From Whole-body Sections Down to Cellular Level, Multiscale Imaging of Phospholipids by MALDI Mass Spectrometry*[

Pierre Chaurand‡, Dale S. Cornett§, Peggi M. Angel, and Richard M. Caprioli¶

Significant progress in instrumentation and sample preparation approaches have recently expanded the potential of MALDI imaging mass spectrometry to the analysis of phospholipids and other endogenous metabolites naturally occurring in tissue specimens. Here we explore some of the requirements necessary for the successful analysis and imaging of phospholipids from thin tissue sections of various dimensions by MALDI time-of-flight mass spectrometry. We address methodology issues relative to the imaging of whole-body sections such as those cut from model laboratory animals, sections of intermediate dimensions typically prepared from individual organs, as well as the requirements for imaging areas of interest from these sections at a cellular scale spatial resolution. We also review existing limitations of MALDI imaging MS technology relative to compound identification. Finally, we conclude with a perspective on important issues relative to data exploitation and management that need to be solved to maximize biological understanding of the tissue specimen investigated. Molecular & Cellular Proteomics 10: 10.1074/mcp.O110.004259, 1–11, 2011.

Since its introduction in the late 90s (1), MALDI imaging mass spectrometry (MS) technology has witnessed a phenomenal expansion. Initially introduced for the mapping of intact proteins from fresh frozen tissue sections (2), imaging MS is now routinely applied to a wide range of different compounds including peptides, proteins, lipids, metabolites, and xenobiotics (3–7). Numerous compound-specific sample preparation protocols and analytical strategies have been developed. These include tissue sectioning and handling (8–14), automated matrix deposition approaches and data acquisition strategies (15–21), and the emergence of in situ tissue chemistries (22–25). Originally performed on sections cut from fresh frozen tissue specimens, methodologies incorporating an in situ enzymatic digestion step prior to matrix application have been optimized to access the proteome locked in formalin-fixed paraffin-embedded tissue biopsies (25–29). The possibility to use tissues preserved using non-cross-linking approaches has also been demonstrated (30–32). These methodologies are of high importance for the study of numerous diseases because they potentially allow the retrospective analysis for biomarker validation and discovery of the millions of tissue biopsies currently stored worldwide in tissue banks and repositories.

In the past decade, instrumentation for imaging MS has also greatly evolved. Whereas the first MS images were collected with time-of-flight instruments (TOF) capable of repetition rates of a few hertz, modern systems are today capable of acquiring data in the kilohertz range and above with improved sensitivity, mass resolving power, and accuracy, significantly reducing acquisition time and improving image quality (33, 34). Beyond time-of-flight analyzers, other MALDI-based instruments have been used such as ion traps (35–37), Qq TOF instruments (38–40), and trap-TOF (16, 41). Ion mobility technology has also been used in conjunction with imaging MS (42–44). More recently, MALDI FT/ICR and Orbitrap mass spectrometers have been demonstrated to be extremely valuable instruments for the performance of imaging MS at very high mass resolving power (45–47). These non-TOF-based systems have proven to be extremely powerful for the imaging of lower molecular weight compounds such as lipids, drugs, and metabolites. Home-built instrumentation and analytical approaches to probe tissues at higher spatial resolution (1–10 μm) have also been described (48–50). In parallel to instrumentation developments, automated data acquisition, image visualization, and processing software packages have now also been developed by most manufacturers.

To date, a wide range of biological systems have been studied using imaging MS as a primary methodology. Of strong interest are the organization and identification of the molecular composition of diseased tissues in direct correlation with the underlying histology and how it differs from healthy tissues. Such an approach has been used for the study of cancers (51–54), neurologic disorders (55–57), and other diseases (58, 59). The clinical potential of the imaging MS technology is enormous (7, 60, 61). Results give insights into the onset and progression of diseases, identify novel sets of disease-specific markers, and can provide a molecular confirmation of diagnosis as well as aide in outcome predic-
tion (62–64). Imaging MS has also been extensively used to
study the development, functioning, and aging of different
organs such as the kidney, prostate, epididymis, and eye lens
(65–70). Beyond the study of isolated tissues or organs, whole-body sections from several model animals such as
leeches, mice, and rats have been investigated (71–74). For
these analyses, specialized instrumentation and protocols are
necessary for tissue sectioning and handling (72, 73). Whole-
body imaging MS opens the door to the study of the local-
ization and accumulation of administered pharmaceuticals
and their known metabolites at the level of entire organisms
as well as the monitoring of their efficacy or toxicity as a
function of time or dose (72, 73, 75, 76).

