Advances in cellular and sub-cellular level localization of lipids and metabolites using two- and three dimensional high-spatial resolution MALDI mass spectrometry imaging

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Advances in cellular and sub-cellular level localization of lipids and metabolites using two- and three dimensional high-spatial resolution MALDI mass spectrometry imaging

by

Maria Emilia Dueñas Fadic

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Analytical Chemistry

Program of Study Committee:
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

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ABSTRACT

This thesis presents efforts in the advancement and application of high-spatial resolution matrix-assisted laser desorption ionization-mass spectrometry imaging (MALDI-MSI) for the mapping of small metabolites and lipids at the cellular and sub-cellular level. The following work presents a number of advances, using both 2- and 3-dimensional MALDI-MSI to enable visualization at the sub-cellular level. The first chapter consists of a general introduction to the technique of MALDI-MSI, and the seventh and final chapter provides a brief summary of the presented work and possible future directions.

The second chapter presents a technology development for the optimization and application of matrix recrystallization to improve lipid ion signals in maize embryos and leaves. Using the optimized recrystallization conditions, the ion signals were improved three times, enhancing the image quality of lipid species with no apparent changes in their localization. Additionally, when methanol was used as a recrystallization solvent, unexpected side reactions were observed between phosphatidic acid and methanol vapor, suggesting recrystallization solvent should be carefully selected to avoid side reactions.

The third chapter presents an application using 5- and 10-μm high spatial resolution MALDI-MSI to explore quantitative fatty acyl distributions of two classes of thylakoid membrane lipids along the developmental gradient of maize leaves in two inbred lines, B73 and Mo17, and the reciprocal hybrid lines, B73xMo17 and Mo17xB73. This study demonstrated that high-resolution MALDI-MSI analysis can be directly applied to multicellular plant tissues to uncover cell-specific metabolic biology that has not been possible using traditional metabolomics methodologies. For example, certain thylakoid membrane lipids (e.g. phosphatidylglycerol (PG) 32:0) show genotype-specific differences in cellular distributions.
Inbred B73 shows preferential localization of PG 32:0 in bundle sheath cells, while a more uniform distribution between bundle sheath and mesophyll cells in inbred Mo17.

The fourth chapter presents the first time MALDI-MSI has been applied for three-dimensional chemical imaging of a single cell using newly fertilized individual zebrafish embryos as a model system. High-spatial resolution MALDI-MSI was used to map and visualize the three-dimensional spatial distribution of phospholipid classes, phosphatidylcholines (PC), phosphatidylethanolamines (PE), and phosphatidylinositols (PI), in the zebrafish embryo. The 3D MALDI-MSI volumetric reconstructions were then used to compare four different normalization approaches to find reliable relative quantification in 2D- and 3D- MALDI MSI data sets. Furthermore, two-dimensional MSI was studied for embryos at different cell developmental stages (1-, 2-, 4-, 8-, and 16-cell stage) to investigate the localization changes of some lipids, revealing heterogeneous localizations of different classes of lipids in the embryo.

The fifth chapter discusses the development of a high-throughput MALDI-MS based metabolomics platform using a microarray of nanoparticles and organic matrices. Five matrices that provide broad metabolite coverage were selected and used to analyze turkey gut microbiome samples. Over two thousand unique metabolite features were reproducibly detected across intestinal samples from turkeys fed a diet amended with therapeutic or sub-therapeutic antibiotics, or non-amended feed. This protocol was applied to fifty two turkey cecal samples at three different time points from the antibiotic feed trial, which allowed distinct metabolite profiles to be discovered.

The sixth chapter presents an on-tissue chemical modification strategy for high-spatial resolution MALDI-MSI. A mass spectrometry imaging methodology was used to selectively enhance the metabolite signals for a sub-metabolome at a time by performing on tissue
derivatizations. Three well-known on-tissue derivatization methods were used: coniferyl aldehyde for primary amines, Girard’s reagent T for carbonyl groups, and 2-picolyamine for carboxylic acids. This proof of concept experiment was applied to cross-sections of maize leaves and roots, and enabled the identification of over five hundred new unique metabolite features. Combined, this approach facilitated the visualization of various classes of compounds, which can eventually allow high-spatial resolution MSI in the metabolomics scale.
CHAPTER 1. MALDI MSI INTRODUCTION

Background

Mass spectrometry imaging (MSI) has become a widely used analytical tool that is seeing applications in many fields, from mapping metabolites and other biomolecules in tissues (Stoeckli et al., 2001, Lee et al., 2012, Korte et al., 2015) to drug research and development (Cobice et al., 2015, Nilsson et al., 2015). Such wide applicability has led to extensive development of sample preparation protocols and instrumental setups. Matrix-assisted laser desorption ionization (MALDI)-MSI is the most widely used technique for cellular and sub-cellular level resolution imaging due to its combination of high-spatial resolution, high sensitivity, and chemical versatility.

A traditional MALDI-MSI experiment requires the application of a chemical matrix, which absorbs the laser energy and promotes desorption and ionization, onto the tissue sample of interest. The surface is interrogated with a laser beam, desorbing and ionizing both matrix and endogenous compounds from the tissue surface. The generated ions are then introduced into a mass analyzer and sorted on the basis of their mass to charge ratios ($m/z$). After acquiring a mass spectrum from an individual spot on the tissue, the sample plate is moved and a series of spectra are obtained by rastering the laser across hundreds of x and y positions. Data analysis software is then used to generate heat maps showing where certain compounds are localized in the sample, which can then be correlated to an optical image of the tissue.

MALDI-MSI offers several advantages compared to traditional tissue imaging techniques that require labeling with a radionuclide or fluorescent tags, considering it requires no information about metabolites beforehand and is chemically non-specific. MSI
allows two-dimensional visualization of the spatial distribution of biomolecules without extraction, purification, separation or labeling of analytes (Passarelli and Ewing, 2013). Moreover, a large number of biomolecules, including unknowns, can be detected simultaneously from a single MSI experiment, which allows for direct sub-cellular mapping of biomolecules in high resolution and high throughput ways.

Typically, the majority of MALDI imaging studies are performed at a spatial resolution between 50 and 200 μm per pixel (Spengler, 2015). However, advances in instrument design and sample preparation have made much higher resolution experiments possible. Consequently, MALDI images with pixel size between 2.5-5 μm have been obtained (Römpp and Spengler, 2013, Feenstra et al., 2017). The pixel size of an MS image is determined by the distance that the sample is moved between two measurements. The step size is usually chosen in the same range as the laser focus diameter, this way avoiding overlapping of analyzed area. Pushing the spatial resolution down to 2.5 μm has allowed for finely detailed studies at a sub-cellular level. Understanding the spatial distribution of molecules at a sub-cellular level is indispensable for elucidating their biological roles.

Since biology occurs in organisms in three dimensions, it is not surprising that 3D imaging has had a noteworthy impact on many challenges in the life sciences (Palmer and Alexandrov, 2015). In the past decade, imaging of intact biomolecules using MSI has expanded to 3D analysis to determine volumetric molecular distribution within tissue specimens, agar plates, and 3D cell cultures (Weaver and Hummon, 2013). The most common method of 3D imaging using mass spectrometry consists of collecting consecutive sections of a sample, analyzing each section individually using traditional 2D MSI, and then stacking and reconstructing a final 3D image (Figure 1.1). Novel tools and protocols for
serial 3D imaging MS have been developed for improved sample preparation, accelerated data acquisition, and computationally intensive analysis of the big data generated. At present, serial 3D imaging MS analysis has become a complex but feasible technique. The future of 3D MSI depends on whether specific applications will be found where the benefits from this advanced technique will outweigh its complexity (Palmer and Alexandrov, 2015).

Figure 1.1 Outline presenting the workflow from imaging single sections to 3D reconstruction. Initially, serial sections are prepared and matrix is applied to all the sections. Each of these sections are imaged by mass spectrometry, yielding a dataset of complete mass spectra at each x and y coordinate. 2D mass spectral images are stacked and aligned creating a 3D reconstruction for each ion.

The objective of this thesis, therefore, is to advance and apply high-spatial resolution MALDI-MSI for the mapping of small metabolites and lipids at the cellular and sub-cellular level. The following work presents a number of advances, using both 2- and 3-dimensional MALDI-MSI to visualize at a sub-cellular level, and addresses a range of topics within the scope of MALDI-MSI: application, sample preparation, and method development.
MALDI-MSI: General Workflow

The workflow for a typical MALDI-MSI experiment of the kind described in this dissertation is illustrated in Figure 1.2, and is described below. Immediately after tissue harvesting, the sample is embedded in a supporting medium to allow for cutting of thin sections and simultaneously flash-frozen in liquid nitrogen. The tissue is placed into a mold and the embedding solution is poured into the mold. The mold is then floated on liquid nitrogen to rapidly freeze the sample and embedding medium. After freezing, the tissue/embedding medium block is transferred to a pre-cooled cryostat (~20°C) and the block is allowed to equilibrate to the cryostat temperature, then thin (10 µm) sections are cryo-sectioned. These sections are collected on adhesive tape and are then freeze-dried to provide an intact, dry tissue section for analysis. Next, a MALDI matrix is applied to the surface of the section either by sublimation-vapor deposition (for organic matrices) or by spraying or sputter coating (for inorganic nanoparticles). Following the matrix application, the sample is inserted into the mass spectrometer, and a series of points over the sample surface are irradiated by a focused laser beam.

Figure 1.2 Sample Workflow for typical MALDI-MSI experiment
Dissertation Organization

This dissertation is divided into seven chapters. The first chapter, above, serves as a general introduction to the technique of MSI and provides context for our effort to develop the technique for the analysis of plant and animal metabolites at a sub-cellular level, in both 2- and 3-dimensions. Chapter two to five are reproductions of papers published in peer-reviewed journals. The second chapter presents an application for optimizing and improving lipid ion signals in plant tissue at high-spatial resolution using matrix recrystallization. Chapter three describes our work on applying 5- and 10-μm high spatial resolution MALDI-MSI to the asymmetric Kranz anatomy of Zea mays (maize) leaves to study the differential localization of two major anionic lipids in thylakoid membranes, phosphatidylglycerols (PG) and sulfoquinovosyldiacylglycerols (SQDG). The fourth chapter presents the first time MALDI-MSI has been applied for three dimensional chemical imaging of a single cell. MALDI-MSI was used to map and visualize the three-dimensional spatial distribution of phospholipid classes, phosphatidylcholine (PC), phosphatidylethanolamines (PE), and phosphatidylinositol (PI), in newly fertilized individual zebrafish embryos. Chapter five details the development of a microarray platform using MALDI-MS. This platform was utilized as a high-throughput method of analysis which was applied to the study of the dynamics of turkey gut microbiome metabolomics with and without antibiotic treatments. The sixth chapter presents on-tissue chemical derivatization strategies to selectively enhance the sensitivity of MSI for metabolite analysis. Finally, chapter 7 summarized the work in this dissertation and provides outlook for future direction and applications that could be explored based upon this research.
References


CHAPTER 2. MATRIX RECRYSTALLIZATION FOR MALDI-MS IMAGING OF MAIZE LIPIDS AT HIGH-SPATIAL RESOLUTION

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Abstract

Matrix recrystallization is optimized and applied to improve lipid ion signals in maize embryos and leaves. A systematic study was performed varying solvent and incubation time. During this study, unexpected side reactions were found when methanol was used as a recrystallization solvent, resulting in the formation of a methyl ester of phosphatidic acid. Using an optimum recrystallization condition with isopropanol, there is no apparent delocalization demonstrated with a transmission electron microscopy (TEM) pattern and maize leaf images obtained at 10 μm spatial resolution.

Introduction

The spatial resolution of matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) has become routinely available at the size of 20 μm and has been demonstrated with a pixel size as small as 2.5 μm (Zavalin et al., 2015). A critical challenge in high-spatial resolution MSI is the limited number of analytes available in a small sampling size. Hence, sample preparation should be carefully selected to enhance ion
signals but not to induce delocalization. The vapor phase sublimation of organic matrix produces very small crystal sizes of around 1 μm or smaller and is most commonly used for high spatial resolution MALDI-MSI (Hankin et al., 2007). As a dry method, it does not induce analyte migration, but often shows poor sensitivity due to a lack of analyte incorporation into the matrix crystal (Bouschen et al., 2010).

Yang and Caprioli demonstrated that a recrystallization step added after sublimation can dramatically increase the sensitivity of proteins while keeping high spatial resolution at 10 μm (Yang and Caprioli, 2011). Since then, the recrystallization method has been adopted for many applications (Roth et al., 2012, Ferguson et al., 2013, Shimma et al., 2013, Guo et al., 2014, González de San Román et al., 2015, Lauzon et al., 2015). Incubation with solvent vapor is most commonly used, but manual or automatic solvent spray is also used (Ferguson et al., 2013, Lauzon et al., 2015). Many of these applications, however, have been performed without testing potential delocalization during recrystallization. In addition, there has been no report for the application to plant tissues, which are fragile and quite different from animal tissues, mostly due to the cell walls and different lipid composition.

In the current study, we adopted and optimized the recrystallization method by Yang and Caprioli (2011) to increase lipid signals in maize embryos and leaves while ensuring no apparent delocalization at high spatial resolution. Side reaction that may occur during recrystallization is a major concern, but has not been reported. Here, we also report unexpected side reactions between phosphatidic acid and methanol vapor.
Experimental

Materials

Isopropyl alcohol (IPA), methanol, water, chloroform and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA) in CHROMASOLV LC-MS or Plus grade. Acetic acid was purchased from Fisher Scientific (Waltham, MA, USA) in glacial certified ACS Plus grade. 1, 5-diaminonaphthalene (DAN, 97%) was purchased from Sigma-Aldrich. Gelatin from porcine skin (300 bloom) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Seeds (Zea mays, L., inbreed B73) were obtained from Dr. Marna Yandeau-Nelson at Iowa State University.

Plant Growth and Harvest

Procedures for corn seed germination and plant growth are described in detail elsewhere (Feenstra et al., 2015, Korte et al., 2015), and briefly described below. A maize seed was imbibed for 10 min and placed with the embryo side facing down on top of moist filter paper in a Petri dish which was placed in a climate-controlled greenhouse. After 28 h of germination, the seed was cut longitudinally, flash-frozen, and then placed in a cryo-mold with gelatin (10% w/v solution). For maize leaf imaging, maize seeds were planted in soil and grown in a greenhouse. Plant seedlings were harvested 11 d after planting and sections of leaves were collected at the midpoint. The fresh maize leaf section was embedded in gelatin before cryo-sectioning.

Sample Preparation

The molds were transferred to a cryostat (CM1850, Leica Microsystems; Buffalo Grove, IL, USA) pre-chilled to -20°C, allowed to thermally equilibrate for 30 min, and sectioned at 10 μm thickness. The cryo-sectioned tissues were collected with Cryo-Jane tape (Leica Microsystems; Buffalo Grove, IL, USA), and attached to a cold microscope glass
slide. The glass slides with sections were vacuum dried and DAN matrix was deposited via sublimation (Hankin et al., 2007) at 140°C for 4 min.

**Recrystallization**

A recrystallization chamber was made of a filter paper, a glass Petri dish, a metal plate and a magnet (Yang and Caprioli, 2011). The sublimated slide was adhered to a stainless steel plate using copper tape, and attached to the underside of the top part of the Petri dish by placing magnets on the exterior of the Petri dish. A piece of filter paper was placed in the bottom part of the Petri dish with 1 mL of a solution. The Petri dish was assembled to form a hydration chamber, sealed using tape, and placed in an incubator for various lengths of time.

**Mass Spectrometry Analysis**

MS imaging data was collected using a MALDI-linear ion trap (LIT)-Orbitrap mass spectrometer (MALDI-LTQ-Orbitrap Discovery, Thermo Scientific; San Jose, CA, USA). The instrument was modified to use an external 355nm Nd:YAG laser (UVFQ, Elforlight Ltd.; Daventry, UK) and a custom optical set-up (Korte et al., 2015). TunePlus and Xcalibur software (Thermo Scientific) were used to define imaging acquisition parameters and for data acquisition. The laser energy used was 83-85% at 60 Hz repetition rate. Maize seed and leaf images were acquired using 150 and 10 μm raster step size with a laser spot size of ~25 and ~9 μm, respectively. The laser spot size was adjusted using a beam expander. Mass spectra were acquired in negative mode using Orbitrap for m/z scan range of 350-1000 at the resolution of 30,000 at m/z 400.

MS/MS spectra were acquired with Multiplex MSI (Feenstra et al., 2015) where each pixel (raster step) was divided into four spiral steps with a raster step size of 100 μm and a spiral step size of 50 μm. The first spiral step was collected with a full Orbitrap mass
spectrum for m/z 350-1000. Data-dependent ion trap MS/MS spectra were collected in the other three steps for the three most abundant ions from the pre-loaded precursor mass list. An isolation window of ±1.0 Da and collision energy of 35% was used. Dynamic exclusion was used with a repeat count of 2, a repeat duration of 30 sec, and an exclusion duration of 180 sec.

Observed and imaged peaks are all deprotonated, [M-H]. Peak assignments were made based on accurate mass values and MS/MS. MS images were generated using ImageQuest (Thermo) with a mass window of ±0.003 Da and without normalization to total ion count.

**Results and Discussion**

**Matrix Recrystallization Optimization**

Cross sections of ~28 h germinated maize embryos were used to optimize recrystallization, which was then applied to maize leaf cross sections at 10 µm resolution. This study was confined to negative ion mode with DAN as a matrix because many small metabolites are readily ionized in negative ion mode, as well as several lipids of our interest as previously published (Korte and Lee, 2014).

First, recrystallization solvents were varied at a fixed temperature and time (39 °C and 1.5 min). Yang and Caprioli obtained optimal recrystallization for MSI of proteins using a small amount of organic solvent in water. In addition to 5% methanol and 5% acetic acid they used, a few more organic solvents in water were tested (5% IPA, 5% acetone, 5% chloroform). As shown in Figure S1, four solvents in water (5% acetone, 5% IPA, 5% methanol, and 5% acetic acid) increased phospholipid ion signals of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositols (PI), and phosphatidylglycerols (PG), as well as a sulfolipid, sulfoquinovosyl diacylglycerol (SQDG).
Further optimization was made for the four solvents by varying incubation time at a temperature slightly lower than the boiling point (i.e., 39 °C, 55 °C, 39 °C, and 80 °C for 5% acetone, 5% IPA, 5% methanol, and 5% acetic acid, respectively). As shown in Figure S2, optimum incubation time was determined as 1.5 min, 2 min, 3-4 min, and 2-3 min, respectively. Ion images were also improved as shown in Figure S3. Overall performance is similar at the optimum conditions, but 5% IPA gave a slightly better result in terms of signal improvement.

![Figure 2.1](image)

**Figure 2.1** Mass spectra of maize embryo section after 0 and 3 min of recrystallization with 5% methanol. Green boxes indicate the newly appeared peaks after recrystallization

In a close look at the mass spectra obtained with 5% methanol, two new peaks (m/z 685.481 and 709.481) appeared after recrystallization (Figure 2.1). No such peaks were found with other solvents. The two compounds were initially assigned as odd number fatty acids, PA 35:2 and PA 37:4, respectively, based on accurate mass search against Metlin database (metlin.scripps.edu). Odd number fatty acids are rare in plants and it is very unlikely recrystallization would induce dramatic signal enhancement only for PAs with odd
number fatty acids. MS/MS experiments were performed (Figure 2.2) which identified these peaks as a methyl phosphoester of PA produced during recrystallization. In addition, the isotope patterns of m/z 685.48 and m/z 709.48 exactly match those of m/z 671.46 and m/z 695.46 further supporting their assignment as methylated PA.

Figure 2.2 MS/MS spectra of m/z 685.481 (top) and m/z 709.481 (bottom) shown in Figure 2.1. * represents fragment from precursor contaminant
Recrystallization with 5% IPA at 55 °C for 2 min was selected as the most optimal condition and used for a high-resolution imaging below. Unlike methanol, phospho-esterification of PA did not occur with IPA. The use of 5% acetone or 5% acetic acid was not further considered because of potential delocalization or side reactions for small metabolite molecules.

**Figure 2.3** (a) MS images of erucic acid and PI 34:2 on a TEM grid pattern without (Top) and with (Bottom) recrystallization. (b) Optical image (Top) and MS images for selected lipids (Middle and Bottom) of a cross-section of maize leaf without and with recrystallization. All MS images were obtained at 10 µm spatial resolution. HMBOA-Glc: 2-hydroxy-7-methoxy-1,4-benzoazin-3-one glucoside.
High-resolution MALDI-MS Imaging

A copper transmission electron microscopy (TEM) grid was used to make fine analyte patterns to test analyte delocalization during recrystallization. Two TEM grids were secured on a glass slide and a solution containing soy lipid extracts and erucic acid (22:1 fatty acid) were sprayed on top of the grids (See Figure S4 for the workflow). After the removal of the grids, the slides were sublimated with DAN, recrystallized with 5% IPA at the optimum condition, and MS imaging data were acquired with 10 μm spatial resolution. **Figure 2.3a** shows MS images of erucic acid and PI 34:2. The ion signals are very low before recrystallization, but after recrystallization there are significant signal improvements with almost no signal outside the grid pattern.

Finally, MS imaging was performed for cross-sections of maize leaf with 10 μm spatial resolution. Representative ion images are shown in **Figure 2.3b** for maysin, PG 34:3, and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (HMBOA-Glc) that have distinct localizations. After recrystallization, the ion signals were improved by a factor of three, thus improving the image quality, but there are no apparent changes in their localizations. It should be noted that ion signals are actually decreased for some molecules, especially caffeic and ferulic acids, as shown in **Figure S5**.

**Conclusion**

Spatial resolution in MALDI-MSI is limited by four major factors: (1) delocalization during sample preparation, (2) matrix homogeneity, (3) laser spot size, and (4) sensitivity. Sensitivity has become a critical factor recently, thanks to many advances in sample preparation and instrumentation. This work demonstrates recrystallization can be performed to improve lipid ion signals on plant tissues while producing minimal or no analyte delocalization appropriate for high-spatial resolution MALDI-MSI. The effect of matrix
recrystallization on very small molecules (< 500 Da) shows mixed results, suggesting caution needs to be taken for these molecules. Most importantly, recrystallization data should be carefully inspected for potential side reactions, as we have demonstrated the occurrence of phosphoester formation between methanol vapor and PA.

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Supplemental Figures

Figure S1. Comparison of several lipid species on maize embryos after recrystallization by various solvents at the same temperature and incubation time (39 °C and 1.5 min). Selected lipid species are compared for their ion intensities (Top) and MS images (Bottom). Optical image is shown for the germinated maize embryo. Images are generated without normalization to TIC. Scale bar represents 2 mm.
Figure S2. Representative lipid ion signals of ~28 h germinated maize embryos recrystallized with four solvents at various incubation time. Note the tissues used were at slightly different development stages between different solvents as shown in Figure S3, and the original amount of lipids might be slightly different. Comparison should be made only between different recrystallization times for the same solvent data set.
Figure S3. MS images of a few representative lipids on maize embryo section at the optimized recrystallization condition for each solvent. Images are generated without normalization to TIC. Scale bar represents 2 mm.
**Figure S4.** Workflow to make TEM grid patterns of a few analytes that were used to test recrystallization.
**Figure S5.** Optical image and MS images of various metabolites in maize leaf cross section obtained at 10 µm spatial resolution without (left) and with (right) recrystallization. HMBOA-Glc: 2-hydroxy-7-methoxy-1,4-benzoazin-3-one glucoside, PG: phosphatidylglycerol, SQDG: sulfoquinovosyl diacylglycerol. Images are generated without normalization to TIC, but the same max scale was used for each metabolite between control and recrystallization.
References


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Abstract

Metabolism in plants is compartmentalized among different tissues, cells and subcellular organelles. Mass spectrometry imaging (MSI) with matrix-assisted laser desorption ionization (MALDI) has recently advanced to allow for the visualization of metabolites at single-cell resolution. Here we applied 5- and 10-µm high spatial resolution MALDI-MSI to the asymmetric Kranz anatomy of Zea mays (maize) leaves to study the differential localization of two major anionic lipids in thylakoid membranes, sulfoquinovosyldiacylglycerols (SQDG) and phosphatidylglycerols (PG). The quantification and localization of SQDG and PG molecular
species, among mesophyll (M) and bundle sheath (BS) cells, are compared across the leaf developmental gradient from four maize genotypes (the inbreds B73 and Mo17, and the reciprocal hybrids B73xMo17 and Mo17xB73). SQDG species are uniformly distributed in both photosynthetic cell types, regardless of leaf development or genotype; however, PG shows photosynthetic cell-specific differential localization depending on the genotype and the fatty acyl chain constituent. Overall, 16:1-containing PGs primarily contribute to the thylakoid membranes of M cells whereas BS chloroplasts are mostly composed of 16:0-containing PGs. Furthermore, PG 32:0 shows genotype-specific differences in cellular distribution, with preferential localization in BS cells for B73, but more uniform distribution between BS and M cells in Mo17. Maternal inheritance is exhibited within the hybrids such that the localization of PG 32:0 in B73xMo17 is similar to the distribution in the B73 parental inbred, whereas that of Mo17xB73 resembles the Mo17 parent. This study demonstrates the power of MALDI-MSI to reveal unprecedented insights on metabolic outcomes in multicellular organisms at single-cell resolution.

