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Advances in MALDI Imaging Mass Spectrometry of Proteins in Cardiac Tissue, Including the Heart Valve

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Abstract

Significant progress has been made for tissue imaging of proteins using matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI IMS). These advancements now facilitate mapping of a wide range of proteins, peptides, and post-translational modifications in a wide variety of tissues; however, the use of MALDI IMS to detect proteins from cardiac tissue is limited. This review discusses the most recent advances in protein imaging and demonstrates application to cardiac tissue, including the heart valve. Protein imaging by MALDI IMS allows multiplexed histological mapping of proteins and protein components that are inaccessible by antibodies and should be considered an important tool for basic and clinical cardiovascular research.

Keywords

protein imaging; imaging mass spectrometry; cardiac; cardiovascular; semilunar valve; extracellular matrix

Introduction

Matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI IMS) is a powerful tool for defining the proteomics of the tissue microenvironment while retaining spatial information. MALDI was developed in the late 1980s and was shown to measure biological molecules spanning from metals to oligonucleotides [1, 2]. Initial experiments using MALDI as source for imaging mass spectrometry probed the on-tissue proteomes of rat pituitary and pancreatic tissue sections, demonstrating that intact proteins could be mapped to their tissue environments [3]. While there are now several sources that can be employed for protein imaging mass spectrometry including DESI [4], LAESI [5], LESA [6], SELDI [7], MALDI IMS stands alone as the most used and advanced platform for investigating the spatial distribution of proteins from tissue.

There are many reviews detailing the process of MALDI IMS and concepts for study planning [8, 9]. MALDI IMS starts with a tissue that has been sectioned and placed on an instrument appropriate substrate such as an indium tin oxide coated microscope slide. After sample preparation aimed at uncovering target molecules, a chemical matrix is sprayed onto the tissue. A UV laser, usually a frequency-tripled Nd:Yag operating at 355 nm, is used to sample the matrix coated tissue. At each point of sampling, the laser energy is absorbed by the matrix and results in rapid desorption and ionization of the matrix coated sample. For tissue imaging, each point of laser sampling generates a mass spectrum with a unique x,y coordinate. Selection of an ion allows one to generate a heat map of a single ion across the tissue. Investigation of proteins in tissue by MALDI IMS does not require prior knowledge of a known sequence and results in highly multiplexed reporting of protein localization from tissue.

While MALDI IMS has been used to map proteins to their histological features from organs including brain [10], kidney [11], liver [12], and lung [13], there is a dearth of information on protein imaging on cardiac tissue in the literatures. In this article, we discuss current approaches to gain and utilize MALDI IMS protein information and highlight strategies with data on cardiac tissue. This article is intended to inform the reader of advances and capabilities for imaging of cardiac tissue by MALDI IMS.

1. Challenges in Protein Imaging of Cardiac Tissue: Biochemical Content of Heart Tissue

The molecular composition of the heart is exquisitely sensitive to hemodynamic forces. Large molecular changes occur in heart development, which is one of the first organs to develop and supports all other organ development. In the mature heart, major anatomical features of the heart such as the ventricle, atria and heart valves each have a unique environment that is continually sensing hemodynamic forces and adjusting biochemical composition to maintain appropriate heart function. Diseases affecting the molecular composition have a feedforward effect on hemodynamics and heart function. A full understanding of the complex interplay between biochemical change and tissue function calls for an approach such as protein imaging, where highly multiplexed expression can be mapped to histological features and integrated with other imaging modalities.

There is an enormous amount of change in protein composition and organization as the heart develops and such changes impact protein imaging strategies. Detection of proteins by MALDI IMS is sensitive to tissue composition, and we use developmental patterns of the heart as window into the biochemical composition of heart tissue; these changes may be repeated in part during disease remodeling. Major extracellular changes include a progression of collagens, elastin, and proteoglycans (Figure 1). Detection of many of these proteins by MALDI IMS can be challenging. Collagens are a main component of the ECM, that from fetal to adult tissue evolve from a loose network of collagenous strands to tightly bound polyproline bundles with multiple disulfide bonds [14, 15]. Collagens are both glycosylated and hydroxylated for stabilization [16], and this can limit detectability through either intact protein imaging or through tryptic peptide production. Elastin has a high level of polymerization that includes modification of lysines to allysine, which limits tryptic activity on lysine residues [17]. Collagen also shows conversion of lysine to allysine, but at a lower level than elastin. Elastin and collagen can be further crosslinked together, either enzymatically or nonenzymatically, dependent on disease or developmental status [18]. Additionally, the heart has a significant population of proteoglycans that fluctuates during development and disease [19, 20]. For protein imaging, glycosylation may decrease detection of intact proteins through the presence of multiple glycoforms which has an effect of diluting the protein signal. Major protein changes occur on the background of a complex carbohydrate population. Glycosaminoglycans such as hyaluronan, constitute the framework for early heart modeling [21]. ECM signaling involves significant interactions between proteoglycans and carbohydrate populations to coordinate heart morphology and function in development and disease [19, 22]. Cell densities show large variations across anatomical features and biological status, requiring imaging strategies capable of efficiently extracting and detecting proteins within areas of low and high cell density. A good example of cell density variation is the semilunar valve, which varies from 1,800 cells/ mm² in pediatric tissue to less than 500 cells/mm² in adult tissue [15]. While changes in heart biology can present a challenge, they also represent an opportunity for protein imaging. Many of these alterations cannot be studied by immunohistochemistry as there are few antibodies available for specific modifications and/or are incapable of reporting protein changes without prior knowledge of a protein sequence. On the other hand, protein imaging by MALDI IMS has resulted in a toolbox of many strategies to mine multiple levels of protein data, all with associated spatial information.

