ABSTRACT

EKELÖF, MÅNS OLOF. Advancements in Infrared Matrix Assisted Laser Desorption Electrospray Ionization Mass Spectrometry Instrumentation and Methodology (Under the direction of Dr. David Muddiman).

This document details work undertaken along several avenues towards improving the analytical potential of infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI). IR-MALDESI is an ion source for mass spectrometry (MS) and mass spectrometry imaging (MSI), originally conceived in the Muddiman group where it has been used and continuously developed since 2006. The heart of the instrument is a mid-IR laser used to sample material from a sample surface directly into the charged aerosol of an electrospray plume. The mechanism of ionization is very similar to conventional electrospray ionization (ESI), dominated by adduct transfer reactions.

Coupled to a commercial Orbitrap Fourier transform mass spectrometry platform, the IR-MALDESI source produces rapid bursts of transient ion current with each laser shot, which is accumulated in an ion trap prior to analysis. A concern with any instrument requiring prolonged confinement of ions is the loss of material due to coulombic repulsion. To measure and reduce this effect, a method for exact synchronization of events was developed, allowing for removal of previously necessary overhead on the trapping time. With synchronization down to the single microsecond timescale, it was possible to measure the actual ion current produced by each laser shot. Measurements of ion current revealed that the peak ion current generally occurs 2-4 ms immediately following a laser ablation event, and that trapping for more than 10 ms yields only minimal increase in sensitivity. In fact, when allowing a wide mass range into the trapping region leads to very significant loss in signal after as little as 20 ms depending on the total ion
population. These results have been used to define new standard methods for IR-MALDESI imaging, resulting in improved sensitivity and reduced variability.

Since the introduction of IR-MALDESI, there have been several updates to the laser technology used for sample desorption. We have evaluated the performance of a novel diode-pumped burst-mode laser, capable of producing mid-IR laser pulses of 1 mJ or more in response to a digital trigger signal, allowing easy synchronization and a distinct improvement over previously used flashlamp-driven lasers. The new laser system reduces the weight and footprint of the source by more than half, and our experiments show that it performs very well in mass spectrometry imaging applications.

Previously published IR-MALDESI methods have been developed specifically for imaging of animal tissue. However, the direct sampling nature of the source makes it an excellent candidate for interfacing mass spectrometry to high throughput direct analysis. The source was adapted to sample from a microtiter plate, and initial experiments with screening targets taken from actual pharmaceutical research indicate that IR-MALDESI can provide sufficient quantitative measurements from samples even in the presence of significant contamination, including added surfactants and inorganic salts. At sample throughput rates at one or more samples per second, we have achieved good analytical performance of confirmatory screening assays, with the analyte specificity of high resolution mass spectrometry.

The combined need for speed of acquisition and maximal information content, for direct analysis as well as imaging, motivated a major new implementation of IR-MALDESI on an ion mobility platform. Using the on-line size-based separation of ion mobility together with the mass specificity of mass spectrometry, structural information about analyte molecules is gained without the sacrifice of sample throughput. Early experiments with IR-MALDESI-IM-MS on a
drift tube ion mobility instrument demonstrate analytical quality on par with previous instrumentation, with the added capacity for isomer distinction.

The here presented improvements to IR-MALDESI instrumentation have yielded a significantly more reliable instrument that is easy and affordable to implement in the research lab.
DEDICATION

In memory of my grandfather, Torsten Svenre, who taught me the importance of studying thermodynamics and the English language.
BIOGRAPHY

Måns Ekelöf was born in Stockholm, Sweden. He received his master’s degree in chemistry and chemical engineering from the royal institute of technology (KTH) in 2013, where he found an interest in mass spectrometry and development of analytical instrumentation. He came to NC State in 2015, where he has since been happily tinkering away under the supervision of Dr. David Muddiman.
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CHAPTER 1
Mass Spectrometry – Generation, Separation and Detection of Ions

1.1 A Brief Introduction to Mass Spectrometry

1.1.1 Theory and History

Mass Spectrometry (MS) is an analytical technique allowing separation of molecules based on mass. By utilizing the fundamental structural property of molecular weight as the mode of separation, MS provides a uniquely informative tool for the analytical chemist.

Since its inception as a technique, all implementations of mass spectrometry have relied on the same basic processes for mass separation. The analytes, which must be present as charged particles or ions, are operated on by an electromagnetic field, imposing a coulombic force on each particle proportional to its charge. Each individual particle accelerates at a rate which is directly proportional to the force, and inversely proportional to its inertial mass. By rearranging these fundamental properties of matter as shown in equation 1.1, it can be seen that the trajectory of any given particle in a given electromagnetic field can be fully described as a function of its ratio of charge to mass. By convention, mass spectrometry normally reports results in units of mass to charge (m/z).

\[ \vec{F} = z(\vec{E} + \vec{v} \times \vec{B}) \]  (1)
\[ \vec{F} = \frac{dm\vec{v}}{dt} = m \frac{d\vec{v}}{dt} \]  (2)
\[ \Rightarrow \frac{d\vec{v}}{dt} = \left( \frac{z}{m} \right) \left( \vec{E} + \vec{v} \times \vec{B} \right) \]  (3)

Equation 1.1– Combination of the Lorentz force law describing forces affecting charged particles in electromagnetic fields, and Newton’s second law of motion describing the motion of non-relativistic bodies. The constant z/m in the final expression is the basis of separation in mass spectrometry.
The first successful MS experiment was reported in 1897 by J.J. Thomson, who used an electrostatic field to deflect the beam of radiation produced by a cathode tube. By measuring the angle of deflection, Thomson was able to infer the existence of a negatively charged particle of discrete mass, much lighter than any known atom. He received the Nobel Prize in physics in 1906 for his discovery of the electron. His mass spectrometer, although he did not know it by that name, established the basic pattern that every mass spectrometer since has followed.

1.1.2 The Mass Spectrometer: Ion Sources

Before any spatial manipulation based on mass-to-charge ratios can take place, the analytes of interest must be electrically charged and capable of unhindered motion, IE placed in a vacuum. In modern mass spectrometry, the target analytes are molecules rather than charged elementary particles, and thus one of the defining features of any mass spectrometer is the choice of strategy for converting sample molecules into gas phase ions.

Loosely, ionization processes can be grouped into two categories; hard and soft ionization. Producing ions from neutral molecules requires a transfer of charge. The energy required to directly ionize a molecule is often enough to dissociate molecular bonds, causing molecules to fragment. Sources that provide ionization with a large degree of fragmentation are referred to as hard sources. The pattern of smaller, charged fragments produced by a particular compound when subjected to hard ionization can act as a fingerprint, providing structural information about the parent molecule.

The most commonly used hard ionization source is known as electron impact ionization (EI). In EI, gas phase samples are exposed to a beam of fast electrons which collide with and excite molecules, leading to a net emission of electrons. EI typically produces cation radicals which undergo rapid fragmentation. It is possible to reduce the degree of fragmentation by
performing the ionization with an intermediary step where a separate reagent gas is subjected to
the electron bombardment, and the sample is ionized though less energetic interactions with the
resultant product ions. This method is known as chemical ionization (CI). EI and CI sources are
suitable for coupling to gas chromatography (GC), and such GC-MS systems are commonly used
for analysis of volatile or volatilizable compounds in a variety of fields, including petrochemistry
and food science.

For relatively small molecules (less than 1 kDa), EI and CI fragmentation patterns are
often distinct enough to provide unambiguous structural determinations. For larger molecules,
the complexity of the fragmentation reaction becomes a limiting factor, as well as the difficulty
of vaporizing such molecules without causing thermal degradation. The first truly soft ionization
sources were introduced in the 1980s, opening up the field of mass spectrometry to the analysis
of intact macromolecules. The common attribute of modern soft ionization techniques is that
analytes acquire charge through chemical transfer of charge carriers, such as acid-base type
proton transfer or metal ion adduction.

1.1.3 The Mass Spectrometer: Mass Analysis

Once ions are formed, they are accelerated in an electrostatic field, designed such that
they are separated on the basis of mass over charge. The device that accomplishes this separation
is known as the mass analyzer, and forms the core of any MS instrument. The simplest mass
analyzer, known as a time-of-flight (TOF) analyzer, consists of a straight tube, across which is
applied an electrostatic potential which accelerates ions axially. Ions of lower inertial mass are
accelerated at a higher rate than heavier ones, which leads to a separation in time of flight which
can be measured by a suitable detector at the end of the flight trajectory. Other mass analyzers
utilize curved or oscillating ion trajectories to separate ions in time (reaching the same detector at
different times) or space (detection at different locations.) A mass analyzer that is used to physically separate out a range of masses and remove all others is sometimes referred to as a mass filter.

In order to use the differences in trajectory for mass determination, it is necessary to minimize gas-phase interactions, meaning that all mass analyzers must operate at pressures low enough to ensure that ions have a mean free path on the order of their total trajectory length. Typical mass analyzers require vacuum pressures between $10^{-5} – 10^{-10}$ torr. Where mass separation is achieved on the basis of differential mobility, an increase in path length also increases the separation between masses, effectively increasing the ability of the instrument to resolve peaks in the mass spectrum.

One of the simplest and most versatile means of controlling ion motion is by total confinement, or trapping, in a potential field. An ion trap can be as simple as a rectilinear box with repulsive potentials applied to all sides, but there are many designs using electromagnetic fields to confine ions in some favorable geometry. To use an ion trap for mass spectrometry, it is necessary to induce mass-dependent motion upon the trapped ions. In traps where ions are confined to oscillating trajectories, it is possible to apply an external RF potential of resonant frequency to excite a particular ion. This is typically used in quadrupole ion traps to scan over a range of such frequencies, selectively destabilizing and ejecting ions based on mass to charge. The ejected ions can either be measured by a detector such as an electron multiplier, or discarded entirely to enable mass filter behavior.

In some configurations, it is possible to use the exciting waveform to increase the amplitudes of ion oscillations without causing ejection, in such a way as to cause ions of similar mass to charge to move together as dense packages of charge. The oscillations of these ion
packages can be detected by the current induced on an electrode. Collecting the induced current over time gives rise to a complex convoluted waveform, with each trapped species contributing a sinusoid of a different frequency. This transient waveform can be converted to a frequency spectrum by means of the Fourier transform. If the frequency is a known function of mass and charge, the mass spectrum can be obtained by a single transformation of the frequency spectrum.

1.2 The IR-MALDESI Source

One approach to soft ionization, electrospray ionization (ESI), was perfected by Fenn in the mid-80s\(^2\), based on a concept previously described by Dole and others\(^3\). In ESI, a high voltage is applied to a solvent, producing ions through electrochemical reactions which migrate in the strong electrical field. At sufficient field strength, the solution spontaneously aerosolizes into a fine spray of charged droplets from which analyte ions can be desolvated and detected by mass spectrometry\(^4\).

