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Metal Oxide Laser Ionization Mass Spectrometry Imaging (MOLI MSI) using Cerium(IV) Oxide

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Abstract

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) is a powerful technique for spatially-resolved metabolomics. A variation on MALDI, termed metal oxide laser ionization (MOLI), capitalizes on the unique property of cerium(IV) oxide (CeO₂) to induce laser-catalyzed fatty acyl cleavage from lipids and has been utilized for bacterial identification. In this study, we present the development and utilization of CeO₂ as an MSI catalyst. The method was developed using a MALDI TOF instrument in negative ion mode, equipped with a high frequency laser. Instrument parameters for MOLI MS fatty acid catalysis with CeO₂ were optimized with phospholipid standards and fatty acid catalysis was confirmed using lipid extracts from reference bacterial strains, and sample preparation was optimized using mouse brain tissue. MOLI MSI was applied to the imaging of normal mouse brain revealing differentiable fatty acyl pools in myelinated and non-myelinated regions. Similarly, MOLI MSI showed distinct fatty acyl composition in tumor regions of a patient derived xenograft mouse model of glioblastoma. To assess the potential of MOLI MSI to detect pathogens directly from tissue, a pseudo-infection model was prepared by spotting *Escherichia coli* lipid extracts on mouse

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Competing interests

C.R.C. is employed by Cobio Diagnostics. In compliance with Harvard Medical School and Partners Healthcare guidelines on potential conflict of interest, we disclose that N.Y.R.A. is a scientific advisor to BayesianDx and inviCRO.

Supporting information

Supporting information includes optimized MOLI MS and MOLI MSI parameters and mass spectra from target plate analysis of additional glycerophospholipid standards using MOLI MS.

brain tissue sections and imaged by MOLI MSI. The spotted regions were molecularly resolved from the supporting mouse brain tissue by the diagnostic odd-chained fatty acids and reflected control bacterial MOLI MS signatures. We describe MOLI MSI for the first time and highlight its potential for spatially resolved fatty acyl analysis, characterization of fatty acyl composition in tumors, and its potential for pathogen detection directly from tissue.

Graphical Abstract

Fatty acid profiling of tissues and cells has been an extensive area of study, dating back nearly a century.^{1–3} Characterization of the fatty acyl composition of lipids contained within biological tissues traditionally involves a number of steps including tissue homogenization, followed by lipid extraction, hydrolysis and chemical derivatization, all prior to analysis.^{4,5} These additional steps are not only time-consuming but also result in the loss of spatial relationships between these molecules, which is of critical importance in the study of diseases with heterogeneous tissue distribution. Accordingly, an analytical method which not only profiles fatty acids, but also preserves their spatial distribution is of considerable value.

Matrix assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) is an emerging powerful analytical technique, which allows the spatially resolved characterization of a wide range of analytes within biological specimens.⁶ In contrast to commonly used imaging techniques, such as immunohistochemistry (IHC), MALDI MSI allows for the untargeted analysis of tissue sections.⁷ Although many of the original applications of MALDI MSI focused on protein analyses,^{8,9} there has been significant progress in utilization of this technique to measure drugs^{10–12} and lipids¹³ in tissue specimens. Furthermore, recent advances in the technology now allow molecular imaging with a spatial resolution of 5 μm , within the range of single cell analysis.¹⁴

Metal oxide laser ionization (MOLI) is a recently described variation on MALDI in which a catalytic metal oxide is used instead of a traditional co-ionizing organic acid matrix to bring about *in situ* cleavage of fatty acids ions from surface-associated lipids.¹⁵ Like MALDI, MOLI utilizes laser energy to generate ions that are measured by a mass spectrometer, most commonly a time of flight (TOF) mass spectrometer.¹⁶ Compared with MALDI, one of the central advantages of MOLI is the absence of matrix ion peaks, which can obscure ions from the biological specimen in the small molecule range ($m/z < 1000$). Most commonly, when MOLI MS is used, analyte ionization of lipids occurs by protonation, or sodiation, due to interactions with the Lewis acid/base sites on the metal oxide. However, as studies continue, it has been found that the ionization mechanism can vary between metal oxides. For certain metal oxides, such as nickel (II) oxide (NiO), the method of ionization is thought to involve

