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Quantitative EFTEM mapping of near physiological calcium concentrations in biological specimens

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Abstract

Although electron energy-loss spectroscopy (EELS) in the scanning transmission electron microscope (STEM) provides high sensitivity for measuring the important element, calcium, in biological specimens, the technique has been difficult to apply routinely, because of long acquisition times required. Here we describe a refinement of the complementary analytical technique of energy-filtered transmission electron microscopy (EFTEM), which enables rapid imaging of large cellular regions and measurement of calcium concentrations approaching physiological levels. Extraction of precise quantitative information is possible by averaging large numbers of pixels that are contained in organelles of interest. We employ a modified two-window approach in which the behavior of the background signal in the EELS spectrum can be modeled as a function of specimen thickness t expressed in terms of the inelastic mean free path λ . By acquiring pairs of images, one above and one below the Ca $L_{2,3}$ edge, together with zero-loss and unfiltered images, which are used to determine a relative thickness (t/λ) map, it is possible to correct the Ca $L_{2,3}$ signal for plural scattering. We have evaluated the detection limits of this technique by considering several sources of systematic errors and applied this method to determine mitochondrial total calcium concentrations in freeze-dried cryosections of rapidly frozen stimulated neurons. By analyzing $0.1 \mu\text{m}^2$ areas of specimen regions that do not contain calcium, it was found that the standard deviation in the measurement of Ca concentrations was about 20 mmol/kg dry weight, corresponding to a Ca:C atomic fraction of approximately 2×10^{-4} . Calcium concentrations in peripheral mitochondria of recently depolarized, and therefore stimulated and Ca loaded, frog sympathetic neurons were in reasonable agreement with previous data.

Keywords

Energy-filtered transmission electron; microscopy (EFTEM); Electron energy-loss spectroscopy (EELS); Plural scattering; Elemental mapping; Calcium; Detection limits

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1. Introduction

Calcium is a biologically important ion that serves as an intracellular messenger for a wide variety of cellular functions [1-3]. In particular, two organelle networks—the mitochondria and endoplasmic reticulum—strongly regulate cytoplasmic calcium levels and play major roles in normal physiological as well as pathophysiological processes within cells [4,5]. Quantitative mapping of calcium distributions in these cellular organelles can help to elucidate the mechanisms of calcium regulation. Whereas most biologically relevant elements are generally present at the $10\text{--}10^3$ mmol/kg level and are therefore reasonably easy to measure, physiological Ca concentrations ($[\text{Ca}]$) are typically in the range of 1–10 mmol Ca/kg dry weight [6] and the element distributions are often heterogeneous. Consequently, detection and quantitation of calcium are challenging. In addition, considering the limitations due to sample preparation techniques [7,8] and electron beam damage, it is preferred to visualize the Ca distribution over a large specimen area, thus generating more information without jeopardizing sensitivity.

Mapping the distribution of calcium at ~ 10 nm spatial resolution in biological specimens is typically achieved using energy-dispersive X-ray spectroscopy (EDXS) [9,10] or electron energy-loss spectroscopy (EELS) in a scanning transmission electron microscope (STEM) [11]. Previously, EDXS has been successfully employed to measure Ca distributions in freeze-dried cryosections, but it is tedious to obtain high spatial resolution (<50 nm) together with high sensitivity since the time required to detect 2 mmol/kg concentrations of Ca is on the order of 100 s for a typical probe current of 5 nA into a 100 nm spot [12]. The STEM/EELS spectrum imaging technique, based on the efficient collection of inelastically scattered electrons [13, 14], in which one or more multichannel spectra are collected at each point in an array of pixels, has been shown to be effective in measuring low levels of calcium in biological cryosections [11,15]. Maps obtained by spectrum imaging can be used to determine concentrations of Ca in specific organelles at the level of parts per million with a significant improvement of sensitivity relative to EDXS. However, even though this technique has proven to be very successful at measuring low $[\text{Ca}]$, the data collection itself takes a relatively long time, ~ 0.5 h, in order to obtain the information from a sample area of less than a $0.5\text{ }\mu\text{m}^2$, and requires a high electron dose of 10^9 e/nm². Therefore, it is beneficial to develop a technique, in which the acquisition of data takes a much shorter time, with a smaller electron dose, and provides information from a larger sample area.

Here we refine a complementary approach to map calcium using energy-filtered transmission electron microscopy (EFTEM) [16,17]. EFTEM is now widely applied to materials science [13,18,19], as well as certain biological systems, e.g., imaging subcellular phosphorus distributions [20-24]. Advances in EFTEM, which provides inelastic images through a broad beam illumination of the specimen, have resulted in improvements in the sensitivity and spatial resolution of elemental mapping. Nevertheless, despite advances in CCD cameras [25], energy filter design [26,27] and flexible data acquisition schemes [28,29], it is still difficult to match the near single atom sensitivity of STEM/EELS. In many biological applications the limiting factor is not the minimum detectable number of atoms but rather the minimum detectable atomic fraction of an element within a specimen region. It is therefore reasonable to consider whether EFTEM mapping can be used to detect low elemental concentrations by averaging or summing large numbers of pixels. For such an averaging scheme to produce reliable results, it is crucial to eliminate thickness-dependent systematic errors in the subtraction of the spectral background from the weak core edge signal.

In biological specimens the calcium $L_{2,3}$ signal in the energy-loss spectrum at around 350 eV constitutes a very small fraction of the background intensity, although the edge has a very distinctive peak at the threshold energy (Fig. 1). Therefore, special attention must be given to

subtracting the signal from the background intensity [30]. Furthermore, it is well known that the shape of the energy-loss spectrum changes with specimen thickness due to plural inelastic scattering and it is essential to take this effect into account when mapping elements that occur at low concentrations in specimens of varying thickness [31-33].

To test the feasibility of EFTEM detection of physiological levels of calcium, we have analyzed freeze-dried cryosections from two complementary preparations of rapidly frozen neurons attached to carbon-coated polymer support films. To correct for thickness changes, we have employed a modified two-window approach [20], in which the behavior of the EELS background is modeled as a function specimen thickness. By acquiring images above and below the Ca L_{2,3} edge, together with zero-loss and unfiltered images, which provide the corresponding thickness maps, we can correct for plural scattering in calcium elemental maps. By eliminating several sources of systematic error and by averaging over ~10⁴ pixel, we show that it is possible to detect concentrations of ~20 mmol Ca/kg dry weight, which is a level of sensitivity easily suitable for studies of calcium-driven disease and injury processes. In the future, we believe that it might be possible to reduce errors by another order of magnitude, thus bringing the technique within the physiological calcium concentration range of most biological systems.

