

Fine structural detection of calcium ions by photoconversion

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Abstract

We propose a tool for a rapid high-resolution detection of calcium ions which can be used in parallel with other techniques. We have applied a new approach by photo-oxidation of diaminobenzidine in presence of the emission of an excited fluorochrome specific for calcium detection. This method combines the selectivity of available fluorophores to the high spatial resolution offered by transmission electron microscopy to detect fluorescing molecules even when present in low amounts in membrane-bounded organelles. We show in this paper that Mag-Fura 2 photoconversion *via* diaminobenzidine oxidation is an efficient way for localizing Ca²⁺ ions at electron microscopy level, is easily carried out and reproducible, and can be obtained on a good amount of cells, since the exposure in our conditions is not limited to the direct irradiation of the sample *via* an objective but obtained with a germicide lamp. The end product is sufficiently electron dense to be detected clearly when present in sufficient amount within a membrane boundary.

Introduction

The ultrastructural localization of a molecule requires that a certain degree of electron-density, resulting in adequate electron scattering, be present in sufficient amount. Binding of heavy atoms can be accomplished easily with regards to proteins, nucleic acids and other cellular components, in some cases with a high degree of specificity.¹ However, the sub-cellular localization of ions is a challenge, since in many cases ions are diffusible and not

easily kept in place after the procedures of sample preparation for electron microscopy (EM). Many ions can offer enough scattering in spite of not being heavy metals, such as manganese, while others necessitate of blocking or precipitating techniques to be visualized. In particular, calcium ions are easily diffusible inside the cell, and even when protein-bound are not easily detectable. The pioneering paper by Tandler *et al.*² demonstrated the presence of calcium ions by EM *via* pyroantimonate precipitation, with a good yield of the final product. The end product of the reaction, however, is probably overrated, since the technique is capable of stabilizing and precipitating calcium and other bivalent cations, such as magnesium, as proved by electron spectroscopic imaging (ESI) analysis.³

Since their introduction by Tsien and coworkers,⁴ calcium studies have used specific Ca²⁺-sensitive fluorochromes, such as Fura-2, Indo-1 and Fluo-4, coupled to wide-field digitized video fluorescence microfluorimetry and confocal microscopy to image acute elevations in intracellular Ca²⁺ concentration ([Ca²⁺]_i) with high spatio-temporal resolution.^{5,6} Cytosolic and nuclear Ca²⁺ signals regulate a multitude of vital cellular processes, such as gene transcription, cell cycle progression, cytoskeletal and chromatin remodelling, and migration.⁷⁻⁹ However, proper Ca²⁺ handling within the endoplasmic reticulum (ER), the most abundant intracellular Ca²⁺ reservoir,¹⁰ is also crucial for cell-fate decisions. The steady-state ER Ca²⁺ level ([Ca²⁺]_{ER}) does not only control synthesis, folding, export and trafficking of proteins,^{10,11} but it also fuels mitochondrial bioenergetics thereby suppressing autophagy.¹²

Low affinity Ca²⁺ indicators, such as Mag-Fura 2, and genetically-encoded probes, such as the bioluminescent protein aequorin and the GFP-based fluorophores camgaros, pericams, and cameleons, have been devised to assess ER Ca²⁺ levels.^{13,14} Nevertheless, these sophisticated tools do not provide any reliable information on Ca²⁺ distribution within the heterogeneous structure of the ER. We propose in this paper a tool for a rapid high-resolution detection of calcium ions which can be used in parallel with other techniques. We have applied a new approach to the detection of intracellular calcium ions. Maranto¹⁵ and Sandell and Masland¹⁶ have proposed the possibility of photoconverting fluorochromes into an electron-dense end product. The technique utilizes the photo-oxidation of diaminobenzidine (DAB) in presence of the emission of an excited fluorochrome, and it has been shown to be efficient on different dyes and conditions.^{17,18} This method combines the specificity of available fluorophores to the high spatial resolution offered by transmission EM to detect fluorescing molecules

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even when present in low amounts in membrane-bounded organelles. The present manuscript sought to exploit DAB photo-oxidation of Mag-Fura 2 for visualizing ER Ca²⁺ distribution in HeLa cells, which are among the most widespread model cell lines used for studying intracellular Ca²⁺ signaling.

