**Response to Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

The paper describes application of x-ray microanalysis to measure calcium levels in mitochondria, a technique in which the authors are well recognized experts. The technique is complex and for an average researcher its details are not obvious. Therefore, a popularization of this method by JoVE is an excellent idea. The technique is very well described.

*Major Concerns:*

None

*Minor Concerns:*

Line 118, the personal protective equipment needed while handling liquid nitrogen in "a nearby Styrofoam bowl" should be explicitly indicated.

Response: Recommended protective clothing and precautions are now specified at the earliest appropriate mention (lines 100-102).

Line 161, is the mentioned "eyelash glued to wooden stick" a homemade piece of equipment? If it is, the authors should be more explicit about manufacturing this piece.

Response: The eyelash probe is indeed homemade. Its construction is now described.

Line 187, describe the "appropriate precautions."

Response: Caveats re: LN2 handling are described at earliest mention (lines 100-102).

Line 233, this sentence is not clear.

Response: Sentence (now lines 240-241) is clarified as follows: “Before imaging, turn off cryoholder control unit and physically disconnect controller cable to avoid image drift due to thermal cycling and/or vibration pickup.“

Line 295, Mg, Cl and S (unlike Na, P, K and Ca) are only in some cases well suited to EDX analysis. Why? What is peculiar about Mg, Cl and S?

Response: Our writing here was ambiguous and open to misinterpretation. Relevant sentences have been rewritten to indicate that there is nothing peculiar about Mg, Cl and S beyond the fact that changes in their concentrations are not often of physiological interest.

Line 435, the <104 nm2 appears to the area indicated by the red dot in Fig. 2 rather than "a typical area of mitochondrial matrix." This should be clarified.

Response (line 443): Confusion avoided by deleting any reference to the size of the “dot”.

Figure 3 could be better described. In Fig. 3, there are two large C and O peaks that are not described in the inset to Fig. 2. Furthermore, the O peak is much larger in the inclusion than in the matrix. The authors should explain why (phosphate?). I understand that the red trace comes from one of the inclusions indicated by the arrows in the lower panel. However, is not clear where from the blue (matrix) trace is coming from. The figure would be improved if in the lower panel, the inclusions are indicated with blue arrows and the matrix areas with red arrows.

Response: Figure 3 is improved and explanation in figure legend enhanced as recommended. One important point of new emphasis is that large P and O peaks in the spectrum of an inclusion reflect the high phosphorus and oxygen content of the precipitates.

In Figure 4, micrographs of CA3 and CA1 regions should be presented for comparison.

Response: Fig. 4 has been revised to include electron micrographs of both CA3 and CA1.

*Additional Comments to Authors:*

N/A

**Reviewer #2:**

*Manuscript Summary:*

This is an excellent methodological manuscript describing the use of electron probe x-ray microanalysis for monitoring total Ca in neurons at suborganellar levels. Many neuroscientists and scientists from other areas interested in subcellular and mitochondrial Ca distribution would greatly benefit from this paper.

*Major Concerns:*

None

*Minor Concerns:*

Results p.7 (line 6 from the bottom) and Fig.2, inset. The text on p.7 used eV as energy units, whereas keV are used in Fig.2. It seems that the text also should use keV (e.g., 3.69 keV). Please correct or clarify.

Response: Typos are fixed.

*Additional Comments to Authors:*

N/A

**Reviewer #3:**

*Manuscript Summary:*

This article presents a detailed methodology that is required for the preparation and analysis of cryosections of biological material for the purpose of measurement of element content for cell physiology.

The manuscript describes the methodology carried out in a laboratory that is highly regarded world-wide for the work carried out there. The work is very well and clearly presented and will be useful to those wishing to learn this methodology.

The title and abstract are appropriate and I feel that anyone following the steps outlined in the procedure will be able to produce cryosections and undertake their analysis, One step that should be clarified is whether the analysis is carried out on the folded grid? This can be easily incorporated into the manuscript as it stands.

Response: Analyses are indeed carried out on the folding grids, as now made clear (line 206).

All critical steps are highlighted and the literature adequately cited.

*Major Concerns:*

Perhaps there should be more emphasis that cryosectioning needs skills and pracrtice.

Response: Agreed. See response to “Major Concerns” of Reviewer #4 for action taken.

*Minor Concerns:*

Can the authors clarify that the analysis takes place on grids that remain folded i.e. the sections remain between two layers of pioloform film

Response: Yes, analysis is performed on folding grids. See response just above.

*Additional Comments to Authors:*

N/A

**Reviewer #4:**

*Manuscript Summary:*

Authors in this article describe a method that allows determining quantitative content of elements in intracellular compartments utilizing electron probe microanalysis (EPMA). This is a powerful technique giving a possibility to determine element content within intracellular organelles. Since the point of measurement in the sample can be correlated with electron microscopic image one can compare the elements distribution between different cell compartments and/or organelles. All steps are described in details, and also the required tools, instruments and material are listed. As an example the authors shows differential calcium accumulation in mitochondria located in neurons of the CA1 and CA3 hippocampal sub-region of organotypic slices subjected to excitotoxic stress.

*Major Concerns:*

Since about 80 nm thick cryo-sections are used for analysis, the handling and processing of biological samples is crucial for successful measurement. I assume that the attached video to this manuscript will help the readers to follow the procedures, however the cutting of 80 nm sections from cell cultures on coverslip deserves more detailed description since this is very tricky and not a generally used procedure.

Response: With regard to the difficulties of cryosectioning, we understand the concerns of Reviewers #3 and #4. In response, Section 2 of the ms. has been expanded to include more details of exactly how cryosectioning is carried out.

*Minor Concerns:*

N/A

*Additional Comments to Authors:*

N/A