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MALDI spatial proteomics: a mini review of approaches and techniques

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Spatial context is becoming increasingly important in the omics disciplines. Spatial proteomics is a diverse field encompassing numerous techniques that provide both the location and identity of proteins in biological samples. Improving upon bulk analyses, spatial proteomics can map peptides and intact proteins within tissue. This review focuses on the application of matrix-assisted laser desorption/ionization (MALDI) in spatial proteomics. Approaches are grouped into two general categories and discussed: protein MALDI MSI and MSI-guided spatial proteomics. A discussion of the workflow for each method is presented, and challenges to each approach are discussed. Recent and technically interesting cases in the literature are presented for each category. This review aims to guide researchers interested in MALDI protein imaging through the strengths, weaknesses, and technical considerations of the many workflows available to them.

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Introduction

Spatial proteomics is a rapidly developing field encompassing a number of analytical techniques that accurately map proteins in biological specimens. Spatial proteomics improves upon the bulk protein analyses of the past by delivering protein identifications while retaining spatial context. To this end, this method is often presented in tandem with lipid and metabolite data from the same tissue, providing a rich understanding of complex biological processes. To highlight the importance of this approach, Nature Methods named this discipline Method of the Year for 2024.[1] While Nature Methods focused on immunohistochemistry-based techniques that fall under the broad field of spatial proteomics, in this manuscript we focus on matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) of proteins. This review will summarize current MALDI and techniques that help identify proteins in defined regions of interest within tissues and biological samples.

In MALDI, a sample is sprayed or co-crystallized with a chemical matrix on a conductive surface. The sample and matrix are then ablated with a laser. The matrix absorbs radiation at the frequency of the laser, causing the analyte to desorb and ionize from the surface, creating a plume of ions and neutrals.[2,3] The ions are then analyzed in a mass spectrometer. MALDI is a "soft" ionization technique that produces minimal fragmentation of ionized analytes and generally results in low charge state ions. Since the effect of an absorbing matrix in laser desorption mass spectrometry (LDMS) was first described by Karas and coworkers in 1985,[4] and the subsequent expansion of the technique to large biomolecules by Tanaka and coworkers in 1988,[5] MALDI has been used to analyze drugs,[6] metabolites,[7] lipids,[8] peptides,[9] and proteins.[10] With the rise of MALDI MSI in the late 1990s, spatial mapping of analytes in biological context became possible.[11] MALDI is now the most widely used technique in MSI.[12] MALDI is often used for spatial lipidomics and metabolomics but is also a useful tool for spatial proteomics. In MALDI MSI, the laser is rastered across the surface of a thinly-sectioned sample (usually in the tens of micrometers), ablating the matrix-coated sample at a pre-defined spatial interval. The mass spectrometer collects a full mass spectrum at each of these ablation spots, and a heatmap can be generated showing the spatial localization across the sample of each ion detected.[13]

MALDI MSI is an ideal method for imaging a broad range of molecular classes.[14] Applied to spatial proteomics, it features unique advantages over spectroscopic techniques as it is multiplexed, analytes are typically unlabeled, and is not limited by the availability of antibodies.[1] While the applications of MALDI MSI to spatial proteomics are many and diverse, approaches generally fall into two broad categories, which will be discussed here: protein MALDI MSI and MSI-guided spatial proteomics.

Protein MALDI MSI involves ionizing proteins or peptides directly from a tissue section; it often involves on-tissue enzymatic digestion to generate peptides for bottom-up approaches, but top-down approaches are used to analyze intact proteins. In MSI-guided spatial proteomics, an initial MSI experiment is used to determine regions of interest within a tissue section. These regions are excised from the sample and collected, often by laser capture microdissection (LCMD), and subjected to liquid chromatography-mass spectrometry (LC-MS) proteomics. This

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general workflow allows the experimenter to correlate MSI data, which is often lipid- or metabolite-based, with spatial proteomic information for a given sample. The choice of whether to pursue protein MALDI MSI or an MSI-guided experiment involving downstream LC-MS proteomics is influenced by the biological question of the researcher and by sample type, size, and fixation state. Regardless of the approach taken, there are many experimental variables that affect the quality of the resulting dataset, and they will be discussed herein.

