APPLICATION NOTE

#62 Sublimation of MALDI Matrix for High Resolution Imaging of Human Eyes

Application & Background

The human eye is made up of several delicate structures that play important roles in healthy vision. The retina exists as part of the central nervous system and is of particular interest in many ocular diseases. It consists of horizontal layers of distinct cell types that transmit neural signals via the optic nerve for further visual processing. The lipid composition of the retina is unique and serves roles in visual signal transduction and protection against injury. Alteration of the lipid profile of the retina and adjacent structures is often observed in diseased tissues, and thus spatially-driven analysis of retina lipids is an important tool for biomedical researchers.

Eye tissue samples require both special sample processing for preserving morphology during sectioning, as well as ultra-high spatial resolution images to resolve the various regions and components. Both fixed and frozen tissues can be used, with specialized processing steps needed for frozen samples to retain structural integrity. In order to achieve ultra-high spatial resolution imaging by MALDI IMS, matrix must be applied in an extremely dry manner. Sublimation, as a solvent-free process, is the gold standard for such specialized matrix preparation.

Experimental

Sample Processing

Whole human donor eyes were frozen on liquid nitrogen vapor less than 6 hours post mortem. The frozen globes were then dissected with coping saw and rotary cutting tool to obtain the anterior segment, optic nerve, and macular region, which were then individually embedded in fish gelatin (**Figure 1**). The globe was processed using a Dremel rotary tool to remove the vitreous humor before backfilling with fish gelatin. Sections (10-12 µm) were cut onto ITO slides and desiccated. More details on eye tissue processing can be found in previous publication.[1]

Matrix Application using the HTX SubliMATE™

Matrix was applied using the HTX SubliMATE™ to ensure that crystals were very small (< 1 µm) and uniform in size. MALDI matrix DHAP was used for positive mode imaging and DAN for negative mode imaging. Both matrices were dissolved in acetone and pipetted onto the Matrix Wafer (**Figure 2**) situated on a level surface. The solvent was let fully evaporate and then the Matrix Wafer was added to the SubliMATE chamber. The top of chamber was cooled to -78°C using a combination of dry ice and acetone, and the bottom element was heated to 130°C. Sublimation time was set to 10 minutes for full coverage of slides with matrix. The SubliMATE™ was then returned to room temperature prior to breaking vacuum to prevent condensation on samples. A summary of sublimation parameters used for both matrices is shown in **Table 1**.

