ESRF	**Experiment title:** Quantitative mapping of Ca, P and Zn during osteosarcoma cell differentiation: intracellular genesis of nano-sized mineral depositions and evolution in extracellular matrix.	**Experiment number**: LS2921
Beamline: ID16A-N	**Date of experiment**: from: 11th February 2021 to: 18th February 2021	**Date of report**: 22nd February 2021
Shifts: 15	**Local contact(s)**: Sylvain Bohic, Dmitry Karpov	*Received at ESRF:*

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Report:

The aim of this proposal is to study the intracellular genesis of the nano-sized mineral depositions and their evolution in the extracellular matrix, mapping the concentration of Ca, P and Zn during osteoblastic differentiation of osteosarcoma SaOS2 cells (at 4 and 10 days). With this experiment we expect to obtain useful information about the role of Ca, P and Zn in bone mineralization process of osteosarcoma cells. To this purpose, X-ray Fluorescence Microscopy (XRFM) measurements and x-ray phase contrast holo-tomography acquisitions were performed to obtain 2D Ca, P and Zn concentration maps at high spatial resolution. Ca, Zn, K and P fluorescence intensity maps are derived from XRFM acquisitions while the cellular volume is obtained by the holo-tomography at nanoscale.

The samples were prepared in the ID16A laboratory and were constituted of frozen hydrated osteosarcoma SaOS2 cells which were induced to differentiate towards osteoblasts using a cocktail containing β-Glycero-phosphate, ascorbic acid, and vitamin D for 4 and 10 days.

During the experiment, computer failure and sample problems have caused a time loss of nearly 4 shifts on the total amount of the 15 shifts scheduled. Remote sample preparation was very complex due to COVID context. Furthermore, cryogenic samples were very fragile probably due to the 200 nm thickness of the Silicon Nitride membranes used, and therefore sample changes were slowed down by the necessity to find samples with intact membranes (see Figure 1). Due to fluorescence detector computer failure, XRFM acquisitions were delayed even though the defective computer was replaced allowing to continue the experiment. Despite problems during the XRFM measurements and sample changes, the staff excellently worked to minimize the downtime and the experiment run smoothly. We succeeded in analysing treated and control samples at both 4 and 10 day after induction. For all samples, a 50 nm or 70 nm resolution XRFM measurement and an holo-tomography were performed at least on one cell (see Figure 2). In some cases (4 days treated, 4 days control and 10 days treated), a 25 nm resolution XRFM was acquired in correspondence of Ca depositions which were highlighted by the previous fluorescence acquisitions. X-ray fluorescence tomography was also carried out to highlight the spatial distribution of some Ca depositions in the treated sample at 4 days. Due to lack of time, this acquisition

was not extended to the remaining samples (a complete list of the acquisitions performed for each sample is available in Table 1).
We started the data analysis, which is quite complex: the fluorescence spectra must be analysed with PyMCA, to obtain the integrated intensity of several fluorescence lines, including calcium, for all the measured samples; in parallel we carried out phase reconstruction using the programs elaborated by the beamline staff, but obtaining reliable quantitative phase retrieval on all the samples requires further efforts and hence, tomographic reconstruction is still in progress. When both fluorescence spectra analysis and phase retrieval procedure will be completed, fluorescence intensity maps derived through XRFM will be normalized with the cellular volume obtained by the holo-tomography allowing us to reach the projected elemental concentration map of the sample. In the following, we show examples of fluorescence intensity maps of cells from control and treated samples at 4 and 10 days (Figure 3, 4, 5, 6). Ca depositions were observed even at 4 day of osteoblastic differentiation and at 10 days after treatment very high Ca intensity values were registered. In both 4 days control and treated samples, Ca depositions could not be appreciated in all the analysed cells highlighting the need to perform multiple acquisitions with respect to the 10 days samples. From a previous work [1], we found out an increase of the number of mineral depositions with a lower volume in differentiating cells with respect to control cells; a significant analysis of this phenomenon requires a large amount of elemental concentration maps for each sample. Since very little is known about the chemical composition and progression of the extracellular Ca-polyphosphates depositions in osteosarcoma cells, and even less during their osteoblastic differentiation, it is necessary to perform more scans for each sample to increase the number of cells analysed. Up to now, acquisitions were performed mostly in the intracellular environment. In order to further characterize the genesis and the evolution of nano-sized mineral depositions, a more accurate mapping of the extracellular matrix is also required.

