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Measurement of total calcium in neurons by electron probe x-ray microanalysis --Manuscript Draft--

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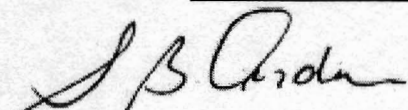
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Measurement of total calcium in neurons by electron probe x-ray microanalysis

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Short Abstract

This paper describes the application of cryo-analytical electron microscopy to the quantitative measurement of total calcium content and distribution at subcellular resolution in physiologically defined biological specimens.

Long Abstract

In this article the tools, techniques and instruments appropriate for quantitative measurements of intracellular elemental content using the technique known as electron probe microanalysis (EPMA) are described. Intramitochondrial calcium is a particular focus because of the critical role that mitochondrial calcium overload plays in neurodegenerative diseases. The method is based on the analysis of x-rays generated in an electron microscope (EM) by interaction of an electron beam with the specimen. In order to maintain the native distribution of diffusible elements in electron microscopy specimens, EPMA requires “cryofixation” of tissue followed by the preparation of ultrathin cryosections. Rapid freezing of cultured cells or organotypic slice cultures is carried out by plunge freezing in liquid ethane or by slam freezing against a cold metal block, respectively. Cryosections nominally 80 nm thick are cut dry with a diamond knife at ca. -160°C , mounted on carbon/piolofom-coated copper grids, and cryotransferred into a cryo-EM using a specialized cryo-specimen holder. After visual survey and location mapping at $\leq 160^{\circ}\text{C}$ and low electron dose, frozen-hydrated cryosections are freeze-dried at -100°C for ~30 min. Organelle-level images of dried cryosections are recorded, also at low dose, by means of a slow-scan CCD camera and subcellular regions of interest selected for analysis. X-rays emitted from ROIs by a stationary, focused, high-intensity electron probe are collected by an energy-dispersive x-ray (EDX) spectrometer, processed by associated electronics, and presented as an x-ray spectrum, that is, a plot of x-ray intensity vs. energy. Additional software facilitates: 1) identification of elemental components by their “characteristic” peak energies and fingerprint; and 2) quantitative analysis by extraction of peak areas/background. This paper concludes with two examples that illustrate typical EPMA applications, one in which mitochondrial calcium analysis provided critical insight into mechanisms of excitotoxic injury and another that revealed the basis of ischemia resistance.

Introduction

Calcium ions are arguably the most important and versatile cell signaling entity in biology, playing an essential role in normal processes as diverse as synaptic transmission and gene expression. On the other hand, calcium is equally important in cell death. In particular, calcium deregulation is a key factor in neuronal injury in stroke, Parkinson's, Alzheimer's and other neurodegenerative diseases^{3,5}. Thus, it is critically important to understand quantitatively how calcium is distributed within cells, and how this changes following physiological or pathophysiological stimuli. This goal is complicated by the fact that calcium is dynamically distributed between two physical states – free in solution or bound to a substrate – and that cellular calcium concentrations change over several orders of magnitude as a consequence of stimulation.

While there are several advanced methodologies available for the analysis of free intracellular calcium, the determination of total calcium concentrations in defined intracellular compartments is realistically limited to one approach, namely, electron probe microanalysis (EPMA). EPMA is a technique that couples an x-ray spectrometer to a transmission electron microscope (TEM). The TEM electron gun focuses a stationary, submicron electron probe on a subcellular region of interest and the element-

specific x-rays emitted as a result of electron bombardment are collected and analyzed (see Refs. 7 and 4 for detailed technical reviews). Advantages of EPMA include single organelle-level resolution and submillimolar sensitivity. In practice, however, EPMA requires specialized cryotechniques and instrumentation for specimen preparation and analysis. Here, the tools, techniques and instruments appropriate for measurements of intracellular calcium using EPMA are described. Intramitochondrial calcium is of special interest on account of the critical role that mitochondrial calcium overload plays in neurodegenerative diseases.

Protocol

The approach described here was developed using specific instruments, tools and software. Because labs will not be using the same experimental setup the approach is generalized where possible.

1. Rapid freezing

The analytical method to be described is absolutely dependent on cryogenic approaches for: 1) the “cryofixation” of cells or tissues in a manner that quantitatively preserves the distribution of diffusible tissue components and chemical elements as they were in live cells at the instant of freezing; and 2) the preparation of ultrathin cryosections suitable for imaging and analysis in a TEM. These techniques are briefly described here, but details necessary to reproduce these procedures in other laboratories are beyond the scope of this article. Interested readers are referred to excellent recent articles^{9,14}.

Caution: This section describes the use of liquefied ethane, which is highly flammable and potentially explosive; appropriate precautions should be taken. Operator must also be familiar with liquid nitrogen (LN₂) safety precautions, including protective lab coat, glasses, and cryo-resistant gloves. Note: Here and throughout, all tools (forceps, etc.) used to handle frozen specimens must be precooled in LN₂ before use to avoid accidental thawing.

Cultured cells on coverslips

1.1) Prepare a plunge freezing device by condensing gaseous ethane at –160° C in the LN₂-cooled central well. Fill to the mark and cover the well with the pivoting aluminum lid when not in use.