A number of excellent reviews on MALDI imaging applied to spatial proteomics and multi-omics exist in the literature.[14–19] Moreover, sub-sections on protein imaging and details of less widely-used MSI protein imaging methods can be found in many broader overviews of MALDI MSI.[20–24] In this review, we will focus on the technical considerations of two widely-used approaches to MALDI protein imaging.

Protein MALDI MSI

Introduction to protein MALDI MSI

Proteins and peptides can be analyzed directly from tissue by MALDI MSI, maintaining the spatial context of a biological sample. Protein MALDI MSI can generally be divided into two approaches: top-down, to detect intact proteins, and bottom-up, in which proteolytic peptides are analyzed.[25] The next two sub-sections will describe each of these approaches.

Bottom-up approaches

In bottom-up approaches, proteins are enzymatically digested on-tissue to their corresponding peptides, which are then ionized and analyzed in an MSI experiment. As peptides are easier to fragment than MALDI-generated protein ions, they are more readily identified by tandem MS (MS/MS).[17]

This on-tissue digestion and subsequent peptide analysis can be applied to both fresh-frozen and formalin-fixed paraffin-embedded (FFPE) tissues. In general, the workflow for bottom-up protein MSI of fresh-frozen tissues involves cryosectioning and depositing the sample on a conductive slide; washing to remove interfering compounds such as lipids and salts as well as fix the proteins;[26] on-tissue enzymatic digestion; incubation; matrix deposition; and MALDI MSI analysis. The workflow for FFPE tissues includes added steps for the removal of paraffin embedding and rehydration through ethanol washes and reversal of formalin-induced crosslinks (antigen retrieval).[27–29] A schematic of the bottom-up protein MALDI MSI workflow can be found in Fig. 1. Typically, a robotic sprayer is used to apply the enzyme directly to the tissue section.[17] Ly *et al.* identified enzyme application and digestion as a major source of variation in MALDI MSI experiments and found that the method can affect spatial resolution. The authors present an improved on-tissue tryptic digestion protocol for FFPE tissues and demonstrate its effectiveness in enabling reproducible MALDI peptide imaging results across multiple sites.[27]

The success of a bottom-up MALDI protein imaging experiment is influenced by the numerous experimental steps listed above. Optimization of both the "wet" methodology and data acquisition are critically dependent on sample type. Recently, Høiem *et al.* optimized MSI detection of peptides in prostate tissue sections by testing the tissue washes, protein denaturation, and tryptic digestion methods involved in the workflow.[30] Using MALDI MSI, González de San Román *et al.* showed the differential distribution of five proteins between the primary and secondary visual cortices of post-mortem human brain sections. The authors used on-tissue digestion and MS/MS at each raster point of the MALDI experiment to identify tryptic peptides which, subjected to database searching, resulted in protein identifications.[31]

Bottom-up analysis is especially prevalent in studies of FFPE tissues, as these samples are ubiquitous in clinical pathology.[29] Due to cross-linking, their intact proteins, even after antigen retrieval, are not fully available for detection by MALDI MSI.[27] Recently, Goncalves *et al.* demonstrated MALDI peptide imaging of FFPE breast cancer tissue cores from 1191 patients. Tandem MS was performed to identify proteins of origin pertaining to highly differentiating tryptic peptides, and the most abundant peptide features were used to train machine-learning models to characterize hormone receptor status in breast cancer tissue.[28] Baltzer *et al.* used bottom-up MALDI peptide imaging, including MS/MS of peptides directly from tissue and subsequent protein identification, to investigate the proteomic effects of leukocyte-poor platelet-rich plasma (LpPRP) injection on synovial tissue of the knee joint prior to surgery. The authors were able to identify discriminating proteins that differentiate between LpPRP-treated and control tissues.[32]

