LipostarMSI

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LipostarMSI: Comprehensive, Vendor-Neutral Software for Visualization, Data Analysis, and Automated Molecular Identification in Mass Spectrometry Imaging

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Supporting Information

ABSTRACT: Mass Spectrometry Imaging (MSI) is an established and powerful MS technique that enables molecular mapping of tissues and cells finding widespread applications in academic, medical, and pharmaceutical industries. As both the applications and MSI technology have undergone rapid growth and improvement, the challenges associated both with analyzing large datasets and identifying the many detected molecular species have become apparent. The lack of readily available and comprehensive software covering all necessary data analysis steps has further compounded this challenge. To address this issue we developed LipostarMSI, comprehensive and vendor-neutral software for targeted and untargeted MSI data analysis. Through user-friendly implementation of image visualization and co-registration, univariate and multivariate image and spectral analysis, and for the first time, advanced lipid, metabolite, and drug metabolite (MetID) automated identification, LipostarMSI effectively streamlines biochemical interpretation of the data. Here, we introduce LipostarMSI and case studies demonstrating the versatility and many capabilities of the software.

KEYWORDS: mass spectrometry imaging, bioinformatics, metabolomics, lipidomics, chemometrics

INTRODUCTION

Mass spectrometry imaging (MSI) provides a powerful and increasingly utilized tool to study both the chemical compositions and spatial distributions of complex samples, most commonly biological tissues.^{1–4} MSI is often applied to study the alterations in endogenous chemical compositions between different tissue types as well as health and disease states.^{5–10} Studying diseased tissues via MSI enables direct visualization of the underlying region-dependent biochemical changes induced by disease. It also enables molecular pathology where tissues are annotated based on spatially-dependent mass spectrometry signatures. This can allow for the identification of chemically distinct regions of tissue that may not be identified with classical histology.^{11–16} In the pharmaceutical industry the main advantages of MSI over imaging techniques used to track drug distributions, i.e., whole-body autoradiography (WBA¹⁷) and microautoradiography (MARG¹⁸), is that with MSI there is no need to radiolabel

compounds, a process that hinders discrimination of drugs and metabolites.¹⁹ As such, MSI is also taking a larger role in the drug discovery pipeline, usually to localize drug and metabolites in tissues, organs or cells, revealing possible toxic effects in the early stage of drug development.^{20–23}

Depending on the scientific question, the nature of the sample, the targeted analyte class, and the spatial resolution required, different desorption/ionization techniques are utilized for MSI with matrix-assisted laser desorption/ionization (MALDI⁹), desorption electrospray ionization,²⁴ and secondary ion mass spectrometry²⁵ being the most popular. Increasingly, these and other ion sources are being coupled with high mass resolving power analyzers to facilitate

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both the resolution of isobaric ion species and their identification via accurate m/z measurements.^{26–29} Alternatively, high-throughput MALDI-time-of-flight systems are being leveraged for rapid tissue classification. Both approaches play a central role in modern MSI for biological tissues as they access many molecular families including metabolites,³⁰ lipids,⁷ peptides,³¹ and glycans.³² Of these, lipids are arguably the most popular targeted analyte, due to a combination of their central roles in many biological processes, the relative ease of detection of several important lipid classes and their simple sample preparation requirements (i.e., no tissue washing or enzymatic digestion is required). Detecting localized changes in lipid composition shows great promise for helping elucidate the molecular mechanisms of disease and providing biological fingerprints that can account for adverse effects (toxicities), therapeutic treatments, and potential biomarkers discovery.^{12,33–37}

