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Enabling Science through European Electron Microscopy

Report on protocols for sample preparation techniques of materials for health

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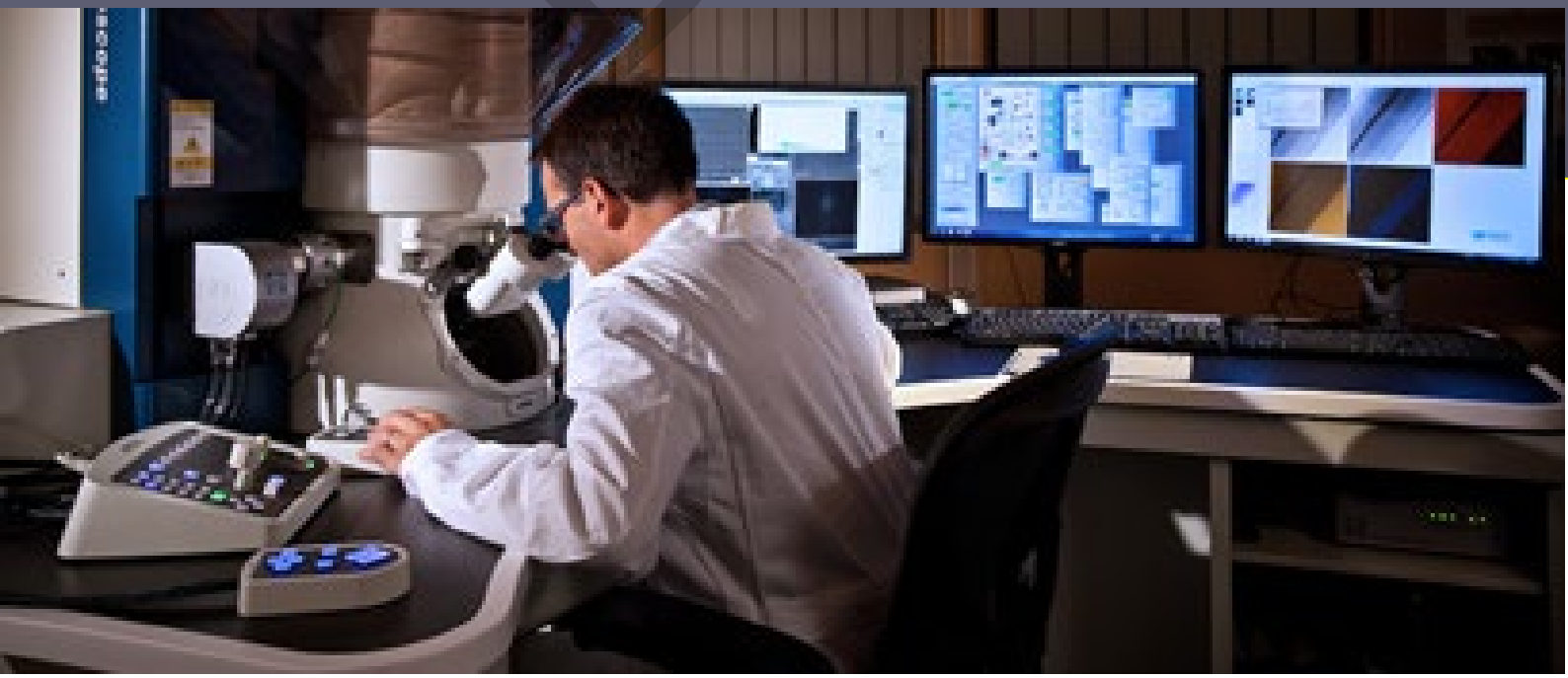

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Version number	Date of release	Author	Summary of changes
V0.1		Vesna Srot	First draft
V1.1	04.04.2023	Peter A. van Aken	Minor changes and approval
V1.2	04.04.2023	Aude Garsès	Minor changes and general review

Excecutive Summary

This report summarises projects, which were performed in STU within WP9 – Materials for Health. Five different projects will be discussed, where various sample preparation protocols that are important for materials for health were optimized or new routines and protocols were developed. The focus has been on optimization and development of sample preparation techniques that can be used for preparation of organic/inorganic materials, biocompatible composites and pharmaceuticals.

