

Mass spectral imaging using AP MALDI ionization source coupled with Sciex® QTOF 6600 analyzer

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1. Introduction

Mass spectral imaging (MSI) is an emerging area of research with significant potential in clinical research, diagnostics and drug discovery. MSI approaches enable direct analysis of biological and clinical specimens with minimal sample preparation. Probing comparative spatial distribution of diverse molecular features including therapeutic drugs in tissues provides a powerful handle for hypothesis generation and verification. Single cell imaging offers a new paradigm in the rapidly emerging systems-based approaches in precision medicine.

Atmospheric pressure matrix assisted laser desorption ionization (AP-MALDI) is a versatile technique further extending the utility of LC-HRMS platforms. AP-MALDI vastly enhances the ease of accessibility closer to real time analysis, especially MSI, at a fraction of cost compared to dedicated vacuum MALDI-MS instruments. Sample handling and ion generation at ambient pressures using the AP-MALDI source offer significant advantages. Rapid interchangeability to exploit the complementarity of ESI and AP-MALDI using the same mass analyzer is an added advantage. Finally, AP-MALDI is compatible with diverse mass analyzers including the high resolution MS and the industry standard triple-quadrupole analyzers.

This application note, written in the form of a protocol, is intended for researchers to readily identify various aspects of the MSI workflow specific to the AP-MALDI analysis on Sciex® QTOF (TripleTOF® 6600) mass analyzer. Figure 1 shows a schematic overview of the AP-MALDI MSI process on Sciex® instruments.



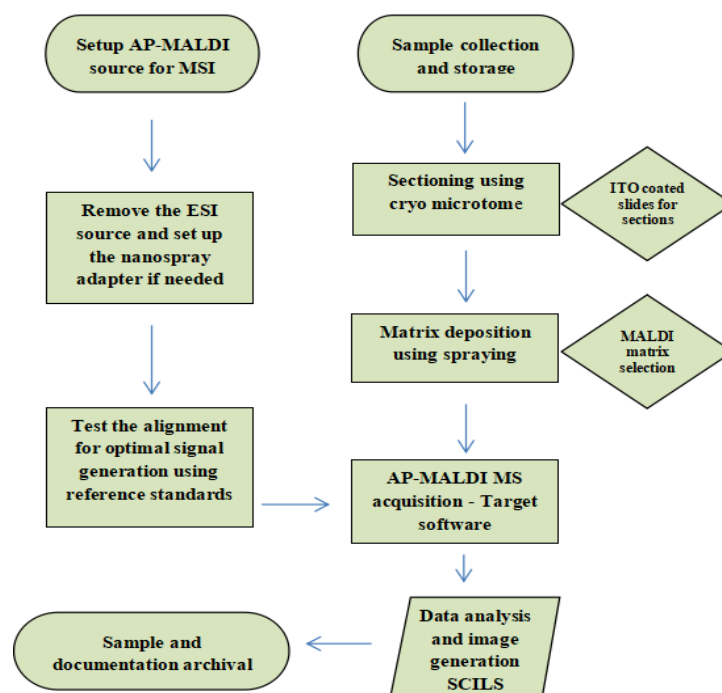


Figure 1 Overview of process steps for AP-MALDI MSI

2. Materials

2.1. Instrumentation

AP-MALDI (ng) UHR ion source (MassTech Inc., Columbia, MD USA) equipped with a 355 nm Nd: YAG laser.

- ◆ AP-MALDI (ng) source's interface is custom made for different mass analyzers and manufacturers. Place an order for a Sciex® compatible AP-MALDI source. Corresponding *capillary extension*, *alignment jig*, *reusable MALDI target plates*, *optical calibration plate*, *MSI adapter* and *connector cables* are included along with MassTech's ion source.

Sciex® QTOF mass analyzer (For ex. TripleTOF® 5600/6600/6600+)

NanoSpray adapter kit for 5600/6600 Systems from Sciex®

NanoSpray source for instrument calibration

- ◆ The nanospray adapter kit is an essential component

Indium tin oxide (ITO) slides (25 mm x 50 mm, resistivity 20 Ω, standard thickness (1.1 mm))

- ◆ ITO slides have to be cut to size to fit the AP-MALDI MSI slide holder if longer than 50 mm – use the conducting side for placing the tissue



Standard 0.3 mm nozzle airbrush with attached 5-7 mL storage cup

N₂ supply or dried compressed air

Standard tubing for connecting N₂ with the sprayer

- ◆ Check for other available options such as HTX TM[®] Sprayer, Sun Collect[®] MALDI sprayer etc.. See existing literature for more details on spraying techniques for optimal results specific to the study context.

