ESRF	**Experiment title:** Correlative super resolution SXRF and STED imaging of biological metals and synaptic proteins in frozen hydrated hippocampal neurons	**Experiment number**: LS-2850
Beamline: ID16A-NI	**Date of experiment**: from: 05/12/2018 to: 09/12/2018	**Date of report**: 20/01/2020
Shifts: 12	**Local contact(s)**: Peter CLOETENS	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

Richard ORTEGA* (CENBG, Univ. Bordeaux, CNRS, Gradignan, France)

Stéphane ROUDEAU* (CENBG, Univ. Bordeaux, CNRS, Gradignan, France)

Florelle DOMART* (CENBG, Univ. Bordeaux, CNRS, Gradignan, France)

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 at the synaptic level. We correlate stimulated emission depletion (STED) microscopy of proteins and synchrotron X-ray fluorescence (SXRF) imaging of trace metals, both performed with 40 nm spatial resolution, on primary rat hippocampal neurons (Figure 1). We achieve a detection limit for zinc of 14 zeptogram (10^{-21} g) per pixel on ID16A beamline. 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-αβ dimer. We observe the co-segregation of copper and F-actin within the nano-architecture of dendritic spines. In addition, zinc chelation causes a decrease in the expression of cytoskeleton proteins in dendrites and spines. Overall, these results indicate new functions for zinc and copper in the modulation of the cytoskeleton morphology in dendrites, a mechanism associated to neuronal plasticity and memory formation (Domart et al., 2019).

Experimental conditions

SXRF and phase contrast imaging (PCI) experiments were performed on the ID16A Nano-Imaging beamline. For the correlative STED-SXRF experiment a 17 keV X-ray beam was chosen to excite 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 silicon nitride membranes holding the neurons (pluge-frozen and freeze-dried) were mounted in vacuum on a piezo nano-positioning stage. An ultra-long working distance optical microscope was used to bring the sample to the focal plane 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 quantitative data treatment of the SXRF data was performed with Python scripts exploiting the PyMCA library, 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 (ng.mm^{-2}) were visualized with ImageJ. The X-ray phase contrast imaging was performed on the same instrument moving the sample a few millimeters downstream of the focus and recording X-ray in-line holograms with a FReLoN CCD based detector located 1.2 m downstream of the focus. The spatial resolution was approximately 30 nm, similar to the STED and SXRF images. The workflow for correlative STED and synchrotron multimodal nano-imaging is presented in Figure 1.

Main conclusions

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. This correlative imaging stands as a solid new tool for the identification of metalloproteins directly in cells by correlating cellular imaging methods at a supramolecular scale. From a biological perspective, given the importance of cytoskeleton proteins in the morphological plasticity of neuronal connections, our results indicate a broad role of copper and zinc in cytoskeleton architecture and provide a better understanding of metals functions in neurons, and may explain why metal dyshomeostasis, in particular copper or zinc depletion, are linked to numerous neuronal disorders.