2. Effect of plural scattering and theoretical detection limits

2.1. Plural scattering and thickness correction

The simplest approach for subtracting the background intensity in EFTEM elemental maps [29,34] is ratio imaging, in which the post-edge intensity, I_{post} , is divided by the pre-edge intensity, I_{pre} . Provided the shape of the background remains constant over the field of interest, the net so-called “two-window” core edge signal is given by

$$S(\Delta, \beta) = I_{post} - k_0 I_{pre}, \quad (1)$$

where Δ is the energy width of the integration window above the core edge and β is the collection semi-angle of the scattered electrons accepted by the imaging filter, as defined by the objective aperture, and k_0 is the ratio of I_{post}/I_{pre} for a specimen in which the element is known to be absent.

When the thickness or composition of the specimen varies over the field of interest this two-window technique becomes invalid. In our previous work [20] we have demonstrated that it is preferable to retain a modified two-window approach, in which we can take into account subtle changes in the background shape as a function of the sample thickness t in units of the total mean free path λ for inelastic scattering, where

$$\frac{t}{\lambda} = \ln \left(\frac{I_{unfilt}}{I_0} \right), \quad (2)$$

where I_{unfilt} is the total unfiltered intensity and I_0 is the zero-loss intensity [29]. We can model the contribution of plural inelastic scattering, which is expressed as a Poisson distribution of multiple scattering events [35-37] by the following expression:

$$I(E) = I_0(E) * \left[\delta(E) + \left(\frac{t}{\lambda} \right) Q(E) + \left(\frac{1}{2!} \right) \left(\frac{t}{\lambda} \right)^2 Q(E) * Q(E) + \dots \right], \quad (3)$$

where $I_0(E)$ is the zero-loss peak intensity, $Q(E)$ is the single scattering distribution normalized to unity over all energy losses, $\delta(E)$ is the delta-function, and the asterisk denotes convolution.

Eq. (3) can be rewritten to obtain the single scattering distribution:

$$Q(E) = (t/\lambda)^{-1} FT^{-1} \left[\ln \left\{ \frac{\widehat{I}(E)}{\widehat{I}_0(E)} \right\} \right], \quad (4)$$

where $\widehat{}$ represents a forward Fourier transform and FT^{-1} an inverse Fourier transform.

Using Eq. (4), we first obtain single scattering distributions for evaporated carbon and cryosection support film by recording spectra from specimen areas of uniform thickness (Fig. 2a). The differences between the two distributions give rise to small but significant differences in the behavior of the k_0 ratios as a function of t/λ .

From the single scattering distribution $Q(E)$, we can calculate the electron energy-loss spectra for different t/λ values, from which it can be observed that the background intensity increases nonlinearly as a function of t/λ , corresponding to the multiple scattering terms in Eq. (3) (Fig. 2b). By integrating the spectral intensities over an appropriate window of 10 eV above and below the Ca $L_{2,3}$ edge at 335 and 348 eV, respectively (Fig. 1), the k_0 ratio as a function of t/λ is calculated.

In Fig. 3 it is seen that the calculated values of k_0 remain relatively constant for both support films until the t/λ value reaches ~ 0.4 , at which point the k_0 values increase as higher order plural scattering terms dominate (Eq. (3)). Since the single scattering distributions for two materials are slightly different, the k_0 curve for carbon support film is 12% steeper above $t/\lambda = 0.35$. To correct for plural scattering in Ca EFTEM mapping, we parameterize the k_0 curves with polynomials, which enables us to calculate thickness-corrected k_0 maps from the measured t/λ images. The behavior of the k_0 curves and their shape differences were discussed in our previous study describing EFTEM mapping of phosphorus [20], in which the physiological atomic fraction of P:C was on the order of 1%. However, when imaging the much lower concentrations of Ca, one must be attentive not only to correct the thickness variations but also to choose the correct k_0 curve.

It is useful to express the concentration of calcium in biological specimens in units of mmol/kg dry weight of the organic matrix. Assuming that the composition in the specimen is $\sim 55\%$ carbon by weight [15] and that negligible carbon is lost during irradiation, the atomic ratio of calcium to carbon $N_{Ca}:N_C$ can be converted to the mmol Ca/kg dry weight. The number of atoms of an element, N , per unit area measured in an energy band Δ inside a collection semi-angle β , can be determined from the net signal $S(\Delta, \beta)$ in Eq. (1) [36-38]:

$$N = \frac{S(\Delta, \beta)}{I_0(\Delta, \beta) \sigma(\Delta, \beta)}. \quad (5)$$

Here, $\sigma(\Delta, \beta)$ is the inelastic scattering cross-section for the core level excitation for the energy band Δ and collection semi-angle β and $I_0(\Delta, \beta)$ is the measured intensity in the transmitted electron beam, including the zero-loss peak for the same energy band and collection angle. We determine $\sigma(\Delta, \beta)$ by means of a spectrum acquired from a dried 1% CaCl solution deposited onto a thin carbon film, and by using EELS reference data from Digital Micrograph (Gatan, Inc., Warrendale, PA). Provided that the specimen thickness is less than the inelastic mean free path λ and that the energy window Δ is less than about 20 eV, most of the low-loss intensity is

contained in the zero-loss peak, in which case $I_0(\Delta, \beta)$ can be approximated by the zero-loss intensity I_0 .

The expression for the calcium concentration [Ca] in the units of mmol/kg dry weight can be written as

$$[\text{Ca}] = 4.5 \times 10^4 \frac{N_{\text{Ca}}}{N_{\text{C}}} \eta, \quad (6)$$

where

$$N_{\text{Ca}} = \frac{I_{\text{post}} - k_0(t/\lambda) I_{\text{pre}}}{I_0(\Delta, \beta) \sigma_{\text{Ca}}(\Delta, \beta)},$$

$$N_{\text{C}} = \frac{I_{\text{pre}}}{I_0(\Delta, \beta) \sigma_{\text{C}}(\Delta, \beta)} \times \frac{1}{f},$$

$$f = \frac{I_{\text{pre}}(t/\lambda) / I_{290}(t/\lambda)}{\lim_{t/\lambda \rightarrow 0} I_{\text{pre}}(t/\lambda) / I_{290}(t/\lambda)},$$

and η is fractional mass loss of carbon due to specimen irradiation.