Top-down approaches

Top-down protein MALDI MSI approaches allow for the analysis of intact proteins, and typically rely on either accurate mass measurements and isotopic pattern or fragmentation of the intact proteins for identification.[25] Fourier-transform (FT) systems, including Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap instruments, are popular for top-down analysis as they offer superior spectral resolution that provides the mass accuracy and isotopic resolution necessary for protein identification. Piga *et al.* demonstrated the ability of Fourier transform ion cyclotron resonance (FT-ICR) MALDI MSI to identify intact proteins in mouse and human pancreas sections. The authors were able to distinguish overlapping isotope envelopes that are convoluted on lower mass resolution systems. To identify small proteins in the samples, the authors used the Uniprot database to generate candidate proteins for mouse and human pancreas, then compared accurate mass and isotope envelopes from the FT-ICR MALDI MSI to simulated isotope envelopes for each species in Bruker Data Analysis software.[33] Zemaitis *et al.* combined a MALDI source with a ultrahigh mass range-enabled Orbitrap for high-resolution accurate mass MALDI MSI and identification of histone proteins in human kidney sections.[34]

Despite their high resolving power, FT systems are typically limited in their detection of large proteins because they tend to have a m/z range that is relatively narrow and skews towards lower m/z values.[35] Time-of-flight (TOF) systems, with their

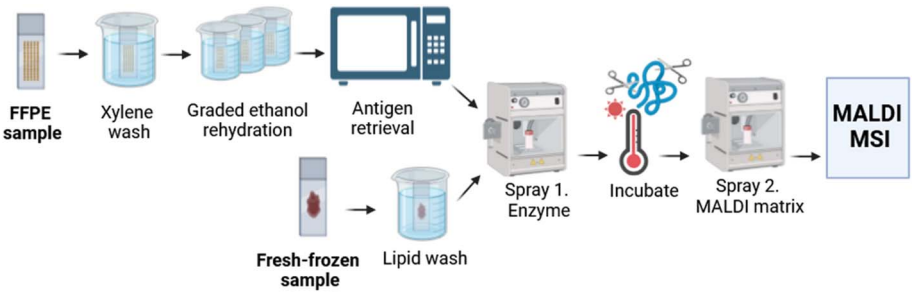

Fig. 1 Overview of a bottom-up proteomics approach to protein MALDI MSI. Sectioned, fresh-frozen samples on conductive slides are washed to remove lipids and salts. FFPE samples must first be deparaffinized by washing in xylene and rehydrating in graded ethanol washes. Formalin-induced crosslinks are reversed by antigen retrieval. At this point in the workflow, both fresh-frozen and FFPE samples are sprayed with enzyme, allowed to incubate in order for digestion to proceed, and sprayed with MALDI matrix before MALDI MSI analysis.

hypothetically unlimited *m/z* range, are therefore a viable option for the detection of larger proteins.[36] While both FT and TOF systems can be used for intact protein MALDI MSI, there is a trade-off involved with each: while FT mass spectrometers provide superior spectral resolution, they suffer from long acquisition times, and while TOF systems provide rapid data acquisition and wide *m/z* ranges, they exhibit comparatively lower spectral resolution. Klein *et al.* recently demonstrated the use of a quadrupole-reflectron time-of-flight (qTOF) mass spectrometer to perform rapid, high-spectral resolution MALDI protein imaging of whole mouse pup and rat brain tissue sections.[35]

Targeted MALDI-IHC

An interesting development in targeted, multiplexed protein MALDI MSI has recently been introduced that incorporates elements of immunohistochemistry (IHC) techniques. This method, dubbed MALDI-IHC, uses photocleavable mass-tags (PC-MTs) to label and map proteins in a biological sample. PC-MTs comprise a peptide mass reporter region connected to a photocleavable linker, with a probe-reactive moiety at the other end (Fig. 2). The PC-MT is reacted with an antibody (probe) which attaches at the reactive moiety end to generate PC-MT-antibodies. An IHC workflow is performed to bind the PC-MT-antibodies to target proteins in tissue. Subsequently, photocleavage with UV light liberates the mass reporter region, and MALDI MSI is performed to map the mass reporter peptides, and subsequently their target proteins.[37] Protein MALDI MSI at high spatial resolution suffers from a decrease in ablated and detectable analyte as the pixel size decreases. MALDI-IHC overcomes this problem through the use of mass reporters and allows for multiplexed, targeted proteomics at small pixel sizes.[37,38] MALDI-IHC has been demonstrated on human tonsil, mouse brain,[37] and breast cancer[37,38] tissue, as well as on small intestinal organoids[39] and single cells.[40,41]
Selection and Application of MALDI Matrix.