The use of lasers to generate ions for mass spectrometry dates back to the 1960s when Honig and Woolston introduced what would later be known as laser desorption/ionization (LDI)\(^5,6\). However, significant fragmentation of molecular ions was observed in LDI experiments. In the late 1980s, it was discovered that mixing the analyte with an energy-absorbing matrix prior to laser desorption and ionization significantly reduced the fragmentation, allowing truly soft, non-destructive ionization\(^7,8\). In matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, ions are formed in chemical reactions between the sample and matrix substance, before (in solution) or directly following (gas-phase) a sampling laser pulse\(^9\). As a consequence, ionization efficiency depends heavily upon the choice of matrix and the method of matrix application.
Rather than relying on this mechanism for ion generation, it is possible to use a secondary excitation source to ionize neutrals desorbed by the sampling laser. This post-ionization approach was described by Beekman and coworkers in 1980\textsuperscript{10}, who successfully used a second laser beam to produce ions from laser ablated neutrals through resonant excitation. Other groups proved the utility of this method for the analysis of biomolecules\textsuperscript{11,12}. More recently, Shiea and coworkers demonstrated the use of an electrospray plume for effective post-ionization in what they called electrospray-assisted laser desorption ionization (ELDI)\textsuperscript{13}. Sampson and coworkers brought this strategy closer to its MALDI roots by re-introducing the use of a resonantly absorbing matrix, a method known as matrix-assisted laser desorption electrospray ionization (MALDESI)\textsuperscript{14}.

In MALDESI and related methods using electrospray for post-ionization, ions are formed through an ESI-like process where charge is imparted through adduct transfer to neutral ablated species from the charged electrospray solvent droplets\textsuperscript{15}. Since charged contaminants are repelled or neutralized by the high surface charge of the electrospray, MALDESI-MS can be thought of as ESI-MS with gas phase sample cleanup prior to ionization, rendering it significantly less sensitive to suppression from stable ionic species.\textsuperscript{16}

1.3 IR-MALDESI and Mass Spectrometry Imaging

Like other ionization methods using spatially sampling probes, MALDESI is inherently suitable for mass spectrometry imaging (MSI), where each mass spectrum is mapped to a spatial location. The main driving force in the field of MSI has since its beginnings been the need to track molecular distributions in biological systems.\textsuperscript{17} MSI has the ability to map a large range of molecules from small metabolites to intact proteins, without the need for chemical or radiological labels. As an inherently destructive technique, laser-based MSI is primarily used for
analysis of biopsied or necropsied animal tissue rather than in-vivo. The spatial resolution is a function of the beam profile of the ablating laser, with smaller ablation volumes allowing resolution of finer details at the cost of lower signal.\textsuperscript{18}

Since MSI is ideally performed without sample cleanup or pre-separation, the chemical complexity of real samples puts a limit on the depth of information that can be extracted. A typical MALDESI scan measures a relatively low number of molecular ions, at most $10^6$ - $10^7$ elementary charges, generated from the ablation of 0.1 - 1 µg of sample depending on laser footprint as well as sample thickness and density. Even by the most optimistic estimate, the transfer efficiency from sample to detector is low – less than a part per trillion of the sampled material being actually measured. For this reason, tissue imaging is limited to metabolites of particularly high numerical abundance or specific ionization efficiency. A high resolving power instrument is crucial, allowing metabolite signals to be resolved from isobaric metabolites as well as the chemical noise; ion of unknown origin and without analytical value. Chemical noise sources include the characteristic background of matrix and solvent clusters in MALDI and ESI respectively, as well as volatile contaminants from the laboratory environment.
1.4 References


CHAPTER 2

IR-MALDESI Method Optimization Based on Time-Resolved Measurement of Ion Yields

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2.1 Introduction

Electrospray post-ionization is an ambient ionization technique where a sample is ionized through interaction with an electrospray plume. The sample can be introduced in a number of different ways. Notable examples of strategies for sample introduction include direct contact with a sample surface, an intersecting aerosol, and the use of sampling probes such as a metal needle or a laser beam. Interfacing electrospray post-ionization with spatially resolved sampling methods makes it suitable for mass spectrometry imaging (MSI) applications, where the recorded ion abundances are mapped back to a physical location.

In matrix-assisted laser desorption electrospray ionization (MALDESI) mass spectrometry, a laser is used as a sample probe to desorb analytes from a surface placed under an orthogonally oriented electrospray cone. In this manner an ESI-like ionization is achieved, where ions are produced mainly through charge transfer in solvent droplets. By employing a mid-infrared (IR) laser, it is possible to use water or ice as an external matrix with the laser wavelength tuned to the 2940 nm absorbance maximum of the O-H stretching mode of water. In IR-MALDESI analysis of biological samples, an externally applied layer of ice is used to provide homogeneous sampling. Keeping samples frozen throughout imaging experiments, which often require several hours, also serves to prevent chemical and enzymatic degradation as well as dehydration.

The IR-MALDESI ion source used throughout this work was coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany). The Q Exactive series utilizes a
bent quadrupole ion trap (C-trap) to accumulate ions prior to injection into the Orbitrap analyzer, making it suitable for use with continuous ion sources such as ESI and APCI.\textsuperscript{9,10} In typical ESI-MS using a Q Exactive Plus, the automatic gain control function (AGC) is used to keep the total trapped charge roughly constant between scans through varying the ion injection time (IT) based on a brief pre-scan of the total ion current (TIC). This has been shown to greatly improve mass measurement accuracy (MMA) combined with a strategy of using known ambient peaks as lock masses for continuous internal re-calibration.\textsuperscript{10}

To measure a pulsed injection from electrospray post-ionization, it is necessary to disable AGC, as ion accumulation must necessarily coincide with the burst of ions from the ionization event. In the IR-MALDESI source, C-trap accumulation is externally triggered, and IT is held at a constant value that is selected to ensure that all generated ions are captured. For animal tissue sections, two mid-IR laser pulses are typically required for complete sampling, which is a prerequisite for absolute quantification.\textsuperscript{11} Using a commercially available 20 Hz laser, suitable injection times fall on the order of 100 ms, two orders of magnitude higher than typical LC-MS analysis using the same instrumentation. As a consequence, during a typical IR-MALDESI analysis sequence using a wide mass window, the C-trap is filled to levels far exceeding its nominal capacity, which is on the order of $10^6$ elementary charges.\textsuperscript{10} The long trapping time leads to the collection of a large fraction of ambient ions of no analytical value, reducing the effective dynamic range of quantitative imaging experiments.

There are two obvious strategies for improving data quality through reducing total trapped charge: lowering trapping times to minimize the collection of ambient ions, and limiting the m/z range allowed into the trap by means of a mass filter. The former has been investigated by Rosen and coworkers, who introduced a high repetition rate IR laser (100 Hz) in order to
reduce the C-trap injection time of a Q Exactive instrument, noting a significant improvement to measured signal at lower injection times.\textsuperscript{12} The latter strategy is suitable for targeted analysis, where broad coverage can be sacrificed for greater sensitivity to a particular ion of interest.

To provide a complete and detailed model of the generation and accumulation of ions from electrospray post-ionization, we here describe a method for synchronizing the Q Exactive Plus ion injection to the laser firing order, and detail several experiments measuring the accumulation of target ions at discrete times after the laser ablation. Using this system, we demonstrate a simple and rapid optimization method for finding both the minimum required accumulation time per ablation event and the maximum total trapping time for a given sample and desired mass range.

\textbf{2.2 Materials and Methods}

\textbf{2.2.1 Materials Used}

Animal tissues were stored at -80 °C until the time of analysis. Sectioning of tissues was done using a Leica (Buffalo Grove, IL, USA) CM1950 cryomicrotome. Tissue sections were thaw-mounted on glass microscope slides for IR-MALDESI analysis. LC-MS grade water and methanol were purchased from Acros Organics (Geel, Belgium). PEG-600 and MS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrogen gas for enclosure purging and humidity regulation was purchased from ARC3 gases (Raleigh, NC, USA).

\textbf{2.2.2 Control Scheme}

To achieve accurate synchronization between the laser ablation event and the start of Q Exactive ion collection, it was necessary to operate the instrument in the “low-latency handshake mode” provided by Thermo Fisher Scientific. In this mode, the instrument responds to an
externally provided trigger signal much faster than the optional “handshake mode”, which introduces a variable delay of up to 100 ms between trigger and response. Detailed diagrams of signal schemes illustrating the difference between the modes are provided as Figure 2.1.

![Diagram of communication schemes](image)

**Figure 2.1** Diagram of communication schemes. Top – Imaging sequence on the Q Exactive in handshake mode. Each trigger signal is followed by a variable and unpredictable delay before start of ion accumulation. The flashlamp pumped OPO laser is run at a constant rate of 20 Hz, and the two laser shots (red lines) are thus not well synchronized with C-trap accumulation. Bottom – Imaging sequence on the Q Exactive in low-latency handshake mode. In this mode, the instrument responds near instantly to the trigger signal, which is externally synchronized to the 20 Hz laser firing order. While low-latency mode is active, the trigger pulse must be provided with a period of approximately $tm < t < 2tm$ where $tm$ is the total time of analysis. At 140,000 nominal resolving power, $tm$ is about 0.5 s.

To achieve the desired behavior in low-latency mode, a set of specialized external circuits were designed and constructed. In this design, a Quantum Composers (Bozeman, MT) 9214 Sapphire square pulse generator was used to control the flash lamp and Q-switch functions of an IR-Opolette 2371 OPO laser (Opotek, Carlsbad, CA, USA) and to provide a timing reference for ion injection triggering. Signal routing was handled by a control unit built around an Arduino
Uno microcontroller (Arduino, Ivrea, Italy). With this system, MS acquisition could be synchronized to a laser trigger with microsecond precision as verified through repeated measurements on a THS-720A oscilloscope (Tektronix, Beaverton, OR). A complete circuit diagram and representative oscilloscope measurements are provided as Figures 2.2 and 2.3.

**Figure 2.2** Complete diagram of external circuits used to keep synchronization between the components of the imaging source. For RLY1, this diagram represents the pinout of LAA110 dual solid state relays. For specifications of Q Exactive in/out circuits, please refer to the latest revision of the instrument manual.
2.2.3 Safety Considerations

The use of a class IV invisible laser for sampling necessitates a number of precautions for safe use. A LAZ-R-SHROUD barrier (Rockwell Laser Industries, Cincinnati, OH) was erected around the IR-MALDESI source at all times during the described experiments to protect other persons working in the laboratory. Additionally, OD 5+ rated protective goggles (Laser Safety Industries, Minneapolis, MN) were kept next to the potential exposure zone and provided to those required to be present in the area with the laser in operation. To minimize the risk of exposure to stray beams, the entire optical path before focusing optics was shielded by anodized aluminum protective screens (Thorlabs, Newton, NJ). All focusing optics were arranged in a protective 1 inch lens tube.

2.2.4 Measurements of ion accumulation and decay from solution

A 0.1 mg/mL solution of polyethylene glycol, average MW 600 Da (PEG-600) in 50:50 methanol:water was analyzed directly from 100 µL microwell plates (Cat. No. 7816 20, Brand GMBH & CO KG, Wertheim, Germany) with the IR laser focused at the center of the meniscus.
in each sample well. The solution was aerosolized with a single laser shot synchronized to the trigger signal for MS acquisition, and the ion injection time (IT) instrument setting was changed between scans. In each experiment, IT was cycled between 1 and 1000 ms in 26 pre-defined steps with each IT setting measured between 40 and 50 times. The complete list of time points is shown in table 2.1. These experiments were repeated for mass windows of 100, 200, 300 and 750 Th centered on the PEG oligomer ion of m/z 564.3596.
**Table 2.1.** Injection time values in milliseconds used for the PEG-600 study (left) and tissue optimizations (right). For the tissue optimization part, a reduced program was used to minimize the amount of tissue needed.

