A Thesis

entitled

Multimodal Spectral Microscopy and Imaging Mass Spectrometry of Biomolecules in Cells and Tissues

by

Yang Xu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Chemistry

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An Abstract of
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Biomolecular imaging enables localization and characterization of biomolecules
at the cellular and tissue levels in order to better understand their roles in biological
organisms. Although a plethora of imaging techniques is available, there is a need to
improve current imaging methodologies and apply them for selective analyses of
complex biological specimens. The work described in this thesis is related to
development and applications of spectral imaging and imaging mass spectrometry (IMS)
methodologies. Spectral imaging uses either labeling dyes or native chromophores to
visualize biomolecules in cells and tissues under a specialized light microscope, while
IMS provides direct detection and analysis of biomolecules on tissue surfaces with
preserved spatial distribution.

In this initial study, multimodal spectral imaging of cells, cellular organelles, and
tissues was performed on a light microscope upon the addition of a transmission
diffraction grating. The instrument concurrently recorded spectral images by fluorescence, darkfield, brightfield, and differential interference contrast (DIC) spectral microscopy. Fluorescence signals originating from fluorescently labeled biomolecules in cells were collected through single and multi-bandpass fluorescence filters, separated by the grating, and imaged using a charge-coupled device (CCD) camera. Cellular components such as nuclei, cytoskeleton, and mitochondria were spatially separated by the fluorescence spectra of the fluorophores present in them, providing detailed multi-colored spectral images of cells. Additionally, the grating-based spectral microscope enabled measurement of scattering and absorption spectra of unlabeled cells and stained tissue sections using darkfield and brightfield spectral microscopy, respectively.

In the second project, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) was utilized to visualize the protein spatial distribution on tissue surfaces. Our study has been focused on imaging of proteins in kidney tissue sections originating from apparently healthy (normal) mice and mice with polycystic kidney disease (PKD). Reconstruction of protein ion maps and identification of proteins by on-tissue enzymatic digestion were performed. MALDI-MS images were correlated with histological features obtained by hematoxylin and eosin (H&E) staining, providing localization of proteins and peptides in different parts of the kidney (renal pelvis, medulla, and cortex). Nano-HPLC was coupled with imaging mass spectrometry to identify proteins in kidney tissue sections. Principle component analysis (PCA) was employed to achieve tissue classification and differentiate between healthy and diseased tissues. Experiments are underway to discover and validate proteins that can become potential biomarkers of PKD.
In summary, we demonstrated that fluorescence, scattering, and absorption spectral images of cells can be acquired by incorporation of a transmission diffraction grating into a light microscope. The present spectral imaging methodology provides a readily affordable approach for multimodal spectral characterization of biological cells and other specimens. A reproducible MALDI-IMS workflow was established and resulted in protein mass distribution maps of normal and PKD kidney tissues. Statistical comparison of MALDI-MS images provides classification of healthy and diseased tissues. Several proteins were localized in tissues and their identity will be further confirmed by HPLC combined with tandem mass spectrometry (MS/MS).
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List of Abbreviations

ACN .................................. acetonitrile

CHCA .................................. alpha-cyano-4-hydroxycinnamic acid

DHB .................................. 2, 5-dihydroxy benzoic acid
DIC .................................. differential interference contrast
DESI .................................. desorption electrospray ionization

ESI .................................. electrospray ionization

HPLC .................................. high-performance liquid chromatography

IMS .................................. imaging mass spectrometry
ITO .................................. indium tin oxide

MALDI ................................. matrix assisted laser desorption ionization
MS .................................. mass spectrometry

SA .................................. sinapinic acid
SIMS .................................. secondary ion mass spectrometry

TFA .................................. trifluoroacetic acid
TIC .................................. total ion count
TOF .................................. time-of-flight
Chapter 1

Overview: Molecular imaging of biological samples

1.1 Introduction

Biomolecular imaging aims to localize and characterize biomolecules at the cellular and tissue levels in order to better understand their roles in biological organisms. Various analytical tools and methodologies with their distinctive principles have been developed to fulfill this goal.\(^1\)\(^-\)\(^2\) Traditional methods of tissue analysis on the protein level require tissue lysis followed by protein extraction through various procedures. In this way, proteins can be analyzed and characterized, but at the same time their spatial distribution information which is important to understand their activities is lost.\(^3\)

Spectral imaging methodology was developed to visualize and characterize biomolecules in cellular organelles, which cannot be readily investigated due to their small sizes and confined localizations. It uses either labeling dyes or native chromophores to visualize biomolecules in cells and tissues under a spectral microscope. Thus, the biomolecules can be characterized through measurement of their emitted, scattered, or transmitted light spectra. Recently, the concept of imaging mass spectrometry was introduced.\(^4\) It has been gaining popularity and undergoing fast development over the last ten years.\(^5\) Utilizing mass spectrometry, direct detection and
analysis of biomolecules on tissue surfaces with preserved spatial distribution was achieved.

1.2 Spectral imaging by light microscopy

Spectral microscopy is a powerful tool, which combines spectroscopy and light microscopy. It enables visualization of samples with high spatial localization and at the same time measures emitted, scattered, or transmitted light from the specimens. A spectral microscope can be built by adding optical filters such as multi-bandpass fluorescence filters, spectral dispersion elements such as a diffraction grating, and signal recording devices such as a CCD camera to a regular high-resolution microscope. The system becomes particularly useful for biomolecular imaging when fluorescence probes are introduced. It enables multi-color analysis of cells and cellular organelles simultaneously. Various applications of fluorescence spectral imaging are reported including analysis of biomolecules in cells and cellular organelles labeled with multiple fluorophores, monitoring fluorescent molecules whose spectra temporally change due to environment, detecting DNA chromosomal aberrations by spectral karyotyping, and spectral identification of single molecules and fluorescent nano-spheres. Besides fluorescence spectral imaging, other forms of spectral imaging were developed such as measuring scattering and absorption spectra from biomolecules, nanoparticles in cells, and dye specimens.

Spectral microscopes are commercially available to scientists for measurement of spectra of cells, cellular organelles, and other specimens. For example, a spectral microscope, incorporating a custom fluorescence multi-bandpass filter cube and an
interferometer for fluorescence spectral imaging, was used for karyotyping of human and mouse chromosomes.\textsuperscript{8-9} There are also inexpensive alternatives to record spectral information from microscopic specimens such as utilizing a diffraction grating. A high-throughput spectral imaging method based on a transmission diffraction grating was used to measure fluorescence spectra of single-molecules and fluorescently labeled cells that flow through a capillary.\textsuperscript{7,10-11} Another setup based on a reflective diffraction grating was developed to record absorption spectra of dyes.\textsuperscript{12} Those cheaper but efficient methods need to be investigated thoroughly to enable high-resolution and high-magnification multimodal spectral imaging of biomolecules in cells and tissues by multi-color fluorescence, darkfield, and differential interference contrast microscopy. Moreover, the spectral imaging methodology needs to be developed to improve the data interpretation.

1.3 Imaging mass spectrometry

Imaging mass spectrometry (IMS) employs mass spectrometry to localize, characterize and identify biomolecules \textit{in situ} from complex biological samples such as tissue surfaces in order to better understand their roles in biological systems.\textsuperscript{13-14} The spatial localization of biomolecules such as endogenous compounds, (e.g., proteins, peptides, and lipids)\textsuperscript{15} and exogenous compounds, (e.g., metabolites and drugs)\textsuperscript{16} can be visualized in terms of their masses. The basic idea is straightforward. Analytes on a surface of solid samples are ionized by various available ion sources and further transferred to a mass analyzer to be separated based on mass, charge, and structure before being measured by a detector. The stage of the target plate where the sample is mounted moves in the x-y plane, and associated mass spectra are generated from known sample
positions. Through registration of sample and detected masses, molecular distribution across the surface can be visualized.

The concept of imaging mass spectrometry was developed 50 years ago when secondary ion mass spectrometry (SIMS) was first used to study semiconductor surfaces. Since then, various mass spectrometry techniques have been invented and have undergone fast development. Besides SIMS, matrix assisted laser desorption ionization (MALDI) and desorption electrospray ionization (DESI) are two other very commonly used desorption/ionization methods in IMS. Other available methods include laser ablation (LA), laser desorption/ionization (LDI), desorption/ionization on silicon (DIOS), and nanostructure-initiator mass spectrometry (NIMS), to name a few.

SIMS is the oldest IMS technique which applies fast moving ion currents and high energy ion beams as primary ions to sample surfaces to generate secondary ions. Those secondary ions are transferred to a mass analyzer, which is typically a time of flight (TOF), and detected with their preserved positions. SIMS is an effective ion source to study small molecules such as elements, metabolites and drugs with high spatial resolution because of focused beam and limited amounts of desorbed and ionized molecules. It requires simple sample preparation procedures. DESI was invented by Cooks and co-workers in 2004. In the ion source, charged microdroplets of solvent desorb analytes from wetted sample surfaces generating secondary droplets. Similarly to ion formation in ESI, most of the ions are multiply charged. The biggest advantage of DESI is its flexibility. DESI operates under atmospheric pressure instead of high vacuum required for SIMS and MALDI and is applicable on various sample surfaces, such as plant tissue, without complex sample preparation procedures.
Among the three major methods, MALDI-MS is the most widely employed methodology, which was developed by Franz Hillenkamp and Michael Karas in the 1980s. The MALDI-MS instrument is equipped with a focused UV or IR laser, to vaporize and ionize analytes from matrix/analyte co-crystals. It allows accurate mass measurement of the analytes in a time-of-flight analyzer as well as the analysis of fragment masses generated from the parent molecule ions under tandem MS. The soft ionization enables the measurement of big molecules such as proteins and peptides. The sensitivity is high (a few femtomole to attomole) and the mass range that can be detected is wide (up to 300 KDa on MALDI-MS instrument used in this study).

The very first publication using MALDI as imaging tool came from the Caprioli group who studied localization of protein and peptide on tissue samples and managed to reconstruct ion map images of those samples. Many new applications have been reported over the years. The most promising one is using information obtained from MALDI-IMS to differentiate tissue samples of diseases such as cancer from normal ones and to discover new disease specific biomarkers. The methodology can facilitate disease diagnosis and early detection.

Although MALDI-MS does not require immunochemical labeling, it is useful to combine it with histological staining for in-depth analysis. Also, imaging drug and drug metabolite distributions in the tissue sample and associated changes in the protein network can help in finding new drug candidates and assessing therapeutic effects.