The selection of MALDI matrix and method of application are critically important to any MSI experiment and must be considered in bottom-up and top-down approaches to protein imaging. Delocalization is a significant challenge in protein MALDI MSI, as the tissue sample undergoes several solvent-based treatments before imaging, as discussed above.[42] Moreover, matrix crystal size after deposition can severely limit spatial resolution. MALDI matrices are typically applied *via* three methods: robotic spotting, spraying (robotic or by airbrush), and sublimation. Because each of these methods results in different crystal sizes for the same matrix, the method of matrix application affects spatial resolution in the final image. Robotic spotting typically leads to the lowest image resolution, limited to 200 μm, while robotic or airbrush spotting typically allows for 10–20 μm resolution, although the high volume of solvent used to spray the matrix can lead to analyte delocalization.[24] In sublimation, the MALDI matrix transitions directly from solid to vapor state under reduced pressure and high temperature conditions, depositing a thin and even layer of matrix on the sample.[43] Sublimation produces smaller crystals than spotting or spraying[44] and prevents delocalization as no solvent is used. However, sublimation can result in lower analyte extraction from the sample than spotting or spraying.[24] Thus, the choice of matrix application method should be guided both by available instrumentation and the biological question (*i.e.*, whether spatial resolution or sensitivity is most important).

The choice of matrix itself is equally important in protein MALDI MSI. α-Cyano-4-hydroxycinnamic acid (CHCA) is a popular matrix in peptide and protein imaging as it produces small crystals, which lead to increased spatial resolution and reduced delocalization.[42] Moreover, CHCA produces low levels of methionine and tryptophan oxidation,[45] simplifying peptide identification post-imaging. While CHCA works well for peptides and small proteins, sinapinic acid is useful in the detection of larger, intact proteins[24,26,46] 2,4,6-trihydroxyacetophenone (THAP) and 2,5-dihydroxybenzoic acid (DHB) exhibit low noise in the low *m/z* range, facilitating detection of small peptides and peptide fragments, and can therefore be used as complementary matrices to CHCA in peptide imaging.[45] THAP was also shown to decrease the signal of phosphorylated peptides by nonphosphorylated peptides in MALDI analysis by Yang *et al.*, but the application of this advantage to MALDI MSI has yet to be demonstrated.[47] Recently, Liu *et al.* demonstrated the utility of caffeic acid as a MALDI matrix for high-molecular-weight proteins approaching 2 00 000 Da on a TOF/TOF system. The authors compared caffeic acid to sinapinic and ferulic acid and found that caffeic acid yields higher protein signals in several animal and plant tissues.[36]