Lewis acid/base interactions between the cation/anion pairs of the metal oxide and the analyte, resulting in the protonation from one surface-bound analyte to another without involving solvent or surface-absorbed water.¹⁷ For others, such as calcium oxide (CaO), analyte ionization occurs due to interactions with Lewis acid/base sites on the metal oxide, and results in a calcium adduct of a fatty acid carboxylate.¹⁵ More recently, cerium(IV) oxide (CeO₂), a rare earth lanthanide, has also been utilized in MOLI MS. Unlike the other metal oxides, CeO₂ demonstrates a unique property of laser induced catalysis of fatty acyl cleavage when applied to phospholipids and is energized by standard lasers found in commonly used and commercially-available MALDI TOF MS instruments.¹⁶ This property of laser-induced cleavage catalysis by CeO₂ provides a considerable opportunity towards various biological and clinical applications in which fatty acid profiling may be needed.^{17,18}

One promising MOLI MS application using CeO₂ involves microbial identification using fatty acid profiling.¹⁹ While conventional MALDI TOF MS has revolutionized the clinical laboratory workflow through the identification of bacterial isolates by their proteomic profile, differentiation and speciation of certain bacteria, including *Acinetobacter*, *Listeria* and *Enterobacteriaceae*, have proven challenging due to intra-species similarities.^{20,21} Fatty acid profiling using MOLI MS has been shown to not only improve genus and species level identification of these organisms, but has the granularity for even strain level identification²² and differentiation of antimicrobial resistant and susceptible isolates,²³ a considerable improvement to current approaches utilized in clinical microbiology. In addition to infectious diseases, there are also several other potential applications for which this could be used.

To date, MOLI MS has been limited to target plate spot analysis. We here introduce the development and optimization of a new method using CeO₂ as a mass spectrometry imaging catalyst, providing metal oxide laser ionization mass spectrometry imaging (MOLI MSI) of tissue sections. This methodology provides an *in situ* platform for the spatially resolved specific chemical characterization of fatty acyl chains. We demonstrate the translational and clinical potential of MOLI MSI with applications to study lipid-rich brain and brain tumor tissue, and the detection of diagnostic odd-chain fatty acyls from bacterial extracts directly from tissue.

Experimental Section

Chemicals and Materials

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were obtained from Avanti Polar Lipids (Alabaster, AL). Cerium (IV) oxide (CeO₂) (<5 μ m, 99.9% purity), and chloroform (CHCl₃) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), and water were obtained from Fisher Chemical (Pittsburgh, PA, USA). Bacterial lipid extracts were obtained from the Clinical Microbiology Laboratory at Brigham and Women's Hospital. ITO-coated glass slides were purchased from Bruker Daltonics (Billerica, MA).

MOLI MS optimization and analysis of bacterial lipids

CeO₂ application and MOLI MS analytical parameters were developed by modifying a previously reported method²² using a MALDI TOF/TOF analyzer in negative ion reflector mode (10.3x2999 V), equipped with a 10 kHz laser (rapifleX with smartbeam™ 3D laser, Bruker Daltonics, Billerica, MA). MS Dried Droplet method was used with 200 shots per raster spot, 2000 total shots per sample, and global laser attenuator offset of 5%. The primary parameters for optimization included laser power and frequency. Additionally, to determine optimal matrix application, CeO₂ was prepared fresh in 2:1 CHCl₃:MeOH (v/v) at the following concentrations: 0, 5, 10, 25, 50 and 100 mg/mL. POPC was prepared at a stock concentration of 25 mg/mL in 2:1 CHCl₃:MeOH (v/v), and diluted prior to analysis to the following concentrations: 0, 0.05, 0.10, 0.25, 0.50 and 1.0 mg/mL. For analysis, 1 µL of CeO₂ solution was spotted onto an MTP 384 ground steel target plate, allowed to dry, and then 1 µL of lipid standard was spotted on top. Spectra were analyzed in FlexAnalysis 4.0.

