DISEASE TISSUE IMAGING AND SINGLE CELL ANALYSIS
WITH MASS SPECTROMETRY

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Cells have been found to have an inherent heterogeneity that has led to an increase in the development of single-cell analysis methods to characterize the extent of heterogeneity that can be found in seemingly identical cells. With an understanding of normal cellular variability, the identification of disease induced cellular changes, known as biomarkers, may become more apparent and readily detectable. Biomarker discovery in single-cells is challenging and needs to focus on molecules that are abundant in cells. Lipids are widely abundant in cells and play active roles in cellular signaling, energy metabolism, and are the main component of cellular membranes. The regulation of lipid metabolism is often disrupted or lost during disease progression, especially in cancer, making them ideal candidates as biomarkers. Challenges exist in the analysis of lipids beyond those of single-cell analysis. Lipid extraction solvents must be compatible with the lipid or lipids of interest. Many lipids are isobaric making mass spectrometry analysis difficult without separations. Single-cell extractions using nanomanipulation coupled to mass spectrometry has shown to be an excellent method for lipid analysis of tissues and cell cultures. Extraction solvents are tunable for specific lipid classes, nanomanipulation prevents damage to neighboring cells, and lipid separations are possible through phase dispersion. The most important aspect of single-cell analysis is that it uncovers the extent of cellular heterogeneity that exists among cellular populations that remains undetected during averaged sampling.
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<td>AGC</td>
<td>Automatic gain control</td>
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<tr>
<td>CAA</td>
<td>Cancer associated adipocyte</td>
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<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
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<tr>
<td>Cer</td>
<td>Ceramide</td>
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<tr>
<td>CID</td>
<td>Collisional-induced dissociation</td>
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<tr>
<td>CPCD</td>
<td>Coupled physical and chemical dynamics</td>
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<tr>
<td>CRM</td>
<td>Charged residual model</td>
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<td>DAPNe</td>
<td>Direct-analyte probe nanoextraction</td>
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<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-Dihydroxybenzoic acid</td>
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<tr>
<td>DIMS</td>
<td>Direct inject mass spectrometry</td>
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<tr>
<td>EI</td>
<td>Electron impact</td>
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<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td>HCD</td>
<td>Higher-energy collisional dissociation</td>
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<td>HILIC</td>
<td>Hydrophilic interaction chromatography</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IEM</td>
<td>Ion evaporation model</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<td>LD</td>
<td>Lipid droplet</td>
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<td>LIT</td>
<td>Linear ion trap</td>
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<td>Description</td>
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<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
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<tr>
<td>MEKC</td>
<td>Micellar electrokinetic chromatography</td>
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<td>MS</td>
<td>Mass spectrometry</td>
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<td>MSI</td>
<td>Mass spectrometry imaging</td>
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<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>NP</td>
<td>Normal phase</td>
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<td>Nanoelectrospray ionization</td>
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<td>Oz-ID</td>
<td>Ozonolysis identification</td>
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<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PEA</td>
<td>Phenethylamine</td>
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<tr>
<td>PESI</td>
<td>Probe electrospray ionization</td>
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<tr>
<td>PG</td>
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<tr>
<td>PL</td>
<td>Phospholipid</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<td>QIT</td>
<td>Quadrupole ion trap</td>
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<tr>
<td>QMF</td>
<td>Quadrupole mass filter</td>
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<tr>
<td>RP</td>
<td>Reverse phase</td>
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<tr>
<td>SFA</td>
<td>Saturate fatty acid</td>
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<tr>
<td>SIMS</td>
<td>Secondary ion mass spectrometry</td>
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<td>SRM</td>
<td>Selected reaction monitoring</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion count</td>
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<tr>
<td>TOF</td>
<td>Time-of-flight</td>
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<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER 1

INTRODUCTION

1.1 Single Cell Analysis

Heterogeneity among populations of the same cell type exists as cell-to-cell variations in cell size, shape, and or chemical composition. Bioanalytical techniques for the extraction of an analyte of interest often involve the homogenization of tissue, whole cell-culture dish lysates, or other population sampling method. These methods results in averaging of data across the population sampled, spatial information is lost, and the inherent cellular heterogeneities go undetected. Cellular heterogeneities are of extreme importance as they are brought about in response to localized environmental changes, the onset of disease states, genetic mutations, and simple variation in time-scales of normal biological processes. Developing methods capable of monitoring and analyzing, and correctly associating them with the correct cause can eventually lead to more accurate disease diagnosis using less invasive methods of testing. Methods of single-cell analysis to study cellular heterogeneity have been developed using chemical and electrical sensors, microscopy, spectroscopy, separations/sorting, and mass spectrometry.

Microscopy is a widely used method for single-cell analysis to monitor morphological and chemical variations in large cellular populations. Label-free methods of optical microscopy allow for cellular morphologies to be analyzed using phase contrast to develop 3-dimensional images of single-cells with 100nm resolution.\[1\] Raman spectroscopy is an important label-free method for single-cell analysis capable

Single cell analysis is an important area of study that is developing very quickly across healthcare based research for single-cell analysis and detection of disease states. An electrochemical sensor designed to identify cancer cells based on alterations in morphology is currently in development. The sensor was used to successfully identify a single cancer cell within a co-culture with normal cells. [8] Identifying malignant cells in co-cultures has also been demonstrated with the use of a pH sensor. Altered energy metabolism, considered to be a hallmark of cancer, has been the focus for the development of a sensor that detects cancer cells in culture by measuring changes in glucose pH. [9]

High-throughput techniques for single-cell analysis within large populations have been developed based on sorting and separation techniques. Flow cytometry is a common method used for cell sorting but little chemical information is garnered. High-throughput single-cell analysis can be achieved using microfluidic devices and capillary electrophoresis (CE). Both of these devices can provide large amounts of chemical and genetic information about the cells. Raman spectroscopy, previously mentioned, can be implemented as a cell sorting technique that uses the chemical signatures identified by Raman signals in a cell.[3] The Raman signatures provide little to no structural information about the molecules that correspond to the signals. Unfortunately, each of
these sorting or separation methods suffer from lost spatial information when larger numbers of cells are separated and sorted into single cells from cell culture or tissues.

Mass spectrometry for single-cell analysis is beneficial for its low limits of detection, high sensitivity, and wide range of detectable biomolecule classes made available through multiple ionization methods and various mass analyzers. [4, 7, 10] Single-cell imaging mass spectrometry methods have been developed using matrix-assisted laser desorption/ionization (MALDI) [4, 11] and secondary ion mass spectrometry (SIMS). [4, 11] Nanomanipulation-coupled to mass spectrometry has been used for the extraction of single cells and organelles from cell culture and tissue samples. [12-14] Single-cell analysis methods are currently being developed with nanomanipulation for the identification of lipid-based biomarkers in biological samples, and will be expanded upon in chapters 4,5,snd 6.

1.2 Lipid Metabolism in Cancer

With the onset of lipidomics using mass spectrometry, lipids have been extensively studied and their roles in cells better understood.[15, 16] Lipids are important biomolecules found to have complex roles in the functionality and structure of cellular membranes, cellular signaling, energy storage, and are found to be vastly diverse and dynamic within the cellular lipidome.[16] Regulation of lipid metabolism is pertinent to maintaining normal healthy function and homeostasis of membranes in cells. There are many diseases associated with the lost regulation of lipid metabolism including obesity, dyslipidemia, diabetes, cardiovascular disease, and cancer.[17]
Reprogramming of metabolic processes within cancer cells is an accepted hallmark of cancer. [18] The increased rate of proliferation experienced by cancer cells is a result of un-regulated metabolism due to oncogenic events taking place in developing cancer cells. [19] Increased amounts of lipid species are required to maintain an increased rate of proliferation. Cancer cells satisfy their requirement for lipids through increased uptake of exogenous lipids or through increased lipid biosynthesis. Either method of lipid accumulation results in an alteration in the lipid profile of a cancer cell relative to its normal cell counterpart and can be used to identify cancerous cells among normal cells. [20, 21] This is the basis for single-cell analysis for lipid based biomarker discovery techniques developed around nanomanipulation-coupled to mass spectrometry. Some of the specific lipid changes that take place as a result of unregulated cancer metabolism have been well documented and provide a general rule of thumb for comparing the lipids of normal and diseased cells.

Fatty acid compositions of cellular lipids are often altered in cancer cells due to increased lipid biosynthesis. When fatty acids are needed to support the increased proliferation rate of cancer cells the post-modification of fatty acids is often cut short. This results in an increased composition of saturated fatty acids (SFA) such as palmitic (FA16:0) or stearic (FA18:0) acids. The newly synthesized fatty acids are then incorporated into larger lipids within the cell, such as phospholipids (PL) or triacylglycerols (TG). [22] Therefore, lipid profiling of suspected cancer and adjacent normal cells showing differences in fatty acid saturation could be used as a lipid indicator for cancerous cells. [17]
Determination of altered lipid metabolism associated with abundances in lipid class is at the forefront of many cancer lipidomic studies, more specifically, determining PL profiles.[22] Breast tumors have been identified as having much higher abundances of phosphatidylcholine (PC) species in their lipid membranes when compared to their normal counterparts.[23] Furthermore, the increased PC content when accompanied with an increase in SFA incorporation was an indicator of an established tumor.[23] Interestingly, the increase in SFA found in membrane lipids is characteristic of all tumors that experience altered lipid metabolism, attributed to reduced chemotherapy efficacy.[24] leads to an increase in membrane rigidity, and a decrease in membrane dynamics.[25] Other cancer types display increases in other PL classes and have been used to identify dysfunction in organelles such as mitochondria. Phosphatidylglycerol (PG) is a PL that also acts as a precursor to cardiolipin, a lipid that is exclusive to the mitochondria. Renal cell and hepatocellular carcinomas have each been shown to have increased PG in their lipid profiles is a strong indicator of improper functioning mitochondria.[26, 27] The extent of altered lipid metabolism and the resulting changes in lipid profiles is still being researched with an end goal of identifying lipid biomarkers that will be useful for early detection of cancer. Coupling these biomarkers to single-cell analysis future diagnostics will be performed on smaller sample sizes resulting in less invasive sampling techniques.

1.3 Lipid Extraction

The diversity of lipid structures leads to various levels of polarity and solvent compatibility for extraction. Therefore, a single method of extraction is not always the
most efficient method for the broadest extraction of lipids in a sample. The development of lipid extraction methods has been a continuing area of research since the late 1950's when the first most widely accepted whole lipid extract methods were introduced by Folch [28] and Bligh and Dyer. [29] These first two methods used a chloroform-methanol extraction of homogenized samples to extract lipids into the organic solvents. [28, 29] Water was used to induce phase separation and remove non-lipids from the extracted lipids. Folch included the water in with the organic solvents [28], however, Bligh and Dyer added water in a second step. [29] These methods were widely accepted for many years but new methods were developed to reduce toxicity and to simplify phase collections. A variation of the Bligh and Dyer and Folch methods was developed replacing chloroform with dichloromethane to reduce the solvent toxicity while achieving similar results. [30] The density of chloroform and dichloromethane cause the organic phase to settle below the organic phase. When extractions are performed in smaller volumes without a separatory funnel, the collection of the organic phase can be difficult. Non-lipid contaminants and proteins settle into an interphase layer that must be circumnavigated to reach the lower organic phase. When this interphase it pierced it is easy to inadvertently collect a small portion into the organic phase collected. Matyash et al. replaced chloroform/dichloromethane with the less dense methy-tert-butyl ether for lipid extractions. [31] With this method the lipids were extracted to the upper organic phase and easily collected without interaction of the interphase layer.

Single-phase lipid extraction techniques have also been developed that do not require a multi-step extraction procedure with the added benefit of reduced solvent
toxicity. Hara and Radin developed a single step method with hexane/isopropanol (3:2) followed with a sodium sulfate aqueous wash that was less toxic and less expensive.[32] The method also extracted fewer non-lipids but was inefficient at extracting gangliosides. Lofgren et al. describes another single-phase extraction method that uses a mixture of butanol/methanol in either a 1:1 or 3:1 ratio called the BUME method.[33] Reports indicate similar performance to the original chloroform/methanol procedures for total lipid extraction. The continued development of solvent based method for lipid extractions that are less toxic and require fewer steps is of great benefit for blossoming single cell analysis methods as the sample volumes are on the microliter to nanoliter scale. These small volumes do not have the luxury of multiple extraction or washing steps.

1.5 References


CHAPTER 2

INSTRUMENTATION

2.1 Mass Spectrometry Instrumentation

The field of mass spectrometry is continually pushing forward to increase the sensitivity, resolution, and robustness of its instruments. While at the same time new methods are always being introduced to expand the breadth of sampling to further increases scientific understanding of complex systems. The three necessary pieces for sample analysis with mass spectrometry still perform the same tasks, only better. An ionization source is still used to initiate gas phase ion formation of analytes. The mass analyzer then selects, traps, and/or separates the analytes based on their mass-to-ratio. Ultimately, the ions are sent to the mass detector to determine the abundance of each ion as they exit the mass analyzer. Only the instrumentation that was used to conduct the research of this dissertation will be presented here.

2.1.1 Quadrupole

Commercial development of the linear quadrupole mass spectrometer provided widespread use through the production of instruments that could be operated by laboratory technicians rather than designers and builders of the instruments. A linear quadrupole is a mass analyzer with four identical-parallel metal rods with hyperbolic or circular cross-sections and configured in a square formation.[34] A quadrupole electric field is created when equal and opposite potentials of direct current (DC) and radio frequency (RF) are applied to oppositely positioned rods. Maintaining a fixed DC/RF ratio while ramping the voltages up or down from a minimum to a maximum value
enables the quadrupole to be used as a scanning mass analyzer. Ions of a specific $m/z$ are associated with a certain set of potentials applied to the DC and RF. At a given set of potentials applied to DC and RF, ions of a single $m/z$ travel to the detector via stable trajectories while other ions endure unstable trajectories and collide with the rods before they reach the detector.