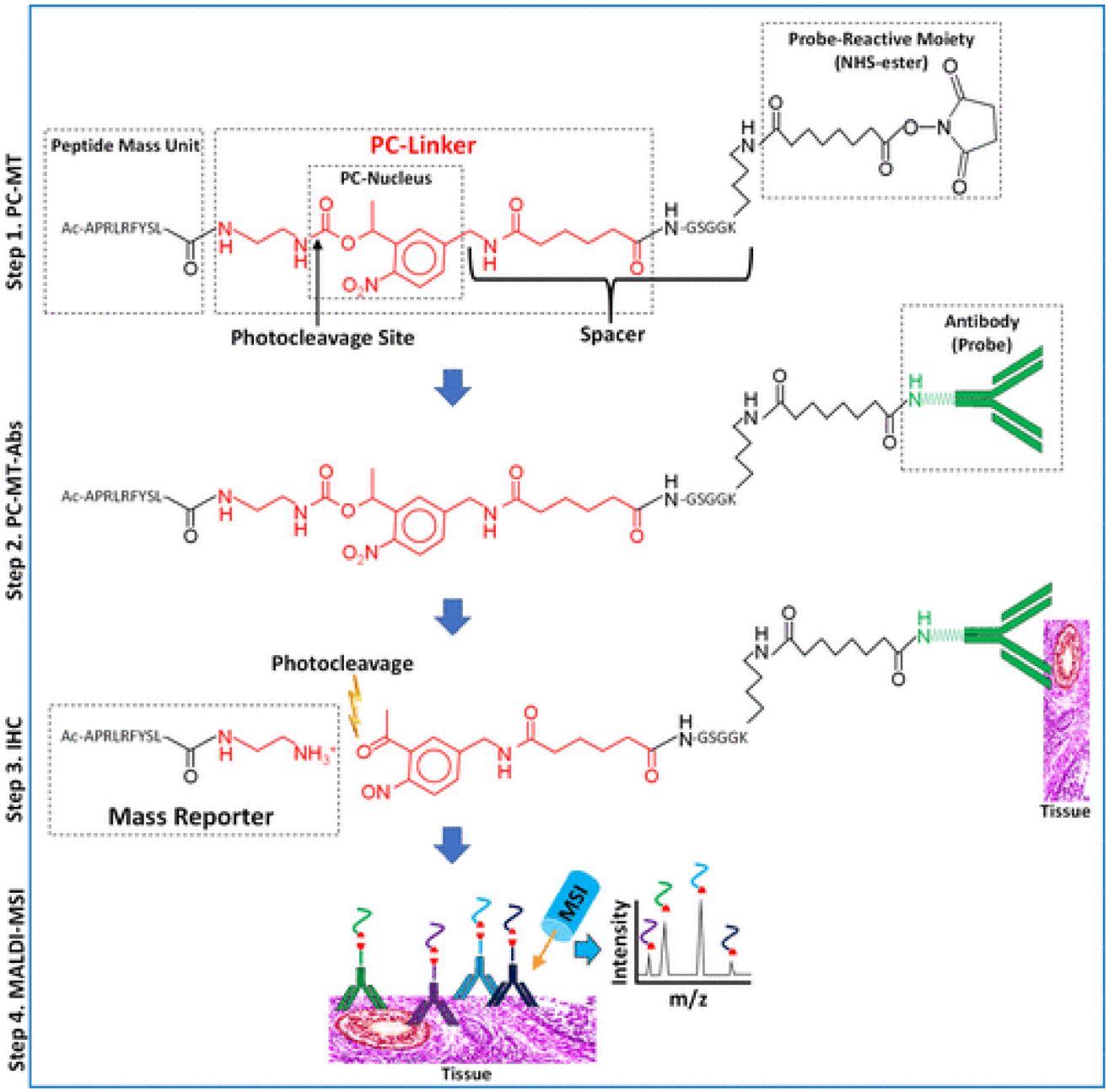

Fig. 2 PC-MTs used for the MALDI-IHC workflow. A peptide mass unit, photocleavable linker, and spacer are attached to a probe-reactive moiety. The PC-MT is reacted with an antibody (probe), which subsequently binds to its target protein. UV-induced photocleavage liberates the mass reporter, allowing it to be detected in a MALDI MSI experiment. Reproduced from ref. 37 with permission from American Chemical Society. Yagnik, G.; Liu, Z.; Rothschild, K. J.; Lim, M. J. Highly Multiplexed Immunohistochemical MALDI-MS Imaging of Biomarkers in Tissues. *J. Am. Soc. Mass Spectrom.*, 2021, **32** (4), 977–988. https://doi.org/10.1021/jasms.0c00473, copyright 2021 AmberGen, Inc. This figure is licensed under CC-BY 4.0.

Challenges in data acquisition and analysis

A major limitation in protein MALDI MSI is its low-throughput nature for identification of peptides and proteins. Tryptic peptides are typically identified by performing spot-wise MALDI-based MS/MS on the *m*/*z* of interest, or by matching the *m*/*z* of detected peptides to LC-MS/MS proteomic data collected from the same tissue. Both of these methods severely hinder the throughput of protein MALDI MSI. Moreover, methods for precursor selection in MALDI MS/MS are limited; most current methods only select one precursor ion per laser raster position.[48] However, in a notable example of targeted protein MSI, the first application of imaging parallel reaction monitoring-parallel accumulation-serial fragmentation (iprm-PASEF) to imaging of tryptic peptides was recently published by Li and coworkers. This workflow allows for the selection of up to 25 peptide precursors, manually chosen from an initial

MALDI trapped ion mobility (TIMS) experiment, to be isolated and fragmented in a subsequent MS/MS experiment.[48]

