



Elemental distribution and sample integrity comparison of freeze-dried and frozen-hydrated biological tissue samples with nuclear microprobe



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ABSTRACT

The analysis of biological samples in frozen-hydrated state with micro-PIXE technique at Jožef Stefan Institute (JSI) nuclear microprobe has matured to a point that enables us to measure and examine frozen tissue samples routinely as a standard research method.

Cryotome-cut slice of frozen-hydrated biological sample is mounted between two thin foils and positioned on the sample holder. The temperature of the cold stage in the measuring chamber is kept below 130 K throughout the insertion of the samples and the proton beam exposure. Matrix composition of frozen-hydrated tissue is consisted mostly of ice. Sample deterioration during proton beam exposure is monitored during the experiment, as both Elastic Backscattering Spectrometry (EBS) and Scanning Transmission Ion Microscopy (STIM) in on-off axis geometry are recorded together with the events in two PIXE detectors and backscattered ions from the chopper in a single list-mode file.

The aim of this experiment was to determine differences and similarities between two kinds of biological sample preparation techniques for micro-PIXE analysis, namely freeze-drying and frozen-hydrated sample preparation in order to evaluate the improvements in the elemental localisation of the latter technique if any.

In the presented work, a standard micro-PIXE configuration for tissue mapping at JSI was used with five detection systems operating in parallel, with proton beam cross section of $1.0 \times 1.0 \mu\text{m}^2$ and a beam current of 100 pA. The comparison of the resulting elemental distributions measured at the biological tissue prepared in the frozen-hydrated and in the freeze-dried state revealed differences in elemental distribution of particular elements at the cellular level due to the morphology alteration in particular tissue compartments induced either by water removal in the lyophilisation process or by unsatisfactory preparation of samples for cutting and mounting during the shock-freezing phase of sample preparation.

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1. Introduction

Determination of the distribution of elements within biological systems at subcellular level down to trace level concentrations is of growing importance for gaining new insights about the highly complex processes that take place within the tissue or the cell. Resolving the distribution and the concentration within the morphological structures of a specific tissue is essential for understanding the mechanisms involved. The preparation of the

specimen in frozen-hydrated state is among the most appropriate methods of preserving the sample integrity for a series of structural and analytical methods [1].

Cryofixation of biological tissues prevents redistribution of the elements stored in tissue compartments by osmosis, and rapid freezing by cryogenics such as propane or isopentane prevents large ice crystal formation, which can destroy cellular organelles. The tissue preservation in the resulting frozen hydrated state thus enables preservation of morphologically intact subcellular structures [2]. The microcrystallinity of the water in the tissue is achieved either by shock-freezing or freezing under high pressure. Resulting tissue appears macroscopically as amorphous stained glass.

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The tissue treatment by lyophilisation has a long history [3] and produces good results for tissue preservation and enables simple handling of the specimens during the analysis.

Elemental mapping of biological tissue could be executed by various methods but with no or limited ability to examine samples of various thicknesses in frozen-hydrated state. Micro-PIXE is one of the least damaging methods for elemental mapping of biological tissue and is therefore also the method of choice for biological samples preserved in frozen-hydrated state. For detailed comparison between various techniques and their ability to process biological samples the reader should refer to earlier published papers [4–7].

2. Experimental

The newly commissioned high brightness negative H-ion beam injection system for our existing tandem accelerator facility has been tuned to deliver optimized brightness with acceptable injection H-ion currents, yielding the measured normalized high-energy proton beam brightness of $14 \text{ A m}^{-2} \text{ rad}^{-2} \text{ eV}^{-1}$ the highest ever reported on tandem accelerators coupled to nuclear microprobes [8].

The high brightness of the new ion source enables the reduction of object slit aperture as well as the reduction of acceptance angle at the nuclear microprobe, resulting in a reduced beam size at selected beam intensity. The first runs with the new ion source enabled us to achieve the beam profile of $800 \times 800 \text{ nm}^2$ at proton currents of 200 pA without any alterations of the existing ion beam optics. This is a promising result for future micro-PIXE analysis on the nuclear microprobe on subcellular level of biological specimens in frozen-hydrated state [1].

Installed cryostat at JSI nuclear microprobe [1] enables us to analyze various types of biological samples in frozen-hydrated state [9] using micro-PIXE/STIM/RBS. Sample load-lock system enables us to quickly insert a sample holder with frozen-hydrated tissue samples onto a cold goniometer head cooled with liquid nitrogen inside the measuring chamber.

