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FEATURE ARTICLE

Discussion point: reporting guidelines for mass spectrometry imaging

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Introduction

Mass spectrometry imaging (MSI) uses biomolecular mass spectrometry techniques to simultaneously record the distributions of molecules directly from tissue samples [1] and within their histological context [2]. MSI is now applied in increasingly diverse biomedical and biological applications, from the identification of clinical biomarkers [3], to the label-free quantification of drugs and metabolites [4], to revealing the molecular cartography of plant tissues [5]. The different focus areas have necessarily led to application-specific approaches, but even within the basic MSI experiment there is still much scope for methodological differences that affect the resulting data [6]. A cursory overview of an MSI experiment includes multiple aspects where differences may arise: tissue processing (e.g., embedding and storage conditions), tissue preparation (e.g., sectioning and matrix application), data acquisition (e.g., ionization method, spatial resolution, and mass analyzer), data processing (e.g., mass spectral processing, intensity normalization, and color scale).

If we consider just the ionization method used for data acquisition, MSI data sets have been recorded using laser desorption ionization (LDI) [7], matrix-assisted desorption/ionization using ultraviolet lasers UV-MALDI [1], MALDI using infrared lasers (IR-MALDI) [8], desorption electrospray ionization (DESI) [9], nano-DESI [10], nanostructure-initiator mass spectrometry (NIMS) [11], secondary ion mass spectrometry (SIMS) [12], and laser ablation–inductively coupled plasma mass spectrometry (LA-ICP-MS) [13]. For example UV-MALDI can be performed under high vacuum or at

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intermediate [14] and atmospheric pressure [15]; utilizing different tissue washes, different matrixes, matrix solutions, and different matrix application methods [16]. Importantly the choice of ionization method, mass spectrometer, and tissue preparation procedure all influence which molecules can be detected and with what sensitivity.

To understand an analysis it is crucial that the methodological context of the results is accurately communicated. This is especially true of emerging technologies, which can be characterized by a high degree of diversity prior to the establishment of standardized protocols. Such analytical data should therefore be accompanied by contextualizing 'metadata', explicitly stating the sample origin, sample processing, and how the analyses (including data processing) were performed. To that end the HUPO Proteomics Standards Initiative (www.psidev.info) has developed guidelines specifying the data and metadata that should be collected for various proteomics workflows, known collectively as the "minimum information about a proteomics experiment" (MIAPE) guidelines [17], and which includes guidelines specifically for mass spectrometry based proteomics [18]. In addition the proteomics community has defined common data formats (including mzML for MS data [19]) and established data repositories to allow exchange, evaluation, and reuse of MS data [20].

This contextualization and standardized/established handling of data are sorely needed in the MSI field. A common data format for MSI has already been established (imzML) [21] and a submission procedure for MSI data to a public data repositories has been described [22]. As for reporting guidelines, we have initiated a discussion within the European network COST Action BM1104 concerning the *minimum* information that is necessary to adequately describe an MSI experiment. In this article we describe these reporting guidelines and illustrate them with selected examples. The purpose is to raise this essential issue and to reach a consensus of which information needs to be included when reporting new MSI data/experiments. In order to enable as wide a discussion as possible we have made the working document available on the www.maldi-msi.org website (under forum, General, MSI MIAPE) [23].

The different experimental paradigms, different goals, and different application areas make it difficult to define a single document that is applicable to all MSI experiments. Instead we first describe the elements of the basic MSI experiment, common to all MSI investigations, before proceeding to the clinical and pharmacological application of MSI, arguably the two principal application areas of MSI. These examples also serve as representative templates for reporting new/different applications.