1.2) Using filter paper wedges, blot plastic coverslips of cultured cells under experimentally appropriate conditions to a thin aqueous film. Blot from the edge so as to avoid touching the tissue; the ideal residual film, determined by trial-and-error, is as thin as possible but not so thin as to evaporate and allow drying of the tissue surface.

1.3) Holding the coverslip by the edge with clamping forceps, quickly immerse in liquid ethane, either manually or using the gravity-powered plunger.

1.4) Place the frozen coverslip in a nearby styrofoam bowl of LN₂, large enough to manipulate the desired number of coverslips into long-term storage containers. Aluminum screw cap cans that don't seize under LN₂ are recommended.

Rapid freezing of cultured brain slices

1.5) Under a dissecting microscope, view and orient an organotypic hippocampal slice, undisturbed and still attached to the culture membrane insert. Place fiducial marks on the membrane near the edge of the slice with a black Sharpie-type pen and photograph.

1.6). Still under the microscope, cut out approx. 5 x 5 mm membrane squares with individual slices centered on the squares. Avoid touching the slice or bending the membrane.

1.7) Mount a membrane-supported slice, cushioned by a ¼"-diameter agar pad, on a custom-designed aluminum disk made to fit a cryoultramicrotome (described below). Rapidly freeze the slice by pneumatically propelling it against an LN₂-cooled sapphire block by means of a customized "slam freezing" device.

1.8) Move to storage as described for cultured cells.

2. Cryosectioning

CAVEAT: Successfully producing dry-cut ribbons of ultrathin sections, while straightforward and logical, requires training, patience and considerable practice.

2.1) Bake out a cryoultramicrotome equipped with a cryo-attachment and cool to -135° C.

2.2) Cut frozen coverslips into approx. 3 x 3 mm squares using a sharp, precooled scalpel. Embed pieces with cultures facing up in a viscous liquid "cryoglue" (a 1:6 mixture of ethanol and 2-propanol¹¹), on standard 3-mm diameter aluminum pins designed to fit the microtome specimen chuck. Avoid leaking cryoglue onto culture surface. Solidify cryoglue by lowering the cryobox temperature to ≤-160° C.

2.2a) For frozen brain slices, securely attach discs directly to a custom-made specimen chuck by means of a screw collar.

2.3) Trim selected areas of the frozen specimens — e.g., cell-rich areas of coverslip pieces or identified areas of slices — to an approx. 250 x 250 µm block face and ~100 µm depth using a diamond trimming tool.

2.4) Dry-cut ribbons of thin sections at ca. -160° C using a 35° diamond cryoknife, essentially as described in Refs. 4 and 9. Cutting speed and knife clearance angle are determined empirically; a good starting point is 0.4-0.6 mm/sec at 9°. The nominal thickness, i.e., specimen advance, of hydrated sections is typically 80 nm, although

sections are actually 1.5-2.0 times thicker, mainly due to compression. For satisfactory sectioning, an antistatic device is essential. Place the ionizing tip of the device 0.5-1 cm from the knife edge and adjust output power until satisfactory ribbons of sections are produced.

2.5) Prepare eyelash probes by gluing (Epoxy) eyelashes to wood applicator sticks. Using such a probe, pick up and transfer sections from the back of the knife onto glow-discharged, carbon-coated pioloform support films cast over 100-mesh folding copper grids and resting on a half-folded indium foil envelope on a working shelf behind the knife. Fold over the top half of the grid and envelope, press with a cold pressing tool.

2.6) Transfer wrapped grids to a convenient grid box and store in an aluminum can as described in step 1.4.

3. Cryotransfer of specimens to the electron microscope

The core instrument for EPMA in this laboratory is an analytical electron microscope operated at 120 kV and equipped for cryomicroscopy, that is, designed with a clean vacuum, specimen-area anticontaminator, a 2k x 2k high-sensitivity digital camera and cryotransfer specimen holder. Check beforehand microscope alignment and operating conditions in both low- and high-magnification modes and confirm a satisfactory column vacuum, ideally $\leq 10^{-7}$ Torr. Tune up as necessary.

3.1) Confirm that the vacuum insulation of the Dewar component of the cryoholder is satisfactory. Pump as necessary.

3.2) Cool the cryoholder to its minimum temperature, at least -160°C , while under high vacuum within the microscope stage; detach the cable connecting the holder to its control box. Tilt the goniometer 45° clockwise before removing the holder to minimize LN_2 spilling onto operator and microscope surfaces.

3.3) Prepare the benchtop cryo-workstation by cooling the integral insulated cup to $\leq 160^{\circ}\text{C}$.

3.4) Transfer (quickly!) the cooled cryoholder from microscope to cryo workstation. Retract the holder's frost shield.

3.5) Retrieve under LN_2 a grid sandwich from storage and place on the working table of the cryo-workstation. A retaining ring for the holder's specimen well is also placed on the table.

3.6) Open the indium envelope and move the enclosed folding grid to the specimen well of the cryoholder. Secure the grid with retaining ring using the spanner tool provided and close the frost shield.

3.7) Quickly remove the cryoholder from the cryo-workstation and insert into the airlock of the microscope and go through the pumping sequence as quickly as possible. On insertion, there should be minimal disruption of the column vacuum.