Task 1: Biodegradable biopolyesters (STU)

Poly-hydroxy-alkanoates (PHAs) are biopolyesters that can be used as scaffold materials in the regeneration of new tissues. They are appropriate for such applications due to their biodegradability, enhanced biocompatibility, mechanical properties, non-toxicity and environmental origin. Their properties can be easily modified through blending with various materials. A new route for TEM sample preparation of these class of materials was developed. Such samples are often unsteady and need to be stabilized prior to cutting. Firstly, a layer of resin was deposited into the mould and left to harden. Afterwards, a piece of sample was placed on the top of the hardened resin and covered with an additional resin layer. In such way the sample is now sandwiched in-between two resin layers (Figure 1).

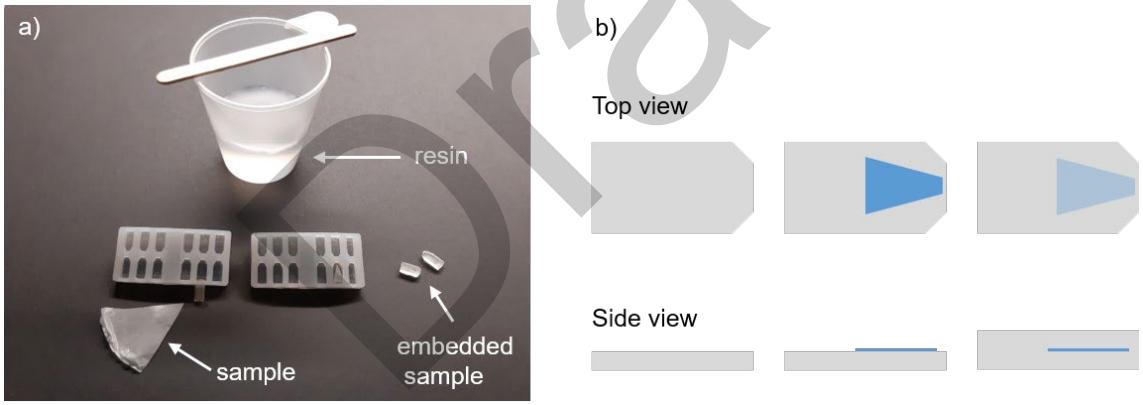

Figure 1: Embedding of the sample in the resin, (a) photo of required components and (b) sketch of the embedding procedure.

Once the resin is completely hardened, the sample can be fixed inside the sample holder (Figure 2a). Corse removal of the block material and trimming of the pyramid is performed with a high-speed milling system Leica EM-TRIM2 (Leica Microsystems, Wetzlar, Germany) (Figures 2 b-c). The block face is created on the tip of the trimmed blocks (Figure 2c). The slices of sandwiched poly-hydroxy-alkanoate samples were cut at room temperature using an ultramicrotome Leica EM-UC6 equipped with a water-filled diamond knife (Diatome, Biel, Switzerland) and collected on transmission electron microscopy (TEM) Cu grids covered with a lacey carbon film, while floating in Ultrapure Millipore water (Figures 3, 4). [1]

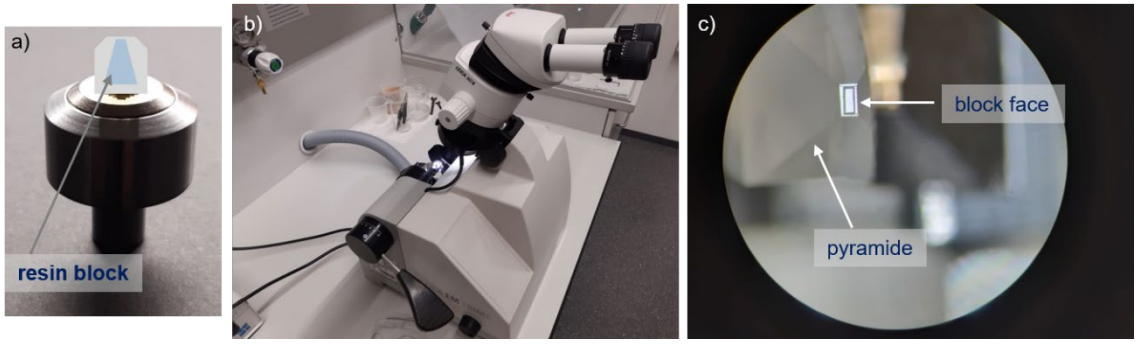

Figure 2: (a) Resin block (with embedded sample) fixed inside the sample holder. (b) Trimming instrument used for coarse removal of material. (c) Resin block shaped into the pyramid with formed block face.