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2.2.5 Measurements of ion yield from ablated animal tissue

The signal rise time following laser ablation was characterized by repeated measurements using 2 ms IT windows delayed in 1 ms increments by 0-25 ms after the ablating laser pulse. A 10 µm section of mouse liver was used as a model tissue. The analysis was otherwise performed as previously described for animal tissue imaging. The average signal of each 2 ms bin was used as an estimate of momentary ion flux. An additional experiment was performed keeping all settings constant while cycling IT between 1 and 250 ms in 22 steps to measure actual ion accumulation at different times after the ablation event.

2.2.6 Method optimization for IR-MALDESI MS Imaging

An injection time cycling experiment with 22 time points between 1 and 250 ms was performed on rat brain and mouse liver tissue samples, sectioned into 25- and 10 µm sections, respectively. For the rat brain, only visually homogeneous areas of grey matter were included in the optimization region of interest. To simulate the conditions of a typical imaging experiment, all tissue analysis was done in the mass range of 250-1000 Th, using a nominal resolving power of 140,000 at m/z 200. Two laser shots were used for sampling, even when the injection time was set too low (<50 ms) to capture material from the second ablation event. The samples were kept between -10 and -8° C throughout analysis, and an ice layer of controlled thickness was deposited according to previously published methodology.

After determining the optimal injection time, sections from the same tissues were immediately imaged. The sections were imaged in two different regions; one with the new optimized setting for low-latency handshake mode operation, and one using a previously described procedure with the instrument in handshake mode using an IT of 110 ms. Directly adjacent regions were imaged this way to allow direct visual comparison.
2.2.7 Data analysis

Abundances measured on the Q Exactive are normally reported in units of ion current, i.e. ion abundance per time unit. This is to ensure that reported abundances correspond to analyte concentration in a directly infused sample when AGC is used. In order to compare abundances from pulsed injections with AGC disabled, it is necessary to multiply the reported value of each scan by the injection time. Whenever abundances are reported in absolute terms throughout this article, the reported values have been multiplied by the injection time in seconds.

To generate imaging information, raw data was converted from .RAW format to .mzml using the MSConvert tool from the ProteoWizard toolkit14, and subsequently from .mzml to .imzml using the imzMLConverter utility15. All ion images were generated using the open-source software package MSiReader16. All images here presented were generated with ±2.5 ppm tolerance.
2.3 Results

2.3.1 Ion overfill and signal decay

A solution of PEG-600 was used as a model system for ion accumulation and decay, providing a distinct and consistent series of oligomer peaks from each laser ablation to simulate the dense spectral population of lipid and metabolite peaks in a biological sample. The normalized ion abundance of the PEG oligomer ion at m/z 564.3596, at the center of each investigated mass range, was collected and converted to units of absolute abundance. A representative IR-MALDESI mass spectrum is shown in Figure 2.4. The average abundance plotted as a function of injection time for different mass ranges is shown in Figure 2.5.

Figure 2.4 Representative 250-1000 Th IR-MALDESI mass spectrum of 0.1 mg/ml PEG-600. Average of 450 scans using one laser shot and an IT of 25 ms.
Figure 2.5 Accumulation of m/z 564.3596 in four mass ranges. Acquired from direct analysis of 0.1 mg/ml PEG-600 using a single laser shot at t=0. One hundred scans were collected at each IT setting. Side-by-side comparison illustrates the loss of signal over time observed for wider mass ranges.

By allowing a larger range of ion masses to enter the C-trap for accumulation, it is possible to induce a loss of signal over time from ions injected at t=0. The observed signal loss can be well described by an exponential decay model as shown in Figure 2.6.
Figure 2.6 Comparison of ion accumulation curves for the PEG oligomer ion of m/z 564.3596 ± 2.5 ppm analyzed directly from 0.1 mg/ml PEG-600 solution. The curves include 95% confidence intervals for all measured points (n=40) as well as an exponential fit to the data points colored in red. The dashed line indicates a peak ion fill of ~3×10^6 in the higher mass ranges. In the lower mass range of 564 ± 50 Th, total fill did not reach a peak value, and no significant decay was observed.

The rate of decay is very similar where observed, and decay onset coincides with a total ion signal of approximately 3×10^6 AU, supporting the hypothesis of Rosen and coworkers that ion exclusion due to overfilling of the C-trap is the most significant cause of signal loss in imaging experiments. The utility of gas-phase fractionation to increase sensitivity is seen in the measurements of a 100 Th mass range, where C-trap fill levels were not exceeded and no decay at all was observed.

A similar but more detailed experiment was performed on animal tissue samples, where the characteristic rates of signal rise was measured for a number of ions. Figure 2.7 shows a comparison of ion accumulation measured as the summation of momentary ion flux in 2 ms IT bins staggered over 25 ms, compared to accumulation data acquired using incremental IT
synchronized to the ablation event. Data is shown for two tissue-specific lipids in the investigated range (200-800 Th). The lipids were putatively assigned as cholesterol [M+H-H2O]+ (m/z 369.3516) and PC(36:4) [M+H]+ (m/z 782.5702).

Figure 2.7 Top – Estimated ion flux of two mouse liver tissue-specific ions in the 200-800 Th mass range. Each bar represents an average of 100 scans using a 2 ms IT window delayed from the laser pulse at t=0 by 0-23 ms. The measured abundances have been normalized to sum to unity. Bottom – Ion accumulation calculated as integrated flux (grey line) in comparison with direct measurement using the IT-cycling method (red line).

This experiment shows that there are very significant differences in the dynamics of ion injection between the two analytes. In this case, using an injection time of 5-10 ms would introduce a very significant systematic bias towards the faster rising species. This unexpected observation must be considered when selecting a suitable metric for method optimization. While reducing accumulation times is generally desirable as a means of minimizing accumulation of ambient background peaks, in so doing one may introduce a bias for fast-rising signals.
2.3.2 Optimizing settings for IR-MALDESI imaging

To demonstrate the utility of trapping time optimization in an imaging application, the signal of cholesterol [M-H2O+H+] at m/z 369.3516 from healthy rat brain was measured in a visually homogeneous region of grey matter. Injection time was cycled between 1 and 250 ms. The accumulation curve and signal comparison are shown in Figure 2.8, suggesting an optimal cholesterol response at 70-80 ms after the first of two 20 Hz laser pulses. Based on this, a 75 ms IT was selected as an estimated optimum for comparison to previously established settings.

The imaging comparison in Figure 2.8 shows an increase in target ion abundance by a factor of 2 with the optimized settings compared to the standard procedure for animal tissue samples (110 ms IT in handshake mode). The whole experiment for empirically selecting a suitable IT was performed in less than 30 minutes using less than 1 mm² of representative sample, which allowed the imaging comparison to be performed on the same section of brain tissue under identical experimental conditions.
Figure 2.8 Accumulation curves of target ions used to determine suitable imaging parameters. Top – Cholesterol (m/z 369.3516 ± 2.5 ppm) abundance acquired from a 25 µm section of mouse brain using two laser pulses at 20 Hz. Error bars represent 95% confidence limits, based on 100 measurements at each time point. Bottom – IR-MALDESI images of cholesterol in rat brain acquired immediately following optimization from the same brain sample. Left side shows an optical image of a different section from the same individual rat for comparison. The ion images show a comparison between the optimized injection time of 75 ms and the previously used setting of 110 ms with the instrument in handshake mode.
Given the previous observation of individual differences in optimal trapping time, it may be necessary to consider more than a single indicator ion to optimize an untargeted experiment. This was tested by simultaneously measuring multiple known tissue-correlated species.

**Figure 2.9** Top - Six known tissue-specific ions acquired simultaneously from a 10 µm section of mouse liver and normalized to unity. Total ion current (TIC) is included to illustrate the relationship between ion fill and ion decay from a pulsed injection. A second laser shot at t=50 ms was used, which can be seen to provide no significant advantage in this case. Bottom – representative IR-MALDESI images comparing the optimized settings to the lab standard method.
The accumulation curves of six lipid ions from IR-MALDESI analysis of mouse liver is shown in Figure 2.9. Of particular note is that the second laser pulse in the sequence, at \( t = 50 \) ms, provides no significant signal increase for this sample. While rise times and decay rates vary significantly between analytes, there is an optimal region between 10-30 ms IT where most ions are present at >75\% of their peak abundance. The average normalized abundance has an apparent maximum at 10 ms IT, which was used for a comparison of imaging settings. The tissue was completely ablated by a single laser pulse, as confirmed by visual inspection after completed analysis. An inherent side benefit of reducing IT is the reduction of ambient peaks in the spectrum, as shown in Figure 2.10. Reducing the collection of parasitic signal automatically improves the dynamic range of the experiment.

**Table 2.2** Average absolute abundance of tissue-specific ions before and after injection time optimization. Averages calculated over the whole tissue region as seen in Figure 2.9.

<table>
<thead>
<tr>
<th>( m/z )</th>
<th>369.3516</th>
<th>269.2265</th>
<th>760.5893</th>
<th>300.2895</th>
<th>716.5246</th>
<th>874.7859</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ms IT</td>
<td>2.20E+03</td>
<td>4.80E+03</td>
<td>8.00E+01</td>
<td>2.80E+02</td>
<td>5.30E+02</td>
<td>1.30E+03</td>
</tr>
<tr>
<td>110 ms IT</td>
<td>1.50E+03</td>
<td>2.70E+03</td>
<td>2.30E+01</td>
<td>2.70E+02</td>
<td>2.60E+02</td>
<td>9.40E+02</td>
</tr>
<tr>
<td>Improvement</td>
<td>46%</td>
<td>78%</td>
<td>260%</td>
<td>3%</td>
<td>104%</td>
<td>38%</td>
</tr>
</tbody>
</table>

Table 2.2 shows a comparison of average ion abundances acquired with either setting over the whole tissue. Two of the selected lipids with the lowest individual signal improvements, \( m/z \) 369.3516 and 300.2895 (putatively cholesterol \([\text{M-H}_2\text{O+H}]^+\) and sphingosine \([\text{M+H}]^+\)) have significantly longer rise times than the other selected ions, which emphasizes the importance of choosing suitable settings when designing untargeted experiments. As a rule of thumb for untargeted IR-MALDESI analysis, injection times may be set to include 25 ms after the last laser pulse required for complete ablation of the sample tissue.
Figure 2.10 Representative IR-MALDESI spectra of the 365-375 m/z range from mouse liver, with cholesterol peak annotated. With the lower injection time, a higher absolute abundance of the target peak is noted. The clear injection time-dependence on abundance of peaks originating from the ambient background highlights the advantage of improved signal to background with lower injection time.
2.4 Discussion

Precisely synchronizing the laser ablation event to the start of ion collection allowed a thorough investigation of when in the process of electrospray post-ionization the measured signal arises, including the surprising observation that there are very significant differences in rise and decay times for different lipids in the same tissue. Signal rise times were found to vary between 5-30 ms after ablation.