2. Sample Preparation for Imaging Intact Proteins from Cardiac Tissue

Imaging mass spectrometry requires customized sample preparation strategies dependent on the target molecule, tissue type and biological status of the tissue. Each aspect of sample preparation must be evaluated, including tissue fixation, specialized treatments of tissue sections, and matrix considerations. Most commonly, MALDI IMS of intact proteins is performed using fresh frozen tissues, as formalin fixed tissues are extensively cross-linked and require many additional processing and washing steps, or requires use of trypsin treatment to generate peptides. For protein imaging of fresh frozen, non-fixed tissue, tissue sections placed on slides must first be rinsed by a series of organic solvents, including Carnoy's solution, and aqueous solvents to remove salts, metabolites and lipids that cause

signal suppression [23–25]. These washes serve to both fix the protein content and remove molecules that suppress protein signal. Selection of additional rinsing or pretreatment solvents depends not only on the type of tissue, desired target protein population, but also the biological status of the tissue, which includes developmental time points or disease state.

One fixation approach that has worked well for obtaining signal from cardiac tissue is fixation by acidified ethanol prior to sectioning [26]. Fresh cardiac tissue is incubated overnight in a cold, acidic solution of ethanol. The tissue then goes through a series of dehydration solutions to be embedded in paraffin wax. Tissue is sectioned, dewaxed and matrix applied for imaging. This particular strategy fixes proteins in place within the organ, prior to sectioning, without crosslinking them, allowing for detection of intact proteins. Advantages are improved histology and longer term storage as the tissue is preserved in wax. Evaluation of chicken heart treated in this manner showed that heart tissue has significant protein gradients throughout the heart with unique proteins found in ventricles, vessels or heart valves. This was one of the first illustrations of the use of MALDI IMS to detect proteomic content from a cross section of heart tissue while retaining localization to histological features [26].

Comparison of tissues with different biological status may be challenging because of the inherent differences in the chemical biology of the tissue. This can affect protein extraction and MALDI matrix crystallization on the tissue. One strategy to minimize chemical differences is to “seed” the tissue with matrix prior to matrix application. Dry seeding has been used as a method of matrix application for lipids [27], whereas wet seeding is a useful tool for protein imaging of tissue [28]. Wet seeding using a saturated solution of sinapinic acid in acidified ethanol may be used to minimize chemistries from different biological time points in cardiac tissue. This process is illustrated in Figure 2 where seeding was used prior to matrix application for protein imaging of cardiac tissue sections from murine embryonic day 14 and adult. Of note, proteins at m/z 8945 and m/z 9980 show similar expression patterns within the ventricles. However, there are several proteins such as m/z 8565 (m/z 8565, putative ubiquitin and m/z 4965, putative thymosin beta 4) that show cardiac alteration between embryonic day 14.5 to adult.

The most common matrix used for intact protein imaging is sinapinic acid. Another matrix, 2,5-dihydroacetophenone, has been shown to be useful to sensitive detection of intact proteins in kidney and brain tissue, but has not been demonstrated on cardiac tissue [29, 30]. Additives may be added to the matrix to improve extraction of protein signal from tissue while maintaining localization. Detergent spiked matrix has been used for detection of protein signal resulting in increased signal from cardiac tissue [31]. Here, the use of 0.05% SDS in the matrix resulted in over half of the peaks showing a significant enhancement in signal. Enhancement due to detergent additives differed for cardiac and brain tissue, highlighting that strategies must be tailored to biological status and organ type for optimal detection of intact proteins by MALDI imaging.

Currently, matrix is applied to the tissue by commercially available automated sprayers. However, matrix application by sublimation has been explored as a tool for protein imaging. This method was originally established as a tool for lipid imaging, and produced a fine

homogenous coating of matrix that allowed high spatial resolution acquisition [32]. Sublimation of protein matrices followed by hydration extracts proteins into the matrix layer for high sensitivity protein imaging [24, 29, 33]. Figure 3 illustrates the use of sublimation for detection of proteins from sheep cardiac tissue. Numerous protein peaks were detected including proteins specific to the valve cusp and hinge (m/z 3194, 11699, and 15045) with some distribution in the ventricle (m/z 5534 and 11258). Sublimation has an advantage in that it can facilitate collection of spatial resolutions of 1 micrometer [29]; however, applying matrix by sublimation in a reproducible manner requires significant user skill and experience with the method, which may limit its broader applicability.

3. Intact Protein Identification

It can be an enormous challenge to identify an intact protein from tissue by MALDI IMS, thus a number of approaches have been developed. Databases have been developed that allow researchers to search for a target m/z previously detected by imaging experiments prior to identification within their tissue of interest [34, 35]. Approaches to identify intact proteins from tissue use combinations of standard proteomic strategies including liquid chromatography, in solution digestions and on-tissue extractions. Protein identification strategies have been developed that span the range of tissue availability, using either large amounts of tissue, serial sections that have undergone sample preparation identical to the imaged section or spatially small regions of tissue for extraction. Protein identification approaches are considered ‘bottom up’ if enzymatic digestion is used to breakdown a protein and identify it by peptide tags. ‘Top down’ approaches fragment intact proteins by tandem mass spectrometry. Top down approaches are especially useful for imaging mass spectrometry as they directly map back to the precursor observed on-tissue and allow detection of protein modifications without prior knowledge [36].

For studies where tissue amount is not a limiting factor, protein identifications are made after isolation of target proteins by chromatography and this is by far the most common approach for protein identification [37–40]. A large amount of tissue, typically ~200 mg, is homogenized and fractionated by liquid chromatography (LC), typically using reverse phase chromatography. Each fraction is analyzed by MALDI MS for the mass-to-charge of the target protein that was observed in the image. The fraction with the protein of interest is then further separated by 1D gel electrophoresis and the protein band of interest cut out for trypsin digestion and identification by LC-MS/MS. An example of this approach was used to identify protein alterations during embryo implantation in a *Plg2g4*^{−/−} model [37]. Five proteins were identified as having statistically significant alterations and subsequently identified as described, including ubiquitin, calyculin (S100A6), calgizzarin (S100A11), and transthyretin. The study reported not only differences in timing of protein expression, but showed gradients of protein expression within the implantation sites [37]. Protein identification by LC fractionation is typically used to target only those proteins that have been shown to have significantly altered expression in imaging studies and does not attempt to identify all protein peaks found in a study.