Basic MSI experiment

The reporting guidelines for the basic MSI experiment cover the technical description of tissue sampling, tissue preparation, MSI data acquisition, visualization, and data processing. They are considered to be the minimum reporting requirements to accurately describe an MSI experiment:

1. Tissue sample(s)
   - (A) Origin—institution, ethical approval.
   - (B) Specimen—species, age, sex, organ; if clinical, indicate if resection specimen or needle core biopsy.
   - (C) Sampling method, postmortem time, stabilization (e.g., rapid heating or focused microwave irradiation), freezing method (e.g., flash frozen by slow immersion in liquid nitrogen cooled isopentane), storage conditions, and storage time.
   - (D) Fixed/fresh/embedded—including method.
   - (E) Morphological classification—including, e.g., WHO classification if analyzing pathological tissue, sampling location, and orientation of sample (e.g., for brain tissues). For studies involving tissue samples from multiple individuals and/or comparing tissue samples from different organs then a summary of the tissue series should be included.
2. Tissue preparation
   - (A) (Cryo)microtome model and cutting temperature.
   - (B) Tissue section thickness.
   - (C) Mounting substrate and mounting method (e.g., thaw mounting onto indium tin oxide coated glass slides).
   - (D) Drying method and time.
   - (E) Tissue wash solutions and procedure (if applicable).
   - (F) On-tissue chemistry (enzymatic digestion, derivatization)—provide reagent solutions and procedure if applicable.
   - (G) Matrix application/ionization enhancement—matrix solution, deposition method and device (if applicable). This section includes the addition of thin metal coatings, nanoparticles, graphite, and other ionization enhancement agents.
   - (H) Quantitation method (internal reference standard, adjacent dilution series, reference standard application method)—provide reagents and procedure if applicable.
3. Optical image
   - (A) Optical image of measured region/regions of interest with scale bar.
   - (B) Staining method.
   - (C) Specify if MSI sample or adjacent section was used for optical image.
4. Data acquisition
   - (A) Pixel size.

(B) Mass analyzer type, model, and laser/ionizing beam parameters. For laser beam spot size an estimate can be obtained by ablating a spot through a thin matrix preparation, e.g., formed by electrospray matrix deposition, using the analytical laser settings.
(C) Ion source used for imaging (if separate system, e.g., DESI source).
(D) Software packages—including versions.
(E) Mass range and polarity.

(a) If MS/MS—isolation window and fragmentation method.
(b) Mass resolution setting if FT-type instrument.

(F) Number of shots per pixel or scanning speed (whichever is applicable).
(G) Scanning pattern (random, left-right, top-down, flyback, meandering)—note these are the same terms defined in the imzML controlled vocabulary [24].
(H) Number of laser shots per pixel (including random walk if applicable).
(I) Oversampling—if applicable provide laser/probe spot size. For constant scanning systems please provide scanning speed, laser repetition rate, and binning width (or number of laser shots).
(J) Representative mass spectrum and MS image.
(K) MSI and histology alignment method.

5. MS pre-processing

(A) Software—including version
(B) Spectra pre-processing

(a) Baseline subtraction algorithm and settings
(b) Smoothing algorithm and settings
(c) Alignment, recalibration—if applicable provide algorithm and settings

(C) Intensity normalization method (e.g., total ion count)
(D) Peak-picking—algorithm and settings
(E) MS data reduction method/binning method if applicable

6. MSI visualization

(A) Peak evaluation method (area, peak height, etc.).
(B) Peak selection range (i.e., 'bin size' or 'bin width', in *m*/*z* or ppm).

(a) Centered on measured or theoretical *m*/*z* value (important for high resolution MS).

(C) Intensity scale for each MS image, and which clearly indicates any contrast adjustments made and if the image uses arbitrary intensity (a.i.) or relative intensity (r.i.). In the ToF SIMS community, which is based on ion-counting technologies, it is established convention to report the *maximum ion count* of the image as a proxy estimate of data quality. The much greater range of mass spectrometry technologies used throughout MSI, the fine details of which determine the S/N typically encountered in an experiment, may make any similar parameter highly context dependent (e.g., vacuum linear MALDI instruments used for protein MSI intrinsically have a lower S/N than lipid analysis using DESI on a high resolution Orbitrap instrument).
(D) Dimension scale bar for each MS image.
(E) Spatial/pixelwise interpolation and image smoothing/binning—provide method if applicable.

7. Compound identification

(A) Identification procedure.

(a) *m*/*z* value only (comparison with theoretical *m*/*z*).
(b) MS/MS on-tissue.
(c) Comparison with additional measurements (e.g., LC-MS/MS) of measured sample (same or adjacent section?); if LC-MS specify extraction method.

