Diabetic Complications Consortium

Application Title: Elucidating Molecular Drivers of Kidney Disease using 3-Dimensional Multimodal Imaging Mass Spectrometry: A Feasibility Study

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1. Project Accomplishments:

During this pilot project we have developed methods for mass spectrometry-based molecular imaging of human kidney tissue biopsies and have demonstrated the ability to visualize differential lipidomic and metabolomic localizations in diabetic tissues. A significant advancement for our MALDI imaging pipelines was the optimization of protocols for working with OCT embedded tissues, a common tissue preservation method for clinical biopsies. This included both sample preparation methods and the application of advanced ion mobility strategies for improving S/N of the MALDI imaging experiment and filtering out OCT-derived chemical noise. We also were able to demonstrate the ability to detect many lipidomic and metabolomic species that showed differential localization between diabetic and age/BMI matched control tissues. Some species, such as glutathione, were shown to be dramatically redistributed in diabetic tissue going from being localized in the glomeruli of normal tissues to more diffuse localization in diabetic nephropathy samples. Further, we uniquely applied NAPA-LDI to image nonpolar lipids and found significant increases in abundance of species including CE(18:2) and LacCer(d34:1) in diabetic tissues. These molecules are not often detected with typical MALDI imaging methods. These studies have resulted in two publications that are currently in preparation and will be used as preliminary data for coming grant submissions focused on molecular drivers of kidney disease.

2. Specific Aims:

AIM 1. Develop a multimodal molecular characterization pipeline for comparing normal and diseased kidney tissue.

Kidney Sample Preparation for Clinical Biopsies. Optimal cutting temperature (O.C.T.) is a common embedding material used for long-term storage of clinical samples, such as needle biopsies and wedge biopsies that were used in these studies. OCT has high concentrations of polyethylene glycol (PEG) polymers that cause analyte signal suppression in matrix assisted laser desporption/ionization (MALDI) imaging mass spectrometry (IMS) experiments. To allow us to work with common clinical biopsies for these studies, we have been working on sample preparation strategies to remove OCT prior to analysis in order to improve the sensitivity of the MALDI IMS experiment. We tested implementing washing protocols prior to matrix deposition (e.g. cold ammonium formate). The washes were successful in increasing the sensitivity of the

IMS experiment by both removing the OCT itself, as well as removing K^+ and Na^+ salts that are commonly adducted to lipids and metabolites during the MALDI processes. We determined th optimal washing procedure for human kidney OCT biopsies to include a combination of ammonium formate and oxalic acid. Normal human kidney biopsies were sectioned on ITO-coated glass slide and washed with 150 mM ammonium formate and 5mM oxalic acid for 30 seconds, followed by two 20-second washes with 150 mM ammonium formate alone. The MALDI matricies 2,5-dihydroxyacetophenone (DHA) and 1,5-diaminonaphthalene (DAN) were used for positive and negative ionization modes, respectively. A comparison of the average mass spectra of unwashed (red) and washed (blue) normal human kidney biopsies can be seen in **Figure 1** (positive ion mode) and **Figure 2** (negative ion mode). For the positive mode analysis, the wash was effective in removing the PEG signal from the mass range *m/z* ~ 1000-2000. However, washing with oxalic acid enhanced matrix-adducted lipid species formation during desorption and ionization leading to increased chemical noise, as evident by the mass spectral signals in the ~ *m/z* 1000 range in the washed tissue analysis. In negative ionization mode, however, the sensitivity for the lipid mass range was enhance by an order of magnitude (**Figure 2**) without significant increases in chemical noise. The wash protocols were also tested on diabetic nephropathy human kidney biopsies, and similar results were found. In addition to introducing wash steps into our protocols we also have developed ion mobility-based protocols to filter out noise/contaminations from OCT, based on gas-phase separations (See Below).