There are a few challenges that need to be addressed in the development and application of IMS. Homogeneousness of the matrix deposited on the sample is the key to obtain good quality mass spectra that accurately represent the molecular composition. MS
imaging results can be altered by an uneven matrix analyte co-crystallization and ion suppression effects, in which some molecules more favorably capture protons in the ionization process and appear in higher abundance in the mass spectrum while the intensities of other ions can be buried in the high background noise. In these cases, spectra cannot represent the true composition of the sample surface. Sample preparation usually takes hours to days during which the analyte of interest might undergo oxidation or degradation. Also the sample preparation process leads to a higher possibility of contamination but can be avoided by careful manipulation. Relocation of molecules of study can happen during sample washing and trypsin digestion incubation period, which decrease quality of the mass spectra. Current sensitivity only allows the most abundant and favored molecules to be detected. Image resolution is also limited by the sensitivity of detector thus subcellular localization of biomolecules has rarely been achieved.\textsuperscript{17}

Imaging mass spectrometry generates large amounts of multi-dimensional data. How to process and interpret the collected data to accurately represent the biological information of samples is very important. For example, normalization of the spectrum is common practice before statistical analysis. But, there are different normalization methods applying distinctively different algorithms.\textsuperscript{24} Choosing the proper normalization requires understanding the sample preparation background, determining the quality of raw data as well as understanding the algorithms behind each method. However normalization of spectra is rarely studied. Because of the variation of the signal intensity from sample to sample, quantitative analysis by IMS is not widely utilized.
1.4 MALDI-IMS system

1.4.1 Introduction

The main component of MALDI IMS system is a MALDI mass spectrometer with a TOF/TOF mass analyzer. The characteristic coupling of the ionization and mass analyzer combination in MALDI allows the spatial information of analytes to be preserved, which is the key aspect of mass spectrometry imaging. The other instrumentation adopted in the MALDI-IMS systems includes sample preparation tools which can either be microspotting or spraying devices for matrix application. To achieve the final goal of identifying individual molecules in images, coupling with a separation tool such as nano-HPLC is an efficient way to simplify the sample mixture composition and increase the detection sensitivity. The offline coupling is enabled by an auto fraction collector such as Proteineer fc (Bruker Daltonics), which collects the sample eluents from the LC to MALDI target plates.

1.4.2 MALDI TOF/TOF mass spectrometry

Mass spectrometry is the technique to measure the molecular masses of charged ions in high vacuum to identify substances in solids, liquids and gases. Introduction of matrix-assisted laser desorption/ionization mass spectrometry in the 1980s provided a soft and efficient ion source, which enables desorption of intact nonvolatile molecules such as proteins and peptides. Mass spectrometers usually contain three main parts: an ion source, a mass analyzer, and a detector. The ionization of analyte molecules is achieved by gaining or losing a proton in the ion source. Therefore, most of the time singly charged ions are formed, while multiply charged ions have been observed for
bigger molecules such as proteins. Usually an intensive UV laser energy is absorbed by the co-crystal of the matrix and the analyte in the high vacuum area of the ion source. By embedding the sample into matrix materials, which are usually small organic acids, sample fragmentation is avoided and high ion intensity is obtained.

Different matrices assist desorption and ionization of different types of molecules. For example, sinapinic acid (SA) is often used for proteins. Alpha-cyano-4-hydroxycinnamic acid (CHCA) is widely used for peptides while 2, 5-dihydroxy benzoic acid (DHB) can be used for peptides and glycopeptides. The laser beam can be focused to a spot with a diameter as small as 10 µm but considering ion yield and sensitivity of the detector, the laser beam diameter is usually between 50 and 200 µm. In MALDI-IMS, the laser focus is one of the factors that determine the imaging spatial resolution of samples such as tissue sections. The target plates are transported into the high vacuum ion source and move on x-y-coordinates according to pointed shot position.

Time-of-flight (TOF) is the mass analyzer associated with MALDI. After the laser hits the analytes, the ions generated in the ion source are accelerated in an electro-static field before passing through a field-free drift region. After acceleration by the voltage \( U \) in the electric field, the kinetic energy of ions is

\[
E = \frac{1}{2}mv^2 = qU
\]

(1)

where \( q \) represent the charge and \( v \) represent the traveling velocity. The velocity can be determined from the following equation:

\[
v = \frac{L}{t}
\]

(2)

where \( L \) is the length of the drift region and \( t \) is flight time. Ions with same kinetic energy but different masses travel at different speed, so they are separated and reach the detector.
at different times in the linear time of flight. Small mass ions reach the detector faster than large mass ions. Ion masses can be determined from the measurement of flight time because ion mass over charge is proportional to time square based on the combination of equations (1) and (2):

\[
\frac{m}{q} = \frac{2Ut^2}{L^2}
\]

Usually, ions with same mass start with some energy spread which will cause a certain amount of peak width. A quadratic or cubic enhanced equation is generated by performing calibration using a standard protein or peptides mix as reference masses. The flight time can be fit into the equation to obtain accurate masses of analyte ions. The energy distribution in the linear mode can be corrected by using a reflector which focuses the ions with the same mass to charge but different kinetic energies.

For analysis of complex samples such as proteins, identification and validation of analytes can be accomplished by tandem MS with LIFT method. Using the LIFT mode, a precursor parent ion is chosen and the precursor ion selector only allows the selected ion mass to pass through the gate while deflecting the other precursor ions. Fragment ions are formed in the ion source during high laser irradiation and electrical field acceleration and have a fraction of the parent ion energy that is proportional to their mass. The kinetic energies of parent and fragment ions are increased (lifted) by the electric field applied in LIFT device and can be detected simultaneously by the detector. For a typical MS spectrum, masses of up to 300 KDa in the linear mode and monoisotopic peaks, which are important for analyte mass confirmation, can be detected in the reflectron mode. Mass resolution of >30,000 can be achieved in the 500 to 4000 Da mass range in the reflectron mode. Fragment MS/MS accuracy is usually below 5 ppm, which allows accurate
determination of protein identity by a MASCOT protein database search (Matrix Sciences).

1.4.3 Matrix coating device: ImagePrep

ImagePrep is an automatic coating device utilized during sample preparation for matrix or digestion enzyme solution spraying on the tissue sample surface. Under an inert environment made by infusion of nitrogen, tiny and even aerosol droplets can be generated by vibration of a spray head through a fine mesh. The size of the droplets is typically 20 to 50 μm in diameter, which is one of the factors that determine imaging resolution. Circles of spray are performed during which the droplets are deposited on the sample surface followed by solvent evaporation. The co-crystals of analytes were formed in the case of matrix spraying. A sensor underneath the sample glass slide detects the thickness and wetness to adjust the spraying time interval. The use of the automatic device ensures the reproducibility and homogeneousness of the sample preparation, which is of high importance to achieve reproducible and high-resolution imaging analyses.

1.4.4 Nano-high-performance liquid chromatography

Nano-HPLC is a separation technique that is often coupled to MALDI-MS to achieve minimal sample consumption and better sensitivity for small amounts of peptide sample extracted from tissue surface compared to traditional LC-ESI. The off-line coupling of nano-LC with MALDI-MS provides a useful workflow for biomolecular imaging. Nano-LC systems consists of a micro autosampler with 1 μL injection loop, a
gradient proportioning micropump, a 1:1000 flow splitter, and a UV detector with 3 nL flow cell. All the capillaries have 20 µm i.d. and minimal dead volume. The separation is usually performed on a 75 µm (i.d.) x 15 cm nano column with tolerance of 2 µg of complex protein digest sample. Usually, the samples are loaded into the plastic glass insert micro vial and placed in the autosampler tray where the temperature is kept around 8 °C. HPLC micropumps deliver the mobile phase, which are typically water and organic solvents such as acetonitrile. Solvents are mixed in a programmed ratio. The solvent mixture then goes into the flow splitter and only 1/1000 of the flow continues to the column and the rest goes to the waste. Upon injection, the autosampler extracts the sample through a needle head to fill the 1 µL loop. By switching the valve, the sample is delivered into the flow line and mixed with the mobile phase. Depending on the hydrophobicity of the molecules, flow rate, and composition change of the mobile phase, analytes interact with the stationary phase which consist of C_{18} particles in the nano column, and elute out at different retention times. The general rule is that the smaller and more hydrophilic molecules elute out faster than the bigger and more hydrophobic molecules, which can be eluted later by increasing organic solvent percent in the mobile phase. The gradient can be programmed and optimized to a specific sample. The separated molecules can be detected by the UV detector and show up in chromatogram. The peak intensity and area can be calculated for quantitative analysis. After the UV detector, the eluates are delivered into a fractioner through a capillary connection.
1.4.5 LC fraction collector: Proteineer fc and AnchorChip target

Proteineer fc (Bruker) is an automated fraction collection system connected to the nano-LC by a fused silica capillary. Continuous LC fractions are collected and evenly distributed on a 384 spot MALDI AnchorChip target plate (Bruker) or a 96 well plate with programmed spotting time and movement pattern. Also, an external syringe pump is coupled to the probe inlet to enable online matrix mixing with the analytes. The AnchorChip is a specially coated stainless steel MALDI target plate with 800 µm hydrophilic anchor spots surrounded by hydrophobic surface. Usually 0.3 to 0.4 µL of sample/matrix mixture is deposited on to one AnchorChip spot. After evaporation of organic volatile solvents, the droplets center themselves at the anchor. Because of the concentrated samples, the sensitivity in the MALDI analysis can increase up to 10 fold. Besides the 384 sample spots, there are 96 calibration spots surrounded by every four sample spots on one AnchorChip plate. During automatic acquisition using the WarpLC program (Bruker), the constant external calibration of spots close to the analyte spots ensures the accuracy of the peak mass-to-charge measurement.27
Chapter 2

Multimodal spectral microscopy imaging of cells and tissues using a transmission diffraction grating on a light microscope

A paper published in Applied Spectroscopy

Dragan Isailovic, Yang Xu, Tyler Copus, Suraj Saraswat, and Surya M. Nauli

2.1 Introduction

Light microscopy and spectroscopy are used in various modalities for the analysis of biomolecular composition and structure of biological cells. For example, fluorescence microscopy is used for localization of fluorescently labeled or natively fluorescent biomolecules in cells, while fluorescence spectroscopy yields information on the spectral properties and amounts of analyzed molecules. Other forms of microscopy (e.g., brightfield, darkfield, phase contrast, and DIC microscopy) and spectroscopy (e.g., UV-VIS spectroscopy) are also used for the analysis of biomolecules in cells. Because microscopy and spectroscopy provide complementary data, it is very useful to combine these two methodologies and simultaneously monitor changes in localization of biomolecules inside biological cells on the basis of their spectral properties.
Spectral imaging, a methodology that couples spectroscopy and microscopy, is a hyphenated technique that has received much scientific attention in the last 10-15 years. The technique is based on microscopes that employ customized optical filters, spectral dispersion elements (e.g., a diffraction grating or an interferometer), or tunable filters (e.g., an acousto-optic or a liquid-crystal tunable filter) to record images as well as spectra of molecules in individual cells and other specimens. Due to the development of a plethora of fluorescent probes, fluorescence spectral imaging became very useful for multi-color analysis of cells and cellular organelles. Compared to conventional microscopes that use various optical filter sets to specifically select fluorophores in cells, fluorescence spectral imaging offers an advantage of simultaneous multi-colored imaging of cells. Applications of fluorescence spectral imaging include: analysis of biomolecules in cells and cellular organelles labeled with multiple fluorophores, monitoring of fluorescent molecules whose spectra temporally change due to environment or fluorescence energy transfer (FRET), labeling of DNA in chromosomes with multi-colored fluorescent dyes for detection of chromosomal aberrations by spectral karyotyping (SKY), and spectral identification of single molecules and fluorescent nanospheres. Other forms of spectral imaging that can measure scattering and absorption spectra from biomolecules, nanoparticles in cells, and dye specimens are also gaining popularity. Hence, spectral imaging finds important applications in biochemical, biophysical, and biomedical research.

