METHOD DEVELOPMENT FOR ROUTINE IMAGING MASS SPECTROMETRY OF SINGLE CELLS GROWN IN CULTURE USING TRANSMISSION GEOMETRY MALDI MASS SPECTROMETRY

By

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LIST OF ABBREVIATIONS

CHCA- α-cyano-4-hydroxycinnamic acid
DHB- 2,5-Dihydroxybenzoic acid
DMEM-Dubelcco’s Modified Eagle Medium
EDTA-Ethlenediaminetetraacetic acid
FBS-Fetal Bovine Serum
GA-Glutaraldehyde
HEK-Human Embryonic Kidney
IMS-Imaging Mass Spectrometry
ITO-Indium Tin Oxide
MALDI-Matrix Assisted Laser Desorption/Ionization
NBF-Neutral Buffered Formalin
PBS-Phosphate Buffered Saline
PFA-Paraformaldehyde
TFA-Trifluoroacetic acid
THAP- 2,4,6-trihydroxyacetophenone
TOF-Time of Flight
Chapter I

Introduction

Matrix Assisted Laser Desorption/Ionization Imaging Mass Spectrometry

Matrix Assisted Laser Desorption/Ionization imaging mass spectrometry (MALDI IMS) technologies have developed rapidly since the technique was first published in 1997. This technology has been used to map the spatial localization of proteins, peptides, lipids, pharmaceuticals, and metabolites. MALDI IMS involves application of an energy absorbant matrix to the surface of a tissue sample and subsequently ionizing the cocrystallized matrix and analytes by striking the surface with a laser pulse in a raster pattern to generate an ion image, as shown in figure 1.

Traditionally, matrices are organic chemicals such as 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), and sinapinic acid. The selection of matrix is made based upon the analyte of interest. Peptides are typically analyzed by CHCA, proteins by sinapinic acid, and lipids by DHB. There are many different matrices that can be applied to each of the analytes, these are just the most common. The method of matrix application is dependent on the resolution required. The size of the crystals formed in the matrix application must be smaller than the desired spatial resolution. Therefore, studies that require high spatial resolution will usually use either sublimation or an automated sprayer.

Either fresh-frozen or formalin-fixed paraffin-embedded (FFPE) tissues of interest are prepared by first sectioning them to between 5 and 10 μm thickness and mounting them onto a MALDI target, traditionally either a conductive metal or glass slide. For fresh-frozen tissues,
samples are then washed using either ammonium acetate or ammonium formate, in order to remove the salt adducts on the lipids\(^2\), for lipid imaging or graded alcohol, to remove lipid and salt contaminants\(^3\), for protein and peptide imaging. Post washing, matrix is applied by a variety of techniques, such as spray coating, sublimation, or acoustic generated droplets. If necessary, the sample may be further rehydrated post matrix application to improve analyte extraction.

![Figure 1: MALDI Imaging workflow. Adapted from Schwamborn et al.\(^4\)](image)

**Ultra High Spatial Resolution MALDI IMS Technologies**

Recently, IMS technologies have moved towards higher lateral spatial resolution. Commercial mass spectrometers have been developed to routinely collect mass spectral images at 15 to 20 \(\mu\)m lateral spatial resolution. Some samples require an even greater spatial resolution because the biological regions of interest are localized in very small regions of the sample. These applications are termed as needing ultra-high spatial resolution, defined as
having spatial resolution at or below 5 μm. For example, a typical mammalian cell has a diameter of 15-30 μm. Another example is a developing mouse embryo, which ranges from 100 μm[EMA#ts01, July, 2012] to 270mm[EMA#ts27, July, 2012] during its development according to EMAP eMouse Atlas Project (http://www.emouseatlas.org). To be able to use MALDI IMS to analyze such small samples, the laser footprint needs to be smaller than the sample being analyzed, and the mass analyzer and detector must be sensitive enough to detect the limited amount of material that is subsequently ionized.

