An External Matrix-Assisted Laser Desorption Ionization Source for Flexible FT-ICR Mass Spectrometry Imaging with Internal Calibration on Adjacent Samples

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Abstract

We describe the construction and application of a new MALDI source for FT-ICR mass spectrometry imaging. The source includes a translational X-Y positioning stage with a 10×10 cm range of motion for analysis of large sample areas, a quadrupole for mass selection, and an external octopole ion trap with electrodes for the application of an axial potential gradient for controlled ion ejection. An off-line LC MALDI MS/MS run demonstrates the utility of the new source for data- and position-dependent experiments. A FT-ICR MS imaging experiment of a coronal rat brain section yields ∼200 unique peaks from m/z 400–1100 with corresponding mass-selected images. Mass spectra from every pixel are internally calibrated with respect to polymer calibrants collected from an adjacent slide.

Key words: Fourier transform, Ion cyclotron resonance, FTMS, Imaging mass spectrometry, INCAS

Introduction

Mass spectrometry (MS) imaging combines molecular (mass) information with spatial information from complex surfaces (e.g., biological tissues) [1–3]. Current instrumental and method developments aim to improve one or both of these facets. Time-of-flight secondary-ion mass spectrometry (TOF-SIMS), stigmatic matrix-assisted laser desorption ionization (MALDI)-TOF ion microscope and highly focused microprobe experiments (via MALDI or SIMS) allow high spatial resolution to sub-micrometer scale [4–13]. On the other hand, high-performance mass spectrometers (e.g., Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers [14] and hybrid linear ion trap (LTQ)-Orbitrap [15], which shall be inclusively referred to as “FT”) are now being used for complementary high mass resolving power and high mass accuracy MS for identification of observed species [16–19]. Here, instrumentation and methodology for a custom-built MALDI source for FT-ICR mass spectrometry imaging are described.

Laser microprobe systems represent the earliest chemical imaging utilizing FT-ICR MS. Early systems used in-cell laser desorption ionization (LDI) for profiling and imaging experiments, with imaging experiments typically in one dimension (i.e., line scans) [20–23]. While these systems generally had excellent spatial resolution, easily less than 10 μm laser spot diameter, the species analyzed were
typically less than $m/z$ 500 and were comprised of mainly metals and metal oxides. These systems relied on sophisticated optics for alignment of the laser beam inside the vacuum system and the operation of a translational stage within the bore of a superconducting magnet was a challenge. However, one such system is currently being used for geomatrix-assisted laser desorption/ionization MS imaging of rock/mineral samples [24].

Ease of access is arguably the largest disadvantage of in-cell ionization techniques. Thus, external laser microprobe ion sources were developed which alleviated some of the above mentioned challenges [25, 26]. Concurrently, the development of MALDI [27–30] for analysis of intact biomolecules by FT-ICR MS was also receiving considerable attention, owing to the advantages of FT-ICR MS over TOF instruments. Many in-cell MALDI FT-ICR systems were developed, though none for MS imaging [31–35]. The development of external MALDI ion sources, typically with the extraction of ions into a multipole storage device, allowed easy access for changing samples and opened the door for higher-throughput MALDI FT-ICR MS [36–43]. Translational X-Y stages addressed the need for large sample plate loading for multi-sample analysis, and these were installed in systems with extraction of ions into a multipole, as described above [44, 45]. The external MALDI source described in this paper is similar in design, with extraction of MALDI-generated ions into a hexapole ion guide and subsequent transfer to a storage octopole. The new Bruker Apollo II dual ESI/MALDI source, which is equipped for MS imaging studies, utilizes a dual ion funnel for collection and focusing of MALDI-generated ions before storage in an external multipole ion trap.

The utility of MALDI FT-ICR for the direct analysis of biological tissues has been demonstrated for peptides from crab neurons [46], crab sinus glands [47], and a wide array of decapod neural tissues [48, 49]. Further, MALDI FT-MS imaging has been used to image peptides and lipids in rat and mouse brain [16, 19, 50] and drugs and metabolites from rat kidney and liver, as well as mouse brain [17]. These studies demonstrate the need for high mass resolving power to resolve isobaric ions and the advantage of high mass accuracy for the identification of analytes and MS/MS fragments.

The instrument described herein presents a flexible platform for high mass resolution and high mass accuracy FT-ICR mass spectrometry imaging. The capabilities of this custom-built instrument—such as workflow-based control software [51, 52], easy implementation of different ICR cell designs [53], and fragmentation by simultaneous electron-capture dissociation infrared multiphoton dissociation (ECD/IRMPD) [54]—expand the possibilities of FT-ICR MS imaging; such possibilities are otherwise not easily implemented on commercial systems.