(B) Mass tolerance used for identification (comparison with theoretical mass (from database), LC-MS, etc.).
(C) Guidelines for performing and reporting the actual LC-MS/MS analyses are available and the reader is kindly referred to these for further information [17].

8. Data analysis

(A) Software package—including version number
(B) Definition/selection of regions of interest based on optical image or MS image (virtual microdissection)
(C) Data analysis algorithm plus parameters

The parameters discussed above are relevant for a wide range of MSI experiments and should be provided (as far as applicable) in any report of MSI data. This first part could serve as a guideline for authors to prepare a manuscript and also for reviewers to evaluate the how well the MSI experiments are described.

The two following examples illustrate the importance of some of these parameters and discuss additional parameters that are relevant to specific applications of MSI. The 'clinical MSI' application serves as an example of a 'mass spectrometrically' untargeted approach, which in general is used to differentiate and characterize different (biological) conditions/states. The 'pharma' application represents a targeted approach in which a known compound (and its metabolites) is imaged and quantified.

Clinical MSI

The clinical application of MSI is highly multidisciplinary often encompassing MSI, histopathology, patient survival, biostatistics, LC-MS/MS-based identification, and independent validation. To date the majority of the work has concerned proteins, in part owing to the widespread commercial availability of MALDI MSI instruments and the ability to use immunohistochemistry for independent validation, but there is a growing body of work concerning clinical lipid analysis [25–27].

As an example of data representation, Fig. 1 shows the histological image and MALDI MS images of a human stomach mucosa tissue section. Figure 1a shows the histological overview of the measured tissue, and clearly communicates the scale and staining method. Figures 1b, c show higher magnification images of specific regions of interest, in this case corresponding to the epithelial cells and glandular cells in the stomach mucosa. Again scale bars are included to ensure that the length scales are clearly communicated to the reader. The MALDI MS images, Fig. 1d–f, show the complementarity and distributions of two small protein ions. The scale bars (parameter 6D) of the images, intensity scales (parameter 6C), *m/z* integration range used to generate images (parameter 6B), and normalization method (parameter 5C) are included. Simple annotations ('M1' and 'M2') are used to indicate the precise origins of the ROI histological images shown in Fig. 1b, c. In this manner the reader can immediately understand how the images were obtained and presented, and place the results in their correct histological context.

The visualization of MS images as shown in Fig. 1 is often only the starting point for further evaluation and analysis of the acquired data. Figure 2 shows examples of the basic data analysis workflow of recently published clinical MSI investigations of proteins, which can be summarized as diagnostic biomarker discovery [28] (A), prognostic biomarker discovery [29] (B), and MSI-based molecular cartography [30] (C). In all examples the MSI data sets are registered to the histological images (the histological images can be recorded from the same section after MSI data acquisition or from adjacent sections). However, in A and B, MSI is subordinated to histology. A histopathological examination is first performed to highlight specific regions of interest. The mass spectra from these regions are then extracted and a representative (mostly average) mass spectrum calculated for each patient tissue and for each histological subtype. In the diagnostic biomarker discovery example this could correspond to tumor-specific mass spectra and those from control tissues. The tumor and control mass spectra may be acquired from different patient tissues, or from different regions of the same patient tissues. Importantly the origin of the tumor and control mass spectra determines which algorithms are appropriate for the subsequent statistical analysis [31]. In the prognostic biomarker discovery example, Fig. 2B, only the spectra from the tumor-specific-mass spectra are extracted and averaged, then statistical tests are applied to identify molecular features associated with patient survival. In both cases MSI is used solely to acquire mass spectra from specific cell types/regions of tissue, i.e., all spatial information from within the selected areas is omitted from the statistical analysis. In contrast in the molecular cartography example, Fig. 2C, MSI is paramount; it is used to identify regions of tissue with distinct molecular signatures, which are then investigated further using microdissection and in-depth proteomics strategies. These analyses may be performed on collections of individual tissue sections, in which each section is defined by its own MSI data set, or a tissue microarray (TMA) in which the entire patient series is contained in one or more blocks [32, 33].