The goal of this project was to develop MALDI IMS technologies for the analysis of biological samples with subcellular spatial resolution. Specifically, this project sought to generate mass spectral images of single or clusters of mammalian cells grown in culture. The instrumentation used in this project was an AB Sciex 4700 modified with a transmission geometry ion source and truncated flight tube. Transmission geometry mass spectrometry is characterized by irradiation of the sample from behind, i.e. the laser irradiates the backside of the sample that is directly affixed to the target, providing a direct path from the laser to ablation to ion optics and the MS analyzer. The primary benefit of the transmission geometry is the ability to focus the laser footprint to less than 5 micrometers by allowing the microscope objective to be closer to the sample. Another benefit is a bit more subtle; by allowing transmission geometry, the incidence angle of the laser is 90 degrees to the sample as well as the detector. Therefore, there is no need to adjust the direction of the ion plume resulting in a maximum amount of sample ions reaching the detector.

The methods that are developed herein were used to generate lipid images from single RKO cells, a common human colon cancer cell, and Human Embryonic Kidney (HEK) 293 cells.

\(^a\) http://www.emouseatlas.org/Databases/Anatomy/Diagrams/ts01/
\(^b\) http://www.emouseatlas.org/Databases/Anatomy/Diagrams/ts27/
\(^c\) For a complete description of the instrument, please see figures 2 and 3 and the instrumental parameters section.
RKO and HEK-293 cells were chosen because they are readily available and are easily cultured.

Method development and specific challenges presented will be addressed and discussed in four defined sections. They are: fixation, visualization, cell adherence, and matrix application.

Materials:

Imaging experiments were performed using 25x75x1.1 mm Indium Tin Oxide (ITO) coated float glass slides (Delta Technologies, Loveland, CO). The MALDI matrix 2’,4’,6’-trihydroxyacetophenone (THAP) was purchased from Sigma Aldrich (St. Louis, MO). Solvents, 10% neutral buffered formalin (NBF), glutaraldehyde, osmium tetroxide, red phosphorous, ammonium acetate, as well as the cresyl violet stain were also purchased from Sigma Aldrich. Tissue-TEK Cryo-OCT was purchased from Fisher Scientific (Suwanee, GA). Trifluoroacetic acid (TFA) was purchased from VWR (Radnor, PA). Dulbecco’s Modified Eagle Medium (DMEM), McCoy Media, 1x phosphate buffered saline (PBS), penicillin-streptomycin, and 0.25 % Trypsin EDTA dissociation buffer were all Gibco brand and purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO). Poly-L-lysine and 4% paraformaldehyde were purchased from Electron Microscopy Sciences (Hatfield, PA). Water used was collected from a Millipore Milli-Q system (Billerica, MA). Rat brains were purchased from Pel-Freez Biologicals (Rogers, AR).
Chapter II

Methods

Instruments and Conditions:

Leica Cryostat

The cryostat used for the preliminary experiments was a Leica CM3050S (Leica Microsystems Ltd., Milton Keynes, UK). The chamber and object temperatures were each set to -18°C. Samples and slides were placed in the cryostat for 10 minutes to equilibrate temperature. Rat brain was sectioned coronally and samples for analysis were sectioned to 9 μm thickness and thaw mounted onto ITO-coated glass slides.

HTX TM-Sprayer

The TM-Sprayer (HTX Technologies, Carborro, NC) is an automated deposition instrument which is used to apply matrix to MALDI targets for MALDI IMS. All TM-Sprayer methods presented used the same conditions. Matrix was dissolved at 5 mg/mL in 90% methanol, 9.7% water, and 0.3% TFA and sonicated before use. An Agilent 1100 HPLC pump controlled the solvent flow rate which was set to 0.2 mL/min. 90% methanol was the solvent that was used to push the matrix solution through the sprayer. The nozzle temperature was set to 120°C. The stage moved at 700 mm/s with 1 mm spacing between adjacent passes, with alternating passes having a 0.5 mm offset, for a total of 4 matrix coats.
Bruker UltrafleXtreme