**Introduction**

Thylakoid membranes play an important organizational role in maintaining the structural integrity of photosystem complexes (Quinn and Williams 1983). The thylakoid membrane is the universal site of the photochemical and electron transport reactions of oxygenic photosynthesis in cyanobacteria and plant chloroplasts. The major lipids within the thylakoid membrane are the neutral galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which together account for approximately 80% of total thylakoid lipids in plant chloroplasts (Kobayashi et al. 2009). The remaining thylakoid membrane lipids are mainly composed of anionic lipids sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Demé et al. 2014, Sakurai et al. 2006); these negatively charged lipids have been shown to
be responsible for the structural and/or functional integrity of photosystems I and/or II (Guskov et al. 2009, Jordan et al. 2001, Sato 2004, Umena et al. 2011).

A number of studies have highlighted that there are significant differences in the fatty acyl chains associated with different thylakoid lipid molecular species (Hsu et al. 2007). These composition patterns can be modified by both the genetic and developmental programs of the organism, and by the environmental stimuli that the organism experiences (Ben Hamed et al. 2005, Murata et al. 2003, Pál et al. 2007). Galactolipids (MGDG and DGDG) are characterized by an exceptionally high trienoic fatty acid content, mainly α-linolenic acid (Duchêne and Siegenthaler 2000, Nishihara et al. 1980), whereas the major lipid species in SQDG are enriched in palmitic acid. PG is a distinct thylakoid lipid, in that its acyl composition vastly differs depending on the plant species.

Unlike C3 plants, Zea mays (maize) employs a highly efficient C4-type photosynthesis that is differentially regulated by cellular compartmentalization of photosynthetic carbon assimilation in the mesophyll (M) cells, and photosynthetic carbon reduction in the bundle sheath (BS) cells, creating a two-cell metabolism system. In addition to the metabolic flux that occurs between these two cell types, ultrastructural and photosystem assays have revealed that the complexity of C4 photosynthesis is also dependent on the composition differences of the chloroplast envelopes and thylakoid membranes (Manandhar-Shrestha et al. 2013). One of these differences is associated with the acyl-chain composition of PG, which appears to be different in the thylakoid membranes of M and BS cells. Specifically, PG with a 16:1 fatty acid at the sn-2 position (e.g., PG 16:0/16:1) is highly enriched in M chloroplasts, and PG with 16:0 at the sn-2 position (e.g., PG 16:0/16:0) is primarily localized in BS chloroplasts (Nishihara, et al. 1980). Moreover, the acyl-chains of PG are correlated with developmental stages as well as chilling
sensitivity. For example, plants with high levels of disaturated thylakoid PGs are shown to be sensitive to chilling (Roughan 1985); however, this is not without debate (Kaniuga et al. 1999).

In plants, grass species serve as an excellent model to study the establishment of various functions relative to the developmental gradient of leaves, in which the youngest cells are situated at the base of the leaf, and the oldest and most mature cells are situated at the leaf tip (Nelson and Langdale 1992). Cellular and photosynthetic differentiation occurs in a controlled manner in a basipetal, or tip-to-base, fashion (Evert et al. 1996, Kirchanski 1975, Leech et al. 1973) with proplastids present at the base of the leaf blade and fully differentiated BS and M chloroplasts present at the tip (Evert, et al. 1996, Majeran and van Wijk 2009, Nelson and Langdale 1992). A number of studies have characterized the transcriptomic and metabolomic changes across the developmental gradient of maize leaves (Li et al. 2010, Pick et al. 2011, Wang et al. 2014). Differential transcriptome (Sharpe et al. 2011, Tausta et al. 2014) and proteome (Majeran et al. 2005, Majeran et al. 2008, Manandhar-Shrestha, et al. 2013) expression have been identified between M and BS cells; however, because these omics studies were performed through the extraction of ground tissue, in situ localization for metabolites or proteins, for example, were not preserved.

Mass spectrometry imaging (MSI) has become a valuable tool for analyzing spatial distributions of a wide range of compounds directly on or within plant and animal tissues (Angel and Caprioli 2013, Jungmann and Heeren 2012, Lee et al. 2012, Sturtevant et al. 2016, Svatos 2010, van Hove et al. 2010, Zaima et al. 2010). Different ionization techniques have been adopted for MSI; however, matrix assisted laser desorption ionization (MALDI) is most attractive in terms of high spatial resolution, sensitivity, and chemical versatility, which is essential for in situ single-cell resolution imaging. The spatial resolution of MALDI-MSI has
become routinely available in the range of 20-30 μm, and has been demonstrated to be as high resolution as 2.5 μm (Zavalin et al. 2015).

Recently, we have established a MALDI-MSI technical platform with a MALDI-linear ion trap Orbitrap mass spectrometer for a high-spatial resolution of 5-10 μm and have demonstrated the visualization of the distribution of a number of different metabolites in cross-sections of maize leaves (Korte et al. 2015). Cell-type specific non-uniform distribution of PG, previously indicated by bulk chloroplast analysis from physically separated BS and M cells (Nishihara, et al. 1980), was confirmed using this single cell direct in situ MSI analysis platform (Korte, et al. 2015). In previous work, we have achieved a 5-μm spatial resolution using an oversampling method with a 9-μm laser spot size. In the present work we used an improved laser spot size of 6-7 μm with minimal oversampling which allows for a clearer image especially for small cells, and applied the high-resolution MALDI-MSI platform to explore quantitative fatty acyl distributions of PG and SQDG along the developmental gradient of maize leaves in two inbred lines, B73 and Mo17, and the reciprocal hybrid lines, B73xMo17 and Mo17xB73 resolution.

**Results**

**Morphology and Lipid Distribution During Leaf Development**

The fatty acid composition of thylakoid membrane lipids was studied at four consecutive developmental zones in the maize seedling leaf: the basal zone, maturing proximal zone, maturing midpoint zone, and mature distal zone, as defined by Li et al. (2010). The overall workflow is shown in Figure S1 and described in detail in the Experimental Procedures section. The third true leaf was harvested from seedlings of inbreds B73 and Mo17, and reciprocal hybrids B73xMo17, and Mo17xB73 11-13 days after imbibition, at a seedling height of 14-18 cm. Each sampled leaf was immediately flash frozen and cryosectioned at four specific positions.
along the developmental gradient of the leaf, as illustrated in Figure S2: (i) basal zone, ~1.5 cm below the ligule of leaf 2; (ii) proximal, ~1.5 cm above the ligule of leaf 2; (iii) midpoint, halfway between proximal end and the leaf tip; and (iv) Distal, ~2 cm from the leaf tip.

High-resolution optical images and anatomical assignments are shown in Figure S3 for the distal and proximal sections of a B73 leaf. In the optical images of Figures 3.1 and 3.2, both inbred lines and the two hybrid lines exhibit the characteristic C4 Kranz anatomy (Brown 1975, Haberlandt 1882), in which concentric rings of BS cells encircle closely spaced veins (vascular bundles, VBs), and are surrounded by M cells. Chloroplasts are mostly located along the boundary of M and BS cells. At the basal zone of the third leaf, Kranz anatomy can be distinguished but is not fully differentiated and few mature chloroplasts are observed (Kirchanski 1975). Because growth rates differ among the four genotypes, leaves from the B73 inbred and the B73xMo17 hybrid were harvested 1-2 days prior to the Mo17 inbred and Mo17xB73 hybrid, such that leaves from each genotype were of similar lengths. Nevertheless, as revealed by the optical images, the sizes of the cells are smaller in inbred Mo17 and hybrid Mo17xB73.

Figures S4-S7 show the MS images for all the molecular species of PG and SQDG obtained at 10-μm spatial resolution, as well as several additional metabolites that are associated with photosynthesis, i.e., chlorophyll a, plastoquinone/plastoquino1, and carotene. Assignments are based on accurate mass measurements, and their identities are also confirmed by separate MS/MS measurements for the lipids (Figures S8 and S9). The presence and cellular distribution of these metabolites and thylakoid membrane lipids were similar in replicate imaging experiments conducted on three independent seedlings. Figures 3.1 and 3.2 present the representative images of three thylakoid membrane lipids (PG 32:0, PG 32:1, SQDG 34:3) and chlorophyll a, comparing between the inbreds and the reciprocal hybrids, respectively.
Figure 3.1 Ten-micron resolution MS images of representative lipids at four developmental sections of the third seedling leaf from inbreds B73 (upper panels) and Mo17 (lower panels). The MS images are of PG 32:0 (m/z 721.503; E-H, E’-H’), PG 32:1 (m/z 719.480; I-L, I’-L’), SQDG 34:3 (m/z 815.501; M-P, M’-P’), and chlorophyll a [sum of fragments at m/z 591.261 (pheophorbide a), 613.232 (chlorophyllide a), and 870.566 (pheophytin a); Q-T, Q’-T’]. The ion signals are normalized to the total ion count and the maximum value of 1.3x10^{-2} was used for all the images. Scale bars: 50 μm.
Figure 3.2 Ten-micron resolution MS images of representative lipids at four developmental sections of the third seedling leaf from reciprocal hybrids B73xMo17 (upper panels) and Mo17xB73 (lower panels). The ion signals are normalized to the total ion count and the maximum values used were $3.0 \times 10^{-3}$, $3.0 \times 10^{-3}$, $1.3 \times 10^{-2}$, and $1.3 \times 10^{-2}$, for PG 32:0, PG 32:1, SQDG 34:3, and Chl a, respectively. Scale bars: 50 μm.
Consistent with the photosynthetic developmental gradient that is visualized by the progression from etiolation at the base to green pigmentation at the tip of the seedling leaf, the ion signal intensity for chlorophyll a increases from the base of the leaf to the tip. MS imaging visualization of the cross-sectional distribution of chlorophyll a indicates that it is highly abundant in BS cells (Figures 3.1 and 3.2). Higher magnification MS images indicate the occurrence of chlorophyll a in M cells (Figures S10 and S11), but it occurs at much lower abundance than in BS cells. In contrast to chlorophyll a that can absorb UV at 355 nm, which is the wavelength of the laser used for these experiments and can thus be ionized even without a matrix, chlorophyll b does not absorb at this wavelength, and could not be detected in these experiments. The fact that chlorophyll a is highly concentrated in the thylakoid membranes of BS cells can be used to guide the localization of SQDGs and PGs between BS and M cells (Kirchhoff et al. 2013). The chlorophyll fluorescence image obtained for a consecutive tissue section agrees well with the MS image, thereby validating our technology (Figure S12). It must be noted that chlorophyll a may act as a matrix itself as a result of its laser absorption, but its effect is expected to be minimum compared with the 1,5-diaminonaphthalene (DAN) matrix according to our experiment on standard samples (Figure S13).

SQDG 34:3 distribution is homogeneous between both the photosynthetic cell types, regardless of genotype and stage of development (Figure 3.1M-P and 3.1M’-P’; Figure 3.2M-P and 3.2M’-P’). In contrast, PGs are differentially distributed among M and BS cells depending on the molecular species, and this non-uniform distribution is affected by genotype, being different among the two inbreds and the hybrids. As we previously reported for the midpoint section (Korte, et al. 2015), in inbred B73, PG 32:0 is primarily located in BS cells (Figure 3.1E-H) and PG 32:1 is almost exclusively present in M cells (Figure 3.1I-L); this
distribution is unaffected by leaf development, but the MS images are clear only for the midpoint and distal sections and are absent or unclear at the basal zone and the proximal end. In Mo17 however, PG 32:1 is not observed in the immature midpoint section as well as the basal and proximal zones, and accumulates to low levels in M cells only in the distal section (Figure 3.1I’-L’). In contrast to B73, PG 32:0 in Mo17 has a broader distribution (Figure 3.1E’-1H’) occurring in both BS and M cells and this distribution is not affected by development. This broader distribution of PG32:0 in Mo17 across developmental stages is very similar to the observed distribution for SQDG 34:3 (Figures 3.1M’-1P’). In the hybrids, the distribution of these lipids generally mirrors the maternal inheritance pattern, with B73xMo17 showing similar patterns to that of the female parent, B73, and in the Mo17xB73 hybrid the pattern is similar to the female parent, Mo17 (Figure 3.2). Specifically, PG 32:0 is located mostly in BS cells for B73xMo17 and in both M and BS cells for Mo17xB73. These distribution patterns are more evident at 5 μm spatial resolution, especially when overlaid with the optical images (Figures 3.4, S10, and S11).

Relative Quantification of Lipids

The relative quantification of each SQDG and PG lipid molecular species was determined by averaging the mass spectra of the MALDI-MSI dataset and then calculating the relative abundance by normalizing with the total ion signal of each lipid class. This relative quantification normalized within the same class has been shown to be a reliable approach to compare different fatty acyl chains within the same lipid class (Horn et al. 2012). To further evaluate the analytical variation of our approach, we performed MALDI MSI analysis of five consecutive sections of maize leaf and compared their relative quantification of SQDG and PG species (Figure S14). As shown in these data, the deviation between consecutive sections is minimal which suggests that the analytical variation in this analysis is negligible compared with
biological variations. In electrospray ionization, it is known that the ionization efficiency of the lipids is predominantly affected by polar head groups and that the length of the acyl chain and the degree of saturation only minimally affects the ionization (Han and Gross 1994, Han et al. 2012). To demonstrate that this is also true in MALDI-MS, we compared MALDI-MS and ESI-MS for total lipid extract of maize leaf. As shown in Figure S15, they showed a similar trend, except for some minor differences in PG 34:4.

Figure 3.3 Quantitative comparison of SQDG (Left) and PG (Right) molecular species from MS images of the distal (A, A’), midpoint (B, B’), proximal (C, C’), and basal zone (D, D’) sections of maize leaves in four genotypes
Figure 3.3 shows the relative abundance of the individual molecular species of SQDG and PG at the four developmental positions of the leaf. SQDG composition is similar across genotypes at each of the leaf positions that are exposed to light (i.e., the proximal, midpoint and distal positions), with SQDG 34:3 being the predominant species (~60-70 mol% in each genotype), SQDG 32:0 comprising 10-20 mol%, and SQDG 34:2 and 36:6 each comprising 5-15 mol%. In the etiolated tissue of the basal zone, however, SQDG 34:2 is the primary thylakoid lipid and comprises 40 mol%, which is significantly higher than any of the exposed portions of the leaf that contain 5-15 mol% (P < 0.001 for all genotypes). SQDG 34:3 is no longer the most dominant species in the basal zone and the relative abundance is significantly less than is found in the leaf zones exposed to light (P < 0.001 for all genotypes). Also notable is the fact that there is no evidence for the presence of palmitoleate (16:1 fatty acyl) containing SQDG species, such as SQDG 32:1 or 34:1. This suggests that the observed SQDG species are composed of 16:0 and 18:x fatty acids (FAs) (x = 0-3). FA 18:2 is most abundant in the basal zone of the leaf (i.e., SQDG 34:2), but is rapidly replaced by FA 18:3 (i.e., SQDG 34:3) as the leaf develops from proximal to distal portions. These observations are supported by complementary MS/MS experiments (Figure S8), and are in agreement with the previous characterization of thylakoid lipids from isolated BS and M cells (Nishihara, et al. 1980). There are some statistically supported genotypic differences in the relative abundances of these species but these are mostly minor differences.

The fatty acid compositional changes in PG are similar to those in SQDG, in that 18:2-containing PG is abundant at the basal zone (i.e. high abundance of PG 34:2), but is gradually replaced by 18:3-containing PG species, correlating with the emergence of the seedling leaf into direct illumination (i.e. an increase of PG 34:3 and a decrease of PG 34:2); however,
accumulation patterns of PG species are more dynamic across the developmental gradient compared with SQDG species. Specifically, the decrease of 34:2 and the increase of 34:3 species are more gradual in PG as development occurs, whereas there are only minimal changes in SQDG for the leaf sections exposed to light. In PG lipids, PG 34:3 is present at trace levels in the basal zone and increases up to 20-40 mol% as the leaf develops, but is not the most predominant species in any of the developmental stages. In contrast, the analogous SQDG 34:3 is the predominant species of SQDG (~60 mol%) in all leaf zones with the exception of the etiolated basal zone, in which it comprises only ~20-40 mol%.

Phosphatidylglycerol (PG) composition also differs from SQDG composition relative to FA 16:1 and FA 16:0-containing species. Low but distinct levels of FA 16:1-containing PG species (i.e., PG 32:1, PG 34:1 and PG 34:4) accumulate as the leaf develops, whereas FA 16:1-containing species are completely absent in SQDG. This is also consistent with MS/MS analysis, which revealed the existence of two species of PG 34:3, 18:3/16:0 and 18:2/16:1 (Figure S9C), whereas SQDG 34:3 is composed of just 18:3/16:0 (Figure S8B). PG species containing two FA 16:0 (i.e., PG 32:0) accumulate at very low levels in the basal zone, and increase to 20-35 mol% in the distal sections, in contrast to SQDG 32:0 which has no or minimal change in the relative abundance across the developmental gradient (P > 0.05 for most pair-wise comparison). In addition, genotypic differences in PG 32:0 accumulation are observed in the proximal sections when comparing the inbred parents with the reciprocal hybrids (P < 0.05), which gradually disappears in later developmental zones, with almost no difference at the distal section. The major contrasts in FA 16:0 and FA 16:1 compositions between PG and SQDG species suggest that such changes may be correlated with the maturity of the photosynthetic cells. It is important to note that PG 36:x species were not detected in this study. They are observed in both ESI-MS
and MALDI-MS analysis of the total extract, but in very low abundance, at <0.5% (Figure S15).

This is attributed to the high detection limit of the high-resolution MS imaging experiment because of the limited sampling volume.

Figure 3.4 Five-micron resolution MS images of each PG molecular species at the midpoint and distal sections of leaf 3 from the four genotypes overlaid with optical images. Scale bars represent 50 μm. Note that the scale bar is bigger for Mo17 and Mo17xB73.
Analysis of Cellular Distributions via High-resolution Mass Spectrometry Images

Close examination of optical microscopic images from leaf cross sections in Figures 3.1 and 3.2 indicates distinct genetics-based size differences between M and BS cells. Specifically, inbred Mo17 and hybrid Mo17xB73 BS cells are 10-20 μm in diameter, whereas BS cells in inbred B73 and hybrid B73xMo17 are twice as large at 20-40 μm in diameter. Because of the small BS cell sizes, 10-μm resolution used in Figures 3.1 and 3.2 is not sufficient to clearly distinguish the cellular distribution of some lipids in Mo17 and Mo17xB73. To clarify the cell type-specific localization of PG species, an MS imaging experiment was performed at 5-μm resolution for the midpoint and distal sections. The MS images of five PG species are compared side-by-side between each genotype and between the two developmental stages (Figure 3.4). For better visualization of the cellular localization of these lipids, the generated MS images are overlaid and aligned with optical microscope images obtained in parallel from consecutive leaf cross sections. The MS images of PG species are also compared with those of SQDG 34:3 and chlorophyll a as shown in Figures S10 and S11 for the midpoint and distal sections, respectively.

For inbred B73 and hybrid B73xMo17, PG 32:1 and PG 34:4 are primarily localized in M cells in both the midpoint and distal sections zones (Figures 3.4E, F, M, N), whereas the other three PGs (PG 32:0, 34:3 and 34:2) are accumulated at low levels in M cells and at much higher levels in BS cells (Figures 3.4I, J, Q, R, U, V). Consistent with the mol% distribution of PG 32:1 data shown in Figure 3.3B’, PG 32:1 is almost absent in Mo17 and Mo17xB73 images at the midpoint (Figures 3.4G, H), and appears to be substituted by PG 32:0, which is present in both M and BS cells (Figures 3.4K, L). PG 32:1 is clearly visible at the distal section for Mo17xB73 (Figure 3.4G) and slightly for Mo17 (Figures 3.4H), and is localized mostly in M cells, like B73 and B73xMo17 (Figures 3.4E, F). PG 32:0, however, is still present in both BS
and M cells for Mo17 and Mo17xB73 (Figures 3.4K, L). For both midpoint and distal sections, the PG 34:2 (Figures 3.4U, X) and PG 34:3 (Figures 3.4Q, T) species show similar localization patterns across all genotypes, in that both species are concentrated in BS cells. The two species that constitute PG 34:3, namely 18:2/16:1 and 18:3/16:0, cannot be distinguished here. PG 34:4 (Figures 3.4M-P) shows localization concentrated in the M cells; this localization pattern is similar across all genotypes in both midpoint and distal sections. Similar distributions between midpoint and distal sections for these PG species further confirm the differential localization is not changing as development occurs.

**MS/MS Imaging of PG 34:3 Molecular Species**

Because the two structural isomers of PG 34:3 (i.e., 18:2/16:1 and 18:3/16:0) could not be resolved via MS imaging alone, it was unclear whether these isomers differed in their abundance or cellular localization. Therefore, to address this question, MS/MS imaging experiments were performed in 5-µm high spatial resolution to distinguish the structural isomers of PG 34:3, and to determine whether their relative quantification or distribution was affected by genotype and/or leaf development. Figure 3.5 compares the relative abundance of PG 18:3/16:0 and PG 18:2/16:1 species that were extracted from MS/MS imaging datasets. For each genotype, PG 18:2/16:1 occurs at minimal levels in the basal zone (~5%), and the abundance increases along the developmental gradient and comprises 30-40% of PG 34:3 at the distal end. Mirroring the above change in PG 18:2/16:1, PG 18:3/16:0 accumulation decreases from ~95 to 60% across the developmental gradient of the leaf. The genotypic differences among these PG molecular species are minor: PG 18:2/16:1 reaches slightly higher levels in the distal section of B73 compared with Mo17xB73 or Mo17 (P = 0.02 and 0.03, respectively).
Figure 3.5 Quantitative comparison of the distribution of PG 18:3/16:0 and PG 18:2/16:1 as affected by leaf development among the four genotypes. These data were extracted from MS/MS imaging data sets of PG 34:3.

In Figure 3.6, 5-μm high-spatial resolution MS/MS images acquired at the midpoint zone show the localization patterns of the two individual isomers that constitute PG 34:3. Irrespective of the genotypes imaged, the cellular localization of PG 18:2/16:1 is evenly distributed between M and BS cells. In contrast, the cellular localization of PG 18:3/16:0 is mostly concentrated within BS cells. This divergence between distributions of PG 18:2/16:1 and PG 18:3/16:0 is in contrast to the localization of PG 32:0 (16:0/16:0) and PG 32:1 (16:0/16:1). As seen in Figure 3.4, PG 16:0/16:1 is mostly present in M cells, whereas PG 16:0/16:0 distribution differs among genotypes with localization to mostly BS cells in B73 and B73xMo17, and both M and BS cells
in Mo17 and Mo17xB73. It suggests that the occurrence of the 16 carbon fatty acid in these lipids is not the only factor that determines their localization.