A core challenge of MSI is the vast amount of data it generates, with routine data sizes for single tissue sections in the GB range (up to 100's of GB for the raw spectral data generated by Fourier-transform-based methods). Such large, multidimensional datasets require significant run-time and memory efficient processing. Further, MSI datasets can suffer from pixel-to-pixel spectral variations, requiring spatially aware pre-processing (e.g., smoothing, denoising); they are biochemically complex and analytically challenging, sometimes showing spectral artefacts introduced during sample preparation (e.g., hotspots) which can alter and compromise data analysis; and they can contain information about the identity of the detected molecules that must be extracted for molecular annotation and biological interpretation.³⁸ Further compounding these challenges is a lack of comprehensive software solutions that cover the entire workflow needed for a complete MSI data analysis. As excellently summarized by Alexandrov,³⁸ a general pipeline MSI data processing comprises five general steps: (1) raw data import; (2) pre-processing; (3) data mining; (4) visualization; and (5) identification. In recent years, a plethora of hardware and vendor-dependent (flexImaging, Bruker; ImageQuest, Thermo Fisher; High Definition Imaging Software, Waters; Imaging MS Solution, Shimadzu) or vendor-neutral (e.g., SCiLS Lab, Bruker; MALDIVision, Premier Biosoft; msIQuant;³⁹ MSiReader;⁴⁰ METASPACE;^{41,42} MassImager⁴³ among others) bioinformatics solutions for MSI have been developed. In addition to these, popular developing and computing environments like R or MATLAB offer MSI data analysis toolboxes.^{44,45} Unfortunately, none of the solutions above cover the entire MSI data analysis and interpretation pipeline yet (Table 1). Arguably, the problem is not the lack of tools for MSI data analysis but the exact opposite: there are many, each one addressing only a partial need of the overall analysis and, taking into account the computational challenges already discussed, even importing the dataset in two or more different environments can be time-consuming, frustrating, and ultimately prevent the full interpretation and exploitation of an MSI analysis.

To address this issue, and building on prior solution developed for the LC–MS community,⁴⁶ we developed LipostarMSI, a comprehensive, vendor-neutral software for MSI that supports both untargeted and targeted data analysis. The workflow supported by LipostarMSI (Figure 1) covers all the steps required for MSI data analysis: raw data processing, histological co-registration, manual drawing, or importing of regions annotated by pathologists, image visualization, a variety

Table 1. Popular MSI Standalone Software and Platform Comparison Based on the General Pipeline MSI Data Processing^{38, a}

	VN	PP	A	ID	MetID	VP	SA
flexImaging		✓				✓	
SCiLS	✓ ^b	✓				✓	✓
ImageQuest		✓				✓	
HDI		✓				✓	✓
MALDIVision	✓	✓				✓	
BioMap	✓	✓				✓	
METASPACE ^d	✓	✓	✓			✓	
msI-Quant ^c	✓	✓				✓	
MSiReader	✓	✓	✓ ^e			✓	✓
MassImager	✓	✓				✓	✓
LipostarMSI	✓	✓	✓	✓	✓	✓	✓

^aVN: vendor neutral; PP: pre-processing; A: accurate mass annotation; ID: MS/MS identification; MetID: prediction and annotation of drug metabolism products; VE: visual processing and image analysis; SA: supervised or unsupervised statistical analysis.

^bExtension for importing imzML files available. ^cSpecifically designed for quantification. ^dFree interaction with dataset not possible.

^eLimited to annotation of putative peaks from user database.

Figure 1. Workflow possibilities in LipostarMSI.

of univariate and multivariate image and spectral analysis tools, and co-localization analysis. For the first time, such steps are also integrated with automated lipid (based on both high mass accuracy and MS/MS data) identification and drug/metabolite annotation, thereby greatly streamlining biochemical interpretation of the data. Finally, given the computational complexity of such datasets and bearing in mind the needs of pharmaceutical and medical applications, special emphasis was given to the optimization of the calculation performance. To this end, all of the algorithms have been optimized and

parallelized to be able to fully exploit the features of the most recent multicore hardware architectures.

In the next sections, we describe how pre-processing, data mining, identification, and visualization can be performed in LipostarMSI. Additional details can be found in the [Supporting Information](#). Finally, with the aim of demonstrating the versatility and the many capabilities of the proposed tool, two real-world case studies are reported for both metabolomic and lipidomic MSI.

■ WORKFLOW AND METHODS

A summary of possible LipostarMSI workflows is shown in [Figure 1](#). Further details are described below and in the [Supporting Information](#).