When operated in RF only mode quadrupoles act as ion guides and transmit all ions within a mass range. Furthermore, in RF only mode quadrupoles are effective at ion transmission across varying pressure regions making them useful for ion transmission from atmospheric pressure ion sources to vacuum regions of mass spectrometers. However, quadrupole ion guides can be limited in effective transmission of large mass ranges. Therefore, variants have been developed using six or eight rods, hexapole and octapole, respectively. These ion guides allow for ions across a larger $m/z$ range to be transmitted. [35]

2.1.2 Ion Trap

The quadrupole ion trap (QIT) also referred to as a 3D trap or Paul trap is based on the same principles of the quadrupole mass filter (QMF) described by Wolfgang Paul, for which he was awarded the Noble Prize in Physics in 1989. [36] The trap is constructed of three hyperbolic electrodes, a ring electrode set between two identical end-cap electrodes. Ions are trapped within a quadrupolar field of three-dimension created by applying RF voltage to the ring electrode while keeping the end-caps at ground. Ions of increasing $m/z$ are ejected from the trap to the detector by ramping up the RF amplitude causing instability of an ion’s axial trajectory. [37, 38] Ion guides are
typically used to direct ions from an ion source into the trap. To increase trapping efficiency, the trap is filled with a dampering gas to collisionally cool the ions and localize them to the middle of the trap. Helium at a pressure around 1 mTorr is typically used to maintain a good balance between mass resolution and trapping efficiency. [38] Ions confined in a trap often suffer from space-charge effects when too many ions are present. To control this, modern instruments implement an automatic gain control (AGC) to limit the maximum number of ions present in a trap at any given time. This in turn can create a competitive environment that can cause ion suppression making the QIT semi-quantitative. [37]

A more recent ion trap, the 2D or linear ion trap (LIT), resembles the quadrupole mass filter more closely than the 3D QIT. Similar to the QMF, previously described, four hyperbolic rods are arranged in a square formation that can be either segmented or fitted with lenses on either end. Ions are kinetically cooled by dampering gas and trapped radially by an applied RF voltage and axially by DC voltages applied to the end segments or lenses. LIT can be used as stand alone mass analyzers or coupled to other mass analyzers. Furthermore, the LIT can be configured to eject ions either axially or radially. Axial ejection of ions through the end lenses/segments occurs when AC voltage is applied between the end lenses and rods. However, this form of ejection is inefficient as only the ions that are near the lens are ejected. Alternatively, radial ejection of ions is through slits cut into two opposing rods within the middle of a segmented LIT. When equal DC potentials are applied to the end segments trapped ions are directed towards the center of the trap. Ramping of the RF potential of the rods ejects ions radially at increasing m/z through the slits. When two detectors are utilized,
one near each slit, the ejection efficiency is near 50%. Some advantages of the LIT over the 3D QIT are increased ion capacity and reduced space-charge effects, both as a result of the larger trap volume. Furthermore, the LIT offers greater trapping efficiency, [39] increased sensitivity, and a larger dynamic range than the 3D QIT. [35]

The dampening gas in the 2D and 3D quadrupole traps acts to kinetically cool the ions, as previously stated, but it is also used as the collisional gas for fragmentation during tandem mass spectrometry experiments, MS/MS. [35] Upon fragmentation of a selected precursor ion all fragments are trapped and can be ejected to the detector or a single fragment mass can be isolated and undergo further fragmentation. This process can be repeated for subsequent fragment ions to perform multiple tandem mass spectrometry experiments, MS^n.

2.1.3 Orbitrap

The most recent type of ion trap, the orbitrap, uses electrostatic fields only to trap ions [40] and is based on the Kingdon trap.[41] Developed and described by Alexander Makarov, [42] the orbitrap was introduced into the commercial market in 2005 by Thermo Electron Corporation. The trap is composed of an inner spindle-shaped electrode surrounded by a barrel-shaped outer electrode.[42] The outer electrode is floated at ground while a constant DC voltage is applied to the inner electrode. Ions requiring a certain kinetic energy are injected at increasing m/z into the space between the two electrodes and immediately begin orbiting around the inner electrode. These ions undergo three types of motion in the trap, radial, angular, and axial oscillations. A mass spectrum is produced by fast Fourier-transform of the frequencies created as ion
packets oscillate along the z-axis. Electrostatic acceleration lens, a linear ion trap, or a C-trap can be used to achieve the successful injection of ions into the orbitrap for analysis. Before injection, ions can undergo precursor fragmentation in the C-trap or in an adjacent octopole collision cell, known as higher-energy collisional dissociation (HCD). Fragmentation can also be performed in the LIT, MS^n, for structural elucidation of ions of interest. Orbitrap instruments boast mass resolution of >500,000 full width half mass (FWHM) and mass accuracy below 2 parts per million (ppm) at m/z 200. Orbitrap instruments can be coupled to ESI and MALDI sources as well as inline with liquid chromatography.

2.1.4 Collisonal-Induced Dissociation (CID)

One method of tandem mass spectrometry (MS/MS) uses the dissociation of ions to obtain structural information, analyze complex mixtures, or for quantitative analysis in specific applications. Collisional-induced dissociation (CID) is a method of ion activation used in tandem mass spectrometry in which ions are fragmented through collisions with target molecules. [43] Generally, a precursor ion is mass selected then electrostatically excited to increase its kinetic energy and allowed to collide with a target molecule of a neutral gas such as nitrogen or helium. The kinetic energy of ions during CID in QIT and linear quadrupole instruments ranges from 1-100 eV and is considered low-energy CID. The collision between a precursor ion and target molecules causes a conversion of precursor ion energy from kinetic to internal. If the internal energy is large enough molecular dissociations at the most substituted site(s) within the precursor ion produces product ions.
Depending on the instrument, MS/MS through CID can be performed in time or in space. MS/MS-in-space instruments employ two mass analyzers physically separated by a collision region, as in the triple quadrupole mass spectrometer. The mass analyzers can be set to scan across a mass range or select for ions of a specific m/z. Inert gas is flowed into the differentially pumped non-discriminant RF-only multipole collision region. QIT instruments operate as MS/MS-in-time instruments where precursor ion mass selection, dissociation, and product ion trapping all take place in the same physical space at different time intervals. A benefit of the QIT instruments is that product ions can be mass selected as precursor ions for additional fragmentation events extending MS/MS to MS\(^n\), where a practical value of \(n\) can reach eight.\[35\] In addition As a result of lost ions during transmission from one physical region to another, sensitivity is lower in MS/MS-in-space relative to MS/MS-in-time.\[36\]

There are four common scan modes that utilize CID for mass spectral analysis: product ion, precursor ion, neutral loss, and selected reaction monitoring (SRM). During a product ion scan the first mass analyzer is set on a specific m/z for fragmentation and all product ions are scanned for in the second analyzer. The first mass analyzer in a precursor ion scan sends all ions across a mass range to the collision region and the second analyzer is set to scan for ions of interest with single m/z. Neutral loss scans require both analyzers to synchronously scan across a mass range. However, the second analyzer is set to scan for product ions that are equal to the m/z of the precursor ion minus an expected neutral loss mass. Product ion, precursor ion, and neutral loss scans can only be performed in MS/MS-in-space instruments. The final scan type, SRM, can be performed in both MS/MS-in-space and MS/MS-in-time instruments. The
first mass analyzer selects a single m/z for the precursor ion of interest while the second analyzer is set for a single known and expected product ion. Quantitative analysis is possible from an SRM scan when using an MS/MS-in-space instrument.[36] SRM is capable of monitoring multiple ion fragmentation events, however, SRM is interpreted as single reaction monitoring, in such a case, multiple reaction monitoring (MRM) is then used to describe the scan used to monitor the dissociation of more than one precursor ion of interest.[36]

CID is routinely used for structural determination of biomolecules when coupled with soft ionization sources (see section 2.2) as parent molecules remain intact during ionization. Intact molecular ions are especially important when analyzing lipid samples to determine lipid profiles. CID has been used for the MS/MS and MSn analysis of lipids leading to known fragmentation pathways providing rhetoric for the confidant identification of unknown lipid species in samples.[44] Product ions of MS/MS analysis of unknown lipids can be used for lipid class identification. Further fragmentation allows for fatty acyl chain length determinations for many lipids classes improving the level of identification. However, numerous isobaric species and structural isomers provide a deeper complexity to identifying the molecular structure of lipids.[45] For example, the molecular structure identification of a glycerophospholipid is complete when the locations of all double bonds along an acyl chain and the stereospecific number (sn) location of the acyl chain on the glycerol backbone have been determined. Using intrap reactions with ozone, Oz-ID provides identifications of double-bond location along an acyl chain [46] and sn positions.[47] Acyl chain sn position has also been determined through the analysis of resulting product ion mass spectra of charge-remote
fragmentation using high-energy CID (>1keV).[48] Recently, structural identifications have been achieved with the use of high-energy CID for MS/MS analysis coupled with subsequent low-energy CID, equating to an MS$^3$ experiment. [49]

2.2 Ionization Sources

The 2002 Nobel Prize in chemistry was awarded for the development of three important methods for the identification of biomolecules, two of which were soft ionization techniques for use with mass spectrometry. Soft ionization allows for the ionization of analytes in their intact molecular form.

2.2.1 Electrospray Ionization

The ground work for electrospray ionization was laid by Malcolm Dole in 1968 during experiments in which Dole attempted to ionize large polymer molecules, however, John Fenn is credited with the development of electrospray ionization[50, 51]. The 2002 Noble Prize in chemistry was awarded to Fenn for his 1984 demonstration of ESI by identifying proteins on low mass instruments (< m/z 4000) as multiply charged ions [35]. Fenn’s work opened the doors for the analysis of nonvolatile small and large molecules, including macromolecules that far exceed the m/z range of an instrument [36]. ESI is compatible with various types of samples delivering solvated ions into the gas phase producing singly and multiply charged ions of analytes that are typically dissolved in a polar solvent [50]. Gas-phase ion generation by ESI results after a series of three events at atmospheric pressure: charged droplet formation, reduction of charged droplet size, and formation of gas-phase ions [50].
Solutions for ESI are prepared by dissolving an analyte in a solvent and adding a charge carrier such as acetic acid or ammonium acetate to increase the electrolyte concentration of the solvent and aid in charge formation of less volatile analytes. Using a syringe pump with a flow rate of 1-20μL delivers the sample through a fused silica capillary that is in contact with a metal needle. A voltage, 3-5.5kV, is applied to the needle inducing charge separation within the solution. As the like-charges build up the solution exiting the silica is distorted and a Taylor cone is formed. As the liquid in the tip of the cone experiences increased charge repulsion the surface tension of the solvent is overcome and a series of multiply-charged droplets burst forth from the cone as a fine spray. A coaxial sheath gas acts to dry the droplets by evaporating the solvent and decreasing the droplet radii until Coulombic fission occurs. The droplets continue to shrink and fission as they are attracted to the counterelectrode situated at the inlet of the mass spectrometer. Before entering the vacuum region of the mass spectrometer the droplets pass through a heated capillary. Droplets are further desolvated by the high heat, and collisions within the capillary take place to break up clusters that may form between ions and neutrals [52]. Droplet fission repeats until single ions are produced from the droplets before entering the vacuum region of the mass spectrometer. The event that describes the formation of gas-phase ions from evaporating charged droplets can be described by two models. The Ion Evaporation Model (IEM), developed by Iribarne and Thomason, suggests that when droplets reach a certain size, charged ions aligned near the surface of the droplet are ejected during fission forming gas-phase ions directly from a solvated droplet. While the charged residual model (CRM) proposes single ions are formed as a result of continued fission and complete desolvation of the
droplet. Large molecules are believed to follow the CRM while smaller molecules associate with the IEM.

2.2.2 Nanoelectrospray Ionization

Wilm and Mann developed Nanoelectrospray ionization (NSI) in the mid 1990’s and reported greater ionization efficiency as well as ion transfer efficiency. [53] NSI shares many similarities to ESI in that a voltage (as low as 600 V [53]) is applied and charged droplets spray from a Taylor cone that undergo the same process of desolvation and fission to form gas-phase ions for introduction into the mass spectrometer. In ESI an external pump delivers solution at a rate of 2-20 µL/min, while NSI uses no external pumps and achieves an electrospray driven flow rate of 20-40 nL/min [53]. The spray orifice diameter for ESI is on the order of 100’s of µm and 1-2 µm for NSI [54]. The smaller orifice coupled to the slower flow rate create initial charged droplets with radii of roughly 200 nm [54], compared to ESI droplets of 1-2 µm [53]. These changes in NSI lead to more efficient gas-phase ion generation, tolerance to increased salt content in solutions, sample volumes in the low nano-liters, and reduced sample preparation required for mass spectral analysis [53-55].

2.2.3 Matrix Assisted Laser Desorption/Ionization

In the late 1980’s, two separate researchers, Hillenkamp and Tanaka, each reported the use of an organic matrix and laser desorption for analysis of proteins using time-of-flight mass spectrometry (TOF-MS). [56] Matrix assisted laser desorption/ionization (MALDI) is a soft ionization technique that creates intact molecular
ions that are predominantly singly charged. Analyte and matrix are co-mixed then spotted on a plate, allowed to dry, and then loaded into the MALDI source. The matrix concentration is much greater than the analyte concentration,[57] typically on the order of 10,000:1. [56, 58] Typically, a UV or IR laser is employed to liberate molecules of both matrix and analyte from the plate during a laser ablation event. The released molecules travel away from the plate at high speeds creating an expanding plume above the plate where primary and secondary ionization mechanisms take place.[59] Surprisingly, as an ionization method that has been in use for nearly three decades no single explanation of the ionization mechanism has been widely accepted. [56] However, two proposed mechanisms have garnered the most attention for their plausibility.[58] The Coupled Physical and Chemical Dynamics (CPCD) model states that neutral analytes undergo charge transfer with protonated molecules in the plume to give protonated analyte ions.[60] A more recently suggested mechanism called the Lucky Survivor model states that matrix molecules form clusters around pre-charged analytes.[61] This model describes large peptides and proteins that have multiple protonation sites arising from sample preparation with strong acids. The multiple protonated sites provide an increased chance of a pre-charged analyte surviving the often-neutralizing cluster dissociation event perpetuated by protonated matrix molecules in the plume.

