

Video Article

Measurement of Total Calcium in Neurons by Electron Probe X-ray Microanalysis

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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/piloloform-coated copper grids, and cryotransferred into a cryo-EM using a specialized cryospecimen holder. After visual survey and location mapping at ≤160 °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.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50807/>

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 references^{7,4} 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 cryoresistant 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. 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.
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.
3. Holding the coverslip by the edge with clamping forceps, quickly immerse in liquid ethane, either manually or using the gravity-powered plunger.
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

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.
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.
7. Mount a membrane-supported slice, cushioned by a ¼ in 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.
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.

1. Bake out a cryoultramicrotome equipped with a cryoattachment and cool to -135 °C.
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.
 - For frozen brain slices, securely attach discs directly to a custom-made specimen chuck by means of a screw collar.
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.
4. Dry-cut ribbons of thin sections at ca. -160 °C using a 35° diamond cryoknife, essentially as described in references^{4,9}. 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 1.5-2.0x 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.
5. Prepare eyelash probes by gluing (with 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.
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 ≤10⁻⁷ Torr. Tune up as necessary.

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

- 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.
- Prepare the benchtop cryoworkstation by cooling the integral insulated cup to $\leq 160^{\circ}\text{C}$.
- Transfer (quickly!) the cooled cryoholder from microscope to cryo workstation. Retract the holder's frost shield.
- Retrieve under LN_2 a grid sandwich from storage and place on the working table of the cryoworkstation. A retaining ring for the holder's specimen well is also placed on the table.
- 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.
- Quickly remove the cryoholder from the cryoworkstation 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.
- Return goniometer to 0° tilt. Reconnect and reenergize the control box and confirm that the specimen temperature is $\leq 150^{\circ}\text{C}$.
- Refill the Dewar of the specimen holder and allow vacuum and temperature to fully recover.

4. Visual Survey of Sections

- Retract the frost shield of the holder to expose specimen and turn on the electron beam.
- Visually evaluate the specimen at low magnification, typically 250X, and low illumination. As illustrated in **Figure 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.
- 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

- Freeze-dry sections by increasing holder temperature to ca. -100°C for ~ 30 min.
- Recool holder to -160°C or below.
- 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.

- Activate the EM in hi-mag mode.
- Choose and image at $\sim 2,000\text{X}$ (as TIFFs, see **Figure 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.)
- 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**.)

- 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 .
- Tilt the holder 20° toward the detector.
- 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.
- Go to spot mode (on some microscopes just converge the beam using second condenser focus) and increase the beam current to ca. 3 nA (as measured with a Faraday cup or similar) into a 100 nm spot.
- Launch the spectrum acquisition software and begin 100 sec acquisitions, which can be viewed live time on the display monitor. Save recorded spectrum (**Figure 2**). The industry standard EMSA format is preferred.
- Shut down the EM and transfer the multiple-spectra file(s) to an (offline) analysis workstation.

8. Analysis of X-ray Spectra

Qualitative analysis

An EDX spectrum (**Figure 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 **Figure 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_{α} at 1.04 keV), P (2.01 keV), K (3.31 keV), and Ca (3.69 keV).

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 **Figure 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}.

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 **Figure 2**).
3. Integrate the continuum between 1.45-1.61 keV. Alternative interference-free regions can be used.
4. Calculate peak/continuum ratios and then concentrations by comparison to standards. Propagate errors throughout the analysis.
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 (**Figure 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 - ~1,300 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 (**Figure 4**, micrograph) - was used to show that toxic stimuli induce much larger calcium elevations in vulnerable CA1 neurons than in resistant adjacent CA3 neurons (**Figure 4**, bar graph). Consequently, CA1 mitochondria exhibit extensive injury and dysfunction, indicating that Ca^{2+} -overload-induced mitochondrial dysfunction is a determining factor in the selective vulnerability of CA1 neurons¹³.