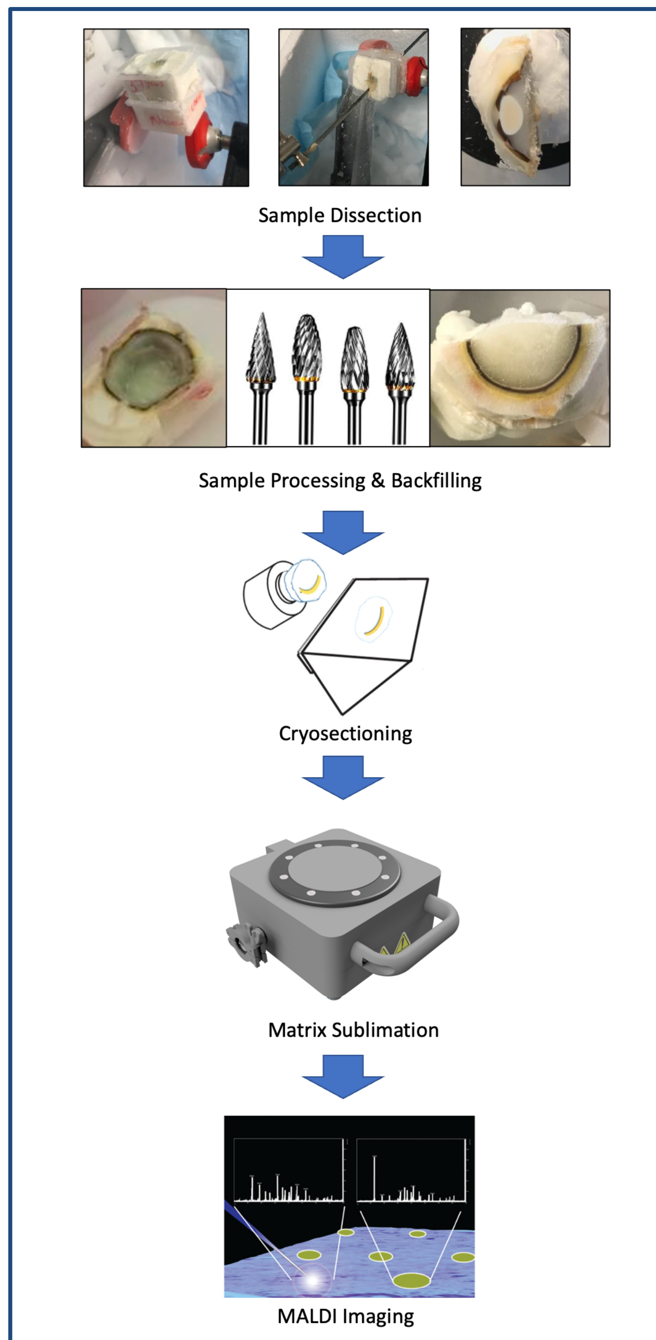

Figure 1. Workflow for frozen whole eye sample processing, matrix sublimation, and MALDI IMS. Figure adapted from Anderson *et al*. 2020 *JASMS*.[1]

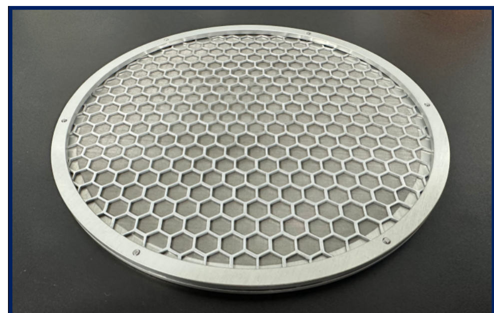

Figure 2. Matrix Wafer for matrix addition. Honeycomb patterning promotes even distribution of matrix solution across wafer prior to sublimation.

Table 1. Parameters used by the HTX SubliMATE™ for matrix deposition.

Matrix	DHAP	DAN
Matrix Concentration	20 mg	15 mg
Top Element Cooling Temp.	-78°C	-78°C
Bottom Element Heating Temp.	130°C	130°C
Sublimation Time	10 minutes	10 minutes

MALDI IMS & Data Analysis

MALDI IMS of lipids was performed at 5 μm spatial resolution using a Bruker timsTOF fleX mass spectrometer. To enable high-precision registration of IMS and optical signals, tissue autofluorescence and reflectance images were acquired from sections before and after IMS experiments. Lipid identifications were performed via LC-MS/MS analysis.

Results

Tissue lipid analysis by MALDI IMS revealed that the delicate architecture of the retina was preserved during sample processing. Individual photoreceptor layers were detected in the peripheral retina (**Figure 3**) and central retina (*data not shown*). Specific lipids were found to be contained within discrete retina cell layers, allowing for MALDI IMS results to show the outer nuclear layer, inner segments, outer segments, and retinal pigment epithelium (RPE). The RPE is a supportive single cell layer and was visualized with a lipid signal at *m/z* 799.6351. Of note, a phosphatidylethanolamine (PE) *m/z* 790.5117 containing docosahexaenoic acid (DHA) was observed localized to the outer and inner segments, **Figure 3B**. Decreased concentration of DHA-containing photoreceptors is characteristic of retinal degeneration, observed in diseases such as age-related macular degeneration.

Beyond the photoreceptor cells of the retina, lipid signals were localized to myelinated and unmyelinated nerve regions of the optic nerve as well as glial cells and blood vessels in the surrounding tissue, **Figures 4-5**. The optic nerve is responsible for transmitting visual information from the specialized cells of the retina to the visual cortex of the brain for higher order visual processing. In **Figure 4B**, unmyelinated nerve bundles of the optic nerve head are visualized by a phosphatidylcholine *m/z* 810.5991.