The selection and application of matrix is an important aspect of any MALDI experiment. MALDI matrices have been categorized into metal or particle, liquid, ionic-liquid, and solid. [56] The properties of the solid category are ideal for this body of research and focus will be on their properties. The choice of matrix is dependent upon
several factors and therefore there is not an individual matrix that fits every application, but in some instances there is a matrix that has been found to perform well across a class of molecule. 2,5-dihydroxybenzoic acid (2,5-DHB) is a commonly used organic solid matrix for large and small biomolecule MALDI experiments [62] and is considered to be the matrix of choice for positive mode MALDI-imaging of lipids. [63] Early MALDI experiments described uniform crystal formation and near

A matrix must have an efficient absorption at the laser wavelength, most commonly 337 nm,[57] which is achieved in organic matrices with an extended aromatic conjugated $\pi$ system to absorb laser energy.[64] The matrix should have a propensity for ionization of the analyte of interest. [63] Stability in the MALDI source is important for longer sampling times like MALDI-imaging experiments.[63] The matrices of the solid category solubilize readily into solvents and form crystals upon evaporation of the solvent. [56] Ideally the resulting crystals should be uniform in their distribution and size to prevent spots of high or low pockets of analyte signal [64-66] that can hinder signal reproducibility. [56] Crystal size is also important and can influence the quality of your data. In MALDI-TOF ions should have the same initial kinetic energy for mass analysis and this is provided with large flat crystals. However, vaporization occurs more readily with smaller crystals. A consideration about MALDI selection that can greatly affect the analysis of low mass analytes, less than $m/z$ 1500, where matrix associated peaks have been shown to interfere with analyte signal. [64, 67, 68] Methods have been employed to reduce matrix-interference in low mass MALDI through matrix additives. [69, 70]

Matrix selection still needs to be considered carefully, however, the application of that matrix requires just as much thought. Keeping focus on the solid matrices, the dried
spot method is a common choice. However, the application of matrix solubilized in a solvent can cause the delocalization of analytes across a tissue sample producing false hot spots. [58] Furthermore, using the dried spot method on MALDI plates can also result in unwanted aggregation and deposition of sample and the spot dries, also known as the coffee-ring effect. So use of the dried spot method should require considerable thought. One method used to overcome the coffee-spot method involves cooling the MALDI plate to roughly 5°C before spotting the co-mixture. [71] Furthermore, solid matrix can be crushed to a fine powder and can be applied by sublimation.

Although MALDI was initially developed using large biomolecules such as proteins and peptides, it has proven to be a successful technique for lipid analysis especially in MALDI-imaging of lipids. [62, 72, 73] MALDI-imaging provides analyte-specific spatial distributions across the surface of a sample. The method provides a means of mapping analytes of interest on the surface of a sample at user defined spatial increments[63]. The laser irradiates the sample while scanning across the tissue at predetermined step sizes while a mass spectrum is generated unique to each position, generating a single pixel. All pixels are compiled using imaging software that is then used to create a heat map of the spatially distributed analyte(s) of interest. MALDI-imaging using this technique is commonly implemented for lipid analysis of biological systems to determine links between normal and diseased health states and how lipid distributions may help identify aberrant cellular activity. [63]
2.3 Nanomanipulation

Chemical analysis of forensic and biological samples through precise localized sampling with nanomanipulation techniques coupled to mass spectrometry has been demonstrated to be a relatively non-destructive technique. [12-14, 55, 74-81] An L200 nanomanipulator, originally developed by Zyvex (Richardson, TX) but currently owned by Thermo Fisher Scientific, was used throughout this research. The nanomanipulator can equipped with up to eight probers, each can be fitted with microgrippers for physical manipulation of cells, microelectrodes for cell membrane characterization by low-impedance measurements, nanospray capillary tips for mass spectrometry analysis, and borosilicate or quartz capillaries or rods for cellular probing. Palladium-coated nanospray capillaries with tip diameters of 1.0 ± 0.2 µm were purchased from New Objective. An in-house P-2000 CO2 laser tip puller from Sutter Instruments is used for pulling capillaries and rods of varying tip diameters. A single joystick with X, Y, and Z directional control, operates all of the probers individually via piezoelectric, with 100nm of resolution in coarse mode and 5.4 nm resolution in fine mode. Total travel distance for each nanopositioner is 28 mm along the Y-axis and 12 mm along both the X and Z-axes. The nanomanipulator can be mounted to an 8X multi-zoom microscope equipped with 2x or 5x long working distance objectives, or to an inverted microscope with 10x, 40x, or 100x objectives. A nitrogen gas pressure injector is coupled to the nanomanipulator for injection of solvent or specific chemistries into cells and aspiration of cellular chemistries when probers are fitted with a capillary tip. The coupling of the pressure injector to the nanomanipulator has been used to demonstrate true single-call chemical analysis of tissue sections, in vitro organelle extractions, and MALDI imaging.
of extracted organelles. The nanomanipulation technique demonstrated in the previous examples was found to be non-destructive to the surrounding sample allowing for subsequent extractions of adjacent cells. Not only limited to biological applications, nanomanipulation has also been shown to be non-destructive in forensic chemical analysis as well as ink analysis of unique documents.

2.4 References


[74] Clemons, K.; Dake, J.; Sisco, E.; Verbeck, G. F.; Trace Analysis of Energetic Materials Via Direct Analyte-Probed Nanoextraction Coupled to Direct Analysis in


to Profile Fatty Acid Composition of Triglyceride Species. Journal of Analytical Oncology 5, 47-54 (2016)
CHAPTER 3
A COMPARATIVE ANALYSIS OF NINE POPULAR SOLVENT-BASED LIPID EXTRACTION METHODS TO IDENTIFY LIPID SPECIFIC AFFINITY FOR DIRECT EXTRACTION AND INJECT MASS SPECTROMETRY TECHNIQUES

3.1 Abstract

Extracting lipids from a variety of biological samples has traditionally been performed using multi-step methods coupled to separation-mass spectrometry techniques. Recently, smaller sample sizes direct analysis, imaging, and single cell extractions can be performed in the sub µL sample volumes. Examining lipids on the sub-µL level provides a glance into localized lipid metabolism and heterogeneity within single healthy and diseased cells, and concentrated regions of tissue. A comparative study of nine solvent systems was conducted to evaluate lipid extraction efficiencies of eight lipid classes to identify systems that extract both polar and non-polar lipids effectively within a single tip technique, Direct-Analyte Probe Nanoextraction (DAPNe). A 1:1 isopropanol:dichloromethane solvent system was found to extract the broader range of lipids, and have a higher affinity for polar lipids than other solvent systems tested. Furthermore, identified here are solvent system affinities for specific individual lipid classes enabling the selected extraction of a particular lipid class rather than a total lipid extraction. The application of this solvent selection is finally demonstrated in a DAPNe single cell analysis of triacylglycerols in differentiated murine adipocytes.
3.2 Introduction

Adipocytes are cells that comprise the majority of adipose tissue and are specialized for the storage of fat as triacylglycerol (TG) within organelles called lipid droplets. Adipocytes play many roles in regulating healthy weight and metabolism through the regulation of fatty acid oxidation, lipolysis, and through regulation of glucose uptake [82]. Through the secretion of signaling molecules, such as adipokines, growth factors, and cytokines, adipocytes exhibit paracrine and endocrine functionality during states of inflammation and disease [82-84]. Adipocytes located within close proximity to tumors have been identified as having a support role within the tumor microenvironment, and therefore, are named cancer-associated adipocytes (CAA). CAA undergo lipolysis and release their stores of TG as free fatty acids that are then transported out of the adipocyte where they become available for uptake by adjacent tumor cells. Recently, the heterogeneity of cellular TG in the lipid profile of healthy and cancerous human breast tissue has been identified [85], as well as among cotton seed cells and even organelles within the same cell [86]. Identifying the reason for this heterogeneity amongst organelles and cells is of great interest, thus it is important to investigate metabolic pathways at the specific site of activity, in which a small sample may be present only.

Single cell analysis of lipids has been achieved through the use of Raman microscopy, however, the information collected is only on the presence of a lipid class and not the specific lipid species [86]. However, mass spectrometry has proven to provide lipid specifics regarding the species, the acyl chain length, and the presence of unsaturation, making it the better choice for single cell analysis. Separation mass
spectrometry has been typically utilized for lipid analysis of tissues and cell cultures. However, if analyzing a single cell, or reduced group of cells, the sampling size for single cell analysis is around 10 µL, which is not conducive for most separation techniques. Separation methods also alter the configuration of lipid chemistry within a sample as the sample interacts with the separatory device. To prevent these unwanted interactions, and maintain the configuration of the cellular chemistry, a direct inject method is preferred for single cell analysis.

To achieve a direct inject method, a nano-extraction of the cell is required and is achieved with the use of a nanomanipulator, which has been described elsewhere[85]. The use of the nanomanipulator has provided the means necessary to isolate and extract single cells and single organelles of both plant and animal origins [85, 86].

The extraction of lipids from biological tissues and whole cells in culture has been well documented with the use of multi-step methods [87-99] have been modified to achieve better yield and extraction efficiency [90, 94-96, 100-111]. Lipid extraction solvent systems have been studied to determine extraction specificity across varying biological sample sets [87, 88, 90, 91, 94, 95, 98, 101, 105, 110]. Due to the diverse lipid composition of varying degrees of the polarity of lipids found in biological samples a universal solvent system has yet to be identified.

Traditionally, lipid extraction solvent systems are composed of an organic solvent combined with a low weight alcohol. The Folch extraction method utilizes a 2:1 chloroform:methanol (CHCl₃:MeOH) mixture [87], and has been widely used since it was first reported in 1957 [88, 89, 93, 96, 98, 105]. Many modified variations of this solvent system have been tested to determine an optimal ratio of CHCl₃: MeOH that provides
the most complete extraction of lipids from a sample [88, 90, 98]. Due to the toxicity and harmful effects of chloroform, dichloromethane has also been utilized for lipid extractions and has shown similar results to chloroform [92, 107].

Many extraction methods currently in use are designed to extract large percentage yields of lipid species through multiple extraction and purification steps [94]. For single cell and small volume (<1 µL) direct inject analysis, multistep extraction methods are not feasible as the solvent volume and overall sample sizes are extremely limited. Therefore, a single step extraction in which a chosen solvent system is capable of extracting multiple lipid classes is optimal for single cell analysis. Furthermore, with the right solvent system specific lipid classes can be extracted over others, specifically to identify the ratio of eicosanoids to fatty acids in cancerous tissue [112-114].

Our lab has been performing single whole-cell and single organelle extractions with CHCl₃:MeOH as a solvent with much success for the extraction of triglycerides. Erickson reported that 70% of the lipids extracted with a 2:1 MeOH:CHCl₃ system are triglycerides, while only 17% are phospholipids [95]. Furthermore, due to its high affinity for neutral lipids a CHCl₃:MeOH system is not efficient at extracting the more polar lipids. Therefore, we have provided an evaluation of the total lipid extraction efficiencies of nine solvent systems based on the number of lipid classes extracted from a standard solution using multi-phase methods. The goal of this study was to identify a highly efficient system capable of extracting non-polar as well as polar lipids, followed by a demonstration of this solvent system in a single-phase nano-extraction of a single cell.
3.3 Methods

3.3.1 Materials

Chloroform (CHCl₃), dichloromethane (CH₂Cl₂), ethanol (EtOH), ethyl acetate (EtOAc), hexane (Hex), isopropanol (IPA), and methanol (MeOH) are of LC grade and were purchased from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. Phosphatidic acid (PtdOh14:0/14:0), phosphatidylcholine (PtdCho 14:0/14:0), phosphatidylethanolamine (PtdEtn 14:0/14:0), triglyceride (TAG 17:0/17:0/17:0) and free fatty acid (FFA) (16:0) were purchased from Sigma Aldrich. Phosphatidylglycerol (PtdGro) (14:0/14:0), phosphatidylserine (PtdSer) (14:0/14:0), and ceramide (Cer d18:1/14:0), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All standards were reconstituted in a 1:1 CHCl₃: EtOH mixture at concentrations of 5mg/mL. To perform single cell DAPNe, an L-200 nanomanipulator (DCG Systems Inc., Fremont, CA) equipped with 1-μm inner-diameter (ID) emitter tips was used.

3.3.2 Sample Preparation

Nine 10mL glass screw cap vials were prepped with a tin foil lining in the cap and labeled. To each vial, 1mL of Phosphate Buffered Saline (PBS) and 20 nmol of each lipid standard were added. Vials were then flushed with nitrogen and placed on ice until extraction. Cell cultures of 3T3-F4882A murine adipocytes were cultured and differentiated according to the methods described by Elfakhani et al. [115].
3.3.3 Solvents

Nine extraction solvent mixtures composed of one organic solvent and one alcohol were selected for evaluation. The solvent mixtures were 1:1 CH$_2$Cl$_2$:MeOH [92], 2:1 [87], 1:1 [88], and 1:2 [90]; CHCl$_3$:MeOH, 1:1EtOAc:EtOH [105], 1:1 CH$_2$Cl$_2$:IPA, 1:1 CHCl$_3$:IPA [95], and 3:2 Hex:IPA [91]. Only glass pipettes and vials were used during the extractions to prevent the introduction of unwanted plasticizers.

3.3.4 Extractions

All total lipid extractions were carried out using the following method. First, 2 mL of the extraction solvent mixture was added to each vial, flushed with nitrogen, and sonicated for 20 minutes. After sonication the sample was returned to ice to allow the solution to separate. The solution was remixed, sonicated for an additional 5 minutes, placed on ice, and 1mL water was added. Vials were inverted 10 times to ensure proper mixing. Once the phases separated, the organic phase was aspirated and placed in a new screw cap vial, flushed with nitrogen, and returned to the ice. A second aliquot of solvent extraction solution was added to the remaining aqueous phase, inverted 10 times, and returned to the ice. The resulting organic phase was again aspirated and added to the first organic phase. The organic phase is dried with nitrogen and the lipids were reconstituted in 1mL of their respective solvent system. Reconstituted samples were then diluted 1:1000 (v:v) in solutions that are comprised of the extraction solvent with 0.1% ammonium acetate added to facilitate analysis via electrospray ionization – mass spectrometry (ESI-MS). The murine preadipocytes were
cultured and differentiated as described by Elfakhani et al. [115] and were extracted following the methods described by Phelps et al. [85].