3.8) Return goniometer to 0° tilt. Reconnect and re-energize the control box and confirm that the specimen temperature is $\leq 150^{\circ}\text{C}$.

3.9) Refill the Dewar of the specimen holder and allow vacuum and temperature to fully recover.

4. Visual survey of sections

4.1) Retract the frost shield of the holder to expose specimen and turn on the electron beam.

4.2) Visually evaluate the specimen at low magnification, typically 250x, and low illumination. As illustrated in Fig 1, sections should be thin and smooth, not folded or overlapping, flat and well attached to the support film, and generally not obscured by grid bars.

4.3) Optionally photograph selected sections. (NOTE: Minimize beam exposure, since frozen-hydrated sections are very susceptible to beam-induced damage.) Use the automated digital goniometer stage to store coordinates of selected sections.

5. Freeze-drying of sections

5.1) Freeze-dry sections by increasing holder temperature to ca. -100°C for ~30 min.

5.2) Recool holder to -160°C or below.

5.3) Before imaging, turn off cryoholder control unit and physically disconnect controller cable to avoid image drift due to thermal cycling and/or vibration pickup.

6. Imaging of cells and organelles

Structural images of freeze-dried sections are obtained at ca. -160°C as low-dose, zero-loss images recorded digitally using a 2k x 2k slow-scan CCD camera controlled by appropriate software.

6.1) Activate the EM in hi-mag mode.

6.2) Choose and image at ~2,000x (as TIFFs, see Fig 2) selected cells and subcellular areas of interest in high-quality sections whose locations were previously recorded and stored. (NOTE: The dried sections are now substantially less fragile and less susceptible to electron beam damage.)

6.3) Evaluate images in order to select regions of interest (ROIs) for x-ray analysis. This step can optionally be performed off-line, in which case the EM can be turned off and the specimen allowed to warm to room temperature.

7. Acquisition of x-ray spectra

X-ray spectra can be recorded using any of several commercial or custom-designed x-ray analysis systems minimally consisting of an energy-dispersive x-ray (EDX) detector, associated pulse-processor electronics and compatible acquisition and display software. (The system used in this lab is described in Table 1.)

7.1) Configure the EM for the x-ray acquisition by inserting the EDX detector into the column (if necessary), withdrawing the objective aperture, and inserting and centering any stray radiation apertures. Adjust the cryoholder to the lowest temperature at which frost contamination of the specimen is avoided, but at least below -100°C .

7.2) Tilt the holder 20° toward the detector.

7.3) Under wide-field imaging conditions, and assuming one intends to analyze the matrix of an individual mitochondria, choose a mitochondrion for analysis, move it to the center of the field and focus.

7.4) Go to spot mode (on some microscopes just converge the beam using second condenser focus) and increase the beam current to ca. 5 nA (as measured with a Faraday cup or similar) into a 100 nm spot.

7.5) Launch the spectrum acquisition software and begin 100 s acquisitions, which can be viewed live time on the display monitor. Save recorded spectrum (Fig 2). The industry standard EMSA format is preferred.

7.6) Shut down the EM and transfer the multiple-spectra file(s) to an (off-line) analysis workstation .

8. Analysis of x-ray spectra

Qualitative analysis

An EDX spectrum (Fig 2, inset) is essentially an x-y plot of the x-ray intensity vs. energy. Spectra contain qualitative and quantitative information about the elemental composition of the analyzed volume, in that the “characteristic” energy of a peak identifies the element giving rise to that peak while the intensity reflects the amount of that element. The characteristic peaks ride on top of a slowly varying background, the “continuum”. (The legend to Fig. 2 further discusses salient details of EDX spectra.) The energy of the peak manifolds for the entire periodic table are defined by the well known electronic structure of elements, thus all EDX software links to a database that automatically identifies components of an analyte. In a physiological context, elements

of general interest that are well suited to EDX analysis include Na ($K\alpha$ at 1.04 keV), P (2.01 keV), K (3.31 keV), and Ca (3.69 keV).

8.1) Take advantage of available software database and peak matching routines to identify major elements in the spectra. In a biological specimen expect to find peaks for Na, Mg, P, S, Cl, K and Ca. (CAUTION: The last two elements overlap!)

Quantitative analysis

Quantitative analysis of EDX spectra consists of extracting the integrated area of identified peaks and converting this value to a concentration. For biological analysis, the established approach is the Hall peak/continuum method^{4,7,10}, which takes advantage of the fact that the intensity of the continuum (defined above and in Fig 2) is proportional to the dry mass of the analyzed volume. Thus, the ratio of peak area/continuum area, when compared to the same ratio in spectra of standards of known composition, specifies the concentration in the targeted cellular compartment. Note that this approach provides concentrations in units of moles per weight, typically expressed as mmol/kg dry weight. This unit is unusual and for interpretation may require additional conversion to, for example, mmol/l wet weight or mmol/mg protein, as described elsewhere^{4,10}.

8.2) Extract peak areas (and error estimates) of biological elements between $Z = 10-20$ (0.5-4.0 keV), i.e., Na, Mg, P, S, Cl, K and Ca, using one of the fitting routines built into analysis software (see Table 1, especially footnote 4). This lab uses Simplex or multiple-least squares fitting. Note that this fitting requires careful attention to resolving the overlap between K and Ca peaks (see Fig 2).