Raw Data Import. In order to facilitate the exchange and processing of MSI data, the MSI community has agreed to use a general data format, namely the imzML format.⁴⁷ LipostarMSI was designed to directly read the imzML format. Nevertheless, native Bruker Solarix FT-ICR and Waters data can be imported. A dedicated mzML-to-imzML converter is also included with the software to convert Thermo Orbitrap data acquired with a Spectrograph MALDI/ESI injector ion source. This is compatible with both conventional MSI data and for data acquired using the DDA-imaging method.⁴⁸ In the latter case, LipostarMSI will also extract all MS/MS data to be used in later lipid identification methods. Additionally, MS/MS data acquired in a second experiment (either as raw data from the major LC-MS vendors⁴⁶ or manually acquired MS/MS from Bruker Solarix FT-ICR) can be automatically incorporated to aid in identifications of MSI-observed ions.

Pre-processing. As widely reported elsewhere,^{38,49} MSI pre-processing requires different algorithms to be executed. In particular, profile spectra require baseline correction, spectrum smoothing, and peak picking. For profile data, the baseline is corrected by using a linear interpolation algorithm, followed by smoothing with the popular Savitzky-Golay algorithm.⁵⁰ Then, a local maxima peak picking algorithm⁵¹ is executed, while filtering out peaks below a minimum signal-to-noise ratio, absolute intensity, and/or percentage intensity with respect to the base peak values, as set by the user ([SI Figures S1.1 and S1.2](#)). For centroid data, or after the peak picking is completed, alignment, isotope clustering and, if needed, mass recalibration and normalization steps can be performed. Similarly to what is described for Lipostar⁴⁶ and other pre-processing workflows,³⁹ pixel spectra must be aligned to form an initial data matrix, where rows represent the pixels and columns represent the m/z entries; m/z tolerance for the alignment process is tunable by the user to handle data acquired at differing mass resolving powers ([SI Figures S1.1 and S1.3](#)). If needed, both linear and non-linear correction functions for spectra recalibration ([SI Figure S1.3](#)) can be applied prior to the alignment.

With the aim of distinguishing real, informative compounds from noise or redundant information, after the alignment several parameters can be adjusted for a preliminary, although not compulsory, data reduction. For instance, a columnwise search for isotopic patterns can be performed. Also co-localization among the m/z images of the isotopic pattern is taken into account for the isotopic clustering ([SI section S1](#)). Additionally, peaks that appear under a given percentage of pixels and/or absolute intensity can be discarded. At this stage, also a minimum spatial chaos can be set to evaluate the spatial information within each detected peak, retaining only the most

structured ones, although the ideal value is dependent on the tissue under investigation and on the scientific question.^{52,53} The effect of the spatial chaos filter on two different datasets is provided in [SI Figure S1.4](#).

Once the dataset is created, normalization can be used to pre-process the data before visualization or statistical analysis. A number of normalization methods are available: from the widely used total ion chromatogram (TIC), to root-mean-square (RMS), or a pre-specified m/z value (see [SI section S2](#)).

All of the above-mentioned data processing parameters can be adjusted based on the instrumentation and acquisition mode used; nevertheless, an “MSI file preview” inspection tool is also available to quickly evaluate the effect of the different parameters and guide the user toward the definition of optimal importing settings.

Data Mining. Statistical analysis is a key step to mine complex MSI datasets. Individual or combinations of m/z values that vary in relative abundance from pixel to pixel, or from sample to sample, can be easily revealed using unsupervised or supervised multivariate statistical procedures. In LipostarMSI, two possible data analysis scenarios apply: the first in which the sample(s) is analyzed without any prior knowledge (exploratory data analysis, EDA) and the second in which, under the guidance of histology, tissue features or “regions-of-interest” (ROIs) are defined and considered for further analysis. For EDA, spatial segmentation,^{54,55} principal component analysis (PCA^{56,57}), and co-localization⁵⁸ algorithms are available. Clustering in the spatial segmentation can be based on the popular k-means (requiring the number of clusters to be specified a priori) or the efficient iterative clustering algorithm bisecting k-means.^{59,60} PCA is a fast, well-known, and widely-used component analysis,^{38,61} which results in score images and corresponding loading images that can be automatically extracted for inspection. Co-localization can be performed on a cluster obtained either from segmentation or to a specific ion density image: the output will be a list of m/z values showing a spatial distribution similar (where similarity is quantified by a Pearson correlation coefficient) to the input. The same multivariate data analysis tools and plots already presented for Lipostar⁴⁶ are available for ROIs analysis. Following histology interpretation and co-registration of histologically annotated images, specific ROIs can be created and processed as single objects in multivariate statistical analyses. ROIs can be manually created or automatically imported from software used for annotation by expert pathologists (e.g., Panoramic Viewer-3DHISTECH, Budapest, Hungary).⁶² Ion signals that emerge as important from the statistical analysis can be visually inspected by automatically creating preferred lists of the relative ion distribution images.