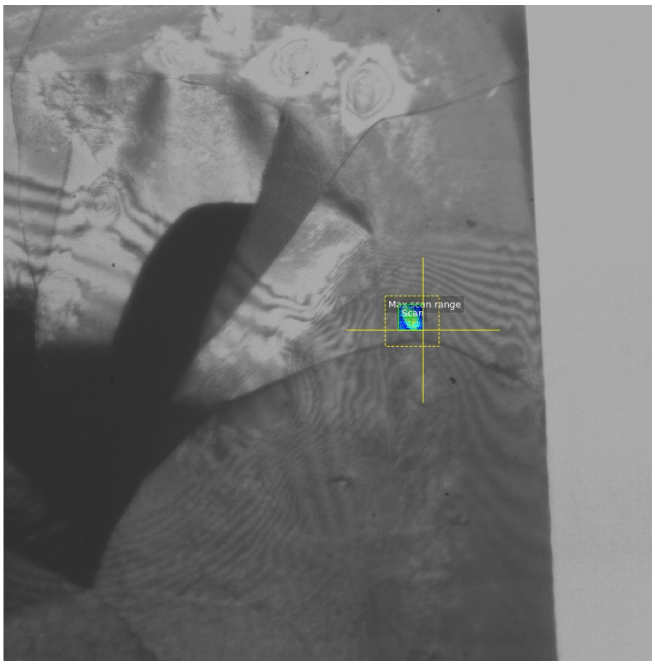

Figure 1 Example of sample with broken membrane. In the yellow square, the cell analysed through XRFM.

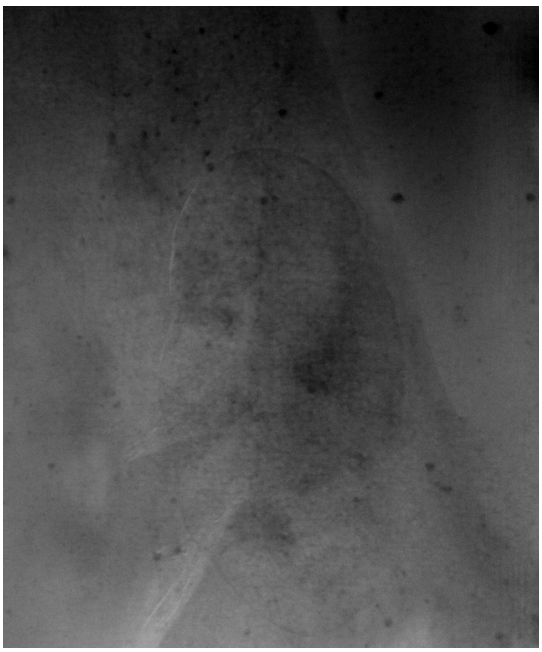

Figure 2 Slice of the x-ray phase contrast holo-tomography acquisition on the control sample at 10 days.

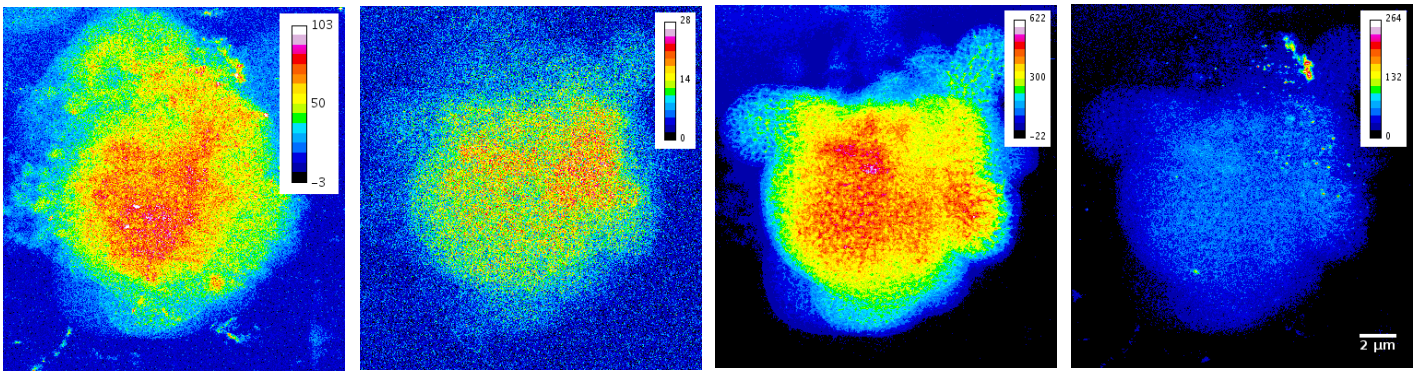

Figure 3 From left to right: fluorescence maps of Zn, P, K and Ca of a 4 days control sample.

