doi:10.1017/S1431927622006018
Microsc. Microanal. 28 (Suppl 1), 2022

3D Visualisation of Biological Species at Hard-Soft Bone Interfaces using Cryo FIB/SEM

Aekta Upadhyay[1*], Angelique Galvani[2], Jean Le Bideau[1], Valerie Geoffroy[2] and Patricia Abellan[1]

[1]Nantes University, CNRS, Jean Rouxel Institute of Materials of Nantes (IMN), F-44000 Nantes, France

[2]Nantes University, INSERM U1229, Regenerative Medicine and skeleton Research Lab (RMES), 44042 Nantes, France

[*]Aekta.Upadhyay@cnrs-imn.fr

The characterization of interfaces between different tissues at high resolution is critical to the understanding of biological mechanisms. Particularly challenging is the characterization of hard-soft interfaces, which exist in different regions such as tooth-enamel interface, bone-fibrous connective tissue interface in the ear and nose [1]. These interfaces exhibit mismatched behaviours in terms of bio (chemical), mechanical and physical properties [2] and thus, imaging them represent specific challenges. Despite the recent development in numerous technologies available for biological samples, the highly contrasting properties at interfaces still create significant challenges for the preparation and imaging of these samples. Techniques that can provide suitable information and analysis of one of the constituent at the interface may not be able to withdraw similar information from the other constituent due to poor sensitivity or damage [3].

Cryogenic scanning electron microscopy (cryo-SEM) combined with focussed ion beam (FIB) can facilitate qualitative and quantitative 3D studies of these interfaces. At cryogenic temperatures, the electron beam images the sample surface while the ion beam mills into the surface to expose the interior of the sample. FIB-milling enables imaging additional planes by creating cross-sections perpendicular to the cryo-fracture surface, thus adding a third imaging dimension to the cryo-SEM [4]. For the development of methodologies to image these interfaces (especially in biological tissues), there are three major challenges. These are: (a) preparation of samples preserving the structural integrity of biological species at both sides of the interface, (b) developing methodologies to acquire images with good resolution despite electron-beam sensitivity, charging effects and low contrast between the different phases and (c) accessing the specific sites of interest buried within the volume of the sample.

For our studies, we have chosen bone as a model system to develop a suitable protocol for simultaneous imaging of hard-soft interfaces. Bone is a complex architecture, which undergoes constant structural and functional optimisation through renewal and repair. Different bone cell types such as osteocytes, osteoblasts and osteoclasts play key role in the bone remodelling process. The cell-to-cell communication within these cell types occurs through extracellular vesicles [2]. In order to develop a better understanding of the bone remodelling and signalling processes, it is important to develop methodologies to visualise the biological species in near native conditions. In all our studies, front leg of three months old wild mice were dissected and chemically fixed immediately, to preserve the near native hydrated state of tissues.

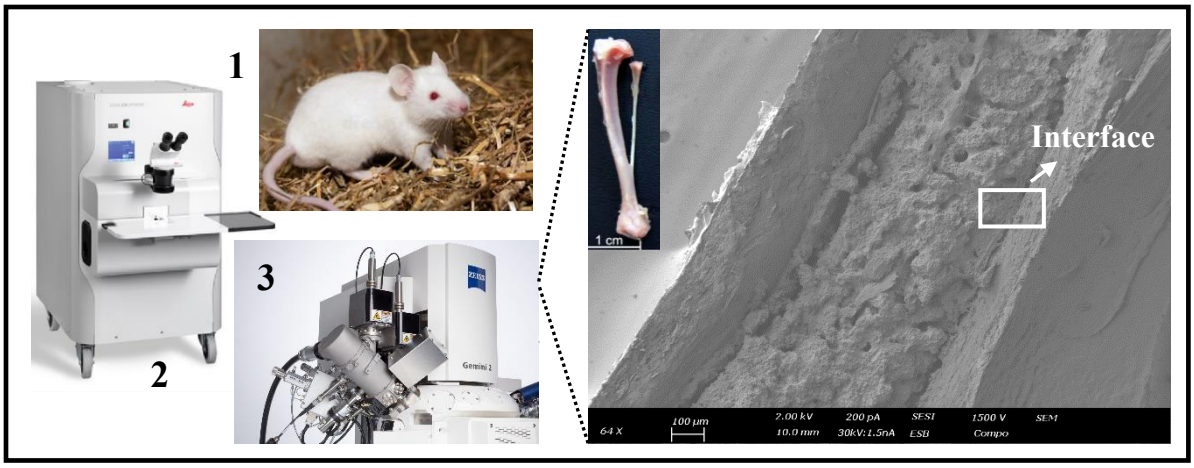

Figure 1: represents the three main aspect of present study: **1**: dissection of rat bone samples, **2**: HPF and **3**: FIB-SEM. Low magnification SEM image of bone showing the interface of hard-soft material (*Right*).

In this presentation, we will cover *challenges and development of suitable method to prepare samples containing hard-soft interface of bone using HPF*. High pressure freezing (HPF) is a cryo-freezing technique, which allows the sample freezing at high pressure of 210MPa, which leads to vitrification of water molecules [5]. This can allow gaining access to specific sites inside the 3-dimensional volume of the bone architecture without ice crystal formation and artefact within the sample. The bone consists of different components such as mineralized and un-mineralized part; they interact differently with the ion beam [6]. Therefore, *optimization of milling parameters, deposition and 3D acquisition* is another challenge for such samples, which will be covered in the presentation (The **Figure 1** represents the three aspects of the present study). These methodologies will open avenues for 3D visualisation of other relevant biological interfaces [8].

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7. The authors would like to thank the financial support by the French National Research Agency (ANR) through the project JCJC VINCI (with reference ANR-20-CE11-0009).

https://doi.org/10.1017/S1431927622006018 Published online by Cambridge University Press