Visualization. Visualization of ion intensity distributions is the core of an MSI data analysis. In LipostarMSI, multiple images (e.g., high-resolution histological or optical images) can be co-registered to the MS images in a semi-automatic fashion to enhance interpretation. A number of image visualization processes are available to optimize this crucial step. Normalization, smoothing, denoising, and hotspot⁶³ removal algorithms can be applied to the visualization for further statistical analysis ([SI section S2](#)). The impact of such image-processing algorithms can be significant,^{63–65} so when developing LipostarMSI emphasis was given to allow the users to evaluate their effect in real-time. Finally, transparency and additive color scales can be adjusted to enhance visualization of both single

Figure 2. Example data analysis pipeline for metabolite MSI of rat kidney tissue. (a) Bisecting k-means segmentation is performed and enables differentiation of medulla (red) and cortex (blue). (b) Optical image of the analyzed tissue section following MSI acquisition and stained with H&E. The medulla region is annotated with a white circle. (c) Regions-of-interest (ROIs) are defined using both annotated regions from stained tissues (ROI1, medulla) and from bisecting k-means segmentation shown (ROI2, cortex/outer stripe). To aid visualization of the regions, ion images for m/z 279.0391 (blue) and m/z 215.0330 (red) are also displayed. From the regions a variety of univariate and multivariate analyses can be performed for both inter- and intrasample comparisons. (d) Peak area for an ion at m/z 215.0330 within ROI1 and ROI2. This ion is automatically identified as the $[M + Cl]^-$ ion of an analyte with molecular formula $C_6H_{12}O_6$, most likely a monosaccharide. Finally, automatic identification of ion signals that co-localize with ROIs (e, f) and m/z values (g) is performed. All images are normalized to the total ion current on a 0–100% intensity scale.

and multiple m/z values as overlay images.⁶⁶ All of the images can be exported in publication-ready quality or added to a report for future inspection and/or communication.

Identification. A critical step toward the interpretation of the observed ion distributions is the structural identification of the many detected molecules. High-mass-resolution MSI is widely used to assign elemental compositions and sum-composition identifications. Nevertheless, accurate mass is often insufficient to uniquely identify a certain molecule or class of molecules;^{48,67} for robust molecular identification MS/MS is also needed.^{68–70} For instance, MS/MS of lipids can provide identification of individual fatty acyl chains and confidently identify lipid class (i.e., identification to the molecular lipid species level). Therefore, for datasets lacking MS/MS, LipostarMSI includes exact mass searching that can be automatically performed for lipids (LIPID MAPS database supported), metabolites (HMDB database supported), drug metabolites (full integration with MetaSite^{71,72} and MassMetaSite,⁷³ Molecular Discovery Ltd.), and any molecule of interest since in-house databases can also be imported. When MS/MS are available, the identification workflow is analogous to the one described for Lipostar⁴⁶ with the only exception that here the identification score is also weighted on the spatial chaos and spatial co-localization of theoretical isotopic pattern (SI Figure S3.1). MS/MS data can either be from the same MSI experiment (e.g., using DDA-imaging), or can be incorporated from a second experiment (either via LC–MS/MS or manually acquired MS/MS). This flexibility is given by the *DB manager*,⁴⁶ a complementary tool to LipostarMSI,

which allows importing and handling of different database formats, generating MS/MS fragment databases (automatically or by proprietary rules of fragmentation), as well as importing raw LC–MS spectra of standards to be used to identify peaks in MSI experiments (SI section S3). Tentative identifications can also be narrowed by assigning certain MS/MS fragments as “mandatory” (SI section S3) for a positive match. Noteworthy, identifications can be approved or recalled based on newly integrated data. As, by default, lipid identification is based on LIPID MAPS databases, caution should be exercised as the LIPID MAPS nomenclature system identifies lipids to a higher level than often supported by the data (e.g., identification of acyl chain and double bond positioning as well as double bond geometry). In this regard the user should factor in recommended guidelines for final reporting of identified species.⁶⁷ Finally, all of the experimental information available (i.e., multiple adducts fragmentation) can be merged in order to minimize false discovery rates, a long-debated issue in MSI lipidomics (SI section S3).^{41,74,75} These identification features in particular make LipostarMSI unique, as it is currently only MSI software capable of automatically supporting lipid identifications with MS/MS data.