On tissue extraction for protein identification may be performed either by simple pipetting, using small amounts from serial sections or using gel-based extraction to remove and

identify proteins from discrete areas of tissue. Pipetting approaches endeavor to extract proteins from discrete locations on tissue sections for subsequent identification [41, 42]. Small amounts (1–3 μL) of solvent are placed onto the tissue, allowed to absorb, followed by pipetting up and down for increased extraction. The extracted proteins are then used for either top down or bottom up protein identifications. Pipetting is a simple approach that does not require specialized equipment, may be used by anyone, and maintains some spatial information. Manipulation of the organic solution concentration of the extraction solvent is a way to extract and enrich for proteins with mass to charge matching those in the imaging study. For tissues with limited available amounts, several tissue sections neighboring the tissue section used for MALDI IMS may be homogenized for protein identification by nanoLC-MS/MS. This approach was applied to investigate intact proteins from calcific lesions in aortic valve stenosis. [43] Two proteins, collagen type 6 alpha 3 (Col6A3) and NMYC downstream-regulated gene 2 (Ndr2) were found increased in lesion sites. Immunohistochemistry confirmed these findings. An alternative method to identify proteins from spatially localized regions is the use of hydrogel extraction [44, 45]. Here, small (3 mm^3) hydrogel cylinders were dried, rehydrated with trypsin solution, placed on target regions of brain tissue sections, and incubated at high humidity. Peptides were extracted from gels by alternating organic and aqueous solvents, followed by analysis by LC-MS/MS, producing peptide tags for protein identification. Spatially directed hydrogel extraction identified over 50 protein groups directly related to the images of intact proteins from brain, allowing additional proteomics data to be mined [44]. For approaches using enzymatic digest after extraction (bottom up protein identification), mapping tryptic peptides back to the intact precursor may be challenging and can result in ambiguous data due to peptide homologies.

Top down proteomic approaches facilitate direct identification of an intact protein by reporting both precursor m/z and fragment information, making it easier to link a precursor back to the imaged tissue. In top down proteomic approaches, intact proteins are separated by LC and fragmented for sequence information without further enzymatic digestion [46]. Proteins may be extracted from the same tissue section by pipette either with matrix after imaging or by homogenization of the tissue section [39, 41, 47]. Typically, an LC-MS run on a small portion of sample (5–10%) is used to determine charge states of target m/z from imaging data and create an inclusion list for fragmentation. Figure 4 shows mapping and identification of an intact protein, ultimately identified as ubiquitin, at m/z 8565, separated by reverse phase C18 and fragmented by electron transfer dissociation. Ubiquitin shows higher abundance in the hinge regions and intima of the aortic wall. Top-down approaches have been combined with bottom up approaches to identify intact proteins and increase proteomic information from tissue [35, 44]. Observations have been that different charge states of intact proteins yield different efficiencies of fragmentation, and that collecting ETD data with another fragmentation method such as CID yields additional sequence information [41]. High mass accuracy protein imaging further increases confidence in linking top down data of the precursor back to the imaging data [48]. With ongoing improvements in sensitivity and data independent approaches, it is anticipated that simultaneous imaging and fragmentation of multiple proteins may be achieved in the very near future.

4. Tryptic Peptide Imaging

Protein information may be obtained from cardiac tissue using tryptic digestion and imaging the peptides [49–51]. This approach has an advantage in that it may be applied to a wide range of preserved tissues, including formalin fixed paraffin embedded (FFPE) tissues, it increases access to higher molecular weight proteins, and maintains a high quality of histology. Tissues are treated by dewaxing and antigen retrieve using citraconic or Tris buffer to disrupt some of the crosslinks [50, 52, 53]. Trypsin is sprayed onto the tissue and allowed to digest in a high humidity chamber, typically a sealed cell culture dish. The chemical matrix α -cyano-4-hydroxycinnamic acid is sprayed onto the tissue followed by imaging. Identification is performed by extracting tryptic peptides from tissue, and gaining sequence information by tandem mass spectrometry, usually through nano LC-MS/MS approaches. Figure 5 shows an example of tryptic peptide imaging used to explore unique protein expression distinct to porcine heart valve and aortic wall. The use of fresh frozen tissue allows detection of modifications on the peptide backbone [54]. Tissue decellularization followed by tryptic digestion has been used to access high molecular weight ECM proteins [55]. Tryptic peptide imaging is considered a powerful way to access protein content from tissue due to versatility in using formalin fixed paraffin embed tissue preservation and the ability to extract information from target protein populations such as extracellular matrix.

5. Post Translational Modifications

Major cardiac mechanisms in disease and health are regulated by protein post-translational modifications (PTMs) [56–58]. Post translational modifications interact with other biomolecules, influencing protein folding, transport, degradation and signaling and are thus considered an important topic for therapeutic research [59, 60]. Major PTMs include phosphorylation, glycosylation, acetylation, ubiquitination, deamidation, all of which may be defined by well-developed chromatography coupled mass spectrometry based strategies [61]. For MALDI IMS, PTMs are typically accessed through sequence information from tryptic peptides or intact proteins [54, 62–64]. Enzymatic approaches may improve detection of PTM sites [63]. Analysis of PTMs by enzymatic release is particularly useful for investigations since enzymatic strategies usually produce minimal signal interference versus chemical approaches. N-Glycan imaging by enzymatically driven N-glycan release is a new tool for glycomics research that reports localization and structural content of N-glycan PTMs [65–69]. N-glycan imaging is performed by spraying the endopeptidase F (PNGase F) onto antigen retrieved tissue to cleave the N-glycans from proteins. Distinct N-glycans are then visualized by routine imaging mass spectrometry, an advantage since lectin antibodies can only report localization of simple sugar moieties or specific sugar motifs that may occur across different N-glycan structures [70]. This approach works well with FFPE tissue, as the proteins remain relatively crosslinked, while the dewaxing further removes lipids and metabolites that may cause ion suppression. N-glycans, including sialylated glycans, do not seem to be affected by formalin fixation. Figure 6 shows an example of N-glycan imaging of porcine aortic tissue with distinctive N-glycans defining valve anatomy and aortic wall regions. Mapping an N-glycan back to a protein structure may require use of PNGase F digestion of the tissue in ^{18}O to definitively mark the site of N-glycosylation [71]. Current

techniques of protein imaging by high mass accuracy followed by top down sequencing should be able to identify sites of glycosylation on a single protein or glycopeptide, but haven't yet been reported. Additional and complementary strategies are needed to fully define the glycome. For fresh frozen tissue, detection of intact proteins may be improved by deglycosylation; removal of glycoforms yields a single peak with improved detection [72].