Cryo-microtome

Desiccator

2.2. Reagents

Acetonitrile (ACN) and methanol, LC-MS or MS grade

Trifluoroacetic acid (TFA)

- ◆ ***Safety note:*** Exercise extreme care while handling solvents, chemicals and reagents as per the respective materials safety data sheets (MSDS) and always wear personal protective equipment as mandated and use a fume hood while transferring. Follow EPA/EHS guidelines for all chemical and biological waste disposal

Appropriate MALDI matrix, for ex. α -Cyano-4-hydroxycinnamic acid (CHCA)

Cryo gel or optimum cutting temperature (OCT) compound

Synthetic peptides mixture either individually mixed or a commercial kit, for ex. Sciex[®] 6-peptide mixture kit (Part number: 4465940)

Small molecule reference standards, for ex. verapamil and midodrine HCL

2.3. Software

Target[®] /Target-Next[®] data acquisition software – please download the latest version from the www.apmaldi.com along with the User Manual.

MT imzML Converter (MassTech Inc.)

Proteowizard[®]

SCiLS[®] – contact MassTech or <https://scils.de/> for a license

Analyst[®] (bundled with MS for instrument control, data acquisition and processing)

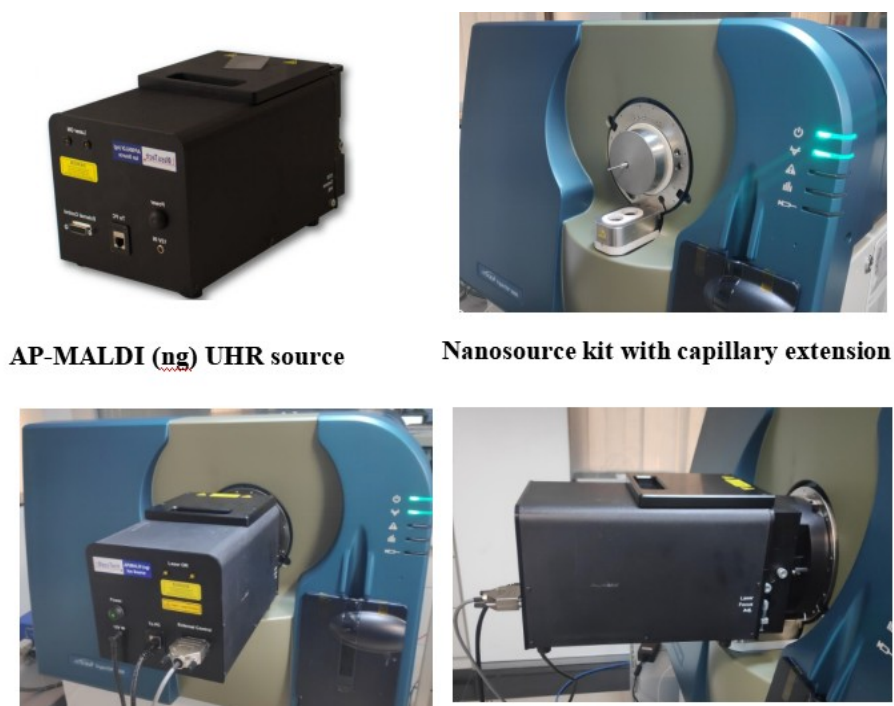


3. Method

3.1. AP MALDI Setup

1. Carefully unbox or remove the AP-MALDI source or if already unboxed, from its place of usual storage.
 - ◆ Go through the checklist and ensure that the shipment contains all the items on the checklist.
 - ◆ Read MassTech's AP MALDI Sciex® manual on Installation, Operation and Maintenance for comprehensive details and troubleshooting (available on www.apmaldi.com). Get familiar with all the connections including mechanical flanges, electrical cables, and power indicators
 - ◆ Read and understand the Safety Procedures section of the User Manual.
 - ◆ ***Safety note: All installation and source changes should be performed with the power connections turned 'OFF'.***
 - ◆ Store the AP-MALDI source carefully when not in use, preferably in an encasement to prevent any damage or misalignment to the internal components.
 - ◆ Exercise extreme care when handling the AP-MALDI source. Mishandling can result in permanent damage to the sensitive optical, electronic and the mechanical components.
 - ◆ For any queries, including service, spares and maintenance questions, please contact MassTech Inc. 6992 Columbia Gateway Dr. Suite 160 Columbia, MD 21046 USA Phone: (443) 539-1758; E-mail: msms@apmaldi.com
2. Remove the existing ESI or any other source from the mass analyzer after venting the vacuum and install the nanospray adapter kit – orifice plate & curtain plate along with the capillary extender.
4. Mount and lock the AP-MALDI source in place as per the instructions in the user manual. Complete the installation by connecting the power, ethernet (computer) and external control cables (to the MS). Power on the source. Figure 2 shows representative images after successful installation: the AP-MALDI ion source, interface with the nanospray adapter kit and capillary extender, and with the source properly connected respectively.
 - ◆ Ensure that the AP-MALDI connector pin is correctly aligned with that of the MS instrument. At the end of correct installation, there should be no interlock errors or activation of any sensor switches (for ex. gas sensor). Ensure the laser is optimally aligned. Read the document detailing AP-MALDI laser alignment using the alignment jig.





AP-MALDI (ng) UHR source

Nanosource kit with capillary extension

Figure 2 AP MALDI imaging ion source connected with the SCIEX 6600 QTOF system

3.2. Reagent preparation

