ESRF	Experiment title: 3D CHARACTERIZATION OF VOCAL-FOLD FIBROUS ARCHITECTURE AND MECHANICS USING REAL-TIME AND MULTISCALE SYNCHROTRON X-RAY MICROTOMOGRAPHY	**Experiment number**: MD-957
Beamline: ID19	**Date of experiment**: from: 13 June 2016 at 08:00 to: 16 June 2016 at 08:00	**Date of report**: 08/09/2016
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Report:

• Objective

Proposal MD-957 aimed at characterizing the macroscale and microscale architectures of fresh human larynges and their vocal-fold tissues, once subjected to anatomical placements and mechanical loadings close to those occurring during a typical phonatory cycle. To our knowledge, no similar study has been previously conducted on excised larynges using multiscale synchrotron X-ray microtomography.

Within this context, two steps were initially planned:

Step 1. To characterize the *changes in the macro- and microstructures of the larynges and their vocal folds depending on the macroscopic stretch applied to the vocalis muscle*, responsible for pitch transitions during phonation. In that step, the vocal folds were attached to the surrounded laryngeal cartilages.

Step 2. To characterize the *mechanical behavior of the vocal fold fibrous networks and their strain-induced microstructure evolutions*, after excision from the surrounded laryngeal cartilages.

To this end, **9 shifts were initially required: 6 for the scans on larynx samples, 3 for those on excised vocal folds. Within the allocated time (6 shifts), Step 1 was successfully accomplished and tests of feasibility for Step 2 were demonstrated**.

The present report describes the sample details, the mechanical set-up developed and used during the experiment, the optical set-up and the first promising results obtained from this session.

• Sample details

The experiments were carried out on 6 fresh *ex vivo* human larynges (see details in Table 1). Anatomical pieces came from donated bodies and were excised at the *Laboratory of Anatomy of the French Alps* (LADAF – UGA, CHU Grenoble). Their excision and transportation are regulated in the frame of Body Donation (after written consent given by the donor prior to death). Safety aspects were regularized with the ESRF Safety Office for Biology and Biochemistry. All samples were confined in sealed and sterilized chambers regulated at proper hygrothermal conditions.

Sample name	N° body	Gender	Age (y)	Weight (kg)	Size (m)	Conservation
Test : Larynx L04	29.2016	Female	90	50	1.45	Frozen after excision
Larynx L05	16.2016	Female	85	40	1.60	Frozen after excision
Larynx L06	17.2016	Female	89	75	1.60	Frozen after excision
Larynx L07	13.2016	Male	81	40	1.60	Frozen after excision
Larynx L08	15.2016	Male	89	80	1.80	Frozen after excision
Larynx L09	21.2016	Female	75	80	1.55	Frozen after excision

Table 1: Details on the ex vivo larynges and the donors

• Mechanical set-ups

Three mechanical set-ups were developed specifically for this proposal and previously tested in the partner's laboratory experiments:

Set-up n°1 - Based on our expertise to analyse the physical properties of phonation in excised larynges (Hanna, 2014), the first set-up was designed to subject the vocalis muscles (fixed onto *fresh* larynges) to a macroscopic stretch, so as to reproduce phonatory placements and elongation of the vocal folds.

Set-up n°2 - The second set-up was a sample handler dedicated to the conditioning of *frozen* vocal-fold samples in ultra-low temperature. This was meant to provide an image reference as compared to *fresh unfrozen* samples.

Set-up n°3 - The third set-up was a tension/compression device developed at 3SR Lab for the 3D *in situ* X-ray imaging of fibrous materials (Viguié et al., 2013). It has been adapted in order to properly subject the *fresh* excised vocal folds to tensile loadings close to that encountered during phonation.

All three set-ups included technical constraints (i) to minimize X-ray artefacts and attenuation, (ii) to confine samples into sealed and sterilized chambers regulated at proper hygrothermal conditions. The required developments were funded by CNRS and UGA. **Set-up n°1 was dedicated to the step 1 (experiments at the larynx scale), and set-up n°2/3 were dedicated to step n°2 (experiments at the excised vocal-fold's scale). Only set-ups n°1 and n°2 could be used during the allocated time.** Both are illustrated in Figure 1 and Figure 2 respectively.

