Review

For reprint orders, please contact: reprints@futuremedicine.com

Biomarkers in Medicine

Drug localizations in tissue by mass spectrometry imaging

Advances in development of mass spectrometry (MS) are successfully utilized for spatial localization of pharmaceutical compounds in these tissue sections. Today MS instruments can be used in imaging mode when the datasets are generated from the surface of the tissue over an array of acquisition positions. This review is focused on the technological developments of matrix-assisted laser desorption/ionization MS imaging (MALDI-MSI) and related sample preparation procedures. MALDI-MSI provides a sensitive and label-free approach for imaging of drugs and their metabolites. Due to these features, MALDI-MSI is expected to become a standard technique in pharmaceutical development providing complementary information to current methods.

Keywords: cryo-microtome • drug localization • MALDI matrix • MALDI-MSI

Ákos Végvári
Clinical Protein Science & Imaging, Department of Biomedical Engineering, Lund University, Sweden; CREATE Health, Lund University, Sweden; Department of Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, TX, USA
akos.vegvari@bme.lth.se
akvegvar@utmb.edu

Since its introduction [1], mass spectrometry imaging (MSI) has emerged to an important technique complementary to other biological imaging methods such as position emission tomography (PET), whole-body autoradiography (WBA) and MRI. MSI offers a reasonably sensitive tool to localize an unmodified active compound and its metabolites simultaneously. This is an enormous advantage in comparison with *in vivo* imaging technologies, like autoradiography, fluorescent imaging, computer tomography (CT) and MRI, presently used in combination with chemically modified substances [2,3].

Various instrumentations and application areas are explored and MSI is currently still under dynamic development. The present review focuses on drug distributions investigated by MSI, in particular using matrix-assisted laser desorption/ionization (MALDI) mass spectrometers. The reason for this restriction is due to the fact that MALDI instruments are the most widely available providing not only the best access to perform MSI but also high sensitivity compared with other ionization procedures, such as secondary ion mass spectrometry (SIMS) [4], desorption electrospray ionization (DESI) [5], laser ablation electrospray ionization (LAESI) [6] or nanostructure initiator mass spectrometry (NIMS) [7].

The initial work on the detection of pharmaceutical compounds directly from tissue using MALDI-MS was published by Troendle *et al.*, in which they profiled sections of a human ovarian tumor treated with the anticancer drug paclitaxel, and of a rat liver tissue spiked with the antipsychotic compound, spiperone [8]. Later on the technique was further developed to map the localization of orally administered antitumor drug substances in rat brain and in mouse tumor tissue using MALDI-MS for sampling the tissue sections in an array of positions with predefined step size [9]. This imaging mode of the MALDI-MS analysis of tissue sections evolved to the typically experimental setup that comprises the following steps: tissue isolation (organ or whole animal), sectioning frozen tissue using a cryo-microtome, deposition of matrix solution onto the tissue surface and MS data acquisition followed by visualization of extracted ion maps as