3.3.5 Mass Spectrometry

Mass spectra are collected using an LCQ Deca XP Plus (Thermo Scientific, Waltham, MA, USA) equipped with an electrospray ionization (ESI) source. Samples are introduced to the ESI source at a flow rate of 3 µL/min using the built in syringe pump. Spray voltages of +3.0 kV and -2.5 kV were employed for positive and negative modes, respectively. In both modes, the capillary temperature was 250°C and sheath gas was set to 8 (arbitrary units). The resulting spectra were analyzed and given a rating of 1 to 6 corresponding to the level of extraction efficiency.

3.3.6 Application

To determine the effectiveness of the 1:1 CH₃Cl:MeOH system in a real application, TG and phosphoglycerolipids of cells in culture were targeted for extraction. Differentiated 3T3-F442A murine adipocytes were cultured for lipid droplet growth. Upon maturation the lipid droplets of single cells were extracted using DAPNe and analyzed though the use of nanospray ionization mass spectrometry (NSI-MS). The lipid profiles were determined through the relative abundance of all of the peaks in the 750 – 1000 Da range. This spectral range was selected to include glycerolipids and phosphoglycerolipids only to determine the effectiveness of extracting both of these lipid classes, as they are important to adipocytes. The lipid profile of each individual cell was then compared against other cells within the same culture dish to determine lipid
heterogeneity. It was determined that lipid heterogeneity was present from cell to cell indicating that the de novo lipid synthesis is dynamic throughout a cell population. The lipid heterogeneity of two murine adipocytes can be seen in Figure 2 when comparing the lipid abundances of each of the spectra. The lipid distribution for each individual cell is the indicator of lipid heterogeneity among the cells.

3.4 Results

3.4.1 Evaluation and Grading

The mass spectra of all the extraction solvents are evaluated for their efficacy in lipid extraction by comparing their relative peak abundances. First the relative abundances are determined, relative noise is subtracted, and finally the relative abundances are adjusted for the noise-subtracted-spectrum and evaluated based on a rating system. The rating system assigns a numerical value of 1 to 6, where 1 corresponds to a relative peak abundance of > 40% while 6 corresponds to <1% abundance. Furthermore, 1%-10% is rated a 5, 10%-20% a 4, 20%-30% a 3, and 30%-40% a 2. The rating system is demonstrated in Figure 1 showing both the 1:1 IPA:CHCl₃ positive spectrum and the EtOAc:EtOH negative spectrum as examples. The summation of all extraction ratings for a single spectrum gives the total score for that extraction solvent system for either positive or negative mode. The overall extraction efficiency score is then determined by adding the positive and negative polarity scores for the solvent system as seen in Table 1.
Figure 3.1  Example of the extraction efficiency rating system, a.) 1:1 IPA:CHCl3 positive spectrum, and b.) 1:1 EA:EtOH negative spectrum.
The first observable trend in Table 1 shows an absence of the TAG peak across all solvent systems in negative mode. However, in positive mode the TAG peak is the most abundant for all the solvent systems except the IPA: Hex system. In negative mode, the MeOH containing systems have a poor efficiency of phospholipid extraction, each with a relative abundance <10% for all phospholipids except PtdGro. In contrast, these same systems extract the same lipids in the positive mode with a relative abundance >10%. Additionally, the MeOH systems extract Cer at or above 20% relative abundance in the negative mode and between 1% and 20% in the positive mode in both polarities.

The three of the four IPA systems extracted all lipid classes except TAG in negative mode and FFA in positive mode. The IPA: Hex system in negative mode has the same extraction efficiencies as the MeOH systems. While in positive mode the IPA: Hex system resembles the other IPA systems, except for its poor extraction efficiency of PtdGro and PtdEtn. Lastly, the EtOAc: EtOH system extracted six of the eight lipid classes at an abundance at least >1%, in negative mode. In the positive mode seven lipid classes were present and four had abundances >40%. Lin et al. reported that the total lipid extraction percentage from egg yolk was the same for both a 2:1 CHCl₃: MeOH mixture and a 2:1 EtOAc:EtOH mixture[105]. However, it was observed that the EtOAc: EtOH system had a higher extraction efficiency than the 2:1 CHCl₃: MeOH system, furthermore, these two systems do not share affinities for specific lipid classes. Differentiated murine adipocytes were extracted as an application of the results of the extraction solvent study. The solvent system selected was the 1:1 CHCl₃: MeOH system due to its high affinity for TAG.
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<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
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<td>1</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
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<td>59</td>
<td>59</td>
<td>63</td>
<td>64</td>
<td>67</td>
</tr>
</tbody>
</table>

Rating System: 1 - excellent, 2 - good, 3 - fair, 4 - poor, 5 - absent from spectrum
The adipocytes are rich in TAG and therefore, the heterogeneity of the cells would be identified within those lipids. Upon examination of the spectra, it was determined that there was in deed heterogeneity among the cells extracted, Figure 2. It should also be noted that the phosphoglycerolipids, PtdCho, were extracted as well. The PtdCho abundance of the cells also demonstrated a heterogeneity, which was also observed, however, the PtdCho abundances also correlated to the size of the cell. As seen in Figure 2 the PtdCho peaks were greater in number and relative abundance for the larger of the two cells, while in the smaller cell there was a smaller ratio of PtdCho to TAG peaks. The results from the application demonstrate the lipid specificity of the solvent system that was selected for the adipocytes.

3.5 Discussion

Overall, the solvent systems were all found to be highly efficient at extracting triglycerides in the positive mode as observed by Erickson [95]. It was also observed that the CH$_2$Cl$_2$ substituted systems had extraction ratings that matched, if not surpassed, their CHCl$_3$ counterparts, as Chen reported [92]. The IPA: Hex system was described by Hara, Radin, and Erickson to perform equally as well as the CHCl$_3$: MeOH systems for the extraction of lipids from animal tissues [91, 95]. Similarly, the IPA: Hex system demonstrated an extraction efficiency that rated within the upper and lower limits of the CHCl$_3$: MeOH system ratings, which supports the literature. Three of the nine solvent systems had overall extraction efficiencies scores that were the same, however, each system displayed differing lipid affinities.
Figure 3.2  Lipid profiles of two differentiated 3T3-F442A adipocyte cells from the same culture dish. There is a considerable size difference between the cells, which is represented by the abundances of the 45:4 PtdCho and 50:2 PtdCho peaks relative to the TAG peaks in the spectra. The larger surface area of cell b has a greater ratio of PtdCho to TAG compared to the smaller surface area of cell a.
These results indicate that the overall extraction efficiency should not be used as a sole indicator of a solvent system’s performance and highlights the usefulness of the individual lipid scores in designing a custom solvent system based on its affinity for individual lipid classes.

The evaporation of the solvent system during the DAPNe lipid extraction under bright field procedure can be reduced by using solvents with higher boiling points resulting in increased evaporative times for the solvents[95]. Although the samples are placed on ice in between sonication and centrifugation steps, the temperature of the sample does rise during these necessary steps. Therefore, choosing solvents with relatively higher boiling points will increase the evaporative time and in turn increase the amount of time a system is in contact with a tissue ultimately increasing the extraction efficiency. Although a multi-step extraction method was used to evaluate these efficiencies, the results of this study still prove beneficial for single-step extraction solvent selection. There may be a need for modification of these systems in order to separate non-lipid contaminants from the extracted sample. This could be achieved with the addition of a miscible volume of water that will help to separate the hydrophilic portions of the extracted sample from the lipids of interest.

This study was intended to identify a solvent system that is best suited for use in a single-step lipid extraction based on a solvent system’s overall lipid extraction efficiency. The individual lipid extraction scores may provide the information needed to design a solvent system that has a higher affinity for lipids of interest while reducing possible interferences caused by other lipid classes. One specific area that will benefit from these results is the nano-extraction of single cell lipids within tissues from adipose
rich regions of the body, such as breast tumors, as the neutral lipids can be excluded from an extraction with the appropriate solvent system.

3.6 Acknowledgments

This work is supported by the Cancer Prevention & Research Initiative of Texas - R-13-HIHR-1

3.7 References


CHAPTER 4

ONE-CELL ANALYSIS AS A TECHNIQUE FOR TRUE SINGLE-CELL ANALYSIS OF ORGANELLES IN BREAST TUMOR AND ADJACENT NORMAL TISSUE TO PROFILE FATTY ACID COMPOSITION OF TRIGLYCERIDE SPECIES\textsuperscript{1}

4.1 Abstract

Breast cancer develops in an adipose rich environment of normal adipocytes that are known to aid in tumor progression through an unknown method of lipid transfer from normal cells to tumor cells. Much research is built around lipid analysis of breast tumor and adjacent normal tissues to identify variations in the lipidome to gain an understanding of the role lipids play in progressing cancer. Ideally, single-cell analysis methods coupled to mass spectrometry that retain spatial information are best suited for this endeavor. However, many single-cell analysis methods are not capable of subcellular analysis of intact lipids while maintaining spatial information. One-Cell analysis is a true single-cell technique with the precision to extract single organelles from intact tissues while not interfering or disrupting adjacent cells. This method is used to extract and analyze single organelles from individual cells using nanomanipulation coupled to nanoelectrospray ionization mass spectrometry. Presented here is a demonstration of the analysis of single lipid bodies from two different sets of breast tumor and normal adjacent tissues to elucidate the fatty acid composition of triglycerides using One-Cell analysis coupled to tandem mass spectrometry. As a result, thirteen

\textsuperscript{1} This entire chapter is reproduced with permission from [Hamilton, J.S., and Verbeck, G.F.: One-cell analysis as a technique for true single-cell analysis of organelles in breast tumor and adjacent normal tissue to profile fatty acid composition of triglyceride species. J. Anal. Oncol. 5, 47-54 (2016)]. Copyright [2016], Lifescience Global
fatty acid species unique to the tumor tissues were identified, five in one set of tissues and eight in the other set.

4.2 Introduction

Breast cancer is the most commonly diagnosed site specific cancer and the second leading cancer-related cause of death in women.[116] Breast tumors reside in an environment favorable for progression. The microenvironment of breast tumors is comprised of vasculature, connective tissue, and adipocytes, which store triglycerides (TG) in an organelle called a lipid body. Adipocytes have been shown to play an important tumor-supporting role by providing tumor cells with a supply of fatty acids released through lipolysis during tumor progression.[117-120] Once these adipocytes begin to undergo lipolysis their lipid body releases its stores, and the cell undergoes a phenotypic change in which it decreases in size and takes on a fibroblast like nature at which time they are then termed cancer-associated adipocytes (CAA).[117] Furthermore, Chajes et al. identified that the lipid composition of the breast adipose tissue is different from other adipose sites in the body.[121] Much research has been focused on changes in serum lipid profiles as an indicator of the onset of breast cancer,[122-127] but this type of analysis does not provide information about the ever-changing tumor microenvironment that is needed to understand localized tumor progression.

Current tissue lipid analysis methods often involve chemical or mechanical lysis of multiple cells followed by multi-step extractions to isolate lipid species from other cellular components. These methods result in lipid profile data that is averaged across multiple cells or even cell types in the case of complex intact tissues. This averaging of
data can overshadow cellular lipid heterogeneity as it pertains to normal cellular development[13] or diseased induced alterations, such as de novo lipogenesis that has been detected early in tumor development.[128] However, with the advent of CAA tumor support, emphasis should be placed on the analysis of cellular lipid heterogeneity within the lipid bodies of the localized cells of the breast tumor and adjacent normal tissues that comprise the complex breast tumor microenvironment. Ultimately, there is a great need for true single-cell analysis that retains spatial localization within tumor tissues to discover lipid biomarkers of emerging disease for early detection, and gain an understanding of cellular processes at the onset of tumor development.

High-throughput single-cell analysis techniques have been developed utilizing capillary electrophoresis, flow cytometry, microfluidics[4, 10, 11] and printed microarrays.[129] However, spatial information is lost as these methods separate groups of cells from culture or tissues down to single cells. Raman is a non-destructive technique capable of single-cell lipid analysis using ratios of single bonded carbons versus double bonded carbons to identify the degree of saturation of fatty acyl chains present in a cell, but not the lipids themselves.[130] Matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) is routinely used for the imaging of lipids of intact tissues with the capability of single-cell spatial resolution, but is still disadvantaged by the lengthy sample preparation times and the use of matrix that may alter or disburse cellular chemistry across a sample during application.[4, 131] Utilizing a focused ion beam, secondary ion mass spectrometry (SIMS) achieves subcellular resolution but causes excessive fragmentation resulting in loss of parent lipid identification and makes interpretation of spectra difficult.[4, 11] Furthermore, SIMS
also requires an involved sample preparation method to remove the cellular membrane to analyze intracellular metabolites.[132] Lastly, Single-probe mass spectrometry has been developed to analyze single-cells with an achieved spatial resolution of roughly 8µm, but with a probe size of 6µm ultimately lacks the ability to analyze subcellular structures and has been described as targeting only cytoplasmic metabolites.[133, 134] Furthermore, the targeting precision of the technique is limited by the X,Y,Z stage’s translational movement 0.1µm.[133]

One-Cell analysis is a method developed by our group as a solution to the problem of lost spatial information during single-cell analysis capable of elucidating subcellular structures and their unique heterogeneities.[12, 13, 77, 79, 135] The One-Cell technique encompasses nanomanipulation for the extraction of targeted subcellular components coupled to nano-electrospray ionization mass spectrometry (NSI-MS) for lipid analysis. One-Cell analysis is capable of individual whole cell extraction as well the targeted extraction of individual organelles. Using a modified nanomanipulator, One-Cell analysis is capable of subcellular spatial resolution with a step size of 100 nm in coarse mode and 10 nm in fine mode. The nanomanipulator is equipped with multiple positioners to perform nanoextraction using quartz rods, micro-pipettes, and metal coated nano-spray capillary emitters. The positioners can also be fitted with end effectors to perform micro gripping and low impedance electrical characterizations. Extraction solvents can be tuned for the optimal extraction of specific analytes removing the need for additional sample cleanup. Cell cultures and tissues remain viable after analysis because extractions are performed in ambient laboratory conditions under a microscope and allowing for adjacent cells to remain unaffected so
that they may undergo further analysis at a later time. Once a cell or organelle is extracted into a capillary, the analyte-containing capillary is then transferred to a nano-electrospray ionization source and analyzed by tandem mass spectrometry. Peaks of interest are selected for further analysis and fragmented. Based on the resulting fragmented peaks along with the calculation of neutral losses, structural information of the peak can be identified. However, this technique is limited in its throughput as each extraction and analysis requires roughly 10 minutes to perform. Furthermore, due to the small extraction volume of 10 µL, mass spectrometry analysis time is limited to two minutes. In lieu of this short analysis window, automated instrument methods have been developed to perform complete analysis of the analytes of interest. Phelps et al. provides a complete description of the apparatus and method in detail.[12]

Previously this technique has been used to identify the organelar heterogeneity of TG from individual lipid bodies extracted from neighboring cells within plant seeds,[77, 79] mammalian cell culture[12, 13, 135] and human breast cancer tissue from a single donor.[12] Phelps et al., through the use of One-Cell, identified a decrease in the 54-acyl carbon TG species of adipocytes extracted from the tumorous breast tissue compared to extracted adipocytes from adjacent normal tissue of that same patient. Interestingly, Guo et al. identified an increase in the free fatty acid (FFA) species 18:1 in cancerous breast tissue compared to normal tissue,[136] and therefore, we hypothesize that the tumorous cells may be consuming 18:1 FA species rather than continuing to store the FA as a fatty acyl constituent of TG species in their respective lipid bodies as a cause for this change in TG distribution. The work presented here demonstrates the use of One-Cell analysis coupled to tandem MS for the determination of the fatty acid
composition of TG in normal and diseased breast tissue lipid bodies to determine if the relative decrease of TG species is a result of altered fatty acid composition due to tumor progression.