Currently, there are a few spectral microscopes commercially available to scientists for measurement of spectra of cells, cellular organelles, and other specimens. For example, a spectral microscope, which is using a custom fluorescence filter cube and
an interferometer for fluorescence spectral imaging, is used for karyotyping of human and mouse chromosomes. Although spectral imaging instruments are essential for measurement of spectra of fluorophores in analyzed cells or colorimetric investigation of tissue specimens, they are not readily affordable to many laboratories. Therefore, several recent studies described alternative but efficient ways to record spectral information from microscopic specimens by a diffraction grating. A high-throughput spectral imaging method based on a transmission diffraction grating was used to measure fluorescence spectra of single-molecules and fluorescently labeled cells that flow through a capillary.\textsuperscript{7, 10-11} Another setup based on a reflective diffraction grating was developed recently to record absorption spectra of dyes, and was featured as a simple and inexpensive alternative to commercially available spectral microscopes.\textsuperscript{12} The presented work outlines a spectral imaging setup based on a transmission diffraction grating that can be used for fluorescence as well as absorption (colorimetric) and scattering (darkfield) spectral imaging of cells.

While transmission gratings have been used in microscopy and spectroscopy for long time, their applications for single-molecule and single-cell fluorescence imaging are recent.\textsuperscript{7, 10-11, 34} However, high-resolution and high-magnification multimodal spectral imaging of cells through a transmission diffraction grating has not been investigated yet. Since most standard light microscopes do not have spectral imaging capabilities, it is challenging to perform imaging of biological cells and other specimens when high-resolution spectral imaging studies are needed. With a significant increase of applications of multi-color fluorescence, darkfield, and DIC spectral imaging, it is important to investigate how a microscope containing an inexpensive transmission
diffraction grating can facilitate detailed multimodal spectral analysis of biomolecules in individual cells. Moreover, the spectral imaging methodology based on the transmission diffraction grating could complement or improve the data obtained by other spectral imaging techniques.

Therefore, we report the development of a multimodal spectral imaging methodology based on a transmission diffraction grating and its application for spectral analysis of biological cells. The methodology is developed for several microscopy modes as well as combinations thereof. Fluorescence spectral imaging of yeast, bovine, and mouse cells, darkfield spectral imaging of human cells, and brightfield and DIC spectral imaging of stained tissue sections, dyed nanoparticles, and free dyes were performed using the presented methodology.

2.2 Instrumentation and methodology

2.2.1 A transmission diffraction grating based light microscope

A transmission diffraction grating disperses the light of various wavelengths (λ) through multiple grooves into spectral orders (n). For example, a transmission diffraction grating used previously for fluorescence spectroscopy of individual cells and single molecules transmits incident light into a zero order image (n=0) and diffracts light mostly into the first order (n=+1) spectrum. It can be expected that this diffraction grating will disperse in a similar way light scattered or transmitted by the cells in darkfield and brightfield or DIC microscopy, respectively. Hence, in spectral imaging mode, light emitted, scattered, or transmitted by the cells will be dispersed by the transmission diffraction grating into the first order spectral images and transmitted into
zero order images that are equivalent to the images of cells without the grating. Wavelengths in the first order spectral images can be determined using the grating equation for normal incident light:

\[ n\lambda = dsin\theta \]  

(1)

where \( d \) represents the grating constant (i.e., the spacing between grooves of a grating). To calculate a wavelength in the first order spectrum, the diffraction angle (\( \theta \)) should be determined from the following equation:

\[ \theta = \tan^{-1}\left(\frac{d_2}{d_1}\right) \]  

(2)

In this equation, \( d_1 \) represents the distance between the grating and the CCD camera, and \( d_2 \) represents the displacement distance between zero order images and the first order spectral image of the cell.

2.2.2 Methodology of multimodal spectral imaging

The schematic diagram shown in Figure 2-1 explains how a conventional light microscope is used for multimodal spectral imaging of cells upon incorporation of a transmission diffraction grating. The grating is positioned between the microscope objective and the CCD camera above a fluorescence filter cube and microscope tube lens. In the fluorescence mode, cellular fluorescence is excited by the light of a mercury lamp and collected through a filter cube containing an excitation filter, a dichroic mirror, and an emission filter. For each cell on the slide, the CCD camera records at the same time zero order images of cells (white dots in Figure 2-1) and first order spectral images corresponding to emission fluorescence spectra of fluorophores that are used to label cells. As expected from the grating equation (Eq. 1), a grating with a higher number of
grooves (i.e. lower value of grating constant, d) will spread the first order image more, but will have better spectral resolution than a grating with a lower number of groves (larger d). Spectral resolution can also be increased if a grating is set further from the CCD camera (larger d1) or if a CCD camera with smaller pixels is used.

Figure 2-1. Schematic diagram of the grating-based spectral microscope. The incorporation of a transmission diffraction grating into the light microscope enables spectral imaging of cells by fluorescence, darkfield, brightfield, and DIC microscopy.

First-order spectral images can be used to determine fluorescence spectra of fluorophores present in cells in the range of wavelengths selected by the emission filter of
a fluorescence filter cube. Wavelengths ($\lambda$) in the first order spectrum are calculated using the diffraction grating equation (Eq. 1) upon calculation of diffraction angles corresponding to these wavelengths (Eq. 2). The displacement distance ($d_2$) between zero-order and first order images is measured by the camera software in pixels, and converted into units of length upon multiplication with the size of a pixel. It is hard to measure precisely exact distance between the camera and the grating ($d_1$) because of the unknown thickness of the camera window. Therefore, the distance between the camera and the grating was calculated by recording the fluorescence signal of a compound with known emission properties (FITC) as described below.

The grating-based spectral microscope can record the spectrum of the light scattered by the cells. To perform this experiment, the fluorescence filter cube (Fig. 2-1) is rotated out of the light path and cells are selectively illuminated through a colored filter (e.g., green bandpass filter) and a darkfield condenser by a halogen lamp, whose light reaches the cells from the bottom of the slide. First order spectral images of cells represent light scattered by the cells upon their illumination with the incident light selected by the filter, and corresponding scattering wavelengths can also be calculated using Equations 1 and 2.

The setup can also measure absorption spectra of dye-labeled cells (e.g., hematoxylin and eosin labeled cells) or other colored specimens that are illuminated through a DIC/brightfield condenser. Instead of peaks with higher intensity than the background, as it is the case in darkfield and fluorescence imaging, absorption bands that appear in the first order spectral images will have a lower intensity than the background due to the absorption of incident light by the cells.
Alternatively, unlabeled or fluorescently labeled cells illuminated through a colored filter (e.g., green bandpass filter) and a DIC/brightfield condenser will absorb the color of light selected by a filter, and the first order spectral images of cells will show up as well. These spectral images are due to selective illumination through a colored filter and higher absorption (i.e. lower transmission) of incident light through cells in comparison to the surrounding medium. Hence, these spectral images do not show up because of absorption of incident light by specific chromophores present in the cells.

2.3 Experimental

2.3.1 Chemicals and materials

Microscope glass slides, coverslips, hematoxylin, cell growth media, and ChromoSphereTM red dyed microspheres (d = 50 µm) were purchased from Thermo Fisher Scientific (Pittsburg, PA). Fluorescent dyes 4',6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), rhodamine 123, and rhodamine isothiocyanate (RITC) were purchased from Sigma (St. Louis, MO). Yeast from Saccharomyces cerevisiae, type II, was also obtained from Sigma. Polysterene nanoparticles with diameter of 800 nm were from Bangs Laboratories. FluoCells® prepared slide #1 containing bovine pulmonary artery endothelial (BPAE) cells and FocalCheck™ fluorescence microscope test slide #3 were from Invitrogen Molecular Probes (Eugene, OR). In the BPAE cells, nuclei were labeled with DAPI, actin filaments in the cytoskeleton were labeled with Alexa Fluor 488-phalloidin, and mitochondria were labeled with MitoTracker® Red CMXRos. FocalCheck™ fluorescence microscope test slide contains blue, green, orange, red, and deep red fluorescent particles with diameters
of 2.5 μm. Human cheek epithelial cells were noninvasively sampled onto a glass slide by a healthy volunteer after receiving consent. Hematoxylin and Eosin (H&E) stained slides were purchased from Carolina Biological Supply (Burlington, NC).

2.3.2 Yeast cell labeling

Yeast cells were washed three times with 1X phosphate buffered saline (PBS) solution. After washing, cells were suspended in PBS, and fluorescent labels were added to the cells in the following concentrations: 1 µL of 1 µg/mL DAPI solution in PBS, 5 µL of a 1 mg/mL FITC solution in DMSO, and 5 µL of a 1 mg/mL RITC solution in DMSO. Cells were incubated with the dyes in the dark for 30 minutes. After the incubation, cells were washed with PBS three times again and re-suspended in 1 mL of PBS solution.

2.3.3 Mouse cell culture and labeling

Mouse vascular endothelial cells were cultured on 22 x 22 mm coverslips in six-well plates filled with Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10 % fetal bovine serum (FBS) and 1 % penicillin. The cells were grown in the incubator under 5% of CO₂ at 39°C. For labeling, 2 ml of media was mixed with 20 µL of 10 µg/mL DAPI solution in water, 10 µL of a 1 mg/mL rhodamine 123 solution in water, and 10 µL of a 0.6 mg/mL RITC solution in water. Dye mixture was added onto the coverslip containing cells, which was rinsed with PBS, and the cells were incubated with the dye at 37°C for 90 minutes. Then, the dye solution was discarded and coverslip was washed twice with PBS and culture medium for 10 minutes.
2.3.4 Preparation of microscope slides

Slides containing yeast cells were prepared after 1 μL of yeast cell suspension in PBS was delivered on a microscope slide and covered with a coverslip. Upon sampling, human cheek epithelial cells were spread on the glass slide and covered with a coverslip. Mouse vascular endothelial cells grown on a coverslip were washed twice with PBS, and coverslip was attached using double sided tape to the microscope slide containing a small drop of PBS and antifade solution. For prolonged storage of slides, the coverslips were sealed around the edges with nail polish. Particle solutions were dispersed in water and sandwiched between a microscope slide and a coverslip. Other slides were used as received from manufactures.