Even after isolation and fragmentation are achieved, protein MALDI MSI is hindered by a lack of bioanalytical tools for identifying fragmented peptide data.[17,49] Common methods of identifying peptides are low-throughput and include searching a limited number of MS/MS spectra from selected peptides in MASCOT,[29] manually annotating spectra, relying on peptide ion *m*/*z* alone, or comparing MSI datasets to LC-MS/MS-generated databases prepared from the same tissue.[49,50] An exciting development in this area is High resolution Informatics Toolbox in MALDI-MSI Proteomics (HIT-MAP), an R package which allows for the identification and quantification of peptides and proteins from MSI data. HIT-MAP performs segmentation of the imaging data (provided in.imzML and.ibd files) and subsequently performs peptide mass fingerprinting analysis, peptide scoring, protein grouping, and scoring. Finally, HIT-MAP generates ion images of identified peptides and proteins.[49]

MSI-guided spatial proteomics

Advantages of MSI-guided spatial proteomics. Beginning a spatial proteomics experiment with untargeted MSI of other biomolecules can provide invaluable biological context. In MSI-guided spatial proteomics, an initial MSI experiment, typically using a MALDI source, provides spatial regions of interest which are subsequently interrogated by chromatography-based proteomic methods. The initial imaging experiment analyzes the spatial distribution of lipids,[51–54] metabolites,[55] or peptides[56–58] on a tissue section. This dataset is used to define distinct regions of interest within the tissue, often by segmentation methods such as k-means clustering. A subsequent method is used to isolate and collect these regions from the tissue. Often, LCMD is used to precisely collect sub-mm^2 regions of the sample.[51] These subsamples then undergo an LC-MS proteomics workflow to identify proteins in each region, and the proteomics data can be used to support or further explain results of the MSI experiment. An overview of the general MSI-guided spatial proteomics workflow can be found in Fig. 3.

This general workflow capitalizes on the spatial information garnered from MSI and the chromatographic separation of LC-MS. Proteomic analysis by MSI alone inherently suffers from a limited dynamic range due to the technique's lack of chromatographic separation and typically only captures the top 5% of abundant proteins.[59] Moreover, MALDI is the most commonly used ionization method in MSI[14,60] and ions generated by this method tend to have lower charge states than ESI-generated ions. A lower charge state results in lower gas-phase fragmentation efficiency and therefore limited sequence coverage in MALDI.[17] MSI-guided spatial proteomics circumvents these limitations by using MSI data to define regions of interest and LC-MS to interrogate their proteomes.

Experimental variables. Many variables affect the outcome of this type of workflow, including slide substrate, the method of enzymatic digestion, and method of excising tissue after the MSI experiment is complete. Groups have shown consecutive MALDI MSI and LCMD on a single polyethylene naphtalate (PEN) slide[51,56] and on a single conductive slide.[52] The choice of slide influences performance of each stage of the workflow, as conductive slides typically work best for MALDI mass spectrometry and PEN slides provide a supportive structure and ensure release of the tissue regions in LCMD.