6. Informatics: Current and Future

Informatics tools for imaging are continually expanding. There are now multiple informatic tools and strategies for analysis of protein images. Sophisticated software, both open-source [73, 74] including Biomap (Novartis) and Datacube [75], as well as commercial (<http://scils.de/research/>), are available for image analysis. These programs allow reporting of molecular data by image segmentation, development of classification signatures and statistical tests reporting relative quantitation between images or between regions in a single image. Data can be flexibly combined within a lab or into multicenter studies [76]. Three-dimensional combination of images is achievable by protein imaging of serial sections. The protein image data are then combined into a volume for three dimensional viewing. Modern informatic approaches and instrumentation have improved the capabilities for performing three dimensional protein imaging [77–79]. Protein images may also be linked to other biomedical imaging modalities [80] [81].

The multiplexed amount of information obtainable by protein imaging facilitates new opportunities to combine image information into “Big Data” from multiple studies, including metabolomics, proteomics, and transcriptomes. A prospect is the capability to easily obtain and combine the multiplexed data into spatially derived networks that drive hypothesis generation in cardiac research as has been done with proteomics and immunohistochemistry [82]. Figure 7 illustrates the use of MALDI IMS and same-tissue section proteomics to derive additional biological information using tissue specific protein interaction networks. An exciting potential is the use of data from tryptic digestion of tissue sections to obtain protein information on pathway signaling; we hypothesize that this data could then be used in targeted imaging approaches to monitor peptide sets representing pathway alterations on-tissue.

7. Perspective

Protein imaging mass spectrometry has become a sophisticated toolbox for cardiac research with access to multiple levels of protein information that are mostly inaccessible by immunohistochemistry. The plethora of strategies is an advantage that allows investigators versatility in accessing topics of interest. Current efforts continually increase the number of proteins detected, simplify protein identification workflows, improve PTM evaluation and advance informatic capabilities. Combining protein imaging strategies with other imaging modalities and ‘Omics data provides exciting new opportunities to explore and understand the systems biology of the heart, down to subanatomical features. Application to clinical specimens is currently underutilized and could be particularly useful in aid in treatment of heart disease, including transplant evaluation. For cardiac research, IMS presents a powerful

and needed technology to mine protein and critical spatial information from tissue, “spatial proteomics”, to improve our understanding of cardiac development and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MALDI	matrix-assisted laser desorption/ionization
DESI	desorption electrospray ionization
LAESI	laser ablation electrospray ionization
LESA	liquid extraction surface analysis
SELDI	surface-enhanced laser desorption ionization
MS	mass spectrometry
LC	liquid chromatography
ETD	electron capture dissociation
CID	collision induced dissociation

References

1. Karas M, Bachmann D, Hillenkamp F. Influence of the wavelength in high-irradiance ultraviolet laser desorption mass spectrometry of organic molecules. *Analytical Chemistry*. 1985; 57:2935–2939.
2. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, Matsuo T. Protein and polymer analyses up to m/z 100 000 by laser ionization time of flight mass spectrometry. *Rapid Communications in Mass Spectrometry*. 1988; 2:151–153.
3. Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Analytical Chemistry*. 1997; 69:4751–4760. [PubMed: 9406525]
4. Takats Z, Wiseman JM, Gologan B, Cooks RG. Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science*. 2004; 306:471–473. [PubMed: 15486296]
5. Nemes P, Vertes A. Laser ablation electrospray ionization for atmospheric pressure, in vivo, and imaging mass spectrometry. *Analytical Chemistry*. 2007; 79:8098–8106. [PubMed: 17900146]
6. Kertesz V, Van Berkel GJ. Fully automated liquid extraction-based surface sampling and ionization using a chip-based robotic nanoelectrospray platform. *Journal of mass spectrometry*. 2010; 45:252–260. [PubMed: 20020414]