1. A 10 mg / mL solution of CHCA is prepared in 70:30 (v/v) ACN: 0.1% TFA in water. Deionized water was used (Sp. Resistivity 18.2 M Ω -cm).
 - ◆ Prepare sufficient quantity taking into consideration the tissue sections / slides and for uniform spraying. Freshly prepared solutions are preferred.
 - ◆ The choice of matrix and its application / deposition will significantly determine the outcome of the MSI experiments. There are several articles that discuss this topic at length. For example, please see *L.R.S. Huizing, et al. Clinical Mass Spectrometry 12 (2019) 7–15* and *Calvano et al. Anal. Bioanal. Chem. (2018) DOI: 10.1007/s00216-018-1014-x*
2. Prepare a solution of angiotensin I 19 pmol/ μ L, bradykinin(2–9 clip) 14 pmol/ μ L, glu1-fibrinopeptide 10 pmol/ μ L for signal optimization. 5 μ L peptide mixture and 5 μ L CHCA are mixed in the ratio 1:1.
3. 1 μ L of thoroughly mixed matrix-analyte solution from above is to be spotted on a previously cleaned regular 192-well MALDI target plate and air dried.
4. Other small molecule reference standards can also be used.



subsequently for testing the signals following AP-MALDI ionization. Atorvastatin, verapamil and mildodrine HCL are excellent and easily available standards used in this protocol.

3.3. Benchmarking

1. Benchmarking of the performance of AP-MALDI hyphenated with the QTOF analyzer is recommended prior to performing MSI described in subsequent steps. This not only ensures optimal signals generated using standard reference materials, but also can be used to both qualitatively and quantitatively assess the performance of both the ion source and the mass analyzer over a period of time. This data will be especially useful for setting quality control, troubleshooting and scheduling timely and routine maintenance.
 2. Start the data acquisition software Target[®]. After the initialization and optical calibration is complete, select the ABI Opti TOF 192 sample plate option for further analysis.
 3. Use the 192 well plate spotted and dried with the reference standards as described in the previous section. It is advisable to spot multiple wells with the reference standards to check reproducibility prior to starting an imaging experiment or even preparing tissue sections.
 4. Laser parameters described in Table 1 below for laser energy, frequency and firing pattern are typically to be used for this experiment. Parameters for the configuring the MS are also given in Table 1. Data generated in this experiment can be visualized using the Analyst[®] software or raw data exported to other freeware.
 5. Figure 3 is representative of the signals obtained for the peptide and small molecule reference standards analyzed. Acceptable S/N can be obtained as seen in the figure for peptide signals at femtomole levels. Similarly, low m/z analytes, verapamil and midodrine HCL – both drugs of importance to the pharmaceutical industry - were detectable at low parts-per-billion levels. These signals were reproducible (at least 6 spots each) and the S/N variations were obtained within 15% relative standard deviation before the system was qualified for MSI.
- ◆ Calibrate the mass analyzer as per established protocols prior to the benchmarking and MSI. Integrity of the reference standards and the matrices along with appropriate matrix crystallization effects are vital for obtaining reproducible signals. Prepare fresh samples in case no signals are obtainable. If sub-optimal signals are generated, check and adjust instrumental parameters including the laser alignment, laser energy and the capillary extender-to-plate surface distance.