4.3 Methods and Materials

Solvents for extraction include Chromosolv® Plus for HPLC chloroform (Sigma Aldrich, St. Louis, MO), 98% ammonium acetate (NH₄OAc) (Sigma Aldrich, St. Louis, MO), and Optima LC/MS methanol (Fisher Scientific, Fairlawn, NJ).

Snap-frozen infiltrative ductal carcinoma tumor and normal adjacent breast tissues from two female donors were purchased from Cureline Inc. (Cureline, West San Francisco, CA). These tissues were cut into slices with a thickness of 80 µm using a CM 1850 cryomicrotome (Leica Microsystems, Buffalo Grove, IL) and placed on glass coverslips for extraction.

4.3.1 Extraction

One-cell analysis extractions were performed using a nanomanipulator equipped with two probers (DCG Systems Inc., Fremont, CA) mounted to an AZ100 microscope (Nikon, Melville, NJ). One prober was fitted with a quartz rod pulled using a P-2000 CO₂-laser micropipette puller (Sutter Instruments, Novato, CA) to ~ 8µm. The second prober was fitted with a 1µm (±0.2µm) tip diameter Pd/Au-coated Econo12 PicoTip™ Emitter (New Objective, Woburn, MA) backfilled with 10 µL of extraction solvent, 1:1 MeOH:CHCl₃ plus 0.1% NH₄OAc. Individual adipocytes were optically identified using the microscope, Figure 1, and then extracted following the procedure described in detail
After extraction the emitters were then transferred to a nano-electrospray source (Proxeon Biosystems, Odense, Denmark) for nano-electrospray ionization mass spectrometry analysis on a Thermo LTQ XL mass spectrometer (Thermo, San Jose, CA).

Figure 4.1 Bright field image of the nano-extraction of a single adipocyte from A) healthy breast tissue showing the emitter capillary tip (b) after entering the adipocyte (a); and B) cancerous tissue also showing the emitter tip (a) entering the adipocyte (b)

4.3.2 Mass Spectrometry Conditions

Triglyceride profiling experiments were analyzed in positive mode with a spray voltage of 1.8kV, a capillary inlet temperature of 225°C, with a mass range of m/z 700-1000. Fatty acid (FA) composition determination experiments were analyzed in positive mode with a spray voltage of 1.8kV, an inlet temperature of 225°C, a mass selection...
window of 1 Da, and a collision induced dissociation (CID) energy of 40%. FA determination mass spectra were collected through CID scans of the isolated peaks with masses corresponding to the 52:4, 52:3, 52:2, 52:1, 54:5, 54:4, 54:3, and 54:2 ammoniated TG species, respectively. FA species were identified as neutral losses due to the fragmentation of the TG ions, [M+NH₄]⁺, resulting in the diacylglycerol (DG) ion, [M+NH₄⁻(RCOOH+NH₃)]⁺ as described by McAnoy et al. [137]

4.3.3 Data Analysis

4.3.3.1 TG Profile

Mass spectra data files were imported and plotted using the PSI-Plot software suite (Poly Software International, Pearl River, NY). The area under the curve was calculated for the individual areas for each of the regions corresponding to the 48-, 50-, 52-, 54, and 56-acyl carbon TG species using the <Calculate Area> function of the software suite. Finally, the ratio of the individual peaks to the total peak area were calculated and graphed to identify alterations in the TG profile as a function of the health state of the tissues.

4.3.3.2 Fatty Acid Composition

The spectra for the CID scans corresponding to the masses of the 52:4, 52:3, 52:2, 52:1, 54:5, 54:4, 54:3, and 54:2 TG species were also imported and plotted using the PSI-plot software suite. The areas for all DG ion peaks were calculated. For each CID spectrum DG peak areas were then converted to relative abundance by dividing
each individual DG ion peak area by the summed area of all DG ion peaks within the same CID spectrum.

4.4 Results

One-cell analysis was performed on adjacent normal and tumor breast tissues from two female donors, each with infiltrative ductal carcinoma. Analysis consisted of nanomanipulation guided subcellular extraction of lipid droplets (LD) from a single adipocyte within a tissue sample. Analysis was performed in replicates of five to show reproducibility and that variations in signal were caused by sample heterogeneity and not by normal instrument variance.

4.4.1 Triglyceride Profiling

As illustrated in Figure 2, the relative abundance averages of the TG peak areas of the normal extractions from the first tissue set were found to be 42.34%, 30.42%, 12.18%, 6.56%, and 5.33%; corresponding to the 52-, 54-, 56-, and 48-acyl carbon TG species. While the diseased tissue extractions relative abundance averages were 43.54%, 26.94%, 16.60%, 8.35%, and 4.56%; representing the 52-, 54-, 50-, 48, and 56-acyl carbon TG species. Within the second tissue set, the extractions from the normal tissue produced relative abundance averages of 42.81%, 26.43%, 16.51%, 7.81%, and 6.44%; corresponding to the 52-, 54-, 50-, 56-, and 48-acyl carbon TG species. Lastly, the diseased tissue extractions had relative abundance averages of
42.71%, 20.05%, 15.67%, 5.84%, 4.35%, 1.67%, and 1.05%; corresponding to the 52-, 54-, 50-, 48-, and 56-acyl carbon species, respectively.

The ratio of the average relative abundance of the 52:54-acyl carbon TG species was calculated for all tissue extracts. The normal and diseased ratios were then compared for each tissue set. Ultimately, relative to the normal tissues there was a decrease of 9.98% and 14.98% in the abundance of the 54-acyl carbon species found.
in the diseased tissues of both tissue set 1 and 2, respectively. This decrease in the 54-acyl carbon species prompted further analysis of the tissues to determine if this change could be a result of disease related alterations in the fatty acyl constituents of the aforementioned TG species.

<table>
<thead>
<tr>
<th>FA</th>
<th>Tumor Avg. Relative Abundance</th>
<th>Tumor Average Deviation</th>
<th>Healthy Avg. Relative Abundance</th>
<th>Healthy Average Deviation</th>
</tr>
</thead>
<tbody>
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<td>1.07%</td>
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<td>20.30%</td>
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<td>0.00%</td>
<td>1.76%</td>
<td>0.38%</td>
</tr>
<tr>
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<td>1.37%</td>
<td>0.00%</td>
<td>0.00%</td>
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<tr>
<td>18:0</td>
<td>9.72%</td>
<td>2.23%</td>
<td>10.98%</td>
<td>1.08%</td>
</tr>
<tr>
<td>18:1</td>
<td>49.96%</td>
<td>2.16%</td>
<td>47.23%</td>
<td>1.14%</td>
</tr>
<tr>
<td>18:2</td>
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<td>2.55%</td>
<td>19.73%</td>
<td>0.92%</td>
</tr>
<tr>
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<td>2.05%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>20:2</td>
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<td>0.95%</td>
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<td>26:0</td>
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</tbody>
</table>

Table 4.2 Relative fatty acid composition of cells extracted from tissue set #2, cells with a gray fill indicate those specific to all tumor cells extracted.
4.4.2 Fatty Acid Composition

Based on the results of the TG profiling data the largest change between the two health states of each tissue set was the decrease in the 54-acyl carbon TG species. To determine if this phenomenon was based on a change in fatty acid composition, a tandem MS/MS experiment was set up. The experiment was conducted in the same manor as the TG profiling experiment, with added CID analysis of the four most abundant individual TG species in each of the 52- and 54-acyl TG ranges.

Within the first set of tissues six FA species (16:0, 16:1, 18:0, 18:1, 18:2, and 20:1) were found to be present in all of the normal and diseased cells extracted from the first tissue set. These six fatty acids account for 100% of the total FA composition of the normal extracted cells and 98.36% in the diseased cells. The remaining 1.64% in the diseased cells was made up of five FA that were unique to all extracted diseased tissue cells, 17:1, 18:3, 20:0, 20:2, and 20:3, Table 1.

Within the second tissue set, four FA species (16:0, 18:0, 18:1, and 18:2) were present in all of the normal and diseased cells extracted, and accounted for 98.24% and 88.64% of the total FA compositions, respectively. The remaining 1.76% of the normal FA composition is made up of 16:1 species, a FA that is absent from the diseased tissue cells extracted. Meanwhile, the remaining 11.36% is comprised of eight FA species (12:0, 17:1, 20:1, 20:2, 20:4, 22:3, 23:0, 26:0) present in all of the diseased tissue cells extracted and not in any of the normal tissue cells extracted, Table 2.
4.5 Discussion

During times of cellular proliferation all cells are known to undergo metabolic variation but normal cells are capable of retaining metabolic regulation, while tumor cells lose metabolic regulation due to mutations in their signaling pathways.[138] It is the loss of regulation that leads to aberrant metabolite production making metabolites, such as lipids, ideal biomarkers for disease detection.

Hilvo et al. conducted a total lipid profiling study using whole tissue extracts to compare changes in tumor and normal breast lipids in which only a few tumor samples were found to have a decrease in TG species relative to their normal tissue.

<table>
<thead>
<tr>
<th>FA</th>
<th>Tumor Avg. Relative Abundance</th>
<th>Tumor Average Deviation</th>
<th>Healthy Avg. Relative Abundance</th>
<th>Healthy Average Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>1.04%</td>
<td>1.07%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>16:1</td>
<td>2.05%</td>
<td>0.75%</td>
<td>1.84%</td>
<td>0.72%</td>
</tr>
<tr>
<td>17:1</td>
<td>0.14%</td>
<td>0.09%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>18:0</td>
<td>11.18%</td>
<td>1.59%</td>
<td>11.29%</td>
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<tr>
<td>18:1</td>
<td>47.70%</td>
<td>2.27%</td>
<td>48.74%</td>
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<tr>
<td>18:2</td>
<td>16.68%</td>
<td>1.79%</td>
<td>17.03%</td>
<td>1.75%</td>
</tr>
<tr>
<td>18:3</td>
<td>0.88%</td>
<td>0.29%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>20:0</td>
<td>0.14%</td>
<td>0.13%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>20:1</td>
<td>0.56%</td>
<td>0.21%</td>
<td>0.58%</td>
<td>0.22%</td>
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<tr>
<td>20:2</td>
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</tbody>
</table>

Table 4.1 Relative fatty acid composition of cells extracted from tissue set #1, cells with a gray fill indicate those specific to all tumor cells extracted.
counterparts.[139] While we do not see a complete decrease in TG, our findings do show a decrease in the relative abundance 54-acyl carbon TG of extracted diseased cells relative to the normal cells, which is consistent with the previous findings of Phelps et al.[12]

We originally hypothesized that the change in 54-acyl TG species abundance would be due to a cancer related consumption of 18:1 FA species. Rather unexpectedly, a set of unique FA species specific to the disease tissue cells were identified, and the 18:1 FA species remained the most abundant FA species in all cells extracted. Furthermore, there was no significant change in the degree of saturation of FA present in the TG species of the normal and diseased tissue cells, however, in a recent study by Guo et al. cancerous breast tissue was found to contain an increased abundance of monounsaturated FFA and a decrease in polyunsaturated FFA species.[136] In a recent total FA analysis of similar tissues, Azordegan et al. extracted lipids from whole tissues representing breast tumor, interface, and adjacent normal tissues to find increases in 18:0 FA species in breast tumors, and decreased abundance of 18:1, 18:2, and 18:3 in the tumor tissues.[140] Whereas in this study, no significant difference in the abundance of 18:0 was found among the tissue types while we saw no significant change in 18:1 and 18:2 species among the two tissues. The FA species 18:3 was only identified in the tumor cells extracted from tissue set one in our study and were not found in any of the other tissue cells extracted.

Of the thirteen unique FA species associated only with the cells extracted from disease tissue (tissue set 1: [17:1, 18:3, 20:0, 20:2, 20:3] and tissue set 2: [12:0, 17:2, 20:1, 20:2, 20:4, 22:3, 23:0, 26:0]) only one, 20:2, is found in both tissue sets. The
18:3 FA species was reported to be found only in breast cancer tissues during a whole tissue extraction comparison of liver, pancreas, and breast cancer tissues to their normal counterparts.[141] The FA species 20:4, may be arachidonic acid depending on the location of its double bonds, however, this was not determined and therefore no claim to its incidence in these cancer tissues can be made. The presence of odd chain fatty acids, 17:1 and 17:2, may be due to an increased peroxidation of lipids in breast cancer patients as reported by Gupta et al.,[142] possibly through alpha oxidation.[143] The remaining FA species (20:0, 20:2, 20:3, 12:0, 20:1, 22:3, 23:0, and 26:0) have all been associated with serum fatty acids in previous cancer related studies[127, 144] and may in fact be related to individual diets of the individuals associated with the tissues samples analyzed. While the importance of these disease specific fatty acid species is currently unknown, their identifications as fatty acyl constituents of stored triglycerides within the lipid bodies of breast tumor cells would not be known without the use of One-Cell analysis.