Materials and Methods

Cell culture

HeLa cells have been cultured in Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 2% L-Glutamine, 1% penicillin and streptomycin, at 37°C air atmosphere with 5% CO₂. After reaching 90% confluence, cells have been plated in 6 wells cell culture plate (1x10⁵ cells per well), or in chamber slides for staining and microscopic examination (8x10³ cells per well). The day after seeding, when cells reached about 90% confluence, the medium has been removed from the wells and replaced with three solutions, one for each experimental condition. The solutions, different in Ca²⁺ concentration, are the following: physiological salt solution (PSS), with the following composition: 150 mM NaCl, 6 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 10 mM Glucose, and 10 mM HEPES; Ca²⁺-free solution (0 Ca²⁺), where Ca²⁺ has been substituted by 2 mM NaCl, and 0.5 mM EGTA was added, whereas the solution with Ca²⁺ more concentrated is similar to PSS in composition except for 120 mM NaCl and 20 mM CaCl₂. All the solutions have been brought to pH 7.4 with an osmolality of 338 mmol/kg, measured with an osmometer (Wescor 5500; Wescor, Inc., Logan, UT, USA).

Intracellular Ca²⁺ labeling and diaminobenzidine-photoconversion

Intracellular Ca²⁺ deposits have been marked by fluorescent dye-based calcium indicator Mag-Fura-2, acetoxymethyl (AM) ester UV light-excitable (Life Technologies, Carlsbad, CA, USA), added to the three calcium solutions to obtain a final concentration of 5 μM. Cells have thus been incubated at 37°C in the dark, for 1 h. Cells cultured on chamber slides, and treated as hereto described, have been analyzed at fluorescence microscopy. An Olympus BX51 microscope (Olympus Italia Srl, Segrate, Italy) was used under the following conditions: 100-W Hg lamp as the excitation source, 330- to 385-nm excitation filter (excf), 400-nm dichroic mirror (dm), and 420-nm barrier filter (bf); 100× Olympus UplanFl oil-immersion objective lens (NA 1.25). Images were recorded with an Olympus Magnifier digital camera system, and stored by the Olympus CellF software.

Instead, cells in plates have been fixed at room temperature for 20 minutes with a fixative solution (2% paraformaldehyde and 1% glutaraldehyde in PBS). Meanwhile, a solution with DAB has been freshly prepared as follows: 20 mg of DAB (Sigma Aldrich, St. Louis, MO, USA) were dissolved in 1 ml DMSO, briefly vortexed and then brought to 30 mL with PBS. Thereafter, cells have been washed twice with PBS and incubated for 3 h under a germicidal UV lamp (Sankyo Denki, Kanagawa, Japan; G30T8, UV-C) with 2 mL DAB solution which is replaced with fresh solution every half an hour.

In parallel, some cells were incubated in the absence of Mag-Fura 2, treated with DAB and processed as above. Moreover, to exclude a possible interference by aldehyde groups, some slides were fixed and then treated with 0.5M NH₄Cl in PBS for 30 min at 4°C, then incubated with DAB and processed as above.

Transmission electron microscopy

Fixed and photoconverted cells have been washed twice with distilled water and postfixed with 2% osmium tetroxide in water for 1 h, at room temperature. Once the osmium was removed and the cells rinsed several times with distilled water, cells have been scraped, centrifuged discarding supernatant, dehydrated in a graded series of ethanol and embedded in LRWhite resin (Sigma Aldrich). Ninety nm ultrathin sections have been cut by a Reichert ultramicrotome, transferred to grids without membrane and visualized by a Zeiss EM900 transmission electron microscope operating at 80kV.

Results

Calcium labeling with Mag-Fura 2 results

in highly fluorescent staining mainly localized in the cytoplasm. After incubation of the cells with 20 mM Ca²⁺ for 1 h, the signal is present in large vesicles in the cytoplasm and around the cell nucleus, as well as in elongated structures probably related to mitochondria (Figure 1a). The faint signal visible in the nucleus is very probably due to the superposition of the cytoplasmic layer surrounding it. When the cells are incubated in PSS, the signal is lower (Figure 1b) and mostly present in the perinuclear vesicles, while the cytoplasmic signal far from the nucleus somehow disappears. In the last condition, without calcium and in the presence of EGTA, some labeling is still visible, although to a much reduced level (Figure 1c). This continuous presence of Ca²⁺ represents very probably the last ions the cell cannot allow to lose, a sort of surviving threshold concentrations. Supplementary Figure 1 confirms that altering extracellular Ca²⁺ concentrations results in a consequent change in ER Ca²⁺ homeostasis, which may have important consequences also on cytosolic Ca²⁺ dynamics. At EM level, the controls did not show any electron dense precipitates, both in the absence of the fluorochrome or after aldehyde blockade (*not shown*); consequently, the end product obtained in the Mag-Fura 2 stained cells is due to the fluorochrome photoconversion. The DAB photoconversion is an efficient way to stabilize the ions and to visualize them. It must be underlined here that we observe a thin section, 60-80 nm thick, corresponding, consequently, only to a fraction of the fluorescent signal.

