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Citation: AIP Conference Proceedings **1365**, 395 (2011); doi: 10.1063/1.3625386

View online: http://dx.doi.org/10.1063/1.3625386

View Table of Contents: http://scitation.aip.org/content/aip/proceeding/aipcp/1365?ver=pdfcov

Published by the AIP Publishing

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Abstract. We present in this paper a novel methodology that combines scanning x-ray fluorescencee microscopy and atomic force microscopy. The combination of these two techniques allows the determination of a concentration map of Mg in whole (not sectioned) cells.

Keywords: magnesium, intracellular imaging, spatial distribution
PACS: 87.64.kd, 87.64.Dz

INTRODUCTION

Magnesium (Mg referring both to ionized and bound cation) is the most abundant divalent cation within cells. It is known to play a crucial structural and regulatory role, including stabilization of membrane bilayers, nucleic acids, and proteins, and modulation of many enzymatic reactions [1]. Despite the enormous amount of data about the biochemistry of Mg, a complete picture of its cellular homeostasis is still lacking.

Experimental evidence suggests that regulation of intracellular magnesium is a finely tuned mechanism controlled not only via Mg influx/efflux through the cell membrane, but also by Mg buffering and compartmentalization in intracellular organelles, such as mitochondria [2]

There is a need for investigative tools to map intracellular Mg in order to elucidate whether its regulatory mechanism stems from mobilization from intracellular stores and/or from the shift between free and bound Mg. Despite recent efforts in applying new live imaging techniques to the field of Mg research [3], an accurate characterization of Mg distribution in the cellular environment is still lacking. Here we used scanning fluorescence x-ray microscopy (SFXM), a highly sensitive method for mapping elements in cells [4]. Accurate mapping of intracellular Mg concentrations by SFXM can be performed on cell sections such as those utilized for electron microscopy. However, measuring a single section does not reveal the distribution of the target element throughout the whole cell. This study addressed the problem of mapping the Mg concentration, and not just the Mg content, in whole cells. Towards this end, we combined SFXM measurements with atomic force microscopy (AFM) measurements on the same cell in order to take into account non-homogeneous thickness of the cell. In this manner, we mapped the distribution of intracellular Mg in dehydrated whole cells and obtained a Mg concentration map.

The 10th International Conference on X-ray Microscopy
AIP Conf. Proc. 1365, 395-398 (2011); doi: 10.1063/1.3625386

EXPERIMENT

Scanning Fluorescence X-ray Microscopy Measurements

SFXM measurements were carried out with the scanning x-ray microscope [5] at the 2-ID-B beamline at the APS [6]. A Fresnel zone plate lens focused a monochromatic 1.5-keV beam to a spot size of about 50 nm onto the sample. The zone plate consists of lithographically fabricated 180-nm-thick gold rings, with an outermost zone width of 30 nm, on a Si_3N_4 membrane. The sample was transversally scanned in the zone plate focus under computer control. At each scan step a full fluorescence spectrum and selected energy regions-of-interest were acquired with an energy-dispersive fluorescence detector (D1), in particular the Mg K line at 1250 eV. Simultaneously the flux transmitted by the sample was measured using a multi-element configured detector (D2). A schematic layout of the experiment is presented in Fig. 1. The sample was slightly tilted (about 10°) with respect to the plane perpendicular to the incoming beam, to improve the fluorescence signal. The fluorescence detector was in the horizontal plane, perpendicular to the incoming beam. The air paths between sample and detectors were filled with flowing He gas, in order to reduce absorption by air. The fluorescence detector, which was oriented in the horizontal plane at about 90° from the incident beam, was an SII model Vortex EX-60 silicon drift diode. X-ray fluorescence spectra from this detector were acquired and digitized by an XIA Saturn DXP multichannel analyzer. The transmitted flux was measured with a custom-built 9-element configured detector [7], which allowed for both absorption and differential phase contrast imaging. The signals from the various detector elements of this system were read out in parallel with charge-sensitive amplifiers and then digitized by computer.

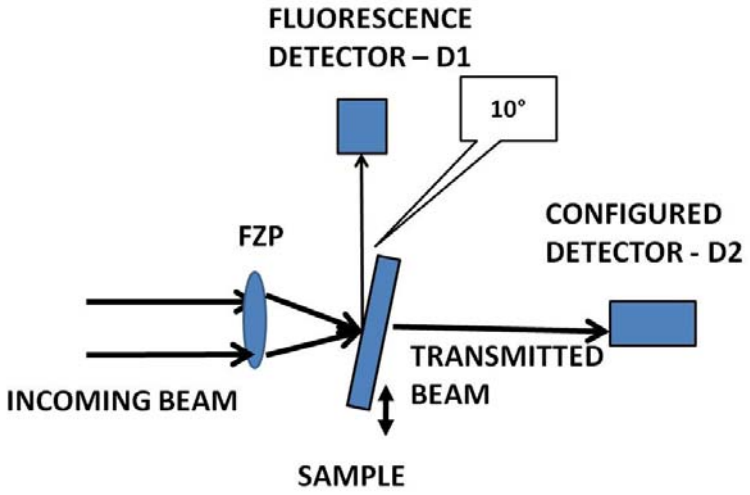


FIGURE 1. Schematic layout of the experiment at 2-ID-B beamline at APS (Argonne). The incoming beam was focused at about 50 nm by a Fresnel zone plate on the sample, which was (x,y) scanned in front of the beam. Fluorescence and transmitted intensity were measured by detectors D1 and D2, respectively. The sample was inclined at about 10° with respect to the plane perpendicular to the incoming beam.