Since the calcium pre-edge intensity corresponds to the tail of the carbon K edge, the calcium pre-edge intensity can be used to estimate the number of carbon atoms provided that the appropriate cross-section and a plural scattering correction factor are included. The plural scattering correction factor f is found by calculating the ratio of I_{pre} to the integrated background subtracted carbon edge intensity at energy loss 290 ± 5 eV as a function of t/λ , and then by dividing this result by the same ratio in the limit of $t/\lambda = 0$. The calculated correction factor is then parameterized as a third-order polynomial in t/λ as shown in Fig. 4. Without the correction factor the number of carbon atoms will be overestimated and thus the calcium concentration will be underestimated.

2.2. Systematic errors

The quantitative scheme for measuring calcium concentrations formulated in Eq. (6) takes account of subtle changes in spectral background shape due to plural scattering, which are incorporated into the t/λ dependence of the k_0 curve. This can be considered as a correction of first-order systematic errors in the standard two-window EFTEM imaging, which does not include treatment of plural scattering effects.

However, there are some additional sources of second-order systematic errors that are more difficult to correct. The error in the calcium signal arising from an error in k_0 is given by

$$\delta S_{\text{sys}}(\Delta, \beta) = \frac{k_0 - k'_0}{k_0} I_{\text{pre}}, \quad (7)$$

where I_{pre} , k_0 , and k_0' are all functions of t/λ , and k_0' represents an errant k_0 value caused by a discrepant matrix material, a shift in energy loss during acquisition of the filtered images, or non-isochromaticity of the imaging filter.

To estimate the Ca detection limits in units of mmol Ca/kg dry weight Eq. (7) is combined with Eq. (6) to obtain

$$\delta[\text{Ca}]_{\text{sys}} \approx 4.5 \times 10^4 \left(\frac{k_0 - k_0'}{k_0} \right) \left(\frac{\sigma_c}{\sigma_{\text{Ca}}} \right) f\eta. \quad (8)$$

We first estimate the error in [Ca] due to neglect of the t/λ dependence of k_0 . The systematic errors in [Ca] as a function of t/λ are displayed in Fig. 5a for 10 constant values of k_0' ranging from 0.85 to 0.94. As discussed in our previous work [20], some of the k_0' values fall outside the range predicted by the k_0 curve in Fig. 3. In short, the optimal k_0' value depends on the thickness range and is determined practically by offsetting the intensity to zero in regions of the specimen that are known not to contain Ca. For example, a k_0' value of 0.85 gives a relatively flat concentration error curve for the t/λ range of 0.02–0.2 resulting in an offset of around 260 mmol Ca/kg dry weight. Also, it is known that the experimentally measured k_0 values obtained from post-edge and pre-edge images can be slightly different from the ones calculated from the modeled spectra due to the electron optical considerations. A slightly different focusing action of the condenser lens due to offset of the microscope accelerating voltage results in a change of spot size with the energy-loss offset and this alters the incident electron flux and displaces the k_0 curve vertically.

A second source of systematic error arises from uncertainty in the shape of the k_0 curve due to variations in the matrix material. Fig. 5b shows the behavior of the systematic error in calcium concentration corresponding to the differences between the two k_0 curves in Fig. 3, which result from using the k_0 curve for carbon to correct the spectral background for the cryosection polymer support film. Beyond $t/\lambda > 0.3$, the error increases monotonically with thickness.

A third contribution to the generation of systematic error results from energy-loss instability or misalignment of the imaging filter. A lack of filter isochromaticity or shifts in energy loss by a few electron volts will give rise to changes in the k_0 curve and thus produce errors in the calcium concentration. To evaluate the error due to an energy shift we model k_0 curves for which we applied an energy shift of several electron volts in the Ca pre-edge and/or post-edge. Fig. 5c shows the behavior of the maximum error as a function of thickness that would occur when the pre-edge image has an energy shift of +2 eV and post-edge image a shift of -2 eV. The initial height of the curve is somewhat arbitrary, as it corresponds to the thickness where [Ca] is zero.

To approximate the detection limits for low calcium concentrations one needs to consider carefully different sources of systematic error. For example, in the thickness regime of $0.2 \leq t/\lambda \leq 0.5$, the systematic error can range from 50 to 200 mmol Ca/kg dry weight due to the uncertainty in the k_0 value (Table 1).

2.3. Statistical errors

Next we consider the statistical errors in the two-window method caused by the finite number of electrons incident on the specimen. We have shown that it is possible to correct for systematic errors in samples as thick as one inelastic mean free path, but we need to take into account the increased statistical error in the extracted core edge signal due to the nonlinear increase in background underlying the core edge. The estimated variance of the signal, $\text{var}(S)$, is given by the sum of the variances of the post-edge intensity, $\text{var}(I_{\text{post}})$, and the variance of the pre-edge

intensity, $\text{var}(k_0 I_{pre})$, and as previously derived [20]. The statistical error in the number of atoms per unit specimen area is then given by

$$\delta N_{stat} \approx \frac{\sqrt{k_0 (1+k_0) I_{pre}}}{I_0 \sigma(\Delta, \beta)}. \quad (9)$$

In the units of mmol Ca/kg dry weight this expression can be written as

$$\begin{aligned} \delta \left(\frac{N_{Ca}}{N_C} \right)_{stat} &= \frac{N_C \delta(N_{Ca}) - N_{Ca} \delta(N_C)}{N_C^2} \approx \frac{\delta(N_{Ca})_{stat}}{N_C}, \\ \delta[Ca]_{stat} &\approx 4.5 \times 10^4 \sqrt{\frac{k(1+k)}{I_{pre}}} \left(\frac{\sigma_C(\Delta, \beta)}{\sigma_{Ca}(\Delta, \beta)} \right) f \eta. \end{aligned} \quad (9)$$

The statistical error is plotted logarithmically as a function of t/λ for different numbers of incident electrons in Fig. 6, from which it is evident that as the number of incident electrons I_0 increases the statistical error decreases with the inverse square root of I_0 . Also it can be seen that the statistical error is relatively constant over a large thickness range and gradually increases for $t/\lambda > 1$.

For thin specimens of relative thickness $t/\lambda = 0.2$ and for a dose of 10^7 incident electrons per nm^2 , Eq. (10) predicts a standard deviation of about 130 mmol Ca/kg dry weight.