8.3) Integrate the continuum between 1.45-1.61 keV. Alternative interference-free regions can be used.

8.4) Calculate peak/continuum ratios and then concentrations by comparison to standards. Propagate errors throughout the analysis.

8.5) Use standard statistical software and formulas to estimate averages that reflect biological variability.

Representative Results

Brain cells typically sustain excitotoxic injury as a result of the pathological neurotransmitter release that occurs under ischemic conditions. EPMA was critical to discovering how the ability of neuronal mitochondria to sequester massive amounts of calcium underlies the mechanism of injury. The electron micrograph in Figure 3 illustrates the appearance of mitochondria in freeze-dried cryosections of cultured hippocampal neurons rapidly frozen after 30 min exposure to an excitotoxic stimulus (100 μ M NMDA). Most mitochondria appear to be structurally damaged, as they are highly swollen and contain small, dark inclusions (red arrows). EPMA, which has resolution sufficient to perform elemental analysis within and exclusive of (the “matrix”)

mitochondrial inclusions (Fig 3, red and blue spectra, respectively), revealed that the inclusions are largely composed of calcium and phosphorus. The extremely high calcium content of these inclusions — ~1300 mmol/kg dry weight, whereas <1 mmol/kg is typical of resting mitochondria — explains their extraordinary calcium-buffering capacity, and why overwhelming this buffering mechanism leads to calcium overload-triggered cell death¹¹.

The hippocampus, an area of the brain critical for learning and memory, is a major site of injury after ischemia. Interestingly, and therapeutically important, the functionally distinct region named CA3 is far less vulnerable to ischemic injury than is the adjacent, synaptically connected CA1 region. EMPA analysis — in conjunction with cryosections of specific regions of hippocampal slices that maintain *in situ* structure and function (Fig. 4, micrograph) — was used to show that toxic stimuli induce much larger calcium elevations in vulnerable CA1 neurons than in resistant adjacent CA3 neurons (Fig 4, bar graph). Consequently, CA1 mitochondria exhibit extensive injury and dysfunction, indicating that Ca²⁺-overload-induced mitochondrial dysfunction is a determining factor in the selective vulnerability of CA1 neurons¹³.

Discussion

The electron microscope-based analytical method presented here allows for the detection, identification, and quantitation of several elements of biological interest, including Na, K, P, and especially Ca. These analyses can be carried out at subcellular, i.e., intra-organelle, resolution owing to the ability to locate and identify structures of interest in high-quality images of cryosections prepared from rapidly frozen specimens. Note that no staining is required to record electron images comparable in structural quality to conventionally fixed, plastic-embedded preparations, even while the location of tissue elements is quantitatively preserved.

Although structural survey images are recorded at low applied electron dose, much higher doses are required to elicit x-ray emission at count rates sufficient to obtain good statistics. Therefore, a microscope useful for EMPA must be able to form a high current focused probe; 2-5 nA into 25-50 nm, suitable for encompassing organelles like ER cisternae or synaptic vesicles, is acceptable. This minimally requires a high-brightness lanthanum hexaboride electron gun. Under these instrumental conditions, calcium at typical tissue concentrations of 1-10 mmol/kg dry weight can be quantitatively analyzed to a standard error of ± 0.1 mmol/kg in ~100 s live time. (The sensitivity of calcium analysis would be greatly improved if it were not for the unfortunate overlap of the major calcium x-ray line with a minor potassium line, deconvolution of which adds significant uncertainty to the integration of the calcium signal (see Fig 1 legend for details).) Note that the boundary conditions described are greatly relaxed when local calcium concentrations are unusually high, as occurs during physiological, and especially pathological, mitochondrial calcium accumulation (Fig 2-3).

Despite numerous successful applications, it is clear that EPMA is not a particularly efficient technique, so there is much incentive to improve data throughput. Three

promising avenues are mentioned here: 1) electron energy loss spectroscopy (EELS) instead of EDX; 2) 2D element maps instead of point probes; and 3) newer, state-of-the-art instrumentation. Rather than collecting emitted x-rays, EELS depends on recording incident electrons that have lost characteristic, element-specific amounts of energy after electron/atom collisions. Statistically, EELS is inherently ~4x better than EDX, and EELS hardware and software are now practically mature for biologists. (See Refs. 6 and 2 for reviews of EELS applications in biology.)

The improved sensitivity offered by EELS — and also by the newer high-throughput Si-drift EDX detectors, see below — allows shorter dwell times per analysis, e.g., milliseconds rather than hundreds of seconds, and therefore the ability to generate digital maps of selected areas in reasonable times. As an example, a 128x128 calcium map can be recorded in approx. one hour¹. Note that this approach, known as “spectrum imaging”^{6,2} provides a complete EELS spectrum at each pixel, so information on several elements is embedded in the recorded “data cube” (x vs. y vs. E).

Lastly, there have been substantive advances in instrument technology and performance since the present system, as described in the Protocol section, was developed over 20 years ago. State-of-the-art technology that would be *de rigueur* in an EMPA lab being setup today would include: 1) a high-brightness field-emission gun that can pump several nanoamps (nA) of current into subnanometer-sized spots; 2) a large area silicon-drift detector for maximized x-ray collection efficiency and throughput⁸; and 3) modern software offering superior flexibility and performance for spectral acquisition, analysis and quantitation. Such software packages are commercially available from most manufacturers; alternatively, an updated and improved version of DTSA software, DTSA II, is available at no cost from NIST (see Table 1, footnote 4). The features just described are on top of any automation and/or robotics available on the base electron microscope.