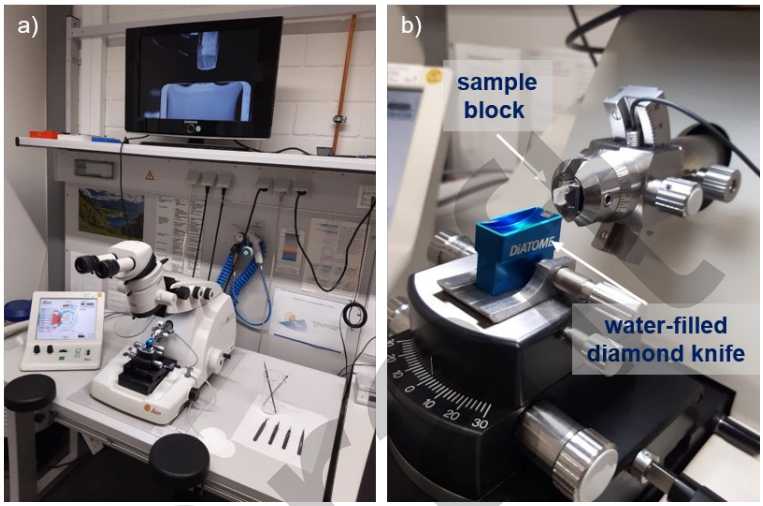

Figure 3: (a) Ultramicrotome with (b) water-filled diamond knife.

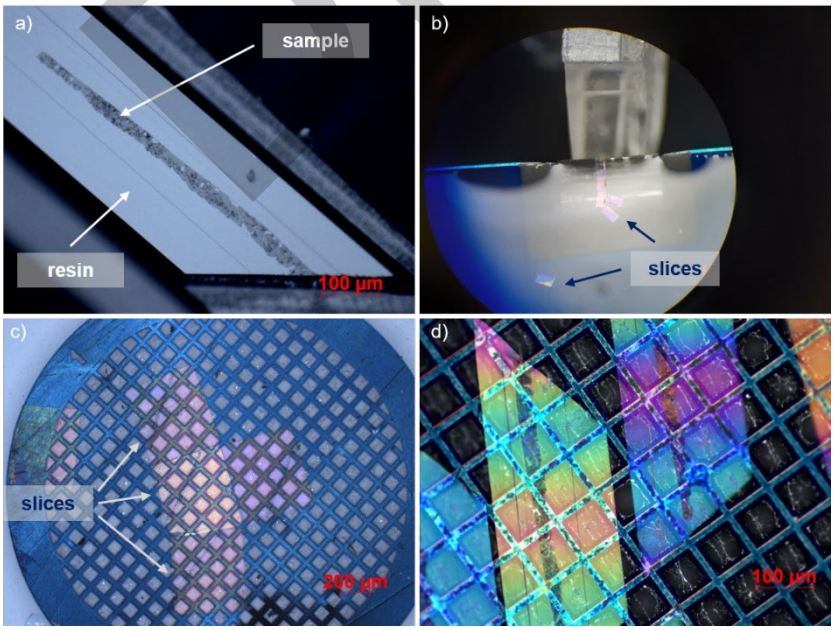

Figure 4: (a) Block face with sandwiched poly-hydroxy-alkanoates sample in resin. (b) Cut slices floating on the ultrapure Millipore water and (c-d) collected on the Cu grid covered with C lacey.

Task 2: Natural biocomposites (STU)

The interplay of the mineral distribution and the orientation of an organic matrix was studied in several naturally appearing systems, where the organic matrix is tightly intertwined with inorganic components. Here, we present the claws of a sea slater.