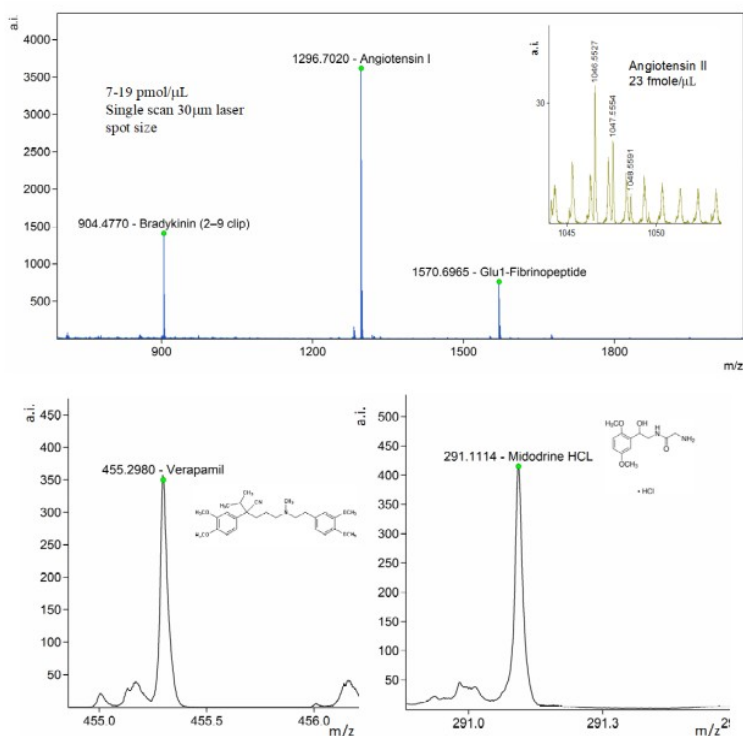


Figure 3 AP MALDI mass spectrum of peptides and small molecule drugs used for benchmarking the signals. Angiotensin II at concentration of 23 fmole/μL was detectable.

3.4. Tissue preparation

1. This protocol assumes fresh frozen tissue samples. Please refer to available literature for using tissue samples prepared differently. For example, see *Ahmed et al. J Clin Pathol (2020) doi:10.1136/jclinpath-2019-205864*
2. Prior to the tissue sectioning, keep the ITO coated slides in cryomicrotome for 15 minutes so that the slides equilibrate. The ITO coated side of the slide could be marked at the edge of the slide with a marker to avoid placing the tissue on the uncoated side.
3. Carefully section the tissue sample to obtain multiple 8-10 μm tissue sections onto the ITO coated slide. Repeat this for all the sample and control tissues, if any.
4. Press below the slide where the tissue is placed using your thumb. This enables uniform fixation of the tissue section on the ITO coated slide. Set aside the slide(s) with the section(s) in the cryomicrotome for 10-15 minutes.
5. Thereafter, place the slides in a slide box and store the tissue sections

at -80 °C. They can alternatively be placed in a cryo-box filled with dry ice, if needed, for transportation between the labs for storage or the sample preparation laboratory and the mass spec analyzer. The sections on the ITO slides must remain at -80 °C until use for MSI.

6. For MSI data acquisition, remove the ITO slide with the tissue section from – 80 °C freezer and keep it in a desiccator for 30 minutes prior to performing subsequent steps.