Table 1 summarizes the cell-specific localization of PG molecular species for the four genotypes from Figures 3.4 and 3.6 (detailed data are presented in Table S1). Quantitative analysis was performed for the intensity ratio of these PG molecular species in unit areas of BS versus M cells, as shown in the parentheses in Table 3.1, which confirms the visual localization of these species. These data together show that there is a common trend for FA16:0-containing PGs (i.e., PG 16:0/16:0, PG 18:3/16:0, PG 18:2/16:0) to accumulate preferentially in BS cells, and for FA16:1-containing PGs (i.e., PG 16:0/16:1 and PG 18:3/16:1) to accumulate preferentially in M cells, although PG 18:2/16:1 is present almost evenly between M and BS cells. These contrasts occur regardless of genotype, except for PG 16:0/16:0, which uniquely shows genotypic differences, evenly distributed between M and BS cells in Mo17 and Mo17xB73 but more concentrated in BS cells in B73 and B73xMo17.

**Figure 3.6** Five-micron resolution MS/MS images of two structural isomers of PG 34:3 at the midpoint section of maize leaves. The ion abundances for PG 18:3/16:0 is determined from the sum of [M−16:0], [M−(16:0−H2O)], and [M−(16:0+glycerol)] and PG 18:2/16:1 is determined from [M−16:1], [M−(16:1−H2O)], and [M−(16:1+glycerol)]. Scale bar: 50 μm.
Table 3.1 Summary of cell-specific localization of phosphatidylglycerol (PG) species.

<table>
<thead>
<tr>
<th>PG 32:1 (16:0/16:1)</th>
<th>B73</th>
<th>B73xMo17</th>
<th>Mo17xB73</th>
<th>Mo17</th>
</tr>
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<tbody>
<tr>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>(0.66-0.72)</td>
<td>(0.23-0.70)</td>
<td>(0.01-0.93)</td>
<td>(0.09-0.78)</td>
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</table>

<table>
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<tr>
<th>PG 32:0 (16:0/16:0)</th>
<th>B73</th>
<th>B73xMo17</th>
<th>Mo17xB73</th>
<th>Mo17</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>BS</td>
<td>M and BS</td>
<td>M and BS</td>
<td>M and BS</td>
</tr>
<tr>
<td>(2.8-9.3)</td>
<td>(1.9-2.8)</td>
<td>(0.70-1.4)</td>
<td>(0.71-1.5)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>PG 34:4 (18:3/16:1)</th>
<th>B73</th>
<th>B73xMo17</th>
<th>Mo17xB73</th>
<th>Mo17</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>(0.46-0.91)</td>
<td>(0.14-0.48)</td>
<td>(0.15-0.44)</td>
<td>(0.22-0.98)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>PG 34:3 (18:2/16:1)</th>
<th>B73</th>
<th>B73xMo17</th>
<th>Mo17xB73</th>
<th>Mo17</th>
</tr>
</thead>
<tbody>
<tr>
<td>M and BS</td>
<td>M and BS</td>
<td>M and BS</td>
<td>M and BS</td>
<td>M and BS</td>
</tr>
<tr>
<td>(1.1-2.4)</td>
<td>(1.2-2.8)</td>
<td>(0.93-1.3)</td>
<td>(1.1-1.4)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>PG 34:2 (18:3/16:0)</th>
<th>B73</th>
<th>B73xMo17</th>
<th>Mo17xB73</th>
<th>Mo17</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>BS</td>
<td>BS</td>
<td>BS</td>
<td>BS</td>
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<tr>
<td>(2.2-3.4)</td>
<td>(2.3-4.8)</td>
<td>(1.6-3.2)</td>
<td>(2.2-5.4)</td>
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<table>
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<tr>
<th>PG 34:0 (18:2/16:0)</th>
<th>B73</th>
<th>B73xMo17</th>
<th>Mo17xB73</th>
<th>Mo17</th>
</tr>
</thead>
<tbody>
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<td>BS</td>
<td>BS</td>
<td>BS</td>
<td>BS</td>
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<td>(6.4-97)</td>
<td>(2.9-87)</td>
<td>(6.9-57)</td>
<td></td>
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</tbody>
</table>

* The numbers in parenthesis represent the range of ion signal ratios in BS versus M cells per unit pixel area obtained from three replicates of midpoint sections. Detailed analysis is presented in Table S1.

Discussion

Different cell types can be separated physically for lipidomic, transcriptomic or proteomic analysis, but the sample processing involved can result in undesirable degradation or cross-contamination of the materials. MALDI-MSI provides distinct advantages for the visualization of metabolite distributions within intact tissues and cellular environments. This is especially important because the molecular distributions of metabolites or lipids may not always be the same, even among the same cell types. For example, 2,4-dihydroxy-7-methoxy-1,4-benzoazin-3-one glucoside (DIMBOA-Glc) and 2-hydroxy-7-methoxy-1,4-benzoazin-3-one glucoside (HMBOA-Glc) are known to be present in M cells of maize, but MALDI-MSI revealed that they are present only in M cells between each pair of vascular bundles (Korte, et al. 2015). This is also observed in the current dataset for all four genotypes (Figures S10, S11).

The mechanisms underlying tissue development and function are fundamental questions of cellular biology. Maize is an excellent model to study the establishment of spatially distributed metabolic functions in which metabolism is facilitated by specialized cellular
differentiation and cellular arrangement. In maize leaves, this is specifically associated with the differentiation of two types of photosynthetic cells, the bundle sheath and the mesophyll, that are characteristic of the Kranz anatomy (Laetsch 1974), facilitating C4 photosynthetic photoautotrophy (Edwards et al. 2001, Hatch 1987, von Caemmerer and Furbank 2003). The chloroplasts within BS and M cells harbor different metabolic capabilities and express different ultrastructures: the BS chloroplasts contain Rubisco and catalyze the Calvin cycle for CO2-fixation, whereas these capabilities are absent from the chloroplasts of M cells (Nelson and Langdale 1992). In contrast, photosystem II (PSII) and granal stacks are absent from BS chloroplasts (Woo et al. 1970).

Taking advantage of the MSI capabilities that we have developed over the past 8 years, here we directly tested the hypothesis that the lipidomes of M and BS cells in maize are differentially affected by cellular development, and that this developmental program is genetically controlled, as determined by the state of the maize genome. Specifically, we applied high spatial-resolution MSI (performed at either 5- or 10-μm resolution) on four developmental stages from leaves of four different maize genotypes, and compared the cellular location and relative abundances of two major anionic thylakoid lipids, SQDG and PG, within mesophyll and bundle sheath cells.

Typical of monocot leaves, the cellular differentiation that establishes the Kranz anatomy is linearly arranged from the base to the tip of the maize leaf, greatly facilitating its molecular and cellular study (Nelson and Langdale 1989). Recent studies have revealed the dynamic nature of cellular differentiation as changes in the transcriptome and proteome along the developmental gradient of the leaf (Li, et al. 2010). These analyses have uncovered a programmed change in the pattern of gene expression along the developmental length of the leaf, which parallels known
morphometric alterations in cellular differentiation that marks the establishment of the Kranz anatomy (Li, et al. 2010). Specifically, in the basal section of the leaf, which is associated with the zone of cell division and elongation, expressed genes are markedly enriched for functions encoding for protein, DNA and cell wall biosynthesis. In the proximal section of the leaf that is associated with the cellular transition from sink to source tissue, the expressed genes are associated with photosynthetic machinery and secondary wall biosynthesis functions. Subsequently in the distal portion of the leaf, which is fully photoautotrophic, expressed genes associated with photosynthetic reactions, including the Calvin cycle, are enriched.

Similar transcriptomic (Tausta, et al. 2014) and proteomic (Majeran, et al. 2008) studies have specifically profiled the gene expression programs of BS and M cells along the developmental gradient of the maize leaf. These studies demonstrated the dynamic differences in gene expression between the two photosynthetic cell types, with more than twice the number of differentially expressed genes at the midpoint section of the leaf, as compared with the mature leaf tip. These molecular profiling studies identified differential expression patterns between M and BS cells, including those of photosystems I and II proteins, and thylakoid and envelope membrane proteins that function as metabolite transporters (Manandhar-Shrestha, et al. 2013). These molecular differences between M and BS cells are consistent with morphological and functional differences between these two cell types, particularly associated with the two chloroplast populations that differentiate the two cell types. These attributes particularly distinguish the photosynthetic capabilities and thylakoid membrane ultrastructures of the two chloroplast types (Hatch 1987).

Integrating the recent data on the dynamics of the transcriptomic and proteomic profiles with the ultrastructural changes associated with the developmental gradients of the two
photosynthetic cell types, led to the hypothesis that there will be lipidomic differences between these two cell types and these will differentially affect the developmental gradient of the leaf. Indeed, earlier physical fractionation-based lipid analyses of isolated chloroplasts provided indications of such lipidome differences among chloroplasts of M and BS cells (Edwards and Black 1971, Nishihara, et al. 1980, Poincelot 1973, Woo, et al. 1970). However, conclusions from these earlier studies were confounded by the use of fractionation methods that included slow digestion processes, and were susceptible to cross-contamination of the M and BS cell fractions (Edwards and Black 1971, Kanai and Edwards 1973, Majeran, et al. 2005, Nelson and Langdale 1992, Poincelot 1973). They were further confounded by the use of more complex analytical technologies, which often needed enzymatic digestion with undefined reagents (e.g. digestion of cell wall components with crude fungal-derived hydrolytic enzyme preparation) or chemical modification reactions of the tissue or the isolated lipids (e.g. the use of positional-sensitive lipases or chemical reagents to characterize lipid molecular species; Christie and Han 2012, Kuksis et al. 1983, Siebertz et al. 1979). The application of modern advances in mass spectrometry has more recently provided enormous progress in the understanding of acyl lipid profiles and lipid metabolism in plants (Gasulla et al. 2013, Li-Beisson et al. 2013, Ramadan et al. 2014, Riedelsheimer et al. 2013, Tarazona et al. 2015, Vu et al. 2014, Welti et al. 2002).

SQDG and PG are chloroplast lipids that are crucial for maintaining chloroplast structure and function, including the stability of the PS II complex in the thylakoid membranes (Kansy et al. 2014, Yu and Benning 2003), and they are essential for the growth and development of photoautotrophs (Yu and Benning 2003). PG could be distinguished from SQDG by the fact that it has at least one 16-carbon fatty acyl chain, either 16:0 or 16:1. We could not determine whether these 16-carbon acyl-chains occur at the sn-1 or sn-2 position, but earlier studies
indicate that both 16:0 and 16:1 prefer the sn-2 position, with 16:1 having the priority if both acyl chains are present (i.e., PG 16:0/16:1; Nishihara, et al. 1980). One clear difference between our data and the earlier study is the presence of PG 18:2/18:3 localized to BS cells (Nishihara, et al. 1980). According to the extract analysis, we do observe PG 36 species but in much lower abundance (Figure S15). Hence, the difference is most likely associated with the difference in genotype (L. var. Honey Buntum) and/or age of tissue (45-day old plants).

The relative abundances of SQDG molecular species determined by MSI is consistent with earlier bulk-extract analysis of this class of lipid: namely, 16:0- and 18:3-containing SQDG lipids account for the majority of this lipid class (Kenrick and Bishop 1986), with 80-90% being SQDG 32:0 and SQDG 34:3. There are only minor differences in the abundance of these lipids among the four genotypes evaluated; however, development dramatically affects their abundance. Particularly, the most abundant SQDG 34:2 in the basal zone is replaced by SQDG 34:3 as the leaf develops. In the third leaf that we assessed for molecular imaging, this cellular differentiation occurs as the leaf expands through the ligule of the second leaf, and becomes directly exposed to illumination. This is marked by the differentiation of proplastids to different types of chloroplasts that are non-uniformly distributed between BS and M cells, and this differentiation is associated with an increase in the size and number of chloroplasts per cell. At this differentiation of the M and BS chloroplasts, granal stacks begin to develop in M chloroplasts, but not in BS chloroplasts (Majeran et al. 2010). Therefore, the observed changes in the distribution of the different molecular species of SQDG may suggest that SQDG 34:3 is mostly associated with thylakoid membranes, whereas SQDG 34:2 is a major component of the chloroplast envelopes.
In the case of PG, PG 34:2 is the dominant molecular species in the basal zone of the leaf, accounting for 80-90% of this lipid class, with essentially no difference among the inbreds and hybrids evaluated (Figure 3.3). As leaf development progresses, the abundance of other PG molecular species (PG 32:0 or PG 34:3) increase and they become most dominant at the midpoint and distal end sections. This is consistent with earlier work that showed the increase of 18:3 and the decrease of 18:2 fatty acyl composition of PG across the developmental gradient of the maize leaf (Roughan 1985). Using the same logic as discussed above for SQDG molecular species, these findings may suggest that PG 34:2 is mostly associated with chloroplast envelopes, and that PG 32:0 and PG 34:3 molecular species are primarily associated with thylakoid membranes. The relative abundance of PG molecular species, however, continuously changes, at least up to the midpoint (Figure 3.3B), whereas SQDG shows only minimal change after the proximal zone (Figure 3.3A). Furthermore, the 16:1 fatty acyl composition of PG continuously increases as the leaf matures, manifested by the increase of PG 32:1 (Figure 3.3B) and PG 34:3 (18:2/16:1) (Figure 3.5) from the basal to distal zones of the leaf. This suggests the importance of PG molecular species, specifically with 16 carbon fatty acyl chains, for the functional development of chloroplasts, not merely anatomical differentiation mostly dictated by 18:2 and 18:3. In addition to the developmental effect on the relative abundances among PG molecular species, there is also a genotype dependence on the distribution of these lipids. This difference among the genotypes is especially prominent at the proximal end of the leaf, and becomes indistinguishable as the leaf expands into the mature stages.

Of significance, there is a difference in the localization of PG molecular species between BS and M cells, which appears to be dependent on the fatty acyl chains, and it is also a trait that is partially influenced by genotype. Such a heterogeneity was previously suggested by the lipid
analysis of physically separated BS and M cells (Nishihara, et al. 1980). This differential localization is not exclusive but is rather preferential, and major PGs are present in both BS and M cells. As summarized in Table 1, PGs with FA16:0 and FA16:1 prefer to localize in BS and M cells, respectively, for all genotypes. The preferential localization of FA16:1-containing PGs in M cells and their increased accumulation in later stages of leaf development may indicate their importance to the development of the PSII complex and granal stacks, which are localized in M cells and most enriched in the distal region of the leaf (Li, et al. 2010, Majeran, et al. 2010). Some PGs are much more homogeneously localized between the two cell types, specifically PG 18:2/16:1 in all four genotypes (Figure 3.6) and PG 16:0/16:0 in Mo17 and Mo17xB73 (Figure 3.4). The homogeneous distribution of these PG molecular species may suggest the importance of PG 18:2/16:1 and PG 32:x in both BS and M cells. Considering the importance of 16:1-containing PGs, it is reasonable to hypothesize that a corresponding desaturase would be differentially expressed in large-scale proteomics or transcriptomics data sets, especially in leaf development and/or between different cell types (Li, et al. 2010, Majeran, et al. 2008). In Arabidopsis, fatty acid desaturase 4 (FAD4) is known to be involved in the formation of $\Delta^{3}_{\text{trans}}$ FA16:1 at the sn-2 position of PGs (Gao et al. 2009). The maize genome includes two homologs of FAD4 in the NCBI BLASTP search, i.e. GRMZM2G175401 and GRMZM2G097509, and both of them are expressed in shoots, especially at the tip of stage-2 leaves of V5 and V7, respectively (Sekhon et al. 2011). Furthermore, GRMZM2G175401 is shown to have a higher expression in M cells than BS cells according to the supplementary data of Li et al. (2010); however, the correlation between the content of 16:1t in PGs and FAD4 is obscure as the reduction of 16:1t has been also reported for other mutants in PG biosynthesis such as pgpp1-I and pgp-1 (Babiychuk et al. 2003, Kobayashi et al. 2015, Lin et al. 2016).
In summary, the work described herein demonstrates that high-resolution MALDI-MSI analysis can be directly applied to multicellular plant tissues to uncover cell-specific metabolic biology that has not been possible using traditional metabolomics methodology. This capability of in situ imaging is specifically valuable for metabolites and lipids because of their highly dynamic nature compared with transcriptome or proteome. Combined with other single cell ‘-omics’ technologies (Dai and Chen 2012, Shalek et al. 2013, Wang and Bodovitz 2010), high-resolution MSI will allow for enhanced systems biological understanding at the single-cell level in the foreseeable future.

Experimental

Plant Growth and Sample Preparation

The overall experimental workflow is illustrated in Figure S1. Maize kernels (Zea mays L. inbreds B73 and Mo17, and reciprocal hybrids B73xMo17 and Mo17xB73) were planted in soil, and grown in a climate-controlled glasshouse at 30% humidity under a diurnal cycle of 16 h of light and 8 h of dark at 27°C and 24°C, respectively. Seedlings were harvested at approximately 13:00 h local time, 11-13 days after planting, when the length of the third leaf was 14-18 cm, as measured from the second ligule. Sections from four positions along the developmental gradient of leaf 3 (Figure S2) were collected from three individual plants of each genotype, at similar positions as described by Li et al (2010). The etiolated basal zone leaf section was collected as a cross-section through the sheathed basal zone of leaf 3 within the whorl, 1.5 cm below leaf ligule 2. The second section of leaf 3, labeled as the proximal section, was collected 1.5 cm above the position of the ligule 2 and at the point of emergence from the whorl, where the leaf begins to unfold and becomes fully exposed to direct illumination. The third section of leaf 3, labeled as the midpoint, was collected halfway between the proximal
section and the leaf tip. The fourth section of leaf 3, labeled as the distal section, was collected ~2 cm from the tip of the leaf and approximately 12-16 cm from the base.

Tissue samples were cryosectioned and prepared for MALDI-MSI as described previously (Korte and Lee 2014). Briefly, fresh maize leaf sections were placed in a cryomold, then submerged in gelatin (10% w/v solution) and immediately frozen with liquid nitrogen. The molds were then transferred to a cryostat (CM1850, Leica Microsystems; Buffalo Grove, IL, USA) that was pre-chilled to -20°C, and the samples were allowed to thermally equilibrate for 30 minutes. Leaf tissue was cryo-sectioned at 10 μm thickness and was then collected with Cryo-Jane tape (Leica Biosystems, http://www.leicabiosystems.com), and attached to pre-chilled glass slides. The prepared slides were placed onto a chilled aluminum block and were vacuum dried while gradually warming to room temperature. After acquiring optical microscope images (Axio Zoom.V16; Carl Zeiss, http://www.zeiss.com), the dried tissues were subjected to matrix deposition by sublimating 1,5-diaminonaphthalene (97%; Sigma-Aldrich, http://www.sigmaaldrich.com) (Hankin et al. 2007) at 140°C for 4 minutes at a pressure of ~50 mtorr.

For the fluorescence image shown in Figure S12, a leaf tissue was cryosectioned at 10-μm thickness and collected on a charged glass slide (Fisher Scientific, http://www.fishersci.com) by thaw mounting. The fluorescence image was obtained with an Olympus BX-60 microscope (http://www.olympus-global.com) with a Jenoptik C-5 camera (http://www.jenoptik.com). A consecutive cross-section was collected with Cryo-Jane tape to compare with the MS image of chlorophyll a.

Mass Spectrometry Analysis

Mass spectrometry imaging data were collected using a MALDI-linear ion trap (LIT)-Orbitrap mass spectrometer (MALDI-LTQ-Orbitrap Discovery; Thermo Scientific,
http://www.thermofisher.com). The instrument was modified to use an external 355-nm frequency tripled Nd: YAG laser (UVFQ; Elforlight Ltd., http://www.elforlight.com). The laser energy used was 83-84% (~1 μJ per pulse) at a 60 Hz repetition rate. The laser optics are similar to those previously described by Korte et al. (2015), but the focus lens was further reduced to 60 or 75 mm and a commercial 10X or 5X beam expander (Thorlabs, http://www.thorlabs.com) was used to reduce the laser spot size. The laser spot size was 9-11 μm for 10 μm-resolution imaging with a 5X beam expander and 6-7 μm for 5 μm-resolution imaging with a 10X beam expander; thus, 5-μm resolution imaging was acquired using an oversampling method. TunePlus and Xcalibur (ThermoFisher Scientific) were used to define imaging parameters and to acquire data, respectively. Mass spectra were acquired with 10 laser shots per spectrum in negative mode using an Orbitrap mass analyzer (resolution of 30 000 at m/z 400) for an m/z scan range of 100-1000.

All lipids were detected as deprotonated ions, [M-H]−. MS images were generated using ImageQuest (ThermoFisher Scientific) with a mass window of ±0.003 Da, and with normalization to the total ion count (TIC) for Figures 3.1, 3.2, and S4-S7, and without normalization for the rest of the figures. Overlays of MS images with the optical microscope images were made using the software module of MATLAB, Image Processing Toolbox 9.3 (MATLAB 2015). MSiReader v.0.09 (Robichaud et al. 2013) was used to obtain the ion signal ratios of PG molecular species between BS and M cells normalized to each cell area. First, the optical images and MS images were overlaid using the MSiImage tool. Then, the region of interest (ROI) for the bundle sheath and mesophyll cells was selected using the polygon drawing tool and the spectra intensity data for each ROI was exported for all PG species. The total
intensity of each PG species at each ROI was then normalized to the area of the ROI for each cell type.

MS/MS imaging was performed using the ion trap analyzer for selected ions using the same conditions as described for MS imaging. An isolation width of 2.0 Da and normalized collision energy of 35 were used. MS/MS images were generated using MSiReader with a mass window of ±0.2 Da and no normalization to the TIC. Quantitative comparison was made by averaging metabolite intensities over the entire imaging area of the maize leaf that contained both mesophyll and bundle sheath cells. Three or more biological replicates of each genotype were analyzed, and the average is reported with standard deviation. Student’s t-tests were performed to calculate pairwise P-values.

**Lipid Extraction and Direct-Infusion ESI-MS**

Total lipids were extracted from maize leaf (inbred B73) as described by Shiva et al. (2013). Lipid samples were then purified by two-phase partitioning with chloroform and 1 M KCl, dried under N₂ and resuspended in chloroform, and stored at -20°C until MS analysis. Three biological replicates were analyzed, and the average is reported with standard deviation. Total lipid extracts were analyzed by direct-infusion ESI-MS on a Fourier Transform Ion Cyclotron Resonance mass spectrometer (7T SolariX FT-ICR MS; Bruker Daltonics, http://www.bruker.com).

**Acknowledgements**

This work was supported by the US Department of Energy (DOE), Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences, and Biosciences. MDY-N and BJN acknowledge the support of the National Science Foundation under Award No. EEC-0813570 and Award No. IOS-1354799, which co-sponsored the development of the genetic
stocks imaged in this study. The Ames Laboratory is operated by Iowa State University under DOE Contract DE-AC02-07CH11358.

Supplemental Figures

**Figure S1.** Overall workflow for MALDI-MSI of maize leaves
Figure S2. Illustration of four positions along the developmental gradient of maize leaf 3 used in this study. The distal section corresponds to 70–90% maturation when compared to the unified development model (UDM) based on the length, while the midpoint section collected at 6-8 cm corresponds to 36-47% maturation.* The proximal section, in contrast, is expected to correspond to only ~15% maturation.