As stated in the protein MALDI MSI section, enzymatic digestion is required to break proteins into peptides, which are more readily analyzed by MS/MS. As in a classic LC-MS proteomics experiment, trypsin is the most commonly used enzyme.[17] How and when this enzymatic digestion occurs can vary greatly from workflow to workflow and with the needs of the researcher. Enzymatic digestion in these experiments can occur on-tissue, or in tubes after regions of interest have been collected (or both). As in the bottom-up protein MSI procedure, on-tissue digestion is typically performed using a robotic sprayer to apply the enzyme directly to the tissue section, allowing the sample to incubate, and applying MALDI matrix before analysis.[17] Dilillo *et al.* demonstrated atmospheric pressure (AP)-MALDI MSI, LCMD and LC-MS proteomics on a single slide of murine brain tissue, using peptide signals from the imaging experiment to guide subsequent separation and proteomic analysis of white and gray matter regions.[56] In this case, proteins were digested to peptides using on-tissue digestion. Peptides were analyzed from the same tissue section with both AP-MALDI imaging before LCMD and with LC-MS once regions were collected. Alberts *et al.* used peptide-based MALDI MSI to identify heterogenous tumor regions within five FFPE breast cancer samples. They found that spatial LC-MS proteomics, guided by the MALDI MSI segmentation, could discriminate between these tumor regions based on their proteomes.[57] Here, enzymatic digestion was first performed on one tissue section by spraying trypsin and incubating. A serial section of the tissue was then subjected to LCMD and the excised regions were digested with trypsin in sample tubes. Recently, Truong *et al.* demonstrated lipid-based MALDI MSI is capable of distinguishing tumor and benign tissue regions in prostate cancer radical prostatectomy cores, and used an LCMD-LC/MS workflow to perform subsequent proteomic analyses of the regions.[53] Here, on-tissue digestion was completely avoided and tissue regions excised by LCMD were enzymatically digested in tubes after lipid MSI. Recent work from Pace *et al.* demonstrates an interesting method of tissue digestion for MSI-guided proteomics that utilizes nanodroplet processing in one pot for trace samples (nanoPOTS) technology. The authors collected tissue samples from LCMD into nanowells to perform enzymatic digestion, followed by high-sensitivity LC-MS. The use of nanoPOTS enables proteomic analysis of small protein samples as it minimizes sample loss due to surface adsorption. The authors were able to correlate the proteomics data with separately gathered metabolomics data from MSI of serial tissue sections.[61] The method, timing, and combination of enzymatic digestion methods ultimately depends on whether the researcher is analyzing peptides or lipids/metabolites by MSI and whether serial sections or the same tissue section will be analyzed by MSI and LC-MS proteomics.

Alternatives to LCMD. While LCMD is a common option in MALDI-guided spatial proteomics, other methods of collecting

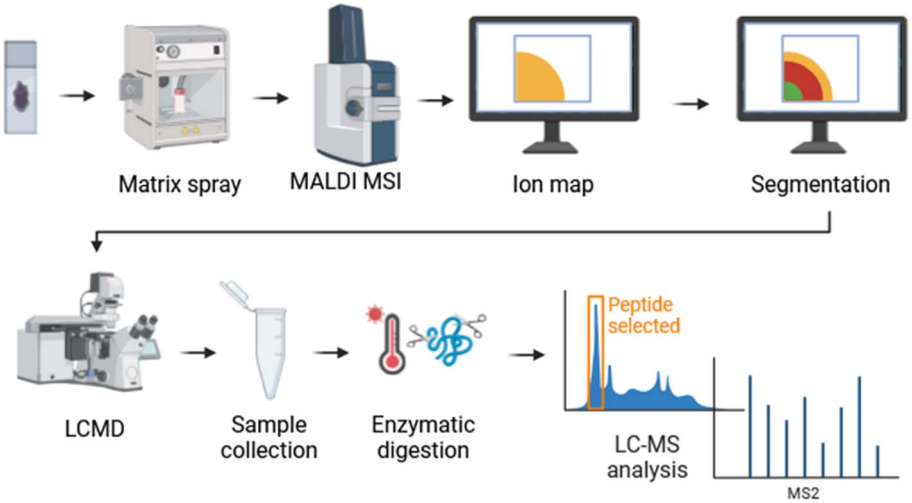

Fig. 3 General workflow of MSI-guided spatial proteomics. A sample mounted on a conductive slide is sprayed with matrix and MALDI MSI is carried out to generate ion images. Segmentation is performed on the dataset to generate regions of interest. These regions are excised (often by LCMD) and collected, then subjected to enzymatic digestion and subsequent LC-MS analysis. Note that in some workflows enzymatic digestion occurs *via* on-tissue digestion prior to application of the MALDI matrix, as shown in Fig. 1. In these cases, the digestion prior to LC-MS analysis is omitted as the sample proteins have already been converted to peptides.

protein or tryptic peptides from the tissue exist. Le Ruhn *et al.* followed peptide-based MALDI MSI with Liquid Extraction Surface Analysis (LESA) to collect peptides from selected regions. They demonstrated that subsequent spatial proteomics of these designated regions can be used to classify gliomas by molecular signatures, expanding on the standard World Health Organization classification guidelines.[58] Ryan *et al.* used LESA to identify proteins from a MALDI MSI experiment in both bottom-up and top-down methods.[62] Delcourt *et al.* followed lipid-based MALDI MSI with both liquid microjunction micro-extraction and parafilm-assisted microdissection to gather regions for top-down proteomics of rat brain.[54]

MSI-guided spatial proteomics combines the spatial information of MSI experiments with the proteome coverage and depth of LC-MS. For this reason, it is a popular approach to spatial proteomics, as evidenced by the breadth of variation in workflows that researchers have developed to fit their own experimental constraints and biological questions.