There is considerable interest in determining the identifi-
cation and localization of small biomolecules such as lipids
in tissues because they are involved in many essential bio-
ological functions including cell signaling, energy storage,
and membrane structure and function. Defects in lipid me-
tabolism play a role in many diseases such as muscular
dystrophy and cardiovascular disease. Phospholipids in tis-
sues have been intensively studied by several groups (37,
40, 77–83). In this respect, for optimal recovery of signal,
several variables such as the choice of matrix for both
imaging and fragmentation, solvent system, and instrument
polarity have been investigated (20, 84). Particularly, the use
of lithium cation adducts to facilitate phospholipid identi-
fication by tandem MS directly from tissue has also been
reported (85). Of significant interest is the recent emergence
of two new solvent-free matrix deposition approaches that
perform exceptionally well for phospholipid imaging analy-
ses. The first approach, described by Hankin et al. (86),
consists in depositing the matrix on the sections through a
sublimation process. The described sublimation system
consists of sublimation glassware, a heated sand or oil bath
(100–200 °C), and a primary vacuum pump (∼5 × 10⁻²
torr). Within a few minutes of initiating the sublimation pro-
cess, an exceptionally homogeneous film of matrix forms on
the section. The thickness of the matrix may be controlled
by regulating pressure, temperature, and sublimation time.
The second approach, described by Puolitaival et al. (87),
uses a fine mesh sieve (≈20 μm) to filter finely ground
matrix on the tissue sections. Agitation of the sieve results
in passage of the matrix through the mesh and the deposi-
tion of a fairly homogeneous layer of submicrometer matrix
crystals of the surface of the sections. The matrix density on
the sections is controlled by direct observation using a
standard light microscope. This matrix deposition approach
was also found to be ideal to image certain drug com-
ponents (88, 89). Both strategies allow very rapid production
of homogeneous matrix coatings on tissue sections with a
fairly inexpensive setup. Signal recovery was found to be
comparable with those obtained by conventional spray dep-
oosition. With the appropriate size sublimation device or
sieve, larger sections with dimensions of several centime-
ters such as those cut from mouse or rat whole bodies can
also be rapidly and homogeneously coated.

Here we present several examples of MALDI imaging MS of
phospholipids from tissue sections using TOF mass spec-
trometers over a wide range of dimensions from whole-body
sections (several centimeters), to individual organs (several
millimeters), down to high spatial resolution imaging of se-
lected tissue areas (hundreds of micrometers) at 10-μm lat-
eral resolution and below. For all of these dimension ranges,
technological considerations and practical aspects are dis-
cussed. In light of the imaging MS results, we also address
issues faced for compound identification by tandem MS anal-
ysis performed directly on the sections. Finally, we discuss
under “Perspective” our vision of the future of the field as well
as the technological improvements and analytical tools that
need to be improved upon and developed.

MATERIALS AND METHODS

Chemicals

Matrix, 2,5-dihydroxybenzoic acid (DHB),¹ was purchased from Sigma-Aldrich and recrystallized in house.

Mouse brains were purchased frozen from Pel-Freez Biologicals (Rogers, AR). One-day-old whole ICR mouse pups and one female ICR adult were a gift from H. Scott Baldwin (Vanderbilt University). All mice were sacrificed according to Vanderbilt University Institutional Animal Care and Use Committee-approved protocols. After sacrifice, (a) the pups were immediately flash frozen by slowly plunging them into liquid nitrogen to avoid shattering, and (b) the heart was dis-
sected from the adult mouse and immediately flash frozen by immer-
sion in liquid nitrogen.

For sectioning, all tissue samples were immobilized with embed-
ding medium, cut on a Leica CM3050 cryostat (Leica Microsystems
GmbH, Wetzlar, Germany), and thaw-mounted on a gold-coated
MALDI target plate (mouse brain) or on indium-tin oxide-coated glass
slides (Delta Technologies, Stillwater, MN) (mouse pup and heart).
Brain sections were cut at a thickness of 10 μm, whereas the mouse
pup sections and adult heart were cut at a thickness of 12 μm.
All sections were allowed to dry in a desiccator prior to matrix applica-
tion. To assess the histological accuracy of the imaging MS data,
serial sections were also collected and stained with hematoxylin and
eosin (H&E) according to standard protocols.