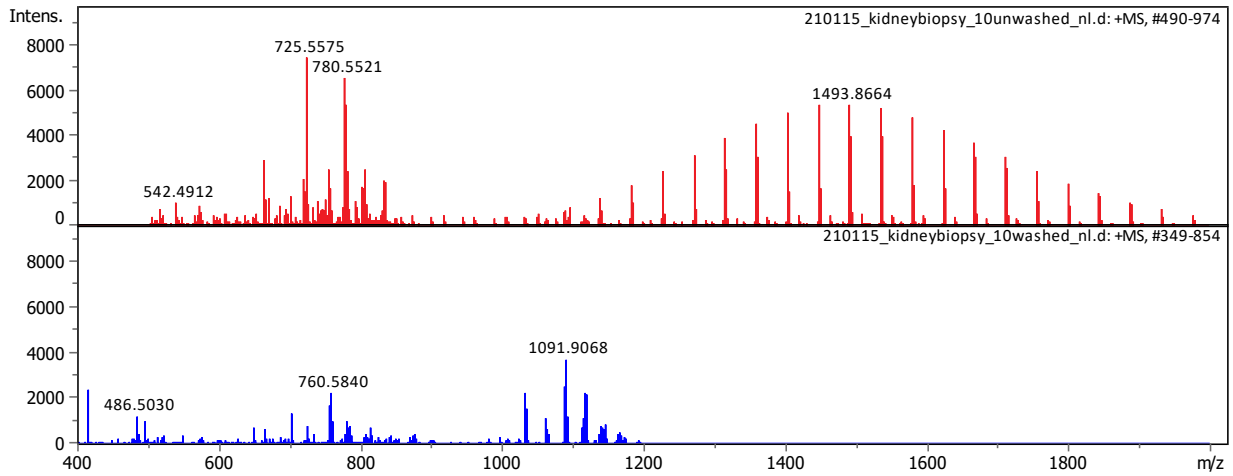

Figure 1. Average mass spectra of unwashed (red) and washed (blue) normal human kidney clinical biopsies in positive ionization mode using DHA matrix.

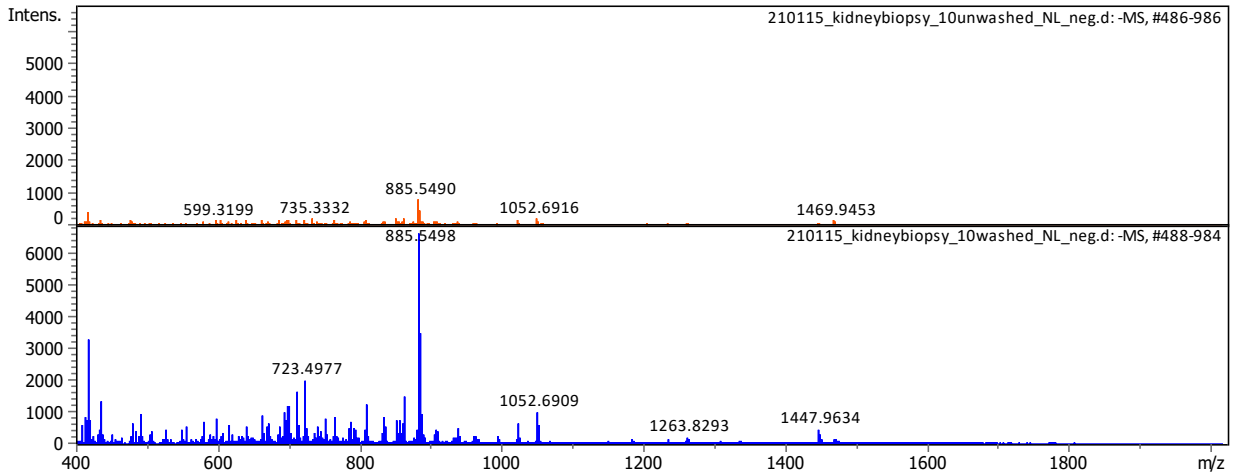

Figure 2. Average mass spectra of unwashed (red) and washed (blue) normal human kidney clinical biopsies in negative ionization mode using DAN matrix.

MALDI TIMS Analysis of Kidney Biopsies. In addition to sample preparation methods, we are also developed instrumental methods to separate lipid and metabolite analyte signals from OCT/chemical noise using trapped ion mobility spectrometry (TIMS). TIMS is a high-resolution ion mobility technology where separations are carried out in the first vacuum stage of a mass spectrometer consisting of an augmented ion funnel with an entrance funnel, TIMS tunnel, and exit funnel. In brief, ions are accumulated in the device, where analytes with differing mobilities are trapped and separated by opposing forces. Moving forward through the funnel, ions are propelled by a carrier gas. In the opposite direction, force is applied to the ions using an axial electric field gradient. To elute trapped ions, the electric field gradient is gradually reduced/scanned resulting in transmission of ions with ascending mobilities (see PMID: 32668145, 25331153, 31593446).