After PSS incubation, the cells show a distinctive positivity (Figure 2a). In some cases it is possible to see the presence of precipitates without the direct association with smooth or rough ER (SER or RER, respectively) membranes, likely related to unbound Ca²⁺ ions (Figure 2b), and electron dense material inside the vesicles is also positive (Figure 2c). A possible suggestion of Ca²⁺ internalization or extrusion is shown in Figure 2 d,e, where the deposits are present on the outer membrane. When the cells are incubated without calcium and in the presence of EGTA, the deposits are extremely reduced, although still present on the outer cell membrane, suggesting the presence of an extruding mechanism (Figure 2f). On the contrary, the diffuse, low density end product is almost absent from the cytoplasm.

At higher Ca²⁺ concentration in the medium (Figure 3a), the dark precipitate is detectable on the inner membrane of cytoplasmic vesicles possibly pertaining to SER, and in discrete foci with no apparent association with vesicles or membranes. The deposits are present within electron dense vesicles (Figure 3b) and electron lucent structure, in some cases associated, and suggesting the idea of pas-

sage of ions from one compartment to another. In other cases, DAB-positive material is present on the membranes of complex systems such as multivesicular bodies (Figure 3c) or on the outer membranes of cytosolic vesicles (Figure 3d). Interestingly, calcium deposits are detectable on the outer cell membrane (Figure 3e) in spots, localized and separated, suggesting the presence of calcium

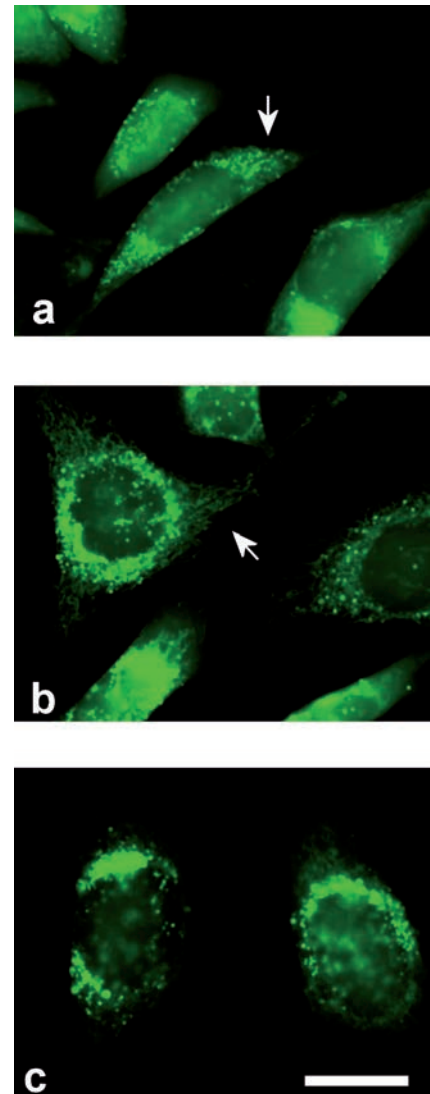


Figure 1. Detection of Ca²⁺ in fluorescence after Mag-Fura 2 staining. a) High Ca²⁺ concentration; the labeling is present in the cytoplasm within vesicles as well as in filamentous structures (arrow) probably related to ER. b) Normal Ca²⁺ concentration; the vesicles are more evident near the nuclear envelope, while the cytoplasm is less labeled with the exception of the filamentous structures (arrow). c) When Ca²⁺ is omitted from the medium, only the perinuclear vesicles are labeled. Scale bars: 10 μm.

channels. In Figure 3f, another example of surface labelling of calcium ions is presented. The end product of the DAB photoconversion is a speckled, dense precipitate of varying size. However, another abundant end product is present as small sized dots, showing a markedly lower electron density (Figure 3f, inset) and a widespread distribution.

Discussion

We show in this paper that Mag-Fura 2 photoconversion *via* DAB oxidation is an efficient

way for localizing Ca^{2+} ions at EM level. Moreover, this could be considered also as a means of stabilizing their presence, thus limiting the loss of ions. As for the method, photoconversion is easily carried out and reproducible, can be obtained on a good amount of cells, since the exposition in our conditions is not limited to the direct irradiation of the sample *via* an objective but obtained with a germicide lamp.