Cell Culture and Fixation

HC11 mouse mammary epithelial cells were cultured in MEM medium (Sigma) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulphate. For the scanning fluorescence x-ray microscopy (SFXM) measurements, cells were plated on 1 × 1 mm^2, 200-nm-thick silicon nitride (Si_3N_4) membrane windows, on a 5 × 5 mm^2 Si frame (Silson, UK), previously sterilized in ethanol. Cells were incubated at 37 °C in 5% CO_2 for at least 24 h before fixation.

At 50-80% confluency, cells were briefly rinsed in 150 mM KCl, fixed in ice-cold methanol/acetone 1:1, and air dried.

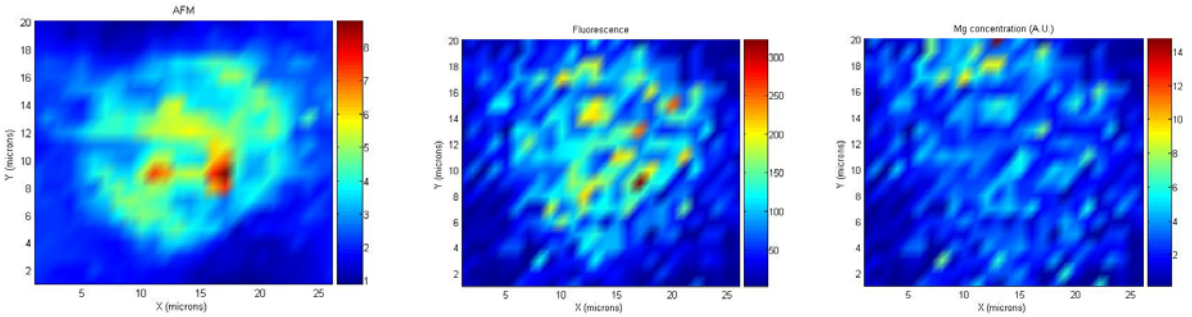
Atomic Force Microscopy Measurements

AFM topography analysis was performed with a Digital Instruments D3100 AFM equipped with a Nanoscope IIIa controller, operating in air. The AFM worked in Tapping Mode at a resonance frequency of about 260 kHz. Commercial monolithic silicon tips were used, with an apex curvature radius in the 5- to 10-nm range and a typical force constant of ~40 N m-1.

The typical square scan size was about 35 μm. Images were collected without any real-time filtering or flattening, and were post-processed using the WSxM 5.0 software.

RESULTS

Figure 2(a) shows the AFM measurements of a typical HC11 cell. Both the x-y and z scales are expressed in microns. This figure shows three peaks in thickness (two of them more pronounced).



(a) (b) (c)

FIGURE 2. (a) Atomic force microscopy topographic map of a typical HC11 cell. The x-y and z scales are in microns. (b) Mg fluorescence intensity map. (c) Mg concentration map obtained normalizing the Mg fluorescence intensity by the AFM measurements.

Figure 2(b) shows the fluorescence map intensity on the same cell shown in Fig. 2(a). An enhancement of intensity in correspondence of the maximum cell thickness can be observed. However, the photons produced in the fluorescence event suffer from the absorption in the cell itself (the so-called self-absorption effect). Because the thickness of the sample is quite inhomogeneous, the self absorption can modify significantly the fluorescence map. To take into consideration this possible source of error, we developed an algorithm that calculates, for each pixel, the path through the cell, and hence the absorption for the given geometrical arrangement (in particular the orientation of the sample with respect to the fluorescence detector). In this particular case the correction did not give significant differences with respect to the fluorescence intensity map.

To obtain the Mg concentration, the fluorescence map—corrected for self absorption—must be normalized by the thickness measured by AFM. Very careful registration procedures between the fluorescence map and the thickness map must be carried out before normalization. To this purpose, MATLAB subroutines have been used. Figure 2(c) shows the result of thickness normalization, which provides a Mg concentration map. As can be seen, the concentration map is quite different from the fluorescence intensity map: indeed the fluorescence intensity is higher in the nucleus region, where the thickness is higher; instead the concentration shows a quite homogeneous distribution through the cell, with a slight enhancement outside the nucleus.

CONCLUSIONS

We here demonstrate that a combination of complementary experimental methodologies, i.e., SFXM and AFM, enables determination of the concentration map of intracellular Mg. Importantly, these data were obtained from a whole cell and not from sections, as with electron microscopy techniques. We showed that the concentration distribution is quite different from the fluorescence distribution, which is directly related to the number of excited atoms. In many biological processes, the local element concentration and not the total element content is the important issue. In this study we used methanol/acetone dehydrated samples; the next step will be to perform experiments in cryo-fixed cells, where subcellular structures are better preserved, and diffusion of elements does not take place. In addition, the procedure for registering the x-ray and AFM measurements can be greatly facilitated, for example, by nano-patterning the sample substrate. We believe that the procedure herein proposed addresses a

general problem related to localization of important constituents and trace elements in subcellular compartments. Thus it can constitute a viable solution to visualize whole cells and to determine elemental concentration with high sensitivity and resolution.

ACKNOWLEDGMENTS

The project PRIN 2007ZT39FN_003 is acknowledged for partial financial support. The CNR-RAS agreement is also gratefully acknowkedged

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