In these studies, an unwashed DN needle biopsy was imaged in positive ionization mode (DHA matrix) with TIMS activated (**Figure 3**). Signal from the embedding material can be seen in different trendlines throughout the entire ion mobility heatmap (**Figure 3**, top), with more prominent peaks in higher mass range < m/z 1000. The lipid analyte signal, on the other hand, occupies a smaller area of the heatmap in the lower mass range (**Figure 3**, red box). The analyte ion mobility-*m/z* region of interest can be isolated from chemical noise during post-processing to improve signal-to-noise (S/N) and sensitivity of molecules of interest. When comparing the mass spectrum of the region of interest (red) with the average mass spectrum of the entire dataset (blue), the isolated region of interest has two-orders of magnitude S/N increase. These methods are particularly useful for analyzing species that cannot be easily remove by implementing a washing protocol or to avoid unnecessary tissue loss/distortion during wash steps.

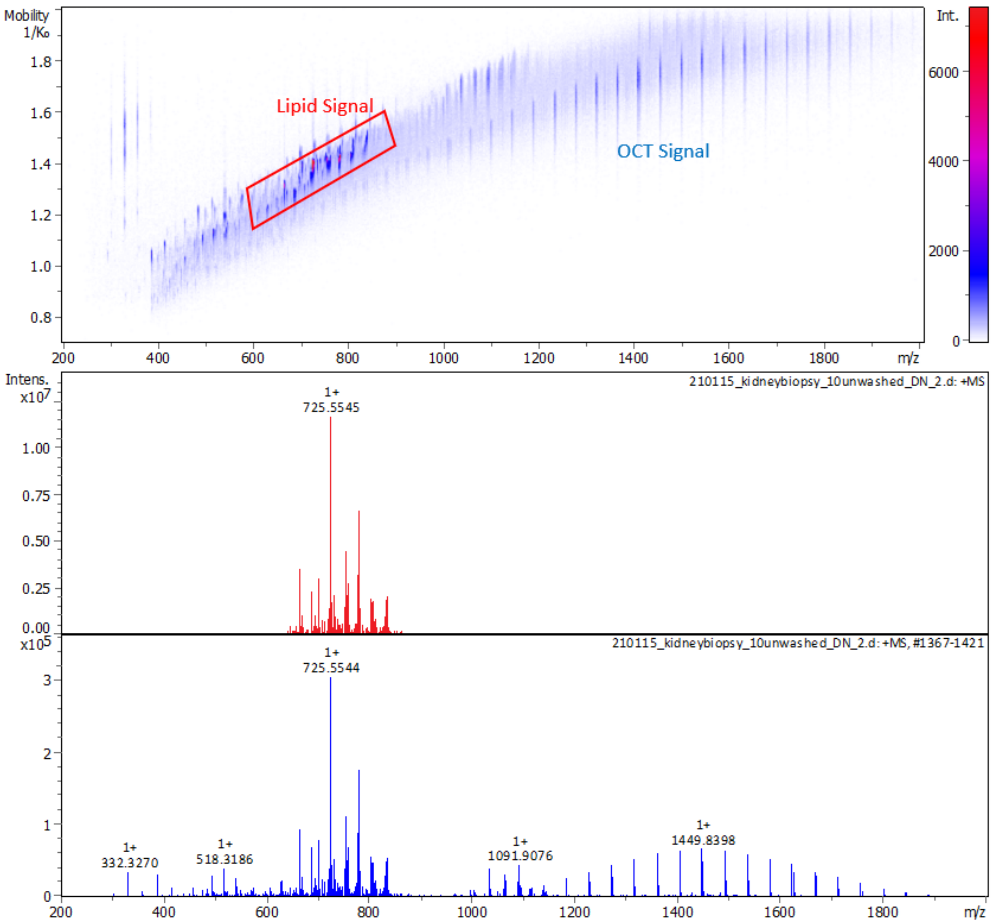

Figure 3. MALDI TIMS data from unwashed human kidney biopsies. The additional ion mobility dimension is able to differentiate chemical noise from lipid and metabolite signals (top). The ion mobility-m/z heat map can be used to extract signals of interest during post-processing providing increased S/N for the selected ions (red) in comparison to the unfiltered data (blue).

