**Editorial comments:**

Please sign your Article and Video License Agreement by hand (do not type).

*Form has been completed*

1. Formatting: 7.3 – Please split into two steps.

*Step 7.3 has been split into two steps as follows:*

7.3.1 Click the ‘Baselines’ tab to apply background correction for the sample. On the phosphorus (P31) image prompted on the screen, select areas of the background using the rectangle draw tool.

7.3.2 To make the background signal more visible, select the graph edit tool (top left of the displayed image) and right click the image. Select ‘Modify Image Appearance’ and change the ‘First Color at Z=’ to a large negative value (eg. -100000). Proceed by clicking ‘Done’.

2. Grammar: 2.5.1.2 – Should be “weigh”

*Has been changed to ‘measured’.*

3. Please remove commercial branding: -Iolite – 6 note, 6.2, 6.4.1, 6.9 -Igor Pro – 6 note

*All references to ‘Iolite’ and ‘Igor Pro’ have been removed. Please note that we were hesitant to remove the references to specific software names in note 6, as they are highly relevant. We have, however, ensured correct references are in place.*

4. Results: Please discuss what the data in Figure 2 mean in the Results section. What is the interpretation?

*We have added the following to the interpretation of Figure 2:*

Elemental maps can be particularly useful when used in conjunction with existing anatomical and functional reference atlases26, where information on the colocalization of metals can the expression of specific metal-binding proteins can provide insight into the function of metals within a brain region, or changes in metal levels in line with an identified disease-related biomolecule.

5. Discussion: Please discuss what the limitations of the method are.

*We have added the following:*

The current major limitations of LA-ICP-MS as an imaging technique for spatially assessing metal distribution are throughput and sensitivity. There is a tradeoff between speed of analysis and spatial resolution5,12, with higher resolution images requiring longer analysis times. The technique is well suited to biological elements at higher concentrations, though elements such as manganese, cobalt and selenium are restricted due to their low abundance in normal tissue and/or limitations in their detection by conventional ICP-MS. New advances in ICP-MS technology, such as the introduction of triple-quadrupole mass analyzers, allow for targeted detection of difficult analytes, such as selenium30 at higher sensitivities31. As a technology-driven procedure, advances in both laser and mass spectrometry design will see this imaging technique continue to evolve, increasing the speed of analysis and sensitivity32.

**Reviewers' comments:**

**Reviewer #1:** *Manuscript Summary:* This is a piece of work that will be very useful to many like me and my groups interesting in bio-imaging.

*Major Concerns:* N/A

*Minor Concerns:*

-section 1, I would suggest adding details about how samples should be prepared prior to sectioning (i.e. from dissection to freezing). There's a comment on fixation and cryoprotection, but some recommendation would help.

*We have expanded Section 1 as follows:*

1.1. Sample preparation and sectioning

Note:Fixation using 4% paraformaldehyde and cryoprotection in 30% sucrose in 0.1 M PBS will result in varying amounts of leaching of metals from tissue. See Hare *et al*19 for specific details. Ensure all samples have undergone identical fixation and cryoprotection steps.

1.1.1 Transcardially perfuse the euthanized animal with ice-cold 0.1 M PBS, pH 7.4 (see the Methods section in Dodt *et al.*20 for details) and remove the brain.

1.1.2 Place the brain in 4% paraformaldehyde overnight to fix the tissue.

1.1.3 Cryoprotect the brain by placing it in 30% sucrose in 0.1 M PBS for 24 hours, then change to fresh 30% sucrose for another 24 hours.19

1.1.4 Freeze the brain in a cryostat at -20˚C for at least one hour.

1.1.5 Mount the brain on a chuck using a suitable mounting medium.

1.1.6 Section the brain on a cryostat using a metal-free disposable blade (*e.g.* polytetrafluoroethylene [PTFE]-coated knives) and mount on a standard microscope slide. The optimal thickness for the section should be approximately 30 μm.

1.2 If using paraffin-embedded samples, section at the desired thickness, float the ribbon onto a warm water bath and mount on standard microscope slides.

Note: Precise effects of long-term fixation and paraffin-embedding of biological samples are not known. As described in 1.1, ensure all samples have undergone identical sample preparation procedures if comparative analysis is intended.

1.2.1 Dewax paraffin-embedded samples by dipping the slide in three changes of xylene, one change each of 100% ethanol, 95% ethanol, 70% ethanol and a minimum of three changes in ISO 3696 or equivalent purified water (hereafter, referred to as ‘water’; see Hare *et al*.21 for a detailed method).

-section 4.2, explain that total integration time of 0.25 s is spot size / scan speed, so readers can adjust to different integration times, spot size and/or scan speed if they wish to do so (although it is mentioned in the introduction; but might be good to remind the reader)

*We have added to the Note:*

For instance, using a 100 μm spot size, a scan speed of 400 μm s-1 with an integration time of 0.25 s will produce images with true pixel sizes. Integration time can be adjusted to improve sensitivity; when increasing integration time to 0.33 s scan speed should be slowed to three times the beam diameter.

 -It is slightly frustrating to see a workflow for Biolite... when it's not available yet.

*We are currently working on making Biolite available, and hope to have the plugin freely available to Iolite users in the next six months.*

*Additional Comments to Authors:* N/A

**Reviewer #2:**

*Manuscript Summary:* N/A

*Major Concerns:* - Is it necessary to ablate the 30 μm of tissue (without ablating the glass microscope slide) to ensure that the same amount of sample is being analysed? How does affect the sample thickness uncertainty to the quantitative values? Please comment.

*We have added to 3.5.2:*

At 30 μm tissue thickness the laser beam does not penetrate the full thickness of this tissue, eliminating any potential contaminant from the microscope support. Normalizing to carbon23 can be used to correct for variation in the amount of tissue ablated.

 - Please comment about the use of internal standards.

*See point above.*

 - Please comment about plasma/laser drift corrections (bracketing quantification method).

*We have added to 7.3.1:*

Selecting as many regions as possible creates an image-wide map of signal/plasma drift to ensure adequate compensation from these confounding factors.

*Minor Concerns:* - All the references should have a consistent style (e.g. all the co-author names should be included)

*All referenced have been corrected.*

 - Quality resolution of Figure 1 is quite poor.

*Figure 1 is 600 dpi, perhaps conversion to PDF reduced resolution?*

*Additional Comments to Authors:* N/A