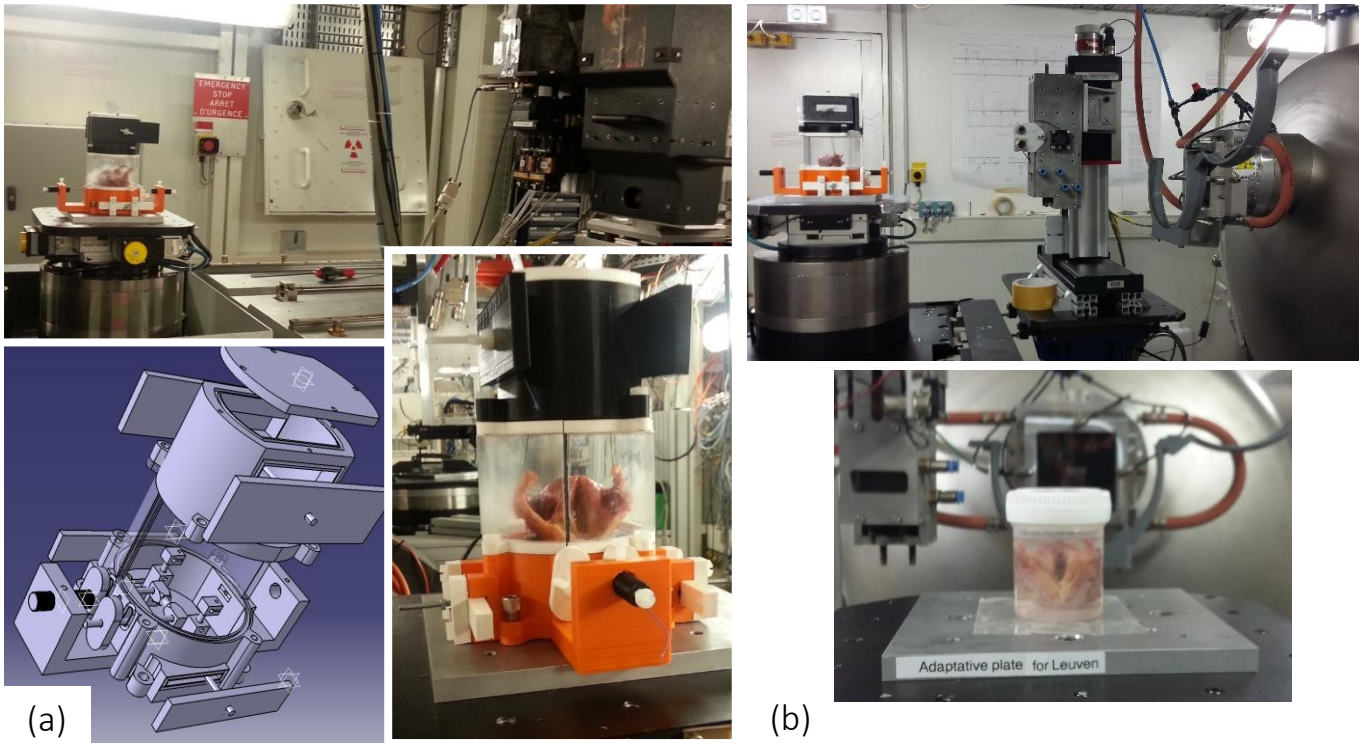

Figure 1: (a) top: Picture of mechanical set-up n°1 positioned onto the ID19 X-ray tomograph, in hutch 1. bottom: 3D view of the set-up for 3D printing processing and zoom on the processed device; (b) top: picture of mechanical set-up n°1 positionned in hutch 2 (mono hutch). bottom: zoom on the sample, immersed in alcohol.

