Imaging Mass Spectrometry for Spatial Metabolomics

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Introduction

Over the past decade, mass spectrometry (MS) has seen major technical advances that have increased the scope, applicability and adoption of the technology in a vast array of research areas (1). The application of MS to biochemical imaging via imaging mass spectrometry (IMS) has emerged as one of the leading spatial analysis technologies in biological systems. There are many recent excellent comprehensive reviews of IMS (1-13). In this article we provide an introduction to matrix-assisted laser desorption ionisation (MALDI) IMS. The 'omics technologies – genomics, transcriptomics, proteomics and metabolomics (and others) – have provided insights into biochemistry, physiology and biology and are at the forefront of discovery in modern systems biology (14). Colloquially, the potential of the genome is described in the statement "What could happen," the transcriptome "What appears to be happening," the proteome "What's doing the work," and the metabolome "What is currently happening and what has already happened." The exquisite specialisation and compartmentalisation of biological systems require spatial approaches to allow examination of "Where things are happening" to unveil the underlying biology.

In the past, spatial analysis has been conducted using a number of different techniques, which can be broadly categorised into two approaches: 1) *in vitro* isolation and extraction of individual tissue/cell types and 2) *in situ*, including *in vivo*, analysis using an imaging approach. The suite of technologies available for *in situ* imaging is enormously powerful and varied; including Fourier transform infra-red spectroscopy, magnetic resonance imaging, electron microscopy, and histochemical and immunolabelling approaches coupled with optical and fluorescence microscopy. Each approach takes advantage of different physical and chemical properties of the underlying tissue to provide unique insights. IMS has a number of advantages which are directly derived from the capabilities of modern MS instrumentation, which provide molecular specificity, high sensitivity, and the ability to measure a broad range of analytes at high mass resolving power with high mass accuracy across wide mass ranges. IMS can provide very high lateral resolution, enabling molecular distinction across fine morphological features within tissues.

Although the concept of using MS for imaging was introduced in 1962 (secondary ion mass spectrometry, SIMS), it was not until the mid-'90s, with the introduction of soft ionisation techniques, in particular MALDI, that IMS began to be applied to the biosciences (15,16). IMS has advanced using a variety of different ion sources and approaches. It has found extensive use in molecular pathology and histology where the technique is used to map the spatial distribution of proteins and small molecules (including drugs, lipids and endogenous metabolites) within tissues (1,12). IMS has been demonstrated to have a number of advantages, including a label-free analysis and the simultaneous multiplex measurement of hundreds to possibly thousands of analytes in a single imaging experiment, providing rich, high density multidimensional data. Combination of IMS with advanced software and data analysis techniques now allows the virtual microdissection and interrogation of the molecular makeup of individual tissues. Lately, advances in spatial resolution have placed IMS at the forefront of single cell metabolomics (17,18).

Imaging Mass Spectrometry

There are four steps in a basic IMS experiment: 1) sample selection and preparation, 2) desorption and ionisation, 3) mass-analysis and 4) image registration and data analysis. Careful control of each is essential to enable generation of high quality images. In particular, sample selection, storage and preparation has a disproportionate impact on the final results; many sample preparation steps or techniques have the potential to contaminate the tissue section with exogenous material, affecting reproducibility, ionisation and image quality. Fundamentally, the IMS process involves placing a suitable tissue section into an ion source, ionising the sample and collecting a series of mass spectra. This series of individual mass spectra is collected in a two dimensional (2D) array across the tissue section or the surface of a tissue using one of a range of different ion sources and mass analysers. The most common approach is a microprobe approach where for each spatial coordinate, a single corresponding mass spectrum is collected. The resulting mass spectra represent the ionisable molecules present as their mass-to-charge ratios *(m/z)* that are then correlated with a high resolution optical image of the tissue or histochemical stain, with each spectrum assigned as an individual pixel for image generation. When the intensity value of each ion is plotted as an intensity map across the 2D array, the resultant reconstructed ion image represents the spatial distribution of the corresponding molecule(s). Three dimensional (3D) approaches are also possible, where serial 2D arrays from sequential tissue sections (or depth profiling) from the one tissue sample are measured and then used for computational reconstruction of a 3D volume.

