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HuBMAP Lung TMC: SRS and Hyperspectral Imaging (HSI) of FFPE Prepared Tissue Sections

 Forked from [SRS: 3D Mapping and Hyperspectral Imaging \(HSI\)](#)

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We use this protocol and it's working

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Keywords: hyperspectral sweep across multiple different raman wavenumber, lipid concentration from distinct raman shift wavenumber, stimulated raman scattering, raman scattering, raman spectrum, hyperspectral imaging, hyperspectral image, distinct raman shift wavenumber, ch region of the raman spectrum, multiple different raman wavenumber, hyperspectral sweep, performing srs imaging, srs imaging, free measurements of specific macromolecule, unsaturated lipid, lipid, imaging, second harmonics generation, ffpe prepared tissue sections this protocol, protocol for stimulated raman scattering, ffpe prepared tissue section, hubmap lung data, lung tissue, sections of lung tissue, ffpe tissue block, hubmap lung tmc, second harmonics generation microscopy, rich tissue region, images of collagen

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Abstract

This protocol presents the acquisition of images via our SRS modality. We utilized a modification of our protocol for stimulated Raman scattering (SRS) to acquire label-free measurements of specific macromolecules in sections of lung tissue processed from FFPE tissue blocks. When performing SRS imaging we acquired total protein, total lipid, unsaturated lipid, and saturated lipid concentration from distinct Raman shift wavenumbers. We also acquired a hyperspectral sweep across multiple different Raman wavenumbers in smaller regions of interest to acquire a hyperspectral image (HSI) of the CH region of the Raman spectrum.

Finally second harmonics generation microscopy was applied to generate images of collagen rich tissue regions.

Some HuBMAP Lung data was generated from FFPE sections following Xenium spatial transcriptomics assay.

Section 1: Tissue Preparation

- 1 Tissue samples were prepared in tissue microarrays or larger ~1×1 cm tissue sections as described in **Xenium HuBMAP Lung FFPE Protocol**
dx.doi.org/10.17504/protocols.io.yxmvmdbk9g3p/v1
The four tissue microarrays and the additional 27 WSI, approximately 1 × 2 cm, FFPE blocks were serially sectioned (5 micron) for use in multiple assay modalities. The fifth section in the serial TMA series and 3rd serial section from the WSI blocks were placed on a Xenium™ slide (Figure 2) and prepared as detailed in the 10X Genomics TissuePreparation Guide (CG000578 Rev C). The TMA block was appropriately sized for the 1×2 cm Xenium™ imaging area. Between 2 and 4 WSI tissue sections were fit into the Xenium™ imaging area of single slides.
Directly following the Xenium spatial transcriptomics data generation, the same 5th section of each FFPE block was passed to the investigators for SRS Imaging as follows:

Section 2: SRS Imaging

- 2 **Start-up SRS microscope system.**
 - 2.1 Power on picoEmerald dual laser system, Si photodiode detector, and Zurich lock-in amplifier.
 - 2.2 Configure picoEmerald laser system: tunable pump from 780-990 nm, 5-6 ps pulse width, and 80 MHz repetition rate and Stokes beam with fixed 1031 nm wavelength, 6 ps pulse width, and 80 MHz repetition rate.
 - 2.3 Adjust average excitation power of both lasers to 500 mW for 3D imaging.
- 3 **Mount microscope slide onto microscope stage.**
 - 3.1 Apply immersion oil to the high-NA (1.4 NA) oil condenser.
 - 3.2 Mount microscope slide onto the condenser.
 - 3.3 Apply a large water droplet onto microscope slide for the water-immersion objective lens.

4 SRS acquisition

- 4.1 Acquired SRS following multiphoton (MPF) and/or second harmonic generation (SHG) imaging to avoid photobleaching.
- 4.2 Olympus FV3000 Software: Select image resolution: 256×256 for 3D mapping, 512×512 for single region of interest and HSI.
- 4.3 Olympus FV3000 Software: Select pixel dwell time and time constant: 20 μ s and 15 μ s, respectively.