7. Bouamrani A, Ternier J, Ratel D, Benabid AL, Issartel JP, Brambilla E, Berger F. Direct-tissue SELDI-TOF mass spectrometry analysis: a new application for clinical proteomics. *Clinical Chemistry*. 2006; 52:2103–2106. [PubMed: 16990423]
8. Angel PM, Caprioli RM. Matrix-assisted laser desorption ionization imaging mass spectrometry: in situ molecular mapping. *Biochemistry*. 2013; 52:3818–3828. [PubMed: 23259809]
9. Norris JL, Caprioli RM. Analysis of tissue specimens by matrix-assisted laser desorption/ionization imaging mass spectrometry in biological and clinical research. *Chemical Reviews*. 2013; 113:2309–2342. [PubMed: 23394164]
10. Andersson, M., Andren, P., Caprioli, RM. Chapter 7: MALDI Imaging and Profiling Mass Spectrometry in Neuroproteomics. In: AO, editor. *Neuroproteomics*. CRC Press/Taylor & Francis; Boca Raton FL, Llc: 2010.
11. Herring KD, Oppenheimer SR, Caprioli RM. Direct tissue analysis by matrix-assisted laser desorption ionization mass spectrometry: application to kidney biology. *Seminars in Nephrology*. 2007; 27:597–608. [PubMed: 18061842]
12. Marquardt C, Tolstik T, Bielecki C, Kaufmann R, Crecelius AC, Schubert US, Settmacher U, Stallmach A, Dirsch O. MALDI imaging-based classification of hepatocellular carcinoma and non-malignant lesions in fibrotic liver tissue. *Zeitschrift für Gastroenterologie*. 2015; 53:33–39. [PubMed: 25594705]
13. Kriegsmann M, Casadonte R, Kriegsmann J, Dienemann H, Schirmacher P, Kobarg JH, Schwamborn K, Stenzinger A, Warth A, Weichert W. Reliable Entity Subtyping in Non-small Cell Lung Cancer by Matrix-assisted Laser Desorption/Ionization Imaging Mass Spectrometry on Formalin-fixed Paraffin-embedded Tissue Specimens. *Molecular & Cellular Proteomics*. 2016; 15:3081–3089. [PubMed: 27473201]
14. Johnson RC, Manasek FJ, Vinson WC, Seyer JM. The biochemical and ultrastructural demonstration of collagen during early heart development. *Developmental biology*. 1974; 36:252–271. [PubMed: 4814567]
15. Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, Schoen FJ. Human Semilunar Cardiac Valve Remodeling by Activated Cells From Fetus to Adult: Implications for Postnatal Adaptation, Pathology, and Tissue Engineering. *Circulation*. 2006; 113:1344–1352. [PubMed: 16534030]
16. Shoulders MD, Raines RT. Collagen structure and stability. *Annual Review of Biochemistry*. 2009; 78:929.
17. Pinnell SR, Martin GR. The cross-linking of collagen and elastin: enzymatic conversion of lysine in peptide linkage to alpha-aminoadipic-delta-semialdehyde (allysine) by an extract from bone. *Proceedings of the National Academy of Sciences*. 1968; 61:708–716.
18. Reiser K, McCormick RJ, Rucker RB. Enzymatic and nonenzymatic cross-linking of collagen and elastin. *The FASEB Journal*. 1992; 6:2439–2449. [PubMed: 1348714]
19. Schroeder JA, Jackson LF, Lee DC, Camenisch TD. Form and function of developing heart valves: coordination by extracellular matrix and growth factor signaling. *Journal of Molecular Medicine*. 2003; 81:392–403. [PubMed: 12827270]
20. Stephens EH, Chu C-K, Grande-Allen KJ. Valve proteoglycan content and glycosaminoglycan fine structure are unique to microstructure, mechanical load and age: relevance to an age-specific tissue-engineered heart valve. *Acta Biomaterialia*. 2008; 4:1148–1160. [PubMed: 18448399]
21. Manasek FJ, Reid M, Vinson W, Seyer J, Johnson R. Glycosaminoglycan synthesis by the early embryonic chick heart. *Developmental Biology*. 1973; 35:332–348. [PubMed: 4274694]
22. Lockhart M, Wirrig E, Phelps A, Wessels A. Extracellular matrix and heart development. *Birth Defects Research Part A: Clinical and Molecular Teratology*. 2011; 91:535–550. [PubMed: 21618406]
23. Seeley EH, Oppenheimer SR, Mi D, Chaurand P, Caprioli RM. Enhancement of protein sensitivity for MALDI imaging mass spectrometry after chemical treatment of tissue sections. *Journal of the American Society for Mass Spectrometry*. 2008; 19:1069–1077. [PubMed: 18472274]
24. Yang J, Caprioli RM. Matrix sublimation/recrystallization for imaging proteins by mass spectrometry at high spatial resolution. *Analytical Chemistry*. 2011; 83:5728–5734. [PubMed: 21639088]

25. Thomas A, Patterson NH, Laveaux Charbonneau J, Chaurand P. Orthogonal organic and aqueous-based washes of tissue sections to enhance protein sensitivity by MALDI imaging mass spectrometry. *Journal of Mass Spectrometry*. 2013; 48:42–48. [PubMed: 23303746]
26. Grey AC, Gelasco AK, Section J, Moreno-Rodriguez RA, Krug EL, Schey KL. Molecular morphology of the chick heart visualized by MALDI imaging mass spectrometry. *The Anatomical Record*. 2010; 293:821–828. [PubMed: 20186963]
27. Puolitaival SM, Burnum KE, Cornett DS, Caprioli RM. Solvent-free matrix dry-coating for MALDI imaging of phospholipids. *Journal of the American Society for Mass Spectrometry*. 2008; 19:882–886. [PubMed: 18378160]
28. Grey AC, Schey KL. Age-related changes in the spatial distribution of human lens α -crystallin products by MALDI imaging mass spectrometry. *Investigative ophthalmology & visual science*. 2009; 50:4319–4329. [PubMed: 19387068]
29. Zavalin A, Yang J, Hayden K, Vestal M, Caprioli RM. Tissue protein imaging at 1 μ m laser spot diameter for high spatial resolution and high imaging speed using transmission geometry MALDI TOF MS. *Analytical and Bioanalytical Chemistry*. 2015; 407:2337–2342. [PubMed: 25673247]
30. Anderson DMG, Spraggins JM, Rose KL, Schey KL. High Spatial Resolution Imaging Mass Spectrometry of Human Optic Nerve Lipids and Proteins. *Journal of the American Society for Mass Spectrometry*. 2015; 26:940–947. [PubMed: 25893273]
31. Angel PM, Mainini V, Magni F, Caprioli RM. Detergent enhancement of on-tissue protein analysis by matrix-assisted laser desorption/ionization imaging mass spectrometry. *Rapid Communications in Mass Spectrometry*. 2011; 25:199–204. [PubMed: 21157864]
32. Hankin JA, Barkley RM, Murphy RC. Sublimation as a method of matrix application for mass spectrometric imaging. *Journal of the American Society for Mass Spectrometry*. 2007; 18:1646–1652. [PubMed: 17659880]
33. Zavalin A, Todd EM, Rawhouser PD, Yang J, Norris JL, Caprioli RM. Direct imaging of single cells and tissue at sub-cellular spatial resolution using transmission geometry MALDI MS. *Journal of Mass Spectrometry*. 2012; 47:1473–1481. [PubMed: 23147824]
34. McDonnell LA, Walch A, Stoeckli M, Corthals GL. MSiMass list: a public database of identifications for protein MALDI MS imaging. *Journal of Proteome Research*. 2013; 13:1138–1142. [PubMed: 24313301]
35. Maier SK, Hahne H, Gholami AM, Balluff B, Meding S, Schoene C, Walch AK, Kuster B. Comprehensive identification of proteins from MALDI imaging. *Molecular & Cellular Proteomics*. 2013; 12:2901–2910. [PubMed: 23782541]
36. Siuti N, Kelleher NL. Decoding protein modifications using top-down mass spectrometry. *Nature Methods*. 2007; 4:817–821. [PubMed: 17901871]
37. Burnum KE, Tranguch S, Mi D, Daikoku T, Dey SK, Caprioli RM. Imaging mass spectrometry reveals unique protein profiles during embryo implantation. *Endocrinology*. 2008; 149:3274. [PubMed: 18403475]
38. Mathur BN, Caprioli RM, Deutch AY. Proteomic analysis illuminates a novel structural definition of the claustrum and insula. *Cerebral Cortex*. 2009; 19:2372–2379. [PubMed: 19168664]
39. Hardesty WM, Kelley MC, Mi D, Low RL, Caprioli RM. Protein signatures for survival and recurrence in metastatic melanoma. *Journal of Proteomics*. 2011; 74:1002–1014. [PubMed: 21549228]
40. Lagarrigue M, Becker M, Lavigne R, Deininger SO, Walch A, Aubry F, Suckau D, Pineau C. Revisiting rat spermatogenesis with MALDI imaging at 20- μ m resolution. *Molecular & Cellular Proteomics*. 2011; 10:M110–005991.
41. Schey KL, Anderson DM, Rose KL. Spatially-directed protein identification from tissue sections by top-down LC-MS/MS with electron transfer dissociation. *Analytical Chemistry*. 2013; 85:6767–6774. [PubMed: 23718750]
42. Longuespée R, Tastet C, Desmons A, Kerdraon O, Day R, Fournier I, Salzet M. HFIP Extraction Followed by 2D CTAB/SDS-PAGE Separation: A New Methodology for Protein Identification from Tissue Sections after MALDI Mass Spectrometry Profiling for Personalized Medicine Research. *Omics: a Journal of Integrative Biology*. 2014; 18:374–384. [PubMed: 24841221]

