ESRF	**Speciation analysis of arsenic chemical forms in cellular ultrastructures**	**Experiment number**: SC-1773
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Inorganic arsenic is a well-documented carcinogen, and human exposure is associated with an increased risk of developing tumours of the skin, bladder, liver, kidney, or lung. The precise mechanisms of arsenic cancer causing effects are unknown. The identity of arsenic species involved in carcinogenesis is ambiguous and metabolization of inorganic species seems to create more toxic arsenic compound by methylation. On the other hand, arsenic trioxide (As_2O_3) exhibits anti-cancer effects and is used for the treatment of acute promyelocytic leukemia. Arsenic trioxide induces apoptosis through a reactive oxygen species-dependent pathway and loss of mitochondrial membrane potential in cells. The aim of this experiment is to attempt to clarify the mechanism of action of arsenic trioxide.

Epidermal mice cells (JB6) and human ovarian cancer cells (IGROV1) were cultured onto 2 µm polycarbonate foils. They were exposed to 9 µM (IC50) and 15 µM As_2O_3 during 24 h or 48 h. Mitochondria were marked with a fluorescent dye (Rhodamine123) and their intracellular location was determined by epifluorescence microscopy and confocal microscopy. Video images were recorded for further identification of cellular organelles. Two sample preparation protocols were performed in order to be compared: 1) cells were cryo-fixed into liquid nitrogen chilled isopentane and stored into liquid nitrogen until analysis in their frozen hydrated state at 150 K; 2) cells were cryo-fixed into liquid nitrogen chilled isopentane and freeze dried at –35°C, then analysed at room temperature.

XANES experiments were performed with a 1.5 x 4.0 μm^2 spatial resolution using ID-22 hard X-ray microprobe with a Kirkpatrick-Baez focusing mirror. This experimental setup provided a photon flux of typically 1.5 x 10^{11} ph/s allowing X-ray absorption spectroscopy to be performed with micrometric resolution. The use of a liquid nitrogen cryo-jet enabled to irradiate the samples in their frozen hydrated state at 150 K. Prior to µ-XANES, a fast synchrotron X-ray fluorescence mapping of arsenic and potassium distributions was performed in order to precisely identify the intracellular organelles. Arsenic chemical speciation was determined locally, by point analysis, into nucleus, mitochondria and cytoplasm by scanning around As absorption K-edge from 11860 to 11910 eV with 0.5 eV energy resolution.

A strong limitation of the µ-XANES method is the damage caused to the biological samples by an intense X-ray irradiation. Comparison of 5 µ-XANES spectrum obtained on the same area either of freeze-dried cells or frozen hydrated cells (Figure 1) shows an important decrease of As fluorescence on freeze dried cells but not on frozen hydrated cells. The irradiation of frozen hydrated cells cooled down to 150 K with a liquid nitrogen cryo-jet induces much less damage than room temperature irradiation of freeze-dried specimens.

An example of µ-XANES spectra within cell compartments (mitochondria, nucleus, and cytosol) is presented in figure 2. Arsenic oxidation state was found similar in each cells compartment. µ-XANES analyses of As chemical standards shown that As absorption edge in cell compartments was between those of As_2O_3 standard and dimethyl-As(V) standard (DMA^V), which were separated by less than 1eV. However µ-XANES did not allow the formal identification of As chemical forms (mixture of both compounds, or other metabolites ?). A further improvement would be the identification of As chemical forms on the same samples when the technology for EXAFS analysis at the micron scale will be available.

This experiment has opened a new field of investigation, the in-situ determination of chemical element oxidation sates within cellular organites. Arsenic oxidation state was the same in the main cellular compartments, including the mitochodria which is the suggested target for As_2O_3 mechanism of cytotoxic action. Thus the mechanism of action of As_2O_3 may not be related to a different redox state of As in the mitochondria. This experiment was also critical to specify that the analysis of frozen hydrated cells cooled down to 150 K is absolutely required for arsenic speciation µ-analysis; the analysis of freeze dried cells at room temperature leading to high specimen damage. In the future, other chemical forms of As (organic and inorganic) should be investigated to obtain a broader view of As interactions with cells, and to explain the known differences in cytotoxicity and carcinogenicity of As compounds.

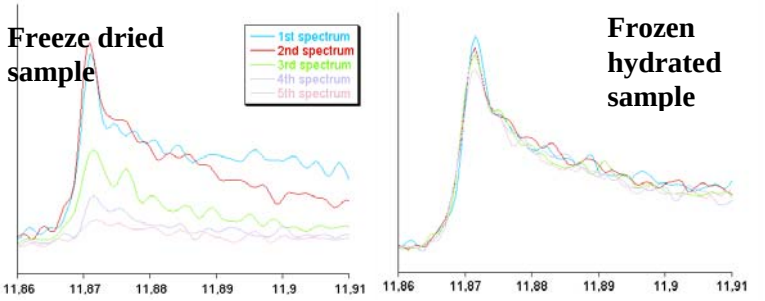

Figure 1. Evolution of µ-XANES spectrum on cell nucleus of each samples preparation (Freeze–dried samples and frozen samples). 5 spectra were done consecutively. Frozen sample are not damaged but freeze dried samples are not stable and dramatically damaged.

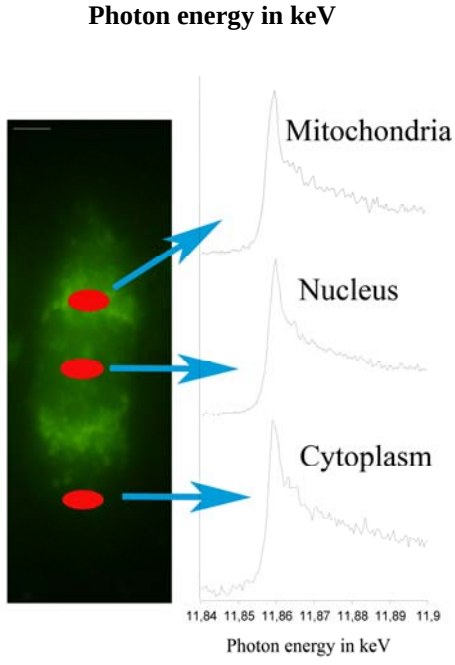

Figure 2. Imaging of fluorescent probe (Rhodamine123) used for localization of mitochodria (green) and µ-XANES spectra in IGROV1 cell compartments. Red spot represent the beam position. µ-XANES spectra suggest a similar oxidation state for As in these 3 compartments.