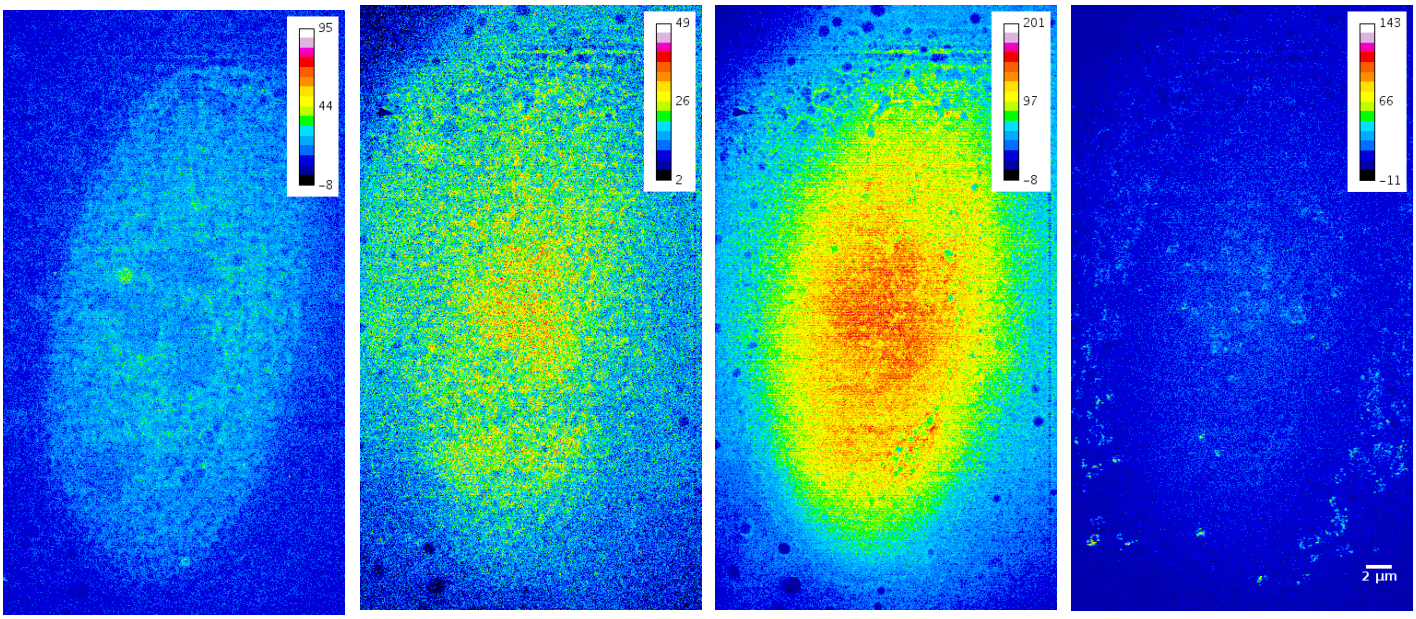

Figure 4 From left to right: fluorescence maps of Zn, P, K and Ca of a 4 days treated sample.