4.6 Conclusion

Due to the complexity of the breast tumor microenvironment, One-cell analysis is an ideal method to extract and analyze the lipids of individual cells and targeted organelles within the breast tumor microenvironment to identify lipids specific to tumor progression. While other methods such as, time-of-flight secondary ion mass spectrometry (ToF-SIMS) and probe electrospray ionization mass spectrometry (PESI-MS) are capable of identifying tumor cells from normal cells in a mixture[132] and determining tumor borders in live mice,[145, 146] respectively, neither is capable of
elucidating organelle specific lipids. Furthermore, the use of One-Cell analysis leaves un-extracted cells intact for future analysis in tissues, or future biochemical analysis within in culture. The current application was tuned for the extraction of triglycerides, but simply changing the extraction solvent and mass spectrometer settings will allow for the analysis of any metabolite of interest.

Future work would ideally be focused on the analysis of more tumor tissues to create a larger data set to discover a breast cancer lipid biomarker present in all breast tumors. One-Cell analysis also needs to be expanded to other lipid classes and metabolites. Ultimately, One-Cell analysis is an ideal technique for the detection of tumor cells during on-site analysis of minimally invasive needle biopsies at the time of collection.

Much work has gone into identifying the pathways involved in obesity related cancer progression such as those relating to adipokines, estrogen, insulin, and pro-inflammatory cytokines.[147] Obesity is often considered to be a state of chronic inflammation that elicits an immune response that is known to create a local environment that is beneficial for the proliferation of adipocytes.[148] However, this increase in proliferation often leaves many cells in the immature preadipocyte form. These preadipocytes are responsible for an increased secretion of pro-inflammatory cytokines, some of which are responsible for the recruitment of immune cells and the promotion of angiogenesis.[148] Many forms of cancer thrive in the presence of adipose tissue[149, 150] and the ability to analyze tumor microenvironments one cell at a time could provide an insight as to how the aforementioned pathways and secreted cytokines affect cells at the forefront of a progressing tumor. The use of One-cell
analysis to gather information pertaining to the intracellular changes resulting from obesity related-cancer pathways would be an integral part in the development of multidisciplinary research teams focused on elucidating the mechanism(s) of obesity-related cancer progression to identify new therapeutic targets and develop personalized treatment plans.

4.7 Acknowledgments

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4.8 References


[143] Jenkins, B.; West, J. A.; Koulman, A.; A Review of Odd-Chain Fatty Acid Metabolism and the Told of Pentadecanoi Acid (C15:0) and Heptadecanoic Acid (C17:0) in Health and Disease. Molecules 20, 2425-2444 (2015)


CHAPTER 5

DAPNE WITH MICRO-CAPILLARY SEPARATORY CHEMISTRY-COUPLED TO MALDI-MS FOR THE ANALYSIS OF POLAR AND NON-POLAR LIPID METABOLISM IN ONE CELL²

5.1 Abstract

The cellular metabolome is considered to be a representation of cellular phenotype and cellular response to changes to internal or external events. Methods to expand the coverage of the expansive physiochemical properties that makeup the metabolome currently utilize multi-step extractions and chromatographic separations prior to chemical detection leading to lengthy analysis times. In this study, a single-step procedure for the extraction and separation of a sample using a micro-capillary as a separatory funnel to achieve analyte partitioning within an organic/aqueous immiscible solvent system is described. The separated analytes are then spotted for MALDI-MS imaging and distribution ratio calculations. Initially, the method is applied to standard mixtures for proof of partitioning. The extraction of an individual cell is non-reproducible; therefore a broad chemical analysis of metabolites is necessary and will be illustrated with the one-cell analysis of a single Snu-5 gastric cancer cell taken from a cellular suspension. The method presented here shows a broad partitioning dynamic range as a single-step method for lipid analysis demonstrating a decrease in ion suppression often present in MALDI analysis of lipids.

5.2 Introduction

The cellular metabolome is a dynamic collection of metabolites considered to be small molecules of less than 1000 Da in molecular mass [151], including sugars, peptides, lipids, and other small molecules of varying hydrophilicity and lipophilicity [152, 153]. The cellular metabolome is considered to be a direct representation of a cell’s phenotype and considered to be a measure of the immediate cellular responses brought about by changes in environment, disease state, or diet [154]. The responsive nature of the metabolome provides the opportunity for biomarker discovery through metabolomics and lipidomics [154-158]. However, the metabolome is an expansive set of molecules that can greatly vary in their physiochemical states making single-platform analysis for polar and non-polar metabolite profiling difficult. The method presented here offers a demonstration of a solution through the simultaneous extraction and partitioning of polar and non-polar lipids in an immiscible solvent system coupled to mass spectrometry imaging (MSI) by matrix assisted laser desorption/ionization (MALDI) for single platform analysis.

Historically, the extraction of lipophilic and hydrophilic metabolites consists of multi-step extraction procedures using immiscible solvent systems of an organic and aqueous solvent to create a biphasic mixture. Lipids and non-polar metabolites partition into the organic phase while polar metabolites partition into the aqueous phase. The phases are then collected separately to undergo further purification or re-extraction to ensure complete metabolite extraction [153]. The Folch and Bligh-Dyer methods are two of the most commonly used biphasic extraction methods and were developed to extract lipids into the organic phase while the aqueous phase was used for sample
purification to remove non-lipids [28, 29]. The Folch method uses a 2:1 chloroform/methanol (CHCl₃/MeOH) solvent system for the extraction of lipids followed by a 0.2 volume addition of water (H₂O) during a washing step to remove non-lipids [28]. The Bligh-Dyer method uses an initial extraction solvent of 1:2 CHCl₃/MeOH followed by the addition of 1:1 CHCl₃/H₂O to induce a biphasic mixture and remove polar and non-lipid contaminants from the organic phase [29]. However, because polar metabolites are extracted into the aqueous phase of the Folch and Bligh-Dyer methods they are useful for metabolomics studies. Biphasic extraction methods using varying ratios ofCHCl₃/MeOH/H₂O for the extraction of polar and non-polar metabolites have been performed on mouse melanoma cells [159], blood parasites [160], bacterial and macrophage cells [161], urine, and plasma [162]. Several other groups used a modified extraction method by replacing CHCl₃ with methyl tert-butyl ether (MTBE) for the extraction of metabolites from plasma [163-165], mouse tissue [166], rat tissue [167], and serum spots, urine, and cerebrospinal fluid [168]. Methods have been developed to extract polar and non-polar metabolites using mono-phasic systems to cut down on time and prepare for single platform analysis [169-171], but these systems often neglect the extraction of neutral lipids, as outlined in a comparison study of twelve such methods by Dietmar et al. [169].

Metabolomics experiments are most commonly performed using nuclear magnetic resonance (NMR) or mass spectrometry (MS) through direct injection of a crude sample or following separation by gas chromatography (GC-MS), capillary electrophoresis (CE-MS), or liquid chromatography (LC-MS) [153, 154, 158, 172]. NMR has several advantages for its use in metabolomics; it is non-destructive, biological
samples can be analyzed in their natural state, results are highly reproducible, and metabolites can be quantified down to the micromolar scale [153, 154, 158]. However, NMR is limited by its sensitivity, as metabolites of sub-micromolar concentrations are not detected [154, 158]. Mass spectrometry (MS) provides picomolar sensitivity, metabolites can be quantified, structural information is provided, isomeric metabolite identifications can be ascertained with tandem MS/MS regarding the detected analytes as compared to NMR. Furthermore, high-resolution MS can provide chemical formulations that can be used for putative matching of unknown metabolites using searchable databases such as the LIPID MAPS [173] and Human Metabolome [174] databases. To increase the selectivity of MS and reduce matrix effects, chromatography techniques such as gas chromatography (GC-MS), capillary electrophoresis (CE-MS), and liquid chromatography (LC-MS) are commonly placed inline with MS. GC-MS separates molecules in the gas phase with excellent reproducible retention. In addition, the consistent reproducible fragmentation patterns created by electron impact (EI) make molecular identification through database matching relatively simple [154]. However, GC-MS is disadvantaged in that only volatile and non-volatile molecules that have been derivitized, are acceptable for GC-MS analysis [153]. CE-MS is best used for the separation of charged polar metabolites [153] and it is suggested that lipids be removed from the sample [175]. Alternatively, with the addition of a charged surfactant to CE, neutral molecules can be separated by micellar electrokinetic chromatography (MEKC) [172]. The reproducibility of CE is considered to be poor, unwanted electrochemical side reactions can take place, and it is not a robust technique [172]. LC-MS is widely used in metabolomics and often incorporates both reversed-phase (RP) and normal-
phase (NP) columns [153, 172]. The commonly used stationary phases of C₈ or C₁₈ in RP columns provides separation of lipids and other non-polar metabolites in the extracted organic phase [172], while NP-LC can employ hydrophilic interaction chromatography (HILIC) to separate the more polar metabolites of the aqueous phase extract [172]. Both RPLC and HILIC can be used to expand the coverage of the metabolome, but two consecutive chromatographic separations increases both analysis time and cost [161, 176]. There are methods being developed to simultaneously separate polar and lipid metabolites using a single column, but thus far only lipids with polar headgroups, such as glycerophospholipids, have been detected [161].

Historically, a separatory funnel has been used to separate polar and non-polar molecules to determine a molecule’s respective partition coefficient [177]. The partition coefficient, log $K_{O/W}$, is a constant that describes a molecule’s affinity for the organic or aqueous phase and is defined as the log of the ratio of a molecule’s concentration in the organic phase to the concentration in the aqueous phase using octanol and water at equilibrium [177]. When immiscible solvents other than octanol and water are implemented the distribution ratio can be calculated to measure the partitioning of a solute. The distribution ratio, $K_D$, measured using the ratio of a molecule’s concentration in the organic to aqueous phase of any immiscible solvent system regardless of equilibrium [177]. Once correlation coefficients based on a solute’s retention factor are determined the distribution ratios calculated by chromatography methods can then be used as predictors of partition coefficients for that solute [177, 178].
Analyses of the metabolome and/or lipidome incorporating biphasic mixtures are most prevalent in the analysis of biofluids and tissues resulting in data that is averaged over many cells. Techniques for the high-throughput analyses of single cells have been developed using printed microarrays [129], flow cytometry, microfluidics, and CE [4, 11, 152]. However, chromatographic separation by microfluidics and CE incurs lengthy run-times or purification steps. Flow cytometry is limited in the breadth of information acquired, and printed microarrays often result in the deposition of multiple cells [129] resulting in data averaged over multiple cells. NMR and Raman spectroscopy analysis of single cells are non-destructive techniques that provide real-time analysis of the dynamic metabolome. Raman is disadvantaged by the lack of molecular identifications it can provide [130] and NMR is disadvantaged by its sensitivity limitations of micromolar analyte detection [154, 158], as previously stated.

Direct analyte-probed nanoextraction (DAPNe) is a method developed by our group for the extraction of trace materials in complex matrices using a nanomanipulation workstation coupled to nanoelectrospray ionization (NSI) for analysis by direct inject mass spectrometry (DIMS) [55, 74-76, 179] The nanomanipulator is mounted on a microscope stage and is equipped with up to four nanopositioners driven by piezoelectric motors. Each of the positioners can be fitted with microelectrodes, microgrippers, nanospray emitters, quartz rods, or glass capillaries. The positioners are controlled by a joystick in the X, Y, and Z-axes with a spatial resolution of 100 nm and 5 nm in coarse and fine modes, respectively. The movement of the positioner allows for the precise extraction of the area of interest reducing matrix effects as well as the need for chromatographic separation or pretreatment of the sample [55, 76].
Recently, DAPNe of one cell has been used to identify lipid heterogeneity within breast tumor and adjacent healthy tissues [12, 81], mammalian cell culture [13], and plant tissues [77, 78]. DAPNe of one cell has been developed for the extraction of individual cells and/or organelles from individual cells and can be coupled to nanoelectrospray ionization (NSI)-MS [12-14, 77, 78, 81], or matrix assisted laser desorption/ionization (MALDI)-Orbitrap-MS to image an extracted sample [14]. Here we describe the use of DAPNe using a glass micro-capillary tip as a separatory funnel coupled to MALDI-MS to image the distribution of solutes from a reaction mixture of phenethylamine analogues and a lipid standards mix, in a CHCl₃/H₂O solvent system to establish proof of principle. Ultimately, the method is demonstrated for the analysis of one cell as an untargeted lipid profiling method to analyze both polar and nonpolar lipids in a single extraction step while reducing ion suppression often found in direct lipid analysis of MALDI samples [180].

5.3 Materials and Methods

5.3.1 Phenethylamine Reaction Mixture

Following the procedure described by Clemons et al.[181], alkylated phenethylamine (PEA) analogues were synthesized using 1-iodomethane, 1-iodoethane, 1-iodopropane, 1-iodobutane, 1-iodopentane, 1-iodohexane, 1-iodoheptane, and 1-iodooctane; all from Sigma Aldrich (Saint Louis, MO, USA). The synthesis resulted in a mixture of secondary and tertiary PEA analogues of varying alkyl moieties with 2-16 total carbons and primary PEA. The final reaction mixture is dissolved in 1:1 CHCl₃:MeOH (HPLC grade, Sigma-Aldrich, St. Louis, MO, USA).

5.3.2 Lipid Standards
Lipid standards including polar brain lipids, triheptadecanoate (17:0/17:0/17:0) (TG 51:0) (both from Avanti Polar Lipids, Alabaster, AL, USA), and triglycerol mix 2:0-10:0 (Sigma-Aldrich, St. Louis, MO, USA) were each dissolved in 1:1 CHCl₃:MeOH (HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) and mixed to a final concentration of 2 µM for each standard.

5.3.3 Snu-5 Cells

Human gastric carcinoma cells, SNU-5 (ATCC® CRL-5973, American Type Culture Collection (ATCC), Manassas, VA, USA), were cultured in suspension producing both individual cells and aggregates of multiple cells. The average diameter of an individual cell was found to be 20 µm through measurements using the Nikon Elements software package (Nikon, Melville, NY, USA). The cells were cultured at 37°C under 5% CO₂ in Iscove’s Modified Dulbecco’s Medium supplemented with 20% fetal bovine serum (all from ATCC, Manassas, VA, USA).

