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Absolute zinc quantification at the sub-cellular level by combined use of hard X-ray fluorescence and phase contrast imaging techniques

Ewelina Kosior¹, Sylvain Bohic^{1,2}, Heikki Suhonen¹, Peter Cloetens¹

¹European Synchrotron Radiation Facility, 6 rue Jules Horowitz, 38000 Grenoble, France

²INSERM U-836, Chemin Fortuné Ferrini, 38706 La Tronche, France

E-mail: ewelina.kosior@esrf.fr

Abstract. Hard X-ray fluorescence microscopy and magnified phase contrast imaging are combined to obtain quantitative maps of the projected zinc mass fraction in whole cell of PC12 cell lines. The experiments were performed on freeze dried cells at the nano-imaging station ID22NI of the European Synchrotron Radiation Facility (ESRF). X-ray fluorescence analysis gives the areal mass of most major, minor and trace elements while quantitative phase contrast imaging provides maps of the projected mass. The combined method was validated on calibration samples by comparison with other alternative techniques such as Atomic Force Microscopy (AFM) and Scanning Transmission Ion Microscopy (STIM). Up to now, absolute quantification at the sub-cellular level was impossible using X-ray fluorescence microscopy but can be reached for the first time with the use of the proposed approach.

1. Introduction

Metals present as trace elements in biological systems play an important role in the cell metabolism [1], [2]. They are involved in important biosynthetic pathways including the conversion of electrochemical to chemical energy, the biosynthesis of DNA and an array of important metabolites [3]. The number of studies on trace elements is growing and nowadays their role in human health both in their natural occurrence and via therapeutic drugs is recognized as crucial. Compounds containing metals are used for the diagnoses and treatment of diseases (gadolinium for imaging, platinum-based drugs for chemotherapy [4]) and to develop nanocomposites (for example functionalized titanium or gold nanoparticles) for gene therapy. Quantitative study of the distribution of trace elements at the sub-cellular level [5] provides important information for the study of the functions and pathways of metalloproteins and therapeutic approaches.

The relevant information about trace elements in biological specimens increases with the sampling resolution. To probe the sub-cellular complexity of metal ion homeostasis mechanisms, one requires sub-micrometer resolution together with a sub-femtogram absolute detection limits due to the decreasing quantity of sample probed. X-ray fluorescence (XRF) analysis with a nanoprobe is the most direct and sensitive method to quantify the distribution of metals and other elements at the sub-cellular level [6], [7]. It provides high sensitivity for transition metals and other relevant trace elements together with the capability of penetrating and mapping whole cells.



Most of the structural details of cells are undetectable in hard X-ray microscopy due to the weak absorption contrast between structures with similar transparency. However the various organelles show wide variation in X-ray refractive index. The technique exploiting the refractive index (its real part) is called phase contrast imaging. Methods based on phase contrast provide complementary information about cells by mapping the projection of the electron density hence to a good approximation the projected mass. Information about the projected mass combined with the average density of the sample allows to calculate its thickness [5], value which can be compared with the one obtained through the use of alternative techniques (STIM, AFM).

2. Methods

2.1. Sample preparation

PC12 cells, a clonal catecholaminergic cell line derived from rat pheochromocytoma [8] were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum, penicillin (25 $\mu\text{g}/\text{mL}$) and streptomycin (25 $\mu\text{g}/\text{mL}$). Cultures were maintained according to standard protocols at 37°C in a 95% humidified incubator with 5% CO_2 as already described [9]. The cells were cultured directly on gelatin gel coated thin polycarbonate foil (2 μm thick). These targets were subsequently fastly cryofixed in liquid nitrogen, chilled isopentane (-160°C) and further lyophilized.

2.2. Experimental setup of ID22NI

X-ray fluorescence analysis was performed at the nano-imaging station ID22NI of the ESRF, using the intrinsic monochromaticity of the undulator of about $\delta\lambda/\lambda \simeq 0.015$. The experimental station is located at a distance of 63 m from the undulator source and at 37 m from the high power slits used as secondary source in the horizontal direction (25 μm slit opening). The synchrotron radiation is focused by an X-ray optical device consisting of two elliptically shaped mirrors acting in two orthogonal planes using the so-called Kirkpatrick-Baez geometry [10]. The mirrors are coated with a graded multi-layer. No other monochromator is used in the setup, resulting in a very high and unique X-ray flux (up to 10^{12} photons/s) at energies between 17 and 29 keV. In this work, the energy of the pink photon beam was set to 17.5 keV. For more details regarding the ID22 beamline refer to [11]. For the description of the techniques (X-ray fluorescence and phase contrast imaging) used at this beamline, refer to [5].