Imaging experiments were performed using a Bruker UltrafleXtreme Time of Flight (TOF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Data was obtained at 250 µm spatial resolution. All experiments were performed in positive ion, reflectron mode. The instrument was optimized for resolution in the range 700 to 1000 Da in 50 shot increments for 200 shots total using a laser repetition rate of 1000 Hz. Mass calibration was performed using 6 mg/mL red phosphorous in 50% methanol. The collected data was analyzed using FlexImaging version 3 (Bruker Daltonics).

Applied Biosystems 4700

An Applied Biosystems (Carlsbad, CA) 4700 MALDI TOF/TOF, hereafter referred to as “the modified 4700”, was modified as described by Zavalin et al. The flight tube was truncated from 1972 mm to 980 mm and the reflectron electrostatic mirrors were removed making the instrument a linear TOF only. The reflection geometry source was supplemented with a transmission geometry source through the back wall of the source chamber, as shown in figure 2. Transmission and reflection geometries are compared in figure 3. Notice that the laser strikes the sample perpendicularly from the back where reflection strikes the front at an angle. The laser used for transmission geometry source is a frequency tripled Nd:YLF with a frequency of 349 nm operating at 100 Hz. The laser footprint is reduced to 1 µm by focusing it through a Mitutoyo ULWD 100X/0.50 objective placed within the vacuum chamber of the source directly behind the target. The spot size was determined by spray coating THAP matrix over the surface of a slide and ablating the matrix using laser pulses using the same conditions as were used for the actual imaging experiments. The distance of the separation between the objective and the sample is controlled using an automated z-stage in order to bring the sample into optical focus, which has been calibrated to be the optimized focal point of the laser as judged using the
connected camera, a Canon EOS 7D digital camera. This instrument was calibrated using the same red phosphorous as the Bruker UltrafleXtreme. Data was analyzed using both Data Explorer (Applied Biosystems) and BioMap (MSImaging, Novartis).

Figure 2: Schematic of transmission geometry ion source as it is used in our system. Figure used from Zavalin et al.\textsuperscript{8}

Figure 3: Transmission versus reflection geometries.
Millipore Milli Q Water System

Water used was provided by a Millipore Milli-Q A10 system. Resistivity of water was 18.0-18.3 MΩ·cm.

Light Microscope

The light microscope used was an Olympus (Shinjuku, Tokyo, Japan) BX-50 operated with a transmission light source.

Cell Culture and Plating Methods:

RKO cells were grown in McCoy’s media and HEK-293 grown in DMEM. All media was supplemented with 10% (v/v) FBS and 1% (v/v) penicillin streptomycin. Cells were removed from cryopreservation and placed in their respective media for 24 hours, after which the media was removed and changed to fresh media. These were allowed to grow to confluence before they were split 1:10 for RKO and 1:3 for HEK-293. Cells were taken through at least two passages before being plated.

One flask of each cell type was drained of media followed by the addition of 3 mL of trypsin EDTA dissociation reagent. For RKO cells, the dissociation reagent was removed after 15-30 seconds and media was washed over the cells for collection. For HEK-293 cells, media was added to the dissociation reagent in order to collect the cells. Cells were counted using a BioRad (Hercules, CA) TC-10 automatic cell counter, and approximately $10^6$ cells were placed onto a petri dish containing an ITO slide. Slides were placed into a cell incubator overnight at 37°C with 5% CO$_2$. Media was removed via suction and the cells were washed twice with 1x PBS. Immediately following the wash, cells were fixed as described in Method Development: Fixation, washed again, and then placed into new petri dishes and placed in the dark to dry.
Method Development:

Fixation:

In order to obtain a mass spectral image from a sample that preserves biological relevance, it is important that the sample retains the features of its dynamic living state. This is an easy feat when handling tissues, where cells are held together through cellular networks. However, single cells do not have a cohesion network so extra care needs to be taken to maintain the morphology of living cells. In addition, if a cell is left unfixed, it will rapidly undergo the apoptotic process which irreversibly changes cells’ biochemistry. Initial attempts at plating cells did not use any fixative. As expected, these cells shrank considerably due to dehydration and showed signs of infection. Therefore, the method of fixation had to be addressed. Most fixatives that have been used for cell microscopy are alcohol-based, but because lipids are alcohol soluble, they would be partially washed away by the use of alcohol-based fixatives. Therefore, aqueous-based fixatives were required to preserve the organization of lipids in the cell. The most prevalent aqueous-based fixatives are aldehydes. In addition, osmium tetroxide has also been used as a lipid stabilizer\textsuperscript{9}, and was considered for its application to this study. Osmium tetroxide has been shown to crosslink lipids, though, so it was not expected to yield favorable results.

To test the application of aldehyde fixatives for lipid imaging, preliminary studies were conducted with rat brain. Brain samples were sectioned at 9 μm-thickness using a Leica cryostat and thaw mounted onto ITO-coated glass slides. The fixatives tested were 4% paraformaldehyde (PFA), 2.5% glutaraldehyde (GA), and 10% neutral buffered formalin (NBF). In addition, a sample from each primary fixative was subsequently fixed with 1% osmium tetroxide. Slides were removed from the cryostat and immediately immersed in the fixative for 10 minutes. Following
fixation, slides were washed twice with 1X PBS and twice with Milli-Q water. Slides treated with osmium tetroxide were immersed in the 1% solution for an additional 10 minutes and subsequently washed as the others. All samples were spray coated with 5 mg/mL THAP in 90% methanol using an HTX TM-Sprayer. Images were collected using a Bruker UltraflexXtreme with a lateral spatial resolution of 250 µm. Figure 4 shows the spectra from PFA, GA, and NBF. Upon inspection, NBF and GA yield similar spectral profiles and intensity; therefore formalin was chosen as the fixative due to its stability and its prevalence as a common fixative. Figure 5 compares formalin fixation with and without osmium tetroxide. As expected, osmium tetroxide yielded poor lipid spectra, most likely due to extensive crosslinking. Table 1 summarizes the used fixatives and the quality of the resulting spectra.

Figure 4: Average lipid spectra from a single hemisphere of coronal sections of rat brain fixed with neutral buffered formalin, glutaraldehyde, and paraformaldehyde. The spectra were normalized using the Root Mean Squared normalization in FlexImaging.
Figure 5: Formalin fixation with and without osmium tetroxide. Spectra presented are averaged from one hemisphere of coronal image. The spectra were normalized using the Root Mean Squared normalization in FlexImaging.

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<th>Primary Fixative</th>
<th>Secondary Fixative</th>
<th>Signal Strength</th>
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<tr>
<td>4% Paraformaldehyde</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>4% Paraformaldehyde</td>
<td>1% Osmium Tetroxide</td>
<td>-</td>
</tr>
<tr>
<td>2.5% Glutaraldehyde</td>
<td>None</td>
<td>+++</td>
</tr>
<tr>
<td>2.5% Glutaraldehyde</td>
<td>1% Osmium Tetroxide</td>
<td>-</td>
</tr>
<tr>
<td>10% Neutral Buffered Formalin</td>
<td>None</td>
<td>+++</td>
</tr>
<tr>
<td>10% Neutral Buffered Formalin</td>
<td>1% Osmium Tetroxide</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1: Summary of fixatives tested and the quality of the resulting spectra.
Using NBF, which is buffered with phosphate buffered saline, introduces a high concentration of salt to the sample. In an attempt to simplify the spectra by decreasing the amount of metal adducts on lipids, tissues were washed 3 times with room temperature, 150 mM ammonium acetate for 3 seconds, after the initial PBS and water washes. Figure 6 shows spectra of tissue fixed in formalin and either washed only with water or washed with water followed by ammonium acetate. Though the wash seemed to show no significant increase in signal, it was added to the protocol in order to assist in the elimination of any salts present.