**Figure S3.** Transverse sections of a B73 maize leaf (distal and proximal sections, respectively) showing the characteristic C4 leaf Kranz anatomy where concentric wreaths of mesophyll and bundle sheath cells surround closely spaced veins, the vascular bundles.
Figure S4. MS images of PGs, SQDGs, and three photosynthesis-related metabolites at the basal zone, proximal, midpoint, and distal sections of B73 inbred maize leaf. Each maximum value for color scheme was adjusted for the best comparison across the sections and between the genotypes. Chl a: chlorophyll a [sum of fragments at m/z 591.261 (pheophorbide a), 613.232 (chlorophyllide a), and 870.566 (pheophytin a)]. PQ: plastoquinone/plastoquinol. Scale bar represents 50 µm.
Figure S5 MS images of PGs, SQDGs, and three photosynthesis related metabolites at the basal zone, proximal, midpoint, and distal sections of B73 x Mo17 hybrid maize leaf. Each maximum value for color scheme was adjusted for the best comparison across the sections and between the genotypes. Chl a: chlorophyll a [sum of fragments at m/z 591.261 (pheophorbide a), 613.232 (chlorophyllide a), and 870.566 (pheophytin a)]. PQ: plastoquinone/plastoquinol. Scale bar represents 50 µm.
Figure S6. MS images of PGs, SQDGs, and three photosynthesis related metabolites at the basal zone, proximal, midpoint, and distal sections of Mo17 x B73 hybrid maize leaf. Each maximum value for color scheme was adjusted for the best comparison across the sections and between the genotypes. Chl a: chlorophyll a [sum of fragments at m/z 591.261 (pheophorbide a), 613.232 (chlorophyllide a), and 870.566 (pheophytin a)]. PQ: plastoquinone/plastoquinol. Scale bar represents 50 µm.
Figure S7. MS images of PGs, SQDGs, and three photosynthesis related metabolites at the basal zone, proximal, midpoint, and distal sections of Mo17 inbred maize leaf. Each maximum value for color scheme was adjusted for the best comparison across the sections and between the genotypes. Chl a: chlorophyll a [sum of fragments at m/z 591.261 (pheophorbide a), 613.232 (chlorophyllide a), and 870.566 (pheophytin a)]. PQ: plastoquinone/plastoquinol. Scale bar represents 50 µm.
Figure S8. MS/MS spectra of (A) SQDG 32:0, (B) SQDG 34:3, and (C) SQDG 36:6 obtained from the midpoint zone of leaf 3 from inbred B73. Major fragment ions represent the loss of the 16:0 and 18:3 fatty acid side chains.
Figure S9. MS/MS spectra of (A) PG 32:0, (B) PG 34:2, and (C) PG 34:3 from the proximal zone of leaf 3 from inbred Mo17, showing two isomers, PG 18:3/16:0 and PG 18:2/16:1. Fatty acid fragments of C18:3 and C18:2 are due to the presence of other phospholipid species (i.e. PE 36:3, PE 36:2) within the isolation mass window used for MS/MS. PGs fragment is predominantly at the sn-2 position*, which is the location of 16:0 and 16:1 FAs†. Hence, the loss of C16:0 and C16:1 ([M-C16:0]-, [M-C16:1]-, ([M-(C16:0-H2O)]-[M-H]-, [M-(C16:1-H2O)]-[M-H]-, and [M-(C16:0 + glycerol)]-[M-H]-) was used for the quantification of PG 18:3/16:0 and PG 18:2/16:1 in Figure 3.5A.


Figure S10. (A-D) Overlay of optical images from the midpoint zone of leaf 3 from B73, B73xMo17, Mo17xB73, and Mo17 with MS images of (E-H) PG 32:1, (I-L) PG 32:0, (M-P) PG 34:4, (Q-T) PG 34:3, (U-X) PG 34:2, (Y-B’) SQDG 34:3, (C’-F’) Chl a, and (G’-J’) HMBOA-Glc. Note that the scale bar is larger for Mo17 and Mo17xB73. Scale bars represent 50 μm.
Figure S11. (A-D) Overlay of optical images at the distal cross-section of B73, B73xMo17, Mo17xB73, and Mo17 maize leaves with (E-H) PG 32:1, (I-L) PG 32:0, (M-P) PG 34:4, (Q-T) PG 34:3, (U-X) PG 34:2, (Y-B') SQDG 34:3, (C'-F') Chl a, and (G'-J') HMBOA-Glc. Note that the scale bar is larger for Mo17 and Mo17xB73. Scale bars represent 50 μm.
Figure S12. Comparison of chlorophyll a localization between (Top) MS and (Bottom) fluorescence images obtained from two consecutive leaf cross-sections. Optical and overlaid images are also shown for the comparison. Considering the slight difference between the two tissues as seen in optical microscope images, MS and fluorescence images are matching quite well. MS and fluorescence images could not be obtained from the same tissue sections. For fluorescence imaging, the tissues needed to be directly attached to glass slides to avoid fluorescence backgrounds. For MS imaging, the tissues needed to be attached with double side tape to maintain tissue integrity during the sample preparation.
Figure S13. MALDI-MS spectra of a mixture of soy PG standard and spinach extract (A) with and (B) without DAN as the matrix. Spinach extract is used as a source of chlorophyll $a$ to study its effect on MALDI ionization. Spinach extract is at the final concentration of 42 mg/mL where the chlorophyll $a$ concentration was 0.5 mM according to UV absorption measurement. The final concentration for soy PG standard was 1.25 mM, calculated assuming the average molecular weight of 800 g/mol. DAN concentration was maintained to be low, 0.5 mM, to make it compatible with that of chlorophyll $a$. Base ion signals are $2.04 \times 10^5$ and $2.87 \times 10^4$ ion counts for panel A and B, respectively, demonstrating MALDI efficiency by chlorophyll $a$ is only about $\sim14\%$ even compared with this very low DAN concentration. When compared with typical DAN concentration, $\sim10$ mM, MALDI efficiency by chlorophyll $a$ was only $1\sim2\%$. 
Figure S14. Reproducibility test of MALDI MSI analysis using five consecutive cross-sections of maize leaf. Relative quantification of (Top) SQDG and (Bottom) PG species.
Figure S15. Comparison of PG and SQDG species from total lipid extract of B73 leaf 3 obtained by (A) ESI-FTICR MS and (B) MALDI-MS. Final concentration of leaf extract was 0.025-0.028 mg/ml in ESI-FTICR MS analysis.
Table S1. Quantitative analysis for cell-specific localization of PG species.

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* The numbers represent the ion signal ratios of each PG molecular species in BS vs M cells per unit pixel area obtained from three replicates of midpoint sections. See the experimental section how it was calculated.

* In this analysis, PG ion signals in BS cells are expected to be slightly over-estimated. Because the chloroplasts in BS cells are mostly localized toward the cellular boundary with surrounding M cells, the MS imaging pixels containing both BS and M cells are mostly considered as if they are BS cells. As a result, some PG signals in M cells might have been counted as those in BS cells. Considering the overestimation of BS cells, the color label for the average value of the three replicates is determined as following.

- **>3** Localization is dominant in BS
- **2-3** Localization is primarily in BS
- **0.8-2** Localization is in both M and BS
- **0.5-0.8** Localization is primarily in M
- **<0.5** Localization is dominant in M
References


CHAPTER 4. 3D MALDI MASS SPECTROMETRY IMAGING OF A SINGLE CELL: SPATIAL MAPPING OF LIPIDS IN THE EMBRYONIC DEVELOPMENT OF ZEBRAFISH

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**Abstract**

The zebrafish (Danio rerio) has been widely used as a model vertebrate system to study lipid metabolism, the roles of lipids in diseases, and lipid dynamics in embryonic development. Here, we applied high-spatial resolution matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry imaging (MSI) to map and visualize the three-dimensional spatial distribution of phospholipid classes, phosphatidylcholine (PC), phosphatidylethanolamines (PE), and phosphatidylinositol (PI), in newly fertilized individual zebrafish embryos. This is the first time MALDI-MSI has been applied for three dimensional chemical imaging of a single cell. PC molecular species are present inside the yolk in addition to the blastodisc, while PE and PI species are mostly absent in the yolk. Two-dimensional MSI was also studied for embryos at different cell stages (1-, 2-, 4-, 8-, and 16-cell stage) to investigate the localization changes of
some lipids at various cell developmental stages. Four different normalization approaches were compared to find reliable relative quantification in 2D- and 3D- MALDI MSI data sets.

**Introduction**

The zebrafish, *Danio rerio*, is a model vertebrate organism for studying and understanding developmental biology, drug discovery, and neurodegenerative diseases (McGrail *et al.*, 2011, van Amerongen *et al.*, 2014, MacRae and Peterson, 2015). Zebrafish, a small tropical aquarium fish native to Southeast Asia, have a unique combination of genetic and experimental embryologic advantages that make them ideal for studying early development. Fertilized zebrafish embryos are accessible to observation and manipulation at all stages of development due to external fertilization and optical clarity (Veldman and Lin, 2008). Furthermore, zebrafish are readily available, inexpensive, hearty, easy to care for, and can lay hundreds of eggs at weekly intervals.

Zebrafish embryos have been used to study lipid metabolism, the roles of lipids in diseases, and lipid dynamics in embryonic development (Carten *et al.*, 2011, Fraher *et al.*, 2016, Pirro *et al.*, 2016). Recently, Fraher *et al.* conducted a lipidomic study using liquid chromatography-mass spectrometry and revealed that cholesterol, phosphatidylcholine (PCs), and triglycerides are the most abundant lipids in the zebrafish embryo. They demonstrated that lipids are processed within the yolk prior to mobilization to the embryonic body (Fraher *et al.*, 2016). Desorption electrospray ionization mass spectrometry (DESI-MS) has also been used for direct MS analysis and imaging of lipids in individual zebrafish embryos across embryonic development (0, 24, 48, 72, and 96 hours post-fertilization) (Pirro *et al.*, 2016). Metabolomics and lipidomics studies in zebrafish are of interest because these compounds have key biological functions, such as serving as energy storage sources, participating in cell signaling, and acting as essential components of cell membranes (Shevchenko and Simons, 2010, Christie and Han,
Exploring how metabolites and lipids are regulated is key to understanding biological pathways and developmental processes occurring in a biological system.

Traditional analytical approaches to study small metabolites and lipids require extensive sample preparation, laborious extractions, derivatizations, and previous knowledge of compounds of interest. Mass spectrometry imaging (MSI) has become a widely used analytical tool for these studies thanks to the recent development in sample preparation protocols and instrumentations (Stoeckli et al., 2001, Lee et al., 2012, Korte et al., 2015a). MSI allows for two-dimensional visualization of the spatial distribution of biomolecules without extraction, purification, separation or labeling of analytes (Passarelli and Ewing, 2013). Moreover, many different classes of compounds, including unknowns, can be detected simultaneously from a single MSI experiment, which allows for direct cellular or sub-cellular mapping of biomolecules in high resolution and a high throughput manner.

Since biology occurs in organisms in three dimensions, it is not surprising that 3D imaging has had a noteworthy impact on many challenges in the life sciences (Palmer and Alexandrov, 2015). Recently, imaging of intact biomolecules using mass spectrometry imaging has expanded to 3D analysis to determine volumetric molecular distribution within tissue specimens, agar plates, and 3D cell cultures (Weaver and Hummon, 2013). The most common method of 3D imaging using mass spectrometry consists of collecting consecutive sections of a sample, analyzing each section individually using traditional 2-dimensional mass spectrometry imaging, and then stacking and reconstructing a final 3D imaging MS data set from the multiple 2-dimensional sets using computational methods (Palmer and Alexandrov, 2015).
Our group has developed high-spatial resolution matrix-assisted laser desorption/ionization (MALDI)-MSI down to 5 μm resolution and utilized this for cellular or sub-cellular level imaging of plant metabolites (Korte et al., 2015a, Feenstra et al., 2017). Here, we present 3D MALDI-MSI of newly fertilized individual zebrafish (Danio rerio) embryos utilizing this high-spatial resolution. This is the first demonstration of 3D MSI for a single cell obtained with MALDI, revealing sub-cellular level localization of various lipid compounds.

TOF-SIMS has been utilized for 3D MSI of single cells (Passarelli et al., 2015, Vanbellingen et al., 2016), especially incorporating depth profiling as a way to achieve z-directional information; however, high mass compounds that can be analyzed by TOF-SIMS have been mostly limited to exogenous drug compounds due to significant fragmentations. In this analysis, we performed 3D MALDI-MSI on single zebrafish zygotes by acquiring MS imaging data set in positive and negative ion mode for alternative slides of 62 consecutive cross-sectional tissue sections. This allows for 3D visualization of more comprehensive lipid species from a single cell. Additionally, four different normalization approaches were compared to determine which of these can provide more representative results when comparing 2D MSI with the 3D volume reconstruction.

Furthermore, full-scan MSI and MS/MS were acquired for embryos at different cell stages (1-, 2-, 4-, 8-, and 16-cell stage) to investigate the changes in phospholipid distribution during the early stages of zebrafish development.

Results and discussions

Lipid Profiles in Three Dimensions in One-cell Stage Zebrafish Embryos

The newly fertilized zebrafish egg is in the zygote period until the first cleavage occurs (Kimmel et al., 1995). In this period, the embryo is at the single-cell stage. Supplementary Figure S1a shows bright-field microscope images of serial cryo-sections of entire fertilized zebrafish embryo at the one-cell stage. Because of the nature and complexity of 3-dimensional
imaging, sample preparation is a critical step in this study as it may be time-consuming and error-prone (Anderson et al., 2016). To diversify the chemical compounds that can be studied, both positive and negative ion mode data have been obtained for the same embryo. Odd number serial sections were analyzed in negative ion mode with DAN as a matrix, and even number serial sections were analyzed in positive ion mode with the binary matrix of DHB/Fe₃O₄ as a matrix. This will not only reduce the sample preparation time involved in the selection of the embryo, cryo-sectioning, and microscope inspection, but also minimize the sample-to-sample variation. Optical images (Supplementary Figure S1b) of a zebrafish embryo at the one- and two-cell stages show the fertilized egg and uncleaved or cleaved blastodisc. The blastodisc and yolk can be readily distinguished in the microscope image, and their sizes are approximately 400 μm and 600 μm, respectively.

The overall workflow for MALDI-MSI of zebrafish embryos is shown in Supplementary Figure S2. Zebrafish embryos were harvested at the one-cell stage, embedded in gelatin, and immediately flash frozen. Then, zebrafish embryos were cryo-sectioned at 10 μm thickness and collected through the entire embryo, from one side to the other, resulting in 62 sections. Optical images were then acquired prior to matrix application. MALDI-MS images were generated for phosphatidylcholine (PC), phosphatidylethanolamines (PE), and phosphatidylinositol (PI) molecular species in the serial sections using positive and negative mode data sets. For the 3D model reconstruction, all the MS images were computationally aligned to each other using the optical images as a guide, regaining the initial spatial relations prior to sectioning. False-colored images for each molecular species were adjusted to the same maxima for each species.
Representative single pixel mass spectra are shown in Figure 4.1 for both ion modes. Spectral regions that are dominated by a particular phospholipid molecular species are highlighted and labeled for PC, PE, and PI. The spatial distribution of PE (22:6_16:0), PI (18:0_20:5), PC (18:1_16:0), and PC (16:0_22:6) were heterogeneous in localization in the different areas of the one-cell embryo (Figures 4.2 and 4.3). Overall, PE and PI are mostly absent or present minimally inside the yolk, while significant amounts of PCs are present, suggesting PCs are stored in the yolk. All three phospholipids are present in high abundance with symmetric distribution inside the blastodisc, as well as the boundary of yolk, which is not surprising considering their role as membrane lipids.

**Figure 4.1** Representative single pixel mass spectra obtained in negative (top) and positive (bottom) ion mode. Spectral regions that are dominated by a particular phospholipid class are highlighted and labeled with the following class: PC, phosphatidylcholine; PE, phosphatidylethanolamine; and PI, phosphatidylinositol. *corresponds to trimethylamine loss (-N(CH₃)₃) from PC head group.
To further illustrate these localizations, cross-section profiles were generated by mapping the signal intensities across the central part of zebrafish embryo as shown in Figure 4.4, where the blastodisc region corresponds to x=0~A, and the yolk region corresponds to x=A~B (Figure 4.4a). PI (18:0_20:5), PC (18:1_16:0), and PC (16:0_22:6) show relatively homogenous distribution inside the blastodisc, whereas PE (22:6_16:0) shows elevated signal at the central region of the blastodisc (Figure 4.4b-c). This slight change in distribution can also be visualized with a 2D-MSI overlay of PI (18:0_20:5), PE (22:6_16:0), and the optical image (Supplementary Figure S3).
Figure 4.2 (a) Odd numbered optical images of fertilized zebrafish embryo at the one-cell stage. False color two-dimensional MALDI-MS images of (b) PE (22:6_16:0) at m/z 762.509 and (c) PI (18:0_20:5) at m/z 883.535. Projected images are shown on the right by overlaying all 2D images. All species were detected as deprotonated ions, [M-H]$^-$. 
Figure 4.3 (a) Even numbered optical images of fertilized zebrafish embryo at the 1-cell stage. False color two-dimensional MALDI-MS images of (b) PC (18:1_16:0) at m/z 798.535 and (c) PC (16:0_22:6) at m/z 844.525. Projected images are shown on the right by overlaying all 2D images. All species were detected as potassiated ions, [M+K]^+.
Figure 4.4 (a) Optical image of fertilized zebrafish embryo showing the arrow to indicate where the line profile is obtained. Line profile of ion intensities for (b) PI (18:0_20:5) and PE (22:6_16:0) and (c) PC (18:1_16:0) and PC (16:0_22:6), obtained from the tissue section 53 and 52, respectively.

Additionally, this approach indicates that PC species in the blastodisc is about four times higher than in the yolk. These distributions were consistent across multiple MALDI-MSI sections (Supplementary Figure S4) as well as other major PE, PI, and PC species (not shown). Although sub-cellular level heterogeneous distribution of PE is clear from this analysis, we were not able to determine which sub-cellular organelles are responsible for this heterogeneity. It is mostly due to the fact that many sub-cellular organelles are being synthesized in a compact space, of which the distinction is beyond the spatial resolution of the current technology.

These species have also been detected in other metabolic studies on zebrafish embryos by GC-MS and LC-MS (Huang et al., 2013, van Amerongen et al., 2014) and via product ion scans using DESI-MS and nESI-MS (Pirro et al., 2016); however, detailed interpretation on the spatial distribution was limited due to their low spatial resolution of 250 µm. The PC and PE species are key markers for organogenesis (Hazel, 1984) while PI species are known to play a crucial role in intracellular signaling, RNA editing, protein phosphorylation, and gene transcription (Pirro et al., 2016), therefore their presence at the one-cell stage is not surprising.
Assignments are based on accurate mass measurements, and their identities are also confirmed by separate MS/MS measurements for lipids (Supplementary Figure S5 and Table S1). Additionally, in order to assign a degree of confidence to the assignments, molecular annotation was performed using the METASPACE (Palmer et al., 2017) annotation engine. Results are publically available at annotate.metaspace2020.eu and are shown in Table S2.

The video animations (Supplementary Videos S1-4) of the 3D distributions of PE (22:6_16:0), PI (18:0_20:5), PC (18:1_16:0), and PC (16:0_22:6) allow for rotation and visualization of the one-cell stage embryo and the yolk. These 3D reconstructions of lipid molecular species provide unprecedented volumetric chemical and spatial distribution of the lipidome in a single zebrafish embryo. Moreover, a 360° rotation visualization of PC (16:0_22:6) (Supplementary Figure S6) reveals that the cross (e.g. 360°) and longitudinal visualization (e.g. 120°) are similar, verifying that the symmetry of the embryo is not affected when viewing that data in a 3D manner.

Comparison of Normalization Strategies for Relative Quantification in Three-dimension MSI of One-cell Stage Zebrafish Embryo

Relative quantification is often achieved by normalizing analyte signals using a reference compound homogeneously present in the sample or summed ion signals from the same class of compounds (Rzagalinski and Volmer, 2016). This procedure minimizes variation of ion signals, and provides a better semi-quantitative representation. Improper normalization, however, may unintentionally distort the relative quantification (Sturtevant et al., 2017). Here, we compare four different normalization procedures to calculate mol% lipid composition from the 3D MALDI-MSI data set for one-cell stage zebrafish embryo. In our previous study on 3D MALDI-MSI analysis of Arabidopsis seeds (Sturtevant et al., 2017), we compared two different normalization methods, which can be expressed in equations (1) and (2).
\[
\hat{I}_{P_i,\text{volume}} = \frac{\sum_z \sum_{x,y} I_{P_i}(x,y,z)}{\sum_i \sum_{x,y} I_{P_i}(x,y,z)} \quad (1)
\]

\[
\hat{I}_{P_i,\text{section}} = \frac{1}{\sum_z N_z} \sum_z \left( \frac{\sum_{x,y} I_{P_i}(x,y,z)}{\sum_i \sum_{x,y} I_{P_i}(x,y,z)} \right) N_z \quad (2)
\]

Here, \(I_{P_i}(x,y,z)\) is an ion signal of lipid species \(P_i\) with fatty acyl composition of \(i\) at a given tissue section of \(z\) and 2D position of \(x\) and \(y\). Each \(\hat{I}_{P_i}\) represents a mol percentage of lipid species \(P_i\) within the same lipid class of \(P\), normalized by different normalization methods. \(N_z\) corresponds to the number of voxels for the tissue section \(z\).

Equation (1) represents a simple intensity average throughout the whole volume. In equation (1), the raw ion intensities for each lipid molecular species were summed together across all the tissue sections, and then normalized to the total ion signals from the same lipid class. This approach assumes that the signal at each voxel/section is a good representation of the molecular abundance in the whole embryo. This approach is easy to calculate but ignores any voxel-to-voxel or section-to-section analytical variation or the change of ionization efficiencies.

Equation (2) represents 2D normalization performed at each section. In equation (2), the raw ion intensities for each lipid molecular species were summed together for each tissue section, then normalized by the same lipid class, and then averaged over the cross-sectional \(z\)-dimension. In this approach, it is assumed that section-to-section variation could be significant, thus normalization needs to be performed at each section. Because each section occupies a different volume (or different number of voxels, \(N_z\)), two-dimensionally normalized mol percentage is weight-averaged across the section by the number of voxels in each section. This approach can minimize section-to-section variation (e.g., difference in matrix deposition) by normalizing at each section before weight-averaging over the entire sections, but it has a limitation because it ignores the quantitative differences between the voxels. By simply summing across a tissue...
section, the tissue or organelle-specific changes of ionization efficiencies are not taken into account. In our study on 3D analysis of Arabidopsis seeds, we could not find any difference between the two methods in the relative quantification of PC molecular species.

The third approach expressed in equation (3) is intended to minimize voxel-to-voxel analytical variation, by normalizing with the summed ion signals of the lipid class at each voxel, then averaging over the entire volume. This approach, however, does not take into account the quantitative difference between the voxels, and considers every voxel in equal weight. As a result, it may exaggerate the contribution from a voxel with low total lipid amount.

\[
\hat{I}_{P_i, \text{voxel}} = \frac{1}{\sum_x}\sum_x \sum_{x,y} \frac{I_{P_i}(x,y,z)}{\sum_i I_{P_i}(x,y,z)}
\]  (3)

The forth normalization indicated in equation (4) is designed to minimize the above limitations, by normalizing with a matrix signal at each voxel before averaging over the entire volume. This approach assumes that matrix signal is a good representation of analytical variation.

\[
\hat{I}_{P_i, m, \text{voxel}} = \frac{\sum_x \sum_{x,y} I_{P_i}(x,y,z)}{\sum_i \sum_x \sum_{x,y} I_{P_i}(x,y,z)}
\]  (4)

The four aforementioned normalization strategies were tested for relative quantification of three lipid classes (PC, PE, and PI) in the entire embryo as shown in Figure 4.5. It is known that the amount of salt or matrix can affect ionization efficiency (Luxembourg et al., 2003). The matrix application method adopted here provides a very homogeneous deposition, and there is a high potassium level in the DHB/Fe$_3$O$_4$ solution. As such, we do not expect any serious normalization artifacts, although the normalization process is expected to correct minor variations in ion signals. Only potassiated adducts are shown here, as they are the dominant species (88–95% of the total abundance of all adducts) and some protonated and sodiated adducts
are indistinguishable (i.e. [PC-36:4+H]+ and [PC-34:1+Na]+ have m/z values of 782.569 and 782.568, respectively). Regardless, the same spatial distribution was obtained for all the adduct species present in positive ion mode (+H, +Na, and +K).

**Figure 4.5** Comparison of the four normalization procedures in mol% calculation for the three lipid species of (a) PE, (b) PI, and (c) PC.

The three lipid classes show subtle but significant differences. The first and third approach are considered as two extremes, ignoring voxel-to-voxel variation of ionization efficiencies or over-emphasizing the variation of ionization efficiency at each voxel level, respectively. The last approach, normalization with a matrix peak at each voxel, seems to provide the best representation and typically gives the middle range values among the four methods.

