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Correlating STED and Synchrotron XRF Nano-Imaging to Explore Metal Functions in Synapses

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Abstract. Zinc and copper are involved in neuronal differentiation and synaptic plasticity but the molecular mechanisms behind these processes are still elusive due in part to the difficulty of imaging trace metals together with proteins at the synaptic level. We correlate stimulated-emission-depletion microscopy (STED) of proteins and synchrotron X-ray fluorescence (XRF) imaging of trace metals, both performed with 40 nm spatial resolution, on primary rat hippocampal neurons. Synchrotron XRF was performed on the ID16A Nano-Imaging beamline at the European Synchrotron Radiation Facility. ID16A beamline provides a pink nanoprobe ($\Delta E/E \approx 1\%$) at two discrete energies: $E = 17$ keV and 33.6 keV. For the correlative STED-SXRF experiments 17 keV was chosen, despite a larger focus at lower energy, as it is more efficient in exciting the XRF of the biologically relevant elements zinc and copper. The X-ray focus with dimensions of 35 nm (H) x 57 nm (V) provided a flux of $3.7 \cdot 10^{11}$ ph/s. Thanks to this experimental setup, we reveal the co-localization at the nanoscale of zinc and tubulin in dendrites with a molecular ratio of about one zinc atom per tubulin- $\alpha\beta$ dimer. We observe the co-segregation of copper and F-actin within the nano-architecture of dendritic protrusions. These results indicate new functions for zinc and copper in the modulation of the synaptic cytoskeleton morphology, a mechanism associated to structural plasticity. From a methodological perspective, the combination of STED super-resolution microscopy and nano-SXRF imaging stands as a solid new tool for the identification of metalloproteins directly in cells by correlating cellular imaging methods at a supramolecular scale.

INTRODUCTION

Understanding the neurological functions of essential trace elements such as copper (Cu) and zinc (Zn) requires to correlate their localization with respect to relevant proteins in neurons. Synchrotron X-Ray Fluorescence (SXRF) imaging using hard X-ray beams, typically above 10 keV energy, is a powerful technique to investigate the cellular localization of metals since it allows the mapping of element distributions in single cells with high analytical sensitivity¹. Using Kirkpatrick–Baez (KB) focusing mirrors, SXRF has reached a spatial resolution of 13 nm on ID16A beamline at the European Synchrotron Radiation Facility (ESRF), while maintaining a high photon flux as required for detecting trace elements². We have previously reported a correlative microscopy approach consisting in labeling organelles or proteins with specific fluorophores for live-cell imaging prior to SXRF imaging³⁻⁴. This correlative approach is limited by the spatial resolution of optical fluorescence microscopy, above 200 nm, which is larger than the spatial resolution achieved today with nano-SXRF and insufficient to resolve synaptic sub-structures. To overcome this limitation we present a method to correlate nano-SXRF with STED (STimulated Emission Depletion microscopy) performed both at 40 nm resolution⁵. With the combination of these two high resolution imaging techniques we observe trace metals co-localization with cytoskeleton proteins at the synaptic level in rat hippocampal neurons.

MATERIAL AND METHODS

Cell culture

Primary rat hippocampal neurons were cultured on silicon nitride (SN) membranes (Silson Ltd) consisting in square silicon frames of 5 x 5 mm² and 200 µm thickness with a central SN membrane of 1.5 x 1.5 mm² and 500 nm thickness. During manufacturing, a second, smaller (0.1 x 0.1 mm²), SN membrane is added in one of the corners of the silicon frame to serve as orientation object. Primary rat hippocampal neurons were dissociated from E18 Sprague-Dawley rat embryos (Janvier labs) and plated on the SN membranes previously treated with 1 mg.ml⁻¹ poly-lysine (Sigma) in a 0.1 M borate buffer pH 8.5. The SN membranes were placed on an astrocyte feeder layer growing on a dish treated by 0.1 mg.ml⁻¹ poly-lysine in a 0.1 M borate buffer pH 8.5, in Neurobasal medium (Gibco) as described in the protocol from Kaech and Banker⁶. At 3-4 days *in vitro* (DIV3-4), cell cultures were treated with 2 µM of cytosine arabinofuranoside (Sigma) to limit the growth of the glial cells. From DIV6 and twice a week, half the Neurobasal medium was removed and replaced by BrainPhys medium (STEMCELL) at 310 mOsm, a culture medium designed to respect neuronal activity for *in vitro* models. To develop dendritic spines, neurons were maintained in culture at 36.5°C in 5% CO₂ atmosphere until DIV15.