Future Medicine part of fsg

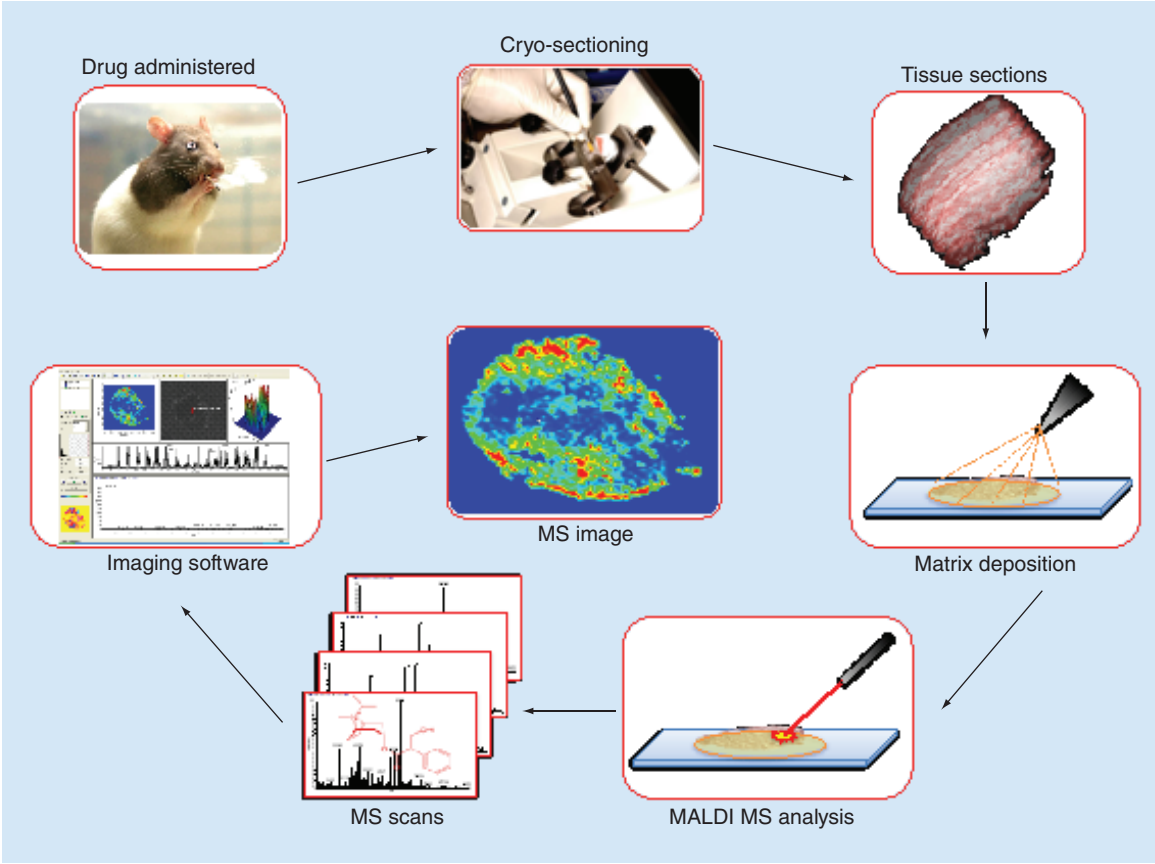

Figure 1. Schematic workflow of the experimental procedure aimed at determining drug localization in tissue sections by MALDI-MSI.

depicted in Figure 1. The majority of the experimental arrangements are focused on typical absorption, distribution, metabolism and excretion (ADME) used for investigation of active compound and its metabolites in animal models. Subsequent studies have been carried out to map a range of compounds in a number of different biological tissues [10,11]. Since then, the MALDI-MSI technology has gain significant attention in the pharmaceutical industry as a potential method for monitoring drug distribution kinetics in animal models [12].

Technical advances & analytical limitations

Instrumentation

Currently, one of the rapidly advancing imaging technologies is MALDI mass spectrometry imaging (MALDI-MSI) [13]. MALDI is the most sensitive desorption/ionization technique offering a broad dynamic mass range and often absolute identification of analytes by MS/MS data acquisition. MALDI-MS is widely used in combination with different pulse laser sources and mass analyzers, of which time-of-flight (ToF) is the most common. Spatial resolution is typically in the range of 50–200 µm (raster step size), essentially limited by the diameter of the laser beam. Selection of practical spatial resolution is dictated by the specimen size ranging from biopsies (0.5–2 mm) to organs (0.5–2 cm) and whole animal (5–15 cm). Considering tissue heterogeneity, many cases cellular or even subcellular spatial resolution is desirable to be able to distinguish details in drug distribution dictated by the localization of target receptors.

Recently, in high spatial resolution experiments 5 and 10 µm raster size was applied [14] that provided superior spatial details of drug localization utilizing accurate mass (≤2 ppm) at high mass resolution (R = 30,000), see Figure 2. High repetition rate lasers have also been introduced to ToF instruments to reduce acquisition time that is usually long considering the high number of data acquisition positions [15].