A liquid chromatography (LC)-MALDI experiment was "imaged" to test the new configuration in a data- and position-dependent MS/MS mode. Half of a coronal rat brain section was imaged to assess the applicability of the system to tissue analysis, where over 200 unique peaks are observed. Polymer calibrant ions are collected from an adjacent glass slide and provide internal calibration over the entire dataset, thus bypassing any difficulties associated with ion suppression following the deposition of calibrants on the tissue surface. In addition, internal calibration of each pixel of the imaging experiment allows confident generation of mass-selected images with narrow (10 mDa) mass windows. In-house developed software is used to produce high mass resolution "datacubes" for easy data navigation and analysis.

Materials and Methods

Sample Preparation

LC-MALDI Acetonitrile (BioSolve, Valkenswaard, NL) and acetic acid (JT Baker, Phillipsburg, NJ, USA) were used without prior purification. Savinase (synthetic bacillus serine protease) was digested with trypsin and CNBr and 5 µL was separated on a LC Packings nanoLC-system (Dionex, Amsterdam, NL) with a C18 PepMap 100 pre-column (internal diameter 300 µm, length 1 mm) and a C18 PepMap 100 analytical column (internal diameter 75 µm, length 15 cm). The eluents were 0.1% formic acid and 5% acetonitrile in water (A) and 0.1% formic acid and 5% water in acetonitrile (B). The LC method was 50 min with an initial 6 min equilibrium period, followed by a gradient of 20 min 0–70% B, a 1 min gradient of 70–95% B, 10 min of 95% B, and 13 min of 5% B. The capillary was coupled to a SymBiot I sample workstation (Applied Biosystems, Foster City, CA, USA) and effluent was spotted at intervals of 30 s and a spacing of 1 mm (10×10 matrix) on a pre-made 2,5-dihydroxybenzoic acid (DHB) MALDI foil (LabConnections, Northborough, MA, USA). The delay time between the start of the LC run and spot deposition was 20 min. Peptide and protein were identified by Mascot database search (www.matrixscience.com).

Rat Brain Section and Polymer Calibrants A 12 µm thick coronal section of rat brain (Harlan Laboratories, Boxmeer, The Netherlands) was prepared in a Microm HM 525 cryomicrotome (Microm International, Walldorf, Germany) at −20 °C and placed on indium–tin oxide coated glass slides (ITO, 4–8 Ω resistance; Delta Technologies, Stillwater, MN). The section was washed with cold (−20 °C) ethanol (70% in H2O) and coated with ~30 layers of a 30 mg/mL solution (1:1 MeOH/H2O (0.2% trifluoroacetic acid)) of DHB with a Bruker ImagePrep matrix deposition device. For internal calibration, an ~156 µM solution of poly(methyl methacrylate) 640 (PMMA) was prepared in a 30 mg/mL solution of DHB and deposited on an ITO-coated glass slide as described above. Lipid assignments were made using LIPID MAPS (LIPID Metabolites and Pathways Strategy; http://www.lipidmaps.org)
with a mass tolerance of 0.01 Da with regards to the average measured mass.

**Instrumentation**

All experiments were performed on a heavily modified Bruker APEX 7.0e FT-ICR mass spectrometer [55]. Figure 1 shows a schematic of the new instrument configuration. A 355 nm Nd:YAG laser (1000 Hz repetition rate; Bright-Solutions, Cura Carpignano, Italy) is coupled to the source through a 3 m tapered fiber optic cable (800–200 μm, Fiberguide Industries, Stirling, NJ, USA). The beam is collimated after the fiber optic cable and aligned through the hexapole rods and conductance limit (4 mm) onto the sample surface, which is ~2 mm from the hexapole entrance. The laser power, start time, and output frequency are controlled from the data station. In addition, a 337 nm N2 laser (Laser Science, Inc., Franklin, MA, USA) can also be coupled to the system. A translational X-Y stage with 10×10 cm range of motion (Fraunhofer USA, Brookline, MA, USA) allows for the analysis of large sample areas [56]. For ESI, the MALDI stage is removed and replaced with an electrodynamic ion funnel [57–59] and operated in nano-ESI mode.