The clinical characteristics are required for the reader to determine how well the patients match. Individual differences are the dominant source of variation in clinical studies, and it is recommended to use a smaller well-matched series than larger but more diverse patient collections. It would be possible to report very poorly a clinical MSI investigation that used patient samples that were identical except for the experimental parameter. Conversely it would also be possible to report very well an investigation with very poorly matched patients. Importantly only with adequate reporting can the readers assess how well they match, and thus correctly contextualize the results and any conclusions drawn.

We propose that the data analysis workflow used for the experiment, as exemplified in Fig. 2, be reported as supplementary information so that the reader can readily understand which data has been recorded, how it has been analyzed, and, importantly, can clearly see the relational aspects between different data types, e.g., histology and MSI.

Figure 3 shows an example of a workflow for a bilateral biomarker discovery study, indicating specific steps in which *ideally* one would strive to use identical criteria and methods in order to match, as much as possible, the MSI experiments in both centers. Dekker et al. recently reported the first fully independent bilateral MSI biomarker discovery experiment [34]; a *carefully defined histology-based biomarker discovery pipeline*, aided by scientific exchange of personnel between both centers, demonstrated that some biomarkers detected by MSI could be robust to changes in MSI methodology. For reproducible results across many sites, in which the scope for methodological differences is much larger, it may be essential to establish standardized methods for each common step of the experiment (see inset of Fig. 3). Part of the motivation for this MSI reporting guidelines discussion paper is to ensure that experiments are reported with sufficient detail so that the results can be compared across different laboratories and reproduced elsewhere. The inclusion of the data analysis workflow is an important step in that it clearly outlines the experimental structure and how the different forms of data interact.

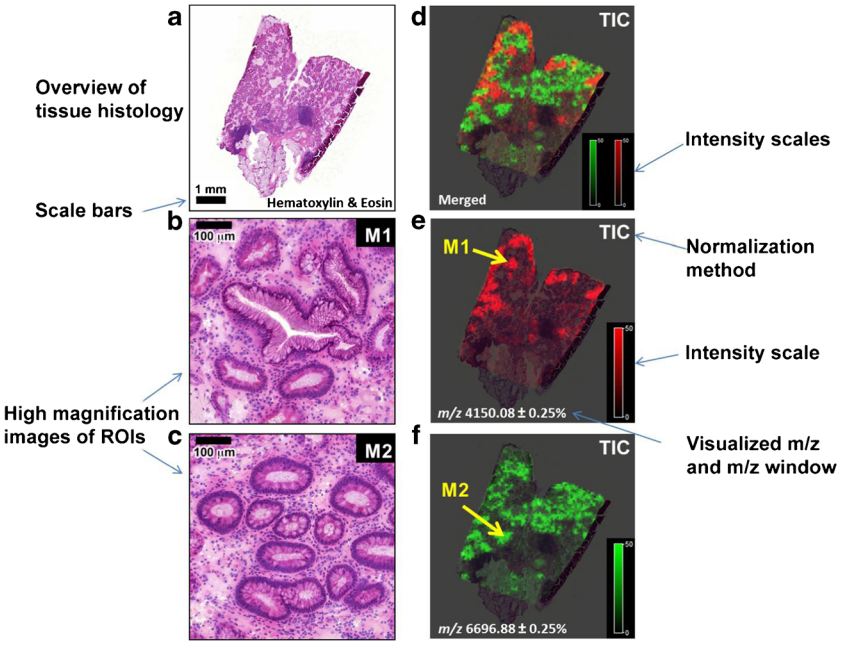

Fig. 1 Representative example of the presentation of an MSI data set. A human stomach mucosa was measured by MALDI MSI at 70-µm pixel size. An overview of the tissue's histology is shown in **a**; the staining method should be provided in the image or in the figure caption as well as a scale bar. Magnifications showing the cellular composition of regions of interest within the tissue should be provided to help explain the differential distributions of mass signals. Here, the magnifications show the presence of epithelial cells (**b**) and glandular cells (**c**) in the stomach mucosa. Visualizations of certain mass signals show precise co-localizations with these cell types (**d**, scale as in **a**). Two of these mass signals are shown more in detail. Whereas *m/z* 4,150.08 showed high correlation with the epithelial cells (**e** and **b**), *m/z* 6,696.88 shows exclusive presence in glandular cells (**f** and **c**). Note the inclusion of the intensity and normalization method in all MS images. MS images were TIC-normalized and are overlain on the optical image without spatial interpolation. Intensity scales are set to 50 % maximum

