



Pushing the boundaries of imaging pharmaceuticals in tissue using MALDI-2

Large scale screening of drugs uncovers the potential of MALDI-2 Imaging to bring pharmaceutical analysis to the next level

Abstract

Spatial analysis of drug distribution presents an opportunity to the pharmaceutical industry for evaluating and understanding the impact of target molecules within tissue. A key method in spatial mapping of small molecules is matrix-assisted laser desorption/ionization mass spectrometry. This technique has found widespread use across many applications due to its versatile application, flexibility, and depth of molecular information. The usefulness of **MALDI Imaging** in the pharmaceutical industry is dependent on the actual ion yield of the target drug in MALDI, and whether those drugs or representative biomarkers are within the limit of detection. When a drug targets specific pathways within the body, efficacy always has an upward trend, improving the intended therapeutic effect. The quantity of drug within the body will naturally decrease over time, owing to metabolism and excretion. Any enhancements that can improve sensitivity are valuable as they allow more accurate detection. The introduction of **MALDI-2 technology**, which utilizes a secondary post-ionization laser, significantly enhances the ionization performance of selected analytes and various molecular classes, including small drug molecules.

Keywords:
MALDI Imaging, MALDI-2,
post-ionization, sensitivity,
pharmaceuticals, drugs,
pharma

Introduction

Matrix-assisted laser desorption/ionization (**MALDI**) **Imaging** has found use in the pharmaceutical industry, particularly for determining drug delivery, distribution, and metabolism [1]. Given the high cost attributed to the development of new drugs, essential information to reduce attrition

of drug candidates is centered around the question "Where has the drug gone?" [2] Naturally, MALDI Imaging provides huge potential to contribute to addressing this question by providing spatial information about drugs and metabolites directly from tissue. MALDI Imaging technology has progressed remarkably in recent years, allowing the imaging of complex biochemical systems at low limits of detection (LOD). Nevertheless, due to a constant increase in drug efficacy, there is a constant need for further improvements in technology and methodology, especially with regard to sensitivity.

In spatially resolved drug studies, any observed boost in signal intensity during a MALDI Imaging experiment can make an important difference towards the usefulness of the technique. In the ideal scenario where increased drug efficacy results in lower concentration doses, increased sensitivity and decreased LOD are often needed to generate meaningful MALDI Imaging data. In these cases, ultimately, improvement in analytical capability can be pivotal for the access to precise and essential knowledge of the spatial distribution of a drug molecule and its metabolites. One way to improve drug detection from model systems when utilizing MALDI Imaging is to incorporate MALDI-2 [3], an ionization method that starts with a conventional MALDI ablation event and then introduces a post-ionization laser event in the resulting MALDI plume. This post-ionization process may increase ion yields for certain analytes drastically, enhancing the performance of traditional MALDI and MALDI Imaging. This unique tool enhances the applicability of MALDI Imaging to drug development, for *in situ* and *ex vivo* studies.

Here, the first systematic study of its kind is presented, evaluating the relative performance of MALDI-2 to conventional MALDI for ~1200 drugs or drug-like compounds spotted on a liver homogenate tissue section. More than 60% of tested compounds showed over a 10-fold enhancement when using MALDI-2, and ~16% showed over a 100-fold enhancement. Such an improvement provides the potential for measurement of drug- and drug candidates in biochemical environments where traditional MALDI would not have generated enough signal, particularly in MALDI Imaging.

Methods

Extensive consideration was given to experimental design to ensure consistent data collection and analysis. This methods section covers these aspects with a high level of detail. Further information can be found here [4] and in the supporting information of the cited text.

Chemicals

2,5-Dihydroxyacetophenone (DHAP) was obtained from Merck (Darmstadt, Germany). Water, methanol (MeOH), and acetonitrile (ACN) were obtained from Carl Roth (Karlsruhe, Germany). The 1196 drug and drug-like compounds were provided from a collection of synthesized or purchased compounds held by GlaxoSmithKline UK (GSK) and then dissolved in MeOH at a concentration of 1 mg/mL. Compounds not soluble in MeOH were dissolved in either ACN or water instead.