To confirm MOLI MS bacterial analysis using the newer generation MALDI MSI platform, we utilized lipid extracts from reference bacterial strains (ATCC #): *Staphylococcus aureus* (25923, 29213, 43330), *Escherichia coli* (35218), and *Klebsiella pneumoniae* (35637). Briefly, bacteria were plated on standard 5% sheep blood agar plates and grown for 24 h at 37°C and 5% CO₂. Following incubation, 1-3 bacterial colonies from each strain (depending on colony size) were collected and bacterial lipids were extracted using Folch extraction² and dried by air. Since we found optimal MOLI MSI performance using 25 mg CeO₂ suspended in 1 mL isopropanol (described in Results), we analyzed bacterial lipid extracts using this solvent system. CeO₂ suspension in IPA (1 µL) was spotted onto the MALDI target plate, allowed to dry, followed by 1 µL of the bacteria extract spotted on top.

Optimization of MOLI MSI

Solvent selection and CeO₂ deposition on mouse brain tissue was optimized to produce an MS image with suitable spatial resolution and peak intensity. Control mouse brains were kindly provided by Dr. Jann Sarkaria (Mayo Clinic, Rochester, MN) and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. Brains were snap-frozen and stored at -80°C. Prior to sectioning, mouse brains were allowed to equilibrate to -20°C for approximately 20 minutes. Whole mouse brains were sectioned coronally at -20°C using a HM 550 (Thermo Scientific) and thaw mounted on ITO-coated glass slides and allowed to dry under vacuum. Several steps of sample preparation were optimized including sample sectioning thickness (4, 8, 12 µm), CeO₂ solvent selection (various combinations of CHCl₃, MeOH, H₂O, ACN and IPA), matrix deposition (spotting/spraying, pre-coating/post-coating), particle suspension method (sonication/vortexing), and temperature of solvent at deposition. For MOLI MSI acquisition, slides with tissue sections were scanned at 2800 dpi prior to CeO₂ application. Tissue sections were imaged at pixel size of 100 x 100 µm, a laser power of 76%, and a frequency of 5 kHz with a Bruker rapifleX MALDI TOF mass spectrometer equipped with a Nd:YAG laser operating at 355 nm. All other instrument settings were the same as the MALDI spot plate analysis method. Imaging data was imported to SCiLS Lab (Bruker) for analysis.

MOLI MSI applications

The optimized MOLI MSI method was then utilized in three applications using mouse brain tissue. In all cases, mice were euthanized by cervical dislocation following CO₂ inhalation and brain was harvested, flash frozen in liquid nitrogen and stored at -80°C. The first application used coronally sectioned normal mouse brains from healthy mice to characterize the lipid fatty acyl pools in different anatomical regions of the brain. The second application involved the imaging of mouse brain containing tumors derived from patient derived xenografts models of glioblastoma (GBM). Intracranial GBM xenograft was generated in a 6-8 week old female athymic nude mouse (Harlan Sprague Dawley Athymic Nude-Foxn1nu mouse, Envigo, Indianapolis, IN) as previously described.²⁴ The final application involved a proof-of-concept for utilization of MOLI MSI to identify bacterial lipids in tissue. To accomplish this, we spotted one hemisphere of control mouse brain with 0.2 µL of *E. coli* lipid extract, thereby simulating a “pseudo-infection.” In all three applications, a freshly sonicated solution of 25 mg CeO₂ suspended in 1 mL of IPA was prepared as described and spotted to completely cover the tissue (~ 5-8 µL/brain section).

Results and Discussion

Optimization of MOLI MS

Before proceeding with MSI application, instrument parameters were optimized for MOLI MS as the instrument and laser frequency used in these studies had not previously been used for MOLI MS. Studies were conducted using glycerophospholipid standards to ensure fatty acid cleavage catalysis could be reproduced on the rapifleX instrument. MOLI MS analysis of POPC resulted in the major spectral peaks m/z 255 and m/z 281, as expected, representing cleaved palmitic acid and oleic acid, respectively (Figure 1A). Analysis of POPE and POPS standards demonstrated similar fragmentation (Supplemental Figure 1). After confirmation of CeO₂-induced fatty acid cleavage, studies using varied lipid concentration, CeO₂ concentration, and laser power were performed using POPC. The most consistent and reproducible signal was found using a laser frequency of 2 kHz operating with laser power between 65-80% and a global attenuator offset of 5%. As seen in Figure 1B, the lowest concentration of CeO₂ that could be used without significant loss of signal intensity is 25 mg/mL (145 mM).