3. Analysis

PC12 cells were imaged by synchrotron X-ray fluorescence and X-ray phase contrast imaging. After scanning the sample and fitting the emitted fluorescence spectra, the intracellular distribution of the accessible chemical elements can be determined at a spatial resolution corresponding to the beam size (100 nm) (Fig. 1). By applying a phase retrieval algorithm [12], the relative phase map was generated. The quantitiveness of the phase imaging was validated through a comparison with AFM and STIM, see details in [5]. In order to combine both (fluorescence and phase contrast) images, corrections to the phase map were applied. The background of the phase map was fitted with a polynomial function to correct for low frequency fluctuations. Both images were aligned with a cross correlation technique and distortion corrections were applied to phase map. All the corrections were performed with the open source software ImageJ and under GNU octave code. The result of corrected phase map is presented on the Fig. 1. Once the phase map was corrected both images were divided pixel by pixel (fluorescence map by corrected phase map) in order to obtain the projected zinc mass fraction in the PC12 cell (Fig. 1). This provides truly quantitative information, not biased by changes in the sample thickness.

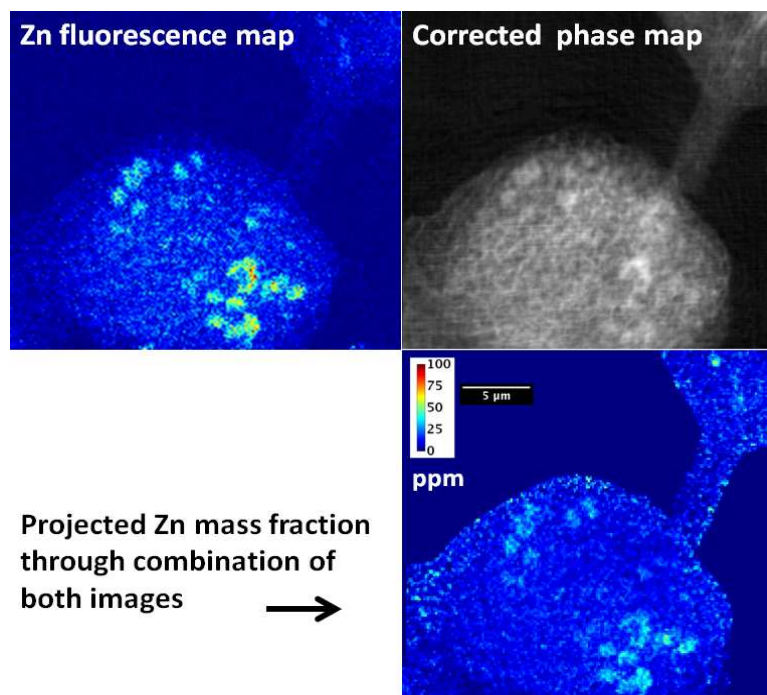


Figure 1. X-ray fluorescence of zinc (upper left) and phase contrast (upper right) image of PC12 cell. Dwell time of scanning was set to 150 ms and step size 150 nm. Both images were divided pixel by pixel in order to access absolute zinc mass fraction (bottom image); the scale is in ppm of dry weight.

4. Discussion

An important application of X-ray fluorescence microscopy is the determination of the distribution of the chemical elements at the sub-cellular level. However, the mapping of true element concentration at the sub-cellular level is a difficult task. Locally, the composition and the density or the thickness of different sub-cellular compartments can vary. To address this problem, X-ray fluorescence sub-cellular mapping and propagation-based X-ray phase contrast imaging for mass normalization were combined, for the first time, to provide high-resolution, truly quantitative, elemental distribution maps in cells [5]. Phase-contrast imaging with a partially coherent beam produces Fresnel interference fringes which makes possible to image samples with small variations in mass density. These variations would be undetectable using absorption contrast imaging.

The projection X-ray microscopy method used in the present work is optimized for relatively high energies (17 keV-29 keV) that match well with efficient excitation of the X-ray fluorescence. As we used a full-field CCD based approach; our method is more dose efficient, provides a larger field of view and is much faster than scanning methods to obtain the phase information (like the one in [13]). For comparison, the phase images are acquired with a few seconds of exposure time and contain 4 million pixels ($1 \mu\text{s}/\text{pixel}$), whereas the fluorescence scanning data requires about 6 hours of exposure time for 100000 pixels ($250 \text{ ms}/\text{pixel}$).

Alternatively scanning differential phase contrast imaging has been performed by measuring the deviation of the beam by the sample with a position sensitive detector [14]. However the results obtained were done at low energy (2.5 keV) that limits the range of elements accessible through X-ray fluorescence. Also Compton to Rayleigh scattering analysis has been used to obtain more accurate X-ray fluorescence quantification in 2D [15] or 3D [16] measurements.

However a limitation in this approach comes from the estimation of the matrix mean atomic number which not necessarily match the sample matrix studied and variation at the sub-cellular scale. In addition the thickness needs to be determined from thickness calibration curves on the basis of the total Compton scatter intensity. On the overall these average values based on Compton signal seem not suited to provide absolute quantification at the sub-cellular level. On the overall this work is encouraging the use of X-ray phase imaging to improve the elemental quantification in 2D and 3D X-ray fluorescence imaging.

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