Matrix Application

Matrix application was performed according to published protocols
(86, 87). Imaging MS data applications were performed on the same
day as sectioning.

Sublimation—Matrix deposition was performed in a sublimation
apparatus (Chenglass Life Science, Vineland, NJ) (supplement-
mental Fig. 1). The gold-coated MALDI plates or glass slides were
mounted on the bottom of the condenser using thermally conductive
double sided tape (3M, St. Paul, MN). The target plate was kept cold
(∼0 °C) by adding ice slush to the reservoir. Approximately 300 mg of
in-house recrystallized 2,5-DHB matrix was deposited in the bottom
of the condenser sleeve. Matrix was distributed evenly to ensure an

¹ The abbreviations used are: DHB, dihydroxybenzoic acid; TIC, total ion current.
even and homogenous matrix coating on the section. The sublimation apparatus was connected to a primary vacuum pump (EZM12, Edward, Tewksbury, MA) via a vacuum connection valve. A cold trap was installed between the primary pump and the sublimation apparatus to capture sublimated matrix particles carried by the vacuum flow. This significantly helps in maintaining a clean and properly functioning vacuum gauge. The condenser apparatus and the bottom flask were sealed via a Viton O-ring. The primary pump was started, and a $5 \times 10^{-7}$-torr vacuum was established in the apparatus. The system was allowed to equilibrate under vacuum for a period of 2 min. After stabilization of vacuum was assured, sublimation was initiated by placing the bottom of the sublimation apparatus in a sand bath heated to $-120^\circ$C. 2,5-DHB sublimation was allowed to progress for $4 \text{ min}$. This resulted in a thin homogeneous 5–10-$\mu$m-thick coating of matrix on the sections. The process was halted by removing the sublimation apparatus from the sand bath and closing the vacuum valve. The sublimation apparatus was allowed to slowly re-equilibrate to atmospheric pressure before removal of the sample plate.

**Dry Coating—**Dry coating was performed as described previously (87) (supplemental Fig. 2). Briefly, a slide with a thawed-mount tissue section was attached by double sided tape to the receiving pan of an automated shaker. Approximately 1 g of finely ground 2,5-DHB was added to a 20-$\mu$m mesh U. S. A. standard stainless steel test sieve (Hogentogler and Co., Columbia, MD) with five glass milling balls. The sieve with 2,5-DHB was attached to the receiving pan, covered, and placed onto an automated shaker (Analysette 3 Spartan, Fritsch, Idar-Oberstein, Germany). The amplitude of the milling balls was manually adjusted to a height of 1 inch, and sieving was performed for a total of 20 min when a uniform coating was observed across the tissue.

**MALDI Imaging Mass Spectrometry**

**Low Spatial Resolution Imaging—**Low spatial resolution imaging MS (100–200 $\mu$m) was performed on a MALDI-TOF/TOF Ultraflex III mass spectrometer equipped with SmartBeam technology operating at 200 Hz (Bruker Daltonics, Billerica, MA). MALDI MS data acquisition was performed in reflectron geometry under optimized delayed extraction conditions (focused at $m/z \sim 800$) with a source accelerating voltage of $\pm 20$ kV. With optimal source settings, a mass resolving power of $M/\Delta M \sim 20,000$ was achieved in the studied mass range for phospholipids between $m/z$ 600 and 1000. External mass calibration was performed using 100 nl of a mixture of standard peptides deposited onto the sample plate after matrix deposition. With both positive and negative polarities, this mixture provides nine calibration points in the mass range between 428 and 1480 Da and allows mass accuracy for phospholipids to be typically better than 40 ppm across a whole-body image. Imaging MS data acquisition and image reconstruction and visualization were performed with FlexImaging 2.1 software.

