ± 20.5%).

The majority of K leaching from brain tissue appeared to occur during the initial overnight fixation in 4% PFA (84.59 ± 0.62% loss of total K), with practically no further leaching (1.08 ± 0.25% loss) by the second sucrose cryoprotection step (Step 2 in Fig. 1). This is surprising, considering the high solubility of K in the brain and its predominant localization to intracellular spaces.[20] Mg, on the other hand, lost 76.46 ± 2.47% of its total mass during Steps 2 and 3. Whilst Ca appeared to remain at its original metal concentration prior to fixation and cryoprotection, closer inspection of metal leaching from and into tissue during the preparation process revealed a pattern of metal loss during PFA fixation and the second cryoprotection step (mean difference = −2.44%; $p < 0.05$) and reuptake during the first sucrose step (mean difference *vs.* PFA = −12.16%; $p < 0.0001$; *vs.* sucrose 2 = 9.73%, $p < 0.0001$). The return to the original metal concentration may be coincidental, and total immersion time may alter the amount of leaching in to or out of tissue. Like K, the solubility of Ca in the brain is expected to be high, reflected in the highly significant metal loss during fixation. Sr displayed a similar pattern of significant uptake during the initial sucrose cryoprotection step (Step 2). This similar pattern of metal uptake may be indicative of the similar chemistries observed between Sr and Ca in the brain.[21] However, without significant leaching into PFA or even the second sucrose immersion phases, total Sr content in the digested tissue was markedly greater than the expected 100% recovery (see Fig. 3). Interestingly, another divalent alkaline earth metal, Mg, did not mirror metal uptake during cryoprotection like Ca and Sr; rather, Mg displayed a consistent

Table 4 Total loss (−)/gain (+) of metal (μg g^{-1} tissue (wet weight) ±1 standard deviation) in each fixation/cryoprotection step ($n = 20$), and final wet weight metal concentration in fixed, cryoprotected brains ($n = 6$)

	PFA	Sucrose 1	Sucrose 2	Digest concentration
Al	−0.0953 ± 0.0471	−0.139 ± 0.0420	−0.129 ± 0.043	0.0164 ± 0.0114
B	0.91 ± 66.7[a]	−167 ± 711[a]	50.1 ± 173.6[a]	0.188 ± 0.010
Ca	−0.969 ± 0.345	1.20 ± 0.50	−0.151 ± 1.78	18.0 ± 10.2
Cu	−0.634 ± 0.050	−0.190 ± 0.034	−0.116 ± 0.046	2.39 ± 0.15
Fe	−1.58 ± 0.12	−0.531 ± 0.141	−0.679 ± 0.319	7.70 ± 0.80
K	−2782 ± 91	−4640 ± 55	−35.6 ± 37.4	7.16 ± 0.51
Mg	−7.32 ± 1.09	−27.5 ± 5.5	−28.6 ± 8.6	17.8 ± 10.7
Mn	−55.9 ± 17.7[a]	−29.4 ± 6.9[a]	−42.0 ± 14.8[a]	0.133 ± 0.008
Ni	−1.84 ± 1.44[a]	−1.54 ± 2.82[a]	0.996 ± 4.02[a]	0.428 ± 0.301
Sr	0.517 ± 1.66[a]	18.0 ± 4.3[a]	5.22 ± 20.23[a]	0.130 ± 0.063
Zn	−0.796 ± 0.180	−1.70 ± 3.84	−0.856 ± 0.573	7.25 ± 0.27

[a] ng g^{-1} tissue (wet weight).