For MALDI analysis, the source chamber is ~5×10^{-5} mbar and a small (0.5–5 V) positive bias voltage is applied to the sample plate (over the hexapole entrance conductance limit). Ions from the surface pass through the hexapole conductance limit and are injected into a short (75 mm) transfer hexapole, pass through a mass-selective quadrupole (ABB Extrel, Pittsburg, PA, USA) before storage (0.1–1 s) in a 170 mm accumulation octopole (with helium cooling gas at ~5×10^{-6} mbar) [60]. External ion accumulation allows for the accumulation of a large number of laser shots at high repetition rate. In addition, calibrants from an adjacent sample [61, 62] can be stored in the octopole while analyte ions are collected, and the entire packet sent to the ICR cell simultaneously. Ejection electrodes are used for efficient extraction of ions into two transfer quadrupoles (253 and 889 mm) (ABB Extrel, Pittsburg, PA) [63, 64]. This improves image contrast by increasing the number of ions observed per pixel and thus the detection efficiency. The instrument can be equipped with either a capacitively coupled cylindrical open ICR cell [53] or a Bruker Infinity cell [65], where ions are cooled with an argon pulse gas [66] before broadband frequency (chirp) excitation [67] and direct-mode detection. An in-house developed workflow-based data station (“AWG3”) is used for experiment control and data acquisition [51, 52].

**Results and Discussion**

The new source was designed around an X-Y translational stage with high position accuracy (~0.4 μm), with a 10×10 cm range of motion for analysis of large samples. The AWG3 workflow-based data station uses command and decision modules, which are termed “nodes”, for easy and flexible design of FT-ICR experiments. Stage control is managed through a new “StageDriver” node in the AWG3 flow-based control software. The absolute position of the sample stage is calibrated by a simple three-point (or more) calibration routine of the sample stage live feed with respect to a scan of the sample plate. Regions of interest with user-defined raster size can be defined by polygonal or rectangular grids. “Stage command” nodes in the workflow define stage movements such as an offset relative to the current position, a “go to position” command, and a decision to wait for the stage to stop movement. The stage position is stored as a variable and can be used for position-dependent experiments. An overview of alternative MS imaging control software for FT and time-of-flight MALDI instruments can be found elsewhere [68].

Figure 2 shows a data-dependent (LC)-MALDI FT-ICR MS/MS “imaging” experiment of a trypsin/CNBr digest of savinase. An optical image of the spotted MALDI foil with an overlay of the UV LC trace is shown in Figure 2a. Mass-selected images of three trypptic peptides are shown in Figure 2b and an overlay of m/z 1200.8464 (red; NPSWSNVQIR) and its y8 product ion (m/z 989.6202, blue; SWSNVQIR) are shown in Figure 2c. Mascot database search resulted in a Mowse probability score of 72 with 45% sequence coverage. The workflow for the (LC)-MALDI LC-MS/MS experiment is shown in Figure 2d. Here, a prescan was collected and evaluated by the PeakPicker node to determine if peaks were present, followed by declustering to mono-isotopic peaks in the ClusterList node. If at least one cluster was found, the stage was offset and the ion corresponding to the most abundant peak from the prescan was isolated by stored waveform inverse Fourier transform (SWIFT) [69] and fragmented by sustained off-resonance irradiation collision-induced dissociation (SORI-CID) [70, 71]. This workflow can be modified to fragment specific target ions (i.e., a selected reaction monitoring experiment) or

![Figure 1. Schematic of the AMOLF 7 Tesla MALDI/ESI FT-ICR mass spectrometer. The source features an X-Y stage with 10×10 cm range of motion for MALDI that can be replaced with an electrodynamic ion funnel for ESI](image-url)
Careful mass calibration is essential for confident generation of mass-selected images from large MS imaging and LC-MALDI datasets. This is especially important for MALDI MS imaging, where the shot-to-shot variation of MALDI, compounded with the spatial variations in analyte concentration on the tissue surface, lead to ion number variations in the ICR cell which shift the measured frequency (and thus the mass) [72–75]. Calibration methods used for MALDI FT-MS imaging include internal calibration on MALDI matrix clusters [17] and lock mass calibration on a known m/z [76].

The MALDI source described herein exhibits no MALDI matrix interferences, so internal calibration by these means is not possible. In addition, the system does not have the ability to closely control the number of ions injected into the ICR cell (i.e., automatic gain control [77–81]). Thus, a strategy for internal calibration on adjacent samples (INCAS) [61, 62] was developed to allow confident internal calibration on every pixel of the dataset. Figure 3 shows a representative workflow for the INCAS strategy. First, internal calibrants (here, PMMA 640) are collected from an adjacent slide and stored in the external octopole ion trap. The X-Y stage is moved to the sample where analytes are collected and stored in the same external octopole ion trap. Thus, both calibrants and analytes can be sent to the ICR cell simultaneously, and...
ion suppression due to the application of calibrants on the sample can be avoided. Here, the application of PMMA 640 on tissue (in the MALDI matrix solution) resulted in no signal for either the polymers or tissue analytes. However, peptide standards applied directly on tissue may prove useful as internal calibrants. In-house data analysis software was used to locate calibrant peaks and to calculate and apply the internal calibration parameters [74].