7. Freshly prepare the matrix solution, CHCA for example as detailed above, in sufficient quantities for uniform spraying.

8. Proceed subsequently to matrix spraying uniformly making sure there is no moisture on the glass slide. Apply a generous amount of matrix to cover the entire area encompassing the tissue section. The slide can be set aside for 3-5 minutes at room temperature after which the matrix coated tissue section is ready for data acquisition.

- ◆ Demarcate an area, say within a fume hood, placing a barrier such as a firmly placed cardboard box to catch spray falling outside of the ITO slide. Covering all nearby surfaces with a surface protector is advisable to avoid the matrix spillage and chemical contamination of surfaces. Practice spray control with DI water or a solvent such as methanol or acetonitrile. Note that there are alternative approaches to matrix deposition and it is advisable to try out and optimize a suitable working protocol.

Table 1: AP-MALDI MS and tandem MS parameters for analysis

AP-MALDI parameters	
Laser energy (%)	50
Laser fire pattern	Constant speed raster motion (flyback)
Laser frequency (Hz)	5000
Spatial resolution (mm)	0.1
Velocity	0.1 mm/sec
MS speed (spectra/seconds)	10
Spectra per pixel	2
MS parameters	
Mass spectrometer	Triple TOF 6600
Scan range (m/z)	100-1500
Ionization mode	Positive
Interface heater temperature (°C)	220
IonSpray voltage floating (V)	4000
Declustering Potential (DP)	70
Acquisition time (min)	Depends on the area of tissue



3.5. AP MALDI MSI

1. Carefully place the matrix-deposited ITO slide with the tissue section onto the AP-MALDI slide adapter. The slide should be fixed firmly using the screws that can be adjusted using a screwdriver.
2. Open the pre-installed Target-Next[®] software tool. The sample plate preview option generates the preview of an entire slide. Approximate time taken to generate a preview is 15 mins. Options on the dialog box of the Target-Next[®] tool can be configured before powering on the source. Choose the appropriate folder location where the data including the spatial information will be saved that is essential for generating 2-D images.
3. Setup the AP MALDI parameters laser frequency as 5000 Hz, laser energy at 85% and firing pattern as constant speed raster motion.
4. Optimize MS acquisition method and measure actual mass spectrometer speed using a small insignificant area of the tissue. Constant injection time is recommended for imaging experiments. Find optimal laser energy for the tissue to be analyzed.
5. Set spatial resolution (MS image pixel size) at 0.1mm, actual mass spectrometer speed in terms of spectra per second (see item 4 above), and desired number of spectra per pixel as 2, and laser energy. Zoom into the area of interest of generated optical preview.
6. Select the tissue area to be analyzed within a boundary of points that form a defined 'polygon'. Prior to starting the data acquisition, review the expected duration of the imaging experiment based on the polygon size and configure acquisition on the mass analyzer accordingly. The imaging area defined by the polygon can be affixed with a name and descriptor in a dialog box. This identifies the imaging area, associated geometry file and the MS data file of the imaged areas by a specific and identifiable nomenclature.
7. Acquisition of the data for the entire area of interest on the tissue section is started using the Target-Next[®] software.
8. Typical parameters to be used for the data acquisition for imaging are shown in Table 1. The m/z range chosen encompasses typical metabolites and lipids of interest. This can be adjusted based on the specific context by a user.
9. Once the imaging data acquisition is completed transfer the MS imaging geometry file and the MS raw data for data processing system. Consult Target-Next[®] manual for additional information.