Live-cell confocal and STED microscopy

Confocal and STED microscopy were performed on a commercial Leica DMI6000 TCS SP8 X microscope. DIV15 neurons cultured on SN membranes and labeled with silicone rhodamine (SiR) fluorogenic probes, SiR-tubulin and SiR700-actin (Spirochrome). Neurons were maintained in the microscope chamber at 37°C in an equilibrated (37°C, 5% CO₂) Tyrode's solution (D-Glucose 1 mM, NaCl 135 mM, KCl 5 mM, MgCl₂ 0.4 mM, CaCl₂ 1.8 mM and HEPES 20 mM) pH 7.4 at 310 mOsm, the osmolarity of the BrainPhys culture medium. For live cell microscopy the SN membrane was mounted in a Ludin chamber. The SN membrane is placed on the glass coverslip of the Ludin chamber, with neurons facing the coverslip to minimize the distance between the objective and the cells to be observed. Confocal and STED images were acquired with a HC-PL-APO-CS2 93x immersion objective in glycerol with a numerical aperture of 1.3 and a scan speed of 400 Hz. SiR fluorogenic probes were excited at 640 nm (670 nm for SiR700) and the signal was detected with a Leica HyD hybrid detector with a window of emission recording from 651 to 680 nm for SiR and from 710 to 750 for SiR700. For STED acquisitions, the fluorescence outside the centre was quenched with a 775 nm pulsed diode laser synchronized with excitation.

Sample preparation for nano-SXRF

Immediately after STED observation the neurons were plunge-frozen. Samples were quickly rinsed in a 310 mOsm ammonium acetate solution prepared with ultrapure water, pH 7.4, to remove extracellular inorganic elements present in Tyrode's solution that would interfere with nano-SXRF element mapping. The osmolarity of Tyrode's and ammonium acetate solutions were measured with a vapor pressure osmometer (VAPRO 5600, Elite) and adjusted to the initial values of the BrainPhys culture medium (310 mOsm). Then the cells were blotted with Whatman paper and plunge-frozen during 20 seconds in 2-methylbutane (Sigma) cooled down at -165°C in liquid nitrogen. Excess 2-methylbutane was carefully blotted with Whatman paper cooled in liquid nitrogen vapors and transferred in the freeze-drier. Neurons were freeze-dried in mild conditions, during 2 days at -90°C and 0.040 mbar in a Christ Alpha 1-4 freeze drier. Then the temperature and the pressure were slowly raised up to room temperature and ambient pressure and the samples were stored at room temperature within a desiccator until synchrotron analysis.

Nano-SXRF imaging

Nano-SXRF imaging was performed on the ID16A Nano-Imaging beamline² at the European Synchrotron Radiation Facility (Grenoble, France). The beamline is optimized for X-ray fluorescence imaging at 20 nm spatial resolution, as well as coherent hard X-ray imaging including in-line X-ray holography and X-ray ptychography. Featuring two pairs of multilayer coated Kirkpatrick-Baez (KB) focusing mirrors, the beamline provides a pink nanoprobe ($\Delta E/E \approx 1\%$) at two discrete energies: $E = 17$ keV and 33.6 keV. Despite the larger focus at lower energy, for the correlative STED-SXRF experiment 17 keV was chosen as it is more efficient in exciting the X ray fluorescence of the biologically relevant elements. The X ray focus with dimensions of 35 nm (H) x 57 nm (V) provided a flux of

3.7 10^{11} ph/s. The focus spot size was determined with a lithographic sample consisting of a 10 nm thick, 20 x 20 μm^2 square of nickel on a 500 nm thick SN membrane. The SN membranes holding the neurons were mounted in vacuum on a piezo nano-positioning stage with six short range actuators and regulated under the metrology of twelve capacitive sensors⁷. An ultra-long working distance optical microscope was used to bring the sample to the focal plane (depth-of-focus $\pm 3 \mu\text{m}$) and to position the STED regions of interest in the X-ray beam. The samples were scanned with an isotropic pixel size of 40 nm, in some cases 20 nm for the scans of smaller size, and 100 ms of integration time. The X-ray fluorescence signal was detected with two custom energy dispersive detectors (Rayspec Ltd) at either side of the sample, holding a total of ten silicon drift diodes. The quantitative data treatment of the SXRF data was performed with Python scripts exploiting the PyMCA library⁸, using the fundamental parameter method with the equivalent detector surface determined by calibration with a thin film reference sample (AXO DRESDEN GmbH). The resulting elemental areal mass density maps (units: $\text{ng}\cdot\text{mm}^{-2}$) were visualized with ImageJ.

RESULTS

Zinc and tubulin are co-localized

We observed Zn and tubulin co-localization in thin dendritic processes as illustrated in Figure 1. A region of interest showing parallel microtubule filaments was selected (Figure. 1a). These parallel tubulin filaments could not be resolved by confocal microscopy (Figure. 1b), but only by STED microscopy (Figure. 1c). Similarly to STED microscopy, nano-SXRF performed on the same region of interest was able to separate the element distributions of the two thin dendritic processes (Figure. 1d-e). The very good superimposition of S and Zn distributions along tubulin imaged by STED was systematically observed. The quantitative analysis of nano-SXRF data for 21 regions showing Zn and tubulin co-localization indicates that the atomic ratio S/Zn is of 43 ± 10 (Figure. 1f). Considering that the amino acid sequence of the rat tubulin- α/β dimer contains 49 sulfur atoms per dimer, from methionine and cysteine residues, the S/Zn ratio in microtubules corresponds to a theoretical tubulin- α/β dimer over Zn ratio of 0.9 ± 0.2 (Figure. 1f).