Ionization and Mass Analysis

IMS relies on the ability to form ions that are then transferred under vacuum and measured by the mass analyzer (**Fig. 1**). Currently, the dominant ion source and approach is MALDI, owing to a range of commercial instruments that display high spatial and mass resolutions,

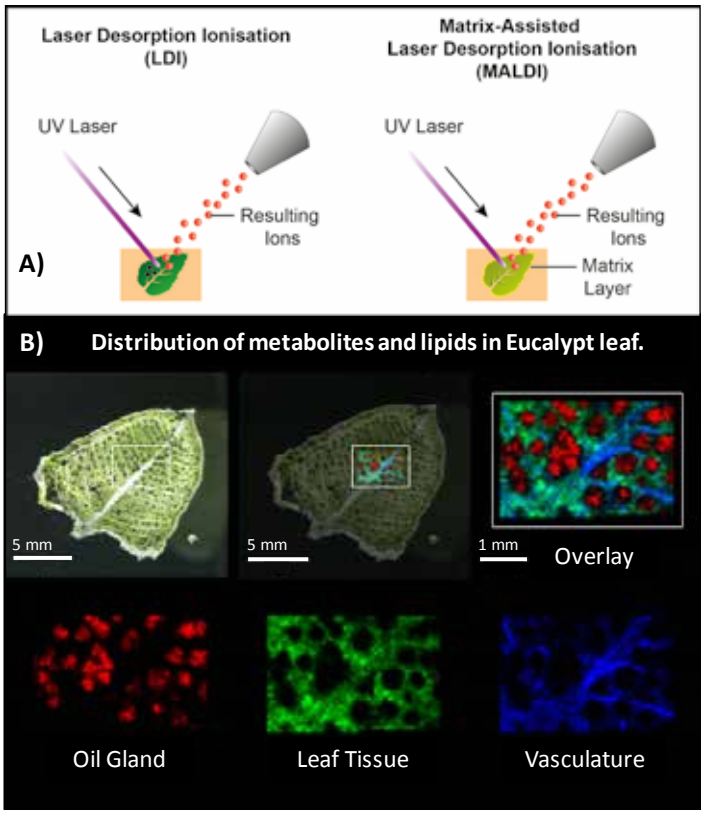

Fig. 1A. Laser desorption ionisation (LDI) approach versus matrix-assisted laser desorption ionisation (MALDI) approach. For LDI, no matrix is applied and ions are generated by directly ablating tissue, relying upon the presence of endogenous UV absorbing molecules to aid desorption. For MALDI, a thin layer of chemical matrix is applied across the surface of the tissue. The matrix preferentially absorbs the UV light, desorbing analytes from the surface.

B. Results showing the distribution of a number of different *Eucalyptus* species metabolites generated using LDI. Metabolites can be mapped to the oil glands (secretory cavities), general leaf tissue and vasculature. Data generated on a 7T Bruker SolariX fourier transform ion cyclotron mass spectrometer. Images generated using FlexImaging 4.1.

ease of use and a broad range of applicability to a variety of biological applications. In practice, lateral resolutions for MALDI instruments are in the 5–50 µm range. The past three to five years has seen an explosion in the availability of different different types of ion sources, particularly specialised sources for ambient ionisation conditions (19). Further, a number of popular alternative ion sources exist, including SIMS, desorption electrospray ionisation (DESI), and laser ablation electrospray ionisation (LAESI). When operating IMS instruments at very high spatial resolutions, there is also a significant trade-off against sensitivity: as the sampling area decreases, the total number of ions decreases, and the overall sensitivity for individual ions decreases. Advanced mass analysers and detectors are now allowing the measurement of very low numbers of ions, which to some degree mitigates losses in sensitivity at high lateral resolution.