Figure 6: Comparison of formalin fixation with and without 150 mM ammonium acetate wash. Spectra presented are averaged from one hemisphere of coronal image. The spectra were normalized using the Root Mean Squared normalization in FlexImaging.
Visualization:

Once the cells were fixed and washed, they were inspected using light microscopy for morphology, approximate count, and relative location. Unstained cells showed little contrast between the ITO surface and the cells, making visual inspection impossible. Therefore, the cells required staining with an agent that is compatible with mass spectrometry, such as cresyl violet.\textsuperscript{10} Cresyl violet is a basic stain, meaning it has a negative ionic charge so it associates with positively charged species.\textsuperscript{11} To stain the cells, 0.5\% (w/v) cresyl violet in water was pipetted to cover the surface of the slide and allowed to sit for 30 seconds before being washed twice with Milli-Q water. Cells stained with cresyl violet provided enough contrast to be visualized via light microscopy and the on-board camera of the modified 4700 mass spectrometer.

Cell Adherence:

A slide was prepared using the methods described herein. Matrix was applied identically to the method presented in the fixative testing, but analysis was performed using the modified 4700. Locating an isolated cell for mass spectral analysis was easily achieved due to the cresyl violet staining; however, obtaining a mass spectrum was challenging. Spectra could be acquired only on the periphery of the cell with a low number of laser shots. As the number of shots increased and the laser location moved from the periphery toward the center of the cell, the cell would catapult from the surface of the slide.

The catapulting issue is similar to desired effect found in Laser Pressure Catapulting technique developed by PALM Microlaser technologies AG (Bernreid, Germany) used in laser capture microdissection. The technique was developed in order to provide for sample preparation that did not introduce contamination. The catapulting process is produced by slightly defocusing the laser and striking the area to be isolated with a single, rapid pulse. This process mimics what occurred with the cells from this study.\textsuperscript{12}
The issue of this catapulting, termed the energy catapult phenomenon, was eliminated using surface chemistries on the ITO surface. The fixation process using formalin forms a Schiff base between a primary or secondary amine, the aldehyde group of formaldehyde, and a carbonyl-containing compound, as described by the Mannich reaction shown in figure 7. This same process was implemented to adhere cells to the surface of the ITO slide using a poly-l-lysine surface modification. These modified slides are commonly used in cell microscopy to assist in cell adherence because the lysine residues contain a primary amine functional group that can react with the formaldehyde group and a carbonyl-containing compound, such as an aspartic or glutamic acid or a carboxy terminus, thus stabilizing the cells on the surface of the slide.

![Figure 7: Generic Mannich Reaction.](image)

Poly-l-lysine was used according to the manufacturer’s recommendations. Briefly, the stock solution was diluted 1:10 with Milli-Q water. ITO slides were cleaned by sonication in ethanol, acetone, and ethanol again. The slides were then dried using 5-second blasts of
compressed air before being placed in the 1:10 dilution for 5 minutes at room temperature. Finally, slides were placed into a slide box and stored in a dessicator to dry overnight.