3. Experimental methods

For acquisition of reference and single scattering spectra, carbon films were deposited on freshly cleaved mica using an Auto 306 evaporator (Edwards Inc., Tewksbury, MA), floated onto water, and picked up on lacy carbon films supported on 400 mesh copper grids (EM Sciences, Hatfield, PA). For CaCl_2 films, a drop of 1% CaCl_2 solution was adsorbed on to the carbon films after they had been glow discharged in air at a pressure of ~500 mtorr. Cultured rat hippocampal neurons, grown on cover slips for 10–17 days *in vitro*, were plunge frozen [8]; primary frog sympathetic neurons maintained in culture as dispersed ganglia were slam frozen against a liquid nitrogen-cooled metal mirror by means of a LifeCell CF-100 freezing machine [7]. Cryosections were prepared using standard procedures [7,8]. In brief, neurons were sectioned (Leica Ultracut S/FCS cryoultramicrotome; Leica, Deerfield, IL) at a temperature of -160°C to a nominal thickness of 80–100 nm (compression effectively doubles the actual thickness), and adhered to carbon/pioloform-coated grids. Grids containing frozen-hydrated sections were cryotransferred into a EM912 Omega electron microscope (Zeiss SMT, Peabody, MA), where they were freeze-dried and imaged. The sections were subsequently stabilized by evaporating a ~5 nm carbon film on both sides of the cryosection. Two complementary neuronal preparations were examined. The first sample consisted of cultured hippocampal neurons that were exposed to lethal concentrations (100 μM for ca. 20 min) of *N*-methyl-D-aspartic acid (NMDA), which induces massive Ca loading, primarily seen as large immobilized deposits of calcium within swollen mitochondria, and leads to excitotoxic cell death. The second sample consisted of frog sympathetic neurons that had undergone quasi-physiological stimulation by membrane depolarization (50 mM K^+ for 2 min, followed by a 5-min recovery period), thus increasing mitochondrial calcium concentrations to high but not pathological levels.

EEL spectra and energy-filtered images were obtained using a Tecnai TF30 transmission electron microscope (FEI Inc., Eindhoven, The Netherlands) operating at an accelerating voltage of 300 kV and equipped with a Tridiem post-column imaging filter (Gatan Inc., Pleasanton, CA) [26]. A 40- μm diameter objective aperture was used for EFTEM imaging and

the output of the 2048×2048 pixel ultrascan cooled CCD array detector was binned to 512×512 pixel to improve the signal-to-noise ratio. EELS data were recorded at 300 kV using an energy dispersion of 0.2 eV. Four spectral segments (zero-loss, plasmon, and two core-loss regions) were acquired each with four different readout times in order to optimize the signal-to-noise ratio. These spectral segments were later spliced together and used to obtain the single scattering distribution for carbon and cryosection polymer support film. Unfiltered and zero-loss (± 5 eV) images were acquired, and t/λ maps were determined by means of Eq. (2) [29]. Energy-selected images were acquired in the vicinity of the Ca $L_{2,3}$ edge using a 10-eV slit width with a pre-edge window at 335 eV and a post-edge window at 348 eV (Fig. 1). The readout time for each energy-selected image was 16–32 s and each image was summed over four readouts. Calcium ratio maps and k_0 maps were obtained by applying the two-window ratio method, as described. For all data acquisition either in spectroscopy or in imaging mode, it was critical for the dark current to be subtracted properly, for the CCD gain to be normalized, and for the Tridiem or GIF100 filter to be tuned to provide an isochromaticity of less than 1 eV. To obtain accurate k_0 and t/λ maps, it was also important to calibrate and position the energy-selecting slide accurately. Images and spectra were acquired and processed by means of Digital Micrograph Software (Gatan Inc., Pleasanton, CA). Specimen drift occurring between successive acquisitions was corrected by manual alignment of the images.

Spectra were spliced, calibrated, and deconvoluted using a computer program written in Matlab (Mathworks Inc., Natick, MA). Around 10–15 spectra were acquired and analyzed from each of the section support material.

Spectral imaging data were acquired using a STEM (VG Microscopes HB501) operating at 100 kV. The sample stage was cooled to -160 °C to minimize contamination and to reduce radiation damage. EEL spectrum images in the range of 250–550 eV were acquired from a small region of approximately 50×50 pixel, 0.2 s/pixel, with a parallel detection EELS (Gatan Model 776 UHV Enfina) using an energy dispersion of 0.3 eV/channel. During acquisition of the spectrum images, annular dark-field STEM images were collected at the end of each scan line and used for drift correction by cross-correlating successive images. The ratio of the Ca:C signal was calculated by subtracting the extrapolated background in the spectra using the “spectrum-imaging analysis” routine in Digital Micro-graph Software.

4. Results and discussion

To validate our quantification and thickness correction scheme we used, as a null control, a multilayered pure evaporated carbon film and measured the calcium concentration [Ca] in units of mmol/kg. The acquired unfiltered and zero-loss images of a specimen region containing 2, 3, and 4 layers of carbon film were used to determine the t/λ map (Fig. 7a), which gave a thickness variation from 0.2 to 0.7 inelastic mean free paths. The thickness map, together with the energy-selected images recorded at energy losses of 348 ± 5 and 335 ± 5 eV, corresponding to the post-edge and pre-edge windows for the Ca $L_{2,3}$ excitation, were used in Eq. (6) to compute [Ca] at each pixel. In Figs. 7b and c we compare two methods of determining [Ca]. The horizontal line profiles were obtained by averaging over 40 pixel in the vertical direction to reduce statistical noise. In Fig. 7b, [Ca] was calculated by using a single k_0 value of 0.85, which corresponds to the observed k_0 value in the control region of the filtered images. It is evident that the thickness effects in [Ca] map are corrected only in the region from which the k_0 value was obtained. The fact that the observed k_0 value is lower than the expected value, indicated by the carbon k_0 curve in Fig. 3, can be explained by variations in condenser illumination when the energy-loss offset is changed [20]. To correct for plural scattering effects across the full range of thicknesses, it is necessary to apply the thickness-dependent k_0 -ratio method that we have reported earlier for quantitative mapping of phosphorus [20]. The calcium concentration calculated using k_0 -curve correction is shown in Fig. 7c. As seen from the

horizontal line profile across the film, fluctuations in [Ca] are about ± 150 mmol Ca/kg. Although on a single-pixel scale this error corresponds to very high (non-physiological) concentrations of calcium, averaging over larger regions greatly improves the detection limits, as illustrated in Fig. 8. The same layered film of pure carbon is used to generate histograms of measured calcium concentrations to establish the detection limits of our technique. When individual pixels are analyzed as shown in Fig. 8a, a Gaussian peak with standard deviation of 275 mmol Ca/kg centered at zero Ca concentration is obtained. As the averaged area increases to 100×100 pixel (Figs. 8b–d), the distributions become narrower, as expected on the basis of the statistical errors.