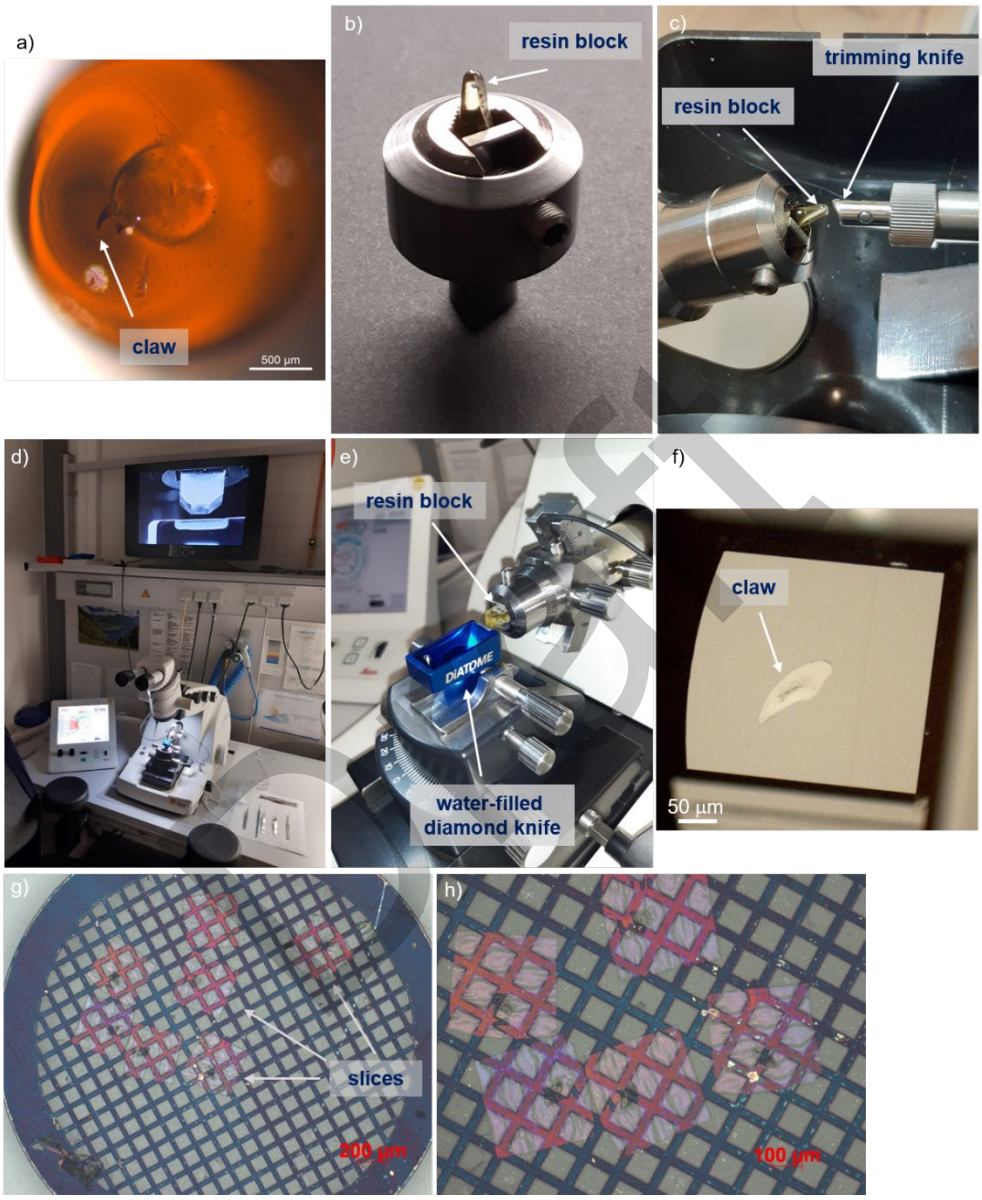

Figure 5: (a) Claw embedded in the resin block and (b) inserted into the sample holder. (c) Trimming the pyramide on the resin block. (d,e) Ultramicrotome with water-filled diamond knife. (f) Prepared block face with visible claw sample. (g-h) Slices deposited onto the Cu grid covered with C lacey.