To confirm that the optimized method could be used for fatty acid characterization of biological samples, we performed MOLI MS on lipid extracts from five clinical bacterial reference strains, including three different *S. aureus* strains, as well as a strain of *E. coli* and *K. pneumoniae* (Figure 1C). MOLI MS profiles produced from analysis of three *S. aureus* strains included characteristically high peaks at m/z 241.4 and m/z 269.4 (presumptively C15:0 and C17:0, respectively), similar to those seen previously in *S. aureus*²³ and consistent with the original reports of branched C15 and C17 fatty acids found in the membrane lipids of Gram-positive organisms.²⁵ Conversely, the Gram-negative *Enterobacteriaceae* strains of *E. coli* and *K. pneumoniae* showed higher relative abundance at m/z 227.4, m/z 267.4, and m/z 295.4 (tentatively assigned C14:0, C17:1 and C19:1) and consistent with the previously published spectra.²² These studies demonstrate the compatibility of using MOLI MS on the newer generation rapifleX instrument with the

previously reported MOLI MS studies which used other MALDI TOF instruments (ultrafleX, microflex).²²

Optimization of MOLI MSI

To optimize MOLI MSI, we first sought to optimize instrument parameters for imaging. Using parameters from the target plate analysis as a starting point for tissue analysis, we optimized laser power, global attenuator offset, detector gain, sample rate, and baseline adjustment offset. The optimal parameters for both MOLI MS (performed on a stainless-steel target plate) and MOLI MSI (performed on tissue mounted on ITO slide) are presented in Supplemental Table 1. These parameters were optimized to increase spectral resolution and decrease background noise. It is important to note that many of these values will change over time as an instrument and the laser ages, though were consistent within our study.

Next, we determined the optimal solvent and CeO₂ concentration for MSI. In the original description of this reagent, a concentration of 100 mg CeO₂ per 1 mL of Folch reagent (2:1 CHCl₃:MeOH)² was used. However, we found that at this concentration of CeO₂ the metal oxide particles quickly aggregated and fell out of suspension. For MSI applications, a homogenous suspension of matrix is critical for more consistent pixel-to-pixel analysis. We found that dissolving CeO₂ in isopropanol achieved a more homogenous and stable suspension of the metal oxide particles, providing the more uniform layer needed for MSI.

We next determined the optimal preparation and application of the CeO₂ mixture for MOLI MSI. Based on our target plate and solvent optimization studies, we used 25 mg/mL of CeO₂ in 100% isopropanol as a MOLI MSI matrix solution. Unlike target plate analysis, however, we found that proper preparation of the CeO₂ solution prior to application on tissue was critical. While CeO₂ did not fully dissolve in any solvents, to achieve an even suspension, the solution was vortexed for 10 seconds until a slurry was formed and sonicated for 20 min. Following sonication, enough cerium oxide solution to cover the brain was spotted (approximately 5-8 μ L per brain). If multiple tissue sections were prepared for analysis, it was found that an additional 10 seconds of sonication was needed prior to spotting each section. We also tested other application methods, such as spraying the CeO₂ solution, to improve spatial resolution. However, we found that manual spraying resulted in more delocalization, and was not consistent due to the relatively rapid precipitation of the CeO₂ in the solvent. Also, we chose not to introduce this reagent into the automated sprayer systems due to concerns it may clog the spray nozzle based on this precipitation. We appreciate the need for appropriate matrix application and the risks of delocalization of analytes associated with the spotted application of matrix, however we assessed the level of delocalization from measurements of the substrate surrounding the tissue and found it to be acceptable, potentially providing a diagnostics compatible method. CeO₂ application methods will be a focus for future work.