Next we apply the thickness-dependent k_0 -ratio correction scheme to map the distribution of calcium in cryosection of dissociated cultured hippocampal neurons that had been subjected to an excitotoxic concentration of NMDA. The general structural features of this model of excitotoxic injury are illustrated in Fig. 9a, which shows a zero-loss image recorded at an incident beam energy of 100 kV. Visible in Fig. 9a are cross-sections of two dendrites (D) containing damaged, often swollen, Ca-loaded mitochondria, glial processes (G) with normal mitochondria, and an artifactually thick band of material across the top of the field. Figs. 9b and c show unfiltered and zero-loss images recorded at an incident beam energy of 300 kV, which give the t/λ map in Fig. 9d with thickness varying from 0.5 to 1.4 inelastic mean free paths. Unlike in sections of plastic embedded cells, in freeze-dried cryosections the thickness variations between adjacent pixels can be large, due to the large differences in water content between organelles, cytoplasm and extracellular space and due to segregation or movement of organic components during freeze-drying. This is mainly due to the fact that when the specimen is freeze-dried, the spaces between organelles previously occupied by ice become empty. To demonstrate the effectiveness of our thickness correction scheme, the specimen was tilted to an angle of 50° relative to the beam direction to achieve larger thickness variations. Figs. 9e and f show the Ca $L_{2,3}$ post-edge and pre-edge images. When a constant value of k_0 is adopted it is evident that the thickness variations give artifactual features in the calcium image, which are reflected in the k ratio map displayed in Fig. 9g. The calculated carbon and calcium maps are presented in Figs. 9h–j in units of atoms/nm², together with horizontal line profiles obtained by averaging over 10 pixel in the vertical direction. The carbon map in Fig. 9h was calculated using the f factor, which corrects for plural scattering effects, and then the signal due to the underlying carbon film was subtracted. The calcium map in Fig. 9i was obtained using an optimized single k_0 value of 0.886. As evident in the line profile, the map reveals spurious Ca intensities that can be traced to the thickness variations in the cryosection. After applying the thickness-dependent k_0 -curve corrections these artifacts in Ca map are eliminated as shown in Fig. 9j. As expected, calcium is now only evident in the mitochondria, which are known to accumulate a massive amount of the element under the pathological conditions of the preparation procedure. Inspection of the uncorrected and corrected Ca map line profiles in Figs. 9i and j reveals that not only the thickness artifacts are removed but also that the apparent Ca content itself is significantly reduced.

To determine the mitochondrial calcium content in units of mmol/kg, four calcium-containing features in Fig. 9j were selected and outlined. There is considerable uncertainty in η , the fractional mass loss of carbon due to electron irradiation, but based on our previous experience for very high exposures, we adopt a value of $\eta = 0.5$ [9,11]. An error in η will affect the absolute but not the relative concentrations. To assess the detection limits and accuracy of the calcium measurements, tissue features that contain carbon but not calcium were also evaluated (e.g., region 5 outlined in Fig. 9j). From these results, which are summarized in Table 2, the standard deviation (σ) for five [Ca] measurements in cytoplasm is 46 mmol/kg.

Next we analyzed a more physiological neuronal preparation, in which intramitochondrial calcium concentrations were significantly lower. Fig. 10 shows the EFTEM mapping of a

region of the cell body of a freeze-dried cryosection of a rapidly frozen frog sympathetic neuron that had undergone quasi-physiological stimulation by depolarizing the membrane (2 min exposure to 50 mM K^+ , followed by 5 min of repolarization) to elevate intracellular calcium levels. Fig. 10a illustrates the cellular context of the mapped area, which is just beneath the plasma membrane and therefore subject to the largest cytosolic calcium transients [7]. The calculated carbon and calcium maps are in units of atoms/nm² and are shown together with horizontal line profiles, averaged over 10 pixels in the vertical direction. In this specimen, the relative thickness, t/λ , varied from 0.25 to 0.9 mean free paths. As in the previous analysis in Fig. 9, we compare two calculated calcium distributions, with and without the thickness corrections applied. In Fig. 10b the carbon map was calculated using the f factor to correct for plural scattering effects, and the background due to the underlying carbon film was subtracted. In Fig. 10c the Ca map obtained using a single optimized k_0 value of 0.855 reveals thickness artifacts that are evident in the horizontal line profile across the image. After applying the k_0 -curve correction (Fig. 10d) thickness artifacts are nearly eliminated and Ca is found only within mitochondria. The seven mitochondria outlined in the image were used to calculate the calcium concentration. In addition, seven areas of cytoplasm that did not contain calcium were analyzed. The results from both mitochondrial and cytoplasmic regions are summarized in Table 3.

The standard deviation of the cytoplasmic [Ca] in Table 3 is 36 mmol/kg. Since cells in their normal physiological states typically contain at least a factor of 10 lower concentrations of calcium, compared to those shown in Table 3, it becomes even more important to correct for thickness variations.

To take full advantage of the EFTEM calcium mapping technique, Fig. 8 shows that is essential to average over an extended specimen area rather than consider individual pixel values. Detection limits of around 200–300 mmol Ca/kg/pixel make it impossible to measure physiological levels of calcium within a single pixel. However, as demonstrated in Fig. 8, averaging larger regions that correspond to cellular organelles such as mitochondria allow precision of the calcium measurement to approach physiological concentrations. Currently, we can detect ~20 mmol/kg, or an atomic fraction of Ca:C of order 2×10^{-4} .

