ESRF	**Experiment title:** Study of the phrenic motor neurons (PhMN) network in the healthy and injured ex-vivo mouse spinal cord using X-ray phase contrast micro-tomography	**Experiment number**: **MD-1041**
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Report:

The phrenic motor neurons (PhMN) pool is located in the cervical region of the spinal cord (levels C3-C5) and connects the bulbar respiratory group to the diaphragm. This explains why traumatic spinal cord injury (SCI) cases affecting the cervical region usually lead to death, being the respiration compromised. In particular, following mid-cervical SCI, initial insult results in immediate neuronal death and axotomy of neuronal projections [1,2]. Acute stages are followed by a period of secondary cell degeneration and a rostro-caudal extension of the lesion along the gray and white matters, which produces additional functional deficits [1-3]. However, information about the 3D spatial arrangement of PhMN in humans and rodents is very poor and restricted to columnar organization extending from C3 to C5. Although the recent and seminal studies from Nicaise et al. on some rodent models of cervical C4 SCI [1,2] mimicking histopathological and functional respiratory outcomes observed in SCI patients, a morphological analysis capable to visualize the integrity of lateral or rostro-caudal projections on long distances is still lacking. Moreover, the experimental procedure proposed in [1,2] limits the study to 2D structural arrangement of PhMN and a 3D reconstruction should be feasible only by sectioning the sample. As recently demonstrated [4], Synchrotron X-ray micro-tomography (SXrPCμT) has revealed to be a very promising tool to visualize the 3D architecture of the mouse spinal cord neuronal network at scales spanning from millimetres to hundreds of nanometers, without contrast agents and without sectioning or other destructive sample preparation procedures. In particular, nerve fibres, axon-bundles, and neuron soma have been imaged and have resulted to be easily distinguishable at the white/gray matter interface in the ventral horn of spinal cord. In such a framework, the aim of this experiment was to employ SXrPCμT in order to non-invasively image the location and connections of the PhMN pool inside the respiratory neural network, with a sufficiently high resolution and contrast not provided by other non-invasive imaging tools currently widely employed (e.g. MRI).

The samples used in this experiment were spinal cords (length: 8 mm, diameter: 2 mm) obtained from 15 euthanized mice (male C57BL/6, 10-12 week old). Five spinal cords were injured, five C4-contused at acute stage (2 days post-injury) and five C4-contused at subacute stage (2 weeks post-injury) [5]. All the 15 spinal cords extracted from the mice were fixed in 2% paraformaldehyde + 0.2% glutaraldehyde. In addition, two weeks before sacrifice, animals received intrapleural injections of retrograde fluorescent tracers (a cocktail of monosynaptic tracer cholera toxin β and transsynaptic tracer wheat-germ-agglutinin), that was helpful for visualizing first or second order respiratory neurons. The experiment was performed in line mode, using a 35.0 keV monochromatic X-ray beam. Tomographic images were acquired at 2.0 m from the sample, using the PCO.5.5 camera with a pixel size of about 3 μm. Samples were included in plastic containers filled with agar-agar and 2000 projections were collected for each tomography. After the application of a proper reconstruction algorithm and a preliminary stage of image analysis, we have obtained promising 3D images of the PhMN network of *ex-vivo* cervical spinal cord. We report our preliminary results in Figure 1 and Figure 2. More specifically, Figure 1 shows the arrangement of neurons in a healthy spinal cord; the availability of these images allowed us to study the spinal cord morphology at a large scale, as well as the neurons distribution at the gray/ white matter interface. Figure 2 highlights the injury, as visible in the neuronal network. These results demonstrate that our experimental approach is able to visualize the 3D spatial organization of neuronal network in the cervical region of spinal cord, giving significant hints for a pre-clinical *ex-vivo* investigation of SCI.

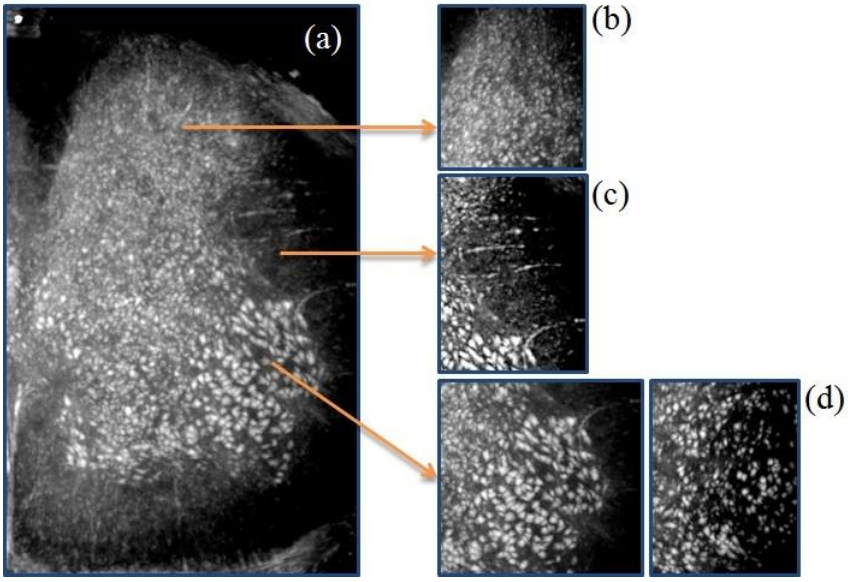

Figure 1: **(a)** A representative 3D reconstruction of a ~400μm-thick volume (axial section) in the cervical region of a healthy spinal cord is reported. The striking contrast between white and gray matter (reversed colors in the figure) allows to image the neuronal cell bodies, appearing as white spots in the gray matter, and the nerve fibers surrounding the gray matter. **(b)** Enlargement of the spinal cord dorsal horn where interneurons are clearly visible (small white spots). **(c)** Enlargement displaying neuron fibers in the region between the dorsal and lateral horns. **(d)** Enlargement of the spinal cord lateral and ventral horns showing a pool of motor neurons (white spots).

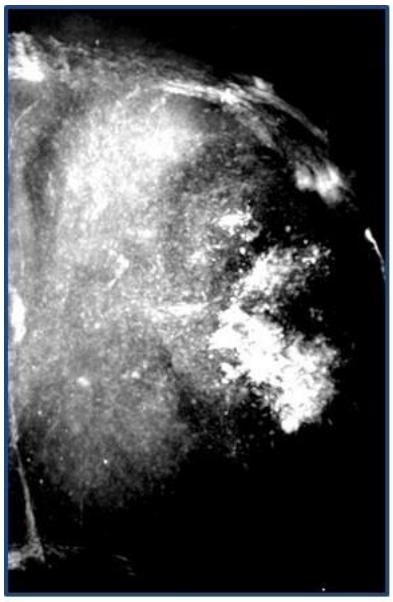

Figure 2: A representative 3D recontruction of a ~400μm-thick volume (axial section) around the same cervical region reported in Figure 1 for an injured spinal cord is displayed. An anomalous distribution of the neuronal network with respect to the healthy case, and the presence of a sort of "bump", can be regarded as marks of the precence of the injury.

References

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[4] Fratini M. et al, SRP 5, 8514 (2015).
[5] Nicaise C. et al. J Neurotrauma 29 (18), 2748-2760 (2012).