In addition to the experimental workflow the significant role of histology, clinical data, and statistics in the clinical application of MSI requires the following additional information to be reported for the experiment to be fully understood:

9. Details for clinical studies/samples

   (A) Clinical characteristics of patient/animal series, e.g., UICC classification for cancer [e.g., pTNM, G (differentiation), R (residual tumor)], patient survival

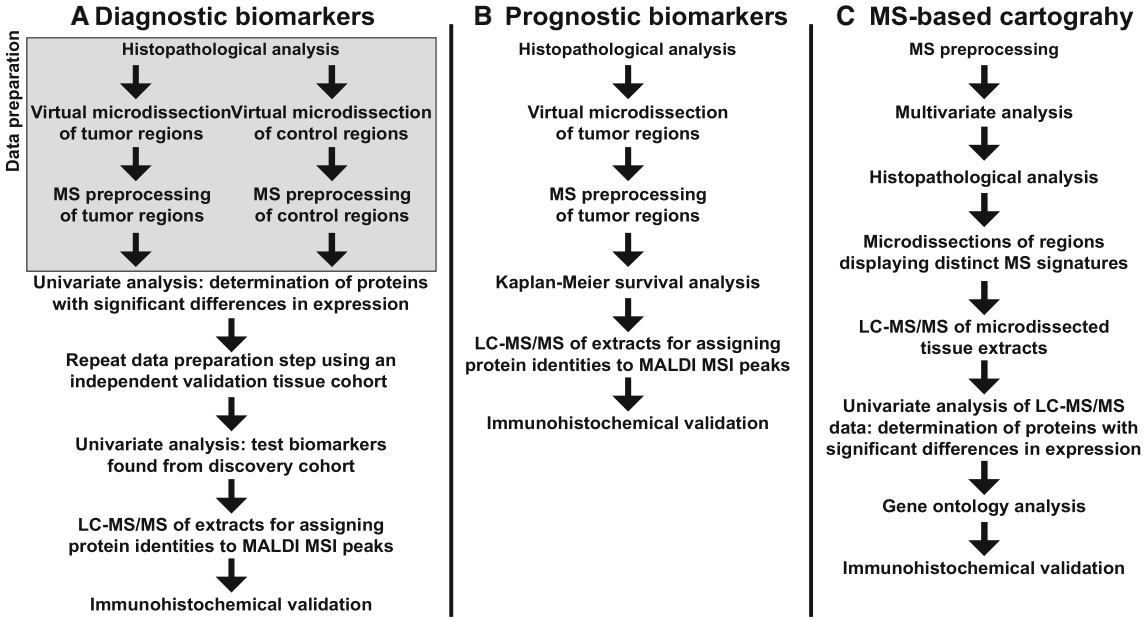

Fig. 2 Examples of data analysis workflows utilized in recent clinical MSI investigations