Cryotome-cut slice of frozen-hydrated biological sample is mounted between two thin foils and positioned on the sample holder. In first experiments, silicon nitride windows were used [1] and later replaced by polymer foils (Mylar or Pioloform) stretched over aluminium frames. In this way, we avoid the Si background peak from the acquired PIXE spectrum, which now enables us to detect and quantify trace elements in the low energy range from F to Cl. In addition, the use of polymer windows results in less demanding sample mounting procedure on the sample holder, as they are less fragile than Si_3N_4 membranes used in first series of measurements. In the same time, the application of Pioloform foils significantly reduces the sample preparation costs. The temperature of the cold stage in the measuring chamber is kept below 130 K throughout the insertion of the samples and the proton beam exposure.

Sample deterioration during proton beam exposure is monitored during the experiment, as both Elastic Backscattering Spectrometry (EBS) and Scanning Transmission Ion Microscopy (STIM) in on-off axis geometry [10] are recorded together with the events in two PIXE detectors and backscattered ions from the chopper [11] in a single list-mode file.

2.1. Sample preparation

Biological samples of *Thlaspi praecox* leaves, studied earlier by Katarina Vogel-Mikuš et al. [3], and water animal *Daphnia magna*, a frequently used ecotoxicological model organism [12,13] were prepared with plunge-freezing in liquid propane cooled by liquid

nitrogen [14]. *T. praecox* leaves were then cut by cryotome with selected thickness of 30 μm . Water animal *D. magna* was left intact. The rapid freezing results in vitreous ice formation, which is desired form of frozen hydrated tissue specimens during the analysis. Samples of terrestrial isopod *Porcellio scaber* were prepared with rapid freezing on pre-cooled plate with liquid nitrogen [15] and also cut on the cryotome with selected thickness of 60 μm . All samples were positioned between two pre-cooled polymer membranes (1 μm thick Mylar or 100 nm thick Pioloform) stretched over two aluminium frames. Encapsulating the sample between two foils may prevent against unavoidable lyophilisation during the exposure of the tissue to the high vacuum conditions. This sandwich-like structure was then placed inside the pre-cooled sample holder blocks, designed to ensure a sufficient thermal contact with the aluminium frames. Sample holder with frozen-hydrated biological tissue was then transported to the measuring chamber through a load-lock transport system [1] and attached to the cold goniometer head inside the measuring chamber cooled with liquid nitrogen.

Biological samples of *T. praecox* leaves [3] and samples of terrestrial isopod *P. scaber* [15] were also prepared for freeze-drying. After lyophilisation samples were mounted on Al holder between two thin layers of Pioloform foil [3].

2.2. Micro-PIXE analysis

Micro-PIXE analysis was done with a nuclear microprobe at the Microanalytical center at Jožef Stefan Institute [16]. Proton beam with the energy of 3 MeV, diameter of 1 μm and the beam current of 100 pA was used for the analysis. For freeze-dried samples, a standard configuration of the sample holder and goniometer head was used [3]. For frozen-hydrated samples, the temperature of the cold goniometer head was kept below 130 K throughout the proton beam exposure. Simultaneous on-off axis Scanning Transmission Ion Microscopy (STIM) [1] was performed to determine beam exit energy from the sample, related to the sample local area density and thickness. Thinning of the sample as well as water evaporation during the high vacuum and proton beam exposure was monitored by the measurements with STIM and RBS method simultaneously with micro-PIXE measurements. Scanning areas were chosen according to the size of the objects of interest. For the analysis of *T. praecox* leaves, scan sizes of $800 \times 800 \mu\text{m}^2$ (Fig. 1) for freeze-dried sample and $1000 \times 1000 \mu\text{m}^2$ (Fig. 2) for frozen-hydrated sample were appropriate to scan the desired details. For the analysis of digestive gland of *P. scaber* (Fig. 3), scan sizes of $550 \times 550 \mu\text{m}^2$ (Fig. 4) for freeze-dried sample and $500 \times 500 \mu\text{m}^2$ (Fig. 5) for frozen-hydrated sample were selected to scan the desired details. For the analysis of *D. magna*, scan sizes of $1200 \times 1200 \mu\text{m}^2$ (Fig. 6), $400 \times 400 \mu\text{m}^2$ and $200 \times 200 \mu\text{m}^2$ (Fig. 7) were selected to scan the desired details of the frozen-hydrated sample. *D. magna* was prepared only as frozen-hydrated sample. Two PIXE spectra were extracted from a pair of X-ray detectors covering the energy range from 0.8 up to 30 keV [11,17], as well as a spectrum from the chopper [11] to obtain corresponding proton dose distributed over the selected area.

3. Results and discussion

Measurements of biological tissue in frozen-hydrated state show no visible degradation during the 8 h period of micro-PIXE measurements. In parallel, on-off axis STIM spectra were recorded in a list mode with OMDAQ acquisition system to acquire information on potential sample thinning due to beam damage, which could be deconvoluted from time evolution of STIM spectra [1]. Leaf cross-sections of *T. praecox* were scanned to obtain the comparison of the micro-PIXE elemental maps (Figs. 1 and 2) in

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