Tissue Sections

All animal studies were ethically reviewed and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Directive 2010/63/EU, and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals. A Fast Prep 96 bead homogenizer (MP Biomedicals, VWR International Ltd., Lutterworth, UK) was used to homogenize rat liver tissue, without additional solvent, using stainless steel lysing beads. The resulting liver homogenate was transferred to a 40 x 26 x 13 mm³ aluminum foil-lined megacasset (Leica Biosystems, Milton Keynes, UK) and frozen on dry ice. The block was stored at -80°C until further use. The liver block was mounted to a cryostat chuck using an aqueous solution of 1% (v/v) carboxymethylcellulose and sectioned to a thickness of 16 µm using a cryostat. Two sections were thaw-mounted onto indium tin oxide (ITO) coated glass microscopy slides. 84 slides were used in total, with 60 discrete compounds applied to each slide using a pipetting robot (Cybio Felix, Jena Analytik UK, London, UK). For each target molecule, 1 µL of the 1 mg/mL stock solution was deposited onto the liver homogenate.

Slides were placed into an [MTP Slide Adapter II](#), as the timsTOF fleX acquisition software, timsControl provides a feature to screen pre-determined wells. Spots were only placed such that they aligned with the predetermined wells to facilitate automated acquisition.

Direct deposition of stock solutions onto the liver homogenate cannot mimic the actual complexity of drug activity, however this technique has previously been optimized to mimic biological complexity compared to direct analysis from a stainless steel MALDI target [5, 6]. It should also be noted that the relatively high concentration compared to the physiological activity of 1 mg/mL was used to ensure drugs were detectable in both modalities.

MALDI Imaging

Samples were brought to room temperature in a desiccator and sprayed with DHAP matrix using standard methodology for MALDI Imaging [4].

Spectra were acquired from m/z 50–2000 in positive and negative ion modes. For MALDI-2, the post-ionization laser delay was set to 10 μ s. Mass calibration was performed using red phosphorus after every new sample and after switching polarities. Laser power optimization was performed independently for MALDI and MALDI-2. For both acquisition settings, further increase in laser power did not lead to an increase in lipid ion signal intensity, suggesting all laser power effect differences between MALDI and MALDI-2 were accounted for.

The built-in automation method in timsControl was used to generate spectra from the spots on the liver homogenate. For each spot, sum spectra were acquired from 100 random positions using 50 laser shots per position within the spot. A different set of random positions was used from the same spot for MALDI and MALDI-2 acquisitions. Endogenous compounds were monitored across all spots to ensure experimental stability.

Data Processing

One sum spectrum per acquisition was exported into mzML format using Compass Software 6.0 (Bruker) and subjected to a single-point recalibration to endogenous tissue components. In positive ion mode, the signal for cholesterol $[M-H_2O+H]^+$ at m/z 369.35158 was used for recalibration, and in negative ion mode the signal for $[PI(38:4)-H]^-$ at m/z 885.54930 was used for recalibration. Expected m/z for the annotations were determined by calculating the neutral compound mass and then $[M]^+$, $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M]$, $[M-H]$, and $[M+Cl]^-$ molecular species. Further, isotope pattern envelopes were calculated based on the chemical composition and theoretical mass resolving power to fit against targets, which had to pass fitting criteria for annotations to be accepted. Further processing details can be found in Soltwisch, *et al.* [4]. Peaks were centroided using the Python package [pyMSpec 0.1.2](#). Further deconvolution of $[M]^{\pm}$ from $[M\pm H]^{\pm}$ was performed when necessary.

Results and Discussion

In a MALDI Imaging experiment, the ability to detect the drug within the LOD is a key metric for the success of an experiment. Often, signal intensities for drug molecules, especially within a complicated biological matrix such as tissue, are comparably low. MALDI-2 addresses this unmet need by mapping the distribution of drugs throughout tissue with increased sensitivity and potentially demonstrating molecular changes in biological pathways through changes in lipids and metabolites. Further, as will be demonstrated, many drug and drug-like molecules cannot be detected without MALDI-2, enabling imaging experiments that are not possible with MALDI alone.