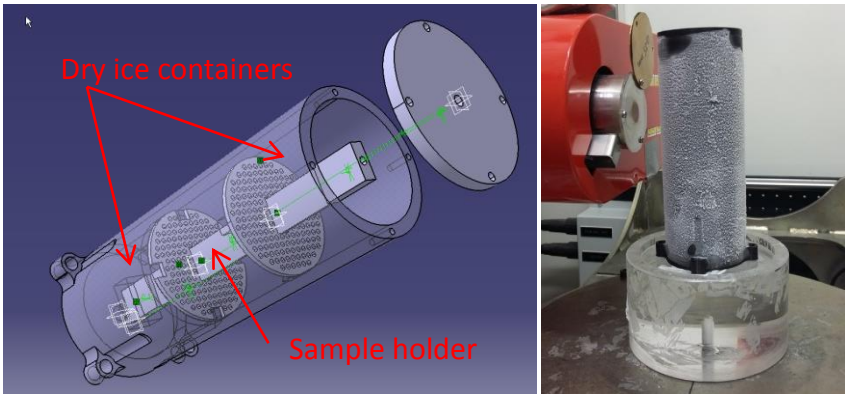

Figure 2: (a) left: 3D view of the set-up n° 2, for 3D printing processing; (b) right: picture of the final device, filled in with dry ice and containing a vocal-fold sample (on 3SR Lab's tomograph).

- **Optical set-ups**

For step 1 (experiments at the larynx scale)

Sample Larynx_04 was dedicated to the preparation and assessment of the optical set-ups. All samples were fixed in the mechanical set-up n° 1 in one static phonatory position, and placed onto the ID19 X-ray tomographs as shown in Figure 1. The **MR imaging set-up** was used.

Vocal-fold constitutive layers vary between 50 μm (epithelium), 1-2 mm (lamina propria) to 8 mm (vocalis muscle) in thickness. Within these layers, collagen and elastin fibers bundles are around 0.5 to 20μm in diameter. Therefore, measurements were planned to be acquired with a *multi-resolution approach*: 12.3μm to visualize the tissue multilayered arrangement; 0.65 μm to focus on the fibrous networks.

First series of successful acquisitions (3 shifts)

A first series of scans was acquired with the mechanical set-up placed in **hutch 1**, so that the distance between the sample and the cameras was around $\mathbf{x_c = 30\ cm}$ (see Fig. 1a). The X-ray beam was adjusted using filters (Al – thickness 2.8mm, Cu – thickness 0.14mm), an average **energy of 65keV** and a wiggler gap

of 95mm. The LUAG500 Scintillator and FRELON caméra (2048x2048 pixel, pix. 14 µm) was used for **half-acquisition at 12.3µm resolution** and **phase retrieval (Paganin) imaging mode** (4900 projections). **Samples L05 to L08 were successfully scanned** in this configuration during 24h (3 shifts). **Two extreme phonatory positions** were acquired sequentially for each sample: at rest in a first time, and at maximal macroscopic stretch of the vocalis muscle in a second time. Typical illustrations of the obtained 3D images are shown in Figure 3. A relevant macroscale description of the strain-induced geometries of the larynx components (cartilages, muscles, soft tissues) for several physiological macroscale configurations is under study thanks to this original database.

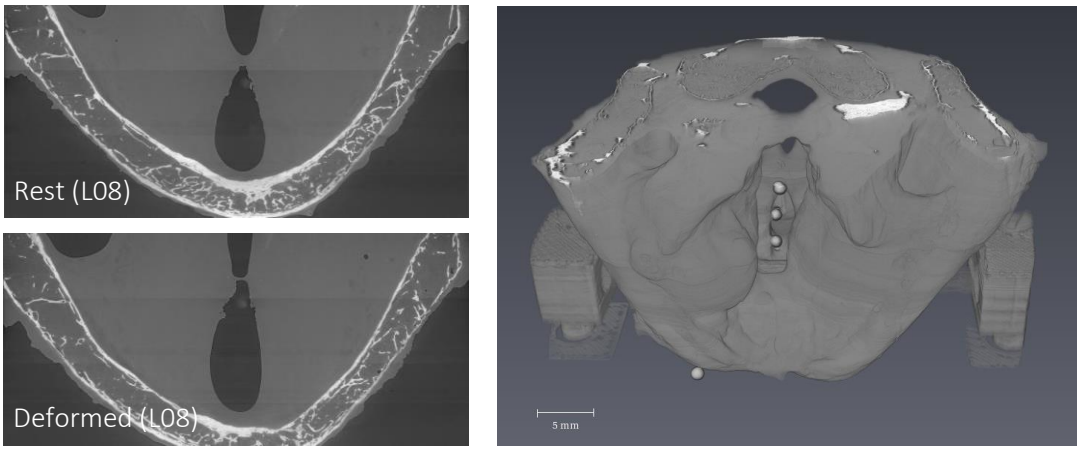

Figure 3: (left) 2D views of L08's vocal-fold plane at two phonatory positions (right) 3D reconstruction of sample L05.

