ESRF	**Experiment title:** **3D nano-XRF imaging of As and Hg in neuronal synapses to assess synaptic toxicity mechanisms of environmental metals in Alzheimer's disease**	**Experiment number**: LS-3068
Beamline: ID16A	**Date of experiment**: from: 06/07/2022 to: 11/07/2022	**Date of report**: 03/03/2023
Shifts: 15	**Local contact(s)**: Marina Eckermann and Peter Cloetens	*Received at ESRF:*
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Preliminary report (data treatment under progress):

We investigated a novel mechanism of metal-induced synaptic toxicity, potentially involved in Alzeimer's disease, based on the interaction of metals with the synaptic cytoskeleton architecture and occurring at environmentally relevant concentrations. We sought to compare the distribution of Hg and As and to determine whether they share common mechanisms of synaptic toxicity. Using synchrotron XRF nano-imaging at ID16A beamline, we mapped the 2D and 3D distribution of chemical elements in dendrites and synaptic compartments of primary rat hippocampal neurons exposed to Hg and As. We correlated synchrotron XRF images with fluorescence cryo-microscopy of cytoskeleton proteins labeled with silicon-rhodamine tags (SiR-tubulin, or SiR-actin), and of PSD95 (Post-Synaptic Density 95) protein labeled with xph20-GFP.

Primary rat hippocampal neurons were transfected with xph20-GFP and cultured *in vitro* for 19 days (DIV19), to allow the development of dendrites and synapses. Neurons were directly cultured onto silicon nitride membranes for synchrotron XRF imaging. Cells were exposed to subcytotxic concentrations (IC10: 10% growth inhibitory concentration) of $HgCl_2$ (40 µM) or $NaAsO_2$ (5 µM). After 24h exposure, cells were washed and labeled with SiR-tags. Finally samples were plunge-frozen in liquid ethane and maintained in liquid nitrogen at all time, during storage, transport, cryo-fluorescence microscopy and nano-SXRF analyses. The correlative microscopy combining optical fluorescence and SXRF was performed in fully cryogenic conditions. This is a new protocol (only a few fluorescence cryo-microscopes are available today in France), difficult to perform, but very effective to correlate the distribution of proteins and metals.

Hg shows a dotted distribution with hot spots along the dendrites (Fig. 1A). The distribution of Hg was compared to the cryo-fluorescence microscopy of PSD95 labeled with xph20-GFP, a protein expressed in the post-synaptic compartment (Fig. 1B). This cryo-correlative protocol allowed the unambiguous comparison of fluorescence microscopy images and SXRF images as illustrated in Fig. 1C. After careful processing of the data, the images show that the Hg hot-spots do not correspond to PSD95-xph20-GFP structures (Fig. 1C). Therefore, Hg is not detected in the post-synaptic density. However, Hg is located in round structures ranging from 0.2 to 2 µm diameter along dendrites. Hg is highly correlated with Zn and S distributions in these structures. The size and chemical composition of these structures suggest a possible lysosomal localisation of Hg, to be confirmed. We have also performed 3D nano-SXRF imaging of these structures but still need to complete the data treatment.

The distribution of As greatly differs from that of Hg. Arsenic is uniformly detected at very low concentration all along dendrites (Fig. 2). There is no strict correlation between the distribution of As and PSD95-xph20-GFP structures but since As is present in the whole volume, it is also present in the post-synaptic structures (Fig. 2). Selenium is detected in round micron-size, yet undefined, structures, and only in neurons exposed to As (not in control or in Hg-exposed neurons). This result suggests a cellular defense mechanism against As-toxicity via the biosynthesis of Se antioxidant proteins.