Poly-l-lysine-modified ITO slides were plated with cells, fixed, stained, and coated with matrix as previously described. In order to co-register optical and ion images, matrix was carefully etched away in the shape of a box. Lipids in the cells were detected using the modified 4700, without observing the catapulting phenomenon. Collecting lipid spectra from a single spot on a single cell does not guarantee the ability to acquire a mass spectral image of a single cell. Therefore, an image was generated from a cluster of cells with a step size of 1.5 µm and a laser spot size of 1µm and visualized using BioMap. Figure 8 shows before and after optical images of an area imaged using the 4700. This shows that the energy catapult phenomenon was eliminated, as the same cells are present in both A and B. More importantly, fixation allowed for the generation of an ion image from the collected mass spectra. Figure 9 shows the generated ion image for \( m/z \) 782 in HEK-293 cells.
Figure 8: Optical images of sampled HEK-293 cells (A) before and (B) after mass spectrometry imaging. Before and after optical images show the same cells, indicating that the energy catapult was eliminated. Figure adapted from Todd et. al.¹⁴
Matrix Application:

In high spatial resolution imaging mass spectrometry, two methods of matrix application are readily used, sublimation\textsuperscript{15} and automated spray coating\textsuperscript{16}. The TM-Sprayer by HTX imaging applies matrix quickly and reproducibly. Therefore, it was the method that was first employed for the analysis of single cells though matrix was applied using sublimation for comparative purposes. THAP was sublimated onto the target as previously described.\textsuperscript{17} Using the same mass spectrometry conditions as used for the TM-Sprayed sample, spectra could not be collected from the sublimated samples. Due to the difference in matrix application methods, the mass spectrometer could require different conditions to yield spectra. Various instrumental conditions were changed and tested, however, spectra could not be generated.
Image Spectra Validation:

In order to verify that the spectra acquired during cellular imaging accurately portray the lipid composition of each of the cell types, lipids were extracted from $2 \times 10^7$ cells in 1 mL of media using the Bligh-Dyer method. A volume of 3.75 mL chloroform:methanol (1:2) was added to 1 mL of cell suspension and vortexed. Then 1.25 mL of chloroform was added followed by vortexing. To this, 1.25 mL of water was added and vortexed again. The solution was centrifuged at 3000 x g for 10 minutes to separate the aqueous (top), protein pellet (central), and organic phases (bottom). The lipid-containing organic phase was collected into a microcentrifuge tube and dried under vacuum. Immediately before analysis, 20 µL of 90% methanol was added to the dried lipid extract. This was then mixed 1:1 with 20 mg/mL THAP in 90% methanol, 9.7% water, and 0.3% TFA. This extract was spotted on the edge of the target containing plated cells and also onto a gold-coated target for analysis using the Bruker UltrafleXtreme. Using the 4700 and identical conditions, spectra were collected from a single plated cell and from the pipetted lipid extract. Figure 10, RKO cells, and figure 11, HEK-293 cells, show the comparison of spectra from the single cell and cell extract. This shows that data collected from a single cell represents the actual species present in the cellular extract. In order to verify that these same species are observed in other instruments, the analysis of cellular extract was repeated using the Bruker UltrafleXtreme, shown in figure 12. The spectra from cell extracts between the 4700, figure 11, and the UltrafleXtreme, figure 12, are quite similar. Most, if not all, peaks present in the UltrafleXtreme data are also present in the 4700 data. This provides evidence that the spectra collected from the 4700 accurately depict the lipid content of the cell.
Figure 10: Spectra from RKO cell extract versus single cell collected using the modified 4700. Figure adapted from Todd et. al.\textsuperscript{14}

Figure 11: Spectra from HEK-293 cell extract versus single cell collected using the modified 4700. Figure adapted from Todd et. al.\textsuperscript{14}
Figure 12: HEK-293 cell extract spectra using Bruker UltraFleXtreme.
Chapter III

Final Method

In summary, imaging single cells for lipids requires optimization and method development in four major areas. They are: fixation, visualization, cell adherence, and matrix application. The process is explained here and summarized in figure 15. ITO coated glass slides were modified with poly-l-lysine by immersion in a 1:10 dilution of the provided stock solution for 5 minutes followed by overnight drying in a desiccator. Approximately $1 \times 10^6$ cells were plated onto the modified slides and allowed to settle overnight. Once the cells had settled, media was removed and replaced with 10% NBF for 10 minutes followed by two washes with PBS, two washes with Milli-Q water, and three washes with 150 mM ammonium acetate. The cells were stained with a 0.5% cresyl violet in water for 30 seconds followed by two water washes. The plate was allowed to dry for about 30 minutes before THAP matrix in 90% methanol, 9.7% water, and 0.3% TFA was spray coated over the surface of the plate using the TM-Sprayer. An area of interest was marked by carefully removing the matrix around the area and high resolution optical images were taken of the cells within this region for co-registration of the ion image. An ion image was finally collected using conditions that had been optimized for the sample.

