EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

Experiment Report Form

	Experiment title: X-ray 3D imaging-based evaluation of therapeutic strategies to slow down neurodegeneration in models of retinal ganglion cell degeneration	**Experiment number**: MD1258
Beamline: ID17	**Date of experiment**: from: 03.02.2022 to: 05.02.2022 (FIRT SESSION, second session scheduled in July 2022)	**Date of report**: 04.03.2022
Shifts: 6 of the15 granted	**Local contact(s)**: Herwig Requardt, Marina Eckermann	*Received at ESRF:*

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Report:

NOTE: This is a preliminary report. This beamtime is split into two experimental sessions. The first one was realized in Febbruary 2022 and the second one is scheduled in July 2022.

- **Objective of the proposal**

The project aims at studying the potential therapeutic effects of idebenone and mGlu1 receptor antagonists in experimental models of glaucoma by using a high-sensitivity microscopic approach based on X-ray phase-contrast micro-CT (X-PCI-CT) for multiscale, post-mortem 3D virtual-histology. Neuroprotection is demanded in eye disorders such as glaucoma, where death of retinal ganglion cells (RGC) takes place through different mechanisms (1-5). Targeting mitochondria and mGlu1 receptors could be a potential disease modifying treatment for optic neuropathies where no treatment is currently available.

- **Scientific and experimental backgroud**

Monitoring the effect of treatments with a non-destructive and full-organ approach is key for the comprehension and the assessment of new therapeutic strategies. X-PCI-CT is a powerful technique for tissue-conserving virtual histology (6,7), sensitive to electron density variations, label-free and multiscale. X-PCI-CT data can serve for quantitative analyses of neuroinflammation markers such as activated microglia, of degenerating shrunk neurons and of vascular features within the retina. Upon standard histology, X-PCI-CT can visualize structures in 3D and allow virtual sectioning in the 3 main axes without cutting, staining and processing every single slice. Furthermore, Synchrotron X-PCI-CT can depict soft tissues with contrast and spatial resolutions that cannot be reached with post-mortem MRI imaging or standard micro-CT. Previous works of our group include the development (8) and application (6,9) of multi-scale synchrotron X-PCI-CT virtual histology of organs from small animal models, of vascular, nervous and anatomical details as well of entire monkey eyes (10).

- **The experiment**

Sample preparation (performed at the IRCCS Neuromed Institute, IT)

We use a genetic model: DBA/2J mice spontaneously developing glaucoma with pigment dispersion (11) and an iatrogenic model: dexamethasone (DEX)-induced glaucoma (12). DBA/2J male mice are treated daily with vehicle and idebenone (10 mg/kg/day) by eye drops, or vehicle and JNJ16259685 (1 mg/kg/day) by using subcutaneously implanted osmotic minipumps (Alzet, model 2004), either in the pre-symptomatic (at 7 months of age) or in the symptomatic phase after the increase of IOP (weekly measured by a tonometer) for 2 months. Mice with DEX-induced glaucoma are treated with idebenone and JNJ16259685 with the same protocol for DBA/2J mice but only after increase of IOP. We will confirm RGC density by immunohistochemistry of Brn-a, and activated microglia by immunoreactivity for Iba-1 in the contralateral eye. Retinal vasculopathy is examined by CD31 immunostaining. Mice are killed at the end of treatments and eyes (3-4 mm in diameter) are dissected out, formalin fixed and paraffin embedded for imaging at the ESRF and immunohistochemistry. The experimental groups of this study are: DBA/2J mice treated with vehicle, idebenone or JNJ16259685 in the pre-symptomatic phase; DBA/2J mice treated with vehicle, idebenone or JNJ16259685 in the symptomatic phase; DEX-induced glaucoma mice treated with vehicle, idebenone or JNJ16259685; mice without glaucoma (controls) treated with vehicle, idebenone or JNJ16259685.
In this experiment, we examined the eyes of the animals groups in the pre-symptomatic phase. The total number of samples was 39. During the 6 shifts of this first session, we managed to image 26 specimens.