The mass analyser is the core component of a MS, enabling determination of the *m/z* of an ion. The type of mass analyser used and the spectral resolution achievable also has a direct impact on the ability to conduct IMS experiments. Types of mass analysers used in IMS instruments include: 1) unit resolution, encompassing quadrupole and ion-trap technologies, typically a linear ion trap; 2) accurate mass, including time-of-flight (TOF) instruments; and 3) Fourier transform (FT), encompassing both Orbitrap and FT ion cyclotron resonance (FT-ICR) instruments. For metabolites, there is a clear need for accurate mass and high mass resolving instruments and/or the use of tandem MS to distinguish between differing metabolites in tissues. Low mass resolution instruments can lead to misidentification or misinterpretation due to an inability to resolve peaks of similar mass. IMS experiments are less sensitive than analyses that orthogonally separate analytes prior to measurement and detection; this is directly due to the complexity of biological tissues, where vast concentration ranges of chemical entities are present with differing chemistries and molecular sizes (e.g. proteins, lipids, organic acids, amino acids, carbohydrates, inorganic ions etc.). For MALDI experiments, the presence of high abundance, low molecular weight ions generated directly from the matrix constitute significant interfering signals. The ability of a MS to distinguish one mass peak from another peak close in mass is described by both mass resolution and resolving power (RP). Higher mass resolution allows easier identification of contributing ions and exclusion of interference from the presence of other chemical entities. Higher mass RP is essential for high mass accuracy, whereby a higher RP allows identification of the centre of the peak and determination of the mass error, with low mass error allowing unambiguous assignment of a molecular formula, aiding in identification. Modern high resolution instruments are capable of <10 parts per million (ppm) mass error for TOF and <2 ppm mass error for FT instruments. Measurements conducted on low mass resolution instruments are typically operated in a targeted tandem MS approach to provide molecular selectivity where specific fragment ions of single analytes are monitored, providing both molecular specificity and increased sensitivity. IMS measurements using higher resolution detectors provide the ability to unambiguously resolve a peak from the complex spectra that are generated, allowing untargeted profiling type techniques.

A hybrid approach that uses ion mobility coupled to mass spectrometry (IM-MS) that first separates ions by their mobility in a carrier gas followed by detection by

MS has recently been developed (20,21 and previous article). IM-MS offers the ability to orthogonally separate ions in the gas phase with similar *m/z* but different shapes via collisional cross section (CCS), providing a number of benefits including better signal-to-noise and the potential to separate isomers according to their shape and charge. The application of IM-MS to MALDI-IMS experiments provides much promise for the analysis of large lipids, peptides and proteins; however, the benefits of IM-MS for small molecule analysis are still being assessed.

Applications

MALDI-IMS has been used extensively in biomedical research with hundreds of studies published. More recently, interest in mapping the distribution of plant metabolites has taken off with around 80 publications focused on plant secondary metabolites, primary metabolism, disease states and environmental interactions. We have developed a number of methods to examine plant metabolites using a laser desorption ionisation (LDI) approach in *Eucalyptus* species (**Fig. 1B**). The LDI approach (22) relies upon the natural abundance of UV-absorbing plant secondary metabolites to enhance ionisation directly from tissue. Using LDI, we have been able to map the distribution of a number of different plant metabolites, including non-volatile oleuropeyl-glucose esters and flavonones, to the extracellular domain of leaf secretory cavities (oil glands) (23). The secretory cavities of *Eucalyptus* species are characterised by their distinctive composition of volatile terpenes and a layer of non-volatile components directly adjacent to highly metabolically active secretory cells. The non-volatile components have been hypothesised to provide a potential buffer against auto-toxic terpenes. Our LDI data show distinct colocalisation of a number of different oleuropeyl-glucose esters to the edges of the secretory cavities of sectioned leaf, confirming previous results generated from fluorescence imaging and laborious manual collection, extraction and MS analysis of secretory cavity content.