2.3.5 Spectral imaging

Cells were imaged using a Nikon Eclipse 80i upright microscope equipped for simultaneous work in the epi-fluorescence and a transmission light microscopy mode (i.e. DIC, darkfield, or brightfield mode). 10X Plan Fluor (numerical aperture, NA = 0.3), 40X Plan Fluor (NA = 0.75), and 100X Oil Plan Apo (NA = 1.4) objectives were used for imaging by fluorescence, DIC, and brightfield microscopy. A 40 X Plan Fluor and a 100X Oil Plan Fluor objective with an iris diaphragm (NA = 0.5-1.3) were used for darkfield microscopy. For fluorescence imaging, the following filter cubes were used: DAPI (excitation filter: 360BP40; emission filter 460BP50), FITC (480 BP40; 535BP50), TRITC (545BP30; 620BP60), Texas Red (560BP55; 645BP75), and triple DAPI/FITC/Texas Red (excitation: 395-410 nm, 490-505 nm, and 560-585 nm; emission: 450-475 nm, 515-545 nm, and 600-652 nm). One high-resolution oil condenser (NA =
1.4) was used for cell imaging by DIC and brightfield microscopy, while another oil condenser (NA = 1.20-1.43) was used for darkfield microscopy. Optics for DIC microscopy included appropriate DIC prisms, a polarizer, and an analyzer. An X-Cite 120 illuminator (EXFO Life Sciences, Mississauga, Canada) coupled to a liquid light guide was used for illumination of cells for fluorescence microscopy, while a halogen lamp was used for illumination of cells in DIC, brightfield, and darkfield microscopy. A 45-mm green bandpass filter (546BP20) was used for selective illumination for darkfield microscopy and occasionally for DIC microscopy. Images of cells were acquired using a Photometrics CoolSNAP ES2 CCD camera (Tucson, AZ). The camera contained an imaging array consisting of 1392 x 1040 pixels, and the size of each pixel is 6.45 x 6.45 µm.

The transmission grating containing the 70 grooves/mm was purchased from Edmund Scientific (Barrington, NJ). The spectral imaging methodology used is similar to the one used previously for fluorescence imaging of single molecules. Briefly, the only modification of a conventional upright light microscope was incorporation of a diffraction grating in the light path. Initially, a transmission grating was placed between the CCD camera and microscope objective on the glass window, which was above the tube lens, at a distance of ~10.5 cm from the camera chip. The grating and magnification of objectives were chosen to observe maximal separation of fluorescent, absorbed, or scattered light within the first order spectrum, while maintaining both the zero and first order spectral image within the same field of view. An additional C-mount tube containing a movable slider that held the grating was machined (University of Toledo, Department of Physics Machine Shop). This slider allowed gradual change of the
distance between microscope objective and the CCD camera between 6 cm and 7 cm. However, all images presented in this manuscript were acquired using 70 grooves/mm grating set at the distance of 10.5 cm from the CCD camera chip. According to manufacturer specification, this grating typically transmits 41% of light into the zero order and 32% of light in the first order (n= +1) at 632 nm, while light diffracted into other orders is negligible. To measure grating transmission at other wavelengths, FocalCheck™ fluorescence test slide was used and regions of interest (ROIs) were imaged around zero and first order images of fluorescent nanoparticles.

2.4 Results and discussion

2.4.1 Fluorescence spectral imaging of yeast cells labeled with multiple fluorescent dyes..

The grating-based spectral microscope can perform spectral differentiation of multiple fluorophores in individual cells and cellular organelles. Figure 2-2 shows the fluorescence spectral image of a yeast cell (diameter ~ 5 μm), labeled with DAPI, FITC, and RITC. These dyes bind to the cell nucleus (DAPI) and cellular proteins (FITC and RITC), and are spectrally distinguished in yeast cell using the grating and a triple filter cube that contains excitation and emission filters specific for all dyes. The dyes can be also spectrally characterized by the grating when excitation and emission filters specific for each dye are used (Fig. 2-3), but this experiment takes longer time because three filter cubes are used interchangeably instead of one triple filter cube. Hence, the diffraction grating setup in combination with a triple filter cube provides simultaneous spectral imaging of all three dyes in a single experiment. The combination of the grating with filter cubes also distinguishes multiple fluorescent dyes that are localized in the same
location (e.g., in a single organelle). For example, FITC and RITC are bound to cellular proteins throughout the cytoplasm of the yeast cell, but they are clearly distinguished by the grating because of differences in their fluorescence emission spectra.

Figure 2-2. First-order spectral image (top left) of a single yeast cell (top right) labeled with DAPI, FITC, and RITC. Fluorescent dyes were excited simultaneously using a triple filter cube, and imaged through a diffraction grating by the fluorescence microscope. Intensity profile across first and zero order spectral images is shown at the bottom. A 40X microscope objective was used for imaging.

Figure 2-3. Compiled first-order spectral images (top left) of a single yeast cell (top right) labeled with DAPI, FITC, and RITC. Fluorescent dyes were excited separately using three filter cubes and imaged through a diffraction grating by a fluorescence microscope.
Additionally, the grating based spectral microscope can be used to characterize fluorophores by the calculations of their emission fluorescence spectra using Equations 1 and 2. To determine the accurate distance between the diffraction grating and the camera chip (d1), the distance between peaks in the intensity profile of the first and zero order images of FITC (fluorescence emission maximum at 525 nm) was measured in labeled yeast cells (Fig.2-2). This distance was 599 pixels (3864 µm) using a grating with 70 grooves/mm (d = 1/70 mm). Using grating equation (Eq.1), it could be calculated that light with a wavelength of 525 nm is dispersed at an angle of 2.100. Further, it was derived using Equation 2 that the distance between the grating and camera was 10.5 cm. This value was used to calculate wavelengths corresponding to fluorescence emission maxima of other dyes. From the intensity profile shown in Figure 2-2, it was found that emission maxima of DAPI and RITC are at 456 nm (517 pixels away from zero-order maximum) and 610 nm (696 pixels away from zero order maximum), respectively. Calculated values closely match emission maxima of these dyes reported previously. Since fluorescence emission maxima of DAPI and RITC are 154 nm and 179 pixels apart, the spectral resolution of the system is ~0.86 nm/pixel. Intensity profiles can be also exported into a spreadsheet and plotted as conventional emission fluorescence spectra (i.e., relative fluorescence intensity vs. wavelength) upon appropriate calculations. Figure 2-4 shows emission spectrum of DAPI, FITC, and RITC in a yeast cell. To plot this spectrum, the maximum intensity of the zero order image (Figure 2-2) was set as zero of the wavelength scale, and wavelengths corresponding to fluorescence spectra of dyes were calculated and plotted along with associated fluorescence intensities.
Figure 2-4. Fluorescence emission spectrum of a single yeast cell labeled with DAPI, FITC, and RITC. Intensity profile across first and zero order spectral images of the cell was measured, and wavelengths were calculated using Equations 1 and 2 considering the zero order maximum as the zero of the wavelength scale.

2.4.2 Fluorescence spectral imaging of fluorescently labeled organelles in BPAE cells

Besides spectral differentiation of multiple fluorescent dyes, the grating based spectral microscope can spectrally image with high resolution dyes bound to various cellular organelles. Figure 4 shows a bovine pulmonary artery endothelial (BPAE) cell whose nucleus (labeled with DAPI), cytoskeleton actin (labeled with Alexa Fluor 488-phalloidin), and mitochondria (labeled with MitoTracker Red) were spectrally and spatially distinguished by the diffraction grating upon illumination through a triple DAPI/FITC/Texas Red filter cube. Therefore, cellular organelles (e.g. nuclei,
cytoskeleton, and mitochondria) can be spectrally imaged in BPAE cells based both on their spectral properties and morphology of organelles. Alternatively, all three fluorophores can be imaged in BPAE cells using the grating setup along with specific filter cubes (Fig. 2-5 A-C). The combination of a grating with filter cubes again provides a way to distinguish multiple fluorescent dyes in a single organelle. For example, the nucleus of a BPAE cell shows up twice in the spectral image (Fig. 2-6) when the grating is used with triple DAPI/FITC/Texas Red filter cube, and it also can be imaged when the grating is used with DAPI and FITC filters separately (Fig. 2-5 A-C). This is probably due to the binding of the green fluorescent Alexa Fluor 488-phalloidin, which is spectrally distinguished from DAPI, to actin present in the nucleus. Again, displacement distances were measured between maxima of the first order and zero order images and used to calculate the wavelengths corresponding to emission maxima of DAPI, Alexa Fluor 488, and MitoTracker Red. These values were 456 nm, 525 nm, and 604 nm, respectively, and are close to emission wavelength of each dye reported by dye manufacturers.
Figure 2-5. A. First-order fluorescence spectral image (top left) of a single BPAE cell (top right) acquired through a green (FITC) filter cube. Fluorescence of actin filaments in the cytoskeleton that were labeled with Alexa Fluor 488-phalloidin was imaged. B. Compiled first-order spectral images (top left) of a fluorescently labeled BPAE cell (top right). Spectral images of MitoTracker labeled mitochondria (red) and DAPI-labeled nucleus (blue) were separated by a diffraction grating upon fluorescent imaging through Texas Red and DAPI filter cubes, respectively. C. Overlap of spectral images of fluorescently labeled cytoskeleton (green), mitochondria (red) and nucleus (blue) of a BPAE cell.
Figure 2-6. First-order fluorescence spectral image (left) of a single BPAE cell (right) excited through triple DAPI/FITC/Texas Red filter cube. A 40X microscope objective was used for imaging.

2.4.3 Spectral imaging of cells using dark field microscopy

The grating based spectral microscope can also record spectral images of cells by darkfield microscopy. In this spectral imaging mode, the grating diffracts light that is scattered by the cells upon their illumination through a darkfield condenser. Figure 2-6 shows a darkfield microscopy image of a human cheek epithelial cell (d~50 µm) and corresponding first order spectral image upon illumination of the cell through a green filter (λ = 546 ± 10 nm). Using the grating equation (Eq.1) and intensity profiles in this image, we calculate that cells scatter green light at the maximum wavelength of 543 nm,
which matches closely the wavelength of the green bandpass filter used for illumination of cells. This experiment indicates that scattering spectral characteristics of biological cells can be measured by the grating based setup.

Figure 2-7. First-order scattering spectral image (left) of a human cheek epithelial cell (right) recorded by darkfield microscopy through a diffraction grating. Cells were illuminated through a green bandpass filter and a 40X microscope objective was used for imaging.

2.4.4 Spectral imaging of stained cells by bright field and differential interference contrast microscopy

Spectral imaging of various chromophores and colored biomarkers is useful for colorimetric investigation of cells and tissues. To test if the grating based setup can be used for measurement of absorption spectra of dyes, colored marker dots were drawn on a glass slide and imaged by brightfield and DIC microscopy. Figure 2-7 shows the measurement of absorption spectra of a red marker dye by brightfield microscopy with the intensity profile shown at the bottom of the image. The absorption maximum of 490 nm was determined for this red dot from the zero order and first order images and the
grating equation. This absorption maximum wavelength corresponds to the blue-green region of the electromagnetic spectrum, and its color is complementary to the red color of the dot supporting the calculation. By imaging the same dot under fluorescent illumination (Figure 2-8), it was found that its emission fluorescence spectrum shows a maximum at 586 nm, indicating that the marker may contain a rhodamine-like fluorophore.

Figure 2-8. First-order image (left) of a red marker dot (right) recorded by brightfield microscopy through a diffraction grating using 10X microscope objective (top). Intensity profile across the image is shown at the bottom.
Figure 2-9. First-order spectral image (left) of a red marker dot (right) recorded by fluorescence microscopy through a diffraction grating using 10X objective. Intensity profile across the image is shown at the bottom.