Difficulties

- Due to the very high absorptive heterogeneity of the larynx (cartilages, soft conjonctive tissue, muscle), the sequential multiresolution imaging mode could not allow the fine microscale characterization (placement, content, orientation, deformation) of collagen and elastin fibrous networks within the vocal folds as expected (fibers bundles being 0.5 to 20µm in diameter). **Several acquisitions at 0.65µm have been tested using the PCO Edge Camera (2500x2100px, pix. 6.5 µm), by changing the energy level (high, low), the sample conditioning (humid, dry), yet without any success to better track the vocal-fold fibrous networks**.
- Unfortunately, time loss was also due to several difficulties not related to the sample or measurement strategy itself (interruption of the beamline for about 4h during the first night)

Second series of successful acquisitions after pre-tests (1,5 shift)

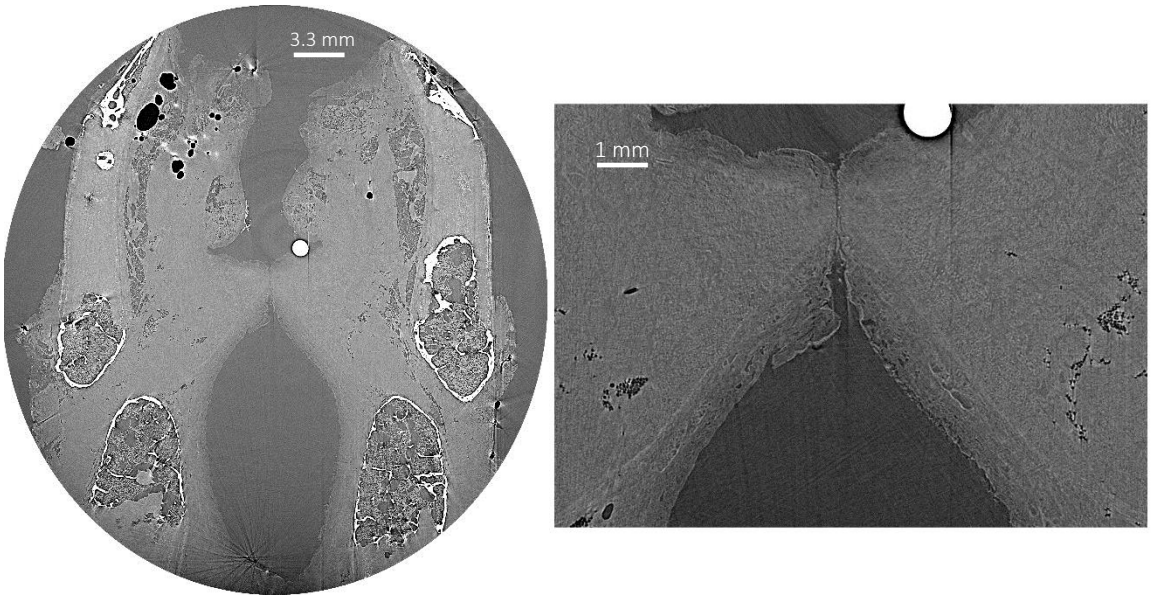

Figure 4: (left) X-ray tomographic image of larynx sample L09 (vertical 2D slice); (right) zoom on vocal-fold area

Facing the difficulties mentionned above, a new procedure has been chosen for the 2nd day : a second series of scans was acquired with the mechanical set-up placed in **hutch 2** ("mono hutch"), so that the distance

between the sample and the cameras was around $\mathbf{x_c}$ **= 11m** (see Fig. 1b). Filters were updated (2.8mm Al, 0.28mm Au, 0.7mm Cu), the average energy was kept at around 60keV, and the wiggler gap at 61mm. **Sample L09** was scanned in this configuration (one static position), using **half-acquisition at 12.3µm and Paganin imaging mode** (4900 projections). This configuration yielded to a **real improvement in the phase contrast imaging**. To limit artefacts on the structures' borders, the sample was immersed in alcohol, as shown in Figure 1b (bottom). Typical obtained images are illustrated in Figure 4.