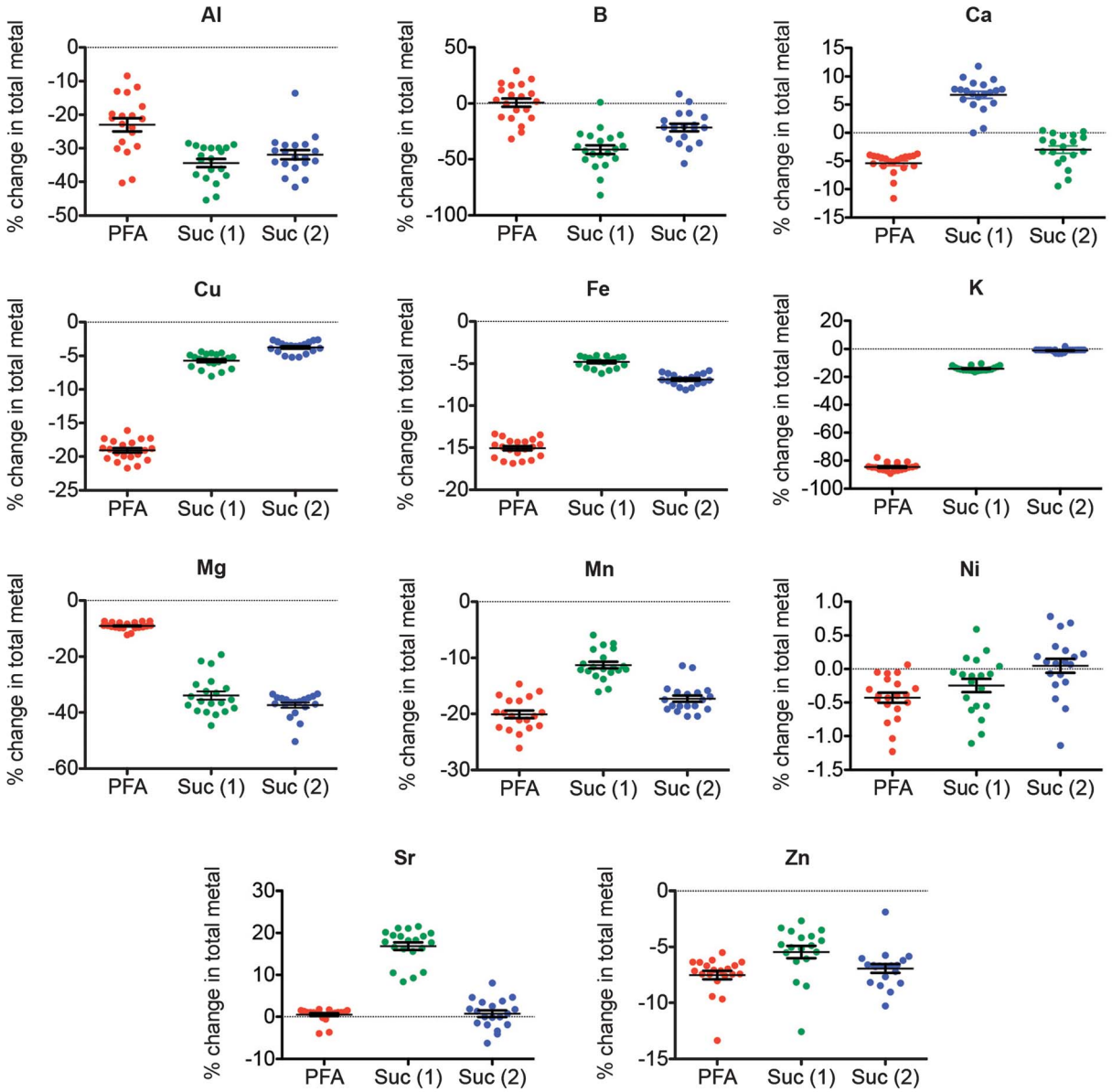

Fig. 2 Percentage (%) change in total brain metal levels through fixation (PFA) and cryoprotection (Suc (1) and (2)) steps for B, Mg, Al, K, Ca, Mn, Fe, Ni, Cu, Zn and Sr, presented as deviation from summed metal content of each step plus digested brain (0%; grey line). Bar represents mean ± standard error of the mean.

leaching from tissue, most marked in the sample cryoprotective Steps 2 and 3, resulting in only 21.90 ± 0.54% of total brain Mg remaining in the final tissue digest.

Less soluble transition- and semi-metals did not leach from the brain tissue to the same extent as the group 1 and 2 metals analysed, though the effect of fixation and cryoprotection was still apparent. Boron, suggested to play a role in cognitive function,[22] lost nearly half its total mass (46.91 ± 1.04%), as did Mn (51.14 ± 1.36%). Such significant Mn loss was surprising, considering labile Mn^{2+} is a potent pro-oxidant[23] and it was expected most ionic Mn would be chaperoned by metalloproteins. However, the ability of Mn^{2+} to substitute Mg^{2+} in numerous biomolecules, as well as its presence in intracellular spaces may account for its similar pattern of loss.[24]