**High Spatial Resolution Imaging—**High spatial resolution imaging MS (25–5 $\mu$m) was performed on a previously characterized homebuilt MALDI-TOF system (48). This system was recently upgraded with a higher performance N2 laser (Marathon 103-PD, LTB Laser-technik Berlin, Berlin, Germany). Briefly, the low divergence of the laser beam, the coaxial laser illumination ion source geometry, and the control of the position by a Piezo stage of the sample plate on the time-of-flight axis achieve on-target surface irradiation of less than 5 $\mu$m in diameter. For all experiments, precise adjustment of the laser energy and number of laser shots per spot was predetermined with respect to desired spatial resolution. MALDI MS data acquisition was performed in linear geometry under optimized delayed extraction conditions (focused at $m/z \sim 800$) with a source accelerating voltage of 15 kV. With optimal source settings, a mass resolving power of $M/\Delta M \sim 2000$ was achieved in the studied mass range for phospholipids between $m/z$ 600 and 1000. Mass calibration was performed with the peptide mixture mentioned above. Imaging MS data were acquired with MMSIT software and visualized in Biomap (http://maldi-msi.org/).

**Approaches to Glycerophospholipid Imaging Analysis**

**Whole-body Imaging MS—**The primary goal of whole-body imaging by MS is to rapidly obtain compound localization throughout the body with sufficient spatial resolution to clearly delineate the different organs present. Several aspects must be considered when imaging whole-body sections from model animals by MALDI MS. Of primary concern are tissue sectioning, overall matrix deposition, and data acquisition time. For smaller whole-body specimens ($\leq 3$ cm in length), standard specimen cryostats can be used as described under “Materials and Methods.” For sectioning larger animals such as adult mice and rats, whole-body cryostats are used. Because of the size of the resulting sections, specialized handling protocols have been adapted (73). To minimize overall processing time, the time taken by the matrix deposition step needs to be taken into account. Most reports of whole-body imaging MS examples shown in the literature involve a very quick matrix deposition step such as manual spray coating (40). In this regard, for whole-body imaging of phospholipids, both matrix sublimation and dry coating methods are ideal approaches. Based on the dimension of the sections to be analyzed, the choice of spatial resolution will significantly affect the total time for data acquisition. A higher pixel density may produce a better looking image but in the end may not give more information. Another significant aspect to consider is the stability of the sample with time, especially in the high vacuum environment of MALDI time-of-flight instruments.

The example shown here relates to the imaging of phospholipids from a 1-day-old mouse pup section. The section is $28 \times 8$ mm$^2$ in dimension. Matrix deposition was performed using the dry matrix coating approach. The coating procedure including setup, agitation, and cleaning took less than 30 min. After dry coating, a relatively homogeneous coating was observed throughout the sections with an 80–90% coating coverage. The percentage of coverage was observed to be tissue- and organ-dependent (Fig. 1A). The stability over time of the 2,5-DHB dry coating under high vacuum conditions was found to be excellent with no significant signal alteration within a 24-h period. Imaging MS data were acquired with a spatial resolution of 200 $\mu$m, representing 5444 pixels. Imaging MS data were acquired by summing the signals from 300 consecutive laser shots per pixel over the course of 5.5 h. Fig. 1B displays a photomicrograph of a serial H&E-stained section where numerous organs can be observed including the eye, brain, lung, liver, stomach, kidney, adrenal gland, and intestines. Fig. 1C presents the sum spectrum resulting from imaging MS data acquisition, displaying a typical phospholipid profile with intense signals from $m/z$ 650 to 900 (87). Fig. 1D presents an overlay of six different ion images localizing in some of the different organs or tissues found throughout the
section. The signal at \( m/z \) 691.04 was overall found ubiquitously in muscles throughout the section; \( m/z \) 770.53 was predominantly found in the eye socket, brain, and lungs; \( m/z \) 783.63 was exclusively observed in the stomach; \( m/z \) 810.60 was found to be very intense in the adrenal gland; and \( m/z \) 844.51 was observed to be very intense in the liver. Note that no normalization or postacquisition processing was performed. For whole-body imaging MS data, variations observed in total ion current (TIC) across the different organs and tissues encountered during the course of analysis may generate misleading ion images. Some tissues of the sections such as bones produce very little signal in the phospholipid mass range. Examples of some of the potential adverse effects of TIC normalization are addressed below for the mouse brain (see supplemental Fig. 4). For some of the observed signals in Figs. 1 and 2 and supplemental Figs. 3 and 4, tentative identifications based on molecular weight may be found elsewhere (20, 40, 80).