■ CASE STUDIES

Data-Mining and Metabolite Identification. Negative-ion mode metabolite data acquired from rat kidney tissue was used to demonstrate several image and spectral analysis features of LipostarMSI. Data import settings are provided in SI section S4. Using a peaklist containing the 100 peaks having

the highest spatial chaos scores, segmentation was performed via bisecting k-means with a radius of three pixels⁷⁶ (Figure 2a). Different clusters are indicated with different colors with the red and blue clusters highlighting the medulla and cortex/outer stripe regions, respectively. This segmentation is interactive until a set depth, allowing the user to explore the different levels of the segmentation. Region annotation can also be performed on histologically stained tissue (Figure 2b) and either manually or automatically (following optical image co-registration) transferred to the MSI data. To compare the mass spectrometry profiles of different tissue, ROIs can be created through a variety of processes, including by manually drawing of the regions, saving a given cluster from the segmentation as an ROI, or via the import of histological annotations as already discussed. Figure 2c shows two ROIs on the MSI dataset along with the overlaid ion images of m/z 215.0330 (red) and 279.03941 (blue). ROI1 corresponds to the medulla and was defined manually following visual correlation with the H&E annotation. ROI2 was automatically defined from the blue cluster shown in Figure 2a and displays higher intensity to the cortex and outer stripe regions.

Ion abundances within different ROIs can be quickly compared to reveal region-dependent signals. For example, in this case study the ion at m/z 215.0330 was revealed to be ~2.5-fold higher in the medulla than cortex/outer stripe regions (Figure 2d). The next analysis step is to reveal tentative identifications of the observed metabolite signals to enable biological interpretation. Metabolite identification is performed using the publicly available HMDB database and accurate m/z matching within user set tolerances (SI section 4), while combination with other metrics such as isotope pattern matching and spatial chaos can be used to help filter the identifications. It should be emphasized that as identification is based on m/z values observed in the MS¹ data, the identification output often provides multiple isomeric and/or isobaric molecules that cannot be distinguished in this analysis alone. Limiting the search space to an m/z tolerance of ± 3 ppm and only $[M - H]^-/[M + Cl]^-$ ions, 307 m/z values returned potential identifications across the m/z 50–500 range. For example, the ion at m/z 215.0330 in Figure 2d was identified as the $[M + Cl]^-$ ion for a number of isomers having the elemental formula $C_6H_{12}O_6$, suggesting this ion arises from a number of possible monosaccharide isomers. This is consistent with earlier observations demonstrating the detection of $[M + Cl]^-$ ions of glucose using the same NEDC matrix.⁷⁷ Of the 307, 26 yielded isotopic pattern scores >80 (many of the peaks correspond to low abundance ions with no detectable isotopes). The full list of identified compounds (limited to maximum 10 unique compounds per m/z) is provided in SI Table S1. This list of m/z values with tentative identifications can be used to create a preferred m/z list to be used for later statistical or image analysis. LipostarMSI also enables rapid identification of ions co-localized to ROIs or m/z values. Figure 2e shows selected species and associated Pearson correlation coefficients found to be co-localized with ROI1 (inner medulla, Figure 2e), ROI2 (cortex/outer stripe, Figure 2f), and m/z 203.1040 (outer stripe, Figure 2g).