More recently, we have developed new matrices and analytical techniques to examine lipids, including glycerophospholipids and unstable glycosphingolipids in mammalian tissues (**Figs. 2, 3**). Using high mass resolution MALDI-IMS on a Bruker Solarix FT-ICR-MS, we have been able to distinguish different lipid classes, including elusive sulfatides that are similar in mass to phosphatidylserine (**Fig. 2**). Gangliosides are high mass glycosphingolipids that are found in relatively high concentrations in neurons and are directly involved in or implicated in a number of different disease states including Tay-Sachs disease, Fabry's disease, Alzheimer's, Guillain-Barré syndrome and during infection by pathogens that cause conditions such as botulism, cholera and influenza, amongst others. Gangliosides are built upon a core ceramide backbone conjugated to a variable oligosaccharide, which is typically made up of a number of hexose units (e.g. galactose, glucose, hexosamines and N-acetyl-hexosamines), and sialic acid (N-acetylneuraminic acid). Previous MALDI approaches using traditional matrices lead to in-source fragmentation and loss of sialic acid residues or do not display sufficient sensitivity to observe very low abundant acetylated species (24,25). Our research has shown an increase in the total number of glycosphingolipids observed and increased sensitivity for low abundant species (**Fig. 3A**). Using an experimental proton sponge analogue to measure lipids in rat brain we have been able to observe the major ganglioside species GM1 and GD1, and an acetylated GD1 species (**Fig. 3B**). In total, we were able to tentatively identify by accurate mass (<5 ppm mass error using SimLipid 4.3 software (Premier Biosoft, Palo Alto, CA, USA)) up to 69 different

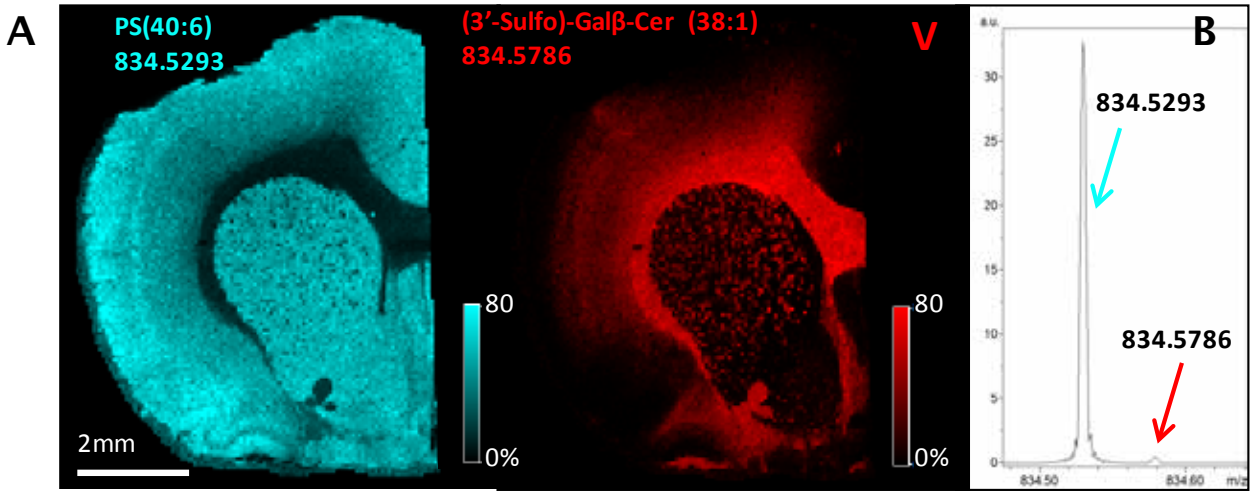

Fig. 2A. Distribution of two lipid species with similar masses in coronal sections of rat brain, displaying vastly different signal intensities and spatial distributions. Tentative identification by accurate mass match (<5 ppm mass error) using SimLipid 4.3 (Premier BioSoft). Blue – PS(40:6) = phosphatidylserine (40:6), *m/z* = 834.5293 (-0.29 ppm mass error); red – (3'-sulfo)-Galβ-ceramide (38:1) = N-eicosanoyl-1-β-(3'-sulfo)-glucosyl-sphing-4-enine, *m/z* = 834.5786 (-1.85 ppm mass error).

B. Mass spectra showing difference in response of PS(40:6) (blue) and (3'-sulfo)-Galβ-ceramide (38:1) (red). Data generated on a 15T Bruker SolariX FT-ICR-MS. Images generated using FlexImaging 4.1.