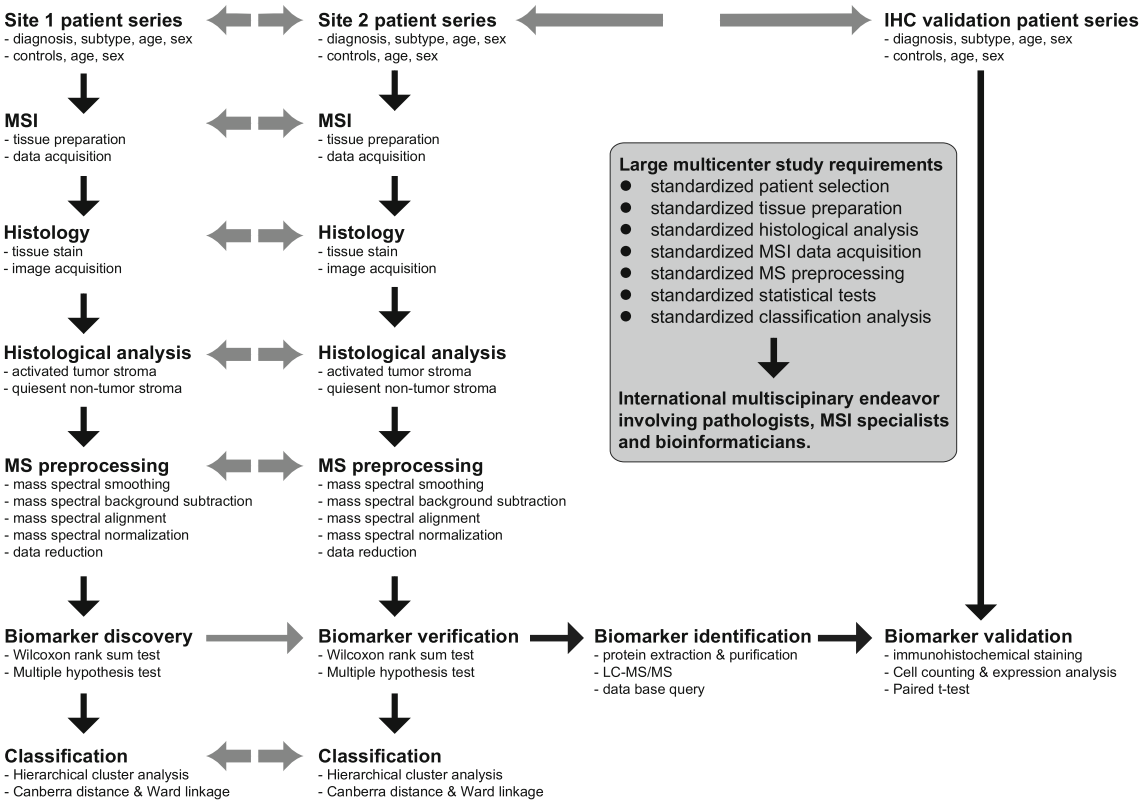

Fig. 3 Example of the workflow of an immunohistochemically validated bilateral study of protein biomarker discovery experiment using MALDI MSI, as used by Dekker et al. for identifying protein biomarkers of stromal activation [34]. The workflow is a variation of the *diagnostic biomarker* data analysis workflow shown in Fig. 2A; in this instance the biomarkers distinguish activated from non-activated stroma within the *same* tissue section, and the experiments were performed in two centers

data, metastasis status, disease-specific subgroups (e.g., ER, PR, and HER2 status for breast cancer)

(B) Histological and MSI images of regions referred to in the manuscript

(C) Statistical analysis

(a) Software package—including version number
(b) Statistical analysis algorithm plus parameters
(c) Data pretreatment if performed (e.g., rank sum normalization)
(d) Statistical significance, confidence intervals, error bars, if applicable

(D) Workflow of data analysis

(E) Validation method if applicable

Pharma

Applications in pharmaceutical research are diverse, including target finding, compound and metabolite distributions [35], and biomarker localization [4, 36]. In common with all these applications is the need to document and report the experiment and its results for it to be understandable and reproducible. While MS imaging data is currently not being used for compound filing, it is applied to drive decisions on compound selection by providing relevant information. A coherent documentation and detailed reporting of MSI data is essential, as it provides the traceability of the decision down to the individual experiment.

In this context it is essential to provide guidelines on the extent of the technical data to report, in order to adequately detail the parameters that are known to affect MSI data, and thus may influence a decision. To provide an example, an image without the addition of an internal reference standard for in situ normalization of the MSI signal may be used to indicate the presence of an analyte at a particular location, but the lack of intensity at another location might just be due to signal suppression and under no circumstances should be interpreted as having low analyte concentration [37]. Figure 4 shows an example of non-normalized MS images (left) in comparison with an image properly normalized to the internal standard (right); it is quickly apparent that the correction of ion suppression provided by the internal reference standard has a significant impact on the observed spatial distributions, and so great care must be taken when interpreting non-normalized MSI data. Källback et al. have compared a number of normalization procedures and demonstrated that the addition of