In a real experiment where reproducible quantitative sampling is desired, the rise time of the slowest rising target analyte effectively dictates the minimum acceptable accumulation time. When the C-trap is filled to higher levels than it is rated for, there is an accompanying loss of signal, which can be modelled as an exponential decay. This relationship between fill rate and signal loss supports the findings of Rosen and coworkers\textsuperscript{12} regarding C-trap accumulation time dependence of signal, and emphasizes the importance of minimizing ion trapping times.

An injection time cycling experiment was designed to find the best injection time settings for a typical imaging experiment. A comparison with experiments performed using previous standard operating procedure greatly favors the optimized timing settings and provides a significantly improved ratio of signal to background, owing to the overall reduced injection times. We recommend including this brief optimization step as a routine procedure for any imaging method using an ion trapping step, as it requires only a few minutes of data acquisition and a small amount of equivalent sample while potentially offering a significant increase in sensitivity. This is essential for applications of MS imaging within the fields of lipidomics and metabolomics, where a large number of analytes of very different abundances are analyzed simultaneously.
2.5 References


11. Bokhart, M. T.; Rosen, E.; Thompson, C.; Sykes, C.; Kashuba, A. D. M.; Muddiman, D. C., Quantitative mass spectrometry imaging of emtricitabine in cervical tissue model


Chapter 3

Characterization of a Novel Miniaturized Burst-Mode Infrared Laser System for IR-MALDESI Mass Spectrometry Imaging

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3.1 Introduction

Infrared matrix-assisted laser desorption ionization (IR-MALDESI) combines the laser sampling of MALDI with the ionization properties of ESI\(^1\), resulting in a very flexible ion source capable of analyzing a wide range of analytes. MALDESI has been shown to work well with a range of laser wavelengths for sampling, from typical MALDI ultraviolet lasers to lasers well into the far-infrared range (\(\lambda > 10 \, \mu\text{m}\))\(^2\). The mid-infrared (IR) range close to 3 \(\mu\text{m}\) has proven particularly useful for the analysis of biological samples such as animal or plant tissues, as endogenous or exogenous water can be used as an efficient energy-absorbing matrix, reducing the need for sample preparation\(^3\). The method of relying entirely on the native mid-IR absorptivity of native water in the sample is often referred to as Laser Ablation Electrospray Ionization (LAESI)\(^4\) rather than ELDI which was originally performed using a 337 nm N2 laser. The use of lasers for sample introduction makes these ion sources inherently suitable for mass spectrometry imaging (MSI), where sampling is regularly spaced across a surface to produce a spatial ion map.\(^5\)

Many commercially available IR lasers suitable as sample probes for mass spectrometry use an optical parametric oscillator (OPO) to frequency-shift a lower wavelength, such as those produced by Nd- or Er-based solid-state pulsed lasers. Such laser systems are typically tunable, allowing access to a number of wavelengths based on experimental needs. OPO-based lasers used to date have employed flashlamp-pumped Nd:YAG lasers that required closed-loop water-
cooling, resulting in relatively large and expensive lasers that need periodic maintenance of the flashlamp and water cooling system. Here we demonstrate the use of a novel miniature, diode-pumped, conductively-cooled OPO laser for MSI instrumentation, suitable for IR-MALDESI, LAESI, ELDI and IR-MALDI applications.

3.2 Experimental

3.2.1 Materials Used

Nitrogen gas was purchased from ARC3 gases (Raleigh, NC, USA). LC-MS (Optima) grade methanol and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). MS-grade formic and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neonatal mouse samples were stored at -80°C prior to analysis, and sectioned into 25 µm sections on the day of analysis using a CM1950 cryomicrotome (Leica, Buffalo Grove, IL, USA).

3.2.2 Prototype Burst-mode Laser

The “burst-mode” 2940 nm laser prototype was developed by JGM Associates, Inc. (Burlington, MA) and is shown in Figure 3.1. This laser is a miniaturized 1064 nm-pumped OPO that converts 1064 nm emission into 1664 nm (signal wave) and 2950 nm (idler wave emission). The 1064 nm laser is a diode-pumped, passively Q-switched solid-state Nd laser made by JGM Associates for handheld LIBS instruments (laser-induced breakdown spectroscopy). The 2950 nm emission is separated from the residual 1064 nm emission and 1664 nm emission using two
dichroic mirrors inside the head; only the 2950 nm emission is emitted and used for laser desorption.

**Figure 3.1.** Photograph of the prototype burst-mode laser system in 50 mm² package.
Figure 3.2 Laser output measured on a LeCroy LC684DXL scope. A) 1664 nm pulse, output from ET-3500 photodetector B) 2950 nm pulse, output from PEM-10.6 photodetector
A temporal waveform for the 1664 nm pulse is shown in Figure 3.2a. This waveform was measured using an ET-3500 InGaAs photodetector (Electro-Optics Technology; Traverse City, MI) having a 25 ps rise/fall time, and a 1.5 GHz bandwidth LeCroy LC684 DXL oscilloscope (Teledyne-LeCroy Inc.; Chestnut Ridge, NY). The waveform for the 2950 nm pulse is expected to be similar, but we didn’t have a detector fast enough to resolve the modulation on the pulse. Figure 2b shows the 2950 nm pulse measured using a PEM-10.6 photoelectromagnetic (PEM) detector (Boston Electronics; Brookline, MA) having a rise/fall time of 1 ns.

Since all of the 2950 nm pulse energy is delivered within a short time compared to the thermal time constant of a typical MSI irradiation area/volume (focal spot diameters in the 10 to 100 micron range), tissue ablation proceeds as if all of the pulse energy were delivered instantaneously. Pulse energy of each 2950 nm pulse is 500 uJ measured at the laser.

The laser generates bursts of such 2950 nm pulses which may include 1 to 15 pulses per burst (user defined) at a maximum burst rate of 4 Hz. Energy of the individual pulses in the burst are all the same, e.g., a 10-pulse burst has total pulse energy of 5 mJ. During each burst, individual pulses are about 100 microseconds apart which means that laser pulse rate during each burst is 10 kHz. This inter-pulse period can be varied by adjusting peak current to the 1064 nm laser’s diode-pump array.

The laser head includes a photodiode that provides a pulsed output signal that is synchronous with generation of the Q-switched 1064 nm laser pulse. (Generation of the 2950 nm pulse is coincident with 1064 nm pulse generation). JGM Associates indicates that the complete diode-pumped 2950 nm laser head can be packaged into a 2”W × 2”L × 0.9”H volume as shown in Figure 3.1. Certain aspects of this laser design are patent-pending.
3.2.3 Incorporation of Laser into Existing IR-MALDESI Platform

The burst-mode laser was integrated into an existing IR-MALDESI research instrument with minimal modification. The laser head was mounted to the optical bench using pedestals (standoffs) that provided little or no conductive cooling into the optical bench. The laser head was essentially convectively air-cooled; a cooling fan was not used.

Control of fire order and synchronization to the ion injection on a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) required the construction of an external control circuit, using an Arduino Uno prototyping platform (Arduino, Ivrea, Italy) to interface the system to an instrument PC, enabling easy adjustment of laser parameters. A complete schematic of the required electronics is provided as Figure 3.3.
Figure 3.3 Circuit diagram for laser controls. The burst-mode laser is monitored and triggered by two general-use logic pins (D2 and D4 in diagram). The Q Exactive is controlled through contact-closure channels as described in the latest version of the instrument operating manual.

Driving current for the laser pump was provided through a D100 laser driver board (Highland Technology; San Francisco, CA), which was set to provide a 150 A peak current pulse in response to a 5 V trigger signal. The number of pulses in each burst was in this way directly controlled by the duration of the trigger signal. The laser pulses were detected using a silicon photodiode (optically filtered to block diode-pump light) inside the laser head and the width and spacing were measured using a THS-720A oscilloscope (Tektronix, Beaverton, OR).

Representative oscilloscope readings to confirm correct laser behavior are shown in Figure 4. The pulse frequency was determined to be 10 kHz (ca. 100 μs pulse spacing) when 150 A of peak diode pump current was used. The pulses representing the individual Q-switched 1064 nm
pulses “ride” on a pedestal of detected 1064 nm laser fluorescence that is scattered around within the laser head, but none of this fluorescence pedestal emission is emitted (does not interact with the sample).

In a diode-pumped passively Q-switched laser, laser pulse rate during each burst of pulses is directly determined by the pumping current to the laser’s diode pump array. A preliminary IR-MALDESI experiment (data not shown) showed no increase in performance when using currents of up to 200 A, so the lower current level of 150 A was selected for use in imaging experiments to minimize ohmic heating. The average energy per pulse was measured to 500 µJ at the laser head using an Ophir PE25-C pyroelectric sensor (Ophir Optronics Ltd.) and up to 14 pulses per burst could be fired at a continuous burst rate of 2 Hz for several hours without degradation in performance or buildup of heat. Over the course of several hours of continuous firing at a fixed trigger pulse width, the pulse count per burst was found to drift slightly (±1 pulse) as the laser reached thermal equilibrium. In order to use the laser for IR-MALDESI imaging with fixed ablation energy, a method for controlling the pulse count per burst was thus necessary. To enable reliable performance for the duration of an imaging experiment, as well as easy adjustment of energy output, the 5 V readout of the photodiode was fed back to the Arduino microcontroller, allowing on-demand triggering of the desired pulses-per-burst regardless of the thermal state of the laser head and diode-pump array.

3.2.4 Performance Evaluation

An imaging experiment was performed in order to evaluate the performance of the miniaturized laser. A 25 µm thick whole-body section of a 2 day old neonatal mouse was imaged in a 10 hour experiment. The laser beam was focused through a single 50 mm EFL (effective focal length) aspheric lens onto the sample stage, and the firing order was synchronized to the
trigger signal of the Q Exactive Plus, run in low-latency handshake mode. Other than a gold-coated turning mirror, no other optics were used between the laser head and 50 mm EFL asphere lens.

An ice layer was deposited over the sample prior to analysis according to previously established protocol\(^3\), stabilized by regulating the humidity of the enclosure using a nitrogen flow controlled by an OMEGA CNiTH-I3222-2 process controller (OMEGA Engineering Inc, Norwalk, CT). The imaging was performed using a previously developed polarity switching MSI method to maximize metabolome coverage\(^7\).

The resulting mass spectra were converted from to .mzml format using the msconvert utility of the ProteoWizard software package\(^8\), and from .mzml to to .imzml\(^9\) format using imzMLConverter\(^10\) for data analysis. The open-source software package MSiReader\(^11,12\) was used to produce all images shown in this work. The resulting dataset was uploaded to the Metaspace annotation platform\(^13\) (http://annotate.metaspace2020.com) for automatic identification of metabolite signals.