Automated Lipid Identification from DDA-Imaging Data. Negative-ion mode data acquired from rat liver tissues was used to evaluate the automated lipid identification abilities of LipostarMSI. Descriptions of the data import and identification parameters are provided in the SI section S4 and Figures S4.1 and S4.2. Briefly, identifications were

supported by both ion trap MS/MS spectra and detection of the precursor ion in the MS data within a 3 ppm tolerance. The publicly available lipid database from LIPID MAPS was used (as the SDF format file) to generate lipid fragments using the proprietary default fragmentation rule set, and further modified to include 22:5 fatty acyl chains using the *DB manager*. Figure 3 shows the summary of unique sum-

Figure 3. Number of deprotonated sum-composition species identified for each lipid sub-class from a rat liver section. Identifications are supported by both high mass accuracy and MS/MS. Several annotated MS/MS spectra are provided in Figure 4.

composition lipid species identified across a variety of sub-classes. The identification data, obtained in only a few seconds, is interactive and allows users to evaluate each identification, view the assigned MS¹ and MS/MS peaks and approve/disapprove as needed (Figure S3.1). We automatically approved all identification with LipostarMSI score >60 (see SI section S3 for further details), giving 95 identifications. Following further manual interrogation six of these were manually disapproved as they corresponded to very low abundance precursor ions assigned to contain unusual fatty acyls (e.g., 17:1) that were not observed in the MS/MS data (only the complementary acyl chain was observed leading to ambiguity given the low resolution MS/MS spectra). This interactive approach enables users to freely interrogate their data and approve only the identification matches wanted. Secondly, some species were identified as both phosphatidylglycerol (PG) and monoacylglycerophosphomonoacylglycerol (LBPA) species. These isomeric lipids cannot be distinguished with MS/MS, and thus, only the PG identification was kept, but the presence of isomeric LBPAs cannot be excluded. The acquired MS/MS spectra enabled automated identification of lipid species to the molecular species level. A screenshot of the identification window enabling viewing of both MS¹ and MS/MS assignments for selected peaks is provided in SI Figure S3.1. Information such as the assigned lipid species, peaks used to confirm the assigned lipid, number of fragment matches, and mass accuracy for the precursor are displayed. For example, Figure 4 shows the corresponding MS/MS (a–d), isotopic pattern (e–h), and ion distribution images of four identified lipids representing four different phospholipid classes. Each corresponds to a low intensity precursor ion having relative intensities between 1–4% in the MS¹ spectrum and the supporting MS/MS fragments are indicated with a red line (also indicated in the software). Note due to the low abundance precursor ions isotopologues beyond the $M + 1$ species were not detected above the peak detection thresholds for $[PE(18:0_18:2)-H]^-$ (Figure 4a) and

Figure 4. Extracted MS/MS spectra (left column), MS¹ isotopic pattern of the precursor ions (middle column), and ion distribution images of four selected (1–4% base peak intensity) lipid species identified to the molecular species level. MS/MS fragments supporting each identification are indicated in red and are also indicated when viewing MS/MS identifications in LipostarMSI (SI Figure 3.1). Each corresponds to a low abundance species with MS¹ intensities 1–4% that of the base peak. Unassigned MS/MS peaks arise from co-isolated isomeric and isobaric lipids that also simultaneously undergo collision-induced dissociation. Scale bar indicates relative intensity. Images are generated following total ion current normalization and hotspot removal (quantile thresholding, high quantile = 99%).

[PI(17:0_20:4)-H]⁻ (Figure 4d). Interestingly, several PI lipids containing odd numbered fatty acyl chains were identified. For example, the bottom panels show the identification information and image of [PI(17:0_20:4)-H]⁻ with evidence of the 17:0 fatty acyl chain provided by the fatty acid anion (m/z 269.3), the neutral loss of a 17:0 acyl chain (m/z 601.3) and a $[17:0+C_3H_5O_3P]^-$ fragment at m/z 405.3. Additional odd chain PI lipids namely LPI(17:0), PI(19:0_20:4), PI(17:0_18:2), PI(17:0_18:1), PI(19:0_20:4) were also identified with direct MS/MS evidence of the odd-chain fatty acyls. Odd-chain fatty acid-containing lipids have previously been observed in human liver tissues using MALDI-MSI.⁷⁸ The full list of identified lipids (top five matches for each m/z value) is provided in SI Table 2. Following lipid identification, a peak list can be created containing only identified peaks that can be used for subsequent analyses (e.g., multivariate statistical analysis), greatly improving the ability to correlate MSI distributions and patterns with the molecules driving these MSI observations.