Due to the fast technological development of mass spectrometers, instrumental designs have provided improved properties in terms of mass resolution (up to 400,000 on FT and Orbitrap instruments), acquisition time and sensitivity. Unfortunately, FT mass spectrometers have not been frequently used for imaging experiments because of the associated high operat-

ing costs. Furthermore, new principles are introduced and applied to MSI, such as ion mobility spectrometry (IMS) that was demonstrated for MALDI-MSI to be able to resolve peaks in gas phase separations. Consequently, IMS has the ability to provide isolation of drug peaks separated from isobaric lipid signals that ToF instruments could not be distinguished [16]. New instruments, like a novel imaging mass microscope (iM-Scope, Shimadzu Corporation) has introduced, which is equipped with both an optical microscope allowing observation of high-resolution morphological images and a hybrid ion trap-ToF mass spectrometer for localization of specific molecules in tissue sections [17].

Sample preparation

The principal purpose of sample preparation in MALDI-MSI is to conserve spatial localization of dosed compounds of interest and include processing steps that fulfill this criterion. In case of isolated organs or biopsies the typical workflow of MALDI-MSI usually begins by sectioning cryopreserved tissue at low temperature for localization of drug compounds [9]. Tissue sections are then carefully collected on MALDI target plates keeping the original morphology of organs. Importantly, routinely employed 'optimal cutting temperature' (OCT) solutions containing water-soluble polymers should be avoided because of profound ion suppression and background signals. Alternative approaches were

