Protein identification in mass-spectrometry imaging

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With a rapidly growing number of biomedical applications of mass-spectrometry imaging (MSI) and expansion of the technique into the clinic, spectrum annotation is an increasingly pressing issue in MSI. Although identification of the species of interest is the key to answering biomedical research questions, only few of the hundreds of observed biomolecular signals in each MSI spectrum can be easily identified or interpreted. So far, no standardized protocols resolve this issue.

Present strategies for protein identification in MSI, their limitations and future developments are the scope of this review. We discuss advances in MSI technology, workflows and bioinformatic tools to improve the confidence and the number of protein identifications within MSI studies.

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Abbreviations: CID, Collision-induced dissociation; ETD, Electron-transfer dissociation; ESI, Electrospray ionization; FT-ICR, Fourier transform ion cyclotron resonance; IHC, Immunohistochemistry; IMS, Ion-mobility spectrometry; LC, Liquid chromatography; LCM, Laser-capture microdissection; MALDI, Matrix-assisted laser desorption/ionization; MS, Mass spectrometry; MSI, Mass-spectrometry imaging; PTM, Post-translational modification; MS/MS, Tandem mass spectrometry; ROI, Region of interest; TOF, Time-of-flight

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1. Introduction

In recent years, technological and methodological advances have brought mass-spectrometry imaging (MSI) to the biomedical field. MSI allows analysis and visualization of peptides, proteins, lipids, metabolites and pharmaceuticals directly from biological tissues and cell samples [1,2]. The technique uses a surface-sampling process, in which mass spectra are collected at discrete locations, according to a predefined Cartesian grid. In this way, the distribution of ions of interest can be mapped.

MSI has several advantages compared to other imaging techniques {e.g., immunohistochemistry (IHC) or positron emission tomography}. It has the capability to detect hundreds of (unknown) compounds simultaneously in one molecular imaging experiment, allowing multiplexed analysis and discovery-based research. As MSI requires no target-specific labeling, unmodified species can be studied. Importantly, in contrast to standard MS analysis, which requires tissue homogenization, MSI leaves the molecular distributions in the tissue intact, so it can be utilized to assess molecular differences between specific cellular regions within tissues.

An increasing number of studies report on applications of MSI in the biomedical field. MSI is used in distribution studies of pharmaceutical compounds and their metabolic products for drug evaluation [3,4] and in (clinical) proteomics applications [5,6]. MSI has already been employed to assist in diagnosis, prognosis and biomarker discovery, when the technique is utilized to construct protein profiles that predict a disease status or progression, to identify molecular patterns for disease prognosis and to assess molecular markers in treatment-response studies [7–9]. Not only can a better fundamental understanding of the molecular processes underlying disease be acquired using MSI, but this knowledge can also aid in the development of new drugs and treatments {e.g., the study of the molecular basis of intratumor heterogeneity is not only expected to lead to improved understanding of tumor biology, but also fits the trend towards personalized medicine [10,11]}.

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The most widely-used ionization technique for MSI is matrix-assisted laser desorption/ionization (MALDI) [12]. MALDI-MSI was introduced in 1997 by Caprioli and co-workers and utilizes a matrix, typically an acidic aromatic compound [13]. As the matrix compound absorbs energy at the wavelength of the laser, exposure of the crystals to laser pulses results in desorption and ionization of the sample. Ions are separated based on their mass-to-charge (m/z) ratio, usually by a time-of-flight (TOF) mass analyzer, which is high-throughput and sensitive, and has a broad mass range [14].

Despite the fast developments in MSI technology and workflows, several challenges still need to be addressed for MSI to become an established tool in biomedical research. Apart from the need for improved mass resolution, spatial resolution and sensitivity of the instruments used for MSI, an important limitation is that only a few of the hundreds of observed signals in each mass spectrum can be easily identified or interpreted. Annotation of ions of interest requires an additional step in the experimental workflow and so far no standardized protocols exist that solve this issue. Identification might be hampered by (unknown) modifications, even when the compound class is known. This holds for example for ions derived from proteins, where post-translational modifications (PTMs), protein isoforms and chemical modifications resulting from sample preparation or proteolysis can hinder interpretation.

However, from the point of view of a biomedical researcher, identification of the species of interest is an essential step to solve a biomedical research question. Although recently studies have been published in which statistical data-analysis tools were used to annotate tissues solely based on their MS profiles [10,11,15], MSI data need to be complemented with information on the nature of the biomolecular species to access the full potential of MSI [16].

With a rapidly growing number of biomedical applications and expansion of MSI into the clinic, spectrum annotation is an increasingly pressing issue in MSI. Present strategies to provide annotation of MSI spectra, their limitations, and newly developed identification strategies are the scope of this review. As proteins are the biomolecules most often probed by MSI in a biomedical context, this review focuses on protein identification in MSI. However, confident chemical assignment of any biomolecular species in MSI spectra faces similar challenges and some of the approaches described here could also be employed in that context.

2. MS-based protein-identification methods

MS is an established analytical technique for protein characterization at both species level and proteome level. Numerous, often very sophisticated, methods of MS-based protein identification have been developed [17,18]. Current MSI has implemented MS-based protein-identification methods in its workflows according to the needs and constraints posed by the technique.

In general, two approaches exist for MS-based protein identification:

(1) ***In a top-down experiment***, identification is performed through intact mass measurement followed by tandem mass spectrometry (MS/MS) analysis. Sequence-specific fragmentation patterns are used for identification through database searching, in which the experimentally-obtained fragments are compared with theoretical fragments. A top-down approach in MALDI-MSI works for small-to-medium-sized proteins up to 7–10 kDa, because large singly-charged molecules will not easily dissociate.

(2) ***In a bottom-up experiment***, a protein or protein mixture is first enzymatically digested. The resulting proteolytic peptides are analyzed by MS (so called peptide-mass fingerprinting), and by MS/MS in the case of a protein mixture. In a bottom-up imaging approach, multiple peptide matches per protein are required for confident identification of the protein.

An ideal MSI experiment consists of automatically triggered MS/MS experiments on proteins or peptides directly from tissue, thereby combining the localization of species with their identification within a single experiment. However, low sensitivity seriously hampers the identification, compared to standard identification approaches using protein extraction followed by gel-based separation or liquid chromatography (LC) coupled to electrospray ionization (ESI) MS/MS. The low sensitivity is caused by ion-suppression effects due to the complex molecular composition of tissue. In addition, ions generated by MALDI typically have only unit charge. The resulting inefficient ion activation of larger ions renders intact proteins too big for direct identification through fragmentation. As a result, efficient MS/MS can only be performed in a mass range of 500–3500 Da on the majority of mass spectrometers used for MSI. Fig. 1 summarizes the protein-identification workflows utilized in MSI.

2.1. Top-down approaches in MSI

Few examples of a top-down approach used in MALDI-MSI can be found in literature. Minerva and co-workers identified several endogenous peptides up to 3.5 kDa using MALDI-TOF/TOF directly on mouse pancreatic tissue [19]. Alternative identification strategies are employed to annotate larger masses, which combine fractionation of tissue extracts by LC, MALDI-MS for fraction selection, followed by ESI-MS/MS [20–22]. In this way, identification of the 8.4-kDa cysteine-rich intestinal protein 1 in breast-cancer tissue was demonstrated [20]. Top-down analysis of the 14+ charge state resulted in

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