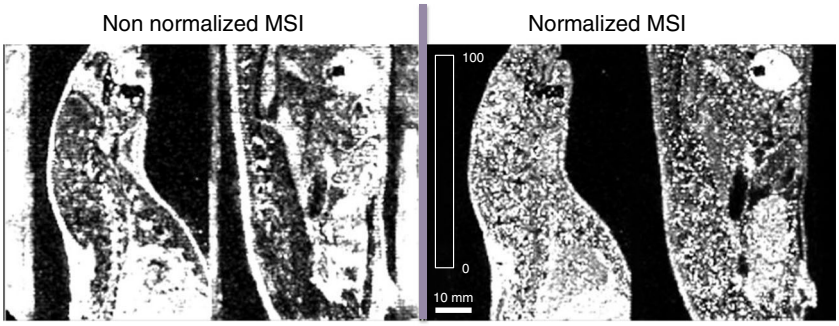

Fig. 4 Non-normalized MS images (*left*) of a compound in whole rat tissue sections after dosing, pixel size 500 μm. Poorly aligned sequential line scans, of a serpentine data acquisition pattern, leads to visible artifacts. Significant MS signals are present outside of the tissue. The images shown on the *right* have been normalized to an internal reference standard (isotopically labeled analogue of the compound uniformly sprayed onto the tissue sections prior to matrix deposition). No MS signals are present outside the tissue. The grainy appearance of the tissue is due to the continuous scan multiple reaction mode (MRM) data acquisition mode, meaning that the ablation location of the compound and its internal reference standard do not match exactly

isotopically labeled reference standards provides the best quantitative performance [38].

The instrument parameters can also have a significant effect on MSI results and therefore need to be provided with the image data. In the example of Fig. 4 the data was acquired in a serpentine measurement pattern (corresponding to parameter 4G) but in which sequential lines were not perfectly aligned, resulting in some visible jitter in the image's structure. The MRM data acquisition mode (corresponding to parameter 4Ea) used to acquire this MSI data set [39] provides a high degree of mass spectral specificity, significantly greater than creating images using the intensity of the precursor ion detected in a TOF analyzer for instance, but the normalized images appear grainy. This is due to the discrete nature of the triple quadrupole instrument used for the experiment. As only a single MS/MS transition can be analyzed at any given moment in time with the employed triple-quad mass spectrometer, the internal reference standard ions are not analyzed from exactly the same position as the compound ions (the laser moved during the dwell time of 50 ms). While the matrix layer in this example was delivered as a relatively dry spray (low flow rate, longer distance to sample), a wet delivery of matrix may provide increased sensitivity (higher flow rate, shorter distance to sample) but can also lead to significant delocalization of analytes. This may be acceptable for a particular experiment but needs to be reported with sufficient information for it to be repeated (sprayer type, flow rate, distances, temperature, solvent composition).

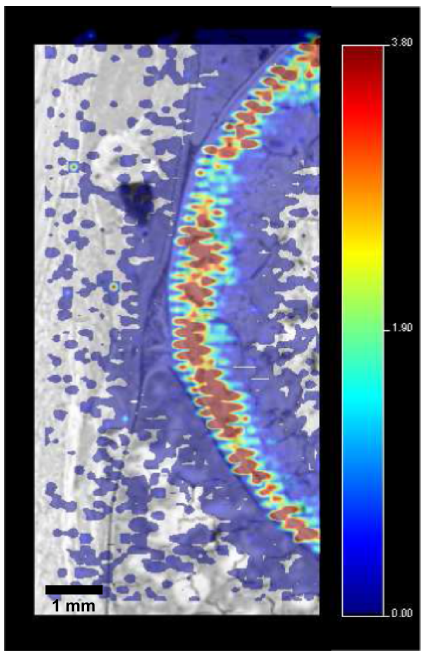

Fig. 5 MS image (*rainbow color scale*) of a compound from the stomach wall of a mouse, overlain on the optical image. Imaging artifacts are clearly visible as a result of the serpentine scanning mode. Pixel size was 100 μm, with a laser beam size of 200 μm, visualized using a linear interpolation. This rastering in overscan mode required high laser intensity, leading to redeposition of analyte outside of the actual tissue region. Accurate quantification of stomach wall concentration is not possible using this data