Absorption spectral imaging of cells and tissue sections labeled with common colorimetric dyes such as hematoxylin and eosin (H&E) can also be performed using the diffraction grating based spectral microscope. While hematoxylin binds to nuclei, eosin binds mostly to intra cellular and extra cellular proteins. Figure 2-10 shows a brightfield image of a part of Amphiuma tissue section containing hematoxylin and the corresponding intensity profile in the image that was used to determine the absorption
spectrum of the dye (Figure 2-11). The wavelengths in the absorption spectrum were calculated considering the point of the lowest intensity in the zero order image as the zero of the wavelength scale. The pixel intensities (I) were converted into transmittance (T = I/I₀) taking the intensity of the background as the intensity of light reaching the sample (I₀), and the absorbance values were further calculated (A = -logT). Figure 2-11 shows that the absorption maximum of hematoxylin is at ~650 nm. This value closely matches the literature value of absorption maximum of hematoxylin bound to a tissue section. Figure 2-12 shows another part of an H&E-stained Amphiuma liver tissue section indicating that spectral properties of multiple cells across tissue section can be measured.

Figure 2-10. First-order absorption image (top left) of a part of an H&E stained *Amphiuma* liver tissue section (top right). Image was recorded by brightfield microscopy through a diffraction grating using a 10X microscope objective. Intensity profile across the first and zero order images is shown at the bottom.
Figure 2-11. Absorption spectrum of hematoxylin stain bound to the *Amphiuma* liver tissue section. Intensity profile was measured across first and zero order spectral images in Figure 2-10, and wavelengths were calculated using Equations 1 and 2 considering the zero order minimum as the zero of the wavelength scale. Absorbance values were calculated as described in the text.
The setup can be used for brightfield or DIC imaging of unlabeled cells that show sufficient contrast between cellular features. As mentioned above, spectral images of unlabeled cells can also be recorded by brightfield or DIC microscopy using a colored filter in the light path. Figure 2-13 shows DIC spectral images of human cheek epithelial cells taken upon their illumination through a green bandpass filter. However, as explained previously, DIC microscopy in this case does not measure absorbance spectra of chromophores present in this cell but rather absorption spectrum of a colored filter that was inserted in the illumination path.
Figure 2-13. Spectral image of human cheek epithelial cells imaged through a green bandpass filter by spectral DIC microscopy using 40X objective

2.5 Evaluation of the multimodal spectral imaging system

2.5.1 Simultaneous use of multiple spectral imaging modes for cell imaging

As shown in Figures 2-5 and 2-7, the grating-based spectral microscope can be used for simultaneous spectral imaging of dye specimens by fluorescence and brightfield microscopy. Simultaneous multimodal imaging of fluorescently labeled cells is possible if cells contain structures, dyes, or particles that have fluorescent properties, scatter the light, and have high absorption coefficients (i.e. if they show up with sufficient contrast in all spectral imaging modes). However, one should be cautious on how data are
interpreted since cellular fluorescence can be excited not only by epi-fluorescence but also by darkfield or DIC microscopy. For example, Figure 2-13 shows an overlap between spectral fluorescence images of a FITC and RITC labeled yeast cell with corresponding spectral images acquired by DIC microscopy. The fluorescence signals of two dyes acquired by epi-fluorescence microscopy were pseudo colored in green and red. They overlapped with a fluorescence spectral image of the cell acquired by DIC microscopy (long streak across other images) and a spectral image of the same cell that was acquired by DIC using a green bandpass filter (monochromatic image next to the FITC image). Therefore, the former spectral image represented cellular fluorescence spectrum instead of absorption spectrum.

Figure 2-14. Compiled first-order spectral images (top left) of a single yeast cell (top right) labeled with FITC and RITC. The image was acquired by fluorescence and DIC microscopy using 40X objective. Fluorescent dyes were excited using FITC and TRITC filter cube, while DIC microscopy images were acquired with and without a green bandpass filter in the illumination path.

The sensitivity of a grating-based setup for absorption spectra measurement is lower than in the case of fluorescence spectral imaging. This can be noticed in imaging of fluorescently labeled BPAE cells, where brightfield and DIC microscopy neither
visualized these cells nor measured absorption spectra of fluorophores in cells (data not shown). However, a spectral microscope containing transmission diffraction grating can be used in favorable cases to simultaneously record fluorescence, absorption, and/or scattering spectra of cells. Besides localization of various probes in the cells, information on spectral properties of biomolecules and labels that are used to tag cells can be determined by these experiments.

2.5.2 Spectral characteristics of the imaging system

The spectral resolution of the described spectral microscope using a grating with 70 grooves per mm was ~0.86 nm/pixel. It is independent on the magnification of the objective used, and the distance between zero and first order spectral images of cells will be the same when objectives of different magnification are used. However, the objective’s magnification and NA will determine spatial resolution of the spectral images because the size and spatial resolution of the first and zero order images are dependent on the objective used. Objectives of higher magnification and numerical aperture are desirable so that the best spatial resolution can be obtained. However, the objective magnification should be carefully selected, because large cells that are magnified too much could overlap with their first order spectra, and this may complicate the spectral analysis.

The presented spectral imaging approach is also useful for imaging of multiple cells that can be spectrally imaged on the slide at once (e.g., Figs. 2-11 and 2-12). A high cell density on a microscope slide may lead to the overlap of spectral images of cells with zero order images of neighboring cells. This can be readily avoided by the preparation of
slides with appropriate cell density. The bigger challenges are to avoid overlap between the first order spectra of cellular organelles that are located at the adjacent positions in the cell and smearing of spectral images due to out of focus light. To address these issues, the deconvolution of spectral images was performed. For example, fluorescently labeled mitochondria in Figure 2-6 are located so close to each other that their spectra will overlap and make spectral images extra wide. The iterative deconvolution of this figure by Image J software improved the spectral image of fluorescently labeled BPAE cells (Fig. 2-15). While algorithms are available for deconvolution of both monochrome and multi-colored images, they are mostly optimized for the deconvolution of fluorescence microscopy images rather than spectral images. Therefore, a potential development of spectral imaging deconvolution software could improve the resolution of spectral images.

Figure 2-15. Deconvoluted first-order fluorescence spectral image (left) of a single BPAE cell (right) excited through a triple filter cube using a 40X microscope objective. Image was deconvoluted using Image J software.
Another challenge, which is common for all spectral imaging methods, is distinguishing fluorophores or chromophores that have similar spectral properties and are present at the same location in the cells.\textsuperscript{39} Currently, this can be done only by combination of spectral imaging with a mathematical procedure called spectral unmixing,\textsuperscript{39} and the development of such procedure for presented spectral imaging method would be very beneficial.

The spectral performance of present system is also dependent on the quality of the grating used. Using fluorescent test slide, we found out that the ratios of the intensities of the first and zero order light diffracted by the grating are: 8, 3.5, 1.6, 1.5, and 0.9 for blue, green, orange, red, and deep red fluorescent nanoparticles, respectively. Tuning of the spectral properties of the grating can help increasing sensitivity of the spectral imaging measurement in fluorescence, scattering, or transmission mode.

2.5.3 Applicability of the spectral imaging system

Preceding examples showed that the spectral imaging setup can be used for imaging of standard fluorescently labeled cells, unlabeled cells, and stained cells. To check performance of the system on more complex specimens, mouse vascular endothelial cells were grown and labeled with DAPI, rhodamine 123 (a dye that labels mitochondria), and RITC. As can be seen in Figure 2-15, fluorescent spectral images of cells containing these three dyes can be readily recorded. Described setup is convenient for spectral characterization of cellular organelles and can be used potentially for quantification of signals coming from different cellular compartments. In present study,
spectral information was acquired through band pass filters, but it is also possible to acquire spectra of multiple fluorophores using long pass filters.\(^7\)

Figure 2-16. First-order fluorescence spectral images (right) of single mouse vascular endothelial cells labeled with DAPI, rhodamine 123, and RITC (left). Images were recorded using DAPI, FITC, and TRITC filter cubes. A 40X microscope objective was used for imaging.

The present setup is extremely sensitive for fluorescence measurements and it could be used for single-molecule fluorescence spectroscopy of highly fluorescent molecules. The sensitivity of the system for scattering light spectral measurement is also high because of low background of darkfield images, but clean slides and coverslips should be used for imaging in order to prevent non-specific scattering. The sensitivity and
signal-to-noise ratio of absorption spectra measurements will depend on the absorption coefficient, thickness, and amount (e.g. concentration) of the dyes present in a specimens.

In order to tune the present system for absorption spectra measurement, we have imaged 50 µm particles labeled with a red dye whose absorption maximum is broad and peaks at 524 nm. Figure 2-16 shows absorption measurement of these nanoparticles and our calculations indicate similar value of absorption maximum. Additionally, nanoparticles with diameter of 800 nm stained with hematoxylin were imaged by the spectral microscope using 100X microscope objective (image not shown). The signal-to-noise ratio of the first order spectrum was not sufficient to precisely measure the absorption maximum, but an image corresponding to absorption maximum of the dye was recorded. Therefore, the sensitivity of the absorption spectral imaging can be tuned by appropriate choice of an objective or a diffraction grating as well as by labeling of samples with appropriate dyes.
Figure 2-17. First-order absorption image (top right) of a red dyed microsphere (top left). Image was recorded by bright field microscopy through a diffraction grating using a 40X microscope objective. Intensity profile across the first and zero order images (blue line) and background spectrum (purple line) are shown at the bottom.

2.6 Conclusion

Multimodal spectral imaging of cells was demonstrated by the incorporation of a transmission diffraction grating into a light microscope. Fluorescence, scattering, and absorption spectral images of cells were acquired with presented spectral imaging methodology. Applications described included fluorescence spectral imaging of yeast and mammalian cells labeled with multiple fluorophores, darkfield spectral imaging of unlabeled human cheek cells, and brightfield and DIC spectral imaging of H&E stained cells, fluorescently labeled cells, dyed particles, and free dyes. The multimodal spectral imaging methodology is readily performed on a conventional light microscope, and can be applied for spectral imaging analysis of cells and other specimens.
Future work will strive to overcome the aforementioned challenges and improve the technique so that it can be used more efficiently for simultaneous spectral imaging of higher number of fluorophores and chromophores in multiple cellular organelles. Despite all challenges, the multimodal spectral imaging methodology is a fast approach for spectral imaging of cells and other specimens. Considering relatively low cost of a transmission grating, it is also an affordable approach for scientists in need of the acquisition of spectral images. Incorporation of the grating into a conventional light microscope is straightforward and potential users can become proficient quickly in application of the described spectral imaging methodology. The methodology is also open to other commonly used applications of microscopic imaging such as optical sectioning of cells and tissues, live cell imaging, and high-throughput cell analysis.
Chapter 3

Imaging mass spectrometry of mouse kidney tissues using MALDI TOF/TOF-MS

3.1 Introduction

Proteins are involved in the complex biochemical processes in a living organism. Identifying them and characterizing their function in biological samples such as tissues, plasma and urine are of primary importance for the comprehensive understanding of their roles in the biological systems and how they are related to human diseases. Various techniques have been developed over the years for proteomics studies. Among them, mass spectrometry is a powerful tool to study the protein sequence, structure, post translational modifications, and protein-protein interactions. Mass spectrometers equipped with soft ionization sources such as ESI and MALDI are widely used in combination with separation techniques. For example, common practices for protein identification involve gel separation and enzyme digestion followed by peptide mass fingerprinting or peptide separation by chromatography coupled to tandem mass spectrometry.

