Title: "*Improvements in biological nano-imaging resolution with innovative sample preparation and optimized X-ray dose strategies*".

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Introduction

Imaging biological tissues plays a crucial role in various aspects of biomedical research and clinical applications. The analysis of histological alterations in tissues is of primary importance in pathology for accurate and robust diagnosis [1]. Advanced imaging techniques have enabled the study of biological tissue anatomy in three dimensions. The three-dimensional visualization of cellular and intracellular structure, of entire (without sectioning) and in the native state and at the nanometer scale is particularly desirable for gaining a more physiologically relevant understanding of disease hallmarks and cellular processes. High-resolution X-ray imaging techniques have become increasingly important for studying biological cells and tissues, offering several key advantages: X-ray imaging allows non-invasive, high-resolution visualization of thick biological specimens, enabling structural imaging of bone, metal implants, and soft tissue cavities (Chen et al., 2012). Coherent diffractive imaging (CDI) is a powerful technique, which more and more befit from ultra-low emittance sources, such as Sirius facility (LNLS-CNPEM). Ptychographic X-ray Computed Tomography (PXCT) has emerged as consistent CDI set up which provides quantitative contrast for subcellular structures observation [3], at tens of nanometers spatial resolution. However, the data resolution of such samples can be limited by radiation damage, a problem very well known for soft matter. At room temperature the ionization of chemical bonds and production of free radicals by X ray irradiation degrade biomolecules structure [4, 5]. Promising studies demonstrates that, in order to circumvent this issue, optimized protocols with resin embedded samples combined with dose optimized experiments allowed for imaging large fragments of brain tissue down to 60 nm resolution [6] (Figure 1). This is just the beginning, since tissues have different cellular compositions and therefore biochemical properties, meaning that sample specific strategies are required. Therefore, embedding media, which can properly infiltrate, preserve the sample integrity and avoid damage still lacks further investigations.

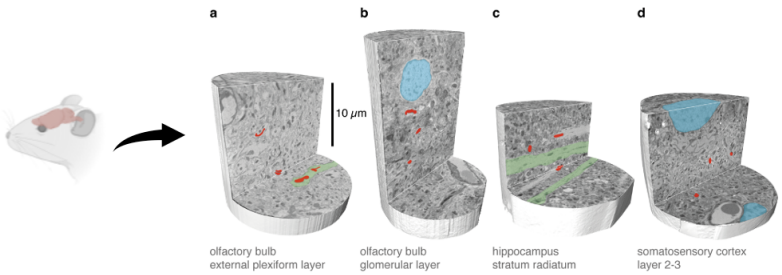

Figure 1. **X-ray ptychography tomograms for different tissues**. Brain mouse tissues imaged with radiation doses of 3.8 x 10^8 Gy, 3.8 x 10^8 Gy, 2.2 x 10^8 Gy, 1.9 x 10^8 Gy). The respective tissue samples were embedded in resin A (EMbed812) (a-c) and resin B (Epon812) (d). Subcellular features including dendrites (green), nuclei (blue) and mitochondria (red) are indicated. Adapted from [6].

State of the art

At present, the resins employed in electron microscopy are well established and specifically tailored for this application, which requires a notably different penetration capability than that of X-rays. Uncertainties remain regarding the optimal methods for preserving the native state, making sample preparation a significant challenge for achieving high-resolution imaging, such as PXCT. From a data collection standpoint, there is a lack of experiments examining the relationship between imparted dose and resolution in tissue samples. Consequently, it is crucial to conduct systematic investigations into effective embedding medias which withstand the ideal imparted dose minimizing the histological structure disruption and yielding higher-resolution state of the art from PXCT. For such end we intend to use cardiac and aorta vessels, since these tissues may demand the nano-architecture knowledge in order to comprehend critical diseases such as coronary disfunction and aortic aneurism.

Aims

The project focuses on creating methods to acquire high-resolution PXCT images while maintaining the original histo-architecture. To accomplish this, the sample will be subjected to resin embedding techniques and evaluated under different experimental conditions at the CATERETE beamline. By utilizing tomographic reconstructions and image segmentation, the best conditions will be determined.

Methods

The proposed strategies to proceed with these studies is summarized at Figure 2 and described in detail below.

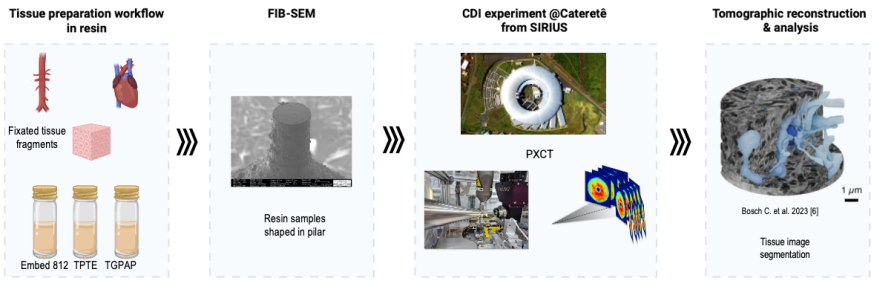

Figure 2. Summary of the experimental workflow to be tested. The different steps are represented, including the sample preparation embedded in resin and data collection at different X-ray doses at CATERETE beamline, from SIRIUS (LNLS-CNPEM).

a) Tissue material

Biological tissues related to heart and vessels will be provided by a collaboration with the Heart Institute (InCor) hospital (Dr. Ayumi Miyakawa). The samples will be dissected according to the group's protocols and the sample will be delivered at the proper chemical fixation agent. The 1 x 1 x 1 mm^3 tissue fragments will be first stained with reduced osmium (2% OsO_4, 3% potassium ferrocyanide, 2 mM $CaCl_2$) and dehydrated with increasing ethanol solutions (75,

90, 2 × 100%), transferred to propylene oxide, and infiltrated with the different resins mixed with propylene oxide in increasing concentrations (25, 50, 75, 2 × 100%). Finally, the resin and polymerized individually for 72 h at 70 °C.

b) Resin embedding

At least 3 distinct embedding polymers will be utilized in this study, Embed 812, TPTE and TGPAP. Each resin is derived from a mixture of monomers at specific ratios, subsequently polymerized at 70°C for approximately 72 hours. Different hardeners will be used.

c) Histology

Prepared embedded samples will be previously analyzed with optical microscopy to check integrity after the infiltration process. The sample blocks will be cut down to 10-15 um-thick with a rotative microtome (Histocore Autocut, Leica) and evaluated with bright field optical microscopy (LMD7, Leica), using the infrastructure from LCRIO (LNLS). The rest of the block will be used for the FIB-SEM samples.

d) FIB-SEM

Samples will be shaped to 30 to 50 um diameter pillars using a Xenon-FIB (Helios 5 PFIB CXE Dualbeam) available at LAM-FIB (LNLS) and transferred to CATERETE metallic pin, using the equipment micromanipulator and fixed with carbon deposition.

e) PXCT

The PXCT data will be collected at the CATERETE beamline. The 3D reconstructions of the samples will be aimed for spatial resolutions of 50 nm. Image segmentation and analysis will be performed using the TEPUI facility at LNLS with Avizo software.

Timetable

	Literature review	Resin embedding	Validation with sample histology	FIB preparation	PXCT measurements	Image analysis
First semester	**x**	**x**	**x**			
Second semester		**x**	**x**	**x**	**x**	**x**

References

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