First, the claw samples were inserted into the conical tube and covered with a resin material (Figure 5a). The viscous liquid resin should be slowly added, to retain the claw sample close to the conical tip. Afterwards, the hardened resin block was

inserted into the sample holder (Figure 5b). Corse removal of the resin block material was done with a trimming knife (Figure 5c) and the block face was created using an ultramicrotome (Figures 5d and 5f). The electron transparent slices of embedded claw samples were cut at room temperature using an ultramicrotome with a water-filled diamond knife (Figures 5d-e) and collected on TEM Cu grids covered with a lacey carbon film (Figures 5g-h).

The structural and compositional features of the claws were studied (Figure 6). The distribution of mineral components in the cuticule was determined with a combination of backscattered electron image (BSE) together with energy-dispersive X-ray spectroscopy (EDX) elemental mapping using scanning electron microscope (SEM). In the claws, the concentration of calcium (Ca) and phosphorous (P) is high in the inner layers of the cuticule. The relatively thick outer layers show elevated amounts of bromine (Br). High-angle annular dark-field (HAADF-) scanning transmission electron microscopy (STEM) imaging of these samples allowed us to determine the organization of organic and mineral components of the cuticule at the nanometer scale. We found the presence of amorphous calcium phosphate in the endocuticule and non-mineralized exocuticule with elevated amounts of Br (Figure 7). [2, 3]

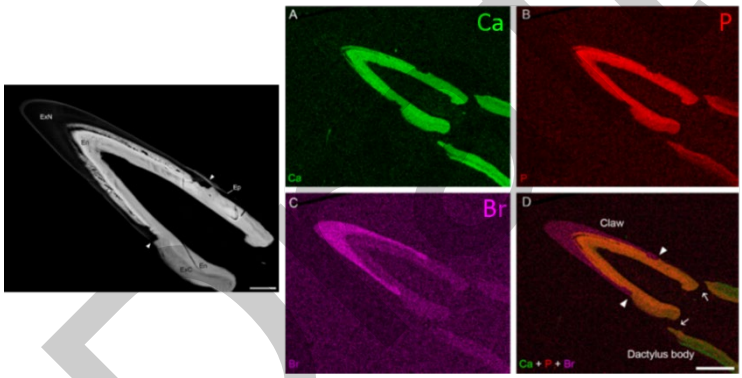

Figure 6: Backscattered electron image of a polished outer claw in longitudinal section with corresponding Ca, P, Br maps obtained by EDX.

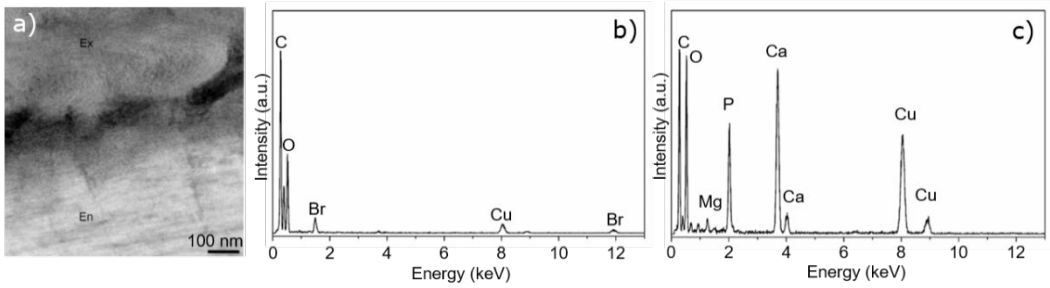

Figure 7: (a) HAADF-STEM image of the cuticule at the base of the outer claw with corresponding EDX spectra measured from the non-mineralized claw exocuticule (b) and claw endocuticule.

Task 3: Pharmaceuticals (STU+CAM)

Tabletting is a process, by which an active pharmaceutical ingredient (API) and excipient powders are compressed into a tablet form. The excipient is often chosen to allow good adhesion of the tablet. However, high pressure and mould shapes may have an impact on the spatial distribution of API/excipient inside the tablet.