One of the latest promising innovations in mass spectrometry driven proteomics is mass spectrometry imaging (MSI). It is a technique that is using mass spectrometry to visualize the spatial distribution of molecular compounds such as proteins, lipids,
metabolites and pharmaceutical compounds by measuring their molecular masses. By MSI, tissues are directly analyzed to uncover the biomolecular distribution in situ and their spatial distribution across the surface is also maintained. By correlating the molecular information with the histological features, the tissue samples can be classified into different types, e.g., normal versus diseased, through statistical analyses.\textsuperscript{42} The use of a MALDI mass spectrometer as a tool to study the localization of peptides and proteins in biological samples was started by Caprioli’s group in late 1990s.\textsuperscript{4} The instrumentation, sample preparation methodologies, and data analysis have been in rapid development ever since. In the field of clinical proteomics, MALDI became a potential tool for the diagnostic of various diseases and is complementary to histological and pathological diagnostic techniques.\textsuperscript{43} Furthermore, the newly developed technique of offline coupling of nano-HPLC and MALDI TOF/TOF can provide confident protein identification.\textsuperscript{44}

Our specific interest is to study proteomics related to polycystic kidney disease (PKD). PKD is a group of genetic kidney disorders resulting in appearance of renal cysts. The cysts are formed in the nephrons and become fluid filled. PKD is one of the most common monogenetic disorders diagnosed in adults. The final stage of PKD results in enlarged kidney and requires renal replacement.\textsuperscript{45} On the proteomics level, limited biomarkers related to the development of PKD, such as PKD1 and PKD2 proteins, have been found and characterized, and the current techniques for diagnostics of PKD mostly rely on immuno-antigen binding. We use normal and PKD mouse kidney tissues as model and utilize MALDI-IMS to compare the protein distributions on normal and PKD tissue samples and to identify proteins that are exclusively present or over expressed in
the diseased tissue, but not in the normal tissue. Furthermore, we tried to differentiate the PKD tissue in the early stage which could not be observed by histological staining.

3.2 Principle of MALDI-IMS

Mouse kidneys are fresh frozen and stored at -80°C before sectioning into 10 to 15 µm thick sections in a cryostat. Tissue sections are mounted on indium tin oxide coated glass slides. These slides are conductive and transparent allowing sample analysis by both MALDI-MS and histology. Tissue slides then undergo a gradient of alcohol washes for stabilization as well as salt and lipid removal. The tissue positions can be marked with a white marker and sample slides can be scanned under regular color scanner for future registration with sample position under laser beam. A light-absorbing matrix is applied to the tissue section. The matrix extracts analytes in tissue sections and remains on the tissue surface after washing. The procedure of matrix deposition can be performed by either microspotting or spraying. After the matrix is sprayed across the tissue surface, the matrix forms co-crystals with analytes. For protein identification, tissues are in situ digested into peptides with trypsin by spraying the enzyme over the tissue surface. Matrixes of choice for peptides are applied in the same way as for protein imaging. The prepared sample slides are mounted on the metal holder and sent into the high vacuum ion source of the MALDI mass spectrometer. Analyte/matrix co-crystals are irradiated by a Nd:YAG laser with wavelength of 355 nm. The desorbed molecules formed singly protonated ions which are accelerated by an electric field and pushed into a field-free high-vacuum drift tube to be separated and detected. Under a programmed procedure, a laser beam with fixed frequency, number of shots and intensity irradiates the
tissue surface according to the raster distance with zig-zag horizontal movement. After a number of shots on each raster spot, a mass spectrum is formed along with spot position. Hundreds to thousands of spectra are generated after each tissue imaging run. Additionally, peptides from enzyme-digested tissues can be extracted by proper solvents. Furthermore, after purification and desalting, the peptide extracted from tissue surface can be separated by nano-HPLC and fractioned and concentrated on specially-coated target plates. The fractions are measured by MALDI TOF/TOF and protein identification can be achieved by combining MS and MS/MS information and searching against protein databases. Figure 3-1 shows the workflow of MALDI mass spectrometry imaging.
Figure 3-1. MALDI mass spectrometry imaging workflow
3.3 Experimental

3.3.1 Chemicals and materials

HPLC grade water, acetonitrile, hydrochloric acid, methanol and glacial acetic acid were purchased from Fisher. Trifluoroacetic acid (TFA) was purchased from Sigma. Sinapinic acid, alpha-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid were purchased from Bruker Daltonics and used without further purification. The protein and peptide calibration standards were also purchased from Bruker Daltonics. Cytochrome C digest standard was from Dionex. Trypsin was purchased from Sigma and dissolved in 50 mM ammonium bicarbonate to obtain a solution with final concentration of 0.05 μg/μL. Hematoxylin Gill 3x and Eosin Y were purchased from Fisher. Histoclear was purchased from National Diagnostics. Mounting medium was purchased from Vector. MilliQ water was collected through water filter system in the pharmacology department.

3.3.2 Animal samples

Mouse kidney tissues of normal and Polycystic Kidney Diseased type were provided by Dr. Surya Nauli’s laboratory (Department of Pharmacology, University of Toledo). Normal and PKD kidney tissues were from 6 month old mice.

3.3.3 Instrumentation and software

The tissue sections were cut on a cryostat (Leica CM1950, Wetzlar, Germany) and mounted onto conductive indium tin oxide (ITO)-coated glass slides (25 mm × 75 mm × 1.1 mm) (Bruker Daltonics, Bremen, Germany). The sections were coated with
matrix using the ImagePrep device (Bruker Daltonics). The peptide separations were performed on a Nano-HPLC system (Ultimate 3000, Dionex) with a C18 PepMap column (75 µm i.d. x 150 mm, Dionex) and fractioned on a 384-spot AnchorChip target plate (Bruker Daltonics, Germany) through Proteineer fc (Bruker Daltonics, Germany). The mass spectrometry analyses were performed on a MALDI TOF/TOF equipped with a Smartbeam laser (UltrafleXtreme, Bruker Daltonics, Germany) in linear, reflectron and LIFT positive ion modes. For each spot 500 laser shots were accumulated at constant laser power with 500 Hz repetition frequency. The laser intensity was adjusted between 70% and 85% before every imaging auto run. The mass detection range was set as 3000 to 20000 Da for imaging of proteins and 800 to 4000 Da for imaging of peptides. The MALDI TOF/TOF was operated using FlexControl (Version 3.3, Bruker) and FlexImaging (Version 3.0, Bruker) for calibration, method parameter setup and image register. Online database search was enabled through MASCOT batch run function in BioTools (Version 3.2, Bruker). Spectra online and post processing of baseline subtraction and peak smooth were achieved by FlexAnalysis (Version 3.3, Bruker) under batch mode. Imaging data analysis was performed in FlexImaging and ClinProTools (Version 2.2, Bruker). Spectra were plotted in OriginPro8 (OriginLab). Nano-HPLC and Proteineer fc were operated using HyStar (Version 3.2, Bruker) and Chromeleon Xpress (Dionex) software. Tandem MS and online protein searching were performed using WarpLC (Version 1.2, Bruker) with embedded MS, MS/MS and BioTools methods. The spotted AnchorChip plate was automatically measured by MALDI TOF/TOF using WarpLC software. After obtaining the MS spectrum of each individual spot, the instrument performed MS/MS analyses adding up to 2000 shots on selected peptide
peaks. A file containing the MS and the MS/MS peak lists was submitted to the MASCOT server (Version 2.3, Matrix Science, UK) against SwissProt database with 0.2 Dalton parent mass tolerance, 0.7 Dalton fragment tolerance, and one missed trypsin cleavage site.

3.3.4 Tissue imaging sample preparation

3.3.4.1 Tissue embedding

Fresh mouse kidney samples were snap-frozen and stored at -80 °C before analysis. By using Cryo-Gel embedding medium, the kidney tissues were set on top of cryomolds by barely touching the medium on the bottom. The process was performed on dry ice to keep samples frozen as well as to solidify the embedding medium for cryostat cutting. The position of the tissue was carefully oriented to ensure vertical sectioning.

3.3.4.2 Tissue sectioning

The cryostat was turned on at least one night before sectioning to reach ideal low temperature, -20 °C. Tissues mounted on cryostat were placed in the cryostat for 20 min to raise the temperature of tissue which can prevent tissue sections from flaking and tearing. ITO coated glass slides (Bruker Daltonics) were placed in the cryostat to cool down as well. Cutting thickness was adjusted to 10 µm for both normal and early stage mouse kidney tissue and 15 µm for late stage PKD tissue after a few test cuttings. The tissues were sliced with a stainless steel blade cleaned of contaminates with a methanol rinse before cutting. The samples were sectioned with fixed thickness from the top to the middle part. The bottom part in contact with embedding medium was avoided. The
sliced thin layers of tissue sections were collected onto the ITO glass slides by applying the conductive side to the tissue and warming it up from the other side with tip of the fingers to make tissue thawed and adhered onto the glass surface. The mounted sections were immediately placed in a vacuum desiccator for 30 minutes to prevent protein oxidation or migration. Then tissue sections were sealed in a plastic bag with Drierite in a dry ice environment for transportation and kept at -80 °C before analysis.

3.3.4.3 Tissue washing

The slides were slowly thawed by placing them at -20 °C for 10 minutes, followed by 10 minutes at 4 °C, and at last 10 minutes at room temperature in a vacuum desiccator. To fix the tissue and remove salts, lipids and other small molecules, the sample slides needed to be washed by a series of alcohol gradients. For protein analysis, the sections were immersed in 70% ice-cold isopropanol for 30 seconds and dried completely in the vacuum desiccator followed by immersion in 95% ice-cold isopropanol twice for 30 seconds. For peptide analysis, the wash by 70% and 90% isopropanol were shortened to 10 seconds, and final wash in 90% isopropanol, 9% glacial acetic acid and 1% HPLC grade H₂O for 30 seconds. Slides were dried in a vacuum desiccator.

3.3.4.4 On-tissue trypsin digestion

400 μL of freshly-made 50 mM ammonium bicarbonate was added to 20 μg of proteomics grade trypsin (Sigma). 200 μL of trypsin solution was sprayed to one tissue slide using ImagePrep by applying the correct spray method and adjusted spray strength. The process was complete after a few spraying cycles of trypsin on to the slide surface.
The sample slides were kept in a sealed box with a Kimwipe wetted with 50/50 methanol/water to keep a moist environment, and was incubated at 37 °C in a CO₂ incubator overnight.

3.3.4.5 Protein and peptide calibration and image scan

Aliquots of protein standard calibration mixture, which contains insulin, ubiquitin, cytochrome C and myoglobin were deposited on tissue slides to calibrate the mass range from 3000 to 17000 Da. For a mass range from 800 to 3000 Da, aliquots of peptide standard calibration mixture, which contains angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39 and somatostatin 28 was applied. Positions of tissue sections and calibration spots were marked by a white marker, which helped locate the position under the laser beam during setting up of the FlexImaging autorun. Tissue slides with marked positions were scanned in regular photo scanner, and the JPEG format of slide images were uploaded into the MALDI controlling computer for online registration with real image under camera in the ion source.