The final method that was developed using HEK-293 cells was also applied to RKO cells to verify that the methods can be applied to other cell types. Figure 13 shows an ion image of the signal at $m/z$ 782 in RKO cells. In an exercise to compare the spectra from both cell types, images were generated from the signals at $m/z$ 725 and 825, which have a differential expression based on cell type. The signal at $m/z$ 725 was found only in RKO cells, while the signal
at $m/z$ 825 was found only in HEK-293 cells. This experiment is shown in figure 14. All of the mass spectral images show the same pattern of spectra within the cytoplasm but a lack of signal in the nuclear area. This appears to arise from the rigidity of the cell membrane caused by the fixation process.

![Figure 13: Ion and optical images of RKO cells. A) Ion image of $m/z$ 782 and B) optical image of sampled area. Lateral spatial resolution is 1.5 µm. Figure adapted from Todd et. al.\textsuperscript{14}](image)
Figure 14: Side by side comparison of images from m/z 825 and 725 in HEK-293 and RKO cells. Ion intensity scale bar for m/z 825 is 0-500, and for m/z 725 is 0-2000. Figure adapted from Todd et. al.

Figure 15: Summary of final method.

1. Modify ITO slide with poly-L-lysine
2. Plate 1 x 10^6 cells onto slide
3. Fix with 10% neutral buffered formalin
4. Wash twice with each of the following
   1. Phosphate buffered saline
   2. Water
   3. 150 mM ammonium Acetate
5. Stain with 0.5% cresyl violet
6. Apply THAP matrix using HTX TM-Sprayer
   1. Mark fiducials
7. Collect optical image using microscope
8. Collect ion image
Chapter IV

Conclusion

This work provides a novel approach to single-cell imaging and represents an unprecedented advancement for the field of IMS by showcasing the strength of high resolution imaging. The ability to attain lateral spatial resolution of 1.5 μm allows for many applications in bioanalytical fields. This work studied lipids in single cells grown in culture, but arguably, the lipid information gathered could have been obtained using lipid extracts and any other instrument. However, the work here proves that this high spatial resolution can be achieved with ease and with striking results. These methods can be applied to many other areas of research, including embryogenesis, cell differentiation, cell migration, and many more. Nevertheless, there are still developments that need to be made. The first issue to be addressed is mass resolution. The 4700 was modified to have a shortened flight tube, resulting in mass resolution far below the instruments original capabilities. Now that the technique has been optimized, a longer flight path could be incorporated, or the mass analyzer switched to one with higher resolution. The spectra in figures 11 and 12 show that the most abundant lipids visible using traditional MS instrumentation are also clearly visible from a single cell using the 4700. The mass resolution of the instrument impedes the ability to determine the lower limit of detection. Close inspection does indicate that the majority of peaks present in figure 12 are also present in figure 11 though they show significant broadening because of the poorer mass resolution.

This work has presented initial method development and optimization towards single cell imaging using a modified transmission geometry MALDI ion source on an Applied
Biosystems 4700. This required a great deal of research in four major areas: fixation, visualization, cell adherence, and matrix application. Once optimized, the final method was applied to cells to obtain ion maps of lipids distinct to single cells. In addition, lipid signals were used to show differences in expression between two cell types. This has provided one of the first applications for ultra-high spatial resolution imaging towards the analysis of single cells in the field of imaging mass spectrometry.