**Intermediate Dimension Imaging MS**—MALDI imaging MS of intermediate dimension sections (~1 cm in length) is often the starting point for most users. Tissue specimens are for the most part easily handled and cut. For the imaging of phospholipids, no organic rinsing protocol is applied as these have been shown to extensively remove the lipid component of sections, although in some cases, brief washes in aqueous solution have been found to be useful (90). After a brief dehydration step in a desiccator, the sections are ready for matrix deposition. Several matrix coating strategies may be considered, ranging from manual or automated spray, droplet array printing, sublimation, or dry deposition. In all cases, coating can be performed within a few hours. Imaging MS of midsize sections is typically performed with resolutions between 250 and 50 \( \mu \)m. The spatial resolution required will greatly affect the overall data acquisition time. Acquisition time may again become critical when considering the stability of the sample, especially in the high vacuum environments of time-of-flight instruments. This is especially true for matrix deposited by sublimation. In this case, the thin layer of matrix coating the section may progressively “back-sublimate” and with time create gradient artifacts as imaging acquisition progresses. From our experience, for sublimated 2,5-DHB with a thickness of ~7 \( \mu \)m, such effects start to be significant for time periods exceeding 24 h.

Fig. 2 and supplemental Fig. 3 present imaging MS results serially acquired from a coronal mouse brain section in positive and negative ionization mode, respectively. Imaging MS data were acquired with a spatial resolution of 100 \( \mu \)m. For imaging MS in the negative mode, to avoid ablation pattern overlaps, the grid array was aligned with an offset of 50 \( \mu \)m both the \( x \) and \( y \) dimensions with respect to the array defined for positive data acquisition. In both cases, to maximize the information, the number of shots per pixel was kept constant at 400. Per pixel, the resulting ablated area was ~40 \( \mu \)m in.
diameter. The oval-shaped brain section analyzed had dimensions of \( \frac{13}{100} \times \frac{8}{26} \) mm; when imaging at a spatial resolution of 100 \( \mu \)m, this represents \( \frac{10,850}{26} \) pixels. Fig. 2A presents a photomicrograph of the section after data acquisition where the matrix ablation pattern is clearly visible. The total acquisition time (positive and negative polarities) was \( \frac{22}{10} \) h. Fig. 2B presents the sum spectrum after acquisition in the positive mode. A typical phospholipid profile in the 650–900 m/z range was observed (86). Fig. 2C presents an overlay of three phospholipid images expressed in different regions of the section. The signal at m/z 734.65 was predominantly found in the cerebral cortex and striatum, m/z 741.59 was exclusively found in the ventricles, and m/z 826.73 was found in part of the striatum and the corpus callosum. Again, signal intensity normalization was not performed. Although normalization by TIC may improve the image contrast for some phospholipid signals, we have observed that even when restricted to the phospholipid m/z range TIC normalization can significantly distort localization. Some effects of TIC normalization are given in supplemental Fig. 4. Whereas in some cases normalization seems to have improved the quality of the image (m/z 660.07 and 776.16), in other cases, obvious variations within the images were observed such as edge effects (m/z 746.66 and 820.66), oversaturation (m/z 772.67), loss of information (m/z 746.66 and 797.67), and of greater concern “fabrication” of information (m/z 820.66). The necessity and effects of imaging MS data set normalization are worthy of additional study, particularly for the relatively narrow phospholipid mass range. Normalization using TIC is not the only strategy that needs to be thoroughly explored (91).

Supplemental Fig. 3 presents the imaging MS results obtained in negative ionization. Although 2,5-DHB is not the optimal matrix for negative phospholipid analysis by MALDI MS, some signals are nevertheless observed. An advantage to using 2,5-DHB is that it is fairly stable under high vacuum conditions for time periods of several hours, allowing collection of large images in both positive and negative modes. Supplemental Fig. 3A presents the sum spectrum obtained after imaging MS data acquisition. Again, signals in the phospholipid range (m/z 650–1400) are observed but with overall less intensity than in the positive ionization mode. Supplemental Fig. 3C presents an overlay of three phospholipid images (computed without normalization) expressed in different regions of the section. The signal at m/z 883.85 was predominantly found in the outer layers of the cerebral cortex and in the striatum, m/z 888.90 was found in the inner layers of the cerebral cortex and was very abundant in the corpus callosum, and m/z 1236.07 was exclusively found in the ventricles.