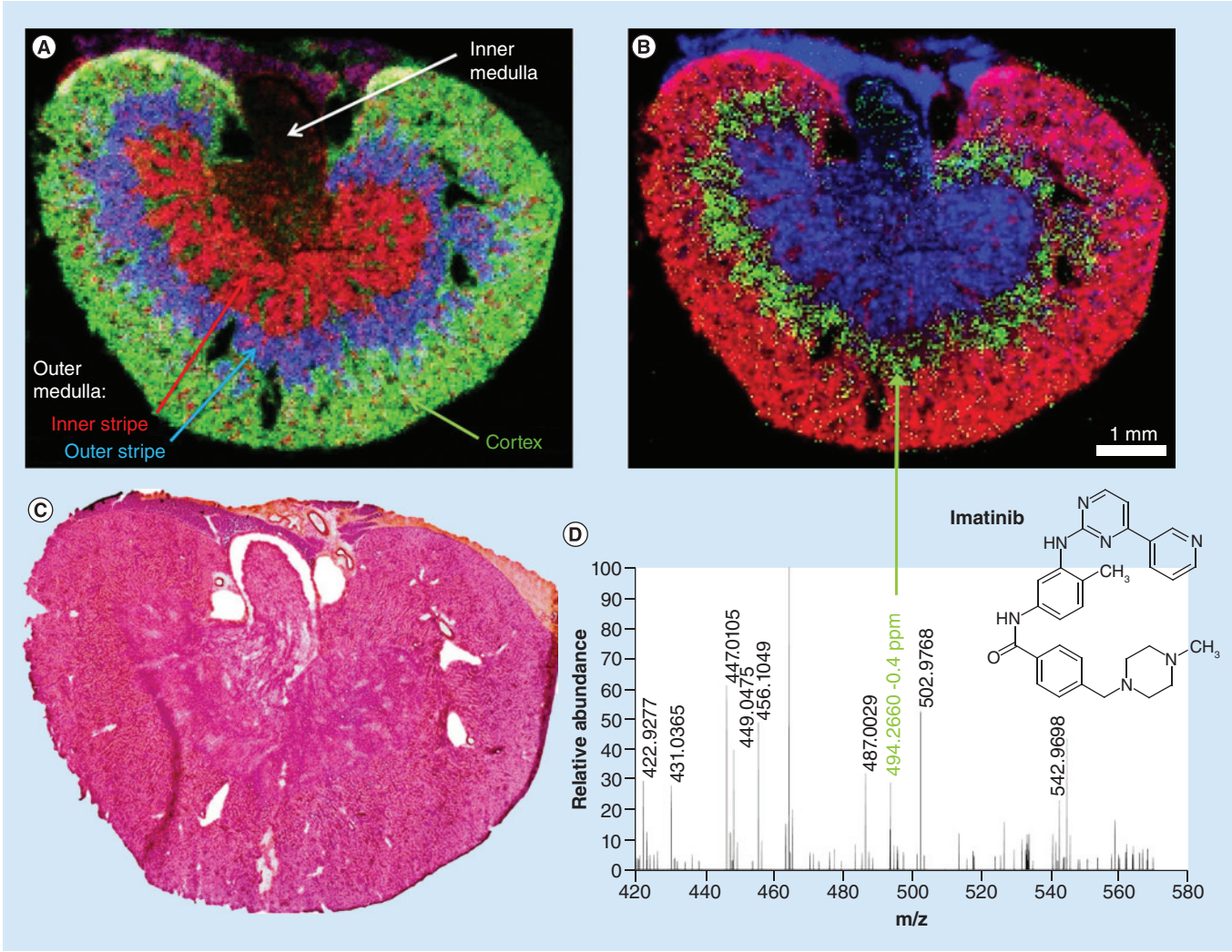

Figure 2. Mouse kidney (A) overlay of selected ion images: in cortex [PC (32:0) + K]+ = 772.5253, in outer stripe of outer medulla [PC (40:6) + K]$^+$ =872.5566, in inner stripe of outer medulla [PC (38:5) + K]$^+$ = 846.5410, 225 9 150 pixels, 35 μm step size, bin width Δm/z = 0.01; **(B)** overlay of selected ion images: in cortex [PC (32:0) + K]$^+$ = 772.5253, imatinib [M + H]$^+$ =494.2662 indicated by the arrow in the outer stripe of outer medulla, in the inner stripe of outer medulla and in the inner meddula [PC (34:1) + H]$^+$ = 760.5851, 225 9 150 pixels, 35 μm step size, bin width Δm/z = 0.01; **(C)** optical image of the investigated mouse kidney section, H&E stained after MS imaging measurement; **(D)** single-pixel mass spectrum of the outer stripe outer medulla of the mouse kidney section. Reproduced with permission from [14]. Springer Science+Business Media.

introduced to sectioning whole body tissues that require embedding medium to fix the animal [18].

Following that, the matrix solution is deposited (either sprayed or spotted) onto the surface of tissue sections in an attempt to extract sufficient amount of analytes from the tissue section and to form small-sized, homogeneous crystals [13]. The choice of matrix compound is crucial, however most applications use traditional matrices, such as 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid. MALDI-MS analysis is then performed, sampling the tissue surface in an array of measuring positions in raster mode, typically collecting full MS spectra and/or additional MS/MS data.

Software solutions for visualization, discovery & quantification

Since MALDI-MS instruments have been designed for general analytical purposes, vendor software packages usually offer simple and limited imaging features. Additionally, the raw data format varies greatly that led to the introduction of a new standard in MSI. This novel format is based on the HUPO-PSI standard 'mzXL' and thus called imaging mzXL (imzXL) [19]. The most important advantage of the imzXL format is that it facilitates comparisons in-between datasets generated on different instruments and that third party software tools can be used following conversion of raw data into imzXL format. Open access software, like MSiReader [20], are important tools to display extracted ion maps of mass signals providing flexible programming options to interpret data correctly. Further information about available software and the imzXL format can be found on the MSImaging website [21].

In most cases when the goal is to determine localization of drug in tissue sections, the expected parent and fragment masses of both the active compound and its metabolites are known. However, the data collected in full scans at high mass accuracy may carry additional valuable information about other endogenous signals that can be accessed using software designed to query the entire dataset to associate new *m/z* values of potential interest with biological structures, or to the spatial distribution of known ions [22]. Newly discovered ions then may be correlated with either specific tissue compartments or the coincident distribution of other ions.

Upon localization of any drug in samples probably the fundamental question is whether the observed signal intensities are mirroring the genuine concentrations of the analyte in tissue sections. Other endogenous molecules, such as lipids as well as salts, can severely suppress ionization of the analytes of interest. Additionally, this known ion suppression effect characteristic of MALDI-MS may vary between organs or even within the type of tissue [23]. Commonly employed methods to compensate for the variations of signal intensities not related to the amounts of compound apply normalizations on either total ion current (TIC), matrix cluster ions or internal standards [24].