2D MALDI-MSI data from a tissue section are often used to estimate lipid molar compositions, ignoring volumetric contribution (Horn et al., 2012). We compared the lipid composition between 3D and 2D MALDI-MSI dataset, using the matrix ion signal at each voxel normalization, to see how good 2D data can represent 3D dataset. Considering the symmetrical shape of zebrafish embryos in single cell stage, a tissue section from the central area is expected to be a good representation of the whole volume. As shown in **Supplementary Figure S7**, there
is no dramatic difference between the 3D molecular compositions of PE species compared to those from two representative tissue sections. However, there are subtle but noticeable differences in their molecular compositions in some cases: i.e., PE-38:6 is 36% in 3D volume and 40% in section 47, and PC-34:1 is 48% in 3D and 52% and 45% in sections 46 and 52, respectively. This suggests that the 2D MALDI-MSI dataset from an optimal and representative tissue section could be a good semi-quantitative representation but may have up to a 10% difference compared to the 3D representation. It is important to note that these observations are valid for symmetric tissue samples like these, and may not be valid for other complex and heterogeneous samples. The mol% of these lipids obtained from 3D MALDI-MSI dataset is relatively in good agreement with those obtained from ESI-MS analysis of total extract (Supplementary Figure S8). Minor difference between MALDI- and ESI-MS datasets are mostly attributed to the difference in electrospray ionization efficiencies between carbon chain length and unsaturation (Han and Gross, 1994, Koivusalo et al., 2001), as well as imperfection in the normalization process used in semi-quantitative analysis in MALDI-MS.

**Lipid Distribution of Zebrafish Embryos in Early Stages Development**

In an effort to better understand how the metabolites may change as the zebrafish embryo develops, a number of embryos at different stages (1-, 2-, 4-, 8-, and 16-cell stage) were evaluated using high-spatial resolution 2D MALDI-MS imaging (Figure 4.6). These images were normalized with a matrix ion signal at each voxel (the forth normalization approach mentioned above). These images revealed spatially distinct areas of different lipid composition. Phosphatidic acids (PA) and PIs are present in the blastoderm region of the embryo while PCs are present in both the yolk and blastoderm. As development occurs, ceramide (Cer) containing lipids and phosphatidylserines (PS) are localized in the cell boundaries [i.e. PS (34:1), PE-Cer(t36:0) and CerP(t34:0)], and sphingomyelins [SM(t34:0)] are present more on the outer
membrane boundary of the embryo. The latter is consistent with the fact that sphingomyelins are an important component of the outer leaflet of mammalian cell membrane (Pandit et al., 2008). This is in contrast to other phospholipids (i.e. PI, PA, PG, and PC) that are present almost homogeneously inside the cells, suggesting they are essential components of not only the plasma cell membranes, but also membranes of various sub-cellular organelles. Lipid content in the yolk decreases at a later stage as they are being consumed for cell development (Fraher et al., 2016), which is especially clear for PCs at 16 cell stage. Due to low signal and poor quality of MS/MS spectra, these compounds have been assigned solely based on accurate mass but tentative identification has been supported by literature.

Conclusions

The work described herein demonstrates, for the first time, that high-resolution MALDI-MSI can be applied for three dimensional chemical imaging of a single cell. We applied this platform to map the three-dimensional spatial distribution of phospholipid classes, phosphatidylcholine (PC), phosphatidylethanolamines (PE), and phosphatidylinositol (PI), for a newly fertilized individual zebrafish. All three phospholipid classes are present with symmetric distribution inside the blastodisc, as well as the boundary of the yolk, but each reveals different localization; PE shows heterogeneous sub-cellular localization highly abundant at the center of blastodisc. These 3D MALDI-MSI volumetric reconstructions were used to compare four normalization strategies and the normalization with the matrix ion signal at each voxel was found to provide the best representation for relative quantification. Using 2D MALDI-MSI, the distribution of phospholipids and ceramide containing lipids were observed in embryos at the 1-, 2-, 4-, 8-, and 16-cell stage revealing heterogeneous localization of different classes of lipids in the embryo. Future studies would reveal the details of embryo development with higher spatial
resolution and visualization of small metabolites, as well as multi-modal imaging with fluorescence microscopy.

Figure 4.6 MALDI-MS images of selected lipid species in early developmental stages of zebrafish embryos. Peak assignments were based on accurate masses, except those marked by asterisk which were confirmed by MS/MS.
Experimental

Chemicals

All the chemicals used in this study, 1,5-diaminonaphthalene (DAN, 97%), 2,5-
dihydroxybenzoic acid (DHB, 98%), isopropanol (LC-MS grade) and gelatin from porcine skin
(300 bloom), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron oxide nanoparticles
(NPs) were synthesized, as previously described (Yagnik et al., 2016).

Zebrafish Embryo Harvesting and Sample Preparation

All experimental protocols were approved by the Iowa State University Institutional
Animal Care and Use Committee (Log # 11-06-6252-I) and are in compliance with American
Veterinary Medical Association and the National Institutes of Health guidelines for the humane
use of laboratory animals in research. The WIK wild type strain was obtained from the Zebrafish
International Research Center (http://zebrafish.org/zirc/home/guide.php). Zebrafish were reared
in an Aquatic Habitat system (Aquatic Ecosystems, Inc., Apopka, FL) and the fish were
maintained on a 14-hr light/dark cycle at 27 °C. Newly fertilized embryos were obtained through
natural mating of adult zebrafish and were collected and maintained at 28.5 °C in fish water
(60.5 mg ocean salts/l). Embryos at the one-cell stage were transferred using a glass pipette into
a Petri dish containing fish water, and embryo development was monitored under a
stereomicroscope.

Upon reaching the desired cell stage (1-, 2-, 4-, 8-, and 16-cell stage), the zebrafish
embryos were embedded in a 10% (w/v) gelatin solution and immediately frozen in liquid
nitrogen, without dechorionation. The molds were transferred to a cryo-stat (Leica CM1850,
Leica Microsystems; Buffalo Grove, IL, USA) set to a temperature of -20 °C and the samples
were allowed to thermally equilibrate for 30 minutes. Zebrafish embryos were cryo-sectioned at
10 µm thickness. Sections were collected on Cryo-Jane tape (Leica Biosystems), and attached to
pre-chilled glass slides. The prepared slides were placed on a chilled (-80 °C) aluminum block and lyophilized under moderate vacuum (~250 mtorr) for 1-1.5 hours. The lyophilizer was then slowly equilibrated to room temperature and atmospheric pressure, and sections were stored in a desiccator until matrix application for MALDI-MS imaging. All sections were imaged within 36 hours of sectioning.

Mass Spectrometry Analysis

Binary matrix of a mixture of DHB and Fe₃O₄ NPs was applied by spraying with an oscillating capillary nebulizer and DAN was applied by sublimation, using the protocol previously reported (Korte et al., 2015b, Feenstra et al., 2016). DAN was applied to odd number serial sections and binary organic-inorganic matrix to even number serial sections. MS imaging data were collected using a MALDI-linear ion trap (LIT)-Orbitrap mass spectrometer (MALDI-LTQ-Orbitrap Discovery, Thermo Scientific; San Jose, CA, USA). The instrument was modified to use an external 355 nm frequency tripled Nd: YAG laser (UVFQ, Elforlight Ltd.; Daventry, UK). The laser energy used was 83-84% (~1 μJ/pulse) at a 60 Hz repetition rate. Laser optics is similar to previously described that allows down to 3-4 μm laser spot size with a 10X beam expander (Feenstra et al., 2017). For this work, the laser spot size of ~7 μm and the raster size of 10 μm was used with a 5X beam expander. TunePlus and Xcalibur software (Thermo Scientific) were used to define imaging parameters and to acquire data, respectively. Mass spectra were acquired with 10 laser shots per spectrum in positive and negative mode using an Orbitrap mass analyzer (resolution of 30,000 at m/z 400) for m/z scan range of 100-1000. Raw mass spectral files acquired from the Orbitrap analyzer were used to generate images using ImageQuest software (Thermo Scientific; San Jose, CA, USA) with a mass window of ±0.003 Da and without normalization. Compound identification was based on accurate mass and confirmed by MS/MS analysis on replicate sections. MS/MS imaging was performed using the ion trap analyzer for
selected ions using the same conditions as described for MS imaging. An isolation width of 2.0 Da and normalized collision energy of 35 were used. Cross-section profiles for MSI were generated by mapping the signal intensity across the zebrafish embryo using MSiReader (v. 0.09; North Carolina State University) (Robichaud et al., 2013).

**Development of 3D Models**

Three-dimensional MALDI-MSI reconstructions was accomplished by collecting MALDI-MSI data sets for consecutive, serial sections of a sample, and reconstructing 3D images of each analyte from the multiple, stacked MALDI-MS images. First, 2D profile images of each specific m/z were produced using ImageQuest and saved in '.TIFF' format; then, all the images were stacked together as a 3D model using TrakEM2 module (Saalfeld et al., 2010, Cardona et al., 2012, Saalfeld et al., 2012) of ImageJ (https://imagej.nih.gov/ij/; version 1.50e). Each 2D image was placed on top of the previous image, and adjusted for x, y position and rotation to properly orient the section relative to the previous sections. To ensure proper orientation and alignment, the 2D images were positioned using a half-transparent overlap with the previous image, and optical images obtained in parallel were used to guide the alignment. Once the 2D MALDI-MS images were stacked, aligned and transformed, Image J was used to visualize a 3D model and create 3D MALDI-MS image videos.

**Acknowledgements**

This work is partially supported by the US Department of Energy (DOE), Office of Biological and Environmental Research.
Figure S1. (a) Bright-field images of serial cryo-sections of entire fertilized zebrafish embryo (*Danio rerio*) at the one-cell stage. The order of the sections is notated at the top left hand corner of each image. (b) Bright-field images of intact zebrafish embryos with the blastodisc, cleavage and yolk labeled.
Figure S2. Overall workflow for MALDI-MSI of zebrafish embryos

3D Reconstruction

Data Analysis and Image Generation

Data Acquisition

Zebrafish breeding

Zebrafish embryo embedding and cryo-sectioning

Matrix application

Figure S3. (a) Optical image, (b) overlay MS image of PI (18:0_20:5) (red) and PE (22:6_16:0) (green), and (c) overlay of both lipids and the optical image obtained from tissue section 53.
Figure S4. Line profile of ion intensities for (a) PI (18:0_20:5) and PE (22:6_16:0) and (b) PC (18:1_16:0) and PC (16:0_22:6), obtained from the tissue section 47 and 46, respectively.

Figure S5. MS/MS spectra of (a) PE (22:6_16:0), (b) PI (18:0_20:5), (c) PC (18:1_16:0), and (d) PC (16:0_22:6) obtained from zebrafish embryo at one-cell stage. PCho: phosphocholine head group. N(CH₃)₃: choline head group. PCho-N(CH₃)₃: Phosphocholine head group with trimethyl amine loss.
Figure S6. 2D visualization of PC (16:0_22:6) sliced along the various angles of 3D MS imaging data set.
Figure S7. Comparison of the mol% of (a) PE, (b) PI, and (c) PC molecular species obtained from 3D and 2D MALDI-MSI datasets, using the matrix ion signal for the normalization at each voxel.
Figure S8. Mol% of (a) PE and (b) PI molecular species obtained by ESI-MS of total extract of one-cell stage zebrafish embryos.
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<th>ID(^a)</th>
<th>m/z</th>
<th>Species</th>
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<th>Supporting MS/MS ions(^b)</th>
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Table S1. continued

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a. Assignments were based on accurate mass search on Metlin and manual MS/MS interpretation.
b. Fragment assignments are shown in parenthesis. N(CH₃)₃: choline head group, PCho-N(CH₃)₃: phosphocholine head group with trimethyl amine loss. 16:0, 18:0, 18:1, 18:2, 18:3, 20:5, 22:6: C16:0, C18:0, C18:1, C18:2, C18:3, C20:5, C22:6 fatty acid, respectively.
Table. S2 Metaspace Annotation

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CHAPTER 5. NANOPARTICLE MICROARRAY FOR HIGH-THROUGHPUT MICROBIOME METABOLOMICS USING MATRIX-ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY


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Abstract

A high-throughput matrix-assisted laser desorption/ionization mass spectrometry (MALDI)-MS based metabolomics platform was developed using a pre-fabricated microarray of nanoparticles and organic matrices. Selected organic matrices, inorganic nanoparticle (NP) suspensions, and sputter coated metal NPs, as well as various additives, were tested for metabolomics analysis of the turkey gut microbiome. Four NPs and one organic matrix were selected as the optimal matrix set: α-cyano-4-hydroycinnamic acid, Fe3O4 and Au NPs in positive ion mode with 10 mM sodium acetate, and Cu and Ag NPs in negative ion mode with no additive. Using this set of five matrices, over two thousand unique metabolite features were reproducibly detected across intestinal samples from turkeys fed a diet amended with therapeutic or sub-therapeutic antibiotics (200 g/ton or 50 g/ton bacitracin
methylene disalicylate (BMD), respectively), or non-amended feed. Among the thousands of unique features, 56 of them were chemically identified using MALDI-MS/MS, with the help of in-parallel liquid chromatography (LC)-MS/MS analysis. Lastly, as a proof of concept application, this protocol was applied to fifty two turkey cecal samples at three different time points from the antibiotic feed trial. Statistical analysis indicated variations in the metabolome of turkeys with different ages or treatments.

**Introduction**

Mass spectrometry (MS) based metabolomics research has seen exponential growth in the past decade (Dettmer et al., 2007). Most MS-based metabolomics experiments make use of liquid chromatography-electrospray ionization (LC-ESI). While chromatographic separation provides comprehensive analysis of a complex mixture, this technique requires long data acquisition times, thus making large-scale analysis challenging due to limitations in time and cost (Wikoff et al., 2009, Dunn et al., 2013, Aretz and Meierhofer, 2016). Matrix assisted laser desorption/ionization (MALDI)-MS has been suggested as an alternative to ESI-MS for high throughput metabolomics analysis (Wang et al., 2008, Fagerer et al., 2013); however, its application has been limited due to: (i) interference from matrix peaks in the low-mass range, (ii) difficulty in compound identification because of the lack of chromatographic separation, and (iii) limited metabolite coverage depending on the choice of matrix. To overcome the first limitation, matrices with no or minimum interferences have been developed, such as nanoparticles (NPs) (Korte et al., 2016) or basic matrices in negative mode (Shroff and Svatoš, 2009, Korte and Lee, 2014), while high-resolution mass spectrometry (HRMS) can partially overcome the lack of separation.

The recent evolution of NPs as MALDI matrices has significantly contributed to small molecule analysis by MALDI-MS (Lu et al., 2017). Most NPs have UV absorption and
can be homogeneously applied, which is useful for imaging applications and to minimize spot-to-spot variation. They are especially useful for the small molecule analysis due to their no or low matrix background peaks (Chiang et al., 2011). Recently, our group has performed a large scale systematic LDI-MS screening of thirteen different NPs, including metal oxide NPs, carbon-based NPs, and metal NPs, for the analysis of two dozen small metabolite molecules (Yagnik et al., 2016). As capping agents were not used for these NPs to avoid contamination from these organic compounds, aggregation of some NPs, especially metals, caused significant loss of ion signals. To eliminate this problem, physical vapor deposition (PVD), commonly known as sputter coating, has been recently used as a means to create NPs in situ, as demonstrated previously for Au, Ag, and Pt (Kawasaki et al., 2012, Dufresne et al., 2013, Dufresne et al., 2016).

Antibiotics are essential to animal health and production. Sub-therapeutic levels of antibiotics have been commonly utilized for growth promotion, but starting in 2017, FDA guidance 209 restricted the off-label and feed-efficiency usage of antibiotics determined to be important for human health (Medicine, 2012). Although antibiotics have long been known to disrupt the gastrointestinal microbiome, the impact on the bacterial population (microbiota) and the metabolomic interplay is poorly understood (Hernandez et al., 2013, Vernocchi et al., 2016, Yan et al., 2016). The relationship between the microbiota and its metabolome is a dynamic system with tremendous individual and temporal variations, which may not be revealed by small sets of data. Large scale studies are necessary to find the correlation between microbiota membership and the gut metabolome in each individual animal. However, the lack of a high-throughput low cost metabolomics platform is a serious bottleneck toward that end.
Here we propose a high-throughput MALDI-MS based metabolomics approach using a set of nanoparticles or matrices in a microarray format. Due to differences in analyte selectivity between matrices, a carefully chosen set of matrices can effectively enhance metabolite coverage, thus allowing diverse classes of small molecule metabolites to be analyzed. While it is impossible to achieve a metabolomic profile as comprehensive as LC-MS based metabolomics, we hypothesize it is possible to grasp some important features with this approach. In the current study, this platform was optimized and then, as a proof of concept, applied to fifty two turkey gut microbiome samples treated with therapeutic or sub-therapeutic dosages of the antibiotic bacitracin methylene disalicylate (BMD).

**Materials and Methods**

**Materials**

Isopropyl alcohol (IPA), methanol (MeOH), water (H₂O), chloroform (CHCl₃), acetonitrile (ACN), piperidine (99%), triethylamine (>99%), ammonium hydroxide, formic acid (>95%), sodium acetate (>99%), and trifluoroacetic acid (TFA, 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); solvents were purchased in CHROMASOLV LC-MS or Plus grade. Organic matrices were purchased from Sigma-Aldrich (St. Louis, MO, USA): 1,5-diaminonaphthalene (DAN, 97%), 2,5-dihydroxybenzoic acid (DHB, 98%), α-cyano-4-hydroxycinnamic acid (CHCA, 99%), and 9-aminoacridine (9AA, 98%) hydrochloride hydrate. The basic form of 9AA was prepared by dissolving the hydrochloride salt in boiling water and adding excess sodium hydroxide to precipitate the free base. The product was isolated by filtration and rinsed several times with cold water, then dried under vacuum. Aluminum-doped zinc oxide (AZO; zinc oxide NPs doped with 2 wt% aluminum oxide, 99.99%, 15 nm) was purchased from US Research Nanomaterials, Inc. (Houston, TX, USA). Iron oxide NPs (Fe₃O₄, 11 nm, no organic capping) and titanium dioxide NPs (TiO₂)
were synthesized as previously described (Yagnik et al., 2016). Characterization of these NPs can also be found in the supplementary information of the work by Yagnik et al. (Yagnik et al., 2016). The sputter targets were purchased from Ted Pella, Inc (Redding, CA, USA): silver (99.99%), gold (99.99%), titanium (99.6%), and copper (99.99%). The µFocus LDI plates (5x16 circles, 600 µm) were purchased from Hudson Surface Technology (Old Tappan, NJ, USA).

**Animal Experiment and Metabolite Extraction**

All animal experiments were performed at the Animal Disease Center, USDA, Ames, IA, USA, following the IACUC protocol (ARS-2016-567). As a part of a larger animal microbiome study (manuscript submitted), two-hundred-and forty day-of-hatch Nicolas turkey poults (Valley of the Moon Hatchery, Osceola, Iowa) were obtained and allowed to co-mingle and acclimate for two weeks. At two weeks of age, birds were randomly separated into one of three rooms to begin antibiotic treatment of 50 g/ton feed or 200 g/ton feed BMD for sub-therapeutic or therapeutic treatments, respectively, or non-medicated diet. Sub-therapeutic BMD was administered continuously for 11 weeks. Therapeutic BMD was given for five weeks, followed by reduction to sub-therapeutic concentration for six weeks. Ten turkeys were sampled from each group after euthanasia, taken at 7, 35, and 78 after the start of the antibiotic therapy. Two hundred mg of cecal contents from each bird were flash frozen in liquid nitrogen for metabolomic analysis. Lyophilized cecal contents (~5 mg) were placed in a 1.5 mL centrifuge tube and then suspended in 1.0 mL of extraction solvent with internal standards (5 μL of 10 mg/mL jasmonic acid, 5 μL of 10 mg/mL 13C- ribitol, 495 μL H2O, 495 μL MeOH). Extraction proceeded with 10 min of vortexing (1,400 rpm), followed by centrifuging for 10 minutes (12,000 rpm). The hydrophilic supernatant was transferred to a
new 1.5 mL centrifuge tube. The samples were dried under vacuum and re-suspended to a final concentration of 1 mg/mL with ACN:H₂O (50:50).

**Workflow for LDI-MS Analysis**

The overall workflow for the experiments and an example of the microarrays used in the initial optimization are illustrated in Fig. 1. Organic or NP matrix solutions were prepared by dissolving or suspending in IPA at a concentration of 10 mM. Organic matrices were vortexed for ~1 min and NP matrix solutions were sonicated for ~1 hr before spotting. For the microarrays made from organic matrix solutions or NP suspensions, 2 µL of matrix solution, followed by 2 µL turkey cecal contents extraction and 2 µL additive solution (optional), were spotted on a µFocus LDI plate (Hudson Surface Technology; Old Tappan, NJ, USA). All of the solutions were spotted on a µFocus LDI plate pre-heated to 45°C to ensure uniform deposition. Spots were completely dried before the next solution was spotted. CHCA, DHB, and Fe₃O₄ were tested for positive mode, and 9AA, DAN, and AZO were tested for negative mode. TiO₂ was used for both ion modes.

For the metal NP microarrays deposited through PVD, 2 µL of cecal extract was spotted first, followed by 2 µL of additive solution (optional), and then metal NPs were sputter coated using a Cressington 108Auto (Ted Pella). The portion of the plate that was not to be sputter coated was covered using a glass slide. Optimized sputter times are 5, 10, 20, and 40 sec for Ag, Au, Ti, and Cu, respectively. Both positive and negative mode were used for all the sputter coated metals.

In order to improve the detection of unique features, three different additives were tested in each polarity. For positive ion mode, 0.1% TFA (v/v), 0.1% formic acid (v/v), and 10 mM sodium acetate were used. For negative ion mode, 1% piperidine (v/v), 1%
triethylamine (v/v), and 10 mM ammonium formate were used for this test. The additives were compared to see which offered the most unique features.

Figure 5.1 (a) The overall workflow of matrix microarray experiment. An extraction was conducted on the turkey cecal sample, followed by spotting (or sputtering) the matrix, extract and additives onto the μFocus LDI plate. High-resolution Orbitrap scans were acquired and the data was analyzed using MSiReader and Xcalibur. (b) Examples of matrix spotted and metal sputtered coated microarrays. For pre-fabricated pipet spotted microarray (plate 1 and 2, in positive and negative ion mode, respectively), 2 μL of matrix, followed by 2 μL turkey cecum extract sample, and 2 μL additive were spotted on a LDI plate. For PVD microarray (plate 3), 2 μL turkey cecum extract sample was spotted first, followed by 2 μL of additive, and then the metal was sputtered.
Mass Spectrometry Analysis

A linear ion trap-Orbitrap mass spectrometer with a MALDI ion source (MALDI LTQ-Orbitrap Discovery; Thermo Scientific, San Jose, CA, USA) was used to acquire data in imaging mode for each circle in the microarray, approximately 300-375 pixels per circle. The instrument was modified to use an external frequency-tripled, diode-pumped Nd:YAG laser operating at 355 nm and 60 Hz (UVFQ; Elforlight Ltd., Daventry, UK). Data were collected using a 150 µm raster step size and a ~30 µm laser spot size. Laser pulse energies were optimized individually for each matrix. MSiReader (Robichaud et al., 2013) and Xcalibur (ThermoFisher Scientific) were used to define imaging parameters and to acquire data, respectively. Mass spectra were acquired with 10 laser shots per spectrum using an Orbitrap mass analyzer (resolution of 30 000 at m/z 400) for an m/z scan range of 50-1000.

Estimation of Potential Metabolites Based on Accurate Mass

The total number of potentially identifiable metabolites (i.e., unique features) were estimated based solely on accurate mass. The mass spectra over the entire circle were averaged and all m/z values and their ion intensities were exported as .csv files using MSiReader. Two levels of filtering were applied to extract only those m/z values that are meaningful. In the first filtering, any m/z values at noise level with an absolute intensity below 500 (signal-to-noise ratio of 20) were removed. In the second filtering, matrix and contamination peaks (background outside the region of interest), as well as 13C isotope peaks, alkali metal adducts in positive mode and a water loss in negative mode, were removed using an in-house Python script within 5 ppm tolerance. MS images were then generated for all remaining m/z values using MSiReader, and inspected to ensure they were not also present in the background. This final mass list was compared to the compound list identified by Metabolon using an LC-MS/MS approach (see next sub-section). For the matching features
based on accurate mass within 5 ppm mass tolerance, MS/MS was performed using the ion-trap analyzer using an isolation width of 2.0 Da. The collision energies were individually optimized for each metabolite. MetFrag (Ruttkies et al., 2016) and CFM-ID (Allen et al., 2014) were used to aide metabolite identification.