High Resolution Imaging MS—High resolution imaging is necessary for analysis of small anatomical features (a few hundreds of micrometers in dimension) within a tissue section with a lateral resolution down to the dimensions of individual cells (\( \leq 10 \mu \)m). The ability to analyze tissue sections at high resolution is primarily dependent upon laser spot size for laser microprobe systems and application of an ultrafine homogeneous coating without inducing analyte lateral migration. To date, only a handful of MALDI instruments capable of acquiring imaging MS data at cellular length scales (\( \leq 10 \mu \)m) have been reported (48–50). Current commercially available instruments are limited to spot sizes of at best 15–20 \( \mu \)m. However,
Fig. 3. High resolution imaging MS of phospholipids in positive ionization mode from a coronal mouse brain section. A, photomicrograph of the section after 2,5-DHB matrix deposition by sublimation and imaging MS data acquisition. Measurements were performed with a lateral resolution of 25 \(\mu\)m over the tip of the ventricle and at 9.6 and 4.8 \(\mu\)m within the striatum. B, magnified view of the tip of the ventricle showing the matrix ablation pattern after data acquisition. C and D, phospholipid ion images acquired with a lateral resolution of 25 \(\mu\)m over the tip of the ventricle. E and F, phospholipid ion images acquired with a lateral resolution of 9.6 \(\mu\)m over the striatum. G, magnified view of the ablation pattern after imaging MS of an area of the striatum with a lateral resolution of 4.8 \(\mu\)m. H and I, phospholipid ion images acquired with a lateral resolution of 4.8 \(\mu\)m over an area of the striatum.

limitations on laser spot size may be compensated for using an oversampling approach (92). In this technique, the raster width is narrower than the laser spot diameter. Because this causes the laser spot to overlap several pixels, full depletion of the matrix is necessary to produce sufficient signal for image calculation before movement to the next adjacent pixel. With oversampling, resolutions may be reasonably reduced to 4 times smaller than the laser spot size. High resolution imaging utilizes the same tissue sectioning procedures as intermediate imaging. However, because the area of interest has finer details than gross anatomy of an organ, matrix coatings are limited to those that produce very homogenous coatings of submicrometer crystals (<1 \(\mu\)m) such as obtained by sublimation (86).

Examples of high resolution imaging MS are given in Fig. 3. Several serial measurements were performed in positive ion mode on selected areas of a coronal mouse brain section coated with 2,5-DHB by sublimation (Fig. 3A). In this case, matrix sublimation was used as the coating technique with respect to the dry coating approach because it gives near complete tissue coverage. Data were acquired using a prototype linear MALDI-TOF mass spectrometer capable of focusing the laser probe on target to better than 5 \(\mu\)m in diameter (supplemental Fig. 5A) (48). The ability to manipulate the stage in the z direction allowed for precise control of laser spot focus onto the sample target. This system is also capable of operating under delayed extraction conditions, and unit mass separation on phospholipid signals can be achieved (supplemental Fig. 5B). Measurements were performed with a lateral resolution of 25 \(\mu\)m over the tip of the ventricle and at 9.6 and 4.8 \(\mu\)m within the striatum. Best results were obtained for laser energy-number of shots combinations for which matrix ablation went through the sample down to the target plate (see Fig. 3, B and G). Ion images obtained from the imaging of the tip of the ventricle are presented in Fig. 3, C and D; the signal at \(m/z\) 741 was found to be specific to the ventricle, whereas the signal at \(m/z\) 734 was found to be uniquely expressed in the surrounding tissues. With an imaging resolution of 25 \(\mu\)m, some pattern is already visible within the striatum (right side of images). This patterning was further explored by imaging over the striatum with lateral resolutions of 9.6 and 4.8 \(\mu\)m. At these resolutions, some signals were clearly found to be expressed within the nerve bundles of the striatum (\(m/z\) 825 and 851; Fig. 3, I and E), whereas others were uniquely detected within the surrounding tissues (\(m/z\) 772; Fig. 3, F and H). A closer observation of the ablation pattern obtained after imaging at 4.8-\(\mu\)m spatial resolution (Fig. 3G) shows regular rows and columns of drilled holes, indicating that no overlap between adjacent pixels occurred. It is also interesting to note that for the brain, when imaging sections at spatial resolutions of 5–10 \(\mu\)m, no obvious phospholipid signal delocalization was observed. This indicates that no delocalization is induced during the freezing, sectioning, mounting, and matrix application steps.