Undoubtedly, the application of internal standards can provide the closest estimation of the endogenous amounts of drugs in tissue if these compounds have ionization properties nearly identical to the analytes. Useful alternatives for internal standards thus include chemically modified structures of the analyte, for instance methylated, typically observed as metabolites in tissue. Furthermore, stable isotope labeled analytes used as internal standards can afford the best resemblance to the endogenous compounds and should be optimally employed in quantitative studies. Isotope-labeled standards, the homologs of compounds of interest, are particularly useful in reliable quantitation of administered drugs as was demonstrated in conjunction with the introduction of a novel software tool recently [25]. Although the availability and cost of synthesis of isotope-labeled compounds are major limitation, it is likely that the MALDI-MSI applications would develop intensively in this direction as quantification has utmost importance in drug localizations in tissue.

Software tools for quantification utilizing different approaches have introduced, such as Quantinetix (ImaBiotech SAS, Loos, France) [26]. Quantinetix supports raw input data of various mass spectrometer vendors, including Thermo Fisher Scientific, Bruker Daltonix and ABSciex and quantification of drug compounds in more than 25 organs in whole body distribution using normalized ion suppression.

Applications

MSI in drug discovery & development

New drugs are often directed to specific organ, where an affinity to a target protein is expected to occur with a defined specificity and selectivity. These drug data are in most cases predictive and not really proven at a molecular level due to the difficulty and challenge to perform these exact measurements. This is particularly the case when a high number of active drug candidates are to be tested. The facts that health organizations like FDA in USA and EMEA in Europe require more and more detailed characterization of novel drug candidates and, moreover, that the numbers of these compounds entering to clinical studies increase drastically, demand experimental capabilities.

During the last decade, significant technological improvements in MS have had a great impact on drug discovery and development. MALDI-MSI has gained attention in applications useful for pharmaceutical industry due to its straightforward approach, which eliminates the need of additional tagging and labeling chemistry steps and its potential to detect and monitor metabolites of drug compounds. The direct analysis of tissue sections using MALDI-MSI is an emerging technology based on a surface sampling process. MALDI-MSI has been used to measure drugs administered locally or systemically in tissue biopsies from both experimental models and human clinical studies at a resolving power of 20–30 µm [27,28]. Currently, MALDI-MSI is used as a standard technology for identification of metabolites [29] and monitoring their metabolic behavior of many drug candidates by measuring their quantities and localization [30–32].

In many cases, the detection of drug compounds in tissue sections is hampered by ion suppression and poor ionization properties, which is a prerequisite for generation of high-quality MS images. In order to improve ionization of endogenous drug molecules chemical derivatization was introduced that increased the LOD significantly [33]. The approach is based on the deposition of a derivatization agent (1,1′-thiocarbonyldiimidazole) onto the tissue section allowing to chemically modifying the target 3-methoxysalicylamine locally prior application of matrix. Due to the efficient reaction, the resulted oxothiazolidine derivative was then analyzed with considerably higher sensitivity by MALDI than the administered analyte itself [33].

The quantitative aspect of MALDI-MSI in terms of accuracy and precision of analysis was studied when correlated with conventional HPLC-MS/MS method [32]. The method proposed to estimate endogenous concentrations of compounds by using a dilution series of drug solution on control tissue section. Despite of the different ionization mechanisms and sensitivity of mass spectrometers, the determined concentrations of administered drug at standard pharmacological dosage by MALDI-MSI agreed well with the levels measured by standard LC-MS/MS.

In vivo disease models in rodents

The prediction of what dose of drug should be given in a Phase I clinical study is probably one of the most crucial steps that the candidate drug needs to manage in the drug development pipeline. These investigations are in most cases outlined by *in vitro* developments and taken further into *in vivo* studies with experimental rodent models and targeted approaches are used to study cell behaviors in *in vivo* whole body biology [34]. By the development of these novel drug testing mod-