**LC-MS/MS based identification of turkey gut microbiome**

Metabolomics analysis was performed by Metabolon (Morrisville, NC) for a selected set of turkey gut microbiome samples (manuscript submitted). In short, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/ultra-performance liquid chromatography (UPLC)-MS/MS methods in positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. All methods utilized a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive HRMS. Compounds were identified with the Metabolon library based on authenticated standards that contain the retention time/index (RI), mass to charge ratio ($m/z$), and chromatographic data (including MS/MS spectral data) for all molecules present in the library.

**Results and Discussions**

**Finding the Optimal Experimental Conditions for Development of Nanoparticle Microarray**

The overall workflow is illustrated in Fig. 1a. Samples were directly deposited on a pre-spotted NP or matrix microarray, which can be done in a high-throughput manner, especially using a multichannel pipettor or robotic autosampler. The microarray was prepared using a µFocus MALDI plate and matrix deposition was done on a heated plate to reduce
signal variation due to the inhomogeneity of matrix crystals. The surface of µFocus MALDI plate is hydrophobic, except for an array of small circles with hydrophilic surfaces, so that samples in aqueous solutions can be focused onto small areas. This study focused on utilizing various NPs as MALDI matrices, as they are particularly useful in high-throughput small molecule analysis due to the few or no background peaks as well as homogeneous application with minimal signal variation (Yagnik et al., 2016). Then, after the microarrays were prepared, MALDI-MS data acquisition was performed in as fast as a few minutes for a set of matrices, and hundreds of samples can be run in a single day without manual interruption using batch mode operation.

The microarrays were optimized using cecal samples from several pooled non-medicated, 93 day old turkeys. Different matrices and additives were tested to find conditions that yielded the most comprehensive coverage of metabolites of interest. Three NPs from our previous study (Fe₃O₄, AZO, TiO₂) (Yagnik et al., 2016), four traditional organic matrices (CHCA, DHB, 9AA, DAN), and four PVD metals (Ag, Au, Ti, Pt) were selected in this preliminary study. Three different additives were tested in each polarity in order to improve the ionization efficiencies of unique features. For positive ion mode, 0.1% TFA, 0.1% formic acid, and 10 mM sodium acetate were used. For negative ion mode, 1% piperidine, 1% trimethylamine, and 10 mM ammonium formate were used for this test.

Venn diagrams comparing the number of unique features detected when using different additives in negative and positive ion modes can be found in Fig. S1. Unique features are defined as having a signal-to-noise ratio of at least 20, and matrix peaks, known contaminations, ¹³C isotope peaks, and multiple adducts (see experimental section for the details) were excluded. As can be seen in Fig. S1, there was little benefit of using additives in
negative mode. However, in positive ion mode the use of 10 mM sodium acetate significantly increased the number of unique features. For example, Fe$_3$O$_4$ and CHCA show ~2 and ~1.5 times more features, respectively, when sodium acetate was used, compared to no additive, due to the efficient formation of sodium ion adducts. **Fig. 2** shows Venn diagrams summarizing the number of unique features for various matrices with 10 mM sodium acetate in positive mode and with no additive in negative mode. In total, over one thousand unique features are identified in each polarity. However, it would take too long for data acquisition if all eight matrices were used in each polarity.

Out of eight matrices in each polarity, the five most effective matrices were chosen from **Fig. 2** in terms of total coverage: Fe$_3$O$_4$, CHCA, and Au in positive ion mode, and Cu and Ag in negative ion mode. In positive mode, Ag has a slightly higher number of unique features than Au, however many of these overlap with those from Fe$_3$O$_4$ (comparison not shown). Additionally, Ag in positive mode results in silver adducts and clusters which can complicate the spectrum. In negative mode, Au has the same number of unique features as Cu, but Cu was selected as many of the Au features overlap with Ag. **Fig. 3a** compares mass spectra from the pooled control sample among the five matrices/NPs represented in a barcode-like pattern to facilitate quick and easy comparison of the distribution of features. The overlap is minimal in the metabolite coverage for each matrix/NP. Cu and Ag have the greatest coverage in the mass range of $m/z$ 100-300, due to the tendency of metabolites effectively ionized in negative mode to have lower masses, while Fe$_3$O$_4$ provides comprehensive coverage over the entire mass range of $m/z$ 100-500. Overall, these data suggest that these five matrices/NPs can be used to detect a wide range of metabolites in the gut microbiome.
Figure 5.2 Venn diagrams displaying the number of unique features observed when comparing different matrices in positive and negative ion mode. The number of unique compounds shown is after the removal of adducts.

Proof of Concept Application using Turkey Microbiome Samples

The optimal experimental conditions were then applied to cecal samples collected from 93 day-old turkeys that were either non-medicated, or treated with sub-therapeutic antibiotics, or therapeutic antibiotics. Fig. 3b compares the total number of unique features for each sample type combined from all five matrices/NPs that were detected consistently in all three biological replicates. As a particular metabolite can be detected with multiple matrices, the reported total numbers of features in each sample type have been carefully screened to remove any duplicate features. Over 1,000 unique metabolite features were detected in each sample type, and several hundred of them are unique to each, suggesting the potential of this approach for covering a diverse range of metabolites. A total of
approximately 2,100 unique features are detected combining all three sample types. The distribution of the total detected features from each sample type for each of the matrices/NPs used is shown in Fig. S2.

Figure 5.3 (a) Microarray MALDI-MS results of turkey microbiome for optimal matrices, shown in a barcode-like pattern (b) Venn diagram displaying the total number of unique features observed across in all five matrices, using the optimized experimental conditions.

Identifying all these compounds is not feasible, especially without chromatographic separation and standard analysis. Therefore we focused our analysis to a handful of compounds that were also detected with in-parallel UPLC-MS/MS based metabolomics performed by Metabolon (metabolon.com; a separate manuscript in preparation) and that were confirmed through MALDI-MS/MS. Metabolon performed extensive metabolomics profiling using four different UPLC-MS/MS runs (two in positive and two in negative mode, optimized for hydrophilic and hydrophobic compounds each) and compared these to over 3,000 entries in an in-house standard library. Selected samples from the 7, 35, and 78 days post-treatment time points for each of non-medicated, sub-therapeutic treated, and therapeutic treated were analyzed (n=6 per condition). A total of 712 unique metabolites were chemically identified in the Metabolon study, while thousands more unique features were unidentified. Among those identified in the Metabolon data, 96 of them match features
shown in Fig. 3b within 5 ppm mass tolerance. MALDI-MS/MS was performed for each of the 96 matching compounds, and 56 of them were confirmed to match with the Metabolon-identified chemical structures. The list of identified compounds is summarized in Table S1 along with fragment ions from MS/MS. Some metabolites have mass differences that are less than what can be isolated by the ion trap. In that case, they are simultaneously fragmented and the combination of exact mass and MS/MS information was utilized to identify the metabolites. Assignments were made based on the presence of 1) a unique precursor ion in the MS spectrum, and 2) at least one corresponding fragment in the MS/MS spectrum. The results of the metabolomics study performed by Metabolon is beyond the scope of the current work and will be presented in a separate publication.

**Mid-throughput Screening Comparing Antibiotic Treatments**

Previous studies using MALDI-MS based metabolomics have targeted specific metabolites and thus have been limited in scope mostly due to low metabolite coverage, resulting from the lack of chromatographic separation (Wang et al., 2008, Miura et al., 2010, Yukihira et al., 2010, Zhang et al., 2013). Here, we use MALDI MS combined with a microarray platform for metabolomics analysis to compare the gut microbiome of different antibiotic treated turkeys. As a proof of concept experiment demonstrating that a nanoparticle microarray based MALDI-MS platform can be used for a large scale high-throughput analysis, this method was applied to a total of 52 turkey gut microbiome samples from three different antibiotic treatment groups (therapeutic, sub-therapeutic, and non-medicated). After two weeks of co-mingling, young turkeys were separated into three treatment groups, and samples were collected at three time periods (7, 35, and 78 days) after the start of treatment (n = 5 or 6 for each condition). We used a microarray MALDI plate with a spot size of 600 µm, and data was collected for about five minutes per spot. The total data acquisition time
was 2.7 days in the current study (52 samples x 5 matrix x 3 analytical replicates x 5 min), but it could be shorted to a half-day for a one-minute data acquisition time using a MALDI plate with smaller focus size. The same number of experiments would take one or two weeks using LC-MS based metabolomics. Additionally, to save data acquisition time, analytical replicates are often ignored as there are multiple biological replicates; however, as discussed below, each individual animal is different and therefore shows slightly different metabolomic profiles, presumably due to their different microbiota membership.

**Figure 5.4** Three-dimensional Partial Least Squares Discriminant Analysis (PLSDA) and Box and Whisker Plots for selected metabolites for (a) Day comparison with Non-medicated Turkeys and (b) Treatment Comparison at Day 7.

The signal intensities for the compounds listed in Table S1 were extracted for each cecal sample, and then uploaded to MetaboAnalyst (http://metaboanalyst.ca) (Chong et al., 2018) for statistical analysis, after averaging over analytical replicates and normalized to internal standard: the sum of $^{13}$C-ribitol adducts in positive mode (m/z 154.080, 176.062, and 192.036 for [M+H]$^+$, [M+Na]$^+$, and [M+K]$^+$, respectively) or jasmonic acid in negative mode (m/z 209.118 for [M-H]$^-$). **Fig. 4a** shows three-dimensional Partial Least Squares Discriminant Analysis (PLSDA) for non-medicated turkeys from the three studied time-
points as well as box and whisker plots for selected differentiating metabolites. Moderate separation is clear between different groups, especially in three dimensional space. Metabolites shown in Fig. 4a (indole-3-carboxylic acid, thymine, equol) have significant increases in day 35, compared to day 7, and then stabilize to lower levels at day 78. It is well known that poultry microbiota changes in membership over time and with host age, so these differences are likely due to aging (Lu et al., 2003, Scupham, 2007). Fig. 4b shows PLSDA of the treatment comparison at the day 7 time point, which also shows moderate but clear separation between groups as well as box and whisker plots. Indole-3-carboxylic acid shows a dramatic increase for therapeutic and sub-therapeutic treatments compared to non-medicated birds, whereas 1-myristoylglycerol and pentadecanoate are decreased, indicating significant changes in gut microbiome metabolites during antibiotic usage. Fatty acids are known to be involved with antibiotics (Zheng et al., 2005, Cheung Lam et al., 2016) and are constituents of bacterial membranes (Crompton et al., 2014), particularly odd-chain fatty acids like pentadecanoate. Indole-3-carboxylic acid has been shown to play a role in intra- and inter-kingdom signaling (Bianco et al., 2006, Defez et al., 2016, Matilla et al., 2018) and regulates expression of genes involved in a variety of processes including metabolism and stress (Bianco et al., 2006). Additionally, microbial-derived indole metabolites have been shown to be important drivers of intestinal health, binding with the host aryl hydrocarbon receptor, modulating immune responses along the mucosa (Zelante et al., 2013). Data like these may help identify bacterial functions that can be targeted to improve animal health. In all cases, the trend of these metabolites is in good agreement with the data provided by Metabolon (not shown).
Statistical analysis shows relatively moderate separation, which is attributed to high variation among the individual turkeys. **Fig. 5** shows an example demonstrating that biological variation is much greater than analytical variation for these metabolites. Selected metabolites from four day 7 non-mediated turkeys which were raised in the same room are compared to show the biological and analytical variations. The standard deviations for each metabolite (i.e. the analytical variation) are very narrow, whereas the differences between the mean values for each animal (i.e. the biological variation) are quite large, especially for certain metabolites. This is typical in animal studies with outbred animals. We hypothesize this is likely due to the variability in the microbiota composition and host genetics, between turkeys. 16S rRNA gene sequence analysis has identified microbiota membership and compositional differences between treatment groups, as well as inter-group variability (data not shown). Correlations between members of the microbiota and metabolites within the metabolome is currently under investigation.
Conclusions

The use of microarray technology is a common analytical platform for other types of analyses, most notably DNA or RNA (Taub et al., 1983, Pollack et al., 1999, Schäferling, 2011). In the field of mass spectrometry, surface-enhanced laser desorption (SELDI), a variation of MALDI-MS, was developed in the early 90s for protein analyses (Hutchens and Yip, 1993). In this technique, biological samples such as blood or urine are spotted onto special microarray surfaces which bind certain proteins whereas any others are washed away. Various surfaces have been used for this purpose, mostly based on chromatographic interactions, although chemically modified reactive surfaces can also be used. This technology has been commercialized by Biorad (http://www.bio-rad.com). SELDI-MS, however, works only for large proteins and there has been no microarray MALDI-MS platform for small metabolite analysis.

In this study, we developed and optimized a pre-fabricated microarray for high-throughput metabolomics analysis. It was determined that the addition of sodium acetate yielded more features in positive mode data than with no additive. Out of an initial eight matrices/NPs in each polarity, a total of five matrices/NPs were selected for inclusion in the final NP microarray platform: Fe$_3$O$_4$, CHCA, and Au in positive ion mode, and Ag and Cu in negative ion mode. We were able to successfully apply our microarray platform to compare a large sample set of non-medicated, sub-therapeutic treated, or therapeutic treated turkey cecal samples 7, 35, and 78 days after the start of antibiotic treatment with our optimized conditions. We detected thousands of unique metabolite features and monitored their changes over time or with antibiotic treatment.

Identification of thousands of unique features is the current bottleneck in this approach. However, we demonstrated it could be partially mitigated through in-parallel LC-
MS/MS metabolomics analysis and MALDI-MS/MS of selected metabolites. While the traditional chromatography-based method gives more comprehensive coverage, the analysis of all samples in a large-scale study with this method would be costly and require extensive data acquisition time. Our high-throughput microarray technology, however, can be used to quickly acquire the large data sets needed for the study of a dynamic system, such as the turkey gut microbiome, and, as shown here, can be used in combination with a chromatographic-based study to enable high-throughput metabolomics experiments.

Acknowledgements

This work was funded by the United States Department of Agriculture- National Institute of Food and Agriculture (USDA-NIFA).
Supplemental Figures

**Fig. S1:** Venn diagrams displaying the number of unique features using different additives in negative and positive ion mode. The number of unique compounds shown is after the removal of adducts.
Fig. S2: Venn diagrams displaying the number of unique features observed for each matrix when comparing non-medicated, sub-therapeutic, and therapeutic treatment turkey microbiome extract, using the optimized conditions. The number of unique compounds shown is after the removal of adducts.
Table S1: Metabolites identified through MALDI-MS/MS analysis

<table>
<thead>
<tr>
<th>m/z</th>
<th>Metabolites</th>
<th>Observed Fragments</th>
<th>Matrix (Polarity)</th>
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<td>choline</td>
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<td>109.030</td>
<td>resorcinol</td>
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<td>methysuccinate/ethylmalonate</td>
<td>95, 85, 69</td>
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<td>tyrosol</td>
<td>(101),91, (77)</td>
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<td>p-hydroxybenzaldehyde</td>
<td>93, 79, 77, 63</td>
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<td>thymine</td>
<td>107, 97</td>
<td>Ag (-)</td>
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<td>128.035</td>
<td>5-oxoproline</td>
<td>110, 84</td>
<td>Ag (-)</td>
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<td>133.030</td>
<td>3-hydroxyphenylacetate</td>
<td>105, 89</td>
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<td>hydroxymybenzoate</td>
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<td>phenylpyruvate-H₂O</td>
<td>(117), 101</td>
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<td>quinolinin-2-one</td>
<td>118, 103</td>
<td>Au (+)</td>
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<td>phenyllactate (PLA)</td>
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<td>4-hydroxymandelate</td>
<td>121, 105</td>
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<td>3-phenylproionate (hydrocinnamate)</td>
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<td>valylglycine</td>
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<td>indole-3-carboxylic acid</td>
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<td>resveratrol-H₂O</td>
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<td>pinosylvin</td>
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<td>dodecanedioate-H₂O</td>
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<td>equol-H₂O</td>
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<td>gamma-glutamylglycine</td>
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<td>resveratrol</td>
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<td>dodecanedioate</td>
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<td>equol</td>
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Table S1 continued

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<td>palmitoleate (16:1)</td>
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<td>hydroxypalmitate</td>
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<td>alpha-tocopherol</td>
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Note: Parentheses in 'Observed fragments' indicate fragments observed only in one matrix.
References


CHAPTER 6.  TOWARDS MASS SPECTROMETRY IMAGING IN THE METABOLOMICS SCALE: INCREASING METABOLIC COVERAGE THROUGH MULTIPLE ON-TISSUE CHEMICAL MODIFICATIONS

A paper to be submitted to *Frontiers in Plant Science*

Maria Emilia Dueñas and Young Jin Lee

Department of Chemistry, Iowa State University, Ames, IA, USA

**Abstract**

Exploring the metabolic differences directly on cells and tissues is essential for the comprehensive understanding of how multicellular organisms function. Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) is an attractive technique towards this goal due to its untargeted analysis capability, high-sensitivity, versatility, and small sampling size. Unfortunately, the lack of chromatographic separation, limited number of molecules, and low ionization yields of metabolites has hindered the ability to sufficiently understand overall metabolomics changes in a biological system. Several on-tissue chemical derivatization approaches have been reported to increase MALDI-MSI signals, mostly targeted for specific compounds of interest. Herein, we adopted this strategy for consecutive tissue sections, to selectively enhance the metabolite signals for a specific functional group at a time. Three well-known on-tissue derivatization methods were used as a proof of concept experiment: coniferyl aldehyde for primary amines, Girard’s reagent T for carbonyl groups, and 2-picolylamine for carboxylic acids. This untargeted approach was applied to leaf and root cross-sections of two different maize genotypes (B73 and Mo17), and enabled the identification of over five hundred new unique metabolite features compared to without
Statistical analysis indicated variation between the different metabolites in the tissue sections, while MS images revealed differences in localization between the genotypes of maize. Combined, this untargeted approach facilitated the visualization of various classes of compounds, demonstrating the potential for untargeted MSI in the metabolomics scale.

**Introduction**

Modern metabolomics technology, led by innovations in mass spectrometry, has allowed for systems biological understanding at the gene, RNA, protein, or metabolite level (Zhang et al., 2012). Most of these studies, however, are performed ignoring the variations between different cell types or even different tissue types. As multicellular organisms, plants and animals achieve their complex living activities through the highly organized metabolic interplay of a three-dimensional array of individual cells and tissues. Thus, understanding the metabolic differences of individual cells directly on tissue is essential for the comprehensive understanding of how multicellular organisms function.

Mass spectrometry imaging (MSI), especially matrix-assisted laser desorption/ionization (MALDI)-MSI, is an attractive technique towards this goal due to its untargeted analysis capability, high-sensitivity, versatility, and small sampling size. Recently, MS images with pixel sizes from 1-15 µm have been obtained (Zavalin et al., 2013, Korte et al., 2015) which has allowed for finely detailed studies at the cellular and sub-cellular levels. Atmospheric pressure scanning microprobe MALDI (AP-SMALDI) has been used to study the brain of D. melanogaster, which enabled the specific localization of neuropeptides at 6-15 µm (Khalil et al., 2017). Transmission geometry laser ablation electrospray ionization (tg-LAESI) MS was used for an in situ analysis of small adherent cell populations, using a spot size of 10-20 µm (Jacobson et al., 2015). Furthermore, Yin et al. demonstrated the power of nanospray desorption electrospray ionization (nano-DESI) MSI for robust imaging of
pancreatic islets with high spatial resolution of ~11 µm (Yin et al., 2018). Visualizing detailed metabolite information at this scale can offer unprecedented details in terms of metabolite composition and localization which can be crucial for elucidating their biological roles.

Our group has established a MALDI-MSI technical platform for 5-10 µm high-spatial resolution and have applied this platform to visualize the distribution of a number of different metabolites in maize leaves (Korte et al., 2015, Dueñas et al., 2017), seeds (Dueñas et al., 2016, Feenstra et al., 2017a), and roots (Feenstra et al., 2017b). Previously, the work of Korte et al. (Korte et al., 2015) revealed that molecular distribution of metabolites and lipids may be heterogeneous even among cells of the same tissue type. More recently, this platform was applied to explore the quantitative fatty acyl distributions of thylakoid membrane lipids along the developmental gradient of maize leaves of four inbred lines of maize (Dueñas et al., 2017). This study demonstrated that certain thylakoid membrane lipids show genotype-specific differences in cellular distribution.

Although MALDI-MSI provides high-spatial resolution information that is unprecedented in traditional metabolomics, the lack of chromatographic separation, matrix-dependent analyte selectively, and the limited number of molecules available in a small sampling size, has hindered the ability to sufficiently understand overall metabolomics changes in a biological system. This is especially significant when the sampling is narrowed to micron-size for cellular and sub-cellular imaging. Despite the fact that cellular or sub-cellular resolution MALDI-MSI has been demonstrated, there are currently multiple obstacles to the full realization of using this technique to visualize metabolites at the metabolomics scale. Recently, Feenstra et al. proposed a MSI methodology that combined
multiplex MSI data acquisition with multiple matrices on consecutive tissue sections (Feenstra et al., 2015). This proof of concept experiment used the matrix-dependent selectivity of analytes to increase the diversity of chemical compounds that could be identified and visualized. Even though this approach could eventually lead to untargeted metabolomics analysis, the number of primary and secondary metabolites that could be visualized was limited due to low abundance, variability in ionization efficiency, and/or sensitivity of some of these metabolites.

On-tissue chemical modifications have been suggested as an alternative approach to improve the ionization efficiency of targeted compounds in MSI. According to a study by Manier et al., 4-hydroxy-3-methoxycinnamaldehyde (CA) was found to be the optimal reagent for the derivatization of amino acids and neurotransmitters (Manier et al., 2014). Girard’s reagent T (GT), a quaternary ammonium acetylhydrazine chloride, has been used to chemical modify carbonyl groups on-tissue, specifically corticosteroids in rat adrenal and mouse sections (Shimma et al.), testosterone in mouse testis (Cobice et al., 2016), and triamcinolone acetonide in human cartilage (Barré et al., 2016). As another on-tissue chemical modification, 2-picolylamine (2-PA) was used to visualize endogenous fatty acids in rat brain tissues (Wu et al., 2016). All of these reactions proceed quickly at room temperature without the need of additional of specific buffers.

In this work, a MSI methodology is used to selectively enhance the metabolite signals for a functional group at a time. This is accomplished by performing on-tissue derivatization using three known chemical reactions: CA for primary amines (Manier et al., 2014), GT for carbonyl groups (Barré et al., 2016), and 2-PA for carboxylic acids (Wu et al., 2016). These reactions, previously used for targeted analysis, are used in combination enabling the
visualization of different classes of compounds in an untargeted manner. In a proof of concept application, this multiple on-tissue derivatization strategy is applied to explore the differences in metabolite coverage in roots and leaves of two different maize genotypes (B73 and Mo17), where single cell resolution imaging capabilities for lipids has been previously demonstrated (Dueñas et al., 2017, Feenstra et al., 2017b). Previously however, the number of other primary and secondary metabolites that could be visualized was limited without derivatization, and only highly abundant compounds were visualized.

**Experimental**

**Materials**

Methanol, water, acetonitrile were purchased from Millipore-Sigma (St. Louis, MO, USA) in CHROMASOLV LC-MS or Plus grade. Girard’s reagent T (GT), 4-hydroxy-3-methoxycinnamaldehyde (CA), 2-picolylamine (2-PA), 2,2-dipyridyl disulfate (DPDS), triphenylphosphine (TPP), 1,5-diaminonaphthalen (DAN), 2,5-dihydroxybenzoic acid (DHB), and potassium acetate were purchased from Millipore-Sigma (St. Louis, MO, USA). Gelatin from porcine skin (300 bloom) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Gold sputter targets were purchased from Ted Pella, Inc. (Redding, CA, USA). B73 and Mo17 inbred maize seeds were obtained from Dr. Marna Yandeau-Nelson at Iowa State University.