3.3.4.6 Matrix coating in ImagePrep

A solution of SA prepared at concentration of 10 mg/mL in 60/40 (v/v) ACN/H₂O with 0.3% TFA was applied to tissue slides for protein analysis and either 25 mg/mL DHB in 50/50 methanol/H₂O with 0.3% TFA or 15 mg/mL CHCA in 50/50 ACN/H₂O with 0.3% TFA was applied to trypsin digested tissue slide using ImagePrep. The crystals formed were observed under a light microscope to determine the matrix coverage and crystal size.
3.3.4.7 Nano-HPLC separation

Cytochrome C from bovine digest standard (4 pmol) was loaded by an autosampler for each run. Fractionation was performed for 120 min with a gradient of buffer A (100% H$_2$O, 0.05% TFA) and buffer B (80/20 (v/v) ACN/ H$_2$O, 0.05% TFA): 0 min, 2% buffer B; 80 min, 50% B; 81-85 min, 90% B; 86-100 min, 2% B. The peptide separations were performed at 25 °C at flow rate of 200 nL/min and signals were detected at 214 nm. The eluates were online mixed with CHCA matrix solution using an external syringe pump with a flow rate of 100 μL/h in a micro tee chamber (Upchurch). Elution fractions were spotted automatically onto the AnchorChip spots with every 15 second interval in Proteineer fc.

3.3.4.8 Providing external matrix for LC fractions

Matrix solution for LC fractions was freshly prepared by mixing 712 μL of 95:5 (v/v) ACN/H$_2$O with 0.1% TFA, 72 μL of saturated CHCA in 90:10 (v/v) ACN/H$_2$O with 0.1% TFA, 8 μL of 10% TFA in H$_2$O and 8 μL of 100mM NH$_4$H$_2$PO$_4$. The solution was loaded to a 1 mL flathead gastight syringe. The syringe pump supplying the matrix was set to a flow rate of 100 μL/h with 15 seconds per fraction spot. An absolute amount of matrix per spot was approximately 417 nL. External calibrants were prepared by mixing saturated CHCA with peptide calibration standard and manual spotting on the 96 calibration anchor spot with 0.1 μL of solution per spot.
3.3.4.9 H&E staining

Hematoxylin and eosin staining was applied on the adjacent sections of tissue samples, which were cut together with the tissue in the cryostat sectioning procedure. The sections were mounted on regular glass slide coated with 3-aminopropyltriethoxysilane and stored at -80 °C before staining. Tissue slides were brought to room temperature and wetted in 70% ethanol. Then tissues were rehydrated with MilliQ water for a few minutes and immersed in hematoxylin solution for a few minutes. Excess stain was washed out by MilliQ water, and 50/50 (v/v) ethanol/H₂O solution containing 0.25% HCl. After a few more rinses by tap water, slides were immersed in eosin for one to two minutes. To remove excess dye, a few MilliQ water rinses were performed. The H&E stained tissue sections were dehydrated in 95% ethanol, then 70% ethanol and fixed in histoclear and mounting medium. Histological study of the kidney tissue cells was performed under light microscope.

3.4 Results and discussion

3.4.1 Normal and PKD kidney tissue protein distribution image

Six adjacent (~100 µm apart) sections of normal mouse kidney tissue sections were cut with 10 µm thickness in a cryostat and mounted on one ITO-coated glass slide. The tissue samples were washed and then sprayed with SA. Four adjacent (~100 µm apart) sections of early stage PKD mouse kidney tissue (no PKD phenotype is visually observed) were treated in the same way as normal tissue samples but mounted on a different ITO slide. MALDI imaging was performed in linear positive mode with calibration error under 60 ppm in the 3000 to 17000 Da mass range. To achieve decent
signal-to-noise, the laser intensity was set to 75% to 85% of its maximum intensity with repetition rate of 500 shots per spot and 100 µm raster resolution. A spectrum of every single spot and their position information were saved. The spectra were online processed with peak smoothing and baseline subtraction in FlexAnalysis. The average spectra of normal and PKD tissue sections were generated in FlexImaging. Figure 3-2 shows the average spectrum of the six normal mouse kidney sections and Figure 3-3 shows the average spectrum of the four PKD mouse kidney sections. All spectra were normalized by dividing every single data point by the most intense peak in the respective spectrum. Under the same sample preparation procedure, spectra from the same type of tissue (normal and diseased) show similar ion peak pattern and relative intensity. The reproducibility of the MALDI-IMS system was confirmed. In the spectra, some ions show relatively high intensity while the other ions were suppressed. In the image construction software FlexImaging, peaks from average and a single spectrum can be selected and the distribution of the peaks can be viewed with single or multiple gradient colored view. Usually, spectra normalization algorithms such as total ion count (TIC) are applied to get images with better contrast. In our analysis, protein distribution images of the normal and PKD tissue sections were constructed by assigning peaks in the normalized average spectrum to scanned images of tissues (Figures 3-4 and Figure 3-5). Because both normal and PKD tissue sections were superficial cutting of the kidney organ, proteins detected are mostly from cortex region of the tissue. In some of the images, medulla region can also be distinguished because those proteins can only be seen in the cortex region and no signals were detected in medulla region, which is then shown in dark. The reasons can be the abundance of the different proteins and favorable
ionization in the ion source. Spectra of normal and PKD tissues were compared as shown in Figure 3-6. While some peaks show up in both spectra, such as m/z 3881, 5444, 8945 and 9980, most of the ion peaks vary in intensity and m/z values. Some peaks with similar m/z and intensity such as m/z 14979 in normal tissue and m/z 15012 in PKD tissue are very likely to be the same protein. But, the mass differences may indicate possible protein modifications, which could be caused by the disease.
Figure 3-2. Average protein spectra (A-F) of six normal mouse kidney tissues
Figure 3-3. Average protein spectra (A-D) of PKD mouse kidney tissues
Figure 3-4. Protein mass images of a normal mouse kidney tissue section
Figure 3-5. Protein mass images of a PKD mouse kidney tissue section
Figure 3-6. Comparison of mass spectra from normal (A) and PKD tissues (B)
3.4.2 On-tissue trypsin digestion and peptide distribution maps

From the protein mass spectra of the kidney tissues, no signals above 16,000 Da were detected because larger proteins are harder to be extracted by the matrix and have lower ionization efficiency. Thus, a lot of proteins with mass over 16,000 Da in the tissues are not readily seen in the protein spectra. The highest protein mass detected by direct protein MS imaging reported is around 80,000 Da, but was achieved by special chemical treatment and complex matrix deposition procedure, which is not widely adopted. Also, direct protein analysis is of little help to identify the protein. To solve the problem, a widely applied method is on-tissue enzyme digestion. Proteomics grade trypsin is usually applied (e.g., by microspotting or spraying) on tissue surfaces, and it cleaves peptide chains at the carboxyl side of lysine and arginine under suitable temperature (37 °C) and humidity. Peptide fragments are formed from the proteins without relocation.

In our study, a few normal kidney and late-stage PKD tissue sections close to the middle of the organ were sliced with 10 µm thickness and mounted on ITO slides. In PKD tissue sections many cysts filled with fluid were formed and a significant part of the tissue structures was missing. The sections were treated with gradient alcohol wash. Trypsin solutions were sprayed on each slide using ImagePrep device and the tissue samples were incubated at 37 °C overnight. CHCA and DHB matrices were applied on different slides of normal tissues and DHB matrix was applied to late-stage PKD tissues. MALDI image runs were performed in reflectron positive mode with calibration error under 15 ppm in the 800 to 3000 Da mass range. Laser power was set as high as 75% to 85% with 500 shots per spot and 100 µm raster as resolution. Average peptide spectra were shown in FlexImaging after each imaging run. Peaks in one average spectrum of
normal kidney tissue with CHCA as matrix were labeled and corresponding peptide distribution map was constructed by assigning the peaks to the scanned image of the tissue section. From Figure 3-7, we can clearly see distinct localization of a few different peptides. For example, a peak with m/z 861.45 is only found in the pelvis, m/z 1358.96 only shows exclusively in the cortex, m/z 1585.08 locates around the medullary cortex junction and m/z 2540.57 shows strong intensity in the medulla.

Figure 3-7. Peptide mass images of a normal mouse kidney tissue section
3.4.3 Histological correlation by H&E staining

Hematoxylin and eosin staining is a common histological method to study the morphology of tissues in pathology labs. Hematoxylin mainly stains nuclei of cells to a purple color and eosin works as a counter stain with a pink color. After staining, changes of nuclei can be observed by a pathologist under a high magnification microscope to diagnose diseases. For example, tubular cluster of cells of glomerulus is the indicator for development of polycystic kidney disease. Also, different regions of a tissue section can be differentiated by color darkness. We performed H&E staining on the adjacent tissue sections to those analyzed by MALDI-IMS. Figure 3-8 shows the selected peptide peaks from the normal mouse kidney tissue. The overlay of the four images showing ions with selected mass-to-charge ratios is in good agreement with H&E staining in terms of localization of these ions and kidney tissue morphology (Figure 3-9). Furthermore, H&E staining can correlate morphology with mass spectrometry results, which helps interpreting MS imaging data.
Figure 3-8. Selected mass distribution image of a normal mouse kidney tissue section

Figure 3-9. Overlay of selected mass images shown in Figure 3-8 and H&E staining of adjacent tissue section
3.4.4 Matrix influence on ionization selectivity

Figure 3-10 shows the average spectrum of two adjacent normal mouse kidney tissue sections. They were digested and prepared in the same way, but sprayed with different matrix. Because of the closeness of the two sections, we assume that the protein composition across the tissue surfaces is very similar. The results show that CHCA and DHB have their own preferences to particular compounds, which resulted in different peak intensities. For example, m/z 1274.9 and 1160.7 are the second and the third most intense peaks in CHCA spectrum, but show much weaker intensity and are buried in the noise in the DHB spectrum. So quantitative analysis of proteins in IMS needs to be performed with care because factors different than the intrinsic composition of samples such as matrices might contribute to ion intensities. More peaks and less noise are observed in the mid-mass range in CHCA samples while more high-mass peak signals were detected from DHB matrix sample. With different matrices, peak m/z 1530 is the most intense in both spectra, indicating that it might be from an abundant protein. If a known analyte of interest is to be detected, different matrices can be screened and selected by their influence on extraction efficiency and ionization preference.
Figure 3-10. Spectra comparison of two adjacent normal mouse kidney tissue sections with matrices CHCA (A) and DHB (B).
3.4.5 Comparison of late-stage cyst PKD and normal tissues

Both normal and late-stage PKD tissue sections were digested by trypsin and sprayed with DHB matrix. Figure 3-11 shows that the two average spectra are very different with much more intense peaks showing up in the PKD sample. Most of the intense signals from both normal and PKD were compared. Only a few peptide peaks are shared by both samples such as m/z 944, 1529 and 1275 Da. However their relative intensity within the samples varies a lot, indicating possible protein over or under expression in the PKD samples. By localizing the peaks in the PKD cyst tissue sections, we found that most of the peaks are from the hollowed area where the cysts were formed. Because of the obvious destruction of tissue morphology by the cysts, it is hard to localize protein for diagnostic purposes. But, through comparing different stages of the PKD disease, MALDI-IMS provides a new angle to look into the disease progression.
Figure 3-11. Average mass spectra and scanned images of a normal mouse kidney tissue (A) and a late-stage PKD mouse kidney tissue (B)
3.4.6 Data processing: Baseline subtraction, spectrum smoothing, peak picking and spectrum normalization

Data processing such as baseline subtraction, peak smoothing and mass list finding is a prerequisite for further analysis of MALDI-IMS data. Because of tissue complexity, lower ionization efficiencies of analytes are observed and higher laser intensities are used than for purified samples. Raw imaging data usually have high baseline with non-Gaussian peak shapes. There are a few different methods available for each processing step in data processing software such as FlexAnalysis, FlexImaging, and ClinproTools. Usually, optimized imaging methods are customer created by the instrument company based on investigated preliminary data. Those methods contain proper data processing algorithms for specific applications.