Representative data are shown in Figure 1, with a comparison of the MALDI and MALDI-2 performance. Three drug-like molecules recorded in positive ion mode are shown on the left, and three drug-like molecules recorded in negative ion mode are shown on the right. The intensity scale (y-axis) for MALDI and MALDI-2 results are the same. In all examples provided, varying degrees of MALDI-2 enhancement in signal intensity over MALDI signal are shown, across the mass range $\sim m/z$ 180–500. In evaluating the positive ions, Figure 1A shows a unique feature of MALDI-2, which is the generation of an intense $[M]^{+\bullet}$ radical cation. Sufficient absorptivity of the analyte substance provided, the direct absorption of two photons from the MALDI-2 laser can lead to a direct photoionization, resulting in the creation of the radical cation. The radical cation is rarely observed without MALDI-2, and in all cases presented here, the protonated ion and radical cation are strongly enhanced using MALDI-2.

Figure 1b shows a simple enhancement of the parent $[M+H]^+$ ion. Another example of radical cation production leading to enhancement can be seen in Figure 1C. In Figure 1D, the $[M-H]^-$ ion of the target is undetectable by the isotope envelope peak-picking algorithm when ionized with MALDI but is readily detected with MALDI-2. Similar to the positive ion mode production of radical anions in negative mode can be seen in Figure 1E-F, though the mechanism for production of negative radical anions is less well understood. While signal intensities for all presented compounds increase, it is worth observing, that molecules shown in Figure 1C and 1D can only be identified using MALDI-2 based on their isotopic envelope. Consequently, only the use of MALDI-2 would uniquely enable the spatial analysis of these compounds.