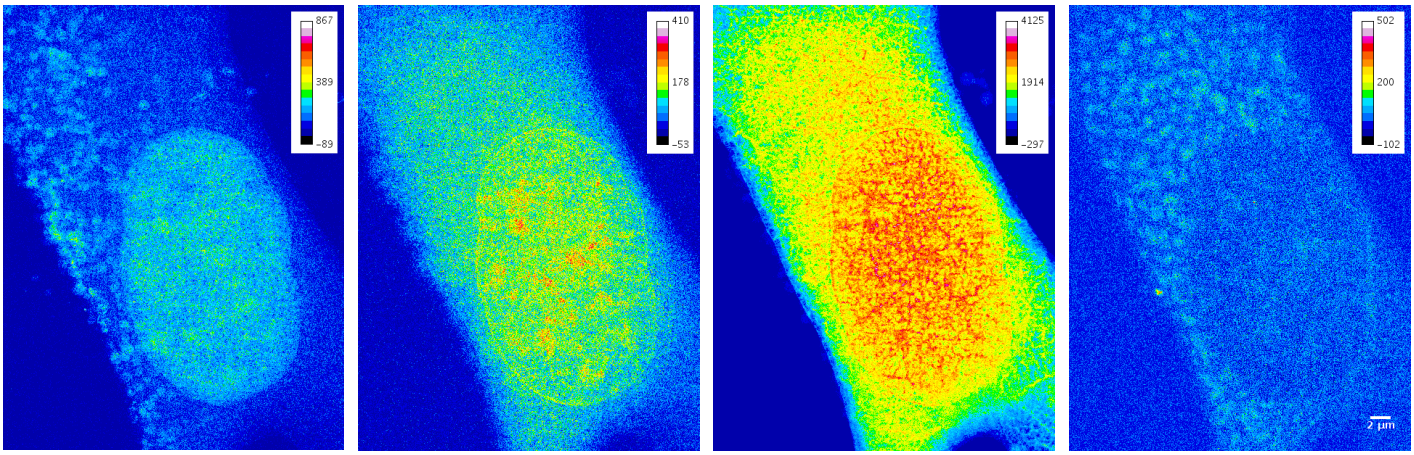

Figure 4 From left to right: fluorescence maps of Zn, P, K and Ca of a 10 days control sample.

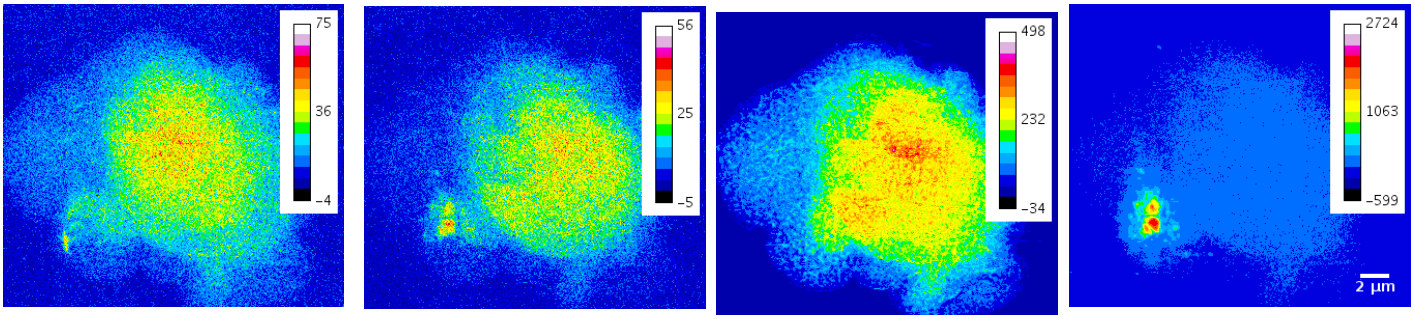

Figure 6 From left to right: fluorescence maps of Zn, P, K and Ca of a 10 days treated sample.

	4 days control	4 days treated	10 days control	10 days treated
XRFM Pixel size 70 nm XRFM Pixel size 25 nm	2 cells 4 regions in 1 cell	2 cells 3 regions per cell	1 cell	2 cells 1 region in 1 cell
Holo-tomography Pixel size 30 nm	1 cell	2 cells	1 cell	1 cell
X-ray fluorescence tomography		2 regions in 1 cell		
Ca depositions	1 noteworthy	Multiple depositions can be appreciated inside the cell and in the extracellular environment near the membrane	1 noteworthy, many depositions can be appreciated in the intracellular matrix	1 noteworthy, many depositions can be appreciated in the intracellular matrix

Table 1 Acquisitions performed for each sample and characterization of Ca depositions observed.

1) PICONE, Giovanna, et al. Analysis of intracellular magnesium and mineral depositions during osteogenic commitment of 3d cultured saos2 cells. *International journal of molecular sciences*, 2020, 21.7: 2368.