43. Mourino-Alvarez L, Iloro I, de La Cuesta F, Azkargorta M, Sastre-Oliva T, Escobes I, Lopez-Almodovar LF, Sanchez PL, Urreta H, Fernandez-Aviles F. MALDI-Imaging Mass Spectrometry: a step forward in the anatomopathological characterization of stenotic aortic valve tissue. *Scientific Reports*. 2016; 6:27106. [PubMed: 27256770]
44. Nicklay JJ, Harris GA, Schey KL, Caprioli RM. MALDI imaging and in situ identification of integral membrane proteins from rat brain tissue sections. *Analytical Chemistry*. 2013; 85:7191–7196. [PubMed: 23829295]
45. Harris GA, Nicklay JJ, Caprioli RM. Localized in situ hydrogel-mediated protein digestion and extraction technique for on-tissue analysis. *Analytical Chemistry*. 2013; 85:2717–2723. [PubMed: 23402265]
46. Catherman AD, Skinner OS, Kelleher NL. Top down proteomics: facts and perspectives. *Biochemical and Biophysical Research Communications*. 2014; 445:683–693. [PubMed: 24556311]
47. Ye H, Mandal R, Catherman A, Thomas PM, Kelleher NL, Ikonomidou C, Li L. Top-down proteomics with mass spectrometry imaging: a pilot study towards discovery of biomarkers for neurodevelopmental disorders. *PloS One*. 2014; 9:e92831. [PubMed: 24710523]
48. Spraggins JM, Rizzo DG, Moore JL, Rose KL, Hammer ND, Skaar EP, Caprioli RM. MALDI FTICR IMS of intact proteins: Using mass accuracy to link protein images with proteomics data. *Journal of the American Society for Mass Spectrometry*. 2015; 26:974–985. [PubMed: 25904064]
49. Groseclose MR, Massion PP, Chaurand P, Caprioli RM. High-throughput proteomic analysis of formalin-fixed paraffin-embedded tissue microarrays using MALDI imaging mass spectrometry. *Proteomics*. 2008; 8:3715–3724. [PubMed: 18712763]
50. Casadonte R, Caprioli RM. Proteomic analysis of formalin-fixed paraffin-embedded tissue by MALDI imaging mass spectrometry. *Nature protocols*. 2011; 6:1695–1709. [PubMed: 22011652]
51. Groseclose MR, Andersson M, Hardesty WM, Caprioli RM. Identification of proteins directly from tissue: in situ tryptic digestions coupled with imaging mass spectrometry. *Journal of Mass Spectrometry*. 2007; 42:254–262. [PubMed: 17230433]
52. De Sio G, Smith AJ, Galli M, Garancini M, Chinello C, Bono F, Pagni F, Magni F. A MALDI-Mass Spectrometry Imaging method applicable to different formalin-fixed paraffin-embedded human tissues. *Molecular BioSystems*. 2015; 11:1507–1514. [PubMed: 25592401]
53. Gustafsson JOR, Oehler MK, McColl SR, Hoffmann P. Citric acid antigen retrieval (CAAR) for tryptic peptide imaging directly on archived formalin-fixed paraffin-embedded tissue. *Journal of Proteome Research*. 2010; 9:4315–4328. [PubMed: 20597554]
54. Wenke JL, Rose KL, Spraggins JM, Schey KL. MALDI Imaging Mass Spectrometry Spatially Maps Age-Related Deamidation and Truncation of Human Lens Aquaporin-0. *MALDI Imaging Mass Spectrometry. Investigative ophthalmology & visual science*. 2015; 56:7398–7405. [PubMed: 26574799]
55. Gessel M, Spraggins JM, Voziyan P, Hudson BG, Caprioli RM. Decellularization of intact tissue enables MALDI imaging mass spectrometry analysis of the extracellular matrix. *Journal of Mass Spectrometry*. 2015; 50:1288–1293. [PubMed: 26505774]
56. Lee A, Oh J, Gorski PA, Hajjar RJ, Kho C. Post-translational Modifications in Heart Failure: Small Changes, Big Impact. *Heart, Lung and Circulation*. 2015; 25:319–324.
57. Pogoda K, Kameritsch P, Retamal MA, Vega JL. Regulation of gap junction channels and hemichannels by phosphorylation and redox changes: a revision. *BMC Cell Biology*. 2016; 17:137.
58. Lincoln J. The cardiac matrix revolution: Post-translational modification of Scleraxis. *Journal of Molecular and Cellular Cardiology*. 2016; 93:106. [PubMed: 26964702]
59. Walsh G, Jefferis R. Post-translational modifications in the context of therapeutic proteins. *Nature Biotechnology*. 2006; 24:1241–1252.
60. Jefferis R. Posttranslational Modifications and the Immunogenicity of Biotherapeutics. *Journal of Immunology Research*. 2016; 2016
61. Olsen JV, Mann M. Status of large-scale analysis of post-translational modifications by mass spectrometry. *Molecular & Cellular Proteomics*. 2013; 12:3444–3452. [PubMed: 24187339]