The EMPA protocol as described has proven itself highly useful for attacking several issues of interest to neuroscientists, helping, for example, to reveal cellular mechanisms of neurodegeneration. Considering the improvements just recommended, EMPA should continue to be a solid contributor to future research.

Figure Legends

Figure 1 – Low-magnification electron micrograph of two ribbons of frozen-hydrated cryosections supported on a pioloform film. Characteristics of a good preparation are illustrated, including flat, well attached, minimally fragmented sections. Grid squares with rips in the support film should be avoided, as the film tears further under the electron beam. Two squares fully appropriate for analysis are indicated by asterisks. Scale bar = 100 μ m.

Figure 2 – Quantitative analysis of organelle elemental composition by EPMA. Low-dose, digital scanning transmission electron micrograph of sympathetic neuron in a freeze-dried cryosection prepared from rapidly frozen control ganglion illustrates the

detail achievable in such specimens. Note the sharp plasma membrane, well preserved stacks of endoplasmic reticulum and abundant mitochondria. *Inset* – EDX spectrum recorded from a typical area of mitochondrial matrix, as indicated by the red dot. Elements corresponding to the major K shell x-ray peaks are identified; in the case of potassium and calcium, two K-shell lines arising from alternative electronic transitions are resolved, indicated as K_{α} and K_{β} according to standard spectroscopic notation. Spectrum illustrates typical distribution of major elements in living neurons. Note the slowly varying continuum radiation, e.g. between 1.5-1.8 or 2.8-3.1 keV, which reflects the mass density of this cellular compartment. Quantitative analysis of calcium in healthy cells is complicated by the presence of relatively high levels of potassium (ca. 500 mmol/kg dry weight, approximately equivalent to 150 mM), which gives rise to overlap of the potassium K_{β} and calcium K_{α} peaks in the EDX spectrum. There are standard algorithms for deconvolving this overlap. Scale bar represents 1 μ m.

Figure 3 – Excitotoxic stimulation induces variable and localized calcium accumulation in individual mitochondria. Digital transmission electron micrograph of freeze-dried cryosection prepared from unfixed, rapidly frozen hippocampal cell culture after excitotoxic stimulation of the NMDA subtype of glutamate receptors (100 μ M NMDA for 30 min) shows numerous mitochondria containing small, naturally electron dense, punctate inclusions (red arrows). Corresponding x-ray spectra demonstrate that toxic stimulation resulted in loss of ion homeostasis, as evidenced by increased Na peak and reduced K peak in the inclusion-free mitochondrial matrix (blue arrows and spectrum). The large Ca peak in the red spectrum reflects strong mitochondrial calcium accumulation localized to characteristic inclusions, which also contain large amounts of phosphorus and oxygen. Average Ca concentration in inclusions and matrices was ~1300 and ~60 mmol/kg dry weight, respectively. Scale bar represents 500 nm.

Figure 4 – Mitochondrial calcium overload is responsible for selective ischemic vulnerability of hippocampal CA1 neurons. *left panel* – Electron micrographs of cryosections of cell bodies in the CA3 and CA1 regions of an organotypic hippocampal slice culture, rapidly frozen under ischemia-mimicking conditions (100 μ M NMDA). *right panel* – EPMA analyses of mitochondrial calcium content shows that chemical ischemia induces much larger calcium elevations in mitochondria of vulnerable CA1 neurons than in adjacent, ischemia-resistant CA3 neurons (*, $p < 0.05$ relative to NMDA-exposed CA1). NMDA-induced calcium overload was abolished by NMDAR antagonist MK-801. Scale bar represents 2 μ m.

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Disclosures

The authors report no conflicts of interest.