Fig. 4 presents the analysis of an adult mouse aortic valve acquired in the positive ion mode at a 9.6-\(\mu\)m lateral resolution. The size of one valve cusp is 500-\(\mu\)m length \(\times\) 15-\(\mu\)m width. The adult mouse aortic valve consists of an outer layer
of endothelial cells with an internal single cell layer of vascular interstitial cells. The valve cusp is essentially three cell layers in thickness so imaging the valve requires exceptional sensitivity as well as high spatial resolution techniques in instrumentation and matrix application. For imaging the aortic valve, a 12-μm cryosection was collected from the heart and coated with 2,5-DHB by sublimation. An area of 540 × 1100 μm encompassing both leaflets, representing 4697 pixels, was imaged over a period of 6.5 h. Laser ablation of sublimated matrix resulted in a circular spot size with an internal diameter of 2.93 ± 0.34 μm impinging into the tissue and an outer diameter of 6.48 ± 0.41 μm (Fig. 4A). These spots did not overlap between pixels, indicating achievement of a cellular level of analysis. Fig. 4B presents the sum spectrum of the image with many intense peaks occurring from m/z 550 to 600 and from m/z 700 to 925, matching a typical phospholipid signature. These data were not normalized to TIC. Fig. 4C presents an H&E staining of a neighboring section. Fig. 4D maps the intensity of the ion signal at m/z 600 occurring in the hinge region of the aortic valve. Ion intensity maps from m/z 741 (Fig. 4E) and m/z 846 (Fig. 4F) are good examples of ion signal occurring in both the hinge region and the cups of the valve. These data pose an exciting application toward investigations involving small anatomical features. For instance, numerous mouse models exist for the study of cardiovascular disorders and defects (93). Further improvements in resolving power, mass accuracy, and sensitivity to such instruments will greatly increase the utility for phospholipid imaging MS analyses.

**PERSPECTIVE**

Interest in imaging lipids from biological tissue sections by MALDI MS is growing, and the recent advances in matrix deposition strategies and instrumentation have significantly contributed to developments in the field. Imaging lipids with MALDI-TOF instruments provides distribution maps of at least the most abundant species, bringing a wealth of new molecular information. However, a typical MALDI lipid profile contains a wide range of molecular species with the presence of numerous species within a nominal isobar. Resolving images from these will require alternative imaging strategies that incorporate high mass-resolving MS instrumentation such as MALDI FT/ICR or Orbitraps in conjunction with high spatial resolution capabilities. Ion mobility mass spectrometry offers...
Although imaging lipids can bring important insight into some extremely complex biological processes, there is a more general question that needs to be addressed. That is, how do we make optimum use of the wealth of information contained within an imaging MS experiment? Strict comparison with the underlying histology may highlight subsets of signals that are differentially expressed. Even after identification of a majority of the compounds involved, we must ask how that information is correlated to the physiological state of the tissue. How does it complement imaging information obtained from proteins? How do we integrate all of that information with previous findings related to the same biological system or disease? New bioinformatics tools need to be developed to rapidly interrogate imaging MS data and, upon compound identification, interface the results with other sets of data using a systems biology approach.

Since the introduction of MALDI imaging MS over a decade ago, tremendous advances in technological development have propelled the discipline to new heights. We foresee that in the next decade MALDI imaging MS will become a primary tool in biological and biomedical research, enabling a better understanding of molecular mechanisms over a wide range of biological systems and facilitating a vast array of new applications.

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† This article contains supplemental Figs. 1–5.
‡ Present address: Dept. of Chemistry, Universite de Montreal, Pavillon Roger-Gaudry, 2900 boul. Edouard-Montpetit, Montreal, Quebec J4X 1T8, Canada.
§ Present address: Bruker Daltonics, 40 Manning Rd., Billerica, MA 01821.
¶ To whom correspondence should be addressed: Mass Spectrometry Research Center, 465 21st Ave. S., 9160 MRB III, Vanderbilt University, Nashville, TN 37232-8575. Tel.: 615-343-9207; Fax: 615-343-8372; E-mail: r.caprioli@vanderbilt.edu.

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