3.2.5 Safety Considerations
The laser system used in this study is a class IV laser of sufficient energy flux to cause harm to humans upon exposure to a focused beam. To protect others working in the same laboratory space, a LAZ-R-SHROUD barrier (Rockwell Laser Industries, Cincinnati, OH) was used to enclose the working area at all times. The greatest risk of stray beam exposure was during alignment of the invisible IR laser beams, and during all such work, OD 5+ glasses were worn by all personnel in the zone of potential exposure. During normal operation the beam was fully enclosed in a 1” beam tube.
3.3 Results and Discussion

3.3.1 Validation of Basic Functionality

The output of the burst-mode laser was monitored through oscilloscope readings during the experiments. The correlation of the trigger signal and photodiode current in feedback controlled bursts was recorded, and representative oscilloscope readouts may be found in Figure 3.4. To use the laser with the Q Exactive Plus in low-latency handshake mode, the signal for acquisition start was synchronized to the laser trigger to within a microsecond.

The laser system was found to produce an oval beam profile approximately 50 µm X × 100 µm Y using the assigned dimensions of the stage motor axes. At optimal alignment, 50% transmission efficiency through the beam path (1 meter) was achieved, yielding a measured 220 µJ per pulse after the final optic. For imaging, 10 pulses per burst were used (2.2 mJ per burst at target, see Figure 3.5), which was found sufficient to completely ablate 25 µm thick tissue as shown in Figure 3.6b.
**Figure 3.4.** Oscilloscope confirmation of laser bursts controlled by active feedback. Top: ten-pulse burst produced by ~1100 µs long trigger signal. Bottom: Single pulse produced by ~200 µs trigger signal. For a user selectable pulse count N, the termination of the trigger pulse is synchronized to the N-th falling edge of the pulse train.

**Figure 3.5** Illustration of the IR-MALDESI test sequence used, showing a comparison between the pulse energy at sample of the burst-mode laser (10 pulses) compared to a single 3 mJ flashlamp pumped laser as used in previously published MALDESI imaging.
3.3.2 IR-MALDESI Imaging

The imaging datasets of whole-body neonatal mouse section were uploaded to Metaspace as Mouse_Wholebody_3 and Mouse_Wholebody_3_Negative. Figure 6A shows two representative lipid distributions acquired in negative mode. At the time of writing, a Metaspace search against the Human Metabolome Database¹⁴ with 10% FDR yields 266 identifications from the positive ion mode data, and 175 identifications from negative ion mode for a total of 441 metabolites. A previously uploaded set from an equivalent tissue section analyzed with a 20 Hz pulsed laser in positive ion mode only, also available on Metaspace as mouse_body_2, produced 414 identifications at 10% FDR.
Figure 3.6 A) Representative images of whole-body neonatal mouse in negative mode. Here showing raw signal from \( m/z \) 281.2486 and 331.2642 (2.5 ppm tolerance), identified as isomers of oleic and andrenic acid, respectively at <5% FDR (Metaspace, HMDB). B) Microscope image acquired with 10× magnification on a LMD7000 (Leica, Buffalo Grove, IL, USA), illustrating the approximately 50 × 100 \( \mu \)m ovaloid shape of the ablation spots.
3.4 Conclusions

A compact diode-pumped burst-mode laser was successfully incorporated into an existing instrument for mass spectrometry imaging. We have demonstrated the suitability of the laser system for full-thickness ablation and sampling of animal tissue in the context of IR-MALDESI. The burst-mode system yields images with quality comparable to that of previous work done with flashlamp pumped Nd:YAG pulsed lasers, but without the requirement for water cooling or large heatsinks. The reduction in size and weight of the laser allows for a substantial reduction in size of the imaging source, and the feedback controlled variable bursts provide a flexible means of adjusting the energy per shot.
3.5 References


Chapter 4

Direct Screening of Enzyme Activity using Infrared Matrix-Assisted Laser Desorption Electrospray Ionization (IR-MALDESI)


4.1 Introduction

High-throughput screening (HTS) is a critical step in the drug discovery process, where the biochemical activities of a large number of drug-like compounds are tested in order to identify potential candidates that exhibit a desired feature, such as binding affinity or inhibitory activity. Over the past two decades many strides have been made in miniaturization and automation of HTS, along with advancements in developing new assays and integrating rapid sampling with sensitive detection.1,2

Some of the most important detection methods in HTS applications are optical assays that rely on UV/Vis or fluorescence spectroscopy for detection.3 These methods are commonly used due to their speed and sensitivity, but suffer from certain limitations. For instance, many assays are not amenable to detection by fluorescence because the substrate(s) or product(s) require tagging. In addition, while fluorescence-based methods are highly sensitive, they are not highly specific and phenomena such as autofluorescence or non-specific tagging have to be accounted for when choosing such methods.3,4

Mass spectrometry-based methods are an emerging technological advancement for application to HTS assays. These techniques are label-free, highly sensitive, and more specific than optical methods, and have been widely used in many phases of drug discovery such as target validation to tracking pharmacokinetics. However, historically their utility for HTS applications have been limited mostly due to the low throughput of HPLC-MS or UPLC-MS
methods, which typically require several minutes of analysis time per sample. In addition, most MS-based methodologies are susceptible to the presence of nonvolatile compounds such as salts, buffers, and detergents that are required in enzymatic assays, and therefore, sample cleanup and desalting are necessary prior to analysis. The RapidFireTM platform (Agilent Technologies, Lexington, MA, USA) is one of the recent advancements in MS-based HTS instrumentation aimed at circumventing the aforementioned limitations. Using RapidFireTM, the analysis times have been reduced to ~10 seconds per sample and the sample is driven through a solid-phase extraction (SPE) cartridge to remove salts and other contaminants prior to injection of eluent into the MS source. Other recent developments in sampling and ionization for high-throughput MS include desorption electrospray ionization (DESI) and acoustic droplet ejection (ADE). These very different methods share the important property of rapid, spatially resolved sampling combined with very high tolerance for non-volatile contamination.

Matrix-assisted laser desorption electrospray ionization (MALDESI) is an ambient ionization method combining features of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). In MALDESI analyses a laser is used to desorb material from the surface of a sample. The plume of ablated material partitions in the charged droplets of an orthogonally oriented electrospray plume, where ions are generated in an ESI-like manner. The most current version of the source is coupled to a high resolving power/accurate mass (HRAM) mass spectrometer, the Q Exactive Plus, and utilizes a mid-Infrared (IR) laser for sample desorption/aerosolization. The mid-IR laser excites the O-H stretching modes of water present in the sample and facilitates the desorption/aerosolization of neutrals from the surface. With a sampling rate limited mainly by the duty cycle of the mass analyzer, IR-MALDESI can achieve analysis times of less than one second per sample, making it inherently suitable for HTS
applications. Typically two laser shots are used to aerosolize the material and the generated ions from both pulses are stored in the C-trap for a fixed accumulation time (75 ms), and subsequently injected into the Orbitrap for mass measurement. The whole process for analyzing a sample with 2 laser pulses takes less than one second; therefore, the contents of a 1536-well plate can be analyzed entirely in ~30 minutes, limited only by the duty cycle of the mass spectrometer. The IR-MALDESI source is coupled to a high resolving power (RP) mass spectrometer, and most analyses are performed at nominal Resolving power (RP) of 140,000. Since most HTS experiments are performed in a semi-targeted manner, the total analysis time can be reduced by performing the experiments at lower resolving powers. Furthermore, the ESI post-ionization in IR-MALDESI allows for direct analysis of analytes from matrices containing high salt and protein concentrations, that are used to perform enzymatic reactions.[13,14]

The features mentioned above make IR-MALDESI an exciting platform technology for HTS analyses since not only the analysis can be performed rapidly, but also compared to other MS-based methods the sample preparation and cleanup steps are drastically reduced or completely eliminated because of the inherent nature of IR-MALDESI ionization process.

Herein, we present an application of HTS analysis using IR-MALDESI by monitoring the enzymatic activity of isocitrate dehydrogenase 1 (IDH1) directly from well plates containing the quenched reactions in buffer. The conversions of isocitrate to α-ketoglutarate (α-KG) and NADP$^+$ to NADPH were measured separately and used to estimate the reaction progress. This work serves as a proof-of-concept study for demonstrating the utility of IR-MALDESI for HTS applications.
4.2 Experimental

4.2.1 Materials

HPLC-grade methanol and water for ESI solvent were purchased from Burdick and Jackson (Muskegon, MI, USA). Acetic acid, magnesium chloride (MgCl2), bovine serum albumin (BSA), DL-isocitric acid trisodium salt hydrate, β-nicotinamide adenine dinucleotide phosphate hydrate (NADP\(^{+}\)), isocitrate dehydrogenase 1 human (IDH1, catalog # I5036), and BRAND® 384-well plates (catalog # BR781620) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Micro-24TM slides were obtained from Prosolia (Indianapolis, IN, USA). Formic acid, DL-dithiothreitol (DTT), 6N hydrochloric acid (HCl), and 1M Tris-HCl buffer (molecular biology grade, pH 8.0) were purchased from Fisher Scientific (Nazareth, PA, USA). The pH of the Tris-HCl buffer was adjusted to 7.5 with hydrochloric acid. All other materials were used without further modifications.

4.2.2 Enzymatic Reactions

The reactions were carried out in an assay buffer of 20 mM Tris-HCl pH 7.5, with 1 mM MgCl2, 1 mM DTT, and 0.01% BSA by weight. The same buffer was used to prepare all stock solutions. All solutions were prepared fresh on the day of analysis.

Reactions were started by thorough mixing of equal parts of solutions containing substrates and enzyme. The reaction scheme along with the final concentrations used in the two experiments are shown in Figure 4.1. Reactions were carried out at ambient temperature and pressure until quenching, which was done by addition of 1/10 reaction volume of 10% formic acid.
Figure 4.1 Schematic of isocitrate dehydrogenase 1 (IDH1) reaction and the concentrations of precursors and enzyme used in two different experiments.

4.2.3 IR-MALDESI Reaction Screening

The IR-MALDESI analyses were performed as previously described in detail. Briefly, the well plates containing the samples were placed on an X-Y positioning stage and adjusted so as to position the liquid surfaces of the wells 5 mm below the inlet of the mass spectrometer. A mid-IR laser tuned to 2940 nm (IR-Opolette 2371, Opotek, Carlsbad, CA, USA) was used to aerosolize material from the sample in each well. The plume of aerosolized material was ionized through interactions with an orthogonal electrospray. A schematic of the IR-MALDESI source as used in this experiment is shown in Figure 4.2.
Figure 4.2 Schematic of IR-MALDESI source adapted for direct analysis of samples from well plates. The zoomed-in region shows the side view of the well plate containing the solution and the laser focused on top of the meniscus.