Lastly, we sought to determine the best workflow for sectioning and applying the matrix to the tissue. In the original description of this reagent, the cerium oxide was spotted first on the plate, followed by spotting the extracted bacterial lipids.²² MOLI MSI was performed by coating the plate prior to tissue sectioning with CeO₂ (pre-coating), coating CeO₂ after tissue mounting (post-coating), as well as both pre- and post-coating the same tissue section.

We found that MOLI MSI of a specimen mounted on a pre-coated slide failed to provide an adequate signal while post-coating provided a strong signal and clearly delineated different neuroanatomical regions (Figure 2). Also, combining pre-coating and post-coating did not provide any additional discernable benefit to post-coating alone. As demonstrated in the mass spectrometry images, different anatomical regions including the corpus collosum could be clearly visualized based on relative abundance of certain fatty acids, thereby providing the first evidence of CeO₂ as an MSI reagent. We then applied the optimized protocol to three different applications on mouse brain tissue.

Application 1: MOLI MSI reveals regional variability in fatty acid composition in mouse brain

In the first application, we utilized the optimized MOLI MSI method to spatially characterize the fatty acid composition of healthy mouse brain, using frozen coronal tissue sections. Regional differences in fatty acid composition are clearly visualized in different neuroanatomical regions (Figure 3). For example, a substantially higher relative intensity of FA 18:1 (m/z 281) and FA 20:1 (m/z 309) is observed in the corpus callosum, a thick tract of nerve fibers that runs below the cerebral cortex. The higher concentration of these fatty acids can be attributed to the high myelin content of these nerve fibers and consistent with previously reported literature.^{26,27} In a study by Sun,²⁶ the myelin fraction contained approximately 4-fold and 10-fold higher levels of FA 18:1 and FA 20:1, respectively, in the fatty acyl pool when compared to human gray matter regions, consistent with our MOLI MSI findings. Conversely, the relative abundance of FA 16:0 (m/z 255), FA 18:0 (m/z 283), FA 20:4 (m/z 303) and FA 18:2 (m/z 279) were found in higher relative abundance in gray matter compared to myelinated regions, also consistent with previous reports.^{26,28} The ability to spatially characterize fatty acid composition will not only be useful to understand brain lipid metabolism,²⁸ but in other tissues such as adipose tissue, where a better understanding of the metabolic changes that occur is needed.^{29,30} Performing complementary lipidomic analysis of the tissue using conventional MALDI MSI would allow the correlation of these fatty acid pools with the parent lipids from which they are cleaved. Together, these provide a more complete picture of the lipid composition of the tissue.

Application 2: MOLI MSI reveals different fatty acids in PDX model of GBM

Altered metabolism is a central hallmark of cancer,³¹ and as such metabolomic characterization of both tumor and microenvironment have been intensive areas of study over the past decade.^{32,33} Particularly, there is mounting evidence that dysregulation in lipid metabolism is important in several cancers, and has implications in anabolic, catabolic, and signaling functions.³⁴ Furthermore, recent evidence highlights that these differences can be used not only to better understand tumor metabolism, but to distinguish tumors as well.³⁵ Accordingly, we sought to utilize MOLI MSI in a PDX model of GBM, to characterize differences in the fatty acid pool of these tumors. As shown in Figure 4, the tumor regions demonstrated a distinct fatty acid signature compared with normal (non-tumor) regions in the brain. Specifically, we found that certain fatty acids including FA 20:1 (m/z 309) were higher in abundance in normal brain, other fatty acids such as FA 18:0 (m/z 283) and FA 18:1 (m/z 281) were found in similar abundance in tumor and non-tumor regions, while

others including FA 16:0 (m/z 255) and FA 18:2 (m/z 279) were considerably elevated in the tumor. The lower apparent levels of FA 18:2 in the non-tumor regions of the PDX-containing brain as compared to the normal brain (Figure 3) is a result of the normalization of this ion to the total ion count, further highlighting the substantial increase of this ion in the tumor. Collectively, MOLI MSI provides a method to characterize a different aspect of tumor metabolomics, in this case fatty acids, and would provide complementary information to other cancer metabolomic approaches. Furthermore, due to the relative heterogeneous nature of human tumor tissue, the ability to perform these analyses *in situ*, would allow characterization of both tumor and non-tumor regions within the same specimen.