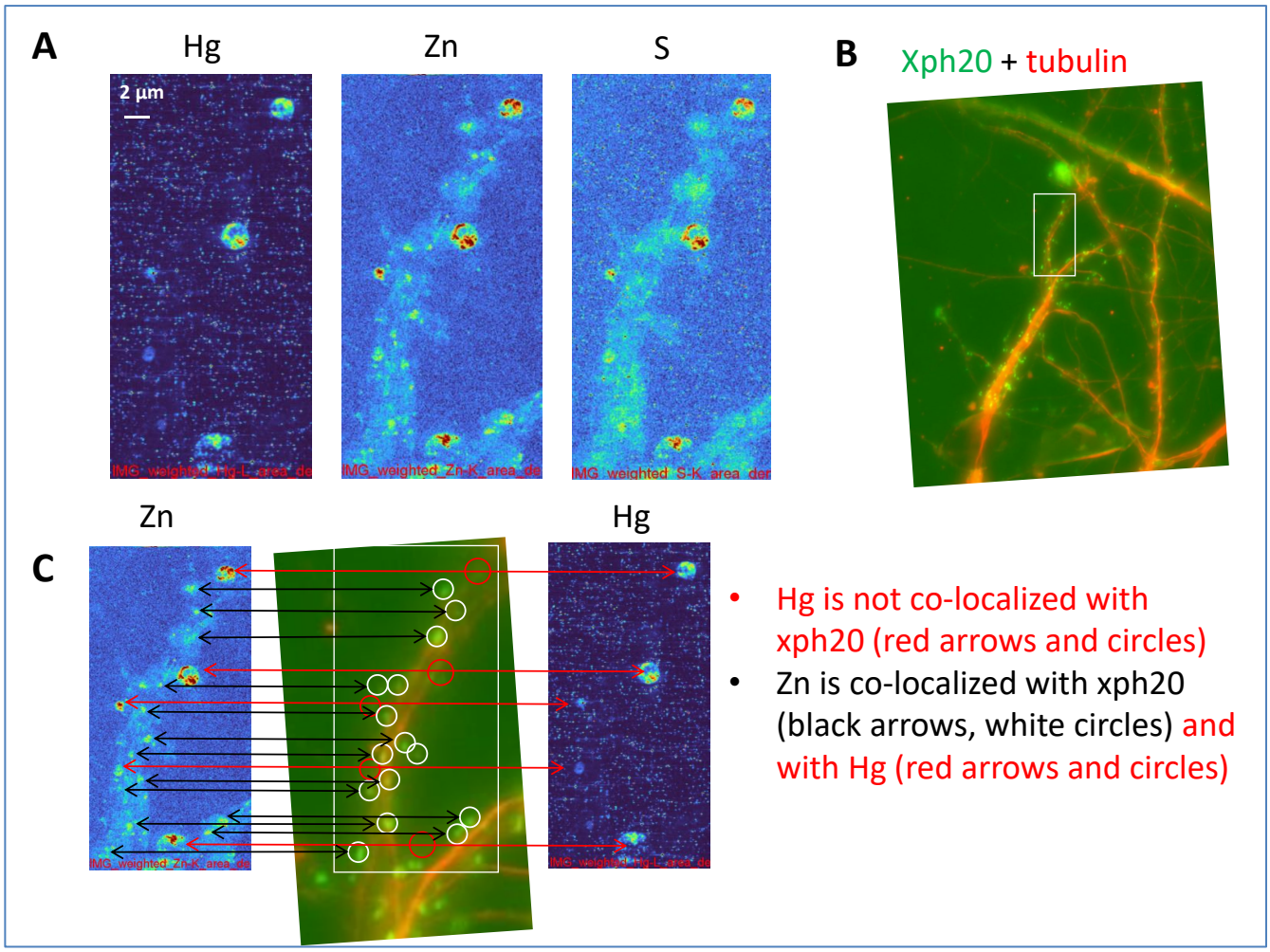

Figure 1. A) Nano-SXRF imaging of Hg, Zn and S along dendrites of a mature hippocampal neuron (DIV19, 19 days in culture) exposed to Hg at subcytotoxic concentration (40 µM, 24h). Scan size = 28 x 15 µm^2, step size = 80 nm; dwell time = 100 ms. Hg is present in hot-spots along the dendrites. Zn and S display a similar distribution. Hg hot-spots are also rich in Zn and S. **B)** Fluorescence cryo-microscopy of a post-synaptic protein (PSD95) labeled with xph20-GFP (green), and of tubulin labeled with SiR-tubulin (red), obtained from the same region as in A) (white frame). **C)** The distributions of Hg and Zn are compared to xph-20 and SiR-tubulin. Hg hot-spots (red circles) do not co-localize with PSD95-xph20-GFP structures (red arrows). Zn is present in the post-synaptic compartments (black arrows and white circles), and is also co-localized with Hg-rich structures.

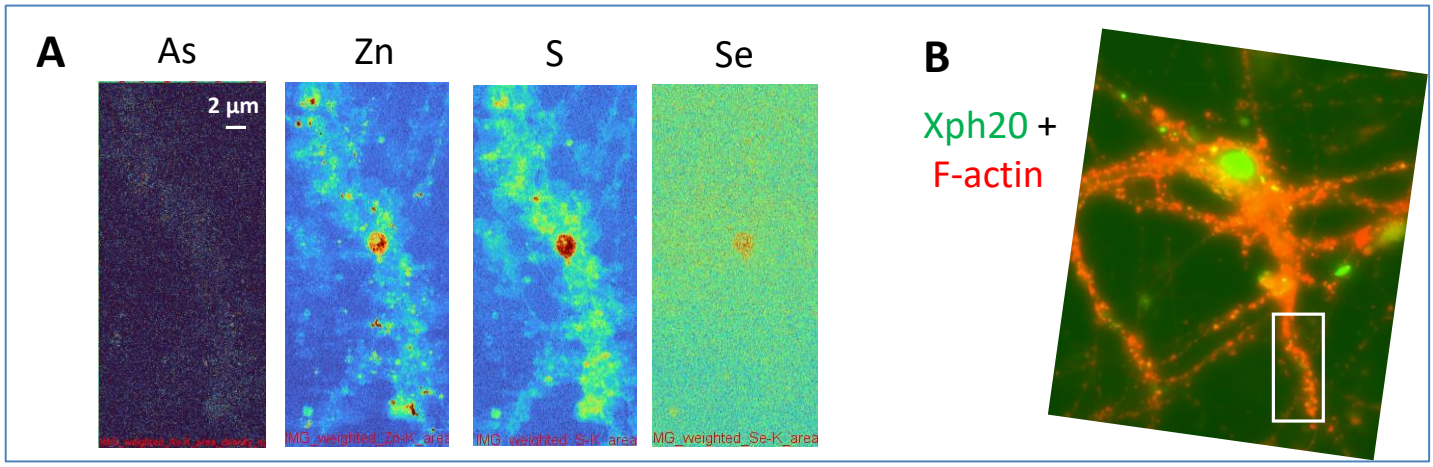

Figure 2. A) Nano-SXRF imaging of As, Zn, S an Se along dendrites of a mature hippocampal neuron (DIV19, 19 days in culture) exposed to As at subcytotoxic concentration (5 µM, 24h). Scan size = 17 x 37 µm^2, step size = 80 nm; dwell time = 100 ms. Arsenic is present at low concentration all along the dendrites. Zn and S are present in postsynaptic compartments (F-actin and xph20 labeling). Se is present only in As exposed neurons, in few hot-spots. **B)** Fluorescence cryo-microscopy of a post-synaptic protein (PSD95) labeled with xph20-GFP (green), and of F-actin labeled with SiR-actin (red), obtained from the same region as in A) (white frame).