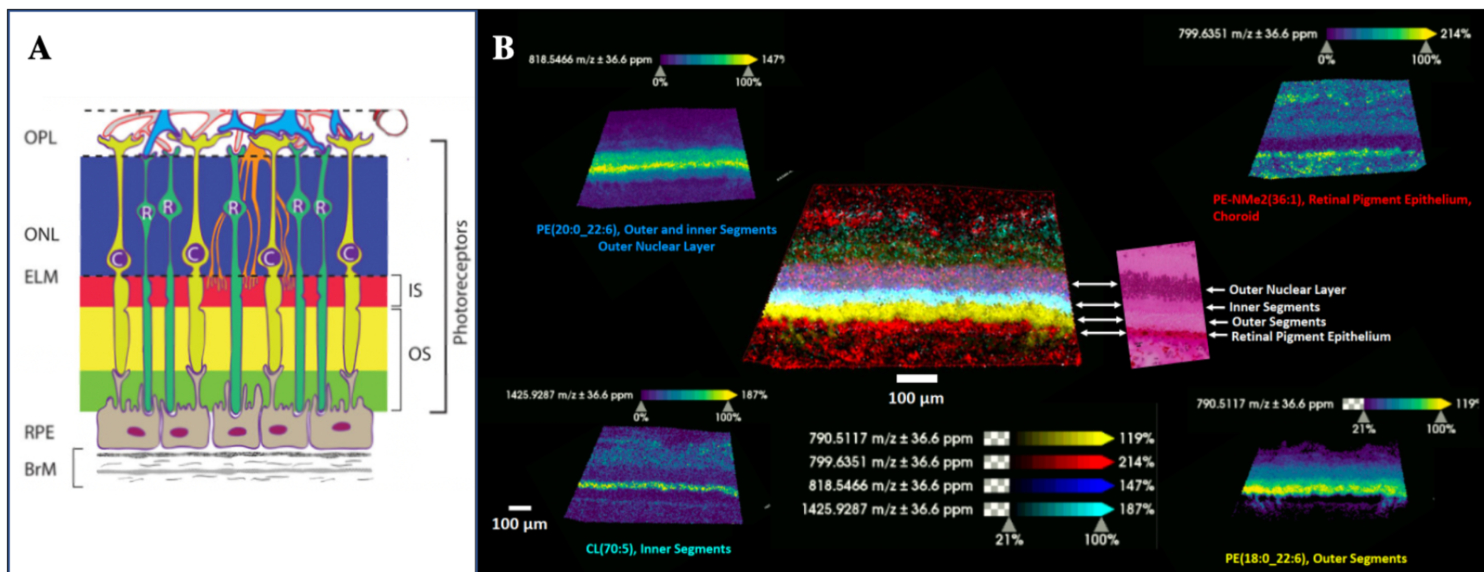

Figure 3. A) Schematic diagram of outer retina with layers *OPL* outer plexiform layer, *ONL* outer nuclear layer, *ELM* external limiting membrane, *RPE* retinal pigment epithelium, *C* cone, *R* rods, *IS* inner segments, *OS* outer segments. Figure adapted from Anderson *et al.* 2020 *JASMS*.[1] B) MALDI IMS data of photoreceptors in peripheral retina. Imaging was performed in negative mode at 5 μm with sublimated matrix DAN. Specific lipid signals correlated with discrete layers include PE(20:0_22:6), OS and IS and ONL; PE-NMe2(36:1), RPE; CL(70:5), IS; PE(18:0_22:6), OS and IS.

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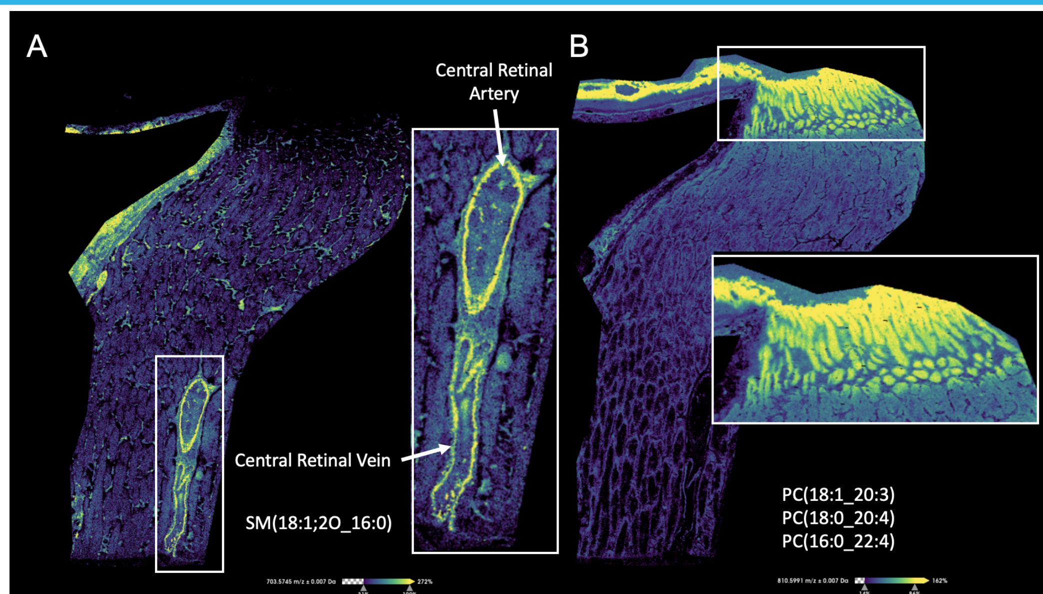

Figure 4. MALDI IMS data of optic nerve and surrounding tissue. Imaging was performed in positive ion mode at 5 µm with sublimated matrix DHAP. A) Lipid signal SM(18:1;2O_16:0) localized to venous structures such as the central retinal artery and central retinal vein are highlighted in the pop-out. B) Lipid signals PC(18:1_20:3), PC(18:0_20:4), and PC(16:0_22:4) localized to unmyelinated nerve fiber bundles extending from the neural retina to the optic nerve head.