The end product is sufficiently electron dense to be detected clearly when present in sufficient amount within a membrane boundary. In these conditions, the blackness of the product stands out from the background. In our

conditions, in fact, Ca^{2+} ions are immediately detectable within the vesicles and the tubules of the SER, while it is much more difficult to see their presence when in the cytosol, due to their low electron density. This is a clear advantage over the conventional imaging of Mag-Fura 2 fluorescence that must be removed by the cytosol in order to study truly ER signals.^{19,20} This is achieved by permeabilizing the plasma membrane with brief applications of antibiotics or detergents, but this procedure may destroy intracellular signaling pathways, dilute cytosolic modulators of ER Ca^{2+} release/sequestration, and disassemble the cytoskeletal elements that maintain the con-

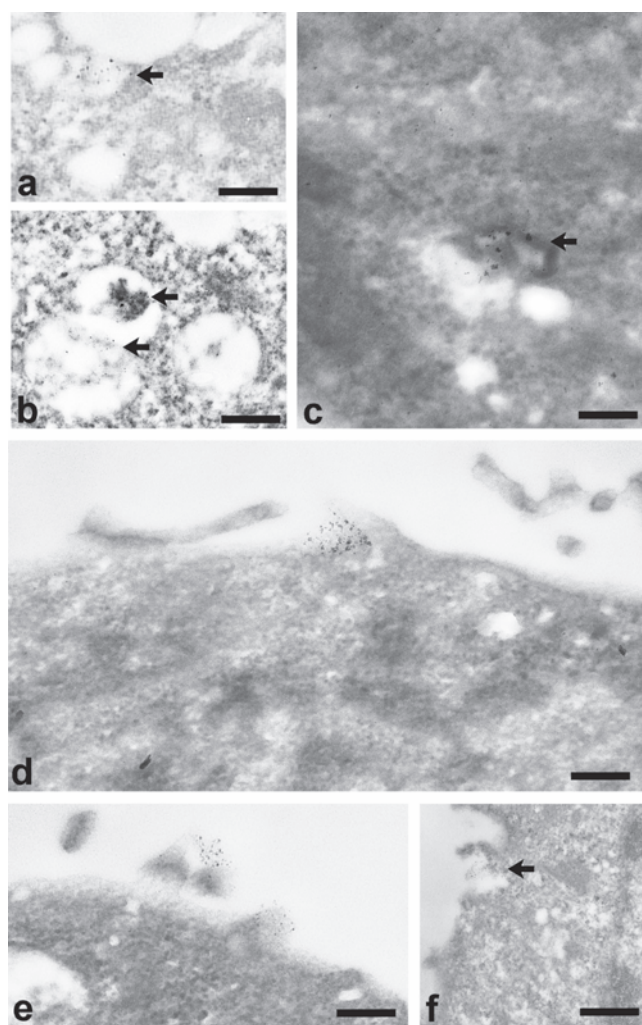


Figure 2. Endoplasmic reticulum photoconversion. When the cell culture medium contains normal Ca^{2+} concentrations, the dense precipitates can be visualized in the cytoplasm (a) in the vicinity of vesicles, or within them (b) or as being released in open structures (c). At the cell surface (d,e) the deposits suggest a process of extrusion/incorporation of Ca^{2+} ions. In (f) very few precipitates are present when Ca^{2+} is not present in the culture medium. Scale bars: a-c) 200 nm; d-e) 100 nm; f) 200 nm.