In our study, all peaks were smoothed by a Gauss algorithm with a smoothing width of 0.1 m/z. Each spectrum was baseline subtracted with TopHat method. Peaks were detected by SNAP algorithm and centered by Centroid algorithm with signal to noise threshold set at 2. Total ion count (TIC) normalization was performed before statistical analyses such as principal component analysis. The normalization aims to remove variance of pixel to pixel signal intensity caused by uneven matrix distribution, ionization efficiency and ion suppression. The benefit from normalization with various algorithms in different cases was demonstrated by Deininger et al. In the TIC algorithm, all spectra are divided by the mean of all data points and all spectra are assumed to have a similar area which is determined in large extent by chemical noise. Normalization can help construct better images, but we need to use it with care because artifacts are created.
3.4.7 Tissue classification by principle component analysis

MALDI imaging generates huge amount of data in multi-dimensions with each point on a tissue sample surface having a position coordinate and a mass spectrum representing peak intensities versus ion m/z values. In order to achieve high spatial resolution, imaging raster is typically set in the 50 μm to 200 μm range. Thus, there are thousands of spectra created after a single tissue section imaging run. While an average spectrum shows a relative protein expression as well as their localization in tissue, further investigation requires looking into every single spectrum and their correlated tissue histology. Such work demands that a person who analyzes the data knows both mass spectrometry and pathology, and is time consuming. Therefore, methodologies based on statistical analysis through math algorithms such as principle component analysis (PCA) and hierarchical clustering have been developed and incorporated into data analysis software such as ClinProTools (Bruker).

PCA basically transforms the MS data into a principal component coordinate system with the goal to show that similar tissue types generate similar spectra. Mass spectra collected for different tissue types and at different tissue locations are variable due to differences in ion intensities at various m/z values. If a mass spectrum shows n peaks, the n peaks can form an n-dimensional coordinate system with each axis representing one peak. If all spectra in one dataset are loaded into this n-dimensional coordinate system and each spectrum is represented by a dot, similar spectra should be
close to each other. New axes of the n dimensional are then formed by rotating and shifting the dataset with the first axis pointing to the direction of the highest variances, second axis pointing to the next highest difference and so on. Usually, first three PCs can represent the most variance in the dataset. The new axes are called principle components. Differences between mass spectra are expressed in terms of principal components and provide a way to represent differences among various samples and sample groups analyzed by mass spectrometry. Principle components are formed by linear combination of ion intensities where contribution of each m/z value to the variation between data sets is determined by loading plots.

In our preliminary PCA study, five random spectra from normal tissue dataset and five random spectra from diseased tissue data set were chosen to perform PCA. Figure 3-12 shows clear separation of the two datasets. Five red dots representing five normal kidney tissue spectra were on the negative side of PC1 while five green dots representing five diseased kidney tissue spectra were on the positive side of PC1. At the same time loading plots correlating to each PCs were created. Figure 13-3 shows the loading plot for PC1 and PC2 of the two datasets. Each spot in the loading plot represents one mass-to-charge value. The distance of dots to the origin of the coordinate system shows how much particular peaks contribute to the separation of the two datasets. The further the dot from the origin of the coordinate system, the more that peak contributes. For example, m/z 5445.20 (close) and 14985.42 (far away) were chosen to demonstrate this methodology. In average protein spectrum of normal and diseased tissue section (figure 3-6), we found that peak 5445.20 appears in both spectra while peak at m/z of 14985.42 is only present in PKD tissue sections.
Hence, PCA provides a robust method for interpretation of MALDI-IMS data, and will be applied in our future MALDI-IMS studies for the statistical analyses of larger and more complex datasets. Potentially, PCA could distinguish healthy from PKD tissues at an early stage without a need to identify proteins. Additionally, loading plots can lead to discovery of proteins that are different in healthy and diseased tissues, which will help finding novel biomarkers of PKD.
Figure 3-12. Principle components (PCs) shown in 3D and 2D display
Figure 3-13. Mass loading plot corresponding to PCA plot shown in Figure 3-12.

3.4.8 Nano-HPLC performance and tandem MS

Coupling nano-HPLC to MALDI-MS through Proteineer fc was tested using cytochrome C trypsin digest standard. Peptide fragments with m/z 795.507, 1168.737, 1296.815, 1433.892 and 1584.846 were selected by peak picking threshold for fragmentation after automatic MS screening. Combined MS and MS/MS results were sent to MASCOT with parameters of all entities, oxidation of methionine, one trypsin cleavage, 0.2 Da MS tolerance and 0.7 Da MS/MS tolerance against SwissProt database. All entries gave confident recognition as cytochrome C from bovine. The LC-MALDI
test with standard protein digest proved the capability of the system. For peptide extract from tissues surface, additional sample purification procedures are needed to maintain the efficient separation.

Figure 3-14. Chromatogram of cytochrome C digests under optimized separation conditions
3.4.9 Critical aspects to obtain high quality data

Careful and considerate tissue sample handling and preparation is the key to obtain high quality tissue imaging data. During the sample preparation process, a few things worth of mentioning are: avoid using OCT based liquid mounting media, which affect the ionization of analytes due to existing polymers. Gelation can be used but the best mounting medium used so far is Gyro-Gel embedding medium, which avoids direct contact with the tissue section. Homogeneous matrix distribution is critical for unambiguous interpretation of mass spectrometry imaging data. Usually uneven or insufficient coverage of matrices on tissue samples can affect resolution, reproducibility and increase the chances of artifacts. To obtain homogeneous matrix coverage, spray head should be checked and adjusted every time before spraying. One easy way to examine the matrix coverage is looking at the crystals formed after spraying under a light microscope. Figure 3-15 shows different matrix coverage on tissue surfaces. The crystal sizes were also measured to determine the imaging data resolution. Typically, there are more than one tissue sections mounted on each ITO glass slides in our case there are four to six sections on one slide. After matrix application, it is best to analyze the sample as soon as possible to avoid sample degradation and oxidation. MALDI ion source usually becomes dirty much faster in mass spectrometry imaging acquisition considering hundreds and thousands of laser shots were applied for each tissue section. Dirty ion source may lower resolution and cause peak shift. Cleaning ion source after two or three imaging runs with ethanol and acetone should be routine practice.
Figure 3-15. Images of matrix/analyte co-crystals with different matrix coverage under a 10x light microscope
3.5 Conclusions

A reproducible MALDI-IMS workflow was established to obtain protein and peptide mass distribution maps of normal and PKD kidney tissues. The imaging MS enabled us to visualize protein and peptide distributions across tissue surfaces. Average spectra of normal and diseased tissue sections were analyzed and compared. Differences in peak mass and intensity were observed. Through construction of tissue images, different regions of the tissues were clearly differentiated by their ion distribution. H&E staining was performed and revealed the morphology of the tissue sections, which was in good agreement with ion map images.

Different matrices were tested to check their impact on ionization efficiency and selectivity of MALDI-IMS. The results showed that CHCA and DHB have their preferences for ionization of different peptide ions. Thus, matrix selection needs to be considered for specific IMS applications. Data processing in MALDI-IMS was discussed and principle component analysis was performed on small imaging datasets. Spectra originating from normal and PKD kidney sections were clearly separated by PCA, indicating that PCA is a valuable tool for analysis of diseased and healthy tissue sections. Some critical aspects to achieve successful MALDI imaging were discussed.

A nano-LC-MALDI workflow for protein identification was established and tested on a standard protein digest. The result showed good separation capability of nano-LC and high-confidence recognition of a test protein after MALDI-MS and MS/MS analysis. The system is ready to be utilized for protein identification after peptide extraction from the surfaces of the tissues and application of tandem MALDI-MS.
Chapter 4

Conclusions

Two imaging methodologies with distinctive principles were utilized in this study to investigate biological samples on cellular and tissue levels. In chapter two, we reported the development of a multimodal spectral imaging methodology based on a transmission diffraction grating and its application for spectral analysis of biological cells. A MALDI-MS imaging system which allows direct detection and analysis of biomolecules on tissue surfaces with preserved spatial distribution was established. Its application for the analysis of normal and diseased kidney tissue samples was described in chapter three.

By the incorporation of a transmission diffraction grating into a light microscope, fluorescence, scattering, and absorption spectral images of cells were acquired. Applications described included fluorescence spectral imaging of yeast and mammalian cells labeled with multiple fluorophores, darkfield spectral imaging of unlabeled human cheek cells, and brightfield and DIC spectral imaging of H&E stained cells, fluorescently labeled cells, dyed particles, and free dyes. The presented multimodal spectral imaging is a low cost alternative methodology for scientists in need of the acquisition of spectral images of cells and other specimens. Incorporation of the grating into a conventional light
microscope is straightforward and potential users can become proficient quickly in application of the described spectral imaging methodology. Future work will strive to overcome the challenges such as first order spectral image smear and spectrum resolution to enable simultaneous spectral imaging of higher number of fluorophores and chromophores in multiple cellular organelles.

A reproducible MALDI-IMS workflow was established, enabling us to visualize protein and peptide distribution across tissue surfaces. The methodology was applied to MS imaging analyses of normal and diseased kidney tissue sample. For the first time, polycystic kidney disease (PKD) was characterized and compared with normal tissue using MALDI-IMS. By analyzing differences in mass spectra, healthy and diseased tissues were characterized and differentiated. Protein and peptide distributions on tissue sample surfaces were constructed and associated with histological staining to reveal the molecular morphology in term of masses. A statistical data analysis was performed and showed potential ability for fast and unambiguous tissue classification. Additionally nano-LC-MALDI MS/MS workflow was established for protein identification and showed good performance.

In future experiments, the established MALDI-IMS system will be utilized to study progression of polycystic kidney disease (PKD) on protein level. MALDI-IMS will provide a different angle to observe the disease development. Principle component analysis and another powerful statistical tool called hierarchical clustering will be applied on larger and more complex imaging datasets. Peptide extracts from healthy and PKD tissue surfaces will be separated and analyzed by tandem MS, leading to simultaneous identification and imaging of potential protein biomarkers in PKD tissues.
References