62. Schey KL, Gutierrez DB, Wang Z, Wei J, Grey AC. Novel fatty acid acylation of lens integral membrane protein aquaporin-0. *Biochemistry*. 2010; 49:9858–9865. [PubMed: 20942504]
63. Jiao J, Miao A, Zhang Y, Fan Q, Lu Y, Lu H. Imaging phosphorylated peptide distribution in human lens by MALDI MS. *Analyst*. 2015; 140:4284–4290. [PubMed: 25943257]
64. Chaurand P, Fouchécourt S, DaGue BB, Xu BJ, Reyzer ML, Orgebin-Crist MC, Caprioli RM. Profiling and imaging proteins in the mouse epididymis by imaging mass spectrometry. *Proteomics*. 2003; 3:2221–2239. [PubMed: 14595821]
65. Powers TW, Jones EE, Betesh LR, Romano PR, Gao P, Copland JA, Mehta AS, Drake RR. Matrix assisted laser desorption ionization imaging mass spectrometry workflow for spatial profiling analysis of N-linked glycan expression in tissues. *Analytical Chemistry*. 2013; 85:9799–9806. [PubMed: 24050758]
66. Powers TW, Neely BA, Shao Y, Tang H, Troyer DA, Mehta AS, Haab BB, Drake RR. MALDI imaging mass spectrometry profiling of N-glycans in formalin-fixed paraffin embedded clinical tissue blocks and tissue microarrays. *PLoS One*. 2014; 9:e106255, 106251–106211. [PubMed: 25184632]
67. Gustafsson OJR, Briggs MT, Condina MR, Winderbaum LJ, Pelzing M, McColl SR, Everest-Dass AV, Packer NH, Hoffmann P. MALDI imaging mass spectrometry of N-linked glycans on formalin-fixed paraffin-embedded murine kidney. *Analytical and Bioanalytical Chemistry*. 2015; 407:2127–2139. [PubMed: 25434632]
68. Briggs MT, Kuliwaba JS, Muratovic D, Everest-Dass AV, Packer NH, Findlay DM, Hoffmann P. MALDI mass spectrometry imaging of N-glycans on tibial cartilage and subchondral bone proteins in knee osteoarthritis. *Proteomics*. 2016
69. Holst S, Heijs B, de Haan N, van Zeijl RJM, Bruijn IH Briare-de, van Pelt GW, Mehta AS, Angel PM, Mesker WE, Tollenaar RAEM. Linkage-specific in-situ sialic acid derivatization for N-glycan mass spectrometry imaging of FFPE tissues. *Analytical Chemistry*. 2016; 88:5904–5913. [PubMed: 27145236]
70. Cummings, RD., Etzler, ME. *Essentials of Glycobiology*. Cold Spring Harbor Press; Woodbury, New York: 2009. Chapter 45: antibodies and lectins in glycan analysis.
71. Angel PM, Lim JM, Wells L, Bergmann C, Orlando R. A potential pitfall in 18O-based N-linked glycosylation site mapping. *Rapid Communications in Mass Spectrometry*. 2007; 21:674–682. [PubMed: 17279607]
72. Chaurand P, DaGue BB, Ma S, Kasper S, Caprioli RM. Strain-based sequence variations and structure analysis of murine prostate specific spermine binding protein using mass spectrometry. *Biochemistry*. 2001; 40:9725–9733. [PubMed: 11583173]
73. Jardin-Mathé O, Bonnel D, Franck J, Wisztorski M, Macagno E, Fournier I, Salzert M. MITICS (MALDI Imaging Team Imaging Computing System): a new open source mass spectrometry imaging software. *Journal of Proteomics*. 2008; 71:332–345. [PubMed: 18644473]
74. Robichaud G, Garrard KP, Barry JA, Muddiman DC. MSiReader: an open-source interface to view and analyze high resolving power MS imaging files on Matlab platform. *Journal of the American Society for Mass Spectrometry*. 2013; 24:718–721. [PubMed: 23536269]
75. Klinkert I, Chughtai K, Ellis SR, Heeren RMA. Methods for full resolution data exploration and visualization for large 2D and 3D mass spectrometry imaging datasets. *International Journal of Mass Spectrometry*. 2014; 362:40–47.
76. Römpf A, Both JP, Brunelle A, Heeren RMA, Laprévote O, Prideaux B, Seyer A, Spengler B, Stoeckli M, Smith DF. Mass spectrometry imaging of biological tissue: an approach for multicenter studies. *Analytical and bioanalytical chemistry*. 2015; 407:2329–2335. [PubMed: 25575583]
77. Trede D, Schiffler S, Becker M, Wirtz S, Steinhorst K, Strehlow J, Aichler M, Kobarg JH, Oetjen J, Dyatlov A. Exploring three-dimensional matrix-assisted laser desorption/ionization imaging mass spectrometry data: three-dimensional spatial segmentation of mouse kidney. *Analytical Chemistry*. 2012; 84:6079–6087. [PubMed: 22720760]
78. Thiele H, Heldmann S, Trede D, Strehlow J, Wirtz S, Dreher W, Berger J, Oetjen J, Kobarg JH, Fischer B. 2D and 3D MALDI-imaging: conceptual strategies for visualization and data mining.

- Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2014; 1844:117–137. [PubMed: 23467008]
79. Anderson DMG, Van de Plas R, Rose KL, Hill S, Schey KL, Solga AC, Gutmann DH, Caprioli RM. 3-D imaging mass spectrometry of protein distributions in mouse Neurofibromatosis 1 (NF1)-associated optic glioma. *Journal of Proteomics*. 2016 pii: S1874–3919(16)30027-6.
80. Seeley EH, Wilson KJ, Yankeelov TE, Johnson RW, Gore JC, Caprioli RM, Matrisian LM, Sterling JA. Co-registration of multi-modality imaging allows for comprehensive analysis of tumor-induced bone disease. *Bone*. 2014; 61:208–216. [PubMed: 24487126]
81. Verbeeck N, Yang J, De Moor B, Caprioli RM, Waelkens E, Van de Plas R. Automated anatomical interpretation of ion distributions in tissue: linking imaging mass spectrometry to curated atlases. *Analytical Chemistry*. 2014; 86:8974–8982. [PubMed: 25153352]
82. Lage K, Møllgård K, Greenway S, Wakimoto H, Gorham JM, Workman CT, Bendtsen E, Hansen NT, Rigina O, Roque FS. Dissecting spatio-temporal protein networks driving human heart development and related disorders. *Molecular Systems Biology*. 2010; 6:381. [PubMed: 20571530]

Figure 1. ECM modeling during semilunar valve development as an example of major ECM changes in the heart. **A)** Ovine valve histology at prenatal day 90 has a loose collagen (pink/lavender) structure. Lighter pink corresponds to the spongiosa, a glycosaminoglycan rich region of the valve. **B)** High magnification at prenatal day 90 shows elastin in short linear or v-shaped strands (red arrows). **(C)** Adult ovine heart valve shows thick compartmentalized collagen (hot pink) with a defined elastin layer facing the inflow (dark purple/black). **D)** High magnification reveals organized elastin (dark purple) layered between the collagen (hot pink) and internal glycosaminoglycans (light pink). **E)** Examples of gene changes during heart development, obtained by searching collagens (COL1A1, COL2A1, COL3A1) elastin, and select prote(Hyaluronan Synthase 2 (HAS2), Heparan Sulfate Proteoglycan 2 (HSPG2), and the chondroitin sulfate proteoglycan (VCAN). Abbreviations: ED- embryonic day; PN1- Postnatal Day 1. Method Details in Supplemental.

Figure 2. Protein imaging of mouse models of cardiac development using matrix seeding to minimize tissue chemistry differences. A) Seeded or unseeded matrix on mouse embryo after imaging. Far left, unseeded; middle, seeded and sampled with higher spatial resolution on cardiac region; far right, seeded. B) spectral comparison of embryonic day 14 and adult cardiac tissue showing good intensity for both, yet differential expression. C) image comparison of cardiac regions. Method Details in Supplemental.

Figure 3.

Protein imaging on ovine valve and cardiac muscle tissue using a sublimation and hydration protocol for matrix application and protein extraction. Sinapinic acid matrix was applied by sublimation followed by hydration a closed cell culture dish with 20% methanol for 5 minutes at 85°C. This protocol is compatible with hematoxylin and eosin (H&E) staining. A) Example signal and images from proteins detected using the protocol. B) Neighboring section from the same valve showing the pulmonary valve leaflet with MALDI matrix on it after imaging at 35 µm step size. Inset shows a high magnification view of laser puncture marks, illustrating that even higher spatial resolution may be achieved. C) H&E staining after removing the matrix in 70% ethanol for 1 minute. No laser marks are observed and cell level details are detected, showing that histological staining may be performed after MALDI IMS. Method Details in Supplemental.

Figure 4.

Protein Identification from sheep cardiac tissue. A) Hematoxylin, eosin and alcian blue stain. Purple-nuclei, pink- cytoplasm and collagen structure, blue- glucosaminoglycans. V- valve; AW-aortic wall. B) MALDI IMS image of ubiquitin, m/z 8565 (M+H), more abundant in valve tissue versus aortic wall. Valve was imaged at 35 μ m step size and aortic wall at 100 μ m step size to manage data collection time. C) Comparison of overall average mass spectra from valve and aortic wall. D) Zoom in on ubiquitin peak at m/z 8565, showing less intensity in aortic wall compared to valve. E) Intact protein fragmentation of m/z 8565,

MH ⁺¹² at m/z 714.4 by ETD, which subsequently identified it as ubiquitin. Method Details in Supplemental.

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Figure 5.

Peptide imaging of formalin-fixed paraffin embedded porcine aortic valve and aortic wall.

A) Grayscale unstained tissue with anatomic designations. B) Image examples observed after trypsin digestion (top) with controls that were not treated with trypsin (bottom). Example images map to the valve cusp and intima of the aortic wall (m/z 743.379), the collagenous fibrosa and hinge region (m/z 1444.540) or the inner aortic wall (m/z 1550.868). C) Combined ion image showing little overlap in false colored images. Black line demarks differences in spatial resolution. Method Details in Supplemental.

Figure 6. N-Glycan imaging of porcine aortic sinus. A) Grayscale unstained neighboring section with anatomic designations. B) Image examples observed after PNGase F digestion with putative glycoform structure (Glycoworkbench 2.0 database; sodiated precursor). C) Combined ion image showing contrasting patterns of N-glycosylation in aortic valve and wall. Method Details in Supplemental.

Figure 7.

An example of using the combination of MALDI IMS and proteomics to drive new hypotheses in cardiac research. A) MALDI IMS was performed on a human heart valve with congenital aortic valve stenosis, resulting in detection of thymosin beta 4 (TMSB4X) protein (identified by electron transfer dissociation, data not shown). B) LC-MS/MS proteomics was done on the same section and a TMSB4X protein-protein interaction network constructed from the tissue specific proteomics. The TMSB4X network reported “Regulation of actin cytoskeleton organization”, (p-value 2.0E-5) and “Regulation of cell migration”, (p-value 1.4E-4) included the proteins integrin linked kinase (ILK) and vinculin (VCL). C) TMSB4X expression by MALDI IMS is circled in red. These regions show abnormal cell migration is associated with TMSB4X as calculated by the protein interaction network. Red arrows - no cells present; circled regions on insets show cell clustering. D) Movat’s pentachrome of the same valve, but adjacent FFPE section used for immunofluorescence staining. Yellow-collagen, blue-glycosaminocans, purple-elastin. E) Co-localization of thymosin beta 4 and ILK associated with abnormal cell migration (elongated nuclei) and not in cells that were not migrating (red arrow). This data suggests that TMSB4X-ILK interactions are linked to abnormal cell migration in CAVS. Method Details in Supplemental.