CONCLUSIONS

In this paper, we have presented LipostarMSI, a novel software tailored for comprehensive MSI data analysis. To demonstrate the versatility and many capabilities of the software we also presented two case studies (metabolite imaging from rat kidney and lipid imaging from rat liver tissue).

LipostarMSI has been developed to successfully process imzML data, therefore being instrument- and vendor-neutral, although some vendor specific format converters are also integrated. It has been designed as a comprehensive and user-friendly software handling all the steps required for MSI data analysis, namely: data import, pre-processing, supervised, and unsupervised data mining for inter- and intrasample comparisons, image co-registration, advanced visualization, and automated lipids/metabolite identification, including MS/MS-based lipid identification. MS/MS spectra can either be acquired in parallel with the MSI acquisition or via separate LC–MS/MS experiments, where identifications are then linked with MSI data via precursor ion (MS¹) matching. We note that, with the generation of suitable precursor and fragmentation ion databases in the *DB manager*, the identification routines can be adapted to other molecular classes widely studied in MSI such as tryptic peptides and glycans. This makes LipostarMSI the only integrated software package to-date capable of supporting both comprehensive MSI data analysis and analyte identification.

With the rapid adoption of MSI and the continuing improvements in throughput and spectral quality, advanced software tools are needed to handle increasing data complexity. In this context, LipostarMSI provides a powerful platform enabling researchers to effectively exploit MSI data and the (bio)chemical information contained in MSI data.

MATERIALS AND METHODS

Sample Preparation. Rat liver was obtained from a control group of rats sacrificed for a colonic anastomotic healing study approved by the local Animal Ethics Committee of the Radboud University. Rat kidney was collected under ethical approval by the review board of Maastricht University, the Animal Ethical committee, the Inspection for Animal Welfare (AVD107002016720), and the Dutch Central Committee of Animal experiments. The 12 μm consecutive sections were cryosectioned at -20°C and thaw-mounted onto indium tin oxide (ITO) coated glass slides (4–8 Ω resistance, Delta Technologies, Loveland, TX). Prior to lipid analysis, the sections were coated with 15 layers of Norharmane matrix with a concentration of 7 mg/mL in 2:1 chloroform/methanol (v/v) at 30°C using the TM-Sprayer M3 (HTX Technologies, Carborro, NC). For metabolite analysis, the sections were coated with 7 layers of NEDC matrix with a concentration of 7 mg/mL in 70% methanol (v/v) at 80°C using the same sprayer system. Details on the used materials can be found in SI S3.

Data Acquisition. Data was acquired using a MALDI/ESI Injector (Spectrograph LLC, Kennewick, WA) coupled to either an Orbitrap Elite (lipid imaging) or a Q-Exactive HF (metabolite imaging) mass spectrometer operating in negative ion mode (both from Thermo Fisher Scientific GmbH, Bremen, Germany). Lipid data (MSI and MS/MS) was acquired using the DDA-imaging method as recently described.⁴⁸ Briefly, MS¹ (MSI) data was acquired at a nominal mass resolution of 240,000 (FWHM @ m/z 400) across the m/z 180–2000 range while MS/MS data was acquired in parallel using the ion trap and a normalized collision energy of 40 (manufacturer units). MSI pixel size was 60 μm . Metabolite data was acquired at a nominal resolution of 240,000 (FWHM @ m/z 200) across the m/z 50–500 range and a pixel size of 50 μm .

Data Analysis. All data were analyzed using LipostarMSI. Further details and descriptions of settings used for preprocessing and identification can be found in SI section S4.

Details on the chemicals and histological staining can be found in the SI section S4.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.9b00034>.

The material includes details on software workflows and algorithms, chemicals, histological staining, data import and lipid identification settings (PDF)

Data import settings (XLSX)

Lipid identification settings (XLSX)

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Notes

The authors declare the following competing financial interest(s): S.T., P.T., and G.C. are the developers of

LipostarMSI. The other authors declare no competing financial interest.

LipostarMSI is freely available for evaluation and then reduced fees apply to non-profit research institutions. <https://www.molhorizon.it/software/lipostarmsi/>.

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