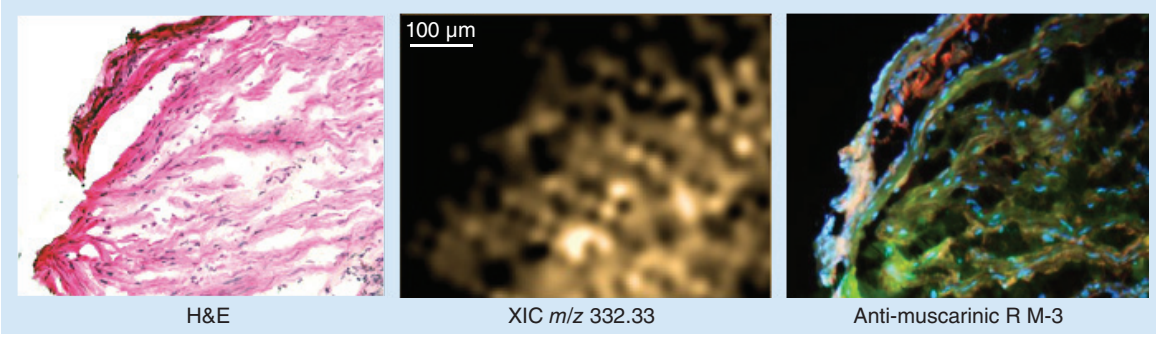

Figure 3. The lateral distribution of an administered drug (ipratropium, m/z 332.33) by MALDI-MSI (in the middle) is compared with the histological image (H&E) and the localization of its target receptor (antimuscarinic receptor M-3 on the right) by immunhistological staining.

els, the long-term goal is to implement methodologies based on MALDI-MSI that allow for a detailed characterization of new drug candidates.

A great achievement of the field was the establishment of whole body analysis of experimental animals dosed with drugs [10]. The methodology is based on standard whole body autoradiography procedures, including embedding and sectioning animals sagittally in order to cover all the organs of interest while administering nonradioactive compounds. Alternatively, rodent models can be utilized to investigate drug distribution and their metabolism in isolated organs or xenograft tumors developed in the experimental animals [28]. For instance, anticancer drugs, such as used in personalized medicine treatments of lung cancer, could be localized acquiring accurate full and fragment mass spectra showing spatial distribution in the target organ. Low concentrations of erlotinib and gefitinib at attomolar levels were realized upon optimization of the method allowing compound read-out at single cell resolution [28]. The distribution of erlotinib and its metabolites were also analyzed in liver, spleen and muscle tissues of rat following 5 mg/kg dosing [35].

The mode of drug action is also an area of great interest to the healthcare and patients, since it is often closely related to the safety profile of the medicines used. Usually in human clinical studies, following MALDI-MSI analysis the tissue sections were stained using routine procedures for hematoxylin and eosin (H&E) and cover slipped. These images were carefully superimposed upon the histological images of each sample using landmarks defined by total ion current images of the tissue sections scanned in the MS mode [36]. When drug imaging is combined with high-resolution histology images of the same sample, the MALDI-MSI drug signals provide an exact visualization of the amount of drug transported to a tissue microenvironment (Figure 3).

Conclusion & future perspective

The concept of drug localization by MSI has been evidently established providing a label-free approach that allows for not only absolute identification of drug molecules and their metabolites but also for quantification. The technology has been introduced to pharmaceutical companies to screen lead compounds in whole body analysis as well as their metabolism in isolated organs at high spatial resolution. These companies actively participate in and drive research to support PK/PD and toxicology studies.

The rapid development of mass spectrometers will continue in the future delivering instrument with higher mass accuracy and mass resolution that can generate spectral data in shorter duty cycles. Beyond technological improvements, the interplay between MSI of drugs and proteomics is expected to be enforced providing *in situ* co-localization of compounds and their receptors as well as identifying novel drug targets.

Financial & competing interests disclosure

The author is grateful for funding support – Innovate Support 2011-03926 – from CREATE Health and for the University of Texas Medical Branch and the Cancer Prevention Research Institute of Texas. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Several MS ionization techniques, including SIMS, DESI, LAESI, NIMS and MALDI, can be employed to acquire imaging of endogenous compounds in tissue sections.
- MALDI-MSI instrumentation is widely available and most frequently accommodated to imaging purposes. Today, many MALDI mass spectrometers can be operated in imaging mode, provided software for data acquisition and visualization available.
- Technological developments of MALDI-MSI instruments offer high spatial and high mass resolution analysis of drug compounds permitting their identification and quantification in tissue sections.
- Software solutions for visualization and quantification are improved greatly. International efforts have resulted in the introduction of a standard data format for MS imaging datasets (imzML).
- Most applications of MALDI-MSI in the field of drug development are based on animal models, investigating isolated organs of interest or performing whole body analysis.
- It is expected that MALDI-MSI will contribute to the understanding of drug action mechanisms and thus the technique will continue to be a standard tool for localization of compounds and metabolites of novel drug candidates.