A rat brain coronal section was used to test the tissue imaging capabilities of the new source to which the INCAS strategy was applied. Figure 4 shows the result of internal calibration for every pixel from a FT-ICR MS imaging experiment of half of the rat brain section. The measured mass accuracies (in parts per million (ppm)) of six common glycerophospholipids (with phosphocholine (PC) and glycerophosphate (PA) head groups) observed in brain [19, 82–86], m/z 760 ([PC 34:1+H]^+), 767 ([PA 38:2+K]^+), 772 ([PC 32:0+K]^+), 782 ([PC 34:1+Na]^+), 798 ([PC 34:1+K]^+), and 804 ([PC 36:4/PE 39:4+Na]^+) were calculated, binned into

![Figure 4](image)

Figure 4. Number of peaks per bin versus mass measurement accuracy (0.1 ppm bin size) for six lipids (m/z 760, 767, 772, 782, 798, and 804) observed in rat brain (N_{peaks}=7499). Internal calibration centers the distribution around zero and narrows the FWHM.

![Figure 5](image)

Figure 5. (a) Mass-selected images of six lipids from a positive-ion MALDI FT-ICR MS imaging experiment of a coronal rat brain section collected at 200 μm raster size. The images are from the range of the stated mass +0.01 Da (e.g., 760.578–760.588). Scale bar=600 μm. (b) Overlay and datacube mass spectrum of two isobaric ions at m/z 806. Blue=806.5114 and red=806.5660. The width of the colored bars indicates the mass range selected for the overlaid images. Pink pixels indicate where these two ions are observed in the same pixel.
$0.1 \text{ m/z}$ bins, and plotted as histograms for internal and externally calibrated datasets. Internal calibration greatly improves the measured mass accuracy and narrows the full width at half-maximum (FWHM) of the histogram, which allows for smaller mass windows for generation of mass-selected images, such as those shown in Figure 5a for these six lipids.

In addition, ~200 unique peaks ($S/N > 5:1$) were observed from $\text{m/z}$ 400–1100, with an average mass resolving power ($\text{m/Δm}_{50\%}$) of 122,000. In-house developed software was used to create a database of the X-Y correlated mass spectra, in which data can be easily navigated and mass-selected images exported [87]. The large number of spectra from an FT-ICR MS imaging experiment, here 1777, requires mass spectral binning in order to reduce the datacube size; here, a bin size of 0.005 Da was used (as determined by the FWHM of the internal calibration shown in Figure 4, where 5 ppm at mass 700 is 0.0035, to ensure small shifts in mass do not distort the peak shape in the datacube binned mass spectrum). The spectra and the red-blue overlay in Figure 5b show two isobaric ions at $\text{m/z}$ 806 which have different spatial distributions (blue = 806.5114 (PC 35:4/PE 38:4) and red = 806.5660 (PC 36:3). The high mass resolving power of FT-ICR MS easily resolves these two ions which differ in mass by ~55 mDa. In addition, the high mass accuracy allows confident assignment of elemental compositions and identifies these peaks as corresponding to distinct glycerophospholipid species. Long detection times for high-resolution FT-MS imaging remain the time-limiting step. Here, transients of 2.6 s (in addition to pulse-gas pump-down delay) were collected in a serial fashion, which presents a significantly longer image acquisition time over MALDI TOF instruments. The move to higher-field superconducting magnets for FT-ICR [88], higher-performance electrostatic FT instruments [89], and interlaced data acquisition strategies (e.g., the collection of analytes during the excite/detect events) will improve the duty cycle for FT-MS imaging studies.

Conclusions

We have constructed a dedicated MALDI source for FT-ICR mass spectrometry imaging. Software updates allow data- and position-dependent workflow-based MS imaging experiments as demonstrated with a (LC)-MALDI MS/MS experiment. Mass-resolved images of lipids from a rat brain section demonstrate the utility of the new source for biological tissue measurements. INCAS allows confident mass assignment without adulteration of the sample surface with calibrants. Isobaric ions with a mass difference of ~55 mDa are mass resolved (over the entire mass spectrum) and display different spatial distributions.

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