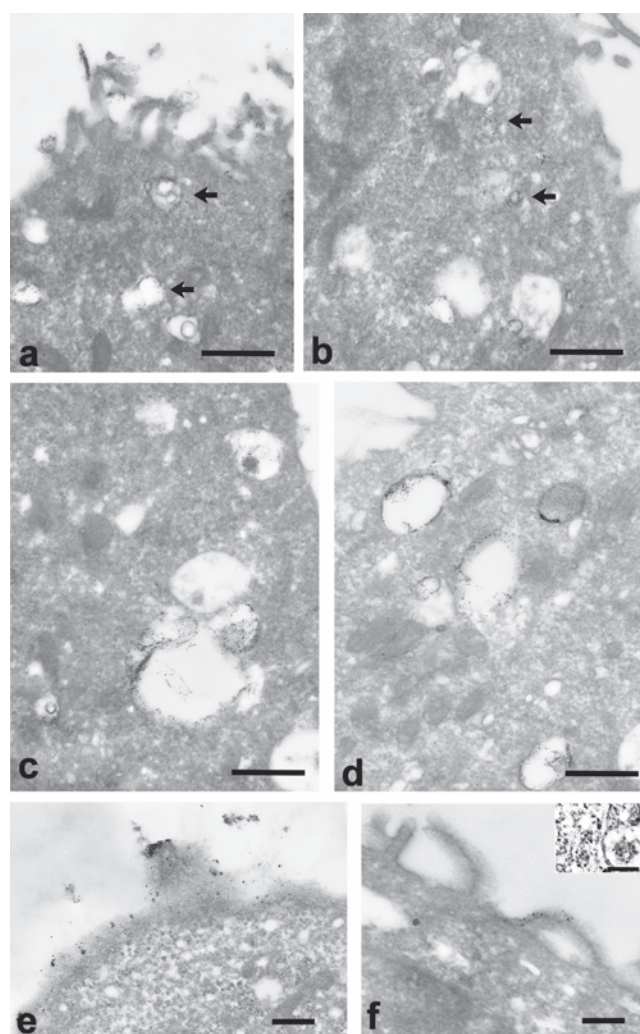


Figure 3. Endoplasmic reticulum photoconversion. At high Ca^{2+} concentration, the end product can be found both in vesicular structures (arrows) as well as on the outer membrane structures (a) or in the cytoplasm nearby vesicles (b, arrows). The end precipitates are also present lining the inner membrane of the ER-derived structures (c, d). Interestingly, the staining is present on the membrane in discrete spots at regular distance (e) or on membrane protrusions (f) as well as diffuse precipitates in the cytoplasm (inset). Scale bars: a-d) 500 nm; e-f) 100 nm; f inset, 100 nm.

tact between cell membrane and ER. Alternatively, the whole-cell configuration of the patch-clamp technique could be exploited to remove the dye, but this experimental approach is rather expensive and challenging, requires highly skilled electrophysiologists and has a quite low throughput. The final yield of photoconverted Mag-Fura 2 is in direct relation with the amount of Ca present in the sample considered. In our condition, the Ca-enriched medium gave a higher end product staining than the normal physiological conditions or than Ca deprivation.

It is interesting that in several cases, dense precipitates could be found in areas suggesting the local accumulation of Ca²⁺ nearby the mouth of plasmalemmal Ca²⁺ channels. Accordingly, such precipitates are evident when extracellular Ca²⁺ concentration is increased, which is consistent with the notion that constitutive Ca²⁺ inflow from the extracellular milieu is enhanced. This unexpected outcome of DAB photooxidation is extremely relevant in light of the master role served by local Ca²⁺ microdomains generated by extracellular Ca²⁺ influx, which regulate cell functions as diverse as cycle progression, proliferation, migration, nitric oxide production, and adenylyl cyclase activity.^{7,21} These findings will be further explored in a work now in progress.

One thing that should be underlined here is that we could not visualize the SER tubule filled with the end product. This could be due to three different causes: i) Ca²⁺ can be dispersed (lost) in the conditions used here; ii) local intraluminal Ca²⁺ concentration is less than expected (which is less probable as it is unlikely to be lower than 100 μM); or iii) DAB photoconversion is not efficient enough for Mag-Fura.

By comparing fluorescence images with EM micrographs, it is clear the difference in the signal coming from vesicles and the elongated filamentous structures, the latter being much less fluorescent.

The relative low yield when comparing fluorescence images is definitely related to the extremely lower amount of end product which can be found within a 60-80 nm thin section in relation to the fluorescence originating from the whole cell. In addition, albeit [Ca²⁺]_{ER} fluctuates between 100 and 500 μM,²⁰ Ca²⁺ accumulates in specific regions of the ER²² that may be missed in our sections. Moreover, releasable Ca²⁺ is actually trapped by oligomers of the Ca²⁺-binding proteins calnexin and calreticulin: these form a concealed Ca²⁺ source that is not in rapid equilibrium with the [Ca²⁺]_{ER}, but is easily accessible to Ca²⁺-releasing channels upon extracellular stimulation.²³ Thus, the lack of SER tubules or cisternae filled with the end prod-

uct of the photo-conversion, *i.e.*, Ca²⁺, is not surprising. Finally, the photoconversion process seems to be efficient, and gives an end product more reasonable than the precipitation technique utilized, for instance, by Tandler *et al.*² In the latter case, an important amount of precipitates were found in the cell and especially in the nucleus; our method gives a much finer end product, and this allows a superior resolution and localization.

In conclusion, this method is a reliable and efficient technique for stabilizing and visualizing Ca²⁺ ions at EM. It gives a high resolution end product thus allowing the fine structural Ca localization. In principle, this approach could provide important qualitative information about the amount and sub-cellular distribution of calcium ions both in cultured cells and, more importantly, in *ex vivo* samples, which are far less amenable to high resolution Ca²⁺ imaging of ER Ca²⁺ levels.

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