Based on previous optimizations\textsuperscript{15}, a 50/50 mixture of MeOH/H\textsubscript{2}O (v/v) containing 1 mM acetic acid was used as the electrospray solvent for analysis in negative ion mode. The IR-MALDESI source was coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The laser beam was focused on top of the meniscus of the solution in the wells and two laser shots (20 Hz) per scan were used to aerosolize the sample solution. Due to the pulsed nature of the ionization the automatic gain control (AGC) function was disabled. The ions generated from the two laser pulses were accumulated in the C-trap for a fixed injection time (IT) of 75 ms before injection into the Orbitrap for mass analysis, as this IT was optimized previously for two laser shots\textsuperscript{16}.
As shown in Figure 4.1, two separate experiments at different concentrations of precursors and enzyme were performed to gauge the dynamic range of the assay. The reaction with lower concentrations is referred to as “Experiment 1” throughout the manuscript, and the term “Experiment 2” refers to the reaction at higher concentrations. Experiment 1 was performed by pipetting 5 μL of reaction mixture at different time points after reaction initiation onto the wells of a Micro-24™ slide and focusing the laser on the droplet. Experiment 2 was performed by pipetting 100 μL of reaction mixture into the wells of a 384-well plate and focusing the laser beam on the meniscus. Once the source geometry was optimized for direct analysis from a 384-well plate the rest of analyses were performed using them. The equivalency of the two methods was confirmed in a separate experiment (not shown).

A custom method consisting of 6 different scan events (Figure 4.3) was developed using the accompanying Xcalibur software (Thermo Scientific, San Jose, CA, USA). The first 4 scans in this method monitored α-KG (m/z 145.0 ± 1 Th), isocitrate (m/z 191.0 ± 1 Th), NADP⁺ (m/z 742.1 ± 1 Th), and NADPH (m/z 744.1 ± 1 Th) in a SIM fashion at a nominal RP of 70,000 (FWHM, m/z 200), and the last two scans measured wider ranges of 75-300 Th and 200-1000 Th at RP of 140,000 (FWHM, m/z 200) to monitor the conversion of isocitrate to α-KG and NADP⁺ to NADPH, respectively.

**Figure 4.3** Schematic of the 6-step method used to screen the activity of IDH1 in Experiments 1 and 2. The first 4 SIM scans were performed at a nominal RP of 70,000 and the last 2 full MS scans at RP of 140,000.
4.2.4 IR-MALDESI HTS Proof-of-Concept

In order to demonstrate the utility of IR-MALDESI, a pseudo-HTS experiment was designed where 10 different wells were analyzed in HTS manner. This experiment was performed in a single blind fashion, where one of the co-authors of this work prepared 10 different solutions (reactions at concentrations used in Experiment 1 along with negative controls), and pipetted them into the wells of a well plate without informing the other co-authors about the contents of each well. Each well was analyzed using a method where m/z regions of 130-200 Th and 735-755 Th were measured alternatingly at a nominal RP of 70,000 to monitor conversion of isocitrate to α-KG and NADP+ to NADPH, respectively. Two laser shots were used to measure each m/z range, and the IT was set to 75 ms for each scan. The first 14 scans (i.e. 7 scans in low m/z range and 7 in high m/z range) were used to calculate percent conversion of isocitrate to α-KG and NADP+ to NADPH in order to predict the content of each well.

4.2.5 Data Analysis

The .RAW files generated by the instrument were processed in the Qual Browser of Xcalibur software (Thermo Scientific, San Jose, CA, USA) and m/z tolerance of ±2.5 ppm was used to generate extracted ion chromatograms (XIC) of analytes. The data was then exported to Microsoft Excel for statistical analysis and visualization.
4.3 Results and Discussion

4.3.1 Screening Enzyme Activity Using IR-MALDES1

Screening enzyme activity is one of the most common applications of HTS. In this study, the percent conversion of precursors to products was used to monitor the activity of enzyme over different time points after starting the reaction (addition of enzyme to substrate mixture). The percent conversion for products was calculated using Equation 4.1:

**Equation 4.1:** \[ \% \text{ Conversion} = \frac{I_{\text{product}}}{I_{\text{product}} + I_{\text{precursor}}} \]

where I is the ion abundance of the analyte of interest. The percent conversion is a more robust measure of enzyme activity than the absolute abundance of the product ion, since even if the ion abundance of analytes varies from one shot to another due to variability in sampling efficiency, the ratio of ion abundances remains constant.
Figure 4.4 Representative mass spectra showing the 4 analytes of interest in IR-MALDESI direct analyses in Experiment 2 at time points 0 and 60 minutes. Parts A and C show the lower m/z range where isocitrate and α-ketoglutarate are observed, and parts B and D show the higher m/z range that include peaks for NADP+ and NADPH.

As mentioned above in the experimental section, a method consisting of six scan events was used to analyze screen enzyme activity in Experiments 1 and 2. The SIM scans were used to confirm the identity and presence of the compounds and the two full MS scans were used to calculate conversion percentages based on the ratio of peaks collected together. Representative mass spectra from the full MS scans in Experiment 2 at 0 and 60 minute time points are shown in Figure 4.4, where the decrease in ion abundance of precursors and concomitant increase in ion abundance of products can be observed. Using the ion abundances at different time points, percent conversions of isocitrate to α-KG and NADP+ to NADPH at different time points for Experiments 1 and 2 were calculated (Figure 4.5). It can be seen that in both reactions the
enzyme activity can be readily monitored. As expected, the reaction proceeded 10 times faster in Experiment 2 compared to Experiment 1 since the concentration of precursors and enzyme was increased by 10-fold. In addition, the similarity of the curves for each set of analytes for both experiments demonstrates the large estimated dynamic range of the developed method.

In order to validate the IR-MALDESI results, conventional UV/Vis analysis of cofactors was performed for Experiment 1 at the beginning and at the final time point (0 and 90 minutes). The UV/Vis spectra (Figure 4.6) show a peak at 260 nm corresponding to NADP$^+$ immediately after starting the reaction. After 90 minutes another peak at 340 nm, corresponding to NADPH, is observed, indicating a successful reaction.

**Figure 4.5** Screening the activity of IDH1 enzyme by monitoring the percent conversion of precursors to products. The top panel shows the conversion rate of isocitrate to α-KG (A) and NADP$^+$ to NADPH (B) in Experiment 1 (low concentration). The bottom panel shows the conversion rate of isocitrate to α-KG (C) and NADP$^+$ to NADPH (D) in Experiment 2 (high concentration). For all analyses the samples were analyzed immediately upon transfer from the reaction mixture to the sample well; however, in Experiment 2 the reaction occurred rapidly enough that the conversion for isocitrate to α-KG was observed to rise from 3% to 7% during the minute-long analysis at t=0 (data not shown).
Figure 4.6 UV/VIS spectra of the reaction mixture in Experiment 1 at time points 0 and 90 minutes. Strong absorption at 260 nm confirms the presence of NADP⁺, and the peak at 340 nm corresponds to NADPH absorption. The spectra were acquired from 2 µL of sample using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).
4.3.2 Suitability of IR-MALDESI for HTS

A marked advantage of IR-MALDESI for HTS is its direct compatibility with a large range of sample preparations including most commonly used well plates, potentially allowing seamless integration of HRAM MS with other screening modalities such as fluorescence assays on the same line. As a measure of assay quality, the Z-factor\textsuperscript{17} of a potential IR-MALDESI screen was calculated using Equation 4.2:

\textbf{Equation 4.2:} \quad Z = 1 - \frac{(3\sigma_+ + 3\sigma_-)}{\mu_- - \mu_+}

where \( \sigma \) is the standard deviation and \( \mu \) is the mean, and + or – signs indicate positive and negative controls, respectively. The Z-factor typically fall within a range of \(-1 \leq Z \leq 1\), with larger values indicating higher suitability of the assay for HTS. The Z-factor for IR-MALDESI direct screening of IDH1 can be calculated by using the percent conversions of precursors at time points of zero and 90 minutes, respectively as negative and positive controls (Figure 4.7). The calculated Z-factor for percent conversion of isocitrate to \( \alpha \)-KG was 0.65, while the Z-factor for percent conversion of NADP\(^+\) to NADPH was less sensitive at 0.41. Together these values demonstrate the ability of the method to separate hits from false positives with high confidence.
Figure 4.7 The calculated percent conversion of isocitrate to α-KG (A) and NADP⁺ to NADPH (B) in Experiment 1, measured continuously over one minute. In each case the Z-factor for each pair was calculated using Equation 2.

4.3.3 IR-MALDESI HTS

To further investigate the utility of IR-MALDESI in a HTS analyses a pseudo-HTS study was designed. The study was performed in a single-blind fashion where one co-author prepared 10 samples and another co-author analyzed the samples directly from well plates, without having any prior knowledge about the identity of the samples. The lower concentrations of precursors and enzyme, similar to Experiment 1, were used since they are closer to those used in biological assays. The percent conversions of isocitrate to α-KG and NADP⁺ to NADPH were used to predict the identity of the samples. In this pseudo-HTS study, IR-MALDESI was able to
correctly predict the identity of each sample, and 8/10 samples were identified with high confidence.

The work presented here demonstrates the potential of IR-MALDESI in HTS applications as a new technological platform that reduces sample preparation steps required prior to analysis. The current rate of 2 samples/second, limited by the need to acquire several scans per well, is competitive with currently available options for MS-based HTS. It is also worth noting that the current IR-MALDESI source has been highly optimized for mass spectrometry imaging experiments of different tissue types\textsuperscript{18}. Therefore, the source geometry (e.g. distance between inlet and emitter tip, distance between the plate and the emitter tip, etc.) need to be optimized by performing a detailed design of experiments (DOE) study\textsuperscript{19} in order to ensure the highest shot-to-shot reproducibility for HTS analyses. Current efforts are focused on designing a thorough DOE study to improve signal reproducibility, as well as identify the best type of well plates, to achieve the ideal single-shot assay sample rate of >5 samples/second.
4.4 Conclusions

Using a prototype instrument with minimal optimization, we have demonstrated the capacity of IR-MALDESI for characterizing the rate of conversion in an enzyme reaction. The quality of the data collected from the model reaction is shown to be sufficient for an activity assay with a throughput of 2.5 seconds per sample, with the potential of up to a tenfold increase given an improvement in ionization efficiency. The results demonstrate the utility of IR-MALDESI as a compact platform for HR/AM-MS, directly compatible with samples from industry standard assays.
4.5 References


Chapter 5
Development of an IR-MALDESI Source for High Throughput Ion Mobility Mass Spectrometry

5.1 Introduction

In the pharmaceutical discovery process as well as within clinical testing, there is a largely unfilled need for versatile instrumentation capable of screening for a wide range of analytes while maximizing throughput. Mass spectrometry is the intuitive choice for such a platform, and modern TOF and FT-MS mass analyzers are certainly capable of providing excellent mass resolution and quantitative performance at rates well under one sample per second.