The three transition metals of particular interest to neurodegenerative disease research (Fe, Cu and Zn)[25,26] all displayed a significant, yet smaller degree of leaching. Zn showed the most marked loss, with the final tissue digest accounting for 68.38 ± 1.05% of total brain Zn, with leaching spread fairly evenly throughout each preparatory step. Cu and Fe retained 7.171 ± 1.45% and 73.39 ± 3.12% of their total content, respectively, with the majority of metal leaching occurring during fixation. It is difficult to speculate on the significance of this metal loss during sample preparation, considering the precise chemical state of leached metals remains unknown. The involvement of metals in neurodegenerative diseases is likely confined to deficiencies in metal metabolism (*i.e.* the metalloproteins responsible for metal chaperoning and activity), rather than the

Table 5 Mean percentage deviation ($n = 20$) of total metal in 4% PFA and 30% sucrose solutions following immersion of brain tissue[a]

	PFA *vs.* Suc 1	Suc 1 *vs.* Suc 2	PFA *vs.* Suc 2
Al	11.42%; ****	−2.49%; ns	8.93%; ***
B	42.09%; ****	−19.84%; **	22.25%; ***
Ca	−12.16%; ****	9.73%; ****	−2.44%; *
Cu	−13.36%; ****	−1.98%; ****	−15.34%; ****
Fe	−10.28%; ****	2.09%; ****	−8.19%; ****
K	−70.49%; ****	−13.02; ****	−83.51%; ****
Mg	24.91%; ****	3.46%; ns	28.27%; ****
Mn	−8.81%; ****	6.00%; ****	−2.81%; **
Ni	−1.82%; ns	−0.29%; ns	−0.48%; **
Sr	−16.37%; ****	16.13%; ****	−0.24%; ns
Zn	−2.06%; **	1.47%; ns	−0.59%; ns

[a] *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns = not significant.

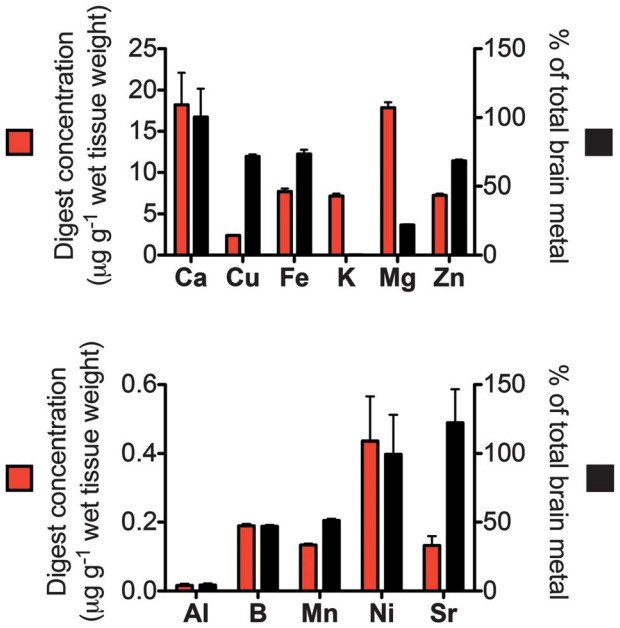

Fig. 3 Concentration (μg g^{-1}) of metal in fixed, cryoprotected and digested tissue (red bar, left axis) and percentage of total brain metal represented by digested tissue (black bar, right axis).

free metal concentration of the 'healthy' brain (assuming, that is, that the source of metal leaching predominantly consists of labile and intracellular material). However, the potent redox activity of free metal species cannot be underestimated, and this loss may impede investigations of murine models of disease intended to impair metal metabolism and induce metal-mediated oxidative stress.[27] Further experiments must be conducted to determine if metal leaching during sample preparation obscures changes to the labile metal pool *inside* the cell. Regardless, the metal loss observed does not render this preparatory technique invalid for studying *in situ* metal content in sections prepared for immunohistochemistry; the narrow range of metal leaching observed for each fixation/cryoprotection step (Fig. 2) suggests the rate of leaching is fairly consistent, and appropriate experimental controls coupled with a precise analytical technique, such as ICP-MS should ensure that small changes in metal levels between experimental groups are observed with sufficient accuracy.[6]

Conclusions

Metal leaching from fixed and cryoprotected murine brain tissue is both marked and significant, the amount of which likely depends on the solubility of the metal involved, particularly group 1 and 2 metals. Less soluble transition metals are less susceptible to metal leaching, yet still display some degree of metal loss during fixation and cryoprotection. Caution should be taken to minimise metal loss during sample preparation, and thus improving preparatory steps for frozen sectioning of tissue should become a priority. However, proper experimental design (*i.e.* proper experimental controls to identify relative changes) does not preclude this method from providing important information about altered metal metabolism.

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