It is interesting to compare our approach of quantitative EFTEM with STEM/EELS analysis. The latter technique provides a complete spectrum at each pixel, and is the method of choice for analyzing small specimen regions. However, in many biological applications it is important to analyze large fields of view rapidly, which is difficult to achieve with current STEM/EELS setups. From the spectral image data acquired with STEM/EELS, it is possible to extract precisely the elemental signal by multiple linear least square fitting (MLLS) of reference spectra [11,39,40]. For our present purpose we can consider the STEM/EELS analysis to provide an accurate measure of the calcium concentration. To assess the accuracy of calcium quantitation in EFTEM, we have analyzed sub-regions of the same cells using STEM/EELS. Fig. 11 shows regions (2 and 6) from the specimen presented in Fig. 10. STEM/EELS spectrum imaging was performed in a dedicated VG Microscopes HB501 STEM at an accelerating voltage of 100 kV, as described previously [11]. The Ca signal was extracted by MLLS fitting of reference spectra, which included the Ca $L_{2,3}$ edge and the C K edge, as well as two plural scattering terms consisting of the C K edge convoluted with 1 plasmon and 2 plasmons, respectively, to take into account changes in background shape as a function of specimen thickness (Fig. 11). Also shown in Fig. 11 are spectra from pixels summed over two areas: one from a Ca-rich inclusion, and the other from a mitochondrion (area labeled 2) occupying ~2500 pixel. The shaded region indicates the Ca $L_{2,3}$ edge signal corresponding to the intensity in the post-edge image of the EFTEM data in Fig. 10 recorded at an accelerating voltage of 300 kV. The spectrum on the right shows a strong Ca $L_{2,3}$ edge, which is easily distinguished from the background, whereas the Ca signal in the second spectrum on the left is barely visible. As expected, the calcium signal-to-noise ratio from a single pixel in the Ca STEM/EELS spectrum

image is much higher than from a single pixel in the background-subtracted EFTEM image. However, it is possible to improve the analytical sensitivity for measuring calcium from EFTEM images by averaging over many pixels, e.g., 2500 pixel for the regions in Fig. 11. Due to residual systematic errors described in Section 2.2, we do not yet have perfect quantitative agreement between the STEM/EELS and the EFTEM data but the analyses agree to within an uncertainty of ~20 mmol/kg. It is still necessary to develop methods for correcting those discrepancies.

In the future by removing other sources of systematic errors, particularly through reduction of energy-loss drift and improved filter isochromaticity, it seems possible that detection limits can be reduced by another order of magnitude. It might also be necessary to assess subtle effects of matrix composition and radiation damage on the EEL spectral shape. If such additional refinements can be made, EFTEM calcium mapping could become a useful technique for mapping physiological calcium levels. Even at the level of sensitivity demonstrated here, EFTEM mapping should be valuable for studying mechanisms of Ca-dependent neuronal damage, which is highly relevant to ischemic injuries.

5. Conclusions

We have developed a refinement to the EFTEM technique that enables quantitative mapping of calcium concentrations in biological structures at near physiological levels. The accuracy of this method depends on correction of systematic errors. It is possible to correct for thickness artifacts in the calcium images by modeling small changes in the ratio of post-edge to pre-edge intensities as a function of specimen thickness. We have demonstrated application of the technique for mapping calcium in cryosections of rapidly frozen neurons. By averaging image intensities over $\sim 10^4$ pixels, it is possible to detect calcium concentrations of ~20 mmol Ca/kg, corresponding to about 200 atomic parts-per-million. It is anticipated that our correction scheme can be refined even further to improve detection limits by correcting for subtle energy drifts and non-isochromaticity patterns resulting from the instability of the energy filter. The EFTEM technique complements STEM/EELS for quantitative analysis of calcium and other elements distributed over large areas of cryosectioned cells.

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References

1. Berridge MJ, Bootman MD, Roderick HL. Nat. Rev. Mol. Cell Biol 2003;4:517. [PubMed: 12838335]
2. Giorgi C, Romagnoli A, Pinton P, Rizzuto R. Curr. Mol. Med 2008;8:119. [PubMed: 18336292]
3. Rizzuto R, Pozzan T. Physiol. Rev 2006;86:369. [PubMed: 16371601]
4. Friel DD. Cell Calcium 2000;28:307. [PubMed: 11115370]
5. Nicholls DG. Cell Calcium 2005;38:311. [PubMed: 16087232]
6. Somlyo AP, Bond M, Somlyo AV. Nature 1985;314:622. [PubMed: 3990795]
7. Pivovarova NB, Hongpaisan J, Andrews SB, Friel DD. J. Neurosci 1999;19:6372. [PubMed: 10414966]
8. Pivovarova NB, Nguyen HV, Winters CA, Brantner CA, Smith CL, Andrews SB. J. Neurosci 2004;24:5611. [PubMed: 15201334]
9. Andrews SB, Pivovarova NB, Leapman RD. Scanning Microsc 1999;13:147.
10. Shuman H, Somlyo AV, Somlyo AP. Ultramicroscopy 1976;1:317. [PubMed: 1028201]
11. Leapman RD, Hunt JA, Buchanan RA, Andrews SB. Ultramicroscopy 1993;49:225. [PubMed: 8475601]