References

1 Spengler B, Hubert M, Kaufmann R. Maldi ion imaging and biological ion imaging with a new scanning UV-laser microprobe. Presented at: *The 42nd Annual Conference on Mass Spectrometry and Allied Topics*. Chicago, IL, USA, 21–26 May 1994.

2 Reyzer ML, Chaurand P, Angel PM, Caprioli RM. Direct molecular analysis of whole-body animal tissue sections by maldi imaging mass spectometry. *Methods Mol. Biol.* 656 285–301 (2010).

3 Sugiura Y, Setou M. Imaging mass spectrometry for visualization of drug and endogenous metabolite distribution: toward in situ pharmacometabolomes. *J. Neuroimmune Pharmacol.* 5(1), 31–43 (2010).

4 Heeren RMA, Mcdonnell LA, Amstalden E, Luxembourg SL, Altelaar AFM, Piersma SR. Why don't biologists use sims?: a critical evaluation of imaging MS. *Appl. Surf. Sci.* 252(19), 6827–6835 (2006).

5 Wiseman JM, Ifa DR, Song Q, Cooks RG. Tissue imaging at atmospheric pressure using desorption electrospray ionization (desi) mass spectrometry. *Angew. Chem. Int. Ed. Engl.* 45(43), 7188–7192 (2006).

6 Nemes P, Barton AA, Vertes A. Three-dimensional imaging of metabolites in tissues under ambient conditions by laser ablation electrospray ionization mass spectrometry. *Anal. Chem.* 81(16), 6668–6675 (2009).

7 Northen TR, Yanes O, Northen MT *et al.* Clathrate nanostructures for mass spectrometry. *Nature* 449(7165), 1033–1036 (2007).

8 Troendle FJ, Reddick CD, Yost RA. Detection of pharmaceutical compounds in tissue by matrix-assisted laser desorption/ionization and laser desorption/chemical ionization tandem mass spectrometry with a quadrupole ion trap. *J. Am. Soc. Mass Spectrom.* 10(12), 1315–1321 (1999).

9 Reyzer ML, Hsieh YS, Ng K, Korfmacher WA, Caprioli RM. Direct analysis of drug candidates in tissue by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* 38(10), 1081–1092 (2003).

10 Rohner TC, Staab D, Stoeckli M. Maldi mass spectrometric imaging of biological tissue sections. *Mech. Ageing Dev.* 126(1), 177–185 (2005).

11 Hsieh Y, Chen J, Korfmacher WA. Mapping pharmaceuticals in tissues using MALDI imaging mass spectrometry. *J. Pharmacol. Toxicol. Methods* 55(2), 193–200 (2007).

12 Prideaux B, Stoeckli M. Mass spectrometry imaging for drug distribution studies. *J. Proteomics* 75(16), 4999–5013 (2012).

13 Shanta SR, Kim TY, Hong JH *et al.* A new combination maldi matrix for small molecule analysis: application to imaging mass spectrometry for drugs and metabolites. *Analyst* 137(24), 5757–5762 (2012).

14 Römpp A, Guenther S, Takats Z, Spengler B. Mass spectrometry imaging with high resolution in mass and space (hr2 msi) for reliable investigation of drug compound distributions on the cellular level. *Anal. Bioanal. Chem.* 401(1), 65–73 (2011).

15 Spraggins JM, Caprioli RM. High-speed maldi-tof imaging mass spectrometry: rapid ion image acquisition and considerations for next generation instrumentation. *J. Am. Soc. Mass Spectrom.* 22(6), 1022–1031 (2011).

16 Trim PJ, Henson CM, Avery JL *et al.* Matrix-assisted laser desorption/ionization-ion mobility separation-mass spectrometry imaging of vinblastine in whole body tissue sections. *Anal. Chem.* 80(22), 8628–8634 (2008).

17 Shimadzu, iMScope TRIO. www.shimadzu.com/an/lifescience/imaging/imscope.html

18 Prideaux B, Staab D, Stoeckli M. Applications of maldi-msi to pharmaceutical research. *Methods Mol. Biol.* 656 405–413 (2010).