X-ray Phase contrast imaging set-up and experimental parameters

The propagation-based X-PCI-CT technique was used with a multi-scale setup. The experiment was performed An X-ray pink beam with mean energy of about 40 keV was set. The PCO.Edge5.5 sCMOS camera was used as imaging sensor coupled with indirect-conversion optics systems affording different magnifications, i.e. voxel sizes of 3.5^3-0.7^3 μm^3. The $3.5^3 \mu m^3$ voxel size was used for a full organ coverage, for centering the samples and targeting the regions of interest to be imaged then with the $0.7^3 \mu m^3$ voxel size. During the CT acquisition, we observed some sample degradation due to the radiation. Thus, the X-ray spectrum was made harder, the photon flux was decreased and the exposure time was increased. These changes partly helped but the did not solve the problem. As a consequence, several dataset are affected by artefacts. A slightly different sample preparation and the use of a cooling system integrated into the CT sample stage (already tested in another experiment) are already foreseen.

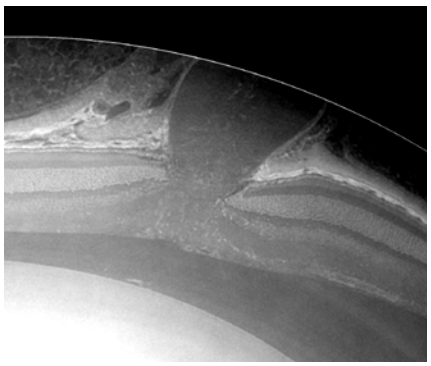

Figure 1 Sagittal view of the optic nerve insertion; on the bottom left the eye lens is visible. Around the insertion of the optic nerve, the retina, choroid and the sclera layers are visible.

- **Data analysis and preliminary results**

The data reconstruction and processing is ongoing. Figure 1 reports an example of preliminary results featuring anatomical details around the optic nerve insertion. Sample preparation plays a pivotal role in the successful visualization of the structures of interest. Strong image artefacts have been observed due to the presence of air in the surrounding medium (paraffin) and due to radiation degradation (heating) and technical solutions to be employed in the next session have been already identified. Parts of these measurements need to be repeated.

In the following data analysis, morphological tissue alterations derived from glaucoma and the treatment, such as RGC density and retinal vasculopathy (vessel analysis) will be quantified via appropriate (semi-) automatic algorithms.

- **Concluding remarks and acknowledgements:**

These first measurements allowed testing technical aspects related to samples preparation and data acquisition. Preliminary results have been promising and demonstrated that the methodology can visualize the entire retinal structure and discriminate its different layers. To complete this research project, an additional beamtime is needed to analysis all the specimens of the different experimental groups and make the study statistically significant. In addition, we plan to use X-ray nanoholotomography to image and quantify lesions at subcellular levels in the retina of mice with increased intra ocular pressure. Two new experimental proposals have been thus submitted recently.

We are grateful for the help provided to us by the local contacts and the teams of ID17. The multiscale CT imaging procedures worked efficiently.

References: 1) Chhetri J & Gueven N. Expert Opin Ther Targets 2016, 1-16. 2) Haefeli et al. PLoS One 2011, 6:e17963. 3) James et al. J Biol Chem 2005, 280:21295-21312. 4) Rauchova et al. Physiol Res 2012, 61:259-265. 5) Liberatore et al. Neuroscience 2017, 363:142-149. 6) Barbone et al. IJROBP 2018, 101:965-984. 7) Bravin et al. Phys Med Biol 2013, 58:R1-35. 8) Mittone et al. J Synchrotron Radiat 2020, 27. 9) Barbone et al. J Neurosci Meth 2020, 339:108744. 10) Mittone et al. Physica Medica, 2018, 51:7-12. 11) John et. Investig Ophthalmol Vis Sci 1998, 39:951-962. 12) Liu et al. Curr Eye Res, 2017, 42:1124-1129.