**Maize Tissue Growth and Harvest**

Procedures for maize root growth is described in detail elsewhere, and briefly described below (Feenstra et al., 2017b). Briefly, a row of seeds, with the embryo facing down, were staggered along the top edge of a moist brown paper towel. The paper towel was rolled tight enough to hold the seeds in place and the bottom of the roll was placed into a 1 L beaker half filled with water. The beaker was placed in the dark and the seeds were allowed
to grow for 10 days. The beaker was monitored periodically to ensure enough water remained inside in order to submerge the lower portion of the paper towel roll. The roots were harvested, 10 days after planting, when the lengths of the primary root was 10-14 cm, as measured from the tip of the root. The area of interest was embedded in a 10% (w/v) gelatin solution in a cryo-mold and flash-frozen in liquid nitrogen until the gelatin was ~80% solidified.

For maize leaf imaging, maize seeds were planted in soil and grown in a climate-controlled greenhouse at 30% humidity under a diurnal cycle of 16 hour of light and 8 hours of dark at 27°C and 24°C, respectively. Plant seedlings were harvested 11 days after planting. The sections of leaves were collected at the midpoint of leaf 3. The fresh maize leaf sections were embedded in gelatin before cryo-sectioning.

**Sample Preparation**

The molds were transferred to a cryostat (CM 1850, Leica Microsystems; Buffalo Grove, IL, USA) pre-chilled to -20°C, and allowed to thermally equilibrate for 30 minutes. Tissue samples were cryo-sectioned at 10 µm thickness, collected with Cryo-Jane tape (Leica Biosystems), and attached to a pre-chilled glass slide. The prepared slides were placed onto a chilled aluminum block and were vacuum dried while gradually warming to room temperature. After acquiring optical microscope images, the dried sample tissue were subject to derivatization using a TM-Sprayer (HTX Technologies, Chapel Hill, NC, USA) or by electrospray deposition (laboratory-constructed system). The TM-Sprayer conditions for the derivatization agent solutions were the following: 30°C spray nozzle temperature, 30 µL/min flow rate, 8 criss-cross and off-set passes, 1200 mm/min spray nozzle velocity. The optimal derivatization reagent concentrations were the following: 20 mg/mL CA (in methanol), 10 mg/mL GT (in methanol with 2% TFA), and 6:30:30 mM 2-PA:TPP:DPDS (in acetonitrile).
For the electrospray deposition conditions, 5 kV was applied to the spray nozzle with the tissue-coated slide held at ground (Wu et al., 2016). A 2 mL/h flow rate and the emitter-to-tissue distance was approximately 3 cm.

A 6.5 mM potassium acetate solution was TM-Sprayed prior matrix deposition using the same method as the derivatized agent solutions. Then, the matrix was deposited by sputter coating (108 Auto Sputter Coater, Ted Pella INC, Redding, CA, USA) gold at 40 mA for 20 seconds, or by TM-Spraying 40 mg/mL DHB (70% Methanol), or 20 mg/mL DAN (in acetonitrile). The TM-Sprayer conditions for the organic matrices were the following: 75°C spray nozzle temperature, 100 µL/min flow rate, 8 criss-cross and off-set passes, and 1200 mm/min spray nozzle velocity. The overall workflow is summarized in Figure 1.

**Figure 6.1** Overall workflow for on-tissue derivatization using MALDI-MSI

**Mass Spectrometry Imaging Analysis**

Mass spectrometry imaging data were collected using a MALDI-linear ion trap (LIT)-Orbitrap mass spectrometer (MALDI-LTQ-Orbitrap Discovery; Thermo Finnigan, San Jose, CA, USA). The instrument was modified to incorporate an external 355 nm frequency tripled Nd: YAG laser (UVFQ; Elforlight, Daventry, UK). The laser optics are similar to those
previously described by Feenstra et al. (Feenstra et al., 2017b) using a commercial 5x beam expander to reduce the laser spot size to <10 µm. Tuneplus and XCalibur (Thermo Fisher Scientific) were used to define imaging parameters and to acquire data, respectively. Maize leaves and roots were acquired using 10 µm raster step size. Mass spectra were acquired with 10 laser shots per spectrum in both negative and positive ion mode using an Orbitrap mass analyzer (resolution of 30000 at m/z 400) for an m/z scan range of 100-1000.

MS images were generated using ImageQuest (ThermoFisher Scientific) with a mass window of ±0.003 Da. MS/MS analysis was performed on an adjacent tissue section prepared identically to the one used for MS imaging. The ion-trap analyzer was used for MS/MS for selected ions using the same conditions are described for MS imaging. The ions shown in this work were analyzed with a mass window of 1.0 Da and normalized collision energy of 35 were used.

Peak assignments were based on accurate mass measurement, tandem MS, and comparison with online databases (METLIN, https://metlin.scripps.edu, and Maize Genetic and Genomics Database, https://cornycb73-v4.maizegdb.org/). Signal intensities for derivatized compounds were extracted from each maize cross-section sample (3 replicates of each genotype/tissue type, 36 total samples) and then uploaded to MetaboAnalyst (http://metaboanalyst.ca) (Chong et al., 2018) for statistical analysis.

Results and Discussion

Finding the Optimal Experimental Conditions for Derivatization Reactions

Reactions to chemically modify three functional groups are shown in Scheme 1. This derivatization strategy was applied to maize tissue cross-sections, for which we have previously demonstrated cellular heterogeneity in single cell resolution but only for abundant metabolites such as lipids, phenolics, benzoxazinone derivatives, sugars, phosphate sugars,
flavonoids, and flavonoid glycosides (Korte et al., 2015, Dueñas et al., 2017). The combination of these three functional groups is expected to cover a majority of metabolite class. For example, a commercially available large metabolite library (MSMLs, Mass Spectrometry Metabolite Library of Standards; IROS Technology, Bolton, MA) contains 634 common metabolic compounds: 211 of them contain primary amines, 262 contain carbonyl groups, and 292 contain carboxylic acids. Out of the 88 compounds that do not have any of these three chemical functionalities, most of them have multiple alcohol groups (i.e. sugars) which can be easily ionized without the need of derivatization.

Scheme 1: a. coniferyl aldehyde (CA) (Manier et al., 2014) and a primary amine, b. 2-picolylamine (P) (Wu et al., 2016) and a carboxylic acid, and c. Girard’s reagent T (GT) (Barré et al., 2016) and a carbonyl group. Derivatization reagents are shown in red.

The experimental conditions such as concentration of derivatization reagent, TM-sprayer flow rate, and number of passes in the TM-sprayer, were optimized based on the number of new unique features observed on the derivatized tissue compared to the underivatized tissue section. Two levels of filtering were applied to extract only those peaks that in fact were unique. In the first filtering, any m/z values at noise level with an absolute
intensity below 500 (signal-to-noise ratio of 20) were removed. In the second filtering, contamination peaks (background outside the tissue area, derivatization related peaks, etc.), as well as repeated adducts were removed, using a 5 ppm tolerance. MS images were then generated for all remaining m/z values using MSiReader (Robichaud et al., 2013), and inspected to ensure they were not present in the underivatized sample and background.

Maize root cross-sections were used to optimize matrix selection and TM-Sprayer conditions for derivatization reactions. Based on our previous experience, DHB and sputter coated gold were selected as potential matrices to test the coverage of metabolite compounds of interest. Sputtered Au has been used to analyze fatty acids and triacylglycerol species on tissues (Tang et al., 2011, Dufresne et al., 2016), and a recent systematic study reveals that Au was effective in providing overall signal intensity across a broad range of metabolites in positive ion mode (Hansen et al., manuscript accepted to JASMS). DHB is well known to have good affinity for a relatively wide variety of compounds including lipids, oligosaccharides, organic acids, sugars, flavonoids, and their conjugates (Gemperline et al., 2014). Moreover, DHB forms small crystal sizes which is ideal for high-spatial resolution MSI (Hankin et al., 2007, Gemperline et al., 2014).

Dozens of new peaks were detected with the on-tissue derivatization reactions. With DHB as a matrix, 25, 85, and 70 unique metabolite features were detected using CA, GT, and 2-PA, respectively. Using gold, 151, 53, and 108 unique features were detected, respectively. Although hundreds of features were revealed with CA and 2-PA as derivatization agents, the features observed using GT with the TM-Sprayer conditions were limited (only 53). Electrospray deposition is known to enhance extraction and derivatization efficiency for certain classes of compounds, while maintaining native spatial resolution of the metabolites.
In particular, microdroplet reaction acceleration is reported in the detection of cortisone using reactive DESI with GT (Badu-Tawiah et al., 2012). Unlike the other two reactions, GT is an acid-catalyzed reaction (Scheme 1C), and the reaction acceleration is attributed to the low pH of the ESI droplets. In electrospray deposition, microdroplets are emitted from a solution placed in contact with one electrode and directed towards the tissue sample, which is in contact with the second electrode. These electrodes have a high potential differences between the two. Electrospray deposition forms a plume of charged microdroplets containing the desired reagent ion and allows solvent to evaporate from the droplets during the deposition (Badu-Tawiah et al., 2012). When using electrospray deposition, the number of unique features observed for the GT derivatization reaction dramatically increased to 365, fourfold than neutral droplets using TM Sprayer, whereas there was no improvement for CA and 2-PA.

Overall, a total of 624 unique metabolite features were detected. Gold was selected as the optimal matrix when using CA and 2-PA as derivatization agents, while DHB was selected as the optimal matrix for GT. Subsequently, various concentrations were tested for each of the derivatization agents and the optimal derivatization concentrations that provided the most comprehensive coverage of metabolite compounds of interest and the highest number of unique features were the following: 20 mg/mL CA (in methanol), 10 mg/mL GT (in methanol with 2% TFA), and 6:30:30 mM 2-PA:TPP:DPDS (in acetonitrile). Adding salt to tissue sections is known to increase the ionization and desorption efficiency of certain compounds (Dufresne et al., 2016), therefore, a 6.5 mM potassium acetate solution was sprayed prior to matrix deposition. The optimal TM-Sprayer conditions were the following: 30°C, 0.03 mL/min, using 8 passes.
Statistical Analysis of Different Genotypes and Tissue Types

As a proof of concept application, this multiple on-tissue derivatization strategy was applied to cross-sections of roots and leaves of two contrasting maize genotypes (inbreds B73 and Mo17). Inbred B73 is derived from the Stiff Stalk Synthetic population generated at Iowa State University, and Mo17 was selected from Lancaster Sure Crop material (Troyer, 2004). These inbreds differ significantly in their genomic structure and this genetic diversity has been translated into metabolic, physiological, and phenotypic differences (Munamava et al., 2004). This approach was used to explore the metabolic differences arising from the genetic and tissue-type differences between these two inbred.

The first step in this analysis was to investigate the differences in metabolite abundance between the tissue-types and inbreds. Partial least squares discriminant analysis (PLSDA), was used to improve the understanding of the chemical information extracted from the on-tissue chemical modifications by simplifying the complex data. Using a large set of data, PLSDA finds features (i.e. m/z values) that increase the variance between different groups and decrease the variance between similar groups. Each group (i.e., B73 root, B73 leaf, Mo17 root, and Mo17 leaf) is plotted as a function of each component based on their similarity; groups closer together have similar features in common while groups far apart are very different. PLSDA, as well as principle component analysis (PCA), are commonly used when analyzing gas chromatography (GC) and liquid chromatography (LC)-MS data (Bajoub et al., 2016). Even though this analysis disregards any spatial information associated with the sample, similar to any GC and LC-MS data, it dramatically simplifies the data and allows us to extract meaningful m/z values that can later be explored with MSI.

For statistical analysis, the raw ion intensities for each tentatively assigned derivatized metabolite were extracted for each tissue type using MSReader (Robichaud et
The intensities were then summed together across all the tissue area, and then averaged over the number of pixels of each tissue. A total of 36 (three biological replicates per genotype, per tissue type, and per derivatization reaction) tissues were collected under identical instrumental settings and environmental conditions. The web-based metabolome process software, MetaboAnalyst 3.0 was utilized for multivariate statistical analysis to explore differences between the roots and leaves and the maize genotypes B73 and Mo17. 

**Figure 2** shows two- and three-dimensional PLSDA. For leaf cross-sections, moderate separation is clear between the different genotypes, B73 and Mo17 (**Figure 2a**), while a more distinct separation is observed with the root cross-sections (**Figure 2b**). The three-dimensional PLSDA for both tissue types and genotypes are shown in **Figure 2c**. Box and whisker plots (**Figure 3**) were constructed by plotting the relative intensities within each sample group. Peak assignments were based on accurate mass measurement, tandem MS, and comparison with online databases (METLIN, [https://metlin.scripps.edu](https://metlin.scripps.edu), and Maize Genetic and Genomics Database, [https://cornyc-b73-v4.maizegdb.org/](https://cornyc-b73-v4.maizegdb.org/)). These plots show clear separation between tissue types but minimal separation between genotypes.

Some metabolites shown in **Figure 3** have a significant higher abundance in the root tissue section compared to leaves, regardless of the genotype [amino acids (alanine, serine, glutamine, etc), pyruvic acid, 1-hexanal, 1-heptanal, jasmonic acid, etc.]. For the case of amino acids (**Figure 3b**), this difference is likely due to the fact that these metabolites are stored in the root and then transported to other parts of the plant (Okumoto and Pilot, 2011). The transportation of nutrients between organs is of fundamental importance in multi-cellular organisms, like maize, for the proper supply of nutrients and removal of unwanted products. Amino acids are considered the main nitrogen carriers. They are localized in specialized cells
and tissues and then transported through vascular systems when needed. For example, glutamine (primary nitrogen assimilation) is used to synthesize other amino acids and nitrogen-rich compounds by transamination, a process that occurs in the root tissue (Okumoto and Pilot, 2011). Once synthesized, amino acids are delivered to the leaves, and other sink organs such as flowers and seed.

**Figure 6.2** Partial Least Squares Discriminant Analysis (PLSDA) for derivatized metabolites using CA, 2-PA and GT for genotype comparison with a. leaves and b. roots; c. three-dimensional PLSDA comparing the different genotypes (B73 and Mo17) and tissue sections (roots and leaves). Red- B73 leaf cross-section, Green- B73 root, Blue- Mo17 leaf, and Cyan- Mo17 root.

Jasmonic acid (JA), a compound that regulates defense and development in plants, and OPC-8:0, a metabolite in the linoleic acid cascade leading to JA (Ainai *et al.*, 2003) also shows a higher increase in roots, regardless of genotypes. These metabolites are oxylipin derived from either enzymatic or autoxidation of free or membrane-esterified fatty acids (Borrego and Kolomiets, 2016). This increase in root versus leaves is in great agreement with
previously reported from Gao et al. (Gao et al., 2007) where they reported a ~0.1 nmol/g FW JA content in B73 leaves compared to ~0.3 nmol/g in roots (Figure 3a).

Conversely, certain carboxylic acid and ketone containing compounds (palmitic acid, oleic acid, stearic acid, dotriacontanal, etc.) show an increase distribution in leaves compared to roots (Figure 3a and c). For example, dotriacontanal is a cuticular wax which not only provides the first barrier protection against UV-radiation and fungi, but also plays a key role in preventing non-stomatal water loss (Hansjakob et al., 2011). Cuticular waxes are the first contact between an airborne pathogen and its post so it is not surprising that this has a higher accumulation in leaf tissue.
High-spatial Resolution Mass Spectrometry Images

Using the information extracted from the PLSDA, on-tissue derivatization high-spatial resolution MSI was used to explore the localization differences of metabolites in maize roots and leaves of inbreds, B73 and Mo17. Maize roots have a unique architecture which allows for an efficient uptake of water and nutrients and provides anchorage (Hochholdinger, 2009). Bright-field microscope images and anatomical assignments are shown in Figure S1 for the cross-section of a B73 root. Maize roots exhibit a central vascular cylinder composed of the pith, xylem vessels (the largest vascular elements that are responsible for the transport of nutrients and water), and the pericycle (the outermost cell layer of the inner cylinder). The ground tissue is made up of a single endodermis layer, multiple layers of cortex tissue and single epidermis cell layer. Maize leaves exhibit the characteristic C4 Kranz anatomy (Haberlandt, 1882, Brown, 1975) in which concentric rings of bundle sheath cells encircle closely spaced veins (vascular bundles) and are surrounded by mesophyll cells (Figure S1).

This multiple on-tissue chemical modification strategy enabled the identification of over five hundred new unique metabolite features compared to without modification. We were able to detect various classes of compounds, which can eventually allow MSI in the metabolomics scale. As shown in Figure S2, 2-PA derivatized free fatty acids were easily detected in positive ion mode data without background contamination, which is a common issue in negative ion mode data. Moreover, using the features in the statistical analysis, twelve different CA derivatized amino acids were compared between genotypes B73 and Mo17 (Figure 4). These metabolites are present in different regions of the maize root and are more abundant in inbred Mo17.
Figure 6.4 Mass spectrometry images (left) and a bar graph (right) comparing the intensity of twelve amino acids in inbreds B73 and Mo17.

Additional derivatized compounds using CA, GT, and 2-PA are shown in Figure 5. All these compounds have been tentatively assigned based on accurate mass measurement with online databases (METLIN, https://metlin.scripps.edu, and Maize Genetic and Genomics Database, https://cornycb73-v4.maizegdb.org/), and are known to be present in B73. As observed in Figure 5, these derivatized metabolites are present on different regions of the tissue. For example, aminobutyric acid and phospho-ethanolamine (Figure 5a) are localized throughout the entire tissue with exception of the xylem. On the contrary, dotriacontanal (Figure 5b) is localized in the epidermis layer, which is not surprising since this metabolite is a cuticular wax that provides the first barrier protection in leaves (Hansjakob et al., 2011). Tandem mass spectrometry (MS/MS) was conducted on selected metabolites and a representative spectrum for each derivatization can be observed in Figure S3 and S4. Most of the dominant ions observed in the spectra are losses from the derivatization agents.
Figure 6.5 Selected derivatized compounds using a. CA, b. GT, and c. 2-PA as derivatization agents. DHICA: dihydroxy indole carboxylic acid; SHCHC: hydroxyl succinylcyclohexadiene carboxylate
Conclusion

Despite the growing popularity of MS-based metabolomics, key challenges remain. Previous studies using MS imaging based metabolomics have targeted specific metabolites, making the analysis limited in scope mostly due to low metabolite coverage (Wang et al., 2008, Zhang et al., 2012). LAESI (Etalo et al., 2015, Kulkarni et al., 2018) and time of flight secondary ion mass spectrometry (TOF-SIMS) (Mas et al., 2008) have shown promise as tools to profile and compare metabolites in different species. In this work, we adopted several on-tissue chemical derivatization reactions in order to facilitate the visualization of metabolites in an untargeted manner. Incorporating on-tissue chemical derivatization as an untargeted approach enabled the identification of over five hundred new metabolite features. Moreover, statistical analysis revealed the variation between metabolites in different tissue sections and between the different genotypes.

This proof of concept experiment suggests that MALDI-MSI in the metabolomics scale is possible. A combination of derivatizations can be used on-tissue to overcome the inherent limitations of samples to allow for more complete metabolite visualization and detection in a single analysis. The current significant challenge are the lack of adequate MS and MS/MS databases and minimizing any side reactions and/or unwanted ion suppression from the derivatization agents. Improvements to databases and sample preparation would further the metabolic coverage of this method. Regardless of these limitations, using on-tissue chemical modifications not only improves the existing MALDI-MS technologies but can be used to advance towards the single-cell metabolomics scale.
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Supplemental Figures

![Figure S1](image)

**Figure S1:** Cross-section of a B73 maize root (left) and leaf (right) with anatomical assignments shown in white. Scale bar are 200 µm and 100 µm, for root and leaf, respectively.
Figure S2: Cross section of maize root a. without derivatization using DAN in negative ion mode [M-H]^−, b. without derivatization using Au in positive ion mode [M+K]^+ and c. with P derivatization using Au in positive ion mode [M+Der+K]^+.

Figure S3: Representative MS/MS spectra for a derivatized compound using a. CA (alanine, m/z 288.20), b. GT (aldehydo-D-galactose, m/z 294.166), and c. P (L-glutamyl 5-phosphate, m/z 318.083). Inset in a. corresponds to FT MS/MS of m/z 161.060, which corresponds to C_{10}H_{10}O_{3}. 
Figure S4: a. MS/MS spectra of selected derivatized GT compounds. The dominant ions observed are [M-59.07]+ and [M-87.07]+ which correspond to [M-N(CH$_3$)$_3$]+ and [M-CO-N(CH$_3$)$_3$]+ losses from GT, respectively. b. MS/MS spectra comparing the fragments from m/z 158.129 in the tissue section (left) and using a pyruvic acid standard (right).
References


CHAPTER 7. SUMMARY AND OUTLOOK

Summary

The work outlined in this dissertation has sought to expand the utility of high-spatial resolution MALDI-MSI for the mapping of small metabolites and lipids at the cellular and sub-cellular level. A matrix recrystallization method demonstrated to improve ion signal on plant tissues, enhancing the image quality of lipid species with no apparent changes in their localization. Using high-spatial resolution MALDI-MSI, the heterogeneous cellular distribution of two classes of thylakoid membrane lipids in maize leaves were investigated. This study demonstrated that MALDI-MSI analysis can be directly applied to multicellular plant tissues to uncover cell-specific metabolic biology. 3D-MALDI-MSI was applied, for the first time, for three dimensional chemical imaging of a single cell using newly fertilized individual zebrafish embryos as a model system. A microarray platform was developed using five optimized matrices for high-throughput MALDI-MS analysis which was then applied to metabolomics studies of turkey gut microbiome samples with and without antibiotic treatment. Finally, multiple on-tissue chemical derivatization strategies were adopted to dramatically improve the sensitivity of MSI for metabolite analysis.

Outlook

In recent years, mass spectrometry has seen significant and exponential progress in metabolomics analysis. Achieving single-cell level metabolomics with MALDI-MSI is beginning to be realized. The extreme complexity of biological samples, and the challenges in identification of unknown metabolites require innovative solutions to allow the continual advancement of metabolomics analysis. Recent growth in this field can be attributed to emerging sampling/ionization methodologies, new software and database packages for data
analysis, multimodal imaging approaches, and applications. This progress along with the work presented in this dissertation offer intriguing possibilities towards cellular and sub-cellular level MS images in the metabolomics scale.

The methodologies presented in this dissertation include the recrystallization of lipids, 3D MSI, the microarray analytical platform, and on-surface chemical modification. These methods could easily be adapted to study a plethora of systems. For example, 3D mass spectrometry imaging can be used in any system to study heterogeneous volumetric localization of metabolites. The microarray platform can be used for any high-throughput and large-scale studies, to compare time-points, different mutants, to study disease state, etc. Different derivatization strategies can be applied and optimized to increase the sensitivity and selectivity of multiple sub-metabolomes.

The work presented in this dissertation represents a good foundation and offer opportunities for extension and refinement. The main limitation of the work presented in Chapter 2 is the matrix optimization. Additional studies to identify recrystallization conditions for other commonly used organic (DAN, CHCA, 9AA, etc.) and/or binary matrices would allow this recrystallization strategy to expand to a wider range of compounds.

Chapter 4 (3D MSI) revealed, for the first time, a volumetric distribution of lipids in a single cell. Essential tools and protocols need to be developed in order to demonstrate the feasibility of 3D imaging MS. For example, producing 3D MSI data requires the handling of large number of tissue sections, which increases the risk of sample-to-sample variation. Moreover, the data can take several days or weeks to acquire, requiring high stability of measurements over time. Computational analysis (registration of individual data sets into a 3D volume data) and interpreting, analyzing and visualizing the data can also be challenging.
These technical limitations are currently being addressed, which will enable 3D-MSI to truly excel.

The high-throughput microarray platform presented in Chapter 5 demonstrated that this methodology can be used to quickly acquire large data sets needed for the study of dynamic systems. Although we were able to detect thousands of unique features with this approach, identification of these metabolites is the current bottleneck. This issue could be addressed through continued development of comprehensive MS/MS databases and further improvement to in-silico fragmentation software. The wealth of information provided by this platform will dramatically increase as more metabolites are able to be confidently identified in a high-throughput manner.