AIM 2. Elucidate molecular drivers of kidney disease through multimodal molecular imaging.

MALDI Imaging Mass Spectrometry. Initial MALDI IMS experiments comparing normal and diabetic nephropathy human kidney tissues were carried out in positive and negative ionization mode with DHA matrix. The tissues were sectioned to 10µm thickness on ITO-coated glass slides and DHA matrix was applied via a robotic sprayer. Measurement regions of roughly the same size were acquired from the cortex of normal and diabetic nephropathy human kidney tissues. Although no significant differences were found in lipid ion intensities, the spatial distributions of some ions were found to differ between healthy and DN tissues. For example in negative ionization mode, the ion with *m/z* 701.52, putatively identified as $[PA(36:1)-H]^-$, was found to localize to the glomeruli in the healthy kidney tissue, but was found throughout the cortex in the diabetic nephropathy example (**Figure 4**).

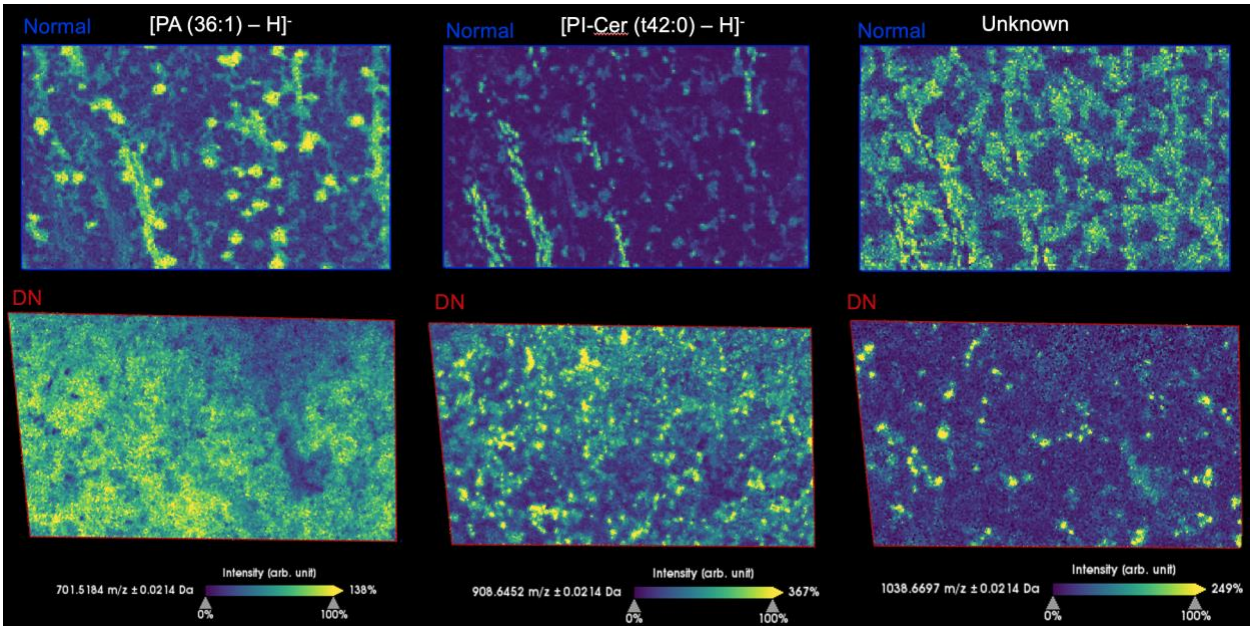

Figure 4. MALDI IMS negative ion mode data collected from normal (top row) and diabetic nephropathy (DN, bottom row) human kidney tissue biopsies. Three example ions of the thousands detected in the analysis are shown to highlight observed differences between healthy and diseased kidney tissue.

We have also made efforts to integrate trapped ion mobility separations in analyzing healthy vs diabetic nephropathy samples. TIMS can be used for gas-phase separation of isobaric/isomeric structures that could remain unresolved on a typical qTOF platform. This is especially important for lipid analysis, where many isomers and isobars can be present at a given nominal mass. In our work as part of this aim, we discovered potential lipid isomers with differential spatial distributions in normal vs. diabetic kidney tissues. For example, using MALDI TIMS we found multiple ion mobility peaks at *m/z* 756.52, tentatively identified as $[PE(36:4(OH))+H]^+$. Although the spatial distributions were similar in normal and DN tissue, the extracted ion mobility ratios are different, indicating that the isomer composition of *m/z* 756.52 is different between healthy and diabetic nephropathy tissues. This has motivated new research paths with current efforts focused on developing and integrating identification strategies in parallel to TIMS separations. We are alos developing methods for detection and imaging of higher mass lipid species, including cardiolipins, sulfated-glycoshpingolipids, and gangliosides.