12. Leapman RD, Andrews SB. *J. Microsc* 1991;161:3. [PubMed: 2016736]
13. Reimer, L. *Energy-Filtering Transmission Electron Microscopy*. Springer; Berlin: 1995.
14. Egerton RF. *Micron* 2003;34:127. [PubMed: 12895484]
15. Leapman RD. *J. Microsc* 2003;210:5. [PubMed: 12694411]
16. Arsenault AL, Ottensmeyer FP. *Proc. Natl. Acad. Sci* 1983;80:1322. [PubMed: 6572390]
17. Arsenault AL, Ottensmeyer FP. *J. Cell Biol* 1984;98:911. [PubMed: 6699092]
18. Hofer F, Grogger W, Kothleitner G, Warbichler P. *Ultramicroscopy* 1997;67:83.
19. Midgley PA, Weyland M. *Ultramicroscopy* 2003;96:413. [PubMed: 12871805]
20. Aronova MA, Kim YC, Zhang G, Leapman RD. *Ultramicroscopy* 2007;107:232. [PubMed: 16979822]
21. Aronova MA, Kim YC, Harmon R, Sousa AA, Zhang G, Leapman RD. *J. Struct. Biol* 2007;160:35. [PubMed: 17693097]
22. Bazett-Jones DP, Leblanc B, Herfort M, Moss T. *Science* 1994;264:1134. [PubMed: 8178172]
23. Bazett-Jones DP, Hendzel MJ, Krulak MJ. *Micron* 1999;30:157.
24. Ottensmeyer FP. *J. Ultrastruct. Res* 1984;88:121. [PubMed: 6400028]
25. Gubbens AJ, Brink HA, Kundmann MK, Friedman SL, Krivanek OL. *Micron* 1998;29:81.
26. Krivanek OL, Friedman SL, Gubbens AJ, Kraus B. *Ultramicroscopy* 1995;59:267. [PubMed: 7571120]
27. Brink HA, Barfels MMG, Burgner RP, Edwards BN. *Ultramicroscopy* 2003;96:367. [PubMed: 12871802]
28. Hunt JA, Kothleitner G, Harmon R. *Microsc. Microanal* 1999;5(Suppl 2):616.
29. Hofer, F.; Warbichler, P. *Transmission Electron Energy Loss Spectrometry in Materials Science and the EELS Atlas*. Vol. second ed.. Ahn, C., editor. Wiley-VCH; Berlin: 2004. p. 159Chapter 6
30. Leapman RD, Rizzo NW. *Ultramicroscopy* 1999;78:251. [PubMed: 10389278]
31. Leapman RD. *Ann. NY Acad. Sci* 1986;483:326. [PubMed: 3551725]
32. Leapman RD, Ornberg RL. *Ultramicroscopy* 1988;24:251. [PubMed: 3281358]
33. Richter K, Haking A, Troester H, Spiess E, Spring H, Probst W, Schultz TP, Witz J, Trendelenburg M. *Micron* 1997;28:407. [PubMed: 9519469]
34. Leapman RD, Jarnik M, Steven AC. *J. Struct. Biol* 1997;120:168. [PubMed: 9417981]
35. Batson PE, Silcox J. *Phys. Rev. B* 1983;27:5224.
36. Egerton, RF. *Electron Energy Loss Spectroscopy*. Vol. second ed.. Plenum Press; New York: 1996.
37. Leapman, RD. *Transmission Electron Energy Loss Spectrometry in Materials Science and the EELS Atlas*. Vol. second ed.. Ahn, C., editor. Wiley-VCH; Berlin: 2004. p. 49Chapter 3
38. Egerton RF. *Ultramicroscopy* 1978;3:243. [PubMed: 695137]
39. Ho R, Feng J, Somlyo AP, Shao Z. *J. Microsc* 2000;197:46. [PubMed: 10620147]
40. Leapman RD, Swyt CR. *Ultramicroscopy* 1988;26:393. [PubMed: 3238813]

Fig. 1. Schematic diagram of an EEL spectrum from a specimen containing 1% calcium in a carbon matrix, showing Ca L_{2,3} pre-edge and post-edge energy windows at 335±5 and 348±5 eV, respectively. The Ca L_{2,3} pre-edge intensity (I_{pre}) and the post-edge intensity (I_{post}) are used to determine the calcium signal by means of the two-window method.

Fig. 2.

EEL spectra from polymer support film for different relative sample thickness t/λ . (a) Experimental spectrum (dashed line) from a film with relative thickness $t/\lambda \sim 0.4$, and single-scattering spectrum (solid line) averaged over many specimen regions. Single-scattering spectrum near the Ca L_{2,3} edge (inset). (b) Reconvoluted modeled spectra as a function of sample thickness for t/λ range of 0.2-2. The inset shows the nonlinear increase in the background intensity due to plural scattering in the vicinity of Ca L_{2,3} edge.

Fig. 3.

Behavior of $k_0 = I_{post}/I_{pre}$ as function of relative sample thickness t/λ for carbon film (solid) and cryosection polymer support film (dashed), calculated by applying Eqs. (3) and (4) to measured spectra. Each curve is parameterized by a polynomial function, from which a thickness-dependent k_0 map can be computed at each pixel. For carbon $k_0 = -0.016 (t/\lambda)^5 + 0.106 (t/\lambda)^4 - 0.286 (t/\lambda)^3 + 0.383 (t/\lambda)^2 - 0.126 (t/\lambda) + 0.886$, and for polymer support film $k_0 = -0.010 (t/\lambda)^5 + 0.067 (t/\lambda)^4 - 0.189 (t/\lambda)^3 + 0.271 (t/\lambda)^2 - 0.089 (t/\lambda) + 0.882$.

Fig. 4.

Carbon plural scattering correction factor f as a function of t/λ . Correction factor is calculated from modeled spectrum by taking ratio of integrated intensity at 335 ± 5 eV to background-subtracted carbon intensity at 290 ± 5 eV, and dividing the result by the ratio of intensities in the limit $t/\lambda = 0$. Without this correction the number of C atoms is overestimated and the Ca concentration is thus underestimated.

Fig. 5. Magnitude of three different sources of systematic error in the calcium concentration, $\delta[\text{Ca}]_{\text{syst}}$ in the units of mmol Ca/kg, as a function of relative specimen thickness t/λ . (a) Error from using a single k_0 value in the range 0.85–0.94 with increments of 0.01. If the error curve is relatively flat over a given range of t/λ , then it is possible to use a single value of k_0 together with an offset to obtain reasonably accurate elemental maps. However, if the error curve is changing over a particular range of t/λ , then it is necessary to use the k_0 -curve correction. (b) Error corresponding to the differences in 2 k_0 curves in Fig. 3, when estimating the calcium concentration by using the k_0 curve for carbon to correct the background due to the polymer support film. (c) Error due to energy-loss instability of the imaging filter, for a relative energy-

loss shift of 2 eV from Ca $L_{2,3}$ pre-edge and post-edge windows. Similar errors occur across the image due to non-isochromaticity of the imaging filter.

Fig. 6.

Logarithmic plot of magnitude of statistical error (one standard deviation) in calcium concentration in units of mmol Ca/kg, as a function of t/λ . Curves are shown for an accelerating voltage of 300 kV for four different numbers of incident electrons (10^5 , 10^6 , 10^7 , and 10^8).

Fig. 7.

Illustration of correction scheme applied to layered evaporated film of pure carbon containing no calcium. The horizontal line profiles below each image are averaged over 40 pixel in the vertical direction. (a) t/λ map calculated from unfiltered and zero-loss images, which gave a thickness variation from 0.2 to 0.7 inelastic mean free paths as seen from the line profile below. (b) Calculated calcium concentration in units of mmol/kg using a single k_0 value of 0.85 ([Ca] uncorrected). (c) Calculated calcium concentration obtained by using k_0 curve ([Ca] corrected). Apparent changes in calcium concentration in the line profiles across the different layer thicknesses in (b) are artifacts due to thickness-dependent change in the background. Application of the k_0 -curve correction in (c) eliminates the thickness artifacts across all carbon layers, leaving only narrow residual features at the boundaries between layers, which are attributed to slight misalignment between the core-edge images and the low-loss images. Fluctuations in calcium concentration are ± 150 mmol/kg along 1 horizontal pixel averaged over 40 vertical pixels.