Further refinement with the on-tissue derivatization strategy described in Chapter 6 is possible and likely to be completed in the future. Unwanted side reactions and excess reagents left on the tissue interfere with the MS analysis. Computation code can be developed to effectively identify derivatized peaks and tentatively assign peaks based on accurate mass, dramatically improving the data analysis pipeline. Current group work is also investigating the possibility of employing new derivatization methods and understanding the mechanism in the reaction. These works will allow more information to be obtained from a single experiment, and demonstrate that MALDI-MSI can be extended to visualize metabolites at the metabolomics scale.

The objective of this work has been to develop MALDI-MSI into a more useful technique for the analysis of metabolites at cellular and sub-cellular cell. As discussed above, much work remains to be done to achieve single-cell metabolomics using MSI. Nonetheless, the methodologies and applications studies demonstrated herein represent advances that
should prove useful in future efforts to gain new insights and improve the understanding of the interplay of metabolites in biological systems.
APPENDIX  CELLULAR AND SUB-CELLULAR LEVEL LOCALIZATION OF MAIZE LIPIDS AND METABOLITES USING HIGH-SPATIAL RESOLUTION MALDI MASS SPECTROMETRY IMAGING

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Abstract

Recent technological advances have pushed the achievable spatial resolution for mass spectrometry imaging (MSI) to cellular and sub-cellular levels. Direct visualization of maize tissues by this tool has provided key insights into the localization of metabolites and lipids. This chapter outlines methodology for sample preparation, data acquisition, and data analysis of maize tissue sections using high-spatial resolution matrix-assisted laser desorption ionization (MALDI)-MSI, as well as the incorporation of a multi-resolution optical system, which allows for simple inter-conversion between different resolution setups (5, 10, and 50 μm imaging).

Introduction

Mass spectrometry imaging (MSI) has become a widely used analytical tool to visualize metabolites, lipids, proteins, and various small molecules directly on plant tissues (Sturtevant, Lee and Chapman 2016, Lee et al. 2012, Takahashi et al. 2015). Such wide
applicability has led to extensive development of sample preparation protocols and instrumental setups for these analyses. Matrix-assisted laser desorption/ionization (MALDI)-MSI is the most widely used technique for cellular and sub-cellular resolution imaging due to its combination of high-spatial resolution, high sensitivity, and chemical versatility. Recent technological advances in instrument design and sample preparation have expanded the capabilities of MALDI-MSI and have made much higher resolution experiments possible. Consequently, MALDI images with pixel sizes from 1-10 μm have been obtained (Zavalin, Yang and Caprioli 2013, Korte et al. 2015a). Pushing the spatial resolution below 10 μm has allowed for finely detailed studies at the cellular and sub-cellular level. Visualizing detailed metabolite information at this scale can offer unprecedented details in terms of localization and metabolite composition of various tissue types which is crucial for elucidating their biological roles.

A typical experimental workflow for MALDI-MSI is provided in Figure A.1 and described in detail in our recent protocol paper (Korte et al. 2015b) and other publications (Römpp and Spengler 2013, Weaver and Hummon 2013). Briefly, the plant tissues are flash-frozen in an embedding medium, cryo-sectioned, and lyophilized. A MALDI-MSI experiment requires the application of a chemical matrix, which absorbs the laser energy and promotes desorption and ionization, onto the tissue sample of interest. The matrix is typically applied by either pneumatic spraying, sputter coating or by solvent-free sublimation, depending on the matrix used. After matrix deposition, the sample is inserted into the instrument and the surface is interrogated with a laser beam, desorbing and ionizing both matrix and endogenous compounds from the tissue surface. The generated ions are then analyzed to produce a mass spectrum. After acquiring a mass spectrum from an individual
spot on the tissue, the sample plate is moved a predefined distance and another spectrum is acquired. This process is repeated over the tissue to acquire spectra at hundreds or thousands of x-y positions. The resulting spectra at each location along with their corresponding x-y coordinates are then used to generate two dimensional maps for ions of interest by converting the ion’s intensity at given pixels into a false color intensity. These ion images can then be correlated to an optical image of the tissue.

**Figure A.1** Typical workflow of a MALDI-MSI experiment The first step is tissue harvesting and cryo-sectioning, followed by vacuum drying, matrix application, and matrix recrystallization (optional). After MSI data acquisition and mass spectral interpretation, the compounds of interest can be visualized in two-dimensional false-color images.
We have established a MALDI-MSI technical platform for 5-10 μm high-spatial resolution and applied this platform to visualize the distribution of a number of different metabolites in cross-sections of maize leaves (Dueñas et al. 2017, Korte et al. 2015a), geminated seeds (Feenstra et al. 2017a) and roots (Feenstra, Dueñas and Lee 2017b).

Previously, in the work by Korte et al. (Korte et al. 2015a), 5 μm spatial resolution was achieved using a 9 μm laser spot size to examine the sub-cellular metabolite distributions in the upper and lower epidermis, bundle sheath, mesophyll, and vasculature (Figure A.2A). This work revealed that molecular distributions of metabolites and lipids may be heterogeneous even among cells of the same tissue type. For example, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc) and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (HMBOA-Glc) are known to be present in mesophyll cells of maize, but these compounds were detected only in interior mesophyll cells between the vascular bundles (Figure A.2A a-d). More recently, we modified our optical system and improved our laser spot size to 6-7 μm (Dueñas et al. 2017), then 3-4 μm (Feenstra et al. 2017b), and applied this to explore quantitative fatty acyl distributions of sulfoquinovosyldiacylglycerols (SQDG) and phosphatidylglycerols (PG) in thylakoid membranes along the developmental gradient of maize leaves of two inbred lines, B73 and Mo17, and the reciprocal hybrid lines, B73 x Mo17 and Mo17 x B73 (Dueñas et al. 2017). This study demonstrated that certain thylakoid membrane lipids (e.g. PG 32:0) show genotype-specific differences in cellular distribution, as shown in Figure A.2B.

Our previous protocol paper focused on how to prepare plant tissue samples for MALDI-MSI and how to perform multiplex MS imaging (Korte et al. 2015b), a method that can simultaneously perform MS/MS along with precursor MS in a single imaging
experiment. Here, we describe a methodology for high-spatial resolution MALDI-MSI using a simple, interchangeable, multi-resolution optical system. Commercially available Galilean configuration beam expanders are used to allow spatial resolutions of 5, 10, and 50 µm, with laser beam size of ~4, ~7, and ~45 µm, which are achieved using 10x, 5x, or no beam expander, respectively (Feenstra et al. 2017b). In addition to the laser optics modification, we describe improved protocols for sample preparation, data acquisition, and data analysis optimized for high-spatial resolution.

![Figure A.2](image)

**Figure A.2** (A) Overlaid MALDI-MS images of a few compounds in maize cross-section obtained with 5 µm spatial resolution: (a) optical images, (b) flavonoids in upper and lower epidermal cell layers, showing rutin and maysin almost exclusively localized in a few upper epidermal cells, (c) PG 34:2 localized in bundle sheath and SQDG in both bundle sheath and mesophyll, and (d) HMBOA-Glc and DIMBOA-Glc in vacuoles of mesophylls, but only between each pair of vascular bundles, and SQDG in chloroplasts (reproduced from ref 5 with permission from Springer) (B) 5 µm resolution MS images of two PG molecular species at the midpoint and distal sections of maize leaves from four genotypes overlain with optical image. Scale bar 50 µm (reproduced from ref 12 with permission from Wiley).
Materials

Tissue Sample Preparation

1. Plant tissues, here with maize seedlings as an example
2. 10% w/v 300 bloom gelatin solution prepared in high-purity water
3. Styrofoam cooler filled with liquid nitrogen
4. Cryo-mold and Cryo-Jane adhesive tape sections
5. Optimal cutting temperature (OCT) compound
6. Cryo-microtome
7. Glass microscope slides and a microscope slide box
8. Microscope with camera
9. Vacuum chamber or lyophilizer with vacuum system
10. Metal block (20x10x1 cm) pre-cooled in a -80 °C freezer for several hours
11. Fine point permanent marker
12. Scotch tape
13. Forceps

Matrix Application by Sublimation

1. Glass sublimation apparatus with flat-bottomed condenser (Chemglass)
2. Temperature-controllable heating mantle
3. Acetone/dry ice slurry
4. MALDI matrix (see Note 1)

Matrix Recrystallization

1. Recrystallization chamber composed of a glass Petri dish, stainless steel plate, and magnets
2. Filter paper
3. Copper tape
4. Electrical tape
5. 5% isopropyl alcohol solution prepared in high-purity water
6. Incubator pre-set at 55 °C

**Laser Optics**

1. Optical table
2. Cage system construction rods
3. Right-angle post clamps
4. Support plates
5. Optical element mounts
6. Laser line mirrors
7. Dichroic mirror
8. Quartz window
9. UV achromatic doublet
10. 1 mm diamond aperture
11. 60 mm focal length near UV focus lens
12. 25/25.4 mm diameter lens mount
13. 10x and 5x beam expander

**Methods**

**Tissue Harvesting, Sectioning, and Drying**

This procedure covers sample preparation including: tissue harvesting, embedding, cryo-sectioning, and drying. Our previous protocol (Korte et al. 2015b) describes a
general method for different sample preparation techniques for MALDI-MSI while the current protocol is optimized for cross-sectional tissue imaging in high-spatial resolution.

1. At the desired developmental stage, harvest the tissue from the plant, place the tissue of interest in a cryo-mold filled with gelatin (10% w/v solution), and immediately flash freeze the sample and gelatin in the mold, by floating on liquid nitrogen.

2. Transfer the mold to a cryostat pre-chilled to -20 °C and allow the sample to thermally equilibrate for at least 30 minutes. Remove the sample from the mold and affix it to the cryostat chuck using a minimum amount of OCT compound.

3. Cryo-section the tissue at the desired thickness (10-20 µm) and collect the tissue on Cryo-Jane adhesive tape. Place the Cryo-Jane tape with section attached face-up on a pre-chilled glass slide, and attach the Cryo-Jane tape to the glass slide by taping both ends to the slide with Scotch tape (see Note 2).

4. Rapidly remove a section from the cryostat and visualize under a microscope to ensure that the sample is intact. If the tissue is completely damaged, run off several sections of the mold in order to reach a different portion of the embedded tissue and repeat step 3 (see Note 3).

5. Once an intact region of interest in the tissue is observed under the microscope, discard this section (see Note 4).

6. Repeat step 3, collecting the desired number of sections and placing them in a microscope slide box, pre-chilled at -20 °C, located inside the cryostat (see Note 5).

7. Keep the tissue sections frozen at all times and store at -80 °C until analysis.

8. When ready for analysis, place the microscope slide box containing the slides with sections onto a chilled aluminum block and immediately place into a vacuum
chamber. Evacuate the chamber. Make sure no condensation is forming on the sample surface during the thaw-vacuum dry process.

9. Dry the sample for 1.5-2 hours (see Note 6).

10. Release the vacuum and remove the samples.

11. Observe a set of consecutive tissue sections under a microscope to find the most intact tissues that will be used for MSI. Make sure the region of interest shows intact tissue with almost no visible cracking or separation of cells.

12. Re-orient the tissue section to be aligned along the x- and/or y-axis for efficient MS imaging (See example in Figure A.3). With the help of forceps, position the tissue section on a glass slide and attach it by taping both ends to the slide with Scotch tape. Thoroughly inspect the slide to make sure the section is flat (see Note 7).

13. Visualize the slide under the microscope and acquire an optical image (see Note 8).

14. On the back side of the glass slide of the optimal section, use a fine point permanent marker to mark the tissue region that will be imaged in MS (see Note 9).

![Figure A.3](image)

**Figure A.3** Importance of positioning tissue sample for high-resolution MSI. In this example, optimal orientation (A) can be covered by 70 pixels whereas suboptimal orientation (B) requires 126 pixels.
Matrix Sublimation

Homogeneous matrix application is important for high-spatial resolution MALDI-MSI (Hankin, Barkley and Murphy 2007, Bouschen et al. 2010). Vapor phase sublimation allows organic matrices to be applied to tissues homogeneously, down to a one-micron scale (see Note 10). Sensitivity is often limited in high-spatial resolution MSI due to the reduced sampling volume. To maximize sensitivity, the choice of matrix and matrix application is critically important.

1. Attach the glass slide with the optimal section to the flat bottom of the sublimation condenser using adhesive tape with the tissue side facing down (Figure A.1). Evenly distribute ~300 mg of organic matrix in the bottom of the lower flask. Assemble the apparatus, and evacuate to <100 mTorr.

2. Make a ~40 mL acetone/crushed dry ice slurry in the condenser reservoir and after 2 minutes, initiate sublimation by placing the sublimation apparatus into a pre-heated heating mantle.

3. After the desired amount of matrix has been deposited (as suggested in Note 10), remove the sublimation apparatus from the heating mantle, wait until the bottom flask is cooled to room temperature, and add an excess of room temperature water to the condenser to warm it to ambient temperature (see Note 11).

4. Disassemble the sublimation apparatus and carefully remove the glass slide and sample from the sublimation condenser.

Matrix recrystallization

This is optional but often necessary to improve ion signals in high-spatial resolution (Dueñas, Carlucci and Lee 2016, Yang and Caprioli 2011, O'Rourke et al. 2015). Assemble a
recrystallization chamber using filter paper, a glass Petri dish, a stainless steel plate, and magnets (Figure A.1).

1. Using copper tape, which improves heat conduction, adhere the sublimated slide to a stainless steel plate.

2. Place the stainless steel plate on the underside of the Petri dish cover facing down and attach by placing magnets on the exterior of the Petri dish.

3. Place a piece of filter paper in the bottom part of the Petri dish and moisten evenly with 1 mL of solution (see Note 12).

4. Assemble the Petri dish to form a hydration chamber by placing the cover on top of the bottom part of the dish.

5. Seal the Petri dish using electrical tape.

6. Place the hydration chamber in an incubator set at 55 °C for 2 minutes. Disassemble hydration chamber and carefully remove slide (see Note 13).

**Laser Optic Modifications**

Here, we describe modifications to the laser optical system of a commercial MALDI-MS system for interchangeable, multi-resolution MSI. The procedure described here is intended for MALDI-LTQ-Orbitrap instruments, but the idea can be generalized to other mass spectrometers. In the work described here, the original nitrogen laser of the MALDI source is bypassed, and an external Nd:YAG laser is used for desorption/ionization. This procedure has been discussed in more detail previously (Korte et al. 2015a), (Feenstra et al. 2017b). A schematic representation showing the modified optical system is presented in Figure A.4.

*Safety warning:* Replacement of the manufacturer’s enclosed laser system with an external laser as described here results in a class IIIB laser system, and appropriate
precautions should be taken, including the use of protective eyewear and appropriate access controls.

1. Use an optical table with threaded holes to enable optical and mechanical system components to be mounted.

2. Position the laser head at a sufficient distance from the mass spectrometer to provide maneuvering space.

3. Place the pinhole along the path. In our current setup, the laser is directly filtered by placing a one millimeter diameter pinhole into the beam path (see Note 14).

4. Place a beam expander after the pinhole. Commercially available beam expanders can be held in place by a simple locking screw or by threading into a holder. Make sure to incorporate the necessary beam expander holders into the optical system to ease the interchange of beam expanders.

5. Place the dichroic mirror along the laser beam path to direct the laser into the instrument while also allowing the MALDI plate to be visualized with the CCD camera (Strupat et al. 2009).

6. Integrate a custom-built lens holder into the source of the instrument to allow focusing of the final lens as close as possible to the MALDI plate. Make sure the distance matches with the focal length. A custom-built lens holder should be manufactured with adjustable components in order to optimally position the final focus lens.

7. Optimize the position of the final focusing lens by systematically adjusting the z-position with the highest magnification (e.g. 10x beam expander) and measuring the resulting laser burn marks under the microscope (see Note 15).
8. To use the interchangeable, multi-resolution optical system described in our recent work (Feenstra et al. 2017b), set up the necessary beam expander holders in a way that each beam expander can be easily exchanged (see Note 16).

9. Each time the beam expander setting is changed, make minor laser alignment to ensure that the laser beam enters the final focus lens with correct alignment and shape.

Figure A.4 Schematic of modified beam-delivery optics (reproduced after minor modification from ref 10 with permission from Springer).

**High-resolution MS Imaging**

Please refer to the instrument guidelines or manual for the operational details of MS imaging. Here, we describe only the basic procedure with a focus on high-spatial resolution
MS imaging. The procedure described here is intended for LTQ-Orbitrap instruments, but the idea can be generalized to other mass spectrometers.

1. Using an optical scanner or an in-source camera, acquire an optical image of the whole slide for later reference.

2. Using the instrument software (Tune Plus), optimize instrumental parameters such as laser energy and number of laser shots. For this purpose, pick a tissue region that is not the region of interest or use a dummy tissue that was prepared in parallel. Save the tune file that contains this information.

3. Generate ablation spots on the tissue outside the imaging area or on the dummy tissue, eject the sample plate, and examine the spots under the microscope to inspect the spot size and shape. If necessary, adjust the position of the pinhole and beam steering mirrors and repeat **steps 2 and 3**.

4. With the aid of the optical image acquired in **step 1**, select the tissue region to be imaged in the MALDI window of Tune Plus. Define the raster step size and save all this information as a MALDI Position file.

5. Set up an instrument method using Xcalibur software and in the Sequence Set-up window of Xcalibur, provide a data file name to be used, destination folder for data, instrument method, and MALDI position file name. Run the sample.

6. Multiplex MS imaging can be performed to obtain simultaneous MS/MS data acquisition. Please refer to our previous method paper for the details (Korte et al. 2015b).
Semi-automatic Registration using MATLAB

Overlaying MS images with the corresponding optical image is an important step for better understanding of the high-resolution MS images. In this section, we explain how to register the two images with a tool in MATLAB. Data processing starts with the raw mass spectra collected by MS imaging measurements, which then needs to be registered with the locations of the sampling points. All the data used in this example were collected from B73 maize leaf tissue cross-sections using MALDI-MS (Figure A.5) (Dueñas et al. 2017).

1. Open ImageQuest (or any MS imaging software of your choice) and choose a \( m/z \) value of interest for visualization. Select a monochromatic color scheme, adjust minimum and maximum scale values, and select ‘None’ in the drop-down box of the smooth option (see Note 17).

2. Save the 2-D MS image as .tiff.

3. Repeat steps 1 and 2 to create ion maps of all \( m/z \) values of interest.

4. Place the optical microscope image and all the 2-D MS images in a folder where the MATLAB program will be running (MATLAB).

5. In the MATLAB command window, load the optical and MS images to the vector matrices in the names of 'fixed' and 'moving', respectively, by typing the following:

   \[
   \text{fixed} = \text{imread('optical\_image.tiff')}
   \]

   \[
   \text{moving} = \text{imread('mz\_image.tiff')}
   \]

Here, 'optical_image.tiff' and 'mz_image.tiff' are the names of the optical image file and MS image file, respectively.

6. Type \texttt{cpselect(moving,fixed)} at the prompt to start the “Control Point Selection Tool” (Figure A.5a). This is a graphical user interface that enables you to select control
points in two related images. The MS image will show on the left side of the toolbox and the optical image on the right.

7. Manually select 15-20 unique features within each image by clicking one after the other (see example on Figure A.5a).

8. Select "Export Points to Workplace" from the pull down menu under "File". Check all the boxes in the pop-up window, and then click "OK". The selected control points in MS images and optical images are stored as 'movingPoints' and 'fixedPoints', respectively, in the temporary memory of workplace.

9. Original MS image vector of 'moving' can be geometrically transformed to the new vector of 'registered' using 'imwarp' command by typing the following (see Note 18):

   ```matlab
   mytform=fitgeotrans(movingPoints, fixedPoints, 'projective');
   Rfixed=imref2d(size(fixed));
   registered=imwarp(moving, mytform, 'FillValues', 0, 'OutputView', Rfixed);
   ```

10. To display the transformed MS image and the overlay image, type the following (see Figure A.5b for blended image).

    ```matlab
    figure, imshowpair(fixed, registered, 'blend');
    figure, imshow(registered);
    ```

11. Save the transformed MS image and overlaid image in the MATLAB graphic window.

12. (Optional) To overlay the optical and MS images with more options, 'Image Fusion' on the 'Wavelet Toolbox Main Menu' can be used. Type `wavemenu` command and select 'Image Fusion'.
13. In the Image Fusion window, upload the optical image and the newly transformed MS image as Images 1 and 2, respectively. Adjust parameters (see Note 19) and click 'decompose' followed by 'apply' to generate 'synthesized image' (see Figure A.5b). Synthesized image can be saved from the pull down menu under 'File'.

**Figure A.5** (A) Screenshot of Control Point Selection toolbox showing features that are unique within images selected and numbered, and (B) Overlay of the optical image with MS image using imshowpair command with 'blend' option vs. Image Fusion of MATLAB.
Notes

1. The matrix should be carefully chosen based on the analyte(s) of interest. For example, 2,5-dihydroxybenzoic acid (DHB) is effective for most lipids in positive mode, and 1,5-diaminonaphthalene (DAN) is useful for most lipids and small molecules in negative mode. 9-aminoacridine (9-AA) is also useful for small molecules in negative mode, especially phosphate compounds.

2. The tissue can also be collected directly via thaw mounting but we prefer to use Cryo-Jane tape to preserve the structural integrity of fragile tissue sections. Cryo-Jane tape cannot be used for MALDI-TOF, as its high-voltage requires conductive surface.

3. Since the tissue goes from -20 °C to room temperature when visualizing under the microscope, the tissue will begin to break and cells will burst. In order to visualize an intact sample, this step must be completed within 10-15 seconds after removing from the cryostat.

4. After the section has been thermalized to room temperature without a proper drying step, this section cannot be used for MSI because the tissue might have been damaged and the internal metabolites might have migrated while viewing under the microscope.

5. Do not view these slides under the microscope. Make sure to acquire multiple sets of consecutive sections.

6. This will give enough time for the metal block to reach room temperature. This is important because if cool, the water from the atmosphere will condense onto the sample, possibly redistributing metabolites.

7. This step is performed to minimize the scanning of the non-tissue area and reduce the total acquisition time.
8. This will be the optical image that will later be used for reference and co-registration with the MS images.

9. This will help locate the area of interest when setting up the instrumental parameters.

10. Nanoparticle matrix (Yagnik et al. 2016) or organic-inorganic binary mixture matrix (Feenstra et al. 2016) cannot be sublimated and should be applied via spraying as previously described (Korte et al. 2015b). The required temperature and time will vary by matrix. The commonly used organic matrices DAN and DHB are sublimated at 140 °C for 3-5 and 5-7 minutes, respectively.

11. If vacuum is broken before the condenser has returned to room temperature, condensation will occur on the sample slide, causing redistribution and/or turnover of metabolites.

12. Solvent composition can be changed depending on the applications, but we found 5% IPA in water to be optimal for lipids and most metabolites (Dueñas et al. 2016).

13. Time and temperature can be varied depending on the application. Too long time or too high temperature can cause re-distribution of small molecules.

14. The purpose of this pinhole is to remove significant non-Gaussian components of the beam. This can be done more efficiently by pre-focusing the laser beam to a very small spot size and using a smaller pinhole (Korte et al. 2015a); however, we found optical alignment more challenging with this setup.

15. The laser spot size should be measured as if in a real tissue, with a Cryo-Jane tape, 10-20 µm thick gelatin layer, and matrix, because of the narrow depth of focus at high magnification. If the final focus lens is inside the vacuum, it might be necessary to vent the system for each adjustment of the focus lens positions.
16. We achieved laser spot size of ~4, ~7, and ~45 µm by using 10x, 5x, or no beam expander, respectively, in our setup.

17. Use of pixelated MS images is preferred for overlaying with microscope optical image.

18. Here, 'mytform' is a geometric transformation defined through the command

   fitgeotrans to convert 'movingPoints' in MS images to 'fixedPoints' in optical images. 'Rfixed' is an imref2d object used to define the new MS image to have the same size as the optical image. Empty image pixels in the new MS image is filled as zero (black).

19. The parameters used in Figure A.5b are the following:

   Wavelet: sym 2

   Level: 2

   Approx: max

   Details: max

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References