The throughput of mass spectrometry is effectively limited by the process of sample introduction. Since real samples rarely come in pure form, but rather as complex mixtures in a matrix of buffer salts, detergents and other additives, some sample treatment is often necessary. ESI-MS analysis typically requires on-line extraction or separation processes to minimize matrix effects, and in practice there is a tradeoff between sensitivity and throughput. Separation techniques such as LC- or CE-MS can provide unmatched sensitivity through combined pre-concentration and matrix removal, but require a time investment of minutes to hours per sample depending on complexity. While sample preparation steps such as filtering or extraction can be effectively parallelized to increase throughput, the crucial sequential steps of ionization and sampling into the mass spectrometer have to be performed at a rate not significantly slower than the scan rate of the mass spectrometer.
5.1.1 Infrared Matrix-Assisted Laser Desorption Electrospray Ionization (IR-MALDESI)

IR-MALDESI is an ambient ionization method, using an infrared laser for sampling and generating ions through interactions between the aerosol of ablated material and charged electrospray droplets. The mechanism of ionization is very similar to ESI or DESI, with ions being products primarily of protonation/deprotonation reactions\(^1\). It is a flexible system suitable for analysis of ESI ionizable analytes from almost any preparation with sufficient IR absorptivity, which makes it suitable for mass spectrometry imaging (MSI) as well as direct analysis. Samples can be liquid or solid, and are typically prepared either on microscope slides or in low-volume microtiter plates for easy insertion. Since IR-MALDESI samples and ionizes material in a single concerted process, it runs very fast, hitting a practical upper limit somewhere around 20 Hz, imposed by the fact that the ion current from a single shot persists for about 50 ms in current instrumentation\(^2,3\).

5.1.2 Ion Mobility Spectrometry

Ion mobility spectrometry (IMS) is a trending analytical separation technique that provides complementary resolution of ions prior to analysis by MS. IMS separates ions based on differences in electrophoretic mobility in an inert gas (typically nitrogen) under the influence of a weak electric field (ca. tens of V/cm). The mobility of each ion, determined as a function of analyte velocity and field strength, is often used to calculate a rough measure of ion size, referred to as the ion’s collision cross section, or CCS, via the Mason-Schamp equation\(^4\): After experimental variables related to gas density (\(N_0\)) and temperature (\(T\)) are accounted for, the CCS of each analyte is calculated as function of the ion’s charge (\(z\)), reduced mass (\(\mu\)), and mobility (\(K_0\)).
Mason – Schamp equation: \[ \Omega = \frac{3}{16} \left( \frac{2 \pi}{\mu k_b T} \right)^\frac{1}{2} z e \]

Because IMS separations occur in the gas phase, each IMS experiment is very rapid (in this work, 60 ms/spectrum). As high-throughput MS workflows cannot rely on chromatographic separation for resolution of analytes prior to mass analysis, IMS provides additional resolution which is easily nested into existing chromatography-free workflows. Because the duration of each IMS scan is very similar to the upper limit in scan speed of MALDESI, these ionization and separation mechanisms can be interfaced in a synchronous manner. Moreover, as separation of isomeric analytes remains challenging in imaging experiments, IMS separation provides complementary resolution for isomers. Provided sufficient difference in molecular structure for each isomer and enough resolving power in the mobility dimension, IMS can separate, identify, and quantify each isomer in a complex sample, provided that analytical standards of each are readily available for comparison.

In addition to isomer separations, recent developments in the precision and reproducibility of mobility measurements have promoted wide scale utility of translatable mobility values (collision cross sections, CCS) across laboratories as an additional metric of ion annotation in untargeted experiments. These values are currently being collected across laboratories with subsequent compilation into libraries of CCS information. For example, recent work from Picache and coworkers have developed an online CCS database for open-access annotation of previously characterized samples\(^5\). Using CCS values as additional ion descriptors, we illustrate in this work that the proposed MALDESI-IM-MS workflow provides exciting analytical utility in both targeted and untargeted experiments.
5.2 Materials and Methods

5.2.1 Materials

Methanol, water, acetonitrile, formic acid and caffeine, of HPLC-grade or higher purity, were obtained from Fisher Scientific (Hampton, NH, USA).

Agilent Low Concentration Tune Mix was used for mass and mobility calibrations.

Triton X100 was obtained from Sigma-Aldrich (St. Louis, MO, USA).

All optics and optomechanical components were purchased from Thorlabs (Newton, NJ, USA).

5.2.2 Instrument Integration

The IM-MS platform used in all experiments was an Agilent 6560 IM-QTOF (Agilent Technologies, Santa Clara, CA). The 6560 sets up ion mobility scans by opening a trapping ion funnel for a preset duration prior to each injection into the linear drift tube. Adapting a pulsed, transient ion source like MALDESI therefore necessitates accurate synchronization of peak ion current to active ion accumulation. The instrument does not natively support external triggering of single scans, so it was necessary to trigger the laser based on the trapping funnel state, which is continuously reported on externally accessible digital outputs.
An Arduino Uno microcontroller (Arduino, Ivrea, Italy) was programmed to track the instrument state on a 200 μs timer running in parallel with the firing sequence of a custom-built pulsed mid-IR laser system (JGMA inc., Burlington, MA). For each shot requested, the firing sequence waits for the appropriate instrument state before sending the trigger signal to the laser. The laser responds to the trigger with a burst of nanosecond-long pulses at 10 kHz, for as long as the trigger signal is asserted or until a maximum of ~15 pulses limited by the capacity of the current supply board (D100B, Highland Technology, San Francisco, CA). The laser activity was measured using a photodiode circuit fed back to the Arduino, allowing easy control of energy output by controlling the number of pulses per burst. The signals involved were monitored using a 4 channel oscilloscope (TBS1064, Tektronix, Beaverton, OR), and once setup, the system could be run at 100% duty cycle indefinitely, IE a single 1 mJ pulse per ion acquisition at a rate of 10-20 Hz (Figure 5.1).

The system, being designed with high throughput analysis and imaging in mind, had to be dimensioned to allow samples to fit under the electrospray axis with a reasonable amount of allowance for travel. To achieve this, a 2” capillary extension was constructed from stainless steel and PEEK. A magnetic identifier was removed from a separate NanoESI source and used to
bypass the instrument interlock. The driving voltage for the electrospray was set at +3500 V using an external high voltage supply, with the instrument-specified capillary voltage held at 0V nominally.

Figure 5.2: Connection diagram showing the major components of the instrument and connections to the Arduino microcontroller board used to control acquisition.

5.2.3 Optimization of IR-MALDESI Ionization

A 1 mg/ml aqueous solution of Triton X100 was used to evaluate the efficiency of IR-MALDESI sampling and ionization. Droplets were manually spotted onto a plate placed at the laser focal plane, and sampled by manual triggering of single shots. The characteristic mass/drift spectrum was used as a basic quality control during adjustments of source settings such as voltage and ESI separation.

After tuning the instrument, a lipid extract of healthy rat liver was prepared using the Folch method\(^7\). To reduce volatility and increase IR absorptivity, the organic, lipid-rich fraction was isolated and diluted 1:1 with methanol to a final composition of approximately 75%
methanol/25% chloroform prior to analysis. Droplets of lipid extract were manually dispensed and analyzed.

5.2.4 Model Assay Design

After optimization of the sampling process, a quantification assay was designed to characterize the dynamic range and carry-over effects. A caffeine solution was prepared by sequential dilution by ½ from 1000 - 16 µg/ml in 50% methanol, and manually dispensed into microtiter plate wells. Each well was analyzed continuously for one minute with one laser shot per IM acquisition, and the results were summed together. The experiment was repeated several times, each with the samples run in order of increasing concentration, to minimize the impact of carry-over. Blanks and IM calibration solutions were run before and after each run. A sample of Coca-Cola Classic (the Coca-Cola Company, Atlanta, GA) of nominal caffeine content 96 µg/ml was procured as a model unknown for the purpose of demonstrating matrix effects. The sample was decarbonated through brief sonication and analyzed in the same way as the calibration solution.

5.2.5 Safety Considerations

The experiment as described involves defeating the safety interlock of an instrument in order to run without a source enclosure. This allows the user to enable a high voltage supply to parts of the instruments exposed to intentional or accidental contact with persons and equipment. The electrospray high voltage supply is likewise exposed.

The laser used for sampling is a class IV invisible pulsed IR laser, and it can output a significant amount of energy at a main wavelength of 2970 nm. By NC State regulations, all personnel required to work in a location with a Class IV laser installed must first undergo laser safety training. Adequate eye protection for each such system is a minimum precaution.
5.3 Results and Discussion

5.3.1 Implementation and Validation of Basic Functionality

The instrument was initially configured to mirror previous parameters for IR-MALDESI analysis on an Orbitrap platform\(^9\). The initial parameters used places the laser axis 1 mm from the electrospray emitter and 5 mm from the inlet capillary. Varying these measurements by up to several millimeters had only very subtle effects on the signal from Triton X100 solution, and so the initial positioning was kept. (Figure 5.3A).

Once the ionization process was verified to work consistently, a lipid extract from rat liver was injected to provide a measure of inter-platform reproducibility. A representative spectrum of the dominating phospholipid signals is shown in Figure 5.3B. The identities and abundances of the main lipid signals are all identical to previous results from the same sample acquired on an Orbitrap system, and the measured m/z ratios agree to within 5 ppm.
Figure 5.3: A) Mass-drift spectrum of 1 mg/ml Triton X-100 at suitable conditions for IR-MALDESI sampling. The characteristic series of repeating peaks between 400-800 m/z was used as a quality reference for tuning the system and verifying that sampling and ionization was working as intended. B) Mass spectrum of Folch method lipid extract of rat liver, with putative identifications. The series of protonated and sodiated phosphatidyl choline cations is ubiquitous in mass spectrometry imaging of animal tissue.

5.3.2 Performance Evaluation for High Throughput Analysis

Results from IR-MALDESI direct analysis of caffeine solutions are shown in Figure 5.4. The calibration curve shows a region is reached at very high sample concentrations. Blanks run immediately after a minute of uninterrupted sampling of the most concentrated samples showed elevated abundances of caffeine, on the order of 1% of the previously measured signal. This carry-over signal could be eliminated through a quick methanol wash of the fused silica emitter tip, indicating that very little carry-over is caused by contamination of the ion optics of the mass
spectrometer itself. The effect could be reduced by attenuating the power of the sampling laser to lower overall signal. Low abundance carry-over is a minor concern for confirmatory screening, but to achieve the greatest potential dynamic range, shielding or active decontamination of the emitter would be necessary.

Using a same-day caffeine calibration curve to calculate the concentration of caffeine in Coca-Cola resulted in an estimate of 50 ug caffeine/mL, an underestimation by a factor of two, due to suppression effects from the sample matrix. In particular, the mass spectra from the undiluted sample are dominated by an intense peak at m/z 203.05, corresponding to sodium adducts of hexose. Using the CCS Compendium recently published from the McLean Lab we could tentatively annotate this feature as a combination of fructose and glucose, with a mass error of ca. 2 ppm and CCS errors of <0.7% (Figure 5.4B). Thus estimating the composition of high fructose corn syrup (100 mg/ml) has no particular analytical value in the context of the experiment, but it serves as a demonstration of the power of IMS to provide rapid separations for isomer distinction in a timeframe compatible with high-throughput screening and mass spectrometry imaging.
Figure 5.4: A) Average integrated abundance of the caffeine [M+H]^+ signal over 50 IM acquisitions per concentration level. Error bars show one standard deviation. B) Single-field calibration curve utilized to convert drift time values to CCS. The drift time measured for [M+H]^+ adduct of caffeine was 17.96 milliseconds, which after conversion to CCS was calculated at 140.5 Å². Previous measurements of caffeine produced an average CCS value of 140.3 Å², which is in very good agreement. C) Drift spectra for caffeine calibration standards, including a solvent blank and undiluted Coca-Cola. D) Drift spectrum of hexoses [M+Na]^+ acquired from Coca-Cola sample. The two-gaussian fit to the shouldered peak gives a drift time separation consistent with database values for fructose and glucose, with an abundance ratio of 68/32.