Relatively little is known about the nanoscale distribution of API and excipient in tablets. This is due to difficulties in preparing thin electron transparent samples representative of a tablet cross section, and the beam-sensitive nature of the samples.

We have prepared electron transparent slices of commercially available ibuprofen using two different routes. Firstly, a piece of a ibuprofen tablet was directly mounted into the specimen holder (Figure 8a). As an alternative preparation route, the tablet was embedded in a resin (Figure 8d). Considering an extreme sensitivity and instability of ibuprofen tablets, the block face was formed with an ultramicrotome (Figures 8b and 8e). In addition, no water was used in any step of the preparation due to water solubility of the tablets. Afterwards, thin slices were cut from the tip of the pyramide and deposited on the Cu grids with C lacey (Figures 8c and 8f). [4]

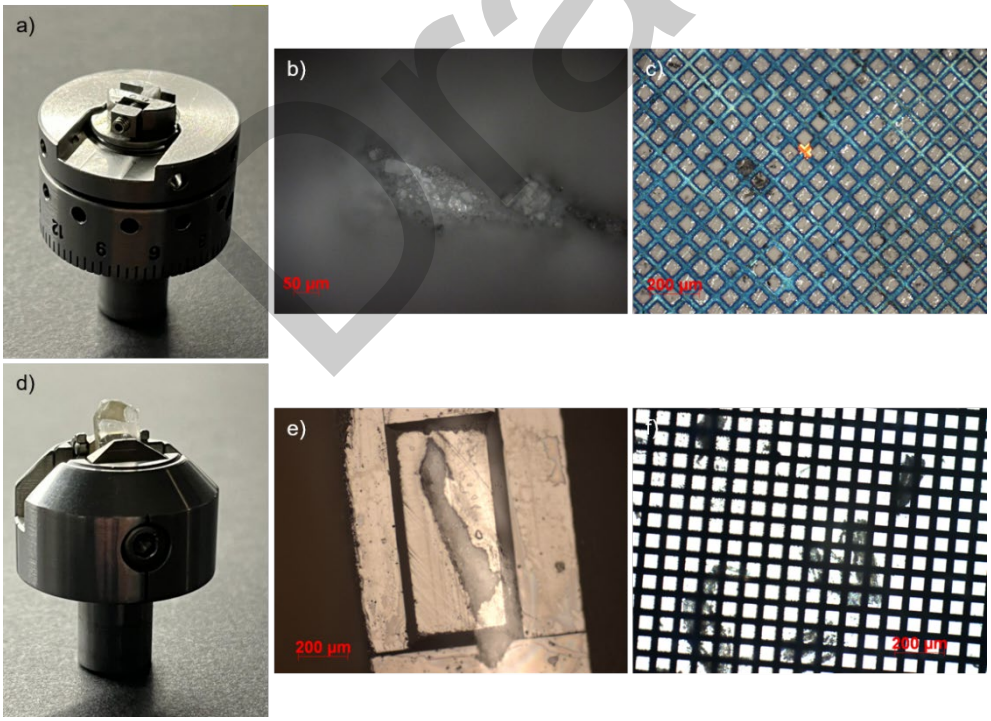

Figure 8: (a) A piece of a pharmaceutical tablet was directly fixed into the holder. (b) A block face was created and (c) thin slices of material were cut by ultramicrotome and deposited onto a Cu grid with carbon lacey. (d) Embedded pharmaceutical tabled in a specimen holder, (e) trimmed block face and (f) thin electron-transparent slices on a TEM grid.

Task 4: Dental pathologies (STU)

Studies of environmental pathologies on enamel are critical for better understanding of the origins of diseases. Focused ion beam (FIB) was used for preparation of such especially sensitive biological samples from site-specific areas. The FIB was operated at 30 kV beam energy. Coarse milling was performed using a beam current of 5 and 3 nA, and was followed by a fine milling using 1 nA. Afterward, the samples were lifted out and fastened to a Cu ring using Pt. FIB lamellae were milled from both sides at an accelerating voltage of 16 kV and 0.25 nA beam current. Several regions of the lamellae were further milled at 8 kV using a beam current of 25 pA, followed by an accelerating voltage of 5 kV and beam current of 16 pA. Final polishing was done at an accelerating voltage of 2kV and beam current 8.9 nA.