Fig. 8.

Histograms of measured calcium concentrations obtained from the layered film of pure carbon shown in Fig. 7. (a) Analysis of individual pixels in image gives a Gaussian peak with standard deviation of 275 mmol Ca/kg centered at a value of zero by applying a small offset to the k_0 curve (see text). Analyses of (b) 20×20 pixel, (c) 50×50 pixel, (d) and 100×100 pixel regions of the multilayer carbon film yield narrower Gaussians. The standard deviation of the measurements in (d) is 20 mmol Ca/kg, corresponding to an atomic fraction of Ca:C of approximately 2×10^{-4} .

Fig. 9.

EFTEM mapping of a freeze-dried cryosection prepared from a culture of dissociated hippocampal neurons exposed to an excitotoxic concentration of NMDA (100 μM for ca. 20 min). (a) “Low-dose” zero-loss image at 100 kV prior to mapping. Field illustrates the biological context of the experiment, including cross-sections of dendrites (D) containing Ca-loaded mitochondria, some unswollen (arrowheads) and some severely swollen (arrow), and glial processes (G), which do not express NMDA receptor and contain only normal mitochondria (arrowhead). (b) Unfiltered image and (c) zero-loss image at 300 kV in the EFTEM, and (d) calculated t/λ map showing thickness variation from 0.5 to 1.4 inelastic mean free paths; for the purpose of demonstration, the specimen has been tilted to an angle of 50° to provide a larger thickness variation in the beam direction. (e) Corresponding Ca L_{2,3} post-edge image, (f) pre-edge image, and (g) k_0 -ratio image. (h) Calculated carbon image, (i) uncorrected calcium image, and (j) thickness-corrected calcium image. Line profiles below the

elemental maps show calcium distributions averaged over 10 pixels in the vertical direction in units of atoms/nm². The f correction factor was applied to the carbon map to correct for plural scattering effects as described in the text. In (i) the Ca map obtained using a single k_0 value of 0.886 reveals spurious intensities that follow the thickness variations in the cryosection. (j) Application of the k_0 -curve correction eliminates the thickness-dependent artifacts and contrast is only seen in those mitochondria that contain punctate, extremely calcium-rich inclusions; selected calcium-containing features, and a region with thickness artifacts removed are outlined and used to calculate concentrations in mmol/kg (Table 2).

Fig. 10.

EFTEM mapping of freeze-dried cryosection of rapidly frozen neuron from frog sympathetic ganglion. (a) “Low-dose” zero-loss image at 100 kV prior to mapping. Field illustrates target region of the cell body (rectangle), which includes a mitochondria-rich region lying 2–4 mm under the plasma membrane (arrow). (b) Carbon map with correction for plural scattering, (c) uncorrected calcium map obtained using a k_0 value of 0.855, and (d) k_0 -curve-corrected calcium map, with their respective horizontal line profiles averaged over 10 pixels in the vertical direction in units of atoms/nm². The relative thickness t/λ across the image varied from 0.25 to 0.9. Mitochondria in these neurons contain much less calcium than in NMDA-stimulated hippocampal neurons (Fig. 9), which makes the thickness correction scheme even more important. The outlined mitochondria in the image are used to calculate Ca concentrations in mmol/kg presented in Table 3.

Fig. 11.

Calculated Ca signal using MLLS fitting from EELS spectrum image recorded in dedicated STEM at an accelerating voltage of 100 kV, and with a pixel size of 3.7 nm. Indicated regions 2 and 6 are from same specimen shown in Fig. 10. The STEM/EELS spectra summed over regions indicated by rectangles are shown below the image. In each spectrum the shaded area indicates Ca $L_{2,3}$ post-edge intensity of the EFTEM image recorded at 300 kV accelerating voltage. STEM-EELS shown on the right is summed over a 100 pixel region containing a calcium-rich deposit. Spectrum on the left summed over a 2500 pixel region containing a mitochondrion shows a barely visible Ca $L_{2,3}$ edge signal.

Table 1Systematic errors in [Ca] in a sample with $0.2 < t/\lambda < 0.5$.

Sources of error	$\delta[\text{Ca}]$ (mmol/kg)
$k_0' = 0.87$	210
k curve error	53
eV shift	115

Table 2

Quantitative analysis of Ca and C maps from hippocampal neuron in the areas of mitochondria and cytoplasm.

	# of pixels, pixel size 9.8nm	C (atoms/nm ²)×10 ³	Ca (atoms/nm ²)	Estimated [Ca] (mmol/kg)
<i>Mitochondria spot #</i>				
1	328	9.69	993	2307
2	649	9.29	405	982
3	474	8.88	622	1578
4	369	8.32	310	839
5	3383	4.77	290	1369
<i>Cytoplasm area #</i>				
6	472	7.5	−17.58	−52.5
7	1767	14.04	−7.68	−12.5
8	3811	5.32	−4.92	−21
9	2793	6.44	19.89	69.5
10	3975	3.12	3.19	23

Cytoplasm areas 8–10 are not shown in Fig. 9.

Table 3

Quantitative analysis of Ca and C maps from cryosectioned frog sympathetic neuron in areas of mitochondria and cytoplasm.

	# of pixels, pixel size 3.9nm	C (atoms/nm ²)×10 ³	Ca (atoms/nm ²)	Estimated [Ca] (mmol/kg)
<i>Mitochondria spot #</i>				
1	1745	6.63	52.17	177
2	2408	5.55	43.32	176
3	1877	5.91	60.36	250
4	2255	5.63	48.15	193
5	5610	3.87	32.46	189
6	4121	5.55	43.18	175
7	1446	8.51	109.74	143
<i>Cytoplasm area #</i>				
8	3657	2.70	6.12	38
9	1627	3.72	0.80	11
10	3773	2.45	6.93	64
11	3115	1.82	−4.04	−50
12	8771	1.92	2.26	27
13	4773	2.10	−0.32	−3.5
14	3725	1.17	1.41	27

Cytoplasm areas 8–14 are not shown in Fig. 10.