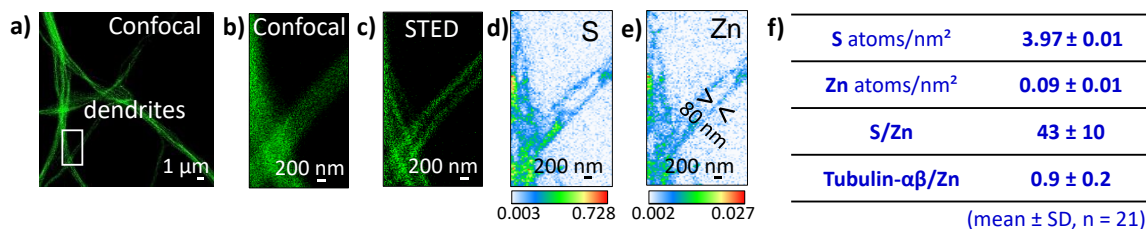


FIGURE 1. Correlative STED and nano-SXRF imaging in dendrites. a) Confocal image of SiR-tubulin in dendritic arbors. b) Confocal SiR-tubulin image of the region framed in (a). c) STED SiR-tubulin image of the region framed in (a). d-e) Sulfur and zinc SXRF maps (ng/mm^2). f) Quantitative data analysis of the number of sulfur and zinc atoms. nm^{-2} for 21 regions of interest centered on thin microtubules (mean \pm SD; n=21).

Copper and F-actin are partially co-localized

Copper distribution is only partially correlated with F-actin localization but corresponds to the highest signal of F-actin in spines (Figure. 2). This observation could be explained by two different mechanisms. Copper might bind directly to F-actin but copper content is below the detection limit in regions containing lower amount of F-actin. Alternatively, copper could interact indirectly with F-actin, through binding to molecules involved in the regulation of F-actin network in the dendritic spine such as cofilin, explaining the partial co-localization with F-actin.

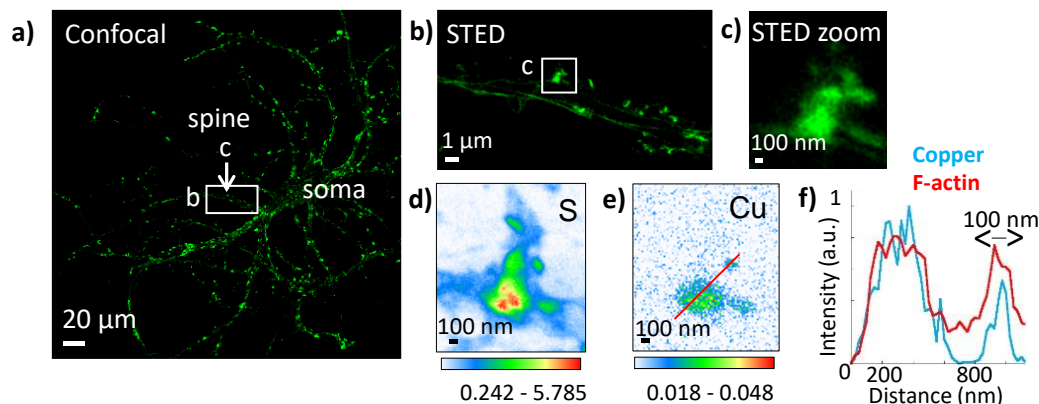


FIGURE 2. Correlative imaging of F-actin, sulfur and copper in a sub-synaptic compartment. a) Confocal imaging of a single primary rat hippocampal neuron labelled with SiR-actin, showing the soma, and numerous dendritic spines rich in F-actin. A single spine has been selected for STED imaging (arrow). b) Super resolution STED imaging of actin in the dendritic region framed in (a). c) Zoom on the single dendritic spine from the STED SiR-actin of the region shown in (a and b). d-e) Sulfur and copper nano-XRF quantitative maps (ng/mm^2) from the STED region of interest. f) Line scans for F-actin and copper normalized distributions along the red line plotted in (e) showing the co-localization of Cu and F-actin.

DISCUSSION AND CONCLUSION

Actin and tubulin are the most widely expressed cytoskeleton proteins and are involved in the formation, plasticity, and stability of synapses. Dynamic polymerization or depolymerization of actin filaments (F-actin) serve as driving force for the formation or retraction of dendritic spines. Microtubules are formed by the polymerization of $\alpha\beta$ -tubulin heterodimers. Microtubules are located mainly in axons and dendrites but also in synapses. Overall, our findings obtained by correlating STED and nano-SXRF suggest that Cu and Zn physiologically interact with neuronal tubulin and F-actin to control synapse formation and stability⁵. From a methodological perspective, the combination of STED super resolution microscopy and nano-SXRF imaging opens numerous perspectives of application to investigate the chemical biology of metals.

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