References

1. Aronova M.A., Kim Y.C., Pivovarova N.B., Andrews S.B. & Leapman R.D. Quantitative EFTEM mapping of near physiological calcium concentrations in biological specimens. *Ultramicroscopy*. **109**, 201-12 (2009).
2. Aronova M.A. & Leapman R.D. Elemental mapping by electron energy loss spectroscopy in biology. *Methods Mol. Biol.* **950**, 209-26 (2013).
3. Bezprozvanny I. Calcium signaling and neurodegenerative diseases. *Trends Mol. Med.* **15**, 89-100 (2009).
4. Fernandez-Segura E. & Warley A. Electron probe X-ray microanalysis for the study of cell physiology. *Methods Cell Biol.* **88**, 19–43 (2008).
5. Gibson G.E., Starkov A., Blass J.P., Ratan R.R. & Beal M.F. Cause and consequence: Mitochondrial dysfunction initiates and propagates neuronal dysfunction, neuronal death and behavioral abnormalities in age-associated neurodegenerative diseases. *Biochim. Biophys. Acta.* **1802**, 122-134 (2010).
6. Leapman R.D. Novel techniques in electron microscopy. *Curr. Opin. Neurobiol.* **14**, 591-8 (2004).
7. LeFurgey A., Bond M. & Ingram P. Frontiers in electron probe microanalysis: application to cell physiology. *Ultramicroscopy*. **24**, 185-219 (1988).
8. Newbury D.E. The new X-ray mapping: X-ray spectrum imaging above 100 kHz output count rate with the silicon drift detector. *Microsc. Microanal.* **12**, 26-35 (2006).
9. Pierson J., Vos M., McIntosh J.R. & Peters P.J. Perspectives on electron cryotomography of vitreous cryo-sections. *J. Electron Microsc (Tokyo)*. **60**, S93-100 (2011).
10. Pivovarova N.B., Hongpaisan J., Andrews S.B. & Friel D.D. Depolarization-induced mitochondrial Ca accumulation in sympathetic neurons: spatial and temporal characteristics. *J. Neurosci.* **19**, 6372-6384 (1999).
11. Pivovarova N.B., Nguyen H.V., Winters C.A., Brantner C.A., Smith C.L. & Andrews S.B. Excitotoxic calcium overload in a subpopulation of mitochondria triggers delayed death in hippocampal neurons. *J. Neurosci.* **24**, 5611-5622 (2004).
12. Ritchie N.W. Spectrum simulation in DTSA-II. *Microsc. Microanal.* **15**, 454-68 (2009).
13. Stanika R.I., Winters C.A., Pivovarova N.B. & Andrews S.B. Differential NMDA receptor-dependent calcium loading and mitochondrial dysfunction in CA1 vs. CA3 hippocampal neurons. *Neurobiol. Dis.* **37**, 403-411 (2010).

14. Zhang P. *et al.* Direct visualization of receptor arrays in frozen-hydrated sections and plunge-frozen specimens of *E. coli* engineered to overproduce the hemotaxis receptor *Tsr*. *J. Microsc. (Oxford)* **216**, 76–83 (2004).