In Figure 9, a piece, cut from a diseased human tooth, is shown. Several FIB lamellas were prepared from an area, where stripes are clearly visible on the tooth surface (yellow rectangle). Bright-field (BF)- STEM images (Figure 10) show a cross-sectional view of such a diseased area showing modified microstructure. [5]

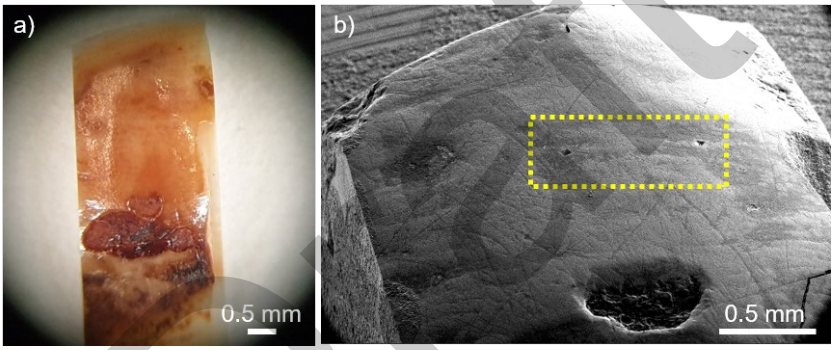

Figure 9: (a,b) Piece of diseased human tooth. (b) FIB lamellas were prepared from an area marked with the yellow rectangle.

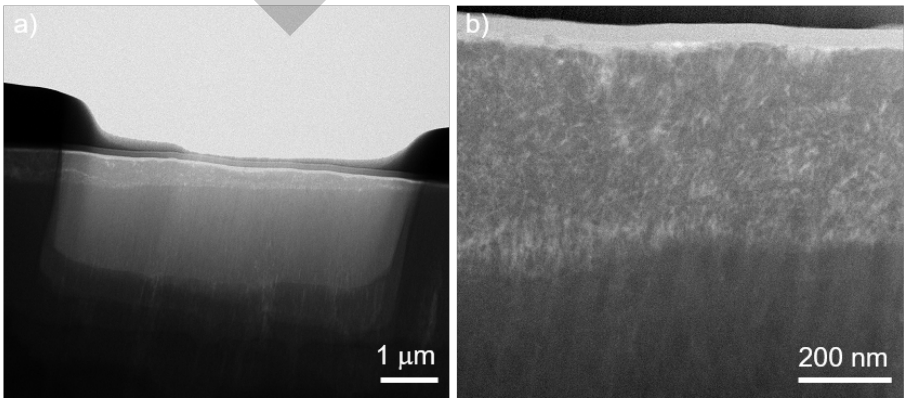

Figure 10: (a,b) BF-STEM images prepared from the site specific areas of a diseased human tooth.

Task 5: Dental tissues (STU)

Composite organic/inorganic materials are highly challenging in terms of sample preparation and TEM investigations due to different physical, chemical and mechanical properties of their constituent elements. Different polishing and milling rates represent a serious limitation that needs to be overcome. It is well known that the quality of TEM samples is often a limiting factor for successful electron microscopy investigations.

The composition of natural organic/inorganic materials could be altered during the ultramicrotomy (UM) preparation due to de-mineralization processes while electron transparent slices are floating on the water. To study whether such processes effect also dental tissues, we have compared samples that were prepared by different methods. We have optimized the preparation of electron-transparent slices from dentine areas in rodent teeth by UM (Figure 11) and a combination of UM, FIB and Nano-mill (NM) ion milling techniques (Figure 12).