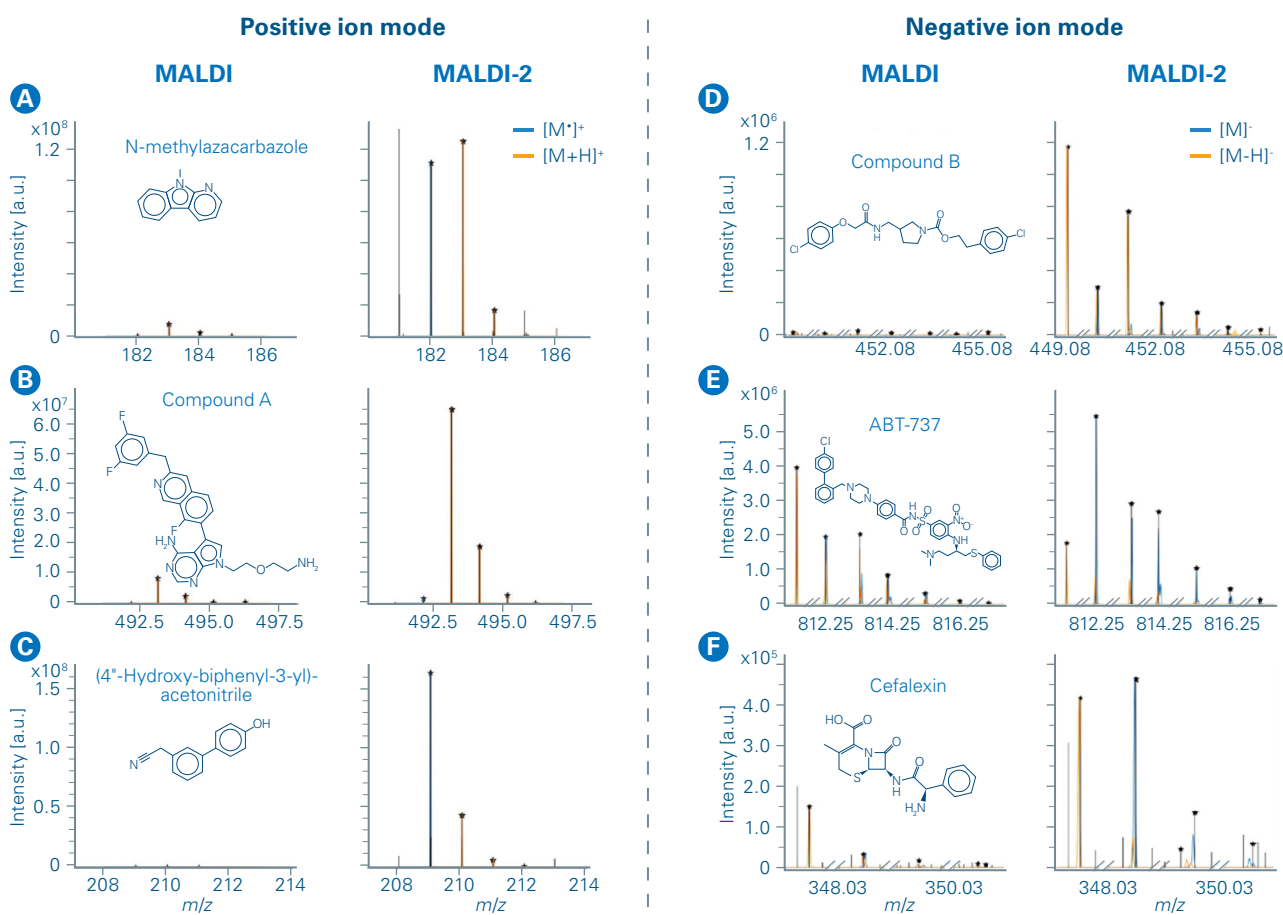


Figure 1. Comparison of centroided mass spectra of six compounds using MALDI and MALDI-2. In positive ion mode examples for (A) N-methylazacarbazole, (B) Compound A and (C) (4'-Hydroxy-biphenyl-3-yl)-acetonitrile are shown, the three negative ion mode examples contain (D) compound B, (E) ABT-737 and (F) cefalexin.