NAPA-LDI IMS

In addition to MALDI IMS, we have been developing silicone nanopost array laser desorption ionization (NAPA-LDI) IMS sample preparation techniques to enhance lipid coverage. NAPA-LDI IMS provides a complimentary lipid coverage to MALDI IMS, as it allows for the ionization of species, including hexosylceramides, lactosylceramide, cholesterol esters, and triglycerides that are not detected with traditional MALDI matrices. Furthermore, NAPA LDI-IMS does not require a matrix application prior to analysis, making it a good candidate for imaging of low-mass analytes, such as metabolites.

To highlight the utility of NAPA-LDI IMS for detecting metabolomic differences observed in diabetic tissues, DN and control human kidney tissues were sectioned, thaw mounted onto NAPA chips, and imaged at 20 μm spatial resolution (**Figure 5**). Ions with *m/z* 330.06 and *m/z* 464.12 were tentatively identified as glutathione and terazosin, respectively. Ion images show their unique localizations to the glomeruli in the normal human kidney tissue, and a distinct reorganization of these molecules to a more diffuse localization within the cortex and higher intensity within the medulla for DN tissue. We also observed distinct lipid reorganization in diabetic kidney tissues. For example, NAPA-LDI images of *m/z* 687.55 and *m/z* 900.59 showed dramatic increases in intensity in the cortex of the kidney relative to normal tissues where these species were detected with low intensity (**Figure 6)**. Based on exact mass and subsequent fragmentation data, the ions were tentatively identified as the [CE(18:2)+K]+ (*m/z* 687.55) and [LacCer(d34:1)+K]+ (*m/z* 900.59). The highlighted lipid and metabolite ions are challenging to detect with traditional MALDI sample preparation techniques, but are readily detected with NAPA-LDI, indicating that the two platforms provide complimentary lipid coverage.

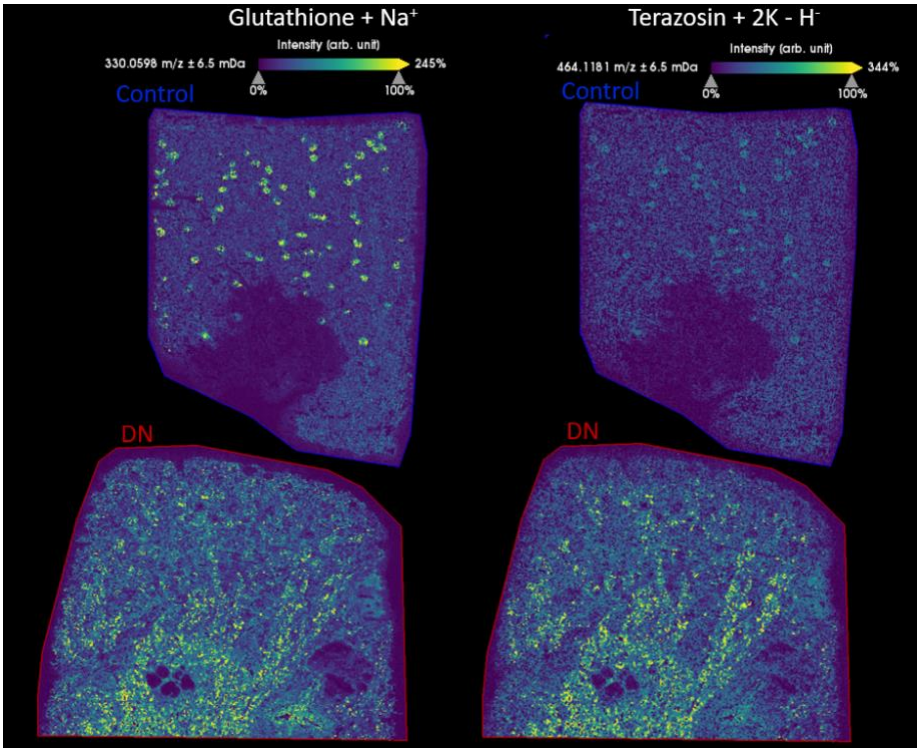

Figure 5. NAPA-LDI IMS of metabolites from control and diabetic nephropathy human kidney tissue. Both ions highlighted here, $[Glutathione+Na]^+$ (*m/z* 330.060) and $[Terazosin+2K-H]^+$ (*m/z* 464.118), were found to localize in the glomeruli of normal tissues and more diffuse localization in the cortex and higher intensity in the medulla of DN tissue.

