

Cryo-imaging of Inflated Frozen Human Lung Sections at -60°C using Multiphoton and Harmonic Generation Microscopy

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Rationale: Lung is a complex gas exchanger with interfacial area (where the gas exchange takes place) is about the size of a tennis court. Respiratory functions of lung are linked to the biomechanical stability of the gas exchange or alveolar regions which directly depends on the spatial distributions of the extracellular matrix such fibrillar collagens and elastin fibers. It is very important to visualize and quantify these fibers at their native and inflated total lung capacity conditions in order to have correct morphometric information in the healthy and diseased states. This can be only achieved in *ex vivo* states by imaging directly inflated frozen lung sections. Multiphoton microscopy, which uses ultra-short infrared laser pulses as the excitation source, produces multiphoton excitation fluorescence (MPEF) signals from endogenously fluorescent proteins (e.g. elastin) and induces specific second harmonic generation (SHG) signals from non-centrosymmetric proteins such as fibrillar collagens in fresh human lung tissues [1-3]. Here we report 3D image data obtained from directly from thick frozen inflated lung specimens (~0.7 millimeter thick) visualized at -60°C without prior fixation or staining. **Objective:** To visualize and quantify the spatial distribution of fibrillar collagens and elastic fibers in inflated frozen lung specimens in healthy and COPD. **Method:** Normal lungs (n=4) donated for transplantation and released for research when no appropriate recipient was identified, and the diseased lung specimens donated for research by patients receiving lung transplantation for very severe COPD (n=4) were prepared as previously described [4]. Lung slices (approximately n=8/lung) evenly spaced between apex and base were examined using multiphoton microscopy while maintained at -60°C using temperature controlled cold stage with a temperature resolution of 0.1°C . Infrared femto-second laser pulses tuned to 880nm, dry microscopic objectives with long working distances, and non-de-scanned detectors/spectrophotometer located in the reflection geometry were used for generating the 3D images/spectral information. At least five 3D stack images (representing $1200\mu\text{m} \times 1200\mu\text{m} \times \sim 700\mu\text{m}$ thick lung volume) with optical slice thickness of approximately $2.5\mu\text{m}$ were captured from each specimen. The volume fractions of fibrillar collagens and the elastin, the alveolar wall thickness, and fiber size distributions were computed from the 3D image data sets. **Results:** In the healthy lung alveoli, the 3D images showed that the anisotropic collagen and isotropic elastin structures were strongly associated and encircling at the walls of the alveoli. In these healthy lungs, the structural features with regard to alveolar shape, wall thickness and fiber distributions were found to be nearly identical from apex to base. In sharp contrast, COPD lung sections in the early stages of alveolar disruptions showed extensive remodeling particularly with regard to fibrillar collagens and considerable dissociation of collagen and elastin fibers with significantly increased alveolar wall thickness compared to the healthy lungs. In addition, COPD lungs showed considerable variations with regard to fibrillation, alveolar wall thickness, alveolar geometry, and fiber distributions from apex to base. **Conclusions:** The SHG and MPEF methods were successfully performed on the inflated rapidly frozen lung specimens at -60°C

without prior fixation or staining. The same tissues subjected to the cryo-imaging can be preserved for other experiments including other modalities of imaging and gene expression studies. We found that this novel imaging approach can provide spatially resolved 3D images with spectral specificities that sensitive enough to identify and quantify the micro-structural details of fibrillar collagens and elastin in alveolar walls in healthy and diseased states [5].

References:

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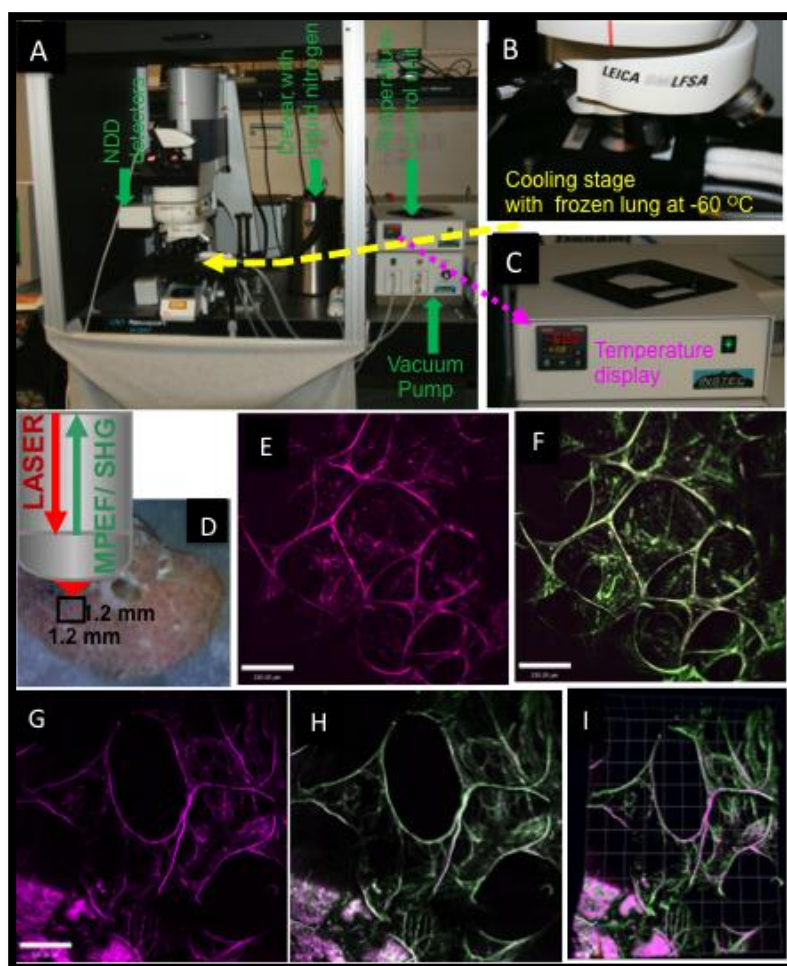


Figure 1: Schematic of multiphoton microscope system capable of detecting both MPEF and SHG signals from inflated frozen lung sections maintained at -60°C [A-D]. The laser beam is focused on the specimen through a long working distance dry objective. The backscattered emissions from the thick tissue samples are collected through the same objective lens and directed to the non-descanned PMT detectors in the reflection geometry. Representative SHG image originating from the fibrillar collagens [E] overlaid with the MPEF image of normal alveolar tissue [F]. These are 3D views representing approximately 1mm thick healthy lung tissue section. Both fibrillar collagens (violet) and predominantly elastin fibers (green) condensed around the alveolar walls [Scale bar: $230\mu\text{m}$]. Representative SHG image originating from the fibrillar collagens [G] overlaid with the MPEF image of disrupted emphysematous alveolar tissues [H] [Scale bar: $230\mu\text{m}$]. A 3D projection is also shown [I]. Slab-like fibrillar collagens are seen emphysematous alveolar tissues.