19 Schramm T, Hester A, Klinkert I *et al.* Imzml – a common data format for the flexible exchange and processing of mass spectrometry imaging data. *J. Proteomics* 75(16), 5106–5110 (2012).

20 Robichaud G, Garrard K, Barry J, Muddiman D. Msireader: an open-source interface to view and analyze high resolving power ms imaging files on matlab platform. *J. Am. Soc. Mass Spectrom.* 24(5), 718–721 (2013).

21 Msimaging, COST Action On MS Imaging – Become Part Of It. http://maldi-msi.org

22 Suits F, Fehniger T, Végvári Á, Marko-Varga G, Horvatovich P. Correlation queries for mass spectrometry imaging. *Anal. Chem.* 85(9), 4398–4404 (2013).

23 Stoeckli M, Staab D, Schweitzer A. Compound and metabolite distribution measured by maldi mass spectrometric imaging in whole-body tissue sections. *Int. J. Mass spectrom.* 260(2–3), 195–202 (2007).

24 Hamm G, Bonnel D, Legouffe R *et al.* Quantitative mass spectrometry imaging of propranolol and olanzapine using tissue extinction calculation as normalization factor. *J. Proteomics* 75(16), 4952–4961 (2012).

25 Källback P, Shariatgorji M, Nilsson A, Andrén PE. Novel mass spectrometry imaging software assisting labeled normalization and quantitation of drugs and neuropeptides directly in tissue sections. *J. Proteomics* 75(16), 4941–4951 (2012).

26 Imabiotech, benefits. www.imabiotech.com/Quantinetix-TM-Maldi-Imaging.html

27 Fehniger TE, Végvári Á, Rezeli M *et al.* Direct demonstration of tissue uptake of an inhaled drug: proof-of-principle study using matrix-assisted laser desorption ionization mass spectrometry imaging. *Anal. Chem.* 83(21), 8329–8336 (2011).

28 Marko-Varga G, Fehniger TE, Rezeli M, Döme B, Laurell T, Végvári Á. Drug localization in different lung cancer phenotypes by maldi mass spectrometry imaging. *J. Proteomics* 74(7), 982–992 (2011).

29 Miura D, Fujimura Y, Wariishi H. *In situ* metabolomic mass spectrometry imaging: recent advances and difficulties. *J. Proteomics* 75(16), 5052–5060 (2012).

30 Benabdellah F, Touboul D, Brunelle A, Laprevote O. *In situ* primary metabolites localization on a rat brain section by chemical mass spectrometry imaging. *Anal. Chem.* 81(13), 5557–5560 (2009).

31 Bouslimani A, Bec N, Glueckmann M, Hirtz C, Larroque C. Matrix-assisted laser desorption/ionization imaging mass spectrometry of oxaliplatin derivatives in heated intraoperative chemotherapy (hipec)-like treated rat kidney. *Rapid Commun. Mass Spectrom.* 24(4), 415–421 (2010).

32 Nilsson A, Fehniger TE, Gustavsson L *et al.* Fine mapping the spatial distribution and concentration of unlabeled drugs within tissue micro-compartments using imaging mass spectrometry. *PLoS ONE* 5(7), (2010).

33 Chacon A, Zagol-Ikapitte I, Amarnath V *et al.* On-tissue chemical derivatization of 3-methoxysalicylamine for maldi-imaging mass spectrometry. *J. Mass Spectrom.* 46(8), 840–846 (2011).

34 Setou M, Saito Y, Hayasaka T, Onoue K, Takizawa Y. Imaging mass spectrometry (ims): a novel imaging tool to visualize the spatial distribution of drug in whole-body sections. *Drug Metab. Rev.* 43 18–18 (2011).

35 Signor L, Varesio E, Staack RF, Starke V, Richter WF, Hopfgartner G. Analysis of erlotinib and its metabolites in rat tissue sections by maldi quadrupole time-of-flight mass spectrometry. *J. Mass Spectrom.* 42(7), 900–909 (2007).

36 Ait-Belkacem R, Sellami L, Villard C, Depauw E, Calligaris D, Lafitte D. Mass spectrometry imaging is moving toward drug protein co-localization. *Trends Biotechnol.* 30(9), 466–474 (2012).