5.4 Conclusions

The combination of a rapid, point-sampling ion source with drift tube ion mobility mass spectrometry is an effective method for maximizing the data content per spectrum without reducing sample throughput. We have presented an implementation of IR-MALDESI-IM-MS designed to be simple to construct using standard parts, and demonstrated its use for high content
direct analysis. Our results show that an instrument constructed on this pattern is well suited for rapid high content screening and IM-MS imaging.
References


6.1 Introduction

Mass spectrometry imaging (MSI) datasets are highly complex, containing abundance and distribution information about thousands of chemical species. As sample probes and ionization techniques have evolved, the information density of untargeted (discovery) MSI data has increased to the point where comprehensive manual interpretation is not practical. Some degree of automation is often employed to extract features of interest in a semi-targeted fashion.

The desired outcome of discovery-type MSI experiments is typically the identification of molecular distributions correlated to some other feature such as a known region of the sample, or the distribution of some known compound such as a disease marker, isotopic label, or a drug. For this type of study, data interpretation comes down to finding images of a particular appearance from a limited search space. This is, in essence, an image recognition problem similar to those of facial recognition or compression quality evaluation in digital image processing.

The gold standard for calculating the perceived similarity of two given images is the structural similarity index (SSIM)\(^2,3\). The SSIM algorithm arose from a need to automatically predict the perceived quality of digital images after compression or other processing. To calculate the SSIM index for a pair of aligned images, each image is subdivided into smaller sub images, typically by generating a small window around each pixel. For each aligned pair of sub images x and y, the arithmetic mean (\(\mu_x, \mu_y\)), standard deviation (\(\sigma_x, \sigma_y\)) and Pearson’s correlation coefficient (\(\sigma_{xy}/\sigma_x\sigma_y\)) are calculated. The mean intensity and standard deviations are
converted into 0-1 scores which are multiplied together to generate the SSIM score as shown in equation 6.1. The final result can be shown either as a map of local similarities, or as a mean SSIM (MSSIM) score for the whole image as shown in equation 6.2.

Equation 6.1: 
$$SSIM(x_i, y_i) = \frac{2\mu_x\mu_y + \sigma_{xy}}{\mu_x^2 + \mu_y^2 + \gamma^2} \times \frac{2\sigma_x\sigma_y + \sigma_{xy}}{\sigma_x^2 + \sigma_y^2} = \text{luminance} \times \text{contrast} \times \text{structure}$$

Equation 6.2: 
$$MSSIM = \frac{\sum_{i=1}^{n} SSIM(x_i, y_i)}{n-1}$$

6.2 Methods

6.2.1 MSiReader Implementation

To apply image recognition methods to real MSI data, the batch processing function of MSiReader\textsuperscript{4,5} was modified to enable correlation scoring for a range of MS images with a given reference image. The SSIM algorithm is included in the MATLAB Image Processing Toolbox (The MathWorks, Inc., Natick, MA, USA). The MATLAB implementation of SSIM calculates the index at each pixel by applying a circular gaussian weighting function of adjustable radius. The combined score at each pixel is then calculated as

Equation 6.3: 
$$SSIM = \text{luminance}(x, y)^\alpha \times \text{contrast}(x, y)^\beta \times \text{structure}(x, y)^\gamma$$

where the weighting constants $\alpha$, $\beta$ and $\gamma$ can be set by the user. They default to 1. An example of SSIM output using 200x200 monochrome images is shown in Figure 6.1, illustrating both the individual components and final scoring (mean SSIM).
In order to test the usefulness of image recognition for real problems, two imaging datasets were produced, selected to be representative of typical work done in our lab. Each image was acquired using IR-MALDESI ionization coupled to a Q Exactive Plus mass spectrometer operating at a nominal resolving power of 140,000 as previously described. The raw data was converted to .imzml using msconvert and imzmlconverter, and loaded into MSiReader for analysis. Normalization to maximum abundance per image was used to ensure matching based
on relative rather than absolute ion abundance for the luminance score. All heatmaps were generated using the “hot” colormap preset in MSiReader.

The image recognition tools used are included in the current open source and stand-alone versions of MSiReader (v. 1.01 or later), available at http://www.msireader.com.

6.2.2 Imaging of Artemisia annua Leaf

The sweet wormwood (Artemisia annua, Chinese: Qinghao) native to China, is notable as the primary natural source of artemisinin, a powerful antimalarial compound, the discovery of which was awarded the 2015 Nobel medicine prize. Artemisinin and other related metabolites (e.g. its precursors and derivatives) are accumulated in glandular trichomes on the leaf surface, the size and density of which depend on spatial positions of leaves and plant ages. The unique chemical composition and localization of glandular trichomes on the leaf surface makes it suitable as a validation system for MSI data analysis.

Leaves on the 15-17th nodes of two-month old A. Annua plants, grown in the NC State phytotron, were collected and affixed to a glass microscopy slide using double-adhesive tape. A 2x2 mm region of interest was imaged in negative mode at a spatial resolution of 50 µm (40x40 scans), in the mass range of m/z 100-400. The molecular ion of intact artemisinin [M-H] observed at m/z 281.1395 was selected as reference for image scoring. The MSiPeakfinder tool was used to pre-generate a list of 332 masses with a 2x or higher abundance ratio in scans from leaf tissue compared to blank scans. This reduced dataset was used to evaluate the effect of the various SSIM parameters (α, β, γ, Gaussian radius).

6.2.3 Imaging of Drug-dosed Macaque Lymph Node

Combinations of antiretroviral therapies (ARVs) have radically improved health outcomes for persons living with HIV. Interruption of these regimens, however, leads to rapid
viral rebound that may result from inadequate penetration of drug into tissues where virus primarily resides such as lymph nodes\textsuperscript{12}. Tissue disposition of the viral entry-inhibitor maraviroc was investigated in the lymph node of a rhesus macaque, an animal model of infection, receiving 270 mg/kg maraviroc dosed twice daily. Since ARV tissue distribution can be highly heterogeneous\textsuperscript{13}, MSI analysis provides a useful tool in identifying ions accumulating in similar patterns to maraviroc that may participate in its trafficking and metabolism within the lymph node.

A 10 µm thick section of dosed lymph node was imaged in positive mode at 100 µm spatial resolution (75x90 scans, or 7.5x9 mm), in the mass range of m/z 200-800. Comprehensive SSIM analysis was performed by binning the whole mass range into evenly spaced non-overlapping bins of 5 ppm width (277259 bins), and subsequently comparing each bin against the reference distribution of maraviroc (m/z 514.3352) using default SSIM weightings. Duplicate hits resulting from the same peak being included in adjacent 5 ppm bins were removed, with only the highest ranked image at a given mass (10 ppm tolerance @ m/z 550) kept for analysis.
6.3 Results and Discussion

6.3.1 Trichome-bound Metabolites in *A. annua*

To find suitable constant parameters for the SSIM algorithm, SSIM evaluation for the *A. annua* sample was performed repeatedly, with the weighting parameters (α, β, γ) varied between 0.5 and 4 individually and pairwise. While changes to the weightings did affect the numerical SSIM score, the rank order was largely unchanged, and so the default weight of 1 to each parameter was used for all data here presented. Similarly, evaluating the SSIM scores with the Gaussian radius parameter varying between 1 and 5 showed only minor effects on the final ranking. It was observed that a small increase in the radius parameter led to significantly lower ranking of images with visible noise, caused either by low absolute ion abundance (shot noise) or significant chemical background noise. We found that a value of 2.25, raised from the default 1.5, resulted in somewhat improved contrast between visually identified “good hits” and “bad hits”, while still assigning high similarity scores to images with moderate levels of chemical noise. These parameters (α=β=γ=1; radius=2.25) were used for all subsequent processing.

The pre-selected set of 332 tissue-correlated peaks was evaluated against the reference m/z 281.1395 (artemisinin), and sorted by similarity as shown in Figure 2. The final ranking correctly identified the reference mass itself as a perfect correlation match, with its own first isotope as a close second. Known artemisinin derivatives including deoxyartemisinin (m/z 265.144, rank 13) and dihydroartemisinin (m/z 283.155, rank 22) were identified as visually similar distributions despite large variance in actual ion abundance. The peak set contained 31 ion masses with the characteristic distribution pattern of artemisinin, which were correctly assigned ranks of 1-31.
**Figure 6.2** Illustration of the image recognition workflow applied to an MSI dataset acquired from IR-MALDESI imaging of A. annua leaf using a known metabolite (Artemisinin, m/z 281.1395) as a reference. All images were internally normalized to a 0-1 abundance scale before processing and visualization. Some selected masses taken from the top 100 best matches are shown with similarity rank.

6.3.2 Drug Distribution in ARV Dosed Tissue

For the drug dosed lymph node section, a comprehensive brute force search was performed, where the SSIM evaluation was performed separately for the mass image of each non-overlapping 5 ppm bin through the whole mass range of m/z 200-800. Performing the evaluation this way required a total of 20 hours of computation time. This represents the most thorough search possible with the method, providing a “worst case” example of computation requirements.

The 500 best unique image matches were batch exported and inspected. The top 20 unique matches yielded images of very high visual similarity, with the top 10 almost indistinguishable from the reference. The exported images were all found to visually outline the
tissue shape in part or whole, with lower ranked and partial images generally ranking lower. This is illustrated in Figure 3, showing a selection of images throughout the correlation range.

![Normalized abundance](image)

**Figure 6.3** Selected output from a comprehensive SSIM search of drug dosed lymph node tissue. The images are shown on the normalized 0-1 abundance scale used for SSIM comparisons, with similarity rank shown in white. The image ranked 1 is that of maraviroc (m/z 514.3352), used as a reference.

We have found SSIM to be a very robust and useful noise filter for images including some blank or off-tissue data. Caution must however be taken not to include so much blank data as to make the contrast between blank and sample dominate the correlation calculations. For images containing very large regions consisting exclusively of blank scans, or where matching to very localized distributions is desired, we recommend limiting the search to a pre-defined region.
of interest. Narrowing the search space this way has the additional effect of reducing processing times proportionately and can be applied for that purpose alone.

6.4 Conclusions

We have here described the implementation and use of an open-source tool using image similarity scoring to extract features of potential interest from high resolving power mass spectrometry imaging datasets. Using the SSIM method for image similarity scoring, the process of semi-targeted discovery can be performed in an automated fashion. Sorting or filtering data by structural similarity effectively reduces complex datasets down to a scale suitable for manual interpretation, and can be used as reproducible pre-processing step for methods where computation time and memory requirements are limiting factors, e.g., principal component analysis.

All the algorithms used have been incorporated into the latest public release of MSiReader through the batch processing interface. The code is distributed under the BSD 3 license and can be freely adapted to other platforms used for analyzing MSI data.
6.5 References