Future directions. The future of MALDI spatial proteomics will be dictated by advances in protein and peptide identification and integration with other "omics" methods (including image co-registration). Already, the MSI community has responded admirably to the challenge of protein and peptide identification. iprm-PASEF promises to aid in targeted imaging proteomics workflows by allowing for the selection and fragmentation of peptide precursors,[48] a feat not possible until the application of this technology. The HIT-MAP R package is a promising advancement in peptide mass fingerprinting of MALDI data, which is often the limiting factor in protein identification.[49] Another recent software development, ImShot, has been developed to identify MALDI MSI peptides and their parent proteins based on a corresponding LC-MS dataset using a novel "most likely peptide" scoring system that ranks the many possible LC-MS peptides matched to a single MALDI *m*/*z* feature.[63] The need for more streamlined, high-throughput solutions for both peptide selection and identification remains, but researchers are rising to the challenge as the popularity of spatial proteomics grows.

Both protein MALDI MSI and MSI-guided spatial proteomics are increasingly combined with other "omics" methods to generate more informative datasets. Schäfer *et al.* recently combined MALDI MSI, LCMD and LC-MS proteomics with spatial transcriptomics to identify, confirm, and map proteins in mouse hippocampus. The authors used the ImShot software to integrate their MALDI MSI and LC-MS/MS data, thereby identifying brain peptides and proteins, and confirmed these identifications with spatial transcriptomics and mining of public data sets.[64] Bharti *et al.* combined MALDI MSI, LC-MS/MS, H&E staining, and two-photon laser scanning microscopy to compare proteomic signatures across regions of differing collagen architecture and nuclei distribution in human colorectal cancer tissue.[65] Zhang *et al.* demonstrated the utility of a deep-learning-based model pre-trained on histopathology datasets to combine H&E and peptide MALDI MSI and ultimately distinguish invasive melanoma from benign growths in patient skin biopsies.[66] Critical to the integration of imaging sets from different modalities, including protein MALDI MSI data, is the development of increasingly precise co-registration methods.[67,68] Shojaei *et al.* recently proposed a feature-based method of multimodal image co-registration, while simultaneously demonstrating the combination of MALDI MSI and laser ablation inductively coupled plasma MSI data.[69] Liang *et al.* described an image fusion model for the correlation of MALDI MSI, immunohistochemistry, and brightfield microscopy images.[70] Spangenberg introduced msiFlow, an open-source software allowing for the co-registration of multimodal imaging data. Notably, msiFlow incorporates many more steps of multimodal co-registration than most open-source packages,

including data import, registration, and analysis.[71] While MALDI spatial proteomics is a valuable tool in its own right, its power as a complementary method when combined with other "omics" and imaging modalities is becoming increasingly apparent.

Conclusion

Spatial information is becoming increasingly important in omics fields, and through improvements in instrumentation and workflows, is becoming increasingly accessible to researchers. Where bulk proteomic analysis of biological samples used to overlook regional and cellular differences, spatial proteomics can now map regions of differential protein expression. The field of spatial proteomics relies upon a number of analytical techniques, with MALDI imaging playing a crucial role in both targeted and untargeted analyses. Broadly, MALDI-based methods can be classified as protein MALDI MSI or MSI-guided spatial proteomics. Advances in these methods have led to increased depth of coverage, accuracy, and spatial resolution when mapping and identifying proteins. Here, we have detailed the main approaches, highlighting examples from the literature and discussing workflows for each. We have included strengths and limitations of the approaches and cited new and exciting developments that will strengthen the field. Spatial proteomics is a rapidly expanding discipline and the applications of MALDI and associated mass spectrometry-based techniques are diverse. It is our hope that this review will serve as a guide for researchers approaching biological questions that require the insight provided by spatial proteomics.

Conflicts of interest

There are no conficts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Acknowledgements

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