5 Large Tissue Section Mapping:

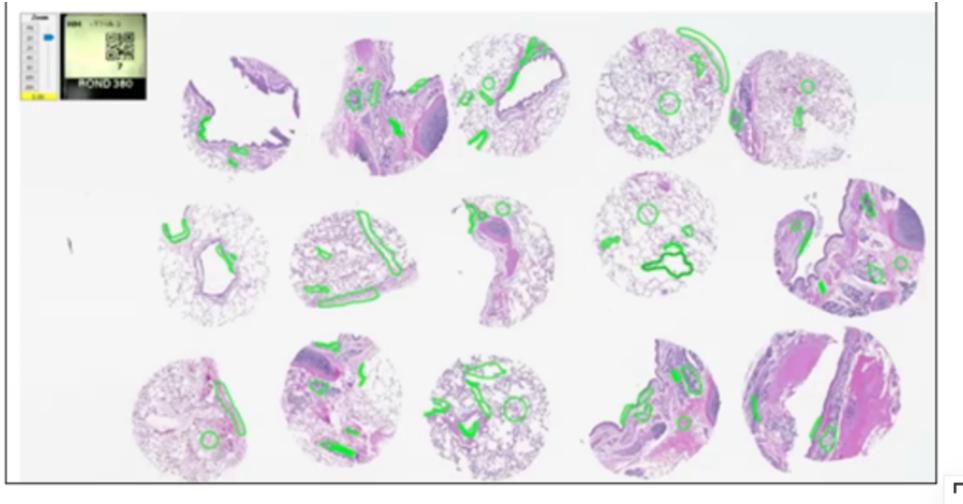
- 5.1 Olympus FV3000 Software: Select "MATL" for mosaic imaging and "Range: ON" to initiate 3D mapping.
- 5.2 Olympus FV3000 Software: Under "Range" window of Fluoview software: Select step size, number of z steps, and top of tissue surface
- 5.3 Olympus FV3000 Software: Under "Map" tab: Use rectangular fitting to create a mosaic map over the x-y region of interest/whole tissue.
- 5.4 Tune pump laser for each macromolecule wavelength between each acquisition:
 1. 791.3 nm for CH₃ asymmetric stretching/total protein (2938 cm⁻¹)
 2. 797nm for CH₂ asymmetric stretching/total lipid (2848 cm⁻¹)
 3. 794.6 nm for saturated lipids (2885 cm⁻¹)
 4. 786.5 nm for unsaturated lipids (3015 cm⁻¹)
- 5.5 Acquire a three-plane z-stack at each tile position within the stitched mosaic image, then compute a maximum-intensity projection across the three z planes to generate the final 2D mosaic image (.oir)

6 Hyperspectral Imaging (HSI):

HSI was performed as described by Zhang, W., Li, Y., Fung, A.A. *et al.* Multi-molecular hyperspectral PRM-SRS microscopy. *Nat Commun* **15**, 1599 (2024).
<https://doi.org/10.1038/s41467-024-45576-6>

1. On laser control panel select hyperspectral sweep and select range of wavelengths: 781.5-806.5nm (3100-2700 cm⁻¹) and choose a step number of at least 60.

2. Olympus FV3000 Software: For HuBMAP Lung TMC project, regions of interest were acquired from tissue cores of 4 tissue microarrays as multiple sequential images to generate hyperspectral stack (.oir files). Example:



Small regions of interests selecting specific lung structures were selected for Hyperspectral Imaging. These images corresponded to similar ROIs selected in TMA serial sections for GeoMx assay by the HuBMAP Lung TMC described in [GeoMx Whole Transcriptome Atlas \(WTA\) Assay with H&E-Guided ROI Selection for FFPE Human Lung Tissue Microarrays \(TMA\)](#)

Section 3: Data pre-processing

7

images were prepared for downstream ratiometric analysis by first removing destriping artifacts. This was done using code published by Fung A.A., Fung, A. H., Li, Z. and Shi, L. Single-Frame Vignetting Correction for Post-Stitched-Tile Imaging Using VISTAmapi, [Nanomaterials 15\(7\): 563, 2025.](#)

Protocol references

- Zhang, W., Li, Y., Fung, A.A. *et al.* Multi-molecular hyperspectral PRM-SRS microscopy. *Nat Commun* **15**, 1599 (2024). <https://doi.org/10.1038/s41467-024-45576-6>
- Fung, A. A., Fung, A. H., Li, Z., & Shi, L. (2025). Single-Frame Vignetting Correction for Post-Stitched-Tile Imaging Using VISTAmapi. *Nanomaterials*, 15(7), 563. <https://doi.org/10.3390/nano15070563>