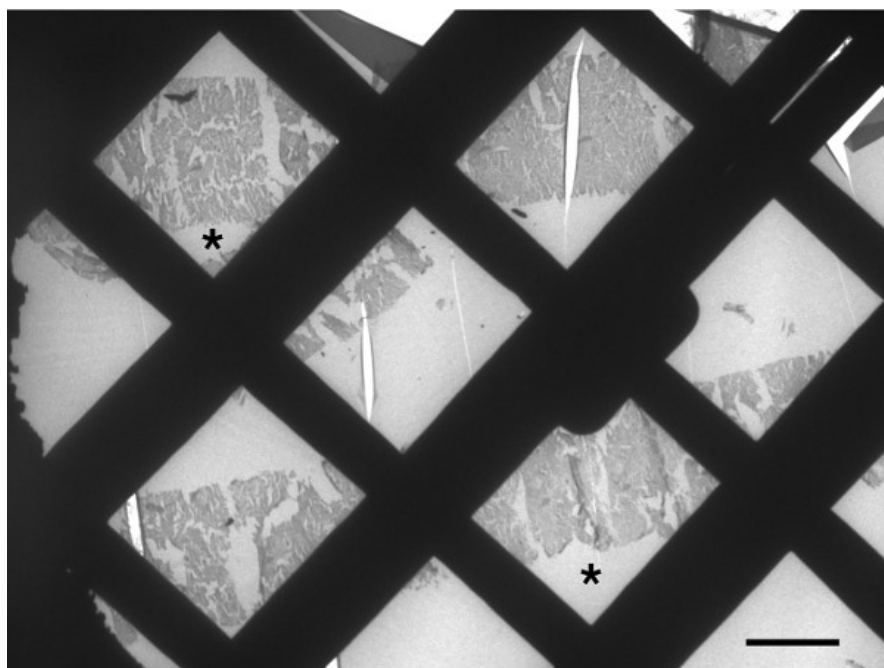


Figure 1. Low-magnification electron micrograph of two ribbons of frozen-hydrated cryosections supported on a pioloform film. Characteristics of a good preparation are illustrated, including flat, well attached, minimally fragmented sections. Grid squares with rips in the support film should be avoided,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

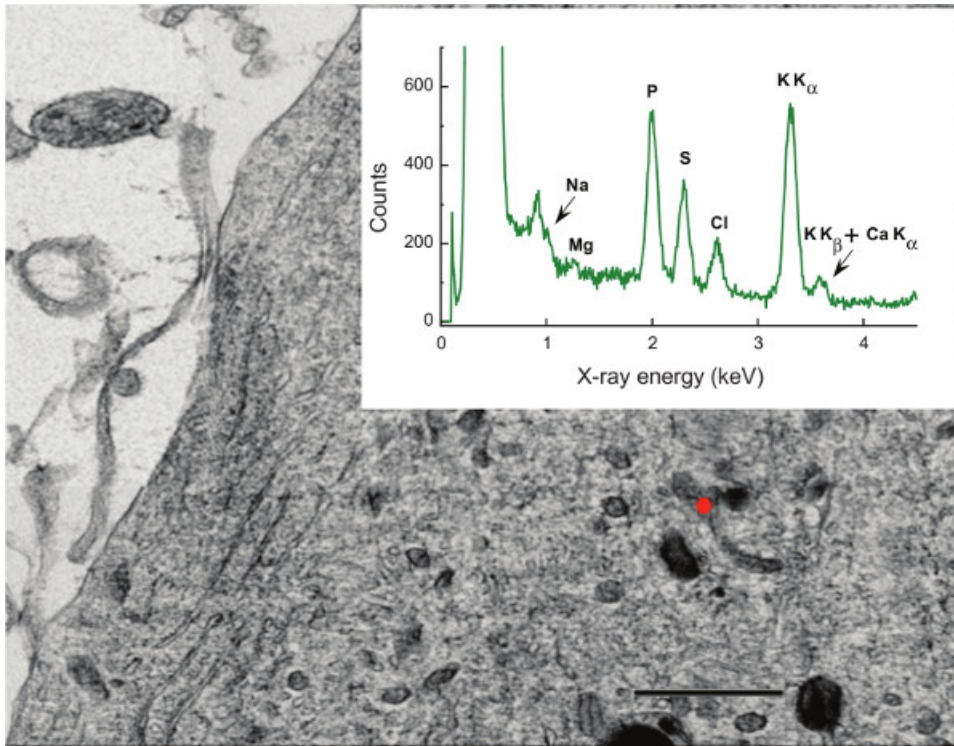


Figure 2. Quantitative analysis of organelle elemental composition by EPMA. Low-dose, digital scanning transmission electron micrograph of sympathetic neuron in a freeze-dried cryosection prepared from rapidly frozen control ganglion illustrates the detail achievable in such specimens. Note the sharp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