Application 3: MOLI MSI can be used to differentiate bacterial lipids within tissue samples

For the final application, we utilized MOLI MSI as a proof-of-concept for pathogen detection in tissue. Pathogen detection from clinical tissue specimens remains a considerable challenge with most current approaches lacking either the speed, sensitivity or specificity needed for rapid and accurate diagnosis. MALDI TOF has the capacity to provide high throughput identification of bacterial, fungal or mycobacterial isolates, with minimal sample preparation and lower reagent costs, though current approaches rely heavily on proteomic signatures from culture extracts. Many microorganisms produce fatty acids not commonly observed in animals.²² We used this principle to demonstrate that MOLI MSI could differentiate bacterial lipids from mammalian tissue. To accomplish this, we spotted one hemisphere of control mouse brain with *E. coli* lipid extract, leaving the other side as a control, simulating a pseudo-infection. Using this model, we were able to identify the fatty acid chains enriched in *E. coli* on the spotted region (Figure 5), most notably in peaks identified as FA 14:0 (m/z 227), FA 17:1 (m/z 267) and FA 19:1 (m/z 295), which are consistent with the elevated spectral peaks from the reference strain of *E. coli* (Figure 1C). Although this model has limitations and is not intended to be physiological, it provides a proof-of-principle that bacterial-specific lipids can be distinguished from mammalian tissue lipids using MOLI MSI. Further investigation to determine the ability of this method to detect and identify pathogens in tissue are ongoing. These include using a wide range of bacteria and other microorganisms in both simulated and infected tissues, utilization of higher spatial and mass resolution for improved sensitivity and specificity, and determination of the limit of detection for different pathogens in a variety of tissue types. The ultimate goal of this application will be to develop a method to directly and rapidly detect pathogens using their unique fatty acyl signatures in infected clinical tissues.

Conclusion and Future Directions

In this study, we present the development, optimization, and application of MOLI MSI to spatially characterize the fatty acid composition of tissue specimens. Tissue sample preparation was designed to be as simple and rapid as possible, while still producing a mass spectrometry image with adequate spatial resolution and was used in multiple applications. Future studies will include application to clinical specimens, including tissues containing tumor and/or pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Optimization of MOLI MS using the rapifleX platform. (A) Mass spectra of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) standard using CeO_2 , confirming anticipated fatty acyl catalysis. (B) Signal intensity of fragmented fatty acyl chains using various concentrations of POPC and CeO_2 . Left panel shows signal intensity of m/z 255 (FA 16:0, palmitic acid); right panel shows signal intensity of m/z 281 (FA 18:1, oleic acid). (C) MOLI MS of extracted lipids from reference bacterial species (and strain numbers), containing CeO_2 -catalyzed fatty acyl fragments.

Figure 2.
MOLI MSI image of FA 20:1 using different applications of CeO_2 .

Figure 3. MOLI MSI of control mouse brain tissue. Coronal sections (12 μ m thickness) of frozen mouse brains were thaw mounted and spotted with a solution of CeO₂ in isopropanol. MOLI-generated fatty acyl pool profiles contained in various neuroanatomical regions are highlighted. MOLI MSI images were acquired using 100 μ m pixel resolution.

Figure 4.
MOLI MSI of mouse brain tissue containing tumor of glioblastoma patient-derived xenograft. Histopathology (H&E stain of tissue): tumor delineated as *T*, Corpus callosum as *CC*. MOLI MSI ions images were acquired using 100 μ m pixel resolution.

Figure 5.
MOLI MSI of a coronal normal mouse brain cryosection spotted with *E. coli* lipid extract.
CeO₂-catalyzed fatty acyl spectra demonstrating localization of bacterial-enriched acyl
groups (red) in spotted hemisphere, compared with normal mouse brain spectra (blue).
MOLI MSI images were acquired using 100 μ m pixel resolution.