Summary: the images acquired during this first session (4,5 shifts) will allow both the knowledge on the complex anatomy and histology of the larynx and the recent micro-mechanical models of the vocal folds to be improved (Miri et al., 2013; Cochereau et al., 2016). In particular, the maximal strain subjected to the tissue in physiological phonatory conditions is currently under study thanks to this first original database (PhD Thesis of T. Cochereau).

For step 2 (experiments at the vocal-fold scale)

For the time left (1,5 shift), the optical set-up has been changed to work at a higher resolution **(0.65µm)** on the excised vocal-fold samples (excision done between day 1 and day 2 at the CHU-Laboratory of Laboratory of Anatomy of the French Alps). The acquisition parameters were finally characterized by: a 19keV energy, the use of a new camera GOLD PCO Edge (powerful equipment yet acquired one week before the measurement), x_c = 50/40mm, number of projections 2000.

A **series tests of feasibility** was then carried out in this configuration, on one *frozen* vocal-fold, one *fixed* vocal-fold sample and one *fresh* vocal-fold sample previously immersed in alcohol (from L04 and L07).
Conclusions were as follows:

- The **investigation of the frozen sample using the mechanical set-up n°2 was not suitable to high-resolution recordings** (where the sample is close to the optics): the temperature of the frozen device was not adapted and had consequences on the focus of the optical set-up, which could not be corrected in time (time loss of about 4h).
- **However, the 16 tests of feasibility to detect the fine microstructure of two vocal-fold samples (under fixed or fresh conditions) have been very successful**: **they all conducted to few, but unprecedented and very promising 3D images of collagen and/or muscular fibrous networks within the vocal folds (see typical results in Fig. 5)**. However, within the allocated time, it was not possible to validate these observations from other samples, nor to stretch *in situ* vocal-fold tissues to observe the deformation of collagen networks (as initially planned).

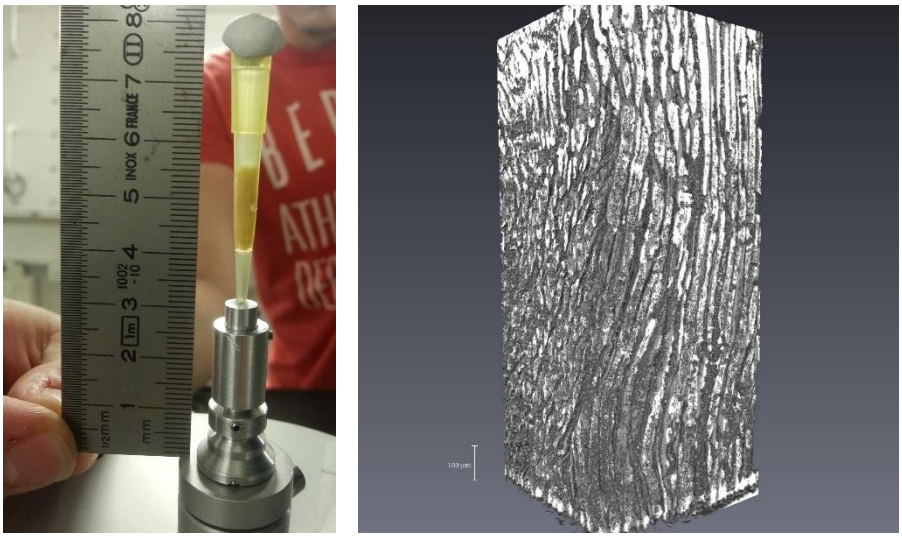

Figure 5: (left) Conditioning of a fresh vocal-fold sample prepared for a test of feasibility in high-resolution acquisition; (right) 3D reconstruction of the collagen fibrous network within the sample's upper layer

References

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