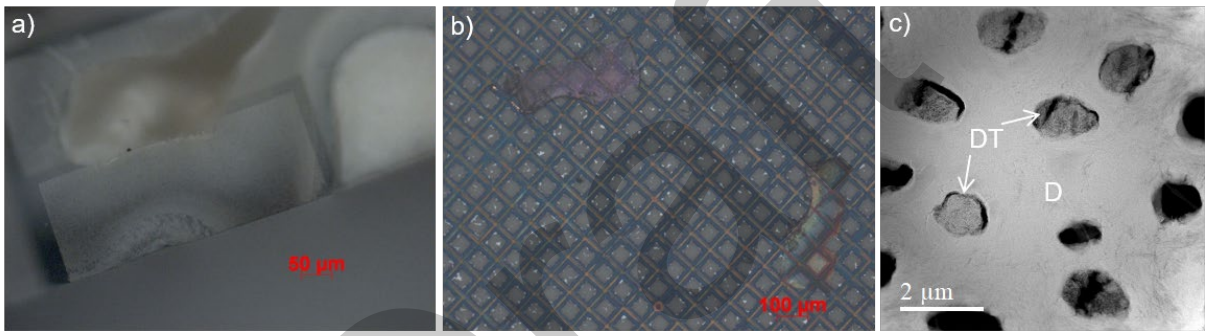

Figure 11: Ultramicrotomy sample preparation of rodent dentine, (a) showing a block face, (b) electron-transparent slices deposited on a Cu grid, and (c) dentinal tubules (DT) in dentine (D).

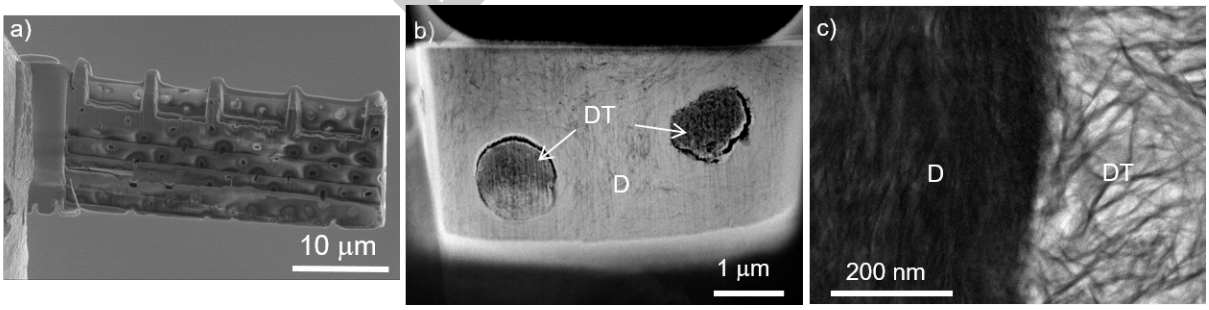

Figure 12: Sample preparation of rodent dentine (D) by a combination of UM, FIB and NM. (a) SEM image of the lamellae, (b) HAADF-STEM image and (c) BF-STEM image.

Table 1: Samples of rodent dentine prepared by UM and combination of UM+FIB+NM characterized by EDX. Quantification was done by using experimentally determined k-factors.

DENTIN (D)	**UM**	**UM+FIB+NM**
Mg (at%)	5.4 ± 0.3	4.8 ± 0.2
P (at%)	31.3 ± 1.6	37.1 ± 1.9
Ca (at%)	58.3 ± 2.9	58.1 ± 2.9

All samples have been characterized by a combination of STEM imaging and EDX (Table 1). Experimentally determined k-factors were used for the quantitative characterization of the dentine composition. We noticed only minor variations in composition when comparing samples prepared by UM and UM+FIB+NM, that could be also attributed to compositional variations in the material. Our results show that samples prepared by UM were not affected by the sample preparation and can be applied for our further measurements. Sample preparation by both approaches, UM and UM+FIB+NM, results in high quality samples exhibiting preserved microstructure and composition. UM preparation offers relatively large areas that can be used for the measurements, but has limited precision. On the other side, FIB prepared samples are site specific, yet the area sizes are incomparably smaller. [6]

References:

[1] Work in progress

[2] "*The mechanical consequences of the interplay of mineral distribution and organic matrix orientation in the claws of the sea slater ligia pallasii*". Vittori M, Srot V, Korat L, Rejec M, Sedmak P, Bussmann B, Predel F, van Aken PA, Strus J, Minerals **11** 1373 (2021).

[3] Work in progress

[4] Work in progress

[5] Manuscript under review

[6] 2 manuscripts in preparation