5.3.4 Extraction

Extractions, Figure 1a, were performed utilizing a modified nanomanipulator workstation (DCG Systems Inc., Fremont, CA, USA) mounted to an AZ100 microscope (Nikon, Melville, NY, USA). A single prober was fitted with a quartz capillary tip pulled using a P-2000 CO₂-laser micropipette puller (Sutter Instruments, Novato, CA, USA) to an internal diameter of 20 µm matching the average diameter of the Snu-5 cells to reduce the chance of damaging the cells and inducing unwanted responses from mechanical shearing during extraction.
Figure 5.1  Illustration representing the DAPNe-micro-capillary separation-MALDI-MS imaging method. Use of DAPNe for the extraction of one cell using a micro-capillary backfilled with 1µL of organic solvent (a), followed by aspiration of 1µL water for metabolite partitioning in an immiscible solvent system. Upon phase separation, (b) the phases are spotted onto a MALDI slide, evaporated, and spotted with matrix for MALDI-MS imaging. The resulting total ion count (TIC) (c) represents the overall shape of the sample spot and (d) the matrix crystal formation after matrix application. The peak selection displays the distribution of that peak to the organic phase (e) or aqueous phase (f).
For analysis of the lipid standards and drug mix (PEA analogues), 1 µL of a standard solution is backfilled into a capillary tip. A 1-µL droplet of 18Ω Milli-Q® water (Millipore, Billerica, MA, USA) is deposited on a clean glass slide. Using the joystick controller the capillary tip was then positioned directly above the water droplet and slowly lowered into the droplet. Using a pressure injector, the water is aspirated into the capillary tip and drawn through the standard solution, Figure 1a. The immiscible layers are allowed to separate before being spotted onto a stainless steel MALDI tissue slide, Figure 1b.

The procedure for the analysis of the suspension cells differs slightly from the standard solutions, 1 µL of the cell suspension was centrifuged to remove the growth media, and cells resuspended in ammonium carbonate buffer until extraction. First, the capillary tip is backfilled with 1 µL of CHCl₃, then positioned adjacent to a suspended Snu-5 cell within a droplet of cell suspension. Once in position, using a nitrogen gas pressure injector, negative pressure, -1.0 psi, is applied to draw the cell into the capillary tip, the cell is left to lyse within the solvent (~5 minutes), then 1 µL of water is aspirated into the capillary tip and the phases allowed to separate before being spotted.

Immediately after the spotted sample has evaporated, a second capillary tip filled with 2 µL of MALDI matrix, 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) (DHB 98%, Sigma-Aldrich, St. Louis, MO) solution in 3:2 acetonitrile/water (ACN/H₂O) (v/v), (HPLC grade, Sigma-Aldrich, St. Louis, MO) is placed into the prober and positioned over the sample spot. Following the method described by Phelps et al.[14], DHB is then spotted.
onto the sample and dried with a stream of nitrogen before being loaded into the MALDI front end for analysis, figure 1b.

5.3.5 MALDI-LTQ-Orbitrap Analysis

MALDI-MS experiments were conducted on a MALDI-LTQ-XL-Orbitrap (Thermo Scientific, San Jose, CA, USA) equipped with a 337 nm N2 laser (MNL 100; Lasertechnik Berlin, Germany). Conditions were as follows: 15 µJ energy per laser shot, 1 laser shot per spectra, laser raster motion was set at a 60 step size for lipid samples and 100 µm for drug sample. Data acquisition was collected in positive ion mode using a mass range of m/z 100–1100, lipids, and m/z 50-600, drug, with a mass resolution of 60,000 (at m/z 400). Data processing was performed using Xcalibur v 2.3 (Thermo Scientific, San Jose, CA). Image processing for the deposited spots was performed with ImageQuest v 1.1, (Thermo Scientific, San Jose, CA) the mass range plotted and linear smoothing applied, figure 1c-e. Mass spectra were plotted using PSI-Plot software (Poly Software International, Pearl River, NY).

5.3.6 Distribution Ratio (K_D)

To evaluate the partitioning of various solutes the distribution ratio was calculated as the ratio of the total ion count of an analyte in the organic phase (chloroform) to the total ion count of the same analyte in the aqueous phase (water). This was performed using the polygonal selection tool of the MSI software suite [182] to highlight each phase of the MALDI-MS images, separately. The mass spectral data for each scan within the selected area was exported and the absolute intensity of a specific peak was summed for each phase. The total intensity for both the organic and aqueous regions
of the image, Table 1, were then used to calculate the distribution ratio, \( K_D \) and the log of the ratio taken, \( \log K_D \) using equations 1 and 2, respectively.

\[
K_D = \frac{[CHCl_3]}{[H_2O]} \quad (1)
\]

\[
\log K_D = \log 10 \left( \frac{[CHCl_3]}{[H_2O]} \right) \quad (2)
\]

5.4 Results/Discussion

During the drying process of the MALDI spots it was determined that the aqueous phase remained in the center of the spot as the organic phase spread outward from the origin of the spotting position. While the center of the MALDI images is the aqueous phase, some aggregation of hydrophobic molecules can be found within this phase due to the density and evaporation rate of the chloroform. During the spotting and phase separations the chloroform was expelled from the capillary tip first followed by the water. The surface tension of the chloroform is such that it does not form a droplet, but rather spreads out radially, in effect, increasing the surface to air ratio causing the chloroform evaporation to speed up. This resulted in the deposition of some chloroform soluble analytes beneath the aqueous phase thus providing the illusion that some organic phase-analytes dispersed into the aqueous phase. This was not corrected for when calculating the distribution ratios.
Table 5.1 Tabulated information for calculation of $K_D$ and peak identification in spectra

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TG – triglyceride; LPA – lysophosphatidic acid; MG – monoacylglycerol; PA – phosphatidic acid;

MGDC - PC – phosphatidylcholine; PE - phosphatidylethanolamine; FA – fatty acid; Cer – ceramide; CE – cholesterol ester ; MGDG - monogalactosyldiacylglycerol;
Figure 5.2  (a-o) MALDI images of selected peaks of phenethylamine (PEA) analogues for the visualization of their respective phase partitioning. (p) The total ion count of the PEA reaction mixture sample spot. Identified peaks of most abundant PEA analogues within an averaged spectrum (q).
5.4.1 Drug Spots

The dispersion of the PEA analogues between the two phases correlates with the total number of carbons in the acyl moieties of the alkylated PEA such that as the carbon number increases, the dispersion from the aqueous phase to the organic phase also increases, as can be seen in Figure 2. The images of Figure 2b-n differ by the addition of a single carbon to the alkyl moieties of the PEA analogue from two to fourteen carbons and a total of 16 carbons in Figure 2o, as denoted in Table 1a. It is important to note that secondary PEA analogues exist within the analytes detected in Figure 2b-h. These secondary species are expected to partition between the aqueous and organic phases despite an increasing alkyl chain as observed in Figure 2g and 2h.

Figure 2a, m/z 122.0970, shows the predominant partition into the aqueous phase of phenethylamine, a primary amine. The secondary and tertiary PEA analogues with two total substituted carbons also partition strongly within the aqueous phase, as seen in Figure 2b, m/z 150.1283. With a total substituted carbon count of seven, Figure 2g, the analytes begin to partition into the organic phase. Figure 2j illustrates a relatively even partitioning of tertiary PEA analogues with ten total carbons in which an ethyl is the shortest alkyl chain. This trend of an increased partition into the organic phase continues as the total number of carbons added to the alkylated PEA analogues increases, Figure 2k-o. The partitioning trend is also expressed by the log $K_D$ values found in Table 1a, which range from -0.708 to 0.824 for m/z 122.0970 and m/z 346.3474, respectively. The drug spot analysis provides evidence for the ability of this method to provide a visual partitioning of molecules with minimal increases in
hydrophobic character based on their increased distribution into the organic phase of the solvent system.

5.4.2 Lipid spot

The dispersion of the triglyceride (TG) standards in the aqueous and/or organic regions within the spots is relative to the fatty acyl chain length of the TG and can be seen in the images of Figure 3 and supported by the log $K_D$ values in Table 1b. The TG (12:0) standard (Figure 3a) was localized in the aqueous phase with a log $K_D$ of -1.11 and the TG (51:0) standard (Figure 3e) is localized more in the organic phase, with a log $K_D$ of 0.33. The polar brain lipid standards were found in both the aqueous and organic phases. Lysophosphatidic acid (LPA) (Figure 3f), with its polar head group, migrated to the aqueous phase and has a log $K_D$ value of -0.91. Monoacylglycerol (MG) (20:2) (Figure 3g) partitions more in the organic phase with a log $K_D$ of 0.995, and is a result of its glycerol backbone and long 20-carbon acyl chain. The distribution of the lipid standards is correlated to both the acyl chain length of the fatty acyl constituents as well as the nature of the head group for the specific lipids described here.

5.4.3 Snu-5 Whole Cell

The MALDI-MS analysis of lipids extracted from an intact individual Snu-5 gastric cancer cell resulted in the detection of multiple lipid classes including glycerolphospholids, ceramides, cholesterol esters, triglycerides, and fatty acids in the positive mode using the MALDI matrix 2,5-DHB. Select peaks of interest were
Figure 5.3  (a-j) Selected peaks of expected lipid species in a lipid standard mixture for visualization of their respective phase partitioning.  (k) The total ion count of the lipid standard mixture sample spot.  (l) Identified peaks of most abundant expected lipid species within an averaged spectrum.
Select peaks of interest were putatively identified through a Lipid MAPS database search of protonated, sodiated, or potassiated neutral and polar lipid species with a tolerance of \( m/z \pm 0.005 \). Distribution ratios for fifteen peaks of interest were calculated, Table 1c; and visual partitioning of those fifteen putative matches are represented as MALDI images, Figure 4a-o. The center of the Snu-5 MALDI spot was determined to be the aqueous phase. An irregular oval shaped black line was drawn around the center region of each MALDI image in Figure 4 to show where the inner aqueous phase meets the outer organic phase.

Of the fifteen peaks reported here, five were found to be phosphatidylcholine making it the most represented lipid class within the single-cell extract. The partitioning of the PC species resulted in an aggregation near where the aqueous and organic phases meet. The calculated log \( K_D \) values of the PC species ranged from -0.161 to 0.318, Table 1c. Four masses of interest were putatively identified as phosphatidylethanolamine; making it the second most represented class of lipids. The PE species appear to partition into both phases and appear to not aggregate within a specific area of the sample spot. The identification of nine glycerophospholipids is not surprising as they are readily associated with cellular membranes and should be expected to account for a larger portion of the cellular lipid population. Contrary to the PE species, two cholesterol ester peaks, m/z 661.533 and 686.564, are shown to aggregate heavily to a single region forming a relatively concentrated pocket along the organic side of the biphasic line within the MALDI spot, Figure 4l and m. It is of interest to note that the hydrophilic triglyceride species, m/z 967.868 and 1009.856, partitioned within the aqueous region of the spot, Figure 4n and o.
Figure 5.4  (a-o) Selected peaks of putatively identified lipid species from the one-cell extraction of a Snu-5 cell for visualization of their respective phase partitioning.  (p) The total ion count of the one-cell extraction sample spot.  (q) Identified peaks of selected putatively identified lipids within an averaged spectrum, with inset (r) of peaks greater than m/z 600.
The partitioning of these neutral lipids can best be explained as a result of the drying process described at the beginning of the results and discussion section.

The identification of Cer (d38:0), Figure 4b, is of interest as it has been reported to be formed in response to apoptosis within a cell [174], and suggests the addition of a quenching step prior to extraction is required. Quenching is performed through pretreatments of a sample with an organic solvent. Dietmar et al. performed a comparison study of twelve metabolite extraction procedures coupled with several quenching methods and determined that a pretreatment with cold 0.9% NaCl is the only acceptable quenching method for mammalian cells as all other methods tested resulted in cell membrane damage that lead to metabolite leakage [169].

Although the lipid species of the Snu-5 one cell analysis were putatively identified through an exact mass search of the LIPID MAPS database [173] with a mass tolerance of 0.005 Da; tandem MS should be performed to elucidate chemical structures for confirmation of identification. Tandem MS can be difficult to perform on single-cell samples due to small sample volumes and limited analysis times. Spotting extracts for MALDI imaging provides an increase in the amount of time that can be spent with a sample when compared to other methods such as NSI-MS, in fact, Phelps et al. reported a 15 fold increase in analysis time when coupling DAPNe to MALDI-MS [14]. Furthermore, Phelps et al. demonstrated the ability to reanalyze the MALDI sample spot using tandem MS to verify the identification of analytes of interest [14]. This increase in time allows for more thoughtful and thorough processing of the data and offers the benefit of multiple MALDI analyses. Unfortunately, due to the length of time between
putative database identification and original analysis, the MALDI spots were not reanalyzed for structural confirmation of putative identifications.

During immiscible solvent extraction, semi-polar metabolites can disperse into both the organic and aqueous phases [153]. If a procedure requires the collection and purification of each phase separately for separation by RP-LC and NP-LC, one or both of the methods may not be suitable for these semi-polar metabolites resulting in their elution in the dead volume preventing their detection in the sample [161]. DAPNe-micro-capillary separatory chemistry-MALDI-MS removes the need for phase collection and purification steps as well as chromatographic separations meaning the whole of the organic and aqueous phases are retained and deposited on the MALDI slide for simultaneous analysis. Furthermore, separation prior to MALDI-MS analysis has been shown to reduce the ion suppression effect of the phosphatidylcholine species on other lipid classes [180]. In a review of MALDI-MS characterization of lipids it was reported that the presence of PC can severely suppress neutral lipids such as triglycerides [180]. However, with the use of solid phase extraction this suppression is significantly reduced and TG species ion intensities are more representative of their actual abundance within a sample. Using DAPNe-micro-capillary separatory chemistry-MALDI-MS for lipid analysis PC, PE, TG and CE lipid species are all identified with ion intensities on the same order of magnitude when normalized to the total ion count. Therefore, the use of immiscible solvents for the partitioning of polar and non-polar lipids during one-cell analysis provides a method for separations when chromatographic methods are infeasible.
5.5 Conclusion

The coupling of DAPNe to micro-capillary separatory chemistry with MALDI-MS imaging demonstrates a technique capable of increasing the range of polar and non-polar molecules that can be analyzed simultaneously using a single step extraction method of one cell without pretreatment or chromatography separations. The spotting of one-cell extracts on a MALDI slide for imaging not only allows reanalysis of the sample but also provides visual confirmation that partitioning of hydrophilic and lipophilic molecules occurs within the micro-capillary during extraction. However, to reduce the unexpected partitioning of hydrophilic molecules within the aqueous phase the MALDI spotting method needs optimization. Furthermore, due to limitations of our instrument, fast polarity switching was not available and therefore analysis was not performed in the negative mode. However, to fully examine the method described here, the analysis of both positive and negative ions will be included in future work, along with performance evaluations of other multiphasic solvent systems for enriched lipid extraction and enhanced biomarker discovery.