3.6. MSI data analysis

1. Convert the raw data files (*.wiff) to mzml file format using the ProteoWizard software.
2. Load the mzml MS raw data file and the MS imaging geometry file on MT imzML Converter software to convert to imzML format.
- ◆ Please go through the SCiLS user manual for familiarization of all the modes of data processing and for details of all the features available.
3. Open SCiLS software and load the imzML file on it. SCiLS converts the imzML data into its own file format *.ibd, *.slx and *.sbd.
4. Once the file is opened on SCiLS windows using an “image area view”, “spectrum view” and “data pan” also appear alongside on the screen. Image area view is used to select different types of tissue views. Spectrum view area displays the acquired spectra.
5. Data pan is used to display and manage the content of the dataset, especially for visualization. Selecting a specific peak (m/z) from the spectrum corresponding to an analyte of interest creates a corresponding image. Each m/z receives a single and specific color for representation if individual gradient is selected. Variations in the chosen gradient is a function of the signal intensity of the specific peak of interest and is indicative of its abundance on the tissue section. Multiple analytes represented by their respective m/z values and corresponding abundances can thus be visualized or overlaid as gradients for further comparison and interpretation.
6. AP-MALDI MSI was performed on tissue sections of a mouse brain sample. The steps described in the previous sections were followed for sample preparation, data acquisition and processing. Figure 4 illustrates the images obtained from this process. The total ion chromatogram of the imaged section is shown in the top panel. The subsequent panels showcase the geo-spatial images reconstructed for the matrix used and specific lipids known to be found in tissues with implications in disease contexts. Diverse lipid classes could be detected using the AP-MALDI MS of which the phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs) are shown in the illustrated figure. Differential distribution of unsaturated and saturated of varying alkyl chain lengths can also be clearly seen from the image profiles of their respective m/z values.

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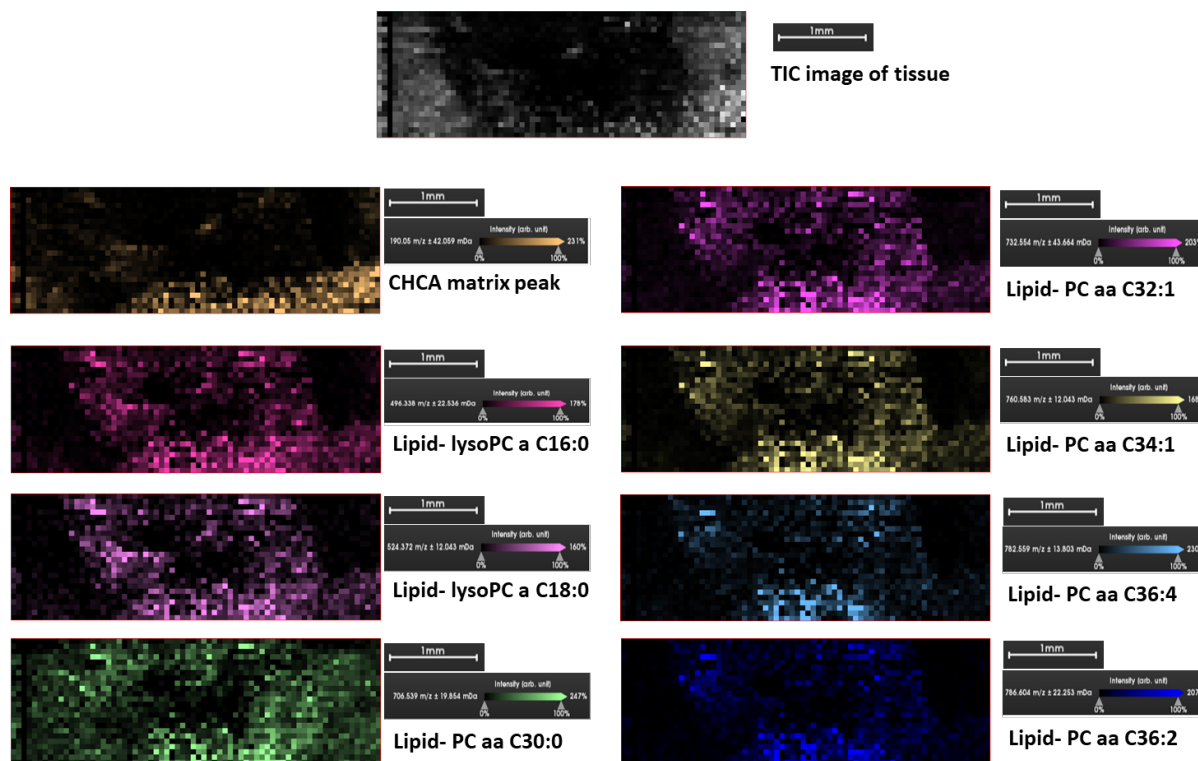


Figure 4 AP MALDI MS based analysis of mouse brain tissue on SCIEX 6600 QTOF



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