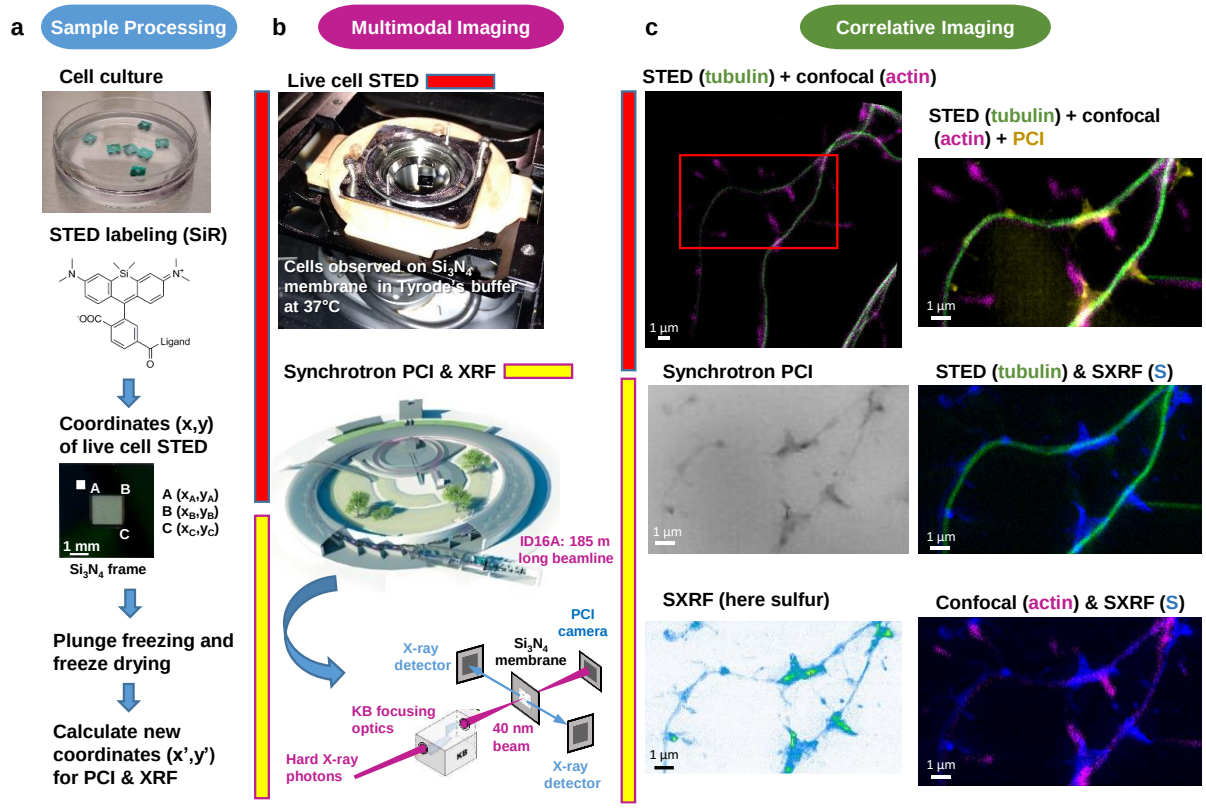

Figure 1 | Workflow for correlative STED and synchrotron multimodal nano-imaging*. **a,** Sample processing. Primary neurons are cultured on silicon nitride membranes and labelled with fluorescent probes designed for STED microscopy such as SiR-tubulin or SiR700-actin. STED microscopy is performed on living cells and orthonormal coordinates (x,y) of regions of interest are recorded relatively to the position of three membrane corners A, B, C identified thanks to an orientation frame. Immediately after STED microscopy cells are plunge-frozen and freeze-dried. New coordinates (x',y') of the regions of interest are calculated to perform XRF and PCI imaging on the synchrotron microscope. **b,** Multi-modal imaging. Live-cell STED and confocal microscopy are performed with a Leica DMI6000 TCS SP8 X microscope equipped with a thermalized chamber. Synchrotron XRF and PCI are carried out on freeze-dried samples at ESRF on beamline ID16A. The KB optics are 185 m away from the X-ray source enabling to focus hard X-rays at 40 nm beam size. **c,** Correlative imaging. Overlay images of STED, confocal, synchrotron PCI and XRF are produced on areas of few tens of µm large with a spatial resolution of 40 nm for STED, 30 nm for PCI and 40 nm for SXRF. Several elemental maps (here sulfur) can be super-imposed with protein distributions (i.e. actin or tubulin) in dendrites and spines at 40 nm spatial resolution.*

Publications

Domart F., Cloetens P., Roudeau S., Carmona A., Verdier E., Choquet D., Ortega R. Correlating STED and synchrotron XRF nano-imaging unveils the co-segregation of metals and cytoskeleton proteins in dendrites, BioRxiv, 810754, **2019**.

Domart F., Synchrotron X-ray fluorescence and super resolution correlative nanoimaging of metals and proteins in synapses of hippocampal neurons. PhD Thesis, University of Bordeaux, France, **2019**.