5.6 References


CHAPTER 6
APPLICATION OF DAPNE WITH MICRO-CAPILLARY SEPARATORY CHEMISTRY-COUPLED TO MALDI-MSI FOR SINGLE-CELL LIPID ANALYSIS OF NORMAL AND MALIGNANT CELLS IN CULTURES

6.1 Abstract

Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) is a useful tool for spatially identifying analytes of interest across a sample’s surface. MALDI-MSI for lipid analysis suffers from ion suppression of neutral lipids in the presence of phosphocholine species. While MALDI-MSI spatial resolution has increased for single-cell analysis through techniques such as oversampling, it can still suffer from decreased sensitivity. Here we describe an application of MALDI-MSI coupled to direct-analyte probe nanoextraction-microcapillary-separatory chemistry (DAPNe-sep-tip-MALDI-MSI) for the extraction of single cells and dispersion of analytes into immiscible solvents to decrease ion suppression and retain sensitivity and spatial information associated with the extracted cell.
6.2 Introduction

Cellular lipids are diverse biomolecules found to play important roles in cellular structure, signaling, energy storage, and metabolism.[183] Many diseases are known to encompass metabolic changes that lead to unregulated lipid metabolism resulting in increased cellular lipid heterogeneity within a seemingly identical cellular population. Cancer cells are known to increase glutamine uptake,[184] which can lead to diacylglycerol accumulation.[185] While increases in reactive oxygen species (ROS) and oxidative stress are also common in many types of cancer,[186] and can affect several lipid classes including phosphocholines,[187] cholesterol esters,[188] sphingomyelins, and ceramides.[189, 190]

Lipid profiling by mass spectrometry is typically performed by extracting the lipids of a large group of cells from culture or tissue. The extraction procedures often require multiple purification and/or preparation steps that can lead to loss of analytes or sample volume. These samples are then analyzed by mass spectrometry either through direct injection or coupled with chromatography. The data obtained from this type of analysis is representative of the averaged lipid profiles of the cells and the individual cellular heterogeneities that exist are not identified.[13] Furthermore, developmental changes or progressive disease-induced alterations cannot be monitored within the sample as it has been consumed during this type of extraction. Therefore, the need for single-cell lipid analysis methods to identify these heterogeneities is of the utmost importance to understanding the altered lipid metabolism and related cellular functions of progressive diseases, such as cancer.
Lipid analysis using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is well established [187, 191, 192] and allows for spatially resolved lipid analysis of tissues,[191, 193] and single cells[131] through MALDI-MS imaging (MALDI-MSI). MALDI-MSI lipid analysis of healthy and diseased tissues and cells has been used as a method to find lipid biomarkers for clinical use [193] and to identify changes in metabolism.[191] MALDI-MSI has some inherent drawbacks regarding single cell imaging as the homogeneity and size of matrix crystals, diffusion of analytes across a sample surface,[131] and laser spot size can all affect spatial resolution. Oversampling has been shown to effectively increase spatial resolution[194], however, an increase in spatial resolution results in a decrease in sensitivity.[131] Furthermore, MALDI-MSI can suffer from ion suppression during lipid analysis of mixed lipid samples,[180] but can be reduced or removed by coupling liquid chromatography to MALDI-MSI (LC-MALDI-MSI);[195] however, digestion of the sample is required and spatial information is lost.

MALDI-DOMS (direct organelle mass spectrometry) was recently introduced as a method to discriminately extract and analyze individual organelles from cells using direct analyte probe nano-extraction (DAPNe) coupled to MALDI-MSI.[14] DAPNe is performed using nanomanipulation with a spatial resolution on the nanometer scale allowing for the precise extraction of individual organelles,[14, 77, 78, 81, 196] single-cell,[13, 197] or analyte of interest[55, 74-76, 80, 179] leaving the remainder of the sample viable for continued culture and/or future analysis. Furthermore, coupling micro-capillary separatory chemistry to DAPNe-MALDI-MS analysis (DAPNe-sep-tip-MALDI-MS) has previously been demonstrated to separate molecules based on their phase
dispersion between two immiscible solvents for analysis by MALDI-MSI.[197] DAPNe-sep-tip-MALDI-MS reduced the ion suppression of lipids by phosphocholine species and through dispersion separated more polar non-lipid contaminants from lipids in a whole cell extraction.

Here we describe the application of DAPNe-sep-tip-MALDI-MSI for the lipid analysis of single cells from a normal murine liver cell line (ATCC® TIB-73™ (CRL-5973)) and its malignant clone in culture (ATCC® TIB-75™ (BNL 1ME A.7R.1) (American Type Culture Collection (ATCC), Manassas, VA, USA)). The malignant TIB-75 cells have an increased baseline of reactive oxygen species (ROS) as compared to the normal TIB-73 cells.[198, 199] The two cell lines were cultured under the same conditions to determine variations in lipid profiles arising from changes in lipid metabolism as a result of malignancy through chemical transformation and in response to increased ROS. Extraction, spotting, and matrix application were performed following the procedure described by Hamilton et al.[197] In brief, a single cell was extracted into a micro-capillary tip containing 1µL of chloroform. After 5 minutes, a 1 µL droplet of water was then aspirated into the capillary tip, phase separation occurred, the sample was spotted onto a MALDI plate, and 2,5-dihydroxybenzoic acid (DHB) applied as matrix by nebulized spraying. Four replicates per cell line, one replicate of the cell culture media, and one replicate of the cell buffer were performed.

Peak detection was conducted using mMass software[200] and used to build a peak list. Detected peaks located outside of the sample spot and peaks associated with cell buffer or growth media were removed from the peak list. The peak list was then searched for against the Lipid MAPS database[173] for putative identifications among
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Table 6.1  Total relative abundances of the seven reported lipid classes for each of the single cell extracts, four TIB-73 cells and four TIB-75 cells. The LPC/PC row represents the lysophosphatidic acid to phosphatidylcholine ratio for each cell extract.
seven lipid classes: phosphocholines (PC), lysophosphocholines (LPC), triacylglycerols (TG), diacylglycerols (DG), cholesterol esters (CE), sphingomyelins (SM), and ceramides (Cer), Table 1. Peaks selected for analysis were all matched at 3 ppm or below and did not overlap with other lipid classes. The peak intensities were summed in the instance where more than one adduct of a lipid species was matched (e.g. \([M+H]^+\) and \([M+Na]^+\)). As a result, 38 lipid species were putatively matched: 8 PC, 4 LPC, 4 TG, 7 DG, 2 CE, 9 SM, and 4 Cer.

For each replicate, the total peak intensity for each lipid class was calculated to determine their relative abundances. Images of the spotted cell extracts were compiled for each of the reported lipid classes, Figure 1 and 2. The averages and standard deviations were calculated for the replicates within each cell type, Table 1. It should be noted that high standard deviations are a result of cell-to-cell variations in lipid abundances and have been shown to indicate cellular heterogeneity. [81, 196]

Within the single cell extractions the SM lipids represented roughly one fourth of the lipids reported with an average of 27.37% and 25.44% in the TIB-73 and TIB-75 cells, respectively. The TIB-75 cells had higher abundances of both PC and LPC compared to the TIB-73 cells. Furthermore, the LPC to PC ratio nearly doubled from 0.36 in the normal cells to 0.61 in the malignant cells. Glycerolipids, both TG and DG, increased in the TIB-75 cells compared to the TIB-73 cells. While the CE species were considerably lower in the malignant cells as compared to the normal cells, 6.68% and 35.75%, respectively. Finally, the abundance of the Cer species were found to be lower in the normal cells, 4.66%, compared to the malignant cells, 10.60%.
Figure 6.1 (a-g) MALDI images of selected peaks of TIB-73 single cell extraction for the visualization of their respective dispersion. (h) The total ion count of the cell extraction. Identified peaks of most abundant ion from each lipid class in Table 1.
Figure 6.2 (a-g) MALDI images of selected peaks of TIB-75 single cell extraction for the visualization of their respective dispersion. (h) The total ion count of the cell extraction. Identified peaks of most abundant ion from each lipid class in Table 1.
To demonstrate the extent of lipid heterogeneity, lipid extractions from whole culture dishes (lysates) were prepared using the Folch method. For continuity, only those lipids identified in the single cell samples were considered for comparison, Table 2. Phosphocholines were the most abundant lipid class reported in the lysates of the normal, 29.03%, and malignant cells, 57.77%. The LPC to PC ratio was 0.07 and 0.06 in the normal cells and malignant cells, respectively. Compared to the single cell extracts the lysate samples of both cell types had lower abundances in the CE and SM species reported. The TG and DG species showed a slight increase in the TIB-73 lysates compared to the single cells, but nothing significant. While, the same TG and DG species in the TIB-75 lysates were one-half and one-third the abundance of the single cells. Lastly, the Cer species in the normal cell lysate were found to be greater than four times that of the normal single cells. The same Cer species in the malignant lysate were found to be less than half that of the malignant single cells.

In the comparison of the single cells to the lysate there were both expected and unexpected results. The LPC to PC ratio is considerably lower in the lysates compared to the ratios found in the single cell extractions for both cell types. The formation of LPC from PC is normally mediated by phospholipase A2 (PLA2), however, in the presence of reactive oxygen species (ROS) the formation of LPC has been shown to increase. Therefore, the ratio was expected to increase in the TIB-75 lysate in response to the previously stated increase in the baseline ROS of these cells. Cholesterol esters have been shown to undergo oxidation to oxy-cholesterol esters in the event of increased oxidative stress. The decrease in abundance of the CE species is evident in both
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</tbody>
</table>

Table 6.2. Total relative abundances of the seven reported lipid classes with single cell abundances averaged for each cell type and compared to the whole culture dish lysates. The LPC/PC row represents the lysophosphatidic acid to phosphatidylcholine ratio for each cell extract.
the lysate and single cell extractions of the malignant cells as compared to the normal cells, which was expected. Sphingomyelinases catabolize sphingomyelins to ceramides and ROS has been shown to increase sphingomyelinase activity in cells. Therefore, the SM abundance is expected to decrease and the Cer species increase in the malignant cells relative to the normal cells. This expected change in abundance is evident in the lysate samples; however, the malignant single cells show a slight increase in the Cer species compared to the normal single cells. Malignant cells often rely on other sources of carbon, such as glutamine, for biomolecule synthesis,\cite{184} which can lead to accumulation of DG in cells.\cite{185} Therefore, the decrease in DG abundance in the lysate of the malignant cells is unexpected, but the TIB-75 single cell extracts do have an increase in DG abundance relative to the normal cells. Lastly, the ion suppression effects of PC species in MALDI lipid analysis can result in the complete absence of TG ion detection. To illustrate the reduced ion suppression from the DAPNe-sep-tip-MALDI-MS method the TG species abundance is reported. It is important to point out that the TIB-75 TG species are still detected despite the significant increase in PC species.

6.3 Conclusion

The DAPNe-sep-tip-MALDI-MS method demonstrated here is capable of single cell imaging without the use of oversampling or modification of the laser spot size. Molecule separation through phase dispersion offers an alternative to chromatography methods while retaining spatial information relative to the cellular population of which individual cells are extracted. Furthermore, DAPNe-sep-tip-MALDI-MS does not suffer
from decreased sensitivity associated with other methods used to increase the spatial resolution of MALDI imaging,[131] The method described here reveals the extent of lipid heterogeneity within a cellular population of phenotypically similar cells that is not identified in the analysis of large groups of cells. Single-cell analysis through DAPNe-sep-tip-MALDI-MS provides an approach to identify biomarkers that may be lost in other MS methods due to ion suppression.

6.4 References


Hamilton, J. S.; Verbeck, G. F.; One-Cell Analysis as a Technique for True Single-Cell Analysis of Organelles in Breast Tumor and Adjacent Normal Tissue to Profile Fatty Acid Composition of Triglyceride Species. Journal of Analytical Oncology 5, 47-54 (2016)


CHAPTER 7

CONCLUSION AND FUTURE WORK

7.1 Single-Cell Extractions

In previous work the extraction of lipid droplets from adipocytes was developed into a streamlined method of cell breaching and organelle extraction with little to no affects on surrounding cells. However, the extraction of whole adherent cells has proven to be difficult in practice. Currently, the best method is to loosen cell adherence with trypsin before selecting cells for extraction. While successful in the extraction of an individual cell the spatial information is lost. Optimization of adherent whole-cell extraction should be a focus for future work. This may be realized with the addition of laser breaching. An addition of a small volume of trypsin in the extraction tip may also prove effective if the mass spectrum does not incur any added noise from the trypsin.

7.2 Automation of the Nanomanipulator

The current procedure for extraction is time consuming and dependent on user ability to prevent breaking of capillary tips while landing on the surface of the analyte to be extracted. Implementing a method to automated the landing in the z-axis would greatly benefit this method and reduce lost sample due to solvent spillage in the event of a broken tip.

7.3 MALDI Plate Modifications

The solvents used for analyte dispersion experiments are immiscible and will repel each other. The use of chloroform and its low surface tension present problems
when trying to reduce spot size and promote lipid aggregation in sample spots. MALDI plates could be modified by etching wells that will contain the sample volume within a space forcing continued phase interaction while maintaining a specific spot size may provide increased analyte dispersion and aggregation of like analytes.