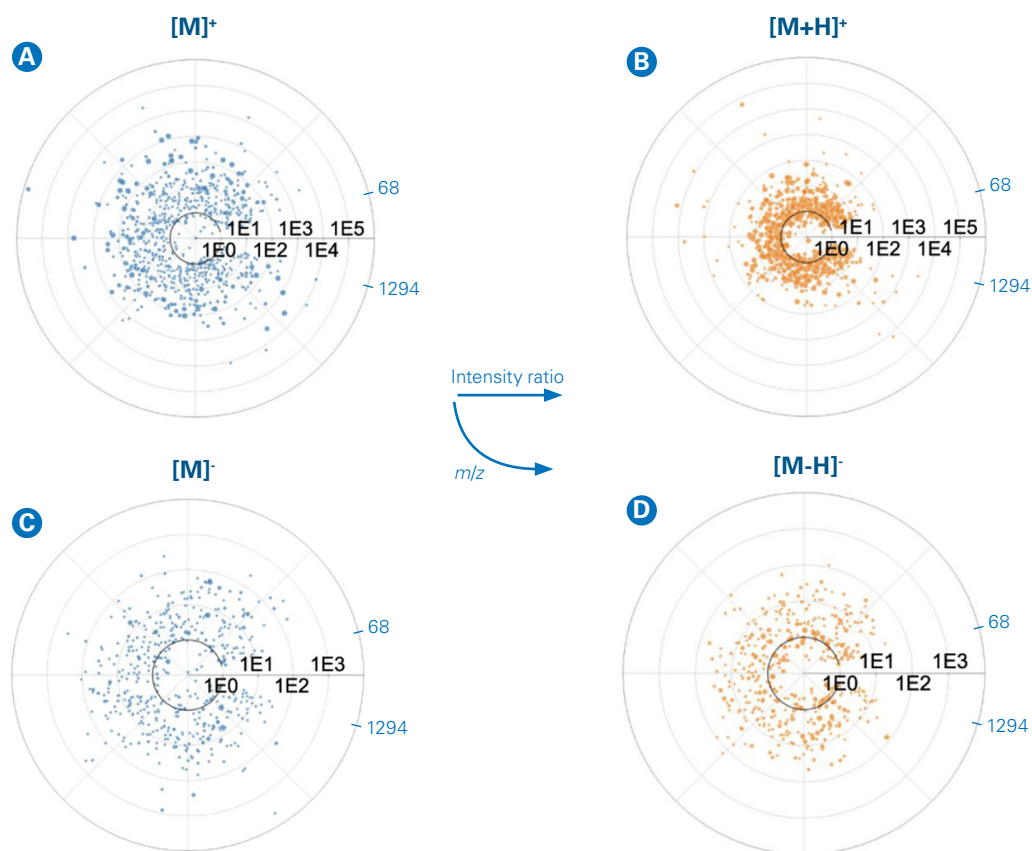


Figure 2. Radial plots of the intensity ratios of MALDI-2/MALDI signals for the ion types of **(A)** [M]⁺, **(B)** [M+H]⁺, **(C)** [M]⁻, and **(D)** [M-H]⁻. Note the lowest and highest *m/z* of 68 and 1294, respectively. The size of the respective markings in the circular axis of the plot corresponds to the signal intensity of the individual peaks.

Similar degrees of enhancement were recorded across many other species tested in this study, with some drug-like compounds showing over a 100,000-fold increase in signal intensity. The general results are summarized in both Figure 2 and Table 1. The data shown in Figure 2 represents the ratio of molecular ion signal intensity in MALDI-2 to the molecular ion signal intensity in MALDI depicted for both positive and negative ion modes. The data reveals meaningful enhancement to both molecular and protonated ions. This is complemented by Table 1, which summarizes percentages of compounds that exhibit a sizeable enhancement, sorted by orders of magnitude. Nearly 60% of analytes studied here show at least a 10-fold increase in signal intensity.

As shown in Table 1, the enhancement achieved for MALDI-2 will vary depending on the analyte, with some analytes benefiting from a 100,000-fold increase in signal and some showing no enhancement at all. Recently, a follow-up study introduced a machine learning-based prediction tool to estimate ionization efficiency for MALDI and MALDI-2 based on molecular structure [7]. This approach helps researchers anticipate whether MALDI-2 will provide a significant benefit for a given compound, reducing trial-and-error and further increasing the practical utility of MALDI-2 in pharmaceutical studies.

Table 1. Number of compounds at various degrees of signal enhancement by using MALDI-2.

Factor	Positive ion mode (841 = 100%)	Negative ion mode (486 = 100%)	Both ion modes (320 = 100%)	Either ion mode (869 = 100 %)
1	799 (95%)	437 (89.9%)	267 (84.3%)	863 (96.3%)
10	427 (50.5%)	171 (35.2%)	36 (11.2%)	510 (56.9%)
100	127 (15.1%)	23 (4.7%)	5 (1.6 %)	145 (16.2%)
1000	34 (4%)	1 (0.2%)	1 (0.3%)	34 (3.8%)
10000	10 (1.2%)	0	0	10 (1.1%)
100000	1 (0.1%)	0	0	1 (0.1%)

Conclusion

- MALDI Imaging is a flexible and established method that provides spatial information on pharmacological and biologically relevant compounds from biological matrices such as tissue.
- MALDI-2 has the potential to improve the ionization efficiency of selected analytes (60% of the ~1200 analytes studied here), and up to a staggering 5 orders of magnitude, allowing researchers to enhance the study of physiologically relevant concentrations of drugs in tissue.
- MALDI-2 provides enhancement across both polarities, though the most significant improvement is seen in acquiring positively charged ions.
- There are selected cases, in which MALDI-2 does not enhance signal intensity. In these cases, increased background signal intensity can effectively reduce the LOD. Consequently, a combination of MALDI and MALDI-2 acquisitions will provide the best coverage and selectivity when analyzing all molecules from tissue.
- Recent work has demonstrated the feasibility of such prediction tools using machine learning, enabling users to estimate ionization behavior and select optimal conditions for MALDI or MALDI-2. This advancement will make MALDI Imaging more accessible and efficient for non-experts and accelerate decision-making in drug development.

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