Quantitative MSI is applied to measure compound and metabolite distributions and is in selected studies used to replace established methods like LC-MS or WBAL [37, 40]. The core result of such a study is a list of tissues or tissue regions with determined concentrations, in which the MS images are often only provided as supplementary information for reference. Accordingly the MSI experiment *and the method of converting the MSI data to quantitative amounts* need to be traceable and adequately reported. This includes the general-case parameters of tissue preparation, data acquisition, MS pre-processing, compound identification (verification that the peak corresponds to the drug of interest), but also the method used for converting the peak intensities into concentrations (for which several different methods have been

reported and which differ in approach, practicality, and accuracy [38, 41]). Accordingly the following information should be provided for quantitative MSI:

10. Details for pharmacological studies
    (A) Software—including version number
    (B) Quantitation method—for example calibration curve determined on-tissue, calibration curve determined on mimetic tissue, calibration curve determined using tissue extinction coefficients.
    (C) Calibration curve
    (D) Validation method

As with the clinical example described above the selection of regions of interest can be performed on co-registered optical images or directly on the MS data (parameter 8B); pixel spacing can range from a few microns [42] to millimeters [39]; these and other parameters have an influence on the accuracy of measurements, particularly for smaller tissue regions where leakage from adjacent regions could distort the reading. An example is provided in Fig. 5, which shows the MSI data of a compound in the stomach wall of a mouse, overlain on the optical image. If one were to use the optical image to define a region of interest on the stomach wall, it would be possible to obtain a concentration value for this tissue. But by visually analyzing the image it is clear that the selected 100-μm raster (parameter 4A) is not of sufficient resolution to localize MS signals to the stomach wall, and consequently is not of sufficient resolution to adequately evaluate the concentration of the compound in this tissue. The oversampling method (laser beam size of 200 μm, parameter 4J) used in this example resulted in significant delocalization of the analyte. By providing the acquisition parameters this image can still be used to deduce information, namely an accumulation of the compound in the stomach wall, but it cannot be accurately quantified.

Concluding remarks

There are many approaches to performing MSI experiments and many factors that are known to affect the results. These range from the generic (e.g., mass and spatial resolution), to those specific for the methodology (the addition of an internal standard is essential for accurate quantification) or application area (careful patient selection for the determination of prognostic markers). Only if this parameter set is available/reported consistently can MSI data become significant for driving science, as otherwise it would just be colorful images. The compilation presented in this manuscript is a first suggestion for reporting guidelines and is meant to initiate a more intensive discussion of this topic in the MSI community. Finally it should be noted that the parameter set cannot be considered fixed, as the technique is always developing and new ionization sources/tissue preparations/mass analyzers are utilized; nevertheless any new developments should be reported in sufficient detail to cover parameters known to affect the results.

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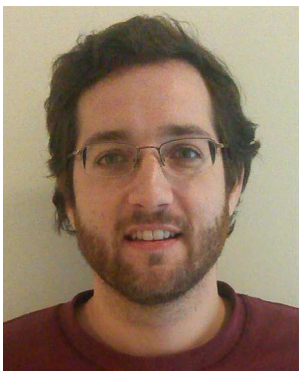

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Garry Corthals' research focuses on solving critical questions in science from a molecular perspective. The research of his group focuses on the development of technologies allied to mass spectrometry and applied to a range of biomedical applications. Innovative research is pursued in four key areas: 1. mass spectrometric tools and allied technologies aimed at sensitive and quantitative analysis of proteins and their posttranslational modifications; 2. separation sciences that can efficiently resolve complex molecular mixtures so their analysis is within the capacity of our analytical technology; 3. apply new chemistries (in methods and molecules) to biomedical applications to reveal molecular functional information of protein networks and protein complexes; and 4. computational methods to assist in the efficient validation and understanding of proteome-scale analysis of biomolecular systems. Most applications for these technologies are in health, disease, food, and forensics. Recently Garry has also established a new area of activity for HIMS in 'Science for Art'.

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