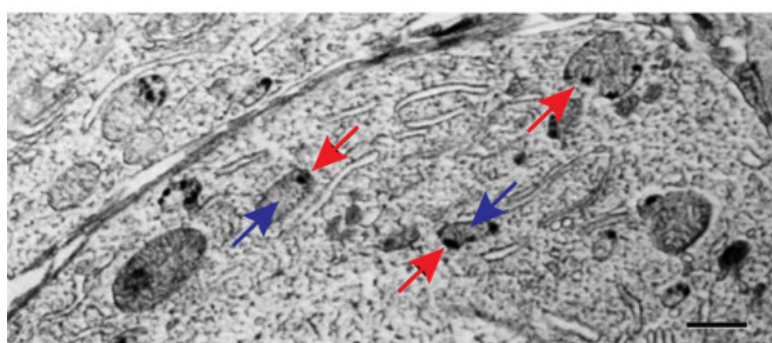
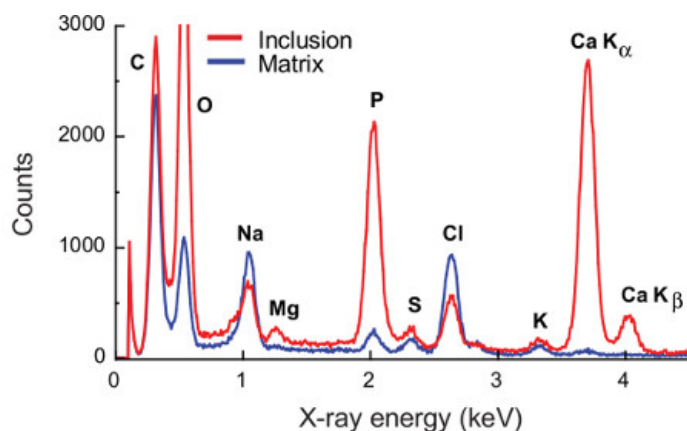


Figure 3. Excitotoxic stimulation induces variable and localized calcium accumulation in individual mitochondria. Digital transmission electron micrograph of freeze-dried cryosection prepared from unfixed, rapidly frozen hippocampal cell culture after excitotoxic stimulation of the NMDA subtype of glutamate 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 ~1,300 and ~60 mmol/kg dry weight, respectively. Scale bar represents 500 nm.

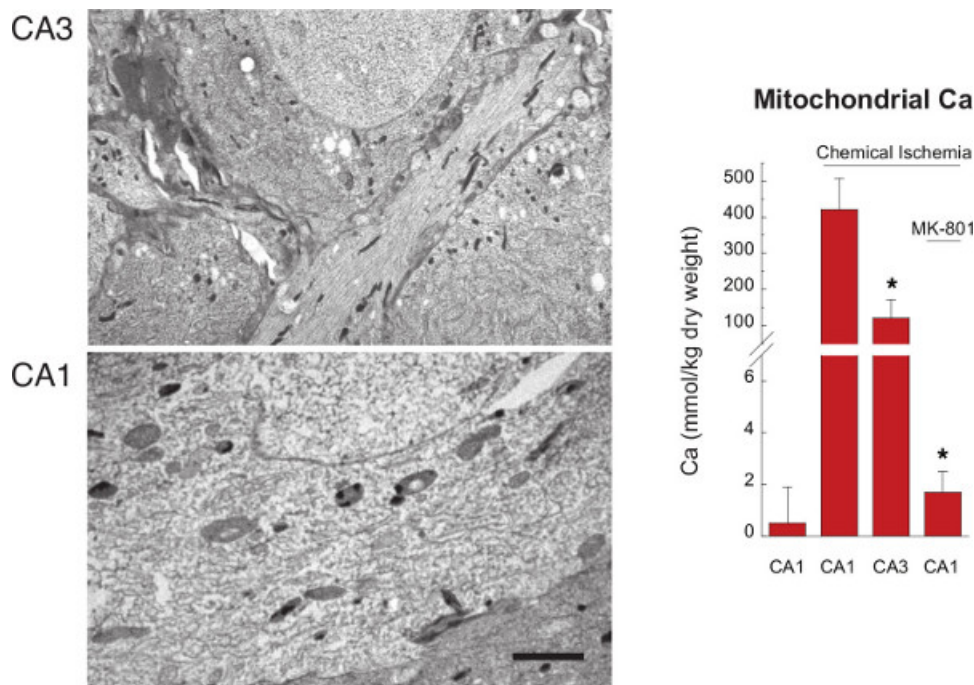


Figure 4. Mitochondrial calcium overload is responsible for selective ischemic vulnerability of hippocampal CA1 neurons. *left panel* - Electron micrographs of cryosections of cell bodies in the CA3 and CA1 regions of an organotypic hippocampal slice culture, rapidly frozen under ischemia-mimicking conditions (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.

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 sec 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 **Figure 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 (**Figures 2-3**).

Despite numerous successful applications, it is clear that EMPA 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 $\sim 4\times$ better than EDX, and EELS hardware and software are now practically mature for biologists. (See references^{6,2} 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 128 x 128 calcium map can be recorded in ~ 1 hr¹. 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.

Disclosures

The authors report no conflicts of interest.

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