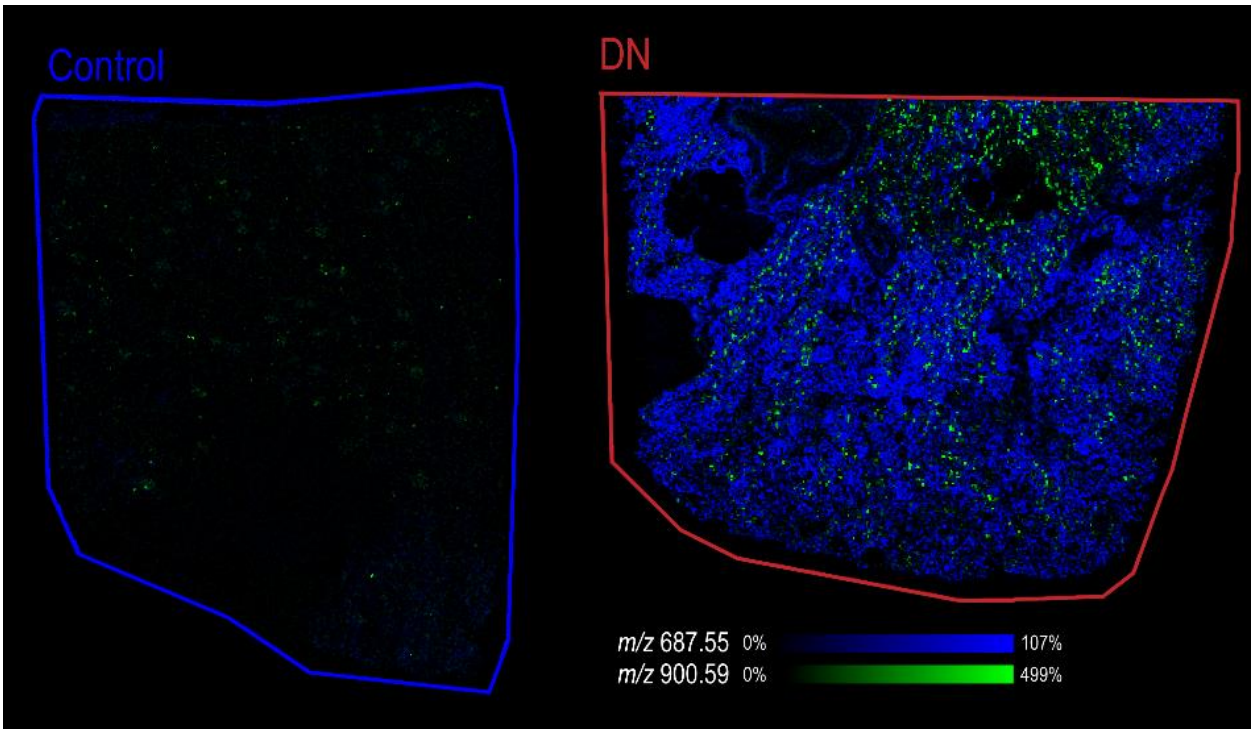

Figure 6. NAPA-LDI IMS of control and diabetic nephropathy human kidney shows two lipid species that localize to different regions of the cortex in the diseased tissue and that are in found with very low intensity in the control tissue. The lipids were tentatively identified as $[CE(18:2)+K]^+$ (m/z 687.55) and $[LacCer(d34:1)+K]^+$ (m/z 900.59).

3. Publications:

We are currently working on two manuscripts related to the work highlighted above. One focused on methods for MALDI IMS of OCT tissues and one on metabolomic and lipidomic differences overserved in diabetic tissues. We anticipate these manuscripts will be submitted later this summer.