These neural cells differ in their lipid signatures from local blood vessels, which are shown in **Figure 4A** by a sphingomyelin *m/z* 703.5745 found in the wall of the central retinal artery and along the path of the central retinal vein. The lipid signal from the unmyelinated nerve bundles in the optic nerve head, **Figure 4B,** quickly disappears at the interface with myelinated axons posterior to the optic nerve head, **Figure 5** (yellow, *m/z* 810.5991). Here the nerve fibers (red, *m/z* 788.6153) rely on myelin for insulation rather than the extensive connective tissue network found anterior to the optic nerve head. Glial cells are also present in this region (blue, *m/z* 753.5902) Together, this complex nervous tissue is essential for structure and function of the visual system.

Conclusions

We demonstrate the use of the HTX SubliMATE™ system for ultra-high spatial resolution imaging of human eye tissue. Delicate structures such as the optic nerve and individual layers of the retina were able to be imaged via MALDI IMS due to high quality sample preparation techniques. Specialized dissection, embedding, and sectioning of whole human eye globes preserved morphology of structures on slide sections to be prepared for MALDI imaging. In order to resolve the small structures and the individual cell layers, a uniform matrix coating consisting of very small crystals was necessary. The HTX SubliMATE™ reproducibly coated samples with MALDI matrix in a solvent-free process, which created a homogenous layer of fine matrix crystals < 1 µm.

Lipids signals could be localized to individual regions of the eye tissue such as the retina cell layers and photoreceptors, neural retina, optic nerve myelinated and unmyelinated nerve regions, and optic nerve vasculature. Such intricate cell regions required ultra-high spatial resolution imaging to observe as unique features. Notably, the single cell layer of retinal pigment epithelium was able to be resolved with this technique.

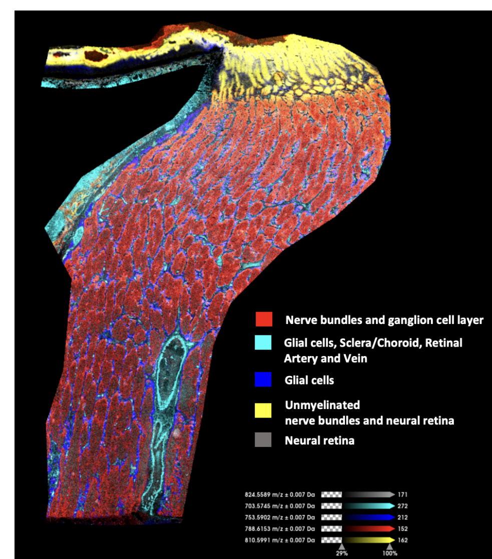

Figure 5. MALDI IMS data of optic nerve and surrounding tissue. Imaging was performed in positive ion mode at 5 µm with sublimated matrix DHAP.

The tissue images and MS data presented here were provided by Drs. David Anderson and Kevin Schey from Vanderbilt University.

References

(1) Anderson DMG, Messinger JD, Patterson NH, Rivera ES, Kotnala A, Spraggins JM, Caprioli RM, Curcio CA, and Schey KL. (2020) Lipid Landscape of the Human Retina and Supporting Tissues Revealed by High Resolution Imaging Mass Spectrometry. *J. Am. Soc. Mass Spectrometry*. 31(12):2426-2436.

(2) Kotnala A, Anderson DMG, Patterson NH, Cantrell LS, Messinger JD, Curcio CA, and Schey KL. (2021) Tissue fixation effects on human retinal lipid analysis by MALDI imaging and LC-MS/MS technologies. *J. Mass Spectrometry*. 56(12):e4798.

(3) Anderson DMG, Spraggins JM, Rose KL, and Schey KL. (2015) High spatial resolution imaging mass spectrometry of human optic nerve lipids and proteins. *J. Am. Soc. Mass Spectrometry*. 26(6):940-947.