Figure 1

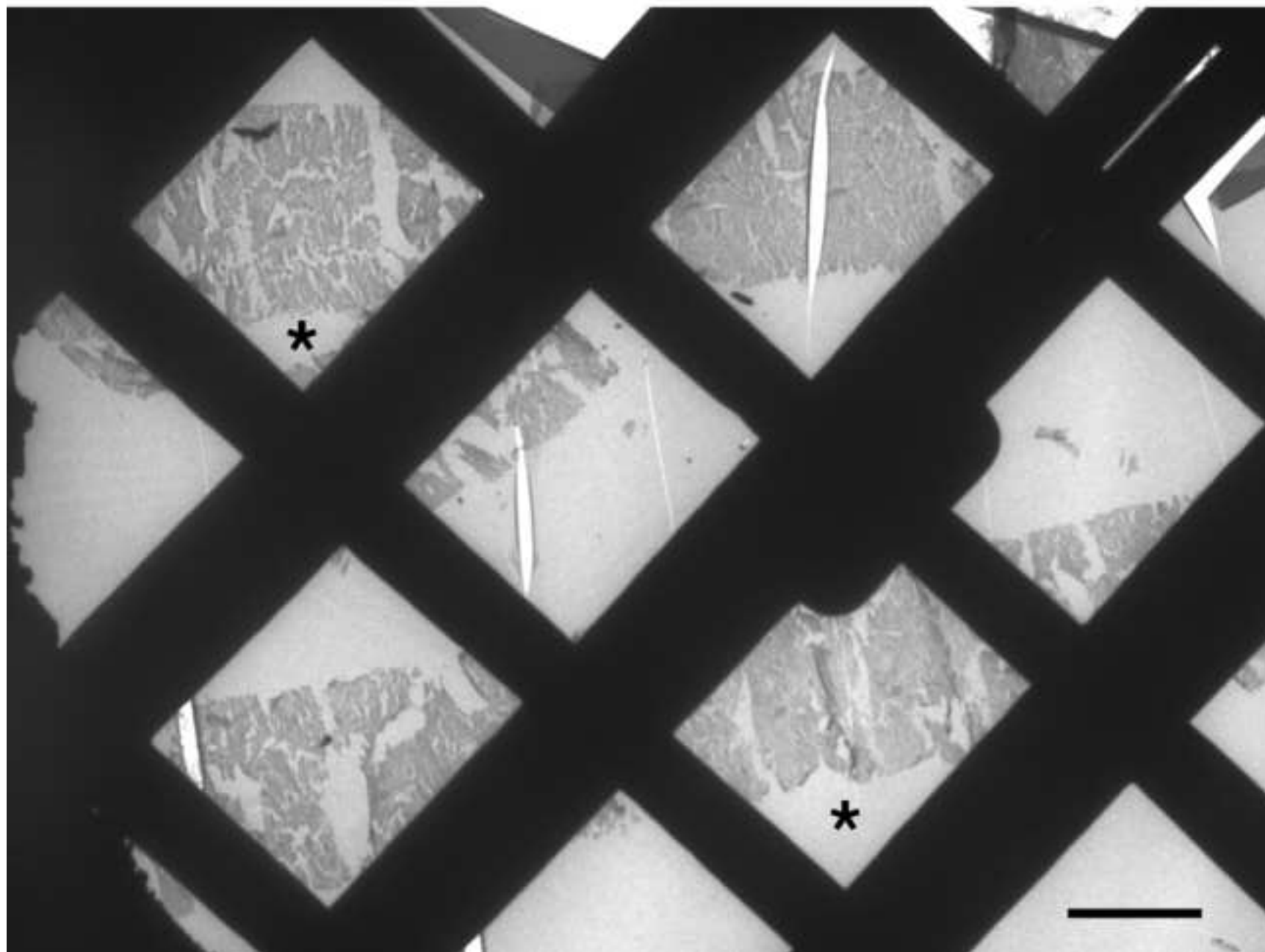


Figure 2

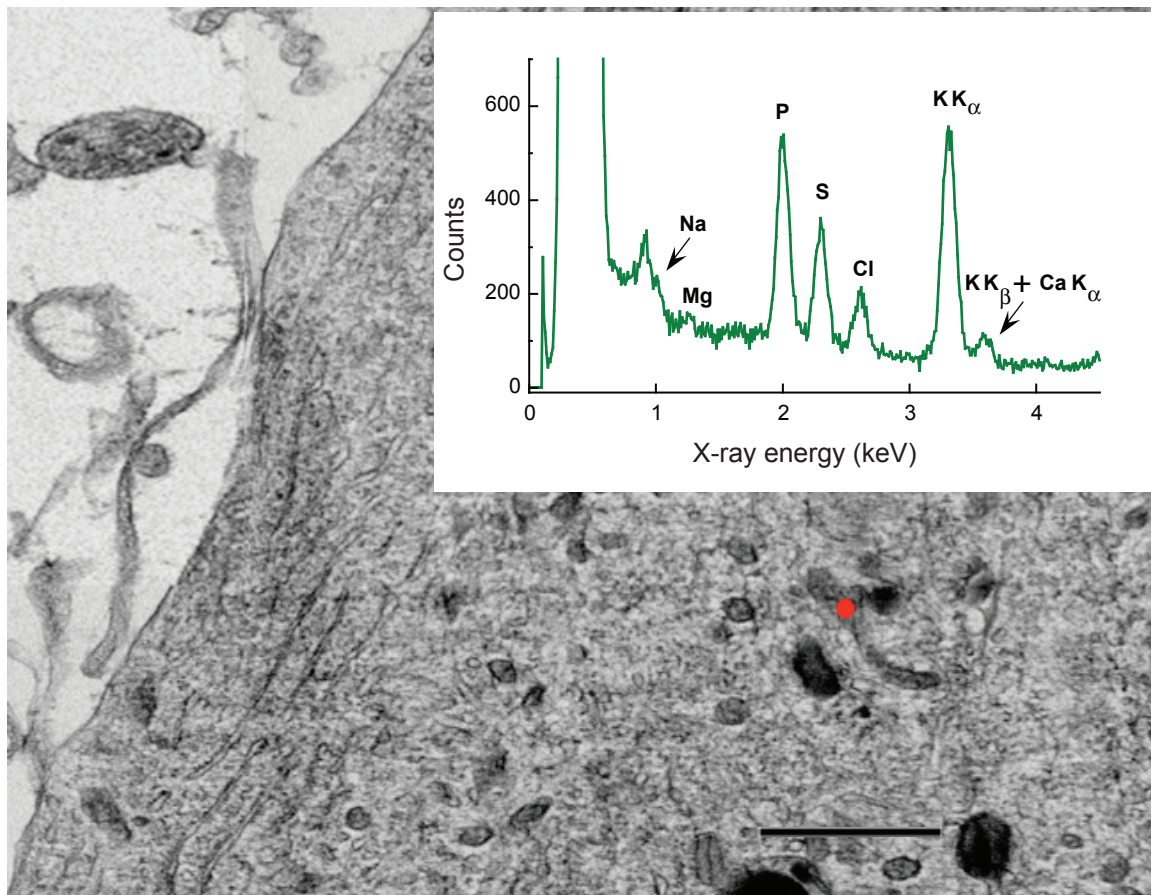


Figure 3

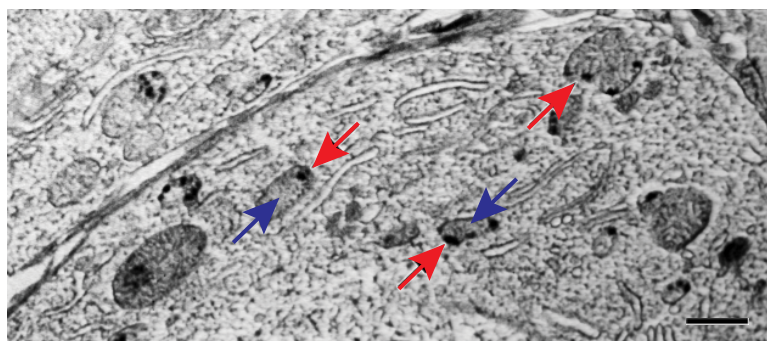
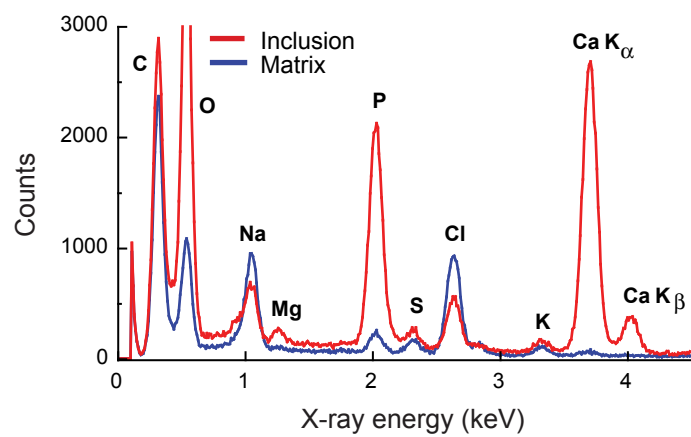
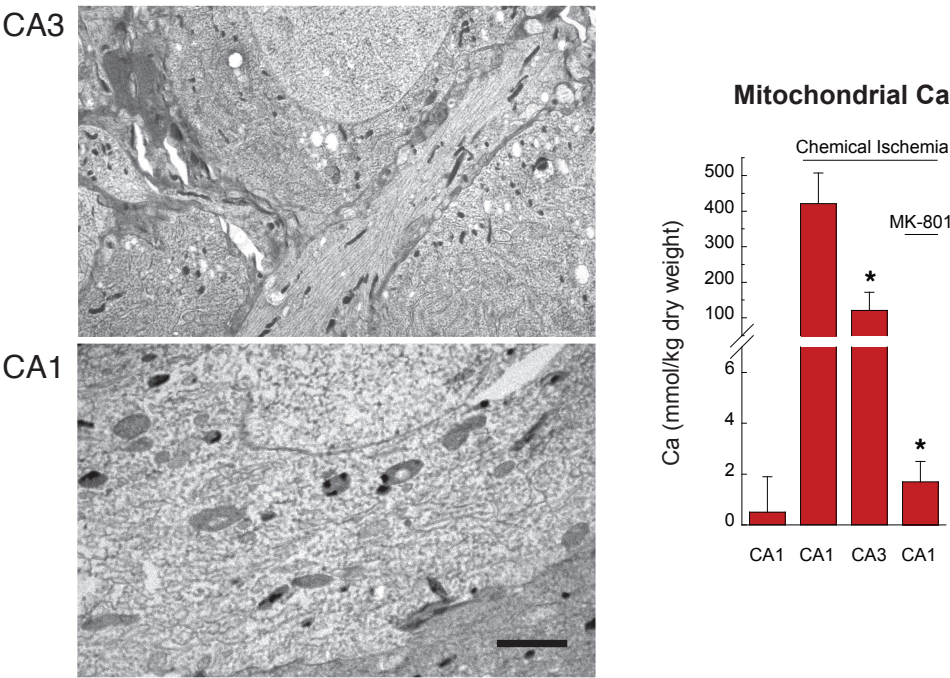


Figure 4



REAGENTS/MATERIALS:

Name	Company	Catalogue No.	Comments
Thermanox plastic coverslips	Thermo Fischer Scientific	72280	
Culture inserts	BD Falcon	353090	For 6-well plates
Cryopins	Leica Microsystems	16701952	Grooved
Wood applicators	EM Sciences	72300	
Folding EM grids	Ted Pella	4GC100/100	100 mesh
Indium foil	Alfa Aesar	13982	0.25 mm thick

EQUIPMENT:

Name	Company	Catalogue/Model No.	Comments
Plunge freezing device	Leica Microsystems	KF-80	
Slam freezing device	LifeCell	CF-100	1)
Ultramicrotome	Leica Microsystems	UC6	
Cryoattachment for microtome	Leica Microsystems	FC6	
Diamond cryotrimming tool	Diatome	Cryotrim 45	
Diamond cryoknife	Diatome	Cryo 35	
Antistatic device	Diatome	Hauf Static Line	
Cryo electron microscope	Carl Zeiss Microscopy	EM912 Omega	
EM cryo specimenholder	Gatan	CT3500	2)
Slow-scan CCD camera, 2K x 2K	Troendle (TRS)	Sharpeye	
Image acquisition software	Olympus SIS	iTEM suite	
ED x-ray detector	Oxford Instruments	Linksystem Pentafet	
Pulse Processor	Oxford Instruments	XP-3	
PCI backplane card	4pi Systems	Spectral Engine II	3)
Desktop computer	Apple	Any OS9-compatible model	
X-ray analysis software	NIST	DTSA, DTSA II	4)
Spreadsheet software	Microsoft	Excel	

1) The CF100 is no longer sold commercially, although the machine is available at many academic facilities, and complete machines or parts can be found on-line.

2) A video tutorial for the CT3500 cryotransfer holder is available at http://www.gatan.com/files/Movies/CT3500_Cryo_transfer_holder.mp4.

3) The SEII is obsolete; the Universal Spectral Engine™ Is a later, PC-compatible product with comparable functionality. 4pi has ceased manufacturing and sales but still provides technical customer support. Used systems are often found on-line.

4) The original DTSA is now obsolete. NIST offers in the public domain an updated successor, DTSA II ¹² (<http://www.nist.gov/mml/mmsd/software.cfm>).

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Author(s):

Natalia B. Pivovarova and S. Brian Andrews

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MS # (internal use):

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 practice.

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