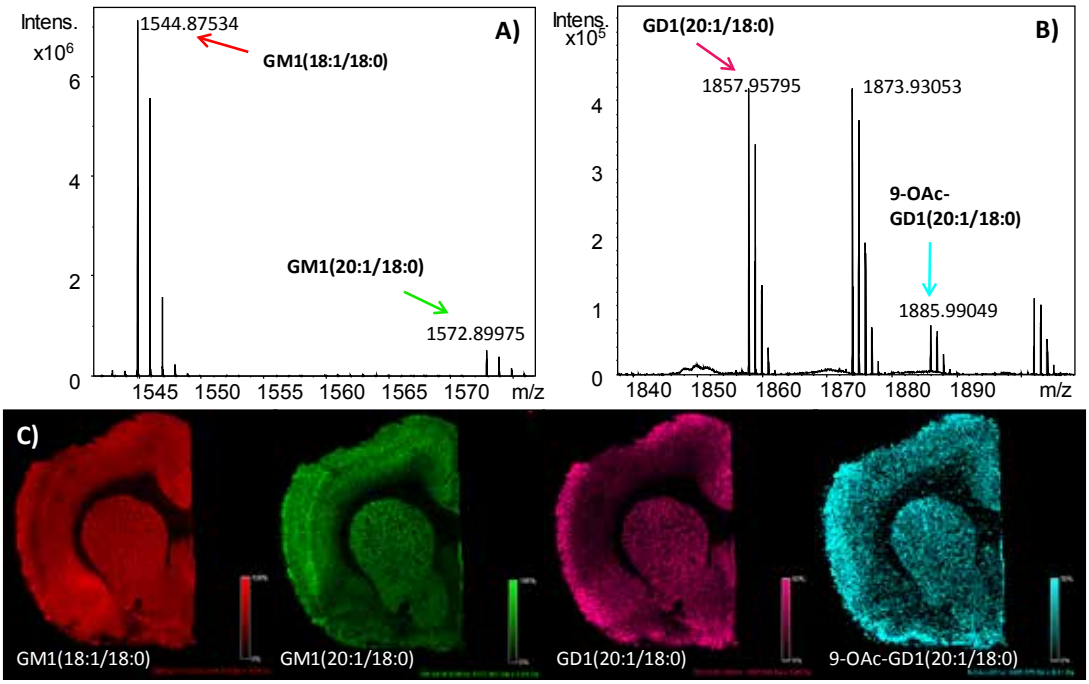

Fig. 3A. Average mass spectra of high mass lipid range, *m/z* 1540–1575, displaying ions for a range of tentatively identified gangliosides (SimLipid 4.3). Red – GM1(18:1/18:0), *m/z* = 1544.875 (-4 ppm mass error); and green – GM1(20:1/18:0), *m/z* = 1572.901 (-1 ppm mass error).

B. Average mass spectra of high mass lipid range, *m/z* 1840-1900, displaying ions for a range of tentatively identified gangliosides (SimLipid 4.3) Pink – GD1(20:1/18:0), *m/z* = 1857.956 (-12 ppm mass error); and blue – 9-Oac-GD1(20:1/18:0), *m/z* = (-7 ppm mass error).

C. Distribution of respective gangliosides in rat brain coronal section. GM1(18:1/18:0) and GM1(20:1/18:0) signal intensity scaled 0–100%, GD1 signal intensity scaled 0–50%, 9-OAc-GD1 signal intensity scaled 0–30%. Data generated on a 15T Bruker SolariX FT-ICR-MS. Images generated using FlexImaging 4.1.

glycosphingolipids, ceramides, sphingomyelin, sphingoid bases and sulfatides, providing much promise for future spatial ganglioside research.

Conclusion

MALDI-IMS has demonstrated applications in a vast range of spatial biochemical and metabolomics research. The application of ultra-high resolution and high mass accuracy MS provides the ability to distinguish molecular species very close in mass and accurately determine molecular formula. The rich, multidimensional and highly dense data are currently providing unique insights into the vast chemical complexity and specialisation found within biological systems that is not possible using other methods. With the technology and approaches now established in the Australian research community the future looks very promising.

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